Characterizing Determinants of BK Polyomavirus-specific Immune Response

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1 Abbreviations

aa	Amino acid
ADCC	Antibody dependent cell cytotoxicity
AIM2	Absent in melanoma 2
ALR	AIM2-like receptor
APC	Antigen presenting cell
BCR	B cell receptor
BKPyV	BK polyomavirus
BKPyVAN	BKPyV-associated nephropathy
BKPyVHC	BKPyV-associated hemorrhagic cystitis
CARD	Caspase recruitment domain
CCR5	Chemokine receptor 5
CLIP	Class II-associated invariant chain peptide
CLR	C-type lectin receptors
CMX001	1-O-hexadecyloxypropyl-cidofovir
CNI	Calcineurin inhibitor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dsRNA	Double-stranded RNA
EBNA1	EBV nuclear antigen 1
EBV	Epstein- Barr virus
EGFP	Enhanced green-fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzime-linked immunospot
ER	Endoplasmic reticulum
EVGR	Early viral gene region
GEq	Genome equivalents
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HPyV	Human polyomavirus

HSCT	Hematopoietic stem cell transplantation
Hsp70	Heat-shock-protein-70
HSV	Herpes simplex virus 1
ICC	Intracytoplasmatic cytokine staining
IEDB	Immune Epitope Database and Analysis Resource
IFN	Interferon
IFN(x)R	IFN (x) receptor
Ii	Invariant chain
IL	Interleukin
IPS-1	IFN-β promoter stimulator-1
IS	Immunosuppressive drug
IRF	IFN-regulatory factor
JCPyV	JC polyomavirus
KSHV	Kaposi's sarcoma associated herpesvirus
KIPyV	KI polyomavirus
KIR	Killer-cell immunoglobulin-like receptor
KTR	Kidney transplant recipient
LANA	Latency associated nuclear antigen
LCMV	Lymphocytic choriomeningitis virus
LTag	Large tumor antigen
LVGR	Late viral gene region
MCC	Merkel cell carcinoma
MCMV	Murine cytomegalovirus
MCPyV	Merkel cell polyomavirus
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
miRNA	Micro-RNA
MPL-A	Monophosphoryl lipid A
MPyV	Murine polyomavirus
MWPyV	Malawi polyomavirus
MXPyV	Mexico polyomavirus
NCCR	Non-coding control region
NLR	NOD-like receptor
NJPyV	New Jersey polyomavirus
NK	Natural killer
OBD	ORI binding domain

ORF	Open reading frame
ORI	Origin of replication
PADRE	Pan HLA-DR binding epitope peptide
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
PLC	Peptide-loading complex
PMNs	Polymorphonuclear leukocytes
PP2A	Protein phosphatase 2A
PRR	Pattern recognition receptors
PyV	Polyomavirus
Rb	Retinoblastoma
RIG-I	Retinoic acid-inducible gene
RLRs	RIG-I like receptor
RPTEC	Renal tubular epithelial cells
RSV	Respiratory syncytial virus
ssRNA	Single-stranded RNA
sTag	Small tumor antigen
STLPyV	Saint Louis polyomavirus
SV40	Simian virus 40
TAP	Transporter associated with antigen processing
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptors
TNF	Tumor necrosis factor
Treg	Regulatory T cell
truncTag	Truncated tumor antigen
TSPyV	Trichodysplasia spinulosa polyomavirus
VLPs	Virus-like particles
WUPyV	WU polyomavirus
α-SNAP	α-Soluble N-ethylmaleimide-sensitive fusion attachment protein

2 Summary

BK polyomavirus (BKPyV) is one of now 13 human polyomavirus (HPyV) species detected in humans. BKPyV is only known to infect humans and seroprevalence rates of more than 90% have been reported in adult populations around the world. Following primary infection, BKPyV persists in the renourinary tract without causing any disease as evidenced by urinary shedding in 5% - 10% of healthy immunocompetent blood donors.

In immunocompromised persons, however, BKPyV can cause significant diseases whereby uncontrolled high-level replication may lead to organ invasive pathologies in kidneys, bladder, lungs, vasculature, and the central nervous system. The most consistently found diseases are BKPyV-associated hemorrhagic cystitis (BKPyVHC) in 5%-20% allogeneic hematopoietic stem cells transplant patients, and BKPyV-associated nephropathy (BKPyVAN) in 1%-15% of kidney transplant patients. BKPyVHC is highly symptomatic with pain, anemic bleeding, and increased mortality. BKPyVAN is asymptomatic except for progressive renal failure and premature return to dialysis. Both entities are characterized by high-level viral replication i.e. with urine BKPyV loads of 8-10 $\log_{10}$ Geq/mL, plasma BKPyV loads often above 4 $\log_{10}$ Geq/mL, and an allogeneic constellation between the virus-infected host cell and the available T-cell effectors. Despite these similarities, the clinical manifestations are strikingly different suggesting relevant, but experimentally undefined differences in pathogenesis. Thus, BKPyVHC typically occurs within 4 weeks after allogeneic HSCT and is confined to the bladder, and typically without kidney involvement. By contrast, BKPyVAN is diagnosed around 3-6 months after kidney transplantation and confined to the kidney allograft without causing cystitis. Although high-level BKPyV replication should be formally amenable to antiviral drug treatment, no effective and BKPyV-specific antiviral therapy is currently available. Therefore, a better understanding of the immune alteration in both diseases has been deemed essential to identify patients at risk and to develop prophylactic, preemptive and therapeutic strategies.

The currently recommended strategy for BKPyVAN is to screen kidney transplant patients for BKPyV replication and to promptly reduce immunosuppressive therapy in those with significant replication to facilitate mounting of BKPyV-specific T cell responses and thereby preventing progression to disease. This manoeuver has been linked to expanding BKPyV-specific T cell responses in the peripheral blood of kidney transplant patients. However, this approach may place patients at risk for

acute rejection episodes that predispose equally well to premature kidney transplant failure. Although the clinical feasibility of reducing immunosuppression and curtailing BKPyV replication has been shown to be effective in prospective cohort studies for many, but not all of kidney transplant patients, this approach has not been possible in allogeneic HSCT patients because of concurrent or imminent graft-versus host disease. Thus, there are significant gaps in the current understanding of the BKPyV–host interaction in the normal host and in the allogeneic setting, which need to be investigated for a more effective and safer management of these significant viral complications.

In this thesis, the interaction of BKPyV and the immune response has been approached from two different angles. In the first project, potential mechanisms of BKPyV immune evasion were studied. Here, we focused on a small accessory protein called agnoprotein encoded as a leader protein in the late viral early region (LVGR). Although HPyV genomes overall show a very similar genome organization, agnoproteins are only found in the genomes of BKPyV and JCPyV that have a kidney tropisms, but not in any of the other 11 presumably non-renotropic HPyVs. We hypothesized that agnoprotein could play a role in immune evasion by downregulating HLA expression. The effects of agnoprotein were studied on HLA class I and II expression *in vitro* by flow cytometry following transfection of primary human renal tubular epithelial cells, which are the viral target of BKPyV-associated nephropathy. In addition, transfected human UTA-6 cells were studied as well as UTA-6 cells bearing a tetracycline-regulated agnoprotein. As control, the effects were compared with the ICP47 protein of Herpes simplex virus-1, which has been previously reported to effectively down-regulate HLA class I. Although both viral proteins share some similarities at the protein level, our results showed that BKPyV agnoprotein did not down-regulate HLA class I or class II molecules. Also, there was not inhibitory effect on the increase of HLA-class I or class-II surface expression following exposure to interferon-γ. By contrast, ICP47 reduced HLA class I surface expression, but not class II. We also evaluated effects of agnoprotein on virus epitope-specific T-cell killing by ^{51}Chromium release assay, however no interference could be observed. We concluded that agnoprotein did not contribute to these types of HLA-dependent immune evasion processes. However, further investigations are needed to understand if agnoprotein could contribute to viral immune escape by other mechanisms.

In the second project, we aimed at better characterizing BKPyV-specific CD8 T cell immunity targeting epitopes encoded in the early viral gene region (EVGR). Selected coding sequences of the BKPyV EVGR were submitted to two web-based computer

algorithms (SYFPEITHI, IEDB) in order to predict immunodominant 9mer epitopes presented by 14 frequent HLA-class I molecules. For an experimental confirmation, 97 different 9mer epitopes were chemically synthesized and tested in 42 healthy individuals. A total of 39 epitopes could be confirmed by interferon-γ ELISpot assay in at least 30% of healthy individuals. Interestingly, most of the 9mer epitopes appeared to cluster in short amino acid stretches, and some 9mer could be presented by more than one HLA class I allele as expected for immunodominant domains. HLA-specific presentation was demonstrated by 9mer- MHC-I streptamers for 21/39 (54%) epitopes. The 9mer dependent T-cell killing by ^{51}Chromium release assay and the CD107a surface detection indicated that the 9mer epitopes could be recognized by cytotoxic T-cells. Moving to a clinically relevant situation, 13 9mer epitopes could be validated in 19 kidney transplant patients protected from, or recovering from, BKPyV viremia. The results suggest that, pending further corroboration in larger patient populations, novel 9mer epitopes can be identified, which are associated with CD8 T cell control of BKPyV replication. Thus the identified immunodominant 9mer T-cell epitopes could be further developed for clinical assays to better predict the risk and the recovery of BKPyV diseases, help guiding immunosuppression reduction, and to develop specific adoptive T-cell therapy or vaccine responses to prevent or treat BKPyV-associated diseases.

3 Introduction

3.1 Virus-host interaction

Viruses are among the smallest of all self-replicating organisms present in nature, being constituted in the most basic cases by a little as a small segment of nucleic acid encapsidated in a protein shell. Viruses do not have their own metabolism, rather they need to parasitize cells and subvert their intracellular machinery in order to replicate and possibly transmit to new potential hosts (Walsh and Mohr 2011). Hosts and their cells, on the other hand, have developed defense mechanisms in order to protect from virus infection and the associated damage resulting from virus replication.

The infection of a specific host cell (cell tropism) depends mainly on the presence of the appropriate receptors on the cell surface, to which the virus must attach in order to gain entry into the cell. Upon cell entry, in case of lytic infection, the cellular replication machinery is redirected, resulting in viral genome replication, with consequent protein synthesis, assembling and packaging into new viral particles, and finally exit the cell. In the case of latent infection, viruses express no or only latency associated genes causing hardly or no damage to the host cell. Latent or persistent, viruses need to avoid immune recognition; therefore they have evolved mechanisms of immune escape, which may involve dedicated immune evasion proteins. As disadvantage, no active virus transmission occurs during latency phase.
Viral evolution, which involves the parallel generation of different viral variants constituting a viral swarm called quasispecies, is important for successful spreading in a given host and counteracting host cell defenses. Rapid generation of viral mutant variants may permit escape from host cell defenses, and more efficient replication capacity (viral fitness) (Domingo 2007, Ojosnegros, Perales et al. 2011).

In the next paragraphs, aspects of how viruses and hosts interact are presented as well as how the immune system responds to infection, where and why the immune system can fail in mounting an efficient response, and how it can overcome these challenges. This knowledge is deemed essential for finding better strategies to prevent viral replication and disease (Ayres AnnuRevImmunol2012).

3.1.1 Host immune response to viruses

Once a virus infects a host, it eventually needs to enter the relevant host cells in order to survive, replicate, and produce viral progeny. The host needs to build up an effective defense mechanism to protect himself against the devastating effects of viral infection, and such protective responses are mediated by the immune system (Hirsch 2005).

In humans as a member of the mammalian species, the immune system is organized in two main compartments, which act in a cooperative and often sequential way:
1) The innate immune system, which detects the presence of “non-self” through germline-encoded pattern-recognition receptors (PRRs), capable of distinguish nonself- molecules from self-molecules (Brubaker, Bonham et al. 2015), and consequently initiates mechanisms aiming at eliminate pathogens. Innate immunity also activates adaptive immune responses.
2) The adaptive immune system, which at the first encounter with the “non-self” entity acts as a second line of defense, is characterized by antigen-specificity and immunological memory. Immunological memory describes the observation that once an antigen is encountered for a second or repeated time, the adaptive immune response is faster and more effective (Zielinski, Corti et al. 2011).

3.1.1.1 Innate immunity

Epithelial barriers on all body surfaces, e.g. on the skin, eyes, in the respiratory or gastrointestinal tract, act as a first line of defense to prevent virus entry and spread within the host. These barriers together with mechanical and biochemical clearance through pH and enzymes are considered part of the unspecific defense. Once the pathogen succeeds in entering through the anatomical barriers, a rapid innate immune response may start immediately (Brubaker, Bonham et al. 2015).
The first innate components include preformed soluble molecules and epithelial secretions: lysozyme that is an antimicrobial enzyme able to digest bacterial cell walls, defensins which are peptides that can lyse bacterial cell membranes, and the complement system, which is constituted by several plasma proteins acting hierarchically and sequentially, targeting pathogens for both direct lysis and phagocytosis by cells of the innate immune.

The cellular effectors of the innate immune response consist of natural killer (NK) cells, macrophages, γ/δ-T lymphocytes, dendritic cells (DCs), polymorphonuclear leukocytes (PMNs) such as neutrophils, basophils, and eosinophils. These cells are able to recognize viruses as non-self. Typical pathogen structures, viral proteins and nucleic acids (named as pathogen-associated molecular patterns-PAMPs) can be distinguished from cellular counterparts by cellular PRR, present either in the cell cytoplasm or on cellular membranes, where they detect viral components.

3.1.1.1.1 Sensors of “non-self” in the innate immune response

Most PRRs can be included into one of five families according to their protein domain homology: there are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-I like receptors (RLRs), and the AIM2-like receptors (ALRs) (Figure 1). According to cellular localization, they can be divided in two main classes: unbound intracellular receptors (NLRs, RLRs, and ALRs) , and membrane-bound receptors (TLRs and CLRs) (Kumar, Kawai et al. 2011). TLRs and CLRs are found at the cell surface or on endocytic compartments. These receptors detect the presence of microbial ligands in the extracellular space and within endosomes. The NLRs, RLRs, and ALRs sense the presence of intracellular pathogens. Sensing of PAMPs by PRRs leads to the production of chemical messages, proinflammatory cytokines and interferons (IFN), that are crucial for initiating and modulating immune responses, and aiming at containing the spread of an initial infection (Takeuchi and Akira 2010)**(Figure 1).**

The RLR family consists of retinoic acid-inducible gene-I (RIG-I) and the melanoma differentiation gene 5 (MDA5). These proteins are composed of two N-terminal caspase recruitment domains (CARD), a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain (Kawai and Akira 2006). RIG-I detects double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA). These types of RNAs are usually not found in the cytoplasm of uninfected cells; rather they are typically products of viral replication. Once RIG-I binds viral RNAs, the CARD domain triggers signaling cascades by interacting with the N-terminal CARD-containing adaptor IFN-β-promoter stimulator 1 (IPS-1), which is located on the mitochondrial membrane, and subsequentially activates the transcription factors IRF-3 and NF-kB, leading to the synthesis of type 1 IFN and other proinflammatory cytokines. It has also been reported that RIG-I can be activated in presence of DNA virus, a template

of dsDNA is converted into dsRNA by RNA polymerase III DNA sensor, activating RIG-I and subsequently leading to the induction of type I IFNs (Chiu, Macmillan et al. 2009).

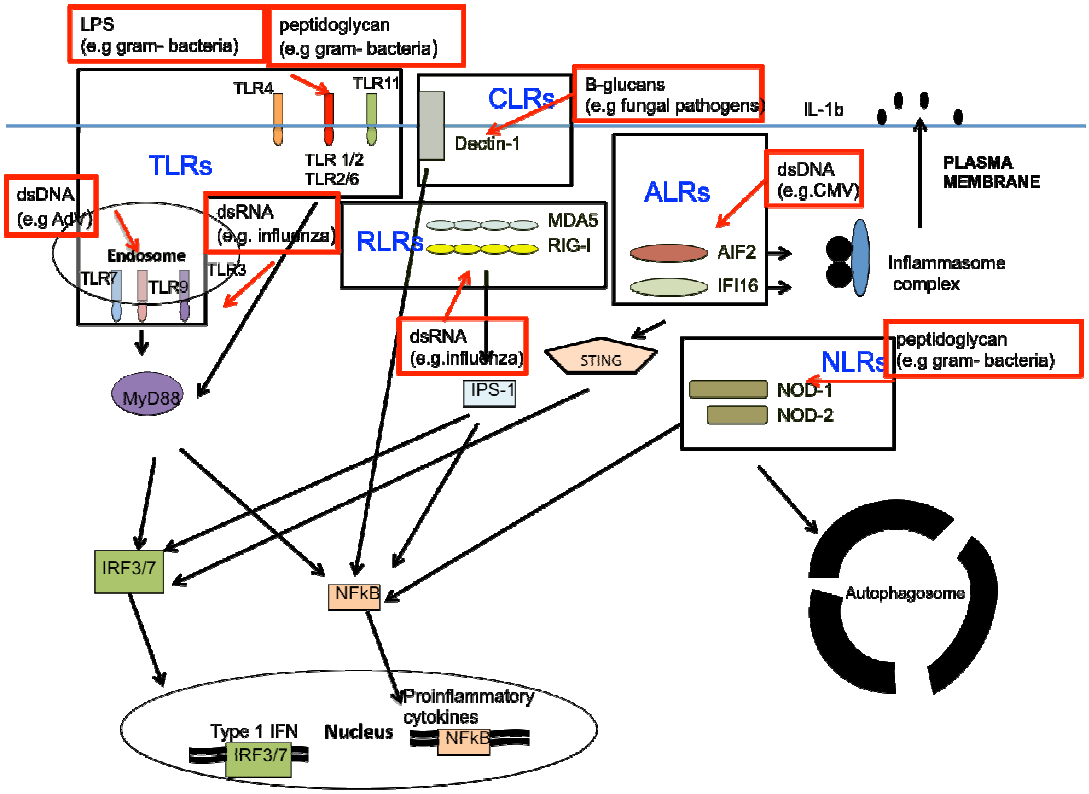

Figure 1: Summary of pattern recognition receptors and activation of innate immune responses.

NOD1 and NOD2 are key members of the NLR family (Saleh 2011). They are cytosolic proteins constituted by C-terminal ligand-binding LRRs, a central NACHT domain, and a single (NOD1) or two (NOD2) N-terminal CARD domains. They are involved in detection of components of bacterial outer membrane. Upon binding, interactions via CARD domain trigger NF-kB pathway and production of proinflammatory cytokines (Park, Kim et al. 2007). However, NOD1 and NOD2 can also eliminate pathogens independently from NF-kB, by inducing autophagy, which is a process in which self-proteins and damaged organelles are degraded in double-membraned vesicles called autophagosomes (Travassos, Carneiro et al. 2010).

The ALRs family includes AIF2 and IFI16, two receptors having a PYHIN domain for protein-protein interactions and a DNA-binding HIN-200 domain, involved in sensing cytoplasmic DNA, as viral DNA viruses. Upon detection of DNA, AIF2 promotes the

inflammasome formation. This multiprotein complex mediates the start of an innate immune response characterized by the secretion of proinflammatory cytokines and a rapid form of cell death (pyroptosis) that contributes to inflammation (Lamkanfi and Dixit 2014). IFI16 activates inflammasome formation, and interacts with STING (stimulator of IFN gene), that by activating IRF3 leads to the production of IFN.

CLRs recognize a wide range of microorganisms, including fungi and bacteria, and all share a characteristic C-type lectin-like domain. Dectin-1 is a member of CLRs and recognizes mainly fungal antigens, upon binding it promotes ligand uptake by phagocytosis and the initiation of a signaling cascade that regulates gene expression and cytokine production.

Other important detectors of viruses are among the membrane-bound toll-like receptors (TLRs), which sense viral glycoproteins, dsRNA, ssRNA, and the CpG sequence in viral DNA (Szabo and Rajnavolgyi 2013). At least 10 TLRs have been identified in humans, characterized by an extracellular domain, a transmembrane domain, and an intracellular Toll/IL-1R homology (TIR) domain.
In particular, TLR1 TLR2, TLR4, TLR5 TLR6, and TLR11 are expressed on the cell surface, while TLR3, TLR7,TLR9 and TLR10 are localized within cytoplasmic compartments, such as endosomes (Matsumoto, Funami et al. 2003, Takeuchi and Akira 2010). TLRs can activate different transcriptional responses depending on which adaptor set is utilized. Among the TLR family members, TLR3, TLR7, TLR8, and TLR9, recognize nucleic acids derived from viruses and TLR9 is the most important in sensing viral DNA, as it can detect CpG DNA sequences, which are characteristic of viral genomes, in fact it has been shown that TLR9 is involved in recognition of DNA viruses, such as hepatitis B virus (HBV), murine cytomegalovirus (MCMV) and Epstein- Barr virus (EBV).
It has been demonstrated the importance of TLR9 also in human polyomaviruses (HPyV), in fact a recent report demonstrated the expression of TLR2, 4, 5, 7 and 9 in Merkel Cell Carcinoma tumor specimen, but a decreased expression of TLR9 correlated strongly with Merkel Cell polyomavirus (MCPyV) positivity (Jouhi, Koljonen et al. 2015). In another study it has been shown that Large T antigen of MCPyV was the responsible for a decreased TLR9 expression, and the same observation was done for BKPyV, although at a lower extent (Shahzad, Shuda et al. 2013).
Sensing of viral DNA induces trafficking of TLR9 from the endoplasmic reticulum to the endolysosome, a subsequent cleavage by proteases present in the endolysosome, and recruitment of a complex of proteins including MyD88, an

adaptor protein displaying a TIR domain. Its activation can trigger a signaling cascade leading to NF-kb translocation into the nucleus and subsequent expression of proinflammatory cytokine genes, or another pathway leading to IRF7 phosphorylation, with consequent upregulation of the expression of type I IFN genes (Takeuchi and Akira 2010).

3.1.1.1.2 Effectors of innate immune activation

Interferons play an important role in the resistance against viral infection by binding to a common cell surface receptor on the infected cell as well as on neighbouring uninfected cells (Akira and Takeda 2004). Interferon family is constituted by distinct proteins grouped into three classes according to their receptor complexes. Type I IFNs includes in humans IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, type II IFN is consists only by IFN -γ, and type III IFN is represented by IFN-λ. Type I IFNs signal through heterodimeric receptor complexes. For type I IFN, the receptor is constituted by the IFN-α receptor 1 (IFNAR1) and IFNAR2 subunits. Type II IFNs signal through heterodimers consisting of IFN-γ receptors 1 (IFNGR1) and 2 (IFNGR2), whereas type III IFN signal through interleukin-10 receptor 2 (IL-10R2) and IFN-λ receptor 1 (IFNLR1) heterodimers. Engagement of both type I and type III IFNs to their receptors triggers phosphorylation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which in turn phosphorylate the receptors and leads to the recruitment and activation of signal transducers and activators of transcription 1 and 2 (STAT1 and 2). A heterodimer constituted by STAT1 and 2 recruits the IFN-regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 (ISGF3). Binding of type II IFN dimers to the IFNGR1/2 leads to phosphorylation of JAK1 and JAK2, and consequent recruitment and phosphorylation of STAT1. Phosphorylated STAT1 homodimers form the IFN-γ activation factor (GAF). Both ISGF3 and GAF translocate to the nucleus and permit expression of genes regulated by IFN-stimulated response elements (ISRE) and gamma-activated sequence (GAS) promoter elements, respectively, resulting in expression of antiviral genes (Schneider, Chevillotte et al. 2014).

In theory all humans cells can synthesize IFNα/β, however some cells show a better ability to produce these cytokines, as the precursors of plasmacytoid dendritic cells (DC). As main function, IFN-α and IFN-β interfere with virus replication in neighboring, not yet infected cells by activating a set of antiviral functions. This includes gene product leading to the destruction of mRNAs and inhibiting the

translation of viral proteins. They can induce major histocompatibility complex (MHC) class I expression in most cell types, and increase the synthesis of MHC class I molecules in newly infected cells, so that they can be more easily recognized by CD8 cytotoxic T cells (Schneider, Chevillotte et al. 2014). IFNα/β can also activate NK cells, and thereby inducing the killing of infected cells during the innate immune response (Bogdan 2000, Jost and Altfeld 2013).

IFN-γ is induced upon stimulation of epitope-specific T cells and NK cells, resulting in cellular immune responses, activation of macrophages and NK cells, it promotes upregulation of Human Leukocyte Antigen (HLA) class I and II expression on B cells and macrophages, and at higher levels induce class II on tissue cells to enhance antigen presentation. IFN-γ is also considered the key cytokine in the T lymphocyte helpers (Th) Th1 immune response, in fact they secrete IFN-γ, which as consequence induces more undifferentiated CD4+ lymphocytes to differentiate into Th1 cells, in a positive feedback loop way, suppressing Th2 cell differentiation.

The IFN-λ family is the most recently discovered group of IFNs, comprising four homologous members. It has been demonstrated that polymorphisms in IFN-λ3 gene, leading to its reduced expression, are associated to decreased replication of CMV and lower rates of clearance of HCV (Egli, Santer et al. 2014). Almost any cell type is able to express IFN-λ 1–3 in response to viruses, but it is mainly produced by DCs (Egli, Santer et al. 2014).

DCs are professional antigen presenting cells (APC), meaning that they can process an antigen and subsequently present it on the cell surface to the T cells. They act as a bridge between the innate and the adaptive immune system. Upon encounter with pathogen they can induce secretion of cytokines (e.g. IFN-α), which in turn can activate eosinophils, macrophages, and natural killer (NK) cells. Following antigen uptake and the respective activation, they migrate to lymphoid organs where, after maturation, they display major histocompatibility complexes with the digested peptides to T cells. The recognition of these MHC-peptide complexes is key to triggering the adaptive immune response. A subset of DCs is constituted by plasmacytoid DCs**.** They are present in the bone marrow and all peripheral organs, and respond to viral infection with a massive production of type I interferons, however, they also can act as antigen presenting cells and control T cell responses.

Macrophages can also present antigens, playing a crucial role in starting the immune response. Monocytes and macrophages are secretory cells, producing enzymes, complement proteins, and regulatory factors such as interleukin-1 (IL-1). Monocytes can migrate to tissues and differentiate into DCs, mainly during inflammation processes.

NK cells are another key component of innate immunity. They display an antigen-independent lytic activity. Their effector functions are the result of the balance of activating and inhibitory signals provided by killer-cell immunoglobulin-like receptors (KIRs) through the interaction with specific HLA class I ligands.

3.1.1.2 Adaptive immunity

d Engagement of such receptors, in the presence of additional signals, activates proliferation, differentiation, and the effector phase of the adaptive immune response. The specificity of adaptive immune response is the result of genetic mechanisms occurring during lymphocyte development in the bone marrow and thymus to generate a wide range of variants of the genes encoding the lymphocyte receptors, known as somatic recombination of variable (V), joining (J), and in some cases, diversity (D) gene segments. The main cellular component of adaptive immunity is constituted by lymphocytes, which develop in the thymus (T lymphocytes), or in the bone marrow (B lymphocytes) and display by receptors with differentiated structure and function.

Lymphocyte antigen receptors, in the form of immunoglobulins on B cells and T-cell receptors on T cells (Aleman, Rahbin et al.), are the means by which lymphocytes detect antigens in their environment. The receptors produced by each lymphocyte are characterized by unique antigen specificity, given by the structure of their antigen-binding site. The range of different antigen specificities in the antigen receptor is due to variation in the amino acid sequence at the antigen-binding site, which is constituted by a variable (V) region of the receptor protein chains. In each chain the V region is linked to an invariant constant (C) region, which can provide effector or signaling functions. In B cells, the rearranged V region is known to undergo additional modification, known as somatic hypermutation, occurring when B cells encounter the antigen and become activated.

3.1.1.2.1 T cells

T lymphocytes mediate cellular immune responses to antigens, recognized by the T cell receptor (Aleman, Rahbin et al. 2013), heterodimeric molecule composed of two trans-membrane glycoprotein chains, α and β. The extracellular portion of each chain consists of two domains displaying a V region and a C region, forming antiparallel β-sheets. The C region is proximal to the cell membrane, followed by a transmembrane region and a short cytoplasmic tail, while the V region is responsible for antigen binding. TCR is noncovalently associated with the nonpolymorphic CD3 proteins forming the TCR complex, needed in the T cell activation signaling. T cells also express on their surface co-receptor molecules, necessary for recognition and activation. During development in the thymus, thymocytes express both co-receptors CD4 and CD8, then after a positive selection process, cells expressing TCRs with potentially useful ligand specificities are identified, and thymocytes resulting from this selection ultimately develop into either CD4 or CD8 expressing cells with a lineage fate determined by the MHC restriction specificity of their TCR. In particular, cells with a MHC class II restricted TCRs differentiate into CD4+ T cells, whereas those receiving signals through MHC class I will become CD8+ T cells. Transcription and nuclear factors are involved in T cell commitment, the most important for CD4+ lineage has been shown to be Th-POK, a zinc finger protein, whereas RUNX transcription factors lead the commitment to CD8+, by binding a sequence in the gene encoding for Th-POK, inhibiting its expression. Also GATA3, an enhancer-binding zinc-finger protein, has an important role in CD4 lineage choice, in fact its sustained expression blocks the generation of CD8+ cells (Singer, Adoro et al. 2008). Once T cells have expressed their receptors and co-receptors, they migrate to the periphery and can be activated upon MHC:peptide encounter. T cells require three collaborative but distinct signals for efficient activation. The first signal is provided by the engagement of the TCR complex to its specific peptide antigen, bound to the MHC molecules on the surface of antigen presenting cells (APC) also through co-receptor molecules, that are CD4 or CD8, expressed at the T cell surface close to the TCR molecule. The second step is a co-stimulatory signal provided by engagement of T cell surface receptor CD28 with the specific ligands on APC, that are B7.1 and B7.2 (CD80, CD86), whereas signaling through the TCR alone without signal two can lead to a state of T cell unresponsiveness that is termed anergy or to apoptosis. Antigen recognition by TCR ultimately induces the synthesis of transcription factors as NFAT (nuclear factor of activated T cells), that activates transcription of IL-2, leading to cell proliferation. Further co-stimulatory signals driving proliferation include

interaction of CD40 ligand (CD154) with CD40 expressed on APCs. Upon activation, CD8+ and CD4+ T cells can exert their effector functions, meaning cytotoxic activity and helper function, respectively.

The cytokines present in the milieu during the activation process lead to CD4+ T cells to differentiate into one of several Th, or peripherally derived regulatory T (Treg) cells subsets, or even cytotoxic CD4+ cells, for example in presence of IFN and IL-12 commitment towards Th1 cells is driven, while IL-4 leads to Th2, transforming growth factor (TGF)-β, IL-6, IL-21 and IL-23 to Th17, while IL-2 and TGF-β induce Tregs, and IL-2 can lead to cytotoxic CD4+ T cells. Each Th subset is determined by a specific gene expression program, under the control of a lineage-defining transcription factors, which is T-bet, member of the T□box family, for Th1 cells, GATA3 for Th2 cell, retinoic acid receptor-related orphan receptor-γt (RORγt) for the Th17 cell lineage, forkhead box P3 (FOXP3) for Treg cells, and eomesodermin (EOMES) for cytotoxic CD4 T cells. The distinct gene expression profile of CD4+ subsets is defined by the signature cytokines that they express, their distinct homing properties and their specialized effector functions (Swain, McKinstry et al. 2012) **(Figure 2).**

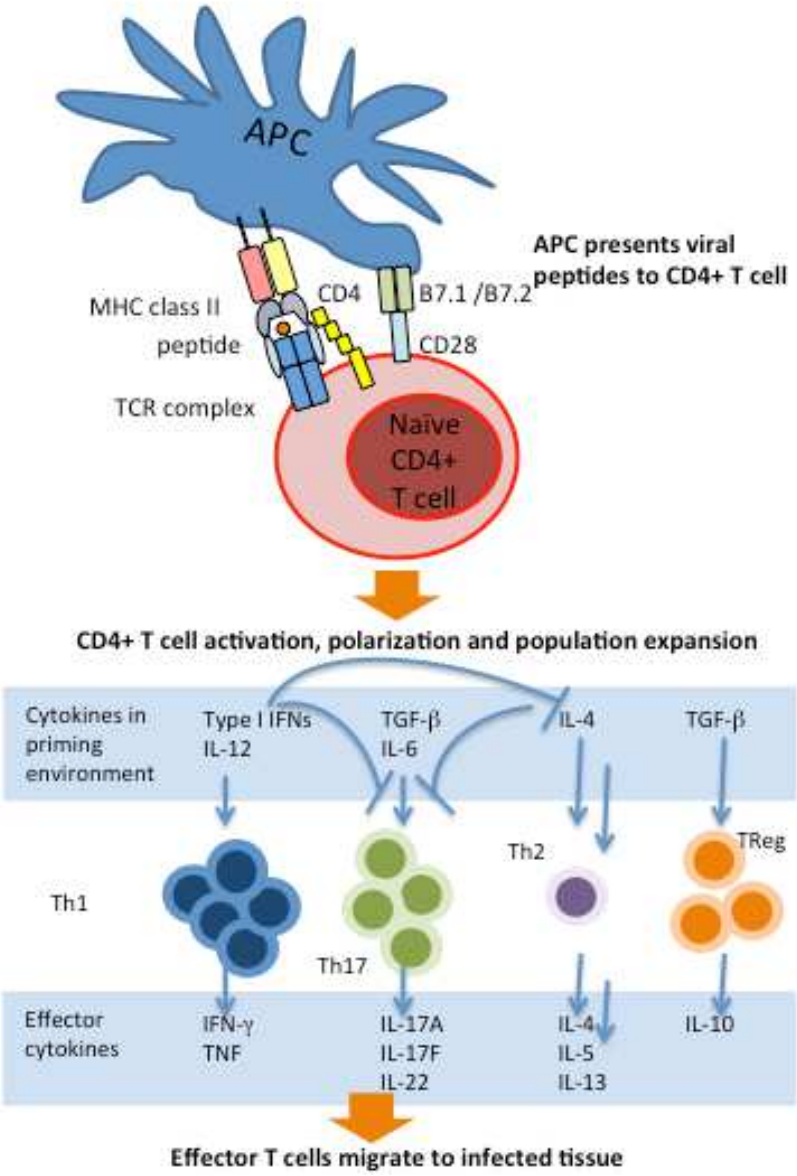

Figure 2: Generation of antiviral CD4+ T cells modified from (Swain, McKinstry et al. 2012).

Typically, Th1 cells are involved in the immune response to intracellular pathogens, as viruses, through the production of IFN-γ, TNF-α, and IL-2, whereas Th2 cells which produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, evoke stronger antibody responses and are involved in the immune responses against extracellular pathogens and parasites, however Th2 cells are demonstrated to contribute to antiviral immunity providing efficient help for the generation of neutralizing antibodies.

T helper subsets have been identified characterized by proinflammatory IL-17 and IL-22 production (Th17), mainly involved in autoimmune diseases, and in resistance to extracellular bacterial and fungal infections. Their role has been also investigated in the context of viral infection, e.g. in case of HBV, respiratory syncytial virus (RSV), and human cytomegalovirus (HCMV). Their proinflammatory properties may be detrimental or beneficial, it depends on the disease pathogenesis, the tissue damage can caused either by direct virus replication or immunopathology. In the first scenario Th17 could be of help in the disease prevention and/or resolution (Feng, Yin et al. 2015, Mangodt, Van Herck et al. 2015, Wunsch, Zhang et al. 2015).

Recently, another CD4+ helper population named Th9 cells has been identified. They secrete IL-9, IL-10 and IL-21, are primed in response to TGF-β and IL-4 and have been shown to contribute to inflammation in several autoimmune disease models. (Dardalhon, Awasthi et al. 2008). Reports indicate Th9 as major contributors to human atopic disease have been linked to the development of asthma and food allergies in humans, and to the pathogenesis of inflammatory bowel disease. Conversely, it has been observed that the number of Th9 in patients with melanoma is reduced compared with healthy individuals, suggesting a protective role (Kaplan, Hufford et al. 2015).

Th22, characterized but the production of IL-22 and TNF-α, have been only recently identified (Trifari, Kaplan et al. 2009). They have been observed to play an important role in epidermal immunity, remodeling and autoimmune diseases (Eyerich, Eyerich et al. 2009), but recent reports have investigated their role in HIV context, in infected patients an impairment of Th22 cells was observed, suggesting a possible protective role (Kim, Nazli et al. 2012).

Regulatory T cells (Tregs) include a heterogeneous group of T lymphocytes that are critical for the control of potentially dangerous autoreactive T cells in the periphery. They play an important role in the immune homeostasis and peripheral self-tolerance. Tregs may not be proper T helper cells, but it is known that inducible Tregs differentiate from the same cell precursor from which Th subsets develop. In viral disease they can suppress immunopathology, but also cytotoxic T lymphocytes (CTL) responses, promoting viral replication. For example, their role in antiviral immunity has been investigated in the context of chronic hepatitis B infection (Peng, Li et al. 2008). In this setting the expression of programmed death ligand 1 (PD-L1) on Tregs of patients induced an inhibitory signal into effector T cells by the

interaction with programmed death-1 (PD-1), correlating with high levels of markers of liver injury, thus suggesting a contribution of progression of HBV infection (Feng, Cao et al. 2015) (Aubert, Kamphorst et al. 2011). Persistent infection by EBV can induce increase of Tregs, as observed in patients suffering from EBV correlated nasopharyngeal carcinoma, such cells can suppress the proliferation of autologous CD4+CD25- T cells preventing viral clearance (Lau, Cheng et al. 2007), while in patients undergoing hematopoietic stem cell transplant and reactivating CMV, Tregs do not inhibit pathogen clearance by effector T cells (Velaga, Ukena et al. 2013).

CD8+ T cells exert their cytotoxic effector function through at least 3 different mechanisms. The first one is the production and calcium dependent release of specialized lytic granules upon recognition of antigen. These granules are modified lysosomes that contain cytotoxic effector proteins. One of these cytotoxic proteins, perforin, polymerizes to form trans-membrane pores in target cell membrane, as a consequence water and salts pass rapidly into the cell, and without integrity of the cell membrane the cell dies rapidly. Other cytotoxic proteins consist of proteases called granzymes, which enter the targeted cell through perforin induced pores, and upon cleavage of intracellular proteins induce apoptosis.
The second way of CD8+ T cell elimination of infected cells is via FasL/Fas (CD95ligand/ CD95) interactions. Activated CD8+ T cells express at their surface FasL which binds to its receptor Fas, that is a member of tumor necrosis factor (TNF) receptor superfamily present on the surface of the target cell. The FasL/Fas interaction induces the activation of the caspases 2 and 8, which also results in apoptosis of the target cell.
The third cytotoxic mechanism of CD8+ T cells consists in secreting cytokines, mainly IFN-γ and TNF-α, contributing to host defense in several ways. IFN-γ directly inhibits viral replication and upregulates expression of MHC class I, increasing the probability that infected cells will be recognized as target cells. IFN-γ also activates macrophages in synergy with TNF-α, recruiting them to sites of infection both as effector cells and as APCs.

T cell response after viral clearance is characterized by a contraction and resolution phase during which the majority of the effector T cells die. These cells enter the third stage, the ‘memory’ phase. Memory T cells are crucial in case of subsequent encounter with the antigen.
In particular T cells in the memory phase can be grouped, according their effector functions, and responsiveness to antigen or cytokines, in effector memory T cells

(TEM) that after being stimulated migrate to the periphery and exert immediate effector function, and central memory T cells (TCM) with lower effector function, but high proliferating potential and differentiation into effector cells upon antigenic stimulation. TCM express CCR7 and CD62L, two homing receptors that are also present in naïve T cells, but differently from those, TCM show higher sensitivity to antigenic stimulation and are less dependent on co-stimulation. Upon TCR engagement, TCM produce mainly IL-2, but after proliferation they efficiently differentiate into effector cells. TEM loose the expression of CCR7, are heterogeneous for CD62L expression, and are characterized by rapid effector function. Some CD8 TEM express CD45RA, and are named as TEMRA showing the largest amount of cytotoxic granules (Sallusto, Lenig et al. 1999).

3.1.1.2.2 B cells

Other essential players in adaptive immunity are B cells, or bone marrow-derived lymphocytes. The maturation of B cells occurs in the secondary lymphoid organs, as the spleen and lymph nodes, where they can encounter antigens either soluble or presented by APCs. The antigen is recognized through the B cell receptor (BCR), composed of membrane-bound immunoglobulin. Immunoglobulins of the same antigen specificity are secreted as antibody by terminally differentiated B cells the plasma cells.

The antibody molecule can exert two distinct functions: binding specifically to molecules from the pathogen that elicited the immune response via the V region, and via the C region it can recall other cells and molecules to eliminate the bound pathogen. Binding by antibody can neutralize viruses and mark pathogens for destruction by phagocytes and complement. Depending on C region, immunoglobulins can be classified in IgM, IgD, IgG, IgA, and IgE, which are each specialized for activating different effector mechanisms. The V region of an antibody generally recognizes only a small region on the surface of a large molecule such as a polysaccharide or protein, termed antigenic determinant or epitope.

B cell maturation following antigen recognition can take place in organized lymphoid structures called germinal centers. Upon binding, the antigen is endocytosed, degraded and presented on the surface to T helper cells. During B and T helper cells interaction, CD40 ligand expressed on T cell surface binds to CD40 on B cell surface, inducing IL-4 and IL-21 production. Sustained B cell activation leads to B cell proliferation, induction of somatic hypermutation, resulting in affinity maturation, and

class-switch recombination in the immunoglobulin locus (Harwood and Batista 2010). Thus, B cell maturation in germinal centers leads to generation of specific, long-lived plasma cells and memory B cells that circulate in the blood or migrate to effector sites to confer protective immunity. B cells can alternatively undergo polyclonal activation and differentiate into short-lived plasma cells producing low-specificity antibodies, important in the early response towards pathogens **(Figure 3).**

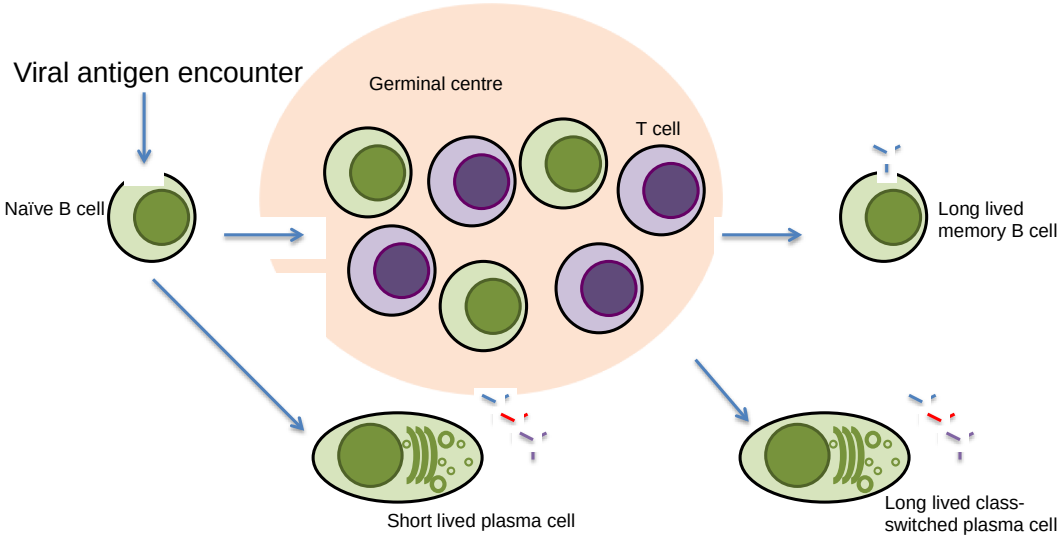

Figure 3: B cell differentiation scheme upon antigenic activation.

Regarding viral immune response, antibody response is mainly directed towards the viral structural proteins. To induce an efficient antiviral response, antibodies have a neutralizing ability, as during this activity antibodies can block viral receptors, interfering with the uncoating of the genomes in endosomes, or causing aggregation of virus particles. However, non-neutralizing antibodies can still activate the complement system, that is an enzymes cascade mediating response against infection, constituted of numerous effector and regulatory components. Complement activation has three main pathways: the classical, lectin, and alternative pathways. Activation of the classical pathway occurs when the fraction C1 binds to antibody or directly to activating surfaces. The lectin pathway is triggered by recognition of carbohydrate residues, found mainly on microbes, by mannose binding lectin, whereas the alternative pathway starts when C3 binds to a suitable activating surface. The three pathways converge into a final common pathway and lead to the formation of a membrane attack complex, which forms pores on the surface of the targeted cell with consequent lysis.

Antibodies can also act by opsonization, a process by which the virus is ingested and destructed by a phagocyte. An antibody-coated virus can be killed by a cytotoxic effector cell also through a non-phagocytic process, characterized by the release of the content of cytotoxic granules or by the expression of cell death-inducing molecules. Effector cells that mediate antibody dependent cell cytotoxicity (ADCC) include NK cells, monocytes, macrophages, neutrophils, eosinophils and dendritic cells.

Producing antibodies is not the sole role that B cells have in anti pathogen immune response, in fact B cells can regulate CD4+ Th cells by secreting cytokines. According to the specific cytokine they can activate different Th subsets and specific functions, as for example they can produce IFN-γ and IL-6 supporting Th1 cells, or secreting IL-2 supporting Th2 pathway and consequently also the humoral response (Shen and Fillatreau 2015).

3.1.1.2.3 Antigen processing and presentation to MHC

To generate an antigen specific response, lymphocytes need to encounter the antigen bound to the MHC molecules for T cell activation, or also in soluble form regarding B cell activation. There are two classes of MHC molecules, MHC class I and MHC class II which have different structures and a distinct expression pattern. The MHC complex is encoded as a group of genes, which in humans is located on chromosome 6. The MHC set of alleles present on chromosome 6, MHC aplotype, is inherited by each parent and co-dominantly expressed in each individual. The MHC class I gene complex includes three loci A, B and C, as also the class II gene complex, DP, DQ and DR. Many alleles of each locus permit thousands of possible assortments.

The MHC class I molecules are expressed in all nucleated cells. The expression levels depend on the cell differentiation and cell activation. The MHC class I molecule consists of a heterodimer of a constant light chain (β 2 microglobulin) which has a domain organization similar to that of an immunoglobulin C domain and a heavy chain which consists of three domains (alpha1, 2 and 3), the latter being linked to a transmembrane helix.

The structure of MHC class I can be divided in two regions: one region is located near the membrane and consists of the β 2 microglobulin and α3 domain spanning

the membrane. The second region consists of domains α1 and α2, which form the edges of a pocket on the surface of the molecule; this is the site of peptide binding **(Figure 4).**

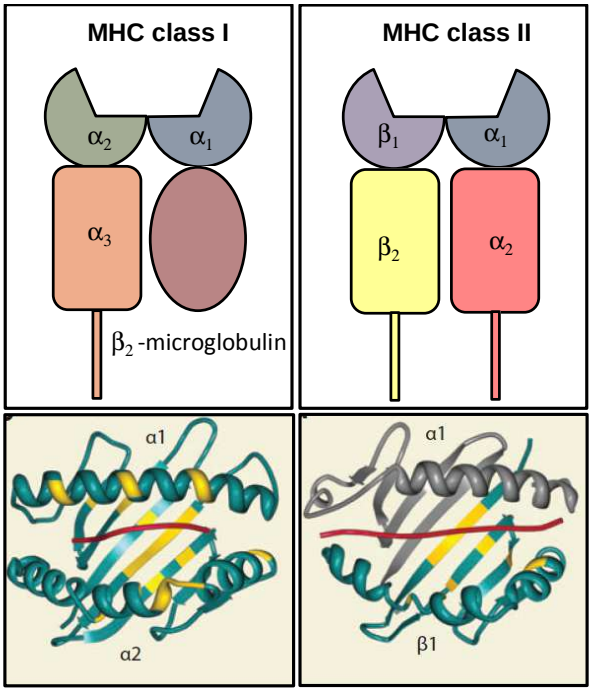

Figure 4: Schematic representation of MHC class I and II domains (upper panels) and the 3D conformation of the peptide binding pockets (lower panels) (adapted from Blum, Wearsch , et al. 2013).

The binding of a peptide epitope to the MHC class I complex is stabilized at both ends of the cleft by the contacts between the free N- and C- terminus of the peptide and the invariant sites present at the edges of all MHC class I molecules **(Figure 5A)**. The peptide lies in an elongated conformation along the groove with a usual length of 8-10 aminoacids. Peptides that can bind to a given MHC allelic variant can share similar amino acid residues at two or three defined positions along the peptide sequence; as the binding of these side chains anchors the peptide to the MHC molecule, such residues are named anchor residues. Both the identity and position and of these residues can vary, depending on the particular MHC class I type, however most of binding peptides have hydrophobic (or sometimes basic) anchor residue at the carboxy terminus **(Figure 6).**

MHC class II molecules are constitutively expressed in professional APCs, such as B lymphocytes, DCs, and macrophages, however MHC class II expression may also be

induced on other cells upon IFN-γ exposure. The MHC class II molecule consists of two trans-membrane glycoprotein chains, α and β. Each chain has two domains, and the two chains together form a four-domain complex. The α1 and β1domains, sites of major polymorphisms of the molecule, form the groove for the peptide binding, which, conversely from MHC class I, have open ends, for this reason peptides binding to MHC class II are longer, at least 13 amino acids **(Figure 5B).** The binding pockets of MHC class II are more permissive in the accommodation of amino acid side chains therefore it is more difficult to define anchor residues. However MHC class II alleles have specific patterns of permissive aa, for example negatively charged aa at the N terminus, and hydrophobic at C terminus, peptides are usually cleaved by peptidases at 13-17 aa length (**Figure 5B and 6**).

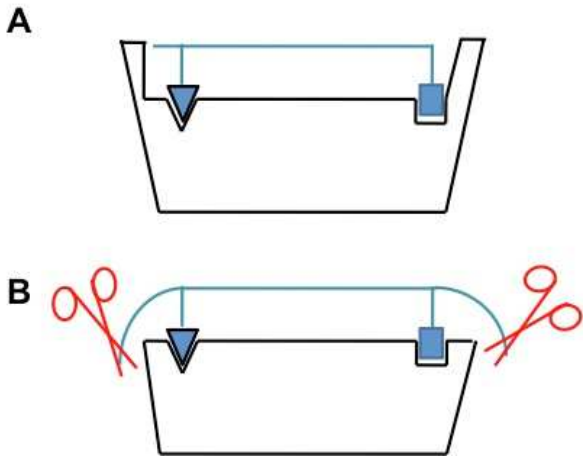

Figure 5: Representation of MHC/peptide complexes.

The 9mer peptide lays in the groove of MHC class I with the N and C termini tightly fixed in the edges, and binding it also with anchor residues (A). The open ends of the MHC class II pocket (B) allow peptides to protrude out of the edges, and degradation occurs at 13-17 aa length. The binding is mediated through the core anchor residues.

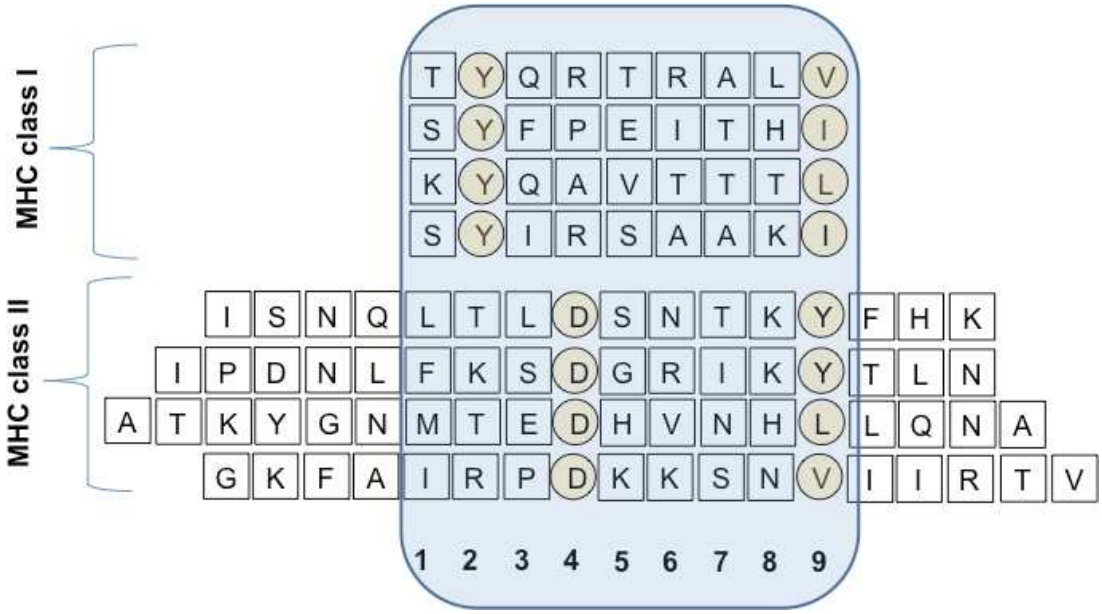

Fig 6 : Peptides binding MHC class I or II molecules.

Upper panel shows different peptides binding to same MHC class I allele, displaying similar anchor residues. Lower panel shows different peptides binding to the same MHC class II allele, with different length, but a similar chore 9mer residues pattern (modified from Immunobiology : the immune system in health and disease by Charles A. Janeway, 5th edition, chapter 3).

Recognition of peptidic epitopes by TCR occurs when they are bound to an MHC molecule and presented on the cell surface of an APC. In APCs, different intracellular pathways and mechanisms are responsible for generating complexes of MHC class I and II molecules with peptide antigens for presentation to T cells **(Figure 7).** Through antigen processing and presentation, T cells are continuously in touch with the intra and extracellular milieu and can detect signs of infection or abnormal cell growth.

Usually MHC class I peptides derive from endogenous proteins marked with ubiquitin for destruction by cytoplasmic protein degradation pathways, and then presented to CD8+ T cells, while MHC class II molecules are usually associated to peptides derived from endocytosis or phagocytosis of proteins and presented to CD4+ T cells. However, it is known that APCs can alert naive $CD8^+$ T cells for presence of neoplastic cells or infected cells through a mechanism called cross-presentation. In this way APCs take up antigens from the extracellular milieu and process them for presentation by MHC class I molecules to CD8+ T cells (Sigal, Crotty et al. 1999). On the other hand, endogenous and viral proteins can generate peptide-MHC class II complexes presented to CD4+ T cells, through a mechanism

involving autophagy. A MHC II restricted CD8+ T cytotoxic response has even been recently observed in the case of monkey vaccination with a cytomegalovirus vector expressing antigens from the simian immunodeficiency virus (Hansen, Sacha et al. 2013).

Once MHC class I molecules are assembled in the endoplasmic reticulum (ER), they are stabilised by chaperone proteins, such as calnexin, calreticulin, Erp57, protein disulfide isomerase, and tapasin. Transporter associated with antigen presentation (TAP), tapasin, MHC class I, ERp57 and calreticulin constitute a complex which is called the peptide-loading complex (PLC). The degradation of most cellular proteins occurs by the ubiquitin-proteasome pathway. The first consists in the conjugation of ubiquitin to the amino group of lysines found in the protein substrate, allowing rapid degradation of the protein by the proteasome. This process creates a very large number of different peptides, depending on the length and sequence of the protein (Rock, York et al. 2004).

Viral infection and consequent production of the immune-modulatory cytokine IFN-γ by activated T helper type 1 $CD4^+$ lymphocytes, $CD8^+$ CTLs and NKs, can induce expression of several constituents of the proteasome system (the so called immunosubunits LMP2 and LMP7), which is then turned into a different proteasome, the immunoproteasome, resulting in an enhanced antigen presentation.

Once peptides are generated from the proteasome, tapasin interacts with the transport protein TAP which translocates them from the cytoplasm into the ER. Peptides transported to the ER are of 8 –16 aa length and therefore may require additional trimming in the ER before they can bind to MHC class I molecules. This is executed by ER aminopeptidases associated with antigen processing. When peptides bind to MHC class I molecules, the chaperones are released and peptide–MHC class I complexes leave the ER for presentation at the cell surface. In some cases, it happens that peptides fail to associate with MHC class I, so they have to be transported back to the cytosol for degradation.

After presentation to the cell surface, MHC class I complexes may dissociate and the heavy chain can be internalised. Once MHC class I molecules are internalised into the endosome, they enter the MHC Class II presentation pathway. Some of the MHC class I molecules can be recycled and present endosomal peptides as a part of the cross-presentation process.

There are two major pathways of antigen processing during cross-presentation, the first one is TAP- and proteasome-dependent, the second one is TAP- and proteasome-independent. In the proteasome-independent cross-presentation, named also vacuolar cross-presentation, acidic lysosomal proteases generate the MHC class I ligands in the endocytic pathway. The vacuolar route of MHC class I cross-presentation is considered to be less effective than proteasome-dependent cross-presentation (Sigal and Rock 2000, Compeer, Flinsenberg et al. 2012).The TAP and proteasome- dependent cross-presentation includes transport of exogenous antigen from the endocytic pathway to the cytosol. DCs export the antigenic material very efficiently from the endocytic vesicles to the cytosol. Other cell subsets involved in cross-presentation are macrophages, endothelial cells, γδT cells, mast cells, and B cells, usually elicited by inflammatory conditions (Adiko, Babdor et al. 2015).

Once the complex peptide and MHC class I molecule is on the surface of the APCs, it interacts with the T cell receptor on the CD8+ T cell surface. Additionally, the CD8 molecule itself interacts with the MHC class I molecule, resulting in a very specific binding. However, in order to fully activate the T cell and consequently induce an epitope specific response, further interactions are needed: the interaction between the costimulatory molecules CD80/86 on the APC and CD28 on the surface of the T cell, and eventually the production of cytokines by the APCs.

Conversely to peptides binding to MHC class I molecules, those that bind MHC class II are usually derived from extracellular proteins, including soluble antigens, antibody- or complement-coated immune complexes, or even cellular debris from dying cells. Such exogenous proteins are taken up by APCs and transported into the endosomal-lysosomal compartment. Before encountering the peptides, MHC class II molecules are assembled in the ER and stabilised by invariant chain (Ii). The complex of MHC class II and Ii is transported through the Golgi into a compartment named as MHC class II compartment, where an acidic pH activates proteases cathepsin S and cathepsin L resulting in the digestion of Ii, leaving a residual class II-associated Ii peptide (CLIP) in the peptide-binding groove of the MHC class II. Finally the CLIP is exchanged for the antigenic peptide degraded in the endosomal pathway. MHC class II molecules loaded with foreign peptide are then transported to the cell membrane to present the antigen to CD4+ T cells. Thereafter, the process of antigen presentation by of MHC class II molecules follows a similar pattern as for MHC class I presentation, with interaction of TCR, CD4 and further costimulatory signals.

Understanding the processes of antigen processing and presentation can provide important insights for novel and more effective vaccine design and therapeutic strategies harnessing T-cell responses.

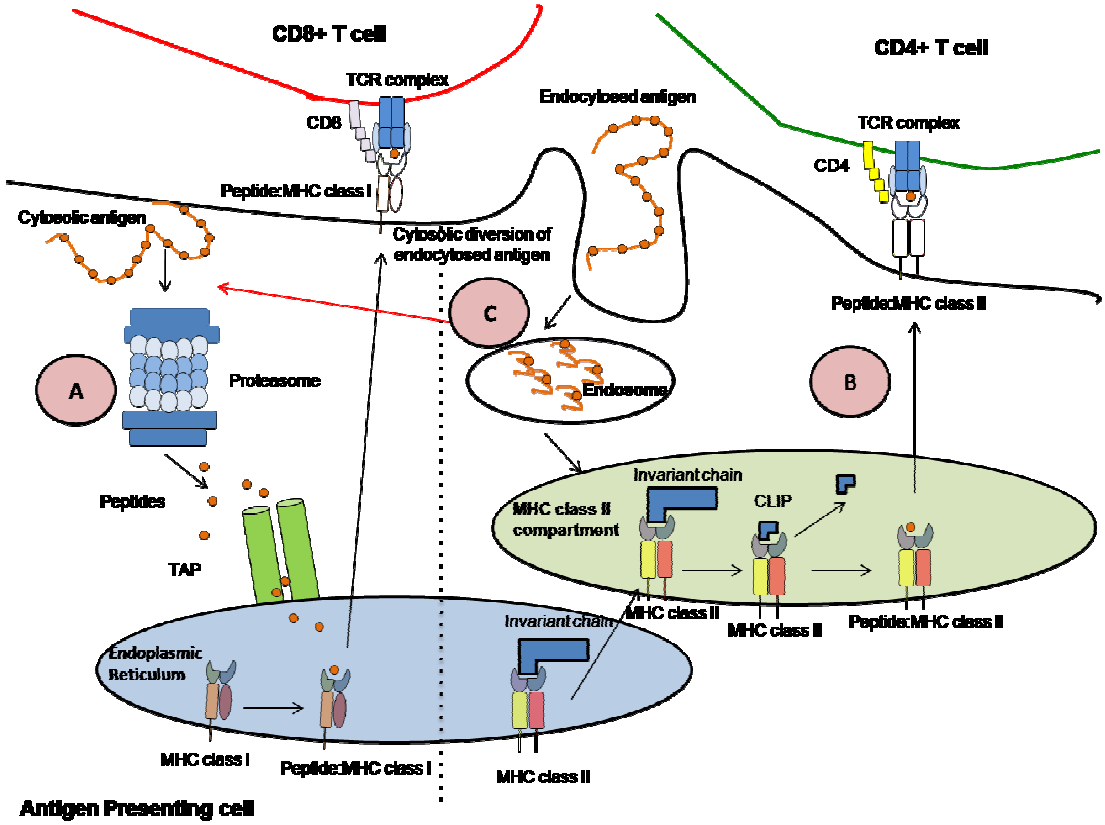

Figure 7. Different antigen-processing pathways for the MHC class I and class II molecules. A MHC class I antigen presentation of endogenous antigens. **B** MHC class II antigen presentation of exogenous antigens, and **C** MHC class I cross presentation of exogenous antigens, modified from (Heath and Carbone 2001).

3.1.2 Viral strategies to escape host immunity

Viruses are obligate parasites, needing host cells for survival. To replicate into the host cell, without being detected and eliminated by the immune system, viruses have developed strategies to evade the immune control and even exploit host proteins for their own life cycle targeting several mechanisms involving both innate and adaptive immunity. Different mechanisms of subverting the innate immune response have been described:

First, as pathogens are recognized by host PRRs in APCs through viral PAMPs, one strategy is to making inaccessible the viral genome by capping it, this is how coronaviruses and Dengue virus subvert RIG-I recognition of their viral RNA.

Second, viruses can interfere with innate signal transduction in order to prevent inflammatory cytokines and INF production. For example HCV protease NS3/ NS4A cleaves an adaptor protein, MAVS, preventing its downstream signaling, and NS4B targets STING (Coccia and Battistini 2015).

Third, APCs can also be targeted through their chemokines receptors, essential to migrate in response of chemokine gradients. HIV gp-120 VP3 loop binds to the chemokine receptor 5 (CCR5) and allows viral entry in CCR5+cells, including DCs (Tamamis and Floudas 2014). Kaposi's sarcoma associated herpesvirus (KSHV) inhibits monocyte differentiation into DCs and reduces DC migration by downregulating CCR6 and CCR7 expression on the cell surface by inducing cytoskeleton modifications (Cirone, Conte et al. 2012).

Fourth, viruses can modulate cytokine and chemokine production. An example is the induction of IL-10 by viruses such as HIV and HCV. The effect is impairment in DC maturation and T cell response, leading to viral persistence.

Many viruses escape from immune control by targeting and inhibiting peculiar mechanisms inside the cells of the innate immune system, that are essential for the activation of an adaptive immune response, i.e. antigen processing and presentation pathway (Klenerman and Hill 2005, Hansen and Bouvier 2009, Boss and Renne 2010, Noriega, Redmann et al. 2012). Antigen presentation includes different steps that can be targeted by viruses **(Figure 8).** Inhibition of the proteasomal processing is mediated by EBV nuclear antigen 1 (EBNA1), because its sequence contains repeated motifs of glycine and alanine, which prevent the protein to be degraded. Similarly, aa sequence of KHSV latency associated nuclear antigen 1 (LANA1) has a sequence rich in glutamine, glutamic acid and aspartic residues which prevents as well its proteasomal degradation (Bennett, May et al. 2005).

Another important mechanism in antigen presentation is the transport of the peptide across the ER, mediated by the TAP complex and requiring ATP. This step is targeted by Herpes Simplex virus 1 (HSV) ICP47 protein, HCMV US6 protein, and EBV BNLF2a. ICP47 is a cytoplasmic membrane associated protein, binding TAP on

the cytoplasmic side, inhibiting peptide binding and translocation. The domain mediating the inhibitory effect is at the N-terminal fragment consisting of 32 residues (Beinert, Neumann et al. 1997, Galocha, Hill et al. 1997, Aisenbrey, Sizun et al. 2006). Differently, US6 binds to TAP at its ER lumen side, while BNLF2a prevents binding of peptide and ATP to TAP.

HCMV US3 directly binds to and inhibits tapasin, essential for the expression of stable MHC class I molecules on the cell surface, causing MHC class I molecules to be retained in the ER. At the same level, Adenovirus E3-19K inhibits the formation of the TAP–tapasin complex, impairing its inclusion in the PLC (Cox, Bennink et al. 1991). Interference with MHC antigen presentation can occur also by inducing degradation of MHC molecules, this is the case of HCMV US2 and US11, which target MHC class I molecules for ER-associated degradation(van der Wal, Kikkert et al. 2002).

KHSV kk5 and kk3 induce rapid endocytosis and degradation of MHC molecules, and downregulate the expression of other cell surface receptors, such as IFN-γ receptors, and MHC class I polypeptide-related sequence A and B (MICA and B), ligands for the NK activating receptors NKG2D, leading to minor control by NK cells (Thomas, Boname et al. 2008). HIV-1 protein nef downregulates the expression of MHC class I and II molecules, and CD4, optimizing viral particle production (Barouch, Faquin et al. 2002).

Adaptive immunity can be targeted also interfering with T cell activation on costimulator signals, for example KHSV kk5 can downregulate CD86 expression on B cells by inducing their endocytosis and consequent degradation, thereby impairing the ability to activate T cells. Interfering with T cell response is another clever mechanism of immune evasion. Viruses such as RSV influence the polarization of CD4+T cells from Th1 phenotype, able to produce antiviral interleukins, to Th2 or Th17 phenotype (Christiaansen, Varga et al. 2015). Herpesviruses HCMV and EBV induce Treg response against latently expressed peptides, maintaining viral persistence. In particular Tregs increase in the peripheral blood of EBV positive patients with nasopharyngeal carcinoma and suppress the proliferation of autologous CD4+CD25- T cells (Lau, Cheng et al. 2007).

During viral latency only a minimal set of genes is expressed, including micro RNA (miRNA), which contribute to immune evasion, targeting viral antigens, which could

be recognized by the immune system. This is the case for Simian virus 40 (SV40) that can express miRNA targeting and therefore repressing the expression of the early protein Large T antigen, which usually elicits strong T cell responses (Sullivan, Grundhoff et al. 2005) . In BKPyVand JCPyV the early region encodes a pre-miRNA that generates two functional miRNAs complementary to the LTag mRNA and posttranscriptionally down-regulate LTag expression. The 3'miRNA was found to target the mRNA of the cellular stress induced ligand ULBP3, ligand for NKG2D a recognition receptor for detection of infected cells, which in NK cells triggers cytotoxicity (Bauman, Nachmani et al. 2011).

Investigating on all mechanisms of viral immune escape is a very important tool to clarify many immunological pathways, and also necessary to understand how to design vaccines able to prime and boost an efficient immune response, and prevent viral related diseases.

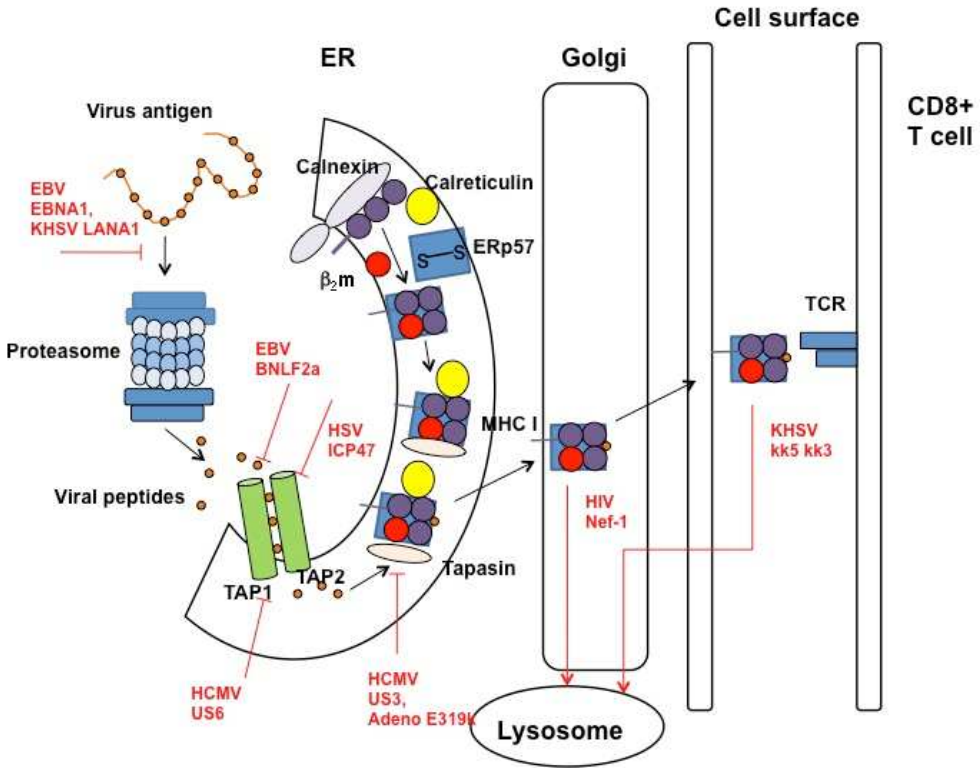

Figure 8: Antigen processing and presentation of peptide:MHC class I and interference by viral proteins modified from (Hansen and Bouvier 2009)

3.1.3 Vaccines

The first vaccine used in humans, live vaccinia virus, was developed by Jenner more than 200 years ago for the control of smallpox. Through the centuries, vaccines have been developed and extensively used in order to induce protective immune responses against a specific pathogen. Jenner vaccination strategy for smallpox consisted in injecting in human an analog non-human specific pathogen. Another strategy has been to use killed organisms, and subsequently obtain inactivated viral components, as in 1885 Louis Pasteur did for rabie vaccine, growing the virus in rabbits, and then weakening it by drying the infected tissues .

Currently, more than 50 different vaccines are licensed by the Food and Drug Administration in the United States, and they are either live, inactivated, toxoid or biosynthetic,(http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093833.htm), 14 are directed against viral pathogens.

Live attenuated vaccines could be produced against measles, mumps, and chickenpox, the advantage of this kind of vaccines is that they are the most similar to a natural infection, as a disadvantage it exists the possibility that an attenuated microbe could revert to a virulent form and cause disease. Such inconvenience can be overcome with the nonliving vaccines, which include Poliovirus vaccine. Nonliving vaccines are in principle prepared growing viruses in continuous cell lines cultures, then the virus is inactivated by chemicals or disrupted with detergents allowing immunization with little or no risk of infection. However some infectious contaminants can remain, that was the case of Poliovirus vaccine contaminated with SV40 virus.

Recombinant DNA technology has helped to produce large quantities of purified protective viral antigens for use in immunoprophylaxis without risk of infection or contamination. With such technique it is possible to express viral proteins in eukaryotic cells of yeast, insect, or mammalian origin. An example of recombinant vaccine is the HBV one, produced by expressing the hepatitis B surface antigen (HBsAg) in yeast cells. The HBsAg assembles into virus-like particles (VLPs), which are highly immunogenic, resulting a very efficient vaccine. Another example are the HPV vaccines based on VLPs derived from HPV-6, -11, -16, and/or -18 subtypes. These vaccines display the L1 recombinant proteins of each subtype, expressed either in yeast or in an insect-cell system. The L1 is the major capsid protein and its *in vitro* expression leads to the assembly of VLPs. Vaccines based on recombinant

proteins show several advantages in terms of safety and production costs, with the disadvantage of weak or poor immunogenicity when administered alone, requiring the use of adjuvants to elicit a more protective and long-lasting immune response.

An alternative and more focused approach to immunization involve the production of synthetic peptides representing immunodominant domains or even single epitopes of viral antigens, that could elicit humoral, cellular, or both immune responses, according to the specific pathogen, in fact protection against extracellular organisms is provided mainly by antibodies, whereas for the control of intracellular organisms, an effective CTL response is also essential. It is well known that during a natural infection, proteasomal degradation and processing of pathogen-derived proteins occur, and epitopes will be presented at the surface of APCs, but not all pathogen-derived proteins and epitopes will induce an effective immune response against the pathogen, indeed some epitopes can induce tolerogenic responses or induce inflammatory response without effective protection (Gibson, Nikolic et al. 2015). For this reason, it is of importance to choose appropriate antigens and epitopes able to induce an effective immune response. It is also important that vaccines should be effective in the majority of the population exposed to the pathogen, therefore epitopes able to induce an effective response in broad range of MHC alleles, or at least in the most frequent ones, need to be considered.

Such considerations lead to the idea that a vaccine should contain pathogen-derived peptides, which can generate an appropriate, all life long lasting, protective immune response in the vast majority of population, therefore an epitope mapping of pathogen derived immunogenic proteins should be performed. Numerous approaches have been developed to identify peptides derived from pathogens proteins that can be recognized by B and T cells, both experimental and predictive (Zhao, Zhang et al. 2013).

Among experimental methods to identify B cell epitopes, one of the most frequently used techniques is the fusion phage display technology (Smith 1985). It consists in cloning the coat protein gene region into a phage, allowing the candidate peptides to be expressed at the phage surface and then screened with sera samples or specific antibodies. Phages with positive binding are analyzed to determine the specific peptides sequences. By phage display technology and by an affinity selection technique called Biopanning, it is possible to produce linear peptides mimicking the

structure of an epitope (mimotopes) and able to induce the antibody response similar to the one elicited by the epitope.

Experimental method to map T cell epitopes have been developed, which utilize several different approaches, such as starting from identification of peptides binding to HLA molecules on solid-phase, to functional assays based on the identification of peptides able to elicitate *in vitro* an epitope-specific T cell response. The most used techniques are enzyme-linked immunospot (ELISpot) assay, allowing to identify T cells producing cytokines in response to an antigenic stimulation, intracytoplasmatic cytokine staining (ICS) and HLA multimers to be used in flow cytometry, lymphoproliferation, and killing assays such as ^{51}Chromium release assays. Such methods should be used in combination to identify HLA specific immunodominant peptides, as some of these can identify functionally immunogenic peptides but only others, such as HLA multimer staining, can identify HLA restricted immune responses.

Predictive approaches are based on algorithm-based software and databases: from the sequences of peptides or even of a whole protein they can predict which epitopes will bind to a specific HLA allele.(e.g. SYFPEITHI, BIMAS)(Schuler, Nastke et al. 2007), others, such as Immune Epitope Database (Vita, Zarebski et al. 2010) offer the user tools to identify T cell epitopes, not only based on HLA-peptide binding, but also on antigen processing and presentation parameters, such as proteasomal cleavage and TAP transport.

It can occur that bioinformatics databases find putative peptide candidates which are virtually able to bind HLA molecules, which may reveal not being immunogenic *in vitro* and *in vivo*, therefore they need to be coupled with experimental approaches.
Once epitopes capable of inducing protective immune responses to the chosen antigen are identified, further considerations need to be done in order to develop a peptide-based vaccine and improve its immunogenicity:

- Using short peptides, which do not require processing, may not always help to obtain a protective long lasting T cell response; it has been demonstrated that vaccination with long peptides, which are taken up by APCs, processed and presented could induce a more sustained CTL response when compared to short peptide vaccination. Moreover longer peptides can contain CD4+ T cell epitopes, eliciting a helper cell response, which can enhance also the CTL response (Kenter, Welters et al. 2008, Welters, Kenter et al. 2008,

Kenter, Welters et al. 2009). Improving CD8+ T cell response can be achieved by adding a synthetic non-natural pan HLA-DR binding epitope peptide (PADRE), which elicits CD4+ T cell responses and consequently enhances CD8+ T cells epitope specific responses (Cong, Mui et al. 2012). The inclusion of B epitopes would give a more complete T cell response, and in case of extracellular pathogen would be required.

- Peptide-based vaccines, as also recombinant vaccines, are poorly immunogenic *in vivo,* therefore they require adjuvants, with properties of immunostimulants and/or vehicles of the antigen.

Adjuvants defined as immunostimulants act on the immune system enhancing the immune responses to antigens. In particular, they can influence cytokine production by activating MHC molecules, costimulatory molecules, or through related intracellular signaling pathways. They comprise TLR ligands as the monophosphoryl lipid A (MPL-A), which triggers TLR4, CpG DNA, acting on TLR9. Adjuvants acting as vehicles, such as emulsions, liposomes, virosomes, or virus like particles (VLPs), target vaccine antigens to the immune system in a more efficient way and control the depot and release of antigens to enhance the specific immune response. Emulsions, including Freund's complete and incomplete adjuvants, Montanide/ISA-51 and MF59 containing squalene, and AS02 (containing squalene, MPL1 and the saponin extract QS-21) create a depot at the injection site, enhancing antigen uptake by prolonged exposure of antigen to the APC. The release of antigen needs to be at low-level, so it can induce a potent immune response, in fact it is known that when a given antigen is used over a wide range of concentrations, intermediate doses induce immunity, and low and high doses induce tolerance.

Peptides can be carried also by liposomes, phospholipid bilayer structures forming small vesicles. Liposomes can efficiently protect the immunogenic peptide from enzymatic degradation, are easily altered to obtain optimum presentation of the antigen, and can be efficiently taken up by APCs due to their particulate nature, however their limitation is a low entrapment efficiency, which could be overcome by conjugating the peptide to the lipids forming the vesicle (Nagata, Toyota et al. 2007). Virosomes, differently from liposomes, have integrated in the liposomal bilayer the influenza surface antigens neuraminidase and hemagglutinin, this feature enhances HLA class I and II presentation and induces an antigen-specific adaptive immune response.

VLPs are constituted by recombinant viral structural proteins that are self-assembled to mimic the conformation of viruses. They have the ideal size (20–100 nm in diameter) to be efficiently taken up APCs, and are able to also stimulated an efficient CTL response because recombinant VLP may gain access to the cytosol, internalization via endogenous processing pathways, thus activating antigen specific CTL by cross-presentation. VLP vaccines for HPV (Gardasil® and Cervarix®) are commercially available.

ISCOMs are immunostimulatory complexes including antigen, cholesterol, phospholipid and saponin. They favour the endosomal and cytosolic pathways for antigen presentation. Also polymers can be used for the delivery of vaccines, as the synthetic polylactides, polyglicolic acid, or the natural chitosan.

Currently in the U.S. some vaccines contain adjuvants, it is the case for vaccines that prevent hepatitis A, hepatitis B, diphtheria-tetanus-pertussis, Haemophilus influenzae type b, HPV, pneumococcus infection which contain aluminium salts, whereas the HPV vaccine Cervarix® is carried by VLPs and added with MPL-A.

In summary, virus-host interactions are very complex, viruses have developed mechanisms to replicate and expand within the host, and in parallel the host has built response mechanisms aiming at clearing the virus from the host cell. Such processes include the recognition of the pathogen, its elimination and the elimination of infected cells through the mediators of the immune system. To counteract the host, viruses target the most important checkpoints in the immune response, and exploit the cellular machinery to evade immunity. Knowledge on the most important issues in virus-host interaction can help and guide the design and development of appropriate tools to monitor, prevent and /or treat virus associated diseases.

3.2 Polyomaviruses

3.2.1 General information

Polyomaviruses belong to the *Polyomaviridae* family, constituted by at least 26 different members, with a broad range of infected host species, including human, monkey, bovines, rabbit, hamster, rat, mouse, and birds species, and recently, the International Committee on Taxonomy of Viruses prompted revisions on its taxonomy based on host range, genetic repertoire, and DNA sequence identity (Johne, Buck et al. 2011) **(Figure 9).** From the single genus Polyomavirus, the *Polyomaviridae* family has been splitted into three genera:

- Avipolyomavirus: this genus is constituted by avian polyomaviruses, characterized by high pathogenicity, and a consensus sequence in the DNA binding domain of the LTag different to the other genera. Some of the avian polyomaviruses have an additional open reading frame (ORF) encoding for a late protein, VP4.
- Wukipolyomavirus: this genus includes the WU polyomavirus (WUPyV), KI polyomavirus (KIPyV), human polyomavirus 6 (HPyV6) and 7 (HPyV7). These viruses share greater DNA homology than with the other mammalian polyomaviruses, especially concerning the late region.
- Orthopolyomavirus: all remaining mammalian polyomaviruses are included in the last genus, as also the human BK polyomavirus (BKPyV), JC polyomavirus (JCPyV), Merkel Cell polyomavirus (MCPyV), and the Simian Virus 40 (SV40).

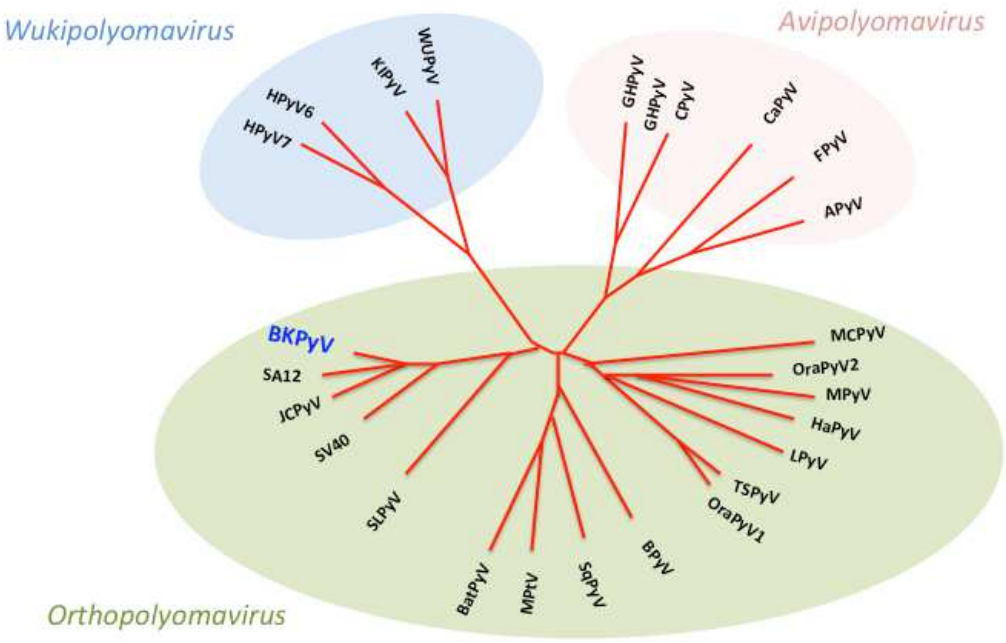

Figure 9: Phylogenetic tree of *Polyomaviridae* family, adapted from (Johne, Buck et al. 2011).

All polyomaviruses share common key features:

- An icosahedral non-enveloped capsid constituted 72 capsomers
- A double-stranded DNA, of a size of approximately 5 kbp.
- A non-coding control region (NCCR) harboring the origin of replication (ORI).
- Transcription starting from one side of the ORI results in the encoding of early non-structural proteins, named tumor (T) antigens.
- The early viral gene region (EVGR) is transcribed into one major transcript, from which the encoded large T antigen (LTag) and the small T antigen (sTag) are generated by alternative splicing, while murine PyV (MPyV) and hamster PyVs also encode a third viral early protein called middle T antigen. In the EVGR of a subset of human and non human PyV (BKPyV, JCPyV, MCPyV, SA12, MPyV and SV40) miRNA sequences have been identified, complementary to LTag sequence. In BKPyV and JCPyV it has been shown to bind the cellular stress induced ligand ULBP3, involved in NK cell mediated lysis (Bauman, Nachmani et al. 2011).

- From the opposite side of the ORI, transcription of late viral gene region (LVGR) generates the encoded structural proteins VP1, VP2 and VP3, whereby VP1 being the most abundant component of VLPs. Of note, only the LVGR of BKPyV, JCPyV, and SV40 encode also a small non-structural protein called agnoprotein upstream of the VP1 ORF.

3.2.2 Human Polyomaviruses

Human polyomaviruses (HPyV) belong either to the *Wukipolyomavirus* or to the *Orthopolyomavirus* genera, in particular WUPyV, KIPyV, HPyV6 and HPyV7 are the members of *Wukipolyomavirus* genus, while JCPyV, BKPyV, MCPyV, *Trichodysplasia spinulosa* virus (TSPyV), HPyV9, HPyV10 (with the two variants Malawi polyomavirus (MWPyV) and MX polyomavirus (MXPyV)), Saint Louis polyomavirus (STLPyV), HPyV12, and New Jersey polyomavirus (NJPyV-2013) all belong to the genus *Orthopolyomavirus* **(Table 1).**

The first two HPyV to be isolated were JCPyV and BKPyV, named after the initials of the patients from whom they were first isolated. JCPyV was isolated from the diseased brain tissue of a patient with PML, while BKPyV was isolated in cytophatically altered urinary epithelial cells shed by a kidney transplant recipient (KTR) (Gardner, Field et al. 1971, Padgett, Rogers et al. 1977). More than 35 years later, from 2007, other HPyV have been discovered not by isolation, but using different molecular identification techniques.

Table 1: Human Polyomaviruses

Name	Year of discovery	First source of isolation	Reference
BKPyV	1971	Urinary epithelial cells from KTR	(Gardner, Field et al. 1971)
JCPyV	1971	Brain tissue	(Padgett, Walker et al. 1971)
KIPyV	2007	Airways samples from patients with respiratory disease	(Allander, Andreasson et al. 2007)
WUPyV	2007	Airways samples from patients with respiratory disease	(Gaynor, Nissen et al. 2007)
MCPyV	2008	Merkel cell carcinoma	(Gaynor, Nissen et al. 2007, Feng, Shuda et al. 2008)
HPyV6	2010	Skin of healthy subject	(Schowalter, Pastrana et al. 2010)
HPyV7	2010	Skin of healthy subject	(Schowalter, Pastrana et al. 2010)
TSPyV	2010	Skin of Trychodysplasia spinulosa patient	(van der Meijden, Janssens et al. 2010)
HPyV9	2011	Serum sample from KTR	(Scuda, Hofmann et al. 2011)
HPyV10	2011	Papillomavirus induced anal condylomata	(Buck, Phan et al. 2012)
STLPyV	2013	Stool samples from diarrhea affected children	(Lim, Reyes et al. 2013)
HPyV12	2013	Liver tissue	(Korup, Rietscher et al. 2013)
NJPyV-2013	2014	Muscle biopsy of a pancreatic transplant recipient	(Mishra, Pereira et al. 2014)

- KIPyVV and WUPyV were identified in airways samples from patients with respiratory disease at the Karolinska Institute and at the Washington University, respectively (Allander, Andreasson et al. 2007, Gaynor, Nissen et al. 2007).

- MCPyV was identified in 2008 in Merkel cell carcinomas (MCC), which is an aggressive form of cutaneous cancer (Feng, Shuda et al. 2008). In MCC cells infected by MCPyV, mutations, insertions, and deletions within LTag result in the expression of truncated LTag before the helicase domain (Duncavage et al., 2009) and a 57 kb T antigen production.

- TSPyV was identified as etiological agent of Trychodysplasia spinulosa, skin disease of severely immunocompromised hosts (van der Meijden, Janssens et al. 2010).
- HPyV6 and HPyV7 were first identified in skin swabs of healthy individuals, while HPyV9 was first identified in serum of a KTR (Schowalter, Pastrana et al. 2010).

- HPyV10 was found in 2012 in condylomas of a patient affected by warts, hypogammaglobulinemia, infections, and myelokathexis syndrome (Buck, Phan et al. 2012).
- MWPyV was found in a feaces sample of a healthy child from Malawi (Siebrasse, Reyes et al. 2012), then MXPyV was identified in stool samples from children with diarrhea in the US (Yu, Greninger et al. 2012). As MWPyV and MXPyV show DNA identity of more than 95% with the previously discovered HPyV10, they can be considered as variants of HPyV10.
- STLPyV was identified most recently in stool samples of children
- HPyV12 in organs of the gastrointestinal tract of patients affected by malignant diseases (Korup, Rietscher et al. 2013).
- HPyV13, the latest member of the HPyV, is also called NJPyV-2013 and has been identified in a muscle biopsy of a pancreatic transplant recipient (Mishra, Pereira et al. 2014).

HPyV are ubiquitous viruses, infecting a large proportion of the human population. Seroprevalence for most of them have been described (Dalianis and Hirsch 2013) showing different age-dependent patterns, suggesting that HPyV are transmitted independently of one another.
HPyV infect their host without causing any apparent primary disease, but they usually persist latent without causing clinical symptoms, however in case of immunosuppression, they can cause severe diseases.

So far, a causative role in human diseases has been demonstrated for JCPyV, BKPyV, MCPyV, and TSPyV. JCPyV is mainly known as causative agent of progressive multifocal leukoencephalopathy in immunocompromised individuals, also related to the immune reconstitution inflammatory syndrome, moreover it is also associated to meningitis, granule cell neuronopathy, and rarely to nephropathy in KTRs. BKPyV can cause nephropathy in KTRs, hemorrhagic cystitis in hematopoietic stem cell transplantation, ureteric stenosis, encephalitis, pneumonia, vasculopathy, bladder cancer, and it might play a role in prostate cancer. MCPyV is recognized as cause of Merkel Cell Carcinoma, while TSPyV is the cause of Trychodysplasia spinulosa. Other HPyV have been found in samples of patients, as HPyV7 was found in samples of lung transplant, but their role in causing diseases has not been fully elucidated.

3.2.3 BK Polyomavirus (BKPyV)

3.2.3.1 Viral genome and proteins

BKPyV, as all PyV, is a non-enveloped, double stranded DNA virus. The ichosaedral virion is about 45 nm in diameter and the capsid consists of 72 capsomers, each composed of 5 molecules of the structural protein VP1 linked to one molecule of VP2 or VP3. The C-terminus of VP1 protein interacts with the neighboring pentamer of VP1, stabilizing the capsid. BKPyV virion encapsidates the genome, which is organized in a circular DNA molecule (about 5 kbp length) packed around the cellular histones H2A, H2B, H3, and H4, forming a minichromosome. The genome, containing about 20 nucleosomes, is organized in three distinct functional regions typical of all PyV: the NCCR, the early viral gene region (EVGR) and the late viral gene region (LVGR) (Imperiale 2007) **(Figure 10).**

The NCCR contains the ORI, TATA- and TATA-like sequences for both early and late viral gene transcription, many DNA- and transcription-binding sites, promoter and enhancer elements as well as binding sites for the LTag, and directs the early and late transcription of replication. NCCR is divided in blocks defined O143, P68, Q39, R63, and S63.

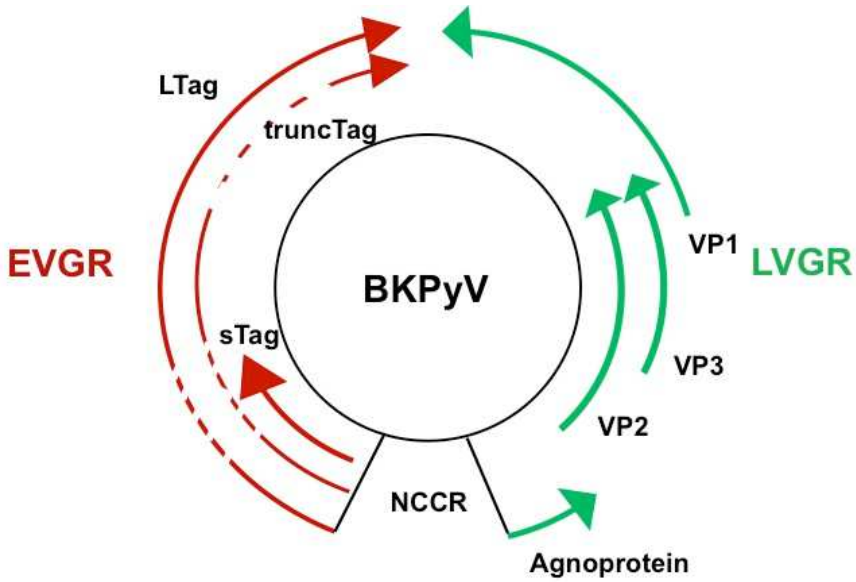

Fig 10: Genome organization of BKPyV

Based on the DNA sequence of the NCCR, BKPyV can be defined as archetype strain, most frequently found in urine of both healthy and diseased people. In immunocompromised patients with high-level BKPyV replication such as nephropathy in renal transplant recipients, the NCCR may have deletions, insertions or duplication of the blocks, leading to rearranged NCCR (Randhawa, Zygmunt et al. 2003, Azzi, De Santis et al. 2006, Olsen, Andresen et al. 2006, Gosert, Rinaldo et al. 2008). Rearranged variants are most often detected in sera and urine of patients with BKPyV associated diseases. These NCCR rearrangements have been shown to increase EVGR expression and replicative capacity (Gosert, Rinaldo et al. 2008, Bethge, Hachemi et al. 2015).

The BKPyV EVGR is on the proximal side of the ORI and encodes for the regulatory proteins LTag (80.5 kDa), sTag (20 kDa), and the truncated tumour antigen (truncTag, 17 kDa), expressed by alternative splicing of a single mRNA transcript.
In BKPyVand JCPyV the EVGR encodes a pre-miRNA that generates two functional miRNAs complementary to the LTag mRNA and posttranscriptionally down-regulate LTag expression. The 3'miRNA was found to target the mRNA of the cellular stress induced ligand ULBP3 (Bauman, Nachmani et al. 2011), recognized by the natural killer receptor NKG2D, thus possibly mediating evasion from NK cell mediated elimination.
LTag is multifunctional regulatory protein of 695 amino acid lengths. It can bind a wide variety of cellular proteins, its structure includes different functional domains that are critical for viral replication, and may be involved also in cell transformation **(Figure 11A).** At the N-terminus there is the J domain, which is a heat-shock-protein-70 (Hsp70) binding domain. Hsp70 is usually activated upon cellular stress, and plays a role in folding and unfolding of proteins. Interaction between the J domain and Hsp70 results in stimulation of the ATPase activity of Hsp70. It has been demonstrated in SV40, that upon recruitment of Hsp70, complexes involving the retinoblastoma (Rb) family proteins p107 and p130 are disrupted. As result the host cell enters the S phase and proliferates. (Sullivan and Pipas 2002, Sullivan, Baker et al. 2004). Next to the J domain of LTag, there is the Rb binding domain, with a conserved LxCxE motif, which acts together with the J domain in preventing pRb interaction with cellular E2F, which would have resulted in activation of transcription of genes involved in DNA replication activities and cell cycle regulatory activities, in particular the transition from G1 phase to S phase(Harris, Christensen et al. 1998).

The ORI-binding domain (OBD) of the LTag is located close to the helicase domain, which are binding to the ORi-sequence of the PyV genome and promote the unwinding of the circular viral DNA for DNA replication. For these processes hydrolysis of ATP is required, and a sequence inside the helicase domain has an ATPase activity. The ATPase domain has a highly conserved sequence among PyV, therefore such sequence has been investigated as a target in the search of anti-polyomavirus compounds (Seguin, Ireland et al. 2012, Topalis, Andrei et al. 2013).
At the outside surface of the helicase domain, at residues 351–450 and 533–626, there is a domain of interaction with the tumor-suppressor p53. LTag engaging p53 interferes with its binding to DNA and prevents host cell apoptosis. Therefore, LTag might play a role in oncogenic transformation (Hirsch 2005, Dalianis and Hirsch 2012, Dalianis and Hirsch 2013, Papadimitriou, Randhawa et al. 2016).

Following the helicase domain, BKPyV LTag contains a C-terminal region that bears some homology to the SV40 C-terminal domain, which can be phosphorylated on threonine 701 and, resulting in an interference with the degradation of the G1–S-specific cyclin E1 and MYC, contributes to cellular growth and proliferation. A C-term host range domain has been described in BKPyV LTag. Such domain, in SV40 is critical for viral replication in the host (Pipas 1992).

Alternative splicing of LTag mRNA can lead to expression of a truncated protein (136 aminoacids), lacking the helicase domain, but preserving the Rb binding domain. In mice models, expression of the truncTag induced cellular transformation in the host (Abend, Joseph et al. 2009).

Among HPyV, the oncogenic properties of BKPyV, JCPyV and MCPyV have been described *in vitro* and in animal models (Moens, Van Ghelue et al. 2007, Borchert, Czech-Sioli et al. 2014, Verhaegen, Mangelberger et al. 2014). For MCPyV, a clear association with cancer is described in its natural host. A particular role has been attributed to the sTag and possibly a truncated form of LTag has been found to be peculiar in Merkel cell carcinoma tumors (Chang and Moore 2012). However, BKPyV may increase the risk of developing renal and prostate cancer (Hirsch 2005, Dalianis and Hirsch 2012, Bulut, Ozdemir et al. 2013, Dalianis and Hirsch 2013, Papadimitriou, Randhawa et al. 2016). In a study conducted in BKPyV-positive prostate cancer patients, stimulation of peripheral blood mononuclear cells (PBMC) with LTag derived peptides resulted in expansion of antigen-specific T cells with suppressive properties (IL-10-secreting, expressing CD4+CD25++CD127−FoxP3+),

leading to the maintenance of a regulatory microenvironment, thus favoring a bad prognosis (Sais, Wyler et al. 2012). In another study, Keller et al evaluated if preoperative antibody response to BKPyV LTag and VP1 could be associated with the risk of biochemical recurrence in patients undergoing prostatectomy for primary prostate cancer and demonstrated that seropositivity to BKPyV LTag significantly reduced the risk of biochemical recurrence, suggesting serology as a prognostic biomarker in prostate cancer.

The sTag of BKPyV contains 172 amino acids and results from an alternative splicing of the same EVGR mRNA transcript of LTag. This smaller early regulatory protein seems to be involved in viral replication and cellular transformation. Its sequence includes a shared domain with LTag, that is the J domain, and a region ranging from aa 83 to 172 containing two zinc-binding motifs formed by cysteine residues configured as CxCxxC (Cho, Morrone et al. 2007), which is responsible for inhibition of serine/threonine protein phosphatase 2A (PP2A), leading to cellular proliferation (Mungre, Enderle et al. 1994, Sontag, Sontag et al. 1997, Sontag and Sontag 2006, Cho, Morrone et al. 2007) (Yuan, Veldman et al. 2002) **(Figure 11B).** It has been recently shown that mutations in the second domain result in lack of binding with PP2A (Cardoso, Diaz et al. 2015).

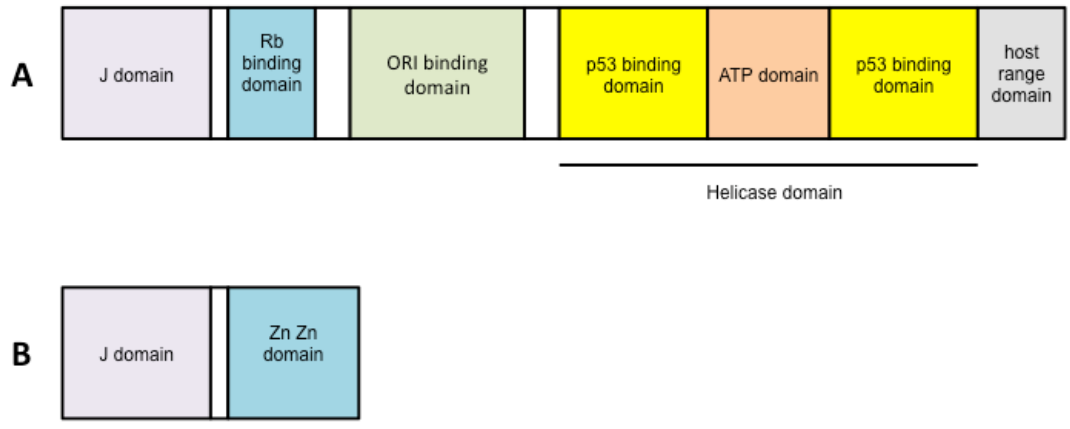

Figure 11: A LTag functional domains. B sTag functional domains

The LVGR of BKPyV is located on the distal side of the ORI, and encodes three structural proteins: VP1 (40.1 KDa), VP2 (38.3 KDa), VP3 (26.7 KDa), and a non-structural protein called agnoprotein (7.4 kDa). The structural proteins VP2 and VP3 derive from the same transcript, whereas VP1 and agnoprotein are translated in

another reading frame from partially overlapping transcripts. VP1 constitutes the majority of the BKPyV virion proteins, which is composed by 72 pentameric capsomers arranged in an icosahedral structure. Inside the capsid, one molecule of either VP2 or VP3 is linked to each VP1 pentamer. VP1 interacts via N-terminus with other VP1 molecules within the same pentamer, while the C-terminus interacts with other pentamers stabilizing the capsid. It is believed that both VP2 and VP3 are important for packing JCPyV and BKPyV genomes into virions as well as for uncoating and delivery of viral genome to the host cell nucleus (Shishido-Hara, Ichinose et al. 2004, Gasparovic, Gee et al. 2006).

The BKPyV agnoprotein is the least conserved protein among mammalian PyV , and in fact, has a corresponding homologue only in JCPyV and SV40 with an aa identity of 60% (Rinaldo, Traavik et al. 1998, Gerits and Moens 2012). In BKPyV wild type (ww), agnoprotein is a 66 aa phosphoprotein (Rinaldo, Traavik et al. 1998), where potential phosphorylation acceptor sites have been mapped to Ser-7, Ser-11, and Thr-21 (Sariyer, Akan et al. 2006, Johannessen, Myhre et al. 2008). Replacing Ser-11 with alanin or aspartic acid leads to lower viral propagation and less protein stability (Johannessen, Myhre et al. 2008). In JCPyV it has been shown that agnoprotein potentially can enhance virus release by acting as a viroporin (viral protein that interacts with membranes leading to modified cell permeability to ions or other small molecules) requirinq the basic Arg-8 and Lys-9 for such activity(Suzuki, Orba et al. 2010).

Agnoprotein is located in the cytoplasm and perinuclear area (Rinaldo, Traavik et al. 1998), and a recent work demonstrated that agnoprotein targets lipid droplets, requiring an amphipathic helix encoded in residues 20-42 (Unterstab, Gosert et al. 2010), however it has been shown that it is also present in the plasma membranes, requiring the aa clusters in the N terminus for such localization (Suzuki, Orba et al. 2010). It has been shown that BKPyV agnoprotein can interact with α-soluble N-ethylmaleimide-sensitive fusion attachment protein (Johannessen, Walquist et al. 2011), and *in vitro* studies demonstrated that such interaction might interfere with the secretory pathway (Johannessen, Walquist et al. 2011).

The secondary structure of JCPyV agnoprotein has been studied in more detail, but because of the high homology, some of the findings are likely to also apply to the BKPyV agnoprotein. This includes an α-helix constituted by hydrophobic residues in aa 17 - aa 42, and this sequence has been shown to mediate *in vitro* agnoprotein

oligomerization (Coric, Saribas et al. 2014). This sequence is also found in the BKPyV agnoprotein, but no functional proof in cells in vivo is available at this point for either viral agnoprotein.

Another potential interaction partner of agnoprotein is the proliferating cell nuclear antigen (PCNA). The interaction with agnoprotein inhibits PCNA-dependent DNA synthesis *in vitro* and reduces host cell proliferation. A possible role in switching off viral DNA replication to allow assembly of genomes and viral capsid proteins into infectious viral particles has been suggested (Gerits, Johannessen et al. 2015).

3.2.3.2 Viral Life Cycle

BKPyV infection begins with the binding of major capsid protein VP1 to the α2,3-linked sialic acid structures at the host cell surface (e.g. GD1b and GT1b gangliosides) whose function is to mediate cellular recognition and cell-to-cell interaction (Dugan, Eash et al. 2005, Low, Magnuson et al. 2006, Imperiale 2007, Dugan, Gasparovic et al. 2008). Both GD1b and GT1b are present on kidney and urinary tract cells, making them the main sites of the viral infection and replication (Low, Magnuson et al. 2006, Tsai and Inoue 2010). However, it has been demonstrated in an *in vivo* study, that a BKPyV genotype binds the host cell surface through a ganglioside-independent pathway (Pastrana, Ray et al. 2013). Murine GM95 cells lacking gangliosides were transduced with BKPyV variants with or without supplementation with exogenous gangliosides GT1b or GD1a or GD1b, BKPyV genotype IV could efficiently enter the cells and supplementation of gangliosides did not affect the process. (Pastrana, Ray et al. 2013).

The next step of cell entry is internalization of the virus via caveolae-mediated endocytosis (Dugan, Eash et al. 2006, Jiang, Abend et al. 2009), transport to the ER, and subsequent capsid disassembly before it can enter into the nucleus. Once the genome enters the nucleus, the EVGR expression begins. LTag starts binding to members of Rb family, inducing the cell to enter the S-phase, then upon binding with p53 LTag interferes with apoptosis. LTag binds to the ORI and unwinds the viral DNA through its helicase activity it promotes DNA replication, by recruiting the host cell enzymes needed for viral replication (DNA polymerase, alpha-primase, topoisomerase I and replication protein A).

After the start of viral DNA replication, LVGR expression dramatically increases as LTag promotes LVGR mRNA transcription from multiple genome copies (gene dosage effect). As VP1, VP2 and VP3 return from the cytoplasm to the nucleus by virtue of the nuclear localization sequences, the assembly of the virions with encapsidated viral DNA takes place, leading to nuclear inclusions and enlargement, and eventual lysis of the cell (Imperiale 2007, Fanning and Zhao 2009). The viral replication capacity is mainly dependent to the NCCR, which offers DNA binding sites to host cell transcription factors and regulators in the promoter/enhancer. In fact, rearrangement of the NCCR results in variants that may have stronger early promoters, produce more LTag and replicate faster in immunodeficient hosts (Gosert, Rinaldo et al. 2008).

3.3 BKPyV and clinical impact

3.3.1 Epidemiology

Primary BKPyV infection occurs at young age (Knowles, Pipkin et al. 2003), and its transmission probably involves the respiratory or oral route (Hirsch and Steiger 2003). The virus persists in the epithelium of the renourinary tract, and it has not been clearly determined if BKPyV can remain as a latent infection, without replication, or if it there is a persistent low-level viral replication. However, in the healthy blood donor population, it has been demonstrated that BKPyV can reactivate in 5% - 10% of the population, and urinary shedding has been observed at low viral loads, without clinical symptoms (Polo, Perez et al. 2004, Egli, Infanti et al. 2009, Kling, Wright et al. 2012). Moreover, a fecal shedding has been observed in 10% of healthy adult individuals (Vanchiere, Abudayyeh et al. 2009).

As BKPyV infection is virtually ubiquitous, its overall seroprevalence is high, reaching rate of 90% and more in the adolescent human population (Knowles, Pipkin et al. 2003, Egli, Infanti et al. 2009, Kean, Rao et al. 2009, Kardas, Leboeuf et al. 2015). Interestingly, the seroprevalence of BKPyV rates decrease after the 5th decade of life, where that of JCPyV still increases indicating different exposure rates during adult life (Egli, Infanti et al. 2009, Hirsch, Kardas et al. 2013, Kardas, Leboeuf et al. 2015). Recent data confirm this observation in an independent population and demonstrate that cellular immune responses show a similar pattern (Knowles, Pipkin et al. 2003, Kean, Rao et al. 2009).

Different BKPyV genotypes can be found among human population. The first BKPyV genotyping schema was based on an epitope region of the VP1 gene, recognizing four main genotypes I-IV (Jin, Gibson et al. 1993) which are also fully distinct serotypes (Pastrana, Brennan et al. 2012, Pastrana, Ray et al. 2013). Additional subtypes within genotype I (Ia, Ib1, Ib2, Ic) and genotype IV (IVa, IVb, IVc) could be identified by nucleotide sequence analysis of all known genome-full-length isolates (Luo, Bueno et al. 2008).

Genotype I is worldwide distributed, genotype IV is mainly detected in East Asia, and genotypes II and III are rarely detected (Zhong, Randhawa et al. 2009). In a study conducted in Switzerland on 400 healthy individuals, the most common found genotype was I, followed by type IV. (Egli, Infanti et al. 2009)

3.3.2 BKPyV-associated diseases

Prevalence and level of BKPyV replication in urine, occasionally observed in the healthy population may increase with pregnancy, and immunodeficiency status including hematopoietic stem cell and renal transplantation (HSCT and KT). The clinical impact of BKPyV is important in immunosuppressed transplant recipients, and the main BKPyV-related diseases are BKPyV-associated nephropathy (BKPyVAN) in KTRs and BKPyV-associated hemorrhagic cystitis (BKPyVHC) in HSCT patients. However, BKPyV can be the ethiological agent for other diseases, such as ureteric stenosis, encephalitis, pneumonia, vasculopathy, bladder cancer, and it might play a role in prostate cancer.

3.3.2.1 BKPyV-associated hemorrhagic cystitis (BKPyVHC)

Hemorrhagic cystitis is characterized by painful haematuria due to inflammation of the urinary bladder mucosa. In HSCT patients, such disease can be caused both by chemotherapy, and by viral infections. BKPyVHC occurs about 50 days post-transplantation and affects up to 15% of HSCT patients. Diagnosis of BKPyVHC should be based on evidence of clinical cystitis, hematuria of grade 2 or more, and high-level BKPyV replication of > 7log10 copies/mL in urine. In about two-thirds of patients with BKPyV-HC, significant BKPyV viremia has been described, which can be used as virological marker of progression and remission (Erard, Kim et al. 2005, Cesaro, Hirsch et al. 2009, Koskenvuo, Dumoulin et al. 2013, Cesaro, Tridello et al. 2015).

Hirsch and co-workers were the first to propose that the pathogenesis of BKPyV-HC might result from a sequence of events (Binet 2000). Accordingly, the first phase occurs pre-transplant, during the conditioning regimen, when the use of chemotherapy especially involving cyclophosphamide, damages the uroepithelium. The regenerating uroepithelial lining in the absence of a functional immune effectors provides the appropriate milieu for BKPyV replication (Hirsch and Steiger 2003). The immunosuppressive regimen is critical by decreasing the BKPyV-specific cellular immunity, providing suitable conditions for extensive high-level viral replication, and leading to cytopathic denudation of the uroepithelial mucosa. In fact, patients excreting with high urine BKPyV loads (about 10^7 BKV copies/ml) are significantly more likely to develop BKPyVHC as compared with those who excreted less (Azzi, Cesaro et al. 1999, Leung, Suen et al. 2001). Post-engraftment invasion of donor cells causes significant inflammation causing extensive mucosal damage and haemorrhage characteristic of severe BKPyVHC (Binet 2000, Hirsch and Steiger 2003).

This model would be compatible with the concept of immune reconstitution diseases as discussed in detail, since most cases of post-engraftment BKPyVHC occur in allogeneic HSCT with graft versus host disease (Hirsch and Steiger 2003, Hirsch 2005). In a study conducted in a cohort of pediatric HSCT (HLA-haploidentical or HLA-matched) patients, the role of BKPyV-specific T cell of the donor in the subsequent development of BKPyVHC in the recipient has been evaluated. The results suggest that BKPyVHC may be associated to a lower virus-specific T cell immunity in the donor (Basso, Algeri et al. 2013). In the light of these data, a prolonged and clinically more prominent manifestation of BKPyVHC in the absence of prominent BKPyV-specific T-cells could be a relevant factor leading to more extensive denudation.

The therapy for BKPyVHC is mainly supportive, consists of bladder irrigation to prevent clot formation, hyperhydration to increase diuresis and urosurgical intervention (Hirsch 2010). Substitution of platelets and erythrocytes has been used as therapeutic approach, with no evident benefit. Some studies report the use of cidofovir, a nucleoside analogue of deoxy-cytidine monophosphate, for the treatment of BKPyVHC, with controversial results since it seems that more than cidofovir, immune suppression and immune reconstitution might play a major role in the

resolution of BKPyVHC (Cesaro, Hirsch et al. 2009, Cesaro, Pillon et al. 2013, Koskenvuo, Dumoulin et al. 2013, Rascon, Verkauskas et al. 2015).

3.3.2.2 BKPyV-associated nephropathy (BKPyVAN)

In the last 15 years, with the discovery of potent immunosuppressive drugs for the anti-rejection therapy in solid organ transplantation, BKPyV has emerged as the most challenging infectious cause of renal allograft dysfunction and graft loss (Binet, Nickeleit et al. 1999, Drachenberg, Beskow et al. 1999, Howell, Smith et al. 1999, Nickeleit, Hirsch et al. 1999, Randhawa, Finkelstein et al. 1999, Binet 2000, Hirsch, Brennan et al. 2005). BKPyVAN was initially reported to cause graft loss in 10% to **>** 80% of cases (Binet, Nickeleit et al. 1999, Drachenberg, Beskow et al. 1999, Randhawa, Finkelstein et al. 1999, Vasudev, Hariharan et al. 2005, Acott and Hirsch 2007, Comoli and Ginevri 2012), but thanks to the improvements in BKPyV monitoring strategies after transplantation and prompt/preemptive therapeutic intervention, a positive impact on graft outcome has been obtained (Brennan, Agha et al. 2005, Ginevri, Azzi et al. 2007, Schaub, Hirsch et al. 2010, Sood, Senanayake et al. 2012) as summarized in (Hirsch, Babel et al. 2014).

BKPyVAN represents a complication associated to high-rate virus replication in the grafted kidney leading to cytopathic damage of the renal tubular epithelium in the renal allograft. Consequently, the virus spreads into the tissue and bloodstream and inflammatory cells infiltrate the interstitium causing tubular atrophy and interstitial fibrosis in the allograft with a subsequent worsening of graft function and eventual graft loss. However, it has been demonstrated by mathematical modelling of viral infection that also urothelial cells are crucial in the pathogenesis of BKPyVAN; results suggest that viral replication starts in the renal tubular epithelial cells but is then carried to the urothelial cell compartment where more than 90% of urine BKPyV loads are generated (Funk 2008). Histopathological data confirm such results, as infected urothelial cells are detected in the bladder of patients with PyVAN (Nickeleit, Hirsch et al. 1999). Also, *in vitro* results obtained in primary human urothelial cells from bladder show high permissivity to BKPyV infection (Li, Sharma et al. 2013).

3.3.2.2.1 Risk Factors

In KTRs BKPyV replication can be caused by a post transplant reactivation of the virus or by transmission from the allograft donor (Bohl, Storch et al. 2005, Schmitt,

Raggub et al. 2014). BKPyV donor serostatus or active replication is therefore one of the risk factors for development of BKPyV viremia in the recipient (Bohl, Brennan et al. 2008). BKPyV replication and consequent progression to BKPyVAN have been correlated to several demographic and clinical parameters (Wiseman 2009, Barraclough, Isbel et al. 2011), with a limited predictive value.

BKPyVAN development results from the interaction of multiple risk factors, where probably the immunosuppression plays a crucial role (Binet, Nickeleit et al. 1999, Hirsch 2002, Hirsch, Knowles et al. 2002, Hirsch, Vincenti et al. 2013, Hirsch, Yakhontova et al. 2015). Other risk factors are correlated with the patient (e.g. older age, male sex, pre-transplant BKPyV negative serostatus, HLA type) (Ramos, Drachenberg et al. 2002, Ginevri, De Santis et al. 2003, Bohl, Storch et al. 2005, Bohl, Brennan et al. 2008, Hirsch and Prestele 2010, Barraclough, Isbel et al. 2011, Hirsch, Vincenti et al. 2013, Masutani, Ninomiya et al. 2013), with the donor (BKVPyV positive serostatus, high number of HLA mismatches) (Bohl, Storch et al. 2005, Awadallah, Duquesnoy et al. 2006, Sood, Senanayake et al. 2013), or with the virus (genotype, NCCR rearrangements, replicative capacity) (Randhawa, Zygmunt et al. 2003, Gosert, Rinaldo et al. 2008, Ramos, Drachenberg et al. 2009, Masutani, Ninomiya et al. 2013).

The use of triple immunosuppressive therapy including steroids, tacrolimus, mycophenolate derivatives appears as major factor associated with uncontrolled viral replication and related disease (Hirsch, Knowles et al. 2002, Borni-Duval, Caillard et al. 2013, Hirsch, Vincenti et al. 2013). In particular the use of tacrolimus rather than cyclosporine has been associated to BKPyV replication (Brennan, Agha et al. 2005, Dharnidharka, Cherikh et al. 2009, Hirsch, Vincenti et al. 2013). In a prospective multicenter randomized trial for the use of tacrolimus vs cyclosporine in combination with mycophenolic acis conducted in 682 de novo kidney transplants suggested a dynamic risk factor evolution for BKPyV viremia. In the first 3 months, no difference between the two CNI was observed, while at 6 and at 12 months, rate and load of viremia were lower in patients randomized to receive cyclosporine, suggesting that differences between the CNIs did not play out early after transplant, but after 6 months, when usually PyVan has already been diagnosed. What seemed to play a role in the early onset of replication was high exposure to steroids (Hirsch, Vincenti et al. 2013). Conversely, in a prospective study conducted in KTRs receiving cyclosporine, or low dose tacrolimus, a lower incidence of BKPyV viremia in the latter group has been observed (Geddes, Gunson et al. 2011).

Some studies also report that induction agents such as ATG or alemtuzumab could be associated to BKPyV infection (Dadhania, Snopkowski et al. 2008, Theodoropoulos, Wang et al. 2013). All these results are in agreement that the use of potent immunosuppressive drugs facilitate the infection or the reactivation of BKPyV, and that in the context of an immunodeficient status, the virus can replicate in an uncontrolled manner, and lead to cytophatic damage to the graft causing nephropathy and eventually graft loss.

It is known that immunosuppressive drugs exert their function by acting on T cell activation at different levels **(Figure 12).** For example cyclosporine, tacrolimus, and sirolimus are also named immunophilin-binding drugs, as they can interact with immunophilins (cyclophilins and FK binding proteins-FKBPs) interefering on the last signal of T cell activation, inhibiting the production of cytokines as IL-2. In particular, cyclosporine engages cyclophilin, while tacrolimus and sirolimus bind other cellular protein including FKBP12. The engagement of cyclosporine and tacrolimus inhibits the protein phosphatase calcineurin and prevents it from activating transcription factors such as NFATc, which is responsible for activation of cytokine genes during the immune response. Upon binding to FKBP12, sirolimus inhibits mammalian target of rapamycin (mTOR) and prevents it from activating the translation of mRNA-encoding proteins needed for cell division, inhibiting T cell division activated by growth factors such as IL-2.

Recently, the direct effects of immunosuppressive drugs on the BKPyV immune effectors and on the virus replication have been analyzed (Egli, Kohli et al. 2009, Hirsch, Yakhontova et al. 2015). In detail, effects of the mTOR inhibitor sirolimus and the CNIs cyclosporine and tacrolimus have been analyzed in in primary human renal tubular epithelial cells. Sirolimus significantly ihibited BKPyV replication at clinical concentration, and such inhibition occurred in the first 24 hours after infection, during viral early gene expression, but not during viral late gene expression. Conversely, tacrolimus favoured BKPyV replication. The knockdown of FKBP12 by siRNA, mimicking its natural binding with tacrolimus, resulted in an increase in BKPyV replication. Results demonstrated that sirolimus and tacrolimus have opposite effects on viral replication, by acting on the same target. Conversely, cyclosporine inhibited viral replication by binding cyclophilin and inhibiting calcineurin (Hirsch, Yakhontova et al. 2015).

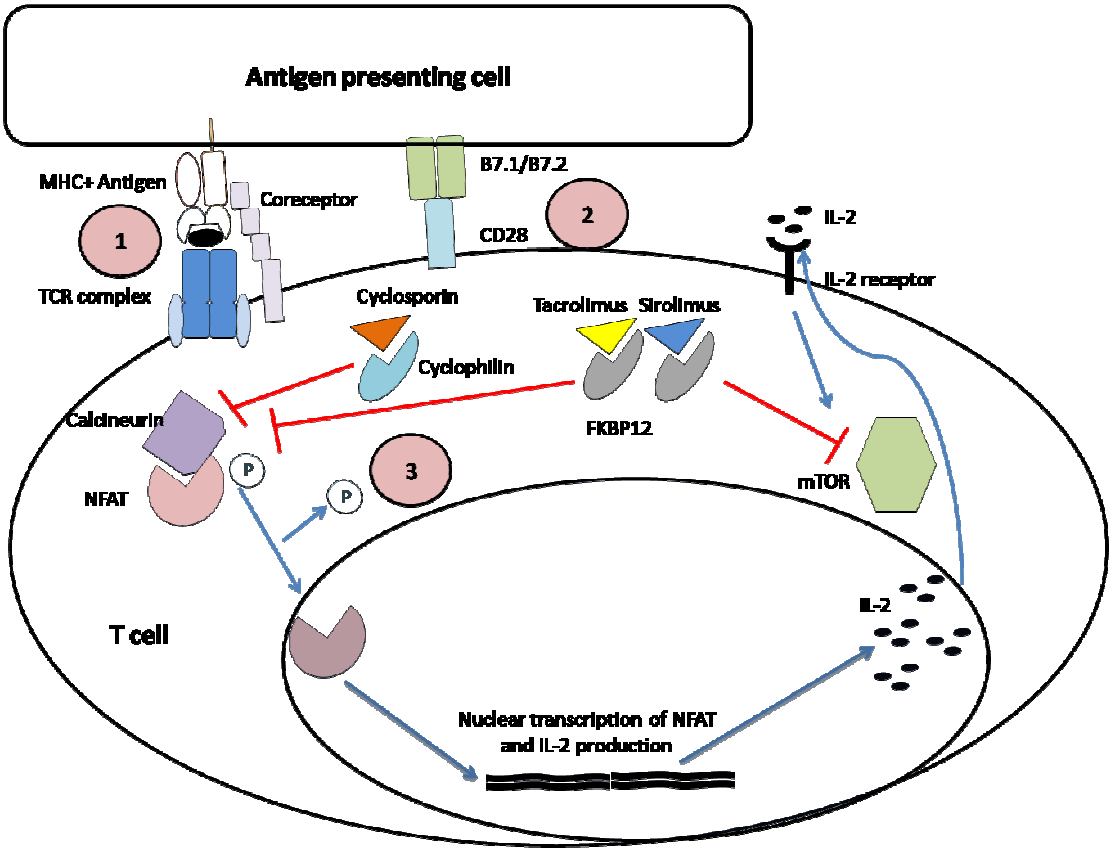

Figure 12: Immunosppressive drugs interference mechanisms with the multiple steps of T cell activation: Signal 1, TCR complex:MHC-peptide engagement; Signal 2, Costimulatory molecules activation; Signal 3; Activation of transcription factors for cytokines production.

Egli et al investigated on the direct effects of immunosuppressive drugs on BKPyV specific T cell immunity on PBMCs of healthy individuals. Adding tacrolimus at clinically relevant concentrations of 3 ng/mL resulted in inhibition of BKPyV specific T cell directed against LTag. Sirolimus did not have any inhibitory effect on T cell activation, however, adding it during BKPyV specific T cell expansion resulted in a decrease in BKPyV specific T cells. The results support the recommendation of reducing IS for treating BKPyV replication and disease, and that a switch to mTOR inhibitors after BKPyV specific T cell activation could be beneficial (Egli, Kohli et al. 2009).

3.3.2.2.2 Diagnosis and monitoring of BKPyV infection

BKPyVAN represents a complication associated with high-level and extremely rapid virus replication in the kidney transplant (Hirsch, Knowles et al. 2002, Hirsch and

Steiger 2003). Monitoring of BKPyV viruria, by urine cytology or quantitative PCR for viral DNA, and monitoring of BK viremia by quantitative PCR, can identify patients at high risk of developing histologically proven PyVAN (Hirsch, Brennan et al. 2005, KDIGO 2009, Hirsch and P. 2013). Genetic studies on intrapatient variants in blood and urine demonstrated that urine and plasma are separate replication compartments, with plasma being directly linked to intragraft replication whereas more than 95% of the urine viral loads appear to be generated from replication in the urothelial cell layer of the pyelon, ureter and bladder (Funk 2008).

BKPyV reactivation can occur early after kidney transplantation, and the rate of patients with viral replication increases in the first 6 months and with a peak at the third month. This paradigm of BKPyV replication and disease has been first described in the Basel group (Nickeleit, Klimkait et al. 2000, Hirsch, Mohaupt et al. 2001, Hirsch, Knowles et al. 2002) and confirmed by many studies around the world (Ginevri, De Santis et al. 2003, Koukoulaki, Grispou et al. 2009).

Testing for BKPyV viruria provides a window period of 6–12 weeks before viremia and nephropathy (Hirsch, Knowles et al. 2002). Viremia detection has a positive predictive value of 30–50% for biopsy-proven PyVAN with a window period of 2–6 weeks (Hirsch, Knowles et al. 2002, Hirsch and Randhawa 2013). The diagnosis of biopsy proven PyVAN requires tissue examination, i.e. by renal allograft biopsy showing positive immunostaining with cross-reactive antibodies to SV40 LTag, hence demonstrating PyV cytopathic changes (PyVAN-A), cytopathic and various degrees of inflammation previously called interstitial nephritis (PyVAN-B), and in late stages increased tubular atrophy and fibrosis (PyVAN-C) (Hirsch, Brennan et al. 2005, Drachenberg and Papadimitriou 2006, KDIGO 2009, Hirsch and P. 2013).

The focal nature of PyVAN and the possible overlap with other pathologies such as T cell mediated rejection can render a histological diagnosis very difficult. In fact, 10%-30% false-negative needle biopsy results have been estimated from a study determining BKPyV loads in urine and plasma and comparing the discordance rate between to biopsy cores (Drachenberg, Papadimitriou et al. 2004, Drachenberg, Papadimitriou et al. 2004). A similar pattern has been suggested when the clearance of histologic BKPyVAN disease has been examined (Menter, Mayr et al. 2013).

Thus, BKPyV viremia is the most predictive assay for the presence of “presumptive” PyVAN (Hirsch, Knowles et al. 2002, Hirsch and Steiger 2003, Hirsch, Brennan et al.

2005), thus it is recommended by current guidelines as the best assay to guide preemptive interventions (Brennan, Agha et al. 2005, Hirsch, Brennan et al. 2005, Drachenberg and Papadimitriou 2006, Ginevri, Azzi et al. 2007, Saad, Bresnahan et al. 2008, KDIGO 2009, Hirsch and P. 2013). Current guidelines suggest a BKVPyV replication screening at least every 3 months during the first 2 years post-transplant, and then annually until the fifth year post-transplant. Using this strategy, at least 80–90% patients at risk for BKPyVAN could be identified before significant functional impairment of the renal allograft occurs (KDIGO 2009, Hirsch and Randhawa 2013, Hirsch, Babel et al. 2014). In association with viral molecular monitoring, analysis of specific immune responses could become instrumental in assisting the surveillance and identification of patients at risk for developing BKPyVAN (Ginevri, De Santis et al. 2003).

3.3.2.2.3 Immune response to BKPyV

The host immune response is crucial in limiting primary infection and controlling viral replication through both innate and adaptive response. The first line of defense, prior to increase in adaptive immune response, is mediated by innate immunity effectors.

3.3.2.2.3.1 Innate Immunity

In BKPyV infection and related diseases innate immunity has not been deeply investigated, but some studies demonstrated the involvement of innate immune response effectors in the context of BKPyV infection and related diseases.

One study by Bohl demonstrated an association between lack of the HLA-C7 allele and sustained BKPyV viremia (Bohl, Storch et al. 2005), suggesting a possible role for inhibitory and activating killer-cell immunoglobulin-like receptors (KIRs) in the control of BKPyV infection, which has been confirmed later by Trydzenskaya (Trydzenskaya, Juerchott et al. 2013). Indeed, in samples from KTRs diagnosed with BKPyVAN, significantly lower frequencies of the activating receptor KIR3DS1 has been found. The protective role of this KIR genotype was demonstrated previously in other viral infections.

PRRs play a crucial role in triggering an efficient innate immune response. In KTRs with BKPyVAN, it has been demonstrated that TLR3 and RIG-I were upregulated, thus probably involved in the antiviral and inflammatory response (Ribeiro, Wornle et al. 2012), also affecting the Tumor Necrosis Factor apha (TNF alpha) and TNF receptor (TNFR) system (Ribeiro, Merkle et al. 2015). Indeed, it has been

demonstrated that BKPyV infection resulted in TNF alpha downregulation, and that PRRs TLR3 and RIG-I could induce an increase in TNFR expression.
Recognition of viruses by PRRs activate signal transduction cascades leading to secretion of IFN, which consequently binds to the IFN receptor expressed on the cell surface, and stimulates type I or II IFN-mediated signaling pathways. As a result, the STATs are phosphorylated and thus dimerize and translocate to the nucleus, where occurs the binding to gene promoters and activation of the transcription of interferon stimulated genes (ISGs), involved in antiviral responses. A recent study demonstrated that expression of BKPyV LTag induces an antiviral state by upregulation of ISGs via STAT1 activation. In particular the first 136 aa of the protein, mapping until the Rb binding domain, are necessary for this mechanism (Giacobbi, Gupta et al. 2015).

Among the key mediators of the innate immune system are also defensins, a family of antimicrobial peptides, which have been demonstrated to be involved in anti-BKPyV innate immune response. An *in vitro* study showed that human defensin 5 (HD5) interacts with the virus inducing aggregation of viral particles into very large, dense masses, and therefore interfering with viral attachment to host cells (Dugan, Maginnis et al. 2008). The ability to mount an efficient innate immune response against viruses is thought to also enable and activate effectors of the adaptive immune responses. Here, DCs act as a bridge between innate and adaptive immunity, uptake, processing and presenting pathogen-derived antigens, thus playing a crucial role in antiviral response. Impairments or deficits in DCs could lead to an ineffective adaptive immune response. A DCs level deficiency has been demonstrated in KTRs with BKPyVAN, but also in patients evaluated before transplant, who subsequently develop post-transplant viremia, underlying the importance of DCs and therefore of innate immunity in BKPyV infection (Womer, Huang et al. 2010).

3.3.2.2.3.2 Adaptive Immunity

In the control of BKPyV replication the interplay between humoral and cellular immunity is very important, with a crucial role for cell-mediated immune response in the kidney transplant patient. A study conducted in healthy individuals revealed that the majority of the population has BKPyV-specific antibodies (Egli, Infanti et al. 2009). It has also been demonstrated the presence of BKPyV specific antibodies with neutralizing activity in immunoglobulins preparations from healthy individuals,

meaning that in principle antibodies could contribute to BKPyV specific immunity (Randhawa, Pastrana et al. 2015), even if they cannot prevent cell to cell viral spread and are not expected to eradicate established viral infection (Egli, Infanti et al. 2009).

In the context of kidney transplantation many observations on BKPyV specific humoral immunity have been done. It has been shown that pre-transplant BKPyV seronegativity or low antibody titer correlates with higher risk of viral replication and consequent nephropathy (Ginevri, De Santis et al. 2003, Smith and McDonald 2006). Furthermore, a low baseline anti-BKPyV IgG titer and a reduced prevalence of IgA in patients with increasing viral loads suggests that pre-existing antibodies could have a protective role in BKPyV activity (Randhawa, Bohl et al. 2008). In cohorts of patients with high rate viruria or viremia, compared to patients without active viral replication, the pattern of BKPyV-specific antibodies was parallel to the level and duration of viral replication (Bohl, Storch et al. 2005, Hariharan, Cohen et al. 2005, Ginevri, Azzi et al. 2007, Leuenberger, Andresen et al. 2007, Bohl, Brennan et al. 2008, Bodaghi 2009, Schachtner, Muller et al. 2011, Schachtner, Stein et al. 2014). However, it has also been shown that presence of BKPyV-specific antibodies does not provide full protection from viral replication and consequent disease. In fact even though there is a correlation between antibodies titers and viral load, no correlation has been observed with viral clearance (Ginevri, Azzi et al. 2007, Bohl, Brennan et al. 2008, Bodaghi 2009).

It is important to underline that BKPyV-specific antibodies are mainly detected in enzyme-linked immunosorbent assay (ELISA) assays using as source of antigen VLPs, which are mainly constituted by VP1 protein. Indeed, when humoral response to VP1 has been compared to humoral response to LTag, different results has been observed. In fact, in KTR VP1 humoral response correlated with recent viruria and viremia, whereas anti-LTag response was associated with emerging BKPyV-specific immune control. (Leuenberger, Andresen et al. 2007, Bodaghi 2009). Furthermore, anti-agnoprotein antibodies were evaluated in healthy individuals and KTRs and compared to anti-VP1 and-LTag antibodies frequency. In healthy individuals anti-agno IgG were observed only in 15% of cases, compared to 41% and 63% of anti-LTag and anti-VP1 antibodies, respectively. In KTRs results were similar, in fact anti-agno antibodies were present in 8% of the samples, while anti-LTag and anti-VP1 antibodies were found in 63% and 80% of samples. In KTRs IgG levels were analyzed in patients, without or with low replication, with high active replication, and with past high replication. As expected, there was a strong increase of anti-LTag

antibodies from patients without replication to active high replication and even more in past replication. An increase was was observed also for anti VP1 from no replication group to high replication, but no correlation with viral replication was observed for anti-agnoprotein antibodies (Leuenberger, Andresen et al. 2007).

Taken together, available data suggest that markers of humoral immunity are not sufficient to give full protection against BKPyV replication, and that a distinction could be made between anti-LTag and anti-VP1 antibodies, as the first seems to have a more protective role. However, the presence an effective T cell mediated response is essential in controlling viral replication.

BKPyV-specific T-cell responses have been investigated in several studies conducted mainly in kidney transplant patients, or in other BKPyV related diseases. In healthy individuals BKPyV- specific T-cells were identified soon in early studies, and also more recently, in healthy individuals, could be identified showing a rather low frequency, when compared to antigens derived from other viruses as HCMV or EBV (Drummond, Shah et al. 1985, Tong, Miller et al. 2005, Egli 2009, Egli, Kohli et al. 2009).

BKPyV-specific T cell responses could be identified either for early and late proteins, with a predominance of CD4+ T cells (Zhou, Sharma et al. 2007). A recent studied showed that BKPyV-specific T-cells are age-dependent, in particular the authors observed a peak in BKPyV-specific T-cells in young individuals, suggesting that BKPyV-specific cellular immunity reflects phases of active BKPyV replication after primary infection in childhood (Schmidt, Adam et al. 2014). The control of BKPyV replication and PyVAN was associated with onset of virus-specific T cell response. In particular, Binggeli et al used overlapping 15mer peptides covering the LTag and the VP1 in ELISpot assays (Binggeli, Egli et al. 2006) and found that in kidney transplant patients, BKPyV-specific T cell responses in patients with decreasing or past BKPyV viral loads were significantly higher compared to patients with increasing or persisting viral loads (Binggeli, Egli et al. 2007). However, the VP1-specific responses were generally stronger compared to the LTag responses, and more likely involved CD4+ T-cells. Conversely, the LTag-specific responses contained a higher percentage of CD8+ T-cells (Binggeli, Egli et al. 2007).

These results were confirmed in a prospective cohort of 45 pediatric patients, as it was observed that kidney recipients during BKPyV reactivation had undetectable

levels of virus-specific T-cell responses. Upon immunosuppression reduction, parallel to declining viral loads in plasma and urine, the frequency of BKPyV-specific T cells increased (Ginevri, Azzi et al. 2007). As sequential blood samples were analyzed, it was observed that T-cell responses directed to LTag emerged slightly later than the anti-VP1-specific T-cell responses and significantly increased at viral clearance (Ginevri, Azzi et al. 2007). Moreover, an early onset of LTag-specific T-cells after transplant was demonstrated to be protective from the risk of viruria and viremia, in a cohort of KTRs, in fact LTag specific CTL activity was analyzed in patients who did not reactivate the virus, viruric, and viremic ones. At 1 month after transplantation a higher CTL activity was observed in patients who did not reactivate the virus, compared to the ones with viral replication throughout the follow up (Comoli, Basso et al. 2009). Similarly, in another cohort of adult patients diagnosed with BKPyVAN, T-cell responses towards 20mer peptides derived from the LTag coincided with clearance of BKPyV viremia (Prosser, Orentas et al. 2008). Chakera et al. reported that a significantly higher BKPyV-specific T-cell response was associated with cleared BKPyV viremia in kidney transplant patients compared to those patients with active replication or without evidence of reactivation, whereby a heterogeneous pattern of responses to the different viral antigens was observed, depending on single patients, with no clear immunodominance of a specific viral protein, moreover the antigens with higher association to viral clearance were VP1 and LTag (Chakera, Bennett et al. 2011).

More recent studies confirmed the previous results, and investigated BKPyV T-cell immunity directed to the other viral proteins. The T-cell responses were evaluated towards early proteins (sTag, and LTag) and late proteins (VP1, VP2, VP3), in patients with a history of BKPyVAN and with BKPyV transient reactivation. Results show that responses could be identified towards all investigated antigens, with a slight predominant immunogenicity to VP3, moreover patients with a history of BKPyVAN demonstrated significantly higher frequencies of IFN-**γ** and IL-2 producing CD4+ T cells (Mueller, Schachtner et al. 2011). However, the role of VP3 responses was later downplayed by the same group, when Schachtner et al showed that patients with self-limited viral reactivation developed BKPyV-specific T cell responses, and cleared the virus in a short time (median: 1 month). In patients diagnosed with BKPyVAN, virus-specific immune response emerged later (median 5 months) after therapeutic interventions (Schachtner, Stein et al. 2014). Notably, immune response towards structural proteins were higher and appeared earlier, whearas anti sTag and LTag T cells could be observed in parallel to viral clearance

(Schachtner, Muller et al. 2011, Schachtner, Stein et al. 2014).

The combined data suggest a pivotal role of BKPyV-specific cellular immunity in the control of viral replication, and has been explored for time points before and after kidney transplantation. Patients with decreasing LTag-specific T cells from pre- to post-transplantation are at significant higher risk of viral replication, as observed in KTRs with or without viremia (Schachtner, Stein et al. 2015). Decreasing of BKPyV specific T cells at 30 days post-transplant had a positive predictive value for early-onset BK viremia of 81.8% (9/11 patients) and sensitivity of 90% (9/10 patients), however detectable pre-transplant BKV-specific T were not a really reliable prognostic factor, as it gives a positive predictive value for early-onset BK viremia of 58.8% (10/17 patients) and sensitivity of 62.5% (10/16 patients). Such observation confirmed all previous data, suggesting that immune response to early proteins seems to be more crucial in viral replication control (Binggeli, Egli et al. 2007, Ginevri, Azzi et al. 2007, Comoli, Basso et al. 2009).

In most of these studies, BKPyV-specific T-cell immunity has been investigated using IFN-γ ELISpot assays (Prosser 2006, Binggeli, Egli et al. 2007, Ginevri, Azzi et al. 2007, Prosser, Orentas et al. 2008, Chakera, Bennett et al. 2011, Schachtner, Muller et al. 2011), which identified IFN-γ producing T cells in response to viral antigenic stimuli, but did not give information about subpopulations. Ginevri et al reported data with functional ^{51}Chromium release assay, identifying CTL responses, which in most of cases corresponded to antigen-specific CD8+ T cells. In particular, they showed that cytotoxic responses occurred at viral clearance and were directed mainly against LTag (Ginevri, Azzi et al. 2007).

Using flow cytometry analysis, Binggeli et al could determine that VP1 mainly induced production of intracellular IFN-γ in CD4+ T cells whereas LTag preferentially stimulated CD8+ T cells (Binggeli, Egli et al. 2007). Trydzenskaya observed that patients with rapid BKPyV clearance showed higher frequency of multifunctional IFN-γ/IL-2/TNF-α and IL-2/TNF-α CD4+ T cells and absence of Th17 CD4+ T cells (Trydzenskaya, Sattler et al. 2011). In more recent studies approaching antigen-specific T cell analysis by multiparameter flow cytometry strategy, it has been possible to provide information on phenotype and functionality of BKVPyV-specific T cells (Weist, Schmueck et al. 2014, Weist, Wehler et al. 2015). They were categorized in CD4+ T helper (coexpressing CD137 and CD154) and cytolytic T cells

based on expression of CD137, GranzymeB and CD4 or CD8 and endowed with IFN-γ/IL-2/TNF-α secretion capacity. The authors show that CD4+ helper cells have mainly an effector memory phenotype, while the cytolytic ones were mostly terminally differentiated effectors, probably deriving from the helper compartment (Weist, Wehler et al. 2015).

Furthermore, an increased number of CD4+ PD-1+ T cells were observed during and after viral clearance, marker that has been associated to functional exhaustion and chronic viremia in other viral infections, such as CMV infection (Dirks, Egli et al. 2013). Conversely to previous studies, no significant role was observed for CD8+ T cells, probably because of different cell stimulation methods, as lymphocytes were stimulated by a mixture of peptides covering the whole sequence of early and late proteins, while in previous studies CD8+ T cells were shown to be stimulated mostly by LTag. Of note, only one study evaluated the possible role of agnoprotein in BKPyV-specific T cell immunity, and results obtained in healthy individuals and KTR showed that in both healthy and immunocompromised individuals agnoprotein-specific T cells were barely detectable. Agnoprotein poor immunogenicity, despite its abundant expression demonstrated in KTR biopsies, could suggest a role in possible viral escape mechanisms (Leuenberger, Andresen et al. 2007).

In the context of kidney transplantation, immunosuppressive therapy may affect virus-specific T cell responses, as such drugs act at diffent levels of T cell activation. Such effects have been studied *in vivo* and *in vitro* (Egli, Kohli et al. 2009, Weist, Wehler et al. 2015). Results show that BKPyV-specific T cells inversely correlated with tacrolimus trough levels, and sirolimus affected only antigen-dependent T cell expansion (Egli, Kohli et al. 2009). The same effects of CNIs on BKPyV-specific T cell activation could be confirmed by Weist et al (Hirsch, Yakhontova et al. 2015, Weist, Wehler et al. 2015).

Taken together, all studies focusing on BKPyV-specific immune response after transplant demonstrated that size, frequency, and possibly also subtypes of the virus-specific T-cell response is inversely correlated with viral replication, and that monitoring viral activity and immune response may provide relevant information to better determine patients at risk for developing BKPyV viremia and progressing to BKPyVAN. In particular, the fact that LTag-specific T cells emerge after viral clearance, when the immune system is efficiently limiting virus replication, strongly suggests that immune responses directed to BKPyV early proteins play a major

protective role from the development of BKPyV-associated diseases, therefore it would be of help in the clinical management of KTRs during their post transplant follow up, a screening of BKPyV-specific T cell responses in order to identify subsets of patients at risk for developing viremia, and monitoring should be continued until the detection of virus-specific T cell response.

3.3.2.2.4 Therapeutic strategies

3.3.2.2.4.1 Immunosuppression reduction

Studies focusing on BKPyV-specific immune response after kidney transplantation help to monitor the course of viral activity and guide pre-emptive interventions. The uncontrolled replication of the virus is allowed by a lack of efficient BKPyV-specific immune response, thus the current therapeutic strategy to prevent the progression to BKPyVAN is the reduction of the immunosuppressive drug regimen in order to restore an effective BKPyV-specific T cell response.

In kidney transplant patients the standard anti-rejection therapy consists usually in CNI, MMF, and steroids. Diverse strategies in different steps have been reported for IS reduction and/or suspension:

1. As first step, dose reduction of the CNI by 25–50%, then reduction of the antiproliferative drug by 50%, followed by discontinuing the latter (Ginevri, Azzi et al. 2007, Schaub, Hirsch et al. 2010, Almeras, Vetromile et al. 2011).
2. Reduction of the antiproliferative drug by 50% followed by reducing CNI by 25–50%, followed by discontinuing the antiproliferative drug (Brennan, Agha et al. 2005).
3. Concurrent reduction of dosages of both CNI and mycophenolate mofetil (Sood, Senanayake et al. 2012, Knight, Gaber et al. 2013).

   Creatinine levels and BKPyV load are tightly monitored during and after these steps, to control the efficacy of the treatment and avoid possible episodes of acute rejection.

Despite preemptive BKPyV viremia-guided multiple steps of IS reduction, a minor quote of patients still progress to overt BKPyVAN.

Similarly, in cases of proven BKPyVAN the first line of therapy is IS reduction. However, additional therapeutic strategies have been reported, but results are still controversial.

3.3.2.2.4.2 Antivirals

Cidofovir *is* a nucleoside analog, licensed by The Food & Drug Administration for the treatment of cytomegalovirus retinitis. *In vitro* experiments demonstrated that it can inhibit viral replication in BKPyV-infected renal proximal tubular epithelial cells (Bernhoff, Gutteberg et al. 2008). *In vivo* administration of cidofovir gives controversial results, since some studies report clinical improvements (Vats, Shapiro et al. 2003, Araya, Lew et al. 2008), whereas others report no demonstrable benefit, with additional risk of nephrotoxicity (Kuypers, Vandooren et al. 2005, Kuypers, Bammens et al. 2009). More recently, a more potent lipid-ester derivative 1-O-hexadecyloxypropyl-cidofovir (CMX001) able to effectively inhibit *in vitro* BKPyV replication either in human renal tubular cells and in urothelial cells (Randhawa, Farasati et al. 2006, Rinaldo, Gosert et al. 2010, Tylden, Hirsch et al. 2015). It has been used in sporadic cases of BKPyVAN resulting in stabilization of creatinine (Reisman, Habib et al. 2014, Papanicolaou, Lee et al. 2015).

Leflunomide is an immunomodulatory drug capable of mitochondrial dihydroorotate dehydrogenase inhibition, leading to pyrimidine depletion and cytostasis, particularly in activated lymphocytes. It has been shown to inhibit BKPyV replication *in vitro* in renal tubular cells (Bernhoff, Tylden et al. 2010). It has been administered as a replacement for discontinued mycophenolic acid during IS reduction (Halim, Al-Otaibi et al. 2014), or in addition to protocols applying at the same time a reduction in IS (Elfadawy, Flechner et al. 2013). In some smaller studies, this has been associated with clinical improvement (Teschner, Gerke et al. 2009), while in other cases this remained without benefits (Krisl, Taber et al. 2012), and in fact additional toxicity (Faguer, Hirsch et al. 2007). However, results from randomized controlled trials are needed to assess the clinical efficacy of the drug.

Fluoroquinolones have been proposed as inhibitors of BKPyV replication via an effect on the helicase activity of LTag but the selectivity index results low *in vitro* (Sharma, Li et al. 2011). No significant improvement has been shown when combined to IS reduction, leflunomide, and human intraveneous immunoglobulins (IVIG) (Halim, Al-Otaibi et al. 2014).

IVIG have been administered which have been shown to contain BKPyV-neutralizing antibodies (Randhawa, Pastrana et al. 2015). Thirty patients with diagnosis of BKPyVAN and not responding to 8 weeks IS reduction, nor leflunomide treatment were infused with IVIG, viral load significantly decreased after treatment and 90%

cleared viremia at 1 year of follow up (Vu, Shah et al. 2015). Larger cohorts and controlled studies would be required to verify the efficacy of such treatments.

3.3.2.2.5 Novel immunotherapeutic strategies

All therapeutic approaches besides immunosuppression reduction do not seem to highly improve graft outcome in presumptive and proven PyVAN, for this reason alternative strategies are needed to boost BKPyV-specific immunity.

3.3.2.2.5.1 Adoptive T cell transfer

It has been extensively demonstrated that regaining BKPyV-specific T-cell immunity to prevent PyVAN is essential, thus protocols of virus-specific adoptive T-cell therapy could be an effective approach (Comoli, Cioni et al. 2013). In an early study a method for *in vitro* generation of BKPyV-specific T cells has been described. PBMCs from BKVPyV seropositive healthy donors and kidney transplant patients were stimulated with dendritic cells pulsed with inactivated virus, in the presence of IL-7 and IL-12. BKPyV-specific T-cells with cytotoxic activity could be obtained, with a high frequency of CD3+ /TCRγδ+ cells displaying an MHC-unrestricted cytotoxicity (Comoli, Basso et al. 2003). The use of inactivated virus is not easily conducted in the context of the requirements for good medical manufacturing practice, which are a prerequisite for the permission to use T-cell therapies.

A recent study demonstrated the possibility of expanding BKPyV-specific T-cells for possible use in adoptive cell transfer, by stimulating PBMCs from healthy donors and transplant patients with monocyte-derived dendritic cells pulsed with overlapping peptide pools covering the whole amino acid sequences encoded in VP1, VP2, VP3, sTag and LTag in the presence of IL-2. CD4+ and CD8+ T cells could be obtained, which responded to restimulation to viral antigens, in particular VP1, LTag and sTag, but without a clear immunodominance. Intracellular production of IFN-γ TNF-α and IL-2 could be observed, suggesting the contribution of both Th1 and Th2 cells (Blyth, Clancy et al. 2011).

So far, no cases of BKPyV-specific adoptive T cell transfer have been published for kidney transplant patients, but there have been clinical studies in HSCT patients (Papadopoulou, Gerdemann et al. 2014) where T cells for adoptive T cell transfer were generated from PBMCs of HSCT donors specific for 12 immunogenic antigens of EBV, Adenovirus, CMV, HHV-6 and BKPyV (VP1 and LTag). From 48 clinical grade T cells preparation obtained after peptidic stimulation including CD8+ and

CD4+ T cells, 14 had activity against all five stimulating viruses (pentavalent), 9 recognized four viruses (tetravalent), 12 were trivalent, 11 were divalent, 1 was monovalent, and 1 failed to recognize any of the targeted viruses. Only 28 of 48 were BKPyV specific. Cells were infused and resulted to be safe and effective in those patients showing viral reactivations (Papadopoulou, Gerdemann et al. 2014).

There are some unique data for a young HSCT recipient affected by JCPyV-related PML successfully treated with JCPyV-specific T cells obtained by stimulating PBMCs from the HSCT donor with a pool of 15mer peptides spanning the whole sequence of JCPyV VP1 and the LTag (Balduzzi, Lucchini et al. 2011), which had been characterized earlier in the Binggeli study in kidney transplant patients (Binggeli, Egli et al. 2007) and another study on JCPyV-specific responses in HIV-AIDS patients with and without PML (Khanna, Wolbers et al. 2009). This expansion method could be extended to BKPyV and might be used in clinical practice.

Another promising method that could be part of the strategy aiming at obtaining antigen-specific T cells for adoptive transfer is streptamer staining and selection. This method derives from HLA-peptide multimers technology, consisting of multimers of HLA molecules bound to HLA-restricted peptides and labeled to fluorophores, which target and allows the detection of HLA-matched antigen-specific T cells. In this way, epitope-specific T cells can be identified and sorted, but cannot be administered for adoptive T cell transfer, as markers remain on the cell surface after staining and therefore could compromise the effector function of the stained T cells. The novelty of streptamers consists in the possibility of reverting the binding of the multimer to the cell thanks to a Strep-tag / Strep-Tactin technology. After the selection of epitope-specific T cells, by adding biotin, the interaction of Strep-tag with Strep-Tactin can be disrupted and purified epitope-specific T cells are free of any marker on their surface, possibly with preserved effector functions.

3.3.2.2.5.2 Epitope mapping and vaccine development

In the perspective of using epitope-specific T cells for adoptive transfer, it would be necessary to identify immunogenic, possibly immunodominant, HLA-restricted epitopes deriving from viral proteins. For this purpose, epitope mapping would be useful. T cell epitope mapping can be performed with different techniques, at different levels, predictive and experimental. Predictive approaches include the use of computer algorithms predicting the processing and/or binding of aminoacidic

sequences to defined HLA alleles. Then, putative epitopes can be tested experimentally with solid phase binding assays, ELISpot assays, killing assays and also multimer staining.

Identification of immunodominant epitopes would be of great interest also in the context of peptide vaccine development to prime and/or boost BKPyV-specific immunity in order to prevent BKPyV-related diseases. Different studies investigated BKPyV-specific T cell immunity identifying immunogenic epitopes within VP1 and LTag sequences, either for HLA class I and II alleles **(Table 2).** Most of studies focused on the highly frequent HLA-A*02 allele, while it would be of interest to identify immunodominant epitopes through the majority of frequent alleles.

In the first BKPyV report on selective epitopes by Krimskaya et al, an immunogenic epitope within VP1 sequence was identified. Candidate peptides selected by algorithm predictions were experimentally verified by ^{51}Chromium release assay and ICC in splenocytes obtained from humanized HLA-A*02 mice. Moreover, HLA restriction was tested in PBMCs samples obtained from 10 healthy individuals (HI) and 1 KTR and stimulated *in vitro* with VP1 peptides. By tetramer and CD107 staining they could identify an HLA-A*02 9mer epitope starting at aa position 108 in VP1 sequence (p108), able to elicit cytotoxic T cell response in 3/10 HI and in the KTR (Krymskaya, Sharma et al. 2005). The same group confirmed the p108 epitope and another epitope at position VP1 144 (p144) in a larger cohort of HI, and observed that such epitopes were cross-reactive with JCPyV (Sharma, Zhou et al. 2006). They later could determine that such epitopes were able to elicit CD4+ cytotoxic T cell responses characterized by secretion of multiple cytokines, in particular IFN-γ and TNFα- (Zhou, Sharma et al. 2007).

VP1-derived p108 epitope was confirmed to be immunodominant also by other investigators, through computer prediction and experimental confirmation with ^{51}Chromium release assay and tetramer staining, Chen at al could identify a cytotoxic T cell response in 50% of HI samples, and identified another HLA-A*02 immunodominant 9mer epitope in 80% of HD (p44). In samples from PyVAN patients the response was more frequently found towards p108 than p44, suggesting a different pattern of response in patients (Chen, Trofe et al. 2006).

Other groups reported epitopes within the LTag sequence. The first study identified an immunodominant epitope specific for HLA-B*07 and –B*08 at N-terminus of the

protein, as a 15mer sequence starting at position 25, also containing a 9mer epitope at position 27. They could also find HLA class II 15mer epitopes restricted for HLA-DRB1*0901 at aa position 154, 139 and 140, and for HLA-DRB1*0301 at position 15. Such epitopes were conserved in JCPyV (Li, Melenhorst et al. 2006). Randhawa et al identified other LTag epitopes in HLA-A*02 HI and KTR. Candidate epitopes selected by algorithm prediction were tested *in vitro* in ELISpot assay and 9mer epitopes at position 362 was found to elicit IFN-γ production in 83% of the tested HI and in 71% KTR. Epitopes at position 406 and 410 were also frequently identified in HLA-A*02 HI and KTR (Randhawa, Popescu et al. 2006) and further confirmed by other studies (Zhou, Sharma et al. 2007, Schneidawind, Schmitt et al. 2010). Provenzano et al characterized in healthy donor samples the immune response to HLA-A*02 specific LTag 9mer epitope starting at position 579, previously identified by Zhou et al. They could find that the epitope elicited an increase in IFN-γ expression and secretion, moreover it could elicit CTL response by CD8+ CD45RA+ T cells (Provenzano, Bracci et al. 2006).

Another study focused on other frequent HLA Class I alleles, in particular HLA-A*01, A*03, and A*24 (Ramaswami, Popescu et al. 2009). The authors could identify numerous immunogenic epitopes after computer prediction, *in vitro* binding and ELISpot assay using PBMCs cultured in presence of a mix of overlapping peptides spanning the whole LTag sequence. A 9mer epitope at position 506 was found in 100% of HI and 67% of HLA-A*03 KTRs. Other epitopes were found with a minor frequence (Ramaswami, Popescu et al. 2009). Unfortunately, other tests would be required to confirm HLA restriction. Thereafter, the same group identified a 15mer epitope able to elicit a T cell response mediated by class II, in different HLA-DRB1 alleles. It is located in the helicase domain of LTag, at position 313 (Ramaswami, Popescu et al. 2011). A recent study characterized the phenotype of epitope-specific T cells, in particular to VP1 p44 and p108, and LTag p579 and p410. They observed the immunodominance of VP1 p108, a low frequency of p579 responses and none for p410. Epitope-specific T cells showed an effector memory phenotype by FACS analysis, not proliferating. They produced IL-2, IFN-γ, TNF-α and expressed low levels of CD107a (van Aalderen, Remmerswaal et al. 2013). All these studies suggest an immunodominance for VP1 epitope p108 in HLA-A*02 individuals, which could be confirmed by several studies and techniques, whereas this was not the case for the BKPyV LTag **(Table 2)**.

Epitope specific T cell responses should be analyzed in KTR and correlated with the

status of BKPyV-related disease, to understand which are more associated to viral clearance or protection from viremia, and studies investigating BKPyV VP1 and LTag epitopes restricted to other frequent HLA types would be required in order to characterize the pattern of BKPyV immune response in the majority of the population. Targeting several HLA types would be of great interest in the context of immunotherapy, giving the possibility of preventing and/or treating diseases in as many people as possible, either by adoptive T-cell therapy and vaccine administration. A very recent paper investigated BKPyV T-cell epitopes by next generation sequencing of the whole viral genome in samples from viremic transplant recipients. The authors found low levels of variants in epitopes sequences, which may suggest that despite conservation, BKPyV variants may encode peptides that can escape the immune response and emerge in case of selection pressure given by an eventual immunotherapy (Sahoo, Tan et al. 2015).

Table 2: Published BKPyV specific epitopes

Reference	BKVPyV protein	aa start position	aa sequence	HLA restriction	Studied population	Frequency	Experimental Methods
(Krymskaya, Sharma et al. 2005)	VP1	108	LLMWEAVTV	HLA-A*02	Humanized mice 10 HI, 1 KTRs	30% HI 100% KTR	$^{-51}$Cr release assay -ICC -tetramer staining -CD107 assay
(Sharma, Zhou et al. 2006)	VP1	44	AITEVECFL	HLA-A*02	11 HI	73%	
	VP1	108	LLMWEAVTV	HLA-A*02	25 HI	20%	
(Chen, Trofe et al. 2006)	VP1	44	AITEVECFL	HLA-A*02	10 HI 10 KTRs with PyVAN	80% HI 100% KTR	$^{-51}$Cr release assay -tetramer staining
	VP1	108	LLMWEAVTV	HLA-A*02	10 HI, 10 KTRs with PyVAN	50%HI 100% KTR	
(Zhou, Sharma et al. 2007)	VP1	108	LLMWEAVTV	HLA-A*02	13 HI	not described	-ICC -CD107 assay
	LTag	362	MLTERFNHIL	HLA-A*02	13 HI	not described	
	LTag	406	VIFDFLHCI	HLA-A*02	13 HI	not described	
	LTag	579	LLLIWFRPV	HLA-A*02	13 HI	not described	
(Randhawa, Popescu et al. 2006)	LTag	362	MLTERFNHIL	HLA -A*02	6 HI, 7 KTRs	83%HI, 71% KTRs	IFN-γ ELISpot assay
	LTag	406	VIFDFLHCI	HLA-A*02	6 HI, 7 KTRs	50%HI, 29% KTRs	
	LTag	410	FLHCIVFNV	HLA-A*02	6 HI, 7 KTRs	50%HI, 29% KTRs	
	LTag	579	LLLIWFRPV	HLA-A*02	6 HI, 7 KTRs	0% HI, 14% KTRs	
(Provenzano, Bracci et al. 2006)	LTag	579	LLLIWFRPV	HLA-A*02	5 HI	100% IFN-γ, 80% ^{51}Cr	HLA binding assay IFN-γ gene expression $^{-51}$Cr release assay Tetramer staining
	LTag	406	VIFDFLHCI	HLA-A*02	5 HI	80% IFN-γ, 100% ^{51}Cr	
	LTag	410	FLHCIVFNV	HLA-A*02	5 HI	80% IFN-γ,50% ^{51}Cr	
	LTag	398	CLLPKMDSV	HLA-A*02	5 HI	80% IFN-γ,	
	LTag	216	KLCTFSFLI	HLA-A*02	5 HI	25% IFN-γ,	
	LTag	472	VVFEDVKGT	HLA-A*02	5 HI	25% IFN-γ,	
	LTag	558	SLQNSEFLL	HLA-A*02	5 HI	25% IFN-γ,	
	LTag	157	TLACFAVYT	HLA-A*02	5 HI	25% IFN-γ,50% ^{51}Cr	
Li 2006	LTag	27	LPLMRKAYLRKCK	HLA-B*07, HLA -B*08	17 HI	not described	ICC, CSFE based cytotoxicity assay
	LTag	15	TLYKKMEQDVKVAHQ	HLA-DRB1*0301	17 HI	not described	
	LTag	139	IYLRKSLQNSEFLLE	HLA-DRB1*0901	17 HI	not described	
	LTag	140	KSLQNSEFLLEKRIL	HLA-DRB1*0901	17 HI	not described	
	LTag	154	TFSRMKYNICMGKCI	HLA-DRB1*0901	17 HI	not described	
(Ramaswami, Popescu et al. 2009, Randhawa, Viscidi et al. 2009)	LTag	506	SVKVNLEKK	HLA-A*03	3 HI, 6 KTRs	100% HI, 67% KTRs	HLA peptide binding assay IFN-γ ELISpot assay
(Ramaswami, Popescu et al. 2011)	LTag	313	PYHFKYHEKHFANAI	restriction none or unknown	27 HI	33%	IFN-γ ELISpot assay ICC
(Schneidawind, Schmitt et al. 2010)	VP1	108	LLMWEAVTV	HLA-A*02	25 HI 7 HSCT HC	75%HSCT HC, 28% HI	IFN-γ and GranzimeB ELISpot assay Tetramer staining
(van Aalderen, Remmerswaal et al. 2013)	VP1	108	LLMWEAVTV	HLA-A*02	15 HI	47%	Tetramer staining
	VP1	44	AITEVECFL	HLA-A*02	15 HI	40%	

4 Aims

With this study we sought to explore BKPyV-specific immune responses, focusing on:

1. Evaluation of BKPyV agnoprotein role in immune escape mechanisms, as downregulation of MHC class I and II molecules.
2. Identification and characterization of immunodominant 9mer-epitope T cell responses within BKPyV EVGR proteome

Providing new insight in BKPyV immunity has the ultimate goal of applying such new knowledge to clinical practice, giving insights for novel therapeutic approaches for preventing and/or treating BKPyV associated diseases.

5 Results

5.1 Comparing Effects of BK Virus Agnoprotein and Herpes Simplex-1 ICP47 on MHC-I and MHC-II Expression

Agnoprotein of BKPyV is abundantly expressed in the late viral life cycle, and localizes mainly to the cytoplasm and perinuclear area, it colocalizes with lipid droplets (Rinaldo, Traavik et al. 1998, Leuenberger, Andresen et al. 2007, Unterstab, Gosert et al. 2010). Among all HPyV, an ORF encoding an agnoprotein has been found only for BKPyV and JCPyV, and several studies have been investigating its function, which at present still remains to be defined.

Potential cellular interaction partners have been proposed following a yeast-2-hybrid screening in order to come a step closer to cellular pathways that agnoprotein might target. Thus, it has been shown that BKPyV agnoprotein can interact with α-SNAP, protein involved in the intra-cellular trafficking and fusing of vesicles in the cell membranes, leading *in vitro* to interference in the cell surface secretion pathway (Johannessen, Walquist et al. 2011). The possible involvement of agnoprotein in vesicle transport is supported by the observation that agnoprotein co-localizes with lipid droplets (Unterstab, Gosert et al. 2010), since other proteins detected in lipid droplets have been linked to intracellular vesicle transport. (Guo, Walther et al. 2008). Agnoprotein has also been demonstrated to interact with PCNA, leading to inhibition of PCNA-dependent DNA synthesis in vitro and consequent reduction of cell proliferation (Gerits, Johannessen et al. 2015).

Despite abundantly expressed, also *in vivo* in florid biopsies of of kidney transplant patients diagnosed with PyVAN, agnoprotein seems to be immunologically ignored. Both, humoral and cellular responses have been investigated towards this protein, but with very low or even absent results. Moreover, differently from LTag and VP1, the rare agnoprotein-specific T cell responses did not correlate with the course of viral replication in patients (Leuenberger, Andresen et al. 2007).

The observations that agnoprotein could negatively influence vescicular transport

and cell proliferation, together with the absence of antigen specific immune responses could suggest a role for agnoprotein in immune evasion mechanisms, maybe involving antigen processing and presentation pathways. In this context, it is of interest to note that histopathology studies reported a decreased HLA class II expression of BKPyV-infected renal tubular epithelial cells compared to the levels found in biopsies showing acute cellular rejection (Nickeleit, Hirsch et al. 2000). Together, these data suggested the hypothesis that maybe a component of BKPyV genome could negatively influence HLA class II expression.

This hypothesis is in line with some considerations regarding the virus-host interaction, that can be derived from the BKPyV and JCPyV biology and epidemiology. BKPyV and JCPyV are viruses successfully adapted to the human host, as evidenced by the fact, that the vast majority of the general human population is infected, yet without any significant clinical manifestations. However, BKPyV and JCPyV are not perfectly controlled in the human host, since there is evidence of asymptomatic persistent and/or intermittent replication in healthy individuals (Egli, Infanti et al. 2009). Indeed, signs of disease are almost exclusively found in special patient groups that are immunocompromised (Hirsch 2005, Hirsch, Kardas et al. 2013, Hirsch, Babel et al. 2014). Persistence is peculiar to viruses having strategies of immune evasion such as members of the Herpesviridae, as EBV, CMV, KHSV and HSV. In BKPyV and JCPyV, a possible mechanism for immune evasion and down-regulation of viral gene expression has been proposed, as a miRNA has been identified in the LVGR, capable of targeting the mRNA of the cellular stress induced ligand ULBP3, with the effect of down-regulating LTag mRNA expression, known to induce an important cell response (Bauman, Nachmani et al. 2011). Taken together, it seems a biologically plausible hypothesis to postulate that BKPyV has evolved one or more mechanisms of immune escape, and that agnoprotein could play a role in such processes.

Several viral proteins have been described able to down-modulate cell surface HLA molecules expression in order to hide from immune system recognition. Notably, the immediate early protein ICP47 (pICP47) from HSV is small viral protein of only 88 aa, similarly small as the BKPyV agnoprotein. The pICP47 has been reported to downregulate HLA class I antigen presentation by specifically binding to TAP, and thereby impairing the presentation of HLA-peptide complexes. The functional domain of HSV-pICP47 appears to map to the aa 2-35 of the N-terminus. Strikingly, the secondary structure of HSV-pICP47 is predicted to contain an amphipathic helix

structure (Galocha, Hill et al. 1997, Aisenbrey, Sizun et al. 2006). Given these similarities between HCV-pICP47 and the BKPyV agnoprotein, we hypothesized that the BKPyV agnoprotein might have a similar immunomodulatory role in BKPyV immune evasion. Therefore, an experimental system was set up to test this hypothesis in vitro. We investigated effects of agnoprotein expression on HLA class I and II (HLA-ABC and –DR) surface expression in transiently and stably transfected cells.

The levels of HLA class I and class II expression of primary human renal tubular epithelial cells (RPTEC) were quantified by flow cytometry in the presence or absence of agno expression. In a second approach, UTA-6 cells co-transfected with plasmids constitutively expressing agnoprotein and the enhanced green-fluorescent protein (EGFP), as well as in UTA-6 cells bearing tetracycline regulated agnoprotein. As a control, the HSV-pICP47 was transfected and its effect on HLA expression was compared. Finally, as the ultimate goal of antigen presentation via MHC class I is the induction of CTL activity, we evaluated the influence of agnoprotein expression on antigen specific CTL on UTA-6 cells expressing tetracycline regulated agnoprotein.

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Research Article

Comparing Effects of BK Virus Agnoprotein and Herpes Simplex-1 ICP47 on MHC-I and MHC-II Expression

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Background. Among human polyomaviruses, only BK virus (BKV) and JC virus (JCV) encode an agnoprotein upstream of VP1 on the viral late transcript. BKV agnoprotein is abundantly expressed late in the viral life cycle, but specific cellular and humoral immune responses are low or absent. We hypothesized that agnoprotein might contribute to BKV immune evasion by downregulating HLA expression, similar to Herpes simplex virus-1 ICP47. Methods UTA-6 or primary human renal proximal tubular epithelial cells (RPTEC) were co-transfected with plasmids constitutively expressing agnoprotein, or ICP47, and enhanced green-fluorescent protein (EGFP). EGFP-gated cells were analyzed for HLA-ABC and HLA-DR expression by flow cytometry. HLA-ABC and HLA-DR expression was also analyzed on UTA-6 bearing tetracycline-regulated agnoprotein or ICP47. Effects of agnoprotein on viral peptide-dependent T-cell killing were investigated using ^{51}Cr release. *Results*. ICP47 downregulated HLA-ABC without affecting HLA-DR, whereas agnoprotein did not affect HLA-ABC or HLA-DR expression. Interferon-γ treatment increased HLA-ABC in a dose-dependent manner, which was antagonized by ICP47, but not by agnoprotein. In UTA-6 cells, agnoprotein expression did neither impair HLA-ABC or -DR expression nor peptide-specific killing impaired by HLA-matched T-cells. *Conclusion*. Unlike the HSV-1 ICP47, BKV agnoprotein does not contribute to viral immune evasion by down-regulating HLA-ABC, or interfere with HLA-DR expression or peptide-dependent T-cell cytotoxicity.

1. Introduction

Human polyomavirus (HPyV) species comprise currently at least 9 members that infect 30 to 90 percent of the general population without severe clinical manifestations [1–6]. In immunocompromised individuals, however, significant pathologies have been linked to HPyVs, for example, nephropathy and haemorrhagic cystitis to BKV, progressive multifocal leukoencephalopathy to JCV, *trichodysplasia spinulosa* to TSPyV and Merkel cell carcinoma to MCPyV [7, 8]. The general architecture of the approximately 5.1 kb circular dsDNA genome is shared among all HPyVs consisting of the noncoding control region separating the early nonstructural genes encoding small and large T antigen and late capsid VP1, -2, and -3 genes [6]. So far, however, only BKV and JCV contain an additional small conserved open reading frame (ORF) in the late gene transcript upstream of the major capsid protein VP1 [9, 10], a feature shared by the simian virus SV40, but not by other HPyVs [11]. The encoded protein of 60–70 amino acids (aa) has been termed agnoprotein, and, despite multiple studies, its major function in the biology of BKV or JCV has not been identified [12–14]. Agnoprotein is abundantly expressed late in the viral life cycle, and localizes predominantly to the cytoplasm both *in vitro* [9, 15] and also *in vivo* as shown in biopsies of BKV-associated nephropathy [16]. In the cytoplasm, BKV agnoprotein shows a reticular distribution but also colocalizes with lipid droplets, the latter being mediated by an amphipathic helix of 20 aa in the central part of the 66 aa BKV agnoprotein [15].

Despite its abundant expression of BKV agnoprotein *in vivo*, agnoprotein-specific humoral and cellular immune responses were low or undetectable in individuals exposed

to BKV [16]. This observation was contrasted by the strong immune responses found for the BKV capsid protein VP1 located on the same transcript or for the amino-terminal domain shared between small and large T antigen [16–18]. Since BKV and JCV persist lifelong after primary infection and are asymptomatically shed in immunocompetent healthy individuals [3], we speculated that agnoprotein might have a role in immune evasion. Interestingly, histopathology studies reported decreased MHC-II expression of BKV-infected renal tubular epithelial cells as a potential marker distinguishing BKV-associated nephropathy from acute cellular rejection [19].

Multiple mechanisms of viral immune evasion have been described. The immediate early protein ICP47 of Herpes simplex virus-1 (HSV-1) has been reported to downregulate HLA class-I antigen presentation by specifically binding to and blocking TAP, the transporter associated with intracellular export of peptides generated from intracellular proteins by the proteasome. Thereby, peptide epitope loading and presentation of MHC-peptide complexes on the cell surface is impaired and decreases immune recognition by cytotoxic T lymphocytes [20]. HSV-ICP47 is a small protein of 88 amino acids, its functional domain maps at the N-terminus, and the secondary structure is predicted to contain amphipathic helix structures [21, 22]. We therefore hypothesized that the BKV agnoprotein might have a similar immunomodulatory role and investigated its effects on HLA-ABC and HLA-DR surface expression in transiently and stably transfected cells.

2. Materials and Methods

2.1. Plasmids. The pCMV-agno plasmid was a kind gift from Dr. Christine Rinaldo, Tromsø, Norway [9]. The hygromycin selectable expression plasmid phTRE-agno was generated by subcloning the agnoprotein coding sequence from pTRE-agno into pTRE2hyg using BamHI and NotI restriction sites. The pCMV-ICP47 and phTRE-ICP47 were generated by replacing the agnoprotein coding sequence with the corresponding sequence encoding ICP47 (a kind gift from Dr. Paul Zajac, University Hospital of Basel, Switzerland). pEGFP-N1 was purchased from BD Biosciences.

2.2. Transfection. Cells were transfected with GeneExpresso 8000 (IV-1047; Lab Supply Mall, Inno-Vita Inc., Gaithersburg, MD, USA), according to manufacturers instructions. Briefly, for a well of a 6-well plate, cells were seeded in 1.5 mL of culture medium; 3 μL of transfection reagent were diluted in 250 μL of OptiMEM (Gibco) and kept for 5 minutes at RT; 4 μg of DNA were diluted in 250 μL of OptiMEM, and then mixed with the diluted transfection reagent for 20 minutes at room temperature before dropwise adding to the cells. After 4 hours of incubation at 37°C and 5% CO_2, medium was replaced. UTA-6 cells were cultured in presence of Tet and G418, unless indicated otherwise.

2.3. Cells. UTA-6 cells [23] were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% Fetal Bovine Serum (FBS, Biochrome AG, Berlin, Germany), 2 mM L-glutamine, supplemented with 500 μg/mL G418 (Geneticin, Sigma) and 1 μg/mL tetracycline (Tet) (Sigma). For inducible protein expression experiments, medium containing 1 μg/mL Tet was replaced with medium without Tet for 48 hours.

Primary human renal proximal tubular epithelial cells (RPTEC; ATCC PCS-400-010, lot 5321) were maintained in epithelial cell medium (EpiCM, ScienCell, Carlsbad, CA, USA) and passaged with Passage Kit 2 (2040002, Provitro GmbH, Berlin, Germany).

UTA-6 agno clones stably expressing the BKV agnoprotein in a Tet-dependent manner were obtained by cotransfecting the hygromcyin-selectable BKV agnoprotein-encoding construct phTRE-Agno and the EGFP encoding construct pEGFP-N1 (Clontech, Mountain View, CA, USA) in a 10 : 1 ratio. One day after transfection, cells expressing EGFP were sorted using a cell sorter (FACSARIA, BD) and seeded in 96 multiwell plates at a limiting dilution of 0.3 cells per 100 μL of DMEM containing 10% FCS supplemented with 500 μg/mL G418 (Geneticin, Sigma), 1 μg/mL Tet (Sigma) and 800 μg/mL hygromycin-B (Calbiochem). Medium was changed every 48 hours for 14 days to select clones. Subsequently the clones were expanded in medium containing 500 μg/mL G418, 1 μg/mL Tet and 200 μg/mL hygromycin-B, tested by immunofluorescence for BKV agnoprotein expression after Tet removal. Clones with a Tet-dependent BKV agnroprotein expression were cryopreserved.

2.4. Interferon-γ Treatment. Recombinant human interferon-γ (IFNγ) was purchased from Peprotech (Rocky Hill, NJ, USA) and diluted in the cell medium at 100 U/mL, 10 U/mL and 1 U/mL. UTA-6 cells (4×10^4) or RPTECs (1×10^5) were seeded in a 24 multiwell plate. Serial dilutions of IFNγ were added to the culture medium and left at 37°C, 5% CO_2 for 48 hours.

2.5. Antiagnoprotein Rabbit Serum. To detect expression of BKV agnoprotein or HSV ICP47, the following antibodies were used: antiagnoprotein rabbit serum (a kind gift from Dr. Christine Rinaldo, Tromsø, Norway) diluted 1 : 800 and anti-ICP47 rabbit serum (a kind gift from Dr. Klaus Frueh, Portland, ON, USA) diluted 1 : 750 in 3% milk/PBS. Cells were seeded onto glass coverslips and, after 2 days, washed with phosphate-buffered saline w/o Ca^{2+} and Mg^{2+} (PBS) and fixed using 4% paraformaldehyde at room temperature for 20 minutes. After two rounds of washing with PBS, the cell membranes were permeabilized with 0.2% Triton at room temperature for 10 minutes, washed again with PBS, and blocked for 15 minutes at 37°C using 3% milk/PBS. Coverslips were then stained with primary antibodies for 60 minutes at 37°C and washed three times in PBS. Then, Hoechst 33342 (0.5 μg/mL; H21492, Invitrogen) and fluorescently labeled secondary antibody (anti-rabbit-Cy3 1 : 2000, 111-165-144, Jackson Immunoresearch, West Grove, PA, USA) were added and incubated for 60 minutes at 37°C. After 3 washes in PBS, the slides were mounted in 90% Glycerol (1.04095, Merck, Darmstadt, Germany) in PBS containing 1% *N*-propyl gallate (P-3130, Sigma) as an antifading agent.

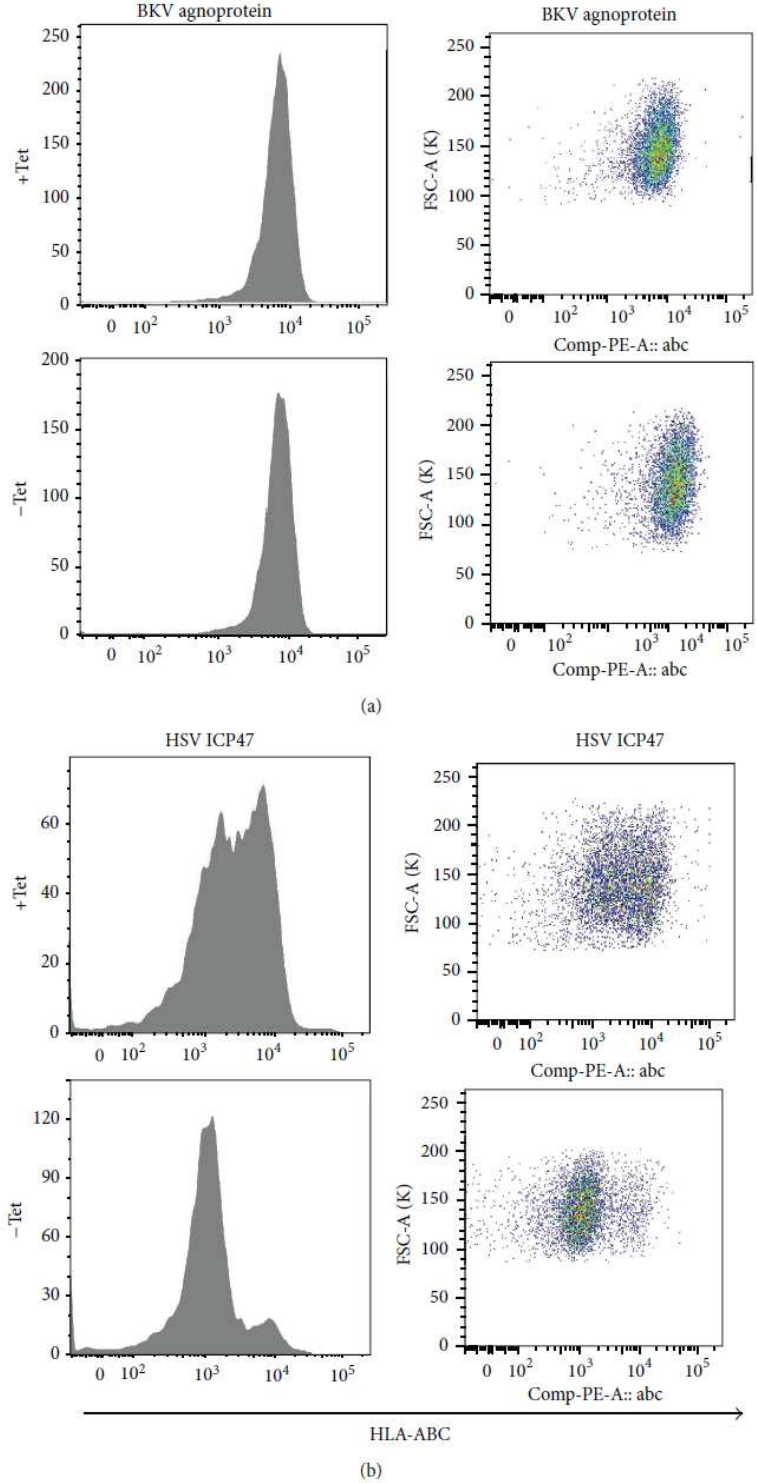

FIGURE 1: HLA-ABC cell surface expression in transfected UTA-6. Cells were cotransfected with pEGFP-N1 and the Tet-off regulated phTRE-agno or pTRE-ICP47 at a 1 : 10 ratio. HLA-ABC expression was analyzed by flow cytometry using labeled antibodies on EGFP-gated UTA-6 cells 48 h after transfection and 24 h after Tet-off induction.

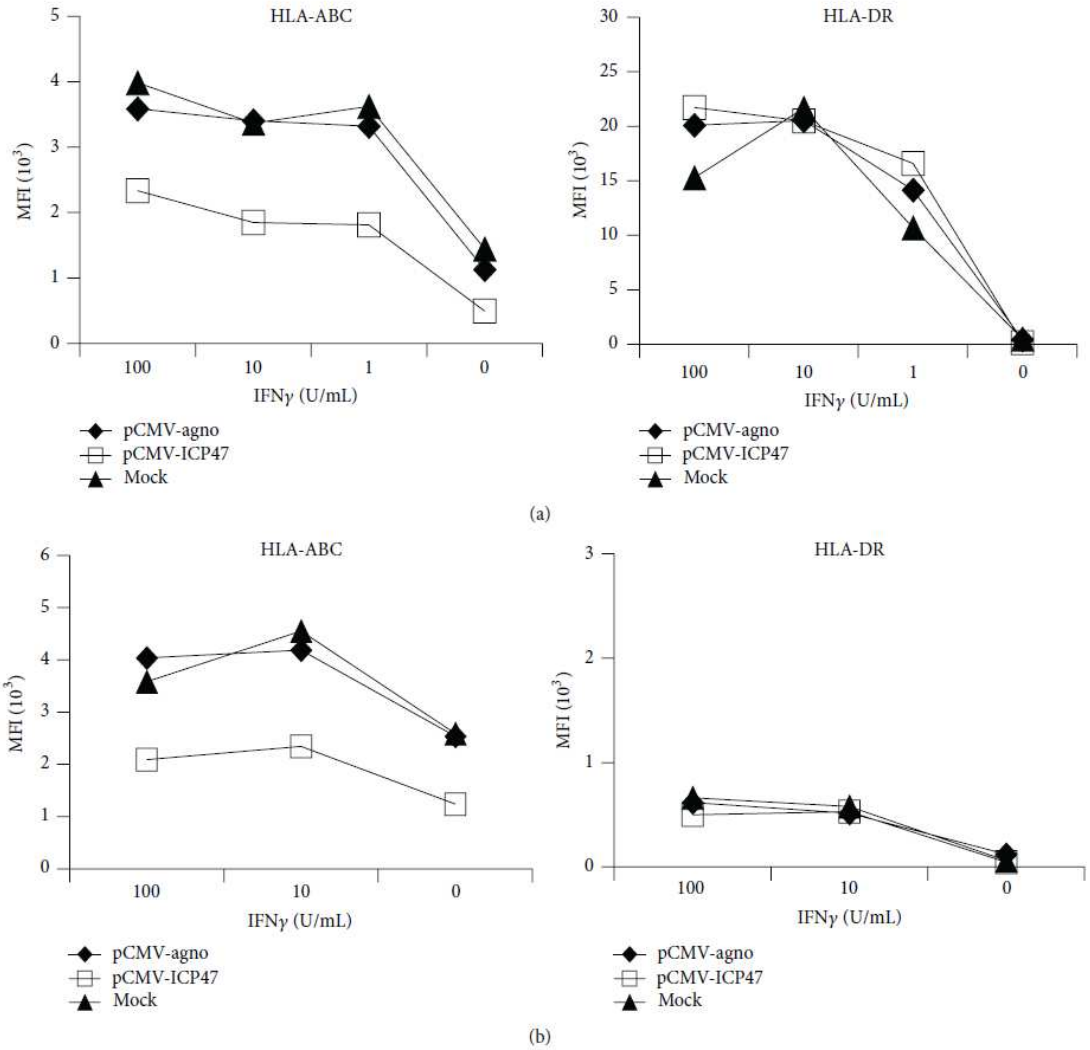

FIGURE 3: (a) HLA-ABC and -DR surface expression in transiently transfected RPTECs. Cells were cotransfected with pEGFP-N1 and either pCMV-agno or pCMV-ICP47 at a 1:10 ratio. pEGFP-N1 alone (mock, ▲) or together with pCMV-agno (♦) or with pCMV-ICP47 (□) as described above. MFI: mean fluorescence intensity. (b) HLA-ABC and -DR surface expression in transiently transfected UTA-6. UTA-6 cells were cotransfected with pEGFP-N1 and either pCMV-agno or pCMV-ICP47 at a 1:10 ratio. pEGFP-N1 alone (mock, ▲) or together with pCMV-agno (♦) or with pCMV-ICP47 (□) as described above. MFI: mean fluorescence intensity.

CMV-driven BKV agnoprotein or HSV-1 ICP47 together with EGFP (Figure 3(b)).

To verify that any potential effect of BKV agno expression could be compared in the same cell population, we analysed selected UTA-6 cell clones that were stably transfected with a Tet-regulated construct and expressed agno in a tightly regulated manner. BKV agno was expressed at different levels depending on the Tet concentrations (Figure 4(a)). After 48 hours of incubation with or without IFNγ, HLA-ABC expression was not significantly altered in the presence or absence of agno expression, and if at all, a slight increase in HLA-DR expression was seen after induction in UTA-6 cells clones (Figure 4(b)).

We next investigated the potential effect of BKV agnoprotein on HLA-peptide-dependent killing by antigen-specific cytotoxic T lymphocytes (CTL). The HLA-0201 positive UTA-6 cells expressing agnoprotein under Tet-off regulation were used as targets and HLA-A0201 specific T cells were used as effectors in a ^{51}Cr release assay. Peripheral blood mononuclear cells (PBMCs) from two HLA-A0201 healthy donors (HD1 and HD2) were stimulated with a 15-mer peptide derived from CMV-pp65 (AGILARNLVPMVATV) containing the well-characterized HLA-A0201 restricted immunodominant nonameric sequence NLVPMVATV. The EliSpot assay of the HD1 and HD2 cell preparations showed a peptide-specific IFN-γ response with the 15-mer and with the

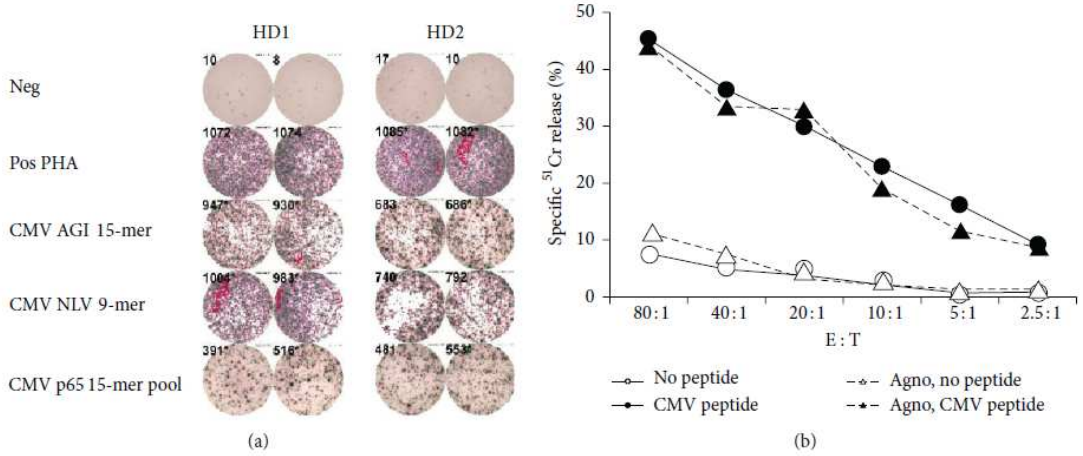

FIGURE 5: (a) IFNγ production by CMVpp65-specific T cells: IFNγ SFUs are shown for 1×10^5 T cells from healthy donor 1 (HD1) and 2 (HD2) challenged with medium (neg), phytohemagglutinin (PHA), CMV-pp65-15 m123, and CMV-pp65-9 m495, CMV-pp65-15 Mp. (b) Effect of Tet-off inducible BKV agnoprotein expression on CTL activity. UTA-6 clone 2C9 cells were cultured for 24 h in the presence of Tet (continuous line) or absence of Tet expressing agnoprotein (dotted line, agno), labeled with ^{51}Cr and then pulsed without (△, ◯) or with (▲,◯) CMV 9-mer peptide NLVPMVATV. CMV antigen-specific T cells from a healthy donor was added in the indicated effector : target ratios and percent release of ^{51}Cr measured.

that impaired recognition of infected cells by virus-specific cytotoxic CD8 T cells must provide significant advantages. Among persisting human DNA viruses, the adenovirus E3-19 K and the cytomegalovirus U3 proteins were shown to interfere with epitope peptide loading via the peptide transporter TAP and tapasin, while the cytomegalovirus US6 protein prevents transport of peptide epitopes to the endoplasmic reticulum side causing MHC-I retention and degradation. On the other hand, the HSV-1 ICP47 causes the decrease in MHC-I surface molecules by blocking peptide transport through binding to TAP on its cytoplasmic side and thereby decreasing immune recognition by cytotoxic T-lymphocytes [20]. BKV and JCV can be regarded as viruses successfully adapted to the human host [25] given the observation that 50 percent to 90 percent of the world's population is infected [3, 5] and 10–40 percent of healthy immunocompetent adults continue to shed these viruses asymptomatically in the urine [3]. These high rates of infection and asymptomatic replication together with the fact that significant BKV and JCV diseases are almost exclusively seen in immunodeficient patients suggest that some immune control is operative in healthy individuals but not to an extent that permits complete suppression and/or elimination from the infected host. Since both BKV and JCV express a highly homologous agnoprotein, a potential role in immune evasion could be hypothesized. We observed that there are some structural similarities between HSV-ICP47 and BKV agnoprotein, as they are both small cytoplasmic proteins, bearing a central domain of an amphipathic helix. Given these similarities and the fact that HSV-ICP47 has been shown to downregulate MHC-I expression, we hypothesized that BKV agnoprotein could play a similar role, contributing to BKV immune evasion. Moreover, it was previously reported that BKV agnoprotein could negatively influence the transport of a temperature-sensitive VSV virus glycoprotein to the cell surface [12]. MHC-I molecules stabilized by loaded peptides are continuously transported from the endoplasmic reticulum to the cell surface for immune display. The results reported here demonstrated that expression of BKV agnoprotein had no discernible influence on HLA-ABC or HLA-DR. Stimulation with interferon-γ resulted in an increase in HLA-ABC expression indicating that relevant quantitative increases in surface HLA-ABC levels could be detected by flowcytometry. Expression of the HSV-1 ICP47 effectively downregulated this interferon-γ response of HLA-ABC, but had no effect on the HLA-DR upregulation in line with the specificity of this effect. By contrast, BKV agnoprotein showed no effect on HLA-ABC or HLA-DR expression, with or without prior interferon-γ stimulation in UTA-6 as well as in RPTECs as the natural host cell target of BKV. The data suggest that BKV agnoprotein does not exert a similar effect on immune evasion as HSV-ICP47. The data are of interest, since clinical pathology data of kidney allograft biopsies suggested that HLA-DR might be reduced in interstitial nephritis due to BKV-associated nephropathy as compared to those showing T cell-mediated interstitial rejection [19].

Results obtained in transiently transfected cells have different limitations, in terms of efficiency of transfection and durability of protein expression. However, our earlier studies on BKV agnoprotein suggested that under these circumstances, infection and transfection provide comparable results regarding the magnitude and the subcellular localization of BKV agnoprotein [15]. The response of stable, inducible clones confirmed the observations in transiently transfected cells. Importantly, under these conditions, HSV-1 ICP47 was able to elicit its specific downregulating effects

on HLA-ABC rendering a severe limitation of our approach unlikely.

As the HLA-ABC and -DR molecules were not numerically reduced on the cell surface, we investigated the possibility of a functional impairment mediated by BKV agnoprotein. To test this hypothesis, we used a well-characterized, immunodominant 9-mer peptide from the CMV-pp65 antigen, which is an immunodominant target of cytotoxic T cell response *in vitro* and *in vivo* in HLA-A0201 positive individuals. As shown, BKV agnoprotein expression failed to interfere with CMV 9-mer NLVPMVATV-dependent killing arguing against a major role of BKV agnoprotein in modulating a functional immune evasion of this kind. In conclusion, we demonstrate that BKV agnoprotein does not modulate surface expression of HLA-ABC and -DR or interfere with HLA-A0201-mediated CTL activity. Thus, other functions and mechanisms of BKV agnoprotein need to be considered which may or may not include a role in immune evasion.

Acknowledgment

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In our experimental model HLA class I and II expression were not affected by agnoprotein. This has been demonstrated both inducing constitutive and tetracycline regulated agnoprotein expression, also when IFN-γ was added to the cells to mimick an inflammatory context and upregulation of HLA molecules. Conversely, as expected, pICP47 expression specifically decreased HLA class I but not II expression.

Possible effects of heterogeneous expression of agnoprotein in cell cultures could be excluded as we obtained the same negative results in UTA-6 cell clones, wich expressed agno in a tightly regulated manner. Finally, CTL activity upon activation by viral antigen presentation was not affected by agnoprotein expression.

None of our results could indeed demonstrate a role for agnoprotein in interfering with antigen processing and presentation pathways, and consequent CTL activity. Our hypothesis was based on previous studies in which immunological ignorance towards agnoprotein was observed (Leuenberger, Andresen et al. 2007). Agnoprotein was also demonstrated to be involved in vescicular transport to the surface, and HLA molecules loaded with peptides are transported from the endoplasmic reticulum to the cell surface for immune display (Johannessen, Walquist et al. 2011). Moreover, in earlier studies PyVAN biopsies were compared to T-cell mediated rejection biopsies in KTRs, and HLA class II was observed to be downregulated in BKPyV infected cells. In a recent study BKPyVAN biopsies were compared with T cell mediated rejection for expression of TAP1 and HLA class I β-2 –microglobulin and no difference has been observed, suggesting that BKPyV does not seem to be involved in this kind of potential immune evasion mechanism (Buettner, Xu et al. 2012).

However, many other checkpoints in the immune system can be targeted by viruses, starting from interfering with viral recognition by innate immunity then impairing recruitment of immune response effectors by modulating production of chemokines, and also targeting adaptive responses by co-stimulation inhibition or induction of Treg responses. For this reason, further approaches to investigate a role of agnoprotein in immune evasion need to be explored.

5.2 Characterization of Immunodominant BK Polyomavirus 9mer-Epitope T-cell Responses

BKPyV is an ubiquitous virus infecting up to 90% of the human population, mainly during childhood, without any clinical symptoms (Knowles, Pipkin et al. 2003, Egli, Infanti et al. 2009, Schmidt, Adam et al. 2014). Disease manifestations appear almost exclusively in immunosuppressed individuals, specifically in transplanted patients receiving IS therapy anti rejection, in which uncontrolled high-level BKPyV viruria and viremia have been identified as markers of progression to disease (Hirsch, Knowles et al. 2002). Despite a number of antiviral therapy have been proposed, the current strategies to manage BKPyV replication and disease is immune suppressive therapy reduction, leading to a restauration of T cell immunity (Hirsch, Brennan et al. 2005, KDIGO 2009, Hirsch and Randhawa 2013). In fact, by reducing immunosuppression, parallel to decrease of viral replication, a significant increase of BKPyV-specific T cell immunity has been observed (Comoli, Azzi et al. 2004, Binggeli, Egli et al. 2007, Ginevri, Azzi et al. 2007, Schachtner, Muller et al. 2011). Thus, an effective virus specific T cell response seems to be crucial in the control of viral replication.

In previous studies T cell responses have been investigated to BKPyV antigens, overlapping 15mer peptide pools encoded in the EVGR and LVGR have been used as stimulus (Binggeli, Egli et al. 2006, Binggeli, Egli et al. 2007, Ginevri, Azzi et al. 2007, Schachtner, Muller et al. 2011, Weist, Schmueck et al. 2014), and it has been observed that responses to LVGR, and in particular to VP1, are prominent compared to EVGR and mainly characterized by CD4+ T cells producing IFN-γ (Binggeli, Egli et al. 2006, Binggeli, Egli et al. 2007, Ginevri, Azzi et al. 2007, Weist, Schmueck et al. 2014) .

The CD8+ T cell responses could be observed (Krymskaya, Sharma et al. 2005, Binggeli, Egli et al. 2006, Provenzano, Bracci et al. 2006, Binggeli, Egli et al. 2007, Ginevri, Azzi et al. 2007, Schachtner, Stein et al. 2015), their characterization demonstrated that they were directed mostly to the EVGR protein LTag and that they strongly correlated with viral resolution, suggesting a protective function. However, because most of the studies used 15mer peptide pools to evaluate BKPyV specific responses, the distinct contribution of CD8+T cells remained undefined. We

decided to better characterize BKPyV-specific CD8+ T cell responses specific for 9mer epitopes. Complementary to current research, we focused on the BKPyV EVGR region. We interrogated the responses in seropositive healthy individuals and attempted confirmation by independent techniques including streptamers, CD107a, and cytotoxic killing assays. The results were then taken to a cohort of pediatric kidney transplant patients for an independent assessment.

viruria and viremia have been identified as markers of progression to PyVAN (25), thus current management strategies recommend screening KTRs for viremia followed by reducing immunosuppression (26–28). In prospective observational studies, this preemptive intervention has been successful, as shown by clearance of viremia and PyVAN in 80–100% of cases, with a low risk of subsequent acute rejection in 0–14% of patients (29–33). BKPyV viremia clearance has been paralleled by increasing BKPyV-specific T cell responses in peripheral blood (30–36). Because BKPyV-specific T cell responses are ≈50- to 100-fold lower than those to cytomegalovirus, these assays have not readily entered clinical practice. Moreover, the risk factors for BKPyV replication and nephropathy vary in different KT studies and include steroid pulses for acute rejection, maintenance immunosuppression such as tacrolimus–mycophenolate versus cyclosporine–mycophenolate, older age of recipients, male sex and a higher number of HLA mismatches (37–45). According to the recent Organ Procurement and Transplantation Network and Scientific Registry of Transplant Recipients report, these risk factors are present in a substantial number of KT patients (46). Moreover, organs from BKPyV IgG–positive donors for recipients with low or undetectable antibodies may face an increased risk (34–48).

We and others investigated cellular immune responses to overlapping peptide pools encoded in the early viral gene region (EVGR) or the late viral gene region (LVGR) of the BKPyV DNA genome (30–50). T cell responses to the LVGR-encoded capsid viral protein VP1 were generally more pronounced than those to EVGR-encoded viral proteins (30–49). Interferon γ (IFN-γ) responses were largely derived from $CD4^+$ T cells and, to a lesser extent, from $CD8^+$ T cells (30–53). Because most of these studies used overlapping 15mer peptide pools (15mP), the contribution of individual $CD8^+$ T cell–restricted epitopes to these responses is largely undefined. With few exceptions, HLA-restricted T cell responses to BKPyV are mostly reported from HLA-A*02 individuals (51–56). To better characterize BKPyV-specific $CD8^+$ T cell epitopes, a bioinformatics approach was chosen to predict 9mer epitopes encoded in BKPyV EVGR and presented by 14 common HLA types in Europe and North America for experimental testing in healthy adult individuals and pediatric KTRs.

Materials and Methods

Healthy participants

Peripheral blood mononuclear cells (PBMCs) were prepared from 42 healthy individual (HIs), consisting of 34 blood donors from the Swiss Red Cross blood donation center in Basel, Switzerland, and from eight other healthy volunteers (Table S1). HLA types were determined with fee-for-service by the Transplantation Immunology Laboratory (Basel). Participants gave written informed consent for the protocol (IRB 267/06), which was approved by the local institutional review board (IRB).

Pediatric KTRs

A total of 118 consecutive pediatric KTRs were referred to the Genova Pediatric Kidney Transplant Program between March 2003 and November 2012. Three were aged >21 years but were still included in the cohort because they were initially received care as children for their end-stage renal disease the Nephrology Unit, IRCCS, Genova, Italy. Cryopreserved PBMCs were analyzed from 19 KTRs protected (i.e. without BKPyV viremia) or recovering from BKPyV replication (Table S2). The study was approved by the local IRB (867/2014).

BKPyV IgG enzyme-linked immunosorbent assay

BKPyV IgG serology was performed using BKPyV VP1-derived virus-like particles, as described previously (4–58).

In silico *epitope prediction*

The Syfpeithi database (59) provided information about HLA class I peptide binding affinity (60), whereas the Immune Epitope Database and Analysis Resource (61) provided a multiparametric prediction based on proteasomal cleavage, TAP transport and HLA class I peptide binding (62). The predictions were limited to HLA-A and -B types present in >5% of the population within Europe or North America (63). For each HLA allele, the 20 epitopes within the BKPyV EVGR sequence displaying the best scores in both algorithms were considered.

BKPyV EVGR–derived peptides

A pool of 180 overlapping 15mP spanning BKPyV EVGR (Dunlop strain) or a pool of 11 longer LPm1-11 peptides (long peptide pool [LPP]) covering immunodominant clusters of predicted BKPyV 9mer epitopes were used for *in vitro* T cell expansion. Cells were restimulated after expansion, as reported (35), using 15mP or a pool of 73 predicted 9mer peptides (9mP). The 9mer peptides were also resuspended in different subpools according to a checkerboard matrix approach, from A to H and from 1 to 9 (called 9msA–H and 9ms1–9). Each of the 73 peptides was present in two subpools. An additional set of 24 9mer peptides that initially were not predicted by computer algorithms and three longer peptides were later synthesized and used to assess "prediction gaps" in EVGR sequence. All peptides were >70% pure and resuspended in dimethyl sulfoxide (10 mg/mL; Eurogentec Deutschland GmbH, Köln, Germany).

In vitro *expansion of T cells*

Freshly isolated or thawed PBMCs were stimulated with LPP or 15mP (200 ng/mL) in 24-well plates and incubated for 7–14 days at 37°C in 5% CO_2 before performing phenotypic and functional assays. Recombinant human IL-2 (20 U/mL; Peprotech, Rocky Hill, NJ) and recombinant IL-7 (5 ng/mL; Peprotech) were added once a week.

Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISpot) assay was performed, as described previously (35). Expanded T cells were cultured without (negative control) or with BKPyV-specific peptides. Cells treated with *Staphylococcus* enterotoxin B (2 μg/mL; Sigma-Aldrich, St. Louis, MO) or phytohemagglutinin-L (PHA; 2 μg/mL; Roche Diagnostics GmbH, Mannheim, Germany) were used as positive control. ELISpot data are averaged in duplicate or triplicate wells with background wells subtracted. Responses greater than background plus 2 standard deviations were considered positive.

MHC streptamer staining

BKPyV-specific T cells were stained with phycoerythrin (PE)- or allophycocyanin-labeled streptamers (IBA GmbH, Göttingen, Germany) composed of ≈8–12 peptide-loaded MHC molecules, allowing better sensitivity than tetramers or pentamers. The cells were then incubated with CD8-PE-Cy7 antibody (BD Biosciences, San Jose, CA) and analyzed on a flow cytometer (FACSCanto; BD Biosciences) using FACSDiva software.

CFSE *proliferation assay*

PBMCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience, Vienna, Austria) before being expanded with BKPyV-derived peptides and analyzed by flow cytometry.

CD107a degranulation assay

Expanded T cells were rechallenged for 5 h with BKPyV 9mer peptides or phorbol 12-myristate 13-acetate (100 ng/mL; Sigma-Aldrich) and ionomycin (1 μg/mL; Sigma-Aldrich) as positive control in the presence of PE-Cy7–labeled CD107a antibody (BD Biosciences) before being analyzed by flow cytometry.

Cytotoxicity assay

Expanded T cells were cocultured for 4 h with ^{51}Cr-labeled autologous PHA blasts pulsed with BKPyV 9mer peptide at different effector:target cell ratios. Counts per minute were taken with a β-counter (TopCount; PerkinElmer, Waltham, MA).

Statistical analysis

Proportions of viremic or viruric KTRs, and proportions of matched or mismatched patient populations were compared using the Fisher exact test with GraphPad Prism version 4.00 (GraphPad Software, La Jolla, CA). Differences corresponding to $p < 0.05$ were considered statistically significant.

Additional material and methods can be found in the supporting information.

Results

Bioinformatic prediction of HLA-A– and HLA-B–binding BKPyV 9mer epitopes

Syfpeithi and IEBD programs were used to predict 20 top-scoring 9mer epitopes encoded in the BKPyV EVGR for each of 14 HLA-A and -B types prevalent in Europe and North America. These HLA types are common in many ethnics groups worldwide, as shown in Table S3. The predictions for each HLA type were visualized relative to the BKPyV EVGR sequence and arbitrarily numbered (Figure 1). Although each HLA type appeared to have its own unique 9mer pattern, there were clearly sequence stretches in which the predicted epitopes appeared to cluster locally as well as across several HLA types by both algorithms (Figure 1). To focus on immunodominant epitopes, a total of 73 predicted 9mer epitopes including epitopes identified in previous studies (Table S4) were selected from different prominent clusters present across most HLA-A and -B types for chemical synthesis and experimental testing. Twenty-four additional 9mer predictions outside of these clusters were synthesized, resulting in a total of 97 9mer-epitope candidates. In addition, 11 longer peptide stretches were selected for domains in which several predicted 9mers overlapped.

Experimental testing of predicted BKPyV 9mer epitopes in HIs

PBMCs were obtained from 42 HIs (median age 46 years) (Table S1) who were BKPyV-IgG seropositive, as defined by the normalized OD_{492nm} of >0.1 at 200-fold dilution (Figure 2), which was previously shown to be very highly sensitive and specific (58). Because of the low BKPyV-specific T cell frequency in PBMCs, an *in vitro* expansion protocol was adopted (35), and PBMCs were stimulated using BKPyV EVGR 15mP or LPP. IFN-γ ELISpot assays were performed before and after expansion using 15mP, LPP, 9mP and 9msP for single-epitope cross-identification.

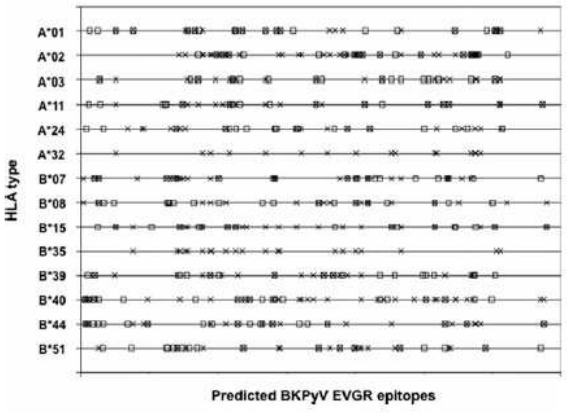


Figure 1: *In silico* prediction of BKPyV EVGR immunogenic epitopes. The graph depicts 20 top-scoring 9mer epitopes predicted in BKPyV EVGR sequences for common HLA-A and -B types in Europe and North America, according to the Immune Epitope Database (X) and Syfpeithi (□) algorithms. BKPyV, BK polyomavirus; EVGR, early viral gene region.

The approach is illustrated in HI-29: PBMCs were either stimulated directly or after expansion using the indicated peptide pools (Figure 3A). IFN-γ ELISpot results showed

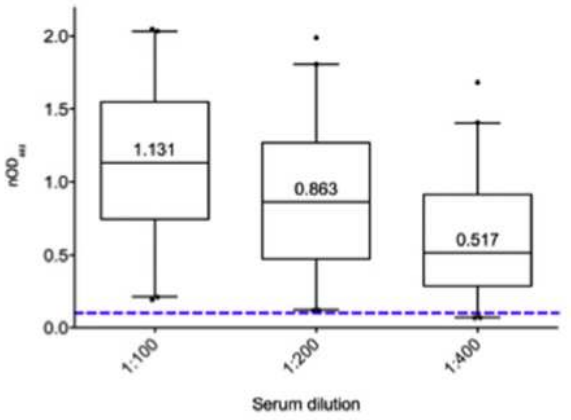


Figure 2: BKPyV IgG serology of 42 healthy individual participants. Normalized BKPyV IgG antibody levels are shown at 1:100, 1:200 and 1:400 dilutions (median, box shows 25th, 75th percentiles; whiskers 5% and 95%). Positive serological status was defined as OD_{492nm} ≥0.100 (dotted line) at the 1:200 dilution. BKPyV, BK polyomavirus; nOD, net optical density.

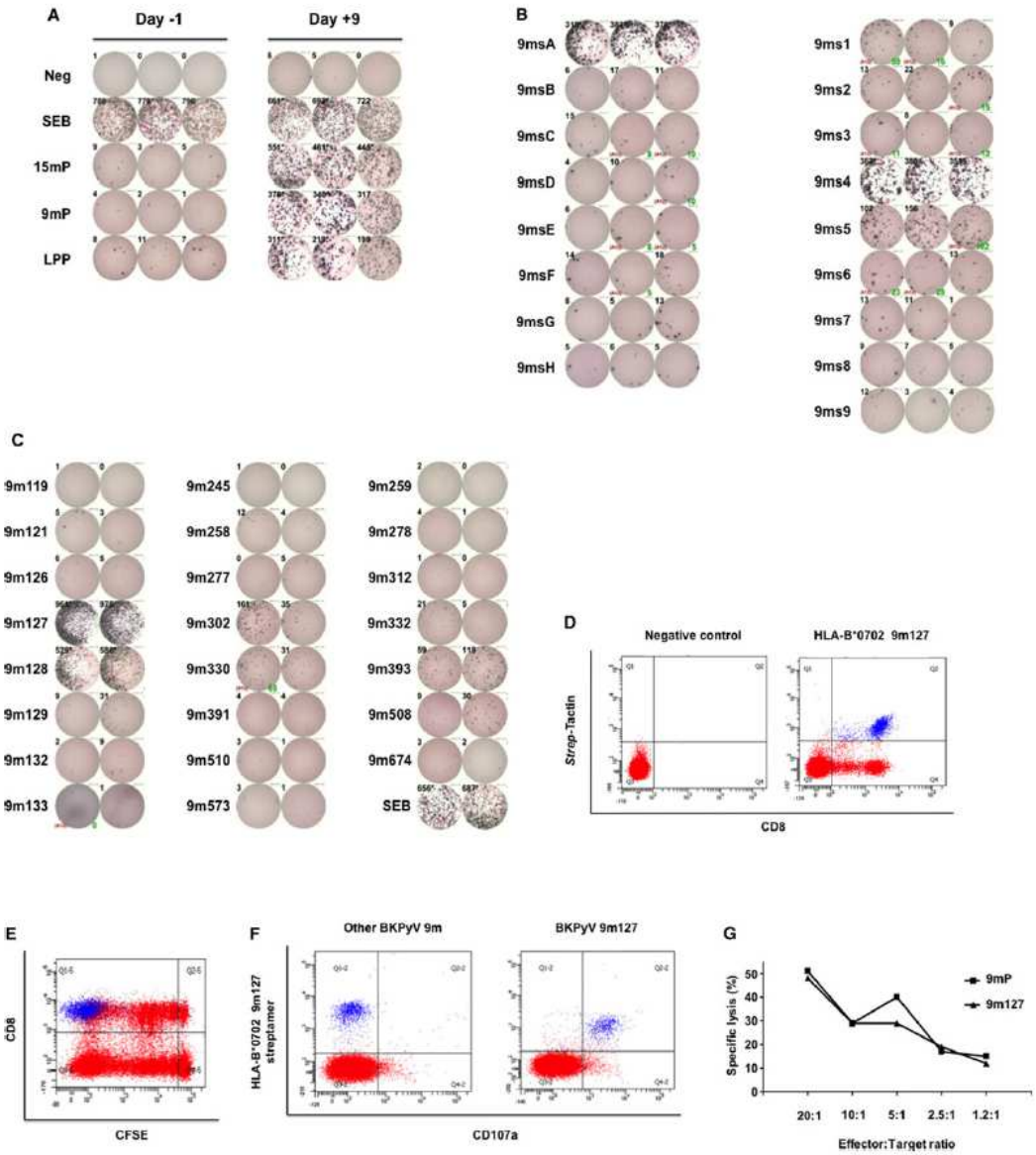


Figure 3: Characterization BKPyV EVGR 9mer-specific immune responses. (A) Interferon γ enzyme-linked immunospot assay using PBMCs directly after isolation from fresh blood (day −1; left panel) or after 9-day expansion with BKPyV EVGR peptides (day +9; right panel). Cells were treated with medium (neg), SEB, 9mP, overlapping 15mP spanning the BKPyV EGFR sequence or with LPP. (B) Rechallenge of 9-day expanded T cells with 9mer subpools 9msA to 9msH, and 9ms1 to 9ms9. Each peptide was present in two subpools for cross-identification. (C) Identification of 9mer epitopes by restimulating expanded cell with single 9mer peptides contained in the subpools eliciting the highest responses (e.g. 9msA, 9ms4, 9ms5). (D) HLA streptamer staining using PE-labeled Strep-Tactin without (left panel) or with (right panel) HLA-B*0702 molecules bearing 9m127 peptide. (E) $CD8^+$ T cell proliferation during *in vitro* expansion. PBMCs were stained at day 0 with CFSE dye (red) that dilutes on cell division (*x*-axis). HLA-B*07–positive 9m127-specific T cells are shown in blue. (F) Epitope-specific degranulation of $CD8^+$ T cells using PE-Cy7-labeled CD107a antibody (red) on 5-h restimulation with 9m127 (right panel) or another BKPyV EVGR peptide (left panel). HLA-B*07 9m127-specific T cells are shown in blue. (G) The 9mer-specific cytotoxic activity of expanded T cells. Autologous phytohemagglutinin-L blasts stained with ^{51}Cr and pulsed with 9mP (■) or 9m127 (▲) were used as target cells and incubated for 4 h with expanded T cells (effector cells). Percentage of target cells lysis (*y*-axis) at the different effector:target cells ratios (*x*-axis) is shown. 9mP, 9mer-peptide pool; 15mP, 15mer peptide pool; BKPyV, BK polyomavirus; CFSE, carboxyfluorescein diacetate succinimidyl ester; EVGR, early viral gene region; LPP, long peptide pool; Neg, negative; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; SEB, *Staphylococcus* enterotoxin B.

that the *in vitro* T cell expansion protocol resulted in a significant increase of BKPyV-specific T cells that responded to both 15mP and 9mP (Figure 3A). Rechallenge with 9mer subpools showed that the highest responses could be attributed to specific subpools, namely, 9msA and 9ms4 (Figure 3B). The single 9mer common to both of these subpools is 9m127. A slightly weaker response was also observed in 9ms5, suggesting some response to eight amino acid–overlapping 9m128 as well. Rechallenge of expanded cells with single 9mer-peptides confirmed that the major response to 9msP was indeed attributable to 9m127, with a response similar to that observed with 9mP or 9msA and 9ms4 and, to a lesser extent, to 9m128 (Figure 3C). Based on the peptide length of nine amino acids, this functional IFN-γ response should originate from $CD8^+$ T cells. To address this directly, expanded T cells were stained with HLA-B*07:02 9m127 streptamer and with CD8 surface marker and analyzed by flow cytometry (Figure 3D). A population of HLA-B*0702–positive 9m127-specific $CD8^+$ T cells (right panel) could be detected, representing 3.9% of the total lymphocyte population.

To address proliferation following peptide stimulation, PBMCs were stained with CFSE before expansion and labeled for CD8 and HLA-B*0702–positive 9m127 streptamer after expansion. CSFE dilution indicated the presence of at least nine divisions of the $CD8^+$ T cell population (Figure 3E, red population). Gating on CCR7 and CD45RA revealed that a majority of $CD8^+$ T cells undergoing seven, eight and nine division were of central or effector memory phenotype, in contrast to naïve $CD8^+$ T cells that did not divide or that divided only once (Figure S1). HLA-B*0702–positive 9m127-specific $CD8^+$ T cells showed the lowest CSFE signals, indicating that these cells had divided close to once per 1–2 days during the expansion period (Figure 3E, blue population).

To correlate HLA-B*0702–positive 9m127-specific $CD8^+$ T cells and degranulation function, expanded T cells were stimulated with 9m127 or another BKPyV peptide (9m259) for 5 h in the presence of CD107a antibody and stained for HLA-B*0702–positive 9m127 streptamers (Figure 3F). Only 9m127 induced degranulation of nearly the entire HLA-B*0702–positive 9m127 $CD8^+$ T cell population.

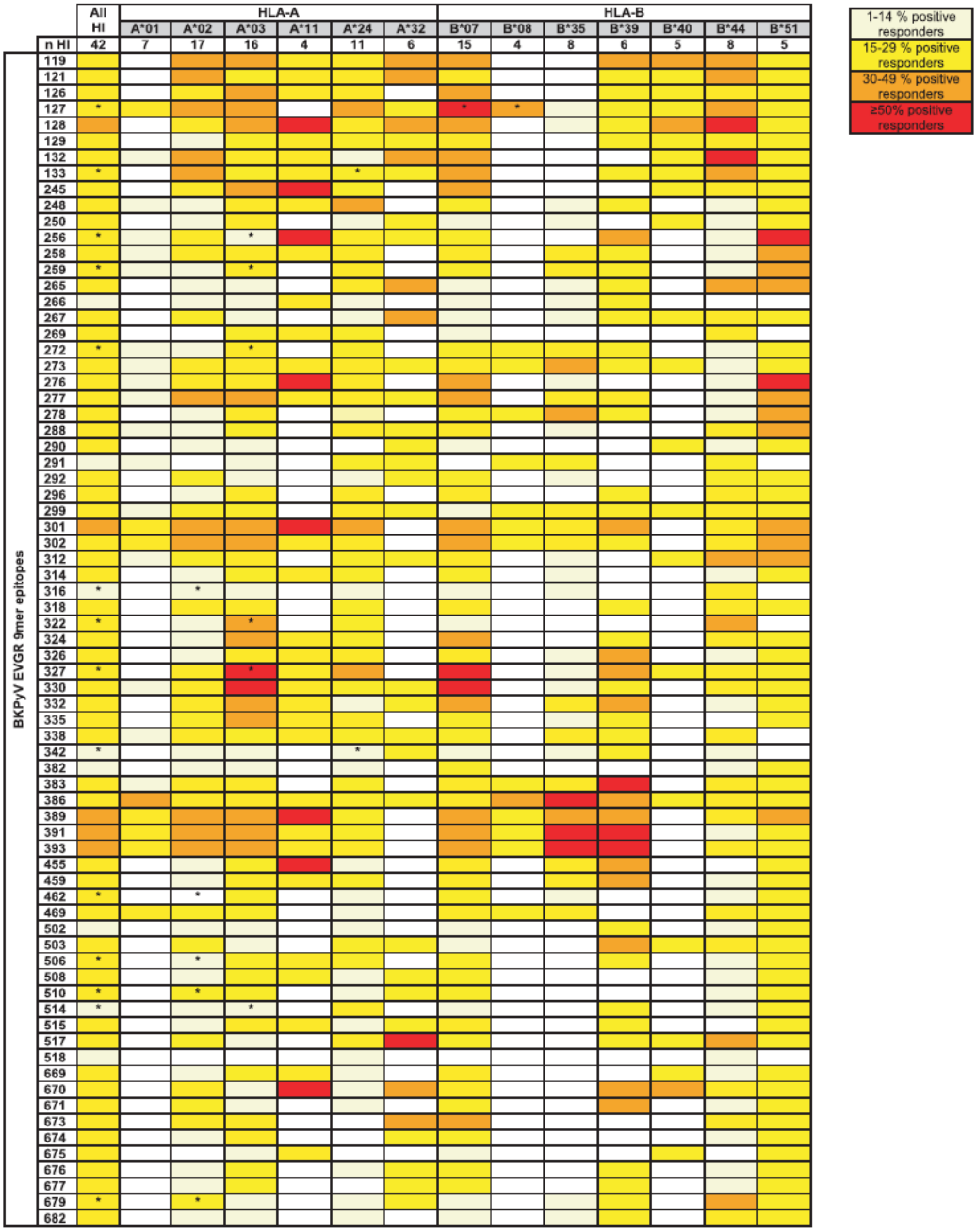
T cell functionality was also investigated in a killing assay in which lytic activity of expanded T cells against autologous ^{51}Cr-labeled PHA blasts pulsed with the single 9m127 or with 9mP was assessed (Figure 3G). The results showed that 9m127 mediates a mean specific lysis of 48% at an effector:target ratio of 20:1. This single 9m127 response was comparable to that mediated by 9mP, in line with an immunodominant BKPyV epitope.

This experimental approach permitted functional identification of candidate 9mer epitopes from BKPyV recognized by $CD8^+$ T cells in strongly BKPyV-seropositive HIs, even if cells were present at a low frequency among PBMCs. In some cases, the responses induced by 15mP and by 9mP did not correlate (data not shown), suggesting the presence of different epitope-specific T cell populations among $CD4^+$ or $CD8^+$ subsets. Testing of expanded PBMCs from BKPyV- and JC polyomavirus (JCPyV)–seronegative HIs remained negative for 9mer responses but showed some residual 15mer responses (Figure S2, left panel). This indicated that this *in vitro* protocol of only 2 weeks expansion did not lead to significant priming of naïve $CD8^+$ T cells, as detected by 9mer responses, but most likely induced proliferation of memory $CD8^+$ T cells. Interestingly, HIs who were seronegative for BKPyV but seropositive for JCPyV showed a response to BKPyV EVGR 15mer and 9mer (Figure S2, right panel). This suggested the presence of LTag crossreactive responses from JCPyV-specific T cells, as discussed previously (35).

In total, 42 HIs were analyzed, and the frequency of responses was summarized according HLA type in a heat map (Table 1). Only two 9mer responses had frequencies of <15% of HIs (9m316, 9m518). Many 9mer-epitope responses could be detected in up to one-third of HIs, but some epitopes had more frequent responses (40 epitopes elicited IFN-γ production in 30–49% of donors; 17 epitopes induced IFN-γ ELISpot responses in >50% of tested participants). The presence of response hotspots supports the notion that not all 9mer epitopes are equally potent, and certain clustering is observed. This is illustrated by the ELISpot results for selected epitopes (Figure 4). The overall results indicated that BKPyV EVGR-specific 9mer T cell responses were heterogeneous in terms of frequency and strength. For some HLA types, positive responses were found more frequently and directed towards more epitopes compared to HLA types. For example, in participants positive for HLA-A*03, -A*11, -B*07, -B*35, -B*39, -B*44 or -B*51, more than one epitope could be identified in ≥50% of HIs (Table 1). Single 9mer epitopes were associated with variable responses in HIs (Figure 4), whereby values of >69 spot-forming units (SFU)/10^6 cells were regarded as strong responses in accordance with a previously defined threshold of protection from BKPyV viremia in KTRs (35).

In summary, 9m301 elicited specific responses in 47% of participants with HLA-B*07 with a median of 292 SFU/10^6 cells. The same 9mer epitope was found to be immunogenic in 35% of donors with HLA-A*02 and 45% with HLA-A*24. In addition, 9m327 induced a median response of 304 SFU/10^6 cells in eight HIs who were positive for HLA-A*03. Overall, 9m330 induced fairly strong responses in 53% of HIs who were positive for HLA-A*03, with a median of 593 SFU/10^6 cells. Moreover, 9m330-specific IFN-γ production could also be detected in some HIs with HLA-B*07 and -B*39. Finally, 9m389 elicited a median response of 299 SFU/10^6 cells in six participants with HLA-A*02 (35%) and elicited IFN-γ production in 50% of participants with HLA-A*11 and 18% with HLA-A*24 (Table 1).

Table 1: BKPyV EVGR 9mer responses in IFN-γ ELISpot assay



All HI = epitope-specific IFN-γ responses in all healthy individuals, regardless of the HLA-type (mean)
n HI= number of healthy individuals in each HLA group

Light-yellow boxes indicate 1–14% positive responders; yellow boxes indicate 15–29% positive responders; orange boxes indicate 30–49% positive responders; red boxes indicate ≥50% positive responders. BKPyV, BK polyomavirus; EVGR, early viral gene region; IFN-γ, interferon γ; HI, healthy individual.
†Mean epitope-specific IFN-γ responses, regardless of the HLA type.
‡Number of HIs in each HLA group.
*Epitope identified in previous publications.

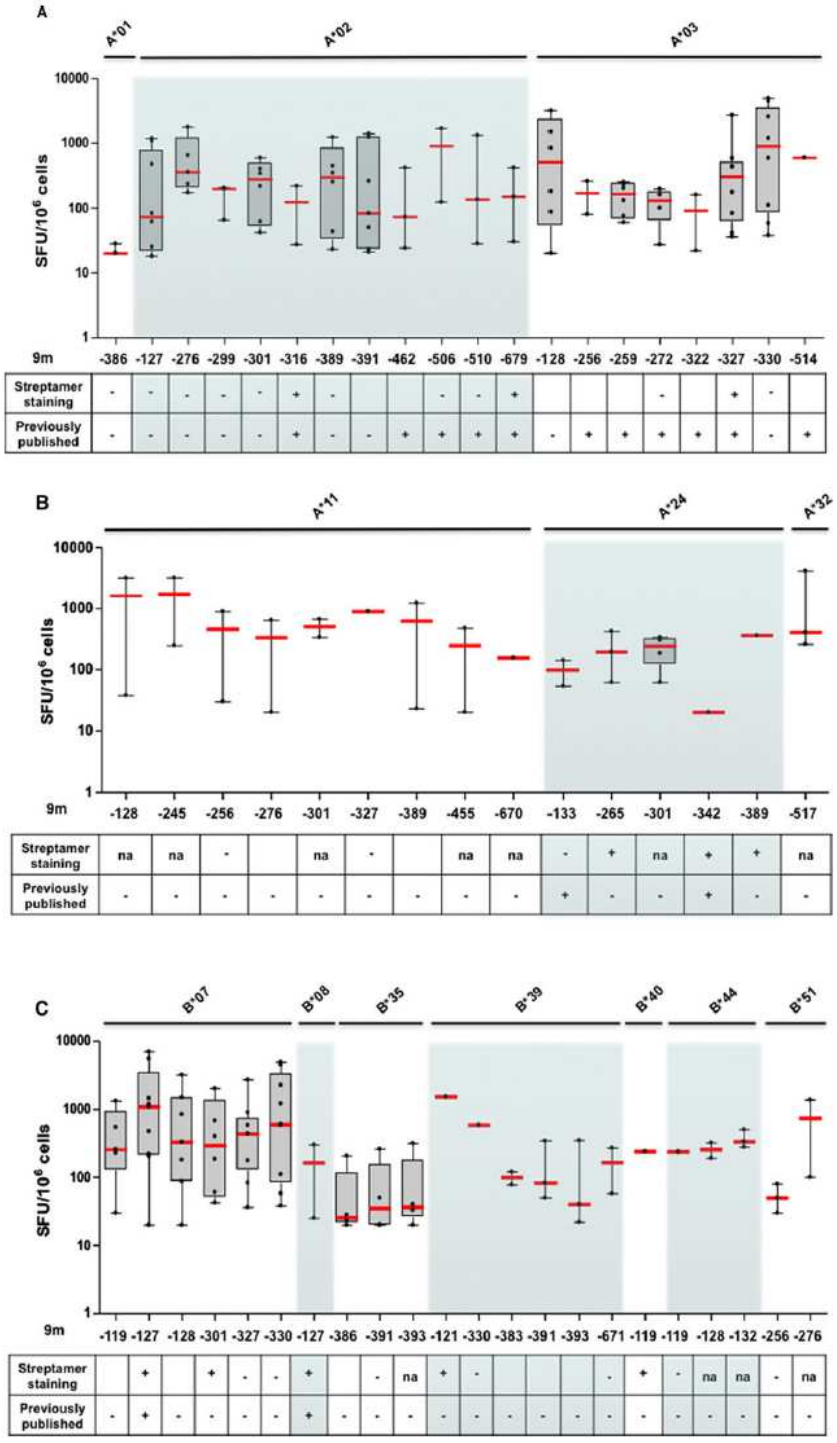


Figure 4: Breadth and strength of BKPyV EVGR epitope-specific immune responses in HIs. The 9mer-epitope responses in interferon γ enzyme-linked immunospot assay (dots) found in at least 40% of HIs, listed in Table 1, with positive streptamer staining or previous publication (median in red, box 25th and 75th percentiles, whiskers 5th and 95th). BKPyV, BK polyomavirus; HI, healthy individual; na, not available; SFU, spot-forming unit.

Table 2: HLA-A and -B specificity of BKPyV EVGR $CD8^+$ T cell responses in HIs

HLA type	Streptamer	Tested HIs (n)	HIs with positive response (%)	Epitope-specific $CD8^+$ cells T cells (mean %)
A*02	316	6	33	0.04
	679	6	50	0.04
A*03	327	5	40	0.01
A*24	265	4	25	0.02
	312	4	25	0.02
	342	7	14	0.04
	389	7	14	0.02
B*07	127	9	89	0.86
	301	1	100	0.23
B*08	127	3	67	0.27
B*35	121	1	100	0.03
	240	3	67	0.16
	391	4	50	0.03
B*39	121	2	50	0.19
	240	2	50	0.04
B*40	119	2	50	0.3
	330	1	100	0.25
	631	1	100	0.17

BKPyV, BK polyomavirus; EVGR, early viral gene region; HI, healthy individual.

In line with the predicted clusters, some areas in the EVGR appeared to be more immunogenic than others (Table 1). The domain spanning from 9m383 to 9m393 could induce frequent responses in participants with HLA-A*02, -A*11, -B*35 and -B*39. The domain from 9m119 to 9m133 appeared to be highly immunogenic across different HLA types. Additional epitope clusters in the 221–240 and 536–645 EVGR areas also appeared to be more immunogenic (Table S5); however, they were tested in only few participants and thus deserve further investigation.

HLA restriction of BKPyV EVGR-specific T cell responses in HIs

To address HLA specificity of IFN-γ–inducing 9mer epitopes, cells were stained with MHC streptamers (Table 2). Some MHC streptamers showed strong staining such as HLA-B*07– and -B*08–positive 9m127 streptamers that were identified in 89% and 67% HIs with mean values of 0.86% and 0.27%, respectively. T cells presenting 9m127 via the HLA-A*02 molecule could not be detected, despite high 9m127-specific IFN-γ T cell responses among HLA-A*02–positive HIs (Figure 4A), suggesting that those responses were not HLA-A*02 restricted. Conversely, HLA-A*02–positive T cells specific for 9m679 could be detected in 50% of tested participants with HLA-A*02, despite a low number of responsive donors in the IFN-γ ELISpot assay (18%).

Of note, HLA-A*03–positive 9m327-specific T cells could be detected in 40% of HIs with HLA-A*03, but 9m327 elicited IFN-γ responses in donors with HLA-A*03, -A*11 and -B*07. HLA-A*24–positive 9m389-specific T cells could be detected in 14% of HIs, but 9m389 elicited IFN-γ responses in donors with HLA-A*02, -A*11 and -A*24. HLA-B*07–positive 9m301-specific T cells could be detected in the only tested participants with HLA-B*07, but 9m301 elicited IFN-γ responses in donors with HLA-A*02, -*A24 and -B*07. Finally, HLA-B*40–positive 9m119-specific T cells could be detected in 50% of HIs with HLA-B*40 but 9m119 elicited IFN-γ responses in donors wit HLA-B*07, -B*40 and -B*44.

Finally, some T cell–activating 9mers were presented by more than one HLA molecule, namely, 9m121 (HLA-B*35 and -B*39), 9m127 (HLA-B*07 and -B*08), and 9m240 (HLA-B*35 and -B*39) (Table 2).

BKPyV EVGR-specific epitope $CD8^+$ T cell responses in pediatric KTRs

To extend the results from HIs to the clinical setting, BKPyV-specific T cell responses were investigated in 19 pediatric KTRs who were protected or recovered from BKPyV viremia (Table S2). Several epitopes identified in HIs could be confirmed in these 19 KTRs (Table 3).

Overall, 9m389 was recognized in 67% of patients with HLA-A*02, with a mean value of 312 SFU/10^6 cells, but HLA-A*02 restriction could not be confirmed. This epitope induced T cell responses in 33%, 50%, 50%, and 40% of patients with HLA-A*11, -A*24, -B*07, and -B*51, respectively, with HLA specificity confirmed for HLA-A*24 and -B*51 molecules. In addition, 9m679 was found to be immunogenic in eight of nine tested patients with HLA-A*02, and specific $CD8^+$ T cells were detectable in 36% of patients with HLA-A*02. ELISpot assays could confirm 9m327 and MHC streptamer staining in KTRs who were positive for HLA-A*01, -A*03, and -A*11. Furthermore, 9m127 elicited T cell responses in 100% of patients with HLA-B*07 and -B*08, and MHC streptamer staining identified $CD8^+$ T cells for one patient with HLA-B*07.

Four 9mer epitopes elicited functional IFN-γ responses in HIs and KTRs and were HLA-specific in both cohorts: 9m127 (HLA-B*07 specific), 9m327 (HLA-A*03 specific), 9m389 (HLA-A*24 specific) and 9m679 (HLA-A*02 specific). Three 9mer responses were found in KTRs but not in HIs (9m302, 9m536, and 9m633).

BKPyV replication prevalence in pediatric KTRs

To evaluate the potential association of BKPyV replication with specific HLA-A and -B types, a prospective cohort of 118 consecutive pediatric KTRs was analyzed, of whom 38 (32%) experienced BKPyV viremia. The rate of BKPyV viremia was not equally distributed across HLA types or mismatches (Figure 5). Although the overall sample size was too small for statistically supported conclusions, patients with HLA-A*01 seemed to have lower rates of viremia compared with the overall population

Table 3: BKPyV EVGR-specific T cell responses in kidney transplant recipients

		IFN-γ ELISpot			MHC-streptamers		
HLA type	BKPyV epitope	Tested KTRs (n)	KTRs with positive response (%)	SFU/10^6 cells (mean)	Tested KTRs (n)	KTRs with positive response (%)	Epitope-specific $CD8^+$ T cells (mean %)
A*01	256	2	50	100	–	–	–
	327	2	100	40	3	67	0.10
	386	–	–	–	3	33	0.02
A*02	316	–	–	–	5	20	0.14
	302	8	12	245	–	–	–
	389	9	67	312	–	–	–
	391	4	25	840	–	–	–
	393	–	–	–	3	33	0.06
	510	10	50	95	–	–	–
	536	–	–	–	4	25	0.14
	679	9	89	324	11	36	0.09
A*03	256	3	30	120	–	–	–
	272	–	–	–	3	67	0.12
	327	3	30	270	1	100	0.05
A*11	256	3	30	120	–	–	–
	276	3	30	465	–	–	–
	327	3	67	270	3	33	0.08
	389	3	33	70	–	–	–
A*24	127	3	33	200	–	–	–
	342	–	–	–	1	100	0.08
	389	4	50	370	2	50	0.02
B*07	119	2	50	130	2	50	0.07
	127	2	100	280	2	50	0.08
	240	–	–	–	1	100	0.03
	301	–	–	–	1	100	0.09
	389	2	50	285	–	–	–
B*08	127	1	100	150	–	–	–
	462	–	–	–	1	100	0.08
	679	–	–	–	1	100	0.07
B*35	240	5	80	410	–	–	–
	391	5	40	590	–	–	–
	633	5	40	145	–	–	–
B*51	256	4	25	180	–	–	–
	389	5	40	135	2	50	0.10
	506	2	100	175	–	–	–

BKPyV, BK polyomavirus; EVGR, early viral gene region; IFN-γ, interferon γ; KTR, kidney transplant recipient; SFU, spot-forming unit.

($p < 0.05$), and mismatching for this allele was present in about half of the KTRs. Similarly, a trend for a lower rate of BKPyV viremia was seen among patients positive for HLA-A*26, -A*28 and -B*57, without a clear association with matching. Interestingly, 60% of nonviremic patients with HLA-B*07 were matched for this particular allele, whereas all viremic patients with HLA-B*07 were mismatched (Figure 5). This observation would be in line with the hypothesis that the absence of HLA-B*07 might increase the risk of viremia if confirmed by an independent study of larger sample size. In contrast, there was a higher proportion of HLA-B*35–matched patients among viremic KTRs than among the nonviremic KTRs ($p < 0.05$). Finally, viremia seemed to be independent of matching for HLA-A*01, -A*24, -A*29, -B*08, and -B*51 types, for which the proportions of matched patients among viremic and nonviremic patients were similar (Figure 5). A higher rate of high-level viruria was found in patients with HLA-A*31 ($p < 0.05$), whereas a trend toward a lower rate was found in patients with HLA-B*57 ($p < 0.05$) and HLA-A*01 ($p = 0.08$) (data not shown). Consequently, although immunogenic properties of some 9mer epitopes could be confirmed in KTRs, a simple association of single mismatching with risk or matching with protection could not be derived from this pediatric cohort.

Discussion

BKPyV-associated nephropathy is now widely recognized as an emerging complication in KT (64–67). Insufficient

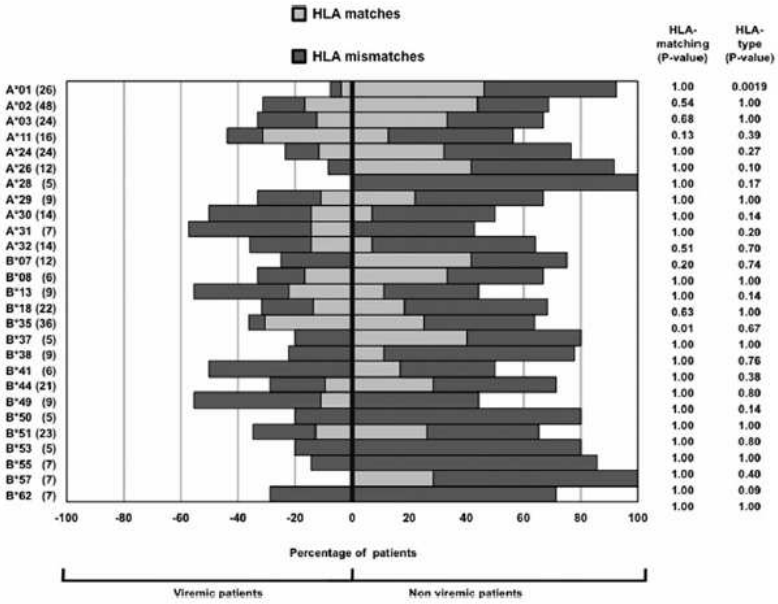


Figure 5: BKPyV viremia and HLA-matching in 118 pediatric KTRs. The percentages of viremic (left side) and non-viremic (right side) KTRs according to HLA type are shown. For each HLA type, the percentage of patients displaying matched (light gray) or mismatched (dark gray) allele with their kidney donor is shown. The number of KTRs with the most common HLA types is indicated at left. BKPyV viremia was analyzed for single HLA types versus the whole population (p-value for HLA type) or by comparing viremia occurrence in matched and mismatched patients (p-value for HLA matching; Fisher exact test). BKPyV, BK polyomavirus; KTR, kidney transplant recipient.

BKPyV-specific T cell control of the recipient over viral replication in donor allograft is suspected as the common denominator and key mechanism (67). Independent single-center studies indicated that reducing immunosuppression was associated with increasing BKPyV-specific T cell responses and coincided with clearance of viremia and nephropathy (30–36). Alternatively, the decline of cellular immunity at 1 month after transplantation has been proposed to identify patients at increased risk of BKPyV viremia, but no or low IFN-γ responses in at least half of patients impeded the predictive value for individual patients (53). Given these experiences and the fact that mostly $CD4^+$ T cell responses were measured by overlapping 15mP, better characterization of BKPyV epitope-specific $CD8^+$ T cell response seems needed to improve the current understanding and clinical utility of BKPyV-specific cellular immunity. Because we observed that BKPyV-specific $CD8^+$ T cell responses are more frequently directed to LTag than VP1 (30,35), we focused on the BKPyV EVGR–encoded epitopes rather than the capsid antigens (68,69). The following aspects emerged from the present study.

First, systematic *in silico* analysis of immunogenic epitopes predicts both immunogenic hotspot clusters and gaps in BKPyV EVGR for each of the 14 major HLA class I types. Predicted areas were similarly clustered across different HLA class I types, and this observation argues for potential immunodominant domains, in which the virus would be particularly susceptible to immune control and selection pressure. Because these domains are present in EVGR-encoding crucial viral regulatory proteins early in the viral replication cycle, at least transient escape from cellular immune control would be particularly important for BKPyV replication (70,71).

Second, the expansion protocol used in the present study has been devised to overcome the low frequency of BKPyV-specific T cell responses to overlapping 15mP (35) and was used successfully in the prospective study of pediatric KTRs (30). Our results demonstrate that functional $CD8^+$ T cell responses can be amplified which selectively target few 9mer epitopes located in predicted immunodominant clusters. CFSE dye dilution and streptamer staining supported this central observation, indicating that these cells were among the most active, dividing approximately once every 1–2 days. The 9mer responses were functionally defined by IFN-γ secretion but could be linked in principle to 9mer-specific cell surface expression of CD107a, a marker of granzyme and perforin degranulation, and to cytotoxic activity in ^{51}Cr release assays.

Third, although certain 9mer epitopes were shown to be selective for specific HLA types by streptamer staining of $CD8^+$ T cells, presentation by different HLA class I types could be observed. This has been demonstrated for HLA types that belong to crossreacting group 1C (e.g. HLA-A*01, -A*03 and -A*11) or 7C (e.g. HLA-B*07 and -B*08) (72).

Fourth, 10 of the 9mer epitopes identified in HIs could be confirmed in an unrelated cohort of 19 pediatric KTRs who were protected or recovering from BKPyV replication. Consequently, predicted and tested immunodominant responses could be linked to the clinically relevant situation of immunosuppressed KTRs.

Interestingly, three additional 9mer responses found in KTRs had been predicted but were not detected in HIs. This might indicate that responses in HIs may be lower and hence less detectable. Conversely, the additional responses in pediatric KTRs might result from extensive exposure to BKPyV replication and the more vigorous immune response described for children (5).

The fact that the major site of BKPyV replication is in donor cells of the renal allograft deserves consideration because this might affect HLA presentation and modify T cell receptor recognition of BKPyV epitopes. Interestingly, in our prospective cohort of 118 pediatric KTRs, single HLA mismatching could not be associated with BKPyV viremia. Although sample size might be an important aspect (73), it could also reflect the fact that other risk factors besides HLA-mismatching including maintenance immunosuppression can promote progression to viremia and nephropathy equally well and thereby contribute to the failing balance between BKPyV replication in the graft and BKPyV-specific T cell control in an individual patient.

Previous studies focused mainly on persons with HLA-A*02 (41–71) and more rarely on epitopes restricted for HLA-A*01, -A*03, -A*11, -B*07 and -B*08 (54,56,74). Most published epitopes could be confirmed in our study, such as the HLA-A*02–restricted epitopes 9m679 and 9m316 or the HLA-B*07– and HLA-B*08–restricted 9m127, validating our approach and underscoring their potential immunodominance. Our approach expands this list to at least 39 mostly new 9mer epitopes, of which 21 were linked by streptamer staining to a range of HLA types. These findings may be of high interest, especially because BKPyV epitope-specific T cells could be sorted using specific MHC streptamers, further expanded *in vitro* after removal of the MHC-streptamer complex and used for adoptive T cell transfer. In addition, some of the epitopes identified in the present study have common sequences with the closely related JCPyV, suggesting that BKPyV-specific T cells might induce cross-protection to JCPyV, which is also associated with disease in some immunocompromised patients.

Our study has several limitations that should be considered for a balanced interpretation of the results. First, not all IFN-γ responses could be linked unambiguously to HLA types present in functionally responding HIs or KTRs by streptamers. There may be several reasons for this, including low T cell frequencies, technical properties of certain streptamers that reduce sensitivity, or the fact that some 9mer epitopes eliciting IFN-γ responses were presented by HLA molecules not predicted in this study, especially in the transplantation context. These aspects might be better addressed as prediction and technology improves. Second, some aspects of BKPyV-specific $CD8^+$ T cell characterization (proliferation during expansion, cytotoxicity, degranulation) could not be performed for all epitopes but could be addressed at least in principle in experimental animal models (75,76). Third, the use of cryopreserved samples instead of fresh PBMCs might have reduced detection of some rare BKPyV-specific T cells, even if immunodominant responses were still detectable. Fourth, the investigation of cellular immune responses in KTRs was done in a relatively small cohort; therefore, the number of identified epitopes was limited. Overall, the results are encouraging to take this approach to larger clinical cohorts and relevant prospective study settings.

We conclude that *in vitro* cellular expansion using 15mP EVGR peptides or longer peptides allowed us to experimentally test the immunogenicity of 9mer epitopes predicted by *in silico* algorithms. We could identify immunogenic 9mer epitopes in both HIs and KTRs and extended the current list to at least 39. Several epitopes were located in clusters and induced specific responses in different common HLA class I types, as expected for immunodominant epitopes. Further studies will have to prove how this information can be best harnessed for clinically relevant immune monitoring and possibly foster adoptive T cell transfer for prophylaxis and therapy in kidney and hematopoietic stem cell transplant patients.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Phenotype of dividing cells during *in vitro* expansion. Peripheral blood mononuclear cells from a

healthy donor were labeled with CFSE before being expanded *in vitro* and stained with CD45RA and CCR7 markers. (A) CFSE content of $CD8^+$ T cells after *in vitro* expansion. Gates P11–P20 allow discrimination of different $CD8^+$ T cell divisions (P11 = no division; P12 = one division; P13 = two divisions, and so forth). (B) CD45RA and CCR7 expression by $CD8^+$ T cells at different division stages. Each panel is gated in one of the 10 gates (P11–P20). CFSE, carboxyfluorescein diacetate succinimidyl ester.

Figure S2: Immune responses of BKPyV-seronegative HIs. PBMCs from HIs who were seronegative for both BKPyV and JCPyV (HI-43, HI-44, HI-45 and HI-46; left panel) or seropositive for JCPyV and seronegative for BKPyV (HI-47, HI-48; normalized enzyme-linked immunosorbent assay net OD (58)) (right panel) were expanded for 14 days in the presence of BKPyV 15mP and restimulated with BKPyV 15mP, LPP or 9mP in interferon γ enzyme-linked immunospot assay. 9mP, 9mer-peptide pool; 15mP, 15mer peptide pool; BKPyV, BK polyomavirus; HI, healthy individual participant; JCPyV, JC polyomavirus; LPP, long peptide pool; OD, optical density; SFU, spot-forming unit.

Figure S1

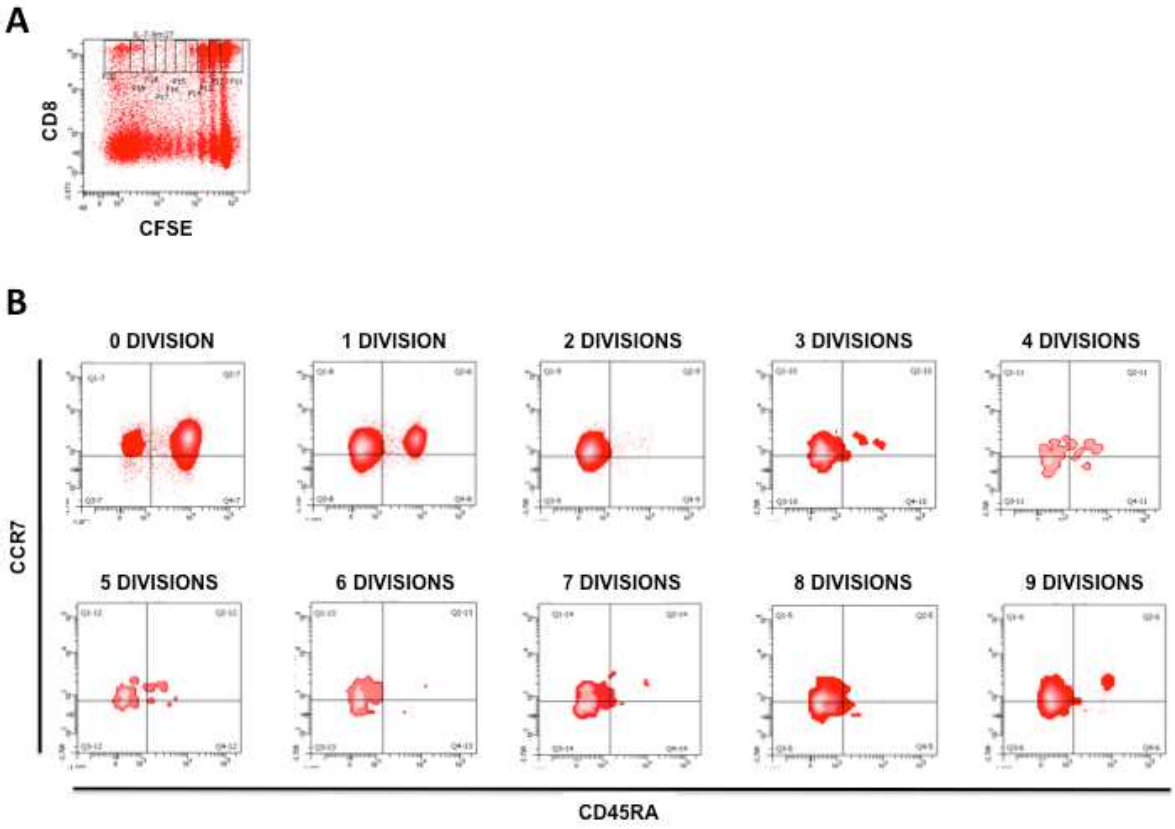
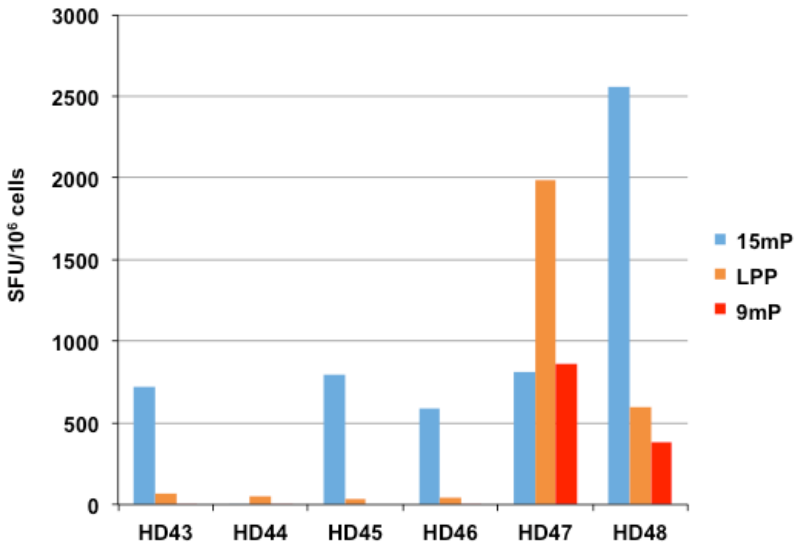


Figure S2



	HD43	HD44	HD45	HD46	HD47	HD48
BKPyV serostatus	-	-	-	-	-	-
JCPyV serostatus	-	-	-	-	+	+
BKPyV OD (1:200)	0.023	0.068	0.050	0.047	0.049	0.033
JCPyV OD (1:200)	0.067	0.065	0.052	0.055	0.850	0.489

Supplementary Table 1: Characteristics of 42 healthy individuals

Healthy Individual	Gender	Age (years)	HLA-A		HLA-B	
HI-1	m	52	2	11	55	51
HI-2	f	41	1	29	8	35
HI-3	f	59	23	28	44	44
HI-4	f	40	3	24	7	35
HI-5	f	53	3	28	40	41
HI-6	m	61	1	28	13	35
HI-7	f	48	24	24	18	62
HI-8	m	60	3	28	35	35
HI-9	f	60	3	26	7	27
HI-10	f	48	3	2	7	44
HI-11	m	52	2	1	8	12
HI-12	f	33	3	26	7	39
HI-13	f	51	26	32	40	44
HI-14	f	57	1	1	5	51
HI-15	f	50	2	24	35	39
HI-16	f	33	3	24	7	65
HI-17	m	33	29	24	55	44
HI-18	m	44	2	11	7	62
HI-19	f	29	1	3	8	35
HI-20	f	36	3	2	7	62
HI-21	m	32	2	30	13	35
HI-22	f	37	2	24	62	39
HI-23	f	24	2	19	63	60
HI-24	m	38	3	11	7	62
HI-25	f	31	2	32	40	44
HI-26	f	53	3	3	7	51
HI-27	nd	nd	3	24	7	15
HI-28	nd	nd	3	32	27	40
HI-29	nd	nd	1	3	7	15
HI-30	nd	nd	3	32	7	13
HI-31	nd	nd	32	33	44	51
HI-32	nd	nd	2	2	13	15
HI-33	f	28	24	29	44	49
HI-34	f	50	2	2	7	15
HI-35	f	27	2	32	15	44
HI-36	f	21	2	2	35	39
HI-37	m	46	2	24	51	55
HI-38	f	34	2	24	39	62
HI-39	f	52	2	2	13	39
HI-40	nd	nd	3	11	7	40
HI-41	f	48	1	24	7	55
HI-42	m	55	3	32	8	15

HI, healthy individual; m, male; f, female; nd, not determined

Supplementary Table 2: Characteristics of 19 pediatric kidney transplant recipients

Patient	Gender	Age (years)	BKPyV high viruria	BKPyV viremia	HLA-A		HLA-B		HLA-DR		HLA-A (donor)		HLA-B (donor)		HLA-DR (donor)	
KTR-1	f	17	-	-	29	33	8	14	3	3	32	33	8	14	1	3
KTR-2	m	21	-	-	29	32	44	51	11	11	2	29	13	44	7	11
KTR-3	m	15	-	-	2	26	39	62	16	17	2	30	13	39	3	16
KTR-4	f	16	+	-	1	31	37	38	7	11	1	24	51	51	4	11
KTR-5	f	17	+	-	2	24	18	35	11	13	33	33	58	58	3	14
KTR-6	f	25	+	-	1	2	51	64	7	8	1	1	64	57	7	7
KTR-7	f	27	+	-	2	3	60	60	4	11	2	32	39	18	11	11
KTR-8	m	26	+	-	2	30	51	53	11	13	2	24	8	39	3	13
KTR-9	m	13	+	-	1	2	50	51	7	13	2	25	18	51	4	4
KTR-10	m	15	+	-	11	26	7	55	14	15	2	23	18	27	15	15
KTR-11	m	14	+	+	2	24	38	41	4	8	25	26	15	35	4	4
KTR-12	m	12	+	+	2	24	51	65	13	14	1	3	8	16	13	7
KTR-13	f	14	+	+	2	30	49	51	7	13	1	11	49	55	11	14

Table S3: HLA allele frequencies in worldwide populations*				
*Data were retrieved from http://www.allelefrequencies.net				
A*01:01			**A*02:01**	
Population	**Frequency (%)**		**Population**	**Frequency (%)**
Ireland South	44,8		Argentina Gran Chaco Western Toba Pilaga	60,0
England North West	38,6		Mexico Mestizo	56,1
Ireland Northern	36,4		Greece pop 8	51,8
USA Caucasian Bethesda	28,7		Argentina Gran Chaco Eastern Toba	46,4
Belgium	29,2		Philippines Ivatan	48,0
Italy North pop 3	26,9		Austria	48,0
Sudan Central Shaigiya Mixed	27,8		Italy North pop 3	53,8
France Southeast	27,7		England North West	50,7
Austria	27,0		Italy Bergamo	46,5
Greece pop 8	25,3		Ireland Northern	46,8
Romania	23,0		Belgium	50,0
USA Philadelphia Caucasian	21,5		USA Caucasian Bethesda	47,8
Saudi Arabia Guraiat and Hail	21,6		USA Philadelphia Caucasian	40,7
Tunisia	21,0		Romania	43,7
India Delhi pop 2	20,0		Ireland South	43,6
Mexico Mestizo	14,6		Brazil Belo Horizonte Caucasian	43,2
Cuba Caucasian	15,7		Argentina Gran Chaco Mataco Wichi	40,9
Brazil Belo Horizonte Caucasian	13,7		Oman	39,8
Argentina Rosario Toba	15,1		France Southeast	38,5
USA African American Bethesda	14,8		Brazil Terena	38,3
Oman	14,4		Argentina Rosario Toba	34,9
Cuba Mulatto	14,3		Taiwan Taroko	36,4

Chile Santiago Mixed	11,0		Saudi Arabia Guraiat and Hail	33,3
Sudan Mixed	10,0		Cuba Caucasian	34,3
South Africa Natal Zulu	8,0		Cuba Mulatto	31,0
Indonesia Java Western	5,1		Tunisia	32,0
Ghana Ga-Adangbe	4,6		Sudan Mixed	33,0
Hong Kong Chinese	2,1		Chile Santiago Mixed	30,0
Taiwan Minnan pop 1	1,0		Taiwan Saisiat	21,6
Singapore Chinese	0,7		Ghana Ga-Adangbe	19,1
			Singapore Chinese	19,5
			Sudan East Rashaida	18,5
			Taiwan Minnan pop 1	17,6
			USA African American Bethesda	16,8
			Taiwan Thao	13,3
			Taiwan Atayal	13,2
			Indonesia Java Western	12,7
			Hong Kong Chinese	11,9
			Sudan Central Shaigiya Mixed	11,1
			Taiwan Hakka	10,9
			Taiwan Pazeh	10,9
			Taiwan Bunun	7,9
			Taiwan Paiwan	7,8
			Taiwan Siraya	7,8
			Taiwan Rukai	6,0
			Taiwan Tsou	5,9
			Taiwan Ami	5,1
			South Africa Natal Zulu	5,0
			Taiwan Puyuma	4,0
			Taiwan Tao	4,0
			India Delhi pop 2	3,3

A*03:01			A*11:01	
Population	**Frequency (%)**		**Population**	**Frequency (%)**
Belgium	30,2		Taiwan Tao	62,0
Austria	28,0		Taiwan Hakka	60,0
Ireland Northern	26,3		Taiwan Minnan pop 1	54,9
England North West	25,5		Taiwan Pazeh	50,9
France Southeast	23,8		Hong Kong Chinese	48,8
USA African American Bethesda	23,8		Singapore Chinese	47,7
Romania	21,8		Taiwan Thao	43,3
Ireland South	21,6		Taiwan Siraya	33,3
USA Philadelphia Caucasian	21,5		Indonesia Java Western	30,1
USA Caucasian Bethesda	20,6		Taiwan Saisiat	21,6
Italy North pop 3	19,2		Taiwan Tsou	21,6
Ghana Ga-Adangbe	19,1		Oman	21,2
Brazil Belo Horizonte Caucasian	17,9		India Delhi pop 2	18,9
Cuba Mulatto	16,7		Taiwan Bunun	18,8
Cuba Caucasian	15,7		Romania	15,8
Greece pop 8	15,7		Italy North pop 3	15,4
Saudi Arabia Guraiat and Hail	14,1		Taiwan Atayal	15,1
India Delhi pop 2	13,3		Ireland Northern	15,0
Chile Santiago Mixed	13,0		Taiwan Taroko	14,5
Tunisia	13,0		Philippines Ivatan	14,0
South Africa Natal Zulu	12,0		USA Caucasian Bethesda	14,0
Sudan Mixed	9,5		England North West	13,1
Oman	9,3		USA Philadelphia Caucasian	12,6
Mexico Mestizo	4,9		Greece pop 8	12,1
Indonesia Java Western	4,7		Ireland South	11,2
Argentina Rosario Toba	3,5		Cuba Caucasian	10,0
Hong Kong Chinese	1,6		Belgium	9,4
Singapore Chinese	1,3		Tunisia	9,0
			France Southeast	8,5
			Taiwan Rukai	8,0
			Mexico Mestizo	7,3
			Austria	6,5
			Saudi Arabia Guraiat and Hail	5,6

			Taiwan Ami	5,1
			Brazil Belo Horizonte Caucasian	4,2
			Taiwan Puyuma	4,0
			Taiwan Paiwan	3,9
			Sudan Mixed	3,5
			Sudan Central Shaigiya Mixed	2,7
			Chile Santiago Mixed	2,0
			Argentina Rosario Toba	1,2

A*24:02			**A*32:01**	
Population	**Frequency (%)**		**Population**	**Frequency (%)**
Taiwan Tsou	98,0		Oman	18,6
Taiwan Paiwan	96,1		Greece pop 8	16,9
Taiwan Rukai	96,0		Sudan East Rashaida	14,8
Taiwan Thao	90,0		Sudan Central Shaigiya Mixed	13,9
Taiwan Puyuma	88,0		France Southeast	13,8
Taiwan Saisiat	86,3		Italy Bergamo	11,9
Taiwan Ami	84,7		Sudan Mixed	11,0
Taiwan Bunun	84,2		Scotland Orkney	10,0
Taiwan Atayal	82,1		Romania	9,8
Taiwan Siraya	78,4		Ireland South	8,8
Taiwan Tao	78,0		USA Philadelphia Caucasian	7,4
Taiwan Taroko	72,7		Belgium	7,3
Taiwan Pazeh	58,2		Cuba Caucasian	7,1
Philippines Ivatan	58,0		Cuba Mulatto	7,1
Taiwan Minnan pop 1	34,3		Morocco Settat Chaouya	6,8
Indonesia Java Western	25,8		Ireland Northern	6,1
Taiwan Hakka	25,5		India Delhi pop 2	5,6
Romania	23,8		Austria	5,5
Greece pop 8	21,7		England North West	5,4
USA Caucasian Bethesda	20,6		South Africa Natal	5,0

			Zulu	
Argentina Gran Chaco Mataco Wichi	20,5		Mexico Mestizo	4,9
France Southeast	20,0		Saudi Arabia Guraiat and Hail	4,7
USA Philadelphia Caucasian	20,0		USA Caucasian Bethesda	4,4
Austria	20,0		Italy North pop 3	3,8
Sudan East Rashaida	18,5		Brazil Belo Horizonte Caucasian	3,2
Chile Santiago Mixed	16,0		USA African American Bethesda	3,0
Brazil Terena	15,0		Tunisia	3,0
England North West	13,8		Cameroon Bakola Pygmy	2,0
Ireland South	13,2		Singapore Chinese	1,3
Tunisia	13,0		Argentina Rosario Toba	1,2
Belgium	12,5		Chile Santiago Mixed	1,0
Saudi Arabia Guraiat and Hail	11,7		Taiwan Minnan pop 1	1,0
Italy North pop 3	11,5		Hong Kong Chinese	0,7
Sudan Mixed	9,5			
Argentina Gran Chaco Eastern Toba	9,5			
USA African American Bethesda	8,9			
Argentina Rosario Toba	5,8			
Sudan Central Shaigiya Mixed	2,7			
India Delhi pop 2	2,2			

B*07:02			B*08:01	
Population	**Frequency (%)**		**Population**	**Frequency (%)**
Ireland South	33,6		Ireland South	35,2
Ireland Northern	31,4		England North West	29,9
USA Caucasian Bethesda	28,7		Ireland Northern	29,8
England North West	27,5		Belgium	23,5
Austria	22,5		USA Caucasian Bethesda	22,5
USA Philadelphia Caucasian	20,7		Oman	21,2
Saudi Arabia Guraiat and Hail	16,6		Austria	21,0
USA African American Bethesda	16,0		Scotland Orkney	17,6
Cuba Caucasian	15,7		France Southeast	16,9
Ghana Ga-Adangbe	13,7		Serbia pop 2	15,7
France Southeast	13,8		Saudi Arabia pop 5	15,2
Italy North pop 3	13,3		Romania	14,1
Brazil Belo Horizonte Caucasian	11,6		South Africa Natal Zulu	14,0
Chile Santiago Mixed	12,0		Brazil Belo Horizonte Caucasian	13,7
Cuba Mulatto	11,9		USA Philadelphia Caucasian	13,3
Mexico Mestizo	9,8		Saudi Arabia Guraiat and Hail	13,2
South Africa Natal Zulu	9,0		USA African American Bethesda	12,8
Greece pop 8	8,4		Morocco Settat Chaouya	12,4
Serbia pop 2	7,8		Cuba Caucasian	11,4
Romania	7,2		Italy Bergamo	9,9
India Delhi pop 2	6,6		Cuba Mulatto	9,5
Sudan Mixed	4,5		Tunisia	9,0
Oman	3,4		Sudan Mixed	7,5
Tunisia	3,0		Greece pop 8	7,2
Argentina Rosario Toba	2,3		Mexico Mestizo	4,9
Singapore Chinese	2,0		Chile Santiago Mixed	4,0
Indonesia Java Western	1,7		Argentina Rosario Toba	3,5
Hong Kong Chinese	0,4		India Delhi pop 2	3,3
			Cameroon Bakola Pygmy	2,0
			Taiwan Siraya	2,0
			Taiwan Hakka	1,8
			Hong Kong Chinese	0,5

B*35:01			B*39:01	
Population	**Frequency (%)**		**Population**	**Frequency (%)**
Italy North pop 3	26,7		Taiwan Saisiat	82,4
Romania	19,3		Taiwan Tsou	43,1
Cuba Mulatto	19,1		Taiwan Taroko	40,0
Serbia pop 2	17,6		Taiwan Atayal	35,8
Oman	15,3		Taiwan Bunun	28,7
Mexico Mestizo	14,6		Taiwan Thao	26,7
Greece pop 8	14,5		Taiwan Rukai	26,0
Austria	13,0		Taiwan Ami	19,4
Argentina Rosario Toba	12,8		Taiwan Puyuma	10,0
USA Caucasian Bethesda	12,4		Taiwan Paiwan	7,8
Ghana Ga-Adangbe	12,2		Sudan East Rashaida	7,4
USA Philadelphia Caucasian	11,1		Taiwan Hakka	7,3
USA African American Bethesda	10,6		Argentina Gran Chaco Western Toba Pilaga	7,1
Ireland Northern	10,2		Philippines Ivatan	6,0
Tunisia	10,0		Singapore Chinese	4,7
France Southeast	9,2		Romania	4,6
Brazil Belo Horizonte Caucasian	8,4		Morocco Settat Chaouya	4,2
Sudan Mixed	8,0		Taiwan Minnan pop 1	3,9
South Africa Natal Zulu	8,0		France Southeast	3,8
India Delhi pop 2	7,7		USA Caucasian Bethesda	3,8
Ireland South	6,8		Taiwan Pazeh	3,6
England North West	6,4		Argentina Gran Chaco Eastern Toba	3,5
Taiwan Siraya	5,9		Serbia pop 2	2,9
Cuba Caucasian	5,7		Hong Kong Chinese	2,8
Singapore Chinese	4,0		Greece pop 8	2,4
Taiwan Pazeh	3,6		Cuba Mulatto	2,4
Hong Kong Chinese	3,2		Ireland South	2,0
Chile Santiago Mixed	3,0		Taiwan Siraya	2,0
Saudi Arabia Guraiat and Hail	2,4		Ireland Northern	1,8
Argentina Gran Chaco Mataco Wichi	2,2		Brazil Terena	1,7
Taiwan Minnan pop 1	2,0		Austria	1,5
Taiwan Paiwan	2,0		England North West	1,3
Taiwan Hakka	1,8		India Delhi pop 2	1,1

			USA Philadelphia Caucasian	0,7
			Saudi Arabia Guraiat and Hail	0,5

B*40:01			**B*44:02**	
Population	**Frequency (%)**		**Population**	**Frequency (%)**
Taiwan Atayal	60,4		Scotland Orkney	26,5
Taiwan Paiwan	58,8		Ireland South	24,8
Taiwan Taroko	58,2		Ireland Northern	24,6
Taiwan Ami	55,1		England North West	19,1
Taiwan Saisiat	54,9		USA Philadelphia Caucasian	16,3
Taiwan Thao	46,7		USA Caucasian Bethesda	16,2
Taiwan Hakka	40,0		France Southeast	15,4
Taiwan Pazeh	40,0		Austria	15,0
Taiwan Bunun	39,6		Belgium	11,2
Taiwan Tsou	35,3		Serbia pop 2	10,8
Taiwan Minnan pop 1	34,3		Cuba Caucasian	10,0
Taiwan Rukai	34,0		Romania	8,6
Philippines Ivatan	30,0		Brazil Belo Horizonte Caucasian	8,4
Singapore Chinese	28,2		Greece pop 8	7,2
Hong Kong Chinese	28,0		Morocco Settat Chaouya	6,8
Taiwan Puyuma	26,0		Italy North pop 3	6,7
Taiwan Siraya	25,5		Chile Santiago Mixed	6,0
USA Caucasian Bethesda	12,4		USA African American Bethesda	4,2
Belgium	12,2		Sudan East Rashaida	3,7
Cuba Mulatto	11,9		Cuba Mulatto	2,4
England North West	11,4		Sudan Mixed	1,5
USA Caucasian	11,0		India Delhi pop 2	1,1
Ireland Northern	10,2		Saudi Arabia Guraiat and Hail	0,5
India Jalpaiguri Toto	10,0		Hong Kong Chinese	0,4
USA Philadelphia Caucasian	8,1			
Indonesia Java Western	7,2			
Japan Hyogo	6,3			

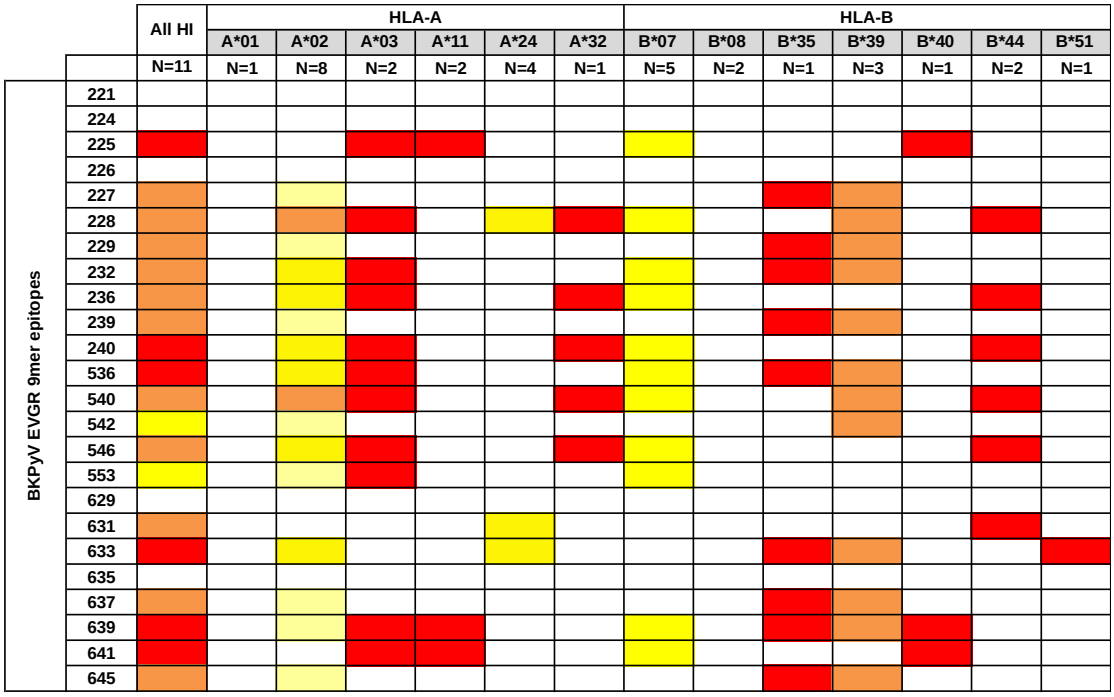
Austria	6,0			
Taiwan Tao	6,0			
Scotland Orkney	5,9			
Brazil Belo Horizonte Caucasian	5,3			
Ireland South	5,2			
France Southeast	4,6			
Romania	4,6			
Cuba Caucasian	4,3			
USA African American Bethesda	4,2			
India Delhi pop 2	3,3			
Serbia pop 2	2,9			
Morocco Settat Chaouya	2,8			
Chile Santiago Mixed	2,0			
Sudan Mixed	1,5			
United Arab Emirates pop 2	1,1			
Saudi Arabia Guraiat and Hail	0,5			

B*51:01	
Population	**Frequency (%)**
Italy North pop 3	46,6
Oman	33,1
Greece pop 8	30,1
Saudi Arabia Guraiat and Hail	26,3
Serbia pop 2	23,5
Romania	20,4
Argentina Rosario Toba	18,6
Singapore Chinese	15,4
Brazil Belo Horizonte Caucasian	14,7
Tunisia	14,0
Cuba Caucasian	12,9
India Delhi pop 2	12,1
Sudan Mixed	11,5
France Southeast	11,5
Austria	10,0
Mexico Mestizo	9,8
Cuba Mulatto	9,5
England North West	9,4
Hong Kong Chinese	8,6
USA Philadelphia Caucasian	7,4
Argentina Gran Chaco Western Toba Pilaga	7,1
Chile Santiago Mixed	7,0
Indonesia Java Western	6,4
Argentina Gran Chaco Eastern Toba	6,0
USA Caucasian Bethesda	5,4
USA African American Bethesda	5,3
Ireland Northern	5,2
Ghana Ga-Adangbe	3,8
Ireland South	3,6
Taiwan Siraya	2,0
Brazil Terena	1,7

Epitope	Amino acid sequence	HLA type	Reference
127	LPLMRKAYL	B*07, B*08	(Li, Melenhorst et al. 2006)
133	AYLRKCKEF	A*24	(2)
256	RTLACFAVY	A*03	(Ramaswami, Popescu et al. 2009)
259	ACFAVYTTK	A*03	(2)
272	ILYKKLMEK	A*03	(2)
316	KLCTFSFLI	A*02	(Provenzano, Bracci et a 2006)
322	FLICKGVNK	A*03	(2)
327	GVNKEYLLY	A*03	(2)
442	PYHTIEESI	A*24	(2)
462	MLTERFNHI	A*02	(Randhawa, Popescu e al. 2006)
506	VIFDFLHCI	A*02	(3)
510	FLHCIVFNV	A*02	(3)
514	IVFNVPKRR	A*03	(2)
679	LLLIWFRPV	A*02	(3)

Table S4: Previously published BKPyV EVGR 9mer epitopes predicted in our study

Table S5: BKPyV 9mer responses in IFN-γ ELISpot assay - additional 24 epitopes



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2. Ramaswami B, Popescu I, Macedo C, Metes D, Bueno M, Zeevi A, et al. HLA-A01-, -A03-, and -A024-binding nanomeric epitopes in polyomavirus BK large T antigen. Hum Immunol. 2009;70(9):722-8.

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4. Randhawa PS, Popescu I, Macedo C, Zeevi A, Shapiro R, Vats AN, et al. Detection of CD8+ T cells sensitized to BK virus large T antigen in healthy volunteers and kidney transplant recipients. Hum Immunol. 2006;67(4-5):298-302.

SUPPLEMENTAL MATERIALS AND METHODS

Assessment of BKPyV viruria and viremia in pediatric KTRs

BKPyV viruria and viremia were measured at predefined time points (1, 3, 6, 9, 12, 18, 24 months after transplantation and yearly thereafter) by the Transplantation & Clinical Virology laboratory in Basel using a quantitative real-time polymerase chain reaction (PCR) as previously described (Hirsch, Knowles et al. 2002, Dumoulin and Hirsch 2011). BKPyV viruria was defined by a urine viral load of ≥2500 genome equivalents (GEq)/mL, high-level BKPyV viruria by ≥7 log10 GEq/mL and BKPyV viremia by ≥1000 GEq/mL. Based on protection and recovery from BKPyV viruria and viremia, PBMCs samples of 19 KTRs were selected and analysed (**Table S2**).

BKPyV IgG ELISA

BKPyV VP1-derived virus-like particles were used as antigen to detect BKPyV IgG as described (Egli, Infanti et al. 2009, Kardas, Sadeghi et al. 2014). Each serum sample was serially diluted 1:100, 1:200 and 1:400 and the optical density (OD) was measured at 492nm. The OD_{492nm} values were normalized to the OD_{492nm} of an internal reference serum, sera with a normalized OD_{492nm}>0.100 at the 1:200 dilution were defined as IgG positive.

Isolation of peripheral blood mononuclear cells from whole blood

PBMCs from anticoagulated blood or from buffy coat preparations were diluted 1:2 in D-PBS w/o Ca^{2+} and Mg^{2+}, and overlaid on Ficoll (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). After centrifugation (room temperature, 800g; 25 minutes (min)), PBMCs were recovered, and washed twice i.e. resuspended in D-PBS w/o Ca^{2+} and Mg^{2}, and centrifuged (RT, 300g, 10min). The cells were counted and resuspended in

culture medium RPMI-1640 supplemented with 5% Human Serum AB and 2mM of L-Ala-Glutamine (all **Sigma-Aldrich Chemie GmbH** Buchs SG, Switzerland) or cryopreserved in culture medium containing 10% DMSO and stored in liquid nitrogen.

In vitro *expansion of T-cells*

Freshly isolated or thawed PBMCs were seeded at a concentration of $2x10^6$/ml in culture medium in 24 well-plate after the number of viable cells was counted using Trypan Blue exclusion. PBMCs were stimulated with LPP or 15mP (200ng/ml), and incubated for 9-14 days at 37℃ 5% CO_2 before phenotypic and functional assays were carried out. Recombinant human IL-2 (20U/ml, Peprotech, Rocky Hill, NJ, USA) and recombinant IL-7 (5ng/ml, Peprotech) were added once a week.

PBMCs obtained from cryopreserved samples from pediatric KTRs were first thawed and resuspended in pre-warmed culture medium. The number of viable cells was counted using Trypan Blue solution. The cells were resuspended at the concentration of $2x10^6$/ml in culture medium, seeded in 24 well-plate and incubated with 200ng/ml 15mP at 37℃ 5%CO_2. Recombinant IL-2 (20U/ml) and recombinant IL-7 (5ng/ml) were added at day 3, before performing phenotypical and functional assays at day 7.

ELISpot assay

PDVF multiscreen filter 96 well plates (MSIPS4W10, Millipore Bedford, MA) were coated with 100µl of anti-IFN-γ mAb 1-D1K (Mabtech, Nacka, Sweden) at 10µg/ml and incubated overnight at 4℃. After three washing steps using PBS, freshly isolated or thawed PBMCs ($2.5x10^5$/well) or expanded T cells ($1x10^5$/well) were seeded in presence of 2µg/ml of BKPyV-specific peptide pools, checkerboard subpools, or single peptides. Cells without added peptide were used as negative

control, whereas cells treated with *Staphylococcus* enterotoxin B (SEB) (2µg/ml; Sigma, Saint Louis, Missouri, USA) or Phytohemagglutinin-L (PHA) (2µg/ml; Roche Diagnostics GmbH, Mannheim, Germany) served as positive control. After incubation for 20-24 hours (h) at 37℃, the plates were washed five times with PBS 0.05% Tween-20 and anti-IFNγ mAb 7-B6-1-Biotin (Mabtech) was added at 1µg/ml for 3h at RT. After washing, Streptavidin ALP (Mabtech) was added at 1µg/ml for 1h at RT. The plates were washed five times with PBS 0.05% Tween-20 and tap water before incubation with SigmaFast BCIP/NBT (**Sigma-Aldrich Chemie GmbH** Buchs SG, Switzerland) for 20min at RT in the dark. Plates were rinsed with water, dried and spots counted with an ELISpot reader (Cellular Technology Ltd Europe, Bonn, Germany). ELISpot data are averaged duplicate or triplicate wells with background wells subtracted. Response >background plus 2 standard deviations were counted as reactive.

MHC-streptamer staining

The presence of BKPyV-specific T-cells was investigated using MHC-streptamers obtained from custom service (IBA GmbH, Göttingen, Germany). Peptide-loaded MHC molecules were incubated with PE- or APC-coupled Strep*Tactin* for 45 minutes on ice before being incubated with 2-10x10^5 cells for 45 minutes on ice. After washing with immunostaining IS buffer (IBA), cells were incubated with CD8-PE-Cy7 antibody (BD Biosciences, San Jose, CA, USA) for 15min on ice, washed with IS buffer and acquired on a flow cytometer (FACSCanto; BD Biosciences) using the FACSDiva software. Gating was performed on live cells using forward scatter and side scatter profiles, and doublets were excluded. Data are reported as percentage of specific populations after subtracting the negative control (PE or APC-coupled Strep*Tactin* alone).

CSFE proliferation assay

PBMCs were resuspended at a concentration of $5x10^6$/ml in PBS containing 5µM carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience, Vienna, Austria). After 15min incubation at RT on a shaker, cells were washed twice with culture medium and resuspended in fresh medium for BKPyV-specific T-cell expansion described above. Cells were stained with specific MHC-streptamers and CD8 as described above and their CFSE content was analysed by flow cytometry.

CD107a degranulation assay

Expanded T-cells were resuspended in fresh medium ($2x10^6$/ml) and seeded in a 96-well plate ($2x10^5$ cells per well). The BKPyV 9mer-peptide of interest was added to the cultures (1µg/ml) for 5h-stimulation at 37℃. Phorbol 12-myristate 13-acetate (PMA; 100ng/ml; Sigma) and ionomycin (1µg/ml; Sigma) were used as positive control, and a BKPyV 9mer-peptide of another HLA specificity was used as negative control. PE-Cy7-labelled CD107a antibody (BD Biosciences) or PE-Cy7-labelled isotype control (BD Biosciences) was added during the whole period of stimulation, whereas monensin (0.3µl per well; BD Biosciences) and brefeldin A (10µg/ml; Sigma) were added for the last 4h only. Cells were then labeled for specific MHC-streptamers and CD8 as described above and analysed by flow cytometry.

Cytotoxicity assay

Specific cytotoxic activity of expanded T-cells was assessed by ^{51}Cr-release assay of autologous PHA-stimulated blasts obtained by culturing PBMCs in the presence of PHA (4µg/ml) for 3-6 days. PHA-blasts were loaded for 1h at 37℃ with 200 µCi ^{51}Cr (Sodium Chromate Hartmann Analytic, Braunschweig, Germany), then pulsed for 1h with 2 µg/ml of 9mer candidate peptides or an unrelated peptide (the melanoma related peptide MAGE-4, kindly provided by Dr Paul Zajac, Department Biomedicine,

University of Basel, Switzerland) used as negative control. Effector T-cells were incubated with $2x10^3$ target cells at different effector:target (E:T) cell ratios for 4h at 37℃ 5%CO_2. Then 50µl of the supernatant was transferred to a lumaplate (Perkin Elmer, Waltham, Massachusetts, USA) and dried. Counts per minutes (cpm) were counted in a β-counter (TopCount, Perking Elmer). Killing data are the average of duplicate wells and calculated as percentage of lysis according to following formula: (Sample cpm-Spontaneous Release cpm)/(Maximum Release cpm/Spontaneous Release cpm)X100, where Spontaneous Release corresponds to ^{51}Cr release by target cells alone and Maximum Release corresponds to ^{51}Cr release by target cells mechanically lysed. Data were considered reliable when Minimum release was less than 50% of Maximum Release.

By SYFPEITHI and IEBD epitope prediction alghoritms, we obtained 20 topscoring 9mer epitopes encoded in the BKPyV EVGR for each of 14 HLA-A and -B types prevalent in Europe and North America, which showed to cluster across several HLA types. From these clusters, 73 predicted 9mer epitopes, including epitopes identified in previous studies, were selected. Additional 24 candidate epitopes locating out of the clusters were also included, for a total of 97 epitopes. Corresponding peptides were synthesized and used for experimental epitope mapping, with also longer peptides corresponding to clusters where 9mer epitopes overlapped.

BKPyV-specific T cells were expanded from PBMCs samples by stimulation with a pool of 15mer overlapping peptides spanning the whole LTag (15mP) sequence, then tested in a IFN-γ ELISpot assay re-stimulating cells with the 15mP and subpools of 9mer peptides according to a checkerboard matrix approach. Positive responses were confirmed by re-challenge with single 9mer peptides. Our current approach of cell expansion was successful and we could elicit BKPyV-specific responses. Out of 97 candidates, we could confirm 39 epitopes present in at least 30% of BKPyV healthy participants with different HLA distribution. The overall results indicated that BKPyV EVGR-specific 9mer T cell responses were heterogeneous in terms of frequency and strength. For some HLA types, positive responses were found with higher frequency and directed towards multiple epitopes; for example, in HLA-A*03, -A*11, -B*07, -B*35, -B*39, -B*44 or -B*51 positive individuals, more than one epitope could be identified in >50% of healthy individuals.

In line with the predicted clusters, some areas appeared to be more immunogenic than others. The area including 9m383 to 9m393 was highly immunogenic in individuals with HLA-A*02, -A*11,-B*35 and -B*39, and also the domain from 9m119 to 9m133 appeared to be highly immunogenic in multiple HLA types.
Since ELISpot assays are functional responses, but cannot attribute the HLA-specificity of the IFN-γ inducing 9mer epitopes, MHC streptamer staining has been performed, and we could determine HLA specificity in 21 of 39 epitopes, with restriction for specific HLA alleles in some cases, while other epitopes could be presented by different HLA class I types, mainly belonging to the same cross reactive epitope groups, which are known to share epitopes specificities.

Furthermore our results demonstrate that with our expansion protocol we could obtain in healthy individuals functional epitope-specific CTL responses, as

demonstrated in killing assay and CD107 staining, and that such cells were actively proliferating, dividing approximately once every 1–2 days. With this successful method it could be possible to confirm our results in and independent cohort of 19 pediatric KTRs who were protected or recovered from BKPyV viremia. Thirteen epitopes could be validated in these 19 KTRs, in fact 10 of the 9mer responses found in HI could be detected also in patients, and additional 3 epitopes were found independently only in KTRs, may be the result of a persistent exposure to the virus concomitant with the stronger immune responses characterizing children (Schmidt, Adam et al. 2014).

The identified epitopes could be used for monitoring virus specific T cell responses in KTRs in combination with BKPyV replication screening, in order to identify patients at risk for BKPyVAN progression, and consequently guide therapeutic interventions. As the observed epitope-specific CD8+ T cells were functional and highly proliferating, the identified epitopes could be used also in the perspective of adoptive T cell transfer or vaccine development.

In the context of kidney transplantation, it has been previously demonstrated that a risk factor for BKPyV viremia is constituted by a high number of HLA mismatches between the recipient and the donor, and also specific HLA types in the recipients (Awadallah, Randhawa et al. 2004, Bohl, Storch et al. 2005, Awadallah, Duquesnoy et al. 2006, Masutani, Ninomiya et al. 2013), therefore we evaluated the potential association of BKPyV replication with single HLA-A and –B alleles in 118 consecutive KTRs, with 38 experienced viremia. We could observe heterogeneous distribution of viremic patients among HLA types, with a significant lower rate of viremic patients in HLA-A*01 patients ($p<0.005$), furthermore single mismatches were correlated to viremia, but no clear association could be observed in any of the analyzed HLA. Unfortunately, the studied cohort could be to small to obtain significant results about HLA types and risk of infection, therefore similar analysis should be performed in larger cohort of patients.

6 DISCUSSION

BKPyV infection can cause disease almost exclusively in immunodeficient patients (Hirsch and Steiger 2003, Rinaldo, Tylden et al. 2013), while in immunocompetent individuals it can persist lifelong after primary infection and can be asymptomatically shed in urines (Knowles, Pipkin et al. 2003, Egli, Infanti et al. 2009, Schmidt, Adam et al. 2014). Viral persistence could be explained by an immune control operating in healthy individuals, able to prevent viral mediated damage, but not to the extent to fully eliminate the virus from the body. In this context there could be the possibility of a viral component acting to subvert immune control, with mechanisms of immune evasion.

Many viruses, in particular those characterized by persistence in the host, target different steps of immune system, from viral recognition to adaptive immune responses, in order to get advantages over the host cell. Several studies have investigated on the immune response to BKPyV trying to identify which effectors could play a major role in the control of viral replication and prevention of disease, identifying T cells, as critical effectors in the control of virus (Binggeli, Egli et al. 2006, Binggeli, Egli et al. 2007, Ginevri, Basso et al. 2007, Comoli, Basso et al. 2009, Schachtner, Muller et al. 2011), therefore a possible targeted checkpoint by BKPyV could be antigen processing and presentation, idea supported by the observation that in biopsies from KTRs with BKPyVAN diagnosis HLA-DR expression seemed lower compared to T-cell mediated rejection (Nickeleit, Hirsch et al. 2000).

One of the peculiar features of BKPyV among all HPyV, shared only by JCPyV, is the expression of agnoprotein, a small viral protein of 66 aa demonstrated to be expressed in BKPyV-infected cells, also in patients diagnosed with BKPyVAN, but the only among all BKPyV proteins not able to elicit an immune response (Leuenberger, Andresen et al. 2007), suggesting a possible role in immune escape mechanisms, idea that could be supported by the observation that agnoprotein could be involved in vesicular transport (Johannessen, Walquist et al. 2011), and the fact that agnoprotein shared a similar structure with a HSV protein, pICP47, known to inhibit HLA-peptide complex presentation to the APC surface by binding TAP (Galocha, Hill et al. 1997, Aisenbrey, Sizun et al. 2006). For all these reasons, we

have compared the effects of agnoprotein and ICP47 on HLA class I and II expression in transfected cells expressing the two proteins constitutively or under tetracycline regulation.

The results showed that BKPyV agnoprotein expression had no inhibitory effect on HLA class I and II expression in our in transiently transfected cells, even by simulating an inflammatory milieu, obtained by adding IFN-γ with a consequent upregulation of HLA expression. Conversely, ICP47 specifically downmodulated HLA class I on the cell surface, whereas no inhibition was observed, as expected, on class II expression. Our results obtained in transiently transfected cells can have some limitations, in terms of efficiency of transfection and durability of protein expression, to overcome this problem, we created stable clones expressing agnoprotein or ICP47 under the tight control of tetracycline, confirming the previous results, thus we could more robustly demonstrated that agnoprotein did not interfere with the quantity of HLA molecules on the cell surface.

To exclude the involvement of agnoprotein on CTL function upon peptide presentation and consequent T cell activation, we evaluated if agnoprotein expression could interfere with antigen specific CTL activity using as antigen a well-characterized, immunodominant HLA-A*02 restricted 9-mer peptide from the CMV-pp65 antigen, but no impairment in killing activity could be observed when agnoprotein was expressed.

From all our results we can conclude that agnoprotein does not seem involved in antigen processing and presentation pathway as it could have been hypothesized by previous studies results demonstrating its low immunogenicity and its involvement in transport of vescicles to the cell surface. Our results do not confirm the clinical pathology data of BKPyVAN biopsies which suggested that HLA class II might be reduced in interstitial nephritis due to the virus (Nickeleit, Hirsch et al. 2000). Conversely, our results would be in agreement with a recent study, where the expression of the HLA class I antigen presentation machinery components was investigated in BKPyVAN biopsies compared to T cell mediated rejection biopsies, and it resulted that infection with BKPyV did not have any effect, thus excluding BKPyV inhibitory role in this immune mechanism (Buettner, Xu et al. 2012).

As the immune response to viruses is the result of many complex processes, against which viruses have evolved immune escape strategies, we cannot exclude that

agnoprotein could have a function targeting a different mechanism, therefore its function needs to be furtherly investigated also at the light of a recent finding that agnoprotein could have an inhibitory effect on cell proliferation, as it has been observed that one of its potential cellular partners is PCNA, and their interaction results in host cell proliferation, suggesting also possible role in switching off viral DNA replication to allow the assembly of genomes and viral capsid proteins into infectious viral particles (Gerits, Johannessen et al. 2015).

Futhermore, it has to be considered that BKPyV could be implicated in immune evasion mechanisms through the expression of a miRNA located in the EVGR, which was found to target the cell stress induced ligand ULBP3 (Bauman, Nachmani et al. 2011), dowregulating killing of infected cells by NK cells, and inhibiting the expression of the early protein LTag.

LTag, is multifunctional regulatory protein of 695 amino acid lengths located in the EVGR, its expression is critical for viral replication, it may be involved also in cell transformation, and it has been clearly demonstrated that immune responses towards such protein are crucial in the control of viral replication, in fact in KTRs with active BKPyV replication LTag-specific T cell responses are barely detectable, and emerge after viral clearance (Binggeli, Egli et al. 2006, Binggeli, Egli et al. 2007, Ginevri, Azzi et al. 2007, Schachtner, Muller et al. 2011), in particular with an important CTL contribution. However, in most of studies T cell responses were measured by overlapping 15mer peptide pools, raising mixed CD4+ and CD8+ T cell responses, so there is the need to understand the specific role of CD8+ T cells in BKPyV replication control.

Identifying and characterizing T cell responses specific for the early viral region is clinical interest, as in a previous study it has been shown that the decline of cellular immunity early after transplantation could identify patients at increased risk of BKPyV viremia, however the fact that no or low T cell responses could be found in almost half of patients gave to the results a very low positive predictive value for individual patients (Schachtner, Stein et al. 2015), needing a more efficient method for monitoring BKPyV specific T cell responses.

In the second part of the project we could identify and characterize immunodominant 9mer-epitope T cell responses within BKPyV EVGR approaching techniques of

predictive and experimental epitope mapping in healthy individuals, thereafter we could transpose our methods to validate epitopes in a cohort of 19 pediatric KTRs.
By *in silico* analysis of T cell epitopes within EVGR, we could identify 97 immunogenic 9mer sequences inside hotspot clusters and gaps for each of the 14 most frequent HLA-A and –B types present in Europe and USA. Predicted sequences were similarly clustered across different alleles, suggesting that maybe those sequences could correspond to domains in which the virus would be particularly susceptible to immune control and selection pressure. However, low levels of variants in epitopes sequences could be observed also in a recent paper in which T cell epitopes were studied by next generation sequence of the viral genome from viremic patients, such observation may suggest that despite conservation, BKPyV variants may encode peptides that can escape the immune response and emerge in case of selection pressure given by an eventual immunotherapy (Sahoo, Tan et al. 2015).

Our protocol was successful in expanding EVGR specific T cells, the obtained CD8+ T cells showed a specificity towards predicted immunodominant clusters, and demonstrated to be highly proliferating, as shown by CSFE dye dilution, streptamer staining, CD107a staining and functional CTL activity by ^{51}Chromium release assay. This approach seemed therefore to overcome the problem of low frequency of BKPyV CD8+ T cell responses to overlapping 15mer peptides.

From 97 candidate, 39 epitopes could elicit specific IFN-γ secretion in at least 30% of the studied healthy individuals by ELISpot assay, however these responses could not be attributed specifically to certain HLA types, therefore we have performed streptamer staining, with the result of 21 epitopes which could be unambiguously presented by the tested HLA types, some of them resulted restricted for a specific allele, whereas others could be presented by different HLA alleles, often within the same cross reactive epitope group.

Results obtained in healthy individuals could be translated to the clinically relevant situation in pediatric KTRs. Ten of the 9mer epitopes identified in healthy participants were confirmed in KTRs, and 3 more epitopes were found only in KTRs, showing differences that could be due to the low frequency of specific T cells in healthy individuals, compared to the higher frequency in patients which were more persistently exposed to the virus. Discrepancies maybe also due to the fact that KTRs are susceptible to many infections, with consequent presentation to T cells of

peptides derived from different viruses, some presenting cross-reactive epitopes (Welsh, Selin et al, 2004).

Comparing our results to previous studies, we could give a more complete panel of immunodominant epitopes across the most frequent HLA alleles, whereas most of the published sequences were studied in HLA-A*02 individuals (Li, Melenhorst et al. 2006, Provenzano, Bracci et al. 2006, Randhawa, Popescu et al. 2006). With our methods we could anyway confirm most of the published sequences, as HLA-A*****02–restricted epitopes 9m679 and 9m316 or the HLA-B*****07– and HLA-B*****08–restricted 9m127, supporting our methods and the potential immunodominance of the epitopes. In particular, these immunodominant 9mer T cell epitopes could be included in to BKPyV T cell immunity monitoring in kidney transplant patients to identify population at risk and help guiding immunosuppression reduction. They can be also used to stimulate T cells for adoptive T cell therapy, as with streptamer technology epitopes specific T cells can be sorted and used in clinical settings (Freimuller, Stemberger et al. 2015), or eventually included in a peptide based vaccine, to prevent virus related diseases. The evaluation of the impact of recipient HLA types and mismatches between the donor and the recipient in kidney transplant context did not allow us to draw any clear conclusion, although a significant lower presence of viremia in HLA-A*01 patients could be observed, the sample size for such analysis may be not large enough.

The study has some limitations, that can underestimate the range of detection of immunodominants epitopes, as the use of cryopreserved rather than fresh samples for KTRs, moreover we could observe that not all responses identified by ELISpot could be linked to at least one particular HLA type by streptamer staining, maybe due to low T cell frequencies, or perhaps such responses should be attributed to HLA types not included in the streptamer screening. Nevertheless, our results are encouraging to take this approach to larger clinical cohorts and relevant prospective study settings.

In conclusion, our results gave new insight on different interesting aspects of BKPyV specific immunity. On one side, we investigated on BKPyV agnoprotein, whose function has been unresolved despite many studies, and we could exclude its involvement in antigen presentation inhibition, despite many observations were concordantly going to this direction. In the second study, we could identify and characterize a large panel of immunodominant epitopes, useful for monitoring BKPyV

specific immune responses in KTRs and possibly used in clinical protocols, or even in vaccine design, all aiming at preventing viral related diseases.

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8 GLOSSARY

Adjuvant: agent which improves the immunostimulation by enhancing antigen presentation and/or by providing co-stimulation signals.

Antibodies: proteins of the immunoglobulin family, either present on the surface of B cells, or secreted in response to antigen stimulation, and binding specifically to epitopes.

Antibody affinity: tendency of an antibody to bind to a specific epitope at the surface of an antigen, reflecting the strength of the interaction.

Antibody avidity: sum of the epitope specific affinities for a given antigen.

Affinity maturation: somatic hypermutation and affinity-based selection of antigen-specific B cells leading antibodies production with increased affinity.

Antigen: any molecule that can be recognized by the immune system.

Antigen presenting cell: cell that intakes antigens by endo- or phagocytosis mechanisms, then processes them into small peptides, and presents them at their surface through MHC molecules to activate T cells.

Autophagy: self-degradative process that is important for balancing sources of energy at critical times in response to nutrient stress. Autophagy has an important role in removing misfolded or aggregated proteins, clearing damaged organelles, such as mitochondria, endoplasmic reticulum and peroxisomes, as well as eliminating intracellular pathogens.

B cell receptor (BCR): Transmembrane receptor of B cells recognizing an epitope;

Capsid: protein shell which surrounds and protects the viral genome. It consists of multiple protein sub-units called capsomers.

Caspases: group of enzymes involved in the apoptotic process. All caspases are produced in cells as catalytically inactive zymogenes and must undergo proteolytic activation during apoptosis. Activation of effector caspases is carried out by an initiator caspases**.** Once activated, the effector caspases are responsible for the proteolytic cleavage of cellular targets, which ultimately leads to cell death.

Chemokine: group of small cytokines involved in immune and inflammatory processes. Chemokines are involved in the development of dendritic, B and T cells, and lymphoid cell trafficking.

Cluster of differentiation (CD): identifies cell surface molecules for immunophenotyping of cells.

Complement system cascade: enzymes cascade mediating the immune response against infection, constituted of several effector and regulatory components. Complement activation has three main pathways: the classical, lectin, and alternative pathways. Activation of the classical pathway occurs when the fraction C1 binds to antibody or directly to activating surfaces. The lectin pathway is triggered by recognition of carbohydrate residues found mainly on bacteria by mannose binding lectin, whereas the alternative pathway starts when C3 binds to a suitable activating surface. The three pathways converge into a final common pathway and lead to the formation of a membrane attack complex (MAC), which forms pores on the surface of the targeted cell with consequent lysis.

Costimulatory molecules: molecules expressed at the surface of antigen presenting cells upon activation and deliver stimulatory signals to T and B cells.

Cytokines: a superfamily of soluble protein mediators and communicators released from cells by specific stimuli. They modulate the differentiation and division of hematopoietic cells and the activation of lymphocytes and phagocytes. They can be mediators of inflammation. In general they exert more than one function.

Cyclosporine: 11 aa cyclic peptide which, bindinding to cyclophilin, inhibits calcineurin phosphatase and T cell activation.

Dendritic Cells (DCs): specialized antigen-processing and presenting cells, endowed with a high phagocytic capability when immature, and acquiring high

cytokine producing capacity as mature cells. DCs regulate T cell responses both in homeostatic and inflammatory conditions. A subset of DCs is constituted by **Plasmacytoid DCs**. They are present in the bone marrow and all peripheral organs, and respond to viral infection with a massive production of type I interferons, however, they also can act as antigen presenting cells and control T cell responses.

Envelope: lipoprotein membrane which can surround viruses, derived from the plasma membrane of the host cell.

Epitope: antigenic determinant of a molecule that binds a cognate T or B cell receptor, triggering the activation of adaptive immune response.

Germinal center: dynamic structure that develops in spleen/nodes in response to an antigenic stimulation. It includes a monoclonal population of antigen-specific B cells that proliferate and differentiate thanks to the interaction with follicular dendritic cells and Th cells. Immunoglobulin class switch recombination, affinity maturation, B cell selection and differentiation into plasma cells or memory B cells essentially occur in germinal centers.

Granzymes: serine proteases found in granules of CTLs, which may enter target cells via perforin pores to activate enzyme involved in DNA degradation and apoptosis.

Immunodominance: highly immunogenicity of an epitope, resulting in a predominant immune response towards it. An epitope is likely to be immunodominat when binds the presenting MHC molecule with high affinity, if the MHC-peptide complex is abundantly expressed on the antigen presenting cell, and if there is a high frequency of specific precursor effector cells. Immune response against different epitopes can be in competion with each other, during persistent infections the immunodominant epitope induces the most significant response, excluding the others.

Immunological memory: the ability to remember an encountered antigen leading to a quicker, and more potent immune response on the subsequent encounters with that antigen.

Infection: the process in which an infectious agent enters the host. Most infections are followed by replication of the microbe in the host.

Infectious disease: clinical manifestation of damage caused by a host-microbe interaction.

Inflammasomes: molecular complexes activated upon cellular infection or stress leading to the maturation of proinflammatory cytokines such as interleukin-1β to start the innate immune defenses.

Isotype switching:
Switch of immunoglobulin expression and production from IgM to IgG, IgA or IgE, occurring during B cell differentiation through DNA recombination

Macrophages: phagocytic cells resident in lymphoid and non-lymphoid tissues. Macrophages display a broad range of pathogen recognition receptors making them efficient at phagocytosis and production of inflammatory cytokines.

Major Histocompatibility complex (MHC): large group of genes dresponsible for antigen presentation to T cells. In humans is also known as Human Leukocyte Antigen (HLA).

MHC restriction: Recognition of antigen by particular MHC molecules.

MicroRNA: piece of about 21-23 bases length of single-stranded RNA binding to a complementary mRNA, leading to a decrease in the production of the corresponding protein.

Monocytes Monocytes are immune effector cells, displaying chemokine receptors and pathogen recognition receptors that mediate migration from blood to tissues during infection. They produce inflammatory cytokines and take up cells and toxic molecules. They can also differentiate into inflammatory DCs or macrophages during inflammation.

Natural killer (NK) cells: Lymphocytes of the innate immune system displaying different inhibitory and activating cell surface receptors which balance their activation and ability to kill target cells and produce cytokines.

Non-self: any antigen that is not normally present in that individual.

Open reading frame: continuous stretch of codons that potentially code for a protein or peptide.

Opsonization: process in which particles, microorganisms, and immune complexes are coated with molecules which allow them to bind receptors on phagocytes, enhancing their uptake.

Paratope: region of a B or T cell receptor that recognizes and binds a cognate epitope

Pathogen: organism able to cause disease in a susceptible host

Phagosome: membrane bound intrecellular vescicle which contain phagocytosed material.

Peptide: Short chain of linked amino acids.

Perforin: Pore-forming molecule related to terminal complement fraction C9, which polyimerize on the target cells to form channels.

Persistent infection: infection in which the host response cannot completely clear the microbe. During persistent infection the microbe can be produced in the cell with no or minimal cytopathic effect thereby enabling long-term infection. Persistent infections may have silent stages, but can also evolve into overt disease, depending on the balance between host and microbe.

Polymorphonuclear leukocytes (PMNs): also known as granulocytes, as they are leukocytes characterized by the presence of cytoplasmic granules. They include neutrophils, basophils, and eosinophils.
Neutrophils are professional phagocytes. They ingest microorganisms, then release proteins (e.g. defensis, proteolytic enzymes) contained in their granules to eliminate them.
Basophils are characterized by granules containing histamine, heparin, peroxidase, important in the inflammatory response.

Eosinophils have granules rich in cationic proteins, they produce toxic oxygen radicals. They are antigen presenting cells, and express both hematopoietic and inflammatory cytokines.

Proteasome : multisubunit enzyme complex essential in degradation of proteins and generation of peptides. It is also important in the regulation of proteins that control cell-cycle progression and apoptosis.

Reactivation: Active viral replication after a period of latency.

Recombinant: Produced by genetic engineering.

Repertoire: the sum of antigen receptors produced by the immune system of an indivividual .

Self (antigen): any antigen normally present in that individual.

Sirolimus: triene macrolide binding to FKBP12, inhibiting mammalian target of rapamycin and IL-2 driven T cell proliferation.

Somatic hypermutation: process that introduces random mutation in the variable region of the B cell receptor locus during B cell proliferation at an extremely high rate.

Somatic recombination: rearrangement of **variable** (V), **diversity** (D) and **joining** (J) gene segments in T and B cell receptors, generating the repertoire of specificities of the adaptive immune response.

Tacrolimus: macrolide antibiotic binding to FKBP12, inhibiting calcineurin phosphatase and T cell activation.

T cell receptor: Transmembrance protein expressed on mature T cell, which specifically recognize MHC-peptide complexes. The receptor consists of a heterodimer responsible for antigen-MHC binding and a cluster of associated membrane-bound polypeptides, the CD3 complex.

Tolerance: acquisition of non-responsiveness to a molecule recognized by the immune system.

Viral replication: process resulting in the production of new viruses. It involves multiple steps, starting from virus attachment to the host cell surface by binding specific receptors, then penetration into the cell, uncoating of the viral genome to permit transcription, then virion assembly consisting in bringing together the new nucleic acid and the structural proteins, and finally the release out of the infected cell.

Viremia: presence of viral genome in the blood.

Viruria: presence of viral genome in the urines.

Virus: the smallest of all self-replicating organisms present in nature, being constituted in the most basic cases by a little as a small segment of nucleic acid encapsidated in a protein shell. Viruses do not have their own metabolism, rather they need to parasitize cells and subvert their intracellular machinery in order to replicate and possibly transmit to new potential hosts.

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10 CURRICULUM VITAE

Europass Curriculum Vitae

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Self-assessment	Understanding		Speaking		Writing
European level (*)	Listening	Reading	Spoken interaction	Spoken production	
ENGLISH	Independent user	Proficient user	Independent user	Independent user	Independent user

FRENCH		Independent user		Independent user		Independent user		Independent user		Independent user
GERMAN		Basic user		Basic user		Basic user		Basic user		Basic user

(*) Common European Framework of Reference for Languages

Technical skills and competences

Laboratory techniques:
Cellular immunology techniques:

- GMP cell production per in vivo use
- Cell culture
- cytofluorimetric analysis
- functional cellular assays: cytotoxicity assays, Elispot assays, proliferation assays

Molecular biology techniques:

- RNA extraction
- PCR and RT-PCR
- transfection with plasmid vectors

Computer skills and competences

Good knowledge of Windows / Word, Excel and PowerPoint programs.
GraphPad prism for statistical analysis

Annexes

List of publications:

1. Cioni M, Leboeuf C, Comoli P, Ginevri F, Hirsch HH. Characterization of Immunodominant BK Polyomavirus 9mer-Epitope T-cell Responses. Am J Transplant. 2015 Dec 12. doi: 10.1111/ajt.13598.

2. S Basso, M Algeri, A Gurrado, M Cioni, I Guido, G Quartuccio, LStrocchio, A Tolva, M Zavattoni, F Baldanti, H Hirsch, M Zecca and P Comol. Role of Donor Virus-specific Immunity in the Onset of BKV-related Hemorrhagic Cystitis after Hemopoietic Stem Cell Transplantation. J Stem Cell Res Ther 2013, S6-007.

3. M Cioni, C Mittelholzer, M Wernli, H Hirsch.Comparing effects of BK virus agnoprotein and herpes simplex-1 ICP47 on MHC-I and MHC-II expression. Clinical and developmental Immunology 2013 ID 626823.

4. A Balduzzi, G Lucchini, H Hirsch, S Basso, M Cioni, A Rovelli, A Zincone, M Grimaldi, P Corti, S Bonanomi, A Biondi, F Locatelli, E Biagi, P Comoli. Polyomavirus JC- targeted T-cell therapy for progressive multiple leukencephalopathy in a hematopoietic cell transplantation recipient. Bone Marrow Transplant. 2011 Jul;46(7):987-92

5. Ginevri F, Azzi A, Hirsch HH, Basso S, Fontana I, Cioni M, Bodaghi S, Salotti V, Rinieri A, Botti G, Perfumo F, Locatelli F, Comoli P.Prospective monitoring of polyomavirus BK replication and impact of pre-emptive intervention in pediatric kidney recipients. Am J Transplant. 2007 Dec;7(12):2727-35.

6. Brignole C, Marimpietri D, Pastorino F, Nico B, Di Paolo D, Cioni M, Piccardi F, Cilli M, Pezzolo A, Corrias MV, Pistoia V, Ribatti D, Pagnan G, Ponzoni M. Effect of bortezomib on human neuroblastoma cell growth, apoptosis, and angiogenesis. J Natl Cancer Inst. 2006 Aug 16;98(16):1142-57

Abstracts:

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1. M Cioni, P Comoli, A Parodi, S Basso, A Gurrado, A Trivelli, A Magnasco, HH Hirsch, F Ginevri. Impact of polyomavirus BK viremia on long-term allograft outcome in pediatric renal transplant recipients. J of Neurovirology 19 (3), 294-295. 5° international Conference on Polyomaviruses and Human Diseases. Stresa, May 9-11 2013.

2. M Cioni, C Mittelholzer, M Wernli, H Hirsch.Comparing effects of BK virus agnoprotein and herpes simplex-1 ICP47 on MHC-I and MHC-II expression. J of Neurovirology 19 (3) 5° international Conference on Polyomaviruses and Human DiseasesStresa, May 9-11 2013.

3. Cioni M, Mittelholzer C, Egli A, Hirsch HH. Characterization of BK Polyomavirus (BKV)-specific T-cell responses in Healthy Blood Donors (HBD) .Swiss Workshop in Fundamental Virology, Thun (CH), August 2011.

4. A Balduzzi, G Lucchini, H Hirsch, S Basso, M Cioni, A Rovelli, A Zincone, M M Grimaldi, P Corti, S Bonanomi, A Biondi, F Locatelli, E Biagi, P Comoli CTL-JCV specific for the treatment of progressive multiple leukoencephalopathy in a recipient of hematopoietic stem cells. HAEMATOLOGICA-THE HEMATOLOGY JOURNAL 2010: g95 (7), S31-S32.

5. Comoli P, Cioni M, Gurrado A, Parodi A, Hirsch H, Basso S ,Fontana F, Barbano G, Locatelli F, Ginevri F. Role of Circulating BKV-Specific Cytotoxic T Cells in Kidney Recipients with BKV Infection. Am J Transplant 2009; 9 (S2). American Transplant Congress, May 2009.

6. M Cioni, S Basso, A Gurrado, R Maccario, S Telli, D Pagliara, V Spartà, F Locatelli, P Comoli. Generazione di linee T cellulari BKV-specifiche per il trattamento della cistite emorragica in riceventi di trapianto allogenico di cellule staminali emopoietiche. XVI° Winter-InterLab Meeting in Pediatric Hematology-Oncology AIEOP Falcade, March 2009.

7. S Basso, M Cioni, A Gurrado, R Maccario, S Telli, D Pagliara, V Spartà, F Locatelli, P Comoli.“Generation of Donor polyomavirus BK-specific T-cell lines for BK-related hemorrhagic cystitis after allogeneic HSCT” Bone Marrow Transplant 2009; 43 (s1). European Bone Marrow Transplantation Group Meeting, Göteborg March 2009.

8. Basso S, Pedrazzoli P, Gurrado A, Cioni M, Secondino S, Schiavo R, Zecca M, Georgiani G, Maccario R, Siena S, Locatelli F, Comoli P. “In vitro generation of autologous EBV LMP2-specific citotoxic T lymphocytes for the treatment of nasopharingeal carcinoma (NPC)” Bone Marrow Transplant 2009; 43 (s1). European Bone Marrow Transplantation Group Meeting, Göteborg March 2009.

9. P Comoli, S Basso, H H. Hirsch, A Azzi, I Fontana, M Cioni, G Botti, V Cappelli, F Locatelli, F Ginevri.“Humoral and Cellular Immunity to Polyomavirus BK Large T and VP1 Antigens after Pediatric Kidney Transplantation”. Am J Transplant 2008; 8 (S2). American Transplant Congress, Toronto, June 2008.