Aus der Klinik III für Hämatologie Onkologie und Transfusionsmedizin
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

In vitro induction and expansion of CD8 positive T cells: a method applicable for the generation of low-frequency antigen specific CD8 positive T cells

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Agnieszka Wieczorek

aus Proszowice

Gutachter/in: 1. Prof. Dr. med. L. Uharek

2. Prof. Dr. med. N. Kröger

3. Prof. Dr. med. M. Stelljes

Datum der Promotion: 22.03.2013

To my parents

Abbreviations

ACT	adoptive cell transfer
ADAP	adhesion- and degranulation-promoting adaptor protein
AML	acute myeloid leukaemia
anti- TGFβ	anti-transforming growth factor-β
AP-1	activator protein 1
APC	antigen presenting cell
APC	allophycocyanine
cSMAC	central supramolecular activation cluster
CD	cluster of differentiation
cDNA	complementary DNA
CMV	cytomegalovirus
CTL	cytotoxic T cell lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
DCs	dendritic cells
DLI	donor lymphocyte infusion
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E:T	effector to target ratio
EBV	Epstein Barr-Virus
ELISpot	enzyme linked immuno-spot technique
FACS	fluorescence activated cell sorting
FADD	Fas-associated protein with death domain
FASL	FAS Ligand
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor
GMP	good manufacturing practice
GvHD	graft versus host disease
GvL	graft versus leukaemia
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus

HEV	high endothelial venules
HIV	human immunodeficiency virus
HLA	human-leukocyte antigen
HSCT	haematopoietic stem cell transplantation
HSV-TK	herpes simplex virus thymidine kinase
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
IFN-γ	interferon γ
IP	inositol phospholipid
LCLs	lymphoblastoid cell lines
LFA-1	lymphocyte function-associated antigen-1
MAPK	mitogen-activated protein kinase
MCs	microclusters
mHAgs	minor histocompatibility antigens
MHC	major histocompatibility complexes
Mig	monokine induced by interferon-gamma
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NK	natural killers
NOD	nonobese diabetic
OKT-3	muromonab-CD3
PBMCs	peripheral blood mononuclear cells
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PGE_2	prostaglandin E_2
PMA	phorbol-12-myristat-13-acetat
pSMAC	peripheral supramolecular activation cluster
RNA	ribonucleic acid
rpm	rounds per minute
SCID	severe combined immunodeficiency
SEREX	serological screening of cDNA expression libraries
TAA	tumour-associated antigens
TAP	transporter associated with antigen processing
T_{CM}	central memory T cells

TCR	T cell receptor
T_{EM}	effector memory T cells
TILs	tumour infiltrating lymphocytes
TNF-α	tumour necrosis factor alpha
TRAIL	TNF-apoptosis-inducing ligand
TREC	T-cell receptor excision circle
VLA-4	very late antigen 4
WT-1	Wilms' tumour antigen 1

I. Introduction

1.1 Specific immunity

1.1.1 Antigen-specific immune response against virus-infected cells and tumour cells

CD8+ T cells play a critical role in the abatement of virus- or tumour-transformed cells. Through their interaction with major histocompatibility complex (MHC) class I they can generate:

1. cytotoxic degranulation, which leads to perforin-mediated lysis (Ito et al. 2001)
2. the induction of apoptosis-inducing proteins including FAS Ligand (FASL) and TNF-apoptosis-inducing ligand (TRAIL) (Croft, Curr Opin Immunol. 1994)
3. target-cell destruction through the release of inflammatory cytokines such as: tumour necrosis factor alpha (TNF-α); interferon γ (IFN-γ).

The fate of a virus-infected or pathologically changed cell is almost always apoptosis initiated by cytotoxic T cell lymphocytes (CTLs), a fate which effectively reduces the risk of neighbouring cells being infected. Perforin and granulysin form pores in the target cell's membrane causing lysis of the cell. The perforin-formed pores create an entrance for CTLs released granzymes which induce apoptosis through activation of caspases.

The second way apoptosis is induced involves the interaction between surface protein of T cells – Fas ligand (CD178) and Fas molecules (CD95) expressed on target cells. Also, two receptors belonging to TNF receptor family – DR4 (TRAIL-R1) and DR5 (TRAIL-R2) are capable of transducing an apoptotic signal in a wide variety of cancer cells (Croft, Curr Opin Immunol. 1994) Moreover, it has been suggested that TRAIL-R2 transduces a signal through Fas-associated protein with death domain (FADD)- and caspase 8-dependent pathway (Croft, Curr Opin Immunol. 1994).

Apart from the purely destructive potential of CTLs which are able to induce apoptosis or antigen-specific lyses, CTLs are also able to release inflammatory cytokines. This explains how an inefficient process of antigen-specific lyses that requires direct physical contact between CTL and target cell can still take over control of infections involving a large number of cells.

The cytokines secreted by antigen-specific CTLs also play a crucial role in adaptive immunity. In this manner, IFN-γ influences monocytes and their differentiation into macrophages which induce the generation of professional antigen presenting cell (APC). Moreover, it was shown that it increases the synthesis and activation of transporter protein associated with antigen processing (TAP) (Sijts et al., Curr Mol Med. 2001), induces entrance in G1-phase and causes apoptosis (Kaplan et al., J Exp Med. 1998), and increases the presentation of virus and tumour antigens via increase of MHC molecules. Through induction of angiogenesis, the inhibitors IP10 and Mig remain the most critical of antitumour indirect mechanisms, and because of the slower growth of tumour cells, this enables the attraction of CD8+ T cells via chemokines (Sun et al., J Immunother 2001). Additionally, the exceptional role of IFN-γ role in virus infections could be shown by inhibition of hepatitis B virus (HBV) transcription and replication in human hepatocytes without cell lysis (Suri et al., J. Hepatol. 2001).

1.1.2 Differentiation of CD8+ T cells

The quality of T cell response is fundamental for determining the disease's outcome. In recent years, due to extensive efforts to link the T cell phenotype with their function, great insight into an incredible phenotypic and functional heterogeneity in T cell populations has been won.

Based on phenotypic and functional attributes, CD8+ T cells can be categorised into different subsets representing distinct differentiation states: naive, effector, memory and memory stem cells (Lanzavecchia and Sallusto, Nature Rev. Immunol., 2002; Appay et al., Nature Med., 2002; Seder and Ahmed, 2003; Sallusto and Lanzavecchia, Nature Med., 2011).

Briefly, naive T cells are those of the T cell population which have not encountered a specific antigen. They are also characterized by a limited proliferative history (low expression of the proliferation markers), long telomeres and a high content of T-cell receptor excision circle (TREC). Moreover, they exhibit diverse specificities towards their TCRs. Naive T cells are able to circulate through the lymph nodes, passing through the high endothelial venules (HEV) (Lanzavecchia and Sallusto, Nat Rev Immunol. 2002). This process is mediated by interaction of CD62L on the T cell with vascular addressins on endothelial cells (Lanzavecchia and Sallusto, Nat Rev Immunol. 2002), and a gradient of the chemokines

CCL19 and SLC, which are recognized by the chemokine receptor CCR7 on the T cell (Lanzavecchia and Sallusto, Nat Rev Immunol. 2002). In the lymph nodes, an activation of naive T cells and initiation of an immunological response take place (Kaech et al., Cell 2002). Because of the expression of various proteins, naive T cells can be also identified by expression of CD27 (a ligand for the TNF-receptor-like protein), CD28 (a co-stimulatory molecule, capable of directing homing capacity of human memory T cells) and CD45RA (isoform of the CD45 molecule).

One of the paradigms defined by Lanzavecchia and Sallusto divides antigen-experienced T cells into central memory T cells (T_{CM}), effector memory T cells (T_{EM}) and effector T cells (Lanzavecchia and Sallusto, Nat Rev Immunol. 2002). This almost purely phenotypical characterisation has been recently amended by extensive usage of multi-parameter flow cytometry.

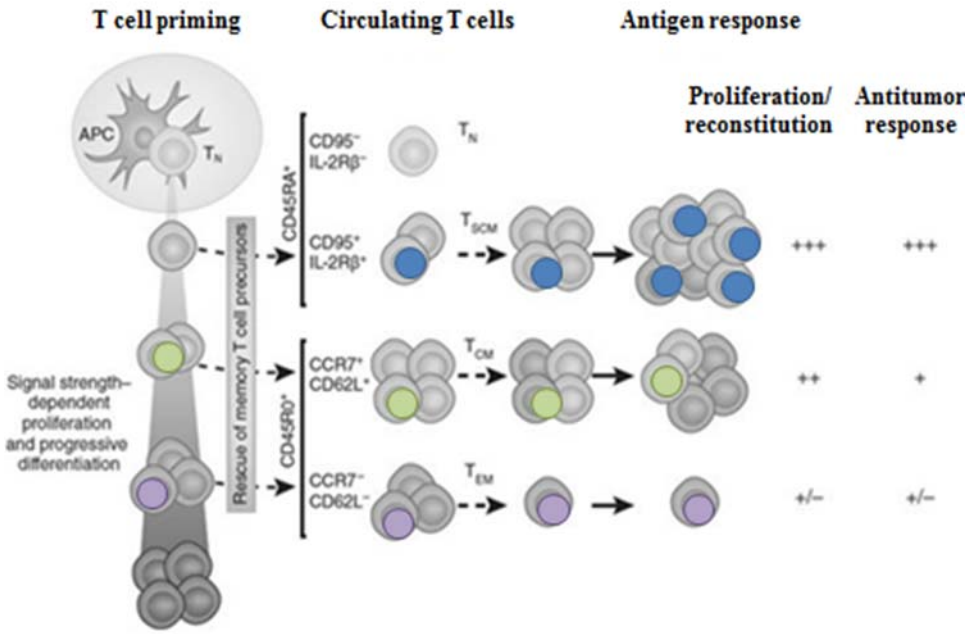


Figure 1 Model of T cell differentiation after priming by antigen-presenting cells (modified and adapted from Nature Med., 2011).

T_{CM}s are those antigen-specific T cells which express cell-surface receptors for homing to secondary lymph organs like CD62L or CCR7. Moreover, they are thought to be long-lived and can serve as precursors for effector T cells upon re-exposure. They are also considered to be multifunctional (so called “triple producers”) and able to produce IFN-γ, TNF and IL-2. T_{EM} cells also belong to an antigen-experienced T cell pool and can upon re-exposure to

antigen, rapidly express their effector traits such as production of IFN-γ and TNF and very rarely IL-2 (so called "double producers"). Furthermore, they can also efficiently migrate to peripheral sites of inflammation by expression of proteins involved in homing to inflamed tissue (such as CCR2, CCR5 or CD49b) (Kaech et al., Cell. 2002; Seder and Ahmed, Nat Immunol. 2003). Continuous antigen stimulation can result in terminally differentiated effector T cells that produce only IFN-γ and are short-lived.

		Naive	*Effector*	*Memory*	
				Effector memory	Central memory
Differential expression of	CD3, CD8	+	+	+	+
	CD45RA	+	-	-	+
	CD45RO	+	-	-	+
Activation and co-stimulation molecules	CD25	+	-	+	+
	CD27	+	+/-	+	+
	CD28	-	++	-	-
	CD57	(+)	++	++	++
	CD58	-	+	++	++
	CD69	-	++	(+)	(+)
	CD95				
	FasL				
Integrins	CD11a	(+)	++	+	+
	CD11b	-	++	-	-
	CD18	(+)	++	+	+
	CD29	+	++	+	+
	CD49d	(+)	++	+	+
	CD49e	(+)	++	+	+
Homing receptors	CD62L	++	+/-	-	++
	CCR7	+	+/-	-	+
Cytokine receptor	IL-2	+	++	++	++
	IL-15Rα	+	++	++	++
	IL-15Rβ	+	-	++	++
	IL-7 Rα				
Effectors function: Cytokine production	IL-2	+	-	+	++
	IL-4	-	-	++	-
	IL-12	-	++++	+++	++
	IFN-γ	-	+	++	+++
	TNF-α				
Effectors function:	cytotoxicity		++++	+++	+++
Cytotoxic mediators	perforin	-	++	(+)	(+)
	granzym B	-	++	(+)	(+)
Frequency of cells in PBMC populations of	healthy	++	+	++	++
	virally infected individuals	+	++	+++	+
Homing capacity	2° lymphoid organs	++++	+/-	+/-	+++
Proliferative capacity	antigen specific	++++	++	++	++++
	homeostatic	-	+/-	++	++++

Table 1 Summary of phenotypical features of naive, effector, central memory and effector memory T cells according to articles published by Sallusto 1999, Appay 2002, Seder and Ahmed 2003.

Recently, existence of an additional subset of memory T cells with stem-like properties was postulated by Gattinoni et al. (Nat Medicine, 2011). The memory stem cells (T_{SCM}) express similar to naive T cells CD45RA, CCR7 and CD62L. Unlike naive T cells, T_{SCM} express CD95, IL-2Rβ, CXCR3 and higher levels of lymphocyte function-associated antigen-1 (LFA-1). T_{SCM} circulate preferentially through lymph nodes and mucosal lymphoid organs, where they can undergo secondary immune response.

1.1.3 Primary and secondary immune response

After positive and negative selection process in the thymus, naive T cells migrate to the periphery where they enter the T cell pool. The recognition of an antigen by the immune system evokes coordinate number of changes. Upon first encounter with a specific antigen, a naive T cell undergoes divisions into many clones acquiring effector phenotype (primary immune response). They are able to secrete cytokines (T helper cells) or kill infected cells (cytotoxic T cells). These processes lead mostly to a rapid clearance of the pathogen within a week's time. The contraction of the effector T cell compartment which follows is due to apoptosis. However, a minority of those cells (5-10%) escapes apoptosis and turn into memory T cells. After entering the antigen-experienced T-cell compartment, memory T cells circulate through the peripheral lymphatics. Accumulated in secondary lymphoid tissues, they can undergo rapid expansion upon re-stimulation by their cognate antigen (during the secondary immune response).

1.1.4 Mechanisms limiting immune responses

Despite the ingenuity of the immune system which protects the host from pathogen invasion or tumour development, it also exerts a selection pressure on virus infected or tumour cells promoting the survival of escape variants.

Viruses have developed a variety of mechanisms to escape immunosurveillance. Through production of viral proteins which actively fight the host immune response it is for example possible for the human immunodeficiency virus (HIV) to undergo rapid antigenic variations or for herpes viruses to survive through latent infection or through modality of the life cycle as is the case in papillomaviruses. Moreover, it is becoming evident that viruses, such as HIV-1 subvert functions of certain cells, for example: dendritic cells (DCs) (van Kooyk et al., Trends Mol Med. 2003).

Similarly, very many viral diseases and tumours – although potentially immunogenic – succeed in escaping control mechanisms. To explain such a phenomenon, Dunn and colleagues formulated a concept called "cancer immunoediting" (Dunn et al., Nat Immunol., 2002). According to this, the tumour escape can be divided into three phases. The first one – the elimination phase – encompasses the original Burnet and Thomas concept of immuno-surveillance (Burnet, Transplant Rev. 1971). The second phase – the equilibrium phase – is one in which the host immune system exerts a selective pressure on tumour cells, thus resulting in survival of less immunogenic variants. The third one – the escape phase – consists of uncontrolled expansion of tumour cells which are insensitive to immunological detection.

There are many possible ways in which a tumour cell can reach the escape phase. To mention only a few of these, we encounter non-activated antigen-presenting cells, a lack of helper T cells, a suppression mediated by regulatory T cells, paucity of CTLs, a low avidity of CTLs' TCR, deficient receptor signalling, low CTLs' lysis capacity or T-helper-2-cell polarized cytokine release. Moreover, the immune response might be blocked by mechanisms including failure of T cells to traffic to tumour sites (high intestinal pressure, defective blood vessels, a lack of lymph vessels), production of immunosuppressive factors by the tumour, or CTL apoptosis on encountering tumour cells. Finally, the tumour cells might acquire resistance to CTL through insufficient release of tumour-associated antigen through the tumour's stroma, loss of tumour antigen expression, reduced expression of co-stimulatory molecules, loss or down-regulation of human-leukocyte antigen (HLA) expression, acquisition of resistance to CTL lysis, or loss of capacity for apoptosis.

1.2 Immunotherapy

1.2.1 Approaches to immunotherapy

Since the introduction of vaccine immunization through Jenner in 1796, immunotherapy has made a tremendous progress. Among many other things, this includes numerous successful applications of active immunotherapy approaches in the form of prophylactic vaccines for acute self-limiting infections such as smallpox, cholera, varicella, mumps, poliomyelitis, and tetanus (Waldmann, Nat Med., 2003).

Also the toxin isolation of diphtheria bacterium by Roux and Yerson in 1888 inaugurated an enormous development in the field of passive immunotherapy. Immunotherapy moved all the way from initial antitoxin-containing antisera, through the usage of immunoglobulins, development of hybridoma technology for monoclonal antibody generation up to the production of antibodies armed with toxins or radio nuclides (Waldmann, Nat Med., 2003).

Despite many undisputable successes in both active as well as passive immunotherapeutic approaches, these therapeutic affords have been less effective in the abatement of chronic infectious diseases and cancer. Until now all efforts to produce a safe and efficient vaccine against human immunodeficiency virus or hepatitis C virus (HCV) have failed. Similarly, despite so many approaches, none of those vaccines so far tested towards non-virus associated cancer have brought any real breakthrough. Nonetheless, it should be mentioned that with the discovery of tumour-associated antigens in late seventies by Baldwin and Moore and the development and use of alternative techniques for its recognition involving biochemical techniques like high performance liquid chromatography and mass spectrometry, cDNA expression cloning, serological screening of cDNA expression libraries (SEREX) genetic linkage analysis, or so called “reverse immunology” with bioinformatic algorithms, we are getting closer to the target.

Up till now, many different series of vaccine formulations have been developed and tested in clinical trials: *ex vivo* peptide-loaded DCs, recombinant viruses or bacteria expressing the antigen as well as toll-like receptor ligands, DNA or RNA vaccines. Because of the rather weak T cell response developed by the majority of vaccinated patients, cancer vaccine approaches have not yet moved beyond clinical trials (Rosenberg, N Engl J Med., 2004).

Almost parallel to the research on cancer vaccines, yet another passive immunotherapeutic approach has been introduced, namely that of adaptive cell transfer (see Chapter 1.4 Adoptive cell transfer).

1.2.2 Haematopoietic steam cell transplantation

One of the greatest advances of immunotherapy is the application of allogenic haematopoietic stem cell transplantation (HSCT). Although this procedure had originally been developed for the prevention of bone marrow toxicity due to very intensive chemotherapy and radiotherapy

regimens, it soon became the only curative therapeutic option for many subtypes of leukaemia.

Subsequent to allogenic stem cell transplantation, there occurs a potent reciprocal immune reaction between donor and recipient which leads to graft versus tumour effect or, in the case of leukaemia to graft versus leukaemia (GvL). Graft versus leukaemia is normally accompanied by graft versus host disease (GvHD). The reason for the presence of both lies in the existence of polymorphisms in endogenous proteins which gives rise to so-called minor histocompatibility antigens (mHAgs). It had been demonstrated that CD8+ mHAgs-specific CTLs can inhibit the growth of leukemic colonies (Falkenburg et al., J Exp Med 1991) and prevent the engraftment of acute myeloid leukaemia (AML) in NOD/SCID mice (Bonnet et al., PNAS 1999). Moreover, the application of donor lymphocyte infusion (DLI) in cases of relapse after HSCT may lead to durable remission. These data proved the potency of the GvL effect (Kolb et al., Blood. 1995). It had also been shown that, apart from CD8+ and CD4+ T cells, the GvL effect is also mediated by natural killer cells (Parham and McQuenn, Nat Rev Immunol. 2003).

Since GvL is accompanied by life threatening GvHD, many attempts have been made to separate them from each other. The first of these, aiming at T cell depleted transplants, ended in a higher rate of relapse (Horowitz et al., Blood. 1990), accompanied by a higher occurrence of infections and a higher incidence of graft failure. On the other hand, antigen-specific CTLs directed towards minor histocompatibility antigens or tumour-associated antigens would, theoretically, reach the tumour site without destroying any of the peripheral tissues.

1.3 Adoptive T cell transfer

As already mentioned in regard to the case of relapse or resistant infection after HSCT, and in many others clinical situations, after an exhaustion of standard procedures, an adoptive T cell transfer seems to be the only experimental therapeutic option for many patients with a viral infection or a tumour disease (Riddell et al., Science 1992; Dudley and Rosenberg, Nat Rev Cancer. 2003).

The first proofs of a successful assignment of that approach based on the application of antigen-specific T cells were published 40 years ago (Delorme and Alexander, Lancet. 1964).

The concept was first applied to patients with a metastatic melanoma by Rosenberg in the late nineties. He demonstrated a successful application of tumour infiltrating lymphocytes (TILs) and IL-2 (Rosenberg et al., N Engl J Med., 1988). This was followed by very optimistic reports from other research groups. In those first reports, the adoptive T cell transfer was claimed to be a very effective and almost adverse side effect-free procedure. Similarly, Epstein Barr-Virus- (EBV) and cytomegalovirus- (CMV) -specific T cells have successfully restored antiviral immunity after HSCT (Riddel et al. Science. 1992; Walter et al., N Engl J Med. 1995).

For EBV-associated lymphoma as well, an adoptive T cell transfer approach proved to have an antiviral activity which prevent developing EBV-associated immunoblastic lymphoma (Rooney et al., Blood, 1998). Unfortunately, among most patients with non-virus-associated tumours, the efficacy of adoptive T cell transfer has remained limited. One of the reasons for this is the lack of a safe, reliable, effective and easily accessible method for the generation of antigen-specific T cells. Despite evident advances in *in vitro* expansion methods, application of tumour infiltrating lymphocytes, incorporation of professional antigen presenting cells, HCMV-infected autologous fibroblasts or EBV-infected lymphoblastoid cell lines (LCLs), most of these methods of generation were unfortunately not successfully applicable on a large enough scale.

Generation of a large amount of highly avid, relatively young specific T cells, capable of proliferation and trafficking to tumour or infection site antigens – and that all within a relatively short time – still remains a challenge.
In principle, there are three main strategies by which T cell generation can be approached:

- *in vitro* enrichment and expansion of *in vivo* primed antigen-specific T cells,
- *in vitro* induction and expansion of naive or less-frequent T cells, and
- *in vitro* generation of T cells having the desired specificity through TCR-gene transduction.

1.3.1 Expansion of *in vivo* primed T cells for adoptive cell transfer (ACT)

Rosenberg and colleagues were one of the first groups, who, through isolation and *in vitro* cultivation of tumour infiltrating lymphocytes were able to successfully carry out this therapeutic approach for a melanoma patient (Rosenberg et al. N Engl J Med. 1988).

According to the data published, upon application of TILs, objective response rates between 49% and 72% were observed. Moreover, in a recent trial involving 25 patients receiving maximum lymphodepletion, seven of the 25 patients (28%) achieved a complete response (Rooney, Curr Opin Immunol. 2009) Although this strategy has yielded a successful application among melanoma patients, it cannot be widely used due to limitations in availability and immunogenicity of the tumour.

One of the methods used for enrichment of antigen-specific T cells is IFN-γ capture assay (Brosterhus et al., Eur J Immunol. 1999). Antigen-specific T cells secrete IFN-γ after peptide stimulation. Since interferon gamma is essential for tumour rejection, a selection of previously activated T cells allows their specific isolation. It could be shown in a mouse model that IFN-γ-positive T cells from tumour-immunized mice are cytolytic and mediate tumour rejection upon adaptive transfer (Becker et al., Nat Med. 2001).

A similar approach which enriches nearly a complete repertoire of antigen-specific T cells is one based on the expression of CD137 by activated T cells (Wolf et al., Blood 2007). CD137-positive selected cells elicit a heterogeneous function including both memory and naive phenotypes. Furthermore, CD137 mediates anti-apoptotic functions that promote T-cell proliferation and T-cell survival.

Apart from the enrichment of activated T cells, several other attempts have been made to isolate T cells of the desired specificity. These are based on MHC-tetramer technology to isolate antigen-specific T cells with a defined T cell receptor (TCR) (Szmania et al., Blood 2001). Although this approach had been claimed to deliver promising results, it has still not yet been used for any routine applications in the clinic (Cobbold et al., J. Exp. Med. 2005). One of the disadvantages here is the single specificity, which poses an increased risk for the selection of tumour escape variants. Moreover, since the enrichment process is not based on T cell function, many of the T cells isolated may be anergic. Additionally, until now, use of tetramer technology has only allowed the isolation of CD8+ T cells. Whether or not the co-isolation of CD4 positive T cells is in fact essential for tumour rejection remains uncertain. Some of the critique towards MHC-tetramer strategy was aroused due to the irreversible binding of TCR, a property which may hamper T cell function. While searching for new strategies which would evade this obstacle, a reversible MHC multimer staining method was introduced (Knabel et al. Nat Med. 2002). This technique makes it possible to isolate only the

desired T cell population, claims to be more efficient than other enrichment methods and seems to be less traumatic for the isolated cells. In contrast to the tetramer isolation strategy, it does not reduce the cytotoxic potential of isolated cells (Neudorfer et al., J Immunol Methods. 2007).

1.3.2 *In vitro* induction and expansion of naive antigen-specific T cells for ACT

Antigen-specific T cells can also be efficiently primed and expanded *in vitro* using different techniques. ACT can be based on protocols which include the generation of memory T cells from naive precursors by patients with insufficient or lacking immune response as well as from patients under immunosupression.

There are many protocols which make use of tumour lysates and successfully prime T cells because of their high immunogenicity (Kurokawa et al., Int J Cancer. 2001; Montagna et al., Int J Cancer., 2004). A similar phenomenon has been observed by induction with fungal or viral lysates (Tramsen et al. Bone Marrow Transplant, 2008). Unfortunately, the possible presence of live tumour cells or pathogens is considered a serious threat. Because of this, they are consistently disapproved by competent authorities as being an optimal antigen source for adoptive T cell transfer.

Another approach for successful priming is based on the application of different antigen-presenting cells such as those of EBV-immortalized B-lymphoblastoid cell lines (B-LCLs) (Rooney et al., Blood 1998), CMV-infected autologous fibroblasts (Riddell et al., Science. 1992), or genetically modified APCs. Unfortunately, also here the potential biohazard resulting from the presence of live viruses may not meet the current good manufacturing practice (GMP) standards.

A special type of APCs is that represented by peptide or antigen-pulsed DCs. Meanwhile, it is possible to generate DCs according to GMP standards. However, most of the protocols require an enormous amount of time and cost consuming generation procedures. Thus, these render themselves almost inapplicable for any clinical trial. In order to approach this problem, several groups like Ho and colleagues (Ho et al., J Immunol Methods. 2006) based their protocol for the generation of Wilms' tumour antigen 1 (WT-1) antigen-specific T cells on short-time DCs.

Another example of using antigen-presenting cells was provided by Schultze and colleagues (Schultze et al., J Clin Invest. 1997). They used CD-40 activated B-cells. Although this is a very effective method which can indeed be applied in those cases where the number of cells is low, they still do not fulfil GMP requirements because they make use of mouse fibroblast cell lines. That very promising approach already developed in 1999, substituting mouse fibroblasts by a dissoluble, trimerised CD40-antibody has not yet been applied clinically.

1.3.3. *In vitro* generation of T cells with gene engineering methods for ACT

1.3.3.1. *In vitro* generation of T cells with defined TCR

The endogenous T cell repertoire for tumour-associated antigens is limited in size or activity by self-tolerance. Because of this, there is a third concept, which is based on gene T cell engineering. Furthermore, there are mainly three different strategies to overcome these limitations which are being tested in the clinic. With the help of lentiviral or retroviral constructs, lymphocytes can undergo transduction with TCR of a desired specificity. In this manner, the size limitation and avidity repertoire of tumour-associated specific T cells could be easily supplemented. Until now, new high-avidity TCRs have been generated basically in two ways: *in vivo* in HLA-transgenic mice (Kuball et al., Immunity, 2005) or *in vitro* by implementation of a phage display system (Li et al., Nature Biotechnology, 2005).

The idea behind the transfer of TCR derived from transgenic mice for HLA molecules is that the TCR repertoire in mice is not affected by negative selection in the thymus. Therefore it will definitely contain high-avidity TCRs and will allow the circumvention of tolerance to tumour-associated antigens (TAA). These ideas, already tested by Sherman and colleagues in a mouse model, seem to be very promising (Sherman et al., Crit Rev Immunol. 1998). Via protein-DNA interactions that utilize bacteriophages, the phage display systems connect proteins with the genetic information. This allows large libraries of proteins (as well as different human TCRs) to be screened and amplified in a process of *in vitro* selection, which is analogous to natural selection.

Apart from TCR display systems, many efforts are being undertaken in the direction of engineering approaches such as TCR gene sequences optimisation, inclusion of murine constant domains, or inclusion of an engineered disulfide bond. All of these can be used to

enhance the expression of the TCR introduced. Additionally, the latter two approaches can also suppress the formation of bispecific TCRs which are composed of endogenous and exogenous TCR chains, likely contributing to the safety of the therapy (Uckert and Schumacher, Cancer Immunol Immunother. 2009)

A second strategy is based on the introduction of chimeric receptors that have antibody-based external receptor structures and cytosolic domains that encode signal transduction molecules of the T cell receptor (Eshhar et al. J.Immunol. Methods, 2001). This approach using T cells expressing a T body receptor specific for a folate-binding protein has recently been tested among ovarian carcinoma patients (Kershaw et al. Clin. Cancer Res., 2006). This trial appeared to be safe, but the expression and persistence of transferred T cells were poor. On the other hand, Lamers and colleagues observed an unexpected serious hepatic toxicity when testing T cells which expressed a T cell body receptor for carbonic anhydrase IX in a group of renal carcinoma patients. Furthermore, this trial shows how important the choice of targets for chimeric antigen receptor is in regard to safety measures.

The third approach involves improving receptor design by optimisation of ligand-binding domain and by incorporation of a co-stimulatory domain into the signalling module (Sadelain et al., Nat Rev Cancer. 2003). These changes should ensure two qualities of the modified T cells. Firstly, T cells can not only be specific, but also capable of being disengaged from the target in order to approach other cancer cells. Secondly, proper co-stimulatory signals delivered upon tumour recognition will avoid induction of anergy or apoptosis (Riley and June, Blood, 2005) and increase T cell resistance towards the tumour microenvironment (Loskog et al., Leukemia, 2006).

1.3.3.2 ACT-*in vitro* generation of T cells expressing the suicide gene

Donor lymphocyte infusion almost became a standard therapy option for patients with a relapse after allergenic T cell transplantation. Nevertheless, apart from the desired GvL effect, a severe and potentially lethal GvHD can occur. In order to keep both of these reactions separated, an approach with herpes simplex virus thymidine kinase (HSV-TK) has been developed. T cells transduced with HSV-TK can be ablated after administration of acyclovir or ganciclovir (Ciceri et al., Lancet Oncol. 2009). The infusion of suicide-gene transduced T cells seems to promote immune reconstitution after HSCT and thereby induce an anti-tumour

effect. Application of DLI elicits an onset of GVHD, which can be abrogated with a ganciclovir-induced elimination of HSV-TK transduced T cells (Ciceri et al., Lancet Oncol. 2009).

1.4 Strategies to augment the outcome of adaptive T cell therapies

A search for improvements in adoptive T cell transfer considers not only T cells which are about to infuse, but also the microenvironment of the host. Until now, several strategies that aim to augment the function of adaptively transferred T cells have been tested. In order to impact persistence of adaptively transferred T cells, different cytokines have been administered. According to recent studies, IL-2 application may on the one hand induce the proliferation and maintenance of effector CTLs. On the other hand, this might actually increase the number of T-regs and deplete memory CTLs. In contrast to IL-2, IL-7 and IL-15 seem to promote persistence of memory CTLs and might even decrease the ratio of T-regs to effector T cells (Ku et al., Science, 2000). Moreover, several other substances have been under investigation: an antibody that mediates blockage of a cell surface molecule (CTLA4) (Sutmuller et al., J.Exp.Med., 2001), an anti-transforming growth factor-β (anti-TGFβ) (Suzuki et al., Clin. Cancer Res., 2004) and anti-programmed death 1 (anti-PD-1) antibodies (Barber et al., Nature, 2006).

An enormous increase in efficacy and a high frequency of adverse affects have led to the application of T cells to lymphopenic hosts. It has been shown that lymphopenic environments can provide advantages for some populations of T cells. Furthermore, by eliminating T-regs and competing cell population, this can enhance the availability of important cytokines such as IL-2, IL-7 and IL-15. Under these conditions, T cells undergo homeostatic expansion more easily and can thus increase their activation status. Alongside investigations concerning the challenging host, several attempts have been undertaken to challenge tumour bed via chemotherapy (Bergmann-Leitner et al., J.Immunol. 2000), chemokine expression, cryoablation and sensitization of tumour stroma (Zhang et al., J.Exp. Med., 2007).

1.5 Aim of the thesis

This thesis should provide an evaluation of a protocol for an induction, activation and expansion of naïve (or low frequent) CD8+ T cells in order to achieve high avidity-specific T

cells for adoptive cell transfer. Moreover, the prepared protocol should meet following standards:

- be conducted in a short time and generate specific cells in HLA.A 204 donors
- generate high number of specific T cells from limited amount of blood (maximum normal donation – 500ml)
- do not contain infectious materials which, by adjusting the protocol to the GMP standards, could be later difficult to substitute.

To address those questions several steps were undertaken:

1. The starting point considered testing different unspecific stimulating factors for their effectiveness during the induction process. It answered the question if the addition of such unspecific stimulators would be advisable.
2. This part was followed by comparison of modified but already existing protocols. The aim of this was to check the effectiveness of those protocols. It considered:
   - various cytokine cocktails,
   - different restimulation modules: considering autologous, allogenic or mixture of autologous and allogenic feeder cells
   - variety of viral antigens: HIV, HBV, HCV
3. The next step compared favorable usage of professional antigen presenting cells with direct addition of peptide to the culture.
4. Experiments following that part should have shown different responsiveness to antigens depending on culture condition. (HIV, HBV, HCV, Ras antigens were tested).
5. The comparison of modified protocols was performed with tumor antigens in order to investigate whether there are major differences between them and viral antigens.
6. From tumor antigens, one was selected and tested under different conditions in order to choose the most suitable environment for the generation process
7. Generation process was conducted under GMP conditions in order to check its applicability.

II. Materials and methods

2.1 Donors

A total number of 10 HLA.A_2-positive healthy, donors were recruited after HLA-typing as suitable for the study. All donors serologically tested HIV-negative, six of them were vaccinated against hepatitis B and two were HCV-seropositive but without any evidence of liver disease in the past. The virological diagnostics using standard serological and molecular techniques were performed by the Institute for Microbiology, Charité, Campus Benjamin Franklin (see Table 1).

Table 1 Donors characteristic.

	No.	Donor	HIV-ELISA	HBs-antigen	anti-HBs	anti-HBc (core-antigen)	Hepatitis C antibodies
Experiments with viral antigens	1	JaHe	neg.	neg.	neg. (0.1)	neg.	neg.
	2	JoZa	neg.	neg.	neg. (10.6)	neg.	neg.
	3	IlKa	neg.	neg.	neg. (0.1)	neg.	neg.
	4	MaSc	neg.	neg.	neg. (45.9)	neg.	neg.
	5	FaGü	neg.	neg.	pos.(>1000)	neg.	neg.
	6	AnNe	neg.	neg.	pos.(>1000)	neg.	neg.
Experiments with TAA	7	DeKu	neg.	neg.	pos. (49.7)	neg.	neg.
	8	InKö	neg.	neg.	pos. (>1000)	neg.	neg.
	9	BäKi	neg.	neg.	pos.(>1000)	pos.	neg.
	10	BaKo	neg.	neg.	pos.(>1000)	pos.	neg.

2.2 Cell lines

2.2.1 T_2 cells

T_2 cells (human-human somatic cell hybrids) were obtained from Prof. Dr. C. Scheibenbogen. This line was originally established by PEG-mediated fusion of the B-lymphoblastoid cell line (B-LCL) with a variant of the T-LCL CEM (Salter et al., Immunogenetics, 1985). This cell line is known for expressing small amounts of HLA.A2 antigen on the cell surface and therefore was used as target in intracellular IFN-γ secretion and ELISpot assay.

2.2.2 K-562 HLA.A2 cells

K-562 HLA.A2 were obtained from Prof. Dr. Huber, Mainz. K-562 is a human chronic myeloid leukemia cell line. Originally established from the pleural effusion of a patient with chronic myeloid leukemia (CML) in 1970. Cells were transduced with HLA.A2 molecules and used as targets in intracellular IFN-γ secretion and ELISpot assay.

2.3 Laboratory equipment

CO_2-Incubator	Heraeus, Germany
Heraeus, Germany	BD, Germany
CellQuest Software	BD,Germany
Magnet Dynal MPC-L	Dynal Biotech GmbH, Germany
Neubauer-counting chamber	LO – Laboroptik GmbH, Germany
Microscope ID 03	Carl Zeiss, Germany
Laminar airflow workbench	Nuaire, USA
Water bath	Dinkelberg GmbH, Germany
Centrifuge Labofuge 400e	Kendro Laboratory Products, USA
Chemiluminescent camera	Raytest, Germany
Software Aida Image Analyzer v3.21	Raytest, Germany

2.4 Plastic and glass materials

Blood bag	Fresenius HemoCare GmbH, Germany
Cell scraper 24 CM	TPP®, Switzerland
Combitips plus 10 ml	Eppendorf-Netheler-Hitz-GmbH, Germany
Disposable Filter Unit 0.2µm Cellulose acetate	Schleider&Schuell MicroScience GmbH, Germany
Stericup ™ – 150 ml	Milipore, USA
Tissue culture plates:	
6, 12, 24, 48, 96 -well Plates	Nunc ™, Denmark
MultiScreen™, Sterile Clear Plates	Millipore, USA
Falcon 14 ml round bottom tube, polypropylene	BD Labware, USA
Falcon 5 ml round bottom tube, polystyrol	BD Labware, USA
Nalgene Cryo 1°C Freezing Container	Nunc™, Denmark
Pipets: 1, 2, 5, 10, 25 ml	Becton Dickenson, USA

Pre-sterilized tips ART 1000 (100-1000µl)	MβP Molecular BioProducts, USA
Pipette tips, 2- 200µl; 100-1000µl	Brand GmbH, Germany
Tissue culture flask 75cm^2	Sarstedt, USA
Tissue culture flask	NUNC^{TM}, Denmark
Reagent Reservoir 50ml	Corning, SA
Reagent Reservoir	Bel-Art, USA

2.5 Reagents and chemicals

Brefeldin A 0.5 mg/ml	Sigma – Aldrich, Germany
CFSE	Invitrogen GmbH, Germany
Biocoll Separating Solution	Biochrom AG, Germany
Bovine Serum Albumin (BSA) 10 mg/ml	Sigma, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Germany
EDTA	Sigma, Germany
Ethanol	Sigma-Aldrich, Germany
FACS-Lysing solution	BD, Germany
FACS-Permeabilizing solution	BD, Germany
Formaldehyde, 37% solution in water	Sigma-Aldrich, Germany
Herpes Buffer 1M 500 m	Biochrom AG, Germany
Human IgG Flebogamma	Griflos GmbH, Germany
NaN_3 (Sodium azide)	Sigma, Germany
Na_2CO_3 Sodium Carbonate	Sigma-Aldrich, Germany
$NaHCO_3$ Sodium Bicarbonate	Sigma-Aldrich, Germany
Penicillin/Streptomycin	Gibco,Invitrogen GmbH, Germany
PBS – Dulbecco o/w Ca^{2++}/Mg^{2++}	Biochrom AG, Germany
Phosphate buffered saline (PBS)	Phosphate buffered saline (PBS)
Protein Transport Inhibitor (containing monensin)	BD Bioscience Pharmingen, USA
Trypan Blue Solution	Sigma-Aldrich, Germany

2.6 Cell culture additives and components

2.6.1 Sera

Foetal Bovine Serum 500ml	Invitrogen GmbH, Germany
Human AB Serum Type AB	Sigma-Aldrich, USA

2.6.2 Cultures' media

RPMI 1640	Biochrom AG, Germany

Iscove Medium 500 ml	Invitrogen GmbH, Germany
Iscove Mod. Dulbecco's 500ml	Invitrogen GmbH, Germany

2.6.3 Freezing media

90% FCS (Final concentration)	Biochrom, Germany
10% DMSO (Final concentration)	Sigma-Aldrich, USA

2.6.4 Antibodies

OKT-3	e-Bioscience, USA
Mouse anti-human CD45RA 0. mg	BD, Germany
Mouse anti human CCR7 0.25mg	BD, Germany

2.6.5 Cytokines

Table 2 Cytokines used for cultivation of dendritic cells, culture cells and cell lines. Manufacturer, stock concentration and final concentration are listed. Cytokines were dissolved in PSA or culture medium and stored at -80˚C or -20˚C.

Cytokine	Stock concentration	Final concentrations	Manufacturer
Human IL-1β	5µg/ml	10ng/ml	CellGenix, Germany
Human IL-2 (Proleukin®)	18 x 10^6IU/ml	30-300IU/ml	Chiron Corporation, USA
Human IL-4	10µg/ml	50ng/ml	Cell Concepts GmbH, Germany
Recombinant human IL-6,	10^5IU/ml	1000IU/ml	R&D Systems, Germany
Recombinant human IL-7,	1µg/ml	20ng/ml	R&D Systems, Germany
Recombinant human IL-10	1µg/ml	10ng/ml	R&D Systems, Germany
Recombinant human IL-12	1µg/ml	0.5; 0.1; 5ng/ml	R&D Systems, Germany
Recombinant human IL-15,	5µg/ml	5ng/ml	R&D Systems, Germany
GM-CSF (Leukine®)	2.8 x 106 IU/ml	100ng/ml	Berlex, USA
PGE_2 (Minprostin E_2)	5µg/ml	1µg/ml	Pharmacia GmbH, Germany
TNF-α	2.7 x 10^3IU/ml	10ng/ml	CellGenix, Germany

2.7 Immunological method material

2.7.1 ELISpot

DAB Peroxidase Substrate (3,3' – Diaminobenzidine)	Sigma-Aldrich, Germany

2.7.2 Cell separation products

CD8 Negative Isolation Kit	Dynal® Biotech GmbH, Germany
Anti-human CD 45RA Particles	BD Bioscience Pharmingen, USA
BD™ IMagnet	BD Bioscience Pharmingen, USA
Dynabeads M450 Rat anti-mouse	Dynal Biotech GmbH, Germany
Dynabeads M450 Sheep anti-mouse	Dynal Biotech GmbH, Germany
MPC-L Magnet	Dynal Biotech GmbH, Germany

2.7.3 Peptides

2.7.3.1 Viral peptides

Table 3 All viral peptides were HLA-A*0201-restricted peptides and were synthesized and HPLC-purified by NMI Technologietransfer (Reutlingen, Germany).

	Epitope		Sequence	Reference
HIV epitopes group	p17 77-85	HIV 1s	SLYNTVATL	Ferrari et al., 2000
	RT 476-484	HIV 2i	ILKEPVHGV	Altfeld et al, 2000
	Gag-433	HIV3f	FLGKIWPS	Cohen et al, 2002
	VPR 59	HIV 5a	AIIRILQQL	Gruters et al, 2002
	Nef 180-189	HIV 6v	VLEWRFDSRL	Ferrari et al., 2000
HBV epitopes group	Core 18-27	HBV1f	FLPSDFFPSV	Livingston et al, 1999
	Env335-349	HBV2w	WLSLLVPFV	Webster et al, 2001
	Pol 455-463	HBV 3g	GLSRYVARL	Webster et al., 2002
	Pol 575-583	HBV 4f	FLLSLGIHL	Livingston et al, 1999
	HBx 52-60	HBV 5h	HLSLRGLFV	Livingston et al, 1999
	HBx 115-123	HBV 6c	CLFKDWEEL	Webster et al., 2002
HCV epitopes group	Core 131-140	HCV 1a	ADLMGYIPLV	Scognamiglio et al., 1999
	NS3 1073-1081	HCV 2c	CINGVCWTV	Cerny et al, 1995
	NS3 1406-1415	HCV 3k	KLVALGINAV	Urbani et al., 2001
	NS4 1789-1797	HCV4s	SLMAFTAAV	Scognamiglio et al., 1999
	NS5B 2594-2602	HCV 5a	ALYDVVTKL	Cerny et al, 1995
	Flu matrix$_{58-66}$	Flu	GILGFVFTL	Bednarek et al., 1991

2.7.3.2 Tumour peptides

Table 4 Tumour peptides used for induction and generation of antigen-specific T cells. All of the following epitopes are HLA-A*0201-restricted peptides and were synthesized and HPLC-purified by Biosyntan GmbH, Berlin. All Ras peptides were HLA-A*0201-restricted peptides and were synthesized and HPLC-purified by NMI Technologietransfer (Reutlingen, Germany).

	Epitope	Sequence	Reference
AML epitopes group	AM 1 183	FLKANLPLL	Maeda et al., 2000
	WT-1 (DB126)	RMFPNAPYL	Oka et al., 2000
	WT-1(WH187)	SLGEQQYSV	Oka et al., 2000
	WT-1 (WH242)	NLGATLKGV	Oka et al., 2000
	WT-1 37	VLDFAPPGA	Smithgall M, 2001
	p53 (65-73)	KLVPDSLYV	Theobald et al., 1995
	p53 (264-272)	LLGRNSFEV	Theobald et al., 1997
	Sur1	LTLGEFLKL	Andersen et al., 2001
	Sur9	ELTLGEFLKL	Schmitz et al., 2000
	pTEN (566-574)	TLCQAALLL	Poetsch et al., 2001
CML epitopes group	b3a2P3	GFKQSSKAL	Nieda et al., 1998
	b3a2P4	SSKALQRPV	Yotnda et al., 1998
Breast tumour epitopes group	cdr 2-1	KLVPDSLYV	Albert et al., 1998
	CEA 571	YLSGANLNL	Zhuet et al., 2000
	HER2 (369)	KIFGSLAFL	Fisk et al., 1995
	Muc 1.1	STAPPVHNV	Brossart et al., 1999
	h-TERT	ILAKFLHWL	Minev et al., 2000
Melanoma epitopes group	Ep-3	ILYENNVITI	Ras et al, 1996
	gp100 (457)	LLDGTATLRL	Kawakami et al., 1995
	MAGE-3	FLWGPRALV	Gaugler et al., 1994
	PRAME (PRA142)	SLYSFPEPEA	Kessler et al., 2001
	PRAME (PRA425)	SLLQHLIGL	Kessler et al., 2001
K-ras epitopes group	Ras12C	KLVVVGACGV	Barbacid et al., 1987
	Ras12D	KLVVVGADGV	Barbacid et al., 1987
	Ras12G	KLVVVGAGGV	Barbacid et al., 1987
	Ras12R	KLVVVGARGV	Barbacid et al., 1987
	Ras12S	KLVVVGASGV	Barbacid et al., 1987
	Ras12V	KLVVVGAVGV	Barbacid et al., 1987

2.7.3.4 FACS – Antibodies

Table 5 FACS antibodies used for detection of cell surface antigens and cytokines. Fluorochrome conjugations: FITC – fluorescein isothiocyanate, PE – phycoerythrin, PerCP - peridinin-chlorophyll-protein, APC – allophycocyanin, Alexa Fluor 647.

Antibody specificity	Fluorochrome conjugation	Isotype	Manufacturer
Anti-human CCR7	Alexa Fluor	Rat IgG2a, k	BD Pharmingen™
Anti-human CD3	PerCP	Mouse IgG1,k	BD Pharmingen™
Anti-human CD8	FITC	Mouse IgG1, k	BD Pharmingen™
Anti-human CD8	APC	Mouse IgG1,k	BD Pharmingen™
Anti-human CD27	FITC	Mouse IgG1,k	BD Pharmingen™
Anti-human CD28	APC	Mouse, IgG1,k	BD Pharmingen™
Anti-human CD45RA	APC	Mouse IgG2b,k	BD Pharmingen™
Anti-human CD45RO	FITC	Mouse IgG2a, k	BD Pharmingen™
Anti-human CD57	FITC	Mouse IgM, k	BD Pharmingen™
Anti-human CD62L	APC	Mouse, IgG1, k	BD Pharmingen™
Anti-human CD62L	PE	Mouse IgG1,k	BD Pharmingen™
Anti-human CD69	APC	Mouse IgG1, k	BD Pharmingen™
Anti-human CD95	FITC	Mouse IgG1,k	BD Pharmingen™
Anti-human 107a	FITC	Mouse IgG1, k	BD Pharmingen™
Anti-human IFN-γ	FITC	Rat Ig1, k	BD Pharmingen™
Anti-human IFN-γ	PE	Mouse IgG1, k	*Miltenyi* Biotec
Anti-mouse IgG1,k	FITC	Mouse IgG1,k	BD Pharmingen™
Anti-mouse IgG, k	APC	Mouse IgG1,k	BD Pharmingen™
Anti-mouse IgG2a, k	FITC	Mouse IgG2a, k	BD Pharmingen™
Anti-mouse IgG 2b, k	APC	Mouse IgG2b, k	BD Pharmingen™
Anti-mouse IgM, k	FITC	Mouse IgM, k	BD Pharmingen™
Anti-rat IgG2a, k	AlexaFluor 647	Rat IgG2a,k	BD Pharmingen™

2.7.3.5 MHC-multimers

Table 6 Pentamers used for antigen-specific T cell receptor staining. All pentamers were designed for HLA.A*0201 epitopes and were synthetised by ProImmune Ltd., United Kingdom.

Epitope	Sequence	Concentration (µg/ml)	Approximate µl needed per test	Reference
HIV 1s	SLYNTVATL	45	11	ProVe™ MHC Pentamer
HIV 2i	ILKEPVHGV	32	16	ProVe™ MHC Pentamer
HIV 5a	AIIRILQQL	34	15	ProVe™ MHC Pentamer
HCV 2c	CINGVCWTV	25	20	ProVe™ MHC Pentamer
HCV 3k	KLVALGINAV	26	19	ProVe™ MHC Pentamer
HCV 4s	SLMAFTAAV	46	11	ProVe™ MHC Pentamer
HBV 1f	FLPSDFFPSV	54	9	ProVe™ MHC Pentamer
HBV 2w	WLSLLVPFV	47	11	ProVe™ MHC Pentamer
HBV 3g	GLSRYVARL	29	17	ProVe™ MHC Pentamer
HBV 4f	FLLSLGIHL	47	11	ProVe™ MHC Pentamer
Ras 12c	KLVVVGACGV	35	14	ProVe™ MHC Pentamer
Ras 12d	KLVVVGADGV	27	18	ProVe™ MHC Pentamer
Ras 12g	KLVVVGAGGV	44	11	ProVe™ MHC Pentamer
Ras 12r	KLVVVGARGV	26	19	ProVe™ MHC Pentamer
Ras 12s	KLVVVGASGV	29	17	ProVe™ MHC Pentamer
Ras 12v	KLVVVGAVGV	31	16	ProVe™ MHC Pentamer
Flu	GILGFVFTL	50	10	Pro5 Pentamer

2. 8 General cell culture methods

2.8.1 Isolation of PBMCs

The peripheral blood mononuclear cells (PBMCs) were collected from fresh heparinized venous blood using density gradient centrifugation according to Boyum's method (Boyum, 1968). This method was based upon the principle that different cell types vary in their density. Due to that fact density gradients are used to separate mononuclear cells (low density) from erythrocytes/ granulocytes (high density) and to separate live cells (low density) from dead ones (high density).

Collected from HLA-A2.1 healthy donors, the blood was diluted with sterile PBS in a ratio of 1:2. 10 ml of Ficoll Hypaque was placed in 50 ml Falcon centrifugation tubes and 40 ml blood suspension was carefully layered. After 40 minutes of centrifugation at 20°C at 400g with no *break* the erythrocytes and granulocytes sediment had settled on the bottom of the tube while PBMC were seen as delicate interphase situated above the Ficoll phase and under the plasma – PBS – phase. The interphase was carefully removed and retained without disturbing the erythrocyte and granulocyte pellet. In order to wash out cytotoxic Ficoll, PBMCs were washed three times and centrifuged at three different speeds: 400g, 300g and 200g. The cells were used immediately for experiments or frozen in 1-2x10^7 in 1 ml in frozen *media* and placed in a cryo-container with isopropanol in a -80°C freezer for 24 hours, and afterwards stored at -196°C in liquid nitrogen.

2.8.2 Counting of cells

In order to determine the cell count, 20 µl of the sample was mixed with 20 µl of trypan blue/ PBS dilution (1:2) and loaded into one of the chambers of the Neubauer slide. All the cells in at least two of the four quadrants in a chamber were counted, including cells placed on two of the four borderlines. The percentage of dead cells was estimated and the concentration of cells was calculated according to the following formula:

Cells/ml = cell count in 2 quadrants x 10^4/ ml x dilution factor

2.8.3 Cryopreservation of cells

Cells must be frozen for several reasons: to conduct experiments other than the day the cells were obtained and, what is more important, to maintain their phenotypic and genotypic character which may be lost during prolonged culture. Moreover, freezing minimizes problems of contamination as well as the overall expense of cells.

The cells to be frozen were pelleted at 1300 rpm and resuspended in a freezing medium consisting of FCS (sterile, heat-inactivated 1h at 56˚C) and 10% dimethyl sulfoxide (DMSO) in final concentration of 1-2 x 10^7. After that they were aliquoted to cryotubes of 1 ml and immediately put into a cryo-container with isopropanol in -80°C freezer. After 24 hours they were placed in liquid nitrogen. The most critical parameter for successful freezing of cells was

first of all the proliferation state of cells at the moment of freezing and the time between resuspending in freezing media and placing in freezer, this was important because of the toxic character of DMSO.

2.8.4 Thawing of cells

The correct thawing of cells is very important for saving their status. If done incorrectly it may induce the cells' decay. Cryovial should be prewarmed in 37˚C water bath for few minutes. Under tissue culture hood, 1 ml of thawing media (7 ml IM, 1 ml AB) should be added to vial and after gentle pipetting, the contents should be transferred to 15 ml Falcon tube with the rest of the thawing media. After spinning at 1300rmp for 10 minutes a supernatant should be discarded and cells resuspended in medium. Antigen-specific cells should be restimulated when thawed.

2.8.5 Radiation of cells

T_2 cells and PBMCs were radiated with 30Gy in order to stop cell proliferation.

2.8.6 Generation of dendritic cells

Dendritic cells were generated from blood monocytes. PBMCs obtained from HLA-A_2 positive donors after adjusting cell concentration at 0.6 x 10^7 were incubated for 2 hours. Plating 2 x 10^7 cells in 3 ml IM in tissues culture flasks was performed due to separate adherent monocytes from the nonadherent lymphocyte rich fraction. After 2 hours of incubation at 37°C in 5% CO_2, nonadherent cells were removed by gentle washing. The monocytes were cultured in IM supplemented with 10% AB serum, 8 ml Herpes Buffer for the next five days. Additionally GM-CSF and IL-4 were added at the final concentration of 10^3 IU/ml each. For the maturation of immature DCs, on the fifth day a following cytokines were added: IL-1β, IL-6, TNF-α and PGE_2. On the seventh day the mature dendritic cells were harvested, washed, centrifuged at 300g for 10 minutes and counted.

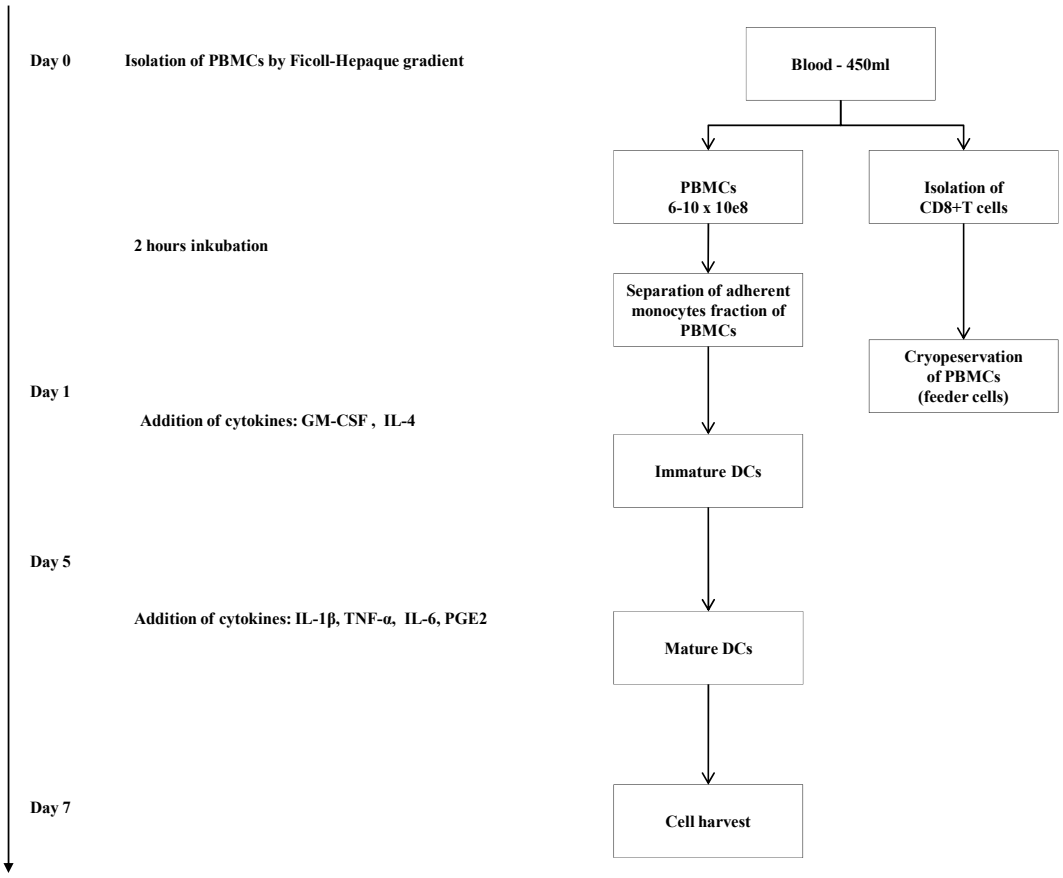


Figure 4 Schema for generation of mature dendritic cells.

2.8.7 Pulsing of T_2, PBMCs and dendritic cells with antigens

T_2 cells, PBMCs and dendritic cells were pulsed with antigens in a concentration of 1 mg/ml for 2 hours in culture medium (IM, 10% AB serum, 8 ml Herpes Buffer). After incubation, cells were washed twice with PBS.

2.8.8 Isolation of naïve T cells

For the untouched isolation of naïve T cells negative Pan T Cell isolation kit from Miltenyi Biotec was used. PBMC suspension was centrifuged for 10 minutes at 300 g and the supernatant was aspired completely. PBMCs were resuspended in 40 µl of PBS with 0.1% BSA per 10^7 total cells. 10 µl of Naive Pan T Cell Biotin-Antibody Cocktail was added to the cell pellet per 10^7 total cells. After mixing, the cells were incubated for 5 minutes at 2-8°C. Afterwards, 30 µl of buffer as well as 20 µl of Naive Pan T Cell MicroBead Cocktail were added per 10^7 cells. Cells were mixed and incubated for an additional 10 minutes at 2−8 °C. Before placing the cell

suspension within a MACS® Column in the magnetic field of a MACS Separator, the volume was adjusted to a minimum of 500 µl of PBS with 0.1% BSA. A flow-through cell population representing the enriched naive pan T cells was collected. After removal of the column from the separator and addition of 0.1% BSA and PBS solution, the magnetically labelled non-naive pan T cells were collected and FACS staining was performed (see Figure 5).

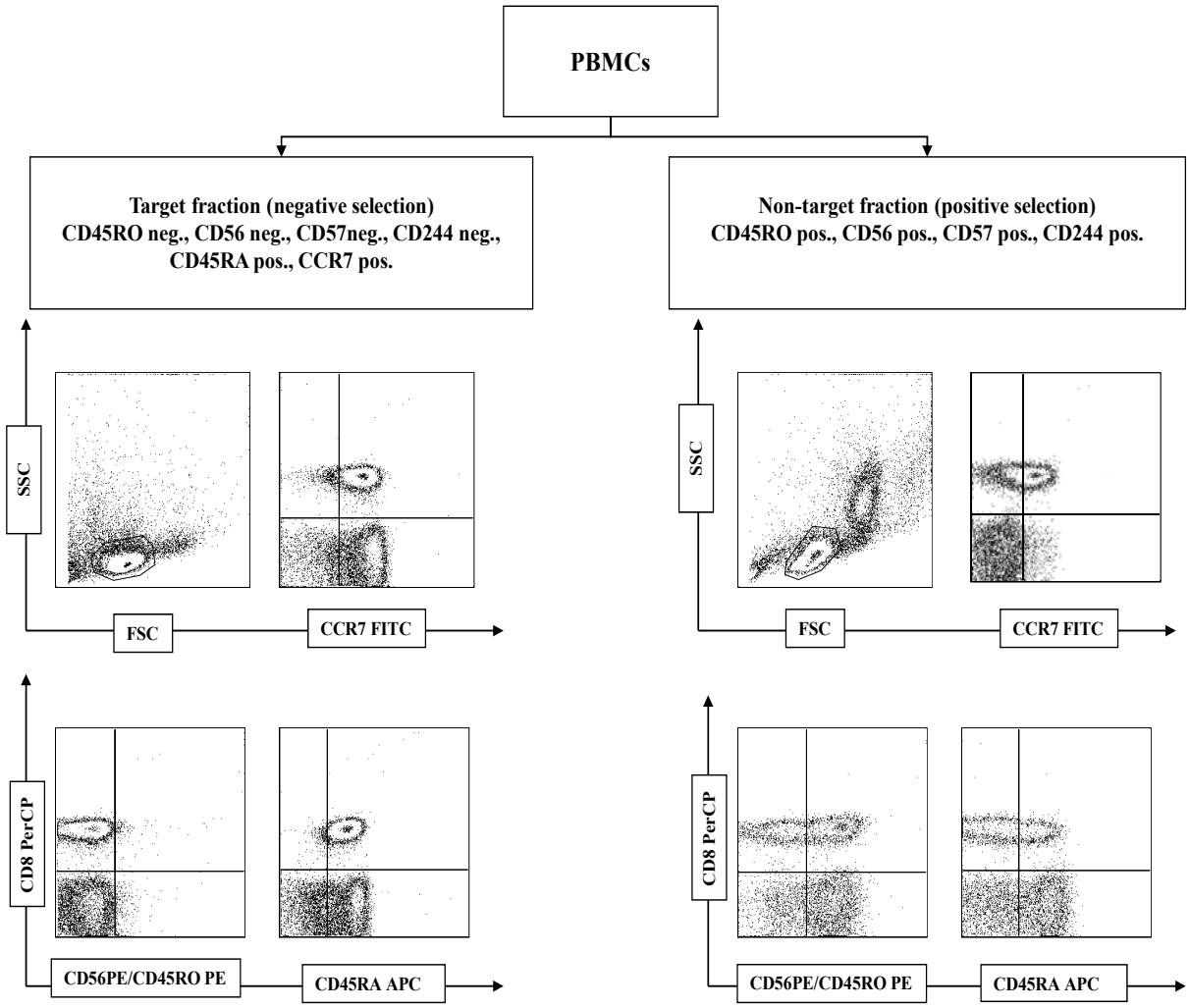


Figure 5 Naïve and memory T cells were isolated with naïve T cell isolation kit from Miltenyi. The untouched target fraction with naïve T cells was predominantly CD45RO negative, CD56 negative, CD57 negative, CD244 negative, CD45RA positive and CCR7 positive. Non-target fraction enriched by positive selection using biotin-conjugated antibodies against CD45RO, CD56, CD57, CD244 contained: memory/ effector T cells, activated T cells, NK cells and monocytes.

2.8.9 Isolation of CD8+ cells

For the untouched isolation of CD8+ cells a negative isolation kit was used. CD8+ cells were enriched from PBMCs by depletion of B cells, NK cells, monocytes, CD4+ cells and granulocytes. PBMCs were resuspended in 100 µl PBS with 0.1% BSA per 1 x 10^7. 20 µl heat-inactivated FCS and 20 µl antibody mix were added. After mixing, the cells were incubated for 15 minutes at 2-8°C. Afterwards cells were washed with PBS with 0.1% BSA (isolation media) and centrifuged for 8 minutes at 500g. Cells were resuspended in isolation media and 200 µl prewashed Depletion Dynalbeads were added. During 15 minutes of incubation at 20°C, cells were gently tilted and rotated. The generated Depletion Dynabeads/cell rosettes were carefully resuspended by pipetting. After increasing the isolation medium, a tube with cells was placed in magnet for 2 minutes and supernatant was transferred to a fresh tube. In order to boost the purity, this procedure was performed twice.

2.9. Protocols for generation of antigen-specific T cells

2.9.1 Stimulation of cells with unspecific stimulators

For unspecific stimulation of T cells the following substances were used: anti – CD3 antibodies, PMA and Ionomycin.

In order to stimulate CD3+ cells, OKT-3 was used. 0.5 ml of 10µg/ml anti-CD3 diluted in PBS was used to coat the antibody tissue plate. The prepared plate was incubated for 24h at 4°C. The antibody solution was then removed and plates were washed twice with PBS and IM with 10% FBS. Cells were incubated for 4 days before the final experiment which was designed to start at the moment of high proliferation of cells. The protocol of the experiment is shown below (see Figure 6).

In the next experiment the same protocol was followed but this time using PMA and Ionomycin instead of OKT-3. Those two substances belong to a different kind of stimulators and were added to the CD8+ cells 2 days before the final experiment in concentration: 25ng/ml of PMA with 1µg/ml of Ionomycin.

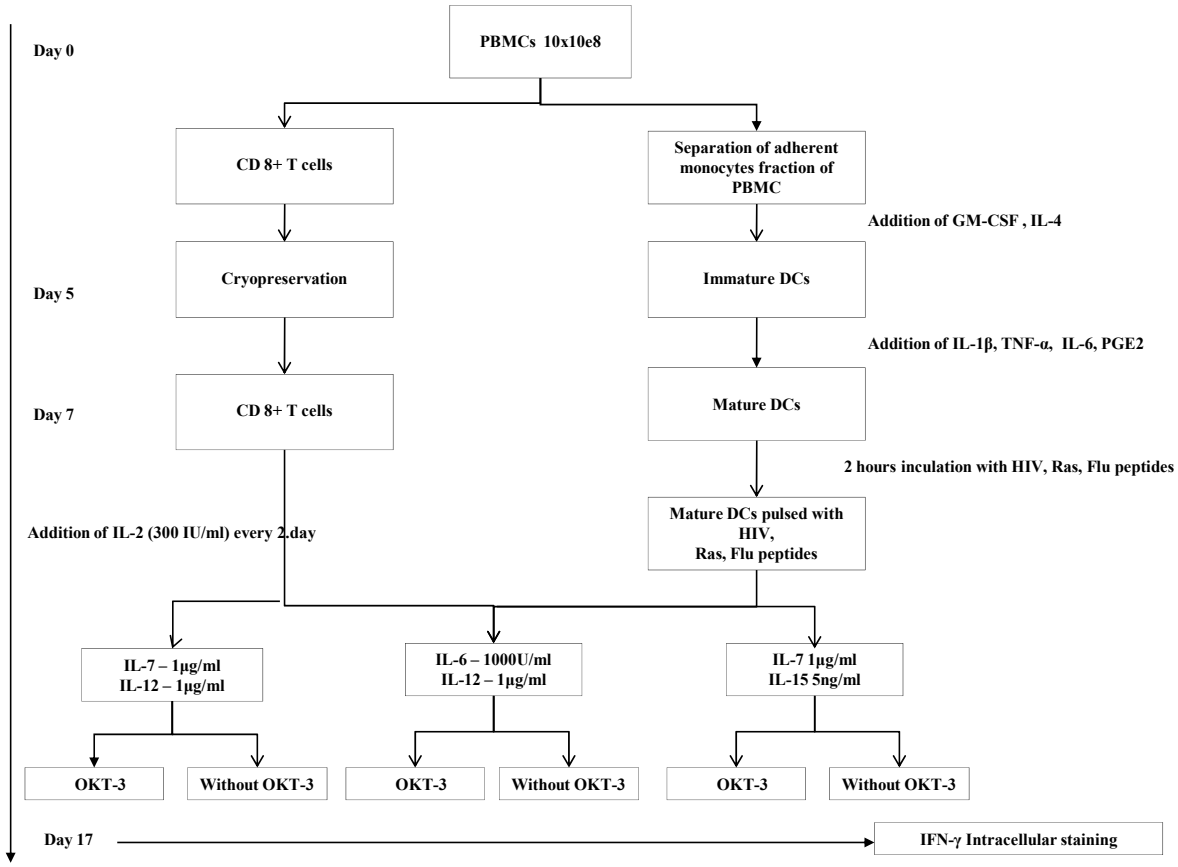


Figure 6 Schema for generation of antigen-specific T-cells by using CD8 positive T cells, peptide-pulsed mature dendritic cells, different cytokines mixtures and OKT-3.

2.9.2 Generation of antigen-specific CD8+ T cells

Cells were cultured at a concentration of 2 to 2.5 * 10^7/ well in 6-well plates. The culture medium was supplemented with one out of four different cytokine cocktails: 1). IL-7 20 ng/ml and IL-12 0.5 ng/ml, 2). IL-7 20 ng/ml, IL-10 10ng/ml and IL-12: 0.1ng/ml, 3). IL-6 1000 U/ml and IL-12 5 ng/ml, 4). IL-7 20 ng/ml (added on day 1) and IL-15: 5 ng/ml (added on day 5.) The cytokine cocktails were added on day 1 and their concentration was adjusted to the final well volume of 5 ml.

Depending on the experiment, cells were stimulated with different poles of antigens, which included various HIV, HBV, HCV antigens. Cell cultures were incubated at 37ºC in 10% CO_2. After 10 days, the *in vitro* primed CTLs were equally split for restimulation with one out of three different antigen-pulsed, irradiated feeder cells: autologous PBMCs, allogeneic PBMCs or a mixture of autologous and allogeneic PBMCs. Feeder cells were added at a culture cell to

feeder cell ratio of 1:2. Restimulation was repeated weekly. After the second and fourth restimulations the intracellular IFN-γ secretion assay was performed.

2.9.3 Generation of viral specific cells – Protocol with DCs

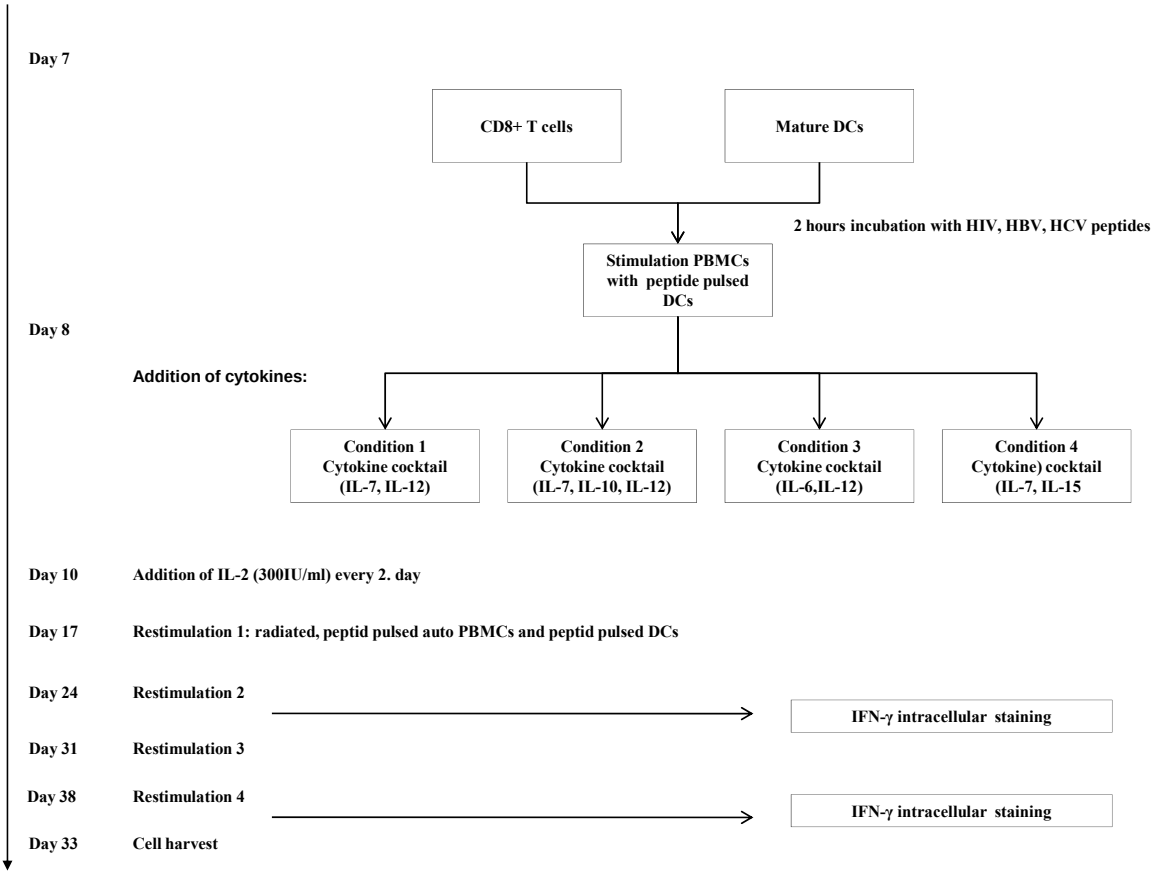


Figure 7 Schema for generation of antigen-specific T-cells with peptide pulsed mature dendritic cells as APCs.

In some experiments, instead of the direct addition of antigen, such as antigen presenting cells (APCs), dendritic cells were used. DCs were generated as described in 2.8.6. Generation of dendritic cells. They were pulsed with antigens at a concentration of 10µg/ml, incubated for 2 h, washed with PBS and added to the isolated CD8+ cells. Apart from stimulation with DCs, the cells were cultured in the same way as described in 2.9.2. Generation of antigen-specific CD8+ T cells.

2.9.4 Restimulation protocols

In long term cultures which were maintained for 38 days, the restimulation protocol was performed every 7 days starting from day 10 or day 17 in cultures with DCs. As feeder cells three different cell populations were used: autologous PBMCs, allogeneic PBMCs and a mixture of autologous and allogeneic PBMCs (1:1).

2.10 Immunological methods

2.10.1 Characterization of antigen-specific cells

Flow cytometry was used to identify different cell populations, to measure the presence of antigen-specific cells, to analyse cell markers and to identify their ability to secrete cytokines after stimulation with a specific antigen. Both antigen and cytokines were stained by antibodies coupled to fluorescent dye. The analysis was performed with a FACS Calibur flow cytometer (Becton Dickinson) equipped with CellQuest^{TM} software (BD Bioscience).

The principle of flow cytometry is based on light scatter and fluorescence to analyse cells or particle flow in a fluid stream one by one through a laser ray (488 nm, 200 mW). The scattered and fluorescent light produced by cells is collected by a system of lenses, mirrors, filters and photodetectors that convert the photon pulses into electronic signals. Through to electronic and computation processing, cells can be measured and up to six parameters can be analysed: in forward scatter which is related to cell size and in side scatter which reflects cell granularity. The fluorochromes which are used to study extrinsic features include: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanine (APC) or AlexaFluor 647.

2.10.2 MHC-multimer staining

Pentamer staining is designed to recognize antigen-specific T cells. The binding affinity of the MHC molecule for the T cell receptor (TCR) varies depending on the allele or peptide combination. Pentamer is a complex of five MHC-peptide molecules that bind to a T cell receptor (TCRs) of a particular specificity.

Bulk cultures were first tested with intracellular IFN – γ staining. Afterwards the positive peptides groups were tested once more with different pentamers. 2-5 x 10^5 cells per staining were used. Cells were washed with a wash buffer (0.1% sodium azide, 0.1% BSA in PBS) and resuspended in rest volume (100µl). According to manufacturer's advice, 0.5µg of pentamer was used per staining. Then cells were incubated in the dark for 15 minutes and washed in the wash buffer. Pro^{TM}Fluortag (8µl) and mouse human anti-CD8 APC antibody were added to each staining. After 30 minutes' incubation, cells were washed twice and fixed. As negative control, unstimulated PBMCs from seronegative individuals were taken.

2.10.3 Immunophenotyping

The immunophenotyping was performed with Flu-specific and HBV- and HCV- specific cultures. Firstly cells were stained with pentamer as described above and afterwards dyed with eight different double monoclonal antibody combinations (see Table 7) Pro condition $5x10^5$ – $1x10^6$ cells diluted in 100µl FACS – buffer were used. After external staining for 20 min at 4°C, cells were washed in FACS Puffer and fixed in 1% paraformaldehyde. Appropriately matched isotype controls were used in each experiment. Finally, surface expression was analysed with a FACSCalibur and data was analysed using the CELLQuest program.

Table 7 The three-color monoclonal antibody panel used to immunophenotype antigen-specific T cell cultures. Each number matches one combination of dyes, numbers 6 to 8 match isotype controls.

No	FITC	PE	APC/ *AlexaFlour 647
1	CD8	Pentamer/ Tetramer	CD69
2	CD27	Pentamer/ Tetramer	CD45RA
3	CD57	Pentamer/ Tetramer	CD28
4	CD45RO	Pentamer/ Tetramer	CCR7 *
5	CD95	Pentamer/ Tetramer	CD62L
6	Mouse IgG2a, kappa	Pentamer/ Tetramer	Mouse IgG2b, kappa
7	Mouse IgG1, kappa	Pentamer/ Tetramer	Mouse, IgG1, kappa
8	Mouse IgG1, kappa	Pentamer/ Tetramer	Mouse, IgG2a, kappa*

2.10.4 ELISpot assay

To determine the frequency of peptide-specific CTL, also enzyme-linked immunospot assay was used. Briefly, Millipore Multiscreen, 96-well plates (Millipore, Bedford, MA) were coated with

mouse anti-human IFN-γ mAb, and the plates washed and blocked with 10% AB serum. Culture cells were resuspended at 5×10^5 per ml and stimulated with antigen-pulsed irradiated T_2 cells for 16–24 hours at 37°C in 5% CO_2. Plates were washed and incubated with the secondary biotinylated anti-IFN-γ monoclonal antibody, 7-B6-1 biotin (Mabtech, Hamburg, Germany) for 2 hours, followed by the addition of avidin-biotinylated peroxidase complex (ExtrAvidin peroxidase complex, Sigma, Deisenhofen, Germany). DAS tablet solution (Sigma-Aldrich, Taufkirchen, Germany) containing peroxidase substrate was used to visualize the spots. The spots were counted using an ELISpot Reader (Version 3.0, Autoimmun Diagnostica, Strassberg, Germany) and expressed as percentage of IFN-γ-producing T cells. A response was defined as positive and specific if a minimum of 10 spots was detected and the number of spots was at least twice as high as in the negative control.

2.10.5 IFN-γ secretion assay

To quantify antigen-specific T cells based on IFN-γ secretion, cells were preincubated with 1 x 10^6 T_2 cells in 1 ml medium pulsed for 1hour at 37°C with the antigen of interest, followed by 5 hours incubation with 10 µg/ml brefeldin A. Addition of brefeldin A during cell activation, which is a protein transport inhibitor, assures accumulation of the specific cytokines within the cell. As negative control, T_2 cells were stimulated with irrelevant antigen and treated as described above.

Following incubation, cells were harvested and washed once with FACS buffer. After discarding the supernatant and resuspending the pellet in remaining volume (~100µl) by tapping 10µl of CD3 PerCP and 5µl of CD8 APC of extracellular antibodies were added. Cells were incubated for 15 minutes on ice in darkness. After adding 2 ml FACS lysing solution, cells were centrifuged. 500 µl FACS permeabilizing solution was added to the pelleted cells and vortexed. Cell membranes were permeabilized with the detergent saponin to facilitate the passage of antibodies through the membrane and staining of intracellular molecules. Permeabilized cells were stained for intracytoplasmatic interferon γ (IFN-γ) FITC. After 30 minutes incubation on ice and in the dark, cells were washed in FACS buffer and fixed with 200µl of PBS +1% formaldehyde. This procedure was followed by FACS analysis.

2.11 Statistics

2.11.1 Statistical analysis

The statistical analysis of T cell proliferation was based on the assumption that the number of cells follows normal distribution. Differences between values were determined by ANOVA model with explicitly defined heterogeneous variance blocks.

In statistical modelling, the percentage of antigen-specific T cells was defined for each individual, by subtracting the percentage of T cells detected among controls stimulated with control peptide (not used during the cultivation process) from the percentage observed when T cells were incubated with culture antigens. It was assumed that the number of antigen-specific T cells (IFN-γ, CD3, CD8+ T cells) followed quasi binomial distribution. That is, binomial because between 0 - lack of cells of this type and 1 – all cells of this type of cells is space for continuity (quasi).

Because not all data were independent (observations were nested within patients) in all models, this fact was incorporated into analysis by treating patients as a random factor.

Expected values presented in the graphs were surrounded with 95% confidence intervals. p-value of < 0.05 was considered statistically significant. All statistical analyses were conducted using SAS software (Version 8.0, SAS Institute Inc, USA). Part of graphics was done by means of GraphPad Prism (Version 5.0., GraphPad Software Inc, USA).

III. Results

3. 1. Generation of antigen-specific cells with unspecific stimulating factors

Our first series of experiments was based on the conviction that successful induction of naïve T cells requires the following elements: stimulation of T cells, presence of professional antigen presenting cells which carry antigen and addition of cytokines which facilitate T cell induction, proliferation and survival.

Generalizing, it can be said that majority of sufficient, long lasting and protective T cell memory response arise on the site of inflammation. In order to simulate that situation *in vitro* we decided to pre-incubate CD8+ T cells with different kinds of activation factors: firstly: OKT-3 (Landegren et al. Eur J Immunol., 1984) and secondly: phorbol 12-myristate 13-acetate (PMA) with Ionomycin (Maraskovsky et al., J Immunol. 1989). As antigen presenting cells, mature DCs were used. They were stimulated with two antigens which represented different groups: HIV –as a viral antigen with no previous memory T cell response and K-ras – as tumor antigen. CD8+ T cells were divided into two groups: cells which were incubated with activation factors or without stimulators. On day 4 of the T cell culture, peptide pulsed mature DCs, as well as cytokines were added. After 10 days cells were harvested and IFN-γ secretion assay was performed. Unfortunately, no antigen-specific T cells were generated (data not shown).

3.2 Generation of viral, antigen-specific cytotoxic lymphocytes in bulk cultures

3.2.1 Antigen pools

While looking for the optimal protocol in regard to the priming and expanding of CD8+ T cells, we investigated four different induction and restimulation protocols. Since the choice of antigen seems to be crucial for priming, several viral peptides of known immunogenicity were chosen (see Material and Methods chapter 2.7.3.1 Viral peptides). In order to enhance chances of successful priming of the cells of low frequencies or specific naïve cells, PBMCs were cultivated in bulk cultures. The chosen antigens represented three groups: HIV, HBV and HCV epitopes. Additionally, HBV epitopes included only core antigens and at the same time

only those which have not been reported as being any part of vaccine against hepatitis B currently available on the market.

Since we wanted to conduct a successful induction of naïve antigen-specific T cells, we collected blood only from healthy donors, which had no hepatic or tumour history and underwent appropriate viral diagnostics as summarized in Materials and Methods (see chapter 2.1. Donors)

3.2.2 Induction and expansion

To systematically investigate the conditions for the *in vitro* priming of CD8+ T cells, we selected four different induction protocols consisting of different cytokine cocktails (see Figure 8).

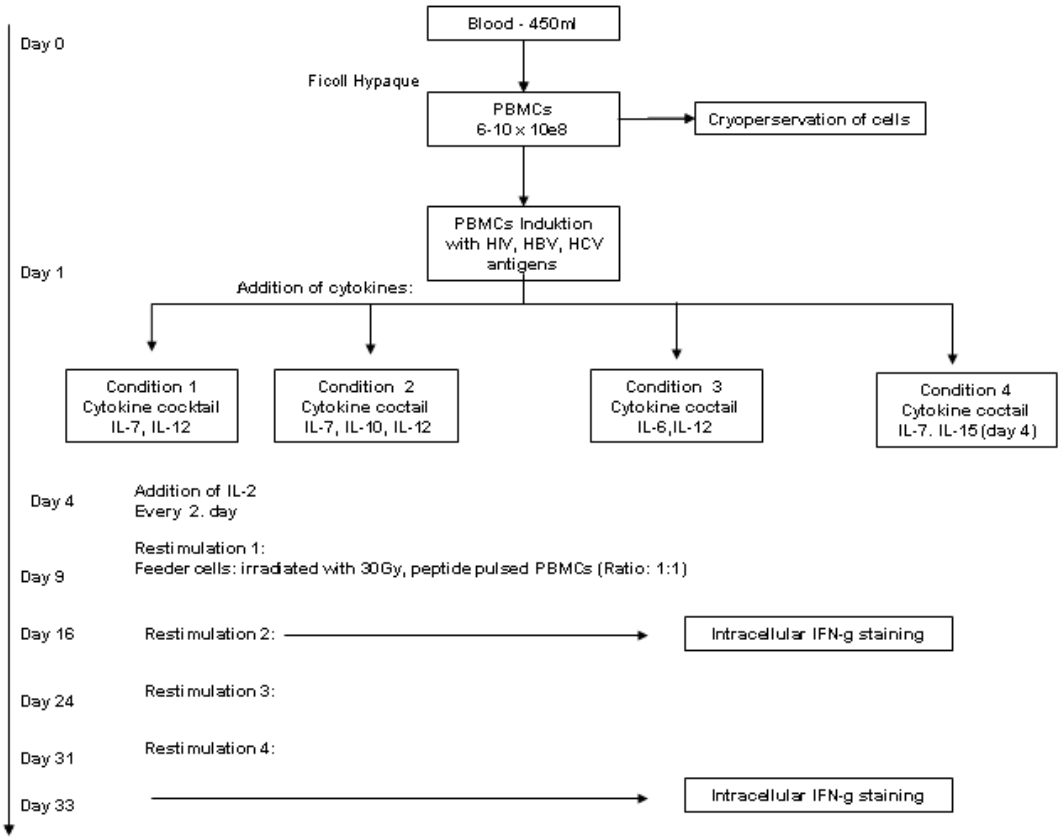


Figure 8 Schematic diagram for the generation and characterization of antigen-specific CD8+ T cells. PBMCs were stimulated with several antigens and cultivated under four different conditions and expanded up to 33 days. Cells under each condition were restimulated weekly with autologenic, allogenic and autologenic/allogenic PBMCs. Characterisation of cultured cells included an IFN-γ secretion assay performed after the 2nd and 4th restimulation. Additionally, for selected, effective experiments, multimer stainings and phenotypings were also performed.

The total number of viable cells was assessed weekly to follow the overall expansion of cells in culture during the four week cultivation. Starting with 1.5-2.0 x 10^7 PBMCs for each condition, the culture was split into three equal parts (4.2-5.5 x 10^6 PBMCs) to allow for the first restimulation with autologenic, allogeneic, or autologenic/ allogeneic feeder cells. The cultures were restimulated weekly. After 33 days, the median number of cells reached 1.5 x 10^7 (range ± 9.16 x 10^6).

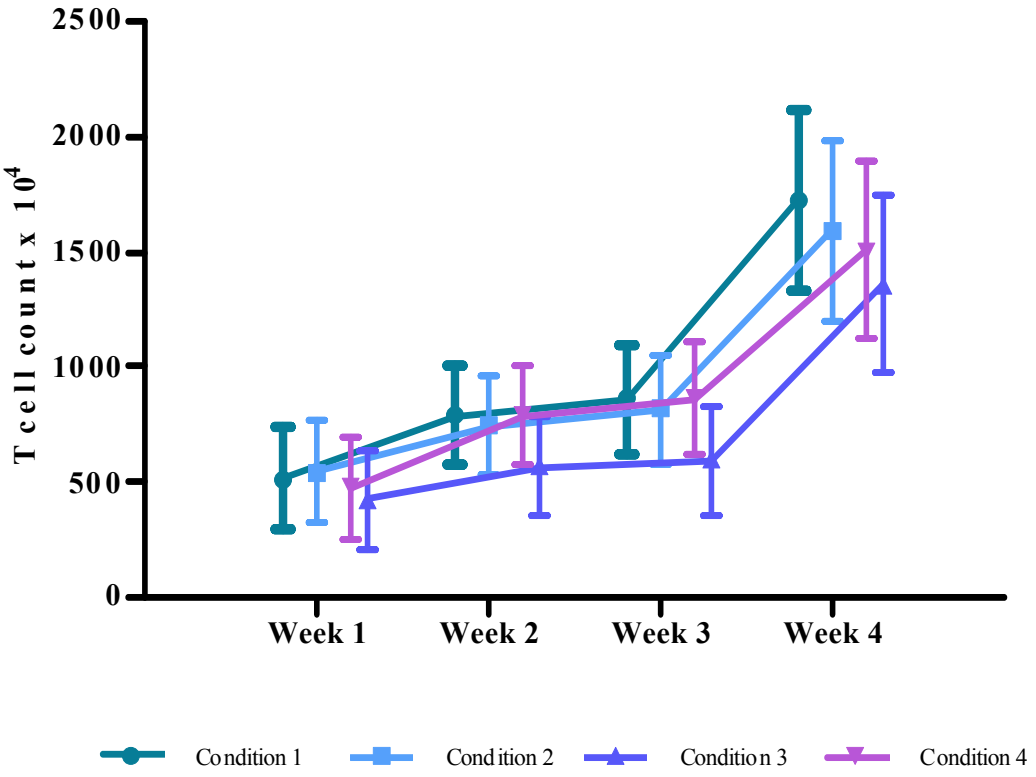


Figure 9 Proliferation curves of four different conditions are shown as least square mean values over a four-week cultivation period. The error bars indicate 95% confidence intervals. Data were analysed using ANOVA model with explicitly defined heterogeneous variance blocks.

Statistical analysis of the proliferation of T cells was based on the assumption that number of cells follows normal distribution. The data were analyzed in the ANOVA model with explicitly defined heterogeneous variance blocks. This was done for two reasons: firstly - in preliminary data overview it was noted that the variance in data grew in time (variance in week 1 is smaller that variance in week 2 and subsequent), secondly - in experiments based on counts it is common that variance is related to the mean. In this approach, time was treated as a categorical factor. And so, the proliferation of T cells is significantly influenced by time ($p < 0.001$) and different conditions ($p = 0.0024$). Interaction between those two factors was not

significant. The calculated least square means counts of each of four conditions (n=5) and the corresponding 95% confidence limits are shown in Figure 9. As illustrated, the proliferation rises with time at an approximate rate of 46.2% per week (39.9 – 52.7%). Values of proliferation for last square were decreased in the following order: condition 1 > condition 2 > condition 4 > condition 3 (see Figure 9).

In order to determine the frequency and functionality of antigen-specific cells, the assessment of antigen-triggered production of IFN-γ was performed after the second and fourth restimulations. For an approximate analysis, T_2 cells were pulsed with a mixture of all antigens used in bulk culture. Although proved negative in some cases after the second restimulation, it still showed a significant proportion of CD3+CD8+ T cells after the fourth restimulation (see Figure 10).

Table 8 The table shows the percentage of antigen-specific CD8+ T cells after the 2nd and 4th restimulations. As a negative control to mixture of peptides used during the induction, the antigen K-Ras antigen was applied.

Condition	Restimulation mode	Peptide mixture (control Ras Ag) after 2nd restimulation	Peptide mixture (control Ras Ag) after 4th restimulation
Condition 1 IL-7, IL-10, IL-12	Autologenic PBMCs	0 (0)	21.48 (0.08)
	Allogenic PBMCs	0.023 (0)	0.745 (0.10)
	auto-/allogenic PBMCs	0 (0)	1.58 (0.11)
Condition 2 IL-7, IL-12	Autologenic PBMCs	0 (0)	7.27 (0.03)
	Allogenic PBMCs	0.04 (0.02)	0.12 (0.09)
	auto-/allogenic PBMCs	0 (0)	0.84 (0.11)
Condition 3 IL-6, IL-12	autologenicPBMCs	0.09 (0)	20.39 (0.05)
	allogenic PBMCs	0.1 (0)	0.59 (0.02)
	auto-/allogenic PBMCs	0 (0)	0.89 (0.01)
Condition 4 IL-7, IL-15 (day 4)	autologenicPBMCs	0 (0)	9 (0.49)
	allogenic PBMCs	0 (0)	0.35 (0.20)
	auto-/allogenic PBMCs	0 (0)	0.69 (0.08)

After 4 weeks of cultivation, least square mean of antigen-specific T cells was about 21 times greater as compared to the 2 weeks' assessment ($p<0.0001$) (see Figure 11). For exact detection, antigens group were split up, and T_2 cells were pulsed with antigens inherent to separate groups (see Figure 12).

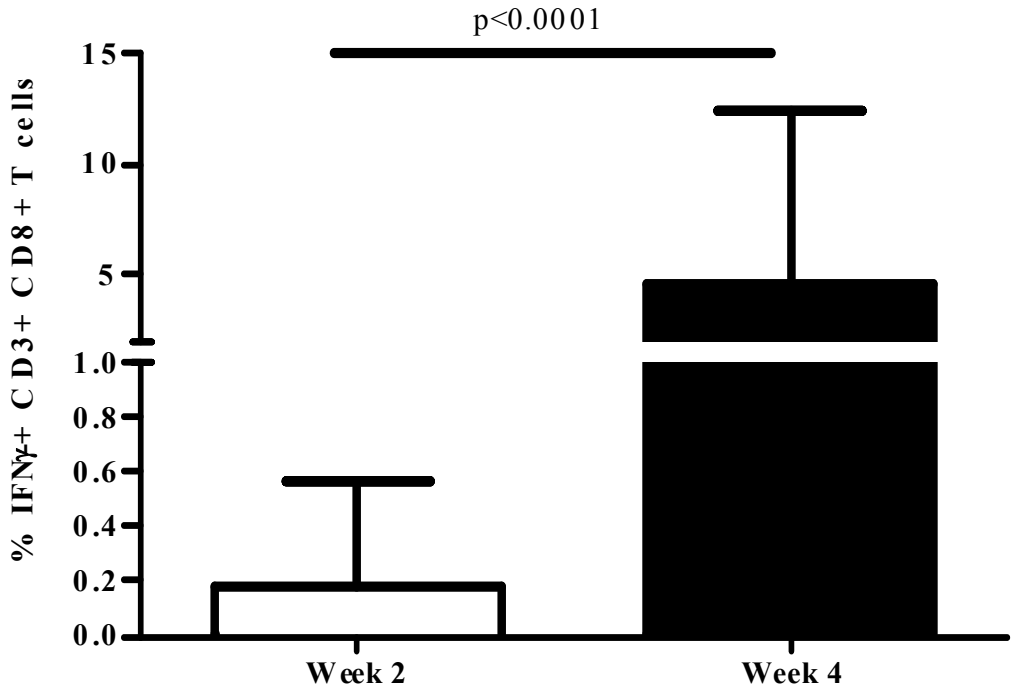


Figure 11 Frequency of antigen-specific T cells was determined by means of IFN-γ secretion assay. T cells were tested against T_2 cells pulsed with a mixture of antigens used in bulk cultures (HIV, HBV, HCV antigens). Bars represent least square means of antigen-specific T cells after second and fourth weeks of cultivation. p-values of <0.05 were considered statistically significant.

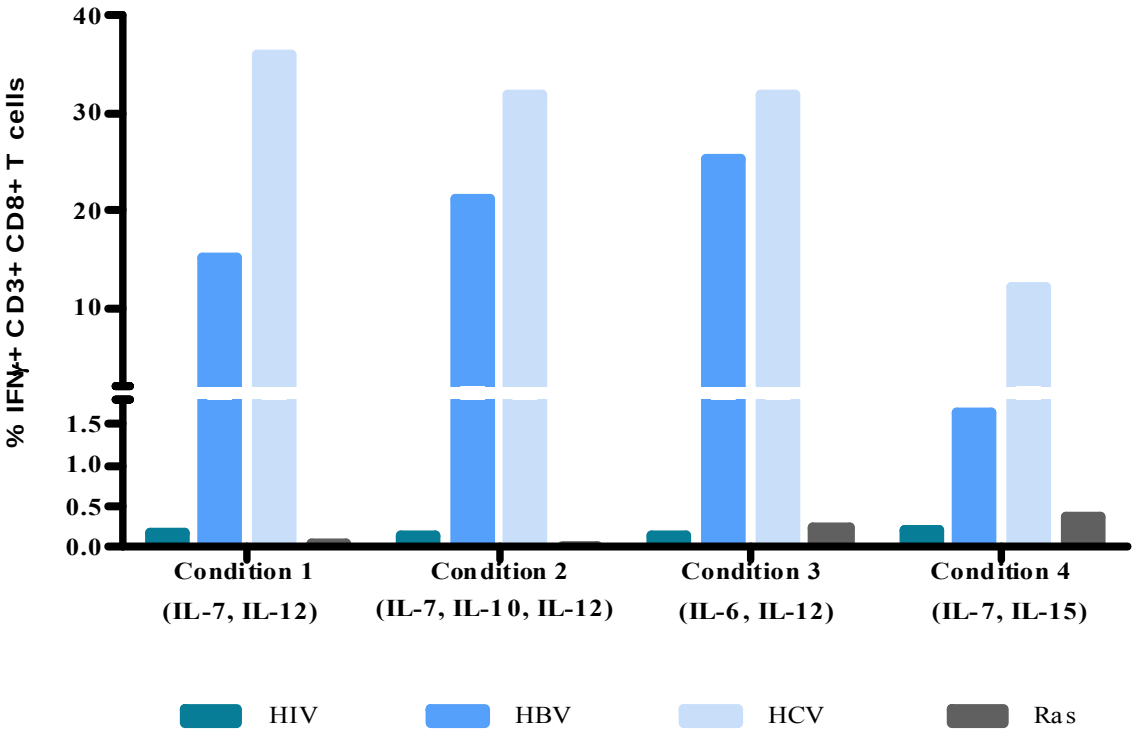


Figure 12 The data here are representative of five independent experiments. The culture condition proved to be positive after the 4th restimulation was tested in the IFN-γ secretion assay once again against following antigen

groups: HIV, HBV, and HCV. The results are shown as the percentage of IFN-γ+ T cells out of CD3+CD8+ T cell in comparison with negative controls (K-Ras antigen).

In order to compare the induction capacities of four different conditions to generate antigen-specific cells, the assessment of antigen-triggered production of IFN-γ was performed after the fourth restimulation. For an approximate analysis, T_2 cells were pulsed with a mixture of all antigens used in bulk culture. Antigen-specific IFN-γ+ CD3+ CD8+ T cells were detected in almost all cultures (see Table 9). However, none of the four conditions used for priming, showed induction capacity which was significantly higher than any of the others (see Table 9 showing the percentage of IFN-γ+ CD3+ CD8+ T cells in the IFN-γ secretion assay in bulk cultures, which were restimulated with A. autologenicfeeder cells, B. allogeneic feeder cells and C. autologenic/ allogeneic feeder cells).

Table 9 The tables show the percentage of IFN-γ+ CD3+ CD8+ T cells in the IFN-γ secretion assay as performed after the 4th restimulation in five independent experiments (see donor 4, 6, 3, 1, 7). Cells were tested against mixture of peptides (HIV, HBV, HCV) used for induction in bulk cultures. K-Ras antigen was used as control. The percentages shown are determined after subtraction of the background (control). Medians and ranges of five experiments were calculated for each condition. Restimulation was performed with A. autologenicfeeder cells, B. allogeneic feeder cells and C. autologenic/ allogeneic feeder cells.

A. Restimulation with autologenic feeder cells

	Donor 4	Donor 6	Donor 3	Donor 1	Donor 7	Median	Range
Condition 1 IL-7, IL-12	16.9	41.4	28.16	21.4	1.2	21.4	1.2 – 41.1
Condition 2 IL-7, IL-10, IL-12	35.16	21.08	0	7.24	1.7	4.47	0 – 35.16
Condition 3 Il-6, IL-12	0	42.7	24.08	20.34	1.16	20.34	0 – 42.7
Condition 4 IL-7, IL-15 (day4)	0.14	38.49	34.34	8.51	6.96	8.51	0.14 – 38.49

B. Restimulation with allogeneic feeder cells

	Donor 4	Donor 6	Donor 3	Donor 1	Donor 7	Median	Range
Condition 1 IL-7, IL-12	0.34	18.71	5.24	0.65	0.11	0.65	0.11–18.7
Condition 2 IL-7, IL-10, IL-12	15.69	17.54	3.7	0.03	0.84	3.7	0.03–17.5
Condition 3 Il-6, IL-12	0	3.97	1.57	0.57	1.84	1.57	0 – 3.97
Condition 4 IL-7, IL-15(day4)	0.03	8.22	4.49	0.15	4.14	4.14	0.03 – 8.2

C. Restimulation with autologenic/ allogenic feeder cells

	Donor 4	Donor 6	Donor 3	Donor 1	Donor 7	Median	Range
Condition 1 IL-7, IL-12	0.05	34.45	31.42	1.47	2.77	2.77	0.05 – 34.5
Condition 2 IL-7, IL-10, IL-12	20.39	9.24	12.68	0.73	1.73	9.24	0.73 – 20.4
Condition 3 Il-6, IL-12	0	11.85	1.69	0.88	20.16	1.69	0 – 11.85
Condition 4 IL-7, IL-15 (day4)	0.17	25.92	9.38	0.61	5.6	5.6	0.17 – 25.9

3.2.3 Induction and expansion with mature dendritic cells

In order to compare the efficiency between the additions of peptide pulsed mature DCs and that peptide alone, two experiments with monocyte-derived DCs were performed (Jonuleit et al., Eur. J. Immunol., 1997). Briefly, PBMCs from healthy HLA A_2 donors were isolated by density determinations on Ficoll-Hypaque. Part of them were used for generation of DCs, the rest was cryopreserved. Obtained on day 7 of the culture, peptide pulsed mature DCs were

used for the priming of CD8+ isolated cells. DCs were used as well for each restimulation cycle next to autologenic PBMCs (see Material and Methods, chapter 2.9.3. Generation of viral specific cells – Protocol with DCs).

Under both sets of conditions, we were able to achieve a significant expansion and induction of antigen-specific CTLs (see Figure 13). As was seen previously, an antigen-specific population was difficult to detect after the second restimulation (see Table 10).

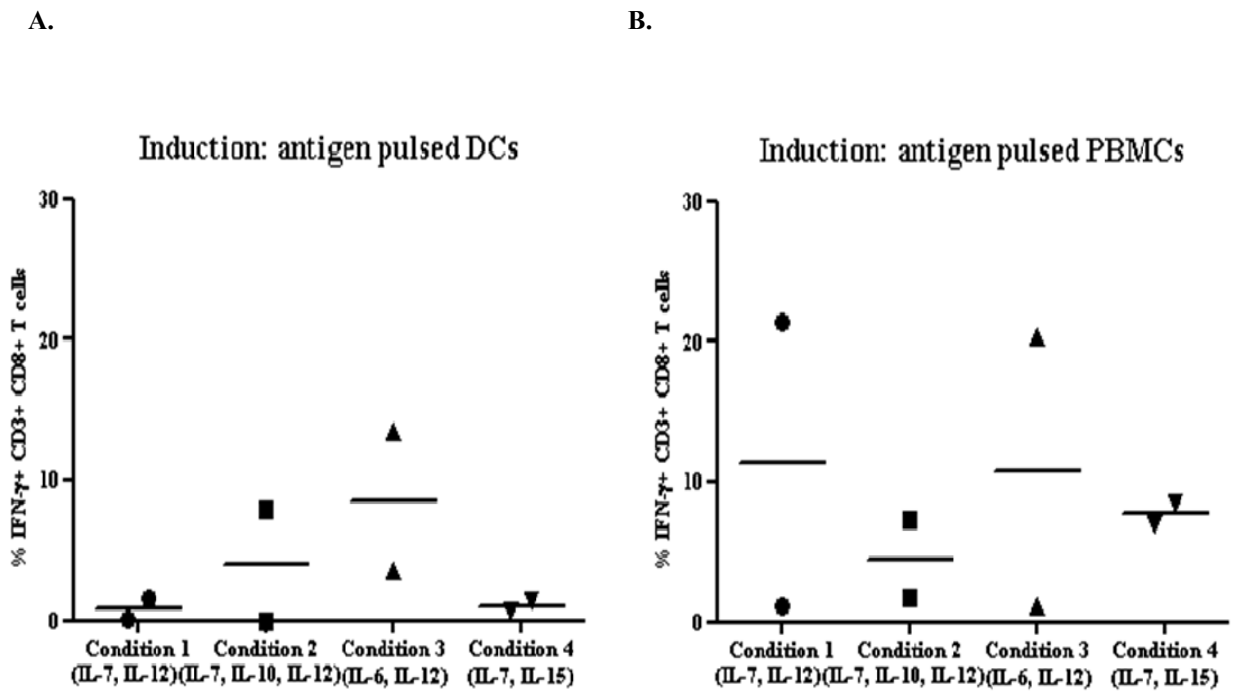


Figure 13 Percentage of IFN-γ expressing cells among the overall population of CD3+CD8+ T cells specific for HBV, HCV epitopes for two experiments with the antigen pulsed PBMCs, and for two experiments (donors 1 and 7) with antigen pulsed DCs. Medians are shown by horizontal lines.

Table 10.A. The table shows the percentage of antigen-specific CD8+ T cells after the 2nd and 4th restimulations in two independent experiments (A – donor 1 and B – donor 7). As a negative control to mixture of peptides used by induction the antigen K-Ras was applied.

A.

Condition	Restimulation mode	Peptide mixture (control Ras Ag) after 2nd restimulation	Peptide mixture (control Ras Ag) after 4th restimulation
Condition 1 IL-7, IL-12	AutologenicPBMCs/ autologenic DCs	0 (0)	0.26 (0.14)
Condition 2 IL-7, IL-10, IL-12	AutologenicPBMCs/ autologenicDCs	0 (0)	0.16 (0.17)
Condition 3 Il-6, IL12	autologenicPBMCs/ autologenicDCs	0 (0)	3.79 (0.22)
Condition 4 IL-7, IL-15 (day4)	autologenicPBMCs/ autologenicDCs	0.04 (0)	1.88 (0.37)

Table 10B. The table shows the percentage of antigen-specific CD8+ T cells after the 2nd and 4th restimulations in two independent experiments (A – donor 1 and B – donor 7). As a negative control to mixture of peptides used by induction the antigen K-Ras was applied.

B.

Condition	Restimulation mode	Peptide mixture (control Ras Ag) after 2nd restimulation	Peptide mixture (control Ras Ag) after 4th restimulation
Condition 1 IL-7, IL-12	autologenicPBMCs/ autologenicDCs	0.42 (0)	2.08 (0.45)
Condition 2 IL-7, IL-10, IL-12	autologenicPBMCs/ autologenicDCs	0.40 (0)	7.90 (0.05)
Condition 3 Il-6, IL12	autologenicPBMCs/ autologenicDCs	0.56 (0)	13.55 (0.22)
Condition 4 IL-7, IL-15 (day4)	autologenicPBMCs/ autologenicDCs	0.06 (0)	0.92 (0.29)

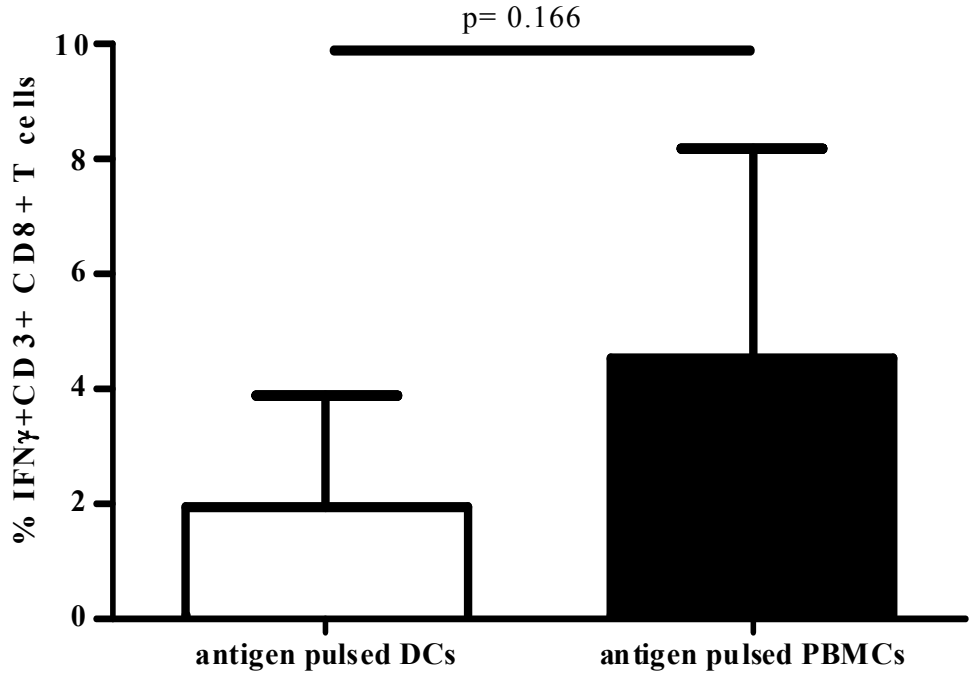


Figure 14 Frequency of antigen-specific T cells was determined by means of IFN-γ secretion assay. Bars show least square means of antigen-specific T cells obtained in cultures in which induction was performed with antigen pulsed DCs or antigen pulsed PBMCs. A p-value of < 0.05 was considered statistically significant

The statistical analysis shows that, least square mean of T cells cultivated with DCs was not significantly higher than least square mean of T cells cultivated with antigen alone ($p = 0.166$) (see Figure 14). In conclusion, we were not able to demonstrate any advantage by the application of mature dendritic cells.

3.2.4 Restimulation

To determine whether the use of allogeneic feeder for restimulation may improve the stimulation and expansion of the antigen-specific CTLs, we compared peptide pulsed autologenic, allogenic and an equal mixture of autologenic and allogenic irradiated PBMCs as feeder cells. Autologenic PBMCs activated and expanded precursor cytotoxic cells in culture more effectively than allogenic and mixture of autologenic and allogenic PBMCs. Cultures that were restimulated with autologenic PBMCs reached the highest specificity with the number of antigen-specific T cells ranging from 8.77% to 21.81% of all viable cells. In contrast, restimulations with allogeneic PBMCs alone yielded the lowest number of antigen-specific T cells (ranging from 5.97% to 7.56%). The numbers of antigen-specific T cells

obtained with a mixture of both autologenic and allogeneic PBMCs were in between those of the two others (ranging from 6.9% to 14.03%).

Table 11 The table shows the percentage of antigen-specific CD8+ T cells after 4th restimulation in three independent experiments determined by means of IFN-γ secretion assay. Restimulation in these sets of experiments was performed with autologenic PBMCs as feeder cells. K-Ras antigen was used as control.

	Donor 4				**Donor 6**				**Donor 3**			
	HIV	**HBV**	**HCV**	**Ras**	**HIV**	**HBV**	**HCV**	**Ras**	**HIV**	**HBV**	**HCV**	**Ras**
Condition 1 IL-7, IL-12	4.10	16.40	11.02	1.80	0.12	1.10	42.73	0.20	0.41	4.43	1.31	0.39
Condition 2 IL-7, IL-10, IL-12	0.13	0.14	25.94	0.62	0.25	2.82	36.10	2.52	9.60	10.0	27.50	6.87
Condition 3 Il-6, IL-12	0.08	0.32	0.26	0.20	1.59	2.70	44.77	2.07	0.35	8.88	29.76	0.33
Condition 4 IL-7, IL-15(day4)	0.14	0.08	0.11	0.06	0.28	0.92	42.73	0.19	0.46	40.57	2.43	0.53

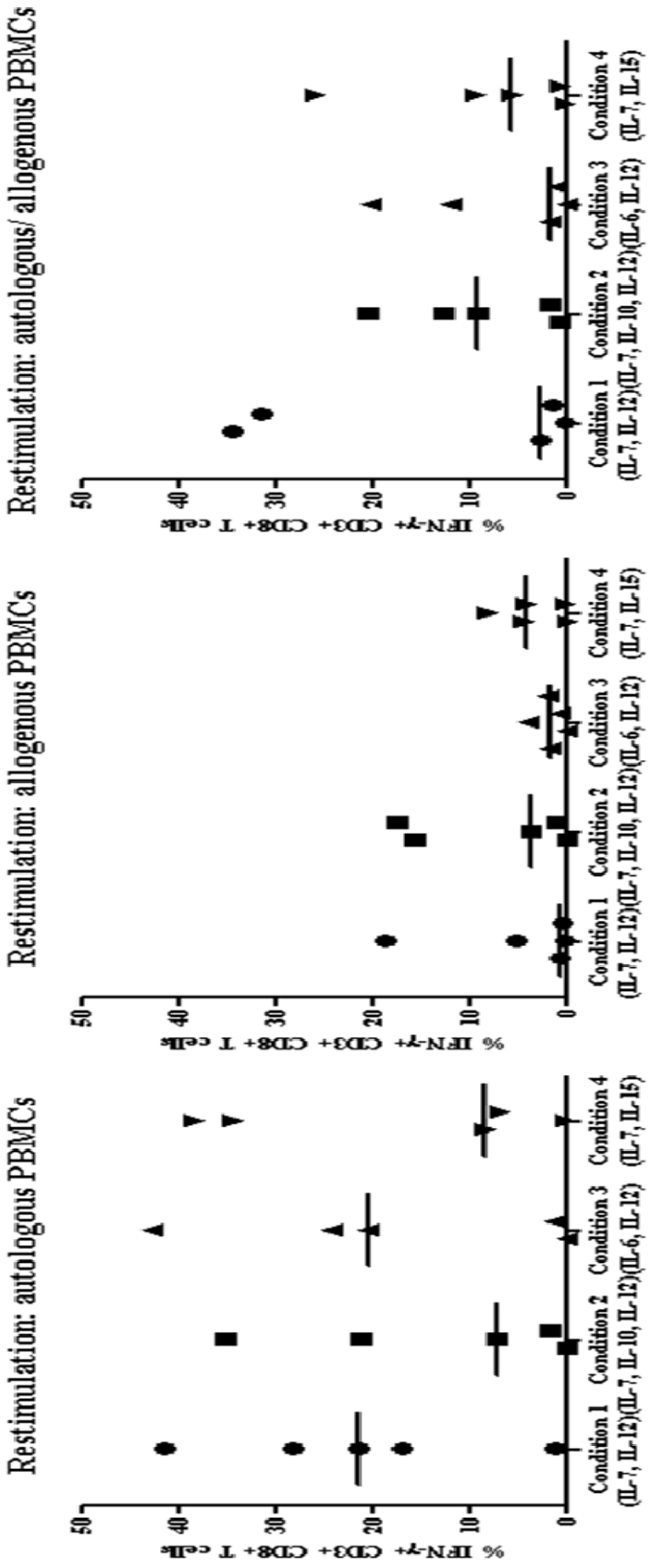


Figure 15 The percentage of IFN-γ+ CD3+ CD8+ T cells in the IFN-γ secretion assay as performed after the 4th restimulation. The results shown here are those from five independent experiments. Medians are shown by horizontal lines.

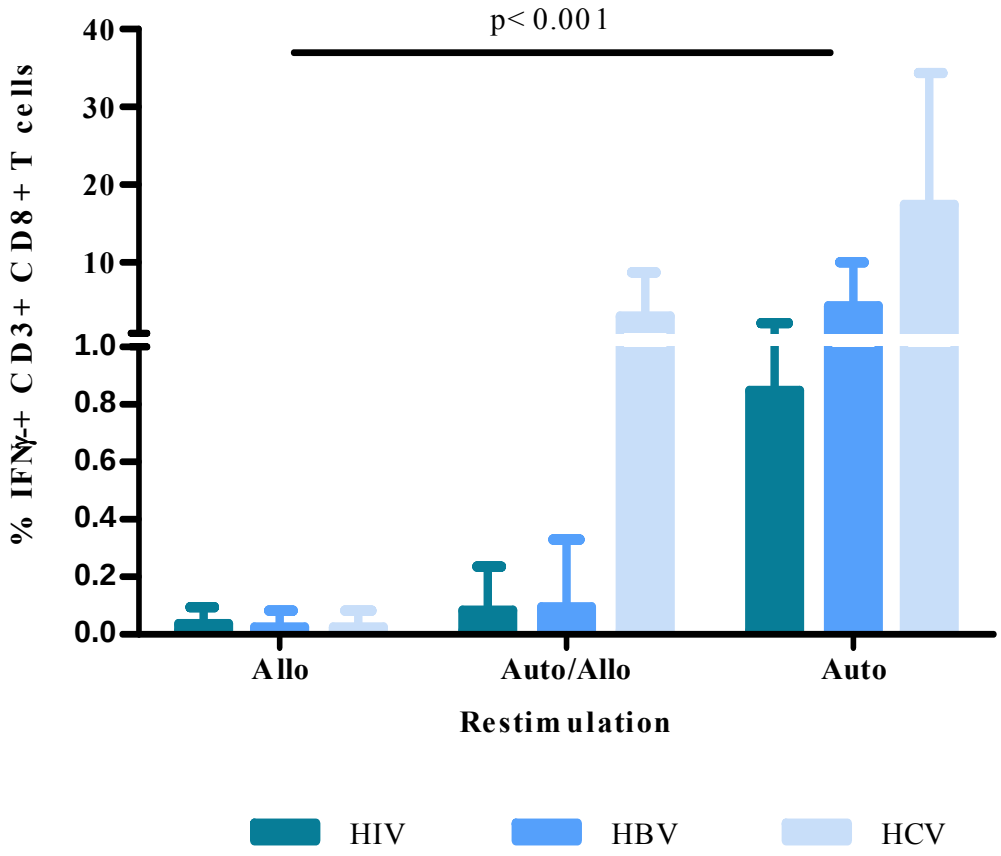


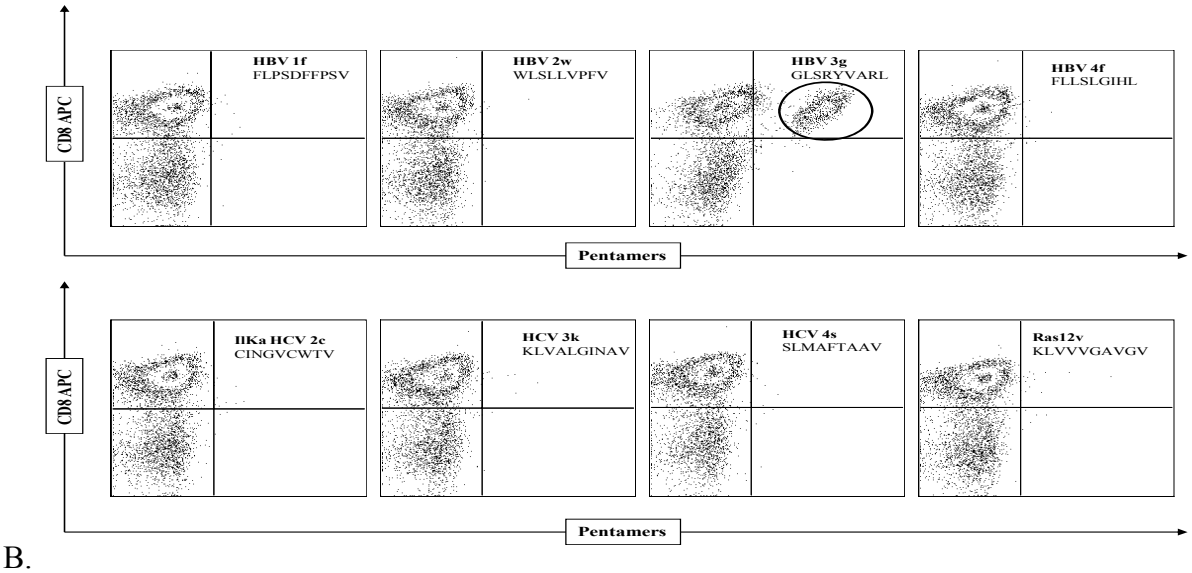
Figure 16 Frequency of IFN-γ CD3+ CD8+ T cells depends significantly on kind of restimulation mode ($p<0.001$) and on antigen ($p<0.001$).

3. 3 Characterization of antigen-specific CTLs

3.3.1 MHC-multimer staining

In addition to the IFN-γ secretion assay, MHC-multimer staining and immunophenotyping were performed in selected cultures with high numbers of positive cells in the IFN-γ assay. We detected antigen-specific CD8 positive populations in both the HBV and HCV groups (see Figure 17).

A.



B.

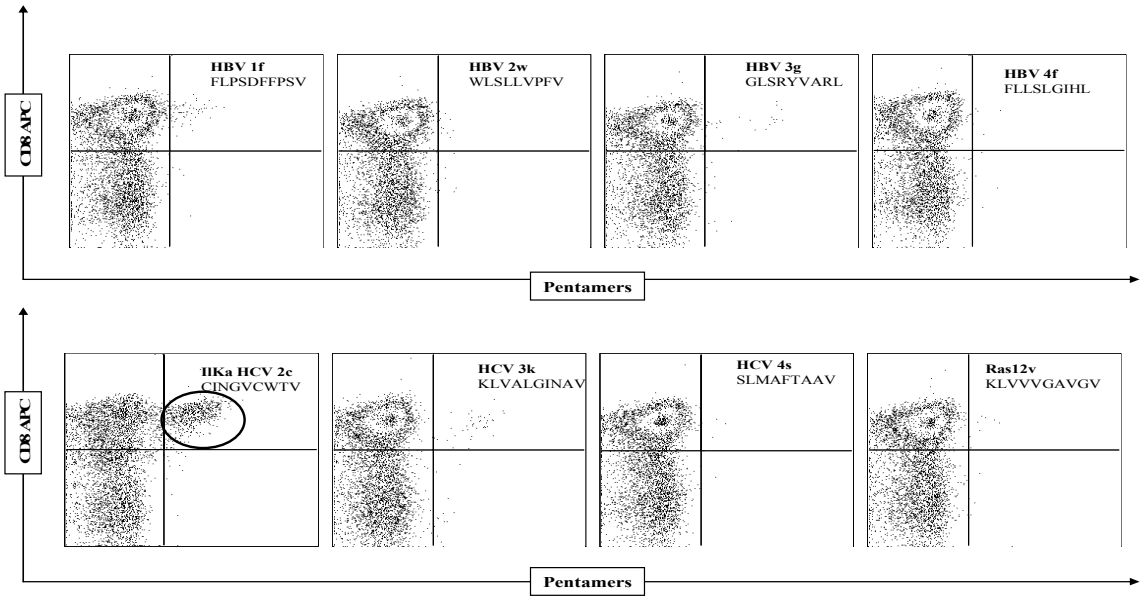


Figure 17 Representative pentamer staining of two antigen-specific T cell cultures. T cells which have been proven to secrete IFN-γ in an antigen-specific manner also have proven to be double-positive for CD8 and pentamer. Figure 17A. In HCV positive bulk culture, HCV2c antigen-specific T cells have been detected. Whereas in Figure 17B HBV INF-γ positive T cells were predominantly specific for HBV3g antigen.

The almost absent unspecific pentamer staining supported the technical validity of the above finding of antigen-specific cells. Interestingly, no specific MHC-multimer positive populations were found for HIV peptides. This may have two possible explanations. Firstly,

the second antibody in the pentamer staining showed some unspecific binding activity revealing positive populations both in tested samples as well as in negative control. Secondly, because of low binding affinity to MHC class I molecules of two HIV peptides, two pentamers compatible with them could not be synthesised (see Figure 18).

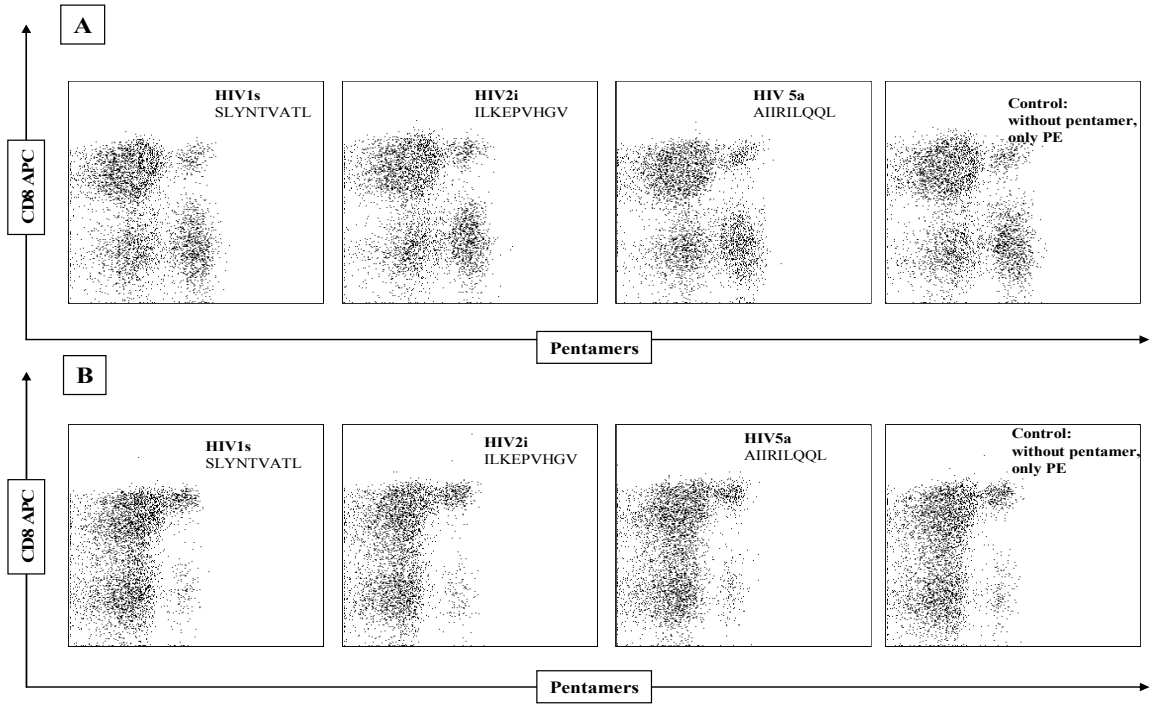


Figure 18 Unspecific staining of HIV pentamers and control (staining without pentamer, only with second antibody: PE antibody) by two different donors (A and B).

3.3.2 Immunophenotyping

To further characterise the antigen-specific cells, cell cultures with a strong pentamer staining were immunophenotyped with a panel of monoclonal antibodies (see II. Material and Methods 2.7.3.4 FACS – Antibodies Table 5). To assess the differentiation state, some HBV- and HCV-specific CD8+ T cells were tested for the expression of CD45RA and CCR7. In the example shown, most of the cells (83% of the HBV-specific T cells and 95% of the HCV-specific T cells) presented CD45 negative, CCR7-positive phenotypes, which is consistent with an effector memory phenotype. A small number of T cells (between 3 and 4%) expressed CCR7 and CD62L, consistent with a central memory phenotype.

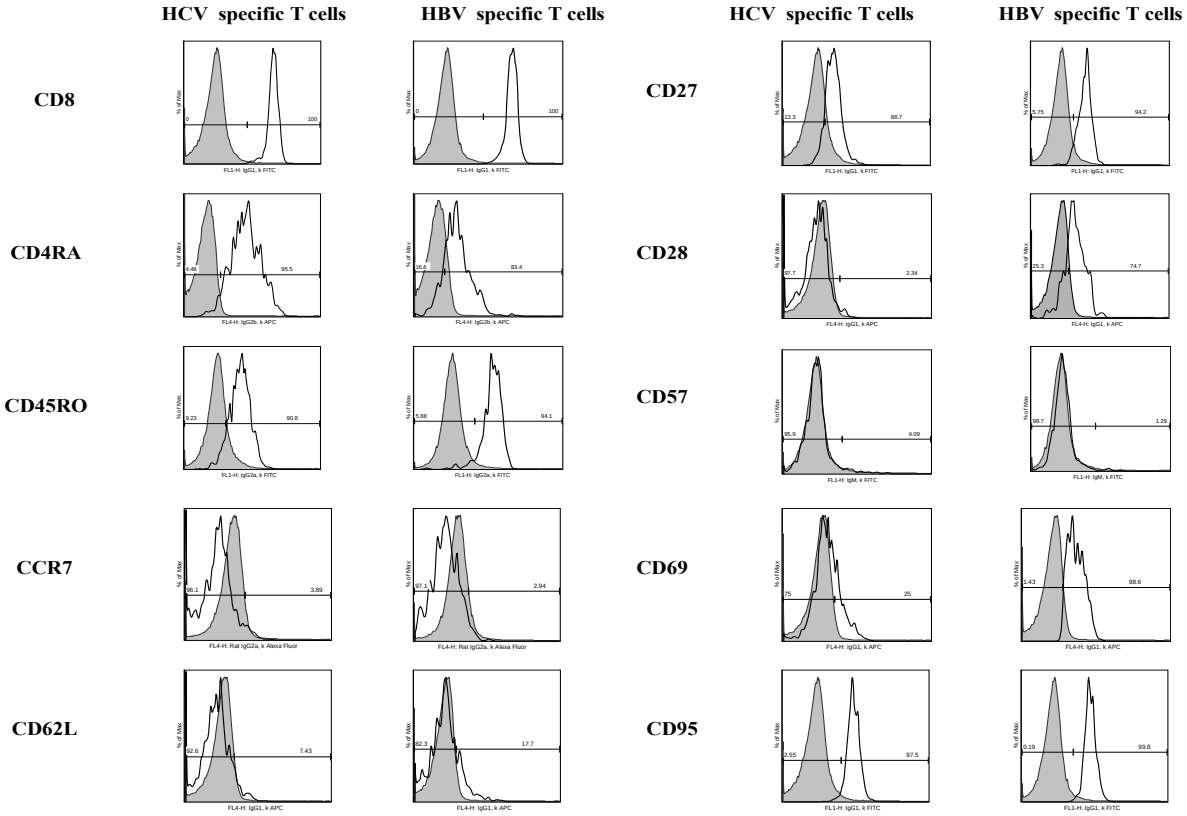


Figure 19 Immunophenotyping of antigen-specific CD8+ T cells. Shown here is a histogram plot where cells were gated on pentamer/tetramer positives and represent two different specific T cell populations: for donor for HCV-specific T cells (HCV 2c CINGVCWTV), and for HBV-specific T cells (HBV 3g GLSRYVARL).

A high expression of costimulatory molecules such as CD27 and CD28 indicated an functionally early phenotype of the cells. CD57, whose expression on $CD8^+$ T cells is associated with proliferation incompetence and replicative senescence, was found in up to 4% of the cells. Expression of CD69, an early activation marker, was considered to occur differentially (between 25 and 90%). Almost all antigen-specific T cells expressed high amounts of CD95, a finding which may indicate increased sensitivity towards CD95-mediated apoptotic cell death *in vitro*.

3. 4 Generation of virus and tumour antigen-specific CTLs in separate antigens cultures

The optimised protocol was used to generate cultures specific for individual viral epitopes. The induction of specific T cells were performed with single viral antigens (HIV, HBV, HCV) and tumour antigens (Ras) in four different conditions provided by original protocol.

Each culture was restimulated weekly with autologenicpeptide pulsed PBMCs, irradiated with 30Gy. After 21 days of expansion, cells were analysed for their specificity in the ELISPOT assay. As shown in Figure 20, the antigens differed in their ability to induce specific responses. For three out of five HIV epitopes, no specific response was observed, whereas all HBV and HCV antigens did induce a T cell response. Also, for tumour-associated epitopes with different Ras mutations, a specific response was observed in two of the three antigen pairs. As a result, we proved that the protocol can not only be successfully applied for generation of viral antigen-specific T cells, but also may be used with tumour antigens (see Figure 20 and 21).

Antigens	Condition 1 IL-7, IL-12	Condition 2 IL-7, IL-10, IL-12	Condition 3 IL-6, IL-12	Condition 4 IL-7, IL-15 (on day 4)
HIV 1s (SLYNTVATL)				
HIV 2i (ILKEPVHGV)				
HIV3f (FLGKIWPS)	+	+	+	
HIV5a (AIIRILQQL)	+			++
HIV6v (VLEWRFDSRL)				
HBV1f (FLPSDFFPSV)		+		
HBV2w (WLSLLVPFV)	++	+		
HBV3g (GLSRYVARL)	++			
HBV4f (FLLSLGIHL)	+	+	+	+
HBV5h (HLSLRGLFV)	+		++	+
HBV6c (CLFKDWEEL)	+	++	+	
HCV1a (ADLMGYIPLV)				+
HCV2c (CINGVCWTV)	+	+	+	+
HCV3k (KLVALGINAV)			+	
HCV4s (SLMAFTAAV)				+
HCV5a (ALYDVVTKL)	+			+
Ras 12C/D (KLVVVGAC (D)GV)	+		+	+
Ras 12G/R (KLVVVGAG(R)GV)				
Ras 12S/V (KLVVVGAS(V)GV)	+	++		

Figure 20 Semi-quantitative analysis of ELISPOT results of two separate experiments: "+": positive response in one of two experiments, "++": positive response in both experiments. A response was considered to be positive if a minimum of 10 spot-forming cells per well could be counted and if the cell count was at least twice as high as that of the control.

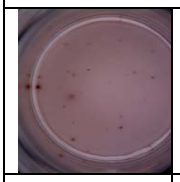
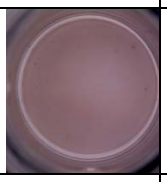
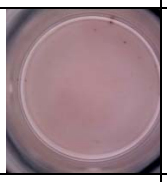
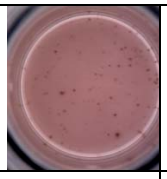
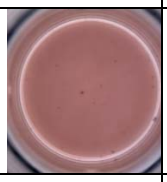
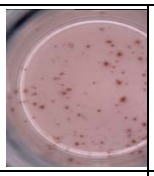
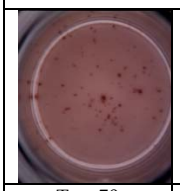
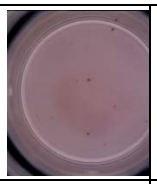
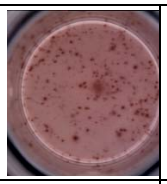
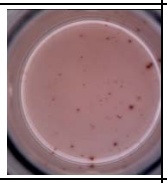
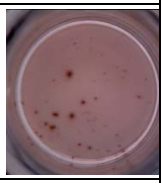
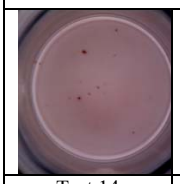
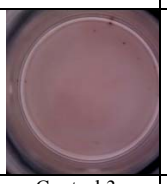
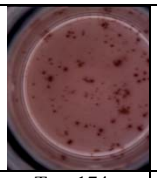
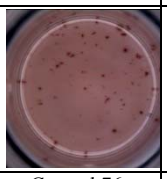
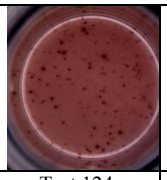
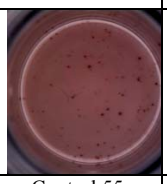
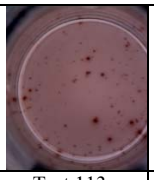
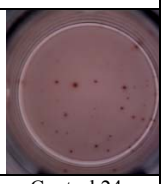
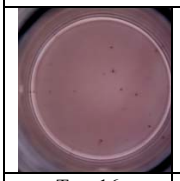
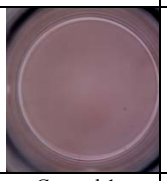
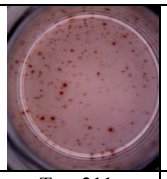
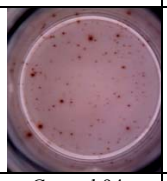
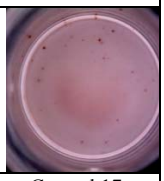
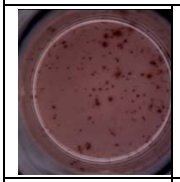
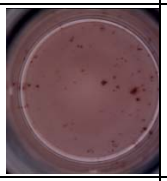
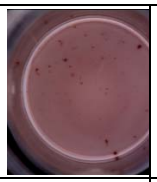
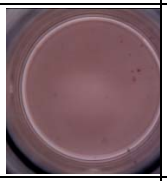
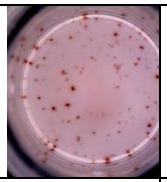
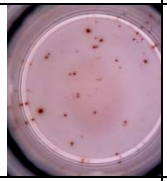
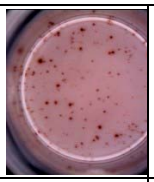
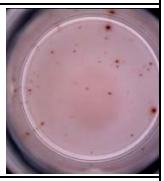
Donor 2/HIV 3f/ 1. Condition		**Donor 2/ HIV 3f/ 3. Condition**		**Donor7/HCV 1a/4. Condition**		**Donor7/HCV 4s/4. Condition**	
							
Test 24	Control: 5	Test 14	Control 3	Test 81	Control 8	Test 156	Control 34
Donor 7/HIV 5a/ 1. Condition		**Donor 2/ HIV 5a/ 4. Condition**		**Donor7/HCV 2c/4.Condition**		**Donor2/HCV 5a/1 Condition**	
							
Test 79	Control 29	Test 10	Control 2	Test 296	Control 39	Test 139	Control 45
Donor 7/ HIV 3f/ 2. Condition		**Donor7/ HIV 5a 4. Condition**		**Donor2/HCV 3k/3.Condition**		**Donor2/HCV 5/ 4. Condition**	
							
Test 14	Control 3	Test 174	Control 76	Test 124	Control 55	Test 113	Control 24
Donor2/ HBV 1f 2.Condition		**Donor2/HBV 4f 4.Condition**		**Donor2/Ras12C/12D 1Condition**		**Donor2/Ras12S/12V 1Condition**	
							
Test 16	Control 1	Test 119	Control 27	Test 211	Control 94	Test 37	Control 17
Donor 7/ HBV 2w/ 2.Conditon		**Donor 7/ HBV5h/ 4.Condition**		**Donor2Ras12C/12D 3Condition**		**Donor7/Ras12S/12V 2.Condition**	
							
Test 180 spots	Control 65	Test 31	Control 7	Test 120	Control 41	Test 161	Control 58
Donor 2/ HBV 3g/ 1.Condition		**Donor 2/ HBV 6c/ 3.Condition**		**Donor2/Ras12C/12D 4Condition**		**Donor7/Ras12S/12V 2.Condition**	
Test 246	Control 88	Test 162	Control 45	Test 181	Control 88	Test 34	Control 12

Figure 21 Representative examples of single experiments. A response was considered to be positive if a minimum of 10 spot-forming cells per well could be counted and if the cell count was at least twice as high as that of the control.

3.5 Generation of virus and tumour antige n-specific CTLs from naïve und memory precursors

To determine whether generated antigen-specific T cells originate from naïve or low frequency memory precursors, PBMCs were depleted of non-naive T cells and NK cells. Both enriched cell populations were cultivated according to the protocol under four different conditions. Enrichment of a pure and fully functional population of naïve T cells is a very challenging task. A few attempts to cultivate a highly pure CCR7 and CD45RA positive T cell population isolated by positive selection failed due to extensive manipulation of the cells (data not shown). Finally, negative selection using naïve T cell isolation kit resulted in gaining a functional and enriched T cell population (see Materials and Methods, chapter 2.8.9 Isolation of naïve T cells).

Table 12A The table shows the percentage of IFN-γ-expressing cells among the CD3+CD8+ T cells specific for HBV, HCV and WT-1 epitopes after 4th restimulation in two independent experiments with naive T cells (A: target fraction) and memory T cells (B: not-target fraction). HA-1 epitope was used as control. Restimulation was performed with autologenic feeder cells. Both fractions of PBMCs (target and non-target fraction) were cultivated for four weeks. An IFN-γ secretion assay performed at the end of the culture revealed antigen-specific T cells only in cultures consisting of naïve T cells. No antigen-specific response was generated from non-naive precursors (see B).

A.

Condition	HBV	HCV	WT-1	HA-1 (control)
Condition 1 IL-7, IL-12	11.26	3.304	6.03	0.177
Condition 2 IL-7, IL-10, IL-12	1.24	3.7	9.068	0.43
Condition 3 Il-6, IL-12	2.91	2.491	23.409	0.13
Condition 4 IL-7, IL-15 (day4)	0.8	2.5	26.34	0.39

B.

Condition	HBV	HCV	WT-1	HA-1 (control)
Condition 1 IL-7, IL-12	0.03	0.4	0.09	0.47
Condition 2 IL-7, IL-10, IL-12	0.197	0.008	0.035	0.21
Condition 3 Il-6, IL-12	0.124	0.006	0.088	0.084
Condition 4 IL-7, IL-15(day4)	0,029	0,049	0,089	0,079

Table 12B The table shows the percentage of IFN-γ expressing cells among the CD3+CD8+ T cells specific for HBV, HCV and WT-1 epitopes after 4th restimulation in two independent experiments with naive T cells (A: target fraction) and memory T cells (B: non-target fraction). HA-1 epitope was used as control. Restimulation was performed with autologenicfeeder cells. Both fractions of PBMCs (target and non-target fraction) were cultivated for four weeks. IFN-γ secretion assay performed at the end of the culture revealed antigen-specific T cells only in cultures consisting of naïve T cells. No antigen-specific response was generated from non-naive precursors (see B).

3.6 Generation of tumour antigen-specific CTLs

To examine whether tumour-associated antigens are also able to induce a specific CD8 response, PBMCs were stimulated under four induction conditions with a pool of tumour antigens. They were cultivated for up to 21 days and restimulated weekly, in most cases with autologenicbut also with a mixture of autologenic/ allogenic and peptide pulsed PBMCs, irradiated with 30Gy. Next, to investigate the immune responses of CTLs, an ELISPOT assay with induced IFN-γ was carried out. As shown in Figure 23, after 21 days of cultivation we observed higher levels of IFN-γ producing cells among all twelve tumour antigens.

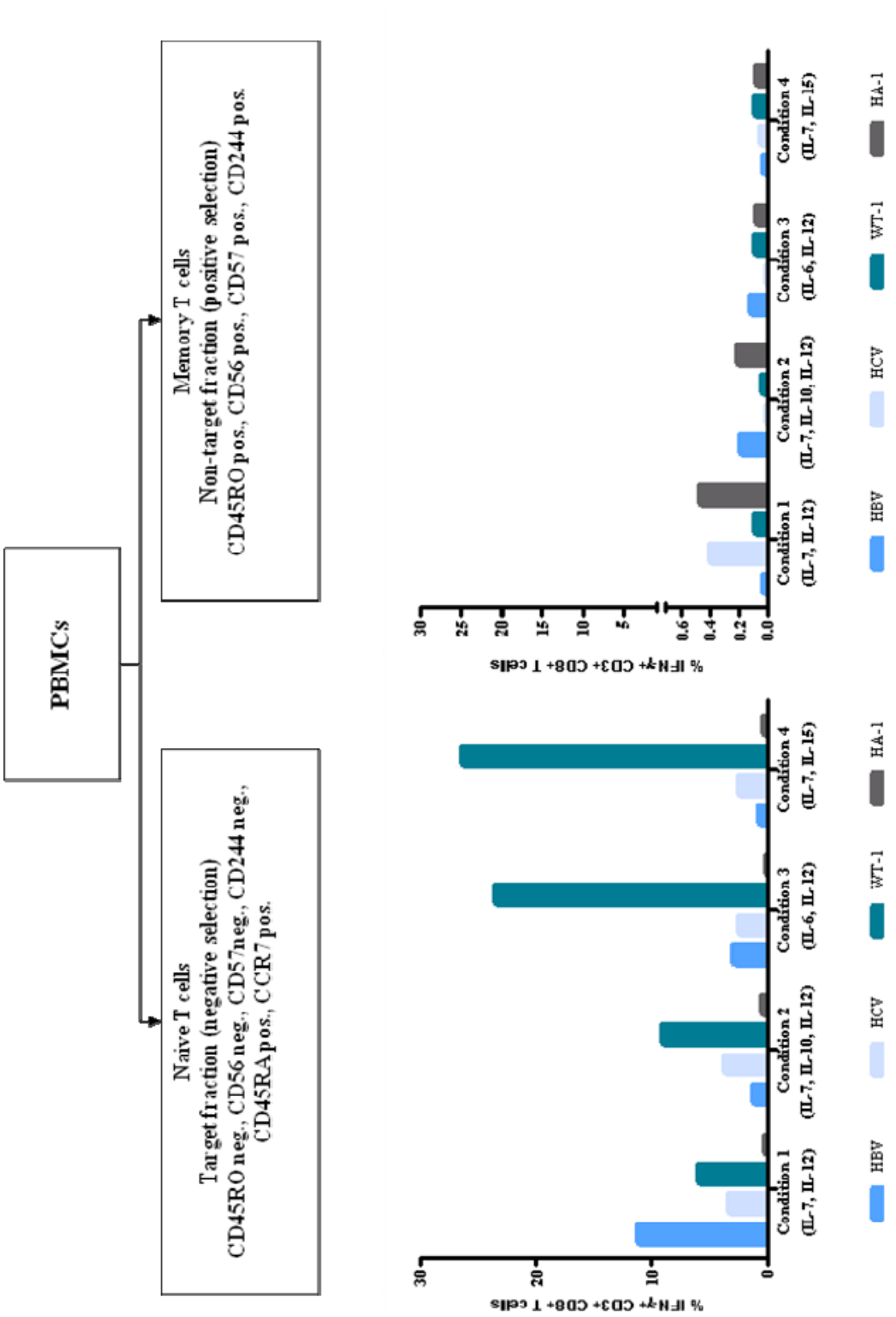


Figure 22 Naïve and memory T cells were isolated with Naïve T cell isolation kit from Miltenyi. Both: untouched target fraction with naïve T cells as well as non-target fraction with memory T cells were cultivated under standard protocol for up to 4 weeks. Afterwards, culture cells were harvested and tested with intracellular IFN-γ secretion assay. Percentages of IFN-γ expressing cells among the overall population of CD3+CD8+ T cells specific for HBV, HCV, WT-1 epitopes are shown above.

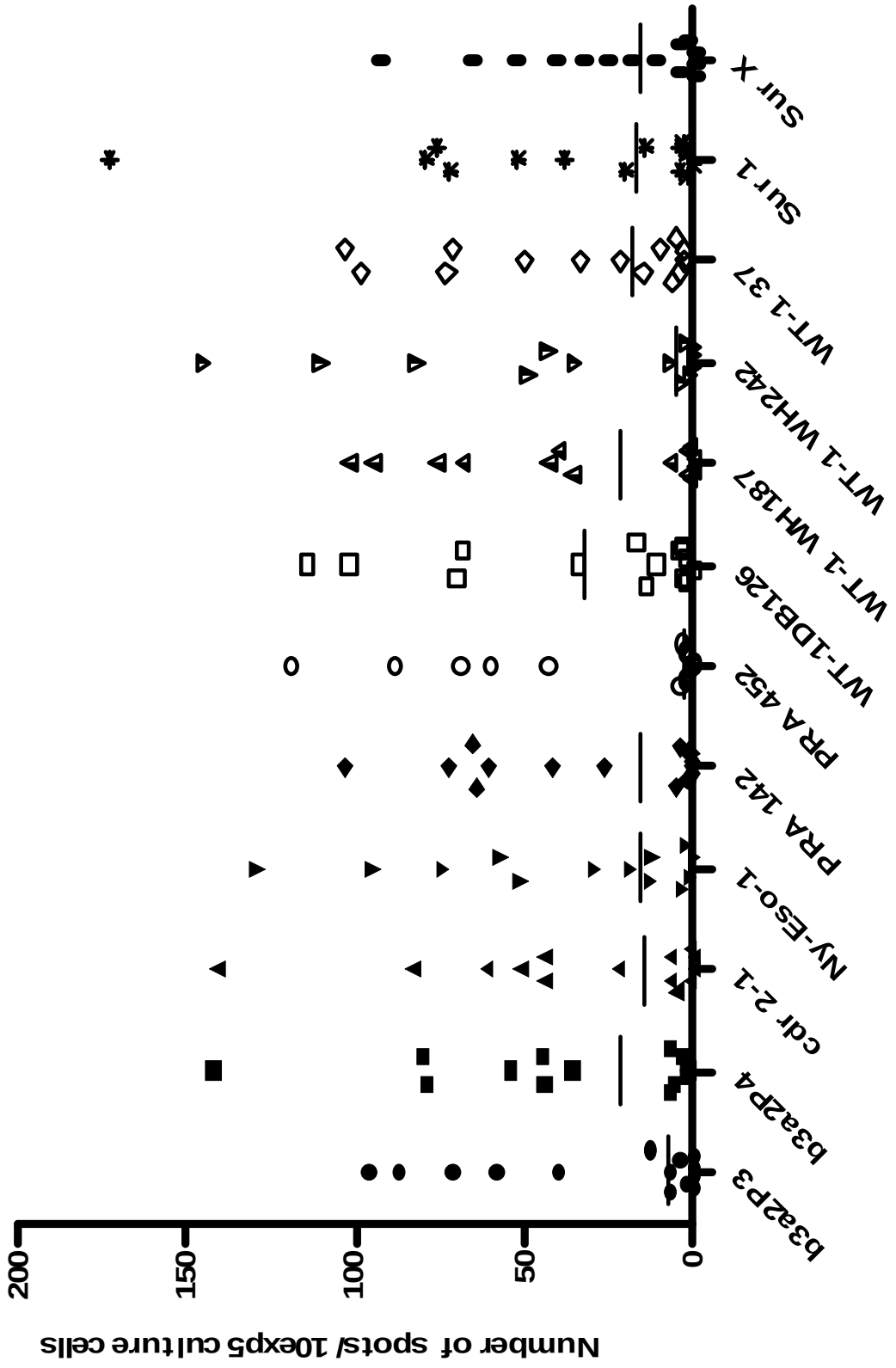


Figure 23 IFN-γ ELISPOT assay. Culture cells were tested after 21 days of culture. This shows the cumulative result of three separate experiments which were carried out as a test for the possible occurrence of induction tumour specific T cells. All in all, 12 antigens were used, and it was possible to generate IFN-γ secreting cells with tumour-associated-antigens.

In subsequent experiments, PBMCs from six healthy donors were cultivated using a pool of antigens according to our protocol. The aim of this was to choose the most effective conditions out of the four used in the original protocol in order to prime and expand tumour antigen-specific T cells. As representatives of tumour antigens three epitopes, of WT-1 were chosen. As control two hepatitis B and two hepatitis C antigens were used in the peptide pool. Most effective results for WT-1 antigens considering specificity of the cultures were achieved by using of IL-6 and IL-12 in the third condition (see Table 13 and Figure 24).

Table 13 The table shows percentage of IFN-γ expressing cells in the overall population of CD3+CD8+ T cells specific for HBV, HCV and WT-1 antigens. HIV antigen was used as negative control.

Antigen group	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
HIV (control)	0.719	0.39	0.09	0.397	0.88	0.11
HBV	1.29	0.138	0.936	0.75	1.21	16.97
HCV	4.79	4.49	1.049	5.167	11.02	11.05
WT-1	33.879	15.2	2.009	13.68	35.3	11.69

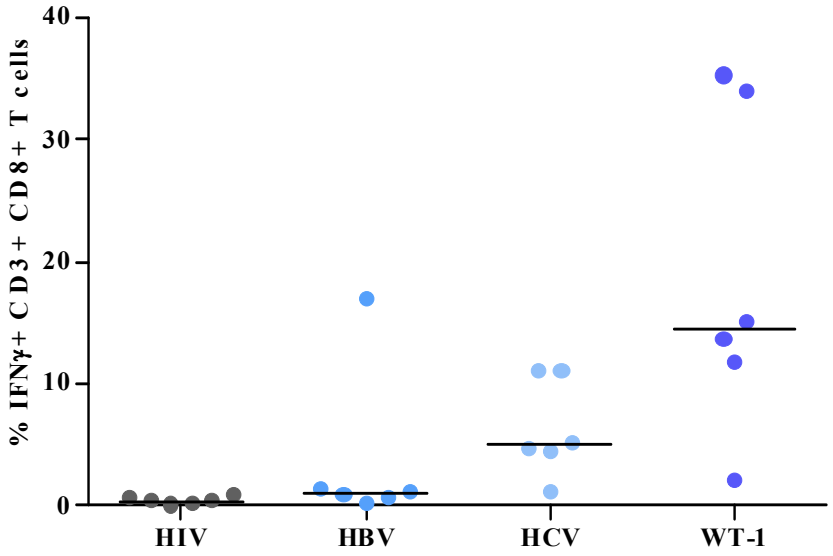


Figure 24 The percentage of IFN-γ+ CD3+ CD8+ T cells in the IFN-γ secretion assay for the third condition as performed after the 4th restimulation. The results shown here are those from six independent experiments. (HIV antigen was used as negative control.)

3.7. Generation of tumour antigen-specific CTLs under GMP conditions

Based on above described experiments, condition three of the original protocol was chosen to be adapted to the GMP conditions. The GMP conformed method was applied by a 26-year-old patient with acute myeloid leukaemia and WT-1 coexpression. In this high-risk patient, after syngeneic bone marrow transplantation, PBMCs were collected from syngeneic donor by leukapheresis. After four weeks cultivation, WT-1-specific T cells (see Figure 25) were cryopreserved. Six months after bone marrow transplant, our patient suffered a relapse of her hematologic malignancy. As part of the individualised experimental conditioning protocol, WT-1-specific T cells were used. The patient received a total of 6 x 10^8 T cells, of which 4,38 x 10^7 were a WT-1-specific T cells. No short- and long-term adverse events were observed. Because of a very high risk of relapse, the patient underwent haploidentical transplantation. As a result, the frequencies of WT-1-specific T cells were not monitored.

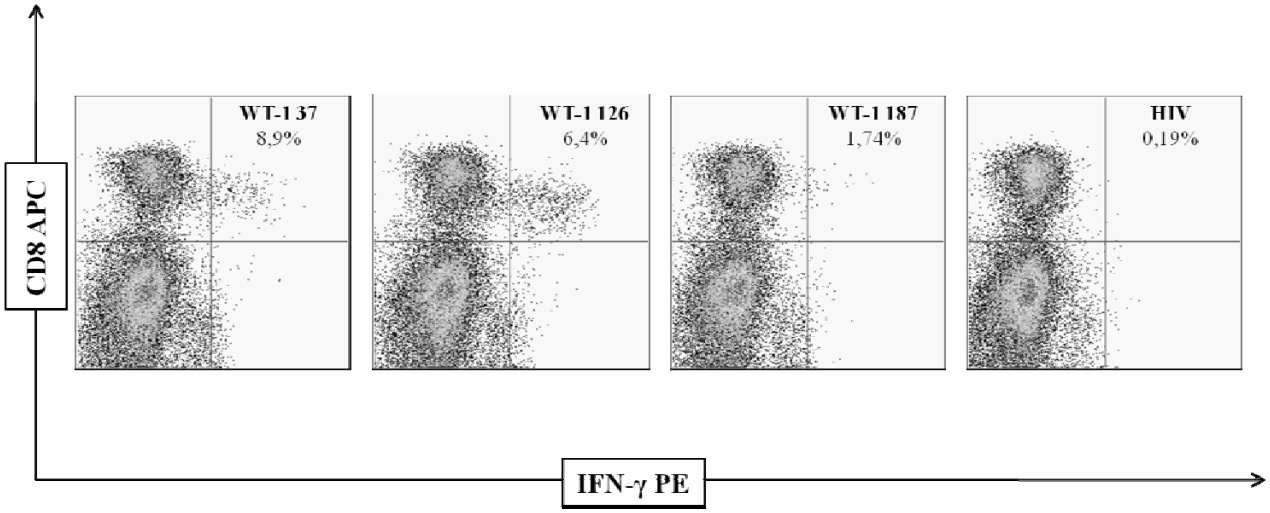


Figure 25 The percentage of IFN-γ + CD3+ CD8+ T cells in the IFN-γ secretion assay for the induction with IL-6 and IL-12 (third condition) and restimulation with autologenicfeeder cells as performed after the 4th restimulation.

IV. Discussion

4.1 Adoptive T cell transfer and its limitations

The adoptive cell transfer of *in vitro* primed and expanded antigen-specific T cells may provide a promising experimental approach for patients with a therapy-resistant viral infection (Walter et al., N. Engl. J. Med. 1995; Pahl-Seibert et al., J. Virol. 2005) or malignant disease (Dudley et al., Nat Rev Cancer, 2003).

There are many successful applications of this concept for the treatment of infection caused by EBV (Rooney et al., Blood 1998) or by CMV (Walter et al., N Engl J Med., 1995) as well as for the treatment of tumour patients or leukaemia patients after haematopoietic stem cell transplantation (Fujita et al., Bone Marrow Transplant 2008). One of the reasons why adoptive T cell transfer still remains an experimental procedure is the lack of solid good manufacture practice protocols for different settings – involving both viral and tumour antigens, as these generate large numbers of T cells from naïve and memory responders.

4.2 Unspecific stimulating factors

The feasibility of successful generation of antigen-specific CTLs from healthy donors has already been demonstrated by several groups. Many of them use mitotic agents such as PHA, CD3 or CD28 ligation in their protocols (Riddell and Greenberg, J. Immunol Methods, 1990). Brenchley at al. (Clin. Exp Immunol 2002) also showed that it is possible to expand activated naïve T cells. According to that data, upon initial stimulation of T cell receptor, the naive CD8+ T cells divide and undergo modulation of superficial antigens. On the basis of those experiences and findings, our alternate hypothesis was that the specific T cell answer can be induced after unspecific stimulation of naive lymphocytes by addition of antigens in the presence of cytokines. We also claim that the induction takes place during a relatively short period of time, up to 7-10 days at most. In view of this finding, the readout assays were conducted at the end of the 10-day cultivation period.

Our first experiments were conducted with unspecific stimulators such as: CD3-ligand and PMA and ionomycin. The antigen panel used for induction was very modest and included only representative antigens. For the induction of a naïve response with virus antigen, we have

chosen human immunodeficiency virus (HIV) epitopes, and for evoking antigen-specific tumour response we used Ras antigen. As control peptide, for inducing memory answer in memory T-cell pool, influenza antigen was used. Unfortunately, neither the so-called "naïve" antigens nor the control antigen was able to induce any antigen-specific response. Summing up, experiments conducted at the beginning of our study did not support our hypothesis that usage of unspecific pre-stimulated T cells may be advantageous.

4.3 Antigen selection. Antigen pools versus overlapping peptide pools

Failure of our initial approach could have been caused by several limiting factors, for example our small repertoire of antigens. In order to optimise the entry conditions, the peptide pool was extended. Since it is known that viral protective and long-lasting memory response can be induced *in vivo*, we decided in the first part of this project to analyse induction of memory response with three different viral antigen pools. To avoid the risk of selecting epitopes that for some reason, such as a broader TCR (cross) reactivity, are much easier to generate a specific T cell response against, we used several epitopes of viral and tumour-associated protein for which a strong reactivity in healthy individuals is neither known nor to be expected. Antigens from HIV, HBV, and HCV were chosen because the exposure to these viruses could be reliably tested in donors.

HIV antigens were chosen because of their possible immunogenicity albeit rather low (Cohen et al., J.Virology, 2002; Ferrari et al., AIDS Research and human retroviruses, 2000), which although *in vivo* is capable of inducing a memory immune response, is in fact mostly an insufficient one.

The next group contained hepatitis B virus epitopes. Because hepatitis B virus infection nowadays can be prevented with adequate vaccination strategy, we used it as a example of adequate immune response. In order to challenge possible complaints about expanding already existing memory T cells, we chose epitopes which are present in vaccines that are not commercially available. These were mainly core antigens (Livingston et al., Human Immun., 1999).

Hepatitis C virus infection very often transforms to a chronic disease, in which a creation of an adequate protective immunological response cannot be achieved. Our HCV antigens consisted

of those to which a response could be detected *in vivo* or was inducible *in vitro* by naïve individuals (Cerny et al., J. Clin. Invest., 1995, Urbani et al., Hepatology 2001).

As already mentioned, we preferably tried to work with a wide range of different HLA.A201 antigens in order to achieve a specific answer. In this way, on the one hand, we increase the probability of successful priming due to the enhanced number of naïve antigen-specific precursors. On the other hand, future usage of polyclonal population of antigen-specific T cells enlarges the numbers of possible targets which will ultimately minimize the chances of virus or tumour escape.

After successful induction of viral antigen-specific response, in the later experiments the protocol was conducted with a group of tumour epitopes. Most of them represented tumour associated antigens, apart from K-Ras antigen or bcl-abl which represented tumour-specific antigens and epitopes derived from fusion antigens. Those epitopes are ubiquitously expressed on the tissues of the healthy individuals and thus *per se* an inducible T cell response should come from naïve or low-frequent precursors.

Also, choosing the "right" antigen for future clinical application seems only at first sight an easy issue. Originally, the protocols which were compared in this project included almost solely tumour lysates (Kurokawa et al., Int J Cancer. 2001; Montagna et al., Int J Cancer., 2004) or immunogenic melanoma antigens (Kaiser et al, Eur. J. Immunology, 2003). The usage of irradiated allogeneic lymphoblastoid cell lines or tumour lysates as the source of antigen may be difficult to reconcile with GMP conditions due to the risk associated with the administration of potentially pathogenic material. For the same reason, fungal or viral lysate – claimed however by many to be non-infectious (Tramsen et al., Bone Marrow Transplant, 2008) - is still not being approved by authorities as an optimal antigen source for an adoptive T cell transfer. Furthermore, the successful in vitro expansion of T cells specific for such tumour antigens as Melan A or tyrosinase may be attributed to an idiosyncrasy of the antigen and/or the precursors reactive to these antigens (Pittet et al., J Exp Med. 1999) rather than the method used to induce and expand them *in vitro*.

At this stage, it was deemed advisable to search for a method which may be widely applicable for different antigens. In our experimental system we used only selected pools of single HLA-

A*0201antigens. But for future clinical trials, in order to apply the generation method to all types of donors, it seems almost a necessity to use overlapping peptide pools.

4.4 Introduction of antigen through antigen presenting cells

Previously published protocols for the generation of antigen-specific T cells use a great variety of antigen-presenting cells. These include donor-derived dendritic cells DCs (Montagna et al., Cancer Res., 2006); RNA loaded DCs (Milano et al., J Immunol Methods. 2007), DCs transduced with adenoviruses (Gulen et al., Int Immunopharmacol. 2008), CD40L transfected fibroblasts (Hoogendoorn et al., Leukemia. 2004), CD40-activated human B cells as an alternative source of highly efficient antigen-presenting cells to generate autologenic antigen-specific T cells for adoptive immunotherapy (Schultze et al., J Clin Invest., 1997), CD40 ligation of loaded DCs (Montagna et al., Blood, 2001) and artificial antigen presenting cells (APCs) (Oosten et al., Blood, 2004).

Peptide-pulsed DCs are commonly used APCs. An understanding of their role in the introduction of antigen-specific response has grown enormously since theirs identification through Steinman and his colleagues in early eighties (Steinman et al., J Exp Med, 1973). It has become clear that not only DCs survey all kinds of tissues for pathogens, but also due to their long dendrites they can recruit and activate many T cells at the same time (Banchereau and Steinman, Nature, 1998). Naturally, many of the scientific groups working in the field of adoptive immunotherapy have put a lot of expectation into those powerful cells. They have been applied both in vaccination (Morse et al., J Transl Med. 2007) as well as in adoptive T cell transfer trials (Palmer et al., Hepatology. 2009). Already *in vivo* DCs display wide immunophenotypical and functional heterogeneity. Since they cannot be effectively collected from peripheral blood, the diversity in protocols for DCs generation has been tremendous. First of all, these are represented by immature and mature DCs (Ito et al., J Gastroenterol Hepatol. 2001). Immature dendritic cells have turned out to be poor activators of T cells and in some cases it seems that they actually preferentially activate regulatory T cells. By contrast, mature DCs have been considered to be potent antigen presenting cells. Because of that we have additionally added them to negatively isolated CD8+ T cells in order to optimise T cell priming. Surprisingly, application of DCs did not show any enhancement of proliferation in comparison to addition of antigen alone in the standard protocol. Within the scope of that experiment parallel to peptide pulsed PBMCs, we have also used peptide pulsed DCs for restimulation.

Although DCs are often claimed to be indispensable, in our case the use of in vitro generated mature DCs for priming did not show any enhancement of specificity or expansion. In our setting, PBMCs seem to be even superior to DCs in the restimulation of T cells. This observation confirms a conclusion made by Ho et al (J. Immunol Methods, 2006). Data on DCs suggest that "fast DCs" instead of mature DCs may be more promising. Cells generated in that manner (Dauer et al., J Immunol Methods. 2005) – although they express the same phenotypical markers (CD86, CD80) – have a distinct morphology and may have distinct stimulatory capacities. Also, a separate experiment (data not shown) comparing our protocol with the addition of antigen alone with one where the antigen was introduced with peptide pulsed fastDCs did not induce a higher number of antigen-specific T cells. Whether "fast DCs" indeed contribute to a higher lytic activity of CTLs *in vitro* remains to be addressed in future studies.

All in all, since DCs generation is a rather complex, time-consuming and very expensive procedure, we would advise using DCs only in exceptional cases when induction cannot be achieved by using simpler methods.

4.5 Bulk cultures (usage of selected T cells subpopulation)

The total T cell clonal diversity, as well as the number of naive antigen-specific precursors, has shown firstly that the number of antigen-inexperienced precursors is very low; in healthy donor PBMCs, it is lower than 10^{-5} (Blattman et al., J Exp Med.2002). Secondly, it seems that response to certain epitopes involves several different T cell clones. Because of very low frequencies of naïve antigen-specific T cells and facing failure our first approach, we decided to use bulk cultures with a large number of cells in order to raise the possibility of successful induction. This approach, proved to be the right strategy. In most cases we used between 1.5 x 10^7 and 2 x 10^7 cells per condition. In some experiments we reduced the outcome number of cells to 2 x 10^6 cells per condition to screen single antigens. In that case as well we received positive results. However, since the number of T cell clones is proportional to the precursor frequency, initial reduction of cell number will hamper the future outcome. This was foremost the case when the number of naïve antigen-specific T cells was very low and the time left for proliferation was not sufficient to expand the existing cells above the number necessary for its detection.
Our standard protocol also led to successful generation of antigen-specific cells when a selected population of naïve T cells was used instead of PBMCs. Although the separation technique needs future optimization, this experiment, conducted as "proof of principle", shows that

specific T cell response was generated from a naïve T cell pool. In contrast to this, memory T cells which underwent similar procedures did not show any antigen-specific response.

Furthermore, this technique of purifying the initial T cell population could be applied in the future to a GMP-grade isolation protocol in order to achieve the highest possible purity. This may be crucial for patients after allogenic stem cell transplantation because of the possible occurrence of a graft versus host disease (GvHD).

4.6 Restimulation

Restimulation, although often very simple, is no doubt one of the most crucial procedures in successful cultivation of specific T cell clones. Feeder cells seem to be a crucial component for restimulation of antigen-specific T cells (Haas and von Boehmer, J Immunol Methods. 1982).

In our project we compared three different modes of restimulation using peptide-pulsed, radiated autologenic, allogenic and a mixture of both types of PBMCs. It turned out that autologenic PBMCs were the best feeder cells in our comparison. Its application resulted in a significantly higher percentage of generated antigen-specific T cells.

The time of application as well as persistence in the culture seems very important. Feeder cells should disappear from the cultures after 5 to 6 days, after radiation. However, in older cultures, feeder cells often remain present, which may act suppressively on cell proliferation. While expanding T cells in long-term culture, it has to be borne in mind that proliferation of T cells is defined and will not cross the Hayflick limit. Usually, it is reported that T cell clones slow down after less than 12 stimulations with the feeder cells and it becomes increasingly difficult to expand the cells. Our experience demonstrated that cells could have been cultivated without any problems even up to the fourth week, the maximal cultivation time reaching eight weeks. Since it is suggested that acquisition of full effector function paradoxically impairs the efficacy of antitumor specific T cells, it may be necessary to adjust the length of the culture accordingly. Moreover, since extended cultivation process enhances the possibility of occurrence of chromosomal aberrations and culture contamination, it should remain as short as possible.

4.7 Cytokine pool

Cytokines play a fundamental role in T cell homeostasis. Despite extensive research into cytokine control of T cell differentiation process, there are still many unanswered questions about appropriate timing and quantity of cytokines applied to *in vitro* cultures. While preparing and uniting different protocols we found out that most of the published protocols use the following interleukins: IL-2, IL-6, IL-7, IL-10, IL-12, IL-15. Unfortunately, they involve many differences in regard to their exact application.

In order to make a comparison between sometimes very distant generation strategies we had to make a few suppositions. Firstly, we assumed that the induction process must take place within the first hours after T cell contact with antigen. Secondly, we assumed that applied cytokines which probably play a supportive role in induction have to be applied within the first 12-24 hours. Thirdly, we decided to compare cytokine cocktails in the belief that addition of single cytokine may not be sufficient.

The first condition in our standard protocol was based on articles published by Montagna et al (Int. J. Cancer, 2004) and Carlsson et al, (The Prostate, 2004) and consisted of IL-7 and IL-12. IL-7 has been proven to be crucial for survival and homeostatic expansion of naïve T cells. This cytokine facilitates proliferation of naïve T cells while maintaining their naïve phenotype, in contrast to proliferation of antigen experienced T cells, which seem to be unaffected by IL-7 (Brenchley et al, Clin.Exp.Immunol, 2002). There exist also some hints suggesting a synergic enhancing effect of both IL-7 and IL-12 on proliferation and CD8+ mediated cytotoxicity (Mehrotra et al, J. Immunology, 1995). Moreover, exogenous application of IL-12 favours generation of antigen-specific CTLs and can induce stable and protective CD8+ T cell response. IL-12 also appears to interfere with IL-2. When used with a low dose of IL-2, it was induced proliferation and differentiation of CD8+ T cells. In contrast to the effect of higher concentrations of IL-2, this seems to trigger the inhibitory potential of IL-12.

The second cytokine cocktail included IL-7, IL-10 (Yee at al, J. Immunology, 1999) and IL-12 (Kieper et al, J. Immunology, 2001). IL-10 has been widely characterized as an immunosuppressive cytokine with pleiotropic effects. Although its major effects include the down-regulation of co-stimulatory and MHC molecule expression as well as pro-inflammatory cytokine production by APCs, when it is directly added to the cultures, it seems to enhance

CD8+ T cell cytotoxicity *in vitro*. Furthermore, application of IL-10 either alone or in combination with other cytokines, such as IL-2, IL-18, and IL-12, makes CTLs more efficient in lysing tumour targets (Maynard and Weaver. Immunol Rev., 2008).

The third cytokine compositions was prepared using IL-6; IL-12 (Kaiser et al, Eur. J. Immunology, 2003). IL-6 is a pro-inflammatory cytokine. At the same time, its anti-inflammatory effect is mediated inter alia through its activation of IL-1ra and IL-10 (Jones et al. J. Immunol., 2005). Exactly how the combination of IL-6 and IL-12 influences T cell population is unclear. Still it is worth mentioning that some groups have used the combination of those two cytokines for successful induction of specific memory CD8+ T cell response. Whether this effect of IL-6 includes its ability to suppress CD4+CD25+ regulatory T cells (Treg) remains open and requires further investigation (Longhi et al., PLoS Pathog., 2008).

The last condition was carried out with addition of IL-7 added on day 1 and IL-15 on day 5. It was based on the belief that as IL-7 is necessary for induction in order to assure proliferation of naïve T cells, IL-15 should also be added to promote the long-term survival of antigen experienced T cells (Surh and Sprent, Immunity, 2008). Although there are several reports maintaining the homeostatic role of IL-15 in T cell homeostasis (Ku et al, Science, 2000), the question remains open whether or not addition of IL-15 to the cultures already supplemented with IL-2 was necessary.

One of the things which linked all of the above-mentioned protocols with many others (Ito et al., J Gastroenterol Hepatol., 2001; Oelke et al. Nat. Med., 2003) was the usage of IL-2. This cytokine, identified by Morgan et al. in 1976, is today almost a canonical T cell grow factor and is commonly used for supplementation of T cell cultures. But because it also drives proliferation and activation of regulatory T cells, it has to be used for an induction within a certain time interval.

A protocol based on those four different cytokine conditions applied in many experiments with different antigen pools showed a great consistency in results. It was not possible to select one widely applicable condition. Apart from tested cytokine cocktails there are still other interesting cytokines like interleukin 18 (IL-18) (Wang et al., J. Immunol., 2009) and interleukin 21 (IL-21) which, due to their promoting the generation of antigen-specific CTLs, are worth analyzing.

4.8 Readout methods

The right choice of an adequate readout method is very important. For our purposes we needed a test which allows us to get an insight into T cell functionality, simultaneously defining its phenotypical markers and last but not least one which is easily applicable. Taking those three things into consideration we decided to choose an IFN-γ intracellular secretion assay. This assay was used as readout in all experiments involving a sufficient number of cells. For those with a limited number of cells, IFN-γ ELISpot assay was applied. In our experimental settings, tetramer or pentamer staining was inapplicable for two reasons. Firstly, due to high cost of synthesis of more than 15 different multimers. Secondly, because of the fact that positive tetramer staining delivers only information on the presence of specific TCR on the cell surface; as shown by several groups (He and Greenberg., Methods Mol Med. 2004).

The decision to utilize IFN-γ intracellular staining assay rather than the ELISpot assay was justified by the possibility that phenotypical characterization concurs with functional analysis. In a manner similar to ELISpot, most the cytotoxic assays also do not enable phenotypical characterization of the effector population. This can lead to false positive results due to cytolytic activity of other cell populations, such as that of natural killer cells (NK cells).

4.9 Characterisation of antigen-specific T cells

A proper characterisation of T cells can become a very demanding and complex issue. In the last few years there have been several innovative publications focusing on T cell characterization (Appay et al. Nat.Med., 2002). Nowadays, according to new trends in characterization of T cells, more attention is paid to cytokine profile of analysed cells (Lamoreaux et al., Nat Protoc. 2006) rather than to its phenotypical affiliation (Lanzavecchia and Sallusto, Nat Rev Immunol. 2002). Such complex analyses are only possible by using multicolour flow cytometry.

In this project we concentrate only on the analysis of the most important features of generated cells. We performed pentamer staining, combined with phenotypical characterisation only for positive in IFN-γ intracellular staining T cell clones. We could demonstrate pentamer, CD3 and CD8 positive populations in both the HBV and HCV antigen groups together with negative

controls. Unfortunately, due to the unspecific staining and lack of two pentamers compatible with the peptides used, no specific response could be shown in the HIV peptide group.
While performing characterisation of selected HBV- and HCV-specific CD8+ T cells we defined a FACS panel including ten phenotypical markers. Apart from cytotoxic T cells marker in the form of CD8, activation markers such as CD27 and CD28 were included. Naïve and memory T cells were distinguished by means of CD45RA, CD45RO, CCR7, and CD62L (Seder and Ahmed, Nature Immun., 2003; Lanzavecchia and Sallusto, Nat Rev Immunol. 2002). A predominant number of HBV- and HCV-specific CD8+ T cells expressed 65-90% of CD45RA negative and CCR7 positive phenotype classifying cell to effector memory phenotype. Additionally, the analyzed expression of CD57, whose expression on CD8+ T cells was shown to be associated with proliferation incompetence and replicative senescence was found only in up to 4% of the pentamer positive T cells. This finding implies that the overwhelming majority of generated cells is functional and capable of proliferation. Expression of an early activation marker CD69 was considered to occur differentially (between 25 and 90%) and requires additional investigation, especially in the course of following restimulations. High expression may indicate an increased sensitivity towards CD95-mediated apoptotic cell death *in vitro*.

4.10 Application of generated antigen-specific T cells

Generation of effective antiviral and anti-tumour adoptive T cell transfer remains a very personalized kind of treatment. In general, this kind of therapy can be applied both in viral and tumour diseases and can be performed in autologenic or allogenic setting. The application of autologenic T cells has been performed in many studies (Riddel et al. Science. 1992; Walter et al., N Engl J Med. 1995; Rooney et al., Blood. 1998). Most of them use antigen-specific T cells already primed *in vitro.* The present protocol tries to establish a system which would enable induction and expansion of antigen-specific T cells from naïve precursors. To summarize, the protocol created can be applied in a situation where protective memory T cell response has not occurred. Secondly, due to the usage of cytokines during the induction there is no need of additional use of professional antigen presenting cells. Thirdly, the method presented does not use any viral transduction system or any transformed cell lines. It is for this reason that the protocol described is easily adaptable to good manufacturing practice conditions. Moreover, the fact that the efficiency of this method usually reaches 40% of IFN-γ secreted antigen-specific T

cells seems satisfactory. This is especially the case, when one considers that the HLA.A*0201 epitopes applied here can be easily replaced with overlapping peptides.

An application of pure, highly specific T cell population would be considered as optimal approach in adoptive T cell transfer. In an autological setting, transfer of T cell culture remnants would not be considered as dangerous or critical for the therapeutic effect originally sought after. This issue seems completely different when considered in an allogenic setting. Transferring larger amounts of alloreactive T cells, parallel to desired T cell population, may cause severe graft versus host disease (GvHD) – with mortal consequences. It has already been shown by application of lymphocyte donor infusions that there is a strict dosage correlation between number of alloreactive T cells and occurrence of GvHD. Because of this, adoptive T cell transfer by patients after hematopoietic stem cell transplantation should be performed only with a highly purified T cell population. This can be achieved by enrichment of antigen-specific T cells in three ways: one, by means of tetramer or streptamer technology (Knabel et al. Nat Med. 2002; Neudorfer et al., J Immunol Methods. 2007), two, by usage of the IFN-γ cytokine capture assay (Brosterhus et al., Eur J Immunol. 1999), and three, via CD4 T cells depletion.

4.11 Adoptive T cell transfer versus autoimmunity

Various homeostatic mechanisms maintain a balance between responsiveness and tolerance. On the one hand, they try to protect the host from uncontrolled immune response. On the other hand they protect the host against potentially harmful autoimmunity. It is autoimmunity which has become a very critical and extensively discussed issue in the context of adoptive T cell transfer. Some immunologists are completely in agreement with each other that an effective antitumor response mostly towards self-antigens is impossible due to mechanisms of self-tolerance induction (Janicki et al.; Cancer Res. 2008). It is claimed that antigen-specific naïve precursors expressing high-affinity T-cell receptors (TCR) are often functionally deleted from the repertoire. Thus, the repertoire of naïve CD8+ T cell precursors remaining will entail only low-affinity TCR. Those opposed to this view point to the spectacular success of a lymphodepletion (Muranski et al., Nat Clin Pract Oncol, 2006), in murine models (Overwijk et al., J Exp Med., 2003; Gattinoni et al. J Exp Med., 2005) as well in humans (Dudley et al., Science, 2002), in which removal of regulatory T cells and competing cell populations creates an optimal environment for transferred cells. In cases where single or repeated lymphodepletion regimens

are contraindicated, it has been postulated that a curative therapy may be achieved by increasing the T-cell dose. Taken together, it appears that there are still many solutions possible for the avoidance of peripheral inhibitory mechanisms.

V. Summary

Reliable methods for the priming and *in vitro* expansion of antigen specific cytotoxic T lymphocyte cells (CTLs) are necessary for the adoptive immunotherapy with low-frequency precursors. Although several methods for the expansion of *in vivo* primed CTLs have been reported, the need for an efficient protocol to prime and expand low-frequency and naive CD8 positive cells *in vitro* still remains a challenge.

Here, we report on a method to efficiently expand *in vitro* low frequency antigen-specific T cells from antigen-naïve individuals. HLA A2 binding peptides of known immunogenicity from human immunodeficiency virus antigens (HIV), hepatitis C (HCV), and hepatitis B core antigens (HBV) were selected to prime *in vitro* naive T cells from healthy volunteers (negative for HIV, HBV, HCV). To optimize the induction procedure, four different cytokine cocktails including IL-6, IL-7, IL-10, IL-12 and IL-15 in various combinations and concentrations were tested. Antigen-pulsed mature dendritic cells (mDCs) did not enhance the priming efficiency when compared to that of the antigen alone. Among three different re-stimulation protocols, the most effective re-stimulation procedure required the use of irradiated, autologous and peptide-pulsed peripheral blood mononuclear cells (PBMCs). The cultures were supplemented with IL-2 from day 3 of the culture on. In most experiments, we were able to generate antigen-specific T cell response as demonstrated by the antigen-specific release of IFN-γ and tetramer staining.

We also applied the method to generate specific CTL cultures with tumour-associated antigens. All twelve screened tumour-associated antigens proved to be sufficiently immunogenic for healthy individuals to generate an antigen specific response. Furthermore, the experiment with enriched memory and naive T cells confirmed our thesis that only the antigen specific T cells can be generated from naive precursors.

Moreover, we applied an optimized method (using a cytokine cocktail including IL-6 and IL-12) which was able to successfully generate WT-1 specific CTLs under GMP conditions. PBMCs were obtained from a healthy steam cell donor, who is a sister of our AML patient. Six months after bone marrow transplant our patient suffered a relapse of her haematological malignancy. As part of the individualised experimental conditioning protocol, WT-1 specific T cells were used. Patients received all in all 6×10^8 T cells, from which 4.38×10^7 WT-1 specific T cells. All short- and long-term adverse events were taken into account.

In conclusion, this simple and effective approach can be utilized to generate CTLs of desired antigen specificity for an adoptive therapy in viral or malignant diseases.

VI. Zusammenfassung

Zuverlässige Methoden zur Induktion und *In-vitro*-Expansion von Antigen-spezifischen T-Zellen bilden die Grundlage zur Etablierung eines suffizienten adoptiven T-Zelltransfers von niedrig-frequenten oder sogar naiven T-Zellen. Obwohl mehrere Methoden zur *In-vitro*-Induktion und Expansion von naiven T-Zellen bereits beschrieben wurden, konnte bislang keine dieser Methoden im klinischen Alltag etabliert werden.

In dieser Arbeit gelang die Etablierung einer Methode zur effizienten *In-vitro*-Expansion von niedrig-frequenten Antigen-spezifischen T-Zellen von gesunden Antigen-naiven Spendern. Es wurden HLA.A2 positive, potenziell immunogene Antigene des Humanen Immundefizienz-Virus, der Hepatitis C und des Hepatitis-B-Core-Proteins ausgewählt und zur In-vitro-Induktion von naiven T-Zellen bei gesunden Spender eingesetzt.

Um die Induktionsprozedur zu optimieren, wurden vier Zytokin-Cocktails mit verschiendenen IL-6, IL-7 IL-10, IL-12 und IL-15 Kombinationen und Konzentrationen eingesetzt. Darüber hinaus wurde die Induktionseffizienz von reifen, Peptid beladenen dendritischen Zellen mit Zugabe von Antigen alleine verglichen. Durch den Einsatz von dendritischen Zellen konnte keine Steigerung der Induktionseffizienz erreicht werden. Als effektivstes Restimulationsprotokoll hat sich die Zugabe von bestrahlten, Peptid beladenen mononukleären Zellen des peripheren Blutes (PBMCs) erwiesen. Zusätzlich wurden die Zellkulturen mit IL-2 ab Tag 3 supplementiert. In überwiegender Mehrheit der Experimente, konnte eine Antigen-spezifische T-Zell Antwort generiert werden. Die spezifische CD3+/CD8+ T-Zellen konnten mittels IFN-γ-Sekretions-Assay sowie MHC-Multimer-Färbung nachgewiesen werden.

Die entwickelte Methode zur Induktion und Expansion Antigen-spezifischer T-Zellen wurde zur Generierung Tumor-spezifischer T-Zellen eingesetzt. Zwölf immunogene Tumor-assoziierte Antigene (TAA) wurden eingesetzt und konnten Antigen-spezifische Antworten bei gesunden Spendern generieren. Um die Induktion einer Antigen-spezifischen T-Zell Antwort durch naive T-Zellen zu beweisen, wurde ein Experiment mit angereichten naiven und Gedächtnis-T-Zellen durchgeführt. Antigen-spezifische T-Zellen konnten aus naiven T-Zellen generiert werden.

Diese verbesserte Methode (mit Einsatz von IL-6 und IL-12) wurde zur Generierung von WT-

1 spezifischen zytotoxischen T-Zellen unter GMP Bedingungen benutzt. PBMCs für diesen Ansatz wurden von einer gesunden Spenderin (Zwillingsschwester einer AML Patientin) gewonnen. Sechs Monate nach allogener Stammzelltransplantation von der Zwillingsschwester wurde ein Rezidiv der vorbekannten AML diagnostiziert. Bei fehlenden standardisierten Therapieoptionen wurden in Rahmen eines Heilversuches diese Tumor-spezifischen T-Zellen eingesetzt. Die Patientin erhielt 6 x 108 T-Zellen, davon 4,38 x 107 WT-1 spezifische T-Zellen. Es wurden keine Nebenwirkungen beobachtet.

Zusammenfassend zeigen die vorgelegten Ergebnisse, dass eine einfache und effektive Induktion und Expansion Antigen-spezifischer T-Zellen gewünschter Spezifität möglich ist und durch adoptiven T-Zelltransfer zur Therapie von viralen sowie Tumorerkrankungen angewendet werden kann.

VII. References

1. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med. 2002 Apr;8(4):379-85.
2. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998 Mar 19;392(6673):245-52.
3. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature. 2006 Feb 9;439(7077):682-7.
4. Becker C, Pohla H, Frankenberger B, et al. Adoptive tumor therapy with T lymphocytes enriched through an IFN-γ capture assay. Nat Med. 2001; 7:1159-1162.
5. Bergmann-Leitner ES, Abrams SI. Differential role of Fas/Fas ligand interactions in cytolysis of primary and metastatic colon carcinoma cell lines by human antigen-specific CD8+ CTL. J Immunol. 2000 May 1;164(9):4941-54.
6. Blattman JN, Antia R, Sourdive DJ, Wang X, Kaech SM, Murali-Krishna K, Altman JD, Ahmed R., Estimating the precursor frequency of naive antigen-specific CD8 T cells. J Exp Med.2002; Mar 4;195(5):657-64.
7. Bonnet D, Warren EH, Greenberg PD, Dick JE, Riddell SR. CD8(+) minor histocompatibility antigen-specific cytotoxic T lymphocyte clones eliminate human acute myeloid leukemia stem cells. Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8639-44.
8. Brenchley JM, Douek DC, Ambrozak DR, Chatterji M, Betts MR, Davis LS, Koup RA. Expansion of activated human naïve T-cells precedes effector function. Clin Exp Immunol. 2002 Dec;130(3):432-40.
9. Brosterhus H, Brings S, Leyendeckers H, et al. Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T-cells based on cytokine secretion. Eur J Immunol. 1999;29:4053-4059.
10. Burnet FM. Immunological surveillance in neoplasia. Transplant Rev. 1971;7:3-25.
11. Carlsson B, Tötterman TH, Essand M.Generation of cytotoxic T lymphocytes specific for the prostate and breast tissue antigen TARP. Prostate. 2004 Oct 1;61(2):161-70.

12. Cerny A, McHutchison JG, Pasquinelli C, Brown ME, Brothers MA, Grabscheid B, Fowler P, Houghton M, Chisari FV. Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. J Clin Invest. 1995 Feb;95(2):521-30.
13. Ciceri F, Bonini C, Stanghellini MT, Bondanza A, Traversari C, Salomoni M, Turchetto L, Colombi S, Bernardi M, Peccatori J, Pescarollo A, Servida P, Magnani Z, Perna SK, Valtolina V, Crippa F, Callegaro L, Spoldi E, Crocchiolo R, Fleischhauer K, Ponzoni M, Vago L, Rossini S, Santoro A, Todisco E, Apperley J, Olavarria E, Slavin S, Weissinger EM, Ganser A, Stadler M, Yannaki E, Fassas A, Anagnostopoulos A, Bregni M, Stampino CG, Bruzzi P, Bordignon C. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. Lancet Oncol. 2009 May;10(5):489-500.
14. Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H, Assenmacher M, Billingham L, Steward C, Crawley C, Olavarria E, Goldman J, Chakraverty R, Mahendra P, Craddock C, Moss PA. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. J Exp Med. 2005 Aug 1;202(3):379-86.
15. Cohen WM, Bianco A, Connan F, Camoin L, Dalod M, Lauvau G, Ferriès E, Culmann-Penciolelli B, van Endert PM, Briand JP, Choppin J, Guillet JG. Study of antigen-processing steps reveals preferences explaining differential biological outcomes of two HLA-A2-restricted immunodominant epitopes from human immunodeficiency virus type 1. J Virol. 2002 Oct;76(20):10219-25.
16. Croft M. Activation of naive, memory and effector T cells. Curr Opin Immunol. 1994 Jun;6(3):431-7.
17. Dauer M, Schad K, Herten J, Junkmann J, Bauer C, Kiefl R, Endres S, Eigler A.FastDC derived from human monocytes within 48 h effectively prime tumor antigen-specific cytotoxic T cells. J Immunol Methods. 2005 Jul;302(1-2):145-55.
18. Delorme EJ, Alexander P. Treatment of primary fibrosarcoma in the rat with immune lymphocytes. Lancet 1964 Jul 18;2(7351):117-20.
19. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA.

Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science. 2002 Oct 25;298(5594):850-4.

20. Dudley ME, Rosenberg SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer. 2003 Sep;3(9):666-75.
21. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002 Nov;3(11):991-8.
22. Eshhar Z, Waks T, Bendavid A, Schindler DG. Functional expression of chimeric receptor genes in human T cells. J Immunol Methods. 2001 Feb 1;248(1-2):67-76.
23. Falkenburg JH, Goselink HM, van der Harst D, van Luxemburg-Heijs SA, Kooy-Winkelaar YM, Faber LM, de Kroon J, Brand A, Fibbe WE, Willemze R, et al. Growth inhibition of clonogenic leukemic precursor cells by minor histocompatibility antigen-specific cytotoxic T lymphocytes. J Exp Med. 1991 Jul 1;174(1):27-33.
24. Ferrari G, Kostyu DD, Cox J, Dawson DV, Flores J, Weinhold KJ, Osmanov S. Identification of highly conserved and broadly cross-reactive HIV type 1 cytotoxic T lymphocyte epitopes as candidate immunogens for inclusion in Mycobacterium bovis BCG-vectored HIV vaccines. AIDS Res Hum Retroviruses. 2000 Sep 20;16(14):1433-43.
25. Fujita Y, Rooney CM, Heslop HE. Adoptive cellular immunotherapy for viral diseases. Bone Marrow Transplant. 2008 Jan;41(2):193-8.
26. Gattinoni L, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, Hwang LN, Yu Z, Wrzesinski C, Heimann DM, Surh CD, Rosenberg SA, Restifo NP.J Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. Exp Med. 2005 Oct 3;202(7):907-12.
27. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C, Wang E, Douek DC, Price DA, June CH, Marincola FM, Roederer M, Restifo NP. A human memory T cell subset with stem cell-like properties., Nat Med. 2011 Sep 18;17(10):1290-7.
28. Gulen D, Abe F, Maas S, Reed E, Cowan K, Pirruccello S, Wisecarver J, Warkentin P, Northam M, Turken O, Coskun U, Senesac J, Talmadge JE. Closing the manufacturing process of dendritic cell vaccines transduced with adenovirus vectors. Int Immunopharmacol. 2008 Dec 20;8(13-14):1728-36.
29. Haas W, Von Boehmer H. Methods for the establishment of continuously growing cytolytic T cell clones. J Immunol Methods. 1982 Jul 30;52(2):137-48.

30. He XS, Greenberg HB. Detection and characterization of virus-specific CD8+ T cells using the tetramer approach. Methods Mol Med. 2004;96:89-96.
31. Ho WY, Nguyen HN, Wolfl M, Kuball J, Greenberg PD. In vitro methods for generating CD8+ T-cell clones for immunotherapy from the naïve repertoire. J Immunol Methods. 2006 Mar 20;310(1-2):40-52.
32. Hoogendoorn M, Wolbers JO, Smit WM, Schaafsma MR, Barge RM, Willemze R, Falkenburg JH. Generation of B-cell chronic lymphocytic leukemia (B-CLL)-reactive T-cell lines and clones from HLA class I-matched donors using modified B-CLL cells as stimulators: implications for adoptive immunotherapy. Leukemia. 2004 Jul;18(7):1278-87.
33. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, Rimm AA, Ringdén O, Rozman C, Speck B, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990 Feb 1;75(3):555-62.
34. Janicki CN, Jenkinson SR, Williams NA, Morgan DJ. Loss of CTL function among high-avidity tumor-specific CD8+ T cells following tumor infiltration. Cancer Res. 2008
35. Ito A, Kanto T, Kuzushita N, Tatsumi T, Sugimoto Y, Miyagi T, Takehara T, Katayama K, Mochizuki K, Hiramatsu N, Kasahara A, Yoshiya I, Sasaki Y, Hori M, Hayashi N. Generation of hepatitis C virus-specific cytotoxic T lymphocytes from healthy individuals with peptide-pulsed dendritic cells. J Gastroenterol Hepatol. 2001 Mar;16(3):309-16.
36. Kaech SM, Hemby S, Kersh E, Ahmed R. Molecular and functional profiling of memory CD8 T cell differentiation. Cell. 2002 Dec 13;111(6):837-51.
37. Kaiser E, Förster R, Wolf I, Ebensperger C, Kuehl WM, Lipp M. The G protein-coupled receptor BLR1 is involved in murine B cell differentiation and is also expressed in neuronal tissues. Eur J Immunol. 1993 Oct;23(10):2532-9
38. Kaplan MH, Wurster AL, Grusby MJ. A signal transducer and activator of transcription (Stat)4-independent pathway for the development of T helper type 1 cells. J Exp Med. 1998 Sep 21;188(6):1191-6.
39. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, Chen CC, Yang JC, Rosenberg SA, Hwu P. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res. 2006 Oct 15;12(20 Pt 1):6106-15.
40. Kieper WC, Prlic M, Schmidt CS, Mescher MF, Jameson SC. Il-12 enhances CD8 T cell

homeostatic expansion. J Immunol. 2001 May 1;166(9):5515-21.

41. Knabel M, Franz TJ, Schiemann M, Wulf A, Villmow B, Schmidt B, Bernhard H, Wagner H, Busch DH. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. Nat Med. 2002 Jun;8(6):631-7.
42. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdonck L, Niederwieser D, van Rhee F, Mittermueller J, de Witte T, Holler E, Ansari H; European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood. 1995 Sep 1;86(5):2041-50.
43. van Kooyk Y, Appelmelk B, Geijtenbeek TB. A fatal attraction: Mycobacterium tuberculosis and HIV-1 target DC-SIGN to escape immune surveillance. Trends Mol Med. 2003 Apr;9(4):153-9.
44. Ku CC, Murakami M, Sakamoto A, Kappler J, Marrack P. Control of homeostasis of CD8+ memory T cells by opposing cytokines. Science. 2000 Apr 28;288(5466):675-8.
45. Kuball J, Schmitz FW, Voss RH, Ferreira EA, Engel R, Guillaume P, Strand S, Romero P, Huber C, Sherman LA, Theobald M. Cooperation of human tumor-reactive CD4+ and CD8+ T cells after redirection of their specificity by a high-affinity p53A2.1-specific TCR. Immunity. 2005 Jan;22(1):117-29.
46. Kurokawa T, Oelke M, Mackensen A. Induction and clonal expansion of tumor-specific cytotoxic T lymphocytes from renal cell carcinoma patients after stimulation with autologous dendritic cells loaded with tumor cells. Int J Cancer. 2001 Mar 15;91(6):749-56.
47. Lamoreaux L, Roederer M, Koup R. Intracellular cytokine optimization and standard operating procedure. Nat Protoc. 2006;1(3):1507-16.
48. Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol. 2002 Dec;2(12):982-7.
49. Lanzavecchia A, Sallusto F. Understanding the generation and function of memory T cell subsets. Curr Opin Immunol. 2005 Jun;17(3):326-32.
50. Li Y, Cu YT, Luo D. Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Nat Biotechnol. 2005 Jul;23(7):885-9.
51. Livingston BD, Crimi C, Fikes J, Chesnut RW, Sidney J, Sette A. Immunization with the HBV core 18-27 epitope elicits CTL responses in humans expressing different HLA-A2 supertype molecules. Hum Immunol. 1999 Nov;60(11):1013-7.

52. Longhi MP, Wright K, Lauder SN, Nowell MA, Jones GW, Godkin AJ, Jones SA, Gallimore AM. Interleukin-6 is crucial for recall of influenza-specific memory CD4 T cells. PLoS Pathog. 2008 Feb 29;4(2):e1000006.
53. Loskog A, Giandomenico V, Rossig C, Pule M, Dotti G, Brenner MK.Addition of the CD28 signaling domain to chimeric T-cell receptors enhances chimeric T-cell resistance to T regulatory cells. Leukemia. 2006 Oct;20(10):1819-28.
54. Maynard CL, Weaver CT. Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. Immunol Rev. 2008 Dec;226:219-33.
55. Mehrotra PT, Grant AJ, Siegel JP. Synergistic effects of IL-7 and IL-12 on human T cell activation. J Immunol. 1995 May 15;154(10):5093-102.
56. Milano F, van Baal JW, Rygiel AM, Bergman JJ, Van Deventer SJ, Kapsenberg ML, Peppelenbosch MP, Krishnadath KK. An improved protocol for generation of immuno-potent dendritic cells through direct electroporation of CD14+ monocytes. J Immunol Methods. 2007 Apr 10;321(1-2):94-106.
57. Montagna D, Maccario R, Locatelli F, Rosti V, Yang Y, Farness P, Moretta A, Comoli P, Montini E, Vitiello A. Ex vivo priming for long-term maintenance of antileukemia human cytotoxic T cells suggests a general procedure for adoptive immunotherapy. Blood. 2001 Dec 1;98(12):3359-66.
58. Montagna D, Schiavo R, Gibelli N, Pedrazzoli P, Tonelli R, Pagani S, Assirelli E, Locatelli F, Pession A, Fregoni V, Montini E, Da Prada GA, Siena S, Maccario R. Ex vivo generation and expansion of anti-tumor cytotoxic T-cell lines derived from patients or their HLA-identical sibling. Int J Cancer. 2004 May 20;110(1):76-86.
59. Montagna D, Daudt L, Locatelli F, Montini E, Turin I, Lisini D, Giorgiani G, Bernardo ME, Maccario R. Single-cell cloning of human, donor-derived antileukemia T-cell lines for in vitro separation of graft-versus-leukemia effect from graft-versus-host reaction. Cancer Res. 2006 Jul 15;66(14):7310-6.
60. Morse MA, Hobeika A, Osada T, Niedzwiecki D, Marcom PK, Blackwell KL, Anders C, Devi GR, Lyerly HK, Clay TM.Long term disease-free survival and T cell and antibody responses in women with high-risk Her2+ breast cancer following vaccination against Her2. J Transl Med. 2007 Sep 6;5:42.
61. Muranski P, Boni A, Wrzesinski C, Citrin DE, Rosenberg SA, Childs R, Restifo NP. Increased intensity lymphodepletion and adoptive immunotherapy - how far can we go? Nat Clin Pract Oncol. 2006 Dec;3(12):668-81.

62. Neudorfer J, Schmidt B, Huster KM, Anderl F, Schiemann M, Holzapfel G, Schmidt T, Germeroth L, Wagner H, Peschel C, Busch DH, Bernhard H. Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. J Immunol Methods. 2007 Mar 30;320(1-2):119-31.
63. Oelke M, Maus MV, Didiano D, June CH, Mackensen A, Schneck JP. Ex vivo induction and expansion of antigen-specific cytotoxic T cells by HLA-Ig-coated artificial antigen-presenting cells. Nat Med. 2003 May;9(5):619-24..
64. Oosten LE, Blokland E, van Halteren AG, Curtsinger J, Mescher MF, Falkenburg JH, Mutis T, Goulmy E. Artificial antigen-presenting constructs efficiently stimulate minor histocompatibility antigen-specific cytotoxic T lymphocytes. Blood. 2004 Jul 1;104(1):224-6.
65. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Dellemijn TA, Antony PA, Spiess PJ, Palmer DC, Heimann DM, Klebanoff CA, Yu Z, Hwang LN, Feigenbaum L, Kruisbeek AM, Rosenberg SA, Restifo NP. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. J Exp Med. 2003 Aug 18;198(4):569-80.
66. Palmer DH, Midgley RS, Mirza N, Torr EE, Ahmed F, Steele JC, Steven NM, Kerr DJ, Young LS, Adams DH.A phase II study of adoptive immunotherapy using dendritic cells pulsed with tumor lysate in patients with hepatocellular carcinoma. Hepatology. 2009 Jan;49(1):124-32.
67. Pahl-Seibert MF, Juelch M, Podlech J, Thomas D, Deegen P, Reddehase MJ, Holtappels R. Highly protective in vivo function of cytomegalovirus IE1 epitope-specific memory CD8 T cells purified by T-cell receptor-based cell sorting. J Virol. 2005 May;79(9):5400-13.
68. Parham P, McQueen KL. Alloreactive killer cells: hindrance and help for haematopoietic transplants. Nat Rev Immunol. 2003 Feb;3(2):108-22.
69. Pittet MJ, Valmori D, Dunbar PR, Speiser DE, Liénard D, Lejeune F, Fleischhauer K, Cerundolo V, Cerottini JC, Romero P. High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. J Exp Med. 1999 Sep 6;190(5):705-15.
70. Riddell SR, Greenberg PD. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. J Immunol Methods. 1990 Apr

17;128(2):189-201.

71. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T-cell clones. Science. 1992; 257:238-241.
72. Riley JL, June CH. The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. Blood. 2005 Jan 1;105(1):13-21.
73. Rooney CM, Smith CA, Ng CY, Loftin SK, Sixbey JW, Gan Y, Srivastava DK, Bowman LC, Krance RA, Brenner MK, Heslop HE. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. Blood. 1998 Sep 1;92(5):1549-55.
74. Rosenberg SA. Shedding light on immunotherapy for cancer. N Engl J Med. 2004 Apr 1;350(14):1461-3.
75. Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol. 2009 Apr;21(2):233-40.
76. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med. 1988 Dec 22;319(25):1676-80.
77. Sadelain M, Rivière I, Brentjens R. Targeting tumours with genetically enhanced T lymphocytes. Nat Rev Cancer. 2003 Jan;3(1):35-45.
78. Schultze JL, Michalak S, Seamon MJ, Dranoff G, Jung K, Daley J, Delgado JC, Gribben JG, Nadler LM.CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. J Clin Invest. 1997 Dec 1;100(11):2757-65.
79. Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol. 2003 Sep;4(9):835-42.
80. Sherman LA, Theobald M, Morgan D, Hernandez J, Bacik I, Yewdell J, Bennink J, Biggs J. Strategies for tumor elimination by cytotoxic T lymphocytes. Crit Rev Immunol. 1998;18(1-2):47-54.
81. Sijts A, Zaiss D, Kloetzel PM. The role of the ubiquitin-proteasome pathway in MHC class I antigen processing: implications for vaccine design. Curr Mol Med. 2001 Dec;1(6):665-76.

82. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med. 1973 May 1;137(5):1142-62.
83. Sun H, Kundu N, Dorsey R, Jackson MJ, Fulton AM. Expression of the Chemokines IP-10 and Mig in IL-10 Transduced Tumors. J Immunother 2001 Mar;24(2):138-143.
84. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity. 2008 Dec;29(6):848-62.
85. Suri D, Schilling R, Lopes AR, Mullerova I, Colucci G, Williams R, Naoumov NV. Non-cytolytic inhibition of hepatitis B virus replication in human hepatocytes. J. Hepatol. 2001; Dec;35(6):790-7.
86. Sutmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP, Toes RE, Offringa R, Melief CJ. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med. 2001 Sep 17;194(6):823-32.
87. Suzuki E, Kapoor V, Cheung HK, Ling LE, DeLong PA, Kaiser LR, Albelda SM. Soluble type II transforming growth factor-beta receptor inhibits established murine malignant mesothelioma tumor growth by augmenting host antitumor immunity. Clin Cancer Res. 2004 Sep 1;10(17):5907-18.
88. Szmania S, Galloway A, Bruorton M, Musk P, Aubert G, Arthur A, Pyle H, Hensel N, Ta N, Lamb L Jr, Dodi T, Madrigal A, Barrett J, Henslee-Downey J, van Rhee F. Isolation and expansion of cytomegalovirus-specific cytotoxic T lymphocytes to clinical scale from a single blood draw using dendritic cells and HLA-tetramers. Blood. 2001 Aug 1;98(3):505-12.
89. Tramsen L, Koehl U, Tonn T, Latgé JP, Schuster FR, Borkhardt A, Uharek L, Quaritsch R, Beck O, Seifried E, Klingebiel T, Lehrnbecher T. Clinical-scale generation of human anti-Aspergillus T cells for adoptive immunotherapy. Bone Marrow Transplant. 2009 Jan;43(1):13-9.
90. Uckert W, Schumacher TN. TCR transgenes and transgene cassettes for TCR gene therapy: status in 2008. Cancer Immunol Immunother. 2009 May;58(5):809-22.
91. Urbani S, Uggeri J, Matsuura Y, Miyamura T, Penna A, Boni C, Ferrari C. Identification of immunodominant hepatitis C virus (HCV)-specific cytotoxic T-cell epitopes by

stimulation with endogenously synthesized HCV antigens. Hepatology. 2001 Jun;33(6):1533-43.

92. Waldmann TA. Immunotherapy: past, present and future. Nat Med. 2003 Mar;9(3):269-77.
93. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038-1044.
94. Wang Y, Chaudhri G, Jackson RJ, Karupiah G. IL-12p40 and IL-18 play pivotal roles in orchestrating the cell-mediated immune response to a poxvirus infection. J Immunol. 2009 Sep 1;183(5):3324-31
95. Wolf M, Kuball J, Ho WY, Nguyen H, Manley TJ, Bleakley M, Greenberg PD. Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. Blood. 2007 Jul 1;110(1):201-10.
96. Yee C, Savage PA, Lee PP, Davis MM, Greenberg PD. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. J Immunol. 1999 Feb 15;162(4):2227-34.
97. Zhang B, Bowerman NA, Salama JK, Schmidt H, Spiotto MT, Schietinger A, Yu P, Fu YX, Weichselbaum RR, Rowley DA, Kranz DM, Schreiber H. Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. J Exp Med. 2007 Jan 22;204(1):49-55.

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version nicht veröffentlicht.

Publications/ Abstracts and Posters

Agnieszka Wieczorek, Carmen Scheibenbogen, Anttonio Pezzuto, Lutz Uharek, Kang-Hun Lee. *In vitro* induction and expansion of CD8 positive T cells: a method applicable for the generation of low-frequency antigen specific CD8 positive T cells. *(Manuscript, submitted)*

Wieczorek A., Lee K.H., Scheibenbogen C., Uharek L. *In vitro* induction and expansion of CD8 positive T cells: a method applicable for generation of low-frequency antigen specific CD8 positive T cells. *(Cancer Immunotherapy (CIMT), 6th Annual Meeting May 2008, Mainz, Poster)*

Wieczorek A, Lee K.H., Thiel E., Uharek L. *In vitro* induction and expansion of CD8 positive T cells: a method applicable for the GMP graded generation of low-frequency antigen specific CD8 positive T cells. (*Deutsche Gesellschaft für Hämatologie und Onkologie, Jahrestagung, Oktober 2008, Vienna, Poster)*

Wieczorek A., Lee K.H., Thiel E., Scheibenbogen C., Uharek L. Efficient GMP graded method for in vitro induction and expansion of low-frequency antigen specific CD8 positive T-cells. *Bone Marrow Transplantation 2010, Vol. 45, Suppl 2, 1-451; abstract P 950. (European Group for Blood and Marrow Transplantation, 36th Annual Meeting, March 2010, Vienna.)*

Acknowledgements

Firstly, I would like to take this opportunity to thank all the people who provided scientific and financial support to make this thesis possible. I must thank Prof. Thiel for allowing me to work in a clinic presided over by him.

Secondly, I would like to thank Prof. Scheibenbogen who gave me the unique opportunity to use the excellent research facilities in her laboratory. My sincere thanks goes also to Prof. Uharek who made it possible to continue work on the project, for giving me possibility of scientific discussion, for his advice and continuous support that I always greatly appreciated.

I am deeply indebted to Dr. Lee, the initiator of my thesis project, for embarking with me on this journey and teaching me how good immunology should be done. He taught me science in a very ambitious, critical and honest way. I appreciate the contribution of his time and ideas.

Furthermore, I would like to express my greatest thanks for help and encouragement to other members of our research group, i.e. Mareen, Christian and Benjamin who I had the pleasure to workwith or alongside – sometimes literally night and day.

My sincere thanks goes to Dr. Jenner and Mrs. Gericke for their guidance, advice and support in dealing with biostatistics.

Special thanks are also for my friend, Uta, who encouraged me to stick to my goal and was very supportive during the last two years.

I want to thank Roman for his critical presence and constant IT support.

I gratefully acknowledge the funding sources that made my work possible. I received a support from the scholarship provided by Berliner Krebsgesellchaft for my second year of the research.
I would also like to thank my parents for general education and for giving me the opportunity to start and pursue an adventure in the field of immunology. Despite their sudden and unexpected death, I still feel their unending encouragement and critical support.

Last but by no m eans least, very special thanks goes to m y beloved sister Justyna for being such a great com panion through the wilderness of our childhood. Your friendship helps m e confront our past and inspires me to look inquisitively and passionately into the future. I miss you very much and I am looking forward to living much closer to you.

Thank you

Eidesstattliche Versicherung

„Ich, Agnieszka, Wieczorek versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema:

> „In vitro induction and expansion of CD8 positive T cells: a method applicable for the generation of low-frequency antigen specific CD8 positive T cells"

selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Betreuer, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst."

22.01.2013 Agnieszka Wieczorek