Polyomavirus BK-Specific Cellular Immune Response in Kidney Transplant Recipients

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„A journey of a thousand miles begins with one step“

(Lao Tse)

FÜR HENRI

STATEMENT TO MY PHD THESIS

This work was carried out from July 2003 to August 2007 under the supervision of Prof. H. H. Hirsch at the Institute for Medical Microbiology of the University of Basel, Switzerland.

My PhD thesis is written in a cumulative format and consists of a general introduction about polyomaviruses, its related diseases and polyomavirus-specific immune response. The cumulative part includes six publications and one manuscript: The first two publications give an overview about polyomavirus related problems in transplant recipients and management of polyomavirus diseases. Three publications elucidate the polyomavirus-specific immune response to different viral proteins. The sixth publication is a case report about a patient suffering from polyomavirus-associated nephropathy. The manuscript is about cytomegalovirus, a latent virus also causing problems in the transplant setting like polyomaviruses. Finally, two chapters about current projects summarize the experiments done until today.

INDEX

ABBREVIATIONS

APC	Antigen presenting cell
BKV and JCV	Human polyomavirus Type 1 (BK virus) and Type 2 (JC virus)
CMV	Cytomegalovirus
CsA	Cyclosporine A
CSF	Cerebrospinal fluids
EBV	Epstein Barr Virus
ESA	ELISpot assay
FK506	Tacrolimus
HAART	Highly active antiretroviral therapy
HC	Hemorrhagic cystitis
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplant
IFN-γ	Interferon-gamma
IRIS	Immune-reconstitution inflammatory syndrome
KT	Kidney transplant
LT	Large T (early protein)
mDC	Mature dendritic cell
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
mRNA	messenger RNA
NCCR	Non-coding control region
NLS	Nuclear localization site
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PML	Progressive multifocal leukoencephalopathy
PP2A	Protein phosphatase 2A
PVAN	Polyomavirus-associated nephropathy
Rb	Retinoblastoma susceptibility protein
SFU	Spot forming units
sT	Small T-antigen (early protein)
VLP	Virus-like particle
VP1	Viral capsid protein 1 (late structural protein)

INTRODUCTION

1. INTRODUCTION

1.1 GENERAL INTRODUCTION TO HUMAN POLYOMAVIRUSES

Polyomaviruses are able to infect, to replicate in and to induce different diseases in natural and accidental hosts. The following chapters focus on the two human polyomaviruses type 1 and type 2 which were first isolated in 1971 and designated BK virus (**BKV**) and JC virus (**JCV**) after the initials of the respective patients (Gardner et al. 1971; Padgett et al. 1971). Other polyomaviruses such as SV40, KI and WU, are also known to infect humans.

Human exposure to polyomavirus SV40 occurred through contaminated poliovirus vaccines in the United States between 1955 and 1963. Because of the carcinogenicity of SV40 in infected rodents, many studies have been performed to determine if there is an association between SV40 infection and cancer in human (Paracchini et al. 2006). SV40 is the best investigated polyomavirus and most findings about polyomavirus properties are due to extensive SV40 research.

Recently the DNA of two new polyomaviruses KI and WU could be isolated by polymerase chain reaction (**PCR**) from respiratory tract samples mainly from children less than 3 years of age (Allander et al. 2007; Gaynor et al. 2007). These two viruses share strikingly similar properties as both do not code for agnoprotein and both are lacking the large turmor (**LT**) /antigen host range domain at the carboxyl terminus, responsible for viral assembly (**Chapter 1.1.2.1**) (Pipas 1992). KI and WU have an amino acid sequence homology of 70% for LT-antigen and 65% for viral capsid protein 1 (**VP1**). In contrast, there is only a low level homology to BKV and JCV (LT-antigen: 49% and 48%, and VP1: 28% and 27%, respectively). Whether polyomaviruses KI and WU are able to induce a disease and if they also have transforming properties needs further investigation.

The genomic organization of polyomavirus BK, JC, SV40, KI, and WU is conserved between these viruses.

1.1.1 Polyomavirus Genomic Organization

BKV and JCV are non-encapsulated, circular double-stranded DNA viruses of approximately 5200 base pairs and are approximately 40-45 nm in diameter. The polyomavirus genome has a relatively simple architecture and can be divided into three parts according to their functions: 1. Non-coding control region (**NCCR**), 2. Early genes (coding for small and large tumor-antigen), and 3. Late genes (coding for viral capsid proteins 1-3 and agnoprotein) (**Figure 1.1 and Table 1.1**).

The NCCR is located between the early and late coding regions and contains the origin of DNA replication (***ori***), the TATA box, LT-antigen binding sites, cellular transcription factor-binding sites, and promoter and enhancer for transcription of early and late genes (Cole et al. 2001; Moens et al. 2001). The BKV and JCV NCCR may undergo DNA rearrangement *in vitro* and *in vivo*, and is associated with BKV nephropathy and PML (Sundsfjord et al. 1990).

The early region is the first part of the genome which is transcribed and translated during the viral life cycle. Small tumor (**sT**) and LT-antigen are produced by alternative splicing of a common precursor messenger RNA (**mRNA**) (Howley et al. 1975; Moens et al. 2001).

The late region is transcribed and translated after the early region and the precursor mRNA is translated to the three capsid proteins VP1-3 and agnoprotein by alternative splicing (Kamen et al. 1980; Moens et al. 2001).

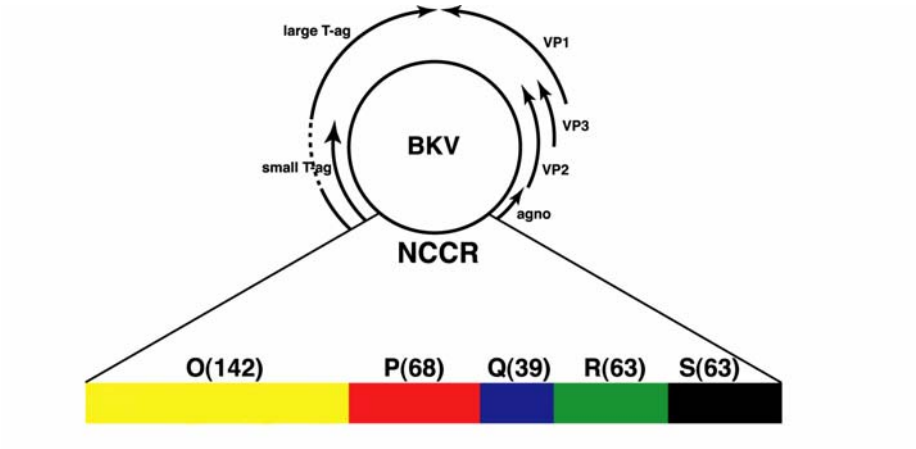

Figure 1.1: Polyomavirus BK genome. The NCCR contains sequence blocks (P, Q, R, S) which serve as regulatory regions, or enhancer elements. The primary transcript from the early region is alternatively spliced in two mRNAs encoding for small and large tumor-antigens. VP1-3 and agnoprotein are the gene products from the late region.
adapted from (Moens et al. 1995; Slavov 2006)

Table 1.1: Polyomaviruses BK and JC genomes.

		Number of bp[1]		Homology[2]
		BKV[3]	JCV[4]	BKV-JCV
Genome (bp)		5153	5130	74%
Early Coding Region	large T-antigen	2088	2067	78%
	small T-antigen	519	519	78%
Late Coding Region	VP1	1089	1065	75%
	VP2	1056	1035	81%
	VP3	699	678	80%
	Agnoprotein	201	216	72%

[1]bp: base pairs, [2]Clustal W pairwise alignment, [3]BKV-Dunlop strain (NC001538), [4]JCV-Mad1 strain (NC001699).

1.1.2 Polyomavirus Proteins

BKV and JCV proteins share high amino acid sequence homology (**Table 1.2**). Each of the proteins are expressed at different time points during the virus life cycle and have different functionalities important for polyomavirus propagation.

Table 1.2: Polyomaviruses BK and JC proteins.

Proteins	Molecular Weight (kDa)	Number of Amino Acids		Sequence Homology[1]	NCBI Accession Number	
		BKV	JCV	BKV-JCV	BKV	JCV
Early Proteins						
Large T-antigen	79	695	688	83%	P03071	AAA82102
Small T-antigen	20	172	172	78%	P03082	AAA82103
Late Proteins						
VP1	40	362	354	78%	P03088	AAA82101
VP2	37	351	344	79%	P03094	AAA82099
VP3	26	232	225	75%	P03094	AAA82100
Agnoprotein	8	66	71	59%	P03085	AAA82098

[1] *Clustal W pairwise alignment*

1.1.2.1 Early Proteins Small and Large T-Antigen and Their Role in Cell Transformation and Autoimmunity

The early protein sT-antigen binds to protein phosphatase 2A (**PP2A**), a host cell enzyme, leading to simultaneous activation of several pathways promoting cell proliferation. PP2A, the only confirmed cellular target of sT-antigen, is critical for cell homeostasis and essential for cell survival, cell cycle regulation, DNA damage response and embryonic development (Janssens et al. 2001; Sontag 2001). Mutation or lower expression levels of PP2A have been found in certain cancers. The absence of sT-antigen does not impair viral replication, but sT-antigen mutants are unable to drive cell proliferation and fail to transform host cells (Moens et al. 2001; Skoczylas et al. 2004; Ahuja et al. 2005; White et al. 2005).

Interest in the transforming ability of polyomaviruses has been triggered by the detection of SV40 DNA sequences in some human tumors and has revealed cell transforming properties of LT-antigen. The binding of LT-antigen to the heat shock chaperone (hsc70), the retinoblastoma family (Rb-family) of tumor suppressors, and to the tumor suppressor p53, contribute to transformation (Ali et al. 2001; Saenz-Robles et al. 2001; Sullivan et al. 2002; Garcea et al. 2003).

The structure of the LT-antigen is shown in **Figure 1.2**. Analysis of this protein revealed different domains and functions during polyomavirus life cycle (**Chapter 1.2.1**). Firstly, LT-antigen serves as a DNA helicase and promotes the assembly and function of host cell proteins. Secondly, it drives the host cell into S-phase by binding and inactivating the retinoblastoma susceptibility protein (**Rb**) family (Dyson et al. 1989; Ewen et al. 1989; Hannon et al. 1993; Harris et al. 1996; Caracciolo et al. 2006). Disruption of the Rb-E2F complex by LT-antigen leads to an increase of E2F activity, a transcription factor facilitating the S-phase progression of the host cell and thus leads to inappropriate cell proliferation (DeCaprio et al. 1988; Dyson et al. 1990; Harris et al. 1996; Krynska et al. 1997). The Rb

motif LXCXE is required to induce high-density cell growths and release of E2F from Rb (Zalvide et al. 1995). E2F release from Rb is also mediated by the J-domain containing the HPDKGG motif which recruits the heat shock protein 70 to the Rb-E2F complex where the action of the Hsp70-mediated ATP hydrolysis liberates E2F (Campbell et al. 1997; Sullivan et al. 2002). The J-domain is not only responsible for cell transformation but is also required for viral DNA replication and virion assembly (Peden et al. 1992; Spence et al. 1994).

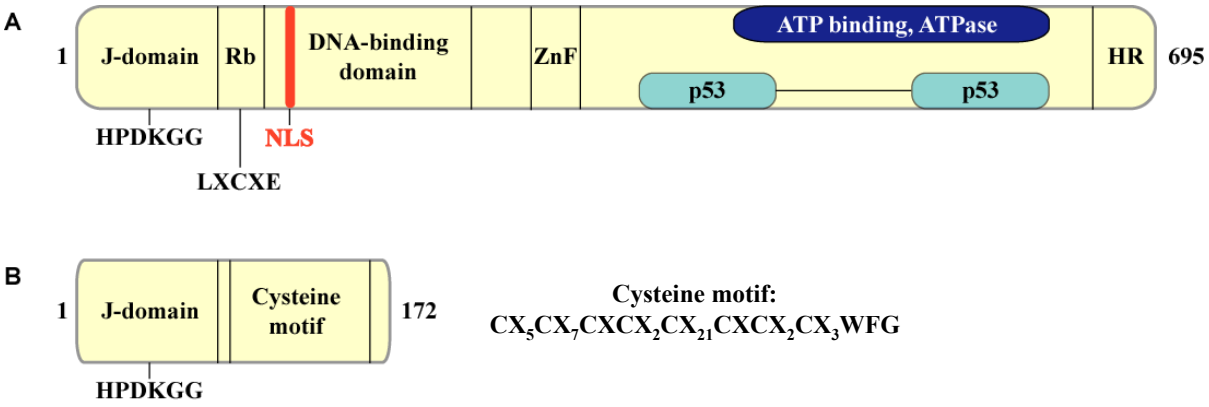

Figure 1.2: A) BKV LT-antigen and **B)** BKV sT-antigen protein structure. J-domain, LXCXE-domain and ATPase-p53-domain are able to bind to host cell proteins, NLS: nuclear localization signal necessary for the nuclear transport, ZnF: zinc finger region modulates DNA-binding specificity of LT-antigen, HR: host range domain is required for viral assembly. N-terminal region is identical for LT- and sT-antigen. The C-terminal region of sT-antigen contains a stretch of cystein residues.
adpted from (Pipas 1992; Caracciolo et al. 2006)

A third target of LT-antigen is the tumor suppressor protein p53, the main mediator of the checkpoint in the cell cycle and initiator of programmed cell death. LT-antigen binds with its ATPase-p53 binding domain and inactivates p53, thus preventing inhibition of the cell cycle and apoptosis (Bollag et al. 1989; Krynska et al. 1997). The zinc finger (**ZnF**) region is located between the *ori* DNA binding and ATPase domains and may play a role in oligomerization of the protein (Loeber et al. 1989). Additionally it functions mainly in the replication of viral DNA and modulates the DNA-bindings specificity of LT-antigen (Pipas 1992; Nemethova et al. 2004). The host range (**HR**) domain is located at the C-terminus of LT-antigen and is required late in viral productive infection and viral assembly (Pipas 1985; Khalili et al. 1988; Stacy et al. 1989). The nuclear localization signal (**NLS**) is the minimal signal sufficient for the nuclear transport of LT-antigen (Zanta et al. 1999).

In vitro studies have shown the ability of BKV sT- and LT-antigen to induce mutations in rodent and human cells (Trabanelli et al. 1998). It could also be shown that LT-antigen not only induce mutations in rodent cells but may also transform these cells into a neoplastic phenotype (Imperiale 2000; Imperiale 2001; Tognon et al. 2003). These rodent cells expressed LT-antigen in their nucleus and the viral DNA was integrated into the cell genome.

Evidence of a possible role for BKV in human cancer has been supported by the detection of BKV-DNA in a range of human tumors such as brain tumors, neuroblastoma, bone tumors, insulinomas, Hodgkin's diseases, Kaposi's sarcoma, urinary tract tumors and genital tumors (Tognon et al. 2003). Clearly, it needs more investigation to prove that BKV infection is a risk factor for the development and/or progression of BKV-associated human tumors as viral DNA detection alone is not evidence enough.

LT-antigen was also linked to the production of autoimmune T- and B-cells directed against nucleosomes, particularly to double stranded DNA (**dsDNA**) and histones. Anti-dsDNA antibody is a marker for systemic lupus erythematosus (**SLE**), a disease with a wide variety of unrelated manifestations. Several steps are necessary to break the self-tolerance and to develop dsDNA-specific B-cells for autoimmunity progression. Different pathways may lead to autoimmunity: 1. Autosensitization by release of true autoantigens due to tissue injury, 2. B- and T-cell activation by cross-reactive viral peptides (mimicry), 3. B- and T-cell activation due to presentation of a self-ligand complexed with a non-self molecule, and 4. T-cell activation due to non-specific bystander activation of autoreactive T-cells (Van Ghelue et al. 2003). Here only the last two points will be addressed to the role of LT-antigen in the context of autoimmunity.

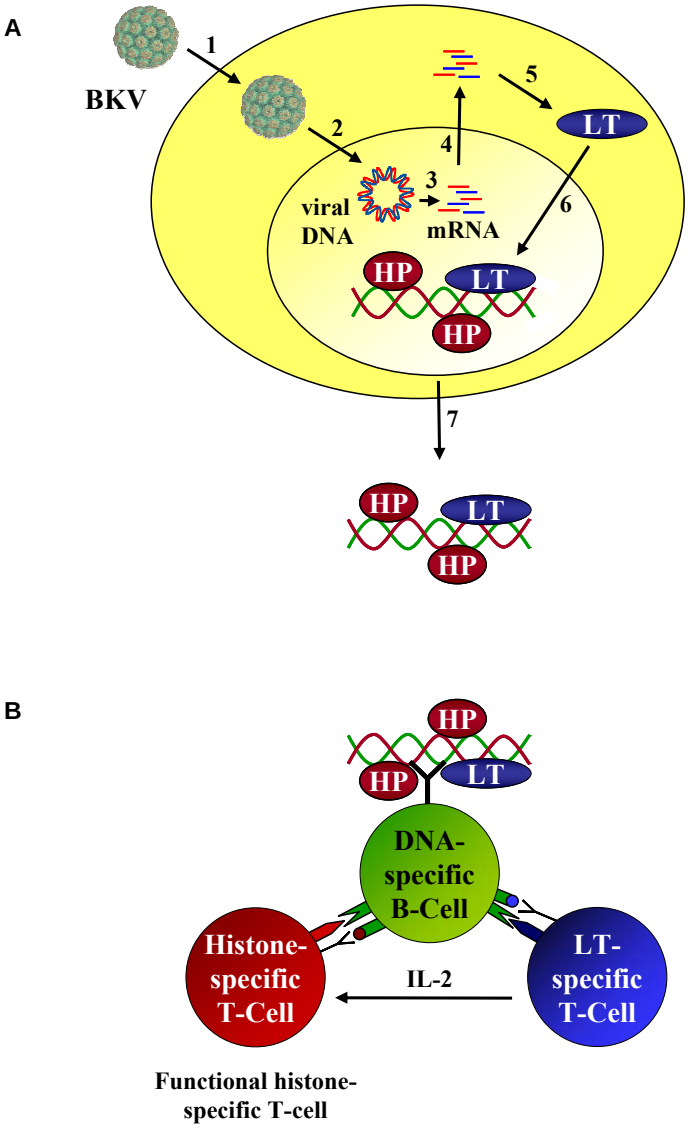

Figure 1.3: A) BKV-infection of a host cell (1), viral-genome release into the nucleus (2). Transcription (3), mRNA transport to the cytoplasm (4) and translation into viral proteins, including LT-antigen (LT) (5). LT-binding to cellular chromatin (6) and liberation from the host cell (7). **B)** The LT-antigen/cellular chromatin-complex can then be recognized, internalized, processed, and presented by B-cells. LT-specific T-cells secrete IL-2 upon recognition and may activate histone-specific T-cells in the microenvironment. LT: LT-antigen, HP: histone protein.
adpted from (Van Ghelue et al. 2003)

Upon host cell infection polyomaviruses start with the production of viral proteins, including LT-antigen which is able to bind host cell nucleosomes (Andreassen et al. 1999; Bredholt et al. 2001). After release of this complex, B-cells may internalize and present both, LT-antigen and histone-derived peptides to T-cells (**Figure 1.3 A**).

Responder LT-antigen-specific T-cells secrete IL-2 upon peptide recognition and may then nonselectively activate autoimmune, histone-specific T-cells which are present in the microenvironment (**Figure 1.3 B**) (Beverly et al. 1992; Jenkins 1992). These histone-specific T-cells may clonally expand if histone-derive peptides are presented by antigen presenting cells (**APC**) and if sufficient costimulatory signals are available. Furthermore, these T-cells then stimulate the DNA-specific B-cells to proliferate and differentiate into an anti-DNA antibody producing plasma cell and result in autoimmunity.

1.1.2.2 Late Proteins VP1-3 and Agnoprotein

The three viral proteins VP1, VP2 and VP3 are expressed late in infection and form a icosahedral capsid which encapsulates the viral DNA. The BKV and JCV capsid contains 360 VP1 monomers arranged in 72 pentameric subunits with each pentamer associated with one peptide of either VP2 or VP3 (**Figure 1.4**) (Liddington et al. 1991).

Figure 1.4: Polyomavirus particle consisting of 72 pentameric subunits of VP1 associated with either VP2 or VP3.
(Slavov 2006)

BKV-VP3 is identical with the VP2 C-terminus (amino acids 120-351) and consists of a DNA binding domain, a nuclear localization signal, and a VP1 interacting domain (**Figure 1.5**). Polyomavirus assembly consists of two phases: 1. The "subvirion" assembly phase, where pentamerized VP1 associates with VP2 and VP3 in the cytoplasm. All three capsid proteins harbor a NLS and the "subvirion" complex is transported into the nucleus. 2. During the "virion" assembly phase, the viral genome is packaged. For virion formation, the capsid proteins are sequentially arranged on the viral minichromosome. It is hypothesized that six DNA binding domains are involved in the packaging of the viral genome: five from the VP1 pentamer and one from either VP2 or VP3. It has been shown that both minor capsid proteins are required for the viral life cycle, including the proper packaging of the genome (Clever et al. 1993; Dean et al. 1995; Gasparovic et al. 2006).

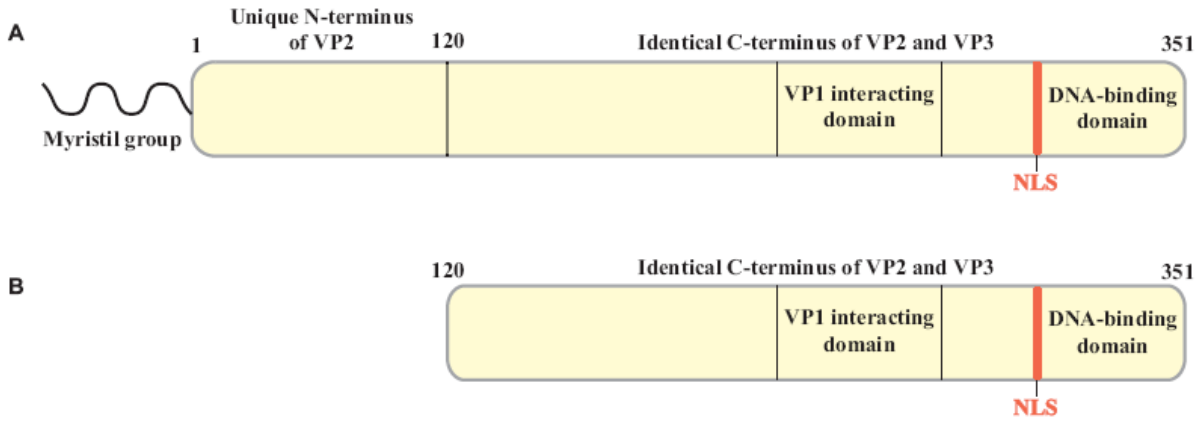

Figure 1.5: A) BKV-VP2 and **B)** BKV-VP3. The C-terminal residue is identical (amino acid 120-351) and consists of a VP1 interacting domain, a NLS and a DNA-binding domain. The unique N-terminus of VP2 is hydrophobic and possesses a fatty acid myristyl group which is added during synthesis. *adapted from (Clever et al. 1991; Daniels et al. 2006; Nakanishi et al. 2006).*

Virus-like particles (**VLPs**) are morphologically similar to natural virus particles, but are not infectious due to the lack of genome. VLPs are composed of VP1 alone and are capable of self-assembly for both BKV and JCV (Chang et al. 1997; Li et al. 2003).

Agnoprotein is a small highly basic protein with multiple roles, such as interacting with cellular proteins, facilitating the efficient packaging of viral capsid proteins, regulating viral transcription and translation. Additionally, agnoprotein was found to interact with LT-antigen and downregulate viral gene expression and DNA replication (Cole 1996; Safak et al. 2001).

1.2 POLYOMAVIRUSES IN IMMUNOCOMPETENT HOSTS

1.2.1 Polyomavirus BK and JC Infection and Life Cycle

The mode of transmission of BKV and JCV is not completely resolved but the infection occurs most probably via oral and/or respiratory routes during pregnancy, transfusion, or transplantation (Hirsch et al. 2003) and occurs asymptomatically in 50%-90% of the population (**Figure 1.6**) (Moens et al. 1995; Knowles et al. 2003).

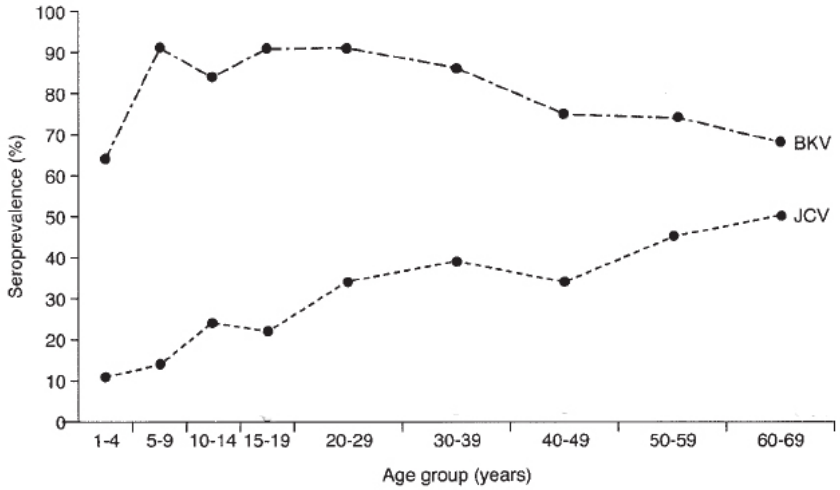

Figure 1.6: Age dependent polyomavirus BK and JC seroprevalence.
adapted from (Knowles et al. 2003)

The infectious life cycle of polyomaviruses can be divided into an early and a late stage. In the early stage, the virus interacts with the surface of the host cell, enters the host cell and starts to replicate the viral DNA. The late stage includes the assembly of new virions and the release of viral progeny which complete the viral replication cycle (**Figure 1.7**).

Both viruses, BKV and JCV, bind sialic acid for the host-cell entry (BKV: terminal α2-3-linked sialic acid, JCV: terminal α2-6-linked sialic acid). While BKV interacts with gangliosides GC1b and GT1b, JCV depends on the serotonin receptor $5HT_{2A}$ to infect cells. Host-cell invasion occurs by caveolae-mediated endocytosis (BKV) or via clathrin-coated pits (JCV) (Dugan et al. 2006; Gee et al. 2006; Low et al. 2006). Upon host cell entry, polyomaviruses are transported to the nucleus by the cytoskeletal transport machinery (Dohner et al. 2005).

In the nucleus, polyomaviruses are uncoated and transcription of the early viral genes, sT- and LT-antigens, is initiated. Subsequent to early transcription is the switch to DNA-replication, initiated by LT-antigen, which binds to the origin of replication. LT-antigen is a strong activator of the late viral promoter (Henson 1995). Expression and nuclear localization of the viral capsid protein VP1-2, leads to the assembly of the virion and DNA packaging. The newly packaged virion progeny is then released by a lytic rupture of the host cell (Clayson et al. 1989; Imperiale 2001).

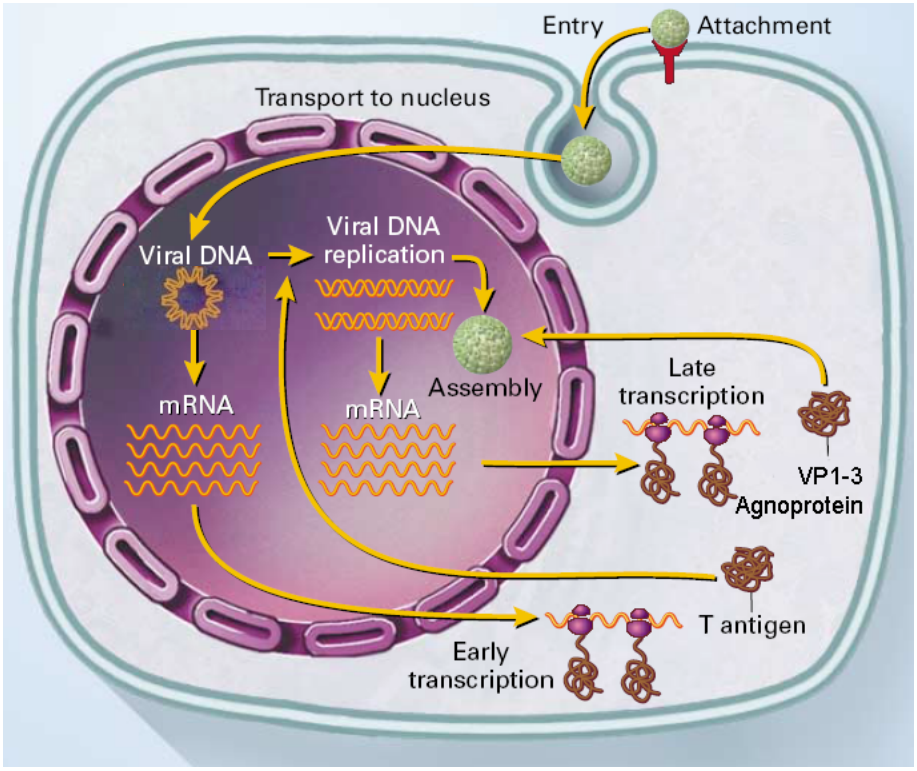

Figure 1.7: Polyomavirus life cycle. Entry into the host cell and viral DNA-replication in the nucleus happens in the early onset of infection. The late onset includes all subsequent events leading to the assembly of virions and the release of viral progeny.
adapted from (Fishman 2002)

1.2.2 Polyomavirus BK and JC Latency and Reactivation

During primary infection (**Table 1.3**), different host cells, including lymphoid tissues, brain, liver, and renourinary tract, are exposed to the polyomaviruses. The most important sites are the renal tubular epithelial cells and the urothelial cell layer where BKV and JCV reside in a nonreplicative latent state and from where they may reactivate. Reactivation in immunocompetent hosts occurs in up to 10% and 40% for BKV and JCV, respectively (Ling et al. 2003; Polo et al. 2004). Such a reactivation is characterized by histologically (decoy cells) and PCR (DNA) detection of the virus in urine. However, viral replication is effectively suppressed in immunocompetent individuals and polyomavirus-associated disease is only seen in severely immunocompromised hosts.

Table 1.3: Polyomavirus infections: terminology.

	Definition
Primary Infection	Initial infection of host with polyomaviruses including viremic spread to permissive tissues with insignificant clinical symptoms.
Latent Infection	Dormant asymptomatic infections of permissive cells (e.g. renal tubular, transitional cells) following the primary infection; virus detection only with molecular techniques.
Virus-Replication	Case with evidence of virus multiplication obtained by detection of infectious virus, virions, structural proteins or their mRNA, or cell-free DNA in nonlatency sites (e.g., plasma or CSF); by cytological analysis (of decoy cells); or by histological analysis.
Virus-Disease	Case with histological evidence of BKV-mediated organ pathology

adapted from (Hirsch et al. 2003; Hirsch 2005; Nickeleit et al. 2006).

1.3 POLYOMAVIRUSES IN IMMUNOCOMPROMISED HOSTS

Diseases caused by polyomaviruses are typically observed in immunosuppressed individuals, who are not able to mount a protective immune response to control the viral replication. BKV-associated diseases are hemorrhagic cystitis (**HC**) in hematopoietic stem cell transplant (**HSCT**) recipients, and polyomavirus-associated nephropathy (**PVAN**) in kidney transplant (**KT**) recipients. JCV is the causing agent for progressive multifocal leukoencephalopathy (**PML**) in human immunodeficiency virus positive (**HIV+**) patients.

1.3.1 Polyomavirus JC: Progressive Multifocal Leukoencephalopathy in HIV+ Patients

PML is a fatal demyelinating disease of the central nervous system in patients with an impaired immune system. JCV is the causing agent of PML and brain biopsies show lesions which may range from small to several millimeters in diameter. Progression of PML results in the enlargement and confluence of these lesions. JCV lytically infects and destroys oligodendrocytes, the myelin-producing cells. The infection of astrocytes ends with an abortive replication and causes morphological changes in those cells, resembling transformed cells (Reiss et al. 2003). Patients with PML suffer from cognitive disturbances, visual impairment, sensory loss, speech and language disturbance (Brooks et al. 1984; Berger et al. 1998).

PML occurs mainly in HIV+ patients, but is also seen in other individuals with impaired immunity: 80% of reported cases have AIDS, 13% suffer from hematological malignancies, 5% are transplant recipients, and 2% have chronic inflammatory diseases (Koralnik 2006). Diagnosis involves brain biopsies which are not always appropriate due to their invasive nature. Detection of JCV DNA in cerebrospinal fluid (**CSF**) by PCR can also be used for diagnosis with a diagnostic specificity of 100% and sensitivity of 80% (Hirsch et al. 1998). In addition, JCV-DNA is detectable in B-lymphocytes in the brain and can be also found in the blood of >95% of PML patients (Houff et al. 1988; Sweet et al. 2002). PML has a significant mortality rate in HIV+ patients and before highly active antiretroviral therapy (**HAART**) the median survival rate of AIDS patients was 3.5 months (Berger et al. 1998; Tassie et al. 1999). After introduction of HAART the median survival rate increased to 2.2 years in 63% of the patients (De Luca et al. 2000; Berenguer et al. 2003).

Recently, it has been shown that JCV was also reactivated in multiple sclerosis patients receiving Natalizumab (Tysabri®). Natalizumab, a monoclonal antibody against integrin, significantly increased the risk for developing PML. The incidence of PML was 1 in 1000 Natalizumab-treated patients, which is substantially lower than the incidence in AIDS patients (Berger et al. 2006).

1.3.2 Polyomavirus BK: Hemorrhagic Cystitis in Hematopoietic Stem Cell Transplant Recipients

HC is a significant complication following HSCT and is characterized by painful hematuria due to hemorrhagic inflammation of the bladder. It is not clear what factors lead to HC but chemo-irradiation, cytopenia, viral infections (Adenovirus, Cytomegalovirus, BKV) and alloimmune reactions may contribute to the disease (O'Reilly et al. 1981).

HC can generally be divided into the early and late onset complications. Early onset is mainly related to uroepithelial toxicity from conditioning regimen (chemotherapy, cyclophosphamide, and busulphan) (Tsuboi et al. 2003). Late onset complications have been

linked to several risk factors as unrelated donors, occurrence of graft-versus-host disease, and virus infections (Seber et al. 1999; Cesaro et al. 2003; El-Zimaity et al. 2004).

First evidences of the role of polyomavirus in complications in HSCT recipients was discovered in the 1980s with the detection of BKV in urine of those patients (O'Reilly et al. 1981; Arthur et al. 1986). Nearly 77% of HSCT recipients shed the BKV into urine, but only 5% - 34% developed HC (Azzi et al. 1999; Priftakis et al. 2003). Later studies showed that those patients suffering from HC had a significantly higher BKV load in urine compared to the asymptomatic immunocompromised patients (**Figure 1.8**) (Bedi et al. 1995; Azzi et al. 1999; Leung et al. 2005). BK viruria in HSCT recipients was as high as 10^8 -10^{10} copies/ml and patients with a BKV load in urine >10^7 copies/ml were at higher risk of developing HC. Not only the amount of BKV in urine is a risk factor but also the dynamics of viruria over time. Eight of 29 patients with HC showed a peak ≥3log higher compared with patients without HC (0/39) (Leung et al. 2005).

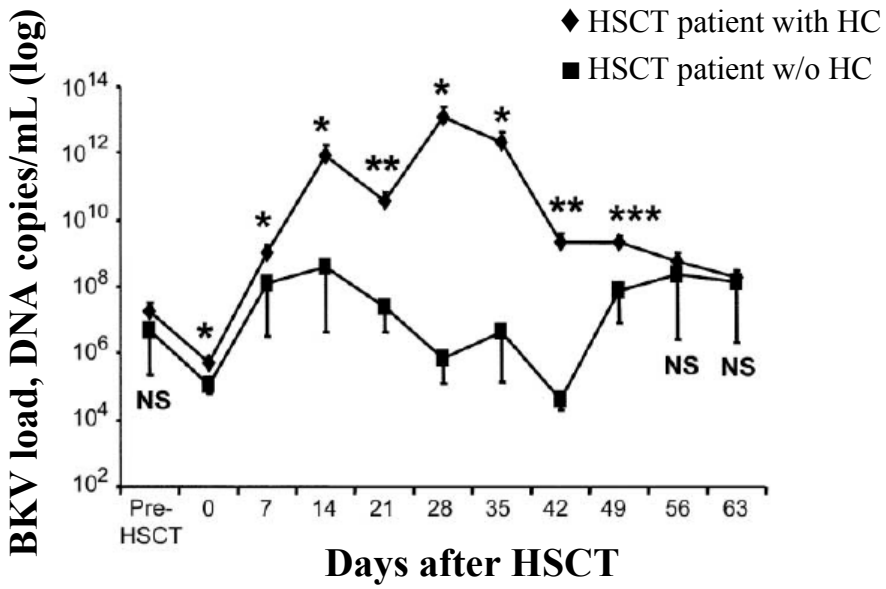

Figure 1.8: Higher BKV load in urine in patient with HC (diamonds) compared to patients without HC (squares). Peaking of BKV load in urine in HSCT patients with HC and low-level BK viruria in HCST patients without HC (NS: not significant, *P<0.05 **P<0.01 ; ***P<0.001).
adapted from (Leung et al. 2005)

HC is rare in allograft recipients and AIDS patients, suggesting that HSCT-specific factors are necessary for causing the disease (Gluck et al. 1994). It can be suggested that pathogenesis of post-engraftment HC is divided into three phases: Phase 1 includes uroepithelial damage caused by chemotherapy/irradiation during the conditioning regimen. This is followed by uroepithelial regeneration which provides an appropriate cellular milieu for BKV-replication. In phase 2, BKV is able to replicate due to the immunosuppressive effects of the conditioning as BKV-specific cellular immunity is decreased. BKV-replication then leads to cytopathic effects and shedding of the virus in the urine. In phase 3, hematopoietic reconstitution leads to the recovery of BKV-specific immunity and this immunological reaction causes extensive mucosal damage and severe HC (Binet 2000; Hirsch et al. 2003; Leung et al. 2005).

1.3.3 Polyomavirus BK: Polyomavirus-Associated Nephropathy in Kidney Transplant Recipients

BKV is an opportunistic pathogen in KT recipients and causes PVAN in up to 10% of the patients with graft loss in up to 80% of the cases (Hirsch 2005). Even though BKV is the main causing agent of nephritis in KT recipients, rare cases of PVAN have been reported due to JCV or SV40 (Kazory et al. 2003; Wen et al. 2004). The role of BKV-replication in non-renal solid organ transplantation is not clearly understood, and the best investigated data come from KT recipients.

1.3.3.1 Risk Factors

The risk factors for developing PVAN in KT recipients are controversial, but it seems that multiple factors are required, including characteristics of the patient (>50 years, male gender, white race, BKV-seronegative status prior transplantation, diabetes), the transplant (HLA-mismatch, prior acute rejection, ischemia), the immunosuppression (triple combination of tacrolimus/mycophenolate mofetil/prednisone, drug level, anti-rejection treatment as anti-lymphocyte globulin), and the virus (serotype, genome mutations, genome rearrangements, immune evasion, and fitness) (**Figure 1.9**) (Hirsch et al. 2003; Hirsch 2005).

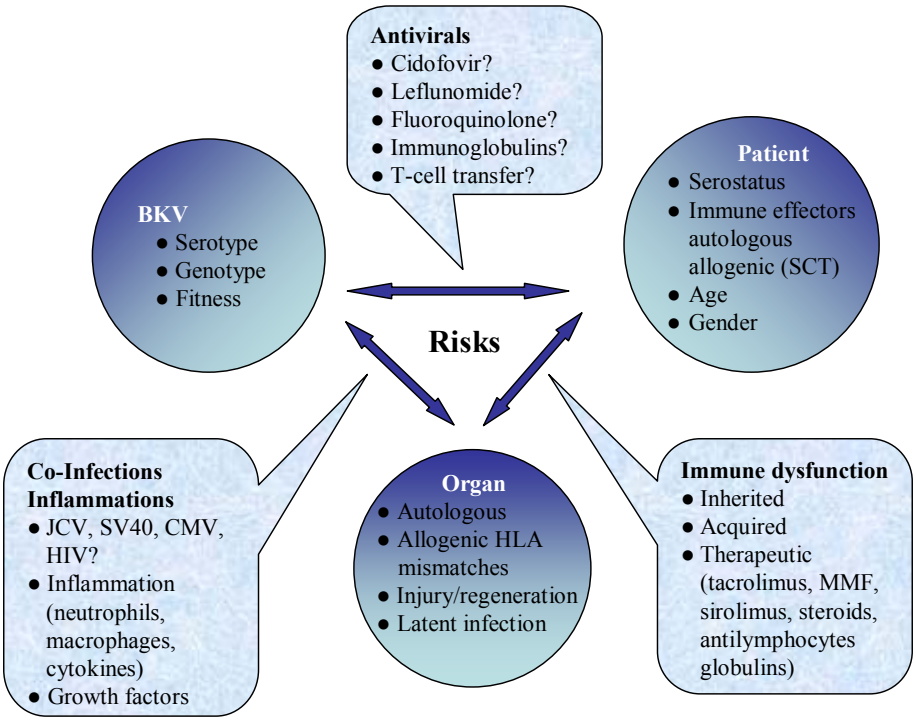

Figure 1.9: Risk factors for PVAN development: multiple factors deriving from the virus, patient and organ are necessary for development of PVAN.
adapted from (Hirsch et al. 2003)

From all of these risk factors, immunosuppression is generally accepted as the key modulator increasing the risk for PVAN. The new potent immunosuppressive drugs, tacrolimus and mycophenolate mofetil (**MMF**), have been significantly associated with BKV-replication and progression to the disease (Brennan et al. 2005).

1.3.3.2 Diagnosis & Monitoring

Different diagnostic tools, such as detection of decoy cells and BKV-DNA in urine and detection of BKV-DNA in plasma, have been used to screen patients for BKV-replication. Measurement of BKV-DNA load in plasma correlates closely with allograft involvement, and monitoring of the BKV-load seems to be suitable for patient monitoring (Nickeleit et al. 2000). KT patients with a BKV plasma load of >10'000 DNA copies/ml are at high risk of developing PVAN (Hirsch et al. 2001; Hirsch et al. 2002).

The road to PVAN includes different stations (**Table 1.4**): 1. Possible PVAN in presence of BK-viruria but absence of -viremia, 2. Presumptive PVAN in the presence of viruria and viremia, and 3. Definitive PVAN with histological evidence of BKV (Hirsch et al. 2005). Definitive PVAN can only be proven by biopsies that have a high specificity but are limited due to their invasive nature, their sampling error because of focal distribution and their frequent misinterpretation as graft rejection (Randhawa et al. 1999). A lack of detectable BKV-DNA in urine practically excludes PVAN due to its high negative predictive value.

Table 1.4: Road to PVAN.

	Urine	Plasma	Biopsy	Definition
Possible	+	-	-	Presence of BK viruria
Presumptive	(+)	+	-	BKV load >10'000 DNA copies/ml in plasma, BKV load >10^7 DNA copies/ml in urine
Definitive	(+)	(+)	+	Histological evidence of allograft involvement

adapted from (Funk 2007, USGEB, poster number 100) and (Hirsch et al. 2005)

Histological outcome of PVAN can be divided into three patterns (A, B or C) according to the progression of the biopsy (**Table 1.5**). Viral cytopathic changes in the kidney result from accumulation of newly formed virions in the cell nucleus and cellular lysis. The following lesions are multifocally distributed in the graft and PVAN progression results from enlargement of these infected foci (**Figure 1.10 A**) (Drachenberg et al. 2003). To confirm the polyomavirus origin of infection, staining for SV40 LT-antigen is usually performed, which cross-reacts with BKV- and JCV-LT-antigen (**Figure 1.10 B**) (Hirsch 2002; Hirsch 2005).

Table 1.5: Histological patterns of PVAN.

A	Viral cytopathic changes only, in near-normal renal parenchyma. Negligible or absent tubular atrophy, interstitial fibrosis, and inflammation.
B	Combination of viral cytopathic changes and focal/multifocal areas of tubular atrophy/interstitial fibrosis/inflammation.
B1	<25% of the core shows tubular atrophy/interstitial fibrosis/inflammation. The majority of the core lacks features of scarring.
B2	26-50% of the core shows tubular atrophy/interstitial fibrosis/inflammation. Significant areas of the biopsy lack features of scarring.
B3	>50% of the core shows tubular atrophy/interstitial fibrosis/inflammation. Only a minority of the core lacks features of scarring.
C	Very scarce viral cytopathic changes in diffusely scarred renal tissue. Extensive tubular atrophy/interstitial fibrosis/inflammation involving the entire tissue core with no residual areas of nonatrophic tubules.

adapted from (Drachenberg et al. 2004)

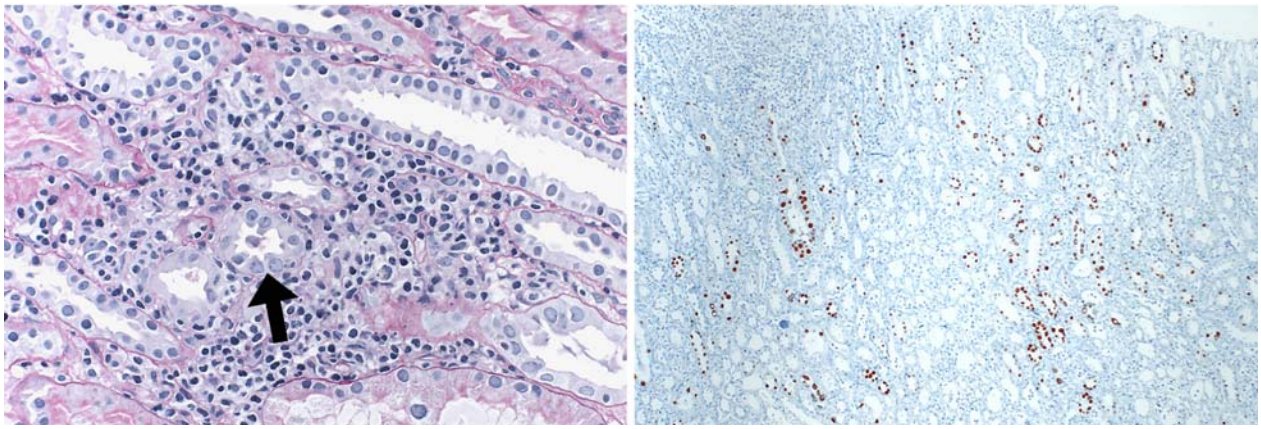

Figure 1.10: A) Focal infiltrates and enlarged nuclei (arrow) in kidney biopsy**, B)** Staining for SV40 LT-antigen which demonstrates numerous viral inclusions (brown) in infected tubular epithelial cells. *(Schaub et al. 2007)*

1.3.3.3 Treatment of PVAN

Current management strategy is to lower immunosuppressive levels to allow immunological clearance of BKV-replication. Lowering the immunosuppressive dosage can be achieved by three different strategies: 1. Reduction (MMF, tacrolimus and cyclosporine), 2. Stopping (MMF), or 3. Switching (from FK506 -> cyclosporine or sirolimus, or from MMF -> leflunomide) of the drug (Hirsch et al. 2002; Wali et al. 2004). However, reduction of immunosuppressants bears the risk for rejection and is histologically difficult to distinguish from immune reconstitution inflammatory syndrome (**IRIS**) (Hirsch et al. 2003). IRIS is a strong inflammatory response due to recovery of different immune cells (NK, T-cells) which attack the abundant viral antigens and polyomavirus-infected cells.

There is no established antiviral therapy to control BKV-replication, but some compounds including cidofovir, leflunomide, quinolone, intravenous immunoglobulines, and lactoferrin have been found to have anti-polyomavirus properties (**Figure 1.11**) (Josephson 2005; Longhi

et al. 2006; Rinaldo et al. 2007). Cidofovir is given intravenously for CMV retinitis treatment in AIDS patients but the use of cidofovir is limited due to nephrotoxicity. In vitro studies revealed that cidofovir is also effective against mouse polyomavirus and SV40 (Andrei et al. 1997). The mechanism by which cidofovir mediates anti-polyomavirus activity is not known. However the effectiveness *in vivo* is unclear and prospective studies are missing.

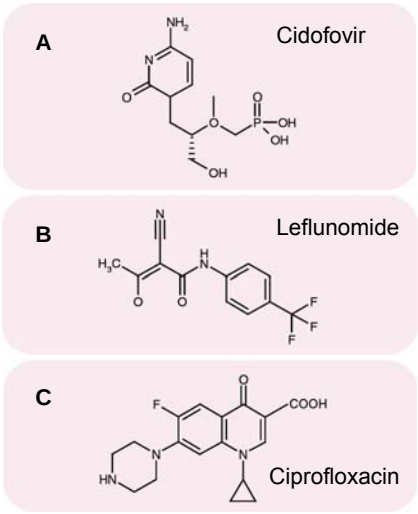

Figure 1.11: Compounds with anti-polyomavirus properties. **A)** Cidofovir, commonly used for CMV treatment, **B)** Leflunomide, approved for use in rheumatoid arthritis, and **C)** Ciprofloxacin, an antibiotic normally used to inhibit the activity of type II bacterial topoisomerase.
adapted from (Rinaldo et al. 2007)

Leflunomide is an antiproliferative agent which limits cellular and humoral immune response. It is approved for use in rheumatoid arthritis but has also been found to have antiviral properties. Leflunomide may cause severe liver injury which occurs mostly within the first 6 months of initiation. In a small study, 17 patients with biopsy-proven PVAN underwent treatment with leflunomide. Those patients with a leflunomide blood level of >40 µg/ml (15/17) cleared the virus or had progressive reduction of BKV. Even though this result looks promising the known risk of toxic effects has to be taken into account (Williams et al. 2005).

The quinolone antibiotic ciprofloxacin has been shown to suppress BKV-replication and thus favors the outcome of HC in HSCT recipients. Quinolones inhibit DNA-gyrase in prokaryotes and suppress BKV-replication. None of the patients with significant BK-viruria suppression developed HC. Nevertheless, these results have to be validated with a larger study group.

The effect of immunoglobulins as anti BK-virals is not clear. In a study with eight patients suffering from PVAN, immunoglobulins were administered and immunosuppression was lowered. In follow-up biopsies only one patient was positive for PVAN and after 15 months of follow up seven out of eight patients had functional grafts (Sener et al. 2006). Unfortunately, it remains unclear whether this outcome was due to the immunoglobulins or due to reduced immunosuppressants.

Lactoferrin is an iron-binding glycoprotein and plays an important role in fighting parasitic, mycotic, bacterial and viral infections. In an *in vitro* assay with Vero (green monkey kidney) cells, Longhi et al. could demonstrate that lactoferrin inhibits BKV infection by directly binding to viral particles (Longhi et al. 2006). The efficacy of all these compounds is controversial and large studies are missing.

1.4 POLYOMAVIRUS-SPECIFIC IMMUNE RESPONSE

The key element of polyomavirus disease is the disruption of the balance of the viral replication and the host immune control. Currently, there is only limited knowledge of the humoral and cellular immune response to BKV and JCV.

1.4.1 Humoral Immune Response

BKV-specific neutralizing antibodies reduce the abundance of infectious virus particles, prevent a systemic spread of the pathogen in vivo and are directed against the viral capsid protein VP1 (Comoli et al. 2006). The exact role of BKV-specific antibodies in a kidney transplant setting is controversial. It seems to be that BKV-seropositivity prior to transplantation does not protect from BKV-replication and subsequent disease, and that antibody titers increase only after resolution of viremia (Comoli et al. 2004; Hariharan et al. 2005). It has been shown that in pediatric KT recipients a BKV-seronegative status is strongly associated with viral replication and progression to PVAN (Ginevri et al. 2003; Smith et al. 2004). The role of antibodies against JCV is not yet clear, but some reports describe a decrease of JCV-DNA in the cerebrospinal fluid (**CSF**) with a parallel increase of anti-VP1 antibody titer (Giudici et al. 2000; Guillaume et al. 2000).

Polyomavirus-specific antibodies clearly deserve more attention, in particular in context of using them as biomarkers to monitor KT recipients.

1.4.2 Cellular Immune Response

A protective immune response to viruses is established upon recognition of viral peptides by CD8+ (responsible for killing the virus-infected cell) and CD4+ effector T-cells (helping B-cells to produce anti-viral antibodies). For viral-peptide recognition by the T-cell receptors, the antigen has to be loaded on APC. APCs present antigens on major histocompatibility complex (**MHC**) molecules which appear in two distinct classes: MHC class I (**MHC I**) or MHC class II (**MCH II**). MHC I are present on all nucleated cells while MHC II are mainly present on specialized APCs. Viral peptides are classically bound on MHC I and recognized by CD8+ T-cells. CD4+ T-helper (**Th**) cells are activated by antigens bound on MHC II and either activate macrophages (**Th1-cells**) or B-cells (**Th2-cells**). Only few BKV- and JCV-epitopes are known to induce CD8+ or CD4+ cellular immune responses **(Table 1.6)**. Epitopes recognized by T-cells may serve as vaccination strategies or adoptive T-cell transfer as a therapeutical option for treating or preventing the viral disease. This has already been demonstrated for HSCT or SOT recipients who received *ex vivo* expanded CMV- or EBV-specific cytotoxic T-lymphocytes (**CTLs**) (Riddell et al. 1992; Papadopoulos et al. 1994; Rooney et al. 1995; Heslop et al. 1996; Comoli et al. 2002). *Ex vivo* expansion has also been developed for BKV-specific CTLs showing that these cells can efficiently lyse BKV-infected cells (Comoli et al. 2003).

Epitope mapping of immunogenic peptides is possible by using peptide libraries for *in vitro* testing, or in a more convenient way, by computer prediction. *In vitro* epitope mapping is very time consuming and expensive, but naturally processed epitopes can be found with this method and it is HLA-independent. Computer prediction may miss some epitopes because algorithms are not available for all HLA-types.

Table 1.6: BKV- and JCV-epitopes inducing a cellular immune response.

Virus	Peptide	Length	Sequence	HLA	Reference
BKV	**LT_{25}**	15aa	GNLPLMRKAYLRKCK	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{27}	9aa	LPLMRKAYL	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{362}	9aa	MLTERFNHIL	HLA-A*02	(Randhawa et al. 2006)
	LT_{406}	9aa	VIFDFLHCI	HLA-A*0201	(Provenzano et al. 2006)
	LT_{410}	9aa	FLHCIVFNV	HLA-A*0201	(Provenzano et al. 2006)
	LT_{553}	15aa	IYLRKSLQNSEFLLE[1]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{557}	15aa	KSLQNSEFLLEKRIL[2]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{579}	9aa	LLLIWFRPV	HLA-A*0201	(Provenzano et al. 2006)
	LT_{613}	15aa	TFSRMKYNICMGKCI[3]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	$VP1_{p44}$	9aa	AITEVECFL[4]	HLA-A*0201	(Chen et al. 2006)
	$VP1_{p108}$	9aa	LLMWEAVTV[5]	HLA-A*0201	(Chen et al. 2006)
JCV	**LT_{552}**	15aa	AYLRKSLSCSEYLLE[1]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{576}	15aa	KSLSCSEYLLEKRIL[2]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{612}	15aa	TFSTMKANVGMGRPI[3]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	$VP1_{p36}$	9aa	SITEVECFL[4]	HLA-A*0201	(Du Pasquier et al. 2003)
	$VP1_{p100}$	9aa	ILMWEAVTL[5]	HLA-A*0201	(Koralnik et al. 2002)

[1-5] *BKV and JCV homologues peptides*

The importance of developing an effective immune response to polyomaviruses was also illustrated for JCV, the causing agent of PML. CTL-response to JCV-VP1 peptide ILMWEAVTL was recognized in five of seven (71%) PML survivors but in zero of six PML progressors (Koralnik 2002). Furthermore, PML patients showed reduced JCV-specific CD4+ T-cell response and restoration was associated with JCV clearance from CSF which indicates the importance of CD4+ response in preventing PML (Koralnik 2002; Gasnault et al. 2003).

Further studies are needed to understand the interplay of the immune response and viral reactivation and replication.

Aim

2. AIM

The aims of the present study were: 1. To elucidate the BKV-specific cellular immune response to LT-antigen, capsid protein VP1 and agnoprotein in healthy blood donor (**HB**) and KT recipients, 2. To illustrate the interplay between BKV-replication and cellular immune response, and 3. To test for cross-stimulation of JCV proteins LT-antigen, VP1 and agnoprotein in the same KT patients, due to the 75% homology to BKV.

To test BKV- and JCV-specific cellular immune response in HB and KT recipients, peptide libraries of three BKV-proteins (LT-antigen, VP1 and agnoprotein) and three JCV-proteins (LT-antigen, VP1 and agnoprotein) were used for PBMC stimulation. The cellular immune response to the different peptides was measured by ELISpot assay, using IFN-γ as a marker for peptide-specific immune response.

SUMMARY OF RESULTS

3. SUMMARY OF RESULTS

Polyomavirus BK is an emerging pathogen in KT recipients. New potent immunosuppressive drugs promote reactivation and replication of BKV and progression towards PVAN. PVAN occurs in up to 10% of the KT recipients with a graft loss in up to 80% of the cases. New potent immunosuppressive drugs, as MMF) and FK506 are risk factors for developing PVAN. As no proven antiviral drugs are available, the only therapy of choice is the reduction of immunosuppressiva in order to regain BKV-replication control (H. H. Hirsch, M. Dickenmann, S. Binggeli, J. Steiger, Schweiz Med Forum 2004; 4:538–541).

BKV-specific cellular and humoral immune response is not well characterized. Recent findings have shown that BKV-seropositive patients prior to transplantation are not protected from BKV-replication. In contrast, BKV-specific cellular immune response correlates with the diagnosis of PVAN (P. Comoli, S. Binggeli, F. Ginevri, H. H. Hirsch, Transplant Infectious Disease Jun 2006; 8(2):86-94, Review).

The aim of this study was to investigate the interplay of BKV-specific immune response and BKV-replication in blood samples of KT recipients. We examined the BKV-specific immune response by ELISpot assay in KT. PBMC of KT recipients were stimulated with BKV LT-antigen and BKV-VP1 peptide libraries. The BKV-specific immune response was measured by the detection of IFN-γ by ELISpot assay. From the results of a pilot study with eight patients we were able to deduce that the dynamics of viral-replication rather than the viral load correlates with a protective immune response (S. Binggeli, A. Egli, M. Dickenmann, I. Binet, J. Steiger, H. H. Hirsch, American Journal of Transplantation, Sep 2006; 6(9):2218-9).

To corroborate this previous observation the BKV-specific cellular immunity in 42 KT recipients and 10 HB were tested. The KT patients were divided into two groups: patient group 1 with an increasing or stable viral load (inc/hi)[1] and patient group 2 with a decreasing viral load or after resolved PVAN (dec)[2]. Indeed patients in group 2 showed a significantly higher immune response upon stimulation with BKV-LT and BKV-VP1 than patients in group 1 (P=0.003, P=0.001, respectively, Wilcoxon, two-sided). Detailed analysis revealed a cut-off of >69 SFU/Mio PBMC for BKV LT-antigen, but not for BKV VP1, with significantly more KT patients from group 2 (dec) than from group 1 (inc/hi). This cut-off has to be validated in a prospective study and also analyzed whether such a cut-off can be used for immunosuppressive reduction guidance.

BKV-specific cell expansion was tested in a short-term culture in the presence of either BKV-LT or -VP1. After 9-day culture, PBMC were restimulated with BKV-LT or -VP1 and the responses were then compared with responses to direct stimulation (without prior cultivation). BKV-LT and -VP1 specific cellular immune responses were significantly higher after 9-day cultivation than after direct stimulation (P=0.002, P=0.003, respectively, Wilcoxon, two sided).

Due to high sequence homology between JCV and BKV, JCV-LT and -VP1 overlapping peptide pools were used to test PBMC-cross recognition. JCV-LT and -VP1 responses were significantly lower than BKV-mediated response (P=0.008, P<0.001, respectively, Wilcoxon, two-sided). Comparison of JCV- and BKV-specific responses after 9-day culture revealed that the BKV-VP1 response was significantly higher than the JCV-VP1 (P=0.016, Wilcoxon, two sided), but no significant difference was observed for LT-antigen (S. Binggeli, A. Egli, S. Schaub, I. Binet, M. Mayr, J. Steiger, H. H. Hirsch, American Journal of Transplantation, Mar 2007; 7:1-9).

[1] Group 1: increasing or persisting plasma BKV loads of $>10^4$ DNA copies/ml and <1.5 log.
[2] Group 2: decreasing viral load >1.5 log.

Agnoprotein, a late viral protein, is highly expressed upon infection. We investigated whether agnoprotein is able to induce a BKV-specific immune response and whether it may serve as a diagnostic marker. Immunostaining revealed that agnoprotein was highly expressed in the cytoplasm of infected cells and was only seen in combination with BKV-LT which is located in the nucleus. Interestingly, BKV-agnoprotein specific cellular and humoral immune responses were scarcely detected in HB or KT recipients. There are only few published studies concerning BKV-agnoprotein, and further investigations are necessary to fully understand the function of agnoprotein during infection. **(**D. Leuenberger, P. A. Andresen, R. Gosert, S. Binggeli, E. H. Ström, S. Bodaghi, C Hanssen Rinaldo, H. H. Hirsch, Clinical and Vaccine Immunology, Aug 2007; 14(8): 959-968).

As no antiviral treatment is available for BKV, the only therapy is the reduction of immunosuppressive drugs in order to regain immunological control over BKV-replication and PVAN. However reduction of immunosuppressants upon PVAN diagnosis bears the risk of rejection or inflammatory response to BKV. It is difficult to distinguish between these two outcomes because specific markers are yet lacking. Therefore, it is pivotal to record the clinico-pathological course of the KT patient in order to correctly diagnose the problem as the therapies are completely different. Measuring the BKV-specific cellular immune response may support and complement other markers, such as PCR analysis and biopsies, to better distinguish between rejection and BKV-specific immune response. (S. Schaub, M. Mayr, A. Egli, S. Binggeli, B. Descœudres, J. Steiger, M. J. Mihatsch, H. H. Hirsch, Nephrology Dialysis Transplantation, Aug 2007; 22(8): 2386-90).

Finding the optimal immunosuppressive drug level is crucial for preventing rejection (under-immunosuppressed) and viral replication (over-immunosuppressed). Our current study showed a cut-off level of 6.65 ng/ml FK506 drug level in blood, dividing those KT patients with and without BKV-replication control (ROC-curve: AUC=0.897, sensitivity=78%, specificity=86%). If this cut-off is validated by a well designed prospective study, it may serve as a guideline to administrate the optimal drug level. (S. Binggeli, 2007, current results).

BKV-specific epitopes have received considerable attention in the last five years. We started with the epitope mapping in a kidney patient with the most common HLA-type: HLA-A*01, HLA-B*08. First screening of BKV-LT revealed ten 15aa long peptides with immunogenic potential. Three of these ten peptides were further investigated for cross-recognition with the homologous JCV-peptides. Even though response to the three JCV-peptides was lower, cellular immune response could be clearly detected. It needs further investigation to find more BKV-specific epitopes and also to test the ability of CD8+ T-cells to kill BKV-antigen presenting cells. (S. Binggeli, 2007, current results).

_____*PUBLICATIONS & MANUSCRIPT*

4. PUBLICATIONS & MANUSCRIPT

4.1 H. H. Hirsch, M. Dickenmann, **S. Binggeli**, J. Steiger
Alte Viren-neue Immunsuppressiva: *liaison dangereuse?*
Schweizerisches Medizinisches Forum, 2004; 4:538-541

4.2 P. Comoli, **S. Binggeli**, F. Ginevri, H.H. Hirsch
Polyomavirus-associated nephropathy: update on BK virus-specific immunity
Transplant Infectious disease, 2006; 8: 86-94

4.3 **S. Binggeli**, A. Egli, M. Dickenmann, I. Binet, J. Steiger H.H. Hirsch
BKV Replication and Cellular Immune Responses in Renal Transplant Recipients
American Journal of Transplantation, 2006, 6(9):2218-9

4.4 **S. Binggeli**, A. Egli, S. Schaub, I. Binet, M. Mayr, J. Steiger, H. H Hirsch
Polyomavirus BK-Specific Cellular Immune Response to VP1 and Large T-Antigen in Kidney Transplant Recipients
American Journal of Transplantation, 2007; 7:1-9

4.5 Stefan Schaub, Michael Mayr, Adrian Egli, **Simone Binggeli**, Bernard Descœudres, Jürg Steiger, Michael J. Mihatsch, Hans H. Hirsch
Transient allograft dysfunction from immune reconstitution in a patient with polyoma BK-virus-associated nephropathy
Nephrology Dialysis Transplantation, 2007; 22(8): 2386-90

4.6 David Leuenberger, Per Arne Andresen, Rainer Gosert, **Simone Binggeli**, Erik H. Ström, Sohrab Bodaghi, Christine Hanssen Rinaldo, Hans H. Hirsch
"Human Polyomavirus type 1 (BK virus) Agnoprotein is abundantly expressed, but immunologically ignored"
The Journal of Clinical Investigation, 2007; 14(8): 959-968

4.7 Egli Adrian, Isabelle Binet, **Simone Binggeli**, Clemens Jäger, Alexis Dumoulin, Juerg Steiger, Urban Sester, Martina Sester, Hans H. Hirsch.
"Cytomegalovirus-specific T-cell Responses in Kidney Transplant Recipients with and without Viral Replication"
American Journal of Transplantation, submitted Aug 2007

FORSCHUNG.CH Schweiz Med Forum 2004;4:538–541 538

Alte Viren – neue Immunsuppressiva: *liaison dangereuse?*

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Zusammenfassung

Polyomaviren wie das BK- und JC-Virus sind entwicklungsbiologisch alte, wenig pathogene Erreger, die sich optimal an ihren menschlichen Wirt angepasst haben. Seit 1995 werden aber Polyomaviren zunehmend für Funktionsstörungen in transplantierten Nieren verantwortlich gemacht. Die mit dem Polyomavirus assoziierte Nephropathie (PVAN) ist bei zirka 5% der Patienten nachweisbar und kann in über 80% zu einem Transplantatverlust führen. Der Grund für diese Häufung ist unklar, fällt aber mit der Einführung von neuen Immunsuppressiva wie Tacrolimus und Mycophenolat-Mofetil zusammen. Derzeit gibt es keine antivirale Therapie. Einzige Behandlungsoption ist eine Reduktion der immunsuppressiven Therapie, um eine verbesserte immunologische Kontrolle der Polyomavirusinfektion zu erreichen. Die Diagnose der PVAN basiert auf der Biopsie, wurde aber meist in einem fortgeschrittenen Stadium gestellt. Möglicherweise ist eine frühzeitige Diagnose kritisch für eine erfolgreiche Intervention. In Basel wurde deshalb prospektiv das Auftreten von virusinfizierten «Decoy-Zellen» im Urin, von BK-Virämie und PVAN bei 78 Patienten untersucht, die eine Basis-Immunsuppression mit Tacrolimus oder Mycophenolat-Mofetil erhielten. Die Ergebnisse zeigten eine Inzidenz von PVAN von 8% (95%, Konfidenzintervall 1–15%). BK-Virus-spezifische Antikörper waren bei 80% vor Transplantation nachweisbar und schützten nicht vor PVAN (Sekundärinfektion). Mögliche Risikofaktoren sind vorgängige Abstossungsepisoden und deren Behandlung. Die BK-Virämie konnte als Surrogatmarker der PVAN zur Diagnose (>10000 Kopien/ml) und als Verlaufsparameter nach Reduktion der Immunsuppressiva verwendet werden.

Einleitung

Für Patienten mit terminaler Niereninsuffizienz gibt es derzeit zwei Behandlungsmöglichkeiten:
- Dialyseverfahren (Hämo- oder Peritonealdialyse) und
- Transplantation einer Spenderniere.

Beide Behandlungsmöglichkeiten sind heute Routine und haben spezifische Vor- und Nachteile. Vergleichsuntersuchungen zeigen aber, dass die Nierentransplantation die Therapie der Wahl ist, da ausser geringeren Kosten zwei wesentliche Vorteile für die betroffenen Patienten bestehen: geringere Mortalität und höhere Lebensqualität. Trotzdem bleiben bei der Transplantation weiterhin hohe Anforderungen an Patienten, Pflege und Ärzte bestehen. Offensichtlich kritische Punkte sind die Organspende (Lebendspende, Leichenspende, Organknappheit, Warteliste), die Planung und Durchführung der Transplantation sowie die interdisziplinäre Nachsorge der Patienten. Die Verbesserung der Nachsorge hat bei Lebend- oder Leichenspende in der Zeit von 1988 bis 1996 zu einem Anstieg des Transplantatüberlebens von 5–10% innerhalb des ersten Jahres geführt [1].

Aus infektiologischer Sicht kann man die Nachsorge grundsätzlich in zwei Abschnitte einteilen: In den ersten Tagen bis Wochen stehen bakteriologische Komplikationen im Zusammenhang mit Chirurgie und Hospitalisation im Vordergrund wie Katheter-, Wund- und Harnwegsinfektionen. In den anschliessenden 6 bis 12 Monaten folgen opportunistische Infektionen, für die einerseits die notwendige immunsuppressive Behandlung, andererseits Eigenschaften von Transplantat und Empfänger massgeblich sind [2]. Infektionen mit Viren stellen diesbezüglich eine besondere Herausforderung dar [3], weil
- die immunologische Kontrolle von Viruserkrankungen zum Teil dieselben Mechanismen verwendet wie immunologische Abstossungsreaktionen;
- bestimmte Viren nach erfolgter Infektion in Körperzellen des Patienten bzw. des Organs versteckt, d.h. latent, bleiben können und dort als «Schläfer» nicht vom Immunsystem erkannt und eradiziert werden;
- Viruslatenz und -reaktivierung klinisch und diagnostisch oft schwer unterscheidbar sind;
- nicht pathogene Begleitreaktivierung gelegentlich schwer von einer eigentlichen Viruserkrankung abgrenzbar ist;
- Viruserkrankungen häufig erst spät diagnostiziert werden;
- antivirale Medikamente nicht verfügbar sind oder, wenn vorhanden, diese nur die Virusreplikation bremsen, nicht aber zu einer Viruseradikation führen.

Ausgehend von latenten Virusinfektionen kann es zum Wiederaufflammen der Virusvermehrung und Organschädigung kommen. Diese Reaktivierung wird von einem intakten Immunsystem meist problemlos wieder unter Kontrolle gebracht. Die zur Vermeidung von Abstossungsreaktionen notwendigen immunsuppressiven Medikamente schränken die immunologischen Kontrollmöglichkeiten ein. Polyomaviren entfalten genau in dieser Situation ihr «krankmachendes» Potential im Sinne einer neuen opportunistischen Komplikation [4, 5].

Alte Viren

Polyomaviren sind bei Wirbeltieren weit verbreitet und zeichnen sich durch eine hohe Wirtsspezifität aus. Der Mensch ist der natürliche Wirt für zwei Polyomaviren, Typ 1 und Type 2, die nach den Initialen der ersten Patienten BK- und JC-Virus genannt werden, von denen sie vor 30 Jahren erstmals isoliert wurden. Etwa 80% der erwachsenen Bevölkerung haben in der Kindheit wahrscheinlich durch familiäre Übertragung Primärinfektionen mit beiden Polyomaviren durchgemacht, ohne dass dabei besondere Zeichen oder Symptome erkennbar wären. Polyomaviren verbleiben lebenslang im Körper. Hauptorte dieser latenten Infektion sind die Nieren und ableitenden Harnwege. Von dort kommt es bei 1–20% der gesunden Erwachsenen zu spontaner Reaktivierung mit nachfolgend meist symptomloser Ausscheidung in den Urin [4]. Hohe Durchseuchung und geringe Pathogenität sprechen für eine entwicklungsbiologisch etablierte Adaptation. Tatsächlich können menschliche Migrationsbewegungen der letzten 100 000 Jahre anhand von Polyomavirus-Geno-Subtypen nachvollzogen werden kann, wie dies für mitochondriale DNA möglich ist. Dies gilt für Wanderungsbewegungen indianischer Ethnien von Afrika über Asien und Alaska nach Amerika und entsprechend Kolonisation und Sklavenhandel für das Auftreten der europäischen und westafrikanischen Genotypen unter der heutigen amerikanischen Bevölkerung. Auf virologischer Ebene zeigt sich die enge Verzahnung von Wirt und Virus nicht nur in einer ausschliesslichen Verwendung von Wirtszellenzymen für die Virusreplikation, sondern auch darin, dass bei der Verpackung des Virusgenoms Histone der Wirtszelle verwendet werden, die sogar in die Virionen «entführt» werden [4].

Neue Immunsuppressiva

Während JC-Virus als Ursache der meist tödlich verlaufenden Hirnerkrankung PML (progressive multifokale Leukoenzephalopathie) bei schwer immungeschwächten Patienten, z.B. 1–5% der AIDS-Patienten, lange bekannt ist, bestand für das BK-Virus lange keine sichere Krankheitsassoziation. Dies änderte sich 1995, als unabhängig in Basel und in einigen Transplantationszentren der USA bei nierentransplantierten Patienten eine Häufung von Polyomavirusinfektionen mit progredientem Befall der transplantierten Nieren beobachtet wurde [6]. Der Grund für die plötzliche Zunahme ist bisher nicht geklärt, aber fast alle Patienten erhielten neue potente Immunsuppressiva wie Tacrolimus und/oder Mycophenolat-Mofetil, die meist sogar kombiniert wurden. Mittlerweile sind auch erste Fälle in Kombination mit Sirolimus aufgetreten [4, 5]. Möglicherweise ist die Potenz dieser neueren Immunsuppressiva ein wichtiger Faktor für das Auftreten der PVAN. Ein einziger Fall von PVAN durch JC-Virus wurde kürzlich beschrieben (<1% aller Fälle) [7]. Die Bedeutung des verwandten Affenvirus SV40, mit dem Anfang 1960 Polioimpfstoffe kontaminiert waren, wird zur Zeit noch kontrovers diskutiert [8].

Antivirale Therapie?

Eine spezifische antivirale Behandlung existiert derzeit nicht. Insbesondere bieten Polyomaviren keine selektiven Angriffspunkte für etablierte antivirale Medikamente, wie man sie für die DNA-Polymerasen der Herpesviren kennt. Ähnlich wie für Zytomegalievirus-Infektionen in der Zeit *vor* Ganciclovir ist die wichtigste Intervention eine Reduktion der immunsuppressiven Behandlung, um eine immunologische Kontrolle der BK-Virus-Replikation zu erreichen [4]. Diese Intervention ist nicht unproblematisch, da sich damit das Risiko für eine immunologische Abstossungsreaktion erhöht [5, 9].

Diagnose der PVAN

Die Diagnose PVAN basiert auf der mikroskopischen Untersuchung einer Gewebsprobe (Biopsie) [10]. Besonders zu Beginn wurde die Diagnose häufig verpasst bzw. erst in einem fortgeschrittenen Stadium gestellt. Möglicherweise ist aber eine frühzeitige Diagnose wichtig für eine erfolgreiche Intervention. In Basel haben wir beobachtet, dass bei Patienten, die eine histologisch dokumentierte PVAN entwickelten, teilweise Wochen vorher BK-Virus im Blut nachweisbar war [11].

Prospektive Untersuchung zu PVAN

Wir haben deshalb prospektiv 78 Patienten, die eine Immunsuppression mit Tacrolimus oder Mycophenolat erhielten, bis zu 140 Wochen (Median 85) nach Nierentransplantation überwacht [12].

Unsere Ergebnisse zeigen, dass
- bei 8% (Streuung 1–15%) eine BK-Virus-Nephropathie erwartet werden kann;
- die Diagnose im Schnitt bis zu 12 Wochen früher gestellt wurde als in den ersten retrospektiven Untersuchungen;
- bei allen Patienten bis zu 5 Wochen vorher eine ansteigende BK-Virus-Menge auf Werte >10000 Kopien/ml im Blut nachweisbar war;
- bei 80% aller Patienten bereits vor Transplantation BK-Virus-spezifische Antikörper nachweisbar waren;
- durch eine Modifikation bzw. dosierte Reduktion der Immunsuppressiva die BK-Virus-Last im Blut abnahm;
- kein Transplantat wegen PVAN oder deren Behandlung verloren wurde [12].

Risikofaktoren für PVAN

Patienten nach durchgemachten Abstossungsreaktionen und entsprechender Behandlung waren signifikant häufiger betroffen. Dies unterstützt die Hypothese, dass PVAN durch das besondere Zusammenwirken von mindestens zwei Komponenten gefördert wird: Gewebeschädigung durch immunologisch-entzündliche Prozesse wie Abstossung sowie durch herabgesetzte Reaktionsfähigkeit des Immunsystems [12].

PVAN-Hypothese

Obwohl eine intensive Immunsuppression einer der wichtigsten Faktoren für PVAN ist, gehen wir davon aus, dass PVAN nicht allein durch eine *liaison dangereuse* mit neuen potenten Immunsuppressiva zustande kommt, sondern durch multiple, sich ergänzende Faktoren, die zusammen eine Nische für diese neue opportunistische Komplikation eines eigentlich relativ harmlosen Erregers bilden [4]:
- zellulär: Tubuluszellschädigung, Regeneration, Zytokinwirkung;
- immunologisch: schwere Immunsuppression mit neuen Immunsuppressiva, HLA-Mismatch, Primoinfektion, neuer Serotyp;
- viral: Primoinfektion, Zweitinfektion mit neuem Serotyp, adaptierter hoch replizierender neuer Genotyp.

Gründe für diese Annahme liegen nicht zuletzt in der Beobachtung, dass PVAN in allogenen Nieren relativ häufig, aber nur selten in autologen Nieren bei anderen Solidorgantransplantationen wie Leber und Herz beobachtet wird, obwohl dieselben Immunsuppressiva bzw. Dosen verwendet werden [5]. Antikörper vor Transplantation schützen nicht vor PVAN. Eine Infektion mit dem BK-Virus-Subtyp des Spenders ist nicht ausgeschlossen, verbessert aber möglicherweise die Reaktionsfähigkeit der Immunabwehr. Umgekehrt ist vorstellbar, dass bei polyomavirusnaiven, also seronegativen, Patienten mit einer durch das Transplantat übertragenen Primoinfektion und gleichzeitiger intensiver Immunsuppression allenfalls keine früheren Abstossungsepisoden vorliegen müssen [5, 13].

PVAN und Abstossung

Die Diagnose von PVAN und gleichzeitiger Abstossung wird in verschiedenen Zentren kontrovers beurteilt. Wird PVAN zusammen mit einer Abstossung diagnostiziert [10], haben wir in Basel gute Erfahrungen mit einem zweiphasigen Vorgehen gemacht: initiale Abstossungsbehandlung, meist mit intravenösen Steroiden als erstem Schritt, gefolgt von einer Modifikation/Reduktion der Basisimmunsuppression [9, 12]. Serumkreatinin und BK-Virämie dienen als Verlaufsparameter.

Für die Praxis

PVAN stellt eine neue opportunistische Komplikation von alten viralen Begleitern nach Nierentransplantation dar, welche die Verbesserung des Transplantatüberlebens der letzten 10 Jahre kritisch reduziert. Wegen der Bedeutung einer frühen Diagnose wird für die Nachsorge die Urinzytologie bzw. die quantitative PCR als dreimonatliches Screening während der ersten zwei Jahre empfohlen. Dies sollte ebenso bei Abklärungen von Funktionsverschlechterungen des Transplantats durchgeführt werden. Bei Patienten mit Zeichen einer Virusreplikation im Urin («possible PVAN») sollte die BK-Virämie im Plasma gesucht und quantifiziert werden. Bei einer auf >10000 Kopien/ml ansteigenden BK-Virämie («presumptive PVAN») sollte die Diagnose mittels Biopsie gesichert werden («definitive PVAN»).

Für die Forschung

Wichtige Ziele sind
- die Bedeutung der Reduktion der Immunsuppression zu evaluieren;
- die immunologischen Mechanismen zu charakterisieren;
- immunologische und/oder antivirale Therapieoptionen zu entwickeln.

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Review article

Polyomavirus-associated nephropathy: update on BK virus-specific immunity

Key words:
polyomavirus; BK virus; nephropathy; T cells; antibody; cellular immunity; CTL; humoral immunity; transplantation; kidney; renal transplantation

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Abstract: The human polyomavirus type 1, also called BK virus (BKV), causes polyomavirus-associated nephropathy (PVAN) in 1–10% of renal transplant recipients, with graft loss in over 50% of cases. The risk factors for PVAN are not conclusively defined and likely involve complementing determinants of recipient, graft, and virus. A central element seems to be the failing balance between BKV replication and BKV-specific immune control, which can result from intense triple immunosuppression, HLA-mismatches, prior rejection and anti-rejection treatment, or BKV-seropositive donor/seronegative recipient pairs. Consistent with this general hypothesis, the timely reduction of immunosuppression in kidney transplant recipients reduced graft loss to less than 10% of cases. However, the BKV-specific humoral and cellular immune response is not well characterized. Recent work from several groups suggest that changes in antibody titers and BKV-specific CD4+ and CD8+ T cells may help to better define the risk and the course of PVAN in renal transplant patients.

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The morbidity and mortality of viral infections is significantly increased in transplant patients. A key reason resides in the overall weakened immune functions following induction and maintenance immunosuppression to prevent immunological injury (rejection) post transplant (1). In addition, specific antiviral immune functions are impaired when virus-infected cells and cellular immune effectors meet in an allogenic transplant constellation. Viral infections with a propensity for latency require continuous immune control to restrict the rate and level of virus reactivation as, by definition, clearance from the host cannot be obtained for such viruses, regardless of antiviral treatment (2). For diagnostic and clinical reasons, it is important to carefully distinguish latent infection from active virus replication or virus disease, of which the latter is

often, but not always, linked to concurrent virus replication (3). Cytomegalovirus and Epstein Barr virus (EBV) are notorious sources of viral complications post transplant owing to intricate latency and replication patterns, multilateral interactions with the host's immune system, and viral pathologies that are only partly susceptible to antiviral treatment (4 6). By contrast, polyomavirus infections have been described only rarely (7, 8), but are now receiving increasing attention in transplant patients as BK virus (BKV) was identified as the major cause of polyomavirus-associated nephropathy (PVAN) in 1 10% of renal transplants with subsequent graft loss in over 50% of cases (9 14). JC virus (JCV), on the other hand, was identified early on as the etiological agent of progressive multifocal leukoencephalopathy, a fatal, demyelinating disease of the central nervous system, which has been observed in 5% of acquired immunodeficiency syndrome (AIDS) patients in the era before antiretroviral combination therapy, but is rare in recipients of solid organ transplants (15 17).

Polyomavirus and epidemiology

BKV and JCV were first isolated in the 1970s and named after the initials of the first patients. Both viruses are specific for humans, but belong to the family of polyomaviridae which are found in a wide range of vertebrates including birds, rodents, cattle, monkeys, and primates. The virions are non-enveloped icosahedral particles of 45 nm diameter that contain the circular double-stranded DNA genome of 5.3 kb. Polyomavirus genomes are 60 75% homologous at the nucleic acid level. Three genomic areas have been distinguished: (i) the non-coding control region harboring the origin of viral DNA replication and transcription/promoter sequences to control viral gene expression; (ii) the early genes encoding the regulatory proteins called the tumor (T)-antigens for their transforming potential; (iii) the late genes encompassing the capsid proteins VP-1, VP-2, and VP-3, and the agnoprotein (14, 18). The host cell specificity of polyomaviruses is mediated by the capsid protein VP-1 interacting with host cell receptors (19) and through cell-specific expression of early and late genes from the non-coding control region (20). Efficient polyomavirus replication depends on the recruitment of cellular factors by the regulatory T-antigens and is modulated by cellular differentiation and external signals. Assembly of polyomavirions occurs in the nucleus and high-level replication releases infectious progeny by host cell lysis.

Serological evidence indicates that BKV and JCV infect 50 90% of the general population during childhood (21, 22). Natural transmission is not resolved and occurs presumably via oral and/or respiratory routes, but also during pregnancy, transfusion, and transplantation (14). During primary replication/viremia, exposure of various cell types is thought to occur, which include lymphoid tissues, brain, liver, and the renourinary tract. The renal tubular epithelial cells and the urothelial cell layer represent clinically often silent, but epidemiologically most relevant, sites of latency and reactivation. In immunocompetent individuals, intermittent urinary BKV and JCV replication is found in $<10\%$ and $<40\%$, respectively (23 27). Data from a large prospective renal transplant study enrolling 700 adult patients indicate that BKV is found in the urine of 6.7% of 344 patients with residual urine production before transplantation, at mean viral loads of 10^5/mL (range 10^4 10^6) (28). Prevalence and level of BKV replication in the urine may increase with pregnancy, kidney disease, immunodeficiency including human immunodeficiency virus (HIV)/AIDS (14), and following transplantation procedures (29 31). However, polyomavirus disease is rare, and almost always associated with specific characteristics of the immune system being either immature, naive, immunosuppressed, or over-responsive (3).

Outline of antiviral immune responses

The risk factors for PVAN are not conclusively defined and likely involve complementing determinants of the triad of recipient, graft, and virus (14). Disruption of the balance between the virus replication and host immune control is generally viewed as a key element of viral pathogenesis (32, 33). In general, primary infections are brought under control by innate responses and non-specific cytotoxic cells (natural killer cells, lymphokine-activated cells, MHC-unrestricted $\gamma\delta+$ T-lymphocytes), followed by increasing antibody titers and epitope-specific human leukocyte antigen (HLA)-restricted T-cell populations. Virus neutralizing antibodies reduce infectious virus particles and interfere with the recruitment of new cellular replication sources.

Non-neutralizing antibodies clear viral antigens and, after processing by antigen presenting cells (APC), stimulate epitope-specific CD4 + T cells. CD4 + T cells respond to viral antigenic peptides presented by HLA class II, and contribute to virus control mainly by producing cytokines that stimulate macrophages, CD8 + T lymphocytes (T helper type 1 response), or B lymphocytes (T helper type 2 response), but some antiviral CD4 + T cells also have cytotoxic activity (34). Most virus-specific cytotoxic T cells are CD8 + and induce perforin-mediated lysis of infected cells once virus-derived peptide epitopes are recognized as 'foreign' on HLA class I molecules on the cell surface. With declining antigen load, the specific lymphocyte populations contract by mechanisms involving apoptosis. However, some virus-specific cells survive and enter the memory pool. These memory cells are able to reactivate when stimulated by antigen rechallenge. The function and frequency of specific memory cells is thought to be critical for control of viral latency state.

Up to now, studies on BKV-induced immune responses have been scarce, and consist mainly of morphological presentation, indirect evidence, and delineated pathophysiological concepts (3, 14), derived from indirect evidence and experimental tumor models (35). The coincidence of PVAN with widespread clinical application of potent triple immunosuppressive regimens that allowed for solid organ transplantation across boundaries of histocompatibility, implies a role of a marked cellular immunodeficiency. In addition, PVAN seems to have a predilection for renal transplants as compared to native kidneys of patients with other transplants. These graft-associated factors might include tissue injury from ischemia/reperfusion, allosensitization, BKV tissue load, and HLA mismatching. Failure to mount protective immunity in case of a primary viral replication, or impairment of functional memory T cell expansion in case of viral reactivation (secondary replication) post transplant, may cause morbidity and mortality even from agents whose pathologic potential in immunocompetent individuals is limited. In addition to lack of protection, BKV replication may be facilitated in the context of local injury/regeneration, as has been shown in experimental murine models (36). Interestingly, antilymphocyte agents used for induction treatment were not significantly associated with BKV replication and PVAN compared to their use in anti-rejection treatment (14). Table 1 lists risk factors related to immunity that have been associated with BKV replication and PVAN (37 52).

Immunity-related risk factors for polyomavirus-associated nephropathy

Risk factors	References
Intense triple immunosuppression	
(Calcineurin-inhibitor, antiproliferative agent, steroid)	(10, 37, 38)
Steroids	(39, 40)
Renal transplantation	(29–31, 41)
HLA-mismatches	(38, 42, 43)
Rejection and anti-rejection treatment	(42, 43)
BKV-seronegative recipient	(44, 45)
BKV-seropositive donor	(8, 46)
BKV new serotype, immune evasion	(47)
HLA-C7 negative donor, negative recipient	(46)
Low number BKV-specific IFN-γ-producing T cells	(48–52)

HLA, human leukocyte antibody; BKV, BK virus; IFN, interferon.

Table 1

BKV-specific humoral immune response

Neutralizing antibodies to BKV and subtype-specific antibodies are directed against determinants on the BKV major capsid protein VP-1. Binding of BKV-VP-1 to glycosylated structures on red blood cells mediates hemagglutination, which has been used to quantify viral titers as well as antibody titers through hemagglutination-inhibition (HAI). The HAI titers correlate well with neutralizing antibody titers and have been used in many early studies describing the age-dependent increase in BKV-specific seroprevalence (53). Using VP-1-based enzyme immunoassays (EIA), a good correlation with HAI titers was found and only limited cross-reactivity between BKV and JCV (54). BKV seroprevalence increased in children until age 10, reaching a seropositivity of 98% for BKV among adults as compared to 72% for JCV (55, 56).

The role of BKV-specific antibody titers was investigated more recently in the setting of PVAN. In a first study using HAI, Hirsch et al. (42) in 2002 found that 77% of adult kidney transplant recipients were BKV seropositive before transplantation with a fairly low median titer of 80 (range 10 1280). The results showed that BKV-seropositive renal transplant recipients were not protected from significant BKV replication and PVAN, particularly in the setting of HLA-mismatches and anti-rejection treatment. A recent

study using VP-1 EIA observed significantly lower antibody titers in adult patients progressing to viremia than in patients with sole viruria (57). Although no cases of PVAN occurred in that study, the data suggested a limited protective effect for early stages of BKV replication. In keeping with this notion, Ginevri et al. (44) in 2003 found that pediatric renal transplant recipients with HAI titers of < 40 had an increased risk for BKV replication and disease post transplant, which was independently confirmed for seronegative recipients by another study using VP-1 EIAs (45). The protective effect may not be attributed simply to neutralizing antibody titers, but rather the titers may represent a surrogate of the BKV-specific memory compartment. Thus, low or absent BKV-specific memory response may be associated with an increased risk of subsequent BKV replication and disease. On the other hand, BKV-seropositive donors were shown to increase the risk for subsequent BKV infection (8, 46). Remarkably, the risk of BKV replication in the recipients was highly correlated with BKV antibody titer in the donors (46). These data not only support the hypothesis of the donor origin of the virus harbored in the transplant, but also suggest that recent infection and a potentially higher tissue viral load may be important factors contributing to the imbalance of BKV replication and immune control.

The course of BKV-specific antibody responses after renal transplantation was found to follow the level and the duration of BKV replication (57, 58). In patients with newly diagnosed BKV replication and PVAN, BKV-specific IgG antibody levels were found to increase significantly in patients with declining BKV viremia or past PVAN (58). Moreover, the transient appearance of BKV-specific IgM antibody levels in some patients correlated with declining BKV viremia, and may serve as an early marker of the recovering immune response after reduced immunosuppression. Unlike IgM, IgG responses are largely dependent on T-cell function, but it has been shown for mice polyomavirus that approximately 10% is mounted in a T-cell independent, but CD40 CD154 involving, manner (59).

BKV-specific cellular immune response

Early studies on the cellular immune response against BKV have been essentially limited to hematopoietic stem cell transplantation because of the association of BKV viruria with hemorrhagic cystitis of late onset (60 62). In 1985, Drummond et al. (62) observed a significant correlation between BKV viruria, increasing antibody titers, and the mounting of a detectable BKV-specific lymphocyte proliferation after engraftment. The affected patients are seropositive before hematopoietic stem cell transplantation (63) and develop very high BKV loads in the urine after conditioning (64 66). Because of scattering BKV loads, however, similarly high levels can be found in patients without hemorrhagic cystitis (65, 67). Moreover, as high-level BKV replication precedes hemorrhagic cystitis, but then manifests upon engraftment, it has been suggested some time ago that the underlying pathogenesis might correspond to an 'immune reconstitution syndrome' (3, 14, 16, 68). This phenomenon of excessive inflammatory response is well known from HIV/AIDS patients after starting antiretroviral therapy (69) and can be elicited by polyomaviruses (70, 71) and may affect any organ including the kidneys (72).

In the setting of kidney transplantation, there is now evidence that failure to mount or expand a cellular immune response is linked to BKV replication and PVAN development. Using ELISpot assays, Comoli et al. (48) found that BKV-specific interferon (IFN)-γ-secreting lymphocytes were detectable at a mean frequency of 151 spot-forming units per 10^6 (range 20 430) in the peripheral blood of healthy seropositive individuals. In contrast, BKV-seropositive pediatric kidney transplant recipients had significantly lower mean frequencies of 6 spot-forming units per 10^6 (range 0 9), whereas no BKV-specific IFN-γ-secreting cells were detectable in patients with the diagnosis of PVAN (48). However, in patients with PVAN treated with reducing immunosuppression, BKV loads in plasma and urine declined and the frequency of IFN-γ-secreting lymphocytes increased to the same level as found for healthy controls. Simultaneously declining serum creatinine concentrations indicated stabilization of allograft function and the absence of PVAN was confirmed in biopsy studies (48). For one patient, a rebounding increase of BKV viremia was noted that was coupled to disappearing BKV-specific IFN-γ-producing peripheral blood mononuclear cells (PBMC), but responded to further reduction of immunosuppression (Fig. 1). The antibody titers, however, remained unchanged at the elevated levels (49). These data indicate that the dynamics of BKV-specific cellular immunity are a mirror image of the BKV loads. The discordance of humor-

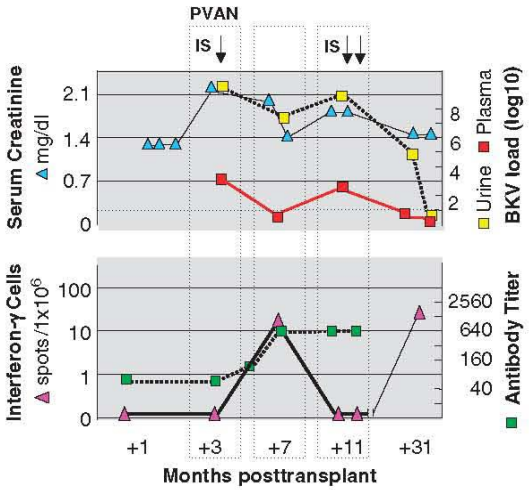

Fig. 1. Monitoring BK virus-specific humoral and cellular immune responses (data from Comoli et al. [48]). PVAN, polyomavirus-associated nephropathy.

al and cellular immune response is important as it demonstrates that the dynamic balance of BKV replication and specific immune response is insufficiently captured by antibody titers alone.

The improving allograft functions in these cases provide evidence against the hypothesis that expanding cellular immunity is a major determinant of polyomavirus disease as proposed for hemorrhagic cystitis (3). However, the success of expanding cellular immunity controlling BKV replication without extensive immunopathologic damage may apply only to early stages of disease. In the case of prolonged high-level viral replication, BKV-mediated cytopathic damage has been estimated to amount to 10^6 tubular epithelial cells per day (73), which may lead to a necrotic granulocytic inflammatory reaction at the site of infection. Further cytotoxic-inflammatory damage and organ compromise could be driven by homing of HLA-restricted effectors. The impact of HLA-restricted T cells may be particularly brisk and thus correspond to an immune reconstitution disease (3, 14) when HLA matching is optimal, which might explain the paradoxical worse graft survival in renal allografts with PVAN and better HLA matches (74). In a mismatched allogenic environment, HLA-unrestricted T cells may cause immune-mediated damage. Indeed, in a recent study, Comoli et al. (75) observed that BKV-specific cytotoxic T-cell lines from kidney graft recipients with PVAN displayed a strong HLA-unrestricted, BKV-directed killing, which could be partly abrogated by experimental depletion of CD3 + /TCRγδ + T cells. Finally, allospecific T-cell responses may emerge by BKV replication in the kidney allograft, which elicit a condition morphologically and molecularly indistinguishable from acute rejection (76) and cause a major dilemma with regard to treatment (77).

To gain further insight into BKV-specific cellular immunity and its role in the genesis and course of PVAN, it will be important to define whether there is a specific pattern of cellular response to BKV antigens. Recently, overlapping peptide libraries for BKV-encoded proteins including large T, small t, VP1, VP2, and VP3 were employed in ELISpot and flow cytometry assays (78). In healthy BKV-seropositive individuals, 18 of 24 (75%) persons tested had more than 10 spot-forming units per 10^6 PBMC to at least one of the BKV peptide pools, but without a clear pattern of immunodominance. Interestingly, 91% of the individuals had a CD4 + mediated response, whereas only 33% generated a CD8 response. Further studies in 10 subjects linked the response to a specific peptide sequence within that pool (78). In another recent study, 15 adult renal transplant patients with BKV viremia were tested for IFN-γ production in PBMC by flow cytometry after stimulation with overlapping peptides spanning the BKV VP-1 (50). CD4 + T-cell responses were detected in the peripheral blood in 7 of 15 patients (47%), but CD8 + T-cell responses were found in only 2 of the 15 patients (13%). In patients with detectable responses, BKV load in serum was >250,000 copies/mL, whereas the BKV load ranged from 900 to 37,000 copies/mL in patients lacking detectable responses in the peripheral blood. It is not clear whether or not patients with higher BKV replication have been diagnosed at a later stage, or whether immunosuppression had been modified before the diagnosis. However, over the next 10 months, both patients with detectable CD8 + T-cell responses lost their allografts (50). These data are intriguing and suggest that some of the BKV-specific responses might be associated with an allospecific response, as discussed. Interestingly, when T-cell lines were generated directly from allograft biopsies after stimulation with irradiated autologous PBMC, interleukin-2 and anti-CD3 stimulation, only VP-1-specific CD8 + T cells were found. In 2 patients, in whom BKV-specific T-cell responses could be measured in PBMC, T-cell lines could be raised from the needle biopsy, but not from the peripheral blood. In some of these patients, the time of biopsy was before the PBMC analysis, suggesting that return into the

circulation could be a sign of recovery from PVAN (50). Although these data are in part contradictory to other reports, and need further study, in particular with respect to the dynamics of BKV replication, the magnitude and diversity of the immune response and the potential of unraveling the interaction of BKV replication and immune responses are nicely demonstrated.

Using a similar strategy with overlapping peptide pools, another study demonstrated the presence of both VP1- and large T-specific cytotoxic T-cell responses in cell lines reactivated from healthy subjects and pediatric kidney transplant recipients (49). Direct testing of PBMC from adult patients with past PVAN by ELISpot assay revealed a higher frequency of BKV-specific IFN-γ responses directed against capsid VP-1 epitopes compared with large T-antigen responses, most of which are generated by CD4 + T cells (Fig. 2) (79). In addition, JCV-derived peptides also elicited a response in these patients, supporting the notion of possible cross-reactivity that can be evaluated using peptide pools.

Definition of the BKV protein peptide sequences recognized by specific T cells will allow adaptation of sensitive tools such as BKV protein tetramers and flow cytometry to quantify cellular immune responses. Using computer-based prediction algorithms, 2 recent studies identified peptides from BKV VP-1 that are recognized in the context of HLA A*0201, and employed the corresponding tetramers to evaluate BKV immunity in kidney transplant patients. Using tetramer painting, Krymskaya et al. (51) reported that BKV VP-1p108-specific CD8 + T cells were detectable at a frequency of 0.7% of CD8 + T cells in the peripheral blood of a renal transplant patient with BKV viremia, but not in another patient with BKV viremia. After *in vitro* stimulation and 2 week culture, expansion up to 24% was observed in the former patient, whereas the latter had low frequencies around 1%, similar to healthy controls. Chen et al. (52) detected BKV VP1p44 and VP1p108-specific CD8 + T cells after *in vitro* culture in the blood of 8/10 (80%) and 5/10 (50%) healthy individuals, respectively. In renal transplant patients with PVAN, CD8 + T cells specific for BKV VP1p44 and VP1p108 were present in the blood of 5/7 (71%) and 6/7 (86%) patients tested, respectively. Patients with high number of CD8 + T cells had lower BKV load in their blood and low anti-BKV antibody titers. Conversely, those who had low cytotoxic T-cell responses had persistently elevated BK viremia and high anti-BKV antibody titers (52). Interestingly, similar responses could be elicited by the homologous JCV-derived peptides calling for a careful dissection of the extent of epitope cross-recognition and possible cross-protection of BKV- and JCV-specific T cells (51, 52, 79).

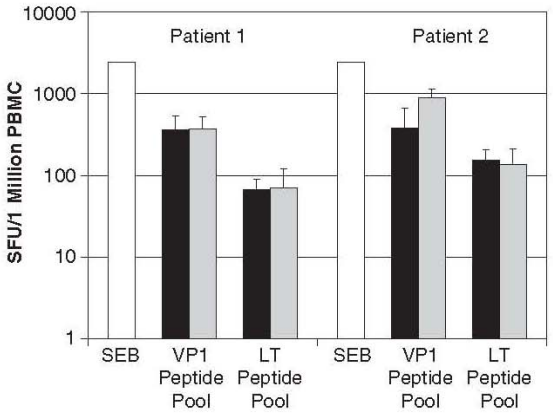

Fig. 2. Interferon-γ production after stimulation of peripheral blood mononuclear cells (PBMC) with peptide pools covering VP-1 and large-T (LT) antigen from BK virus (black bars) and JC virus (grey bars) in kidney transplants after polyomavirus-associated nephropathy (data from Binggeli et al. (79). SEB, *Staphylococcus* endotoxin B.

Perspective

Monitoring of specific immunity has gained widespread consideration for diagnosis and management of viral infections complementing viral load measurements in immunocompromised hosts. EBV DNA-emia is observed in 20 30% of graft recipients post transplant, but only a fraction eventually develops post-transplant lymphoproliferative disease (PTLD). Likewise, EBV seronegativity is a risk factor for PTLD development, yet only some seronegative recipients present with disease. In a recent paper, Smets et al. (80) showed that EBV-seronegative graft recipients with high viral loads have a much higher probability to develop PTLD when unable to mount EBV-specific T-cell responses. Thus, by measuring EBV-specific T-cell frequency, it may be possible to proceed to a finer characterization of a subgroup of patients at risk, in whom preemptive therapy may be justified. Similarly, assessment of BKV-specific T-cell frequency might allow us to identify patients who are more likely to progress to PVAN among those with positive

viremia or even high-level viruria. Similarly, measurement of BKV-specific immunity might provide rational guidance to the reduction of immunosuppressive drugs. However, the best parameter for monitoring specific immunity still needs to be defined. Thus, prospective studies of combined virological and immunological monitoring are warranted to assess the usefulness of this strategy.

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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.

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Letter to the Editor

Table 1: Plasma BKV load and large T- and VP1-specific interferon-γ production in peripheral blood mononuclear cells

Patient	Age/Sex	Serum creatinine (μmol/L)	Immunosuppression	BKV load plasma (copies/mL)	BKV replication dynamics	Δ log10	PBMC ELISpot (IFNγ SFU/10^6 cells)		CD4+ T cells		CD8+ T cells	
							LT	VP1	LT Flow cytometry IFNγ (%)	VP1	LT	VP1 Flow cytometry IFNγ (%)
1	36/F	113	Sir/MMF ↓	51 100	Decreasing	−3	542	392	0.16	0.11	0.04	0.06
2	61/F	141	Sir/MMF ↓	300	Decreasing	−3	97	373	0.04	0.10	0.02	0.07
3	26/M	104	CyA/Aza	944	Decreasing	−2	67	444	neg	neg	neg	neg
4	53/M	115	CyA/MMF/Pre	9600	Decreasing	−0.5	49	227	0.03	0.02	neg	neg
5	50/M	344	CyA/Tac/Lef	38 400	Stable	+0.3	10	13	neg	0.01	neg	neg
6	45/M	155	CyA/MMF/Pre	98 000	Stable	−0.2	37	156	neg	0.01	neg	neg
7	50/F	203	CyA/MMF/Pre	0	Cleared PVAN		75	276	0.02	0.07	0.09	0.07
8	65/M	Hemodialysis	No	0	Graft loss post-PVAN		20	47	0.03	0.11	0.02	0.05

Aza = azathioprine; CyA = cyclosporine; Lef = leflunomide; MMF = mycophenolate mofetil; Tac = tacrolimus; Pre = prednisone; Sir = sirolimus; PVAN = polyomavirus-associated nephropathy; LT = BKV large T-antigen; VP1 = BKV viral capsid protein-1; SFU = mean spot-forming units; IFNγ = interferon-γ; neg = negative (<0.01%).
BKV load was determined by real-time PCR as described with a cut-off of 300 c/mL plasma. ELISpots were performed in triplicate for interferon-γ detection after overnight stimulation of 2.5×10^5 PBMC with 2 μg/mL of 15-mer peptide pools of 11 amino acid overlap spanning LT or VP1 versus negative control medium. Intracellular cytokine staining for IFN-γ detection and flow cytometry was performed after 6 h stimulation with LT or VP1 peptide pools and addition of brefeldin A.

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

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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

March 3–October 10, 2006, were enrolled with written informed consent (Ethics Committee of the Basel Cantons 235/06) if there was evidence of present or past plasma BKV viremia (for summary, see Table 1). Where indicated, immunosuppression was either increased, decreased or remained unchanged in the preceding 8 weeks of analysis. Cidofovir was not used in any patient. Leflunomide had been used in the one patient who subsequently suffered renal allograft loss [1/42 (2.3%)]. All study participants were seropositive for BKV and JCV as determined by testing of 1:400 diluted plasma by ELISA format for IgG against BKV- or JCV-VP1 virus-like particles purified from Sf9 insect cells.

According to our previous analysis of plasma BKV load dynamics in KT patients taking into account sampling density, individual fluctuation and the variation coefficient of 29.6 (13,22), we considered plasma BKV load changes of $>1.5\log_{10}$ significant for the purpose of this study. KT patients were divided into two groups according to the changes in plasma BKV load at the time of PBMC testing for BKV-specific interferon-γ (IFN-γ) production. Group 1 included all KT patients with increasing plasma BKV loads ($n = 5$) or persisting BKV loads with past BKV loads of $>10^4$ cp/mL and $<1.5\log_{10}$ decline ($n = 17$) at the time of PBMC testing (Table 1). Group 2 included KT patients with ongoing BKV viremia at the time of PBMC testing, but declining plasma BKV loads of $>1.5\log_{10}$ decline ($n = 13$) or past BKV viremia ($n = 7$) at the time of PBMC testing (Table 1), the latter with histologically confirmed PVAN at median 20 months earlier (range 5–75 months).

Collection of blood cells and plasma

Human PBMC of BKV-seropositive HD and KT patients were isolated using CPT™ tubes (Becton Dickinson, Allschwil, Switzerland). The cells were washed, counted and either used directly for IFN-γ ELISpot assay (ESA) or for antigen stimulation and 9-day culture, or cryopreserved in 10% DMSO/90% FCS (SIGMA, Buchs, Switzerland). PBMC for ESA were diluted in RPMI/5% human serum/1% glutamax/1% penicillin streptomycin (R5AB, SIGMA). Patient plasma and urine were used to detect BKV- and JCV-DNA by real-time PCR.

Quantitative PCR for BKV- and JCV-DNA detection

BKV- and JCV-DNA were isolated with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and quantified by real-time PCR (TaqMan/7700, Stratagene Mx4000 or BioRad iCycler) as described previously (27). The following primers were used for BKV-LT amplification: BKV forward; AGC AGG CAA GGG TTC TAT TAC TAAAT (26 mer), BKV reverse; GAA GCA ACA GCA GAT TCT CAA CA (23mer), Fam-Tamra-labelled BKV probe; AAG ACC CTA AAG ACT TTC CCT CTG ATC TACACC AGT TT (38 mer), JCV forward; CTA AAC ACA GCT TGA CTG AGG AAT G (25mer), JCV reverse; CAT TTA ATG AGA AGT GGG ATG AAG AC (26mer), Fam-Tamra-labelled JCV probe; TAG AGT GTT GGG ATC CTG TGT TTT CAT CAT CAC T (34 mer). Primers and probes were obtained from Eurogentech (Geneva, Switzerland). The linear range of the real-time PCR is 10^2–10^8 cp/assay and the limit of detection was 300 cp/mL.

BKV and JCV overlapping peptide pools

Overlapping peptide pools spanning large LT and VP1 coding sequences from the BKV Dunlop strain and from the JCV Mad 1 strain were used for PBMC stimulation. The pools consisted of 180 (BKV-LT), 88 (BKV-VP1), 170 (JCV-LT) and 86 (JCV-VP1) peptides of 15 amino acids (aa) in length overlapping by 11aa (Eurogentech) and dissolved in DMSO (SIGMA).

ESA for IFN-γ detection

BKV- and JCV-specific cellular immune responses were determined by measuring IFN-γ upon stimulation of PBMC. For ESA, 96-well multiscreen filter plates (MSIPN, Millipore, Volketswil, Switzerland) were coated with 100 µL (10 µg/mL) coating IFN-γ antibody (1-D1K, Mabtech, Hamburg, Germany) and incubated overnight at 4°C. PBMC were seeded in triplicate at 2.5×10^5/well with or without peptides (2 µg/mL) and at 2.5×10^4/well with *Staphylococcus enterotoxin B* (SEB; SIGMA, 1 µg/mL) as positive control, and incubated overnight at 37°C/5% CO_2.

Plates were washed five times with PBS/0.05% TWEEN 20 (SIGMA) and incubated for 2 h at room temperature with 100 µL (1µg/mL) biotinylated detection IFN-γ antibody (7-B6-1 biotin, Mabtech), washed five times and incubated for 1 h at room temperature with 100 µL (1µg/mL) Streptavidin ALP (Mabtech). Spots were developed by adding 100 µL BCIP/NBT (Calbiochem, Luzern, Switzerland) for 10 min. IFN-γ spots were counted by using an ELISpot reader (AID, Büron, Switzerland). The number of spot-forming units/10^6 PBMC (SFU) per well was calculated from triplicates after subtractions of negative control.

Nine-day expansion of PBMC

PBMC (2×10^6/mL) were distributed into 24-well plates and incubated overnight at 37°C/5% CO_2. Nonadherent cells were recovered, washed and adjusted to 2×10^6 cells/mL. Adherent monocytes were detached by scraping/pipetting, adjusted to 2×10^6 cells/mL and stimulated with 5 µg/mL BKV-LT and -VP1 for 2 h at 37°C/5% CO_2. Monocytes were washed and 10^5 cells/mL were added to 2×10^6 of nonadherent cells. After 9 days at 37°C/5% CO_2, ESA was performed as described above using 10^5 cells for peptide restimulation or 2.5×10^4 for SEB per well.

Intracellular cytokine staining and flow cytometry

BKV-specific IFN-γ production was quantified using intracellular cytokine staining and flow cytometry as published by Sester et al. (28) after stimulation with BKV-LT or -VP1 peptide pools (2 µg/mL, Eurogentech). Medium alone served as negative control and stimulation with SEB (1 µg/mL, SIGMA) as positive control. At least 30 000 $CD3^+$ cells were acquired and analysed on a FACS-Canto (Becton Dickinson). The frequency of BKV-specific cellular immune responses was determined for each antigen and expressed as percent of IFNg+ cells in $CD3^+CD4^+$ or $CD3^+CD8^+$ gated lymphocyte populations, respectively.

Statistical methods

Nonparametric statistical tests were performed using the SPSS (Version-14) to account for sample size and non-normal distribution. For comparison of the study groups, two-sided Mann–Whitney U-Test was used for calculating the p value. The correlation between paired samples was evaluated by the two-sided Wilcoxon signed rank sum test. Data were expressed either as mean ± standard deviation (SD) or as median and range. Differences with p values <0.05 were considered as statistically significant. For better comparison, box plots are shown with median and interquartile range over data scatters, with outliers above or below the whiskers.

Results

BKV-specific cellular immune response in HD

PBMC from BKV-seropositive HD were stimulated using peptide pools covering the entire BKV large LT and VP1, respectively. IFN-γ secretion was detected by ESA. The median spot-forming units (SFU)/10^6 PBMC (SFU) were 24 (range 15–95) for LT peptide pools and 25 (range 7–113) for VP1 peptide pools (Figure 1). No statistical difference was observed between LT and VP1 induced responses in HD ($p = 0.626$).

Table 1: BKV-replication and -specific cellular immune response in KT patients

	Patient tested	Age (years)	Sex (f/m)	S-Creat conc (μmol/L)	Weeks post-TX	IS	Urine BKV-load (cp/mL)	Plasma BKV-load (cp/mL)	Plasma ΔBKV-load (Δlog cp/mL)	Plasma ΔBKV-load (weeks)	BKV-LT (SFU/Mio PBMC)	BKV-VP1 (SFU/Mio PBMC)
Group 1 increasing (n = 5)	206	64	f	147	450	unc	1.00E + 08	3.00E + 02	1.41	7	0	8
	214	40	f	88	54	unc	3.00E + 02	2.06E + 03	0.84	2	4	19
	175	61	m	144	23	unc	1.00E + 08	1.84E + 06	2.94	11	16	0
	91	36	m	136	21	inc	2.62E + 07	1.93E + 07	2.07	10	25	56
	134	62	f	150	163	inc	1.03E + 04	3.00E + 02	0.82	1	37	84
	Mean	53		133	142		4.52E + 07	4.23E + 06	1.62	6	16	33
	SD	13		26	182		5.11E + 07	8.47E + 06	0.90	5	15	35
	Median	61		144	54		2.62E + 07	2.06E + 03	1.41	7	16	19
	Range	36–64		88–150	21–40		3.00E + 02–1.00E + 08	3.00E + 02–1.93E + 07	0.82–2.94	1–11	0–37	0–84
Stable (n = 17)	160	54	m	217	22	unc	2.74E + 07	2.39E + 03	1.94	102	0	12
	8	51	m	166	298	unc	n.a.	2.12E + 03	−1.46	8	3	7
	4	64	m	n.a.	56	unc	n.a.	2.77E + 05	−0.27	41	8	120
	153	54	m	235	15	dec	1.00E + 08	9.91E + 04	0.32	8	9	92
	112	57	f	156	n.a.	unc	3.00E + 02	4.38E + 05	−0.05	24	12	6
	203	52	m	139	27	unc	1.00E + 08	6.75E + 03	0.34	15	16	28
	62	72	m	126	21	unc	1.00E + 08	3.53E + 04	−0.50	11	19	44
	145	70	f	320	218	unc	5.43E + 04	2.25E + 03	0.59	24	19	49
	202	52	m	133	23	unc	1.00E + 08	5.14E + 03	0.46	11	24	31
	196	52	m	132	13	unc	3.06E + 07	1.49E + 04	0.00	1	28	12
	88	36	m	136	11	dec	n.a.	1.63E + 05	−1.38	3	37	156
	66	72	m	106	26	unc	1.00E + 08	6.55E + 05	1.76	16	40	243
	116	26	m	105	332	unc	2.14E + 06	3.68E + 03	−0.41	16	45	423
	97	54	m	111	20	unc	8.31E + 07	1.12E + 04	0.23	8	49	227
	237	46	f	130	313	unc	3.43E + 03	2.65E + 04	1.95	6	49	157
	73	72	m	107	35	inc	7.15E + 07	7.28E + 04	−0.81	25	65	259
	172	21	m	192	105	dec	3.10E + 07	6.96E + 03	−0.06	34	120	256
	Mean	53		157	96		5.33E + 07	1.07E + 05	0.16	21	32	125
	SD	15		58	120		4.38E + 07	1.85E + 05	1.01	24	29	121
	Median	54		135	27		5.13E + 07	1.49E + 04	0.00	15	24	92
	Range	21–72		88–320	11–332		3.00E + 02–1.00E + 08	2.12E + 03–6.55E + 05	−1.46–1.95	1–102	0–120	6–423
Total group 1 (n = 22)	Mean	53		151	107	unc	5.12E + 07	1.04E + 06	0.49	17	28	104
	SD	14		53	133	unc	4.45E + 07	4.10E + 06	1.15	22	27	114
	Median	54		136	27	unc	3.10E + 07	1.31E + 04	0.33	11	22	53
	Range	21–72		105–320	11–40	unc	3.00E + 02–1.00E + 08	3.00E + 02–1.93E + 07	−1.46–2.94	1–102	0–120	0–423
Group 2 decreasing (n = 13)	164	54	m	257	27	dec	7.25E + 04	1.66E + 03	−2.10	19	8	72
	208	64	f	171	462	unc	1.85E + 04	3.00E + 02	−1.41[1]	5	11	45
	167	54	m	150	36	unc	1.70E + 05	3.00E + 02	−2.84	29	15	101

Continued.

Table 1: Continued.

	Patient tested	Age (years)	Sex (f/m)	S-Creat conc (μmol/L)	Weeks post-TX	IS	Urine BKV-load (cp/mL)	Plasma BKV-load (cp/mL)	Plasma ΔBKV-load (Δlog cp/mL)	Plasma ΔBKV-load (weeks)	BKV-LT (SFU/Mio PBMC)	BKV-VP1 (SFU/Mio PBMC)
	54	37	f	103	152	dec	1.04E + 04	3.00E + 02	−4.34	20	29	107
	183	61	m	n.a.	38	dec	n.a.	2.92E + 05	−1.66[1]	4	29	223
	187	61	m	155	44	dec	6.22E + 05	6.44E + 03	−3.33	10	69	293
	100	54	m	117	28	unc	n.a.	3.00E + 02	−2.42	8	80	105
	92	59	m	206	21	unc	2.17E + 07	1.93E + 04	−2.20	12	93	293
	25	54	f	172	376	unc	5.40E + 03	3.00E + 02	−1.31[1]	10	109	373
	126	51	f	137	71	unc	2.96E + 06	2.43E + 02	−2.03	30	121	317
	186	61	m	163	42	unc	3.49E + 05	4.43E + 03	−3.49	8	268	840
	182	61	m	202	36	dec	1.00E + 08	1.57E + 05	−1.94[1]	3	465	1432
	49	37	f	104	140	dec	8.99E + 06	1.12E + 04	−2.77	8	524	392
	Mean	55		161	113		1.23E + 07	3.80E + 04	−2.45	13	140	353
	SD	9		45	143		2.98E + 07	8.75E + 04	0.88	9	172	386
	Median	54		159	42		3.49E + 05	1.66E + 03	−2.20	10	80	293
	Range	37–64		103–257	21–462		5.4E + 03–1.00E + 08	2.43E + 02–2.92E + 05	−4.34–1.31	3–30	8–524	45–1432
After PVAN (n = 7)	119	26	m	116	356	(dec)/unc	1.57E + 05	3.00E + 02	−3.60[2]	28	20	147
	141	66	m	n.a.	n.a.	no	n.a.	3.00E + 02	−4.69[2]	73	20	47
	38	38	f	113	46	(dec)/unc	3.00E + 02	3.00E + 02	−4.32[2]	22	24	53
	16	54	f	203	349	(dec)/unc	7.55E + 03	3.00E + 02	−2.41[2]	9	75	276
	139	41	m	217	340	(dec)/unc	3.00E + 02	3.00E + 02	−3.32[2]	19	93	412
	129	62	f	141	141	(dec)/unc	4.50E + 03	3.00E + 02	−4.43[2]	33	97	373
	55	37	f	105	156	(dec)/unc	3.70E + 03	3.00E + 02	−4.33[2]	24	400	816
	Mean	46		149	231		2.89E + 04	3.00E + 02	−3.88	30	104	303
	SD	14		49	134		6.31E + 04	0.00E + 00	0.81	21	135	269
	Median	41		129	248		4.10E + 03	3.00E + 02	−4.32	24	75	276
	Range	26–66		105–217	46–356		3.00E + 02–1.57E + 04	3.00E + 02–3.00E + 02	2.41–4.70	9–73	20–400	47–816
Total group 2 (n = 20)	Mean	52		157	151		7.95E + 06	2.48E + 04	−2.95	19	128	336
	SD	12		45	148		2.44E + 07	7.19E + 04	1.09	16	157	343
	Median	54		153	71		7.25E + 04	3.00E + 02	−2.80	16	78	285
	Range	26–66		103–257	21–462		3.00E + 02–1.00E + 08	2.43E + 02–2.92E + 05	−4.34–4.70	3–73	8–524	45–1432
Total (n = 42)	Mean	52		154	128		3.08E + 07	5.59E + 05	−1.04	18	76	214
	SD	13		49	140		1.42E + 07	2.98E + 06	2.11	19	120	274
	Median	54		141	45		1.38E + 06	4.06E + 03	−1.06	11	29	114
	Range	21–72		88–320	11–462		3.00E + 02–1.00E + 08	2.43E + 02–1.93E + 07	−4.70–2.94	1–102	0–524	0–1432

f = female; m = male; S-Creat = serum-creatinine; unc = unchanged; dec = decreased within the preceding 8 weeks; inc = increased within the preceding 8 weeks; no = no immunosuppression; (dec)/unc = decreased after diagnosis; unchanged with the preceding 8 weeks; cp/mL = copies per milliliter urine or plasma; LT = BKV-large T-antigen; VP1 = BKV-VP1 capsid protein; SFU/Mio = spot-forming units per million PBMC; n.a. = not available.

[1] Decreasing >2 $\log_{10}$ in previous episode.

[2] Maximum decrease in previous episode.

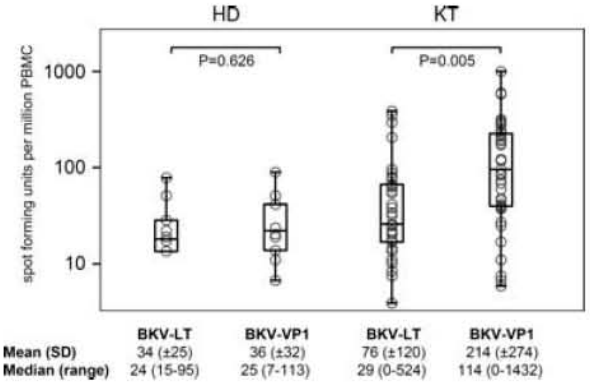


Figure 1: IFN-γ response of PBMC after stimulation with BKV-LT and -VP1 peptides by ESA. PBMC of HD and KT patients were stimulated with BKV-LT and -VP1 overlapping peptide pools and IFN-γ secretion was quantified by ESA. Box plots show median and interquartile range superimposed over scatter (Wilcoxon, two-sided).

BKV-specific cellular immune response in KT patients

We tested PBMC from BKV-seropositive KT patients with ongoing or past BKV viremia who attended outpatient nephrology clinics between March and October 2006 (Table 1). Stimulation with BKV-LT and -VP1 peptides elicited 29 [median, range (0–524)] and 114 [median, range (0–1432)] SFU, respectively (Figure 1). Overall, the SFU cellular immune responses to BKV-VP1 antigens were higher than the LT-specific response ($p = 0.005$) which was also true at the individual level for 38 of the 42 KT patients (91%). When KT patients were grouped according to the level of plasma BKV load, no significant differences were observed between patients with BKV loads $<10^3$, 10^3–10^5, $>10^5$ cp/mL (Figure 2).

To identify the contribution of $CD4^+$ and $CD8^+$ T cells in PBMC, we performed intracellular cytokine staining and flow cytometry after direct stimulation with BKV-LT or -VP1 peptides. In 21 KT patients (13 with >100 IFN-γ SFU/10^6 PBMC to VP1 peptides), we found IFN-γ positive cells at frequencies above 0.01%. Although the overall responses were low, mean IFN-γ responses to BKV-VP1 tended to be higher for $CD4^+$ T cells than for $CD8^+$ T cells ($p = 0.038$), whereas the difference did not reach significance for BKV-LT peptides ($p = 0.304$) (Figure 3). However, $CD8^+$ T cells were more likely to respond to BKV-LT than to -VP1 peptides ($p = 0.033$).

To investigate the association of plasma BKV load dynamics and immune responses, we divided KT patients in two groups: group 1 consisted of 22 KT patients with increasing ($n = 5$) or persistent plasma BKV loads ($n = 17$) at the time of PBMC testing. Group 2 consisted of 20 KT patients with plasma BKV loads decreasing on an average >2$\log_{10}$ cp/mL ($n = 13$) or with past PVAN ($n = 7$) at the time of PBMC testing (Table 1, see Section 'Study Participants and Methods'). Although the number of IFN-γ SFU were scattered in both groups for both antigens, BKV-LT-specific responses in group 1 were significantly lower than in group 2 [median 22 (0–120) vs. 78 (8–524); $p = 0.003$]. Similarly, BKV-VP1-specific responses in group 1 patients were significantly lower than in group 2 [median 53 (0–423) vs. 285 (45–1432) SFU/10^6 PBMC; $p = 0.001$ (Figure 4)]. Comparison with HD did not allow identifying a cut-off for KT patients with a protective cellular immune response. However, 69 SFU/10^6 PBMC for BKV-LT antigens, but not for VP1 antigens, identified significantly more KT-patients of group 2 with decreasing plasma BKV loads than of group 1 with increasing plasma BKV loads (12 of 20 vs. 1 of 22, respectively; $p = 0.019$, Fisher's exact test, two-sided).

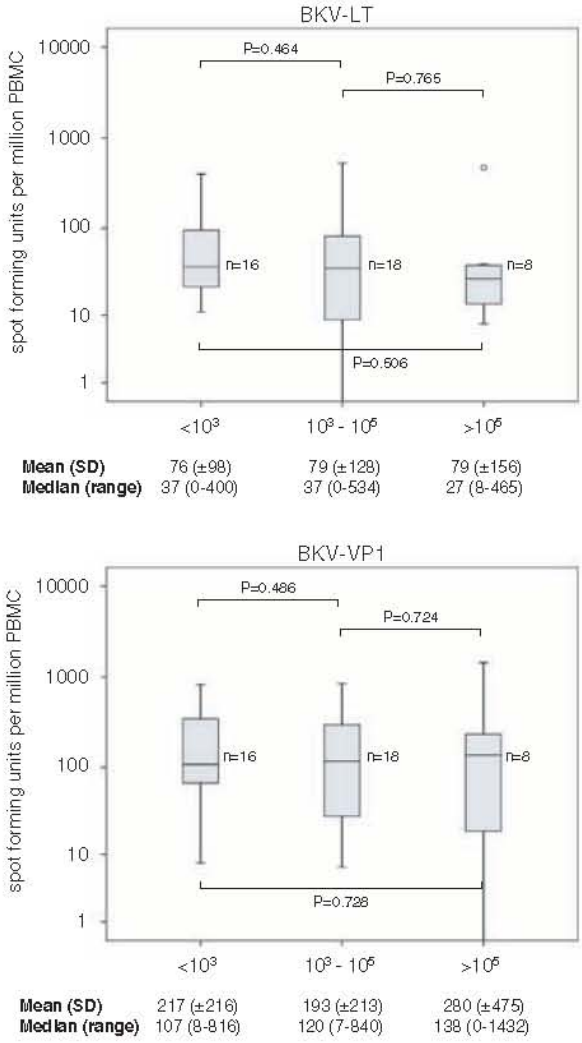


Figure 2: IFN-γ response of PBMC of KT patients with low or high plasma BKV load. KT patients were divided into groups of $<10^3$, 10^3–10^5 and $>10^5$ cp/mL and compared for SFU by ESA obtained after stimulation with BKV-LT and -VP1 peptides.

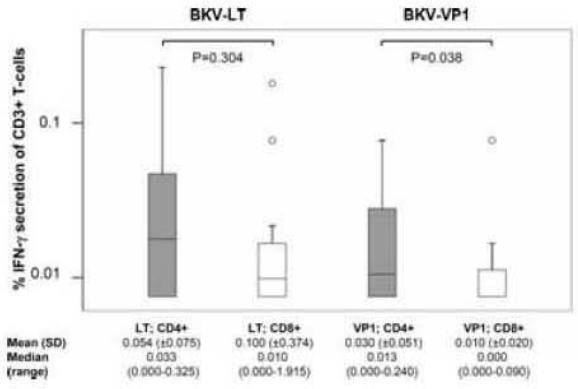


Figure 3: Intracellular IFN-γ response in $CD4^+$ T cells and $CD8^+$ T cells of KT patients after stimulation with BKV-LT and -VP1 peptides. PBMC of KT patients were stimulated with indicated peptides and gated for $CD4^+CD3^+$ and $CD8^+CD3^+$ lymphocytes and analysed for intracellular IFN-γ production using flow cytometry (Wilcoxon, two-sided).

BKV-specific cell expansion by short-term culture

To examine the ability of T cells able to expand in response to BKV-specific antigens in vitro (after wash out of immunosuppression), we stimulated PBMC of 24 KT patients (12 from each group) with autologous activated mononuclear cells primed with BKV-LT or -VP1 peptides. After 9 days of *in vitro* culture, cells were restimulated with BKV-LT or -VP1 peptides and tested for IFN-γ production by ESA. In most cultures, we observed a significant expansion of BKV-LT- and -VP1-specific responses ($p = 0.002$ and 0.033, respectively) compared to the SFU obtained prior to expansion

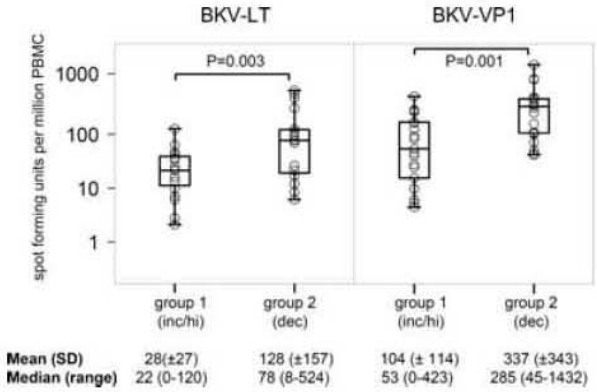
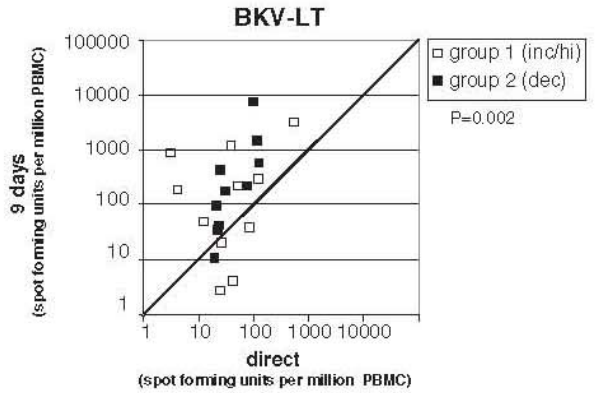


Figure 4: IFN-γ response of PBMC and plasma BKV load dynamics in KT patients. PBMC of KT patients with increasing or persisting BKV loads (group 1) or with decreasing (mean $>2\log_{10}$) or past plasma BKV load (group 2) were stimulated with indicated peptides and IFN-γ production was measured by ESA (Wilcoxon, two-sided).



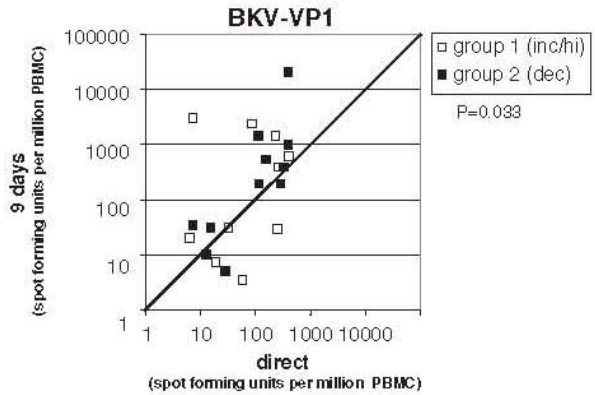


Figure 5: IFN-γ responses of PBMC after direct stimulation or after 9-day culture and restimulation. PBMC of KT patients were stimulated with BKV-LT or -VP1 peptides and cultured for 9 days. Cells were restimulated with indicated BKV peptides and IFN-γ SFU were quantified by ESA. The SFU responses post-culture were compared with preculture responses obtained by direct stimulation (Wilcoxon, two-sided).

(Figure 5). When we compared the SFU obtained after in vitro expansion of T cells from KT patients grouped according to plasma BKV load dynamics (group 1, inc/hi; group 2, dec), BKV-LT-specific expansion yielded significantly higher SFU in KT patients with declining plasma BKV loads (group 2; $p = 0.013$) than in group 1 KT patients with increasing or high plasma BKV loads (group 1; $p = 0.064$). In contrast, no significant difference was observed for expanding BKV-VP1-specific responses of both groups ($p = 0.084$ and 0.286, respectively).

Cross-stimulation with JCV peptide pools

As BKV and JCV share 75% DNA sequence homology, we wondered, if JCV-LT and -VP1 overlapping peptide pools were able to elicit a comparable IFN-γ response as BKV-LT and -VP1 peptides. When PBMC from 40 KT patients were stimulated directly with JCV-LT and -VP1 peptides, mean

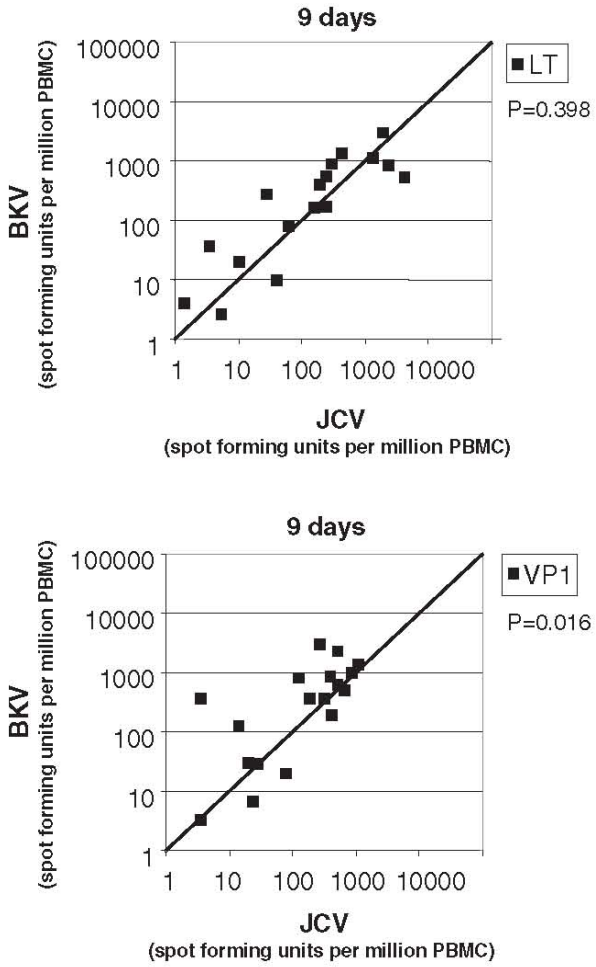


Figure 6: Cross-stimulation with homologous JCV-LT and -VP1 peptides. IFN-γ responses to BKV- and JCV-LT (top) and -VP1 (bottom) were compared after 9-day culture with BKV peptides (top panel: restimulation with JCV-LT, $p = 0.398$; bottom: restimulation with JCV-VP1; lower, $p = 0.016$; Wilcoxon, two-sided).

49 (±60; median 27, range 0–255) (±59) SFU and mean 118 (±169; median 47, range 0–868) SFU/10^6 PBMC were found, respectively. The JCV-peptide mediated response was significantly lower when compared to the corresponding BKV-mediated response ($p = 0.008$ and $p < 0.001$, respectively).

When JCV-LT or -VP1 peptides were used to restimulate T cells after BKV-specific 9-day expansion in vitro, the overall BKV-VP1 restimulation responses were significantly higher compared to JCV-VP1-specific responses ($p = 0.016$), but few individual exceptions of higher JCV responses were noted (Figure 6). In contrast, no significant difference was observed between BKV-LT and JCV-LT restimulation responses ($p = 0.398$) (Figure 6).

Discussion

A failing balance between BKV replication and BKV-specific cellular immune functions has been suspected as the common denominator of PVAN pathogenesis (15). Likely, this balance can be perturbed at different points of the patient, graft and virus interaction (3) which may account for controversial results on some of the implicated risk factors such as type of immunosuppression, BKV serostatus of donor and recipient, HLA mismatches or antirejection therapy. However, plasma BKV load is now widely accepted as surrogate marker of this failing balance and the risk of PVAN in KT patients (2,13,20). Also, plasma BKV loads not only provided first estimates of cytopathic damage (2,13,20), but also indicated the average efficacy of reduced immunosuppression as the current key treatment (22). Accordingly, clearance of plasma BKV viremia in patients with 10^5–10^7 cp/mL requires 7–11 weeks, once the immune system has started to curtail BKV replication (22). Despite the versatility of BKV loads in clinical screening and monitoring, individual courses are often difficult to predict. Instead, BKV-specific cellular immune responses in PBMC have been suggested as a more direct measure of this failing balance complementing BKV loads in KT patients (15,17). The results of the present study indicate that BKV-seropositive KT patients with increasing or persisting BKV loads show significantly lower IFN-γ SFU for both, BKV-LT and -VP1 peptides than KT patients with mean 2.45$\log_{10}$ decreasing or past plasma BKV loads. Thereby, the dynamics of plasma BKV replication are mirrored by emerging BKV-specific cellular immune responses in PBMC. Previous reports suggested that in some KT patients, VP1-specific cellular immune responses were preferentially detectable in patients with high plasma BKV loads of $>10^5$ cp/mL (25). Although BKV-specific cellular immune responses are detectable in some of these patients, we observed no significant differences between patients with plasma BKV loads above or below 10^5 cp/mL. Our results suggest that replication dynamics, not replication levels correlated with emerging BKV-specific cellular immune responses.

The increasing responses to defined BKV early gene (LT-) and late gene (VP1-) antigens reported here extend previous findings in paediatric KT patients using whole virus preparations (17) or VP1-based responses with peptide pools (25) or tetramer staining limited to single HLA-0201 binding epitopes in adults (24,29). Our data further suggest that BKV-LT and -VP1 responses seem to differ in several respects. First, the median number of IFN-γ SFU to BKV-VP1 was significantly higher than to BKV-LT ($p = 0.005$) which was also true at the individual level for 38 (91%) of the 42 KT patients tested. In line with our previous observation (30), VP1 responses seem to be a more sensitive measure of cellular immune responses in PBMC which can be detected at an earlier time point. Preliminary data on five KT patients with serial testing support this notion which requires further study in a prospective fashion.

Second, flow cytometry indicated that higher IFN-γ frequencies to BKV-VP1 largely resulted from $CD4^+$ T-cells as compared to $CD8^+$ T cells ($p = 0.038$). While part of the $CD4^+$ predominance is likely to reflect the preferential presentation and recognition of 15mers peptides in the MHC-II context, it should be noted that 15mer peptides can elicit predominant $CD8^+$ T-cell responses as shown for cytomegalovirus IE1 pp72 (31). Moreover, among $CD8^+$ T cells, BKV-LT-derived peptide responses were more frequent than VP1 responses. Together, our data suggest that mounting BKV-specific cellular immune responses involved differential antigen recognition with VP1 responses occurring earlier in PBMC with higher $CD4^+$ T-cell frequencies than LT responses.

The specificity of the BKV antigen responses in KT patients became apparent by significantly lower IFN-γ responses elicited by homologous JCV-LT and -VP1 peptide pools. Since all of our KT patients were also JCV-seropositive, the differential BKV responses most likely reflect recent boostering by antigen exposure as captured by plasma BKV loads. Comparison of JCV and BKV responses after BKV peptide-stimulated in vitro expansion indicated that overall IFN-γ responses were significantly higher for BKV supporting the view of an only limited cross-reactivity at the level of the major capsid protein. In contrast, no significant differences were obtained for the BKV-LT and JCV-LT responses after 9-day BKV-LT stimulated expansion. These results are intriguing and suggest the possibility that cellular immune control via LT at the level of infected host cells might be more conserved between human polyomaviruses, whereas capsid responses seem to be more type-specific as expected for neutralising antibody responses. Clearly, more detailed analyses are needed, but given the considerable homology between BKV and JCV at the genome level and reports about humoral and cellular cross-reactivity (10,15,32), our results are a first indication that direct ESA with PBMC are likely to capture specific cellular immune responses against human polyomaviruses. In addition, specific T-cell expansion from PBMC should be feasible for adoptive therapy of BKV-seropositive KT patients with increasing BKV replication and corresponding risk of PVAN.

Although these results are appealing, some limitations require further study. First, considerable scatter of IFN-γ responses was observed for both, BKV-LT and -VP1 responses in KT patients. Similar scattering, albeit at lower median levels was observed in non-immunosuppressed BKV-seropositive individuals (HD), none of whom had evidence of BKV or JCV in urine or plasma at the time of sampling and who must be considered protected. This precluded defining a simple threshold of BKV-specific immunological protection in KT patients. Most likely, scattering of BKV-specific cellular immune responses represent individual factors including variable past and present exposure to BKV antigens as approximated by extent and duration of BKV replication and concurrent immunosuppression. Indeed, PVAN has been diagnosed at plasma BKV loads scattering over three orders of magnitude (33). Second, the interplay of immunosuppression, plasma BKV loads and emerging BKV-specific cellular immunity is difficult to dissect. Not unexpected, immunosuppression had been decreased more frequently in our KT patients showing declining BKV loads if only the preceding 8 weeks were taken into account. Although all patients in this study were BKV-seropositive, and presumably had BKV-specific memory cells, expansion and (re-)acquisition of BKV-specific immunocompetence is likely to differ among KT patients depending on the net state of immunosuppression. Possibly, individual longitudinal changes of BKV-specific immune responses together with plasma BKV loads might be more predictive than single point measurements. However, with a cut-off of 69 SFU for BKV-LT antigens, but not for VP1 antigens, we identified significantly more KT patients with $>2\log_{10}$ decreasing plasma BKV loads ($p = 0.013$, Fisher's exact test, two-sided). If such a cut-off is validated in future studies, single measurements of plasma BKV load together with IFN-γ responses by ESA might allow to stratify the risk of PVAN and to guide reducing immunosuppression. Third, BKV immune responses in peripheral blood may not adequately represent antiviral control and risk of allosensitization at the site of disease, i.e. in renal allografts. This issue may require the characterization of T cells present in allografts although sampling and *in vitro* expansion are potential causes of bias. Nevertheless, our data indicate that plasma BKV loads and BKV-specific LT and VP1 responses provide first information on the dynamic balance between virus and immune system which can be generated in clinically relevant settings with potential impact for patient management. Well-designed prospective studies are needed to corroborate the findings of our cross-sectional study to investigate the role of expansion and contraction dynamics of BKV-specific T-cell responses and to better dissect the relevant factors involved.

Acknowledgment

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Human Polyomavirus Type 1 (BK Virus) Agnoprotein Is Abundantly Expressed but Immunologically Ignored[▽]

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Impaired BK virus (BKV)-specific immunity is a key risk factor of polyomavirus-associated nephropathy. We hypothesized that BKV agnoprotein might constitute an important immune target, as it is highly expressed after infection in vitro. We demonstrate abundant expression of BKV agnoprotein in vivo by immunostaining of kidney transplant (KT) biopsy specimens. Antibody responses to the recombinant affinity-purified BKV agnoprotein, large tumor (LT), and VP1 antigens in 146 sera from 38 KT patients and in 19 sera from 16 healthy donors (HD) were compared by enzyme immunoassay. In HD, low titers of anti-agnoprotein immunoglobulin G (IgG) were found in 15% of sera, compared to 41% for anti-LT antigen and 63% for anti-VP1. No anti-BKV IgM was detectable. In KT patients, anti-agnoprotein IgG and IgM were found in 8% and 3.6% of sera, compared to 63% and 18% for anti-LT IgG and IgM and 80% and 41% for anti-VP1 IgG and IgM, respectively. Anti-LT antigen and anti-VP1, but not anti-agnoprotein, activities increased during and after BKV viremia in KT patients. To investigate specific cellular immune responses, we compared levels of gamma interferon production in peripheral blood mononuclear cells (PBMC) of 10 HD and 30 KT patients by enzyme-linked immunospot assay. In HD, the median numbers of gamma interferon spot-forming units per million PBMC for the agnoprotein, LT antigen, and VP1 peptides were 1, 23, and 25, respectively, whereas the responses in KT patients were 2, 24, and 99, respectively. We conclude that BKV agnoprotein, though abundantly expressed in vivo, is poorly recognized immunologically.

The human polyomavirus BK virus (BKV) is the primary etiological agent of polyomavirus-associated nephropathy (PVAN), which causes irreversible graft loss in 1 to 10% of kidney transplant (KT) patients (15, 31). BKV was first discovered in 1970 in the urine of a KT patient with the initials B.K. who had ureteric stenosis and abundant decoy cell shedding (9). However, BKV asymptomatically infects 60 to 90% of the human population (23) and establishes a state of nonreplicative infection in the renourinary tract (13, 15). Intermittent low-level urinary replication with BKV loads of ≤10e6 per ml is detected in 5% of immunocompetent individuals, whereas high-level replication with BKV loads of ≥10e7 per ml is found in 20 to 60% of immunosuppressed patients (15). In KT patients, high-level urine BKV replication is found in 30%, which may be followed by BKV viremia in 13% and by histologically confirmed PVAN in 8% of patients (18). The risk factors for PVAN are not conclusively defined and likely involve complementing determinants of the triad of recipient, graft, and virus (17). Disruption of the balance between the BKV replication and host immune control is generally viewed as a key element of PVAN pathogenesis (5). In the absence of validated antivirals, reducing maintenance immunosuppression represents the primary treatment option for presumptive or definitive PVAN (3, 39).

BKV belongs to the genus *Polyomavirus* of the *Polyomaviridae* family, along with the related human polyomavirus JC virus (JCV) and simian virus 40 (SV40). The genomes are 70% homologous and consist of a circular double-stranded DNA of about 5,300 bp which can be divided into the noncoding control region (NCCR), containing the origin of replication and promoters of gene transcription, and the early and late gene regions (21, 37). The early genes encode two regulatory proteins called the small tumor and large tumor (LT) antigens. The late genes comprise genes encoding the viral capsid proteins VP1, VP2, and VP3, as well as a small open reading frame encoding a basic protein of 66 amino acids at the 5′ end of the VP1 mRNA. Studies of BKV infection of human endothelial cells and various cell lines demonstrated that BKV agnoprotein is abundantly expressed in the cytoplasm, with perinuclear accumulations (12, 34, 35). BKV agnoprotein, as well as the closely related JCV and SV40 agnoproteins, is expressed in a defined interval after early LT antigen expression, together with VP1 (24, 26, 28, 35). The function of BKV agnoprotein is not well defined, but data from BKV, JCV, and SV40 studies indicated roles in capsid assembly, virion egress, cell cycle regulation, viral replication, and gene expression. In vivo data on agnoprotein expression have been reported only for JCV replicating in brain tissue of cases with progressive multifocal leukoencephalopathy (29). Similar in vivo data are

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TABLE 1. Characteristics of study participants

Characteristic	Value for:					Statistical comparison
	HD	KT patient group[a]				
		All	A	B	C	
No. of subjects	16	38[b]	16	27	23	
Age (yr) at drawing of first sample						
Median	39.4	52.8	48.0	52.3	53.9	NS[e] (HD vs all)
Range	27.4–59.0	17.2–71.1	18.1–58.2	17.2–71.1	25.7–69.1	
No. (%) male	8 (50.0)	24 (63.2)	10 (62.5)	17 (63.0)	10 (43.5)	NS (HD vs all)
Time (wk) posttransplantation[c]						
Median		30.3	9.4	25.5	120.3	$P = 0.003$ (A vs B); $P < 0.0001$ (A vs C and B vs C)
Range		2.3–462.1	2.3–380.7	4.3–449.9	14.0–462.1	
BKV plasma load (log cp/ml)						
Median	Not detectable	4.0	3.0	4.7	3.0	$P < 0.0001$ (A vs B and B vs C); NS (A vs C)
Range		3.0[d]–7.7	3.0–3.7	3.0–7.7	3.0–3.8	

[a]A, no/low-level replication; B, high-level replication; C, past replication.
[b]For some kidney transplant recipients, samples were drawn before the onset of, during, and after high-level BKV replication. Therefore, this number is smaller than the total for groups A, B, and C.
[c]Values are for all the samples in the corresponding subject group.
[d]Set limit of detection; also if no plasma sample was tested.
[e]NS, not significant.

lacking for BKV agnoprotein, except for an isolated fatal human immunodeficiency virus (HIV)/AIDS case with meningoencephalitis, pneumonitis, nephritis, and disseminated BKV replication (2). In our ongoing study to identify relevant targets of the immune system controlling BKV replication in KT patients (1), we hypothesized that agnoprotein might represent an important antigen, given the conserved nature of the protein and the abundant expression pattern in vitro. We have therefore assessed the expression of BKV agnoprotein in vivo in kidney biopsy samples from KT patients and have compared both the humoral and cellular immune responses against this viral protein in KT patients and in healthy controls.

MATERIALS AND METHODS

Study participants. Plasma and urine samples were obtained, with informed consent according to the protocol of the Ethics Committee of the Basel Cantons, from 16 healthy donors (HD) and 38 KT patients (Table 1). Samples from KT patients were divided into three groups according to the present and past levels of BKV viremia and/or viruria: group A comprised all samples from patients without high-level BKV replication ("no/low-level replication"); group B comprised all samples from patients with BKV viremia and/or viruria of >10e4 or >10e7 copies/ml, respectively ("high-level" replication); and group C comprised samples from those patients who had documented high-level BKV replication with definitive or presumptive PVAN (16) earlier but in whom this had decreased by >1.5 $\log_{10}$ by the time of sample drawing (past "high-level" replication).

Quantitative PCR for BKV DNA detection. Measurements of BKV viral load in plasma and urine were performed according to standard real-time PCR protocols. Briefly, DNA was isolated with the QIAamp DNA minikit (QIAGEN, Hilden, Germany) and quantified by real-time PCR (TaqMan/7700, Stratagene Mx4000, or Bio-Rad iCycler) as described previously (19).

For the detection of BKV in biopsy specimens, cells were scraped under sterile conditions, incubated at 95°C for 10 min, transferred to ice, and then treated with 5 μl of proteinase K (5 mg/ml) at 55°C for 3 to 5 h, followed by enzyme inactivation at 95°C for 10 to 15 min. Subsequently, the DNA was quantified by real-time PCR using an ABI Prism 7900 HT sequence detector (Applied Biosystems, Rotkreuz, Switzerland). The following primers were used for BKV LT antigen amplification: PYV.for (5′-TAGGTGCCAACCTATGGAACAGA-3′) and PYV.rev (5′-GAAAGTCTTTAGGGTCTTCTACC-3′). The BKV probe was 5′-FAM-CATTAAAGGAACTCCACCAGGACTCCCACTC-TAMRA-3′.

Infection and immunofluorescence microscopy. Renal proximal tubule epithelial cells (Cambrex CC-2553) were infected with BKV (Dunlop) as described previously for human umbilical vein epithelial cells (10). At 2 days postinfection, cells were washed with phosphate-buffered saline (PBS) and fixed with methanol. Subsequently, cells were incubated at 37°C with primary and secondary antibodies for 30 min. The following antibodies were used: monoclonal SV40 T antibody (Pab416; Calbiochem, San Diego, CA) (dilution, 1:100) and rabbit antiserum directed against BKV agnoprotein (anti-BKV agnoprotein serum) (dilution, 1:800) (14, 35). The secondary antibodies were conjugated with AlexaFluor 568 or 488 (1:500; Molecular Probes, Inc., Eugene, OR). Images were collected using a Nikon TE2000 microscope equipped and processed with NIS Elements Basic Research software version 2.2 (Nikon Corporation).

Staining of kidney biopsy specimens for BKV protein expression. Samples were screened for the presence of BKV agnoprotein and LT antigen by in situ immunohistochemical staining. Briefly, 3-μm tissue sections were dewaxed twice in xylene, followed by rehydration in a graded series of ethanol solutions. Nonenzymatic antigen retrieval was performed by heating the sections in 0.1 M citrate buffer (pH 6.0), first at 900 W for 3 min and then at 90 W for 30 min. After cooling in cold water for 15 min, the slides were washed in APK wash solution (Ventana Medical Systems Inc., Tucson, AZ). Endogenous peroxidase was quenched, and tissues were permeabilized by the incubation of sections in methanol containing 1.1% H_2O_2 at 37°C for 4 min and then washed in APK wash solution at room temperature (RT) for 5 min.

For immunostaining of agnoprotein, sections were incubated with the primary antibody at 37°C for 1 h, using the anti-BKV agnoprotein serum (14, 35) diluted 1:600. As a negative control, a set of parallel sections were incubated with rabbit preimmune serum. EnVision+ horseradish peroxidase (Dako, Glostrup, Denmark) was applied as a secondary antibody to the sections at RT for 30 min. The sections were developed with liquid diaminobenzidine+ substrate-chromogen (Dako) and counterstained with Shandon's instant hematoxylin (Shandon Inc., Pittsburgh, PA). Finally, the sections were mounted in Eukitt (Kindler GmbH, Freiburg, Germany) after dehydration in a graded series of ethanol solutions and in xylene.

For double immunostaining to detect LT antigen and agnoprotein simultaneously, sections were first incubated with the LT antigen-cross-reacting anti-SV40 LT antigen clone PAb416 (1:400 dilution) at 37°C for 30 min. Envision+

horseradish peroxidase and liquid diaminobenzidine+ substrate-chromogen were added as described above. Then, after being washed, the slides were incubated with the anti-BKV agnoprotein serum (1:600 dilution) as described above. Envision alkaline phosphatase (Dako) was added as a secondary antibody to the sections, with incubation at RT for 30 min. Finally, the sections were developed with Vector-red (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin, before being mounted in Eukitt.

Generation of recombinant baculoviruses expressing GST-BKV fusion proteins. The sequence encoding the BKV agnoprotein was amplified by PCR from the urine of a KT patient who was shedding archetype NCCR BKV serotype 1. The following primers were used: Agno-for (5′-TTTTGGATCCACCATGGTT CTGCGCCAGCTG-3′) and Agno-rev (5′-TTTTGCGGCCGCTAGGAGTCTT TTACAGAGTCT-3′). The resulting amplicon was cloned into the pTRE2pur plasmid (Clontech, Mountain View, CA), yielding pTRE-agno, and verified by sequencing. The entire BKV agnoprotein gene was again amplified by PCR, using pTRE-agno as the template and the primers Agno-TEV-for (5′-TTGGA TCCCCGAAAACCTGTATTTTCAGGGCATGGTTCTGCGCCAGCTGTC-3′) and Agno-rev. The resulting amplicon was cloned into the multiple cloning site of the pFastBacGST plasmid, digested with BamHI and NotI, cloned in frame with the gene coding for glutathione *S*-transferase (GST) (yielding pFastBacGST-BKV-agno), and confirmed by restriction analysis and sequencing. The coding sequences of the entire BKV VP1 and the amino-terminal 15.6-kDa domain of BKV LT antigen (amino acids 1 to 133) were amplified using the primers BKV-LTD1-for (5′-GCGCGGATCCCCGAAAACCTGTATTTTCAGGGCATGGATAAGTT CTTAACAGGGAAGA-3′) and BKV-LTD1-rev (5′-TTTTCTCGAGTTACTTTC TTTTTTTTTTGGGTGGTGTTG-3′) for LT antigen and BKVP1-f (5′-GCGCG GATCCCCGAAAACCTGTATTTTCAGGGCATGGCCCCAACCGGCCCCA ACCAAAAGAAAAGGA-3′) and BKVP1-r (5′-TTTTCTCGAGTTAAAGCATT TTGGTTTGCA-3′) for VP1 antigen to generate comparably sized BKV LT domain 1 (LTD1), consisting of the amino-terminal 133 amino acids containing the sequences of the J domain, the Rb binding and nuclear localization sequences, and full-length VP1 amplicons that could be inserted in frame downstream of GST in pFastBacGST by using the restriction enzymes BamHI-XhoI and BamHI-HindIII, yielding pFastBacGST-BKV-LTD1 and pFastBacGST-BK-VP1 (S. Bodaghi and H. H. Hirsch, unpublished data).

Expression and purification of recombinant antigens GST-BKV agnoprotein, GST-BKV LTD1, and GST-BKV VP1. Recombinant baculoviruses containing the coding sequence for the GST-BKV fusion proteins were generated using the Bac-to-Bac expression systems (Invitrogen Ltd., Carlsbad, CA) according to the manufacturer's instructions. Recombinant bacmids were isolated and used for transfection of Sf9 insect cells with the Cellfectin reagent. Recombinant baculovirus was recovered after 48 h and used for subsequent infection for Sf9 cells. Sf9 cell lysates were prepared between days 3 and 5 postinfection. GST fusion proteins (GST-BKV agnoprotein, GST-BKV VP1, and GST-BKV LTD1) and GST were purified by glutathione affinity chromatography using glutathione Sepharose 4 fast flow (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Protein expression and purity were assessed by polyacrylamide gel electrophoresis, Coomassie blue staining, Western blotting using the anti-GST antibody B-14 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or enzyme-linked immunosorbent assay using the anti-BKV agnoprotein serum (14), the cross-reacting SV40 LT Pab416, or the BKV VP1 antiserum (35).

Enzyme immunoassay (EIA) with GST-BKV fusion proteins. Standard 96-well EIA plates with high-level coating properties were used for coating for 16 h overnight at 4°C. The wells were washed five times with 0.1% Tween 20, treated with blocking buffer (PBS [pH 7.4], 4.0% bovine serum albumin, 0.1% Tween 20) at RT for 2 h, and washed three times. The wells were incubated for 1 h at RT with 100 μl of diluted patient sera, washed five times, incubated with the secondary antibodies at RT for 1 h, and washed again five times before application of the secondary anti-human immunoglobulin G (IgG) or anti-human IgM antibodies (Sigma-Aldrich), washing, and detection using *o*-phenylenediamine hydrochloride (Sigma-Aldrich, St. Louis, MO) for 30 min at RT by adding 1 N sulfuric acid. Optical densities (ODs) were measured using an automated plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 492 nm. Equivalents of 1.0 pmol of antigen/well, serum dilutions of 1:400, and dilutions of anti-IgG and -IgM antibodies of 1:10,000 were found to yield minimal background. Affinity-purified GST was run as a negative control and subtracted from the GST-BKV LTD1, GST-BKV VP1, and GST-BKV agnoprotein signals. For every sample, the OD was determined by subtraction of the GST background applied in parallel. The cutoff was defined as two standard deviations the GST background level taken as the negative control. Therefore, all OD values of <0.0400 were deemed to be negative. Values from 0.0400 to 0.0999 were classified as + (borderline positive), values of >0.1000 as 1+ (positive), and values of >1.0000 as 2+ (strongly positive). For each antigen, all samples tested for IgG were run on the same day, as were all samples tested for IgM. For confirmatory testing, we included three selected samples as references, which were negative, strongly positive, and borderline positive, indicating variation coefficients of <10%, which can cause minimal shifts in the groups.

Testing patient sera for agnoprotein antibodies on UTA-6 agnoprotein-expressing cells. UTA-6 cells (8) were transfected with pTRE-agno in the absence of tetracycline. After 44 h, cells were transferred to wells of 10-well glass slides and incubated at 37°C in a CO_2 atmosphere for 4 h. Cells were washed in PBS, fixed in cold 100% acetone for 5 min, and air dried. As a negative control, naive UTA-6 cells were treated in parallel. For immunofluorescence, cells were incubated with patient sera diluted in PBS-bovine serum albumin (1:40 or 1:160) at RT for 1 h, washed in PBS, incubated with fluorescein isothiocyanate-labeled anti-human IgG diluted 1:3,200 (bioMerieux, France) as before, washed, mounted with *N*-propyl gallate in glycerin, and viewed with a Nikon E800 epifluorescence microscope.

BKV overlapping peptide pools. Overlapping peptide pools which covered the entire coding sequence of the BKV LT antigen (ppLT), VP1 (ppVP1), and agnoprotein (ppAgno) as defined by the sequence of the BKV Dunlop strain (GenBank accession no. V01108) were used for peripheral blood mononuclear cell (PBMC) stimulation. The pools consisted of 180 (LT antigen), 88 (VP1), and 16 (agnoprotein) peptides with 15 amino acids each and 11-amino-acid overlaps (Eurogentec, Geneva, Switzerland), dissolved in dimethyl sulfoxide.

ESA for IFN-γ. The PBMCs for enzyme-linked immunospot assay (ESA) were obtained from 10 HD and 30 KT patients as described previously (1). PBMCs were isolated in CPT tubes (Becton-Dickinson, Allschwil, Switzerland). The cells were washed, counted, and stored frozen in fetal calf serum containing 10% dimethyl sulfoxide (Sigma-Aldrich). For ESA, PBMCs were diluted in RPMI 1640–5% human serum–1% Glutamax-1% PenStrep (R5AB) (Sigma-Aldrich). The BKV-specific cellular immune response was determined by measuring gamma interferon (IFN-γ) upon stimulation of PBMCs in an ESA. Ninety-six-well multiscreen filter plates (MSIPN; Millipore, Volketswil, Switzerland) were coated with 100 μl (10 μg/ml) mouse anti-human IFN-γ monoclonal antibody (1-D1K; Mabtech, Hamburg, Germany) and incubated overnight at 4°C. PBMCs were seeded at a concentration of 2.5×10^5/well (2.5×10^4/well for the positive control) and incubated overnight at 37°C in 5% CO_2 with 2 μg/ml pooled BKV antigens (ppLT, ppVP1, and ppAgno), a negative control (R5AB), and 1 μg/ml *Staphylococcus* enterotoxin B (Sigma-Aldrich) as a positive control. ESAs were performed in triplicate. After incubation, the plates were washed with PBS with 0.05% Tween 20 (Sigma-Aldrich), incubated at RT for 2 h with 100 μl (1 μg/ml) detection biotinylated mouse anti-human IFN-γ monoclonal antibody (7-B6-1 biotin; Mabtech), washed again, and incubated at RT for 1 h with 100 μl streptavidin ALP (1 μg/ml; Mabtech). Spots were developed by treatment with 100 μl 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Calbiochem, Lucerne, Switzerland) per well for 10 min, washed 5 times with H_2O from both sides, and dried in the dark. IFN-γ spots were counted by using an ESA reader (AID, Büron, Switzerland). The number of spots per well was determined by subtracting the negative control value.

Statistical methods. Statistical analyses were performed using XLSTAT 2006 software (Addinsoft, New York, NY). Comparisons between immune responses to different antigens were performed using the Kruskal-Wallis test for nonparametric dependent samples, with the Bonferroni adjustment for pairwise comparisons. Comparisons between immune responses to a single antigen in the different subject groups were performed using the Friedman test for nonparametric independent samples, again with the Bonferroni adjustment for pairwise comparisons.

RESULTS

BKV agnoprotein is abundant in the cytoplasm of infected renal tubular cells. We investigated the patterns of expression of agnoprotein and LT antigen in renal primary proximal tubular epithelial cells at 2 days postinfection with BKV strain Dunlop (Fig. 1A). BKV agnoprotein was located predominantly in the cytoplasm of renal proximal tubular epithelial cells (green fluorescence), whereas LT antigen was detected exclusively in the nucleus (red fluorescence). The cells were positive either for LT antigen alone (Fig. 1A, cell 1), for LT antigen and low-level cytoplasmic agnoprotein (cell 2), or for high-level cytoplasmic agnoprotein (cell 3) but were never positive for agnoprotein without LT an-

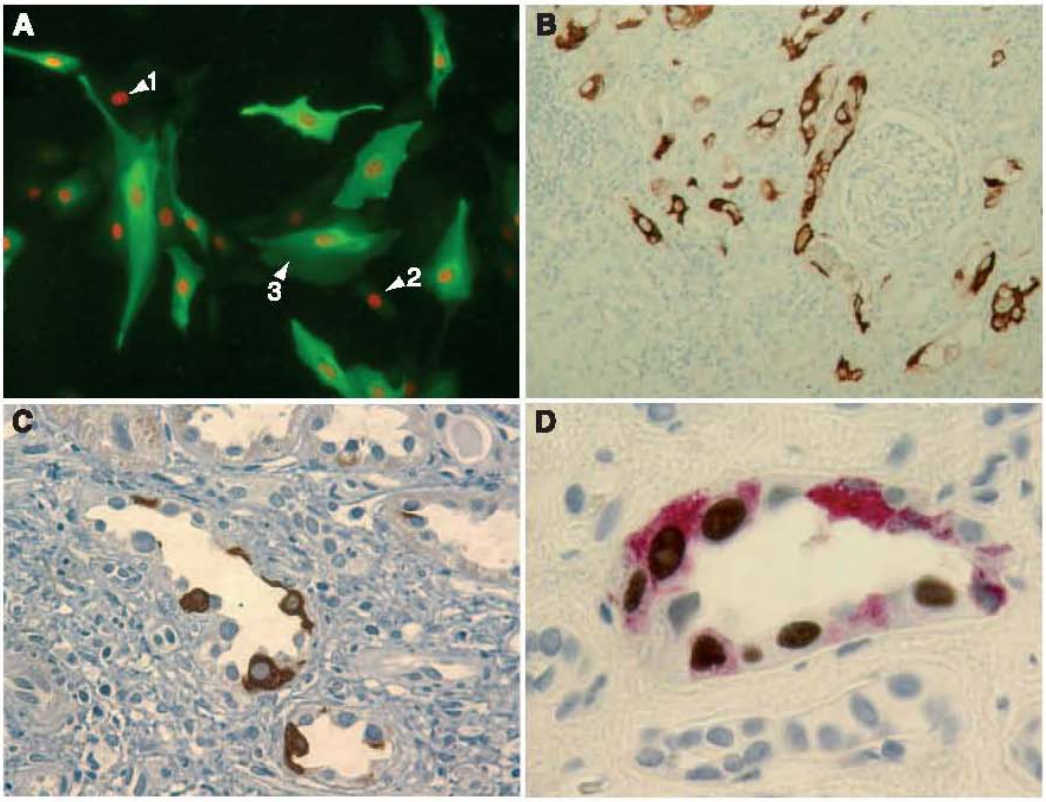

FIG. 1. BKV agnoprotein expression in kidney tubular epithelial cells. (A) Indirect immunofluorescence of primary kidney proximal tubular epithelial cell culture infected with BKV strain Dunlop and stained at 48 h postinfection with rabbit anti-BKV agnoprotein serum (green) and anti-SV40 LT Pab416 (red). Arrows indicate cells with LT antigen (cell 1), LT antigen plus a little agnoprotein (cell 2), and LT antigen plus abundant agnoprotein (cell 3) (note that there are no cells with agnoprotein alone). (B) Immunohistochemistry of renal allograft biopsy specimen stained with anti-BKV agnoprotein serum (brown). Magnification, ×100. (C) Immunohistochemistry of renal allograft biopsy specimen stained with anti-BKV agnoprotein serum (brown). Magnification, ×400. (D) Immunohistochemistry of renal allograft biopsy specimen double stained with anti-BKV agnoprotein serum (red) and anti-SV40 LT Pab416 (brown). Magnification, ×1,000.

tigen. The data demonstrate that BKV agnoprotein is abundantly expressed in the cytoplasm of renal primary proximal tubular epithelial cells infected in vitro, as expected for the BKV late gene.

To investigate the expression of BKV agnoprotein in vivo, we examined 16 needle biopsy specimens from renal transplant recipients with BKV viruria. PCR analysis of DNA extracted from the paraffin-embedded specimens revealed the presence of BKV DNA sequences in 6 of the 16 samples (38%). Immunohistochemical analysis revealed agnoprotein expression in 4 of the 6 BKV PCR-positive biopsy specimens (67%) but in none of the BKV PCR-negative renal tissues (overall frequency, 25%). Agnoprotein-expressing cells frequently showed a focal distribution surrounded by neighboring negative areas which often contained inflammatory infiltrates as in PVAN B (Fig. 1B). Single immunostaining for BKV agnoprotein revealed predominant staining of the cytoplasm of renal tubular epithelial cells with unstained, typically enlarged nuclei (Fig. 1C). Double immunostaining for LT antigen and BKV agnoprotein (Fig. 1D) showed the exclusive nuclear staining of LT antigen and a range of intensities for agnoprotein in the cytoplasm of infected cells, similar to the picture obtained by in vitro infection of renal primary proximal tubular epithelial cells (Fig. 1A). Indeed, some cells were positive for LT antigen but negative for agnoprotein (Fig. 1D), while different levels of agnoprotein expression were observed in adjacent cells of the same tissue sample (Fig. 1D), all of which were positive for LT antigen as expected for different stages of the early and late phases of the BKV replication cycle.

BKV agnoprotein elicits only poor IgG and IgM responses compared to LT antigen and VP1. To investigate the antibody response against BKV agnoprotein by EIA, we generated GST fusion proteins with the full-length BKV agnoprotein, the amino-terminal 133 amino acids of LT antigen, and the full-length major capsid protein VP1 for high-level expression in insect cells and convenient purification via glutathione affinity chromatography. As shown for GST-BKV agnoprotein by polyacrylamide gel electrophoresis and Western blotting with anti-GST (Fig. 2A), GST fusion proteins were prominently expressed and highly enriched by single-step glutathione-Sepharose 4B, with some limited amounts of smaller proteins corresponding to endogenous GST and breakdown products. Using purified GST-BKV agnoprotein, GST-BKV LTD1,

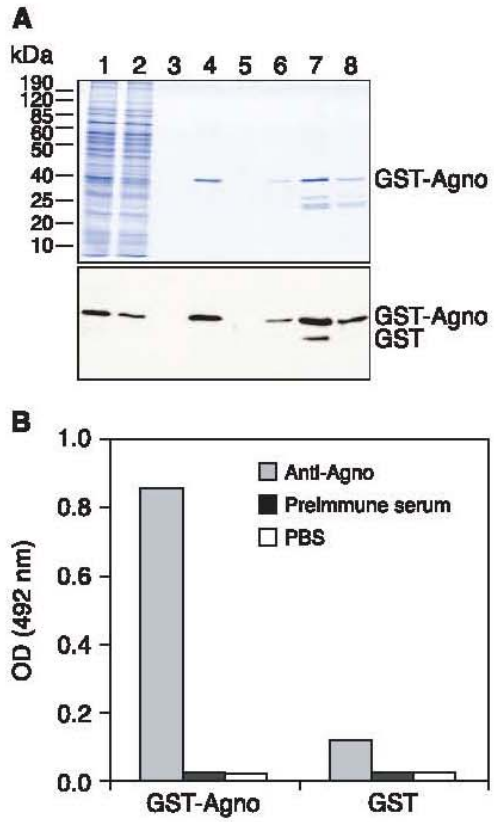

FIG. 2. GST-BKV agnoprotein purification from Sf9 cells and EIA. (A) Coomassie blue staining and detection of purified GST-agnoprotein by Western blotting by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, cell lysate; lane 2, flowthrough; lane 3, wash; lane 4, Sepharose beads after elution; lanes 5 to 8, elution fractions 1 to 4, respectively. GST-agnoprotein was detected with monoclonal anti-GST antibody (1:5,000) and goat anti-mouse antibody conjugated to horseradish peroxidase (1:10,000). (B) A 96-well enzyme-linked immunosorbent assay plate was coated with GST-agnoprotein or GST as a control in PBS, pH 7.4 (1 pmol/well). A polyclonal rabbit anti-BKV agnoprotein serum was used as the primary antibody at a dilution of 1:20,000. Rabbit preimmune serum and PBS (pH 7.4) were used as negative controls. All assays were performed in triplicate.

GST-BKV VP1, and GST alone, we adapted antigen coating conditions, serum dilutions, and dilutions of secondary antibodies in order to obtain optimal signal-to-noise ratios (Fig. 2B and data not shown).

We tested 146 plasma samples from 38 KT patients as well as 19 samples from 16 HD for IgG and IgM activity. The characteristics of these subject groups are shown in Table 1. No significant differences were found concerning age or gender distribution. None of the HD had detectable viremia, and only one HD had detectable low-level viruria (6×10^3 copies/ml) in one of the samples (frequency, 1/19 [5%]). By contrast, KT patients had a median plasma BKV load of 10^4 copies/ml. Not unexpectedly, the median time of sampling of KT patients posttransplantation increased from group A (9 weeks) to group B (26 weeks) to group C (120 weeks; $P < 0.001$).

The results of the antibody responses found in the study participants (Table 2; Fig. 3) can be summarized as follows. Only 4 of 54 individuals had no detectable anti-BKV antibodies, yielding an overall BKV seropositivity rate of 92.6%, which is in the range of published results for other serological assays (5, 22). IgG responses were more frequent and with higher OD values in KT patients than in HD, particularly with ongoing or past high-level BKV replication, and were more pronounced against the BKV VP1 capsid protein than against the BKV LT antigen. Anti-VP1 IgG was detected in 12/19 (63%) HD and in 117/146 (80%) KT patients. Anti-LTD1 IgG were detected in 8/19 (42%) HD and in 91/146 (62%) KT patients. By contrast, IgG responses against BKV agnoprotein were found in only 3/19 (15%) HD and 11/146 (7.5%) KT patients. When borderline positive results were regarded as seronegative, only 1/165 samples (1%) had detectable anti-agnoprotein IgG, compared to 60/165 (41%) and 78/165 (53%) samples for anti-LTD1 IgG and anti-VP1 IgG, respectively. Finally, IgG responses were generally more frequently detected than IgM responses for any of the three antigens and had higher OD values. In sera from HD, no IgM responses against any of the three BKV antigens could be found. In sera from KT patients, IgM responses were most frequently found in group B patients with ongoing high-level replication (Table 2). Anti-agnoprotein IgM responses were rarely detected in 6/165 (3.6%) samples tested, 5 of which were weakly positive and 1 (0.6%) of which was positive.

Among KT patients, the percentage of positive samples and the median OD values for anti-LTD1 responses increased from group A ("no/low-level" replication) (56% and 0.0433) to group B ("high-level" replication) (59% and 0.0499) and group C (past "high-level" replication) (74% and 0.1863) ($P = 0.01$), with the strongest difference between groups A and C ($P = 0.008$). The anti-VP1 IgG response was increased in KT patients from group A (72%; 0.0676) to group B (83%; 0.1708) ($P < 0.008$) and was lower in group C (79%; 0.0820) but did not reach statistical significance. By contrast, anti-agnoprotein responses remained low in all groups, with no statistically significant changes (percentages of positive samples and median ODs: group A, 8% and 0.0006; group B, 6% and 0.0064; and group C, 10% and 0.0027). In all subgroups, the response to agnoprotein was significantly lower than that to any of the other antigens ($P < 0.0001$ for all comparisons). The anti-LTD1 response was significantly higher than the anti-VP1 response only in group C. To confirm the poor anti-agnoprotein IgG response by a different technique based on native agnoprotein, we tested 30 sera from KT patients by indirect immunofluorescence of agnoprotein-expressing UTA-6 cells. Only 1 of 30 patient sera (3.3%) was found to be reactive at a serum dilution of 1:40.

The IgM responses showed no clear tendency for anti-LTD1 responses, whereas anti-VP1 responses increased significantly during the active PVAN phase and subsided afterwards (percentages of positive samples for groups A, B, and C, 24%, 55%, and 39%, respectively; median OD values for groups A, B, and C, 0.0214, 0.0446, and 0.0250, respectively; $P < 0.008$ for the increase in group B, but the subsequent decrease in group C did not reach statistical significance). Again, anti-agnoprotein responses remained very low throughout this time and were significantly lower than responses to any of the other antigens.

BKV agnoprotein elicits poor cellular immune responses. To test whether the BKV agnoprotein elicited cellular immune

TABLE 2. Antibody activity against purified recombinant BKV proteins

Group (no. of subjects)	Antibody (no. of samples tested)	Protein	No. (%) of samples that were[a]:				Median OD at 492 nm (interquartile range)
			Negative	Borderline positive	Positive	Strongly positive	
HD (16)	IgG (19)	LTD1	11 (57.9)	5 (26.3)	3 (15.8)	0	0.0322 (0.0128–0.0688)
		VP1	7 (36.8)	4 (21.1)	7 (36.8)	1 (5.3)	0.0744 (0.0320–0.1380)
		Agnoprotein	16 (84.2)	1 (5.3)	2 (10.5)	0	0.0120 (0.0050–0.0201)
	IgM (19)	LTD1	19 (100)	0	0	0	0.0088 (0.0043–0.0131)
		VP1	19 (100)	0	0	0	0.0099 (0.0026–0.0133)
		Agnoprotein	19 (100)	0	0	0	0.0046 (0.0003–0.0076)
KT patient groups[b]							
A (16)	IgG (25)	LTD1	11 (44.0)	7 (28.0)	6 (24.0)	1 (4.0)	0.0433 (0.0238–0.1018)
		VP1	7 (28.0)	12 (48.0)	5 (20.0)	1 (4.0)	0.0676 (0.0319–0.0958)
		Agnoprotein	23 (92.0)	2 (8.0)	0	0	0.0006 (0.0000–0.0145)
	IgM (25)	LTD1	20 (80.0)	3 (12.0)	2 (8.0)	0	0.0146 (0.071–0.0316)
		VP1	19 (76.0)	4 (16.0)	2 (8.0)	0	0.0214 (0.0103–0.0284)
		Agnoprotein	23 (92.0)	2 (8.0)	0	0	0.0002 (0.0000–0.0044)
B (27)	IgG (83)	LTD1	34 (41.0)	18 (21.7)	14 (16.9)	17 (20.5)	0.0499 (0.0193–0.5721)
		VP1	14 (16.9)	14 (16.9)	55 (66.3)	0	0.1708 (0.0633–0.2854)
		Agnoprotein	78 (94.0)	4 (4.8)	1 (1.2)	0	0.0064 (0.0000–0.0147)
	IgM (83)	LTD1	62 (74.7)	15 (18.1)	6 (7.2)	0	0.0156 (0.0031–0.0389)
		VP1	37 (44.6)	17 (20.5)	29 (34.9)	0	0.0446 (0.0226–0.1556)
		Agnoprotein	79 (95.2)	3 (3.6)	1 (1.2)	0	0.0016 (0.0000–0.0063)
C (23)	IgG (38)	LTD1	10 (26.3)	6 (15.8)	15 (39.5)	7 (18.4)	0.1863 (0.0425–0.7489)
		VP1	8 (21.1)	13 (34.2)	17 (44.7)	0	0.0820 (0.0488–0.2115)
		Agnoprotein	34 (89.5)	4 (10.5)	0	0	0.0027 (0.0000–0.0103)
	IgM (38)	LTD1	32 (84.2)	3 (7.9)	3 (7.9)	0	0.0107 (0.0047–0.0212)
		VP1	23 (60.5)	6 (15.8)	9 (23.7)	0	0.0250 (0.0095–0.0805)
		Agnoprotein	38 (100.0)	0	0	0	0.0020 (0.0000–0.0062)
All (38[c])	IgG (146)	LTD1	55 (37.7)	31 (21.2)	35 (24.0)	25 (17.1)	0.0578 (0.0215–0.5187)
		VP1	29 (19.9)	39 (26.7)	77 (52.7)	1 (0.7)	0.1144 (0.0549–0.2222)
		Agnoprotein	135 (92.5)	10 (6.8)	1 (0.7)	0	0.0042 (0.0000–0.0139)
	IgM (146)	LTD1	114 (78.1)	21 (14.4)	11 (7.5)	0	0.0142 (0.0041–0.0309)
		VP1	79 (54.1)	27 (18.5)	40 (27.4)	0	0.0360 (0.0132–0.1038)
		Agnoprotein	140 (95.9)	5 (3.4)	1 (0.7)	0	0.0016 (0.0000–0.0056)
All subjects (54)	IgG (165)	LTD1	66 (40.0)	36 (21.8)	38 (23.0)	25 (15.2)	0.0570 (0.0185–0.3585)
		VP1	36 (21.8)	43 (26.1)	84 (50.9)	2 (1.2)	0.1093 (0.0541–0.2147)
		Agnoprotein	151 (91.5)	11 (6.7)	3 (1.8)	0	0.0058 (0.0000–0.0148)
	IgM (165)	LTD1	133 (80.6)	21 (12.7)	11 (6.7)	0	0.0127 (0.0041–0.0265)
		VP1	98 (59.4)	27 (16.4)	40 (24.2)	0	0.0277 (0.0103–0.0896)
		Agnoprotein	159 (96.4)	5 (3.0)	1 (0.6)	0	0.0018 (0.0000–0.0066)

[a] Antibody activity was measured as the OD at 492 nm in an enzyme-linked immunosorbent assay at a serum dilution of 1:400 and was classified as follows: negative, <0.0400; borderline positive, 0.0400 to 0.0999; positive, 0.1000 to 0.9999; and strongly positive, >0.9999.

[b] A, no/low-level replication; B, high-level replication; C, past replication.

[c] For some KT patients, samples were drawn before the onset of, during, and after the resolution of PVAN. Therefore, this number is smaller than the total for groups A, B, and C.

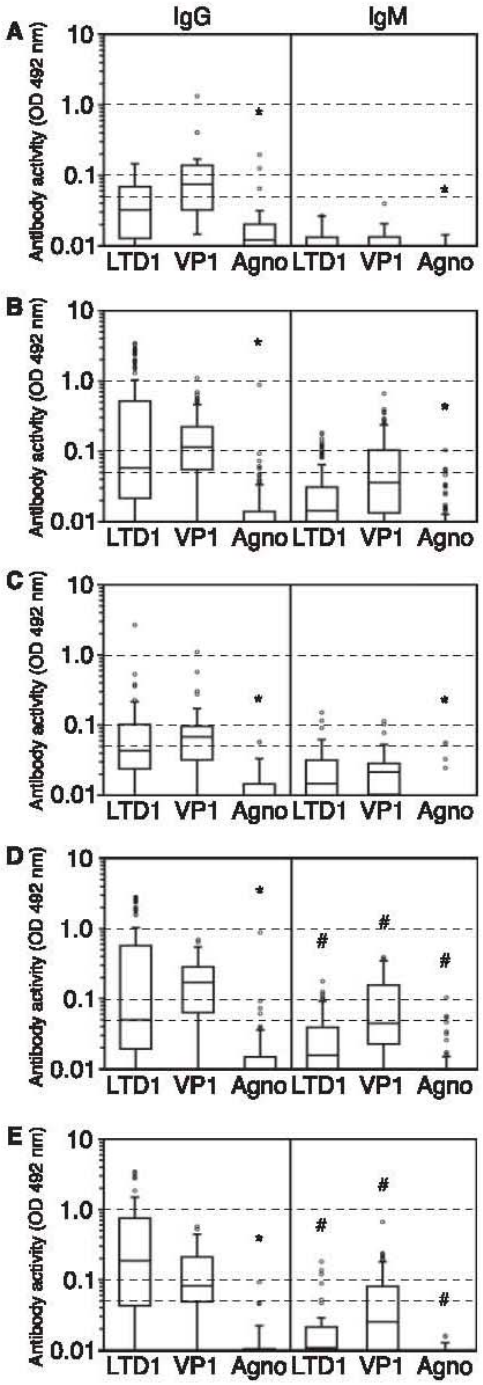

FIG. 3. Antibody responses to BKV proteins in HD and KT patients. Box-and-whiskers plots, with the length of each box corresponding to the interquartile range and the upper and lower boundaries of the box representing the 75th and 25th percentiles, respectively, are shown. The line in the box indicates the median value. Dashed horizontal lines show the different cutoff levels for antibody activity. *, significant difference ($P = 0.02$) for anti-agnoprotein activity compared to activity against LTD1 or VP1; #, significant activity difference ($P < 0.02$) for all the antigen groups (Kruskal-Wallis test). (A) Anti-BKV antibody activity in healthy donors (19 samples from 16 participants). (B) Anti-BKV antibody activity in all renal transplant recipients (146 samples from 38 patients). (C) Anti-BKV antibody activity in renal transplant recipients without evidence of BKV replication or prior to the development of PVAN (25 samples from 16 patients). (D) Anti-BKV antibody activity in renal transplant recipients with ongoing PVAN (83 samples from 27 patients). (E) Anti-BKV antibody activity in renal transplant recipients after resolution of PVAN (38 samples from 23 patients).

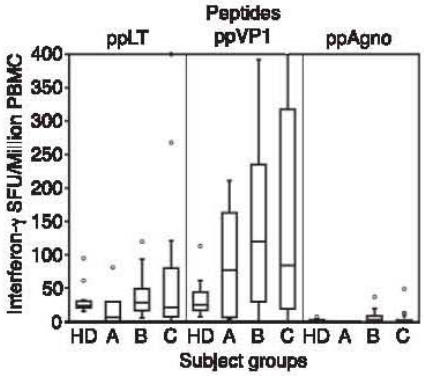

FIG. 4. BKV agnopeptide-specific cellular immune response. IFN-γ responses after stimulation of PBMCs with BKV LT antigen, VP1, and agnoprotein peptides are shown. Results are for HD ($n = 10$), KT patients without high-level BKV replication (group A; $n = 4$), KT patients with high-level BKV replication (group B; $n = 19$), and KT patients after high-level replication (group C; $n = 25$). Agnoprotein responses were significantly lower than LT antigen and VP1 responses ($P < 0.05$ by the Kruskal-Wallis test).

responses, we analyzed 10 PBMC samples from 10 HD and 48 PBMC samples from 30 KT recipients (Fig. 4). ESAs were performed to detect IFN-γ secretion as described previously (1), using *Staphylococcus* enterotoxin B as a positive control (median of 8,080 spot-forming units [SFU] per million PBMCs). In HD, the median number of IFN-γ SFU for LT antigen peptide stimulation was 23 (interquartile range, 20 to 30), that for VP1 peptides was 25 (17 to 44), and that for agnoprotein peptides was 1 (0 to 2). In KT patients, the median number of IFN-γ SFU for LT antigen peptides was 24 (interquartile range, 8 to 76), that for VP1 peptides was 99 (18 to 246), and that for agnoprotein peptides was 2 (0 to 5). Thus, the response to agnoprotein peptides was significantly lower, even after adjusting for protein length by dividing the number of SFU by the number of 15-mer peptides per protein pool ($P < 0.05$ by the Kruskal-Wallis test).

Time courses of immune responses in selected patients. To explore the evolution of humoral and cellular immune responses to different BKV proteins over time, we identified three patients with sufficient sampling density to show different patterns of antibody activity changes before, during, and after high-level BKV replication and PVAN.

Patient 1 (Fig. 5A) is a 49-year-old female who developed definitive PVAN at 7 weeks posttransplantation and had a prolonged course of high-level BKV replication, defined as a plasma BKV load of >10e4/ml (16) over more than 35 weeks. The plasma BKV load peaked at 1.16×10^6 copies/ml at 27

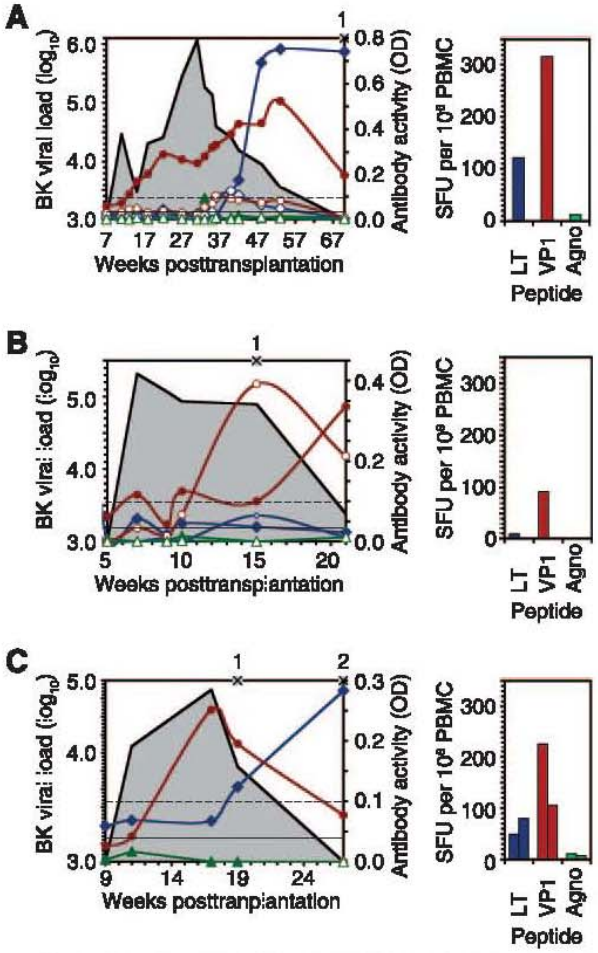

FIG. 5. BKV-specific antibody titers and cellular immune response in KT patients with PVAN. Left panels show plasma BKV load (black line, log/ml, left *y* axis) and antibody activity (OD at 492 nm, right *y* axis) for anti-VP1 (brown lines) (IgG, solid circles; IgM, open circles), anti-LT antigen (blue lines) (IgG, solid diamonds; IgM, open diamonds), and anti-agnoprotein (green lines) (IgG, solid triangles; IgM, open triangles) as well as the time points (×) of ESAs. Right panels show IFN-γ ESAs after stimulation with LT antigen peptides (blue bars), VP1 peptides (brown bars), and agnoprotein peptides (green bars) at the time points indicated in the left panels. (A) Patient 1 (49-year-old female), with definitive PVAN diagnosed at 7 weeks posttransplant. (B) Patient 2 (54-year-old male), with definitive PVAN at 5 weeks posttransplant. (C) Patient 3 (53-year-old male), with definitive PVAN at 9 weeks posttransplant.

weeks posttransplantation. Initially, she had only a weakly positive IgG anti-VP1 antibody response, which over the next weeks increased without any effect on BKV load. At about 34 weeks posttransplantation, increases in anti-VP1 IgM were noted, followed by anti-LT antigen IgM. The increase in the IgM antibody activities heralded a further increase in anti-VP1 IgG (peak at 54 weeks posttransplantation), followed by a steep rise of anti-LT antigen IgG from week 41 to a plateau at week 54 posttransplantation. Simultaneously with the latter changes in anti-BKV responses, the BKV load dropped to 1.36×10^5 at week 37 and decreased further below the threshold of detection at 71 weeks posttransplantation. ESA at 71 weeks posttransplantation showed strong anti-VP1 and anti-LT antigen responses (317 and 121 spots, respectively). The agnoprotein-specific antibody and cellular responses were very low and undetectable, respectively.

Patient 2 (Fig. 5B) is 54-year-old male who developed PVAN just 5 weeks posttransplantation and had a intermediate course of high-level BKV replication over 15 weeks. The BKV viral load peaked at 2.08×10^5 copies/ml at 7 weeks posttransplantation and then remained stable at 1×10^5 copies/ml until week 16, before it dropped to 2.39×10^3 copies/ml at week 22. Anti-VP1 IgG was detectable at low levels from the first sample on, but it started to increase only after week 15, after anti-VP1 IgM peaked. In contrast to the case for patient 1, anti-LTD1 responses remained low or undetectable throughout, as did the anti-agnoprotein antibodies. ESA was performed at week 16, when the BKV viral load was still high, and found a moderate IFN-γ response to VP1 peptides (49 spots), while the LT-specific response was still low (19 spots). Agnopeptide responses were negative.

Patient 3 (Fig. 5C) is a 53-year-old male who developed PVAN at 9 weeks after transplantation and had a relatively short course of high-level BKV replication over 8 weeks. His BKV viral load peaked at 7.90×10^4 copies/ml at 18 weeks posttransplantation and steadily declined thereafter. He was weakly positive for anti-VP1 antibodies, which increased in parallel with the BKV viral load rise and peaked at 18 weeks. Anti-LT IgG antibodies remained low until 20 weeks posttransplantation and started to increase at the same time that the BK viral load started to do so. No BKV-specific IgM was detected. The cellular immune response was assayed at week 20, revealing a strong VP1- and LT antigen-specific IFN-γ response with 227 and 49 SFU, respectively. At 28 weeks, LT antigen-specific IFN-γ responses had slightly increased, while the anti-VP1 response had dropped (80 and 105 SFU, respectively). At both time points, agnoprotein-specific IFN-γ responses were low (12 and 10 SFU, respectively).

DISCUSSION

In this study, we demonstrate that BKV agnoprotein is expressed in vivo in renal tubular epithelial cells of needle biopsy samples from KT patients with histologically defined PVAN. The cellular staining pattern in vivo is indistinguishable from the one found upon BKV infection of primary renal tubular epithelial cells in vitro (Fig. 1), where BKV agnoprotein is abundant in the cytoplasm and enriched in the perinuclear area, as reported previously (35). Of note, agnoprotein expression in vitro and in vivo was found exclusively in cells positive for the early viral gene product LT antigen. By contrast, LT antigen was detectable in the nuclei of cells in which agnoprotein ranged from undetectable to maximal cytoplasmic staining, as expected for sequential progression from viral early to late gene expression. The asynchronous side-by-side quality of different stages of the polyomaviral replication cycle in vivo is typically seen in tissue culture after spread and secondary infection of susceptible cells in vitro. This interpretation is in line with ultrastructural studies of PVAN demonstrating the uptake of polyomavirus particles (7). Alternatively, asynchronous

replication could result from differential reactivation of latent BKV infection and progression from early to late gene expression in response to qualitative or quantitative differences in (micro)environmental costimuli. Since agnoprotein is an abundant late, but nonstructural, viral protein of the lytic BKV infection cycle, staining of renal biopsy specimens may be an interesting marker of viral cytopathic damage to renal tubular epithelia. Further investigations are certainly warranted at this point.

Despite the abundant expression of BKV agnoprotein in vivo, we did not find a corresponding level of humoral and cellular immune responses. Anti-agnoprotein IgG was detected in only 15% and 7.5% of HD and KT patients, respectively, which was significantly lower than the 63% and 80% seropositivity for VP1 and the 42% and 62% seropositivity for LTD1, respectively. In adult populations similar to our study groups, BKV seroprevalence rates of between 60 and 90% have been reported using assays which detect antibody responses directed against the major capsid protein VP1 by EIA or by inhibiting its interaction with receptor-like glycosylated surface structures of type O erythrocytes, i.e., by hemagglutination inhibition (18, 23, 25, 32). The higher VP1-type response may partly be due to the fact that the host immune system is more readily and even systemically (re)exposed to viral surface proteins which typically contain neutralizing epitopes (11). Accordingly, standard serological assays for other viruses such as herpesviruses or parvoviruses are commonly based on glycoproteins of enveloped viruses or capsid proteins of nonenveloped viruses, respectively. In keeping with this notion, anti-LT antigen responses were lower than anti-VP1 responses but still significantly higher than anti-agnoprotein responses. A technical problem seems unlikely to explain this difference, since the applied antigen was well detectable by the rabbit anti-BKV agnoprotein serum. To rule out effects of a potentially nonnative conformation of agnoprotein epitopes in the GST fusion protein, we investigated anti-agnoprotein IgG by indirect immunofluorescence. In line with the EIA results, only 1/30 KT patient sera (3.3%) was reactive using agnoprotein-expressing UTA-6 cells. The smaller size of the agnoprotein and a potentially lower antigenicity could be contributing factors, but the LT antigen used was truncated after the amino-terminal 133 amino acids (LTD1), which should reduce the impact of size differences on detection. When we corrected the response rate for the antigen size, anti-agnoprotein responses were still lower than those against VP1 and far lower than those against LTD1.

The anti-BKV response pattern in nonimmunosuppressed individuals was similar to that in KT patients with no/low-level BKV replication (group A). In particular, we found no IgM responses to any of the three BKV antigens in HD sera. By contrast, KT patients with ongoing high-level replication (group B) had the highest IgG responses to VP1 and also the most prominent IgM responses. Interestingly, the median anti-LTD1 responses in group B KT patients were also increased relative to those in HD or group A KT patients, but those in KT patients with past high-level BKV replication (group C) were significantly higher (but with a slight decline in anti-VP1 IgG). These data indicate that anti-LT antigen IgG responses increase later than anti-VP1 responses, coinciding with emerging immune control of BKV replication. This is highlighted by the time courses demonstrating that anti-LT antigen responses evolve significantly later than anti-VP1 responses, at a time of declining plasma BKV load. In fact, testing before week 39 in patient 1 and before week 17 in patient 3 detected only anti-VP1 responses. Thus, the time courses underline the absence of anti-agnoprotein responses and reduce the likelihood that the time points of the cross-sectional sampling for anti-agnoprotein responses were merely suboptimal.

Our serological studies are further complemented by the poor IFN-γ responses elicited by agnoprotein peptide pools (median, 2 SFU; range, 0 to 5) compared to LT antigen (median, 24; range, 8 to 76) or VP1 (median, 99; range, 18 to 246) peptide pools, even after correction for peptide pool size. Although specific IFN-γ-producing $CD8^+$ T cells have been detected for other viruses, e.g., cytomegalovirus (38), we cannot exclude the possibility that the use of overlapping 15-mers may be suboptimal for the detection of agnoprotein-specific major histocompatibility complex class I-restricted $CD8^+$ T-cell responses. On the other hand, we would expect a significant agnoprotein-specific major histocompatibility complex class II-restricted $CD4^+$ T-cell response in the fairly sensitive ESA format. Taken together, humoral and cellular immune responses to the abundant BKV agnoprotein are rare and likely reflect its poor immunogenicity. Moreover, anti-agnoprotein immune responses seem to have no apparent importance in overcoming BKV viremia and PVAN.

Only a few studies have examined the presence of antibodies against the major regulatory protein LT antigen. Antibodies to BKV LT antigen have been detected by indirect immunofluorescence in 1.15% of 952 neoplasia patients, 4.40% of 113 renal transplant recipients, and 0.80% of healthy controls (6). In another study, antibodies to LT antigen were found in 29.1% of 103 BKV-infected renal transplant recipients and were absent in BKV-negative patients (27). These results show that not only virions but also remnants of infected cells are processed by antigen-presenting cells (20). It remains to be seen if anti-LT antibody titers can be used as a surrogate for BKV replication control.

The low immunogenicity of BKV agnoprotein is in contrast to the HIV protein Tat, consisting of 86 to 101 amino acids. Anti-TAT antibodies have been found in only 10 to 14% of HIV type 1 (HIV-1)-positive patients (4) and were predictive of nonprogression both in HIV-1 (33)- and HIV-2 (36)-positive patients. Consequently, HIV Tat has been considered for use as a candidate anti-HIV vaccine (30). At this point, we can only speculate about the underlying mechanism of the poor immunogenicity of agnoprotein, which might involve host as well as viral factors. Since abundant agnoprotein expression is tightly restricted to switching to late gene expression, it is tempting to hypothesize that efficient immune recognition of agnoprotein would significantly curtail the replication and spread of polyomavirus in its host population. Our results indicate that agnoprotein may not be a sensitive tool for monitoring the immune response in KT patients, but its potential as a candidate anti-BKV vaccine cannot be conclusively ruled out.

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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

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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

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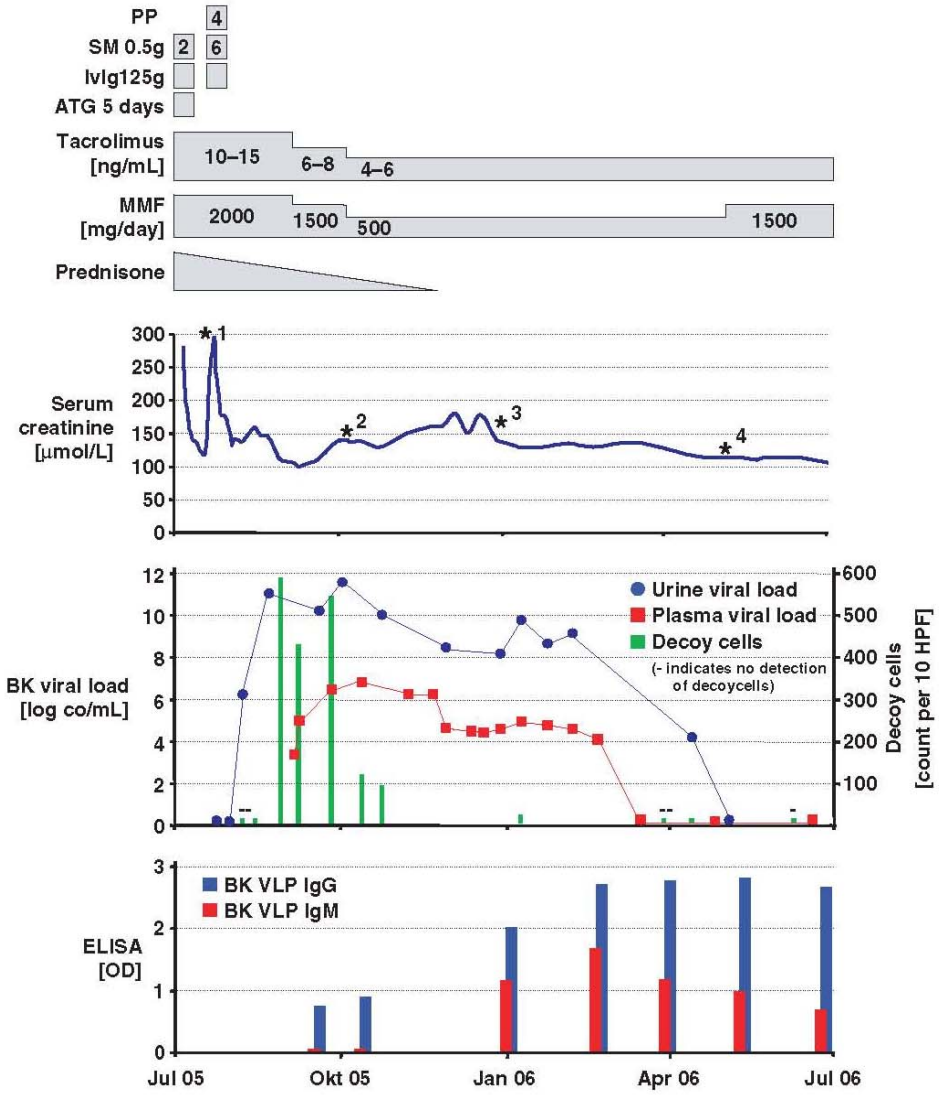

Fig. 1. Course of immunosuppressive therapy, allograft function, BK-virus activity and BK-virus serology within the first year after transplantation. Stars with appended numbers in the serum creatinine graphic indicate allograft biopsies. The corresponding allograft histologies are shown in Figure 2. PP, plasmapheresis; SM, solumedrol i.v.; IvIg, intravenous immunoglobulins; BK-virus-specific IgG and IgM antibodies were measured in human sera using virus-like particles (VLP) as antigens coated to microtitre plates. BK-virus-like particles were purified by density gradient centrifugation of lysates from Sf9 insect cells infected with recombinant BK-virus-VP1 Bac-to-Bac expression vectors (Invitrogen). Antigen coating and serum dilutions provided optimal OD results at 50 ng and 1: 400, respectively (S. Bodaghi and H.H. Hirsch, unpublished data).

copies/ml. The diagnosis of 'presumptive' PVAN was made [1] and immunosuppression reduced (tacrolimus trough levels from 10 15 ng/ml to 6 8 ng/ml, MMF dose from 2 to 1.5 g/day, further tapering of steroids). Three weeks later, a second allograft biopsy was performed. Dense focal lymphohistiocytic infiltrates were seen, affecting 20% of the cortical and 35% of the medullar area with mild tubulitis. There were numerous SV40 antigen-positive tubular epithelial cells in the medulla and in the cortex (Figure 2; picture 2A and 2B). At this time-point, BK-viral loads were 3.9×10^{11} copies/ml in urine and 5.1×10^{6} copies/ml in plasma, respectively. The diagnosis of PVAN pattern B was made and immunosuppression further reduced (tacrolimus trough levels to 4 6 ng/ml, MMF dose to 0.5 g/day, steroids were tapered out over 8 weeks).

BK-viraemia remained constantly above 10^6 copies/ml for 8 weeks, but then started to decline by two log10 units to a plateau of around 4×10^4 copies/ml. Within this time period, serum creatinine concentrations slowly rose from 140 to 180 µmol/l. A third allograft biopsy was performed, which showed massive diffuse lymphohistiocytic infiltrates affecting 100% of the cortical and medullar area with severe tubulitis. There were only few SV40 antigen positive tubular epithelial cells in the medulla and the cortex (Figure 2; picture 3A and 3B). A presumed diagnosis of BK-virus specific immune reconstitution was made.

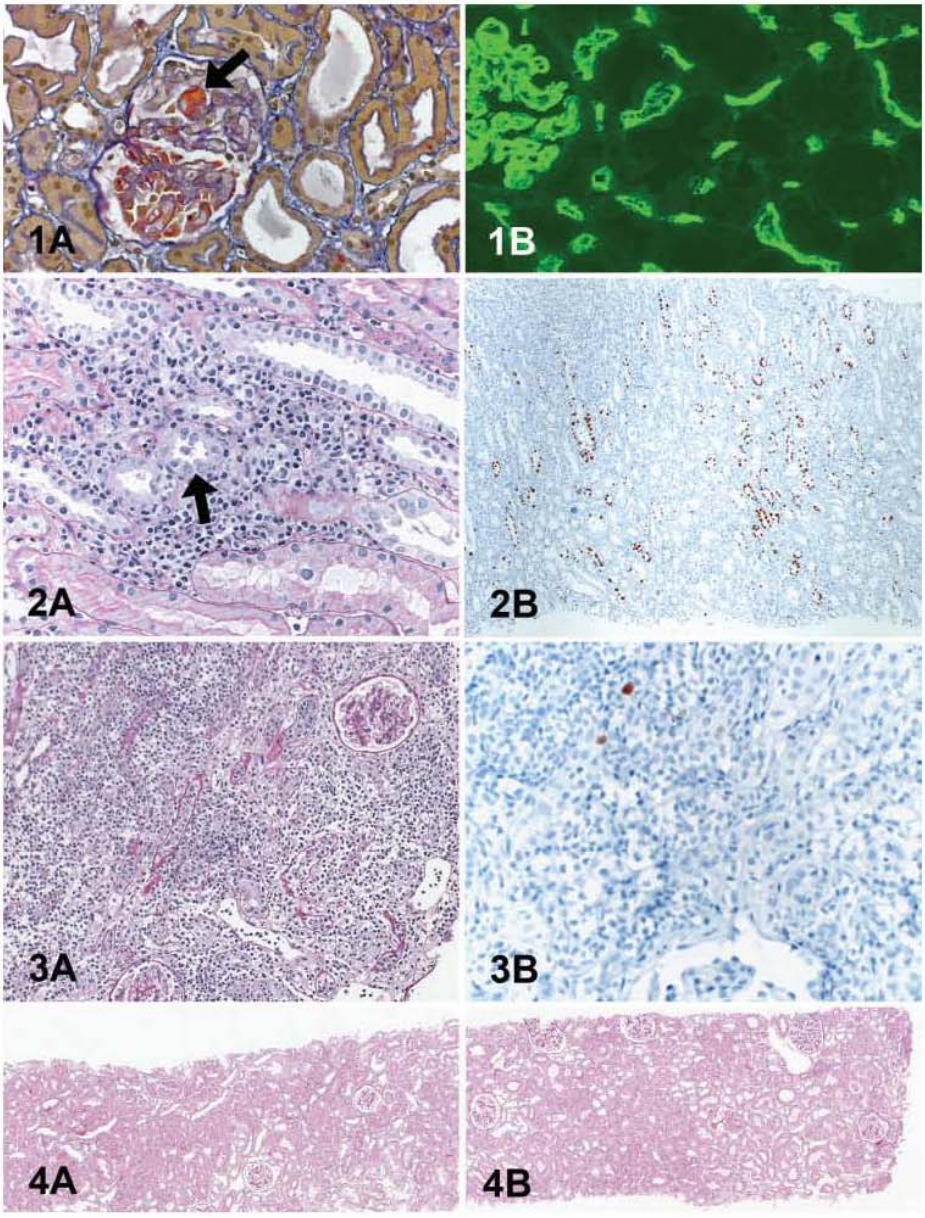

Fig. 2. Allograft histology graded according to the Banff classification [16] and a recently proposed semi-quantitative assessment for PVAN [1]. All allograft biopsies consisted of two cores obtained with a 16-gauge needle. **First biopsy**, 21 days post-transplant (cy0, i0t0, ci0ct0): the tubulointerstitial compartment was completely normal, but there were fibrin thrombi in peripheral glomerular capillary loops (arrow) consistent with thrombotic microangiopathy (**1A**). Peritubular capillaries (PTC) were diffuse positive for C4d by immunofluorescence (IF) (**1B**). Staining for SV40 antigen by immunohistochemistry was negative. **Second biopsy**, 3 months post-transplant (cy3, i2t1, ci0ct0): dense focal lymphohistiocytic infiltrates in the cortical and medullary interstitial space with mild tubulitis. Note enlarged nuclei (arrow) without clear-cut inclusion bodies (**2A**). Glomeruli and arteries were completely normal. Staining for SV40 antigen revealed numerous infected tubular epithelial cells in the cortex and medulla (**2B**). Staining for C4d in PTC and HLA-DR in tubular epithelial cells were negative. **Third biopsy**, 6 months post-transplant (cy1, i3t3, ci0ct1): massive diffuse lymphohistiocytic infiltrates affecting the whole cortical and medullary space with severe tubulitis (**3A**). Immune phenotyping of the infiltrate revealed a very dominant fraction of T-cells (CD3+) with equal amounts of CD4+ and CD8+ cells, few macrophages (CD68) and few B-cells (CD20). Glomeruli and arteries were completely normal. Only few tubular epithelial cells were positive for SV40 antigen (**3B**). Staining for C4d in PTC and HLA-DR in tubular epithelial cells were negative. **Fourth biopsy**, 10 months post-transplant (cy0, i0t0, ci0ct0): both biopsy cores showed perfectly normal renal tissue (**4A** and **4B**). No tubular epithelial cells were positive for SV40 antigen. Staining for C4d in PTC and HLA-DR in tubular epithelial cells were negative.

Immunosuppression consisting of tacrolimus with trough levels of 4–6 ng/ml and MMF 0.5 g/day was maintained and no additional therapy introduced. Retrospective analysis of serum samples at this time-point revealed newly produced BK-virus specific IgM together with increasing IgG antibodies (Figure 1, bottom panel) consistent with an emerging BK-virus specific immune response.

Ten weeks later, BK-viraemia became negative, followed by clearance of the virus in the urine. Serum creatinine declined and stabilized at 110–120 μmol/l. A follow-up biopsy showed normal allograft tissue without any tubulointerstitial infiltrates, no tubular atrophy and interstitial fibrosis and no SV40 antigen positive tubular epithelial cells (Figure 2; picture 4A and 4B). Testing for BK-virus-specific T-cells by interferon-γ ELISpot at this time-point revealed a response against BK-virus large T and VP1 proteins. Intracellular staining for interferon-γ showed a prominent large T- directed CD8+ T-cell response consistent with an established cellular immune response as reported previously (Figure 3) [9].

Discussion

The majority of patients with PVAN demonstrate tubulointerstitial inflammation [6,10], which can either indicate concurrent allograft rejection or an inflammatory response to the BK-virus. The relative contribution of these two entities is unknown and specific single markers to separate them are currently lacking. The clinico-pathological course of our patient is intriguing and may serve as an index case of BK-virus-specific immune reconstitution syndrome.

At the time of the third allograft biopsy showing diffuse massive interstitial infiltrates and severe tubulitis, circumstantial evidence suggested that acute rejection was less likely as the cause. First, extensive infiltrates were coincident with a significant decline of BK-virus replication, indicated by a decreasing plasma BK-viral load of >2 log10 units and a substantial clearance from the allograft based on a significant reduction of SV40 antigen-positive tubular epithelial cells. Second, the increase of serum creatinine was only moderate, despite severe tubulointerstital pathology. Third, staining of tubular epithelial cells for HLA-DR in frozen sections was available, which served as an adjunctive tool to differentiate between tubulitis due to rejection and PVAN. HLA-DR positivity is a typical finding in Banff Ia/Ib rejection whereas it is mostly negative in PVAN, as in this case and described earlier [11]. However, the strongest argument against an alloimmune-related inflammation was the clinico-pathological course. Indeed, massive tubulointerstitial infiltrates developed after immunosuppression was significantly lowered, but they subsequently resolved completely while maintaining the same low immunosuppression without having applied any rejection treatment. A drug-induced interstitial nephritis is also very unlikely, because no drugs were added or discontinued within this time frame, with the exception of tapering out prednisone and reducing tacrolimus and MMF. Therefore, BK-virus-specific immune reconstitution is the most probable explanation for the observed clinico-pathological course, and to the best of our knowledge there is no consistent alternative diagnosis.

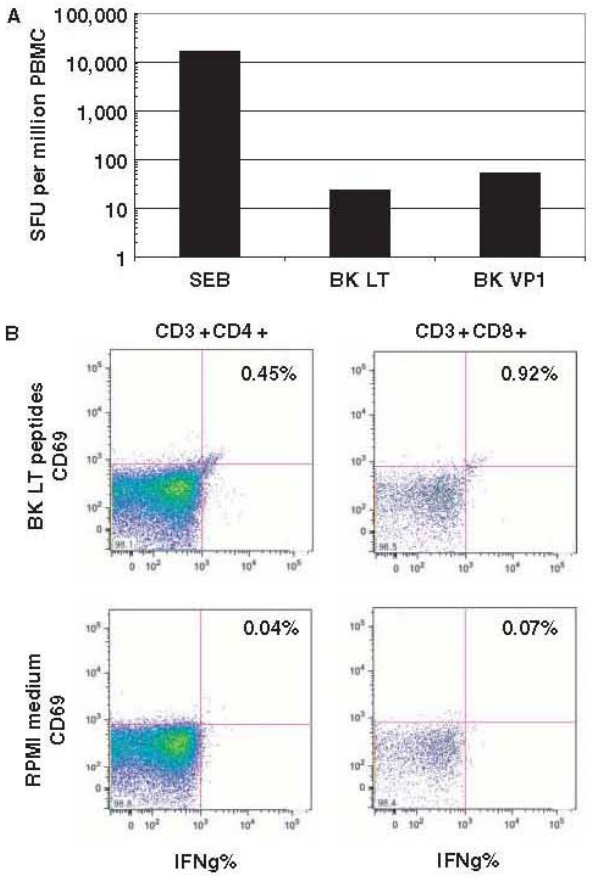

Fig. 3. BK-virus-specific interferon-γ production in PBMC at resolution of PVAN (i.e. at the time point of the fourth allograft biopsy). The 15-mer peptide pools of 11 amino acid overlaps spanning BK-virus large T-antigen (LT) and VP1 capsid protein (VP1) were used for PBMC stimulation and interferon-γ detection by ELISpot assay (**A**) or by intracellular cytokine staining/flow cytometry (**B**) as described previously [9]. *Staphylococcus enterotoxin B* (SEB, SIGMA, Buchs, Switzerland, 1 μg/ml) and cell culture medium served as positive and negative control, respectively. The number of spot forming unit (SFU) per well was calculated from triplicates after subtractions of negative control. By flow cytometry, at least 30 000 CD3+ cells were acquired and analysed on a FACS-Canto (Becton Dickinson). The frequency of BK-virus-specific cellular immune responses was determined for each antigen and expressed as percent of interferon-γ positive cells among CD3+CD4+CD69+ or CD3+CD8+CD69+ gated lymphocytes, respectively.

Support for this interpretation comes from retrospectively measured BK-virus-specific antibody titres as a surrogate marker of the emerging immune response to the BK-virus, as has been reported previously [12]. A marked increase of BK-virus specific IgM and IgG was noted following the decline of plasma BK-virus load at the time of severe tubulointerstitial inflammation. Longitudinal analysis of BK-virus specific cellular immune responses or characterization of cellular infiltrates in the allograft would have been of particular interest in this case [13], but were not available. However, we analysed the frequency of BK-virus specific interferon-γ producing

T-cells by ELISpot and flow cytometry after resolution of PVAN, which were consistent with an established cellular immune response against the BK-virus and in line with our previous observation [9]. The reported case suggests that integration of longitudinal dynamics of BK-virus replication together with allograft function, histology and possibly markers of virus-specific cellular and humoral immunity may currently be the most valuable parameter for differentiation of tubulointerstitial allograft rejection *vs* a BK-virus-specific immune response.

An interesting finding in this case was the detection of completely normal renal tissue in the last biopsy despite severe tubulointerstitial inflammation and many BK-virus infected tubular epithelial cells in previous biopsies. This finding is surprising and may indicate that the end-stage of PVAN characterized by progressive tubular atrophy and interstitial fibrosis is the result of ongoing tubular cell necrosis unbalanced by regeneration [10]. Therefore, the good outcome in the reported case could be related to an early and efficient clearance of the BK-virus, or to a high capacity of the allograft from a 7-year-old donor to sustain injury and to regenerate. Although this single case suggests that even severe inflammation due to immune reconstitution may not be harmful, the value of immunomodulatory drugs including steroids and leflunomide as a treatment option to limit the extent of inflammation is not clear yet. Notably, the immune reconstitution syndrome is well-known in HIV/AIDS patients and steroids are often considered when the inflammation becomes damaging to an organ [14,15].

In conclusion, we report a case consistent with BK-virus-specific immune reconstitution after reduction of immunosuppression for treating PVAN, leading to transient severe tubulointerstitial inflammation and moderate allograft dysfunction. Clearly, no general recommendations regarding diagnosis and treatment of BK-virus specific immune reconstitution syndrome can be made based on this single case. Prospective studies with standardized management are required to validate our findings and to further elucidate aetiology and treatment of tubulointerstitial infiltrates in patients with PVAN.

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Conflict of interest statement. None declared.

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4.7 CYTOMEGALOVIRUS-SPECIFIC T-CELL RESPONSES IN KIDNEY TRANSPLANT RECIPIENTS WITH AND WITHOUT VIRAL REPLICATION

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Running head: Cytomegalovirus-specific T-cells after Kidney Transplantation
Key words: CMV, drug resistance, ganciclovir, T-cells, transplantation, UL97 mutation

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ABSTRACT

Cytomegalovirus (CMV) seronegative kidney transplant (KT) recipients (R-) of kidney-transplants from seropositive donors (D+) are at higher risk for CMV-replication and ganciclovir (GCV)-resistance than R+ KT patients. To assess the role of CMV-specific T-cells in R+ KT, we studied interferon-γ-producing T-cell frequencies in peripheral blood mononuclear cells of 73 KT patients (25 D+R-, 48 R+) undergoing routine testing for CMV-replication. Median T-cell responses to CMV-lysate, CMV-pp72 and -pp65 peptide pool-specific antigen preparations were higher in R+ KT than D+R- KT patients, but lower than in CMV seropositive healthy donors ($P<0.05$). CMV-lysate induced higher frequencies than pp65 and pp72 peptide pools, but favored CD4+ over CD8+ T-cell responses. By contrast, pp72 favored CD8+ responses, while pp65 responses were comparable in both subsets. T-cell responses were lower in R+ KT with than without concurrent CMV-replication (pp65 median CD4+ 0.00% vs. 0.03%, $P=0.001$; CD8+ 0.01% vs. 0.03%; $P=0.033$). CMV pp65-specific CD4+ T-cell frequencies >0.03% allowed to identify R+ KT patients free from concurrent and from future CMV-replication within 2 months ($P=0.021$). GCV-resistant CMV-replication occurred in 3/48 R+KT patients all of whom had pp65-specific CD4+ T-cells <0.03%. The data suggest that CMV-pp65-specific CD4+ T-cells >0.3% may indicate protection from CMV replication and GCV-resistance in R+ KT recipients.

INTRODUCTION

Potent immunosuppressive drug regimens have led to a significant decline of acute and chronic immune reactions in solid organ transplant (SOT) recipients, with increasing graft survival across HLA mismatches (Dharnidharka et al. 2004; Meier-Kriesche et al. 2006). However, complications associated with impaired immunity have become more frequent (Fishman et al. 1998; Opelz et al. 2006). Cytomegalovirus(CMV) is a notorious infection posttransplantation exerting significant direct and indirect effects on graft and patient survival, despite the availability of validated strategies for prophylactic, preemptive and therapeutic intervention (Reinke et al. 1999; Ljungman et al. 2002; Opelz et al. 2004). The risk of CMV-replication and disease after SOT is highest in seronegative recipients (R-) of seropositive donor (D+) organs and in seropositive (R+) patients exposed to T-cell depleting agents (Kusne et al. 1999). This suggests that CMV-specific immunity provides a certain degree of protection despite maintenance immunosuppression. Thus, prophylaxis with antiviral drugs such as ganciclovir (GCV) or valganciclovir (ValGCV) is recommended (Paya et al. 2004; Preiksaitis et al. 2005). However, CMV D+R- SOT are at an increased risk of GCV-resistant CMV-replication and disease (Limaye et al. 2000). We hypothesize that the risk may also be increased in R+ SOT with low CMV-specific T-cells. Cytotoxic CD8+ T-cells are thought to play a major role in terminating CMV-replication, but CMV-specific CD4+ T-cells have been linked to long-term antiviral control (Sester et al. 2001; Bunde et al. 2005). In clinical studies, responses to CMV-lysate antigens or peptides covering CMV immediate-early (e.g. pp72) and late (e.g. pp65) proteins have been used to monitor cellular immunity, but have rarely been compared. Here, we studied T-cell responses to different CMV antigens in recently KT patients and searched for UL97 mutations in cases with persisting CMV-replication.

PATIENTS AND METHODS

PATIENT POPULATION

A total of 73 consecutive adult CMV D+R-, D+R+ or D-R+ KT patients (n=73) were enrolled in this prospective cross-sectional study. Patients were included if CMV-replication was submitted to the laboratory for routine testing of CMV in the blood by PCR and/or pp65 antigenemia. CMV testing was done bi-weekly for the first 4 months, then monthly until months 6, to guide preemptive therapy, or weekly for cases where CMV had been suspected and that had been found positive (**Table 1**). D-R- patients were not included (as not routinely tested). Patients were assigned to CMV replicating and non-replicating subgroups according to whether or not CMV was detected. At the time of laboratory testing for CMV-replication and cellular immune responses, triple immunosuppression was administered in 42 patients (58%; CMV-replication in 7 cases), dual immunosuppression in 24 cases (33.3%, CMV-replication in 5 cases) and monotherapy was used in 2 cases, 1 with CMV-replication. Tacrolimus was used in 51 patients (71%) with mean trough levels 8.71ng/mL, 13 showing CMV-viremia (P=0.038; chi-squared). Mycophenolate mofetil was used in 41 patients (57%) with mean trough levels of 2.54ug/mL, 6 showing CMV-viremia (P=0.311; chi-squared). Sirolimus was used in 18 patients (25%; mean trough levels 6.92ng/mL, no CMV-replication), cyclosporine A in 10 patients (14%; mean trough levels 288ng/mL, no CMV-replication); azathioprine in 14 patients (19%, CMV-replication in 3 cases); leflunomide in 3 patients (4.2%; CMV-replication in 3 patients); prednisone in 41 patients (57%; mean dosing 9.3mg/day; CMV-replication in 7 cases). Thirty non-immunosuppressed healthy donors (HB) served as control group (17 seropositive HB+: median age 31, range 22-48, 8 males; 13 seronegative; HB-: median age 32, range 27-60, 9 males). Participants were enrolled in Basel and in St. Gallen according to the protocol approved by the IRB (299/06).

Table 1: Characteristics of KT patients with and without ongoing CMV-replication.

	CMV D+R- (n=25, m=50)			**CMV D+R+/D-R+ (n=48, m=79)**		
CMV replication (n/total)[a]	all 25	yes (9/25)	no (22/25)	all 25	yes (6/48)	no (42/48)
Age, median years	60	63	59	49	53	43
(range)	(18-71)	(25-71)	(18-71)	(21-73)	(43-70)	(21-73)
Gender (m/f)	15/10	6/3	13/9	33/15	5/1	29/14
Sample date postTx						
median weeks	18	30	15	27	21	17
(range)	(2-383)	(6-41)	(2-383)	(2-314)	(5-138)	(2-314)
Induction (n)[b]	19	7	12	36	3	33
(%)	(76.0)	(77.8)	(54.5)	(75.0)	(50.0)	(76.7)
T-cell depleting induction (n) [c]	1	0	1	4	0	4
(%)	(4.0)	(0.0)	(4.5)	(8.3)	(0.0)	(9.3)
AR therapy (n)[d]	8	2	6	21	3	18
(%)	(32.0)	(22.2)	(27.3)	(43.8)	(50.0)	(41.9)
T-cell depleting AR therapy (n)[e]	3	0	3	11	2	9
(%)	(12.0)	(0.0)	(13.6)	(22.9)	(33.3)	(20.9)
1st CMV load, mean (c/ml)[a]	-	109’900	<300	-	49’588	<300
1st CMV peak, mean (c/ml)[a]	-	181’811	<300	-	90’009	<300
1st CMV clearance time weeks, median (range)[a]	-	14 (4-37)	-	-	18 (7-106)	-
2nd CMV replication (n/total)[a]	-	1/9	5/22	-	1/6	7/42
2nd CMV load, mean (c/ml)[a]	-	288’001	9’323	-	1’489	72’581
2nd CMV peak, mean (c/ml)[a]	-	1’240’000	37’260	-	3’387	311’071
2nd CMV clearance time weeks, median (range)[a]	-	30 (-)	8 (1-30)	-	22 (-)	3 (1-30)

AR: acute rejection, ATG: anti-thymoglobuline, n: numbers of patients, m: number of measurements.
1st CMV episode was either primary (in R-) or secondary replication (in R+). 2nd CMV episode was either primary (in remaining R-) or secondary replication (in R+) after determination of CMV-specific cellular immunity, after the 1st CMV episode (follow-up ≥3 months).
[a] Patients are grouped according to CMV replication at time-point of T-cell response measurement (1st CMV episode). 14 patients were measured after seroconversion increasing the numbers to 22.
[b] Numbers of recipients with induction treatment.
[c] Numbers of recipients with induction treatment based on T-cell depleting antibodies (ATG).
[d] Numbers of treated AR episodes.
[e] Numbers of patients with AR therapy based on T-cell depleting antibodies (ATG).

CMV DIAGNOSTIC ASSAYS

IgG CMV-serology (AxSym™ Assay, Abbott, Baar, Switzerland) was used according to manufactures instructions to identify CMV seropositive and seronegative individuals. CMV-replication was quantified by standard commercial tests of pp65 antigenemia assay (CINAkit™, Argene, Varilleg, France) in peripheral blood mononuclear cells (PBMC) or by quantitative realtime-PCR. In all patients with CMV-antigenemia testing, CMV DNA load in whole blood was quantified by CMV-specific real-time PCR. The extraction of DNA was performed according to the QIAamp DNA MiniKit™ (QIAGEN, Hombrechtikon, Switzerland). CMV-replication was quantified by real-time PCR using the primers TTT TTT CTA GGC GCT TCC GA and ACA CTG CGG CTT TGT ATT CTT TAT C, and the Fam-Tamra labeled probe AGG CGA AGC CGG CGA CGA in validated routine assay. Primers and probes were obtained from Eurogentec (Geneva, Switzerland). The linear range of the real-time PCR is 10e2 to 10e8 cp/mL. The limit of detection was 300 copies (cp)/mL, and 1000 cp/mL were used as routine diagnostic cut-off value. All assays were performed in triplicates. For quantification, a standard curve was constructed from defined copy number of the cloned targets using 10e6 (10e8/mL), 10e4 (10e6/mL), 10e2 (10e4/mL) per 5 uL. DNA specimens or controls were added as 5 uL to 20 uL containing 300nM of the respective primer pair, 200nM of the respective probe and 12.5 uL of the 2-fold concentrated commercially obtained Mastermix containing the AmpliTaq polymerase, dNTP mix with dUTP replacing dTTP, Uracyl-N-gylcosylase (AMPerase, Roche) to yield final volume of 25µL. Each DNA sample was analyzed after being spiked with 1000 copies of the target pCMV1 to monitor for inhibition. The temperature profile consisted of preincubation at 37°C, 15 min to allow for enzymatic decontamination of synthetic uracyl-containg amplicons, followed by 95°C; 15 min for hot-start activation and 45 cycles of 95°C; 15 sec; 60°C; 60 sec, followed by 7 min at 72°C. In case of inhibition or unclear results, the DNA was extracted once again and assayed as described. Each PCR assay contained routinely non-template controls in triplicates as well as one contamination control of human blood donor serum which was taken through the entire process of DNA extraction and assayed in triplicates.

Clinical GCV-resistance was defined as persistent CMV-replication despite adequate antiviral treatment for >7 days or as breakthrough replication during prophylaxis (Preiksaitis et al. 2005). Genotypic resistance was diagnosed when known mutations in the CMV UL97 gene were identified using cycle sequencing after a nested PCR strategy with TGC TGC ACA ACG TCA CGG TAC ATC and AAA CAG ACT GAG GGG GCT ACT as outer primers (10 min at 95°C; then 40 cycles of 30 sec at 95°C, 30 sec at 50°C, 1 minute at 72°C and 7 minutes extension at 72°C) followed by amplification of two fragments with CGT TGG CCG ACG CTA TCA AAT TTC and ACA GCT CCG ACA TGC AAT AAC G (348bp), as well as GTG GGT AAC GTG CTG GGC TTT TG and GTG GGT TTG TAC CTT CTC TGT TGC (518bp). (10 min at 95°C; then 40 cycles of 30 sec at 95°C, 30 sec at 50°C, 1 minute at 72°C and 7 minutes extension at 72°C). Final concentrations in a 50µL reaction volume were 1uM primer, 200nM dNTP, 1x Pwo buffer, 1U of Pwo polymerase (Roche, Basel, Switzerland). The respective amplicons were isolated from preparative gel electrophoresis for cycle sequencing. If UL97 sequences indicated multiple CMV mutants, the amplicons were cloned in pGEM3Zf+ plasmid (Promega, Madison, USA), and clones were sequenced.

QUANTIFICATION OF CMV SPECIFIC T CELLS

The measurements of the T-cell responses were 2 ±1.2 times (median 2, range 1-6) in the D+/R- group and 1.7 ±1.4 (median 1, range 1-8) in the R+ group (P=n.s.). The frequency of CMV-antigen-specific interferon-gamma (IFNγ) producing T-cells by intracellular cytokine staining was carried out according to a previously published protocol (Sester et al. 2001) except that peripheral PBMC instead of whole blood were used. PBMC were taken before medication and tested for CD69+ IFNγ+ response in CD4+CD3+ and CD8+CD3+ T-cells after stimulation with three different CMV-antigens: 1) Cell-lysate preparations from CMV infected fibroblast cell cultures (4ug/ml, Virion, Rüschlikon, Switzerland), 2) peptide pool covering the immediate early protein 1 (pp72), and 3) peptide pool covering the late gene tegument protein (pp65). The peptide pools consisted of 15 amino acids (aa) long peptides with 11aa overlaps and were used in a final concentration of 2ug/ml (Eurogentech, Geneva, Switzerland). Non-infected fibroblast-lysate preparations served as negative control and *Staphylococcal enterotoxin B* (SEB, 1ug/ml, Sigma, Buchs, Switzerland) as positive control.

PBMC were recovered either from citrate anti-coagulated CPT^{TM}-vacutainers (BD, Allschwil, Switzerland) or from EDTA blood using Lymphprep^{TM} (Axis Shield, Oslo, Norway). PBMC were washed twice in phosphate-buffered saline (PBS) and stimulated with CMV-antigens in presence of αCD28/αCD49d (1ug/ml, Becton Dickinson, Allschwil, Switzerland) for total 6 hours in RPMI 1640 medium (Sigma) with 10% fetal calf serum, 1% penicillin/streptomycin (Gibco, Basel, Switzerland) and 1% glutamax (Gibco). After two hours brefeldin A (10ug/ml, Sigma) was added to prevent IFNγ secretion. Cells were washed once with PBS (without Ca^{2+} and Mg^{2+}, pH 7.2), fixed first with 4%, then with 1% paraformaldehyde, permeabilized with 0.1% saponin in PBS and stained at room temperature in the dark with following antibodies: αCD3, αCD4, αCD8, αCD69 and αIFNγ (all Becton Dickinson). At least 30'000 CD3+ lymphocytes were analyzed with FACSCanto (Becton Dickinson). The frequency of CMV-specific T-cells was analyzed for each antigen and was expressed as percentage of CD69 and IFNγ double positive CD4+CD3+ or CD8+CD3+ cells. Negative controls were subtracted to determine the antigen-specific frequency.

DATA ANALYSIS

Data were summarized as mean ±standard deviations (±SD) or as median and ranges where appropriate. When Kolmogorov-Smirnov Z Test indicated lack of normal distribution, nonparametric tests were used such as the Mann-Whitney U-test, Spearman's rho correlation analysis, and 2-related Wilcoxon test. Categorical markers were analyzed by Fisher's exact or Pearson's chi-square test. Binary logistic regression's default and receiver operator characteristics (ROC) analysis with Youlden's Test was used to determine cut-off levels of T-cell responses. P-values <0.05 were considered as statistically significant. For statistical analysis, we used the SPSS 13th version package (SPSS, Chicago, USA).

RESULTS

CMV-SPECIFIC CELLULAR IMMUNE RESPONSE IN KT PATIENTS

PBMC from 73 KT patients undergoing routine testing for CMV replication were analyzed for CMV-specific T-cell responses **(Figure 1, Table 1**). Nine of 25 D+R- KT patients had primary CMV-replication at the time of the first CMV-test and a total of 14 seroconverted during the follow-up. Six of 48 R+ KT patients had secondary CMV-replication (reactivation) at the time of first testing, increasing to a total of 13 during the follow-up. The longer time to clear CMV-viremia is remarkable and resulted in part from CMV-persistence due GCV-resistance and the more sensitive PCR on whole blood as compared to serum.

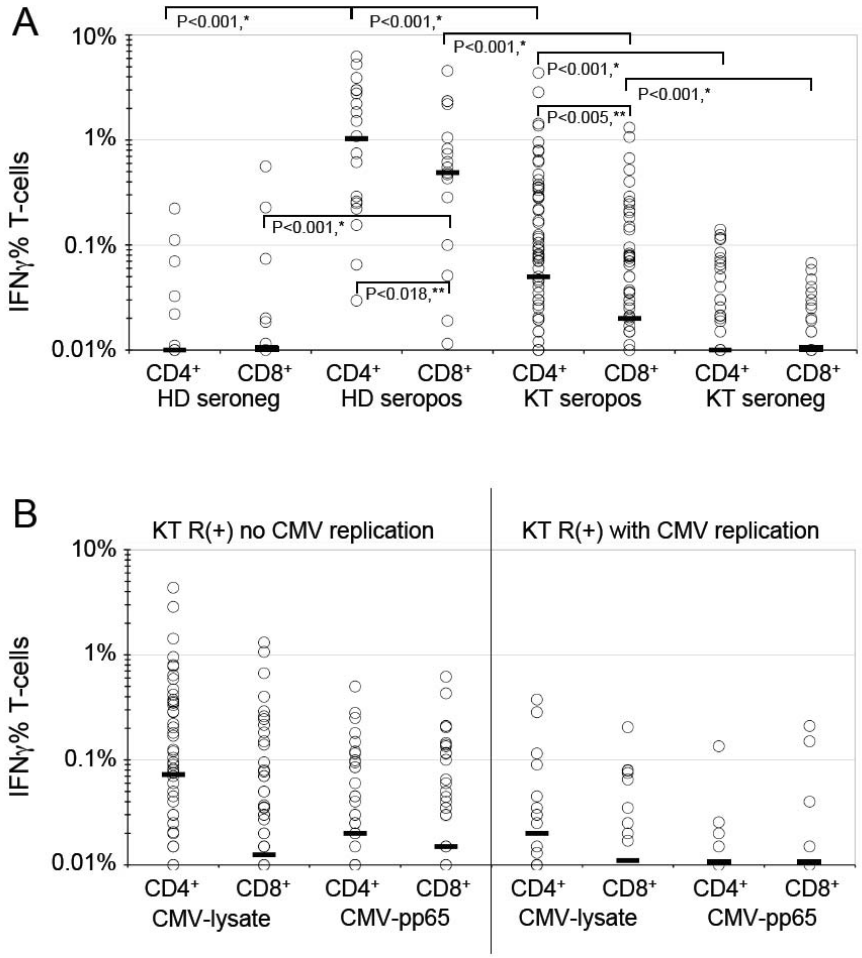


Figure 1: IFN-γ positive CMV-lysate specific T-cells in HB and KT patients. **A)** HB and KT patients according to CMV serostatus. **B)** CMV seropositive KT patients according to CMV replication.
X-axis indicates CD4+ or CD8+ T-cell subsets. Y-axis shows % of IFN-γ positive T-cells after specific stimulation with CMV-lysate. Black bars indicate median values. P-values were calculated using non-parametric Mann-Whitney U-test (*) or Wilcoxon Test (**).

CMV-seropositive R+ had significantly higher CD4+ and CD8+ T-cell responses than D+R- KT patients for all CMV-antigens tested (Mann-Whitney U-test, P<0.05, **Table 2**) except for pp72 stimulated CD8+ T-cells where the significance level was not reached (P=0.056). In R+ KT patients, T-cell responses were generally higher for CMV-lysate (CD4+ 0.05%, CD8+ 0.02%) than for the peptide pools of pp72 (CD4+ 0.04%, CD8+ 0.05%) or pp65 (CD4+ 0.02%, CD8+ 0.02%). CMV-lysate elicited higher CD4+ than CD8+ T-cell responses (Wilcoxon signed rank test p <0.005, two-tailed), while no significant differences for CD4+

vs. CD8+ T-cell responses were observed for pp65 peptides (P=0.183, two-tailed) or pp72 peptides (P=0.255, two-tailed, **Figure 1A, Table 2**).

When we compared HB+ and R+ KT-patients, the CMV-specific IFNγ T-cell frequencies in seropositive R+ KT patients were significantly lower than in seropositive HB+ for all tested CMV-antigens (Mann Whitney U-test, p <0.05), except for pp72-specific CD4+ T-cells where the level of significance was barely missed (P=0.0545). In HB+, the median CMV-specific T-cell responses were significantly higher than in HB- for all CMV-antigens tested. CMV-lysate elicited higher responses (CD4+ 1.03%, CD8+ 0.49%) than pp65 peptides (CD4+ 0.11%, CD8+ 0.21%) and pp72 peptides (CD4+ 0.08%, CD8+ 0.20%). Of note, CMV-lysate induced CD4+ T-cell responses were higher than respective CD8+ T-cell responses (Wilcoxon signed ranks test, P=0.018, two-tailed). No significant differences between CD8+ and CD4+ T-cell subsets were found for pp65 responses (P=0.528, two-tailed), while a trend for higher CD8+ responses was observed for pp72 peptides (P=0.051, two-tailed). Overall, the different antigens elicited cellular immune responses as expected for the CMV serostatus of HB. But for some of the CMV seronegative patients, a CMV-specific T-cell response above the technical cut-off of 0.01% was measured. Taken together, CMV-specific T-cell frequencies to CMV-lysate or -pp65 antigens were significantly higher in R+ KT patients than in R- KT patients, but lower than in non-immunosuppressed HB+ (**Figure 1A**).

Table 2: CMV-antigen specific IFN-γ producing CD4+ and CD8+ T-cells in HB and KT patients.

		Healthy donors			Kidney transplant patients		
CMV-antigen	**T-cell**	**HB+ n=13, m=19 median (range)**	**HB- n=17, m=17 median (range)**	**P-value[1]**	**D+R+/D-R+ n=48, m=79 median (range)**	**D+R- n=25, m=50 median (range)**	**P-value[1]**
Lysate	$CD4^+$	1.03% (0.03%-6.19%)	0.01% (0.00%-0.22%)	<0.001	0.05% (0.00%-4.35%)	0.01% (0.00%-0.14%)	<0.001
	$CD8^+$	0.49% (0.00%-4.57%)	0.00 (0.00%-0.56%)	<0.001	0.02% (0.00%-1.31%)	0.00% (0.00%-0.07%)	0.003
pp72	$CD4^+$	0.08% (0.01%-2.83%)	0.00% (0.00%-0.03%)	0.001	0.04% (0.00%-0.36%)	0.02% (0.00%-0.26%)	0.027
	$CD8^+$	0.20% (0.00%-3.47%)	0.00% (0.00%-0.03%)	0.002	0.05% (0.00%-0.68%)	0.03% (0.00%-0.34%)	0.056
pp65	$CD4^+$	0.11% (0.00%-4.70%)	0.00% (0.00%-0.02%)	0.018	0.02% (0.00%-0.50%)	0.01% (0.00%-0.55%)	0.013
	$CD8^+$	0.21% (0.00%-2.80%)	0.00% (0.00%-0.20%)	0.009	0.02% (0.00%-0.62%)	0.00% (0.00%-0.25%)	0.001

Data are % of total $CD3^+$ T-cells (30'000). CMV: Cytomegalovirus, HB: healthy blood donors, KT: kidney transplant, n: number of patients, m: number of measurements. CMV seronegative KT patients had a D+R- constellation. CMV seropositive KT patients included both D+R+ and D-R+ constellations. [1]Significant if P-value <0.05, Mann-Whitney U-test

COMPARING IFNΓ RESPONSES TO DIFFERENT CMV ANTIGENS

CMV-lysate specific CD4+ and CD8+ T-cell responses correlated well with pp65-specific CD4+ and CD8+ T-cell responses (Spearman's rho 0.564, 2-tailed, P<0.001; and Spearman's rho 0.514, 2-tailed, P<0.001, respectively). No correlation was found between pp65- and pp72-specific CD4+ T-cells (Spearman's rho -0.133, 2-tailed, P=0.347), or between CMV-lysate specific and CMV-pp72-specific CD8+ T-cell or CD4+ T-cell frequencies (Spearman's rho 0.091, 2-tailed, P=0.530; and (Spearman's rho -0.263, 2-tailed P=0.065). These results suggest that CMV-lysate and -pp65 responses are largely equivalent in PBMC stimulated T-cell subsets with overlapping specificities of KT patients, while CMV-pp72 seems to stimulate a different T-cell population.

CMV-SPECIFIC CELLULAR IMMUNE RESPONSES IN SEROPOSITIVE KT PATIENTS WITH CMV-REPLICATION

To investigate T-cell responses and their association with antiviral control, we compared the frequency of CMV-specific IFNγ responses in R+ KT patients with and without concurrent CMV-replication. R+ KT patients with concurrent CMV-replication had significantly lower CMV-specific CD4+ and CD8+ T-cell responses compared to R+ KT patients without concurrent CMV-replication (P=0.033; **Figure 1B, Table 3**). We noted two exceptions being CD8+ T-cell responses to CMV-lysate (Mann-Whitney U-test, P=0.189) and CD4+ T-cell responses to pp72-peptides (P=0.291), which seemed to reflect the antigen-specific subpopulation bias described earlier **(Table 3)**. We conclude that the data point to an inverse correlation of CMV-specific T-cell responses and viral replication, which was most consistently captured by the pp65-specific CD4+ T-cell responses.

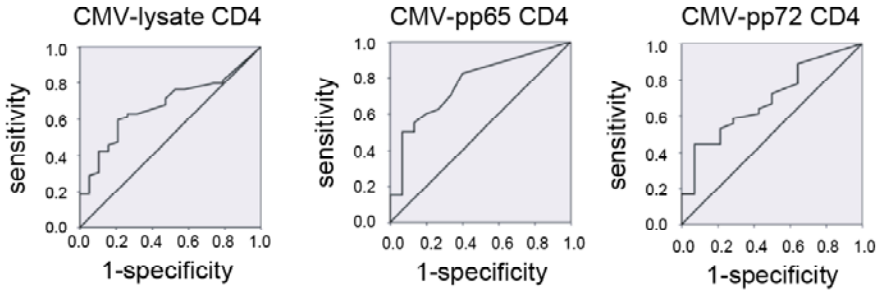
Table 3: CMV-antigen specific IFN-γ producing CD4+ and CD8+ T-cells of R+ KT patients with or without concurrent CMV replication.

CMV-antigen	T-cell	CMV replicating (n=6/48) median (range)	CMV non-replicating (n=42/48) median (range)	P-value[1]
Lysate	CD4+	0.02% (0.00%-0.38%)	0.08% (0.00%-4.35%)	0.011
(m=79)	CD8+	0.01% (0.00%-0.21%)	0.02% (0.00%-1.31%)	0.189
pp72	CD4+	0.04% (0.00%-0.22%)	0.04% (0.00%-0.36%)	0.291
(m=50)	CD8+	0.03% (0.00%-0.24%)	0.07% (0.00%-0.68%)	0.019
pp65	CD4+	0.00% (0.00%-0.14%)	0.03% (0.00%-0.50%)	0.001
	CD8+	0.01% (0.00%-0.21%)	0.03% (0.00%-0.62%)	0.033

Data are % of total $CD3^+$ T cells (30'000). CMV: cytomegalovirus, R: recipient, n: number of patients, m: number of measurements. CMV seropositive KT patients included both, D+R+ and D-R+. [1]Significant if P-value <0.05, Mann-Whitney U-test.

To identify an IFNγ T-cell frequency associated with protection from CMV-replication, we conducted ROC analyses. For CMV-lysate, IFNγ-positive CD4+ T-cell frequencies of >0.1% **(Figure 2)** were associated with absence from concurrent CMV-replication. For CD8+ T-cells, frequencies of >0.09% were indicated, but the area under the curve (AUC) was only 0.566 (specificity: 95%, sensitivity: 25%, Fisher exact test P=0.05) For CMV-pp65, the respective threshold values for CD4+ **(Figure 2)** and CD8+ T-cells were >0.03% (AUC

CD8+: 0.659, specificity: 80%, sensitivity: 55%, Fisher exact test P=0.04). For CMV-pp72, the cut-offs for CD4+ was >0.07% (Figure 3) and CD8+ T-cells was >0.09% (AUC CD8+: 0.69 specificity: 93%, sensitivity: 45%, Fisher exact test P=0.011), respectively. Thus, CMV pp65-specific responses were lower than the ones observed for CMV-lysate or -pp72, but provided a best discrimination as captured by the highest ROC AUC.



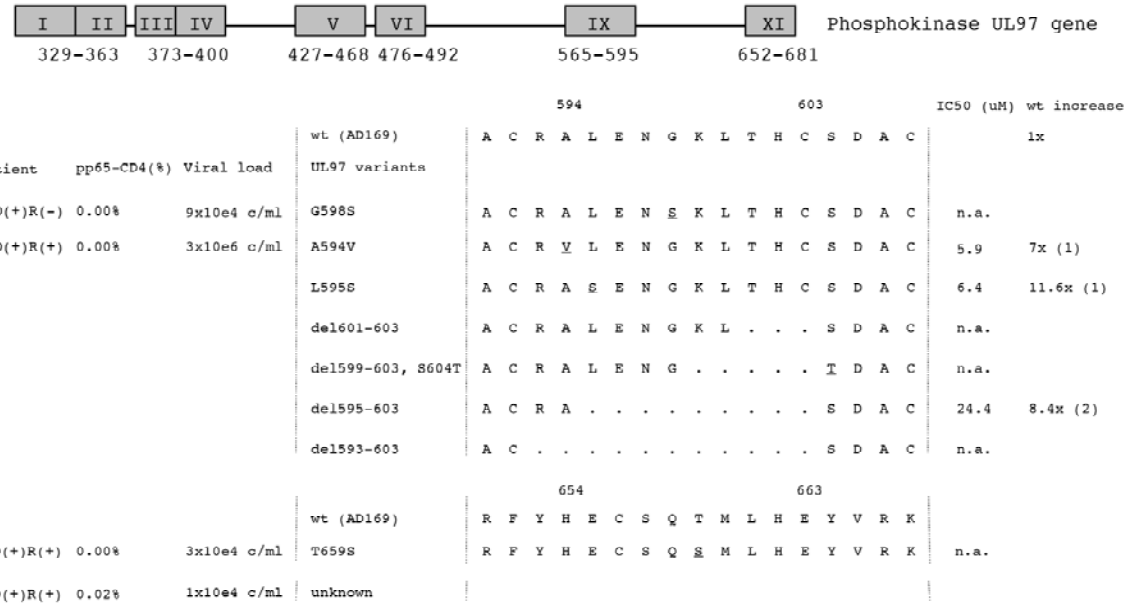
AUC	0.675	0.765	0.69
Threshold	0.1%	0.03%	0.09%
Sensitivity	45%	50%	45%
Specificity	84%	93%	93%
p-value	0.020	0.003	0.011

Figure 2: ROC curve analysis - CMV-specific CD4+ T-cell frequency protecting from concurrent CMV replication. ROC curves show CD4+ T-cell thresholds which protects from concurrent CMV-replication for different CMV-antigens tested.
AUC, Area under the ROC curve; P-value<0.05 is significant, Fisher exact.

To investigate the degree of protection for subsequent CMV-replication episodes, we tested occurrence of CMV-replication during the follow-up period of 3 months. Seven of 43 R+ patients without CMV-replication at the initial time-point of measurement had subsequent CMV-replication at a median 8 weeks later (range: 6 to 56 weeks) (**Table 1**) and pp65-specific CD4+ T-cell frequencies were significantly lower compared to R+ without subsequent CMV-replication (median 0.01% vs. 0.03%, Mann-Whitney U-test P=0.021). Using the threshold of 0.03% for pp65-specific CD4+ T-cell frequencies, a level >0.03% was correlated with protection from subsequent CMV-replication (<2 months, AUC: 0.763, specificity: 100%, sensitivity: 47%, Fisher exact test: P=0.036). We could not identify a similar cut-off for pp65-specific CD8+ T-cell responses or any of the CMV-lysate or -pp72 responses (data not shown).

GANCICLOVIR-RESISTANCE AND CMV-SPECIFIC CELLULAR IMMUNE RESPONSES

Among the patients studied, one of 25 D+R- KT patients (4%) developed clinical resistance to GCV treatment which was confirmed by the identification of the CMV UL97 mutation G598S, in line with the data from the literature (Limaye et al. 2000). In R+ KT patients, however, clinical resistance to GCV-treatment was identified in 3 out of 48 (6.25%) cases, which is significantly higher than previously noted (Limaye et al. 2000). In these R+ patients, CMV-specific T-cells IFNγ responses were <0.03% for CMV-pp65 antigens. The data and genotype of the CMV-UL97 sequences are summarized including 6 variants that were detected in one patient with three novel in frame deletions (**Figure 3**).



Patient	pp65-CD4(%)	Viral load	UL97 variants	594 603	IC50 (uM)	wt increase
			wt (AD169)	A C R A L E N G K L T H C S D A C		1x
1 D(+)R(-)	0.00%	9x10e4 c/ml	G598S	A C R A L E N S K L T H C S D A C	n.a.	
2 D(+)R(+)	0.00%	3x10e6 c/ml	A594V	A C R V L E N G K L T H C S D A C	5.9	7x (1)
			L595S	A C R A S E N G K L T H C S D A C	6.4	11.6x (1)
			del601-603	A C R A L E N G K L . . . S D A C	n.a.	
			del599-603, S604T	A C R A L E N G . . . . . T D A C	n.a.	
			del595-603	A C R A . . . . . S D A C	24.4	8.4x (2)
			del593-603	A C . . . . . S D A C	n.a.	
				654 663		
			wt (AD169)	R F Y H E C S Q T M L H E Y V R K		
3 D(+)R(+)	0.00%	3x10e4 c/ml	T659S	R F Y H E C S Q S M L H E Y V R K	n.a.	
4 D(+)R(+)	0.02%	1x10e4 c/ml	unknown			

Figure 3: Identified UL97 mutated variants. Boxes indicate gene clusters in phosphokinase UL97 gene and show mutations found in 70% of GCV resistant clinical isolates. The entire UL97 region was sequenced (amino acids 329-681). Letters indicate amino acids; underlined letters are changed amino acids due to gene sequence mutations. GCV-inhibitory concentrations 50% (IC50) associated with mutations are listed as uM and -fold increase over wild type (wt) AD169 (1) or Towne (2) strains.

DISCUSSION

Clinical studies have linked the absence of CMV-specific T-cells in SOT recipients to an increased risk of CMV replication and disease. This is most obvious for CMV D+R- SOT patients (Paya et al. 2004). CMV R+ patients may also develop CMV-replication and disease, albeit at lesser frequencies (Sester et al. 2001; Bunde et al. 2005; Sester et al. 2005) which might reflect differences in CMV-specific immunity. Our systematicly comparison of CMV-lysate, -pp65 and -pp72 responses in one of the largest number of R+KT patients indicated quantitative and qualitative differences in the response profiles. First, CMV-lysate responses were higher than the responses elicited by overlapping 15-mer peptide pools covering pp65 or pp72. Contribution of alloresponses to fibroblast cells could not account for this difference as since responses to non-infected fibroblast lysate were generally low and always subtracted from the individual CMV-lysate responses. More likely, and inline with the most recent results by Gerna and colleagues (Lilleri et al. 2007), these quantitative differences may reflect the wider range of CMV-antigens contained in CMV-lysate preparations compared to peptides restricted to pp65 or pp72. Second, CMV-lysate favored CD4+ T-cell responses, whereas CMV-pp72 peptides favored CD8+ T-cell responses. The preferential CD4+ response to CMV-lysate has been reported previously by Sester and collaborators (Sester et al. 2001; Sester et al. 2002) and may result from uptake, processing and preferential presentation of CMV-lysate antigens in an MHC-class II context. CMV derived 15mer peptides might be more eligible for direct binding and processing to 8- to 10mer peptides on MHC-class I molecules. However, IFNγ responses to pp65-derived CMV 15mers appeared equally frequent in both CD4+ and CD8+ T-subpopulations. This difference between pp72 and pp65 suggests that sequence-encoded differences in epitopes and processing are likely to be operating as well. We conclude that in our series, overlapping peptides covering pp65 provided the best

stimulus to induce IFNγ-responses in both CD4+ and CD8+ subpopulations to assay directly for CMV-specific T-cells in an HLA-independent clinical setting.

Regarding the risk of CMV viremia, we found that R+ KT patients with concurrent CMV-replication had significantly lower CMV-specific T-cell frequencies in PBMC than R+ KT patients without concurrent CMV-replication. ROC curve analysis indicated that pp65-specific CD4+ T-cells frequencies of >0.03% were associated with absence of CMV-replication, with higher AUC than respective thresholds for pp72 or CMV-lysate. Although CD8+ T-cells are known to be required to terminate CMV replication, pp65-directed T-cell responses may reflect long-term CMV-protection. By contrast, it can be speculated that pp72-specific responses operate in short-term replication clearance mediated by CMV-specific CD8+ T-cells, but this hypothesis requires further study.

Previous studies identified a correlation of >0.25% CMV-lysate specific CD4+ T-cells with the absence of CMV disease in patients early after transplantation in long-term kidney and heart transplant recipients (Sester et al. 2001; Sester et al. 2005). For CMV-pp72, CD8+ T-cell frequencies above >0.4% at any time during the first months after lung and heart transplantation has been associated with a decreased risk of CMV disease, but not with CMV replication (Bunde et al. 2005). An important caveat in defining thresholds resides in the dynamic aspect of the virus – host balance, which is exquisitely sensitive to changes in immunosuppressive treatments, particularly anti-rejection treatment. In patients with concurrent CMV replication, it cannot be decided whether a lower number of CD4+ T-cells is the cause or the consequence of CMV-replication. Clearly, positive CMV PCR in blood identifies patients at higher risk for CMV-associated complications, and CMV-replication dynamics are helpful to predict the further course (Mattes et al. 2005; Funk 2007). However, we found that frequencies above the threshold of 0.03% were predictive of CMV viremia-free time for the following 8 weeks with a specificity of >93%. This observation may have implications in the specific situation where negative CMV PCR results are difficult to interpret with regard to future risk. Our data suggest that the detection of CMV-pp65 specific CD4+ T-cell frequencies of >0.03% might indicate a reduced risk of future CMV replication in stable KT patients (Dickenmann et al. 2001; Dickenmann et al. 2004). With this limitation in mind, CMV-specific CD4+ T-cells might serve as a dynamic marker of protection when >0.03% and, if validated in future studies, may be useful to complement CMV load diagnostics.

Lack of CMV-specific immunity has been associated with an increased risk of GCV-resistance CMV-replication (Limaye 2002). Although the improved bioavailability of ValGCV appears as a significant safeguard against GCV-resistance, outpatients with changing renal function are at an increased risk of suboptimal dosing and emergence of antiviral resistance under oral antivirals. In our study, clinical GCV-resistance as defined by Preiksaitis et al (Preiksaitis et al. 2005) occurred in 1/25 (4%) D+R- KT patients, at a rate comparable with the study by Limaye and colleagues (Limaye et al. 2000). By contrast, the frequency of GCV-resistance in our R+ KT patients was significantly higher as described previously with 3/48 (6.25%). In three of four cases, we confirmed clinical CMV-resistance genotypically by identifying mutations in the CMV-UL97 encoding phosphotransferase mutated variants. UL97 mutations have been reported rarely in R+ SOT patients to date, but not in KT patients yet (Lurain et al. 2002; Boivin et al. 2005). Among UL97 mutations, A594V and L595S was identified in 30% and 13.3% of all cases, respectively, whereas T569I and G598S mutations were rare (Chou et al. 2002; Comoli et al. 2002; Gilbert et al. 2002; Lurain et al. 2002; Gilbert et al. 2005). Intermittently negative CMV-loads have been observed in these patients, yet pp65-specific CD4+ T-cell frequencies remained <0.03% over prolonged periods. Although a low CMV-specific CD4+ T-cell response cannot be used as exclusive parameter to predict the

emergence of GCV-resistance in R+ KT patients, its use as risk factor may warrant further investigations.

The limitations of our study are the cross-sectional approach and the relatively small sample size of KT patients. In addition, variations associated with the laboratory techniques may preclude the adoption of our threshold values by other institutions without further standardization. Nevertheless, our data were obtained from studying KT patients in the routine clinics and therefore may warrant further studies in larger, preferably prospective settings. In summary, monitoring of CMV-specific T-cell frequencies may identify R+ KT patients at risk for CMV-replication and resistance. Provided further corroborating evidence, thresholds may enter clinical validation and improve decisions concerning duration and dosage of antiviral prophylaxis.

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CURRENT PROJECTS:

PRELIMINARY RESULTS AND OUTLOOK

5. CURRENT PROJECTS

5.1 Influence of Immunosuppressive Drugs Cyclosporine and FK506 on BKV-Specific Cellular Immune Response in Kidney Transplant Recipients

5.2 Epitope Mapping of BKV-Proteins: LT, VP1 and Agno of a HLA-A*01, HLA-A*03, HLA-B*08, HLA-B*51, HLA-DR*19, HLA-DR*11 Kidney Patient with Past Polyomavirus- Associated Nephropathy

5.1 INFLUENCE OF IMMUNOSUPPRESSIVE DRUGS CYCLOSPORINE AND FK506 ON BKV-SPECIFIC CELLULAR IMMUNE RESPONSE IN KIDNEY TRANSPLANT RECIPIENTS

INTRODUCTION

Polyomavirus BK (**BKV**) is the causing agent of polyomavirus associated nephropathy (**PVAN**) in up to 10% of the kidney transplant (**KT**) patients with a graft loss in up to 80% of cases (Hirsch 2005). The increase of incidence for PVAN correlates with the administration of the new potent immunosuppressiva (**IS**) such as FK506 and MMF (Brennan 2004). As no antiviral treatments are available, the only therapy is IS reduction in order to regain BKV-specific immunity for the control of viral replication. We have recently evaluated a cut-off of 69 spot forming units (**SFU**) per million PBMC for BKV-LT specific cellular immune response by ELISpot assay (**ESA**). This cut-off included most of the KT recipients with decreasing or absent plasma BKV loads indicating replication control by BKV-specific immunity (Binggeli et al. 2007). The balance between BKV-specific cellular immune response and control of viral replication may be shifted in favour of viral replication by intense IS. Therefore, adjustment of IS drug levels are crucial for BKV-replication control and have to be evaluated individually for each patient.

Our aim was to establish a system that allowed us to evaluate correlations between IS drug levels and the BKV-specific immune response in KT patients, including dose dependence of IS, especially with regard to the previously defined cut-off of >69 SFU/10^6 PBMC.

STUDY PARTICIPANTS AND METHODS

Forty-two KT patients[3] (median age 54 years, range 21–65) were enrolled from March 3 to October 10, 2006, with written informed consent (Ethics Committee of the Basel Cantons 235/06). Administration of IS drugs (mg/day) and IS drug levels in plasma (ng/ml) are summarized in **Table 1**. According to our previous analysis of plasma BKV load dynamics in KT patients, sampling density and individual fluctuations, we considered plasma BKV load changes of >1.5log10 units as significant for the purpose of this study. KT patients were divided into two groups according to the changes in plasma BKV load at the time of PBMC testing for BKV-specific interferon-g (IFN-γ) production. **Group 1** included all KT patients with increasing plasma BKV loads (n=5) or persisting BKV loads with past BKV loads of >10^4 copies (cp)/mL and <1.5log10 decline (n = 17) at the time of PBMC testing. **Group 2** included KT patients with ongoing BKV viremia at the time of PBMC testing, but declining plasma BKV loads of >1.5log10 decline (n = 13) or past BKV viremia (n = 7) at the time of PBMC testing, the latter with histologically confirmed PVAN at a median of 20 months (range 5–75 months) (Binggeli et al. 2007).

ELISPOT ASSAY (ESA) FOR IFN-γ DETECTION

BKV- and JCV-specific cellular immune responses were determined by measuring IFN-γ secretion upon stimulation of PBMC with viral overlapping peptide pools (**OPP**) in an ESA. For this 96-well multiscreen filter plates (MSIPN, Millipore, Volketswil, Switzerland) were coated with 100 µL (10 µg/mL) coating IFN-γ antibody (1-D1K, Mabtech, Hamburg, Germany) and incubated over night at 4°C.

[3] These patients were the same participants in the study of Binggeli et al. 2007. Data were used to perform first analysis for IS experimental set up.

PBMC were seeded in triplicates at 2.5×10^5/well with or without peptides (2 μg/mL) and at 2.5×10^4/well with *Staphylococcus enterotoxin B* (SEB; SIGMA, 1 μg/mL) as positive control, and incubated over night at 37°C/5% CO_2. Plates were washed five times with PBS/0.05% TWEEN 20 (SIGMA) and incubated for 2 h at room temperature with 100 μL (1 μg/mL) biotinylated detection IFN-γ antibody (7-B6-1 biotin, Mabtech), washed five times and incubated for 1 h at room temperature with 100 μL (1 μg/mL) of Streptavidin ALP (Mabtech). Spots were developed by adding 100 μL of BCIP/NBT (Calbiochem, Luzern, Switzerland) for 10 min. IFN-γ spots were counted by using an ELISpot reader (AID, Büron, Switzerland). The number of spot forming units (**SFU**) 10^6 PBMC per well was calculated from triplicates after subtraction of the negative control.

CMV-pp65 OPP was used in order to establish new protocols as CMV-specific cell frequency is higher than to BKV-specific cell frequency and thus easier to detect.

STATISTICAL METHODS

Nonparametric statistical tests were performed using SPSS (Version-14) to account for sample size and non-normal distribution of data. Comparison of the study groups was performed by the two-sided Mann–Whitney U-Test. Correlations between paired samples were evaluated by the two-sided Wilcoxon signed ranks sum test. Data were expressed either as mean ± standard deviation (**SD**) or as median and range. Correlation between IS and BKV-specific cellular immune responses were calculated using Spearman's rho correlation coefficient (one-sided). Only P-values <0.05 were considered to be statistically significant. Box plots depict the median and the interquartile ranges, outliers are plotted above or below the whiskers.

RESULTS

IS LEVEL IN KT PATIENTS

Table 1: Study participants and immunosuppressive drugs.

	Age years	CsA[3] mg/day	FK506[4] mg/day	MMF[5] mg/day	Sirolimus mg/day	CsA[3] ng/ml	FK506[4] ng/ml	MMF[5] ng/ml	Sirolimus ng/ml
Group 1[1] N=22									
Mean	54	150	5	976	3	224	8	3	5
SD	±14	±50	±3	±441	±2	±186	±2	±2	±2
Median	54	150	5	1000	3	187	8	2	5
Min	21	100	2	360	1	60	5	1	3
Max	72	200	12	2000	4	426	12	6	7
Group 2[2] N=20									
Mean	52	173	3	875	3	116	5	3	5
SD	±12	±77	±2	±358	±1	±41	±2	±2	±2
Median	54	168	2	875	3	136	5	2	5
Min	26	100	2	250	1	61	1	1	3
Max	66	300	8	1500	4	153	8	7	8

[1]Group 1: KT patients with increasing or high stable high viral load, [2]Group 2: KT patients with decreasing viral load or past PVAN, [3]CsA: cyclosporine A, [4]FK506: tacrolimus, [5]MMF: mycophenolate mofetil.

KT patients were divided into two groups: group 1 included patients with an increasing or stable high plasma viral load and group 2 included patients with a decreasing plasma viral load or after resolved PVAN. IS administration in mg/day is listed on the left side of **Table 1** and IS concentration in ng per ml (ng/ml) plasma is listed on the right side of the table.

COMPARISON OF DIFFERENT IMMUNOSUPPRESSIVE DRUGS

Comparison of group 1 (inc/hi) and group 2 (dec) showed no differences in the administration of IS drugs per day (mg/ml) of cyclosporine (**CsA**), tacrolimus (**FK506**), mycophenolate mofetil (**MMF**), and sirolimus. Detection of IS in plasma revealed only for FK506 a significantly higher concentration (**Table 2**).

Table 2: Comparison of group 1 (inc/hi) and group 2 (dec) and IS drug level administered per day (mg/day) and in plasma (ng/ml).

	CsA[1] mg/day	FK506[2] mg/day	MMF[3] mg/day	Sirolimus mg/day	CsA[1] ng/ml	FK506[2] ng/ml	MMF[3] ng/ml	Sirolimus ng/ml
P-value[4]	0.905	0.122	0.670	1.000	0.548	0.005	0.963	0.730

[1]CsA: cyclosporine A, [2]FK506: tacrolimus, [3]MMF: mycophenolate mofetil, [4]P-value: significant if <0.05, Mann-Whitney U-Test, two-sided.

CORRELATION OF IS DRUG LEVEL AND BKV-SPECIFIC CELLULAR IMMUNE RESPONSE

We tested the correlation of IS drug level in plasma and BKV-specific cellular immune response of KT patients by calculating Spearman's rho coefficient (r_s, one-sided). Immune response of BKV-LT and -VP1 was either correlated with CsA, FK506, MMF or sirolimus. Except for BKV-VP1/FK506 (P=0.002), no correlation could be found (**Figure 1**). However, the clustering of group 1 (inc/hi) data points towards the right side of the panel suggested a FK506 cut-off to be critical for BKV-replication control.

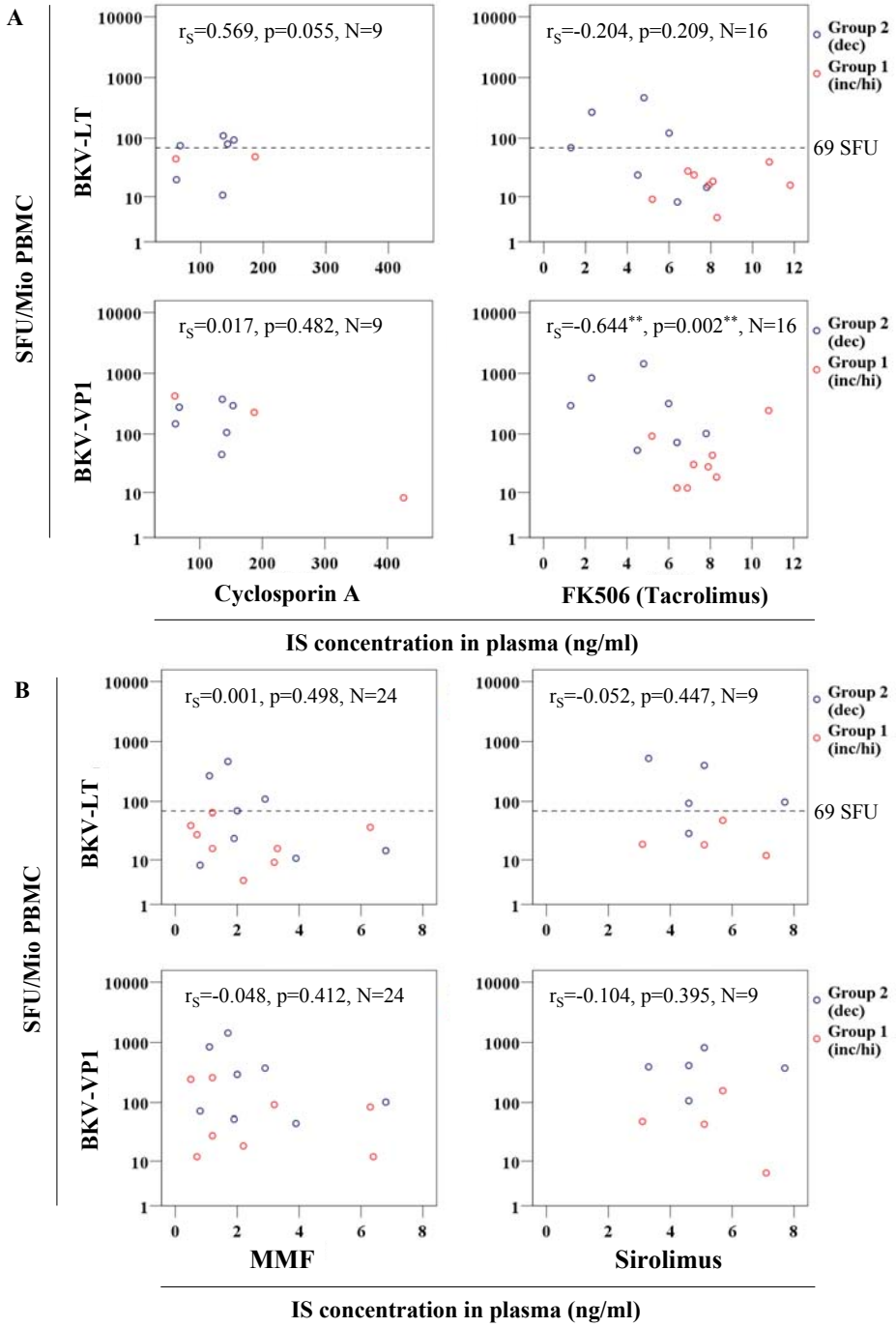

Figure 1: Correlation between different IS drug levels in plasma and BKV-specific cellular immune response to LT and VP1 in KT patients. **A)** Cyclosporine A and tacrolimus, **B)** Mycophenolate mofetil and sirolimus. Patients with an immune response >69 SFU/10^6 PBMC were mostly able to control BKV-replication (Binggeli et al. 2007). Negative values indicate a negative correlation, r_s: Spearman's rho coefficient (one sided), **: P<0.005 (significant). Missing data points are zero.

A ROC-curve analysis revealed a cut-off of 6.65 ng/ml FK506 with a sensitivity of 78% and specificity of 86% (AUC: 0.897). KT patients receiving FK506 (16/42, 14.3%) were divided into two groups: those with <6.65 ng/ml and those with >6.65 ng/ml FK506 concentration in plasma. Patients who with a plasma level of >6.65 ng/ml had a significantly lower BKV-VP1 specific immune response (30 SFU/10^6 PBMC, range 0-243 SFU/10^6 PBMC) than those patients with FK506 plasma level <6.65 ng/ml (193 SFU/10^6 PBMC, range 12-1432SFU/10^6 PBMC) (P=0.038, Mann-Whitney U-Test, two-sided) (**Figure 2**). No difference could be found for BKV-LT (P=0.442). From 16 KT patients receiving FK506, 9 were in group 1 (inc/hi) and 7 were in group 2 (dec). Almost all KT patients with increasing BK-viral load (7/9, 78%) had a >6.65 ng/ml FK506 plasma concentration whereas only one KT patient (1/7, 14%) with decreasing BK-viral load had a FK506 plasma level >6.65 ng/ml.

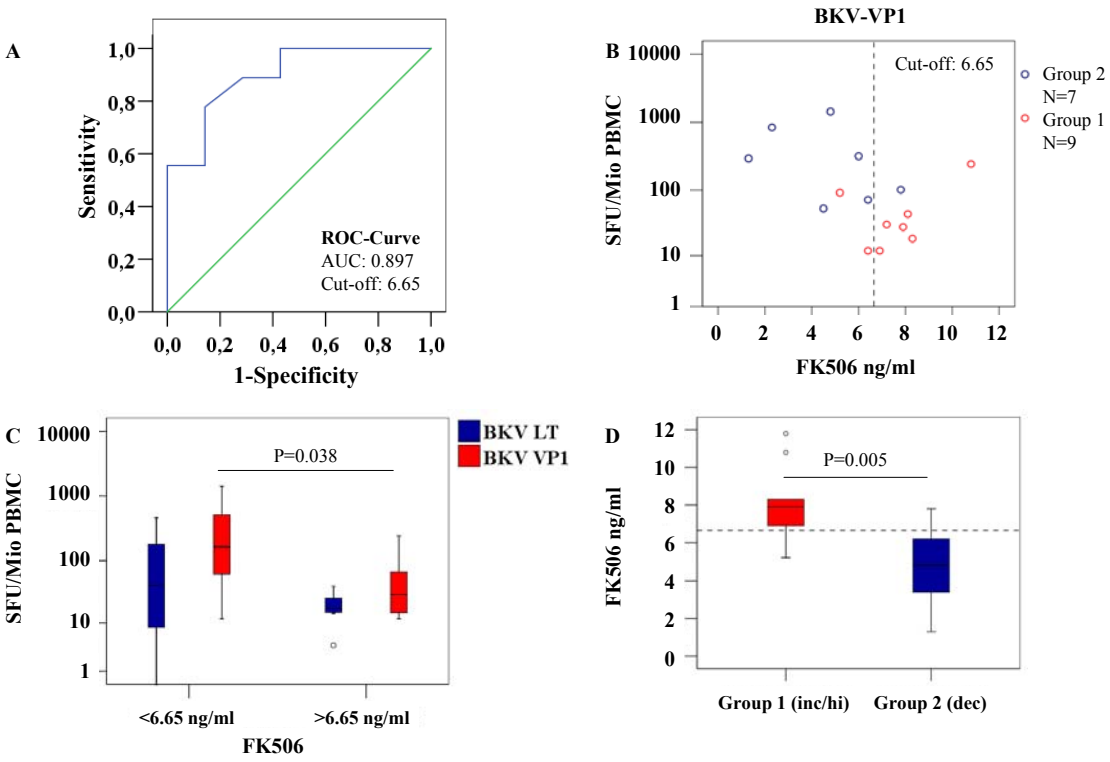

Figure 2: A) ROC-curve analysis: AUC: 0.897 and **B)** cut-off of 6.65 ng/ml for FK506 plasma level. **C)** BKV-VP1 response was significantly lower in patients of group 1 than group 2 (P=0.038). No differences for BKV-LT (P=0.442). **D)** KT patients in group 1 (inc/hi) had a higher FK506 level in blood than patients in group 2 (dec) (P=0.005), Mann-Whitney U-Test, two-sided.

ESTABLISHMENT OF ESA FOR BKV-SPECIFIC CELLULAR IMMUNE RESPONSE MEASUREMENT UNDER INFLUENCE OF IS

Since FK506 seems to correlate with the cellular immune response, we established an ESA for this calcineurin inhibitor. CsA was included for the ESA establishment as it also targets the calcineurin phosphatase. The influence of IS drugs on the cellular immune response was measured by detection of IFN-γ secreting cells upon stimulation of PBMC from KT patients with BKV-LT and -VP1 overlapping peptide libraries. FK506 and CsA were added to PBMC in a serial dilution.

For the establishment of the method, following tests had to be performed: 1. IS serial dilution, 2. Precoating vs. non precoating of the ELISpot membrane with IS, 3. Prestimulation with antigen vs. non prestimulation, 4. Stimulation with BKV-VP1 in BKV-seropositive HB, and 5. Stimulation of CD4+ or CD8+ depleted PBMC with CMV-pp65.

IS SERIAL DILUTION & PRECOATING

Serial dilution and precoating of ELISpot membrane for 2h with FK506 or CsA was performed in the same experiment. PBMC were stimulated with 2 ug/ml SEB over night. During this time PBMC were incubated with either FK506 (0, 10, 20, 40, 80, 120, 160, or 200 ng/ml) or CsA (0, 250, 500, 1000, 1500, 2000, 2500 or 4000 ng/ml). Results are either given in SFU/10^6 PBMC or in percent for cell reactivity. In general, precoating of ELISpot plates with CsA or FK506 induced a lower immune response compared to ELISpot plates which were not precoated with CsA or FK506 (**Figure 3**). Already a FK506 concentration of 10 ng/ml showed very low cell reactivity (2%).

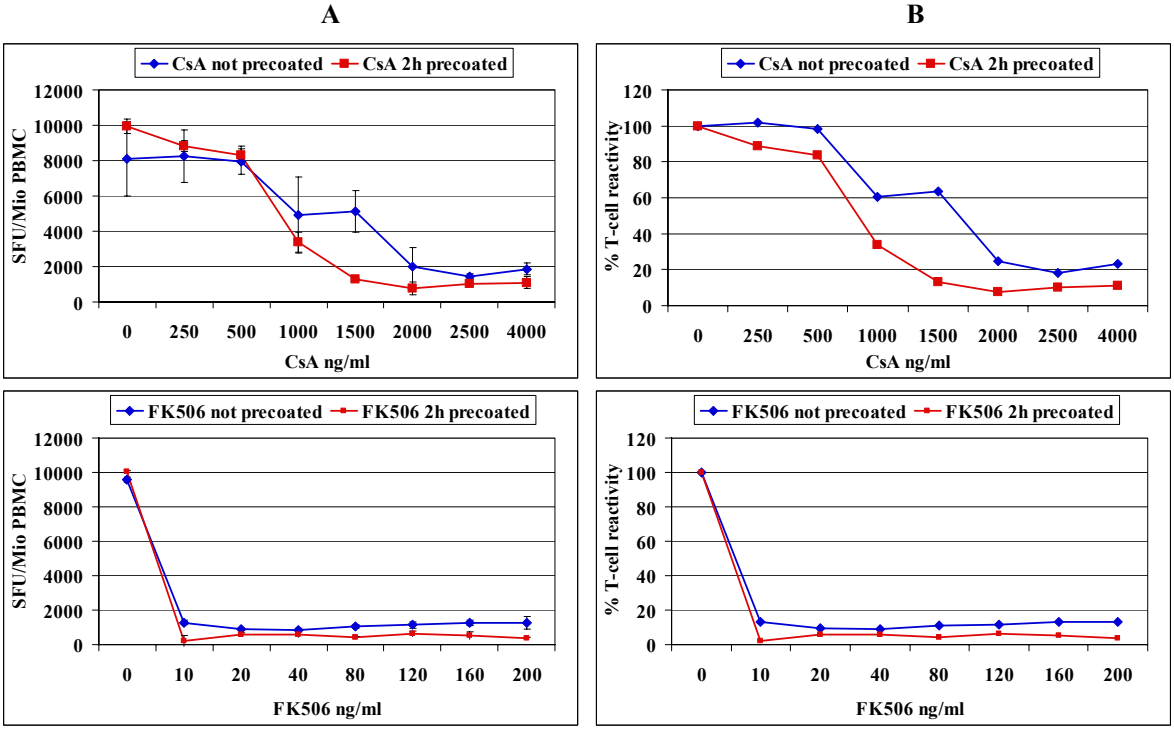

Figure 3: Serial dilution of CsA and FK506. PBMC were stimulated with SEB over night and simultaneously incubated with IS drugs with different concentrations. ELISpot plates were either preincubated with the according IS or added together with the peptides. **A)** SFU/10^6 PBMC for CsA and FK506, **B)** Percent cell reactivity depending on IS drug concentration.

PRESTIMULATION WITH ANTIGEN

As FK506 concentration proved to be too high in the first set-up, the same experiment was repeated in the range of 0-15 ng/ml FK506. Simultaneously, it was tested if immune response to SEB is higher if PBMC were prestimulated for 4h before adding FK506 (no data available for CsA) (**Figure 4**). Immune response increased from 0 ng/ml to 2 ng/ml (2h precoating: 804 SFU/10^6 PBMC - 898 SFU/10^6 PBMC, 0h precoated: 748 SFU/10^6 PBMC - 926 SFU/10^6 PBMC) and decreased to 390 SFU/10^6 (2h precoated) and 192 SFU/10^6 PBMC (0h precoated), respectively. Four hours preincubation with SEB induced a higher response than without preincubation.

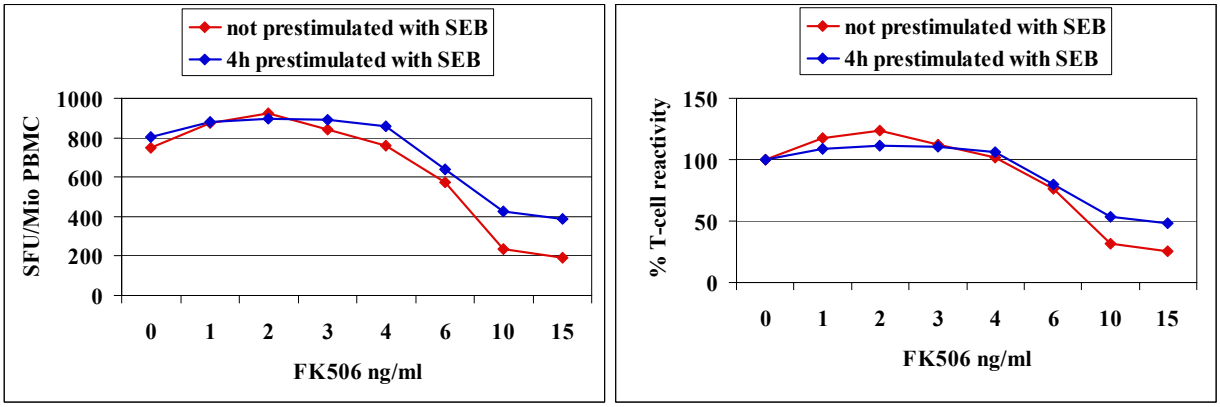

Figure 4: Serial dilution of FK506 with a concentration of 0 - 15 ng/ml. PBMC were either prestimulated for 4h with SEB before FK506 was added (blue line) or SEB was added together with FK506 (red line).

STIMULATION WITH BKV-VP1

The BKV-VP1 specific immune response was tested with following conditions: 2h preincubation of ELISpot plates with CsA or FK506, PBMC stimulation with BKV-VP1 over night without prestimulation. BKV-VP1 specific response decreased from 162 SFU/10^6 PBMC to 42 SFU/10^6 PBMC with increasing CsA concentration (0-3000 ng/ml) and from 83 SFU/10^6 PBMC to 5 SFU/10^6 PBMC with increasing FK506 concentration (0-15 ng/ml) (**Figure 5**).

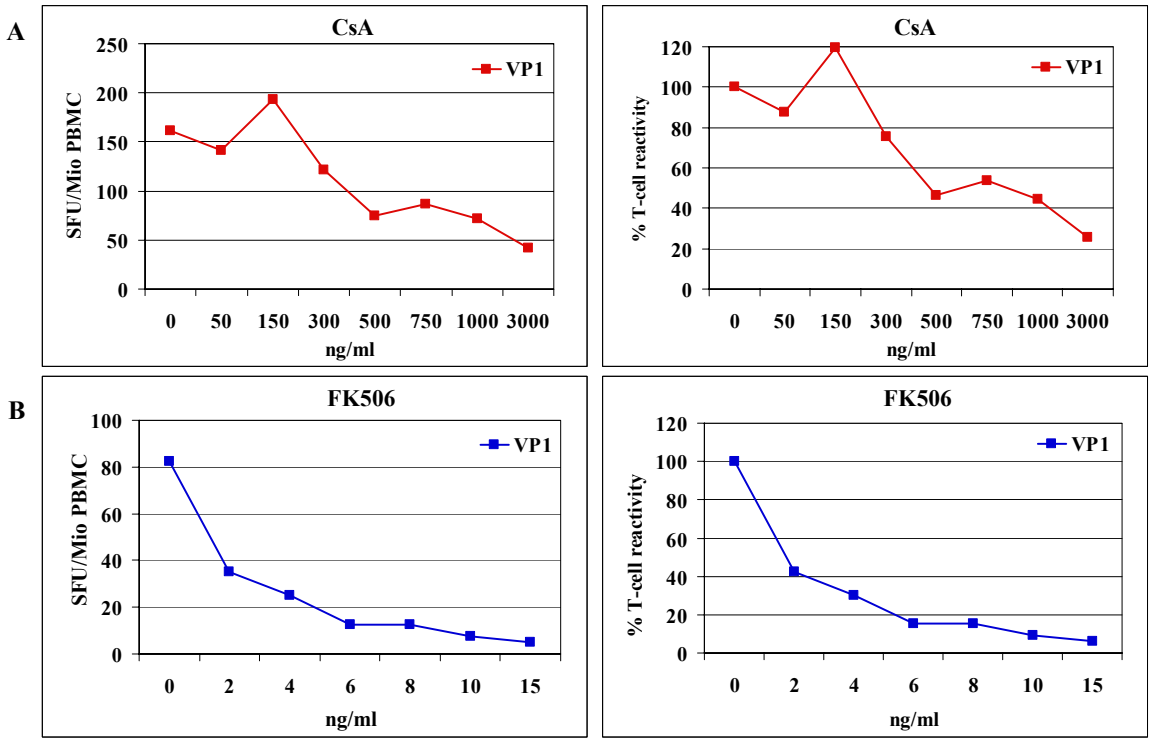

Figure 5: Serial dilution of **A)** CsA and **B)** FK506. PBMC were stimulated with BKV-VP1 over night and simultaneously incubated with IS drugs with different concentrations.

CD4+ AND CD8+ DEPLETED PBMC

To distinguish between CD4+ and CD8+ T-cell derived IFN-γ response, PBMC were depleted by MACSbeads and then stimulated with CMV-pp65. Additionally not depleted (total) PBMC were also stimulated. IFN-γ secretion of total PBMC was highest (CsA: 856 SFU/10^6 PBMC, FK506: 770 SFU/10^6 PBMC) followed by PBMC without CD8+ (blue line; CsA: 420 SFU/10^6 PBMC, FK506: 418 SFU/10^6 PBMC) and PBMC without CD4+ (red line; CsA: 94 SFU/10^6 PBMC, FK506: 90 SFU/10^6 PBMC). Cell reactivity was similar for total PBMC, PBMC CD4- and PBMC CD8- (**Figure 6**).

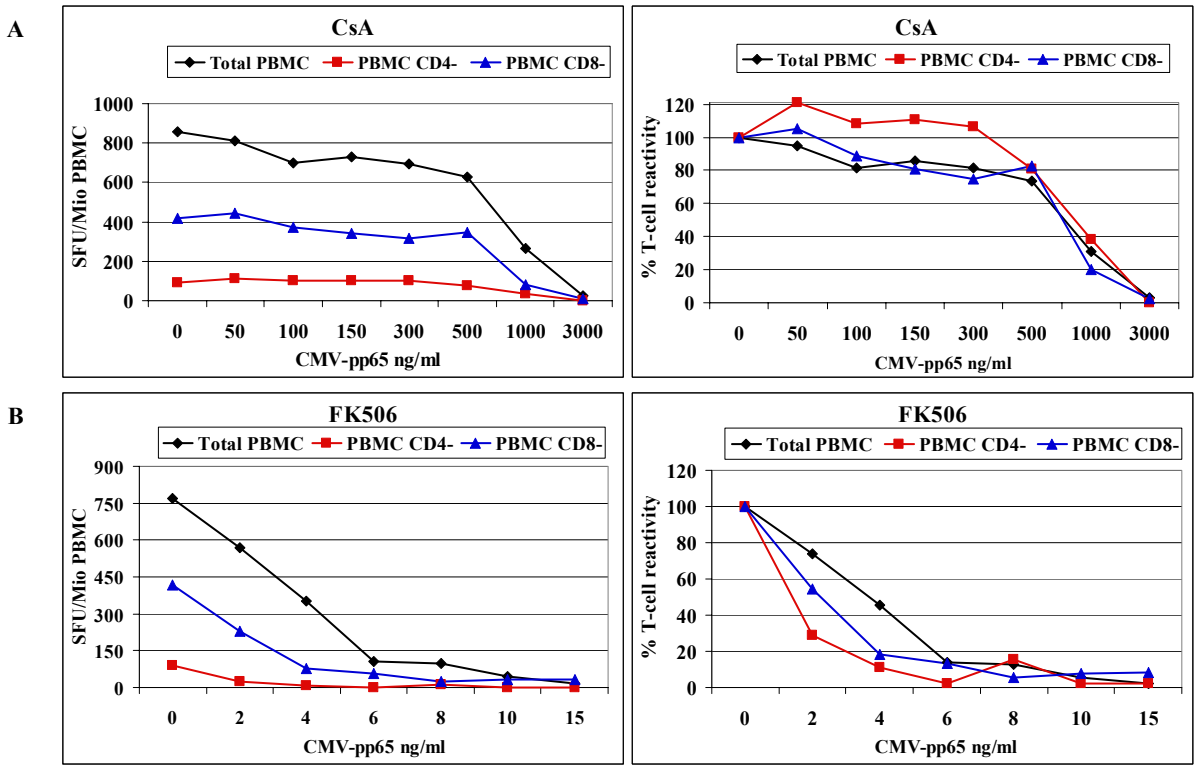

Figure 6: CD4+ (red line) and CD8+ (blue line) depleted PBMC. Immune response of total PBMC (black line) was highest for both, CsA and FK506 treated cells. CD4+ depleted PBMC immune response was lowest for CsA and FK506.

FINAL PROTOCOL

Taking all results into account, following protocol can be followed to test the influence of IS on BKV-specific cellular immune response: 1. Preincubate ELISpot plates for 2h with either CsA or FK506, 2. Add PBMC together with the desired antigen and incubate over night. The next day, wash and develop ELISpot plates (**Figure 7**). BKV-LT and -agnoprotein have not been tested yet and remain open for establishment.

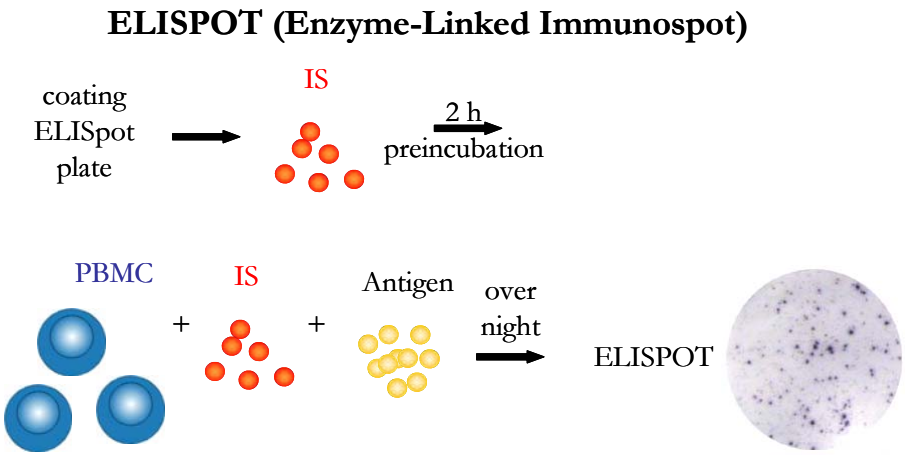

Figure 7: ELISpot plates are first incubated with CsA or FK506 for 2h and then PBMC together with the antigen are added and incubated over night. Next day the ELISpot plate can be washed, developed and analyzed.

DISCUSSION

Immunosuppression is crucial for antirejection treatment in the transplant setting. Unfortunately, one of the side effects might be loss of viral-replication control. It is difficult to find the optimal immunosuppressive dose level for each patient, and the optimal dose also varies over time as drug concentration in the first months posttransplantation is highest and is decreased afterwards.

We hypothesize a negative correlation for IS and immunity. In our study we could show that FK506 plasma concentration had a significantly negative correlation with the BKV-VP1 specific immune response (r_s=-0.644, P=0.002). Sester and colleagues recently reported that a higher drug level of CsA and FK506 correlated with an increased incidence of CMV-replication and -complication not only in KT-patients but also in lung transplant recipients (Sester et al. 2005).

Our study is by far not completed and tests with other immunosuppressants like MMF and sirolimus have to be performed to evaluate the influence of each drug on the BKV-specific immune response. Furthermore, the preliminary cut-off of 6.65 ng/ml FK506 plasma level has to be confirmed by a larger study cohort and may serve as a marker for therapeutical decisions.

5.2 EPITOPE MAPPING OF BKV-PROTEINS: LT, VP1 AND AGNO OF A HLA-A*01, HLA-A*03, HLA-B*08, HLA-B*51, HLA-DR*19, HLA-DR*11 KIDNEY PATIENT WITH PAST POLYOMAVIRUS- ASSOCIATED NEPHROPATHY

INTRODUCTION

Virus-specific cellular immunity is crucial for the control of viral-replication and the prevention of disease (Redpath et al. 2001). Latent viruses like Epstein Barr Virus (**EBV**), cytomegalovirus (**CMV**), and polyomaviruses BK and JC (**BKV, JCV**) require continual surveillance by the immune system. Certain epitopes of viral peptides loaded on MHC class I and class II are recognized by CD8+ and CD4+ T-cells, respectively. Upon recognition, CD4+ and CD8+ T-cells have different modes of action: while CD4+ T-cells activate either macrophages (Th1-cells) or B-cells (Th2-cell), CD8+ T-cells kill the infected cells by releasing perforin and granzymes (Kagi et al. 1996; Edwards et al. 1999; Barry et al. 2000).

Viral epitopes recognized by T-cells may serve for adoptive T-cell transfer (**Figure 1**) and treatment of chronic viral infections, virus-mediated diseases, and virally-induced cancers. Adoptive T-cell transfer remains as a therapy option particularly for viruses resistant to antivirals (Walter et al. 1995; Heslop et al. 1996).

Polyomavirus BK may cause polyomavirus-associated nephropathy (**PVAN**) in up to 10% of kidney transplant (**KT**) patients with graft loss in up to 80% of the cases (Hirsch 2005). Antiviral treatment is not available and adoptive T-cell transfer may serve as an alternative therapeutical option. *Ex vivo* expansion of BKV-specific CD8+ T-cells was recently shown to be efficient in lysing BKV-infected cells (Comoli et al. 2003). In the last five years, several BKV- and also JCV-epitopes from early protein large T (**LT**) -antigen and viral capsid protein 1 (**VP1**) were published for different HLA-types (**Table 1**).

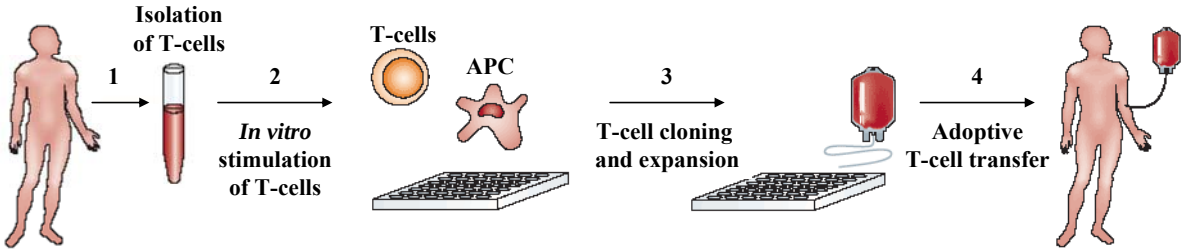

Figure 1: Adoptive T-cell transfer. 1. Blood drawing and T-cell isolation, 2. *In vitro* stimulation of T-cells with antigen pulsed dendritic cells, 3. T-cell clonal expansion over several weeks, and 4. Adoptive T-cell transfer into the patient.
Adapted from (Bleakley et al. 2004)

The aim of our study was to perform epitope mapping of BKV-LT, -VP1, and -agnoprotein for the most common HLA-type A*01-HLA-B*08 (Mori et al. 1997). For this we used blood samples from a kidney patient with past PVAN and HLA type A*01 and B*08. We used the computer program SYFPEITHI to predict BKV-LT, -VP1 and -agnoprotein epitopes for HLA-type A*01, A*03, and B*08 in order to compare these epitopes with those found by *in vitro* epitope mapping. As BKV and JCV show high DNA and protein homology (**Table 2**), JCV-LT, -VP1 and -agnoprotein homologous peptides were also tested *in vitro*.

Table 1: BKV- and JCV-epitopes recognized by T-cells.

Virus	Peptide	Length	Sequence	HLA-Type	Reference
BKV	**LT_{25}**	15aa	GNLPLMRKAYLRKCK	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{27}	9aa	LPLMRKAYL	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{362}	9aa	MLTERFNHIL	HLA-A*02	(Randhawa et al. 2006)
	LT_{406}	9aa	VIFDFLHCI	HLA-A*0201	(Provenzano et al. 2006)
	LT_{410}	9aa	FLHCIVFNV	HLA-A*0201	(Provenzano et al. 2006)
	LT_{553}	15aa	IYLRKSLQNSEFLLE[1]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{557}	15aa	KSLQNSEFLLEKRIL[2]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{579}	9aa	LLLIWFRPV	HLA-A*0201	(Provenzano et al. 2006)
	LT_{613}	15aa	TFSRMKYNICMGKCI[3]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	$VP1_{p44}$	9aa	AITEVECFL[4]	HLA-A*0201	(Chen et al. 2006)
	$VP1_{p108}$	9aa	LLMWEAVTV[5]	HLA-A*0201	(Chen et al. 2006)
JCV	**LT_{552}**	15aa	AYLRKSLSCSEYLLE[1]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{576}	15aa	KSLSCSEYLLEKRIL[2]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	LT_{612}	15aa	TFSTMKANVGMGRPI[3]	HLA-B*0702 HLA-B*08	(Li et al. 2006)
	$VP1_{p36}$	9aa	SITEVECFL[4]	HLA-A*0201	(Du Pasquier et al. 2003)
	$VP1_{p100}$	9aa	ILMWEAVTL[5]	HLA-A*0201	(Koralnik et al. 2002)

[1-5] *BKV and JCV homologous peptides*

Table 2: BKV and JCV DNA and protein sequence homology.

	LT-antigen	VP1	Agnoprotein
DNA	78%	75%	72%
Protein	83%	78%	59%

[1]*Clustal W pairwise alignment*

PILOT STUDY: PARTICIPANTS AND METHODS

STUDY PARTICIPANTS

A 66 year old male kidney patient, with HLA-type: A*01, A*03, B*08, B*51, DR*11, DR*15 was enrolled for this study. The reason for kidney failure is of unknown etiology but might be associated with the use of phenacetin. Kidney transplantation occurred after four months of hemodialysis. First evidence of BKV-replication was seen 2 months posttransplantation with the detection of decoy cells in urine (502/10 HPF[4]) and viral load in plasma (85'660 DNA copies/ml). Definitive PVAN was confirmed by kidney biopsy 5 months after transplantation (plasma viral load: 13'116'320 copies/ml). Nephrectomy occurred 22 months posttransplantation and the patient returned to hemodialysis.

As a control group served 22 HLA-typed healthy blood donors (**HB**) from the Blutspendezentrum in Basel, Switzerland (**Table 3**) which shared only one HLA-type with the KT patient.

[4] hpf: high power fields

Table 3: HLA-type of HB (numbers in red indicate the same HLA-type as for the patient).

	HLA Type			
No.	**HLA-A**	**HLA-A**	**HLA-B**	**HLA-B**
1	1	28	35	37
2	3	0	35	0
3	2	68	51	60
4	3	32	21	35
5	1	35	5	0
6	2	68 (28)	8	44 (12)
7	2	29	51	44
8	1	26	60	65
9	3	0	5	7
10	1	24 (9)	7	62 (19)
11	3	11	7	35
12	24	32	51	60
13	11	28	51	53
14	3	68(28)	14	35
15	2	26(10)	8	65(14)
16	3	10	5	7
17	3	26	7	39
18	1	2	62(15)	57(17)
19	1	11	27	57(17)
20	1	2	7	62
21	25(10)	-	8	-
22	24	0	8	13

COLLECTION OF BLOOD CELLS AND PLASMA

PBMCs of the patient and of the HLA-typed HB were isolated using CPT™ tubes (Becton Dickinson, Allschwil, Switzerland). They were washed, counted, and either used directly for IFN-γ ELISpot (**ESA**), for antigen stimulation and 9-day culture, or cryopreserved in 10% DMSO/90% FCS (SIGMA, Buchs, Switzerland). PBMC for ESA were diluted in RPMI/5% human serum/1% glutamax/1% penicillin streptomycin (**R5AB**, SIGMA).

BKV AND JCV OVERLAPPING PEPTIDE POOLS

Overlapping peptide pools (**OPP**) spanning either large LT, VP1 or agnoprotein coding sequences from the BKV-Dunlop strain and from the JCV-Mad 1 strain were used for PBMC stimulation. The pools consisted of 180 (BKV-LT), 88 (BKV-VP1), 14 (BKV-agnoprotein), 170 (JCV-LT), 86 (JCV-VP1), and 15 (JCV-agnoprotein) peptides of 15 amino acids (**aa**) in length with an overlap of 11aa (Eurogentec, Germany).

COMPUTER PREDICTION SYFPEITHI

The computer prediction program SYFPEITHI (www.syfpeithi.de) was used to predict epitopes for BKV-LT antigen, -VP1, and -agnoprotein for HLA-A*01, HLA-A*03, and HLA-B*07. No information was available for HLA-B*51, HLA-DR*11, and HLA-DR*15. SYFPEITHI T-cell epitope prediction is based on published motifs and considers the amino acids in the anchor and auxiliary anchor position. The score is calculated as following: amino acids of a certain peptide are given a specific value depending on their anchor position. Ten points are given for ideal anchors, 6-8 points for unusual anchors, 4-6 for auxiliary anchors and 1-4 for preferred residues. Negative effect on the binding ability has a negative value between -1 and -3 points. The maximal achievable score is 36 (for comparison, the very well-known epitope GILGFVFTL from influenza A scores 30).

EPITOPE MAPPING STRATEGY

Single peptides were pooled in two ways: 1. The "complete pool" containing all peptides from one protein (BKV-LT; 180 peptides/pool, BKV-VP1; 88 peptides/pool, BKV-agnoprotein; 14 peptides/pool, and JCV-LT; 170 peptides/pool, JCV-VP1; 86 peptides/pool, JCV-agnoprotein; 15 peptides/pool), 2. "Subpools" containing 10 LT- or VP1-peptides/pool (**Figure 2**). As agnoprotein contains only 14 peptides for BKV and 15 for JCV peptides, these peptides were therefore tested individually and not in subpools. For BKV-LT 38 subpools and for BKV-VP1 19 subpools were tested in total.

PBMC were first stimulated with BKV-peptide subpools for CD4+ T-cell mapping. Then we analyzed them further for single peptide activity. CD8+ T-cells were isolated from PBMC and mapped separately by stimulation with peptide loaded dendritic cells. Then, we tested single peptides to determine the minimal required peptide length (**Figure 3**).

BKV-LT 10x10 Pool										
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										
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 10x10 Pool										
POOL	1	2	3	4	5	6	7	8	9	10
A	1	2	3	4	5	6	7	8	9	10
B	11	12	13	14	15	16	17	18	19	20
C	21	22	23	24	25	26	27	28	29	30
D	31	32	33	34	35	36	37	38	39	40
E	41	42	43	44	45	46	47	48	49	50
F	51	52	53	54	55	56	57	58	59	60
G	61	62	63	64	65	66	67	68	69	70
H	71	72	73	74	75	76	77	78	79	80
I	81	82	83	84	85	86	87	88		

Figure 2: Subpools of BKV-LT and BKV-VP1 containing 10 peptides per pool. Numbers or letters in red indicate subpool name, numbers in black are single peptide numbers (e.g. BKV-LT pool A contains peptides number 89-98).

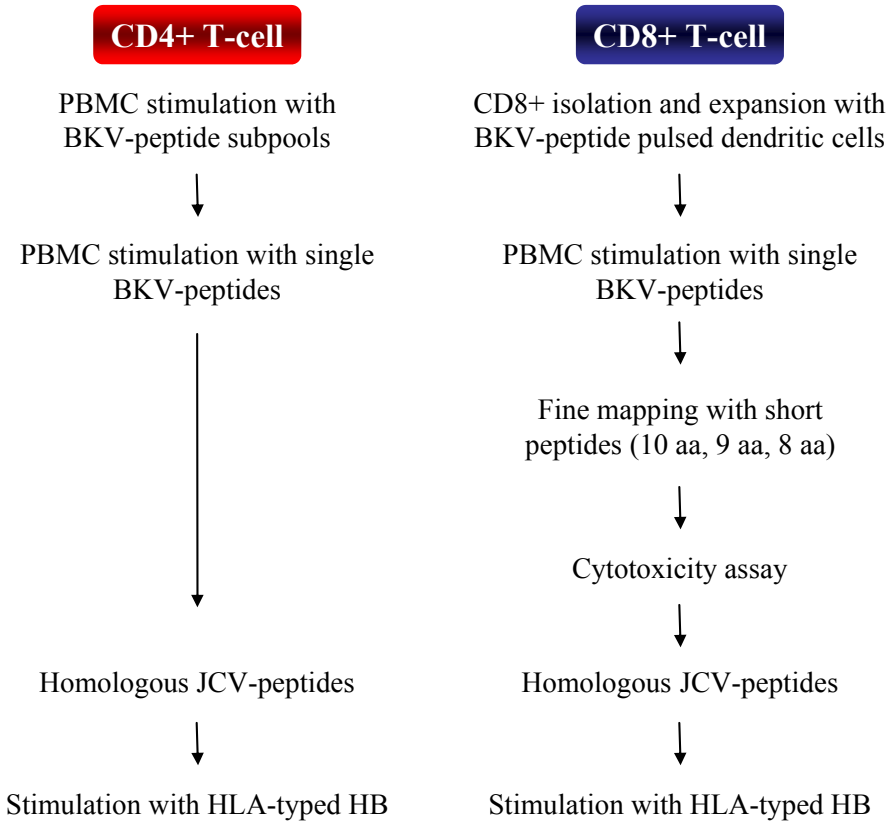

Figure 3: Strategy for epitope mapping. Testing of subpools restricts the number of experiments and saves patient's PBMC. Peptide subpools with a positive response can be further analyzed and tracked to single peptides. The functionality of CD8+ T-cells can be tested by a cytotoxicity assay. Cross-recognition with JCV single peptides has also to be considered.

ESA FOR IFN-γ DETECTION

BKV- and JCV-specific cellular immune responses were determined by measuring IFN-γ secretion upon stimulation of PBMC with viral OPP. For ESA, 96-well multiscreen filter plates (MSIPN, Millipore, Volketswil, Switzerland) were coated with 100 µL (10 µg/mL) of coating IFN-γ antibody (1-D1K, Mabtech, Hamburg, Germany) and incubated over night at 4°C.

PBMC were seeded in triplicates at 2.5×10^5/well with or without peptides (2 µg/mL) and at 2.5×10^4/well with *Staphylococcus enterotoxin B* (SEB; SIGMA, 1 µg/mL) as positive control, and incubated over night at 37°C/5% CO_2. Plates were washed five times with PBS/0.05% TWEEN 20 (SIGMA) and incubated for 2 h at room temperature with 100 µL (1 µg/mL) of biotinylated detection IFN-γ antibody (7-B6-1 biotin, Mabtech), washed five times and incubated for 1 h at room temperature with 100 µL (1 µg/mL) of Streptavidin ALP (Mabtech). Spots were developed by adding 100 µL of BCIP/NBT (Calbiochem, Luzern, Switzerland) for 10 min. IFN-γ spots were counted by using an ELISpot reader (AID, Büron, Switzerland). The number of spot forming units (**SFU**) per 10^6 PBMC was calculated from triplicates after subtraction of the negative control levels.

NINE-DAY EXPANSION OF PBMC

PBMC (2×10^6/mL) were distributed into 24-well plates and incubated over night at 37°C/5% CO_2. Nonadherent cells were recovered, washed and adjusted to 2×10^6 cells/mL. Adherent monocytes were detached by scraping/pipetting, adjusted to 2×10^6 cells/mL and stimulated with 5 µg/mL BKV-OPP for 2 h at 37°C/5% CO_2. Monocytes were washed and 10^5 cells/mL were added to 2×10^6 of nonadherent cells. After 9 days at 37°C/5% CO2, ESA was performed as described above using 10^5 cells/well for peptide restimulation or 2.5×10^4/well for SEB stimulation.

CD8+ AND CD14+ CELL ISOLATION

PBMC (125 Mio/ml) were mixed with 20 ul of MACSbeads conjugated to αCD8 or αCD14 antibodies (Miltenyi Biotec, Germany) and incubated for 20 minutes. CD8+ or CD14+ cells were then isolated by using magnetic separation columns (Miltenyi Biotec). Isolated CD8+ T-cells were resuspended in R5AB (SIGMA) and CD14+ in RPMI/10% fetal calf serum/1% glutamax/1% penicillin streptomycin (**R10**, SIGMA).

DENDRITIC CELL MATURATION AND CD8+ T-CELL EXPANSION

Isolated CD14+ cells (500'000 cells/ml R10) were incubated with 1000 U/ml of IL-4 and 800 U/ml of GM-CSF for 7 days. On day seven 1 µg/ml LPS was added over night for dendritic cell maturation. Maturation was tested by FACS analysis (switch from CD14+ to CD14- and from CD83- to CD83+). Mature dendritic cells (**mDCs**: $5x10^6$ cells/ml) were pulsed with 5 µg/ml OPP and incubated for 2h at 37°C/5% CO_2.

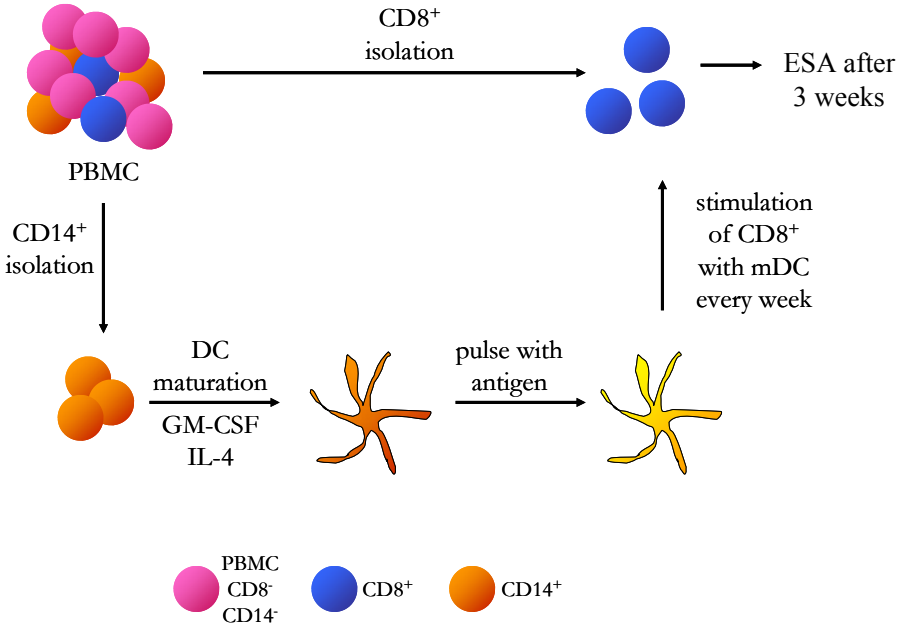

Figure 4: Schematic of CD8+ T-cell expansion. CD8+ and CD14+ cells are isolated from PBMC. CD14+ cells are maturated to dendritic cells and pulsed with viral peptides to stimulate and expand CD8+ T-cells.

After pulsing, mDCs were irradiated for 610 seconds at 2500 rads. Peptide-pulsed mDCs were then added to isolate CD8+ T-cells in a ratio of 1:20. CD8+ T-cells were incubated for 3 weeks and restimulated weekly with fresh peptide-pulsed mDCs (**Figure 4**). CD8+ T-cells response was tested after each week of expansion.

RESULTS

PREDICTIONS WITH SYFPEITHI

BKV-LT, -VP1 and -agnoprotein sequences from the Dunlop strain were analyzed by the SYFPEITHI computer program for potential T-cell epitopes of HLA-A*01, -A*03, and -B*08. Epitopes with a score of ≥20 are listed in **Table 4** and were sorted by BKV-protein, HLA-type, and number of amino acids. In total 110 BKV-LT, 53 BKV-VP1 and 10 BKV-agnoprotein epitopes were found by this method.

Table 4: BKV-LT, -VP1, and agnoprotein epitopes predicted by computer program SYFPEITHI. Peptides with a score ≥20 are listed in the table.

BKV-VP1 (Total 53)

Pos	HLA-A*01 nonamers	score
342	P G D P D M I R Y	27
213	P S R N E N A R Y	20
Total	2	

Pos	HLA-A*01 decamers	score
80	S P E R K M L P C Y	26
191	N T D H K A Y L D K	20
Total	2	

Pos	HLA-A*01 11 - mers	score
159	P L E M Q G V L M N Y	26
276	G T Q Q W R G L A R Y	24
79	D S P E R K M L P C Y	21
313	R T Q R V D G Q P M Y	21
Total	4	

Pos	HLA-A*03 nonamers	score
135	K V H E H G G G K	28
289	I R L R K R S V K	26
26	K L L I K G G V E	25
308	D L I N R R T Q R	25
164	G V L M N Y R S K	24
290	R L R K R S V K N	23
27	L L I K G G V E V	22
8	G E C P G A A P K	21
108	L L M W E A V T V	21
127	L N L H A G S Q K	21
165	V L M N Y R S K Y	21
259	S L Y V S A A D I	21
61	E N L R G F S L K	20
Total	13	

Pos	HLA-A*03 decamers	score
288	K I R L R K R S V K	33
126	M L N L H A G S Q K	30
21	P V Q V P K L L I K	23
26	K L L I K G G V E V	23
107	N L L M W E A V T V	23
164	G V L M N Y R S K Y	22
230	N V P P V L H V T N	21
284	A R Y F K I R L R K	21
331	E V R V F D G T E R	21
340	R L P G D P D M I R	21
128	N L H A G S Q K V H	20
188	Q V M N T D H K A Y	20
244	V L L D E Q G V G P	20
295	S V K N P Y P I S F	20
Total	14	

Pos	HLA-B*08 octamers	score
3	P T K R K G E C	20
15	P K K P K E P V	20
79	D S P E R K M L	20
191	N T D H K A Y L	20
284	A R Y F K I R L	20
290	R L R K R S V K	20
Total	6	

Pos	HLA-B*08 nonamers	score
252	G P L C K A D S L	27
28	L I K G G V E V L	25
62	N L R G F S L K L	22
283	L A R Y F K I R L	21
290	R L R K R S V K N	21
3	P T K R K G E C P	20
15	P K K P K E P V Q	20
24	V P K L L I K G G	20
37	E V K T G V D A I	20
91	T A R I P L P N L	20
158	E P L E M Q G V L	20
349	R Y I D K Q G Q L	20
Total	12	

BKV-LT-Antigen (Total 110)

Pos	HLA-A*01 nonamers	score
603	R L D S E I S M Y	26
545	Q I D F R P K I Y	25
155	R T L A C F A V Y	24
165	T K E K A Q I L Y	24
269	V S W K L I T E Y	22
234	Y S A L T R D P Y	21
77	W S S S E V P T Y	20
Total	**7**	

Pos	HLA-A*01 decamers	score
308	K K D Q P Y H F K Y	26
164	T T K E K A Q I L Y	22
225	K G V N K E Y L L Y	20
Total	**3**	

Pos	HLA-A*01 11 - mers	score
283	C E D V F L L L G M Y	28
163	Y T T K E K A Q I L Y	24
459	T F E L G V A I D Q Y	24
75	G T W S S S E V P T Y	21
224	C K G V N K E Y L L Y	21
608	I S M Y T F S R M K Y	21
Total	**6**	

Pos	HLA-A*03 nonamers	score
221	F L I C K G V N K	31
499	R D Y L D G S V K	29
171	I L Y K K L M E K	28
341	A V D T V L A K K	27
231	Y L L Y S A L T R	26
439	G L L D L C G G K	24
509	N L E K K H L N K	24
603	R L D S E I S M Y	24
272	K L I T E Y A V E	23
350	R V D T L H M T R	23
465	A I D Q Y M V V F	23
520	Q I F P P G L V T	23
175	K L M E K Y S V T	22
452	N L P M E R L T F	22
505	S V K V N L E K K	22
568	R I L Q S G M T L	22
28	P L M R K A Y L R	21
155	R T L A C F A V Y	21
158	A C F A V Y T T K	21
288	L L L G M Y L E F	21
551	K I Y L R K S L Q	21
3	K V L N R E E S M	20
Total	**22**	

Pos	HLA-A*03 decamers	score
475	D V K G T G A E S K	28
28	P L M R K A Y L R K	26
175	K L M E K Y S V T F	26
326	I I F A E S K N Q K	25
299	N V E E C K K C Q K	24
170	Q I L Y K K L M E K	23
272	K L I T E Y A V E T	23
273	L I T E Y A V E T K	23
542	F V R Q I D F R P K	23
171	I L Y K K L M E K Y	22
397	C L L P K M D S V I	22
457	R L T F E L G V A I	22
551	K I Y L R K S L Q N	22
12	E L M D L L G L E R	21
220	S F L I C K G V N K	21
278	A V E T K C E D V F	21
520	Q I F P P G L V T M	21
3	K V L N R E E S M E	20
51	K M K R M N T L Y K	20
236	A L T R D P Y H T I	20
287	F L L L G M Y L E F	20
323	A N A I I F A E S K	20
341	A V D T V L A K K R	20
498	L R D Y L D G S V K	20
677	H L C K G F Q C F K	20
Total	**25**	

Pos	HLA-B*08 octamers	score
417	V P K R R Y W L	35
563	F L L E K R I L	29
164	T T K E K A Q I	28
28	P L M R K A Y L	26
126	P P K K K R K V	26
547	D F R P K I Y L	26
368	H I L D K M D L	25
508	V N L E K K H L	23
551	K I Y L R K S L	23
613	F S R M K Y N I	23
49	E D K M K R M N	22
346	L A K K R V D T	22
399	L P K M D S V I	22
453	L P M E R L T F	22
599	E W K E R L D S	22
429	I D S G K T T L	21
503	D G S V K V N L	21
514	H L N K R T Q I	21
597	I V E W K E R L	21
620	I C M G K C I L	21
35	L R K C K E F H	20
51	K M K R M N T L	20
97	N E K W D E D L	20
128	K K K R K V E D	20
225	K G V N K E Y L	20
347	A K K R V D T L	20
Total	**26**	

Pos	HLA-B*08 nonamers	score
346	L A K K R V D T L	33
164	T T K E K A Q I L	32
417	V P K R R Y W L F	31
126	P P K K K R K V E	27
135	D P K D F P S D L	26
27	L P L M R K A Y L	24
280	E T K C E D V F L	23
399	L P K M D S V I F	23
445	G G K A L N V N L	23
534	V P K T L Q A R F	23
49	E D K M K R M N T	22
201	P H R H R V S A I	22
265	E T K Q V S W K L	22
361	M L T E R F N H I	22
599	E W K E R L D S E	22
128	K K K R K V E D P	21
368	H I L D K M D L I	21
514	H L N K R T Q I F	21
35	L R K C K E F H P	20
127	P K K K R K V E D	20
225	K G V N K E Y L L	20
Total	**21**	

BKV-Agnoprotein (Total 10)

Pos	HLA-A*01 nonamers	score
		all ≤12
Pos	**HLA-A*03 nonamers**	**score**
5	QLSRQASVK	35
52	STTALPAVK	21
8	RQASVKVGK	20
Total	**3**	

Pos	HLA-A*01 decamers	score
		all ≤12
Pos	**HLA-A*03 decamers**	**score**
55	ALPAVKDSVK	32
13	KVGKTWTGTK	28
4	RQLSRQASVK	26
51	KSTTALPAVK	21
45	SVDGKNKSTT	20
Total	**5**	

Pos	HLA-A*01 11 - mers	score
		all ≤12
Pos	**HLA-B*08 octamers**	**score**
47	DGKNKSTT	20
49	KNKSTTAL	20
Total	**2**	

BKV-SPECIFIC CELLULAR IMMUNE RESPONSE OF PBMC

BKV-LT, -VP1 and -agnoprotein complete pools were used for PBMC stimulation. BKV-specific cellular immune response was measured by IFN-γ secretion after stimulation over night and quantified by ESA. Cellular immunitye was strongest for BKV-VP1 (189 SFU/10^6 PBMC), followed by BKV-LT (40 SFU/10^6 PBMC) and BKV-agnoprotein (5 SFU/10^6 PBMC) (**Figure 5**).

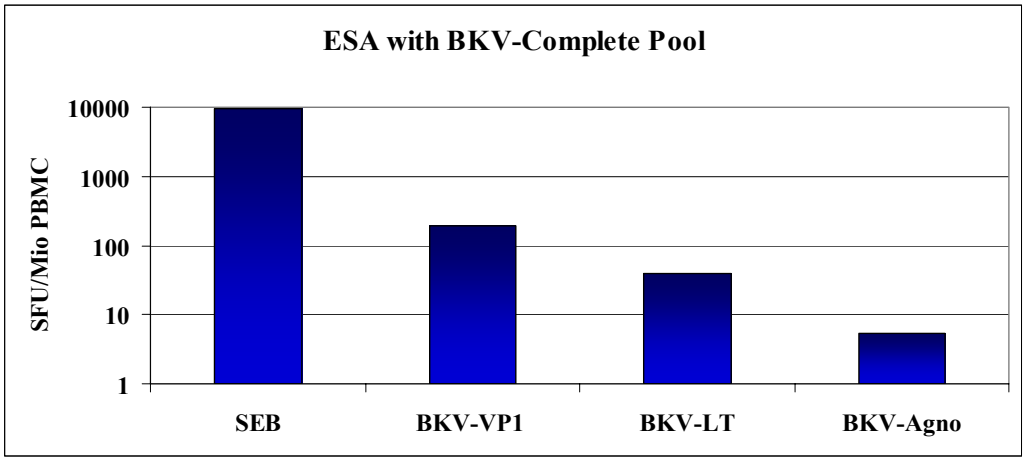

Figure 5: PBMC stimulation with different BKV-OPP. BKV-VP1:189 SFU/10^6 PBMC, BKV-LT: 40 SFU/10^6 PBMC, and BKV-agnoprotein: 5 SFU/10^6 PBMC.

EPITOPE MAPPING OF BKV-LT-ANTIGEN

In a second step BKV-LT subpools were used for PBMC stimulation. A total of 38 BKV-LT subpools were tested for cellular immune response by ESA. Subpools inducing ≥8 SFU/10^6 PBMC (corresponds to one standard deviation above background: 4 SFU/10^6 PBMC ±4) were considered for further analysis. Totally 10/38 (26%) subpools fulfilled this criteria: A, H, O, 6, 7, 8, 11, 12, 15, 16 (**Figure 6**).

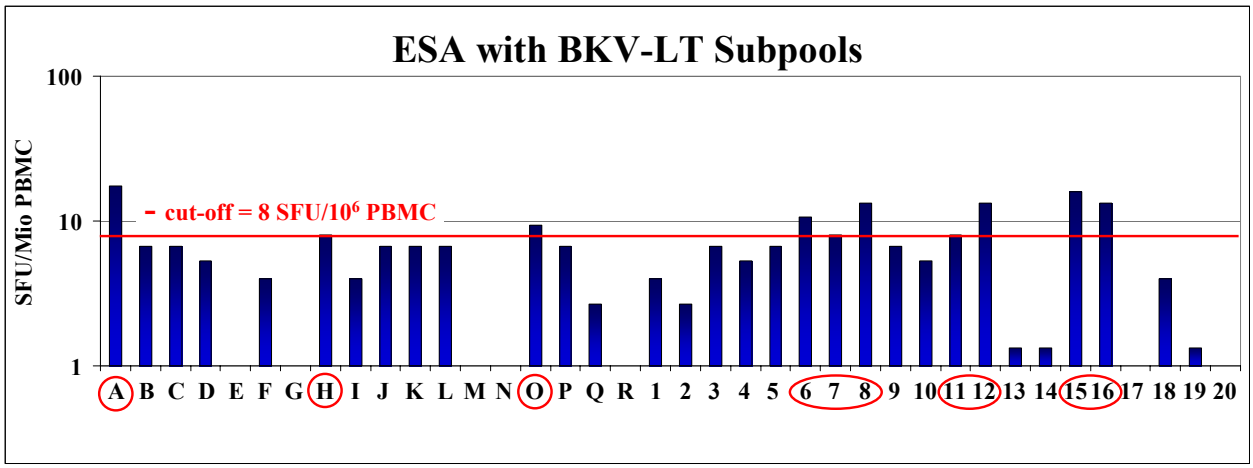

Figure 6: PBMC stimulation with different BKV-LT subpools. Subpools inducing a response ≥8 SFU/10^6 PBMC (cut-off: one standard deviation above background) were considered for further analysis.

Consulting the BKV-LT subpool board revealed 10/180 (6%) potentially immunogenic BKV-LT single peptides: pp94, pp95, pp96, pp164, pp165, pp166, pp229, pp230, 233, and pp234 (**Figure 7**).

A

BKV-LT 10x10 Pool										
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										
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

B

pp94: RAAWGNLPLMRKAYL
pp95: GNLPLMRKAYLRKCK
pp96: LMRKAYLRKCKEFHP
pp164: VEECKKCQKKDQPYH
pp165: KKCQKKDQPYHFKYH
pp166: KKDQPYHFKYHEKHF
pp229: NSEFLLEKRILQSGM
pp230: LLEKRILQSGMTLLL
pp233: LLLLLIWFRPVADFA
pp234: LIWFRPVADFATDIQ

Figure 7: A) Potentially immunogenic single peptides (numbers in bold) identified at the intersection of two subpools (highlighted in yellow). **B)** Peptide sequences for the ten peptides.

For testing single BKV-LT peptides, PBMC were cultivated for 9-days with pp94, pp95, pp96 and then restimulated with single peptides to reach the ESA detection limit. We tested pp94, pp95, and pp96. In addition, we investigated whether peptide cross-restimulation is possible and cultivated PBMC with either pp94, pp95 or pp96 and restimulated with pp94 (for pp95[5] and pp96), pp95 (for pp94 and pp96), and pp96 (for pp94 and pp96) (**Figure 8**).

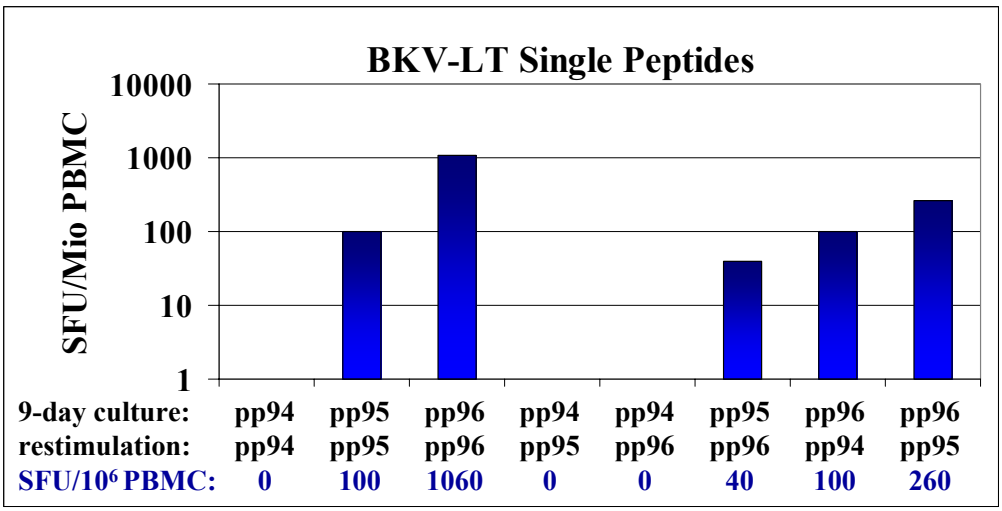

Figure 8: BKV-LT single peptides pp94, pp95, pp96 were used for 9-day cultivation of PBMC and restimulation for ESA.

No detectable immune response was measured after 9-day cultivation with pp94 and restimulation with either pp94, pp95 or pp96.

[5] 9-day cultivation with pp95 and restimulation with pp94 was not performed due to lack of appropriate cell numbers.

BKV-AGNOPROTEIN SINGLE PEPTIDES

PBMC stimulation with single peptides of BKV-agnoprotein was below the detection limit of ESA (data not shown). Therefore, PBMC were incubated for 9-days with agnoprotein complete pool and restimulated for ESA. BKV-agnoprotein specific response was detectable for complete pool and all single peptides except pp8 (**Figure 9**).

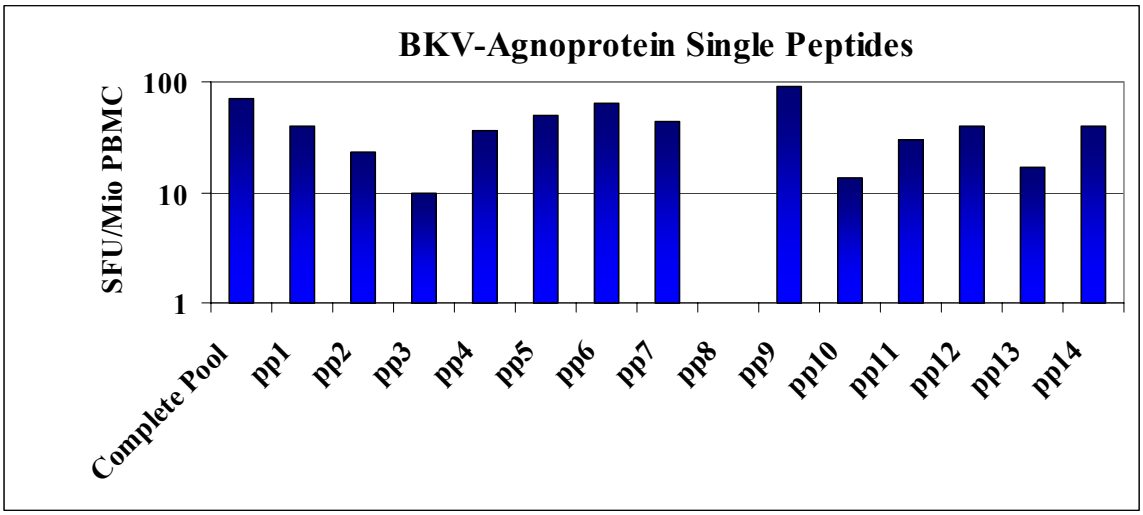

Figure 9: PBMC stimulation with different BKV-agnoprotein single peptides. Except for pp8 all peptides induced detectable responses (≥8 SFU/10^6 PBMC).

Table 5: BKV-agnoprotein specific immune response to single 15aa long peptides.

Agno	SFU/10^6 PBMC	% of CP[1]	Sequence
CP[1]	70	100%	Complete Pool
pp1	40	57%	MVLRQLSRQASVKVG
pp2	23	33%	LSRQASVKVGKTWT
pp3	10	14%	ASVKVGKTWTGTKK
pp4	37	53%	KVGKTWTGTKKRAQR
pp5	50	71%	TWTGTKKRAQRIFIF
pp6	63	90%	TKKRAQRIFIFILEL
pp7	43	61%	AQRIFIFILELLLEF
pp8	0	0%	FIFILELLLEFCRGE
pp9	90	129%	LELLLEFCRGEDSVD
pp10	13	19%	LEFCRGEDSVDGKNK
pp11	30	43%	RGEDSVDGKNKSTTA
pp12	40	57%	SVDGKNKSTTALPAV
pp13	17	24%	KNKSTTALPAVKDSV
pp14	40	57%	TTALPAVKDSVKDS

[1] *CP: complete pool*

Response to the agnoprotein complete pool (70 SFU/10^6 PBMC) was defined as maximal response (100%). Response to single peptide pp9 was higher (90 SFU/10^6 PBMC) than response to complete pool. All other peptides induced a response between 0 and 63 SFU/10^6 PBMC (0-90%) (**Table 5**).

CROSS-RECOGNITION WITH JCV-HOMOLOGUES PEPTIDES

To test if JCV-peptides were also recognized by BKV-specific cells, complete pools of JCV-LT, -VP1, -agnoprotein, and homologous JCV single peptides (pp245, pp246 pp247) were used for restimulation after 9-day expansion with the BKV-complete pool and with BKV-peptides (pp94, pp95, pp96), respectively (**Figure 10**).

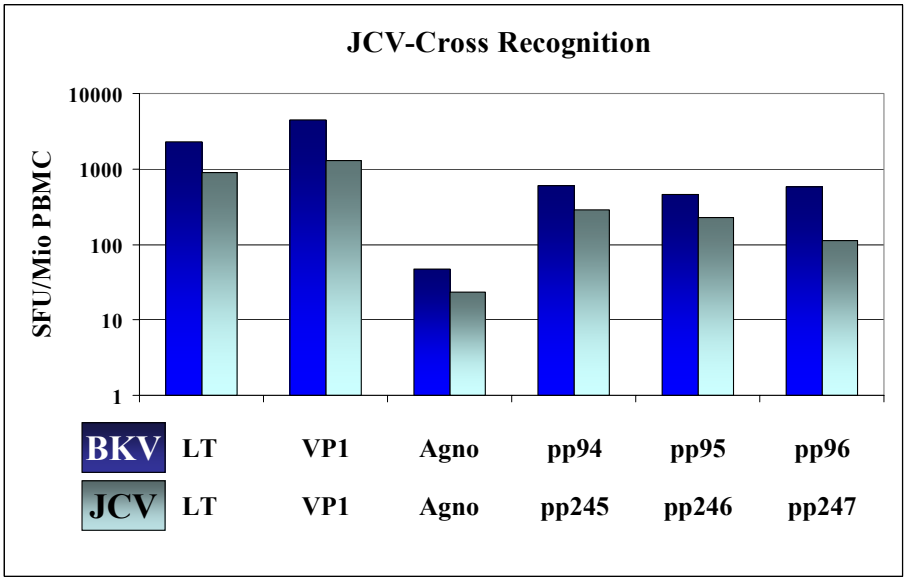

Figure 10: PBMC stimulation BKV-OPP and their JCV-homologues after 9-day cultivation. Response to BKV-antigens were always higher when compared to JCV-antigens.

BKV- and JCV-VP1 complete pools induced the highest immune response (4460 SFU/10^6 PBMC and 1293 SFU/10^6 PBMC, respectively) followed by LT (2280 SFU/10^6 PBMC and 900 SFU/10^6 PBMC, respectively) and agnoprotein (47 SFU/10^6 PBMC and 23 SFU/10^6 PBMC, respectively). Cellular immune response after stimulation with BKV-single peptides pp94, pp95 and pp96 (600 SFU/10^6 PBMC, 463 SFU/10^6 PBMC, and 577 SFU/10^6 PBMC, respectively) was always higher compared to JCV-homologous peptides pp245, pp246 and pp247 (283 SFU/10^6 PBMC, 230 SFU/10^6 PBMC, and 113 SFU/10^6 PBMC, respectively).

CD4+ AND CD8+ T-CELL DEPLETION

In order to differentiate between IFN-γ secretion from CD4+ and CD8+ T-cells, either cells were positively selected by MACSbeads. CD4+ or CD8+ depleted PBMCs were stimulated with either the BKV-LT or -VP1 complete pool. Lowest IFN-γ secretion was detected in CD4+ T-cell depleted PBMC (BKV-LT 51 SFU/10^6 PBMC and BKV-VP1 80 SFU/10^6 PBMC, respectively). CD8+ T-cell depleted PBMC responded to 132 SFU/10^6 PBMC (BKV-LT) and 520 SFU/10^6 PBMC (BKV-VP1) and non-depleted PBMC responded to 97 SFU/10^6 PBMC (BKV-LT) and 280 SFU/10^6 PBMC (BKV-VP1) (**Figure 11**).

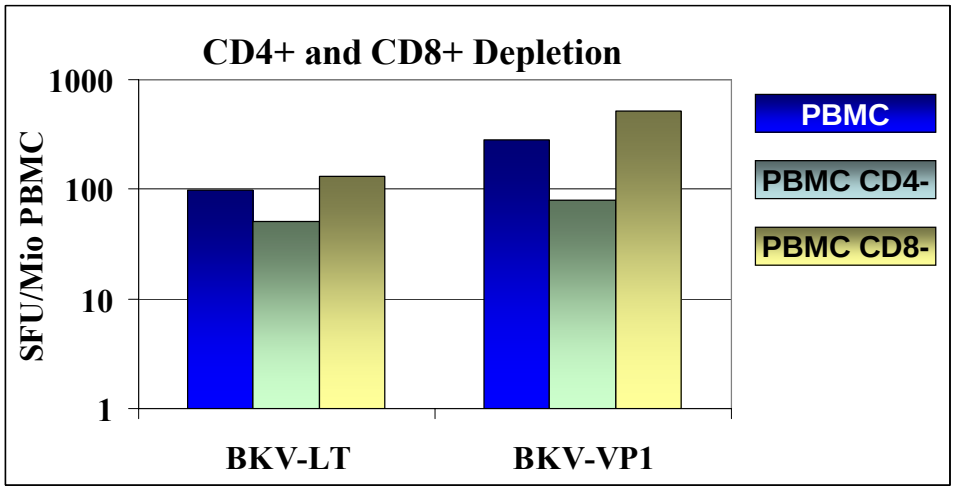

Figure 11: PBMC were depleted from either CD4+ or CD8+ T-cells and stimulated with BKV-LT or -VP1. Response was lowest after CD4+ T-cell isolation.

CD8+ CELL ISOLATION BY MACSBEADS

To establish CD14+ cell maturation and CD8+ T-cell expansion, PBMC from CMV-seropositive HB and CMV-pp65 OPP were used. For measuring the CD8+ T-cell specific cellular immune response, these cells were positively selected out of PBMC using MACSbeads. Additionally, CD14+ cells were also positively isolated and maturated to dendritic cells (**mDC**). The CMV-pp65 complete pool was used to pulse mDC for antigen presentation. CD8+ T-cells were co-cultured for three weeks with peptide pulsed mDC (restimulation occurred every week with freshly peptide pulsed mDC).

Dendritic cell maturation was observed by the switch of CD14+/CD83- cells to CD14-/CD83+ cells using cellular staining and FACS analysis. For maturation, LPS was added over night at day 7 (**Figure 12**).

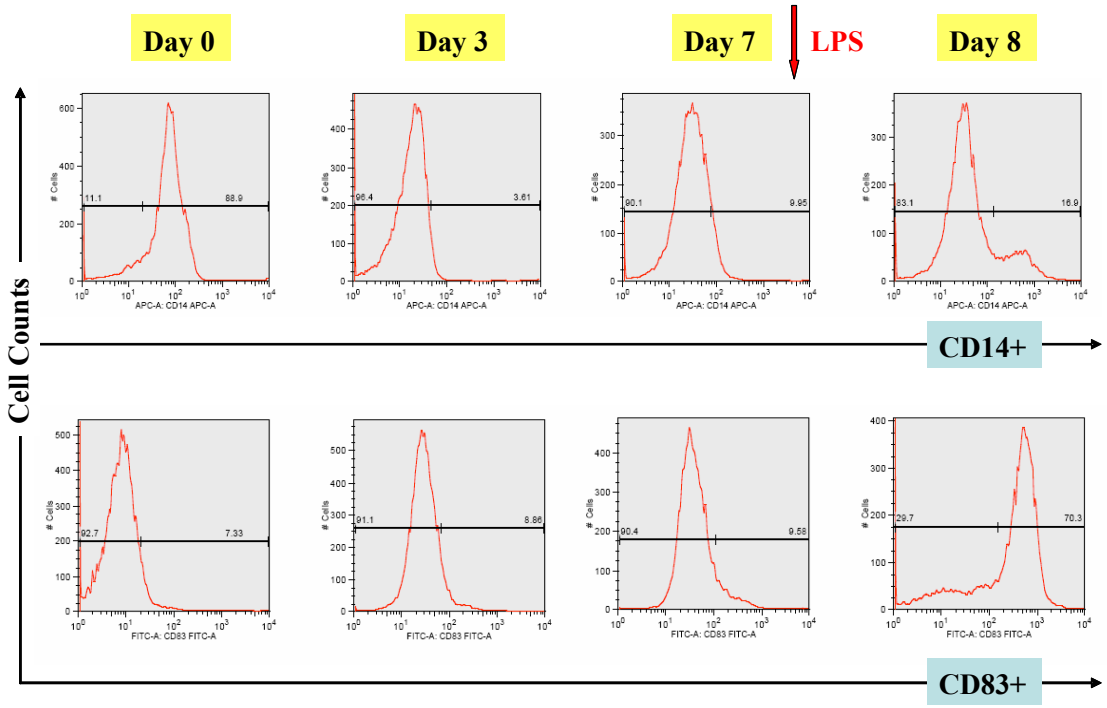

Figure 12: CD14+ cell isolation from PBMC. CD14+ switched from CD83- to CD83+ cells from day 7 to day 8 after adding LPS.

CD8+ T-CELL EXPANSION WITH CMV-PP65

The CMV-pp65 complete pool was used to pulse mDC for CD8+ T-cell expansion over 3 weeks. For background control, following cells were seeded: pulsed mDC alone, CD8+ T-cells alone (not expanded), CD8+ T-cells not expanded but stimulated with mDC-pp65 and CD8+ T-cells alone (expanded) (**Figure 13**). Over three weeks, CD8+ T-cells could be expanded 60x (week 0: 123 SFU/10^6 PBMC, week 1: 2383 SFU/10^6 PBMC, week 2: 5597 SFU/10^6 PBMC, and week 3: 7340 SFU/10^6 PBMC).

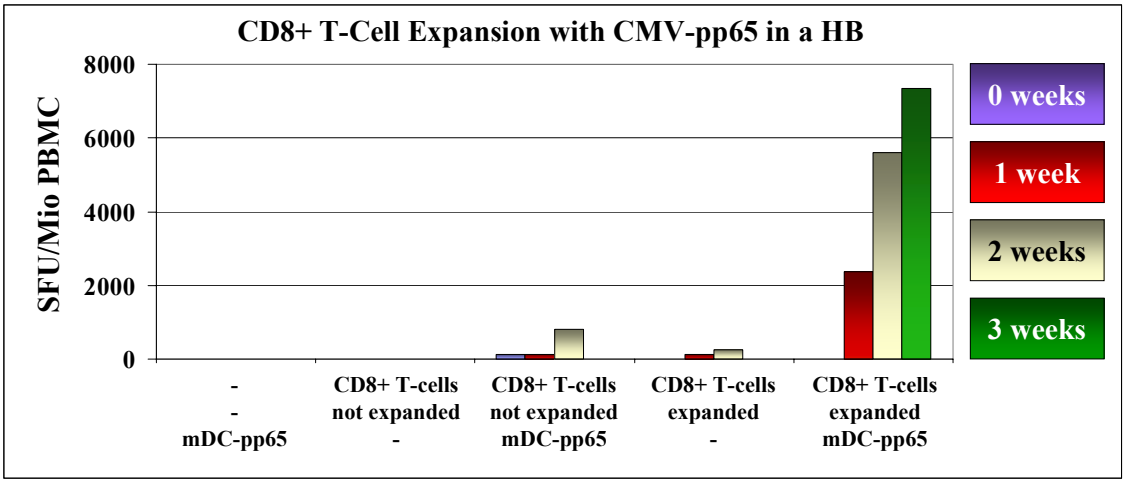

Figure 13: CD8+ T-cell expansion with CMV-pp65 over three weeks. IFN-γ response increased 60x from week 0 (123 SFU/10^6 PBMC) to week 3 (7340 SFU/10^6 PBMC).

DISCUSSION

The knowledge of immunogenic BKV-epitopes may provide valuable information regarding vaccine development and for the development of therapies based on adoptive T-cell transfer. Our aim was to map BKV-LT, -VP1, and -agnoprotein epitopes in the most common HLA-types HLA-A*01, HLA-B*08 (Mori et al. 1997). Epitope mapping is very time consuming and expensive. Therefore, epitope prediction by SYFPEITHI is generally preferred for identification of new epitopes. The disadvantage of this method lies in the restricted availability of HLA-types to test. Furthermore, even with the very well known HLA-type A*02, some epitopes will be missed as computer predictions are not 100% reliable.

Adoptive T-cell transfer with HLA-type dependent epitopes can only be applied to patients with the appropriate HLA-type. A HLA-type independent approach can be achieved by using overlapping peptide pools or virus infected cell lysate for T-cell transfer, but these methods are expensive and time consuming, respectively.

Using ESA as an *in vitro* method for epitope mapping, we found ten potentially immunogenic epitopes for BKV LT-antigen. Further investigations are needed to evaluate the minimal amino acid length inducing an immune response in CD8+ T-cells. Three of these ten BKVLT peptides (pp94, pp95, and pp96) were further tested for cross-recognition with the homologous JCV-peptides (pp245, pp246, and pp247). It was not of surprise that these JCV-LT peptides were also able to induce an immune response as they share 12 of 15 aa with their BKV-LT homologous peptides. Interestingly, the responses to BKV-VP1 complete pool was 3.4 fold higher than to JCV-VP1 complete pool (4460 SFU/10^6 PBMC and 1293 SFU/10^6 PBMC, respectively), The differences between BKV and JCV LT-antigen was only 2.5 fold. This can be explained by the higher protein homology for LT (83%) than for VP1 (78%). In contrast, agnoprotein shows only a protein homology of 59% but BKV-agnoprotein is able to induce a response which is only 2 fold higher compared to JCV-agnoprotein. One explanation can be that only the N-terminal part is important for T-cell recognition. The N-terminus of agnoprotein has a higher homology (84%, amino acids 1-33, Clustal W) compared to the C-terminus (39%, amino acids 34-71, Clustal W).

Our data show that we were able to expand BKV-specific cells during 9-day cultivation and to measure the immune response by ESA. Current results were encouraging and suggested that this project needs more work to track down the different BKV-epitopes. Additionally, a cytotoxicity assay needs to be established to test CD8+ T-cell functionality, and investigation of JCV cross-recognition has to be performed, too.

DISCUSSION

6 DISCUSSION

6.1 Therapeutic Options for BKV-Replication Control in Kidney Transplant Recipients

PVAN is a complication predominantly seen in kidney transplant recipients, with graft loss in up to 80% of patients with advanced stages (Hirsch 2005). It is generally accepted that BKV reactivation and progression to PVAN in KT recipients is associated with impaired cellular immunity. Our knowledge about the risk factors and impaired BKV-specific immune response is limited, making the management of PVAN very difficult. Furthermore, the mechanisms of BKV-reactivation are not yet clear, and it also remains obscure why some KT recipients develop PVAN while others on the same immunosuppressive regimen do not. The only proven therapy to control BKV-replication is by decreasing the dose of immunosuppressives (Trofe et al. 2006). The effect of immunosuppression reduction can be monitored by BKV load measurements in plasma.

The case report **"Transient allograft dysfunction from immune reconstitution in a patient with polyoma BK-virus-associated nephropathy"** (Schaub et al. 2007) shows how important it is to closely monitor BKV loads in a patient after immunosuppressive dose reduction and to observe the clinico-pathological course for therapeutical decisions to achieve a good outcome. This patient showed tubulointerstitial inflammation which may either derive from allograft rejection or from inflammatory response to the BKV. Because of different observations such as BKV-load decline in plasma, only moderate increase of serum creatinine and significant reduction of polyomavirus antigen-positive tubular epithelial cells, it was concluded that the inflammation derived from BKV-specific immune reconstitution and not from rejection. Therapeutical decision is very crucial for the outcome of the kidney allograft as rejection and reconstitution syndrome are treated in opposed ways.

Reduction of immunosuppressive drug doses is very effective in regaining control over BKV-replication in 11%-20% of the patients. However, reducing IS inevitably increases the risk for rejection (Ramos et al. 2002). For each patient, a certain range may exist where the degree of immunosuppressants is neither too high (risk for viral-replication/-reactivation and disease) nor too low (risk for rejection) (**Figure 5.1**). This range may vary among patients due to personal factors (genetic background, metabolization rate, etc.), the amount and way drugs are administered (oral vs. intra venous) as well as the state of the allograft (cadaveric versus living donation, ischemia time, surgical injuries).

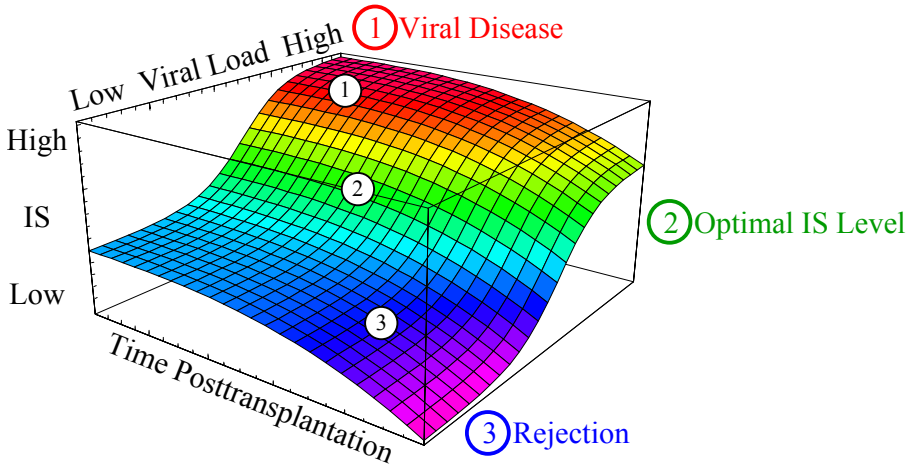

Figure 5.1: Schematic representation of the interplay between immunosuppression (IS), infection and rejection. IS is highest directly after transplantation. The higher the IS dose, the higher the risk for viral reactivation and disease (red area). The lower the IS, the higher the risk for rejection (blue-violet area). Optimal level (green area) of IS with minimal complications.

In general we expect that the higher the immunosuppressive drug concentration, the lower the immune response. Sester and colleagues showed that the frequency of CMV-specific T-cells in lung transplant recipients correlated negative with higher levels of immunosuppressive drugs and with increased CMV complications (Sester et al. 2005). Although our data generally reproduced this pattern, the only significant negative correlation was found for FK506 and BKV-VP1 specific cellular immune response (r_s=-0.644, P=0.002) **("Influence of Immunosuppressive Drugs Cyclosporine A and FK506 on BKV-Specific Cellular Immune Response in Kidney Transplant Recipients"**) (S. Binggeli, current results). There maybe different reasons for this result: 1. Sampling bias: the number of cases is too low. This needs further studies with more KT patients and probably a denser sampling scheme; 2. Misfit: the association between IS and immunity is not a linear correlation and thus cannot be described by Spearman's rho coefficient, as this test looks only for linear correlations. Also this would need more investigation with a higher sample size; 3. Wrong surrogate marker: measuring the BKV-specific response is not an adequate marker for testing immunosuppressive drug level; 4. Complexity: as too many factors play a role and we cannot disentangle them. KT patients usually receive more than one immunosuppressant for antirejection treatment and nephrotoxicity of other drugs may also influence the outcome.

To find a correlation between BKV-specific immune response and IS concentration, an *in vitro* assay may shed light on this question because single immunosuppressants can be tested. In line with the a priory hypothesis that IS reduces immune response, our preliminary data show a decrease of immune response with increasing levels of IS, independent of the antigen (SEB, BKV-VP1 or CMV-pp65). Further studies are needed to reproduce this result with other IS such as MMF and sirolimus.

We found that FK506 plasma levels achieved in a KT patient ranged from 5-12 ng/ml. Analysis of the BKV-VP1 specific response in combination with FK506 revealed a cut-off of 6.65 ng/ml FK506 plasma level. Most KT patients above this cut-off were not able to control BKV-replication. Our pilot experiments suggest further studies to clarify, whether or not elevated levels of FK506 were associated with more frequent BKV replication. If other IS than FK506 play a role for BKV-replication control needs further analysis.

6.2 Biomarker for the Monitoring of Kidney Transplant Recipients

In the clinics as well as in the pharmaceutical industry, there is great interest in identifying new biomarkers for: 1. Prognostic purposes to monitor whether and how fast patients develop PVAN, 2. Stratification purposes, to predict the outcome of the disease after therapeutic intervention, 3.Evaluating the efficacy of therapeutic interventions for individual patients, and 4. Using it as a non invasive marker for differentiation between immune reconstitution inflammatory syndrome (**IRIS**) and rejection. It would be advantageous to have a prognostic marker to define which patient is at risk for rejection before the immune response is strong enough to attack the allograft. Although biopsies are the "gold standard", non-invasive methods are preferred because biopsies are expensive, locally restricted, bear the risk for complications, and are frequency limited.

The ELISpot technique represents such a non-invasive and more direct method to measure the cellular response than staining for HLA-DR positive tubular cells in biopsies is possible by using. In **"Polyomavirus BK-Specific Cellular Immune Response to VP1 and Large T-Antigen in Kidney Transplant Recipients"** (Binggeli et al. 2007) we could demonstrate that KT recipients with an increasing or stable high BKV load in plasma had a significantly lower immune response to the two proteins BKV-LT and -VP1 than with patients that had a decreasing viral load or past PVAN (P=0.003 and P=0.001, respectively, Mann Whitney U-test, two sided). This finding may complement the previously defined threshold plasma viral

load of >10'000 DNA copies/ml and thereby enlarge the pool of diagnostic markers to identify patients at high risk for PVAN. Hammer et al. recently reported that patients with a plasma BKV-DNA load of >250'000 copies/ml and detectable VP1-specific CD8+ T-cell response, are at high risk for PVAN development and graft loss. The authors speculate that VP1-specific CD8+ T-cell in plasma may represent a direct threat for allograft survival (Hammer et al. 2006). However, we were able to detect a BKV-specific cellular immune response not only to VP1 but also to LT-antigen in patients with a plasma viral load <250'000 copies/ml by ESA. Furthermore, we could illustrate that a single measurement of the viral load in plasma does not correlate with the cellular immune response to BKV. Viral dynamics rather than a snapshot measurement of viral load matters. These findings strongly indicate the need for serial PCR in plasma for measurements to guide clinical management of PVAN in KT patients (Funk et al. 2006; Lacey 2007).

A cut-off of 69 SFU per million PBMC for BKV-LT was shown to include most patients with a decreasing viral load (P=0.013, Fisher's exact test, two-sided) and could be used as a marker to define patients who have regained a protective cellular immune response against BKV. BKV-LT antigen is a better discriminator for the group with decreasing viral load than BKV-VP1 and the cut-off of 69 SFU per million PBMC may also be useful for monitoring the effect of immunosuppressant reduction. However, a well-designed prospective study needs to be performed to confirm this cut-off.

Measuring of the cellular immune response by ESA for identification of a rejection marker looks promising. In this context it is tempting to speculate that maybe it is not the cut-off that is important for predicting rejection, but rather the dynamics of how quickly the patient is able to regain a competent immune system (comparable with BKV load dynamics for risk for PVAN). Clearly, this needs further investigation.

6.3 Polyomavirus JC and Cross-Recognition

All study participants (N=42) were also JCV-seropositive. Although BKV and JCV share high protein homology for LT (83%) and VP1 (78%), response to BKV-peptides was specific and significantly higher than responses to JCV-peptides (P=0.008 and P<0.001, respectively). The higher response to BKV-antigen is most likely due to a resent viral exposure. After 9-day cultivation the cellular immunity response to BKV-VP1 was significantly higher than response to JCV-VP1 (P=0.016), indicating a limited cross-recognition. In contrast, in vitro expansion with LT-antigen showed no significant differences after restimulation with either BKV- or JCV-LT. It is, therefore, tempting to speculate that immune response to LT-antigen is more conserved between BKV and JCV as for VP1.

We also found cross-recognition with 15aa long LT-antigen single peptides by epitope mapping **("Epitope Mapping of BKV-Proteins: LT, VP1 and Agnoprotein"**) (S. Binggeli, current results). The decision to map BKV-LT first and not BKV-VP1 was due to the fact that: 1. LT is higher conserved between BKV and JCV, 2. BKV-LT is a better discriminator for cellular immune response in KT patients with a decreasing viral load (Binggeli et al. 2007), and 3. LT-antigen is an early protein and has important functional properties and maybe essential in respect to mounting a protective cellular immune response. From an evolutionary point of view, it makes sense that a CD8+ T-cell response detects and destroys virus infected cells well before they burst and liberate their viral progeny. Thus, BKV-LT which is an early protein should be recognized before the late protein VP1 and may play an important role in mounting an BKV-specific cellular immune response.

As the BKV-LT overlapping peptide pool consists of 180 peptides (15aa in length with 11aa overlaps), it is more time and cost efficient to pool these peptides by 10 peptides per pool (subpools) rather than to test each peptide individually. PBMC stimulation with 38

different peptide subpools revealed 10 subpools inducing a BKV-LT specific cellular immune response (>8 SFU/10^6 PBMC). The peptide board showed ten single peptides (pp94, pp95, pp96, pp164, pp165, pp166, pp230, pp233, pp234) with the potential for inducing a BKV-LT specific cellular immune response. Starting with pp94, pp95 and pp96, those single peptides showed highest response to pp96 and no response to pp94. Surprisingly, in a later experiment pp94 was able to induce an immune response in the same range as for pp95 and pp96. This inconsistency could be due to ESA variation or inappropriate addition of pp94.

Testing for cross-recognition with the homologous JCV single peptides indicated strong recognition despite a lower response suggesting a higher specificity for BKV-peptides. The question whether these peptides are able to induce an immune response also in CD8+ T-cells remains to be open for a future project. Furthermore, tracking the 15aa long peptide to the minimal required size of 8aa, 9aa or 10aa is also subject for future analysis.

The use of BKV-specific epitopes for adoptive T-cell transfer is a promising attempt for therapeutical options and more cost and time efficient than using viral lysate or complete peptide pools. Specific epitopes may target CD8+ T-cells directly and may also be more efficient in T-cell expansion. On the other hand, one depends on the knowledge of immunogenic epitopes and certain HLA-types. This can be circumvent by BKV-lysate or -complete pool which are HLA-independent. BKV-LT epitopes may also serve for KT patient monitoring. In addition, these may help to better distinguish those patients with high risk for PVAN as proposed by Hammer and colleagues (Hammer et al. 2006).

As no proven antiviral treatments are available BKV-specific T-cell expansion and adoptive T-cell transfer may serve as an alternative to the reduction of immunosuppression in KT patients with increasing BKV-replication and increased risk for PVAN.

6.4 Antiviral Treatment

However, new compounds which target BKV are desirable. In contrast to other latent viruses like CMV or EBV, there are no proven antiviral drugs available for BKV-treatment. Antivirals are especially desirable in the context of multi-organ transplants as reduction of immunosuppressive concentration may increase the possibility of rejection in the non-kidney allograft or in patients who are not able to control their BKV load after immunosuppressive reduction. One natural antiviral compound, IFN-γ, can be produced by different immune cells (natural killer cells, CD4+, and CD8+ T-cells) and is secreted upon virus infections (Boehm et al. 1997). Abend et al. recently reported the *in vitro* inhibitory effect of IFN-γ on BKV gene expression and replication in primary human renal proximal tubular epithelial cells. Furthermore, IFN-γ inhibited the level of the viral progeny >50x (Abend et al. 2007). Whether IFN-γ also inhibits BKV-replication *in vivo* needs more investigation.

Other observations of anti-BKV properties of cidofovir, leflunomide, quinolone, intravenous immunoglobulines, and lactoferrin, have been reported but the reports are controversial (**Chapter 1.3.3.3**) (Josephson 2005; Longhi et al. 2006; Rinaldo et al. 2007). More research is necessary to understand the mechanisms by which these compounds act and whether there is potential in further developing them to strong and effective antiviral drugs.

In **"Human Polyomavirus type 1 (BK virus) Agnoprotein is abundantly expressed, but immunologically ignored"** it has been shown by immunostaining of KT biopsies that agnoprotein is abundantly expressed but does not induce a humoral or cellular immune response (Leuenberger et al. 2007). Even though agnoprotein is not suitable for immune response measurement it could still be an interesting target for antivirals. It is already known that BKV-agnoprotein is phosphorylated (Rinaldo et al. 1998) and a recent study analyzed the effect of JCV-agnoprotein mutants. JCV-agnoprotein has three potential targets for serine/threonine-specific protein kinase C: Ser7, Ser11 and Thr21 **(Figure 5.2)** (Sariyer et al.

2006). Conservative substitution of these phosphorylation sites resulted in a viable virus and viral particle release from infected cells, but the virus was not able to propagate as the viral capsid showed a deficiency in DNA-content. Thus, the viral life cycle ended with an abortive replication cycle. Targeting the phosphorylation site of agnoprotein could be an interesting approach for new antiviral drugs. The phosphorylation sites are conserved between JCV and BKV and we can assume that BKV would also be impaired in propagation if agnoprotein is not phosphorylated. To the best of my knowledge there are no published reports concerning BKV-agnoprotein and the potential consequences of interfering with its phosphorylation sites.

Interestingly CMV possesses three tegument proteins which are phosphorylated (pp65, pp71, pp150) and it is speculated that inhibiting of protein phosphorylation by leflunomide may interfere with tegument assembly (Waldman et al. 1999). Whether leflunomide is also able to inhibit BKV-agnoprotein phosphorylation and thus virus propagation needs further investigation.

```
BKV    MVLRQLSRQASVKVGKTWTGTKKRAQRIFIFILELLLEFCRGEDSVDGKN
JCV    MVLRQLSRKASVKVSKTWSGTKKRAQRILIFLLEFLLDFCTGEDSVDGKK
              7    11          21

BKV    KS-TTALPAVKDSVKDS----
JCV    RQRHSGLTEQTYSALPEPKAT
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Figure 5.2: Agnoprotein sequence and potential phosphorylation sites (Ser7, Ser11, Thr21). Accession number: BKV: P03085, JCV: AAA82098, Clustal W alignment of BKV-Dunlop and JCV-MAD1 strains.

BKV-agnoprotein is a very short 66aa long protein and only 14 peptides are necessary to cover the whole protein with a peptide library consisting of 15aa in length with 11aa overlaps. Therefore, single agnoprotein peptides were used for PBMC stimulation after 9-day expansion with complete agnoprotein overlapping peptide pool (**OPP**). The BKV-specific cellular immune response was quantified by ESA. From all 14 peptides, pp8 and pp9 stood out, as pp8 is not able to induce any response and response to pp9 was higher (90 SFU/10^6 PBMC) than response to the complete pool (70 SFU/10^6 PBMC). The single peptide pp8 posseses a long stretch with hydrophobic amino acids which might be unfavorable for binding and recognition by MHC and T-cells. Even though pp9 is the neighboring peptide sharing 11aa, the BKV-specific response of pp9 was very high. This discrepancy between pp8 and pp9 immune response could be due to more favorable peptide binding by the groove binding sites of MHC-class II due to amino acid sequence or three dimensional structure.

The association between agnoprotein and the cellular immune response is not understood; Further investigation is also needed to understand whether agnoprotein may serve for therapeutic purposes.

6.5 Limitation of Polyomavirus-Specific Immune Response Measurement by ELISpot Assay

ESA is a very sensitive and convenient assay to measure IFN-γ secretion of PBMCs. However, like every method, ESA has its limitations. In the current study **"Epitope Mapping of BKV-Proteins: LT, VP1 and Agno of a HLA-*A01, HLA-A*03, HLA-B*08, HLA-B*51, HLA-DR*19, HLA-DR*11 Kidney Patient with Past Polyomavirus Associated Nephropathy"** ESA was used to measure BKV-specific cellular immune response of either

PBMC or BKV-specific expanded CD8+ T-cells for epitope mapping (S. Binggeli, current results).

With ESA the source of IFN-γ secreting cells can not be traced back. As natural killer (NK) cells, CD4+ and also CD8+ T-cells secrete IFN-γ upon stimulation, it can not be distinguished how much each cell contributes to the IFN-γ response. Thus only the overall IFN-γ secretion can be measured. One way to determine the IFN-γ source is cell isolation. CD4+ or CD8+ depleted PBMC can be used to estimate how much each cell contributes to the IFN-γ response. CD4+ T-cell depletion showed a lower response upon stimulation with BKV-LT and -VP1 (51 SFU/10^6 PBMC, 80 SFU/10^6 PBMC) than not depleted PBMC (97 SFU/10^6 PBMC, 280 SFU/10^6 PBMC) and CD8+ depleted PBMC (132 SFU/10^6 PBMC, 520 SFU/10^6 PBMC), indicating that the IFN-γ response is derived mostly from CD4+ T-cells. As we are using 15aa long peptides for PBMC stimulation we could expect a good response of CD4+ T-cells for two reasons: 1. Optimal peptide length for CD4+ T-cell recognition is between 13aa and 17aa, and, 2. The peptides are loaded on MHC-class II via the exogenous pathway (Rudensky et al. 1991). This argues against an optimal peptide presentation and recognition for CD8+ T-cells, as these cells recognize 8-10aa long peptides loaded on MHC-class I via the endogenous pathway (Rammensee 1995).

We observed a higher immune response in CD8+ T-cell depleted PBMC (LT: 132 SFU/10^6 PBMC, VP1: 520 SFU/10^6 PBMC) than in the whole PBMC (LT: 97 SFU/10^6 PBMC, VP1: 280 SFU/10^6 PBMC). This discrepancy may be due to the increased concentration of CD4+ T-cells after CD8+ T-cell depletion as always the same cell concentration was seeded per well for stimulation. In order to distinguish between CD4+ and CD8+ T-cell derived IFN-γ secretion, it would be advantagous to use intracellular cytokine staining, or, when using ESA, positively selecting T-cells and stimulating them with peptide pulsed mDCs. Both methods would exclude a NK-cell derived IFN-γ response. However, the disadvantage of using a FACS assay is that a low frequency of IFN-γ secreting T-cells may fall under the detection limit (Karlsson et al. 2003; Tassignon et al. 2005).

Measuring the IFN-γ response of isolated CD8+ T-cells is very difficult for BKV-peptides as T-cell frequency is mostly under the detection limit (unpublished data). Therefore, IFN-γ detection is only possible with a method sensitive enough to trace IFN-γ at very low levels or to expand BKV-specific CD8+ T-cells to reach a higher frequency. For our purposes we positively selected CD8+ T-cells out of PBMC and expanded them with viral peptides. To establish T-cell expansion in our laboratory, we used PBMC from a HB and CMV-pp65 peptide library (**Appendices 10.3**). After three weeks, we were able to expand CMV-pp65 specific CD8+ T-cells 60x (week 0: 123 SFU/10^6 PBMC, week 3: 7340 SFU/10^6 PBMC). Of course, this method has to be adapted for KT recipients and as well for BKV-LT, -VP1 and -agnoprotein. To complete this experiment it could also be established for CD4+ T-cells.

Whether the cellular immune response of PBMC mirrors the event in the organ remains to be investigated. It can be speculated that BKV-specific cellular immune response of PBMC reflects the action in the kidney, as the kidney is highly supplied with blood. On the other hand, much of the T-cell response may be undetectable in the peripheral blood due to T-cell homing.

6.6 Concluding Remarks

In conclusion, it is of high interest to find new biomarkers for monitoring patients after KT transplantation in order to prevent PVAN and for better patient management. A stable graft function prolongates its survival, and measurement of the BKV-specific cellular immune response is one approach for identifying patients at risk for PVAN and supplementing BKV-load measurement by PCR.

We could show that a BKV-LT specific cellular immune response above 69 SFU/10^6 PBMC correlates with the decline of BK-viral load in plasma and thus with BKV-replication control. Furthermore, we found that a FK506 plasma concentration higher 6.65 ng/ml significantly increases the risk for BKV-replication. Both cut-offs have to be validated in a well designed prospective study. If these cut-offs are confirmed, they may serve as good biomarkers for patient monitoring and as guideline for therapeutical decisions.

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7. REFERENCES

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CURRICULUM VITAE

9. CURRICULUM VITAE

CURRICULUM

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EDUCATION AND PROFESSIONAL EXPERIENCES

07/2003-present **PhD thesis** at the Institute for Medical Microbiology, University of Basel, Switzerland, in the "Transplantation Virology" group of Prof. Hans H. Hirsch.
Title: "BKV-Specific Cellular Immune Response in Renal Transplant Recipients"

Achievements: As part of a new research group I established successfully a broad variety of immunological assays and protocols for human cell cultivation including: flow cytometry analysis, enzyme-linked immunospot (ELISpot) assay, T-cell isolation and expansion for epitope mapping.
In a cross-sectional study I was responsible for the experimental setup, patient recruitment, organization of patient material, communication with the hospital and analysis of the results including statistical analysis with the software SPSS (see publication list 01/2007 and 07/2006).

12/2002-02/2003 **Laboratory assistant** at the Institute for Medical Microbiology, University of Basel, Switzerland, in the "Molecular Diagnostics" group of PD Dr. Thomas Klimkait.

Achievements: In this position I learned how to work in a diagnostic environment and how to deal with patient material. I qualitatively and quantitatively analyzed different viruses (HIV, HBV and HCV) in patient samples by Cobas Amplicor. Furthermore, I generated a tool to facilitate the analysis of PCR results.

09/2001-03/2003 **Master thesis** (formerly called diploma thesis) at the Biozentrum, University of Basel, Switzerland, in the "Microbiology and Cell Biology" group of Prof. Christoph Dehio.
Title: "Genome-Wide Gene Expression Pattern of *Bartonella henselae* during Macrophage Infection Analyzed by Microarray Technology"

Achievements: Microarray technology was established in this laboratory. I was responsible for cell culture (murine macrophages), the bacterial in vitro infection model, mRNA isolation, cDNA synthesis, hybridization on microarray chips and gene expression analyses. During this time I supervised undergraduate students attending the microbiology course and held lectures about microarray technology.

10/1998-03/2003 **Study of Molecular Biology** (Biology II) at the Biozentrum, University of Basel, Switzerland

02/1998-07/1998 **Administrative assistant** at Kiosk AG, Muttenz, Switzerland

08/1994-11/1997 **Gymnasium: Typus C** (high school: science) Münchenstein, Switzerland

08/1991-06/1994 **Commercial School** Münchenstein, Switzerland

LANGUAGES

German	Native language
English	Fluently spoken and written
Spanish	Good knowledge spoken, basic knowledge written
French	Basic knowledge spoken and written

COMPUTER LITERACY

Software Packages Office 2000 and XP: Word, Excel, Power Point
LaTex (word processing)
Adobe: Illustrator and Photoshop (presentation and picture editing)
SPSS (statistical software): certificate for basic & advanced level
Gene Spring (gene expression analysis)

Operating Systems Windows WIN98, 2000, and XP
MacIntosh OS9 and OSX

TEACHING AND SUPERVISION

07/2003-present Introduction into dealing with human cells, flow cytometry and ELISpot technique. Support for creating diagrams and analysis of results with the Excel and SPSS programs.

09/2001-03/2003 Supervision of 3rd-year M.Sc. students during the microbiology course.

PRESENTATIONS

04/2007 Annual Congress SGAI-SSAI (Swiss Society for Allergology and Immunology), Basel, Switzerland
Poster presentation: "Polyomavirus BK-Specific Cellular Immune Response to VP1 and Large T-Antigen in Kidney Transplant Recipients"
Simone Binggeli, Adrian Egli, Stefan Schaub, Isabelle Binet, Michael Mayr, Juerg Steiger, Hans H. Hirsch

07/2006 WTC (World Transplant Congress), Hynes Convention Centre, Boston, Massachusetts, USA
Oral Presentation: "BKV-Specific Cellular Immune Response to VP1 and Large T-Antigen after Polyomavirus-Associated Nephropathy"
Simone Binggeli, Adrian Egli, Juerg Steiger, and Hans H. Hirsch

04/2005 8th annual meeting of ESCV (European Society for Clinical Virology), Geneva, Switzerland
Poster Presentation: " Characterization of CMV-Specific Immune Response using 4-Color FACS Assay"
Simone Binggeli, Adrian Egli, Michael Mayr, Juerg Steiger, Martina Sester, and Hans H. Hirsch

03/2005 17th meeting of the swiss immunology PhD students, Schloss Wolfsberg, Ermatingen, Switzerland
Oral Presentation: " Characterization of CMV-Specific Immune Response using 4-Color FACS Assay"
Simone Binggeli, Adrian Egli, Michael Mayr, Juerg Steiger, Martina Sester, and Hans H. Hirsch

PUBLICATION LIST

05/2007 Egli Adrian, Isabelle Binet, **Simone Binggeli**, Clemens Jäger, Alexis Dumoulin, Juerg Steiger, Urban Sester, Martina Sester, Hans H. Hirsch.
"Cytomegalovirus pp65-specific T-cell responses predicting viral reactivation in renal transplantation"
American Journal of Transplantation, submitted Aug 2007

05/2007 David Leuenberger, Per Arne Andresen, Rainer Gosert, **Simone Binggeli**, Erik H. Ström, Sohrab Bodaghi, Christine Hanssen Rinaldo, Hans H. Hirsch.
"Human Polyomavirus type 1 (BK virus) Agnoprotein is abundantly expressed, but immunologically ignored"
Clinical and Vaccine Immunology, Aug 2007; 14(8); 959-968

04/2007 Stefan Schaub, Michael Mayr, Adrian Egli, **Simone Binggeli**, Bernard Descœudres, Jürg Steiger, Michael J. Mihatsch, Hans H. Hirsch.
"Transient allograft dysfunction from immune reconstitution in a patient with polyoma BK-virus-associated nephropathy"
Nephrology Dialysis Transplantation, Aug 2007; 22(8): 2386-90

01/2007 **Simone Binggeli**, Adrian Egli, Stefan Schaub, Isabelle Binet, Michael Mayr, Juerg Steiger, Hans H. Hirsch.
"Polyomavirus BK-Specific Cellular Immune Response to VP1 and Large T-Antigen in Kidney Transplant Recipients"
American Journal of Transplantation, May 2007; 7(5):1131-9

07/2006 **Simone Binggeli**, Adrian Egli, Michael Dickenmann, Isabelle Binet, Juerg Steiger, Hans H. Hirsch.
Letter to the editor: "BKV Replication and Cellular Immune Responses in Renal Transplant Recipients"
American Journal of Transplantation, Sep 2006; 6(9):2218-9

06/2006 Patrizia Comoli, **Simone Binggeli**, Fabrizio Ginevri, Hans H. Hirsch.
"Polyomavirus-associated nephropathy: update on BK virus-specific immunity"
Transplant Infectious Disease Jun 2006; 8(2):86-94. Review

04/2004 Hans H. Hirsch, Michael Dickenmann, **Simone Binggeli**, Juerg Steiger.
"Alte Viren – neue Immunsuppressiva: *liaison dangereuse?*"
Schweiz Med Forum 2004; 4:538–541

APPENDICES

10. APPENDICES

10.1 PEPTIDE LIBRARY BKV DUNLOP STRAIN

10.1.1 Polyomavirus BK-Large T-Antigen (NCBI: P03071)

89	MDKVLNREESMELMD	134	YSVTFISRHMCAGHN
90	LNREESMELMDLLGL	135	FISRHMCAGHNIIFF
91	ESMELMDLLGLERAA	136	HMCAGHNIIFFLTPH
92	LMDLLGLERAAWGNL	137	GHNIIFFLTPHRHRV
93	LGLERAAWGNLPLMR	138	IFFLTPHRHRVSAIN
94	RAAWGNLPLMRKAYL	139	TPHRHRVSAINNFCQ
95	GNLPLMRKAYLRKCK	140	HRVSAINNFCQKLCT
96	LMRKAYLRKCKEFHP	141	AINNFCQKLCTFSFL
97	AYLRKCKEFHPDKGG	142	FCQKLCTFSFLICKG
98	KCKEFHPDKGGDEDK	143	LCTFSFLICKGVNKE
99	FHPDKGGDEDKMKRM	144	SFLICKGVNKEYLLY
100	KGGDEDKMKRMNTLY	145	CKGVNKEYLLYSALT
101	EDKMKRMNTLYKKME	146	NKEYLLYSALTRDPY
102	KRMNTLYKKMEQDVK	147	LLYSALTRDPYHTIE
103	TLYKKMEQDVKVAHQ	148	ALTRDPYHTIEESIQ
104	KMEQDVKVAHQPDFG	149	DPYHTIEESIQGGLK
105	DVKVAHQPDFGTWSS	150	TIEESIQGGLKEHDF
106	AHQPDFGTWSSSEVP	151	SIQGGLKEHDFSPEE
107	DFGTWSSSEVPTYGT	152	GLKEHDFSPEEPEET
108	WSSSEVPTYGTEEWE	153	HDFSPEEPEETKQVS
109	EVPTYGTEEWESWWS	154	PEEPEETKQVSWKLI
110	YGTEEWESWWSSFNE	155	EETKQVSWKLITEYA
111	EWESWWSSFNEKWDE	156	VSWKLITEYAVETK
112	WWSSFNEKWDEDLFC	157	KLITEYAVETKCEDV
113	FNEKWDEDLFCHEDM	158	EYAVETKCEDVFLLL
114	WDEDLFCHEDMFASD	159	ETKCEDVFLLLGMYL
115	LFCHEDMFASDEEAT	160	EDVFLLLGMYLEFQY
116	EDMFASDEEATADSQ	161	LLLGMYLEFQYNVEE
117	ASDEEATADSQHSTP	162	MYLEFQYNVEECKKC
118	EATADSQHSTPPKKK	163	FQYNVEECKKCQKKD
119	DSQHSTPPKKKRKVE	164	VEECKKCQKKDQPYH
120	STPPKKKRKVEDPKD	165	KKCQKKDQPYHFKYH
121	KKKRKVEDPKDFPSD	166	KKDQPYHFKYHEKHF
122	KVEDPKDFPSDLHQF	167	PYHFKYHEKHFANAI
123	PKDFPSDLHQFLSQA	168	KYHEKHFANAIIFAE
124	PSDLHQFLSQAVFSN	169	KHFANAIIFAESKNQ
125	HQFLSQAVFSNRTLA	170	NAIIFAESKNQKSIC
126	SQAVFSNRTLACFAV	171	FAESKNQKSICQQAV
127	FSNRTLACFAVYTTK	172	KNQKSICQQAVDTVL
128	TLACFAVYTTKEKAQ	173	SICQQAVDTVLAKKR
129	FAVYTTKEKAQILYK	174	AVDTVLAKKRVDTL
130	TTKEKAQILYKKLME	175	TVLAKKRVDTLHMTR
131	KAQILYKKLMEKYSV	176	KKRVDTLHMTREEML
132	LYKKLMEKYSVTFIS	177	DTLHMTREEMLTERF
133	LMEKYSVTFISRHMC	178	MTREEMLTERFNHIL

...Continued

Polyomavirus BK-Large T-Antigen (NCBI: P03071)

179	EMLTERFNHILDKMD	224	ARFVRQIDFRPKIYL
180	ERFNHILDKMDLIFG	225	RQIDFRPKIYLRKSL
181	HILDKMDLIFGAHGN	226	FRPKIYLRKSLQNSE
182	KMDLIFGAHGNAVLE	227	IYLRKSLQNSEFLLE
183	IFGAHGNAVLEQYMA	228	KSLQNSEFLLEKRIL
184	HGNAVLEQYMAGVAW	229	NSEFLLEKRILQSGM
185	VLEQYMAGVAWLHCL	230	LLEKRILQSGMTLLL
186	YMAGVAWLHCLLPKM	231	RILQSGMTLLLLLIW
187	VAWLHCLLPKMDSVI	232	SGMTLLLLLIWFRPV
188	HCLLPKMDSVIFDFL	233	LLLLLIWFRPVADFA
189	PKMDSVIFDFLHCIV	234	LIWFRPVADFATDIQ
190	SVIFDFLHCIVFNVP	235	RPVADFATDIQSRIV
191	DFLHCIVFNVPKRRY	236	DFATDIQSRIVEWKE
192	CIVFNVPKRRYWLFK	237	DIQSRIVEWKERLDS
193	NVPKRRYWLFKGPID	238	RIVEWKERLDSEISM
194	RRYWLFKGPIDSGKT	239	WKERLDSEISMYTFS
195	LFKGPIDSGKTTLAA	240	LDSEISMYTFSRMKY
196	PIDSGKTTLAAGLLD	241	ISMYTFSRMKYNICM
197	GKTTLAAGLLDLCGG	242	TFSRMKYNICMGKCI
198	LAAGLLDLCGGKALN	243	MKYNICMGKCILDIT
199	LLDLCGGKALNVNLP	244	ICMGKCILDITREED
200	CGGKALNVNLPMERL	245	KCILDITREEDSETE
201	ALNVNLPMERLTFEL	246	DITREEDSETEDSGH
202	NLPMERLTFELGVAI	247	EEDSETEDSGHGSST
203	ERLTFELGVAIDQYM	248	ETEDSGHGSSTESQS
204	FELGVAIDQYMVVFE	249	SGHGSSTESQSQCSS
205	VAIDQYMVVFEDVKG	250	SSTESQSQCSSQVSD
206	YMVVFEDVKGTGAE	251	SQSQCSSQVSDTSAP
207	VFEDVKGTGAESKDL	252	CSSQVSDTSAPAEDS
208	VKGTGAESKDLPSGH	253	VSDTSAPAEDSQRSD
209	GAESKDLPSGHGINN	254	SAPAEDSQRSDPHSQ
210	KDLPSGHGINNLDSL	255	EDSQRSDPHSQELHL
211	SGHGINNLDSLRDYL	256	RSDPHSQELHLCKGF
212	INNLDSLRDYLDGSV	257	HSQELHLCKGFQCFK
213	DSLRDYLDGSVKVNL	258	LHLCKGFQCFKRPKT
214	DYLDGSVKVNLEKKH	259	KGFQCFKRPKTPPPK
215	GSVKVNLEKKHLNKR	260	DTSAPAEDSQRSDPH
216	VNLEKKHLNKRTQIF	261	APAEDSQRSDPHSQE
217	KKHLNKRTQIFPPGL	262	EDSQRSDPHSQELHL
218	NKRTQIFPPGLVTMN	263	RSDPHSQELHLCKG
219	IFPPGLVTMNEYPV	264	DPHSQELHLCKGFQC
220	PGLVTMNEYPVPKTL	265	SQELHLCKGFQCFKR
221	TMNEYPVPKTLQARF	266	LHLCKGFQCFKRPKT
222	YPVPKTLQARFVRQI	267	CKGFQCFKRPKTPPP
223	KTLQARFVRQIDFRP	268	FQCFKRPKTPPPK

10.1.2 Polyomavirus BK-VP1 (NCBI: P03088)

1	MAPTKRKGECPGAAP	45	TITPKNPTAQSQVMN
2	KRKGECPGAAPKKPK	46	KNPTAQSQVMNTDHK
3	ECPGAAPKKPKEPVQ	47	AQSQVMNTDHKAYLD
4	AAPKKPKEPVQVPKL	48	VMNTDHKAYLDKNNA
5	KPKEPVQVPKLLIKG	49	DHKAYLDKNNAYPVE
6	PVQVPKLLIKGGVEV	50	YLDKNNAYPVECWVP
7	PKLLIKGGVEVLEVK	51	NNAYPVECWVPDPSR
8	IKGGVEVLEVKTGVD	52	PVECWVPDPSRNENA
9	VEVLEVKTGVDAITE	53	WVPDPSRNENARYFG
10	EVKTGVDAITEVECF	54	PSRNENARYFGTFTG
11	GVDAITEVECFLNPE	55	ENARYFGTFTGGENV
12	ITEVECFLNPEMGDP	56	YFGTFTGGENVPPVL
13	ECFLNPEMGDPDENL	57	FTGGENVPPVLHVTN
14	NPEMGDPDENLRGFS	58	ENVPPVLHVTNTATT
15	GDPDENLRGFSLKLS	59	PVLHVTNTATTVLLD
16	ENLRGFSLKLSAEND	60	VTNTATTVLLDEQGV
17	GFSLKLSAENDFSSD	61	ATTVLLDEQGVGPLC
18	KLSAENDFSSDSPER	62	LLDEQGVGPLCKADS
19	ENDFSSDSPERKMLP	63	GVGPLCKADSLYVS
20	SSDSPERKMLPCYST	64	PLCKADSLYVSAADI
21	PERKMLPCYSTARIP	65	ADSLYVSAADICGLF
22	MLPCYSTARIPLPNL	66	YVSAADICGLFTNSS
23	YSTARIPLPNLNEDL	67	ADICGLFTNSSGTQQ
24	RIPLPNLNEDLTCGN	68	GLFTNSSGTQQWRGL
25	PNLNEDLTCGNLLMW	69	NSSGTQQWRGLARYF
26	EDLTCGNLLMWEAVT	70	TQQWRGLARYFKIRL
27	CGNLLMWEAVTVQTE	71	RGLARYFKIRLRKRS
28	LMWEAVTVQTEVIGI	72	RYFKIRLRKRSVKNP
29	AVTVQTEVIGITSML	73	IRLRKRSVKNPYPIS
30	TEVIGITSMLNLHA	74	KRSVKNPYPISFLLS
31	IGITSMLNLHAGSQK	75	KNPYPISFLLSDLIN
32	SMLNLHAGSQKVHEH	76	PISFLLSDLINRRTQ
33	LHAGSQKVHEHGGGK	77	LLSDLINRRTQRVDG
34	SQKVHEHGGGKPIQG	78	LINRRTQRVDGQPMY
35	HEHGGGKPIQGSNFH	79	RTQRVDGQPMYGMES
36	GGKPIQGSNFHFFAV	80	VDGQPMYGMESQVEE
37	IQGSNFHFFAVGGEP	81	PMYGMESQVEEVRVF
38	NFHFFAVGGEPLEMQ	82	MESQVEEVRVFDGTE
39	FAVGGEPLEMQGVLM	83	VEEVRVFDGTERLPG
40	GEPLEMQGVLMNYRS	84	RVFDGTERLPGDPDM
41	EMQGVLMNYRSKYPD	85	GTERLPGDPDMIRYI
42	VLMNYRSKYPDGTIT	86	LPGDPDMIRYIDKQG
43	YRSKYPDGTITPKNP	87	PDMIRYIDKQGQLQT
44	YPDGTITPKNPTAQS	88	RYIDKQGQLQTKML

10.1.3 Polyomavirus BK-Agnoprotein (NCBI: P03085)

1	MVLRQLSRQASVKVG
2	LSRQASVKVGKTWT
3	ASVKVGKTWTGTKK
4	KVGKTWTGTKKRAQR
5	TWTGTKKRAQRIFIF
6	TKKRAQRIFIFILEL
7	AQRIFIFILELLLEF
8	FIFILELLLEFCRGE
9	LELLLEFCRGEDSVD
10	LEFCRGEDSVDGKNK
11	RGEDSVDGKNKSTTA
12	SVDGKNKSTTALPAV
13	KNKSTTALPAVKDSV
14	TTALPAVKDSVKDS

10.2 PEPTIDE LIBRARY JCV MAD1 STRAIN

10.2.1 Polyomavirus JC-Large T-Antigen (NCBI: AAA82102)

240	MDKVLNREESMELMD	283	YKKLMEKYSVTFISR
241	LNREESMELMDLLGL	284	MEKYSVTFISRHGFG
242	ESMELMDLLGLDRSA	285	SVTFISRHGFGGHNI
243	LMDLLGLDRSAWGNI	286	ISRHGFGGHNILFFL
244	LGLDRSAWGNIPVMR	287	GFGGHNILFFLTPHR
245	RSAWGNIPVMRKAYL	288	HNILFFLTPHRHRVS
246	GNIPVMRKAYLKKCK	289	FFLTPHRHRVSAINN
247	VMRKAYLKKCKELHP	290	PHRHRVSAINNYCQK
248	AYLKKCKELHPDKGG	291	RVSAINNYCQKLCTF
249	KCKELHPDKGGDEDK	292	INNYCQKLCTFSFLI
250	LHPDKGGDEDKMKRM	293	CQKLCTFSFLICKGV
251	KGGDEDKMKRMNFLY	294	CTFSFLICKGVNKEY
252	EDKMKRMNFLYKKME	295	FLICKGVNKEYLFYS
253	KRMNFLYKKMEQGVK	296	KGVNKEYLFYSALCR
254	FLYKKMEQGVKVAHQ	297	KEYLFYSALCRQPYA
255	KMEQGVKVAHQPDFG	298	FYSALCRQPYAVVEE
256	GVKVAHQPDFGTWNS	299	LCRQPYAVVEESIQG
257	AHQPDFGTWNSSEVP	300	PYAVVEESIQGGLKE
258	DFGTWNSSEVPTYGT	301	VEESIQGGLKEHDFN
259	WNSSEVPTYGTDEWE	302	IQGGLKEHDFNPEEP
260	EVPTYGTDEWESWWN	303	LKEHDFNPEEPEETK
261	YGTDEWESWWNTFNE	304	DFNPEEPEETKQVSW
262	EWESWWNTFNEKWDE	305	EEPEETKQVSWKLVT
263	WWNTFNEKWDEDLFC	306	ETKQVSWKLVTQYAL
264	FNEKWDEDLFCHEEM	307	VSWKLVTQYALETKC
265	WDEDLFCHEEMFASD	308	LVTQYALETKCEDVF
266	LFCHEEMFASDDENT	309	YALETKCEDVFLLMG
267	EEMFASDDENTGSQH	310	TKCEDVFLLMGMYLD
268	ASDDENTGSQHSTPP	311	DVFLLMGMYLDFQEN
269	ENTGSQHSTPPKKKK	312	LMGMYLDFQENPQQC
270	SQHSTPPKKKKKVED	313	YLDFQENPQQCKKCE
271	TPPKKKKKVEDPKDF	314	ENPQQCKKCEKKDQ
272	KKKKVEDPKDFPVDL	315	CKKCEKKDQPNHF
273	VEDPKDFPVDLHAFL	316	KCEKKDQPNHFNHHE
274	KDFPVDLHAFLSQAV	317	KDQPNHFNHHEKHYY
275	VDLHAFLSQAVFSNR	318	NHFNHHEKHYYNAQI
276	AFLSQAVFSNRTVAS	319	HHEKHYYNAQIFADS
277	AVFSNRTVASFAVY	320	HYYNAQIFADSKNQK
278	SNRTVASFAVYTTKE	321	AQIFADSKNQKSICQ
279	VASFAVYTTKEKAQI	322	ADSKNQKSICQQAVD
280	AVYTTKEKAQILYKK	323	NQKSICQQAVDTVAA
281	TKEKAQILYKKLMEK	324	ICQQAVDTVAAKQRV
282	AQILYKKLMEKYSVT	325	AVDTVAAKQRVDSIH

...Continued

Polyomavirus JC-Large T-Antigen (NCBI: AAA82102)

326	VAAKQRVDSIHMTRE	369	KRTQVFPPGIVTMNE
327	RVDSIHMTREEMLV	370	VFPPGIVTMNEYSVP
328	SIHMTREEMLVERFN	371	GIVTMNEYSVPRTLQ
329	TREEMLVERFNFLLD	372	MNEYSVPRTLQARFV
330	MLVERFNFLLDKMDL	373	SVPRTLQARFVRQID
331	RFNFLLDKMDLIFGA	374	TLQARFVRQIDFRPK
332	LLDKMDLIFGAHGNA	375	RFVRQIDFRPKAYLR
333	MDLIFGAHGNAVLEQ	376	IDFRPKAYLRKSLS
334	FGAHGNAVLEQYMAG	377	RPKAYLRKSLSCSEY
335	GNAVLEQYMAGVAWI	378	YLRKSLSCSEYLLEK
336	LEQYMAGVAWIHCLL	379	SLSCSEYLLEKRILQ
337	MAGVAWIHCLLPQMD	380	SEYLLEKRILQSGMT
338	AWIHCLLPQMDTVIY	381	LEKRILQSGMTLLLL
339	CLLPQMDTVIYDFLK	382	ILQSGMTLLLLLIWF
340	MDTVIYDFLKCIVL	383	GMTLLLLLIWFRPVA
341	VIYDFLKCIVLNIPK	384	LLLLIWFRPVADFAA
342	FLKCIVLNIPKKRYW	385	IWFRPVADFAAAIHE
343	IVLNIPKKRYWLFKG	386	PVADFAAAIHERIVQ
344	IPKKRYWLFKGPIDS	387	FAAAIHERIVQWKER
345	RYWLFKGPIDSGKTT	388	IHERIVQWKERLDLE
346	FKGPIDSGKTTLAAA	389	IVQWKERLDLEISMY
347	IDSGKTTLAAALLDL	390	KERLDLEISMYTFST
348	KTTLAAALLDLCGGK	391	DLEISMYTFSTMKAN
349	AAALLDLCGGKSLNV	392	SMYTFSTMKANVGMG
350	LDLCGGKSLNVNMPL	393	FSTMKANVGMGRPIL
351	GGKSLNVNMPLERLN	394	KANVGMGRPILDFPR
352	LNVNMPLERLNFELG	395	GMGRPILDFPREEDS
353	MPLERLNFELGVGID	396	PILDFPREEDSEAED
354	RLNFELGVGIDQFMV	397	FPREEDSEAEDSGHG
355	ELGVGIDQFMVVFED	398	EDSEAEDSGHGSSTE
356	GIDQFMVVFEDVKGT	399	AEDSGHGSSTESQSQ
357	FMVVFEDVKGTGAES	400	GHGSSTESQSQCFSQ
358	FEDVKGTGAESRDLP	401	STESQSQCFSQVSEA
359	KGTGAESRDLPSGHG	402	SQCFSQVSEASGAD
360	AESRDLPSGHGISNL	403	FSQVSEASGADTQEN
361	DLPSGHGISNLDCLR	404	SEASGADTQENCTFH
362	GHGISNLDCLRDYLD	405	GADTQENCTFHICKG
363	SNLDCLRDYLDGSVK	406	ENCTFHICKGFQCF
364	CLRDYLDGSVKVNLE	407	TFHICKGFQCFKKPK
365	YLDGSVKVNLERKHQ	408	CKGFQCFKKPKTPPP
366	SVKVNLERKHQNKRT	409	CFKKPKTPPPK
367	NLERKHQNKRTQVFP		
368	KHQNKRTQVFPPGIV		

10.2.2 Polyomavirus JC-VP1 (NCBI: AAA82101)

1	MAPTKRKGERKDPVQ	45	VQSQVMNTEHKAYLD
2	KRKGERKDPVQVPKL	46	VMNTEHKAYLDKNKA
3	ERKDPVQVPKLLIRG	47	EHKAYLDKNKAYPVE
4	PVQVPKLLIRGGVEV	48	YLDKNKAYPVECWVP
5	PKLLIRGGVEVLEVK	49	NKAYPVECWVPDPTR
6	IRGGVEVLEVKTGVD	50	PVECWVPDPTRNENT
7	VEVLEVKTGVDSITE	51	WVPDPTRNENTRYFG
8	EVKTGVDSITEVECF	52	PTRNENTRYFGTLTG
9	GVDSITEVECFLTPE	53	ENTRYFGTLTGGENV
10	ITEVECFLTPEMGDP	54	YFGTLTGGENVPPVL
11	ECFLTPEMGDPDEHL	55	LTGGENVPPVLHITN
12	TPEMGDPDEHLRGFS	56	ENVPPVLHITNTATT
13	GDPDEHLRGFSKSIS	57	PVLHITNTATTVLLD
14	EHLRGFSKSISISDT	58	ITNTATTVLLDEFGV
15	GFSKSISISDTFESD	59	ATTVLLDEFGVGPLC
16	SISISDTFESDSPNR	60	LLDEFGVGPLCKGDN
17	SDTFESDSPNRDMLP	61	FGVGPLCKGDNLYLS
18	ESDSPNRDMLPCYSV	62	PLCKGDNLYLSAVDV
19	PNRDMLPCYSVARIP	63	GDNLYLSAVDVCGMF
20	MLPCYSVARIPLPNL	64	YLSAVDVCGMFTNRS
21	YSVARIPLPNLNEDL	65	VDVCGMFTNRSGSQQ
22	RIPLPNLNEDLTCGN	66	GMFTNRSGSQQWRGL
23	PNLNEDLTCGNILMW	67	NRSGSQQWRGLSRYF
24	EDLTCGNILMWEAVT	68	SQQWRGLSRYFKVQL
25	CGNILMWEAVTLKTE	69	RGLSRYFKVQLRKRR
26	LMWEAVTLKTEVIGV	70	RYFKVQLRKRRVKNP
27	AVTLKTEVIGVTSLM	71	VQLRKRRVKNPYPIS
28	KTEVIGVTSLMNVHS	72	KRRVKNPYPISFLLT
29	IGVTSLMNVHSNGQA	73	KNPYPISFLLTDLIN
30	SLMNVHSNGQATHDN	74	PISFLLTDLINRRTP
31	VHSNGQATHDNGAGK	75	LLTDLINRRTPRVDG
32	GQATHDNGAGKPVQG	76	LINRRTPRVDGQPMY
33	HDNGAGKPVQGTSFH	77	RTPRVDGQPMYGMDA
34	AGKPVQGTSFHFFSV	78	VDGQPMYGMDAQVEE
35	VQGTSFHFFSVGGEA	79	PMYGMDAQVEEVRVF
36	SFHFFSVGGEALELQ	80	MDAQVEEVRVFEGTE
37	FSVGGEALELQGVLF	81	VEEVRVFEGTEELPG
38	GEALELQGVLFNYRT	82	RVFEGTEELPGDPDM
39	ELQGVLFNYRTKYPD	83	GTEELPGDPDMMRYV
40	VLFNYRTKYPDGTIF	84	LPGDPDMMRYVDKYG
41	YRTKYPDGTIFPKNA	85	PDMMRYVDKYGQLQT
42	YPDGTIFPKNATVQS	86	RYVDKYGQLQTKML
43	TIFPKNATVQSQVMN		
44	KNATVQSQVMNTEHK		

10.2.3 Polyomavirus JC-Agnoprotein (NCBI: AAA82098)

1	MVLRQLSRKASVKVS
2	LSRKASVKVSKTWS
3	KASVKVSKTWSGTKK
4	KVSKTWSGTKKRAQR
5	TWSGTKKRAQRILIF
6	TKKRAQRILIFLLEF
7	AQRILIFLLEFLLDF
8	LIFLLEFLLDFCTGE
9	LEFLLDFCTGEDSVD
10	LDFCTGEDSVDGKKR
11	TGEDSVDGKKRQRHS
12	SVDGKKRQRHSGLTE
13	KKRQRHSGLTEQTYS
14	RHSGLTEQTYSALPE
15	LTEQTYSALPEPKAT

10.3 PEPTIDE LIBRARY CMV AD169 STRAIN

10.3.1 CMV-pp65 (NCBI: DAA00112)

1	MESRGRRCPEMISVL	47	AFVFPTKDVALRHVV	93	FTSQYRIQGKLEYRH
2	GRRCPEMISVLGPIS	48	PTKDVALRHVVCAHE	94	YRIQGKLEYRHTWDR
3	PEMISVLGPISGHVL	49	VALRHVVCAHELVCS	95	GKLEYRHTWDRHDEG
4	SVLGPISGHVLKAVF	50	HVVCAHELVCSMENT	96	YRHTWDRHDEGAAQG
5	PISGHVLKAVFSRGD	51	AHELVCSMENTRATK	97	WDRHDEGAAQGDDDV
6	HVLKAVFSRGDTPVL	52	VCSMENTRATKMQVI	98	DEGAAQGDDDVWTSG
7	AVFSRGDTPVLPHET	53	ENTRATKMQVIGDQY	99	AQGDDDVWTSGSDSD
8	RGDTPVLPHETRLLQ	54	ATKMQVIGDQYVKVY	100	DDVWTSGSDSDEELV
9	PVLPHETRLLQTGIH	55	VIGDQYVKVYLESF	101	TSGSDSDEELVTTER
10	HETRLLQTGIHVRVS	56	DQYVKVYLESFCEDV	102	DSDEELVTTERKTPR
11	LLQTGIHVRVSQPSL	57	KVYLESFCEDVPSGK	103	ELVTTERKTPRVTGG
12	GIHVRVSQPSLILVS	58	ESFCEDVPSGKLFMH	104	TERKTPRVTGGGAMA
13	RVSQPSLILVSQYTP	59	EDVPSGKLFMHVTLG	105	TPRVTGGGAMAGAST
14	PSLILVSQYTPDSTP	60	SGKLFMHVTLGSDVE	106	TGGGAMAGASTSAGR
15	LVSQYTPDSTPCHRG	61	FMHVTLGSDVEEDLT	107	AMAGASTSAGRKRKS
16	YTPDSTPCHRGDNQL	62	TLGSDVEEDLTMTRN	108	ASTSAGRKRKSASSA
17	STPCHRGDNQLQVQH	63	DVEEDLTMTRNPQPF	109	AGRKRKSASSATACT
18	HRGDNQLQVQHTYFT	64	DLTMTRNPQPFMRPH	110	RKSASSATACTSGVM
19	NQLQVQHTYFTGSEV	65	TRNPQPFMRPHERNG	111	SSATACTSGVMTRGR
20	VQHTYFTGSEVENVS	66	PFMRPHERNGFTVL	112	ACTSGVMTRGRLKAE
21	YFTGSEVENVSVNVH	67	RPHERNGFTVLCPKN	113	GVMTRGRLKAESTVA
22	SEVENVSVNVHNPTG	68	RNGFTVLCPKNMIIK	114	RGRLKAESTVAPEED
23	NVSVNVHNPTGRSIC	69	TVLCPKNMIIKPGKI	115	KAESTVAPEEDTDED
24	NVHNPTGRSICPSQE	70	PKNMIIKPGKISHIM	116	TVAPEEDTDEDSDNE
25	PTGRSICPSQEPMSI	71	IIKPGKISHIMLDVA	117	EEDTDEDSDNEIHNP
26	SICPSQEPMSIYVYA	72	GKISHIMLDVAFTSH	118	DEDSDNEIHNPAVFT
27	SQEPMSIYVYALPLK	73	HIMLDVAFTSHEHFG	119	DNEIHNPAVFTWPPW
28	MSIYVYALPLKMLNI	74	DVAFTSHEHFGLLCP	120	HNPAVFTWPPWQAGI
29	VYALPLKMLNIPSIN	75	TSHEHFGLLCPKSIP	121	VFTWPPWQAGILARN
30	PLKMLNIPSINVHHY	76	HFGLLCPKSIPGLSI	122	PPWQAGILARNLVPM
31	LNIPSINVHHYPSAA	77	LCPKSIPGLSISGNL	123	AGILARNLVPMVATV
32	SINVHHYPSAAERKH	78	SIPGLSISGNLLMNG	124	ARNLVPMVATVQGQN
33	HHYPSAAERKHRHLP	79	LSISGNLLMNGQQIF	125	VPMVATVQGQNLKYQ
34	SAAERKHRHLPVADA	80	GNLLMNGQQIFLEVQ	126	ATVQGQNLKYQEFFW
35	RKHRHLPVADAVIHA	81	MNGQQIFLEVQAIRE	127	GQNLKYQEFFWDAND
36	HLPVADAVIHASGKQ	82	IFLEVQAIRETVEL	128	KYQEFFWDANDIYRI
37	ADAVIHASGKQMWQA	83	EVQAIRETVELRQYD	129	FFWDANDIYRIFAEL
38	IHASGKQMWQARLTV	84	IRETVELRQYDPVAA	130	ANDIYRIFAELEGVW
39	GKQMWQARLTVSGLA	85	VELRQYDPVAALFFF	131	YRIFAELEGVWQPAA
40	WQARLTVSGLAWTRQ	86	YDPVAALFFFDIDL	132	AELEGVWQPAAQPKR
41	LTVSGLAWTRQQNQW	87	VAALFFFDIDLLLQR	133	GVWQPAAQPKRRRHR
42	GLAWTRQQNQWKEPD	88	FFFDIDLLLQRGPQY	134	PAAQPKRRRHRQDAL
43	TRQQNQWKEPDVYYT	89	IDLLLQRGPQYSEHP	135	PKRRRHRQDALPGPC
44	NQWKEPDVYYTSAFV	90	LQRGPQYSEHPTFTS	136	RHRQDALPGPCIAST
45	EPDVYYTSAFVFPTK	91	PQYSEHPTFTSQYRI	137	DALPGPCIASTPKKH
46	YYTSAFVFPTKDVAL	92	EHPTFTSQYRIQGKL	138	GPCIASTPKKHRG

10.4 USEFUL LINKS

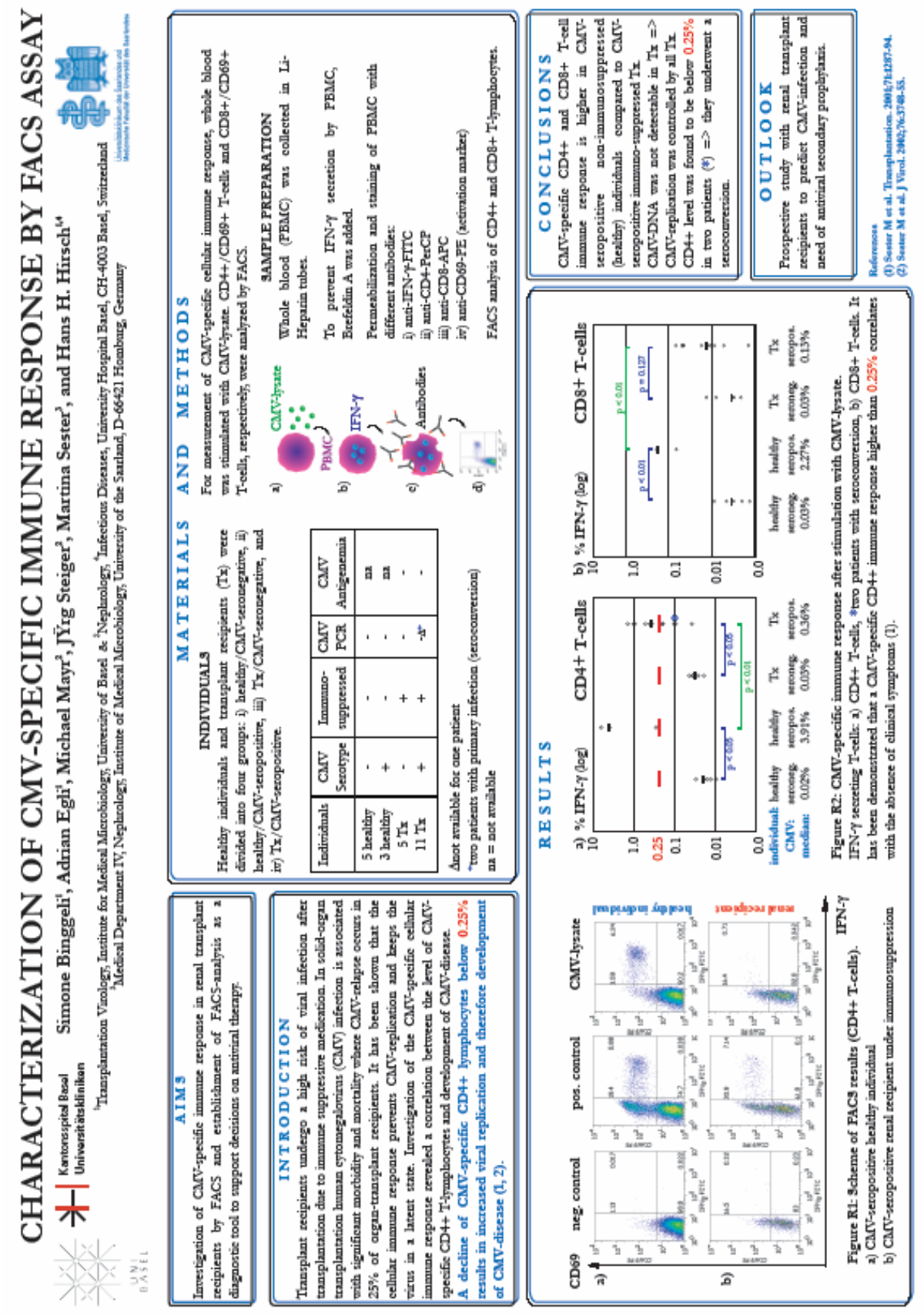
10.4.1 Swisstransplant
www.swisstransplant.org

10.4.2 National Center for Biotechnology Information (NCBI)
www.ncbi.nlm.nih.gov

10.4.3 Database for Peptide Motifs
www.syfpeithi.de
www-bimas.cit.nih.gov/molbio/hla_bind

10.5 POSTER PRESENTATIONS

10.5.1 8th Annual Meeting of ESCV (European Society for Clinical Virology), Geneva, Switzerland



10.5.2 Annual Congress SGAI-SSAI (Swiss Society for Allergology and Immunology), Basel, Switzerland