Aus dem

Interfakultären Institut für Zellbiologie der Universität Tübingen
Abteilung Immunologie

A consensus pool of T-cell epitopes from adenoviral antigens

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

März, Léo

2022

Dekan:	Professor Dr. B. Pichler
1. Berichterstatter:	Professor Dr. S. Stevanović
2. Berichterstatterin:	Professorin Dr. K. Schilbach-Stückle
3. Berichterstatterin:	Professor Dr. S. Jonjic
Tag der Disputation:	10.05.2022

Abbreviations

aa	amino acid
ADP	adenovirus death protein
AF	amplification factor
APC	antigen-presenting cell
ARD	acute respiratory disease
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium
BSA	bovine serum albumin
C	constant immunoglobulin-like domain
CD	cluster of differentiation
CDR	complementary-determining region
CLIP	class II-associated invariant chain peptide
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DBP	single-stranded-DNA-binding protein
DC	dendritic cell
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISpot	enzyme-linked immunospot
ER	endoplasmic reticulum
ERAAP	endoplasmic reticulum aminopeptidase associated with antigen processing
FACS	fluorescence-activated cell sorting
FC	final HAdV HLA class II-peptide cocktail
FCS	fetal calf serum
G-CSF	granulocyte-colony stimulating factor
HAdV	human adenovirus
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
ICAM	intercellular adhesion molecule
ICS	intracellular cytokine staining

IMDM	Iscove's modified Dulbecco's medium
IFN	interferon
Ig	immunoglobulin
Ii	MHC class II-associated invariant chain
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
MHC	major histocompatibility complex
MLP	major late promoter
NAB	neutralizing antibody
NK cell	natural killer cell
NPC	nuclear pore complex
Orf	open reading frame
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononucleotic cell
PBS	phosphate buffered saline
Pen/Strep	Penicillin/Streptomycin
PHA	phytohaemagglutinin
PKR	RNA-dependent protein kinase
PLC	peptide-loading complex
PMA	phorbol 12-myristate 13-acetate
POI	peptide of interest
RID	receptor internalization and degradation complex
rpm	rounds per minute
TAP 1/2	transporters associated with antigen processing-1/2
TCM	T cell medium
TCR	T-cell receptor
T_H	T-helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
TP	terminal protein
T_{reg}	regulatory T cell
V	variable immunoglobulin-like domain

Table of contents

List of tables

List of figures

1 Introduction

1.1 The immune system

Humans are constantly surrounded by pathogens. Nevertheless, symptomatic infections rarely occur in a functioning immune system. The anatomic barriers of our body prevent most pathogens from entering and infecting our organism. These are the skin, the oral mucosa, the respiratory epithelium, and the epithelium of our gastrointestinal tract. These barriers contain both specific and unspecific protecting factors against many pathogens, such as the acidic milieu in the stomach or the presence of immunoglobulin (Ig) A on the mucosal epithelium. If a pathogen overcomes the biochemically protected anatomic barriers, it is encountered by the immune system. It is categorized into the innate and adaptive immune system, both being essential for the cooperative elimination of pathogens. These two systems can both be divided into a cellular and a humoral part.

1.1.1 The innate immune system

The innate immune system is evolutionary older. Its receptors recognize patterns that are common and exclusive to pathogens. Receptors of the innate immune system have a limited variety and specificity. The advantage is a faster immune response compared to the adaptive immune system, especially when encountering a pathogen for the first time. The innate immune response is essential for the initiation of a later adaptive immune response.

The cellular component of the innate immune system consists of natural killer cells (NK cells), dendritic cells (DCs), macrophages, granulocytes, and mast cells. NK cells induce apoptosis in cells with reduced expression of human leukocyte antigen (HLA) class I molecules. HLA molecules display peptides on the cell surface. They are essential for the activation and effector function of the cells of the adaptive immune system (see chapter 1.1.2.2). The identification of cells with reduced HLA molecule expression is referred to as the recognition of “missing self”. (1, 2) A downregulation of surface HLA class I molecules is induced in neoplastic or virus-infected cells. (3)

Both DCs and macrophages are antigen-presenting cells (APCs). DCs reside in epithelial tissues that are inherently exposed to the environment. They constantly survey the extracellular compartment by phagocytosis and induce an adaptive immune response by antigen presentation to B and T cells in draining lymph nodes. After proteasomal

degradation of phagocytosed proteins, resulting peptides are loaded onto HLA class II molecules and presented on the cells surface. After activation, DCs present the antigen-derived peptides and migrate into lymph nodes where they are essential for T-cell activation. DCs can present peptides from extracellular antigens on HLA class I molecules, which is called cross-presentation. This process can be critical for the priming of naïve cluster of differentiation (CD) 8-positive T cells. (4)

Macrophages are tissue-resident cells. Their progenitors before migration from blood to tissue are called monocytes. Macrophages phagocytose pathogens present in their environment and initiate an immune response by the early secretion of inflammatory molecules. (5) When T cells recognize pathogen-derived peptides presented on a macrophage, they induce degradation of material contained inside endosomal vesicles. This process is of importance, as some pathogens could otherwise evade the immune system through their ability to survive inside phagocytotic vesicles of macrophages. (6)

The granulocytes are a group of immune cells, harboring a high density of granules in their cytoplasm. They can be divided into neutrophils, eosinophils, and basophils. They have a live span of a few days. Neutrophils are the most abundant white blood cells in the peripheral blood. They are essential for the elimination of all sorts of microorganisms by phagocytosis and efficient destruction in their endosomal compartment. Basophils and eosinophils are of importance in the fight against parasites.

Mature mast cells are tissue-resident cells. A proinflammatory mediator contained in their cytosolic granules is histamine. The release of histamine leads to increased permeability of blood vessels and facilitates the recruiting of effector molecules and cells to the site of inflammation.

The humoral part of the innate immune system consists of antimicrobial proteins and the complement system. The complement factors are plasma proteins that can interact with antibodies, opsonize pathogens in the absence of antibodies and kill pathogens by forming a membrane-attack complex.

1.1.2 The adaptive immune system

The adaptive immune system is the evolutionary younger part of the immune system. The cellular component of the adaptive immune system consists of B cells and T cells. B cells produce antigen-specific antibodies after activation. Naïve B cells are activated in the lymph node *via* their B-cell receptors when recognizing an antigen. A subgroup of T cells assists the activation of B cells when recognizing the same antigen. Antigen-specific antibodies represent the humoral part of the adaptive immune system. They consist of a Fab and an F_C part. Fab contains the antigen-binding site, whereas F_C binds to F_C-receptors on phagocytes. The binding of the fragments to their counterpart enables phagocytic cells to phagocytose and eliminate pathogens harboring the recognized antigen efficiently. Another critical task of antibodies is the fast and efficient neutralization of antigens. This mechanism is essential for vaccines against toxins. The tetanus vaccine is one example where neutralizing antibodies (NABs) protect the organism from the harmful effects of even very low concentrations of this efficient toxin.

T cells modulate the immune response by the secretion of immunomodulatory molecules such as cytokines, kill infected or mutated cells and orchestrate the effector functions of the cells of the adaptive and innate immune system. They are divided into $CD4^+$ and $CD8^+$ T cells. Each T cell carries its antigen-specific T-cell receptor (TCR).

TCRs and the antibodies produced by activated B cells are very specific. The specificity makes the elimination of pathogens more effective when compared to the innate immune response. The TCRs depend on the presentation of particular peptides by HLA molecules to be activated. The enormous number of epitope-specific TCRs enables the recognition of almost every antigen. During infection, T cells that carry the appropriate antigen-specific receptor get activated by the interaction of their TCR with an HLA-peptide complex and multiply. The activation and multiplication enable the clearance of the pathogen harboring the mentioned antigen. After infection, the populations of antigen-specific T cells are reduced. Some of the antigen-specific T cells survive and remain in the organism as memory T cells, which leads to a long-lasting immunity. In the event of reinfection, memory T cells can mount a faster and stronger answer against the pathogen. This mechanism is used for active vaccination. The development of an immune memory is restricted to the adaptive immune system.

1.1.2.1 Generation of HLA-peptide complexes

For their activation and effector function, T cells depend on the presentation of peptides by HLA molecules on the cell surface. There are two sources of peptides that can be processed and presented by HLA molecules. Firstly, HLA class I molecules present peptides originating from intracellular proteins. These can be proteins from the natural turnover of the cell or viral proteins in case of a viral infection. Intracellular proteins are cleaved by the proteasome, and resulting peptides can be loaded onto HLA class I molecules. Every nucleoid human cell can process peptides and to present them on HLA class I molecules. This makes it possible for the cells of the adaptive immune system to detect intracellular infections or neoplastic changes.

Furthermore, peptides can be presented on HLA class II molecules when they originate from phagocytosed proteins. The phagocytosed proteins are cleaved, and some of the resulting peptides loaded onto HLA class II molecules. This pathway is mostly restricted to cells of the immune system, especially the subgroup of APCs to which DCs, macrophages, and B cells belong. It enables the activation of the adaptive immune system when infected with an extracellular pathogen.

1.1.2.2 The HLA

The HLA gene locus is located on chromosome 6, with over 200 identified genes. In other vertebrates, the HLA is also referred to as major histocompatibility complex (MHC). It encodes several proteins that are essential for the immune system, as the HLA class I and HLA class II molecules but also other proteins necessary for peptide-processing. (7) HLA molecules link the innate and adaptive immune system.

As the human DNA has a pair of each chromosome, one from each parent, every human individual is a carrier of two HLA gene regions. The HLA molecules encoded on chromosome 6 are highly polymorphic. Each HLA allotype has its peptide-binding preferences. HLA alleles are codominantly expressed, with most individuals being heterozygous for each HLA. Another feature of HLA molecules is their polygeny, which further increases the range of peptides that can be bound and presented on the cell surface of an individual. This ensures that peptides of almost every antigen can be bound and presented by at least one HLA allotype. As of April 2013, it had been reported that there were 7,089 HLA class I alleles and 2,065 HLA class II alleles. (8) As of September 2018, it has been reported that there are 14,800 HLA-I alleles and 5,288 HLA-II alleles. (9) This

increase shows the rising research interest in the understanding of this complex gene region.

Three groups of classical HLA class I molecules have been characterized: HLA-A, HLA-B, and HLA-C. HLA class I molecules consist of a polymorphic α-chain and a monomorphic β_2-microglobulin. The α-chain consists of three Ig-like domains, named α_1, α_2, and α_3, which are encoded in the HLA class I gene region. The peptide binding cleft of the HLA class I molecule is formed by the folded α_1- and α_2-chains. These regions are highly polymorphic. They are responsible for the diversity and binding preferences of different HLA allotypes. The α_3 region is the domain of the HLA class I molecule, which interacts with the CD8 receptor of the T cell and is, therefore, less variable. (10) The β_2-microglobulin is the second chain of the HLA class I molecule. It is encoded on chromosome 15. Each HLA gene region encodes one α-chain for each of the three HLAs. Thus, when accounting codominant expression and heterozygosity for each allele, six different classical HLA class I alleles can be carried by an individual.

The length of the peptides presented by HLA class I molecules is limited by strong hydrogen bonds between terminal residues and the peptide carboxyl and amino termini at both ends of the binding groove. (11) Usually, around eight to ten amino acid (aa) long peptides are presented by HLA class I molecules. (12-14) On healthy cells, the presented self-peptides are not recognized by TCRs of immune response-inducing T cells and do not trigger an activation of the immune system. Autoimmune diseases occur when self-peptides are recognized by cells of the immune system and trigger an immune response against healthy cells. HLA class I molecules are essential for the elimination of intracellular infections, such as viral infections. Another important pathogenesis in which HLA class I molecules are crucial is in the state of neoplasia. Neoplasia occurs when a cell accumulates mutations in its DNA, which can then lead to changes in the proteome. These changes can be presented on HLA class I molecules. Peptides originating from somatic mutations of neoplastic cells can trigger an immune response against the mutated cells. When the recognition and destruction of mutated cells do not work correctly, it can result in cancer.

Some viruses, such as cytomegalovirus (CMV) and human adenovirus (HAdV), reduce the surface expression of HLA class I molecules and interfere with peptide-loading onto

HLA class I molecules, thereby minimizing the number of viral peptides presented by infected cells. (15, 16) As mentioned earlier, the immune system does not only eliminate cells with HLA-presented viral epitopes but also cells with reduced or absent expression of HLA molecules. (1)

In contrast, HLA class II molecules are mostly expressed by cells of the immune system, especially APCs. In the presence of proinflammatory molecules, HLA class II expression is induced on epithelial cells. (17) The features of polygeny, codominant expression, and polymorphism are comparable to HLA class I alleles. There are three peptide-binding HLA class II alleles present in each HLA class II gene locus: HLA-DR, HLA-DP, and HLA-DQ. Each of these genes encodes an α- and a β-chain, which together form an HLA class II molecule. Both chains contribute to the peptide-binding groove. In some individuals, the HLA gene region encodes two different HLA-DR β-chains, which can both pair with the DR α-chain. This leads to a second HLA-DR molecule encoded in one HLA class II gene locus. The DR α-chain does not vary between individuals and is functionally monomorphic. (18-20) The core peptide length bound to HLA class II molecules is similar to the peptide length bound to HLA class I molecules (21), with the difference that the binding pocket is not limited on both ends. Thus, longer peptides can be bound and presented by HLA class II molecules. (22-24) Peptides presented on HLA class II molecules mostly originate from protein fragments that were taken up from the extracellular compartment by phagocytosis or pinocytosis. Some of the peptides presented by HLA class II are self-peptides derived from cytosolic proteins, which are degraded in the natural process of autophagy. (25)

1.1.2.3 Peptide processing and presentation

All somatic cells are continually degrading and replacing proteins by newly synthesized proteins. Generally, they are post-translationally modified *via* ubiquitination, for degradation by the 26S proteasome. When a virus infects a somatic cell, it will use the protein biosynthesis of the host cell to reproduce itself. Some viral proteins which are present in the cytosol after synthesis are post-translationally modified with ubiquitin as well and cleaved into fragments by the adenosine triphosphate dependent 26S proteasome. (26) The proteasome consists of the 20S proteolytically active core particle and two 19S regulatory particles. In the presence of interferon-gamma (IFN-γ), some subunits of the proteasome are replaced by more effective ones to form the

immunoproteasome. (27) The fragments generated by the proteasome are then transported from the cytosol into the endoplasmic reticulum (ER) lumen by the transporters associated with antigen processing-1 and -2 (TAP1, TAP2). TAP1 and TAP2 are located in the membrane of the ER and transport peptides with a length of eight to 16 aa. (28) The TAP-1 and TAP-2 genes are encoded within the HLA gene locus. The lumen of the ER is the site where the peptides are first brought into contact with the HLA class I molecules that are translocated into the ER lumen during synthesis. This translocation during synthesis is crucial to prevent the HLA class I molecules from already binding to peptides in the cytosol. The assembly of the HLA class I molecules takes place in the ER lumen, so that only peptides which were transported into the ER by TAP1 and TAP2 are loaded onto the HLA class I molecules. Due to the length restriction of both TAP proteins and the binding groove of HLA class I molecules, most peptides need to be shortened. This is done by an enzyme called endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP). ERAAP trims peptides at their amino-terminal end and shortens them to fit into the binding-groove of the HLA class I molecule. (29) Further chaperones assist peptide-loading onto HLA class I molecules. When the newly synthesized α-chain of the HLA class I molecule is translocated into the ER, it binds to calnexin, which holds it in a partially folded state. As soon as the β_2-microglobulin binds to the partly folded α-chain, it dissociates from calnexin and is associated with the peptide-loading complex (PLC). The PLC consists of four components. First, there is calreticulin, which has a function comparable to that of calnexin. Another component is tapasin, which links the partly folded HLA class I molecule to the TAP. In close location to the TAP is where the HLA class I heterodimer waits for a peptide fitting into its binding-groove to be transported from the cytosol into the ER lumen. The third component is a thiol oxidoreductase named ERp57, which remodels the disulfide bond in the α_2 domain of the HLA class I molecules to optimize the peptide loading process. The final component is TAP. The PLC does not only assist the peptide loading onto HLA class I molecules, but also facilitates the exchange of low affinity bound peptides by peptides with higher affinity. This process is referred to as peptide editing. The HLA class I molecule is released from the PLC only when it has bound a peptide. The released HLA-peptide complex is then transported to the cell surface by the trans-Golgi network. It fuses with the cell membrane to be scrutinized by $CD8^+$ T cells (Figure 1). (27, 30) The HLA

molecules are only stable if they bind a peptide. When the peptide dissolves from the HLA class I molecule, the β_2-microglobulin dissolves from the α-chain as well. This usually ensures that peptides of the extracellular compartment do not replace the peptides which are bound by the HLA class I molecule.

However, some cells are also capable of presenting peptides derived from proteins that were taken up by phagocytosis on HLA class I molecules. This pathway - called cross-presentation - is restricted to APCs and primarily performed by DCs. (30)

The peptides presented on HLA class II molecules on APCs originate from proteins that were degraded in intracellular vesicles. (Figure 2) These are mostly self-proteins taken up from the extracellular compartment by phagocytosis, which leads to the presentation of self-peptides on HLA class II molecules. (25) The most common processing-pathway of HLA class II peptides begins with the uptake of the particles from the extracellular compartment into endocytic vesicles. The receptor-mediated endocytosis of B cells is triggered by proteins that bind to the membrane-bound Ig-like receptor. Macrophages and DCs constantly internalize extracellular material by phagocytosis, receptor-mediated endocytosis, or macropinocytosis. The extracellular material is internalized into endosomes, which are not accessible to the proteases of the cytosol. These vesicles contain acidic proteases, such as the cysteine proteases Cathepsin S and Cathepsin L. As the endosomes advance into the inner of the cell, they become more acidic, thereby activating the acidic proteases. The activated acidic proteases degrade the intravesical proteins. The α- and β-chains of HLA class II molecules are directly translocated into the ER lumen during synthesis. The newly-synthesized HLA class II molecules are joined by the MHC class II-associated invariant chain (Ii) to prevent the HLA class II peptide-binding groove from being occupied by peptides present in the ER lumen. (21) The Ii is a glycoprotein that spans the ER membrane with its amino terminus residing in the cytosol and its carboxy-terminus residing in the ER lumen. It contains a trimerization region close to its carboxy terminus. The Ii groups to stable trimers. The class II-associated invariant chain peptide (CLIP) - a domain of the Ii in close location to the trimerization domain - resides in the binding groove of an HLA class II molecule. Each of the three CLIPs of the trimer occupies the peptide-binding groove of an HLA class II α:β heterodimer. In the ER, HLA class II α- and β-chains and the Ii are bound by calnexin.

Calnexin releases these components, as soon as a nonameric complex, consisting of three Ii, three α-chains, and three β-chains, is formed. The HLA class II molecules can only leave the ER in a nonameric complex. This prevents the binding of HLA class II molecules to peptides in the ER and the transport of HLA class II molecules with its peptide-binding groove occupied by anything else than the CLIP of the Ii out of the ER. When a vesicle containing the nonameric complex buds off the ER, it is delivered to a low-pH endosome, with which it fuses. This is the compartment in which the peptide loading onto the HLA class II molecule can occur. After fusion, the activated acidic proteases first cleave the trimerization domain from the Ii, generating three 22-kDa Ii, named LIP22. In the next step, LIP22 is further cleaved to the 10-kDa fragment LIP10 by cysteine proteases. LIP10 retains the HLA class II molecule in the endosomal compartment. When LIP10 is cleaved, CLIP remains in the peptide-binding pocket of the HLA molecule. The HLA class II molecule is bound and stabilized by HLA-DM for the exchange of the CLIP by a peptide. HLA-DM resembles HLA class II molecules but does not have a peptide-binding pocket. It is mainly found in the endosomal compartment, where it binds to the α-chain of HLA class II molecules. This binding facilitates the exchange of CLIP or peptides with low affinity by other peptides by inducing changes in the structure of the HLA class II molecule. This process is comparable to peptide editing in HLA class I molecules. Another HLA class II-like molecule, which is present in the late endosomal compartment, is HLA-DO. Just like HLA-DM, it cannot bind and present peptides. It binds to HLA-DM and thereby reduces the effectiveness of peptide-loading onto HLA class II molecules. The α- and β-chains of HLA-DM and HLA-DO are both encoded in the HLA class II gene locus, with the HLA-DM α- and β-chains located close to the HLA-DOA gene locus. In the presence of IFN-γ, the transcription of HLA-DMA, HLA-DMB, and HLA-DOA is increased, but not of HLA-DOB. This leads to a predominance of HLA-DM molecules in late endosomes, compared to HLA-DO, and a more effective peptide loading onto HLA class II molecules during infection. (19)

It is of great importance that the HLA class II molecules bind peptides with high affinity to ensure that the peptide can stay bound to the HLA molecule on the cell surface for as long as possible. It can take up to days until the HLA class II-peptide complex encounters a TCR that recognizes it. If the antigenic peptide dissolves from the HLA class II molecule before its TCR counterpart recognizes it, no immune response can be triggered. After

binding a peptide, the HLA class II-peptide complex is transported to the cell surface, where it is presented to the extracellular compartment to be scrutinized by $CD4^+$ T cells (Figure 1). (31)

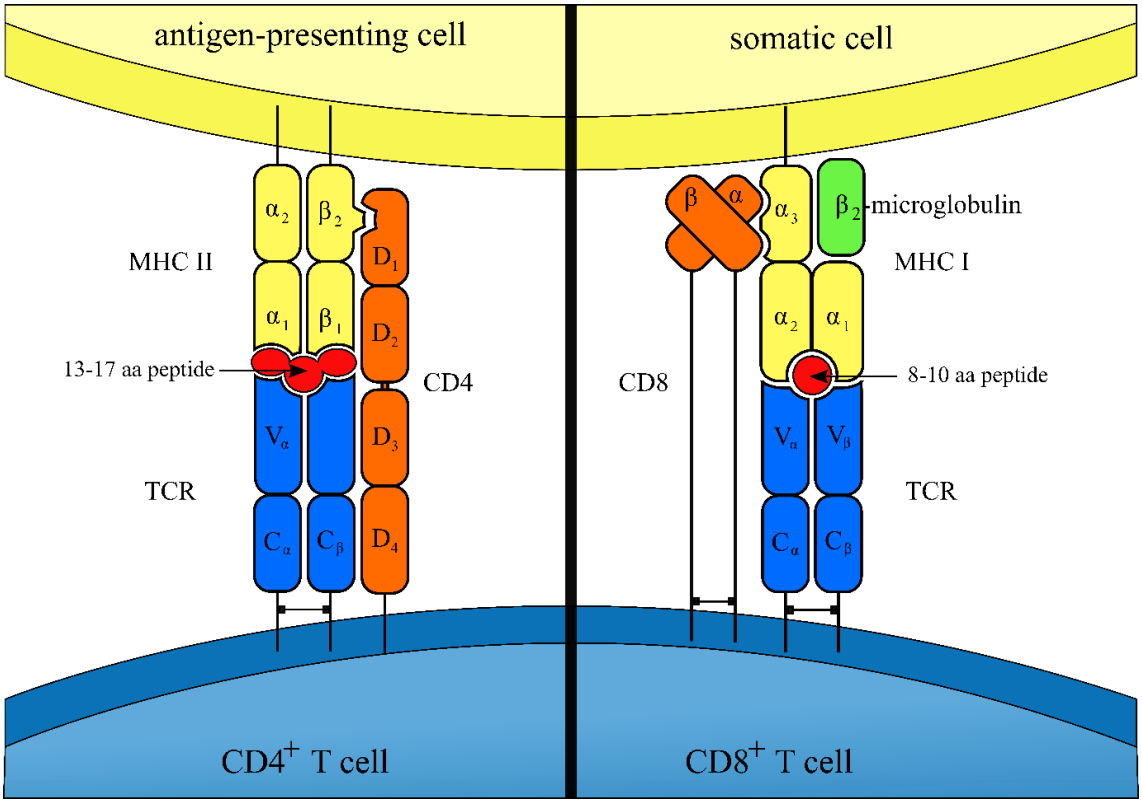


Figure 1: Interaction of TCR with HLA-peptide complex: TCR of $CD4^+$ T cell interacts with the HLA class II-peptide complex on APCs. The CD4 receptor interacts with the β2 domain of the HLA class II molecule (left-hand side). TCR of $CD8^+$ T cell interacts with the HLA class I-peptide complex on somatic cells. The CD8 receptor interacts with the α3 domain of the HLA class I molecule (right-hand side). (10) The peptide, bound by the HLA class II molecule, can protrude from the binding pocket, whereas the binding pocket of the HLA class I molecule is limited to both ends. (11) The peptides bound by HLA class II molecules are longer than peptides bound by HLA class I molecules. The CD3 co-receptor is not illustrated (see Figure 3). Adapted from (19).

HLA molecules are only stable if they bind a peptide. As soon as the peptide dissolves from the peptide-binding groove, the HLA molecule is degraded. However, HLA class II molecules seem to be more stable without a peptide bound to them, than it is the case for HLA class I molecules. (32) The consequences of an HLA class II peptide replaced by another peptide of the extracellular compartment does not have a significant impact, since it is their purpose to present peptides from the extracellular compartment. As the peptides presented on HLA class I molecules are derived from intracellular proteins, the extracellular replacement of a self-peptide by an epitope could induce the elimination of

a healthy cell. Nevertheless, this process also seems to occur as it is a widely used technique to screen for immunogenic T-cell epitopes by adding synthetic peptides to cell cultures. It is not yet clear if those synthetic peptides replace other peptides bound by HLA molecules or if there are HLA molecules with empty binding pockets on the cell surface to which the synthetic peptides can bind.

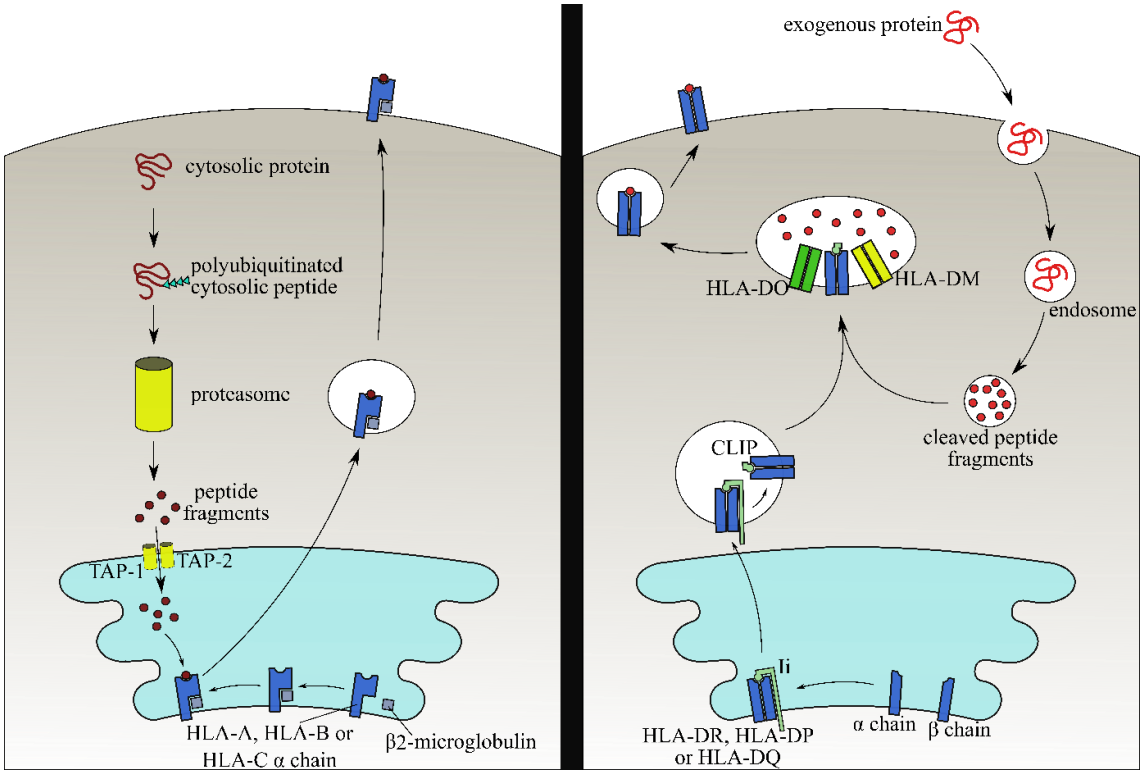


Figure 2: Peptide processing pathways: The processing of HLA class I peptides is illustrated on the left-hand side. Cytosolic proteins are marked for degradation by the proteasome via polyubiquitination. The cleaved peptides are transported into the ER, where they are loaded onto HLA class I molecules with the help of the PLC (not illustrated). The HLA class I α-chain and the β2-microglobulin are directly translated into the ER, where they are assembled. The HLA class I-peptide complex is then transported in a vesicle to fuse with the cell membrane. The processing of HLA class II peptides is illustrated on the right-hand side. An exogenous peptide is taken up into the endosomal compartment from the extracellular compartment. As the endosomal content becomes more acidic, the acidic proteases get activated and cleave the contained protein into fragments. In parallel, the HLA class II α- and β-chains are translated into the ER lumen where a nonamer is formed by three α-chains, three β-chains and three Ii (illustrated as one trimer). This nonamer buds off the ER in a vesicle. The Ii is cleaved stepwise until only CLIP remains in the peptide-binding groove. The vesicle containing the CLIP-loaded HLA class II molecule can then fuse with the late endosome containing the cleaved peptides. With the help of HLA-DM and HLA-DO, the CLIP gets replaced by a processed peptide. The HLA class II-peptide complex is then transported to the cell surface.

1.1.2.4 T cells

T cells are a subset of lymphocytes. They form the cellular part of the adaptive immune system together with the B cells. Their names originate from the tissue, from which the naïve effector cells are released. These are the thymus in the case of the T cells and the bone marrow for the B cells. B cells can present peptides that were taken up by receptor-mediated endocytose on HLA class II molecules, as described in 1.1.2.3. They produce immunoglobulins when activated. The DNA of each B cell encodes a unique immunoglobulin antigen-binding site. The B cell displays many copies of a transmembrane receptor, with this exact antigen-binding site - the B-cell receptor - on its cell surface. When activated, the B cell produces immunoglobulins that are released and can tag the antigen for degradation or neutralize it.

T cells cannot produce soluble forms of the TCR but display the TCR on their cell surface. Most T cells carry a TCR that consists of an α- and β-chain. Some T cells carry a special TCR which is made up of a γ- and δ-chain and is therefore called γ:δ TCR. These cells do not recognize antigenic peptides bound by HLA molecules. Their role is not yet completely understood. There is some evidence that γ:δ T cells can recognize lipids and other non-peptide ligands that are common to pathogens without the help of the classical HLA molecules. The function of this T cell subset is in between innate and adaptive immunity. (19)

T cells that express the classical α:β TCR have different effector functions. They are grouped into three categories: cytotoxic T lymphocytes (CTLs), T-helper cells (T_H) and regulatory T cells (T_{regs}). CTLs express the co-receptor CD8 on their cell surface. CD8 amplifies the sensitivity of the TCR for HLA class I-peptide complexes on somatic cells about 100-fold by binding to a conserved region of the HLA molecule. (10) CTLs detect cells that present peptides of an intracellular pathogen or neoplastic changes on HLA class I molecules and induce apoptosis in those cells. Apoptosis can be induced by receptor-mediated pathways or by the release of perforin and granzyme B from cytotoxic granules from the CTLs cytoplasm. They are also known to secrete the cytokines IFN-γ and tumor necrosis factor (TNF). IFN-γ induces an increase of HLA class I production and expression on surrounding cells and the production of factors that are essential for peptide processing and loading onto HLA molecules. It also attracts macrophages to the site of infection and activates them to serve as APCs and effector cells. The secretion of

TNF can lead to apoptosis of infected cells by binding to TNFR-1 and the activation of macrophages.

T_H and T_{regs} express CD4, which sensitizes the TCR for HLA class II-peptide complexes. There are four different types of T_H cells: T_H1, T_H2, T_H17, and T_{FH}. T_H1 cells are essential for the clearance of pathogens that can evade or survive in the endosomal compartment of macrophages. If an effector T_H1 cell recognizes a peptide on an HLA class II molecule, it secretes IFN-γ, which signals the macrophage to kill its ingested pathogen. The T_H2 cells play a crucial role in the elimination of extracellular parasites. After activation, T_H2 cells secrete the cytokines interleukin (IL) -4, IL-5, and IL-13, which lead to an activation of eosinophils and mast cells, and to class switching of B cells to produce IgE. The eradication of extracellular bacteria and fungi acquires the help of T_H17 cells. The cytokines IL-17 and IL-22, which they produce on activation, lead to high production of granulocyte-colony stimulating factor (G-CSF) in stromal cells and increased secretion of antimicrobial peptides on the epithelial barrier, respectively. G-CSF enhances the proliferation of hematopoietic precursor cells and activates neutrophil granulocytes at the site of infection. The activation of T_H17 cells promotes an isotype switch in B cells, which leads to increased production of opsonizing IgG antibodies. T_{FH} cells are crucial during any type of infection. They play a unique role in the activation of naïve B cells, the support of isotype switching and affinity maturation in activated B cells and plasma cells. The main cytokine they produce is IL-21. (19)

Whereas all the described effector T cells help to clear pathogens from the body, T_{regs} play a negative modulatory role in the adaptive immune response. They suppress T cell responses by producing immunosuppressive agents such as IL-10 and are essential for the prevention of autoimmune reactions.

1.1.2.5 The α:β T-cell receptor

The TCR is a highly specific receptor for antigen recognition. It is established by the T cell during its development in the thymus. Every T cell carries many copies of the TCR with a unique antigen-binding site on its cell surface. The TCR consists of an α- and a β-chain. Each chain contains a variable (V) and a constant (C) immunoglobulin-like domain. A disulfide bond links the two chains. The amino terminal V regions of the α- and the β-chain form the unique antigen-recognition site. The carboxy-terminal C region

consists of a short cytosolic tail, some transmembrane polypeptides which anchor the TCR in the cell membrane, and an extracellular part. (Figure 3) The V region of the α- and β-chain each contains three loops with hypervariable regions called complementary-determining regions (CDRs). The high diversity of the TCR is created by somatic recombination of TCR gene segments.

The V_α region of the TCR is encoded by the joint of one V_α to a J_α segment, of which there are 70 and 61, respectively. There is only one gene segment for the C region of the α-chain. The V_β region is formed by a V(D)J recombination. There are 52 V_β genes which are joined with either one $D_{\beta1}$ gene and one of six $J_{\beta1}$ linked to a $C_{\beta1}$ gene segment or one $D_{\beta2}$ gene segment linked to one of seven $J_{\beta2}$ gene segments and a $C_{\beta2}$ gene segment. The $C_{\beta1}$ and $C_{\beta2}$ gene segments are almost homologous and do not differ in their function. (33) The number of possible combinations is referred to as combinational diversity.

The joint region is further modified by the insertion of P- and N-nucleotides. P-nucleotides are palindromic sequences, generated on both ends, which are later joined. Besides, N-nucleotides are added by the terminal deoxynucleotidyl transferase. N-nucleotides are non-template-encoded and added randomly. After the addition of N-nucleotides, the DNA single strands are joined at points where some of the antisense N-nucleotides fit together. N-nucleotides that do not match are replaced, and the missing nucleotides are filled into gaps. The diversity that is created in the joining region of the V exon is called junctional diversity.

The CDR1 and CDR2 of the V regions are located within the V gene segment and are less variable than the CDR3 region. The CDR3 loop is encoded within the end of the V gene segment, the joining segment of the V(D)J joint, and the beginning of the J gene segment. This leads to high diversity in this region. The CDR3 loop of the α- and β-chain forms most of the antigen-binding site, and the high variability in this region is needed to be able to cover the vast number of possible peptide epitopes, which can be presented by HLA molecules. The CDR1 and CDR2 loops mostly form the contact site to the HLA molecule.

The high diversity in the TCR of developing T cells can also lead to the generation of T cells that could harm the organism. Thus, there is a need for the selection of T cells in the

thymus before the naïve T cells can be released. First, the TCR of CD4 and CD8 double-positive T cells must recognize the HLA molecules of the organism. If the interaction of the TCR of the double-positive T cell with the HLA molecules is too strong or too weak, apoptosis is induced in the T cell. If the interaction of a TCR with either HLA class I or HLA class II molecules is successful, the T cell will receive a survival signal. Furthermore, it will receive a signal to downregulate CD4 expression in case of effective interaction with HLA class I molecules or a signal for downregulation of CD8 expression in case of successful interaction with an HLA class II molecule. This selection is termed positive selection.

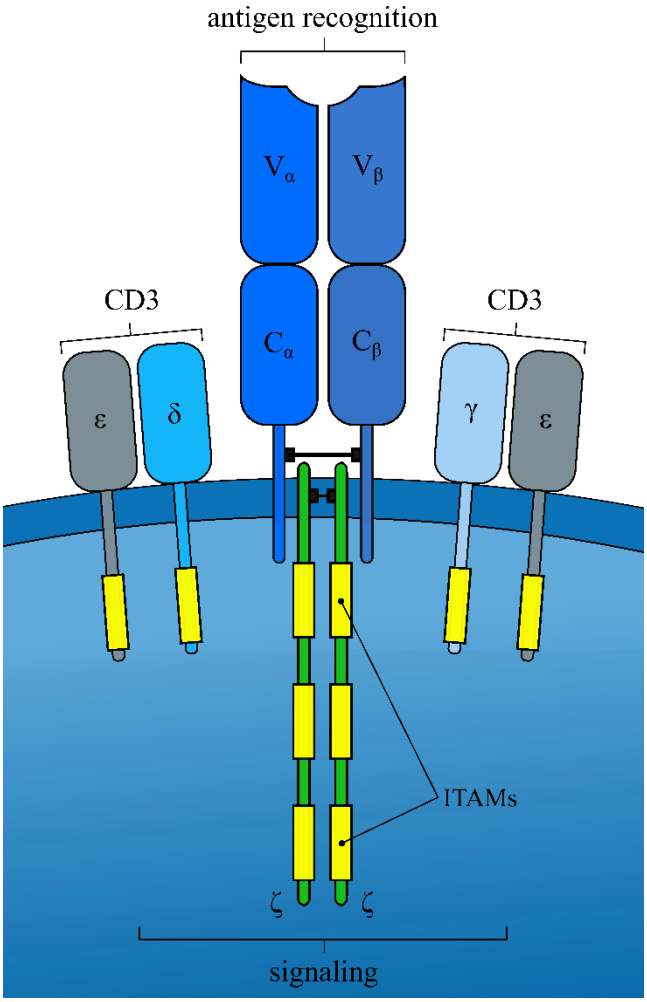


Figure 3: TCR complex, consisting of an α:β TCR and a CD3 complex. The CD3 complex is consisting of two ε-chains, one δ-chain, and one γ-chain, with each chain containing one ITAM, as well as a homodimer of ζ-chains, each containing three ITAMs. Adapted from (19).

After selecting the thymocytes for HLA interaction, the single positive CD4 or CD8 T cells that express a TCR that recognizes self-antigens are eliminated. If the TCR of a single-positive T cells binds to self-antigens presented on HLA molecules, apoptosis is induced. This is a crucial step in the prevention of autoimmune diseases and referred to as negative selection. Nevertheless, some of these cells receive a survival signal to become T_{regs}.

An antigen can only be recognized by a TCR if bound to an HLA molecule on the surface of another cell. This recognition, however, is not enough for the activation of the T cell. As the molecular binding between the TCR and the HLA molecule is not very strong and the

half-time before dissociation often too short for antigen recognition, the CD4 and CD8 molecules are essential for the increase of the time of interaction between the TCR and the HLA-peptide complex. (Figure 1)

Another co-receptor is the invariant CD3 molecule, which is specific for T cells and essential for cell activation after epitope recognition. The CD3-complex is formed by CD3γ, CD3δ, CD3ε, and a disulfide-linked ζ-homodimer. The ζ-chains have a short extracellular domain. The intracellular domain contains three immunoreceptor tyrosine-based activation motifs (ITAMs), which activate intracellular cascades by the phosphorylation of tyrosine residues of the ITAMs when the TCR recognizes an antigen. Each of the CD3γ, CD3δ, and CD3ε chains contain an extracellular Ig-like domain. The intracellular tail of these molecules contains an ITAM as well. The CD3-complex is formed by a CD3ε:CD3γ heterodimer, which is associated with the β-chain of the TCR, as well as an ζ-homodimer which is related to the α-chain of the TCR. (Figure 3)

1.1.2.6 T-cell maturation, activation, and differentiation

The early progenitors of T cells reside in the bone marrow before they migrate to the thymus to develop their TCR and undergo negative and positive selection. Naïve T cells need to be activated before they can contribute to the immune response. (Figure 4) After their development, selection, and release from the thymus, they circulate between the blood, the lymph, and the secondary lymphatic organs, where they constantly screen APCs for antigen recognition to become effector cells. The most potent activators of naïve T cells are DCs. After DC activation by toll-like receptors (TLRs) and other pathogen-recognition receptors, they take up pathogens and migrate into secondary lymphoid tissue to present the antigen to cells of the adaptive immune system. The secondary lymphoid tissue is the location where naïve T cells are primed. T cells are activated when they recognize a peptide presented by an HLA molecule. They then require a co-stimulatory signal, which is mediated by a receptor, present on the surface of activated DCs. If a T cell recognizes a peptide presented on a non-activated DC on which the co-stimulatory receptor is not present, the T cell is driven into apoptosis. This mechanism prevents T cells that recognize self-peptides from triggering an autoimmune reaction. The most studied receptors on DCs, which signal the T cell to survive are B7.1

(CD80) and B7.2 (CD86). These co-stimulatory molecules can bind to CD28 present on naïve T cells. (Figure 4) The third signal required for T-cell differentiation is the activation of cytokine receptors, which are present on the T cell's surface. Cytokines can be either produced by the activated DC, which directly interacts with the T cell or by other cells of the immune system. The T cell subset into which the activated T cell differentiates depends on the surrounding cytokine milieu. Naïve T cells that carry CD8 need to recognize a peptide, which is presented by an HLA class I molecule. As somatic cells do not have co-stimulatory molecules on their cell surface, they cannot activate naïve T cells. Due to the cell tropism of most pathogens, some do not infect DCs. Therefore, it is essential that DCs also present peptides that were taken up by phagocytosis on HLA class I molecules to be able to activate naïve $CD8^+$ T cells in the process of cross-presentation. As there is only one group of $CD8^+$ effector T cells, the cytokine milieu in which they are activated is not of such importance as it is the case for $CD4^+$ T cells. Nevertheless, they need the presence of IL-2 to confirm an infection and trigger their proliferation and differentiation into CTLs.

The differentiation of $CD4^+$ T cells into effector cells depends on the presence of specific cytokines in their surroundings. The presence of IFN-γ and IL-12 leads to the differentiation of a naïve $CD4^+$ T cell (T_H0) into T_H1 effector cells. In the case of the presence of IL-4, the T_H0 cell will become an effector T_H2 cell. A differentiation into T_H17 cells is triggered when the predominant cytokines are IL-6 and IL-23. The presence of IL-6 is also vital for the development of T_{FH} cells. The T_H0 is driven into differentiation to T_{regs} in the presence of the transforming growth factor-β but the absence of IL-6. The presence or absence of IL-6 determines if the T_H0 cell differentiates into an immune-stimulatory effector T_H17 cell or an immune-suppressive effector T_{reg}. Each of these effector T cells produces the cytokines, which stimulates its differentiation. This results in a positive feedback loop.

After activation and differentiation, the antigen-specific T cells amplify for four to five days. During this period, they stay in the lymph node. Afterwards, the effector cells are released from the lymph node and attracted to the site of infection by cytokines. They can induce receptor-mediated apoptosis in infected cells on which they recognize an HLA class I-peptide complex, in the case of CTLs. Effector T_H cells assist B cells and

macrophages in producing antibodies or destroying the pathogens contained in their vesicles, respectively. Effector cells do no longer need a co-stimulatory signal when recognizing an epitope presented on an HLA molecule.

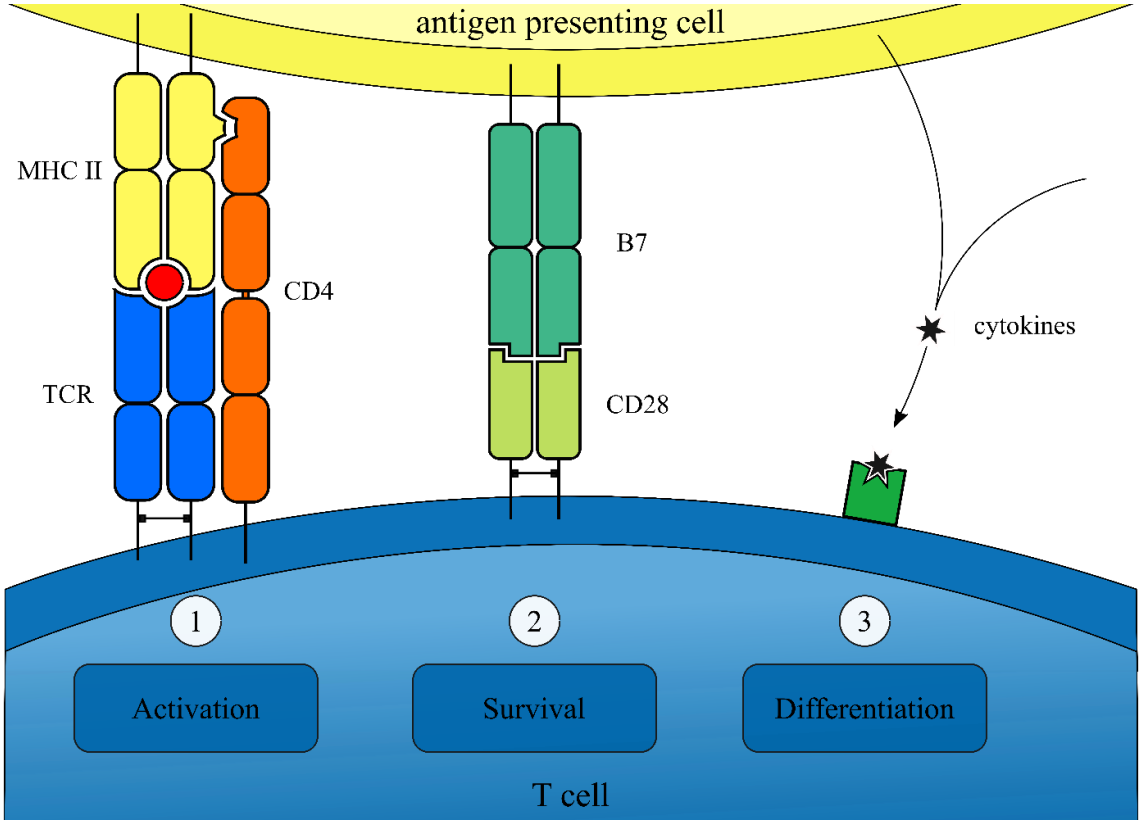


Figure 4: The three signals needed to turn a naïve T cell into an effector T cell. 1. TCR needs to recognize an HLA-peptide complex with the help of CD4 or CD8 to get activated. 2. The T cell needs to get a receptor-mediated survival signal by the APC, which can be mediated by the binding of B7 on the APC's surface to CD28 on the T cell's surface. 3. The T-cell surrounding cytokine milieu stimulates specific cytokine receptors, which leads to the transcription of certain differentiation factors inside the T cell and its differentiation. Adapted from (19).

After the clearance of the pathogen, most effector cells are driven into apoptosis. Some of the effector T cells remain as so-called memory cells. They can mount a faster and more potent immune response to the pathogen in case of reinfection, as they do not need the time-consuming co-stimulatory signals of DCs for reactivation. The presence of these cells is the reason for the effectiveness of active vaccination, besides the production of NABs.

1.2 The human adenovirus

The adenovirus is a non-enveloped, double-stranded DNA virus with an icosahedral capsid. It was named after the tissue from which it was first isolated in 1953: the adenoid tissue. (34)

Five defined genera have been described: Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus, and Ichtadenovirus. The genus of Ichtadenovirus was recently discovered when a fish adenovirus did not fit into any of the four previously defined genera. (35, 36) Until now, there is only one identified Ichtadenovirus serotype.

The HAdV belongs to the genus of the Mastadenovirus in the family of the Adenoviridae. Up to date, there are almost 90 types known to be able to infect humans, categorized into seven species (A to G). (37, 38) Species B is sometimes divided into the species B1 and B2. (39) The characterization of species into serotypes was exclusively done by serum cross-neutralization and hemagglutination up to type 51. (40) The categorization into types is recently performed by more specific criteria such as complete genome sequence analysis. (41) New types are continually emerging. HAdV can infect a broad spectrum of cells. Different serotypes have a specific cell tropism. Adenovirus serotypes which belong to the same species are associated with similar symptoms in the host organism. (42-44)

Table 1: Serotypes in association with type of infection. Adapted from reference (42)

HAdV species	Serotype	Type of infection
A	12,18,31	gastrointestinal, respiratory, urinary
B1	3,7,16,21	keratoconjunctivitis, gastrointestinal, respiratory, urinary
B2	11,14,34,35	gastrointestinal, respiratory, urinary
C	1,2,5,6	respiratory, gastrointestinal including hepatitis, urinary
D	8-10,13,15,17,19,20,22-30,32,33,36-39,42-49,51	keratoconjunctivitis, gastrointestinal
E	4	keratoconjunctivitis, respiratory
F	40,41	gastrointestinal
G	52	gastrointestinal

1.2.1 Structure of the virion

As mentioned in 1.2, the adenovirus is a non-enveloped, double-stranded DNA virus with an icosahedral capsid. The diameter of the capsid is 926 Å without the protruding fiber proteins, which vary in length from 120 Å to 315 Å between different serotypes. (45) The structure of the virion contains a capsid and a core. The capsid is the outer surface of the virus. The main capsid proteins, especially the hexon protein, are preferred targets of the immune system. (46) The capsid is directly exposed to the immune system before the virus enters its host cell, and a high amount of capsid proteins needs to be produced for virus replication.

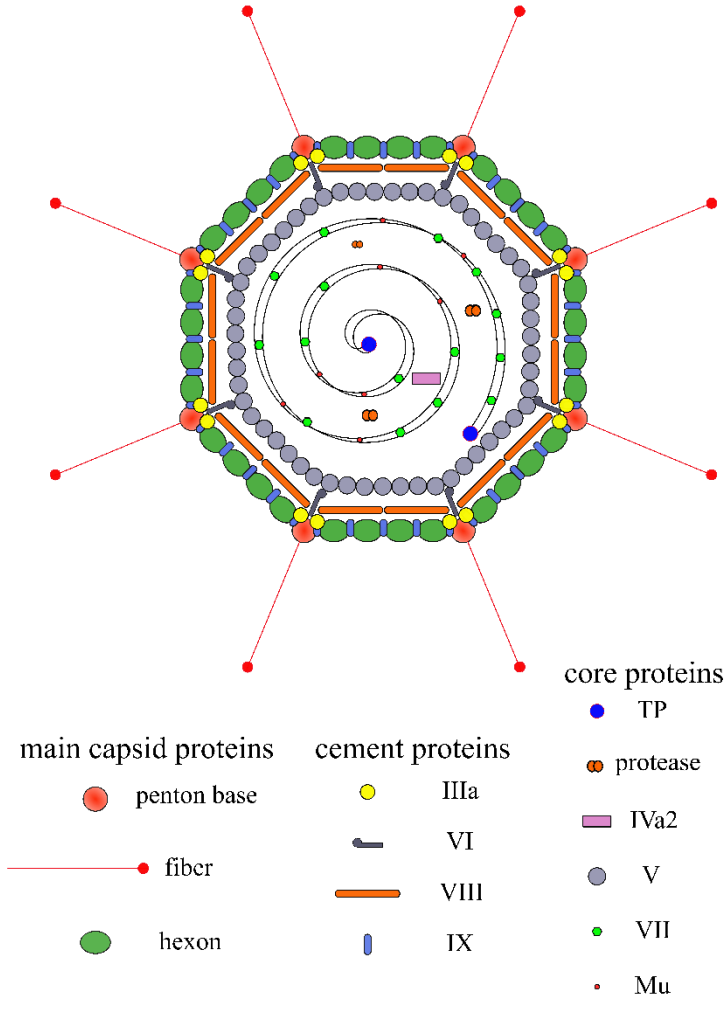


Figure 5: Structure of adenovirus virion. The capsid consists of main capsid proteins (penton base, fiber and hexon) associated with the cement proteins (IIIa, VI, VIII, IX). The core which is located inside the capsid contains the virion DNA, the terminal protein (TP), proteins IVa2, V, VII, Mu and a 23K virion protease.

The main proteins that form the capsid are the hexon protein (protein II), the penton protein (protein III), and the fiber protein (protein IV). A capsid contains 240 hexon capsomeres and 12 penton complexes. One hexon capsomere is a trimer of protein II. Each penton complex consists of a pentamer of protein III and a trimeric fiber protein with a final knob. The penton complex is essential for receptor-mediated uptake by the host cell. Other capsid-associated proteins are the proteins IIIa, VI, VIII, and IX. These

proteins help to stabilize the junctions of the main capsid proteins and are referred to as cement proteins. (45)

The core contains the genome and six more polypeptides: V, VII, X (Mu), IVa2, the terminal protein (TP), and the 23K virion protease. The proteins V, VII, X, TP, and IVa2 are closely associated with the DNA and are essential for DNA condensation. In contrast, the protease is necessary for virion assembly, cleavage of precursor proteins, and virus entry into host cells. (43, 47)

1.2.2 Genome structure and functions

The human adenoviral genome is ranging from 34 to 36-kilobase pairs in length and contains inversed terminal repeats of a length of 36 to more than 200 base pairs. (48, 49) Each of its 5' termini is covalently bound to a TP. (50) The genome encodes over 40 regulatory and structural proteins, which are all transcribed by RNA polymerase II. From those 40 proteins, 12 structural proteins were identified in the final virion. (37, 51) The viral DNA of HAdV also encodes two viral-associated RNAs: VA RNA I and VA RNA II. The adenoviral genes are grouped into early and late genes, depending on their time point of transcription. The reference is, whether they are transcribed before or after DNA replication.

1.2.2.1 Early transcription units

The early genes consist of four cassettes: E1 to E4. The transcript products of the early region model the host cell pathways, enabling viral reproduction, as well as evading the immune response. E1 is often divided into E1A and E1B.

The E1A gene region is transcribed directly after the viral DNA entered the nucleus and referred to as immediate early transcript. The primary function of these proteins is to make the host cell metabolism more receptive to virus replication by driving the host cell into the S-phase of the cell cycle. The p53 level is raised by enhancing its transcription (52) and inhibiting its degradation (53). This leads to the transcription of the other viral gene regions. (54)

E1B encodes two proteins: E1B-19k and E1B-55k. They antagonize some of the apoptotic pathways which are enhanced by E1A. As the E1A transcription products lead to an

increase of p53 concentration, E1B-55k downregulates p53 expression levels by multiple methods: I) it directly binds to the gene locus inhibiting p53 transcription (55), II) E1B-55k induces polyubiquitination of p53 followed by degradation by the proteasome. The downregulation of p53 inhibits apoptosis. (56, 57) The E1B-19k protein also hinders apoptosis. It can inhibit both p53-dependent and p53-independent apoptosis. This is achieved by direct binding of E1B-19k to the pro-apoptotic molecules *Bax* and *Bak*. It can also block the pathways of death-receptor signaling. (58)

The E2 genes are divided into E2A, which encodes the single-stranded-DNA-binding protein (DBP), and E2B, which encodes the precursor TP and Pol. These proteins are essential for viral DNA replication. (59) E2A has an alternative promoter that is activated during the phase of DNA replication when most DBP is needed.

Polypeptides encoded in the E3 gene region can hinder the killing of the infected host cell mediated by CTLs. The adenoviral protein E3-gp19k forms a complex with HLA class I molecules in the ER where it is retained. This inhibits viral epitope presentation to and recognition by CTLs. (16) The receptor internalization and degradation complex (RID) enhances the internalization and degradation of death-receptors, such as Fas and TNF-related apoptosis-inducing ligand (TRAIL) receptors. (60-62) Furthermore, RID also inhibits the downstream pathways of these death receptors. (63) The presence of the E3-14.7k protein makes the cell resistant against TNF-mediated cytolysis. (64)

Another protein encoded within E3 is the adenovirus death protein (ADP). It is synthesized in a low amount in the early stages of infection and a higher amount at later stages. The ADP induces lysis of the host cell, which leads to the release of reproduced viruses. (65, 66)

The E4 gene encodes at least six polypeptides, which are organized in open reading frames (Orfs): E4 Orf1 to E4 Orf6/7. The function of each Orf protein is not yet completely understood. The deletion of the E4 region has a high impact on viral replication, whereas the inhibition of single Orfs does not have mentionable effects. The Orf3 and Orf6 proteins are the best studied. Viruses that lack both Orf3 and Orf6 proteins are not able to reproduce properly. A defect in one of the two proteins can mostly be

compensated by the other. They both enhance the gene expression of late viral mRNAs and are essential for efficient viral DNA synthesis. (67, 68)

The genome of each adenovirus encodes at least one VA RNA. The HAdV C serotypes encode two of them. The VA RNAs are transcribed by RNA polymerase III. Their function is to inhibit the innate immune response. The best-known mechanism by which this is achieved is the downregulation of the RNA-dependent protein kinase (PKR) by VA RNA I. PKR is induced by IFN and can sense viral genomic contamination. The interaction of VA RNA I with PKR prevents its autophosphorylation, which is required for its activation. Activated PKR hinders the initiation of translation and thus the protein biosynthesis of viral proteins. (69)

1.2.2.2 Delayed early and late transcription units

The gene IX is transcribed in the intermediate phase of infection. It encodes the protein IX, which is translated from a non-spliced mRNA. It functions as a transcription factor, especially for the major late promoter (MLP), besides being a structural protein associated with the hexon protein. (70)

Protein IVa2 is a transcription factor that is transcribed from the IVa2 gene during the intermediate phase of infection. It raises the activity of the MLP and thereby the transcription of late transcription units. (71) IVa2 is also associated with virion assembly and encapsulation of the viral genome. (72) Furthermore, it has non-specific DNA binding properties. (73)

The activity of the MLP is increased during the late phase of infection. It leads to the transcription of the late units. These contain the information for most structural proteins and proteins, essential for virion assembly. The late transcription genes can be grouped into five units (L1-L5). L1 encodes the 55k protein and precursor protein IIIa (pIIIa). The transcription products of L2 are the penton protein, precursor protein VII (pVII), protein V and precursor protein X (pX). L3 contains the genomic information for the precursor protein VI (pVI), the hexon protein, and the virion protease. L4 encodes the 100k protein, the 22k protein, the 33k protein, and the precursor protein VIII (pVIII). L5 only encodes the fiber protein. The precursor proteins need to be cleaved by the viral protease to be functional. (74) Except for protein IX, the primary transcript of any given adenoviral polypeptide is spliced at least once during the synthesis of its final mRNA. (75)

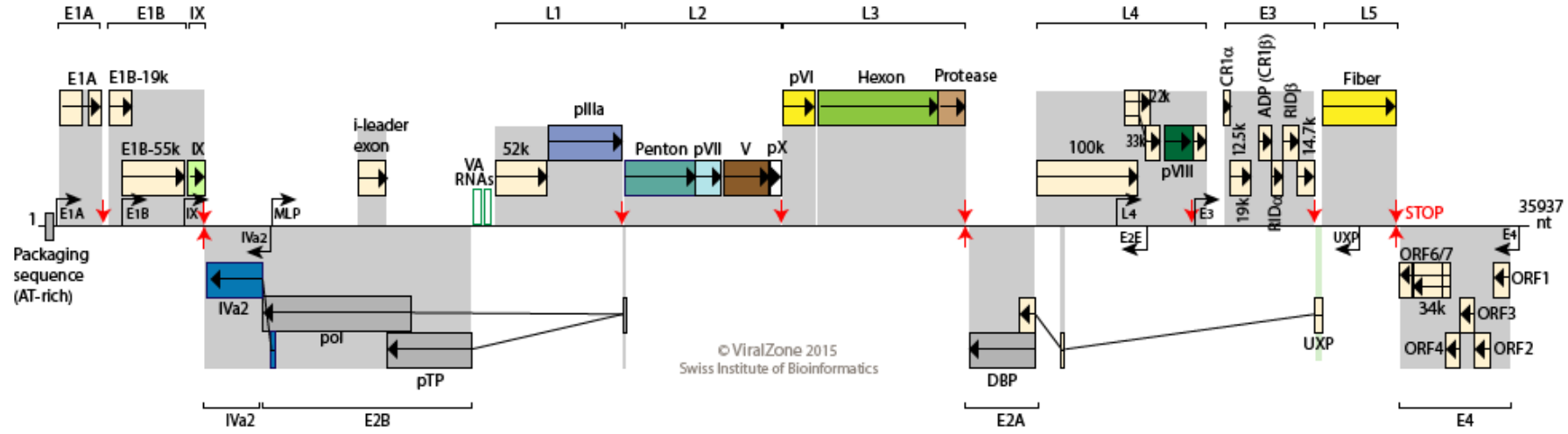


Figure 6: Genome of HAdV C serotype 2. Gene regions with encoding proteins. The p in front of polypeptides means that a precursor protein, which needs to be cleaved for functionality, is synthesized. From (76).

1.2.3 Viral entry into host cells and gene delivery

The fiber knob protein of most HAdV serotypes binds to the coxsackie adenovirus receptor on the host cell. (77, 78) Most fiber proteins of the B genera bind to CD46 except for the serotypes 3 and 7. (79, 80) Serotype 3 attaches to the receptors CD80 and CD86. (81) Some of the HAdV species D bind to sialic acid residues on the target cell. (82) The receptors to which the fiber knob can bind determines the cell tropism of the serotype. (83) The fiber protein always mediates the attachment to the host cell. A further interaction necessary for viral entry occurs between an arginine, glycine, aspartic acid sequence in a loop of the penton base with integrins on the host cell membrane. The interaction with α_v-integrins facilitates the uptake of the virion into an endosomal compartment. (84)

The entry process into the host cell differs between serotypes. Due to the focus of this work on HAdV2, the entry mechanism of this serotype is discussed in more detail. The virus, except for the fiber, which dissociates from the capsid, is taken up into the endosomal compartment after the arginine, glycine, aspartic acid sequence motif of the penton base binds α_v-integrins. (85) The next dissociation step is the loss of the penton base in the endosome. This process correlates with the time of evasion from the endosomal compartment into the cytosol and is mediated by the interaction of the penton base with α_v-integrins. Only 5% of the virions which undergo receptor-mediated endocytosis are destroyed in lysosomes, highlighting the adenoviral efficiency of endosomal evasion. (86) At this time point, the 23k protease, which is needed for activation of precursor proteins and essential for virus reassembly, is reactivated. The reactivation of the 23k protease leads to the degradation of protein VI. Protein VI is a capsid-stabilizing protein. Its degradation is an essential step for the destabilization and further disassembly of the capsid. (47)

The proteins IIIa, VIII, and XI are detached from the capsid (87) during the microtubule-dependent transport of the partly dissociated viral particles (88) through the cytoplasm to the nuclear membrane.

The last step of virion disassembly takes place at the nuclear membrane, where the hexon proteins dissociate after the virus particle has docked to the nuclear pore complex (NPC). The NPC enables the transports of viral DNA and protein VII into the nucleus. (89)

1.2.4 Immune response to human adenovirus infection

1.2.4.1 Innate immune defense

First, the HAdV must overcome the epithelial barriers to be able to infect the organism. These barriers have mechanical ways to protect themselves. Examples are the mucociliary clearance or the acceleration of air when coughing. The mucus layer on different epithelia contains antimicrobial molecules, such as defensins and cathelicidins, which reduce the infectivity of HAdV (90) and are upregulated in the presence of HAdV to mediate chemotaxis (91). The mucus also contains molecules that bind to sialic acid residues and thereby compete for host cell-attachment with adenoviral fiber proteins.

Epithelial cells are the primary site for HAdV infection and replication. They produce and release antimicrobial peptides and cytokines when infected, inducing local inflammation, and attracting cells of the immune system to the site of infection. In infected epithelial cells, the expression of intercellular adhesion molecule (ICAM) -1 is elevated to facilitate the CD18-dependent attachment of neutrophils (92).

Phagocytotic cells of the innate immune system play a pivotal role in the initiation of an immune response by the secretion of IFN-γ, TNF, and IL-6. They can also directly eliminate HAdV by phagocytosis. Macrophages engulf and eliminate viral particles upon a potential viral entry and thereby prevent the infection of epithelial cells. They secrete different cytokines shortly after engulfing viral particles to stimulate other immune cells. (5) They are also able to emit the antimicrobial molecules mentioned earlier. The activation of macrophages is most likely triggered by the presence of capsid proteins but can also be mediated by viral DNA when sensed by TLR-9 (93). DCs show less phagocytotic activity than macrophages, but a higher efficiency in antigen-presentation, which makes them crucial for the initiation of an adaptive immune response. Furthermore, DCs also secrete cytokines in the early phase of an adenoviral infection (94). HAdV capsid proteins are an essential trigger for DC activation, comparable to the activation of macrophages (95). The activation of TLRs by viral DNA also leads to DC activation (93). Neutrophils need to be attracted to the site of infection by cytokines, such as IL-8 produced by macrophages and endothelial cells. After activation, neutrophils produce IL-8 themselves, thereby amplifying the immune response. Neutrophils internalize HAdV that is opsonized by complement factors or bound by IgG. (96) Unfortunately, IL-8 may facilitate the entry of some HAdV serotypes into epithelial cells by inducing the

expression of receptors on the cell's surface (97). Another group of lytic leukocytes that are involved in the clearance of an adenoviral infection are NK cells. (98)

The main cytokines involved in HAdV infections are IL-1, IL-6, IL-8, IL-12, interferons, and TNF. IL-1 is important during the early inflammation response, in the presence of HAdV. (99) It contributes to the production of IL-8 (100) and enhances the expression of ICAM-1 on endothelial cells at the site of infection (101). IL-6 levels increase early during infection compared to other acute-phase proteins such as CRP. (102) IL-6 plasma-concentrations correlate with the severity of HAdV infections. This effect is predominant in HAdV infections compared to influenza and respiratory-syncytial-virus infections. (103) The IL-8 serum-concentration of pediatric patients with HAdV pneumonia correlates with clinical outcomes (104). IL-12 is an essential link between the innate and the adaptive immune system. Its presence leads to the differentiation of T_H0 cells to T_H1 cells and the induction of IFN-γ production in T and NK cells (105). IFN-γ, a type II IFN, increases the expression of HLA-peptide complexes. Type I IFN (IFN-α and IFN-β) are secreted by virus-infected cells. The secretion can be mediated by both TLR-dependent and TLR-independent pathways, depending on the type of infected cells. The decreased immune response in type I IFN-receptor knockout mice demonstrated the importance of type I IFN in the combat of adenoviral infections. (106)

1.2.4.2 Adaptive immune response

APCs and cytokines initiate the adaptive immune response. Macrophages and DCs can activate naïve lymphocytes, with DCs being more efficient. When DCs recognize pathogen-associated molecular patterns (PAMPs), transcription factors, such as NF-κB, are activated. In the case of HAdV, the capsid proteins and the viral DNA are essential PAMPs. NF-κB is the best characterized inflammatory transcription factor. The activation of NF-κB leads to the transcription of inflammatory molecules, which trigger the maturation of DCs. Phagocytosis, peptide-processing, and -presentation on both HLA class I and HLA class II molecules are increased during maturation. Mature DCs migrate to lymph nodes where they are scrutinized by T and B cells. Activation of T or B cells occurs when they recognize the epitope presented by a DC (see chapter 1.1.2.6). After activation $CD8^+$ T cells differentiate to $CD8^+$ CTLs. CTLs leave the lymph node and are attracted to the site of infection, where they lyse infected cells. During HAdV infection, the predominant $CD4^+$ T cells are T_H1 cells, which help macrophages to clear the

pathogens present in their endosomal compartment. (107) The highly cross-reactive T cells (see chapter 1.2.7) play a crucial role in clearing HAdV infections, as can be seen in T-cell-depleted patients in which those infections are associated with high morbidity and mortality (see chapter 1.2.6). The activation of B cells with T_H1 help leads to an Ig class switch and the production of NABs. NABs are efficient in the viral elimination in case of reinfection with the same serotype but are not very cross-reactive. (108) They preferably target the hyper-variable loops on the outer surface of the hexon protein or the fiber knob. (109, 110)

After infection, some of the effector T and B cells become memory cells. These cells can mount a fast and strong response to HAdV in case of reinfection, without the need for reactivation by APCs. A difference between NAB and memory T cells regarding the elimination of pathogens is the high cross-reactivity of memory T cells.

1.2.5 Epidemiology

Human adenoviral infections belong to the most frequent infections in humans. However, there is no necessity for serology during infection, as they cause a variety of symptoms that cannot always be distinguished from other self-limited viral infections. Furthermore, there would be no consequence drawn from positive testing, as only symptomatic treatment is available and necessary. The only HAdV infection in healthy individuals that requires further investigations and must be, namely, reported in Germany is endemic keratoconjunctivitis, as it is extremely contagious. (111)

The spread of adenovirus can occur via fecal-oral or aerosol droplet transmission. (112-114)

Adenoviral infections are endemic in our pediatric population. They are a common cause of upper and lower respiratory tract infections, especially in children from six months to five years of age. (115-117) Some studies state that HAdV causes 5-10 % of respiratory infections in children. (117-119) Although HAdV species C does not bare many serotypes, they are the cause of most infections, with serotype HAdV2 being most frequent. (120-122) *Flomberg et al.* could detect a proliferative response to HAdV2 antigen mediated by $CD4^+$ T cells in the peripheral blood mononucleotic cell (PBMC)

cultures of 29 out of 30 healthy individuals (107). A study by *Sukdolak et al.* identified HAdV-specific antibodies in 196 of 204 healthy individuals (96%) (123). Otherwise, healthy patients infected with non-C serotypes often show more severe symptoms than when infected with C serotypes (121). This can explain the higher prevalence of other serotypes than C in the hospital setting compared to the general population. Due to the limited success of adenovirus as a vector, studies analyzing the prevalence of NABs against certain adenovirus strains were performed. Most studies screened the sera for the presence of antibodies against HAdV2 and HAdV5 serotypes, as they are preferred gene vectors. In a study of a pediatric population in northeast China, anti-HAdV2 and anti-HAdV5 antibodies were present in 59.6% and 43.3% of the tested children, respectively. (124) Anti-HAdV5 antibodies could be identified in 114 out of 114 sera samples from healthy adults in India. (125) An international study showed that 85.2% of the participants had anti-HAdV5 antibodies (126). Another observation confirmed that over 85% of the adult population had been exposed to HAdV C. (122)

Besides children, military recruits are also at risk for adenoviral infections. The most common serotypes to affect military recruits were HAdV4, HAdV7, and HAdV14. (127, 128) The symptoms can vary from fever, cough, sore throat, and chills in mild cases, to pneumonia and acute respiratory disease (ARD) with fatal outcomes. (129-131) Because of the high morbidity of these serotypes in military recruits, a live, enteric-coated vaccine against HAdV4 and HAdV7 was established, which dramatically reduced the occurrence of HAdV4 and HAdV7 associated ARD in this cohort. (132, 133) In 1999, the vaccine was stopped, which led to a new rise in cases of ARD in military recruits. (130) It was reintroduced in 2011 with great success. (134, 135)

After a primary infection, HAdV can persist in human cells without causing symptoms. HAdV preferably persists in T lymphocytes. (136, 137) *Garnett at al.* could detect DNA from HAdV subgroup C in 79% of the tonsils from 35 individuals, removed during routine tonsillectomies. Only 6% of the samples tested directly after removal contained the infectious virus. Weeks to months after explant, the number of infectious viral particles in the same tissue increased. (136) They later demonstrated that latent infections could be reactivated in case of activation of the infected host cells. (138) Adenoviral DNA was also isolated from the gut, indicating another location for persistent HAdV infection.

Viral DNA was predominantly found in lymphocytes. (139) One mechanism by which lytic viral replication is inhibited and persistent infection can be induced is by mimicking of IFN pathways, which block the transcription of the immediate-early gene E1A. (140)

1.2.6 Adenoviral infections in immunocompromised patients

Most viral infections in immunocompetent individuals are usually self-limited and are effectively controlled by our immune system. In the state of acquired immunodeficiency after solid organ transplantation, stem cell transplantation, or symptomatic HIV infection, viral infections can lead to high viremia and solid organ infection with high morbidity and mortality. (141-144) The most common viral infections affecting immunocompromised patients are CMV, HAdV, and Epstein-Barr virus (EBV). (145)

Adenoviral infections are predominant in children and are dangerous to pediatric patients in the early period after stem cell transplantation. (144) *Baldwin et al.* showed that 21% of pediatric patients were HAdV positive following hematopoietic stem cell transplantation (HSCT), compared to 9% of adult patients after HSCT. (146) Allogeneic HSCT recipients have a higher risk of adenoviral infection than autologous HSCT recipients. (147) The source of infection can be the patient's environment as well as a reactivation of a persistent infection, which makes it very difficult to prevent (137). The HAdV C species is the most prevalent to harm patients with a hampered immune system (148). Only a symptomatic treatment of adenoviral infections is currently available. In some studies, the intravenous infusion of antiviral agents such as ganciclovir or cidofovir seemed to be a promising approach after early detection of HAdV in immunocompromised patients. The therapy could lead to the clearance of viremia, even in patients defined as high risk. (149-152) Unfortunately, these drugs were shown to be nephrotoxic and could lead to kidney failure. (153, 154)

The outcome of patients infected with HAdV after HSCT strongly correlates with their T-cell immunity. Most infections after HSCT occur in the first 100 days after transplantation when the immune system is not yet reconstituted. (146) *Feuchtinger et al.* saw that patients who have reconstituted their T-cell count, but lack adenovirus-specific T cells succumb to the disease. In contrast, patients with adenovirus-specific T cells have significantly better chances of clearing the infection. (155) These findings indicate how

important virus-specific lymphocytes are to control viral infections. The administration of live vaccines is not indicated for immunocompromised patients. Thus, the live vaccine against the HAdV serotypes 4 and 7 is neither a preventional nor a treatment option in patients with a weakened immune system.

A promising approach in the therapy of adenoviral infections in immunocompromised patients is the adoptive transfer of virus-specific T cells. (156) *Feucht et al.* demonstrated that the infusion of antigen-specific T cells did not show an increase of graft-versus-host disease, but a significant reduction of adenoviral-associated death in patients who responded to the therapy. (157) *Feuchtinger et al.* reported one case of graft-versus-host-disease following the adoptive transfer of virus-specific $CD4^+$ T cells and $CD8^+$ T cells in nine patients. Five of six evaluable patients could clear their HAdV load. (158)

1.2.7 Homology of - and immune cross-reactivity between different human adenovirus serotypes

Especially the central part of the adenovirus genome contains genus-common genes. Genus-specific genes are preferably located at the terminal regions of the genome, referred to as E1 and E4 in HAdV. Comparative analysis showed that 16 genus-common genes are present in all adenoviruses. Their gene products are mainly responsible for DNA replication, DNA encapsulation, or structural components of the virion. (48)

Here, the focus will be on the homology of HAdV, as the infection of other adenoviruses is not relevant in humans.

Most homology between serotypes can be seen within the same subgroup (see Table 2). As there is only one serotype currently known for HAdV E and HAdV G, no DNA comparison is possible within those subgroups. The DNA homology within subgroup C is the highest, ranging from 99% to 100%. The homology in subgroup A is the lowest with 48% to 69%. In contrast, the DNA homology of serotypes from different subgroups is less than 20%. (98)

Nevertheless, this cross-subgroup homology is enough for cells of the adaptive immune

Table 2: DNA homology within subgroups (115)

HAdV subgroup	DNA homology [%]
A	48-69
B1	89-94
B2	
C	99-100
D	94-99
E	-
F	62-69
G	-

system to be highly cross-reactive. The adaptive immune response against HAdV5 from subgroup C was shown to lead to the activation of subgroup-cross-reactive CTLs. *In vitro* generated HAdV5 specific CTLs were able to lyse fibroblasts infected with HAdV from subgroups A, B, D, and E. The blocking of HLA class I molecules significantly reduced autologous cell lysis.

In contrast, the HLA class II block led to a decreased reduction of cell lysis. (159) Furthermore, the PBMCs of individuals who were seronegative for the uncommon serotype HAdV35 had a specific proliferative $CD4^+$ response after the exposure to HAdV35 antigens, which indicates a cross-reactivity from an earlier HAdV infection with another serotype. (107) *Heemskerk et al.* demonstrated that out of 11 HAdV5 specific $CD4^+$ T cell clones, two were only cross-reactive to cells infected with other HAdV C serotypes, whereas four clones were as well able to lyse cells infected with HAdV B serotypes. Finally, five clones could lyse cells infected with HAdV A, HAdV B, and HAdV C serotypes. Furthermore, HLA-DR and HLA-DP are essential for adenoviral antigen presentation and initiation of an immune response to adenovirus. (160) Other groups showed that both $CD4^+$ and $CD8^+$ cells, which target conserved as well as variable hexon regions, are highly cross-reactive. (161, 162)

The capsid proteins, especially the hexon protein, are stated as preferred targets of the immune system. (163, 164) The homology in the hexon proteins of HAdV serotypes is approximately 80%. (165) In the hexon protein, serotype-specific epitopes are located on the surface of the hexon. In contrast, most conserved regions, which are essential for the formation of the capsid, are located on the inner surface. (165, 166) The differing antigen location explains why antibodies against specific serotypes are less cross-reactive compared to the cellular immune response. T-cell epitopes that are derived from the

conserved regions of the hexon proteins on the inner surface of the capsid can be presented on HLA molecules and recognized by cells of the adaptive immune system (161). In contrast, the antibodies mostly target the more variable outer surface of the virus capsid. (109)

1.3 Aims of this thesis

HAdV is a widespread pathogen. The majority of HAdV infections are subclinical in healthy individuals but are associated with high morbidity and mortality in immunocompromised patients (141, 142, 145, 147), primarily pediatric patients following stem-cell transplantation (144, 146). Until today there is no satisfying treatment option. However, the outcome of the patient depends on the presence of adenovirus-specific T cells (155). The adoptive T-cell transfer of adenovirus-specific T cells could reconstitute a long-lasting immune response and positively influence the outcome of patients with low side-effects (156-158). Therefore, viral epitopes with which specific memory T cells can be selected, and stimulated must be identified. The T-cell response to HAdV is not yet completely understood. In previous work, the search for adenoviral epitopes was mostly focused on conserved regions of the hexon protein to increase the probability of the presence of epitope-specific T cells in the peripheral blood and to take advantage of adenovirus-specific T-cell cross-reactivity. (163, 167-170) The identification of viral epitopes facilitates the selection of virus-specific T cells. The presence of epitope-specific memory T cells is restricted by the individual HLA-genotype and depends on the previous infection with the pathogen. Consequently, stimulating T-cell epitopes have to be selected for each patient. This process is time and cost-intensive.

This work aims to create an HLA class I cocktail consisting of already identified HLA class I epitopes from the HAdV subgroup C. This cocktail should elicit an immune response in as many as possible randomly selected PBMC cultures. As subgroup C infections are most prevalent, all tested peptides are derived from proteins of one of the subgroup C serotypes. Compared to HLA class I epitopes, not much research has been done concerning the identification of HLA class II epitopes. The second aim was, therefore, to predict promiscuous HLA class II epitopes from the 46 reviewed HAdV2 proteins from the UniProt database (171), with the help of the prediction tool SYFPEITHI (172). The predicted promiscuous HLA class II epitope candidates and previously identified HLA class II epitopes were synthesized, screened for immunogenicity *via* enzyme-linked immunospot (ELISpot) screening assays, and confirmed as HLA class II epitopes by intracellular cytokine staining (ICS). Finally, we designed a cocktail that should lead to the stimulation of $CD4^+$ T cells in all tested, randomly selected PBMC cultures without prior HLA class II typing nor HAdV serology.

2 Materials and methods

2.1 Materials

2.1.1 Devices

Analytical balance MC1 research RC 210 S	Sartorius, Göttingen, Germany
Cell culture hood Technoflow	Biosciences, Zizers, Switzerland
Centrifuge Labofuge 400	Heraeus Instruments, Waltham, USA
Centrifuge Megafuge 1.0 R	Heraeus Instruments, Waltham, USA
ImmunoSpot® S6 Ultra-V-Analyzer	Cellular Technology, Shaker Heights, USA
Fluorescence-activated cell sorting (FACS) Canto II	Becton Dickinson, Franklin Lakes, USA
Freezer -20° C	Liebherr, Bulle, Switzerland
Freezer -80° C	Sanyo, Moriguchi, Japan
Nalgene® Mr. Frosty freezing container	Sigma-Aldrich, St. Louis, USA
Ice machine AF30	Scotsman, Milan, Italy
Laboratory Microscope DMLS	Leica, Wetzlar, Germany
Multi-channel pipette (200 µl)	Abimed, Langenfeld, Germany
Neubauer improved counting chamber	Marienfeld Superior, Lauda-Königshofen, Germany
One-channel pipettes (1 µl, 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Abimed, Langenfeld, Germany
Peptide Synthesizer ABI433A	Applied Biosystems, Foster City, USA
Peptide Synthesizer Activo-P11	Activotec, Comberton, United Kingdom
Peptide Synthesizer Liberty Blue	CEM, Kamp-Lintfort, Germany
Pipet boy Integra	Biosciences, Zizers, Switzerland
Refrigerator 4°C	Liebherr, Bulle, Switzerland
Vortex MS1 Minishaker	IKA-works, Wimington, USA

Water bath	GFL, Burgwedel, Germany

2.1.2 General material

96-well-plates	Multiscreen® HTS Filter Plates Millipore®
96-well-plates, U-bottom	Corning Incorporated, Corning, USA
6-well-plates	Becton Dickinson, Franklin Lakes, USA
Cryotubes (2 ml)	Greiner Bio One, Frickenhausen, Germany
Disposable pipettes (5 ml, 10 ml, 25 ml)	Becton Dickinson, Franklin Lakes, USA
FACS tubes (5 ml)	Becton Dickinson, Franklin Lakes, USA
Falcon tubes (15 ml, 50ml)	Becton Dickinson, Franklin Lakes, USA
Pipette tips (10 µl, 200 µl, 1000 µl)	Starlab, Ahrensburg, Germany
Reaction vials (0.5 ml, 1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Sterilization filter 0.22 µm	Corning Incorporated, Corning, USA
Steritop filter units 250 ml	Millipore, Billerica, USA
Syringes (2 ml, 5 ml, 10 ml, 20 ml)	B. Braun, Melsungen, Germany

2.1.3 Chemicals

Acetonitrile	Thermo Fisher Scientific, Carlsbad, USA
Aqua-Live-Dead	Thermo Fisher Scientific, Carlsbad, USA
β-mercaptoethanol	Roth, Karlsruhe, Germany
5-bromo-4-chloro-3-indolyl phosphate -nitro blue tetrazolium (BCIP/NBT) tablet	Sigma-Aldrich, St. Louis, USA
Biocoll separating solution	Biochrom, Berlin, Germany

Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, USA
Brefeldin A	Sigma-Aldrich, St. Louis, USA
double distilled water (ddH_2O)	in-house production
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt, Germany
Dulbecco's phosphate buffered saline (PBS) w/o Mg^{2+} and Ca^{2+}	PAA Laboratories, Pasching, Austria
Ethanol	Liquid Production, Flintsbach, Germany
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe, Germany
FACS-Clean	Becton Dickinson, Franklin Lakes, USA
Fetal calf serum (FCS)	PAA Laboratories, Pasching, Austria
Formaldehyde	Sigma-Aldrich, St. Louis, USA
Human plasma (heat-inactivated)	in-house production, pooled
Iscove's Modified Dulbecco's Medium (IMDM)	Lonza, Cologna, Germany
Penicillin/Streptomycin (Pen/Strep)	PAA Laboratories, Pasching, Austria
Phytohaemagglutinin (PHA)	Sigma-Aldrich, St. Louis, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, St. Louis, USA
Saponin	Sigma-Aldrich, St. Louis, USA
Sodium azide	Sigma-Aldrich, St. Louis, USA
Tween 20	Merck, Darmstadt, Germany
Trypan blue	Merck, Darmstadt, Germany

2.1.4 Buffers and solutions

FACS buffer	2% FCS
	2 mM EDTA
	in PBS

Freezing medium	10% DMSO in FCS
PBS BSA	0.5% BSA 2 mM EDTA in PBS, sterile filtered
PBS-EDTA	2 mM EDTA 0.02% sodium azide in PBS
PBS Tween	0.05% Tween 20 in PBS
Permwash buffer	0.5% BSA 0.1% Saponin S-7900 in PBS
T cell medium (TCM)	10% human serum 1% Pen/Strep 0.05% β-ME [0.1 M] 0.05% Gentamycin in IMDM
Thawing medium	1% Pen/Strep 0.05% β-ME [0.1 M] 0.05% Gentamycin 1 x DNAse [3 µg/ml] in IMDM
Trypan blue solution	0.05% trypan blue 0.02% sodium azide in PBS

2.1.5 Enzymes

DNAse I	Roche, Basel, Switzerland
ExtrAvidin Alkalinphosphatase	Sigma-Aldrich, St. Louis, USA

2.1.6 Antibodies

2.1.6.1 Antibodies for ICS

CD8-PerCP	Biolegend, San Diego, USA
CD4-APC-Cy7	Biolegend, San Diego, USA
INFγ-PE	Biolegend, San Diego, USA
TNFα-PacificBlue	Biolegend, San Diego, USA

2.1.6.2 Antibodies for IFN-γ ELISpot:

Anti- IFN-γ 1-D1K [2 μg/ml]	MabTech, Nacka Strand, Sweden
Anti- IFN-γ 7-B6-1 [0.3 μg/ml]	MabTech, Nacka Strand, Sweden

2.1.7 Softwares

FlowJo version 10	Becton Dickinson, Franklin Lakes, USA
ImmunoSpot® software	Cellular Technology, Shaker Heights, USA

2.1.8 Peptides

2.1.8.1 HLA class I peptides

Table 3: Tested HLA class I peptides, all derived from HAdV2 proteins

Sequence	HAdV2 Source Protein (UniProt-ID)	Position	HLA-restriction	Recogni-tion rate [%]	Positive/ tested PBMCs	Publication
LTDLGQNLLY	CAPSH	901-910	A*01	95.7	66/69	(173-175)
STDVASLNY	CAPSP	76-84	A*01	56.3	9/16	(176)
LLDQLIEEV	E1A	19-27	A*02	67.6	50/74	(175)
RLLPGVFTV	DPOL	733-741	A*02	45.8	11/24	(163)
YVLFEVFDVV	CAPSH	968-977	A*02	48.3	14/29	(163)
FLAPKLYAL	DPOL	1074-1082	A*02	45.8	11/24	(163)
ALYLPDKLK	CAPSH	494-502	A*03	53.1	17/32	*in house*
RLMETRGKK	DPOL	1024-1032	A*03	40.5	15/37	*in house*
VIYGPTGCGK	PKG1	168-177	A*03	46.4	13/28	*in house*
TYFSLNNKF	CAPSH	37-45	A*24	72.7	40/55	(177)
EPRSGGIGTL	CAP8	177-186	B*07	62.5	10/16	*in house*
EPTLLYVLF	CAPSH	928-936	B*07	40.0	6/15	(178)
RPKLVPAIL	E1A	206-214	B*07	50.0	8/16	*in house*
VPATGRTLVL	LEAD	26-35	B*07	66.7	2/3	(174)
FRKDVNMVL	CAPSH	601-609	B*08	68.8	11//16	(170, 179)
IEEFVPSVY	CAP8	189-197	B*18	50.0	8/16	*in house*
AEIEGELKCL	DNB2	276-285	B*40	100	8/8	*in house*
FEPPTLHEL	E1A	38-46	B*40	87.5	14/16	*in house*
CEDRASQML	E1B55	350-358	B*40	75.0	12/16	*in house*
PEIHPVVPL	E1A	241-249	B*40	43.8	7/16	*in house*
NEPVSTREL	SF33K	154-163	B*44	87.5	7/8	*in house*
NESHCGVLVEL	SHUT	236-246	B*44	75.0	6/8	*in house*
NEIGVGNNF	CAPSH	466-474	B*44	66.7	16/24	*in house*
QESPATVVF	E1B55	28-36	B*18; B*40; B*44	50.0; 50.0; 75.0	8/16; 8/16; 18/24	*in house*

2.1.8.2 HLA class II peptides

Table 4: Published HLA class II epitopes and previously tested epitopes from earlier non-promiscuous HLA class II predictions

Sequence	Source protein (UniProt-ID)	Position	HLA-restriction	Publication
VDCYINLGARWSLDY	CAPSH_ADE02	530-544	DR1	(162)
TETLTKVKPKTGQEN	CAPSH_ADE05	423-437	DR1	(162)
GTAYNALAPKGAPNP	CAPSH_ADE05	118-132	DR1	(162)
EWNFRKDVNMVLQSS	CAPSH_ADE02	598-612	DR3/DR4	(162)
ATFFPMAHNTASTLE	CAPSH_ADE02	633-647	DR4/DR7	(162)
THDVTTDRSQRLTLR	CAPSH_ADE02	53-67	DR3	(162)
SQWYETEINHAAGRV	CAPSH_ADE05	209-223	DR4	(162)
FKKVAITFDSSVSWP-NH2	CAPSH_ADE02	734-748	DR7	(162)
DEPTLLYVLFEVFDV	CAPSH_ADE02	927-941	DR	(163, 169)
RPSFTPRQAILTLQT	CAP8_ADE02	159-173	Class II	*in house*
TGNMGVLAGQASQLN	CAPSH_ADE02	354-368	DR	(162)
GASIKFDSICLYATF	CAPSH_ADE02	621-635	DR3	(162)
PGSYTYEWNFRKDVN	CAPSH_ADE05	576-590	DR4	(162)
ENGWEKDATEFSDKN	CAPSH_ADE05	436-450	DR4	(162)
GNNFAMEINLNANLW	CAPSH_ADE02	471-485	DR4	(162)
GWAFTRLKTKETPSL	CAPSH_ADE02	693-707	DR4	(162)
TLRFIPVDREDTAYS	CAPSH_ADE02	65-79	DR4	(162)
DFYFHHINSHSSNWW	DPOL_ADE02	247-261	DR	*in house*
NIALYLPDKLKYNPT	CAPSH_ADE02	492-506	DR	*in house*
LFEVFDVVRVHQPHR	CAPSH_ADE02	935-949	DR	*in house*
VNQFYMLGSYRSEAD	DPOL_ADE02	528-542	DR	*in house*
LKSVYGDTDSLFVTE	DPOL_ADE02	1006-1020	DR	*in house*
RAFVSEWSEFLYEED	DPOL_ADE02	982-996	DR	*in house*
YPTYLGILREPLYVY	DPOL_ADE02	670-684	DR	*in house*
CGMYASALTHPMPWG	DPOL_ADE02	687-701	DR	*in house*
FPEWRCVAREYVQLN	DPOL_ADE02	803-817	DR	*in house*
WRFLWGSSQAKLVCR	E1BS_ADE02	28-42	DR	*in house*
MHLWRAVVRHKNRLL	E1BS_ADE02	120-134	DR	*in house*
RCSMINMWPGVLGMD	E1B55_ADE02	217-231	DR	*in house*
CGCFMLVKSVAVIKH	E1B55_ADE02	329-343	DR	*in house*
AELFPELRRILTINE	E1B55_ADE02	80-94	DR	*in house*
GDDFEEAIRVYAKVA	E1B55_ADE02	164-178	DR	*in house*
SVMLAVQEGIDLLTF	E1A_ADE02	69-83	DR	*in house*
PKLVPAILRRPTSPV	E1A-ADE02	207-221	DR	*in house*
PQKFFAIKNLLLLPG	CAPSH_ADE02	579-593	DR	(162)

Table 5: Tested 15mer peptides from promiscuous HLA class II epitope prediction containing more than five core sequences, only TLRFIPVDREDTAYS has previously been published as adenoviral epitope

Sequence	Source protein (UniProt-ID)	Position	HLA-restriction
LNRFVNTYTKGLPLA	PKG1_ADE02	353-367	DR
LATVPSIATTSAPQA	PKG2_ADE02	70-84	DR
ASWFRMVVDGAMFNQ	E434_ADE02	142-156	DR
IHPVVPLCPIKPVAV	E1A_ADE02	243-257	DR
YIMTPDMTEELSVVL	E4RF1_ADE02	79-93	DR
ISPFIKLTSTHSANK	Y172_ADE02	31-45	DR
LLDLINILQSIVVQE	PKG3_ADE02	242-256	DR
KDLLTDFKAFAARFS	UXP_ADE02	48-62	DR
ILGLLALAAVCSAAK	E3GL_ADE02	5-19	DR
LPLLIPLIAAAIGAV	COR10_ADE02	53-67	DR
RGIFCVVKQAKLTYE	E3145_ADE02	46-60	DR
QKLVLMVGEKPITVT	E3145_ADE02	77-91	DR
LDQLIEEVLADNLPP-NH2	E1A_ADE02	20-34	DR
CAVVDALDRAKGEPV	Y137_ADE02	48-62	DR
RQVMDRIMSLTARNP-NH2	CAP3_ADE02	28-42	DR
IGAVPGIASVALQAQ	COR_ADE02	64-78	DR
YLKVMVRDTFALTHT	DPOL_ADE02	491-505	DR
TPKLILSNSLSGSSS	Y215_ADE02	75-89	DR
VRRVLRPGTTVVFTP-NH2	CORE5_ADE02	72-86	DR
MRVIISVGSFVMVPG	E4RF2_ADE02	67-81	DR
LVSYLGILHENRLGQ	SHUT_ADE02	386-400	DR
TLVLAFVKTCAVLAA	LEAD_ADE02	32-46	DR
VTAFRCIIQGHPRGP-NH2	Y215_ADE02	149-163	DR
TLRFIPVDREDTAYS	CAPSH_ADE02	65-79	DR

Table 6: Peptides that were predicted to contain four cores of the top 2%in the promiscuous SYFPEITHI prediction and were categorized as good binders to HLA class II molecules by NetMHCIIpan 2.0, none of the peptides has previously been published as adenoviral epitope

Sequence	Source protein (UniProt-ID)	Position	HLA-restriction
VSKFFHAFPSKLHDK	PKG1_ADE02	292-306	DR
KNRLLLLSSVRPAII	E1BS_ADE02	130-144	DR
TLLYLKYKSRRSFID	E3GL_ADE06	140-154	DR

Table 7: Elongated HLA class I peptide, showing $CD4^+$ T cell stimulation in previously performed ICS assay, the peptide has not previously been published as adenoviral epitope

Sequence	Source protein (UniProt-ID)	Position	HLA-restriction
TFYLNHTFKKVAITF	CAPSH_ADE02	727-741	DR

Table 8: Previously described HLA class II epitopes from EBV and HAdV were used as positive control peptide cocktails in ELISpot assays

Sequence	Source protein (UniProt-ID)	Position	HLA-restriction	Publication
GTAYNALAPKGAPNP	CAPSH_ADE05	117-131	DR1	(162)
TFYLNHTFKK	CAPSH_ADE02	726-735	A2	(168, 174)
RSPTVFYNIPPMPLPPSQL	EBNA2_EBVB9	277-295	DRB3	*in house*
PRPVSRFLGNNSILY	GP350_EBVB9	268-282	DR	*in house*
IAEGLRALLARSHVERTTDE	EBNA1_EBVB9	481-500	-	(180)

Table 9: Negative control peptide for HLA class II immunogenicity-testing

Sequence	Source protein (UniProt-ID)	Position	HLA-restriction	Publication
ETVITVDTKAAGKGK	FLNA_HUMAN	1669-1683	DR3	(180, 181)

2.1.9 Donors

Table 10: HLA-A and HLA-B genotype of donors used for immunogenicity-testing of previously tested HLA class I epitopes

Donor	HLA-A		HLA-B	
2226	A*03	A*31	B*27	B*62
2255	A*30	A*33	B*13	B*51
2256	A*26	A*69	B*18	B*35
2257	A*24	A*28	B*13	B*55
2264	A*02	A*25	B*44	B*62
2269	A*02	A*24	B*44	B*62
2271	A*03	A*68	B*07	B*60
2272	A*02	A*24	B*61	B*62
2273	A*02	A*11	B*44	B*55
2400	A*23	A*24	B*44	B*35
2405	A*24	A*33	B*44	B*65
2407	A*03	A*26	B*27	B*41
2412	A*32	A*32	B*27	B*62
2413	A*02	A*03	B*35	B*62
2414	A*02	A*02	B*07	B*41
2422	A*11	A*23	B*38	B*55
2423	A*02	A*24	B*44	B*55
2424	A*03	A*24	B*35	B*35
2511	A*02	A*24	B*35	B*60
2512	A*11	A*33	B*75	B*57
2515	A*26	A*26	B*38	B*57
2516	A*02	A*25	B*44	B*62
2526	A*11	A*32	B*18	B*44
2527	A*24	A*26	B*49	B*62
2528	A*02	A*29	B*44	B*62
2532	A*02	A*02	B*35	B*62
2533	A*03	A*32	B*44	B*61

Table 11: HLA-A and HLA-B genotype of donors used for immunogenicity-testing of promiscuously-predicted, already published or previously in house tested epitopes from earlier non-promiscuous HLA class II predictions

Donor	HLA-A		HLA-B	
1264	A*01	A*02	B*13	B*08
1283	not typed	not typed	not typed	not typed
1307	A*01	A*30	B*18	B*57
1311	A*01	A*02	B*08	B*60
1315	A*01	A*32	B*27	B*64
1316	A*02	A*02	B*07	B*08
1318	A*01	A*28	B*08	B*51
1328	A*01	A*01	B*55	B*60
1329	A*01	A*02	B*35	B*35

Donor	HLA-A		HLA-B	
1336	A*01	A*02	B*57	B*60
1376	A*01	A*26	B*07	B*08
1386	not typed	not typed	not typed	not typed
1398	not typed	not typed	not typed	not typed
1431	A*02	A*02	B*07	B*07
1445	A*03	A*24	B*07	B*49
1446	A*01	A*02	B*60	B*62
1735	not typed	not typed	not typed	not typed
1745	not typed	not typed	not typed	not typed
1762	not typed	not typed	not typed	not typed
1763	not typed	not typed	not typed	not typed
1764	not typed	not typed	not typed	not typed
1765	not typed	not typed	not typed	not typed
1774	not typed	not typed	not typed	not typed
1778	not typed	not typed	not typed	not typed
1791	not typed	not typed	not typed	not typed
1874	not typed	not typed	not typed	not typed
1877	not typed	not typed	not typed	not typed
1952	A*03	A*28	B*38	B*62
1967	A*03	A*24	B*18	B*27
2074	A*02	A*02	B*41	B*62
2086	A*03	A*33	B*07	B*27
2100	A*02	A*02	B*35	B*44
2136	A*02	A*11	B*35	B*44
2137	A*02	A*02	B*38	B*56
2141	A*23	A*32	B*44	B*61
2159	A*02	A*29	B*27	B*37
2163	A*02	A*03	B*07	B*44
2176	A*01	A*24	B*08	B*62
2226	A*03	A*31	B*27	B*62
2255	A*30	A*33	B*13	B*51
2274	A*02	A*02	B*44	B*44
2275	A*02	A*02	B*44	B*56
2276	A*03	A*11	B*38	B*62
2281	A*02	A*66	B*27	B*41
2284	A*02	A*33	B*14	B*14
2285	A*02	A*31	B*18	B*55
2292	A*02	A*25	B*18	B*35
2297	A*02	A*29	B*44	B*58
2413	A*02	A*03	B*35	B*62
2469	A*02	A*02	B*44	B*62
2479	not typed	not typed	not typed	not typed
2481	A*11	A*32	B*55	B*61
2483	A*03	A*30	B*13	B*62
2484	A*23	A*23	B*49	B*49
2488	A*02	A*31	B*44	B*57
2489	A*02	A*23	B*44	B*58

Donor	HLA-A		HLA-B	
2490	A*02	A*03	B*18	B*27
2492	A*01	A*32	B*08	B*13
2494	A*03	A*24	B*35	B*57
2497	A*02	A*02	B*49	B*57
2500	A*26	A*26	B*58	B*60
2501	A*03	A*03	B*35	B*55
2503	A*24	A*28	B*35	B*44
2506	A*02	A*29	B*07	B*38
2507	A*02	A*02	B*18	B*47
2509	A*02	A*03	B*18	B*55
2510	A*02	A*29	B*44	B*57
2534	A*02	A*02	B*07	B*57
2536	A*02	A*26	B*39	B*44
2537	A*02	A*26	B*35	B*38
2540	A*02	A*02	B*44	B*60
2543	A*24	A*29	B*44	B*63
2550	A*01	A*26	B*38	B*55
2552	A*25	A*28	B*18	B*44
2553	A*03	A*03	B*35	B*55
2561	A*24	A*25	B*61	B*62
2563	A*01	A*26	B*08	B*45
2564	A*24	A*28	B*35	B*62
2567	A*01	A*26	B*62	B*64
2570	A*02	A*25	B*44	B*62
2573	A*01	A*11	B*08	B*14
2574	A*01	A*28	B*08	B*60
2575	A*02	A*29	B*08	B*27
2577	A*01	A*30	B*08	B*61
2580	A*25	A*28	B*14	B*18
2585	A*02	A*32	B*39	B*51
2588	A*02	A*31	B*56	B*62
2590	A*02	A*02	B*18	B*44
2592	A*02	A*23	B*50	B*62
2595	A*03	A*31	B*14	B*27
2599	A*02	A*32	B*27	B*51
2600	A*29	A*33	B*07	B*57
2601	A*02	A*03	B*07	B*08
2603	A*02	A*23	B*13	B*44
2604	A*01	A*01	B*44	B*55
2605	A*01	A*03	B*08	B*38
2606	A*02	A*30	B*13	B*60
2610	A*02	A*32	B*44	B*51
2611	A*02	A*02	B*08	B*60
2612	A*24	A*32	B*56	B*63
2614	A*01	A*31	B*35	B*57
2615	A*02	A*28	B*44	B*44
2616	A*03	A*03	B*35	B*62
2620	A*24	A*24	B*39	B*41

Donor	HLA-A		HLA-B	
2631	A*03	A*26	B*27	B*41
2633	A*02	A*03	B*35	B*35
2634	A*01	A*24	B*35	B*62
2636	A*03	A*31	B*18	B*37
2639	A*01	A*02	B*08	B*60
2640	A*02	A*32	B*08	B*38
2642	A*23	A*25	B*44	B*62
2643	A*23	A*32	B*38	B*44
2644	A*02	A*29	B*07	B*07
2646	A*24	A*26	B*08	B*49
2647	A*02	A*02	B*18	B*62
2648	A*02	A*03	B*35	B*60
2652	A*02	A*03	B*07	B*51
2653	A*02	A*26	B*07	B*38
2654	A*30	A*33	B*63	B*42
2655	A*01	A*25	B*18	B*62
2657	A*02	A*29	B*07	B*44
2658	A*28	A*32	B*44	B*62
2659	A*02	A*02	B*44	B*57
2660	A*02	A*34	B*14	B*45
2661	A*24	A*24	B*18	B*35
2662	A*02	A*28	B*57	B*62
2665	A*02	A*29	B*44	B*62
2666	A*01	A*02	B*62	B*62
2668	A*24	A*26	B*49	B*70
2670	A*02	A*31	B*41	B*44
2671	A*01	A*01	B*37	B*60
2676	A*01	A*31	B*08	B*35
2677	A*01	A*02	B*08	B*18
2679	A*01	A*02	B*08	B*18
2743	not typed	not typed	not typed	not typed

2.2 Methods

2.2.1 Prediction of promiscuous HLA class II epitopes

SYFPEITHI was used to predict possible immunogenic epitopes. It uses an algorithm based on a position-specific scoring matrix. The position-specific scoring matrix is based on an HLA-specific peptide motif, derived from isolated natural HLA-ligands. As each HLA allotype bears a different peptide-binding motif with specific anchor preferences (21), SYFPEITHI scores each aa depending on the frequency of this aa at an anchor position and its chemical and physical properties. The score ranges from +15 if the aa is favorable to the HLA-peptide binding to -3 if the aa is disadvantageous to HLA-peptide binding at this position. Finally, the score for each aa is summed up. The summation results in a peptide-specific score that correlates with the binding-probability of the peptide to the HLA allotype of interest. (172)

The binding of HLA class II molecules to peptides is less specific when compared to HLA class I molecules. Furthermore, HLA class II molecules have an open-ended peptide-binding groove which permits the binding of peptides with certain core regions, but different length variants (22-24). Thus, HLA class II-bound peptides can contain different core sequences and are thereby sometimes not restricted to only one HLA allele product but can bind to several ones (Figure 7). Because of this phenomenon, described as promiscuity, promiscuous peptides can be predicted to bind to different HLA class II allotypes.

HLA class II binding prediction continues to be a challenge as the binding motifs are not yet completely understood, and the binding sites are not as unambiguous as it is the case for HLA class I molecules. (21, 182) HLA class II allele products pose a higher complexity, due to the contribution of both the α- and β-chains to the binding groove. All these permutations need to be considered for the prediction of peptide-binding to HLA class II gene products. These circumstances have made it hard to assign peptide sequences to HLA class II allotypes and vice versa. Although the phenomenon of promiscuous epitopes is well characterized, no satisfying tool for promiscuous epitope prediction has been published to this time point.

Hence, we tried to establish a way of predicting promiscuous HLA class II epitopes with a focus on identifying promiscuous peptides rather than identifying peptides with high binding-probabilities for single HLA class II allotypes.

We first selected the top-2% of the predicted peptides of a protein for each of the HLA class II molecules for which a prediction can be performed with SYFPEITHI: HLA-DRB1*01:01, HLA-DRB1*03:01, HLA-DRB1*04:01, HLA-DRB1*07:01, HLA-DRB1*11:01, and HLA-DRB1*15:01. If multiple peptides had the same score as the lowest-rated peptide of the top-2% rated peptides, all peptides with this score were selected. In the second step, the selected peptides were sorted by their position in the protein. As the peptides are to be 15 aa long and the HLA binding core is presumed to be nine aa long (24, 183), there is a possibility of several HLA binding cores to be present in a 15mer peptide. Under the assumption that the HLA binding core is flanked by three aa on each side, predicted peptides that differ less than seven aa regarding their position in the protein must contain both HLA binding cores in their sequence (Figure 7). We aimed for peptides that contain most cores within the top-2%-selected peptides. This workflow was applied to all 46 HAdV2 proteins from the Swiss-Prot database. (171)

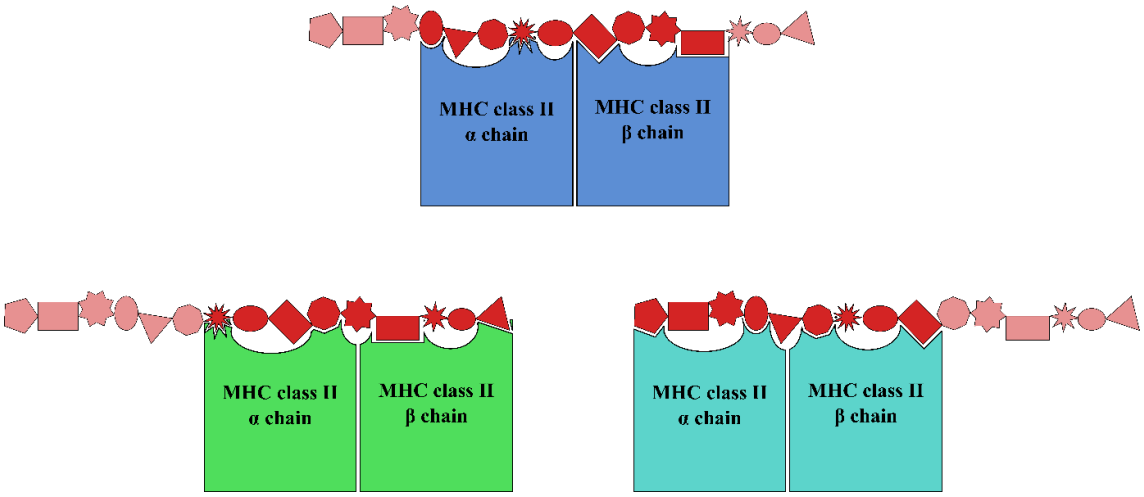


Figure 7: Promiscuous 15mer peptide containing HLA binding cores allowing binding to different HLA class II molecules (represented by different colors). The anchor positions of HLA class II molecules are at positions 1, 4, 6, and 9 of the binding core sequence (184). The aas of the core sequence are colored in dark red, with the flanking residues in light red.

Finally, all potential promiscuous epitopes were sorted by the number of HLA binding cores of the top-2%-selected peptides they were predicted to contain. Peptides containing the same number of predicted HLA-binding cores were sorted by the %-max score, as the

predictable maximum score is different for each HLA allotype (see Table 12). The %-max score sets a relation between the scores a peptide obtained for all the predicted binding cores it contains and the maximum score possible for the respective HLAs. The percentage of the maximum possible score is calculated for all the cores present in the peptide and not for the score this exact 15mer peptide gets for all the HLA allotypes it is predicted to bind.

Table 12: Maximum attainable scores for predictable HLA-DRB1 allotypes with SYFPEITHI and maximum scores reached by HAdV2 peptides

HLA	Maximum attainable score	The maximum computed score for HLA class II peptides
HLA-DRB1*01:01	43	36 (3x)
HLA-DRB1*03:01	40	37 (2x)
HLA-DRB1*04:01	28	28 (71x)
HLA-DRB1*07:01	34	34 (4x)
HLA-DRB1*11:01	38	32 (1x)
HLA-DRB1*15:01	34	34 (18x)

All the promiscuous peptides predicted by SYFPEITHI were also screened with the prediction tool NetMHCIIpan version 2.0, available in October 2016. NetMHCIIpan 2.0 predicts the binding affinity of the HLA-peptide complex, depending on the expected concentration of a peptide in nM, needed to bind 50% of the HLA molecules. This concentration is referred to as nM IC50 value. (185) The lower the nM IC50 value, the higher the predicted affinity of the peptide to the HLA allotype. The peptide is then categorized as a strong binder, weak binder or no binder to a certain HLA class II allotype, if the nM IC50 is below 50 nM, in between 50 nM and 500 nM or above 500 nM, respectively. The aa sequence of each 15mer peptide predicted to contain more than four of the top-2% cores was screened for the binding affinity to the six HLA class II allotypes, for which the promiscuous epitope prediction with SYFPEITHI was performed, by NetMHCIIpan 2.0. (186)

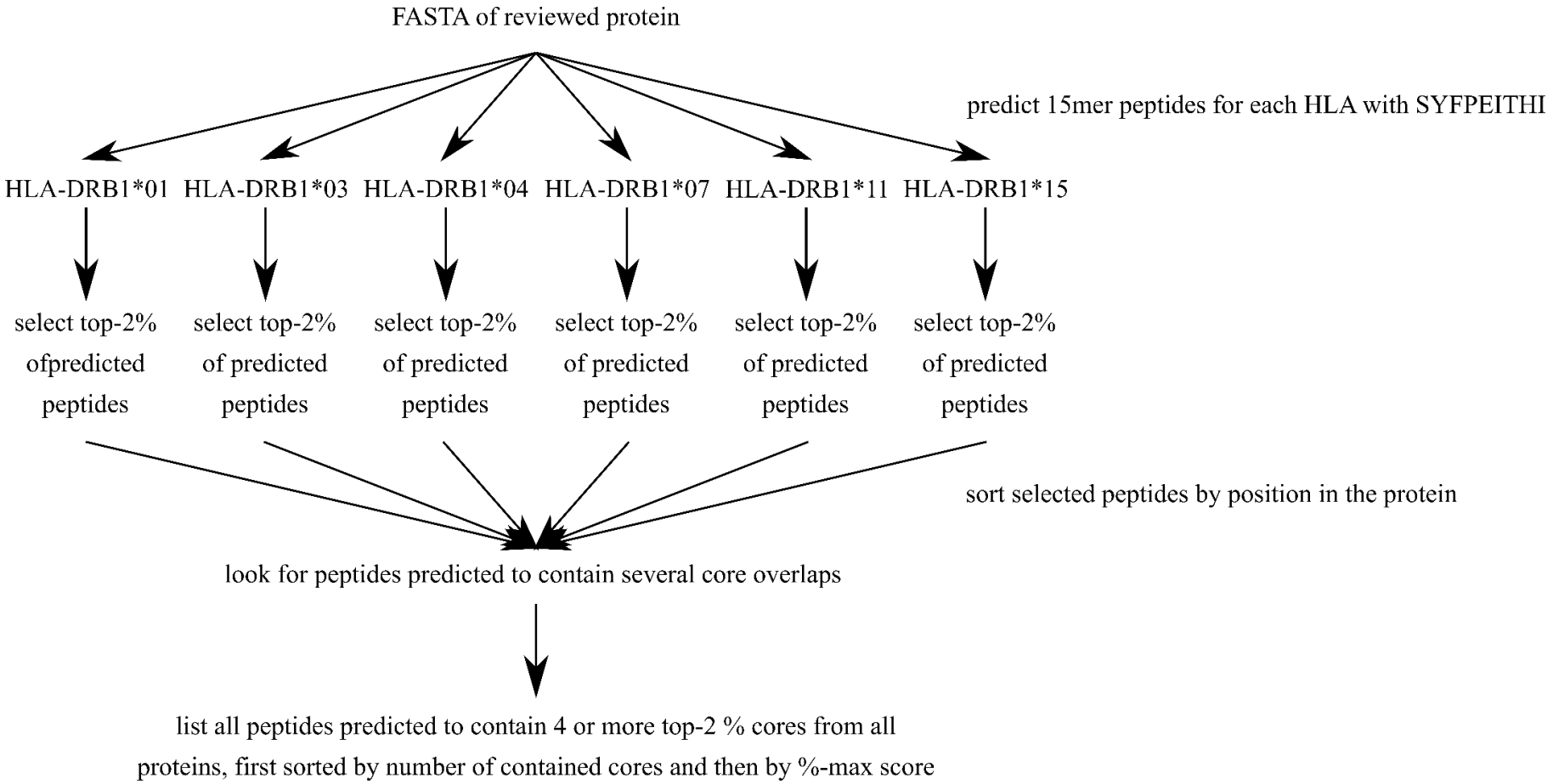


Figure 8: Flowchart for promiscuous epitope prediction shown for one protein. Adapted from (186).

2.2.2 Peptide synthesis

The peptide synthesis was performed on-site by solid-phase Fmoc-synthesis with the peptide synthesizing machines ABI433A, EPS221, and Liberty Blue. The identity of the peptides was confirmed by mass spectrometry, and their purity analyzed *via* high-performance liquid chromatography. Quality control was performed with the help of Ulrich Wulle, Nicole Bauer, and Patricia Hrstić.

2.2.3 PBMC's isolation from buffy coat

The PBMC isolation was performed by density gradient centrifugation. The buffy coats were received from the Institute for Clinical and Experimental Transfusion Medicine at the University Hospital of Tübingen. Informed written consent was obtained in accordance with the Declaration of Helsinki. The isolation and immune testing of PBMCs was approved by the Ethik-Kommission an der Medizinischen Fakultät der Eberhard-Karls-Universität und am Universitätsklinikum Tübingen (Project No. 507/2017B01: „Entnahme von Blut und Zellapheresen bei gesunden Probanden für Forschungszwecke. a) Vollblut b) Thrombozytenkonzentrate c) Leukozytenkonzentrate").

The buffy coat is first transferred into a culture flask, which is then filled to 120 ml with PBS. Four 50 ml falcon tubes are prepared, containing 15 ml of Ficoll each. Then 30 ml of the diluted buffy coat are slowly layered on top of the Ficoll. There should be two separate phases at the end of the layering process. Subsequently, the four falcon tubes are centrifuged for 20 min at 2000 rounds per minute (rpm) with the break turned off to prevent the liquid layers from mixing. Ficoll has a density between the lighter lymphocytes and the heavier erythrocytes and granulocytes.

The centrifugation leads to the sedimentation of the erythrocytes and granulocytes on the bottom of the falcon tube, followed by the Ficoll layer, which is covered by the Lymphocytes. PBS and the plasma contained in the buffy coat cover the lymphocyte layer.

Afterwards, the lymphocytes are isolated by removing the PBS-plasma and lymphocytes layer and transferring the lymphocytes layer to a new falcon tube.

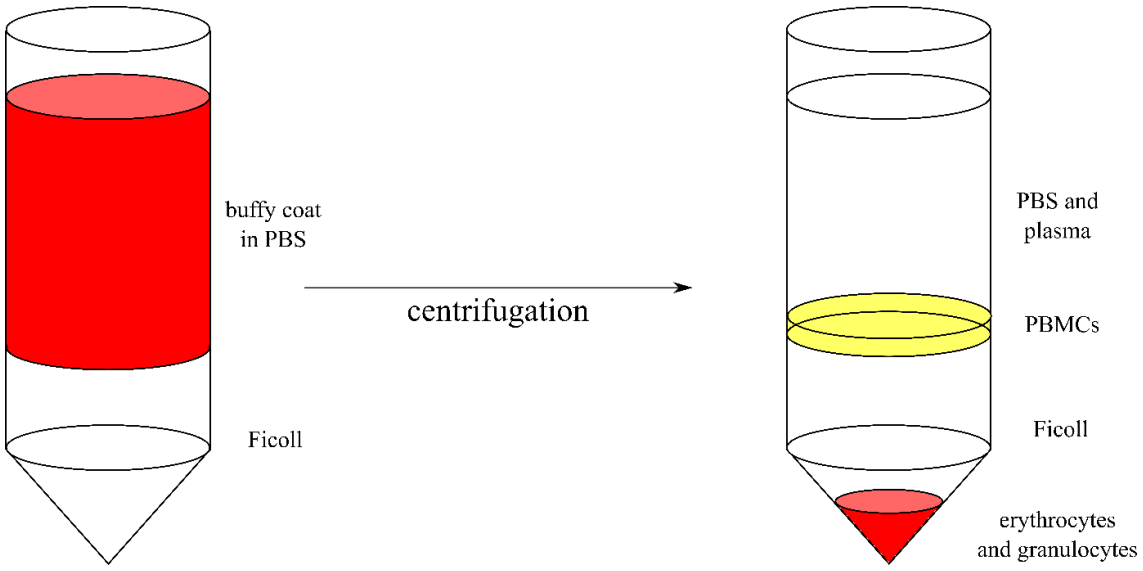


Figure 9: PBMC isolation. Separation by gradient centrifugation

The lymphocytes are then washed twice with PBS and centrifuged at room temperature with 1400 rpm for 6 minutes after the first and 1300 rpm for 8 minutes after the second dilution in PBS. After the second washing step, the PBMCs are diluted in 40 ml PBS and counted (see 2.2.4). The last centrifugation step is done at 4°C with 1300 rpm for 8 minutes. Finally, the PBMC's are resuspended in 90% FCS with 10% DMSO to get a final concentration of 5 x 10^6 PBMCs/ml and stored in aliquots of 1 ml at -80°C.

2.2.4 Cell counting

Cell counting was performed with an improved Neubauer chamber. The Neubauer chamber is the most common method for cell counting with high accuracy. (187)

The cells that need to be counted are diluted in trypan blue. The ratio of trypan blue and medium containing the cells depends on the estimated cells per milliliter. In our setting, the dilution factor was 10. After the cells are diluted with trypan blue and well mixed, the dilution is pipetted onto the Neubauer chamber. The counting is performed on two opposed big squares. When using the formula below, the cell concentration can be determined by inserting the averaged cell number per square.

$$cells/\mu l = \frac{average\ cell\ count\ per\ big\ square * dilution\ factor * 10}{\mu l}$$

The total cell count is computed by multiplying the cells per µl by the total volume [µl] containing the cells.

2.2.5 12-day stimulation

The concentration of epitope-specific T cells in the PBMC cultures, especially for HAdV-specific T cells, is usually low. (123) Therefore, it is helpful to cultivate T cells for 12 days in screening assays before performing an ICS or ELISpot.

During the 12-day stimulation, cell cultures were incubated at 37°C and 7.5% CO_2 with peptides of interest (POIs) and stimulated with IL-2 on day 3, day 6 and day 8 of the 12-day-protocol.

On day one, an aliquot containing $1x10^8$ cells was thawed and resuspended in 10 ml of ATM at 4°C. After centrifugation at 1400 rpm for 7 minutes with the Megafuge 1.0 R, the supernatant was poured off and the pellet resuspended in 10 ml of ATM at room temperature to dilute the DMSO contained in the freezing medium. After the next centrifugation step, the pellet was resuspended in 6 ml of TCM at 37°C. Two ml per well of cell suspension of one donor was then plated onto three wells of a 6-well plate and incubated at 37°C and 7.5% CO_2.

On day two, the peptides were added to the cells with a final concentration of 1 µg/ml per peptide for HLA class I peptides and 5 µg/ml per peptide for HLA class II peptides. All three wells were stimulated with a pool containing ten peptides: nine test peptides plus one negative peptide. One well was additionally stimulated with a positive cocktail, which was adapted during the experiments, in the single-epitope screening setting. The positive cocktail was only added to one well to prevent the suppression of a reaction to the single peptides.

On days three and six, 0.5 ml, and on day eight, 1 ml of TCM containing IL-2 was added per well to obtain a final concentration of 20 U/ml of IL-2 in the culture. On day 10, the cells were fed with fresh TCM. On the 12th day, the cells were prepared for ICS or ELISpot assay.

2.2.6 IFN-γ-ELISpot assay

The IFN-γ-ELISpot assay is an indirect method of detecting activated PBMCs with high sensitivity. In this work, it was used to screen for immunogenic HAdV2 epitopes. The ELISpot assay provides a quantitative estimation of the number of IFN-γ producing cells activated by a test peptide or peptide pool. As PBMCs consist of different cell types ($CD8^+$ T cells, $CD4^+$ T cells, and NK cells) that can produce IFN-γ (188), the ELISpot assay does not provide information regarding the source of the IFN-γ production. This question was later answered by performing an ICS. Only cell responses to epitopes that can bind to at least one of the PBMC donor's HLA molecules and are recognized by a significant number of TCRs can be detected.

In our setting the 96-well ELISpot plates were pre-coated with 100 µl of Human IFN-γ capture antibody (1-D1K) suspension in PBS at a concentration of 2 µg/ml at 4°C overnight. On the next morning, the suspension was poured off, and the wells were washed twice with 200 µl of IMDM. Afterwards, the wells were incubated with 50 µl of TCM at 37°C with 7.5% CO_2 for 2 hours. In parallel, the PBMC cultures of a donor were harvested and pooled. After centrifugation at 1400 rpm for 7 minutes, the pellet was resuspended in 10 ml of TCM at 37°C. The cells were counted before the next centrifugation step with the same parameters as before. The pellet was resuspended in TCM to a final concentration of 10^7 cells/ml. After 2 hours of incubation, 50 µl of TCM containing 500.000 cells, and 50 µl of test solution were added per well. The test solution was either a single-peptide solution containing a test or negative peptide, a peptide cocktail, or a PHA solution. The test peptides and negative peptides were added to a final concentration of 1 µg/ml per well for HLA class I peptides and 2,5 µg/ml per well for HLA class II peptides. The final concentration of PHA as an unspecific positive control was 10 µg/ml. After the cell suspension, and the test solution were added, the 96-well ELISpot plate was incubated at 37°C and 7.5% CO_2 for 20 to 22 hours. By adding the POIs, the cells bearing an epitope-specific TCR are activated and secrete IFN-γ as a result. IFN-γ binds to the human IFN-γ capture antibody (1-D1K) on the membrane. After 20 to 22 hours of incubation, the cells and peptides were washed off. The 96-well plate was washed twice with 200 µl of PBS Tween per well, followed by two washing steps with 200 µl of ddH_2O per well. Thereafter, each well was washed three times with 200 µl of PBS Tween.

Next, the 96-well plate was incubated for two hours at room temperature with 100 µl of detection antibody per well. The detection antibody is a biotinylated anti-human IFN-γ antibody (7-B6-1), which binds to a different region of the 1-D1K-bound IFN-γ. The concentration of the detection antibody suspension was 0.3 µg/ml in PBS BSA. After 2 hours, the 96-well plates were washed six times with 200 µl PBS Tween.

Afterwards, the 96-well plates were incubated for 1 hour at room temperature with 100 µl per well of ExtrAvidin alkaline phosphatase solution with a concentration of 1 µl/ml in PBS BSA. It binds to the biotin-coupled detection antibody. In parallel, a BCIP/NBT tablet was dissolved in 10 ml of ddH_2O in the dark. After the incubation, each well of the 96-well plate was washed three times with 200 µl of PBS Tween, followed by three washing steps with 200 µl of PBS. The BCIP/NBT solution was filtered, and 50 µl of the solution added per well. BCIP/NBT is a substrate of alkaline phosphatase. The reaction leads to a change of color whereby the soluble BCIP/NBT is converted into a blue dye, which settles down on the membrane. When the reaction is stopped after 7-12 minutes by adding ddH_2O to wash off the excess of the substrate, the dye remains bound to the membrane indicating the presence of an epitope-specific T cell. The 96-well ELISpot plates were read out with the ImmunoSpot® S6 Ultra-V-Analyzer and analyzed with the integrated ImmunoSpot® software after at least 24 hours of drying at room temperature.

To estimate the number of memory T cells in the blood, an *ex vivo* ELISpot assay can be performed. In this setting, the PBMCs are thawed, washed, and plated onto the membrane after resting in TCM for a day. When an ELISpot assay is performed with fresh cells, one can see how many antigen-specific cells in the peripheral blood recognize the epitope. This can help identify promising epitopes for clinical approaches. (156)

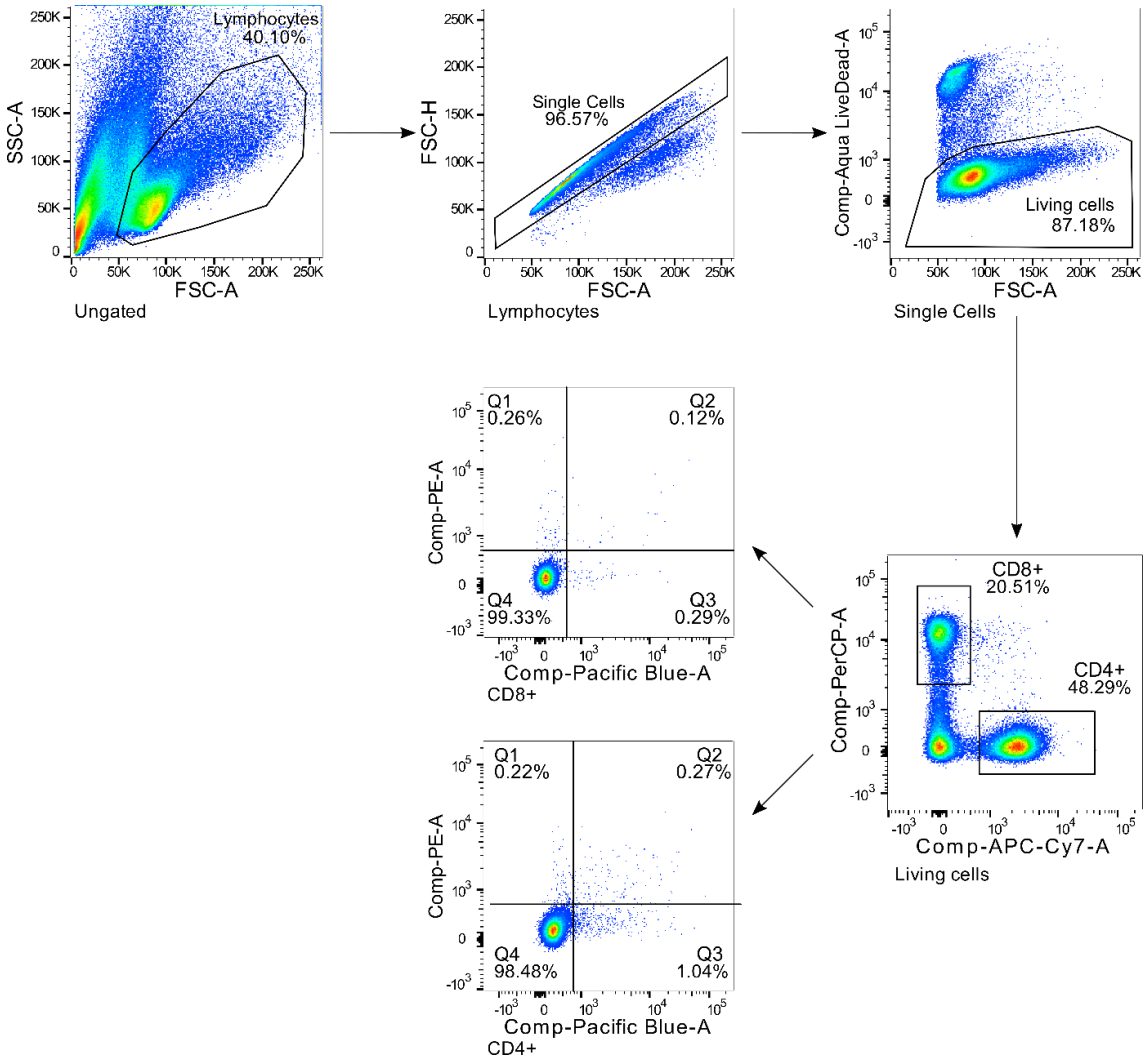
2.2.7 Intracellular Cytokine Staining

The ICS is employed to further characterize the cell type that is activated by a particular epitope in the IFN-γ-ELISpot assay. It is less sensitive than the IFN-γ-ELISpot assay. Therefore, further information is most likely to be obtained from PBMCs of donors with a strong response to the POI in a previous ELISpot assay. Prior to the ICS, the cells were stimulated for 12 days with the POI, the negative control peptide and IL-2 as described under chapter 2.2.5. On the 13th day, the cells were plated into five wells of a

round-bottomed 96-well-plate to a concentration of 0.5-1x10^6 cells in 50 µl TCM per well. After resting for at least three hours, 50 µl of TCM containing the POI was added to two wells, 50 µl of TCM containing the negative peptide to two other wells and 50 µl of TCM containing PMA and Ionomycin to the last well, as a positive control. Finally, 50 µl of TCM containing Brefeldin A and Golgi stop solution were added to every well. The final concentration of the POI and the negative peptide was 25 µg/ml. The final concentration in the positive control was 5 µl per ml and 1 µl per ml for PMA and Ionomycin, respectively. The final concentration for Brefeldin A and Golgi-Stop solution was 10 µg per ml and 6.7 µg per ml in all wells, respectively. The cells were then incubated for 12-14 hours at 37°C and 7.5% CO_2 before being stained.

During the staining process, all steps were performed at 4°C to prevent the cells from internalizing their surface receptors. All incubations had a duration of 20 minutes at 4°C in the dark. Each washing step was followed by centrifugation with Megafuge 1.0 R at 4°C and 1800 rpm for 2 minutes. First, the incubated cells were centrifuged, the supernatant poured off, and the pellet resuspended in 150 µl of PBS-EDTA. This washing step was repeated after another centrifugation. After the supernatant was poured off, the pellet was resuspended in 50 µl of Aqua live/dead - PBS-EDTA solution (1:200). The cells were then incubated. Subsequently, the cells were centrifuged, supernatant poured off, and the cells resuspended in 150 µl of PBS-EDTA, before the next centrifugation. Afterwards, the extracellular membrane-bound molecules CD4 and CD8 had to be stained. Therefore, the cell pellet was resuspended in 50 µl of FACS buffer containing the antibodies CD8-PerCP and CD4-APC-Cy7, both in a concentration of 1:100, followed by incubation. After the incubation, 150 µl of FACS buffer was added per well before centrifugation. After centrifugation, the supernatant was poured off. In the next step, 100 µl of Cytofix/Cytoperm was applied per well, followed by another incubation. The permeabilization of the cell membrane allows the staining of the intracellular molecules: IFN-γ and TNF. After incubation, the cells were washed with 150 µl of Permwash buffer before adding 50 µl of Permwash buffer per well containing the antibodies INFγ-PE and TNFα-PacificBlue in a concentration of 1:200 and 1:120, respectively. The cells were then incubated. Finally, the cells were washed twice with 150 µl of Permwash buffer before being resuspended in 200 µl of FACS buffer. Flow cytometric analysis was

performed using a FACS Canto II cytometer and the software FlowJo version 10. The gating strategy is illustrated in Figure 10.



<u>Figure 10:</u> FACS gating strategy for ICS in a negative control: SSC-A (sideward scatter) against FSC-A (forward scatter) shows the size and the granularity of the cells. Lymphocytes are then gated to single cells, followed by the gating of living cells. Afterwards, $CD4^+$ and $CD8^+$ cells are gated. In this parallel analysis, one can compare the percentage of single, living T cells of the CD4+ or CD8+ linage that produced TNF and IFN-γ when stimulated with the POI, compared to the respective negative control.

3 Results

3.1 Comparison of two HLA class I-epitope cocktails

The immunogenicity of two HLA class I-epitope cocktails was compared in IFN-γ-ELISpot assays. They consisted of published (163, 170, 173-179) and *in house* tested immunogenic HLA class I epitopes derived from HAdV2 proteins. We wanted to compare the frequency of an immune response in PBMC cultures of healthy donors against two different HLA class I-epitope cocktails: one cocktail containing 24 another containing only 15 epitopes.

3.1.1 Generation of the HLA class I-epitope cocktails

We generated a prioritized list of HLA class I-restricted epitopes from adenoviral antigens, previously screened for immunogenicity in ELISpot assays *in house*. For some HLA allotypes, more than one peptide was prioritized. We designed two peptide cocktails, as seen in Table 13 and Table 14. The first cocktail, termed "24-peptides-cocktail", contained 24 HLA class I epitopes (Table 13). The second cocktail only contained the epitopes with the highest recognition rates for each HLA allotype covered by the 24 peptides. Some HLA allotypes were covered with only one epitope.

Table 13: Peptides of 24-peptides-cocktail

Sequence	Source Protein (UniProt-ID)	HLA-restriction
LTDLGQNLLY	*CAPSH_ADE02*	*A*01*
STDVASLNY	*CAPSP_ADE02*	*A*01*
FLAPKLYAL	*DPOL_ADE02*	*A*02*
RLLPGVFTV	*DPOL_ADE02*	*A*02*
LLDQLIEEV	*E1A_ADE02*	*A*02*
YVLFEVFDVV	*CAPSH_ADE02*	*A*02*
RLMETRGKK	*DPOL_ADE02*	*A*03*
VIYGPTGCGK	*PKG1_ADE02*	*A*03*
ALYLPDKLK	*CAPSH_ADE02*	*A*03*
TYFSLNNKF	*CAPSH_ADE02*	*A*24*
EPRSGGIGTL	*CAP8_ADE02*	*B*07*
EPTLLYVLF	*CAPSH_ADE02*	*B*07*
RPKLVPAIL	*E1A_ADE02*	*B*07*
VPATGRTLVL	*LEAD_ADE02*	*B*07*
FRKDVNMVL	*CAPSH_ADE02*	*B*08*
IEEFVPSVY	*CAP8_ADE02*	*B*18*
CEDRASQML	*E1B55_ADE02*	*B*40*
FEPPTLHEL	*E1A_ADE02*	*B*40*
PEIHPVVPL	*E1A_ADE02*	*B*40*
AEIEGELKCL	*DNB2_ADE02*	*B*40*
NEIGVGNNF	*CAPSH_ADE02*	*B*44*
QESPATVVF	*E1B55_ADE02*	*B*44*
NEPVSTREL	*SF33K_ADE02*	*B*44*
NESHCGVLVEL	*SHUT_ADE02*	*B*44*

Table 14: Peptides of 15-peptides-cocktail

Sequence	Source Protein (UniProt-ID)	HLA-restriction
LTDLGQNLLY	*CAPSH_ADE02*	*A*01*
LLDQLIEEV	*E1A_ADE02*	*A*02*
YVLFEVFDVV	*CAPSH_ADE02*	*A*02*
VIYGPTGCGK	*PKG1_ADE02*	*A*03*
ALYLPDKLK	*CAPSH_ADE02*	*A*03*
TYFSLNNKF	*CAPSH_ADE02*	*A*24*
EPRSGGIGTL	*CAP8_ADE02*	*B*07*
VPATGRTLVL	*LEAD_ADE02*	*B*07*
FRKDVNMVL	*CAPSH_ADE02*	*B*08*
IEEFVPSVY	*CAP8_ADE02*	*B*18*
FEPPTLHEL	*E1A_ADE02*	*B*40*
AEIEGELKCL	*DNB2_ADE02*	*B*40*
NEIGVGNNF	*CAPSH_ADE02*	*B*44*
QESPATVVF	*E1B55_ADE02*	*B*44*
NEPVSTREL	*SF33K_ADE02*	*B*44*

In contrast, other HLA allotypes had to be covered with two or even three epitopes with lower recognition rates, resulting in a cocktail containing 15 epitopes. We named it "15-peptides-cocktail". The recognition rates of the epitopes are listed in Table 3.

3.1.2 Testing of the HLA class I-epitope cocktails in IFN-γ ELISpot assays

The 12-day stimulation protocol was performed prior to the ELISpot assays. Cells from each donor were split into equal amounts to allow the stimulation of half of the cells with the 24-peptides-cocktail and the other half with the 15-peptides-cocktail. Thus, the competitive suppression of T-cell responses to epitopes of the 15-peptides-cocktail by epitopes, with a higher affinity to the HLA molecules, contained only in the 24-peptides-cocktail was prevented.

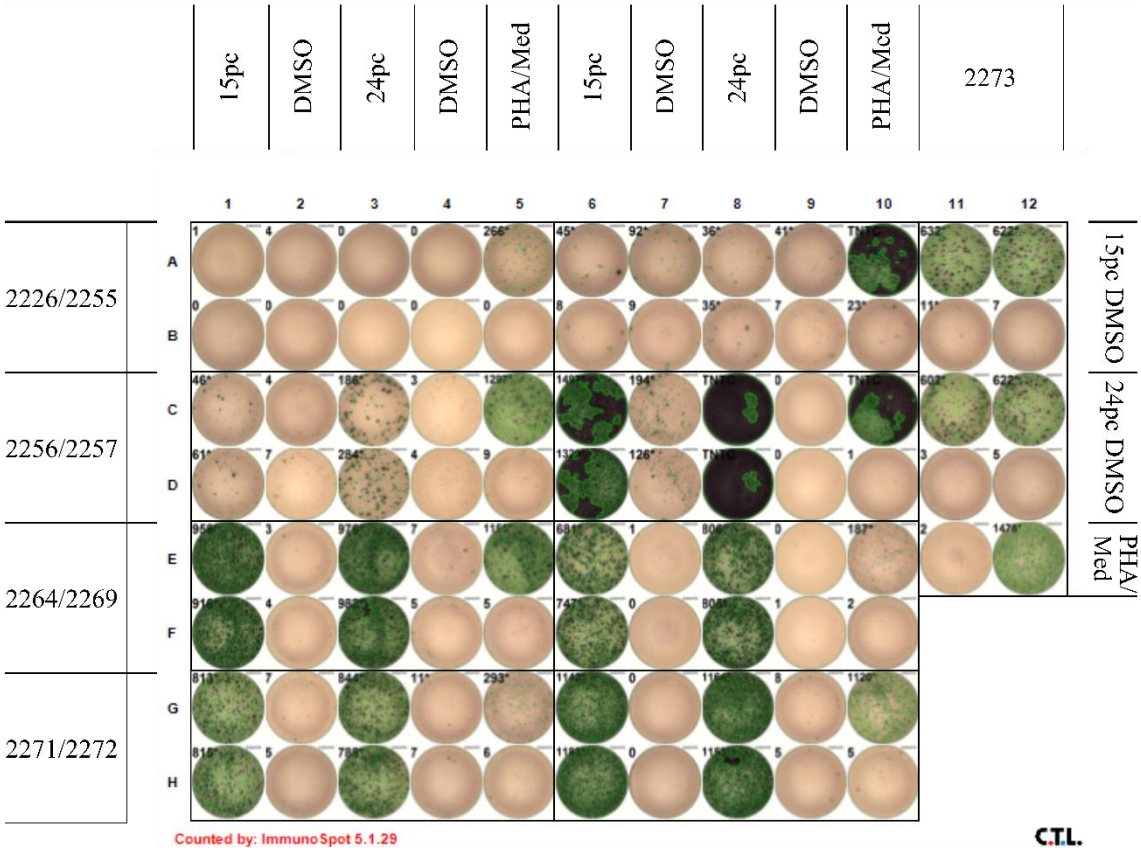


Figure 11: 96 well IFN-γ-ELISpot plate after restimulation of PBMCs from 9 donors. Donor 2273 read from top to bottom. (15pc=15-peptides-cocktail, 24pc=24-peptides-cocktail, DMSO=Dimethyl sulfoxide, PHA=Phytohaemagglutinin, Med=T-cell medium)

TCM with DMSO was used as a negative control in the same DMSO concentration as in the wells, stimulated with the cocktails. Both cocktails were compared in three different

ELISpot assays with PBMCs of nine donors each. No serologic knowledge regarding the HAdV status of the tested PBMCs was acquired. The donors were typed for HLA-A and HLA-B.

PBMCs of 27 donors were tested this way. PBMCs of the donors 2526 and 2532 could not be taken into consideration due to differing spot counts in doublets and an overreactive response, respectively. The 24-peptides-cocktail elicited a positive IFN-γ response in 22 out of 25 PBMC cultures surmounting to a positive reaction in 88% of the tested PBMC cultures. The 15-peptides-cocktail led to a positive IFN-γ response in 20 out of 25 PBMC cultures resulting in a recognition rate of 80%.

The 24-peptides-cocktail was always able to stimulate the PBMCs of donors, which showed a response to the 15-peptides-cocktail. The PBMCs of the donors 2412 and 2512 did not show a reaction to the 15-peptides-cocktail, whereas the 24-peptides-cocktail was able to induce an IFN-γ secretion in both cultures.

The PBMCs from the donors 2226, 2255, and 2413 were unresponsive to both HLA class I cocktails. The HLA class I alleles of donor 2255 consists of rare HLAs. Peptides of the cocktails covered not even one HLA allotype. From the four typed HLA class I alleles of donor 2226, only the HLA-A*03 allotype was covered by peptides contained in the cocktails. The HLA typing of donor 2413 showed that both HLA-A allotypes were covered with peptides of reasonable recognition rates. Still, no activation could be observed.

The HLA class I allotypes of the donors 2412, 2422, 2512, and 2515 were not covered by peptides contained in the cocktails. Nevertheless, the 24-peptides-cocktail was able to elicit an immune response in the PBMCs of all four of them. The 15-peptides-cocktail was able to stimulate the PBMCs of the donors 2422 and 2515.

3.2 HLA class II epitopes

The second and main aim of this work was to generate an HLA class II epitope cocktail. Therefore, it was necessary to determine an algorithm for the prediction of promiscuous HLA class II epitopes. Presumably, one peptide could stimulate several individuals expressing differing HLA class II allotypes. By creating a cocktail that consists of several promiscuous epitopes covering frequent HLA class II-allotypes, almost every donor should be able to present at least one epitope contained in the cocktail. The advantage of HLA class II epitopes compared to HLA class I epitopes is that peptides can be longer as the binding groove of HLA class II molecules is not limited by hydrogen bonds at both ends (22-24) as it is the case for HLA class I molecules (11). Nevertheless, the core sequence is also nine aa long. Thus, epitopes exceeding a length of nine aa can be identified, which can contain several core sequences for different HLA class II allotypes. (186)

3.2.1 Prediction of promiscuous HLA class II epitopes from adenoviral antigens

All 46 reviewed HAdV2 proteins from the Swiss-Prot database (171) were subjected to promiscuous epitope prediction with SYFPEITHI (172) (as described in 2.2.1) against the HLA class II allotypes: HLA-DRB1*01:01, HLA-DRB1*03:01, HLA-DRB1*04:01, HLA-DRB1*07:01, HLA-DRB1*11:01, and HLA-DRB1*15:01. (186) These cover all the alleles that are carried by at least 20% of the population in Germany, except for HLA-DRB1*13, see Table 15.

Table 15: Allele frequencies of the HLA-DRB1 alleles in Germany, with a sample size of 11,407. (189)

HLA allele	% of individuals that have the allele	Allele frequency
DRB1*01	21.0	0.1111
DRB1*03	20.0	0.1055
DRB1*04	24.6	0.1316
DRB1*07	23.6	0.1259
DRB1*08	6.4	0.0325
DRB1*09	1.9	0.0095
DRB1*10	1.7	0.0085
DRB1*11	23.5	0.1253
DRB1*12	3.7	0.0186
DRB1*13	24.1	0.1287
DRB1*14	6.1	0.0309
DRB1*15	26.4	0.1421
DRB1*16	4.8	0.0243

In total, 12,291 potential 15mer epitopes can arise from the 46 reviewed HAdV2 proteins of the Swiss-Prot database. The synthesis and ELISpot testing of this high number of epitopes is not practicable. Therefore, a prediction was performed to identify the most promising epitope candidates prior to peptide synthesis and testing. (186) The results of the prediction are shown in Table 16.

3.2.2 Screening of single HLA class II epitopes from HAdV2

Four groups of peptides were tested in single-peptide ELISpot-screening assays to detect immunogenic HLA class II-peptides. The first group of tested peptides contained either previously published epitopes (162, 163, 169) or epitopes derived from HAdV2 and HAdV5 from earlier non-promiscuous predictions performed *in house*. These 35 peptides are listed in Table 4. The second group consisted of peptides after *in silico* prediction of promiscuous epitopes from HAdV2, as described in 2.2.1. Peptides which contained six and more core sequences of the top-2% prediction were synthesized and further investigated. These 24 peptides are listed in Table 5. The peptide TLRFIPVDREDTAYS appears in both the lists of predicted epitope candidates and already published epitopes. All other peptide sequences are exclusive to one listing. The third group consisted of HAdV2 peptides with a core-overlap of four that were predicted as good binders by NetMHCIIpan 2.0 for the same HLA allotypes as the ones for which the SYFPEITHI-prediction was performed. KNRLLLLSSVRPAII was predicted as a strong binder to all six HLA class II molecules for which the prediction was performed. The peptides VSKFFHAFPSKLHDK and TLLYLKYKSRRSFID were categorized as strong binders to all the HLA class II allotypes for which the prediction was completed except for HLA-DRB1*03, for which they were predicted as weak binders. These three peptides are listed in Table 6. The last group consisted of one HAdV2 epitope candidate with the sequence TFYLNHTFKKVAITF. The aa sequence TFYLNHTFKK was predicted as an HLA class I epitope but was shown to stimulate mainly $CD4^+$ T cells in ICS in previous work, indicating an HLA class II restriction. The sequence of this 10mer peptide was identified in the promiscuous prediction of the top-2% predicted peptides, before the selection step for overlaps, and elongated to the 15mer peptide TFYLNHTFKKVAITF on its C-terminal end. This epitope candidate is listed in Table 7.

All peptides were tested in single-peptide ELISpot-screening assays after a 12-day stimulation (see 2.2.5). (186)

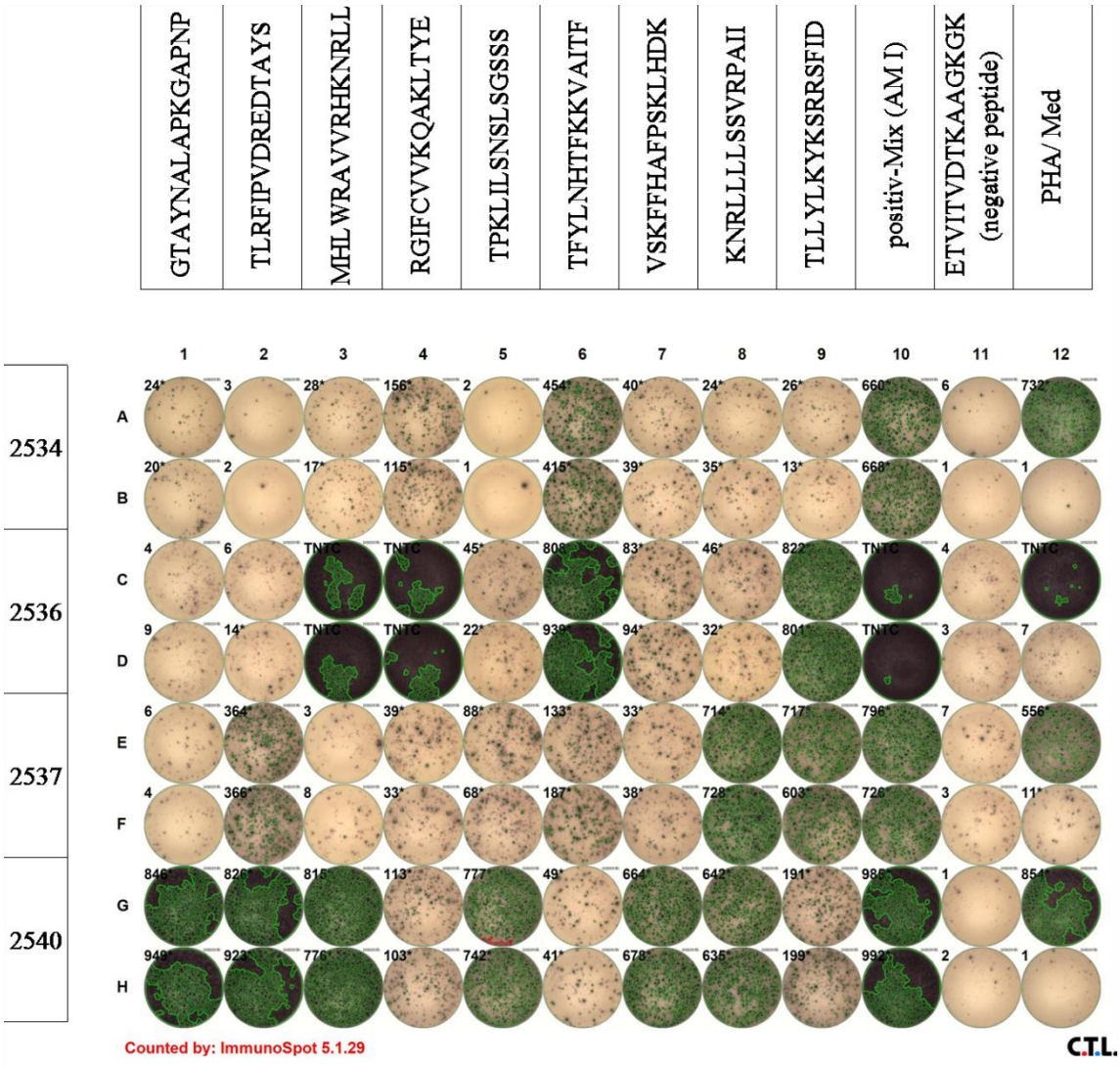


Figure 12: 96-well-plate after screening for immunogenic HLA class II epitopes via IFN-γ ELISpot assay. Immune responses are considered positive when the average spot count per well exceeds ten spots, and when the average spot count is higher than 3x the average spot count of the negative control. The spot count was set to 2,000 spots when too-numerous-to-count (TNTC). The first adenovirus-derived class II cocktail was used as a positive control.

Peptides eliciting positive responses in the PBMCs of healthy donors were tested in further assays. Peptides with recognition rates below 25% did not undergo further testing.

Table 16: ELISpot results of predicted promiscuous peptides: Peptides were sorted by the number of predicted binding, then by %-max score. ‘‘x’’ in response rate column indicates peptides that could not be synthesized. Red colour intensity rises with response rate. (186)

Number of best 2% cores	Sequence	Source protein	Position	HLA-DRB1 coverage	%-max Score	Response rate	Positive/tested
9	I H P V V P L C P I K P V A V	E1A_ADE02	243	1,4,7,11,15	66%	13%	1/8
8	T L R F I P V D R E D T A Y S	CAPSH_ADE02	65	3,4,7,11,15	79%	62%	13/21
8	Y I M T P D M T E E L S V V L	E4RF1_ADE02	79	3,4,7,15	75%	0%	0/8
7	I S P F I K L T S T H S A N K	Y172_ADE02	31	1,4,7,11,15	80%	0%	0/8
7	L L D L I N I L Q S I V V Q E	PKG3_ADE02	242	1,4,7,15	78%	13%	2/15
7	C V G W L G V A Y S A V V D V	E4RF4_ADE02	18	1,4,7,15	74%	x	
7	I L G L L A L A A V C S A A K	E3GL_ADE02	5	1,4,11,15	71%	23%	3/13
7	K D L L T D F K A F A A R F S	UXP_ADE02	48	1,3,4,11,15	71%	38%	6/16
7	L P L L I P L I A A A I G A V	COR10_ADE02	53	1,4,7,15	70%	71%	17/24
7	Q K L V L M V G E K P I T V T	E3145_ADE02	77	1,4,7,11,15	66%	15%	2/13
7	L D Q L I E E V L A D N L P P	E1A_ADE02	20	1,3,4,7	66%	15%	2/13
7	R G I F C V V K Q A K L T Y E	E3145_ADE02	46	1,3,4,11,15	65%	75%	18/24
7	C A V V D A L D R A K G E P V	Y137_ADE02	48	1,3,4,7,11	62%	0%	0/8
6	M R V I I S V G S F V M V P G	E4RF2_ADE02	67	1,4,7,15	81%	19%	3/16
6	L N R F V N T Y T K G L P L A	PKG1_ADE02	353	1,4,7,11,15	78%	46%	11/24
6	L V S Y L G I L H E N R L G Q	SHUT_ADE02	386	1,3,4,7,11,15	74%	0%	0/8
6	R Q V M D R I M S L T A R N P	CAP3_ADE02	28	1,4,7,11,15	73%	67%	16/24
6	I G A V P G I A S V A L Q A Q	COR10_ADE02	64	3,4,7,15	73%	54%	13/24
6	V W L V V F Y F G C L S L T V	LEAD_ADE02	76	4,7,11,15	72%	x	
6	T L V L A F V K T C A V L A A	LEAD_ADE02	32	1,4,7,11,15	72%	71%	17/24
6	L A T V P S I A T T S A P Q A	PKG2_ADE02	70	1,4,7,11,15	71%	13%	2/15
6	L A T V P S I A T T S A P Q A	SF33K_ADE02	70	1,4,7,11,15	71%	13%	2/15
6	V T A F R C I I Q G H P R G P	Y215_ADE02	149	1,4,11,15	70%	0%	0/15
6	I F V L L I F C A L P V L C S	E3RDB_ADE02	6	1,3,11,15	67%	unusable	
6	T P K L I L S N S L S G S S S	Y215_ADE02	75	1,4,7,15	66%	43%	10/23
6	Y L K V M V R D T F A L T H T	DPOL_ADE02	491	3,7,11,15	65%	25%	4/16
6	A S W F R M V V D G A M F N Q	E434_ADE02	142	1,3,7,11,15	65%	0%	0/8
6	V R R V L R P G T T V V F T P	CORE5_ADE02	72	3,4,7,11	64%	13%	1/8

From the 28 predicted peptides described in Table 16, the peptides VWLVVFYFGCLSLTV and CVGWLGVAYSAVVDV could not be synthesized. The peptide IFVLLIFCALPVLCS was unusable due to an unsatisfying purity. The sequence LATVPSIATTSAPQA appears in the PKG2 and SF33K protein sequence. This left 24 different peptides predicted as promiscuous epitopes to be tested in single-peptide ELISpot-screening assays. Six (25.0%) of the predicted peptides could not elicit an immune response in any PBMC culture. Eighteen peptides (75.0%) were immunogenic as they lead to a stimulation of at least one PBMC culture. Six out of 24 peptides (25.0%) could be recognized by T cells in more than 50% of the tested PBMCs and are therefore termed immunodominant epitopes. (186)

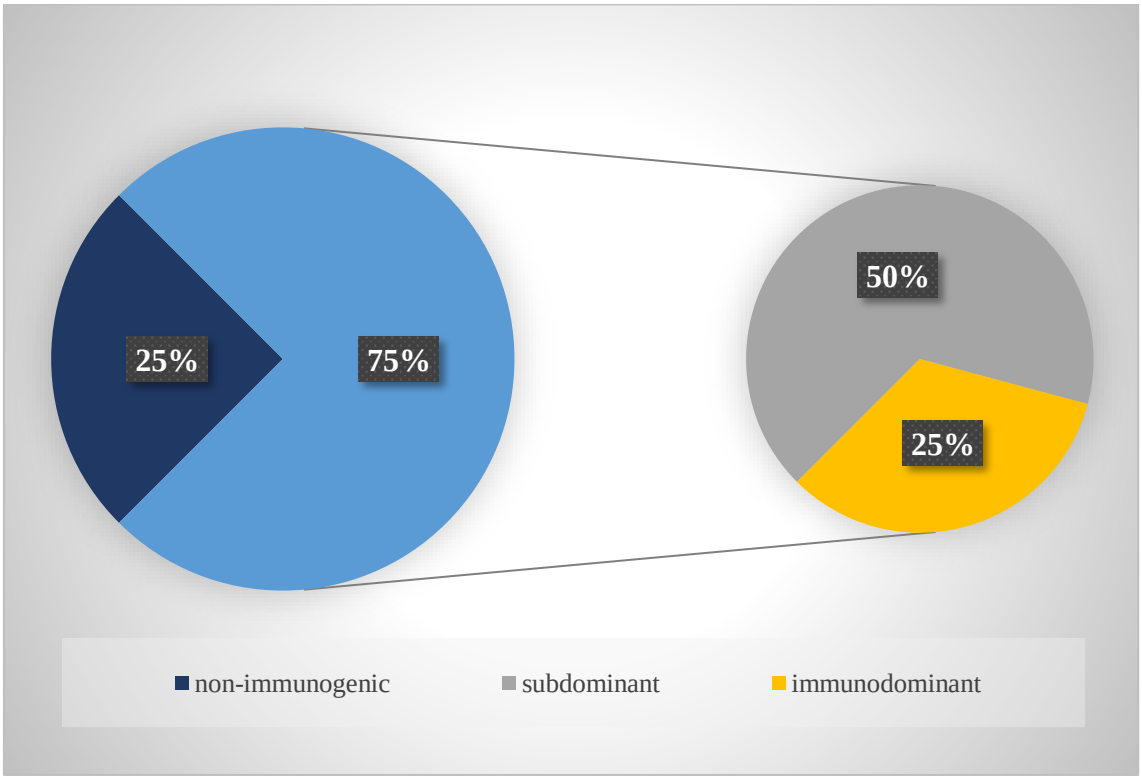


Figure 13: Immune response to predicted promiscuous peptides. From the 24 predicted peptides which could be synthesized, 25% did not elicit an immune response in any tested PBMC culture. 75% of the synthesized peptides triggered an immune response in at least one PBMC culture and are therefore termed immunogenic epitopes. 50% of the synthesized peptides had recognition rates between 0% and 50% and are termed subdominant epitopes. 25% of the synthesized peptides had recognition rates higher than 50% and therefore categorized as immunodominant epitopes. Adapted from (186).

Table 17: ELISpot results of already published HLA class II epitopes and epitopes from earlier non-promiscuous *in house* predictions derived from HAdV2 and HAdV5. (186) Red colour intensity rises with Recognition rate.

Sequence	Source protein	Position	HLA-restriction	Recognition rate	positive/tested
VDCYINLGARWSLDY	CAPSH_ADE02	530-544	DR1	17%	1/6
TETLTKVKPKTGQEN	CAPSH_ADE05	423-437	DR1	13%	1/8
GTAYNALAPKGAPNP	CAPSH_ADE05	118-132	DR1	36%	5/14
EWNFRKDVNMVLQSS	CAPSH_ADE02	598-612	DR3/DR4	19%	4/21
ATFFPMAHNTASTLE	CAPSH_ADE02	633-647	DR4/DR7	14%	2/14
THDVTTDRSQRLTLR	CAPSH_ADE02	53-67	DR3	0%	0/6
SQWYETEINHAAGRV	CAPSH_ADE05	209-223	DR4	21%	3/14
FKKVAITFDSSVSWP-NH2	CAPSH_ADE02	734-748	DR7	17%	1/6
DEPTLLYVLFEVFDV	CAPSH_ADE02	927-941	DR	54%	13/24
RPSFTPRQAILTLQT	CAP8_ADE02	159-173	Class II	0%	0/6
TGNMGVLAGQASQLN	CAPSH_ADE02	354-368	DR	40%	2/5
GASIKFDSICLYATF	CAPSH_ADE02	621-635	DR3	23%	3/13
PGSYTYEWNFRKDVN	CAPSH_ADE02	592-606	DR4	45%	5/11
ENGWEKDATEFSDKN	CAPSH_ADE05	436-450	DR4	0%	0/5
GNNFAMEINLNANLW	CAPSH_ADE02	471-485	DR4	17%	1/6
GWAFTRLKTKETPSL	CAPSH_ADE02	693-707	DR4	10%	1/10
TLRFIPVDREDTAYS	CAPSH_ADE02	65-79	DR4	62%	13/21
DFYFHHINSHSSNWW	DPOL_ADE02	247-261	DR	0%	0/6
NIALYLPDKLKYNPT	CAPSH_ADE02	492-506	DR	48%	10/21
LFEVFDVVRVHQPHR	CAPSH_ADE02	935-949	DR	33%	7/21
VNQFYMLGSYRSEAD	DPOL_ADE02	528-542	DR	13%	1/8
LKSVYGDTDSLFVTE	DPOL_ADE02	1006-1020	DR	14%	2/14
RAFVSEWSEFLYEED	DPOL_ADE02	982-996	DR	13%	1/8
YPTYLGILREPLYVY	DPOL_ADE02	670-684	DR	0%	0/8
CGMYASALTHPMPWG	DPOL_ADE02	687-701	DR	0%	0/8
FPEWRCVAREYVQLN	DPOL_ADE02	803-817	DR	13%	1/8
WRFLWGSSQAKLVCR	E1BS_ADE02	28-42	DR	38%	6/16
MHLWRAVVRHKNRLL	E1BS_ADE02	120-134	DR	68%	15/22
RCSMINMWPGVLGMD	E1B55_ADE02	217-231	DR	38%	5/13
CGCFMLVKSVAVIKH	E1B55_ADE02	329-343	DR	0%	0/6
AELFPELRRILTINE	E1B55_ADE02	80-94	DR	0%	0/6
GDDFEEAIRVYAKVA	E1B55_ADE02	164-178	DR	0%	0/6
SVMLAVQEGIDLLTF	E1A_ADE02	69-83	DR	0%	0/6
PKLVPAILRRPTSPV	E1A-ADE02	207-221	DR	0%	0/6
PQKFFAIKNLLLLPG	CAPSH_ADE02	579-593	DR	86%	18/21

Out of the 35 already published HLA class II peptides and peptides, derived from HAdV2 and HAdV5 proteins, from previous non-promiscuous HLA class II epitope predictions, eleven did not generate an immune response in any tested PBMC culture. Twenty of the tested peptides led to an IFN-γ secretion in at least one PBMC culture, but in less than 50% of the tested PBMC cultures. Only four out of 35 peptides led to a significant IFN-γ secretion in over 50% of the tested PBMC cultures. The peptides DEPTLLYVLFEVFDV (163, 169), TLRFIPVDREDTAYS (162), and PQKFFAIKNLLLLPG (162) are already published epitopes. The epitope MHLWRAVVRHKNRLL was discovered *in house* in a previous non-promiscuous HLA class II epitope prediction. The epitope TLRFIPVDREDTA is a predicted epitope candidate that is already published and therefore appears in both statistics. (186)

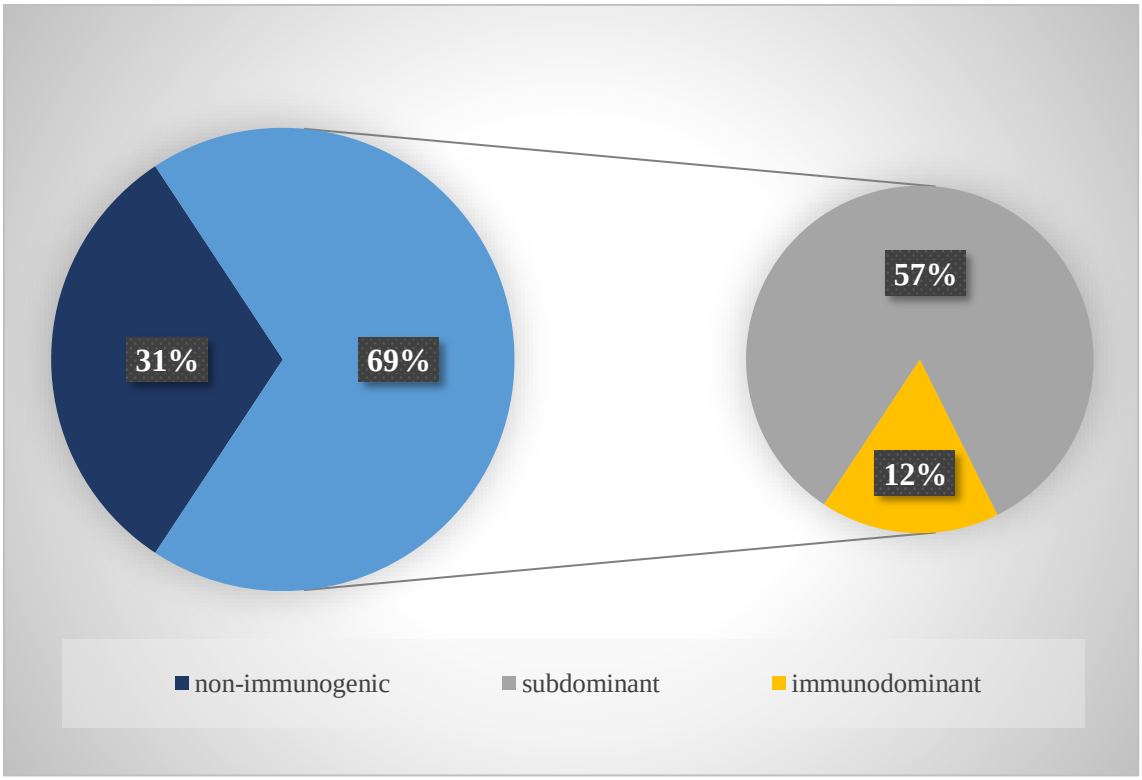


Figure 14: Immune response to already published HLA class II epitopes and epitopes derived from HAdV2 and HAdV5 from previous non-promiscuous HLA class II epitope predictions. From the 35 tested peptides, 31% did not lead to an immune response in any tested PBMC culture. 69% of the peptides triggered an immune response in at least one PBMC culture and are therefore categorized as immunogenic epitopes. 57% of the tested peptides had recognition rates in between 0% and 50% and are termed subdominant epitopes. 12% of the tested peptides had recognition rates higher than 50% and are therefore categorized as immunodominant epitopes.

The peptides listed in Table 18 and Table 19 were synthesized and screened for immunogenicity. They were discovered after the first cocktail, only containing HLA class II adenoviral epitopes, was designed. All three peptides were categorized as strong binders to at least five of the six HLA class II allotypes for which the prediction was performed by NetMHCIIpan 2.0. The response rates in tested PBMC cultures range from 65% to 80% (see Table 18). (186)

Table 18: ELISpot results of three peptides, which contained only four binding cores from the top 2% prediction results but were categorized as strong binders to at least five of the six screened HLA class II allotypes by NetMHCIIpan 2.0 (186) Red colour intensity rises with Recognition rate.

Sequence	Source protein	Position	HLA-restriction	Recognition rate	positive/tested
VSKFFHAFPSKLHDK	PKG1_ADE02	292-306	DR	80%	16/20
KNRLLLLSSVRPAII	E1BS_ADE02	130-144	DR	70%	14/20
TLLYLKYKSRRSFID	E3GL_ADE02	140-154	DR	65%	13/20

The elongated 10mer peptide TFYLNHTFKK resulted in the 15mer peptide TFYLNHTFKKVAITF (Table 19).

Table 19: ELISpot assay results of TFYLNHTFKKVAITF (186)

Sequence	Source protein	Position	HLA-restriction	Recognition rate	positive/tested
TFYLNHTFKKVAITF	CAPSH_ADE02	727-741	DR	85%	17/20

It led to an IFN-γ secretion in 85% of the tested PBMC cultures in ELISpot screening assays. (186)

3.2.3 Evolution of the HLA class II epitope cocktails

In total, four different HLA class II peptide cocktails were tested in ELISpot-screening assays. The first cocktail contained four peptides: two EBV-derived HLA class II epitopes, one already published HLA class II HAdV epitope (162), and the 10mer epitope TFYLNHTFKK (168, 174) derived from HAdV2 (Table 8). Due to the unsatisfactory low recognition rate of 70.4% (19/27) after being tested in 27 PBMC cultures, we added the EBV HLA class II epitope IAEGLRALLARSHVERTTDE (Table 8) to the first HLA class II cocktail. We examined the resulting cocktail in two further ELISpot assays and saw positive results in 15 out of 16 tested PBMC cultures of healthy donors (93.75%).

Subsequently, we created an epitope cocktail only containing HAdV2-derived HLA class II epitopes (Table 20) as we observed positive response rates in single-peptide ELISpot screenings of randomly selected PBMCs.

Table 20: Positive peptide cocktail with 15mer HAdV peptides

Sequence	Source protein (UniProt-ID)	Position	HLA-restriction
EWNFRKDVNMVLQSS	CAPSH_ADE02	598-612	DR3/DR4
DEPTLLYVLFEVFDV	CAPSH_ADE02	927-941	DR
TLRFIPVDREDTAYS	CAPSH_ADE02	65-79	DR4
LNRFVNTYTKGLPLA	PKG1_ADE02	353-367	DR
NIALYLPDKLKYNPT	CAPSH_ADE02	492-506	DR
MHLWRAVVRHKNRLL	E1BS_ADE02	120-134	DR
PQKFFAIKNLLLLPG	CAPSH_ADE02	579-593	DR
LPLLIPLIAAAIGAV	COR10_ADE02	53-67	DR
RGIFCVVKQAKLTYE	E3145_ADE02	46-60	DR
RQVMDRIMSLTARNP-NH2	CAP3_ADE02	28-42	DR
IGAVPGIASVALQAQ	COR10_ADE02	64-78	DR
TLVLAFVKTCAVLAA	LEAD_ADE02	32-46	DR

We included epitopes already tested in ELISpot screening assays, with recognition rates ≥ 50%. The resulting epitope cocktail was employed as a positive control for ELISpot assays to come. Peptides of this cocktail that were not yet tested in single peptide ELISpot assays in 20 PBMC cultures of healthy donors were further confirmed in parallel.

3.2.4 Intracellular cytokine staining shows $CD4^+$ T cell response elicited by HAdV epitopes

This work aimed to design a peptide cocktail only containing HLA class II epitopes that stimulate $CD4^+$ T cells to produce IFN-γ.

ICS was performed to further characterize the T-cell population, which produced IFN-γ in the ELISpot-screening assays. An ICS is less sensitive than an IFN-γ ELISpot assay but more specific as it can provide information about the cell lineage that is producing IFN-γ. We selected PBMCs of two different donors per peptide that showed strong responses to the POI in the single-peptide ELISpot-screening assays. PBMCs of the selected donors were thawed and incubated with the POI and a negative peptide. After a 12-day stimulation protocol, we performed an ICS. A peptide was considered as HLA class I or class II epitope, when the average of the IFN-γ and TNF double positive cells was ≥0.1% in the $CD8^+$ or $CD4^+$ T cell population, respectively. Secondly, the percentage of IFN-γ and TNF double-positive population resulting from the POI had to be at least three times the average of IFN-γ and TNF double positive population resulting from the negative peptide.

3.2.4.1 ICS results of the 12 peptides from the first adenoviral HLA class II cocktail

By performing an ICS, we saw that 10 of the 12 peptides from the first cocktail activated CD4+ T cells primarily. The peptide LPLLIPLIAAAIGAV was the only one that activated mainly $CD8^+$ T cells. In the PBMC cultures of the donors 1764 and 2492, neither a specific $CD4^+$ nor a specific $CD8^+$ T-cell activation could be detected when incubated with EWNFRKDVNMVLQSS. The peptide EWNFRKDVNMVLQSS was published as an HLA class II epitope, but we were not able to confirm this. (162) A positive response to IGAVPGIASVALQAQ could be detected in the $CD4^+$ as well as in the $CD8^+$ T-cell population of the PBMCs of donor 2163, with a stronger $CD4^+$ response.

Table 21: ICS results of single peptides of the first adenoviral HLA class II epitope cocktail tested in PBMCs of two different donors that showed positive responses in ELISpot screening assays, each. Percentages of INF-γ and TNF double positive cells of the $CD8^+$ and $CD4^+$ T-cell fraction are calculated by subtracting the average percentage of the double positive cells of the negative stimulation from the average percentage of double positive cells after stimulation with the POI. ELISpot count is calculated by subtracting the average spot count of the PBMCs incubated with the negative control from the average spot count of the PBMCs incubated with the POI. Color coding: light green when $CD4^+$ stimulation, light orange when $CD8^+$ stimulation.

IGAVPGIASVALQAQ	2163			2176		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	3.76%	0.92%	741	1.60%	0.00%	659
RGIFCVVKQAKLTYE	2488			2494		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	4.23%	0.00%	686	6.83%	0.00%	1976
PQKFFAIKNLLLLPG	2490			2494		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	46.57%	0.00%	1961	21.58%	0.00%	1976
MHLWRAVVRHKNRLL	1336			2494		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	14.75%	0.10%	1890	0.67%	0.01%	320
LPLLIPLIAAAIGAV	2275			2509		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	0.28%	1.38%	1986	0.05%	3.99%	811
RQVMDRIMSLTARNP-NH2	2509			2510		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	0.57%	0.00%	176	0.23%	0.00%	821
DEPTLLYVLFEVFDV	2481			2484		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	2.62%	0.16%	571	0.16%	0.00%	455
TLRFIPVDREDTAYS	2494			2540		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	2.72%	0.04%	279	5.01%	0.15%	873
NIALYLPDKLKYNPT	2573			2574		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	0.25%	0.00%	139	0.18%	0.00%	212
EWNFRKDVNMVLQSS	1764			2492		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	0.00%	0.00%	667	0.12%	0.14%	576
LNRFVNTYTKGLPLA	2507			2510		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	4.36%	0.00%	645	4.40%	0.01%	426
TLVLAFVKTCAVLAA	2100			2503		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	0.33%	0.00%	97	16.12%	0.00%	742

DEPTLLYVLFEVFDV did not elicit a positive response in the $CD4^+$ T-cell population of the PBMCs of donor 2484. Nevertheless, a difference between the $CD4^+$ and $CD8^+$ T-cell populations of the PBMCs of donor 2484 can be observed when incubated with DEPTLLYVLFEVFDV, indicating an HLA class II molecule restriction. The PBMCs of donor 2481 showed strong activation of $CD4^+$ T cells when incubated with DEPTLLYVLFEVFDV.

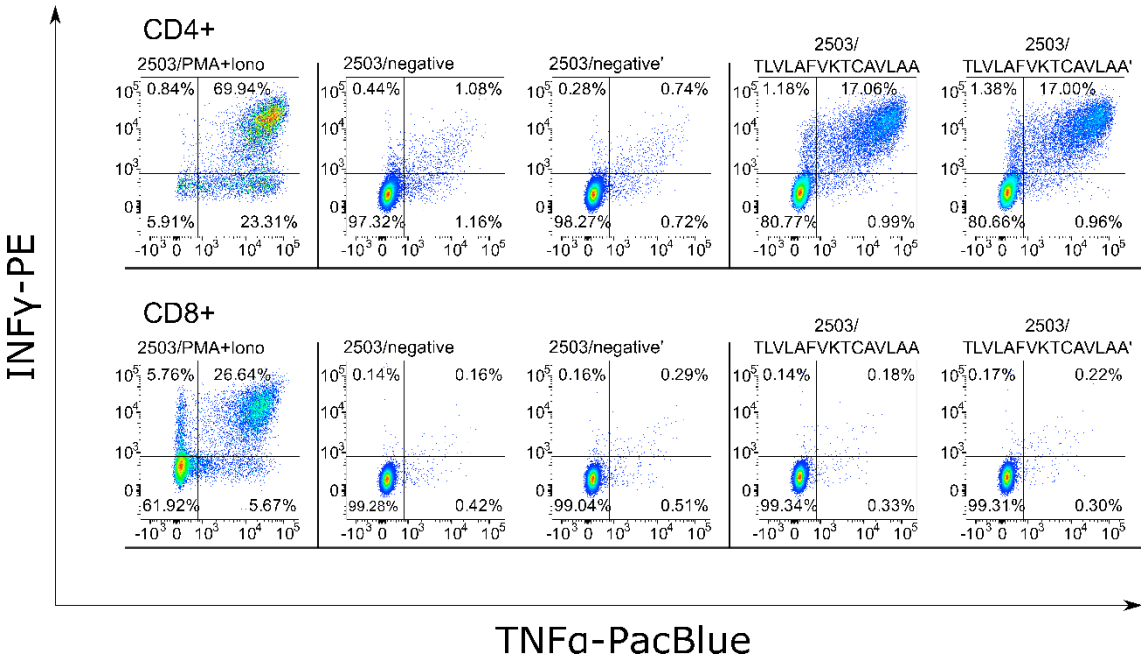
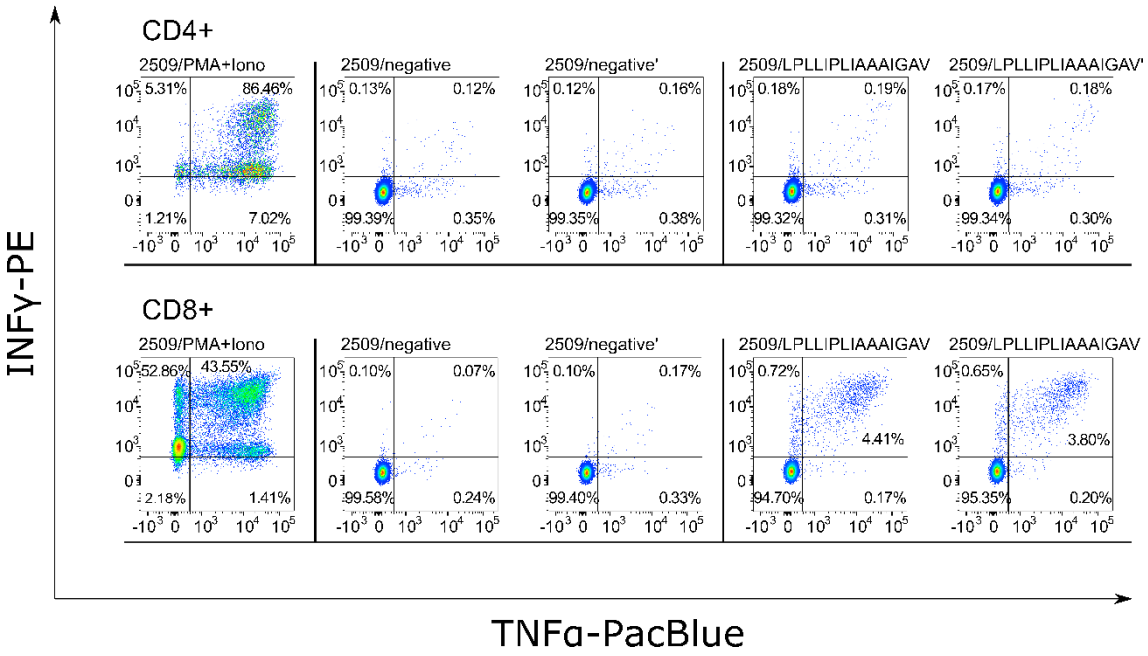


Figure 15: Last gate of the ICS gating strategy shown in Figure 10 for the peptide TLVLAFVKTCAVLAA in donor 2503. The upper row shows the gate of $CD4^+$ T cells, the lower row $CD8^+$ T cells. The first column is the positive control with PMA/Ionomycin. The second and third columns are the negative controls. The two right columns show the response to the POI.

Ten of the 12 tested peptides from the first adenoviral HLA class II cocktail showed a similar response to the one shown in Figure 15. There is a large population of $CD4^+$ T cells that produce TNF and IFN-γ, when stimulated with TLVLAFVKTCAVLAA, compared to the incubation with the negative control peptide (17.0% vs. 0.9%). There is no significant difference in the $CD8^+$ T-cell population between the incubation with the negative control peptide and TLVLAFVKTCAVLAA, indicating that TLVLAFVKTCAVLAA binds to HLA class II molecules. The HLA class II-peptide

complex can interact with the TCR and the CD4 co-receptors and thereby induce TNF and IFN-γ production in $CD4^+$ T cells.



<u>Figure 16:</u> Last gate of the ICS gating strategy shown in Figure 10 for the peptide LPLLIPLIAAAIGAV in the donor 2509. The upper row shows the gate of $CD4^+$ T cells, the lower row $CD8^+$ T cells. The first column is the positive control with PMA/Ionomycin. The second and third columns are the negative controls. The two right columns show the response to the POI.

Figure 16 shows that the peptide LPLLIPLIAAAIGAV activates $CD8^+$ T cells. When looking at the $CD8^+$ T-cell population, an increase of INF-γ and TNF production in response to LPLLIPLIAAAIGAV is visible compared to the negative control. The PBMCs stimulated with LPLLIPLIAAAIGAV do not differ from the $CD4^+$ T-cell population when compared to the PBMCs incubated with the negative peptide.

3.2.4.2 ICS results of the four later synthesized peptides, not yet present in the first HAdV HLA class II epitope cocktail

The four peptides TFYLNHTFKKVAITF**,** VSKFFHAFPSKLHDK, KNRLLLLSSVRPAII, and TLLYLKYKSRRSFID were tested subsequently in single peptide ELISpot screening assays with the first HAdV HLA class II cocktail as positive control and showed recognition rates of above 50%. To see if they could be added to the HLA class II epitope cocktail, an ICS was performed to prove their HLA class II restriction.

Table 22: ICS results of later discovered HLA class II epitopes tested in PBMCs of two different donors, which showed positive responses in ELISpot screening assays, each. Percentages of INF-γ and TNF double positive cells of the $CD8^+$ and $CD4^+$ T-cell fraction are calculated by subtracting the average percentage of the double positive cells of the negative stimulation from the average percentage of double positive cells after stimulation with the POI. ELISpot count is calculated by subtracting the average spot count of the PBMCs incubated with the negative control from the average spot count of the PBMCs incubated with the POI. Color coding: light green when positive for $CD4^+$ stimulation.

TFYLNHTFKKVAITF	2536			2601		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	2.72%	0.00%	870	18.63%	0.26%	1988
VSKFFHAFPSKLHDK	2540			2601		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	0.51%	0.00%	670	5.08%	0.43%	1988
KNRLLLLSSVRPAII	2537			2603		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	2.86%	0.00%	716	0.06%	0.00%	1905
TLLYLKYKSRRSFID	2536			2606		
	$CD4^+$	$CD8^+$	ELISpot	$CD4^+$	$CD8^+$	ELISpot
	0.42%	0.06%	808	0.22%	0.02%	1938

All four peptides were shown to activate $CD4^+$ T cells in the ICS assay. The peptides TFYLNHTFKKVAITF, VSKFFHAFPSKLHDK, and TLLYLKYKSRRSFID activated $CD4^+$ T cells in both tested donors. The $CD4^+$ T-cell population of the PBMCs of donor 2537 showed a strong response to KNRLLLLSSVRPAII. In contrast, no stimulation of the $CD4^+$ T-cell population of the PBMCs of donor 2603 was detectable when incubated with KNRLLLLSSVRPAII. The average of double positive cells of the $CD4^+$ T-cell population of donor 2603 was 0.85% when incubated with KNRLLLLSSVRPAII compared to 0.01% of the $CD8^+$ T cell-population. It was categorized as an HLA class II epitope because of the strong response of the $CD4^+$ T-cell population in the PBMC culture of donor 2537 to KNRLLLLSSVRPAII.

3.2.5 ELISpot results of 15mer peptide LPLLIPLIAAAIGAV in single peptide screening

The peptide LPLLIPLIAAAIGAV was shown to activate $CD8^+$ T cells *via* ICS assay. The TCR of $CD8^+$ T cells usually interacts with HLA class I molecules. A typing for HLA-A and HLA-B alleles was performed in advance by the blood bank of the University of

Tübingen for all donors whose PBMCs were used for the ELISpot testing of LPLLIPLIAAAIGAV.

Table 23: HLA-A and HLA-B typing and ELISpot results after 12-day stimulation protocol for donors tested with LPLLIPLIAAAIGAV

Donor	HLA-A		HLA-B		ELISpot result
2274	A*02	A*02	B*44	B*44	-
2275	A*02	A*02	B*44	B*56	+
2276	A*03	A*11	B*38	B*62	-
2281	A*02	A*66	B*27	B*41	+
2284	A*02	A*33	B*14	B*14	-
2285	A*02	A*31	B*18	B*55	+
2292	A*02	A*25	B*18	B*35	+
2297	A*02	A*29	B*44	B*58	+
2497	A*02	A*02	B*49	B*57	+
2500	A*26	A*26	B*58	B*60	+
2501	A*03	A*03	B*35	B*55	+
2503	A*24	A*28	B*35	B*44	-
2506	A*02	A*29	B*07	B*38	+
2507	A*02	A*02	B*18	B*47	+
2509	A*02	A*03	B*18	B*55	+
2510	A*02	A*29	B*44	B*57	+
2561	A*24	A*25	B*61	B*62	-
2563	A*01	A*26	B*08	B*45	+
2564	A*24	A*28	B*35	B*62	-
2567	A*01	A*26	B*62	B*64	+
2570	A*02	A*25	B*44	B*62	+
2573	A*01	A*11	B*08	B*14	+
2574	A*01	A*28	B*08	B*60	+
2575	A*02	A*29	B*08	B*27	-

3.2.6 Generation of the final HLA class II epitope cocktail from adenoviral antigens

The selection criteria for the peptides in the final HAdV HLA class II-peptide cocktail (FC) was a recognition rate above 50% after being tested in PBMCs of at least 20 different donors. Some PBMCs were overreactive and could not be considered. All the epitopes of the FC were proven to be HLA class II-restricted *via* ICS. (186)

LPLLIPLIAAAIGAV was removed from the cocktail as it activated $CD8^+$ T cells. The peptides EWNFRKDVNMVLQSS, LNRFVNTYTKGLPLA, and

NIALYLPDKLKYNPT were removed from the first HAdV HLA class II cocktail as well since their recognition rates dropped below 50%.

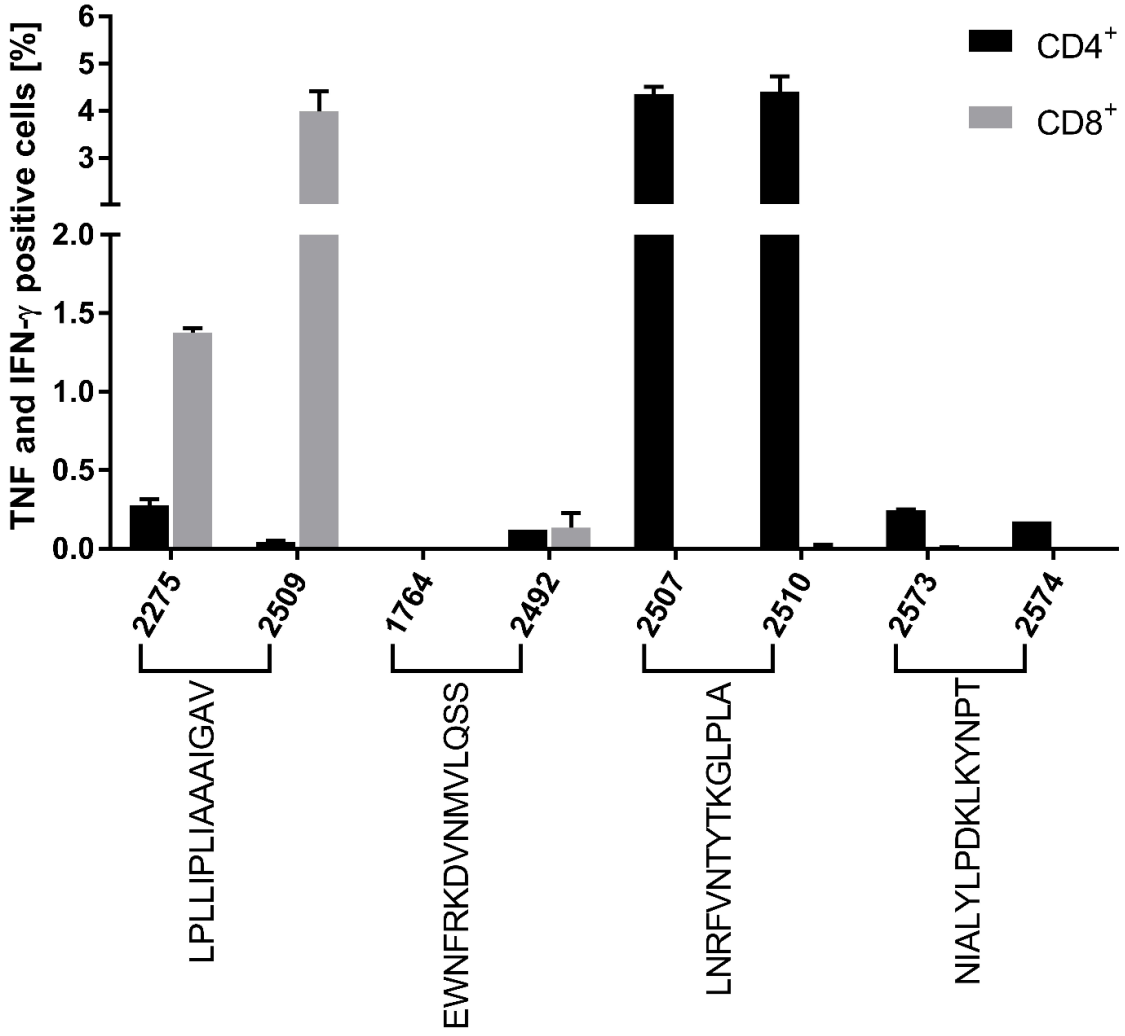


Figure 17: ICS results of epitopes removed from the first HAdV HLA class II cocktail. Comparison of the percentage of single, viable $CD4^+$ T cells that are double positive for IFN-γ and TNF secretion and the rate of single, viable $CD8^+$ T cells that are double positive for IFN-γ and TNF after stimulation with POI. The average percentage of double positive cells of the negative controls was subtracted from the rate of double positive cells of each well incubated with the POI. Each peptide was tested in two PBMC cultures.

These four peptides were replaced by the four subsequently characterized epitopes TFYLNHTFKKVAITF**,** VSKFFHAFPSKLHDK, KNRLLLLSSVRPAII, and TLLYLKYKSRRSFID. They all showed high recognition rates in single peptide ELISpot

screening assays (see tables 18 and 19) and were all proven to be HLA class II-restricted *via* ICS (see table 22).

PBMCs of 44 healthy donors tested in ELISpot assays with the first HAdV HLA class II epitope cocktail could all be stimulated to secrete IFN-γ. The peptide cocktail has a recognition rate of 100% so far. The PBMCs of the donor 2413, which could not be stimulated by either of the HAdV HLA class I epitope cocktails were stimulated by the first HAdV HLA class II cocktail.

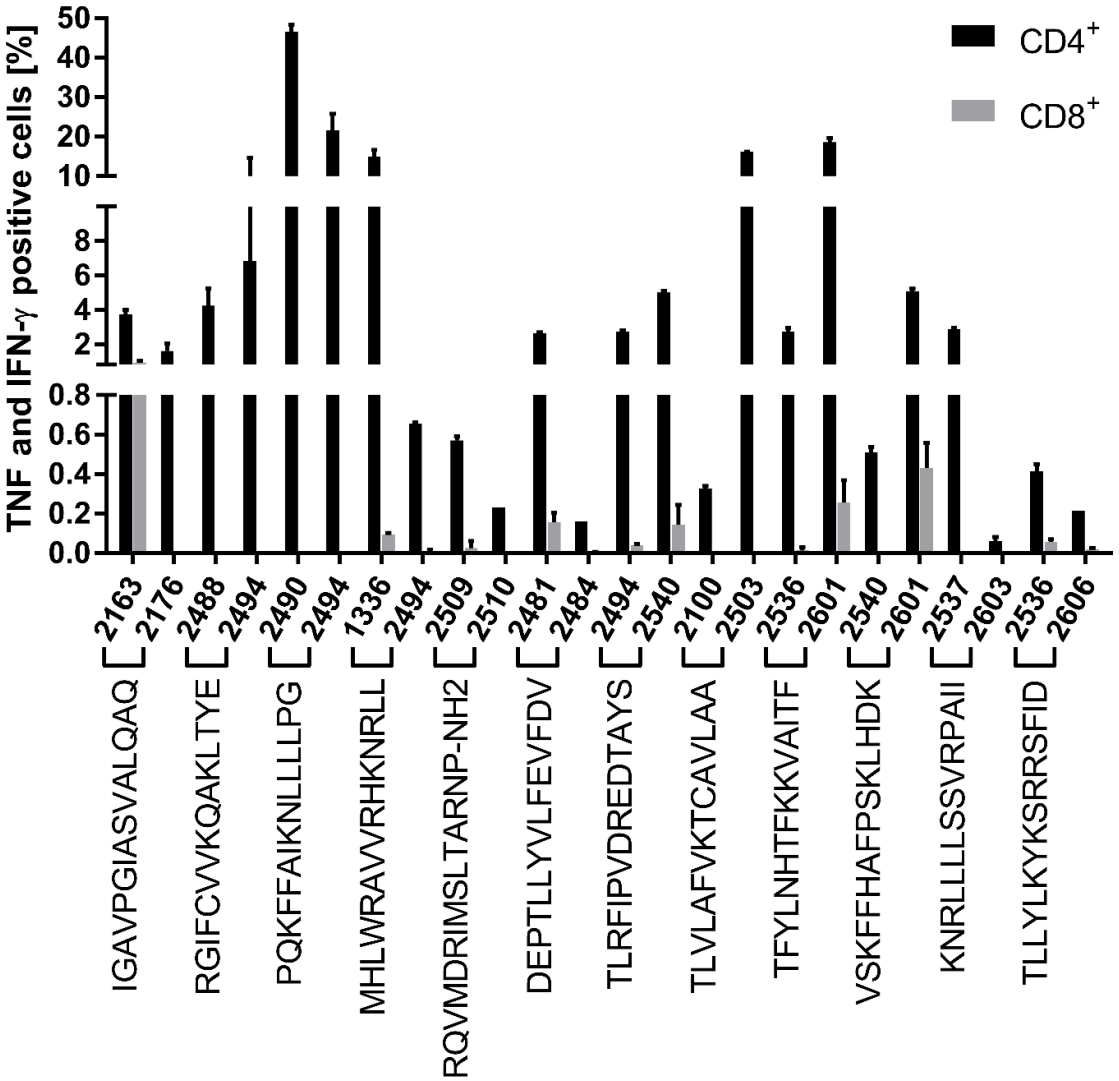


Figure 18: ICS results of epitopes from the final cocktail. Comparison of the percentage of single, viable $CD4^+$ T cells, double positive for IFN-γ and TNF secretion with the percentage of single, viable $CD8^+$ T cells, double positive for IFN-γ and TNF after stimulation with the POI. The average percentage of the double positive cells of the negative controls was subtracted from the percentage of double positive cells of each well incubated with the POI. Each peptide was tested in two PBMC cultures. (186)

The final adenoviral HLA class II cocktail consisted of 12 peptides, all showing recognition rates of at least 50% in single peptide ELISpot screening assays. Moreover, they were all proven to stimulate $CD4^+$ T cells and could thereby be categorized as HLA class II epitopes. (186)

Table 24: Epitopes contained in the final cocktail. Color coding: In dark grey, the four epitopes that were added and replaced LPLLIPLIAAAIGAV, EWNFRKDVNMVLQSS, LNRFVNTYTKGLPLA, and NIALYLPDKLKYNPT (replaced epitopes are not shown). In light grey and white are peptides that were already contained in the first HAdV HLA class II cocktail.

Sequence	Source protein (UniProt-ID)	Position	HLA-restriction
DEPTLLYVLFEVFDV	CAPSH_ADE02	927-941	DR
TLRFIPVDREDTAYS	CAPSH_ADE02	65-79	DR
MHLWRAVVRHKNRLL	E1BS_ADE02	120-134	DR
PQKFFAIKNLLLLPG	CAPSH_ADE02	579-593	DR
RGIFCVVKQAKLTYE	E3145_ADE02	46-60	DR
RQVMDRIMSLTARNP-NH2	CAP3_ADE02	28-42	DR
IGAVPGIASVALQAQ	COR10_ADE02	64-78	DR
TLVLAFVKTCAVLAA	LEAD_ADE02	32-46	DR
TFYLNHTFKKVAITF	CAPSH_ADE02	727-741	DR
VSKFFHAFPSKLHDK	PKG1_ADE02	292-306	DR
KNRLLLLSSVRPAII	E1BS_ADE02	130-144	DR
TLLYLKYKSRRSFID	E3GL_ADE02	140-154	DR

3.2.7 Testing of the final HAdV HLA class II cocktail in ELISpot assay, after 12-day stimulation

The final HAdV HLA class II cocktail was tested in an ELISpot screening assay in PBMC cultures of healthy donors. Twenty-nine of the 32 tested PBMC cultures were selected randomly. The PBMCs of the donors 2226, 2255, and 2413 were chosen because they did not respond to any of the HAdV HLA class I epitope cocktails.

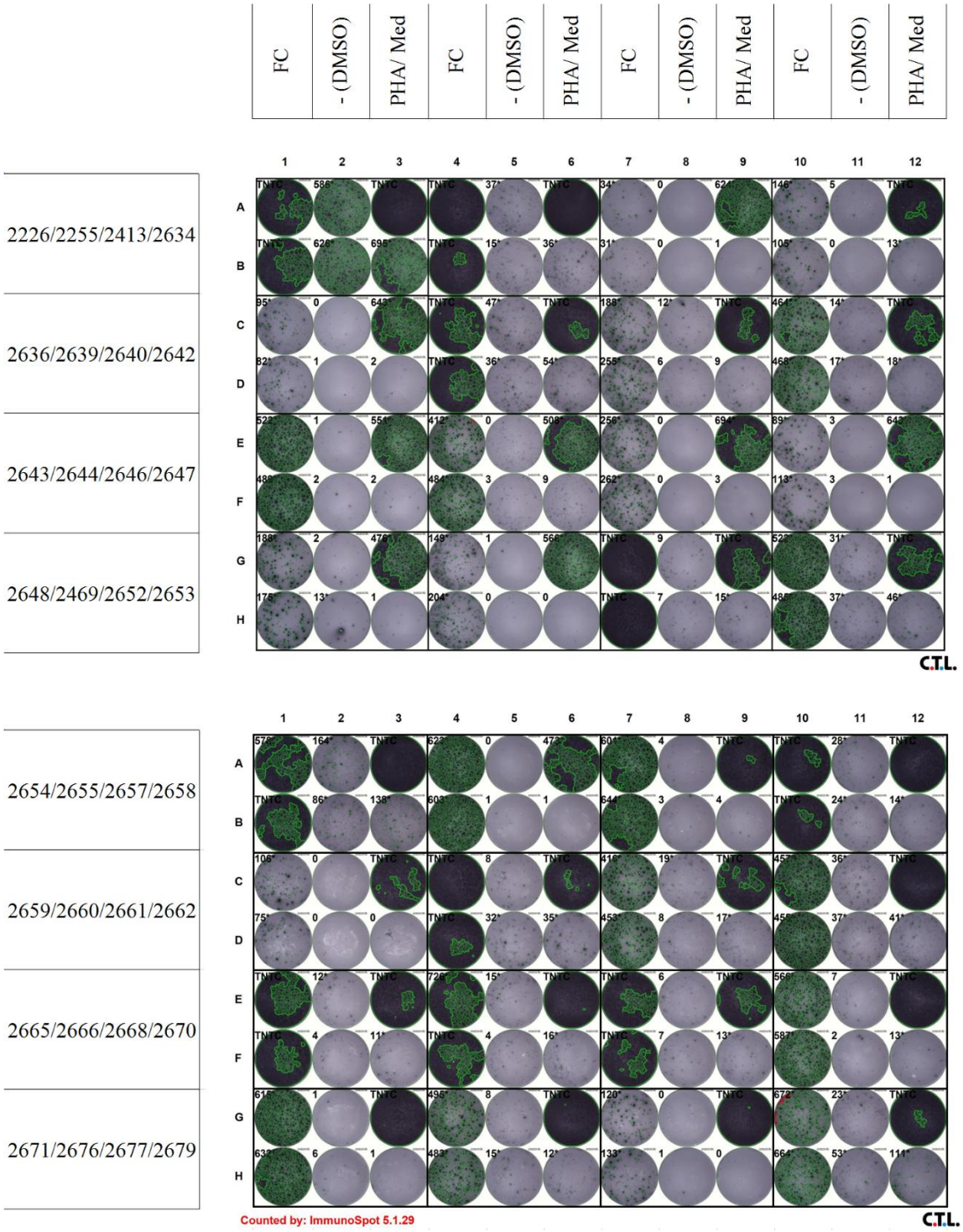


Figure 19: ELISpot assays testing the final HAdV HLA class II peptide cocktail (FC). DMSO was used as a negative control in the same concentration as it was present in the wells incubated with the POI. PHA was used as positive control alternately with TCM as a negative control. A black box separates donor samples. The spot count was set to 2,000 spots when too-numerous-to-count (TNTC). PBMCs of sixteen donors could be tested on one 96-well ELISpot plate. In total, 32 donors were tested. The EliSpot result of donor 2657 is shown as a representative ELISpot in (186).

Unfortunately, the PBMCs of donor 2226 in ELISpot #18 were overreactive and could not be considered (see Figure 19).

The FC could stimulate 31 out of 31 PBMC cultures from healthy donors. (186) The PBMCs of donors that did not respond to the HLA class I cocktails were responsive to the final HAdV HLA class II cocktail.

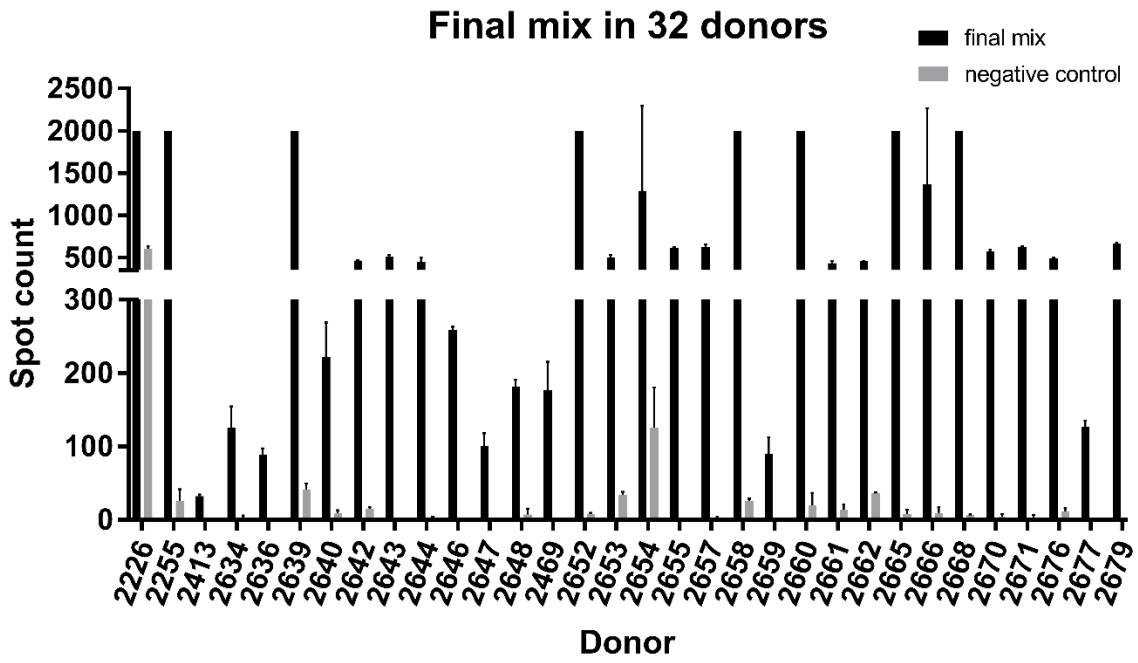


Figure 20: Comparison of spot counts of final cocktail in PBMC cultures of 32 different donors after a 12-day stimulation protocol. Black bars depict spot counts after stimulation with the final cocktail. Grey bars represent spot counts of negative control.

3.2.8 *Ex-vivo* ELISpot assay with ten epitopes with highest recognition rates from the final cocktail

The POIs are added to the cells before several IL-2 stimulations during the 12-days stimulation, to ensure that the T cells recognizing the POI proliferate. Therefore, the ELISpot-screening assay only indicates if the healthy donors have any T-cell clones recognizing the POI.

In an *ex vivo* ELISpot assay, the cells are thawed and analyzed after one day of resting. As a result, an *ex vivo* ELISpot is less sensitive but indicates the number of T cells recognizing the POI instantly.

In our ELISpot assay setting, there are always approximately 500,000 cells per well. From the difference of spot counts between the PBMCs stimulated with the negative control peptide and the PBMCs stimulated with the POI, one can derive how many T cells recognize a particular peptide. Another conclusion that can be drawn from the results of an *ex vivo* ELISpot assay is how well memory T cells are amplified during the 12-day stimulation protocol. One prerequisite is that the same number of cells is seeded into each well for all ELISpot assays. In this case, the amplification factor (AF) of specific memory T cells is calculated as the ratio of the difference between the average spot count obtained from the negative control peptide (NP) and the average spot count obtained with the POI in an ELISpot screening assay after the 12-day stimulation protocol and the same difference obtained from the *ex vivo* ELISpot results.

$$AF = \frac{\frac{POI_{12}\ 1 + POI_{12}\ 2}{2} - \frac{NP_{12}\ 1 + NP_{12}\ 2}{2}}{\frac{POI_{ex}\ 1 + POI_{ex}\ 2}{2} - \frac{NP_{ex}\ 1 + NP_{ex}\ 2}{2}}$$

The *ex vivo* ELISpot assay was performed with the ten peptides from the final HAdV HLA class II cocktail with the highest recognition rates. The PBMC cultures were selected so that each peptide was incubated with the PBMCs of at least one donor that showed reasonable response rates after the 12-day stimulation protocol. Therefore, not all peptides had already been tested in all donors they were incubated with, in the *ex vivo* setting. The AF can only be calculated if the PBMCs of the donor were tested positive following a 12-day stimulation.

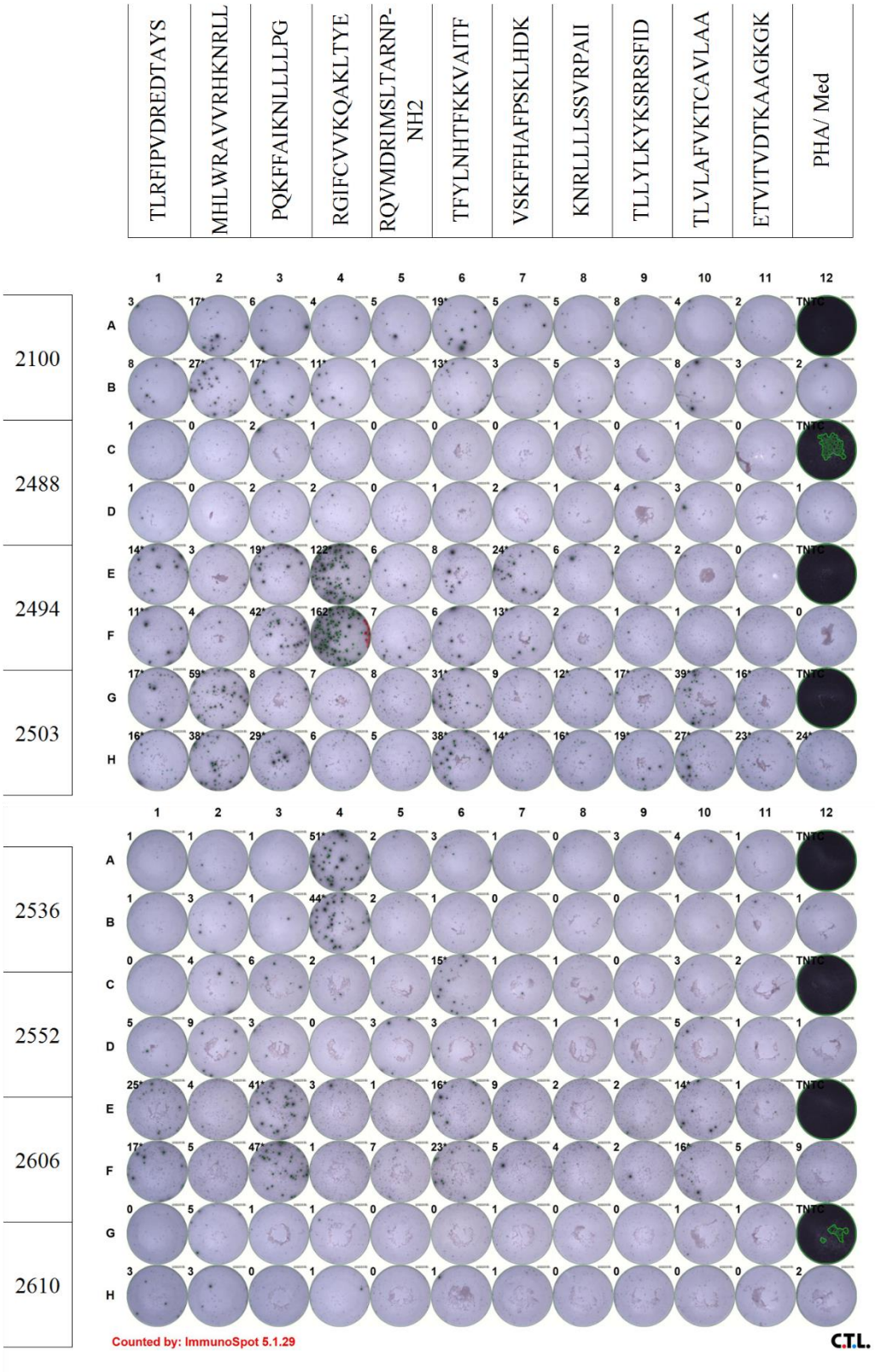


Figure 21: Results of the ex vivo ELISpot assay. In row 1-10 are the ten epitopes with the highest recognition rates of the 12 peptides from the final HAdV HLA class II cocktail. Row 11 was stimulated with the negative control peptide. Row 12 is incubated alternately with PHA as unspecific positive control and TCM as a negative control.

When no significant stimulation of the PBMCs of a donor by a peptide could be observed in the *ex vivo* ELISpot assay, the spot count was set to ten to compute the AF.

Five different constellations are possible when comparing the ELISpot results after the 12-day stimulation protocol with the results of the *ex vivo* ELISpot assay. First, epitopes have already been screened in both 12-day stimulation ELISpot assay and *ex vivo* ELISpot in the same donor. When the peptides could stimulate the PBMCs of a donor after the 12-day stimulation protocol, the result of the *ex vivo* ELISpot assay can be positive or negative. These are the two cases in which an AF can be calculated. When there was no detectable stimulation by a peptide after a 12-day stimulation protocol, the *ex vivo* ELISpot assay is likely negative as well. However, most of PBMCs were not tested in both the *ex vivo* and the 12-day simulation ELISpot assay settings. Nonetheless, a positive response can be observed in an *ex vivo* ELISpot assay without knowing the result of the 12-day stimulation. No AF can be calculated when the peptides have not been screened for recognition by the PBMCs of a donor in the *ex vivo* setting and the 12-day stimulation protocol.

Peptide / Donor	TLRFIPVDREDTAYS	MHLWRAVVRHKNRLL	PQKFFAIKNLLLLPG	RGIFCVVKQAKLTYE	RQVMDRIMSLTARNP-NH2
2100				66	64
2488		67	74	69	
2494	107	32	66	14	
2503					13
2536		200		43	
2552	58	70			
2606					
2610					

Peptide / Donor	TFYLNHTFKKVAITF	VSKFFHAFPSKLHDK	KNRLLLLSSVRPAII	TLLYLKYKSRRSFID	TLVLAFVKTCAVLAA
2100					44
2488					
2494					
2503					74
2536	87	9	4	81	
2552	70				
2606	117	194	194	194	
2610	198	70	198	51	

IFN-µ secretion of PBMCs can be observed after 12-day stimulation, as well as in *ex vivo* setting
IFN-µ secretion of PBMCs can be observed after 12-day stimulation, but not in *ex vivo* setting
IFN-µ secretion of PBMCs can neither be observed after 12-day stimulation, nor in *ex vivo* setting
IFN-µ secretion of PBMCs can be observed in *ex vivo* setting, but PBMCs not tested for activation after 12-day stimulation
IFN-µ secretion of PBMCs can not be observed in *ex vivo* setting, PBMCs not tested after 12-day stimulation with POI

Figure 22: Different peptide-PBMC constellations in ex vivo ELISpot assay. AF is computed when comparable to the same peptide-PBMC constellation in the 12-day stimulation result.

Thirty-five peptide-PBMC constellations were tested in both the 12-day stimulation and in *ex vivo* setting. All six peptide-PBMC constellations, which were negative after the 12-day stimulation protocol, were also negative in the *ex vivo* setting (orange in Figure

22). Of the twenty-nine peptide-PBMC constellations that showed a positive response after the 12-day stimulation protocol, only five (17.2%) of the constellations showed a positive reaction in the *ex vivo* setting (red in Figure 22). In return, this means that in 29 tested peptide-PBMC constellations in which activation of the PBMCs was detected in the 12-day stimulation setting, no stimulation was observed in 24 out of 29 (82.8%) of the constellations in the *ex vivo* setting (blue in Figure 22).

From the 45 PBMC-peptide constellations that were not tested in the ELISpot-screening setting, seven (15.6%) of the PBMC cultures could be driven to a detectable IFN-γ secretion in the *ex vivo* setting (yellow in Figure 22). In contrast, no stimulation could be detected in 38 (84.4%) of the constellations (grey in Figure 22).

Overall, we observed an average AF of 87. However, we see a high variation, with AF ranging from four to 200.

3.2.9 Homology between different adenovirus strains

3.2.9.1 Uniprot-BLAST for identical sequence

Epitope sequences included in the FC share homologies with reviewed protein sequences of at least two different HAdV serotypes and some even across genera. (186)

Table 25: Uniprot blast of the 12 peptides of the final cocktail. The 12 peptides from the FC were queried for exact sequence overlap in proteins from the UniProt database. The blast was performed with uniprot.org (171). Only reviewed sources were considered.

Sequence	Appearance in serotypes
DEPTLLYVLFEVFDV	ADE02; ADE05; ADE12; ADE40; ADE41
TLRFIPVDREDTAYS	ADE02; ADE05
MHLWRAVVRHKNRLL	ADE02; ADE05
PQKFFAIKNLLLLPG	ADE02; ADE05; ADE08; ADE09; ADE31; ADE40; ADE41
RGIFCVVKQAKLTYE	ADE02; ADE06
RQVMDRIMSLTARNP-NH_2	ADE02; ADE05
IGAVPGIASVALQAQ	ADE02; ADE05
TLVLAFVKTCAVLAA	ADE02; ADE05
TFYLNHTFKKVAITF	ADE02; ADE05
VSKFFHAFPSKLHDK	ADE02; ADE05; ADE07; ADE12; ADE40
KNRLLLLSSVRPAII	ADE02; ADE05
TLLYLKYKSRRSFID	ADE02; ADE06

The blast for the epitopes of the FC shows that there are conserved regions - highly recognized in healthy donors. The epitope PQKFFAIKNLLLLPG is present in seven out of the 14 reviewed sequences of the UniProt database (171). (186)

Some epitopes only appear in the protein aa sequence of two different serotypes. In some cases, the reviewed protein sequence is only available for a low number of serotypes.

3.2.9.2 Alignment of epitopes from the final HLA class II cocktail with reviewed proteins from different HAdV serotypes

We performed an alignment of reviewed HAdV proteins with epitopes from the FC to see if this could explain the high cross-reactivity of adenovirus-specific T cells. In rare cases, the alignment does not give more information than the peptide blast, because some proteins have only been sequenced from two serotypes with similar peptide sequences. In other alignments, it is possible to detect protein sequences from different serotypes that share peptide sequences with epitopes of the FC. The aligned proteins are CAPSH, PKG1, E1BS, E3GL, E3145, CAP3, COR10, and LEAD. We aligned 14 reviewed CAPSH protein sequences from different HAdV serotypes. The reviewed sequence of five different HAdV serotypes was available for the PKG1 protein. For the E1BS protein, the reviewed sequences for seven different HAdV serotypes were available. For the proteins E3GL, E3145, CAP3, COR10, LEAD the available reviewed protein sequences for seven, five, three, two, and two different HAdV serotypes were present in the UniProt database (171), respectively. (186)

```
sp|P68978|E3GL_ADE02      YKLWPPQKC--LENTGTFCSTALLITALALVCTLLYLKYKSRRSFIDEKKMP  159
sp|P68979|E3GL_ADE06      YKLWPPQKC--LENTGTFCSTALLITALALVCTLLYLKYKSRRSFIDEKKMP  159
sp|P04494|E3GL_ADE05      YKLWPPQNC--VENTGTFCCTAMLITVLALVCTLLYIKYKSRRSFIEEKKMP  160
sp|P11323|E3GL_ADE03      YDLWPPSKENIVAFSIAYCLVTCIITAIICVCIHLLIVIRPRQSNEEKEKMP  172
sp|P35771|E3GL_ADE1A      YSLWPPSKDNIVTFSIAYCLCACLLTALLCVCIHLLVTTRIKNAN-NKEKMP  166
sp|P68981|E3GL_ADE35      YSLWPPSKDNIVTFSIAYCLCACLLTALLCVCIHLLVTTRIKNAN-NKEKMP  166
sp|P68980|E3GL_ADE1P      YSLWPPSKDNIVTFSIAYCLCACLLTALLCVCIHLLVTTRIKNAN-NKEKMP  166
                          *.****.:   :  : ::*  : ::*.:  **  * :  : :.:  :::***
```

<u>Figure 23:</u> Alignment of the reviewed protein sequences from the protein E3GL of the HAdV serotypes 1A, 1P, 02, 03, 05, 06, and 35. The aa sequence of the epitope TLLYLKYKSRRSFID in position 140-154 of the aa sequence of E3GL of HAdV2 is highlighted in blue. Color coding indicates the chemical property of the aa. "*" indicates perfect alignment. ":" indicates high similarity. "." indicates weak similarity.

It is visible that some positions of the HAdV2-derived epitope barely differ from the peptide sequence of other HAdV serotypes. The alignments of the epitope regions of the other source proteins of the FC epitopes were also performed (see appendix).

4 Discussion

4.1 Two HLA class I epitope cocktails in comparison

When we compared the stimulation of PBMC cultures by two HLA class I cocktail – one containing 24 HLA class I epitopes, the other containing 15 HLA class I epitopes – we could detect an IFN-γ secretion in 22 out of 25 PBMC cultures when incubated with the 24-peptides-cocktail and in 20 out of 25 PBMC cultures when incubated with the 15-peptides-cocktail.

We show that the 24-peptides-cocktail was able to stimulate all PBMC cultures, which the 15-peptides-cocktail was able to stimulate. No suppression or competitive effects between peptides could be observed.

As the cells were cultivated and counted separately, it cannot be concluded that one cocktail was able to activate more PBMCs of the donor because of a higher number of spot counts.

The fact that the PBMCs from 22 out of 25 tested donors reacted to the 24-peptides-cocktail shows that these donors bear HAdV-specific $CD8^+$ memory T cells, indicating a previous HAdV encounter. The HLA class I epitopes were all derived from HAdV2. Due to the high homogeneity between different HAdV serotypes, it is impossible to conclude against which adenovirus serotype the immune response was mounted from the experiments performed in this work. We can envision that the donors could have had contact with HAdV2 or an entirely different serotype with shared epitopes. Nevertheless, it is very unprobeable that all 22 donors from whom the tested PBMCs were isolated already had had contact with HAdV2. This indicates a high adenoviral cross-reactivity of CTLs, as demonstrated previously by *Smith et al.* (159) and *Hutnick et al.* (161).

No HAdV serology was performed for the donors from whom the PBMCs were obtained. The lack of a previous encounter of adenoviral antigens would explain why the PBMCs of three donors could not be stimulated by either one of the HLA class I peptide cocktails. This would suggest that there are no memory T cells with adenovirus-antigen specificity present in the peripheral blood of those donors. Another explanation could be that the immune system of the donor encountered and cleared an adenoviral infection, but that the memory T cells recognize different epitopes than those present in the cocktails.

The existence of HLA supertypes can explain the positive immunogenic response of the PBMCs of donors with HLA gene products that are not predicted to be covered by peptides in the cocktail. To this time point, there are nine defined HLA-I supertypes, including 80% of the over 900 recorded HLA-A and HLA-B alleles. Those HLA supertypes are characterized by similar binding restrictions. (190) This could explain why epitopes that were not restricted to the HLA class I allotypes of a donor could activate some of the PBMCs by supertype cross-reactivity. Another explanation could be that some epitopes present in the cocktails can interact with HLA-C molecules, for which the donors were not typed, and thereby elicit an immune response.

4.2 HLA class II epitopes

The *ex vivo* adoptive T-cell transfer with predominantly T_H1 phenotype was shown to be highly effective in clearing viremia and markedly reduced mortality in patients with therapy-refractory HAdV infection following HSCT. (157) This indicates the importance of the identification of HAdV-derived HLA class II epitopes for the selection of $CD4^+$ T cells as a treatment option.

The high coverage of the predicted HLA class II peptides shows that they play a crucial role in the clearance of adenovirus infections. All tested PBMCs showed a response to the epitope cocktail, irrespective if it was the first HAdV-derived HLA class II cocktail, which also contained one 15mer HLA class I epitope, or the FC, only containing 15mer class II epitopes. These were 44 out of 44 PBMC cultures for the first HAdV HLA class II cocktail, and 31 out of 31 tested PBMC cultures for the final HAdV HLA class II cocktail. Except for the PBMCs of donor 2413, no other PBMC isolates were tested with both cocktails. There are two requirements for a positive result in an ELISpot assay: first the ability of the peptide to bind to an MHC molecule expressed by the donor's cells and secondly, the presence of memory T cells, which were generated during a previous encounter of the antigen from which the peptide is derived, that recognize the HLA-peptide complex. This means that all 74 donors expressed at least one MHC class II molecule capable of binding to one or more epitopes of the cocktails and that they all previously had had contact with adenoviral antigens, which led to the evolution of memory T cells. These were able to recognize at least one of the cocktail epitopes presented on an HLA molecule expressed by the donor's cells. These results also depict the high HAdV infestation.

4.2.1 Promiscuity of HLA class II epitopes and effectiveness of *in silico* prediction

Every HLA class II epitope present in the FC has a recognition rate of at least 50%. A prerequisite for a detectable response in the ELISpot assay is the ability of the peptide to bind to an HLA molecule expressed by the donor's cells. The tested healthy donors were not typed for their HLA class II alleles. As shown in Table 15, the HLA-DRB1*15:01 has the highest allele frequency of 0.1421 in Germany, with 26.4% of the individuals carrying it. This enforces the suggestion that peptides with recognition rates of above 50% are very

probable to be promiscuous epitopes by being able to bind to at least two different HLA class II gene products. As the characterized promiscuous epitopes have recognition rates to up to 85.7%, they are likely to be able to bind to more than two different HLA class II molecules. (186)

Due to the length of peptides bound by HLA class II molecules and their ability to extend out of the binding groove at both the N- and C-terminal ends (22-24), peptide-binding motifs cannot be determined as easily when compared to HLA class I molecules. Hence, HLA class II epitope prediction is still challenging. Although promiscuous binding of HLA class II epitopes is known, there is no satisfying prediction tool for promiscuous HLA class II epitopes. (182) The identification of promiscuous epitopes brings the advantage of non-single-HLA-restricted binding. The identified promiscuous epitopes can thereby lead to T cell activation in individuals carrying different HLA alleles. This leads to higher coverage of peptide recognition in a defined population when compared to single-HLA-restricted epitopes.

The 24 top-ranked epitope candidates from the promiscuous epitopes prediction were synthesized and tested in ELISpot assays. Eighteen (75.0%) were shown to be immunogenic. Six epitopes (25.0%) were identified as immunodominant epitopes. Twelve (50.0%) epitopes were categorized as subdominant epitopes. Six peptides (25%) did not lead to a detectable immune response. This means that 25.0% of the predicted promiscuous epitopes could bind to present HLA class II molecules and stimulate T cells in more than half of the PBMC cultures of healthy donors for which neither an HLA class II typing nor a HAdV-serology was performed. All three epitopes that were selected due to the combined SYFPEITHI/NetMHCIIpan 2.0 criterias were shown to be immunodominant epitopes. As these three epitopes were the only epitope candidates in the prediction to fit the criteria it seems to be efficient, but too strict. Too many promiscuous epitopes would have stayed undetected by only applying the combined criteria. A negative ELISpot result does not allow the conclusion that the predicted peptide could not bind to HLA class II molecules of cells present in a culture. There is a possibility that the peptide could be presented by HLA class II molecules but not be recognized by a TCR. Some of the already published peptides that showed high recognition rates in single-peptide ELISpot assays did not appear in our prediction. (186)

LPLLIPLIAAAIGAV was later shown to stimulate $CD8^+$ T cells. This indicates that it somehow binds to HLA class I molecules. Still, this approach seems to be very effective in the prediction of promiscuous HLA class II epitopes with high coverage rates.

The fact that the prediction was performed for HLA-DR molecules also brings the advantage that one MHC gene locus can encode more than one β-chain. Some β-chains are pseudogenes that are characterized by different defects. The HLA-DRB4 gene may be functional and is sometimes translated into a gene product that can pair with the conserved α-chain. (18, 20) Accordingly, some people can express four different HLA-DR molecules, which further increases the probability of a promiscuous epitope to be presented by one of the expressed HLA-DR molecules.

4.2.2 Homology leads to high cross-reactivity

The peptides in both HLA class II cocktails are derived from proteins from HAdV2. It is improbable that every donor already had had contact with HAdV2. The fact that the peptides, only derived from HAdV2, were able to stimulate the PBMCs of all 74 donors can be explained by T-cell cross-reactivity. The HAdV2-derived epitopes are conserved among multiple HAdV serotypes with an identical (see table 25) or very similar aa sequence (see alignment). Especially HAdV2 and HAdV5 share a lot of epitopes, which implies that the infection of a donor with another HAdV serotype could lead to a positive recall response when stimulated with an epitope derived from another serotype, as already demonstrated by *Flomenberg et al.* (107). (186)

The anchor positions of HLA class II molecules are only occupied by a few aas at specific locations of the peptide sequence. (184) Table 25 shows the peptide sequences that were blasted against the peptide sequence of other HAdV serotypes. Peptides with changes in one or more aas were not listed. Even leucine replaced by isoleucine or the other way around were not considered. As T-cell cross-reactivity against peptides with some aa changes can be observed, the high homogeneity between HAdV serotypes is probable to lead to cross-reactivity between different serotypes. Cross-reactivity seems to be rather limited by the interaction of the TCR to the HLA-peptide complex than by the peptide binding to the HLA molecule. (191) The HLA molecules, as well as the TCR CDR3 loop, were shown to undergo conformational changes when binding a peptide. The

modification of the peptide-MHC-complex interface to the TCR enables the recognition of different epitopes by one TCR. (192) *Kohm et al.* described the mechanism of molecular mimicry, which assumes that peptides sharing fundamental structural and chemical features can explain T-cell cross-reactivity. (193) This would implicate that two peptides from different HAdV serotypes differing in one aa with similar biochemical and physical characteristics could both lead to activation of the same TCR when assuming their ability to bind to the same HLA molecule. An exchange of an aa in other than the anchor position is even less likely to prevent HLA binding and T-cell activation. Contrary to the molecular mimicry theory, other workgroups state that T cells can cross-react to peptides with low similarity. *Wooldridge et al.* showed that a single autoimmune T-cell receptor could recognize a peptide differing from the sequence of the index peptide at seven of ten positions. They also computed the ability of more than $1x10^6$ different decameric peptides to activate the T cell *via* its TCR in the context of one HLA molecule. (194) As they investigated an autoimmune-reactive TCR, the results cannot be transferred on normal-functioning TCRs one-to-one. Cross-reactivity, to such an extent, is probable to trigger autoimmune-reactions.

The flanking aas were shown to influence peptide binding to HLA molecules and TCR interaction with the peptide-HLA complex (195-197). Both of these processes have direct effects on T-cell activation. The extent of the impairment of the flanking aa residues on T-cell activation is not yet understood. Therefore, it is hard to guess about the influence of aa polymorphisms between different HAdV serotypes, located in the flanking residues of epitopes, on T-cell cross-reactivity.

The alignments of the epitopes from the FC with other serotypes show single aa polymorphisms and make adenovirus-specific T-cell cross-reactivity very probable, consistent with previous work (107, 159-162). (186)

4.2.3 Immunodominant HLA class II epitopes could be identified in other proteins than the hexon *via* promiscuous *in silico* prediction

Most immunogenic adenoviral epitopes that have been characterized until now are derived from the hexon protein (163, 167-170). Not much effort has been invested in discovering epitopes derived from other adenoviral source proteins. In 2016 the penton

protein was characterized as a ``*second immunodominant target in human adenovirus infection´´* by *Tischer et al.* (176). Lately, *Gunther et al.* were able to identify an immunodominant HLA class I-restricted peptide epitope derived from the adenoviral 13.6 K protein (174). In our promiscuous epitope prediction, all HAdV2 proteins were treated equally. This led to the identification of immunodominant epitopes from other source proteins than the hexon and penton proteins. Of the seven newly discovered epitopes from the promiscuous prediction, which made it into the FC, without accounting the 10mer peptide TFYLNHTFKK elongated to the 15mer TFYLNHTFKKVAITF and the already published epitope TLRFIPVDREDTAYS, none is derived from neither the hexon nor the penton protein. All the HAdV hexon protein-derived peptides were already discovered earlier by performing non-promiscuous HLA class II epitope-predictions. Nevertheless, the peptides identified *via* non-promiscuous HLA class II epitope-predictions are very probable to be promiscuous HLA class II epitopes due to the high recognition rates in single-peptide ELISpot screening assays. The 10mer peptide TFYLNHTFKK, contained in TFYLNHTFKKVAITF, was characterized when screening for HLA class I epitopes. Other proteins that were already screened for immunogenic epitopes *in house* are the E1B-19kDa protein, the E1B-55kDa protein, the Early E1A protein, and the adenoviral DNA polymerase (see Table 17). The seven immunodominant epitopes which were discovered *via* promiscuous epitope prediction are derived from seven different adenoviral proteins from six different gene regions. The peptide KNRLLLLSSVRPAII is derived from the E1B gene product E1B-19k, which is an antiapoptotic mediator (58). The epitope sequences TLLYLKYKSRRSFID and RGIFCVVKQAKLTYE are derived from E3 gene products: the E3-19kDa protein and the E3-14.7kDa protein, respectively. Both proteins reduce CTL-mediated cytolysis in different ways. (16, 64) VSKFFHAFPSKLHDK is derived from the packaging protein I, which is encoded in the IVa2 gene. This protein is essential for the transcription of packaging proteins as well as the translocation of the viral DNA into preformed capsids. Its absence leads to the production of empty capsids. (198) The source protein of the epitope TLVLAFVKTCAVLAA is the I-leader protein, which is transcribed from the I-leader gene (see Figure 6). The remaining epitopes are both derived from late gene products. The peptide RQVMDRIMSLTARNP-NH2 is derived from the pre-protein or protein IIIa, which is encoded in the L1 gene locus. It is one of the cement proteins that

help to stabilize the capsid (see Figure 5). (45) The aa sequence of the epitope IGAVPGIASVALQAQ is present in pre-core protein X, also known as pMu. It is a core protein encoded in the L2 gene locus and associated with the viral DNA (199).

These findings indicate that immunodominant epitopes are derived from many different adenoviral proteins, which are transcribed from early, intermediate, and late genes. By performing a promiscuous epitope prediction, a wider range of proteins can be screened for immunogenicity. (186)

4.2.4 LPLLIPLIAAAIGAV activates $CD8^+$ $CD4^-$ T cells

We saw that the peptide LPLLIPLIAAAIGAV mainly stimulates $CD8^+$ $CD4^-$ T cells by performing an ICS assay. Usually, 8mer to 10mer peptides occupy the binding groove of HLA class I molecules (12-14). Due to the length of LPLLIPLIAAAIGAV, we did not expect binding to HLA class I and activation of $CD8^+$ T cells. Nevertheless, long peptides have been reported to bind to HLA class I molecules (200-202). Some workgroups analyzed the structure of longer peptides bound to HLA class I molecules. They observed longer peptides ‘bulging’ out of the binding groove. (201, 202) This may allow LPLLIPLIAAAIGAV binding to HLA class I molecules. The HLA class I-peptide complex could then activate $CD8^+$ T cells.

The recognition rate of LPLLIPLIAAAIGAV was 70.8%, with a positive response in PBMC cultures of 17 out of 24 donors. If LPLLIPLIAAAIGAV can be presented on HLA class I molecules, it is improbable that it is restricted to one HLA class I allotype. The feature of promiscuity was primarily attributed to HLA class II epitopes. *Frahm et al.* demonstrated the promiscuity of HLA class I epitopes (203). The donors tested with LPLLIPLIAAAIGAV were typed for their HLA-A and HLA-B alleles. A typing for HLA-C was not performed. As can be seen in Table 23, there is no specific HLA-A or HLA-B restriction limited to one HLA allotype that can be concluded from the results of the 24 tested PBMC isolates. All PBMC isolates recognizing LPLLIPLIAAAIGAV were typed for one of the following HLA class I alleles: HLA-A*01, HLA-A*02, HLA-A*03, or HLA-A*26. If the peptide can bind to HLA-A molecules, it has to have the ability to bind to different ones as the PBMCs of donors 2275, 2497 and 2507, typed for two HLA-A*02 alleles, the PBMCs of donor 2500, typed for two HLA-A*26 alleles and the

PBMCs of donor 2501, typed for two HLA-A*03 alleles could all be stimulated to IFN-γ secretion by LPLLIPLIAAAIGAV. If the PBMCs of a donor who is typed homozygous for an allele did not show a response to the peptide, such as donor 2274, who is typed for two HLA-A*02 alleles, it could not be excluded that the peptide can bind to the HLA allotype. There is still the possibility that the HLA molecule can present the peptide but that the individual, or more specifically the PBMC isolate, lacks a T cell with the epitope-specific α:β TCR.

Another explanation could be that LPLLIPLIAAAIGAV is restricted to HLA-C allele products. Nevertheless, this cannot explain the recognition rate of over 70%.

Furthermore, LPLLIPLIAAAIGAV could have the ability to bind to HLA class I and HLA class II molecules. *Shao et al.* identified a uveitis-associated epitope that was able to bind to HLA class I and HLA class II molecules and stimulate $CD8^+$ and $CD4^+$ T cells (204). *May et al.* found a mutated Wilms' tumor 1 oncoprotein, which was shown to activate $CD4^+$ and $CD8^+$ T cells (205). These works are consistent with our ICS results of the PBMCs of donor 2163 incubated with the epitope IGAVPGIASVALQAQ, which indicates stimulation of the $CD4^+$ as well as the $CD8^+$ T-cell population. As ICS by LPLLIPLIAAAIGAV was only tested in the PBMCs of two different donors, we were able to confirm its ability to activate $CD8^+$ T cells by binding to HLA class I molecules of these donors. In the ELISpot assays performed with PBMC isolates from other donors, it can also have led to an IFN-γ secretion by the activation of $CD4^+$ T cells *via* binding to HLA class II molecules.

4.2.5 Results of *ex vivo* ELISpot assay

The relevance of the 12-day stimulation protocol in the screening setting can be seen when comparing the results of the ELISpot screening setting with those of the *ex vivo* ELISpot setting. Only 17.2% of the positive PBMC responses after the 12-day stimulation protocol could be detected in the *ex vivo* setting when retesting the same PBMC-peptide constellation. *Godkin et al.* found a decreasing number of IFN-γ secreting cells over time when performing an *ex vivo* ELISpot with hepatitis C-derived HLA class II epitopes on the PBMCs of donors who had cleared hepatitis C. Interestingly, the number of IFN-γ secreting cells increased over time when performing an ELISpot assay after a previous

stimulation protocol. They stated that the *ex vivo* ELISpot and the ELISpot after the stimulation protocol detected different populations of $CD4^+$ memory T cells. (206) This could explain the strong responses to single HAdV epitopes in ELISpot assays after the stimulation protocol compared to the poor *ex vivo* responses. This phenomenon was also observed by *Sukdolak et al.* when studying the frequencies of HAdV-specific T cells in PBMCs of 204 healthy donors. Concordant to our results, the frequencies of especially HAdV-specific T cells in the PBMCs of seropositive donors were low or undetectable in the *ex* vivo setting when compared to the results obtained for EBV and CMV. Furthermore, the flow cytometric determined frequencies of HAdV-specific T cells were low. After performing a stimulation protocol, an increased number of HAdV-specific T cells were identified in tested PBMCs. (123)

The number of T cells that are specific for a particular epitope can be meager in the peripheral blood of healthy donors. The frequencies of HAdV-specific memory T cells were shown to be exceptionally low when compared to other viruses (123). There are frequently non-specifically activated, INF-γ secreting cells when performing an ELISpot assay. They can be detected as background in the negative control. The T cells detected in an ELISpot assay are memory T cells. Due to the high diversity of the TCRs the organism has only a low amount of epitope-specific T cells when not infected with the pathogen. In case of an infection with a pathogen that carries the antigen recognized by the TCR, these cells are amplified and reduced after infection. Otherwise, the number of leukocytes in the peripheral blood would be too high. The cut off for a positive result in the ELISpot assay was set to a spot count of at least ten and threefold the spot count of the negative control. Therefore, the presence of epitope-specific T cells can stay undetected in the PBMC cultures of healthy donors in the *ex vivo* ELISpot setting when there are less than ten test-epitope-specific T cells per 500.000 PBMCs. When performing a 12-day stimulation, epitope-specific T cells are amplified and can be detected in an ELISpot assay. The ELISpot assay after the 12-day stimulation protocol takes much longer to prepare than the *ex vivo* ELISpot assay. The advantage is that even a meager amount of epitope-specific T cells can be detected. Without performing the time-intense 12-day stimulation protocol during the screening for recognition of single epitopes, epitope-specific T cells in 82.8% of the PBMC cultures shown to contain epitope-specific

T cells after the 12-day protocol screening would have stayed undetected if only performing *ex vivo* ELISpot assays.

During the 12-day stimulation protocol, the epitope-specific cells are amplified. This explains why none of the PBMC cultures in which a response to an epitope was detectable after the amplification protocol, could be stimulated to an IFN-γ secretion when incubated with the same epitope *ex vivo*.

The PBMC cultures consist of different subpopulations of immune cells. In the *ex vivo* setting, the proportions of the different cells are comparable to the situation in the peripheral blood of healthy individuals. PBMC cultures contain lymphocytes, monocytes, and DCs. In healthy individuals, the majority of the PBMCs are lymphocytes. They represent 70-90% of the PBMCs, followed by monocytes (10-20%). DC only represent 1-2% of the PBMCs. The lymphocytes can be categorized into $CD3^+$ T cells, B cells, and NK cells, with frequencies of 70–85%, 5–10%, and 5–20%, respectively. The $CD3^+$ T cells can further be divided into $CD4^+$ and $CD8^+$ T cells. Usually, the $CD4^+$: $CD8^+$ ratio is around 2:1. (207) Peptides that were tested in the *ex vivo* setting were mainly activating $CD4^+$ T cells. As there were about 500.000 PBMCs in each well and the percentage of $CD3^+$ $CD4^+$ T cells is approximately between 30% and 50%, the number of cells that could possibly recognize the epitope was in between 250.000 and 150.000 per well. This indicates that the percentage of epitope-specific T cells must be at least 0.004% of the $CD3^+$ $CD4^+$ T cell population to be detected in an *ex vivo* setting. When the PBMCs of donor 2494 were incubated with the epitope RGIFCVVKQAKLTYE *ex vivo*, the activated cells represented approximately 140 out of 500.000 after subtracting the unspecific activation. This would mean that 0.06% to 0.09% of the $CD3^+$ $CD4^+$ cells of the PBMCs could recognize the epitope. Another observation is that the PBMCs of this donor showed strong responses to seven out of nine single epitopes in the ELISpot screening assay after a 12-day stimulation protocol. A symptomatic infection would have excluded the donor from a blood donation. As the *ex vivo* response was observed to diminish over time (206), a recently cleared adenoviral infection before blood donation could explain the high frequency of adenovirus-specific $CD3^+$ $CD4^+$ cells in the PBMC culture of donor 2494 when compared to the results obtained from the *ex vivo* incubation of the PBMCs of other donors with adenoviral epitopes. In search of epitopes for clinical

approaches, the *ex vivo* ELISpot result is an important selection criterion, besides a high recognition rate in ELISpot screening assays. PQKFFAIKNLLLLPG led to a detectable INF-γ secretion in three out of eight PBMC cultures *ex vivo*. The epitopes TFYLNHTFKKVAITF, RGIFCVVKQAKLTYE, and TLRFIPVDREDTAYS detectibly activated two out of eight PBMC cultures. These findings render those epitopes most interesting for single epitope clinical approaches. Nevertheless, some findings state that the efficacy of adoptive T-cell transfer is independent of the transfused virus-specific T-cell dose, indicating that the HAdV specific T cells can proliferate *in vivo*, after transfusion. (158) This implicates that a positive result after a screening ELISpot assays would be enough to render an epitope a potential candidate for therapeutic approaches.

5 Outlook

Adenoviral infections still are a serious threat, not to healthy, but immunocompromised individuals. (141, 142, 144) Until now, there is no evidence-based therapy or vaccine against this pathogen.

Adoptive T-cell transfer seems to be a promising approach to this problem (170, 208), as the presence of virus-specific T cells is known to be essential for survival and viral clearance (155). The risk of graft-versus-host disease is reduced when only transferring virus-specific T cells instead of unselected donor lymphocytes (158). As some SCT donors are seronegative for HAdV, no HAdV-specific memory T cells can be isolated from the donor's peripheral blood. Before stem cell isolation from the peripheral blood, the donors receive G-CSF for stem cell mobilization, which was shown to have long-term negative effects on T-cell functionality. (209) The isolation of PBMCs before G-CSF application may circumvent this second problem. Nevertheless, in both cases, third-party donors can be considered. (210)

For the selection of virus-specific T cells, the knowledge of immunodominant peptides is required. By detecting more and more peptides for different HLA class I allotypes *via* reverse immunology approaches, an increasing but not yet satisfying number of epitopes are now identified. The identification of HLA class II epitopes *via* reverse immunology approaches is even less satisfying, as the identification of peptide-binding motifs for epitope prediction algorithms is impeded by the length of peptides interacting with the open-ended binding groove of HLA class II molecules. (182)

With our approach of predicting promiscuous HLA class II epitopes derived from HAdV2, we were able to identify six immunodominant and twelve subdominant epitopes. The designed HLA class II cocktail led to a detectable stimulation in all tested PBMC cultures. The single-peptides, as well as the peptide cocktail, can be used for the isolation of adenovirus-specific T cells in clinical approaches for adoptive T-cell transfer.

To facilitate the promiscuous epitope prediction, the performed peptide-selection algorithm can be integrated into a prediction tool to reduce the time-consuming manual identification and men-made errors of the promiscuous epitope prediction procedure. By using SYFPEITHI, the prediction process had to be performed separately for each protein.

This is feasible for pathogens with a limited number of proteins and, consequently, possible epitopes, such as HAdV, but very time-consuming for pathogens with larger genomes. SYFPEITHI 2.0 is currently being developed. It allows the parallel prediction of epitopes for several proteins. This can already reduce the time invested into promiscuous prediction to a large extent.

We could not observe a dependence of the recognition rate of the predicted epitopes, neither on the %-max score nor on the number of containing cores they were predicted for (data not shown). Only peptides that were predicted to contain at least six cores were synthesized and tested. The epitope MHLWRAVVRHKNRLL that was discovered earlier *in house* and proven to be an immunodominant epitope was predicted to contain 5 top-2% cores and would also have been identified in further testing. Peptides that were predicted to contain four or less core sequences but were classified as good binders by NetMHCIIpan 2.0 showed high recognition rates as well. This means that there are certainly more immunodominant promiscuous epitopes further down the list, which were not yet synthesized and tested. Therefore, further testing of predicted peptides can be useful to identify new immunogenic epitopes for clinical approaches.

Furthermore, the understanding of HLA class II epitope-binding to HLA molecules should be investigated in more detail. As the PBMC donors had not been typed for their HLA class II alleles, the ICS results allow the conclusion that the epitopes were able to bind to HLA class II molecules, but not to which ones. Whether the epitopes identified in this work are promiscuous and to which HLA allotypes they are restricted can only be proven by the testing of HLA class II-typed donors.

Further on, the approach of promiscuous epitope prediction should be validated by the prediction of promiscuous epitopes for other viruses where reverse immunology may be a promising approach, as for EBV or CMV.

6 Summary

In immunocompromised patients, an adenoviral infection can turn into a fulminant progression of the disease, which often leads to death. Adoptive T-cell transfer was identified as a promising treatment approach. For the selection of virus-specific T cells, the knowledge of immunodominant epitopes is required. Within the scope of this project, HAdV-derived epitopes were identified and further investigated. We aimed to design one HLA class I and one HLA class II epitope cocktail that should lead to an immune response in ELISpot assays with randomly selected PBMC cultures. We compared the immune response to two HLA class I cocktails: one contained 24 HLA class I epitopes and the other contained the 15 epitopes with the highest recognition rates. The 24-peptides-cocktail elicited a IFN-γ response in 22 out of 25 PBMC cultures (88%). The 15-peptides-cocktail led to a detectable IFN-γ response in 20 out of 25 PBMC cultures (80%). The 24-peptides-cocktail was always able to stimulate the PBMCs of donors, which were responsive to the 15-peptides-cocktail.

To date, there is no satisfying tool for promiscuous HLA class II epitope prediction. We performed a promiscuous epitope prediction on all 46 reviewed HAdV2 proteins from the Swiss-Prot database with SYFPEITHI and NetMHCIIpan 2.0. The top-ranked peptides and already published epitopes were synthesized and screened for immunogenicity in ELISpot assays. We designed an HLA class II peptide cocktail containing the 12 most immunogenic epitopes, proven to stimulate $CD4^+$ T cells *via* ICS. Four epitopes are already published, seven of the epitopes were first identified by our prediction, and one epitope is an elongated HLA class I 10mer showing $CD4^+$ T-cell stimulation in ICS. The designed HLA class II cocktail could stimulate 31 out of 31 tested PBMC cultures (100%) in ELISpot assays. The assumption that the hexon protein is the main target of the immune response can be questioned as seven immunodominant HLA class II epitopes newly-discovered by the promiscuous prediction are derived from other proteins than the hexon. We identified an effective method for the prediction of promiscuous HLA class II epitopes. The combined SYFPEITHI/ NetMHCIIpan 2.0 prediction resulted in the synthesis of 27 peptides, from which nine (33.3%) were identified as immunodominant and twelve (44.4%) were categorized as subdominant epitopes. One of the dominant epitopes is already published. Six peptides (22.2%) were non-immunogenic. One immunodominant epitope was revealed as an HLA class I epitope in ICS.

7 Zusammenfassung

Bei immungeschwächten Patienten verläuft eine Infektion mit dem Adenovirus oft tödlich. Der adoptive T-Zell-Transfer wurde als vielversprechender Behandlungsansatz ausgemacht. Für die Selektion virusspezifischer T-Zellen ist die Kenntnis immundominanter Epitope erforderlich. Im Rahmen dieses Projekts wurden HAdV-Epitope identifiziert und weiter untersucht, mit dem Ziel einen HLA-Klasse-I- und einen HLA-Klasse-II-Epitop-Cocktail zu entwerfen, die in ELISpot-Assays in möglichst vielen zufällig ausgewählten PBMC-Kulturen zu einer Immunantwort führen. Wir verglichen die Immunantworten auf zwei HLA-Klasse-I-Cocktails: Einer enthielt 24 HLA-Klasse-I-Epitope, der andere die 15 Epitope mit den höchsten Erkennungsraten. Der 24-Peptid-Cocktail führte in 22 von 25 (88 %) und der 15-Peptid-Cocktail in 20 von 25 PBMC-Kulturen (80 %) zu einer Immunantwort. Der 24-Peptid-Cocktail war immer in der Lage die PBMCs von Spendern zu stimulieren, die auf den 15-Peptid-Cocktail ansprachen.

Bis heute gibt es kein zufriedenstellendes Werkzeug für die Vorhersage promiskuitiver HLA-Klasse-II-Epitope. Wir führten mithilfe von SYFPEITHI und NetMHCpan 2.0 eine promiskuitive Epitopvorhersage für alle 46 HAdV2-Proteine der Swiss-Prot-Datenbank durch. Die bestbewerteten Peptide und bereits publizierten Epitope wurden synthetisiert und in ELISpot-Assays untersucht. Wir entwarfen einen HLA-Klasse-II-Peptidcocktail, mit den 12 immunogensten Epitopen, die im ICS nachweislich CD4+ T-Zellen stimulieren. Vier Epitope sind bereits publiziert, sieben der Epitope wurden erstmals durch unsere Vorhersage identifiziert. Bei einem Epitop handelt es sich um ein verlängertes 10mer-Peptid, welches eine CD4+ T-Zellstimulation im ICS zeigte. Der HLA-Klasse-II-Cocktail führte in allen 31 getesteten PBMC-Kulturen (100 %) zu einer Immunantwort. Die Annahme, dass das Hexon-Protein das Hauptziel der Immunantwort ist, kann in Frage gestellt werden, da alle sieben immundominanten HLA-Klasse-II-Epitope, die durch die promiskuitive Vorhersage entdeckt wurden, von anderen Proteinen als dem Hexon abgeleitet sind. Wir konnten eine effektive Methode zur Vorhersage von promiskuitiven HLA-Klasse-II-Epitopen etablieren. Die kombinierte SYFPEITHI/ NetMHCIIpan 2.0-Vorhersage führte zur Synthese von 27 Peptiden, von denen neun (33,3 %) als dominant (eines bereits publiziert) und 12 (44,4 %) als subdominante Epitope ausgemacht wurden. Sechs Peptide (22,2 %) waren nicht immunogen. Eines der immundominanten Epitope erwies sich im ICS als HLA-Klasse-I-Epitop.

8 Publication

Parts of this thesis have been published. All experiments described in the thesis were performed by the author. The reference for the publication in the text is (186).

Broad and Efficient Activation of Memory CD4+ T Cells by Novel HAdV- and HCMV-Derived Peptide Pools

Alexander Höttler†, Léo März†, Maren Lübke, Hans-Georg Rammensee and Stefan Stevanović

† indicates shared first authorship

Reference:

Höttler A, **März L**, Lübke M, Rammensee H-G and Stevanović S (2021) Broad and Efficient Activation of Memory CD4 + T Cells by Novel HAdV- and HCMV-Derived Peptide Pools. Front. Immunol. 12:700438. doi: 10.3389/fimmu.2021.700438

9 Literature

1. Kusunoki Y, Kyoizumi S, Honma M, Kubo Y, Ohnishi H, Hayashi T, Seyama T. NK-mediated elimination of mutant lymphocytes that have lost expression of MHC class I molecules. J Immunol. 2000;165(7):3555-63.
2. Shifrin N, Raulet DH, Ardolino M. NK cell self tolerance, responsiveness and missing self recognition. Semin Immunol. 2014;26(2):138-44.
3. Maudsley DJ, Pound JD. Modulation of MHC antigen expression by viruses and oncogenes. Immunol Today. 1991;12(12):429-31.
4. Schildknecht A, Miescher I, Yagita H, van den Broek M. Priming of CD8+ T cell responses by pathogens typically depends on CD70-mediated interactions with dendritic cells. Eur J Immunol. 2007;37(3):716-28.
5. Zsengeller Z, Otake K, Hossain SA, Berclaz PY, Trapnell BC. Internalization of adenovirus by alveolar macrophages initiates early proinflammatory signaling during acute respiratory tract infection. J Virol. 2000;74(20):9655-67.
6. Nguyen L, Pieters J. The Trojan horse: survival tactics of pathogenic mycobacteria in macrophages. Trends Cell Biol. 2005;15(5):269-76.
7. Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. Nature. 1999;401(6756):921-3.
8. Wang M, Claesson MH. Classification of human leukocyte antigen (HLA) supertypes. Methods Mol Biol. 2014;1184:309-17.
9. http://hla.alleles.org/
10. Salter RD, Benjamin RJ, Wesley PK, Buxton SE, Garrett TP, Clayberger C, Krensky AM, Norment AM, Littman DR, Parham P. A binding site for the T-cell co-receptor CD8 on the alpha 3 domain of HLA-A2. Nature. 1990;345(6270):41-6.
11. Matsumura M, Fremont D, Peterson P, Wilson I. Emerging principles for the recognition of peptide antigens by MHC class I molecules. Science. 1992;257(5072):927-34.
12. Madden DR, Gorga JC, Strominger JL, Wiley DC. The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. Nature. 1991;353(6342):321-5.
13. Rammensee HG, Falk K, Rotzschke O. Peptides naturally presented by MHC class I molecules. Annu Rev Immunol. 1993;11:213-44.
14. Rotzschke O, Falk K. Naturally-occurring peptide antigens derived from the MHC class-I-restricted processing pathway. Immunol Today. 1991;12(12):447-55.
15. Halenius A, Hauka S, Dolken L, Stindt J, Reinhard H, Wiek C, Hanenberg H, Koszinowski UH, Momburg F, Hengel H. Human cytomegalovirus disrupts the major histocompatibility complex class I peptide-loading complex and inhibits tapasin gene transcription. J Virol. 2011;85(7):3473-85.
16. Hermiston TW, Tripp RA, Sparer T, Gooding LR, Wold WS. Deletion mutation analysis of the adenovirus type 2 E3-gp19K protein: identification of sequences within the endoplasmic reticulum lumenal domain that are required for class I antigen binding and protection from adenovirus-specific cytotoxic T lymphocytes. J Virol. 1993;67(9):5289-98.
17. Wosen JE, Mukhopadhyay D, Macaubas C, Mellins ED. Epithelial MHC Class II Expression and Its Role in Antigen Presentation in the Gastrointestinal and Respiratory Tracts. Front Immunol. 2018;9:2144-.
18. Andersson G. Evolution of the human HLA-DR region. Front Biosci. 1998;3:d739-45.
19. Murphy KM, Mowat A, Weaver C. Janeway's immunobiology. London: Garland Science; 2017.
20. Bontrop RE, Otting N, de Groot NG, Doxiadis GG. Major histocompatibility complex class II polymorphisms in primates. Immunol Rev. 1999;167:339-50.
21. Rammensee HG, Friede T, Stevanoviic S. MHC ligands and peptide motifs: first listing. Immunogenetics. 1995;41(4):178-228.

22. Chicz RM, Urban RG, Lane WS, Gorga JC, Stern LJ, Vignali DA, Strominger JL. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature. 1992;358(6389):764-8.
23. Rudensky A, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA, Jr. Sequence analysis of peptides bound to MHC class II molecules. Nature. 1991;353(6345):622-7.
24. Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL, Wiley DC. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature. 1994;368(6468):215-21.
25. Dengjel J, Schoor O, Fischer R, Reich M, Kraus M, Müller M, Kreymborg K, Altenberend F, Brandenburg J, Kalbacher H, Brock R, Driessen C, Rammensee H-G, Stevanovic S. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc Natl Acad Sci. 2005;102(22):7922-7.
26. Nandi D, Tahiliani P, Kumar A, Chandu D. The ubiquitin-proteasome system. J Biosci. 2006;31(1):137-55.
27. Kloetzel PM. The proteasome and MHC class I antigen processing. Biochim Biophys Acta. 2004;1695(1-3):225-33.
28. Ritz U, Seliger B. The transporter associated with antigen processing (TAP): structural integrity, expression, function, and its clinical relevance. Mol Med. 2001;7(3):149-58.
29. Kanaseki T, Blanchard N, Hammer GE, Gonzalez F, Shastri N. ERAAP synergizes with MHC class I molecules to make the final cut in the antigenic peptide precursors in the endoplasmic reticulum. Immunity. 2006;25(5):795-806.
30. Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. Immunol Rev. 2005;207:145-57.
31. Watts C. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. Nat Immunol. 2004;5(7):685-92.
32. Reich Z, Altman JD, Boniface JJ, Lyons DS, Kozono H, Ogg G, Morgan C, Davis MM. Stability of empty and peptide-loaded class II major histocompatibility complex molecules at neutral and endosomal pH: comparison to class I proteins. Proc Natl Acad Sci U S A. 1997;94(6):2495-500.
33. Rowen L, Koop BF, Hood L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. Science. 1996;272(5269):1755-62.
34. Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med. 1953;84(3):570-3.
35. Benko M, Elo P, Ursu K, Ahne W, LaPatra SE, Thomson D, Harrach B. First molecular evidence for the existence of distinct fish and snake adenoviruses. J Virol. 2002;76(19):10056-9.
36. Kovacs GM, LaPatra SE, D'Halluin JC, Benko M. Phylogenetic analysis of the hexon and protease genes of a fish adenovirus isolated from white sturgeon (Acipenser transmontanus) supports the proposal for a new adenovirus genus. Virus Res. 2003;98(1):27-34.
37. Smith JG, Wiethoff CM, Stewart PL, Nemerow GR. Adenovirus. Curr Top Microbiol Immunol. 2010;343:195-224.
38. Robinson CM, Singh G, Lee JY, Dehghan S, Rajaiya J, Liu EB, Yousuf MA, Betensky RA, Jones MS, Dyer DW, Seto D, Chodosh J. Molecular evolution of human adenoviruses. Sci Rep. 2013;3:1812.
39. Segerman A, Arnberg N, Erikson A, Lindman K, Wadell G. There are two different species B adenovirus receptors: sBAR, common to species B1 and B2 adenoviruses, and sB2AR, exclusively used by species B2 adenoviruses. J Virol. 2003;77(2):1157-62.
40. Huang GH, Xu WB. [Recent advance in new types of human adenovirus]. Bing Du Xue Bao. 2013;29(3):342-8.
41. Seto D, Chodosh J, Brister JR, Jones MS, Members of the Adenovirus Research C. Using the whole-genome sequence to characterize and name human adenoviruses. J Virol. 2011;85(11):5701-2.

42. Ghebremedhin B. Human adenovirus: Viral pathogen with increasing importance. Eur J Microbiol Immunol (Bp). 2014;4(1):26-33.
43. Russell WC. Adenoviruses: update on structure and function. J Gen Virol. 2009;90(Pt 1):1-20.
44. Abzug MJ, Levin MJ. Neonatal adenovirus infection: four patients and review of the literature. Pediatrics. 1991;87(6):890-6.
45. Saban SD, Silvestry M, Nemerow GR, Stewart PL. Visualization of alpha-helices in a 6-angstrom resolution cryoelectron microscopy structure of adenovirus allows refinement of capsid protein assignments. J Virol. 2006;80(24):12049-59.
46. Moskalenko M, Chen L, van Roey M, Donahue BA, Snyder RO, McArthur JG, Patel SD. Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. J Virol. 2000;74(4):1761-6.
47. Greber UF, Webster P, Weber J, Helenius A. The role of the adenovirus protease on virus entry into cells. EMBO J. 1996;15(8):1766-77.
48. Davison AJ, Benko M, Harrach B. Genetic content and evolution of adenoviruses. J Gen Virol. 2003;84(Pt 11):2895-908.
49. Dhingra A, Hage E, Ganzenmueller T, Bottcher S, Hofmann J, Hamprecht K, Obermeier P, Rath B, Hausmann F, Dobner T, Heim A. Molecular Evolution of Human Adenovirus (HAdV) Species C. Sci Rep. 2019;9(1):1039.
50. Rekosh DM, Russell WC, Bellet AJ, Robinson AJ. Identification of a protein linked to the ends of adenovirus DNA. Cell. 1977;11(2):283-95.
51. Lehmberg E, Traina JA, Chakel JA, Chang RJ, Parkman M, McCaman MT, Murakami PK, Lahidji V, Nelson JW, Hancock WS, Nestaas E, Pungor E, Jr. Reversed-phase high-performance liquid chromatographic assay for the adenovirus type 5 proteome. J Chromatogr B Biomed Sci Appl. 1999;732(2):411-23.
52. Hale TK, Braithwaite AW. The adenovirus oncoprotein E1a stimulates binding of transcription factor ETF to transcriptionally activate the p53 gene. J Biol Chem. 1999;274(34):23777-86.
53. Grand RJ, Turnell AS, Mason GG, Wang W, Milner AE, Mymryk JS, Rookes SM, Rivett AJ, Gallimore PH. Adenovirus early region 1A protein binds to mammalian SUG1-a regulatory component of the proteasome. Oncogene. 1999;18(2):449-58.
54. Boyer TG, Martin ME, Lees E, Ricciardi RP, Berk AJ. Mammalian Srb/Mediator complex is targeted by adenovirus E1A protein. Nature. 1999;399(6733):276-9.
55. Yew PR, Liu X, Berk AJ. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev. 1994;8(2):190-202.
56. Cardoso FM, Kato SE, Huang W, Flint SJ, Gonzalez RA. An early function of the adenoviral E1B 55 kDa protein is required for the nuclear relocalization of the cellular p53 protein in adenovirus-infected normal human cells. Virology. 2008;378(2):339-46.
57. Querido E, Blanchette P, Yan Q, Kamura T, Morrison M, Boivin D, Kaelin WG, Conaway RC, Conaway JW, Branton PE. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev. 2001;15(23):3104-17.
58. White E. Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. Oncogene. 2001;20(54):7836-46.
59. Rittner K, Schultz H, Pavirani A, Mehtali M. Conditional repression of the E2 transcription unit in E1-E3-deleted adenovirus vectors is correlated with a strong reduction in viral DNA replication and late gene expression in vitro. J Virol. 1997;71(4):3307-11.
60. Chin YR, Horwitz MS. Adenovirus RID complex enhances degradation of internalized tumour necrosis factor receptor 1 without affecting its rate of endocytosis. J Gen Virol. 2006;87(Pt 11):3161-7.
61. Fessler SP, Chin YR, Horwitz MS. Inhibition of tumor necrosis factor (TNF) signal transduction by the adenovirus group C RID complex involves downregulation of surface levels of TNF receptor 1. J Virol. 2004;78(23):13113-21.

62. Lichtenstein DL, Doronin K, Toth K, Kuppuswamy M, Wold WS, Tollefson AE. Adenovirus E3-6.7K protein is required in conjunction with the E3-RID protein complex for the internalization and degradation of TRAIL receptor 2. J Virol. 2004;78(22):12297-307.
63. McNees AL, Garnett CT, Gooding LR. The adenovirus E3 RID complex protects some cultured human T and B lymphocytes from Fas-induced apoptosis. J Virol. 2002;76(19):9716-23.
64. Gooding LR, Sofola IO, Tollefson AE, Duerksen-Hughes P, Wold WS. The adenovirus E3-14.7K protein is a general inhibitor of tumor necrosis factor-mediated cytolysis. J Immunol. 1990;145(9):3080-6.
65. Tollefson AE, Scaria A, Hermiston TW, Ryerse JS, Wold LJ, Wold WS. The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. J Virol. 1996;70(4):2296-306.
66. Lichtenstein DL, Toth K, Doronin K, Tollefson AE, Wold WS. Functions and mechanisms of action of the adenovirus E3 proteins. Int Rev Immunol. 2004;23(1-2):75-111.
67. Tauber B, Dobner T. Molecular regulation and biological function of adenovirus early genes: the E4 ORFs. Gene. 2001;278(1-2):1-23.
68. Weinberg DH, Ketner G. Adenoviral early region 4 is required for efficient viral DNA replication and for late gene expression. J Virol. 1986;57(3):833-8.
69. Meng H, Deo S, Xiong S, Dzananovic E, Donald LJ, van Dijk CW, McKenna SA. Regulation of the interferon-inducible 2'-5'-oligoadenylate synthetases by adenovirus VA(I) RNA. J Mol Biol. 2012;422(5):635-49.
70. Lutz P, Rosa-Calatrava M, Kedinger C. The product of the adenovirus intermediate gene IX is a transcriptional activator. J Virol. 1997;71(7):5102-9.
71. Pardo-Mateos A, Young CS. Adenovirus IVa2 protein plays an important role in transcription from the major late promoter in vivo. Virology. 2004;327(1):50-9.
72. Tyler RE, Ewing SG, Imperiale MJ. Formation of a multiple protein complex on the adenovirus packaging sequence by the IVa2 protein. J Virol. 2007;81(7):3447-54.
73. Yang TC, Maluf NK. Characterization of the non-specific DNA binding properties of the Adenoviral IVa2 protein. Biophys Chem. 2014;193-194:1-8.
74. Anderson CW. The proteinase polypeptide of adenovirus serotype 2 virions. Virology. 1990;177(1):259-72.
75. Zhao H, Chen M, Pettersson U. A new look at adenovirus splicing. Virology. 2014;456-457:329-41.
76. https://viralzone.expasy.org/ 2019
77. Jakubczak JL, Rollence ML, Stewart DA, Jafari JD, Von Seggern DJ, Nemerow GR, Stevenson SC, Hallenbeck PL. Adenovirus type 5 viral particles pseudotyped with mutagenized fiber proteins show diminished infectivity of coxsackie B-adenovirus receptor-bearing cells. J Virol. 2001;75(6):2972-81.
78. Roelvink PW, Lizonova A, Lee JG, Li Y, Bergelson JM, Finberg RW, Brough DE, Kovesdi I, Wickham TJ. The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. J Virol. 1998;72(10):7909-15.
79. Gaggar A, Shayakhmetov DM, Lieber A. CD46 is a cellular receptor for group B adenoviruses. Nat Med. 2003;9(11):1408-12.
80. Marttila M, Persson D, Gustafsson D, Liszewski MK, Atkinson JP, Wadell G, Arnberg N. CD46 is a cellular receptor for all species B adenoviruses except types 3 and 7. J Virol. 2005;79(22):14429-36.
81. Short JJ, Pereboev AV, Kawakami Y, Vasu C, Holterman MJ, Curiel DT. Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. Virology. 2004;322(2):349-59.
82. Arnberg N, Edlund K, Kidd AH, Wadell G. Adenovirus type 37 uses sialic acid as a cellular receptor. J Virol. 2000;74(1):42-8.

83. Martin-Fernandez M, Longshaw SV, Kirby I, Santis G, Tobin MJ, Clarke DT, Jones GR. Adenovirus type-5 entry and disassembly followed in living cells by FRET, fluorescence anisotropy, and FLIM. Biophys J. 2004;87(2):1316-27.
84. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell. 1993;73(2):309-19.
85. Nakano MY, Boucke K, Suomalainen M, Stidwill RP, Greber UF. The first step of adenovirus type 2 disassembly occurs at the cell surface, independently of endocytosis and escape to the cytosol. J Virol. 2000;74(15):7085-95.
86. Greber UF, Willetts M, Webster P, Helenius A. Stepwise dismantling of adenovirus 2 during entry into cells. Cell. 1993;75(3):477-86.
87. Greber UF, Singh I, Helenius A. Mechanisms of virus uncoating. Trends Microbiol. 1994;2(2):52-6.
88. Suomalainen M, Nakano MY, Keller S, Boucke K, Stidwill RP, Greber UF. Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus. J Cell Biol. 1999;144(4):657-72.
89. Greber UF, Suomalainen M, Stidwill RP, Boucke K, Ebersold MW, Helenius A. The role of the nuclear pore complex in adenovirus DNA entry. EMBO J. 1997;16(19):5998-6007.
90. Nguyen EK, Nemerow GR, Smith JG. Direct evidence from single-cell analysis that human {alpha}-defensins block adenovirus uncoating to neutralize infection. J Virol. 2010;84(8):4041-9.
91. Gregory SM, Nazir SA, Metcalf JP. Implications of the innate immune response to adenovirus and adenoviral vectors. Future Virol. 2011;6(3):357-74.
92. Stark JM, Amin RS, Trapnell BC. Infection of A549 cells with a recombinant adenovirus vector induces ICAM-1 expression and increased CD-18-dependent adhesion of activated neutrophils. Hum Gene Ther. 1996;7(14):1669-81.
93. Hensley SE, Amalfitano A. Toll-like receptors impact on safety and efficacy of gene transfer vectors. Mol Ther. 2007;15(8):1417-22.
94. Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, Joshi B, Tazelaar J, Wilson JM. Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol Ther. 2001;3(5 Pt 1):697-707.
95. Molinier-Frenkel V, Lengagne R, Gaden F, Hong SS, Choppin J, Gahery-Segard H, Boulanger P, Guillet JG. Adenovirus hexon protein is a potent adjuvant for activation of a cellular immune response. J Virol. 2002;76(1):127-35.
96. Cotter MJ, Zaiss AK, Muruve DA. Neutrophils interact with adenovirus vectors via Fc receptors and complement receptor 1. J Virol. 2005;79(23):14622-31.
97. Kotha PL, Sharma P, Kolawole AO, Yan R, Alghamri MS, Brockman TL, Gomez-Cambronero J, Excoffon KJ. Adenovirus entry from the apical surface of polarized epithelia is facilitated by the host innate immune response. PLoS Pathog. 2015;11(3):e1004696.
98. Routes JM. IFN increases class I MHC antigen expression on adenovirus-infected human cells without inducing resistance to natural killer cell killing. J Immunol. 1992;149(7):2372-7.
99. Cartmell T, Southgate T, Rees GS, Castro MG, Lowenstein PR, Luheshi GN. Interleukin-1 mediates a rapid inflammatory response after injection of adenoviral vectors into the brain. J Neurosci. 1999;19(4):1517-23.
100. Schwarz YA, Amin RS, Stark JM, Trapnell BC, Wilmott RW. Interleukin-1 receptor antagonist inhibits interleukin-8 expression in A549 respiratory epithelial cells infected in vitro with a replication-deficient recombinant adenovirus vector. Am J Respir Cell Mol Biol. 1999;21(3):388-94.
101. Chang CH, Huang Y, Issekutz AC, Griffith M, Lin KH, Anderson R. Interleukin-1alpha released from epithelial cells after adenovirus type 37 infection activates intercellular adhesion molecule 1 expression on human vascular endothelial cells. J Virol. 2002;76(1):427-31.
102. Nijsten MW, de Groot ER, ten Duis HJ, Klasen HJ, Hack CE, Aarden LA. Serum levels of interleukin-6 and acute phase responses. Lancet. 1987;2(8564):921.

103. Kawasaki Y, Hosoya M, Katayose M, Suzuki H. Correlation between serum interleukin 6 and C-reactive protein concentrations in patients with adenoviral respiratory infection. Pediatr Infect Dis J. 2002;21(5):370-4.
104. Mistchenko AS, Diez RA, Mariani AL, Robaldo J, Maffey AF, Bayley-Bustamante G, Grinstein S. Cytokines in adenoviral disease in children: association of interleukin-6, interleukin-8, and tumor necrosis factor alpha levels with clinical outcome. J Pediatr. 1994;124(5 Pt 1):714-20.
105. Chehimi J, Trinchieri G. Interleukin-12: a bridge between innate resistance and adaptive immunity with a role in infection and acquired immunodeficiency. J Clin Immunol. 1994;14(3):149-61.
106. Zhu J, Huang X, Yang Y. Innate immune response to adenoviral vectors is mediated by both Toll-like receptor-dependent and -independent pathways. J Virol. 2007;81(7):3170-80.
107. Flomenberg P, Piaskowski V, Truitt RL, Casper JT. Characterization of human proliferative T cell responses to adenovirus. J Infect Dis. 1995;171(5):1090-6.
108. Ye X, Xiao L, Zheng X, Wang J, Shu T, Feng Y, Liu X, Su W, Wang Q, Li C, Chen L, Feng L. Seroprevalence of Neutralizing Antibodies to Human Adenovirus Type 4 and 7 in Healthy Populations From Southern China. Front Microbiol. 2018;9:3040.
109. Bradley RR, Lynch DM, Iampietro MJ, Borducchi EN, Barouch DH. Adenovirus serotype 5 neutralizing antibodies target both hexon and fiber following vaccination and natural infection. J Virol. 2012;86(1):625-9.
110. Bradley RR, Maxfield LF, Lynch DM, Iampietro MJ, Borducchi EN, Barouch DH. Adenovirus serotype 5-specific neutralizing antibodies target multiple hexon hypervariable regions. J Virol. 2012;86(2):1267-72.
111. Krause G. Meldepflicht für Infektionskrankheiten. Dtsch Arztebl International. 2007;104(41):2811-.
112. Yun HC, Prakash V. Transmission of adenovirus serotype 14 in the health care setting. Clin Infect Dis. 2008;46(12):1935-6.
113. Cheng J, Qi X, Chen D, Xu X, Wang G, Dai Y, Cui D, Chen Q, Fan P, Ni L, Liu M, Zhu F, Yang M, Wang C, Li Y, Sun C, Wang Z. Epidemiology and transmission characteristics of human adenovirus type 7 caused acute respiratory disease outbreak in military trainees in East China. Am J Transl Res. 2016;8(5):2331-42.
114. Couch RB, Knight V, Douglas RG, Jr., Black SH, Hamory BH. The minimal infectious dose of adenovirus type 4; the case for natural transmission by viral aerosol. Trans Am Clin Climatol Assoc. 1969;80:205-11.
115. Walls T, Shankar AG, Shingadia D. Adenovirus: an increasingly important pathogen in paediatric bone marrow transplant patients. Lancet Infect Dis. 2003;3(2):79-86.
116. Barnadas C, Schmidt DJ, Fischer TK, Fonager J. Molecular epidemiology of human adenovirus infections in Denmark, 2011-2016. J Clin Virol. 2018;104:16-22.
117. Xie L, Zhang B, Xiao N, Zhang F, Zhao X, Liu Q, Xie Z, Gao H, Duan Z, Zhong L. Epidemiology of human adenovirus infection in children hospitalized with lower respiratory tract infections in Hunan, China. J Med Virol. 2018.
118. Yun BY, Kim MR, Park JY, Choi EH, Lee HJ, Yun CK. Viral etiology and epidemiology of acute lower respiratory tract infections in Korean children. Pediatr Infect Dis J. 1995;14(12):1054-9.
119. Chen HL, Chiou SS, Hsiao HP, Ke GM, Lin YC, Lin KH, Jong YJ. Respiratory adenoviral infections in children: a study of hospitalized cases in southern Taiwan in 2001--2002. J Trop Pediatr. 2004;50(5):279-84.
120. Binder AM, Biggs HM, Haynes AK, Chommanard C, Lu X, Erdman DD, Watson JT, Gerber SI. Human Adenovirus Surveillance - United States, 2003-2016. MMWR Morb Mortal Wkly Rep. 2017;66(39):1039-42.
121. Koren MA, Arnold JC, Fairchok MP, Lalani T, Danaher PJ, Schofield CM, Rajnik M, Hansen EA, Mor D, Chen WJ, Ridore M, Burgess TH, Millar EV. Type-specific clinical

characteristics of adenovirus-associated influenza-like illness at five US military medical centers, 2009-2014. Influenza Other Respir Viruses. 2016;10(5):414-20.
122. Schmitz H, Wigand R, Heinrich W. Worldwide epidemiology of human adenovirus infections. Am J Epidemiol. 1983;117(4):455-66.
123. Sukdolak C, Tischer S, Dieks D, Figueiredo C, Goudeva L, Heuft HG, Verboom M, Immenschuh S, Heim A, Borchers S, Mischak-Weissinger E, Blasczyk R, Maecker-Kolhoff B, Eiz-Vesper B. CMV-, EBV- and ADV-specific T cell immunity: screening and monitoring of potential third-party donors to improve post-transplantation outcome. Biol Blood Marrow Transplant. 2013;19(10):1480-92.
124. Yu B, Wang Z, Dong J, Wang C, Gu L, Sun C, Kong W, Yu X. A serological survey of human adenovirus serotype 2 and 5 circulating pediatric populations in Changchun, China, 2011. Virol J. 2012;9:287.
125. Pilankatta R, Chawla T, Khanna N, Swaminathan S. The prevalence of antibodies to adenovirus serotype 5 in an adult Indian population and implications for adenovirus vector vaccines. J Med Virol. 2010;82(3):407-14.
126. Mast TC, Kierstead L, Gupta SB, Nikas AA, Kallas EG, Novitsky V, Mbewe B, Pitisuttithum P, Schechter M, Vardas E, Wolfe ND, Aste-Amezaga M, Casimiro DR, Coplan P, Straus WL, Shiver JW. International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials. Vaccine. 2010;28(4):950-7.
127. Binn LN, Sanchez JL, Gaydos JC. Emergence of adenovirus type 14 in US military recruits--a new challenge. J Infect Dis. 2007;196(10):1436-7.
128. van der Veen J, Oei KG, Abarbanel MF. Patterns of infections with adenovirus types 4, 7 and 21 in military recruits during a 9-year survey. J Hyg (Lond). 1969;67(2):255-68.
129. Dudding BA, Wagner SC, Zeller JA, Gmelich JT, French GR, Top FH, Jr. Fatal pneumonia associated with adenovirus type 7 in three military trainees. N Engl J Med. 1972;286(24):1289-92.
130. Potter RN, Cantrell JA, Mallak CT, Gaydos JC. Adenovirus-associated deaths in US military during postvaccination period, 1999-2010. Emerg Infect Dis. 2012;18(3):507-9.
131. Lewis PF, Schmidt MA, Lu X, Erdman DD, Campbell M, Thomas A, Cieslak PR, Grenz LD, Tsaknardis L, Gleaves C, Kendall B, Gilbert D. A community-based outbreak of severe respiratory illness caused by human adenovirus serotype 14. J Infect Dis. 2009;199(10):1427-34.
132. Gaydos CA, Gaydos JC. Adenovirus vaccines in the U.S. military. Mil Med. 1995;160(6):300-4.
133. Barraza EM, Ludwig SL, Gaydos JC, Brundage JF. Reemergence of adenovirus type 4 acute respiratory disease in military trainees: report of an outbreak during a lapse in vaccination. J Infect Dis. 1999;179(6):1531-3.
134. Broderick M, Myers C, Balansay M, Vo S, Osuna A, Russell K. Adenovirus 4/7 Vaccine's Effect on Disease Rates Is Associated With Disappearance of Adenovirus on Building Surfaces at a Military Recruit Base. Mil Med. 2017;182(11):e2069-e72.
135. Radin JM, Hawksworth AW, Blair PJ, Faix DJ, Raman R, Russell KL, Gray GC. Dramatic decline of respiratory illness among US military recruits after the renewed use of adenovirus vaccines. Clin Infect Dis. 2014;59(7):962-8.
136. Garnett CT, Erdman D, Xu W, Gooding LR. Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes. J Virol. 2002;76(21):10608-16.
137. Kosulin K, Geiger E, Vecsei A, Huber WD, Rauch M, Brenner E, Wrba F, Hammer K, Innerhofer A, Potschger U, Lawitschka A, Matthes-Leodolter S, Fritsch G, Lion T. Persistence and reactivation of human adenoviruses in the gastrointestinal tract. Clin Microbiol Infect. 2016;22(4):381 e1- e8.
138. Garnett CT, Talekar G, Mahr JA, Huang W, Zhang Y, Ornelles DA, Gooding LR. Latent species C adenoviruses in human tonsil tissues. J Virol. 2009;83(6):2417-28.

139. Roy S, Calcedo R, Medina-Jaszek A, Keough M, Peng H, Wilson JM. Adenoviruses in lymphocytes of the human gastro-intestinal tract. PLoS One. 2011;6(9):e24859.
140. Zheng Y, Stamminger T, Hearing P. E2F/Rb Family Proteins Mediate Interferon Induced Repression of Adenovirus Immediate Early Transcription to Promote Persistent Viral Infection. PLoS Pathog. 2016;12(1):e1005415.
141. Rubin RH, Tolkoff-Rubin NE. Viral infection in the renal transplant patient. Proc Eur Dial Transplant Assoc. 1983;19:513-26.
142. Nebbia G, Chawla A, Schutten M, Atkinson C, Raza M, Johnson M, Geretti A. Adenovirus viraemia and dissemination unresponsive to antiviral therapy in advanced HIV-1 infection. AIDS. 2005;19(12):1339-40.
143. Martinez OM, Villanueva JC, Lawrence-Miyasaki L, Quinn MB, Cox K, Krams SM. Viral and immunologic aspects of Epstein-Barr virus infection in pediatric liver transplant recipients. Transplantation. 1995;59(4):519-24.
144. Leen AM, Bollard CM, Myers GD, Rooney CM. Adenoviral infections in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2006;12(3):243-51.
145. Ramamurthy M, Kannangai R, Abraham AM, Sridharan G. Viral Infections in Immunocompromised Hosts. Proc Natl Acad Sci India Sect B Biol Sci. 2012;82(1):95-109.
146. Baldwin A, Kingman H, Darville M, Foot AB, Grier D, Cornish JM, Goulden N, Oakhill A, Pamphilon DH, Steward CG, Marks DI. Outcome and clinical course of 100 patients with adenovirus infection following bone marrow transplantation. Bone Marrow Transplant. 2000;26(12):1333-8.
147. Blanke C, Clark C, Broun ER, Tricot G, Cunningham I, Cornetta K, Hedderman A, Hromas R. Evolving pathogens in allogeneic bone marrow transplantation: increased fatal adenoviral infections. Am J Med. 1995;99(3):326-8.
148. Al Qurashi YM, Guiver M, Cooper RJ. Sequence typing of adenovirus from samples from hematological stem cell transplant recipients. J Med Virol. 2011;83(11):1951-8.
149. Williams KM, Agwu AL, Dabb AA, Higman MA, Loeb DM, Valsamakis A, Chen AR. A clinical algorithm identifies high risk pediatric oncology and bone marrow transplant patients likely to benefit from treatment of adenoviral infection. J Pediatr Hematol Oncol. 2009;31(11):825-31.
150. Bruno B, Gooley T, Hackman RC, Davis C, Corey L, Boeckh M. Adenovirus infection in hematopoietic stem cell transplantation: effect of ganciclovir and impact on survival. Biol Blood Marrow Transplant. 2003;9(5):341-52.
151. Yusuf U, Hale GA, Carr J, Gu Z, Benaim E, Woodard P, Kasow KA, Horwitz EM, Leung W, Srivastava DK, Handgretinger R, Hayden RT. Cidofovir for the treatment of adenoviral infection in pediatric hematopoietic stem cell transplant patients. Transplantation. 2006;81(10):1398-404.
152. Muller WJ, Levin MJ, Shin YK, Robinson C, Quinones R, Malcolm J, Hild E, Gao D, Giller R. Clinical and in vitro evaluation of cidofovir for treatment of adenovirus infection in pediatric hematopoietic stem cell transplant recipients. Clin Infect Dis. 2005;41(12):1812-6.
153. Vora SB, Brothers AW, Englund JA. Renal Toxicity in Pediatric Patients Receiving Cidofovir for the Treatment of Adenovirus Infection. J Pediatric Infect Dis Soc. 2017;6(4):399-402.
154. Ljungman P, Ribaud P, Eyrich M, Matthes-Martin S, Einsele H, Bleakley M, Machaczka M, Bierings M, Bosi A, Gratecos N, Cordonnier C, Infectious Diseases Working Party of the European Group for B, Marrow T. Cidofovir for adenovirus infections after allogeneic hematopoietic stem cell transplantation: a survey by the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant. 2003;31(6):481-6.
155. Feuchtinger T, Lucke J, Hamprecht K, Richard C, Handgretinger R, Schumm M, Greil J, Bock T, Niethammer D, Lang P. Detection of adenovirus-specific T cells in children with adenovirus infection after allogeneic stem cell transplantation. Br J Haematol. 2005;128(4):503-9.

156. Feuchtinger T, Lang P, Hamprecht K, Schumm M, Greil J, Jahn G, Niethammer D, Einsele H. Isolation and expansion of human adenovirus-specific CD4+ and CD8+ T cells according to IFN-gamma secretion for adjuvant immunotherapy. Exp Hematol. 2004;32(3):282-9.
157. Feucht J, Opherk K, Lang P, Kayser S, Hartl L, Bethge W, Matthes-Martin S, Bader P, Albert MH, Maecker-Kolhoff B, Greil J, Einsele H, Schlegel PG, Schuster FR, Kremens B, Rossig C, Gruhn B, Handgretinger R, Feuchtinger T. Adoptive T-cell therapy with hexon-specific Th1 cells as a treatment of refractory adenovirus infection after HSCT. Blood. 2015;125(12):1986-94.
158. Feuchtinger T, Matthes-Martin S, Richard C, Lion T, Fuhrer M, Hamprecht K, Handgretinger R, Peters C, Schuster FR, Beck R, Schumm M, Lotfi R, Jahn G, Lang P. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol. 2006;134(1):64-76.
159. Smith CA, Woodruff LS, Rooney C, Kitchingman GR. Extensive cross-reactivity of adenovirus-specific cytotoxic T cells. Hum Gene Ther. 1998;9(10):1419-27.
160. Heemskerk B, Veltrop-Duits LA, van Vreeswijk T, ten Dam MM, Heidt S, Toes RE, van Tol MJ, Schilham MW. Extensive cross-reactivity of CD4+ adenovirus-specific T cells: implications for immunotherapy and gene therapy. J Virol. 2003;77(11):6562-6.
161. Hutnick NA, Carnathan D, Demers K, Makedonas G, Ertl HC, Betts MR. Adenovirus-specific human T cells are pervasive, polyfunctional, and cross-reactive. Vaccine. 2010;28(8):1932-41.
162. Serangeli C, Bicanic O, Scheible MH, Wernet D, Lang P, Rammensee HG, Stevanovic S, Handgretinger R, Feuchtinger T. Ex vivo detection of adenovirus specific CD4+ T-cell responses to HLA-DR-epitopes of the Hexon protein show a contracted specificity of T(HELPER) cells following stem cell transplantation. Virology. 2010;397(2):277-84.
163. Olive M, Eisenlohr L, Flomenberg N, Hsu S, Flomenberg P. The adenovirus capsid protein hexon contains a highly conserved human CD4+ T-cell epitope. Hum Gene Ther. 2002;13(10):1167-78.
164. Sumida SM, Truitt DM, Lemckert AA, Vogels R, Custers JH, Addo MM, Lockman S, Peter T, Peyerl FW, Kishko MG, Jackson SS, Gorgone DA, Lifton MA, Essex M, Walker BD, Goudsmit J, Havenga MJ, Barouch DH. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. J Immunol. 2005;174(11):7179-85.
165. Crawford-Miksza L, Schnurr DP. Analysis of 15 adenovirus hexon proteins reveals the location and structure of seven hypervariable regions containing serotype-specific residues. J Virol. 1996;70(3):1836-44.
166. Russell WC. Update on adenovirus and its vectors. J Gen Virol. 2000;81(Pt 11):2573-604.
167. Leen AM, Christin A, Khalil M, Weiss H, Gee AP, Brenner MK, Heslop HE, Rooney CM, Bollard CM. Identification of hexon-specific CD4 and CD8 T-cell epitopes for vaccine and immunotherapy. J Virol. 2008;82(1):546-54.
168. Leen AM, Sili U, Vanin EF, Jewell AM, Xie W, Vignali D, Piedra PA, Brenner MK, Rooney CM. Conserved CTL epitopes on the adenovirus hexon protein expand subgroup cross-reactive and subgroup-specific CD8+ T cells. Blood. 2004;104(8):2432-40.
169. Tang J, Olive M, Champagne K, Flomenberg N, Eisenlohr L, Hsu S, Flomenberg P. Adenovirus hexon T-cell epitope is recognized by most adults and is restricted by HLA DP4, the most common class II allele. Gene Ther. 2004;11(18):1408-15.
170. Zandvliet ML, Falkenburg JH, van Liempt E, Veltrop-Duits LA, Lankester AC, Kalpoe JS, Kester MG, van der Steen DM, van Tol MJ, Willemze R, Guchelaar HJ, Schilham MW, Meij P. Combined CD8+ and CD4+ adenovirus hexon-specific T cells associated with viral clearance after stem cell transplantation as treatment for adenovirus infection. Haematologica. 2010;95(11):1943-51.
171. https://www.uniprot.org/ [Internet]. 2017 [cited September 2016].

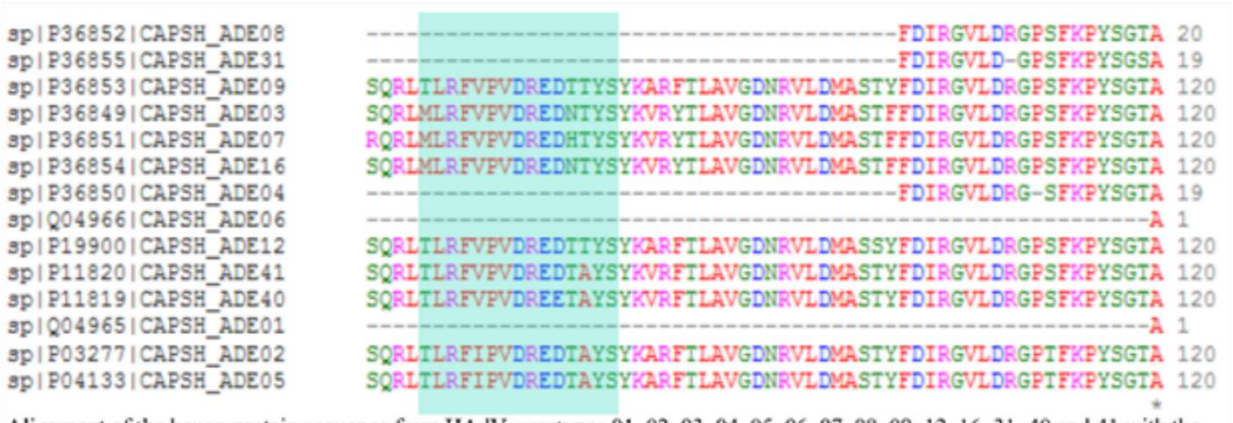
172. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics. 1999;50(3-4):213-9.
173. Dorrie J, Krug C, Hofmann C, Muller I, Wellner V, Knippertz I, Schierer S, Thomas S, Zipperer E, Printz D, Fritsch G, Schuler G, Schaft N, Geyeregger R. Human adenovirus-specific gamma/delta and CD8+ T cells generated by T-cell receptor transfection to treat adenovirus infection after allogeneic stem cell transplantation. PLoS One. 2014;9(10):e109944.
174. Gunther PS, Peper JK, Faist B, Kayser S, Hartl L, Feuchtinger T, Jahn G, Neuenhahn M, Busch DH, Stevanovic S, Dennehy KM. Identification of a Novel Immunodominant HLA-B*07: 02-restricted Adenoviral Peptide Epitope and Its Potential in Adoptive Transfer Immunotherapy. J Immunother. 2015;38(7):267-75.
175. Keib A, Mei YF, Cicin-Sain L, Busch DH, Dennehy KM. Measuring Antiviral Capacity of T Cell Responses to Adenovirus. J Immunol. 2019;202(2):618-24.
176. Tischer S, Geyeregger R, Kwoczek J, Heim A, Figueiredo C, Blasczyk R, Maecker-Kolhoff B, Eiz-Vesper B. Discovery of immunodominant T-cell epitopes reveals penton protein as a second immunodominant target in human adenovirus infection. J Transl Med. 2016;14(1):286.
177. Imahashi N, Nishida T, Ito Y, Kawada J, Nakazawa Y, Toji S, Suzuki S, Terakura S, Kato T, Murata M, Naoe T. Identification of a novel HLA-A*24:02-restricted adenovirus serotype 11-specific CD8+ T-cell epitope for adoptive immunotherapy. Mol Immunol. 2013;56(4):399-405.
178. Zhu F, Xu H, Tsao A, Margolis DA, Keever-Taylor CA. Generation of cytotoxic T-cell lines using overlapping pentadecapeptides derived from conserved regions of the adenovirus hexon protein. J Gen Virol. 2010;91(Pt 6):1577-89.
179. Keib A, Gunther PS, Faist B, Halenius A, Busch DH, Neuenhahn M, Jahn G, Dennehy KM. Presentation of a Conserved Adenoviral Epitope on HLA-C*0702 Allows Evasion of Natural Killer but Not T Cell Responses. Viral Immunol. 2017;30(3):149-56.
180. Icheva V, Kayser S, Wolff D, Tuve S, Kyzirakos C, Bethge W, Greil J, Albert MH, Schwinger W, Nathrath M, Schumm M, Stevanovic S, Handgretinger R, Lang P, Feuchtinger T. Adoptive transfer of epstein-barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. J Clin Oncol. 2013;31(1):39-48.
181. Rammensee HG, Wiesmuller KH, Chandran PA, Zelba H, Rusch E, Gouttefangeas C, Kowalewski DJ, Di Marco M, Haen SP, Walz JS, Gloria YC, Bodder J, Schertel JM, Tunger A, Muller L, Kiessler M, Wehner R, Schmitz M, Jakobi M, Schneiderhan-Marra N, Klein R, Laske K, Artzner K, Backert L, Schuster H, Schwenck J, Weber ANR, Pichler BJ, Kneilling M, la Fougere C, Forchhammer S, Metzler G, Bauer J, Weide B, Schippert W, Stevanovic S, Loffler MW. A new synthetic toll-like receptor 1/2 ligand is an efficient adjuvant for peptide vaccination in a human volunteer. J Immunother Cancer. 2019;7(1):307.
182. Nielsen M, Lund O, Buus S, Lundegaard C. MHC class II epitope predictive algorithms. Immunology. 2010;130(3):319-28.
183. Kampstra ASB, van Heemst J, Janssen GM, de Ru AH, van Lummel M, van Veelen PA, Toes REM. Ligandomes obtained from different HLA-class II-molecules are homologous for N- and C-terminal residues outside the peptide-binding cleft. Immunogenetics. 2019;71(8-9):519-30.
184. Hammer J, Valsasnini P, Tolba K, Bolin D, Higelin J, Takacs B, Sinigaglia F. Promiscuous and allele-specific anchors in HLA-DR-binding peptides. Cell. 1993;74(1):197-203.
185. Nielsen M, Justesen S, Lund O, Lundegaard C, Buus S. NetMHCIIpan-2.0 - Improved pan-specific HLA-DR predictions using a novel concurrent alignment and weight optimization training procedure. Immunome Res. 2010;6:9.
186. Höttler A, März L, Lübke M, Rammensee H-G, Stevanović S. Broad and Efficient Activation of Memory CD4+ T Cells by Novel HAdV- and HCMV-Derived Peptide Pools. Front Immunol. 2021;12(2691).

187. Camacho-Fernandez C, Hervas D, Rivas-Sendra A, Marin MP, Segui-Simarro JM. Comparison of six different methods to calculate cell densities. Plant Methods. 2018;14:30.
188. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol. 2007;96:41-101.
189. http://www.allelefrequencies.net/. 2003.
190. Sidney J, Peters B, Frahm N, Brander C, Sette A. HLA class I supertypes: a revised and updated classification. BMC Immunol. 2008;9:1.
191. Lee JK, Stewart-Jones G, Dong T, Harlos K, Di Gleria K, Dorrell L, Douek DC, van der Merwe PA, Jones EY, McMichael AJ. T cell cross-reactivity and conformational changes during TCR engagement. J Exp Med. 2004;200(11):1455-66.
192. Borbulevych OY, Piepenbrink KH, Gloor BE, Scott DR, Sommese RF, Cole DK, Sewell AK, Baker BM. T cell receptor cross-reactivity directed by antigen-dependent tuning of peptide-MHC molecular flexibility. Immunity. 2009;31(6):885-96.
193. Kohm AP, Fuller KG, Miller SD. Mimicking the way to autoimmunity: an evolving theory of sequence and structural homology. Trends Microbiol. 2003;11(3):101-5.
194. Wooldridge L, Ekeruche-Makinde J, van den Berg HA, Skowera A, Miles JJ, Tan MP, Dolton G, Clement M, Llewellyn-Lacey S, Price DA, Peakman M, Sewell AK. A single autoimmune T cell receptor recognizes more than a million different peptides. J Biol Chem. 2012;287(2):1168-77.
195. Arnold PY, La Gruta NL, Miller T, Vignali KM, Adams PS, Woodland DL, Vignali DA. The majority of immunogenic epitopes generate CD4+ T cells that are dependent on MHC class II-bound peptide-flanking residues. J Immunol. 2002;169(2):739-49.
196. Holland CJ, Cole DK, Godkin A. Re-Directing CD4(+) T Cell Responses with the Flanking Residues of MHC Class II-Bound Peptides: The Core is Not Enough. Front Immunol. 2013;4:172.
197. Moudgil KD, Sercarz EE, Grewal IS. Modulation of the immunogenicity of antigenic determinants by their flanking residues. Immunol Today. 1998;19(5):217-20.
198. Ostapchuk P, Almond M, Hearing P. Characterization of Empty adenovirus particles assembled in the absence of a functional adenovirus IVa2 protein. J Virol. 2011;85(11):5524-31.
199. Anderson CW, Young ME, Flint SJ. Characterization of the adenovirus 2 virion protein, mu. Virology. 1989;172(2):506-12.
200. Macdonald WA, Purcell AW, Mifsud NA, Ely LK, Williams DS, Chang L, Gorman JJ, Clements CS, Kjer-Nielsen L, Koelle DM, Burrows SR, Tait BD, Holdsworth R, Brooks AG, Lovrecz GO, Lu L, Rossjohn J, McCluskey J. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. J Exp Med. 2003;198(5):679-91.
201. Probst-Kepper M, Hecht HJ, Herrmann H, Janke V, Ocklenburg F, Klempnauer J, van den Eynde BJ, Weiss S. Conformational restraints and flexibility of 14-meric peptides in complex with HLA-B*3501. J Immunol. 2004;173(9):5610-6.
202. Tynan FE, Burrows SR, Buckle AM, Clements CS, Borg NA, Miles JJ, Beddoe T, Whisstock JC, Wilce MC, Silins SL, Burrows JM, Kjer-Nielsen L, Kostenko L, Purcell AW, McCluskey J, Rossjohn J. T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. Nat Immunol. 2005;6(11):1114-22.
203. Frahm N, Yusim K, Suscovich TJ, Adams S, Sidney J, Hraber P, Hewitt HS, Linde CH, Kavanagh DG, Woodberry T, Henry LM, Faircloth K, Listgarten J, Kadie C, Jojic N, Sango K, Brown NV, Pae E, Zaman MT, Bihl F, Khatri A, John M, Mallal S, Marincola FM, Walker BD, Sette A, Heckerman D, Korber BT, Brander C. Extensive HLA class I allele promiscuity among viral CTL epitopes. Eur J Immunol. 2007;37(9):2419-33.
204. Shao H, Peng Y, Liao T, Wang M, Song M, Kaplan HJ, Sun D. A shared epitope of the interphotoreceptor retinoid-binding protein recognized by the CD4+ and CD8+ autoreactive T cells. J Immunol. 2005;175(3):1851-7.

205. May RJ, Dao T, Pinilla-Ibarz J, Korontsvit T, Zakhaleva V, Zhang RH, Maslak P, Scheinberg DA. Peptide epitopes from the Wilms' tumor 1 oncoprotein stimulate CD4+ and CD8+ T cells that recognize and kill human malignant mesothelioma tumor cells. Clin Cancer Res. 2007;13(15 Pt 1):4547-55.
206. Godkin AJ, Thomas HC, Openshaw PJ. Evolution of epitope-specific memory CD4(+) T cells after clearance of hepatitis C virus. J Immunol. 2002;169(4):2210-4.
207. Kleiveland CR. Peripheral Blood Mononuclear Cells. In: Verhoeckx K, Cotter P, Lopez-Exposito I, Kleiveland C, Lea T, Mackie A, et al., editors. The Impact of Food Bioactives on Health: in vitro and ex vivo models. Cham (CH)2015. p. 161-7.
208. Feuchtinger T, Richard C, Joachim S, Scheible MH, Schumm M, Hamprecht K, Martin D, Jahn G, Handgretinger R, Lang P. Clinical grade generation of hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. J Immunother. 2008;31(2):199-206.
209. Toh HC, Sun L, Soe Y, Wu Y, Phoon YP, Chia WK, Wu J, Wong KY, Tan P. G-CSF induces a potentially tolerant gene and immunophenotype profile in T cells in vivo. Clin Immunol. 2009;132(1):83-92.
210. Eiz-Vesper B, Maecker-Kolhoff B, Blasczyk R. Adoptive T-cell immunotherapy from third-party donors: characterization of donors and set up of a T-cell donor registry. Front Immunol. 2012;3:410.

10 Appendix

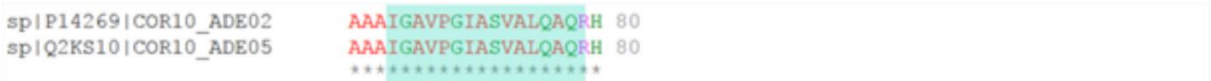
10.1 Alignment



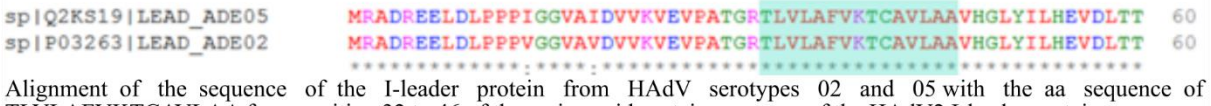
Alignment of the hexon protein sequence from HAdV serotypes 01, 02, 03, 04, 05, 06, 07, 08, 09, 12, 16, 31, 40 and 41 with the aa sequence of TLRFIPVDREDTAYS from position 65 to 79 of the amino acid protein sequence of the HAdV2 hexon protein

Alignment of the hexon protein sequence from HAdV serotypes 01, 02, 03, 04, 05, 06, 07, 08, 09, 12, 16, 31, 40 and 41 with the aa sequence of PQKFFAIKNLLLLPG from position 579 to 593 of the amino acid protein sequence of the HAdV2 hexon protein. Adapted from (186).

Alignment of the hexon protein sequence from HAdV serotypes 01, 02, 03, 04, 05, 06, 07, 08, 09, 12, 16, 31, 40 and 41 with the aa sequence of DEPTLLYVLFEVFDV from position 927 to 941 of the amino acid protein sequence of the HAdV2 hexon protein

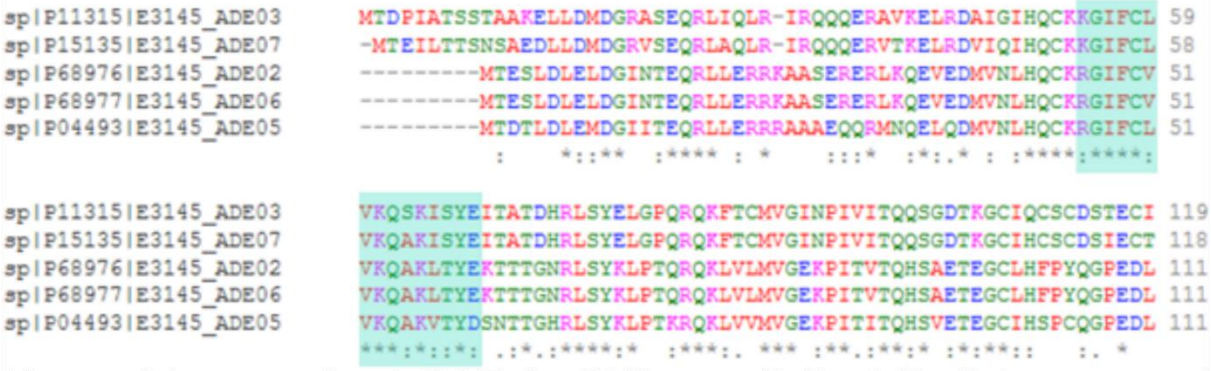


Alignment of the sequence of the protein X from HAdV serotypes 02 and 05 with the aa sequence of IGAVPGIASVALQAQ from position 64 to 78 of the amino acid protein sequence of protein X from HAdV2.



Alignment of the sequence of the I-leader protein from HAdV serotypes 02 and 05 with the aa sequence of TLVLAFVKTCAVLAA from position 32 to 46 of the amino acid protein sequence of the HAdV2 I-leader protein.

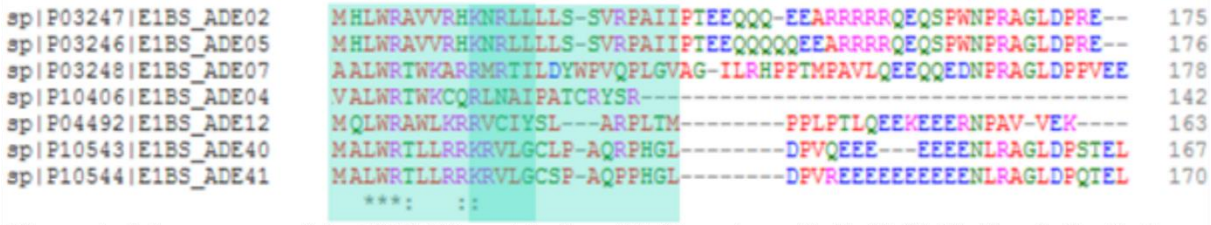
Alignment of the sequence of protein pIII from HAdV serotypes 02, 05 and 12 with the aa sequence of RQVMDRIMSLTARNP from position 28 to 42 of the amino acid protein sequence of protein pIII from HAdV2.



Alignment of the sequence of protein E3-14,7k from HAdV serotypes 02, 05 and 12 with the aa sequence of RGIFCVVKQAKLTYE from position 46 to 60 of the amino acid protein sequence of protein E3-14,7k from HAdV2. Adapted from (186).

Alignment of the sequence of the Packaging protein I from HAdV serotypes 02, 05, 07, 12 and 40 with the aa sequence of VSKFFHAFPSKLHDK from position 292 to 306 of the amino acid protein sequence of Packaging protein I from HAdV2.

Alignment of the sequence of the hexon protein from HAdV serotypes 01, 02, 03, 04, 05, 06, 07, 08, 09, 12, 16, 31, 40 and 41 with the aa sequence of TFYLNHTFKKVAITF from position 727 to 741 of the amino acid protein sequence of the hexon protein from HAdV2.



Alignment of the sequence of the E1BK-19k protein from HAdV serotypes 02, 04, 05, 07, 12, 40 and 41 with the aa sequence of MHLWRAVVRHKNRLL and KNRLLLLSSVRPAII from position 120 to 134 and 130 to 144 of the amino acid protein sequence of the E1B-19k protein from HAdV2.

Erklärung zum Eigenanteil der Dissertationsschrift

Die Arbeit wurde in der Abteilung für Immunologie am Interfakultären Institut für Zellbiologie (IFIZ) in Tübingen unter Betreuung von Prof. Dr. Stefan Stevanović realisiert. Teile der Dissertationsschrift wurden veröffentlicht.

Sämtliche Versuche wurden nach Einarbeitung durch Dr. Maren Lübke von mir eigenständig durchgeführt. Die Produktion sowie die Qualitätskontrolle der Peptide wurden von Ulrich Wulle, Nicole Bauer und Patricia Hrstić ausgeführt.

Der Versuchsaufbau wurde in Rücksprache mit Prof. Dr. Stefan Stevanović und Dr. Maren Lübke konzipiert.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Löwenstein, den 10.07.2021

Léo März

Danksagung

Als aller erstes möchte ich mich bei Prof. Dr. Stefan Stevanović für seine Immunologie-Vorlesung in meinem 5. Semester bedanken, welche überhaupt erst mein Interesse an der Immunologie geweckt hat. Außerdem möchte ich mich für die Aufnahme in seine Arbeitsgruppe, die hervorragende Betreuung und die vielen guten Denkanstöße und Diskussionen bedanken.

Des Weiteren möchte ich mich bei Hans-Georg Rammensee für die Bereitstellung eines Arbeitsplatzes, die vielen konstruktiven Beiträge und die ansteckende Begeisterung für die Forschung im Bereich der Immunologie bedanken.

Mein nächster Dank gilt Dr. Maren Lübke für die engagierte Betreuung, ihre fortwährende Geduld, Antworten auf die anfangs gehäuft auftretenden banalen Fragen und die Durchsicht der Arbeit.

Ein weiterer Dank gilt Ana Marcu, Paul Gratz und Dr. Michael Ghosh für viele wissenschaftliche sowie nicht-wissenschaftliche - hilfreiche, als auch weniger-hilfreiche, aber dennoch immer witzige - Unterhaltungen. Auch möchte ich hier die Durchsicht der Arbeit von Dr. Michael Ghosh und Ana Marcu erwähnen.

Ulrich Wulle, Nicole Bauer und Patricia Hrstić möchte ich einen Dank für die fortwährende Produktion, sowie die Qualitätskontrolle der Peptide aussprechen.

Auch möchte ich Alexander Höttler danken, mit dem der Austausch über unterschiedliche Methoden und der Vergleich der Ergebnisse der HAdV Epitope mit den CMV Epitopen stets sehr lehrreich und produktiv war.

Allen anderen Elchen, mit denen mir die Zusammenarbeit, sowie die Feiern immer unglaublich viel Spaß gemacht und die alle ihren Teil zu der unheimlich angenehmen Arbeitsatmosphäre beigetragen haben, möchte ich ebenfalls danken.

Außerdem möchte ich hier noch das SFB685 für die finanzielle Unterstützung, sowie die vielen interessanten Vorträge erwähnen.

Der letzte Dank gilt meiner Familie, die mich jederzeit unterstützt.