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In vitro and in vivo characterization of the Cytomegalovirus and Polyomavirus BK specific immune response

Inauguraldissertation

zur Erlangung der Würde eines Doktors der Philosophie
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Dr. med. Adrian Egli
aus Basel, BS und Wald, ZH, Schweiz

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Prof. Dr. A. Rolink (Fakultätsvertreter)
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Dekan

Prof. Dr. Eberhard Parlow

Für meine Eltern und David

Was sagst du, Freund? das ist kein kleiner Raum:
Da sieh nur hin! du siehst das Ende kaum.

Ein Hundert Feuer brennen in der Reihe;

man tanzt, man schwatzt, man kocht, man trinkt, man liebt -

nun sage mir, wo es was Bessers gibt!“

(Wolfgang Goethe, Faust)

Index

Abbreviations

A

aa	Amino acid
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
AR	Acute rejection
ART	Anti-retroviral therapy

B

BCIP	5-Bromo-4-chloro-3-indolylphosphate
BFA	Brefeldin A
BKV	BK virus (Polyomavirus BK)
BSA	Bovine serum albumin

C

CD	Cluster of differentiation e.g. CD4+
CDV	Cidofovir
CMV	Cytomegalovirus
CsA	Cyclosporin A
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte

D

D	Donor
DC	Dendritic cell
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid

E

EBV	Epstein Barr Virus
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ESA	Enzyme-linked immunospot assay (Elispot)

F

FACS	Fluorescence activated cell sorting
FK506	Tacrolimus, also see “Tac”

G

gB	Glycoprotein B
GCV	Ganciclovir
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte and macrophage colony-stimulating factor
GranB	Granzyme B
GvHD	Graft versus host disease

H

HAART	Highly active anti-retroviral therapy
HC	Haemorrhagic cystitis
HD	Healthy blood donor
HEK	Human embryonic kidney (cell line)
HHV6	Human Herpes virus 6
HHV8	Human Herpes virus 8
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation

I

i.v.	Intravenous
IE	Immediate early (phase of cytomegalovirus gene expression)
IFN	Interferon e.g. alpha, beta, or gamma
Ig	Immunoglobulin
IL	Interleukin e.g. IL-17
IRIS	Immune reconstitution inflammatory syndrome, also IRS
ISG	Interferon stimulated genes

J

JCV	JC virus (Polyomavirus JC)

K

KCl	Potassium chloride
KIV	KI virus, Polyomavirus KI (Karolinska Institute)
KT	Kidney transplant patient

L

LPS	Lipo-polysaccharide
LT	Large T (early protein)
Lys	Lysate

M

Mϕ	Macrophage
mDC	Mature dendritic cell
MHC	Major histocompatibility complex
MPyV	Merkel cell carcinoma associated Polyomavirus
MPA	Mycophenolate acid
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin

N

NaCl	Sodium chloride
NBT	p-Nitroblue Tetrazoliumchlorid
NCCR	Non-coding control region
NK cell	Natural killer cell
NOD	Nucleotide-binding oligomerization domain, NOD-like receptors
NLS	Nuclear localization signal

O

OD	Optical density

P

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Perf	Perforin
PML	Progressive multifocal leukoencephalopathy
pp	Phosphoprotein
pp65	CMV capsid protein (late protein)
pp72	CMV immediate early 1 protein (immediate early protein)
PVAN	Polyomavirus associated nephropathy
PyV	Polyomavirus

R

R	Recipient
Rapa	Rapamycin, also see “Sir”
Rb	Retinoblastoma susceptibility protein
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (cell medium)
RPTEC	Renal proximal tubular epithelial cells (cell line)

S

SEB	Staphylococcal enterotoxin B (super antigen)
SFU	Spot forming unit
Sir	Sirolimus, also see “Rapa”
siRNA	Small interfering RNA
SOT	Solid organ transplantation
sT	Small T antigen (early protein)
SV40	Simian virus 40

T

Tac	Tacrolimus, also see FK506
TCR	T-cell receptor
TEM	Effector memory T-cell
Th1	T helper cell type 1
Th2	T helper cell type 2
TMB	Tetramethylbenzidine
TLR	Toll like receptor
TREC	T-cell receptor excision cycle
T-reg	T regulatory cells
TRIS	Tris-hydroxymethyl-amino methane

U

UL	Unique long domain, gene sequence in CMV genome e.g. UL97

V

VLP	Virus like particle
VP1	Viral capsid protein 1 (late structural protein)

W

wt	Wild-type
WUV	WU virus, Polyomavirus WU (Washington university)

Miscellaneous

+	Positive
-	Negative
3D	3 dimensional

1. Introduction

The deceptively small size and simple structure of viruses feints their diverse and fascinating interactions with the infected host cells and the corresponding antiviral host responses. During my PhD thesis, I focused on three main aspects of these complex interactions for Cytomegalovirus (CMV) and Polyomavirus BK (BKV):

(I) Measurement of CMV and BKV-specific immune response in healthy individuals and kidney transplant recipients

(II) Determination of immunological risk factors associated with virus replication and development of virus-associated diseases

(III) Identification of immuno-dominant responses to CMV and BKV protein and epitopes

CMV and BKV have an significant impact on transplantation with considerable morbidity, graft dysfunction or loss, and even mortality (Hirsch and Steiger 2003; Egli, Binggeli et al. 2007; Fishman 2007).

A deeper understanding of the basic mechanisms of virus recognition by the immune system and the nature of the resulting immune response in healthy individuals vis-à-vis immunosuppressed patients may help (i) to improve current immunological monitoring assays; (ii) to further identify risk groups for virus replication; (iii) to quantify risks for progression to virus diseases; (iv) to guide antiviral and immunosuppressive therapies; and (v) to develop new therapeutic strategies such as vaccines and adoptive T-cell transfer (Hoffmeister, Kiecker et al. 2003).

1.1. General aspects of virus and host interaction

According to Darwin's principle "survival of the fittest", viruses require sufficient replication for transmission to new potential hosts. The host cells have developed respective defence mechanisms to prevent damage from virus replication. Adaptation of the virus and host occurred co-evolutionary over time and our current view of virus infections result from old evolutionary

established companionship and new challenges due to new opportunity regarding transmission as well as gaps in the immune defence..

Viruses are obligate intracellular pathogens. Importantly, the host and particularly cell tropism are dependent on several factors:

(I) Availability of cell surface receptors for virus entry.
(II) The specificities of the replication machinery in infected cells, built through a conglomerate of the hosts' transcriptome, proteome, and cytokinome.
(III) The immunological pressure presence at the site of replication.

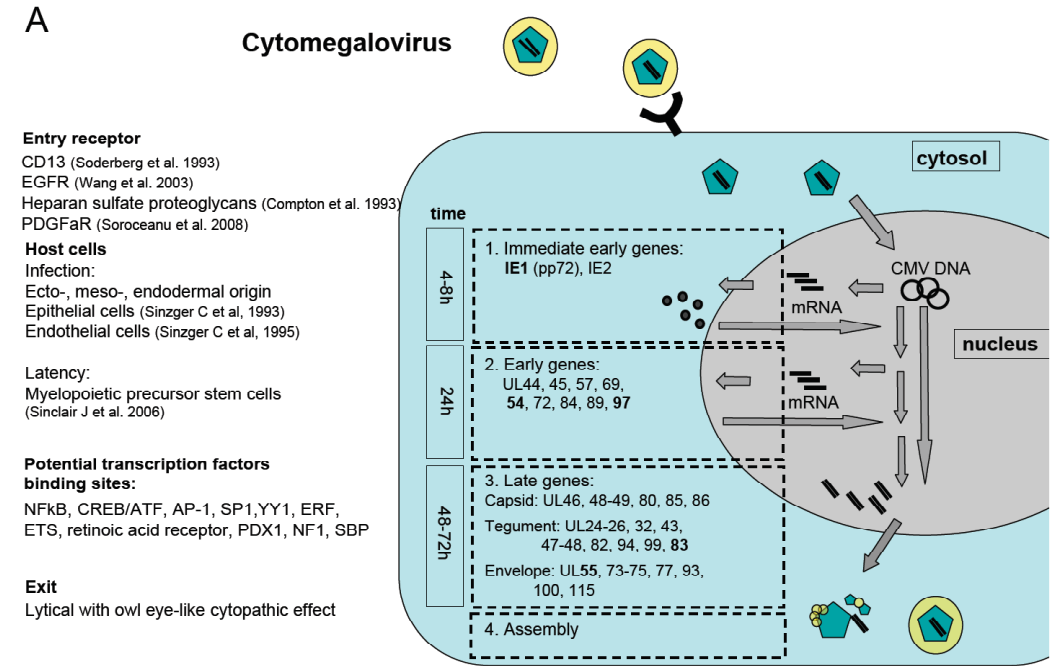
Interestingly, viral cell tropism is not static. Tropism can adapt during the course of infection, to recruit further replication seites or to establish latency (Este, Babrera et al. 1999; Mosier, Picchio et al. 1999).

The first step is viral entry into a host cell. In case of a lytic infection, the cellular replication machinery is redirected for production of more virus particles. The viral genome is replicated, required proteins are built, assembled, and packaged into the newly formed viral particles. Finally, viruses exit cells either via lytical or non-lytical mechanisms. In the case of an abortive infection, the virus may not replicate in the cell due to suboptimal tropism or killing by the host immune response. In the case of a latent infection, viruses only express latency associated proteins and the host cell remains intact.

Figure 1 shows the described pathways of virus replication from cell entry to production and assembly of virus proteins, and virus exit during CMV and BKV infection (**Figure 1**, further details can be found in chapter 1.3.1. and 1.3.2.).

The virus either (I) replicates actively with high viral loads and it becomes cleared by the host immune response. Or, (II) after the primary round of

replication the virus establishes latency or a low replicating state with no clearance, and builds equilibrium together with the host immune response.



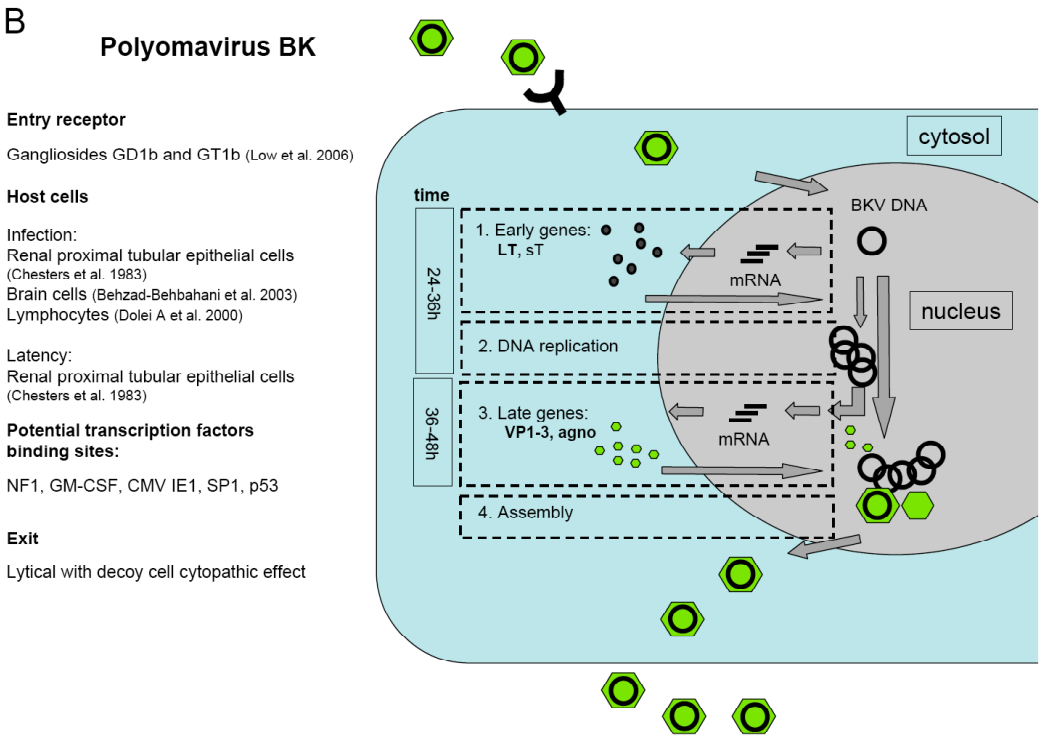


Figure 1. Replication cycle of Cytomegalovirus (**A**) and Polyomavirus BK (**B**). Left text box indicates: entry receptor, host cells infected and transcription factor binding site, and exit mechanisms.
Right scheme shows: cellular mechanism of replication with time scale for the key processes.

1.1.1. Virus latency and reactivation

A virological definition of latency is the infection of host cells with harboured full-length virus genomes, but without replication. During latency, viruses need to avoid immune recognition and initiation of defence mechanisms. This can be achieved by significantly lower antigen presentation and expression of a special set of latency associated proteins. As a cost, no active virus transmission occurs during the latent phase.
From the clinical point of view, latency is infection of a person without disease.

When the host is in “danger” for example during sepsis, trauma, or elective surgery, higher levels of transcription factors are expressed. These transcription factors bind to host gene promoters, but may activate viral promoter and reactivate replication of latent viruses (Sinclair and Sissons 2006; Miller, Rashid et al. 2007; von Muller, Klemm et al. 2007; Limaye, Kirby et al. 2008; Sharma, Gerlitz et al. 2008; Yan, Huang et al. 2008).
Examples of important transcription factors are NFkB and AP-1 (Ha, Park et al. 2004; Lopez-Maury, Marguerat et al. 2008; Montalvo-Jave, Escalante-Tattersfield et al. 2008). These factors may also be activated in the setting of transplantation, either in the donor due to brain damage and ischemia, or in the recipient by the condition leading to failure of the organ to be transplanted or by the surgical procedure. Down-modulation of host cell transcription factors pre-transplantation with siRNA may be a promissing future treatment option to avoid virus reactivation in ischemic phases (Zhen, Lian et al. 2007).

The general course for virus reactivation in transplantation results from activating factors (cytokines, ischemia, etc) and loss of adwquate immune control.

1.1.2. Host immune response

Throughout evolution, viruses and hosts have been engaged in an evolutionary arms race (Domingo 2007). From the evolutionary standpoint, viral infection places host cells under an important pressure to build up an efficient defence mechanism against these obligate intracellular parasites (Cooper 1976; Klein 1982; Tarikudanathan and Sayegh 2007; Cooper 2008; Ferrer.Admetlla, Bosch et al. 2008; Lanier 2008; Tarlinton 2008). Differentiation of self from non-self is the key function of the immune system and executed through the innate and adaptive immune response by inducing an inflammatory reaction (Metchnikoff 1883; Zinkernagel and Doherty 1974).

1.1.2.1. Innate immune response

The hallmark of the innate immunity is its limited but predefined specificity to divide between self and non-self, allowing fast responses yet without immunological memory.

The innate immune response is built upon cellular and humoral effectors.
The first line of cellular defence is the epithelial barrier on skin, in the respiratory and gastrointestinal tract. These barriers contain anti-microbial proteins and more specialized cells (Gallo, Murakami et al. 2002).
Most host cells, but especially professional antigen presenting cells (APC) such as DCs, B-cells and macrophages, can recognize pathogen-associated molecular patterns (PAMPs). Typical pathogen structures (non-self) such as basic lipids and sugar structures or characteristic DNA and RNA motives are recognised by Toll like receptors (TLR, (Arancibia, Beltran et al. 2007; Bowie 2007; Schröder and Bowie 2007)), Mannose-binding-lectin, scavenger receptors, NOD-like receptors (Rietdijk, Burwell et al. 2008), and the inflammosome complex (Lee and Kim 2007; Muruve, Petrilli et al. 2008).

The activation of PAMP receptors lead to a release of humoral effectors of the innate immune system such as interferon alpha or beta, and other inflammatory cytokines leading to a anti-viral or -bacterial state of neighbouring cells (Barton and Medzhitov 2002). Additionally co-stimulatory

molecules for T-cells are up-regulated and antigens processed via MHC. Thereby, the APC builds an important bridge from the innate to the adaptive immune response (**Figure 2** for overview of factors of the innate immune system).

Other humoral effectors of the innate immune system consists of soluble factors such as kinin-, coagulation-, and complement-system, which have an antimicrobial effect (Tomlinson 1993; van der Poll 2001). Especially the complement system is able to recruit further inflammatory cell, is part of the opsonization process and able to induce killing via building the membrane attack complex in antibody-marked pathogens (Bhakdi and Tranum-Jensen 1991).

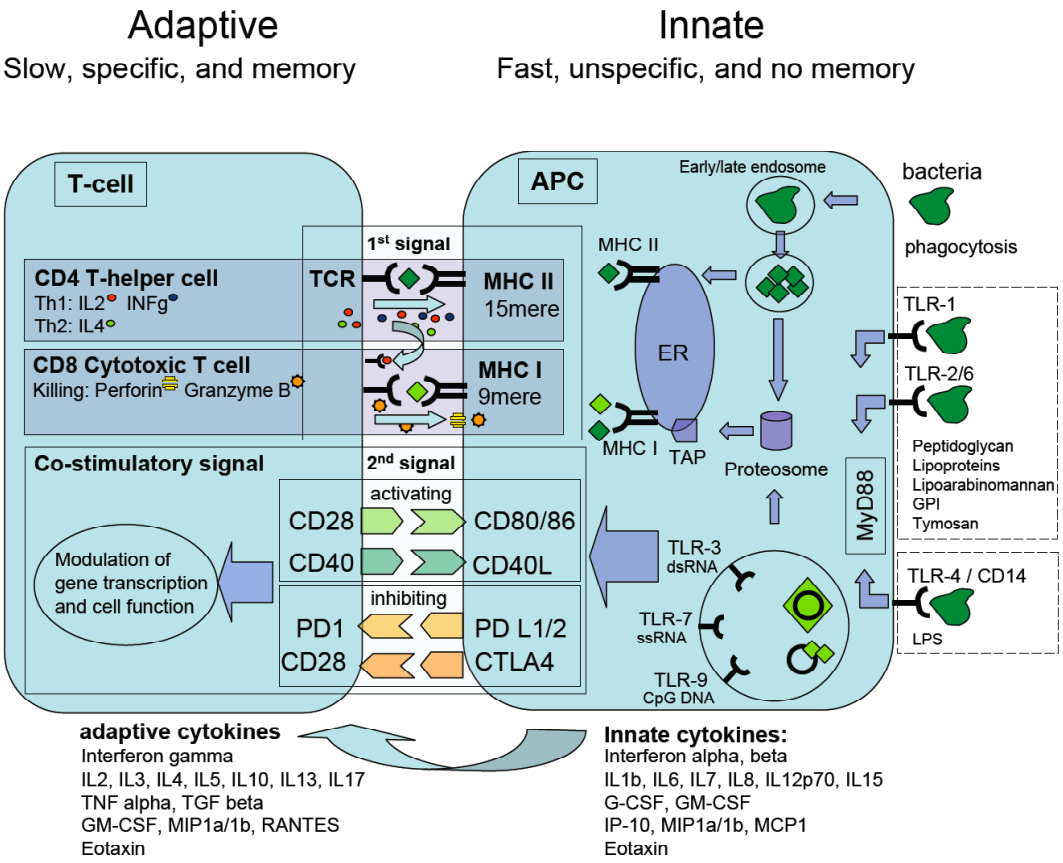


Figure 2. Overview of adaptive (T-cell) and innate (APC) interactions of the immune system. 1st signal: MHC/peptide presentation to T-cell receptor. 2nd signal: Co-stimulation. The central role of dendritic cells between innate and adaptive immune mechanisms is shown.

Another important factor is RNA interference (siRNA) expressed in almost every host cell. Cellular siRNA is already found in prokaryotic cells (Lu, Gan et al. 2008). Within the host cell, siRNA down-modulates foreign viral DNA or RNA via dimer formation (Gottwein and Cullen 2008).

1.1.2.2. Adaptive immune response

The hallmark of the adaptive immune system is its high specificity and its ability to build up an immune response (non-self) faster upon reencounter (immunological memory).

The adaptive immune response is built on cellular and humoral effectors.

Cellular adaptive immune response is engaged by T-cells and B-cells. Two main subset of T-cells exist: CD8+ cytotoxic and CD4+ helper T-cells.
Every host cell constantly presents intra-cellular 9mer peptides via major histocompatibility complex I (MHC I) to CD8+ T-cells. Professional APCs additionally present extra-cellular 15mer peptides via MHC II to CD4+ T-cells.

Peptide presentation is a highly complex process, and also very important for the understanding of epitope mapping in general, therefore the main features are summarized in the next paragraphs.

The main steps of peptide processing are (i) cleavage of the antigen, (ii) loading on the MHC molecule, and (iii) presentation and interaction with a corresponding T-cell receptor (see **Figure 3**).

In the cytosol, a large multi-catalytic protease complex, the proteasome, performs protein degradation of cytosolic proteins. The proteasome consists of 28 sub-unites, arranged in four stacked rings, each of seven subunits. Interferons induce parts of the proteasome (KNP2 and LMP7). An interferon-induced proteasome switches its specificity to an increased cleavage of polypeptides after hydrophobic residues, and reduced cleavage after acidic

residues (Goldberg, Cascio et al. 2002; Rock, York et al. 2002). This may lead to an epitope switch during infection and therefore has important consequences for diagnostic and therapeutical targeting.

For extracellular proteins, protein cleavage happens in the endosome, which becomes increasingly acidic. The endosomes contain cysteine proteases as cathepsins B, D, S, and L.

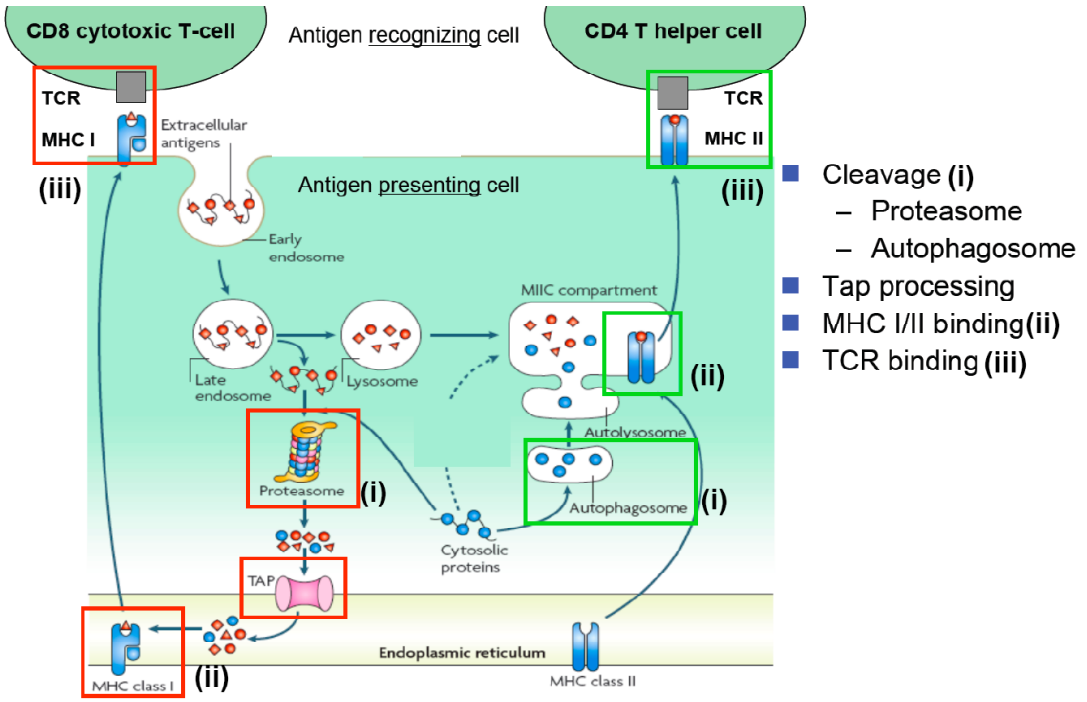


Figure 3. Function of the antigen presenting cells during peptide processing: (i) Protein cleavage, (ii) epitope loading on MHC and (iii) processing on the cell surface (adapted from Felix and Allen, 2007 Nature Review Immunology)

As the MHC molecule trans-locates during synthesis into the lumen of the endoplasmic reticulum (ER) and on the one side, and all proteins are produced and cleaved in cytosol on the other side, there is the requirement for transport of peptides from cytosol to the ER. This transport system is called *t*ransporters *a*ssociated with antigen *p*rocessing -1 and -2 (TAP-1 and -2). The TAP complex prefers peptides between 8 and 16 amino acids with hydrophobic or basic residues at the carboxy-terminus, and has a bias against

proline in the first three amino terminal residues (Uebel and Tampe 1999; Lankat-Buttgereit and Tampe 2002).

Binding of a peptide to MHC is an important step for the assembly of a stable MHC molecule. For MHC I, following steps through the cascade of peptides (calnexis, calreticulin, and tapsin), the MHC I complex finally binds to TAP with the cytosolic cleaved peptide. Only the fully folded trimmed MHC class I molecule and its bound 9mer peptide are then stable and leave the ER via Golgi to the cell surface.
For MHC II molecule, the peptide is also important for stabilisation. However, before binding a 15mer sequence, the MHC II needs to avoid unspecific binding within the ER. This is mediated by the MHC class II-associated invariant chain. The invariant chain lays within the MHC binding grooveand is then cleaved within the less acidic endosome to be replaced by a 15mer peptide fragment. Finally the MHC II-peptide complex is transported to the cell surface (Lagaudriere-Gesbert 2002; Williams, Peh et al. 2002).

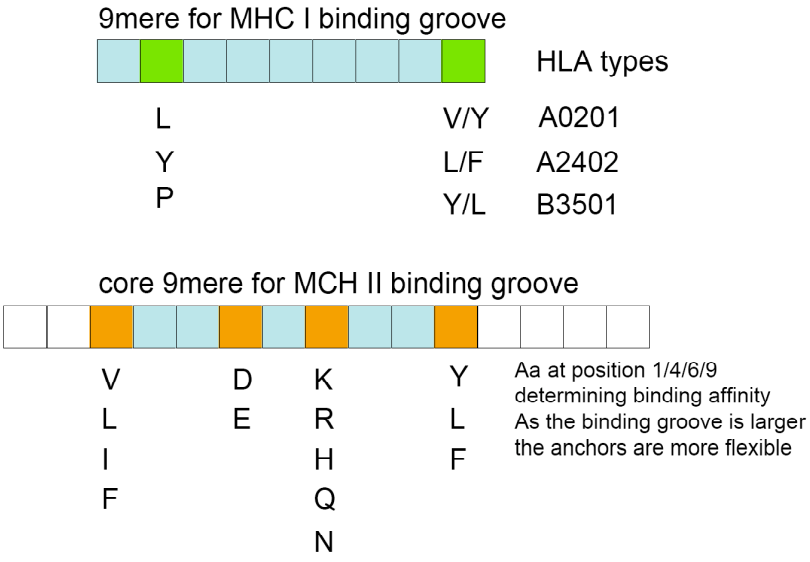


Figure 4. Peptide binding to MHC I (green), and MHC II (orange).

The binding of peptides to the MHC molecule is mediated through specific amino acid anchor residues. The amino acid side chain anchors the peptide by binding to pockets that lie in the peptide-binding groove. Polymorphisms in

the MHC molecule define the amino acid chain structure of the pockets and the pockets' binding specificities. Thus, these polymorphisms determine the particular HLA type and the consequent peptide to be presented (see **Figure 4**). For example HLA A0201 prefers a leucin and valine at the 2nd and 9th position of a 9mer epitope for binding into a MHC I molecule, whereas HLA B0702 prefers a prolin at the second position.

T-cells recognize MHC-bound peptides via their T-cell receptor (TCR) (Zinkernagel and Doherty 1974). The TCR is highly specific for only one determined self or non-self amino acid sequence.

Stimulated T-cells engage several functions in dependance of their subtype:
CD8+ T-cells may induce killing of APCs via Granzyme B and Perforin. CD4+ T-cells mainly produce supporting cytokines. Three subtypes of CD4+ T-helper cells exist: Th1-cells release mainly IL2, TNFα and INFγ, which support CD8+ T-cells; and Th2-cells release IL4, 5, and 13 leading to stimulation of B-cells, IgG switch to IgE and inhibition of macrophage activity. Newly discovered Th17-cells release IL17, although their role in infection control is yet controversial (Jin, Zhang et al. 2008).

The role of dedicated anti-viral T-cells in controlling virus replication is important and development of posttransplant virus disease was correlated to reduced amounts of virus-specific T-cells (Zinkernagel and Althage 1977; Sester, Gartner et al. 2005). The in vivo function of virus-specific T-cells and the protective effect normally is linked to in vitro induced cytokine production. Cytokines can be measured as a release with Elispot assay, or the intracellular accumulation with flow cytometry. Epitope-specific T-cell can be stained with tetramers (see **Figure 6**).
As the immune responses may lead to dangerous collateral damage to the host, it must be tightly controlled.

(i) In the thymus, T-cells with an auto-reactive or excessively strong binding TCR are positive selected by induction of apoptosis. Negative selection acts on T-cells with weakly binding TCR (Plamer 2003; Miosge and Zamyska 2007; Naeher, Daniels et al. 2007).

(ii) In the periphery the expanded pathogen-specific T-cell clone fraction needs to constrict and differentiate into a smaller set of memory T-cells after clearing the pathogen. (Lan, Mackay et al. 2007; Sakaguchi, Yamaguchi et al. 2007). The contraction phase is influenced by the eradication of the pathogen and therefore anergy to T-cells due to less stimulation, induction of apoptosis and external control through regulatory T-cells (T-regs). Similar to cytotoxic T-cells, T-regs also carry a pathogen-specific TCR, but produce down-modulating cytokines such as IL10 or TGFbeta.

Another important part of the adaptive immune response is the production of antibodies by B-cells. The membrane-bound immunoglobulin (B-cell receptor) may also be released into plasma or tissue. Depending on the maturation state of the B-cell, the location of the B-cell and cytokine profile present, IgA, IgD, IgE, IgG or IgM are produced. Mature B-cells release antibodies with a higher binding affinity to an epitope (affinity) (Davies and Chacko 1993).
Immunoglobulins may bind to viruses and inhibit infection of new host cells (neutralizing) or binding to bacteria and help during phagocytosis (opsonization). Although, not all antibodies produced are neutralizing.

1.1.3. Virus adaptation

Like pointed out before, the host's immune responses place viruses under pressure to adapt. In general terms, adaptation to a new biological environment is based on the accumulation of mutations over a specific time-span (Thommes and Hubscher 1990; Drake and Holland 1999; Domingo, Biebricher et al. 2001; Friedberg, Walker et al. 2006). During continuous infection, antiviral treatment and antiviral immune response, a heterogeneous pool of viral genomes (quasi species) evolves in the host. This enables selection for various elegant escape mechanisms (Smith and Inglis 1987; Lopez-Bueno, Mateu et al. 2003; Domingo 2006). This viral positive selection includes complement activation blockade, inhibition of MHC presentation, interference with ubiquitin-dependent proteolysis, interaction with apoptosis pathways, production of cytokines, chemokines and receptor homologues

(Ploegh 1998; Alcami 2003)
Escape mutations from neutralizing antibodies and from T-cell recognition can even be observed in vivo (Borrow, Lewicki et al. 1997; Ciurea, Klenerman et al. 2000).
Due to its random nature, most mutations lead to a severe loss of function, known as viral negative selection(Müller and Bonhoeffer 2003). This is reflected in the frequency of any particular mutant among all possible variants depending on its replication capacity (Domingo 2006). The reduced fitness of a mutated strain can revert to the original sequence, when the selective antibody or T-cell pressure is no longer present (Borrow, Lewicki et al. 1997). However, this possibility only occurs rarely in complex genomic rearrangements, in accordance with Muller's ratchet hypothesis (Chao 1990).

The rate of evolution can be indicated as nucleotide substitutes per year in an organism (Shankarappa 1999). A lower rate of evolution probably indicates a better adaptation to the host, and potentially is higher in latency-associated pathogens.

Over the course of evolution, latent viruses perfectly adapt to the response of the immune system and vice-versa. Nowadays, transplantation medicine challenges the equilibrium of host and virus by putting the graft as a new player into the game. The impact of immune suppression and the presentation of self and non-self peptides via allogenic MHC molecules shifts the established balance between virus and host.

1.2. Aspects of virus and host interaction in the transplant setting

The potent and long lasting immunosuppression in solid organ transplantation has led to a reduction of acute rejection episodes in recent years (Meier-Kriesche, Li et al. 2006). Most probably due to a reduction of virus-specific immune control, in transplant recipients a higher virus-associated morbidity and mortality has been observed (Fishman 2007). Registry data of paediatric kidney transplant patients showed an increase of infections from 20.4% to 30.8% from 1982 to 2002 (Dharnidharka, Stablein et al. 2004). Similarly, in

adults above 50 years of age, infection rates during the first year post-transplant increased from 48% to 69% (Dharnidharka, Caillard et al. 2006). Important players beside viral characteristics are the host and graft. All three form a complex interactive unit, influenced by external factors as trough levels of immunosuppression, antiviral treatment, or co-infections **(Figure 5).**

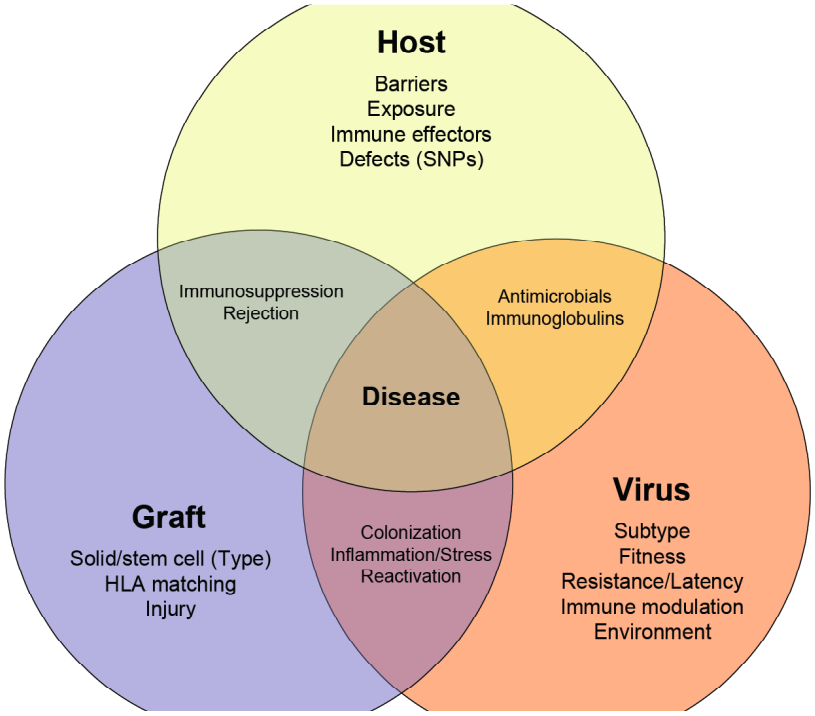


Figure 5. Host-Graft-Virus interaction. Yellow circle: Host factors; red circle: virus factors; blue circle: graft factors. Interacting fields are indicated with colour changes.

1.2.1. Risk profiling

Pre- and post-transplantation the donor, recipient and graft are screened for their respective contribution to infection risk depending on recipient and donor patient history, epidemiological situations of pathogens, patients age, type of transplantation, and signs and symptoms of deceased donors ((Fishman 2007), see **Table 1**). The risk profiles define post transplantation surveillance strategies such as anti-viral prophylaxis or preemptive strategies (Preiksaitis,

Brennan et al. 2005), and limitations or exclusion to transplantation programs (ID Community of Practice 2004).

Recipient	
	Patient history
	Vaccination
	Epidemiology
	Immune defects: humoral or cellular syndromes, splenectomy, thymectomy
	Diagnostics
	Chest x-ray
	Serological immune response (IgG and IgM)
	T. pallidum
	HIV
	Herpes viruses: CMV, EBV, HSV, VZV
	Hepatitis viruses: HBV, HCV
	(Polyomaviruses BK and JC)
	Strongyloides, Coccoides, Histoplasmosis
	Cellular immune response (INFγ positive T-cells)
	Viruses: CMV, EBV, BKV
	TBC (T-spot or Mantoux skin test)
Donor	
	Patient history
	Epidemiology
	Diagnostics
	Serological immune response (IgG and IgM)
	T. pallidum
	HIV
	Herpes viruses: CMV, EBV, HSV, VZV
	Hepatitis viruses: HBV, HCV
	(Polyomaviruses BK and JC)
	Toxoplasmosis
	West nile virus
	Microbiological testing of blood and urine

Table 1. Screening parameter for graft recipient and donors pre- and post transplant (adapted from Fishman, NEJM 2007)

The patient's risk assessment for infection is based on serology and genome detection of the pathogen in urine, plasma or biopsy material. However, this is often inaccurate and shows the following problems (Fishman 2007).

First, serological responses are three to four weeks delayed to acute infection, often are not directly correlated with protection from virus replication, may

cross-react to other similar viruses, and even may be false negative. Additionally, for newer pathogens standardized ELISA assays, clear definition of cut-offs, and baseline characteristics in healthy and immunosuppressed individuals are missing, and sero- or genotype specific gaps may be present. The influence of immunosuppression on serology data is not yet sufficiently examined.

Second, genome amplification methods, on the other hand, require sufficient isolation of DNA or RNA in a sample. A “latent” virus, which is not replicating can be difficult to detect. The present amount of viral loads does not allow to fortune future dynamics.

If an actively replicating virus is found, a graft usually disqualifies for transplantation. Unknown pathogens can show fatal outcomes as recently shown with LCMV infection (Allander, de Lamballerie et al. 2008). Without sequence information available, only high throughput techniques may be able to identify the unknown pathogen in reasonable time.

Due to these problems, new testing methods are needed to further assess the risk of posttransplant reactivation and progression to virus disease. Virus-specific cellular immune assays may answer several of the open questions, and in combination an accurate risk assessment may be performed (Ljungman 2006).

1.2.2. Virus-specific immune assays

In general, three immunological assay types can be used to quantify and characterize a virus-specific immune response after stimulation of peripheral blood mononuclear cells (PBMC) with antigens:

(i) Elispot assay to measure secretion of cytokines by detecting spot forming units (SFU) per million PBMC

(ii) Intracellular cytokine staining and flow cytometry to detect accumulated cytokines in T-cells with addition of surface phenotyping with CD4+/CD8+, memory or effector marker description

(iii) Tetramer assays to detect single virus-epitopes T-cells depending on the HLA context (also flow-cytometry based).

For Elispot and FACS assays usually lysates from infected cell cultures or overlapping peptide libraries are used to stimulate the virus-specific immune responses in vitro (**Figure 6**).

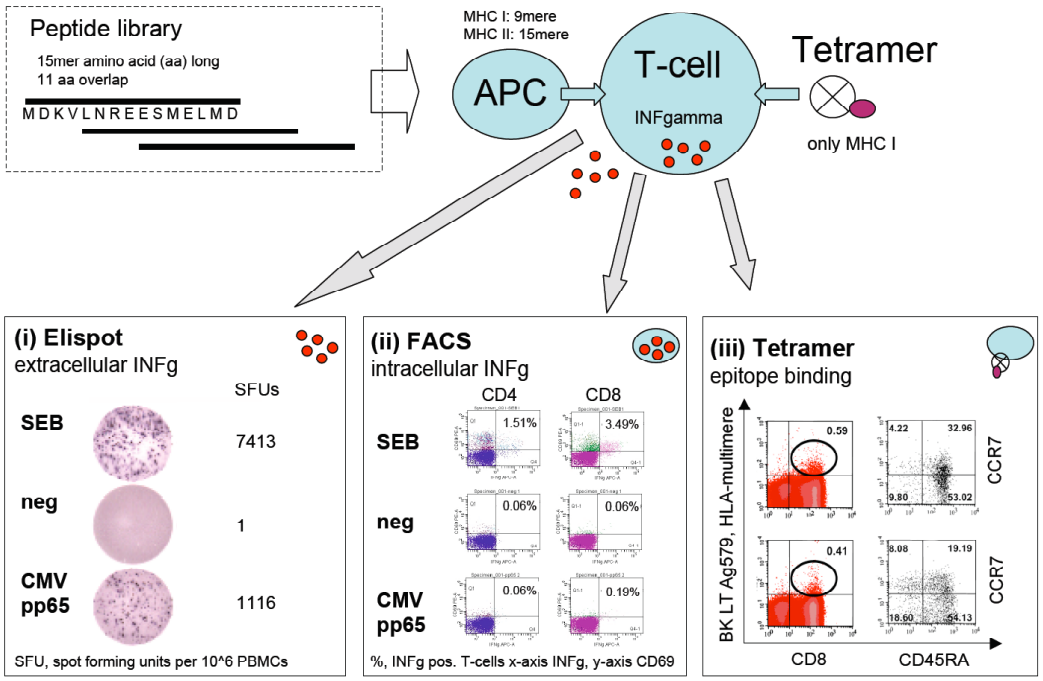


Figure 6. Overview of assays used to measure virus-specific T-cells. Upper part: Difference between natural processed epitopes via APCs and artificial tetramer loading. Lower part: Three read out systems to measure - (i) Elispot, (ii) FACS and (iii) Tetramer binding.

In Tetramer-assays streptavidin-fluorophore labeled MHC-I with a single 9mer bound can be used to stain T-cells harbouring the respective T-cell receptor. In this case, the precise epitope for one particular HLA-type must be known.

A literature research on established cellular immune assays in daily clinical routine to assess the risk of patients for virus replication and progression to disease was rather disappointing (PubMed search September 2008). Hardly any laboratory has defined, published and in routine used cut-off levels, above which patients were protected. Such a cut-off, similar to a viral titer, would be

indeed very useful for assessing patients risk. Exceptions are CMV and BKV-specific immune monitoring assays (see later in **chapter 1.3.1. and 1.3.2.**).

Designing or improving a virus-specific immune assay requires cautiousness at several points. Generally, for a stimulatory antigen-pool, not all viral proteins or potential epitopes can be used. Mainly for cost reasons, a peptide library usually shows a 4aa gap between peptides. Therefore some important HLA relevant epitopes may be missed (HLA gap). Even in commercial tests such as the Quantiferon^{TM} or T-spot^{TM} assay for CMV or tuberculosis, no manufacturer information is available on this topic (http://www.cellestis.com/, http://www.oxfordimmunotec.com/eu/). The identification of immuno-dominant peptides could further reduce the amount of peptides used (Sylwester, Mitchell et al. 2005). Similar to serological test, cellular immune assays may also show a cross-reactive potential (Hamilton, Gravell et al. 2000; Kreijtz, de Mutsert et al. 2008).

Effects of immunosuppression on the monitoring assay should also be considered. The trough level inhibits the allo-reactive and the virus-specific immune response. Immune responses should be set into the context with the specific immunosuppressive agent of the patient.
Drugs such as Calcineurin inhibitors (Tacrolimus and Cyclosporin A) have a direct effect on the cytokine production of T-cells. Anti-proliferation drugs (Mycophenolic acid, rapamycin or leflunomide) act on proliferation capacities after stimulation (Halloran 2004) (**Figure 7**).

Improvement of the current immune monitoring assays could be done, by comparison of healthy individuals to immunosuppressed patients, each with or without virus replication and progression to disease. This would allow identifying patients at risk and guide treatment. Further, immuno-dominant regions could be identified and baselines set for vaccine development.

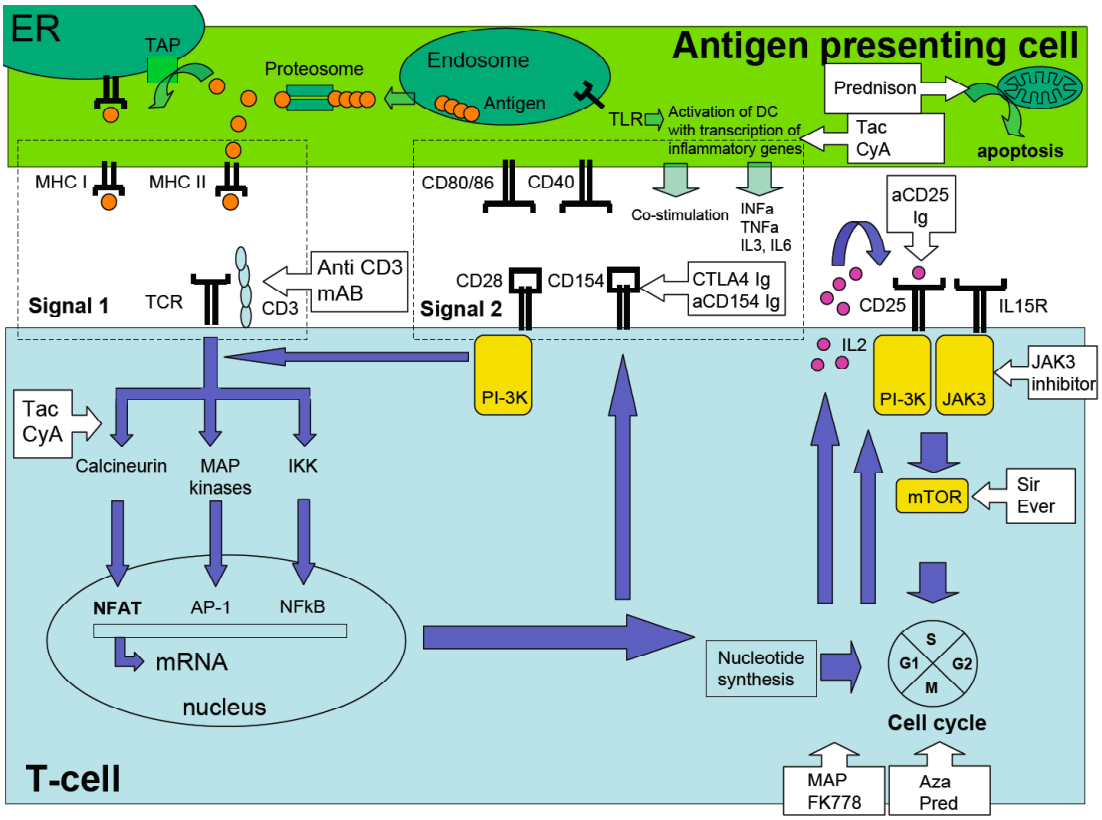


Figure 7. Immunosuppressive drugs and their function on antigen presenting cells and T-cells. Green: Antigen presenting cell. Blue: Responding T-cell. Intracellular signalling pathways are described and interactions with immunosuppressive drugs (white arrows) are shown.

1.3. Cytomegalovirus and Polyomavirus BK post-transplantation

1.3.1. Cytomegalovirus

Cytomegalovirus (CMV) belongs to the group of DNA viruses and the family of herpesviridae (Baltimore 1971). Beside CMV seven other human herpes viruses exist: Herpes simplex type 1 and 2, Epstein-Barr, Varicella zoster, Human Herpes virus 6, 7, and 8.

1.3.1.1. Virus genome and proteins

CMV has a linear double-stranded DNA genome of about 235'000 base pairs with more than 200 open reading frames, coding for at least 59 proteins. In

the nucleus a circular DNA matrix serves as transcription template (Dunn, Chou et al. 2003; Dolan, Cunningham et al. 2004).

CMV latency, reactivation and replication is a tightly regulated process with coordinated expression of immediate-early, early and late genes (see **Figure 1**). Immediate early proteins, such as IE-1 (pp72) or IE-2 are central regulators of viral gene expression. Early gene proteins such as UL97 phosphokinase and UL54 DNA polymerase facilitate viral genome replication. Late proteins such as the tegumentum associated pp65 and glycoprotein B (gB) include structural proteins found in the viral capsid, matrix and envelope.

CMV is transmitted via saliva, body fluids, cells, and tissues. The seroprevalence depends on socioeconomic status and lies between 30-70% in Western Europe and North America (Staras, Dollard et al. 2006). Following primary CMV replication in seronegative individuals, CMV establishes latency in CD34+ myeloid progenitor cells as a major site (Sinclair and Sissons 2006).

CMV reactivation may occur in “danger” situations to the host, due to common shared transcription factors of host cells and viruses. Reactivation of CMV as a bystander during sepsis is a classical example (Limaye, Kirby et al. 2008). Important CMV transcription factors are: NF1, CREB/ATF, NFκB p50, and AP1 (He and Weber 2004; Sinclair and Sissons 2006; Lee, Klase et al. 2007). In critical ill patients commonly immune functions are severely suppressed and a phase of uncontrolled CMV replication may be followed.

CMV has evolved various highly adapted immune evasive strategies against almost every key step in host immune responses (see **Table 2**).

Despite all these fascinating immune evasive strategies, in a healthy immunocompetent host reactivation normally does not occur. If the host is immunosuppressed, uncontrolled and progressed CMV replication may lead to various direct and indirect CMV effects and diseases (Egli, Binggeli et al. 2007).

Principle	CMV factors involved	Ref
Cytokine effects	IL-10 like cytokine	Spencer, 2002
MHC-I interference	• US2 and 11 re-translocate the MHC from ER to cytosol	Wal 2002
	• US3 binds MHCI and causes ER retention	Ahn 1996
	• US6 inhibits peptide transport and ATP hydrosis	Hewitt 2001
MHC-II interference	not known	
DNA	CMV DNA around histones mimics host DNA	Grassi 2003
Interference with co-stimulation	• UL144 mimics inhibitory co-signalling function of herpes simplex virus entry mediator	Poole 2006 Sester 2008
	• CMV infected cell may up-regulate PD1 ligand	
siRNA	• miRNA of UL112 down modulates MIC-B, a NK cell Ligand	Grey, 2007 Reeves, 2007
	• β2.7 binds to mitochondrial enzyme complex 1	Dölken, 2007
NK cell escape	• UL18 decoy MHC-I like molecules	Beck, 1988
	• UL40 activates the inhibitory CD94/NKG2A receptor	Tomasec, 2000
	• UL141 retains CD155, a Ligand for the activating NK receptors DNAM-1 and TACTILE	Tomasex, 2005 Kubin, 2001
	• UL16 binds ULBP1 and 2 which are NKG2D ligands	Wills, 2005
	• UL142 down-modulates MIC-A	
INF reduction	• UL83 (pp65) deletion induces higher INFβ and ISGs	Browne, 2003
ISG interference	• IE2 interferences with NFkB	Abate, 2004
	• HCMV pTRS1 and PIRS1 block PAS mediated eIF2a phosphorylation and reduce RNA degradation by RNase L	Tylor, 2006
JAK/Stat signal-transduction	IE 72kDA and Stat2 association prevents ISGF3 DNA binding	Paulus, 2006
Site of replication	almost every cells may replicate CMV	

Table 2. Viral escape strategies of Cytomegalovirus

1.3.1.2. Risk assessment and immunological monitoring

Management procedures for CMV are well defined (Preiksaitis, Brennan et al. 2005). CMV seronegative recipients (R-) of a CMV seropositive donor graft (D+) and R(+) patients with anti-rejection or T-cell depleting induction

treatment are at high risk for CMV primary infection and uncontrolled replication of reactivated CMV under immune suppression (Hodson, Jones et al. 2005; Kalil, Levitsky et al. 2005; Strippoli, Hodson et al. 2006).
This is in line with the importance of CMV-specific T-cells to effectively control CMV replication and inhibit progression to disease (Sester, Sester et al. 2001; Lacey, Gallez-Hawkins et al. 2002; Bunde, Kirchner et al. 2005; Lacey, La Rosa et al. 2006; La Rosa, Limaye et al. 2007; Egli, Binet et al. 2008). The state of CMV-specific immune controls together with local microenvironment determines how CMV causes organ-invasive disease in intestines (40%), liver (20%), lungs (10%), kidneys (5%), eyes (1%) and the central nervous system (1%).
Therefore, high-risk patients profit from an intense CMV surveillance with prophylactic or preemptive treatment strategy. Both strategies have shown to significantly reduce CMV replication and progression to disease (Humar, Mazzulli et al. 2005). Interestingly, 25% to 50% of CMV D+R- do not seroconvert within one year (Lowance 1999; Humar, Mazzulli et al. 2005; Khoury 2006). Low-level CMV replication with insufficient treatment may be an important problem, leading to a poor long-term graft outcome because of chronic inflammation within the graft (Pérez-Sola, Castón et al. 2008). Still, duration and dosage of prophylaxis and preemptive treatment is still an open question.

The risk of developing drug resistance is another important aspect of high-risk patients with low CMV-specific immune control. Most cases of GCV-resistance development are described for the D+R- high-risk group (Limaye, Corey et al. 2000; Boivin, Goyette et al. 2005). Most mutations (>90%) accumulate in the phosphotransferase UL97. The remaining 10% occur within the CMV DNA polymerase UL54 (Gilbert, Bestman-Smith et al. 2002; Gilbert and Boivin 2005). To avoid resistance, adequate antiviral dosing is critical (Boivin, Goyette et al. 2004). The second group of high-risk patients, R+ with T-cell depleting treatment, additionally supports the role for sufficient CMV-specific T-cell control. These patients are often not well enough monitored, due to their R+ and thereby wrongly supposed protective state. However,

these patients show as well a considerable risk for GCV resistance development (see results part (Egli, Binet et al. 2008)).

Important to highlight is the unknown time-span and dosage of prophylactic treatment (3 or 6 months), microbiological surveillance after CMV replication, and treatment with Ganciclovir (GCV) and ValGCV after eradication of active replication. This is where CMV-specific immune response could help to identify the risk for future CMV replication, GCV-resistance development and progression to disease.

1.3.2. Polyomavirus BK

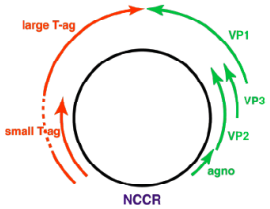
With the introduction of high dosage immunosuppression regimens in transplantation medicine and the onset of AIDS pandemic, polyomaviruses BK and JC were no longer under immune pressure. BKV and JCV started replicating at higher levels and used there chance to make a “new” pathogen (Hirsch 2005).

1.3.2.1. Virus genome and proteins

The genome of all human polyomaviruses is about 5.3kb in size and builds a circular double-stranded DNA encoding early and late proteins (Yang and Wu 1979; Frisque, Bream et al. 1984; Cubitt 2006; Allander, Andreasson et al. 2007; Gaynor, Nissen et al. 2007) (**Figure 8**). Newly discovered human polyomavirus WU, KI and Mantel cell carcinoma polyomavirus (MPyV) will not be discussed, due to their unclear role in immuno-suppressed hosts (Allander, Andreasson et al. 2007; Gaynor, Nissen et al. 2007; Feng, Shuda et al. 2008). Early gene proteins are: the regulatory large tumor antigen (LT-ag) and the small T antigen. Late gene proteins are: the capsid VP-1, -2 and -3 and the agno-protein.

BKV agno protein is degraded by phosphokinase C (PKC), followed by higher LT expression with higher viral loads in cell culture (Johannessen, Myhre et al. 2008).

The transcription of early and late viral genes is driven by the non-coding control region (NCCR), which contains also the origin of DNA replication and several transcription factor binding sites (Moens, Johansen et al. 1995; Gosert, Rinaldo et al. 2008). The replication cycle of BKV is shown in detail in **Figure 1**.



		Number of bp		Number of Aa		Homology	
		BKV	JCV	BKV	JCV	Gene	Aa
Genome		5133	5130			74%	
Early coding region	**LTag**	2088	2067	695	688	78%	83%
	sTag	519	519	172	172	78%	78%
Late coding region	**VP-1**	1089	1065	362	354	75%	78%
	VP-2	1056	1035	351	344	81%	79%
	VP-3	699	678	232	225	80%	75%
	agno	201	216	66	71	72%	59%

Figure 8. BKV and JCV genome organisation of early and late genes, encoded proteins and homology.

Within the NCCR, the main harboured transcription factor binding sites are: SP1, GRE, GM-CSF, NF1, p53, and CMV-IE1 (Moens, Johansen et al. 1995). In clinical studies, grafts from deceased donors with longer phases of ischemia showed persistent Polyomavirus BK replication and poorer graft outcomes compared to living donor kidneys (Wadei, Rule et al. 2006). This may be due to reactivation based on up-regulation of transcription factors during ischemia.

A complex genome adaptation to a new host situation is the development of rearrangements in the NCCR (rr-NCCR, deletions or insertions) post-transplantation. rr-NCCR BKV shows higher transcription of early genes followed by increased late genes transcription. Thereby production of viral particles (high viral fitness) is increased with faster development of cytopathic

effects in cell culture (Gosert, Rinaldo et al. 2008). Interestingly, in immunocompetent hosts, rr-NCCR BKV strains can be found only exceptionally in cases of primary infection or pregnancy (Flaegstad, Sundsfjord et al. 1991; Markowitz, Eaton et al. 1991; Egli, Infanti et al. 2008). This implicates a disadvantage within a competent immune system. Probably, due to more interference with the host immune system, those viruses are cleared faster, not causing harm (**Figure 9**).

BKV immune evasive strategies might be mediated through siRNA down-modulation of host immune factors, but also own BK LT early gene transcripts for further silencing. Gosert et al. have described a down-regulating effect of co-transfected agno DNA on LT expression (Gosert, Rinaldo et al. 2008). Grinde et al. analyzed mRNA expression profiles during BKV infection and found a down modulation of NFkB, IRF3, ATF2 in HUV-EC cells (Grinde, Gayorfar et al. 2007). siRNA activity was described for Polyomavirus SV40 (Sullivan, Grundhoff et al. 2005; Gottwein and Cullen 2007; Sullivan 2008). Another interesting factor might be the up-regulation of co-stimulatory factors in infected cells. Starke et al. have described a up-regulation of PD1 ligand in BKV-infected RPTECs (Starke, Lindenmeyer et al. 2008). PD1 ligand has a inhibitory function on T-cells and thereby probably virus-infected cells are protected from killing (for more information on PD1 see results and discussion part).

1.3.2.2. Virus transmission and latency

The transmission strategies of polyomaviruses BK and JC are highly efficient. Up to 80% of the world population is infected with BKV and 70% with JCV (Knowles, Pipkin et al. 2003; Stolt, Sasnauskas et al. 2003; Lundstig and Dillner 2006).

Transmission of BKV and JCV happen at different time-points. BKV is usually transmitted during childhood within a relative short time period. JCV transmission happens until the age of 40 (Knowles, Pipkin et al. 2003; Stolt, Sasnauskas et al. 2003). Different routes are suspected: oral and respiratory

(Sundsfjord, Spein et al. 1994), vertical (Shah, Daniel et al. 1980; Stolt, Kjellin et al. 2005), as well as via cells and tissues, especially in transplantation (Sundsfjord, Spein et al. 1994; Dolei, Pietropaolo et al. 2000; Dorries, Sbiera et al. 2003).

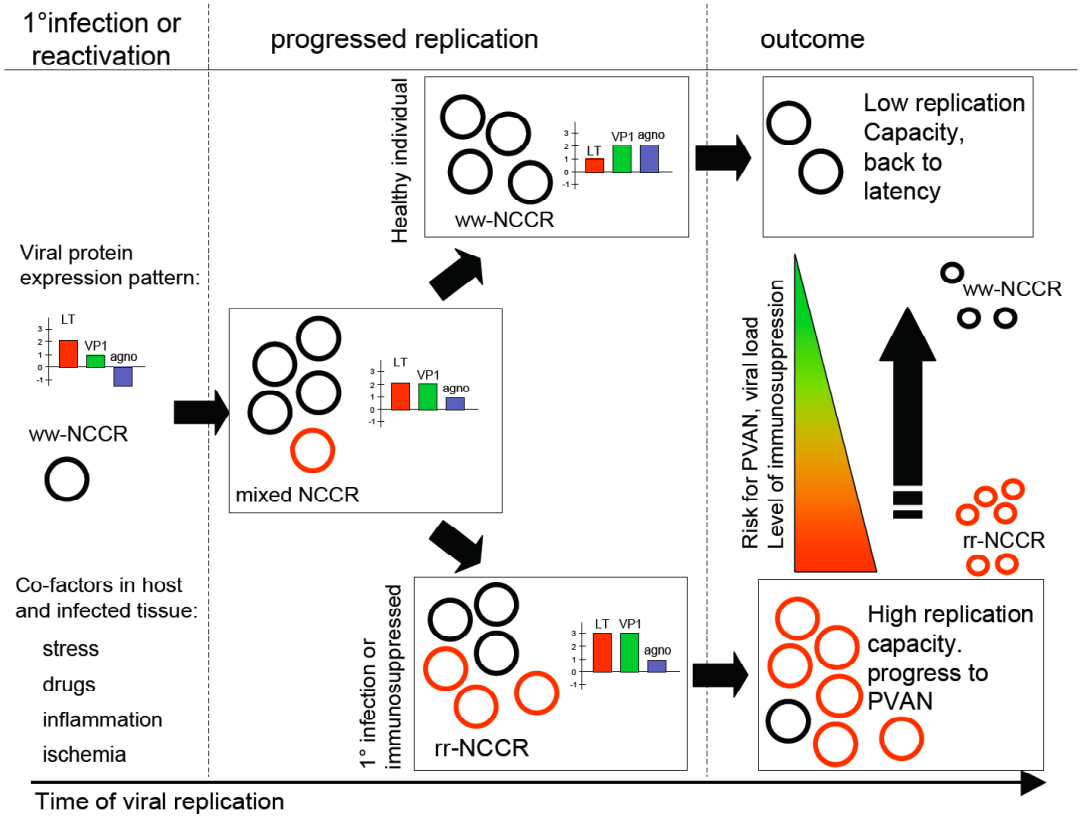


Figure 9. BKV replication in healthy and immunosuppressed hosts. Left part: situation of primary infection or reactivation. Right part: Depending on competence of the immune system, different virus replication patterns can be observed (adapted from Gosert et al. JEM 2008). Red, green and blue bars hypothesis expression profiles of BKV proteins.

Most probably BKV and JCV entry happens through inoculation of a sufficient dosage of infectious particles into the gastrointestinal tract. BKV most probably uses Gangliosides GD1b and GT1b as receptor for host cell entry (Low, Magnuson et al. 2006). JCV requires alpha2-6 linked sialic acid (Komagome, Sawa et al. 2002) or serotonergic 5HT2AR receptor (Elphick, Querbes et al. 2004). Probably JCV enters into circulation via the tonsils. The alpha2-6 linked sialic acid has been found in oligodendrocytes, astrocytes, as

well as B- and T-cells in spleen and tonsils (Eash, Tavares et al. 2004). In about 40% of tonsils samples from children and adults JCV DNA was found (Monaco, Jensen et al. 1998; Kato, Kitamura et al. 2004). Most probably during primary infection BKV is shed in stool. 38.3% of stool samples from children were tested positive for BKV, whereas JCV has not been found (Vanchiere, Nicome et al. 2005).

During primary infection a first phase of BKV and JCV replication leads to the establishment of a latent non-replicative infection in the reno-urinary tract (Chesters, Heritage et al. 1983; Dorries and ter Meulen 1983). In healthy individuals BKV and JCV are intermittently reactivated. In urine BKV can be found in 0-31.5% and JCV in 20-57.4% of samples (Kitamura, Aso et al. 1990; Ling, Lednicky et al. 2003; Rodrigues, Pinto et al. 2007; Rossi, Delbue et al. 2007; Zhong, Zheng et al. 2007; Egli, Infanti et al. 2008).

JCV can be detected especially in older age-groups (Kitamura, Aso et al. 1990; Zhong, Zheng et al. 2007). In peripheral blood mononuclear cells (PBMC) the results are contradictory. Ling et al. did not find virus in PBMC (Ling, Lednicky et al. 2003). Dolei et al. found 21.6% of samples positive for BKV and 0.9% for JCV using an ultra sensitive nested PCR (Dolei, Pietropaolo et al. 2000). Dorries et al. showed that granulocytes may serve as the predominant reservoir harbouring JCV DNA in blood cells (Dorries, Sbiera et al. 2003). There is a need to further define the baseline characteristics in healthy individuals.

BKV or JCV disease in healthy individuals is a rare event. During BKV primary infection, single cases of hemorrhagic cystitis and nephrotic syndrome occurred (Hashida, Gaffney et al. 1976; Nagao, Iijima et al. 1982; Saitoh, Sugae et al. 1993). In a study of 131 children with suspected meningitis or encephalitis 3.8% of CSF samples were positive for BKV, 1.5% for JCV. Additionally 20 healthy control patients examined for lower back pain were negative (Behzad-Behbahani, Klapper et al. 2003).

1.3.2.3. Polyomavirus associated nephropathy

With introduction of high dose and prolonged immunosuppression polyomaviruses BK and JC came into our focus by causing kidney graft malfunction and loss (Shah, Daniel et al. 1974; Jung, Krech et al. 1975; Gardner, MacKenzie et al. 1984; de Silva, Bale et al. 1995; Purighalla, Shapiro et al. 1995; Bachman 1999; Nickeleit, Hirsch et al. 1999; Randhawa and Demetris 2000; Hirsch, Knowles et al. 2002). Polyomavirus associated nephropathy (PVAN) is the major complication linked to high-level BKV replication affecting up to 10% of kidney transplant recipients with graft loss in up to 50% (Hirsch 2005) **(Figure 10)**.

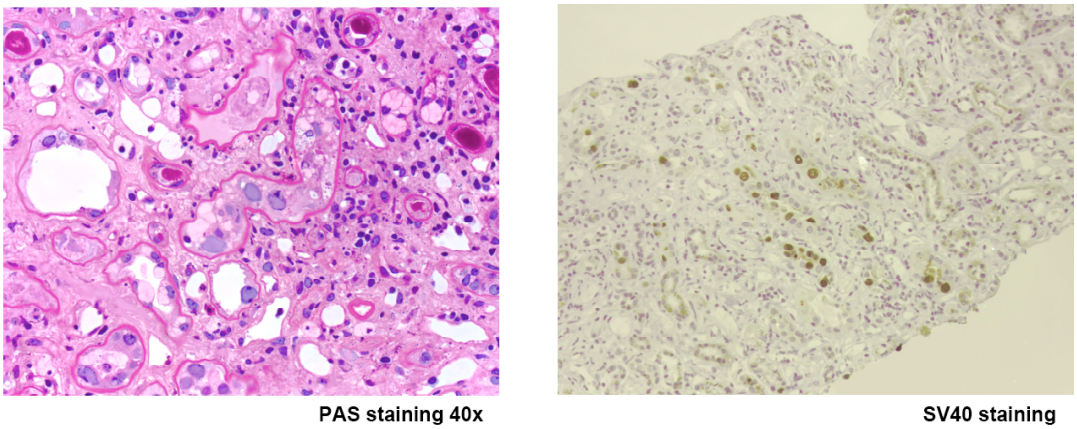


Figure 10. Histology pictures of PVAN in an autologous kidney after lung transplantation. Left picture: PVAN stage B with inflammation, degenerated tubuli and cytopathic effects of enlarged cells due to BKV replication. Right picture: Immune SV40 LT staining with cross-reactive antibody (brown). (Egli, Hirsch and Johnson, unpublished case report)

Virus detection in plasma serves as a surrogate marker for polyomavirus associated nephropathy in transplant recipients (Hirsch and Steiger 2003).

BKV shedding can be found in about 30% of kidney transplant recipients with about 1000fold higher viral loads compared to healthy individuals (Hirsch, Knowles et al. 2002; Drachenberg, Hirsch et al. 2007; Funk, Gosert et al. 2007). About 1/3 of patients with BKV positive urine sample also show BKV

positive plasma samples. BKV in plasma may increase to several logs and serves as an important surrogate marker to assess the risk of PVAN **(Figure 11)**. The negative predictive value for PVAN of BKV in plasma is almost 100%, the positive predictive value is about 50% (Hirsch and Steiger 2003).
Not only the presence of BKV is important but also the viral load. Patients with PVAN have higher viral loads than patients without PVAN (28000c/mL vs. 2000c/mL) (Hirsch, Knowles et al. 2002). If BKV in plasma exceeds 4logs c/mL, PVAN should be considered (high sensitivity and specificity >95%) (Drachenberg, Hirsch et al. 2005). JCV is rarely detected in plasma (14.2%, mean 2000 c/mL) (Drachenberg, Hirsch et al. 2007).
Dynamic examination after surgical removal of PVAN-containing allografts showed a rapid drop of plasma BKV loads, suggesting that the majority of BKV is derived from replication within the graft. Calculated plasma viral half-life of 1-2h implies viral turnover of more than 99% per day and a tubular epithelial cell loss of about 10e6 cells per day (Funk, Steiger et al. 2006; Funk 2007).
Long lasting replication of BKV in an immuno-suppressed host leads to accumulation of NCCR rearrangements (R^2=0.64, p<0.001). First, rearrangements appeared in urine, followed by plasma. In comparison to non-rearranged transplanted patients, those patients show 20fold higher viral loads (medium 20'000 c/mL versus medium 440'000 c/mL), and probably a faster progression to PVAN (Gosert, Rinaldo et al. 2008).

After transplantation Polyomaviruses BK uses its chance of reduced BKV-specific immune control to replicated uncontrolled, and to accumulate mutations, thereby gain higher viral fitness with a even increasing replication state.

1.3.2.4. Risk assessment and immune monitoring of BKV proteins

Knowledge on the immunological differences of BKV proteins will allow improving our current immunological monitoring assays, help to identify

patients at risk for prolonged BKV replication an progression to Polyomavirus associated nephropathy, and to focus on vaccine target regions.

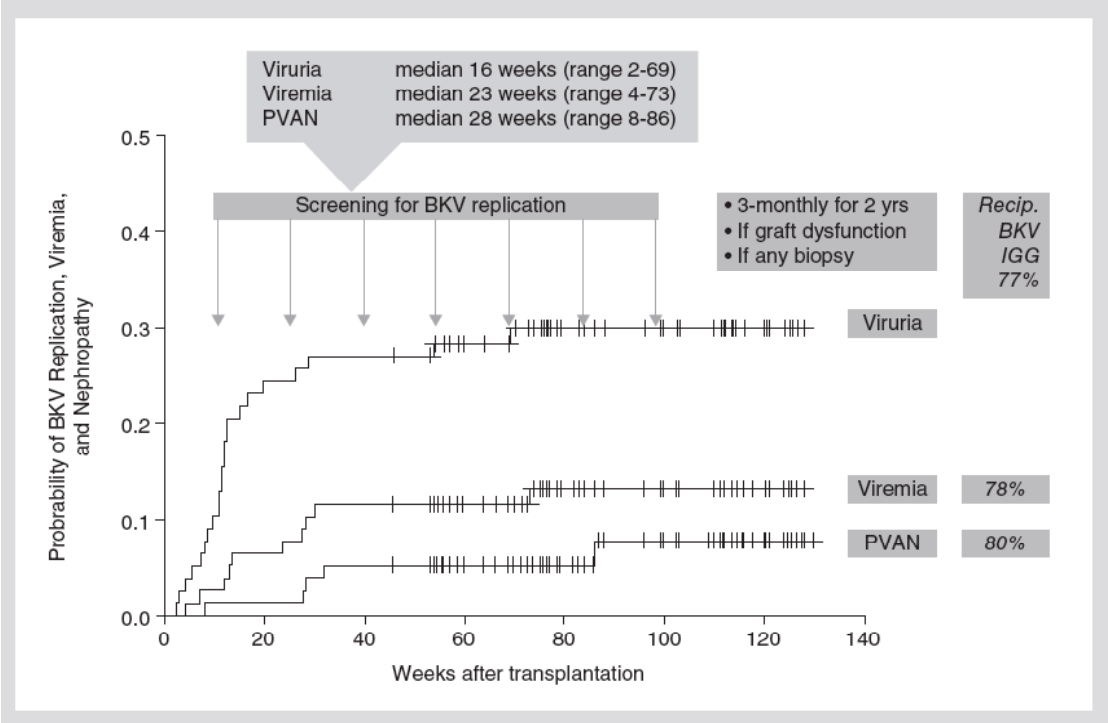


Figure 11. Virus replication after kidney transplantation. Different probabilities for virus replication in urine and plasma and development of PVAN is shown. (adapted from Hirsch et al. NEJM 2002)

1.3.2.4.1. BKV Large T protein

The LT antigen is relative conserved. Probably due to highly important functional sites it does not show large sequence differences between virus strains (Hatwell and Sharp 2000).

The homology between BKV-LT and JCV-LT is the highest for all polyomavirus proteins. This homology also has an impact on cellular immune assays (see results (Binggeli, Egli et al. 2007)). Four BKV-LT epitopes have been identified with cross-reaction to JCV-LT (see **Table 3**).

BKV large T-antigen				
Position	Sequence	HLA type	Comment	Reference
25	GNLPLMRKAYLRKCK	B0708		Li 2006
57	TLYKKMEQDVKVAHQ	DRB1 0301		Li 2006
157	TLTable 4ACFAVYT	A0201		Provenzano 2006
362	MLTERFNHIL	A0201		Randhawa 2006
406	VIFDFLHCI	A0201		Provenzano 2006, Randhawa 2006
410	FLHCIVFNV	A0201		Provenzano 2006, Randhawa 2006
553	IYLRKSLQNSEFLLE	B08	JCV cross	Li 2006
557	KSLQNSEFLLEKRIL	B08	JCV cross	Li 2006
579	LLLIQFRPV	A0201	JCV cross	Provenzano 2006, Randhawa 2006
613	TFSRMKYNICMGKCI	DRB1 0901	JCV cross	Li 2006
BKV VP1 epitopes				
Position	Sequence	HLA type	Comment	Reference
p44	AITEVECFL	A0201	JCV cross	Sharma 2006, Koralnik 2002, Li 2006, Chen 2006
p108	LLMWEAVTL	A0201	JCV cross	Sharma 2006, Koralnik 2002

Table 3. Confirmed BKV LT and VP1 epitopes

In kidney transplant recipients, the decrease of BKV loads in plasma was associated to a higher extent with BKV-specific cellular immune response against BKV LT peptide pools compared to VP1 (Binggeli, Egli et al. 2007; Ginevri, Azzi et al. 2007).

1.3.2.4.2 BKV capsid proteins

The role of neutralizing antibodies to BKV capsid proteins is controversial.
In healthy individuals, increasing BKV and JCV urine loads, and in kidney transplant recipients also plasma loads, are associated with an increasing BKV and JCV VLP IgG responses. This indicates that plasma IgG levels do not correlate with a direct protective effect, but a recent exposure to virus

antigens (Hariharan, Cohen et al. 2005; Randhawa, Gupta et al. 2006; Ginevri, Azzi et al. 2007; Egli, Infanti et al. 2008).
The theoretically higher immunological pressure on the surface antigens, VP1 to 3, should be refleted in higher tendency to adapt and mutate. In JCV, different genes were compared according to variances between strains. Indeed, the VP1 gene showed the highest inter-strain variance (Hatwell and Sharp 2000). Therefore, a VP1 based immune assay or vaccine should cover a larger spectrum of epitopes within one serotype and also contain epitopes from other strains to cover circulating viruses and escape mutants.

BKV VP1-specific T-cells have been used to monitor immune response. However, several differences compared to LT-specific immune response have been observed (see results). Two HLA A0201 VP1 epitopes (AITEVECFL and LLMWEAVTL) have been described (Koralnik, Du Pasquier et al. 2002; Sharma, Zhou et al. 2006; Chen, Trofe et al. 2008). Both epitopes show a cross-stimulatory effect on JCV (**Table 3**).

1.3.2.4.3. BKV agno protein

Although, BKV agno protein is abundantly expressed in the host cell during latency, the agno-specific immune response is weak. Hardly an antibody or cellular response can be detected (Leuenberger, Andresen et al. 2007). During latency this may be due to binding to the nuclear membrane protein HP1a, which is described for JCV agno (Okada, Endo et al. 2001; Safak, Barrucco et al. 2001) or rapid degradation through PKC-associated phosphorylation (Johannessen, Myhre et al. 2008). Thereby, probably agno-epitopes are not bound to MHC molecules and presented on the cell surface to T-cells (**Figure 12**).

2. Aims and Hypothesis

The main aim was to characterize in vivo and in vitro immune responses against Cytomegalovirus and Polyomavirus BK in healthy individuals and kidney transplant recipients.

The three main hypotheses were:

(i) Healthy blood donors show a high seroprevalence and competent virus-directed immune control with no plasma replication.

(ii) Kidney transplant patients with recent, but cleared reactivation show higher humoral and cellular immune response. During virus replication, the amount of virus-specific T-cells negatively correlates with the amount of virus detected in plasma.

(iii) Identification of immunodominant regions with virus proteins will allow improving current immunoassays.

For CMV, we aimed (i) to compare antigen-specific T-cells in healthy and immunosuppressed patients with or without concurrent and/or future CMV replication and development to GCV-resistant CMV-replication, (ii) to identify a threshold level for protection using CMV-specific T-cells as risk marker. We examined (iii) the potential of a single pp65-epitope (RQY) to stimulate immune response in kidney transplant recipients. Finally, (iv) cytokine profiling was used to further characterize CMV-specific immune response and the impact of the co-stimulatory PD1-molecule.

For BKV, we aimed to (i) define the baseline characteristic of virus replication and humoral immune response in healthy blood donors, (ii) to compare BKV-specific immune responses (LT and VP1) in kidney transplant recipients with increasing or decreasing plasma BKV loads. A threshold of protection was defined. Further, CD4 and CD8 BKV-specific immune responses were examined. (iv) The impact of immunosuppressive drugs in CMV and BKV-specific T-cells was examined. (v) Finally HLA A- and B-associated epitopes were identified and corresponding immune response was characterized.

3. Patients, Material and Methods

3.1. Patients and healthy blood donors

All healthy blood donors and transplant recipients gave informed consent on the study protocols. The ethical committee of Basel approved all protocols used.

Healthy blood donors (HD) were recruited from voluntary laboratory personal or from blood donation centre in Basel (SRK, swiss red cross). Serology was determined and reserve aliquots were stored. HLA type determination was done by inclusion to the stem cell donor database in Basel and is based on serological and DNA HLA typing (Prof. Dr. Jean-Marie Tiercy, CHUV, Lausanne).
Transplant recipients were recruited at several time points post transplant mainly form the nephrology department at the university of Basel (Prof. Dr. J. Steiger) and Kantonsspital St. Gallen (Dr. I. Binet).
Data on HLA type and mismatches, virus serology, past viral replication, concurrent trough levels of immunosuppressive drugs, lymphocyte counts, and anti-viral treatment was registered in a database for further analysis.

3.2. Material

3.2.1. Chemicals

6 well cell culture plate	Cellstar (Ref: 657160)
24 well cell culture plate	Cellstar (Ref: 662160)
aHLA-ABC	DakoCyto (Ref: M0736) anti human cloneW6/32
aHLA-DP/DQ/DR	DakoCyto (Ref: M0775) anti human clone CR3/43

Antibodies, all from Becton Dickinson (Allschwil, Switzerland), except otherwise marked

a7-AAD	PerCP (Ref: 559925)
aCCR7	PE (Ref: 552176) CD197

aCD1a	APC (Ref: 559775)
aCD3	PerCP (Ref: 345766), PE (Ref: 345765), FITC (Ref: 345763), APC (Ref: 555335)
aCD4	APC-Cy7 (Ref: 341115), APC (Ref: 555349), PerCP (Ref: 345770)
aCD8	PE-Cy7 (Ref: 335822), APC (Ref: 345775), PE (Ref: 555367)
aCD14	APC (Ref: 555399), APC-Cy7 (Ref: 333951), FITC (Ref: 555397)
aCD16	FITC (Ref: 335035), PE (Ref: 332779)
aCD19	APC (Ref: 345791)
aCD25	PE-Cy7 (Ref: 557741) Clone M-A251
aCD27	FITC (Ref: 340424)
aCD28	Pure (Ref: 348040). PE (Ref: 348047)
aCD45	PerCP (Ref: 345809)
aCD45RA	FITC (Ref: 555488), PE (Ref: 555489)
aCD45RO	APC (Ref: 340438), FITC (Ref: 555492)
aCD49d	pure (340976)
aCD56	FITC (REF: 345811), PE (Ref:345812)
aCD69	PE (Ref: 341652)
aCD80	
aCD83	FITC (Ref: 556910), APC (Ref: 551073)
aCD86	PE (Ref: 555665)
aCD94	PE (Ref: 555889)
aCD107a	FITC (Ref: 555800)
aCD107b	FITC (Ref: 555804)
aCD279	PE (Ref: 557946), PD-1
aFoxP3	Biolegend PE (Ref:320108), FITC (Ref: 320106) Clone 206D
aGranzyme B	PE (Ref: 558132)
aIL2	FITC (Ref: 340448)
aIL10	PE (Ref: 559330)
aINFγ	APC (Ref: 341117), FITC (Ref: 340449), PE (Ref: 340452)

aNKB1 PE (Ref: 555967)

Antigens

All *peptides* are from Eurogentec (Köln, Germany). A library of single peptides (15 amino acid length with 11aa overlap) was ordered and pooled. Pool and Sub-pools (with 10 peptides) were formed and used for epitope mapping.

All *virus lysates* and cell culture control lysates from Virion (Rüschlikon, Switzerland), except homemade BKV Lysate (lysate of vero-cells with freezing and thawning and ultra-sonification).

BK LT pool 50ug/mL (dissolved in 85% DMSO)
BK VP1 pool 50ug/mL (dissolved in PBS)
BK agno pool 200ug/mL (dissolved in PBS)
BK LT cluster 25mer (see appendix)
BK LT cluster 15mer (see appendix)
BK LT cluster 9mer (see appendix)
BKV Lysate, wwNCCR, unknown conc. (dissolved in PBS)
BKV Lysate, rNCCR, insertion, unknown conc. (dissolved in PBS)
BLV Lysate, rNCCR, deletion, unknown conc. (dissolved in PBS)

CMV Lysate 1-3.5% 1mg/mL stock, (dissolved in PBS, EC 0.1mg/mL)
CMV Ctrl Lysate stock
CMV pp65 pool 50ug/mL (dissolved in 85% DMSO)
CMV pp72 pool 50ugmL (dissolved in PBS)

EBV Lysate 1-5%
EBV Ctrl Lysate
HSV1/2 Lysate 1-3%
HSV1/2 Ctrl Lysate

JC LT pool 50ug/mL (dissolved in PBS)
JC VP1 pool 50ug/mL (dissolved in PBS)
JC agno pool 200ug/mL (dissolved in PBS)

RSV Lysate 1-2%
RSV Ctrl Lysate

SEB 100ug/mL dissolved in PBS (Ref: S4881-5MG), Sigma Aldrich

VZV Lysate 2-5%
VZV Ctrl Lysate

BFA	(Ref: B7651-25MG), Sigma Aldrich (Buchs, Switzerland)
BSA	(Ref: A9647-500G), Sigma Aldrich
Cryo tubes	CryoS tubes 2mL, Greiner Bio-one (Frickenhausen, Germany)
CPT tubes	Citrate Plasma (Ref: 362782), Becton Dickinson
Cytokines	
IL2	Peprotech (Ref: 200-02-10)
IL4	Peprotech (Ref: 200-04-10)
GM-CSF	Peprotech (Ref: 300-3-10)
DMSO	Fluka, >99.5%, (Ref: 41644)
EDTA	(Ref: E-7889), Sigma Aldrich
Elispot plates	Multiscreen HTS MSIPN4WSO, Millipore (Molsheim, Germany)
Elispot coating	
human INFγ	mAB 1.D1K (Ref: 3420-3-5000), Mabtech
human Granzyme B	mAB GB10(Ref: 3845-3-1000)
human TNFα	mAB TNFα-I
human IL2	mAB IL2-I (Ref: 3440-3-1000)
human Perforin	mAB Pf80/164 (Ref: 3465-3-1000)
Elispot catching	
human INFγ-biotin	mAB 7-B6-1-biotin (Ref: 3420-6-1000)
human INFγ-ALP	mAB 7-B6-1-ALP
human Granzyme B-biotin	mAB GBII-biotin (Ref: 3485-6-250)

human TNFα-biotin	mAB TNFα-II
human Il2-biotin	mAB IL2-II-biotin (Ref: 3440-6-1000)
human Perforin-biotin	mAB Pf344-biotin (Ref: 3465-6-250)
Elispot development	
Streptavidin-ALP	(Ref: 3300-10)
Streptavidin-HRP	(Ref:3310-9)
Elispot staining	
NBT	(Ref: 484235-250), VWR
BCIP	(Ref: 203788-100), VWR
NBT/BCIP mixture	
TMB	Substrate (Ref: 3651-2), Mabtech
Ethanol	>99.8% (Ref: 02860) 2.5l, Fluka, Sigma Aldrich
FACS tubes (blear)	5mL Polypropylene round bottom (Ref: 352063), Becton Dickinson (Allschwil, Switzerland)
FACS tubes (clear)	5mL Polystyrene round bottom (Ref: 352058), Becton Dickinson
Falcon tubes 15ml	(Ref: 1888271), Becton Dickinson
Falcon tubes 50ml	(Ref: 227261), Becton Dickinson
FACS Flow	wash buffer for FACS machine, (Ref: 342003), Becton Dickinson
FCS	Biochrom AG, 100% (Ref: S0115)
Filters	
mini for Elispot dilution	Spartan 13 0.2 RC, (Ref: 10463040) Whatman (Dassel, Germany)
large for cell medium	Nalgene 500mL 0.4uM, (Rochester, USA)
Falcon	50mL, Millipore Steriflip (Ref: SCGP00525)
FoxP3 Perm Buffer	(Ref: 421403), Biolegend
Human sera	(Ref: H45200-100), Sigma Aldrich
Immunosuppressive drugs	
Tacrolimus	Alexis (Lausen, Switzerland)
Cyclosporin A	Alexis (Lausen, Switzerland)

Leflunomide	A77 1726 Lef, 25mg, (Ref: 430-096-M025) Alexis (Lausen, Switzerland)
Rapamycin	Alexis (Lausen, Switzerland)
KCl	Sigma Aldrich
KH_2PO_4	Sigma Aldrich
L-Glutamine	(Ref: G7513-100), Sigma Aldrich

MACS, magnetic separation micro beads

CD4	human (Ref: 120-000-440)
CD8	human (Ref: 120-000-244)
CD14	human (Ref: 120-000-305)
NaCl	Sigma Aldrich
Na_2HPO_4	Sigma Aldrich
Penicillin Streptavidin	(Ref: P0781-100), 100mL, Sigma Aldrich

Peptides (virus and other antigens)

see antigens

RPMI-1640 cell medium	(Ref: B8758), Sigma Aldrich
Saponin	(Ref: S7900-100G), Sigma
Tryptan-blue 0.5%	
Tween 20	(Ref: 93773), Fluka, Sigma Aldrich

3.2.2. Solutions

Autologous cell media, store at 4° for 1 months
25ml plasma from donor (heat inactivated, centrifuged 1650g 20', EC 5%)
500mL RPMI
filtered with Millipore, 50mL, 0.45uM

BFA, stored at -20°
1mL 70% Ethanol
1mg BFA (EC 1mg/mL)

BSA 20% stock solution, store at -20°C
200mL PBS
40g BSA (EC 20%)

DC expansion medium, stored at 4° for max 1 month
500mL RPMI
25mL human sera (EC: 5%)
5mL L-Glutamine (EC: 1%)
5mL Penicillin Streptavidin (EC: 1%)
125uL GMCSF (200ug/mL, EC: 50ng/mL)
100uL IL4 (1mg/mL EC 0.2ug/mL or 1000U/mL)

EDTA 500mM stock solution
100mL PBS
114.62g EDTA (EC: 500mM)

Elispot antibody dissolving solution, stored at 4°
995mL PBS
5mL FCS (EC: 0.5%)

Elispot developing solution, for immediate use
11mL TRIS
100uL NBT
200uL BCIP

Elispot wash for Elisa washer, store at 4°
1000mL PBS
0.5mL Tween 20 (EC: 0.05%)

FACS Co-stimulation solution, stored at 4°C
200uL aCD28 (1.0mg/mL, EC 100ug/mL)
400uL aCD4+9d (0.5mg/mL, EC 100ug/mL)
1400uL PBS

FACS wash, store at 4°

950mL PBS

50mL FCS (EC: 5%)

5g BSA (EC: 0.5%)

filtered with Millipore, 50mL, 0.45uM

Freezing media, store at 4°

900ml FCS

100ml DMSO (EC: 10%)

MACS solution, store at 4°

485mL PBS

12.5mL 20% BSA (EC 0.5%)

2mL 500mM EDTA (EC 2mM)

Permeabilizing solution, for immediate use

99mL FACS buffer

1mL Saponin 10% (EC: 0.1%)

Phosphate buffered saline (PBS 10x stock, pH7.2), stored at RT

80g NaCl

2g KCl

11.5g Na_2HPO_4 (or 14.41g $Na_2HPO_4x2H_20$)

2g KH_2PO_4

R5 medium, stored at 4° for max 1 months

465mL RPMI

25mL human sera (EC: 5%)

5mL Penicillin Streptavidin (EC: 1%)

5mL L-Glutamin (EC: 1%)

filtered with Millipore, 50mL, 0.45uM

R10 medium, stored at 4° for max 1months
440mL RPMI
50mL FCS (EC: 10%)
5mL Penicillin Streptavidin (EC: 1%)
5mL L-Glutamin (EC: 1%)
filtered with Millipore, 50mL, 0.45uM

Saponin 10%, store at 4° for max 1 week
10mL PBS
1g Saponin

TRIS, store at room temperature
121,1g Tris
800mL MilliQ
add HCl to pH (70mL for 7,4; 60mL for 7.6; 42mL for 8.0)

3.2.3. Machines

Centrifuges:

Eppendorf	5810R, large
Eppendorf	5415, small
Elisa washer	Labsystem well-wash, Ascent, Bioconcept, (Allschwill, Switzerland)
Flow cytometer	Canto I, Becton Dickinson, (Allschwil, Switzerland)
	Calibur, Becton Dickinson, (Allschwil, Switzerland)
Elispot reader	CTL Ltd. (USA)
Luminex	Bioplex 200, Biorad (Reinach, Switzerland)

3.2.4. Software

Adobe Photoshop CS, Version 8.0
Adobe Illustrator CD, Version 11.0
Ch3D Viewer, Version 4.1.

EndNote, Version 9.0
Excel for Mac, Version 11.5.2.
FACS Diva Software, Version 4.0
Flow Jo, Version 3.4.
Mac OS, Version 10.3.9.
Powerpoint for Mac, Version 11.5.1.
Prism, Version 5.0a
SPSS, Version 13.0
Swiss PdB Viewer, Version 3.9.b1
Word for Mac, Version 11.5.0

3.2.5. Websites

http://www.ncbi.nlm.nih.gov/sites/entrez?otool=unibaslib
http://www.ncbi.nlm.nih.gov/gv/mhc/main.cgi?cmd=init
http://www.immuneepitope.org/home.do
http://www.syfpeithi.de/

3.2.6. Biological specimen

3.2.6.1. Urine and Plasma

Native untreated urine was used for PCR detection. Aliquots of 4mL were stored at 4° for max two days or frozen at -20°, if batch-analysis was performed later.

EDTA- or Citrate-Plasma was used for PCR detection and serology assays. EDTA-plasma was obtained from whole blood sample by centrifugation (10' at 3000g). Citrate-Plasma was obtained from CPT Vacutainer^{TM} tubes according to the manufacturers protocol. Plasma was stored at 4° or frozen at -20°, if batch-analysis was performed later.

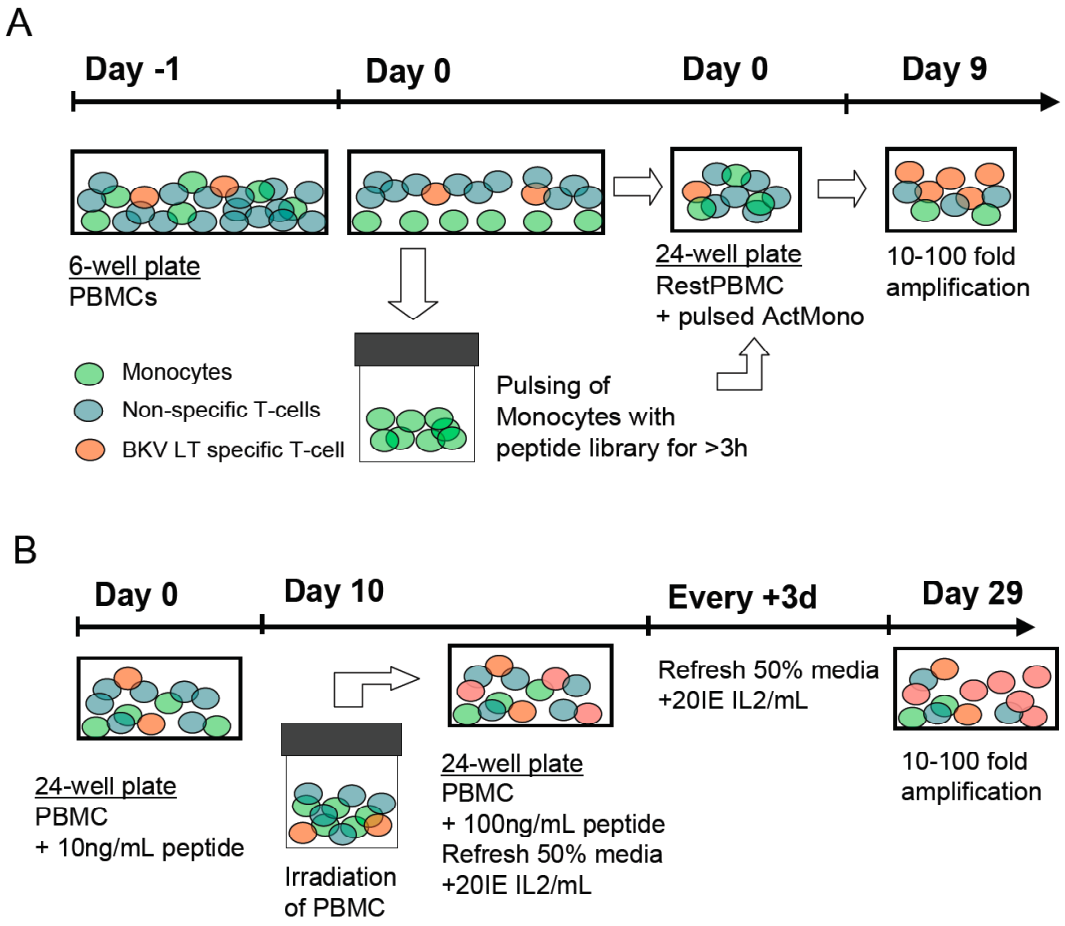
3.2.6.2. Peripheral blood mononuclear cells (PMBC)

Peripheral blood mononuclear cells (PBMC) were used for detection of virus-specific T-cells. PBMCs could be either directly isolated using the CPT-Vacutainer system or from EDTA whole blood with a ficoll gradient purification (detailed protocol see appendix). After counting PBMCs (Neubauer 0.1mm) were directly used or frozen at -80C° in freezing media. Within one week the cells were transferred to liquid nitrogen.

3.3. Methods

3.3.1. T-cell expansion cultures

For a detailed comparison of the different expansion methods evaluated please see appendix or **Figure 12**.



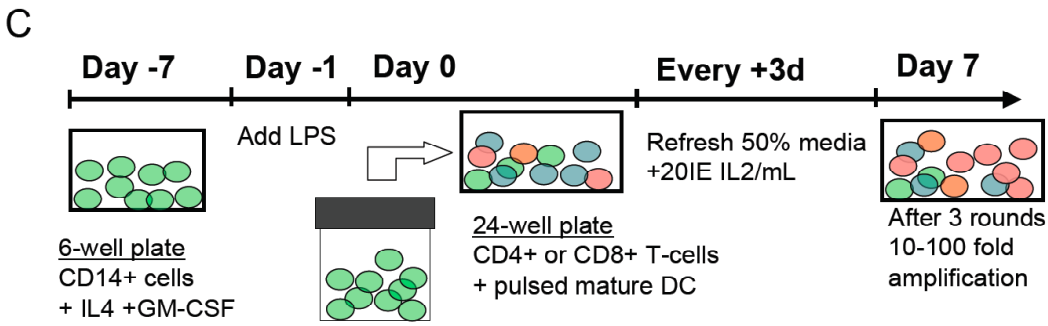


Figure 12. Overview of expansion protocols. ActMono 9days (A), GMP approved expansion 29days (B) and separate expansion 21days (C)

3.3.1.1. ActMono 9 days expansion protocol

The concept of this method is to separately pulse activated monocytes (ActMono) with virus antigen preparation and add to PBMCs (restPBMC) in 1:10 ratio for 9day. Virus-specific T-cells will amplify in a short expansion culture.

At day -1: Three mL of the donor PBMC cell suspension (2 Mio/mL) were put into a 6 well plate. Activated monocytes adhered overnight to the well bottom.

The following day: RestPBMCs were floating on top and removed. ActMono were detached by strong pipetting. Both populations were counted and concentrated to 4Mio per mL. ActMonos were pulsed for >3-4h with an antigen solution at a final concentration of 5ug/mL and washed afterwards. 0.5mL of restPBMCs (4Mio/mL) were transferred into a 24well plate. After pulsing, ActMonos were added in a concentration of 1:10 in 500uL per well. RestPBMCs and ActMonos were incubated for 9days. After 7days medium starts turning yellow, indicating cell division.

Figure 13 illustrates the cell subsets of adherent ActMonos and restPBMCs. Additionally the increasing amount of virus-specific T-cells is shown after 9days of expansion. This expansion protocol was mainly used for Epitope mapping.

3.3.1.2. GMP-approved 28 days expansion protocol

The concept of this method is to co-cultivate antigen and PBMCs during the expansion. With two stimulation phases and increasing antigen concentrations, a more balanced CD4+ and CD8+ T-cell expansion should be obtained. This protocol is based on GMP standards without antibiotics and only autologous cell medium.

Two Mio PBMCs/well/mL are put into a 24 well plate. RPMI with autologous plasma served as expansion medium. The antigen is added at a final concentration of 10ng/mL.

After 10 days of expansion the cells are re-pulsed with irradiated PBMCs and 100ng peptide/mL. Every third day, half of the old medium is replaced with fresh medium containing 20IE IL2 per mL. After 28days expanded cells can be used.

3.3.1.3. Dendritic cell based 21 days expansion protocol

The concept of this method is to separately expand CD4+ and CD8+ T-cell cultures and pulse them with mature dendritic cells (DC). T-cell cultures are stimulated and re-stimulated 3 times in total.

At day-7: CD14 positive cells are isolated with magnetic beads. CD14 negative fraction is frozen. CD14 positive fraction is put into culture with GM-CSF and IL4 for one week.

At day -1: LPS is added to finally mature DCs.

At day 0: CD14- fraction is thawn and CD4+ and CD8+ T-cell fractions are magnetically separated. Mature DCs are checked in FACS (see **Figure 14**) and added at a ratio of 1:10 to the T-cells.

At weekly intervals new matured DCs are used to re-stimulate the expanding T-cells. In a 3 and 4 day interval half of the medium is changed and fresh medium with 100IE IL2/mL added.

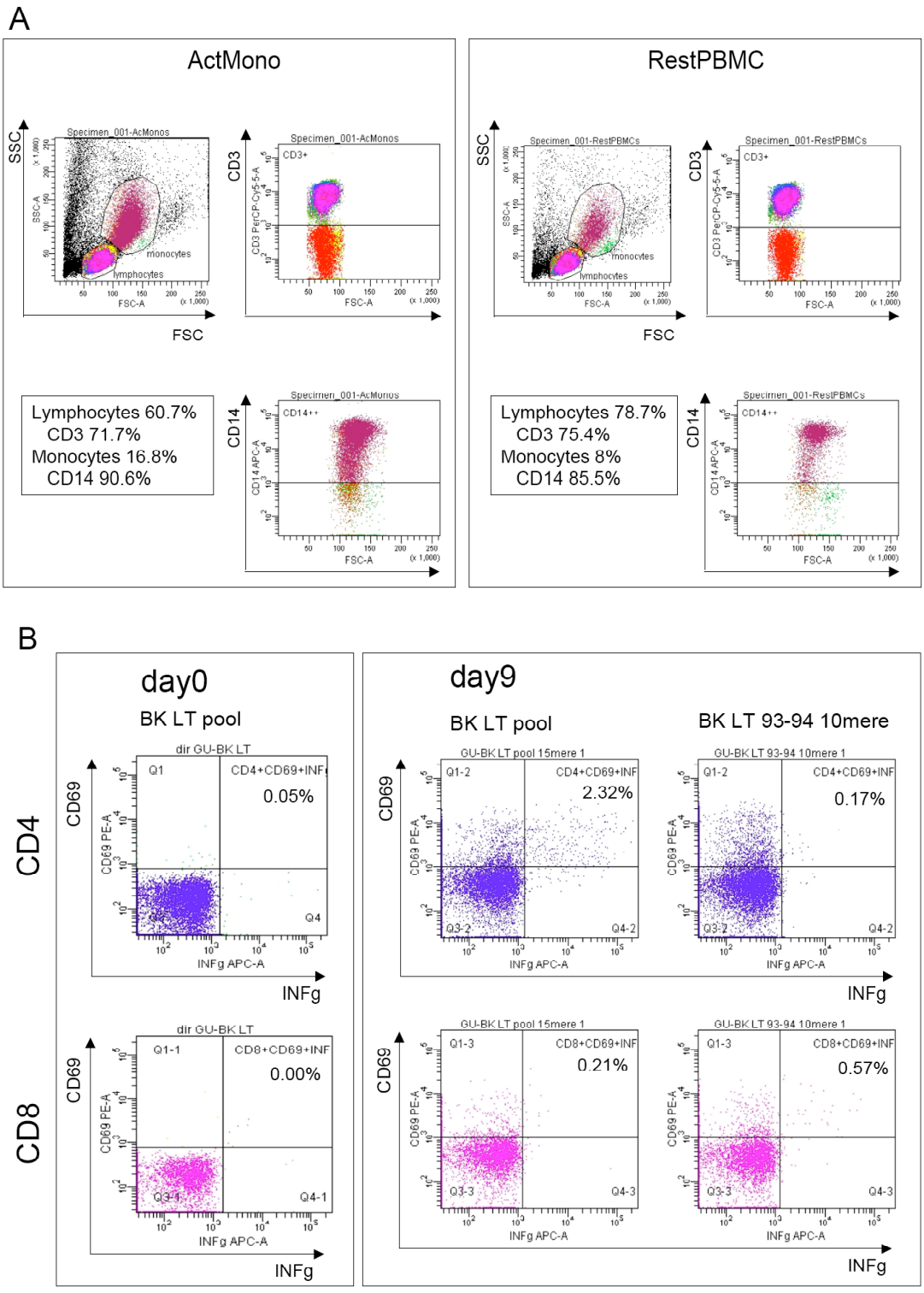


Figure 13. (A) CD3 and CD14 subsets after overnight adherence. (B) BK LT expansion over 9 days with re-challenging.

3.3.2. Detection of virus-specific T-cells

3.3.2.1. FACS

For virus-specific stimulation with FACS analysis 0.25 to 1x10e6 PBMCs were stimulated with positive controls (SEB or PHA), negative controls (medium, or cell lysate), virus peptides (BKV LT and VP1 pool, JCV LT and VP1 pool, and CMV pp65 and pp72 pool) or virus cell culture lysate for 6 hours or overnight in presence of Brefeldin A and of a co-stimulatory cocktail (aCD28 and aCD49).

The stimulation was stopped with adding EDTA, followed by two washing steps. Cell were fixed and permeabilized (see protocol in appendix).

As surface markers anti-CD3, anti-CD4, anti-CD8, anti-CD16/CD56, anti-CD45RA, anti-CD45RO, anti-CCR7, and anti-PD1 for cell sub-typing was used. The activation state was determined with anti-CD69, anti-CD25, anti-IL10, anti-IL2, and anti-INFγ.

The measurement was done with a flow cytometer (For a detailed protocol see appendix).

General concentration and time kinetic experiments are shown in **Figure 15.**

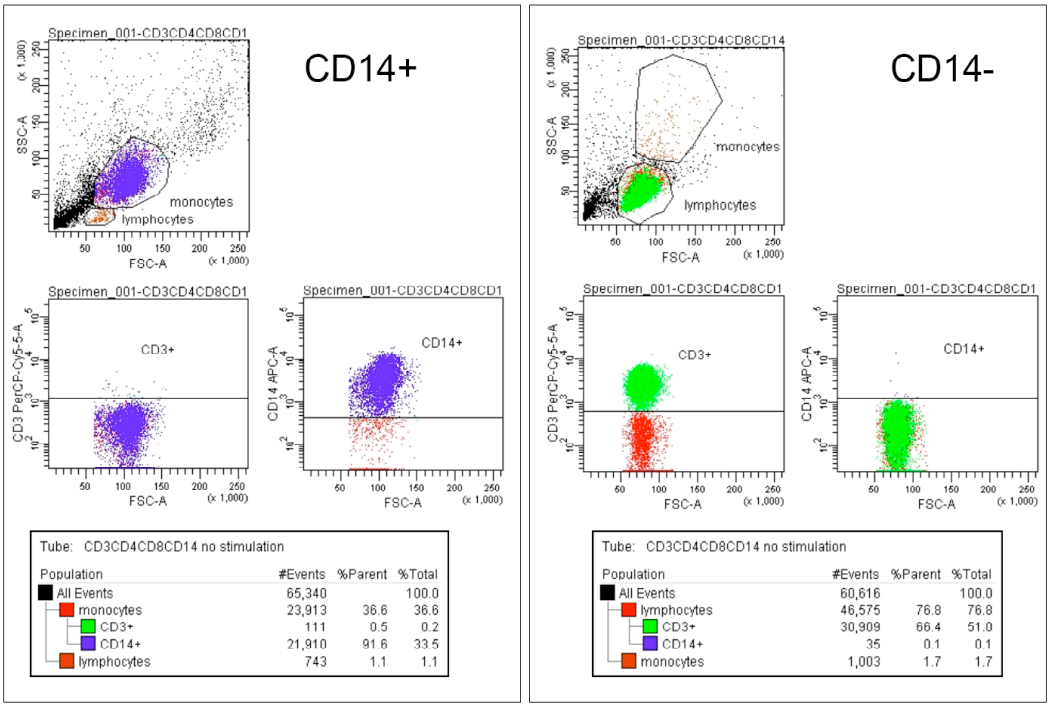


Figure 14. CD14+ and CD14- subsets after MACS separation

In general 30'000-50'000 events (CD3 positive cells) were acquired and further analysed in a gating hierarchy (see **Figure 16**). Percent of INFγ or IL2 and CD69 positive cells of all CD4+ helper or CD8+ cytotoxic T-cells is indicated. The mean fluorescence intensity indicates the amount antibody or cytokine per cell. Further details on the protocol can be found in the appendix.

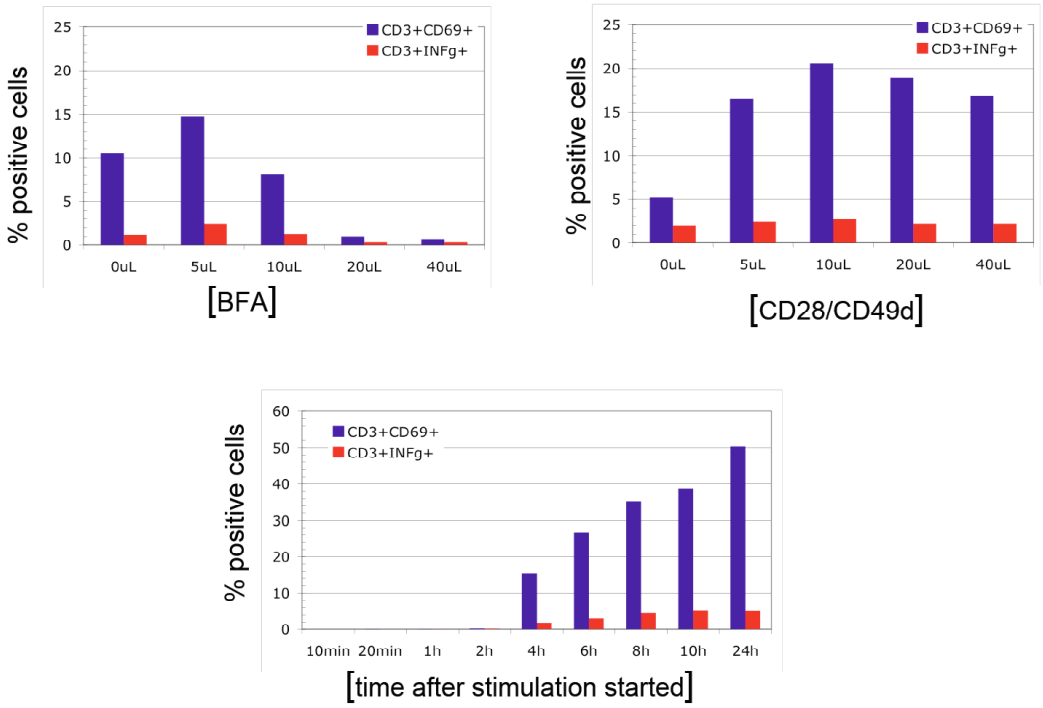


Figure 15. All experiments were performed with SEB on various healthy blood donors. Top left: BFA dilution (1mg/mL, stock) with different end-concentrations into 500uL media and effect on INFγ production after 6h is shown; Top right: CD28/CD49d dilution (1mg/mL, stock) and co-stimulating effects on T-cells after 6h is shown; Bottom: and time kinetics of INFγ and CD69 expression.

3.3.2.2. Elispot

Microtiter-plates were coated with anti-INFγ, anti-IL2, or anti TNFα for detection of the respective cytokine production of stimulated PBMCs. 2.5x10^5 PBMCs and 1-2ug/mL peptide are added for 16-20h to each well of the microtiter-plate. Unspecific antibody bindings are washed away and a biotinylated secondary antibody was added for 2 hours. Afterwards the plate is washed again and an antibody with Streptavidin bound alkaline-phosphatase is added for 1 hour and a chromogenic substrate (NBT/BCIP) induces a coloured spot corresponding to one cytokine producing T-cell. The plates were read in an Elispot reader (For a detailed protocol see appendix). The SFUs are normalized to 1Mio PBMCs per well for comparison. **Figure 17** explains the method.

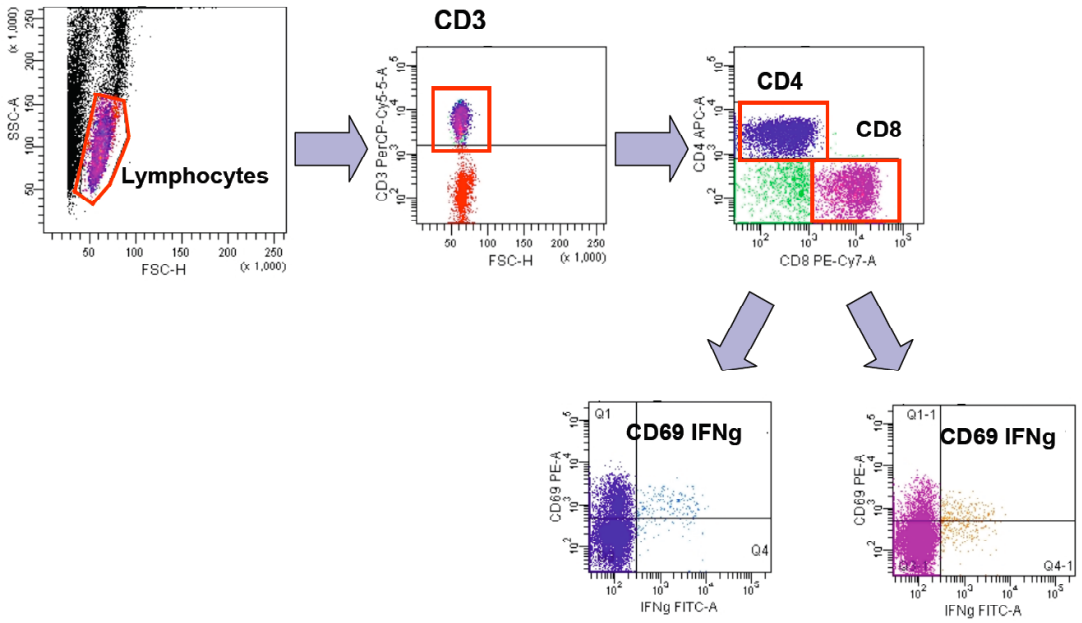


Figure 16. Gating strategy for virus-specific T-cells.

3.3.2.3. Cell separation: CD4+ - CD8+ - CD14

For specific expansion of CD4+/CD8+ T-cells or separation of T-cell subsets in leukemic patients magnetic separation of T-cells was used.

An antibody against a phenotypic marker tagged with a magnetic bead is used to directly separate the corresponding T-cell subset (positive selection).

Briefly, 10 Mio PBMCs are re-suspended in 1mL MACS buffer. 100ul of the magnetic beads are added, followed by incubation on ice for 20 minutes. The

non-marked cells are separated and the marked cells remain in a magnetic column. Finally, the attached marked cells of interest were eluted out with removing the magnetic field. Cell purity reached up to 99% (see **Figure 14**).

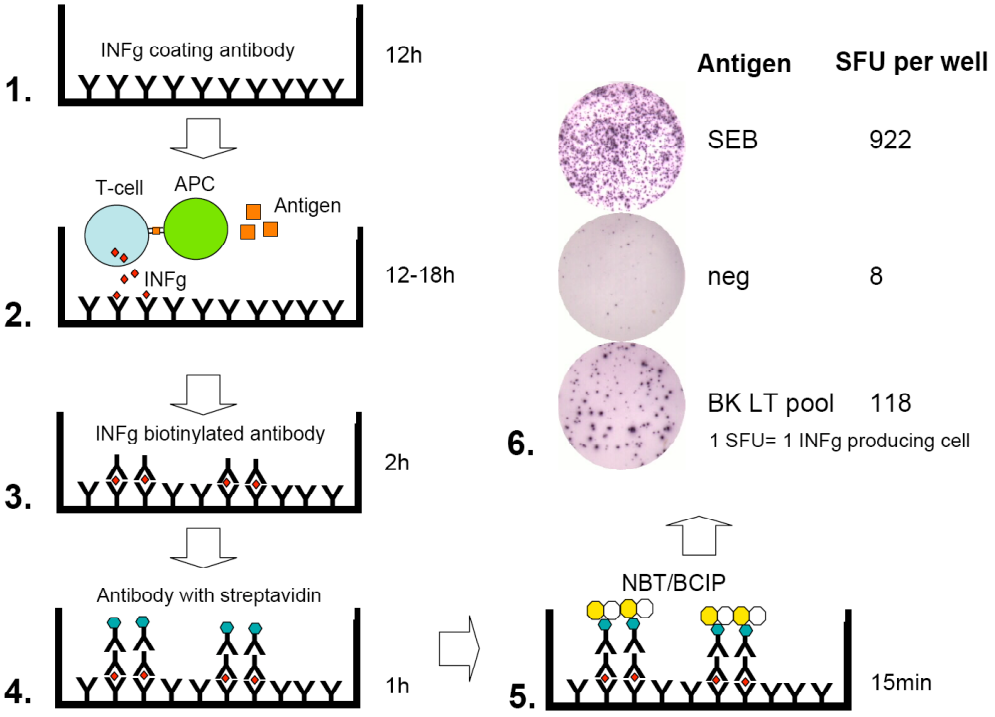


Figure 17. Procedure of Elispot plate coating, stimulating and developing

3.3.3. PCR protocols

Several PCR protocols were used to determine copy numbers of CMV, BKV and JCV per mL of specimen. Additionally, Polyomavirus BK and JC NCCR were determined and rearrangements sequenced, BKV VP1 region was sequenced for serotype determination, and CMV UL97 phosphotransferase gene was sequenced for detection of GCV resistance.

Table 4 gives an overview of all used PCR probes and primers. Detailed protocols can be found in the appendix part.

Description	Sequence
Quantification	
BKV-LT-forward	5'-TAGGTGCCAACCTATGGAACAGA-3'
BKV-LT-reverse	5'-GAAAGTCTTTAGGGTCTTCTACC-3'
BKV-probe	FAM-CATTAAAGGAACTCCACCAGGACTCCCACTC-TAMRA
JCV-LT-forward	5'-CTAAACACAGCTTGACTGAGGAATG-3'
JCV-LT-forward	5'-CATTTAATGAGAAGTGGGATGAAGAC-3'
JCV-probe	FAM-TAGAGTGTTGGGATCCTGTGTTTTCATCATCACT-TAMRA
CMV-IL10-forward	5'-TTTTTTCTAGGCGCTTCCGA-3'
CMV-IL10-reverse	5'-ACACTGCGGCTTTGTATTCTTTATC-3'
CMV-probe	FAM-AGGCGAAGCCGGCGACGA-TAMRA
CMV-UL97	
CMV outer1	5'-TGCTGCACAACGTCACGGTACATC-3'
CMV outer2	5'-AAACAGACTGAGGGGGCTACT-3'
CMV part1 forward	5'-CGTTGGCCGACGCTATCAAATTTC-3'
CMV part1 reverse	5'-ACAGCTCCGACATGCAATAACG-3'
CMV part2 forward	5'-GTGGGTAACGTGCTGGGCTTTTG-3'
CMV part2 reverse	5'-GTGGGTTTGTACCTTCTCTGTTGC-3'
NCCR	
BKV outer 1, BKTT5	5'-GAGCTCCATGGATTCTTC-3'
BKV outer 2, BKTT6	5'-CCAGTCCAGGTTTTACCA-3'
BKV inner 1, BKTT7	5'-CCCTGTTAAGAACTTTATCCATTT-3'
BKV inner 2, BKTT8	5'-AACTTTCACTGAAGCTTGTCGT-3'
JCV outer 1	5'-AGGCCTAATAAATCCATAAGCTCCA-3'
JCV outer 2	5'-GTTCCACTCCAGGTTTTACTAACTT-3'
JCV inner 1	5'-TTTTAGCTTTTTGCAGCAAAAAATTA-3'
JCV inner 2	5'-CCTGGCGAAGAACCATGGCCAG-3'
VP1 sequencing	
BKV outer 1	5'-GTGCAAGTGCCAAAACTACTAATAA-3'
BKV outer 2	5'-TGCATGAAGGTTAAGCATGCTAGT-3'
BKV inner 1	5'-CAACCAAAAGAAAAGGAGAGTGTC-3'
BKV inner 1	5'-TCCTCCACCATGCTCATGCACT-3'

Table 4. Primer and probes for PCR protocols

4. Publications and Manuscripts

4.1. Overview

2006:

Binggeli S., **Egli A.,** Dickenmann M., Binet I., Steiger J., Hirsch H. H.
BKV replication and cellular immune responses in renal transplant recipients.
Am J Transpl 2006, 6:2218-2219

2007:

Egli A., Binggeli S., Bodaghi S., Dumoulin A., Funk G. A., Khanna N., Leuenberger D., Gosert R., Hirsch H. H.
Cytomegalovirus and polyomavirus BK posttransplant.
Nephrol Dialysis Transpl 2007, 22:(Sup 8): 72-82.

Binggeli S., **Egli A.,** Schaub S., Binet I., Mayr M., Steiger J., Hirsch H. H.
Polyomavirus BK-specific cellular immune response to VP1 and large T-antigen in kidney transplant recipients.
Am J Transpl 2007, 7(5): 1131-1139.

Schaub S., Mayr M., **Egli A.,** Binggeli S., Desceudres B, Steiger J., Mihatsch M., Hirsch H.H.
Transient allograft dysfunction from immune reconstitution in a patient with polyoma BK-virus-associated nephropathy.
Nephrol Dialysis Transpl 2007, 22(8): 2386-2390

2008:

Egli A., Bergamin O., Müllhaupt B., Seebach J.D., Müller N.J., Hirsch H.H.
Cytomegalovirus-associated chorioretinits after liver transplantation: case report and review of the literature.
Transpl Infect Dis. 2008, 10(1): 27-43

Egli A., Binet I., Jäger C., Dumoulin A., Steiger J., Sester, U., Sester M., Hirsch H.H.
Cytomegalovirus-specific T-cell responses and viral replication in kidney transplant recipients
J Trans Med, 2008, 6:29: doi:10.1186/1479-5876-6-29

Egli A., Dumoulin A,. Kiss, D., Hirsch H.H.
Neue Diagnostik in der Transplantationsmedizin: Cytomegalovirus (CMV)-spezifische T-Zellfrequenz und Ganciclovir-Resistenzanalyse.
Swiss Medical Forum, 2008, 8(34): 619

Egli A., Dumoulin A., Köhli S., Hirsch H.H.
Polyomavirus BK after kidney transplantation – Role of molecular and immunological markers.
Trends in Transplantation 2008, in press

Gosert R., Rinaldo, Ch.H., Funk G.A., **Egli A.,** Ramos E., Drachenberg C.B., Hirsch H.H.
Polyomavirus BK with rearranged non-coding control region emerge *in vivo* in renal transplant patients and increase viral replication and cytopathology.
J Exp Med. 2008, 205(4): 841-52

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J Inf Dis 2008, in press

4.2. Manuscripts

4.2.1. BKV replication and cellular immune responses in renal transplant recipients

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Letter to the Editor

BKV Replication and Cellular Immune Responses in Renal Transplant Recipients

To the Editor:

Hammer and colleagues report on BKV-specific T-cell responses in peripheral blood mononuclear cells (PBMC) of 15 viremic kidney transplant recipients (1). After stimulation with overlapping peptides spanning the viral capsid protein VP1, interferon-γ (IFNγ)-producing CD4+ T cells were observed in 7 patients (47%), including 2 patients (13%) with a CD8+ T-cell response. All patients with a detectable T-cell response had plasma BKV loads of >250 000 copies (c)/mL. Both patients with VP1-specific CD8+ T cells were the only ones to lose their grafts during follow-up. The authors concluded that high BKV load of >250 000 c/mL correlated with peripheral BKV-specific T-cell responses and that BKV-specific CD8+ responses indicated a risk for subsequent allograft loss (1).

Using ELISpot and flow cytometry for intracellular cytokine staining, we detected BKV-specific PBMC responses in renal transplant patients with plasma BKV loads clearly below 250 000 c/mL. In patient 1 (51 100 c/mL), both large T-antigen (LT)- and VP1-specific IFNγ-producing PBMC were detectable by ELISpot (542 and 392 SFU/10^6 PBMC). By flow cytometry, this response included LT- and VP1-specific CD4+ T cells (0.16% and 0.11%) and CD8+ T cells (0.04% and 0.06%), respectively. A similar BKV-specific cellular immune profile was found in patient 2 (300 c/mL). Patient 3 (944 c/mL) is remarkable for clear responses by ELISpot analysis which could not readily be attributed to CD4+ or CD8+ T cells. This discordance may reflect differences in sensitivity and/or an early phase of cellular immune effectors including NK-cells (2). In patient 4 (9600 c/mL), LT- and VP1-specific CD4+ T-cell, but no CD8+ T-cell responses were detectable. In patients 5 and 6 with persisting BKV viremia (38 400 and 98 000 c/mL), only very low responses were detectable by ELISpot, but none by flow cytometry. Overall, we found no correlation of BKV-specific cellular immune responses and the level of plasma BKV load. However, all patients with detectable cellular responses had a decline of BKV loads in the preceding 4–12 weeks. Interestingly, LT- and VP1-specific responses were not always concordant. Similarly, LT-specific T-cell responses were seen in patient 7 with stable allograft function after clearing BKV viremia and polyomavirus-associated nephropathy (PVAN) more than 12 months ago, and in patient 8 being on hemodialysis after allograft loss due to PVAN 15 months ago (Table 1).

In our series, VP1-specific CD8+ T-cell responses were not associated with poor allograft function. This discrepancy may be due to lower immune effector frequencies which, apart from technical differences, could reflect the stage of PVAN, immune reconstitution dynamics after reducing immunosuppression or other factors. Indeed, Hammer et al. were able to raise VP1-specific CD8+ T cells from allograft biopsies from patients without PBMC responses, all of whom maintained allograft function (1). It is unclear why the authors concluded that these cells originated in the donor rather than from efficient homing of recipient T-cells to the site of replication. It is well recognized that duration and levels of plasma BKV loads are important surrogates of viral tissue damage (3–5), which might also determine specific immune-mediated and possibly allospecific collateral damage. Thus, careful investigation of the dynamics of BKV replication, together with the qualitative and quantitative patterns of BKV-specific cellular immune responses, seems to be needed to elucidate the balance of virus and immune response and to identify the most informative tests.

S. Binggeli[a], A. Egli[a], M. Dickenmann[b], I. Binet[c], J. Steiger[b] and H. H. Hirsch[a,d]
[a]Transplantation Virology, Department of Clinical & Biological Sciences, Institute for Medical Microbiology, University of Basel, Basel, Switzerland
[b]Transplantation Immunology and Nephrology, University Hospital Basel, Basel, Switzerland
[c]Division of Nephrology, Kantonsspital St. Gallen, Switzerland
[d]Infectious Diseases & Hospital Epidemiology, University Hospital Basel, Basel, Switzerland

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NDT
Nephrology Dialysis Transplantation

Cytomegalovirus and polyomavirus BK posttransplant

Adrian Egli[1], Simone Binggeli[1], Sohrab Bodaghi[1], Alexis Dumoulin[1], Georg A. Funk[1], Nina Khanna[1,2], David Leuenberger[1], Rainer Gosert[1] and Hans H. Hirsch[1,2]

[1]Transplantation Virology, Institute for Medical Microbiology, Department Clinical & Biological Sciences, University of Basel, Switzerland and [2]Division of Infectious Diseases and Hospital Epidemiology, University Hospital of Basel, Switzerland

Abstract

Virus replication and progression to disease in transplant patients is determined by patient-, graft- and virus-specific factors. This complex interaction is modulated by the net state of immunosuppression and its impact on virus-specific cellular immunity. Due to the increasing potency of immunosuppressive regimens, graft rejections have decreased, but susceptibility to infections has increased. Therefore, cytomegalovirus (CMV) remains the most important viral pathogen posttransplant despite availability of effective antiviral drugs and validated strategies for prophylactic, preemptive and therapeutic intervention. CMV replication can affect almost every organ system, with frequent recurrences and increasing rates of antiviral resistance. Together with indirect long-term effects, CMV significantly reduces graft and patient survival after solid organ and hematopoietic stem cell transplantation. The human polyomavirus called BK virus (BKV), on the other hand, only recently surfaced as pathogen with organ tropism largely limited to the reno-urinary tract, manifesting as polyomavirus-associated nephropathy in kidney transplant and hemorrhagic cystitis in hematopoetic stem cell transplant patients. No licensed anti-polyoma viral drugs are available, and treatment relies mainly on improving immune functions to regain control over BKV replication. In this review, we discuss diagnostic and therapeutic aspects of CMV and BKV replication and disease posttransplantation.

Keywords: cytomegalovirus; BK virus; prophylaxis; resistance; T-cells; transplantation; viral infections

Introduction

The key challenge after transplantation is the recognition of alloantigens by immune effectors. The resulting acute and chronic immune reactions cause transient and lasting damage with decreasing organ function and graft loss. In recent years, potent immunosuppressive protocols significantly improved graft survival in solid organ transplantation (SOT) by reducing rejections, across HLA mismatches [1]. However, as illustrated by registry data of 7500 pediatric kidney transplant patients, decreasing hospitalization rates in the first 2 years posttransplant for acute rejection from >30% in 1982 to ~12% in 2002 were paralleled by increasing hospitalization rates for infections from 20.4% to 30.8% [2]. Similarly, infection rates increased in adult kidney transplant recipients of >50 years from 48% to 69% during the first year post-transplantation [3]. In hematopoietic stem cell transplantation (HSCT), summary data from the European Bone Marrow Transplantation on 14 403 HLA-identical siblings with early leukemia indicated a declining mortality due to infections within the first 12 months between 1980 and 2002 from 6% to 1% which in part reflected reduced toxicity of induction and conditioning protocols [4]. However, virus attributed mortality largely persisted, with older age and T-cell depletion as significant risk factors [4].

Virus replication and disease posttransplant results from complex interactions of patient, graft and virus determinants (Figure 1) which are modulated by the net state of immunosuppression [5,6]. Transplant patients are at high risk for acute, typically respiratory viruses transmitted according to their activity in the community. By contrast, viruses persisting in patients or in transplants reactivate in an almost time table-like sequence of first Herpes simplex, then cytomegalovirus (CMV), and varicella-zoster virus [5]. Herpes simplex and varicella-zoster virus are conveniently suppressed by well tolerated drugs like acyclovir and famciclovir peri- and post-transplantation. For CMV, markers of virus-specific cellular immune functions are considered

Correspondence to: Hans H. Hirsch, MD, MSc Transplantation Virology, Institute for Medical Microbiology, Department of Clinical & Biological Sciences, Petersplatz 10, CH-4003 Basel, Switzerland. Email: Hans.Hirsch@unibas.ch

4.2.3. Polyomavirus BK-specific cellular immune response to VP1 and large T-antigen in kidney transplant recipients

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Polyomavirus BK-Specific Cellular Immune Response to VP1 and Large T-Antigen in Kidney Transplant Recipients

S. Binggeli[a], A. Egli[a], S. Schaub[b], I. Binet[c], M. Mayr[b], J. Steiger[b] and H. H. Hirsch[a,d,*]

[a] *Transplantation Virology, Institute for Medical Microbiology, University of Basel, Basel, Switzerland*
[b] *Division of Transplantation Immunology & Nephrology, University Hospital Basel, Basel, Switzerland*
[c] *Division of Nephrology, Kantonsspital St. Gallen, St. Gallen, Switzerland*
[d] *Infectious Diseases & Hospital Epidemiology, University Hospital Basel, Basel, Switzerland*
* *Corresponding author: Hans H. Hirsch, hans.hirsch@unibas.ch*

Polyomavirus BK (BKV) is the primary cause of polyomavirus-associated nephropathy (PVAN) in kidney transplant (KT) recipients. Using ELISpot assays, we compared the frequency of interferon-γ (IFN-γ) secreting peripheral blood mononuclear cells (PBMC) after stimulation with overlapping peptide pools covering BKV large T-antigen (LT) and VP1 capsid proteins (VP1). In 10 healthy donors, LT and VP1 responses were low with median 24 (range 15–95) and 25 (7–113) spot-forming units/10^6 PBMC (SFU), respectively. In 42 KT patients with current or recent plasma BKV loads, median LT and VP1 responses of 29 (0–524) and 114 (0–1432) SFU were detected, respectively. In KT patients with decreasing or past plasma BKV loads, significantly higher median BKV-specific IFN-γ responses were detected compared to KT patients with increasing or persisting BKV loads [LT: 78 (8–524) vs. 22 (0–120) SFU, p = 0.003; VP1: 285 (45–1432) vs. 53 (0–423) SFU, p = 0.001, respectively]. VP1-specific IFN-γ responses were higher and more likely to involve $CD4^+$ T cells, while $CD8^+$ T cells were more frequently directed against LT. Stimulation with JCV-specific VP1 and LT peptides indicated only low-level cross-recognition. The data suggest that control of BKV replication is correlated with differentiated expansion of BKV-specific cellular immune responses.

Key words: BK virus interstitial nephritis, kidney transplantation, nephropathy, polyoma, polyomavirus, T cells

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Introduction

Polyomavirus-associated nephropathy (PVAN) is currently the most challenging infectious cause of kidney transplant (KT) failure (1) affecting 1–10% of patients with graft loss in >50% (2–6). The human polyomavirus type 1, called BK virus (BKV) (7), is the primary etiologic agent, although few cases have been attributed to the closely related JC virus (JCV), the primary cause of progressive multifocal leucoencephalopathy (8,9). BKV seroprevalence rates increase during childhood exceeding 90% in adults worldwide (10). BKV persists in a nonreplicative latent stage in the renourinary tract, and reactivation and low-level replication is seen intermittently in 5% of nonimmunosuppressed individuals at comparatively low levels of $<10^6$ copies (cp)/mL (11,12). High-level urinary shedding at $>10^7$ cp/mL occurs in 20–60% of transplant and other immunocompromised patients, and may be followed by viremia in KT patients at risk for PVAN (13,14). Although the risk factors of PVAN are not unequivocally defined and may include partially complementary determinants of patient, graft and virus (3), impaired BKV-specific antiviral immune control is viewed as key factor (15). Different studies have implicated BKV-seropositive donors, seronegative recipients, HLA-mismatching, HLA-C7 negativity of donor or recipient, intensity of maintenance immunosuppression and antirejection treatment (15–19). Accordingly, decreasing of immunosuppressive drugs may be followed by clearing of BKV replication in pre-emptive settings (20) as well as in cases with histologically defined PVAN (13,21–23). In such patients, BKV-specific T-cell responses become increasingly detectable among peripheral blood mononuclear cells (PBMC) (17,24,25). In a pilot study of five patients, we observed that declining BKV loads in plasma were associated with increasing cellular immune responses against BKV early gene large T-antigen (LT) and late VP1 capsid protein (VP1) in PBMC of KT patients (26). We sought to further evaluate this observation and to compare it with responses in BKV-seropositive healthy donors (HD).

Study Participants and Methods

Study participants

HD were 39 years old (median; range 28–53, seven males, three females) without BKV or JCV in plasma or urine. KT patients (median age 54 years, range 21–65) attending outpatient nephrology clinic during

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Case Report

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Transient allograft dysfunction from immune reconstitution in a patient with polyoma BK-virus-associated nephropathy

Stefan Schaub[1], Michael Mayr[1], Adrian Egli[2], Simone Binggeli[2], Bernard Descœudres[1], Jürg Steiger[1], Michael J. Mihatsch[3] and Hans H. Hirsch[2,4]

[1]Clinic for Transplantation Immunology and Nephrology, University Hospital Basel, [2]Transplantation Virology and Molecular Diagnostics, Department of Clinical and Biological Sciences, Institute for Medical Microbiology, University of Basel, [3]Institute for Pathology, University Hospital Basel and [4]Clinic for Infectious Diseases & Hospital Epidemiology, University Hospital Basel, Basel, Switzerland

Keywords: immune reconstitution syndrome; polyoma BK-virus nephropathy; renal allograft rejection

Introduction

During the last 10 years, polyoma BK-virus associated nephropathy (PVAN) has emerged as a serious complication in renal transplant recipients [1]. Due to the establishment of an accurate non-invasive screening procedure measuring polyomavirus BK-viraemia, BK-viruria and decoy cells in urine, PVAN can be diagnosed at early stages [2]. This allows for timely therapeutic intervention, which has significantly reduced the incidence of severe PVAN courses including graft loss [3,4].

Management of PVAN is mainly based on a reduction of the immunosuppressive drugs, while the impact of anti-viral therapy is not yet clear [5]. This strategy bears the inherent risk that allograft rejection may arise, which is difficult to differentiate from an immune response to the BK-virus, because both entities can present as morphologically and molecularly indistinguishable, with interstitial infiltrates and tubulitis [1,5–7]. Therefore, more data regarding the natural course of PVAN under reduced immunosuppression might be helpful to illuminate the scope of post-intervention responses.

Case report

A 37-year-old woman had end-stage renal failure due to a nephropathy of unknown origin. She was highly sensitized as a consequence of two blood transfusions and two pregnancies (peak CDC-PRA 78%, peak FlowPRA^{TM} class I 93%, FlowPRA^{TM} class II negative). After being on haemodialysis for 9 years, she received a kidney from a 7-year-old deceased donor. There were three HLA-mismatches (recipient: HLA-A3/24, B7/55, DR4/13; donor: HLA-A2/24, B7/38, DR11/13) and the recipient had two donor-specific HLA-antibodies (DSA) detectable in three historic sera (A2 and B38; determined by FlowPRA^{TM} single-antigen flow-beads). Flow-cytometric T- and B-cell cross-matches were positive with historic sera, but negative with the current one. The patient was considered to be at high risk for rejection and received an induction therapy consisting of polyclonal anti-T-lymphocyte globulin (ATG-Fresenius) as well as intravenous immunoglobulins (IvIg) [8]. Maintenance immunosuppression consisted of tacrolimus, mycophenolate mofetil (MMF) and steroids.

Figure 1 summarizes the course of immunosuppressive therapy, allograft function and BK-virus activity; Figure 2 demonstrates the histology of the four allograft biopsies obtained in this patient. The allograft had an immediate good function and serum creatinine dropped to 140 µmol/l by day 10 post-transplant. On day 21, serum creatinine rose to 308 µmol/l and the first allograft biopsy was obtained. The diagnosis of antibody-mediated rejection was made based on the presence of thrombotic microangiopathy, diffuse C4d-staining in peritubular capillaries and reappearance of both remote DSA (A2 and B38) in high quantities (Figure 2; picture 1A and 1B). The patient received another course of IvIg, six steroid pulses and four plasmapheresis treatments. Subsequently, serum creatinine declined to 110 µmol/l.

Eight weeks post-transplant, increasing BK-virus replication in the urine was noted along with the appearance of numerous decoy cells, followed by plasma BK-viral loads persisting above 10 000

Correspondence and offprint requests to: Stefan Schaub, MD, MSc, Transplantation Immunology and Nephrology, University Hospital Basel, Petersgraben 4, 4031 Basel, Switzerland.
Email: schaubs@uhbs.ch

4.2.5. Cytomegalovirus-associated chorioretinits after liver transplantation: case report and review of the literature

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Case report

Cytomegalovirus-associated chorioretinitis after liver transplantation: case report and review of the literature

A. Egli, O. Bergamin, B. Müllhaupt, J.D. Seebach, N.J. Mueller, H.H. Hirsch. Cytomegalovirus-associated chorioretinitis after liver transplantation: case report and review of the literature. Transpl Infect Dis 2008: **10**: 27–43. All rights reserved

Abstract: A cytomegalovirus (CMV) donor positive/recipient negative liver transplant recipient developed CMV syndrome with presumed colitis 2 weeks after discontinuing the standard 3 months of valganciclovir prophylaxis. Treatment with intravenous ganciclovir (GCV) reduced, but did not clear, CMV replication. A CMV UL97 mutation (M460V) conferring GCV resistance was identified. Reduction of immunosuppression was followed by rapidly rising lymphocyte counts as well as by clearance of CMV viremia and of clinical symptoms. However, bilateral chorioretinitis was diagnosed 2 weeks later and treated with foscarnet and cidofovir. Then, right eye vitritis occurred necessitating vitrectomy due to a partially rhegmatogeneous retinal detachment. Because chorioretinitis–vitritis after rising lymphocyte counts and clearance of CMV viremia was strongly suggestive of an immune reconstitution syndrome (IRS)-like disease, we investigated CMV-specific T-cells in the peripheral blood available during follow-up. We found strong $CD8^+$ but only low $CD4^+$ T-cell responses (4.77% vs. <0.1%) to the CMV immediate early pp72, while responses to CMV-lysate or CMV-pp65 ($CD4^+$ <0.01%; $CD8^+$ <0.01%) were low. Over 16 weeks of follow-up, pp72-specific $CD8^+$ responses declined, while responses to pp65 gradually increased ($CD4^+$ 0.16%; $CD8^+$ 0.76%) indicating a slowly adapting CMV-specific cellular T-cell response. Review of 12,653 published liver transplant patients identified only 14 (0.1%) reported cases of CMV-associated chorioretinitis at a median 41.7 weeks post transplant. CMV-associated opthalmologic complications late post transplantation may possibly involve 2 different entities of cytopathic retinitis and IRS-like chorioretinitis–vitritis.

A. Egli[1], O. Bergamin[2], B. Müllhaupt[3], J.D. Seebach[4], N.J. Mueller[5], H.H. Hirsch[1,6]

[1]Transplantation Virology, Institute for Medical Microbiology, University of Basel, Basel, Switzerland, [2]Department of Ophthalmology, University Hospital Zürich, Zürich, Switzerland, [3]Clinic of Gastroenterology and Hepatology, University Hospital Zürich, Zürich, Switzerland, [4]Department of Internal Medicine, Laboratory for Transplantation Immunology, University Hospital Zürich, Zürich, Switzerland, [5]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zürich, Zürich, Switzerland, [6]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Basel, Basel, Switzerland

Key words: cytomegalovirus; ganciclovir; resistance; immune reconstitution syndrome; liver transplantation; CMV late disease; chorioretinitis; immune reconstitution inflammatory syndrome; retinitis; vitritis

Correspondence to:
Hans H. Hirsch, MD, MS, Transplantation Virology, Institute for Medical Microbiology, University of Basel, Petersplatz 10, CH-4003 Basel, Switzerland
Tel: 41 61 267 3225
Fax: 41 61 267 3283
E-mail: hans.hirsch@unibas.ch

Authorship: A.E. wrote the paper, performed immunologic tests and analyzed data; O.B., B.M., and J.S. provided clinical data and wrote the paper; N.M. and H.H.H. identified the case, designed the study, and wrote the paper.

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Despite the high incidence of cytomegalovirus (CMV) viremia and disease in organ transplant recipients, intraocular complications are rarely observed. This is in marked contrast to advanced human immunodeficiency virus (HIV) infection, where CMV chorioretinitis was a frequent clinical manifestation of CMV disease in the era before highly active antiretroviral therapy (HAART) became widely available (1). In some HIV patients, however, worsening of CMV chorioretinitis is observed after initiation of HAART, despite of effective antiviral suppression of CMV. This paradoxical clinical course has been referred to as immune reconstitution inflammatory syndrome (IRS) and was linked to recovering CD4 T-cell counts after effectively inhibiting HIV replication (2–4). Initial $CD4^+$ T-cell counts are often <100 cells/mm^3 and subsequently rise >2–4-fold within several weeks after HAART. Activation, expansion,

CMV, cytomegalovirus; GCV, ganciclovir; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; IFNγ, interferon gamma; IRS, immune reconstitution syndrome; PBMC, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; ValGCV, valganciclovir.

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Research

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Cytomegalovirus-specific T-cell responses and viral replication in kidney transplant recipients

Adrian Egli[1], Isabelle Binet[2], Simone Binggeli[1], Clemens Jäger[2], Alexis Dumoulin[1], Stefan Schaub[3], Juerg Steiger[3], Urban Sester[4], Martina Sester[4] and Hans H Hirsch*[1,5]

Address: [1]Transplantation Virology, Institute for Medical Microbiology, University of Basel, Petersplatz 10, 4003 Basel, Switzerland, [2]Nephrology, Kantonsspital St. Gallen, Switzerland, [3]Nephrology and Transplantation Immunology, University Hospital of Basel, Petersgraben 4, 4031 Basel, Switzerland, [4]Medical Department IV, Nephrology, and Department of Virology, University of Saarland, Homburg, Germany and [5]Infectious Diseases and Hospital Epidemiology, University Hospital of Basel, Petersgraben 4, 4031 Basel, Switzerland

Email: Adrian Egli - adrian.egli@unibas.ch; Isabelle Binet - Isabelle.Binet@kssg.ch; Simone Binggeli - simone.binggeli@stud.unibas.ch; Clemens Jäger - Clemens.Jaeger@kssg.ch; Alexis Dumoulin - alexis.dumoulin@unibas.ch; Stefan Schaub - schaubs@uhbs.ch; Juerg Steiger - jsteiger@uhbs.ch; Urban Sester - urban.sester@uniklinikum-saarland.de; Martina Sester - martina.sester@uniklinikum-saarland.de; Hans H Hirsch* - hans.hirsch1@unibas.ch

* Corresponding author

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Abstract

Background: Cytomegalovirus (CMV) seronegative recipients (R-) of kidney transplants (KT) from seropositive donors (D+) are at higher risk for CMV replication and ganciclovir(GCV)-resistance than CMV R(+). We hypothesized that low CMV-specific T-cell responses are associated with increased risk of CMV replication in R(+)-patients with D(+) or D(-) donors.

Methods: We prospectively evaluated 73 consecutive KT-patients [48 R(+), 25 D(+)R(-)] undergoing routine testing for CMV replication as part of a preemptive strategy. We compared CMV-specific interferon-γ (IFN-γ) responses of CD4+CD3+ lymphocytes in peripheral blood mononuclear cells (PBMC) using three different antigen preparation (CMV-lysate, pp72- and pp65-overlapping peptide pools) using intracellular cytokine staining and flow cytometry.

Results: Median CD4+ and CD8+T-cell responses to CMV-lysate, pp72- and pp65-overlapping peptide pools were lower in D(+)R(-) than in R(+)patients or in non-immunosuppressed donors. Comparing subpopulations we found that CMV-lysate favored CD4+- over CD8+-responses, whereas the reverse was observed for pp72, while pp65-CD4+- and -CD8+-responses were similar. Concurrent CMV replication in R(+)-patients was associated with significantly lower T-cell responses (pp65 median CD4+ 0.00% vs. 0.03%, p = 0.001; CD8+ 0.01% vs. 0.03%; p = 0.033). Receiver operated curve analysis associated CMV-pp65 CD4+ responses of > 0.03% in R(+)-patients with absence of concurrent (p = 0.003) and future CMV replication in the following 8 weeks (p = 0.036). GCV-resistant CMV replication occurred in 3 R(+)-patients (6.3%) with pp65- CD4+ frequencies < 0.03% (p = 0.041).

Conclusion: The data suggest that pp65-specific CD4+ T-cells might be useful to identify R(+)-patients at increased risk of CMV replication. Provided further corroborating evidence, CMV-pp65 CD4+ responses above 0.03% in PBMCs of KT patients under stable immunosuppression are associated with lower risk of concurrent and future CMV replication during the following 8 weeks.

Neue Diagnostik in der Transplantationsmedizin: Cytomegalovirus-(CMV-)spezifische T-Zellfrequenz und Ganciclovir-Resistenzanalyse

Adrian Egli[a], Alexis Dumoulin[a], Denes Kiss[b], Hans H. Hirsch[a,c]

[a] Transplantationsvirologie, Institut für Medizinische Mikrobiologie, Universität Basel, [b] Nephrologie, Kantonsspital Liestal, [c] Infektiologie und Spitalhygiene, Universitätsspital Basel

Die CMV-spezifische zelluläre Immunität ist nach Transplantation infolge der Immunsuppression reduziert [1]. Die immunologisch unkontrollierte CMV-Replikation ist eine Gefahr für Rezipient und Transplantat und muss mit Virostatika wie (Val-)Ganciclovir (GCV) behandelt werden [2]. Fluoreszenz-aktiviertes Cell Sorting und Elispot-Assay-(FACS-)Analysen erlauben die Quantifizierung der CMV-pp65-spezifischen zellulären Immunantwort im peripheren Blut. CMV-Gensequenzierungen können Mutationen, die mit GCV-Resistenz assoziiert sind, nachweisen.

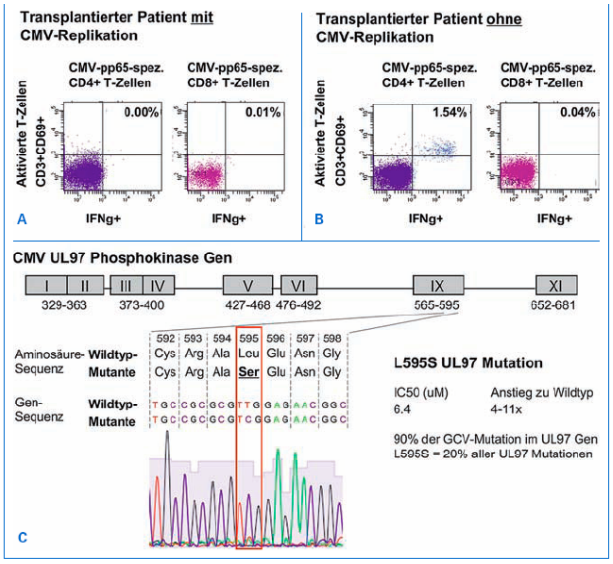

Abbildung 1

A FACS-Analyse mit CMV-pp65-spezifischen CD4- und CD8-T-Zellen, 375 Wochen nach Transplantation, 3 Wochen vor der Resistenzentwicklung. CD3-markierte T-Zellen, CD69 und IFNg sind Aktivierungsmarker von T-Zellen.

B FACS-Analyse eines nierentransplantierten Patienten ohne CMV-Replikation.

C Sequenzierung des CMV-UL97-Gens, verantwortlich für Ganciclovir-Resistenz (Mutation L595S). Vergleich Wildtyp gegen Mutante (L595S). Darstellung des CMV-UL97-Phosphokinase-Gens mit konservierten Domänen (graue Box). IC50, 50% inhibitorische Konzentration.

Der Fall

Ein 66jähriger Patient (CMV sero+) leidet sechs Jahre nach Nierentransplantation (Donor CMV+) an einer CMV-Reaktivierung mit Kolitis und Retinitis. Die initiale Therapie (ValGCV 2× 450 mg/d, Kreatinin-Clearance 30 ml/min) musste infolge Leukopenie gestoppt werden. Die Immunsuppression ist gering (MMF 2,0–4,4 ng/ml und 7,5 mg Prednison), dennoch ist die CMV-pp65-spezifische T-Zellfrequenz sehr tief (0,00% CD4 und 0,01% CD8; Referenz von Nierentransplantierten ohne CMV-Replikation 0,03% CD4 und 0,03% CD8 [Median]) [3]. Ein Indiz für eine unzureichende zelluläre Abwehr.

Zwei Wochen später steigt die CMV-Viruslast trotz erneuter Val GCV (1× 450 mg) auf 5×10^5 c/ml an. Die *Sequenzierung* des UL97-Gens bestätigt eine mit *GCV-Resistenz* assoziierte Mutation (L595S) (Abb. 1 📷). Nach Erhöhung von ValGCV (auf 2× 450 mg/d, Kreatinin-Clearance 30 ml/min) sistiert die CMV-Replikation, parallel verbessern sich Diarrhoe und Visus.

FACS-Analyse und CMV-Sequenzierung finden den Weg in die klinische Praxis [4]. Diese neuen Methoden können wertvolle Entscheidungshilfen bei komplexen und gefährlichen Krankheitsverläufen darstellen.

Korrespondenz:
Prof. Dr. Hans H. Hirsch
Transplantationsvirologie
Universität Basel
Petersplatz 10, CH-4003 Basel
hans.hirsch@unibas.ch

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4.2.8. Polyomavirus BK after kidney transplantation – Role of molecular and immunological markers

Trends in Transplant. 2008;2:XX-XX

Polyomavirus BK after Kidney Transplantation – Role of Molecular and Immunologic Markers

Adrian Egli[1], Alexis Dumoulin[1], Sabrina Köhli and Hans H. Hirsch[1,2]

[1]Transplantation Virology, Institute for Medical Microbiology, Department Clinical & Biological Sciences, University of Basel, Basel, Switzerland; [2]Division of Infectious Diseases and Hospital Epidemiology, University Hospital of Basel, Basel, Switzerland

Abstract

Polyomavirus-associated nephropathy in kidney transplantation is viewed as a complication of prolonged, intense immunosuppression, which disrupts the balance between antiviral immune control and polyomavirus replication. The prevalence rate ranges from 1-10% in kidney transplant programs around the world, with graft failure and return to dialysis in 50%. Most cases are caused by the human polyomavirus BK, which asymptomatically infects more than 80% of the general population. Reactivation of BK virus replication and high urine viral loads precedes increasing plasma BK viral loads and histologically defined as well as clinically manifest disease. Quantitative real-time polymerase chain reaction protocols have proven valuable as surrogate markers to follow the course of polyomavirus-associated nephropathy and for preemptive reduction of immunosuppression. As these assays enter clinical routine diagnostic laboratories, quality control becomes important. Testing of BK virus-specific antibodies and T-cells is currently being explored for a better characterization of the virus/host balance. The BK virus-like particles IgG in enzyme-linked immunosorbent essays are recognized as sensitive indicators of recent BK virus exposure. However, no humoral immune responses have been identified as correlating with protection from BKV viremia or disease. The BK virus-specific T-cells are generally of low frequencies in the peripheral blood of both healthy donors and kidney transplant patients alike, but significantly increase at the time when plasma BK virus loads, hence representing relatively late indicators of regaining control. We discuss the currently available data on molecular and immune markers regarding promises and caveats. (Trends in Transplant. 2008;2:XX-XX)

Corresponding author: Hans H Hirsch, Hans.Hirsch@unibas.ch

Key words

Transplantation. Viral infections. Polyomavirus. JC virus. BK virus. Polyomavirus associated nephrology. T-cells. Epitope mapping. Vaccine. Adaptive T-cell transfer.

Correspondence to:
Hans H Hirsch
Transplantation Virology, Institute for Medical Microbiology
Department of Biomedicine, University of Basel
Petersplatz 10, CH-4003 Basel, Switzerland
E-mail: Hans.Hirsch@unibas.ch

4.2.9. Polyomavirus BK with rearranged non-coding control region emerge *in vivo* in renal transplant patients and increase viral replication and cytopathology

JEM ARTICLE

Polyomavirus BK with rearranged noncoding control region emerge in vivo in renal transplant patients and increase viral replication and cytopathology

Rainer Gosert,[1] Christine H. Rinaldo,[2] Georg A. Funk,[1] Adrian Egli,[1] Emilio Ramos,[3] Cinthia B. Drachenberg,[4] and Hans H. Hirsch[1,5]

[1]Transplantation Virology and Molecular Diagnostic Laboratory, Institute for Medical Microbiology, Department of Biomedicine, University of Basel, CH-4003 Basel, Switzerland
[2]Microbiology and Infection Control, University Hospital of North Norway, 9038 Tromsø, Norway
[3]Department of Medicine and [4]Department of Pathology, University of Maryland Transplant Center, Baltimore, MD 21201
[5]Infectious Diseases and Hospital Epidemiology, University Hospital Basel, 4031 Basel, Switzerland

Immunosuppression is required for BK viremia and polyomavirus BK–associated nephropathy (PVAN) in kidney transplants (KTs), but the role of viral determinants is unclear. We examined BKV noncoding control regions (NCCR), which coordinate viral gene expression and replication. In 286 day–matched plasma and urine samples from 129 KT patients with BKV viremia, including 70 with PVAN, the majority of viruses contained archetypal (ww-) NCCRs. However, rearranged (rr-) NCCRs were more frequent in plasma than in urine samples (22 vs. 4%; $P < 0.001$), and were associated with 20-fold higher plasma BKV loads (2.0×10^4/ml vs. 4.4×10^5/ml; $P < 0.001$). Emergence of rr-NCCR in plasma correlated with duration and peak BKV load ($R^2 = 0.64$; $P < 0.001$). This was confirmed in a prospective cohort of 733 plasma samples from 227 patients. For 39 PVAN patients with available biopsies, rr-NCCRs were associated with more extensive viral replication and inflammation. Cloning of 10 rr-NCCRs revealed diverse duplications or deletions in different NCCR subregions, but all were sufficient to increase early gene expression, replication capacity, and cytopathology of recombinant BKV in vitro. Thus, rr-NCCR BKV emergence in plasma is linked to increased replication capacity and disease in KTs.

CORRESPONDENCE
Hans H. Hirsch:
Hans.Hirsch@unibas.ch

Abbreviations used: dat, days after transfection; geq, genome equivalent; HEK, human embryonic kidney; KT, kidney transplant; LTag, large T-antigen; NCCR, noncoding control region; PVAN, polyomavirus BK–associated nephropathy; RFP, red fluorescent protein; RPTEC, renal proximal tubular epithelial cell.

Polyomavirus BK–associated nephropathy (PVAN) has emerged as the most challenging infectious cause of irreversible kidney transplant (KT) failure (1, 2). PVAN is diagnosed in up to 10% of KT patients around the world, causing premature graft loss in the 6–60 mo after transplant (3, 4). Histologically, progression from a mainly cytopathic pattern (PVAN A) to extensive cytopathic/inflammatory changes of interstitial nephritis (PVAN B) is associated with increasing graft loss from <10 to 50%, exceeding 80% for PVAN C when tubular atrophy and fibrosis predominate (5). Histological studies have demonstrated extensive BKV replication in the urothelial cell layer (6); however, unlike in bone marrow transplant patients, BKV-associated hemorrhagic cystitis is rarely encountered in KT patients, despite high urine viral loads (7). The emergence of PVAN is remarkable in view of the 50 yr of experience in kidney transplantation and the basically unchanged high prevalence of BKV infection in the general population (8–10). The "net state of immunosuppression" seems crucial for PVAN pathogenesis and reflects the use of more potent immunosuppressive drugs synergizing with other factors, such as older age, negative BKV recipient and positive BKV donor status, higher number of HLA mismatches, and prior rejection episodes (7, 11–14). The role of viral determinants is presently unclear. As no antiviral drug of proven efficacy is available (15), current treatment is based on reducing immunosuppression to regain immune control over BKV replication and disease (16, 17). This maneuver bears the risk of rejection and entry into a vicious cycle with eventual graft loss (18).

The online version of this article contains supplemental material.

The Journal of Experimental Medicine

4.2.10. An universal HLA class I and II restricted immunogenic polypeptide from HCMV pp65 promotes the expansion of CD4+ and CD8+ effector T-cells across a wider range of HLA specificities

Journal of Cellular and Molecular Medicine

JOURNAL OF Cellular AND Molecular Medicine
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A HCMV pp65 polypeptide promotes the expansion of CD4+ and CD8+ T cells across a wide range of HLA specificities

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Keywords:	peptide cross-presentation, immunotherapy, vaccine, Human Cytomegalovirus

4.2.11. Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors

Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors

Adrian Egli[1], Laura Infanti[2], Alexis Dumoulin[1,3], Andreas Buser[2], Jacqueline Samaridis[1], Christine Stebler[2], Rainer Gosert[1], and Hans H. Hirsch[1,3,4*]

[1]Transplantation Virology, Institute for Medical Microbiology, Departement Biomedizin, University of Basel, Switzerland,

[2]Blutspendezentrum SRK beider Basel, Basel, Switzerland

[3] Division of Diagnostics, Institute for Medical Microbiology, University of Basel, Switzerland,

[4] Infectious Diseases and Hospital Epidemiology, University Hospital of Basel, Switzerland

Running head: Polyomavirus infection and replication in healthy blood donors.

Key words: Polyomavirus, BKV, JCV, serology, viral replication, NCCR, blood donors, serotype, shedding, transmission

Conflict of interest: No conflict of interest for any author.

***Correspondence:**

Hans H Hirsch MD, MSc

Transplantation Virology, Institute for Medical Microbiology, Department Biomedicine

University of Basel, Petersplatz 10

CH-4003 Basel, Switzerland

phone: +41 61 267-3262; fax: +41 61 255-3283 email: Hans.Hirsch@unibas.ch

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5. Summary and discussion of the key results

5.1. Cytomegalovirus

Effective control of CMV replication requires a sufficient CMV-specific cellular immune control through T-cells (Sester, Sester et al. 2001; Lacey, Gallez-Hawkins et al. 2002; Bunde, Kirchner et al. 2005; Sester, Gartner et al. 2005). Reduction of CMV-specific T-cells may lead to uncontrolled CMV replication in kidney transplant recipients causing CMV-associated diseases in graft and recipient. To reduce virus replication and progression to disease in high-risk patients, a prophylactic or preemptive intervention strategy is common practice (see introduction). Long-term Ganciclovir application under persistent virus replication and immunosuppression increases the risk to select for GCV-resistant CMV replication (UL54 or UL97 mutations) (Limaye, Corey et al. 2000).

5.1.1. Cytomegalovirus-specific T-cell responses and viral replication in kidney transplant recipients (Egli, Binet et al. 2008)

Published immune assays vary according CMV antigen preparation used. We asked, which is best preparation to stimulate CMV-specific T-cells and, whether CMV-specific T-cells can be used after kidney transplantation to predict the risk of future CMV replication episodes and development of GCV-resistant CMV replication.

The aim was to compare the amount of INFγ producing CMV-specific T-cells in healthy blood donors versus kidney transplant recipients using several antigen preparations to detect CMV-specific T-cells (virus-lysate from cell culture and pp65/pp72 peptide pools). We further aimed to characterize R+ patients with or without concurrent CMV replication and GCV-resistance associated mutations. Follow-up of virus detection in blood revealed patients with future CMV replication

We hypothesised, that kidney transplant patients show a lower amount of CMV-specific T-cells compared to healthy blood donors. Additionally, that R+

kidney transplant recipients with low CMV-specific T-cells show a risk for concurrent and future CMV-replication and development of GCV-resistance.

We examined 13 CMV-seronegative and 17 CMV-seropositive healthy donors, and 25 D+R- and 48 R+ kidney transplant (KT) patients.
We determined CMV-specific CD4+ and CD8+ T-cell response to CMV lysate, pp65- and pp72-peptide pools (15mer library with 11aa overlap) with flow cytometry. Concurrent and future CMV replication was checked during the next 3 months with PCR and pp65-antigenemia. GCV-associated mutations were searched with sequencing the CMV-UL97 gene.

CMV seropositive healthy blood donors showed significant higher CMV-specific T-cell responses towards all tested antigen-preparations compared to R+ KT patients (see publication Table 2). CMV-lysate and CMV-pp65 stimulated both CD4+ and CD8+ T-cells, whereas CMV-pp72 seemed to induce a stronger CD8+ response.
As expected, R+ KT patients showed significant higher CMV-specific T-cell responses compared to D+R- KT patients (see **Figure 18**).

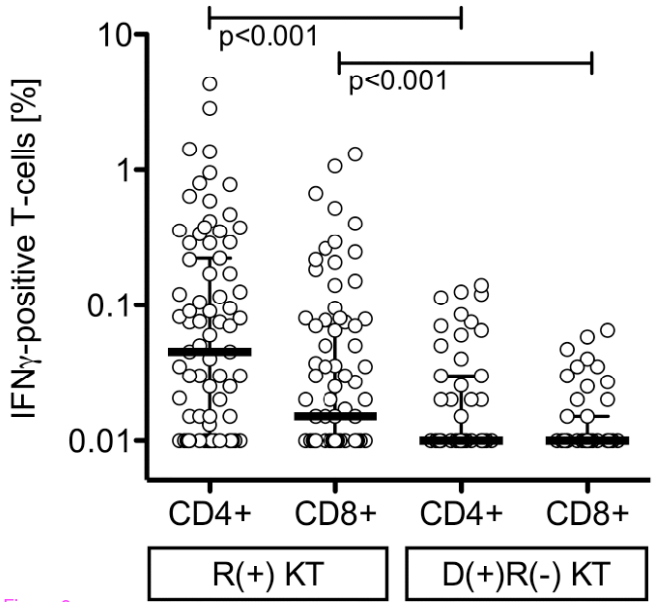


Figure 18. CMV-lysate specific immune responses in R+ KT versus D+R- KT patients.

In total, 9/25 D+R- and 6/48 R+ kidney-transplant patients showed a concurrent CMV replication at time of sampling (see publication Table 1). Overall, R+ KT patients with concurrent CMV replication showed significant lower amounts of CMV-specific T-cells compared to patients without CMV replication (see **Figure 19**). For example median values of pp65-specific CD4+ T-cells in R+ patients with concurrent CMV replication: 0.00% versus without replication: 0.03%, p<0.0001 (MWU).

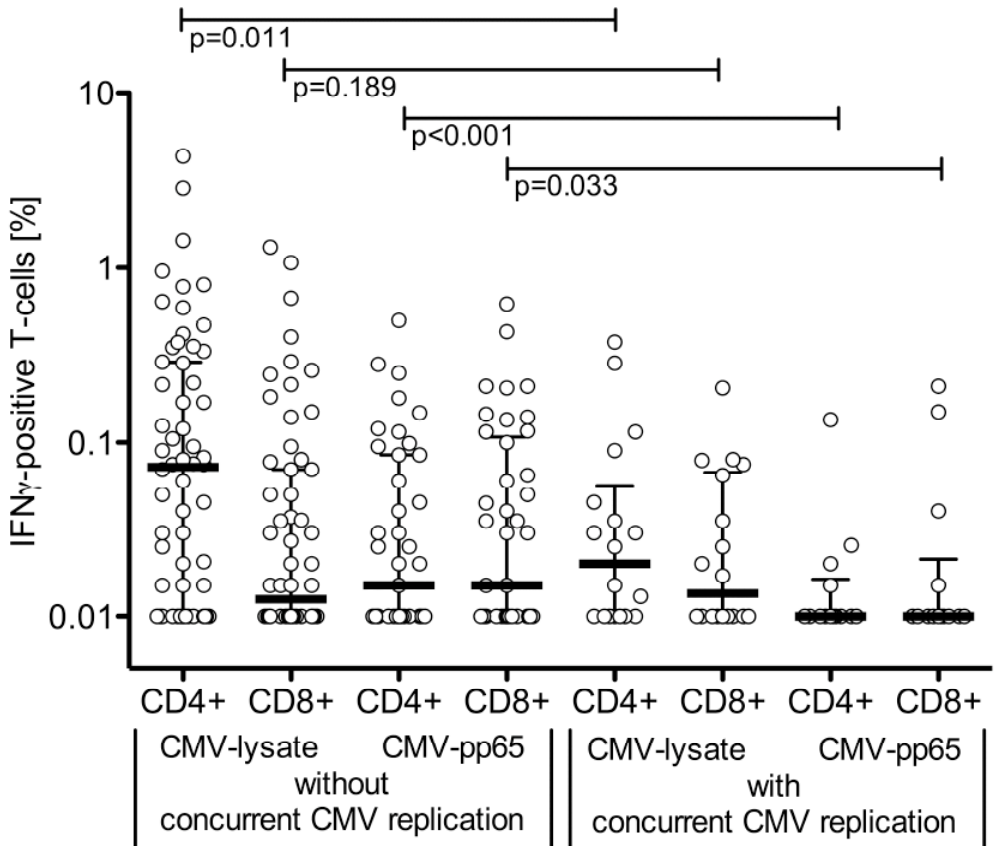


Figure 19. R+ patients with and without concurrent CMV replication and corresponding immune responses.

Receiver operating curve (Roc) analysis identified a cut-off value for the risk of concurrent and future CMV replication and development of GCV resistance. Patient with a CMV-pp65 specific CD4+ T-cell response < 0.03% CD4+ T-cells showed significantly more often concurrent and future CMV replication within the following 8 weeks (p=0.036). Additionally, these patients developed more often GCV-resistance (p=0.041). For future replication the sensitivity

was 50% and the specificity 93%. The positive predictive value was 95% and the negative predictive value was 40% (see **Figure 20**).

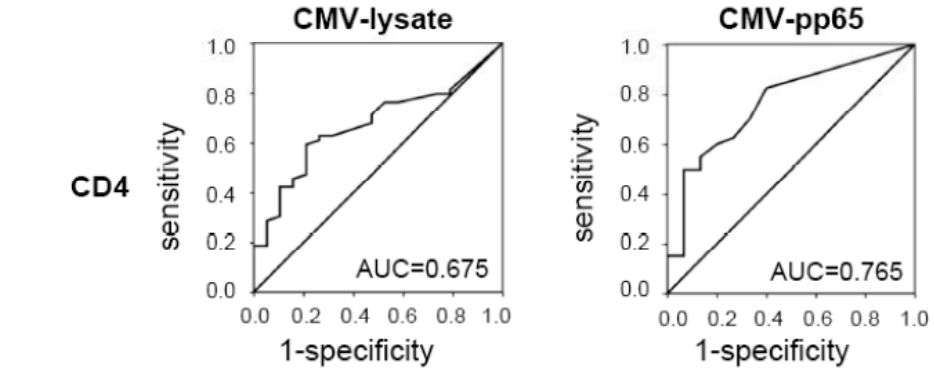


Figure 20. Roc analysis to define threshold of protection from concurrent CMV replication

Bunde et al. reported that higher pp72-specific CD8+ T-cell frequencies were associated with a decreased risk of CMV disease during the first month after heart or lung transplantation (AUC 0.719, specificity 100%, sensitivity 50%, p=0.012) (Bunde, Kirchner et al. 2005). Sester et al. associated the amount of CMV-lysate specific CD4+ T-cell to episodes of CMV pneumonitis in lung transplant recipients, without defining a clear cut-off (Sester, Gartner et al. 2005). Clearly, increasing calcineurin inhibitor levels, anti-rejection treatments particularly with anti-lymphocyte agents and steroid pulses are known to perturb antiviral immune control with lowered CMV-specific T-cell responses (Sester, Gartner et al. 2005; Lilleri, Zelini et al. 2007) and subsequent CMV replication (Dickenmann, Cathomas et al. 2001; Dickenmann, Kabulbayev et al. 2004). With this limitation in mind, CMV pp65-specific CD4+ T-cells might serve as a dynamic marker of protection for patients on stable immunosuppression complementing CMV load diagnostics in centers using a preemptive strategy.

We found 3 R+ patients (6.3%) with GCV resistance. Those were the first described cases in literature. We concluded that GCV resistant CMV replication in R+ may be an underestimated problem and determination of CMV-specific T-cells could help to identify patients at risk. This may be helpful to guide treatment duration of prophylactic or preemptive strategy, where decision on stopping of treatment may be difficult.

5.1.2. Cytomegalovirus-associated chorioretinitis after liver transplantation: case report and review of the literature (Egli, Bergamin et al. 2008)

In some HIV patients, despite of effective antiviral suppression of CMV after initiation of HAART worsening of CMV chorioretinitis is observed, This paradoxical clinical course is known as immune reconstitution inflammatory syndrome (IRS) and was linked to recovering CD4+ T-cell counts after effectively inhibiting HIV replication (Karavellas, Lowder et al. 1998; Hirsch, Kaufmann et al. 2004). Activation, expansion, and persistence of specific T-cell clones are postulated as the patho-mechanism.

We were confronted with the case of a 51-year old CMV D+R- liver transplanted women with primary CMV replication and development of GCV-resistant CMV replication and progression to CMV colitis. Due to persistent GCV-resistant CMV replication, immunosuppression was significantly lowered and lymphocytes increased rapidly. Three weeks after successful viral clearance, the patient suddenly developed chorioretinitis. We wondered whether this could be due to an immune reconstitution syndrome and CMV-specific T-cell associated collateral damage.

Clinical examination of both eyes showed IRS-like chorioretinitis patterns. Still, 8 months after CMV clearance, we could detect an astonishing high amount of CMV-pp72 specific CD8+ T-cells. This high response slowly decreased over the next year of follow-up (**Figure 21**). We hypothesized that during CMV replication pp72-specific CD8+ T-cells clonally expanded and were weakly controlled.

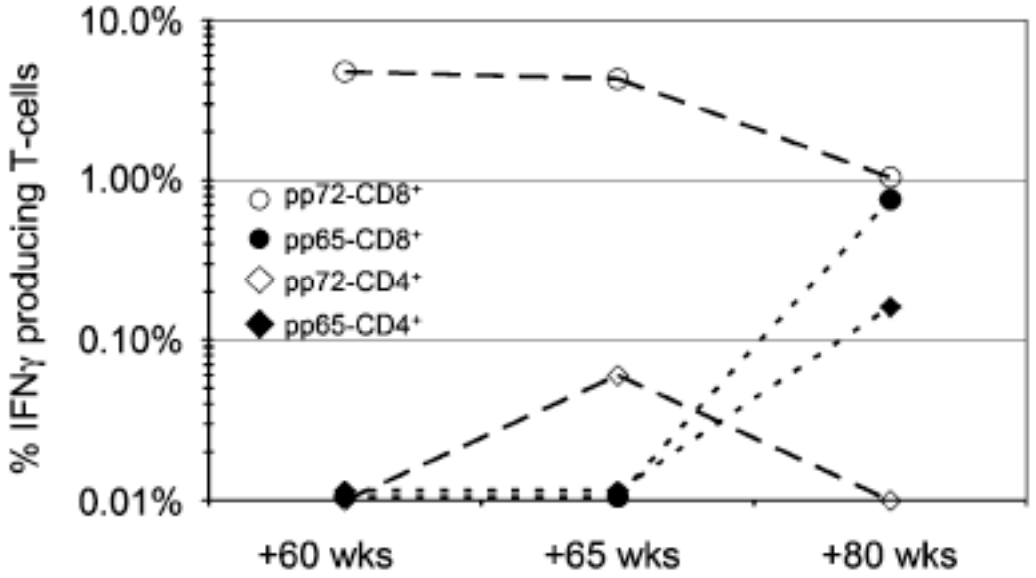


Figure 21. Follow-up of CDM-specific immune responses posttransplant.

We wondered, how often CMV-associated chorioretinitis occurs in post-liver transplantation and reviewed all PubMed accessible papers from 1987 to 2007. In 12'653 liver transplant recipients included, CMV-replication was reported in 25.6% at 6.7±4.4 (mean, SD) weeks, CMV-syndrome in 2.1% at 10.4±7.1 weeks, and organ invasive disease in 10.6% at 8.8±6.8 weeks post transplant, respectively (see publication Table 1). The leading entities were CMV hepatitis, pneumonitis, and enteritis. Instead, CMV-chorioretinitis was reported in only 14 patients (0.1%) and diagnosed at a mean of 101±127 weeks (median 41.7 weeks, range 8-338) post transplant (see publication Table 2). Chorioretinitis occurred substantially later than other CMV diseases such as colitis or pneumonitis. Time of occurrence and course suggested that two patho-physiologic mechanisms might be operative. The first pattern had an earlier onset with considerable damage at the retina suggesting a strong virus-mediated cytopathic component and seemed to respond better to intravenous GCV. The second pattern was seen at later time points when immunosuppression is typically lower and responses to intravenous GCV seemed not as successful.

So far, only for fungal infection IRS-like disease has been described in solid organ transplant recipients (Singh, Lortholary et al. 2005; Singh, Lortholary et al. 2005). We concluded that after significant reduction of immunosuppression

and unexpected CMV-like disease in the absence of viral replication IRS should also be considered in SOT patients.

5.1.3. A HCMV pp65 polypeptide promotes the expansion of CD4+ and CD8+ T-cells across a wide range of HLA specificities (Provenzano, Sais et al. 2008)

As noted before, CMV-pp65 specific T-cell response seems to be valuable as marker to assess the risk for CMV replication. Identification of immuno-dominant epitopes within CMV-pp65 may be helpful to improve diagnostic tests and further identify important virus regions for vaccine development.

M. Provenzano and S. Spagnoli (university of Basel, Switzerland) computer predicted with Syfpeithi algorithm a 16mer sequence of pp65 with potential to stimulate various HLA types (RQYDPVAALFFFDIDL). Spagnoli's group tested the peptide in healthy individuals. Our group aimed to examine the potential immune stimulating role in kidney transplant recipients. In total, we examined 10 kidney transplant recipients (8 R+, 1 D-R- and 1 D+R- with primary infection).

We expanded separately CD4+ and CD8+ RQY-specific T-cells with peptide pulsed DC over 3 weeks (see material and methods, and appendix).

We could show, that the RQY 16mer was recognized by most kidney transplanted patients. Even more, RQY could stimulate both CD4+ and CD8+ T-cells. Single 9mer epitopes were identified for particular HLA types with showing a specific INFγ and IL2 production (See publication Table 3 A and B). We concluded, that RQY probably could be used as a immuno-dominant sequence together with other immuno-dominant peptides to improve current monitoring assays or to stimulate immune responses in adoptive T-cell transfer or vaccine development.

5.1.4. PD1-expression on CMV specific T-cell response and influence on cytokine profiles (unpublished results, Egli, Sester et Hirsch)

Co-stimulatory signals are highly important to induce a antigen-specific T-cell response. Activating or inhibitory signals further modulate the immune response (introduction and **Figure 3**). Recently, co-stimulatory molecules became targets of immunosuppressive agents (introduction and **Figure 7**., (Sharpe and Abbas 2006; Vincenti and Kirk 2008)). However, a CD28-blocking antibody showed unexpected stimulatory effects in a phase 1 trial with severe side-effects (Suntharalingam, Perry et al. 2006).
The role of inhibiting co-stimulatory effects on the function of virus-specific T-cells is a fascinating and yet weakly examined field of research. In latent virus infection the up-regulation of PD1 as an inhibitor of T-cell function seems to be an important escape pathway for viruses. During several viral infections such as Hepatitis B, LCMV, and HIV, PD1 was up-regulated on CD8+ virus-specific T-cells and were associated with impaired T-cell function and even worse disease outcome (Barber, Wherry et al. 2006; Day, Kaufmann et al. 2006; Urbani, Amadei et al. 2006). Blocking PD1 could theoretically lead to a restoration of T-cell function and probably clearance of virus replication and even elimination of latent infected cells.

Sester et al. examined the effect of PD1-expression on IL2 and INFγ production of CMV-specific CD4+ T-cells in kidney transplant. Blockade of the PD1 molecule was induced with binding PD1 ligand to an inhibitory antibody. Beside, increasing INFγ and IL2 production of CMV-specific CD4+ T-cells, also a significant increase in cell-proliferation capacities was observed (Sester, Presser et al. 2008).

We have extensively characterized the amount of INFγ and to some extend also IL2 production of CMV-specific T-cells. We wondered, what the role of other cytokines in kidney transplant patients is. Additionally, as cytokine profiling could help to further evaluate PD1 blockade as a potential anti-viral target, we decided to examine supernatant from T-cell expansion cultures and compare cytokine profile in PD1 blocked and non-blocked samples.

Supernatants of short-term expanded T-cell cultures stimulated with CMV lysate (for 5 days) or SEB (for 3 days) were harvested by Sester et al. (University of Saarland, Homburg, Germany). Baseline PD1 expression level was defined as either high or low, depending on the FACS mean florescence signal of the PD1 staining (Sester, Presser et al. 2008).
In total we examined three healthy blood donors, four kidney transplant recipients with high PD1 expression and five with low PD1 expression. During expansion an inhibitory antibody binding to PD1-ligand and thereby blocking PD1 signalling and an isotype control without effect on PD1-ligand were added. We measured the amount of cytokines in the cell supernatant with a luminex technology. This method allows detecting 27 different cytokines per 50uL of supernatant

First we compared the amount of cytokines produced by different antigen preparations. SEB strongly induced cytokine production with significant higher amounts compared to CMV-lysate. However, IL-1ra, -8, -9, -10, -13, -17, IP10, INFγ, MIP1b, Rantes, and TNFα responded to CMV lysate.
Then, we examined the general increase of cytokine production in different patient groups. Overall, the baseline production of cytokines (isotype treated samples) from CMV-specific T-cells was higher for healthy blood donors compared to kidney transplant patients (**Figure 23**).

On the other, we observed a higher increase of almost all cytokine in kidney transplant patients with PD1 blockade. Cytokines such as, IL4, IL6, IL17, INFγ MCP1, MIP-1a/-1b and TNFα increased by more than 5 fold compared to non treated cell cultures. Even more, kidney transplant recipients with high PD1 expression showed an even stronger fold increase of cytokine release (See **Figure 22**) compared to low PD1 expressing patients.

We conclude that various cytokines may be stimulated with CMV lysate and SEB. PD1 inhibitory antibodies lead to an unselective increase of T-cell function, but interestingly only in CMV-specific T-cells. As a potential treatment for persistent virus, especially CMV replication in a transplant

setting, this needs to be very carefully evaluated, due to potential of graft rejection induction.

The weakness of the so far done study certainly is the low number of persons examined. However, as kidney transplant recipients are very heterogeneous, further studies could be extremely expensive. Therefore we would based on the current results, focus on the key cytokines.

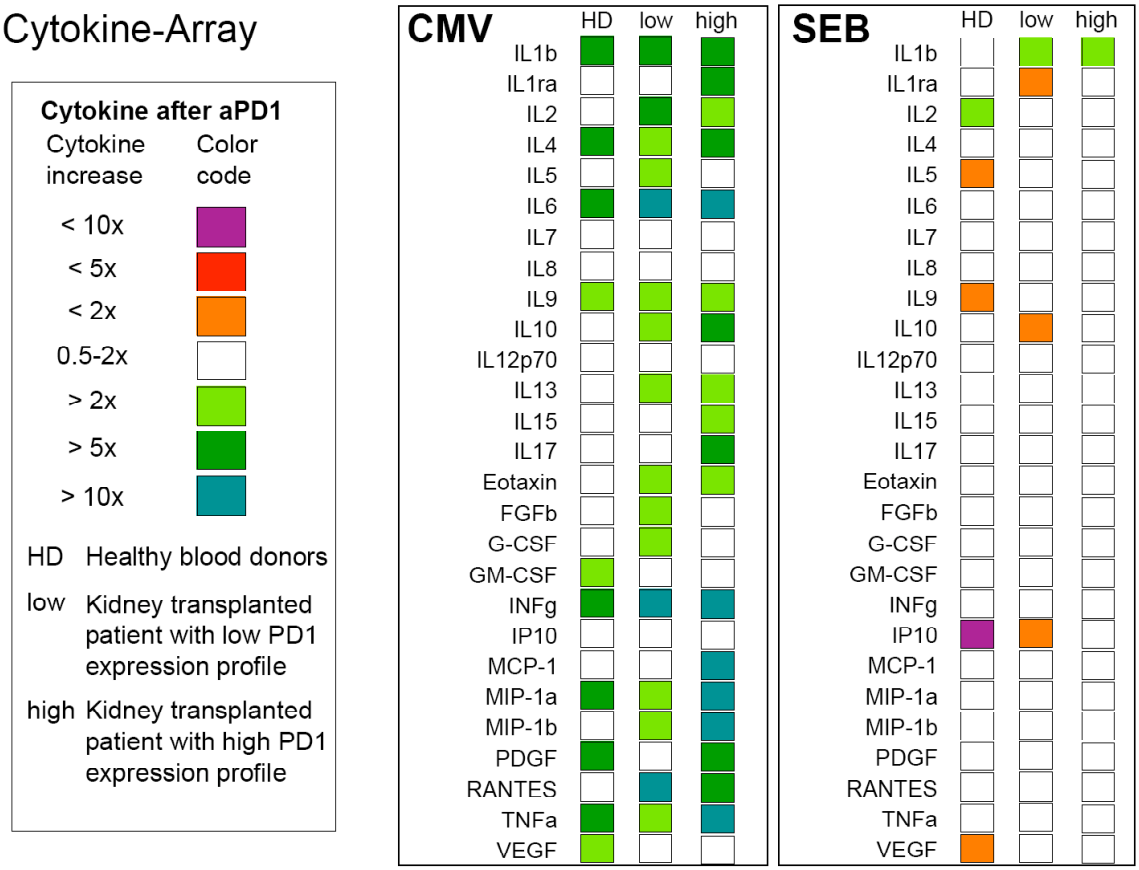
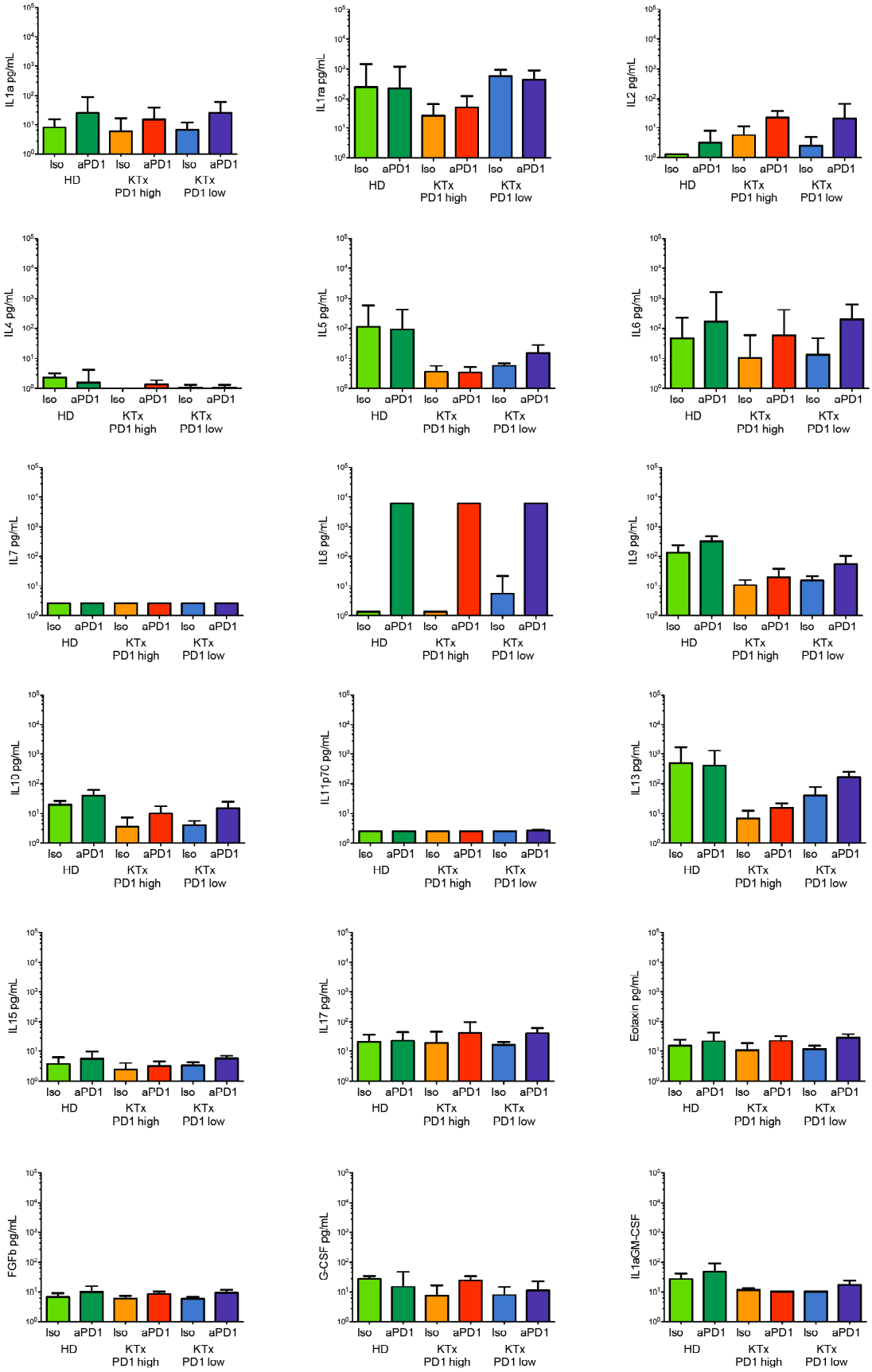


Figure 22. Cytokine array. Colour indicates the fold increase or decrease after treatment with blocking PD1-ligand antibody. From bright green to blue increasing cytokine levels were measured. From orange to purple decreasing cytokine levels were measured.


IL1a pg/mL
IL1ra pg/mL
IL2 pg/mL
IL4 pg/mL
IL5 pg/mL
IL6 pg/mL
IL7 pg/mL
IL8 pg/mL
IL9 pg/mL
IL10 pg/mL
IL11p70 pg/mL
IL13 pg/mL
IL15 pg/mL
IL17 pg/mL
Eotaxin pg/mL
FGFb pg/mL
G-CSF pg/mL
IL1aGM-CSF
Iso aPD1 Iso aPD1 Iso aPD1
HD
KTx PD1 high
KTx PD1 low

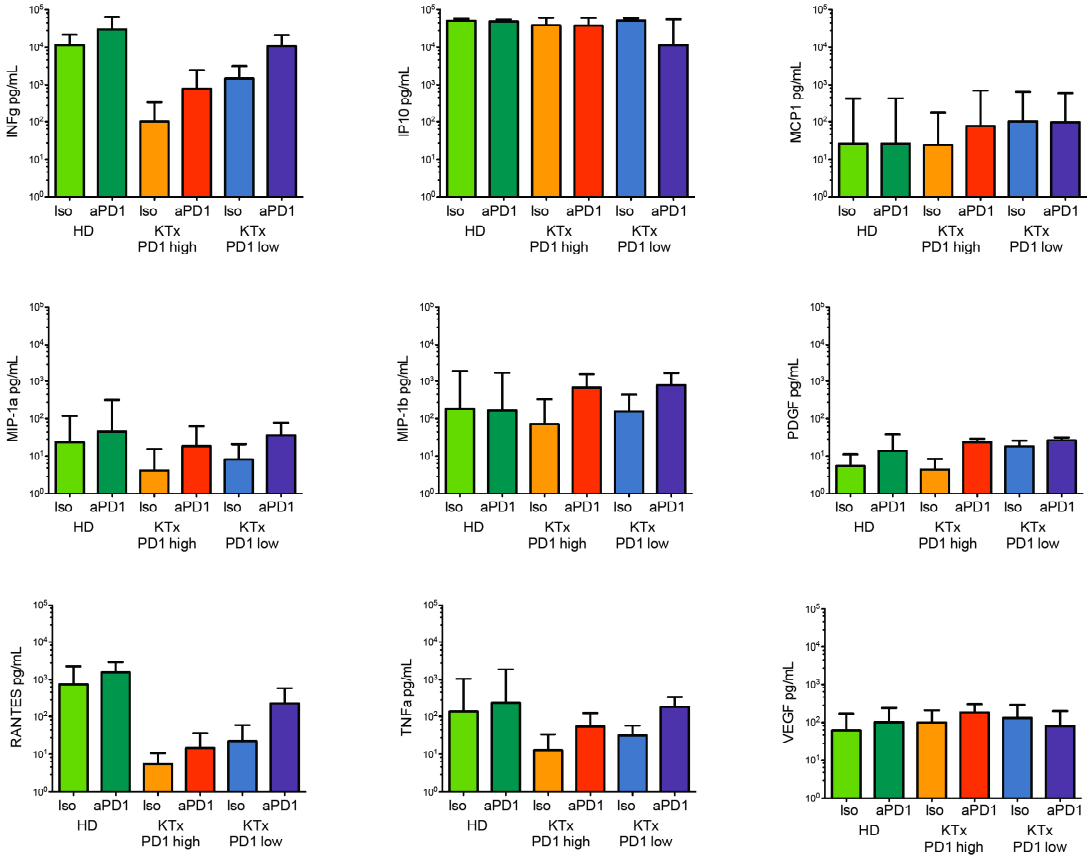


Figure 23. Cytokine profile. healthy blood donors (HD), green; kidney transplant recipients (KTx) with high PD1 expression, red; KTx with low PD1 expression, blue. Darker colour shaded bars indicate treatment with anti-PD1-ligand antibody.

5.1.5. Impact of immunosuppression on CMV-pp65 and BKV-LT specific T-cells (unpublished results, Egli, Köhli et Hirsch)

Prolonged and high levels of immunosuppression un-specifically down-modulates the immune system's function. Allo-reactive as well as virus-specific T-cells are impaired in effector function as e.g. INFγ release or proliferation capacity (see introduction and **Figure 7**). Dosage of immunosuppression is based on clinical observations and measurement in plasma of through levels. Sester et al. showed, the impact of Calcineurin inhibitors on CMV-lysate specific T-cells. However, in their studies only calcineurin inhibitors were tested and the stimulating antigen was a whole virus-lysate from fibroblast cells (Sester, Gartner et al. 2005).

We wondered, what the impact of calineurin inhibitors (CsA and Tac) and anti-proliferative drugs (Rapa and Lef) on CMV-pp65 specific T-cells and BKV-LT specific T-cells may be.

PBMCs from healthy blood donors were isolated and pre-incubated for 3h with respective immunosuppressives at different concentrations. Treated cells were added to the antigen solutions (SEB, CMV-pp65 and BK-LT) in a standard Elispot or FACS assay overnight. The amount of virus-specific T-cells on different dosages was assessed as T-cell reactivity. The value of INFγ releasing T-cells without immunosuppressive treatment was set as 100%.

We first tested the direct effect of Calcineurin inhibitors (Tac and CsA) on SEB, CMV-pp65 and BKV-LT stimulated PBMCs. As shown in **Figure 24**, a significant dose dependent inhibition on virus-specific T-cell reactivity (INFγ production) could be observed for SEB, CMV-pp65 and BKV-LT.

For anti proliferative agents, Rapamycin and Leflunomide no effect was observed for SEB and CMV-pp65. However, for BKV LT we found a dose-dependent decrease (**Figure 25**). This effect could have been due to higher variance in a lower amount of BKV-specific T-cells compared to CMV-pp65 specific T-cells or to a toxic effect of DMSO, which was the dilution medium for BKV LT.

Previously, we tested the effect of DMSO on the production of INFγ. Above 3% we found an inhibitory effect (data not shown). The concentration used in the Rapamycin and Leflunomide assays was always below 1%. To reduce assay variance due to a low amount of BKV LT specific T-cells, we used BKV-LT expanded T-cells. With expanded BKV-specific T-cells no effect of Rapamycin and Leflunomide was found (**Figure 26.**).
expansion

We then tested the effect of rapamycin during expansion. A standard ActMono expansion was performed and during time of monocyte pulsing different concentrations of rapamycin were present in the 24well plate. After

9days, we observed a yellow medium only in wells with low rapamycin concentration, which indicates higher rate of cell division. Elispot assay was used to detect the amount of INFγ, IL2 and TNFα producing BKV-specific T-cells (**Figure 27**). The amount of INFγ releasing BKV-specific T-cells expressed in SFUs/1Mio PBMCs could be reduced to half at 8ng/mL. With the same concentration IL2 decreased to 10%. This indicates a certain hierarchy among cytokines. The strong decrease of IL2 producing T-cells may have an impact on the CD8+ T-cell population as well.

We confirmed that calcineurin inhibitors directly affect the INFγ release of CMV and BKV-specific T-cells. For CsA, a slight stronger decrease of INFγ release in BKV-LT-specific T-cells could be observed compared to CMV-pp65 specific T-cells. Additionally, in the direct essay, we observed a rapamycin-specific INFγ blockade of BKV LT-specific T-cells, but most probably due to a combined toxic effect. Rapamycin and Leflunomid, as antiproliferative substances, show in expanded T-cells no effect on INFγ release, but a clear effect in proliferation capacity over time.

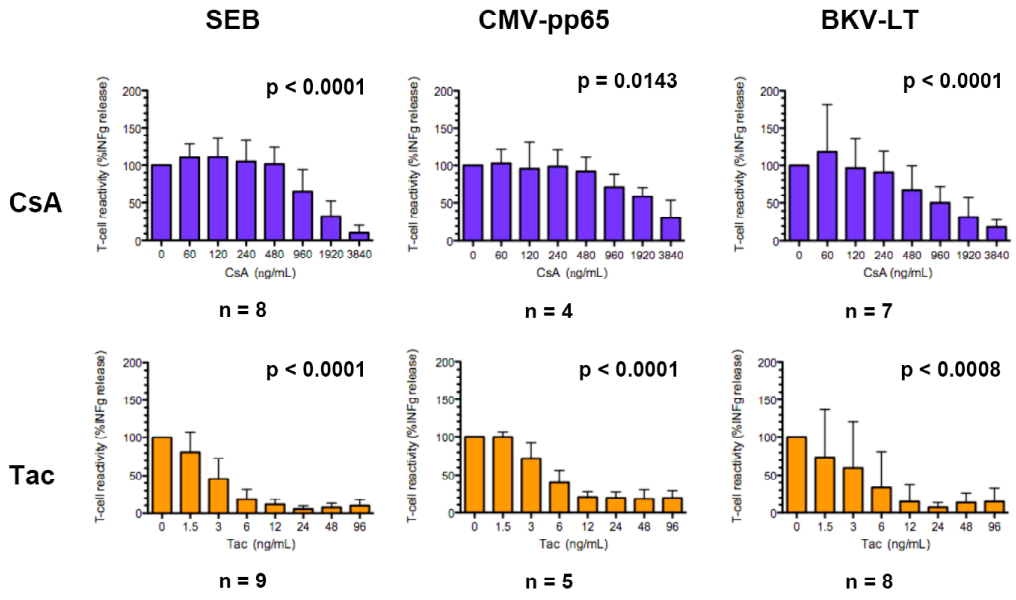


Figure 24. Dose dependant effect of calcineurin inhibitors on SEB, CMV-pp65 and BKV-LT stimulated T-cells.

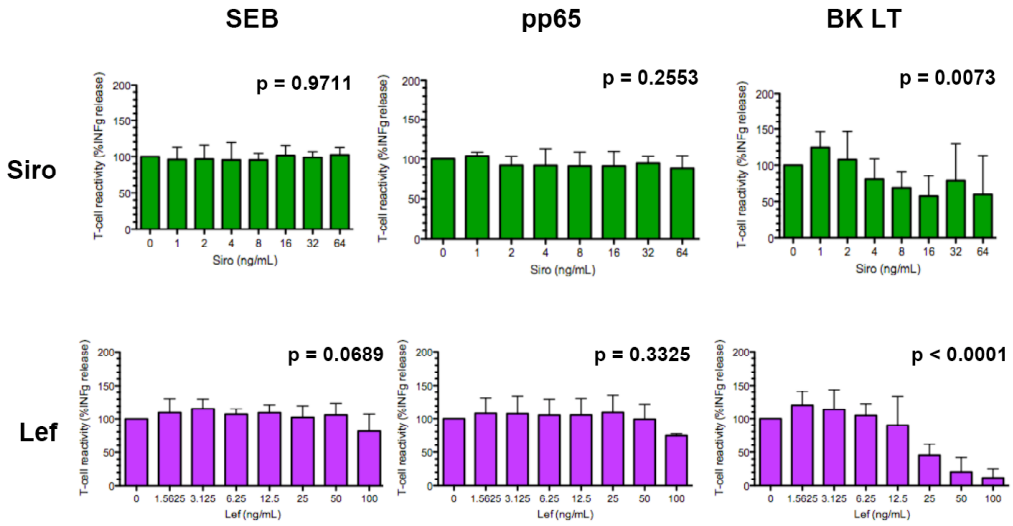


Figure 25. No dose dependant effect of rapamycin or leflunomide on SEB and CMV-pp65, but dose dependence on BKV-LT stimulated T-cell

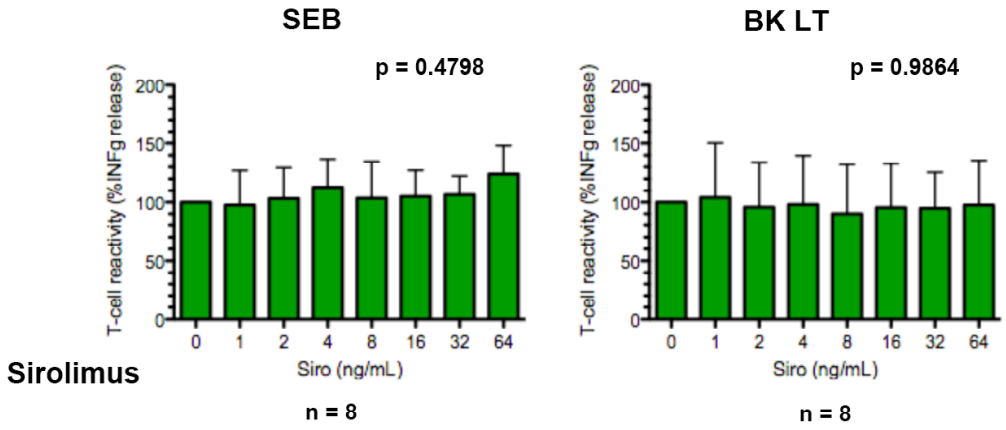


Figure 26. No dose dependent effect of rapamycin after BKV-LT specific

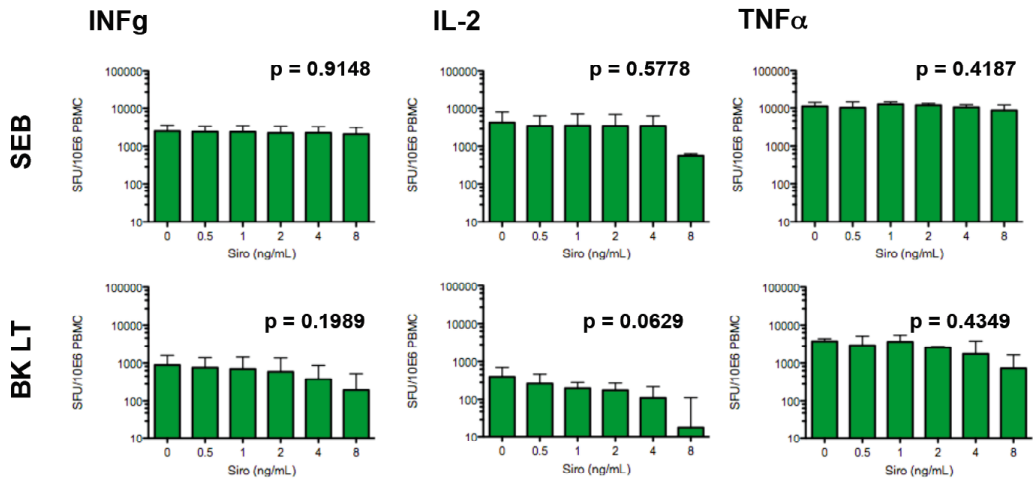


Figure 27. No significant dose dependent effect on amount of cytokine producing BKV-specific T-cells treated with rapamycin during expansion.

Our laboratory examines the impact of rapamycin on BKV replication in infected RPTEC cells. A strong inhibitory effect with reduction by more than 1 log could already be observed at 1ng/mL (M. Wernli, and Hans H. Hirsch unpublished results). As BKV-specific T-cell function is reduced at higher levels, this could have a high clinical impact. If confirmed with further experiments and in clinical studies, Rapamycin might become a choice of treatment of kidney transplant recipients with progressive and prolonged BKV replication.

Guiding immunosuppressive therapy with levels of immunosuppressive drugs according to the risk of virus replication and rejection episodes is not sufficient (Sampson, Dunn et al. 2008). However measuring virus-specific T-cells could be more successful. Using the amount of virus-specific T-cells to measure the impact of immunosuppression requires knowledge of the particular through level. However, only a small fraction is unbound in blood. CsA has only 2% in a unbound fraction, whereas the rest is bound to proteins in blood or distributed in tissues (Akhlaghi and Trull 2002). This means that a higher concentration of immunosuppression may be present at various sites of virus replication. Even so during tissue inflammation the drug metabolism and thereby concentration may additionally change (Pellequer, Weissenborn et al. 2007). Further studies should clearify the impact of tissue concentrations on T-cell functions.

5.2 Polyomavirus BK

Similar to CMV, control of BKV replication requires a sufficient BKV-specific cellular immune control through T-cells (Comoli, Basso et al. 2003; Chen, Trofe et al. 2006; Comoli, Binggeli et al. 2006; Hammer, Brestrich et al. 2006; Binggeli, Egli et al. 2007; Ginevri, Azzi et al. 2007; Egli, Dumoulin et al. 2008, in press). Reduction of BKV-specific T-cells may lead to uncontrolled and progressed BKV replication in kidney transplant recipients causing

Polyomavirus-associated nephropathy. As no specific antiviral treatment is available, the only treatment option is reduction of immunosuppression to regain BKV-specific effector T-cells. However, this harbour a significant risk for induction of graft rejection (Hirsch and Steiger 2003).

5.2.1. Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors (Egli, Infanti et al. 2008)

Polyomavirus BK and JC primary infections happen in early age and latency in kidney is established there after (Knowles, Pipkin et al. 2003; Stolt, Sasnauskas et al. 2003). In healthy donors and immunosuppressed patients, the virus may reactivate and intermittent shedding in urine is observed. Studies on humoral factors and virus replication would give detailed insight in mechanisms of viral transmission and risk for reactivation and disease. Nevertheless, most studies examined humoral and shedding aspects only in transplant patients or weakly defined and questionable immunocompetent individuals. Our aim was to set the baseline characteristics of BKV and JCV serology and viral replication in healthy blood donors.

We consecutively enrolled 400 healthy blood donors from the blood donation center Basel (SRK, Swiss Red Cross) from February 2007 to January 2008. Participants were enrolled into four age decades of 100 persons each consisting of 50 females and 50 males (age 20-29, 30-39, 40-49, and 50-59 years, total 200 male and 200 females). IgM and IgG activity against BKV and JCV-VLP were determined as described before (Leuenberger, Andresen et al. 2007). Standard PCR was used to determine the BKV and JCV loads (Hirsch, Knowles et al. 2002).

Overall BKV seroprevalence and antibody activity was significant higher than JCV seroprevalence and antibody activity (82% vs. 57.7%, 0.6288OD vs 0.4439OD) (**Figure 28A**). In contrast, viral shedding was more common for JCV and also with higher viral loads compared to BKV (18.8% vs. 7%, 4.644 logs c/mL vs. 3.51 logs c/mL) (**Figure 28A**). Viral rearrangements of the non-coding control region occur rarely (BKV 0%, JCV <1%). This supports our hypothesis of accumulation of rr-NCCR in immunosuppression (see

introduction). No virus was detected in plasma. The role of virus detection in PBMC was controversial (Dolei, Pietropaolo et al. 2000; Ling, Lednicky et al. 2003).

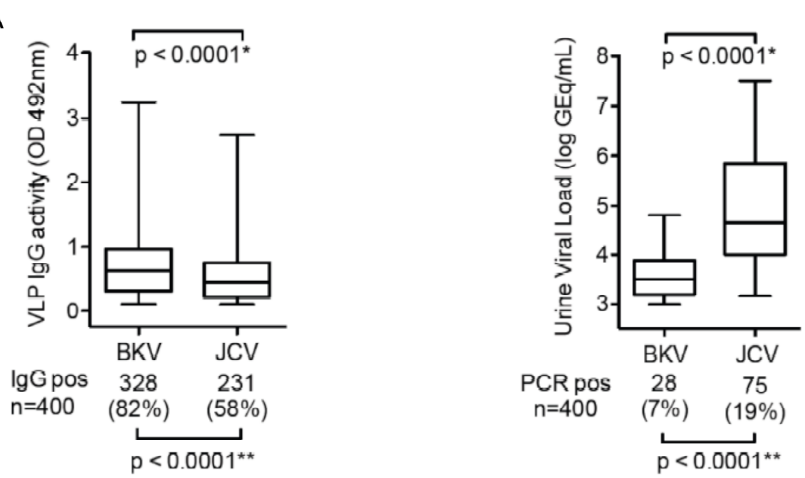
Assuming an average individual is producing one litre of urine a day containing 2'500 c/mL of BKV and 100'000 c/mL of JCV. In a large city with 1'000'000 inhabitants for example Zurich in Switzerland, $2.5x10^{11}$ copies of BKV and $2x10^{13}$ copies of JCV would be shed daily. Indeed, human polyomaviruses can be found in various environmental compartments (Bofill-Mas, Pina et al. 2000; Bofill-Mas, Formiga-Cruz et al. 2001; Bofill-Mas and Girones 2003). Due to the ubiquitary presence of those viruses, they could be used as marker for water quality control or to study human population history by analysing genome alterations (Sugimoto, Hasegawa et al. 2002; Ikegaya, Zheng et al. 2005).

Female showed higher BKV IgG activities than men. Men more often shed both viruses, however viral loads were comparable. With increasing age, we observed a significant decrease of BKV seropositive individuals with significant BKV IgG activity. For JCV a strong opposite trend was found.

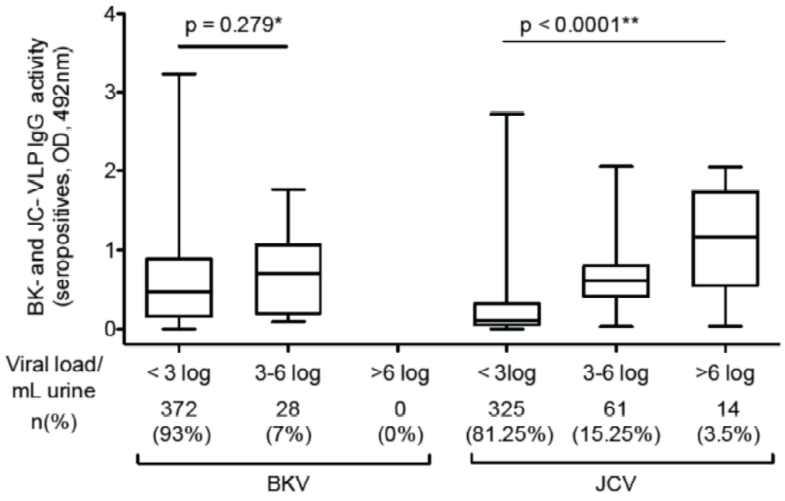
A correlation of JC-VLP IgG activity and JCV loads in urine was found, whereas a trend was observed for BKV (**Figure 28B**). We concluded that IgG activity may serve as a marker for recent antigen exposure and serological immune response may not protect from virus shedding. This was also observed in kidney transplant recipients (Randhawa 2006; Ginevri, Basso et al. 2007; Leuenberger, Andresen et al. 2007). BKV and JCV sero-double positive individuals showed significant less JCV replication in urine compared to JCV single sero positive. This may indicate a cross-protective effect of BKV-specific immunity on JCV replication. For the cellular BKV-specific immune response cross recognition was shown for peptide pools and single epitopes (Egli, Dumoulin et al. 2008, in press).

BKV specific IgG activity was significant lower in BKV non-serotype 1 shedding individuals. This indicates the importance for serotype-specific humoral assays (**Figure 28C**).

A


VLP IgG activity (OD 492nm)
p < 0.0001*
BKV
JCV
IgG pos
n=400
328
(82%)
231
(58%)
p < 0.0001**
Urine Viral Load (log GEq/mL)
p < 0.0001*
BKV
JCV
PCR pos
n=400
28
(7%)
75
(19%)
p < 0.0001**

B


BK- and JC- VLP IgG activity
(seropositives, OD, 492nm)
p = 0.279*
p < 0.0001**
Viral load/
mL urine
n(%)
< 3 log
3-6 log
>6 log
< 3log
3-6 log
>6 log
372
(93%)
28
(7%)
0
(0%)
325
(81.25%)
61
(15.25%)
14
(3.5%)
BKV
JCV

C

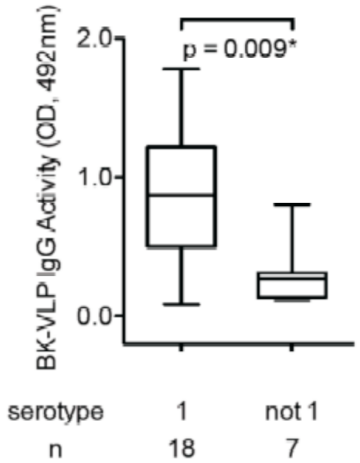

BK-VLP IgG Activity (OD, 492nm)
p = 0.009*
serotype
n
1
18
not 1
7

Figure 28. BKV and JCV serologic IgG activities and BKV and JCV replication in urine is shown (A). Correlation of BKV and JCV activities and virus replication detected in urine is shown (B). Comparison of BKV IgG activities within the individuals shedding serotype 1 or non-serotype 1.

5.2.2. Polyomavirus BK-specific cellular immune response to VP1 and Large T-Antigen in Kidney Transplant Recipients (Binggeli, Egli et al. 2007)

Measurement of BKV-specific T-cells could allow to better profile the risk of kidney transplant patients to replicate BKV and develop PVAN.

We aimed to compare healthy individuals to kidney transplant patients with increasing or decreasing BKV plasma loads. We wanted to compare as well BKV-VP1 and LT peptide pools, and the corresponding potential to expand T-cells in culture. Finally, we were interested in examinations concerning the cross-reactive potential between BKV and JCV.

In total, 10 healthy blood donors without BKV detection in urine or plasma, and 42 kidney transplant recipients (characteristics in publication Table 1) were examined in a cross-sectional study design.
Healthy blood donors compared to kidney transplant recipients showed lower amounts of BKV-VP1 and -LT specific T-cells. This might be due to recent virus reactivation. Interestingly, the immune response in kidney transplant patients and especially against BKV-VP1 is widely scattered (**Figure 29**). The population of kidney transplant recipients is heterogeneous according basic diseases, amount of BKV loads, immunosuppression, etc. Especially for VP1, as postulated, infection with other serotypes or JCV might influence the immune response. Additionally, at the time-point of this study, we were not able to determine BKV serology. As 20% are sero negative, this might add to the scattered immune response.

For CMV, a difference of virus proteins to stimulate either CD4+ or CD8+ T-cells has been observed. We wondered, if the same is true for BKV. In a direct FACS analysis, in healthy individuals normally no BKV-specific T-cells

can be detected due to the sensitivity of the assay (data not shown). However, in transplant patients with recent reactivation, we observed a higher VP1-specific CD4+ T-cell response compared to CD8+ T-cells (**Figure 30**).

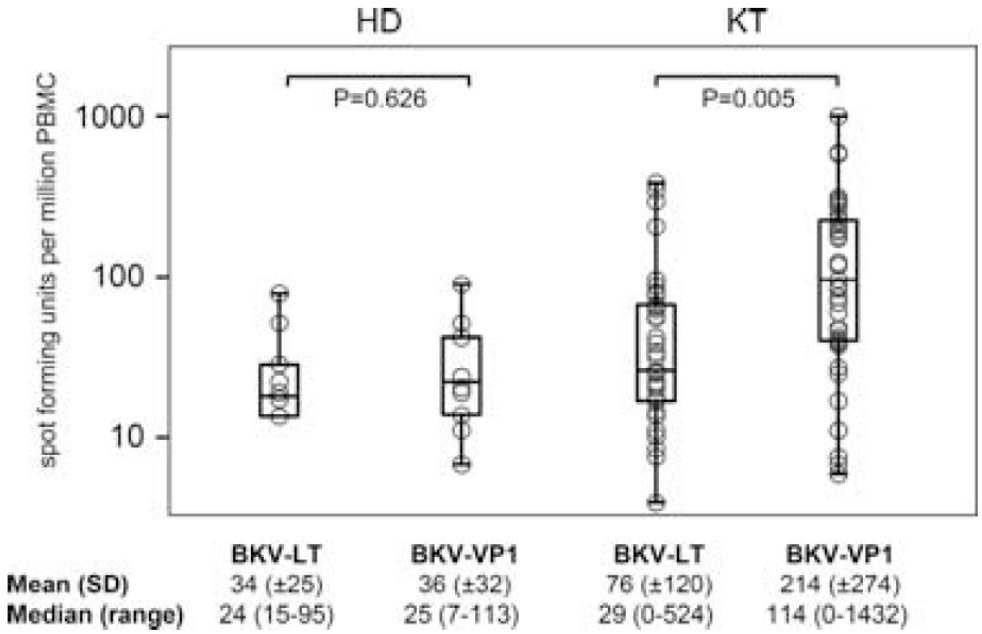


Figure 29. BKV VP1 and LT specific T-cell responses in healthy blood donors and kidney transplant recipients.

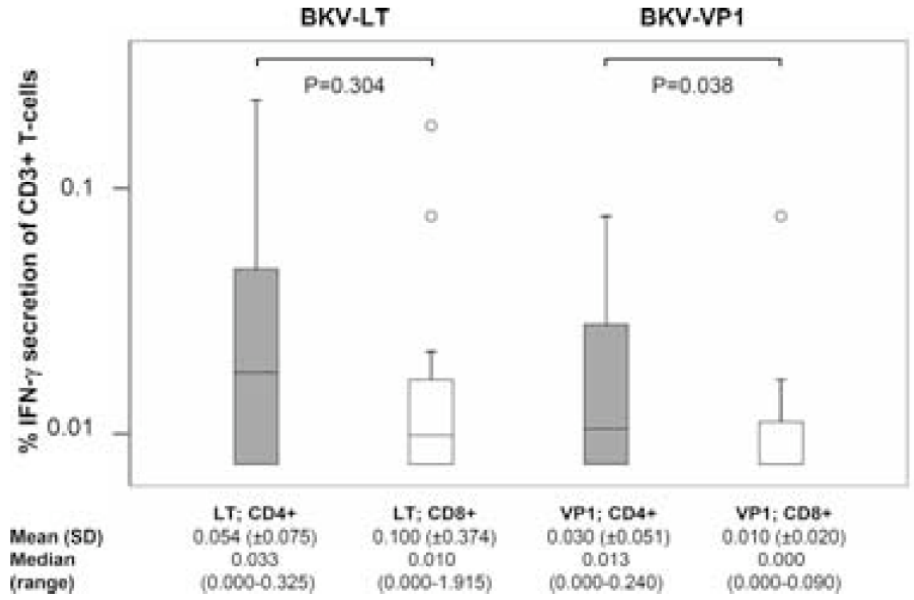


Figure 30. FACS analysis of BKV-specific CD4+ and CD8+T-cells in kidney transplant recipients

We grouped the patients according to their viral load dynamics. A change by more than 1.5 logs within 2 weeks was assessed to be significant. Patients with increasing or high viral loads showed significant lower BKV-specific T-cells compared to patients with decreasing or cleared viral loads (LT: 22 vs. 78 SFU/1Mio PBMC; VP1: 53 vs. 285 SFU/1Mio PBMC (**Figure 31**)).

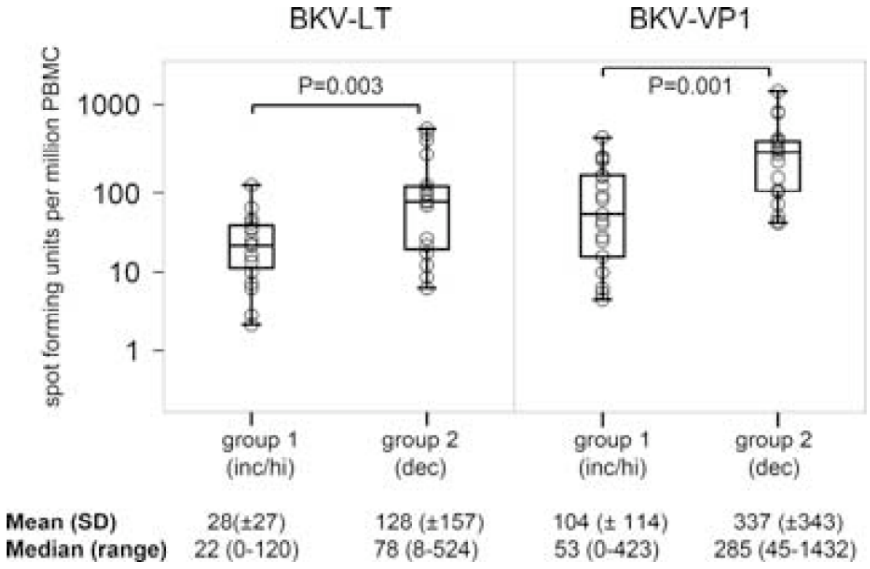


Figure 31. Comparison of patients with increasing (group 1) or decreasing (group 2) viral loads according to BKV LT and VP1 specific immune response. Interestingly, patients with a BKV LT specific immune response above 69 SFU/1Mio PBMCs were significantly more often associated with decreasing BKV loads. A similar cut-off for VP1 could not be calculated. Suggesting that, BKV LT specific T-cell response more be more valuable as monitoring marker.

We then checked the expansion potential of BKV-specific T-cells for 9days with ActMono protocol. The amount of expanded cells was compared to pre-expansion amount. Both patient groups, with increasing and decreasing viral loads, showed a significant increase of BKV-specific immune responses in culture. This indicates, the presence of BKV-specific T-cells, probably due to

higher immunosuppression no sufficient immune response could be mounted in the direct assay. Interestingly re-challenging after 9 days showed that LT-specific T-cell might have a higher expansion capacity. (Binggeli, Egli et al. 2007) (**Figure 32**). Ginevri et al. examined in a prospective study the BKV-specific immune response at different BKV-associated events, such as increasing, decreasing, or clearing BKV load in plasma. Interestingly, Ginevri et al. found no significant difference according INFγ production between BKV-LT and VP1, however a significant stronger killing activity was seen for BKV-LT specific T-cells. In their protocol used, BKV-specific T-cells were expanded before testing this could be an explanation for the difference to our results. During expansion probably CD8+ T-cells, which favour BKV LT show a higher killing activity. Whereas, BKV-VP1 stimulated T-cells as well to produce INFγ, however, more CD4+ T-cells without killing activity (Ginevri, Basso et al. 2007). The role of BKV-specific CD4 T-cells with killing activities remains controversial (Zhou, Sharma et al. 2007).

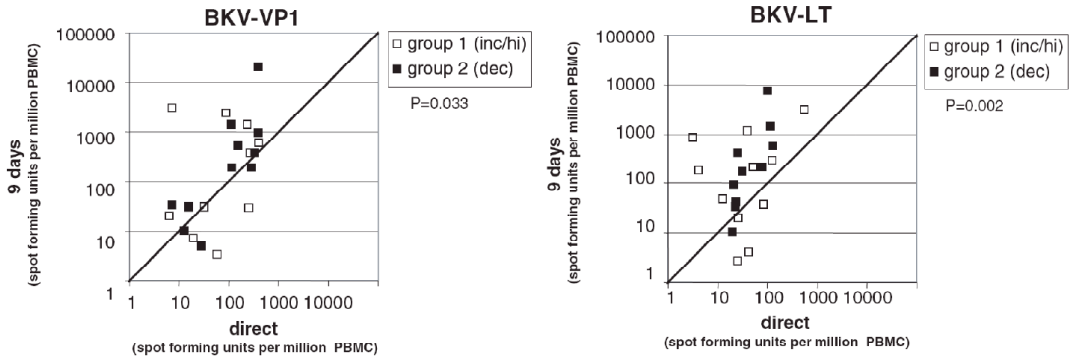


Figure 32. Direct Elispot versus 9day expanded BKV-specific T-cell cultures.

In this study we have shown, that BKV LT and VP1 can be used to monitor BKV-specific immune responses. BKV LT specific immune response seems to be better correlated with protection from BKV replication. The amount of response is also associated with BKV load dynamics and might be used to predict future viral loads.

5.2.3. BKV epitop mapping (unpublished results, Egli et Hirsch)

Epitope mapping is the identification of immunodominant regions (epitopes) within a larger antigen-sequence. The identified epitopes (9 or 15mer) are presented via MHC molecules in a strict HLA background to the T-cells (see introduction). Knowledge of the BKV epitopes will help to further improve our immunological BKV monitoring assays. Probably identified BKV epitopes could be used in a vaccine.

Most vaccines nowadays in use were developed based on conventional techniques as for example repeated passages in various tissue cultures to reduce virulence (Ellis 2004). For the BKV large T protein, a safety issue may concern the biological activity of SV40-LT and its potential role in cancer development. Therefore, we avoid using the whole BKV-LT protein in a vaccine. Epitope mapping may reduce the sequence necessary to a minimum.

Two methods of epitope mapping exist: (i) Computer algorithms to predict potential epitopes and (ii) experimental screening of peptide sub-pools with in vitro stimulation and detection of immuno dominant sub-pools and/or single peptide with INFγ detection assays.

We have decided not only predicting single eptiope for single HLA types, as mostly HLA A0201 were identified so far, but focused to screen the whole LT protein for several HLA types. Cluster regions, covering more HLA types and inducing a CD4+ and CD8+ T-cell response would be of great value.

Computer prediction

Several computer prediction algorithms may be used to simulate in silico single or combined parts of the processing (see Introduction and **Table 5**).

Name	Description	URL
Antjien	Binding to MHC ligands, TCR-MHC complexes, T cell epitopes, TAP	http://www.jenner.ac.uk/antijen/

BIMAS	Predicts half-time of dissociation to HLA class I molecules	http://www-bimas.cit.nih.gov
CEP	Conformational Eptiope Prediction Server	http://202.41.70.74:8080/cgi-bin/cep.pl
EpiMatrix		http://tbhiv.biomed.brown.edu//
EpiMHC	Peptides that bind to MHC molecules	http://bio.dfci.harvard.edu/epimhc/
Epipredict	Predicts HLA-class II restricted T-cell epitopes	http://www.epipredict.de/index.html
IEBD	Integrating TAP, proteosomal cleavage, MHC	http://www.immuneepitope.org
JenPrep	Immunological protein-peptide interactions	http://www.jenner.ac.uk/JenPep/
MAPPP	Possible antigenic peptides to be processed and finally presented on cell surfaces	http://www.mpiib-berlin.mpg.de/MAPPP/expertquery.html
MHCBN	MHC binding, non-binding peptides, epitopes	
MIF antigenicity	Predicts antigenic sites in proteins	http://immunax.dfci.harvard.
NetChop	Cleavage sites of the human proteasome	http://www.cbs.dtu.dk/services/NetChop
PapRoc	Proteasomal cleavages	http://www.paproc.de/
PreDep	Genomic, transcriptomics and proteomic data	http://margalit.huji.ac.il/
ProPred	Predicts MHC Class-II binding regions	http://www.imtech.res.in/raghava/mhcbn/
Superficial	Identification of potential epitopes or binding sites	http://bioinformatics.charite.de/superficial/
Syfpeithi	T-cell epitope and MHC ligand publication	http://www.syfpeithi.de

Table 5. List of computer prediction algorithms

For computer prediction, we decided to use a combination of proteasome cleavage, TAP processing and MHC class I loading. IEBD database (www.immuneepitope.org, (Peters, Sidney et al. 2005)) fulfilled these criteria. MHC II binding prediction was also calculated with IEBD database. Syfpeithi algorithm was used as a comparison to IEBD (www.syfpeithi.de). However, Syfpeithi only predicts the binding affinity between an amino acid sequence and a corresponding MHC molecule, which could be a disadvantage of this algorithm.

As the HLA type is absolutely essential for the MHC presentation of immuno-dominant peptide sequences, first, the HLA distribution of Europe, Basel, and the United states was analysed with the NCBI database (http://www.ncbi.nlm.nih.gov/gv/mhc) (**Figure 33**). For those HLA types above 5% in population, the MHC I epitopes predicted would be of interest.

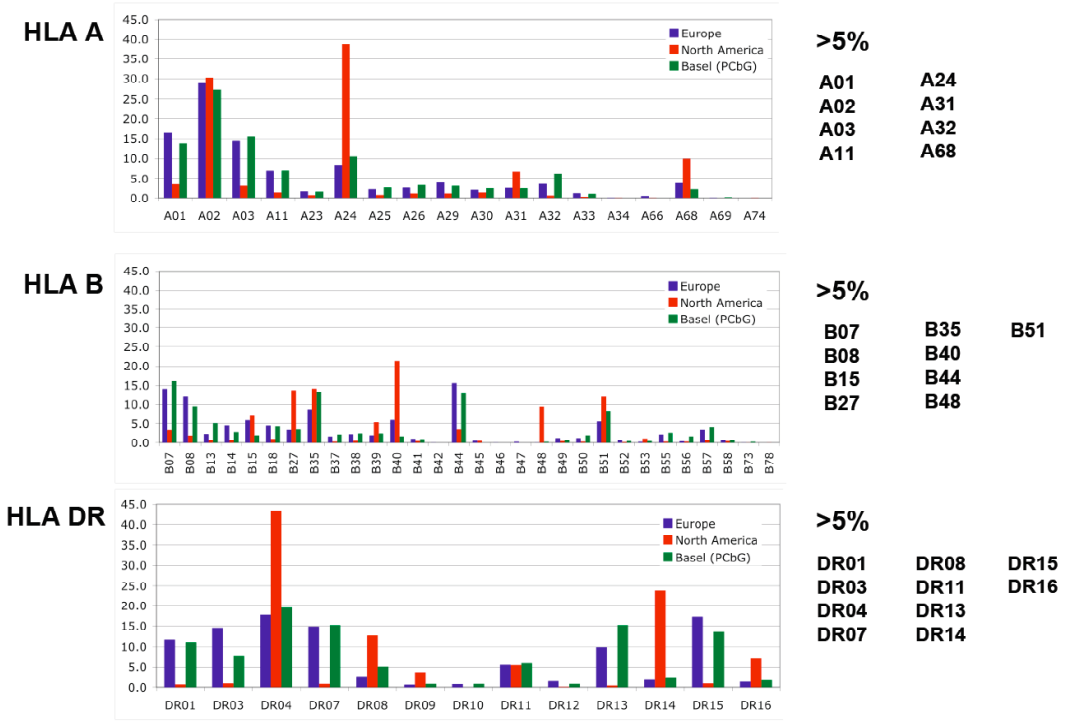


Figure 33. HLA distribution in Europe (blue), North American (red) and Basel, Switzerland (green).

BKV LT protein prediction

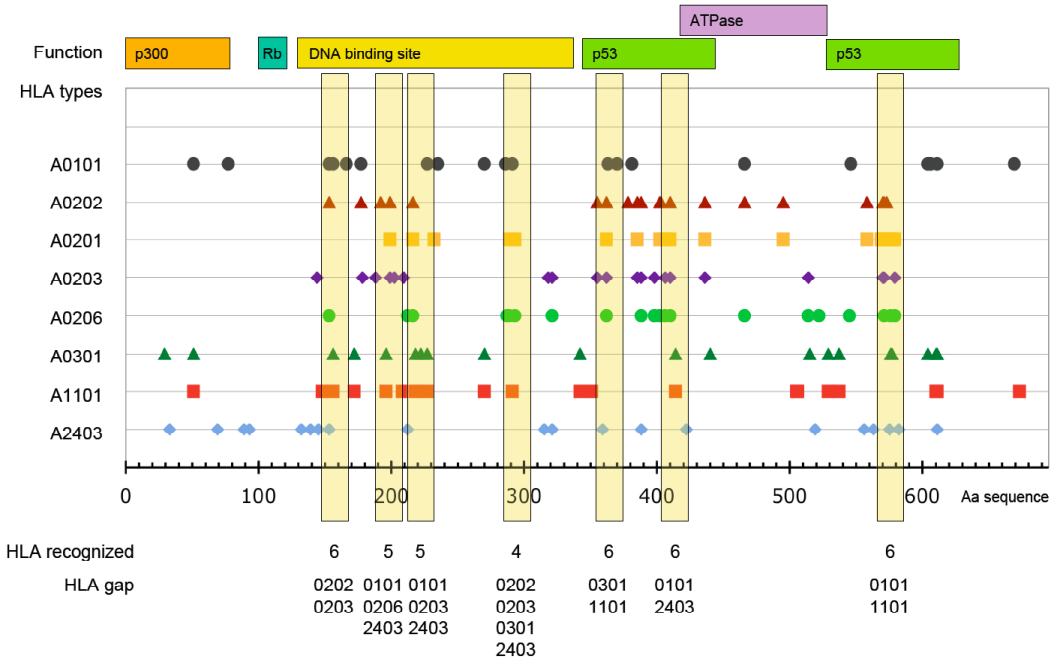
The amino acid sequence of BKV LT (695aa) was entered into the algorithm software and the HLA type of interest was chosen. The algorithm gives several scores to compare the identified epitopes with each other. We focused on the best 20 predicted epitopes with an integrative overall score (combination of proteosomal cleavage, Tap processing and MHC I binding). IEBD MHCII prediction is based only on MHC II binding affinity. Additionally, we also predicted the best 20 epitopes with the Syfpeithi algorithm. Syfpeithi as well is only based on MHC I binding affinity.

The position of the first amino acid of a predicted epitope was entered into a excel file and a overlay of all identified epitopes for all possible HLA combinations was performed to find clusters of epitopes (See **Figure 24 A-F** for method). For 14 HLA A, 14 HLA B and 10 HLA DQ types the BK LT amino acid sequence was entered and the top 20 epitopes were considered for further analysis.

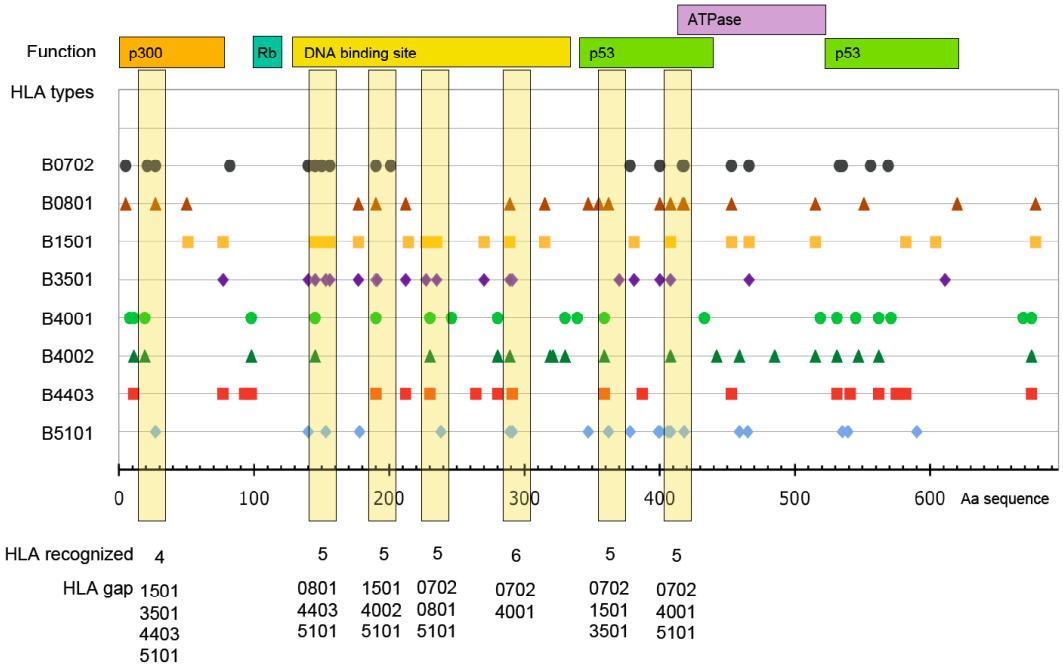
Then the clusters were aligned to find super clusters within HLA A, B or DR. Super clusters were defined as a region, where an epitope sequence recognizing more than 4 different HLA types. These regions covers a high variance of HLA types in the population and would be of value for monitoring and vaccine-development.

A slight difference between Syfpeithi and IEBD prediction was found, especially when single amino acid sequences within a cluster of interest was compared. This might be of relevance when single tetramers are design for testing epitopes. However, over 80% of the predicted epitopes were identical by both algorithms (see **Figure 34**). Most predicted clusters lay within the DNA binding site and the p53-binding region. Rb and ATPase activity site was spared.

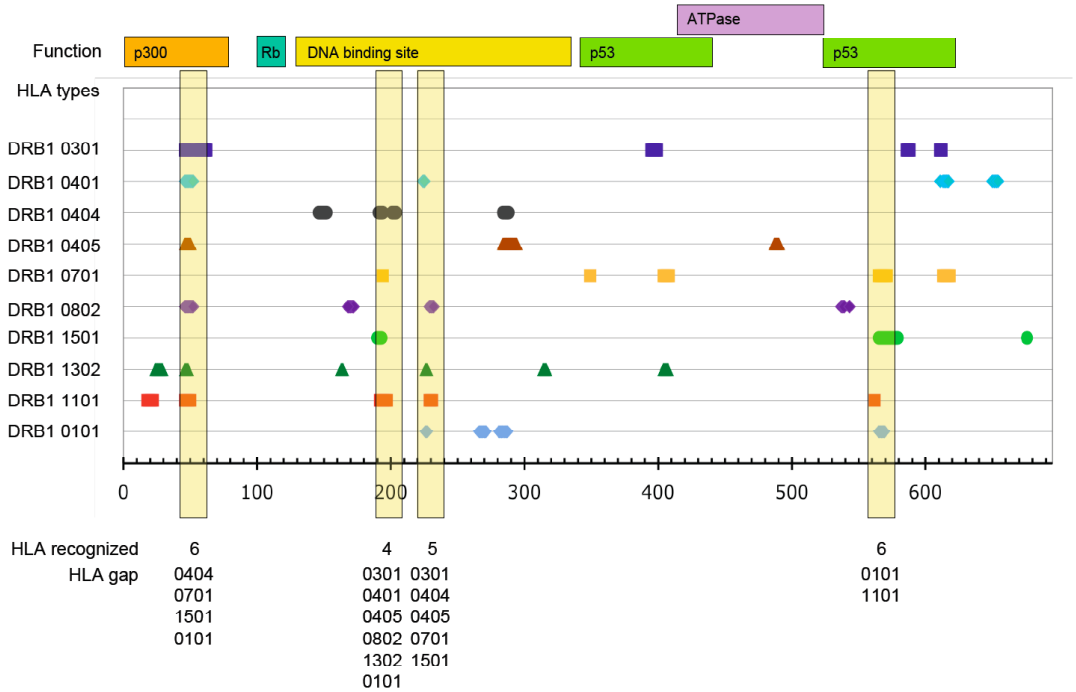
HLA A, IEBD (Proteasome cleavage, TAP & MHC I loading)



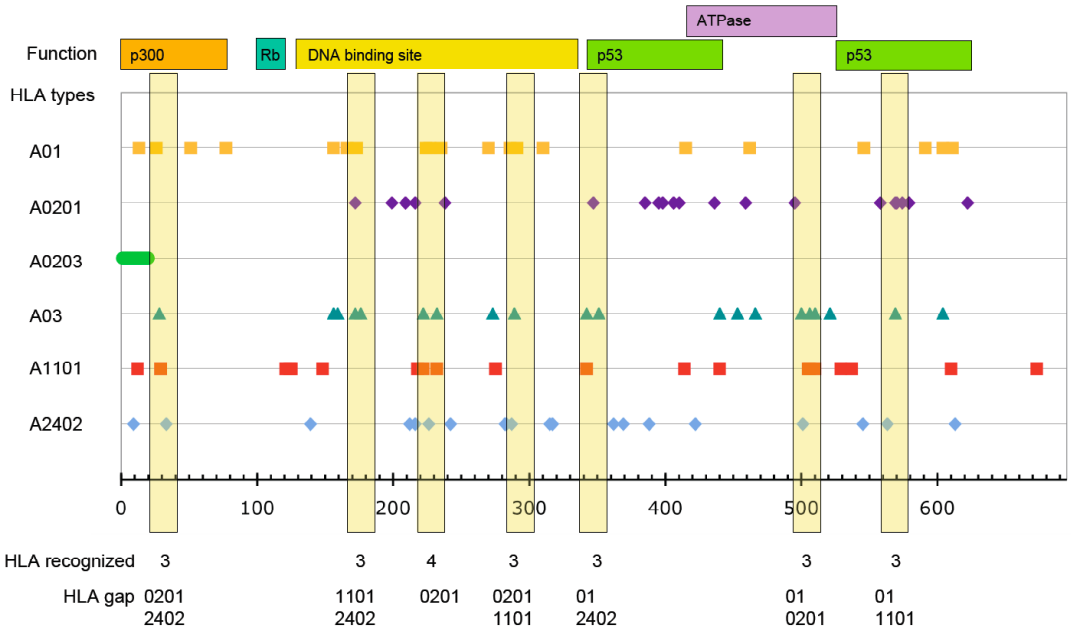
HLA B, IEBD (Proteasome cleavage, TAP & MHC I loading)



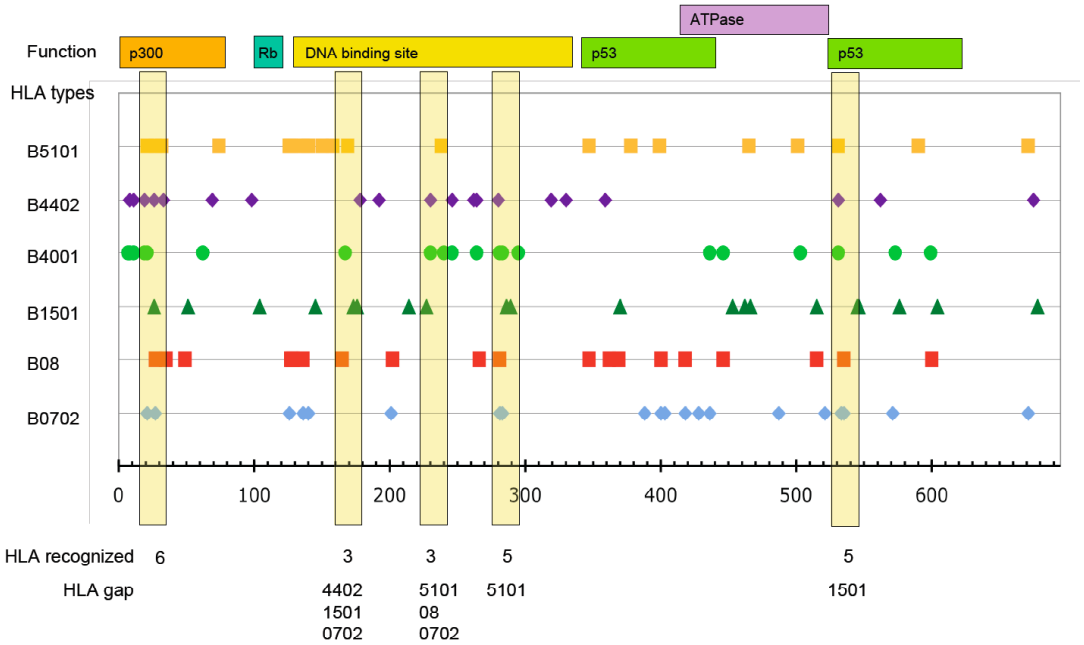
HLA DRB1, IEBD (MHC II loading)



HLA A, Syfpeithi (MHC I loading)



HLA B, Syfpeithi (MHC II loading)



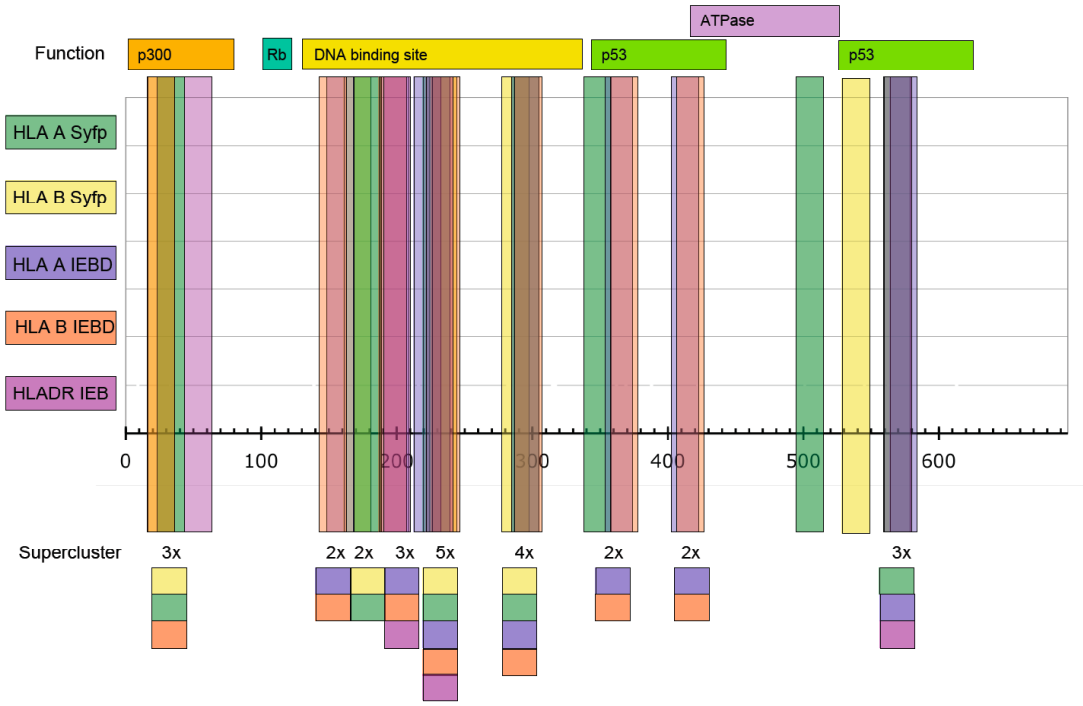


Figure 34. Prediceted epitopes for HLA A, B and DR with IEBD and Syfpeithi are shown. Last graphic shows an overlay analysis to identify super cluster regions.

As longer sequences are not practical for production and harbour a certain danger on biological activities, we identified 15mer and 25mer amino acid sequences within super-cluster regions. Within the cluster-15 and 25mer we checked the amount of 9mer sequences representing particular single epitopes of HLA A and B. 15mer and longer corresponding 25-26meres were ordered for experimental verification of immune stimulation potential (see **Table 6**). **Table 7** shows the predicted 9mers and recognized single HLA types, which we would use in a future checkerboard attempt. **Table 8** shows potential tetramer peptides for single HLA types represented in the cluster regions identified.

Position (aa)	Sequence	length	region
010 to 033	LERAAWGNLPLMRKA	15aa	p300
026 to 040	NLPLMRKAYLRKCKE	15aa	p300

145 to 159	HQFLSQAVFSNRTLA	15aa	DNA binding site
153 to 167	FSNRTLACFAVYTTK	15aa	DNA binding site
172 to 186	ILYKKLMEKYSVTFI	15aa	DNA binding site
190 to 204	MCAGHNIIFFLTPHR	15aa	DNA binding site
212 to 226	NFCQKLCTFSFLICK	15aa	DNA binding site
222 to 235	FLICKGVNKEYLLYS	15aa	DNA binding site
232 to 246	YLLYSALTRDPYHTI	15aa	DNA binding site
281 to 295	ETKCEDVFLLLGMYL	15aa	DNA binding site
287 to 301	VFLLLGMYLEFQYNV	15aa	DNA binding site
357 to 371	MTREEMLTERFNHIL	15aa	p53 binding site
404 to 418	DSVIFDFLHCIVFNV	15aa	p53 binding site
565 to 579	LLEKRILQSGMTLLL	15aa	p53 binding site
573 to 587	SGMTLLLLLIWFRPV	15aa	p53 binding site
019 to 043	LERAAWGNLPLMRKAYLRKCKEFHP	25aa	p300
145 to 167	HQFLSQAVFSNRTLACFAVYTTKEK	25aa	DNA binding site
162 to 186	AVYTTKEKAQILYKKLMEKYSVTFI	25aa	DNA binding site
188 to 212	RHMCAGHNIIFFLTPHRHRVSAINN	25aa	DNA binding site
212 to 237	NFCQKLCTFSFLICKGVNKEYLLYSAL	27aa	DNA binding site
227 to 250	GVNKEYLLYSALTRDPYHTIEESI	24aa	DNA binding site
280 to 304	VETKCEDVFLLLGMYLEFQYNVEEC	25aa	DNA binding site
355 to 378	LHMTREEMLTERFNHILDKMDLIF	24aa	p53 binding site
402 to 426	KMDSVIFDFLHCIVFNVPKRRYWLF	25aa	p53 binding site
565 to 590	LLEKRILQSGMTLLLLLIWFRPVADF	26aa	p53 binding site

Table 6. Predicted superclusters, which cover different HLA types. Position within BKV LT protein, peptide length and function region is indicated

Position	Sequence	Length	HLA Type predicted	Algorithm	Region
19	LERAAWGNL	9aa	B4401	Syf	p300
21	RAAWGNLPL	9aa	B0702, B5101, B4001	Syf, IEBD	p300
26	NLPLMRKAY	9aa	A01, B4402, B1501	Syf	p300
27	LPLMRKAYL	9aa	B5101, B08, B0702, B0801, B0801, B5101	Syf, IEBD	p300
28	PLMRKAYLR	9aa	A03	Syf	p300
29	LMRKAYLRK	9aa	A0301, A1101	Syf, IEBD	p300
32	KAYLRKCKE	9aa	B5101	Syf	p300
33	AYLRKCKEF	9aa	A2403, A2402,B4402	Syf, IEBD	p300
145	HQFLSQAVF	9aa	A2403, B1501,B4002, B4001, B3501, B1501, B0702	Syf, IEBD	DNA
148	LSQAVFSNR	9aa	A1101	Syf, IEBD	DNA
150	QAVFSNRTL	9aa	B5101, B0702	Syf, IEBD	DNA
156	RTLACFAVY	9aa	A0301, A0101, A1101, A03, B3501, B1501	Syf, IEBD	DNA
158	LACFAVYTT	9aa	B5101	Syf	DNA
159	ACFAVYTTK	9aa	A03	Syf	DNA

165	TTKEKAQIL	9aa	B08	Syf	DNA
166	TKEKAQILY	9aa	A0101, A01	Syf, IEBD	DNA
167	KEKAQILYK	9aa	none		DNA
169	KAQILYKKL	9aa	B5101	Syf	DNA
172	ILYKKLMEK	9aa	A1101, A0301, A0201, A03	Syf, IEBD	DNA
173	LYKKLMEKY	9aa	A01, B1501	Syf, IEBD	DNA
176	KLMEKYSVT	9aa	A03	Syf	DNA
177	LMEKYSVTF	9aa	A0202, A0101,B3501, B1501, B0801	Syf, IEBD	DNA
178	MEKYSVTFI	9aa	B4402, B5101	Syf, IEBD	DNA
188	RHMCAGHNI	9aa	A0203	IEBD	DNA
192	AGHNIIFFL	9aa	A0202	IEBD	DNA
190	MCAGHNIIF	9aa	B4403, B4001. B3501, B0801	IEBD	DNA
191	CAGHNIIFF	9aa	B3501	IEBD	DNA
196	IIFFLTPHR	9aa	A1101, A0301	IEBD	DNA
199	FLTPHRHRV	9aa	A0201, A0202, A0203	Syf, IEBD	DNA
201	TPHRHRVSA	9aa	B0702	IEBD	DNA
202	PHRHRVSAI	9aa	A0203, B08	Syf, IEBD	DNA
212	NFCQKLCTF	9aa	A0206, A2403, B4403, B0801, B3501	IEBD	DNA
214	CQKLCTFSF	9aa	B1501	Syf, IEBD	DNA
216	KLCTFSFLI	9aa	A0201, A0202, A0206, A2402	Syf, IEBD	DNA
218	CTFSFLICK	9aa	A0301, A1101	Syf, IEBD	DNA
222	FLICKGVNK	9aa	A0301, A03, A1101	Syf, IEBD	DNA
224	ICKGVNKEY	9aa	A01	Syf	DNA
226	KGVNKEYLL	9aa	A2402	Syf	DNA
227	GVNKEYLLY	9aa	A0301, A1101, A01, B1501, B3501	Syf, IEBD	DNA
230	KEYLLYSAL	9aa	B4001, B4002, B4402, B4403	Syf, IEBD	DNA
232	YLLYSALTR	9aa	A0201, A03, A1101	Syf, IEBD	DNA
235	YSALTRDPY	9aa	A01, A0101, B3501	Syf, IEBD	DNA
238	LTRDPYHTI	9aa	B5101	Syf, IEBD	DNA
242	PYHTIEESI	9aa	A2402	Syf	DNA
282	TKCEDVFLL	9aa	A2402	Syf	DNA
283	KCEDVFLLL	9aa	A2402, B0702, B4001	Syf	DNA
286	DVFLLLGMY	9aa	A01, A0101, B1501	Syf, IEBD	DNA
287	VFLLLGMYL	9aa	A0206, A2402	Syf, IEBD	DNA
289	LLLGMYLEF	9aa	A0201, A03, B1501, B5101, B4002, B0801, B3501	Syf, IEBD	DNA
291	LGMYLEFQY	9aa	A1101, A01, A0101, B5101, B4403, B3501	Syf, IEBD	DNA
293	MYLEFQYNV	9aa	A1101, A0201	IEBD	DNA
355	LHMTREEML	9aa	A0203, B0801	IEBD	p53
359	REEMLTERF	9aa	A2403, B4403, B4002, B4001, B4402	Syf, IEBD	p53
362	MLTERFNHI	9aa	A0201, A0202, A0203, A2402, B0801, B08	Syf, IEBD	p53
369	HILDKMDLI	9aa	A2402, B08	Syf, IEBD	p53
402	KMDSVIFDF	9aa	A0201, A0202	IEBD	p53

403	MDSVIFDFL	9aa	A0202, B0702	Syf, IEBD	p53
406	VIFSFLHCI	9aa	A0203, A0206, A0201, B5101	Syf, IEBD	p53
410	FLHCIVFNV	9aa	A0201, A0203, A0202	Syf, IEBD	p53
408	FDFLHCIVF	9aa	B5101, B4002, B1501, B0801, B3501	Syf, IEBD	p53
414	IVFNVPKRR	9aa	A1101	Syf, IEBD	p53
415	VFNVPKRRY	9aa	none		p53
417	NVPKRRYWL	9aa	B0801, B0702	IEBD	p53
418	VPKRRYWLF	9aa	B08. B5101, B0702	Syf, IEBD	p53
569	RILQSGMTL	9aa	A0201	Syf, IEBD	p53
570	ILQSGMTLL	9aa	A0201, A0203, A0202	Syf, IEBD	p53
571	LQSGMTLLL	9aa	A0206, A0201, A0202, B4001	Syf, IEBD	p53
573	SGMTLLLLL	9aa	A0202, B4001	Syf, IEBD	p53
574	GMTLLLLLI	9aa	A0201	Syf, IEBD	p53
575	MTLLLLLIW	9aa	A2403, B4403	IEBD	p53
576	TLLLLLIWF	9aa	A0301, A0206, A0201, B4403	IEBD	p53
577	LLLLLIWFR	9aa	A0201, A0301	IEBD	p53
579	LLLIWFRPV	9aa	A0201, A0203, A0206	Syf, IEBD	p53
582	IWFRPVADF	9aa	A2403, B4403, B1501, B2403	Syf, IEBD	p53

Table 7. Identified 9mers with corresponding predicted HLA types. In red already published epitopes. Prediction method and functional region is indicated.

A0101	RATING	A0201	RATING	A0301	RATING
NLPLMRKAY	BB**	ILYKKLMEK	AA	PLMRKAYLR	AA
RTLACFAVY	AAA	FLTPHRHRV	AAA	LMRKAYLRK	BB**
TKEKAQILY	AAA	KLCTFSFLI	AA	RTLACFAVY	AA
LYKKLMEKY	BB**	YLLYSALTR	BB	ACFAVYTTK	AA
LMEKYSVTF	BBB	LLLGMYLEF	BB	ILYKKLMEK	AAA
ICKGVNKEY	BB**	MYLEFQYNV	BB	KLMEKYSVT	AA
GVNKEYLLY	BB**	MLTERFNHI	AA	IIFFLTPHR	BB**
YSALTRDPY	AA	KMDSVIFDF	BBB	CTFSFLICK	BBB
DVFLLLGMY	BB**	VIFSFLHCI	AAA *	FLICKGVNK	AAA
LGMYLEFQY	BB**	FLHCIVFNV	AAA *	GVNKEYLLY	BB**
		RILQSGMTL	AA	YLLYSALTR	AAA
		ILQSGMTLL	AAA *	LLLGMYLEF	AA
		LQSGMTLLL	BB	TLLLLLIWF	BB**
		GMTLLLLLI	AA	LLLLLIWFR	BB**
		TLLLLLIWF	BB		
		LLLLLIWFR	BB		

	LLLIWFRPV AAA ***	

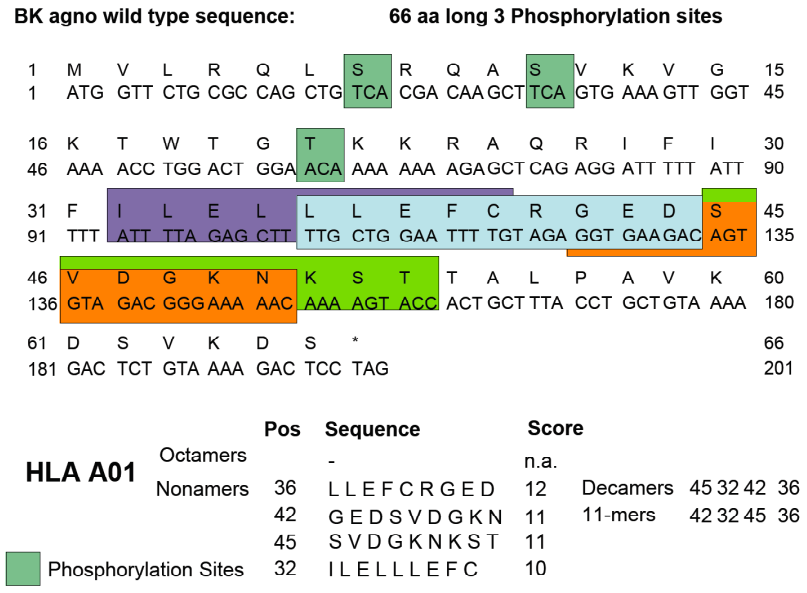
A1101	RATING	A2402	RATING	B0702	RATING
LMRKAYLRK	AAA	AYLRKCKEF	AAA	RAAWGNLPL	BBB
LSQAVFSNR	AA	HQFLSQAVF	BBB	HQFLSQAVF	BBB
RTLACFAVY	BBB	KLCTFSFLI	BB**	QAVFSNRTL	BBB
ILYKKLMEK	AAA	KGVNKEYLL	BB**	TPHRHRVSA	AA
IIFFLTPHR	AA	PYHTIEESI	AA	KCEDVFLLL	BBB
CTFSFLICK	AAA	TKCEDVFLL	BB**	NVPKRRYWL	BBB
FLICKGVNK	AAA	KCEDVFLLL	BB**	VPKRRYWLF	AA **
GVNKEYLLY	BBB	VFLLLGMYL	AA		
YLLYSALTR	AA	REEMLTERF	BBB		
LGMYLEFQY	BBB	MLTERFNHI	BB**		
MYLEFQYNV	BBB	HILDKMDLI	BB**		
IVFNVPKRR	AA	MTLLLLLIW	BBB		
		IWFRPVADF	BBB		

B0801	RATING	B3501	RATING	B4001	RATING
LPLMRKAYL	AAA	HQFLSQAVF	BB**	RAAWGNLPL	BBB
TTKEKAQIL	AAA	RTLACFAVY	BB**	HQFLSQAVF	BBB
LMEKYSVTF	BBB	LMEKYSVTF	BB**	MCAGHNIIF	BBB
MCAGHNIIF	BBB	MCAGHNIIF	BB**	KCEDVFLLL	BBB
PHRHRVSAI	AA	CAGHNIIFF	BB**	REEMLTERF	BBB
NFCQKLCTF	BB	NFCQKLCTF	BB**	LQSGMTLLL	BBB
LLLGMYLEF	BB	GVNKEYLLY	BB**	SGMTLLLLL	BBB
LHMTREEML	BB	YSALTRDPY	BB**		
MLTERFNHI	AA	LLLGMYLEF	BB**		
HILDKMDLI	AA	LGMYLEFQY	BB**		
FDFLHCIVF	BBB	FDFLHCIVF	BB**		
NVPKRRYWL	AA				
VPKRRYWLF	AAA				

Table 8. Potential tetramer epitopes for single HLA types represented in cluster regions. Rating allows to decide on the particular binding affinity on a company algorithm (Proimmune, England, done by Sara Allwood.) AAA, highest probability to bind; AA, fair good probability; BB, could be tried; BBB, will not bind. ** Must provide supporting evidence of binding before acceptance of these rated sequences by ProImmune to attempt as pentamer; *** Successfully synthesised this specificity before; * Predicted binding similar to that of LLLIWFRPV (Previously made as pentamer).

BKV agno protein prediction

The potential role of BKV agno in replication was discussed in the introduction. We wondered if any potential epitope lay within one of the three potential phosphorylation sites of the protein (S7, S11 and T21) (Johannessen, Myhre et al. 2008). Therefore, we computer predicted epitopes for HLA A01 with wildtype agno and phophorylated agno. Phosphorylation of agno protein may influence the natural processing via MHC. Syfpeithi prediction revealed that several epitopes contain the S11 and T21 amino acids, but most epitopes lay within the second part of the agno protein (see **Figure 35**).



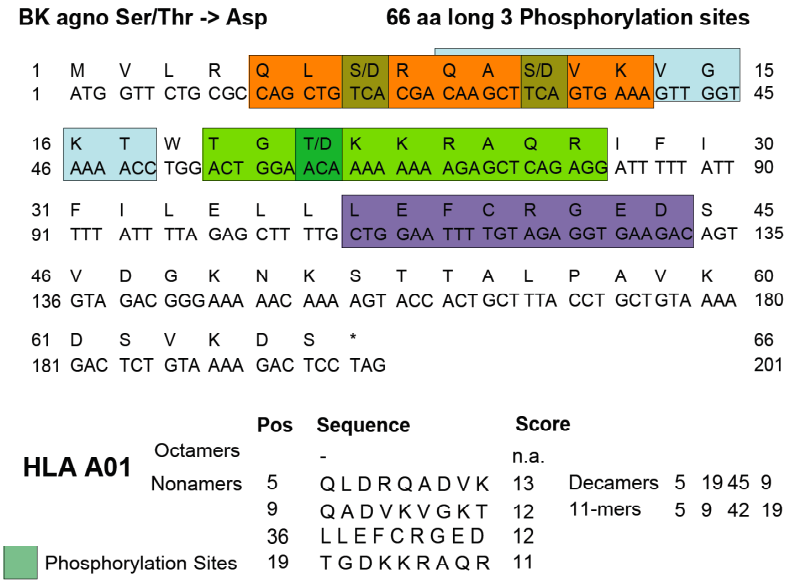


Figure 35. Wild type agno compared to phosphorylated agno and the influence on predicted epitopes.

The MHC binding affinity scores predicted by Syfpeithi indicates a weak binding. This could be an explanation for the bad immuno induction potential. However, other HLA types should be predicted and set into context with the processing of other BKV epitopes. Additionally single patients with high agno responses should be compared to “ordinary” patients without a agno specific immune response.

Experimental results and confirmation of predicted epitopes

To verify, if the in silico predicted regions correlate with in vitro immunology, we checked the BK LT protein using a checkerboard approach. This would verify immuno-dominant sequences and reveal unpredicted regions, which would haven been probably missed by the computer prediction alone.

First, laboratory personal was tested for humoral and cellular BKV- and JCV-LT, -VP1 and -agno-specific immune response. PBMCs of BKV seropositive healthy individuals were isolated and BKV LT-specific T-cells were expanded for epitope mapping. Donor characteristics can be seen in **Table 9**.

Age	Gender	BKV serology	JCV serology	HLA A	HLA B	HLA DR
31	male	+	(+)	24/29	44/55	07/14
30	male	+	-	02/30	13/35	11/13
34	female	+	+	02/03	07/62	04/15
50	male	(+)	+	01/02	08/40	11/13
42	male	(+)	+	02/11	07/62	04/15

Table 9. Showing healthy donor characteristics for epitope mapping. (+) weak serological response.

In principle the ActMono assay was used to amplify virus-specific T-cells (see material and methods and **Figure 11A**).

Every sub-pool consists of 10 single peptides (see appendix for sub-pool specifications). Within the checkerboard, at the cross-points of sub-pools a higher immune response indicates immuno dominant peptides (**Figure 36**).

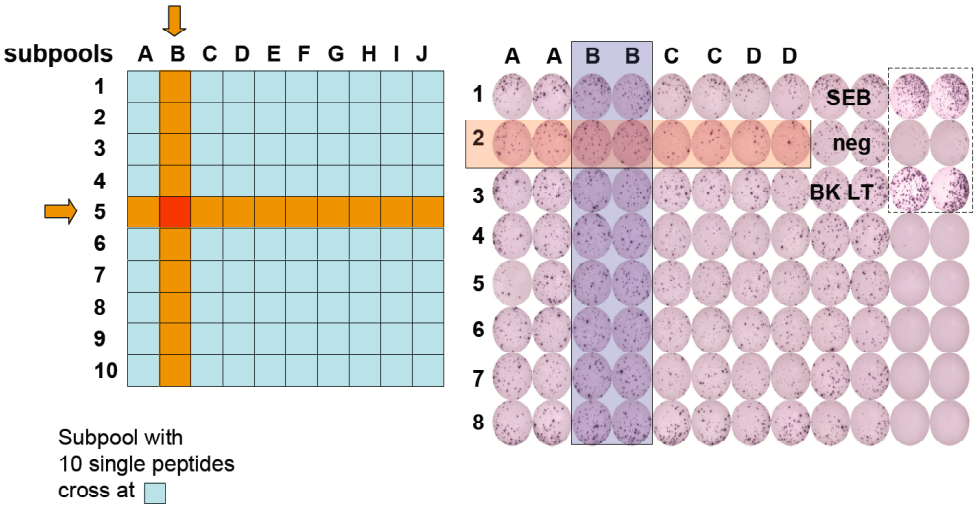


Figure 36. Checkerboard design. Every sub-pool of the 15mer total library contained 10 single peptides. We had in total a sub-pool set from A to J

crossing with 1 to 10 and a set K to R crossing with 11 to 20. Sub-pool containment can be seen in the appendix.

Another way to verify predicted clusters was comparison to already published epitopes. We found than 80% of published peptides were included in our cluster-regions. Two HLA DR1 epitopes (0901: TFSRMKYNICMGKCI AND 0301: TLYKKMEQDVKVAHQ), and one HLA B08 epitope (IYLRKSLQNSEFLLE) were not in our super-cluster (Li, Melenhorst et al. 2006).

Phenotypic characteristics during expansions with BKV LT-pool

Before expansion the amount of BKV -specific T-cells in the peripheral blood is low. In Elispot between 50 and 150 SFU/1Mio PBMCs can be detected, corresponding to 0.005% of PBMC. The sensitivity of flow cytometry normally is too low to detect any BKV specific response without expansion. Using the ActMono expansion method, the amplification efficacy was between 10 and 100 fold. SFUs reached between 500 and 5000 SFUs/1Mio PBMC. In FACS analysis 0.2 to 3% of BKV LT-specific T-cells could be detected after expansion. CD4+ and CD8+ T-cells were in a ration of 2:1. However, further specific memory marker were not yet tested.

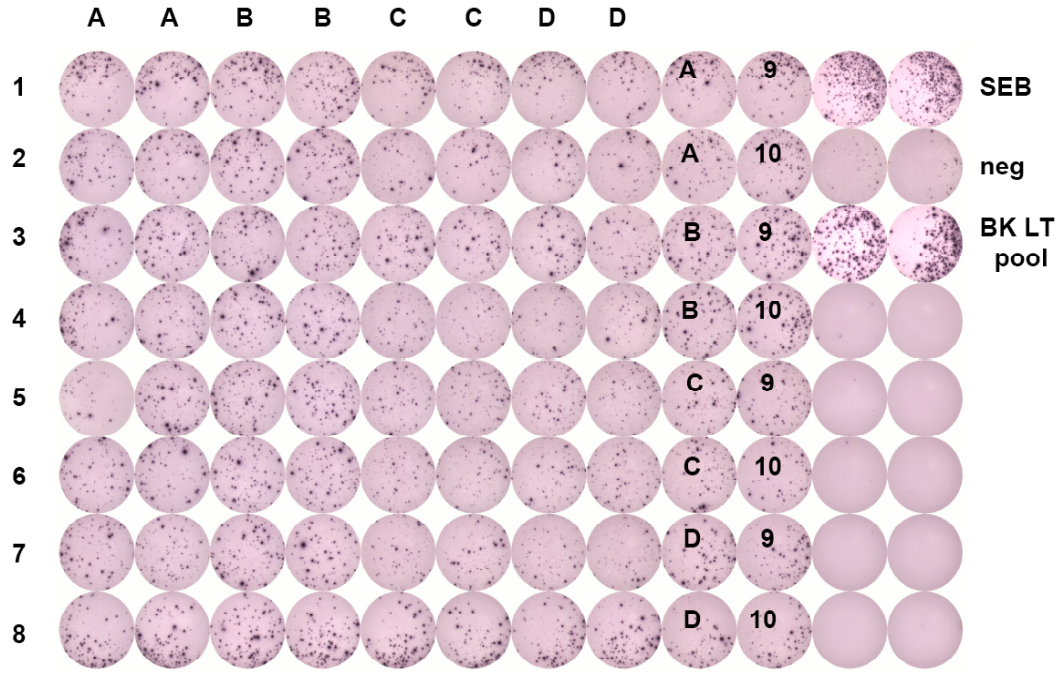
Checkerboard approach for BK LT 15mer library

BKV LT specific T-cells of 5 healthy individuals were expanded over nine days using a peptide pool containing the whole BK LT library (179 15mers). After expansion the virus-specific cells were frozen and new round of expansion with fresh blood was started. After having roughly 50 x 10e6 expanded T-cells we thawn the cells and pooled them for each individual.

Elispot was performed according to standard protocol except using 100'000 expanded T-cells per well. Content of peptide sub-pools can be seen in the appendix. Sub-pools were plated in duplicates onto the Elispot plate (see **Figure 36** for an example).

On every plate staphylococcal enterotoxin B and BK LT pool served as a positive control. Additionally, a background control with cell medium was run

in parallel. The double value of the mean background wells served as a definition for a positive immune response (green marked wells in **Figure 37-38)**.

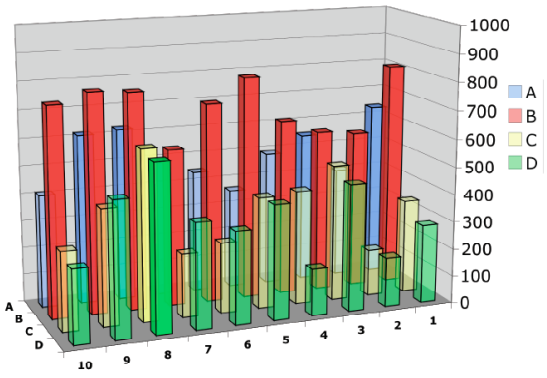


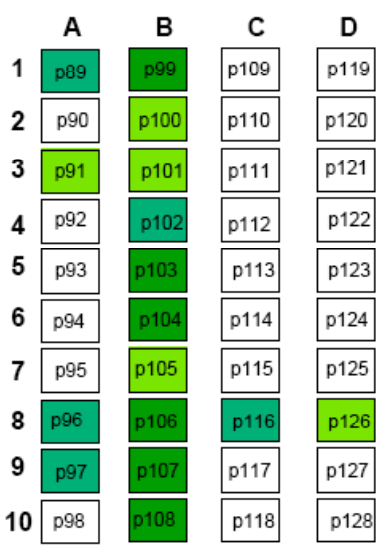
	SFU per Mio PBMC
SEB	4725
neg	0
BK LT	2610

	A	B	C	D
1	635	810	340	285
2	405	575	170	180
3	550	590	495	460
4	490	640	415	170
5	365	805	405	415
6	445	720	255	335
7	495	570	230	380
8	625	780	615	600
9	615	790	420	485
10	410	755	285	265

Positive response: >2x background 510

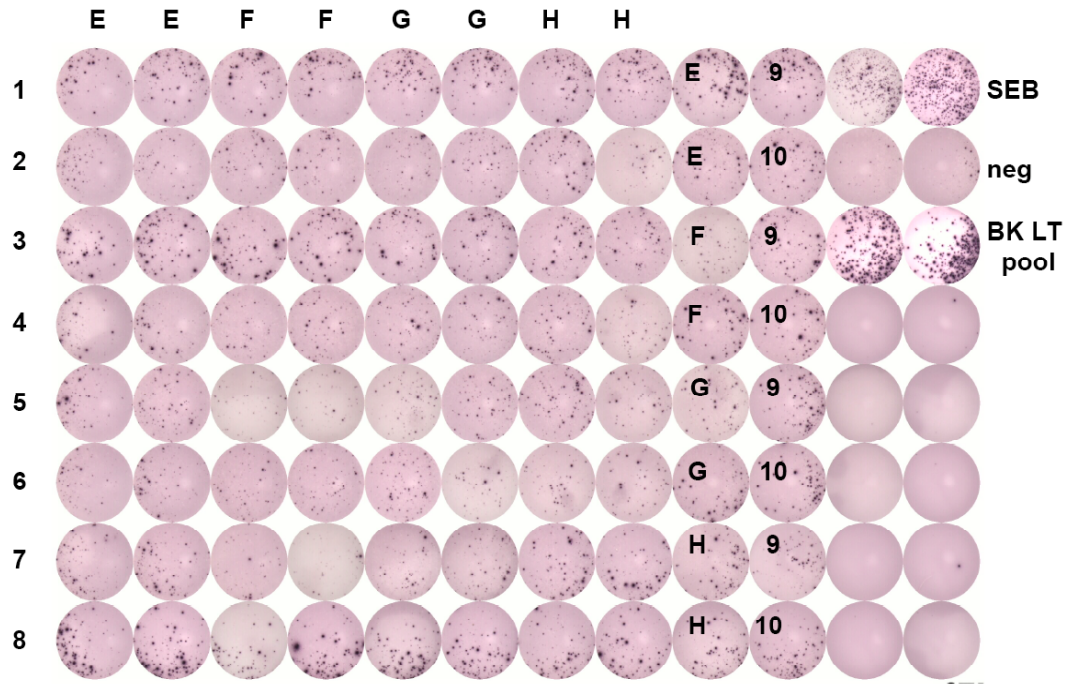
+ 0 - 100 SFU
+ 100 - 200 SFU
+ 200 - 300 SFU





Code	Sequence	Predicted	HLA Type
p89	MDKVLNREESMELMD		
p91	ESMELMDLLGLERAA		
p96	LMRKAYLRKCKEFHP		
p97	AYLRKCKEFHPDKGG		
p99	FHPDKGGDEDKMKRM		
p100	KGGDEDKMKRMNTLY		
p101	EDKMKRMNTLYKKME	yes	DR1101/(1302)
p102	KRMNTLYKKMEQDVK		
p103	TLYKKMEQDVKVAHQ		
p104	KMEQDVKVAHQPDFG		
p105	DVKVAHQPDFGTWSS		
p106	AHQPDFGTWSSSEVP		
p107	DFGTWSSSEVPTYGT	yes	B35(01)
p108	WSSSEVPTYGTEEWE	yes	B35(01)
p116	EDMFASDEEATADSQ		
p126	SQAVFSNRTLACFAV		

Figure 37. Checkerboard of peptide sub-pools A-D and 1-10.



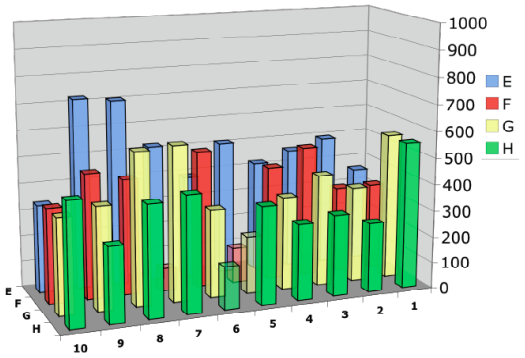
SFU per Mio PBMC

SEB	3980
neg	0
BK LT	2695

Positive response: >2x background 240

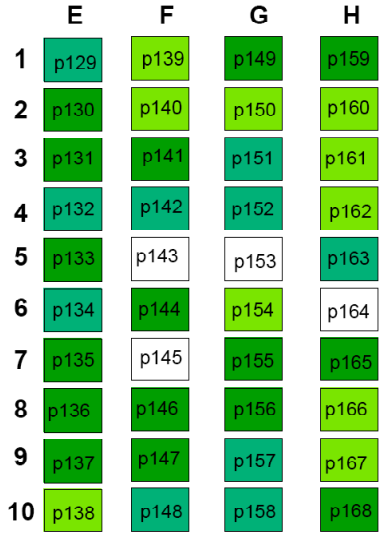
- + 0 - 100 SFU
- + 100 - 200 SFU
- + 200 - 300 SFU

	E	F	G	H
1	360	330	555	555
2	495	330	365	265
3	460	500	425	310
4	420	435	355	290
5	510	135	220	370
6	390	520	335	160
7	520	85	585	440
8	705	440	575	420
9	720	475	390	285
10	335	360	365	460



	E	F	G	H
1	p129	p139	p149	p159
2	p130	p140	p150	p160
3	p131	p141	p151	p161
4	p132	p142	p152	p162
5	p133	p143	p153	p163
6	p134	p144	p154	p164
7	p135	p145	p155	p165
8	p136	p146	p156	p166
9	p137	p147	p157	p167
10	p138	p148	p158	p168

Code	Sequence	Predicted	HLA Type
p129	FAVYTTKEKAQILYK		
p130	TTKEKAQILYKKLME	yes	DR1302
p131	KAQILYKKLMEKYSV	yes	A0201 (Syf)
p132	LYKKLMEKYSVTFIS	yes	B35(01)
p133	LMEKYSVTFISRHMC	yes	B35(01)
p134	YSVTFISRHMCAGHN		
p135	FISRHMCAGHNIIFF	yes	B35(01) 2x
p136	HMCAGHNIIFFLTPH	yes	B35(01) 2x
p137	GHNIIFFLTPHRHRV	yes	A0201,DR1101
p138	IFFLTPHRHRVSAIN	yes	A0201,DR1101
p139	TPHRHRVSAINNFCQ		
p140	HRVSAINNFCQKLCT		
p141	AINNFCQKLCTFSFL		
p142	FCQKLCTFSFLICKG	yes	A0201
p144	SFLICKGVNKEYLLY		
p146	NKEYLLYSALTRDPY	yes	B35(01),DR1101
p147	LLYSALTRDPYHTIE	yes	B35(01)
p148	ALTRDPYHTIEESIQ		



Code	Sequence	Predicted	HLA Type
p149	DPYHTIEESIQGGLK		
p150	TIEESIQGGLKEHDF		
p151	SIQGGLKEHDFSPEE		
p152	GLKEHDFSPEEPEET		
p154	PEEPEETKQVSWKLI		B35(01)
p155	EETKQVSWKLITEYA	yes	B3501
p156	VSWKLITEYAVETK	yes	B35(01)
p157	KLITEYAVETKCEDV		
p158	EYAVETJCEDVFLLL		
p159	ETKCEDVFLLLGMYL		
p160	EDVFLLLGMYLEFQY	yes	A0201, B3501 2x
p161	LLLGMYLEFQYNVEE	yes	A0201 2x
p162	MYLEFQYNVEECKKC	yes	A0201
p163	FQYNVEECKKCQKKD		
p165	KKCQKKDQPYHFKYH		
p166	KKDQPYHFKYHEKHF		
p167	PYHFKYHEKHFANAI		
p168	KYHEKHFANAIIFAE	yes	DR1302

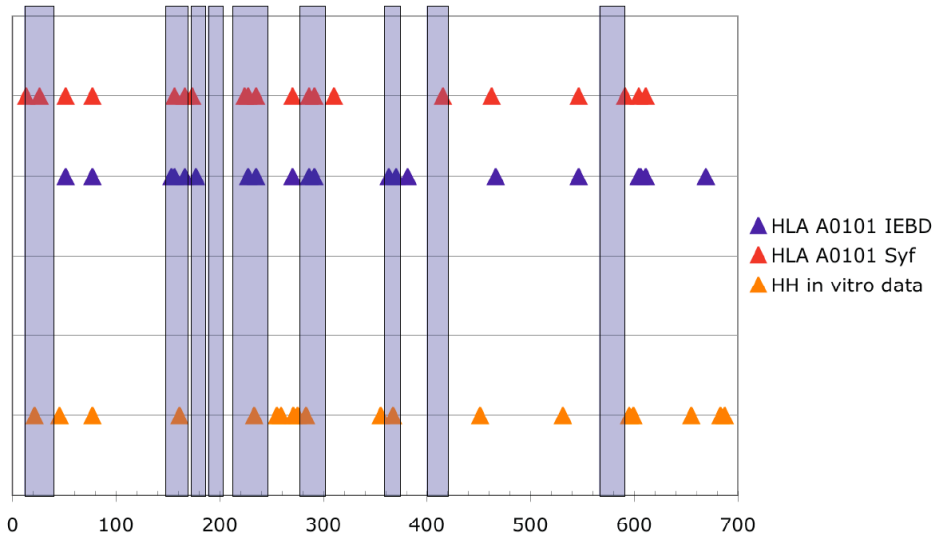
Figure 38. Checkerboard of peptide sub-pools E-H and 1-10.

Checkerboard results of sub-pools I-J/1-10, K-N/11-20 and O-R/11-20 can be seen in the appendix part. The same approach was done for four other healthy individuals.

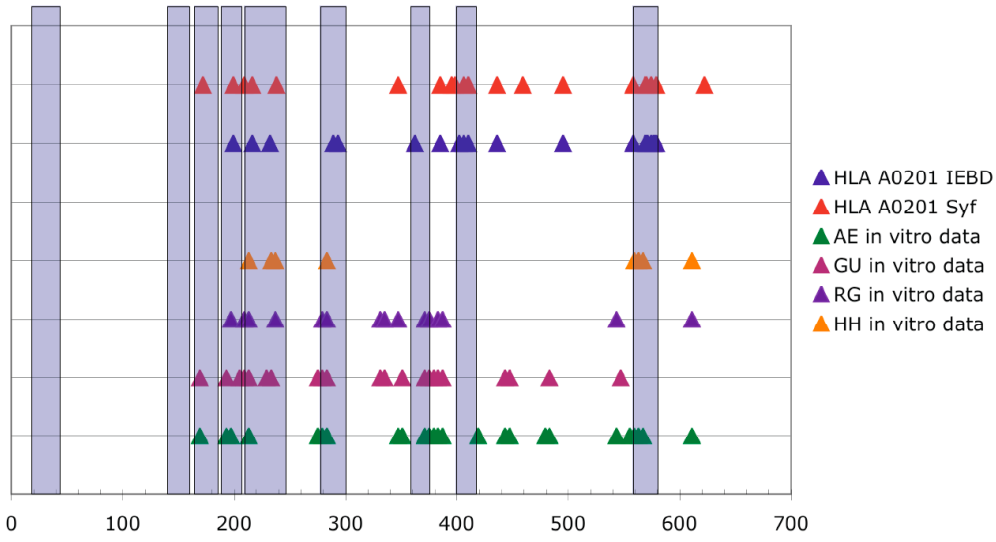
Then, the database was again consulted to check, if the positive response was also computer predicted at a crossing sub-pools. If so, this single peptide was entered into a excel file and compared with other individuals with the same HLA type and also with the overall predicted peptides for this HLA type. For example in Figure 37, sub-pool B crossing with sub-pool 9 identified p107 to be immunodominant with 790 SFU/1Mio PBMCs. Indeed this 15mere harbour the 9mere epitope WSSSEVPTY, which is immunodominant for HLA B3501 and matched with the HLA type of the blood donor.

Overall a high concordance between the predicted IEBD and Syfpeithi epitopes and the in vitro generated data existed for various HLA types (**Figure 39**).

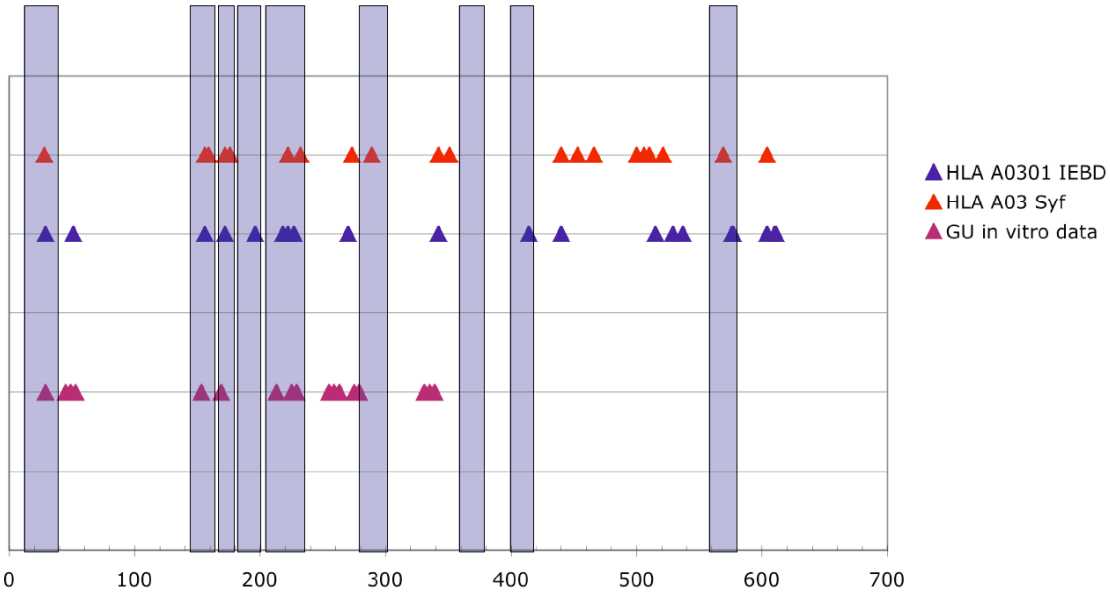
HLA A0101



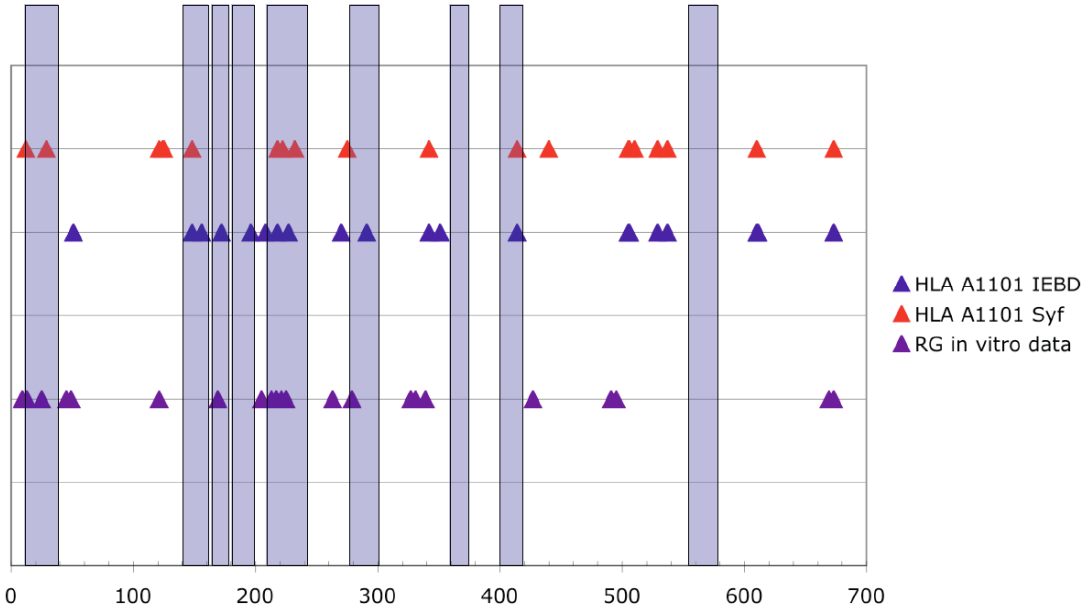
HLA A0201



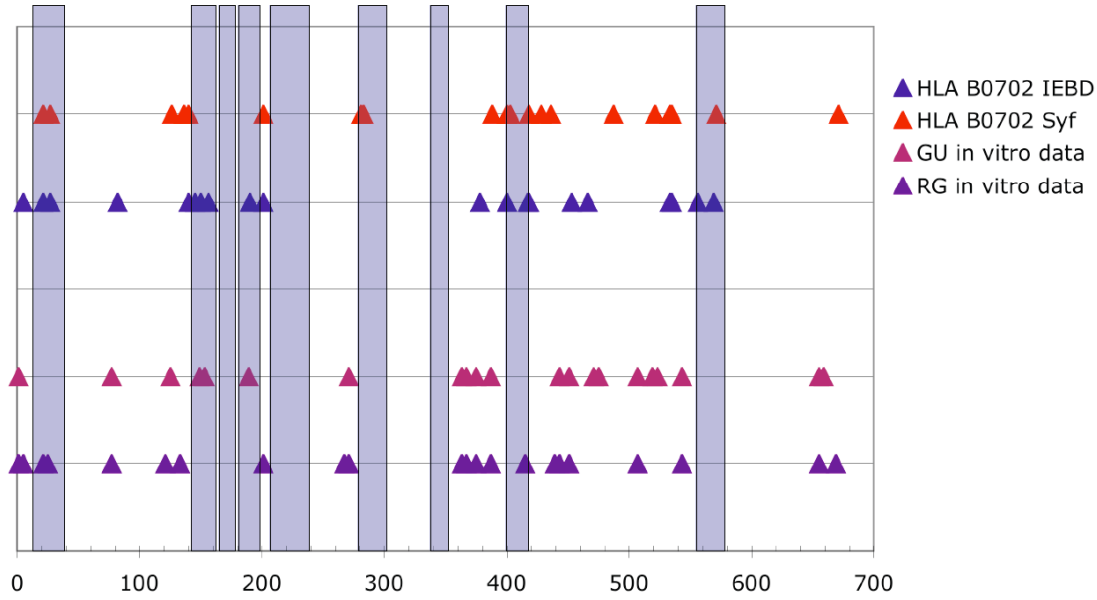
HLA A0301



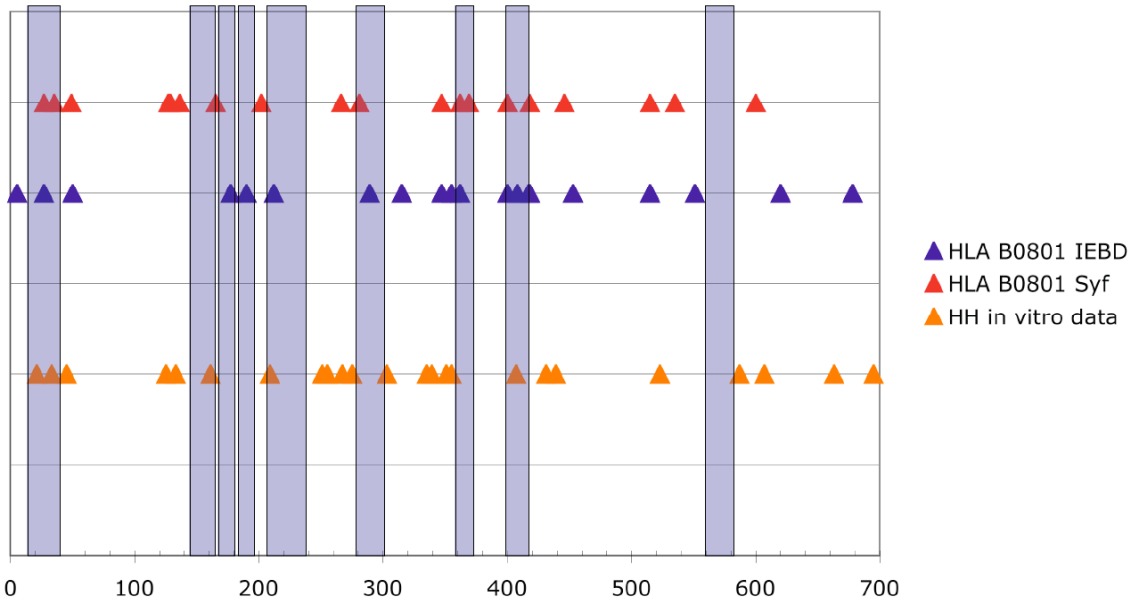
HLA A1101



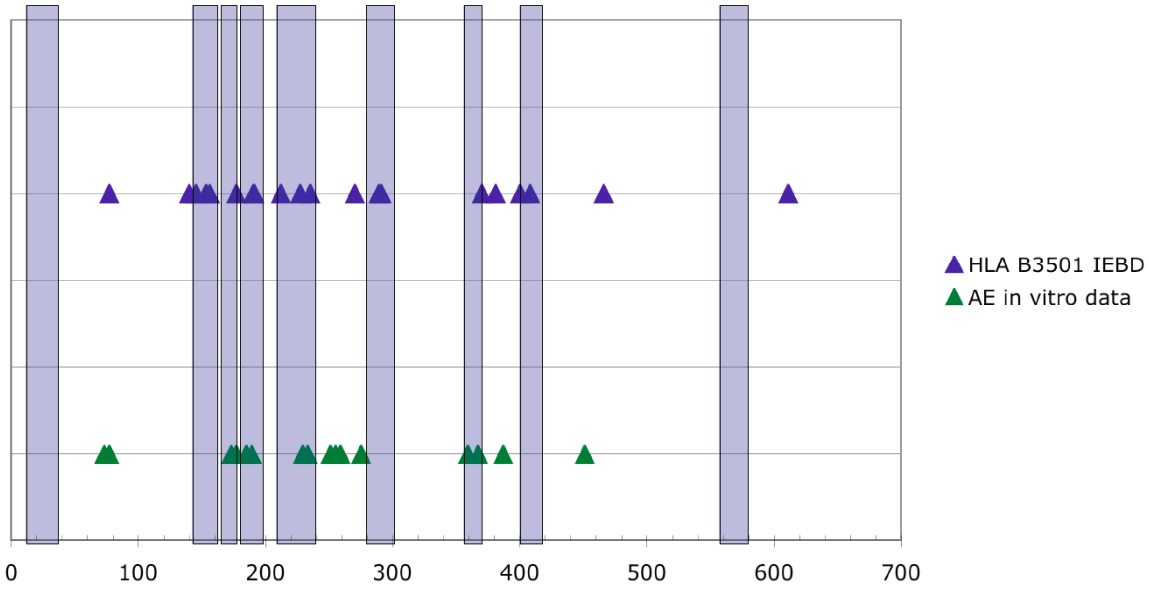
HLA B0702



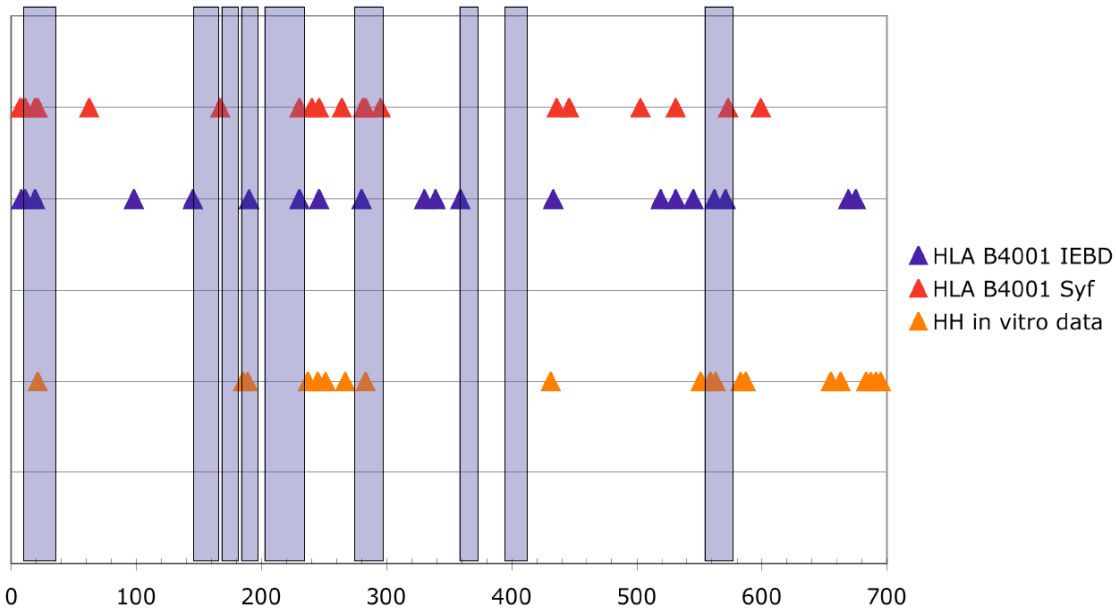
HLA B0801



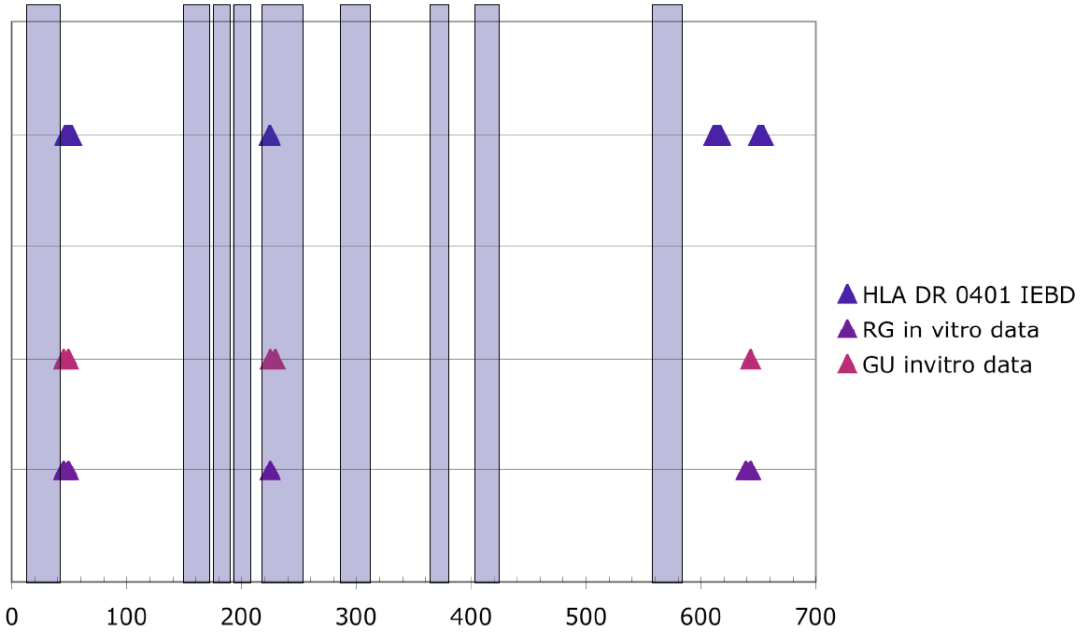
HLA B3501



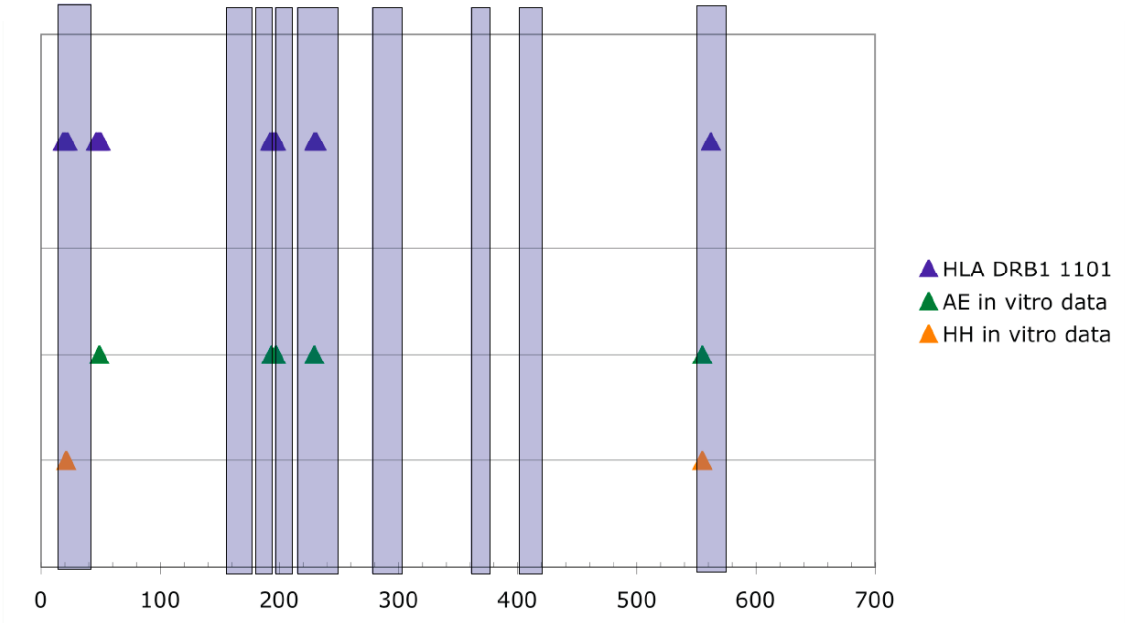
HLA B4001



HLA DRB 0401



HLA DRB 1101



HLA DRB 1501

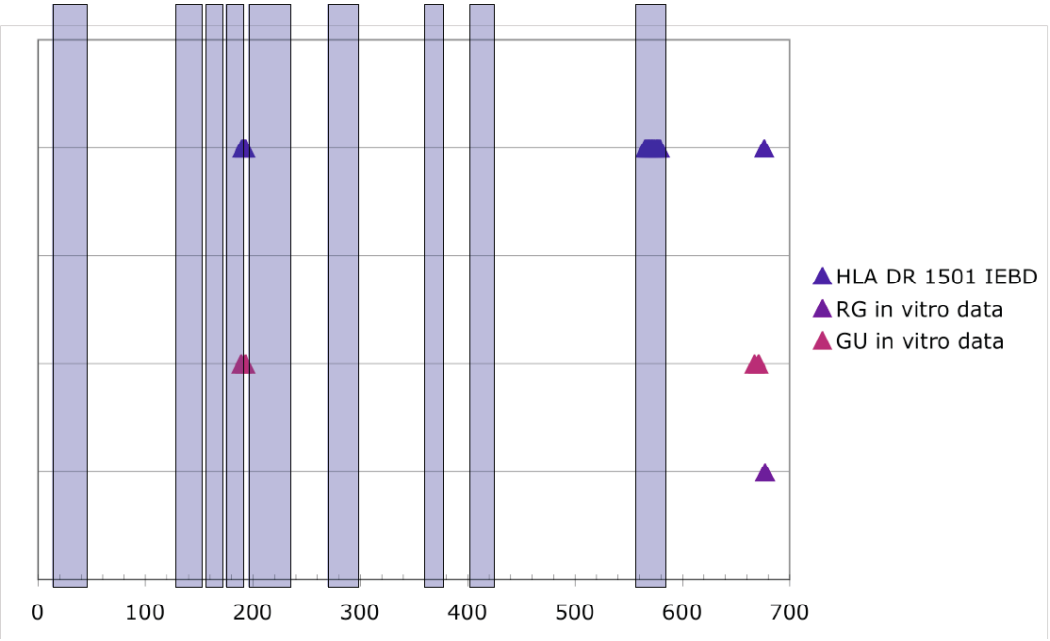


Figure 39. Overlay of predicted Syfpeithi and IEBD epitopes and checkerboard peptides with high activity for different HLA types.

Usage of predicted 15mer and 25mer peptides

The next step was to test the predicted 9-, 15-, and 25mer peptides for functionality on expansion. The predicted 9-, 15-, and 25mers were pooled separately. We performed an ActMono expansion with pulsing the monocytes for 4h with the 15mers and 25mers pools. Re-challenging in Elispot and FACS was after 9days with the corresponding predicted 9-, 15-, and 25mers pools.

Results from the flow cytometry indicated for individual RG (HLA A 02/11 B 07/62) a strong induction of BKV-specific CD8+ T-cells, when challenged with the 9mers (See **Figure 42**). In this individual the re-challening with single predicted 15mers in Elispot revealed that 3 peptides were particular immunodominant (NLP, LER, MTR). Based on the CD8 response in FACS, these 15mer harbour HLA A or B epitopes. When compared with the database we could identify indeed 3 new not published epitopes: HLA A0201: MLTERFNHI; HLA B0702: RAAWGNLPL; HLA B0702: LPLMRKAY. Elispot plates can be seen in **Figure 40**. However, the RAAWGNLPL epitope is

accordning to the pro-immune house prediction assay no good epitope for binding to HLA B0702 MHC I molecules.

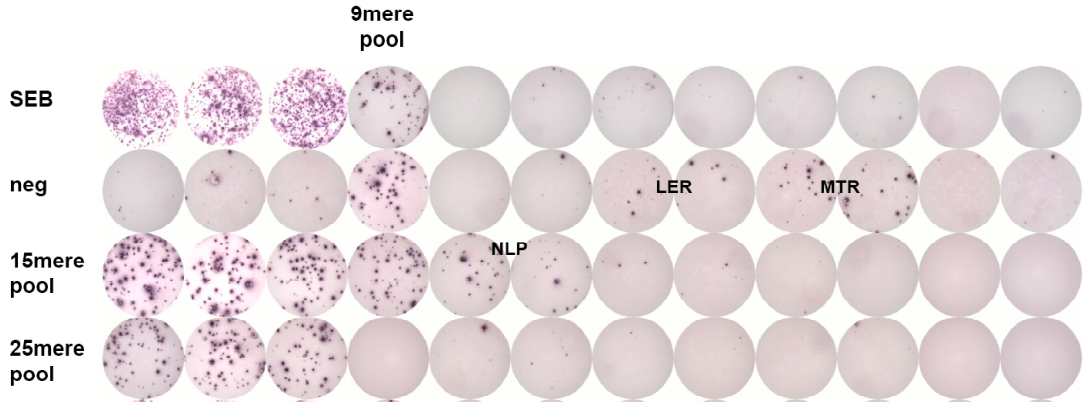
Results from flow cytometry of individual AD (HLA A24/29 B44/55) a weaker BKV-specific CD8+ response compared to CD4+ response in flowcytometry (**Figure 43**). However, the 9mer pool in Elispot responded very strongly. Four single 15mers were strong positive, harbouring the 9meres for HLA A2402 or B4402: AYLRKCKEF, for A2402: PYHTIEESI, for A2403: HQFLSQAVF, and for DR0701: LPLMRKAY (**Figure 41**).

9mer tetramers

Tetra/Pentamer staining is a technique to measure the amount of T-cells binging to a particular epitope. The identified epitopes is artificially loaded on a nanoparticle coated with MHC molecule of a certain HLA type. A T-cell with a TCR specific for the epitope will bind to the tetramer, which is tagged with PE-fluorescence marker (introduction and **Figure 5**). In peripheral blood the spectra of specific T-cells should be highly biased, because only those T-cells, which were previously stimulated will patrol. Stimulation, however, is based on the corresponding MHC with the particular HLA background.

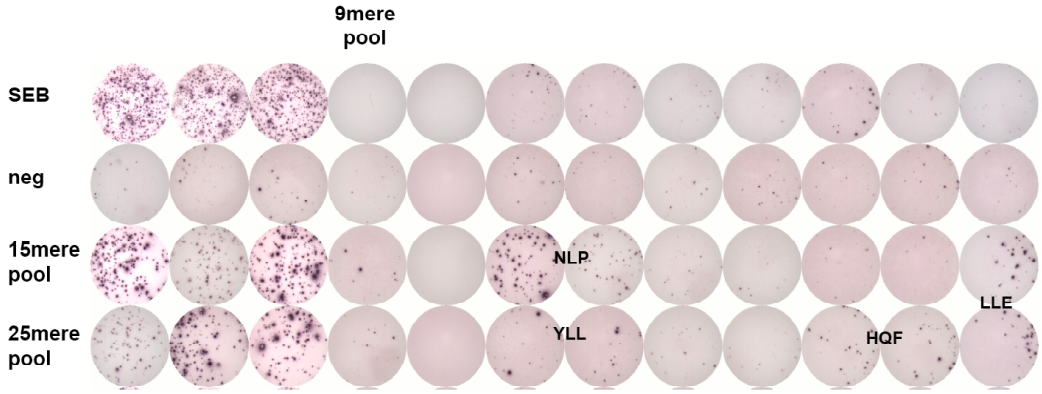
To further identify if epitope-specific T-cells were present in the expanded culture, we ordered pentamers (Proimmune, England) of the corresponding HLA types covering the 15mer and 25mer predicted region. However, so far these pentamers are not yet tested. **Table 8 and 9** show a list of identified tetramers with the corresponding HLA type.

In general, the HLA DR distribution was more focused on certain regions and not as widely spread than HLA A or B epitopes within the protein. Therefore certainly several HLA DR epitopes will be missed using more focused sequences. On the other hand, our laboratory and others have previously shown, that especially the BKV-LT specific CD8+ T-cells response seems to be important in controlling viral replication (Comoli, Binggeli et al. 2006; Hammer, Brestrich et al. 2006; Binggeli, Egli et al. 2007; Ginevri, Azzi et al. 2007). Under this aspect, anyway we would favour HLA A or B epitopes.



NLP: LPLMRKAYL B0702
LER: RAAWGNLPL B0702
MTR: MLTERFNHI A0201

Figure 40. Elispot after 9d expansion with predicted 15mer pool. Re-challenging with predicted 9-, 15-, and 25mer pools and single 15mers. NLP, LER, MTR responded particular good. HLA A 02/11 B 07/62



NLP: AYLRKCKEF A2402, B4402
YLL: PYHTIEESI A2402
HQF: HQFLSQAVF A2403
LLE: LPLMRKAY DR0701

Figure 41. Elispot after 9d expansion with predicted 15mer pool. Re-challenging with predicted 9-, 15-, and 25mer pools and single 15mers. NLP, LER, MTR responded particular good. HLA A24/29 B44/55

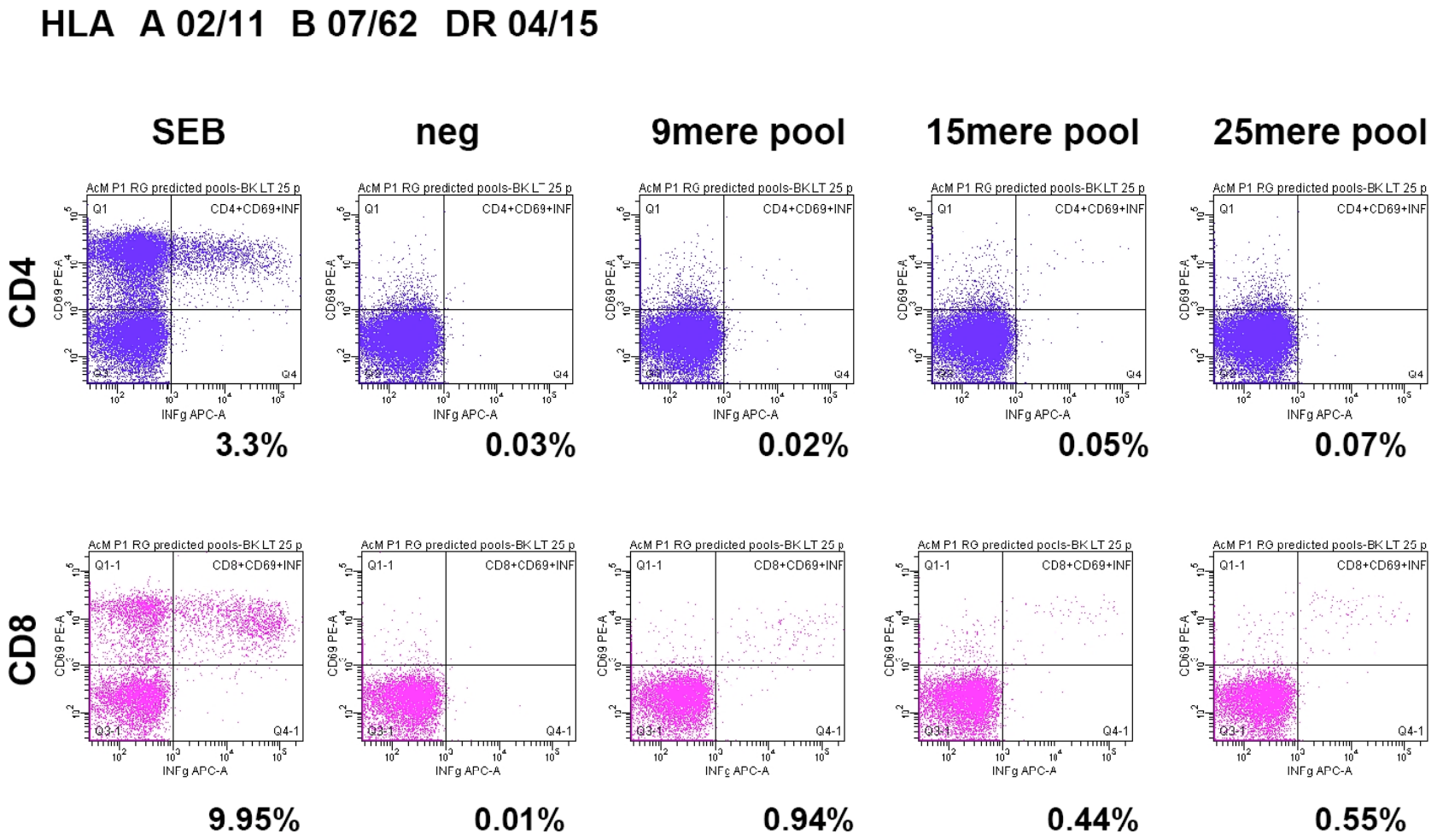


Figure 42. Flowcytometry analysis after expansion with 25mer pools and re-challenge with shorter fragments pools.

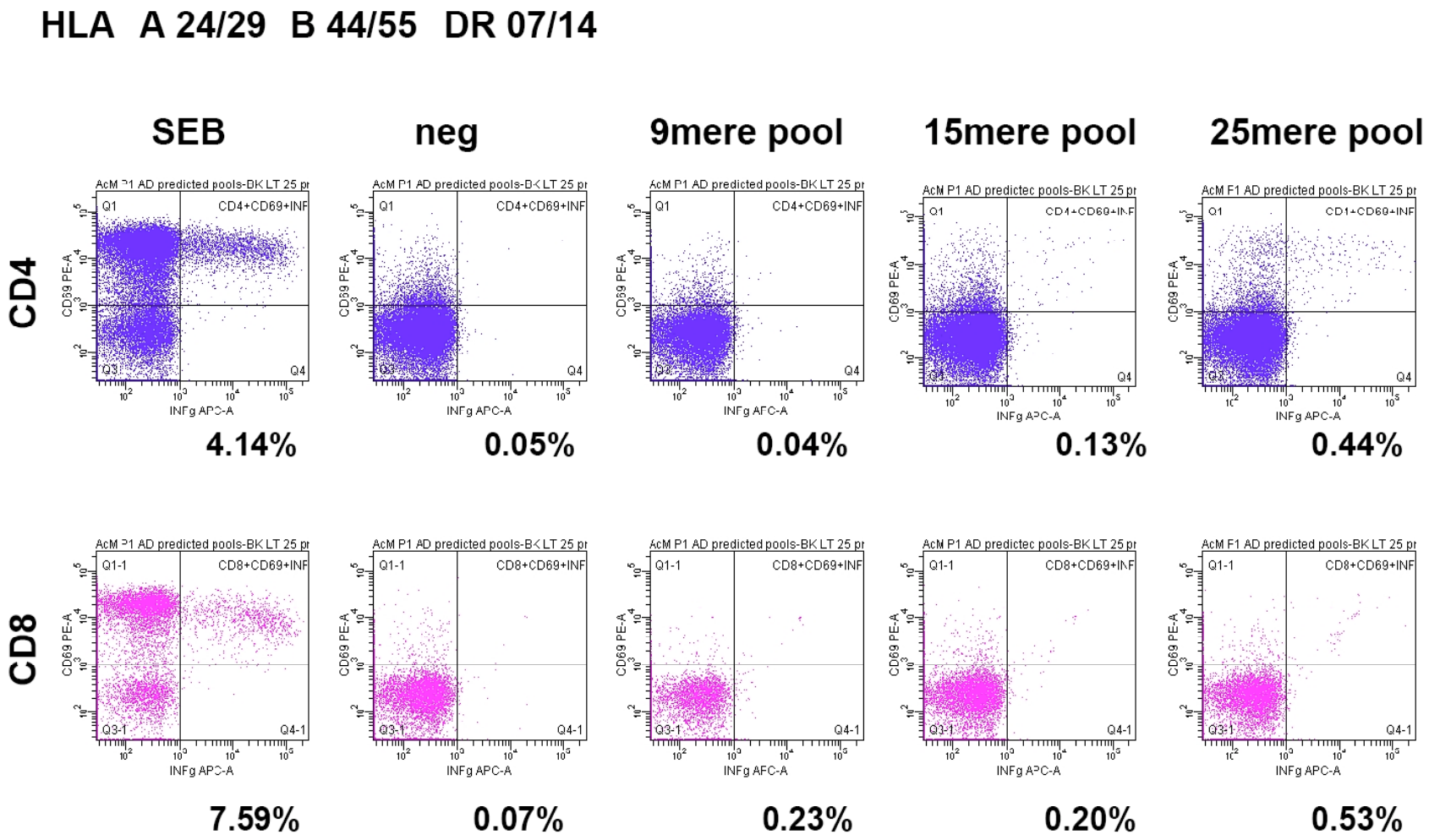


Figure 43. Flowcytometry analysis after expansion with 25mer pools and re-challenge with shorter fragments pools.

As the proteasome shows different cleavage specificities when it is interferon induced, another critical point could be the potential differences in epitopes processing during infection. During active BKV replication indeed the spectra of T-cell is wider scattered (Comoli, Basso et al. 2003). However, Gineveri et al. have shown, that BKV LT specific immune response after viral clearance shows highest killing activity (Ginevri, Azzi et al. 2007). Based on this we could assume that memory T-cell clones will persist recognizing the responsible epitopes. Our assays targets memory T-cells response should catch the important epitopes..

The high homology of more than 80% between BKV LT and JCV LT explains the high immunological cross-reactivity between both viruses. Binggeli et al. showed that BKV-specific expanded cells could be re-challenged with a JCV-LT peptide library. For JCV LT a higher immune response was examined compared to BKV VP1 expanded T-cells re-challenged with JCV-VP1 (Binggeli, Egli et al. 2007). This cross-reactive potential was more specifically examined on single epitope level. Indeed, four BKV LT epitopes have shown so far cross-.reactive potential to JCV LT (TFSTMKYNICMGKCI, IYLRKSLQNSEFLLE, LLIQFRPV, KSLQNSEFLLEKRIL) (Li, Melenhorst et al. 2006; Randhawa, Popescu et al. 2006).

Cross-reactivity could be a problem for a cellular immune assays, but most probably due to high double seropositivity, this cannot be avoided. However, a pre-serological testing could clarify BKV or JCV latent infection. In a vaccine, a cross protective effect could be beneficial.

We have observed, that JCV and BKV double-seropositive individuals shed significantly less often JCV in urine compared to single JCV-seropositives blood donors (Egli, Infanti et al. 2008). However, the impact of this observation in kidney transplant recipients is yet not examined.

6. Conclusions

During my PhD thesis several aspects of the interaction of Cytomegalovirus and Polyomavirus BK with the host's immune system were examined (see list of publications). The overall aim was to compare immune response in healthy individuals and kidney transplant recipients with or without viral replication.

In healthy individuals, Polyomaviruses BK and JC infect 80% and 60%, respectively. For CMV seroprevalences may reach up to 80%. Intermittent virus shedding in urine is observed for BKV in 7%, JCV in 19% and CMV in 0%. However, no virus replication in plasma was detected. Posttransplant, mainly due to prolonged immune suppression the amount and function of CMV- and BKV-specific T-cells is impaired. Calcineurin inhibitors lead to a direct reduction of INFγ production of virus-specific T-cells, whereas anti-proliferative immunosuppressives reduce the expansion capacity in a dose-dependent manner. This may be a major reason for uncontrolled virus replication.

The humoral response reflects the amount of recent antigen exposure and does not directly indicate protection from virus replication. Virus-specific cellular immune responses would probably allow to assessing the risk of future replication.

Overall the importance of CMV and BKV specific T-cells posttransplant in controlling virus replication was examined. For both viruses we could calculate a protective threshold of virus-specific T-cells. CMV-pp65 specific CD4 T-cells above 0.03% were significantly associated with no CMV replication during the next eight weeks. Additionally, below this cut-off, CMV seropositive recipients developed more often GCV-resistant CMV replication. During BKV replication, patients with more than 69 BKV-LT specific T-cells per 1 Mio PBMCs were significantly more often showing decreasing BKV loads in plasma.

As virus-specific T-cells seem to be crutial in reducing virus replication, and reduction of immune suppression harbours the risk of acute rejection, a

booster vaccine could be a new therapeutic option. A booster vaccine could probably elevate the amount of virus-specific T-cells above a critical threshold of protection from disease, despite effects of immune suppression.

We tried to identify immunodominant regions with the CMV pp65 and BKV LT proteins. We used a combined approach of computer prediction algorithms and experimental verification. Epitope mapping of BKV LT with computer prediction revealed several clusters, which could be immunodominant and also potentially be processed and recognized in various HLA backgrounds.
The identified cluster regions were synthesised as 15 and 25mers. Expansion and re-stimulation with predicted epitopes could so far confirm the HLA A and B-specific prediction of single 9mers covered by the larger 15 and 25mer sequences. However, other HLA types need to be tested for direct stimulation and expansion potential of the predicted epitopes. Additionally, tetramer staining should be performed for verification.
Based on this research, we will be able to improve current immune monitoring and probably a high-specific peptide-based vaccine against BKV LT could be developed and be used to increase the amount of BKV-LT specific T-cells.

Another potentially therapeutic agent could be the blockade of PD1 ligand. PD1 expression in chronic virus infection lead to impaired CD8+ T-cell function. CMV-specific CD4 T-cells treated with an inhibitory antibody against PD1 ligand, and thereby activating CD4+ T-cells, lead to a increase of the expansion capacity. We have shown, that the anti-PD1 ligand antibody increases various cytokines. This could be also tested for BK virus.

Measurement of virus-specific T-cells may replace serological assays in the future, due to a better correlation to effective antiviral control, which can be used as monitoring tool during infection and post-vaccination.

6. Outlook

6.1. PD1-expression on CMV specific T-cell response and influence on cytokine profiles

Overall PD1 blockade, and thereby activation of CMV-specific T-cells seems to lead to an unspecific up-regulation of almost any cytokine.
This could be a safety issue when an antibody blocking PD1 would be used therapeutically. There certainly is a theoretical risk for inducing graft rejection by stimulating and activating allo-reactive T-cells. Interestingly, only virus-specific T-cell responded to the antibody treatment, whereas SEB treated cells did not up-regulate their cytokines. As SEB will stimulate a wide range of T-cells as well allo-reactive T-cells, we would conclude that anti-PD1 treatment is only beneficial in virus-specific T-cells. However, this needs to be tested by stimulating T-cells with allo-antigen preparations.

Virus infections may probably lead to an up-regulation of PD1-ligand expression on the host cell surface. This could be a general protective mechanism for viruses as they are thereby protected from T-cell killing. The exact patterns of PD1-ligand expression should be examined in various cell lines with and without virus infection such as CMV and BKV. In kidney biopsies with BKV nephropathy a significant up-regulation of PD1-ligand mRNA was observed (Starke, Lindenmeyer et al. 2008).

We could use infected RPTECs as antigen presenting cells to CD8 T-cells and check the influence of PD1 blockade on killing of virus infected T-cells.

If indeed the effect on virus-specific T-cells is dominating, a PD1 blockade could be tested in a mouse model to check for virus clearance.

6.2. Effect of immunosuppression on CMV-pp65 specific and BKV-LT specific T-cells

As shown in the ongoing research part, we first concentrated on virus-specific PBMCs and the influence of different immunosuppressive compounds.

However, many open aspects remain and even more questions were generated with this set of experiments.

An interesting, although confusing observation was the dose dependent decrease of INFγ-release by BKV-LT specific T-cells with Rapamycin and Leflunomid, but not with SEB and pp65 stimulated T-cells. Possible explanations were:
(i) An immunosuppressive effect of DMSO. (ii) The low amount of BKV-LT specific T-cells may influence the performance of the assay. Indeed, we noted no more dose depending effects when using expanded BKV-specific T-cells. (iii) Probably very speculative, BKV-specific T-cells behave differently and due to intracellular protein patterns more sensitive to mTOR.

A new batch of BKV LT peptides should be ordered in a higher concentration, thereby further reducing DMSO concentration.
To examine potential differences in mTOR profiles, a selection of BKV-specific T-cell could be performed by FACS sorting. Then we could check for activation/expression state of several mTOR-associated proteins.

We would like to examine the effect of immune suppression on several T-cell subtypes. First, we focus on ordinary subsets as CD4+ and CD8+ T-cells, and naïve and memory compartments. Then, we concentrate more specifically on T-cells with high co-stimulatory marker expressions as CD28 and PD1, as these cells may be particular important for immune control.

When immunosuppression is too high, virus replication may happen uncontrolled. Measurement of trough levels of immunosuppressive drugs overall seem to be too unspecific for several reasons. First, trough levels are only an indirect marker, whereas T-cell function would be directly assessing the effects of immunosuppression. Plasma levels may additionally be highly dependent on individual patients characteristics, as e.g. age, gender, fat distribution, other drugs, kidney or liver function, liver enzyme patterns and/or polymorphisms. Therefore, we would like to use BKV and CMV-specific immune response as an immune marker for too much immune suppression in

the near future. On the other hand, a panel of HLA-graft matched antigens could be indicators for allo-reactivity and danger of graft rejection. This will allow to titrate the immune suppression not only on trough levels which may be different for single individuals but to set immune suppression on the real in vivo immune function.

We have chosen INFγ as a common read out cytokine. However, hardly any data is available on influence of immunosuppression on other cytokine profiles. Especially IL2 and TNFα showed in our hands a dose dependent decrease, but faster than INFγ. Therefore, we would like to examine the hierarchy of cytokine production under immune suppression.

6.3. BKV epitop mapping

6.3.1. Further computer prediction

With computer algorithms several mega- and super-cluster regions within the BK LT protein were predicted. JC LT epitopes could also be predicted and may possibly be at a similar position within the protein (p300, DNA-binding site, and p53-binding region). Dependent on the cross-recognition potential between two epitopes, a vaccine could thereby cover both polyomaviruses at once.
Additionally the BKV-VP1 and BKV-agno protein could be further computer predicted. These new epitopes could be used in a combined immuno monitoring test or vaccine with early and late protein targets. As already discussed, a vaccine based on VP1 alone, may be more difficult to design.

Potential side effects of a vaccine, especially unwanted induction of allo-reactivity post-vaccination, could be discovered by a BLAST of the identified predicted sequences. This could reveal virus-epitope and host-protein-epitope similarities. This could be understood as a molecular mimicry and selective advantage of a virus, because, theoretically T-cell recognizing host-proteins should be positive selected. On the other side, this may open the field of virus-induced autoimmune diseases.

As BKV-LT specific IgG response correlates with virus clearance in plasma, we wonder, if this is a effect of neutralizing antibodies, or just a bias of selected antibodies, due to help of BKV-LT specific T-cells. As BKV-LT specific T-cells seem to be especially important for viral clearance, and infected host cells are killed, the new epitopes are more available for the immune system. Our ELISA assay uses different parts of BKV LT - LTD1, -D2 and -D3, LT D1 is approximately 120aa (1-120), LT D2 100a (120-280), and LTD3 420aa (280-695). Interestingly, the antibody epitope prediction algorithm from IEBD revealed that the LT D1 showed highest potential to bind IgGs (Emini surface accessibility scale, Parker hydrophilicity prediction, and linear epitope prediction; results not shown). Leuenberger et al. confirmed this experimentally, where LT D1 showed highest antibody activity (Leuenberger, Andresen et al. 2007). When we would base ELISA assays designs on predicted antibody-antigen sequences, we could probably optimize our current assays and increase as well sensitivity and specificity.

6.3.2. Further experimental testing

Five healthy individuals spanning several HLA types have been so far epitope mapped with the 15mer sub-pools (A-R, 1-20, see appendix) from the whole LT-peptide pool in a checkerboard attempt. We compared predicted single epitopes of the corresponding HLA type with the checkerboard results and showed, that a high match between predicted activity and experimental results exists. However, we have planed to further check more different HLA combinations with this approach.
This will not only improve our comparison of predicted and verify epitopes but also show if the identified super-clusters, which are our candidate sequences for a monitoring tool and vaccine, harbour enough relevant epitopes for a sufficient booster of the immune response.

The next step would be to further use the computer predicted 15mer and 25mer sequences. In two of five individuals the expansion with a pool of

predicted 15mer and 25mer sequences was preliminary tested. The re-challenge with a 9mer pool and FACS analysis supported the potential of the predicted sequences to induce a CD8+ T-cell based immune response.
We plan to further sub-pool the 9mers for a corresponding 15mer. Thereby we will be able to identify single 9mer epitopes for one HLA type.

When we have identified single epitopes we would like to verify these with pentamer staining. This would allow to easily screening a larger patient collective for relevant epitopes.

As we have tested so far only healthy blood donors, we would extend our examination also on blood sample from kidney transplant recipients at several stages of disease. We would compare patients with ongoing high, low and cleared BK viremia. Therefore we could further verify, which epitopes in a transplant recipient are more essential for successful virus control.

Based on these results of the computer predicted and immunological experiments, we could design a vaccine-vector with either containing the genetic sequence of the identified BKV sequence or load DC with the peptides.

In a phase-1 trial, we would test the vaccine preparations in a mouse model to check for side effects and immune response.
In a phase-2 trial, we could vaccine a group of BKV seronegative healthy individuals and check for the induction of an immune response with our monitoring tools for humoral and cellular immune response.
In a phase-3 trial, we would vaccine seronegative and seropositive kidney transplant recipients before transplantation with several vaccine schemes (3 vs. 1 time point; several time intervals pre- and posttransplant). We would compare the immune response and measure viral reactivation in a vaccinated group and compare it to a non-vaccinated group.
In a second phase-3 trial, we could examine the long-term benefitial effect of such a protein vaccine. The main target would be to reduce PVAN.

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Transplantation Virology Lab (alphabetical order):

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Dr. S. Bodaghi
Dr. A. Dumoulin
Dr. G.A. Funk
A. Glaser
Dr. R. Gosert
A. Hirsch
Dr. N. Khanna
S. Köhli
C. Mistl
Dr. G. Unterstab
J. Samaridis
M. Wernli

Molecular Diagnostic:
L. Balaj-Jones
Ch. Bauer
J. Curti
N. Doppler

S. Girardin
R. Ledermann
S. Marlot
H. Atici
R. Reuter
I. Ziekau
M. Zain

Serology:
Dr. I. Steffen

Immunology Research Group
Prof. P. Erb

Technical staff
A. Hermann
A. Schäuble
Dr. C.A. Levy

Collaborators:

Nephrology and Transplantation Immunology, Basel
PD Dr. M. Dickenmann
Dr. M. Mayr
Dr. S. Schaub
Prof. J. Steiger

Nephrology Department, Kantonsspital St. Gallen
Dr. I. Binet
Dr. C. Jäger

Nephrology Department, Kantonsspital Liestal
Dr. D. Kiss

Infectious Diseases and Hospital Epidemiology, Zurich
PD Dr. N. Müller

Virology and Nephrology, University of Saarland, Homburg
PD Dr. M. Sester
Dr. U. Sester

Pediatric Research Lab, Policlinico San Matteo, Pavia
Dr. P. Comoli

Pediatric Immunology, Innsbruck, Austria
Prof. Dr. L. Zimmerhackl
PD Dr. M. Prelog

Infectious Disease Department, Calgary, Canada
PD Dr. A. Johnson

9. Curriculum vitae

1. PERSONALIEN

Name:	Egli
Vorname:	Adrian
Geburtsdatum:	26.09.1978
AHV-Nr.:	307.78.388.118
Titel:	Dr. med., PhD Student
Zivilstand:	ledig
Muttersprache:	Deutsch
Bürgerort:	Basel
Nationalität:	Schweiz

2. ADRESSE

PLZ/Wohnort:	4052 Basel
Strasse:	Lehenmattstrasse 149
Tel. privat:	0041 61 / 321 44 12
Tel. mobil:	0041 76 / 570 15 81

3. SCHULBILDUNG

Primarschule	Therwil	1985-1990
Sekundarschule (PG)	Therwil	1990-1994
Gymnasium	Oberwil	1994-1997
Uni Basel (Med. Fakultät)	Basel	1998-2004

4. WEITERE AUS- UND WEITERBILDUNG

Sprachaufenthalte:	England	(Bournemouth)	1997
	Frankreich	(Hyeres)	1998
Einzeltutoriat	Reinach	Pädiatrie	2001-2002
Wahlstudienjahr:			
Bezirksspital Dornach	Dornach	Innere Medizin	08/02-10/02
PUK	Basel	Psychiatrie	11/02-12/02
Spital St.Maria	Visp	Chirurgie	01/03-03/03
Kantonsspital	Liestal	HNO	04/03
Kantonsspital	Liestal	Pathologie	05/03
Kantonsspital	Liestal	Urologie	06/03
Spital Uster	Uster	Gynäkolgie	07/03
Kantonsspital	Bruderholz	Radiologie	08/03
Kantonsspital	Basel	Dermatologie	09/03

Assistenzarztstellen:

Innere Medizin Spital Dornach 2005
aktuelle Anstellung:
Transplantations-Virologie - MD PhD Programm ab 2006 bis 2008
Institut für Medizinische Mikrobiologie, Uni Basel

5. BISHERIGE TÄTIGKEITEN:

allgemeine:

BP Schweiz Büroarbeiten	Birsfelden	1995
Kantonale Verwaltung Baselland Büroarbeiten	Liestal	1996
Spitalrekrutenschule Spitalsoldat	Romont	1998
Regionalspital Laufenburg Praktikumsstelle	Laufenburg	1998
Internetcafé Cyberzone Kundenbetreuung	Basel	1997-1999
St. Claraspital Pflegeassistent	Basel	1998-2003

Studentische Vertretung im Fakultätsrat Basel 2001-2004

nach dem Studium:

Betreuung Einzeltutoriat Innere Medizin, Dornach 2005

Gruppentutor Themenblock Infektiologie 2007
Betreuung Einzeltutoriat Forschung 2007

Gruppentutor Themenblock Infektion und Abwehr 2008
Betreuung einer Masterstudentin im Hirsch Lab 2008

6. MEDIZINISCHE DISSERTATION

Suizidalität und Depression: Suizide in der Familie und der sozialen Umgebung. (Promotion Frühjahr 2005) bei Prof. Dr. med. Asmus Finzen

7. PHILOSOPHISCH-NATURWISSENSCHAFTLICHE DISSERTATION

Cytomegalovirus und Polyomavirus (BKV) - spezifische zelluläre Immunantwort bei gesunden Blutspendern und nierentransplantierten Patienten bei Prof. Dr. phil Antonius Rolink und Prof. Dr. med. Hans H. Hirsch

8. MITGLIEDSCHAFTEN

- Verein der Schweizerischen Assistenz- und Oberärzte (VSAO, seit 2004)
- Verein der Basler Assistenz- und Oberärzte (VBAO, seit 2004)
- Federation Medicorum Helveticorum (FMH, seit 2004)
- Alumini der Universität Basel (seit 2004)
- Alumni der Medizinischen Fakultät Basel (seit 2004)
- Swiss MD PhD Association (SMPA, seit 2005)
- Amercian Society of Transplantation (AST, seit 2006)

9. REFERENZEN:

Prof. Dr. med. Hans H. Hirsch, Transplantationsvirologie, Unispital Basel
Prof. Dr. med. Manuel Battegay, Chefarzt Infektiologie, Unispital Basel
Prof. Dr. med. Jürg Steiger, Chefarzt Nephrologie, Unispital Basel
PD Dr. med. Nicolas Müller, Oberarzt Infektiologie, Unispital Zürich
Frau Dr. med. A. Koelz, ehemalige Chefärztin Innere Medizin, Spital Dornach

10. GRANTS

Lichtenstein Stiftung Basel, 2006-2008 52000.-

11. BISHERIGE WISSENSCHAFTLICHE TÄTIGKEITEN

A) Artikel

2006:
Egli A., Meier M., Kurth F., Simonius F., Koelz A.
Steinharte Beine – massive dystrophe Kalzinose. Swiss med Forum 6, 485, 2006.

Binggeli S., **Egli A.,** Dickenmann M., Binet I., Steiger J., Hirsch H. H.
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2007:
Egli A., Binggeli S., Bodaghi S., Dumoulin A., Funk G. A., Khanna N., Leuenberger D., Gosert R., Hirsch H. H.
Cytomegalovirus and polyomavirus BK posttransplant. Nephrol Dialysis Transpl 2007 22:(Supl8) 72-82.

Binggeli S., **Egli A.,** Schaub S., Binet I., Mayr M., Steiger J., Hirsch H. H.
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Schaub S., Mayr M., **Egli A.,** Binggeli S., Desceudres B, Steiger J., Mihatsch M., Hirsch H.H.
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2008:
Egli A., Bergamin O., Müllhaupt B., Seebach J.D., Müller N.J., Hirsch H.H.

Cytomegalovirus-associated chorioretinits after liver transplantation: case report and review of the literature. Transpl Infect Dis. 2008 Feb;10(1):27-43. Epub 2007 Dec 17

Egli A., Binet I., Jäger C., Dumoulin A., Steiger J., Sester, U., Sester M., Hirsch H.H. Cytomegalovirus-specific immune response in R(+) kidney transplanted patients with CMV replication, J Trans Med, 2008, 6:29: doi:10.1186/1479-5876-6-29

Egli A., Dumoulin A,. Kiss, D., Hirsch H.H.
Neue Diagnostik in der Transplantationsmedizin: Cytomegalovirus (CMV)-spezifische T-Zellfrequenz und Ganciclovir-Resistenzanalyse. Swiss Medical Forum 2008

Egli A., Dumoulin A., Köhli S., Hirsch H.H.
Polyomavirus BK after kidney transplantation – Role of molecular and immunological markers, Trends in Transplantation 2008 (in press)

Gosert R., Rinaldo, Ch.H., Funk G.A., **Egli A.,** Ramos E., Drachenberg C.B., Hirsch H.H.
Polyomavirus BK with rearranged non-coding control region emerge *in vivo* in renal transplant patients and increase viral replication and cytopathology. J Exp Med. 2008 Apr 14;205(4):841-52. Epub 2008 Mar 17

Provenzano M., Sais G., Bracci L., **Egli A.,** Anselmi M., Viehl C.,,Schaub S., Hirsch H.H., Marincola F.M., Spagnoli G.C.
A universal HLA class I and II resticted immunogenic polypeptide from HCMV pp65 promotes the expansion of CD4++ and CD8++ effector T-cells across a wider range of HLA specificities. (JCM 2008, in press)

Egli A., Stebler Ch., Infanti, L., Dumoulin A., Buser A., Samaridis J., Bodaghi S., Gosert R., Hirsch H.H.
Polyomavirus BK (BKV) and JC (JCV) replication in plasma and urine of healthy blood donors (JID 2008 in press)

B) Kongressbeiträge

2006:

14th Immunocompromised Host Society Meeting, Crans Montana, Switzerland (2 Poster):

- **Egli. A., Müllhaupt, B., Seebach, J. D., Müller, N. J., Hirsch, H. H.**
  CMV specific T cells in a liver transplant recipient with severe CMV retinitis and immune reconstitution syndrome.
- **Egli, A., Binggeli, S., Steiger, J., Binet, I., Hirsch, H. H.**
  CMV specific T cells in seropositive kidney transplant patients with recurrence.

World Transplantation Congress (WTC), Boston, USA (1 Oral Presentation):

- **Egli, A., Binggeli, S., Steiger, J., Binet, I., Hirsch, H. H.**
  CMV-specific T-cells in seropositive kidney transplant patients with recurrence.

2007:

Biovalley Meeting, Basel, Switzerland (2 Poster):

- **Egli A., Jäger C., Binggeli S., Binet I., Dumoulin A., Mayr M., Schaub S., Steiger J., Sester M., Hirsch H.H.**

Cytomegalovirus (CMV)-specific T-cells and CMV replication in kidney transplant patients

- **Egli A., Bodaghi S., Gosert R., Samaridis J., Stebler Ch., Infanti L., Hirsch H.H.**
  Polyomavirus BK (BKV) and JC (JCV) replication in plasma and urine of healthy blood donors

Jahresversammlung Schweizerische Gesellschaft für Allergologie und Immunologie, Basel, Switzerland (1 Oral Presentation):

- **Egli A., Dumoulin A., Binggeli S., Funk G.A., Funk G.A., Schaub S., Mayr M., Sester M., Jäger C., Binet I., Steiger J., Hirsch H.H.**
  Cytomegalovirus-specific T-cells in Seropositive Kidney Transplant Patients with CMV Replication

Jahresversammlung Schweizerische Gesellschaft für Infektiologie und Pädiatrie, Zürich, Switzerland (1 Awarded Poster und 1 Oral Presentation)

- **Egli A., Jäger C., Binggeli S., Binet I., Dumoulin A., Mayr M., Schaub S., Steiger J., Sester M., Hirsch H.H.**
  Cytomegalovirus (CMV)-specific T-cells and CMV replication in kidney transplant patients

16th Meeting of Paediatric Research of Central European Countries, Innsbruck, Austria (2h Lecture on Molecular Techniques in Pediatric Research)

- **Egli A., Hirsch H.H.**
  FACS Analysis for Virus-specific cellular Immunity

European Virology Congress, Nürnberg, Germany (1 Poster)

- **Egli A., Jäger C., Binggeli S., Binet I., Dumoulin A., Mayr M., Schaub S., Steiger J., Sester M., Hirsch H.H.**
  Cytomegalovirus (CMV)-specific T-cells and CMV replication in kidney transplant patients

4th International Polyomavirus Congress, Barcelona, Spain (1 Oral Presentation)

- **Egli A., Stebler Ch., Infanti L., Bodaghi S., Gosert R., Hirsch H.H.**
  Polyomavirus BK (BKV) and JC (JCV) replication in plasma and urine in healthy blood donors (BD)

2008:

KIT2008, Innsbruck, Austria (1 Oral Presentation)

- **Egli A., Stebler Ch., Infanti L., Bodaghi S., Gosert R., Hirsch H.H.**
  Polyomavirus BK (BKV) and JC (JCV) replication in plasma and urine in healthy blood donors (BD)

ATC Toronto 2008 (1 Poster)

- **Egli A., Stebler Ch., Infanti L., Bodaghi S., Gosert R., Hirsch H.H.**
  Polyomavirus BK (BKV) and JC (JCV) replication in plasma and urine in healthy blood donors (BD)

C) Vorträge/Seminare

2006:

Tuesday Seminar (24. Oktober), Institut für Medizinische Mikrobiologie
Cytomegalovirus-specific T cells in Seropositive Kidney Transplant Patients with Recurrence

2007:
Dienstagsclub Infektiologie (6. November), Universitätsspital Basel
Cytomegalovirus-spezifische T-Zell Immunität nach Nierentransplantation: Implikationen für Prophylaxe und Management

Nephrologie Fortbildung (19. November), Universitätsspital Basel
Late Cytomegalovirus reactivation after kidney transplantation: New diagnostic approaches in routine care

2008:
Tuesday Seminar (24. April), Institut für Medizinische Mikrobiologie
Polyomavirus BKV-specific cellular T-cells i- approaches to epitope mapping

D) Publizierte Abstracts

2006:
Egli A., Müllhaupt B., Seebach J. D., Müller N. J., Hirsch H. H.
CMV specific T cells in a liver transplant recipient with severe CMV retinitis and immune reconstitution syndrome (Abstract 32). Int J Inf Dis 10, (S1), 17, 2006.

Egli A., Binggeli S., Steiger J., Binet I., Hirsch H. H.
CMV specific T cells in seropositive kidney transplant patients with recurrence (Abstract 23).
Int J Inf Dis 10, (S1), 13, 2006.
Egli A., Binggeli S., Steiger J., Binet I., Hirsch H. H.
Cytomegalovirus-specific T-cells in seropositive kidney transplant patients with recurrence (Abstract 459). Transpl 82,1 (S3), 267-8, 2006.

2007:
Egli A., Dumoulin A., Binggeli S., Sester M., Binet I., Steiger J., Hirsch H.H.
Cytomegalovirus-specific T cells in seropositive kidney transplant patients with recurrence. Swiss Med Weekly 2007 137(S7)

2008:
Egli A., Stebler Ch., Infanti L., Bodaghi S., Gosert R., Hirsch H.H.
Polyomavirus BK (BKV) and JC (JCV) replication in plasma and urine in healthy blood donors Am J Transpl, 20

10. Appendices

1. Peripheral blood mononuclear cells

Gain PBMCs from CPT tubes (spin 1650g for 20' and wash twice with PBS) or lymphprep separation (spin 800g 20' and wash twice) as described in the manufactures protocols.

2. Stimulation of the PBMCs

Stimulation always in dull polypropylen FACS tubes

Pre-lay 1ug/mL aCD28 and 1ug/mL aCD49d (10uL per tube)
Per stimulation 0.5 - 2 Mio PBMCs dissolved in 500uL R5 are used

Add antigen

SEB (100ug/mL)	neg	peptides (100ug/mL)	Lysate
10uL per tube (ec 2ug/mL)	-	10uL per tube (ec 2ug/mL)	16uL

Caveat: Virus lysate often has a very varying concentration (1-5mg/mL) and batches should be tested out, as well as control lysates from corresponding cell cultures.
If peptides are used add Brefeldin A right from the beginning (10ug/mL ec). Stock 500ug/mL, add 10uL per tube

Vortex

Incubation for 30' 37°C at 7%CO_2
Vortex
Spin 5' at 1100rpm

Incubation for 1h 30' at 37°C at 7%CO_2
Add 10ug/mL (ec) BFA to lysate stimulated tubes

Vortex

Incubation overnight at 37°C at 7% CO_2

3. Fixation of the cells

Prewarm 4% PFA
Add 100uL per tube EDTA (20mM)
Vortex each tube for 10''(!)

Incubation for 15' at room temperature

Add 2mL FACS wash

Spin 10’ 450g at RT

Discard supernatent with vacuum

Add 300uL 4% PFA to each tube for 5’ (!)

Stop fixation by adding 3mL FACS wash

Spin 10’ 450g at RT

Discard supernatent with vacuum

Dissolve pellet and rest fluid in 200uL 1% PFA

4. Staining

Put cells into new clear FACS tubes

Add 2mL of 0.1% Saponin (Permeabilizing solution)
Cave for FoxP3 staining specific perm solution from the company should be used

Incubate for 10’ at RT

Spin 10’ 450g at RT

Discard supernatent with vacuum

Add antibody solution as indicated in the “staining modul” section.

Incubate in dark at RT for 30-45’

Add 3mL FACS wash

Spin 10’ 450g at RT

Discard supernatent with vacuum

Add 200uL 1%PFA

Measurement at FACS or store at 4°C

Staining modules

Standard (INFγ and IL2)

CD3	(PerCP)	10uL
CD4	(APC Cy7)	5uL
CD8	(PE Cy7)	5uL
CD69	(PE)	20uL
INFγ	(APC)	10uL
IL2	(FITC)	20uL
Saponin	10%	1uL

Memory marker (CD45RA and CCR7)

CD3	(PerCP)	10uL
CD4	(APC Cy7)	5uL
CD8	(PE Cy7)	5uL
CCR7	(PE)	20uL
INFγ	(APC)	10uL
CD45RA	(FITC)	20uL
Saponin	10%	1uL

T regulatory cells (CD25 and IL10)

CD3	(PerCP)	10uL
CD4	(APC Cy7)	5uL
CD25	(PE Cy7)	20uL
INFγ	(APC)	10uL
IL10	(PE)	20uL
Saponin	10%	1uL

Monocytes

CD3	(PerCP)	10uL
CD4	(APC Cy7)	5uL
CD8	(PE Cy7)	5uL
CD14	(APC)	10uL
CD83	(FITC)	20uL
CD86	(PE)	20uL
Saponin	10%	1uL

Direct T-cell measurement protocol - Elispot assay

(Protocol Version 3.0, Dr. Adrian Egli, University of Basel)

1. Coating plate with primary antibody

R5 medium= RPMI, +5% hi human sera, + 1% PenStrep (Gibco) +1% L-Glutamine

Pre-wet PVDF (Millipore MSIPN4510) plates with 40uL 50% EtOH
Incubate for 30sec to 1min
Wash plates 3x with 200ul sterile PBS

Dilute INFγ antibody (Mabtech 1-D1K 1mg/mL) in sterile PBS
=> 1:100 (10ug/mL)
Add 100ul/well
Tap plate
Incubate plate at 4°C overnight (or >3h at 37°C)

2. Blocking

Remove antibody 1-D1L
Wash plates 1x with 100ul sterile PBS
Wash plates 3x with 200ul sterile PBS

Add 200uL R5 medium
Block 3h at 37°C
Remove medium

3. Stimulation

Dilute Antigen according to table below
Distribute 100il antigen per well into PVDF plate

Pos Ctrl (1ug/ml, ec)	neg Ctrl	Antigen (2ug/mL, ec)	Antigen (2ug/mL, ec)
98ul R5 2 ul SEB (100ug/mL)	100ul R5	98ul R10 2ul Antigen (200ug/mL)	92ul R5 8ul Antigen (50ug/mL)

(Cave: if antigen is diluted in DMSO make a serial dilution to detect an inhibitory concentration cut-off level.)

Dilute PBMCs to 2.5x10^6 cells /mL in R5
Distribute 100ul cell suspension into PDVF plate

Incubate at 37°C overnight for 15 to 24h
Cave depending on cytokine release adapt incubation time

4. Secondary Antibody

Remove cells
Wash plates 5x in 200ul PBS + 0.05% Tween 20

Dilute 2nd INFγ antibody (Mabtech 7-B6-Biotin 1mg/mL) 1:1000 (1ug/mL) in PBS + 0.5% FCS
Add 100ul per well
Incubate 2-3h at room temperature

Wash 5x in 200ul PBS + 0.05% Tween 20
Dilute strepatvidin-AP 1:1000 (1ug/mL endconc) in PBS +0.5% FCS
Add 100ul per well
Incubate for 1h

Wash 5x in 200ul in PBS + 0.05% Tween 20
Peal off under-drain
Rinse plate from both side with water
Flick off water

5. Development

11ml Tris ph9.5
200ul BCIP (Calbichem)
100ul NBT (Calbiochem)
Filter 0.45um

Add 100ul per well
Develop for 15min

Stop reaction by rinsing the plate with water
Rinse plate from both side
Flick off water
Air dry plates overnight in dark

Analyse spots next day
Responses are positive if they exceed 3x SD and mean of the negative ctrl.

T-cell expansion protocol - ActMono

(Protocol Version 2.0, Dr. Adrian Egli, University of Basel)

1. Day -1

Monocyte Cultivation

Resuspend PBMC in R5AB
Distribute 3ml of $2x10^6$ PBMC/ml in a 6 well plate
(or 1ml of $2x10^6$ PBMC/ml in a 12 well plate).
Incubate overnight at 37° in 5%CO_2 atmosphere

2. Day 0

Seperation

With 5ml pipette take supernatent away (=RestPBMC), wash once with R5AB and also add it to the RestPBMC.

With 1000ul pipette detache adherent cells (ActMono), wash once with R5AB and also add it to the ActMono. Count both.
Dilute RestPBMC (500ul = 10^6 RestPBMC)

Monocyte pulsing

Resuspend ActMono $4x10^6$/ml

Pulse 500ul of ActMono (=$2x10_6$ cells) with

5ug/ml *complete pool*	10ug/ml *peptide pool or single peptides*
50ul of 50ug/ml	25ul of 200ug/ml
12.5ul of 200ug/ml	

Plate RestPBMC in 24well (at $4x10^6$/ml) 500uL per well.
Incubate in incubator until pulsed ActMonos are added.

Incubate for 2h at 37° at 5% CO_2
Wash in R5AB about 6ml
Centrifuge at RT, 450g, 5min
Resuspend pellet in 5000ul R5AB (=100'000ActMono/500ml)

Stimulation

Transfer 500ul ActMono into 24well plate
Add 500ul RestPBMC (=10^6 cells) -> 1:10 dilution ratio
Incubate für 9d at 37° at 5% CO_2

3. Day 9

Elispot

Transfer cells into 15ml Falcon
Wash the cell with R5AB

Count them. Dilute them according to viral peptides used.
CMV normally has a very well expansion: 100'000 PBMC/well

T-cell expansion protocol - Expansion protocol "Provenzano"

(Protocol Version 2.0, Dr. Adrian Egli, University of Basel - adapted from M. Provenzano, Zurich, Switzerland)

1. Dendritic cell culture

Always 7 days before pulsing T-cells
Day -7 Magnetic separation

Transfer 10 Mio PBMCs in 15mL Falcon tube
Spin 10' 450g at 4°C

Add 900uL MACS wash solution
Add 100uL aCD14 MACS beads

Incubate on ice for 20'
slightly shake after 10' 1x

Add 10mL of MACS wash solution

Spin 10' 450g at 4°C

Dissolve pelett in 5mL

Magnetic separation:
Use MACS separation columns (LS) in MACS separator

Pre-wet columns with 3mL
Load 5mL of magnetic bead-tagged cell solution on to columns
tip: wash again with 5mL to harvest the rest in the Falcon tube
Wash with another 5mL of MACS wash solution
= CD14 negative fraction

Remove magnetic field

Wash LS columns 2x with 5mL MACS wash solution
Use two times punching tool to remove attached cells
= CD14 positive fraction

Spin 10' 450g 10min at RT

Dissolve in 2mL R5 medium
Count

Freeze CD14 negative fraction for usage at Day 0

CD14 positive fraction:
2Mio CD14+ in 4mL DC medium in 6 well plate

Incubate for 7 days at 37°C 7%CO_2

Check for CD14 maturation at day 0 and 7.

Day -1

Add 4uL LPS (1mg/mL) per well
Slightly shake

2. Peptide loading on mDCs

Day 0
Harvest mature DCs for loading with peptides

Transfer into a 15mL Falcon
Pulse 5x10^6 CD14+ cells with 10ug/mL single peptides or 5ug/mL peptide pool for 4 h at 37°C 7%CO_2

After pulsing irradiate DCs 2500rad 610''

Prepare for Day +7 new DC culture with autologous fresh DCs
(same protocol)

3. Stimulation of virus-specific T-cells

Thawn CD14 negative fraction from week before

Repeat magnetic separation with aCD4 and aCD8 magnetic bead antibodies as performed with aCD14

Count CD4 positive and CD8 positive cell fractions

Put 1Mio CD4 or CD8 into a 24 well plate. 1Mio per well per mL R5 medium

Add 0.1-0.2 Mio pulsed DCs to each well.

Remove at **Day 4, 5, 6:** 50% of medium and add fresh medium with 20U IL2/mL
At **day 6**: add 4uL LPS to new DC culture

Day 7: Repeat pulsing of expanding T-cell cultures with mDCs

Remove at **Day 10, 11, 12**: 50% medium and add fresh medium with 100U IL2/mL

Use cells at **Day 14**:
Count and perform FACS or Elispot analysis

T-cell expansion protocol - Expansion protocol "Comoli"

(Protocol Version 2.0, Dr. Adrian Egli, University of Basel - adapted from P. Comoli, Pavia, Italy) GMP approved

1. Day 0

2 Mio PBMC/well/mL in autologous RPMI (standard R5 except 10% heat inactivated and 1700g centrifuged donor plasma) in 24 well plate
Add 10ng/mL (end concentration) of peptide pool into the culture

Incubation for 10days at 37° with 5% CO_2

2. Day 10
Repulse with 1Mio PBMCs (irradiated at 3500rad).
Add 100ng/mL (end concentration) of peptide pool into the culture

Remove 50% of old medium by pipetting half away
Add fresh fresh medium with IL2 (ec 20IE/mL)

3. Day 15, 19, 22, 26

Remove half of medium and add fresh medium with IL2 (ec 20IE/mL)

4. Day 29

Harvest cells at day 29
Use INFγ detection assay

Cytomegalovirus proteins:

CMV pp65 peptide library (15aa long peptides, 11aa overlap)

	EGT code	N-term	Sequence	C-term
1	P0510130	H2N	MESRGRRCPEMISVL	CONH2
2	P0510131	H2N	GRRCPEMISVLGPIS	CONH2
3	P0510132	H2N	PEMISVLGPISGHVL	CONH2
4	P0510133	H2N	SVLGPISGHVLKAVF	CONH2
5	P0510134	H2N	PISGHVLKAVFSRGD	CONH2
6	P0510135	H2N	HVLKAVFSRGDTPVL	CONH2
7	P0510136	H2N	AVFSRGDTPVLPHET	CONH2
8	P0510137	H2N	RGDTPVLPHETRLLQ	CONH2
9	P0510138	H2N	PVLPHETRLLQTGIH	CONH2
10	P0510139	H2N	HETRLLQTGIHVRVS	CONH2
11	P0510140	H2N	LLQTGIHVRVSQPSL	CONH2
12	P0510141	H2N	GIHVRVSQPSLILVS	CONH2
13	P0510142	H2N	RVSQPSLILVSQYTP	CONH2
14	P0510143	H2N	PSLILVSQYTPDSTP	CONH2
15	P0510144	H2N	LVSQYTPDSTPCHRG	CONH2
16	P0510145	H2N	YTPDSTPCHRGDNQL	CONH2
17	P0510146	H2N	STPCHRGDNQLQVQH	CONH2
18	P0510147	H2N	HRGDNQLQVQHTYFT	CONH2
19	P0510148	H2N	NQLQVQHTYFTGSEV	CONH2
20	P0510149	H2N	VQHTYFTGSEVENVS	CONH2
21	P0510150	H2N	YFTGSEVENVSVNVH	CONH2
22	P0510151	H2N	SEVENVSVNVHNPTG	CONH2
23	P0510152	H2N	NVSVNVHNPTGRSIC	CONH2
24	P0510153	H2N	NVHNPTGRSICPSQE	CONH2
25	P0510154	H2N	PTGRSICPSQEPMSI	CONH2
26	P0510155	H2N	SICPSQEPMSIYVYA	CONH2
27	P0510156	H2N	SQEPMSIYVYALPLK	CONH2
28	P0510157	H2N	MSIYVYALPLKMLNI	CONH2
29	P0510158	H2N	VYALPLKMLNIPSIN	CONH2
30	P0510159	H2N	PLKMLNIPSINVHHY	CONH2
31	P0510160	H2N	LNIPSINVHHYPSAA	CONH2
32	P0510161	H2N	SINVHHYPSAAERKH	CONH2
33	P0510162	H2N	HHYPSAAERKHRHLP	CONH2
34	P0510163	H2N	SAAERKHRHLPVADA	CONH2
35	P0510164	H2N	RKHRHLPVADAVIHA	CONH2
36	P0510165	H2N	HLPVADAVIHASGKQ	CONH2
37	P0510166	H2N	ADAVIHASGKQMWQA	CONH2
38	P0510167	H2N	IHASGKQMWQARLTV	CONH2
39	P0510168	H2N	GKQMWQARLTVSGLA	CONH2

40	P0510169	H2N	WQARLTVSGLAWTRQ	CONH2
41	P0510170	H2N	LTVSGLAWTRQQNQW	CONH2
42	P0510171	H2N	GLAWTRQQNQWKEPD	CONH2
43	P0510172	H2N	TRQQNQWKEPDVYYT	CONH2
44	P0510173	H2N	NQWKEPDVYYTSAFV	CONH2
45	P0510174	H2N	EPDVYYTSAFVFPTK	CONH2
46	P0510175	H2N	YYTSAFVFPTKDVAL	CONH2
47	P0510176	H2N	AFVFPTKDVALRHVV	CONH2
48	P0510177	H2N	PTKDVALRHVVCAHE	CONH2
49	P0510178	H2N	VALRHVVCAHELVCS	CONH2
50	P0510179	H2N	HVVCAHELVCSMENT	CONH2
51	P0510180	H2N	AHELVCSMENTRATK	CONH2
52	P0510181	H2N	VCSMENTRATKMQVI	CONH2
53	P0510182	H2N	ENTRATKMQVIGDQY	CONH2
54	P0510183	H2N	ATKMQVIGDQYVKVY	CONH2
55	P0510184	H2N	VIGDQYVKVYLESF	CONH2
56	P0510185	H2N	DQYVKVYLESFCEDV	CONH2
57	P0510186	H2N	KVYLESFCEDVPSGK	CONH2
58	P0510187	H2N	ESFCEDVPSGKLFMH	CONH2
59	P0510188	H2N	EDVPSGKLFMHVTLG	CONH2
60	P0510189	H2N	SGKLFMHVTLGSDVE	CONH2
61	P0510190	H2N	FMHVTLGSDVEEDLT	CONH2
62	P0510191	H2N	TLGSDVEEDLTMTRN	CONH2
63	P0510192	H2N	DVEEDLTMTRNPQPF	CONH2
64	P0510193	H2N	DLTMTRNPQPFMRPH	CONH2
65	P0510194	H2N	TRNPQPFMRPHERNG	CONH2
66	P0510195	H2N	PFMRPHERNGFTVL	CONH2
67	P0510196	H2N	RPHERNGFTVLCPKN	CONH2
68	P0510197	H2N	RNGFTVLCPKNMIIK	CONH2
69	P0510198	H2N	TVLCPKNMIIKPGKI	CONH2
70	P0510199	H2N	PKNMIIKPGKISHIM	CONH2
71	P0510200	H2N	IIKPGKISHIMLDVA	CONH2
72	P0510201	H2N	GKISHIMLDVAFTSH	CONH2
73	P0510202	H2N	HIMLDVAFTSHEHFG	CONH2
74	P0510203	H2N	DVAFTSHEHFGLLCP	CONH2
75	P0510204	H2N	TSHEHFGLLCPKSIP	CONH2
76	P0510205	H2N	HFGLLCPKSIPGLSI	CONH2
77	P0510206	H2N	LCPKSIPGLSISGNL	CONH2
78	P0510207	H2N	SIPGLSISGNLLMNG	CONH2
79	P0510208	H2N	LSISGNLLMNGQQIF	CONH2
80	P0510209	H2N	GNLLMNGQQIFLEVQ	CONH2
81	P0510210	H2N	MNGQQIFLEVQAIRE	CONH2
82	P0510211	H2N	IFLEVQAIRETVEL	CONH2
83	P0510212	H2N	EVQAIRETVELRQYD	CONH2
84	P0510213	H2N	IRETVELRQYDPVAA	CONH2

85	P0510214	H2N	VELRQYDPVAALFFF	CONH2
86	P0510215	H2N	YDPVAALFFFDIDL	CONH2
87	P0510216	H2N	VAALFFFDIDLLLQR	CONH2
88	P0510217	H2N	FFFDIDLLLQRGPQY	CONH2
89	P0510218	H2N	IDLLLQRGPQYSEHP	CONH2
90	P0510219	H2N	LQRGPQYSEHPTFTS	CONH2
91	P0510220	H2N	PQYSEHPTFTSQYRI	CONH2
92	P0510221	H2N	EHPTFTSQYRIQGKL	CONH2
93	P0510222	H2N	FTSQYRIQGKLEYRH	CONH2
94	P0510223	H2N	YRIQGKLEYRHTWDR	CONH2
95	P0510224	H2N	GKLEYRHTWDRHDEG	CONH2
96	P0510225	H2N	YRHTWDRHDEGAAQG	CONH2
97	P0510226	H2N	WDRHDEGAAQGDDDV	CONH2
98	P0510227	H2N	DEGAAQGDDDVWTSG	CONH2
99	P0510228	H2N	AQGDDDVWTSGSDSD	CONH2
100	P0510229	H2N	DDVWTSGSDSDEELV	CONH2
101	P0510230	H2N	TSGSDSDEELVTTER	CONH2
102	P0510231	H2N	DSDEELVTTERKTPR	CONH2
103	P0510232	H2N	ELVTTERKTPRVTGG	CONH2
104	P0510233	H2N	TERKTPRVTGGGAMA	CONH2
105	P0510234	H2N	TPRVTGGGAMAGAST	CONH2
106	P0510235	H2N	TGGGAMAGASTSAGR	CONH2
107	P0510236	H2N	AMAGASTSAGRKRKS	CONH2
108	P0510237	H2N	ASTSAGRKRKSASSA	CONH2
109	P0510238	H2N	AGRKRKSASSATACT	CONH2
110	P0510239	H2N	RKSASSATACTSGVM	CONH2
111	P0510240	H2N	SSATACTSGVMTRGR	CONH2
112	P0510241	H2N	ACTSGVMTRGRLKAE	CONH2
113	P0510242	H2N	GVMTRGRLKAESTVA	CONH2
114	P0510243	H2N	RGRLKAESTVAPEED	CONH2
115	P0510244	H2N	KAESTVAPEEDTDED	CONH2
116	P0510245	H2N	TVAPEEDTDEDSDNE	CONH2
117	P0510246	H2N	EEDTDEDSDNEIHNP	CONH2
118	P0510247	H2N	DEDSDNEIHNPAVFT	CONH2
119	P0510248	H2N	DNEIHNPAVFTWPPW	CONH2
120	P0510249	H2N	HNPAVFTWPPWQAGI	CONH2
121	P0510250	H2N	VFTWPPWQAGILARN	CONH2
122	P0510251	H2N	PPWQAGILARNLVPM	CONH2
123	P0510252	H2N	AGILARNLVPMVATV	CONH2
124	P0510253	H2N	ARNLVPMVATVQGQN	CONH2
125	P0510254	H2N	VPMVATVQGQNLKYQ	CONH2
126	P0510255	H2N	ATVQGQNLKYQEFFW	CONH2
127	P0510256	H2N	GQNLKYQEFFWDAND	CONH2
128	P0510257	H2N	KYQEFFWDANDIYRI	CONH2
129	P0510258	H2N	FFWDANDIYRIFAEL	CONH2

130	P0510259	H2N	ANDIYRIFAELEGVW	CONH2
131	P0510260	H2N	YRIFAELEGVWQPAA	CONH2
132	P0510261	H2N	AELEGVWQPAAQPKR	CONH2
133	P0510262	H2N	GVWQPAAQPKRRRHR	CONH2
134	P0510263	H2N	PAAQPKRRRHRQDAL	CONH2
135	P0510264	H2N	PKRRRHRQDALPGPC	CONH2
136	P0510265	H2N	RHRQDALPGPCIAST	CONH2
137	P0510266	H2N	DALPGPCIASTPKKH	CONH2
138	P0510267	H2N	GPCIASTPKKHRG	COOH

CMV pp72 peptide library (15aa long peptides, 11aa overlap)

	EGT code	N-term	Sequence	C-term
1	P0510821	H2N	MESSAKRKMDPDNPD	CONH2
2	P0510822	H2N	AKRKMDPDNPDEGPS	CONH2
3	P0510823	H2N	MDPDNPDEGPSSKVP	CONH2
4	P0510824	H2N	NPDEGPSSKVPRPET	CONH2
5	P0510825	H2N	GPSSKVPRPETPVTK	CONH2
6	P0510826	H2N	KVPRPETPVTKATTF	CONH2
7	P0510827	H2N	PETPVTKATTFLQTM	CONH2
8	P0510828	H2N	VTKATTFLQTMLRKE	CONH2
9	P0510829	H2N	TTFLQTMLRKEVNSQ	CONH2
10	P0510830	H2N	QTMLRKEVNSQLSLG	CONH2
11	P0510831	H2N	RKEVNSQLSLGDPLF	CONH2
12	P0510832	H2N	NSQLSLGDPLFPELA	CONH2
13	P0510833	H2N	SLGDPLFPELAEESL	CONH2
14	P0510834	H2N	PLFPELAEESLKTFE	CONH2
15	P0510835	H2N	ELAEESLKTFEQVTE	CONH2
16	P0510836	H2N	ESLKTFEQVTEDCNE	CONH2
17	P0510837	H2N	TFEQVTEDCNENPEK	CONH2
18	P0510838	H2N	VTEDCNENPEKDVLA	CONH2
19	P0510839	H2N	CNENPEKDVLAELVK	CONH2
20	P0510840	H2N	PEKDVLAELVKQIKV	CONH2
21	P0510841	H2N	VLAELVKQIKVRVDM	CONH2
22	P0510842	H2N	LVKQIKVRVDMVRHR	CONH2
23	P0510843	H2N	IKVRVDMVRHRIKEH	CONH2
24	P0510844	H2N	VDMVRHRIKEHMLKK	CONH2
25	P0510845	H2N	RHRIKEHMLKKYTQT	CONH2
26	P0510846	H2N	KEHMLKKYTQTEEKF	CONH2
27	P0510847	H2N	LKKYTQTEEKFTGAF	CONH2
28	P0510848	H2N	TQTEEKFTGAFNMMG	CONH2
29	P0510849	H2N	EKFTGAFNMMGGCLQ	CONH2

30	P0510850	H2N	GAFNMMGGCLQNALD	CONH2
31	P0510851	H2N	MMGGCLQNALDILDK	CONH2
32	P0510852	H2N	CLQNALDILDKVHEP	CONH2
33	P0510853	H2N	ALDILDKVHEPFEEM	CONH2
34	P0510854	H2N	LDKVHEPFEEMKCIG	CONH2
35	P0510855	H2N	HEPFEEMKCIGLTMQ	CONH2
36	P0510856	H2N	EEMKCIGLTMQSMYE	CONH2
37	P0510857	H2N	CIGLTMQSMYENYIV	CONH2
38	P0510858	H2N	TMQSMYENYIVPEDK	CONH2
39	P0510859	H2N	MYENYIVPEDKREMW	CONH2
40	P0510860	H2N	YIVPEDKREMWMACI	CONH2
41	P0510861	H2N	EDKREMWMACIKELH	CONH2
42	P0510862	H2N	EMWMACIKELHDVSK	CONH2
43	P0510863	H2N	ACIKELHDVSKGAAN	CONH2
44	P0510864	H2N	ELHDVSKGAANKLGG	CONH2
45	P0510865	H2N	VSKGAANKLGGALQA	CONH2
46	P0510866	H2N	AANKLGGALQAKARA	CONH2
47	P0510867	H2N	LGGALQAKARAKKDE	CONH2
48	P0510868	H2N	LQAKARAKKDELRRK	CONH2
49	P0510869	H2N	ARAKKDELRRKMMYM	CONH2
50	P0510870	H2N	KDELRRKMMYMCYRN	CONH2
51	P0510871	H2N	RRKMMYMCYRNIEFF	CONH2
52	P0510872	H2N	MYMCYRNIEFFTKNS	CONH2
53	P0510873	H2N	YRNIEFFTKNSAFPK	CONH2
54	P0510874	H2N	EFFTKNSAFPKTTNG	CONH2
55	P0510875	H2N	KNSAFPKTTNGCSQA	CONH2
56	P0510876	H2N	FPKTTNGCSQAMAAL	CONH2
57	P0510877	H2N	TNGCSQAMAALQNLP	CONH2
58	P0510878	H2N	SQAMAALQNLPQCSP	CONH2
59	P0510879	H2N	AALQNLPQCSPDEIM	CONH2
60	P0510880	H2N	NLPQCSPDEIMAYAQ	CONH2
61	P0510881	H2N	CSPDEIMAYAQKIFK	CONH2
62	P0510882	H2N	EIMAYAQKIFKILDE	CONH2
63	P0510883	H2N	YAQKIFKILDEERDK	CONH2
64	P0510884	H2N	IFKILDEERDKVLTH	CONH2
65	P0510885	H2N	LDEERDKVLTHIDHI	CONH2
66	P0510886	H2N	RDKVLTHIDHIFMDI	CONH2
67	P0510887	H2N	LTHIDHIFMDILTTC	CONH2
68	P0510888	H2N	DHIFMDILTTCVETM	CONH2
69	P0510889	H2N	MDILTTCVETMCNEY	CONH2
70	P0510890	H2N	TTCVETMCNEYKVTS	CONH2
71	P0510891	H2N	ETMCNEYKVTSDACM	CONH2
72	P0510892	H2N	NEYKVTSDACMMTMY	CONH2
73	P0510893	H2N	VTSDACMMTMYGGIS	CONH2
74	P0510894	H2N	ACMMTMYGGISLLSE	CONH2

75	P0510895	H2N	TMYGGISLLSEFCRV	CONH2
76	P0510896	H2N	GISLLSEFCRVLCCY	CONH2
77	P0510897	H2N	LSEFCRVLCCYVLEE	CONH2
78	P0510898	H2N	CRVLCCYVLEETSVM	CONH2
79	P0510899	H2N	CCYVLEETSVMLAKR	CONH2
80	P0510900	H2N	LEETSVMLAKRPLIT	CONH2
81	P0510901	H2N	SVMLAKRPLITKPEV	CONH2
82	P0510902	H2N	AKRPLITKPEVISVM	CONH2
83	P0510903	H2N	LITKPEVISVMKRRI	CONH2
84	P0510904	H2N	PEVISVMKRRIEEIC	CONH2
85	P0510905	H2N	SVMKRRIEEICMKVF	CONH2
86	P0510906	H2N	RRIEEICMKVFAQYI	CONH2
87	P0510907	H2N	EICMKVFAQYILGAD	CONH2
88	P0510908	H2N	KVFAQYILGADPLRV	CONH2
89	P0510909	H2N	QYILGADPLRVCSPS	CONH2
90	P0510910	H2N	GADPLRVCSPSVDDL	CONH2
91	P0510911	H2N	LRVCSPSVDDLRAIA	CONH2
92	P0510912	H2N	SPSVDDLRAIAEESD	CONH2
93	P0510913	H2N	DDLRAIAEESDEEEA	CONH2
94	P0510914	H2N	AIAEESDEEEAIVAY	CONH2
95	P0510915	H2N	ESDEEEAIVAYTLAT	CONH2
96	P0510916	H2N	EEAIVAYTLATAGVS	CONH2
97	P0510917	H2N	VAYTLATAGVSSSDS	CONH2
98	P0510918	H2N	LATAGVSSSDSLVSP	CONH2
99	P0510919	H2N	GVSSSDSLVSPPESP	CONH2
100	P0510920	H2N	SDSLVSPPESPVPAT	CONH2
101	P0510921	H2N	VSPPESPVPATIPLS	CONH2
102	P0510922	H2N	ESPVPATIPLSSVIV	CONH2
103	P0510923	H2N	PATIPLSSVIVAENS	CONH2
104	P0510924	H2N	PLSSVIVAENSDQEE	CONH2
105	P0510925	H2N	VIVAENSDQEESEQS	CONH2
106	P0510926	H2N	ENSDQEESEQSDEEE	CONH2
107	P0510927	H2N	QEESEQSDEEEEEGA	CONH2
108	P0510928	H2N	EQSDEEEEEGAQEER	CONH2
109	P0510929	H2N	EEEEEGAQEEREDTV	CONH2
110	P0510930	H2N	EGAQEEREDTVSVKS	CONH2
111	P0510931	H2N	EEREDTVSVKSEPVS	CONH2
112	P0510932	H2N	DTVSVKSEPVSEIEE	CONH2
113	P0510933	H2N	VKSEPVSEIEEVAPE	CONH2
114	P0510934	H2N	PVSEIEEVAPEEEED	CONH2
115	P0510935	H2N	IEEVAPEEEEDGAEE	CONH2
116	P0510936	H2N	APEEEEDGAEEPTAS	CONH2
117	P0510937	H2N	EEDGAEEPTASGGKS	CONH2
118	P0510938	H2N	AEEPTASGGKSTHPM	CONH2
119	P0510939	H2N	TASGGKSTHPMVTRS	CONH2

120	P0510940	H2N	GKSTHPMVTRSKADQ	COOH

CMV pp65 RQY 16mer

RQYDPVAALFFFDIDL

CMV pp65 RQY spanning 9mers

RQYDPVAAL
QYDPVAALF
YDPVAALFF
DPVAALFFF
PVAALFFFD
VAALFFFDI
AALFFFDID
ALFFFDIDL

Polyomavirus BK proteins:

BKV LT peptide library (15aa long peptides, 11aa overlap)

	EGT	N-term	Sequence	C-term
89	P0510627	H2N	MDKVLNREESMELMD	CONH2
90	P0510628	H2N	LNREESMELMDLLGL	CONH2
91	P0510629	H2N	ESMELMDLLGLERAA	CONH2
92	P0510630	H2N	LMDLLGLERAAWGNL	CONH2
93	P0510631	H2N	LGLERAAWGNLPLMR	CONH2
94	P0510632	H2N	RAAWGNLPLMRKAYL	CONH2
95	P0510633	H2N	GNLPLMRKAYLRKCK	CONH2
96	P0510634	H2N	LMRKAYLRKCKEFHP	CONH2
97	P0510635	H2N	AYLRKCKEFHPDKGG	CONH2
98	P0510636	H2N	KCKEFHPDKGGDEDK	CONH2
99	P0510637	H2N	FHPDKGGDEDKMKRM	CONH2
100	P0510638	H2N	KGGDEDKMKRMNTLY	CONH2
101	P0510639	H2N	EDKMKRMNTLYKKME	CONH2
102	P0510640	H2N	KRMNTLYKKMEQDVK	CONH2
103	P0510641	H2N	TLYKKMEQDVKVAHQ	CONH2
104	P0510642	H2N	KMEQDVKVAHQPDFG	CONH2
105	P0510643	H2N	DVKVAHQPDFGTWSS	CONH2
106	P0510644	H2N	AHQPDFGTWSSSEVP	CONH2
107	P0510645	H2N	DFGTWSSSEVPTYGT	CONH2
108	P0510646	H2N	WSSSEVPTYGTEEWE	CONH2
109	P0510647	H2N	EVPTYGTEEWESWWS	CONH2
110	P0510648	H2N	YGTEEWESWWSSFNE	CONH2
111	P0510649	H2N	EWESWWSSFNEKWDE	CONH2
112	P0510650	H2N	WWSSFNEKWDEDLFC	CONH2
113	P0510651	H2N	FNEKWDEDLFCHEDM	CONH2
114	P0510652	H2N	WDEDLFCHEDMFASD	CONH2
115	P0510653	H2N	LFCHEDMFASDEEAT	CONH2
116	P0510654	H2N	EDMFASDEEATADSQ	CONH2
117	P0510655	H2N	ASDEEATADSQHSTP	CONH2
118	P0510656	H2N	EATADSQHSTPPKKK	CONH2
119	P0510657	H2N	DSQHSTPPKKKRKVE	CONH2
120	P0510658	H2N	STPPKKKRKVEDPKD	CONH2
121	P0510659	H2N	KKKRKVEDPKDFPSD	CONH2
122	P0510660	H2N	KVEDPKDFPSDLHQF	CONH2
123	P0510661	H2N	PKDFPSDLHQFLSQA	CONH2
124	P0510662	H2N	PSDLHQFLSQAVFSN	CONH2
125	P0510663	H2N	HQFLSQAVFSNRTLA	CONH2
126	P0510664	H2N	SQAVFSNRTLACFAV	CONH2
127	P0510665	H2N	FSNRTLACFAVYTTK	CONH2

128	P0510666	H2N	TLACFAVYTTKEKAQ	CONH2
129	P0510667	H2N	FAVYTTKEKAQILYK	CONH2
130	P0510668	H2N	TTKEKAQILYKKLME	CONH2
131	P0510669	H2N	KAQILYKKLMEKYSV	CONH2
132	P0510670	H2N	LYKKLMEKYSVTFIS	CONH2
133	P0510671	H2N	LMEKYSVTFISRHMC	CONH2
134	P0510672	H2N	YSVTFISRHMCAGHN	CONH2
135	P0510673	H2N	FISRHMCAGHNIIFF	CONH2
136	P0510674	H2N	HMCAGHNIIFFLTPH	CONH2
137	P0510675	H2N	GHNIIFFLTPHRHRV	CONH2
138	P0510676	H2N	IFFLTPHRHRVSAIN	CONH2
139	P0510677	H2N	TPHRHRVSAINNFCQ	CONH2
140	P0510678	H2N	HRVSAINNFCQKLCT	CONH2
141	P0510679	H2N	AINNFCQKLCTFSFL	CONH2
142	P0510680	H2N	FCQKLCTFSFLICKG	CONH2
143	P0510681	H2N	LCTFSFLICKGVNKE	CONH2
144	P0510682	H2N	SFLICKGVNKEYLLY	CONH2
145	P0510683	H2N	CKGVNKEYLLYSALT	CONH2
146	P0510684	H2N	NKEYLLYSALTRDPY	CONH2
147	P0510685	H2N	LLYSALTRDPYHTIE	CONH2
148	P0510686	H2N	ALTRDPYHTIEESIQ	CONH2
149	P0510687	H2N	DPYHTIEESIQGGLK	CONH2
150	P0510688	H2N	TIEESIQGGLKEHDF	CONH2
151	P0510689	H2N	SIQGGLKEHDFSPEE	CONH2
152	P0510690	H2N	GLKEHDFSPEEPEET	CONH2
153	P0510691	H2N	HDFSPEEPEETKQVS	CONH2
154	P0510692	H2N	PEEPEETKQVSWKLI	CONH2
155	P0510693	H2N	EETKQVSWKLITEYA	CONH2
156	P0510694	H2N	VSWKLITEYAVETK	CONH2
157	P0510695	H2N	KLITEYAVETKCEDV	CONH2
158	P0510696	H2N	EYAVETKCEDVFLLL	CONH2
159	P0510697	H2N	ETKCEDVFLLLGMYL	CONH2
160	P0510698	H2N	EDVFLLLGMYLEFQY	CONH2
161	P0510699	H2N	LLLGMYLEFQYNVEE	CONH2
162	P0510700	H2N	MYLEFQYNVEECKKC	CONH2
163	P0510701	H2N	FQYNVEECKKCQKKD	CONH2
164	P0510702	H2N	VEECKKCQKKDQPYH	CONH2
165	P0510703	H2N	KKCQKKDQPYHFKYH	CONH2
166	P0510704	H2N	KKDQPYHFKYHEKHF	CONH2
167	P0510705	H2N	PYHFKYHEKHFANAI	CONH2
168	P0510706	H2N	KYHEKHFANAIIFAE	CONH2
169	P0510707	H2N	KHFANAIIFAESKNQ	CONH2
170	P0510708	H2N	NAIIFAESKNQKSIC	CONH2
171	P0510709	H2N	FAESKNQKSICQQAV	CONH2
172	P0510710	H2N	KNQKSICQQAVDTVL	CONH2

173	P0510711	H2N	SICQQAVDTVLAKKR	CONH2
174	P0510712	H2N	AVDTVLAKKRVDTL	CONH2
175	P0510713	H2N	TVLAKKRVDTLHMTR	CONH2
176	P0510714	H2N	KKRVDTLHMTREEML	CONH2
177	P0510715	H2N	DTLHMTREEMLTERF	CONH2
178	P0510716	H2N	MTREEMLTERFNHIL	CONH2
179	P0510717	H2N	EMLTERFNHILDKMD	CONH2
180	P0510718	H2N	ERFNHILDKMDLIFG	CONH2
181	P0510719	H2N	HILDKMDLIFGAHGN	CONH2
182	P0510720	H2N	KMDLIFGAHGNAVLE	CONH2
183	P0510721	H2N	IFGAHGNAVLEQYMA	CONH2
184	P0510722	H2N	HGNAVLEQYMAGVAW	CONH2
185	P0510723	H2N	VLEQYMAGVAWLHCL	CONH2
186	P0510724	H2N	YMAGVAWLHCLLPKM	CONH2
187	P0510725	H2N	VAWLHCLLPKMDSVI	CONH2
188	P0510726	H2N	HCLLPKMDSVIFDFL	CONH2
189	P0510727	H2N	PKMDSVIFDFLHCIV	CONH2
190	P0510728	H2N	SVIFDFLHCIVFNVP	CONH2
191	P0510729	H2N	DFLHCIVFNVPKRRY	CONH2
192	P0510730	H2N	CIVFNVPKRRYWLFK	CONH2
122	P0510731	H2N	NVPKRRYWLFKGPID	CONH2
123	P0510732	H2N	RRYWLFKGPIDSGKT	CONH2
124	P0510733	H2N	LFKGPIDSGKTTLAA	CONH2
125	P0510734	H2N	PIDSGKTTLAAGLLD	CONH2
126	P0510735	H2N	GKTTLAAGLLDLCGG	CONH2
127	P0510736	H2N	LAAGLLDLCGGKALN	CONH2
128	P0510737	H2N	LLDLCGGKALNVNLP	CONH2
129	P0510738	H2N	CGGKALNVNLPMERL	CONH2
130	P0510739	H2N	ALNVNLPMERLTFEL	CONH2
131	P0510740	H2N	NLPMERLTFELGVAI	CONH2
132	P0510741	H2N	ERLTFELGVAIDQYM	CONH2
133	P0510742	H2N	FELGVAIDQYMVVFE	CONH2
134	P0510743	H2N	VAIDQYMVVFEDVKG	CONH2
135	P0510744	H2N	YMVVFEDVKGTGAE	CONH2
136	P0510745	H2N	VFEDVKGTGAESKDL	CONH2
137	P0510746	H2N	VKGTGAESKDLPSGH	CONH2
138	P0510747	H2N	GAESKDLPSGHGINN	CONH2
139	P0510748	H2N	KDLPSGHGINNLDSL	CONH2
140	P0510749	H2N	SGHGINNLDSLRDYL	CONH2
141	P0510750	H2N	INNLDSLRDYLDGSV	CONH2
142	P0510751	H2N	DSLRDYLDGSVKVNL	CONH2
143	P0510752	H2N	DYLDGSVKVNLEKKH	CONH2
144	P0510753	H2N	GSVKVNLEKKHLNKR	CONH2
145	P0510754	H2N	VNLEKKHLNKRTQIF	CONH2
146	P0510755	H2N	KKHLNKRTQIFPPGL	CONH2

147	P0510756	H2N	NKRTQIFPPGLVTMN	CONH2
148	P0510757	H2N	IFPPGLVTMNEYPV	CONH2
149	P0510758	H2N	PGLVTMNEYPVPKTL	CONH2
150	P0510759	H2N	TMNEYPVPKTLQARF	CONH2
151	P0510760	H2N	YPVPKTLQARFVRQI	CONH2
152	P0510761	H2N	KTLQARFVRQIDFRP	CONH2
153	P0510762	H2N	ARFVRQIDFRPKIYL	CONH2
154	P0510763	H2N	RQIDFRPKIYLRKSL	CONH2
155	P0510764	H2N	FRPKIYLRKSLQNSE	CONH2
156	P0510765	H2N	IYLRKSLQNSEFLLE	CONH2
157	P0510766	H2N	KSLQNSEFLLEKRIL	CONH2
158	P0510767	H2N	NSEFLLEKRILQSGM	CONH2
159	P0510768	H2N	LLEKRILQSGMTLLL	CONH2
160	P0510769	H2N	RILQSGMTLLLLLIW	CONH2
161	P0510770	H2N	SGMTLLLLLIWFRPV	CONH2
162	P0510771	H2N	LLLLLIWFRPVADFA	CONH2
163	P0510772	H2N	LIWFRPVADFATDIQ	CONH2
164	P0510773	H2N	RPVADFATDIQSRIV	CONH2
165	P0510774	H2N	DFATDIQSRIVEWKE	CONH2
166	P0510775	H2N	DIQSRIVEWKERLDS	CONH2
167	P0510776	H2N	RIVEWKERLDSEISM	CONH2
168	P0510777	H2N	WKERLDSEISMYTFS	CONH2
169	P0510778	H2N	LDSEISMYTFSRMKY	CONH2
170	P0510779	H2N	ISMYTFSRMKYNICM	CONH2
171	P0510780	H2N	TFSRMKYNICMGKCI	CONH2
172	P0510781	H2N	MKYNICMGKCILDIT	CONH2
173	P0510782	H2N	ICMGKCILDITREED	CONH2
174	P0510783	H2N	KCILDITREEDSETE	CONH2
175	P0510784	H2N	DITREEDSETEDSGH	CONH2
176	P0510785	H2N	EEDSETEDSGHGSST	CONH2
177	P0510786	H2N	ETEDSGHGSSTESQS	CONH2
178	P0510787	H2N	SGHGSSTESQSQCSS	CONH2
179	P0510788	H2N	SSTESQSQCSSQVSD	CONH2
180	P0510789	H2N	SQSQCSSQVSDTSAP	CONH2
181	P0510790	H2N	CSSQVSDTSAPAEDS	CONH2
182	P0510791	H2N	VSDTSAPAEDSQRSD	CONH2
183	P0510792	H2N	SAPAEDSQRSDPHSQ	CONH2
184	P0510793	H2N	EDSQRSDPHSQELHL	CONH2
185	P0510794	H2N	RSDPHSQELHLCKGF	CONH2
186	P0510795	H2N	HSQELHLCKGFQCFK	CONH2
187	P0510796	H2N	LHLCKGFQCFKRPKT	CONH2
188	P0510797	H2N	KGFQCFKRPKTPPPK	CONH2
189	P0510798	H2N	DTSAPAEDSQRSDPH	CONH2
190	P0510799	H2N	APAEDSQRSDPHSQE	CONH2
191	P0510800	H2N	EDSQRSDPHSQELHL	CONH2

192	P0510801	H2N	RSDPHSQELHLCKG	CONH2
193	P0510802	H2N	DPHSQELHLCKGFQC	CONH2
194	P0510803	H2N	SQELHLCKGFQCFKR	CONH2
195	P0510804	H2N	LHLCKGFQCFKRPKT	CONH2
196	P0510805	H2N	CKGFQCFKRPKTPPP	CONH2
197	P0510806	H2N	FQCFKRPKTPPPK	COOH

BKV LT peptide library sub-pools A-R, 1-20

BKV-LT 10x10 Pool (100 ug/ml)

POOL	1	2	3	4	5	6	7	8	9	10
A	89	90	91	92	93	94	95	96	97	98
B	99	100	101	102	103	104	105	106	107	108
C	109	110	111	112	113	114	115	116	117	118
D	119	120	121	122	123	124	125	126	127	128
E	129	130	131	132	133	134	135	136	137	138
F	139	140	141	142	143	144	145	146	147	148
G	149	150	151	152	153	154	155	156	157	158
H	159	160	161	162	163	164	165	166	167	168
I	169	170	171	172	173	174	175	176	177	178
J	179	180	181	182	183	184	185	186	187	188

BKV-LT 10x10 Pool (100 ug/ml)

POOL	11	12	13	14	15	16	17	18	19	20
K	189	190	191	192	193	194	195	196	197	198
L	199	200	201	202	203	204	205	206	207	208
M	209	210	211	212	213	214	215	216	217	218
N	219	220	221	222	223	224	225	226	227	228
O	229	230	231	232	233	234	235	236	237	238
P	239	240	241	242	243	244	245	246	247	248
Q	249	250	251	252	253	254	255	256	257	258
R	259	260	261	262	263	264	265	266	267	268

BKV VP1 peptide library (15aa long peptides, 11aa overlap)

	EGT	N-term	Sequence	C-Term
1	P0510539	H2N	MAPTKRKGECPGAAP	CONH2
2	P0510540	H2N	KRKGECPGAAPKKPK	CONH2
3	P0510541	H2N	ECPGAAPKKPKEPVQ	CONH2
4	P0510542	H2N	AAPKKPKEPVQVPKL	CONH2
5	P0510543	H2N	KPKEPVQVPKLLIKG	CONH2
6	P0510544	H2N	PVQVPKLLIKGGVEV	CONH2
7	P0510545	H2N	PKLLIKGGVEVLEVK	CONH2
8	P0510546	H2N	IKGGVEVLEVKTGVD	CONH2

9	P0510547	H2N	VEVLEVKTGVDAITE	CONH2
10	P0510548	H2N	EVKTGVDAITEVECF	CONH2
11	P0510549	H2N	GVDAITEVECFLNPE	CONH2
12	P0510550	H2N	ITEVECFLNPEMGDP	CONH2
13	P0510551	H2N	ECFLNPEMGDPDENL	CONH2
14	P0510552	H2N	NPEMGDPDENLRGFS	CONH2
15	P0510553	H2N	GDPDENLRGFSLKLS	CONH2
16	P0510554	H2N	ENLRGFSLKLSAEND	CONH2
17	P0510555	H2N	GFSLKLSAENDFSSD	CONH2
18	P0510556	H2N	KLSAENDFSSDSPER	CONH2
19	P0510557	H2N	ENDFSSDSPERKMLP	CONH2
20	P0510558	H2N	SSDSPERKMLPCYST	CONH2
21	P0510559	H2N	PERKMLPCYSTARIP	CONH2
22	P0510560	H2N	MLPCYSTARIPLPNL	CONH2
23	P0510561	H2N	YSTARIPLPNLNEDL	CONH2
24	P0510562	H2N	RIPLPNLNEDLTCGN	CONH2
25	P0510563	H2N	PNLNEDLTCGNLLMW	CONH2
26	P0510564	H2N	EDLTCGNLLMWEAVT	CONH2
27	P0510565	H2N	CGNLLMWEAVTVQTE	CONH2
28	P0510566	H2N	LMWEAVTVQTEVIGI	CONH2
29	P0510567	H2N	AVTVQTEVIGITSML	CONH2
30	P0510568	H2N	TEVIGITSMLNLHA	CONH2
31	P0510569	H2N	IGITSMLNLHAGSQK	CONH2
32	P0510570	H2N	SMLNLHAGSQKVHEH	CONH2
33	P0510571	H2N	LHAGSQKVHEHGGGK	CONH2
34	P0510572	H2N	SQKVHEHGGGKPIQG	CONH2
35	P0510573	H2N	HEHGGGKPIQGSNFH	CONH2
36	P0510574	H2N	GGKPIQGSNFHFFAV	CONH2
37	P0510575	H2N	IQGSNFHFFAVGGEP	CONH2
38	P0510576	H2N	NFHFFAVGGEPLEMQ	CONH2
39	P0510577	H2N	FAVGGEPLEMQGVLM	CONH2
40	P0510578	H2N	GEPLEMQGVLMNYRS	CONH2
41	P0510579	H2N	EMQGVLMNYRSKYPD	CONH2
42	P0510580	H2N	VLMNYRSKYPDGTIT	CONH2
43	P0510581	H2N	YRSKYPDGTITPKNP	CONH2
44	P0510582	H2N	YPDGTITPKNPTAQS	CONH2
45	P0510583	H2N	TITPKNPTAQSQVMN	CONH2
46	P0510584	H2N	KNPTAQSQVMNTDHK	CONH2
47	P0510585	H2N	AQSQVMNTDHKAYLD	CONH2
48	P0510586	H2N	VMNTDHKAYLDKNNA	CONH2
49	P0510587	H2N	DHKAYLDKNNAYPVE	CONH2
50	P0510588	H2N	YLDKNNAYPVECWVP	CONH2
51	P0510589	H2N	NNAYPVECWVPDPSR	CONH2
52	P0510590	H2N	PVECWVPDPSRNENA	CONH2
53	P0510591	H2N	WVPDPSRNENARYFG	CONH2

54	P0510592	H2N	PSRNENARYFGTFTG	CONH2
55	P0510593	H2N	ENARYFGTFTGGENV	CONH2
56	P0510594	H2N	YFGTFTGGENVPPVL	CONH2
57	P0510595	H2N	FTGGENVPPVLHVTN	CONH2
58	P0510596	H2N	ENVPPVLHVTNTATT	CONH2
59	P0510597	H2N	PVLHVTNTATTVLLD	CONH2
60	P0510598	H2N	VTNTATTVLLDEQGV	CONH2
61	P0510599	H2N	ATTVLLDEQGVGPLC	CONH2
62	P0510600	H2N	LLDEQGVGPLCKADS	CONH2
63	P0510601	H2N	GVGPLCKADSLYVS	CONH2
64	P0510602	H2N	PLCKADSLYVSAADI	CONH2
65	P0510603	H2N	ADSLYVSAADICGLF	CONH2
66	P0510604	H2N	YVSAADICGLFTNSS	CONH2
67	P0510605	H2N	ADICGLFTNSSGTQQ	CONH2
68	P0510606	H2N	GLFTNSSGTQQWRGL	CONH2
69	P0510607	H2N	NSSGTQQWRGLARYF	CONH2
70	P0510608	H2N	TQQWRGLARYFKIRL	CONH2
71	P0510609	H2N	RGLARYFKIRLRKRS	CONH2
72	P0510610	H2N	RYFKIRLRKRSVKNP	CONH2
73	P0510611	H2N	IRLRKRSVKNPYPIS	CONH2
74	P0510612	H2N	KRSVKNPYPISFLLS	CONH2
75	P0510613	H2N	KNPYPISFLLSDLIN	CONH2
76	P0510614	H2N	PISFLLSDLINRRTQ	CONH2
77	P0510615	H2N	LLSDLINRRTQRVDG	CONH2
78	P0510616	H2N	LINRRTQRVDGQPMY	CONH2
79	P0510617	H2N	RTQRVDGQPMYGMES	CONH2
80	P0510618	H2N	VDGQPMYGMESQVEE	CONH2
81	P0510619	H2N	PMYGMESQVEEVRVF	CONH2
82	P0510620	H2N	MESQVEEVRVFDGTE	CONH2
83	P0510621	H2N	VEEVRVFDGTERLPG	CONH2
84	P0510622	H2N	RVFDGTERLPGDPDM	CONH2
85	P0510623	H2N	GTERLPGDPDMIRYI	CONH2
86	P0510624	H2N	LPGDPDMIRYIDKQG	CONH2
87	P0510625	H2N	PDMIRYIDKQGQLQT	CONH2
88	P0510626	H2N	RYIDKQGQLQTKML	COOH

BKV agno peptide library (15aa long peptides, 11aa overlap)

	EGT	N-Term	Sequence	C-Term
198	P0510807	H2N	MVLRQLSRQASVKVG	CONH2
199	P0510808	H2N	LSRQASVKVGKTWT	CONH2
200	P0510809	H2N	ASVKVGKTWTGTKK	CONH2

201	P0510810	H2N	KVGKTWTGTKKRAQR	CONH2
202	P0510811	H2N	TWTGTKKRAQRIFIF	CONH2
203	P0510812	H2N	TKKRAQRIFIFILEL	CONH2
204	P0510813	H2N	AQRIFIFILELLLEF	CONH2
205	P0510814	H2N	FIFILELLLEFCRGE	CONH2
206	P0510815	H2N	LELLLEFCRGEDSVD	CONH2
207	P0510816	H2N	LEFCRGEDSVDGKNK	CONH2
208	P0510817	H2N	RGEDSVDGKNKSTTA	CONH2
209	P0510818	H2N	SVDGKNKSTTALPAV	CONH2
210	P0510819	H2N	KNKSTTALPAVKDSV	CONH2
211	P0510820	H2N	TTALPAVKDSVKDS	COOH

BKV LT predicted 15 mere

Pos	N-Term	Sequence	C-Term
010 to 033	H2N	LERAAWGNLPLMRKA	CONH2
026 to 040	H2N	NLPLMRKAYLRKCKE	CONH2
145 to 159	H2N	HQFLSQAVFSNRTLA	CONH2
153 to 167	H2N	FSNRTLACFAVYTTK	CONH2
172 to 186	H2N	ILYKKLMEKYSVTFI	CONH2
190 to 204	H2N	MCAGHNIIFFLTPHR	CONH2
212 to 226	H2N	NFCQKLCTFSFLICK	CONH2
222 to 235	H2N	FLICKGVNKEYLLYS	CONH2
232 to 246	H2N	YLLYSALTRDPYHTI	CONH2
281 to 295	H2N	ETKCEDVFLLLGMYL	CONH2
287 to 301	H2N	VFLLLGMYLEFQYNV	CONH2
357 to 371	H2N	MTREEMLTERFNHIL	CONH2
404 to 418	H2N	DSVIFDFLHCIVFNV	CONH2
565 to 579	H2N	LLEKRILQSGMTLLL	CONH2
573 to 587	H2N	SGMTLLLLLIWFRPV	CONH2

BKV LT predicted 25mer

Pos	N-Term	Sequence	C-Term
019 to 043	H2N	LERAAWGNLPLMRKAYLRKCKEFHP	CONH2
145 to 167	H2N	HQFLSQAVFSNRTLACFAVYTTKEK	CONH2
162 to 186	H2N	AVYTTKEKAQILYKKLMEKYSVTFI	CONH2
188 to 212	H2N	RHMCAGHNIIFFLTPHRHRVSAINN	CONH2
212 to 237	H2N	NFCQKLCTFSFLICKGVNKEYLLYSAL	CONH2
227 to 250	H2N	GVNKEYLLYSALTRDPYHTIEESI	CONH2

280 to 304	H2N	VETKCEDVFLLLGMYLEFQYNVEEC	CONH2
355 to 378	H2N	LHMTREEMLTERFNHILDKMDLIF	CONH2
402 to 426	H2N	KMDSVIFDFLHCIVFNVPKRRYWLF	CONH2
565 to 590	H2N	LLEKRILQSGMTLLLLLIWFRPVADF	CONH2

BKV LT predicted 9mer

Position	N-Term	Sequence	C-Term
19	H2N	LERAAWGNL	COOH
21	H2N	RAAWGNLPL	COOH
26	H2N	NLPLMRKAY	COOH
27	H2N	LPLMRKAYL	COOH
28	H2N	PLMRKAYLR	COOH
29	H2N	LMRKAYLRK	COOH
32	H2N	KAYLRKCKE	COOH
33	H2N	AYLRKCKEF	COOH
145	H2N	HQFLSQAVF	COOH
148	H2N	LSQAVFSNR	COOH
150	H2N	QAVFSNRTL	COOH
156	H2N	RTLACFAVY	COOH
158	H2N	LACFAVYTT	COOH
159	H2N	ACFAVYTTK	COOH
165	H2N	TTKEKAQIL	COOH
166	H2N	TKEKAQILY	COOH
167	H2N	KEKAQILYK	COOH
169	H2N	KAQILYKKL	COOH
172	H2N	ILYKKLMEK	COOH
173	H2N	LYKKLMEKY	COOH
176	H2N	KLMEKYSVT	COOH
177	H2N	LMEKYSVTF	COOH
178	H2N	MEKYSVTFI	COOH
188	H2N	RHMCAGHNI	COOH
192	H2N	AGHNIIFFL	COOH
190	H2N	MCAGHNIIF	COOH

191	H2N	CAGHNIIFF	COOH
196	H2N	IIFFLTPHR	COOH
199	H2N	FLTPHRHRV	COOH
201	H2N	TPHRHRVSA	COOH
202	H2N	PHRHRVSAI	COOH
212	H2N	NFCQKLCTF	COOH
214	H2N	CQKLCTFSF	COOH
216	H2N	KLCTFSFLI	COOH
218	H2N	CTFSFLICK	COOH
222	H2N	FLICKGVNK	COOH
224	H2N	ICKGVNKEY	COOH
226	H2N	KGVNKEYLL	COOH
227	H2N	GVNKEYLLY	COOH
230	H2N	KEYLLYSAL	COOH
232	H2N	YLLYSALTR	COOH
235	H2N	YSALTRDPY	COOH
238	H2N	LTRDPYHTI	COOH
242	H2N	PYHTIEESI	COOH
282	H2N	TKCEDVFLL	COOH
283	H2N	KCEDVFLLL	COOH
286	H2N	DVFLLLGMY	COOH
287	H2N	VFLLLGMYL	COOH
289	H2N	LLLGMYLEF	COOH
291	H2N	LGMYLEFQY	COOH
293	H2N	MYLEFQYNV	COOH
355	H2N	LHMTREEML	COOH
359	H2N	REEMLTERF	COOH
362	H2N	MLTERFNHI	COOH
369	H2N	HILDKMDLI	COOH
402	H2N	KMDSVIFDF	COOH
403	H2N	MDSVIFDFL	COOH
406	H2N	VIFSFLHCI	COOH
410	H2N	FLHCIVFNV	COOH
408	H2N	FDFLHCIVF	COOH

414	H2N	IVFNVPKRR	COOH
415	H2N	VFNVPKRRY	COOH
417	H2N	NVPKRRYWL	COOH
418	H2N	VPKRRYWLF	COOH
569	H2N	RILQSGMTL	COOH
570	H2N	ILQSGMTLL	COOH
571	H2N	LQSGMTLLL	COOH
573	H2N	SGMTLLLLL	COOH
574	H2N	GMTLLLLLI	COOH
575	H2N	MTLLLLLIW	COOH
576	H2N	TLLLLLIWF	COOH
577	H2N	LLLLLIWFR	COOH
579	H2N	LLLIWFRPV	COOH
582	H2N	IWFRPVADF	COOH

Polyomavirus JC proteins:

JCV LT peptide library (15aa long peptides, 11aa overlap)

	EGT	N-Term	Sequence	C-Term
240	P0510369	H2N	MDKVLNREESMELMD	CONH2
241	P0510370	H2N	LNREESMELMDLLGL	CONH2
242	P0510371	H2N	ESMELMDLLGLDRSA	CONH2
243	P0510372	H2N	LMDLLGLDRSAWGNI	CONH2
244	P0510373	H2N	LGLDRSAWGNIPVMR	CONH2
245	P0510374	H2N	RSAWGNIPVMRKAYL	CONH2
246	P0510375	H2N	GNIPVMRKAYLKKCK	CONH2
247	P0510376	H2N	VMRKAYLKKCKELHP	CONH2
248	P0510377	H2N	AYLKKCKELHPDKGG	CONH2
249	P0510378	H2N	KCKELHPDKGGDEDK	CONH2
250	P0510379	H2N	LHPDKGGDEDKMKRM	CONH2
251	P0510380	H2N	KGGDEDKMKRMNFLY	CONH2
252	P0510381	H2N	EDKMKRMNFLYKKME	CONH2
253	P0510382	H2N	KRMNFLYKKMEQGVK	CONH2
254	P0510383	H2N	FLYKKMEQGVKVAHQ	CONH2
255	P0510384	H2N	KMEQGVKVAHQPDFG	CONH2
256	P0510385	H2N	GVKVAHQPDFGTWNS	CONH2
257	P0510386	H2N	AHQPDFGTWNSSEVP	CONH2
258	P0510387	H2N	DFGTWNSSEVPTYGT	CONH2

259	P0510388	H2N	WNSSEVPTYGTDEWE	CONH2
260	P0510389	H2N	EVPTYGTDEWESWWN	CONH2
261	P0510390	H2N	YGTDEWESWWNTFNE	CONH2
262	P0510391	H2N	EWESWWNTFNEKWDE	CONH2
263	P0510392	H2N	WWNTFNEKWDEDLFC	CONH2
264	P0510393	H2N	FNEKWDEDLFCHEEM	CONH2
265	P0510394	H2N	WDEDLFCHEEMFASD	CONH2
266	P0510395	H2N	LFCHEEMFASDDENT	CONH2
267	P0510396	H2N	EEMFASDDENTGSQH	CONH2
268	P0510397	H2N	ASDDENTGSQHSTPP	CONH2
269	P0510398	H2N	ENTGSQHSTPPKKKK	CONH2
270	P0510399	H2N	SQHSTPPKKKKKVED	CONH2
271	P0510400	H2N	TPPKKKKKVEDPKDF	CONH2
272	P0510401	H2N	KKKKVEDPKDFPVDL	CONH2
273	P0510402	H2N	VEDPKDFPVDLHAFL	CONH2
274	P0510403	H2N	KDFPVDLHAFLSQAV	CONH2
275	P0510404	H2N	VDLHAFLSQAVFSNR	CONH2
276	P0510405	H2N	AFLSQAVFSNRTVAS	CONH2
277	P0510406	H2N	AVFSNRTVASFAVY	CONH2
278	P0510407	H2N	SNRTVASFAVYTTKE	CONH2
279	P0510408	H2N	VASFAVYTTKEKAQI	CONH2
280	P0510409	H2N	AVYTTKEKAQILYKK	CONH2
281	P0510410	H2N	TKEKAQILYKKLMEK	CONH2
282	P0510411	H2N	AQILYKKLMEKYSVT	CONH2
283	P0510412	H2N	YKKLMEKYSVTFISR	CONH2
284	P0510413	H2N	MEKYSVTFISRHGFG	CONH2
285	P0510414	H2N	SVTFISRHGFGGHNI	CONH2
286	P0510415	H2N	ISRHGFGGHNILFFL	CONH2
287	P0510416	H2N	GFGGHNILFFLTPHR	CONH2
288	P0510417	H2N	HNILFFLTPHRHRVS	CONH2
1	P0510418	H2N	FFLTPHRHRVSAINN	CONH2
2	P0510419	H2N	PHRHRVSAINNYCQK	CONH2
3	P0510420	H2N	RVSAINNYCQKLCTF	CONH2
4	P0510421	H2N	INNYCQKLCTFSFLI	CONH2
5	P0510422	H2N	CQKLCTFSFLICKGV	CONH2
6	P0510423	H2N	CTFSFLICKGVNKEY	CONH2
7	P0510424	H2N	FLICKGVNKEYLFYS	CONH2
8	P0510425	H2N	KGVNKEYLFYSALCR	CONH2
9	P0510426	H2N	KEYLFYSALCRQPYA	CONH2
10	P0510427	H2N	FYSALCRQPYAVVEE	CONH2
11	P0510428	H2N	LCRQPYAVVEESIQG	CONH2
12	P0510429	H2N	PYAVVEESIQGGLKE	CONH2
13	P0510430	H2N	VEESIQGGLKEHDFN	CONH2
14	P0510431	H2N	IQGGLKEHDFNPEEP	CONH2
15	P0510432	H2N	LKEHDFNPEEPEETK	CONH2

16	P0510433	H2N	DFNPEEPEETKQVSW	CONH2
17	P0510434	H2N	EEPEETKQVSWKLVT	CONH2
18	P0510435	H2N	ETKQVSWKLVTQYAL	CONH2
19	P0510436	H2N	VSWKLVTQYALETKC	CONH2
20	P0510437	H2N	LVTQYALETKCEDVF	CONH2
21	P0510438	H2N	YALETKCEDVFLLMG	CONH2
22	P0510439	H2N	TKCEDVFLLMGMYLD	CONH2
23	P0510440	H2N	DVFLLMGMYLDFQEN	CONH2
24	P0510441	H2N	LMGMYLDFQENPQQC	CONH2
25	P0510442	H2N	YLDFQENPQQCKKCE	CONH2
26	P0510443	H2N	ENPQQCKKCEKKDQ	CONH2
27	P0510444	H2N	CKKCEKKDQPNHF	CONH2
28	P0510445	H2N	KCEKKDQPNHFNHHE	CONH2
29	P0510446	H2N	KDQPNHFNHHEKHYY	CONH2
30	P0510447	H2N	NHFNHHEKHYYNAQI	CONH2
31	P0510448	H2N	HHEKHYYNAQIFADS	CONH2
32	P0510449	H2N	HYYNAQIFADSKNQK	CONH2
33	P0510450	H2N	AQIFADSKNQKSICQ	CONH2
34	P0510451	H2N	ADSKNQKSICQQAVD	CONH2
35	P0510452	H2N	NQKSICQQAVDTVAA	CONH2
36	P0510453	H2N	ICQQAVDTVAAKQRV	CONH2
37	P0510454	H2N	AVDTVAAKQRVDSIH	CONH2
38	P0510455	H2N	VAAKQRVDSIHMTRE	CONH2
39	P0510456	H2N	RVDSIHMTREEMLV	CONH2
40	P0510457	H2N	SIHMTREEMLVERFN	CONH2
41	P0510458	H2N	TREEMLVERFNFLLD	CONH2
42	P0510459	H2N	MLVERFNFLLDKMDL	CONH2
43	P0510460	H2N	RFNFLLDKMDLIFGA	CONH2
44	P0510461	H2N	LLDKMDLIFGAHGNA	CONH2
45	P0510462	H2N	MDLIFGAHGNAVLEQ	CONH2
46	P0510463	H2N	FGAHGNAVLEQYMAG	CONH2
47	P0510464	H2N	GNAVLEQYMAGVAWI	CONH2
48	P0510465	H2N	LEQYMAGVAWIHCLL	CONH2
49	P0510466	H2N	MAGVAWIHCLLPQMD	CONH2
50	P0510467	H2N	AWIHCLLPQMDTVIY	CONH2
51	P0510468	H2N	CLLPQMDTVIYDFLK	CONH2
52	P0510469	H2N	MDTVIYDFLKCIVL	CONH2
53	P0510470	H2N	VIYDFLKCIVLNIPK	CONH2
54	P0510471	H2N	FLKCIVLNIPKKRYW	CONH2
55	P0510472	H2N	IVLNIPKKRYWLFKG	CONH2
56	P0510473	H2N	IPKKRYWLFKGPIDS	CONH2
57	P0510474	H2N	RYWLFKGPIDSGKTT	CONH2
58	P0510475	H2N	FKGPIDSGKTTLAAA	CONH2
59	P0510476	H2N	IDSGKTTLAAALLDL	CONH2
60	P0510477	H2N	KTTLAAALLDLCGGK	CONH2

61	P0510478	H2N	AAALLDLCGGKSLNV	CONH2
62	P0510479	H2N	LDLCGGKSLNVNMPL	CONH2
63	P0510480	H2N	GGKSLNVNMPLERLN	CONH2
64	P0510481	H2N	LNVNMPLERLNFELG	CONH2
65	P0510482	H2N	MPLERLNFELGVGID	CONH2
66	P0510483	H2N	RLNFELGVGIDQFMV	CONH2
67	P0510484	H2N	ELGVGIDQFMVVFED	CONH2
68	P0510485	H2N	GIDQFMVVFEDVKGT	CONH2
69	P0510486	H2N	FMVVFEDVKGTGAES	CONH2
70	P0510487	H2N	FEDVKGTGAESRDLP	CONH2
71	P0510488	H2N	KGTGAESRDLPSGHG	CONH2
72	P0510489	H2N	AESRDLPSGHGISNL	CONH2
73	P0510490	H2N	DLPSGHGISNLDCLR	CONH2
74	P0510491	H2N	GHGISNLDCLRDYLD	CONH2
75	P0510492	H2N	SNLDCLRDYLDGSVK	CONH2
76	P0510493	H2N	CLRDYLDGSVKVNLE	CONH2
77	P0510494	H2N	YLDGSVKVNLERKHQ	CONH2
78	P0510495	H2N	SVKVNLERKHQNKRT	CONH2
79	P0510496	H2N	NLERKHQNKRTQVFP	CONH2
80	P0510497	H2N	KHQNKRTQVFPPGIV	CONH2
81	P0510498	H2N	KRTQVFPPGIVTMNE	CONH2
82	P0510499	H2N	VFPPGIVTMNEYSVP	CONH2
83	P0510500	H2N	GIVTMNEYSVPRTLQ	CONH2
84	P0510501	H2N	MNEYSVPRTLQARFV	CONH2
85	P0510502	H2N	SVPRTLQARFVRQID	CONH2
86	P0510503	H2N	TLQARFVRQIDFRPK	CONH2
87	P0510504	H2N	RFVRQIDFRPKAYLR	CONH2
88	P0510505	H2N	IDFRPKAYLRKSLS	CONH2
89	P0510506	H2N	RPKAYLRKSLSCSEY	CONH2
90	P0510507	H2N	YLRKSLSCSEYLLEK	CONH2
91	P0510508	H2N	SLSCSEYLLEKRILQ	CONH2
92	P0510509	H2N	SEYLLEKRILQSGMT	CONH2
93	P0510510	H2N	LEKRILQSGMTLLLL	CONH2
94	P0510511	H2N	ILQSGMTLLLLLIWF	CONH2
95	P0510512	H2N	GMTLLLLLIWFRPVA	CONH2
96	P0510513	H2N	LLLLIWFRPVADFAA	CONH2
97	P0510514	H2N	IWFRPVADFAAAIHE	CONH2
98	P0510515	H2N	PVADFAAAIHERIVQ	CONH2
99	P0510516	H2N	FAAAIHERIVQWKER	CONH2
100	P0510517	H2N	IHERIVQWKERLDLE	CONH2
101	P0510518	H2N	IVQWKERLDLEISMY	CONH2
102	P0510519	H2N	KERLDLEISMYTFST	CONH2
103	P0510520	H2N	DLEISMYTFSTMKAN	CONH2
104	P0510521	H2N	SMYTFSTMKANVGMG	CONH2
105	P0510522	H2N	FSTMKANVGMGRPIL	CONH2

106	P0510523	H2N	KANVGMGRPILDFPR	CONH2
107	P0510524	H2N	GMGRPILDFPREEDS	CONH2
108	P0510525	H2N	PILDFPREEDSEAED	CONH2
109	P0510526	H2N	FPREEDSEAEDSGHG	CONH2
110	P0510527	H2N	EDSEAEDSGHGSSTE	CONH2
111	P0510528	H2N	AEDSGHGSSTESQSQ	CONH2
112	P0510529	H2N	GHGSSTESQSQCFSQ	CONH2
113	P0510530	H2N	STESQSQCFSQVSEA	CONH2
114	P0510531	H2N	SQCFSQVSEASGAD	CONH2
115	P0510532	H2N	FSQVSEASGADTQEN	CONH2
116	P0510533	H2N	SEASGADTQENCTFH	CONH2
117	P0510534	H2N	GADTQENCTFHICKG	CONH2
118	P0510535	H2N	ENCTFHICKGFQCF	CONH2
119	P0510536	H2N	TFHICKGFQCFKKPK	CONH2
120	P0510537	H2N	CKGFQCFKKPKTPPP	CONH2
121	P0510538	H2N	CFKKPKTPPPK	COOH

JCV VP1 peptide library (15aa long peptides, 11aa overlap)

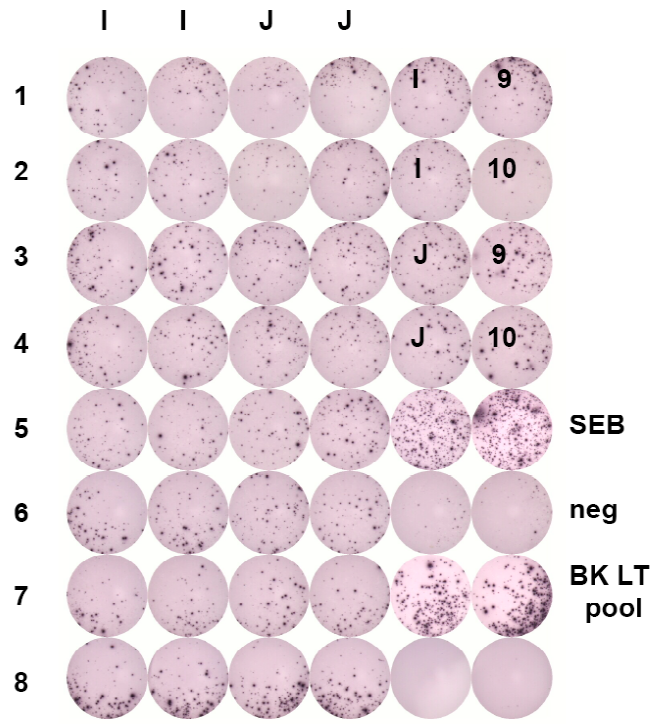
	EGT	N-Term	Sequence	C-Term
139	P0510268	H2N	MAPTKRKGERKDPVQ	CONH2
140	P0510269	H2N	KRKGERKDPVQVPKL	CONH2
141	P0510270	H2N	ERKDPVQVPKLLIRG	CONH2
142	P0510271	H2N	PVQVPKLLIRGGVEV	CONH2
143	P0510272	H2N	PKLLIRGGVEVLEVK	CONH2
144	P0510273	H2N	IRGGVEVLEVKTGVD	CONH2
145	P0510274	H2N	VEVLEVKTGVDSITE	CONH2
146	P0510275	H2N	EVKTGVDSITEVECF	CONH2
147	P0510276	H2N	GVDSITEVECFLTPE	CONH2
148	P0510277	H2N	ITEVECFLTPEMGDP	CONH2
149	P0510278	H2N	ECFLTPEMGDPDEHL	CONH2
150	P0510279	H2N	TPEMGDPDEHLRGFS	CONH2
151	P0510280	H2N	GDPDEHLRGFSKSIS	CONH2
152	P0510281	H2N	EHLRGFSKSISISDT	CONH2
153	P0510282	H2N	GFSKSISISDTFESD	CONH2
154	P0510283	H2N	SISISDTFESDSPNR	CONH2
155	P0510284	H2N	SDTFESDSPNRDMLP	CONH2
156	P0510285	H2N	ESDSPNRDMLPCYSV	CONH2
157	P0510286	H2N	PNRDMLPCYSVARIP	CONH2
158	P0510287	H2N	MLPCYSVARIPLPNL	CONH2
159	P0510288	H2N	YSVARIPLPNLNEDL	CONH2
160	P0510289	H2N	RIPLPNLNEDLTCGN	CONH2

161	P0510290	H2N	PNLNEDLTCGNILMW	CONH2
162	P0510291	H2N	EDLTCGNILMWEAVT	CONH2
163	P0510292	H2N	CGNILMWEAVTLKTE	CONH2
164	P0510293	H2N	LMWEAVTLKTEVIGV	CONH2
165	P0510294	H2N	AVTLKTEVIGVTSLM	CONH2
166	P0510295	H2N	KTEVIGVTSLMNVHS	CONH2
167	P0510296	H2N	IGVTSLMNVHSNGQA	CONH2
168	P0510297	H2N	SLMNVHSNGQATHDN	CONH2
169	P0510298	H2N	VHSNGQATHDNGAGK	CONH2
170	P0510299	H2N	GQATHDNGAGKPVQG	CONH2
171	P0510300	H2N	HDNGAGKPVQGTSFH	CONH2
172	P0510301	H2N	AGKPVQGTSFHFFSV	CONH2
173	P0510302	H2N	VQGTSFHFFSVGGEA	CONH2
174	P0510303	H2N	SFHFFSVGGEALELQ	CONH2
175	P0510304	H2N	FSVGGEALELQGVLF	CONH2
176	P0510305	H2N	GEALELQGVLFNYRT	CONH2
177	P0510306	H2N	ELQGVLFNYRTKYPD	CONH2
178	P0510307	H2N	VLFNYRTKYPDGTIF	CONH2
179	P0510308	H2N	YRTKYPDGTIFPKNA	CONH2
180	P0510309	H2N	YPDGTIFPKNATVQS	CONH2
181	P0510310	H2N	TIFPKNATVQSQVMN	CONH2
182	P0510311	H2N	KNATVQSQVMNTEHK	CONH2
183	P0510312	H2N	VQSQVMNTEHKAYLD	CONH2
184	P0510313	H2N	VMNTEHKAYLDKNKA	CONH2
185	P0510314	H2N	EHKAYLDKNKAYPVE	CONH2
186	P0510315	H2N	YLDKNKAYPVECWVP	CONH2
187	P0510316	H2N	NKAYPVECWVPDPTR	CONH2
188	P0510317	H2N	PVECWVPDPTRNENT	CONH2
189	P0510318	H2N	WVPDPTRNENTRYFG	CONH2
190	P0510319	H2N	PTRNENTRYFGTLTG	CONH2
191	P0510320	H2N	ENTRYFGTLTGGENV	CONH2
192	P0510321	H2N	YFGTLTGGENVPPVL	CONH2
193	P0510322	H2N	LTGGENVPPVLHITN	CONH2
194	P0510323	H2N	ENVPPVLHITNTATT	CONH2
195	P0510324	H2N	PVLHITNTATTVLLD	CONH2
196	P0510325	H2N	ITNTATTVLLDEFGV	CONH2
197	P0510326	H2N	ATTVLLDEFGVGPLC	CONH2
198	P0510327	H2N	LLDEFGVGPLCKGDN	CONH2
199	P0510328	H2N	FGVGPLCKGDNLYLS	CONH2
200	P0510329	H2N	PLCKGDNLYLSAVDV	CONH2
201	P0510330	H2N	GDNLYLSAVDVCGMF	CONH2
202	P0510331	H2N	YLSAVDVCGMFTNRS	CONH2
203	P0510332	H2N	VDVCGMFTNRSGSQQ	CONH2
204	P0510333	H2N	GMFTNRSGSQQWRGL	CONH2
205	P0510334	H2N	NRSGSQQWRGLSRYF	CONH2

206	P0510335	H2N	SQQWRGLSRYFKVQL	CONH2
207	P0510336	H2N	RGLSRYFKVQLRKRR	CONH2
208	P0510337	H2N	RYFKVQLRKRRVKNP	CONH2
209	P0510338	H2N	VQLRKRRVKNPYPIS	CONH2
210	P0510339	H2N	KRRVKNPYPISFLLT	CONH2
211	P0510340	H2N	KNPYPISFLLTDLIN	CONH2
212	P0510341	H2N	PISFLLTDLINRRTP	CONH2
213	P0510342	H2N	LLTDLINRRTPRVDG	CONH2
214	P0510343	H2N	LINRRTPRVDGQPMY	CONH2
215	P0510344	H2N	RTPRVDGQPMYGMDA	CONH2
216	P0510345	H2N	VDGQPMYGMDAQVEE	CONH2
217	P0510346	H2N	PMYGMDAQVEEVRVF	CONH2
218	P0510347	H2N	MDAQVEEVRVFEGTE	CONH2
219	P0510348	H2N	VEEVRVFEGTEELPG	CONH2
220	P0510349	H2N	RVFEGTEELPGDPDM	CONH2
221	P0510350	H2N	GTEELPGDPDMMRYV	CONH2
222	P0510351	H2N	LPGDPDMMRYVDKYG	CONH2
223	P0510352	H2N	PDMMRYVDKYGQLQT	CONH2
224	P0510353	H2N	RYVDKYGQLQTKML	COOH

JCV agno peptide library (15aa long peptides, 11aa overlap)

	EGT	n-term	Sequence	C-term
225	P0510354	H2N	MVLRQLSRKASVKVS	CONH2
226	P0510355	H2N	LSRKASVKVSKTWS	CONH2
227	P0510356	H2N	KASVKVSKTWSGTKK	CONH2
228	P0510357	H2N	KVSKTWSGTKKRAQR	CONH2
229	P0510358	H2N	TWSGTKKRAQRILIF	CONH2
230	P0510359	H2N	TKKRAQRILIFLLEF	CONH2
231	P0510360	H2N	AQRILIFLLEFLLDF	CONH2
232	P0510361	H2N	LIFLLEFLLDFCTGE	CONH2
233	P0510362	H2N	LEFLLDFCTGEDSVD	CONH2
234	P0510363	H2N	LDFCTGEDSVDGKKR	CONH2
235	P0510364	H2N	TGEDSVDGKKRQRHS	CONH2
236	P0510365	H2N	SVDGKKRQRHSGLTE	CONH2
237	P0510366	H2N	KKRQRHSGLTEQTYS	CONH2
238	P0510367	H2N	RHSGLTEQTYSALPE	CONH2
239	P0510368	H2N	LTEQTYSALPEPKAT	COOH

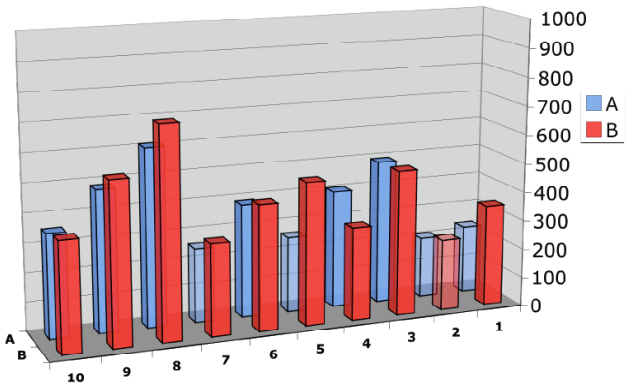


	SFU per Mio PBMC
SEB	5235
neg	0
BK LT	2335

Positive response: >2x background 310

- + 0 - 100 SFU
- + 100 - 200 SFU
- + 200 - 300 SFU

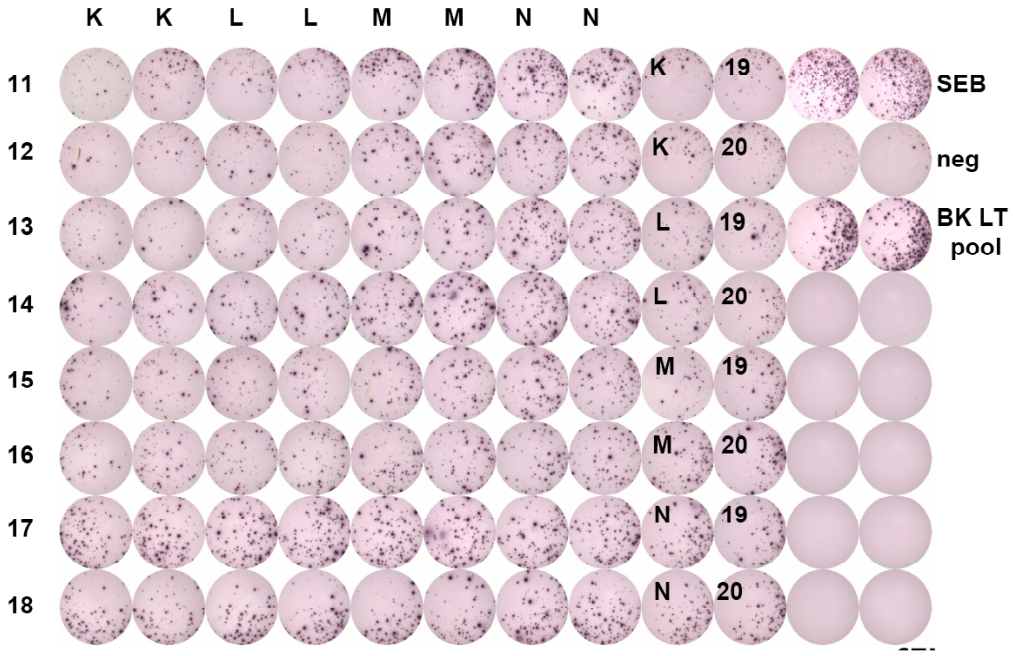
	I	J
1	235	350
2	210	245
3	495	500
4	405	320
5	260	490
6	390	430
7	255	315
8	610	720
9	485	555
10	355	375



	I	J
1	p169	p179
2	p170	p180
3	p171	p181
4	p172	p182
5	p173	p183
6	p174	p184
7	p175	p185
8	p176	p186
9	p177	p187
10	p178	p188

Code	Sequence	Predicted	HLA Type
p171	FAESKNQKSICQQAV		
p172	KNQKSICQQAVDTVL		
p174	AVDTVLAKKRVDTL		
p176	KKRVDTLHMTREEML		
p177	DTLHMTREEMLTERF		
p178	MTREEMLTERFNHIL	yes	A0201
p179	EMLTERFNHILDKMD	yes	A0201
p181	HILDKMDLIFGAHGN	yes	B3501
p182	KMDLIFGAHGNAVLE		
p183	IFGAHGNAVLEQYMA	yes	B3501
p184	HGNAVLEQYMAGVAW	yes	A0201(+Syf)
p185	VLEQYMAGVAWLHCL	yes	A0201(+Syf)
p186	YMAGVAWLHCLLPKM	yes	A0201(Syf)
p187	VAWLHCLLPKMDSVI	yes	A0201(2xSyf)
p188	HCLLPKMDSVIFDFL	yes	A0201(+Syf) B3501

Figure 26. Checker-board of peptide sub-pools I-J and 1-10.



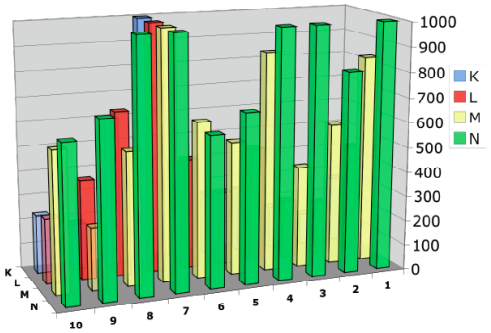
SFU per Mio PBMC

SEB	4665
neg	0
BK LT	1705

Positive response: >2x background 290

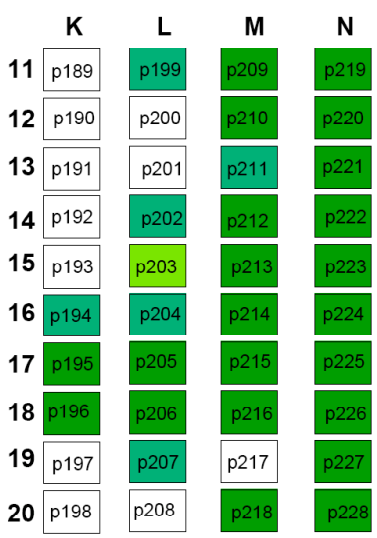
- + 0 - 100 SFU
- + 100 - 200 SFU
- + 200 - 300 SFU

	K	L	M	N
11	270	440	835	1170
12	125	210	570	810
13	170	230	410	1020
14	260	465	880	1025
15	250	295	535	680
16	395	445	625	605
17	1005	1355	1135	1040
18	605	660	535	1000
19	185	400	250	700
20	235	260	565	625



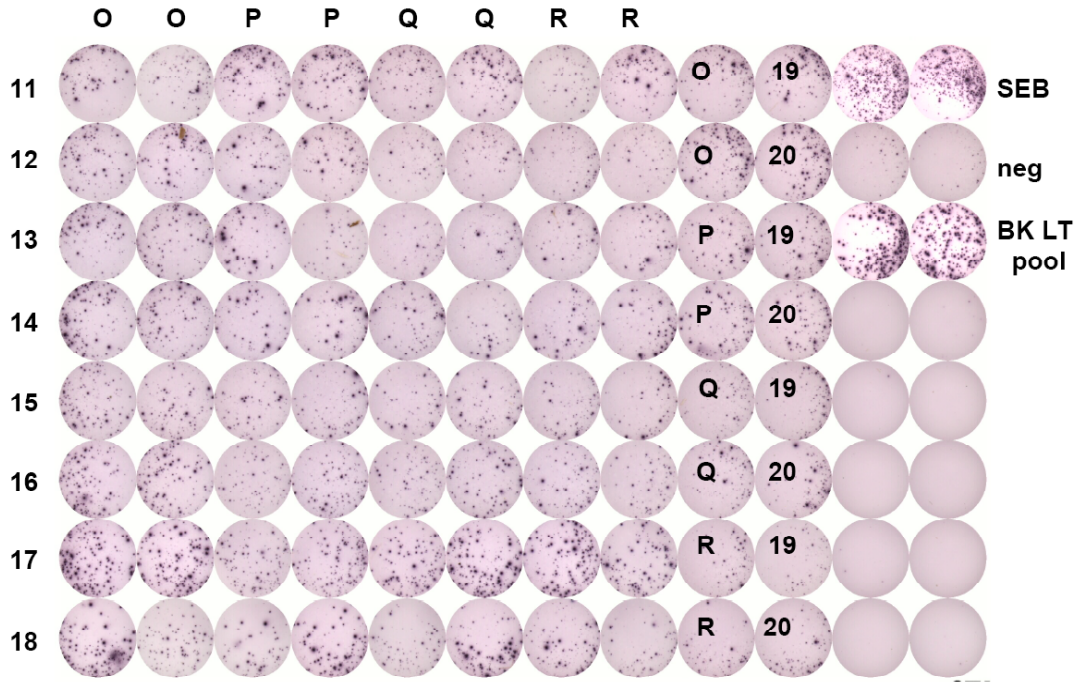
	K	L	M	N
11	p189	p199	p209	p219
12	p190	p200	p210	p220
13	p191	p201	p211	p221
14	p192	p202	p212	p222
15	p193	p203	p213	p223
16	p194	p204	p214	p224
17	p195	p205	p215	p225
18	p196	p206	p216	p226
19	p197	p207	p217	p227
20	p198	p208	p218	p228

Code	Sequence	Predicted	HLA Type
p194	RRYWLFKGPIDSGKT		
p195	LFKGPIDSGKTTLAA		
p196	PIDSGKTTLAAGLLD	yes	A0201(+Syf)
p199	LLDLCGGKALNVNLP		
p202	NLPMERLTFELGVAI	yes	A0201(Syf)
p203	RTLTFELGVAIDQYM	yes	A0201(Syf)
p204	FELGVAIDQYMVVFE	yes	B3501
p205	VAIDQYMVVFEDVKG		
p206	YMVVFEDVKGTGAE		
p207	VFEDVKGTGAESKDL		
p209	GAESKDLPSGHGINN		
p210	KDLPSGHGINNLDSL		
p211	SGHGINNLDSLRDYL	yes	A0201 (+Syf)
p212	INNLDSLRDYLDGSV	yes	A0201 (+Syf)
p213	DSLRDYLDGSVKVNL		
p214	DYLDGSVKVNLEKKH		
p215	GSVKVNLEKKHLNKR		



Code	Sequence	Predicted	HLA Type
p216	VLNEKKHLNKRTQIF		
p218	NKRTQIFPPGLVTMN		
p219	IFPPGLVTMNEYPV		
p220	PGLVTMNEYPVPKTL		
p221	TMNEYPVPKTLQARF		
p222	YPVPKTLQARFVRQI		
p223	KTLQARFVRQIDFRP		
p224	ARFVRQIDFRPKIYL		
p225	RQIDFRPKIYLRKSL		
p226	FRPKIYLRKSLQNSE		
p227	IYLRKSLQNSEFLLE	yes	A0201 (+Syf)
p228	KSLQNSEFLLEKRIL	yes	A0201 (+Syf)

Figure 27. Checker-board of peptide sub-pools K-N and 11-20.



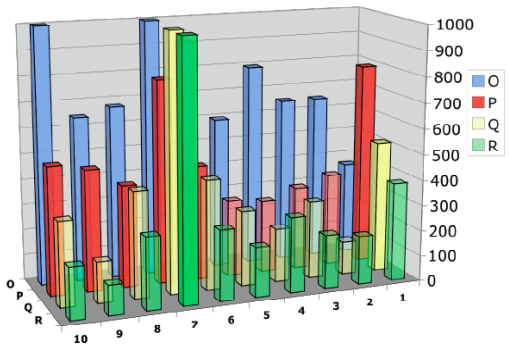
SFU per Mio PBMC

SEB	5230
neg	0
BK LT	2880

Positive response: >2x background 440

- + 0 - 100 SFU
- + 100 - 200 SFU
- + 200 - 300 SFU

	O	P	Q	R
11	365	795	515	385
12	650	365	130	190
13	650	325	305	210
14	795	285	210	295
15	595	300	295	195
16	910	450	430	275
17	1450	795	1045	1085
18	675	400	415	280
19	645	475	160	115
20	1000	500	330	200



	O	P	Q	R
11	p229	p239	p249	p259
12	p230	p240	p250	p260
13	p231	p241	p251	p261
14	p232	p242	p252	p262
15	p233	p243	p253	p263
16	p234	p244	p254	p264
17	p235	p245	p255	p265
18	p236	p246	p256	p266
19	p237	p247	p257	p267
20	p238	p248	p258	p268

Code	Sequence	Predicted	HLA Type
p230	LLEKRILQSGMTLLL	yes	A0201 2x+Syf DR1101
p231	RILQSGMTLLLLLIW	yes	A0201 3x+Syf
p232	SGMTLLLLLIWFRPV	yes	A0201 4x+Syf
p233	LLLLLIQFRPVADFA	yes	A0201 2x
p234	LIWFRPVADFATDIQ		
p235	RPVADFATDIQSRIV		
p236	DFATDIQSRIVEWKE		
p237	DIQSRIVEWKERLDS		
p238	RIVEWKERLDSEISM		
p239	WKERLDSEISMYTFS		
p244	ICMGKCILDITREED	yes	A0201 (Syf)
p245	KCILDITREEDSETE		
p247	EEDSETEDSGHGSST		
p248	ETEDSGHGSSTESQS		
p249	SGHGSSTESQSQCSS		
p255	EDSQRSDPHSQELHL		
p265	SQELHLCKGFQCFKR		

Figure 28. Checker-board for peptide pools O-R and 11-20

Published articles