Efficient *in vitro* priming of tumor- and virus-specific $CD8^+$ T cells with calibrated artifical APCs

Effizientes *in vitro priming* von tumor- und virusspezifischen $CD8^+$ T-Zellen mit kalibrierten künstlichen antigenpräsentierenden Zellen

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Meinen Eltern und meinem Bruder Ronald

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1 General Introduction

1.1 The immune system

Immunity (derived from immunitas: Latin for exemption from civic duties and prosecution) means protection from disease and especially infectious disease. Cells and molecules involved in such protection constitute the immune system and the response to the introduction of a foreign agent is known as the immune response. Not all immune responses protect from disease; some foreign agents, such as the allergens found in house dust mite, cat dander or rye grass pollen, cause disease as a consequence of inducing an immune response. Constantly dealing with natural enemies 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, or tumor development (Figure 1.1). Likewise some individuals mount immune responses to their own tissues as if they were foreign agents. Thus, the immune response can cause the autoimmune diseases common to man such as multiple sclerosis, diabetes, rheumatoid arthritis or myasthenia gravis. Most individuals do not suffer from autoimmune diseases because they have developed tolerance towards their own (self) tissues. We are constantly being exposed to infectious agents and yet, in most cases, we are able to resist these infections. It is our immune system that enables us to resist infections. The immune system is composed of two major subdivisions, the innate immune system and the adaptive immune system. 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), $\gamma\delta$ T cells, B-1B cells and the complement system. In addition, the innate immune system also has anatomical features that function as barriers to infection. Although immune system consits of tow arms which have distinct functions, there is interplay between these systems (i.e., components of the innate immune system influence the adaptive immune system and *vice versa*).

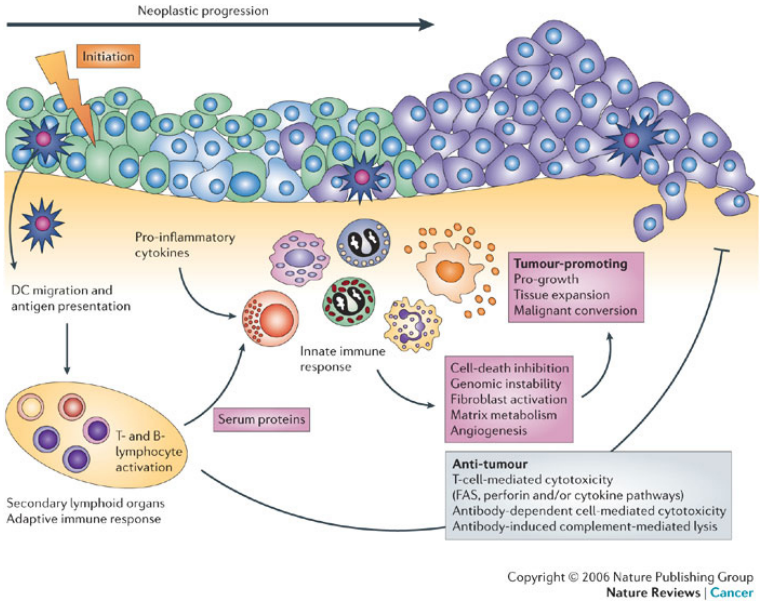

Figure 1.1: **A model of innate and adaptive immune-cell function during inflammation-associated cancer development.**
Antigens that are present in early neoplastic tissues are transported to lymphoid organs by dendritic cells (DCs) that activate adaptive immune responses resulting in both tumor-promoting and antitumor effects. The pathways that regulate DC trafficking during early cancer development and the exact nature of the antigen(s) remains to be established. Activation of B cells and humoral immune responses results in chronic activation of innate immune cells in neoplastic tissues. Activated innate immune cells, such as mast cells, granulocytes and macrophages, promote tumor development by the release of potent pro-survival soluble molecules that modulate gene expression programs in initiated neoplastic cells, culminating in altered cell-cycle progression and increased survival. Inflammatory cells positively influence tissue remodelling and development of the angiogenic vasculature by production of pro-angiogenic mediators and extracellular proteases. Tissues in which these pathways are chronically engaged exhibit an increased risk of tumor development. By contrast, activation of adaptive immunity also elicits antitumor responses through T cell-mediated toxicity (by induction of FAS, perforin and/or cytokine pathways) in addition to antibody-dependent cell-mediated cytotoxicity and antibody-induced complement-mediated lysis.

During evolution, the innate immune system evolved much earlier than the adaptive immune system. However, as the receptors involved in innate immune sytem are of restricted diversity, it is not as flexible as the adaptive immune system. Additionaly, it can not generate an immunological memory. Adaptive immunity also known as acquired

immune response is based on clonal selection of antigen-specific effector lymphocytes and on the generation of memory cells to prevent reinfection [1, 2]. The adaptive immune system requires some time to react to an invading organism, whereas the innate immune system includes defenses that, for the most part, are constitutively present and ready to be mobilized upon infection. Using receptors whose specificities do not have to be genetically encoded, the adaptive immune sytsem is able to respond to high numbers of foreign antigens. The adaptive immune response is characterized by the humoral immunity (latin: humor - moisture, fluid) and the cellular immunity. Humoral immunity can protect against extracellular antigens and toxins supplying antibodies which are secreted by B lymphocytes activated by antigen-specific T helper cells (TH2). Intracellular pathogens (viruses, intracellular bacteria) were defended by the cellular immunity as well as tumors. Cellular immunity is mainly based on cytotoxic T lymphocytes (CTLs) and inflammatory TH1 cells. For activation of CTLs interactions between professional antigen-presenting cells (APCs), most notably dendritic cells (DCs) and TH1 cells are necessary. 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 take place between innate and adaptive immunity.

1.2 Major histocompatibility complex

1.2.1 MHC molecules

The Major Histocompatibility complex (MHC) contains at least 128 functional genes, more than 20% of which have functions in immunity, and is the most gene-dense region of the human genome. The Major Histocompatibility Complex (MHC) is a set of molecules displayed on cell surfaces that are responsible for lymphocyte recognition and antigen presentation. In humans it is called human leukocyte antigens (HLA). The MHC molecules control the immune response through recognition of "self" and "non-self" and, consequently, serve as targets in transplantation rejection. The class I and class II MHC molecules belong to a group of molecules known as the Immunoglobulin Supergene Family, which includes immunoglobulins, T cell receptors, CD4, CD8, and others. The Major Histocompatibility Complex is encoded by a large, highly polymorphic gene cluster, located on human chromosome 6 (Figure 1.2).

This region is usually divided into class I, II and III antigenic region.

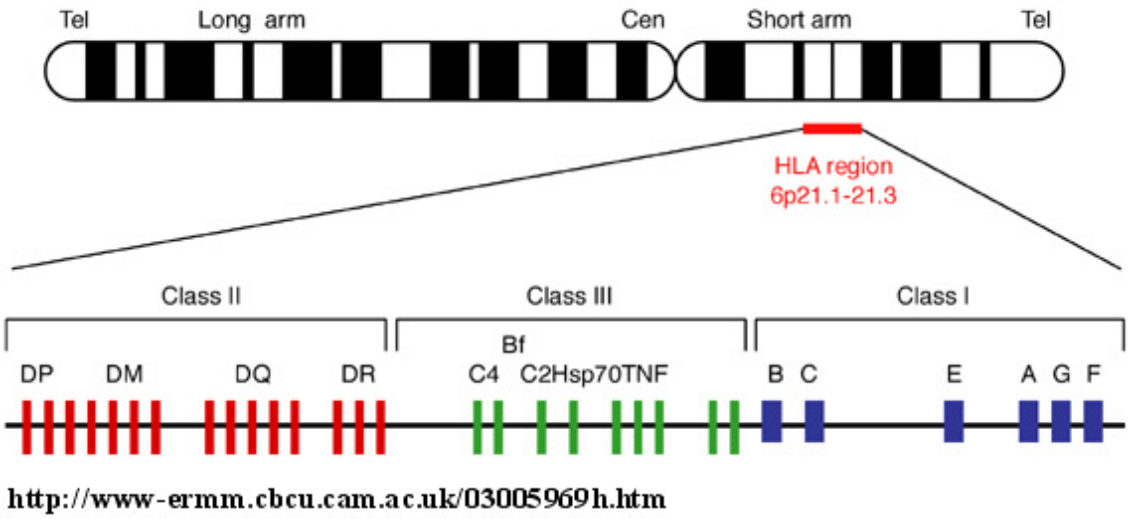

Figure 1.2: **Gene map of the human leukocyte antigen (HLA) region.** The HLA region spans 4 x 10^6 nucleotides on chromosome 6p21.1 to p21.3, with class II, class III and class I genes located from the centromeric (Cen) to the telomeric (Tel) end. HLA class I molecules restrict $CD8^+$ cytotoxic T lymphocyte function and mediate immune responses against 'endogenous' antigens and virally infected targets, whereas HLA class II molecules are involved in the presentation of 'exogenous' antigens to T helper cells. The HLA class III region contains many genes encoding proteins that are unrelated to cell-mediated immunity but that nevertheless modulate or regulate immune responses in some way, including tumor necrosis factor (TNF), heat shock proteins (Hsps) and complement proteins (C2, C4)

There are two major classes of MHC molecules, both of which consist of an α and a β chain, but from different sources. MHC class I molecules (MHC I) consist of one membrane-spanning α chain (heavy chain) produced by MHC genes which is non-covalently linked to one β chain (light chain or β2-microglobulin (β2m)) produced by the β2-microglobulin gene. In humans there are three different heavy chains: HLA-A, HLA-B and -C. The α chain has a short cytoplasmic C-terminal part, a transmembrane domain and three extracellular domains (α1-α3). The two N-terminal domains α1-α2 form a peptide binding groove which is closed at both ends and accommodates the presented peptide with a length of 8-10 amino acids. Whereas MHC class II molecules (MHC II) consist of two membrane-spanning chains, α and β, of similar size and both

produced by MHC genes. Class II molecules in humans are divided into HLA-DR,-DQ and DP. 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. Many proteins are present in more than one genetic variant in human populations: the differences between such proteins are known as polymorphisms, and most polymorphic proteins are found in two or three variant forms. The classical HLA class I molecules are present in almost 2000 different variants (Table 1.1). Polymorphism on this scale is unknown in any other protein and is believed to reflect selection for resistance to disease under pressure from highly mutable pathogens. There are a large number of genetic variants (alleles) at each genetic locus. Crucially many of these alleles are represented at significant frequency ($>$ 1%) in the population, and in addition the alleles generally differ from one another by many (up to 30) amino acid substitutions. Thus the evolution of diversity in peptide binding is driven by the diversity and mutability of the infectious agents that threaten the survival of animals.

Table 1.1: Number of known HLA alleles as of January 2008 (data from the Anthony Nolan Trust, HLA informatics group (http://www.anthonynolan.com/HIG)

Gene locus	Number of alleles
HLA-A	630
HLA-B	979
HLA-C	338

1.3 Antigen processing

1.3.1 Mechanisms of MHC class I restricted antigen processing

MHC class I molecules present peptides from cytosolic proteins [3]. The peptides are generated by exo- and endoproeolytic events during the physiologocal protein turnover which leads to different lenght variants. A large multienzyme complex called ubiquitin-proteasome generates C-termini of T cell epitopes. Proteasomes are multicatalytic and multi-subunit enzyme complexes which represent approx. 1% of the total cell protein and occur as the major proteolytic component in the nucleus and cytosol of all eukaryotic cells. The essential function of proteasomes is the proteolysis of misfolded or

nonfunctional proteins or of usually regulatory proteins designed for rapid degradation. Another function of proteasomal degradation of a multiplicity of cellular or viral proteins is the generation of peptide ligands for major histocompatibility (MHC) class I molecules which are required for T cell-mediated immune response. Proteasome targets are usually marked for proteasomal degradation by attachment of oligomeric forms of ubiquitin (Ub). Ub is a highly conserved protein of 76 amino acids in length, which is covalently coupled to target proteins. The conjugation of Ub molecules results in the formation of "poly-Ub chains". In general, multimers of four Ub molecules are required in order to function as a signal for degradation by the proteasome. Ubiquitination itself is reversible, and Ub molecules can be removed again from the target molecule by a multitude of Ub hydrolases. The 26S proteasome is a 2.5 megadalton (MDa) multienzyme complex which consists of approximately 31 subunits [4]. The proteolytic activity of the proteasome complex is provided by a core structure, the 20S proteasome. The 20S proteasome forms a complicated multienzyme complex consisting of 14 nonidentical proteins, which is arranged in two alpha and two beta rings in an .alpha.beta.beta.alpha. order [4]. The substrate specificity of the 20S proteasome comprises three essential proteolytic activities: a chymotrypsin-like (cleavage after hydrophobic residues), a trypsin-like (cleavage after basic residues) and a caspase-like (cleavage after acidic residues) [5] which are located in the beta subunits δ/Y, Z , and X. The enzymic activities of the 20S proteasome are controlled by attachment of the 19S regulatory subunits which together form the active 26S proteasome particle. The 19S regulatory subunits are involved in the recognition of polyubiquitinated proteins and in the unfolding of target proteins. The 26S proteasome activity is ATP-dependent and degrades almost exclusively only polyubiquitinated proteins. The catalytically active beta subunits of the 20S proteasome (δ/Y, Z ,X) may be replaced by γ-interferon-inducible MHC-encoded low molecular weight protein 2 (LMP2), MECL-1 and low molecular weight protein 7 (LMP7) which then form the "immuno proteasome" [6]. The immuno active-active form of the proteasome is able to enhance the generaton of peptides presented on MHC I. The peptides generated are transported into the ER using the transporter associated with antigen processing (TAP) [7]. In the ER MHC I heavy chain-β2m 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 [8].

The accumulated MHC I heavy chain, β2m and peptide complexes are then transported to the cell surface where they interact with $CD8^+$ T cells (Figure 1.3).

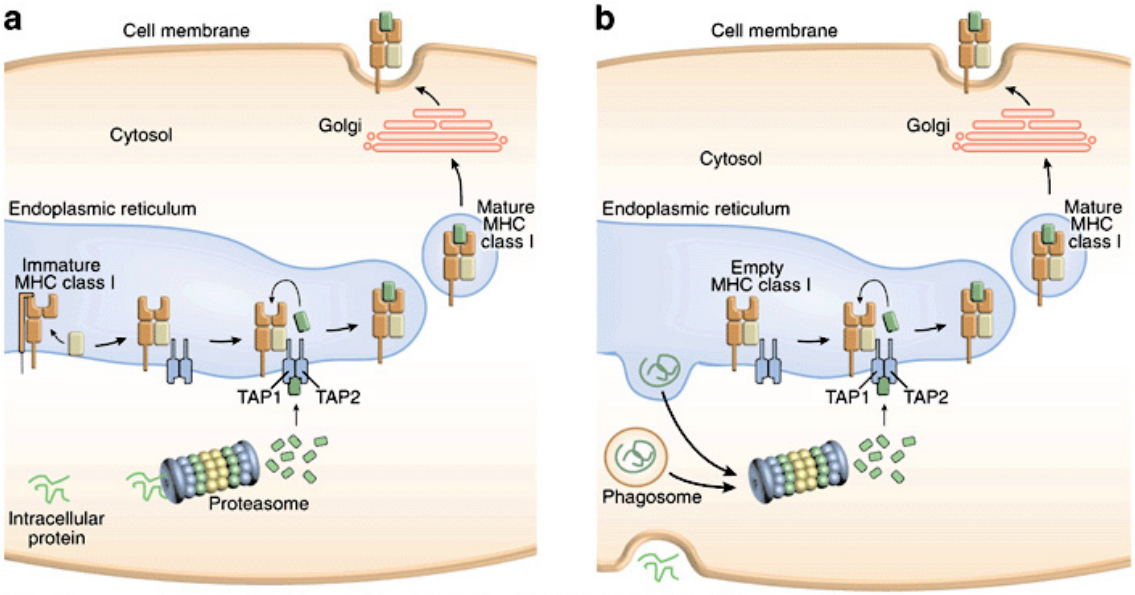

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Figure 1.3: **MHC class I antigen processing and antigen presentation pathways.** In general, MHC class I presented peptides are derived from intracellular proteins (a). These are degraded by the proteasome and transported through the transporter associated with antigen processing (TAP) in to the endoplasmic reticulum. There, newly synthesized MHC class I molecules are stabilized by calnexin until β2 microglobulin binds to the complex. The partial folded MHC class I complex binds to the TAP complex, and, after binding of peptide, the peptide/MHC complex is transported through the Golgi apparatus to the cell surface. Alternatively, exogenous proteins are phagocytosed, and endocytosed antigens may exit the endosomal pathway into the cytosol, either before or after processing, where they can enter the classical MHC class I presentation pathway (b). These proteins are retro-transported out of the endoplasmic reticulum and degraded by the proteasome. The degraded peptides can now enter the normal pathway through the TAP complex.

1.3.2 Mechanisms of MHC class II restricted antigen processing

MHC class II molecules present peptides from exogenous proteins acquired by endocytosis or from internalized plasma membrane proteins to $CD4^+$ T cells. Once an antigen is internalized, it is degraded into peptides within compartments of the endocytic

processing pathway. The endocytic pathway appears to involve increasingly acidic compartments: early endosomes (pH 6-6.5); late endolysosomes (pH 4.5-5). Internalized antigen moves from early to late endosomes, encountering hydrolytic enzymes and a lower pH. Lysosomes contain a unique collection of more than 40 acid dependent hydrolases: Within the compartments of the endocytic pathway, the antigen is degraded into oligopeptids of about 13-18 amino acid residues, which bind to class II MHC molecules [9]. MHC class II α- and β-chains are synthesized into the ER and transported in association with the trimeric invariant chain (li) to the MHC class II loading compartment. The invariant chain contains sorting signals in its cytoplasmic tail that directs the transport of the class II MHC complex from the trans-Golgi network to the endocytic compartments [10]. The class II MHC invariant chain complexes are transported from ER through Golgi complex and trans-Golgi network to the endosomes. As the proteolytic activity increases in endosomes the invariant chain is gradually degraded. However, a short fragment of the invariant chain termed CLIP (class II associated invariant chain peptide) remains bound to the class II molecule. CLIP physically occupies the peptide binding groove of the class II MHC molecule, but within the loading compartment it is replaced by the later presented peptide. Two nonclassical class II MHC molecules, called HLA-DM and HLA-DO play a role in the removal of CLIP and in the subsequent loading of class II molecules with antigenic peptides [11]. Summarizing, 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 1.3). 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 [12]. Intracellular proteins, in contrast, can be presented by MHC class II molecules [13]. It was shown that autophagy plays a role in the MHC class II restricted presentation of peptides from intracellular proteins [14]. Autophagy is the degradation of intracellular components in lysosomes and is therefore relevant in the endosomal/lysosomal degradation pathway.

1.4 T lymphocytes

Compared to the MHC complex, T cells are also very diverse in their phenotype, their function and in the target structures they recognize. Both $CD4^+$ and $CD8^+$ T cells can only recognize antigen when it is presented with a self MHC molecule on the membrane of an antigen presenting cell. This attribute is called self-MHC restriction. The self MHC restriction of $CD8^+$ T cells was first demonstrated by R. Zinkernagel and P. Doherty in 1974 [15]. In their experiments, mice were immunized with lymphocytic choriomeningitis (LCM) virus; several days later, the animals´ spleen cells, which included T cells killed only syngeneic virus infected target cells. Early in development, T lymphocytes are able to differentiate in T cells expressing $\alpha\beta$-T cell receptors ($\alpha\beta$-TCR) or $\gamma\delta$-T cell receptors. $\gamma\delta$-T cells are supposed to recognize heat-shock proteins and non-peptidic antigens such as sphingolipids bound to nonclassical MHC molecules such as CD1 and, additionally, might have regulatory effects. $\alpha\beta$ T cells (NK T cells, $CD8^+$ T cells or $CD4^+$ T cells) recognize processed antigen presented MHC molecules on the surface of cells. The decision whether to become a $CD8^+$ T cell or a $CD4^+$ T cell is made during the positive selection within the thymus. 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 with TCR which neither binds to MHC class I nor MHC class II undergo apoptosis. Negative selection removes T cells expressing a TCR which recognizes self-peptides to prevent self-destruction. Anyhow, several antigens presented in tissues or organs are not present in the thymus. Such self-reactive T cells recognizing peptides from those antigens become anergic in the periphery, because these T cells lack additional signals needed during the first antigen contact. All T cells passed positive and negative selection in the thymus can distinguish between self and non-self or malignant antigens and are named naive T cells which circle through the body via the blood and lymph streams. Contact between a professional APC, such as dendritic cells, B cells or macrophages, via MHC/peptide complex and a costimulatory second signal leads to the activation of the T cell, a process called priming. After this priming process 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 [16] or Fas-ligand interaction [18; 19] (Figure 1.4). TH1 cells secrete IFNγ and interleukin 2 (IL-2) [17] leading to activation of macrophages and $CD8^+$ T cells. TH2 cells support a humoral immune response by secretion of IL-4 [18] 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 [19]. These cells were named regulatory T cells (Treg). They are able to inhibit T cell reactions, a process which is not completely understood until now.

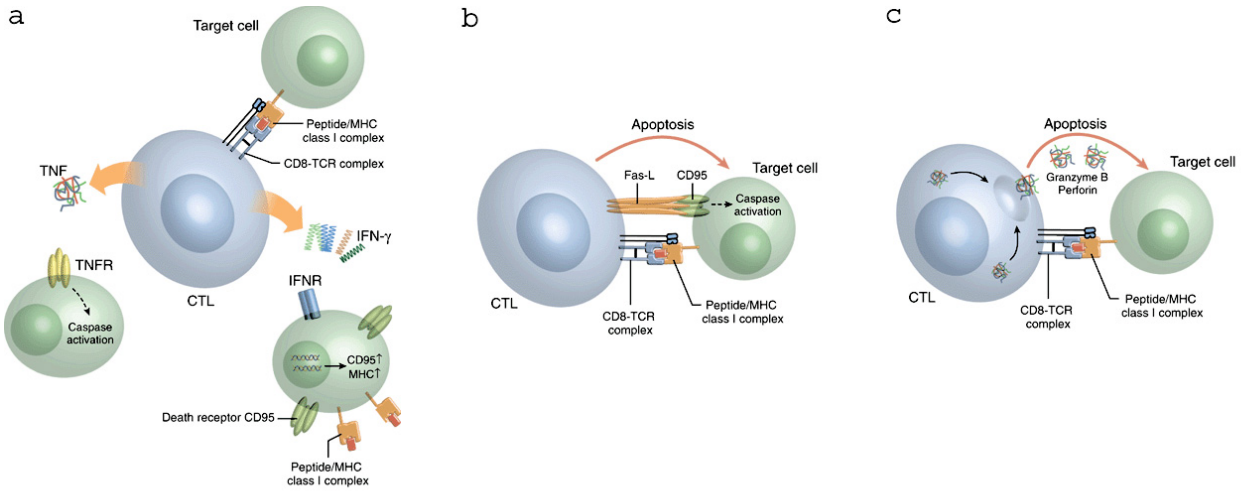

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Figure 1.4: **CTL-mediated cytotoxicity.**
(a) Indirect killing of target cells by release of tumor necrosis factor-alpha and IFN-gamma. (b) Induction of apoptosis in target cells via death receptor triggering. (c) Direct killing by release of granzyme B and perforin into the intercellular space between CTL and target cell. TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; IFNR, IFN receptor; Fas-L, Fas ligand.

1.5 Immunotherapy in cancer

1.5.1 Tumor associated antigens

The existence of structures distinguishing tumors from normal self is the indispensable basis for cancer immunotherapy. Nearly all possibilities for tumor associated antigens one can think about have been found. This is not surprisingly if one concerns, that every

alteration occuring during tumorigenesis at the protein level can potentially cause recognition by T cells, provided that the altered protein can be processed and presented by MHC molecules. Table 1.2 shows a classification of tumor rejection antigens (*adapted from Rammensee et al.) [20]. Knowledge about which of such tumor-associated antigens (TAAs) are expressed by the specific tumor, how they may arise, and what the advantages and restrictions are for different types of TAAs, is an important condition for the design of suitable therapeutic approaches. Despite the identification of a considerable number of TAAs, the number of identified TAAs is still far away from beeing sufficient for substantial immunotherapeutical approaches. One reason therefore is, that the so far known TAAs are restricted to very few cancer species and HLA alleles and this leads to the question how many percent of all patients fit to the vaccine.

Table 1.2: classes from 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]	+	+

1.5.2 Clinical trials

Most cancer patients are treated with some combination of surgery, radiation, and chemotherapy. Radiation and chemotherapy have the disadvantage of destroying healthy as well as malignant cells. Patients often suffer from severe toxicity and lack of specificity of the therapy towards tumor cells. New therapeutic concepts are being developed in addition to the standard therapy. The number of different types of vaccines used in clinical trials until now is immense. Several studies verified that the immune system can be manipulated to specifically recognize and eliminate tumor cells [21, 22]. During the last years, many different approaches have been used to activate the immune system towards the tumor. One of the most promising strategies deals with the immunization with peptides of proteins produced by the tumor in combination with adjuvants such as GM-CSF or keyhole-limpet hemocyanin (KLH) [23], Interleukin 2 [24], Montanide ISA-51 [25], tumor associated [26] or artificial MHC class II ligands [27]. Dendritic cells (DCs) also play an important role in activating the immune sytem, e.g. transfection of dendritic cells with total RNA [28] or RNA coding for defined tumor antigens [29], or DCs loaded with proteins [30, 31] or MHC class I peptides from tumor associated antigens [32]. Moreover, there are approaches using hybrid cells designed by fusion of tumor cells and professional antigen presenting cells [33, 34] irradiated allogenic [35] or autologous tumor cells [36] which are manipulated to become more immunogenetic. Recombinant viruses used as transporters for DNA coding for tumor rejection [37] or autologous tumor-derived heat shock proteins [38]. All vaccines made from autologous tumors have the advantage that the number of antigens is as high as the molecular changes within this tumor. Other advantages are that the vaccine can be applied independent of the HLA-type and may include T helper cell epitopes. But these advantages are faced by some serious drawbacks: in some cases, the amount of the vaccine is related to the tumor size, and therefore, limited with respect to the number of possible repeated vaccinations; 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 [39–41].

1.5.3 Adoptive transfer of Lymphocytes

Despite the attractiveness of active *in vivo* induction of anti-tumor T cell reactions, adoptive T cell transfer has so far been the more succesfull approach [42]. While allogenic T cell transfer has the advantage of mediating an associated graft-versus-tumor (GvT) effect by reactions against tumor minor histocompatibility antigens [43], this is accompanied by the risk of severe graft-versus-host disease (GvHD) in the patient [44]. Therefore, autologous approaches are often employed, such as the isolation of tumor-infiltrating lymphocytes (TILs) followed by an *in vitro* expansion of tumor-reactive TILs and reinfusion into the patient [45, 46]. 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 [47, 48]. Sources of such T cells varies from cells generated by several rounds of *ex vivo* stimulation to cells isolated from autologous tumor tissue [45, 49, 50]. Adoptive transfer not only plays a role in tumor regression but also in the protection of viral diseases such as HCMV or EBV. Adoptively transferred HCMV-specific CTL clones [51, 52] or T cell lines [53] have successfully protected patients at risk from HCMV disease. $CD4^+$ helper T cells also play an important role in orchestrating the effector function of anti-tumor T cell responses [54]. Therefore the identification of $CD4^+$ T cell epitopes derived from tumor-associated antigens has recently been a major focus of attention [55, 56]. Alltogether, due to their specificity directly coupled to potent effector functions, T cells are an attractive means for tumor immunotherapy.

1.6 Altered peptide ligands

Peptide vaccinations have some limitations due to the restriction of peptides to their specific HLA-type, the limited number of known TAA peptides, or the possibly short half-life of MHC:peptide complexes on DCs after loading, which may cause dissociation of a large proportion of MHC:peptide complexes already on the way to draining lymph nodes. The latter point has been addressed by various attempts to modify natural MHC ligands in order to increase their affinity to MHC proteins [57, 58]. The modified peptides are then so called altered peptide ligands(APLs); this can be achievd either through the modification of amino acid residues involved in MHC binding, or those that are predicted to contact the TCR [59, 60]. For instance, the immunogenicity of the human melanoma

peptides, Melan-A/MART-1 and gp100, and that of a prostate-specific antigen peptide has been improved by amino acid substitutions at anchor positions that increase peptide MHC class I binding affinity [57, 61, 62]. These peptides showed enhanced binding to the HLA-A2 molecule and enhanced stability of the peptide-MHC complex that results in improved *in vitro* T cell activation.

1.7 Analysis of antigen-specific T cell responses

1.7.1 Characterization of T cells recognizing tumor antigens

The usually low frequency of T cells specific for an antigen is a big problem for the analysis of antigen-specific T cell responses. The analysis of T cell responses under *in vitro* or *in vivo* conditions is a special case for "immune monitoring". The frequency of naive peripheral T cells against a given antigen has been estimated between 4 x 10^{-8} and 2 x 10^{-5} [63–65] but may increase during T cell responses up to 6 x 10 $^{-1}$ among $CD8^+$ T cells [66]. Any ideal T cell assay would be a direct assay covering this full dynamic range with high linearity. Furthermore, one has to think about different functionalities and pathways of activation between naive, effector, and memory T cells. One may divide T cell assays into functional versus specificity assays. 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. 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.

1.7.2 Functional T cell assays

Effector or memory T cells respond by exerting an "effect" after they got in contact with antigen presented on MHC complexes which are expressed by target cells. Such effects may be proliferation, cytokine expression, expression of activation markers, degranulation, target cell cytotoxicity and/or trogocytosis. 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 or 5-bromo-2´-deoxyuridine (BrdU) into newly generated DNA [67]. 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). Furthermore, it is possible to prelabel cells with a stable covalent dye, such as 5,6-carboxylfluorescein diacetate succinimidyl ester (CFSE) [68]. After each cell division, in response to antigen recognition, CFSE content will be approximately reduced by 50% and could be measured directly by flow cytometry. A variety of cytokine expression assay have been reported up to now for the detection of antigen-specific cells. All these assays can be performed specifically for different cytokines, depending on the expected functionality of the cells. Thereby, the most important cytokine for the detection of activated CD8$^+$ or CD4$^+$ T cell responses is IFN-γ. Bulk cytokine expression assays on a protein level are classic sandwich enzyme linked immunosorbent assays (ELISAs) [69] or similar protein detection assays such as the cytometric bead assay [70], which allow simultaneaius or "multiplexed" detection of several cytokines within one sample. On mRNA level, quantitative real time polymerase chain reaction (qRT-PCR) has been exploited as a sensitive method to detect antigen-specific T cells, 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 [71]. For single based cytokine assays, three methods exist:cytokine capture [72] assay, enzyme linked immunospot (ELISpot) assay [73, 74] and intracellular cytokine staining [75]. In cytokine caption assays, cells are labeled before hand with a "capture matrix", i.e. anti-cytokine antibodies non covalently coupled to the cell surface. After stimula-

tion, secreted cytokines are captured on the cell surface and can be later szained with fluorescently labeled second anti-cytokine antibody. This method can be combined with live cell sorting, this is due to the undestructive nature of this method. The ELISpot assay is nearly similar to ELISA but on a single-cell level. A nitrocellulose-bottomed microtiter plate is non covalently linked to 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. Upon stimulation recognition of the antigen leads to release of cytokine of interest, which is then bound by the antibody coated on the plate and forms small "invisible" spots on the membrane. Cytokine release is visualized after removal of the cells by an enzyme-linked second anti-cytokine 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. Colored spots can be counted by a computer system. The assay detects only T cells that are preactivated in vivo, since naive T cells do not secrete cytokines upon shortterm stimulation. Thus, the ELISpot assay is useful to measure number and functionality of antigen-specific T cells *ex vivo*. 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. Cytokines are usually rapidly secreted and diffuse away from the secreted cell. While performing intracellular cytokine staining, cells are incubated during antigen challenge with specific inhibitors (most often Monensin or Brefeldin A) that are able to block cellular secretory pathways and lead to an intracellular enrichment of the cytokine. 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 and fixation of the cells. The assay can be performed with PBMCs [76], whole blood [77, 78], lymph nodes, or other biologic fluids. Therefore, a total incubation period of 6 hours is optimal for achieving high levels of cytokine-secreting cells for IL-2, IL-4, IFN-γ, and TNF-α, as well as for achieving maximal cytokine staining intensity [77]. 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 [79]. 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 [80]. 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 [81] 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) [82, 83] 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 by a radiochemical compound, with $^{51}Cr(VI)O_4{}^{2-}$, 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 [84]. Labeled target cells are incubated with effector cells ($CD8^+$ T cells) for several hours. When cytotoxicity occurs, ^{51}Cr will be released from the target 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. Trogocytosis is an only recently described phenomenon of all lymphocytes. Within minutes surface material from the antigen presenting cell to the effector cell is transferred during formation of a biological synapse. But the mechanism and biological function of this effect are still not clear [175]. However, it has been already exploited to detect antigen presenting $CD8^+$ T cells [85, 86]. For auch an assay target cells have to be engineered by coupling green fluorescent protein (GFP) to the cognate HLA molecule. When pulsed with antigen and coincubated with HLA antigen-specific T cells, these aquire HLA-GFP from the target cell and become fluorescent and thus detectable by flow cytometry.

1.7.3 Specificity assays

Oppositional to their functionality, antigen-specific T cells can be detected solely by the presence of the cognate T cell receptor on their cell surface. For a quantification of antigen-specific T lymphocytes with regard to their specificity the analysis via labeled multimeric MHC:peptide complexes (also called tetramers) [87] 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 β2-microglobulin and a specific peptide ligand. Purified MHC/peptide monomers are multimerized via streptavidin, an avidin-like protein from *Streptomyces avidinii* which has four binding sites for biotin with very high affinity [88], to form soluble tetrameric complexes (Figure 1.5). MHC/peptide tetramers will bind T cells bearing MHC/peptide-specific T cell receptors and can be detected by flow cytometry (Figure 1.5). Moreover, phenotypic characterization of cells detected by tetramer staining is enabled by possible parallel detection of co-expressed cell surface antigens [89]. During the last years, multimeric MHC/peptide reagents have shed a new light on T cell responses, as they offered for the first time a window to the whole T cell response against a given T cell epitope, independent of function, but 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 [90–92] or concentration of tetramers. Moreover, MHC/peptide tetramers also interact with the CD8/CD4 co-receptor and may therefore influence binding of anti-CD8 antibodies [93, 94] 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 [95–97].

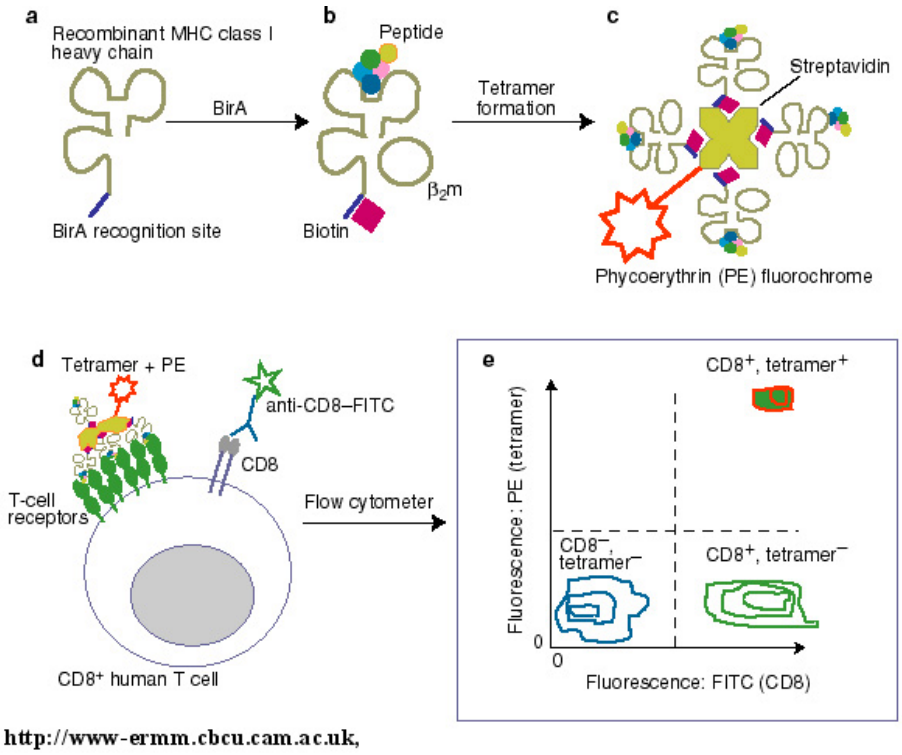

Figure 1.5: **Tetramer analysis to detect T lymphocytes (T cells) that have specific T cell receptors on their cell surface.**
(a) Soluble versions of the heavy chain of major histocompatibility complex (MHC) class I molecules are synthesized in *Escherichia coli bacteria*. (b) The molecules adopt an appropriate conformation following the addition of $\beta 2$ microglobulin ($\beta 2$m) and a synthetic peptide that represents the epitope that is recognized by the T cell receptor (TCR) of interest. This peptide is able to bind to the MHC molecule. In addition, the enzyme BirA is used to attach a biotin molecule to the specific BirA-recognition sequence, which has been incorporated into the carboxyl terminus of the MHC molecule. (c) Four MHC-biotin complexes are linked to a single streptavidin molecule, using the specific biotin-avidin interaction, to form a tetramer. The streptavidin molecule is 'tagged' with a fluorochrome (e.g. phycoerythrin; PE). (d) Tetramers are mixed with the cell population that is to be analyzed (e.g. total peripheral blood mononuclear cell (PBMC) populations or $CD8^+$ T lymphocytes (T cells)). Only T cells that have TCRs that are capable of binding to the particular MHC:peptide combination that is present in the tetramer are able to bind the tetramer; thus, such cells will become labelled with the PE fluorochrome (shown in red on the graph in (e)). A monoclonal antibody that is specific for a T cell marker and is tagged with a different fluorochrome (e.g. fluorescein isothiocyanate (FITC), shown in green) can also be used. (e) The cells are then analyzed using flow cytometry; the proportion of the $CD8^+$ T cell population that stains positively with the tetramer can be determined (top, right-hand quadrant)

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

Using antigen-specific T cells in adoptive immunotherapy requires injection of large amounts of previously amplified antigen-specific T cells. Classically, expansion of naive T cells is achieved by using *in vitro* derived professional antigen-specific cells, such as autologous monocyte derived dendritic cells [98] or autologues activated B lymphocytes [99]. It is important to separate T cells specific for an antigen of desire out of a whole T cell population, therefore it is possible to stimulate and amplify T cells either in an antigen-independent manner or by stimulation with specific antigen. Mixed T cell populations can be separated using fluorescence activated- or magnetic-affinity cell sorting (FACS/MACS) systems [87, 100–102] or populations can be cloned by limiting dilution [103]. Antigen independent amplification of T cells can be achieved using polyclonal mitogens or stimulatory antibodies, usually in combination with cytokines like IL-2 [104]. Several antibodies able to support T cell stimulation are known, e.g. anti-CD3 [104] or antibodies directed against costimulatory antigens such as CD28 [105], 4-1BB [106] or CD27 [107, 108], respectively. Polyclonal mitogens can be concanavalin A (Con A) [109] or phytohaemagglutinin (PHA) [110]. For stimulation and proliferation of antigen-specific T cells *in vitro* several approaches can be used. However, the use of autologous professional antigen presenting cells is hampered by the fact that current methods for theit isolation/generation are expensive, time-consuming, difficult to standardize and can only yield relatively low cell numbers. Different groups tried to overcome these limitations by using artificial antigen presenting cells (aAPCs), like allogeneic tumor cells, e.g. K562, which could be transfected with costimulatory and adhesion molecules, such as CD80, 4-1BBL, LFA-3 and ICAM-1 [111]. Possible effects of allostimulation are avoided by transfection of single peptide-HLA complexes into HLA-deficient cells [208]. A different approach relies on the use of isolated recombinat MHC/peptide complexes (monomers). These complexes are coated on a surface, which can be lipid vesicles[112], plastic microspheres [113, 114], or even HLA-deficient cell surfaces [102].

1.8 The Human Cytomegalovirus (HCMV)

1.8.1 Cytomegalovirus and the Immune system

The human cytomegalovirus (HCMV) is a member of the β-herpesvirus family 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-infected cells typically become enlarged (cytomegalia) [115] and eventually develop nuclear and cytoplasmic inclusions that are characteristic for HCMV infection. *In vivo* the virus targets a wide variety of cells including epithelial and endothelial cells, fibroblasts, smooth muscle cells, and peripheral blood leukocytes, which include monocytes and granulocytes [115]. Furthermore, there is intrauterine (congenital infection), perinatal (cervix secretions) and postnatal (breast milk) virus transmission, [116–119] which occurs also by saliva and sexual contact [120]. 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 [121] whereas the status in developing countries is much higher [121]. HCMV infection rarely causes symptomatic disease in healthy, immunocompetent individuals but manifests itself as a lifelong persistent 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 [122, 123]. Thus, the virus appears to be a major cause of morbidity and mortality in immunocompromised persons. The reactivation of HCMV in these patients leads to a variety of maladies such as HCMV-associated pneumonitis, retinitis pneumonia [124, 125] as well as gastro-intestinal erosions and ulcer [126–128]. Furthermore, severe aetiopathology are described in children infected intrauterine [129–131]. 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 [132].

After primary HCMV infection CMV IgM antibodies are produced before IgG antibodies

and persist for a few months. HCMV IgG antibodies are produced after secondary and primary infections and persist lifelong [133]. This persistence is the reason hat IgG antibodies are used as a sign that the patient had an HCMV infection in the past. Both innate and adaptive arms of the immune response are of importance for the control of HCMV infection. The importance of the humoral immunity is suggested by the clinical observation that without humoral immune response the patient will not clear the virus. 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 [134–136]. Another argument for a role of antibodies in the clearance of the virus is the effectiveness of prophylactic administration of CMV immunoglobulin in seronegative recipients of kidneys from seropositive donors in preventing morbidity and mortality associated with HCMV [137, 138]. The importance of the humoral immune response is also demonstrated by the finding that HCMV-specific antibodies reduce the generation of pp65 positive granulocytes by inhibiting uptake of pp65 by granulocytes from infected endothelial cells *in vitro* [139]. Nevertheless, antibodies are not typically sufficient to control HCMV infection. The cellular immune response by CD4 helper T lymphocytes, CD8 cytotoxic lymphocytes and Natural Killer (NK) cells are the most important adaptive immune components. [140–142]. The production of IFNγ by NK cells facilitates the expansion of antigen-specific TH cells which are critical for HCMV control [122, 143]. 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 [144, 145]. 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 [53, 146–148]. 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 [149]. 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 [51]. By contrast, virion proteins were known to be presented even in the absence of viral replication [150], and additionally, pp65 itself was found to interfere with IE1 presentation [151]. Therefore, research focussed on pp65 for several years [152, 153]. 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 [154]. To date, both pp65 and IE1 are considered dominant T cell targets [155, 156]. 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 [157, 158]. 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. Cytomegalovirus has developed several mechanisms to evade cellular immune response [159, 160]. For example the virus can prevent HLA class I loaded with viral peptides to be delivered to the cell surface. This makes the infected cell invisible to CD8 cytotoxic T lymphocytes. Also the virus encodes a glycoprotein homologous to class I MHC antigens to prevent attack by NK cells. The characterization of the immune response against HCMV turns 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 [161]. HCMV has been associated with increased subsequent risk for acute rejection in kidney and other solid organ transplant recipients [162]. Whether the risk is conferred by the reduction of immunosuppressive medication used to treat HCMV infection or is a direct immunomodulating effect of the virus, is not known. However, experiments in immunocompromised bone marrow transplant recipients [53, 163–165] 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.

1.8.2 Vaccine strategies against HCMV

The development of vaccines against infectious agents are one big feature of immunology and are of high success in diseases such as polio, measles, hepatitis B and tetanus [166]. Anyhow, many infectious agents still evade the immune system and lead to severe infections. HCMV is still 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 [167, 168]. 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. $CD8^+$ and $CD4^+$ $\alpha\beta$-T cells provide an immunologic memory response which may have major significance with regard to protective immunity to cytomegalovirus infection [169]. A model with murine cytomegalovirus (MCMV) suggests a critical role of $\alpha\beta$-T cells in HCMV infection. Although MCMV is genetically distinct from HCMV, the pathogenesis of infection in immunosuppressed mice is similar to that for human CMV [170, 171]. Several methodologies 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 [172] emphasizing the importance of a strong HCMV-specific cellular immunity in persistent HCMV infection. Moreover, strategies to isolate and expand clonal populations of $\alpha\beta$-T cells with defined specificity for viral antigens became well-established and support 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 $\alpha\beta$-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 [173]. This first study on adoptive transfer has proved safety and effectiveness of the method to restore $CD8^+$ HCMV-specific cytotoxic T cell immunity. A very promising strategy to expand HCMV-specific CTLs for allogeneic applications from cord blood, is described by Park et al., [174]. Another setting uses dendritic cells which are pulsed with peptide or protein, or HCMV-specific $CD4^+$ T cells [52] to induce HCMV-specific $CD8^+$ T cell responses for the treatment of HMCV infection. Furthermore, genetically modified T cells are used for adoptive immunotherapy offering the potential to be safe and efficient [175]. 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.

1.9 Aims of the thesis

As peptide-based immunotherapy is restricted by many different HLA-types of patients it was important to identify T cell epitopes with distinct HLA restrictions. Current strategies require the time consuming generation of antigen presenting cells (APCs), usually dendritic cells (DCs), for stimulation protocols. One aim of this thesis was the development of a method for fast and reproducible *in vitro* priming and expansion of $CD8^+$ T cells specific for peptides from tumor-associated antigens. To achieve this goal, T cell priming experiments with artificial antigen presenting cells (aAPCs), coated with different amounts of costimulatory molecules and HLA-peptide complexes, as well as experiments to analyse the specificity and functionality of the induced T cells, were performed. Using aAPCs coated with only one costimulatory antibody (anti-CD28) and one HLA-peptide complex for *in vitro* priming of cytotoxic T cells, which was established and published by Steffen Walter et al.; we tried to further improve this promising method by using a second costimulatory molecule (anti-4-1BB) coated on the surface of the aAPC. Therefore different titration assays had to be performed in order to find the optimal condition for this priming strategy.
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 sich as adoptive transfer of HCMV-specific T cells. Nevertheless, a major limitation of adoptive immunotherapy is the availability of HCMV-specific CTLs in HCMV seronegative blood donors. Thus, this thesis also aimed at the induction and expansion of HCMV-specific $CD8^+$ T cells from the blood of HCMV-seronegative donors. Priming experiments performed with the help of artificial aAPCs coated with costimulatory antibodies and HLA-peptide complexes lead to successful *in vitro* primimg of HCMV-specific T cells, which would be a great progress in the treatment of HCMV infection.

1.10 References

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2 Results and Discussion, Part 1: Artificial antigen presenting cells - a highly capable resource for priming of antigen-specific CD8^+ T cells

2.1 Potent costimulation of human CD8 T cells by anti-4-1BB and anti-CD28 on synthetic artificial antigen presenting cells

This manuscript has been composed by Despina Rudolf*, Tobias Silberzahn*, Steffen Walter, Dominik Maurer, Johanna Engelhard, Dorothee Wernet, Hans-Jörg Bühring, Gundram Jung, Byoung S. Kwon, Hans-Georg Rammensee and Stefan Stevanović. The author of this thesis has performed the experiments leading to Figures 2.1A inset, 2.2, 2.3 A-D, and 2.4. Parts of this chapter are published in Cancer Immunol Immunother 57:175-83(2008).

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2.1.1 Abstract

The *in vitro* generation of cytotoxic T lymphocytes (CTLs) for anticancer immunotherapy is a promising approach to take patient-specific therapy from the bench to the bedside. Two criteria must be met by protocols for the expansion of CTLs: high yield of functional cells and suitability for good manufacturing practice (GMP). The antigen presenting cells (APCs) used to expand the CTLs are the key to achieving both targets but they pose a challenge: Unspecific stimulation is not feasible because only memory T cells are expanded and not rare naïve CTL precursors; in addition, antigen-specific stimulation by cell-based APCs is cumbersome and problematic in a clinical setting. However, synthetic artificial APCs which can be loaded reproducibly with MHC-peptide monomers and antibodies specific for costimulatory molecules could resolve these problems. The purpose of this study was to investigate the potential of complex synthetic

artificial APCs in triggering the costimulatory molecules CD28 and 4-1BB on the T cell. Anti-4-1BB antibodies were added to an established system of microbeads coated with MHC-peptide monomers and anti-CD28. Triggering via CD28 and 4-1BB resulted in strong costimulatory synergy. The quantitative ratio between these signals determined the outcome of the stimulation with optimal results when anti-4-1BB and anti-CD28 were applied in a 3:1 ratio. Functional CTLs of an effector memory subtype ($CD45RA^-$ $CCR7^-$) were generated in high numbers. We present a highly defined APC platform using off-the-shelf reagents for the convenient generation of large numbers of antigen-specific CTLs.

2.1.2 Introduction

CD8 T cells are key effectors in the battle against viral pathogens. In cancer immunology, they help to fight tumor cells. Many tumor-associated T-cell epitopes have been defined and this has enabled the design of highly specific anti-cancer regimens [1]. Due to the lack of functional tumor-specific cytotoxic T lymphocytes in many cancer patients, there is a need for the development of methods to prime and expand tumor-specific CTLs. Adoptive immunotherapy is an approach which involves *in vitro* priming and expansion of T lymphocytes with subsequent *in vivo* infusion of expanded CTLs. Dendritic cells have been used as APCs for the *in vitro* priming of CTLs [2]. This method involves the laborious differentiation of autologous peripheral blood monocytes or $CD34^+$ hematopoietic precursors. A problem of this strategy is the limited number of dendritic cells that can be generated. Furthermore, only dendritic cells which are fully mature can prime T cells; immature dendritic cells induce tolerance [3]. The advent of artificial APCs has raised hopes that these shortcomings might soon be overcome. Many different artificial APCs have been designed-either cell-based or synthetic artificial APCs. First, artificial APCs were developed for the unspecific expansion of T cells. These approaches took advantage of anti-CD3 and anti-CD28 antibodies which were added to T cells [4] and used to decorate cell lines such as K562 [5] or microbeads [6]. However, these methods are unsuitable for anti-cancer immunotherapy when rare precursor T cells need to be primed and expanded in an antigen-specific fashion. Thus, methods for antigen-specific T cell priming and expansion have been established. Various examples of cell-based strategies have been presented: they include HLA-A2-negative B cells loaded with HLA-A2 MHC-

peptide monomers [7], mouse fibroblasts retrovirally transduced with an HLA-peptide complex plus the accessory proteins B7.1, ICAM-1, and LFA-3 [8] and insect cells transfected with single-chain HLA, CD54 and CD80 [9]. These protocols have shown that the generation of antigen-specific T cells by employing artificial APCs is feasible. However, the use of such artiWcial APCs in a GMP setting is problematic because cellbased reagents are difficult to implement in GMP protocols. In contrast, synthetic artificial APCs, which are made from a limited number of "off-the-shelf" components, could be more suitable for the clinic. Previous studies have established protocols that involve microbeads loaded with MHC-peptide molecules in monomeric [10] or Ig-coupled dimeric forms [11], and anti-CD28. Clearly, the engineering of artificial APCs is not only a qualitative problem (in terms of the receptors on the T cell to be triggered) but also a quantitative challenge since APCs *in vivo* display a carefully controlled expression pattern of costimulatory molecules. This issue was neglected during the design of cell-based artifical APCs when cell lines were engineered to express different costimulatory molecules (see above).To address these issues, we hypothesized that the use of further stimulatory antibodies in addition to anti-CD28 could render synthetic artificial APCs more powerful, especially if the stimulatory signals were applied in an optimised ratio. To demonstrate this, we took advantage of a strictly controlled system of microbeads loaded with defined amounts of MHC-peptide monomers and anti-CD28 antibodies that was previously used to prime and expand CTLs [10, 12]. To assess possible synergistic effects between costimulatory antibodies, anti-4-1BB was added to the system in the present study. This antibody triggers the tumor necrosis factor (TNF) receptor family member 4-1BB (CD137) [13]. Its ligand 4-1BBL is expressed by APCs such as activated B cells and dendritic cells [14]. The costimulatory molecule 4-1BB is upregulated within 24 h after T cell activation [15] and signals independently from CD28 [16]. 4-1BB signalling primarily induces CD8 T cell proliferation *in vitro* and leads to the amplification of cytotoxic T cell responses *in vivo* [17]. 4-1BB was characterized as a T cell survival signal [18] which may be due to its anti-apoptotic effects [19]. Several studies have shown anti-tumor and antiviral effects of 4-1BB costimulation [20–23]. 4-1BBL was demonstrated to be a valuable costimulatory factor for the unspecific expansion of T cells via anti-CD3 and anti-CD28 [24, 25]. In this study, we found a synergy between anti-CD28 and anti-4-1BB antibodies during T cell priming and expansion that was dependent on the antibody ratio; we report the highest percentage of peptide-specific CTLs generated by antigen-specific *in vitro* priming so far. The CTLs displayed an eVec-

tor memory phenotype and were functional, thereby proving their value for therapeutic approaches. An effective protocol for the *in vitro* generation of CTLs is provided and the importance of highly defined, synthetic artificial APCs is highlighted.

2.1.3 Material and Methods

Peptides and MHC-peptide monomers

The Melan-A-derived peptide ELAGIGILTV [26] was synthesized using standard Fmoc/tBu chemistry [27]. Biotinylated recombinant HLA-A*0201 molecules and fluorescent MHC-tetramers were produced as described previously [28]. Briefy, fluorescent tetramers were generated by incubating biotinylated HLA monomers with streptavidin-PE or streptavidin-APC (Molecular Probes, Leiden, The Netherlands) at a 4:1 molar ratio.

Biotinylated antibodies

The antibodies mouse IgG2a anti-human CD8 Ab OKT-8, mouse IgG2a anti-human CD28 Ab 9.3, mouse IgG1 antihuman 4-1BB Ab 4B4-1 (Becton Dickinson Biosciences (BD), Heidelberg, Germany) or [29] were biotinylated using sulfo-N-hydroxysuccinimidobiotin (Perbio Science, Bonn, Germany) as recommended by the manufacturer.

Synthetic artificial APCs

About 5.6 μm-diameter streptavidin-coated polystyrene particles (BangsLabs, Fishers, IL, USA) were resuspended at 4 x 10^6 microbeads per ml in PBE (PBS/BSA/EDTA) was PBS (phosphate-buffered saline) (BioWhittaker, Verviers, Belgium) containing 0.5% of bovine serum albumin (Sigma, Aldrich) and 2 mM EDTA (Roth, Karlsruhe, Germany), containing 200 pM biotinylated MHC-peptide monomer and a total antibody concentration of 20 nM and incubated at room temperature for 30 min. After washing, the synthetic artifcial APCs were stored at 4°C prior to use.

***In vitro* priming and expansion of human CD8 T cells**

Fresh HLA-A*02^+ buffy coats or leukapheresis products were used to isolate PBMCs by standard gradient separation. CD8 T cells were MACS-enriched by biotinylated OKT-8 antibody and Streptavidin-Microbeads (Miltenyi-Biotec, Bergisch-Gladbach, Germany). Stimulations were initiated in 96-well plates with 1 x 10^6 responder cells plus 2 x 10^5

microbeads in 250 μl of T cell medium [10] complemented with 5 ng/ml human IL-12 p70 (PromoKine,Heidelberg, Germany). After 3-4 days of incubation at 37°C, fresh medium with 80 U/ml human IL-2 (Chiron, Emeryville, CA, USA) was added and cells were incubated for another 3-4 days. This stimulation cycle was performed three/four times.

CFSE-based proliferation assay and cloning

To evaluate the proliferative response to the MelanA antigen, 70 x 10^6 $CD8^+$ T cells were labeled with 5μM CFSE (5/6-carboxyXuorescein diacetate, succinimidyl ester, Molecular Probes, Leiden, The Netherlands) and incubated for 10 min at 37°C in the dark. To stop labeling, an equal volume of T cell medium complemented with 20% heat-inactivated fetal bovine serum (FCS) (PAA, Cölbe, Germany) was added for another 20 min. Cells were then washed in T cell medium four times. About 10^6 CFSE-labeled cells were seeded into individual wells of a 96-well culture plate, synthetic artificial APCs were added as indicated. $CD8^+$ T cells were incubated at 37°C, 5% CO_2. Antigen-stimulated CFSE labeled $CD8^+$ T cells were analyzed by flow cytometry on a four-color FACSCalibur cytometer (BD).

Flow cytometry

Tetrameric analyses were performed with tetramer-PE/APC plus antibody CD8 PerCP clone SK1 (BD). Cells were incubated with the antibody at 4°C for 20 min in the dark, followed by 30 min incubation with fluorescent MHC tetramers at the same conditions. After washing, cells were analyzed by flow cytometry on a four-color FACSCalibur cytometer (BD). Total specific cell numbers per sample could be calculated after FACS analysis: (specific cells counted) x (microbeads added)/(microbeads counted). For phenotyping CCR7 staining was performed using rat hybridoma supernatant 3D12 (kindly provided by R. Förster, Anova) and donkey anti-rat (Fab´)2-PE fragments (Jackson ImmunoResearch, West Grove, PA, USA) or hCCR7 PE (R&D, Wiesbaden, Germany). After blocking with 10% heat-inactivated mouse serum (CC pro, Neustadt, Germany), cells were further stained with CD45RA-FITC (BD) and tetramer-APC. Analysis of surface costimulatory molecules, CD137 (4-1BB) and CD28 staining was performed using anti-human CD137-PE (BD) and anti-human CD28-FITC (Immunotools, Friesoythe).

Generation of T cell lines

Sorting was done with a FACSVantage cell sorter. Sorted tetramer-positive cells were expanded by PHA-L, IL-2, and feeder cells (irradiated LG2-EBV and irradiated allogeneic PBMCs) as described before [10].

T cell assays

Intracellular IFN-γ staining was done as previously described [23]. Briefly, T cells were stimulated with 10 μg/ml peptide for 6 h in the presence of Golgi-Stop (BD). Cells were analyzed using a Cytofix/Cytoperm Plus kit (BD) and an IFN-γ-PE or IFN-γ-FITC antibody (BD). After staining, cells were analyzed on a four-colour FACSCalibur (BD). Cytotoxicity was tested in a standard 4 h ^{51}Cr release assay using 3,000 target cells per well. Percentage of specific lysis was calculated as follows: (experimental release - spontaneous release)/(total release - spontaneous release) x 100.

2.1.4 Results

Synergy between anti-CD28 and anti-4-1BB in CD8 T cell costimulation is most pronounced when applied in a specific ratio To assess the stimulative capacity of anti-CD28, anti-4-1BB and a potential synergy of the antibodies, beads were coated with MHC-peptide-monomer and loaded either with a single antibody or with both antibodies at different ratios. First, the proportion of antigen-specific CTLs prior to stimulation was determined by flow cytometry (Fig. 2.2 A): in an ideal setting with a purity of 100% CD8$^+$ enriched T cells, 0.1% antigen-specific CTLs correspond to a total number of 1,000 antigen-specific CTLs per well (10^6 CD8-enriched cells were used per well). After three rounds of stimulation, moderate T cell priming and expansion were detected with a single costimulatory antibody (Fig. 2.1 A). However, combining both antibodies resulted in a higher percentage of tetramer-positive cells. A 3:1 ratio between anti-4-1BB and anti-CD28 increased the percentage of tetramer-positive cells to values of up to 48% after three rounds of stimulation. In the experiment shown in Fig. 2.1a, about 147,000 tetramer-positive CTLs were detected using the optimal 3:1 ratio of the costimulatory antibodies compared with an average of 21,000 CTLs when using anti-CD28 only. *in vitro* stimulation of CD8$^+$ T cells from six other healthy donors confirmed that a ratio of 3:1 of anti-4-1BB to anti-CD28 was superior to anti-CD28 alone (Fig. 2.1 A inset):

on average, a fivefold higher percentage of specific CTLs was obtained. When either anti-CD28 or anti-4-1BB were replaced by an isotype control, no synergistic effect was observed (data not shown). Thus, any effects due to antibody dilution could be excluded.

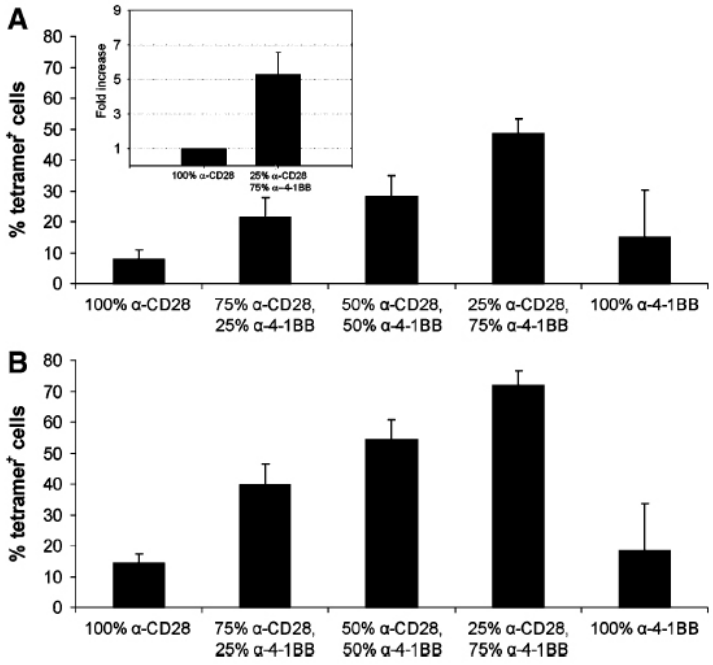

Figure 2.1: **Anti-CD28 and anti-4-1BB on MHC-coated microbeads synergize in T cell priming and expansion**.
Quintuplicates of CD8-enriched T cells of a healthy HLA-A2-positive donor were stimulated three times (A) or four times (B) *in vitro* with A*0201/ELAGIGILTV-coated microbeads loaded with either anti-CD28, anti-4-1BB or a combination of both. Positive T cells were detected by tetramer staining. Repeated stimulation of CD8-enriched T cells of different donors demonstrated that on average a fivefold higher percentage of tetramerspecific T cells can be obtained by costimulation with anti-4-1BB/anti-CD28 in a 3:1 ratio if compared to anti-CD28 alone
(inset Fig. 2.1 A)

Elevated levels of antigen-specificc CTLs after four rounds of stimulation

Usually three rounds of stimulation are performed during *in vitro* priming. Here, we demonstrate that a further round of stimulation can result in a much higher percentage of antigen-specific cells. For the optimal ratio between anti-CD28 and anti-4-1BB, values of up to 72% antigen-specific T cells were monitored (Fig. 2.1 B).

To our knowledge this is the highest value of antigenspecific cells observed for *in vitro* priming experiments.

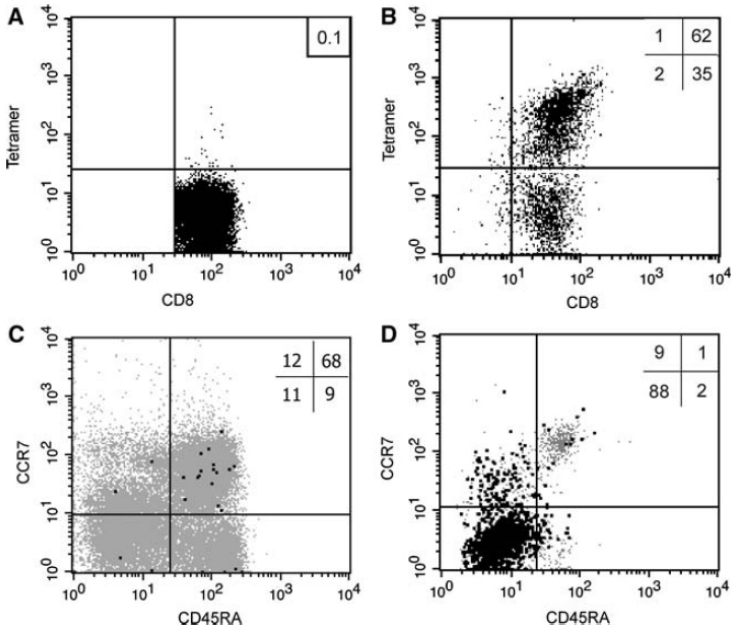

Figure 2.2: **Highly elevated levels of antigen-specific effector memory CTLs after stimulation**.
(A) PBMCs of a healthy HLA-A2-positive donor were stained ex vivo with CD8-PerCP and tetramer to detect the frequency of antigen-specific T cells before stimulation. Due to unspecific tetramer binding to B cells, only $CD8^+$ cells are shown. (B) CD8-enriched T cells of the same donor were stimulated four times *in vitro* with A*0201/ELAGIGILTV-coated microbeads loaded with anti-CD28 and anti-4-1BB in a 1:3 ratio. Positive T cells were detected by tetramer staining. (C) T cells were stained with CD8-PerCP, CD45RAFITC, CCR7 (rat hybridoma supernatant and donkey anti-rat-PE) and tetramer-APC to detect the percentage of tetramer-positive cells and their phenotype. The percentage of $tetramer^+$ $CD8^+$ lymphocytes per quadrant is indicated. Tetramer-positive cells are displayed in black (highlighted), tetramer-negative cells in grey. Assays were performed ex vivo (C) and after four rounds of stimulation (D).

Priming of naïve T cells by synthetic artificial APCs results in the generation of CTLs of the effector memory phenotype

For phenotype analysis, cells were stained with tetramer and appropriate antibodies. In one stimulation process, 62% tetramer-positive T cells were detected after four rounds of stimulation (Fig. 2.2 B). Of these tetramer-positive cells, 88% were effector memory (CCR7$^-$ CD45RA$^-$), 9% central memory (CCR7$^+$ CD45RA$^-$), 2% effector (CCR7$^-$ CD45RA$^+$) and 1% naïve T cells (CCR7$^+$ CD45RA$^+$) (Fig. 2.2 D). These results were confirmed after stimulation and after phenotyping T cells of a second donor, where 80% of effector memory, 10% central memory, 9% effector and about 1% naïve T cells were obtained. To compare the phenotype of these tetramer$^+$ specific cells with tetramer$^+$ cells prior to stimulation we also analyzed PBMCs of the same donors ex vivo. One exemplary result is shown in Fig. 2.2 C. About 70% of tetramer$^+$ cells analyzed ex vivo were of a naïve phenotype (CCR7$^+$ CD45RA$^+$), as expected from previous reports [30].

The generated CTLs are functional and exert effector functions in a specific fashion

For functional analysis, tetramer-positive T cells generated by the optimal antibody ratio were sorted and three cell lines were established. Functionality of the T cells was demonstrated by intracellular IFN-γ staining and chromium release assay. After stimulation with the Melan-A-expressing HLA-A*02$^+$ melanoma cell line MeWo, all T cell lines produced IFN-γ (one representative experiment is shown in Fig. 2.3 A), IFN-γ production was also observed after stimulation of the T cell lines with either ELAGIGILTV-loaded T2 cells (Fig. 2.3 B) or the Melan A expressing HLA-A*02$^+$ melanoma cell line Mel-CG1 (Fig. 2.3 C). (On average, 30-50% of tetramer-specific CTLs produce IFN-γ (Fig. 2.3 A-C), while irrelevant targets (target cell line loaded with HIV-peptide) did not stimulate IFN-γ production (Fig.2.3 D, T2 cells loaded with HIV-peptide). These results correspond well with data of generated virus specific CTLs (Rudolf D et al., submitted) and emphasize our notion that tetramer staining indicates specificity but not functionality of T cells. Furthermore, the same T cell line lysed the Melan-A-positive targets specifically and efficiently (MeWo, Mel-CG-1 and ELAGIGILTV-loaded T2 cells, but there was no lysis of control targets (BV-173 cells and T2 cells loaded with an HIV-peptide) (Fig. 2.3 E). To exclude unspecific lysis of the melanoma cell lines, we tested T cell lines generated from the sorted tetramer-negative cells. Neither ELAGIGILTV-loaded T2 cells nor MeWo cells were lysed by the control cell lines (data not shown). This indicates that the tetramer-positive CTLs killed the melanoma cells in an antigen-specific fashion.

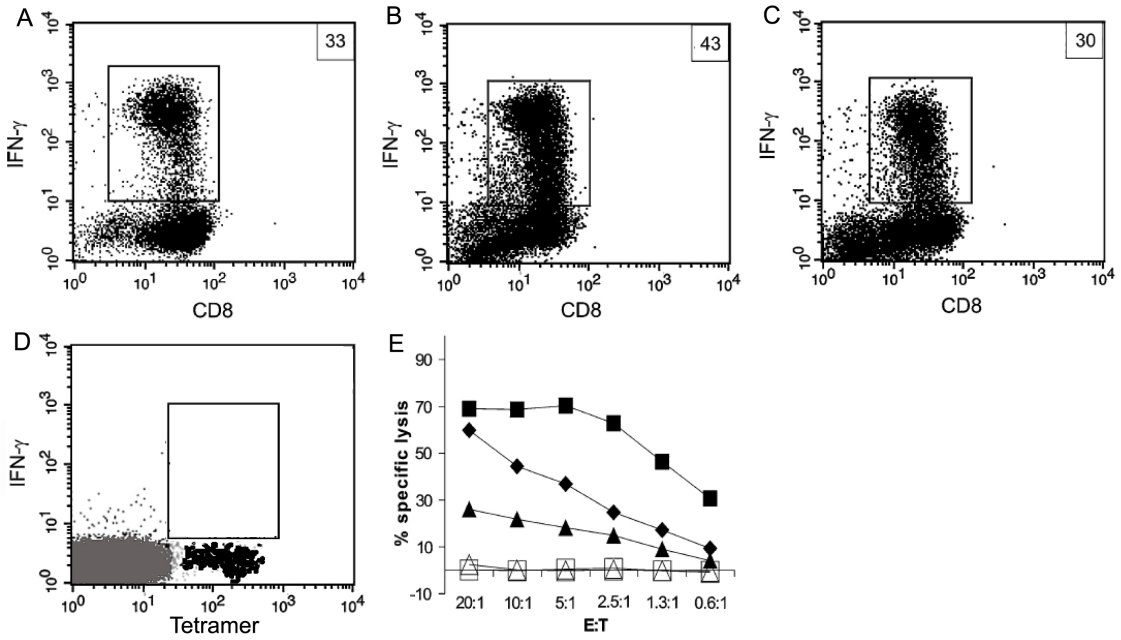

Figure 2.3: **Tetramer-positive cells are fully functional cytotoxic T lymphocytes.**
Intracellular IFN-γ staining of sorted tetramer-positive T cells stimulated with (A) HLA-A*02-positive Melan-A-positive MeWo cells. (B) ELAGIGILTV-loaded T2 cells, (C) HLA-A*02-positive Melan-A-positive Mel-CG1 cells. The percentage of IFN-γ producing CD8-positive T cells among tetramer-positive cells is indicated. (D) Target cells (T2 cells) loaded with irrelevant HIV-peptide do not induce secretion of IFN-γ (Tetramer-positive cells are displayed in black (highlighted), tetramer-negative cells in grey). (E) The killing activity of the same T cell lines was tested against ELAGIGILTV-loaded TAP-deficient T2 cells (filled square), the HLA-A*02-positive Melan-A-positive melanoma cell lines MeWo (filled diamond) and Mel-CG-1 (filled triangle). ILKEPVHGV-loaded T2-cells (HIV-peptide, open square) and the HLA-A*02-positive leukemia cell line BV-173 (open triangle) were used as control. One of three independent experiments with comparable results is shown

CD8, CD28, and 4-1BB surface marker expression during stimulation of CD8$^+$ T cells with aAPCs

To assess cell division and surface expression of the costimulatory molecules CD28 and 4-1BB during *in vitro* stimulation, naive CD8$^+$ T cells were labeled with CFSE and then stimulated with A*0201/ELAGIGILTV-coated microbeads loaded with anti-CD28

and anti-4-1BB in a 1:3 ratio. After three stimulations, cells were stained with tetramer and appropriate antibodies. The CFSE labeling indicates that within the $CD8^+$ T cell population, $tetramer^+$ T cells proliferated almost exclusively during stimulation. The activation was accompanied by a slight down regulation of the CD8 surface molecule in part of the T cells (Fig. 2.4 A). Expression of 4-1BB was low in $CD8^+$ T cells before stimulation, as analyzed by ex vivo staining. After three rounds of stimulation, 4-1BB was expressed again at low levels in tetramer-positive cells while tetramer-negative cells displayed higher 4-1BB expression (Fig. 2.4 B-C): only 10% of tetramer-positive cells expressed 4-1BB compared to 90% of tetramer-negative cells expressing 4-1BB on their surface. The expression of the costimulating molecule CD28 was determined after *in vitro* stimulation: 44% of $CD8^+$ T cells expressed CD28 after stimulation; and 63% of this $CD28^+$ population were $tetramer^+$ T cells (Fig. 2.4 D-E). In summary, the data reveal that when used in a defined ratio, anti-CD28 and anti-4-1BB boost T cell priming and expansion by synthetic artificial APCs. As only a specific ratio of the antibodies resulted in optimal stimulation, this study stresses the importance of defined artificial APCs for *in vitro* T cell priming and expansion.

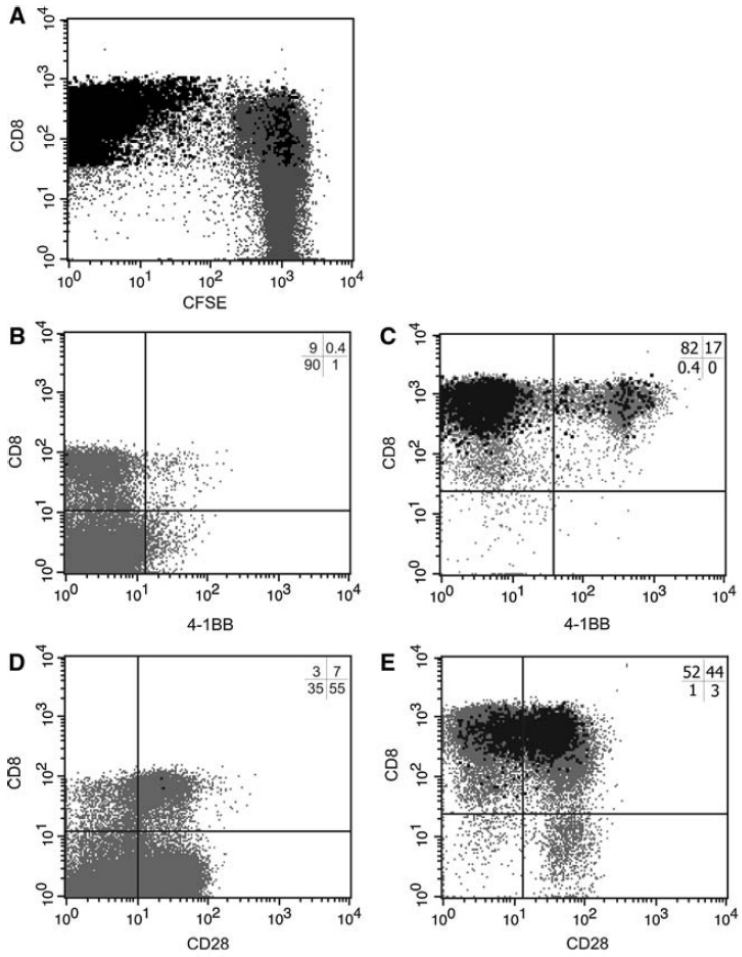

Figure 2.4: **Expression of surface markers CD8, 4-1BB and CD28 during stimulation**.
CD8 enriched T cells of a healthy HLA-A2-positive donor were labeled with CFSE and stimulated three times with A*0201/ELAGIGILTV-coated microbeads and an optimized ratio of costimulatory antibodies. Cell division was assessed at various time points by monitoring the CFSE intensity of these cultures, followed by staining with anti-CD8 and tetramer staining. a only tetramer$^+$ CD8$^+$ lymphocytes proliferate during stimulation with artificial APCs. Proliferation is accompanied by a slight decrease of surface CD8. 4-1BB expression is generally low prior to stimulation (B). After three stimulations, 4-1BB surface expression is low in tetramer$^+$ T cells compared to tetramer$^-$ T cells (C). In contrast, CD28 expression appeared high in tetramer$^+$ CD8$^+$ lymphocytes after stimulation (E) but significant expression was also determined ex vivo (D). The percentage of PBMCs per quadrant is indicated in all panels. Tetramer-positive cells are displayed in black (highlighted), tetramer-negative cells in grey

2.1.5 Discussion

The main goal of this study was demonstrate that synthetic artificial APC could be used as a tool in adoptive cancer immunotherapy if the stimulatory capacity of this APC platform is improved. A parallel comparison recently demonstrated that synthetic bead-based aAPC are superior to dendritic cells [10]. Here, we introduce an additional costimulating compound and also consider the ratio of costimulatory triggers and its eVect on the outcome of stimulation, which is an issue that has been neglected until now. Biotinylated MHC-peptide complexes and biotinylated antibodies were coupled onto streptavidin-coated microbeads. We used a well-described CTL epitope for the study, namely the modiWed Melan-A-derived peptide ELAGIGILTV [26] that has already been used as a target in melanoma immunotherapy. T cell stimulation resulted in greatly increased populations of antigen-specific CTLs upon stimulation with anti-CD28 and anti-4-1BB. The peak of 48% positive CTLs (1,47,000 cells in absolute numbers) after three rounds of stimulation was reached upon stimulation with artificial APCs coated with anti-4-1BB and anti-CD28 in a 3:1 ratio. Thus, approximately 10 ml of peripheral blood gave rise to nearly 1,50,000 antigen-specific CTLs. The large majority of the generated CTLs displayed an effector memory phenotype which had been observed previously in other *in vitro* priming experiments [10, 12]. They can be distinguished from central memory T cells by their lack of the CCR7 chemokine receptor. Effector memory T cells were shown to migrate to peripheral tissues and to exert an immediate effector function, thereby providing protective memory [31–33]. The effector function of the generated CTLs was assessed by two functional assays: The CTLs were shown to be capable of antigen-specific IFN-γ production and target cell lysis. Unspecific lysis of cells, which would contradict usage in adoptive immunotherapy, was not detectable. On average, 30-50% of tetramer$^+$ T cells obtained after three rounds of stimulation were able to secrete IFN-γ. Other groups achieved higher amounts of IFN-γ secreting cells, specific for the same modified Melan A peptide ELAGIGILTV, only after enrichment using an IFN-γ antibody [11] or after cloning. Bulk cultures or T cell lines usually encompass different subsets with different effector functions [34], including even dysfunctional cells [35]. The risk of inducing dysfunctional T cells during strong and rapid expansion has been mentioned before [36]. CD28 is critical for initial T cell expansion, whereas 4-1BB/4-1BBL signalling impacts T cell numbers much later in the response and is required for the survival and/or responsiveness of the memory CD8 T cell pool. Upon engagement

with 4-1BB ligand, 4-1BB can offer a CD28-independent costimulatory signal leading to CD4/CD8 T cell expansion, cytokine production, development of CTL effector function, and prevention of activated induced cell death [16]. 4-1BB is hardly detectable on the surface of freshly isolated human peripheral blood T cells but the expression of 4-1BB is inducible on human T cells. The 4-1BB molecule has been shown to be transiently expressed after TCR engagement in a precise and narrow window of time [14, 15]. *in vitro* the expression peaks at ≈48 h post-activation, is down-regulated and subsequently remains at a low, almost constant level [13]. We have been unable to detect significant 4-1BB expression on the tetramer-positive T cells due to the low level and transient nature of its expression. Members of the TNFR family, including 4-1BB are known to influence cell survival by activating the NF-κB pathway [37, 38], which in turn can lead to upregulation of Bcl-xL as well as cellular inhibitors of apoptosis 1 and 2. CD28 as well as members of the TNFR family influence Bcl-xL expression [19, 39–41]. A variable percentage of unstimulated thymocytes are $CD28^+$ but this is found in high density only on the $CD3^+$ (bright) CD4/CD8 single positive population. In our experiments, 63% of activated tetramer-specific $CD8^+$ T cells expressed CD28 after three weeks of stimulation. CD28 expression increases upon cell activation after 12-24 h and persists for at least 6 days [42]. CD28 appears to be responsible for a first spate of survival signals and this can then be sustained by inducible costimulatory pathways such as 4-1BB. In the light of these findings, to perform optimal T cell priming we used artificial APCs coated with anti-4-1BB and anti-CD28 in a 3:1 ratio. We were able to demonstrate that this artificial system is highly effective for *in vitro* priming. Adoptive immunotherapy of cancer relies on the priming and expansion of rare CTL precursors that are usually naïve $CD8^+$ T cells. Obviously, these cells would be lost in a setting of unspecific T cell expansion via anti-CD3 signals where memory T cells have a growth advantage. Hence, antigen-specific T cell priming and expansion is needed for adoptive immunotherapy of cancer. Until now, most artificial APC approaches for antigen-specific T cell stimulation have relied on genetically engineered cell lines. Clearly, this is a suboptimal solution in clinical immunology where standardised reagents are needed. In this context, synthetic artificial APCs are preferable for three reasons: First, as no living cells are employed, synthetic artificial APCs are true "off-the-shelf" reagents; culturing APCs is more cumbersome than using synthetic agents. Synthetic artificial APCs can be produced ad libitum within the shortest time: biotinylated antibodies, MHC-peptide monomers and microbeads are commercially available and can be stored for long periods of time. They

can be tailored to suit since all stimulatory parameters can be adjusted. In addition, synthetic artificial APCs are convenient. Prior to *in vitro* stimulation, sufficient APCs for all three rounds of stimulation are prepared and stored at 4°C. Each week, an aliquot is transferred to the stimulation experiment and sufficient numbers of APCs from one batch are therefore available for the entire stimulation procedure. Second, the culture of cell-based artificial APC lines requires repeated screenings to ensure the quality of the lines. This is an obstacle in establishing immunotherapy as a routine procedure. An APC system that requires no maintenance operations will support the use of immunotherapy for a greater number of patients. For patients with progressive disease in particular it is vital to provide an APC system that is both readily available and reliable. Third, cellular APCs are defined to a lesser extent than synthetic approaches. In our view, this is a drawback of cell-based APCs, such as dendritic cells or genetically engineered cell lines. It is not only cumbersome and expensive to generate dendritic cells, it is also difficult to produce fully-mature dendritic cells reproducibly that are necessary for *in vitro* priming especially for different donors. Cell based artificial APCs usually express the molecules involved in the T cell priming process as transgenes. Hence, the number of stimulatory molecules at the cell surface is unknown and, more important, it is not possible to tune the expression of costimulatory molecules in order to provide the optimal ratio for stimulation. Another issue is the avidity of *in vitro* generated CTLs. It was shown that only a low concentration of MHC-peptide monomers on artificial APCs resulted in high-avidity T cells [26]. Conversely, a high concentration of MHC-peptide monomers resulted in low-avidity T cells. This finding is often not taken into consideration when cell-based artificial APCs transfected with HLA molecules are used [8, 43]. The amount of "signal 1" that these systems provide is undefined and may vary according to culture conditions. Interestingly, just the combination of two costimulatory signals resulted in an average of 72% positive CTLs after four stimulations, which to our knowledge is the highest value observed in an antigen-specific *in vitro* priming experiment. Hence, synthetic artificial APCs are not only convenient and versatile but also powerful tools for T cell priming and expansion. In sum, the introduction of complex yet defined synthetic APCs should allow the convenient generation of large numbers of high-avidity CTLs by an optimized ratio of MHC:peptide complexes and costimulatory triggers for both therapeutic and experimental use.

Acknowledgments

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2.2 CD8$^+$ T cell responses to MUC1.mod (APL)

2.2.1 Introduction

Mucins are high molecular weight glycoproteins that are found almost exclusively on the apical surface of many glandular epithelia including the gastrointestinal, respiratory, urinary, and reproductive tracts [44–47]. MUC1 is a type I transmembrane glycoprotein, consisting of a very large (1000-2200 amino acids) extracellular domain comprised of a series of 20-25 amino-acid tandem repeats(VNTR)(PDTRPAPGSTAPPAHGVTSA) [48, 49], a 31-amino-acid transmembrane domain, and a short (72 amino acids) cytoplasmic tail. MUC1 primarily functions in lubrication and hydration of epithelia and protection from microbial attack. However, the large extended extracellular domain of MUC1 also appears to play both adhesive and antiadhesive functions and contributes to decreased immune response. More recently, MUC1 has been implicated in signal transduction due to interactions of its highly conserved cytoplasmic tail with several signal transducing molecules [44]. Aside from its normal physiological roles, MUC1 also has been implicated in progression of numerous types of cancer including breast, colon, lung, gastric, and pancreatic cancers [44, 47, 50]. MUC1 expression in tumors is greatly increased and accompanied by altered glycosylation and aberrant expression patterns that become more diffuse when compared to the normal apically restricted pattern.This overexpression was shown to correlate with aggressiveness of malignancy and poor prognosis [51, 52], makes MUC1 an attractive and broadly applicable target for immunotherapeutic strategies [53–59]. Moreover, MUC1 is proposed to help tumor cells evade host defenses by attenuating immune responses and to promote metastasis through a loss of cell-cell and cell-extracellular matrix (ECM) contact [47]. Peripheral CD8$^+$ T cells recognize peptide antigens that are presented in the groove of MHC class I molecules on

APCs [60]. Recently,the definition of MHC class I allele-specific motifs allowed the definition of epitopes contained within a given antigen and provided new opportunities for the development of vaccine therapies [60–62]. However, to date, with a few exceptions (melanoma-associated antigens), there is only limited information about the identity of CTL epitopes presented by human malignant cells [63]. *in vitro* immunization methods using epitopes derived from selfantigens have often proved unsatisfactory because of the low affinity of the elicited CTLs and, consequently, the lack of a sufficient recognition of naturally processed antigens by these CTLs [64, 65]. Presentation of antigens by professional APCs may be critical for the effectiveness of an induced immune response, and the nature of the APCs can determine the outcome, ranging from immunity to tolerance [66]. For this thesis we used the The HLA-A*0201 restricted epitope STAPPVHNV ($MUC1_{950-958}$), which wasidentified by Brossart et al.; [67]. In an attempt to overcome the relatively poor binding of the natural MUC1 peptide to HLA-A*0201, we synthesized single amino acid modificated peptide analogues; through the modification of amino acid residues involved in MHC binding, we changed the natural HLA-A*0201 restricted epitope sequence S**T**APPVHNV into S**L**APPVHNV and performed in *vitro* priming experiments with the use of artificial antigen presenting cells.

2.2.2 Material and Methods

HLA-B*0201 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 STAPPVHNV ($MUC1_{950-958}$) and SLAPPVHNV ($MUC1.mod_{950-958}$). Biotinylated recombinant HLA-B*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 (Becton Dickinson). 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, Colbe, 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 streptavidin (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% formaldehyde (Merck) in PFEA. Cells were analysed on a four-colour FACSCalibur cytometer (Becton Dickinson).

2.2.3 Results and Discussion

For the generation of MUC1.mod$_{950-958}$ 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-A*0201 monomer. As determined by tetramer analysis, stimulation with HLA-A*0201 monomer coated mircrospheres led to generation of specific cytotoxic T cells with corresponding specificity (Fig. 2.5 D), as CTLs generated with beads coated with the modified HLA-A*0201 restricted epitope SLAPPVHNV, were also specific for the natural epitope STAPPVHNV (Fig. 2.5 C,E). No staining was observed with an irrelevant tetramer (data not shown). Also no staining was obtained after stimulating the CD8$^+$ T cells with the beads covered with less immunogenic natural peptide STAPPVHNV (MUC1$_{950-958}$) (data not shown). No antigen-specific T cells were detected after ex vivo staining of PBMCs (Fig. 2.5 A,B), consequently one can say that detected antigen-specific T cells resulted from efficient *in vitro* priming. These results suggest that the MUC1.mod peptide analogue SLAPPVHNV may be more immunogenic than the natural peptides in HLA-A*0201 donors and should thus be considered as a candidate for future peptide-based vaccine trials. Moreover, analogues with greater immunogenicity than their natural counterparts may be useful in shortening the stimulation time required to obtain the large numbers of peptide-specific effector CTL populations required for adoptive transfer therapy.

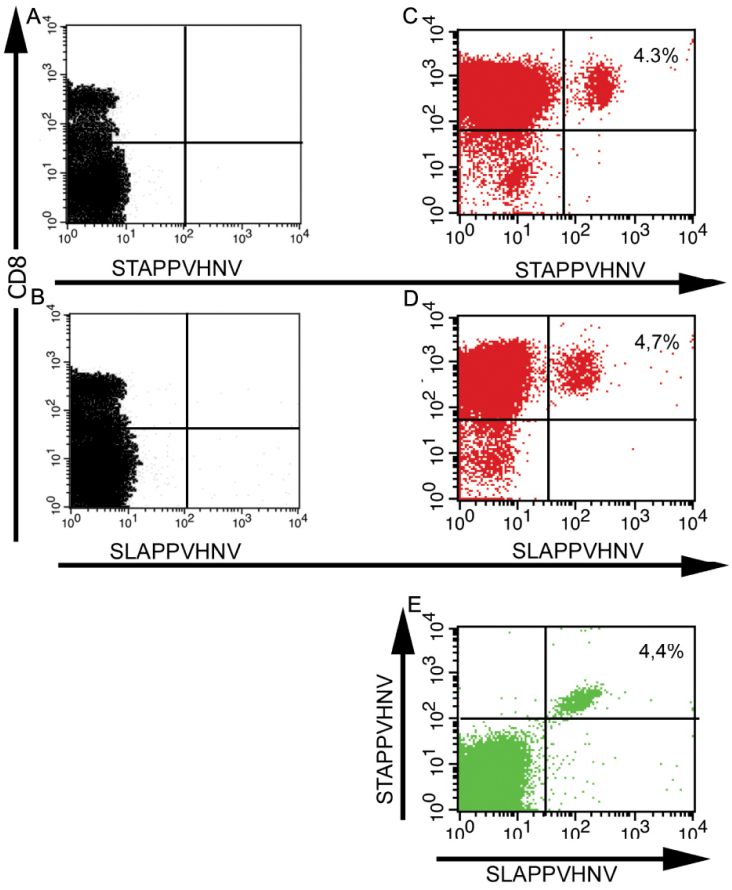

Figure 2.5: **Representative tetrameric analyses of bead stimulated CD8$^+$ T cells**.
CD8$^+$ T cells of 5 HLA-A*0201 positive donors were stimulated 3 times with artificial antigen presenting cells, loaded with costimulatory antibodies and with altered peptide ligand HLA-A*0201 monomer SLAPPHVNV. (A,B) PBMCs of a healthy HLA-A2-positive donor were stained ex vivo with CD8-PerCP and tetramer to detect the frequency of antigen-specific T cells before stimulation. CD8-enriched T cells of the same donor were stimulated four times *in vitro* with A*0201/SLAPPVHNV-coated microbeads loaded with anti-CD28 and anti-4-1BB. Positive T cells were detected by tetramer staining. (C-E) Cells were stained with CD8-PerCP, SLAPPVHNV (MUC1.mod $_{950-958}$) tetramer-APC and natural ligand STAPPVHNV (MUC1$_{950-958}$) tetramer-PE. Percentage of tetramer$^+$ cells within CD8$^+$ T cell population is indicated in each plot.

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3 Results and Discussion, Part 2: Efficient priming of virus-specific cytotoxic T cells *in vitro*

3.1 Synthetic artificial APCs: Fast and reproducible *in vitro* priming of HCMV-specific T cells from seronegative donors

This manuscript has been composed by Despina Rudolf, Maria-Dorothea Nastke, Dominik Maurer, Dorothee Wernet, Christian Sinzger, Hans-Georg Rammensee, and Stefan Stevanović. The author of this thesis has performed the experiments leading to Figures 3.1, 3.2, 3.3, 3.4 and 3.5

Running title: Artificial priming of HCMV CTL from seroneg. donors

Keywords CTLs · Artificial antigen presenting cells (aAPCs) · HCMV· *in vitro* priming

3.1.1 Abstract

Human Cytomegalovirus (HCMV) is frequently virulent after stem cell transplantion and remains a significant cause of morbidity and mortality after reactivation. In healthy HCMV-seropositive individuals HCMV-specific $CD8^+$ T cells typically represent 1-2% of peripheral $CD8^+$ T cells. Recovery of HCMV-specific cytotoxic T cell (CTL) responses after stem cell transplantion is correlated with an improved outcome of HCMV disease, an observation supporting adoptive transfer strategies using HCMV-specific $CD8^+$ T cells. However, *in vitro* priming and expansion of $CD8^+$ T cells from peripheral blood of HCMV-seronegative donors has rarely been successsful. To overcome this limitation, we used a priming strategy based upon artificial antigen presenting cells (aAPCs). Microspheres coated with defined amounts of MHC molecules and costimulatory antibodies such as anti-4-1BB and anti-CD28 can serve as highly effective artificial APCs capable to prime and expand human $CD8^+$ T cells *in vitro*. Here we demonstrate *in vitro* priming

of $CD8^+$ T cells specific for four immunodominant CTL epitopes derived from the pp65 antigen of HCMV and restricted by HLA-A*0201 or HLA-B*0702. We isolated $CD8^+$ T cells from HCMV-seronegative donors, stimulated and expanded these for 3 weeks with aAPCs and succeeded in the generation of high numbers of HCMV-specific $CD8^+$ T cells able to lyse HCMV-infected targets.

3.1.2 Introduction

Human Cytomegalovirus (HCMV) is responsible for a variety of disease syndromes in children and adults. While HCMV infection is normally asymptomatic in immunocompetent individuals [1], this herpesvirus infection still accounts for serious morbidity and sometimes fatal infections in immunocompromised patients, in particular recipients of solid-organ or hematopoetic cell allotransplants and individuals with advanced AIDS [2–4]. Intensified immunosuppression or T cell depletion increasingly performed for unrelated and mismatched or haploidentical stem cell transplantation (SCT) further increases the incidence of and mortality from HCMV infection [5, 6]. Prophylactic strategies are accompanied by myelosuppression, nephrotoxicity, or both, resulting in an increased risk for bacterial or fungal infection [7, 8]. In addition, more and more clinical HCMV strains become resistant to current standard antiviral therapy [9], underlining the need for alternative modalities to prevent and treat HCMV infections. HCMV infection cannot be eliminated by the immunocompetent host, although the titer of the virus in the mucosa and peripheral blood is reduced after acute infection and the virus becomes undetectable. Thus, persistence and latency are established. The individual cell-mediated immune responses to HCMV largely rely on T cells. High frequencies of HCMV-specific CTLs can be detected in peripheral blood of healthy HCMV-seropositive donors [10, 11] and there is a direct correlation between the regeneration of HCMV-specific CTL responses with an improved outcome of HCMV disease [12]. Based on these findings, the concept of adoptive immunotherapy has been developed:[13] Adoptive transfer of HCMV-specific CTL clones [14, 15] or T cell lines [16] has successfully protected patients at risk from HCMV disease. The first study on adoptive transfer of $CD8^+$ HCMV-specific T cell clones in allogeneic hematopoetic cell transplant recipients has proven safety and effectiveness of this approach to restore HCMV-specific cytotoxic T cell immunity [17]. Furthermore, several studies have outlined the significance of antiviral effector functions of helper T cells in maintaining CTL responses after adoptive transfer [18, 19] and their

capacity to produce antiviral cytokines [20, 21]. 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. Nevertheless, a major limitation of adoptive immunotherapy is the availability of HCMV-specific CTLs as current strategies require the time consuming generation of antigen-presenting cells (APCs), usually dendritic cells (DCs), for stimulation protocols. Furthermore, expansion of HCMV-specific CTL is feasible in HCMV-seropositive donors but is rarely successful in HCMV-seronegative individuals [22]. Another strategy to expand HCMV-specific CTLs for allogeneic applications is usage of cord blood that contains many antigen-inexperienced T cells like in HCMV-seronegative donors [23]. However, the possibility of generating HCMV-specific T cells from cord blood is limited by its availability. To overcome this limitation, we demonstrate in this study a highly effective approach for the generation and expansion of HCMV-specific $CD8^+$ T cells: Microspheres coated with defined amounts of HLA:peptide complexes and costimulatory molecules serve as artificial APCs to stimulate blood cells of HCMV-seronegative donors that do not show detectable frequencies of HCMV-experienced T cells *ex vivo*. Our strategy was successful in priming and expansion of CTLs specific for different epitopes of HCMV presented by HLA allotypes HLA-A*02 and HLA-B*07.

3.1.3 Material and Methods

Donors

Leukapheresis products 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 leukapheresis products using standard gradient separation (Lymphocyte Separation Medium, PAA Laboratories, Pasching, Austria) and cryopreserved in fetal calf serum (FCS) (PAA Laboratories) with 10% DMSO (Merck, Darmstadt, Germany) at -80°C until further use.

Peptide, refolding, fluorescent tetramers and artificial antigen presenting cells

Peptides from HCMV pp65 (Swiss-Prot Accession No. P06725) were synthesized by standard Fmoc chemistry using a Peptide Synthesizer 433A (Applied Biosystems, Darmstadt,

Germany) or alternatively using the Economy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany). Immunodominant HLA class I restricted peptides were selected according to published CTL epitope sequences [24]. Peptides were TPRVTGGGAM (HCMV $pp65_{417-426}$) [25] RPHERNGFTVL (HCMV $pp65_{265-274}$) [26], MLNIPSINV (HCMV $pp65_{120-128}$) [27] and NLVPMVATV (HCMV $pp65_{495-503}$) [28]. Synthetic peptides were dissolved at 10 mg/ml in DMSO (Merck), diluted 1:10 in ddH_2O and aliquots stored at -80°C. Biotinylated recombinant MHC class I molecules and fluorescent MHC tetramers were produced as described [29]. 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. Microspheres used for aAPC preparation were streptavidin coated polystyrene particles of 5.60 μm diameter 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 either high density (HD, 20 nM) or low density (LD, 200 pM) and two costimulatory antibodies, anti human CD28 Ab 9.3 (5 nM)[30] and anti 4-1BBL Ab (15 nM, Becton Dickinson). Suspension was incubated at 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.

Reagents and media

T cell medium consisted 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 20μg/ml gentamycin (all BioWhittaker). PFEA (PBS/FCS/EDTA/NaN3) was PBS supplemented with 2% heat inactivated FCS (PAN Biotech, Aidenbach, Germany), 2 mM EDTA (Roth) and 0.01% sodium azide (Merck). TSB (tetramer staining buffer) was PBS (BioWhittaker/Cambrex) containing 50% FCS (PAA), 2 mM EDTA (Sigma Aldrich) and 0.02% NaN3 (Merck).

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

$CD8^+$ T cells were magnetically enriched from 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 CD8

sorted cells plus 2 x 10^5 coated beads per well in 200 μl T cell medium. 5 ng/ml human IL-12 p70 (R&D Systems, Wiesbaden, Germany) was added with beads. Per donor, 20 wells with high density loaded microspheres and 40 wells with low density loaded beads were used. After 3-4 days of 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 on day 21 by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany).

Generation of T cell lines

Sorting was done with a BD FACSAria cell sorter. Sorted tetramer-positive cells were expanded in the presence of PHA-L, IL-2, and feeder cells (irradiated LG2-EBV and irradiated allogeneic PBMCs) as described before [31].

Tetrameric analysis

Cells were washed in PBEA (PBS containing 0.5% protease-free BSA, 2 mM sodium EDTA (both Sigma-Aldrich) and 0.01% sodium azide (Merck) and stained with CD8-PerCP clone SK1 antibody (Becton Dickinson) in the same buffer for 20 min at 4°C. 100 μg/ml unlabeled streptavidin (Sigma-Aldrich) was included in same incubation period. Cells were washed in PFEA 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%formaldehyde (Merck) in PFEA. Cells were analysed on a four-colour FACSCalibur cytometer (Becton Dickinson).

Combined tetramer staining/intracellular IFN-γ staining

After 3 rounds of stimulation 10^6 $CD8^+$ T cells were incubated with autologous PBMCs pulsed with 5 μ/ml peptide at a ratio of 1:1 for 6 h. Golgi-Stop (Becton Dickinson) was added for the final 4 h of incubation. Cells were analysed using a Cytofix/Cytoperm Plus kit (Becton Dickinson) plus Abs IFNγ-FITC (Becton Dickinson), CD8-PerCP clone SK1 (Becton Dickinson) and fluorescent MHC tetramer-PE. Stimulation with PMA/Ionomycin was used as a positive control. After staining, cells were analysed on a four-colour FACSCalibur.

IFN-γ ELISPOT assay

Multiscreen HA plates (Millipore, Bedford, MA) were coated with 5 μg/ml of monoclonal Ab anti-human IFN-γ (1-D1K; Mabtech, Stockholm, Sweden) in PBS overnight at 4°C. Unbound Ab was removed by three washings with PBS. After blocking the plates with RPMI 1640/10% heat-inactivated human serum (1 h, 37 °C), PBMCS at 10^6 cells/well were seeded in triplicates. Antigenic peptides were added at a final concentration of 1 μg/ml. Control wells contained PBMCs, PBMCs in the presence of HIV peptide. Culture medium was RPMI 1640 medium (Bio Whittaker) at a final volume of 200 μl/well. Cells were incubated at 37°C in 5% CO_2 in a water-saturated atmosphere. After a culture period of 26 h, cells were removed by six washings with PBS/0.05% Tween 20 (PBS/T). Captured cytokine was detected by incubation for 2 h at 37 °C with biotinylated mAb anti-hIFN-γ (7-B6-1; Mabtech) at 2 μg/ml in PBS/0.5% BSA. After washing the wells six times with PBS/T, Avidin-Peroxidase Complex (1/100; Vectastain Elite Kit; Vector, Burlingame, CA) was added for 1 h at room temperature. Unbound complex was removed by three successive washings with PBS/T and three with PBS alone. Peroxidase staining was performed with 3-amino-9-ethyl-carbazole (Sigma) for 4 min and stopped by rinsing the plates under running tap water. Spot numbers were automatically determined with the use of CTL Immunospot S3 Analyzer (S3A). To calculate the number of $CD8^+$ T cells responding to a particular peptide, the mean numbers of spots induced by HIV peptide alone were subtracted from mean spot numbers induced by peptide-loaded APC.

HCMV infection of fibroblasts

HLA-B7-positive human foreskin fibroblasts (HFF) were cultured in MEM containing 2.4 mmol/l glutamine, 100 μg/ml gentamicin, and 5% fetal calf serum (MEM5). Fibroblasts were used for experiments between passages 10 and 25. Contamination of cells by mycoplasma strains was tested by staining of DNA with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma Aldrich). Cells were discarded if mycoplasma contamination was detected. HFF were infected at a multiplicity of infection of 5 infectious units per cell with HCMV strain AD169 for 60 min at 37°C. Subsequently, virus preparations were removed, cells were washed with fresh media and maintained at 37°C until used for immunological assays. For negative controls, cells were mock-infected in the same way using medium without virus.

^{51}Chromium release assay

Standard 5 h ^{51}Cr release assay was performed using either 10^4 peptide loaded, B*07-positive HCMV-infected (strain AD169) fibroblasts or HCMV-infected fibroblasts without peptide loading. Unspecific lysis was evaluated by using HLA-matched uninfected fibroblasts (mock fibroblasts) as control. The ^{51}Chromium release experiment was also done with peptide loaded JY cells (Epstein-Barr virus-transformed human B lymphoblastoid cell line; HLA-A*0201, B*0702), or as a control with JY cells without peptide. 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 additional 4 hours at 37°C, 50 μl of supernatant were transferred to a lumaplate (PerkinElmer, Rodgau-Jügesheim, Germany) and read-out was performed by scintillation counting (1450 Microbeta Plus, PerkinElmerTM life science). Specific lysis was calculated by 100 x (experimental release - spontaneous release)/(total release - spontaneous release).

3.1.4 Results

Priming *in vitro* by aAPCs induces antigen-specific CD8$^+$ T cells

For the generation of HCMV-specific cytotoxic T cells (CTLs), MACS-enriched CD8$^+$ T cells from HCMV-seronegative donors were stimulated for three weeks in the presence of IL-12, IL-2, and artificial antigen presenting cells (aAPCs). Each donor was tested before stimulation for the presence of HCMV-specific CD8$^+$ T cells; none of the donors appeared positive in such ex vivo assays (Figure 3.1).

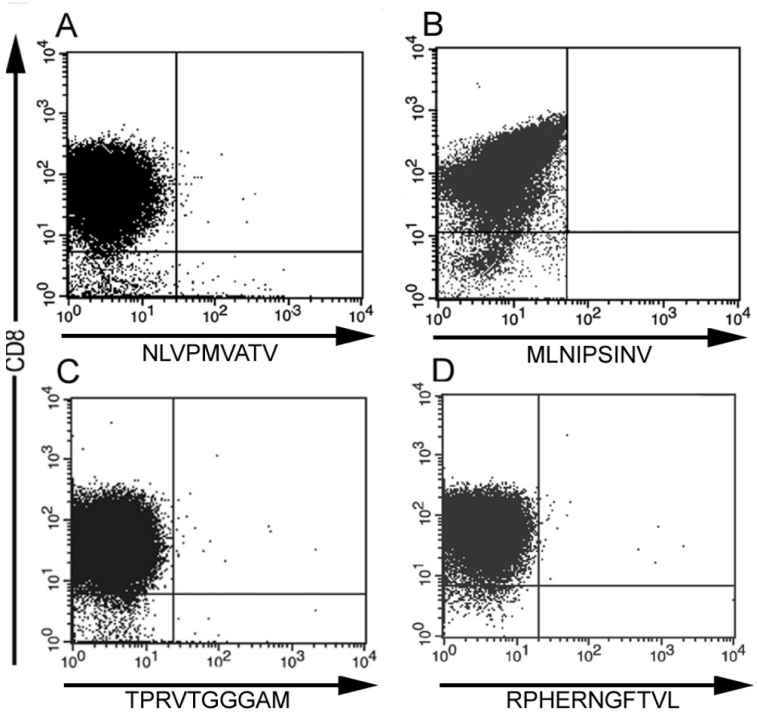

Figure 3.1: ***Ex vivo* HLA-tetramer staining of PBMCs of a healthy HLA-A*02, -B*07-positive, HCMV-seronegative donor.**
PBMCs were stained with CD8-PerCP and HLA-B*0702/TPRVTGGGAM tetramer-PE to determine ex vivo frequency of antigen-specific T cells before stimulation. Since class I tetramers tend to bind unspecifically to B cells, PBMCs were costained with CD19. No unspecific binding of tetramers to CD19$^+$ cells was observed. HLA-tetramer staining was also performed using HLA-B*0702/RPHERNGFTVL, HLA-A*0201/NLVPMVATV and HLA-A*0201/MLNIPSINV tetramer-APC without the detection of any tetramer-positive CD8$^+$ cells. Data shown are representative of 4 independent experiments per donor.

We also confirmed these data by ELISPOT assay (Figure 3.2). A total of 1 x 10^6 PBMC were distributed in eight respectively six wells with the adequate peptide and incubated for 26 hours. As expected none of the tested HCMV-serongetive donors (Figure 3.2 A) was tested positive, in contrast to the tested seropositive donors (Figure 3.2B). As positive control PHA-L was used, as negative contol PBMCs incubated with HIV peptide or medium alone.

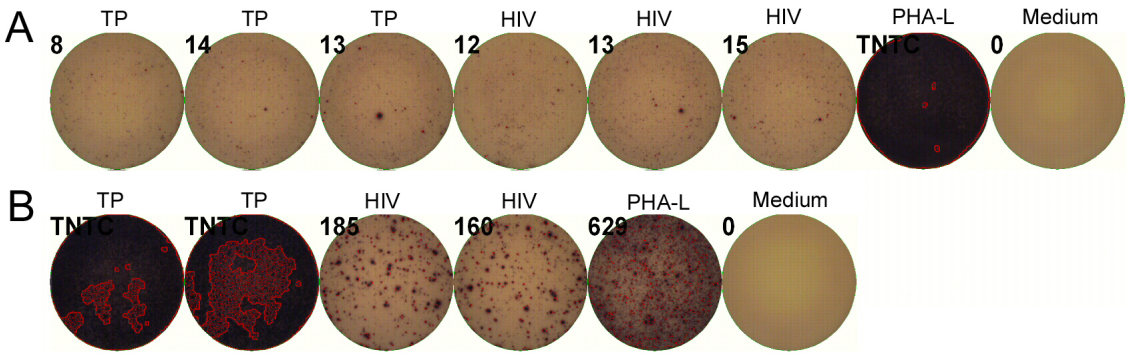

Figure 3.2: ***Ex vivo* IFN-γ-ELISPOT response of HCMV-pecific T cells from HLA-B*0702 restricted peptide TPRVTGGGAM.**
The number of T cells secreting IFN-γ in response to the HLA-B*0702 restricted peptide TPRVTGGGAM peptide and the HIV peptide in unstimulated PBMC from healthy subjects was examined. The response to the HIV peptide was used as a negative control and subtracted from the number of T cells secreting IFN-γ in response to the HCMV peptide. Results from one HCMV-seronegative donor (A) and one HCMV-seropositive donor (B) are shown. IFN-γ secreting T cells are indicated in each well. No IFN-γ producing cells could be obtained within the PBMC fraction from the HCMV-seronegtive blood donor (A), whereas T cells within the PBMC population from the HCMV-seropositive donor secreted IFN-γ in a high amount compared to negative controls (B).

Experiments were performed using aAPCs coated with HLA-B*0702/TPRVTGGGAM monomer or with B*0702-restricted RPHERNGFTVL but also with either HLA-A*0201-restricted NLVPMVATV or HLA-A*0201/MLNIPSINV monomers. $CD8^+$ T cells of 24 different healthy HCMV-seronegative donors were tested for the presence of specific T cells after three weeks of stimulation. T cells specific for NLVPMVATV were observed in 4/5 HLA-A^*02^+ donors tested (Figure 3.3 A, B). T cells restricted to MLNIPSINV, also presented on HLA-A*0201, were detected in 2/5 donors (Figure 3.3 C, D), whereas 3 of 5 donors tested were identical with donors tested for HLA-A*0201/NLVPMVATV specific T cells. Among 11 HCMV-seronegative donors expressing HLA-B*0702, 8 showed $CD8^+$ T cells specific for TPRVTGGGAM (Figure 3.3 E, F) and 4/6 donors had detectable CTL populations specific for RPHERNGFTVL (Figure 3.3 G, H). Our data reflect the degree of immunodominance of HCMV $CD8^+$ T cell epitopes, since response rates agree well with results obtained from PBMCs of healthy donors [24].

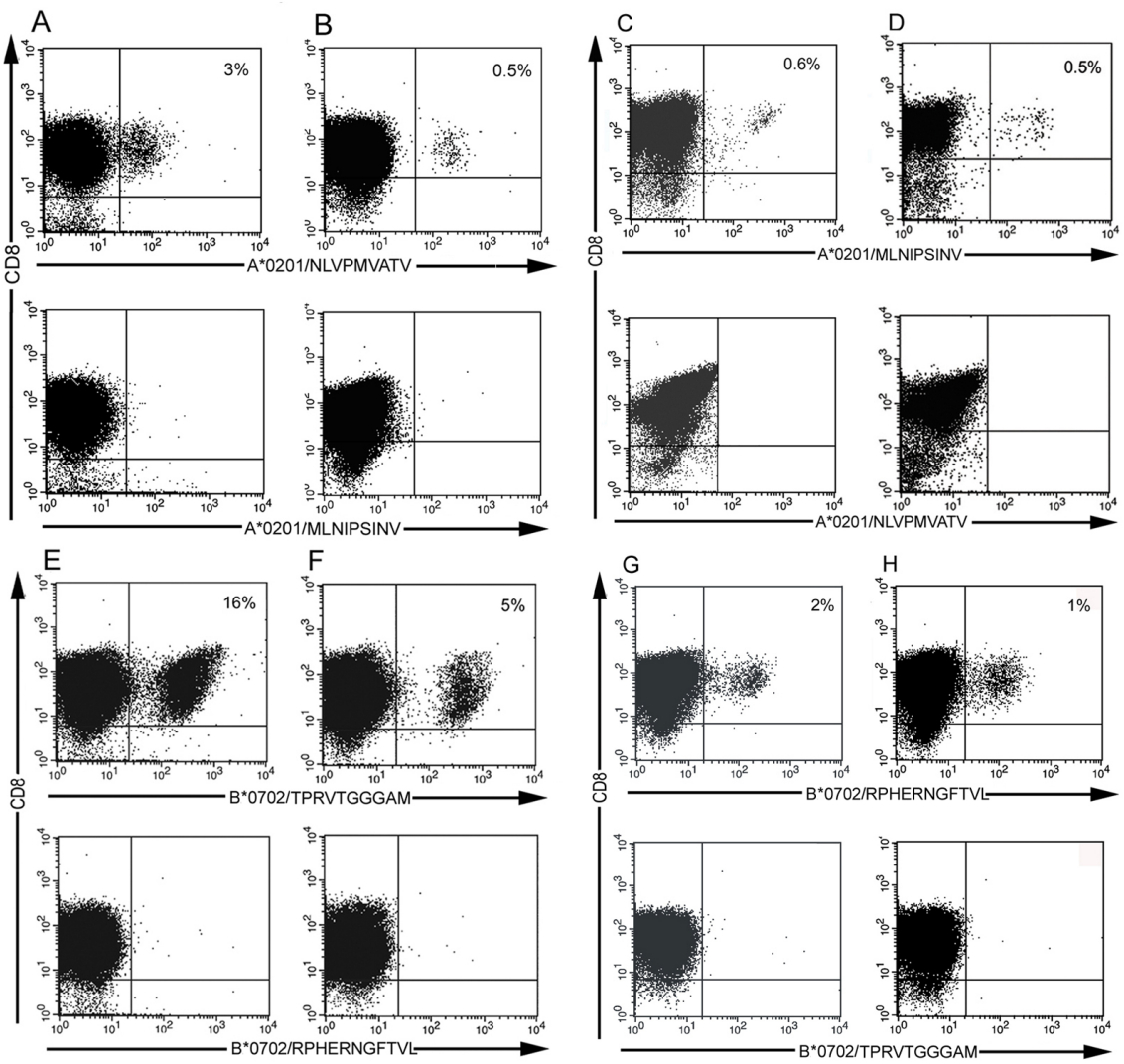

Figure 3.3: **Tetrameric analyses of bead stimulated CD8$^+$ T cells restricted to HLA-A*0201 or HLA-B*0702, from an HCMV-seronegative donor.** Representative data for stimulations of 20 wells with high density (HD, 20 nM) monomer coated beads and 30 wells with low density (LD, 0.2 nM) monomer coated beads are shown. The following HLA tetramers were used in the different panels: (A,HD; B,LD) A*0201/NLVPMVATV tetramer-APC and irrelevant A*0201/MLNIPSINV tetramer-PE, (C,HD; D,LD) A*0201/MLNIPSINV tetramer-PE and irrelevant A*0201/NLVPMVATV tetramer-APC, (E,HD; F,LD) B*0702/TPRVTGGGAM tetramer-PE and irrelevant B*0702/RPHERNGFTVL tetramer-APC, (G,HD; H,LD) B*0702/RPHERNGFTVL tetramer-PE and irrelevant B*0702/TPRVTGGGAM tetramer-APC. Additionally, cells were stained with anti-CD8-PerCP in all panels. Percentage of tetramer$^+$ cells within the CD8$^+$ T cell population is indicated in each plot. Results of stimulations using HLA-A*0201 restricted epitopes are representative of 4/5 donors for NLVPMVATV and 3/5 donors for MLNIPSINV. Similarly, results of stimulations using HLA-B*0702 restricted epitopes are representative for 8/11 independent experiments for the pp65 epitope TPRVTGGGAM and for 4/6 donors stimulated with RPHERNGFTVL.

Efficacy of *in vitro* priming and expansion

In this study we performed experiments using two loading strategies and refer to them as high density (HD) or low density (LD) stimulations. In each experiment we stimulated 20 wells that contained MACS-enriched $CD8^+$ T cells with HD aAPCs. After three weeks of stimulation 20-40% of the wells were positive. For LD stimulations we used 40 wells and usually 10-12% of them were positive (Figure 3.4). In comparison, stimulations of $CD8^+$ T cells from HCMV-seropositive donors with aAPC usually leads to $HCMV^+$ T cells in 100% of stimulated wells (data not shown). Stimulation with aAPCs led to induction of $CD8^+$ T cells with desired specificities as determined by tetramer staining (upper panels of Figure 3.3). The generated T cells were highly specific as no staining with irrelevant tetramers was observed (lower panels of Figure 3.3). *in vitro* priming with HD aAPCs yielded populations of 0.6-15% specific $CD8^+$ T cells while after LD stimulations 0.2-5% of stimulated cells from HCMV-seronegative donors were tetramer-positive. In comparison, percentages of HCMV-specific T cells varied between 0.6-62.3% after stimulation of $CD8^+$ T cells from seropositive donors ($n = 8$); the values were significantly higher than the percentages obtained with seronegative donors ($p=0.005$, Mann Whitney Test (data not shown)). In all stimulations, the number of specific T cells obtained after 3 weeks of stimulation with aAPC appeared donor dependent. Tetramer staining intensities, however, did not differ between HD and LD stimulations; thus T cell receptor avidities were similar in all stimulations.

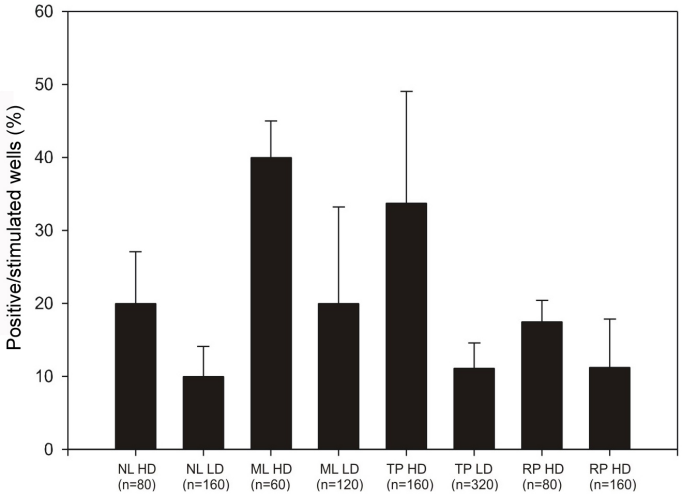

Figure 3.4: **Statistical assembly over the entire stimulations of CD8-enriched T cells with 4 different HCMV epitopes.**
$CD8^+$ T cells of 24 healthy HCMV-seronegative donors were tested for the presence of specific T cells after three weeks of stimulation. Stimulation experiments were performed using aAPCs coated with HLA-A*0201/NLVPMVATV (NL) or HLA-A*0201/MLNIPSINV (ML) monomers but also with either HLA-B*0702-restricted TPRVTGGGAM (TP) monomer or with HLA-B*0702-restricted RPHERNGFTVL (RP). Mean values of tetramer-positive wells are depicted in vertical bars for each HCMV-epitope, error bars represent the SEM. Total numbers of stimulations are indicated under each bar.

Functional characterization of *in vitro* stimulated $CD8^+$ T cells

$CD8^+$ T cells specific for HLA-B*0702/TPRVTGGGAM were analyzed on day 21 for their functional capacity to produce IFN-γ. Therefore, tetramer staining in combination with intracellular IFN-γ-staining was performed after stimulating specific T cells with peptide-loaded autologous PBMCs. As shown in Figure 3.5, $CD8^+$ T cells recognizing the epitope TPRVTGGGAM presented by HLA-B*0702 were capable of producing IFN-γ. Double staining revealed that on average, 30-50% of tetramer-specific CTLs produce IFN-γ, which emphasizes our previous notion that tetramer staining indicates specificity but not functionality of T cells [32, 33]. The vast majority of specific $CD8^+$ T cells after stimulation was $CCR7^-$ and $CD45RA^-$ indicating an effector memory phenotype (data not shown).

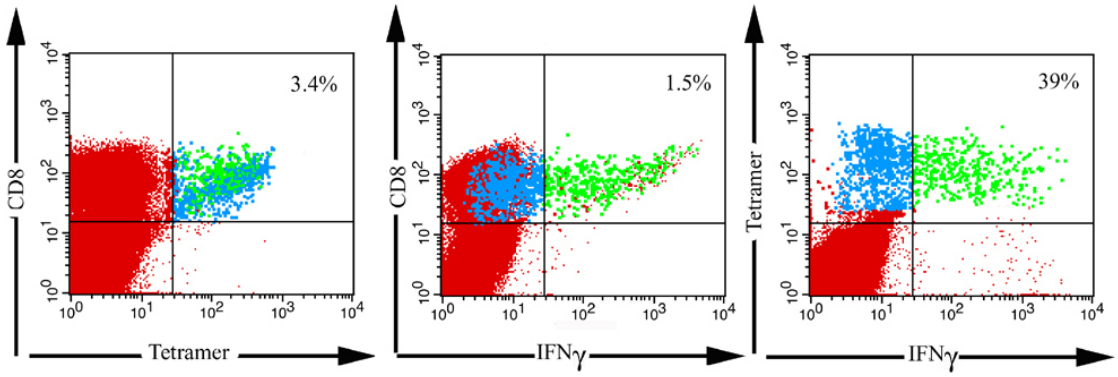

Figure 3.5: **Functionality of CD8$^+$ T cells specific for peptide TPRVTGGGAM.** Intracellular IFN-γ-staining combined with B*0702/TPRVTGGGAM tetramer-PE and CD8-PerCP surface staining. Tetramer$^+$ CD8$^+$ T cells capable of producing IFN-γ are highlighted in blue. Non-IFN-γ producing tetramer$^+$ CD8$^+$ T cells are highlighted in green. These data are representative for one of three independent experiments.

To further characterize the functionality of the generated CD8$^+$ T cells, cells were polyclonally expanded and used in a ^{51}Cr release assay to analyse their ability to lyse antigen presenting target cells. HCMV-specific CTLs did not only efficiently lyse JY cells with the cognate peptide antigen (Figure 3.6), but also allogeneic fibroblasts, peptide pulsed or infected with the HCMV strain AD169 presenting the endogenously processed epitope. The antigen specificity of the generated T cells for the HCMV pp65 peptide TPRVTGGGAM could be further confirmed by the lack of lysis of mock fibroblasts and JY cells without peptide. HCMV pp65-infected fibroblasts positive for HLA-B*0702 were used as antigen presenting target cells either loaded with peptide TPRVTGGGAM or unloaded, respectively. Specific lysis of both target cell lines could be detected by measurement of ^{51}chromium release in the supernatant of cells after 5 hours of incubation. TPRVTGGGAM-specific T cells were able to lyse target cells that naturally processed the pp65 antigen, reflecting a highly effective functionality, while HLA-matched uninfected fibroblasts (mock fibroblasts) as controls were not affected (Figure 3.6).

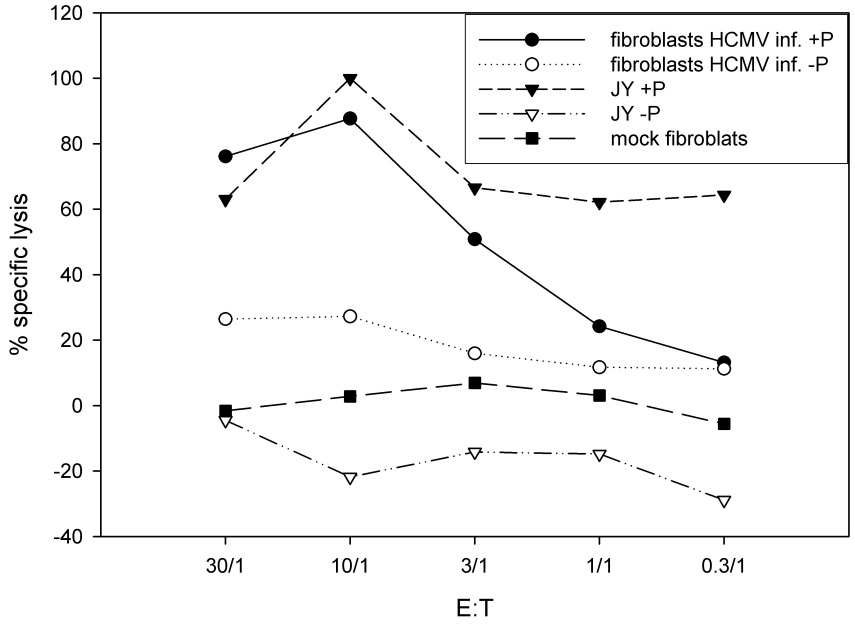

Figure 3.6: **Specific lysis of HCMV-infected cells by TPRVTGGGAM-specific T cells.**
Standard 5 h ^{51}Cr release assay was performed to analyse the lytic activity of *in vitro* primed and expanded T cells. HLA-B*0702 expressing fibroblasts were infected with HCMV and used as target cells either loaded with peptide TPRVTGGGAM or without peptide, as well as JY cells loaded with peptide TPRVTGGGAM. Unspecific lysis was evaluated using HLA-matched uninfected fibroblasts (mock fibroblasts) or JY cells without peptide.

3.1.5 Discussion

It has been demonstrated that T cell immunity is crucial to prevent HCMV reactivation, which underlines the chances of T cell based approaches in therapeutic settings. Antigen-specific CD8$^+$ T cell lines in particular have been shown to mediate protective immunity in patients who are at risk of HCMV disease, e.g. recipients of solid-organ or hematopoetic cell allotransplants [23]. Here, we present a highly efficient T cell priming methodology to generate functional CD8$^+$ T cells specific for different HCMV epitopes from HCMV-seronegative healthy donors. Biotinylated MHC:peptide complexes and biotinylated antibodies coupled onto streptavidin-coated microbeads served as artificial antigen presenting cells. For this study we used well-established immunodominant CTL epi-

topes [24], HLA-B*0702/TPRVTGGGAM, HLA-B*0702/RPHERNGFTVL, and HLA-A*0201/NLVPMVATV as stimulatory antigens as well as one subdominant epitope [24], HLA-A*0201/MLNIPSINV. A maximum of 16% tetramer-positive $CD8^+$ T cells was obtained after three rounds of stimulation with artificial APCs coated with anti-4-1BB and anti-CD28 in a 3:1 ratio, and with different concentrations of HLA:peptide complexes. Previous studies have demonstrated the generation of antigen-specific $CD8^+$ T cells of different affinities using different amounts of MHC monomers loaded onto the beads [31]. High-avidity T cells specific for tumor-associated epitopes are preferentially generated with a low concentration of MHC:peptide complexes, whereas low-avidity T cells result from stimulations with aAPCs densely coated with monomers [31]. Stimulating with a low MHC:peptide complex density will result only in proliferation of the high-avidity clones. High density beads, instead will lead to the stimulation of many low- and few high-avidity clones. The intensity of HLA-tetramer staining has been accepted as a direct measure of the affinity of TCR for the MHC-peptide-complex [34–36] and extrapolated to be an indication of the actual sensitivity of the CTL response to MHC:peptide density [37]. In contrast to Walter et al.[31] we could not observe that CTLs generated by low-density beads bound higher amounts of HLA-tetramers during tetramer analysis. This may be due to the fact that viral T cell epitopes represent "nonself" structures against which high-avidity TCRs should be available, while tumor antigens are derived from "self" and high-avidity TCRs should have been depleted during thymic selection. We could observe that aAPCs coated with the immunodominant HCMV pp65 peptide TPRVTGGGAM served as highly efficient stimulators for CTL responses from healthy donors. Although dendritic cells have been described as professional antigen presenting cells and useful tools for the stimulation of antigen-specific T cells *in vitro*, their generation is time consuming and valuable sources of cells are lost. Recent studies in our laboratory directly compared both, DCs and artificial APCs, for their efficacy as antigen presenting cells. We have shown that bead-based aAPCs are superior to dendritic cells in *in vitro* T cell priming [31, 32]. Artificial antigen presenting cells were shown to be effective stimulators not only in recall of T cell responses but also in direct priming of antigen-specific T cells. The large majority of the generated CTLs displayed an effector memory phenotype, which had been observed previously in other *in vitro* priming experiments [31, 38]. They can be distinguished from central memory T cells by their lack of the CCR7 chemokine receptor. Effector memory T cells have been shown to migrate to peripheral tissues and to exert an immediate effector function,

thereby providing protective memory [39–41]. The effector function of CTLs recognizing the epitope TPRVTGGGAM from HCMV pp65 was analysed by two functional assays: it could be demonstrated that the primed CTLs were capable of antigen-specific IFN-γ production and target cell lysis. On average, 30-50% of tetramer$^+$ T cells obtained after three rounds of stimulation were able to secrete IFN-γ. Furthermore, unspecific lysis of targets cells, which would interfere with application in adoptive immunotherapy, was not detectable. Efficient induction and expansion of HCMV-specific CTLs from seronegative individuals holds much promise for treatment of HCMV reactivation in recipients of allogenic stem cell transplantants. Nevertheless, there are still limitations in the use of HCMV epitopes in immunotherapy. The strikingly high representation of HCMV-reactive memory T cells in HCMV-seropositive subjects contrasts sharply with the almost nonexistent HCMV reactivity of T cells from HCMV-seronegative subjects. Data from Sylwester et al.[42], suggest that cross-reactivity is infrequent to nonexistent for CD4$^+$ T cells and is uncommon for CD8$^+$ T cells among the many hundreds of total HCMV epitopes. However, this finding does not mean that CD8$^+$ T cell cross-reactivity is clinically insignificant but is a limiting factor in the design of immunotherapeutic approaches covering all patients. Therefore, it is of striking importance to develop efficient and fast methodologies to generate antigen-specific T cells *in vitro*. Until now, most artificial APC approaches for antigen-specific T cell stimulation have relied on genetically engineered cell lines. Clearly, this is a suboptimal solution in clinical immunology where standardised reagents are needed. A recent study demonstrated that *in vitro* generation of virus-specific CTLs from naive human cord blood lymphocytes is feasible [24]. However, this approach is limited by the availability and the amount of cord blood lymphocytes. Taken together, artificial APCs provide several advantages compared to dendritic cells: They are a synthetic resource with commercially available tools that are easy to store. No further quality controls are necessary in contrast to using cellular APCs where levels of presented surface antigens, viability as well as vitality of the cells need to be tested reiteratively. Considering these facts, artificial APCs are an adjustable handy system that offers the possibility to align involved components and define optimal conditions for *in vitro* stimulation processes making them essential tools for *in vitro* priming and expansion of antigen-specific T cells.

Acknowledgements

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3.2 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 viruses or intracellular pathogens.
One aim of this thesis was the development of a fast and reproducible method to prime and expand functional antigen-specific $CD8^+$ effector T cells. We used the modified HLA-A*0201 restricted epitope ELAGIGILTV as a model system. Priming experiments were performed using artificial antigen presenting cells with defined MHC densities. Different settings for coating aAPCs had to be tested until we came up with an optimized ratio of 3:1 for costimuatory antibodies anti-4-1BB and anti-CD28, which enabled *in vitro* priming of high- or low-avidity effector T cells. To further characterize the T cell responses, we used fluorescently labeled MHC I tetramers as well as intracellular cytokine staining and ^{51}Cr release assay. Induced CTLs were of an effector memory phenotype and therefore fully functional. Repeated stimulation of CD8-enriched T cells of different donors pointed out that on average a fivefold higher percentage of tetramer-specific T cells could be obtained by costimulation with anti-4-1BB/anti-CD28 in a 3:1 ratio if, compared to anti-CD28 alone. The optimal ratio between anti-CD28 and anti-4-1BB, values of up to 72% of antigen-specific T cells were monitored. Similar results could be obtained for the HLA-A*0201 restricted epitope SLAPPVHNV referred to tetramer staining. T cells specific for this altered peptide ligand recognize the cognate peptide SLAPPVHNV derived from the tumor antigen MUC1.
HCMV infection is a major cause of death and disease in immunocompromised patients, especially organ transplant recipients, haemodialysis patients, cancer patients, people receiving immunosuppressive drugs and HIV-patients. Therefore, it is of utmost remaining interest to research for innovative approaches to generate antigen-specific T cells. The developed artificial priming method was highly effective in priming and expanding HCMV-specific $CD8^+$ T cells from freshly isolated $CD8^+$ T cells from HCVM-inexperienced healthy donors. As expected the induced effector T cells were capable of lysing target cells efficiently and secreted INF-γ.
The efficiency of such a highly controlled T cell stimulation system holds great promise for future therapeutic settings.

3.3 Zusammenfassung

T-Zellen sind wichtige Effektoren bei der Abwehr von Krankheitserregern im menschlichen Organismus. $CD8^+$ T-Zellen erkennen Peptide, die von MHC-Klasse I-Molekülen präsentiert werden und zerstören Zellen, die durch Viren oder intrazelluläre Erreger infiziert sind. Die vorgelegte Arbeit hatte zum Ziel, eine Methode zu entwickeln, mit der es möglich ist schnell und reproduzierbar $CD8^+$ T-Zellen gegen tumorassozierte Antigene zu induzieren und diese zu expandieren. Als Modellsystem wurde das veränderte HLA-A*0201 restringierte Epitop ELAGIGILTV, das vom Tumorantigen MelanA/Mart1 abstammt, genutzt. *Priming*-Experimente wurden mit Hilfe von Antigen-präsentierende Zellen mit kontrollierter MHC-Beladung durchgeführt. Es mussten verschiedene Beladungsstrategien für die künstlichen Antigen-präsentierenden Zellen ausgetestet werden, bis schließlich das optimale Verhältnis an kostimulatorischen Antikörpern von 3:1 (anti-4-1BB zu anti-CD28) ermittelt werden konnte. Mit dieser Kombination war es möglich *in vitro* gezielt hoch- oder niedrig-avide Effektor-T-Zellen zu generieren. Um die Funktionalität der T-Zellantworten näher zu beschreiben wurden Tetramerfärbungen, intrazelluläre Zytokinfärbungen und ^{51}Chrom-Freisetzungstests durchgeführt. Die erzeugten T-Zellen wiesen einen Effektor-Phänotyp auf und waren voll funktionstüchtig. Durch wiederholte Stimulationsversuche mit T-Zellen verschiedener Spender konnte gezeigt werden, dass 5-fach größere Tetramer-spezifsche T-Zellpopulationen mit einer 3:1-Kombination der kostimulatorischen Antikörper, im Vergleich zu anti-CD28 alleine, erzielt werden konnten. Außerdem konnten nach Stimulation T-Zellpopulationen mit bis zu 72% Tetramer-spezifischer T-Zellen damit erhalten werden. Ähnliche Ergebnisse konnten auch mit dem HLA-A*0201 restringierten Epitop SLAPPVHNV beobachtet werden, auch wenn hier noch die funktionalen Tests ausstehen. Die Infektion mit dem Humanen Cytomegalievirus (HCMV) ist eine der Hauptursachen für den Tod bzw. schwere Krankheitsverläufe, bei immunsupprimierten Patienten. Daher ist es immer noch von dringender Notwendigkeit, innovative Ansätze zur Generierung von Antigen-spezifischen T-Zellen in großer Zahl zu entwickeln. Die zuvor beschriebene *Priming*-Methode war auch bei der Induktion und Expansion von HCMV-spezifischen $CD8^+$ T-Zellen, die zuvor aus Virus-unerfahrenen gesunden Spender isoliert wurden, sehr effektiv. Die induzierten Zellen wiesen wie erwartet eine Effektorfunktion auf. Das hohe Maß an Effizienz und Kontrolle dieses Systems könnte sich bei zukünftigen immuntherpeutischen Anwendungen auszahlen.

3.4 References

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4 Appendix

4.1 Abbrevations

aAPC	artificial antigen presenting cell
Ab	antibody
APC	allophycocyanin
APC	antigen presenting cell
β2m	β_2-microglobulin
BCIP/NBT	5-bromo-4-chloro-3-indolylphosphate toluidine und nitroblue tetrazolium
BSA	bovine serum albumin
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate, succinimidyl ester
CLIP	class II-associated invariant-chain peptide
CMV	cytomegalovirus
Cr	chromium
CTL	cytotoxic T lymphocyte
DC	dendritic cell
ddH_2O	bidest water
DMSO	dimethylsulfoxid
DRiPs	Defective Ribosomal Products
EBV	epstein barr virus
EDTA	ethylendiaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ELISpot	enzyme linked immunospot assay
ER	endoplasmatic retikulum
EtOH	Ethanol
FACS	fluorescence activated cell- sorting
FCS	fetal calf serum

FITC	fluoresce inisothiocyanate
FSC	forward scatter
GvHD	graft versus host disease
HCMV	human cytomegalovirus
HD	high density
HEPES	4-(2-Hydroxyethyl)-piperazin-1-ethan-sulfonsäure
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HS	human serume
IFN	interferon
Ig	immungolobulin
IL	interleukin
KIR	killer cell inhibitory receptor
LCMV	lymphocytic choriomeningitis virus
LD	low density
MACS	magnetically activated cell sorting
MDa	megadalton
MHC	major histocompatibility complex
MW	molecular weight
n	number of experiments
NK cells	natural killer cells
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
pH	potentia Hydrogenii
PHA	phytohemagglutinin
PMA	1-(4-methoxyphenyl)propan-2-amine
PMSF	phenylmethylsulfonyl fluoride
RPMI	Roswell Park Memorial Institute

qRT-PCR	quantitative real time polymerase chain reaction
RT room	temperature
SEM	standard error of the mean
SSC	side scatter
TAA	tumor associated antigen
TAP	transporter associated with antigen processing
TBS	tris buffered saline
TCR	t cell receptor
TNF	tumor necrosis factor
TSB	tetramer staining buffer

4.2 Publications

Rudolf D.*, Silberzahn T.*, Walter S., Maurer D., Engelhard J., Wernet D., Bühring H.J., Jung G., Kwon B.S., Rammensee H.-G., Stevanović S. (2008)
Potent costimulation of human CD8 T cells by anti-4-1BB and anti-CD28 on synthetic artificial antigen presenting cells
Cancer Immunol Immunother. 57,175-83

Rudolf D., Nastke M.D., Maurer D., Wernet D., Sinzger C., Rammensee H.-G., Stevanović S.
Synthetic artificial APCs: Fast and reproducible in vitro priming of HCMV-specific T cells from seronegative donors
(submitted Journal of Immunology)

Weinzierl A.O., **Rudolf D.**, Hillen N., Tenzer S., van Endert P., Schild H., Rammensee H.-G., Stevanović S.
Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry
(submitted European Journal of Immunology)

Weinzierl A.O, **Rudolf D.**, Maurer D., Wernet D., Rammensee H.-G., Stevanović S. and Klingel K.
*Identification of HLA-A*01- and HLA-A*02-restricted $CD8^+$ T-cell epitopes 2 shared among group B enteroviruses*
(submitted Journal of Virology)

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4.4 Curriculum Vitae

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06/2005 - 02/2008	PhD student under the supervision of Prof. Dr. Stevanović Title of the thesis: "Efficient in vitro priming of tumor- and virus-specific $CD8^+$ T cells with calibrated artifical APCs" University of Tübingen Institute for Cell Biology Department of Immunology Head: **Prof. Dr. Hans-Georg Rammensee**

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04/2006	Cancer Immunotherapy 2006 meets Strategies for Immune Therapy, Mainz, Deutschland, Poster Präsentation: *In vitro* priming of HCMV-specific T cells from seronegative donors; T cell epitopes derived from RCC antigen Apolipoprotein 1
05/2006	ENII-EFIS conference; Capo Caccia (Sardinien), Italien, Poster Präsentation: *In vitro* priming of HCMV-specific T cells from seronegative donors.
09/2006	1st joint meeting of national European societies of immunology under the auspices of EFIS/ 16th European congress of immunology - ECI; Paris, Frankreich, Poster Präsentation: *In vitro* priming of HCMV-specific T cells from seronegative donors.