T cell epitopes
from viral and tumor associated antigens:
Induction and Analysis of antigen-specific T cells

T-Zellepitope
von viralen und tumor-assoziierten Antigenen:
Induktion und Analyse von antigen-spezifischen T Zellen

Dissertation

der Fakultät für Biologie
der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

vorgelegt
von

Maria-Dorothea Nastke
aus Potsdam
2005

Tag der mündlichen Prüfung: 21.10.2005
Dekan: Prof. Dr. F. Schöffl
1. Berichterstatter: Prof. Dr. H.-G. Rammensee
2. Berichterstatter: Prof. Dr. S. Stevanović

Meinen Eltern und meinem Bruder Sebastian

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1 General Introduction

1.1 The immune system – an overview

During evolution the immune system developed into a highly effective network to prevent higher organisms from severe damage caused by pathogens, e.g. viruses, bacteria, fungi and other parasites. Due to a complex system of humoral and cellular defence mechanisms the immune system is able to discriminate between self and non-self antigens. The defence mechanism or the so-called immune response can be subdivided into two different principles interacting with each other - the innate and the adaptive immunity. The function of the innate immunity is based on the recognition of pathogen-associated molecular patterns (PAMPs) by preformed receptors and effector cells. The components of an innate immune response are inflammatory cells such as macrophages and neutrophils, natural killer cells (NK cells), γ:δ T cells, B-1 B cells and the complement system. As these components are constitutively present in the body the innate immune response provides a first line of defence for the organism controlling infections in the first four days before an initial adaptive immune response takes place. However, the innate immunity is inflexible using receptors with restricted diversity and it does not generate an immunological memory.

Adaptive immunity also known as acquired immune response is based on clonal selection of antigen-specific effector lymphocytes [1; 2] and on the generation of memory cells to prevent reinfection. Furthermore, this system is able to respond to high numbers of foreign antigens using receptors whose specificities do not have to be genetically encoded. The adaptive immune response is characterized by the humoral immunity (latin: umor – moisture, fluid) and the cellular immunity. Humoral immunity can protect against extracellular antigens and toxins providing antibodies which are secreted by B lymphocytes activated by antigen-specific T helper cells (TH2). Cellular immunity protects from intracellular pathogens (viruses, intracellular bacteria) and tumors and is mainly based on cytotoxic T lymphocytes (CTLs) and inflammatory TH1 cells. Activation of CTLs depends on interaction with professional antigen-presenting cells (APCs), most notably dendritic cells (DCs) and TH1 cells (Figure 1). After interaction by pathogenic structures with PAMP-receptors on the surface, DCs become activated and are able to take up antigen in the periphery,

travel to peripheral immune organs and present antigen on MHC molecules to T cells. Therefore, one may say that DCs lie at the interface between innate and adaptive immunity.

To encounter the dangers of daily life in form of viruses, bacteria, fungi and other parasites, the immune system is facilitated with a great variety of different mechanism of defense. These, unfortunately, come along with the risk for various malfunctions, e.g. allergy, autoimmune diseases or tumor development. For this purpose the major aim of research is to achieve a better and more detailed understanding of the immune system.

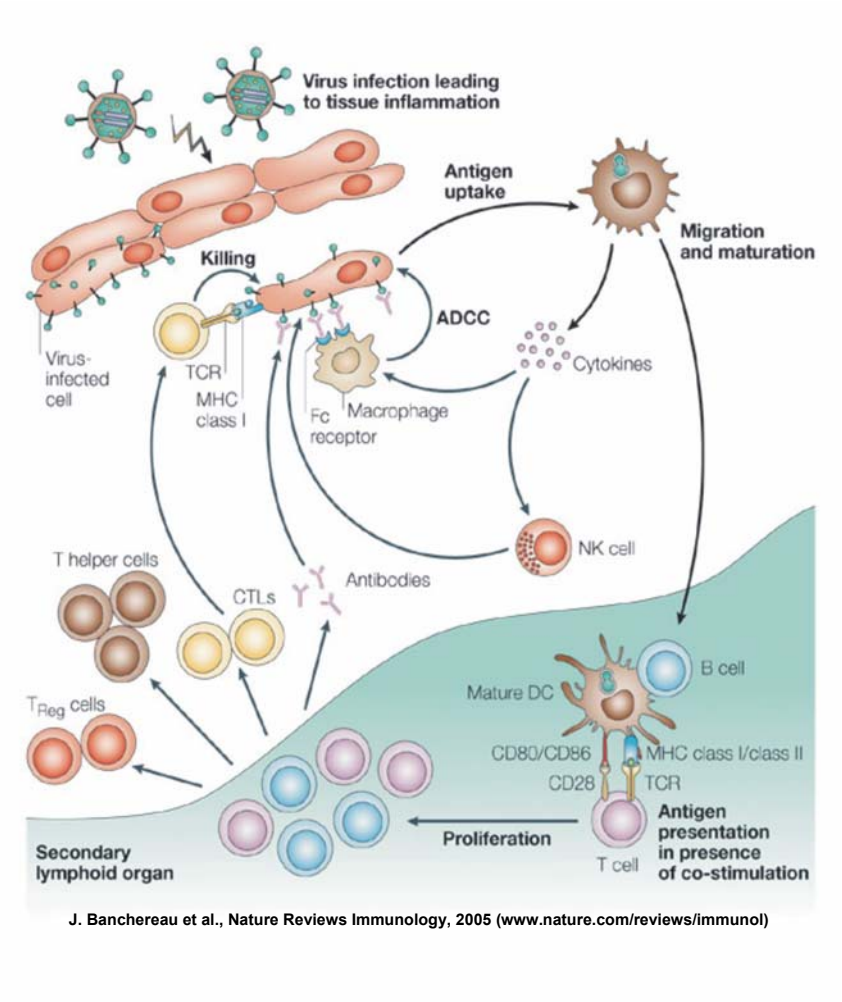

Figure 1. Dendritic cells take up antigens in peripheral tissues, become matured and migrate to draining lymphnodes. After processing of the antigen dendritic cells express MHC/peptide-complexes and co-stimulatory molecules. In the lymphnodes T cells are primed and B cells are activated after contact with antigen presenting DCs leading to initiation of an adaptive immune response.

1.2 Major histocompatibility complex molecules and antigen processing

T lymphocytes recognize antigens presented on major histocompatibility complex (MHC) molecules [3] found on the surface of all nucleated cells. MHC molecules - called human leukocyte antigens (HLA) in humans - are encoded by a large, highly polymorphic gene cluster on chromosome six in humans, which is usually divided into a class I, II, and III antigenic region. MHC class I molecules are heterodimers composed of a heavy α-chain which is non-covalently linked to β2-microglobulin (β2m). The α-chain has a short cytoplasmic C-terminal part, a transmembrane domain and three extracellular domains (α_1-α_3). The two N-terminal domains α_1-α_2 form a peptide binding groove which is closed at both ends and accommodates the presented peptide with a length of 8-10 amino acids. Non-covalent hydrophobic and ionic interactions between the peptide and the α-chain mainly take place between so-called pockets in the α_1-domain and the anchor amino acids of the ligand and involve the N- and C-termini of the peptide.

MHC class I molecules present peptides generated by normal cellular degradation of endogenously occurring proteins [4]. A large multienzyme complex - called ubiquitin-proteasome - generates precursor peptides that have a correct C-terminus and may have N-terminal extensions of several amino acids [5] which are further trimmed by aminopeptidases found either in the cytosol [6] or in the endoplasmic reticulum (ER) [7]. The 20S core proteasome with its three constitutive proteolytic subunits (δ/Y, Z and X) shows three different proteolytical activities: a chymotrypsin-like (cleavage after hydrophobic residues), a trypsin-like (cleavage after basic residues) and a caspase-like (cleavage after acidic residues) [8]. In the presence of interferon γ (IFNγ) the constitutive proteasome can be converted into immunoproteasome by exchange of the subunits (δ/Y, Z and X) which harbor the active sites by immunosubunits called MECL1, LMP2 and LMP7 [9]. The newly formed immunoproteasome is able to enhance the generation of peptides presented on MHC class I [10]. The peptides generated are transported into the ER using the transporter associated with antigen processing (TAP) [11]. In the ER MHC I heavy chain-β_2m heterodimers are loaded with peptides through interactions in the peptide-loading complex which consists additionally of the transmembrane glycoprotein tapasin, the chaperone calreticulin and the thiol oxidoreductase ERp57 [12]. The accumulated

MHC I heavy chain, β_2m and peptide complexes are then transported to the cell surface where they interact with $CD8^+$ T cells.

The structure of MHC class II molecules, in humans HLA-DR, -DQ, -DP, overall strongly resembles class I, although it consists of two polymorphic membrane-spanning chains, the α-chain and the β-chain with two domains each. In contrast to MHC class I molecules, the peptide binding groove is open at both ends what allows binding of longer peptides with 10-15 amino acids. MHC class II molecules present peptides from exogenous proteins acquired by endocytosis or from internalized plasma membrane proteins to $CD4^+$ T cells. Processing of exogenous antigens and peptide loading take place in the same compartment in contrast to the MHC class I processing pathway. MHC class II α- and β-chains are synthesized into the ER and transported in association with the trimeric invariant chain (Ii) to the MHC class II loading compartment. There, the class II-associated invariant-chain peptide (CLIP), a part of the invariant chain which occupies the peptide binding groove of the MHC class II molecules, is replaced by the later presented peptides. This exchange is catalyzed and regulated by HLA-DM and HLA-DO [13].

Recapitulating, in a classical view, MHC class I molecules present peptides from intracellular source proteins, whereas MHC class II molecules present antigenic peptides from exogenous and membrane proteins (Figure 2). However, alternative pathways for delivering peptides have been described. It is known that MHC class I molecules are able to present peptides derived from exogenous antigens by a process called cross presentation [14]. Intracellular proteins, in contrast, can be presented by MHC class II molecules [15]. It was shown that autophagy plays a role in the MHC class II-restricted presentation of peptides from intracellular proteins [16]. Autophagy is the degradation of intracellular components in lysosomes and is therefore relevant in the endosomal/lysosomal degradation pathway.

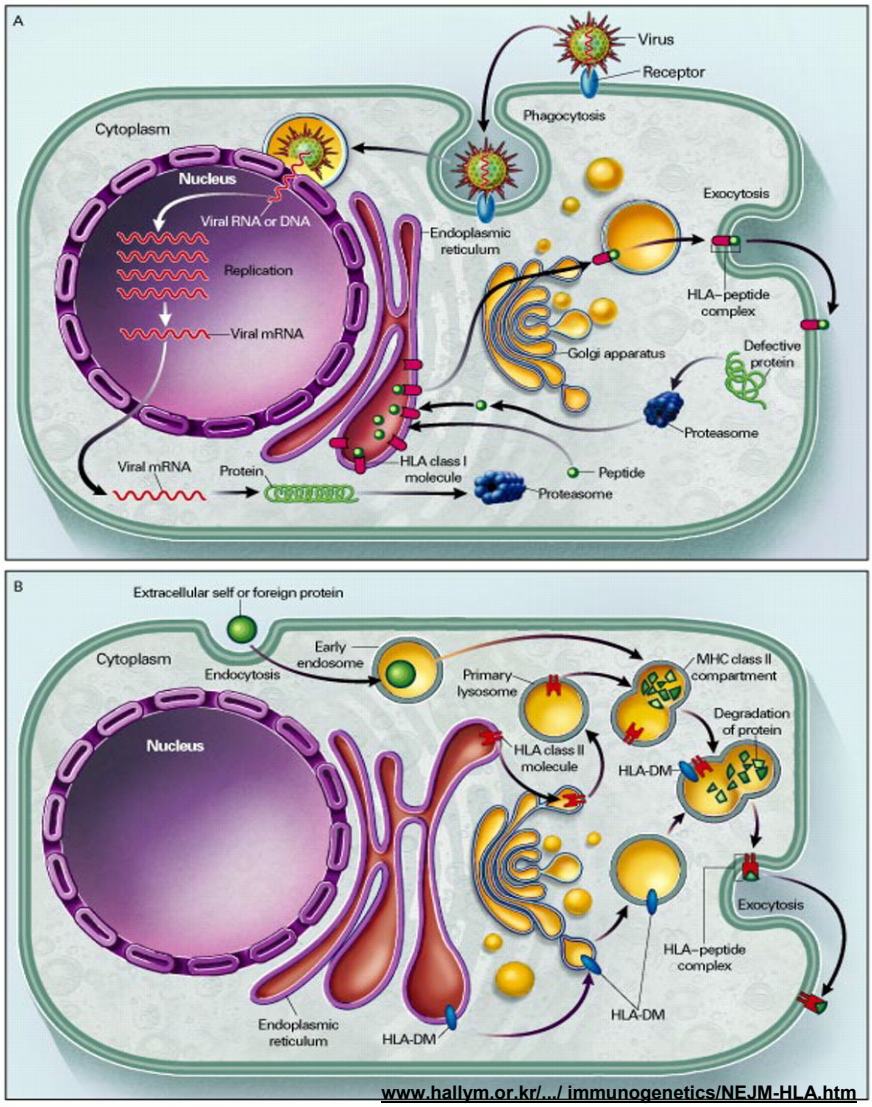

Figure 2. (A) Defective proteins in the cytosol are degraded into peptides in proteasomes. A vast majority of proteins are degraded by proteasomes shortly after their synthesis, presumably due in large part to their inability to achieve a functional state. These polypeptides are termed defective ribosomal products (DRiPs). Peptides are transported into the ER, where they are loaded onto newly synthesized class I molecules. The HLA–peptide complexes are exported by way of the Golgi apparatus to the surface of the cell. (B) shows the processing of extracellular proteins. Self or foreign proteins are taken up by endocytosis (or phagocytosis) and sequestered into endosomes. Class II molecules synthesized in the endoplasmic reticulum are delivered by way of the Golgi apparatus into primary lysosomes, which fuse with the early endosomes to form the major-histocompatibility-complex (MHC) class II compartment. Enzymes brought into this compartment by the lysosomes degrade the engulfed proteins into peptides. HLA-DM molecules synthesized in the endoplasmic reticulum and delivered into the MHC class II compartment by transport vesicles help load the peptides onto the class II molecules. Additionally, peptides can also derive from intracellular antigens, which can be presented on MHC class II molecules through autophagy. The HLA–peptide complexes are then exported to the surface of the cell.

1.3 T lymphocytes

Early in development, T lymphocytes are able to differentiate in T cells expressing αβ T cell receptors (αβ-TCR) or γδ T cell receptors. γδ T cells are supposed to recognize heat-shock proteins and unorthodox nucleotides and phospholipids bound to non-classical MHC molecules such as CD1 and, additionally, might have regulatory effects. αβ T cells recognize processed antigen presented on major histocompatibility complex (MHC) molecules on the surface of cells. T cells expressing the αβ-TCR are $CD8^+$ T cells, $CD4^+$ T cells or NK T cells. Double positive ($CD4^+$ $CD8^+$) T cells pass through the thymus during development where they undergo a positive selection by self MHC molecules. Binding to a MHC class I molecule results in loss of CD4 expression, therefore the T cell becomes CD8 positive. If the T cell binds to MHC class II molecules it becomes CD4 positive. T cells binding to neither self MHC class I nor class II molecules die. Negative selection removes T cells expressing a TCR which recognizes self-peptides to prevent self-destruction. Nevertheless, some antigens presented in tissues or organs are not present in the thymus. Self-reactive T cells recognizing peptides from those antigens become anergic in the periphery. This is due to a lack of additional signals needed during the first antigen contact. Thus, T cells can distinguish between self and non-self or malignant antigens.

T cells that survived as well positive as negative selection in the thymus are named naive T cells which circle through the body via the blood and lymph streams. Contact between a professional APC providing a suitable MHC/peptide complex and a co-stimulatory second signal leads to the activation of the T cell, a process called priming. Activated T cells proliferate and differentiate into armed effector T cells, either cytotoxic T lymphocytes (CTLs), in the case of $CD8^+$ T cells, or TH1 and TH2 cells, in the case of $CD4^+$ T cells. CTLs are able to destroy their target cells via the induction of apoptosis, either using perforin and granzymes [17] or Fas-ligand interaction [18; 19]. TH1 cells secrete IFNγ and interleukin 2 (IL-2) [20] leading to activation of macrophages and $CD8^+$ T cells. In turn, TH2 cells support a humoral immune response by secretion of IL-4 [21] what leads to an activation of antibody secreting B cells and the complement system.

Another T cell population was described which are $CD4^+$ and express constitutively CD25 [22]. These cells were named regulatory T cells (T_{reg}). They are able to inhibit T cell reactions, a process which is not completely understood until now.

1.4 The Human Cytomegalovirus

The human cytomegalovirus (HCMV) is a ß-herpesvirus which is characterized by its strict host specificity. It provokes an acute infection followed by a lifelong persistence of the virus in the human organism with episodes of endogenous reactivation [23]. HCMV infects the host via mucosal contacts or parenterally via infected cells in the blood. Furthermore, there is intrauterine (congenital infection), perinatal (cervix secretions) and postnatal (breast milk) virus transmission, [24-27] which occurs also by saliva and sexual contact [28].

HCMV infection is common worldwide. The prevalence increases continuously after the childhood. In developed countries 10% - 20% of all children and 40% - 100% of adults are HCMV-seropositive [29] whereas the status in developing countries is much higher [29].

Generally HCMV causes only minor symptoms in immunocompetent individuals, [30] mostly in terms of mononucleosis [24]. After this initial response, healthy individuals carry the virus as a latent infection. Evidences for a major role of T cells in the immune control of this persistent infection are provided by the uncontrolled viral replication and HCMV end-organ diseases observed in immunocompromised individuals with severely impaired T cell functions like transplant recipients or AIDS-patients [31; 32]. Thus, the virus appears to be a major cause of morbidity and mortality in immunocompromised persons. In these patients the most frequent manifestation of severe uncontrolled HCMV reactivation is retinitis [33-36], hepatitis [37; 38] and pneumonia [39; 40] as well as gastro-intestinal erosions and ulcer [41-43].

Furthermore, severe aetiopathology are described in children infected intrauterine [23; 44; 45]. Congenital infection is associated with a range of clinical manifestation, but relatively few infected infants are severely ill at birth. In many, the signs and symptoms may be subtle or non-specific during the newborn period and during early childhood, even as the child experiences progressive hearing damage and as serious consequences of infection, including mental retardation, cerebral palsy, and impaired vision, become apparent [46]. HCMV is one of the most important causes, infectious or non-infectious, of deafness in children [44].

1.4.1 The control of HCMV by the immune system

Both innate and adaptive arms of the immune response are of importance for the control of HCMV infection. Within the adaptive immune response, both T cells and antibodies have been shown to protect from acquisition of HCMV or from serious disease in different settings [47-49]. Some studies suggest that transplacentally acquired or passively administered antibody protects against HCMV disease in neonates and transplant recipients. Antibodies, presumably capable of neutralizing infectivity, can have a role in protection against infection or disease [30; 50]. Nevertheless, antibodies are not typically sufficient to control HCMV infection. Virus-specific T cell immunity is the most important adaptive immune component.

HCMV can not be eliminated by the host immune response, although the titer of the virus in the mucosa and the peripheral blood is reduced and the virus becomes undetectable – persistence and latency are established. Once infection has occurred, the role of the immune system is to suppress replication and to establish and maintain the balance such, that reactivations of the virus remain subclinical. The characterization of the immune response against HCMV turn out to be quite difficult because most HCMV infections are asymptomatic making it impossible to examine the early phases of host response to the virus [28]. However, experiments in immunocompromised bone marrow transplant recipients [51-54] demonstrate that CMV-specific cell-mediated immunity is essential to control the disease. The innate immune response may augment the adaptive immune response, and the magnitude of the initial adaptive immune response is important in determining the numbers of antigen-specific memory T cells.

Natural killer cells (NK cells) may also be mentioned to play an important role in the passage from innate immune response to adaptive immunity against HCMV infection. The production of IFNγ by NK cells facilitates the expansion of antigen-specific TH cells which are critical for HCMV control [32; 55]. During initial infection, the frequence of HCMV-specific $CD8^+$ T cells raises to a very high level. After suppression of viral replication many of HCMV-specific T cells die, but compared to other common viral pathogens, the numbers of circulating T cells that recognize HCMV peptides remain quite high [56; 57]. HCMV-specific HLA class I-restricted T cell responses are known to be essential for successful resolution of the infection and maintenance of long-term control of HCMV replication [58-61]. Two HCMV proteins

serve as key target antigens for HCMV-specific T cells: a late matrix protein (pp65; UL83) that is abundant throughout HCMV infection and an immediate early 1 (IE1; UL123) antigen protein that is indispensable for viral replication [62]. For a certain time IE1 was not considered a relevant target because reports had suggested that it was not efficiently presented on MHC class I after infection [63]. By contrast, virion proteins were known to be presented even in the absence of viral replication [64], and additionally, pp65 itself was found to interfere with IE1 presentation [65]. Therefore, research focussed on pp65 for several years [66; 67]. In 1999, IE1 became again of interest as a T cell target after IE1-specific $CD8^+$ T cells were detected in infected individuals at frequencies at least comparable to those of $CD8^+$ T cells specific for pp65 [68]. To date, both pp65 and IE1 are considered dominant T cell targets [69; 70]. All in all, many of the antigen-specific $CD8^+$ T cells recognize peptides of the pp65 or IE1 protein, although the spectrum of antigen recognition may include other viral proteins [71; 72]. HCMV glycoprotein B and other viral glycoproteins are targets of IgG, IgM, and IgA antibodies and are also recognized by T cells [73].

1.4.2 Vaccine development against HCMV

Vaccines against infectious agents are one hallmark of immunology and are of high success in diseases such as polio, measles, hepatitis B and tetanus [74]. Nevertheless, many infectious agents still evade the immune system and lead to severe infections. HCMV causes a spectrum of disease syndromes in children and adults. HCMV is a cause of mononucleosis in immunocompetent individuals and is a well-known cause of serious morbidity and sometimes fatal infections in immunocompromised patients especially recipients of solid-organ or hematopoetic cell allografts and individuals with advanced AIDS [75; 76]. Antiviral drugs have constituted an important advance for prevention or resolution of HCMV infections especially with regard to early posttransplant time. Nevertheless, long-term control of the persistent virus in the host will depend on the ability to raise an adequate immune response to the perspective pathogen. Studies in animal models of experimental infection and human studies have shown that the induction and perpetuation of virus-specific $CD8^+$ and $CD4^+$ T cells is sufficient and often essential for the resolution of infection [77; 78]. Moreover, $CD8^+$ and $CD4^+$ αβ-T cells provide an immunologic

memory response which may have major significance with regard to protective immunity to cytomegalovirus infection [79]. First studies which suggested a critical role of αβ-T cells in HCMV infection used a model with murine cytomegalovirus (MCMV). MCMV is genetically distinct from HCMV, but the pathogenesis of infection in immunosuppressed mice is similar to that for human CMV [80; 81]. In humans several studies examined the relationship between quantitative deficiency of virus-specific αβ T cell responses and progressive human HCMV infection in allogeneic hematopoetic cell transplant and solid organ transplant recipients [32].

Several technologies were developed for determining antigen-specific T cells, e.g. intracellular cytokine staining or staining with tetrameric MHC class I/peptide complexes. These have further confirmed the role of HCMV-specific T cells in controlling HCMV infection – in healthy HCMV-seropositive individuals, up to 40% of all T cells in the peripheral blood can be specific for HCMV [82] emphasizing the importance of a strong HCMV-specific cellular immunity in persistent HCMV infection. Furthermore, strategies to isolate and expand clonal populations of αβ-T cells with defined specificity for viral antigens became well-established and benefits adoptive cellular immunotherapy. For adoptive cellular immunotherapy, $CD8^+$ HCMV-specific T cell clones were cultured *in vitro* by cyclic stimulations of the T cells in presence of donor-derived γ-irradiated feeder cells and Interleukin-2 (IL-2) to promote numeric expansion. Clones that were αβ-TCR^+, $CD3^+$, $CD8^+$, and $CD4^-$, and recognized epitopes derived from structural virion proteins and presented by MHC class I were selected for intravenous administration to the recipient [83]. This first study on adoptive transfer of $CD8^+$ HCMV-specific T cell clones in allogeneic hematopoetic cell transplant recipients has proved safety and effectiveness of the method to restore $CD8^+$ HCMV-specific cytotoxic T cell immunity.

Further studies for the treatment of HMCV infection make use of dendritic cells which are pulsed with peptide or protein, or HCMV-specific $CD4^+$ T cells what was shown to be effective in inducing HCMV-specific $CD8^+$ T cell responses and reduction in HCMV viral DNA load [84]. New perceptions made in the field of the generation of antigen-specific T cells offered better conditions for adoptive immunotherapy. These include use of monocytes, dendritic cells, B lymphoblastoid cell lines as presenting cells of antigen delivered by pulsing with exogenous peptides or proteins [85], or of antigen processed endogenously after transduction with viral vectors. Moreover,

genetically modified T cells are used for adoptive immunotherapy offering the potential to be safe and efficient [86; 87].
As described above, protective responses to infectious agents like HCMV rely largely on T cells. Therefore, not only adoptive transfer of T cells is an approach for antiviral immunotherapy. Also the use of a peptide-based vaccine may be in focus of discussion. A main part of this thesis has the major objective to identify and characterize T cell responses specific for epitopes of HMCV which can be used to design a synthetic HCMV vaccine.

1.5 Immunotherapy in cancer

1.5.1 Clinical trials

Currently available therapeutic measures like radiotherapy and chemotherapy often suffer from severe toxicity and lack of specificity of the therapy towards tumor cells. In the last decades understanding of the immune response to cancer have led to new opportunities for the development of effective immunotherapies against cancer. Several clinical trials demonstrated that the immune system can be manipulated to specifically recognize and eliminate tumor cells [88; 89]. There are numerous strategies to activate an immune reaction against growing cancer whereby dendritic cells (DCs) play an important role, e.g. transfection of dendritic cells with total RNA [90] or RNA coding for defined tumor antigens [91], or DCs loaded with proteins [92; 93] or MHC class I peptides from tumor associated antigens [94]. Simply peptides in association with adjuvants such as GM-CSF or keyhole-limpet hemocyanin (KLH) [95], Interleukin 2 [96], Montanide ISA-51 [97], tumor associated [98] or artificial MHC class II ligands [99] can also be used to enhance a T cell response. Furthermore, there are approaches using hybrid cells designed by fusion of tumor cells and professional antigen presenting cells [100; 101], irradiated allogenic [102] or autologous tumor cells [103] which are manipulated to become more immunogenetic, recombinant virus used as transporters for DNA coding for tumor rejection [104] or autologous tumor-derived heat shock proteins [105].
By the expression of tumor antigens tumor cells may stand out from their surrounding. Many tumor antigens were identified which are either tumor specific

meaning they only appear in tumor tissue, for example cancer-testis antigens, mutated antigens and tumor-virus antigens, or tumor associated antigens which are highly overexpressed in tumors but can also be found in normal tissue, as for example differentiation antigens. Table 1 shows a classification of tumor rejection antigens.

Table 1: Classes of tumor antigens*

class	**examples**	**recognized by**	
		T cells	**antibodies**
Point mutation of normal gene	CDK4 [106]	+	-
frame shift mutation of normal gene	TGFβRII [107]	+	-
anti sense transcript of normal gene	RU2AS [108]	+	-
expressed intron of normal gene	N-acetylglucosaminyltransferase V [109]	+	-
fusion protein caused by translocation	BCR-ABL [110]	+	-
fusion protein caused by posttrans. mod.	gp100 [111]	+	-
altered posttranslational modification	Tyrosinase [112]	+	-
cancer/embryonic antigen	CEA [113; 114]	+	+
overexpressed antigens – protein	Her2/neu, MUC1 [115; 116]	+	+
overexpressed antigens – non-protein	ganglioside GD3 [117]	-	+
cancer testis antigen	NY-ESO-1 [118]		
	MAGE family [119]	+	+
oncogenes	Ras [120-122]	+	+
tumor suppressor genes	p53 [123; 124]	+	+
differentiation antigen	Tyrosinase [125; 126]	+	+
	gp100 [127; 128]	+	+
viral proteins	HPV E7 [129; 130]	+	+

* adapted from Rammensee et al. [131]

Vaccines against cancer aim to induce tumor-specific effector T cells that are able to reduce the mass of the tumor, as well as tumor-specific memory T cells that can control tumor relapse.

1.5.2 Lymphocytes and tumor regression

Identification of defined tumor antigens in humans [132; 133] facilitates the development of adoptive T cell therapy. An innovative strategy for vaccination should combine as well induction of therapeutic T cell immunity in form of tumor-specific effector T cells as protective T cell immunity in form of tumor-specific memory T cells which can control tumor relapse [134-136]. Several studies have been shown that cytotoxic T cells are able to recognize tumor rejection antigens on tumor cells and therefore may contribute to tumor regression [137; 138]. $CD4^+$ helper T cells also play an important role in orchestrating the effector function of anti-tumor T cell responses [139] therefore the identification of $CD4^+$ T cell epitopes derived from tumor-associated antigens has recently been a major focus of attention [140; 141]. All in all, to elicit a long lasting anti-tumor immune response the effector CTL response should be accompanied by effector $CD4^+$ T cells [136; 139]. Moreover, studies have shown that $CD4^+$ T cells are able to induce an anti-tumor immune response without any CTL effector function mostly causes by indirect effects of IFNγ [142-145] or by induction of antibody production and therefore tumor immunity [146].

1.6 Analysis of antigen specific T cell responses

As mentioned above monitoring of the immune response occurring during autoimmunity, infection, transplantation or after vaccination is of major interest for immunologists. The normally low frequency of T cells specific for an antigen is a steady problem for the analysis of antigen specific T cell responses. The frequency of naive peripheral T cells against a given antigen has been estimated between $4x10^{-8}$ and $2x10^{-5}$ [147-149] but may increase during T cell responses up to $6x10^{-1}$ among $CD8^+$ T cells [150]. Furthermore, one has to think about different functionalities and pathways of activation between naive, effector, and memory T cells. Assays to measure T cell responses are characterized by several features: assay accuracy and precision, limit of detection, limit of quantitation, specificity, linearity and range, ruggedness, robustness, and system suitability. Accuracy refers to the correctness and exactness of the test result. It is defined as the closeness of a test result to the true value and can only be calculated by comparison with a standard. But definition of standards for T cell assays is difficult. Nevertheless, the use of commonly occuring

antigens and the generation of T cell clones specific for positive control antigens such as cytomegalovirus and influenza may provide standards for determining the accuracy of T cell methodologies to be evaluated. Precision or reproducibility of an assay is defined as the closeness of a test results to another when using the same specimen. This parameter is expressed as a standard deviation and variation coefficient of multiple samples making known positive controls indispensable. The detection limit of a method is the capacity to detect small amounts of a substance with a certain reliability and can be assessed by spiking a negative sample with known quantities of well-characterized T cells, e.g., CMV clones in a CMV negative HLA matched donor. The detection limit of a method is distinct from its sensitivity, which is the proportion of true positives among positive values (e.g. high sensitivity equates to a low amount of false negatives). Sensitivity of an assay is the parameter most affected by background noise. Specificity reflects the ability to measure true negatives as negative (e.g. high specificity equates to a low amount of false positives).

A reliable method to measure T cell responses should be able to maintain accuracy, precision, sensitivity, and specificity despite changes in external factors such as technicians, instruments, or reagents.

1.6.1 Functional T cell assays

T cells respond with measurable effects after they got in contact with antigen presented on MHC complexes which are expressed by target cells. These effects can be cytokine expression, expression of activation markers, proliferation, degranulation, target cell cytotoxicitiy and/or trogocytosis.

Several methods exist for the detection of cytokines expressed by antigen specific T cells. All these assays can be performed specifically for different cytokines, depending on the expected functionality of the cells. Thereby, interferon γ (IFNγ) is one of the most important cytokine for the detection of activated $CD8^+$ or $CD4^+$ T cells.

Bulk cytokine expression assays on a protein level are classic sandwich enzyme linked immunosorbent assays (ELISAs) [151] or similar protein detection assays such as the cytometric bead assay [152]. These methods allow simultaneous detection of several cytokines within one sample. On mRNA level, quantitative reverse

transcription polymerase chain reaction (qRT-PCR) has been exploited. This assay is based on the principle that amplification of cDNA by polymerase chain reaction follows a strict mathematical equation whereby with each cycle of amplification two copies are made from each individual oligonucleotide species [153].

Three methods exist to enumerate single cytokine-secreting T cells in response to specific (peptide) antigens: enzyme linked immunospot (ELISpot) assay [154; 155], intracellular cytokine staining [156] and cytokine capture [157].

The ELISpot assay is based on the principle of the ELISA. A Nitrocellulose-bottomed microtiter plate is coated with an antibody that binds the cytokine of interest. Antigen-specific T cells, either unseparated PBMCs or $CD8^+$ or $CD4^+$ T cells are incubated in the plate together with the antigen to test. Recognition of the antigen leads to release of cytokine of interest, which is then bound by the antibody coated on the plate. Cytokine release is visualized by an enzyme-labeled detection-antibody and a corresponding chromogenic substrate which has to be non-soluble to precipitate at the bottom of the well. Cytokine secreting T cells become visible as colored spots. The ELISpot assay requires a short-term stimulation with synthetic peptide. Therefore, T cells are stimulated *in vitro*. Nevertheless, the assay detects only T cells that are preactivated *in vivo*, since naive T cells do not secrete cytokines upon short-term stimulation. Thus, the ELISpot assay is useful to measure number and functionality of antigen-specific T cells ex *vivo*. Results can be expressed in terms of relative frequencies of antigen-specific T cells what enables a direct comparison of the strength of *in vivo* T cell responses between groups of patients and between different clinical trials [158-160].

Intracellular cytokine staining is based on direct detection of intracellular cytokine expression with fluorochrome-conjugated anti-cytokine antibodies after short periods of activation with antigens. The assay can be performed with PBMCs [161], whole blood [162; 163], lymph nodes, or other biologic fluids. Intracellular cytokine detection raises the problem that cytokines are usually rapidly secreted and diffuse away from the secreting cell. Therefore, a total incubation period of 6 hours is optimal for achieving high levels of cytokine-secreting cells for IL-2, IL-4, IFNγ, and TNFα, as well as for achieving maximal cytokine staining intensity [162]. Cytokine secretion is disrupted usually during the final 4 hours of incubation by the addition of drugs inhibiting cytokine secretion such as monensin or brefeldin A [164]. After staining with

surface markers like anti-CD8 or anti-CD4, cell membranes are permeabilized using non-ionic detergents, followed by intracellular staining of cytokine of interest.

In cytokine capture assays, cells are labeled with a capture matrix like anti-cytokine antibodies which are non-covalently coupled to the cell surface. After stimulation, secreted cytokines are captured on the cell surface and can be later stained with a fluorescently labeled second anti-cytokine antibody. Because this method does not affect viability it is very useful if a live cell sorting is performed.

Activated T cells express proteins which are not found on resting T cells. Common activation markers are: CD69, CD25 and HLA-DR (in human T cells). Single-cell assays have been employed that use flow cytometric staining of the surface markers on activated T cells [165]. Disadvantages of this method are narrow, distinct time windows in which the molecules are expressed and the fact that these surface markers are also found on subpopulations of *ex vivo* isolated T cells in the absence of antigen [166].

A very important *in vitro* parameter for *in vivo* function is T cell proliferation. The proliferation of T cells in response to *in vitro* stimulation is commonly determined by a radioactive method which is based on incorporation of [^{3}H]thymidine into newly generated DNA [167]. This method is widely applied but has some disadvantages. First, the method requires radioactive facilities. Additionally, this technology gives only information on the overall proliferative responses, but does not give any information about the specific cell subsets involved in these responses.

Alternative methods to measure T cell proliferation are the assessment of CD38 expression on T cells analyzed by flow cytometry and an enzyme-linked immunosorbent assay (ELISA) based on 5-bromo-2´deoxyuridine (BrdU) incorporation into newly synthesized DNA of proliferating T cells [168]. Furthermore, it is possible to prelabel cells with fluorescent dye 5,6-carboxylfluorescein diacetate succinimidyl ester (CFSE) [169]. Proliferation, in response to antigen recognition, results in reduction of CFSE intensity what can be measured directly by flow cytometry.

Antigen-specific cytotoxic T cells (CTLs) can be detected by degranulation. Activation of CTLs leads to the fusion of cytotoxic granula with the cell membrane followed by release in the extracellular environment. Proteins like CD107a/b which are normally found on the membrane of the granula, become transiently located to the cell membrane. This effect is strongly associated to cytotoxicity and can be used to

detect and sort antigen-specific $CD8^+$ T cells [170] by a single-cell based assay that employs staining cells during and after activation with fluorescent anti-CD107a/b antibodies and flow cytometry.

Another effect of functionality of activated $CD8^+$ T cells is the ability to lyse target cells. The first described method for the detection of CTLs is the ^{51}Chromium-release assay (CRA) [171; 172] which can be performed either on fresh cells [Plata, 1987; Walker, 1987; Riviere, 1989] by measuring effector CTL activity, or on CTL lines [Walker, 1987; Nixon, 1988] by evaluating memory CTL activity. Target cells are labeled with $^{51}Cr(VI)O_4^{2-}$, a radiochemical compound which is able to enter cells through anion transport systems. In the cytoplasm it is subsequently reduced to $^{51}Cr(III)$. As Cr(III) will reside inside the cell, cellular accumulation of ^{51}Cr is greatly facilitated [173]. Labeled target cells are incubated with effector cells ($CD8^+$ T cells) for several hours. If the effector cells recognize antigen presenting target cells they will lyse them, ^{51}Cr will be released from the cell and becomes detectable in the supernatant. This assay is very useful for the determination of direct cytotoxicity of T cells but is inadequate for *ex vivo* studies that require the quantification of antigen-specific $CD8^+$ T cells in a mixed lymphoid population. A standard CRA does not determine CTL frequencies except if used in a limiting dilution analysis.

Recently, Snyder et al. described a new-single cell based assay to measure the frequency of antigen-specific functional CTLs [174] – the “Lysispot”. In this assay, target cells are transfected with a beta-galactosidase vector and coincubated with effector cells on top of flat membrane, which is non-covalently linked to an anti-galactosidase antibody. Release of beta-galactosidase after lysis is fixed as a spot on the membrane that can be visualized by the inherent enzymatic activity of beta-galactosidase or by means of an enzyme-linked second anti-galactosidase antibody in correlation with a substrate.

During trogocytosis performed by all lymphocytes, surface material from the antigen presenting cell to the effector cell is transferred but the mechanism and biological function of this effect are unknown until now [175]. However, it is an effect which can be used for the detection of antigen presenting $CD8^+$ T cells [175; 176]. For this purpose, target cells are labeled by coupling green fluorescent protein (GFP) to HLA molecule expressed by the target cell, loaded with antigen and incubated with antigen-specific T cells. These aquire HLA-GFP from the target cell and become fluorescent and thus detectable by flow cytometry.

1.6.2 Assays to determine specificity of T lymphocytes

For a quantification of antigen-specific T lymphocytes with regard to their specificity the analysis via fluorescent multimeric MHC-peptide reagents (also called tetramers) [177] is useful. These are capable of identifying and enumerating antigen-specific T cells and also provide functional information when combined with other methodologies. Tetramers are prepared by *in vitro* folding MHC heavy chain in the presence of β_2-microglobulin and a specific peptide ligand. Briefly, recombinant MHC class I heavy chain that incorporate a short C-terminal substrate peptide for BirA-mediated biotinylation are produced in *Escherichia coli*, purified from inclusion bodies, folded in the context of synthetic peptides and β2-microglobulin and biotinylated. Based on the specific application, fluochrome-labeled streptavidin, an avidin-like protein from *Streptomyces avidinii* which has four binding sites for biotin with high affinity [178], is added to purified MHC/peptide monomers to form soluble tetrameric complexes (Figure 3). MHC/peptide tetramers will bind T cells bearing MHC/peptide-specific T cell receptors and can be detected by flow cytometry (Figure 3). Moreover, phenotypic characterisation of cells detected by tetramer staining is enabled by possible parallel detection of co-expressed cell surface antigens [179]. MHC class II-peptide tetramers have also been developed for the assessment of $CD4^+$ T cell responses [180-182].

But even if these multimeric MHC/peptide reagents offered new possibilities to analyse whole T cell responses against a given epitope, there are also some limitations. First, MHC/peptide tetramers stably bind to TCR exhibiting a certain minimal avidity. Hence, functional T cells may be missed in this assay depending on the staining conditions, e.g. temperature [183-185] or concentration of tetramers. Moreover, MHC/peptide tetramers also interact with the CD8/CD4 coreceptor and may therefore influence binding of anti-CD8 antibodies [186; 187] what may lead to peptide independent binding and ultimately false positive results. Secondly, MHC/peptide tetramers are also known to bind other receptors such as killer cell immunoglobulin-like receptors (KIRs) or immunoglobulin-like transcripts (ILTs) expressed on natural killer cells [188-190].

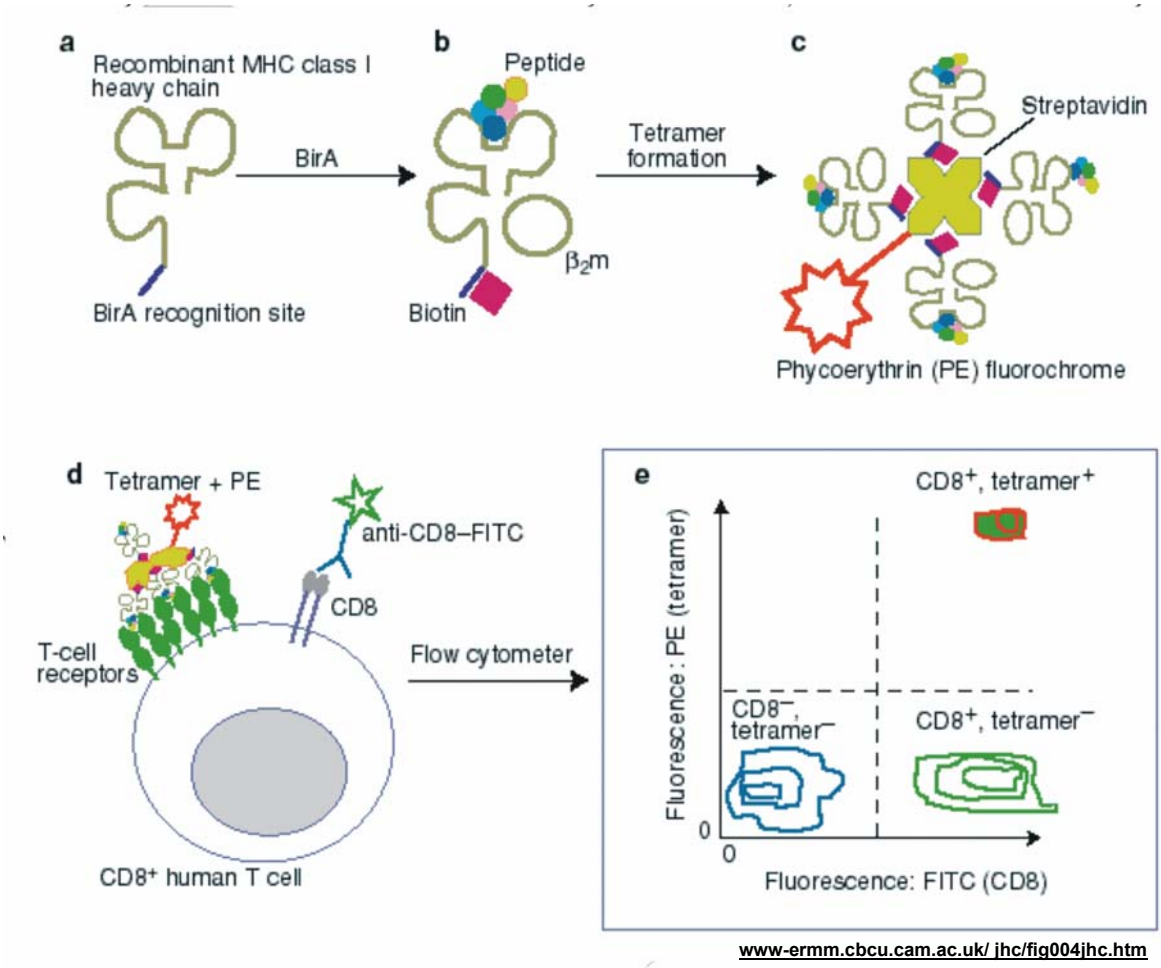

Figure 3. (a) Soluble heavy chain of MHC class I molecules are synthesised in *Escherichia coli*. (b) The molecules adopt an appropriate conformation following the addition of β_2 microglobulin (β_2m) and a synthetic peptide. Enzyme BirA is used to attach a biotin molecule. (c) Four MHC–biotin complexes are linked to a single streptavidin molecule labeled with a fluorochrome to form a tetramer. (d) Tetramers are mixed with the T cell population. Only T cells that have TCRs capable of binding to the particular MHC–peptide combination present in the tetramer are able to bind the tetramer. Such cells become labelled with the fluorochrome [shown in red on the graph in (e)]. A monoclonal antibody specific for a T cell marker and tagged with a different fluorochrome (shown in green) can be used. (e) Cells are analyzed by flow cytometry.

1.7 *In vitro* stimulation of antigen-specific T cells

The use of antigen specific T cells in adoptive immunotherapy is correlated with injection of large amounts of previously amplified antigen specific T cells. Therefore, it is important to separate T cells specific for an antigen of desire out of a whole T cell population. It is possible to stimulate and amplify T cells either in an antigen

independent manner or by stimulation with specific antigen. In mixed T cell populations, composition may change after antigen independent stimulation because not all subpopulations will divide and survive in the same way. However, mixed T cell populations can be separated using fluorescence activated- or magnetic affinity cell sorting (FACS/MACS) systems [191-194] or populations can be cloned by limiting dilution [195]. Antigen independent amplification of T cells can be achieved using polyclonal mitogens or stimulatory antibodies, usually in combination with cytokines like IL-2 [196]. Several antibodies able to support T cell stimulation are known, e.g. antiCD3 [196] or antibodies directed against costimulatory antigens such as CD28 [197], 4-1BB [198] or CD27 [199; 200], respectively. Polyclonal mitogens can be concanavalin A (Con A) [201] or phytohaemagglutinin (PHA) [202].

For stimulation and proliferation of antigen-specific T cells *in vitro* several approaches can be used. Memory T cells can be stimulated with peptides [203] or proteins loaded on non-professional antigen presenting cells within autologous primary cells, autologous derived B-lymphoblastoid cell lines or allogeneic cell lines. Expansion of naive T cells, called *in vitro* priming, can be achieved using autologous monocyte derived dendritic cells [204] or autologous activated B lymphocytes [205] loaded with antigens, e.g. peptides [206]. Nevertheless, generation of these professional antigen presenting cells is expensive, time-consuming and only low numbers can isolated and generated. Therefore, different approaches were developed using artificial antigen presenting cells (aAPCs). Allogeneic tumor cells, e.g. K562, can be transfected with costimulatory and adhesion molecules, such as CD80, 4-1BBL, LFA-3 and ICAM-1 [207]. Possible effects of allostimulation are avoided by transfection of single peptide-HLA complexes into HLA-deficient cells [208].

In another approach to design aAPCs, recombinant MHC/peptide complexes (monomers) are coated on plastic microspheres [209; 210], lipid vesicles [211] or HLA deficient cell surfaces [194].

1.8 Aims of thesis

HCMV is a cause of severe diseases in immunocompromised individuals such as transplant recipients or AIDS patients, therefore, it is very important to search for new opportunities in vaccine development. As HCMV is controlled by $CD8^+$ and $CD4^+$ T cell responses in immunocompetent persons, detailed knowledge of T cell responses against HCMV is essential for the development of a HCMV vaccine. One aim of this thesis was the investigation of natural frequencies of $CD8^+$ and $CD4^+$ T cells in healthy donors. A further field of interest was the characterization of immunodominant features or any hierachies between the epitopes identified. As peptide-based immunotherapy is restricted by many different HLA-types of patients it was also important to identify T cell epitopes with distinct HLA restrictions.

Several clinical trials could show that the immune system can be manipulated to specifically recognize and eliminate tumor cells [212; 213]. Peptide based immunotherapy is capable to raise cytotoxic T cells which are able to recognize tumor rejection antigens on tumor cells and are thus able to contribute to tumor regression. Therefore, it is very important to enlarge knowledge of anti-tumor T cells and their epitopes. Thus, another aim of this thesis was the induction and analysis of $CD4^+$ and $CD8^+$ T cells specific for peptides from tumor-associated antigens.

1.9 References

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2 Results and Discussion, Part 1: T cell epitopes of HCMV

2.1 Major contribution of codominant CD8 and CD4 T cell epitopes to the Human Cytomegalovirus-specific T cell repertoire

This manuscript has been composed by Maria-Dorothea Nastke*, Leah Herrgen*, Steffen Walter, Dorothee Wernet, Hans-Georg Rammensee and Stefan Stevanović. The author of this thesis has performed the experiments leading to Figures 1, 2, 3, and 4 and Table 1, 2, 3. Parts of this chapter are published in *Cellular and Molecular Life Science* 62, 77-86 (2005).

* These two authors contributed equally to this work.

Summary

Human cytomegalovirus (HCMV) infection or reactivation is a cause of morbidity and mortality in immunocompromised individuals. In immunocompetent individuals, in contrast, HCMV is successfully controlled by specific CD8 and CD4 T cells. Knowledge of CD8 and CD4 T cell epitopes from HCMV and their immunodominant features is crucial for the generation of epitope-specific T cells for adoptive immunotherapy and for the development of a peptide-based HCMV vaccine. Therefore, we investigated the natural frequencies of a large number of CD8 and CD4 T cell epitopes, including 10 novel ones. We determined several epitopes as immunodominant. Surprisingly, no clear hierarchies were found for CD8 T cell epitopes, indicating codominance. These results will be valuable for adoptive transfer strategies and support initiatives towards development of a peptide-based HCMV vaccine.

Introduction

Human cytomegalovirus (HCMV) infection is normally asymptomatic in immunocompetent individuals [1]. However, primary HCMV infection [1; 2] or reactivation of HCMV from latency [3] are causes of significant morbidity and mortality in immunocompromised individuals such as transplant recipients. In non-immunocompromised seropositive individuals, HCMV-specific cytotoxic T lymphocytes (CTLs) are present in high frequencies in the peripheral blood [4], and there is a direct correlation between the recovery of HCMV-specific CTL responses with an improved outcome of HCMV disease [5]. The concept of adoptive immunotherapy was derived [6], and the adoptive transfer of HCMV-specific CTL clones [7; 8] or HCMV-specific T cell lines [9] has successfully protected patients at risk from HCMV disease. The importance of T helper cells (TH) in primary HCMV infection [10] as well as for expansion of memory CD8 T cells [11] has lately been emphasized. Furthermore, several studies have outlined the significance of antiviral effector functions of TH cells in maintaining CTL responses after adoptive transfer [12; 13] and their capacity to produce antiviral cytokines [10; 14]. These findings underline the importance of T cell immunity in the control of HCMV infection and the relevance of T-cell-based approaches in therapeutic settings.

Knowledge of HCMV epitopes is crucial not only for monitoring of antiviral immunity but also for the in vitro generation of antiviral CTLs for possible application in adoptive immunotherapy. Several studies have already been successful in the identification of HCMV epitopes [14-29]. According to Sercarz et al. [30], immunodominant epitopes are defined as those that account for the bulk of the global specific T cell response within one individual. However, we use this term to refer to the frequency of responders among individuals carrying the restricting allele, while we refer to codominance as the responsiveness of a single donor against more than one epitope derived from a given protein. Identification of a sufficiently large number of CTL and TH epitopes as well as knowledge of immunodominance of individual epitopes and epitope hierarchies on individual MHC alleles is pivotal for development of an HCMV vaccine. In addition, the use of well-defined peptides for the generation of HCMV-specific T cells for adoptive immunotherapy is preferential to the use of ill-defined viral lysates, which have so far mostly been employed for this purpose [9; 31]. We performed large-scale screenings of HCMV-seropositive donors for

determination of immunodominance of known CTL and TH epitopes and for identification of novel CTL and TH epitopes. Our experiments focused on pp65 as the major target of human CTL reactions, but we also included other HCMV antigens with reported CTL responses. In the course of this study, we were able to determine the immunodominant features of a great number of CTL and TH epitopes. Furthermore, we identified six novel CTL and four novel TH epitopes.

Material and Methods

Donors

Buffy coats were obtained from healthy blood bank donors of known major histocompatibility complex (MHC) class I and II types and of known HCMV serostatus. The local Ethics Committee approved this study. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats using standard gradient separation (Lymphocyte Separation Medium, PAA Laboratories, Pasching, Austria) and cryopreserved in fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria) with 10% DMSO (Merck, Darmstadt, Germany) at –80°C until further use.

Peptides, recombinant MHC molecules and fluorescent tetramers

Peptides from HCMV pp65, pp50 and IE1 were synthesized by standard Fmoc chemistry using a Synergy Personal Peptide Synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) or alternatively using the Econ- omy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany). HLA class I restricted peptides were either selected according to epitope prediction using the SYFPEITHI database [32] or according to published CTL epitope sequences. A panel of 15–20mer peptides constituting potential HLA-DR epitopes from pp65 and IE-1 for CD4 T-cell-epitope screening was selected according to several HLA-DR peptide motifs [32], http://www.syfpeithi.de]. Peptide pools for CD4 T-cellepitope screening consisted of three to five peptides with single peptide concentrations of 5 µg/ml. Peptides were dissolved at 10 mg/ml in DMSO (Merck), diluted 1:10 in ddH2O and aliquots stored at –80°C. Biotinylated recombinant MHC class I molecules and fluorescent MHC tetramers for immunodominance studies and verification of predicted CD8 T cell epitopes were produced as described earlier [33]. Briefly, fluorescent tetramers were generated by coincubating biotinylated HLA monomers with streptavidin-PE or

streptavidin- APC (Molecular Probes, Leiden, the Netherlands) at a 4:1 molar ratio. For all HLA-B*44 peptides, HLA-B*4405 monomers were used. The used HLA-B*2705 tetramers had a cysteine-to-serine mutation in position 67.

Reagents and media

T cell medium consisted of RPMI 1640 containing HEPES and L-glutamin (Gibco, Paisley, UK) supplemented with 10% heat-inactivated human serum (PAA, Cölbe, Germany), 50 U/ml penicillin, 50 µg/ml streptomycin and 20 µg/ml gentamicin (all BioWhittaker, Verviers, Belgium). PBE was PBS (BioWhittaker, Verviers, Belgium) containing 0.5% of bovine serum albumin (Sigma Aldrich) and 2 mM EDTA (Roth, Karlsruhe, Germany). PFEA was PBS supplemented with 2% heat inactivated FCS (PAN Biotech, Aidenbach, Germany), 2 mM EDTA (Roth) and 0.01% sodium azide (Merck, Darmstadt, Germany). TSB (tetramer staining buffer) was PBS (BioWhittaker/Cambrex) containing 50% FCS (PAA), 2 mM EDTA (Sigma Aldrich) and 0.02% NaN3 (Merck).

Peptide stimulation and intracellular IFNγ staining

Cryopreserved PBMCs were thawed, washed two times in PBE, resuspended at 107 cells/ml in T cell medium and cultured overnight to reduce unspecific interferon-γ (IFN γ) production [34]. On the next day, PBMCs were stimulated with 5 µg/ml peptide for 6 h in the presence of Golgi-Stop (Becton Dickinson) for the final 4 h of incubation. Intracellular IFNγ staining was performed for the analysis of CD4 T cell responses because it allows for combining IFNγ and CD4 staining. Cells were analyzed using a Cytofix/Cytoperm Plus kit (Becton Dickinson, Heidelberg, Germany) plus Abs IFNγ - PE (Becton Dickinson), CD4-fluoroscein isothiocyanate (FITC) (Immunotools, Friesoythe, Germany) and/or CD8-PerCP clone SK1 (Becton Dickinson). After staining, cells were analysed on a three-color FACSCalibur (Becton Dickinson).

Tetramer staining

2 x 10^6 thawed PBMCs were stained with CD8-PerCP clone SK1 antibody (Becton Dickinson) and incubated at 4°C for 20 min in the dark, followed by 30 min incubation with fluorescent MHC tetramers at 4°C in the dark. After washing, cells were resuspended in 1% paraformaldehyde in PFEA. Cells were analysed by flow cytometry on a four-color FACSCalibur cytometer (Becton Dickinson).

Combined tetramer staining/intracellular IFNγ -staining

Two different conditions were used to perform this assay provided enough PBMCs were available. PBMCs were stained with tetramer before and after stimulation with peptide (condition 1), and with tetramer only after stimulation with peptide (condition 2), as reported previously [35]. Staining procedures were performed as described above using IFNγ-FITC (Becton Dickinson), CD8-PerCP clone SK1 (Becton Dickinson) and fluorescent MHC tetramer-PE.

Phenotyping of antigen-specific $CD8^+$ T cells

Thawed and washed PBMCs were stained with CD8- PerCP clone SK1 (Becton, Dickinson) plus either CD45RA-FITC (Becton Dickinson) or CD27-FITC, CD28-FITC, CD45RO-FITC, CD57-FITC, CD62LFITC, respectively (all Coulter-Immunotech, Hamburg, Germany). For staining with CCR7, cells were first labelled with rat hybridoma supernatant 3D12 (kindly provided by R. Förster, Anova). After washing, cells were incubated with FITC-labelled donkey anti-rat F(ab´)2 fragments (Jackson Immunoresearch Laboratories, West Grove, PA), and blocking with heat-inactivated mouse serum (CC pro) was performed. After staining with antibodies, cells were washed and labelled with tetramers diluted to 2 µg/ml in TSB. Stained cells were washed and fixed in PFEA containing 1% formaldehyde (Merck). Cells were analysed on a threecolor FACSCalibur (Becton Dickinson).

^{51}Chromium release assay

Standard 4 h ^{51}Cr release assay was performed using T2 cells and Awells as target cells which were used to load with either 100 nM NLVPMVATV-peptide (HCMV $pp65_{495-503}$) or 100 nM ILKEPVHGV-peptide (HIV-1 $RT_{476-484}$) as negative control, respectively. Furthermore, negative controls without peptide were used. Target cells, peptides and 100 µCi $Na_2{}^{51}CrO_4$ (NEN Amersham Bioscience, Freiburg, Germany) were coincubated for 1 hour at 37°C/5% CO_2. After target cells were added to effector cells in a 96 well plate and incubated for additionally for 4 hours at 37°C. 50 µl of supernatant was transferred to a lumaplate (PerkinElmer, Rodgau-Jügesheim, Germany) and read-out was performed by scintillations counting (1450 Microbeta Plus, PerkinElmer™ life science). Specific lysis was calculated by 100 x (experimental release - spontaneous release)/(total release - spontaneous release).

IFNγ ELISpot assay

IFNγ ELISpot assays were performed for the analysis of CD8 T cell responses if no tetramers of the respective HLA molecule were available. 96-well nitrocellulose plates (MHABS4510, Millipore, Bedford, MA) were coated with a mouse anti-human IFNγ antibody (5 µg/ml, Becton Dickinson). 10^6 thawed PBMCs/well were stimulated with 5 µg/ml peptide for 24 h, and secreted IFNγ was detected using biotinylated anti-IFNγ mAb (1 µg/ml), streptavidin-alkaline phosphatase conjugate (all Becton Dickinson) and BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate toluidine and nitroblue tetrazolium, Sigma Aldrich, Steinheim, Germany). The plate was washed with water and dried overnight. IFNγ-producing cells were detected as purple spots on the nitrocellulose membrane of each well. Plates were analysed using the Immunospot Image Analyzer (series 1) and ImmunoSpot Software Version 3.2e (both Cellular Technology, Cleveland, OH).

Results

Immunodominance of $CD8^+$ T-cell epitopes

PBMCs of 67 healthy HCMV seropositive donors were tested ex vivo with respect to the frequency of specific T cells in their peripheral blood. Reactivity of $CD8^+$ T cells to peptides was determined by IFNγ-ELISpot, and specificity of T cell receptors by tetramer staining. From our experience, frequencies down to 0.01% among $CD8^+$ T cells can be clearly distinguished from background by tetramer staining and 0.01–0.001% by IFNγ-ELISpot, respectively. HLA tetramers of several allotypes were prepared containing HCMV peptides derived from pp65, pp50 and IE- 1, respectively, to determine the frequency of responders among donors positive for the restricting HLA allele. Epitopes were considered immunodominant if more than 75% of seropositive donors showed a T cell response with at least six or more donors tested; epitopes with values detected below this average were regarded as subdominant. Peptides tested are listed together with references in table 1. Here we determined five CTL epitopes presented by three different HLA alleles (HLA-A*0101, HLA-A* 0201 and HLA-B*0702) to be immunodominant. Among 15 HCMV seropositive donors expressing HLA-A1, 14 showed CD8 T cells specific for pp65 363–373 and 8/10 donors had high frequencies of CTLs specific for peptide pp50 245–253 (examples in fig. 1A). T cells specific for NLVPMVATV (pp65 495–503) were

observed in all HLA-A2$^+$, HCMV-seropositive donors tested (examples in fig. 1B), but not in HCMV-seronegative donors, indicating this epitope to be the most immunodominant CTL epitope. T cells restricted to pp65 120–128, also presented by HLA-A*0201, were detected in 5/10 donors (examples in fig. 1B). Furthermore, immunodominance was determined for two epitopes presented by HLA-B*0702 (pp65 417–426, pp65 265–275) as can be seen in fig. 1C. Apart from these immunodominant epitopes, we also noticed six CTL epitopes which occurred in less than 50% of donors tested (A*0201: MLNIPSINV, VLEETSVML; A*0301: TTVYPPSSTAK; A*6801: FVFPTKDVALR; B*44: QEFFWDANDIY, SEHPTFTSQY). For several peptides, including pub-lished CTL epitopes [15; 20; 24], no CTL response could be detected in the HCMV-seropositive donors tested (table 1).

Table 1. Summary of CTL epitopes analysed

HLA	Protein	Position	Sequence	Reference	n [c] Tetramer	n [c] IFNγ
A*0101	**pp65**	**363-373**	**YSEHPTFTSQY** [d]	**[26]**	**14/15**	n.t.
	pp50	**245-253**	**VTEHDTLLY** [d]	**[17]**	**8/10**	n.t.
	pp50	274-253	RGDPFDKNY	predicted	0/6	n.t.
A*0201	**pp65**	**495-503**	**NLVPMVATV** [d]	**[29]**	**13/13**	n.t.
	pp65	120-128	MLNIPSINV	[28]	5/10	n.t.
	pp65	522-530	RIFAELEGV	[24]	0/6	n.t.
	IE1	316-324	VLEETSVML	[22]	2/6	n.t.
A*0301	pp150	945-955	TTVYPPSSTAK	[26]	1/12	n.t.
A*1101	pp65	16-24	GPISGHVLK	[20]	0/5	n.t.
	pp65	501-509	ATVQGQNLK	[24]	0/3	n.t.
A*2402	pp65	341-349	QYDPVAALF	[15]	0/6	n.t.
A*6801	pp65	186-196	FVFPTKDVALR	[26]	1/5	n.t.
	pp65	183-191	TSAFVFPTK [b]	this paper	see Table 5	
	pp150	794-802	VTSTPVQGR	predicted	0/5	n.t.
A*69	pp65	86-94	EVENVSVNV [a]	this paper	see Table 5	
B*0702	**pp65**	**417-426**	**TPRVTGGGAM** [d]	**[20]**	**11/13**	n.t.
	pp65	**265-275**	**RPHERNGFTVL** [d]	**[26]**	**10/11**	n.t.
	IE1	310-317	RVLCCYVL	predicted	0/19	n.t.
B*13	pp65	211-219	TRATKMQVI [a]	this paper	see Table 5	

B*1501	pp65	355-363	LLLQRGPQY	predicted	0/3	0/3
	pp65	73-81	NQLQVQHTY	predicted	0/3	0/3
	pp65	361-369	PQYSEHPTF	predicted	0/3	0/3
	pp65	505-513	GQNLKYQEF	predicted	0/3	0/3
	pp65	502-510	TVQGQNLKY	predicted	0/3	0/3
	pp65	319-327	LLMNGQQIF	predicted	0/3	0/3
	pp65	74-82	QLQVQHTYF	predicted	0/3	0/3
	pp65	215-223	KMQVIGDQY	[24]	0/3	0/3
	pp65	173-181	NQWKEPDVY	predicted	n.t.	0/3
	pp65	223-231	YVKVYLESF	predicted	n.t.	0/3
B*2705	pp65	539-547	RRRHRQDAL	predicted	0/2	0/2
	pp65	373-381	YRIQGKLEY	predicted	n.t.	0/2
	pp65	264-272	MRPHERNGF	predicted	n.t.	0/2
	pp65	39-47	TRLLQTGIH	predicted	n.t.	0/2
	pp65	257-265	TRNPQPFMR	predicted	n.t.	0/2
	pp65	358-366	QRGPQYSEH	predicted	n.t.	0/2
	pp65	418-426	PRVTGGGAM	predicted	n.t.	0/2
	pp65	3-11	SRGRRCPEM	predicted	n.t.	0/2
B*40	pp65	42-50	KEVNSQLSL [a]	this paper	see Table 5	
B*44	pp65	511-521	QEFFWDANDIY	predicted	0/10	1/10
	pp65	267-275	HERNGFTVL	[24]	0/10	0/10
	pp65	232-240	CEDVPSGKL	[24]	0/10	0/10
	pp65	364-373	SEHPTFTSQY	[24]	1/10	5/10
B*51	pp65	116-123	LPLKMLNI [a]	this paper	see Table 5	
	pp65	114-123	YALPLKMLNI [a]	this paper	see Table 5	

[a/b] Epitopes were identified by intracellular IFNγ staining[(a)] or tetramer staining[(b)] in donors who had reacted to corresponding long peptides (see Table 4). n.t. = not tested

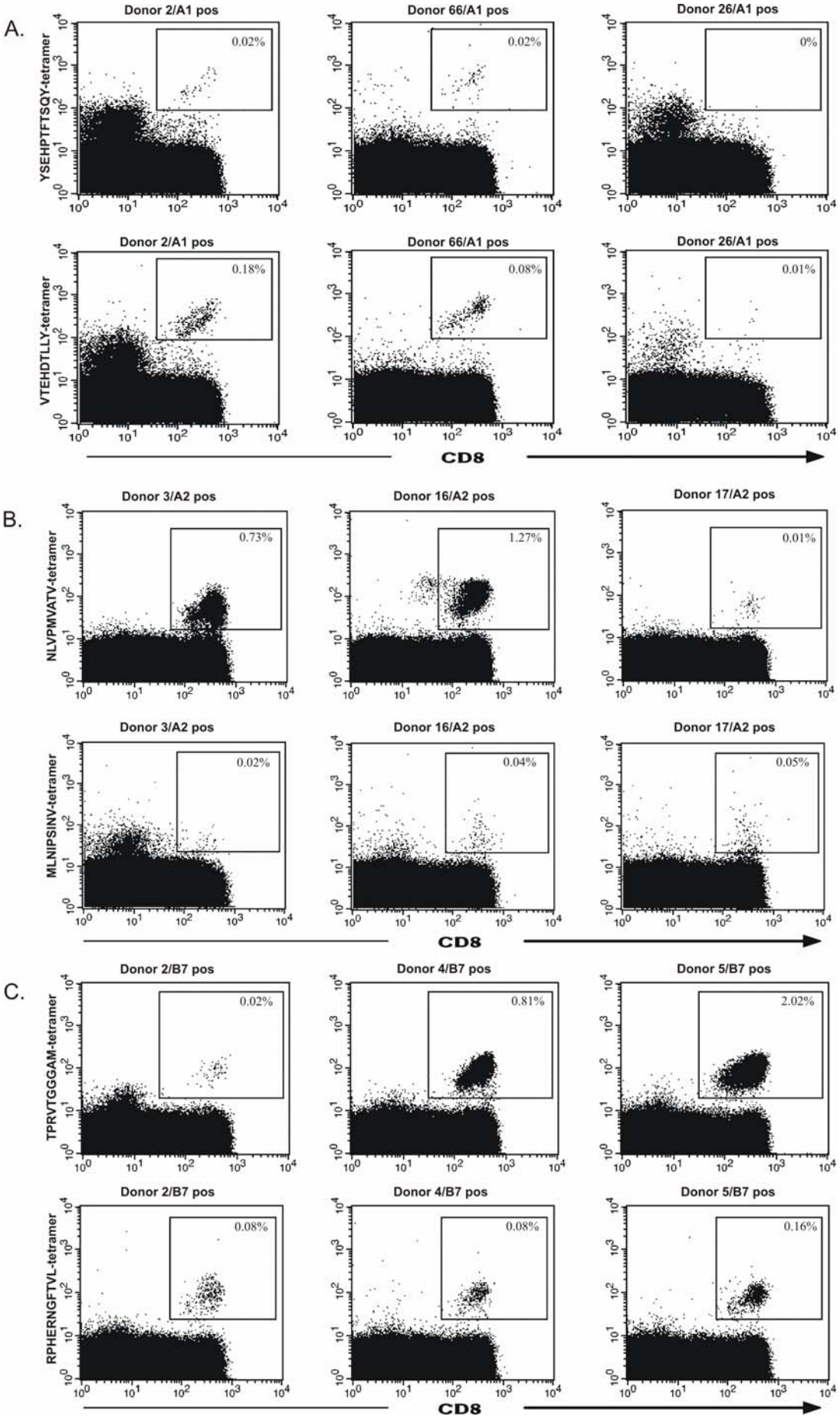
A.
Donor 2/A1 pos
Donor 66/A1 pos
Donor 26/A1 pos
YSEHPTFTSQY-tetramer
0.02%
0.02%
0%
Donor 2/A1 pos
Donor 66/A1 pos
Donor 26/A1 pos
VTEHDTLLY-tetramer
0.18%
0.08%
0.01%
CD8
B.
Donor 3/A2 pos
Donor 16/A2 pos
Donor 17/A2 pos
NLVPMVATV-tetramer
0.73%
1.27%
0.01%
Donor 3/A2 pos
Donor 16/A2 pos
Donor 17/A2 pos
MLNIPSINV-tetramer
0.02%
0.04%
0.05%
CD8
C.
Donor 2/B7 pos
Donor 4/B7 pos
Donor 5/B7 pos
TPRVTGGGAM-tetramer
0.02%
0.81%
2.02%
Donor 2/B7 pos
Donor 4/B7 pos
Donor 5/B7 pos
RPHERNGFTVL-tetramer
0.08%
0.08%
0.16%
CD8

Figure 1. Representative tetrameric analyses for alleles HLA-A*0101 (*A*), HLA-A*0201 (*B*) and HLA-B*0702. PBMCs were stained with HCMV-tetramer-PE, CD8-PerCP and CD19-FITC. Percentages refer to tetramer+ T cells within the $CD8^+$ population. (A) Binding to HLA-A*0101 tetramers YSEHPTFTSQY (pp65 363–373) and VTEHDTLLY (pp50 245–253) in same donors. (B) Binding of HLA-A*0201 NLVPMVATV-tetramer (pp65 495–503) and MLNIPSINV-tetramer (pp65 120–128) in HLA-A2 positive donors. (*C*) Parallel detection of T cells binding to HLA-B*0702 tetramers TPRVTGGGAM (pp65 417–426) and RPHERNGFTVL (pp65 265–275).

Investigation of hierarchy among immunodominant epitopes

Frequencies of CTLs specific for immunodominant epitopes were compared in donors having at least two of the alleles HLA-A1, -A2 or -B7 in common. As can be seen in table 2, T cells specific for different CTL epitopes were detected in parallel. Moreover, strong responses to one epitope did not prevent the detection of lower frequencies of CTLs specific for other epitopes, as can be seen in donor 5 and donor 16. The detection limit of tetramer staining is described as 0.01%. Nethertheless, in two donors we observed distinct $CD8^+$ T cell populations lower than 0.01%. Overall, no clear hierarchy was observed in that CTL populations specific for several immunodominant T cell epitopes in parallel were frequently observed. Therefore, codominance among these epitopes, especially for B*0702 restricted ones, appeared to be a common feature.

Table 2. Frequencies of immunodominant pp65 epitopes in HCMV seropositive donors as determined by tetramer staining

Donor	HLA type of donors tested	YSEHPTFTSQY (A*0101)	NLVPMVATV (A*0201)	TPRVTGGGAM (B*0702)	RPHERNGFTVL (B*0702)
BD 1	A1 A3 B7 B8	0.01		<0.01	0.12
BD 2	A1 A28 B7 B49	0.02		0.02	0.08
BD 3	A1 A2 B8 B35	0.01	0.70		
BD 4	A2 B7 B13		0.85	0.81	0.08
BD 5	A1 B7 B55	0.01		2.02	0.16
BD 8	A11 A24 B7			0.21	0.69
BD 9	A2 A3 B7 B51		0.03	1.31	0.42
BD 10	A1 A2 B18 B62	0.04	0.03		
BD 11	A1 A23 B7 B49	0.07		<0.01	<0.01
BD 13	A3 B7 B35			1.10	0.14
BD 15	A3 A11 B7 B62			0.03	0.03
BD 16	A1 A2 B8 B44	0.32	1.26		
BD 17	A2 A3 B7 B35		0.01	0.29	0.57

Functional and phenotypic characterization of $CD8^+$ T cells restricted to immunodominant epitopes

$CD8^+$ T cells specific for immunodominant epitopes were analysed for their functional capacity to produce IFNγ. Therefore, tetramer staining in combination with intracellular IFNγ staining was performed with PBMCs of donors already tested with the tetramers. As shown in table 3, $tetramer^+$ $CD8^+$ T cells recognizing epitopes presented by HLA-A*0101, -A*0201 or -B*0702 were often capable of producing IFNγ. Surprisingly, no IFNγ secretion by T cells specific for the immunodominant epitope pp65 363–373 presented by HLA-A*0101 could be detected in three independent experiments.

Table 3. IFNγ responses to immunodominant HCMV epitopes

HLA tested	Protein/position	Sequence	n [a]
A*0101	pp65 363-373	YSEHPTFTSQY	0/6
	pp50 245-253	VTEHDTLLY	3/6
A*0201	pp65 495-503	NLVPMVATV	7/8
B*0702	pp65 417-426	TPRVTGGGAM	7/9
	pp65 265-275	RPHERNGFTVL	7/9

[a] n refers to number of individuals showing IFNγ response against this epitope/total number of individuals with corresponding alleles tested.

HCMV tetramer$^+$ CD8$^+$ T cells were investigated for their phenotypic attributes with several surface markers; CCR7, CD45RA, CD45RO, CD27, CD57, CD62L and CD28 (Figure 2). CTLs specific for HCMV antigens were analysed by tetramers pp65 495-503 (A*0201), pp65 415-429 and pp65 265-275 (both B*0702). CD8$^+$ T cells detected by HCMV tetramers expressed CD45RA$^+$ CCR7$^-$ CD27$^+$ and CD28^{low} and found to represent so-called TEMRA cells (CD45RA$^+$ effector memory T cells) which display phenotypic features that are intermediate between naive and effector T cells [36; 37]. Similar phenotypes have already been described for circulating memory CTLs specific for other persisting viruses such as EBV [38]. The low expression of CD28 indicates that these cells are senescent reflecting frequent restimulation *in vivo* [39].
After polyclonal expansion cells were used in a ^{51}Cr-release assay to analyse their ability to lyse antigen presenting target cells. Peptide NLVPMVATV (HCMV pp65$_{495-503}$) was loaded on either TAP-deficient HLA-A*0201 positive T2 cells, or on human LCL HLA-A*0201 Awell cells. In both cases it was possible to provoke lysis of the target cells measured by release of ^{51}chromium in the supernatant. Used effector NLVPMVATV specific T cells were able to kill antigen presenting target cells with a specific lysis of 40%-80% (Figure 3). No lysis was observed if effector cells were incubated with targets either loaded with irrelevant peptide ILKEPVHGV-peptide (HIV-1 RT$_{476-484}$) or without peptide.

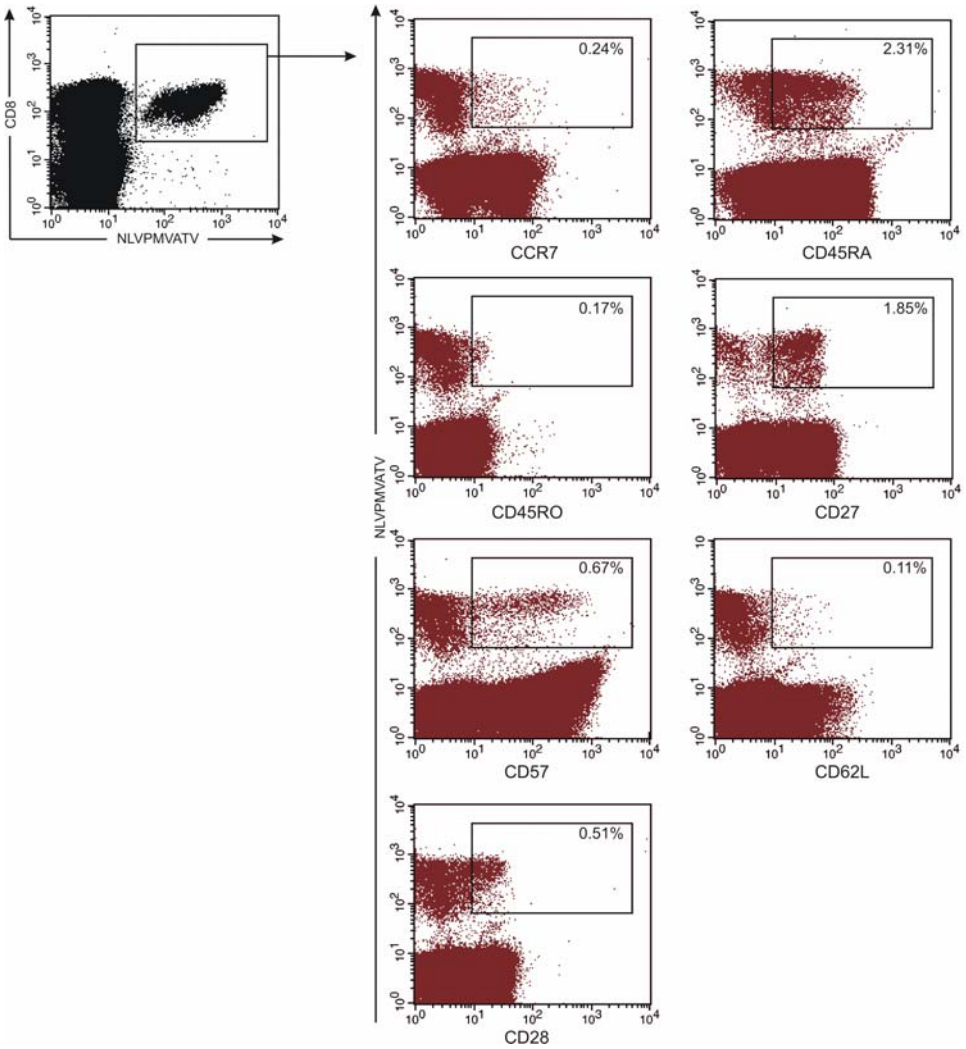

Figure 2. Investigation of phenotypic attributes of HCMV tet$^+$ cells. PBMCs were stained with HCMV tetramer-PE, CD8-PerCP and phenotypic marker labelled to FITC. Percentages present tetramer$^+$/marker$^+$ T cells within the CD8$^+$ population.

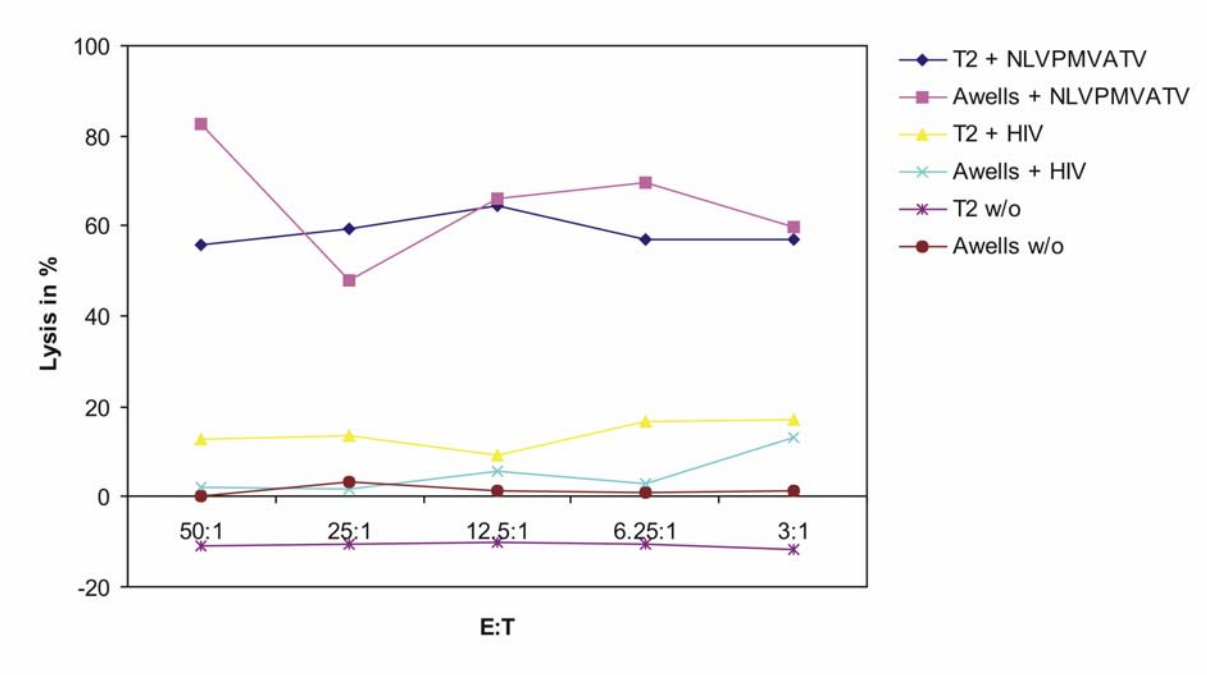

Figure 3. Specific lysis by HCMV pp65$_{495-503}$ specific T cells measured by ^{51}Cr release. Targets cells were loaded with either NLVPMVATV-peptide (HCMV$_{495-503}$), ILKEPVHGV-peptide (HIV-1 RT$_{476-484}$) or without peptide.

T cell receptor downregulation after peptide activation

HLA/peptide complexes (tetramers) are nice tools for the for detection of antigen-specific CD8$^+$ T cells but several questions have been raised about functional significance of the observed antigen-specific T cells [40-43]. Studies in a murine model of lymphocytic choriomeningitis virus (LCMV) infection in which CD4$^+$ T cell help was deficient revealed that the circulating tetramer-staining CD8$^+$ cell population was functionally defective and unable to mediate protection [43]. To address this problem, Appay et al. developed a method that combines the precision of tetramer quantification with intracellular cytokine staining to get detailed information about individual CD8$^+$ T cell function. Combination of these two assays raised a problem: activation procedures to stimulate cytokine production also leads to downregulation of the T-cell receptor (TCR) [44], with which the tetrameric complexes interact. Figure 4 shows staining of peripheral blood mononuclear cells (PBMCs) from three healthy HCMV positive donors with known HLA type with different HCMV tetramers. Stimulation of the cells with peptide led to significant downregulation of the TCR assessed by the intensity of tetramer staining in a CD8$^+$ population (Figure 4, left panel). This problem can be solved by tetramer staining of the cells before activation

(Figure 4, right panel). However, PBMCs stained with tetramers before activation showed also reduced levels of tetramer$^+$ CD8$^+$ T cells compared with levels of antigen-specific CD8$^+$ T cells detected in a single tetramer staining (data not shown). This is probably due to internalization of tetramers [45].

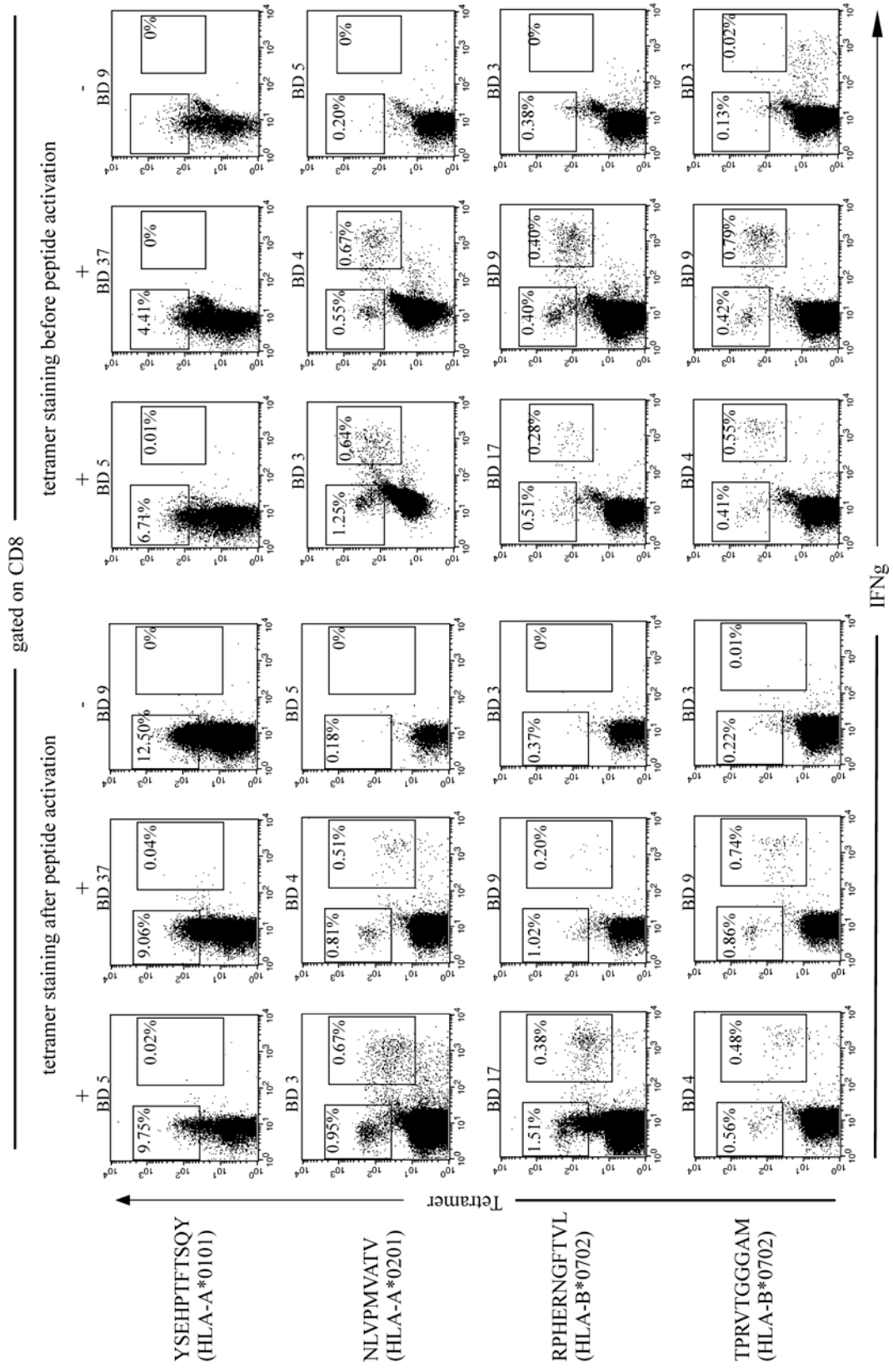
gated on CD8
tetramer staining after peptide activation
tetramer staining before peptide activation
+
-
BD 5
BD 37
BD 9
BD 3
BD 4
BD 17
9.75%
0.02%
9.06%
0.04%
12.50%
0%
0.95%
0.67%
0.81%
0.51%
0.18%
0%
1.51%
0.38%
1.02%
0.20%
0.37%
0%
0.56%
0.48%
0.86%
0.74%
0.22%
0.01%
6.71%
0.01%
4.41%
0%
0.20%
0%
1.25%
0.64%
0.55%
0.67%
0.38%
0%
0.51%
0.28%
0.40%
0.40%
0.13%
0.02%
0.41%
0.55%
0.42%
0.79%
Tetramer
IFNg
YSEHPTFTSQY
(HLA-A*0101)
NLVPMVATV
(HLA-A*0201)
RPHERNGFTVL
(HLA-B*0702)
TPRVTGGGAM
(HLA-B*0702)

Figure 4. Tetramer staining and cell activation. PBMCs from HCMV-seropositive donors were stained with tetramer either after incubation with specific HCMV peptide or before addition of the activators. Percentages of tetramer positive cells within the $CD8^+$ population are shown. (+) Donors are positive for the HLA-molecule given on the left side. (-) Donors are negative for the HLA-molecule given on the left side.

Identification of novel CD8 T cell epitopes

Among several strategies for identification of CD8 T cell epitopes, both verification of predicted epitopes and epitope identification by large-scale screens with partially overlapping 15–20mer peptides [39] have been successfully employed. As these two strategies may complement each other, we utilized both for identification of CD8 T cell epitopes. In this context, the nature of non-CD4 T cell mediated reactions observed during screens with 15–20mer peptides was further investigated as to their identity as CD8 T cell responses directed against shorter peptides included in longer ones, probably after in vitro processing. Non-CD4-mediated reactions were observed after stimulation of PBMCs from 14 HCMV-seropositive donors, but not with PBMCs from HCMV-seronegative donors. Altogether, IFNγ-producing non-CD4 T cells specific for 14 peptides from pp65 and IE-1 were detected (data not shown). As some of these long peptides contained known CD8 T cell epitopes, we assumed that the observed non-CD4-mediated T cell reactions were CD8 T cell responses. Therefore, considering the MHC class I alleles of reacting donors, potential CD8 T cell epitopes were selected from 15–20mer peptides according to peptide motifs of HLA allotypes [32]. Depending on the availability of the heavy chains of MHC class I alleles for tetramer synthesis, which can be restrictive, short peptides were either used for tetramer synthesis and tetramer staining plus anti CD8-PerCP antibody, or tested for their capacity to give rise to specific CD8 T-cell-mediated reactions by intracellular IFNγ-staining plus CD8-PerCP antibody. Peptides capable of eliciting non-CD4 T-cellmediated reactions and corresponding CD8 T cell epitopes are summarized in table 4. Potential CD8 T cell epitopes were tested in donors who had reacted to the corresponding long peptides and who were positive for the potentially restricting allele (one donor for each long peptide except two donors for pp65 109–123). IFNγ-producing CD8 T cells were observed in one donor for each novel CD8 T cell epitope, respectively. This refers to peptides pp65 86–94 (EVENVSVNV, potentially A*69 restricted), pp65 116–123 (LPLKMLNI) and pp65 114–123 (YALPLKMLNI, both

potentially B*51 restricted), pp65 211–219 (TRATKMQVI, potentially B*13 restricted) and IE-1 42–50 (KEVNSQLSL, potentially B*40 restricted), while restriction could be assessed unequivocally by tetramer staining for peptide pp65 183–191 (TSAFVFPTK) for which an A*6801 tetramer had been produced.

Table 4. Non-CD4 reactions to 15-20mer peptides from pp65 and IE-1 and CD8 reactions to corresponding 8-11mer peptides.

Peptide sequence	Protein/Position	(Potential) MHC class I restriction	Reference
	pp65		
ISVLGPISGHVLKAV	12-26		
GPISGHVLK	16-24	A*1101	[20]
EVENVSVNVHNPTGR	86-100		
EVENVSVNV [a]	*86-94*	*A*69*	*this paper*
MSIYVYALPLKMLNI	109-123		
LPLKMLNI [a]	*116-123*	*B*51*	*this paper*
YALPLKMLNI [a]	*114-123*	*B*51*	*this paper*
PLKMLNIPSINVHHY	117-131		
IPSINVHHY	123-131	B*3501	[19]
VYYTSAFVFPTKDVA	180-194		
TSAFVFPTK [b]	*183-191*	*A*6801*	*this paper*
TSAFVFPTKDVALRH	183-197		
FVFPTKDVALR	186-196	A*6801	[26]
FPTKDVAL	188-195	B*3502	
VCSMENTRATKMQVI	205-219		
TRATKMQVI [a]	*211-219*	*B*13*	*this paper*
GPQYSEHPTFTSQYRI	360-375		
YSEHPTFTSQY	363-373	A*0101	[26]
HPTFTSQYRIQGKLE	366-380		
FTSQYRIQGKL	369-379	A*2402	[26]
	IE1		
QTMLRKEVNSQLSLG	37-51		
KEVNSQLSL [a]	*42-50*	*B*40*	*this paper*
RVLCCYVLEETSVMLAKRPLI	310-330		
VLEETSVML	316-324	A*0201	[22]

[a] Specific CD8 T cells were detected by intracellular IFNγ staining.

[b] Specific CD8 T cells were detected by tetramer staining.

Novel CD8 T cell epitopes with their position in protein and potential MHC class I restriction are printed in italic.

Responses to known and novel CD4 T cell epitopes

PBMCs of 30 healthy HCMV-seropositive and 5 healthy HCMV-seronegative donors were stimulated with 15 peptide pools each containing three to five 15–20mer peptides from pp65 or IE-1 which represented potential HLA-DR epitopes. Reactivity to peptides was assessed by intracellular IFNγ staining plus CD4-FITC antibody. Peptide specificity of reactions observed after stimulation with peptide pools was determined by splitting of pools and testing reactions to single peptides. None of five seronegative donors showed any specific reaction after stimulation with peptide pools. Among 30 seropositive donors, 25 showed CD4 T cell reactions to at least one peptide. Most donors specifically reacted to one or two peptides; reactions to more than three peptides in a single donor were observed less frequently. Altogether, 15 peptides from pp65, but none from IE-1, were able to elicit CD4 reactions. Many of these peptides have previously been reported to constitute CD4 T cell epitopes and are listed in table 5. However, four of these peptides have not been described as CD4 T cell epitopes so far, pp65 109–123 (MSIYVYALPLKMLNI), pp65 191–205 (KDVALRHVVCAHELV), pp65 269–283 (RNGFTVLCPKNMIIK) and pp65 339–353 (LRQYDPVAALFFFDI). HLA-DR restriction for novel CD4 T cell epitopes was assigned according to overlapping expression of alleles in reacting donors. If no potential HLA-DR restriction could be assigned, as was the case for peptides pp65 191–205 and pp65 269–283, the number of reacting donors out of 30 HCMV-seropositive donors is given in brackets. In addition to determination of CD4 T cell epitopes, immunodominance values were evaluated for each of the CD4 T cell epitopes investigated in this study.

Table 5. CD4 T cell reactions to 15mer peptides from pp65.

(Potential) DR restriction	Peptide position	Peptide sequence	Reference	n[b]
DRB1*01	117-131[a] (1)	PLKMLNIPSINVHHY	[25]	4/12
DRB1*01	166-180	LAWTRQQNQWKEPDV	[21; 25]	1/12
DRB1*01	510-524[a] (3)	YQEFFWDANDIYRIF	[18; 21; 25]	6/12
DRB1*03	510-524[a] (3)	YQEFFWDANDIYRIF	[18; 21; 25]	3/4
DRB1*01	512-524[a] (3)	EFFWDANDIYRIF	[18; 21; 25]	1/12
DRB1*03	512-524[a] (3)	EFFWDANDIYRIF	[18; 21; 25]	3/4
DRB1*03	250-264	VEEDLTMTRNPQPFM	[25]	1/4
DRB1*04	283-299[a] (1)	KPGKISHIMLDVAFTSH	[21; 25]	3/6
DRB1*07	283-299[a] (1)	KPGKISHIMLDVAFTSH	[21; 25]	3/5
DRB1*04 DRB1*13	370-384	TSQYRIQGKLEYRHT	[25]	4/6
	370-384	TSQYRIQGKLEYRHT	[25]	4/10
*DRB1*07*	*109-123*	*MSIYVYALPLKMLNI*	*this paper*	*2/5*
DRB1*07	180-194	VYYTSAFVFPTKDVA	[25]	2/5
*DRB1*07*	*339-353*	*LRQYDPVAALFFFDI*	*this paper*	*3/5*
DRB1*11	360-375	GPQYSEHPTFTSQYRI	[18; 21; 23; 25]	5/12
DRB1*11	366-380	HPTFTSQYRIQGKLE	[18; 21; 23; 25]	6/12
DRB1*13	366-380	HPTFTSQYRIQGKLE	[18]	1/10
DRB1*15	39-53	TRLLQTGIHVRVSQP	[21; 23; 25]	4/5
not assigned	*191-205*	*KDVALRHVVCAHELV*	*this paper*	*(2)*
not assigned	*269-283*	*RNGFTVLCPKNMIIK*	*this paper*	*(1)*

[a] Peptides with a potential DR restriction(s) that can not account for all of the observed responses are marked. The number of additional reacting donors negative for the potentially restricting DR allele(s) is given in brackets.

[b] n refers to the number of individuals showing responses against this epitope/ total number of individuals with corresponding allele tested

Novel CD4 T cell epitopes with potential HLA-DR restriction are printed in italic.

Discussion

We investigated the CD8 and CD4 T cell responses of 67 HCMV-seropositive donors against reported and predicted HCMV-derived peptides. We identified six new HLA class I restricted and four new HLA class II restricted epitopes, and confirmed previously reported epitopes [15; 17; 20; 22; 24; 26; 28; 29]. We could determine immunodominance, as defined above, for each of two epitopes restricted to HLA-A* 0101 and HLA-B*0702, respectively, and for one HLA-A*0201 restricted epitope. For HLA-A*0201 and for -B*0702, the detected immunodominant epitopes have their origin in the same protein and were detected with similar frequencies in different donors. In view of this observation, we analysed the hierarchy among the frequently recognized epitopes detected in donors carrying HLA-A1, -A2 and/or -B7. Surprisingly, no clear hierarchy was observed among these epitopes tested. The observation that two different epitopes derived from the very same antigen and presented by the same HLA class I molecule appear to be codominant is in sharp contrast to hierarchy studies of viral CTL epitopes in mouse models [46]. The epitope pp65 364–373 was predicted according to the HLA-B*4402 motif. However, donors were only typed with low resolution (HLA-B44). Earlier reports suggested, that this epitope is restricted to HLA-B*4403 [24]. HLA-B*4402 and -B*4403 are two major HLA-B*44 subtypes in white individuals [47; 48] and HLA-B*4403 is the most frequent HLA-B*44 subtype in Japanese individuals [49-51]; the peptide repertoires presented by both subtypes are overlapping by 95% [52]. Furthermore, we were able to define six new CD8 T cell epitopes. Restriction of these novel epitopes could be determined unequivocally by tetramer staining for peptide pp65 183–191, which is A*6801 restricted. Studies aiming at the unambiguous determination of the five remaining epitopes restriction and at confirming these peptides identities as natural CD8 T cell epitopes from HCMV are currently in progress. Furthermore, due to rareness of restricting alleles, immunodominance values of individual novel CD8 T cell epitopes have not yet been assessed but are the subject of ongoing studies. This approach for identification of novel CD8 T cell epitopes from rare alleles may prove valuable for complementing other strategies, such as verification of predicted epitopes for frequent alleles. We identified four novel CD4 T cell epitopes. However, the identity of these peptides as natural CD4 T cell epitopes still needs to be confirmed, and their HLA-DR restriction has to be assessed unequivocally. Both aspects are the subject

of ongoing studies. All other peptides listed in table 5 have been reported previously as CD4 T cell epitopes [25].

In most cases, HLA-DR restriction of known CD4 T cell epitopes was in accordance with earlier reports [18; 21; 23; 25]. However, this was not the case for peptide pp65 370–384, where DRB1*04 and/or DRB1*13 restriction seems to be more plausible than the proposed DRB1*03 restriction. The reduction of immunodominance of peptide pp65 512–524 as compared with peptide pp65 510–524 on DRB1*01 (6/12 vs. 1/12) but not on DRB1*03 (3/4 vs. 3/4) may be explained by the binding motif of DRB1*01. DRB1*01 has a preference for hydrophobic residues in positions 1, 4, 6 and 9 which are provided by peptide pp65 510–524 but not by peptide pp65 512–524. On the other hand, peptide binding to DRB1*03 favours aspartate in position 4, which is supplied by D516 and is therefore independent of the first two amino acids in peptide pp65 510–524. Immunodominance values were approximately equal to those determined in the study of [25]. In general, immunodominance values of CD4 T cell epitopes are considerably lower than those of CD8 T cell epitopes, some of which are capable of inducing responses in up to 100% of donors. In contrast to CD8 T cell epitopes, responses on one allele are not focused on a single epitope but seem to be spread over several epitopes, indicating a preference for codominance. In view of this fact, CD4 T cell epitopes with high codominance values were identified for nearly all frequent DR alleles. Such CD4 T cell epitopes should be valuable for therapy and vaccine development.

The observed CD4 and CD8 T cell responses were investigated for their interrelation. To this end, number and strength of CD4 and CD8 T cell responses were compared in 11 donors already tested. No obvious interrelationship could be assessed. CD8 T cell responses to various numbers of peptides and of varying strengths could be observed in donors independent of number and strength of CD4 T cell responses and even in donors in which no CD4 T cell responses could be observed. Reactions restricted to HLA-DQ and -DP were not the topic of this study.

In summary, we characterized six dominant CTL epitopes from both pp65 and IE-1. Furthermore, four new TH epitopes from pp65 were found. For novel and reported CD8 T cell epitopes, as well as for CD4 T cell epitopes, levels of immunodominance and codominance were determined.

Acknowledgement

Thanks to Patricia Hrstić for expert technical assistance and Prof. Dr Hermann Einsele for critically reading the manuscript. We also thank Dirk Busch for the HLA-A*0301 construct, Hiroeki Sahara and Noriyuki Sato for the HLA-A*2402 construct, and Simon Kollnberger and Paul Bowness for the HLA-B* 2705 C67S construct used for MHC I tetramers. Rat hybridoma supernatant 3D12 specific for human CCR7 was a kind gift of R. Förster. This work was supported by grants from the European Union (QLRT-2001-00620 EPI-PEP-VAC and LSHB-CT-2004- 503319 ALLOSTEM).

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3 Results and Discussion, Part 2: Tumor Associated Antigens

3.1 Unexpected abundance of HLA class II presented peptides in primary renal cell carcinomas

The following manuscript has been composed by Jörn Dengjel*, Maria-Dorothea Nastke*, Gitsios Gitsioudis, Oliver Schoor, Florian Altenberend, Margret Müller, Björn Krämer, Anna Missiou, Martina Sauter, Jörg Hennenlotter, Arnulf Stenzl, Hans-Georg Rammensee, Karin Klingel, and Stefan Stevanović.
The author of this thesis has performed the experiments leading to Figures 2, 3, and 4 and Table 3.
* These two authors contributed equally to this work.

Summary

In the absence of inflammation, expression of MHC class II molecules is mainly restricted to cells of the immune system. To our surprise, we were able to isolate and characterize hundreds of class II peptides directly from primary dissected solid tumors, especially from renal cell carcinomas. Infiltrating monocytes expressed MHC class II molecules as well as tumor cells and in addition, tumor cells showed upregulation of several interferon gamma-induced gene products. Our list of identified peptides contains ligands from several tumor-associated antigens, including IGFBP3 and MMP7. The latter bound promiscuously to HLA-DR molecules and were able to elicit $CD4^+$ T cell responses in healthy donors. Thus, our direct approach will rapidly expand the limited number of T helper epitopes from tumor associated antigens.

Introduction

$CD4^+$ helper T cells play an important role in orchestrating the effector function of anti-tumor T cell responses [1] and for this reason the identification of $CD4^+$ T cell epitopes derived from tumor associated antigens (TAA) has recently been a major focus of attention [2; 3]. Even in the absence of cytotoxic T lymphocyte (CTL) effector cells, helper T cells in the mouse can inhibit tumor angiogenesis via IFNγ [4] and counteract tumor progression via the induction of an Ab response [5]. In contrast to HLA class I ligands, only a small number of class II ligands of TAA have been described. Since HLA class II molecules are constitutively presented on cells of the immune system alone [6], the possibility of isolating class II peptides directly from primary tumors as opposed to class I ligands [7] has not been considered viable. Therefore, numerous strategies to target antigens into the class II processing pathway of antigen presenting cells (APCs) have been described, for example the incubation of APCs with the antigen of interest to enable it to be taken up, processed and presented [8].

In order to identify HLA class II ligands from TAA for their use in peptide-based immunotherapy, we attempted to isolate HLA-DR-presented peptides directly from dissected solid tumors, in particular from renal cell carcinoma (RCC), which have been reported to be able to express class II molecules [9]. Even if the majority of tumor cells were class II negative, with state-of-the-art mass spectrometers it should be possible to identify class II peptides from minimal numbers of tumor cells, from infiltrating leukocytes possibly cross-presenting TAA, and from stromal cells.

The reasons for concentrating on RCC are the following: Around 150,000 people worldwide are affected by RCC each year, resulting in approximately 78,000 deaths per annum [10]. If metastasis is diagnosed, the one-year survival rate decreases to approximately 60% [11], underlining the dissatisfactory therapeutic situation. Because RCC seems to be an immunogenic tumor, as indicated by the existence of tumor-reacting and tumor-infiltrating CTL [12], clinical trials have been initiated to develop peptide-based anti-tumor vaccinations. However, due to the lack of helper T cell epitopes from TAA, molecularly defined vaccines usually comprise class I ligands only.

We were able to isolate class II ligands from ten RCC, three colorectal carcinomas (CCA) and one transitional cell carcinoma (TCC, urothelial carcinoma). Selected

ligands of TAA promiscuously binding to HLA-DR molecules were found to be recognized by $CD4^+$ T cells.

Material and Methods

MHC class II immunohistology

Tumors were fixed in 4% phosphate-buffered formaldehyde, embedded in paraffin, stained with hematoxylin-eosin and examined by light microscopy. Diagnosis of the RCC was carried out according to routine histopathological and immunohistological investigations [13].

For immunohistological detection of MHC class II molecules or CD68 molecules, respectively, 5 µm paraffin-embedded tissue sections were pretreated with 10 mM citrate buffer, pH 6, followed by incubation either with a mouse anti-HLA-DR alpha-chain mAb (clone TAL.1B5, 1:50) or CD68 Ab (Clone PGM1, 1:50) (DAKO, Hamburg, Germany) or mouse IgG1 (2 µg/ml, BD Biosciences Pharmingen, San Diego, USA) and visualized using the Ventana iView DAB detection kit (Nexes System, Ventana Medical Systems, Illkirch, France). Tissue sections were counterstained with hematoxylin and finally embedded in Entellan.

Elution and molecular analysis of HLA-DR bound peptides

Frozen tumor samples were processed as previously described [14] and peptides were isolated according to standard protocols [15] using the HLA-DR specific mAb L243 [16].

Natural peptide mixtures were analyzed by a reversed phase Ultimate HPLC system (Dionex, Amsterdam, Netherlands) coupled to a Q-TOF I mass spectrometer (Waters, Eschborn, Germany), or by a reversed phase CapLC HPLC system coupled to a Q-TOF Ultima API (Waters) as previously described [17]. Fragment spectra were analyzed manually and automatically.

Gene expression analysis by high-density oligonucleotide microarrays

RNA isolation from tumor and autologous normal kidney specimens as well as gene expression analysis by Affymetrix Human Genome U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA) were performed as described previously [18]. Data were analyzed with the GCOS software (Affymetrix). Pairwise

comparisons between tumor and autologous normal kidney were calculated using the respective normal array as baseline. For RCC149 and RCC211 no autologous normal kidney array data were available. Therefore, pooled healthy human kidney RNA was obtained commercially (Clontech, Heidelberg, Germany) and used as the baseline for these tumors.

Maturation of DCs

DCs were prepared using blood from healthy donors. Briefly, PBMCs were isolated using standard gradient centrifugation (Lymphocyte Separation Medium, PAA Laboratories GmbH, Pasching, Austria) and plated at a density of 7×10^6 cells/ml in X-Vivo 15 medium. After 2 hours at 37°C, non-adherent cells were removed and adherent monocytes cultured for 6 days in X-Vivo medium with 100 ng/ml GM-CSF and 40 ng/ml IL-4 (AL-ImmunoTools, Friesoythe, Germany). On day 7 immature DCs were activated with 10 ng/ml TNF-α (R&D Systems, Wiesbaden, Germany) and 20 µg/ml poly(IC) (Sigma Aldrich, Steinheim, Germany) for 3 days.

Generation of antigen-specific $CD4^+$ T cells

10^6 PBMCs per well were stimulated with 2×10^5 peptide pulsed (5 µg/ml) autologous DCs. Cells were incubated in 96-well plates (7 wells per donor and per peptide) with T-cell medium: supplemented RPMI 1640 in the presence of 10 ng/ml IL-12 (Promocell, Heidelberg, Germany). After 3 to 4 days of co-incubation at 37°C, fresh medium with 80 U/ml IL-2 (Proleukin, Chiron Corporation, Emeryville, CA, USA) and 5 ng/ml IL-7 (Promocell) was added. Restimulations were done with autologous PBMCs plus peptide every 6 to 8 days.

Intracellular IFNγ staining

After 3 and 4 rounds of stimulation, PBMCs were thawed, washed twice in PBS, resuspended at 10^7 cells/ml in T-cell medium and cultured overnight. On the next day, PBMCs, pulsed with 5 µg/ml peptide, were incubated with effector cells in a ratio of 1:1 for 6 h. Golgi-Stop (Becton Dickinson, Heidelberg, Germany) was added for the final 4 h of incubation.

Cells were analyzed using a Cytofix/Cytoperm Plus kit (Becton Dickinson) and CD4-FITC- (Immunotools), IFNγ-PE- and CD8-PerCP clone SK1-antibodies (Becton Dickinson). For negative controls, cells of seven wells were pooled and incubated

either with irrelevant peptide or without peptide, respectively. Stimulation with PMA/Ionomycin was used for positive control. Cells were analyzed on a three-color FACSCalibur (Becton Dickinson).

Results and Discussion

HLA class II expression by RCC

Under normal, non-inflammatory conditions class II molecules should only be expressed by cells of the hematopoietic system and by the thymic epithelium [6]. The situation changes during inflammation. MHC II expression can be induced in most cell types and tissues by IFNγ [19]. As RCC incidence is often accompanied by inflammatory events [20; 21], class II molecules are indeed expressed in the vicinity of or by tumors, as has been reported [22].

We analyzed HLA class II expression of ten RCC specimens comprising histological clear cell and papillary renal carcinoma by immunohistochemical staining and found that all investigated samples revealed class II positive tumor cells. As exemplified in Figure 1A, a pronounced HLA class II expression was often detected at the margin of the tumor. In these areas we observed a close spatial correlation of HLA positive tumor cells with tumor infiltrating immune cells as illustrated by the visualization of CD68-positive macrophages in a serial tissue section (Figure 1B). In RCC revealing a more papillary architecture, the expression of HLA class II molecules was more evenly distributed throughout the tumor (Figure 1C, E, G). The comparison of the HLA class II and CD68 immunohistochemical staining patterns in serial tissue sections clearly demonstrates that in addition to macrophages, tumor cells also express HLA class II (Figure 1C, D and E, F). It has been shown that IFNγ producing $CD4^+$ Th1 cells as well as NK cells infiltrate RCC [23]. As class II positive tumor cells were found predominantly in outer parts of dissected tumors, one could speculate that leukocytes attracted by the tumor produce IFNγ which acts on neighboring malignant cells.

Additionally, we investigated class II expression by comparative gene expression analysis using oligonucleotide microarrays. With this technique we were able to asses the overall class II expression in the dissected tumors regardless of the expressing cell types. We analyzed differential expression in four tumors, RCC149, RCC180 RCC190, and RCC211, compared with normal reference kidney. In all four

tumors HLA class II genes were overexpressed (Table 1). One possible reason for this might be an induced expression by IFNγ and for this reason we looked for other genes known to be upregulated by interferons [24]. Interestingly, a considerable number of such genes were found to be overexpressed in one or more tumor samples. Table 1 shows interferon-inducible genes which were upregulated reproducibly in all four samples, in accordance with our earlier findings [25]. Among them are LMP2, LMP7, and MECL1 - proteins which are exchanged against constitutive proteasomal subunits to form the immunoproteasome, a hallmark process in an interferon-rich environment. Additionally, IFNγ was directly assessed by quantitative real-time RT PCR (TaqMan). The tumors displayed in Table 1 showed a 5- to 60-fold IFNγ mRNA overexpression compared with their autologous normal RNA samples (data not shown). Thus our results indicate that IFNγ might play an important role in RCC and be the reason for abundant class II expression.

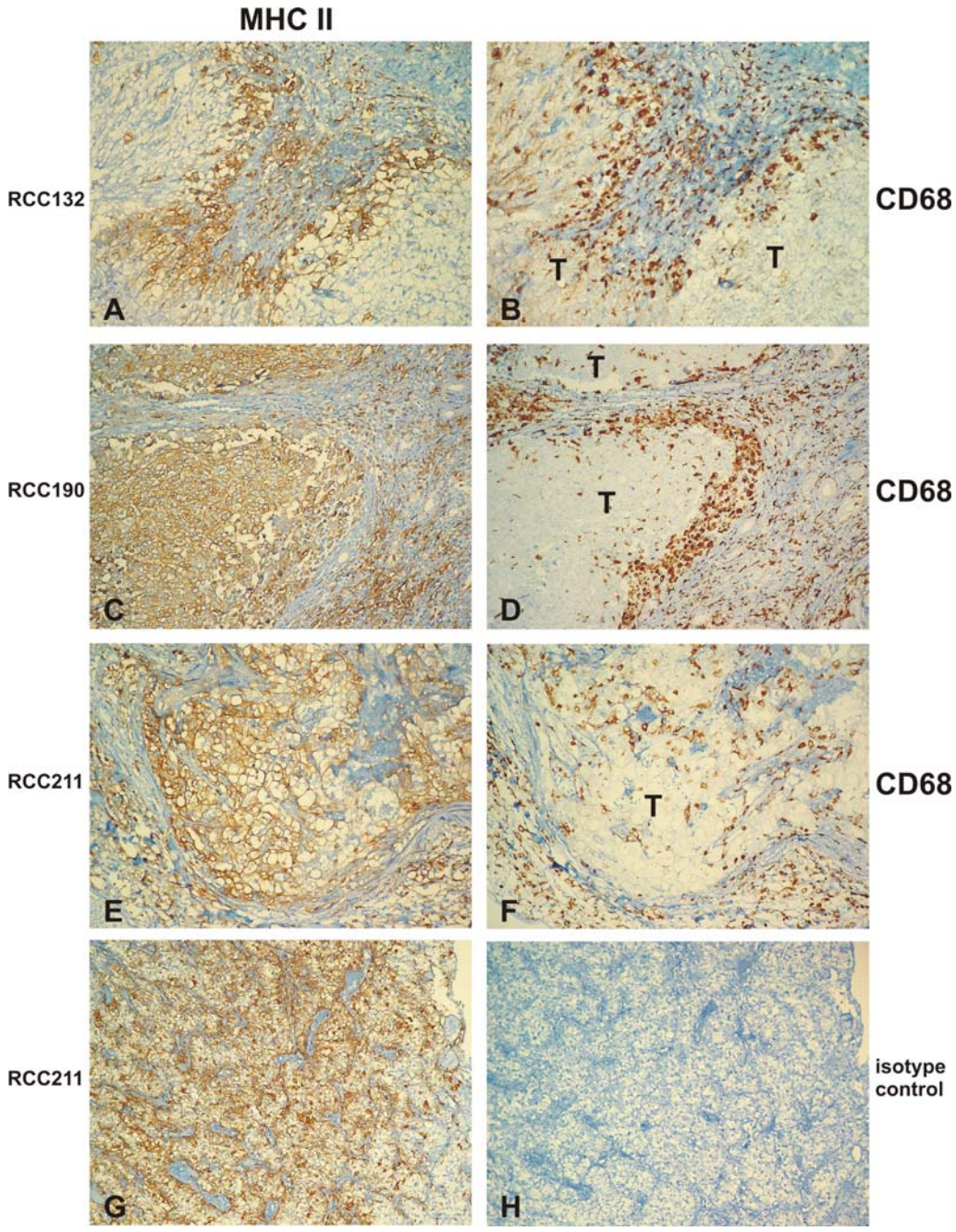

Figure 1: Expression of HLA class II molecules in RCC of three patients. Whereas in the tumor of patient RCC132 the HLA positive cells were preferably localized at the margin (A, B) the HLA class II expression patterns of the tumors from patient RCC190 and RCC211 revealing a more papillary structure were more evenly spread (C, E, G). The visualization of $CD68^+$ macrophages (B, D, F) in serial tissue sections illustrates a close spatial relationship of tumor-infiltrating mononuclear immune cells and HLA II expressing tumor cells. Incubation with mouse IgG instead of specific antibodies consistently revealed negative staining results (H). Capital T marks the tumor.

Table 1: mRNA expression of interferon-inducible genes.

Expression in tumor samples was compared with autologous normal kidney (RCC180, RCC190) or pooled healthy kidney (RCC149, RCC211). All genes showed an "increase" in the change-call algorithm of the GCOS software for all four tumors and have been described as interferon-inducible.

Gene Symbol	Entrez GeneID	Gene Title	-fold overexpression tumor vs. normal			
			RCC149	RCC180	RCC190	RCC211
HLA-DPA1	3113	major histocompatibility complex, class II, DP alpha 1	3.5	3.7	4.9	13.9
HLA-DPB1	3115	major histocompatibility complex, class II, DP beta 1	2.6	2.5	2.8	14.9
HLA-DQB1	3119	major histocompatibility complex, class II, DQ beta 1	4.3	4.0	6.5	5.3
HLA-DRB1	3123	major histocompatibility complex, class II, DR beta 1	1.2	1.9	2.8	4.3
CXCL10	3627	chemokine (C-X-C motif) ligand 10	1.1	3.2	10.6	24.3
FCGR1A	2209	Fc fragment of IgG, high affinity Ia, receptor for (CD64)	6.5	2.6	12.1	29.9
IFI16	3428	interferon, gamma-inducible protein 16	8.6	3.0	4.3	11.3
IFI44	10561	interferon-induced protein 44	2.8	1.4	2.5	2.8
OAS1	4938	2',5'-oligoadenylate synthetase 1, 40/46kDa	3.5	2.3	2.6	5.3
PSMB8	5696	proteasome subunit, beta type, 8 (LMP7)	2.6	4.3	6.1	6.5
PSMB9	5698	proteasome subunit, beta type, 9 (LMP2)	4.3	7.5	6.5	16.0
PSMB10	5699	proteasome subunit, beta type, 10 (MECL1)	3.2	2.5	5.3	13.0
SP100	6672	nuclear antigen Sp100	4.0	1.1	1.5	2.8
TAP1	6890	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	2.5	2.8	6.5	8.0
VCAM1	7412	vascular cell adhesion molecule 1	5.7	5.3	3.2	12.1

HLA-DR ligands isolated from dissected carcinomas

Class II peptides from solid tumors have so far not been isolated and identified. We analyzed ten different RCC, three CCA and one TCC and were able to isolate HLA-DR ligands from all samples, 452 peptides in total (Table 2). Table 3 shows a representative list of peptides and corresponding source proteins identified from RCC190. The specimens differed in their HLA genotypes, in weight and in the number of identified ligands. There was no correlation between tumor weight and number of identified ligands. Peptide source proteins could be divided into two groups. On the one hand, ligands which should be presented by leukocytes were found, such as peptides from complement components C3, C4A, C4 binding protein alpha, and CD14, and Fc fragment of IgG binding protein. On the other hand, we found peptides probably presented by tumor cells from overexpressed TAA, for example from vimentin, matrix metalloproteinase 7, eukaryotic translation elongation factor 1 alpha 1, and nicotinamide N-methyltransferase. This observation is in accordance with immunohistochemistry data (Figure 1) and demonstrates that class II positive tumor cells and infiltrating leukocytes were present in analyzed specimens and that the eluted peptides were very likely derived from these distinct cell types.

In order to identify peptides from TAA, we compared ligand source proteins with overexpressed genes detected by microarray analysis of tumors [26; 27]. We identified a peptide from insulin-like growth factor binding protein 3, $IGFBP3_{166-181,}$ on RCC190. In addition, two variants of this peptide, $IGFBP3_{169-181}$ and $IGFBP3_{169-184}$, were found on TCC108. From the same tumor a peptide from matrix metalloproteinase 7, $MMP7_{247-262}$, could be isolated (Table 2). At the mRNA level, MMP7 was overexpressed in 13 and IGFBP3 in 22 of 23 analyzed RCC (data not shown) and both have been described to be tumor-associated [28-30]. To test these peptides for their immunostimulatory capacity by *in vitro* T cell priming experiments, the shortest variant of the IGFBP3 peptides, $IGFBP3_{169-181}$, and the MMP7 peptide were used.

Table 2: HLA-DR peptide sequences. Peptides were isolated from solid tumors (RCC, TCC, CCA). Shown are peptide sequences, source tumors and corresponding HLA-DR genotypes, source proteins, and Entrez GeneIDs of source proteins.

RCC211 (B1*07, B1*08, B4)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
LSALEEYTKKLNTQ	APOA1	335
KIAFSATRTINVPL	C1QB	713
YSIFSYATKRQDNE	CRP	1401
LMHPDALEEPDDQNRI	FCGR2B	2213
NLRFLATTPNSL	FN1	2335
SNTDLVPAPAVRILTPE	GDF15	9518
LASVSTVLTSKYR	HBA2	3040
LVVYPWTQRF	HBB	3043
VVYPWTQRF	HBB	3043
VRFDSDVGEY	HLA-DRB1	3123
INEQWLLTTAKNL	HP	3240
KDSTYSLSSTLTLSK	IGKC	3514
GSEMVVAGKLQDRGPD	ITIH4	3700
GKIVDLVKELDR	SERPINA1	5265
KAVLTIDEKGTEA	SERPINA1	5265
PYFPTTARKL	SLC4A4	8671
GAYKAIPVAQDLN	SPP1	6696
GAYKAIPVAQDLNAPS	SPP1	6696
NGAYKAIPVAQDLNAP	SPP1	6696
NGAYKAIPVAQDLNAPS	SPP1	6696

RCC190 (B1*11, B1*15, B3, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
WISKQEYDESGPSIVHRKCF	ACTG1	71
LKKYLYEIARRHP	ALB	213
TLVEVSRNLGKVG	ALB	213
STPTLVEVSRNLGKVG	ALB	213
TPTLVEVSRNLGKVG	ALB	213
TPTLVEVSRNLGKVGSK	ALB	213
VSTPTLVEVSRNLGKVG	ALB	213
STPTLVEVSRNLGKVGS	ALB	213
TPTLVEVSRNLGKVGS	ALB	213
EKSKEQLTPLIKKAGTELVNF	APOA2	336
YPKSLHMYANRLLDHR	APOB	338
DYPKSLHMYANRLLDHR	APOB	338
EPYYKMQTRAGSRE	C1R	715
APPSGGPGFLSIERPDSRPP	C4B	721
APPSGGPGFLSIERPDSRPPR	C4B	721
LTSVSVFVDHHLAPS	C4B	721
GFGPIYNYKDTIVFK	C4BPA	722
FGPIYNYKDTIVFK	C4BPA	722
SPDPSIYAYDNF	CALR	811
EPPVIQNPEYKGEWKPRQIDNPD	CALR	811

RCC190 (B1*11, B1*15, B3, B5)

Peptide Sequence	Gene Symbol	Entrez GeneID
GVIKVFNDMKVRKS	CFL1	1072
GVIKVFNDMKVRK	CFL1	1072
DGVIKVFNDMKVRK	CFL1	1072
APGYLAITKKVAVPY	CPE	1363
ASVDLKNTGREEFLTA	FCGBP	8857
GNHQFAKYKSFKVADE	FCN1	2219
GVSHFFRELAEEKREG	FTL	2512
VSHFFRELAEEKREG	FTL	2512
TPDAMKAAMALEKK	FTL	2512
FVMGVNHEKYDN	GAPD	2597
TGVFTTMEKAGAHL	GAPD	2597
TGVFTTMEKAGAH	GAPD	2597
STGVFTTMEKAGAH	GAPD	2597
STGVFTTMEKAGAHL	GAPD	2597
ISWYDNEFGYSNRVVDLMAHMASKE	GAPD	2597
GTGASGSFKLNKKAASGEAKPKA	HIST1H1C	3006
GTGASGSFKLNKKAASGEAKPK	HIST1H1C	3006
DVGVYRAVTPQGRPD	HLA-DQB1	3119
DVGEFRAVTELGRPD	HLA-DRB1	3123
HPLHSKIIIIKKGHAK	IGFBP3	3486
DKDLFKAVDAALKK	KNG1	3827
KDKTYSYLNKLPVK	NPC2	10577
ILVIKMGVAAHKKSHEESHKE	S100A8	6279
LILVIKMGVAAHKKSHEESHKE	S100A8	6279
VIKMGVAAHKKSHEESHKE	S100A8	6279
ILVIKMGVAAHKKSHEESH	S100A8	6279
MIEQNTKSPLFMGKVVNPTQK	SERPINA1	5265
LMIDQNTKSPLFMGKVVNPTQK	SERPINA1	5265
AGPHFNPLSRKHGGPK	SOD1	6647
GPHFNPLSRKHGGPK	SOD1	6647
DPQTFYYAVAVVKKDSG	TF	7018
DPQTFYYAVAVVKKDS	TF	7018

RCC186 (B1*04, B1*11, B3, B4)

Peptide Sequence	Gene Symbol	Entrez GeneID
QEYDESGPSIVHRK	ACTG1	71
FGGPGTASRPSSS	VIM	7431
YASSPGGVYATR	VIM	7431
SLYASSPGGVYATR	VIM	7431

RCC180 (B1*13, B1*14, B3)

Peptide Sequence	Gene Symbol	Entrez GeneID
AQVIILNHPGQISAG	EEF1A1	1915
VIILNHPGQISAG	EEF1A1	1915
LVVYPWTQRF	HBB	3043
VVYPWTQRF	HBB	3043

RCC163 (B1*07, B1*13, B3, B4)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
ATGTIQEDYLREL	MRCL3	10627
FGEKLNGTDPEDVIRNAF	MRCL3	10627
FQVDNNNRLL	A2M	2
INEQWLLTTAKNL	HP	3240
KVNVDEVGGEALGRL	HBB	3043
LVVYPWTQRF	HBB	3043
VNVDEVGGEALGRL	HBB	3043
VVYPWTQRF	HBB	3043
WGKVNVDEVGGEALGRL	HBB	3043
WISKQEYDESGPSIVHRKCF	ACTG1	71
YDNEFGYSNRVVDL	GAPD	2597

RCC158 (B1*01, B1*16, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
DVVYALKRQGRTLYGFGG	HIST1H4A	8359
IDINEDAEGEAA	PAMCI	9182
VHLTPEEKSAVTALWGKVNVDEVGGEALGRL	HBB	3043
LVVYPWTQRF	HBB	3043
LVVYPWTQRFFES	HBB	3043
VVYPWTQRF	HBB	3043
SPMYSIITPNILR	C3	718
SPMYSIITPNILRL	C3	718
YSIITPNILR	C3	718
VLSPADKTNVKAAWGKVGAHAGEYGAEALERM	HBA2	3040
LASVSTVLTSKYR	HBA2	3040
WVIKTEMGKVL	CUBN	8029

RCC157 (B1*07, B1*16, B4, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
RVAPEEHPVL	ACTB	60
VEFSSGLKGMSL	ATP5A1	498
ASVDLKNTGREEFLTA	FCGBP	8857
VLSPADKTNVKAAWGKVGAHAGEYGAEALER	HBA2	3040
ASVSTVLTSKYR	HBA2	3040
AAHLPAEFTPAVH	HBA2	3040
VLSPADKTNVKAAWGKVGAHAGEYGAEALERM	HBA2	3040
VLSPADKTNVKA	HBA2	3040
SVSTVLTSKYR	HBA2	3040
EVGGEALGRL	HBB	3043
VHLTPEEKSAVTAL	HBB	3043
VHLTPEEKSAVT	HBB	3043
LVVYPWTQRF	HBB	3043
VHLTPEEKSAVTALWGKVNVDEVGGEALGRL	HBB	3043
KVNVDEVGGEALGRL	HBB	3043
VDEVGGEALGRL	HBB	3043
GKVNVDEVGGEALGRL	HBB	3043
LVVYPWTQR	HBB	3043
VYPWTQRF	HBB	3043

RCC157 (B1*07, B1*16, B4, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
VDPENFRLLG	HBB	3043
WGKVNVDEVGGEALGRL	HBB	3043
VVYPWTQRF	HBB	3043
VDEVGGEALGRLL	HBB	3043
GKVNVDEVGGEALGRLL	HBB	3043
WGKVNVDEVGGEALGRLL	HBB	3043
VVYPWTQR	HBG2	3048
IKEEHVIIQAE	HLA-DRA	3122
IKEEHVIIQAEF	HLA-DRA	3122
ESRAQLGGPEAAKSDETAAK	HSPB1	3315
FRDGDILGKYVD	HSPE1	3336
VDGEPLGRVSFEL	PPIA	5478
RQDVVRIVGEY	PPM2C	54704

RCC155 (B1*03, B1*04, B3, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
WISKQEYDESGPSIVHRKCF	ACTG1	71
ISKQEYDESGPSIVHRKCF	ACTG1	71
GPGLIYRQPNCDDPE	AHSG	197
GVDEVTIVNILTNRSNAQ	ANXA2	302
EVTIVNILTNRSN	ANXA2	302
EVTIVNILTNRSNAQ	ANXA2	302
VPYVIVPLKTGLQE	C3	718
DNELQEMSNQGSK	CLU	1191
NELQEMSNQGSK	CLU	1191
WGALATISTLEAVR	CREG	8804
EETVITVDTKAAGKGK	FLNA	2316
ISWYDNEFGYSNRVVDL	GAPD	2597
VHLTPEEKSAVTALWGKVNVDEVGGEALGRL	HBB	3043
LVVYPWTQRF	HBB	3043
VVYPWTQRF	HBB	3043
GSHSMRYFDTAMSRPG	HLA-B	3106
NTILPDARDPAFK	LAMP1	3916
VADKIQLINNMLDK	PGK1	5230
VPSIKFCLDNGAK	PGK1	5230
LDFLKAVDTNRASVG	PLXDC2	84898
KAVLTIDEKGTEAA	SERPINA1	5265
KAVLTIDEKGTEA	SERPINA1	5265
APEEIIMDRPFLFVVR	SERPINE1	5054
GVVHVITNVLQPPA	TGFBI	7045
GVVHVITNVLQPP	TGFBI	7045
TTTQLYTDRTEKLRPE	TGFBI	7045
NGVVHVITNVLQPP	TGFBI	7045
TNGVVHVITNVLQPPA	TGFBI	7045
TNGVVHVITNVLQPP	TGFBI	7045

RCC149 (B1*01, B1*13, B3)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
SGTLVLLQGARGFA	CD14	929
VSGTLVLLQGARGFA	CD14	929
VSGTLVLLQGARG	CD14	929
LVVYPWTQRF	HBB	3043
APSAILPLPGQSVER	ITIH4	3700
ASSLVIIWGIL	KIAA0877	23333
SPERPFLAILGGAKVADK	PGK1	5230
PAILSEASAPIPH	SDCBP	6386
FGGPGTASRPSSS	VIM	7431

RCC132 (B1*04, B1*08, B4)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
TPTLVEVSRNLGK	ALB	213
FPKAEFAEVSKLVTD	ALB	213
PKAEFAEVSKLVTD	ALB	213
DVPKWISIMTERSVPH	ANXA2	302
IQHFQAMVKALEKE	APLP2	334
IQHFQAMVKALEKEA	APLP2	334
TFQMKKNAEELK	APOA4	337
GLTFQMKKNAEELK	APOA4	337
EGLTFQMKKNAEELKA	APOA4	337
EGLTFQMKKNAEELK	APOA4	337
GLTFQMKKNAEELKA	APOA4	337
EPGTWKISARFSDG	C4B	432395
ILCVTKAREGSE	CASP14	23581
IKGKINSITVDNCKK	CAP1	10487
NTGVISVVTTGLDR	CDH1	999
VPRYLPRPANPDE	CDH1	999
ETGWLLLNKPLDR	CDH3	1001
DPSPSPVLGYKIVYKPVG	COL12A1	1303
RPKDYEVDATLKSLNN	COL1A2	1278
LPGETLTYVWKIPER	CP	1356
DNGFFKILRGQDH	CTSB	1508
LPQSIVYKYMSIRSDRSVPS	EFEMP1	2202
SGKYDLDFKSPDDPSR	ENO1	2023
IPGHLNSYTIKGLKPG	FN1	2335
LNSYTIKGLKPG	FN1	2335
LTGYRVRVTPKEKTGP	FN1	2335
TGYRVRVTPKEKTGPM	FN1	2335
ITGYIIKYEKPGSPP	FN1	2335
LVVYPWTQRF	HBB	3043
VVYPWTQRF	HBB	3043
FVRFDSDAASPR	HLA-C	3107
KDILEEKRAVPDR	HLA-DPB1	3115
DILEEKRAVPDR	HLA-DPB1	3115
YVGKKQLVEIEK	HPR	3250
APTLTLYVGKKQLVEIEK	HPR	3250
SYAYSLKNQIGDKEK	HSPA5	3309
YAYSLKNQIGDKEK	HSPA5	3309

RCC132 (B1*04, B1*08, B4)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
AYSLKNQIGDK	HSPA5	3309
ESYAYSLKNQIGDKEK	HSPA5	3309
GIFEVKSTAGDTH	HSPA8	3312
KHKVYACEVTHQG	IGKC	3514
GSEMVVAGKLQDRGPDV	ITIH4	3700
KGSEMVVAGKLQDRGPD	ITIH4	3700
MVVAGKLQDRGPD	ITIH4	3700
KGSEMVVAGKLQDRGPDV	ITIH4	3700
FKGSEMVVAGKLQDRGPDV	ITIH4	3700
GSEMVVAGKLQDRGPD	ITIH4	3700
QGSYVPLLRDTDSS	LISCH7	51599
DAIWNLLRQAQEK	LTF	4057
APYKLRPVAAEVYG	LTF	4057
GLAPYKLRPVAAEVYG	LTF	4057
KPPQYIAVHVVPDQ	MIF	4282
KDKTYSYLNKLPVK	NPC2	10577
QRESYELKVVAADRGSPS	PCDH1	5097
EPHRHSIFTPETNPRAG	PLG	5340
LDFLKAVDTNRAS	PLXDC2	84898
DPGVLDRMMKKLDTNSD	S100A11	6282
FHQYSVKLGHPDT	S100A9	6280
DLQNFLKKENKNEKVIE	S100A9	6280
DLQNFLKKENKNEK	S100A9	6280
DLQNFLKKENKNEKV	S100A9	6280
DLQNFLKKENKNEKVI	S100A9	6280
HQYSVKLGHPDT	S100A9	6280
TQGKIVDLVKELDRD	SERPINA1	5265
TQGKIVDLVKELDR	SERPINA1	5265
GTQGKIVDLVKELDR	SERPINA1	5265
TQGKIVDLVKELDRDT	SERPINA1	5265
QGKIVDLVKELDRDT	SERPINA1	5265
IVDLVKELDR	SERPINA1	5265
IVDLVKELDRDT	SERPINA1	5265
GTQGKIVDLVKELDRDT	SERPINA1	5265
QGKIVDLVKELDR	SERPINA1	5265
EAIYDICRRNLDIERP	TUBA6	84790
ELDEKAVRPG	VTN	7448
VVSSIEQKTEGAEKK	YWHAZ	7534
VSSIEQKTEGAEKK	YWHAZ	7534

TCC108 (B1*11, B1*15, B3, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
GNRIAQWQSFQLEGG	A2M	2
KPKDPTFIPAPIQAKTSPV	AGT	183
STPTLVEVSRNLGKVG	ALB	213
TPTLVEVSRNLGKVG	ALB	213
PTLVEVSRNLGKVG	ALB	213
VSTPTLVEVSRNLGKVG	ALB	213
STPTLVEVSRNLGKVGSK	ALB	213

TCC108 (B1*11, B1*15, B3, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
IPEDSIFTMADRGECVPG	AMBP	259
TPPSAYGSVKAYTNFDAER	ANXA2	302
TPPSAYGSVKAYTNFDAERD	ANXA2	302
TPPSAYGSVKAYTNFDAERDA	ANXA2	302
AYGSVKAYTNFDAER	ANXA2	302
GIAEFWINGTPLVK	APCS	325
GIAEFWINGTPLVKKG	APCS	325
YPKSLHMYANRLLDHR	APOB	338
YPKSLHMYANRLLD	APOB	338
EPYYKMQTRAGSR	C1R	715
EPYYKMQTRAGSRE	C1R	715
AGYALAQMGRLKGPLL	C3	718
GGPGFLSIERPDSRPP	C4B	721
APPSGGPGFLSIERPDSRPPR	C4B	432395
APPSGGPGFLSIERPDSRPP	C4B	721
FGPIYNYKDTIVFK	C4BPA	722
GPIYNYKDTIVFK	C4BPA	722
GFGPIYNYKDTIVFK	C4BPA	722
DHIGIISFPDFKIPSNPR	C5	727
VPAQLLVGALRVLAYSR	CD14	929
VSDGVIKVFNDMKVRKS	CFL1	1072
VSDGVIKVFNDMKVRK	CFL1	1072
SDGVIKVFNDMKVRK	CFL1	1072
DGVIKVFNDMKVRK	CFL1	1072
GVIKVFNDMKVRKS	CFL1	1072
SDGVIKVFNDMKVRKSSTPE	CFL1	1072
GVIKVFNDMKVRK	CFL1	1072
TPDTIRRFQSVPAQPG	CLTC	1213
AGLLSTYRAFLSSH	COL15A1	1306
AAGLLSTYRAFLSSH	COL15A1	1306
APGYLAITKKVAVPY	CPE	1363
SAPGYLAITKKVAVPY	CPE	1363
GAFSVYSDFLLYK	CTSB	1508
GPVEGAFSVYSDFLLYKS	CTSB	1508
GPVEGAFSVYSDFLLYK	CTSB	1508
VPDDRDFEPSLGPVCPFR	DCN	1634
GPLGITGFLGPLFFL	est	-
SDSIQWFHNGNLIPT	FCGR2B	2213
GATYNIIVEALKDQ	FN1	2335
TPDAMKAAMALEKK	FTL	2512
TGVFTTMEKAGAHL	GAPD	2597
APMFVMGVNHEKYDN	GAPD	2597
STGVFTTMEKAGAHL	GAPD	2597
GVFTTMEKAGAH	GAPD	2597
STGVFTTMEKAGAH	GAPD	2597
DAPMFVMGVNHEKYDN	GAPD	2597
GKHVLFVNVAAYCGLAA	GPX6	257202
GKPMIIYKGGTSR	GSN	2934
VGVYRAVTPQGRPD	HLA-DQB1	3119
GVYRAVTPQGRPD	HLA-DQB1	3119
DVGVYRAVTPQGRPD	HLA-DQB1	3119

TCC108 (B1*11, B1*15, B3, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
RLEEFGRFASFEAQGAL	HLA-DRA	3122
LEEFGRFASFEAQG	HLA-DRA1	3122
FQTLVMLETVPRSG	HLA-DRB1	3123
DVGEYRAVTELGRPD	HLA-DRB1	3123
DVGEFRAVTELGRPD	HLA-DRB1	3123
TAPKLLIYSNNQRPS	Ig lambda	-
FPPSSEELQANKATLVCL	Ig lambda	-
HSKIIIIKKGHAK	IGFBP3	3486
HSKIIIIKKGHAKDSQ	IGFBP3	3486
LPGTAPKLLIYSNNQRPSG	Ig lambda	-
GPEDNVVIIYLSRAGNPE	ISLR	3671
AISDYVFNTASLVYHEE	LBP	3929
ISDYVFNTASLVYHEE	LBP	3929
DDIIVNWVNETLRE	LCP1	3936
NDDIIVNWVNETLRE	LCP1	3936
SNPVDILTYVAWKISGFPK	LDHA	3939
NPVDILTYVAWKLSGFPK	LDHAL6A	160287
TPQNFLAVLRGDAEA	LGMN	5641
LDLSFNQIARLPSGLPV	LUM	4060
DLSFNQIARLPSGLPV	LUM	4060
DLSFNQIARLPSG	LUM	4060
SQDDIKGIQKLYGKRS	MMP7	4316
QEKNIMLYKGSGLWSR	MRC1	4360
DGRAYTAISHIPQPA	NID2	22795
EGLFSLVARKLSRP	NNMT	4837
ITGKWFYIASAFRNEE	ORM1	5004
LGEFYEALDCLRIPK	ORM1	5004
HGNQIATNGVVHVIDR	POSTN	10631
AHGRINPYMSSPCH	RPL17	6139
EMINPYRNLPLA	SLC7A5	8140
GPHFNPLSRKHGGPK	SOD1	6647
AGPHFNPLSRKHGGPK	SOD1	6647
GNAIFTFPNTPVK	SQRDL	58472
TPLPLIKPYSGPRLPP	TAF9	6880
EDPQTFYYAVAVVKKDSG	TF	7018
ERPTYTNLNRLISQ	TUBA4	80086

CCA156 (B1*01, B1*03, B3)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
APSAILPLPGQSVER	ITIH4	3700
LVAYYTLIGASGQR	C3	718
SPMYSIITPNILR	C3	718
SPMYSIITPNILRL	C3	718
YSIITPNILR	C3	718
YSIITPNILRL	C3	718
VSGTLVLLQGARGFA	CD14	929
DTSYVSLKAPLTK	CRP	1401
IEKFEKEAAEMGKG	EEF1A1	1915
KNGFVVLKGRPCK	EIF5A	1984

CCA156 (B1*01, B1*03, B3)

Peptide Sequence	Gene Symbol	Entrez GeneID
ASVDLKNTGREEF	FCGBP	8857
ASVDLKNTGREEFL	FCGBP	8857
ASVDLKNTGREEFLTAF	FCGBP	8857
VDLKNTGREEF	FCGBP	8857
VDLKNTGREEFL	FCGBP	8857
EETVITVDTKAAGKGK	FLNA	2316
ETVITVDTKAAGKGK	FLNA	2316
TGDAYVILKTVQLRNG	GSN	2934
LVVYPWTQRF	HBB	3043
TAEILELAGNAARDNK	HIST3H2A	92815
MFYVDLDKKETVWH	HLA-DPA1	3113
SPERPFLAILGGAKVADK	PGK1	5230
DTKVYTVDLGRTVT	PIGR	5284
WAAEVISDARENIQR	SAA1	6288
KAVLTIDEKGTEA	SERPINA1	5265
NTDPYQLMNAVNTLDR	SULF2	55959

CCA164 (B1*11, B1*15, B3, B5)

Peptide Sequence	Gene Symbol	Entrez GeneID
TPTLVEVSRNLGKVG	ALB	213
STPTLVEVSRNLGKVG	ALB	213
TPTLVEVSRNLGKVGSK	ALB	213
TPPSAYGSVKAYTNFDAERDA	ANXA2	302
SKEQLTPLIKKAGTE	APOA2	336
GLADASLLKKV	ATXN10	5814
KMDPIISRV	BTG2	7832
EPYYKMQTRAGSRE	C1R	715
HEPYYKMQTRAGSRE	C1R	715
APPSGGPGFLSIERPDSRPP	C4A	720
GGPGFLSIERPDSRPP	C4A	720
GVIKVFNDMKVRK	CFL1	1072
DGVIKVFNDMKVRK	CFL1	1072
FLFLDRTYV	CUL4B	8450
ASVDLKNTGREEFLT	FCGBP	8857
VDLKNTGREEFLTA	FCGBP	8857
ASVDLKNTGREEFLTAF	FCGBP	8857
NTGREEFLTA	FCGBP	8857
ASVDLKNTGREEFL	FCGBP	8857
ASVDLKNTGREEF	FCGBP	8857
ASVDLKNTGREEFLTAFLQN	FCGBP	8857
YSKAYPRLLISSL	FCGBP	8857
ASVDLKNTGREEFLTA	FCGBP	8857
FLDPDRHFL	FLJ46072	286077
NPAEFVVNTSNAGAG	FLNA	2316
TGVFTTMEKAGAHL	GAPD	2597
STGVFTTMEKAGAHL	GAPDH	2597
TGVFTTMEKAGAH	GAPDH	2597
GVFTTMEKAGAHL	GAPDH	2597
DVGVYRAVTPQGRPD	HLA-DQB1	3119
LEEFGRFASFEAQG	HLA-DRA	3122

CCA164 (B1*11, B1*15, B3, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
DVGEFRAVTELGRPD	HLA-DRB1	3123
ILLDIKTRL	KRT20	54474
DLSFNQIARLPSG	LUM	4060
NEEEIRANVAVVSGAP	SDCBP	6386
AGPHFNPLSRKHGGPK	SOD1	6647
DPQTFYYAVAVVKKDSG	TF	7018
GKKEYLIAGKAEGDG	TIMP2	7077
FLYDDNQRV	TOP2A	7153

CCA165 (B1*13, B1*15, B3, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
LKKYLYEIARRHP	ALB	213
YPKSLHMYANRLLDHR	APOB	338
TPKIQVYSRHPAENGK	B2M	567
TPKIQVYSRHPAEN	B2M	567
RTPKIQVYSRHPAENGK	B2M	567
RTPKIQVYSRHPAENG	B2M	567
RTPKIQVYSRHPAEN	B2M	567
GPGEQQKRKIVLDPSGSMN	BF	629
LPKPPKPVSKMRMATPLLMQ	CD74	972
DGVIKVFNDMKVRK	CFL1	1072
GVIKVFNDMKVRK	CFL1	1072
DGVIKVFNDMKVR	CFL1	1072
AQVIILNHPGQISA	EEF1A1	1915
AQVIILNHPGQISAG	EEF1A1	1915
TAQVIILNHPGQIS	EEF1A1	1915
TAQVIILNHPGQISAG	EEF1A1	1915
VIILNHPGQISAG	EEF1A1	1915
VIILNHPGQIS	EEF1A1	1915
SVDLKNTGREEFLTA	FCGBP	8857
YSKAYPRLLISSL	FCGBP	8857
NTGREEFLTA	FCGBP	8857
VDLKNTGREEF	FCGBP	8857
ASVDLKNTGREEFLTAFLQN	FCGBP	8857
ASVDLKNTGREEFLTAF	FCGBP	8857
NTGREEFLTAF	FCGBP	8857
KNTGREEFLTA	FCGBP	8857
NTGREEFLTAFLQN	FCGBP	8857
ASVDLKNTGREE	FCGBP	8857
SVDLKNTGREEF	FCGBP	8857
DLKNTGREEFLTA	FCGBP	8857
LKNTGREEFLTA	FCGBP	8857
ASVDLKNTGREEF	FCGBP	8857
ASVDLKNTGREEFL	FCGBP	8857
ASVDLKNTGREEFLTA	FCGBP	8857
VDLKNTGREEFLTA	FCGBP	8857
FVMGVNHEKYDN	GAPD	2597
DAPMFVMGVNHEKYDN	GAPD	2597
SDVGEFRAVTELGRPD	HLA-DPB1	3115
DVGEFRAVTELGRPA	HLA-DPB1	3115

CCA165 (B1*13, B1*15, B3, B5)		
Peptide Sequence	**Gene Symbol**	**Entrez GeneID**
DVGVYRAVTPQGRPD	HLA-DQB1	3119
SDVGVYRAVTPQGRPD	HLA-DQB1	3119
DVGVYRAVTPQGRPV	HLA-DQB1	3119
LEEFGRFASFEAQG	HLA-DRA	3122
VPPEVTVLTNSPVELREP	HLA-DRA	3122
VPPEVTVLTNSPVELREPN	HLA-DRA	3122
DVGEFRAVTELGRPDA	HLA-DRB1	3123
DVGEFRAVTELGRPD	HLA-DRB1	3123
DVGEYRAVTELGRPV	HLA-DRB1	3123
YGGGNYGPGGSGGSGGYGGRS	HNRPA2B1	3181
VEIIANDQGNRITPS	HSPA5	3309
LPREKYLTWASRQEPS	IGHA2	3494
HGNQIATNGVVHVIDR	POSTN	10631
IYRNTGTEAPDY	SELENBP1	8991
IPLEIRFLHNPDAAQG	SELENBP1	8991

Table 3: HLA-DR ligands isolated from RCC190.

Shown are the core sequences of HLA-DR ligands isolated from RCC190 (HLA-DRB1*11, DRB1*15, DRB3, DRB5).

Gene Symbol	Entrez GeneID	Peptide Sequence	Gene Titel
ACTG1	71	WISKQEYDESGPSIVHRKCF	actin, gamma 1 propeptide
ALB	213	LKKYLYEIARRHP	albumin precursor
ALB	213	TLVEVSRNLGKVG	albumin precursor
ALB	213	TPTLVEVSRNLGKVGS	albumin precursor
APOA2	336	EKSKEQLTPLIKKAGTELVNF	apolipoprotein A-II precursor
APOB	338	YPKSLHMYANRLLDHR	apolipoprotein B precursor
C1R	715	EPYYKMQTRAGSRE	complement component 1, r subcomponent
C4B	721	APPSGGPGFLSIERPDSRPP	complement component 4B proprotein
C4BPA	722	FGPIYNYKDTIVFK	complement component 4 binding protein, alpha
CALR	811	SPDPSIYAYDNF	calreticulin precursor
CALR	811	EPPVIQNPEYKGEWKPRQIDNPD	calreticulin precursor
CFL1	1072	GVIKVFNDMKVRK	cofilin 1 (non-muscle)
CPE	1363	APGYLAITKKVAVPY	carboxypeptidase E precursor
FCGBP	8857	ASVDLKNTGREEFLTA	Fc fragment of IgG binding protein
FCN1	2219	GNHQFAKYKSFKVADE	ficolin 1 precursor
FTL	2512	VSHFFRELAEEKREG	ferritin, light polypeptide
FTL	2512	TPDAMKAAMALEKK	ferritin, light polypeptide
GAPD	2597	FVMGVNHEKYDN	glyceraldehyde-3-phosphate dehydrogenase
GAPD	2597	TGVFTTMEKAGAH	glyceraldehyde-3-phosphate dehydrogenase
GAPD	2597	ISWYDNEFGYSNRVVDLMAHMASKE	glyceraldehyde-3-phosphate dehydrogenase
HIST1H1C	3006	GTGASGSFKLNKKAASGEAKPK	H1 histone family, member 2
HLA-DQB1	3119	DVGVYRAVTPQGRPD	major histocompatibility complex, class II, DQ beta 1 precursor
HLA-DRB1	3123	DVGEFRAVTELGRPD	major histocompatibility complex, class II, DR beta 1 precursor
IGFBP3	3486	HPLHSKIIIIKKGHAK	insulin-like growth factor binding

			protein 3
KNG1	3827	DKDLFKAVDAALKK	kininogen 1
NPC2	10577	KDKTYSYLNKLPVK	Niemann-Pick disease, type C2 precursor
S100A8	6279	VIKMGVAAHKKSHEESHKE	S100 calcium-binding protein A8
SERPINA1	5265	MIEQNTKSPLFMGKVVNPTQK	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
SOD1	6647	GPHFNPLSRKHGGPK	superoxide dismutase 1, soluble
TF	7018	DPQTFYYAVAVVKKDS	transferrin

Antigen-specific CD4$^+$ T cells against IGFBP3$_{169-181}$ and MMP7$_{247-262}$

The peptides IGFBP3$_{169-181}$ and MMP7$_{247-262}$ were analyzed with respect to their binding characteristics by the SYFPEITHI class II epitope prediction algorithm (www.syfpeithi.de). The HLA-DR genotypes of the source tumors were HLA-DRB1*11 and DRB1*15 (Table 2) in both cases. According to the epitope predictions for DRB1*1101 and DRB1*1501, both peptides received a high binding score for HLA-DRB1*11 (data not shown). To generate antigen-specific CD4$^+$ T cells and to test the peptides for promiscuous binding, PBMCs of 4 healthy donors with different HLA-DR alleles (Figure 2), one of them carrying DRB1*1101, were stimulated using peptide-pulsed autologous DCs. In addition, the peptide CCND1$_{198-212}$, a known T cell epitope [31], was used as positive control. As a read-out system for the generation of antigen-specific CD4$^+$ T cells, IFNγ levels were assessed by flow cytometry. T cells were analyzed after the third and fourth weekly stimulation by intracellular IFNγ staining plus CD4-FITC and CD8-PerCP staining to determine the percentage of IFNγ-producing cells in specific T-cell subpopulations. In all experiments, stimulations with irrelevant peptide and without peptide were performed as negative controls. IFNγ response was considered as positive if the percentage of IFNγ producing CD4$^+$ T cells was more than two-fold higher compared with negative controls [32].

In three of four donors we were able to generate specific CD4$^+$ T cells for both peptides (Figure 2). T cell responses could not be observed in donor 4 after any stimulation. In donor 1, 0.05% to 0.1% (Figure 3) IFNγ producing CD4$^+$ T cells were detected in all seven stimulation attempts after the third stimulation with peptide IGFBP3$_{169-181}$. These T cells could be expanded in most cases by an additional round

of stimulation to 0.09% to 0.13%. IFNγ-producing $CD4^+$ T cells specific for the peptide $IGFBP3_{169-181}$ were also observed in donor 2 and donor 3, with maximal frequencies of 0.05% and 0.07% IFNγ producing $CD4^+$ T cells.

Donors 1, 2, and 3 also showed $CD4^+$ T cells reactive to peptide $MMP7_{247-262}$. The highest frequencies of IFNγ producing $CD4^+$ T cells specific for the MMP7 peptide were found in donors 1 and 2, respectively. Donors 1, 2, and 3 showed IFNγ responses to peptide $CCND1_{198-212}$, which has already been described as an MHC class II-restricted T cell epitope [33].

Thus, peptides from IGFBP3, MMP7, and CCND1 are promiscuous HLA class II binders that are able to elicit $CD4^+$ T cell responses in three out of four healthy donors carrying different HLA alleles. If the HLA alleles of the two tumor patients from which the IGFBP3 and MMP7 peptides were derived are compared to those of the four healthy donors, it seems very likely that the peptides are presented by HLA-DRB1*01, HLA-DRB1*04 and HLA-DRB1*11. All three allotypes have a glycine residue at position 86 and an aspartic acid residue at position 57 of their β chains (see www.anthonynolan.com/HIG). Therefore, they have very similar binding characteristics for their binding pockets P1 and P9 [34]. For peptide $CCND1_{198-212}$, a T cell epitope known to be presented by HLA-DRB1*0401 and HLA-DRB1*0408 [35], the same holds true. Donor 4 carries HLA-DRB1*0318 and HLA-DRB1*1401, alleles with peptide motifs that probably differ from those described above. This could explain why it was not possible to elicit T cell responses against the two peptides using cells from this donor.

The identification of helper T cell epitopes of TAA remains an important task in anti-tumor immunotherapy. Until now, different strategies for the identification of class II peptides from TAA have been carried out, ranging from the incubation of APCs with the antigen of interest in order to be taken up and processed [36] to various transfection strategies with fusion proteins [37]. All these methods are very time-consuming and it often remains unclear if the identified ligands are presented *in vivo*. We could show that it is possible to isolate class II ligands from dissected solid tumors, thus identifying the peptides which are presented by tumors and surrounding tissue *in vivo*. Among the source proteins several housekeeping and immunorelevant proteins were present. However, peptides from TAA could also be detected, leading to a straightforward approach for the identification of *in vivo* relevant class II ligands of TAA.

We identified three ligands accounting for one core sequence from IGFBP3 and one ligand from MMP7. We found these proteins to be overexpressed in renal cell carcinomas, in addition, they have been described as tumor-associated [38-40]. These peptides bound promiscuously to HLA class II molecules and were able to activate $CD4^+$ T cells from different healthy donors. Thus, our approach will be helpful in the identification of new class II peptide candidates from TAA for use in clinical vaccination protocols.

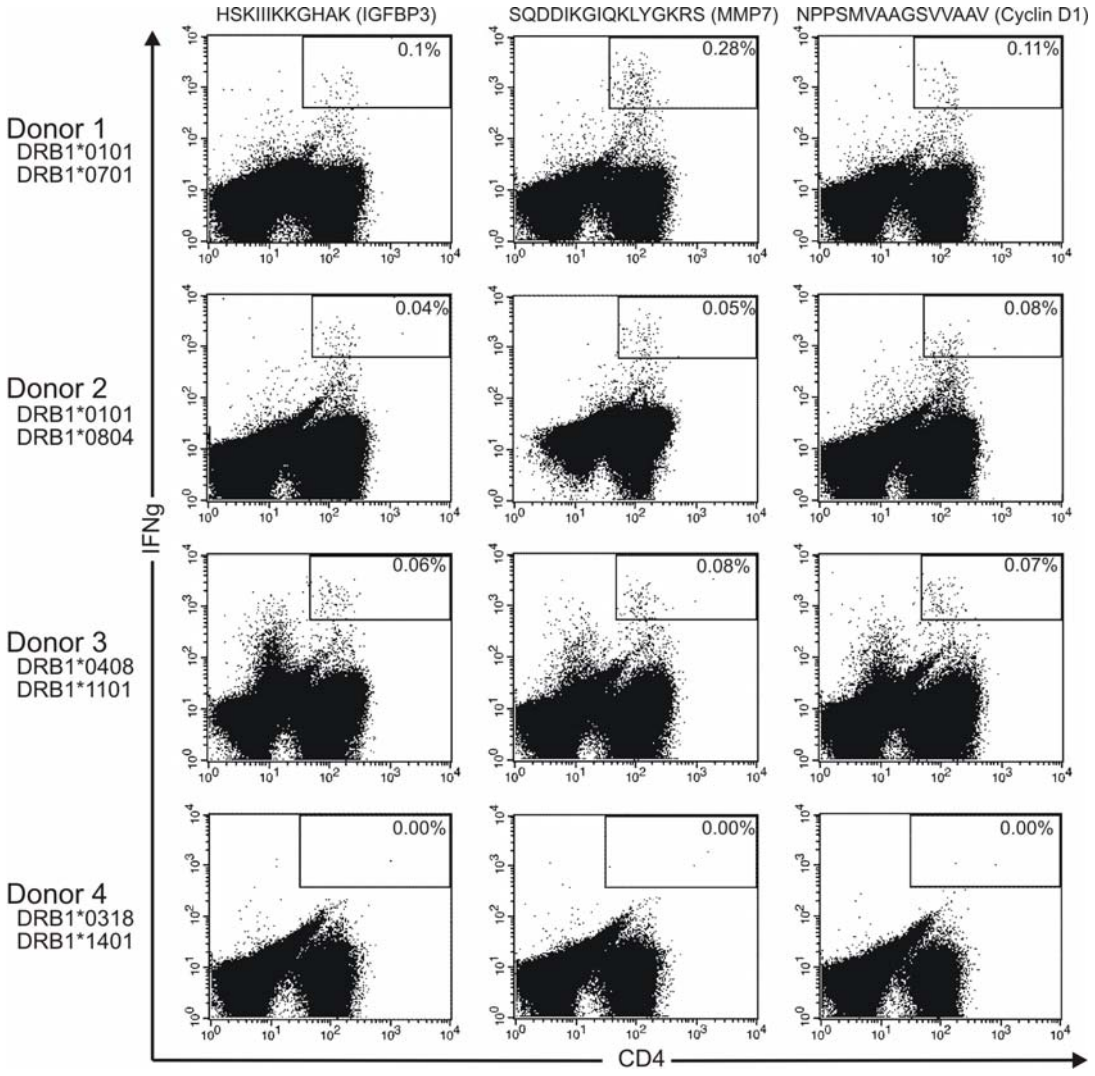

Figure 2: $CD4^+$ T cells specific for $IGFBP3_{169-181}$, $MMP7_{247-262}$.and $CCND1_{198-212}$. Shown are representative dot blots of intracellular IFNγ staining against CD4-FITC.

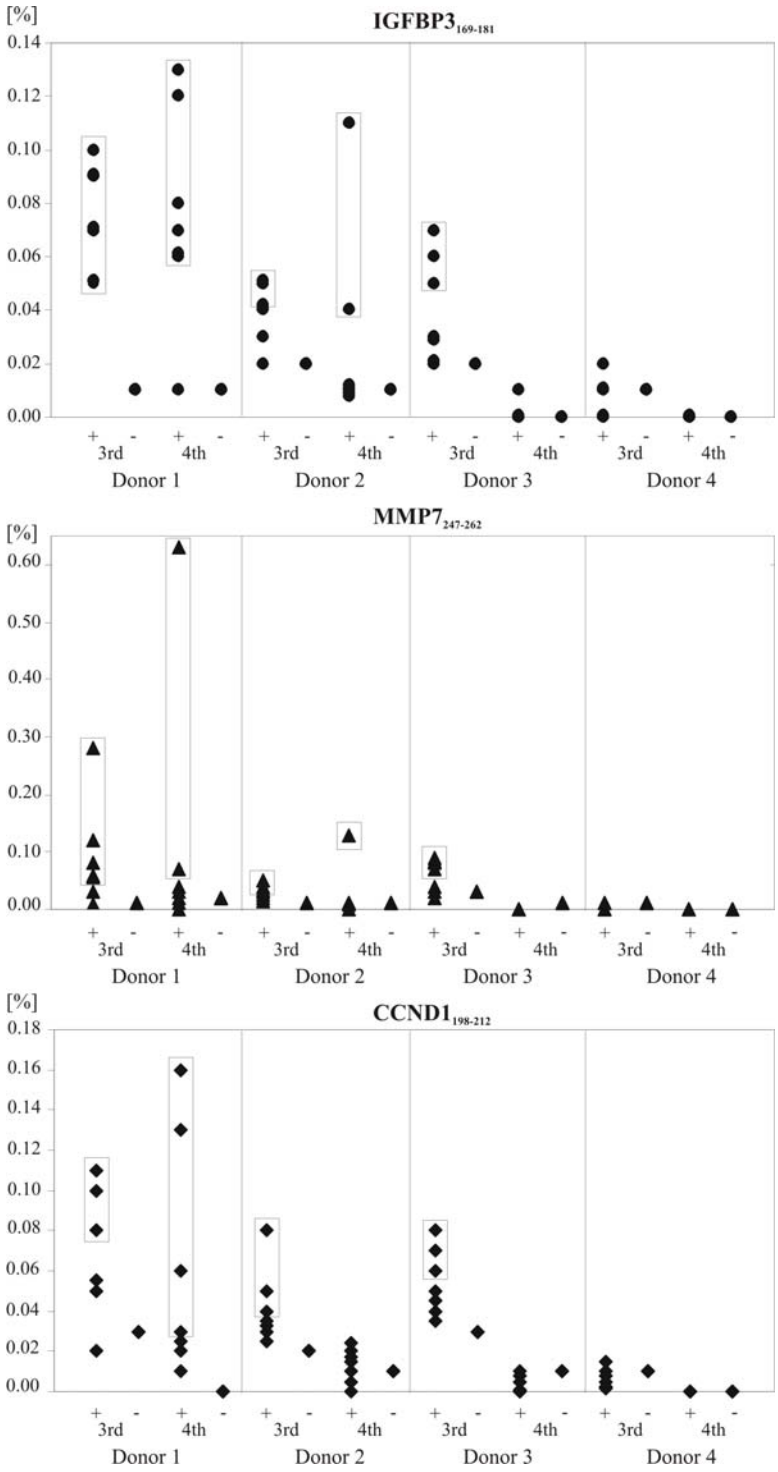

Figure 3: Schematic illustration of antigen-specific IFNγ producing CD4$^+$ T cells detected in each donor and for each peptide.

Shown is the percentage of IFNγ producing CD4$^+$ T cells for each donor and peptide used for stimulation. Cells were incubated in 96-well plates – 7 wells per donor and per peptide. Boxed are values considered as positive: percentage of IFNγ producing CD4$^+$ T cells was more than two-fold higher compared with negative control without peptide. Percentages of IFNγ producing CD4$^+$ T cells detected after stimulation with irrelevant peptide correlated with values after stimulation without peptide, with the exception of Donor 1 after the 3rd stimulation with IGFBP3$_{169-181}$. However, this effect was not seen anymore after the 4th stimulation.

Non-CD4 responses

Interestingly, IFNγ-producing $CD8^+$ T cells were detected in two donors after stimulations with MHC class II peptides, in particular in donor 3, but also to a lesser extent in donor 1 (Figure 4). These observations suggest the presence of CD8 T cell responses directed against shorter class I epitopes included in the long class II peptides, probably after *in vitro* processing. MHC class I predictions were performed using the program SYFPEITHI [41]. Potential CD8 T cell epitopes are listed in Table 4.

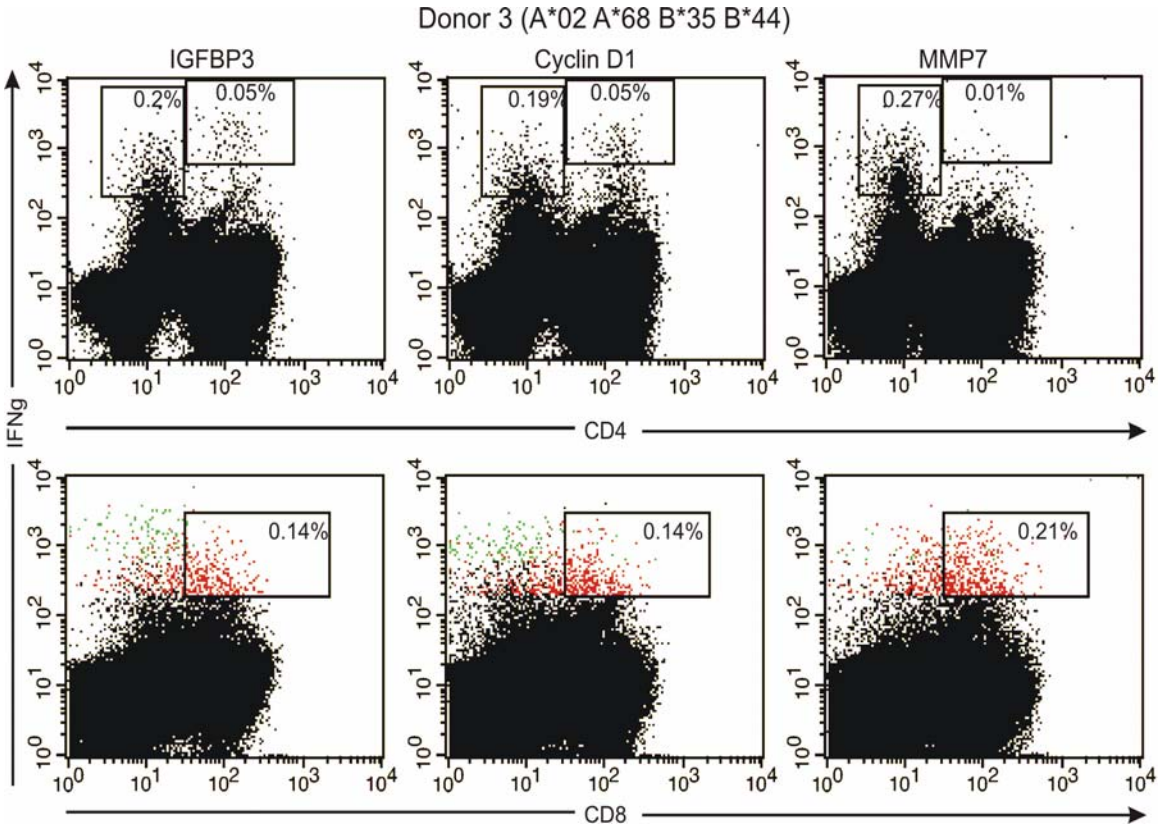

Figure 4. $CD4^+$ and $CD8^+$ T cells specific for IGFBP3, CCND1 and, MMP7. Shown are representative dot blots (one of seven) of intracellular IFNγ staining against CD4-FITC and CD8-PerCP. Non-CD4 IFNγ-reponses were gated and are visualized in red. IFNγ-responses of $CD4^+$ T cells are highlighted in green.

Table 3. Non-CD4 reactions to 15-20mer peptides and potential class I epitopes.

Peptide sequence	Protein/position	(potential) MHC class I restriction	score
	IGFBP3		
HSKIIIIKKGHAKDSQ			
IIIKKGHAK		A*0301	28
IIIKKGHAK		A*1101	19
IIIIKKGHA		A*0201	17
	CCND1		
NPPSMVAAGSVVAAV			
VAAGSVVAAV		A*0201	26
AAGSVVAAV		A*0201	23
AAGSVVAAV		B*5101	22
PPSMVAAGSV		B*0702	20
SMVAAGSVV		A*0201	20
AAGSVVAAV		B*0702	19
AGSVVAAV		B*5101	17
	MMP7		
SQDDIKGIQKLYGKRS			
SQDDIKGIQKLY		A*0101	29
QDDIKGIQKLY		A*0101	27
DIKGIQKL		B*0801	24
DIKGIQKLY		A*2601	24

3.2 $CD4^+$ T cells specific for a peptide derived from the tumor antigen survivin

Introduction

BIRC5, also known as survivin is a unique member of the inhibitor of apoptosis (IAP) protein family. In contrast to other IAPs, survivin is undetectable in normal adult tissues, yet it is abundantly expressed in fetal tissue and in a variety of human tumors including lung, colon, breast, prostate, pancreatic, and gastric carcinomas as well as in high-grade lymphomas and neuroblastomas [42-45]. Survivin is cell cycle regulated and involved in both the control of programmed cell death (apoptosis) and the regulation of cell division [46; 47]. As survivin is highly expressed in cancer tissues it may be a new target for tumor therapy such as peptide-based immunotherapy. For the indentification of MHC class II ligands from survivin for use in peptide-based immunotherapy, we predicted a 15mer peptide of the protein – TLGEFLKLDRERAKN, $survivin_{97\text{-}111.}$ The predicted peptide bound promiscuously to HLA-DR molecules as detected by IFNγ-producing $CD4^+$ T cells after *in vitro* stimulation. The identified peptide will be useful as candidate peptide for vaccination studies for tumor immunotherapy.

Material and Methods

Peptide

Peptide (TLGEFLKLDRERAKN, $survivin_{97\text{-}111}$) was predicted according to HLA-DR peptide motifs [http://www.syfpeithi.de] and synthesized by standard Fmoc chemistry using an Economy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany).

Maturation of DCs

All steps have already been described in chapter 3.1, part Material and Methods.

Generation of antigen-specific $CD4^+$ T cells

See chapter 3.1, Material and Methods.

Intracellular IFNγ staining

See chapter 3.1, Material and Methods.

Results and Discussion

As can be seen in Figure 1, IFNγ-producing $CD4^+$ T cells were detected after stimulation with $survivin_{97\text{-}111}$ in three of four donors, whereby all donors carried different HLA alleles (Table 1). Thus, the peptide from survivin is a promicuous binder being able to activate $CD4^+$ T cells from healthy donors. After the fourth round of stimulation only donor 1 showed $CD4^+$ activated T cells, maybe due to a suboptimal stimulation protocol. Hence, it was tested if fresh autologous PBMCs used for the fourth restimulation lead to a longer survival of the T cells stimulated. The use of fresh isolated PBMCs as antigen presenting cells extend T cell survival (data not shown).

The verification of novel MHC class II ligands from tumor-associated antigens, which were predicted by epitope prediction programs, is possible with *in vitro* stimulation protocols using peptide loaded DCs as antigen-presenting cells. The identified new MHC class II peptide may be useful in already established clinical vaccination protocols.

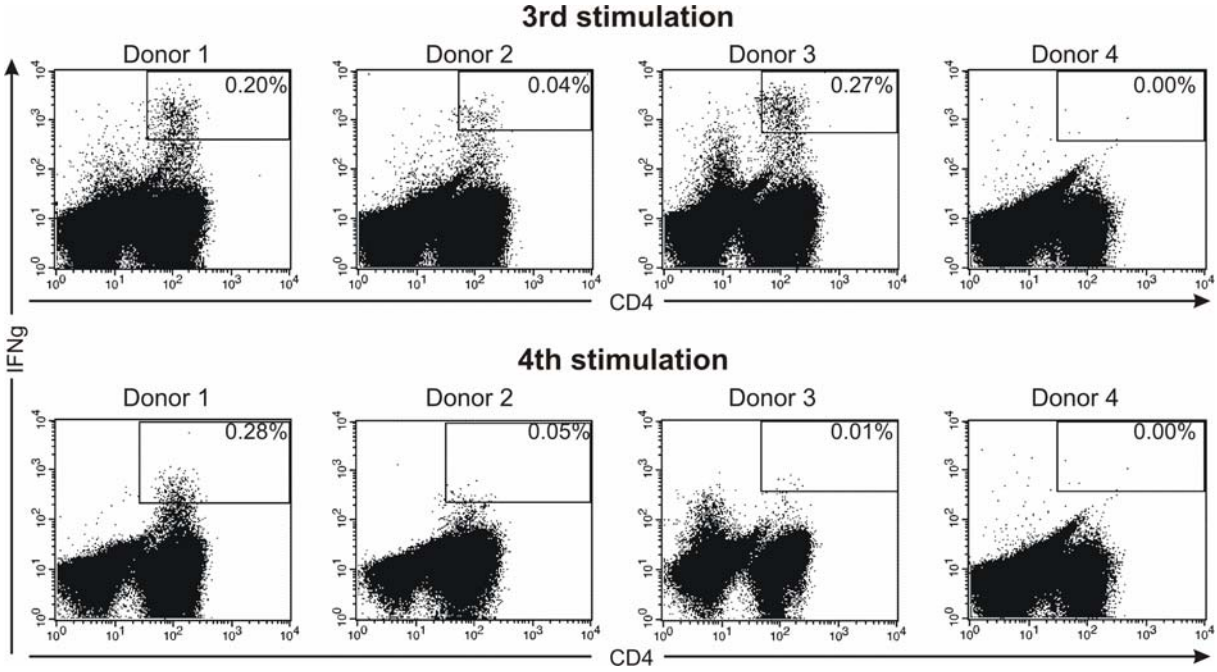

Figure 1. CD4+ T cells specific for survivin$_{97\text{-}111}$. Seven wells per donor were used for stimulation with survivin peptide. Shown are representative dot plots (one of seven) of intracellular IFNγ staining against CD4-FITC after the third and the fourth stimulation.

Table 1. HLA typings of donors tested

	MHC class II
Donor 1	DRB1*0701 DRB1*0101
Donor 2	DRB1*0101 DRB1*0804
Donor 3	DRB1*0408 DRB1*1101
Donor 4	DRB1*0318 DRB1*1401

Acknowledgements

Thanks to Patricia Hrstić for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 510 and 685 and Graduiertenkolleg 794), the NGFN2 (BMBF 0313311) and the Deutsche Krebshilfe (10-2189-St 2).

3.3 CD8 T cell responses to VEGF (vascular endothelial growth factor)

Introduction

VEGF is a potent and specific mitogen for vascular endothelial cells that is capable of stimulating angiogenesis during embryonic development but also during tumor formation. Moreover, VEGF is a key regulator of the process of angiogenesis, which makes it attractive as therapeutic target. Several clinical studies have demonstrated that cancer vaccines designed to target epitopes which are derived from tumor-associated antigens and recognized by cytotoxic T cells are able to raise immunological and clinical responses, e.g. in renal cell carcinoma (RCC), melanoma, prostate and breast cancer. However, these immunotherapeutical approaches are restricted by the limited number of known tumor antigens and epitopes, and HLA restrictions. Aim of this study was the investigation of immunogenicity of peptide SRFGGAVVR ($VEGF_{-114\ to\ -106}$) as a T-cell epitope by generation of specific $CD8^+$ T cells using *in vitro* priming with artificial antigen presenting cells.

Materials and Methods

Donors

HLA-B*2705 positive buffy coats were obtained from healthy blood bank donors. PBMCs were isolated using standard gradient separation (Lymphocyte Separation Medium, PAA Laboratories, Pasching, Austria).

Peptide, refolding, fluorescent tetramers and MHC coated microspheres

Peptides for MHC refoldings were synthesized using standard Fmoc chemistry. Peptides were SRFGGAVVR ($VEGF_{-114\ to\ -106}$) and NLVPMVATV (HCMV $pp65_{495-503}$). Biotinylated recombinant HLA-B*2705 or HLA-A*0201 molecules were produced as previously described [48; 49]. Monomers were fluorescence labeled by coincubating biotinylated HLA monomers with streptavidin-PE or streptavidin-APC (Molecular Probes, Leiden, The Netherlands) at a 4:1 molar ratio.

Microspheres were 5.60 µm diameter streptavidin coated polystyrene particles with a binding capacity of 0.064 µg biotin-FITC/mg microspheres (Bangs Laboratories, Fishers, Illinois, USA). Microspheres were washed and resuspended at 2 x 10^6/well in PBE [PBS (BioWhittaker/Cambrex Bio Science, Verviers, Belgium) supplemented with 0.5% protease-free BSA and 2 mM sodium EDTA (both Sigma-Aldrich, Taufkirchen, Germany)] containing biotinylated MHC in two different densities (high density (HD): 20 nM; low density (LD): 200 pM) and costimulatory antibodies anti human CD28 Ab 9.3 (5 nM) [50] and anti 4-1BBL Ab (15 nM (kindly provided by B.S. Kwon, Korea). Suspension was incubated by room temperature for 30 min while agitating. Coated beads were washed three times, resuspended in PBE and stored for up to 4 weeks at 4°C.

Antigen-specific *in vitro* stimulation of human $CD8^+$ T cells

CD8 T cells were magnetically enriched by negative depletion of PBMCs using biotinylated anti-CD8 Ab (5 µg/ml) and streptavidin-coated MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

In vitro stimulations were performed in 96-well plates with 1 x 10^6 sorted $CD8^+$ cells plus 2 x 10^5 coated beads per well in 200 µl T cell medium consisting of RPMI 1640 containing HEPES and L-glutamine (Gibco, Paisley, UK) supplemented with 10% heat-inactivated human serum (PAA, Cölbe, Germany), 50 U/ml penicillin, 50 µg/ml streptomycin and 20 µg/ml gentamicin (all BioWhittaker, Verviers, Belgium). 5 ng/ml human IL-12 p70 (R&D Systems, Wiesbaden, Germany) was added with beads. Per donor, 30 wells with high density loaded microspheres and 30 well with low density loaded beads were performed. After 3-4 days coincubation at 37°C, fresh medium and 80 U/ml human IL-2 (Chiron, Ratingen, Germany) was added and cells were further incubated at 37°C for 3-4 days. Stimulation was repeated twice before analysing cells by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany).

Tetrameric analysis

Cells were washed in PBEA (PBS containing 0.5% protease-free BSA, 2 mM sodium EDTA (both Sigma-Aldrich, Taufkirchen, Germany) and 0.01% sodium azide (Merck, Darmstadt, Germany) and stained with CD8-PerCP clone SK1 antibody (Becton Dickinson, Heidelberg, Germany) in the same buffer for 20 min at 4°C. 100 µg/ml

unlabeled streptavidine (Sigma-Aldrich, Taufkirchen, Germany) was included in same incubation period. Cells were washed in PFEA ((PBS containing 2% FCS (PAN Biotech, Aidenbach, Germany), 2 mM sodium EDTA (Sigma-Aldrich) and 0.01% sodium azide (Merck)) and tetramer stained at 4°C for 30 min in PFEA. Fluorescent MHC tetramers were always used at concentrations of 5 µg/ml. After washing, stained cells were resuspended in 1% paraformaldehyd (Merck) in PFEA. Cells were analysed on a four-colour FACSCalibur cytometer (Becton Dickinson).

Results and Discussion

For the generation of $VEGF_{-114\ to\ -106}$ specific cytotoxic T cells, enriched $CD8^+$ T cells were stimulated for three 7-9 day rounds in the presence of IL-12 with beads coated with costimulatory antibodies and HLA-B*2705 monomer or HLA-A*0201 monomer. As determined by tetramer analysis, stimulation with HLA-B*2705 monomer coated mircrospheres led to specific cytotoxic T cell generation with corresponding specificity (Figure 1, left panel). No staining was observed with an irrelevant tetramer (Figure 1, right panel). Stimulations with artificial antigen presenting cells were highly effective as 55-80% of high density stimulated wells and 30-50% of low density stimulated cells were positive. No antigen-specific T cells were detected after *ex vivo* staining of PBMCs, consequently one can say that detected antigen specific T cells resulted from efficient *in vitro* priming. Therefore, MHC coated microspheres are useful tools for *in vitro* priming and expansion of antigen specific T cells.

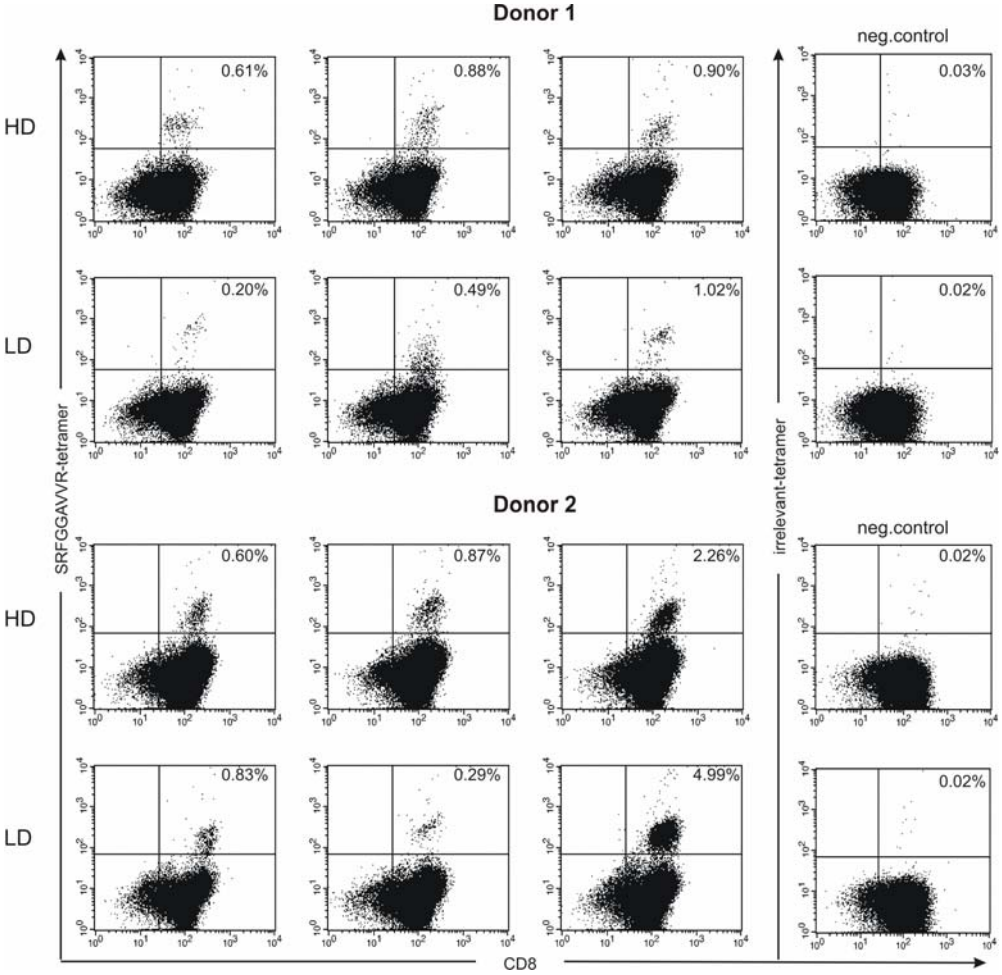

Figure 1. Representative tetrameric analyses of bead stimulated CD8$^+$ T cells. CD8$^+$ T cells of two HLA-B*2705 and HLA-A*0201 positive donors were stimulated 3 times with artificial antigen presenting cells. Per donor, 30 wells with high density loaded beads and 30 wells with low density loaded beads were performed. Cells were stained with CD8-PerCP Ab, SRFGGAVVR ($VEGF_{-114\ to\ -106}$) tetramer-PE or irrelevant NLVPMVATV (HCMV $pp65_{495-503}$) tetramer-APC (right panel). Percentage of tetramer$^+$ cells within CD8$^+$ T cell population is indicated in each plot.

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Summary

T cells are important effectors in the defense of human pathogens entering the organism. $CD8^+$ T cells recognize peptides which are presented by MHC class I molecules and lyse cells which are infected by virus or intracellular pathogens. Moreover, they are able to destroy cancer cells. $CD4^+$ T cells recognize peptides from exogenous proteins acquired by endocytosis or from internalized plasma membrane proteins which are presented on MHC class II. $CD4^+$ T cells play an important role in the defense of bacteria and activation of the humoral immune response.

The human cytomegalovirus (HCMV) is a cause of morbidity and mortality in immunocompromised persons. In contrast, in immunocompetent individuals HCMV is successfully controlled by specific $CD8^+$ and $CD4^+$ T cell responses. One aim of this thesis was the analysis of $CD8^+$ and $CD4^+$ T cell responses against HCMV to identify T cell epitopes which could be used for the development of a vaccine against the virus. *Ex vivo* analyses were performed with blood of healthy donors of different HLA-types using recombinant peptide/MHC I complexes and/or intracellular cytokine staining to characterize T cell epitopes of HCMV. New T cell epitopes of several HLA restrictions could be identified. Furthermore, some were analysed with respect to immunodominant features and hierarchies among T cell epitopes identified. Furthermore, *in vitro* experiments were used to identify new T cell epitopes, e.g. stimulation of PBMCs from healthy donors with peptide loaded dendritic cells (DCs). Knowledge of T cell epitopes and their immundominant features can be useful for the generation of antigen-specific T cells for adoptive immunotherapy or for the development of a peptide-based vaccine against HCMV.

A further aim of this thesis was the induction and analysis of $CD8^+$ and $CD4^+$ T cell responses against tumor-associated antigens. MHC class II ligands identified by mass spectrometric methods were used to perform *in vitro* stimulations. For $CD8^+$ T cell stimulation, artificial antigen presenting cells (aAPCs) were used, for generation of antigen specific $CD4^+$ T cells, DCs were loaded with peptides and used as APCs. Several new helper T cell epitopes from different tumor-associated antigens were identified.

Zusammenfassung

Zur Abwehr humanpathogener Organismen sind T-Zellen unerlässlich. $CD8^+$ T-Zellen erkennen Peptide, die auf MHC Klasse I präsentiert werden und stellen Abwehrreaktionen gegen Viren und intrazelluläre Erreger, aber auch gegen Krebs, bereit. $CD4^+$ T-Zellen erkennen MHC Klasse II-präsentierte Peptide, die aus dem Inneren von Vesikeln stammen und spielen somit eine Rolle bei der Abwehr von Bakterien, aber auch bei der Anregung des humoralen Immunsystems über Aktivierung von B-Zellen.

Das humane Cytomegalievirus (HCMV) verursacht bei Patienten mit geschädigtem oder nicht voll ausgebildetem Immunsystem häufig schwere bis tödlich verlaufende Infektionen. Bei immunkompetenten Individuen hingegen wird das Virus erfolgreich durch spezifische $CD8^+$ und $CD4^+$ T-Zellen kontrolliert. Ein Ziel dieser Arbeit war es, $CD8^+$ und $CD4^+$ T-Zellantworten gegen das humane Cytomegalievirus (HCMV) zu untersuchen, um T-Zellepitope für mögliche Impfstoffe zu identifizieren. Zur Charakterisierung von T-Zellepitopen aus HCMV wurden zum einen *ex vivo*-Analysen in gesunden Spendern verschiedener HLA-Typisierung unter Verwendung rekombinanter Peptid/MHC I-Komplexe oder intrazellulärer Zytokinfärbung durchgeführt. Neue T-Zellepitope zahlreicher HLA-Restriktionen konnten identifiziert werden. Einige wurden zusätzlich hinsichtlich ihrer Immundominanz oder Hierarchie untereinander beleuchtet. Auch *in vitro*-Experimente, beipielsweise die Stimulation von gesunden Spender-PBMCs mit Peptid-beladenen dendritischen Zellen (DCs), trugen zur Identifizierung neuer T-Zellepitope bei. Dieses Wissen um T-Zellepitope und ihre immundominanten Eigenschaften kann nützlich sein für die Generierung antigen-spezifischer T-Zellen für adoptive Immuntherapien oder für die Entwicklung einer Peptid-basierenden HCMV-Vakzine.

Neben viralen T-Zellantworten war ebenso die Induktion und Analyse von $CD8^+$ und $CD4^+$ T-Zellantworten gegen tumorassoziierte Antigene Bestandteil dieser Arbeit. Mittels massenspektrometrischer Verfahren identifizierte MHC Klasse II-Liganden wurden für *in vitro* Stimulationen eingesetzt. Artifizielle Antigen-präsentierende Zellen wurden als Werkzeuge für die Stimulation von Antigen-spezifischen $CD8^+$ T-Zellen genutzt, während $CD4^+$ T-Zellen mittels Peptid-beladener dendritischer Zellen stimuliert wurden. Dies führte zur Identifizierung neuer T-Zellepitope aus verschiedenen Tumor-assoziierten Antigenen.

Abbrevations

aAPC	artificial antigen presenting cell
Ab	antibody
APC	allophycocyanin
APC	antigen presenting cell
BIRC5	Baculoviral IAP repeat-containing 5
BrdU	5-bromo-2´deoxyuridine
β2m	beta-2-microglobulin
BSA	bovine serum albumin
CCA	colon carcinoma
CCND1	Cyclin D1
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate, succinimidyl ester
Cr	chromium
CRA	^{51}chromium release assay
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
EBV	epstein barr virus
EDTA	ethylendiaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ELISpot	enzyme linked immunospot assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFP	green fluorescence protein
HCMV	human cytomegalovirus
HD	high density
HEPES	4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonacid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	interferon
Ig	immunglobulin
IGFBP3	insulin-like growth factor binding protein 3
IL	interleukin
LCL	lymphoblastoid cell line
MACS	magnetically activated cell sorting
MHC	major histocompatibility complex
MMP7	matrix metallo proteinase 7
NK	natural killer cell
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PHA	phytohaemagglutinin
PMA	phorbol myristate acetate
qRT-PCR	quantitative reverse transcriptase-PCR
RCC	renal cell carcinoma
RNA	ribonucleic acid
RPMI	roswell park memorial institute
RT	room temperature
TAA	tumor associated antigen
TAP	transporter associated with antigen processing
TCR	t cell receptor
TEMRA	$CD45^+$ effector memory T cells
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

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(submitted)

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Unexpected abundance of HLA class II presented peptides in primary renal cell carcinomas
(submitted)

[#] These authors contributed equally to this work.

Danksagung

Ein herzliches Dankeschön an Prof. Dr. Stefan Stevanović für die exzellente Betreuung meiner Doktorarbeit, das stets offene Ohr und die ständige Bereitschaft zur Diskussion. Im Besonderen möchte ich mich bedanken für die einzigartig freundliche Atmosphäre in seiner Arbeitsgruppe und die wunderbaren EPI-PEP-VAC-Touren.

Prof. Dr. Hans-Georg Rammensee danke ich sehr für sein stetiges Interesse an meiner Arbeit, den zahlreichen Denkanstößen und den obligatorischen Clubs, die mich sowohl im fachlichen Sinne sehr gefördert haben als auch im Vortragen vor Publikum.

Riesendank an Katharina Kreymborg und Oliver Schoor für eine wunderschöne gemeinsame Bürozeit und auch für zahlreiche Ratschläge sowohl fachlicher als auch privater Art. Ninchen, unserer „schlagender T-Zellorden“ im Kabuff wird mir unvergessen bleiben und ich freu mich auf die Neubegründung!

Natürlich möchte ich mich auch bei Despina Rudolf, Dominik Maurer und Mathias Schuler bedanken, die mir in den letzten Monaten treue Bürogefährten waren und wirklich für jeden Spaß zu haben waren.

Einfach Danke für alles an die gesamte Stevanović-Crew!

Großen Dank an alle Mitarbeiterinnen und Mitarbeiter der AG Rammensee für die stets sehr unkomplizierte Hilfsbereitschaft und den wissenschaftlichen Austausch und die täglich immer wieder auftauchenden leckeren Sachen im Sozialraum.

Ein ganz besonderes Dankeschön verdient Katrin, die mich nun schon seit dem ersten Tag des Studiums begleitet und zu den stärksten und fröhlichsten Persönlichkeiten gehört, die ich kenne. Ohne Dich hätte das Studium sicher nur halb soviel Spaß gemacht.

Ein großes Dankeschön an Stefan W. für die viele Hilfe nicht nur in Computerfragen.

Den größten Dank verdienen jedoch meine Eltern, auf deren Unterstützung ich stets zählen konnte und die mir all dies hier ermöglicht haben. Basti, dir danke ich für viele wertvolle Tipps und ein immer offenes Ohr.

Curriculum Vitae

Maria-Dorothea Nastke

Geburtsdatum: 29.Dezember 1978

Familienstand: ledig

Staatsangehörigkeit: deutsch

Schulische Laufbahn

1985 - 1991	Grundschule Otto Nagel, Bergholz-Rehbrücke
1991 - 1998	Wolkenberg Gymnasium, Michendorf b. Potsdam
Juni 1998	Abitur

Studium - Biologie

Sept. 1998 – Sept. 2000	**Grundstudium der Biologie** Universität Gießen
Sept. 2000 – Jan. 2003	**Hauptstudium der Biologie** Universität Tübingen
Jan. 2003	**Diplom** in Biologie, „sehr gut“ Fächer: Zellbiologie/Immunologie, Virologie, Parasitologie
Mai 2002	**Diplomarbeit** Universität Tübingen, Institut für Zellbiologie, Abteilung Immunologie Leitung: Prof. Dr. H.-G. Rammensee Arbeitsgruppe: Prof. Dr. S. Stevanović „Vergleich verschiedener Stimulations- und Nachweismethoden für zytotoxische T-Zellen“

Promotion

Jan. 2003 – Sept. 2005 **Doktorarbeit**
Universität Tübingen, Institut für Zellbiologie, Abteilung Immunologie

Leitung: Prof. Dr. H.-G. Rammensee
Arbeitsgruppe: Prof. Dr. S. Stevanović
„T-Zellepitope von viralen und tumor-assoziierten Antigenen: Induktion und Analyse von antigen-spezifischen T Zellen”