

**Unconventional T lymphocytes -
recombinant MHC molecules pave the way**

**Unkonventionelle T Lymphozyten -
rekombinante MHC-Moleküle eröffnen neue Wege**

D I S S E R T A T I O N

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2005

vorgelegt von
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Tag der mündlichen Prüfung:

1. April 2005

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1 General Introduction

1.1 Overview of the immune system

The term *immunity* (Latin *immunitas* - freedom from public service) is used to describe a feature that is inherent to all kingdoms of life: the capability to resist infection by at least some pathogens. Even in prokaryotes, mechanisms (restriction endonucleases and corresponding methylases) exist that can differentiate between self (self DNA) and foreign (foreign DNA), and eliminate the latter. The *immune system* of mammals is much more complex and is the sum of all organs, tissues, cells and molecules that are actively involved in the process of immunity. As the beginning of the science of immunity, i.e. *immunology*, 1796 is often cited. In this year, Edward Jenner, an English physician, injected an eight-year old boy with fluid extracted from a pustule derived from relatively harmless cowpox (*vaccinia*). Upon exposition to highly dangerous smallpox (*variola*), the boy survived. Although Jenner published his observation in 1798, the mechanisms required to its explanation were not discovered before the second half of the 20th century. Today, immunology may arguably be one of the best understood fields of medicine.

One can subdivide the immune system either topologically into *central* versus *peripheral* or alternatively, by principles of mechanism, into *innate* versus *adaptive*. The central immune system is responsible for the generation of highly specialized white blood cell (*leukocyte*) types that encompass most functions of immunity. B-lymphocytes and other cell types are generated in the bone marrow (or in chicken, in the *bursa fabricii* - hence the term B-lymphocytes), whereas mature T-lymphocytes are provided by the thymus. The peripheral immune system involves organs where these cell types interact with pathogens and derived substances, i.e. the spleen, the lymph nodes, and the gut associated lymphoid tissues (GALT).

The innate immunity system provides a first line of defence for the organism. It can react very rapidly (0-96 h) to invading pathogens via preformed effectors and effector cells that recognize pathogen-associated molecular patterns (PAMPs) which are common to distinct groups of pathogens. Its components are inflammatory cells such as granulocytes, macrophages, mast cells, the complement system, natural killer cells and subsets of B- and T-lymphocytes,

including $\gamma:\delta$ - and NK1.1^+ NK- T cells. During evolution, the innate immune system evolved much earlier than the adaptive immune system. However, as the receptors involved in innate immunity are of restricted diversity it is not as flexible as the adaptive immune system. Additionally, it can not generate an *immunological memory* (see below).

During an ongoing infection, the adaptive immune system responds later than the innate immune system. However, it is much more flexible as it can respond to a very high number of foreign structures (antigens) using receptors which specificities do not have to be genetically encoded. Furthermore, it provides immunological memory, i.e. it mounts an enduring immune response specific for the invading pathogen that will ensure more rapid elimination during the next encounter. The existence of the immunological memory has been known since very long but its mechanism remained unknown. In the 1950s, however, Macfarlane Burnet proposed the *clonal selection* theory. He postulated the existence of many different preformed cells in the body with single antigen specificity. Upon antigen contact, only those cells expressing, by chance, the corresponding specificity would be activated and become clonally expanded, leading to immunity against the given antigen. At the time Burnet reported his ideas, the cells which may be responsible for the clonal selection in the adaptive immune system were entirely unknown. We know today that these are *B- and T-lymphocytes*, which represent the *humoral* and *cell-mediated* adaptive immune system, respectively.

Humoral immunity (latin *umor* - moisture, fluid) provides antibody responses produced by activated B lymphocytes that can protect against extracellular antigens mainly via three mechanisms: *neutralization* (direct inactivation of antigen via antibody binding), *opsonisation* (engulfment of antigen-antibody conjugates by phagocytic cells) and *complement activation* (indirect inactivation of antigen via soluble factors that mediate destruction of antigen-antibody conjugates). The cell-mediated immune system can protect from intracellular pathogens (viruses, intracellular bacteria) and tumors by the help of T lymphocytes. T lymphocytes are able to differentiate between normal and altered cells by the use of their *T-cell receptor* (TCR). Most T cells express one of two types of receptor, the $\alpha:\beta$ or the $\gamma:\delta$ TCR. $\alpha:\beta$ T cells recognize processed antigen presented on major histocompatibility complex (MHC) molecules on the surface of

cells (see 1.2 and 1.3). T lymphocytes are also responsible for the coordination of many functions of the immune system, including B lymphocytes. To become themselves activated, T cells have to recognize antigen first on the surface of professional *antigen presenting cells* (APCs), most notably *dendritic cells* (DCs). These cells have the ability to take up antigen in the periphery, travel to peripheral immune organs and present antigen on MHC molecules to T cells. In order to perform this task, DCs have to be activated first by pathogenic structures using their PAMP-receptors. Therefore, one may say that DCs lie at the interface between innate and adaptive immunity. Other professional APCs include activated B-lymphocytes and macrophages.

Although other types of T lymphocytes recognizing different antigens exist within the innate immune system, the following text of the general introduction will focus on the interaction of $\alpha:\beta$ T cells recognizing classical MHC molecules.

1.2 Antigens recognized by T lymphocytes

T lymphocytes recognize antigen in the context of MHC molecules. This *MHC restriction* was first elucidated 1974 by Peter Doherty and Rolf Zinkernagel, who demonstrated that cultures of mouse T cells that killed cells infected with the lymphocytic choriomeningitis virus (LCMV) were unable to kill equally infected cells that differed only in their expressed MHC molecules [1].

In humans, MHC molecules are also called Human Leukocyte Antigens (HLA). They are encoded on a large, highly polymorphic gene cluster on chromosome six, which is usually divided into a class I, II and III antigenic region. So-called classical MHC molecules, which are linked to antigen recognition by most T cells of the adult body, lie within the class I and II region.

1.2.1 MHC class I molecules

The structure of an MHC class I molecule, HLA-A2, was first resolved using x-ray crystallography by the group of Don Wiley [2].

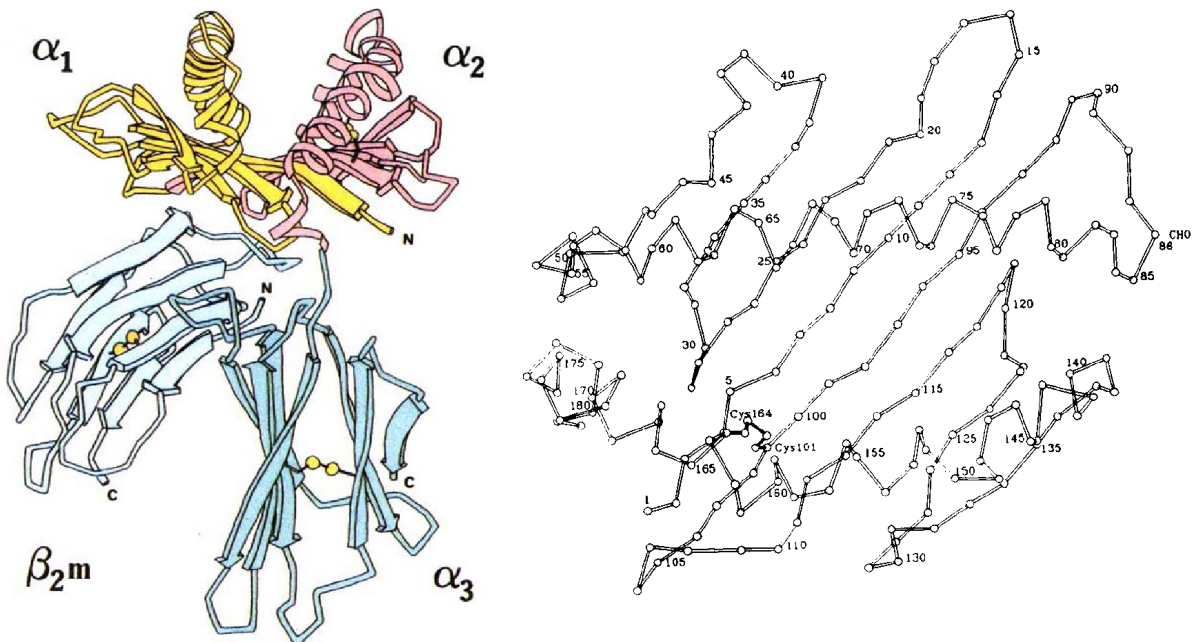


Figure 1: ELCH 1.0 Schematic representation of the secondary structure of HLA-A2 (left panel) and α -carbon backbone of the α_1 and α_2 domain (as viewed from the top). From [2].

The human HLA class I molecule was found to have one attached oligosaccharide residue (which was known not to be required for cellular expression) and three disulfide bonds. The protein is a heterodimer of a highly polymorphic membrane-spanning α (or "heavy") chain (43 kDa) and the invariable β_2 -microglobulin (12 kDa). The heavy chain folds into three domains. While α_3 and β_2m adopt an immunoglobulin-like fold, the α_1 and α_2 domains fold into a single structure consisting of two α -helices forming a groove on top of a sheet of eight antiparallel β strands. In the original report on the structure [2], the presence of an "extra electron density" in that groove was noticed. This was immediately suggested to represent a mixture of *peptide fragments* (antigenic or self) which are presented by HLA class I. This material has been subject to considerable biochemical investigation [3,4]. Today it is known that MHC class I molecules present peptides of generally 8-12 amino acids length within their binding groove. Although peptides are mostly bound via their amino- and carboxy-termini, distinct MHC class I molecules have binding preferences for distinct peptides, due to preferred binding of peptide "anchor amino acids" to residues in the binding groove of the MHC molecule. These sites are generally hotspots of MHC polymorphism and polygeny. In humans, three classical HLA I loci are described, HLA-A, -B and -C.

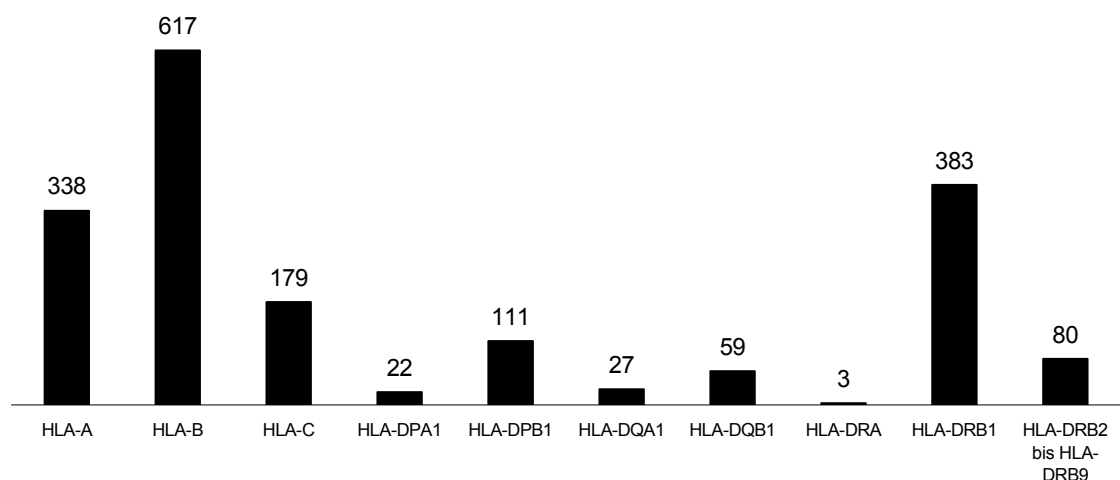


Figure 2: Reported allelic variants of classical HLA loci (including silent mutations, excluding pseudogenes) as of December 2004. Derived from the IMGT/HLA Sequence Database (<http://www.ebi.ac.uk/imgt/hla/>).

At the time the nature of antigen presented on MHC I molecules was revealed, it had been already suggested that these peptides are derived from the cellular protein turnover machinery. Indeed, it could be shown that abrogation of the main cytosolic protein degradation machinery in eukaryotes, the *ubiquitine-proteasome system*, diminishes MHC class I antigen presentation [5,6]. The constitutive 20S proteasome in humans is a 770 kD protein multimer of the structure $\alpha_7\beta_7\beta_7\alpha_7$, forming four rings of 7 homologous subunits per ring. Its inner rings contain each three proteolytically active subunits: β_1 , β_2 and β_5 . Under the influence of immunostimulation via IFN- γ , they are exchanged to the alternative subunits $i\beta_1$, $i\beta_2$ and $i\beta_5$ (LMP2, LMP7 and MECL-1), from which two are encoded in the MHC, forming the immunoproteasome [7,8], which specificity differs from the constitutional proteasome. The 20S proteasome can combine with one or two 19S cap subunits to form the 26S proteasome, which may again differ in specificity from the 20S proteasome [9]. Proteasomal degradation products were biochemically analysed and found to often contain the correct C-terminus for MHC I presentation, but to be N-terminally extended [10,11]. These and other observations using minigenes [12] led to the hypothesis that the proteasome generates N-terminally extended longer precursors of MHC class I ligands. To gain access to MHC molecules, peptides need to be transported into the endoplasmic reticulum. This ATP-dependent transport is mediated by the transporter associated with antigen processing (TAP), a heterodimer of the structure TAP1:TAP2 [13]. In humans, TAP can transport peptide of size and specificity favourable for MHC ligands [14,15], but does also transport N-terminally extended precursors [16]. Extensive experimental evidence now suggests that peptide trimming occurs in the cytosol and endoplasmic reticulum by further proteases [17-20]. It should be also noted that different mechanisms of antigen processing for MHC I have been suggested, such as peptide splicing reported recently [21,22].

Assembly of the MHC I complex is initiated in humans by association of the ER-chaperone calnexin with nascent MHC I α chains. After binding to β_2 -microglobulin, calnexin is exchanged to another chaperone complex, calreticulin and Erp57. This multiprotein complex is associated to TAP via tapasin, where peptide binding occurs. Fully folded peptide:MHC (pMHC) complexes are then released and transported to the cell surface via the Golgi apparatus [23].

1.2.2 MHC class II molecules

The first structure of an MHC class II molecule by x-ray crystallography, HLA-DR1, was also reported by the group of Don Wiley [24]. Overall the structure strongly resembles class I, although it consists of two polymorphic chains, α (34 kD, membrane-spanning) and β (29 kD) with two domains each. The peptide binding cleft is formed by the α_1 and β_1 domains and accounts for most of the structural differences between class I and class II. Due to the more open conformation of the latter, binding of longer peptides can be accommodated. Contrary to MHC class I, where larger peptides may be bound by "bulging" out of the cleft, class II peptides extend out of both ends of the site. Indeed, peptides that bind to MHC class II vary greatly in length averaging 15 amino acids [25]. Sequence analysis of these peptides revealed that they are usually nested sets representing N- and C-terminal extensions of a core sequence. In contrast to MHC class I, binding of peptides is mostly mediated via the interaction of its backbone and the binding cleft. Although binding pockets also exist for MHC class II, they appear to be more permissive than those of MHC class I, and definition of binding motifs for MHC class II alleles has proven to be more difficult.

Due to early observations that inhibition of endosomal pathways abrogated MHC class II antigen presentation, it had been suggested since long that MHC class II molecules differ from MHC class I in presenting peptides derived from the endosomal-lysosomal cellular compartment [26,27]. MHC class II ligands are generated by acidic proteases resident in this compartment, most notably *cathepsins* [28]. To be able to reach MHC II molecules, which are formed in the endoplasmic reticulum, class II molecules are protected first by binding the trimeric *invariant chain* (Ii). Assembly in the ER is assisted by the chaperone calnexin [29]. The nonameric complex $(Ii)_3(\alpha:\beta)_3$ is then targeted via Golgi [30] to the low-pH late endosomal compartment where Ii is cleaved by acidic proteases to leave only a truncated form of Ii, the class II-associated invariant-chain peptide (CLIP) [31]. Exchange of CLIP with MHC class II ligands is assisted by the non-classical MHC class II molecule HLA-DM [32]. This molecule also facilitates the continuous dissociation and binding of peptides from MHC class II, sometimes called "peptide editing", thereby favouring peptide ligands of high avidity.

1.2.3 Cross-talk between antigen processing pathways

In the classical view stated above, MHC class I presents intracellular antigens derived from the cytosol, whereas MHC class II presents antigens taken up from the outside of the cell or from invaders parasiting the endosomal compartment. In this simplistic view, these two classes of MHC are then recognized by different T cells, $CD8^+CD4^-$ and $CD8^-CD4^+$, respectively. However, indications for strong cross-talk between antigen presentation pathways are abundant. It is known that naive $CD8^+$ T cells generally need professional APCs for priming. But not all intracellular pathogens infect APCs [33]. Furthermore, there have been reports of $CD8^+$ T-cell responses to antigens restricted to the extracellular compartment [34]. The putative pathway that may allow presentation of exogenous antigens in the context of MHC class I is often referred to as *cross-presentation*, derived terms are *cross-priming* and *cross-tolerance*. Cross-priming was first reported in experiments involving priming against minor histocompatibility antigens that had to be transferred to APCs of a different MHC haplotype [35,36]. Since then, the nature of the cross-presenting cell, the mechanism of cross-presentation and its biological role *in vivo* have been subject to considerable debate (as reviewed in [37]).

On the other hand, MHC class II presentation of peptides derived from internally synthesized proteins has also been reported and was referred to as the *endogenous pathway of antigen presentation by MHC class II molecules* [38]. Its mechanism includes autophagy and is also subject of recent studies ([39] and Jörn Dengjel, unpublished data).

1.3 T lymphocytes

Even in retrospective, it is rather difficult to define an event that may be considered as the "discovery of T cells". In 1942, at a time when the immune system was believed to be entirely mediated by phagocytic cells and antibodies, Karl Landsteiner investigated the phenomenon of *delayed type hypersensitivity* reaction (DTH). His experiments to transfer it from affected to unaffected animals via serum failed, and for unknown reasons he decided to alternatively inject isolated white blood cells. He observed that he could render the recipient animal

contact sensitive to the same antigen as the donor with this method [40] (luckily, the guinea pigs must have been closely related). Spurred by World War II, several reports were made in the following years on the nature of cells that mediate tissue allograft rejection. In 1944, Peter Medawar made the observation that in contrast to autografts, skin allografts of rabbits are rejected in a reaction that involved leukocyte invasion of the graft [41].

Until the second half of the 20th century, the thymus (possibly from greek $\theta\upsilon\mu\omicron\sigma$ - life force, soul) remained the last major organ of the body whose function was still to be resolved. Between 1961 and 1962, Jaques Miller made several important discoveries on the function of this organ. First, he reported that thymectomized newborn mice kept under normal conditions invariably died later in life [42], showing a marked deficiency in circulating lymphocytes and high susceptibility to infection. Second, neonatally thymectomized mice also failed to reject skin grafts from unrelated mice [43], focusing the immunologist interest on lymphocytes leaving the thymus (later termed T lymphocytes). Finally, he reported a comparable role of the thymus after birth by demonstrating the failure of thymectomized adult mice to recover immune function after total body irradiation [44]. Identification of T cells as a distinct population became possible a few years later since the discovery of the mouse θ antigen (later termed Thy-1) [45-47].

1.3.1 The T-cell receptor

Whereas the genetic structure of the T-cell antigenic receptor was already determined in the mid-1980s, its spatial structure was described about ten years latter by X-ray-crystallography [48-50].

An $\alpha:\beta$ T-cell receptor (Figure 3) consists of two different polypeptide chains, the membrane-spanning T-cell receptor α and β chains. Each chain consists of two immunoglobulin-like domains, an amino-terminal variable V and a constant C region. The antigen-binding site is formed by the 6 complementary-determining region (CDR) loops of the V_α and V_β regions. On the surface of T cells, the $\alpha:\beta$ T-cell receptor is part of a much larger T-cell receptor complex involving several accessory molecules, most notably the coreceptors CD8 or CD4, which can directly co- interact with MHC I or MHC II, respectively, and the accessory chains

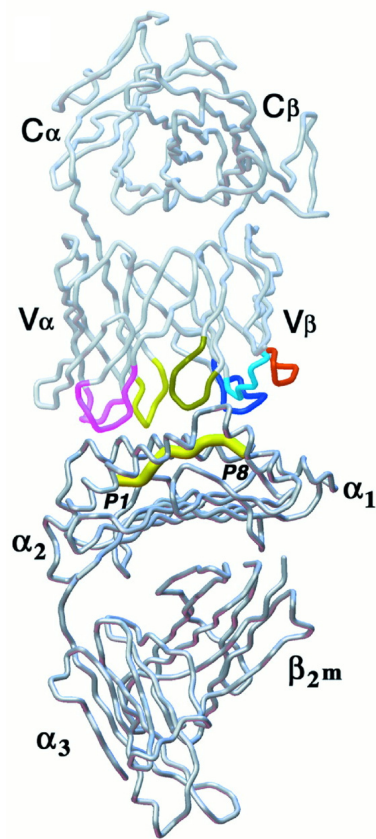


Figure 3: Backbone tube representation of the mouse 2C α : β TCR (top) complexed with the MHC class I molecule H2-K^b-SIINFEKL (bottom) [50]. The CDRs of the T-cell receptor are indicated in color:

α chain CDR1	light purple
α chain CDR2	dark purple
α chain CDR3	yellow
β chain CDR3	green
β chain CDR2	dark blue
β chain CDR1	light blue
β chain HV4	orange

CD3 δ , CD3 ϵ , CD3 ζ that are required for the complex intracellular signaling events that may result in T-cell activation (reviewed in [51]).

The genetic structures that underlie the generation of diversity within the α : β T-cell receptor repertoire were reported 1984 in a series of remarkable publications from the groups of Mark Davis and Tak Mak [52-64]. In strong resemblance to the B-cell receptor, diversity of the T-cell receptor is generated through rearrangement of many genetically encoded segments (V- and J- elements for V α , V- D- and J- elements for V β). This process, which is called *somatic recombination*, is mediated during T-cell development by an enzyme complex that contains the *recombination-activation genes* RAG-1 and RAG-2.

1.3.2 Development of T cells

The developmental stages of T cells in the mouse and human thymus are believed to be relatively well understood (as reviewed in [65,66]). Human T cells derive from a common lymphoid progenitor originating from the bone marrow that

migrates to the thymus, where further development takes place. Anatomically, the thymus consists of many lobules, separated by *trabeculae*, each being surrounded by a *capsula* and divided into an outer region, the *cortex* and an inner region termed *medulla*. Progenitor cells entering the thymus are first to be found in the junction between cortex and medulla. As they migrate through the cortex they differentiate into committed T-cell precursors (pre-T1) expressing for the first time T-cell markers such as CD1a, CD2, intracellular CD3 ϵ , CD7 and RAG-1, but lacking the cell surface CD3:T-cell receptor complex and the co-receptors CD4 or CD8 (hence the term *double-negative* thymocytes). While these cells proliferate and migrate back into the medulla, they differentiate first into CD4⁺CD8⁻ early immature single-positive cells (pre-T2 CD4ISP) and finally into CD4⁺CD8⁺ *double-positive* thymocytes. During these early development steps, rearrangement of the β , γ , and δ loci by the RAG-1:RAG-2 complex occurs. Apparently, commitment to the α : β or γ : δ lineage is determined by the receptor that is first productively expressed. If the β -locus was successfully rearranged in the first place, it pairs with a so-called surrogate α chain, pT α , to form the pre-T-cell receptor (β :pT α) of double positive α : β committed thymocytes, stopping further rearrangement but triggering proliferation. After several rounds of cell division, RAG-1:RAG-2 is reexpressed and productive rearrangement of the α locus leads to the differentiation into CD4⁺CD8⁺ TCR α : β ⁺ cells. However, the majority of TCR α : β ⁺ T cells will die by apoptosis, only a small fraction is rescued by a low-affinity interaction of the TCR with self-peptide-MHC complexes expressed on thymic epithelial cell, a process known as *positive selection*. T cells also have to undergo the process of *negative selection* that leads to clonal deletion of all thymocytes recognizing self-peptide-MHC complexes in the thymus with high avidity. At this stage, most α : β T cells lose expression of one coreceptor, becoming either CD4 or CD8 *single-positive* thymocytes. In the classical view, most α : β T cells recognizing MHC class I are destined to lose expression of CD4, whereas T cells recognizing MHC class II will become CD8 single positive cells. The nature of negative selection, which forms the fundamental basis of *central tolerance*, has recently received renewed interest due to the report of unexpected promiscuous gene expression in medullary thymic epithelial cells [67].

1.3.3 Homeostasis and priming of naive T cells

Mature T cells leaving the thymus have been termed recent thymic emigrants (RTEs) [68]. Human RTEs are characterized by the cell surface phenotype CD1A⁻CD3⁺CD27⁺CD28⁺CD45RA⁺CD45RO⁻CD62L⁺CD69⁻. A more recently described marker for RTEs are TCR excision circles (TRECs) [69]. TRECs are episomal DNA circles that are generated during excisional rearrangement of TCR genes and are non-repetitive, so they become diluted upon T-cell proliferation.

It is not surprising that RTEs show a very similar phenotype as compared to circulating naive T cells. Expression of adhesion molecules like CD62L and chemokine receptors like CCR7 contribute to the homing capacity of these lymphocytes to the peripheral lymphoid organs. Indeed, these cells continuously recirculate between the blood and the lymphatic tissues. Although the thymus is continuously releasing RTEs throughout much of the life, and T-cell clones expand upon antigenic stimulation during infection, the total number of T cells in the body remains approximately the same. On the other hand, if a host may become lymphopenic, T cells will proliferate vigorously until normal cell numbers are reached. These phenomena, called basal and acute homeostasis, have been subject to intensive investigations during the last years, especially in the mouse system. Mainly two factors have been discussed as a basis for homeostasis, competition for cytokines and for self-peptide-MHC complexes.

Table 1: Recent results on the requirements for CD8⁺ homeostasis in mice.

	naive CD8⁺ T cells	memory CD8⁺ T cells
basal homeostasis	IL-7 obligatory [70,71] self-MHC required [72,73]	IL-15 obligatory [74] self-MHC independent [72]
acute homeostasis	IL-7 obligatory [74] IL-15 not involved [71,74] self-MHC required [73]	IL-7 and/or IL-15 obligatory [74] self-MHC independent [75]

Eventually, naive T cells may encounter a pMHC complex that binds to their TCR with high avidity. For a productive outcome of this interaction, the pMHC complex should be expressed on the surface of an activated professional APC. The initiation

of the APC:T cell contact will be mostly mediated by adhesion molecule pairs, such as ICAM-1:LFA-1, ICAM-2:LFA1, DC-SIGN:ICAM-3 or LFA-3:CD2. Adhesion molecules also form the periphery of the so-called immunological synapse [76], the highly organised contact spot of the two cells. In the center of this synapse, binding of the MHC to the TCR complex takes place. According to the Bretscher-Cohn two-signal hypothesis, one of the fundamentals of immunology, [77], a second co-stimulatory signal will be required to activate naive T cells (reviewed in [78,79]). Activation requirements of previously primed T cells are less stringent. Undoubtedly, the best-characterised and most important example of costimulation is signalling via the CD28:B7 superfamily, most notably via CD28 on T cells and B7-1 (CD80) or B7-2 (CD86) on the APC. Other examples include CD27 on naive T cells and CD70 on APCs. However, once a naive T cell is getting activated, it expresses a "second line" of accessory proteins that may sustain costimulation, such as 4-1BB, ICOS and OX40, or act as negative regulators, such as CTLA-4. These receptors may bind to 4-1BBL, LICOS, OX40L and B7 on the APC, respectively. Signalling is also bi-directional, best characterised by activated CD4⁺ T-helper cells that express CD40L, which binds to CD40 on APCs, and leads to further activation of the latter [80,81]. The additionally activated APCs are prone to express higher levels of MHC and costimulatory ligands, but also more inflammatory cytokines, such as IL-12, which in turn may be important as a third signal for T-cell priming [82].

There are many reports suggesting that long-lasting activation of CD8⁺ T cells always requires CD4⁺-mediated help directed against the same antigen (recently reviewed by [83]). The cellular mechanism has been allocated to a supposed "ménage à trois" of an APC, CD8⁺ and CD4⁺ T cell. IL-2 from CD4⁺ T cells binding to the same APC has been suggested to act on the CD8⁺ T cell, and the above mentioned activation of APCs via CD40L from CD4⁺ T cells was taken as evidence. However, recent data has been in conflict with this view [84], so the last word on CD4⁺ help may not have been spoken.

Naive T cells that become successfully primed enter the cell cycle undergoing rapid proliferation [85]. They express several activation markers such as CD69, CD25 (the IL-2 receptor α chain) and secrete IL-2, an autocrine growth factor for

the dividing T cell. Costimulation during priming is essential for the synthesis of IL-2, antigen recognition without costimulation rather inactivates naive T cells, inducing a state of anergy (reviewed in [86]). Proliferating T cells may finally differentiate into an array of possible effector T cells (see below). Effector T cells express a different repertoire of adhesion molecules that facilitates their homing to the periphery, where infection takes place. In addition, a fraction of proliferated T cells will form memory T cells that last long after effector T cells have declined.

1.3.4 Effector functions of T cells

Effector CD4⁺ T cells are traditionally divided into T_H1 or T_H2 helper T cells which differ in their cytokine expression (reviewed in [87]): T_H1 cells secrete predominantly IFN- γ and TNF- α . They activate macrophages, induce B cells to synthesize opsonizing antibodies and may be important in the control of cancer via antiangiogenesis [88,89]. They can also induce target cell apoptosis via expression of FasL. On the other hand, T_H2 cells activate B cells by secreting IL-4 and IL-5, which are then induced to make neutralizing antibodies. It is now assumed that the cytokine profile during early infection, as well as the strength and duration of antigen during priming determines the differentiation of CD4 T cells into T_H1 or T_H2 effector cells (reviewed in [90]).

A mucosal subset of CD4⁺ T cells with immunosuppressive effector functions that predominantly secrete TGF- β and IL-10 has been designated T_H3 [91]. Similarly, CD4⁺ type 1 T regulatory cells (T_R1) have been reported that differentiate in the presence of IL-10 and secrete predominantly IL-10 by themselves [92]. Both T_H3 and T_R1 are believed to be derived from conventional naive α : β CD4⁺ T cells under tolerogenic conditions.

CD4⁺CD25⁺ cells were originally described as a cell population in mouse spleen and lymph nodes with immunosuppressive function [93]. Recently, many groups suggested that these cells constitute an unique subset of T cells, termed innate regulatory T cells (or simply T_{Reg}) distinct from T_H3 or T_R1 cells that may be already generated in the thymus and mediate suppression of other T cells in an so-far uncharacterized, cell-contact-dependent manner (as reviewed by [94,95]).

Conventional textbook knowledge states that naive CD8⁺ T cells differentiate into cytotoxic T lymphocytes (CTLs) that can rapidly induce apoptosis on target cells expressing cognate antigen. For this effector function, T cells rely on several mechanisms (reviewed in [96]), most notably perforin and granzymes which are stored in lytic granules of the CTL, and expression of FasL. However, CTLs can also express cytokines upon activation, particularly T_H1-like cytokines such as IFN- γ and TNF- α , that increase MHC expression on target cells and activate macrophages. By the use of these mechanisms, CTLs play a very important role for the control of viruses, intracellular bacteria and presumably cancer. CD8⁺ T cells expressing T_H1-like or T_H2-like cytokines are also sometimes referred to as T_C1 and T_C2, respectively [97]. Similar to T_R1 CD4⁺ T cells, regulatory CD8⁺ cells (T_R) that suppress immune functions via TGF- β have also been described under tolerogenic environments [98].

1.4 Analysis of antigen specific T-cell responses

The ability to analyse T-cell responses against a given antigen is essential. For example, *in vitro* experiments which are aimed at identifying T-cell epitopes always call for such assays as an end point. Monitored *in vivo* immune responses occurring during infection, transplantation, autoimmunity or after vaccination require determination of T-cell activation, too. The analysis of T-cell responses under such circumstances is a special case of or “immune monitoring” or “immunomonitoring”. This term has appeared in the literature since 1975 [99] and has been used since then for the recording and analysis of patient immunological parameters.

A general problem for the analysis of antigen specific T-cell responses is the usually low frequency of T cells occurring against a given antigen (like so many areas of biology, a needle-in-a-haystack kind of problem). The frequency of naive peripheral T cells against a given antigenic epitope has been estimated between 4×10^{-8} and 2×10^{-5} [100-102] but may increase during T-cell responses up to 6×10^{-1} among CD8⁺ T cells [103]. Any ideal T-cell assay would be a direct assay covering this full dynamic range with high linearity. Biological and technical problems arise. Naive, effector and memory may display different functions and requirements for

activation. Very large quantities of sample material would be necessary, usually human peripheral blood, which is not available in unlimited amounts. Additionally, for a biological assay, exceptional good signal to noise ratio would be necessary. So one does not wonder, although a wide variety of T-cell assays has been reported, the "ideal" assay does not yet exist.

One may divide T-cell assays into *functional* versus *specificity* assays. Within these two groups, tests can be categorized whether they will directly yield a discrete number of positive / negative cells within a sample (*single cell based assays*) or a different overall parameter for a sample (*bulk assays*). Bulk assays may be combined with the principle of limiting dilution, which will then indirectly lead to cell number information.

1.4.1 Functional T-cell assays

Effector or memory T cells encountering antigen, i.e. cognate pMHC complexes, on a target cell, respond by exerting an "effect", which may be proliferation, cytokine expression, expression of activation markers, degranulation, target cell cytotoxicity and/or trogocytosis. One can exploit these effects by challenging a T-cell sample *in vitro* with target cells expressing defined pMHC complexes and measuring the effect.

Proliferation assays can be realized as classical incorporation assays such as [³H]-thymidine [104] or 5-bromo-2'-deoxyuridine (BrdU) uptake [105], which are bulk assays that measure proliferation by incorporation of labeled nucleotide analogs into genomic DNA.

Within single cell based proliferation assays, cells are labeled first with a stable covalent dye, such as 5-(or 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) [106]. After each cell division, CFSE content of the cell will be approximately reduced by 50 %. By flow cytometry, number of cells that have been divided and are still alive as well as the number of cell divisions can be determined.

A variety of *cytokine expression* assays have been reported up to now for the detection of antigen specific cells. All these assays can be made specific for different cytokines, depending on the expected functionality of the cells. Arguably the most important cytokine for the detection of T_H1-like responses is IFN- γ which is strongly expressed after activation of T_H1-like CD8 and CD4 cells and against which favourable monoclonal antibodies have been raised in the past.

Bulk cytokine expression assays on a protein level are classic sandwich enzyme linked immunosorbent assays (ELISAs) [107] or similar protein detection assays such as the cytometric bead assay [108], which allow simultaneous or "multiplexed" detection of several cytokines within one sample. On a mRNA level, quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) has been exploited as a sensitive bulk method to detect antigen specific T cells [109].

Single cell based cytokine assays are intracellular cytokine staining [110], cytokine capture [111] and enzyme linked immunospot (ELISPOT) assays [112,113]. Cellular cytokine staining methods raise the problem that 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, which can be fluorescently labeled after permeabilization and fixation of the cells. In cytokine capture assays, cells are labeled beforehand with a "capture matrix", i.e. anti-cytokine antibodies non-covalently coupled to the cell surface. After stimulation, secreted cytokines are captured on the cell surface and can be later stained with a fluorescently labeled second anti-cytokine antibody. As this method does not require destruction of the cell, it can be combined with live cell sorting. ELISPOT is conceptually very similar to ELISA, albeit on a single-cell level. T cells and target cells are coincubated on top of a flat membrane, which is non-covalently linked to an anti-cytokine antibody. Upon stimulation, cells will secrete cytokine, which diffuse slowly and form an invisible cytokine "spot" bound to the membrane. After removal of the cells, the spot is visualized by an enzyme linked second anti-cytokine antibody that is able to form an insoluble dye from a substrate which will precipitate on the membrane. Spots can be counted manually or computer-aided.

Besides cytokines, activated T cells express other proteins that are not found on resting T cells, but which are not secreted. Common *activation markers* for T cells are the surface molecules CD69, CD25 and (for human T cells) HLA-DR. Single-cell assays have been employed [114] that use flow cytometric staining of these molecules for the detection of antigen specific T cells. Such methods are, however, hampered by the fact that these molecules are only expressed during narrow, distinct time windows and that they are also found on subpopulations of *ex vivo* isolated T cells in the absence of antigen [115].

Degranulation is a new methodology for the detection of antigen specific cytotoxic T lymphocytes. When such cells are activated, cytotoxic granula fuse with the cell membrane and release their content to the extracellular environment. Proteins, e.g. CD107a/b, which are normally found on the granule membrane become transiently located to the cell membrane. This effect, which is strongly associated to cytotoxicity, can be exploited to detect and sort antigen specific CD8⁺ cells [116] by a single-cell based assay that employs staining cells during and after activation with fluorescent anti-CD107a/b antibodies and subsequent flow cytometry.

Cytotoxicity assays are used to detect target cell lysis by an effector cell, which can be a cytotoxic T cell. Among bulk cytotoxicity assays, the oldest assay, but which may still be the most common method, is the ⁵¹Cr release assay [117]. Target cells are labeled by a radiochemical compound, ⁵¹Cr(VI)O₄²⁻, which is able to enter cells through anion transport systems and is subsequently reduced in the cytoplasm to ⁵¹Cr(III) by agents such as glutathione. As Cr(III) will reside inside the cell, cellular accumulation of ⁵¹Cr is greatly facilitated [118]. Labeled target cells are then washed and coincubated with effector cells. When cytotoxicity occurs, target cell ⁵¹Cr will be released from the cell and becomes detectable in the supernatant. This method can be modified into a competitive assay by including a second, unlabeled, target cell population ("cold target inhibition"). Disadvantages are the fact that not all target cell types are able to sufficiently uptake ⁵¹Cr(VI) and the cumbersome handling of radioisotopes. Therefore, different target cell release assays have been proposed [119], which measure e.g. the release of cytoplasmic proteins. A different method for cytotoxicity involves labeling the target cell with a covalent dye such as CFSE and, after coincubation

with effector cells, detecting target cell apoptosis and / or necrosis by using fluorescent markers and flow cytometry [120]. As this method is unable to give direct counts of functional effector cells, it should be taken as a bulk assay.

However, a new single-cell based functional assay has been proposed to directly measure the frequency of antigen specific functional cytotoxic T lymphocytes [121]. For this "Lysis spot" assay, target cells are transfected with a beta-galactosidase vector and coincubated with effector cells on top of a flat membrane, which is non-covalently linked to an anti-galactosidase antibody. Upon lysis, target cells will release beta-galactosidase, which will then form an invisible spot on the membrane. After removal of the cells, this spot is visualized by the inherent enzymatic activity of beta-galactosidase or by means of an enzyme-linked second anti-galactosidase antibody capable of forming an insoluble dye from a substrate. Spots are then counted manually or computer-aided.

Trogocytosis is an only recently described phenomenon of all lymphocytes. Within minutes during formation of a biological synapse, transfer of cell surface material from the antigen presenting cell to the effector cell occurs. The mechanism and biological function of this effect are currently unknown [122]. However, it has been already exploited to detect antigen presenting human CD8⁺ cells [123]. In such a single-cell based assay, target cells have to be engineered such as that they express the cognate HLA molecule coupled to green fluorescent protein (GFP). When pulsed with antigen and coincubated with specific T cells, the later acquire HLA-GFP from the target cell and become fluorescent, which can be detected by flow cytometry.

1.4.2 Specificity assays

As opposed to their functionality, antigen specific T cells can also be detected solely by the presence of the cognate T-cell receptor on their cell surface. This can be achieved via binding of labeled pMHC complexes, pMHC microarrays or indirectly via analysis of the T-cell receptor rearrangement.

Since recombinant pMHC class I complexes with defined peptide content have become technically achievable [124], *labeled pMHC complexes* were tested as a method to detect antigen specific CD8⁺ T cells directly in a single-cell assay via flow cytometry. However, early trials have been hampered by the fast off-kinetics of the pMHC:TCR interaction [125]. This problem has been overcome using labeled multimerized pMHC complexes [126]. In this report, multimerisation was achieved by replacing the transmembrane and cytoplasmic domain of the MHC heavy chain with a substrate peptide for the enzyme BirA ligase. After *in vitro* refolding of the three polypeptide chains to their native conformation and purification, BirA ligase catalyzes transfer of a biotin molecule to a specific lysine epsilon amino acid residue within the substrate peptide sequence [127]. After removal of excess biotin, biotinylated pMHC complexes are multimerized via streptavidin, an avidin-like protein from *Streptomyces avidinii*, which exhibits four binding sites for biotin with very high affinity [128]. Fluorescent labeling is usually accomplished by covalent modification of the streptavidin residue. Although stoichiometry of the multimeric pMHC / streptavidin complexes derived from such protocols is rather poorly defined, they are often called MHC tetramers. Later deviations of this method include biotinylated pMHC monochains instead of trimolecular complexes [129], MHC dimers [130], MHC pentamers [131] or reversible binding pMHC multimers [132]. Later were devised to facilitate sorting of "untouched" CD8⁺ T cells via FACS. Additionally, methods have been employed to magnetically enrich antigen specific CD8⁺ T cells via multimerized pMHC complexes [133]. During the last years, labeled pMHC class I complexes 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 epitope, independent of function. The development of class I reagents was not fully paralleled for MHC class II, although similar tools were devised [134,135]. This may reflect our limited knowledge of CD4 T-cell epitopes, the lower extent of expansion of specific CD4⁺ T-cell precursors compared to their CD8⁺ counterpart, the heterogeneity of CD4⁺ TCR affinities and/or increased difficulties in synthesizing stable recombinant MHC class II reagents. But also for MHC class I multimers, caveats have to be taken into account. First, pMHC does not only bind to the α : β T-cell receptor, but also interacts with the CD8 / CD4 coreceptor. As a consequence, MHC class I multimers and antibodies specific for CD8 can affect each other [136,137]. The

MHC-CD8 interaction can lead to peptide independent binding and ultimately false positive results. Point mutations have been employed to the MHC molecule as a workaround [138,139]. pMHC:TCR interactions are temperature dependent, with consequences for staining protocols [140]. Finally, MHC class I molecules are known to be ligands for molecules unrelated to the TCR/CD8/CD3 complex, i.e. natural killer cell receptors, such as immunoglobulin-like transcripts (ILTs) or killer cell immunoglobulin-like receptors (KIRs) [141-143]. pMHC multimer binding to non-CD8⁺ T cells is therefore often observed but frequently mistaken as a technical artefact.

pMHC microarrays have only been demonstrated very recently [144]. In this report, spots of defined pMHC I multimer complexes were immobilized on a two dimensional matrix and incubated with a preparation of T cells. Binding of individual T cells specific for the spotted pMHC complexes was then detected microscopically. In principle, this test may allow multiplex detection of a large number of antigen specificities within one sample. However, it remains to be proven whether the sensitivity of this methodology can compete with other established T-cell assays.

In principle, *analysis of the T-cell receptor rearrangement* allows quantitation of antigen specific T-cell clones. This is most often achieved by the "Immunoscope" approach which employs design of clonotypic primers specific for the TCR rearrangements of specific T-cell clones [145]. These primers can then be utilized for measuring their frequency by quantitative PCR on blood. Although this bulk assay does not result in direct cell numbers, it is very sensitive due to the amplification involved in the PCR process. The main disadvantages are a) the fact that T-cell clones specific for a given antigen have to be identified and isolated by different means beforehand and b) the oligoclonality of most T cell responses. Although the Immunoscope has been combined with MHC multimer sorting to facilitate isolation of T-cell clones [146], these hinderances have precluded so far the routine application of this method.

1.4.3 Limiting Dilution Assays

Bulk assays have the disadvantage that they do not result in direct cell numbers. However, when a bulk assay has the ability to discriminate between the presence or absence of at least one antigen specific T cell, it can be combined with the principle of limiting dilution. In this method, a heterogeneous T-cell population that contains an unknown frequency of antigen specific cells is diluted to ever smaller cell numbers in a multiwell plate. At one point of the dilution, some wells will receive one or more specific T cells, and some wells will not. These wells have then to be discriminated by employing the bulk assay. From the resulting data, the original frequency of antigen specific cells can be calculated using the statistical Poisson distribution function [147,148]. In practice, a powerful amplification step has to be included (usually proliferation after *in vitro* stimulation with antigen) to allow detection of single cells in one well. Therefore, this assay is strongly biased by detecting only the frequency of those antigen specific cells that have a high *in vitro* proliferation potential and do exert the specific function that is measured by the functional assay. So, although it has been employed successfully to compare the frequency of antigen specific T cells before and after *in vivo* priming [149], these counts are usually a strong underestimation when compared to frequencies determined by direct assays.

1.4.4 Comparison of different T-cell assays

When comparing different T-cell assays, one would have to evaluate systematically several parameters, such as sensitivity, dynamic range, type of information (specificity versus functionality; single cell versus bulk assay), time / cost per sample, robustness and weighting them for the specific application the assay is required for. Such comparisons have been reported, with varying degrees of sophistication [150-152]. Some aspects have emerged for the most common methods. Among direct single cell assays, MHC tetramer staining and IFN- γ ELISPOT have been reported to be more sensitive (detection limit 10^{-4} - 10^{-5}) than cytokine staining techniques. Among bulk assays, IFN- γ qRT-PCR (having a comparable sensitivity as ELISPOT or tetramer staining) appears to be superior to

ELISA or ^{51}Cr release techniques. Not surprisingly, the highest sensitivity is associated with limiting dilution assays [102]. This is currently the only method which is sensitive enough to allow quantification of normal precursor frequencies. However, it is also the most laborious and costly method and will result in strong underestimation of real frequencies. All assays that involve flow cytometry, including MHC tetramer staining, are hampered by the relatively low throughput that is technically achievable. Nevertheless, they have a great potential for very small trials via their possibility to further characterize the antigen specific cells. For medium size or larger trials IFN- γ ELISPOT is so far the most commonly applied assay, largely due to the relatively high sensitivity combined with the high throughput of this single-cell based assay.

1.5 *In vitro* manipulation of antigen specific T cells

Although methods exist that allow detection and quantification of very low T-cell frequencies *ex vivo*, their subsequent detailed characterisation requires large numbers of these cells with high purity. Detection protocols including limiting dilution also require *in vitro* amplification steps. Finally, clinical concepts of adoptive immunotherapy [153] have been suggested that involve injection of large numbers of previously amplified antigen specific T cells into patients to counteract infectious or malignant diseases. Therefore, there is need for methods to amplify antigen specific T cells *in vitro*. Such methods are hampered by the general fact that proliferation capacities of most primary tissue cell types, including human T cells, are limited and strictly regulated under normal conditions.

One can categorize these methods in assays to purify antigen specific T cells, antigen independent amplification methods and amplification methods that involve stimulation by specific antigen.

1.5.1 Purification of antigen specific T cells

If it is desirable to separate among a mixed T-cell population those which are specific for a given antigen, one has to consider different T-cell detection assays

that do not interfere with cellular viability and allow labeling the cell, which is then used as a signal to sort cells by FACS or magnetically. Alternatively or additionally, one may employ limiting dilution to derive T-cell clones.

FACS or magnetic sorting of living antigen specific cells has been reported first by exploiting functional assays after antigen stimulation. For this purpose, upregulation of *activation markers* such as CD25 or CD69 after antigen stimulation, *cytokine capture assays* [111], *degranulation assays* [116] and *trogocytosis* [123] have been suggested. On the other hand, *MHC class I and II multimers* have been employed to sort antigen specific T cells independent of functionality [126,133,138,154]. Lately, reversible binding MHC class I multimers, so called *streptamers* [132] were suggested for sorting of antigen specific T cells, as binding of conventional MHC multimers may change the properties of the sorted cells.

Cloning by limiting dilution is the oldest method to achieve expanded cell cultures of T cells with single specificity [155]. For this purpose, mixed cell populations are diluted to very small numbers per well and cultured in the presence of lethally irradiated feeder cells, mitogen or antigen. However, it is hampered by drawbacks. It is time-consuming as one does need to screen very large numbers of grown clones for correct specificity, especially when the precursor frequency was not very high (< 10%). Furthermore, T-cell clones may differ significantly in their phenotype from each other and from the polyclonal T-cell response found among the precursor cells.

1.5.2 Antigen independent amplification of T cells

Some substances can induce mitosis in most T cells independent of clonal origin and antigen specificity. Their use has greatly facilitated cultivation of T lymphocytes. However, in mixed populations, composition may change after antigen independent stimulation, as not all subpopulations will divide and survive at the same rate. For highly enriched or cloned T-cell population, however, this fact becomes less important. Antigen independent amplification of T cells is most

often achieved by employing polyclonal mitogens or stimulatory antibodies, usually in combination with mitogenic cytokines, most often IL-2.

Most *polyclonal mitogens* are lectins such as phytohaemagglutinin (PHA) [156], concanavalin A (Con A) [157] or pokeweed mitogen [158] that activate leukocytes by cross-linking glycoproteins on the cellular surface. The most commonly applied polyclonal mitogen for human T cells is PHA, a lectin extracted from the red kidney bean (*Phaseolus vulgaris*). PHA is a tetrameric protein consisting of five isolectins (L₄E₀, L₃E₁, L₂E₂, L₁E₃, L₀E₄), with the purified isolectin L₄E₀ (PHA-L or leucoagglutinin) being the most potent isoform for lymphocyte activation [159].

Antibodies that can induce T-cell stimulation have been reported, mostly against the T-cell receptor complex molecule CD3, often in combination with antibodies against costimulatory antigens such as CD28. Antibodies are usually immobilized on a solid substrate (cell culture dish, microspheres or even cell lines) to induce maximal cross-linking of target structures on lymphocytes [160,161]. More recently, such systems have become commercially available. Protocols that involve stimulation with anti-CD3 and high doses of IL-2 [162] have been employed in the clinic to expand tumor specific autologous T cells to very high numbers sufficient for successful adoptive transfer therapies [153].

1.5.3 T-cell amplification by stimulation with specific antigen

In vivo, naive antigen specific T cells are activated by professional antigen presenting cells, most importantly dendritic cells, whereas memory T cells can be activated by non-professional APCs. When driving antigen specific T cell populations into proliferation *in vitro*, approaches that attempt to mimic nature are used. Stimulation of memory T-cell populations with peptide [163], protein or encoding nucleic acids mostly employ non-professional antigen presenting cells within autologous primary cells, autologous derived B-lymphoblastoid cell lines or allogeneic cell lines, which are often defective of antigen processing to avoid allostimulation. Nevertheless, antigen presentation in the absence of important antigen processing pathways is a well-known phenomenon [164].

Classically, expansion of naive T cells is achieved by using *in vitro* derived professional antigen specific cells such as autologous monocyte derived dendritic cells [165] or autologous activated B lymphocytes [166]. Some groups have reported the use of other autologous cell types for such *in vitro* priming experiments such as activated T cells, that do also express various costimulatory molecules [167]. However, the use of autologous professional antigen presenting cells is hampered by the fact that current methods for their isolation/generation are expensive, time-consuming, difficult to standardize and can only yield relatively low cell numbers. Very different approaches have been employed to overcome this obstacle by using artificial antigen presenting cells (aAPCs). Many groups have put effort into transfecting allogeneic tumor cells with costimulatory and adhesion molecules, such as CD80, 4-1BBL, LFA-3 and ICAM-1. To avoid allostimulation in these settings, HLA-deficient cells have been used that were transfected with single peptide-HLA complexes [168]. Such cells lines are potent *in vitro* aAPCs, but have to be established beforehand for each given pMHC complex. A different approach relies on the use of isolated recombinant pMHC complexes. These complexes are coated on a surface, which can be lipid vesicles [169], plastic microspheres [170-172], or even HLA deficient cell surfaces [173]. Interestingly, it has been suggested that MHC with impaired lateral mobility may be more efficient in stimulating the TCR when compared to MHC molecules floating in a lipid bilayer [173]. aAPCs have been used in the past to study the biology of T-cell activation [82,171,173,174].

It has been reported that pMHC density during priming will strongly influence the avidity of the resulting T cell population [175]. As a part of this dissertation, the author investigated such a system with the ability to control pMHC-density, predetermining T-cell avidity.

1.6 References

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Chapter 2

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Cutting Edge: Predetermined avidity of human CD8 T cells expanded on calibrated MHC / anti CD28 coated microspheres

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Journal of Immunology 171: 4974-4978 (2003)

The author of this thesis has performed all the experiments leading to figure 1, 2, 3B, 4, S1 and S2 and wrote the manuscript.

Cytotoxic CD8 T cells are key effectors in the immunotherapy of malignant and viral diseases. However, the lack of efficient methods for their in vitro priming and expansion has become a bottleneck to the development of vaccines and adoptive transfer strategies. Synthetic artificial antigen presenting cells (aAPCs) are now emerging as an attractive tool for eliciting and expanding CTL responses. We show that by controlling the MHC density on aAPCs, high or low avidity tumor-directed human CTL lines can be raised effectively in vitro if costimulation via CD28 and IL-12 is provided. Compared to low avidity CTL lines, high avidity CTLs need 100-1000 fold less peptide for activation, bind more MHC tetramers, and, as expected, are superior in recognizing tumor cell lines expressing antigen. We believe that the possibility to raise antigen specific T cells with predetermined avidity will be crucial for the future use of aAPCs in immunotherapeutical settings.

Introduction

CD8 T cells play a central role in the host defense against viral infections and are thought to be key effectors in immunotherapy of malignant diseases. There are two main reasons to elicit and expand CTLs *in vitro*. First, following "reverse immunology", candidate T cell epitopes are first identified and their immunogenicity validated subsequently (1). Second, for adoptive transfer, large numbers of antiviral or antitumor CTLs are expanded *ex vivo* (2). However, the currently applied approaches to elicit primary *in vitro* CTL responses usually involve differentiation of DCs from the patient's blood, which is time-consuming, expensive and limited by the obtainable cell numbers.

It would, therefore, be highly desirable to have artificial antigen presenting cells at one's disposal for *in vitro* experiments. Although some studies have used tumor cell lines transfected with MHC:peptide complexes and costimulatory molecules for this purpose (3), the most consequently controlled aAPC would involve coating of synthetic surfaces, usually cell-sized plastic microspheres, with purified MHC plus costimulatory molecules. This is basically a very old idea (4) and several groups have relied on it in order to investigate the events that lead to the activation of T cells, mostly in the mouse system (5, 6, 7). Soluble MHC reagents were also used for this purpose (5), but found to be less potent when directly compared to immobilized MHC. Yet, it was only very recently (8) that aAPCs were shown to be able to fully induce *in vitro* priming of human CD8 T cells and to sustain long term cellular proliferation, as required for any immunotherapeutical approach. This last study, however, used "empty" HLA-Ig fusion proteins coated on microspheres and subsequent peptide loading. The efficiency of this process, and thus the functional antigen density on aAPCs, will strongly depend on the affinity of an individual peptide to the MHC molecule. This precluded an easy control of the number of MHC:peptide complexes on the aAPC. It is known, however, that the antigen dose will influence the avidity of the responding T cell population (9), with high avidity CTLs being superior in adoptive transfer experiments.

In this study, we rely on preformed MHC:peptide complexes coupled by biotin:streptavidin biochemistry to the surface. This systems allows the exact control of the MHC density on aAPCs which enables us to selectively elicit high- or low avidity antigen specific CTL responses with high efficiency from healthy

human individuals. Furthermore, we identify the factors that are necessary for successful *in vitro* priming with aAPCs.

Materials and Methods

Peptides, recombinant MHC molecules, fluorescent tetramers and MHC coated microspheres

Peptides in this study (10, <http://www.syfpeithi.de/>) were synthesized using standard Fmoc-chemistry. The peptide library consisted of an approximately equimolar mixture of 6912 nonapeptides with the structure Y, L/M, A/I/L/Y, E/G/P, G/K/L, I/V/L, A/V/P/H, E/K/S/T, V/L. Biotinylated recombinant A*02 molecules and fluorescent MHC tetramers were produced as described previously (11). The costimulatory mouse IgG2a anti human CD28 Ab 9.3 (12) was biotinylated using Sulfo-N-Hydroxysuccinimidobiotin as recommended by the manufacturer (Perbio Science, Bonn, Germany). As a negative control, biotinylated mouse IgG2a Ab G155-178 (Becton Dickinson Biosciences, Heidelberg, Germany) was used. For generation of aAPCs, 5.6 µm diameter streptavidin coated polystyrene particles with a binding capacity of 0.064 µg biotin-FITC/mg microspheres (Bangs Laboratories, Fishers, Illinois/USA) were resuspended at 2×10^6 particles/ml in buffer containing biotinylated MHC and Abs at indicated concentrations and incubated at room temperature for 30 min.

Antigen-specific in vitro stimulation of human CD8 T cells

PBMCs were isolated from fresh buffy coats using standard gradient separation. When indicated, untouched CD8 T cells were MACS enriched by negative depletion (Miltenyi Biotec, Bergisch Gladbach, Germany).

To compare DC to bead stimulations, monocyte derived human DCs were generated as previously described (13) and activated with 10 ng/ml human TNF- α (R&D Systems, Wiesbaden, Germany) + 1 µg/ml human prostaglandin E₂ (Sigma-Aldrich, Taufkirchen, Germany) for 2-3 days. Mature DCs were predominantly CD14⁻ CD40⁺ CD80⁺ CD83⁺ CD86⁺ and HLA-DR^{hi} (data not shown). For restimulations after priming with DCs, cryopreserved autologous PBMCs were used.

In vitro stimulations were initiated in 24 well plates with 5×10^6 responder cells plus 1×10^6 beads or 1×10^6 irradiated APCs per well in 1.5 ml T cell medium which consisted of RPMI 1640 containing 25 mM HEPES (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10 % heat inactivated human AB serum (CC pro, Neustadt/W., Germany), 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 20 µg/ml gentamycin (all BioWhittaker/Cambrex Bio Science, Verviers, Belgium). If not stated otherwise, 5 ng/ml human IL-12 p70 (R&D) was added with APCs or microspheres. After 3-4 days coincubation at 37 °C, fresh medium and 20 U/ml human IL-2 (R&D) was added and cells were incubated for 3-4 days. This stimulation cycle was repeated twice.

Antigen specific T cell enrichment and expansion

FACS sorting of bead stimulated cells was performed on a FACSVantage after staining with PE tetramers and Abs CD8-APC clone SK1 and CD4-FITC (BD).

Alternatively, bead stimulated cells were restimulated with irradiated T2 cells pulsed with 5 µM peptide as described above and IFN- γ^+ cells were enriched by MACS (Miltenyi).

Sorted cells were cultured in the presence of 5×10^5 cells/ml irradiated fresh allogenic PBMCs, 5×10^4 cells/ml irradiated LG2-EBV cells, 150 U/ml IL-2 and 0.5 µg/ml PHA-L (Roche Diagnostics, Mannheim, Germany). Cells were further expanded in T-cell medium containing 150 U/ml IL-2.

Cell surface / intracellular cytometric analysis

Tetrameric analyses were performed with fluorescent MHC tetramers plus Abs CD4-FITC and CD8-PerCP clone SK1 on a four-color FACSCalibur (BD). Total specific cell numbers per sample were calculated by FACS analysis as follows after adding a defined number of microspheres to each sample: (specific cells counted) \times (microspheres added) / (microspheres counted).

Intracellular cytometry was performed using a Cytofix/Cytoperm Plus kit with Abs CD4-FITC, IFN- γ -PE + CD8-PerCP and analysed on a FACSCalibur cytometer (BD).

Cytotoxicity assay

Cytotoxicity was tested in a standard 4 h ^{51}Cr release assay using 3000 target cells per well. % specific lysis was calculated as follows: (experimental release - spontaneous release) / (total release - spontaneous release) \times 100.

Results and Discussion

MHC / anti-CD28 coated microspheres are powerful tools for in vitro priming

Streptavidin linked 5.6 μm polystyrene microspheres could be easily coated with biotinylated MHC molecules and costimulatory anti-CD28 Ab. Moreover, labeling, as indicated by immunofluorescence, remained almost constant over a storage period of 4 weeks (data not shown). Therefore, such particles have immense practical advantages over the use of DCs, which can only be generated in limited amounts in a time-consuming manner.

To test the capacity of beads as APCs, human CD8 T cells were stimulated for three 7-9 day rounds in the presence of IL-12 with beads coated with anti-CD28 Ab plus 10 nM A*02 bound epitopes derived from a viral antigen (CMV pp65), a modified self antigen (Melan-A) or a tumor derived self antigen (MET proto-oncogene). As determined by tetramer analysis, stimulation with beads led in all cases to a specific CTL expansion with the correspondent specificity (Fig. 1, *middle panel*). There was no staining with an irrelevant tetramer (Fig. 1, *right panel*). When stimulated with beads containing irrelevant MHC molecules, a specific tetramer⁺ population was visible only in the case of the virus recall antigen (Fig. 1 *left panel*, the donor in this experiment was HCMV seropositive). The stimulation with the modified self antigen from Melan-A was especially efficient, which was also confirmed in experiments with PBMCs from different donors (later figures and data not shown). This is consistent with the common observation of relatively high precursor frequencies in the blood of healthy A*02⁺ donors against this peptide. However, as these cells appear to be naive (14) and require professional APCs to be expanded *in vitro* (15), our findings indicate that beads were capable of efficient *in vitro* priming.

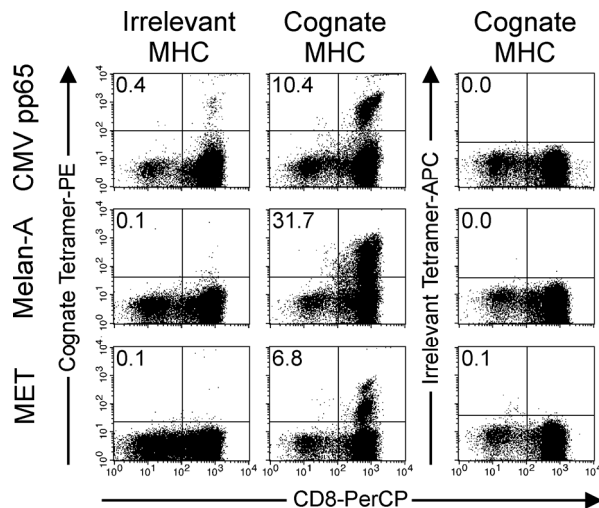


FIGURE 1. Tetrameric analysis of microsphere driven expansions. Enriched CD8 T cells of one A*02⁺ healthy donor were stimulated 3 times with beads coated in the presence of 10 nM CD28 Ab plus either 10 nM of an irrelevant A*02 complex (*left panel*) or A*02 refolded with indicated antigens (*middle and right panel*). All T cell lines were surface stained with CD8-PerCP Ab, cognate tetramer-PE (*left and middle panel*) and irrelevant A*02/ILKEPVHGV tetramer-APC (*right panel*). Percentage of tetramer⁺ cells among CD8⁺ lymphocytes is indicated in each plot.

Microsphere expanded CTLs are functional

The expanded T cells were functional, since they specifically expressed IFN- γ upon restimulation with peptide, as indicated by intracellular cytokine staining. Interestingly, this was also the case when beads were used for CTL priming that were coated with an A*02/peptide library, indicating that aAPCs may also be useful for stimulating with complex antigen mixtures (data not shown).

To investigate the cytotoxic capabilities of expanded CD8 T cells, fractions from T cell lines were sorted and subsequently expanded using mitogen and IL-2. T cells proliferated strongly for at least 3 months under such conditions (data not shown). Specific cytotoxicity was shown for all tested CTLs and was confined to the tetramer⁺ or IFN- γ ⁺ fraction (Fig. 2).

CTL priming by low MHC density aAPCs requires costimulation and IL-12

These data indicated that bead expanded CTLs were antigen specific and functional. However, several of these CTL lines did not recognize target cells

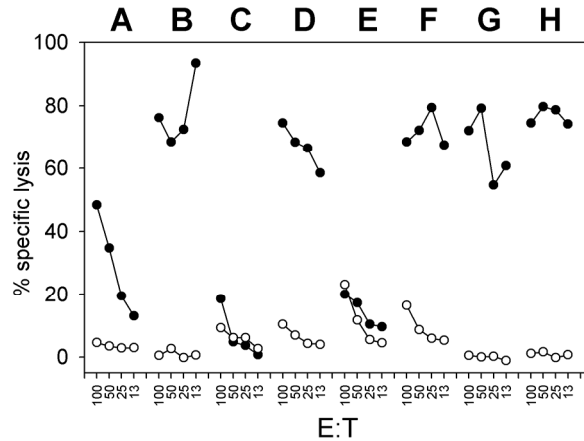


FIGURE 2. Cytotoxic activity of sorted fractions derived from microsphere expanded T cell lines. Enriched CD8 T cells of one A*02⁺ healthy donor were stimulated 3 times with beads labeled in the presence of 10 nM CD28 Ab plus 10 nM of A*02 refolded with either the viral peptide NLVPMVATV (A-E), the modified self peptide ELAGIGILTV (F), the self peptide YVDPVITSI (G) or a 6912 peptides library (H). The unsorted T cell line (A), the tetramer⁺ sorted cells (B, F, G), the tetramer⁻ sorted cells (C), the IFN- γ ⁺ sorted cells (D, H) or the IFN- γ ⁻ sorted cells (E) were expanded using mitogen as detailed in *Materials and Methods*. Specific killing of T2 cells loaded with 5 μ M cognate peptide (filled circles) or irrelevant peptide (open circles) is shown. Cognate peptide was always the peptide or peptide library used for priming.

endogenously processing antigen (data not shown). As an explanation, we speculated that beads coated with 10 nM MHC (high density beads) as used for the above experiments may lead to the preferential expansion of low avidity CTLs. To test this hypothesis, we titrated the specific MHC molecules during bead coating. Efficient expansions were still observed using 100 fold less of the specific MHC molecules (low density beads) than for above experiments (Fig. 3A, *left plot*). The results were similar when total MHC concentrations were kept constant by adding an MHC library (Fig. 3A, *right plot*) as a "filler".

All stimulations described above were performed in the presence of CD28 Ab on aAPCs and exogenous IL-12. To identify obligatory factors for priming, we varied stimulation conditions (Fig. 3B). Using high density beads, T cell lines could be generated even in the absence of costimulatory CD28 Ab or exogenous IL-12, although efficiency was greatly reduced when both were missing. For low density beads, however, the presence of CD28 Ab as well as exogenous IL-12 was obligatory for successful *in vitro* priming. To compare efficiency with a well

established protocol, this was done in parallel with one stimulation by peptide pulsed autologous mature DCs and restimulations by autologous peptide pulsed PBMCs. Compared to stimulations by DCs / PBMCs, MHC coated high or low density beads were at least as efficient or even superior, especially in terms of total numbers of specific cells present. Exogenous IL-12 p70 also enhanced CTL responses initiated by DCs + PBMCs, which is well in accordance to previous studies describing importance of IL-12 p70 in CD8 T cell priming (16). These data support a 3 signal model for the priming of human CD8 T cells that has been suggested previously for mice (7). Nevertheless, signals 2 + 3 (ligation of costimulatory receptors as CD28 and inflammatory cytokines as IL-12) may become redundant in the presence of an extremely strong signal 1, as shown previously for mice (6).

During tetramer analysis, we noted that CTLs generated by low density beads bound higher amounts of MHC tetramers (Fig. 3C). According to some previous studies, this could indicate higher avidity of CTLs (17), although this may not always be the case (18).

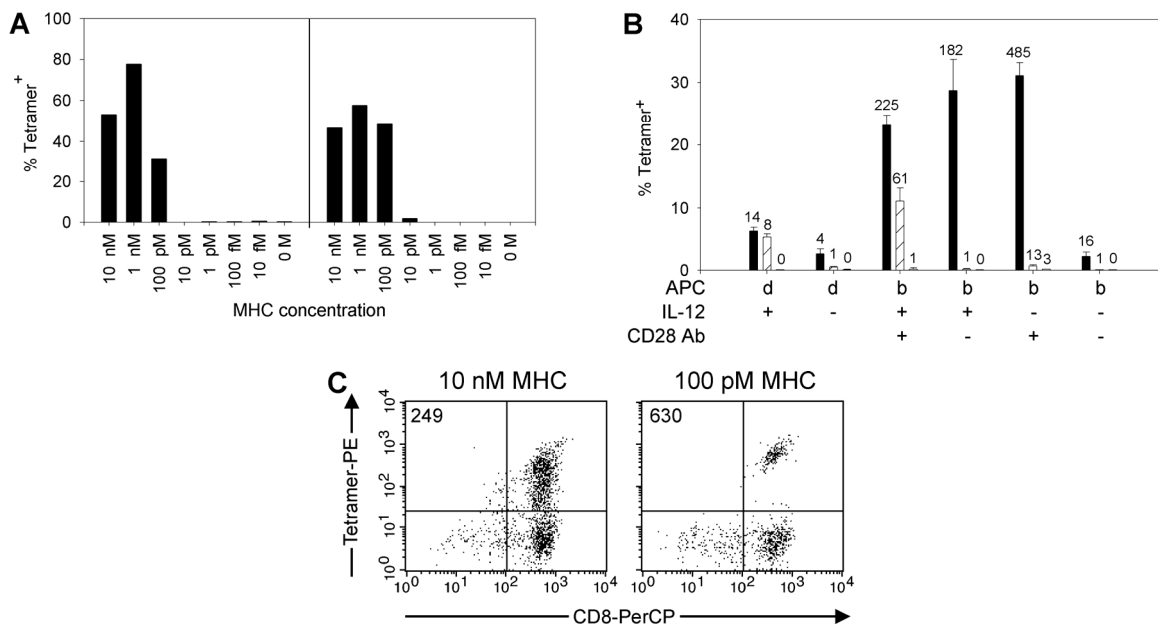


FIGURE 3. Expansion of CD8 T cells using different MHC densities. A, PBMCs of one A*02⁺ healthy donor were depleted from adherent cells and stimulated 3 times with beads labeled in the presence of 10 nM CD28 Ab plus varying concentrations of A*02/ELAGIGILTV (*left panel*), optionally with the addition of A*02/library to reach a constant MHC concentration of 10 nM

(right panel). Shown percentage of specific tetramer⁺ cells among CD8⁺ CD4⁻ lymphocytes in 1 representative out of 3 independent experiments from different donors was determined by staining cells with CD4-FITC, CD8-PerCP, cognate tetramer-PE and irrelevant A*02/ILKEPVHGV tetramer-APC. B, PBMCs of another A*02⁺ donor were depleted from adherent cells and stimulated 3 times. APCs were either autologous DCs and PBMCs (d) or beads (b). 5 ng/ml IL-12 p70 was added (+) or omitted (-) at each stimulation. Abs coated on beads were 10 nM CD28 Ab (+) or 10 nM IgG2a control Ab (-). DCs were pulsed with 5 μM (solid bars), 500 nM (dashed bars) or 0 M (open bars) peptide ELAGIGILTV. MHC coating on beads with A*02/ELAGIGILTV was 10 nM (solid bars), 100 pM + 10 nM A*02/library (dashed bars) or only 10 nM A*02/library (open bars). Cells were stained with Abs CD4-FITC, CD8-PerCP, cognate tetramer-PE and irrelevant A*02/ILKEPVHGV tetramer-APC. Bars represent mean and SD of the percentage of specific tetramer⁺ cells among CD8⁺ CD4⁻ lymphocytes from 3 stimulations. The bar belonging to stimulation with 10 nM A*02/ELAGIGILTV, + IL-12 and - CD28 Ab was derived from only 2 stimulations. Numbers above each bar indicate mean absolute number of specific tetramer⁺ CD8⁺ CD4⁻ lymphocytes per well as described in *Materials and Methods*. C, Tetramer stainings of two T cell lines marked in (A) with asterisks. Plots are gated on CD4⁻ lymphocytes. Numbers in each plot represent mean PE tetramer fluorescence of cells in the upper right quadrant.

MHC density during priming influences avidity of resulting CTLs

To clarify the question of the avidity of CTLs primed by high or low density beads, the amount of antigenic peptide was titrated on target cells in a standard ⁵¹Cr release assay (Fig. 4A). The T cell line generated with high density beads needed approximately 10 nM of peptide for recognition whereas the line generated with low density beads was of much higher avidity and needed only picomolar amounts of peptide for an efficient recognition. Therefore, the antigen density of aAPCs influenced the overall avidity of the responding T cell population. As we used "filler" MHC molecules on the bead surface to ensure a constant overall MHC density, this effect is likely due to less cognate antigen recognition and not due to less CD8 binding. The most ready explanation for this finding is that the naive CD8 T cell precursor pool consisted of different clones with a broad spectrum of avidities. High avidity clones are likely to be rarer for a given MHC:peptide complex. When stimulating with a low determinant density, only the high avidity clones will proliferate. A high antigen dose will lead to the stimulation of many low and few high avidity clones. Furthermore, high avidity clones may be overtly stimulated by high antigen densities and thus die due to exhaustion effects. Overall, these processes would explain the observed results.

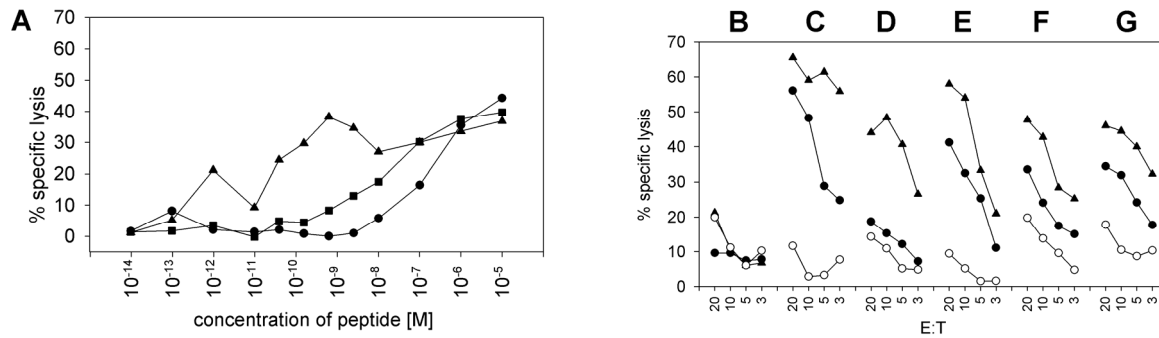


FIGURE 4. Cytotoxic analysis of CD8 T cells expanded with different MHC densities. A, PBMCs of one A*02⁺ healthy donor were depleted from adherent cells and stimulated 3 times with beads labeled in the presence of 10 nM CD28 Ab plus A*02/ELAGIGILTV at a concentration of 10 nM (circles), 1 nM (squares) or 100 pM (triangles) with addition of A*02/library to reach always a total MHC concentration of 10 nM. T cell lines were incubated with ⁵¹Cr labeled T2 cells loaded with varying amounts of peptide ELAGIGILTV at a constant E:T ratio of 4:1 based on the number of tetramer⁺ CD8⁺ CD4⁻ cells. B-G, Enriched CD8 T cells of another A*02⁺ healthy donor were expanded by 3 stimulations with beads labeled in the presence of 10 nM CD28 Ab plus A*02/ELAGIGILTV at a concentration of 10 nM (filled circles), 100 pM (triangles) or 0 M (open circles) with addition of A*02/library to reach always a total MHC concentration of 10 nM. T cell lines were incubated with varying ⁵¹Cr labeled target cell lines at indicated total E:T ratios. Frequency of tetramer⁺ CD8⁺ cells in both A*02/ELAGIGILTV stimulated CTL lines was 40 %. Target cell lines were Melan-A⁻ A*02⁺ JY cells pulsed without (B) or with (C) 5 μM peptide, a Melan-A⁺ A*02⁺ cell line from our dermatology department without (D) or with (E) 5 μM peptide, or Melan-A⁺ A*02⁺ MeWo cells without (F) or with (G) 5 μM peptide.

Finally, we tested whether bead generated T cell lines could also recognize tumor cells expressing Melan-A and A*02 (Fig. 4B-G). One of such tumor cell lines was solely recognized (Fig. 4D) and one other cell line was much better recognized (Fig. 4F) by T cells primed with low density aAPCs. Recognition was specific, as shown by an irrelevant T cell line from the same donor (Fig. 4B-G) and an irrelevant Melan-A⁻ A*02⁺ target cell line (Fig. 4A).

Concluding remarks

By controlling the surface density of MHC molecules on coated microspheres, we were able to prime at will high or low avidity tumor directed human CD8 T cells *in vitro*. Since our method is highly efficient, it could be useful for clinical tumor immunotherapy. MHC coated microspheres provide the most rigorously controlled

antigen presenting "cells" available and are a powerful tool that allows new insights in key parameters necessary for effective T cell responses.

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Supplementary material for Walter S et al: Cutting Edge: Predetermined avidity of human CD8 T cells expanded on calibrated MHC / anti CD28 coated microspheres (not included in publication)

Materials and Methods

Cells

The TAP-deficient cell line T2 (HLA-A*0201^{low}) (ATCC # CRL-1992), the EBV-transformed lymphoblastoid B cell lines JY (HLA-A*0201⁺ Melan-A⁻) (ECACC # 94022533) and LG2-EBV (HLA-A*0201⁻) (1), the melanoma cell line MeWo (HLA-A*0201⁺ Melan-A⁺) (ATCC # HTB-65) and a melanoma cell line from the Department of Dermatology, University of Tübingen (HLA-A*0201⁺ Melan-A⁺) were used in this study. HLA typed fresh buffy coats were provided by the blood bank, University of Tübingen, as approved by the local ethical committee.

Peptides, recombinant MHC molecules, fluorescent tetramers and MHC coated microspheres

Peptides for MHC refoldings and cellular assays were synthesized using standard Fmoc-chemistry. Peptides in this study were NLVPMVATV from HCMV pp65 495-503 (2), ILKEPVHGV from HIV-1 RT 476-484 (3), ELAGIGILTV as a modification

from Melan-A/MART-1 26-35 (4, 5), YVDPVITSI from MET proto-oncogene 654-662 (6) and an approximately equimolar library of 6912 nonapeptides with the structure Y, L/M, A/I/L/Y, E/G/P, G/K/L, I/V/L, A/V/P/H, E/K/S/T, V/L.

Biotinylated recombinant HLA-A*0201 molecules lacking the transmembrane domain and being biotinylated at the carboxy terminus of the heavy chain were produced as previously described (7, 8). Fluorescent tetramers were generated by coincubating biotinylated HLA-A*0201 with streptavidin-PE or streptavidin-APC (Molecular Probes, Leiden, The Netherlands) at a 4:1 molar ratio.

The purified costimulatory mouse IgG2a anti human CD28 Ab 9.3 (9) was chemically biotinylated using Sulfo-*N*-hydroxysuccinimidobiotin under conditions recommended by the manufacturer (Perbio Science, Bonn, Germany). As a negative control, irrelevant biotinylated mouse IgG2a Ab G155-178 (Becton Dickinson Biosciences, Heidelberg, Germany) was used. Microspheres used 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). For microsphere handling, a 0.2 μm sterile filtered PBS/BSA/EDTA buffer consisting of of PBS (BioWhittaker/Cambrex Bio Science, Verviers, Belgium) supplemented with 0.5 % protease-free BSA and 2 mM sodium EDTA (both Sigma-Aldrich, Taufkirchen, Germany) was used. For coupling to biotinylated molecules, microspheres were washed and resuspended at 2×10^6 particles/ml in buffer containing biotinylated MHC and / or antibodies at described concentrations. Binding was allowed at room temperature for 30 min while agitating. Coated beads were washed three times, resuspended in above buffer and stored for up to 4 weeks at 4 °C before use. Bead coating was assayed with F(ab')₂ fragment goat anti mouse IgG + IgM PE (Jackson ImmunoResearch, West Grove, Pennsylvania/USA) and anti human β -2-M R-PE (Cymbus Biotechnology, Chandlers Ford, UK) in a standard immunofluorescence experiment.

Antigen-specific in vitro stimulation of human CD8 T cells

PBMCs were isolated from fresh buffy coats using standard gradient separation medium (Linaris, Wertheim-Bettingen, Germany or PAA Laboratories, Linz, Austria). When indicated, untouched CD8 T cells were magnetically enriched by negative depletion using a CD8 T cell isolation kit (Miltenyi Biotec, Bergisch

Gladbach, Germany) according to the manufacturer's conditions, which resulted in a purity of CD8⁺ TCR $\alpha\beta$ ⁺ cells of more than 80%.

To compare dendritic cell to bead stimulations, dendritic cells were derived from human monocytes as previously described (10) with minor modifications. Briefly, PBMCs were allowed to adhere to plastic for 60 minutes at 37 °C in DC medium which consisted of X-Vivo 15 supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 20 µg/ml gentamycin (all BioWhittaker). Non-adherent cells were removed by washing, centrifuged and resuspended at 1×10^7 cells/ml in T cell medium which consisted of RPMI 1640 containing 25 mM HEPES (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10 % heat inactivated human AB serum (CC pro, Neustadt/W., Germany), 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin and 20 µg/ml gentamycin (all BioWhittaker). 10 U/ml human IL-2 and 2.5 ng/ml human IL-7 (both R&D Systems, Wiesbaden, Germany) were added to non-adherent cells which were kept at 37 °C for up to 9 days. Adherent cells were given DC medium containing 100 ng/ml human GM-CSF (Leukomax; Novartis Pharma GmbH, Nuremberg, Germany) plus 40 ng/ml human IL-4 (R&D) and differentiated into immature dendritic cells at 37 °C. At day 3, fresh DC medium containing IL-4 and GM-CSF was added. At day 6, cells were matured by adding fresh DC medium containing 10 ng/ml human TNF- α (R&D) and 1 µg/ml human prostaglandin E₂ (Sigma). Cells that had lost adhesion were collected at day 9, irradiated with 33 Gy using a 1000 Elite gammacell (MDS Nordion, Ottawa, Canada) and used as professional APCs. Mature DCs were mostly CD14⁻ CD40⁺ CD80⁺ CD83⁺ CD86⁺ and expressed high levels of HLA-DR (data not shown). For restimulations after priming with dendritic cells, cryopreserved autologous PBMCs were thawed, washed, irradiated as described above and used as non-professional APCs.

Peptide loading was performed at 37 °C in X-Vivo 15 medium containing indicated concentrations of peptide for approx. 2 hours. APCs were washed 5 times and resuspended in T cell medium. Coated microspheres stored at 4 °C were washed 2 times in T cell medium.

In vitro stimulations were initiated in 24 well plates with 5×10^6 responder cells plus 1×10^6 APCs or microspheres per well in 1.5 ml T cell medium. If not stated otherwise, 5 ng/ml human IL-12 p70 (R&D) was added with APCs or microspheres. After 3-4 days coincubation at 37 °C, fresh medium and 20 U/ml

human IL-2 (R&D) was added and cells were further incubated at 37 °C for 3-4 days. This stimulation cycle was repeated twice.

Sorted cells were cultured in 24 well plates in the presence of 5×10^5 cells/ml 33 Gy irradiated fresh allogenic PBMCs, 5×10^4 cells/ml 210 Gy irradiated fresh LG2-EBV cells, 150 U/ml IL-2 and 0.5 µg/ml PHA-L (Roche Diagnostics, Mannheim, Germany). Cells were further expanded in T-cell medium containing 150 U/ml IL-2.

Cell surface / intracellular cytometric analysis

For tetrameric analyses, cells were washed in PBS/BSA/EDTA containing 10 mg/ml sodium azide (Merck, Darmstadt, Germany) and stained at 4 °C for 20 minutes in the same buffer containing Abs CD4-FITC and CD8-PerCP clone SK1 (both from Becton Dickinson). After microsphere stimulation experiments, 100 µg/ml unlabeled streptavidine (Sigma) was included. Cells were washed in PBS containing 2 % heat-inactivated FCS (PAN Biotech, Aidenbach, Germany), 2 mM sodium EDTA and 10 mg/ml sodium azide and tetramer stained at 4 °C for 30 minutes in PBS/FCS/EDTA/Azide but including 50 % FCS. Tetramer reagents were always used at MHC concentrations of 5 µg/ml. Stained cells were washed extensively in PBS/FCS/EDTA/Azide and fixed with 1 % formaldehyde (Merck). Cells were analysed on a four-color FACSCalibur (Becton Dickinson). Total specific cell numbers per sample were calculated by adding a defined number of microspheres to each sample and counting specific cell numbers as well as microsphere numbers by FACS. Therefore, (total specific cell number) = (number of specific cells counted) x (number of microspheres added) / (number of microspheres counted).

Peptide pulsing of stimulator cells for intracellular cytometry was performed as described above. Approximately 1×10^5 effector and stimulator cells were cultured in 96 well plates in T cell medium. After 1-2 hours, GolgiStop (Becton Dickinson) was added and incubation was continued for 4-5 hours. Thereafter, cells were permeabilized and stained using the Cytofix/Cytoperm Plus kit and anti-CD4-FITC, anti-IFN- γ -PE, and anti-CD8-PerCP, according to the manufacturer's recommendations (Becton Dickinson). Cytometric analysis was performed using a FACSCalibur cytometer.

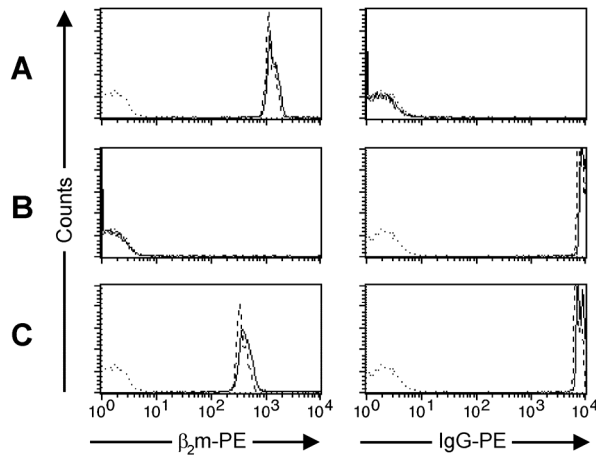


FIGURE S1. Surface coating and stability of microspheres. Beads were labeled in the presence of 10 nM A*02/YVDPVITSI (A), 10 nM CD28 Ab (B) or both (C). Untreated beads (dotted line), freshly coated beads (solid line) or beads coated four weeks before analysis (dashed line) were stained with Abs against human β_2m (left panel) or mouse IgG + IgM (right panel).

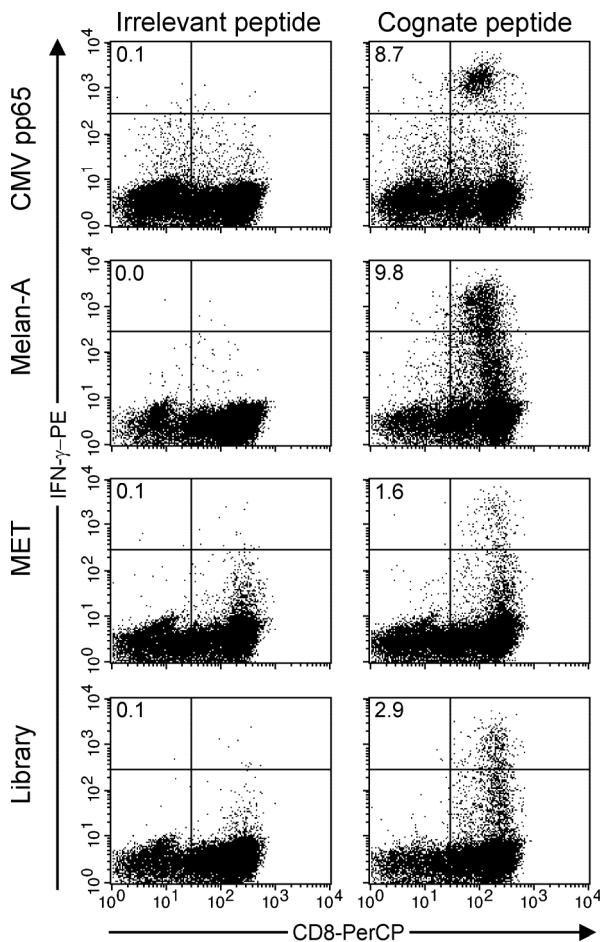


FIGURE S2. IFN- γ expression of T cell lines generated by microsphere driven expansions. Enriched CD8 T cells of one A*02⁺ healthy donor were stimulated 3 times with beads labeled in the presence of 10 nM CD28 Ab plus 10 nM of A*02 refolded with indicated antigens. After a 4-5 hours restimulation of T cell lines with 5 μ M irrelevant peptide (left panel) or cognate peptide (right panel) loaded on T2 cells, cells were labeled with Abs CD4-FITC, CD8-PerCP and IFN- γ -PE. Plots were gated on CD4⁺ lymphocytes and numbers in each plot indicate the percentage of IFN- γ high expressing cells among CD8⁺ CD4⁺ lymphocytes.

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Chapter 3

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HLA class I restricted CD4⁺ T cells in congenital viral infection

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submitted for publication

The author of this thesis has performed all the experiments leading to both figures and wrote the manuscript except the clinical case report.

MHC class I and II restricted antigens are usually recognized by CD8⁺ and CD4⁺ T cells, respectively. Using HLA multimer analysis, we report functional circulating CD4⁺ and CD8⁺ T cells both specific for the same cytomegalovirus peptide bound to HLA-A*0101 in a case of congenital infection. This finding will change our understanding of T cell responses induced by severe viral infections in newborns.

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T cells are key effectors and orchestrators of the adaptive immune system. Mature α/β T cells are usually divided into $CD8^+ CD4^-$ versus $CD8^- CD4^+$ cells. T cells with a $CD8^+ CD4^-$ phenotype are associated with the recognition of short (8-12 amino acids) peptides bound to MHC class I molecules, acting as cytotoxic T lymphocytes (CTLs). $CD8^- CD4^+$ T cells usually recognize longer (10-15 amino acids) peptides bound to MHC class II molecules and are viewed as T helper cells (T_H). However, there have been indications that this may be an oversimplification. First, $CD8^+ CD4^+$ T cells have been described in humans¹ as mature T cells containing MHC class I and II specificities. Second, $CD8^+ CD4^-$ as well as double-negative T cells specific for MHC class II have been described in $CD4^{-/-}$ mice^{2,3}. Finally, there have been sporadic reports of $CD8^- CD4^+$ T cells with MHC class I restriction in mice and men^{4,5,6,7,8}. These studies have been performed after long-term *in vitro* stimulation, leaving open the question whether these cells occur *in vivo*. Here, using HLA class I multimers⁹, we show for the first time the simultaneous detection of circulating functional $CD8^+ CD4^-$ and $CD8^- CD4^+$ α/β T cells specific for the same HLA class I restricted CMV epitope in a patient with acute congenital viral infection.

The term infant (BW 3215 g, HLA-A1⁺ B7⁺ B58⁺) presented classical symptoms of congenital CMV infection immediately after birth. Virus was detected in blood, urine and CSF. Inherited immunodeficiency syndroms have been ruled out as well as co-infection with HIV. Gancyclovir (GCV) treatment was initiated on day 2 p.p. and finished on day 135 p.p. After cessation of GCV repeated episodes of CMV infection with chorioretinitis and pneumonia occurred within the first year of life, while thrombopenia, hepatitis and encephalitis resolved. The child survived with severe eye and CNS damages remaining in a chronic CMV carrier status.

We investigated the CMV specific CTL response of this patient at the age of 14 months using HLA class I multimer analysis of *in vitro* peptide stimulated PBMCs (see **Supplementary Methods** online). After one stimulation, $CD8^+$ lymphocytes binding to the A*0101 multimer loaded with the immunodominant peptide YSEHPTFTSQY from CMV pp65 363-373 (A1/CMV) could be detected among patient PBMCs (data not shown). Immune responses to two immunodominant B*0702 restricted CMV peptides were not detectable in the patient. Surprisingly, we found also a $CD8^-$ population binding to A1/CMV after one stimulation. This finding was reobserved after a second stimulation (**Fig. 1a**). In parallel stimulations

with a negative control peptide, we observed no expansion of CD8⁺ or CD8⁻ A1/CMV⁺ cells (data not shown). We then investigated the phenotype of A1/CMV binding CD8⁻ and CD8⁺ cells (**Fig. 1b** and **Supplementary Methods** online).

Binding of HLA multimers was peptide specific, since A1/CMV⁺ cells were not costained by an HIV-1 peptide-A*0101 multimer. Therefore, it is unlikely that multimer binding occurred via natural killer cell receptors. A1/CMV⁺ CD8⁺ and CD8⁻ cells were mostly CD3⁺ CD56⁻ TCR α/β ⁺ TCR γ/δ ⁻ but differed in their CD4 expression. A1/CMV⁺ CD8⁺ cells contained a minor subpopulation that coexpressed CD4, whereas all A1/CMV⁺ CD8⁻ cells were CD4⁺. These data indicate that CD8⁺ and CD8⁻ A1/CMV⁺ cells appear phenotypically as *bona fide* CD8⁺ and CD4⁺ α/β T cells, respectively.

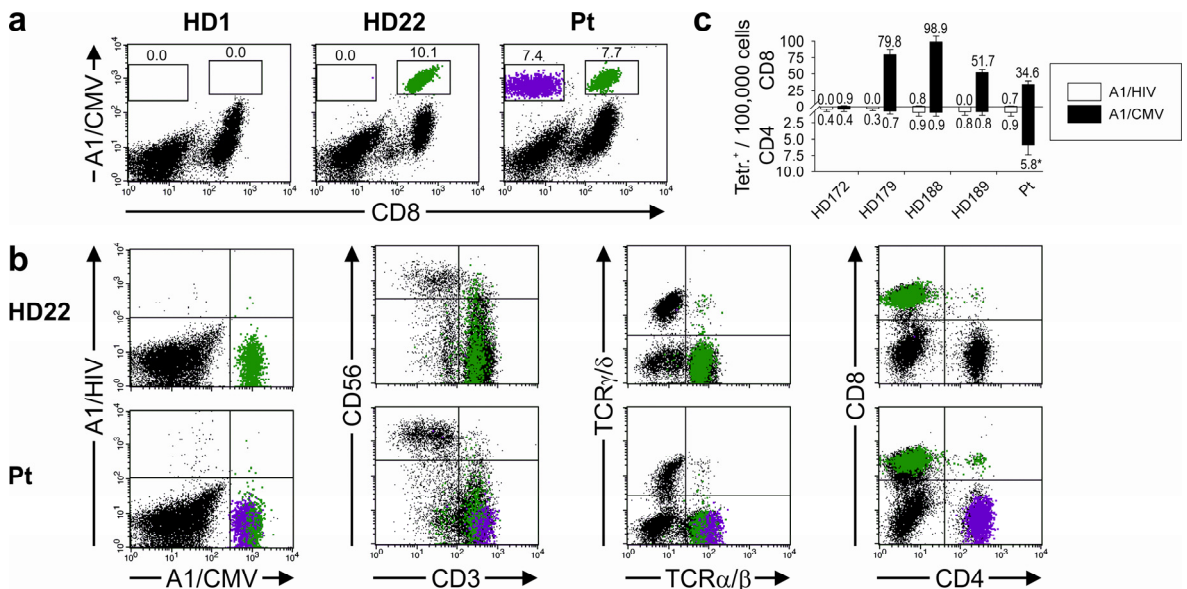


FIGURE 1 Phenotype and frequencies of HLA-A*0101 restricted CMV specific T cells. (a) HLA class I multimeric analysis of 2 x peptide stimulated PBMCs from negative control HLA-A1⁻ CMV⁺ donor HD1, positive control HLA-A1⁺ CMV⁺ donor HD22 and HLA-A1⁺ CMV⁺ patient (Pt). Numbers represent percentages of A1/CMV⁺ cells among lymphocytes. (b) Phenotype of A1/CMV⁺ CD8⁺ (green) or CD8⁻ (magenta) cells among lymphocytes from positive control donor HD22 and patient after 2 peptide stimulations. (c) *Ex vivo* detection of A1/HIV⁺ or A1/CMV⁺ CD8⁺ CD4⁻ (upper half) or CD8⁻ CD4⁺ (lower half) lymphocytes from negative control HLA-A1⁺ CMV⁻ donor HD172, positive control HLA-A1⁺ CMV⁺ donors HD179, HD188, HD189, and HLA-A1⁺ CMV⁺ patient. Bars and error bars represent number and estimated standard error of multimer⁺ among 100,000 CD8⁺ CD4⁻ or CD8⁻ CD4⁺ lymphocytes. *The frequency of patient A1/CMV⁺ CD8⁻ CD4⁺ cells was significantly higher compared to each control donor or to A1/HIV⁺ CD8⁻ CD4⁺ frequencies in all donors (P<0.01, two-sided Fisher's exact test).

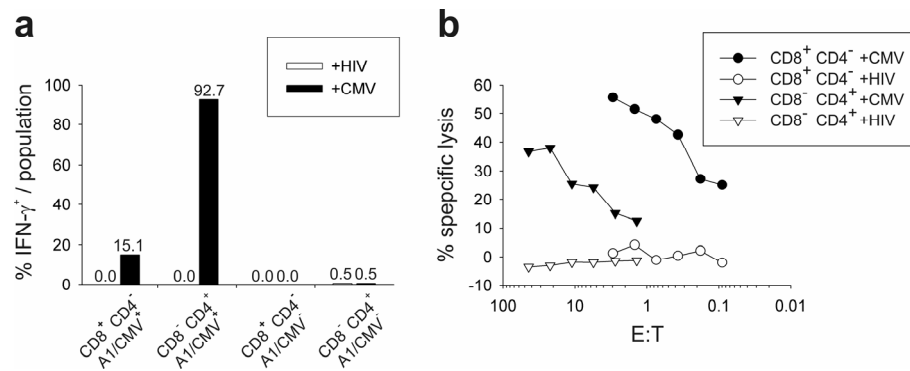


FIGURE 2 Functional properties of A1/CMV⁺ CD8⁺ and CD8⁻ cells. (a) Effector cells were stimulated at 37 °C for 6 h with the HLA-A1⁺ B-cell line COX loaded with an A*01 restricted peptide from HIV-1 or CMV. % IFN- γ cells among indicated populations were determined by intracellular cytokine staining and normalized to the frequencies of the indicated cell populations determined by multimer staining. (b) Effector cells were stimulated at 37 °C for 4 h with the allogeneic ⁵¹Cr labeled HLA-A1⁺ B-cell line COX loaded with an A*01 restricted peptide from HIV-1 or CMV at 37 °C for 6 h. E:T ratios were normalized to the frequencies of the indicated cell populations determined by multimer staining.

To investigate the expression of the T_H1/T_C1 cytokine IFN- γ and the cytotoxic capacity of A1/CMV specific subpopulations, we derived T-cell lines by HLA multimer-guided FACS sorting from stimulated patient PBMCs and expansion with irradiated allogeneic feeder cells, PHA, and IL-2. All CD4⁺ T-cell lines were more than 99.9 % free of A1/CMV⁺ CD8⁺ cells and vice versa (data not shown and **Supplementary Methods** online). T-cell lines were rechallenged with A1⁺ cells loaded with HIV or CMV peptide and expression of IFN- γ was determined (**Fig. 2a** and **Supplementary Methods** online). The majority of A1/CMV⁺ CD4⁺ and a subpopulation of multimer binding CD8⁺ cells specifically expressed IFN- γ under these conditions. TNF- α yielded similar results (data not shown). In a parallel ⁵¹Cr release experiment, both CD8⁺ and CD4⁺ cells displayed specific cytotoxicity against A1⁺ allogeneic cells pulsed with peptide (**Fig. 2b** and **Supplementary Methods** online). However, lysis was about 50 x higher in CD8⁺ compared to CD4⁺ cells. Peptide pulsed A1⁻ cells were invariably not killed and A1/CMV⁻ CD8⁺ or CD4⁺ T-cell lines from the patient displayed no specific cytotoxicity against the CMV peptide (data not shown).

One may argue that CD4⁺ A1/CMV⁺ cells reported here are an *in vitro* artefact, e.g. due to a changed coreceptor expression after prolonged cell culture. To be

able to detect circulating A1/CMV specific T cells *ex vivo*, we used modified HLA class I multimers with reduced background binding¹⁰ (**Fig. 1c** and **Supplementary Methods** online). These frequency counts indicate that among patient PBMCs, A1/CMV⁺ CD8⁺ CD4⁻ and CD8⁻ CD4⁺ were present.

The occurrence of CD4⁺ T cells specific for CMV has been demonstrated in congenital CMV infection but without analysing their HLA restriction¹¹. Our data support the hypothesis that the repertoire of circulating CD4⁺ CD8⁻ α/β T cells does also contain HLA class I restricted cells. For the first time, the presence of such cells could be shown directly *ex vivo*. CD8⁻ HLA class I multimer⁺ cells have been described previously¹² but were found to bind multimers in a non-HLA restricted, non-peptide dependent manner. In contrast, CD8⁻ cells shown here do bind HLA dependent of peptide and their specificity was further confirmed by functional assays. Within the same patient, cells of CD8⁺ CD4⁻ phenotype were found for the identical CMV epitope and HLA restriction. This observation goes along with the lack of T cells specific for immunodominant B*0702 epitopes. As this patient suffers from CMV infection, it remains unclear whether the found cells play a beneficial role for the control of the disease, as has been suggested previously in mice⁵. Functionally, CMV specific CD4⁺ and CD8⁺ cells were consistent with conventional T_H1 and CTLs, respectively. This study demonstrates that HLA class I restricted CD4⁺ and CD8⁺ cells can be detected and discriminated using HLA multimers. Furthermore, it highlights the importance not to overlook CD4⁺ cells in monitoring vaccines which are aimed at HLA class I restricted immune responses. Determining the *in vivo* function of HLA class I restricted, CD4⁺ T cells will be a challenge for the future

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Supplementary material for Walter S et al: HLA class I restricted CD4⁺ T cells in congenital viral infection

Cells, Peptides and MHC multimers. PBMCs were prepared from heparinized patient blood or HLA typed fresh buffy coats provided by the blood bank, University of Tübingen. PBMCs were isolated using standard gradient separation techniques and cryopreserved until use. Informed consent was obtained from the parents for all investigations. COX (HLA-A1⁺, ECACC # 85102902), LCL721.45 (HLA-A1⁻, as described previously¹) and LG2-EBV (a kind gift of P. van der Bruggen, Brussels) are human B-lymphoblastoid cell lines. Peptides for MHC refoldings and cellular assays were synthesized using standard Fmoc-chemistry. Peptides in this study were A*0101 restricted YSEHPTFTSQY and B*0702 restricted RIPHERNGFTVL, and TPRVTGGGAM from HCMV pp65 363-373², 265-275³ and 417-426⁴, respectively. As negative controls, A*0101 restricted peptide GSEELRSLY from HIV-1 gag p17 70-78⁶ and B*0702 restricted APRTVALTA from

HLA-DPB1*0101 9-17⁵ were used. Biotinylated recombinant HLA-A*0101 molecules were prepared analogous to HLA-A*0201 molecules described previously^{7,8}. Biotinylated recombinant HLA-A*0101 molecules with reduced background binding were generated by introducing the point mutation A245V in the α_3 domain as has been described⁹. Fluorescent multimers were generated by coincubating biotinylated HLA molecules with streptavidin-PE or -APC (Molecular Probes, Leiden, The Netherlands) at a 4:1 molar ratio

Multimer analysis of stimulated PBMCs. Thawed PBMCs were washed and cultured overnight in T-cell medium, which consisted of RPMI 1640 containing 25 mM HEPES (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10 % heat inactivated human AB serum (CC pro, Neustadt/W., Germany), 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin and 20 μ g/ml gentamycin (all Cambrex Bio Science, Verviers, Belgium). 5 μ g/ml of each CMV peptide YSEHPTFTSQY, RIPHERNGFTVL and TPRVTGGGAM was added to the stimulation sample. Negative control sample was supplemented with 5 μ g/ml peptide APRTVALTA. Cells were given fresh medium plus 20 U/ml recombinant human IL-2 (Chiron, Emeryville, California/USA) 3-4 days later and incubation was continued for 3-4 days. This protocol was repeated weekly for a maximum number of three stimulations. For multimer staining, an aliquot of cells was removed 7 days after the last stimulation, washed and resuspended in buffer consisting of PBS (Cambrex) supplemented with 0.5 % protease-free BSA, 2 mM sodium EDTA (both Sigma, Taufkirchen, Germany) and 10 mg/ml sodium azide (Merck, Darmstadt, Germany). When indicated, cells were incubated with mouse anti human CD3 antibody OKT3 (a kind gift of Gundram Jung, Tübingen), washed and stained with FITC labeled goat anti mouse F(ab')₂ fragments (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania/USA). After blocking with 10 % heat inactivated mouse serum (CC pro), cells were washed and stained with labeled antibodies as indicated, which were CD8-PerCP clone SK1, TCR α/β -FITC clone WT31, TCR γ/δ -PE (all Becton Dickinson, Heidelberg, Germany), CD4-FITC and CD56-PE (all Coulter Immunotech, Marseille, France). Cells were washed in PBS containing 2 % heat-inactivated FCS (PAN Biotech, Aidenbach, Germany), 2 mM sodium EDTA and 10 mg/ml sodium azide and multimer stained at 4 °C for 30 minutes in the same buffer but including 50 % FCS. Multimer

reagents were always used at MHC concentrations of 5 µg/ml. Washed cells were fixed in PBS/FCS/EDTA/Azide containing 1 % formaldehyde (Merck) and cytometry was performed on a four colour FACS Calibur cytometer (Becton Dickinson).

Sorted T cell lines. For sorting, cells were stained as above under aseptic conditions using Abs CD8-APC (clone SK1, Becton Dickinson), CD4-FITC (Immunotech), and multimer-PE. Sorting of A1/CMV^{+/-} CD8^{+/-} CD4^{+/-} cells as indicated was performed on a FACS Vantage (Becton Dickinson) and sorted cells were cultured in 24 well plates in T-cell medium in the presence of 5 × 10⁵ cells/ml 33 Gy irradiated fresh allogeneic PBMCs, 5 × 10⁴ cells/ml 210 Gy irradiated fresh LG2-EBV cells, 150 U/ml IL-2 and 0.5 µg/ml PHA-L (Roche Diagnostics, Mannheim, Germany). Cells were further expanded in T-cell medium containing 150 U/ml IL-2.

Intracellular cytokine staining. Approximately 1 × 10⁵ stimulator cells COX per condition were incubated in X-Vivo 15 medium (Cambrex) supplemented with 5 µg/ml indicated peptide at 37 °C for 2 hours. Cells were resuspended in T-cell medium with effector T cells as indicated at an approximate effector to target ratio of 1:1. After one hour, GolgiStop (Becton Dickinson) was added and incubation was continued for 5 hours at 37 °C. Cells were permeabilized and stained using the Cytofix/Cytoperm Plus kit and Abs CD4-PerCP, CD8-APC, IFN-γ-FITC and TNF-α-PE according to the manufacturer's recommendations (Becton Dickinson). Analysis was performed using a FACS Calibur cytometer. % IFN-γ expressing cells among indicated populations was calculated from IFN-γ intracellular and MHC multimer staining experiments as follows: (% IFN-γ expressing cells among lymphocytes of indicated CD8/CD4 phenotype) / (% indicated multimer^{+/-} population among lymphocytes of indicated CD8/CD4 phenotype) × 100.

Cytotoxicity assay. Target cells COX or LCL721.45 were loaded with 10 µg/ml peptide at 37°C for 90 minutes. Cytotoxicity was then tested in a standard 4 h ⁵¹Cr release assay using 3000 target cells per well. % specific lysis was calculated as follows: (experimental release - spontaneous release) / (detergent release - spontaneous release) × 100. Indicated were specific effector to target

ratios determined as follows: (ratio of microscopically counted effector per target cells from ^{51}Cr release experiment) * (% indicated population among lymphocytes from multimer staining experiment) / 100.

Ex vivo multimer analysis. Thawed PBMCs were washed and resuspended in PBS/FCS/EDTA/Azide. Cells were then incubated with Abs CD4-FITC (Immunotools, Friesoythe, Germany) and CD8 PerCP for 20 minutes at 4 °C. Cells were washed and resuspended in PBS/FCS/EDTA/Azide containing 50 % FCS and stained with point mutated multimers HLA-A*0101 A245V loaded with peptides YSEHPTFTSQY (PE) and GSEELRSLY (APC) at MHC concentrations of 5 µg/ml per multimer for 30 minutes at 4 °C. Washed cells were fixed as above and analyzed on a FacsCalibur cytometer. Proportion p of multimer binding cells among n lymphocytes of indicated CD8/CD4 phenotype was determined. Number of multimer binding cells among 100,000 lymphocytes of indicated CD8/CD4 phenotype was calculated as $(p \times 100,000)$ and estimated standard error of this number was calculated as follows: $100,000 \times (p \times (1 - p) / n)^{1/2}$

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Chapter 4

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High frequencies of functionally impaired cytokeratin 18-specific CD8+ T cells in healthy HLA-A2+ donors

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submitted for publication

The author of this thesis has performed all the experiments leading to figure 1, 2, 4B, 4C and Table 1. Furthermore, he contributed to experiments leading to figure 3, 5 and 6 and writing the manuscript.

Combining cell surface phenotyping with functional analysis, human CD8⁺ T cells have been divided into several subsets which are being studied extensively in diverse physiological situations, such as viral infection, cancer and ageing. In particular, so-called terminally-differentiated effector cells possess a CD45RA⁺CCR7⁻CD27⁻CD28⁻ phenotype, contain perforin and in different models have been shown to exert direct *ex vivo* killing and to release interleukins upon both antigen nonspecific or specific stimulation. Using HLA-class I multimers, we have identified a high frequency of peripheral CD8⁺ T cells which recognize a peptide derived from the self-protein cytokeratin 18 presented by the HLA-A*0201 molecule. These cells can be detected in approximately 15% of the HLA-A2-positive healthy donors tested. A detailed analysis revealed that they must have divided extensively *in vivo*, have an effector cell phenotype and express various natural killer cell inhibitory receptors. Interestingly, however, they remained unresponsive to antigen-specific stimulation *in vitro*, in terms of cytotoxicity and cytokine secretion. Thus, cytokeratin 18-specific cells constitute a frequently encountered, new CD8⁺ T lymphocyte subpopulation without classical effector status and so far with unknown function.

Introduction

Many recent studies have investigated the heterogeneity of human CD8⁺ peripheral T lymphocytes. The description of subpopulations and stages of differentiation is mainly based on the expression pattern of various cell-surface receptors and on functional properties such as proliferation, cytokine secretion or cytotoxicity. Thus, CD45RA/RO and CD27 were at first used to distinguish naïve cells (CD45RA⁺CD27⁺) from memory cells (CD45RA⁻CD27⁺), which both lack immediate cytolytic function, and effector cells (CD45RA⁺CD27⁻), with high *ex vivo* cytotoxic potential ¹. CD8⁺ subpopulations can also be defined using the costimulatory receptor CD28, with CD8⁺CD28⁻ cells preferentially exerting *ex vivo* redirected cytotoxicity (which correlates with intracellular perforin expression) and representing an *in vivo* expanded population with short telomeres ¹⁻³. More recently, differential expression of the lymph node homing receptor CCR7 was used to subdivide CD8⁺ T cells into four subpopulations: naïve (CD45RA⁺CCR7⁺), central memory (CD45RA⁻CCR7⁺), effector memory (CD45RA⁻CCR7⁻), and effectors, or terminally-differentiated effector cells (CD45RA⁺CCR7⁻). Each of these subsets presents different functional properties, in terms of *in vitro* proliferative capacity in response to TCR-mediated signals or interleukins, cytotoxic potential and interleukin secretion ^{4,5}. Combining the expression of the latter antigens with expression of the costimulatory receptors CD27 and CD28 allowed the identification of additional subsets with properties of both naïve and effector T cells ⁶. A growing number of markers, such as CD57 ⁷, inhibitory/activatory natural killer cell receptors ⁸⁻¹⁰, or interleukin and chemokine receptors ^{11,12}, is now available to refine further the definition of human functional CD8⁺ T-cell subpopulations.

Taking advantage of tetramer technology, antigen-specific T cells are now being studied in different situations such as viral infections, ageing and cancer. Analysis of CD8⁺ T lymphocytes specific for HLA-class I epitopes from persistent viruses such as HIV, EBV and HCMV has showed that their distribution in the naïve/memory/effector compartments, as defined with the surface markers described above, differs for these different viruses ^{13,14}. On the functional level, and following the observations made with antigen-nonspecific populations, effector and effector memory virus-specific cells, in contrast to naïve or central memory

cells, possessed poor proliferative capacity but high antigen-specific cytotoxicity^{13,15}. However, some studies show that terminally-differentiated effector CD8⁺ cells, CD45RA⁺/CD27⁻/CD28⁻/CCR7⁻ can, at least under certain conditions, proliferate vigorously^{12,16}, whereas CCR7⁺ cells can exert immediate effector functions¹⁷. It was also observed that effectors can revert to a central memory, CD45RA⁻CCR7⁺ phenotype, while maintaining effector functions¹⁸. All these and other reports suggest that the CD8⁺ subpopulations are intimately linked, or form a continuum, and indeed their lineage relationships are currently debated^{13,14,19}. Further dissection of antigen-specific, CD8⁺ functional subsets is certainly a crucial step for the understanding of the CD8 lineage differentiation process and eventually its manipulation for therapeutic purposes. Because the detectable frequency of peripheral tumor antigen-specific CD8⁺ T cells is generally extremely low in cancer patients, few studies have been so far able to analyse simultaneously phenotype and function of such CD8⁺ lymphocytes *ex vivo*. The best studied examples are HLA-A*0201-restricted, Melan-A-specific cells. In the blood of healthy donors, these cells are present at a surprisingly high frequency and possess a naïve phenotype (CD45RA⁺CCR7⁺), whereas they are CD45RA⁻CD45RO⁺CCR7⁻ and show direct *ex vivo* activity (cytotoxicity and IFN- γ production) in a significant proportion of melanoma patients²⁰⁻²².

We have recently identified by mass spectrometry a nonameric oligopeptide derived from the cytokeratin 18 protein (CK18 365-373) as a natural HLA-A2 ligand found on primary renal cell carcinomas. Using HLA-A*0201 multimers refolded with this peptide, we detected A2/Ker-binding CD8⁺ T cells at relatively high frequencies in some healthy donor PBMC²³. We now report on the phenotypic and functional characterisation of this particular CD8⁺ subpopulation. In contrast to Melan-A-specific CD8⁺ T cells found in healthy donors, the A2/Ker-binding cells have a phenotype previously associated with terminally-differentiated effector cells capable of immediate function. However, we were unable to detect production of IFN- γ or potent cytotoxic activity upon *ex vivo* peptide-specific stimulation. We propose that these “dysfunctional” CK18-specific cells represent a new subset or differentiation stage of T lymphocytes with unknown function(s), suggesting that CD8⁺ effector cells are functionally heterogeneous and might be further divided into distinct subsets.

Methods

Cells and culture media

Blood samples were obtained after informed consent from healthy volunteers or fresh buffy coats provided by the Blood Bank, University of Tübingen, and HLA-typing was performed serologically. Peripheral mononuclear cells (PBMC) were isolated using a standard Ficoll gradient separation (Ficolite H, Linaris, Wertheim-Bettingen, Germany) and used either immediately or cryopreserved in heat-inactivated fetal calf serum (FCS, PAA Laboratories, Linz, Austria) containing 10% DMSO. T-cell culture medium consisted of RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine (Invitrogen, Karlsruhe, Germany), supplemented with 10% heat-inactivated human AB serum (CC pro, Neustadt/W., Germany), 50 U/mL penicillin, 50 µg/mL streptomycin and 20 µg/mL gentamycin (Cambrex, Verviers, Belgium). The TAP-deficient cell line T2 (HLA-A2^{low}) was maintained in culture in RPMI with 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin and 10% FCS.

Peptides and fluorescent multimers

Peptides for HLA-class I multimers and functional assays were synthesized using standard Fmoc-chemistry, dissolved in bidistilled water containing 10% DMSO at 1 mg/ml, aliquoted and stored at -80°C before use. Purity was checked by reverse-phase HPLC and was always > 80%. The cytokeratin 18 365-373-derived peptide ALLNIKVKL (Ker) has recently been described²³. Viral-derived peptides NLVPMVATV (CMV) from HCMV pp65 495-503, GILGFVFTL (Flu) from Influenza M1 58-66, LLDFVRFMGV (EBV1) from EBV EBNA-6 284-293, GLCTLVAML (EBV2) from EBV IE63 259-267, CLGGLTMV (EBV3) from EBV LMP2 426-434, ILKEPVHGV (HIV) from HIV-1 RT 476-484, as well as YLLPAIVHI (p68) from human helicase p68 and ELAGIGILTV (Mel) as a modification of Melan/MART-1 26-35 are all well-documented HLA-A*0201 epitopes or ligands (www.syfpeithi.de). Abbreviations given in brackets were used to identify peptides throughout the text.

Biotinylated recombinant HLA-A*0201-peptide monomers were produced essentially as previously described²⁴. Fluorescent multimers were generated by coincubating biotinylated HLA-peptide monomers with streptavidin-PE or

streptavidin-APC (Molecular Probes, Leiden, The Netherlands) at a 4:1 molar ratio.

Multimer staining and phenotypic analysis

Staining of cells with multimers and fluorochrome-labeled monoclonal antibodies (mAb) was performed as follows: PBMC were thawed in FACS buffer (PBS 2% FCS, 2 mM EDTA and 0.02% NaN₃), washed and generally 1 x 10⁶ cells were stained for 20 min with anti-CD8PerCP (clone SK1, Becton Dickinson, Heidelberg, Germany) plus either mAb CD19FITC, CD11aFITC, CD45RAFITC, TCR α/β FITC (all Becton Dickinson), CD27FITC, CD28FITC, CD45ROFITC, CD57FITC, or CD62LFITC (all Coulter-Immunotech, Hamburg, Germany). After washing, cells were labeled with diluted multimers at 4°C for 30 min (HLA concentration 5 μ g/mL) in FACS buffer containing 50% FCS. For Figure 1A, cells were stained as previously reported, using multimers and mAb CD8PerCP plus CD4FITC²³.

For staining of the surface marker CCR7, PBMC were labeled first with the rat hybridoma supernatant 3D12 (kindly provided by R. Förster, Hannover, Germany), washed and incubated with FITC- or PE-labeled donkey anti-rat F(ab')₂ fragments (Jackson ImmunoResearch Laboratories, West Grove, PA). After blocking with 10% heat-inactivated mouse serum (CC pro) for 10 min, cells were washed and stained with fluorochrome-labeled mAb and multimers as detailed above.

For staining of the inhibitory killer cell lectin-like receptor G1 (KLRG1), cells were first incubated with Polyglobin 2.5% (Bayer, Leverkusen, Germany) then with the mouse hybridoma supernatant 13A2 (kindly provided by H. Pircher, Freiburg, Germany) which was visualised using a rabbit anti-mouse F(ab')₂-FITC antibody (Jackson). Blocking and further incubation steps were performed as above. Other mAb CD94/NKG2A, KIR2DL1, KIR2DL2 and ILT2 (kindly provided by A. Moretta, Genova, Italy) were labeled using a goat anti-mouse-FITC polyclonal (DakoCytomation, Untermüli, Switzerland) and blocking was performed with mouse IgG1 and IgG2a isotype matched mAb (Coulter-Immunotech) followed by multimers A2/Ker-PE plus A2/CMV-APC staining and finally incubation with CD8APC-Cy7 (SK1).

Analysis of perforin expression was done by staining the cells with mAb CD8PerCP and multimers first, fixing and permeabilizing them with Cytotfix/Cytoperm and incubating with a FITC-coupled mAb directed against

perforin (Becton Dickinson) in permeabilizing buffer PBS containing 0.5% BSA, 0.1 % Saponin and 0.02% NaN_3 .

All samples were fixed in FACS buffer containing 1% formaldehyde and three or four-color analysis was performed on FACSCalibur or FACSVantage cytometers equipped with the CellQuest Pro software (Becton Dickinson).

Cytokine production

Intracellular cytokine stainings were performed using a standard procedure: briefly, $1-2 \times 10^6$ total PBMC per condition were stimulated in 200 μL T-cell culture medium in the presence of GolgiStop (Becton Dickinson), 5 $\mu\text{g}/\text{mL}$ of various synthetic peptides for 13 h or 50 ng/mL phorbol myristate acetate (PMA) together with 1 μM Ca^{2+} ionophore ionomycin (both Sigma-Aldrich, Taufkirchen, Germany) for 2 h at 37°C and 5% CO_2 . Cells were then washed and stained with CD8PerCP and multimers, followed by a permeabilization step with Cytofix/Cytoperm and staining for intracellular cytokine production using an anti-IFN- γ FITC mAb (Becton Dickinson) diluted in permeabilizing buffer. After the last incubation step, cells were washed and analyzed by FACS.

Analysis of IFN- γ mRNA production by quantitative real-time PCR was performed essentially as described²⁵. Briefly, thawed PBMC were plated at 1×10^6 cells per condition in 500 μl medium and allow to recover overnight at 37°C. Synthetic peptides were then added at 5 $\mu\text{g}/\text{mL}$ for 3 h before RNA extraction using Trizol (Invitrogen Lifetechnologies, Karlsruhe, Germany). cDNA was synthesised using random hexamer primers (Amersham Biosciences, Freiburg, Germany) and M-MLV reverse transcriptase (Promega GmbH, Mannheim, Germany) in the presence of 10 U RNasin (Roche Diagnostics, Mannheim, Germany). Real-time PCR was run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) in duplicates for IFN- γ mRNA and CD8 mRNA, using Taqman PCR master mix (Applied Biosystems), specific primers and fluorescent probes. Data analysis was performed as reported previously using the delta cycle threshold method (ΔC_T)²³.

Cell sorting for cytolytic assay and TREC analysis

Cryopreserved PBMC were thawed, washed and allowed to recover by overnight incubation. CD8^+ T cells were magnetically enriched by negative selection using a

CD8 T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enrichment was always > 80%.

For the cytolytic assays, CD8⁺ cells were stained with multimers A2/Ker-PE and A2/CMV-APC for 25 min at 4°C. CD8FITC mAb (clone SK1) was then added for 20 additional min at 4°C. After washing, multimer-positive or negative cells were sorted on a FACSVantage, and incubated overnight in T-cell medium. T2 cells were preloaded with 5 µg peptide for 1 h and labeled with 100 µCi Na₂⁵¹CrO₄ (Hartmann Analytic, Braunschweig, Germany). 1000 T2/well were used at different effector to target ratios in a standard 6 h ⁵¹Cr release assay. Spontaneous (T2 in medium alone) and maximal (T2 in 1% Triton X100) chromium release was determined and % specific lysis was calculated as follows: (cpm experimental release – cpm spontaneous release) / (cpm total release – cpm spontaneous release) × 100.

For the T-cell receptor excision circle (TREC) analysis, CD8⁺ MACS-enriched cells were stained as described above with multimer A2/Ker-PE and CD8APC-Cy7 (SK1). In parallel, a fraction of these CD8⁺ cells was stained with anti-CCR7 3D12 antibody, goat anti-rat-APC (Becton-Dickinson) and mAb CD8APC-Cy7 and CD45RA-ECD (Immunotech). Naïve (CD45RA⁺CCR7⁺), effector (CD45RA⁺CCR7⁻) and CK18-specific CD8⁺ populations were then sorted on a FACSVantage. DNA was extracted and analysis of TREC content was performed in duplicate by quantitative real-time PCR using specific primers. C_T values differed by less than 0.35 between replicates. Quantification was achieved using serial dilutions of a standard plasmid containing the TREC sequence ²⁶.

Results

HLA A*0201/Ker-binding CD8⁺ T cells are frequently detected in healthy donor PBMC

We have recently described a subpopulation of human peripheral CD8⁺ T cells present in some healthy individuals and binding specifically to HLA-A*0201 multimers folded with one peptide derived from the normal self-protein cytokeratin 18 (A2/Ker)²³. To further characterize this population, we have now tested 33 HLA-A2⁺ donors, 5 of whom possessed cells binding to the A2/Ker multimers with a frequency ranging from 0.05% (HD93) to 0.55% (HD7) of the total CD8⁺ cell population. Staining intensity varied between donors, as exemplified in Figure 1 by HD92 and HD93. In contrast, no specific staining was detectable in 20 HLA-A2⁻ controls (Figure 1A and data not shown).

Figure 1

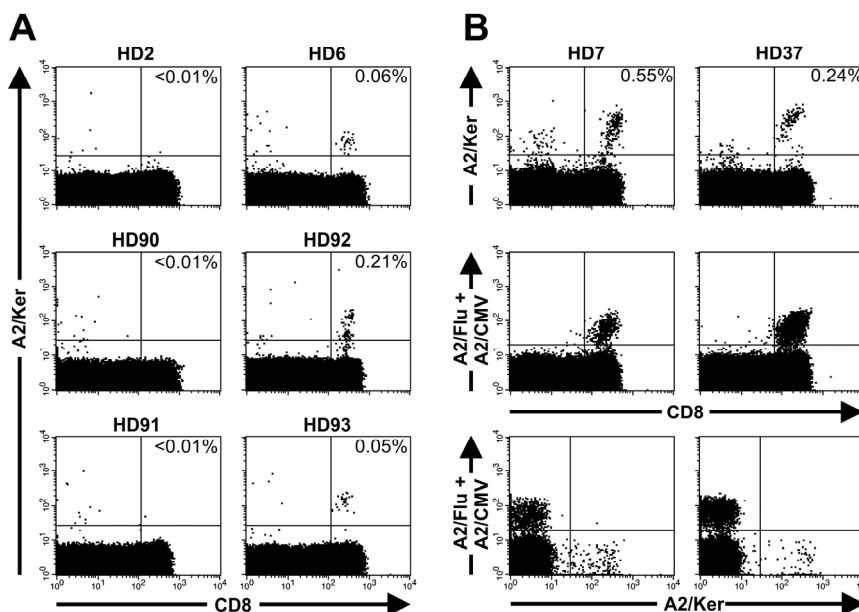
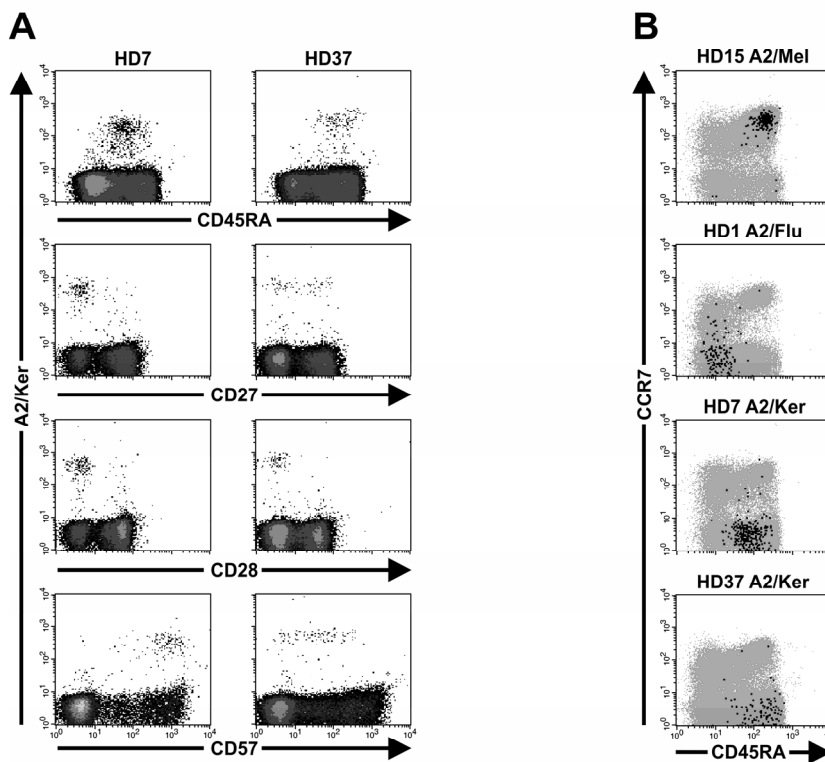


Figure 1. Ex vivo detection of CK18-specific CD8⁺ T cells in healthy donors. (A) PBMC from HLA-A2⁺ (HD2, HD6, HD90, HD92, HD93) or HLA-A2⁻ (HD91) healthy donors were stained with mAb CD8PerCP, CD4FITC and multimers A2/Ker-PE plus A2/CMV-APC. Cells are gated on CD4⁻ lymphocytes. Numbers represent percentage of A2/Ker multimer positive cells among CD8⁺ lymphocytes. (B) PBMC from HLA-A2⁺ healthy donors HD7 and HD37 were stained with mAb CD8PerCP, CD19FITC plus multimers A2/Ker-PE, A2/CMV-APC and A2/Flu-APC. Cells are gated on CD19⁻ (upper and middle panel) or CD19⁻CD8⁺ lymphocytes (lower panel). Numbers represent percentage of A2/Ker multimer positive cells among CD8⁺ lymphocytes.

As shown in Figure 1B for the two additional donors HD7 and HD37, A2/Ker-binding CD8⁺ T cells were not concomitantly stained with a mix of two multimers specific for viral-derived epitopes, suggesting that the reactivity observed was specific for the CK18-peptide. A2/Ker binding was reproducible with 3 batches of separately synthesized peptides and multimers. Furthermore, HLA-A*0201/Ker multimers bearing the point mutation A245V that leads to reduced CD8 dependent binding also retained specific staining (data not shown). Finally, a stable frequency of A2/Ker-specific cells was detected in several donors at different times over more than one year. To summarize, we observed that CK18-specific, HLA-A*0201-restricted CD8⁺ T-cell populations could be detected in about 15% of HLA-A2⁺ healthy donors. Two of these, HD7 and HD37 (0.55% and 0.24% of the CD8⁺ T cells binding to A2/Ker, respectively) were selected for further characterisation. A2/Ker binding was reproducible with 3 batches of separately synthesized peptides and multimers. Furthermore, HLA-A*0201/Ker multimers bearing the point mutation A245V that leads to reduced CD8 dependent binding also retained specific staining (data not shown). Finally, a stable frequency of A2/Ker-specific cells was detected in several donors at different times over more than one year. To summarize, we observed that CK18-specific, HLA-A*0201-restricted CD8⁺ T-cell populations could be detected in about 15% of HLA-A2⁺ healthy donors. Two of these, HD7 and HD37 (0.55% and 0.24% of the CD8⁺ T cells binding to A2/Ker, respectively) were selected for further characterisation.

A2/Ker-binding CD8⁺ lymphocytes have a phenotype of antigen-experienced T cells

With the notable exception of HLA-A*0201/Melan-A-specific cells, the frequencies of A2/Ker multimer-binding CD8⁺ cells are much higher than what has been estimated for single-epitope specific naïve T cells (< 1/100,000 CD8⁺ T cells)^{20,27}. A panel of surface markers associated with naïve or antigen-experienced lymphocytes was tested in combination with multimer staining in HD7 and HD37 (Figure 2 and Table 1). CK18-specific cells expressed the tyrosine phosphatase isoform CD45RA, although at different levels (CD45RA^{dim} for HD7 and CD45RA^{high} for HD37), but mainly lacked the costimulatory receptor CD28 (Figure 2A). This is indicative of antigen-experienced, but not naïve CD8⁺ T cells.

Figure 2**Figure 2. CK18-specific CD8⁺ cells have a phenotype of antigen-experienced T lymphocytes.**

(A) PBMC from donors HD7 or HD37 were stained with mAb CD8PerCP and FITC labeled mAb against T-cell surface antigens CD45RA, CD27, CD28 or CD57, followed by multimer staining with A2/Ker-PE and A2/CMV-APC. Cells are gated on CD8⁺ lymphocytes. Data shown for the CD45RA staining were derived from a separate experiment. (B) PBMC from HLA-A2⁺ donors HD15, HD1, HD7 and HD37 were stained with CD8PerCP, CD45RAFITC, CCR7/donkey anti rat-PE, followed by multimer staining with A2/Mel-APC, A2/Flu-APC or A2/Ker-APC. Cells shown in grey are gated on CD8⁺ lymphocytes whereas highlighted cells shown in black represent multimer-positive CD8⁺ lymphocytes. HD37 staining was performed in a distinct experiment.

Variable lack of expression of the costimulatory receptor CD27 and upregulation of the antigen CD57 further strengthened this observation. Recently, the expression of both CD45RA and the chemokine receptor CCR7 has been employed to differentiate between naïve (CD45RA⁺CCR7⁺), central memory (CD45RA⁻CCR7⁺), effector memory (CD45RA⁻CCR7⁻) and effector (CD45RA⁺CCR7⁻) CD8⁺ T cells. Whereas Melan-A-specific cells from HD15 and influenza-specific CD8⁺ cells from HD1 possessed a phenotype of naïve and effector memory cells, respectively, it was clear that A2/Ker-binding cells had a predominantly effector phenotype, i.e. CD45RA⁺CCR7⁻ in HD7 and HD37 (Figure 2B), as well as in several other donors tested (data not shown). Other mAb were used to complement this

immunophenotyping and results are summarized in Table 1. CK18-specific cells were T-cell receptor (TCR)- α/β -positive T cells expressing high levels of CD11a and intracellular perforin, but low levels of CD62L. Their phenotype was clearly different from that of Melan-A-, influenza- or CMV-specific cells from the same donors. Interestingly, they were not identical in the two donors tested, as judged by the variable expression of CD45RA, CD27 and CD57.

Table 1. Phenotype of peripheral A2/Mel, A2/Flu, A2/CMV and A2/Ker binding CD8⁺ T cells in several healthy donors

	Donors and HLA multimers					
	HD15	HD1	HD7	HD37	HD7	HD37
	A2/Mel	A2/Flu	A2/CMV	A2/CMV	A2/Ker	A2/Ker
TCR α/β ⁺	100	100	100	100	100	100
CD45RA ⁺	96	6	38	54	59	83
CCR7 ⁺	94	8	3	3	4	4
CD11a ^{hi}	21	94	99	99	98	98
CD27 ⁺	99	94	55	29	14	50
CD28 ⁺	93	94	21	11	11	10
CD45RO ⁺	18	88	56	n.t.	40	n.t.
CD57 ⁺	2	10	68	71	89	36
CD62L ⁺	52	9	5	n.t.	6	n.t.
Perforin ⁺	8	8	73	88	91	76

Numbers represent percentage of surface or intracellular antigen expressing cells among HLA multimer⁺ CD8⁺ lymphocytes. Data presented derived from separate experiments and are representative of at least two independent stainings for each population.

n.t. not tested

To confirm that CK18-specific cells are effector cells which had previously encountered antigen *in vivo*, we performed a quantitative analysis of T-cell receptor excision circle (TREC) levels within populations of sorted cells. As TREC are a non-replicative byproduct of the T-cell receptor rearrangement and are diluted upon cell divisions, a low TREC level is indicative of a large number of cell divisions in the T cell's past ²⁶. Results obtained for donor HD7 are shown in Figure 3 and demonstrate that A2/Ker-binding sorted cells carry very small amounts of TREC, equivalent to autologous CD45RA⁺CCR7⁻ effectors but much less than autologous CD45RA⁺CCR7⁺ naïve cells.

In conclusion, these data show that cytokeratin 18-specific CD8⁺ T cells have a phenotype and a replicative history which has been described before for so-called "terminally-differentiated effector cells", at least in the two donors tested.

Figure 3

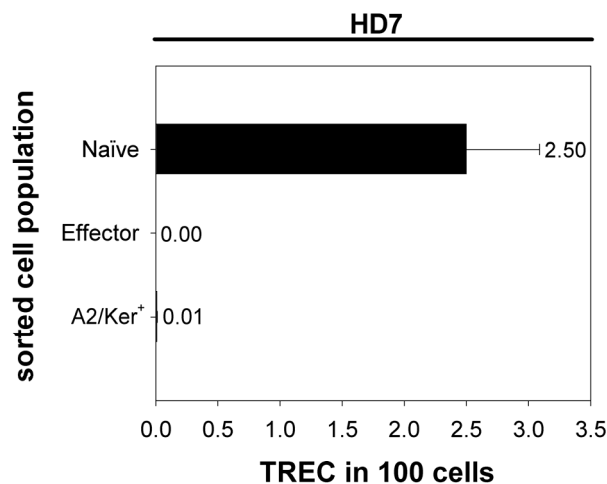


Figure 3. CK18-specific CD8⁺ cells contain low levels of TREC. PBMC from HD7 were sorted into naïve (CD45RA⁺CCR7⁺), effector (CD45RA⁺CCR7⁻) and A2/Ker binding CD8⁺ T cells. TREC levels were determined by quantitative real-time PCR and normalized for 100 cells of each subpopulation. Bars represent mean of TREC values obtained for two consecutive cell sortings of each CD8⁺ T lymphocyte subset. DNA extraction and duplicate real time PCR amplification were performed in parallel. One of two representative experiments performed with two PBMC samples obtained at different time points is shown.

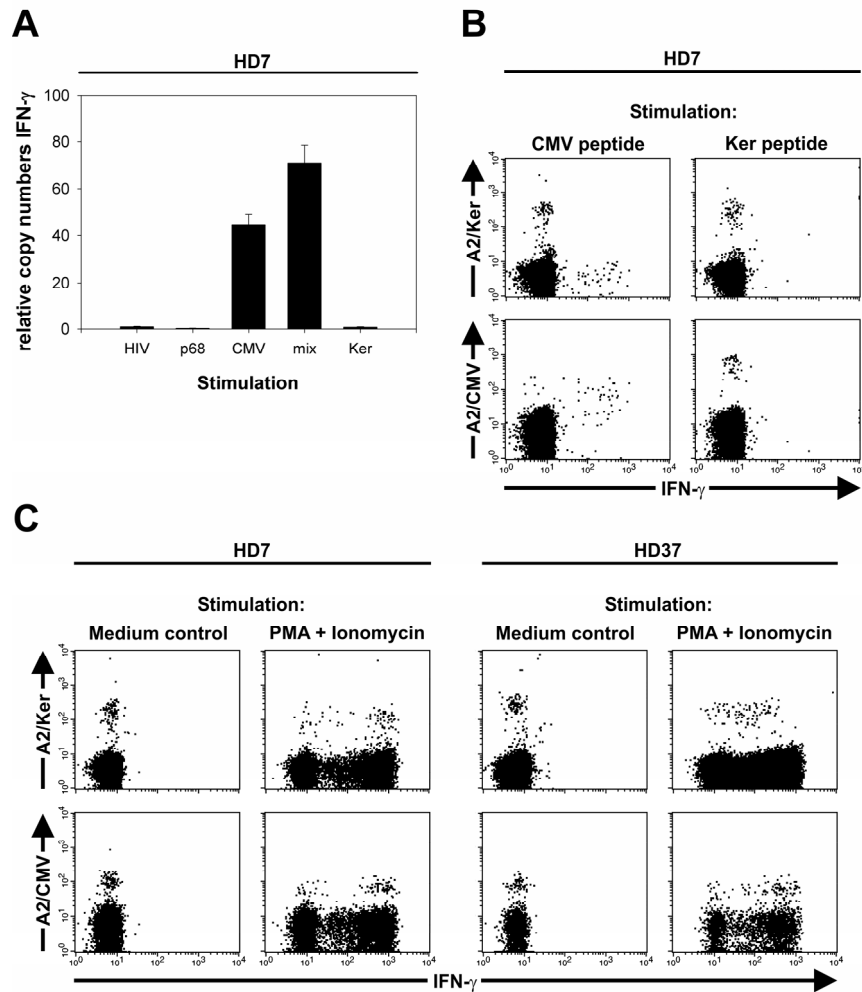
Figure 4

Figure 4. CK18-specific CD8⁺ cells do not produce IFN- γ upon peptide stimulation. (A) PBMC from donor HD7 were stimulated for 3 h with peptides HIV, p68, CMV, a viral-derived epitope mix (antigens CMV, Flu, EBV1, EBV2 and EBV3) or the peptide Ker. Duplicate results are expressed for each stimulation in fold increase IFN- γ mRNA copy numbers normalized to CD8 mRNA, relative to the same ratio obtained with the control stimulation, i.e. PBMC cultivated in the presence of the HIV peptide, which is set to 1. (B) PBMC from the same donor were stimulated as described in the methods section with either the CMV or the Ker peptides for 13 h. (C) PBMC from donors HD7 and HD37 were incubated for 2 h without stimulus or with a combination of PMA and ionomycin. After stimulation, cells were stained with CD8PerCP, IFN γ FITC and multimers A2/Ker-PE and A2/CMV-APC. Dot plots are gated on CD8⁺ lymphocytes.

CK18-specific CD8⁺ T cells are dysfunctional in terms of cytokine production and specific killing

Terminally-differentiated effector CD8⁺ T cells have been reported to display immediate cytotoxicity and rapid production of cytokines such as IFN- γ after

stimulation. We therefore evaluated the functionality of A2/Ker specific cells. However, no IFN- γ production upon specific CK18 peptide stimulation was detectable, neither at the transcriptional (i.e. production of specific mRNA, Figure 4A) nor protein (Figure 4B) levels. As expected, autologous virus-specific, especially CMV-specific, HLA-A*0201-restricted T cells responded to specific peptide stimulation in the same assays, upregulating mRNA and for the majority of them, producing IFN- γ as shown in Figure 4B. Additionally, we observed that the intensity of the CMV-multimer staining decreases notably after contact with the CMV peptide (Figure 4B, lower panel), most probably reflecting the internalization of the TCR (which is known to occur following activation of T cells). In contrast, no obvious TCR downregulation was observed for A2/Ker-stained cells after CMV or Ker peptide stimulation. The absence of IFN- γ , as well as TNF- α , production in the presence of the Ker peptide was also observed for donor HD37 (data not shown). To test whether CK18-specific cells completely lack the ability to synthesize IFN- γ , we stimulated PBMC from donors HD7 and HD37 with the TCR-independent T-cell activator, PMA and ionomycin. Under these conditions, CK18-specific cells were able to produce IFN- γ , although to a lesser extent than CMV-specific cells in donor HD37 (Figure 4C).

The high intracellular perforin content of cytokeratin 18-specific cells observed previously (Table 1) suggested that they are cytolytic T lymphocytes (CTLs). We therefore isolated these cells from donor HD7 by multimer-guided FACS sorting and tested them *ex vivo* against peptide pulsed, HLA-A*0201-expressing T2 target cells. In contrast to CMV-specific CTLs sorted in parallel from the same donor, CK18-specific cells displayed no detectable cytotoxicity after 6 or 21 h (Figure 5 and data not shown).

Thus, although possessing a phenotype generally associated with immediate effector function, cytokeratin 18-specific CD8⁺ cells were unable to exert direct killing or to produce IFN- γ after peptide-specific stimulation, at least under the *in vitro* conditions tested. We conclude that these cells are apparently functionally impaired and represent a so far undescribed new subpopulation of peripheral CD8⁺ T cells.

Figure 5

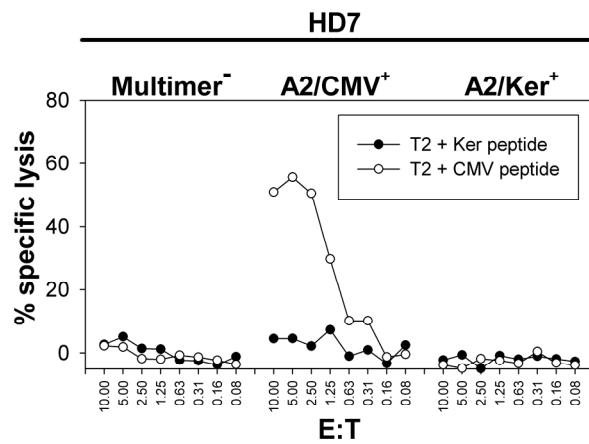


Figure 5. CK18-specific CD8⁺ cells do not kill peptide-loaded targets ex vivo. CD8⁺ T lymphocytes from donor HD7 were sorted using multimers into non multimer-binding, CMV-specific and Ker-specific populations. Specific cytotoxicity was measured in a standard 6h ⁵¹Cr release assay using peptide-pulsed T2 target cells at the indicated effector to target ratios.

Figure 6

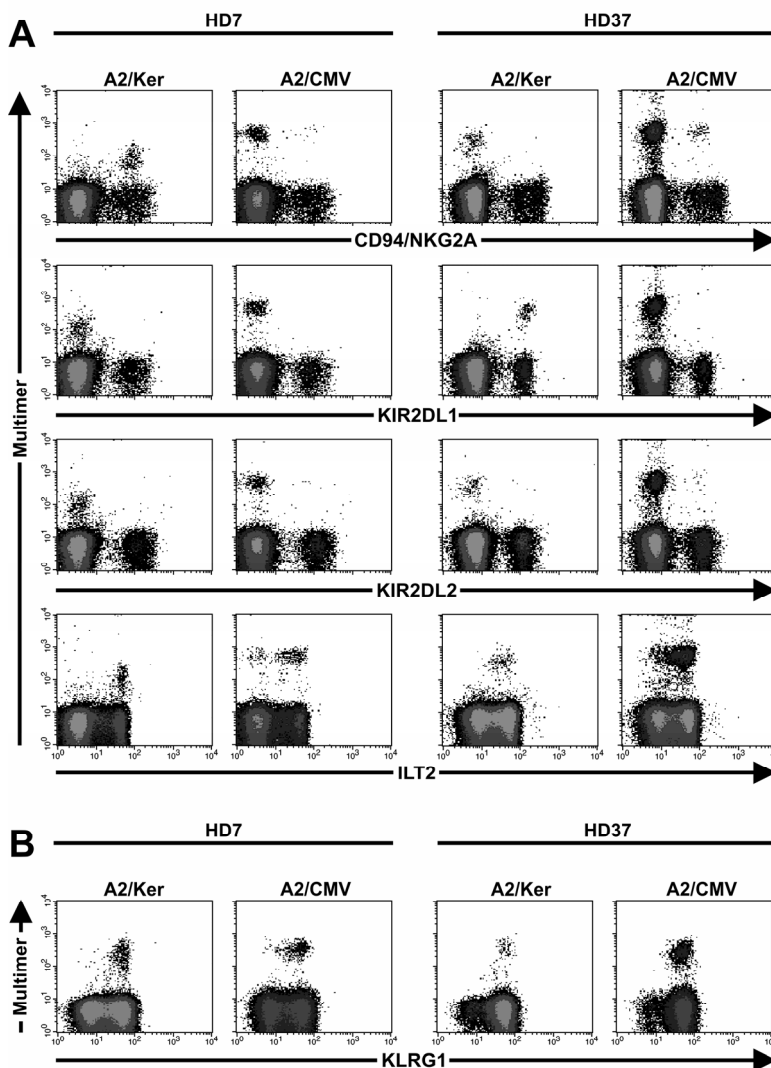


Figure 6. CK18-specific CD8⁺ T cells express natural killer cell inhibitory receptors. PBMC from donors HD7 or HD37 were stained with (A) mAb against CD94/NKG2A, KIR2DL1, KIR2DL2 and ILT2, plus CD8APC-Cy7, and (B) mAb against KLRG1 followed by CD8PerCP and multimers A2/Ker-PE plus A2/CMV-APC as indicated in Methods. Dot plots are gated on CD8⁺ cells and results obtained for the CMV-specific and Ker-specific populations are shown for each donor. Data shown in (A) for HD7 and HD37 derive from separate experiments, and were reproduced at least two times.

CK18-specific CD8⁺ T cells express natural killer cell inhibitory receptors

A possible explanation for the lack of appropriate functional responses of CK18-specific CD8⁺ cells could be the cell-surface expression of inhibitory receptors for widespread ligands, eg. HLA molecules, found on natural killer (NK) cells and some subsets of CD8⁺ T cells. CD94/NKG2A and KIR2DL1 (p58.1) were indeed found to be expressed by most of the A2/Ker-binding cells from donors HD7 and HD37, respectively (Figure 6A). These receptors were present in only a small subpopulation of total CD8⁺ cells and were absent from the cell-surface of most of the CMV-specific cells in these two donors. We also detected uniform expression of the Ig-like transcript 2 (ILT2) by CK18-specific T cells, as well as the majority of the CMV-specific cells. Finally, all CK18-specific cells coexpressed the killer cell lectin-like receptor G1 (KLRG1), as did the CMV-specific cells from the same donor (Figure 6B), but not Melan-A-specific cells present in another healthy donor (data not shown). There was no staining using mAb directed against the inhibitory receptors KIR2DL2 (p58.2), KIR3DL1 (p70), ILT5 or NKRP1A on either CK18- or CMV-specific cells, whereas these two subpopulations were equally weakly recognized by a mAb directed against the activatory receptor 2B4 (Figure 6A and data not shown). Thus, A2/Ker-binding cells express a different pattern of inhibitory NK receptors than CMV-specific cells from the same donors, and this pattern is distinct among individuals, at least in the two donors tested.

Discussion

Among the subpopulations of CD8⁺ peripheral T lymphocytes, CD45RA⁺ cells that lack the expression of markers CCR7, CD27 or CD28 have been shown to exert direct *ex vivo* redirected killing and to secrete inflammatory cytokines such as IFN- γ or TNF- α after non-specific stimulation^{1,4}. Because of this immediate cytotoxicity and their poor ability to proliferate, in certain *in vitro* models at least, they have been thought to represent the final stage of CD8⁺ T-cell differentiation and have been called terminally-differentiated effectors. The cytolytic function of such cells has been confirmed in studies in which viral- (in primed healthy donors), or more rarely, tumor antigen-specific cells (in some tumor bearing patients), were detected by HLA-class I multimer staining combined with cell-surface phenotyping for CD45RA, CD27, CD28 or CCR7 and functional assessment^{13,15,21,22}. However, the variable proliferative capacity and phenotypic changes occasionally observed for CD8⁺ effectors upon stimulation suggest that this population might be more heterogeneous than initially assumed and could have functions other than merely representing a pool of cells “ready to kill”^{12,18}. In the present study, we have characterized a new antigen-specific subpopulation of CD8⁺ T cells which recognizes a peptide derived from the protein cytokeratin 18 and presented by HLA-A*0201. A2/Ker multimer-binding cells could be directly identified at a significant frequency in the PBMC of 5/33 HLA-A2⁺ healthy donors, apparently independent of age (at least, between 35 and 62) or sex, but were absent from all 20 HLA-A2⁻ controls tested (Figure 1 and data not shown).

A2/Ker-binding cells have the distinct profile of antigen-experienced effectors i.e. possess a CD45RA⁺CD28⁻CCR7⁻ phenotype and contain relatively few TREC, as expected from cells that have divided *in vivo*²⁶. However, in HD7 and HD37, differences in the expression of the markers CD45RA, CD27 and CD57 were observed, suggesting slightly different stages of differentiation for A2/Ker-binding cells in these two donors (Table 1). Since CK18-specific cells also express a significant level of intracellular perforin, it was unexpected to find that they were anergic to *in vitro* antigenic (peptide-specific) stimulation, in terms of IFN- γ production (mRNA and protein) and also of killing of peptide-loaded targets after cell-sorting (Figures 4 and 5). It was unlikely that this was due to low affinity, since

we observed that (i) the CK18 peptide binds readily to HLA-A*0201 and (ii) there was no response using titrated concentrations of peptide up to 100 μ M (data not shown). In further intracellular staining experiments using whole PBMC of donors HD7 and HD37, we found no production of the cytokines TNF- α , IL-2, IL-4, and also no IL-10, upon peptide stimulation of the A2/Ker-binding CD8⁺ cells (data not shown). This indicates that they most probably do not belong to the Tc2 subset. Moreover, these cells were unable to proliferate in response to peptide stimulation and low dose hIL-2, whereas A2/CMV-binding cells from the same donors did divide after contact with the specific CMV peptide, as demonstrated in CFSE labeling experiments (data not shown). Thus, under these test conditions, CK18-specific CD8⁺ T cells appeared to be unresponsive to antigen-specific stimulation, whereas IFN- γ production and a significant degree of cell division following activation by PMA/ionomycin, anti-CD3 mAb or PHA was observed (Figure 4C and data not shown).

Different mechanisms could explain this impairment, including active immunoregulation by regulatory T cells or the expression of NK cell inhibitory receptors. It is known that effector CD8⁺ cells do express a large range of inhibitory (but also activatory) molecules. In mouse and human models, blocking of some of these receptors with specific antibodies has been reported to release effector function and induce target cell-killing²⁸⁻³⁰. We found that CK18-specific cells carry several inhibitory receptors. Remarkably, the heterodimer CD94/NKG2A (for HD7) and KIRDL1 (HD37) were not expressed by autologous, functional, CMV-specific cells, and only by a fraction of the whole CD8⁺ T-cell population. These two inhibitory receptors have been reported to interact with HLA-E and HLA-C molecules, respectively. Moreover, ILT2 and KLRG1 were also, but not exclusively, present on the surface of CK18-specific cells from both donors. However, blocking antibodies against ILT2 (HP-F1, kindly provided by Dr. M. Lopez-Botet) and CD94/NKG2A (Y9, gift from Dr. A. Moretta) did not reveal reconstitution of specific antigenic peptide-stimulated IFN- γ production from A2/Ker-binding cells in total PBMC of HD7 and HD37. The broad expression of their ligands, in particular HLA-E, on PBMC might explain this result. Alternatively, NK receptors such as KIR2DL1 (for HD37), KLRG1, or others, could also participate in the downregulation of T-cell reactivity. Finally, CK18-specific CD8⁺ T

cells might exert an undefined function, similar to antigen nonspecific, CD8⁺CD28⁻ peripheral T cells, which have been described to display immunosuppressive activities^{31,32}. The isolation of CK18-specific T-cell clones should support further functional studies.

Our experiments do not exclude the possibility, however, that the cytokeratin 18 peptide is an altered ligand or an antagonist peptide. Crossreactivity of self-ligand with virus-derived epitopes has been described^{33,34}, and experiments based on peptide analogues or combinatorial libraries could help to resolve the issue in this case^{34,35}. Nevertheless, the CK18 365-373 peptide described here constitutes a genuine, most probably relatively abundant HLA-ligand, which was found in HLA extracts obtained from different tumor cells such as fresh renal cell tumors²³ and an EBV-transformed cell line transfected with a CK18-encoding construct (S. Stevanović, unpublished data).

Antigen nonresponsive CD8⁺ T cells have already been described in certain physiological situations. Using HLA-class I multimers, peripheral expansions of CD28⁻ CMV- or EBV-specific T cells (up to 25% of the total CD8⁺ repertoire) have been observed in healthy donors of a very advanced age (>85)^{36,37}. Most of these cells are in fact anergic and dysfunctional, i.e. do not respond to peptide-specific *in vitro* stimulation but do respond to PMA/Ionomycin in terms of cytokine production, and in addition they also express KLRG1. A similar situation is observed in HIV patients, in which only a fraction of the viral epitope-specific T cells produces IFN- γ upon peptide stimulation³⁸. A low level of perforin expression was also observed in these cells^{38,39}. A population of tyrosinase-specific CD8⁺ T cells, as detected by multimer staining of PBMC from a melanoma patient, has also been reported to be anergic to *ex vivo* specific stimulation. These cells were CD45RA⁺CD27⁻CD28⁻CD57⁺, consistent with a differentiated effector phenotype, but they neither killed antigen-presenting cells, nor did they produce IFN- γ or TNF- α , even after exogenous interleukin-2 was added to the assay⁴⁰. Finally, Melan-A-specific effector cells present in melanoma lesions are also unable to secrete IFN- γ upon antigenic challenge *ex vivo*, express variable amount of perforin and granzyme B, but regain functionality after short-term culture with IL2 and IL7⁴¹. Thus, apparently dysfunctional CD8⁺ T cells can be detected during viral infection, tumor progression and in the elderly, all situations of possible chronic antigenic stimulation. A progressive functional impairment of virus-specific CTLs has been

reported to occur in persistent LCMV infection ⁴². It has also been hypothesized that accumulation of antigen-specific, dysfunctional CD8⁺ T cells could actually participate in a more extensive T-cell dysfunction ^{37,43}. Some other mouse studies propose that the development and maintenance of similar, antigen-nonresponsive (i.e. tolerant) CD8⁺ T cells could be due to the absence of proper costimulatory signals ⁴⁴⁻⁴⁶. In this regard, it is interesting to note that cytokeratin 18 constitutes an intermediate filament protein expressed in normal single-layered epithelia such as liver, colon or kidney, and it is tempting to hypothesize that anti-CK18 effector CTLs should be tolerized under normal conditions. Why CK18-specific cells could be detected in some, but not all healthy individuals remains to be investigated. Recently, it has been reported that the protein is released during cell death, distinct forms being produced by apoptotic versus necrotic cells ⁴⁷. Different individuals may differ in this respect. On the other hand, CK18 is a known tumor marker associated with malignancies of various origins, such as renal cell, bladder or breast carcinoma ^{23,48,49}. Because the CK18-derived peptide was eluted from HLA molecules in kidney tumors, we are currently searching for CK18-specific cells in HLA-A2⁺ renal cell carcinoma patients. It is also known that autoantibodies recognizing the CK18 protein are produced in several abnormal physiological conditions, such as autoimmune hepatitis and nonallergic asthma ^{50,51}. Thus, immune reactivity against the cytokeratin 18 protein develops under diverse apparently disease-associated circumstances. Investigations in relevant pathologies should help to characterize this newly-defined, self-specific CD8⁺ T-cell subset further.

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Summary

T cells are central orchestrators and effectors of the adaptive immune system. CD8⁺ T cells that recognize peptide antigens presented on MHC class I molecules are believed to play a central role in fighting viral infections, intracellular pathogens and cancer. The use of recombinant peptide-HLA class I complexes that mimic the natural ligands of human CD8⁺ T cells should greatly facilitate the manipulation and analysis of such cells, allowing further insight in their biology and opening therapeutic applications.

To permit the rapid access to cytotoxic CD8⁺ T cells with defined properties, artificial antigen presenting cells with defined MHC densities were devised that enable *in vitro* priming of high- or low-avidity effector T cells at will. High-avidity T cells required more stringent costimulatory conditions during priming but were clearly superior in recognizing tumor cells expressing antigen. The efficiency and high degree of control of such a system may be of great potential for future immunotherapeutic settings.

Using fluorescent MHC I multimers to analyze T-cell responses during infection and in healthy individuals, it was found that these tools allow the detection of unconventional T cells that would have remained unidentified by the use of conventional methods.

First, in a patient with congenital CMV infection, it was found that circulating functional CD4⁺ and CD8⁺ T cells specific for the same viral peptide bound to an HLA class I molecule can coexist. This unexpected finding will extend the view of T-cell responses against viral antigens and poses questions on the role of MHC class I restricted CD4⁺ T cells *in vivo*.

Finally, the detailed *ex vivo* characterisation of another unexpected cell population was reported. These CD8⁺ T cells, found in a significant proportion of healthy HLA-A2⁺ donors, are apparently specific for a peptide from the self-protein cytokeratin 18. Albeit showing the phenotypical hallmarks of antigen-experienced effector CD8⁺ cells, these cells did neither lyse cognate target cells nor did they express any tested cytokine upon antigen recognition. These findings suggest that the subset of circulating human CD8⁺ T cells with the effector phenotype may be more heterogenous than previously thought and comprise cells without classical effector function and so far with unknown function.

Zusammenfassung

T-Zellen stehen als Effektoren und Organisatoren im Zentrum des adaptiven Immunsystems. CD8⁺ T-Zellen, die Peptide auf MHC Klasse I erkennen, wird eine zentrale Rolle bei der Abwehr von Viren, intrazellulären Erregern und Krebs zugesprochen. Die Verwendung von rekombinanten Peptid-HLA I Komplexen, welche die natürlichen Liganden humaner CD8⁺ T-Zellen imitieren, sollte die gezielte Beeinflussung und Analyse solcher Zellen erleichtern. Dadurch werden neue Einsichten in Biologie und therapeutische Anwendung dieser Zellen erhofft.

Um zytotoxische CD8⁺ T-Zellen mit definierten Eigenschaften zur Verfügung zu haben, wurden künstliche antigenpräsentierende Zellen mit kontrollierten MHC-Dichten erdacht. Damit konnten *in vitro* gezielt hoch- oder niedrigavide Effektor-T-Zellen generiert werden. Hochavide T-Zellen benötigten striktere kostimulatorische Bedingungen, waren jedoch bei der Erkennung von antigenpräsentierenden Tumorzellen überlegen. Das hohe Maß an Effizienz und Kontrolle dieses Systems könnte sich bei zukünftigen immuntherapeutischen Anwendungen auszahlen.

Mittels fluoreszenter MHC I Multimere wurden T-Zellantworten während Infektionen und bei gesunden Spendern untersucht. Dabei zeigt sich, dass diese Werkzeuge den Nachweis von unkonventionellen T-Zellen ermöglichen, die mit gewöhnlichen Werkzeugen unerkannt bleiben würden.

In einem Patienten mit kongenitaler CMV-Infektion wurde gezeigt, dass funktionelle CD4⁺ und CD8⁺ T-Zellen spezifisch für dasselbe virale Peptid auf HLA I im Blut koexistieren können. Diese unerwartete Entdeckung erweitert unser Wissen über T-Zellantworten gegen virale Antigene und wirft neue Fragen zur Funktion von MHC Klasse I restringierten CD4⁺ T-Zellen *in vivo* auf.

Schließlich wurde die genaue *ex vivo*-Charakterisierung weiterer unerwarteter Zellen berichtet. Diese CD8⁺ T-Zellen, die in einem großen Anteil von gesunden HLA-A2⁺ Spendern auftreten, scheinen spezifisch für ein Peptid des körpereigenen Proteins Zytokeratin 18 zu sein. Trotz typischer Merkmale von antigen-erfahrenen Effektor-T-Zellen waren sie weder zur Lyse noch zur Expression von Zytokinen nach Antigenkontakt fähig. Diese Ergebnisse legen nahe, dass zirkulierende humane CD8⁺ T-Zellen mit Effektorphänotyp heterogener zusammengesetzt sind, als bisher angenommen wurde, und Zellen ohne Effektorstatus enthalten. Die Funktion solcher Zellen ist bisher unbekannt.

Abbreviations

aAPC	artificial antigen presenting cell	ILT	immunoglobulin-like transcript
Ab	Antibody	Ker	cytokeratin
APC	antigen presenting cell	KIR	killer cell inhibitory receptor
APC	allophycocyanin	KLRG1	killer cell-lectin like receptor G1
ATP	adenosine-5'-triphosphate	LCL	lymphoblastic cell line
β 2m	beta-2-microglobulin	LCMV	lymphocytic choriomeningitis virus
BSA	bovine serum albumin	LFA	lymphocyte function associated antigen
CD	cluster of differentiation	MACS	magnetically activated cell sorting
CDR	complementary-determining region	Mel	Melan-A
CFSE	carboxyfluorescein diacetate, succinimidyl ester	MHC	major histocompatibility complex
CK	Cytokeratin	NK	natural killer
CLIP	class II-associated invariant-chain peptide	NKRP1A	natural killer cell receptor P1A
CMV	cytomegalovirus	PAMP	pathogen-associated molecular pattern
CNS	central nervous system	PBMC	peripheral blood mononuclear cell
CTL	cytotoxic T lymphocyte	PBS	phosphate buffered saline
DC	dendritic cell	PCR	polymerase chain reaction
DNA	desoxyribonucleic acid	PE	phycoerythrin
DTH	delayed-type hypersensitivity	PerCP	peridinin chlorophyll protein complex
EBV	epstein barr virus	pH	potentia hydrogenii
EDTA	ethylenediaminetetraacetic acid	PHA	phytohaemagglutinin
ELISA	enzyme linked immunosorbent assay	PMA	phorbol myristate acetate
ELISPOT	enzyme linked immunospot assay	pMHC	peptide-major histocompatibility complex
ER	endoplasmic reticulum	Q-RT-PCR	quantitative reverse transcriptase - PCR
FACS	fluorescent activated cell sorting	RAG	recombination-activation gene
FCS	fetal calf serum	RNA	ribonucleic acid
FITC	fluorescein isothiocyanate	RPMI	roswell park memorial institute
GALT	gut associated lymphoid tissue	RT	room temperature
GCV	Ganciclovir	TAP	transporter associated with antigen processing
HD	healthy donor	TCR	t-cell receptor
HEPES	4-(2-Hydroxyethyl)-piperazin-1-ethansulfonsäure	TNF	tumor necrosis factor
HIV	human immunodeficiency virus	TREC	t-cell receptor excision circle
HLA	human leukocyte antigen		
HPLC	high performance liquid chromatography		
ICAM	intercellular adhesion molecule		
IFN	Interferon		
Ig	Immunoglobulin		
IL	Interleukin		

Academic Teachers

Prof. Bisswanger, Prof. Bock, Prof. Bohley, Prof. Christen, Prof. Eisele, Prof. Fröhlich, Prof. Gauglitz, Prof. Grabmayr, Dr. Günzl, Prof. Hagenmaier, Prof. Hamprecht, Prof. Hanack, Prof. Kaiser, Prof. Koeffler, Dr. Mayer, Prof. Mecke, Prof. Nakel, Prof. Ninnemann, Prof. Oberhammer, Prof. Pfeiffer, Prof. Pommer, Prof. Poralla, Prof. Probst, Prof. Rammensee, Prof. Reutter, Prof. Schild, Prof. Staab, PD Dr. Steinle, Prof. Stevanović, PD Dr. Stoeva, Prof. Voelter, Prof. Wegmann, Prof. Weser, Prof. Wohlleben.

Danksagung

Mein besonderer Dank gilt Prof Dr. Stefan Stevanović für die geduldige Betreuung meiner Arbeit. Er ließ mich frei arbeiten und ich fand in ihm einen Ansprechpartner, der immer Zeit für meine Fragen hatte und mich in allen Punkten unterstützt hat. Ich habe die entspannte Atmosphäre in seiner Arbeitsgruppe sehr zu schätzen gelernt.

Bei Prof Dr. Hans-Georg Rammensee möchte ich mich besonders für sein großes Interesse an meiner Arbeit und viele hilfreiche Diskussionen bedanken, sowie für die große Freundlichkeit, mit der ich in seiner Abteilung aufgenommen wurde.

Dr. Cécile Gouttefangeas gilt ebenfalls mein besonderer Dank. Ohne die zahllosen Diskussionen und nützlichen Ratschläge, die ich von Ihr erhalten habe, wäre diese Arbeit so nicht möglich gewesen. Bei PD Dr. Alexander Steinle und PD Dr. Gundram Jung möchte ich mich ebenfalls sehr für den gedanklichen Austausch bedanken.

Ich möchte mich bei allen weiteren Nutzern des Raumes 3.025, insbesondere Jochen Probst und Dr. Steve Pascolo die bedanken. Sie waren gute Ansprechpartner und halfen mit, die klimatischen Bedingungen beim Arbeiten zu ertragen. Vielen Dank auch insbesondere an Oliver Schoor, Dr. Patrice Decker, Thea Nastke, Tobias Silberzahn, Stefan Welte und Katrin Wiemann für das angenehme Klima in der Abteilung, während und nach der Arbeitszeit.

Bei den Ex-Hiwi's Leah Herrgen und Johanna Engelhard möchte ich mich sehr für die geduldige Kultivierung von Bakterien und Aufreinigung von Proteinen bedanken. Denselben Dank möchte ich auch an Nicole Arnold und Beate Pömmmerl richten. Und ohne Patricia Hrstics exzellente Peptide wäre keine Rückfaltung möglich gewesen.

Vielen Dank an Lynne Yakes besonders dafür, meine vielen Kuriersendungen entgegen genommen zu haben.

Ohne Gerhard Hörr, Claudia Falkenburger und Franziska Löwenstein würde im Labor nichts funktionieren ! Vielen Dank für die Unterstützung im Hintergrund.

Ich möchte mich bei allen Kooperationspartnern bedanken, die zu dieser Dissertation beigetragen haben, besonders bei Prof. Dr. Dorothee Wernet für HLA-typisierte Buffy coats, Prof. Dr. Hans-Jörg Bühring für Zellsortierungen sowie Dr. Johannes-Peter Haas für Patientenproben. Sven Koch, Dr. Graham Pawelec, Dr. Eddy Roosnek, Dr. Alfred Zippelius, Gilles Bioley und Prof Dr. Pedro Romero haben mir mit Reagenzien, T-Zellmethoden und Diskussionen sehr geholfen.

Vielen Dank an Anja und Peter für die guten Kontakte, die uns zu allen Zeiten geblieben sind.

Schließlich möchte ich mich bei meinen Eltern, meiner Schwester und meinem Schwager bedanken. Sie waren zu jeder Zeit für mich da.

Lieben Dank an Doreen, besonders für die letzten Monate während des Schreibens !

Publications

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Scholarships and awards

2002 – 2003	Scholarship of the "Reinhold Beitlich Stiftung", Tübingen
2001 – 2003	Scholarship of e-fellows.net
1996 – 2001	Scholarship of the "Studienstiftung des Deutschen Volkes"
1996	Award as best high school senior student
1996	Award of the chemical industry for high school performances

Curriculum Vitae

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01/2002 – 01/2005	PhD thesis at the Institute for Cell Biology, Dept. of Immunology, University of Tübingen supervised by Prof. Dr. S. Stevanović, title: Unconventional T lymphocytes - recombinant MHC molecules pave the way
01/2002	Diploma in Biochemistry
05/2001 – 01/2002	Diploma thesis at the Institute for Cell Biology, Dept. of Immunology, University of Tübingen supervised by Prof. Dr. S. Stevanović, title: Monitoring of T cell responses in the peripheral human blood using MHC tetramers
03/2000 – 05/2001	Graduate studies in Biochemistry at the University of Tübingen
09/1999 – 03/2000	Practical research course at the University of California, L.A., supervised by Prof. Dr. P. Koefler
10/1996 – 09/1999	Undergraduate studies in Biochemistry at the University of Tübingen
1995 – 1996	Compulsory military service in Ellwangen/Jagst.
1986 – 1995	Friedrich-Schiller-Gymnasium, Fellbach (comprehensive secondary school)
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