# Functional and Phenotypic Characterization of Virus-specific CD8+ T Cells in Humans

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Für meine Eltern,

mit all ihrer Liebe und Unterstützung

Für Malte,

mein Fels in der Brandung

# Zusammenfassung

In vielen persistierenden Virusinfektionen wie der chronischen Hepatitis C sind die Virus-spezifischen CD8+ T-Zellen funktionell erschöpft. Genaue Mechanismen, welche diese Erschöpfung beeinflussen oder sie möglicherweise überwinden können sind bisher nur wenig bekannt. Wir beschreiben hier den Phänotyp und die Funktionsfähigkeit HCV-spezifischer CD8+ T-Zellen in Patienten mit chronischer HCV Infektion und die Veränderung dieser Charakteristika hervorgerufen durch ein neues therapeutisches Peptid-Vakzin genannt IC41. In einigen Patienten konnte eine Änderung des Gedächtniszellphänotyps erreicht werden. Allerdings wurde keine Verbesserung der Funktionsfähigkeit dieser Zellen durch das Vakzin IC41 erreicht, da der Impfstoff in seiner Wirkung zu schwach zu sein schien um die während einer persistierenden Infektion bestehende funktionelle Erschöpfung der HCV-spezifischen CD8+ T-Zellen überwinden zu können. Da Immunantworten im menschlichen Immunsystem durch ein weit verzweigtes Netzwerk kostimulatorischer Moleküle reguliert und kontrolliert werden, waren wir bestrebt den allgemeinen Einfluss kostimulatorischer Moleküle auf Virus-spezifische CD8+ T-Zellen zu untersuchen und deren mögliche Rolle während der funktionellen Erschöpfung zu ermitteln. Es wurde bereits vorher gezeigt, dass das kostimulatorische Molekül PD-1 ein inhibierendes Signal auf Virus-spezifische CD8+ T-Zellantworten liefert und eine wichtige Rolle in der funktionellen Erschöpfung von CD8+ T-Zellantworten spielt. Wir zeigen hier, dass im Gegensatz zu anderen Publikationen die Expression von PD-1 auf CD8+ T-Zellen in Patienten mit chronischer HCV oder HBV Infektion und autoimmune-induzierter Hepatitis nicht signifikant erhöht war. Weiterhin konnten wir keine direkte Korrelation zwischen der Expression von PD-1+ auf CD8+ T-Zellen und klinischen Markern der Lebererkrankung feststellen. Da die Blockierung der Signaltransduktion von PD-1 nicht bei allen Individuen eine Verbesserung der Immunantworten erzielte, war es unser Ziel ein weiteres kostimulatorisches Molekül ausfindig zu machen, das möglicherweise die Immunantworten funktionell erschöpfter Virus-spezifischer CD8+ T-Zellen beeinflusst. 2B4 ist ein kostimulatorischer Rezeptor, dessen Einfluss auf CD8+ T-Zellantworten bisher wenig verstanden ist. Wir konnten zeigen, dass sowohl CD8+ T-Zellen als auch NK-Zellen und Monozyten 2B4 exprimierten. CMV-pp65- und EBV-BMLF1-spezifische CD8+ Gedächtnis-T-Zellen gesunder Individuen exprimieren 2B4 in hohem Maße, wohingegen Influenza-A (IV)-spezifische CD8+ T-Zellen nur eine geringe Expression aufwiesen. Die Expressionsrate von 2B4 auf CD8+ T-Zellen war in Patienten mit akuter HBV- oder HCV-Infektion als auch während chronischer HCV-oder HBV-Infektion und autoimmun-induzierter Hepatitis signifikant erhöht. Stimulation von 2B4 aber nicht das Blockieren des Gegenrezeptors CD48 führte zu einer Verstärkung der Degranulation Antigen-spezifischer CD8+ T-Zellen. Aufgereinigte 2B4+ CD8+ T-Zellen zeigten eine deutliche Fähigkeit zur Degranulation, wohingegen 2B4- CD8+ T-Zellen dazu nicht fähig zu sein schienen. Während kein Effekt von anti-2B4- und anti-CD48-Antikörpern auf die Zytokinproduktion

beobachtet wurde, wurde die Proliferation Antigen-spezifischer CD8+ T-Zellen durch Zugabe von anti-2B4 in einigen gesunden Individuen deutlich erhöht. Im Gegensatz dazu führte die Inkubation mit anti-CD48-Antikörper zu einer deutlichen Abnahme der Proliferation CD8+ T-Zellen *in vitro*.

Außer den Einflüssen vom Phänotyp, der Funktionalität und der Expression kostimulatorischer Moleküle, sind auch andere Faktoren wie die Epitophierarchie und die Diversität des T-Zellrezeptorrepertoires entscheidend für Virus-spezifische CD8+ T-Zellantworten. Aus diesem Grunde haben wir in Patienten mit akuter HCV Infektion die allgemeine Hierarchie HCV-spezifischer CD8+ T-Zellen analysiert sowie das T-Zellrezeptorrepertoire HCV NS3-1073-spezifischer CD8+ T-Zellen untersucht, da dieses Peptid Bestandteil des Impfstoffes IC41 war. HCV-spezifische CD8+ T-Zellantworten wiesen eine starke inter-individuelle Variabilität in der Frequenz und eine ausgeprägte Epitophierarchie auf. Außerdem zeigten sich deutliche Unterschiede im T-Zellrezeptorrepertoire HCV NS3-1073-spezifischer CD8+ T-Zellen, welche in allen getesteten Proben polyklonal waren. Die Ergebnisse der Aminosäuresequenzierung der dominanten Vβ7-Kette dieser HCV NS3-1073-spezifischen CD8+ T-Zellen ließen eine stark fokussierte Klonalität dieser CD8+ T-Zellen erkennen und alle Klone wiesen ein gemeinsames, häufig auftretendes Aminosäuremotiv in der CDR3-Region auf. Weitere Experimente werden zurzeit durchgeführt um die Zahl der Patientenproben zu erweitern und um eine mögliche Assoziation eines Aminosäuremotivs mit einer schützenden CD8+ T-Zellantwort aufzudecken.

# Abstract

During many persistent virus infections like chronic HCV, virus-specific CD8+ T cell responses are functionally exhausted. Detailed mechanisms which impact or which can possibly overcome this functional exhaustion are only poorly defined. We describe here the phenotype and functionality of HCV-specific CD8+ T cells in patients with persistent HCV infection and the changes induced by therapeutic a novel peptide vaccination called IC41. In some patients a shift of the memory phenotype towards effector cells could be achieved. The functionality of HCV-specific CD8+ T cells, however, could not be improved as the vaccine IC41 seems to be too weak to overcome the functional exhaustion of HCV-specific CD8+ T cells during persistent infection. As immune responses are regulated and controlled by a tight network of costimulation, we aimed to elucidate the role of certain costimulatory molecules on virus-specific CD8+ T cell responses in general and their possible implication in T cell exhaustion. The inhibitory molecule PD-1 was shown before to negatively influence antigen-specific CD8+ T cell responses and to be implied during functional exhaustion. Here, we show that, controversial to some other reports, PD-1 expression was not significantly upregulated on total CD8+ T cells in patients with persistent HCV or HBV infection or auto-immune-induced hepatitis. Also, we failed to find a direct correlation between the ratio of PD-1+ CD8+ T cells and clinical markers of liver disease. As blockade of PD-1 signalling was not able to improve immune responses in every patient sample, we looked for another costimulatory molecule possibly involved in the regulation of immune responses of functionally exhausted CD8+ T cells. The molecule 2B4 is a costimulatory receptor whose role during CD8+ T cells responses is still poorly understood. Analysing the expression of 2B4 in healthy individuals we found its expression on a fraction of CD8+ T cells, NK cells and monocytes. Virus-specific CD8+ memory T cells recognizing CMV-pp65 and EBV-BMLF1 but not CD8+ T cells specific for Influenza-A (IV) showed high expression of 2B4. Levels of 2B4 expression on CD8+ T cells was significantly upregulated in patients with acute HCV or HBV infection, persistent HCV and HBV infection as well as patients suffering from autoimmune-induced hepatitis. Stimulation of 2B4 but not blocking the counter-receptor CD48 *in vitro* using monoclonal antibodies led to enhancement of degranulation of CD8+ T cells. Where purified 2B4+ CD8+ T cells showed a strong increase of degranulation upon stimulation, 2B4- CD8+ T cells seem to be unable to degranulate. While no effect on the cytokine production could be observed, proliferation of antigen-specific CD8+ T cells was enhanced by anti-2B4 in some but not all healthy individuals tested. On the contrary, treatment with anti-CD48 led to a marked decrease of proliferation of CD8+ T cells.

Besides effects of factors like the phenotype, function and expression of costimulatory molecules on virus-specific CD8+ T cells, the epitope hierarchy of CD8+ T cell responses and the diversity of the T cell receptor repertoire are important determinants for CD8+ T cell responses.

Therefore, we analysed in patients with acute HCV infection the hierarchy of the T cell receptor repertoire of HCV-specific CD8+ T cells in general as well as the specific repertoire of HCV NS3-173 specific CD8+ T cells, as this peptide was included in the vaccine. HCV-specific responses of patients with acute HCV infection showed a great inter-individual variation in frequency and epitope hierarchy. Also, the T cell receptors used for recognition of HCV NS3-1073 varied remarkably and usually were polyclonal. Analysis of the amino acid sequence of a dominant T cell receptor Vβ-7 chain found revealed a strongly narrowed clonality of T cells making up the HCV NS3-1073 specific CD8+ T cell response in all samples analysed. The sequence of the CDR3 region showed a common amino acid motif to be found in all clones analyzed. Further experiments are under way to expand the number of patient samples tested in order to determine a possible amino acid motif associated with protective immune responses against HCV.

Schlagworte: Virus-spezifische CD8+ T-Zellen, Hepatitis C Virus, Kostimulation, Impfung

Keywords: Virus-specific CD8+ T cells, Hepatitis C Virus, costimulation. Vaccination

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# Attributions

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Cell sorting was conducted by C. Reimers and Dr. M. Ballmaier from the central Sorting Facility of MHH.

Sequencing of plasmid inserts for T cell receptor analysis was done by the AGOWA AG, Berlin, Germany.

# Abbreviations

| | |
|---|---|
| % | percent |
| α | alpha |
| β | beta |
| γ | gamma |
| µ | micro ($10^{-6}$) |
| | |
| ab | Antibody |
| ALT | Alanine Aminotransferase |
| ag | antigen |
| APC | Antigen presenting Cell |
| AST | Aspartate Aminotransferase |
| BD | Blood donor (healthy individual) |
| bp | base pairs (nucleotide) |
| BSA | Bovine Serum Albumin |
| CD | Cluster of Differentiation |
| cDNA | complementary DNA |
| CTL | cytotoxic T lymphocyte |
| d | day |
| D | Diversity region (T cell receptor) |
| DC | Dendritic Cell |
| DMSO | Dimethyl Sulfoxide |
| DNA | Desoxyribonucleid Acid |
| e.g. | for example |
| ELISpot | Enzyme Linked Immunosorbent Spot |
| FACS | Fluorescence Activated Cell Sorter |
| FcγR | Fc gamma Receptor |
| FCS | Foetal Calf Serum |
| Fig. | Figure |
| g | gram |
| h | hour |
| H | healthy (individual) |

| | |
|---|---|
| HBV | Hepatitis B Virus |
| HCV | Hepatitis C Virus |
| HIV | Human Immunodeficiency Virus |
| HLA | Human Leukocyte Antigen |
| Ig | Immunoglobulin |
| IFN | Interferon |
| IL | Interleukin |
| IU | International Units |
| l | litre |
| Iono | Ionomycin calcium salt |
| J | Joining region (T cell receptor) |
| m | milli ($10^{-3}$) |
| mAb | monoclonal Antibody |
| MFI | Mean Fluorescence Intensity |
| MHC | Major Histocompatibility Complex |
| ml | millilitre |
| mRNA | messenger Ribonucleid Acid |
| NK | Natural Killer |
| PBMC | Peripheral Blood Mononuclear Cell |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| Pent | Pentamer/Pentameric Complex |
| PHA | Phytohaemagglutinin |
| PMA | Phorbol 12-myristate 13-acetate |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| SEB | Staphylococcal Enterotoxin B |
| TCR | T Cell Receptor |
| Tet | Tetramer/Tetrameric Complex |
| $T_H$ | T Helper Cell |
| TNF | Tumor Necrosis Factor |
| U | Unit |
| V | Variability region (T cell receptor) |
| WHO | World Health Organisation |

# 1 Introduction

## 1.1 The human immune system

The human organism is confronted with various kinds of pathogens such as bacteria, viruses, fungi and parasites invading the body through the gastrointestinal and the respiratory tract and sometimes via blood transmission. The role of the immune system is to detect these pathogens, inhibit their spreading in the organism and finally eliminate them. The immune system disposes of abnormal or degenerate cells of the body in order to maintain the general structure of the organism. At the same time, the immune system must be able to distinguish “own” from “foreign” without destroying natural and necessary microbial flora within the body or prevent overreaction against harmless substances.

The immune system is composed of different kinds of soluble molecules (e.g. complement system, cytokines and chemokines) and various immune cells, each with its own specificity and function (see Figure 1). The human immune system can be divided into two major components, the innate and the adaptive immune system.

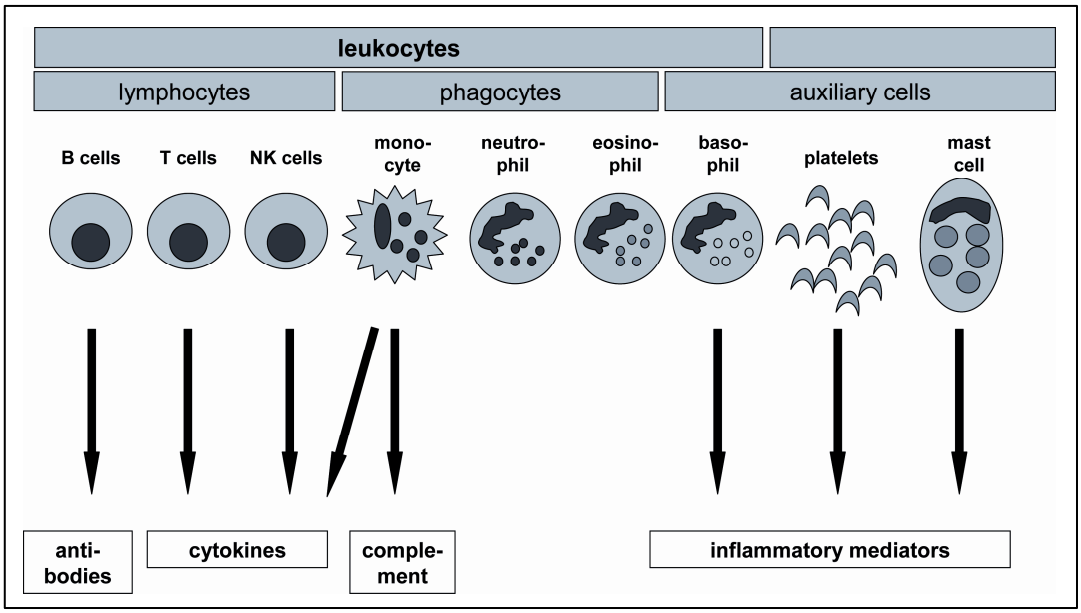



**Figure 1: Cells and mediators of the immune system.** Cellular components of the immune system can be divided into different cell populations and cell subsets. Upon activation those cells produce – besides other molecules - various soluble mediators (Figure adapted from (Male, DK 2006))

### 1.1.1 The innate immune system

The innate immune system can act quickly against pathogens and forms a first defence which inhibits spreading of the infection. Additionally to mechanical barriers like epithelial layers

and the mucosa, it comprises soluble factors such as the complement system, inflammatory mediators, receptors against pathogen-associated molecules (e.g. Toll-like receptors) and cytokines. The cellular components are made up of phagocytes (macrophages and monocytes), mast cells, granulocytes and Natural Killer (NK) cells.

Pathogens can enter the organism through the respiratory tract, the gut or other mucosal surfaces. Located in the tissues directly beneath these surfaces various innate immune cells reside and form a first line of defence. Tissue-residing phagocytes eliminate pathogens by endocytosis and digest them in endosomes combating fast spreading of the infection. Dendritic cells (DCs) and other phagocytes engulf entering pathogens and release cytokines and chemo attractants. These activate and recruit other innate immune cells like neutrophils and Mast Cells to the site of infection. Pathogen fragments are displayed on the surface of DCs bound to Major Histocompatibility (MHC) Molecules (Yewdell, JW and Bennink, JR 1999). Therefore they are classified as Antigen Presenting Cells (APCs) and are capable of activating the adaptive immune system. Further, they start secreting antiviral cytokines (e.g. IFN$\alpha$ and IFN$\beta$) which in turn activate other immune cells. The activated DCs carrying the pathogen migrate to the lymph nodes where they activate and prime more immune cells of the innate immune system like Natural Killer (NK) cells. NK cells are able to recognize infected or degenerated cells in the body and eliminate them through exhibiting cellular cytotoxicity. Their mechanism of identifying target cells does not occur by recognition of specific antigens like in the case of the T cells. Instead, the activity of NK cells is regulated through the presence or absence of various molecules on the target cell surface, which are ligands to respective activating or inhibitory receptors on the NK cells.

### 1.1.2 The adaptive immune system

Activation of the innate immune system is accompanied by the initiation of the adaptive immune response. The cellular part of the adaptive immune system includes B and T cells, both of whom carry individual specific receptors on their cell surface. In contrary to the innate immune system, these immune cells identify definite antigenic structures (epitopes). They are able to divide between foreign (antigens) and self (self-antigens) and are capable of recognizing an almost unlimited variation of epitopes (Zinkernagel, RM and Rosenthal, KL 1981).

One special characteristic of the adaptive immune system is the ability to generate long lasting memory cells, which are able to become activated once more quickly and exert effector functions rapidly upon reinfection. How exactly and at which point during the initial immune response (e.g. if during the initiation of the immune reaction or rather towards the final contraction phase) the generation of memory cells takes place, is not known in detail (Welsh, RM et al. 2004).

## 1.2 T cells

T cells are like all lymphocytes generated in the bone marrow, but in contrary to B cells, the differentiation of T cells takes place in the thymus, a fact which led to the naming of this lymphocyte population. In the thymus the T cell receptor (TCR) of the immature T cells are tested for recognition of self-antigens. T cells which are reactive against autoantigens are eliminated, a process called negative selection (Palmer, E 2003). There is evidence that additional negative selection also occurs in the periphery, as survived auto-reactive T cells get anergic and downregulate their TCR. Moreover, T cells with a TCR which fails to bind to self MHC molecules also get eradicated from the T cell population (positive selection) (Starr, TK et al. 2003).

T cells are divided into two subgroups according to the expression of either CD4 or CD8 molecules on their cell surface. Both subgroups recognize short antigenic peptides, which are bound to MHC-Molecules and presented on the surface of APCs and other cells of the body. CD4+ T cells are also called T helper cells, because they support CD8+ T cells and B cells in their activation and function by secreting cytokines and chemokines (Janeway, C 2005). They recognize peptides of 13-24 amino acids in length bound to MHC molecules of the class 2 (MHC-II). CD8+ T cells on the other hand recognize to MHC-class I molecules (MHC-I) which bear shorter antigenic peptides of eight to ten amino acids. CD8+ T cells are also known as Cytotoxic T Lymphocytes (CTLs), because the binding of an CD8+ T cell to a peptide:MHC-complex matching to the T cell receptor on the surface of the CD8+ T cells will activate their cytolytic function and consequently lead to the killing of virus-infected or degenerated cells of the body (Male, DK 2006).

### 1.2.1 The αβ T cell receptor

The T cell receptor of the common and most prominent αβT cell population is a heterodimer composed of two protein chains, one α and one β chain, linked by disulfide-bondages. Its major function is to recognize antigen and to initiate signal transduction within the T cell, a process usually leading to activation of the cell. Besides a short cytoplasmic tail containing an immunoreceptor tyrosine activation motif (ITAM) and a transmembrane domain, the major part of the TCR is made up by the extracellular domains of constant and variable regions. While the constant regions are conserved and serve for maintaining the general three dimensional structure and sites of binding the MHC molecule, the variable regions are creating the antigen-specific regions and are highly variable. The regions of greatest variability however, are the complementarity determining regions (CDRs). In the three dimensional folding of the TCR protein they are clustered together at the centre to form the antigen-binding site (Bentley, GA and Mariuzza, RA 1996; Garcia, KC et al. 1996). Both hypervariable CDR3 regions of the α and the β chain are located in the very centre of the antigen-binding site and make contact with the antigen.

For expression on the cell surface the TCR must form a complex with CD3 molecules to form a complete, stable and functional receptor and to elicit proper signal transduction.

The generation of the TCR takes place during the early differentiation in the thymus. The TCR is generated by somatic rearrangement of gene segments, the so called V(D)J recombination. This process is irreversible and a T cell displays this fixed TCR during its existence. A highly diverse TCR repertoire is created by the rearrangement of these main gene loci, the Variable (V), Diversity (D) and Joining (J) segments, of which multiple variants exist. During the process of V(D)J recombination a functional V(D)J gene is generated from randomly chosen gene segments which are further fused to a Constant (C) gene segment to build the final somatic TCR gene locus. Because of multiple gene variants existing in the V, D and J gene loci, the resulting rearranged genes are highly diverse. In humans the V gene loci consists of 32 different genes, 24 of which are expressed and functional.

Additional variability is created during the V(D)J recombination, the joining of the gene segments is an imprecise mechanism and nucleotides are randomly inserted or get lost during this fusion process (Jung, D and Alt, FW 2004). Furthermore, the combination of a $\beta$ with a $\alpha$ chain locus results in even greater variability of the TCR receptor. The high variability of the CDR3 region is due to additional and randomly insertion of non-templated nucleotides by the responsible transferase during the recombination process. Overall, it is estimated that the generation of about $10^{22}$ different TCR molecules is possible and after positive and negative selection in the thymus about $10^{12}$ different TCRs are thought to be present in humans (Arstila, TP et al. 1999). This explains why genetically identical twins cannot have identical TCR repertoires due to the stochastic nature of TCR repertoire selection. Thus every individual being has its own private TCR repertoire with public characteristics (Welsh, RM 2006).

### 1.2.2 T cell activation and effector functions

When a T cell encounters and APC bearing the appropriate MHC:peptide-complex on its cell surface, they form a more or less stable bondage - a so called “immunological synapse” - according to the affinity strength of the TCR to the peptide presented (Grakoui, A et al. 1999). It is thought that TCR can aggregate, which will lead to a stronger activation of the T cell. However, only the binding of the TCR to the MHC:peptide-complex alone is not sufficient to lead to T cell activation. For a full activation of T cells additional signals are required, the so called costimulation (see also 1.4). Upon proper and adequate antigenic stimulation a CD8+ T cell will become activated, mature to effector T cells, proliferate and secrete cytokines like Interferon-gamma (IFN$\gamma$), and IL-2. Most importantly, the CD8+ T cell is able to exert cytotoxicity and kill the identified target cell by inducing apoptosis, therefore CD8+ T cells are also called Cytotoxic T Lymphocytes (CTLs). First of all, CD8+ T cells harbour intracellular lytic granules containing

perforin, granzyme A and granzyme B. These molecules are inserted into the target cell membrane forming multimeric pores leading to cell lysis. Another mechanism of CTLs to lyse the target cell is via the Fas-FasLigand pathway. After activation of the T cell the production and contents of perforin and granzymes increase as well as the expression of FasL on their surface. Additionally, the molecule TRAIL (TNF-receptor related apoptosis inducing ligand) is upregulated (Mirandola, P et al. 2004).

The antigen-specific activation of a CD8+ T cell results in maturation, cytokine production and vast proliferation of the immune cells. After clearing of the infection however, most of the activated effector CTLs undergo apoptosis. Only a small fraction of these antigen-specific CD8+ T cells remain in the body forming memory cells, which can easily become activated again upon reinfection and regain effector functions (Harty, JT and Badovinac, VP 2008). How and when exactly during the course of the immune response these memory CD8+ T cells emerge, remains unclear.

### 1.2.3 Characteristics and phenotype of memory T cells

The ability of generating immunological memory is a keystone of the adaptive immune response (Ahmed, R and Gray, D 1996; Welsh, RM et al. 2004). It is also a feature by which it is distinguished from the innate immune system where the generation of memory cells has not been proven yet (Cerwenka, A and Lanier, LL 2001).

Upon infection with a pathogen, naïve antigen-specific cells mature to effector cells and expand rapidly. After clearing the infection, the immune reaction is silenced and most of the effector cells die by apoptosis. Some of cells, however, remain in the host in relatively high numbers constituting immunologic memory and preventing the host from reinfection. Which factors are necessary and involved in the maturation to long-living memory cells still remains unclear. It seems however, that first a high expression intensity of the IL7R defines T cells that will develop to memory cells (Kaech, SM et al. 2003) that the generation of fully functional memory CD8+ T cells and B cells is dependent on CD4+ T cell help during the initial priming (Janssen, EM et al. 2003; Shedlock, DJ and Shen, H 2003).

Resting memory cells confer protection from reinfections with the same pathogen. Resting memory T cells undergo slow homeostatic proliferation in the periphery and are thereby dependent on IL-7 and IL-15 (Prlic, M et al. 2003; Tan, JT et al. 2002). Resting memory T cells have a low threshold for activation, easily produce cytokines and display cytotoxicity upon short T cell receptor stimulation. Further, memory T cells are less dependent of Costimulatory signals than naïve T cells (Dutton, RW et al. 1998). Thus, the generation of memory T cells is a hallmark for the development of vaccines.

Memory T cells are comprised of different subsets. An established concept is distinguishing between naïve, effector ($T_E$), central memory ($T_{CM}$) and effector memory ($T_{EMRA}$) cells (Bachmann, MF et al. 1997; Sallusto, F et al. 1999). The antigen inexperienced naïve T cell completely lacks immediate effector functions and needs first activation and maturation steps to gain the quality of a CTL. $T_{CM}$, have a high proliferative potential but are rather weak in exerting cytotoxicity and cytokine production. They are able to patrol the lymph nodes due to expression of respective homing markers and adhesion molecules (Judge, AD et al. 2002; Yajima, T et al. 2002). Complete activation of a CD8+ T cell through antigen contact leads to the generation of fully matured $T_E$ cells, which are regarded as the classical effector T cells. They circulate in the periphery and are capable of rapid effector functions such as cytokine production (e.g. IFN$\gamma$ or TNF$\alpha$) and cytotoxicity (Masopust, D et al. 2001; Sallusto, F et al. 1999). $T_{EMRA}$ cells are like $T_{CM}$ undergoing slow homeostatic proliferation maintaining a small but constant pool of long living memory cells, which can easily turn into $T_E$ cells upon antigen contact (Sallusto, F et al. 2004; Weninger, W et al. 2001).

Phenotypical identification of memory T cells is possible via analysing the expression of the molecules CCR7 and CD45RA. (see

Figure 2) CD45RA is a splicing variant of the T cell receptor associated molecule CD45, a tyrosine phosphatase, which is crucial for antigen-induced TCR-mediated T cell activation (Pingel, JT and Thomas, ML 1989). The RA isoform is expressed by naïve T cells and after first antigen contact through the T cell receptor the expression changes to CD45RO (Schwinzer, R et al. 1992). The chemokine receptor CCR7 (CD197) has been shown to be important for homing of T cells to lymph nodes (Baekkevold, ES et al. 2001) and its expression disappears following antigen recognition and T cell activation. Different stages of CD8+ T cell maturation are shown to be associated with differential expression levels of CCR7 and CD45RA (Hamann, D et al. 1997; Sallusto, F et al. 2000).

| | CCR7 | CD45RA | CD62L | CD127 | Proliferative capacity | cytotoxicity | Cytokine production |
|---|---|---|---|---|---|---|---|
| naïve | + | + | + | - | - | - | - |
| $T_{CM}$ | + | - | + | + | ++ | - | - |
| $T_E$ | - | - | - | - | +/++ | ++ | ++ |
| $T_{EMRA}$ | - | + | - | + | + | +/++ | + |

**Figure 2: Memory phenotype of CD8+ T cells.** Basic distinction of memory T cells can be done by determining the expression of various cell surface molecules. Expression patterns of four common markers (CCR7, CD45RA, CD62L and CD127) as well as an overview about basic effector functions are displayed.

Another pair of cell surface markers can be used to identify the maturation status of CD8+ T cells. CD62L (L-selectin) is an adhesion molecule which is expressed on the majority of lymphocytes and is required for the homing of lymphocytes to lymphoid tissues (Spertini, O et al. 1992) Especially antigen-experienced $T_{EMRA}$ cells express CD62L for patrolling the peripheral lymphoid organs (Tedder, TF et al. 1990). CD127 is a high-affinity receptor for IL-7 and stimulation by this cytokine is necessary for homeostatic proliferation of T cells and survival of memory T cells in the absence of antigenic stimulation (Kaech, SM et al. 2003).

The process of differentiation to memory cells is not quite clear and different models have been proposed (Wherry, EJ and Ahmed, R 2004). Apparently, the strength of the antigenic stimulus and the presence of costimulatory signals influence the maturation into the different memory subsets (Masopust, D and Ahmed, R 2004; Rocha, B and Tanchot, C 2004; Sallusto, F and Lanzavecchia, A 2001). As well it seems that a transition between the memory subsets is possible as cells can lose or regain expression of CD45RA or CCR7 (van Leeuwen, EM et al. 2005; Wills, MR et al. 1999).

### 1.2.4 Costimulatory molecules

In order to explain the self-tolerance of the immune system the 2-signal hypothesis was first proposed in 1970 (Bretscher, P and Cohn, M 1970) to explain the elimination of self-reactive B cells. Later, this hypothesis was expanded to T cells, where it was proposed that T cells require besides the first stimulus by the appropriate antigen a secondary signal delivered by the APCs

(Lafferty, KJ and Cunningham, AJ 1975). As mentioned above, the mere contact of the T cell receptor to its appropriate antigen bound to a MHC molecule is not sufficient to activate the T cell and induce immune responses in naïve T cells. This was first shown in 1987 where chemically fixed APCs not able to provide secondary signals were used to stimulate antigen-specific T cells, which resulted in a functional non-responsiveness of the T cells (Jenkins, MK and Schwartz, RH 1987). Similarly, it was further shown that the absence of costimulation during antigen contact drives a T cell into anergy, a possible mechanism for maintaining peripheral tolerance (Jenkins, MK 1992; Jenkins, MK et al. 1990; Schwartz, RH 1990).

Costimulatory molecules do not only deliver positive signals to the respective T cell, but there are as well other molecules which act negatively upon the activity of immune cells. Thus, it is thought that nearly all processes within the immune system are regulated by a fine balance between activatory and inhibitory costimulating molecules. These regulators play a role for the activation, differentiation and expansion of T cells, their survival, the influence the B cell help and are involved in regulation of peripheral tolerance (Redmond, WL and Sherman, LA 2005). Especially naïve T cells require costimulation occurring simultaneously to the antigen-specific signal to lead to activation of the T cell. One of the best known costimulatory molecules expressed on T cells are the molecules of the B7-CD28 family. CD28, which interacts with B7.1 (CD80) or B7.2 (CD86) on the APC, will lead to proper activation of the T cell during antigen recognition (Greenwald, RJ et al. 2005). Later during the course of the immune response, CD28 on the T cell surface will be replaced by CTLA-4, which delivers negative signals to the T cells and is crucial to downregulate the immune reaction (Gribben, JG et al. 1995).

In humans the regulation of immune cell activity is only partially well understood. Few data exist about the function of costimulatory molecules and the role and consequence of expression of others is yet unknown.

#### 1.2.4.1 PD-1

The molecule Programmed Death-1 (PD-1, CD279) is a member of the B7-CD28 family. It was first described as a molecule involved in apoptotic processes (Ishida, Y et al. 1992). PD-1 is expressed on T cells upon activation and TCR stimulation as well as on B cells and myeloid cells (Dong, H et al. 1999). Two ligands for PD-1 are known, they were termed PD-L1 (B7-H1) and PD-L2 (B7-DC). Their expression is not only restricted to cells of the immune system, PD-L1 can be found on T and B cells, myeloid DCs, kidney, heart and in the foetal liver (Dong, H et al. 1999; Freeman, GJ et al. 2000) and its expression significantly increases on T cells following activation (Agata, Y et al. 1996). PD-L2 is expressed on DC, liver, heart, lung, pancreas and placenta and found in lower levels in spleen, thymus and lymph nodes (Latchman, Y et al. 2001; Tseng, SY et al. 2001).

The effects of PD-1 signalling on the immune system became clear in mouse models deficient for PD-1 (PD-1-/-). These knock-out mice were suffering from different autoimmune

disorders of peripheral organs and developed lupus erythematodes-like disease (Nishimura, H et al. 1999) and suffered from severe cardiomyopathy (Nishimura, H and Honjo, T 2001). Several studies in mice and with human cell samples showed that PD-1 is a regulatory molecule inhibiting T cell function such as proliferation (Carter, L et al. 2002; Freeman, GJ et al. 2000; Shin, T et al. 2005) or cytokine production (Mazanet, MM and Hughes, CC 2002). PD-1 was also shown to be involved in induction of peripheral tolerance to T cells thereby controlling autoimmune diseases (Probst, HC et al. 2005; Zhang, Y et al. 2006). Upon contact with DCs human T cells upregulate PD-1 expression (Ferlazzo, G et al. 2002) and treatment of human DCs in vitro with monoclonal antibodies against PD-L1 enhanced the activity and cytokine production of T cells upon antigen stimulation (Brown, JA et al. 2003) and increased the T cell stimulatory function of the DCs resulting in the rescue of T cells from anergy (Selenko-Gebauer, N et al. 2003). The liver as an immunologic organ also expresses ligands to PD-1 (Muhlbauer, M et al. 2006) and can thereby induce tolerance to T cells.

More recently the role of PD-1 during viral infections was investigated and drew the PD-1 molecule into closer attention of immunologists. In a mouse model of LCMV infection it could be shown that functionally exhausted (for Exhaustion see ) antigen-specific CD8+ T cells were positive for PD-1 and that this functional impairment could be overcome by blocking the interaction of PD-1 with its ligand PD-L1 restoring full functionality of the antigen-specific T cells (Barber, DL et al. 2006). Shortly after that, two reports showed that this may also be the case in patients infected with the Human Immunodeficiency Virus (HIV). Here as well, almost all HIV-specific CD8+ T cells expressed PD-1. The function of HIV-specific CD8+ T cells could be partially restored by *in vitro* treatment with anti-PD-L1 causing stronger proliferation and cytokine production (Day, CL et al. 2006; Trautmann, L et al. 2006). Interestingly, the expression level of PD-1 on total and antigen-specific CD8+ T cells correlated positively with the viral load. Similar findings were then reported as well for chronic HCV infection. Again blocking PD-1:PD-L1 interaction with antibodies was able to restore the functionality of the otherwise exhausted CD8+ T cells. Again, the expression intensity of PD-1 on HCV-specific T cells correlated with the HCV viral load (Penna, A et al. 2007). (Golden-Mason, L et al. 2008; Urbani, S et al. 2008)

#### 1.2.4.2 2B4 and CD48

Another regulator of immune function is the molecule 2B4 (CD244) which is expressed on all human NK cells and a subset of αβCD8+ T cells, as well as on basophiles, monocytes, γδT cells and CD56+ CD3+ T cells (Garni-Wagner, BA et al. 1993; Romero, X et al. 2004; Valiante, NM and Trinchieri, G 1993) but is absent from B cells and CD4+ T cells (Romero, X et al. 2004; Speiser, DE et al. 2001; Valiante, NM and Trinchieri, G 1993). It belongs to the Ig-superfamily and is closely related to the CD2 subfamily of molecules (Brown, MH et al. 1998; Mathew, PA et al. 1993). 2B4 is a transmembrane protein with four cytoplasmatic ITSMs (immunoreceptor tyrosine-based switch motifs) (McNerney, ME et al. 2005) which become phosphorylated by Src kinases upon ligation. Subsequently, SH2 domain containing proteins (SHP) bind to the ITSM.

The most important protein of this group for 2B4 signalling seems to be the SLAM (signalling lymphocyte activation molecule) and the SAP molecule (SLAM-associated protein) (Chen, R et al. 2004).

2B4 was originally identified as an activating receptor on mouse NK cells and a subset of T cells conveying non-MHC-restricted cytotoxicity (Garni-Wagner, BA et al. 1993; Mathew, PA et al. 1993). A high-affinity counterreceptor for 2B4 in humans as well in mice was shown to be CD48, a molecule of the CD2 family (Brown, MH et al. 1998; Latchman, Y et al. 1998). The cross-linking of 2B4 on murine NK cells augmented redirected killing of target cells (Sivori, S et al. 2000). Besides augmenting cytotoxicity, 2B4 stimulation of mouse cells *in vitro* stimulated as well IFNγ secretion and granule exocytosis (Garni-Wagner, BA et al. 1993; Lee, KM et al. 2006). Experiments using TCR-transgenic mice demonstrated an impact of 2B4 on CD8+ T cells increasing the killing of tumour cells bearing the respective antigen (Lee, KM et al. 2003).

In humans there is evidence that 2B4 acts as well as a costimulatory molecule regulating different processes in the immune system. On NK cells it enhances lytic activity (Sivori, S et al. 2000; Tangye, SG et al. 2000a; Valiante, NM and Trinchieri, G 1993) and IFNγ production (Chuang, SS et al. 2000) upon cross-linking with monoclonal antibodies. There is evidence that on human NK cells 2B4 is functioning as a coreceptor for other activating NK cell receptors like NKp46 (Sivori, S et al. 2000). Not as much is known about the role of 2B4 on T cells. Several reports did not find an activating function of 2B4 on human CD8+ T cells (Nakajima, H et al. 1999; Tangye, SG et al. 2000a). Other more recent reports give evidence for a role of 2B4 on CD8+ T cells regulating killing of EBV-infected cells (Dupre, L et al. 2005). In mice the interplay between 2B4 on NK cells and CD48 on T cells was able to enhance T cell proliferation (Assarsson, E et al. 2004). Again, 2B4 in mice is upregulated upon activation and stimulation of 2B4 leads to a lesser proliferation and cytokine production of previously activated TCR-transgenic CD8+ T cells but had no effect on naïve T cells (Kambayashi, T et al. 2001). Similarly, analysis of human CD8+ T cells showed an association of 2B4 expression with activation and maturation status (Speiser, DE et al. 2001).

CD48 is a pan-leukocyte antigen expressed on lymphoid and myeloid cells (Kato, K et al. 1992). In response to IFNs and during EBV infection CD48 gets upregulated (Tissot, C et al. 1997; Yokoyama, S et al. 1991). Initially it was assumed that CD48 is merely a ligand for 2B4, but several reports show that CD48 itself also has an immunomodulatory function influencing T cell proliferation in mice and enhancing TCR-mediated function through cytoskeletal reorganization (Kambayashi, T et al. 2001; Moran, M and Miceli, MC 1998). Further complication of the interplay between 2B4 and CD48 arises due to observations that 2B4 and CD48 are both expressed on the same cell and cell subsets. Some reports demonstrate that 2B4-CD48 interaction can occur among single T cells and as well between T cells and NK cells influencing the immune response (Assarsson, E et al. 2004; Kambayashi, T et al. 2001; Messmer, B et al. 2006). Recently, it has

been shown that besides PD-1 2B4 was up regulated in gene chips analysing exhausted CD8 T cells from mice infected with LCMV clone 13 (Wherry, EJ et al. 2007).

The general assumption of 2B4 being an activating receptor in the immune system however was challenged by the finding that 2B4 knock-out mice showed an increased killing of CD48+ target cells in vivo, whereas the restoration of 2B4 diminished the lysis of the CD48+ target cells (Lee, KM et al. 2004). Comparably, CD48+ tumour cells were less efficiently lysed by 2B4+ NK cells and this inhibition was abolished in 2B4-deficient mice (Vaidya, SV et al. 2005). These findings are in line with the observation that patients suffering from X-linked lymphoproliferative disorder (XLP) display a substantial defect in cell lysis of EBV-infected cells by NK cells and CD8+ T cells (Coffey, AJ et al. 1998). This disorder results from a inheritable mutation in the gene encoding for the SAP molecule rendering it non-functional (Sayos, J et al. 1998). Subsequently it was shown that the absence of functional SAP molecule abrogated the activating costimulation delivered by 2B4 and instead ligation of 2B4 rendered an inhibitory signal (Parolini, S et al. 2000). This was caused by recruitment of SHP-1 and -2 molecules for downstream signalling, which results in an inhibitory pathway (Morra, M et al. 2001). These findings are in line with a report demonstrating that the four different ITSM contained in the cytoplasmic tail of 2B4 bind different downstream signalling molecules (e.g. SLAM/SAP or SHP-1/2) thereby modifying the nature of the signal delivered (Eissmann, P et al. 2005).

Further discrepancies in the signalling elicited in NK cells by 2B4 ligation comes from studies with immature human NK cells. Here, binding of 2B4+ immature NK cells to CD48 on DCs present in lymphoid organs resulted in a diminished IFN$\gamma$ production and cytotoxicity. This inhibition of immature NK cell function agrees with the finding that immature NK cells do not express SAP, a molecule shown to be crucial for positive signalling of 2B4 (Chen, R et al. 2004). This fact is regarded as a mechanism preventing cytolytic activity of immature NK cells against autologous cells, as in early stage of development the inhibitory receptors are not expressed on NK cells (Sivori, S et al. 2002). Subsequently published data show that human DCs differentially express CD48 according to their differentiation status and localization (Morandi, B et al. 2005). Myeloid DCs (mDCs) bear CD48 expression on their cell surface, like also DCs from primary lymphoid organs do. Plasmacytoid DCs (pDCs) on the other hand do not harbour CD48 on their cell surface and likewise DCs from inflamed lymph nodes as well are negative for CD48. Furthermore it was shown that on NK cells isolated from lymph nodes 2B4 displayed an inhibitory function, while on peripheral NK cells 2B4 ligation delivered an activating signal. As for the initiation of an immune response a close interplay between DCs and NK cells in the lymph nodes occurs which influences the cytotoxic capacitiy and cytokine production of NK cells, the fact that in reactive lymph nodes DCs are not expressing CD48 is most likely due to the necessary activation of NK cells during inflammation processes and therefore the inhibitory signal of 2B4 ligation on lymph node residing NK cells needs to be absent.

## 1.3 Viral immunology

### 1.3.1 Viruses

During their life humans are exposed to various kinds of pathogens. Besides bacteria, parasites and fungi, viruses are frequent pathogens that invade the organism. Viruses are defined as small genetic elements, e.g. DNA or RNA fragments, which can also exist in an extracellular state. Here, the viral genome is surrounded by a protein hull (capsid) and some viruses are additionally enclosed by a membrane consisting of a lipid bilayer with embedded viral proteins, which is called envelope. By means of this extracellular state viruses are capable of leaving their original host cell and newly infect other cells. Upon attaching to a new host cell, only the viral genome is inserted and during this intracellular state virus replication occurs. A viral genome can replicate independently of the host cell chromosomes, but the process of its replication is dependent on the metabolic system of their host cells, which provides the molecular - and also in part enzymatic - components required. By exploiting the host cells nucleic acid synthesis mechanism the viral genome replicates and using the host cell's protein synthesis system the viral capsids are generated. Subsequently, the viral proteins are assembled, the viral genome is packed and the new viruses are released from their host cell.

Frequently, viral genomes encode only for a limited number of viral proteins, which are crucial for their replication and are not present in the host cells. Those genes usually comprise proteases or polymerases and components of the capsid and envelope. Viruses are classified by means of the presence of an envelope and the type of nucleic acids building the viral genome. According to the latter, viruses are divided into double- and single-stranded RNA- or DNA-viruses. Within that there is additional separation into positive- and negative-stranded viruses.

### 1.3.2 Immune responses in viral infections

In contrast to bacteria, which – in most of the cases – are extracellular pathogens, viruses are replicating and "hiding" within the cells of the organism. For this reason the elimination of a viral infection occurs through the complete disposal of the infected cell, the inhibition of viral replication and the prevention of new infection of other cells. Every cell containing a nucleus also bears MHC-class I molecules on their cell surface displaying fragments of every intracellular protein present. Cytotoxic immune cells have different mechanisms of detecting virus infected cells, either in an antigen-specific manner (CD8+ T cells) or by the presence or absence of various surface molecules (NK cells) (Biassoni, R et al. 2001). The elimination of the infected cell occurs by induction of apoptosis by several different mechanisms like through secretion of pro-apoptotic molecules (granzymes, perforin), expression of apoptosis-inducing cell surface

molecules (Fas/FasLigand; CD40/CD40Ligand, TNF-related Apoptosis Inducing Ligand, TRAIL) or cytokines (TNFα/TNF receptor) (Mirandola, P et al. 2004).

Another important process in the defence against viruses is the production and secretion of antiviral cytokines. The most important cytokines in the antiviral immune response are IFN-α, IFN-β, IL-12 and IFN-γ (Salazar-Mather, TP and Hokeness, KL 2006). IFNs are able to inhibit viral replication and additionally enhance the adaptive immune response by activating macrophages, CTLs and NK cells, enhancing MHC-molecule expression and directing the CD4+ T cell response to a $T_H1$ profile. IFN-α/β is produced in the early phase of infection by pDCs and the virus infected cell itself and is elicited by Toll-like receptor signalling. IL-12 promotes activation of NK cells as well as initiating the TH1 cytokine responses and increasing antiviral activity of macrophages. TNFα acts as well by activation of macrophages and cytototoxic cells and enhances expression of MHC-molecules. Furthermore it binds to TNF receptors present on infected cells inducing apoptosis.

### 1.3.3 T cell response to viruses

Directly after infection anti-viral cytokines such as IFN-1α/β are secreted by DCs and NK cells. Soon afterwards, antigen-specific CD4+ and CD8+ T cells get activated and migrate to the lymph nodes. Here they interact with DCs bearing the respective antigen. They mature to effector T cells and undergo drastic expansion. During this exponential phase of clonal antigen-specific T cell expansion cells can divide up to 15 times (Welsh, RM et al. 2004) (see Figure 3). After clearing of the virus and disappearance of antigen, the contraction of T cells takes place. Effector T cell clones die by apoptosis limiting and abrogating the immune response. During the following phase the number of virus-specific memory T cells remains stable through homeostatic proliferation with the total number of virus-specific cells being higher than before the initial infection. As describes above, antigen-specific memory T cells develop and may persist in the body for years (see 1.2.3). Upon reinfection, a second expansion phase occurs, reaching same peak height of CD8+ T cell numbers.

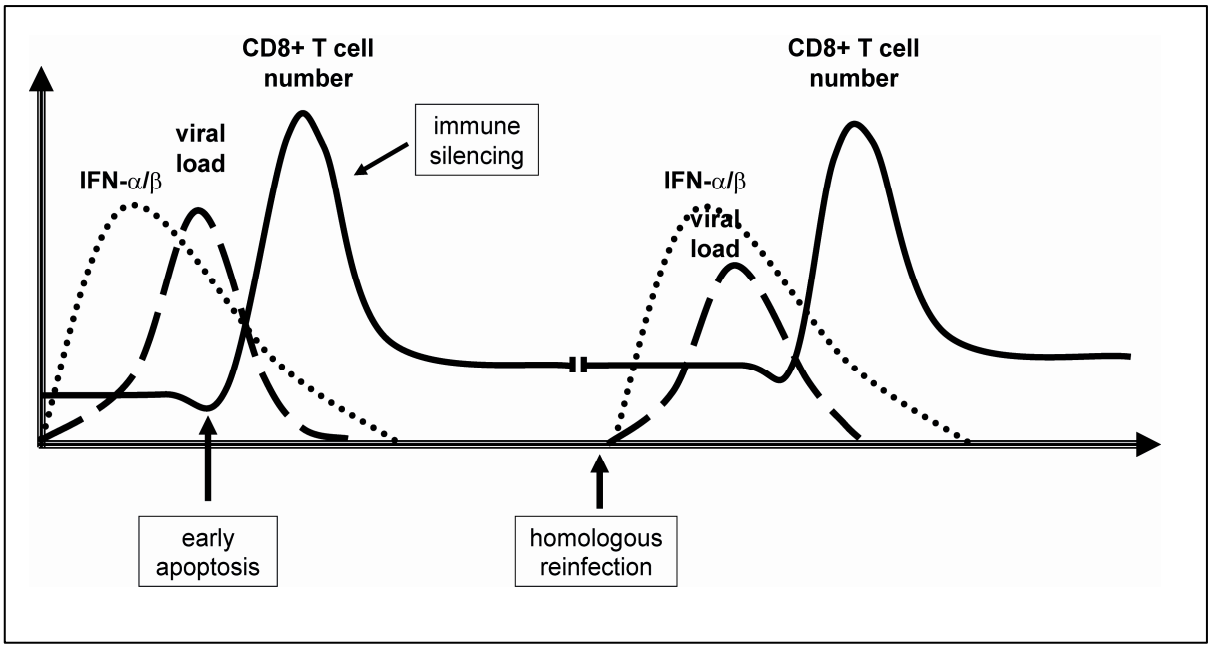



**Figure 3: T cell responses in viral infection.** Directly after infection with a virus, the viral load increases (dashed line) and cells of the innate immune system produce IFN-α/β (dotted line) to inhibit viral replication and activate the immune system. Some days after infection and in some cases after an initial slight decline of CD8- T cells numbers (early apoptosis), virus-specific CD8+ T cells expand and the number of CD8+ T cells in the periphery rises (black line). After disappearing of the antigen, the immune response is silenced. Most effector cells die by apoptosis except a few remaining memory cells. Upon homologous reinfection, IFN-α/β levels rise again and memory T cell responses are elicited, causing a lower and earlier declining viral load. (Figure adapted from (Welsh, RM et al. 2004)

Some viral infections can induce an initial lymphopenia preceding the expansion phase. The reduction of cell numbers occurs within all lymphocyte subsets and seems to be strongly influenced by the rise of IFNα/β secretion in response to viruses (McNally, JM et al. 2001). Especially memory CD8+ T cells are sensitive towards apoptosis and their numbers were shown to decrease substantially during this early phase of apoptosis. It is thought that the induced lymphopenic environment leaves space for the following vigorous expansion of virus-specific CD8+ T cells (Welsh, RM et al. 1997). Attrition of CD4+ T cells though does not seem to occur in case of viral infection (Vargas, AL et al. 2001).

### 1.3.4 T cell exhaustion in viral infections

While in acute viral infection immune cells and T cells in particular extensively display effector functions with strong proliferation, robust cytokine responses and cytotoxicity, the opposite can be the case in chronic infections. In a setting of persistent LCMV infection in mice it was first described that virus-specific T cells are functionally exhausted. That means that antigen-specific T cells are present and detectable but fail to display the regular effector functions characteristic during acute infections (Zajac, AJ et al. 1998). In following studies it became clear

that this exhaustion is a graded process and that loss of effector functions follows a hierarchy (Wherry, EJ et al. 2003a). The cytotoxic potential, IL-2 secretion and proliferation capacity are the first to be impaired while IFNγ production is still detectable in a late stage of exhaustion, although to a much lesser extent. The lack of CD4 T cell help and a high antigen-load seem to be directly linked to the grade of exhaustion of CD8+ T cells (Fuller, MJ and Zajac, AJ 2003; Matloubian, M et al. 1993) (compare Figure 4 ).

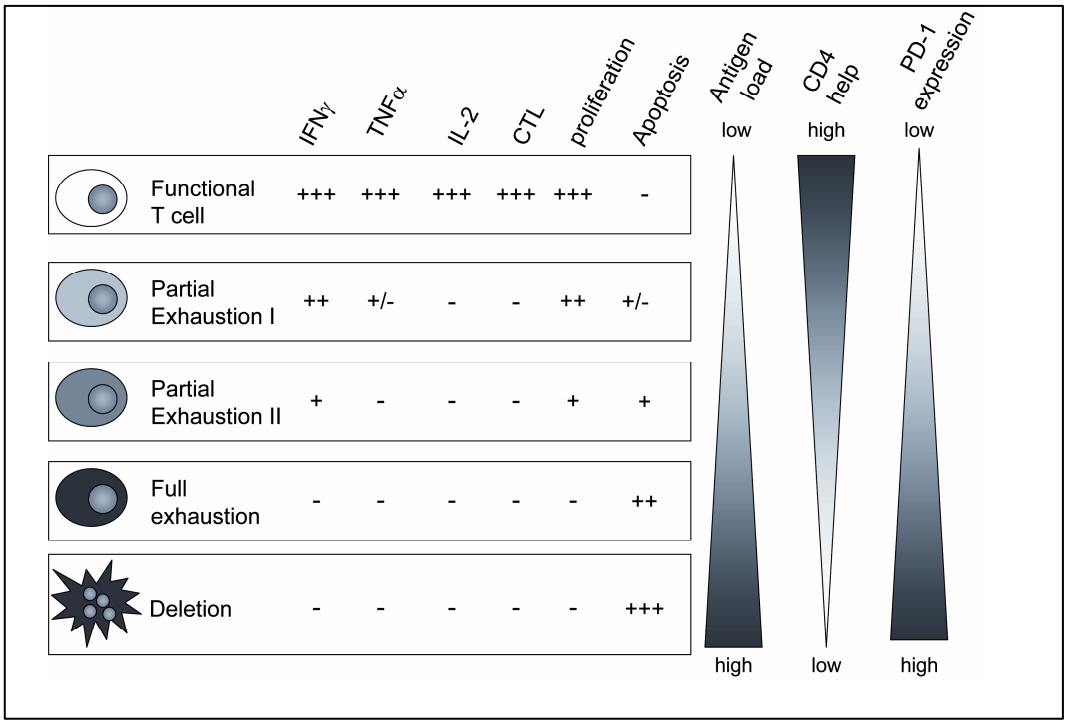



**Figure 4: Characteristics of and influences on CD8+ T cell exhaustion.** While a functional CD8+ T cell is capable of immediate effector functions, these abilities gradually get lost during the different stages of T cell exhaustion. Here, a hierarchy can be seen with cytotoxicity and IL-2 production the first to disappear. Also, with rising levels of exhaustion apoptosis rate of T cells increases up to complete deletion of the cell. Some parameters are known to be linked with or influence T cell exhaustion, such as the antigen load, PD-1 expression and CD4+ T cell help. (Figure adapted from (Freeman, GJ et al. 2006))

The phenomenon of T cell exhaustion is also described for viral infections in humans. Likewise as shown in mouse models with different infecting viruses, HIV-specific T cells in humans display similar grades of exhaustion (Kostense, S et al. 2002; Shankar, P et al. 2000). Comparably, during chronic HCV infection virus-specific CD8+ T cells show varying grades of functional impairment (Gruener, NH et al. 2001; Wedemeyer, H et al. 2002).

The ways by which T cell exhaustion is induced or maintained is only little understood. High antigen-load was shown to be one factor influencing the functionality of T cells. But besides this, other mechanisms must be involved in the regulation of immune responses. As CD4 T cell help was also shown to have an impact on CD8+ T cell responses, it is conclusive that apart from cytokines additional signals delivered may play a role. Consequently, a group of molecules come

into focus which are known to be relevant in the control of the immune system, the so called costimulatory molecules (see 1.2.4). The particular understanding how T cell exhaustion is induced or may be prevented is of interest for the development of effective vaccines.

### 1.3.5 T cell receptor repertoire

Antigen-recognition of CD8+ T cells is mediated by its unique T cell receptor (TCR) which binds to the respective antigen presented on MHC-class I molecules. The TCR is generated by somatic recombination of the TCR genes (see 1.3.1). As this is a random process and the TCR variability is even increased by additional insertion or deletion of nucleotides during the rearrangement process. Altogether, it is estimated that in humans $10^{22}$ different TCR sequences are possible and that after the selection of T cells occurring in the thymus during maturation about $10^{7}$-$10^{8}$ different TCR variants exist in humans (Arstila, TP et al. 1999). Thus, the TCR repertoire existent in different individuals cannot be the same, even in genetically identical mice or identical twins (Bousso, P et al. 1998; Somma, P et al. 2007). This is based on the random selection of the different TCR genes for the $\alpha$ and $\beta$ chain of the TCR and as well in the repertoire of MHC molecules expressed in the individual as the recognition of MHC molecules play a critical role in the positive selection of T cells in the thymus which, as well, is a random process (Gulwani-Akolkar, B et al. 1991; Welsh, RM and Selin, LK 2002).

The TCR repertoire of a given immune response to one HLA-restricted epitope is alike within a group of individuals, displaying dominant V$\beta$ usages. This phenomenon is called “public specificity”. Still, between the individuals the amino acid sequence and the length of the antigen-binding sites (CDRs) of a single V$\beta$ chain will differ, especially regarding the highly variable CDR3 region. This fact is referred to as “private specificity” (Welsh, RM 2006).

## 1.4 Hepatitis C

### 1.4.1 The Hepatitis C Virus

HCV was discovered in 1989 and belongs to the family of Flaviviridae and is a positive-stranded RNA virus (Choo, QL et al. 1989). It is of 50-60 nm size with an envelope surrounding the nucleocapsid. Embedded in the envelope are viral proteins (E1 and E2), which can associate to surface molecules of host cells like CD81, Claudin-1, LDL and Scavenger Receptor B1 (Koutsoudakis, G et al. 2007; Scarselli, E et al. 2002; von Hahn, T et al. 2006). The HCV genome consists of a single-stranded RNA molecule with positive orientation and approximately 9500 nucleotides in size. The genome is flanked by non-coding regions at its 3’ and 5’ end, which were shown to be crucial for virus replication (Honda, M et al. 1996; Ito, T et al. 1998). It encodes for a

single open-reading frame (ORF) and is translated into a polyprotein of about 3010 amino acids size. The viral RNA serves upon entry into the cytosol as mRNA, viral proteins are synthesized and cellular and viral proteases cleave the resulting polyprotein (Grakoui, A et al. 1993). The proteins generated are grouped into structural (Core, E1, E2 and p7) and non-structural (NS2, NS3, NS4a and NS4b, NS5a and NS5b) proteins. NS2 and NS3 have protease function and NS5b is a RNA-dependent RNA-polymerase essential for viral replication. The Core and envelope proteins are translated at the Endoplasmatic Reticulum (ER), the core protein binds the viral RNA and new viral particles are formed. The release of new virions occurs by budding of these complexes from the ER membrane and secretion from the cell (Wolk, B and Wedemeyer, H 2008).

The Hepatitis C virus has a high mutation rate resulting from the inaccuracy of the viral RNA-polymerase with mutation rate of $10^{-5}$ to $10^{-4}$ per nucleotide. As a result of this mutability six different genotypes of HCV exist (genotype 1-6) with each consisting of different quasispecies (e.g. genotype 1a, 1b, 3a etc.) The prevalence of each genotype differs according to geographical distribution. The dominating genotypes in Europe and North America are 1a, 1b and 3a, whereas in Asia most people are infected with genotype 1b or 6 and in Africa genotype 4 is widely spread. As well, the success of standard therapy is dependent of the infecting genotype. Furthermore, the high mutability of HCV often leads to so called escape mutations, where viral epitopes targeted by the host immune system undergo mutations and thereby evade immune recognition (Kuntzen, T et al. 2007; von Hahn, T et al. 2007).

HCV is primarily hepatotrophic, but it may also replicate in lymphocytes (monocytes and Dendritic Cells), although to a lesser extent (Hu, Y et al. 2003). HCV itself is not cytopathic, the occurring liver damage is caused by the host immune system (Rehermann, B and Nascimbeni, M 2005). Infection occurs mainly parenterally through direct contact with blood, blood products or contaminated needles (Kubitschke, A et al. 2007). In rare cases, but sexual and perinatal transmission can also occur (Dienstag, JL 1997).The prevalence of HCV infection worldwide is about 3%. Worldwide 130 million people are estimated to be infected with the Hepatitis C virus (HCV) with additional 3 to 4 million new infections per year (WHO 2004). In some developing countries in Asia, Africa and South America the HCV prevalence can exceed 10%. The burden in developed countries is lower with anti-HCV frequencies of 0.5-2% of its population in Europe and North America (Alter, MJ 2007). The diagnosis of HCV infection is done by detection of either HCV specific antibodies or direct testing for HCV RNA by PCR, which is also used for assessing of the efficacy of antiviral therapy.

### 1.4.2 Natural course of Hepatitis C Virus infection

Clinical symptoms of viral hepatitis experienced by patients are uniform regardless of the infecting virus strain (e.g. Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C Virus

(HCV), Hepatitis D (HDV) and Hepatitis E virus (HEV)). After a variable incubation period of a few days up to several weeks, unspecific symptoms like fatigue, fever, headache, nausea and abdominal pain precede the clinical onset of acute hepatitis, which can be characterized by jaundice and liver pain. The liver tissue is inflamed and increases in size (hepatomegalie) which causes liver pain in some patients. Clinical data show rise of liver enzymes in blood serum, such as Alanin Aminotransferases (ALT), Aspartate Aminotransferases (AST) and Bilirubin.

If the virus cannot be cleared during the acute phase of disease, HBV, HCV and HDV the infection may take a chronic course. Many of the initial symptoms like fatigue, loss of appetite and joint pain persist. However, the course of HCV infection is highly variable between patients. Beginning with different clinical features during the onset of infection, equal differences of disease outcome exist during persistent infection. While some patients may remain completely asymptomatic for decades despite chronic infection, others progress to liver cirrhosis and some of them even develop liver cancer. The incidence of persistency varies between the different hepatitis viruses and in case of HCV it can be as high as 90% of cases (50-90%). In contrast, HAV and HEV infections never become chronic. Frequent complications of chronic viral hepatitis are liver cirrhosis, which can lead to the development of hepatocellular carcinoma (HCC) in 0,5-2% of cases per year (compare Figure 5.). Viral hepatitis is one of the most frequent causes of liver transplantation. An estimated 1.5 million people die each year from HBV or HCV induced clinical complications (www.who.com)

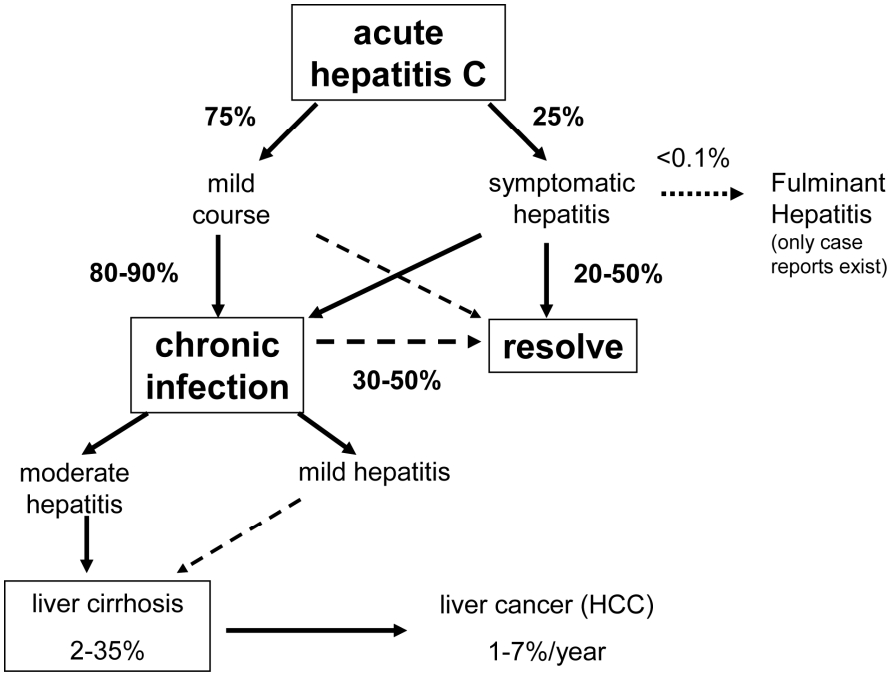



**Figure 5: Natural course of Hepatitis C infection.** Of all patients getting infected with HCV, only one fourth take a symptomatic course of disease, some of which spontaneously resolve infection. For the others, the viral infection take a chronic course just like the majority of those patients without symptomatic acute phase do. Few of chronic HCV carriers are able to clear the infection during therapy. The hepatitis caused by HCV infection can lead in a long term to liver cirrhosis and can also induce liver cancer.

So far, there is no vaccine against HCV available and the rather expensive standard treatment of HCV infection consists of rather unspecific drugs such as interferon-alpha in combination with ribavirin. This treatment is also associated with sometimes severe side effects (Manns, MP et al. 2006). Since the discovery of HCV treatment with interferon alpha-based schedules could be optimized leading nowadays to sustained virological response rates of nearly 60% However, many patients cannot be treated due to contraindications giving an unmet need for new antiviral treatment concepts. Novel drugs are currently tested in phase I/II trials comprising inhibitors of viral replication, viral attachment to the cell and such processes as the assembly of new viruses or the release of new virions by budding at the cellular membrane (Pawlotsky, JM et al. 2007). Another promising treatment option pursued is the development of immunotherapies such as a therapeutic HCV vaccine.

### 1.4.3 Role and function of CD8+ T cells in HCV infection

Most of the data on role and function of CD8+ T cells in HCV infection are documented from patients with chronic and – in fewer cases - acute infection, as suitable in vitro infection models

are lacking and the only existing animal model so far is the infection of chimpanzees. Data from acute HCV infection and animal models suggest a key role of T cell responses for the outcome of HCV infection (Bowen, DG and Walker, CM 2005; Day, CL et al. 2002; Grakoui, A et al. 2003; Lechner, F et al. 2000b; Shoukry, NH et al. 2003). A vigorous CD8+ T cell response targeting multiple epitopes was shown to be beneficial for eradication of the virus (Cooper, S et al. 1999; Cox, AL et al. 2005; Gruner, NH et al. 2000; Lechner, F et al. 2001; Lechner, F et al. 2000a). Still, the CD8+ T cell response is obviously not the only factor determining and influencing the outcome of HCV infection. CD4+ T cell help is important as the cytokine profile present during infection affects the antiviral CTL immune response and without adequate help of $T_H$ cells the immune response appears to get lost marked by a decline in anti-viral CD8+ T cell responses. (Grakoui, A et al. 2003; Thimme, R et al. 2001). Here, a $T_H1$ profile with production of IFN$\gamma$ and TNF$\alpha$ is advantageous for eradication of the virus because it supports the CTL response. A $T_H2$ response on the other hand was shown to be associated with a chronic progression of the infection (Missale, G et al. 1996).

In contrast to the acute phase of Hepatitis C, the CD4+ and CD8+ T cell response is functionally impaired during persistent HCV infection (see 1.3.4 T cell exhaustion). HCV-specific CD8+ T cells have a decreased IFN$\gamma$ production, proliferation and cytotoxicity and CD4 + T cell responses are weak displaying a Th2 profile and reduced proliferation (Gerlach, JT et al. 1999; Wedemeyer, H et al. 2002) and the expression of FasL and granzyme content of CD8+ T cells is reduced (Cerny, A and Chisari, FV 1999). Very few HCV epitopes were shown to be targeted by T cells during chronic infection by both CD8+ and CD4+ T cells (Lauer, GM et al. 2004) which was shown to be the case for peripheral as well as for liver-residing T cells (Grabowska, AM et al. 2001; He, XS et al. 1999; Wong, DK et al. 1998). Also, the maturation of HCV-specific seems to be altered, they display a naïve-like phenotype being CD45RA+, CCR7+, CD27+ and CD28+ (Appay, V et al. 2002; Wedemeyer, H et al. 2002). Additionally, in chronic HCV infection an unusual high percentage of regulatory CD4+ CD25+ T cells could be detected which can negatively influence proliferation and cytokine production (Boettler, T et al. 2005; Chang, KM 2007). Other cells of the immune system were also shown to be impaired in function during chronic HCV infection. Besides NK cells which get inhibited through binding of HCV core protein to the surface molecule CD81 (Crotta, S et al. 2002; Tseng, CT and Klimpel, GR 2002), the activity of DCs seem to be altered regarding maturation status and T cells priming capacities (Bain, C et al. 2001; Kanto, T et al. 1999; Sarobe, P et al. 2003; Wedemeyer, H et al. 2002). However, these issues remain controversial as different observations were made by other groups describing normal DC function in chronic HCV infection (Longman, RS et al. 2004; Piccioli, D et al. 2005)

In summary, much is known about the role of CD8+ T cells in viral infections, but despite the constantly growing knowledge about their function, many things about the regulation of CD8+ T cell activity and induction of functional exhaustion remains unclear.

### 1.4.4 HCV vaccination

The treatment of hepatitis C infection has greatly improved during the last years but still there is no general cure possible (Manns, MP et al. 2006). The costs for the treatment of hepatitis C virus infection as well as of disease complications, e.g. liver cirrhosis, liver cancer and the need for transplantation, are immense (Kim, WR 2002; Kim, WR et al. 2002; Wong, JB 2006).

Until nowadays there is no vaccine for hepatitis C available. Different strategies and different concepts of vaccines have been developed during the last years. Many studies have evaluated HCV-specific immune responses in rodents while few studies in chimpanzees and man are available (Ahlen, G et al. 2005; Chen, JY and Li, F 2006; Encke, J et al. 2005; Firbas, C et al. 2006; Folgori, A et al. 2006a; Forns, X et al. 2000; Pancholi, P et al. 2003). One of the first approaches in man was conducted in 1997 using a protein-based vaccine (Nevens, F et al. 2003). Besides this also plasmid DNA encoding for HCV proteins and the usage of HCV pseudoparticles were tested as possible vaccine candidates (Jeong, SH et al. 2004; Langhans, B et al. 2006; Langhans, B et al. 2004; Pancholi, P et al. 2003).

Vaccination against HCV includes two different concepts based on the clinical setting. One concept is the usage as a preventive vaccine for the treatment of healthy people to avoid new infections. The other concept deals with the implementation as a therapeutic vaccine for the treatment of already infected people to combat an existing chronic infection. The latter aims to stimulate the immune system, which in chronic infection often is exhausted and impaired in its function regarding cytokine secretion, proliferation, cytotoxicity and antibody production (Gruener, NH et al. 2001; Lauer, GM et al. 2004; Wedemeyer, H et al. 2002).

So far, therapeutic vaccine studies were not successful to overcome impaired T cell function in chronic infections. One important question is if it is possible at all to stimulate pre-existing antigen-specific T cells or to induce and recruit new naïve T cells from the thymus (Vezys, V et al. 2006). Preventive vaccinations against HCV so far were able to induce immune responses in healthy people including the generation of antigen-specific T cells, but these immune responses are rather weak and it is questionable if they are protective in an infectious setting or not.

Still in both scenarios it is of great interest to know which type of T cell is induced by the vaccine and to get more information on the maturation and effector status of T cells. Therefore it is important to characterize T cells in these ongoing clinical vaccination trials in order to understand the effects of a vaccine on the immune system, determining its efficacy and to identify the cell type and differentiation status which is needed to gain an optimal preventive or therapeutic effect.

## 1.5 Aim of this thesis

Of the many different pathogens humans are exposed to many are viruses. The course of a viral infection is varying strongly and is dependent on the kind of virus. However, infections with the same virus in different individuals also show a great variation ranging from mild symptoms to severe pathogenesis and even cancer formation. The outcome of a viral infection can hardly be predicted and few of the factors involved or determining it are known. Additionally, therapies for viral infections are expensive and linked with strong side effects. Therefore the desire is to prevent new infections and a vaccination is a general practice to achieve immunologic memory. However, vaccines are not available for all known viral and also bacterial pathogens. Furthermore, for some individuals, preventive vaccination does not lead to the generation of protective immunity for unknown reasons.

The aim of this thesis was the functional and phenotypic characterisation of virus-specific T cells of the human immune system. The objective was to further elucidate the functionality of immune cells and immune responses. Furthermore, it is of desire to identify factors influencing the course and the outcome of viral infections. Besides the recently described inhibitory molecule PD-1, special emphasis was laid on the costimulatory molecule 2B4 and its counter-receptor CD48, whose roles in the human immune system is only poorly understood. Infection of humans with the Hepatitis C Virus served as a model of infection and the impact of immunological memory on immune responses and course of infection was another focus of this work in order to improve existing and develop new vaccines.

The key questions of this thesis were as follows:

1. What are the phenotype and the functional characteristics of HCV-specific CD8+ T cells during persistent infection? Can a therapeutic vaccine lead to a change of the phenotype and functionality of these cells?
2. What is the expression and the role of PD-1 on the function of virus-specific CD8+ T cells?
3. How is the expression of 2B4 and CD48 in healthy individuals and patients with persistent hepatitis virus infection? Does the expression of these molecules have an impact on the function of CD8+ T cells and which role do they play in the regulation of immune responses?
4. How is the immunodominance of different HCV epitopes targeted during acute HCV infection? And what is the T cell receptor repertoire of CD8+ T cells specific for the

dominant HLA-A2 restricted HCV epitope NS3-1073? And how may the immunological memory emerged from previous infections influence the immune responses and the clinical outcome of other virus infections like HCV?

# 2 Materials and Methods

## 2.1 Monoclonal Antibodies

Mouse anti-human fluorochrome-conjugated monoclonal antibodies were purchased as follows:

Beckman Coulter (Fullerton, CA, USA): PECy5-labelled and purified anti-2B4 (clone C1.7), FITC-labelled and purified anti-CD48 (clone J4.57), IOMark Beta Test® Kit for detecting TCR Vβ chains and anti-CD8-PCy7 (clone SFCI21Thy2D3).

R&D Systems (Minneapolis, MN, USA): anti-CCR7-FITC (Clone 150503).

BD Pharmingen (Becton Dickinson, Heidelberg, Germany): anti-CD3 (clone UCHT1), anti-CD4-APC (clone RPA-T4), anti-CD8 (clone RPA-T8), anti-CD14 (clone M5E2), anti-CD19 (clone HIB19), anti-CD25 (clone M-A251), anti-CD27 (clone M-T271), anti-CD28 (clone CD28-2), anti-CD38 (clone HIT2), anti-CD40 (clone 5C3), anti-CD45RA (clone HI100), anti-CD56 (clone NCAM16.2), anti-CD62L (clone Dreg 56), anti-CD107a (clone H4A3), anti-CD107b (clone H4B4), anti-CD127 (clone hIL-7R-M21), anti-CD152 (clone BNI3), anti-HLA-A2 (clone BB7.2), anti-PD-1 (clone MIH4), anti-IFNγ (clone B27), anti-TNF (MAb11), IgG-1 κ isotype controls (clone MOPC-21), IgG2a k isotype control (clone MOPC-173), mouse IgG-2b k isotype control (clone 27-35), purified anti-CD3 (clone HIT3a), purified anti-CD28 (clone CD28-2).

eBioscience (San Diego, CA, USA): anti-PDL1 functional grade purified (clone MIH1), FC-Receptor binding inhibitor

## 2.2 HLA-A2-restricted Peptides

Antigenic HLA-A2 restricted peptides were purchased from ProImmune Ltd., Oxford, UK. Purity of all peptides was >98%. Lyophilized peptides were resuspended in sterile endotoxin-free DMSO as stock solution. Final DMSO concentration during T cell culture never exceeded 0.1%. Amino acid sequences of the specific peptides and concentrations used for CD8+ T cell stimulations are given in Table 1. The final concentration of the peptide used is based on titration experiments.

| epitope | amino acid sequence | final concentration used (µg/ml) |
|---|---|---|
| CMV-pp65 495-504 | NLVPMVATV | 1 |
| EBV-BMLF 1 259-267 | GLCTLVAML | 1 |
| Flu-MA 58-66 | GILGFVFTL | 0,1 |
| HBV core 18-27 | FLPSDFFPSV | 10 |
| HBV envelope 183-191 | FLLTRILTI | 10 |
| HBV polymerase 573-581 | FLLSLGIHL | 10 |
| HBV surface 185-194 | GLSPTVWLSV | 10 |
| HCV-NS3 1073-1081 | CINGVCWTV | 10 |

**Table 1: Amino acid sequences and concentration of antigenic peptides used for in vitro stimulation of PBMCs.**

## 2.3 HLA-A2 restricted MHC Class I Complexes

Pro5®-MHC Class I Pentamers were purchased from ProImmune Ltd, (Oxford, UK). PE-labelled Pentameric Complexes used were specific for CMV-pp65 495-504 (NLVPMVATV), EBV-BMLF1 259-267 (GLCTLVAML), Influenza A Matrix (Influenza-A (IV)) Protein 58-66 (GILGFVFTL), HBV-core 18-27 (FLPSDFFPSV), HBV-envelope 183-191 (FLLTRILTI), HBV-polymerase 573-581 (FLLSLGIHL) or HBV-surface 185-194 (GLSPTVWLSV). PE- or APC-labelled Class I iTag$^{TM}$ MHC Tetramers containing HCV-NS3 1073-1082 (NS3-1073) derived peptide (CINGVCWTV) were purchased from Beckman Coulter Inc. (Fullerton, CA, USA). MHC Class I Tetramers specific for HCV NS3 1406-1415 (KLVALGINAV), HCV core 35-44 (YLLPRRGPRL), HCV core 131-140 (ADLMGYIPLV), HCV core 178-187 (LLALLSCLTV) and HCV NS5 2594–2602 (ALYDVVTKL) were purchased from ProImmune Ltd. (Oxford, UK).

## 2.4 Isolation and long-term storage of peripheral blood mononuclear cells (PBMC)

Heparinized whole blood samples from healthy volunteers (H) and HBV/HCV patients as well as leukocyte filters from healthy blood donors (BD) obtained from the MHH blood donation centre were used as a source for Peripheral Blood Mononuclear Cells (PBMCs). Isolation was done using standard Ficoll Density Centrifugation method (BioColl, Biochrom AG, Berlin, Germany). Cells were either used directly ex vivo for flow cytometric analyses and in vitro stimulations or were cryo-preserved for long-term storage in liquid nitrogen. Freezing medium contained 60% FCS (Foetal Calf Serum; PAA, Pasching, Austria), 30% RPMI (Invitrogen, Karlsruhe, Germany) and 10% DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, MO, USA).

## 2.5 Determination of HLA-status

Testing of PBMC samples for HLA-A2 was performed from whole blood. Erythrocytes of 500µl whole blood were lysed by addition of 5ml Erythrocyte Lysis Buffer (150mM $NH_4CL$, 10mM $KHCO_3$, 0.1mM EDTA, all Sigma-Aldrich) for 10 minutes at room temperature. After washing with PBS cells were stained for HLA-A2 and CD3 for 10 minutes at 4℃ in the dark. After one more washing step with PBS cells were analysed for HLA-A2 expression by flow cytometry.

## 2.6 HCV Vaccine Study

### 2.6.1 The peptide vaccine IC41

The IC41 HCV vaccine (Intercell AG, Vienna, Austria) consists of peptide antigens and the adjuvant Poly-L-Arginine, both synthesized by chemical means. The vaccine harbours highly promiscuous T-helper epitopes and 5 HLA-A2 restricted CTL epitopes. The 5 synthetic peptides in this vaccine (Ipep83, 84, 87, 89, 1426) are derived from HCV genotype1 core23-44 and 132-140, NS3-1073-1081 and 1248-1261, and NS41764-1786, their amino acid sequences are conserved in the most prevalent HCV genotypes 1a (100%, 100%, 83%,100%, 100% for the respective 5 peptides), 1b (98%, 90%, 15%, 94%, 88%) and 2 (91%, 96%, 13%, 91%, 87%). Only individuals positive for HLA-A2 were enrolled in the study. The prevalence of this marker is 45-50% within Caucasians.

Treatment groups were as follows: group D (0.5mg peptide/0.25mg adjuvant); group E (0.5mg peptide/0.5mg adjuvant); group F (2.5mg peptide/0.25mg adjuvant); group G (2.5mg peptide/1.25mg adjuvant); group H (2.5mg peptide/2.0mg adjuvant); group I (5.0mg peptide/0.5mg adjuvant); group K (5.0mg peptide/2.0mg adjuvant). Control groups: group B (0.0mg peptide/2.0mg adjuvant); group C (5.0mg peptide/0.0mg adjuvant); group M (0.0mg peptide/0.0mg adjuvant).

### 2.6.2 Patient population

The patients cohorts studied here have been presented in detail previously. In brief, 108 HLA-A2-positive healthy controls had been vaccinated four times with the peptide vaccine IC41 in a phase I study (see Figure 6A), while further 20 received saline. This study showed that vaccination with IC41 was safe and was able to induce HCV-specific T cell responses. The mean age of the subjects who received IC41 was 28±8 years (range 18-49), 46% were female. The mean age of the individuals receiving saline was 29±8 years (range 19-49) and 25% were female.

The second cohort consisted of 60 HLA-A2-positive patients chronically infected with the hepatitis C virus with most of them being nonresponder or relapser to a previous course of interferon alpha-based antiviral treatment. These patients were also vaccinated with IC41 but have received 6 vaccinations (see Figure 6B) in four week intervals (Wedemeyer, H et al. 2006). Here the mean age of patients receiving IC41 was 46.1 years (range 23-63) and 44.4% being female. The patients of the control group were of a median age of 47.3 years (range 23-65) with 33.3% females.

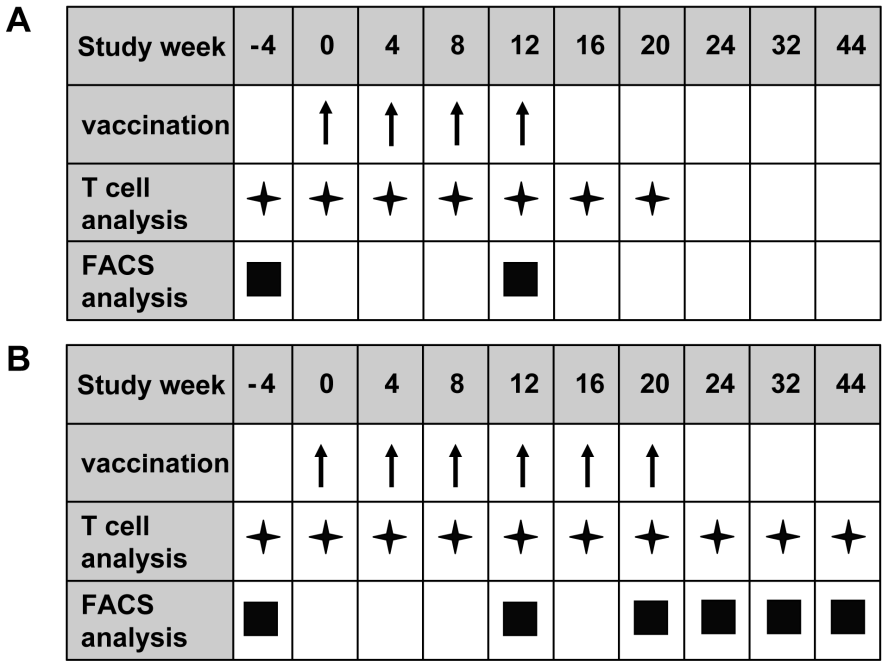



**Figure 6: Scheme of vaccination with IC41**. The schedule for vaccinations applied to (A) healthy volunteers and (B) chronic HCV infected patients are displayed. Arrows indicate vaccinations, crosses represents blood drawing and analysis of T cell function. Closed squares represent time points of FACS analyses performed.

## 2.7 Analysis of PBMC phenotypes

FACS analyses (Fluorescence Activated Cell Sorting) using PBMCs was either performed directly *ex vivo* after density centrifugation or from frozen samples or with cells after *in vitro* cultures. $3x10^5$ cells were stained with monoclonal fluorochrome-conjugated antibodies in 96-well U- or V-bottom plates after washing with FACS-Buffer (PBS + 2% FCS + 0,01% sodium azide) for 15 minutes at 4°C in the dark. Respective contr ol stainings for determining unspecific binding of the antibodies were performed for each staining using respective fluorochrome-conjugated Isotype controls. Afterwards, cells were washed with and resuspended in FACS-Buffer and analysed in a flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany) within 30

minutes. Analysis of FACS data was performed using FlowJo Software (TreeStar Inc., San Diego, CA, USA).

## 2.8 Detection of antigen-specific cells using MHC-class I Tetrameric/Pentameric Complexes

Detection of antigen-specific CD8+ T cells was performed using HLA-A2-restricted human MHC-class I Tetrameric or Pentameric Complexes.

After washing steps with FACS-Buffer, freshly isolated or frozen PBMCs or cells from *in vitro* culture were stained with the respective Pentameric or Tetrameric Complexes for 20 minutes at room temperature in the dark. After addition of respective monoclonal antibodies and further incubation at 4℃ for 15 minutes cells were washed and analysed by flow cytometry within 30 minutes.

## 2.9 Isolation of cells by cell sorting

Purified CD8+ T cell populations were obtained by cell sorting using a BD FACSAria (Becton Dickinson, Heidelberg, Germany) belonging to the central Sorting Facility of MHH. Purity of cell populations obtained was higher than 95%.

For isolation of 2B4-positive and 2B4-negative CD8+ T cells freshly isolated PBMCs from healthy individuals were stained for 2B4 and CD8 prior to cell sorting. Purified CD8+2B4+ and CD8+2B4- cells were then washed and resuspended in AB-medium and used for subsequent readouts such as survival assay and degranulation assays.

## 2.10 In vitro stimulation of PBMCs

Frozen PBMCs were thawed in a water bath and PBS (Phosphate Buffered Saline) was added drop wise to prevent drastic concentration changes and guarantee optimal vitality of cells and washed twice. $3x10^5$ frozen human PBMCs per well were cultured in AB-medium (RPMI-1640 (Invitrogen, Karlsruhe, Germany) with 10% human AB-Serum (Cambrex, East Rutherford, NJ USA) and 100U/ml Penicillin/Streptomycin (PAA, Pasching, Austria) in 96-well U-bottom plates for 5 days at 37℃ and 5% CO2. The following substances were added as unspecific stimulants. IL-2 (Invitrogen,), PHA (Phytohaemagglutinin, Sigma-Aldrich), 1µg/ml purified anti-human CD3 and CD28 or SEB (Staphylococcal Enterotoxin B; Sigma-Aldrich). Medium alone served as a negative control. Each condition was set as duplicates. After the in vitro stimulation, cell surface expression of PD-1, 2B4 and CD48 were analysed by flow cytometry as described above (2.6).

## 2.11 Antigen-specific stimulation of PBMCs in vitro

Fresh or frozen PBMCs were resuspended in AB-medium, Human recombinant IL-2 (Invitrogen, Karlsruhe, Germany) was added in concentrations of 5U/ml if applicable. $3x10^5$ PBMCs per well and condition were cultivated in 96-well U-bottom plates in duplicates. Medium without peptides served as a negative control. Respective antigenic peptides were added at optimal concentrations as mentioned above. After stimulation for 7 days at 37°C + 5%$CO_2$, functional readouts were performed as described below.

## 2.12 In vitro blocking of signalling pathways with monoclonal antibodies in vitro

For analysing the effect of blocking the interaction of 2B4 with its counterreceptor CD48 and between PD-1 and its ligand PDL-1 on the function of antigen-specific and general CD8+ T cell responses, monoclonal antibodies were added to the cell culture at a concentration of 5µg/ml each. In parallel, respective antigenic peptides were added at the indicated concentrations to the cell culture. Lyophilized purified monoclonal anti-human 2B4 and anti-human CD48 antibodies were resuspended in sterile PBS. Functional grade monoclonal anti-human PDL-1 antibody was obtained from eBioscience (San Diego, CA, USA)

## 2.13 Proliferation analysis of PBMCs using CFSE

Proliferation of PBMCs was analysed in vitro by standard CFSE (5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester) dilution assays. Fresh or frozen PBMC samples were stained prior to *in vitro* culture with 2µM CFSE (Sigma-Aldrich) in PBS+0.2% BSA (Serva AG, Heidelberg, Germany) for 7 minutes at 37°C. Reaction was stopped by adding equal volumes of FCS and subsequent washing steps with PBS. CFSE-labelled PBMCs were resuspended in AB-medium containing 5U/ml IL2 if applicable and stimulated with respective antigenic peptides at given concentrations at 37°C. PHA (1µg/ml) was added as positive control and medium alone served as a negative control. Each condition was set up in duplicates. After 7 days, proliferation of PBMCs was analysed after staining with Pentameric or Tetrameric Complexes and respective monoclonal antibodies by flow cytometry.

## 2.14 Intracellular cytokine staining of PBMCs

Thawed or fresh PBMCs or cells derived from in vitro culture were analysed for cytokine production by intracellular cytokine staining (ICS). $3x10^5$ cells per condition (each in duplicates) were stimulated with respective stimulants or peptides for one hour at 37°C. 0.1µg/ml PMA (Phorbol 12-myristate 13-acetate, Sigma-Aldrich) and 1mM Ionomycin (Sigma-Aldrich) served as a positive control. After addition of 2.5mM Brefeldin A (Sigma-Aldrich) cells were further

incubated for 5 hours at 37℃. After washing with FACS-Buffer cells were stained with Pentameric/Tetrameric Complexes and monoclonal antibodies as described above. Cells were washed again and fixated using BD CytoFix solution (Becton Dickinson) for 20 minutes at 4℃. Subsequently, cells were washed with BD Perm/Wash Buffer (Becton Dickinson). Anti-human IFN-γ and TNF monoclonal antibodies were added in combination with Fc-Receptor Blocking Reagent for 30 minutes at 4℃ in the dark. Prior to flow cytometric analysis cell were washed again and resuspended in BD Perm/Wash Buffer.

## 2.15 IFNγ EliSpot

Interferon-gamma (IFNγ) ELISpot assays were performed as described before (Firbas, C et al. 2006). Briefly, ELISpot filtration plates were coated with 10µg/well anti-human IFNγ monoclonal antibody over night. Plates were washed and blocked with medium and human AB-serum. $2x10^5$ previously thawed PBMCs were co-cultivated with individual peptides (10µg/ml) for 16h. Each condition was set up as triplicates. After washing, second biotynilated anti-human IFNγ antibody was added for 2h and after washing Streptavidin-alkaline phosphatase (1.2µg/ml) was added. Developing of assay was performed with BCIP/NBT alkaline phosphatase substrate and reaction stopped after appropriate time. The results were considered as positive, if at least 15 Spots/$1x10^6$ PBMCs were formed and stimulated control showed three fold higher numbers of spots as the negative controls. Calculation of IFNγ/Tet+-ratios were performed by dividing the number of spots formed in response to NS3-1073 or CMV-pp65 by the absolute number of respective Tet+ CD8+ T cells as analyzed by FACS.

## 2.16 Annexin-V staining of lymphocytes

To determine the amount of pre-apoptotic or apoptotic cells detection of Annexin-V was conducted using the FITC Annexin-V Detection Kit (Becton Dickinson) according to manufacturer's protocol. Briefly, cells were stained for cell surface marker such as CD8 as described above. Cells were then washed once with Annnexin-V Detection Buffer and FITC Annexin-V Reagent was added. After an incubation period of 15 minutes at 4℃, cells were analysed by flow cytometry.

## 2.17 Degranulation assay

To assess whether PD-1 2B4 or CD48 signalling has any influence on the cytotoxicity of CD8+ T cells, degranulation of cells was determined by staining for CD107a and CD107b. Frozen cells were thawed and rested over night at 37℃. Purified CD8+ 2B4+ and CD8+ 2B4- cells were used directly after sorting. $3x10^5$ cells were seeded in AB-medium without IL-2 in 96-well U-bottom plates. Each condition was set up as duplicates. Purified anti-human CD3 and

CD28 were added in a concentration of 1µg/ml to mimic TCR stimulation. SEB (1µg/ml) and medium alone as positive and negative control, respectively. In parallel, FITC-conjugated anti-CD107a/b were added to the cell culture. Respective blocking antibodies specific for 2B4 and CD48 were added to the cell culture. After incubation for 2h at 37°C, Brefeldin A (2.5mM) was added to prevent further degranulation. After brief further incubation, cells were washed and stained with anti-CD8 and again with anti-CD107a/b and analysed by flow cytometry.

To analyse antigen-specific CD8+ T cells for degranulation, cells were stimulated for 7 days in vitro with antigenic peptides alone or with additional anti-2B4 and anti-CD48 antibodies (see above). At day 7 $1x10^5$ peptide-pulsed T2 cells were added to the cell culture together with FITC-conjugated anti CD107a/b and anti-2B4 or anti-CD48 antibodies if applicable. After an incubation period for 2 hours at 37°C, Brefeldin A was added a nd cells stained with respective Pentameric complexes, CD8 and CD107a/b as described above. Degranulation of cells was analysed by flow cytometry.

## 2.18 T cell survival

In order to investigate the influence of 2B4 and CD48 signalling on T cell survival, frozen PBMCs from healthy individuals were thawed and rested over night at 37°C. $3x10^5$ PBMCs per well were incubated in presence of anti-2B4, anti-CD48 or both, respectively. Each condition was set up as duplicates. Percentages of T cell populations were analysed at day 1, 3 and 5 and Annexin-V expression was determined after 5 days of incubation via flow cytometry.

As well, sorted CD8+2B4+ and CD8+2B4- T cells were incubated with anti-CD48 for 5 days at 37°C. Medium alone served as a negative control. Survival of cells was determined by assessing numbers of live cells. Acquisition time during flow cytometry was limited to 1 minute.

## 2.19 T cell receptor analysis

Determination of Vβ chain usage of HCV-NS1073-specific CD8+ T cells in HLA-A2+ patients with acute HCV infection was performed in two ways. Flow cytometric analysis was done using frozen PBMC samples of four patients. Total PBMCs were stained with APC-labelled HCV-NS1073-specific Tetrameric Complexes as described above. After incubation at room temperature for 20 minutes, TCR Vβ-chains were detected using the IOTest® Beta Mark antibody kit (Beckman Coulter). Staining of TCR Vß chains was performed according to manufacturer's protocol and CD8+ T cells detected using CD8-PCy7 antibody (Beckman Coulter). Cells were then analysed by flow cytometry within 30 minutes.

The second method of Vβ chain analysis of HCV-NS1073-specific CD8+ T cells was performed using PCR (Polymerase Chain Reaction) technique. For one acute HCV patient sorting of HCV-NS1073-specific CD8+ T cells was possible directly *ex vivo*. For the same patient and two others it was possible to generate short-term T cell lines *in vitro* from frozen PBMC

samples. Total PBMCs were stimulated in IL2-medium (5U/ml) with HCV-NS1073 antigenic peptide at a concentration of 10µg/ml for 7 days. Cultured cells were then stained with HCV-NS1073-specific Tetrameric complexes and anti-CD8 antibodies as described above. HCV-specific cells were subsequently sorted in the Central Sorting Facility of MHH as described. Non-antigen-specific cells were collected as a negative control. Sorted cells were resuspended in PBS containing RNA Protect Reagent® (QiaGen GmbH, Hilden, Germany) and RNA was isolated using the RNEasy Micro Kit (QiaGen) according to manufacturer's protocol.

The isolated total RNA was transcribed and cDNA generated using the SuperScriptIII Reverse Transcriptase (Invitrogen) and oligo-$dT_{12-18}$ primers as indicated. Amplification of the different Vβ chains during PCR was carried out in a 25µl setup containing 2mM dNTPs each, 1U HotMaster Taq® DNA Polymerase (5 Prime Inc., Hamburg, Germany), 1x Hotmaster Taq® Buffer containing 2.5µM Mg2+, 20nM of Cβ reverse primer and 20nM of specific Vβ forward primer (primers selected from the ImMunoGeneTics Information System http://imgt.cines.fr and adapted from (Puisieux, I et al. 1994); for sequences see Table 2: Primer for human TCR V□ chain analysis. Designation, characteristics and nucleic αχιδ sequences for human T cell receptor V□ chain-specific primers are given. Primers adapted from (Puisieux, I et al. 1994)). PCR amplification was performed in an Eppendorf thermocycler (MasterCycler®, Eppendorf, Hamburg, Germany) starting with initial 2 minute denaturation step at 94°C, followed by 30 cycles consisting of 20 seconds at 94°C, 30 seconds annealing at 58-62°C (temperature gradient, see Table 2) and 20 seconds elongation at 68°C and a final elongation step for 10 minutes at 68°C. PCR amplification products were visualized in a 2% agarose gel containing Ethidium bromide.

| Primer name | length | orientation | Sequence | annealing temperature (℃) |
|---|---|---|---|---|
| Vbeta 1 | 24 | forward | CCG CAC AAC AGT TCC CTG ACT TGC | 62 |
| Vbeta 2 | 24 | forward | GGC CAC ATA CGA GCA AGG CGT CGA | 62 |
| Vbeta 3 | 24 | forward | CGC TTC TCC CGG ATT CTG GAG TCC | 62 |
| Vbeta 4 | 24 | forward | TTC CCA TCA GCC GCC CAA ACC TAA | 58 |
| Vbeta 5.6 | 24 | forward | AGC TCT GAG CTG AAT GTG AAC GCC | 57 |
| Vbeta 6.2 | 24 | forward | TCT CAG GTG TGA TCC AAA TTC GGG | 57 |
| Vbeta 7 | 24 | forward | CCT GAA TGC CCC AAC AGC TCT CTC | 61 |
| Vbeta 8 | 24 | forward | CCA TGA TGC GGG GAC TGG AGT TGC | 62 |
| Vbeta 9 | 24 | forward | TTC CCT GGA GCT TGG TGA CTC TGC | 61 |
| Vbeta 10 | 24 | forward | CCA CGG AGT CAG GGG ACA CAG CAC | 62 |
| Vbeta 11 | 24 | forward | TGC CAG GCC CTC ACA TAC CTC TCA | 61 |
| Vbeta 12.2 | 24 | forward | TGT CAC CAG ACT GGG AAC CAC CAC | 61 |
| Vbeta 13.3 | 24 | forward | CAC TGC GGT GTA CCC AGG ATA TGA | 59 |
| Vbeta 14 | 24 | forward | GGG CTC GGC TTA AGG CAG ACC TAC | 62 |
| Vbeta 15 | 24 | forward | CAG GCA CAG GCT AAA TTC TCC CTG | 59 |
| Vbeta 16 | 24 | forward | GCC TGC AGA ACT GGA GGA TTC TGG | 62 |
| Vbeta 17 | 24 | forward | CTG CTG AAT TTC CCA AAG AGG GCC | 61 |
| Vbeta 18 | 24 | forward | TGC CCC AGA ATC TCT CAG CCT CCA | 59 |
| Vbeta 19 | 24 | forward | TCC TCT CAC TGT GAC ATC GGC CCA | 61 |
| Vbeta 20 | 24 | forward | TCT CAA TGC CCC AAG AAC GCA CCC | 62 |
| Vbeta 21.3 | 24 | forward | TCC AAC CTG CAA GGC TTG ACG ACT | 58 |
| Vbeta 22 | 24 | forward | AAG TGA TCT TGC GCT GTG TCC CCA | 59 |
| Vbeta 23 | 24 | forward | GCA GGG TCC AGG TCA GGA CCC CCA | 62 |
| Vbeta 24 | 24 | forward | CCC AGT TTG GAA AGC CAG TGA CCC | 62 |
| Cbeta | 30 | reverse | CTT CGA ATT CCA GAG GAC CTG AAC AAG GTG | 68 |

**Table 2: Primer for human TCR Vβ chain analysis.** Designation, characteristics and nucleic acid sequences for human T cell receptor Vβ chain-specific primers are given. Primers adapted from (Puisieux, I et al. 1994)

For two patients the amino acid sequence of the dominating TCR Vb7 PCR products were analysed by subcloning using the TOPO TA Cloning Kit (Invitrogen). 1µl of PCR product was ligated into the pCR2.1® cloning vector and transformed into TOP10® chemically competent E Coli cells. These were spread on agar plates containing 100µg Ampicillin (Gibco) and 40µg β-Galactose (Sigma-Aldrich). After overnight incubation at 30℃ white colonies containing plasmids with inserted cDNA fragments were picked for each clone and again expanded over night in liquid media containing ampicillin. The following day plasmids were isolated using the PureLink 96 Plasmid Miniprep Kit (Invitrogen) according to manufacturer's instructions. Sequencing of cDNA inserts was performed by the AGOWA AG, Berlin, Germany, using standard M13 primers. Obtained CDR3 nucleotide sequences and CDR3 lengths were analysed using the SeqMan Software (DNAStar, Madison, WI, USA). Translation into amino acid sequences was performed with EditSeq Software (DNAStar).

## 2.20 Statistical analyses

For descriptive means statistics are expressed as mean values ± standard deviations. Two-tailed unpaired Student's T tests were performed. *P* values of <0.05 were considered as significant. Chi-square or Kruskall-Wallis Tests were used to assess differences in the frequencies of Tetramer-positive or IFNγ-positive CD8+ T cells and strength of immune responses between experimental groups.

# 3 Results

## 3.1 Functional and phenotypic characterization of peptide-vaccine-induced HCV-specific CD8+ T cells in healthy individuals and chronic hepatitis C patients

Standard therapy of HCV infection consists of Interferon-alpha in combination with other antiviral drugs such as ribavirin. Despite good optimization of treatment and medication and a mounting success rate of therapy with a sustained virological response rate of up to 60% in chronic HCV patients, an entire cure of all HCV patients is not yet possible (Manns, MP et al. 2006). Therefore, other ways of treating chronic HCV infection are desired and the range of therapy strategies and medication widens steadily.

One approach is based on therapeutic vaccination of chronic HCV infected individuals with the aim to stimulate the natural immune response against the virus. But not only is the cure of infected patients is an important goal in combating HCV. The prevention of new infection is equally crucial and the only means of achieving this is the implication of a preventive vaccine. As mentioned before (see 1.3.2), various vaccines have been tested, but none of them were successful. The reason why these different vaccinations were not able to induce a reduction of HCV-RNA in persistent infected patients is an important issue in order to improve existing and design new vaccination strategies. In the context of a peptide vaccination trial carried out we had the rare opportunity to examine function and phenotype of HCV-specific CD8+ T cells in both patients with chronic hepatitis C and healthy anti-HCV negative individuals. It was possible to analyze the efficiency of the peptide vaccine in terms of first changing the functional capacities of HCV-specific CD8+ T cells in chronic HCV infected patients. Secondly, we determined the potency of the vaccine to induce an hopefully protective immune response in healthy persons (Schlaphoff, V et al. 2007).

### 3.1.1 Phenotype and function of vaccine-induced HCV-specific CD8+ T cells in healthy individuals

Recently it was shown that HCV-specific CD8+ T cells can be induced in healthy subjects by vaccination with IC41 (Firbas, C et al. 2006). In the present study, we further characterized phenotype and function of vaccine-induced HCV-specific CD8+ T cells. After the four vaccinations (week 12; see Figure 7), HCV-specific CD8+ T cells could be detected in 24 of 108 healthy individuals (22%) vaccinated with various doses of IC41. Responses were mainly

restricted to the epitope HCV NS3-1073 with frequencies of vaccine-induced HCV-specific CD8+ T cells between 0.04% and 0.37% of CD8+ T cells. For the present phenotypic analysis we only considered responses with more than 0.04% Tetramer-positive (Tet+) cells of total CD8+ T cells to ensure sufficient number of events (n=13).

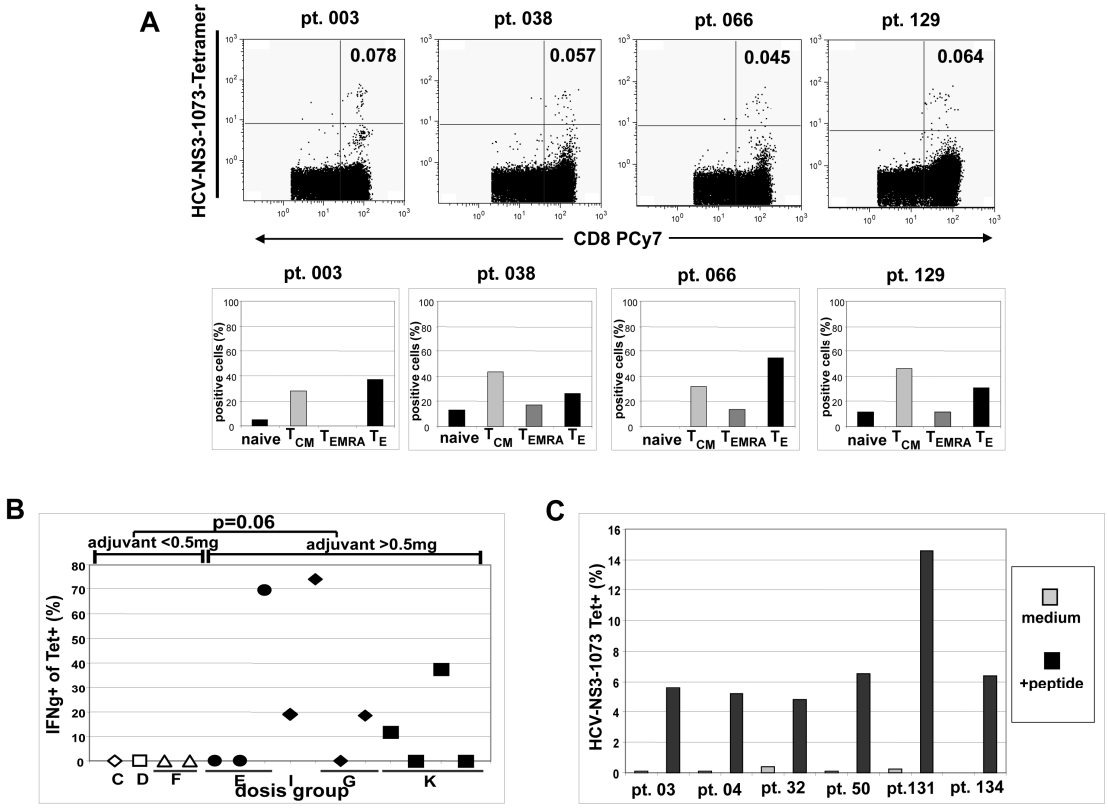



**Figure 7: Functional and phenotypic characterization of HCV-specific CD8+ T cells after vaccination of healthy individuals.** (**A**). Analysis of cryopreserved PBMCs of individual healthy subjects for HCV-specific CD8+ T cells with MHC class I restricted Tetramers vs. CD8 as shown by FACS dot plots (upper panel). The lower panel shows the phenotypic distribution of HCV-specific CD8+ T cells as analyzed by cell surface staining for CCR7 and CD45RA, gated on Tetramer+ CD8+ cells, data are shown in percent. (**B**). IFNγ production of HCV-specific CD8+ T cells upon antigenic stimulus. Cryopreserved PBMCs were tested in an ELISpot against HCV-NS3-1073 peptide for IFNγ production ex vivo. Data are shown in percent calculated as the ratio of SFU and the absolute number of HCV NS3-1073-specific cells. The healthy individuals of different groups are additionally sorted according to the doses of adjuvant applied with the peptide vaccine, the amounts were either below (open symbols) or above (full symbols) 0.5mg total. The data show a clear correlation of IFNγ production with the amount of adjuvant given. (**C**). Proliferation capacity of HCV-specific CD8+ T cells. Cryopreserved PBMCs of healthy individuals were thawed and frequencies of Tetramer+ CD8+ T cells were determined ex vivo. After 7 days of stimulation in vitro with the respective peptides expansion of HCV-specific CD8+ T cells was analyzed again by tetramer staining. Values given are expressed as percentages of HCV-Tetramer+ CD8+ cells of total CD8+ T cells.

Figure 7 shows individual phenotypes of vaccine-induced HCV-specific CD8+ T cells in healthy individuals (mean values see Figure 11 ). Overall, CD45RA- CCR7+ central memory ($T_{CM}$) and CD45RA- CCR7- effector ($T_E$) populations were found with mean values of 45% (range 15.2% to 64%) and 36% (range 9% to 69%) of cells, respectively, whereas the amount of $T_{EMRA}$ (CD45RA+ CCR7-) and naïve-like T cells (double positive) was low (mean of 7.8% and 10.7%,

respectively). However, there was no obvious correlation between the amount of $T_{CM}$ or $T_E$ cells and the strength of the CD8+ T cell response, peptide dose or administration of adjuvant. The frequency of naïve and $T_{EMRA}$-like cells, however, was relatively constant throughout all subjects (range 4-16% and 0-17.4%, respectively).

The ability of HCV-specific CD8+ T cells to produce IFNγ in response to specific peptides was determined by ELISpot assays. We also calculated the ratio between ELISpot-SFU (spot forming units) and the absolute number of HCV-specific Tet+ cells present in the culture as measured by flow cytometry. Interestingly, not all patients with detectable HCV-Tet+ cells had positive IFNγ responses in the ELISpot assay as only 6 out of 15 individuals tested positive. The IFNγ/Tet+-ratio ranged between 11% and 73% in individuals with IFNγ spots (see Figure 7). Higher doses of poly-L-Arginine in the vaccine (0.5mg and more) were associated with a higher IFNγ/Tet+-ratio as compared to those individuals who received less adjuvant (0.25mg and 0mg; p=0.06). In contrast the amount of peptide (0.5mg, 2.5mg or 5mg) was not associated with the IFNγ/Tet+-ratio (compare Figure 7).

The proliferative capacity of HCV-specific CD8+ T cells after antigen exposure was tested in a 7d in vitro culture (Figure 7). All of the six patient samples analyzed showed a strong proliferation upon stimulation with the HCV peptides. An up to 70fold increase in the percentage of Tetramer-positive cells (gated on CD8+ T cells) could be observed on day 7. In vitro, the memory phenotype of these cells changed to a dominant $T_E$ phenotype during the antigen stimulation (data not shown).

### 3.1.2 Phenotype and function of preexisting HCV-specific CD8+ T cells in chronic hepatitis C patients

HCV-specific CD8+ T cell of patients with chronic hepatitis C infection enrolled in this study were investigated before start of vaccination. As expected (Lauer, GM et al. 2004; Wedemeyer, H et al. 2002), the frequencies of tetramer-positive cells in these patients were generally found to be rather low (range 0.01% to 0.04% of CD8+ cells), except for two patients with 0.07% (pt. 4001) and 0.3% specific cells. Memory phenotypes showed great inter-individual variations between different individuals (Figure 8) with a dominant $T_{CM}$ phenotype in some patients and a dominant $T_{EMRA}$ population in others. In contrast to vaccine-induced HCV-specific CD8+ T cells in healthy individuals, only in very few cases $T_E$ cells were the major population. Overall, the four populations based on CCR7/CD45RA staining showed a rather equal distribution of mean values ($T_{CM}$ 27.6%; naïve 19.5%; $T_{EMRA}$ 25.4% and $T_E$ 27.8%).

IFNγ/Tet+-ratios were low in chronically infected patients, as only two out of 17 individuals showed IFNγ producing Tet+ cells before vaccination (see Figure 8).

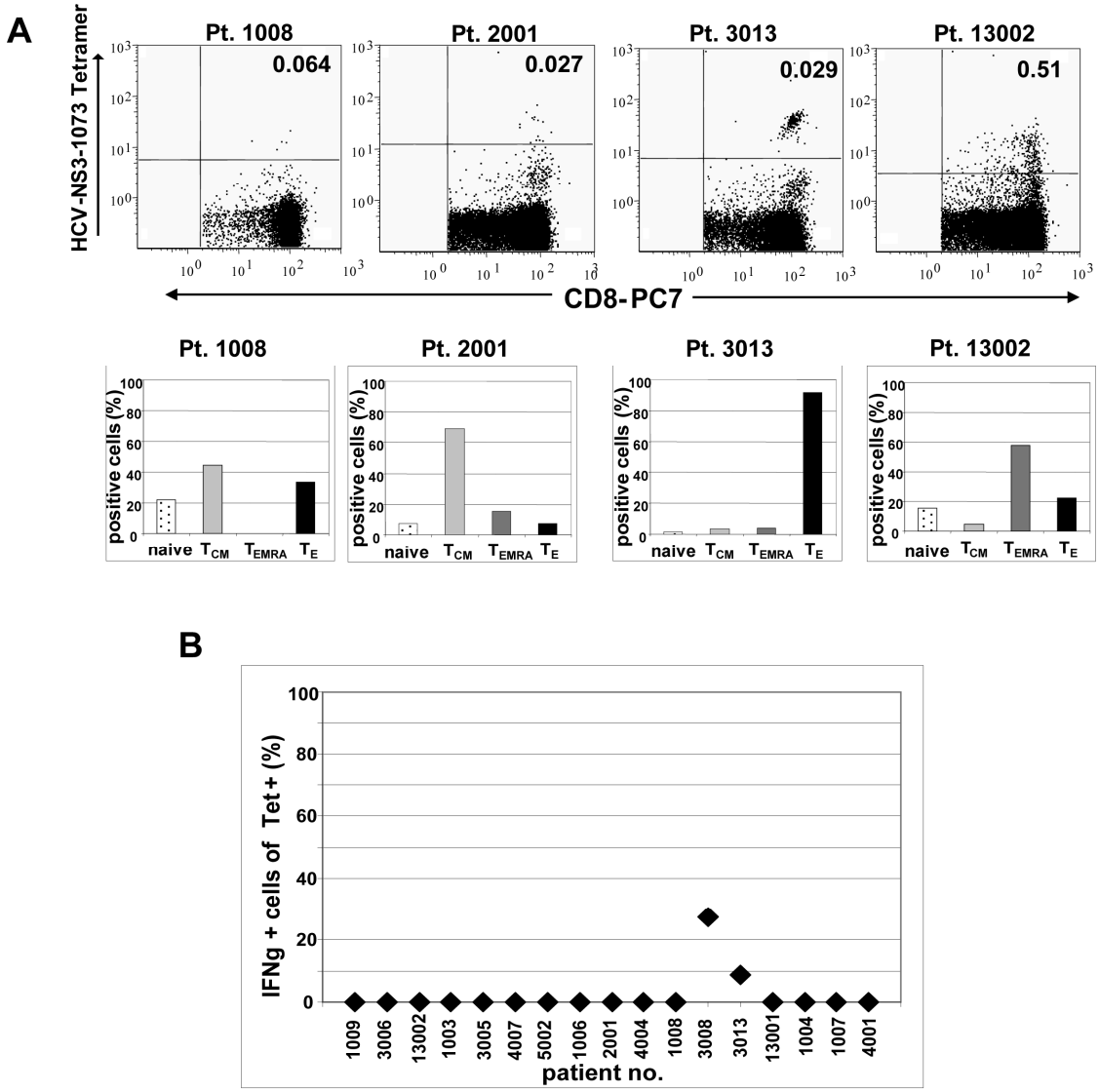



**Figure 8: Phenotype and function of HCV-specific CD8+ T cells in chronic hepatitis C infection.** (**A**) Analysis of cryopreserved PBMCs of individual chronic HCV patients for HCV-specific CD8+ T cells with MHC class I restricted Tetramers vs. CD8 as shown by FACS dot plots (upper panel). The lower panel shows the phenotypic distribution of HCV-specific CD8+ T cells as analyzed by cell surface staining for CCR7 and CD45RA, gated on Tetramer+ CD8+ cells, data are shown in percent. (**B**) IFNγ production of HCV-specific CD8+ T cells upon antigenic stimulus. Cryopreserved PBMCs were tested in an ELISpot against different HCV-peptides for IFNγ production ex vivo. Data are shown in percent calculated as the ratio of SFU and the absolute number of HCV-specific cells. Pt =patient

### 3.1.3 Characteristics of CMV-specific CD8+ T cells in healthy individuals and chronic HCV patients

Several of the healthy individuals which were vaccinated with IC41 also had CMV-specific CD8+ T cells. Thus, we had the chance to compare phenotypes and function of CMV-pp65 and HCV-NS3-1073 specific CD8+ T cells. These CMV-specific cells showed all a dominating $T_E$ and $T_{EMRA}$ phenotype (see Figure 9). Also the IFNγ production upon antigenic stimulation with CMV

peptide during an ELISpot assay revealed a significant response in 6 out of 15 individuals (40%, see Figure 9:)

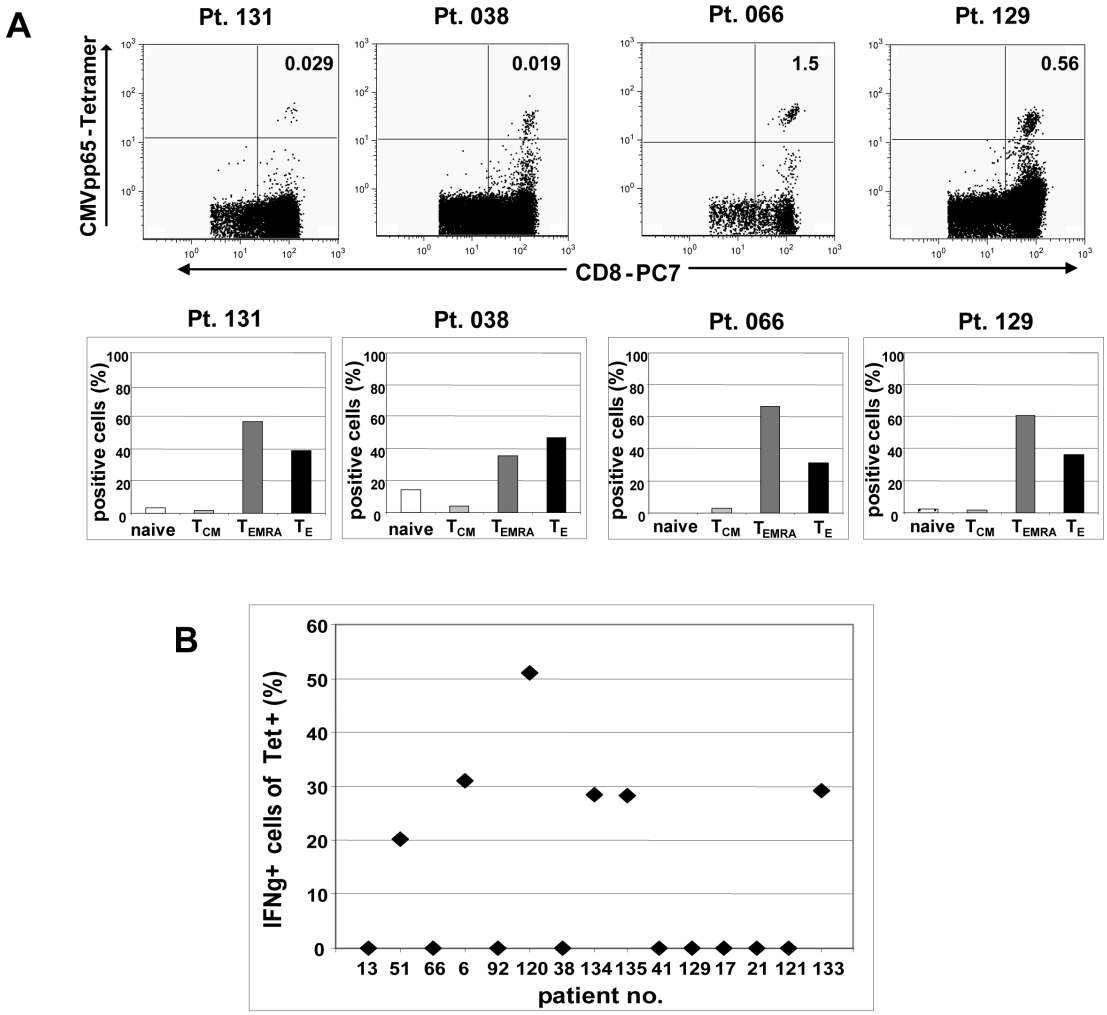



**Figure 9: Functional and phenotypic characterization of CMV-specific CD8+ T cells in healthy individuals.** (**A**) Analysis of cryopreserved PBMCs of individual healthy subjects for CMV-specific CD8+ T cells with MHC class I restricted Tetramers vs. CD8 as shown by FACS dot plots (upper panel). The lower panel shows the phenotypic distribution of CMV-specific CD8+ T cells as analyzed by cell surface staining for CCR7 and CD45RA, gated on Tetramer+ CD8+ cells, data are shown in percent. (**B**) IFNγ production of CMV-specific cells upon antigenic stimulus. Cryopreserved cells were thawed and tested in an ELISpot assay against CMV-pp65-peptides for IFNγ production. Data are shown in percent calculated as ratio of SFU and the absolute number of respective CMV-Tet+ CD8+ cells for individual patients. Pt = patient

Of the 14 chronic HCV patients which were included in the phenotypic analysis, 9 individuals also tested positive for CMV-Tetramers (64%). As shown in Figure 10Aand Figure 11B and F, the phenotypes of CMV-specific cells were different from those of HCV-specific CD8+ T cells as CMV-specific T cells showed a uniform dominant $T_E/T_{EMRA}$ phenotype in all patients (see Figure 10). 50-96% of the CMV-specific cells were CCR7-negative (mean 83%); while HCV-specific cells were CCR7-negative in only 53% (p=0.018). In contrast to some previous studies (Lucas, M et al. 2004), the phenotype of CMV-specific cells in HCV patients (mean percentages $T_E$ 35.5%;

$T_{EMRA}$ 47.2%) did not differ from CMV-specific cells in healthy individuals (mean percentages $T_E$ 39.5% $T_{EMRA}$ 50.1%; see Figure 11A and F).

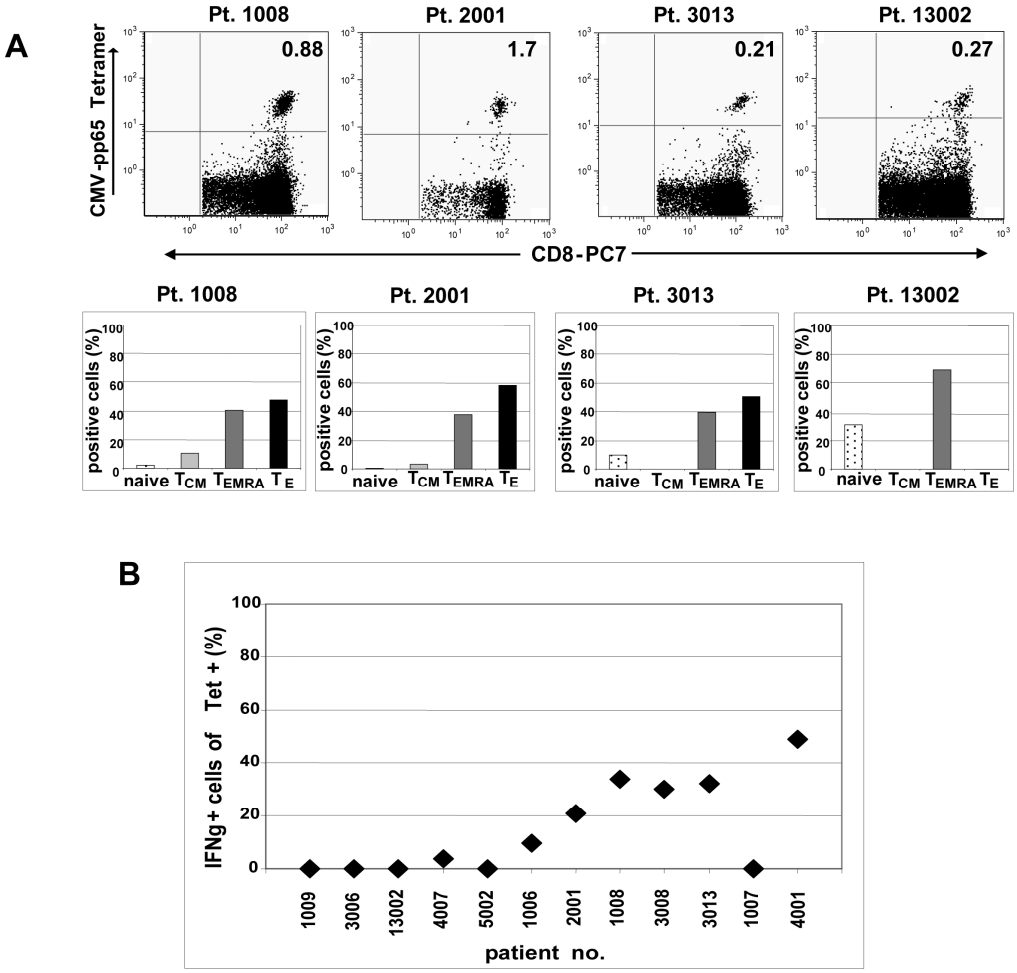
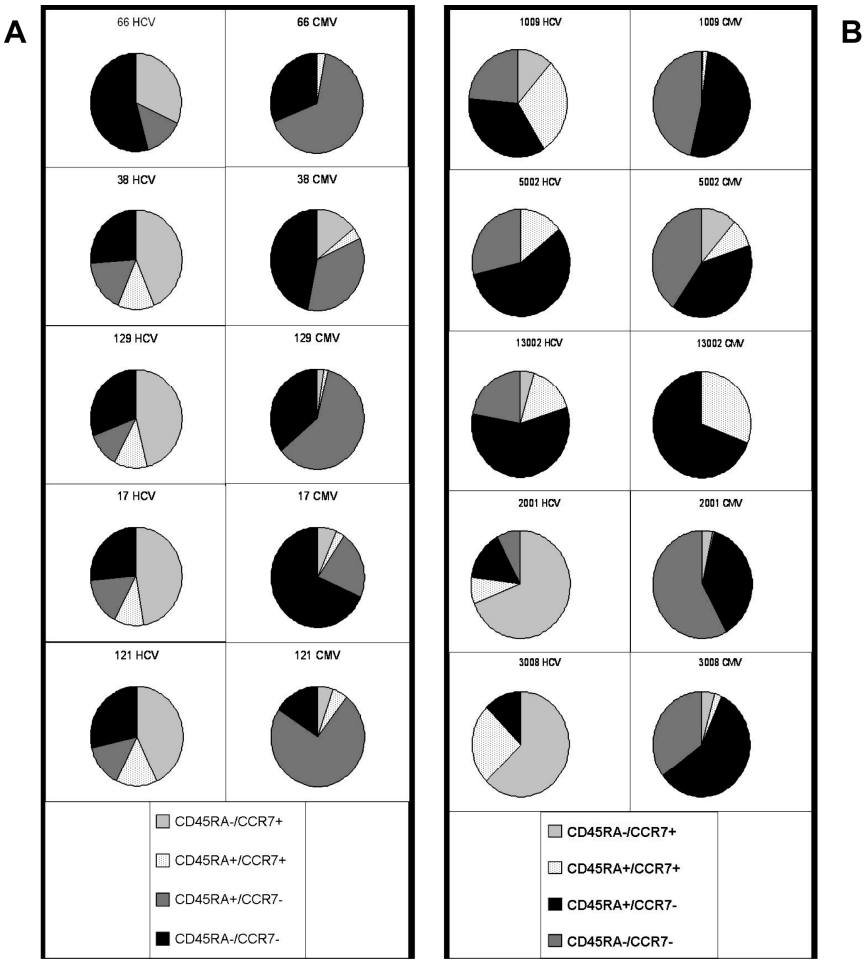



**Figure 10: Characteristics of CMV-specific CD8+ T cells in chronic hepatitis C patients.** (**A**). Analysis of the phenotype of CMV-pp65-specific CD8+ T cells with MHC class I restricted Tetramers vs. CD8 (upper panel). The lower panel shows the results of cell surface staining of CMV-specific T cells for CCR7 and CD45RA. Four patients are shown as representatives. (**B**). IFNγ production capacity of CMV-specific CD8+ T cells. Cryopreserved PBMCs were thawed and tested in an ELISpot assay against CMV-pp65-peptides for IFNγ production ex vivo. Values are shown in percent calculated as ratio between SFU and absolute numbers of respective CMV-Tet+ cells for individual patients. Pt = patient

CMV-specific T cells in HCV patients were functional as 7 of 12 patients with CMV-Tet+ cells (58%) patients showed specific IFNγ ELISpot responses (Figure 10), which was significantly more frequent than HCV-specific responses (p=0.016). The IFNγ/Tet+-ratio was higher for CMV-specific cells than for HCV-specific CD8+ T cells (mean values 2.1 vs. 14.9; p=0.011).

### 3.1.4 Characteristics of vaccine-induced HCV-specific CD8+ T cells in chronic hepatitis C during and after peptide vaccination with IC41

The peptide vaccine IC41 was not only administered to healthy individuals but also to patients chronically infected with hepatitis C (Wedemeyer, H et al. 2008). The absolute number of HCV-specific Tet+ CD8+ T cells increased during vaccination in 15 patients (25%) as shown in Figure 12A. Patients with high baseline frequencies of HCV-Tet+ CD8+ T cells showed only a minor increase in Tet+ frequencies therewith not fulfilling the criteria of being considered as a "FACS-responder" in that trial (Firbas, C et al. 2006). After the fourth vaccination a clear shift in memory phenotypes of HCV-specific cells could be seen in patients with low baseline frequencies of HCV-specific T cells. While mean percentages of naïve-like and $T_{EMRA}$ cells declined during vaccination, cells with a $T_{CM}$ or $T_E$ phenotype increased in patients with an increase tetramer frequency (see Figure 12B) but not in patients who had already at baseline a high frequency of HCV-specific CD8+ T cells detectable (Figure 12D). In contrast, no significant changes in the phenotype of CMV-specific cells were observed during vaccination (data not shown).



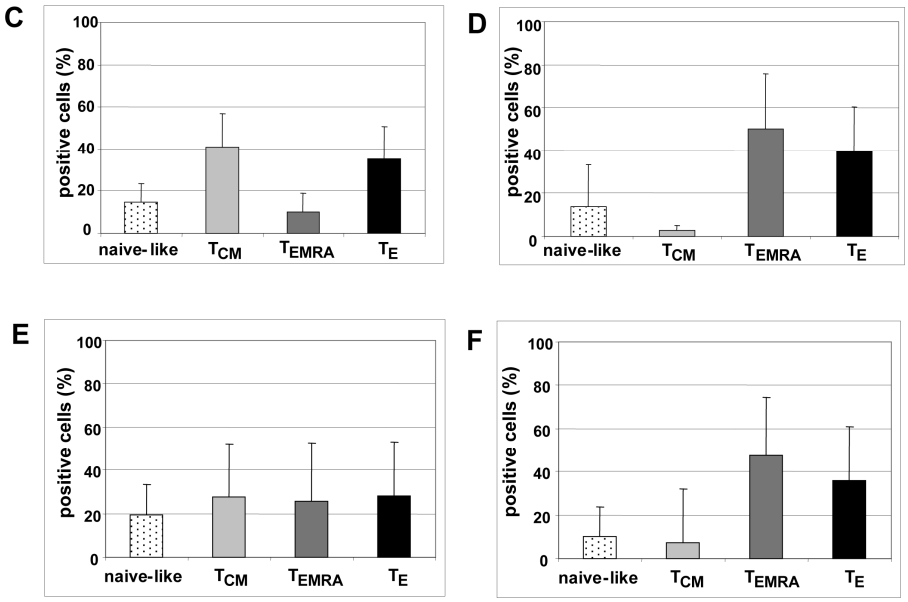



**Figure 11: Comparison of memory phenotypes of HCV- and CMV-specific CD8+ T cells in healthy individuals and chronic HCV patients.** (**A**) Distribution of the four different memory phenotypes naive-like (white area), TCM (light grey), TEMRA (dark grey) and TE (black area).The left row shows the phenotypes of HCV-specific CD8+ T cells, the right column shows the phenotype of CMV-specific CD8+ T cells in healthy individuals after vaccination. Five examples each are shown as representative, size of areas is according to the distribution as analyzed by flow cytometry. (**B**) Memory phenotypes of HCV-specific (left column) and CMV-specific (right column) CD8+ T cells in patients with chronic hepatitis C infection.. Five examples each are shown as representative, size of areas is according to the distribution as analyzed by flow cytometry. (**C**) Mean distribution of memory phenotypes of HCV-specific CD8+ T cells in healthy individuals after peptide vaccination (n=13). (**D**) Mean distribution of memory phenotypes of CMV-specific CD8+ T cells in healthy individuals (n=11). (**E**) Average distribution of memory phenotypes of HCV-specific CD8+ T cells in chronic HCV patients (n=10). (**F**) Average distribution of memory phenotypes of CMV-specific CD8+ T cells in chronic HCV patients (n=10).

Although the frequency of Tet+ cells increased in some patients being associated with a phenotype change, no significant changes in IFNγ production per cell was observed (Figure 12E). Also, no obvious correlation of HCV-RNA levels and HCV-phenotype with IFN$\tilde{\gamma}$/Tet+-ratios could be seen (data not shown).

During follow-up (week 32 and 44) HCV-specific cells showed a backshift in memory phenotype with a loss of $T_E$ and an increase of $T_{EMRA}$ (see Figure 12C and D). Patients with high HCV-Tet+ CD8+ T cell numbers at baseline displayed already at baseline memory phenotypes with a strong and dominant $T_E$ population. As well, this phenotype did not change during and after the vaccination (Figure 12E).

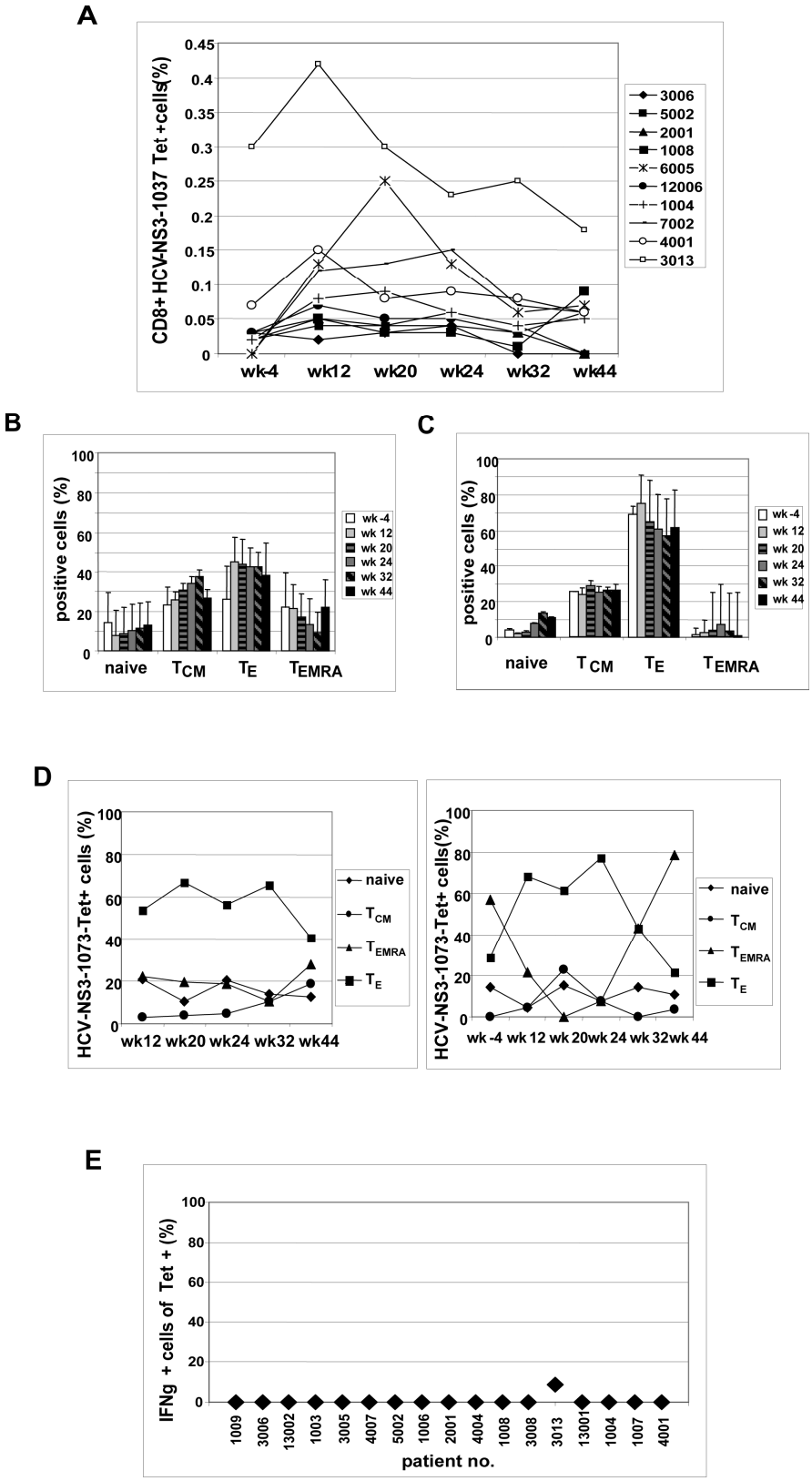



**Figure 12: Evolution of HCV-specific CD8+ T cell numbers in chronic HCV patients during and after vaccination.** (**A**) Changes in the percentages of HCV-specific CD8+ T cells in chronic HCV patients during vaccination. Values represent individual values of HCV-specific T cells in percent, gated on CD8+ cells. 10 patients are shown as representatives. (**B**) Memory phenotype of HCV-specific T cells of chronic HCV patients during course of vaccination. Data are shown as mean values of percentages, gated on CD8+ Tet+ cells (n = 10). (**C**) Phenotypes of HCV-specific CD8+ T cells in chronic HCV patients with high number

of Tet+ CD8+ T cells at baseline. Chronic HCV patients who displayed a high percentage of Tet+ cells before start of vaccination usually displayed an already strong TE phenotype which did not change during vaccination. Mean values of three patients are shown, gated on CD8+ Tet+ cells. (**D**) Change of memory phenotype during vaccination in individual patients. The phenotypes of two patients are shown as examples for the evolution of memory phenotypes of HCV-specific CD8+ cells. The left picture represents a patient with minor changes during vaccination (patient no. 6005) and the right picture shows a patient with significant changes of the phenotype and reversion after end of vaccination (patient no. 5002). (**E**) IFNγ production of HCV-specific CD8+ T cells upon antigenic stimulus after the sixth vaccination. Cryopreserved PBMCs were tested in an ELISpot against different HCV-peptides for IFNγ production ex vivo. Data are shown in percent calculated as the ratio of SFU and the absolute number of HCV-specific cells. No change of cytokine production by peptide vaccination could be achieved in the chronic HCV patients.

Although a change in the memory phenotype of HCV-specific CD8+ T cells of chronic HCV infected patients could be observed, peptide vaccination was not able to provoke a functional modification of those cells. Thus, the vaccine approach using IC41 was too weak to overcome functional exhaustion.

Based on these findings, we further aimed to investigate other factors potentially being involved in the regulation of CD8+ T cells. One mechanism of regulating immune responses is the signaling pathway of PD-1. This inhibitory molecule was described to decrease CD8+ T cell function. Therefore we aimed to clarify the role of PD-1 in viral infections with the aim to more insight about the optimal phenotype of CD8+ T cells generated through vaccination.

## 3.2 Expression and function of PD-1 CD8+ T cells

The molecule PD-1 (programmed death 1) was described to be involved in negative regulation of T cell function (Brown, JA et al. 2003; Carter, L et al. 2002; Freeman, GJ et al. 2000) (see 1.2.4.1). Early after the report that the PD-1:PDL pathway seems to be responsible for the functional impairment of T cells during chronic viral infection in mice we started investigating the expression and role of PD-1 on CD8+ T cells of different antigen-specificities.

### 3.2.1 Expression of PD-1 on leukocytes in healthy individuals

On human PBMCs derived from healthy individuals PD-1 expression could hardly be detected and was generally rather low in intensity. A smaller fraction of CD8+ and CD4+ T cells were found to be positive for PD-1 (see Figure 13A and B). CD56 + CD3+ Natural Killer T (NK-like T) cells as well as CD56+ CD3- Natural Killer (NK) cells were negative for PD-1 (Figure 13C and D). Similarly, also monocytes and B cells showed no expression of PD-1 in healthy individuals (Figure 13D and E).

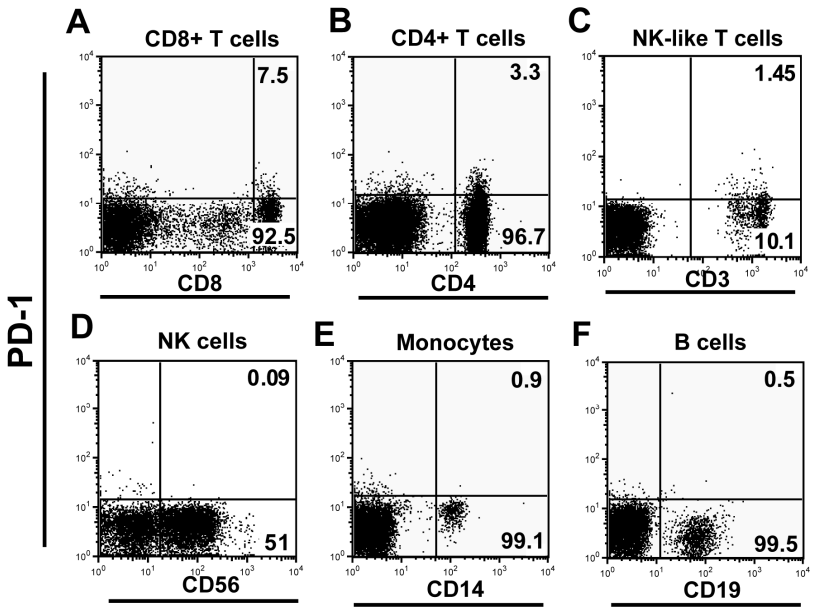



**Figure 13 Expression of PD-1 on different leukocyte populations in healthy individuals.** Freshly isolated PBMCs form healthy volunteers were analyzed for the expression of PD-1 on different leukocyte subsets. (**A**) The representative FACS plots show that PD-1 was expressed on CD8+ and (**B**) CD4+ T cells. (**C**) CD56+ CD3+ NK-like T cells and(**D**) CD56+ CD3- NK cells however, were negative for PD-1, as were also CD14+ (**E**) Monocytes and(**F**) CD19+ B cells.

On CD8+ T cells the presence of PD-1 varied greatly between different individuals, while in some PD-1 expression was as low as 2-5% of total CD8+ T cells, it reached up to 40% in others (compare Figure 14).

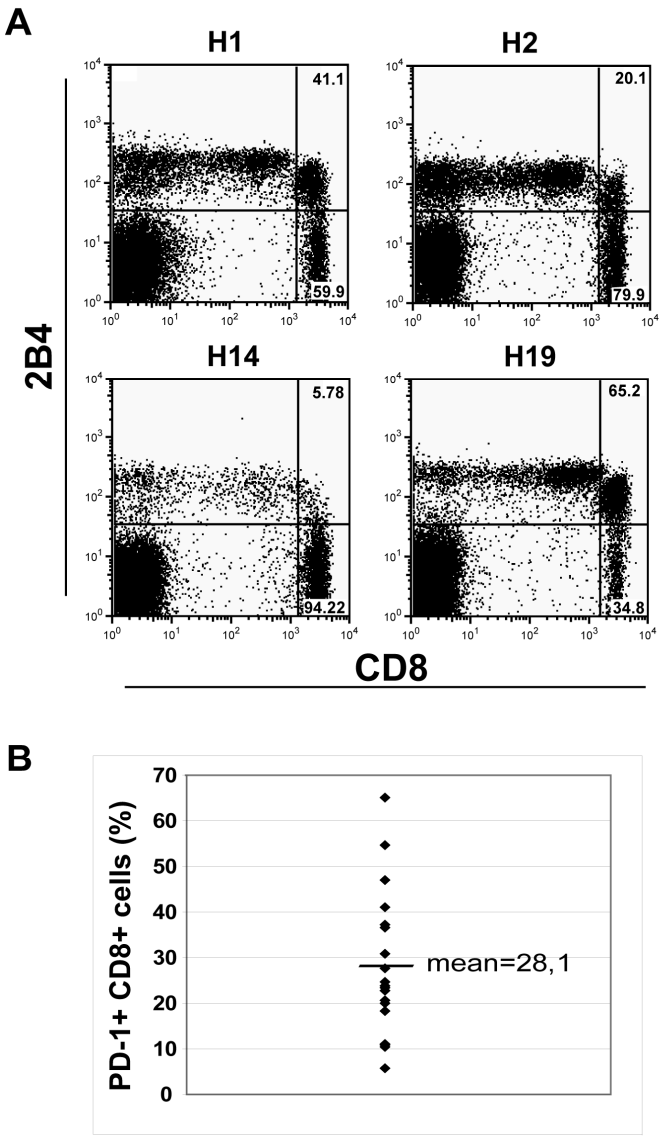



**Figure 14: Variability of PD-1 expression on total CD8+ T cells in healthy volunteers.** CD8+ T cells from healthy individuals were analyzed for the presence of PD-1. (**A**) Four different straining are shown as representative.. (**B**) The expression of PD-1 on CD8+T cells showed a great variability between different individuals (n=20). For analysis cells were gated on lymphocytes according to size and granularity and subsequently on CD8+ T cells. H = healthy

On antigen-specific CD8+ T cells a similar variability was found dependent on the individual and the antigen-specificity. CMV-pp65-specific CD8+ T cells displayed a uniformly high expression of PD-1 and the same held true for EBV-BMLF1 specific CD8+ T cells (Figure 15). In case of Influenza-A (IV)-specific CD8+ T cells however, differences in PD-1 expression between individuals were more obvious and the level of PD-1 was rather low (Figure 15).

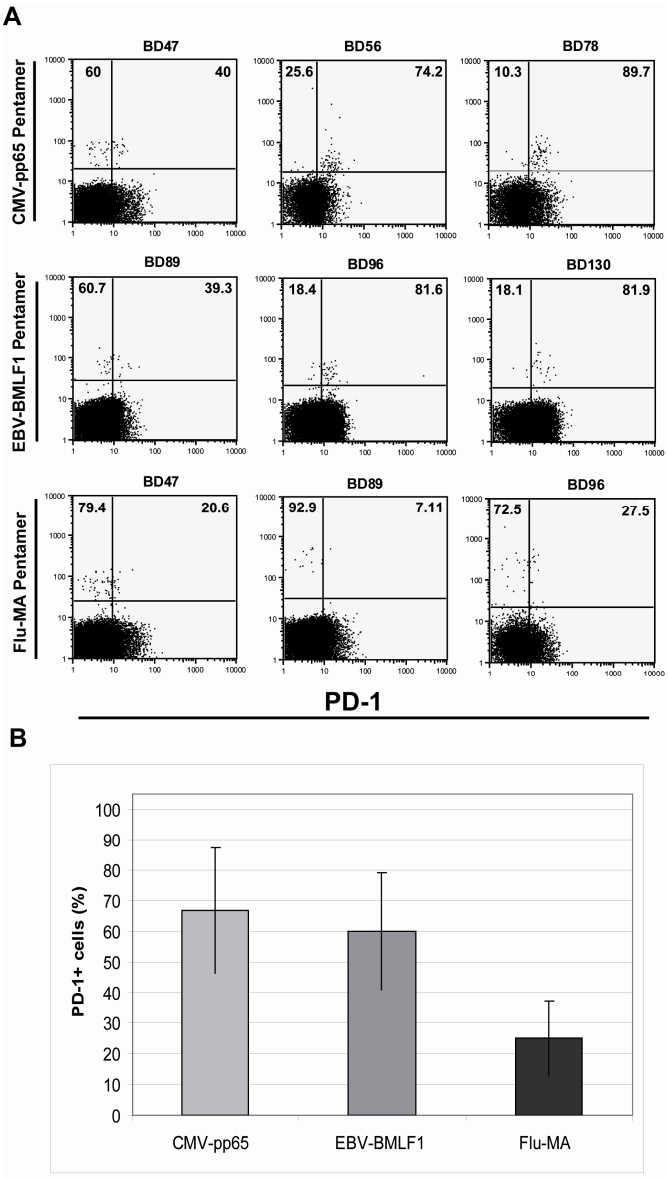



**Figure 15: PD-1 expression on antigen-specific CD8+ T cells in healthy individuals.** (**A**) CD8+ T cells specific for either CMV-pp65 (A; n= 6), EBV-BLMF1 (B; n=13) or Influenza-A (IV) (C; n=13) were analyzed for the expression of PD-1. Individual expression patterns of PD-1 are shown as representatives. (**B**) Influenza-A (IV)-specific CD8+ T cells showed a low mean level of PD-1, whereas CMV- and EBV-specific CD8+ T cells displayed a uniformly high mean expression. BD = blood donor.

PD-1 expression increased after *in vitro* stimulation on CD8+ T cells. Different unspecific stimuli like mitogens and pathogenic components were able to induce PD-1 on CD8+ and CD4+ T cells (Figure 16). Accordingly, also antigen-specific stimulation caused an increase of PD-1 on the respective antigen-specific CD8+ T cell (Figure 17).

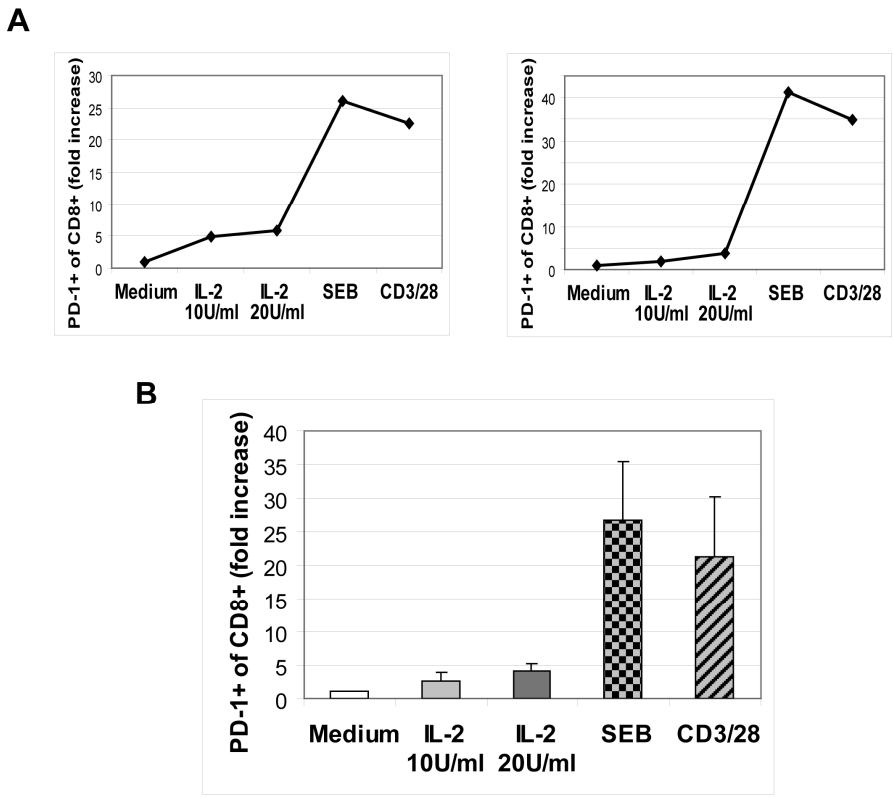



**Figure 16: Induction of PD-1 expression after unspecific stimulation.** PBMCs from healthy individuals were treated in vitro with different bacterial components like LPS, SEB, PHA and anti-CD3/CD28 for 5 days. (A) Strength of increase of PD-1 expression varied between individuals (B). In general, IL-2 induced a concentration-dependent elevation of PD-1 expression. SEB and anti-CD3/28 were found to be the strongest stimulators of PD-1 expression.

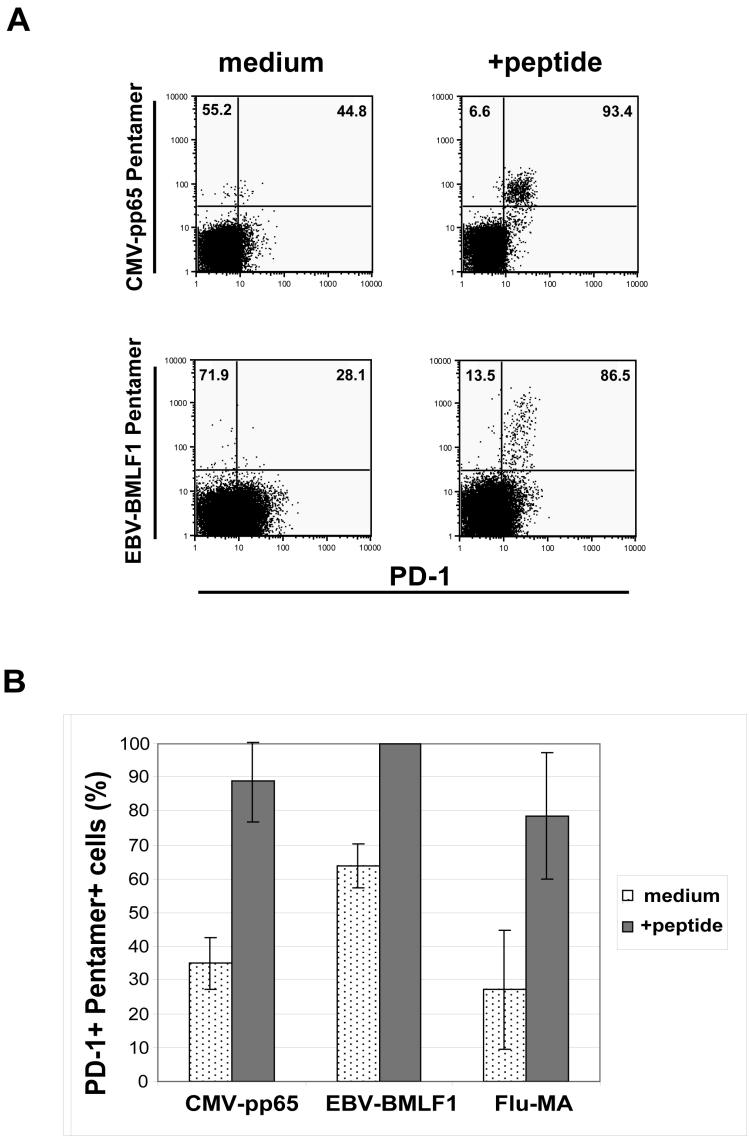



**Figure 17: Increase of PD-1 expression after antigen-specific stimulation.** PBMCs of healthy HLA-A2-positive individuals were stimulated in vitro with antigenic peptides for 5 days. (**A**) The stimulation led to an increase of PD-1 expression on antigen-specific cells regardless of their specificity. Individual examples for PD-1 expression on antigen-specific cells with and without peptide stimulation are shown. (**B**) Stimulation with peptides induced upregulation of PD-1 expression on CMV-pp65- , EBV-BMLF1- and Influenza-A (IV)-specific CD8+ T cells (n=4; n=5 and n=7, respectively).

### 3.2.2 Blockade of PD-1:PDL pathway partially enhances CD8+ T cell function

It has been shown that blockade of PD-1:PDL interaction was able to at least partially overcome functional exhaustion of antigen-specific CD8+ T cells during persistent viral infection. Similarly, we were able to enhance proliferation of virus-specific CD8+ T cells in some but not all healthy individuals by blocking PD-1:PDL1 interaction.. Addition of anti-PDL antibody during

peptide-specific stimulation of PBMC *in vitro* resulted in increased expansion of CMV-pp65-, EBV-BMLF1- and Influenza-A (IV)-specific CD8+ T cells in few cases, but overall the enhancing effect on cell expansion was low in healthy individuals (Figure 18).

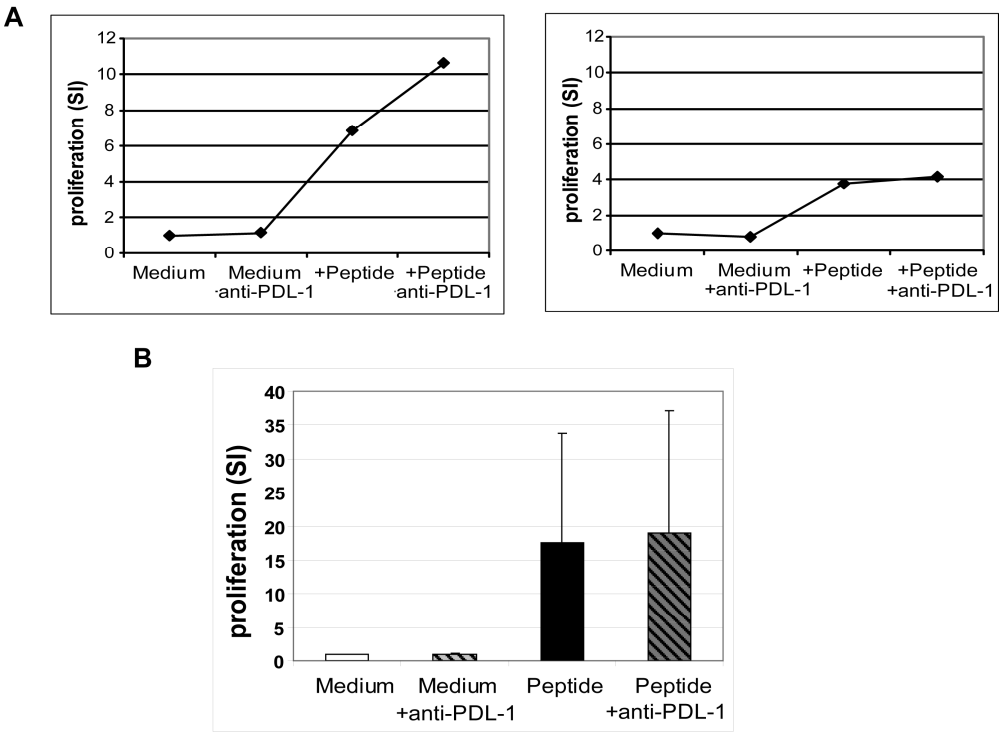



**Figure 18: Effect of PD-1:PDL blockade on cell proliferation in healthy individuals.** PBMCs form healthy volunteers were stimulated in vitro with antigenic peptides and in presence of anti-PDL1 antibody for blocking PD-1 signalling. (**A**) In few cases an enhanced proliferation of antigen-specific CD8+ T cells was seen. (**B**) In the majority of healthy individuals tested (n= 15) however, the effect was only marginal.

Likewise, improvement of antigen-specific CD8+ T cell proliferation could be achieved in some patients with acute HBV- or HCV infection (Figure 19). This was, however, not the case for all individuals tested and some showed no response to PD-1:PDL blockade.

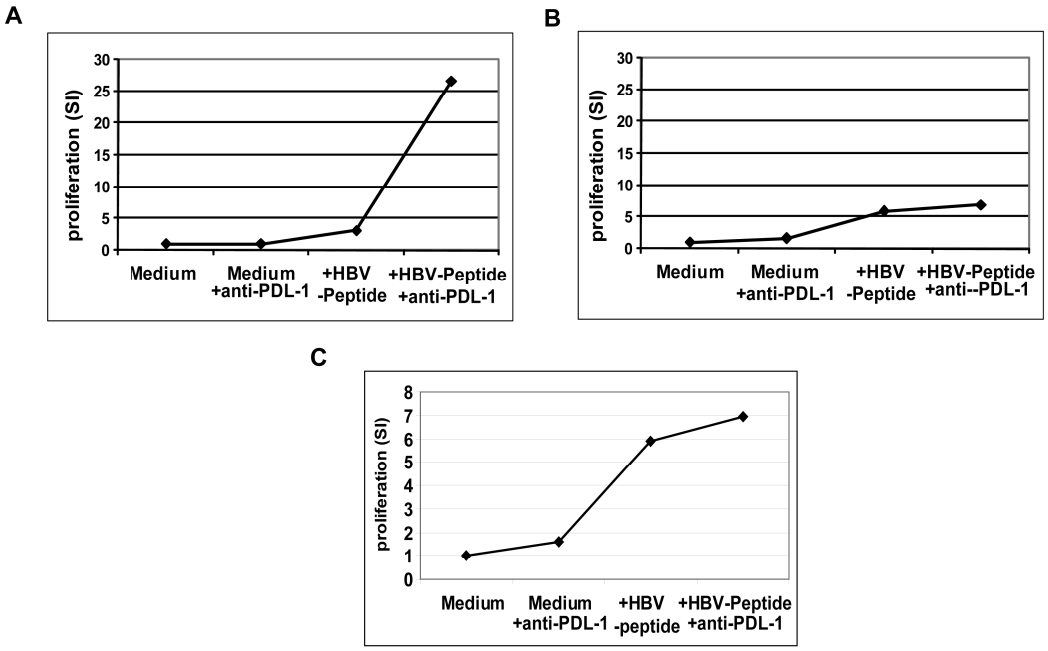



**Figure 19: Effect PD-1 blocking on proliferation in patients with acute hepatitis virus infections.** Patients acutely infected with HBV (**A, C**) or HCV (**B**) were stimulated in vitro with antigenic peptides under blocking of PD-1:PDL interaction. The effect on antigen-specific proliferation differed between individuals with some showing strong enhancement (**A**) and others displaying only mild increase (**B, C**).

Blocking PD-1:PDL *in vitro* during peptide stimulation of virus-specific CD8+ T cells was not able to change the cytotoxic capacity as measured by degranulation nor cytokine secretion (IFN$\gamma$ and TNF$\alpha$) in healthy individuals (Figure 20)

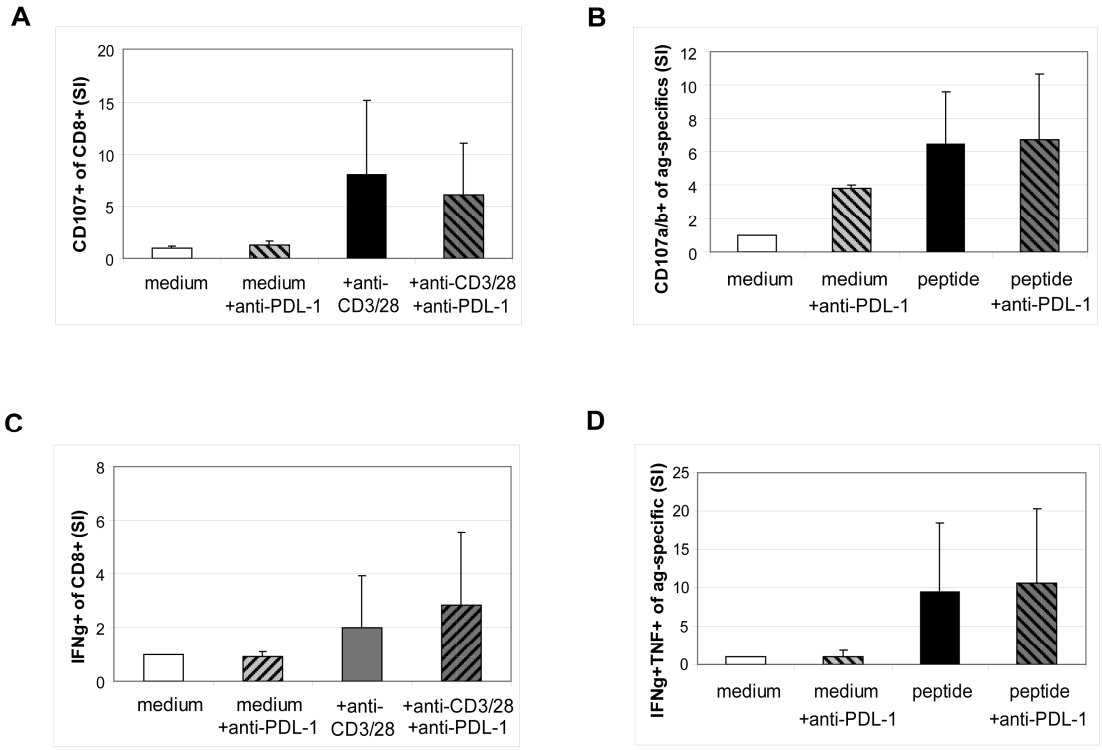



**Figure 20: Effect of PD-1:PDL blocking on cytotoxicity and cytokine production in healthy individuals.** Inhibiting PD-1 signalling showed no effect on (**A, B**) the cytotoxic capacity nor on (**C, D**) cytokine production in healthy individuals. This observation was made as well during TCR stimulation with anti-CD3/28 (**A, C**; n=12) and during antigen-specific stimulation using peptides (**B, D**; n= 4).

### 3.2.3 PD-1 expression in viral hepatitis and correlation with clinical parameters

As the PD-1:PDL pathway was shown to be involved in regulating immune responses and to deliver negative regulatory signals, it is likely that it also plays a role in impaired function of HCV-specific T cells during chronic infection. Indeed it was shown in a mouse model system that during a persistent viral (LCMV) infection PD-1 is involved and is upregulated on CD8+ T cells (Barber, DL et al. 2006). Likewise, it was of interest whether similar observations can be made during viral infections in humans. This seems to apply at least in the case of HIV, as two reports published shortly afterwards demonstrated that in HIV infected patients PD-1 expression on antigen-specific is high as well as on total CD8+ T cells (Day, CL et al. 2006; Trautmann, L et al. 2006). Furthermore, a positive correlation between PD-1 expression and viral load suggested a possible mechanism responsible for the typical severe immune dysfunction occurring during AIDS.

In order to investigate a possible role of PD-1 during HCV infection and to clarify if the high PD-1 expression reported for HIV infection is a general phenomenon during viral infections or if it

is specific for HIV, PBMC obtained from acutely and chronically HCV infected patients were isolated and stained *ex vivo* for PD-1 expression on T cells.

During the early phase of acute HCV infection a bigger part of CD8+ T cells expressed PD-1 on their cell surface (Figure 21A, left). Similarly, CD4+ T cells as well showed a high expression of PD-1 which was equal to or even slightly higher than on CD8+ T cells (compare Figure 21A and Figure 21B). Notably, some cell populations which were shown to be negative for PD-1 in healthy individuals (like NK-like T cells) showed a partial PD-1 expression during acute HCV infection (Figure 21C). However, NK cells, monocytes and B cells remained to be negative for PD-1 (Figure 21 D, E and F).

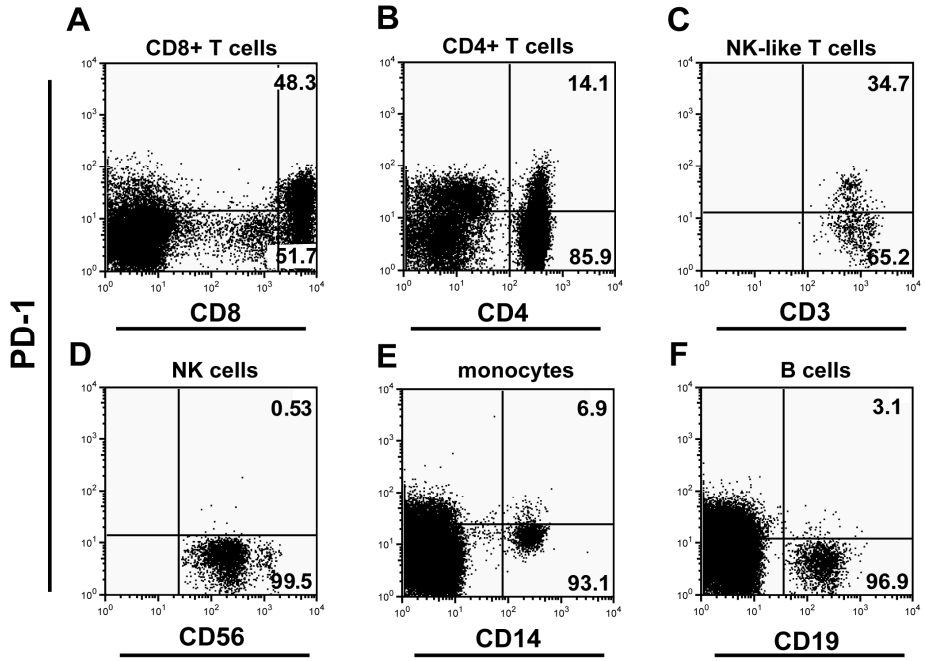



**Figure 21: PD-1 expression on different leukocyte subsets in patients with acute HCV infection.**
Total PBMC of patients with acute HCV were analyzed for expression of PD-1. Pictures shown are representative for 9 patients with acute hepatitis B or C infection. PD-1 expression was strongly increased on (**A**) CD8+ and (**B**) CD4+ T cells. (**C**) Different to healthy individuals some CD56+CD3+ NK-like T cells also showed expression of PD-1, but (**D**) not NK cells, (**E**) monocytes and (**F**) B cells.

Similarly, when investigating PD-1 expression on antigen-specific CD8 T cells in patients with acute HBV or HCV infection they were found to be almost uniformly positive for PD-1 (Figure 22).

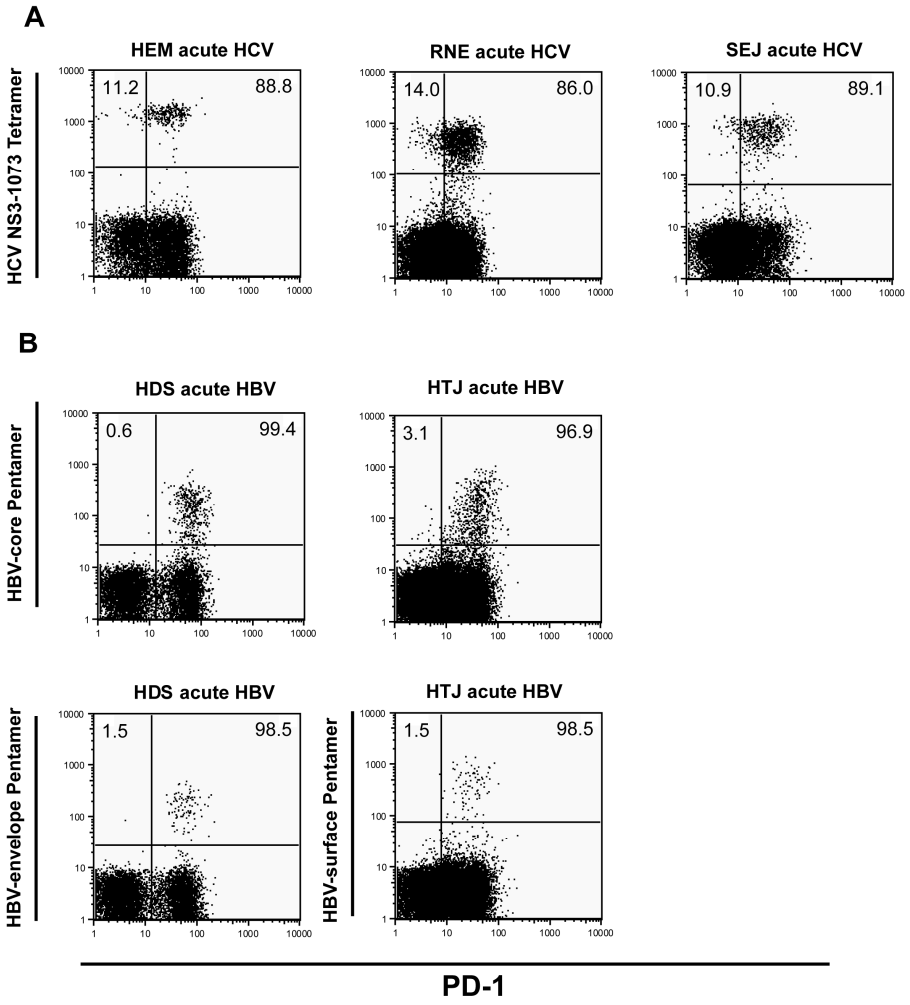



**Figure 22: PD-1 expression on antigen-specific CD8 + T cells in acute HBV and HCV infection.** PBMCs from patients with (**A**) acute HCV or(**B**) HBV infection were stained ex vivo with respective HCV-Tetramers or HBV-Pentamers for PD-1 expression. During acute symptomatic phase high numbers of antigen-specific CD8+ T cells were found with all of them being PD-1 positive.

In chronic HCV and chronic HBV infection PD-1 expression of total CD8+ T cells was highly variable between individuals and was not significantly higher as compared to healthy individuals like seen in case of acute HCV infection (Figure 23).

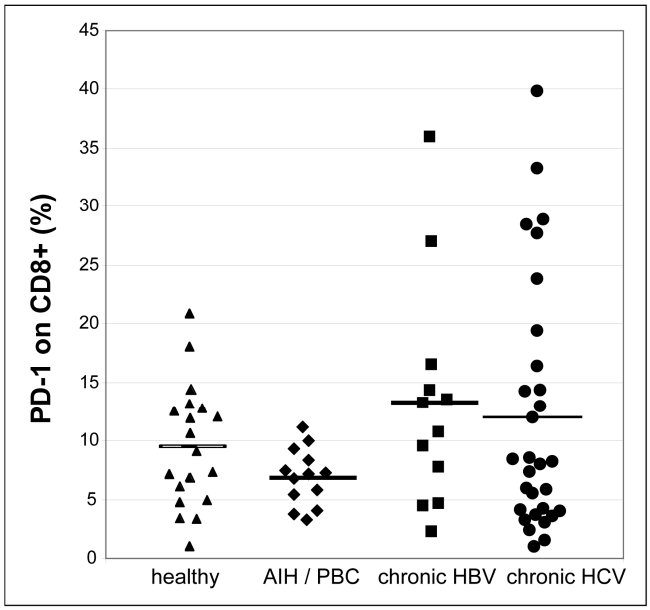



**Figure 23: PD-1 expression on CD8+ and CD4+ T cells.** PBMCs from healthy individuals (**left;** n=20), patients with non-viral auto-immune induced hepatitis (**second left;** n=12), chronic HBV patients (**second right;** n=12) and chronic HCV patients (**right;** n=32) were stained ex vivo for PD-1 expression. The pictures show individual expression levels of PD-1 in percent of total CD8+ T cells. Cells were gated on lymphocytes according to size and granularity.

Like on CD8+ T cells, also no significant increase of PD-1 expression could be seen on CD4+ T cells in chronic HCV or HBV infected and neither in cases of patients with autoimmune-induced hepatitis. Only for patients with symptomatic acute viral hepatitis infection a marked elevation of PD-1 on CD4+ as well as on CD8+ T cells was observed (p=0.02 and p=0.002, respectively; see Figure 24A, B). Similar results were obtained for PD-1 expression on CD56+ CD3+ NK-like T cells where only in acute HBV and HCV infected patients an increase of PD-1 could be seen (p=0.048; Figure 24C). NK cells however, did not gain expression of PD-1 in any of the patient groups analysed (Figure 24D). Monocytes from acute infected patients also showed a tendency of higher PD-1 expression. No significance could be reached due to reduced sample numbers (Figure 24E). On B cells again raised levels of PD-1 expression were observed in acute infected patients (p=0.005). Here, it appears that also B cells have increased PD-1 expression in chronic HCV patients (p=0.016). Lack of significance is again due to low sample numbers in case of chronic HBV infected patients, which also show higher mean levels of PD-1 expression (Figure 24F).

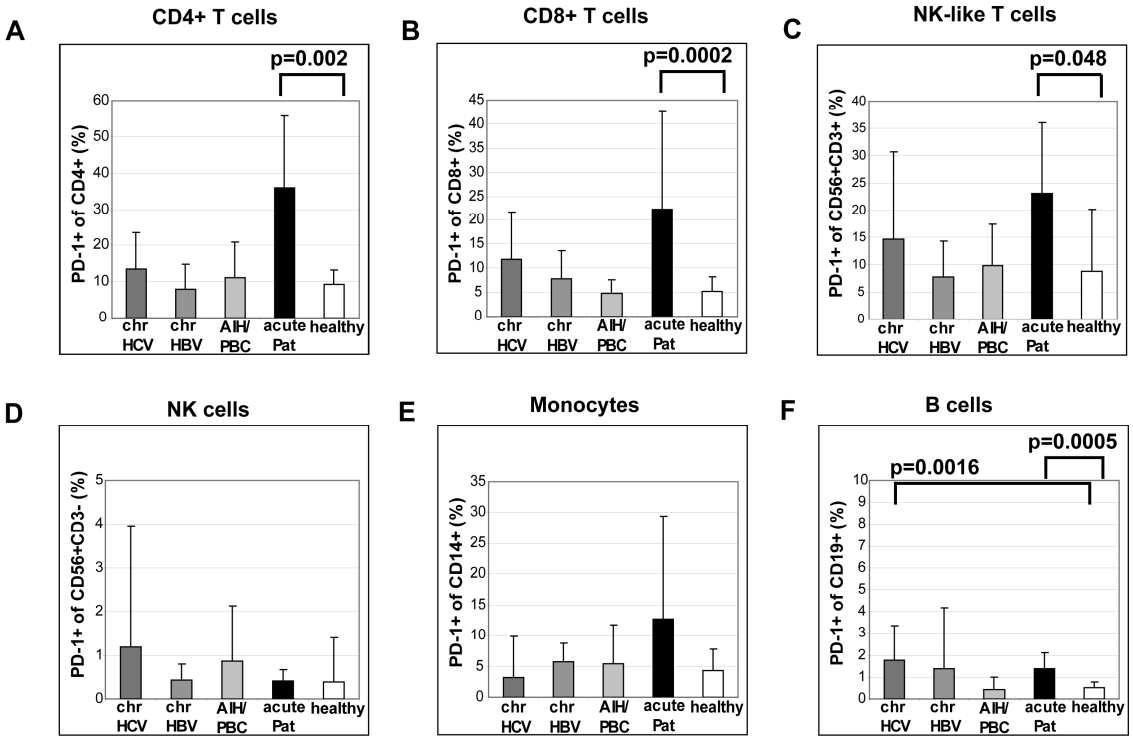



**Figure 24: Comparison of PD-1 expression on different leukocyte subsets between healthy individuals and patients with hepatitis.** PBMCs from healthy individuals (n=20) as well as from patients suffering from chronic HCV (n=42), chronic HBV infection (n=12), autoimmune-induced hepatitis (n=13) or acute HCV or HBV infection (n=6) were analysed ex vivo for expression of PD-1 on various leukocyte subsets. (**A**) Significant increase of PD-1 expression as compared to healthy individuals was found on CD4+ T cells and (**B**) CD8+ T cells in patients with acute infection. (**C**) The same applied for NK-like T cells. (**D**) NK cells however did not display increased expression of PD-1 in either of the groups analysed. (**E**) Monocytes again showed a trend towards elevated PD-1 expression in patients with acute infection. Lack of significance is due to low sample number. (**F**) PD-1 expression on B cells was significantly increased in acutely infected as well as in chronic HCV patients. Lack of significance in case of chronic HBV infection is due to low sample number.

Because of this rather big inter-individual differences of PD-1 expression in patients, a possible association with clinical parameters was investigated which might explain this variability. However, no correlation of PD-1 expression on total CD8+ or CD4+ T cells with clinical parameters associated with liver disease, like ALT or AST levels, fibrosis score or bilirubin levels or with plasma viral load, was observed (Figure 25C-H). Additionally, no correlation of PD-1 expression on CD8+ T cells existed with general gender or age (Figure 25A and B). Neither biochemical values of hepatitis activity (ALT and AST; Figure 25D and E), nor bilirubin as a marker of liver detoxification (Figure 25F) nor histological staging (data not shown) were associated with expression levels of PD-1 on CD8+ T cells. Moreover, HCV and HBV viral load (Figure 25G and H) were not correlated to the amount of PD-1+ CD8+ T cells in these patients.

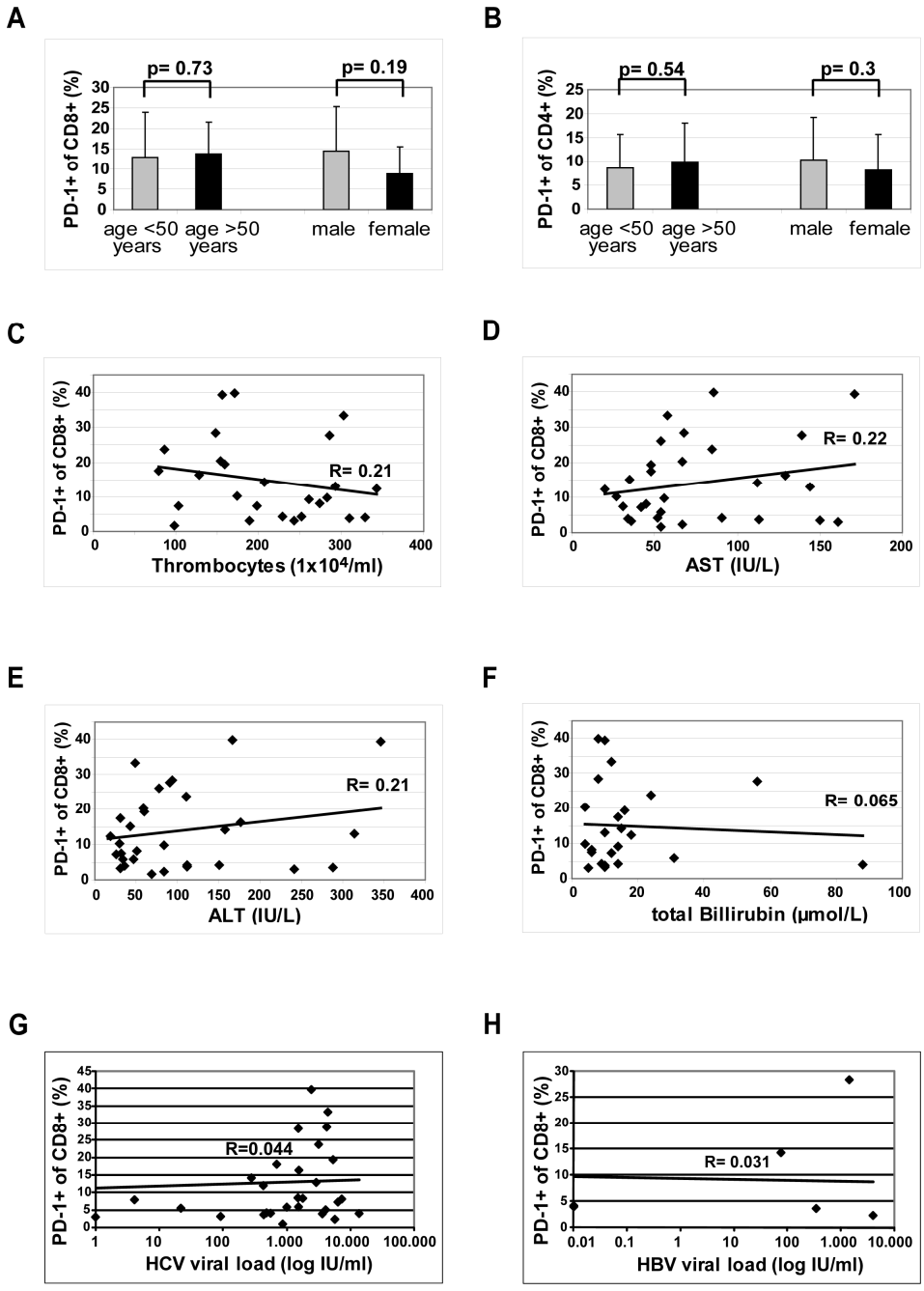



**Figure 25: Correlation of PD-1 expression on CD8+ T cells with clinical parameters in patients with chronic hepatitis infections.** PD-1 expression levels on CD8+ T cells in chronic HCV (left column) and chronic HBV infected patients (right column) was correlated with non-clinical factors such as (**A, B**) age and gender. Correlation of PD-1 expression on CD8+ T cells with various clinical parameters of liver disease such as (**C**) thrombocytes, (**D**) AST, (**E**) ALT, (**F**) total bilirubin levels (**G** and **H**) and plasma viral load showed no significance..

## 3.3 Expression and function of 2B4

In some cases the blockade of the PD-1:PDL pathway improved virus-specific CD8+ T cell response to a similar extent. However, this was not the case in every individual tested. As well, enhancement of HIV-specific CD8+ T cell proliferation could not be achieved for all patients (Day, CL et al. 2006; Trautmann, L et al. 2006).

Analysis of gene expression profiles of exhausted virus-specific CD8+ T cells in mice revealed an alteration of additional signalling pathways (Wherry, EJ et al. 2007). One of those molecules displaying higher expression during exhaustion is the costimulatory molecule 2B4. So far, few data have been published about the role of 2B4 on CD8+ T cells during viral infection. Discrepancies in the signalling elicited by 2B4 ligation are described in the literature with inhibiting functions in some cases and activating capacities in other settings (see 1.2.4.2).

In order to investigate the role of 2B4 as a costimulatory molecule in the immune system and the consequences of its expression on immune cells, we first sought to analyse the expression pattern of 2B4 on the various leukocytes to be found in human peripheral blood. Further, we aimed to analyse a possible role of 2B4 during antigen-specific and general immune responses of CD8+ T cells by blocking the interaction with the counter-receptor CD48.

### 3.3.1 Expression of 2B4 on human lymphocytes

Isolated human peripheral blood mononuclear cells (PBMCs) were stained directly ex vivo for 2B4 in combination with different other markers identifying the main subpopulations of leukocytes. 2B4 expression was found on a small subset of CD8+ T cells (Figure 26). On the contrary, 2B4 expression was hardly detected on CD4+ T cells with not more than 5% being positive (see Figure 26B). In line with the initial description of 2B4 as a molecule involved in NK cell cytotoxicity, all CD56+ CD3- NK cells were found to express this marker (see Figure 26D). Here it is noteworthy that the expression intensity of 2B4 – measured by the Mean Fluorescence Intensity (MFI) of the anti-2B4 antibody- differs between the two subpopulations of NK cells which can be identified according to their level of CD56 expression. The so called $CD56^{bright}$ NK cells, a small fraction of NK cells regarded as a mainly cytokine secreting cell, were found to be lower in 2B4 expression as compared to the $CD56^{dim}$ NK cells who are exhibiting strong cytotoxicity. Interestingly, the majority of the CD56+ CD3+ NK-like T cells also showed to be positive for 2B4 expression (Figure 26C). Similarly, also all monocytes present in the peripheral blood expressed 2B4 on their surface (see Figure 26E). B cells, however, like the CD4+ T cells, expressed 2B4 only in low amounts (Figure 26F).

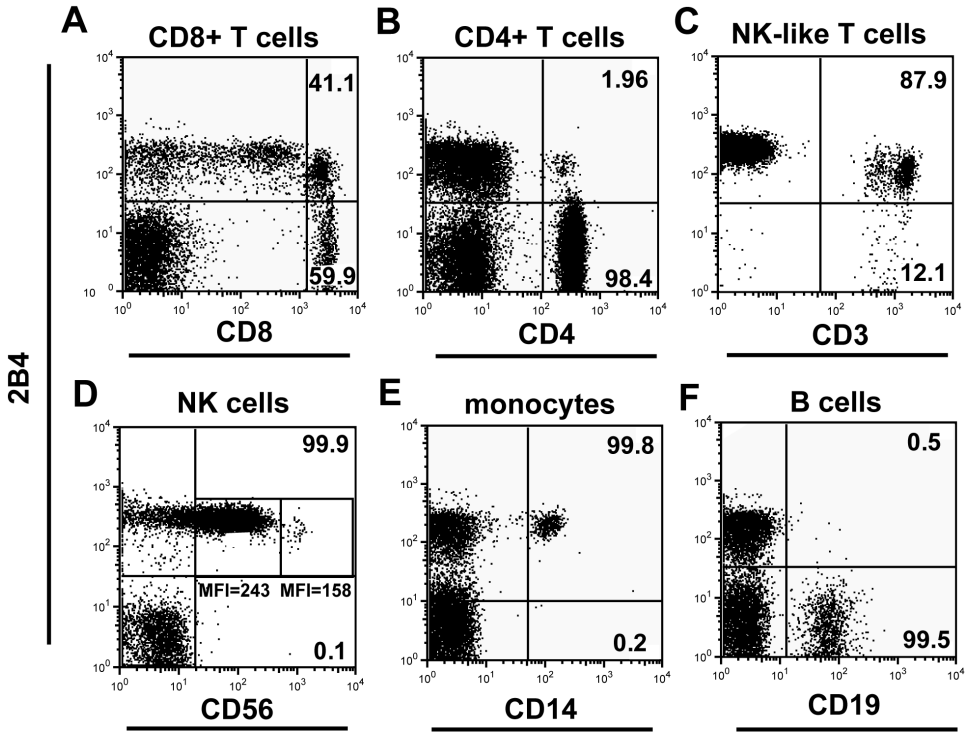



**Figure 26: Expression of 2B4 on different leukocyte subpopulations.** On freshly isolated PBMC from healthy individuals 2B4 expression was analyzed by flow cytometry by simultaneous staining for common molecules identifying the different subpopulations like (**A**) CD8+ T cells, (**B**) CD4+ T cells, (**C**) CD56+CD3+ NK-like T cells, (**D**) CD56+CD3- NK cells, (**E**) CD14+ Monocytes and (**F**) CD19+ B cells.

On CD8+ T cells expression of 2B4 varied between individuals, ranging from 5% to 65% (see Figure 27). The reason for these differences is not known, however, no correlation with gender or age could be found (data not shown).

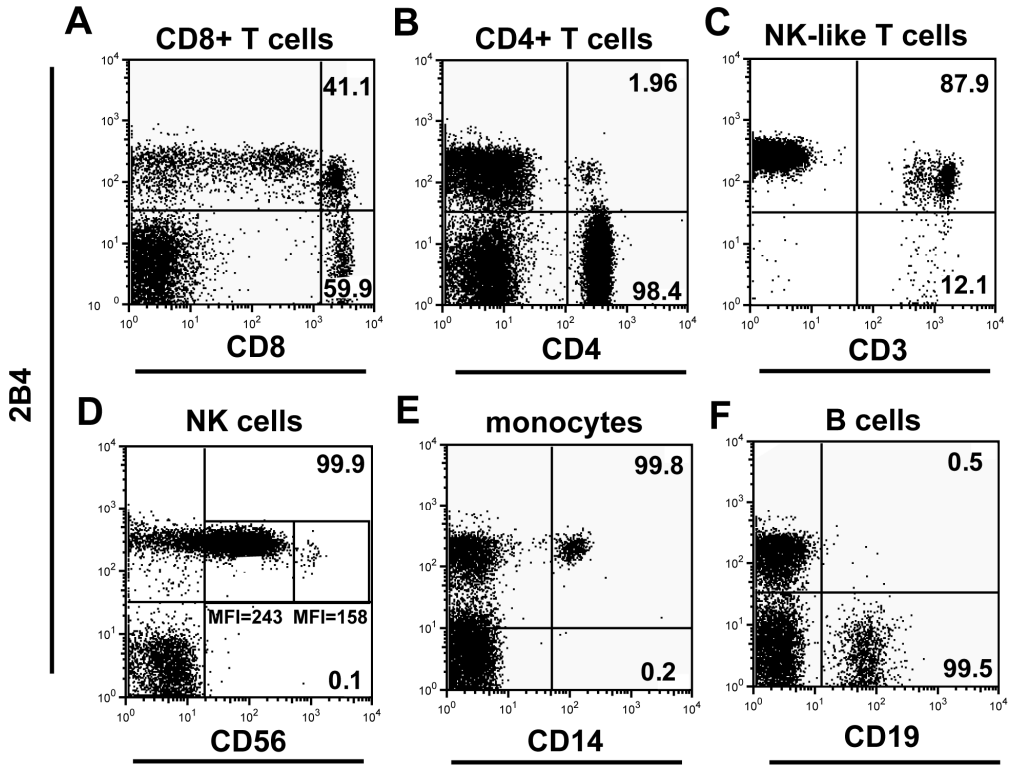



**Figure 27: 2B4 expression on CD8+ T cells in healthy individuals.** Freshly isolated PBMCs form healthy volunteers were stained for 2B4. (**A**) Representative staining for 2B4 expression on CD8+ T cells. Gating was performed by selecting CD8+ T cells within the lymphocyte population. (**B**) Between different individuals 2B4 expression on CD8+ T cells varied greatly ranging from 5.6% to 65% of total CD8+T cells (n=20).

### 3.3.2 Expression of CD48 on lymphocytes

For induction of 2B4 signalling, the molecule has to ligate to its counterpart in order to get cross-linked and evoke downstream signal transduction. The counter receptor of 2B4 in humans was identified as CD48 (Brown, MH et al. 1998). Its expression was found on all leukocytes including T cells, B cells, NK cells and monocytes (compare Figure 28A). Consequently, also antigen-specific CD8+ T cells were expressing CD48 (Figure 28B) without any apparent difference in the expression levels (not shown). Nevertheless, within the lymphocyte population few cells were be found which are clearly negative for CD48 expression. The amount of CD48-negative lymphocytes again varied between individuals (see Figure 28C). These CD48-negative cells did not bear the common markers identifying the different subpopulations of human leukocytes (data not shown) and could, therefore, not be assigned to any of the above mentioned cell populations. It is possible that these cells are representatives of a cell population like granulocytes or Dendritic Cells or might be of a special maturation or activation status.

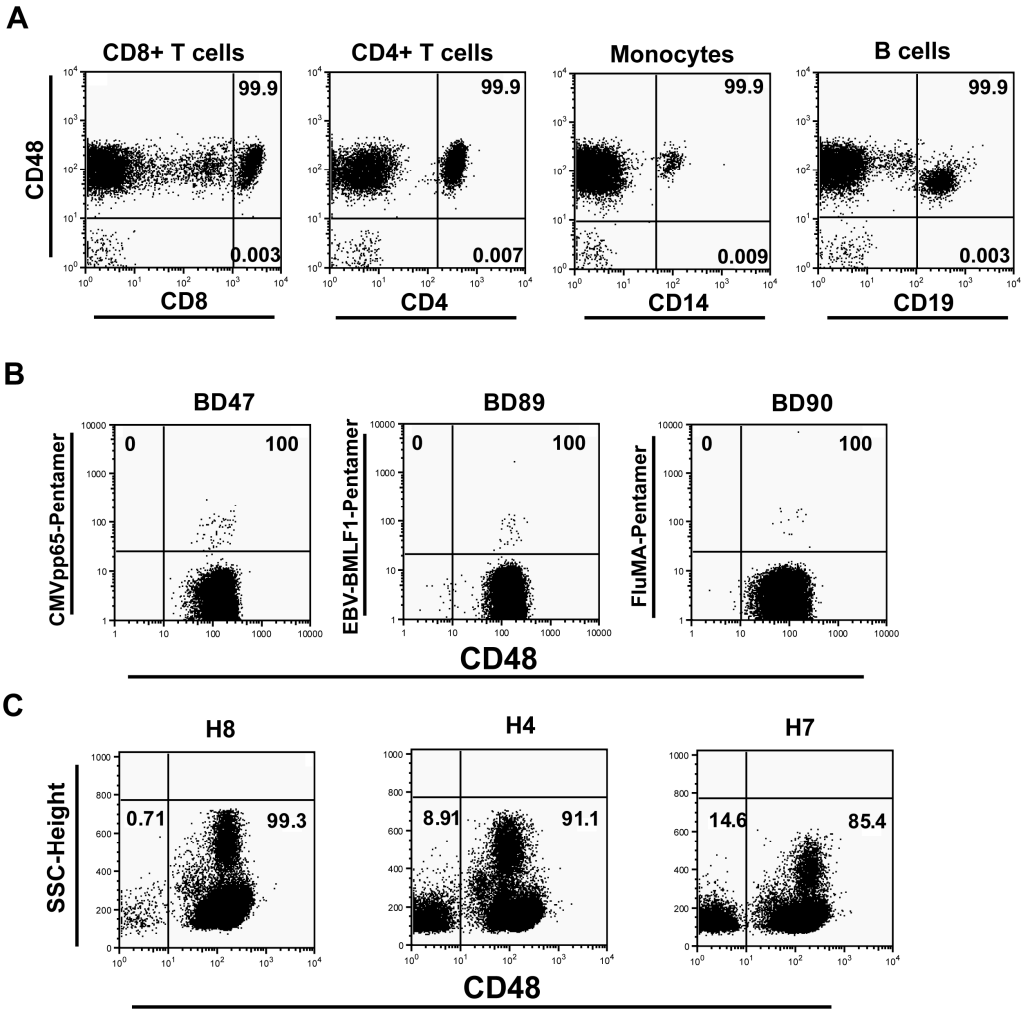



**Figure 28: Expression of CD48 in healthy individuals.** Freshly isolated PBMCs from healthy volunteers were analysed for expression of CD48. (**A**) All cells of the leukocyte population were found to be positive for this molecule. (**B**) Consequently, antigen-specific CD8+ T cells as well were positive for CD48 and no difference in the expression intensity was found (not shown). (**C**) However, a small fraction of CD48-neagtive cells were present in the leukocyte population. Pictures of one representative staining are shown in each case.

### 3.3.3 Expression of 2B4 on antigen-specific CD8+ T cells

When analysing 2B4 expression on CD8+ T cells recognizing different epitopes, a general pattern could be found within cells of the same specificity. CMV-pp65-specific CD8+ T cells as well as cells recognizing the EBV-BMLF1 epitope displayed high expression of 2B4 of approximate 80% being positive (see Figure 29A,B and D). Rather low levels of 2B4 expression were found in case of Influenza-A (IV)-specific CD8+ T cells. These cells displayed a low to intermediate expression of 2B4 in most individuals, but some also showed high expression of 2B4 (see Figure 29C, D). Interestingly, this expression pattern on the various antigen-specific CD8+ T cells was similar to that found for PD-1 (see Figure 15).

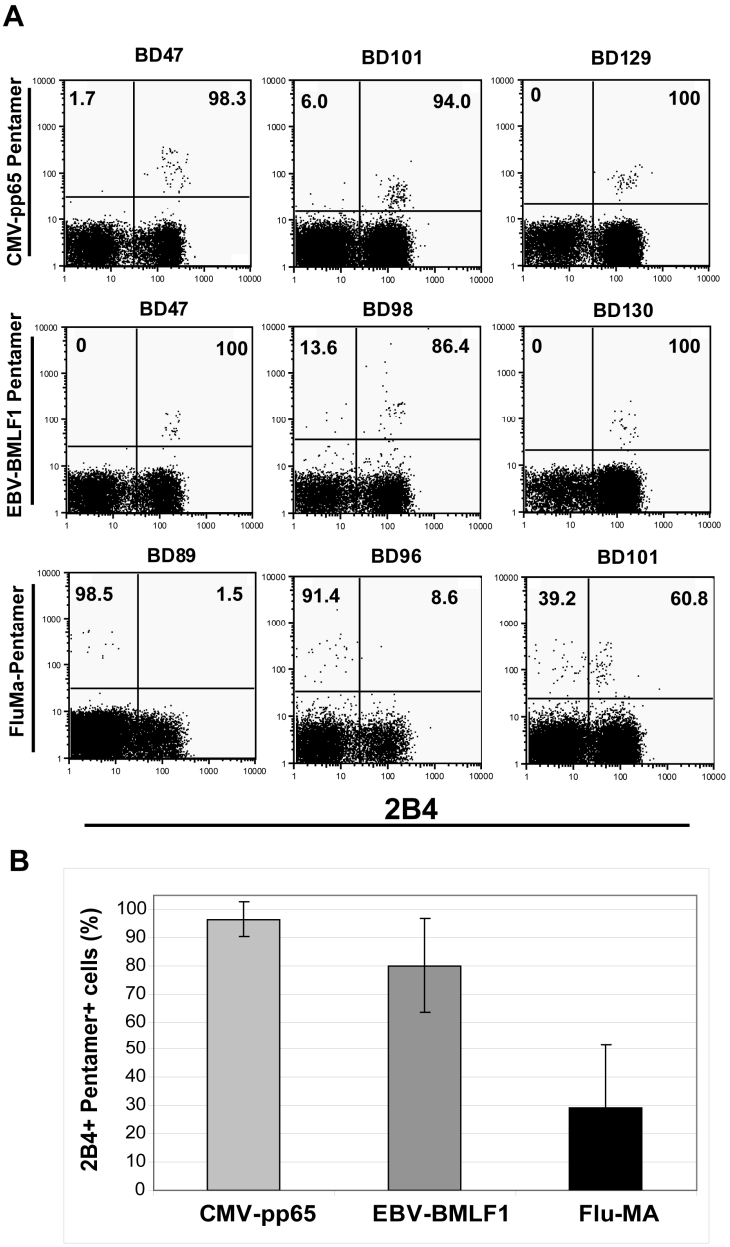



**Figure 29: Expression of 2B4 on antigen-specific CD8+ T cells in healthy individuals.** The presence of 2B4 and CD48 on (**A**) CMV-pp65-specific, EBV-BLMF-1-specific and Influenza-A (IV) specific CD8+ T cells was assessed in healthy individuals using Pentamer technology. Three pictures from healthy blood donors are shown as representatives. (**B**) In the mean, a clear difference in 2B4 expression was seen on CD8+ T cells with different antigen-specificities. CMV-pp65 (n=7) and EBV-BMLF1 (n=13) specific CD8+ T cells were almost 100% positive, while Influenza-A (IV) specific cells (n=15) displayed rather low amounts of 2B4.

### 3.3.4 Phenotype associated with 2B4+ CD8+ T cells

To further elucidate 2B4 expression on CD8+ T cells we thought to examine the coexpression of various other markers which are either expressed upon activation or which characterize the memory status of cells. When gating on 2B4+ CD8+ T cells and analysing the expression of CCR7 and CD45RA, these cells displayed an effector phenotype. Analysing the

total CD8+ T cell population it showed that CD8+ T cells being positive for CCR7 did not express 2B4 on their surface (Figure 30A). Therefore, according to this means of identifying memory cells, 2B4+ CD8+ T cells belong to the effector population of $T_{EMRA}$ or $T_E$ cells (Figure 30B).

Analysing the expression of two other molecules commonly used for determination of the memory status of T cells CD62L and CD127, the picture seen here was concordant with the one above (Figure 30A). Among CD8+ T cells almost all 2B4+ cells were negative for CD127. CD62L on the other hand was also present on 2B4+ CD8+ T cells and so the same $T_E$/$T_{EMRA}$ phenotype showed as with CCR7 and CD45RA.

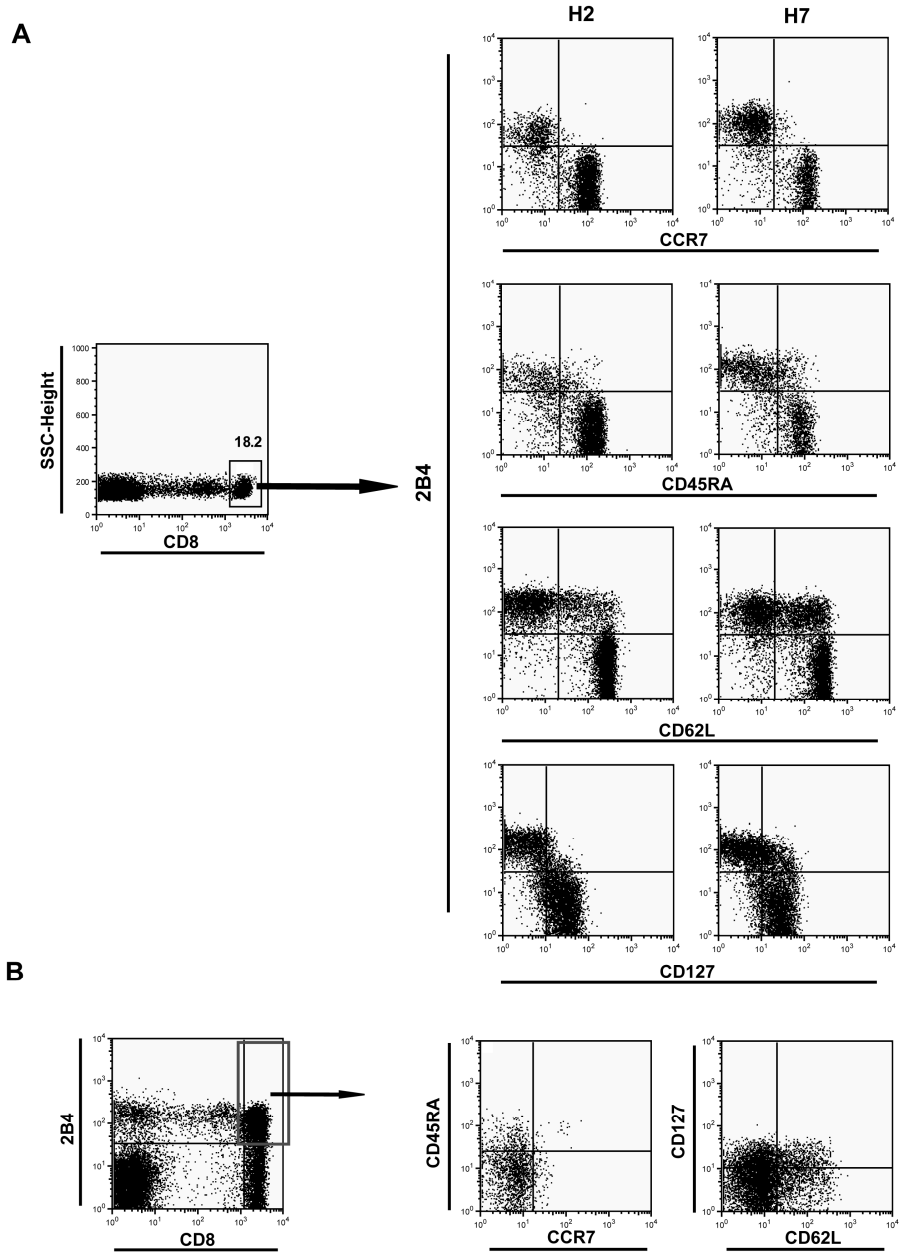



**Figure 30: Coexpression of memory markers and 2B4 on CD8+ T cells.** In healthy individuals the memory phenotype of 2B4+ CD8+ T cells was analysed by simultaneous staining for CD45RA and CCR7 or CD62L and CD127, respectively. (**A**) Total CD8+ T cells showed a reciprocal expression of 2B4 and CCR7 as well as of 2B4 and CD127, but they did express CD45RA and CD62L simultaneously. (**B**) 2B4+ CD8+ T cells were evaluated for the expression of memory markers.

As shown before, 2B4 expression on CD8+ T cells increased upon unspecific and antigen-specific stimulation. Consequently, the question arose to whether a correlation exists between expression of 2B4 and other well known T cell activation and maturation markers like CD38, CD25, CD27 and CD28, CD40 and CTLA-4. The molecules CD27 and CD28 were found to be expressed by 2B4+ CD8+ T cells confirming again the memory status of effector/memory cells (Figure 31A, B). Few of the 2B4+ CD8+ T cells were positive for CD38 (Figure 31C) while no expression of CD152, CD40 and CD25 was seen (Figure 31D, E and F) as the cells from healthy individuals are usually not in an activated status.

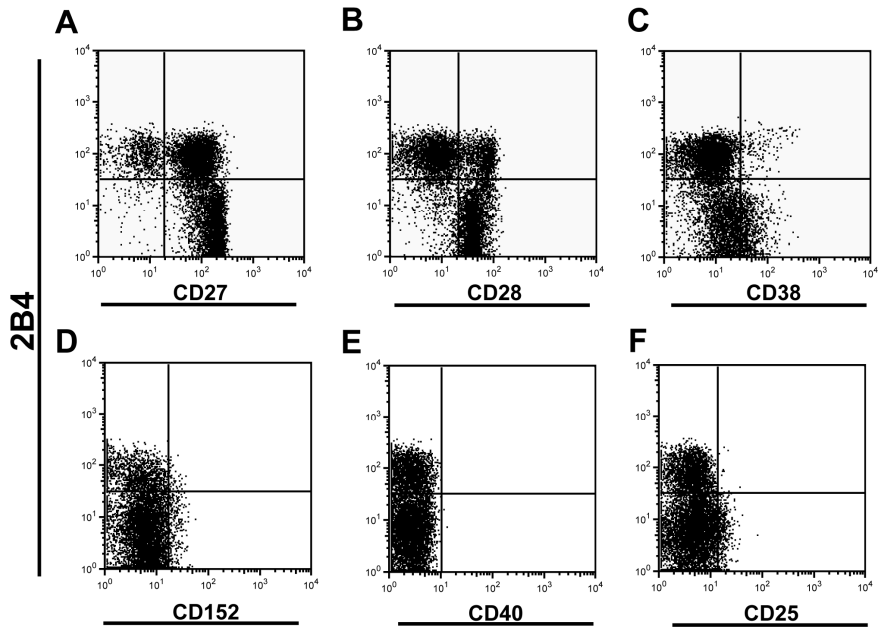



**Figure 31: Coexpression of phenotypic activation-induced and memory markers on 2B4+ CD8+ T cells in healthy individuals**. CD8+ T cells of healthy volunteers were analysed for the coexpression of molecules associated with maturation like (**A**) CD27, (**B**) CD28 or activation such as (**C**) CD38, (**D**) CD152 (CTLA-4), (**E**) CD40, (**F**) and CD25. Cells were gated on CD8+ T cells within lymphocyte population.

### 3.3.5 Influence of different stimuli on 2B4 and CD48 expression on CD8+ T cells

As the 2B4 molecule is generally regarded to have activating function in the immune system, the question arose whether different specific and unspecific stimulants were able to induce 2B4 expression on CD8+ T cells. After an *in vitro* culture of 5-6 days, CD8+ T cells showed a clear upregulation of 2B4 upon stimulation with IL2 (see Figure 32). This effect was also concentration dependent with higher IL2-concentrations inducing a stronger upregulation. Likewise, a T cell receptor stimulation using anti-CD3 and anti-CD28 monoclonal antibodies led to an increase of expression. Interestingly, also SEB caused an elevation of 2B4 on the cell surface of CD8+ T

cells (Figure 32). Notably, all the stimulants mentioned above were not able to markedly alter or induce any expression of 2B4 on CD4+ T cells (not shown).

Nevertheless, besides changes on 2B4 expression also a modification on the expression intensity of CD48 could be observed. As usually all lymphocytes express CD48, the effect was only noticeable through the Mean Fluorescence Intensity (MFI) of the anti-CD48 antibody. Comparable to the upregulation of 2B4, CD48 expression increased in the samples containing IL-2, SEB or CD3/CD28 (see Figure 32).

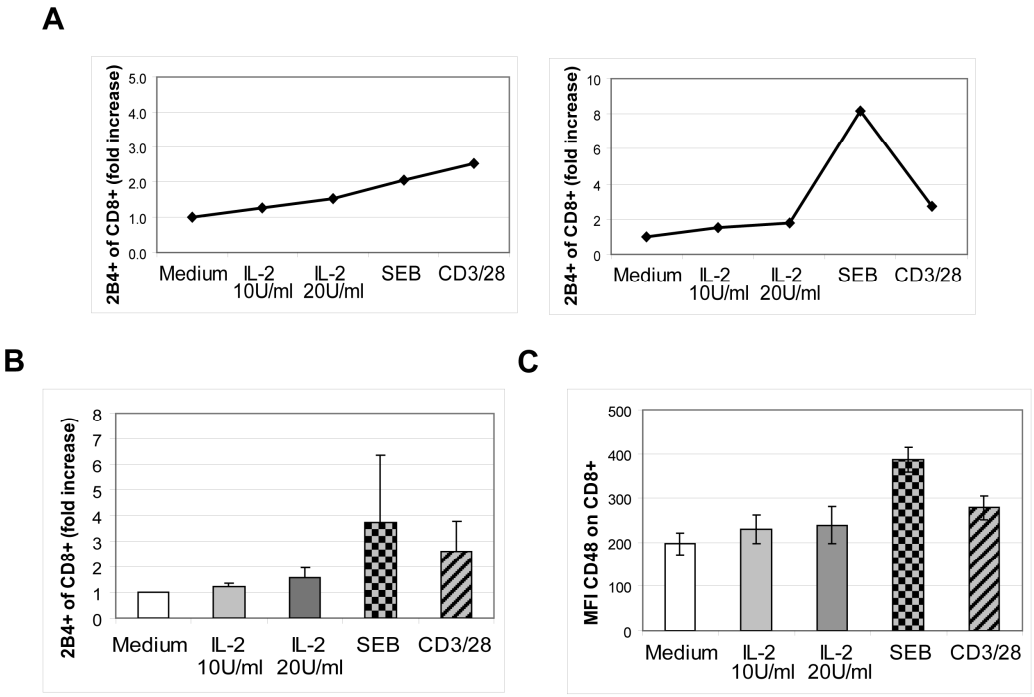



**Figure 32: Alteration of 2B4 and CD48 expression through unspecific stimuli.** PBMC from healthy individuals were stimulated in vitro for five days with various substances including IL-2, SEB or anti-CD3/CD28. After five days incubation cells were analysed for 2B4 and CD48 expression by flow cytometry. 2 examples are shown as representative. (**A**) Strength of 2B4 upregulation varied between different individuals with some showing strong increase of 2B4 expression through SEB (right side). (**B**) An overview of 2B4 expression (n=12) and (**C**) CD48 MFI of at least ten independent experiments showed a clear induction of 2B4 by IL-2 and anti-CD3/CD28, while the expression intensity (MFI) of CD48 experienced a notable increase by all of those stimulants tested and was strongest after stimulation with SEB (n=12).

As to be expected, antigen-specific stimulation of CD8+ T cells caused the respective antigen-specific cells to express 2B4, which was absent in the *ex vivo* staining and in the medium control (see Figure 33). The expression of 2B4 on non-antigen-specific CD8+ T cells did not change upon peptide stimulation. When looking at the expression of the counter receptor CD48, we also found this to be upregulated specifically on the antigen-specific cells. Here, the expression levels (analysed by comparing the MFI) was higher as on the non-specific CD8+ T cells.

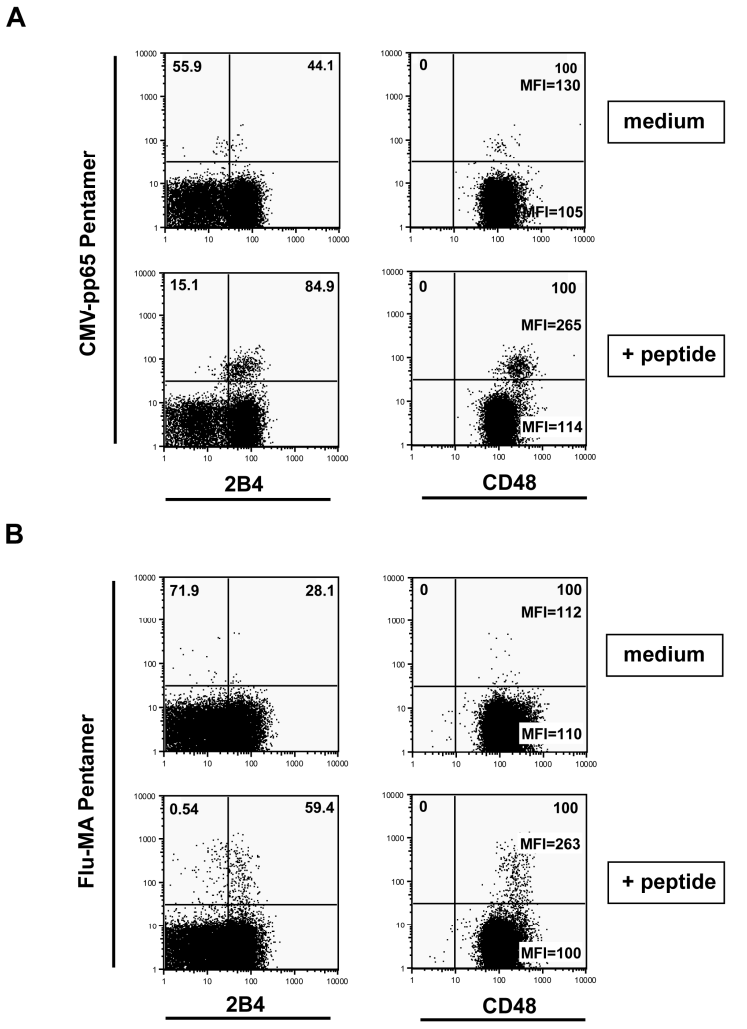



**Figure 33: Induction of 2B4 and CD48 expression after antigen-specific stimulation.** After in vitro stimulation of PBMCs from healthy individuals for five days with antigenic peptides cells were analysed for 2B4 and CD48 expression. (**A**) Expression of 2B4 increased on antigen-specific CD8+ T cells regardless of their specificity. (**B**) Notably, the expression of CD48 increased as well marked by a higher expression level per cell and therewith raised Mean Fluorescence Intensity (MFI). (C) In the mean a clear increase of PD-1 expression on antigen-specific CD8+ T cells was observed regardless of the specificity (CMV: n=4; EBV: n=5; Influenza: n=7).

### 3.3.6 Effect of anti-2B4 and anti-CD48 antibodies on CD8+ T cell cytotoxicity

The most essential function of CD8+ T cells is exerting cytotoxicity for eliminating virus infected or degenerate cells. During chronic HCV infection this vital function is impaired and cytotoxicity is one of the first effector functions to be affected during T cell exhaustion. Further, 2B4 is expressed on all NK cells and cytotoxic functions of NK cells increased if stimulated (Bhat, R et al. 2006). Therefore, the effect of 2B4- and CD48 signalling on cytotoxic capacity measured by degranulation of CD8+ T cells was investigated *in vitro*.

Stimulation of total PBMCs from healthy volunteers with anti-CD3/28 resulted in increased degranulation of CD8+ T cells in vitro (p= 0,02). The presence of anti-2B4 led to a slight increase in degranulation, which could already be seen in the absence of further stimuli (Figure 34A). In combination with anti-CD3/28 it elevated degranulation of CD8+ T cells in some individuals (Figure 34B), although this was lacking significance when combining data for all subjects studied (p=0,5). Inhibition of CD48 ligation by adding anti-CD48 antibody, however, did not have any influence on degranulation of CD8+ T cells.

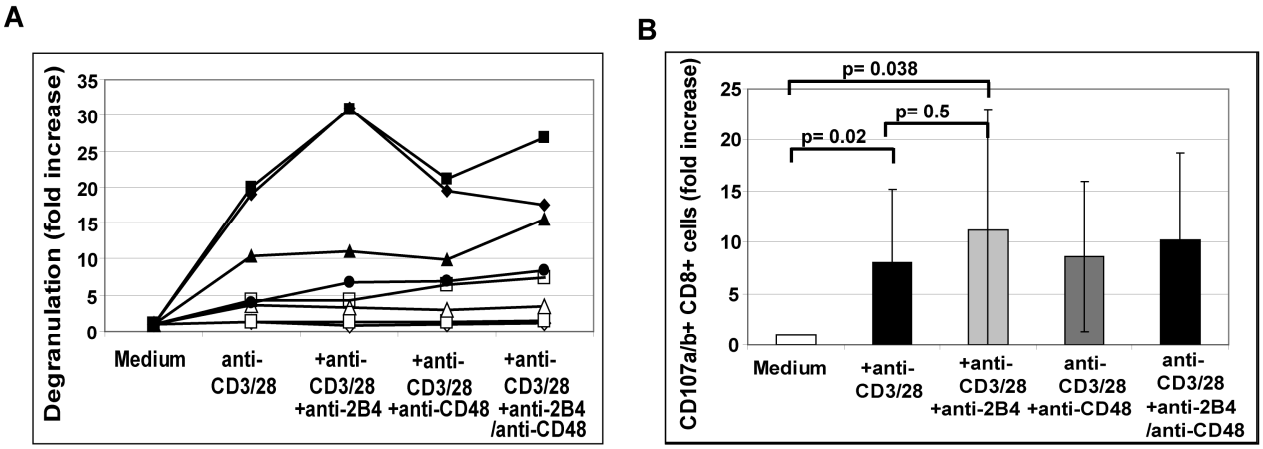



**Figure 34: Degranulation of CD8+ T cells after treatment with anti-2B4 or anti-CD48.** Total PBMCs from healthy individuals were stimulated ex vivo with anti-CD3/28 and anti-2B4 or anti-CD48. (**A**) Already without stimulus (medium) the anti-2B4 antibody was able to increase the degranulation slightly: The lines represent individual changes in degranulation. (**B**) TCR stimulation by anti-CD3/28 increased degranulation of CD8+ T cells significantly and experienced an even stronger elevation in presence of anti-2B4. Blocking CD48 signalling, however, did not have any effect on the cytotoxic capacity (n=12).

As CD8+ T cells act in antigen-dependent manner, it was of interest if 2B4 is capable of increasing the degranulation under these circumstances. Incubation of PBMCs from healthy individuals ex vivo with antigenic peptides in combination with anti-2B4 led to a marginal increase in degranulation of antigen-specific CD8+ T cells in some cases (Figure 35, upper row). In other individuals on the other hand, a slight non-significant decline of degranulation was observed (lower row).

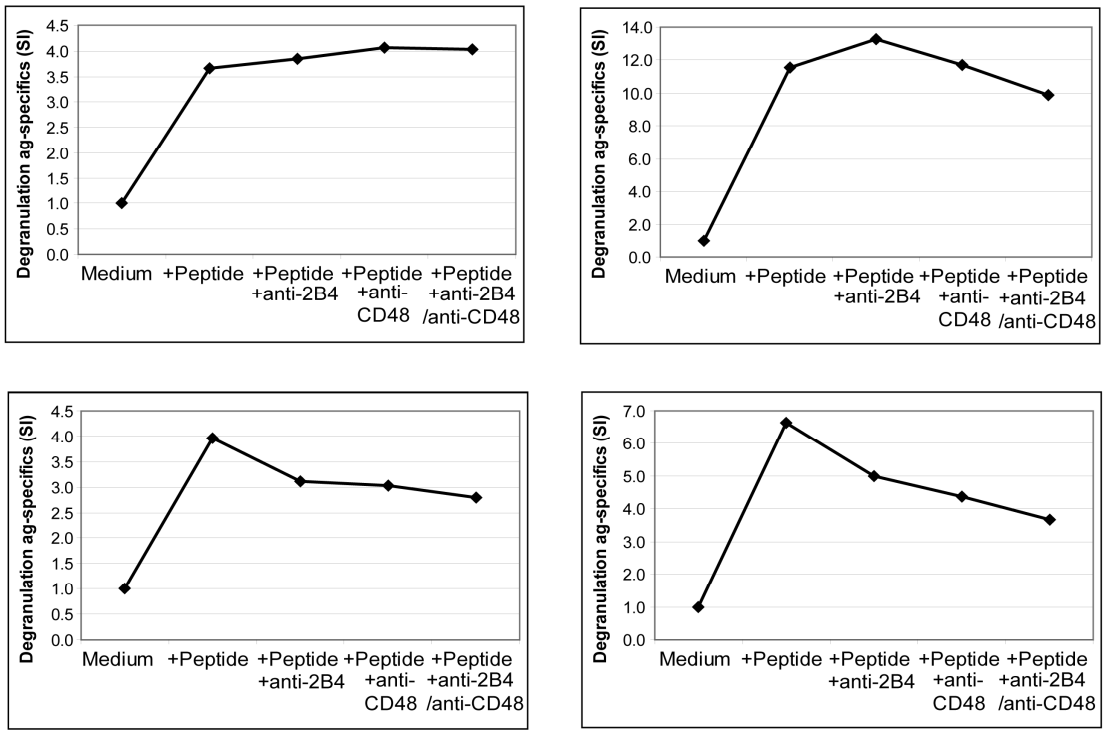



**Figure 35: Degranulation of antigen-specific CD8+ T cells upon peptide stimulation.** PBMCs of healthy individuals were tested ex vivo for degranulation upon antigenic stimulation. The presence of anti-2B4 or anti-CD48 showed a slight non-significant increase of degranulation in some healthy persons (upper row). In other individuals a slight decline of degranulation could be observed in presence of anti-2B4 or anti-CD48 (lower row).

To further clarify a possible influence of 2B4 on the cytotoxic capacity of CD8+ T cells, sorted 2B4+ and 2B4- CD8+ T cells were stimulated *in vitro* with anti-CD3/28 and their degranulation capacity was determined. Interestingly, a clear difference regarding degranulation could be seen between 2B4+ and 2B4- CD8+ T cells. While 2B4- T cells displayed no degranulation at all upon TCR stimulation (Figure 36, dotted line) and the amount of CD107a/b on their cell surfaces was comparable to that found in the medium controls, the opposite was the case for 2B4+ cells. Here, a clear and strong increase of CD107a/b positivity induced by anti-CD3/28 was observed (black line).

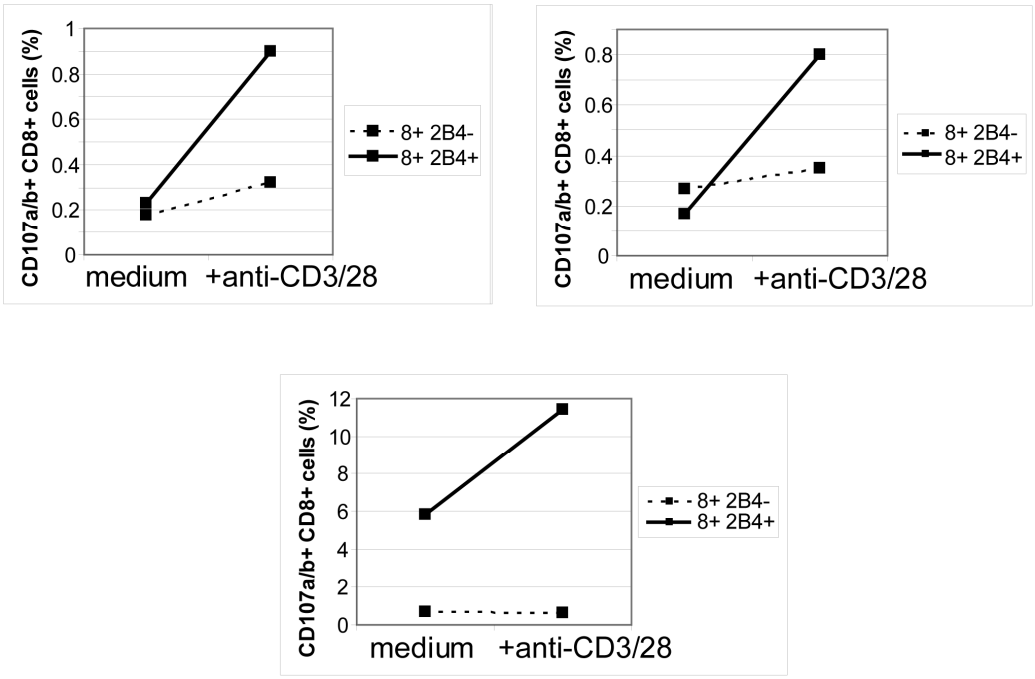



**Figure 36: Degranulation capacity of sorted 2B4+ and 2B4- CD8+ T cells.** PBMCs from healthy individuals were sorted for 2B4+ and 2B4- CD8+ T cells and tested for their degranulation capacity upon stimulation with anti-CD3/28. It shows clearly that 2B4- CD8+ T cells (**dotted line**) have a limited to no ability to degranulate, while it is strongly enhanced in 2B4+ CD8+ T cells (**black line**).

### 3.3.7 Effect of anti-2B4 and anti-CD48 antibodies on cytokine production of CD8+ T cells

An important function of antigen-specific CD8+ T cells is the production of cytokines which activate the immune system and have antiviral activity. INFγ and TNFα are two of the most important cytokines produced by CD8+ T cells. Also, the production of cytokines is one feature to get lost during functional exhaustion of T cells. Thus, the effect of 2B4- and CD48- signalling on cytokine production of CD8+ T cells was investigated.

Total PBMCs of healthy individuals were stimulated *in vitro* using anti-CD3/28 in the presence of anti-2B4 and anti-CD48 antibody or both. IFNγ production of total CD8+ T cells showed no change upon treatment with neither anti-2B4 nor anti-CD48 (Figure 37A). As well, no change could be observed for the production of TNFα (Figure 37B).

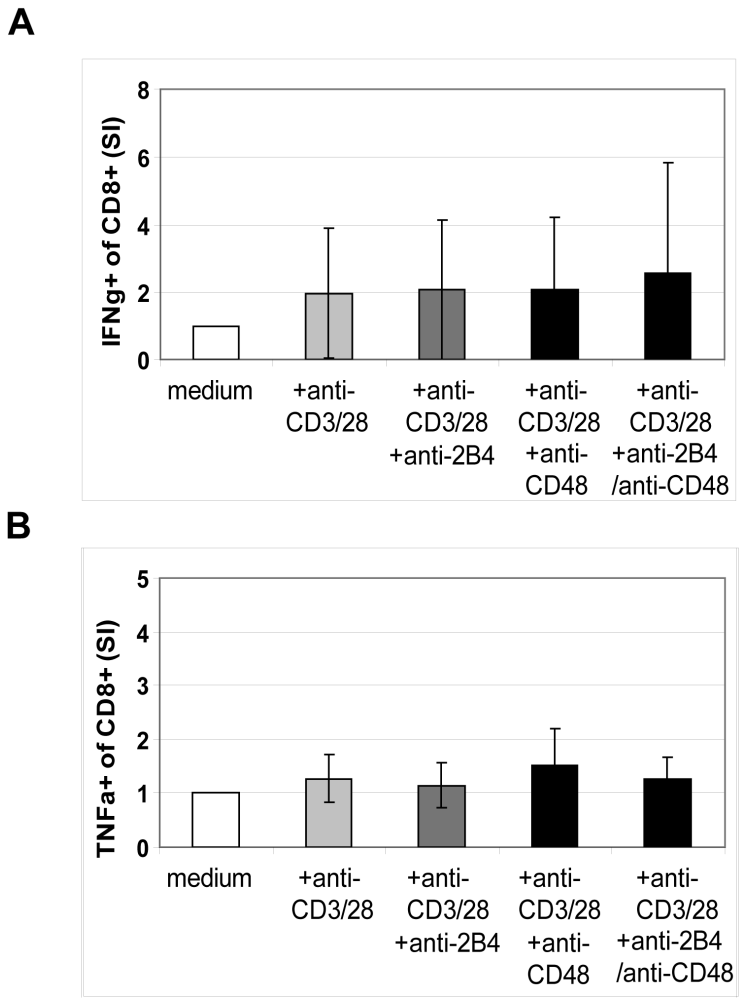



**Figure 37: IFNγ and TNFα production of CD8+ T cells during treatment with anti-2B4 and anti-CD48 in healthy individuals.** Total PBMCs from healthy individuals were stimulated in vitro with anti-CD3/28 in the presence of anti-2B4 and anti-CD48. (**A**) No alteration regarding production of IFNγ or (**B**) TNF could be seen. Figures represent mean stimulation indices of 12 independent experiments.

Treatment of T cells with anti-CD3/28 is mimicking TCR stimulus and it seems that 2B4 and CD48 signalling do not influence cytokine production of CD8+ T cells in this setting. Still, as CD8+ T cell act in an antigen-dependent manner, the effect of 2B4 and CD48- signalling on the cytokine production during antigen-specific stimulation was investigated. Here, the results are similar as no change in the production of IFNγ and TNFα by antigen-specific CD8+ T cells could be observed. Anti- 2B4 or anti-CD48 antibodies were not able to influence cytokine production of antigen-specific CD8+ T cells, neither without antigenic stimulus (Figure 38A) nor upon stimulation with peptides (Figure 38B).

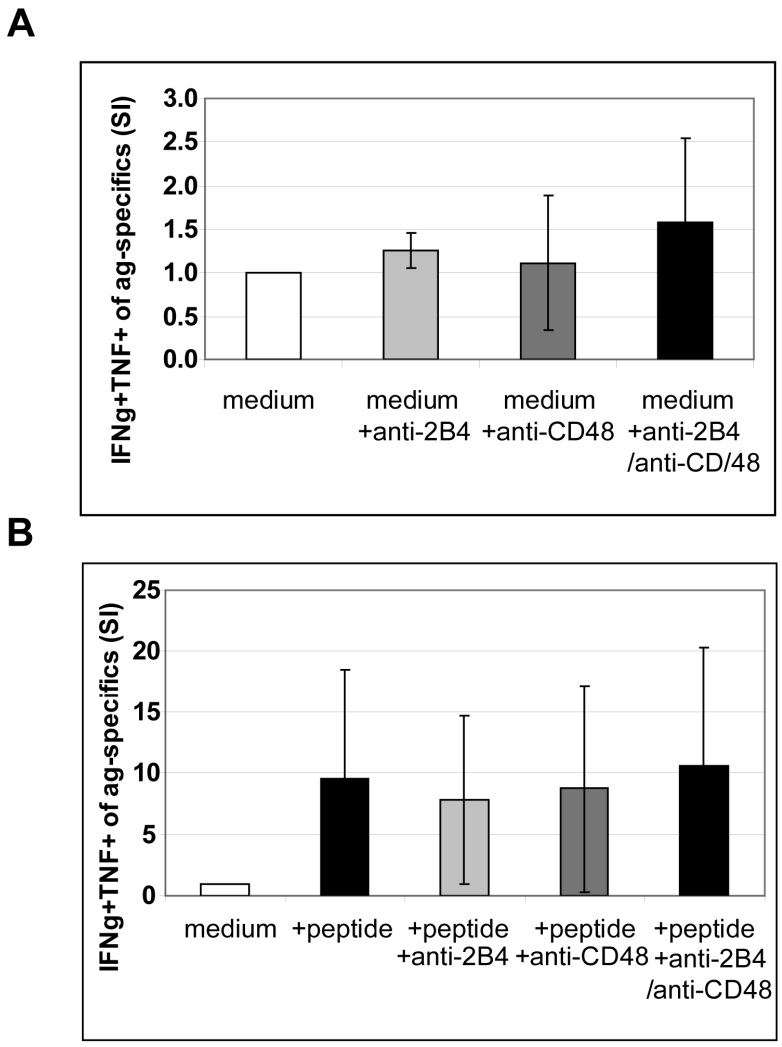



**Figure 38: Cytokine production of antigen-specific CD8+ T cells in presence of anti-2B4 or anti-CD48.** PBMCs from healthy individuals were stimulated ex vivo with antigenic peptides together with anti-2B4, anti-CD48 or both. (**A**)The monoclonal antibodies were not able to induce any change of IFNγ and TNF production by antigen-specific CD8+ T cells in the absence of peptides or (**B)** during antigen-specific stimulation. Antigen-specificities used here included CMV-pp65 (n=2) and Influenza-A (IV) (n=4).

### 3.3.8 Role of 2B4 and CD48 for proliferation of CD8+ T cells

In order to analyse the influence of 2B4 and CD48 ligation on the proliferation of CD8+ T cells, PBMCs from healthy individuals were treated *in vitro* with anti-2B4, anti-CD48 or both, respectively. Additional stimulation was mediated through antigenic peptides in the culture. Upon stimulation with peptides derived from different viruses, human PBMCs are expanding *in vitro*. In order to analyse a possible effect of 2B4 stimulation, anti-2B4, anti-CD48 or both antibodies were added to the cell culture. Proliferation of CD8+ T cells was examined in two ways. One was done by staining with the respective Pentameric Complexes and analysing the percentages of antigen-

specific CD8+ T cells after *in vitro* stimulation. Another method was done by analysing the total CD8+ T cell proliferation by CFSE-dilution after culture.

The presence of antigenic peptides led to a pronounced enumeration of antigen-specific CD8+ T cells after 7 days, regardless of the specificity examined (e.g. CMV, EBV or Flu). The effect of addition of anti-2B4 antibody to the cell culture, however, had various consequences depending on the PBMC sample and the antigen-specificity of the cells examined. Again, while there was an enhancing effect of anti-2B4 addition to be seen in some individuals, no alteration in terms of amplification of antigen-specific CD8+ T cells could be seen in others. On average, additional 2B4-stimulation had no beneficial effect on proliferation on CMV-pp65 specific CD8+ T cells in all individuals tested (see Figure 39A). Increase of proliferation of EBV-BMLF1-specific cells was observed (Figure 39B), although this was lacking statistical significance. The influence of 2B4-stimulation was even stronger on Influenza-A (IV)-specific CD8+ T cells with a clear increase of mean proliferation (Figure 39C). Still, inter-individual differences have to be taken into account.

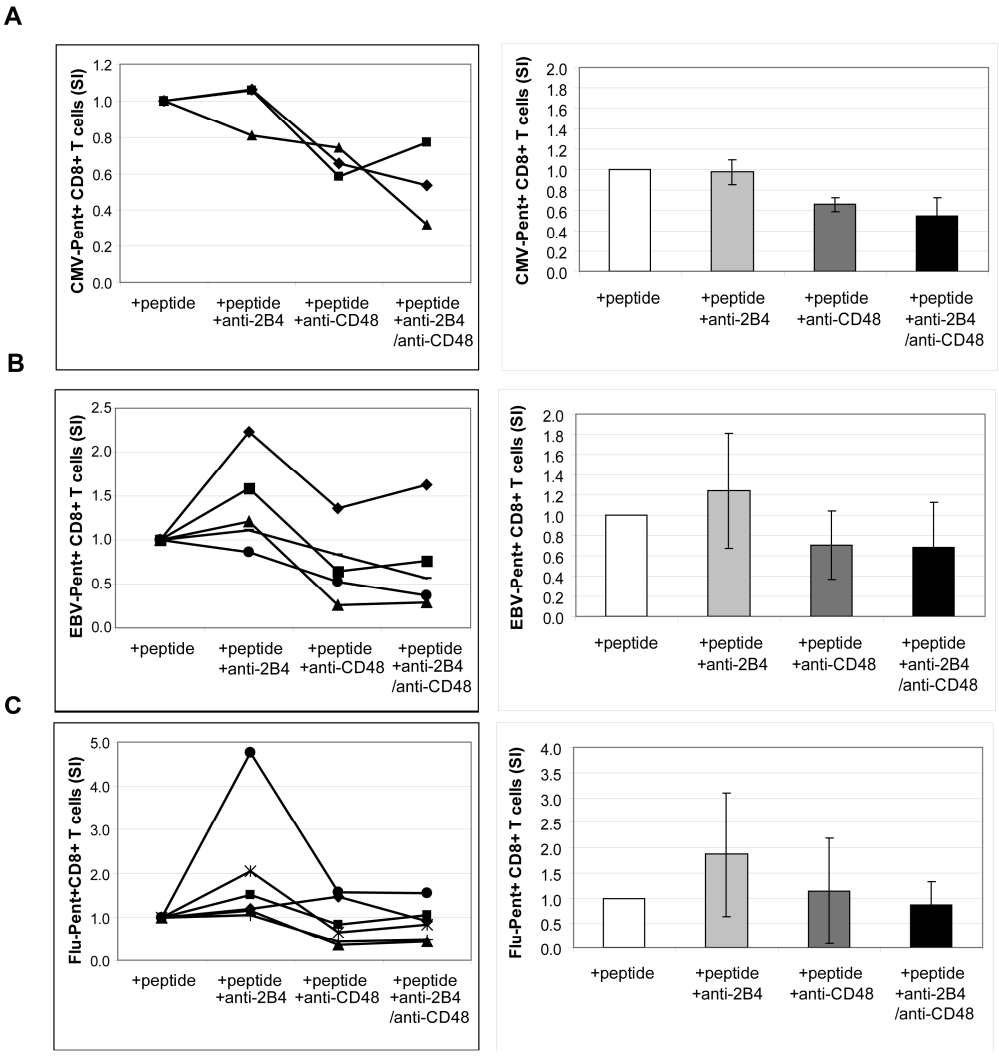



**Figure 39: Effect of anti-2B4 and anti-CD48 on the proliferation of antigen-specific CD8+ T cells in healthy.** The left graphs show representative individual expansion of antigen-specific CD8+ T cells, whereas the right graphs represent average numbers. Addition of anti-2B4, anti-CD48 or both in parallel to antigen-stimulation in vitro showed no effect on the proliferation on CMV-pp65-specific CD8+ T cells (**A**; n=5). In case of EBV-BMLF1-specific CD8+ T cells increased proliferation of antigen-specific cells could be seen in some individuals (B, left), which was also apparent in the average (**B**, right; n=12). The same observation also applied for Influenza-A (IV)-specific CD8+ T cells (**C**; n=13).

The blocking of CD48 ligation through monoclonal antibodies, however, showed an almost uniform effect of reducing the proliferation of CD8+ T cells during stimulation with anti-CD3/28 and with antigenic peptides (see Figure 38 and Figure 39). Furthermore, this influence was already present without additional stimulus (Medium +anti-CD48) and was also observed regarding the proliferation of total CD8+ T cells during antigen-stimulation. Here, proliferation of CD8+ cells was lower as compared to medium or peptide alone (Figure 40). Further, this negative effect on cell proliferation was not overcome by parallel addition of anti-2B4 antibody.

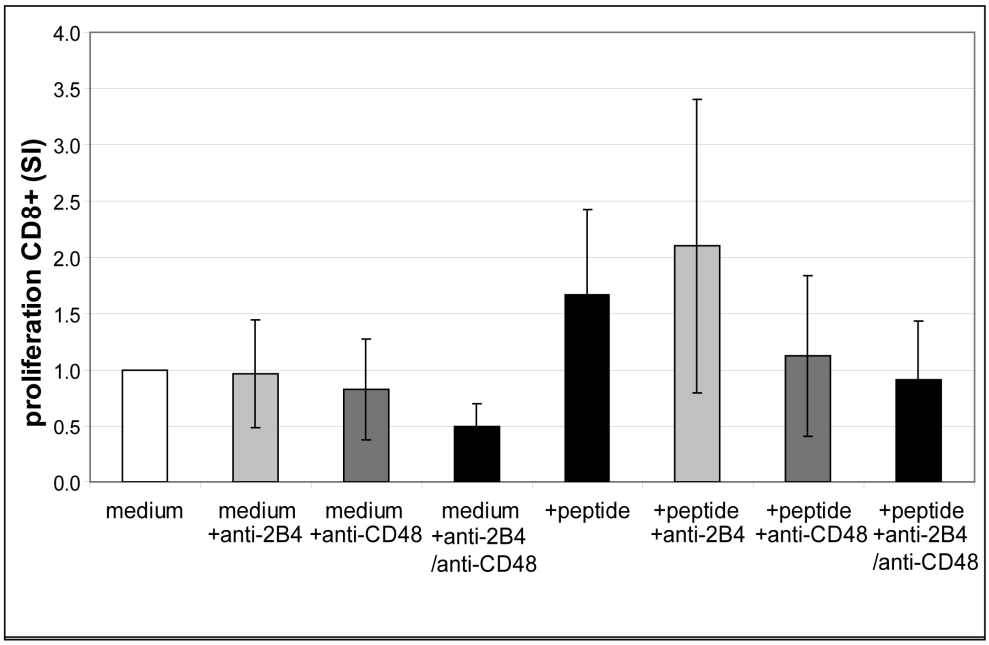



**Figure 40: Proliferation of CD8+ T cells during antigen-specific stimulation.** Proliferation of total CD8+ T cells during antigen-specific stimulation as well shows a slightly enhancing effect of anti-2B4 during cell culture. Further, the negative effect of anti-CD48 on cell proliferation can be seen even in the medium samples without the presence of peptides (n=9).

The reason for lower number of antigen-specific CD8+ T cells during antigen-stimulation in the presence of anti-CD48 seems not to be based on a decreased proliferation of antigen-specific CD8+ T cells *per se*. The percentages of proliferated antigen-specific cells (CFSE-low) did not change in all the samples analysed (Figure 41).

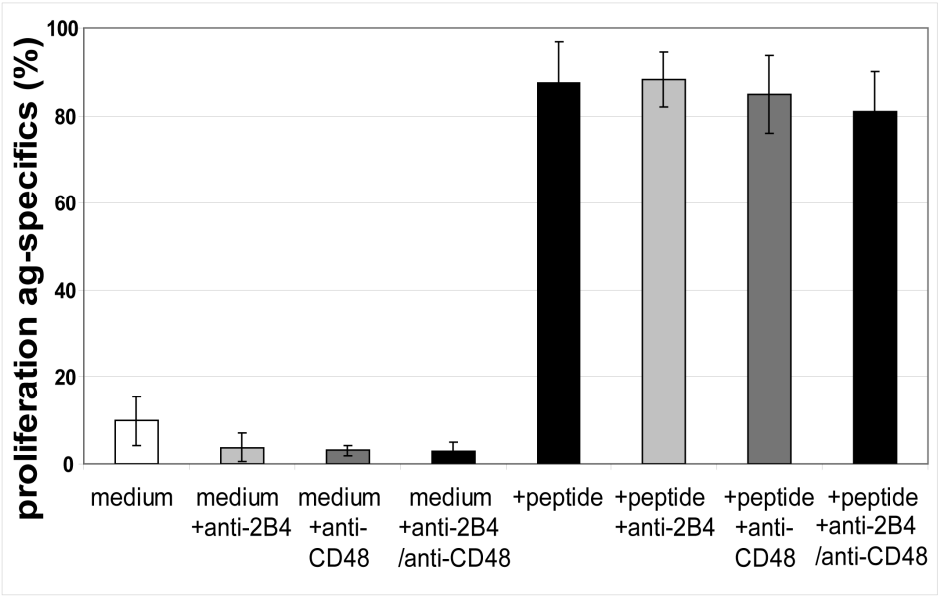



**Figure 41: Proliferation of antigen-specific cells during peptide-stimulation.** The proliferation of antigen-specific CD8+ T cells upon peptide-stimulation did not change upon addition of anti-2B4 or anti-CD48 antibody to the cell culture (n=6). Nearly all antigen-specific cells showed signs of cell division by the loss of CFSE during culture.

Interestingly, the influences of anti-2B4 or anti-CD48 antibody observed during antigen-specific stimulation did not apply for pre-activated antigen-specific CD8+ T cells. When anti-2B4 and anti-CD48 antibodies were added at day 4 after previous stimulation with antigenic-peptides in absence of these antibodies, no change in the expansion of cells occurred (Figure 42). Any effect of anti-2B4 could not be seen as well as the reducing effect of anti-CD48 observed before.

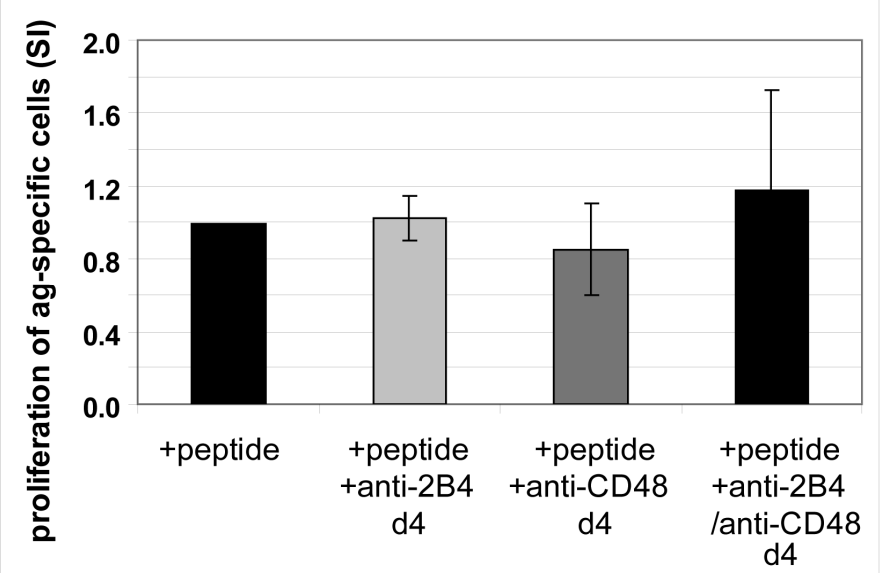



**Figure 42: Effect of addition anti-2B4 and anti-CD48 at day 4 after previous antigen-stimulation.** PBMCs from healthy HLA-A2 positive individuals were stimulated with peptides for four days in vitro before addition of anti-2B4 or anti-CD48. After incubation for more four days amplification of antigen-specific CD8+ T cells was determined by flow cytometry. The enhancing effect of anti-2B4 as seen before was absent in thin setting as well as the negative effect of anti-CD48 on cell expansion (n= 5).

In line with this finding is the observation that in parallel to peptide stimulation treatment of PBMCs from patients with acute HCV or HBV infection with anti-2B4 or anti-CD48 antibody *in vitro* was not able to influence the proliferation of antigen-specific CD8+ T cells in a positive or negative manner.

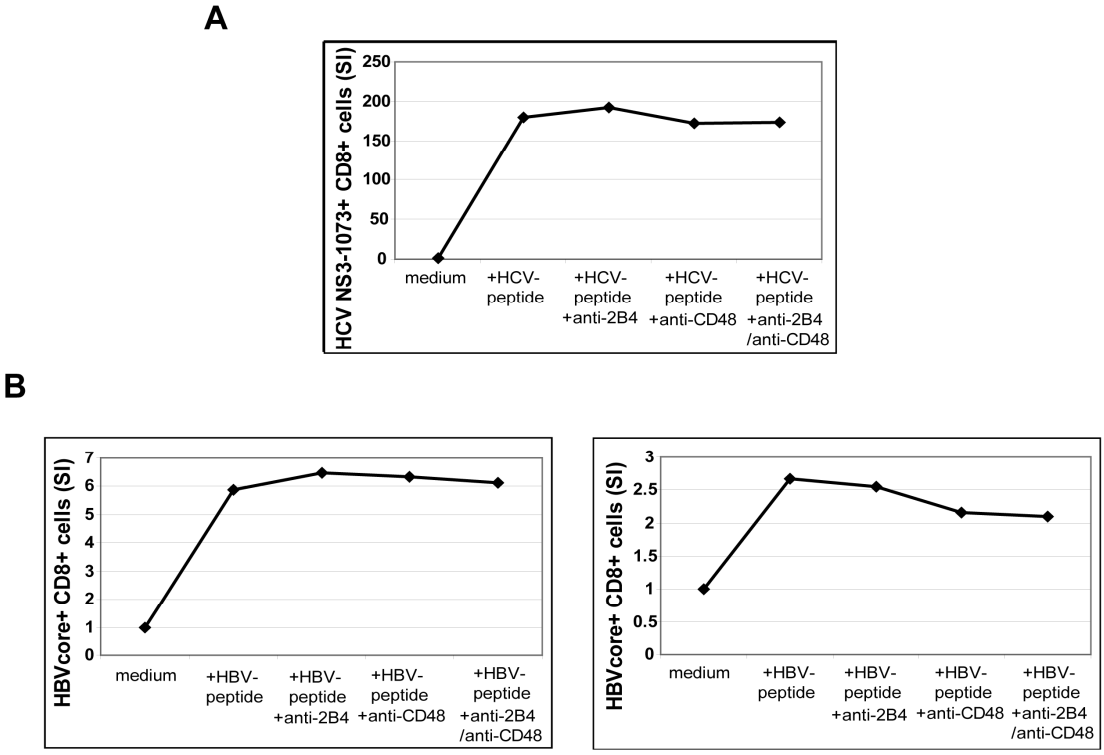


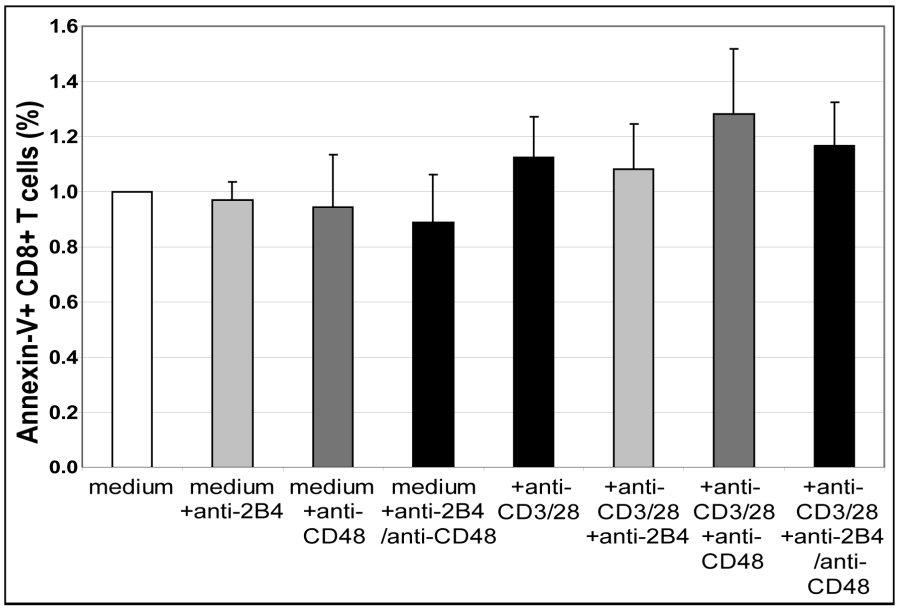

**Figure 43: Effect of anti-2B4 and anti-CD48 on expansion of antigen-specific CD8+ T cells in patients with acute hepatitis infection.** PBMCs from patients with acute HCV (**A**) or HBV infection (**B**) were stimulated in vitro with antigenic peptides in the presence of anti-2B4, anti-CD48 or both. No enhancing or decreasing effect of the monoclonal antibodies on the expansion of antigen-specific CD8+ T cells could be seen.

### 3.3.9 Role of 2B4 and CD48 in cell survival

Addition of anti-CD48 during general and antigen-specific stimulation *in vitro* led to a decreased expansion of total CD8+ and amplification of antigen-specific CD8+ T cells. Still, all antigen-specific CD8+ T cells were able to proliferate as measured by CFSE-dilution. Therefore, the question arose whether the lower number of expanded antigen-specific cells could be due to enhanced apoptosis.

Whole PBMCs from healthy individuals were stimulated for 24 hours with anti-CD3/28 and together with anti-2B4 or anti-CD48 antibody. Subsequently, the Annexin-V expression of CD8+ T cells was analysed to determine the rate of apoptosis occurring under the given circumstances. Here, percentages of Annexin-V+ CD8+ T cells was elevated already in the presence of anti-CD3/28, but no further increase of Annexin-V+ CD8+ T cells could be observed when adding anti-2B4 or anti-CD48 or both (Figure 44A). However, the amount of dead cells, as measured according to the changes in size and granularity, showed an elevation in the presence of anti-CD48 during cell culture (Figure 44B).

**A**




**B**

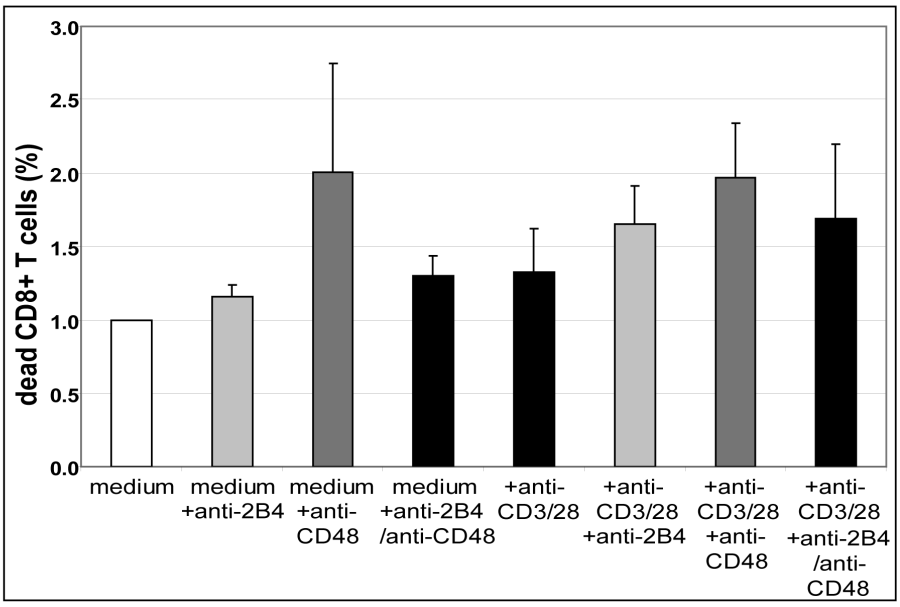



**Figure 44: Influence of anti-2B4 and anti-CD48 on cell survival in vitro.** PBMCs from healthy individuals were stimulated in vitro in the presence of anti-2B4, anti-CD48 or both. (**A**) After 24 hours a marginal increase in Annexin-V content of CD8+ T cells could be observed. (**B**) Also a slight rise in dead CD8+ T cells analysed by changes in size and granularity of the cells could be seen in the presence of anti-CD48 in cell culture; (n=7)

To further clarify the role of 2B4 on cell survival of CD8+ T cells per se and determine a possible negative influence of the anti-CD48 antibody itself, PBMCs from healthy individuals were sorted into 2B4+CD8+ and 2B4-CD8+ T cells. Obtained purified cells were cultured for 6 days *in vitro* in the presence or absence of anti-CD48. The absolute count of remaining live cells showed a clear difference between 2B4- and 2B4+ CD8+ T cells *in vitro*. The total numbers of cells acquired after a fixed acquisition time was about three times higher in the 2B4-CD8+ group

(Figure 45A). As $2x10^5$ cells were seeded initially and the average amount of cells acquired in the 2B4- group was almost equal, it clearly shows that the difference in the cell number is not due to increased proliferation of the 2B4- cells but rather due to an enhanced cell death of the 2B4+ cells.

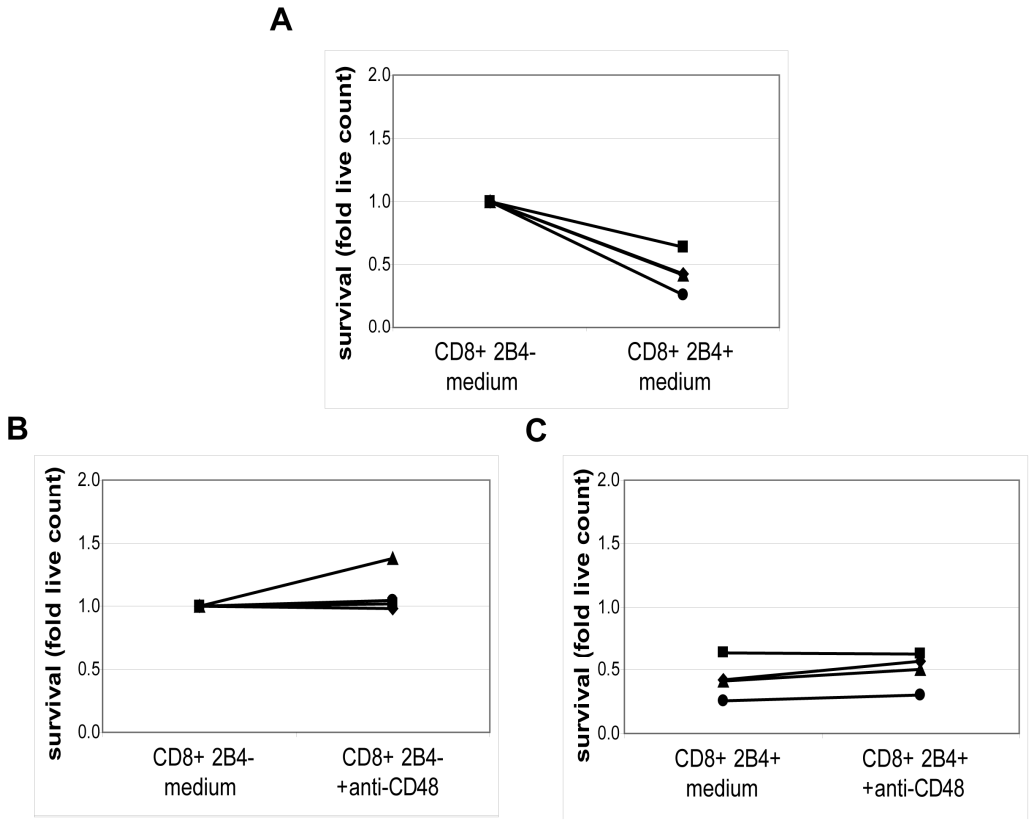



**Figure 45:** Survival of sorted 2B4- and 2B4+ CD8+ T cells in vitro. Purified CD8+ T cells from healthy individuals being either 2B4+ or 2B4- were cultured for 6 days in vitro. Subsequently, total cell numbers of live cells were determined by flow cytometry. (**A**) It showed that the survival of 2B4- cells was greatly enhanced as compared to (**B**) 2B4+ cells. (**C**) Further a possible toxic effect of the anti-CD48 antibody per se could be excluded as there was no difference in total live cell numbers when anti-CD48 antibody was added to 2B4- or 2B4+ CD8+ T cells.

Further, a negative effect on cell survival of the anti-CD48 antibody itself can be excluded, as there is no difference to be seen between the samples with and samples without addition of anti-CD48 to the cell culture (Figure 45B and C).

### 3.3.10 Expression of 2B4 in viral hepatitis infection and association with clinical parameters

Some viral infections can develop a persistency and this is often associated with functional exhaustion of T cells (see 1.3.4). Besides investigating the function of 2B4 and CD48 on CD8+ T cells, it was also of interest to elucidate a possible involvement of these molecules in T cell

exhaustion. Hepatitis C Virus infection takes a chronic course in most of the individuals infected. *Ex vivo* expression of 2B4 on the different leukocyte subsets was performed using PBMCs from chronic HCV and HBV infected patients.

During chronic HCV infection, the expression of 2B4 on leukocytes populations examined showed the same pattern as in healthy individuals. Likewise, a subpopulation of CD8+ T cells showed expression of the molecule on their cell surface, whereas CD4+ T cells did not (compare Figure 46A and B). The majority of CD56+ CD3+ NK-like T cells showed 2B4 expression (Figure 46C) and also CD56+ CD3- NK cells were uniformly positive for 2B4 with the expression intensity being lower on $CD56^{bright}$ cells (Figure 46D). While 2B4 expression was found on monocytes, it was absent on B cells (Figure 46E and F). Mean expression levels of CD48 did not show any difference in any of the patient cohorts, still some patients did show increased numbers of CD48- cells on an individual level (data not shown).

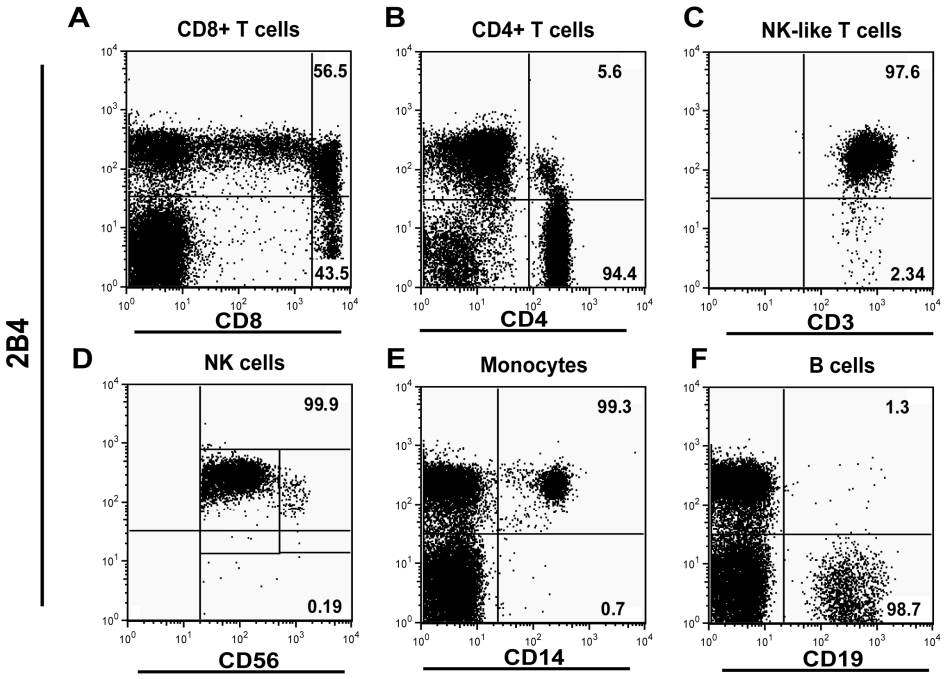



**Figure 46: Expression of 2B4 on leukocyte subsets in chronic HCV infection.** PBMCs from chronic HCV patients were stained ex vivo for expression of 2B4 on various leukocyte subsets. (**A**) While CD8+ T cells do express 2B4, (**B**) it is almost not present on CD4+ T cells. (**C**) NK-like T cells display high expression of 2B4 (**D**) as also do NK cells. (**E**) Similar to healthy individuals, Monocytes are positive for 2B4, but (**F**) B cells lack expression of the molecule. Stainings of one chronic HCV infected patient is shown as representative.

In comparison with PBMCs of healthy individuals it became obvious that in chronic HCV and HBV infected patients a greater proportion of CD8+ T cells were positive for 2B4 ($p=0.0038$ and $p=0.007$, respectively; see Figure 47). However, this was not only the case for chronic HCV and HBV infection, but also applied in case of autoimmune-induced hepatitis (AIH/PBC; $p=0.016$).

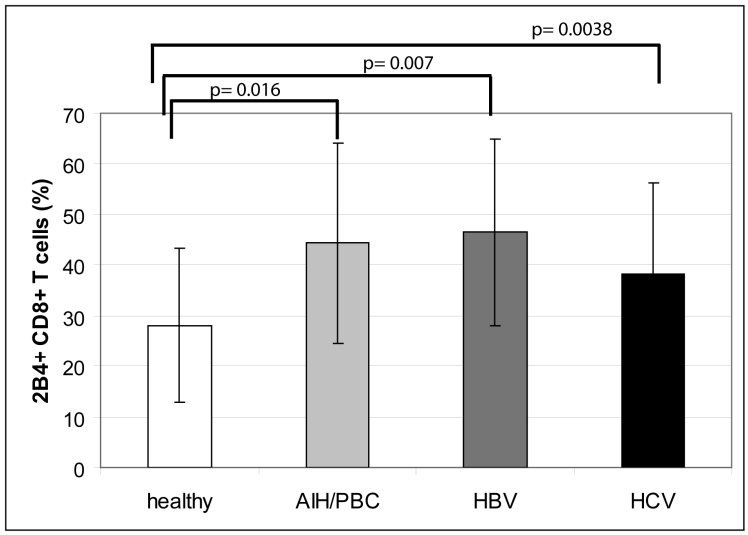



**Figure 47: Comparison of 2B4 expression on CD8+ T cells in healthy individuals and patients with liver disease.** PBMCs from healthy volunteers (n=20) displayed lower expression of 2B4 on CD8+ T cells ex vivo as compared to patients suffering from autoimmune-induced hepatitis (AIH/PBC; n=12), chronic hepatitis B (n=13) and chronic hepatitis C infection (n=35).

Though CD4+ T cells hardly expressed 2B4 on their cell surface, expression levels were found to be elevated in all patient groups analysed except acute viral hepatitis infection (Figure 48A) as compared to healthy individuals. On CD8+ T cells, however, expression of 2B4 was significantly increased in patients with acute virus infection (Figure 48B). While 2B4 expression on NK cells was not altered in any of the patient groups analysed (Figure 48D), 2B4 expression on NK-like T cells appeared to be decreased in patients with acute HBV or HCV infection. However, due to low sample number these differences were not significant (Figure 48C). 2B4 expression on monocytes showed no alteration in any of the patients analysed (Figure 48E). Regarding B cells, it appeared that 2B4 expression was increased in patients with chronic HCV infection (p=0.017) and acute virus infection (p=0.003; Figure 48F). Interestingly, this was not the case for patients with chronic HBV infection.

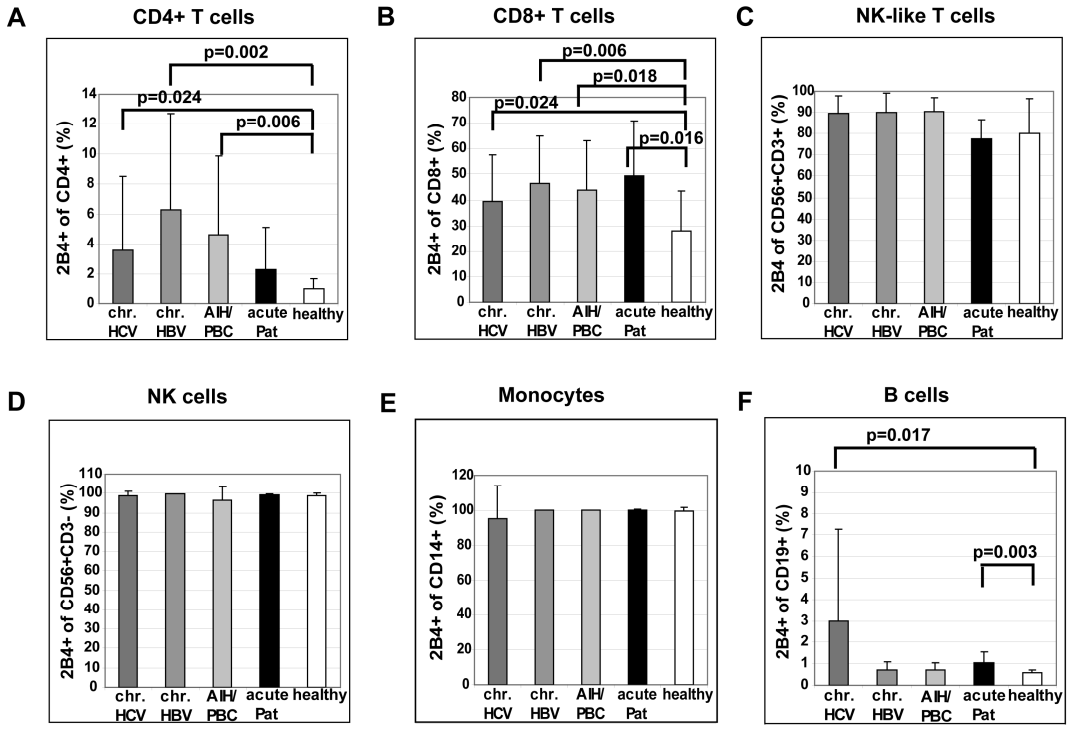



**Figure 48: Comparison of 2B4 expression on various leukocyte subsets in healthy individuals and patients with hepatitis.** PBMCs form healthy individuals (n=20) as well as from patients suffering from chronic HCV (n=42), chronic HBV infection (n=12), autoimmune-induced hepatitis (n=13) or acute HCV or HBV infection (n=6) were analysed ex vivo for expression of 2B4 on various leukocyte subsets. (**A**) Significant increase of 2B4 expression as compared to healthy individuals was found on CD4+ T cells and (**B**) CD8+ T cells in almost all patient groups analysed. (**C**) NK-like T cells however showed a trend to lower expression in acutely infected patients, but lacks significance due to low sample number. (**D**) NK cells however did not display an altered expression of 2B4 in either of the groups analysed.(**E**) The expression of 2B4 on monocytes was not altered in any of the patient groups studied. (**F**) B cells however, again showed a tendency towards increase of 2B4 expression in chronic HCV infected patients as well as acute hepatitis virus infected patients.

As we showed before that antigen-specific CD8+ T cells upregulate 2B4 upon peptide stimulation in vitro, the question arose whether this also applies *in vivo*. Therefore, PBMCs from HLA-A2+ patients with acute HBV and HCV infection were analysed *ex vivo* for 2B4 expression on HCV- and HBV-specific cells. Similar to PD-1, almost all HCV-specific CD8+ T cells (Figure 49A) as well almost all as HBV-specific CD8+ T cells (Figure 49B) expressed high levels of 2B4 during the acute phase of infection.

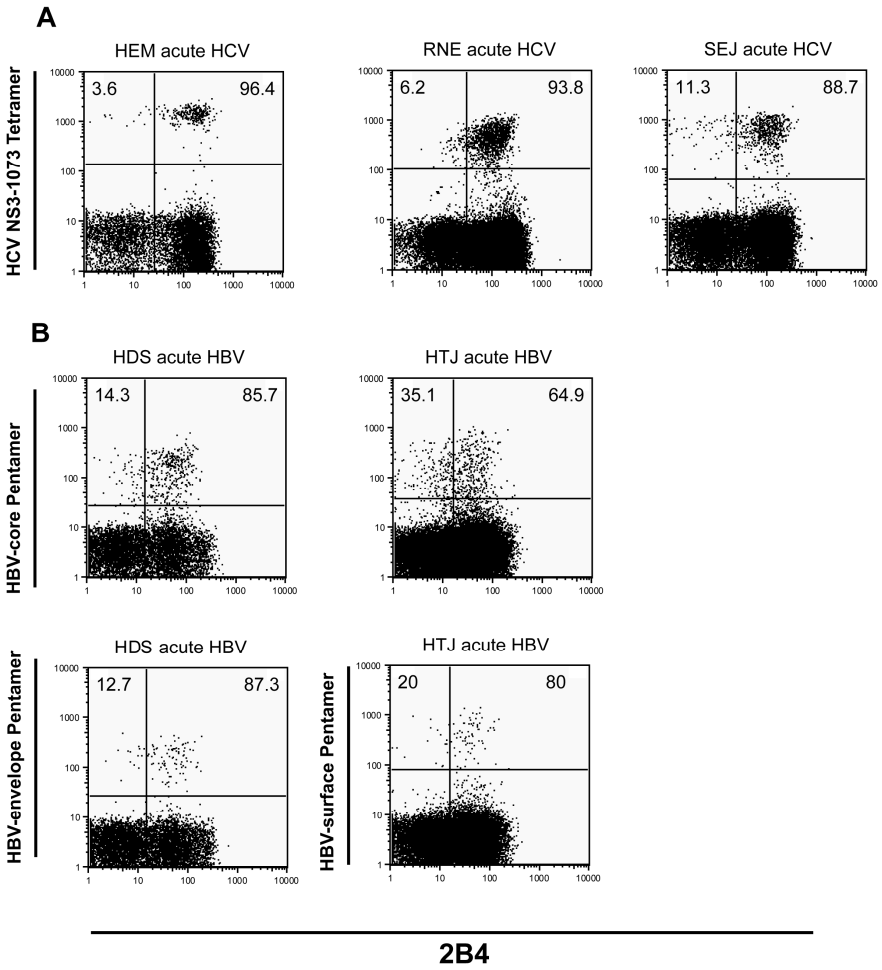



**Figure 49: 2B4expression on antigen-specific CD8+ T cells during acute HCV and HBV infection.** PBMCs from patients with (**A**) acute HCV and(**B**) HBV infection displayed high ex vivo levels of 2B4 expression. Numbers indicated represent percentages of 2B4 expression gated on antigen-specific CD8+ T cells.

Therefore, we questioned, whether 2B4 expression levels on CD8+ T cells in chronic hepatitis patients showed any correlation with clinical parameters of liver disease. However, statistical analyses revealed that neither the numbers of thrombocytes, AST or ALT or total billirubin levels, nor serum viral load correlated in any means with the expression levels of 2B4 on CD8+ T cells (see Figure 50).

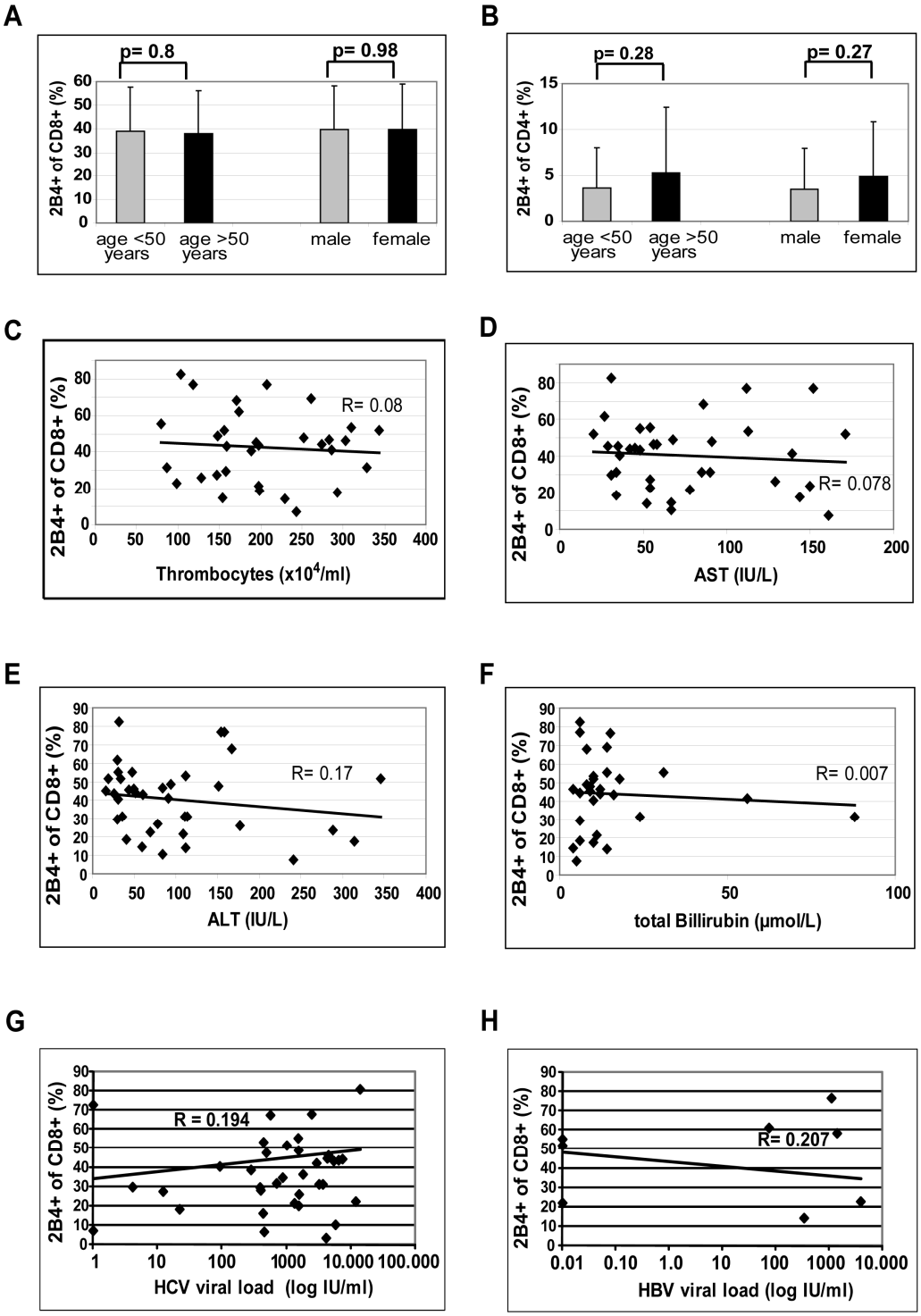



**Figure 50: Correlation of 2B4 expression on CD8+ T cells with clinical parameters of liver disease.** Non-clinical characteristics like (**A**) age and (**B**) gender were not correlated with the expression levels of 2B4 on CD8+ T cells. Clinical parameters of liver disease like (**C**) numbers of thrombocytes, (**D**) AST, (**E**) ALT or (**F**) total billirubin levels showed no correlation to the percentages of 2B4+ CD8+ T cells in patients suffering from liver disease (AIH/PBC, chronic HBV or chronic HCV infection). (**G**, **H**) Further, no association of serum viral load was seen in case of chronic HCV or chronic HBV infected patients.

## 3.4 Hierarchy and T cell receptor repertoire of HCV-specific CD8+ T cell responses during acute HCV infection

In the preceding investigations of this thesis we focused on phenotypic and functional characterization of virus-specific, in particular HCV-specific CD8+ T cells. These were important investigations in order to aim for the goal to develop an HCV vaccine. However, the question if all patients can potentially mount an epitope-specific response to given peptides is a crucial point for the success of a peptide based vaccine. The characteristics of such epitope-specific responses can vary between individuals regarding breadth and epitope hierarchy (Kim, SK et al. 2005; Lauer, GM et al. 2004). Furthermore, epitope specific responses can also lead to pathology (Welsh, RM and Fujinami, RS 2007).Thus, in order to develop a promising vaccine (such as IC41) we need to get further insights of epitope specific CD8+ T cell responses. Besides functional and phenotypic markers we need to evaluate such epitope hierarchies, the polyclonality of a given epitope response and their specific T cell receptor repertoires. We focus on the HLA-A2 restricted HCV NS3-1073 response because this is part of the IC41 vaccine and the most dominant HCV epitope. Factors like the immunodominance also have to be taken into account when developing such vaccines.

### 3.4.1 Hierarchy of epitopes recognized during acute HCV infection

Total PBMCs from four patients with acute symptomatic HCV infection were analysed *ex vivo* for the frequency of antigen-specific CD8+ T cells recognizing various HLA-A2-restricted HCV epitopes. The dominance of viral epitopes targeted during acute HCV infection varied between individuals and the frequency of CD8+ T cells specific for the dominant NS3-1073 epitope differed as well (Figure 51A). While for some patients it was the dominant epitope of all epitopes investigated here, while another patient showed a dominance of the HCV core epitope (Figure 51B). This variability in immune hierarchies was not restricted to HCV, as epitopes targeted by CD8+ T cells during acute HBV infection displayed a similar variability. Again, the frequency of CD8+ T cells recognising the prominent HBV core epitope differed greatly between the patients analysed (Figure 51C) with each patient showing his own individual pattern of epitope dominance (Figure 51D). Thus, variability of immune hierarchies seems to be a general phenomenon during immune responses which we may take into account if we consider peptide based vaccines.

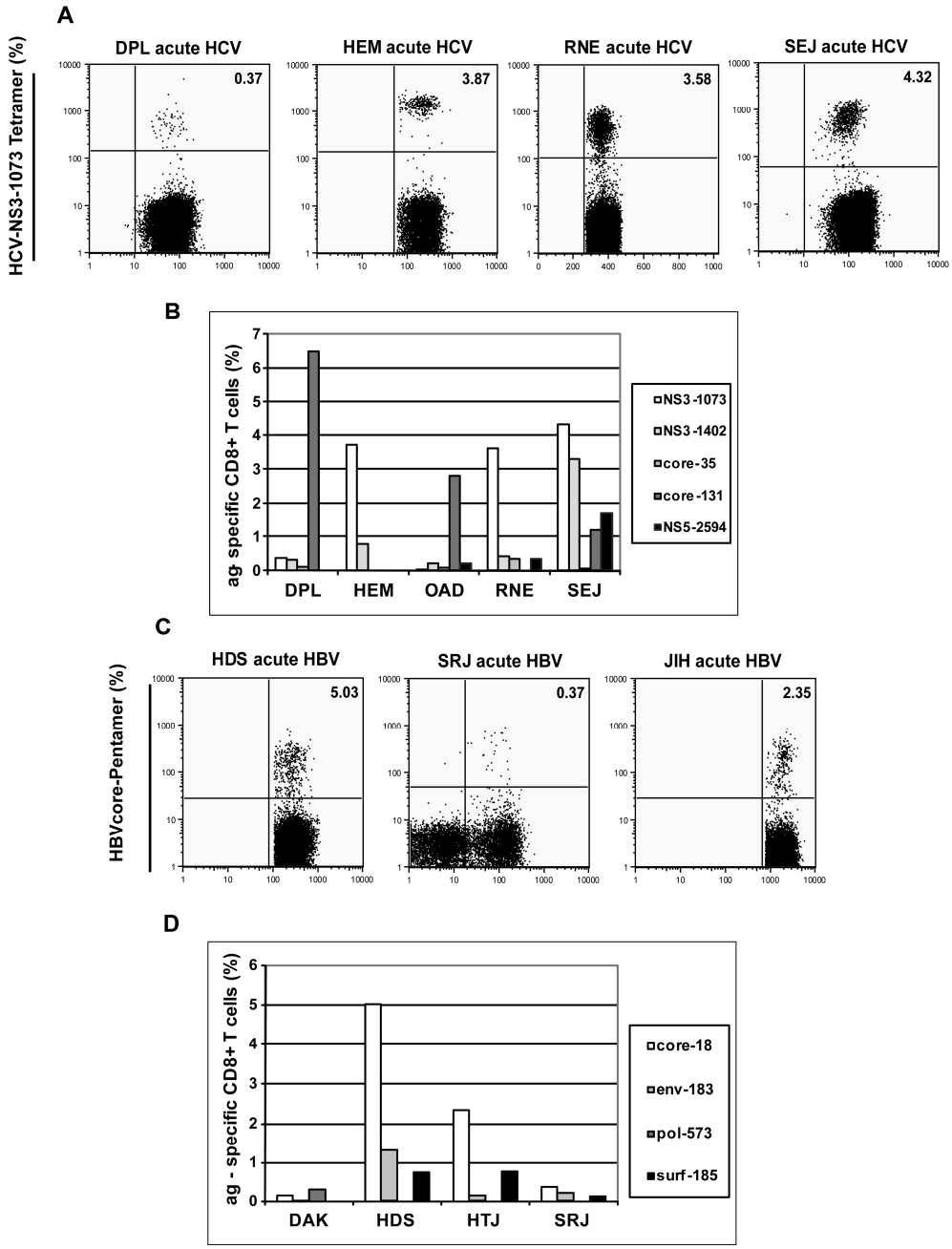



**Figure 51: Epitope hierarchy of virus-specific CD8+ T cells in patients with acute HCV of HBV infections.** PBMCs from patients with acute symptomatic HCV and HBV infection were analysed ex vivo for the frequency of CD8+ T cells targeting various known HLA-A2 restricted epitopes. (**A**) During acute HCV infection, the frequency of CD8+ T cell recognising the dominant NS3-1073 epitope differs greatly between different individuals and (**B**) each patient displays an individual hierarchy of epitopes targeted. (**C**) A similar observation can be made for patients with acute HBV infection. The dominant HBV core18-27 epitope is targeted differently in each patient and (**D**) each individual also displays an individual hierarchy of epitopes targeted.

### 3.4.2 TCR Vβ chain usage of CD8+ T cells recognizing the HCV NS3-1073 epitope

The Vβ chain usage of CD8+ T cells specific for the HCV NS3-1073 epitope was analysed in patients with acute HCV infection. In one case, the frequency of NS3-1073 specific CD8+ T cells was high enough (4.16% of total CD8+ T cells) to perform *ex vivo* sorting of these cells and Vβ chain usage was the determined through PCR. In this case, altogether nine different Vβ chains (Vβ6.2; Vβ5.6; Vβ4; Vβ13; Vβ22; Vβ7; Vβ2; Vβ3 and Vβ8) were found to be used by the NS3-1073 specific CD8+ T cells isolated (Figure 52).

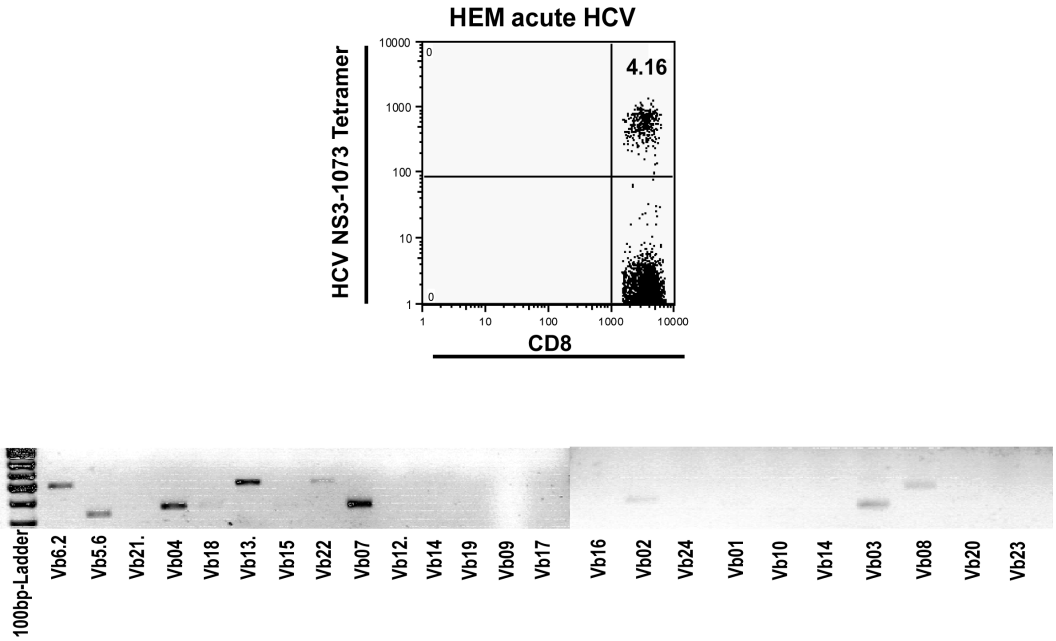



**Figure 52: Ex vivo TCR Vβ chain analysis of HCV NS3-1073 specific CD8+ T cells during acute HCV infection.** HCV NS3-1073 specific CD8+ T cells were sorted ex vivo from a patient with acute HCV infection. Total mRNA was isolated and cDNA generated. Vβ chain usage was then determined using PCR. Altogether nine different Vβ chains appeared to be positive.

In case of this particular acute HCV patient and three others, TCR Vβ usage of NS3-1073 specific CD8+ T cells was again determined *ex vivo* by staining with the respective Tetramer and using monoclonal antibodies identifying altogether 24 different Vβ chains. Interestingly, Vβ chain usage was different in all four patients analysed displaying different Vβ hierarchies within the NS3-1073 specific CD8+ T cell population (see Figure 53). Further, a short-term T cell culture stimulating with NS3-1073 peptide was set up from these patients. Expansion of NS3-1073-specific T cells was successful in all but one patient (DPL). After 7 days, antigen-specific CD8+ T cells were isolated by cell sorting and Vβ chain usage was then determined by PCR.

For the acute HCV infected patient HEM, results from the *ex vivo* Vβ analysis by PCR are comparable to the pattern seen using the antibody staining (see Figure 52). Importantly, the Vβ repertoire of HCV NS3-1073 specific CD8+ T cells displayed a similar Vβ usage after the stimulation *in vitro* as seen *ex vivo* (Figure 53). This in line with other data showing that repertoires of epitope specific CD8+ T cells after short term *in vitro* culture are similar to those found *ex vivo* (Naumov, YN et al. 2003).

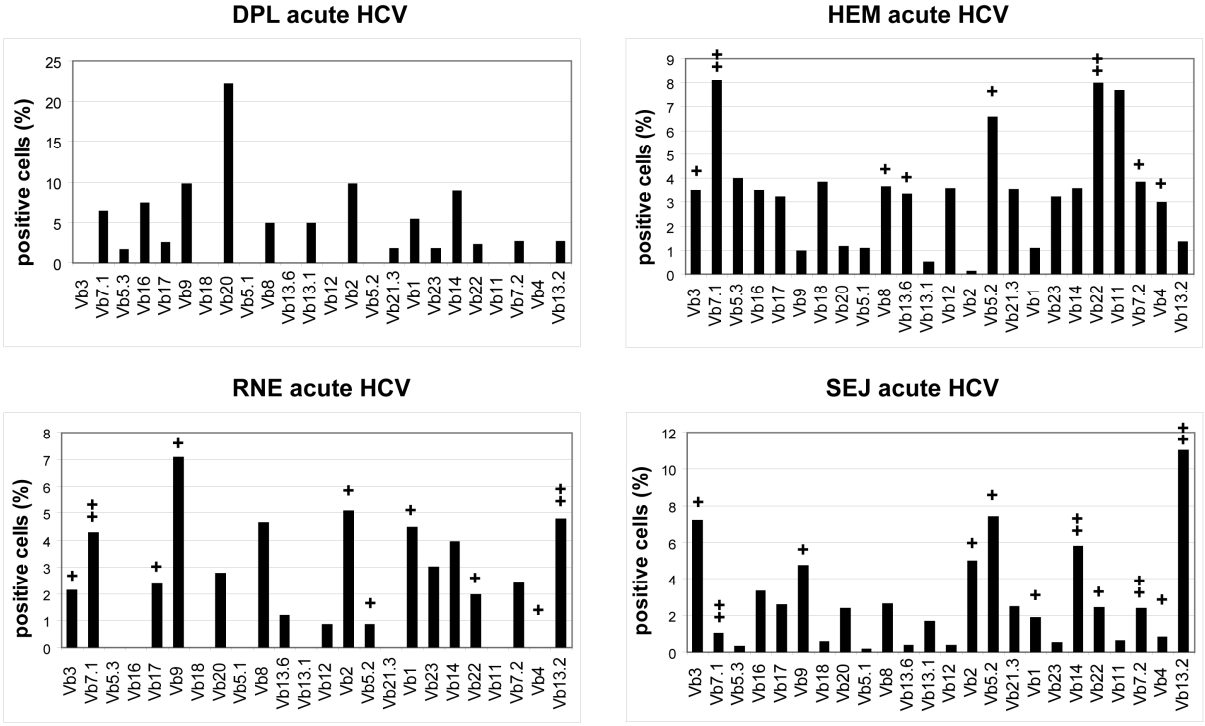



**Figure 53: Comparison of Vβ chain usage by HCV NS3-1073 specific CD8+ T cells ex vivo and at day 7 after antigen-specific T cells culture.** TCR Vβ chain usage of NS3-1073 specific CD8+ T cells was determined ex vivo in four patients with acute HCV infection using monoclonal antibodies (bars). Generation of NS3-1073 specific CD8+ T cell lines was successful for three patients (except patient DPL). After 7 days of culture antigen-specific cells were purified by cell sorting. Subsequently, Vβ chain usage was determined by PCR. Positive signals in the PCR for the each Vβ chain is indicated by crosses above the respective bars, one cross stands for a weakly to normal intense band, while two crosses indicate strong dominant bands.

In case of HEM (*ex vivo*) and SEJ (day 7) the most dominant Vβ chain was Vβ7. Therefore, the Vβ7 amplificate was chosen for subcloning and sequencing of the TCR. Sequence analysis of the CDR3 regions, which is important for peptide recognition, showed a narrowed TCR repertoire with one clone representing 50% of the total T cell receptor repertoire (see Figure 54). However, both individuals analyzed used different clonotypes for their HCV NS107 response. Nevertheless, the clonotypes showed some similarities indicating a motif for the peptide recognition as the sequence analysis revealed a similarity in the amino acids used at specific positions of the CDR3 (see Figure 54, underlined letters). In every clone analysed a Glycine was

found at the third or fourth position of the CDR3 region and many sequences revealed an additional Lysine as the first amino acid.

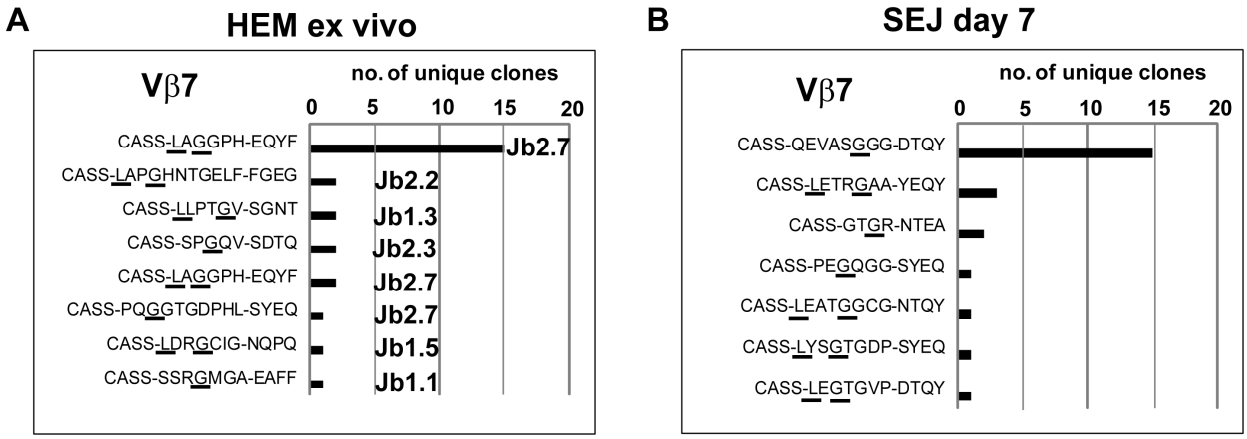



**Figure 54: Clonality and amino acid sequence of Vβ7-CDR3 regions from HCV NS3-1073 specific CD8+ T cells.** Vb7-specific PCR amplificates from sorted HCV NS3-1073 specific CD8+ T cells were ligated into the TOPO-pCR2 vector and transformed into TOP10 *E. coli* cells. Sequencing of plasmids carrying the inserts was performed and analysed for the amino acid sequence of the CDR3 region. The results show a narrowed TCR repertoire with one strongly dominating clone. Also, the samples showed common amino acids at position 1 and 3 or 4 of the CDR3 region (underlined letters). Further, the TCRs often displayed usage of the Jβ 2.3 or 2.7 chains as indicated.

# 4 Discussion

## 4.1 HCV Vaccine Study

An important issue in the field of Hepatology is the development of an effective preventive vaccine against hepatitis C. Inducing a HCV-specific immune response may, however, be effective for the treatment of patients with persistent HCV infection. So far, no vaccine showed significant therapeutic efficacy. Also, the trial with the therapeutic vaccine IC41 described in this thesis was not effective in reducing viral titers in a significant number of patients. However, the vaccine was able to induce HCV-specific immune responses. In order to advance the concept of therapeutic vaccines we need further insights in the quality of the induced immune response. The first issue we addressed was the phenotypic characterization of the T cell responses induced by the peptide vaccine.

Vaccination aims to induce a functional immune response capable of preventing an infection ("prophylactic vaccine") or clearing a pathogen ("therapeutic vaccination"). We had the unique chance to investigate the phenotype and function of HCV-specific CD8+ T cells in well characterized cohorts of individuals vaccinated with the novel candidate vaccine IC41. IC41 consists of three CD4+ as well as five CD8+ HCV epitopes and the adjuvant poly-L-Arginine (Firbas, C et al. 2006). IC41 has been tested in healthy volunteers and chronic HCV patients. So far, very few data on the phenotype and function of vaccine induced CD8+ T cells after vaccination in man are available (Amanna, IJ et al. 2006; Caccamo, N et al. 2006; Hueman, MT et al. 2007; Monsurro, V et al. 2002). We here show that the peptide vaccine IC41 induced HCV-specific IFN$\gamma$-producing T cells in healthy individuals, however, in the far majority of cases, most individuals with chronic HCV infection did not show functional HCV-specific CD8+ T cells before or after vaccination. Nevertheless, vaccination of patients with chronic hepatitis C was able to change phenotypic characteristics of HCV-specific CD8+ T cells.

The desired "optimal" phenotypes and function of antigen-specific CD8+ T cells may differ between therapeutic and prophylactic vaccination. A therapeutic vaccine should drive existing antigen-specific cells into maturation towards the effector ($T_E$) phenotype producing cytokines, exerting cytotoxicity and thereby help to eradicate the pathogen (van Leeuwen, EM et al. 2006). The goal of a prophylactic vaccine is the induction of antigen-specific T cells, which are capable of establishing a long lasting immunological memory. Several studies have investigated phenotypes and function of virus-specific CD8+ T cells in viral infections. In acute infection the phenotypes of antigen-specific T cells have been shown to be similar between different pathogens with dominant CCR7-negative effector T cells. (Appay, V et al. 2002; van Leeuwen,

EM et al. 2006). However, after resolving the acute infection or during chronic stages, phenotypes and function of virus-specific CD8+ T cells can differ dramatically among different viral infections (Powell, DJ, Jr. and Rosenberg, SA 2004). For example, in HIV infected individuals HIV-specific T cells display mainly a $T_{CM}$ phenotype (Appay, V et al. 2002; Chen, G et al. 2001; Papagno, L et al. 2002; van Leeuwen, EM et al. 2007). On the contrary, T cells specific for EBV or CMV have been described to be mainly of the $T_{EMRA}$ phenotype in persistent infection (Appay, V et al. 2002; Chen, G et al. 2001; Gamadia, LE et al. 2001; Hislop, AD et al. 2001; Lucas, M et al. 2004) and tuberculosis-specific T cells were shown to be $T_{CM}$ cells (Caccamo, N et al. 2006). We here confirm for HCV-specific CD8+ T cells from chronic HCV patients previous reports demonstrating rather heterogeneous phenotypes with different distributions of $T_{CM}$, $T_E$ and $T_{EMRA}$ cells. In particular HCV-specific $T_{EMRA}$ cells are functionally impaired cells (Gruener, NH et al. 2001; Lauer, GM et al. 2004; Lechner, F et al. 2000a; Wedemeyer, H et al. 2002).

Considering this context of heterogeneous phenotypes of antigen-specific CD8+ T cells in different phases of viral infections, we believe that our comprehensive phenotypic and functional analysis of HCV- and CMV- specific CD8+ T cells is of interest for the development of therapeutic and prophylactic vaccines. This is the very first study investigating vaccine-induced HCV-specific CD8+ T cells responses in more detail. In these analyses we found that (i) HCV-specific CD8+ T cells induced by peptides vaccination of healthy individuals are able to expand rapidly upon antigen exposure in vitro and that the induction of IFNγ producing effector memory T cells was dependent on the administration of the adjuvant Poly-L-Arginine, and that (ii) peptide vaccination with IC41 of chronic hepatitis C patients increased the frequency of HCV-specific CD8+ T cells in the peripheral blood with altered phenotypes in some but not all patients.

HCV-specific CD8+ T cells induced by the peptide vaccine IC41 showed two dominant populations of a $T_{CM}$ and $T_E$ phenotype in healthy individuals. Few studies have investigated phenotype and function of vaccine-induced CD8+ T cells in humans for other pathogens. Ag-specific CD8+ T cells induced after vaccinia vaccination displayed mainly an effector phenotype early after vaccination changing to a more $T_{CM}$ phenotype during further follow-up (Rock, MT et al. 2005). Similarly, vaccination of a healthy individual with an HIV-vaccine resulted in generation of antigen-specific CD8+ cells of a $T_{EMRA}$ and $T_{CM}$ memory phenotype (Betts, MR et al. 2005). In our study, the ability of tetramer-positive cells to produce IFNγ did depend on the dose of the adjuvant Poly-L-Arginine administered. However, we could not observe a clear correlation between the Poly-L-Arginine dose and different phenotypes of HCV-specific CD8+ T cells. Moreover, no follow-up samples were available in our phase I study and thus we could not investigate whether a similar evolution of HCV-specific T cells occurred as described for vaccinia-specific CD8+ T cells (Rock, MT et al. 2005).

While several studies on therapeutic vaccination have been performed in individuals with malignancies (Perambakam, S et al. 2006; Powell, DJ, Jr. and Rosenberg, SA 2004; Yagi, H et al. 2006), there is only very limited data on therapeutic vaccination in infectious diseases

(Hafalla, JC et al. 2006; Hammarlund, E et al. 2003; Rock, MT et al. 2005). Vaccination of mice against malaria for example was able to induce functional CD8+ T cells (Hafalla, JC et al. 2006). In humans detectable antigen-specific CD8+ T cells responses could be detected up to 75 years after vaccination against smallpox virus (Dunne, PJ et al. 2002). In the present study the peptide vaccination of chronic HCV patients with IC41 led to increased frequency of HCV-specific CD8+ T cells detectable in the peripheral blood. This was accompanied by a shift towards a CD45RA-negative stage for some but not all patients. Expression kinetics of CCR7 and CD45RA on T cells are not linear with maturation but eventually can vary in different phases of viral infections. It has been shown that expression of CCR7 can be regained by CD8+ T cells and therefore a conversion from a CCR7- $T_E$ stage to a CCR7+ $T_{CM}$ phenotype is possible (van Leeuwen, EM et al. 2005). Similarly, regaining CD45RA expression is possible for CD8+ but not for CD4+ T cells (Dunne, PJ et al. 2002; Wills, MR et al. 1999). In our study, vaccination led to a transient loss of CD45RA on HCV-specific CD8+ T cells. Carrasco et al suggested that the time elapsing until re-expression of CD45RA is dependent on the strength and time passed since last antigenic stimulus (Carrasco, J et al. 2006; Wherry, EJ et al. 2003b). This would imply that the stronger the specific stimulation by the vaccine is the higher would be the percentage of CD45RA loss. It is of notion that the phenotype found in chronic HCV patients during vaccination resembles that found in acutely infected HCV patients (Lauer, GM et al. 2002; Wiegand, J et al. 2007). Moreover, chronic hepatitis C patients in this study with high frequencies of HCV-specific CD8+ T cells at baseline – representing patients with putative high initial stimulus leading to a strong adaptive immune response (Rehermann, B and Nascimbeni, M 2005) displayed already at a double negative $T_E$ stage and consequently, no further phenotype change could be induced by vaccination.

Although the vaccine clearly induced a change in frequency and phenotype of HCV-specific CD8+ T cells, the capacity of CD8+ T cells to produce IFNγ in an antigen-specific manner did not increase in parallel in those patients. This would explain the lack of significant antiviral effect in these patients, since IFNγ production by CD8+ T cells is considered to be crucial to control viral replication. Thus our data did not reveal a clear association between IFNγ production and CD45RA expression in CCR7- CD8+ T cells implying that phenotyping alone with the markers CCR7 and CD45RA is not sufficient and direct investigation of cytokine will still be required in future clinical trials. Our data are also in line with other reports on human lymphocytes failing to show a correlation between the level of CD45RA expression and cytokine production by tumor-specific CD8+ T cells (Carrasco, J et al. 2006). Nevertheless, to see clinical effects on viral load, the vaccine will definitely need to be stronger and the induction of IFNγ producing CD8+ T cells will be required.

Several different adjuvants have been developed during the last three decades with different modes of action and efficacies. While many substances have been shown to act through activation of Toll-like receptors, this is not the case for the adjuvant used here. Poly-L-Arginine is

a cationic poly-amino acid, supporting the shuttling of extracellular antigen into the cytoplasm of APC (and hence into class I pathway of antigen processing) thus facilitating a faster transport of antigens applied subcutaneously to draining lymph nodes (Luhrs, P et al. 2002; Mattner, F et al. 2002). Future studies will have to investigate to what extent other adjuvants might induce similar changes in CD8+ phenotypes and induction of interferon gamma as described here for Poly-L-Arginine.

One additional finding of our study was that CMV-specific CD8+ T cells were not impaired in function or phenotype in chronic hepatitis C. There had been some debate whether HCV infection also influences the function of non-HCV-specific T cells. While tetanus-specific CD4+ T cell responses have frequently been described to be normal in HCV patients (Missale, G et al. 1996; Takaki, A et al. 2000), a recent report from the UK showed a difference in phenotype and impaired functions of CMV-specific CD8+ T cells in chronic hepatitis C patients as compared with non-infected individuals (Lucas, M et al. 2004). In contrast to that study we did not observe a major difference in phenotype or cytokine production of CMV-specific CD8+ T cells between healthy controls and chronic HCV patients. This data support the clinical observation that CMV infection does not take a different course in HCV-positive individuals. However, CMV-specific T cells clearly showed a different phenotype as compared to HCV-specific T cells both naturally induced as well as vaccine induced - highlighting the complexity of T cell maturation in response to different antigens and pathogens as previously described in more detail (Appay, V et al. 2002).

In conclusion, therapeutic vaccination may offer novel chances in the treatment of chronic hepatitis C as phenotype and function of HCV-specific T cells can be altered. An obstacle for a therapeutic vaccine however, is that it has to encounter an exhausted HCV-specific CD8+ T cell repertoire during a persistent virus infection (Gruener, NH et al. 2001; Wedemeyer, H et al. 2002). The peptide vaccine used here was too weak to overcome the functional exhaustion of HCV-specific CD8+ T cells. The importance of costimulatory molecules has become more apparent within the last years and many investigations are being made on this field. Still, only few things are known about the role and the mechanism of costimulatory molecules. Thus, our next aim was to characterize HCV-specific T cell responses accordingly. Due to recent publications describing the role of the costimulatory molecule PD-1 in functional exhaustion of virus-specific CD8+ T cells we analyzed the expression and function of PD-1 on virus-specific CD8+ T cells. Further, we extended our investigations to another costimulatory molecule 2B4 which was as well upregulated during functional exhaustion of CD8+ T cells in mice.

## 4.2 Role of costimulatory molecules during T cell exhaustion

Immune responses of CD8+ T cells are strictly regulated through various and diverse mechanisms. T cell receptor stimulus alone is not sufficient to activate a naïve T cell, instead if the appropriate Costimulatory signals are missing during antigen-specific stimulation, these T cells are thought to become anergic or undergo apoptosis (Jenkins, MK and Schwartz, RH 1987; Walker, LS and Abbas, AK 2002). By this the peripheral T cell tolerance towards auto-antigens is maintained. Also, an ongoing immune reaction must be kept under control and be terminated once the infection has subsided. In all these processes Costimulatory molecules are thought to play a crucial role. Even the antigen-experienced memory T cells seem to require certain Costimulatory signals to become reactivated, although to a far lesser extent than naïve T cells (London, CA et al. 2000).

Functional exhaustion of CD8+ T cells is a gradual process displaying a certain hierarchy in which effector functions are impaired. In early stages of T cell exhaustion functions like IL-2 production and cytotoxicity are already diminished, while TNF$\alpha$ and IFN$\gamma$ production and proliferative capacities become impaired at later stages (see 1.2.4). During several persistent viral infections it could be shown that functional exhaustion of virus-specific CD8+ T cells is taking place. In a mouse model of chronic LCMV infection T cells are functionally impaired, while this is not the case for the acute and resolving variant of LCMV infection (Zajac, AJ et al. 1998). Also, the same takes place during chronic infection in humans. During HIV as well as chronic HBV and HCV infection CD8+ T cells are functionally impaired and lack most of the main effector functions (Gruener, NH et al. 2001; Pantaleo, G and Koup, RA 2004; Rehermann, B and Nascimbeni, M 2005; Wedemeyer, H et al. 2002). While during acute HCV infection HCV-specific CD8+ T cells show vigorous and robust effector functions, a loss of those is associated with progressing to persistent infection (Cox, AL et al. 2005; Folgori, A et al. 2006b).

The regulation of CD8+ T cell activity through costimulatory molecules and especially the factors involved in establishing and maintenance of functional exhaustion are only poorly understood. Still, despite much research is done on the control of CD8+ T cell function, many questions remain unanswered. The major goal of this research is to analyse the impact of costimulatory molecules on T cell activity and their possible implication during functional exhaustion.

### 4.2.1 Role of PD-1 on CD8+ T cell function

One factor involved in the impairment of CD8+ T cell function has been identified in a mouse model using persistent LCMV infection. This study showed an upregulation of the inhibitory molecule PD-1 in functionally exhausted virus-specific CD8+ T cells by gene expression analysis in a gene array (Barber, DL et al. 2006). Further, blockade of the interaction of this Costimulatory molecule with its ligands by monoclonal antibodies led to an increased proliferation of the exhausted virus-specific CD8+ T cells and decrease of plasma viral load. Subsequently, the role of PD-1 during functional impairment of human CD8+ T cells was shown for HIV infection. Expression of PD-1 on CD8+ T cells correlated with the viral load and reduced functionality of those cells. In vitro blocking of PD-1:PDL interaction resulted in enhanced proliferation and cytokine production of HIV-specific CD8+ T cells (Day, CL et al. 2006; Trautmann, L et al. 2006).

Consequently, the question arose to the role of PD-1 in other persistent virus infections like HCV. Also, a general implication of PD-1 expression on CD8+ T cells and the consequences are of interest. Therefore, the expression levels and expression pattern of PD-1 on various leukocyte subsets was studied in healthy individuals and patients with different hepatic diseases. Consequence of blocking PD-1:PDL-1 interaction on function of virus-specific CD8+ T cells was analysed in healthy volunteers and patients with acute hepatitis infection.

Expression of PD-1 was found to be generally low in intensity as well as the amount of PD-1+ cells were few in healthy individuals. CD8+ as well as CD4+ T cells did express PD-1 but to an individually varying degree and with higher expression on CD8+ T cells. While PD-1 could be found in low amount on NK-like T cells and weakly on monocytes, it was completely absent on NK cells and B cells. Similar results for healthy individuals were recently described elsewhere (Golden-Mason, L et al. 2008).

The question arose to PD-1 expression levels on virus-specific CD8+ T cells in healthy individuals. As CMV, EBV and Influenza are common viral infections in humans, CD8+ T cells specific for these viruses can be found in many healthy persons. Studying PD-1 expression on these cells revealed differences regarding the antigen-specificity. While CMV- and also EBV-specific CD8+ T cells displayed high expression of PD-1 in most individuals, it was only found on the minority of Influenza-A (IV) specific CD8+ T cells. Again, these findings are in line with other publications showing that CD8+ T cells specific for CMV-pp65 and EBV-BMLF1 are positive for PD-1 (He, XH et al. 2008; Sauce, D et al. 2007; Trautmann, L et al. 2006) although also opposite findings were reported for CMV-pp65 (He, XH et al. 2008; Penna, A et al. 2007).

The amount of PD-1 expression on virus-specific CD8+ T cells in healthy individuals can be set into correlation with the course of the respective infections. Influenza-A Virus causes acute infections and is resolved in the vast majority of patients. Consequently, Influenza-A (IV) specific CD8+ T cells are memory cells not experiencing ongoing exposure to antigen and thus PD-1 expression on CD8+ T cells recognizing this virus is low. In the case of CMV and EBV, however,

persistent infections can develop in humans and induce other kinds of diseases in some patients (Koch, S et al. 2007; Maia, DM and Peace-Brewer, AL 2000). Consequently, CD8+ T cells specific for these viruses show elevated expression of PD-1 in some but not all patients. This finding could be based on different reasons. On the one hand it is possible that the T cells are continually stimulated by their specific antigen which is still presented by APCs due to the latent infection. On the other hand it is not yet fully known if antigen-specific T cells can lose the expression of PD-1 once it is gained. However, the former might be the case as also antigen-specific memory CD8+ T cells of either specificity analysed here can be found that are negative for PD-1 (e.g. Influenza-A (IV) specifics).

It has been shown before, that PD-1 expression is induced on lymphocytes upon stimulation of cells (Agata, Y et al. 1996; Nishimura, H and Honjo, T 2001). Similarly, an increase of PD-1 expression could be seen in our settings on total CD8+ T cells after unspecific stimulation with IL-2 to a marginal extent and strongly upon anti-CD3/28 stimulation or through SEB. As well, other reports show upregulation of PD-1 through INF$\alpha$ or PMA (Agata, Y et al. 1996)0. Likewise, we could show a significant increase of PD-1 after peptide stimulation in vitro on the respective antigen-specific CD8+ T cells.

In this context it is not surprising to find that HCV- and HBV-specific CD8+ T cells show high expression of PD-1 in patients with acute infection. Because of viremia and effective antigen presentation by APCs, T cells were strongly activated through stimulation with viral antigens. Also, PD-1 expression on total CD8+ and CD4+ T cells was significantly upregulated. As well, an increase of PD-1 on NK-like T cells as observed. In case of monocytes and B cells no distinct change of PD-1 expression was seen. The reason for the elevated PD1-1 expression on T cells and NK-like T cells might be due to the general inflammatory reaction taking place and the massive secretion of cytokines or due to the abundance of antigen presentation. Other reports show as well a high expression of PD-1 on T cells during the early phase of acute hepatitis virus infection irrespective of the outcome of infection (Kasprowicz, V et al. 2008). However, it seems that maintenance of PD-1 expression on antigen-specific CD8+ T cells is associated with a chronic progression of infection (Urbani, S et al. 2006). It is often believed that the presence of PD-1 on these HCV-specific CD8+ T cells is the reason for a chronic course of infection. It remains unclear, however, if it is not merely a marker of chronicity and maintained because of constant antigen-specific stimulation of the specific CD8+ T cells. Likewise, in a HCV infection model using chimpanzees, HCV-specific CD8+ memory T cells showed normal functional capacity despite expression of PD-1 (Bowen, DG et al. 2008). It is possible that PD-1 represents a mechanism in the immune system to control and abrogate ongoing immune response to prevent an overwhelming and possibly destructive immune reaction causing immunopathology. Indeed, PD-1 knock-out mice suffer from severe forms of autoimmune diseases (Nishimura, H and Honjo, T 2001; Nishimura, H et al. 1999). Moreover, it could be shown in LCMV infection of

PDL-1 knockout mice that the lacking of inhibitory and regulative PD-1 signalling led to an increased immunopathology (Barber, DL et al. 2006).

During chronic HCV and HBV infection we could find no significant increase of PD-1 expression on total CD8+ and CD4+ T cells. This finding is in contrast to other reports showing a significant increase of PD-1 on T cells during chronic HCV infection on antigen-specific as well as on non-HCV specific CD8+ T cells (Golden-Mason, L et al. 2007; Yao, ZQ et al. 2007) where also a correlation with treatment outcome could be observed (Golden-Mason, L et al. 2008). Still, other reports were not able to show a marked increase of PD-1 on total CD8+ T cells during chronic HCV infection (Penna, A et al. 2007) and also describing a reduction of PD-1 expression despite persistent infection (Urbani, S et al. 2006). In an HCV infection model of chimpanzees it was recently shown that PD-1 expression on memory CD8+ T cells of animals with resolved infection varied between different animals (Bowen, DG et al. 2008). The degree of PD-1 expression on T cells found in this study was also highly variable between different individuals. In contrast to HIV infection, where a clear association between PD-1 expression and plasma viral load was reported, no correlation of PD-1 expression levels on CD8+ T cells or CD4+ T cells with clinical marker of liver disease or with viral load could be observed in our study. Still, opposite to HIV infection, no general immune dysfunction exists during chronic HCV infection and non-HCV specific T cell responses were described to be normal in patients with chronic HCV infection. However, a link between PD-1 expression on HCV-specific CD8+ T cells and plasma viral load was reported (Penna, A et al. 2007), which is to be expected regarding the finding that antigen-specific stimulation leads to upregulation of PD-1 on the respective CD8+ T cell. In the present study, HCV-specific CD8+ T cells in patients with persistent infection was not carried out because of very low numbers of HCV-specific CD8+ T cells that can be found. Another discrepancy in expression levels of PD-1 was found concerning NK cells. While a recent report shows a significant increase of PD-1 on NK cells during chronic infection which is correlated to treatment response (Golden-Mason, L et al. 2008), no expression of PD-1 on NK cells could be seen in our study. Neither in healthy individuals, nor in patients with chronic HBV or HVC infection, autoimmune hepatitis patients nor during acute HBV or HCV infection could an expression of PD-1 on NK cells be observed. The differences in the expression levels of PD-1 on T cells during chronic HCV infection seen in the various studies might be due to a difference in the patient cohorts or based on differences in experimental procedures. However, further investigations are needed to clarify this issue.

Many studies showed an impact of PD-1:PDL blockade on the functionality of virus-specific CD8+ T cells during chronic infection. In healthy individuals treatment with anti-PDL1 during antigen-specific stimulation in vitro led only to an enhanced proliferation of CD8+ T cells in some but not all individuals tested. This, of course, is to be expected as T cells are in no exhausted state in healthy persons. Still, in few cases an increase of amplification of antigen-specific CD8+ T cells was observed, whereas in others even a decrease of proliferation was the case. The enhancement or decline of antigen-specific CD8+ T cell proliferation could not be set into

correlation with the *ex vivo* expression levels of PD-1 on the respective cells (data not shown). The mechanisms and reasons behind these controversial findings are not easily elucidated. In those cases where an increase of proliferation occurs, it is of course possible that these cells are in some kind of “exhaustion” or rather immune control. As for the decline of proliferation, several reasons might be possible. First, different effects of PDL absence on immune function have been described. On the one hand PD-1 is described as an inhibitory receptor and blocking PD-1:PDL interaction led to enhanced immune responses. Like the reports concerning the role of PD-1 in HIV or HCV infection (Day, CL et al. 2006; Penna, A et al. 2007; Trautmann, L et al. 2006), similar findings have been made in mice (Latchman, Y et al. 2001; Maier, H et al. 2007). However, initial descriptions of the ligands PDL-1 and PDL-2 described them as signal transducers and stimulatory molecules and the lack of expression in mice led to decreased cytokine production and proliferation (Dong, H et al. 1999; Shin, T et al. 2005). Further, is it possible that other receptors than PD-1 for PDL-1 exist (Dong, H et al. 2002). Another aspect to be considered regarding the consequences of PD-1 signalling is that the inhibition of immune cell function for example can be overcome by IL-2 (Carter, L et al. 2002). Also, it must be considered that virus-specific CD8+ T cells are fully functional during acute hepatitis virus infection despite expression of PD-1. Therefore it is likely, that other molecules exist whose signalling is superior to that of PD-1. This was shown for example for CD28 and IL-7 signalling (Bennett, F et al. 2003).

The lack of any effect of PD-1:PDL blocking on degranulation and cytokine production observed in this study might again be due to the fact that cells from healthy individuals are usually not functionally exhausted. Positive effects of PD-1 blocking on cytokine production by lymphocytes have been described for IFN$\gamma$ and IL-10 (Brown, JA et al. 2003). In functional exhausted CD8+ T cells blockade of PD-1 was able to enhance IFN$\gamma$, IL-2, IL-10 and TNF$\alpha$ production as well as degranulation (Golden-Mason, L et al. 2007; Penna, A et al. 2007).

However, it is unlikely that PD-1 represents the ultimate control element absolutely controlling immune responses. Instead, as shown before, certain costimulatory signals cannot be influenced by PD-1 (Bennett, F et al. 2003) and PD-1 signaling itself is sensitive towards IL-2 stimulation (Carter, L et al. 2002). In this regard it is not surprising to find that the effect of PD-1 blockade on function of antigen-specific CD8+ T cells during functional exhaustion was very variable with some patients showing a strong increase of CD8+ T cell function and others none to only weak improvement (compare (Day, CL et al. 2006; Penna, A et al. 2007; Trautmann, L et al. 2006). There must be an intricate interplay of immune regulation and the fine balance of this regulation may be very important in the early immune response. Thus we aimed to consider further molecules in our analyses. During LCMV infection in mice the analysis of gene expression of functionally exhausted CD8+ T cells showed that besides PD-1 and CTLA-4 expression of the costimulatory molecule 2B4 is as well strongly induced (2B4 shows an 11 fold increase, while

PD-1 expression is 6.5 fold upregulated; see (Wherry, EJ et al. 2007). Thus, we extended our analysis beyond PD-1 and characterized the role of 2B4 and its ligand CD48 and the consequences of their expression.

### 4.2.2 Expression of 2B4 and CD48 and their role on CD8+ T cell function

Many mechanisms regulate the activity of immune cells. Few of them are known and the mode of action is not quite clear for the most of them. As the function and role of 2B4 in the human immune system is still quite obscure, we intended to investigate its implementation on CD8+ T cell function in general. The further aim of these investigations is to elucidate a possible implication during CD8+ T cell exhaustion.

Expression of 2B4 on human leukocytes showed a distinct pattern with those cells being positive for 2B4 which are able to exert some kind of cytotoxicity. In healthy individuals, CD8+ T cells showed a partial expression of 2B4 with varying percentages. On the contrary, CD4+ T cells displayed no to very low amounts of 2B4 *ex vivo*. On the contrary, all NK cells and the vast majority of NK-like T cells were positive for 2B4. Interestingly, 2B4 expression intensity on NK cells differed within the subpopulations. $CD56^{dim}$ NK cells showed high expression levels with the MFI being the highest among the leukocyte populations. $CD56^{bright}$ NK cells on the other hand expressed lower amounts of 2B4 and the MFI was approximately as high as that of CD8+ T cells. This observation is in agreement with the description that $CD56^{dim}$ NK cells are those cells exerting cytotoxicity, while $CD56^{bright}$ NK cells are strong producers of cytokines but display lower cytotoxicity (Jacobs, R et al. 2001). Similarly, 2B4 was found on monocytes but was absent on B cells. Comparable findings have been reported before with an agreeing expression of 2B4 on leukocytes (Nakajima, H et al. 1999; Romero, X et al. 2004; Valiante, NM and Trinchieri, G 1993).

In healthy individuals 2B4 expression on virus-specific CD8+ T cells showed a clear and distinct pattern. CD8+ T cells specific for CMV-pp65 and EBV-BMLF1 displayed an almost complete expression of 2B4 on the cell surface. On the contrary, Influenza-A (IV) specific cells only expressed low amounts of 2B4. With these findings we could confirm other data showing the same levels of 2B4 expression on CMV- and Influenza-A (IV) specific CD8+ T cells (Speiser, DE et al. 2001). The fact that EBV-specific CD8+ T cells were 2B4+ is fitting to the description that CD8+ T cells are important for the elimination of EBV infection (Sharifi, R et al. 2004). Interestingly, the expression pattern of 2B4 on virus-specific CD8+ T cells investigated in healthy

individuals is comparable to the levels of PD-1 expression found (see above). Again, the expression of 2B4 can be based in the fact that CMV as well as EBV might cause persistent infection leading to a continuing antigen-stimulation of the T cells, while Influenza establishes an acute resolving infection. Like for PD-1 it is not known whether a CD8+ T cell can lose 2B4 expression or if it is fixed on the cells. *In vitro* studies suggest however, that 2B4+ cells do not downregulate the expression (Speiser, DE et al. 2001).

In order to get a clearer idea about a possible role and function of 2B4, coexpression of various cell surface molecules marking the memory or activation status of T cells on 2B4+ CD8+ T cells were analysed. 2B4+ CD8+ T cells displayed a definite memory phenotype of effector ($T_E$) or effector memory ($T_{EMRA}$) cells, as they lack CCR7 and CD127, but do partially express CD45RA and CD62L. Similar descriptions were made before (Speiser, DE et al. 2001) and fits with the observation that 2B4 is a marker of memory CD8+ T cells (Valiante, NM and Trinchieri, G 1993). 2B4+ CD8+ T cells produce IFNγ more rapidly than 2B4- CD8+ T cells and only 2B4+ cells in the lymphocyte population express Granzyme-B and Perforin (Speiser, DE et al. 2001; Valiante, NM and Trinchieri, G 1993). Consistent with the memory phenotype of 2B4+ CD8+ T cells as effector/effector memory cells was the expression pattern of CD27 and CD28, where both molecules were expressed on 2B4+ CD8+ T cells. In healthy individuals 2B4+ CD8+ T cells did not express activation markers like CD40, CD152 (CTLA-4) or CD25. Nevertheless, expression of these molecules is not reciprocal, as CD8+ T cells can express both 2B4 and CD40 or CD152 as seen in patients with acute hepatitis virus infection (data not shown).

Unspecific stimulation with IL-2, SEB or anti-CD3/28 led to a marked increase of 2B4 expression on CD8+, but not on CD4+ T cells. Analysing the expression of CD48 we found that the expression levels on T cells increased as well, as indicated by an elevated MFI. Similarly, peptide stimulation also raised cell surface expression of 2B4 on the respective antigen-specific CD8+ T cells and as well the MFI of CD48. This shows that 2B4 and CD48 might have a definite role on the function of activated CD8+ T cells and could be implicated in the control of T cell function. Further, it supports the aspect of 2B4 being a marker for effector cells.

In this study, we showed for the first time the expression of 2B4 on leukocytes in hepatitis patients. The general expression pattern of 2B4 in the patient cohorts analysed is similar to the one found in healthy individuals. However, a significant increase of 2B4 expression on CD8+ and in part on CD4+ could be seen in all patient cohorts in comparison to healthy individuals. The strongest upregulation of 2B4 on CD8+ could be found in patients with acute hepatitis virus infection. Notably, the mean value of CD8+ T cells expressing 2B4 during chronic HCV infected patients was slightly less as compared to chronic HBV infection. However, the increase of 2B4 on total CD8+ T cells was not only virus-induced as the expression is also elevated in patients with autoimmune-induced hepatitis. However, an association neither with clinical parameters of liver disease (e.g. ALT or AST) nor with the plasma viral load could be observed in hepatitis patients. As discussed before, 2B4 expression on CD8+ T cells was also induced by unspecific stimuli like

IL-2 and SEB. Therefore, it is likely that the rise of 2B4 expression on patient T cells was caused by cytokines produced by the immune system during inflammation. Thus, expression of 2B4 on leukocytes might not serve as a clinical marker of hepatitis. Notably, elevated expression of 2B4 on lymphocytes has been described before in case of HIV infection (Peritt, D et al. 1999). Interestingly, in this report it was demonstrated that 2B4 expression levels on CD8+ T cells correlated inversely with CD4+ T cell count and patients with high initial 2B4 expression on CD8+ T cells progressed faster to severe stages of the disease than patients with low initial levels of 2B4.

The only known ligand for human 2B4 is described as CD48 (Brown, MH et al. 1998), a member of the CD2 family. It lacks a cytoplasmatic domain but is linked to the membrane by a glycosylphosphatidylinositol (GPI)-anchor (Garnett, D et al. 1993). Thereby, it is still able to evoke signal transduction by association of different intracellular phosphatases (Solomon, KR et al. 1996). In accordance to (Reiser, H 1990) we could detect the presence of CD48 on all leukocyte subsets to be found in the peripheral blood. Consequently, also the antigen-specific CD8+ T cells analysed are positive for CD48. However, a small fraction of immune cells could be found in the peripheral blood which did not express CD48. It was not possible to assign these CD48- cells to any of the leukocyte subsets identified in our study. Therefore, the CD48- cells might represent for example monocyte-derived Dendritic cells, as they were shown to down-regulate CD48 upon maturation (Morandi, B et al. 2005). The amount of those CD48- cells varies between individuals. In the mean no difference exists between healthy individuals and hepatitis patients. Still, some of the patients display elevated amounts of CD48- cells. One patient analysed suffered from a fulminant HBV infection with severe clinical symptoms of liver disease and in this case, many CD48- cells could be found in the peripheral blood (data not shown). Unfortunately, it was not possible to correlate the amount of CD48- cells found in our patient cohort with clinical parameters of liver disease due to missing data. Also, it cannot be excluded that these differences are simply due to a divergence in the experimental procedure. However, other reports show that infection of T cells with HIV *in vitro* led to a decreased CD48 expression of the respective cells (Ward, J et al. 2007). Further investigations are needed to analyse a possible link in this regard.

In order to analyse the functional impact of 2B4 expression on CD8+ T cells, key features of CD8+ T cell function were analysed *in vitro* with PBMCs from healthy individuals. Additionally, incubation with monoclonal anti-2B4 and anti-CD48 antibody or a combination of both was carried out. Concerning anti-2B4 it is of importance to know that this particular antibody has an activating function through ligation of the 2B4 molecule as the binding site of the antibody used here involve the same amino acid residues as the ligand binding site (Mathew, A et al. 2005).

One crucial effector function of CD8+ T cells is the exertion of cytotoxicity. In vitro stimulation of total PBMCs from healthy individuals with anti-CD3/28 led to a significant increase of degranulation of CD8+ T cells. Addition of anti-2B4 caused a further increase of degranulation

in most but not all experiments. Blocking of CD48 alone on the other hand had no effect, and absence of CD48 signalling was not able to diminish the enhancing effect of anti-2B4. Regarding degranulation of CD8+ T cells induced by antigen-specific stimulation though, no additional stimulation could be achieved by anti-2B4. It is possible, that for antigen-specific signals other additional stimulants are required in order for 2B4 showing an additional enhancement. This has been demonstrated before for human NK cells, where the enhancing effect of 2B4 ligation on NK cell cytotoxicity was dependent on the simultaneous costimulation with other NK cell receptors (Bryceson, YT et al. 2006). Similar requirements might be necessary as well for CD8+ T cells.

The fact that 2B4 expression definitely does have an effect on CD8+ T cell cytotoxicity could be demonstrated with sorted 2B4+ and 2B4- CD8+ T cells. While 2B4+ cells showed a clear increase of degranulation through anti-CD3/28 stimulation *in vitro*, CD8+ T cells negative for 2B4 expression were almost completely unable to degranulate upon this stimulation. 2B4 ligation was before reported to have an enhancing effect on the cytotoxic capacity of human NK cells (Chuang, SS et al. 2000; Tangye, SG et al. 2000a). In the case of CD8+ T cells the results demonstrated were controversial with some reporting no effect of 2B4 of CD8+ T cells (Nakajima, H et al. 1999) or enhance cytotoxicity (Messmer, B et al. 2006). Still, our findings are in agreement with another publication showing that all lymphocytes expressing perforin and granzyme-A are 2B4+ (Speiser, DE et al. 2001). Accordingly, this might be founded on the fact that 2B4+ CD8+ T cells represent effector cells capable of rapid effector functions (Speiser, DE et al. 2001) or expression of 2B4 might be a (molecular) prerequisite for CD8+ T cells for exerting cytotoxicity.

Cytokine production is another key feature of CD8+ T cells in the defence against virus infections. IFN$\gamma$ and TNF$\alpha$ represent two of the most important cytokines secreted by CD8+ T cells. Therefore, the effect of 2B4 and CD48 signalling on cytokine production of CD8+ T cells was determined. However, no effect of 2B4 ligation or blockade of CD48 signalling on neither IFN$\gamma$ nor TNF$\alpha$ production could be seen. Human NK cells were shown to increase IFN$\gamma$ production upon 2B4 ligation (Chuang, SS et al. 2000; Stark, S and Watzl, C 2006) but in the case of CD8+ T cells further experiments are needed to elucidate any possible implication of 2B4 on cytokine production.

Addition of anti-2B4 to the cell culture during peptide stimulation in vitro caused an enhanced amplification of antigen-specific CD8+ T cells in some but not all healthy individuals analysed. In 8 out of 18 samples (45%) an increase in the percentage of antigen-specific CD8+ T cells could be observed, while in 7 out of 18 cases (39%) no change of T cell expansion could be achieved. Astonishingly, as well the enhancing effect of anti-2B4 seen in some and the negative effect of anti-CD48 on proliferation observed in all samples tested was absent when the antibodies were added *in vitro* to pre-activated cells at day 4. Therefore, it seems as signalling of 2B4 and the absence of CD48 signalling only has an influence during the initial reactivation of memory cells. This would be quite plausible as 2B4+ CD8+ T cells were shown to be

effector/memory T cells. However, more experiments are needed to confirm these observations. There are several possible explanations for the observed differences regarding the effects of anti-2B4 and anti-CD48 antibodies on CD8+ T cell proliferation. First, it is possible that it depends on the activation status of the cell. Adequate other additional stimulation might be needed for 2B4 to have an activating effect on the proliferation (see Figure 55A). Comparable results have been documented for human NK cells, where it was found that IL-2 costimulation is required for 2B4 to enhance function of resting NK cells, but this was not necessary for activated cells (Endt, J et al. 2007). Also, it was reported that other coreceptors were able to modulate the signalling induced by 2B4 on NK cells (Tangye, SG et al. 2000a). As for the decrease of proliferation the structure of the intracellular domain of 2B4 has to be considered. It comprises four ITSMs (Immunoreceptor Tyrosine-based Switch Motif) and various signal phosphatases and downstream-signalling molecules were shown to associate. The major part of signal transduction occurs via recruitment of SAP (SLAM-associated adaptor protein) which elicits an activating signal (Chen, R et al. 2004; Tangye, SG et al. 2000b). But also other phosphatases can be recruited such as SHP-1, SHP-2 and Csk. In this case, the signal transduced upon 2B4 engagement leads to inhibition of immune responses (Eissmann, P et al. 2005; Parolini, S et al. 2000). Usually, SAP association is dominant and supersedes binding of the other molecules (Eissmann, P et al. 2005). But the lack of the SAP molecule consequently leads to signalling via the other pathways and hence to an inhibitory signalling (see Figure 55B). These divergent events were first realized in patients with X-linked lymphoproliferative disease (XLPD) where SAP is mutated and cannot associate to the cytoplasmic domain of 2B4. Thus, SHP-1 and SHP-2 are recruited for signal transduction leading to inhibition of NK cell responses (Parolini, S et al. 2000; Sayos, J et al. 2000). Therefore, the on the one hand activating and on the other hand reducing effect of 2B4-ligation on antigen-specific CD8+ T cell proliferation could be explained by involvement of different signalling phosphatases.

Presence of anti-CD48 during peptide stimulation in vitro led to a marked decrease of CD8+ T cell and antigen-specific CD8+ T cell proliferation in all samples analysed. Comparable phenomena were already described in experiments performed 20 years ago, where the treatment of lymphocytes with anti-CD48 as well caused decreased immune responses of T cells (Reiser, H 1990). Also, CD48 knock-out mice display severe impairment of CD4+ T cell activation (Gonzalez-Cabrero, J et al. 1999). The first assumption for the reason behind that could be the lack of 2B4 signalling during CD48 block (see Figure 55C). It is likely that the signals evoked by 2B4 are crucial for the survival of 2B4+ T cells *in vitro* and possibly *in vivo*. But one argument against this is that the presence of the cross-linking and therewith 2B4-activating antibody is not able to prevent the decreased proliferation of T cells seen *in vitro*. Of course it is still possible that the signalling induced by ligation through the antibody is not as strong as the one induced by ligation with CD48. However, one has to bear in mind that in addition to 2B4 also the ligation of CD48 induces signal transduction (see Figure 55D). Therefore, the observed effects resulting from addition of anti-CD48 antibody to the cell culture might also be due to a lack of CD48 signal transduction which is usually induced upon ligation with 2B4. Further, it is of importance that

another counter-receptor for CD48 besides 2B4 exists (see Figure 55E). CD2 binds with lower affinity than 2B4 (Sandrin, MS et al. 1993), but its expression can be found throughout the immune system. T cells, as well as NK cells (but not B cells) express CD2 on their surface (Sewell, WA et al. 1987). It is feasible that the reduced proliferation of T cells during CD48 blocking is due to lack of CD2/CD48 ligation (see Figure 55F). Which of these two molecules must evoke signal transduction is not clear yet. Supported is this possibility by the report demonstrating that CD2 activated T cells synergistically to a TCR stimulus (see Figure 55E; (Bierer, BE et al. 1988) and anti-CD2 antibodies induce T cell unresponsiveness *in vivo* (see Figure 55F; (Guckel, B et al. 1991)). Further experiments will be needed to answer these issues.

Still, another possibility of reduced numbers of antigen-specific CD8+ T cells and CD8+ T cell proliferation during CD48 blocking is enhanced cell death occurring. Staining for Annexin-V positive cells after 24 hours of anti-2B4 or anti-CD48 treatment showed no strong increase of apoptotic cells in vitro. However, a slight though non-significant rise of dead CD8+ T cells could be observed. Therefore, an additional enhanced death of cells in parallel to an induced unresponsiveness is likely to occur. Probably a significant change in the amount of apoptotic or dead cells can be seen after a longer time period. Also, many of the apoptotic or dead cells might have got lost after culture due to the washing procedure of cells during FACS staining.

Interestingly, different results were achieved concerning the survival of T cells using purified 2B4+ and 2B4- CD8+ T cells. In the 2B4- samples far more cells were found to be alive as compared to the 2B4+ ones. Considering the numbers of cells seeded initially, the conclusion can be drawn that the 2B4- fraction of cells showed better survival in vitro (and not enhanced proliferation) than the 2B4+ cells, whose numbers were significantly decreased. As the difference between the samples is the lack or the presence of 2B4 on CD8+ T cells, suggesting a direct or indirect role of 2B4. There are several possible explanations for this phenomenon. (i) 2B4 itself elicits an intracellular signal leading to cell death. Although there is no difference to be seen in the presence of anti-CD48 (and therewith lack of the ligand) one has still to bear in mind that anti-2B4 antibody is still present (from the cell sorting) and it shown to induce signalling of 2B4. Further, there are several signalling pathways possible upon 2B4 ligation and which pathway (e.g. activating or inhibitory) is employed depends on the circumstances. It is feasible that an inhibitory signalling is evoked here and other additional signals like IL-2 or costimulation through cell contact provided by other - in this setting absent - cells (e.g. NK cells or monocytes) is necessary for an activating signalling. It was shown before that 2B4 signalling is influenced by other costimulatory signals (Endt, J et al. 2007; Tangye, SG et al. 2000a) and interaction between CD48 on NK cells and 2B4 on CD8+ T cells can occur and enhances CD8+ T cell proliferation (Assarsson, E et al. 2004) as well as between T cells themselves (Lee, KM et al. 2003). (ii) As 2B4+ but not 2B4- CD8+ T cells are capable of rapid degranulation, it could be possible that the reduced survival is to be attributed to an increased spontaneous cytotoxicity among the CD8+ T cells. (iii) 2B4 itself can act as a ligand for CD48 thereby inducing signalling leading to cell death could be possible. However, other results from this study as well as data

reported by others suggest an activating signalling of CD48 his possibility can be ruled out. Also, as addition of anti-CD48 to the cells should be blocking this interaction, this possible negative effect on cell survival should be abolished. But as no differences could be seen in all samples when adding anti-CD48 antibody in vitro, the assumed negative signalling of CD48 can be excluded. (iv) As 2B4+ CD8+ T cells are shown to be effector/memory cells, it can be assumed that additional other signals are required for the cells to survive. As, again, these signals may be delivered through contact with other cells (e.g. NK cells or monocytes) and, again, these cells are absent in the cell culture this might drive the 2B4+ cells into apoptosis. 2B4- CD8+ T cells on the other hand much likely represent naïve or central memory cells and they might not need additional stimulation for survival.

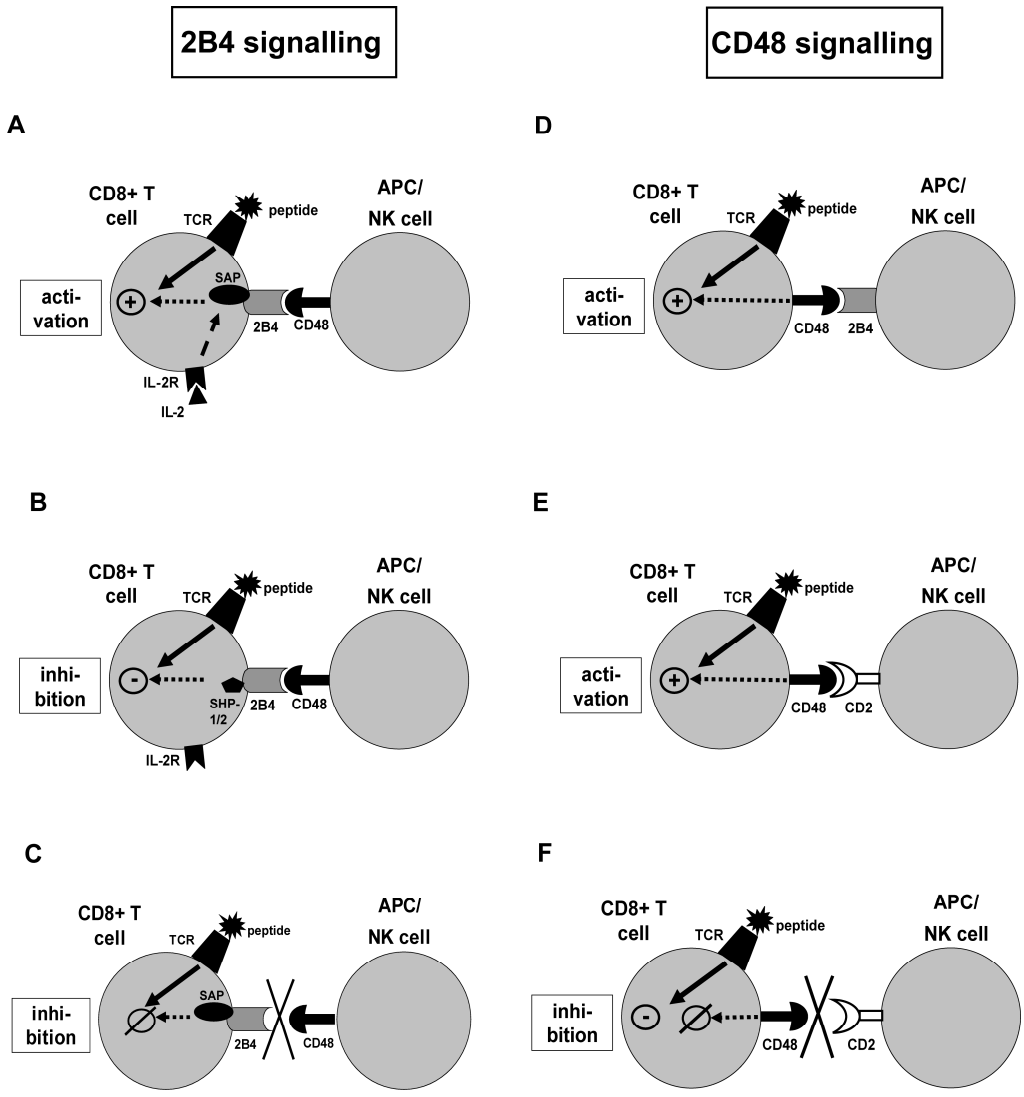



**Figure 55: Schemes of possible regulation of 2B4 and CD48 signalling pathways.** Various possibilities for interactions and modifications of signalling of 2B4 (left column) and CD48 (right column) are shown. (A) Besides the activating signal delivered by 2B4 upon ligation with CD48 on neighbouring cells involving SAP, also inhibitory signals can be mediated. (B) In the absence of other costimulatory signals like IL2 ligation of 2B4 results in involvement of SHP phosphatises and inhibition of CD8+ T cell function. (C)

Diminished proliferation of CD8+ T cells upon treatment with anti-CD48 antibody might be due to lack of2B4 ligation through CD48. (D) Ligation of CD48 is assumed to mediate activating signals on CD8+ T cells. (E) However, CD48 also is able to bind to CD2 also resulting in an activation of CD8+ T cells. (F) The presence of anti-CD48 might cause reduced proliferation in vitro as binding to CD2 in blocked and hence stimulation is missing.

Interestingly, the negative effect of the anti-CD48 antibody seen during culture of total PBMCs did not occur when culturing sorted 2B4+ or 2B4- CD8+ T cells. The mechanism behind this is indistinct. Again, one could argue that other (co)stimuli are missing in this setting. Possibly, other signals taking place between different cells among the PBMCs elicit signals that in the absence of CD48 signalling evoke inhibitory functions and that those are lacking due to the purification of CD8+ T cells. More experiments will be needed to clarify this issue and answer the questions satisfactorily. However, a possible toxic effect of the anti-CD48 antibody causing the reduced survival of lymphocytes can be ruled out by the sorting experiments. Here, addition of anti-CD48 to neither 2B4- nor 2B4+ CD8+ T cells caused a change in the survival of cells.

The function of 2B4 in the immune system seems to be – like almost all costimulators are – tightly regulated. The network of interaction between different molecules and signals is very wide and diffuse and many things seem to be dependent on one another. The implication of 2B4 and CD48 during T cell exhaustion is still not clear. However, some findings are to be kept in mind and might point towards a possible involvement of 2B4 during T cell exhaustion. The first aspect is of course the divergent signalling pathways which can take place upon 2B4 ligation. If the intracellular molecule SAP responsible for an activating effect is lacking, others (e.g. SHP-1 and SHP-2) take its place and hence lead to an inhibiting signal. It could quite be possible that SAP expression is altered during persistent infection of functional exhaustion. The gene expression analysis of exhausted virus-specific CD8+ T cells interestingly shows that while 2B4 expression is strongly upregulated, the level of SAP expression remained stable in these cells and the amount of SHP-2 increased (Wherry, EJ et al. 2007). It was shown that, at least in mice, a functional SAP is crucial for T cell responses during viral infections (Wu, C et al. 2001). However, one has to keep in mind here that the signalling occurring upon 2B4 ligation apparently differs significantly in mice and humans.

Second, in this regard it is also interesting to know that SAP expression of human NK cells can be distinctly regulated. During the development of NK cells when all other inhibitory receptors are not yet present, SAP is not expressed and hence 2B4 delivers inhibitory signal keeping the NK cell cytotoxicity against autologous cells at bay (Sivori, S et al. 2002). NK cells residing in the decidua were also shown to lack SAP expression and consequently 2B4 mediated negative signalling (Vacca, P et al. 2006). In case of human NK cells IL-2 was shown to enhance expression of SAP thereby leading to a positive signalling of 2B4 and IL-2 activated NK cells could be activated by 2B4 ligation alone without requirement of additional other costimulation (Endt, J et al. 2007). It is likely that similar regulations also apply in case of CD8+ T cells. If accordingly IL-2 stimulation increases the SAP content in CD8+ T cells as well, the report

demonstrating 2B4+ CD8+ T cells being almost unable to produce IL-2 (Speiser, DE et al. 2001) again shows the tight control and interaction of costimulatory signals in the immune system. Therefore, 2B4+ CD8+ T cells are dependent on IL-2 being produced by other cells. In a situation like persistent infection and functional exhaustion IL-2 production is one of the first effector functions getting lost (Freeman, GJ et al. 2006).

Third, as CD48 is necessary for 2B4 ligation (as no other ligand for 2B4 seems to exist), expression of CD48 has to be considered. It seems to depend on the respective conditions and DCs in inflamed lymph nodes downregulate expression of CD48 (Morandi, B et al. 2005).

In summary, it could be demonstrated that 2B4 expression and signalling definitely has an impact on the function of human CD8+ T cells. Due to the tight control and many other costimulators having an influence on 2B4 signalling, the effect of 2B4 ligation can be very divergent and is not easy to be elucidated. Many more experiments need to be done to clarify the impact of other costimulatory receptors on 2B4. Also, it would be interesting to analyse a gene expression profile of exhausted CD8+ T cells in humans. Experiments are currently under way analysing the functional role of 2B4 during persistent viral infections. As during viral and auto-immune-induced hepatitis expression of 2B4 is increased, it might be implicated as a possible target for intervention. However, very few things are known about the consequences and regulations of 2B4 expression and signalling yet. Also, a possible implication of 2B4 as a treatment option will be difficult due to the very complex regulation of 2B4 activity and possible harmful effects on the patients need to be considered.

## 4.3 Hierarchy and clonality of HCV NS3-1073 specific CD8+ T cells

We have demonstrated that phenotypes of virus-specific CD8+ T cells show a distinct pattern and that costimulatory molecules like PD-1 and 2B4 can influence the function of these cells. However, also the specific T cell repertoire of such epitope specific CD8+ T cell responses may be important when developing peptide vaccines.

Due to the random generation of the TCR and the existence of multiple gene variants of the D, V and J segments, a unique set of TCRs exist for each individual and even genetically identical twins do not display the same TCR repertoire. Viral proteins give rise to many different epitopes with potential MHC-binding capacity. Interestingly, the T cell response is directed to only a limited number of epitopes and within these a certain hierarchy exists. These immunodominances are based on several aspects. The selection of epitopes to be targeted is influenced by the processing of the viral proteins within the APCs. Also, not all of those peptides generated have similar binding abilities to the MHC molecule and as well the avidity of the resulting MHC:peptide complex to the TCR differs. Further and most importantly, there is a limitation of TCR availability in the host, the contact of a T cell with an APC presenting the respective antigen occurs randomly and those T cell clones stimulated first will dominate and influence the immune response (Selin, LK et al. 2006).

We have shown here and it was demonstrated before that the hierarchy of epitopes targeted during an ongoing infection varies considerably between individuals. For example, it is known that not every HLA-A2+ patient mounts a dominant HCV NS3-1073 response and in some patients this epitope is hardly targeted. However, there is also record of two patients that displayed a strong dominance of the HCV NS3-1073 specific CD8+ T cell responses and this was associated with severe pathogenesis causing fulminant hepatitis. Despite this vigorous immune response these patients were not able to clear the infection and hence progressed to persistency (Urbani, S et al. 2005). These data are of importance for the development of a vaccine. The responses induced by the peptides vaccine such as the IC41 should induce a protective immune response in every individual. However, also in our analyses different patients with acute HCV-infection had varying HCV NS3-1073 responses and epitope hierarchies. Reasons for these different epitope hierarchies which may explain this finding are (i) the availability of specific T cells with a given T cell receptor repertoire; (ii) the strength of the antigen stimulation occurring *in vivo* and (iii) the proliferation of pre-existing cross-reactive memory T cells. Thus, especially analyses of T cell repertoires of epitope specific responses may become important for the design of vaccines

Besides the hierarchy of the epitope targeted during an immune response, there will also be a preferential Vβ chain usage of the TCRs recognizing a given epitope. Similarities exist also in the amino acid motifs of the CDR3 regions, a fact described as 'Public specificity' (Selin, LK et al. 2006). Still, the stochastic process of nucleotide insertions during the TCR gene rearrangement and the random selection of gene segments during V(D)J rearrangement leads to varying TCR availabilities. Together with the random process of T cells encountering the respective DC and the fact that early activated T cells will influence the development of other virus-specific T cells leads to a unique TCR usage in each individual. Such each individual displays its 'Private specificity' of TCR usage during epitope recognition (Welsh, RM 2006). Analysing the Vβ chain usage of HCV NS3-1073 specific CD8+ T cells of the different acute HCV infected patients revealed rather broad and individual TCR repertoires for all patients. Public Vβ chains can be found used by all samples analysed (e.g. Vβ7.1, 9, 20, 2 and 4). But also, each patient displays private Vβ chains (e.g. Vβ3, 17, 8, 5.2, 22 and 13.2) which are unique to the given individual.

We further analysed the clonality of the dominant T cell receptor repertoire of the HCV NS3-1073 specific CD8+ T cell response. Therefore, the nucleotide and amino acid sequence of the most prominent Vβ7 chain was performed for selected samples. Interestingly, we found a strongly narrowed TCR repertoire with one clone being highly dominating. Usually the TCR repertoire and the clonality of a primary given antigen-specific CD8+ T cell population is rather broad with multiple clones and several of them being equally represented (Cornberg, M et al. 2006a; Naumov, YN et al. 2003). However, this is not the case in the samples analysed here. This is reminiscent to data from small animal models showing that the proliferation of cross-reactive T cells caused a narrow repertoire and furthermore lead to highly variable immune responses (Cornberg, M et al. 2006a; Kim, SK et al. 2005). A similar narrowed TCR repertoire with a single clone dominating was demonstrated in mice. Here, after a primary infection with Pichinde Virus (PV) CD8+ T cells specific for one epitope was broad and polyclonal. However, after a subsequent infection with Lymphocytic Choriomeningitis Virus (LCMV), cross-reactive CD8+ T cells initially recognizing a Pichinde Virus epitope were expanding vigorously and dominated the virus-specific immune response (Cornberg, M et al. 2006b). In this case, the TCR repertoire of these cross-reactive CD8+ T cells was narrowed with one clone making up most of the clones participating in the immune response. A similar setting could be possible in this case of acute HCV infection. It is likely that a TCR does not only recognize one fixed peptide, but many different epitopes which resemble each other in their amino acid sequences. Memory T cells – in contrast to naïve T cells – can be activated by TCR signals far weaker than the initial stimulus (Veiga-Fernandes, H et al. 2000) and have an activation advantage towards naive T cells. Therefore, it is possible for memory T cells to get activated by cross-reactive epitopes which have a far lower affinity to their TCR (Curtsinger, JM et al. 1998; Pihlgren, M et al. 1996). For example, in a mouse model system a previous recovered challenge with LCMV delivered protective immunity to a subsequent otherwise lethal infection with Vaccinia Virus (Chen, HD et al. 2001). T cells specific for an otherwise subdominant epitope of VV were able to also

recognize LCMV epitopes mediating protection (Kim, SK et al. 2005). A prerequisite for cross-reactivity lies in the apparent degeneracy of T cell recognition. The concept of Molecular mimicry describes this phenomenon of alternate peptide interaction with the same TCR and the potential implication of autoimmune injuries. Additionally, the identical TCR can recognize other cross-reactive epitopes through so called Alternative recognition. Here, the contact with peptide amino acid residues occurs with different determinants of the TCR as during the initial recognition (Welsh, RM and Selin, LK 2002).

In case of HCV and HBV few is known about the TCR repertoire, single data available originate from studies performed in chimpanzees (Meyer-Olson, D et al. 2003; Meyer-Olson, D et al. 2004). Equally, data from human HCV infection exist which demonstrate the presence of cross-reactive T cells recognizing HCV epitopes (Kennedy, PT et al. 2006; Urbani, S et al. 2005; Wedemeyer, H et al. 2001). For the development and the application of an HCV peptide vaccine factors like the individual HCV epitope hierarchy as well as the presence of possible cross-reactive CD8+ memory T cells are important aspects to be considered. It may be possible that a peptide vaccine induces the proliferation of cross reactive memory T cells and not naïve T cells. This may either be protective or may lead to the development of pathogenesis. A narrow TCR repertoire as one possible result may also lead to immune escape after HCV infection. Thus, the usage of such peptide vaccines need to be carefully considered and possibly even be evaluated individually.

## 4.4 Summary

The aim of this thesis was to analyze the phenotype and function of epitope-specific CD8+ T cells in chronic virus infection with special focus on Hepatitis C Virus.

- During persistent infection the majority of HCV-specific CD8+ T cells displayed resting/immature $T_{EMRA}$ memory phenotype with low frequency and low cytokine production capacities. A change in the memory phenotype towards an effector status but no improvement of effector functions could be achieved by therapeutic peptide vaccination. In healthy individuals HCV-specific CD8+ T cells could be induced by vaccination which displayed a $T_{CM}$ or $T_E$ phenotype and were also capable of IFNγ production and strong proliferation. However, if these cells can mediate protective immunity is not clear.

- In healthy individuals expression of PD-1 *ex vivo* was generally low and a distinct expression pattern on antigen-specific CD8+ T cells could be observed. While PD-1 expression on T cells markedly increased during acute hepatitis virus infection, no significant difference in patients with persistent hepatitis virus infection could be seen. Blocking PD-1:PDL1 interactions by monoclonal antibodies resulted only in a marginally enhanced proliferation of antigen-specific CD8+ T cells in healthy individuals.

- In contrast, expression of 2B4 on T cells *ex vivo* was significantly increased in patients with liver disease in comparison to healthy volunteers, but no direct correlations with clinical markers were found. 2B4 showed a distinct pattern of expression on different leukocyte subsets and also on the different virus-specific CD8+ T cells analysed. Further, expression of 2B4 was demonstrated to have a definite impact on CD8+ T cell function. The ability of degranulation seemed to be restricted to 2B4+ CD8+ T cells and ligation of 2B4 with monoclonal antibodies led as well to increased degranulation of CD8+ T cells. The effect on CD8+ T cell proliferation showed to be diverse and seemed to some degree to be dependent on the antigen-specificity in case of 2B4. While anti-2B4 antibodies led to an increase of proliferation in some individuals, antibodies targeting the 2B4 counter-receptor CD48 always caused a reduced proliferation.

- The hierarchy of HCV epitopes showed to differ individually with varying epitopes being dominant. Also the Vβ chain usage of the often dominant HCV NS3-1073 epitope displayed different repertoires between individuals. Interestingly, the clonality of the dominating T cell clones using the Vβ7 chain showed to be strongly narrowed. However, it was not possible to identify any possible influence of cross-reactive memory CD8+ T cells on the HCV-specific immune response.

In conclusion, the results demonstrated in this thesis give novel insights into the very complex regulation of CD8+ T cell responses towards viruses in humans. Various factors like the phenotype and expression of costimulatory molecules influence CD8+ T cell function and a very wide network of interactions regulate such responses. Further variation can be seen on an individual level as virus-specific CD8+ T cell responses like those against HCV showed a great variability especially on the T cell receptor level. All these aspects have to be considered in the development of new vaccines, as possible harmful impacts on the patients need to be prevented.

# 5 List of Figures

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# Publication list

## Journal Articles

**Schlaphoff V**, Klade CS, Jilma B, Jelovcan SB, Cornberg M, Tauber E, Manns MP, Wedemeyer H; IC41 Study Group (2007). "Functional and phenotypic characterization of peptide-vaccine-induced HCV-specific CD8+ T cells in healthy individuals and chronic hepatitis C patients." Vaccine. **17**;25: 6793-806.

Wiegand J, Cornberg M, Aslan N, **Schlaphoff V**, Sarrazin C, Kubitschke A, Buggisch P, Ciner A, Jaeckel E, Manns MP, Wedemeyer H. (2007). "Fate and function of hepatitis-C-virus-specific T-cells during peginterferon-alpha2b therapy for acute hepatitis C." Antivir Ther **12**(3): 303-16

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PV Suneetha, **V. Schlaphoff**, C Wang, K Stegmann, SK Sarin, M.P. Manns, M Cornberg, H Wedemeyer (2008). "PD-1 Controlled Proliferation of Antigen Specific CD8 T-cells can be Impaired By Using Peptide Pools." submitted

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## Poster Presentations

**V. Schlaphoff**, S.B. Jelovcan, J. Wiegand, M. Cornberg, B. Jilma, M. P. Manns, H. Wedemeyer and C. S. Klade. „Phenotypic characterisation of HCV-specific CD8+ T cells after HCV-peptide vaccination vs. acute and chronic hepatitis C" 12th International Symposium on Hepatitis C and Related Viruses, October 2-6, 2005 in Montréal, Canada

**V. Schlaphoff**, S.B. Jelovcan, J. Wiegand, M. Cornberg, B. Jilma, M. P. Manns, H. Wedemeyer and C. S. Klade. „Phenotypic characterisation of HCV-specific CD8+ T cells after HCV-peptide vaccination vs. acute and chronic hepatitis C" 57th Annual Meeting of the American Associationfor the Study of Liver Diseases, October 27-31, 2006 in Boston, USA

**V. Schlaphoff**, N.A.H. Ho, S. V. Pothakamuri, M. P. Manns, H. Wedemeyer and M. Cornberg. "Lack of correlation between PD-1 expression on T-cells and clinical parameters from patients with chronic hepatitis C". Accepted as Poster Presentation for the 43rd Annual Meeting of the European Association for the Study of the Liver (EASL), April 23 – 27, 2008 in Milan, Italy.