Immune responses to BK polyomavirus in healthy donors and renal transplant recipients

By
Nafraa Fallatah
PhD student /virology
ID 201237992

Supervisors
Prof William Paxton
Dr Steve Christmas
Dr Ian Hart
Dr Georgios Pollakis

Department of Clinical Infection, Microbiology & Immunology, Institute of Infection and Global Health, The Ronald Ross Building
The University of Liverpool
2020

Declaration

I, Nafraa Fallatah, declare that this thesis represents my own work which has been done after registration for the PhD degree at University of Liverpool, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications, except where otherwise indicated.

Signature: Nafraa F Date:

Abstract

Background: BKV is a human polyomavirus, a member of the Polyomaviridae family that includes JC virus and SV40. Within the general population the virus infects the host with no clear symptoms displayed by infected individuals. The virus possesses a high seroprevalence reaching 80% within the general population and with more than 19% of healthy subjects shedding BK virus in their urine. After primary infection, which is mainly sub-clinical, the virus persists in the urinary tissue and establishes latency. In immunocompetent individuals the virus persists in the urinary and renal epithelium in its established latent form. However, in individuals with impaired immune surveillance such as in patients receiving immune-suppressive therapies, as prescribed following organ transplant, the virus can reactivate. In kidney transplant recipients (KTR), 30-40% of patients develop BKV viremia while 1-10% progress to BK virus nephropathy. This project investigated host cellular and humoral immune responses to BK virus and to monitor viral genotypes in infected individuals and correlate these variant parameters with disease severity and progression.

Methods: Urine, plasma, and PBMCs were collected from post kidney transplant recipients and healthy volunteers. Real-time PCR was used to identify subjects secreting BKV in their urine and sandwich ELISAs used to measure BK-specific immunoglobulin G antibody and soluble HLA-G levels in serum samples from healthy and post kidney transplant recipients. To detect cellular immune responses mounted against BKV, INF-γ secreting cells were quantified and HLA-G expression was measured using ELISPOT and surface staining assays, respectively. A nested PCR assay was developed and used to identify BKV genotypes among patients.

Results: BKV specific cellular immune responses were compared between healthy subjects and those where BKV was identified in urine. Immunocompetent subjects with detected virus showed higher cellular responses than negative subjects, while in immunocompromised KTR individuals receiving immunosuppressive therapy it was identified that the presence of virus, as detected by viral DNA, was associated with the induction of anti BKV IgG responses but did not associate with the induction of BKV specific cellular immune responses. However, when immune-suppressive therapies were reduced cellular immune responses were found to be restored.

Investigation of HLA-G cell surface protein expression on PBMCs of patients with BKV infection suggested that monocytes expressed more HLA-G than other leucocyte subsets. Additionally, patients with active BKV infection showed dramatic increases in soluble HLA-G expression levels in plasma in comparison to the control group, while no difference was observed in soluble HLA-G levels between the KTR groups tested.

Pre-transplant anti BKV IgG levels were similar in patients with post-transplant BK viremia and those who never demonstrated BKV viremia. The level was stable in BKV negative patients during follow up, whilst in viremic patients a significant increase in BKV IgG levels associated with development of viremia in parallel with reduction in immunosuppressive therapy. Further, we observed that the presence of HLA-B44 and HLA-DR15 was significantly associated with increased risk of viremia among the recipients.

Finally, by studying the influence of BKV genotypes on development of viremia, genotype Ib-2 was found to be the most prevalent genotype among our patients, with no observed significant correlation between genotype and incidence of BKV viremia.

In conclusion: BKV cellular immune responses, without a detectable level of anti-BKV antibodies, could be associated with protection against BKV reactivation. Moreover, HLA-G expression on some immune cellular subsets increased after primary virus infection and may play a role in virus immune evasion during infection. Pre-transplant anti BKV IgG responses could not predict the risk of post-transplant viremia. Kidney transplant recipient individuals with HLA-B44 and HLA-DR15 alleles were shown to be more susceptible to developing BKV viremia. The majority of BKV patients were shown to be infected with the Ib-2 genotype and no differences were found between virus genotype and virulence.

Acknowledgements

I wish to express my gratitude to the Saudi cultural bureau which funded my study and who helped in starting this journey.

Also, all thanks to my supervisors, Dr Stephen Christmas, Prof. William Paxton and Dr Georgios Pollakis who convincingly guided and encouraged me to finish this project even when the road got tough after my main supervisor's retirement. Also, special thanks to the transplant unit staff at Royal Liverpool Hospital, especially Edmund Gregg and Sally Heyworth and Dr Ian Hart from the Clinical Virology lab, without forgetting Dr Laura Bonnett who helped in the statistics. Without this persistent help the goal of this project would not have been achieved.

Also, big thanks and appreciation to all the healthy donor participants from the Ronald Ross building and the transplant patients from the Royal Liverpool University Hospital who agreed to take part in this study.

Finally, I wish to acknowledge the support and great love of my family, my husband, Anwar; my kids Aldanah and Abdulaziz, and all my other friends. They kept me going and this work would not have been possible without their input.

Table of Contents

List of Figures

List of Tables

List of abbreviations

Ab	Antibody
APC	Antigen presenting cell
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bp	Base pair
BKV	BK polyomavirus
BKPyV	BK polyomavirus
BKyAN	BK Polyomavirus-associated nephropathy
CD	Cluster of differentiation
CR	cytoplasmic reticulum
CMV	Cytomegalovirus
CNI	Calcineurin inhibitors
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DC	Dendritic cells
EDTA	Ethylenediamine tetra acetic acid
ER	endoplasmic reticulum
ELISA	Enzyme linked immunosorbent assay
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
HLA-G	Human Leucocyte Antigen-G
HLA-F	Human Leucocyte Antigen-F
HLA-E	Human Leucocyte Antigen-E
HIV	Human immunodeficiency virus
HVRR	Hypervariable regulatory region
INF-γ	Interferon Gamma
IgM	Immunoglobulin M

IgG	Immunoglobulin G
IVIG	Intravenous immunoglobulin
KTR	kidney transplant recipient
mAb	monoclonal Antibody
mRNA	messenger ribonucleic acid
MHC	Major Histocompatibility Complex
ml	Millilitre
MLPC	Mixed Lymphocyte Peptide Culture
mM	Millimolar
mTOR	Mammalian target of rapamycin
µg	Microgram
µL	Microlitre
ng	Nanogram
NK	Natural Killer Cells
NCCR	non-coding control region
LTag	large T-antigen
LVGR	late viral gene region
O.D.	Optical Density
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PyVAN	Polyomavirus associated nephropathy
qPCR	Quantitative polymerase chain reaction
RT	room temperature
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
SNPs	Single Nucleotide Polymorphisms
SOT	solid organ transplantation
sTag	small T antigen
TBE buffer	Tris/Borate/EDTA

VP1	major capsid protein 1
VP2	minor capsid protein 2
VP3	minor capsid protein 3

CHAPTER ONE

1 Introduction and background

Introduction

BKV is a member of the polyomavirus family similar to other polyomaviruses such as JC virus and SV40 virus, which are closely related to BKV (Figure 1.1). The virus is mainly acquired in early childhood with no symptoms or flu like symptoms shown (G. R. Ambalathingal, R. S. Francis, M. J. Smyth, C. Smith, & R. Khanna, 2017; W. A. Knowles, 2006); in the presence of proper immune surveillance, it then establishes latency with transient low levels of urinary shedding.

In immunocompromised patients, such as renal transplant recipients, BKV reactivates due to the introduction of immune suppressive drugs that weaken host immunity. In fact, the lack of proper immunity leads to the host's inability to control BKV replication, induction of BKV viremia often leading to polyomavirus-associated nephropathy (BKyAN) (H. H. Hirsch et al., 2005; Reploeg, Storch, & Clifford, 2001).

Yet, forty years after discovery of the virus, no effective antivirus treatment is approved for BKV associated disease (Bridges, Donegan, & Badros, 2006; Ganguly et al., 2010; Snydman et al., 2002). The only management strategy in most kidney transplant centres is reduction of immunosuppressive treatments in order to retain some of the host immunity while taking care to avoid graft rejection. This raises the need of better understanding of BKV pathogenesis and the host immunity mechanism controlling virus replication and development of effective treatments that prevent BKV disease and loss of graft following kidney transplant.

BKV reactivation in renal recipient is still puzzling transplant physicians. since 1971, several studies have tried to demonstrate recipient BKV reactivation risk factors to identify those that could be eliminated or modified pre-transplant. Unfortunately, there is conflict and inconsistency within the literature.

Here, we studied immune responses induced against BKV in immunocompetent subjects and renal transplant recipients. In addition, we considered the pre-transplant risk factors that may influence or eliminate viral replication after transplantation, aiming to identify risk factors, monitoring and modification strategies that may influence in BKV disease progression.

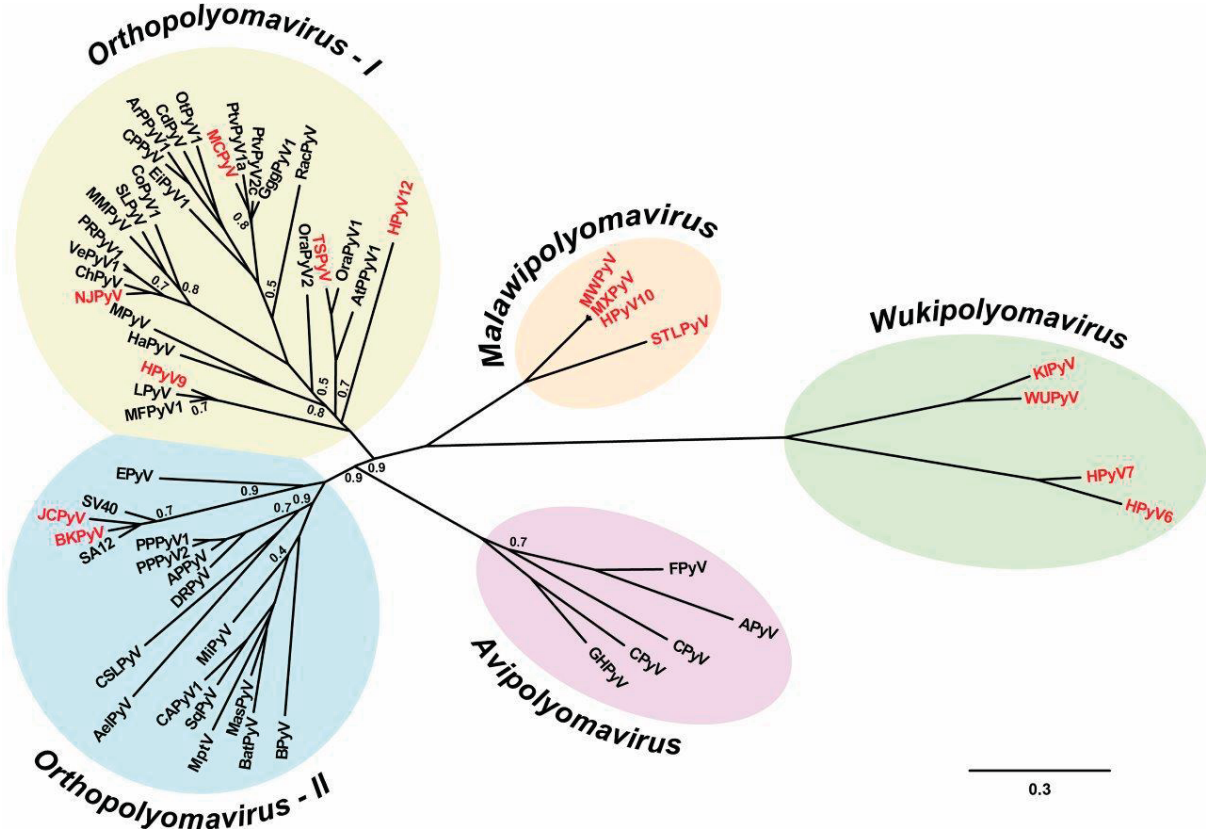


Figure 1.1. Phylogenetic tree of the polyomavirus family.

- *Phylogenetic tree Contains all polyomavirus species know11502007541150200754n until June 2014, including Human polyomaviruses highlighted in red (From(Herman F Wunderink et al., 2019)) .*

1.1 The virus

1.1.1 BKV history

The polyomavirus family was accidentally discovered in 1953 by Ludwig Gross while studying murine leukaemia virus (MLV) and named as polyomaviruses due to their ability to cause multiple(poly)tumours (oma) in mice (Gross, 1953; Krumbholz, Bininda-Emonds, Wutzler, & Zell, 2009). Before 1999, polyomaviruses were classified under *Papovaviridae* that includes human papillomaviruses until they were reclassified into a separate family *polyomaviridae* (Feltkamp, Kazem, van der Meijden, Lauber, & Gorbalenya, 2013). The family of *Polyomaviridae* includes more than 30 species of viruses; only thirteen of them infect humans, including: BK, JC, KI, WU, Merkel cell polyomavirus, H6, H7, H9, H10, H12, STL,

trichodysplasia spinulosa-associated polyomavirus, and NJ (Lamarche et al., 2016). Twelve of them were isolated in the period between 2007-2017 (Gheit et al., 2017; Korup et al., 2013; Lim et al., 2013; Mishra et al., 2014).

BK polyomavirus was discovered in 1971 in London from a urine sample of a transplant recipient who received a kidney from his brother. Initially, the agent was mistaken for a wart virus, however after inoculating the agent in cell culture and evaluating its cytopathic effects (CPE) the isolate was identified as BKV, after the initials of the patient (S. D. Gardner, Field, Coleman, & Hulme, 1971).

1.1.2 Virion and Genome

Polyomaviruses are small non-enveloped viruses with a major capsid protein called VP1; the virion consists of 72 pentameric VP1 capsomers arranged in T=7 that form an icosahedral structure. The VP1 protein is essential for virus entry by attaching to the host receptors (Hurdiss D et al., 2016; Liddington et al., 1991). Deep inside the capsid, there are additionally two minor proteins VP2 and VP3 which are believed to result in genome packaging into the virion, virus uncoating and delivery to the host nucleus (Shishido-Hara, Ichinose, Higuchi, Hara, & Yasui, 2004) (Figure 1.2).

The capsid proteins are encoded by a circular dsDNA genome of 5100-5300bp that can resist heat up to 50 °C for 30min with major effects on its infectivity (Alalawi, Kossi, Jenkins, & Halawa, 2020) and divided into three functional regions; first, the non-coding control region (NCCR), hypervariable region that includes different binding sites that aid in the initiation and regulation of the other regions' transcription. It also includes a hypervariable regulatory region (HVRR) that is divided into five consecutive blocks; O-P-Q-R-S O142, P68, Q39, R63, and S63, where the numbers indicate the sequence length in bp (Olsen et al, 2009; Figure 1.3).

Second, the early coding region is approximately 2400 bp in length and encodes for a large tumour antigen (TAg) as well as a small tumour antigen (tAg) that accumulate in the nucleus to help in virus replication. During virus replication, TAg generates a complex that binds to the ori and facilitates transcription of the late coding region, while tAg also aids in viral replication, cell cycle progression and cellular transformation (Baez, Brandão Varella, Villani, & Delbue, 2017; Dugan,

2008; Rinaldo, Tylden, & Sharma, 2013). Additionally, it is worth mentioning that all polyomaviruses do not encode a viral DNA polymerase enzyme, but completely rely on enzymes and transcription factors of the host and expressed in infected cells. Therefore, activated cell signals and growth factors are essential for virus replication (Khalili & Stoner, 2004).

Finally, the late coding region is approximately 2300 bp in size and includes genes encoding the virus's three structural proteins VP1, VP2 and VP3 in addition to the non-structural protein agnoprotein that is only found in BKV and SV40.

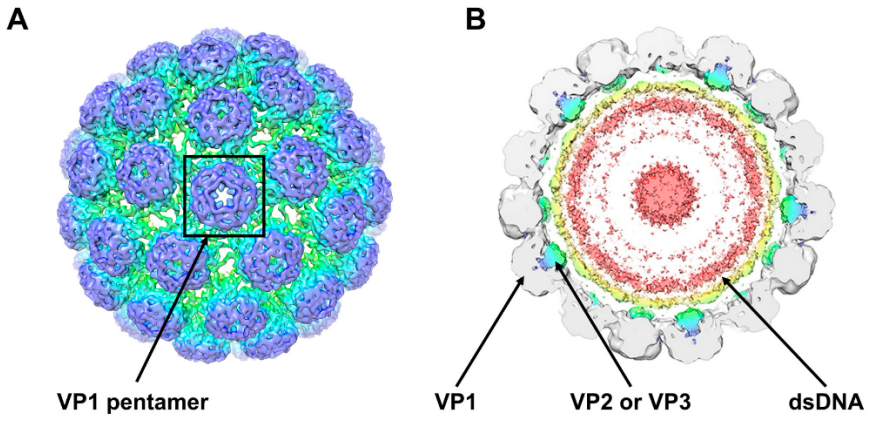


Figure 1.2. Polyomavirus BK structure

VP1 major capsid protein and VP2, VP3 proteins surrounding the dsDNA molecule. Adapted from Helle et al., (2017).

1.1.3 *Genotypes and subtypes*

Based on nucleotide sequence analysis of genomes from all isolates, BKV was classified into four main genotypes (I-IV). Subtype I is considered as the most prevalent genotype with approximately 80% distribution worldwide, followed by subtype IV with 15% distribution and being mainly detected in Asia while the other subtypes are rarely found. However, due to BKV diversity and difficulties in identifying viral strains into the mentioned groupings, additional sub-categorisation was needed within the original four genotypes (Pastrana et al., 2013).

Based on the phylogenetic tree analysis subtype I can be divided into four subgroups: Ia, Ib-1, Ib-2 and Ic. Subtype IV is sub-typed into six different subgroups: IVa1, IVa2, IVb1, IVb2, IVc1 and IVc2. (Figure 1.4). BKV diversity

may have an impact on development of BKVN in kidney transplant recipients. In Japan, a study in 2004 found that most BKV genotypes isolated from renal transplant recipients were subgroup I, while samples from bone marrow transplant recipients were identified as belonging to genotype Ic (Takasaka et al., 2004). Moreover, it has been noticed that some BKV genotypes show different virus properties in vitro; some variants exhibit a greater cytopathic effect than others (Tremolada et al., 2010), but it is still unclear whether some genotypes could possess biological properties that may influence progression of BKV nephropathy in post kidney transplant recipients. A study by Schwarz et al, found that BKV subtype IV may influence progression of BKVN (Schwarz et al., 2016). Furthermore, in Brazil, Rafael and colleges proposed that the BKV 1a genotype may have a potential risk associated with BKVN progression (R. B. Varella, Zalona, Diaz, Zalis, & Santoro-Lopes, 2018).

Even though there is not enough supporting evidence, BKV genotype diversity could be linked to its geographical distribution with different cellular tropisms or even pathological impacts and where genotypes could correlate with specific diseases.

A previous study demonstrated a link between human populations and BKV subtype I subgroups (Ib-2 and Ic); it suggested that subgroup Ib-2 is widespread among Europeans, whereas Ic is more restricted to north-east Asians, with the findings indicating a possible geographical separation of European and Asian BKV strains (Ikegaya et al., 2006). Moreover, there is different classification of BK virus variants based on the sequence diversity within the NCCR region, rearranged (rr) and archetype (ww) (Olsen, Hirsch, & Rinaldo, 2009). Ultimately, further work is needed to elucidate the roles of different BKV genotypes and their influence in BKVN progression.

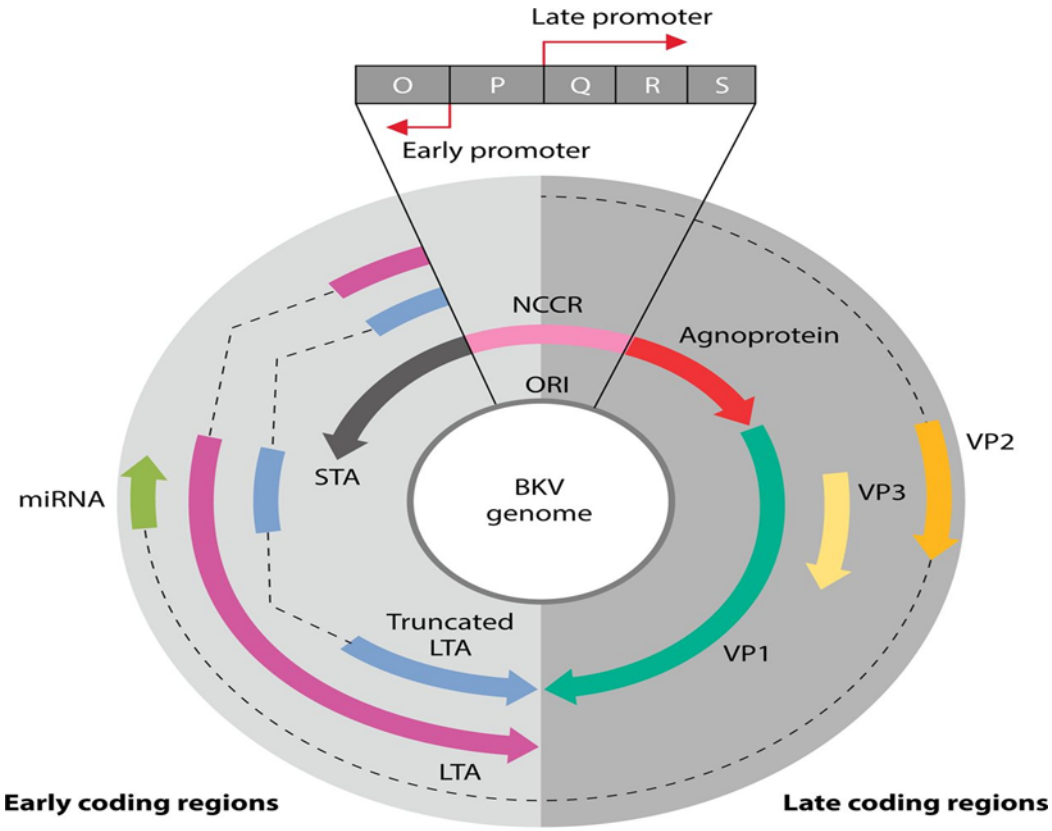


Figure 1.3. BKV genome organization.

The transcription of both early and late coding regions proceeds in a bidirectional way from the origin of replication (ORI) within the noncoding control region (NCCR) The late coding regions encode structural proteins (VP1, VP2, and VP3), in addition to the non-immunogenic agnoprotein, whereas, the early coding regions transcribe the tumorigenic proteins (LTA and STA) (G. Ambalathingal, R. Francis, M. Smyth, C. Smith, & R. Khanna, 2017)

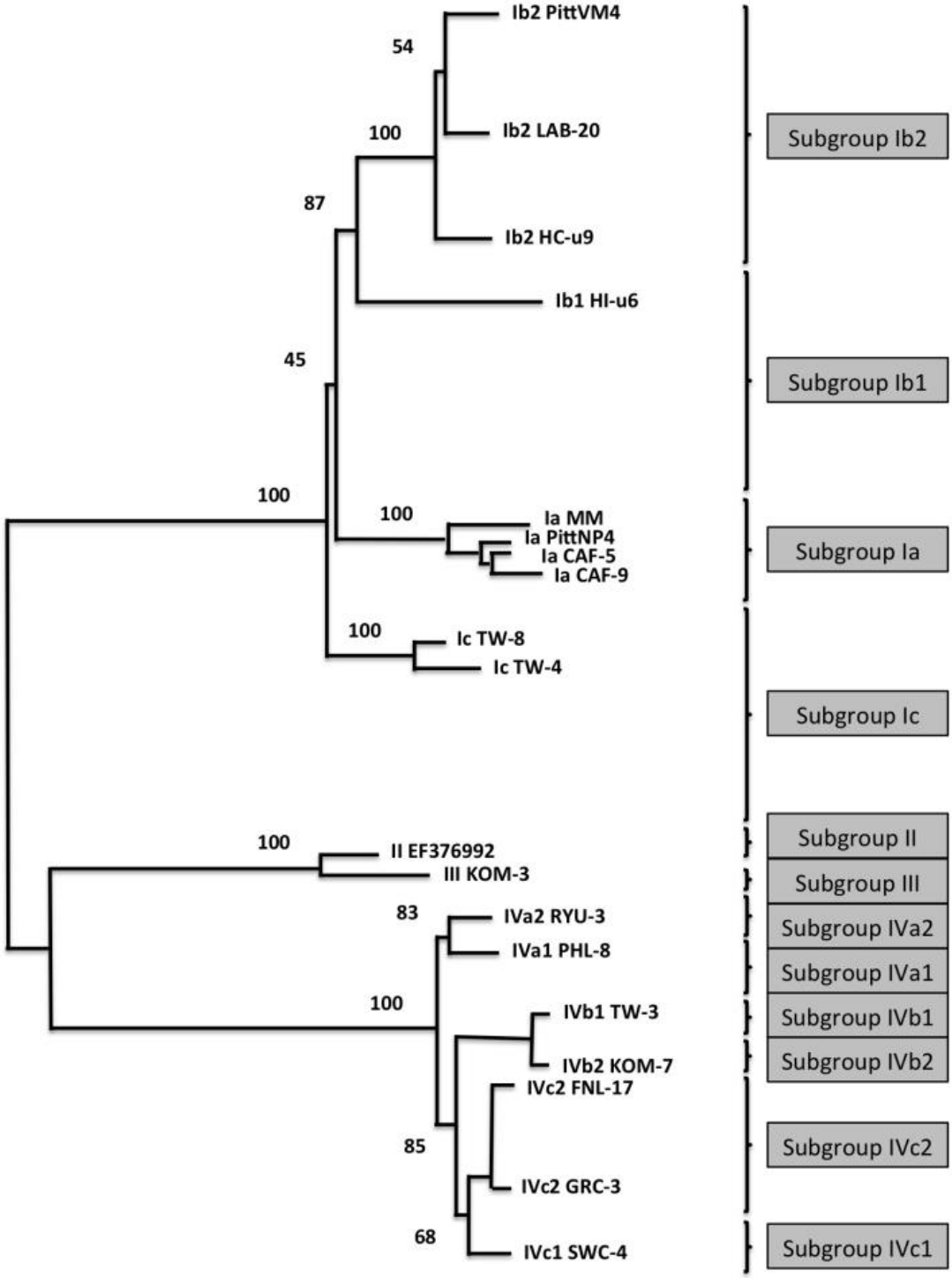
1.1.4 *Life cycle*

Unlike other polyomaviruses that utilise clathrin-mediated viral entry, BKV invades the target cell via caveolae-mediated endocytosis to reach the cytoplasmic reticulum (CR) by using 2 ganglioside receptors: GT1b and GD1b to bind to host cells; these receptors are present on kidney and urinary tract cells making them the main sites of viral infection and replication (Zaman, Ettenger, Cheam, Malekzadeh, & Tsai, 2014) (Figure 1.5). In the CR the virus is partially uncoated by reduction and isomerization of VP1 protein disulphide bonds. Subsequently, the virion translocates to the cytosol using microtubules and transits through the endoplasmic reticulum (ER) for capsid rearrangement.

To pass through the nuclear pore (40-45 nm), the virion must be partially disassembled into approximately 39 nm size particles. After viral DNA entry to the host cell nucleus via the nuclear pore complex (Yogo, Sugimoto, Zhong, & Homma, 2009), it either starts replication or proceeds to latency; in fact this next step is dependent on the host cellular immune status.

Regarding replication, the virus DNA initiates transcription of both the LTag and sTag genes (Kean, Rao, Wang, & Garcea, 2009), followed by Late genes that encode for the structural proteins and transcription of VP1, VP2 and VP3 (H. H. Hirsch & Steiger, 2003). After assembly of the capsid with newly synthesized DNA, the new virion is released extracellularly after cell lysis (Barth et al., 2017).

.



.

Figure 1.4.BKV genotypes and subtypes

Constructed phylogenetic tree illustrates BKV genotypes and subtypes. Adapted from (Kardas, 2015).

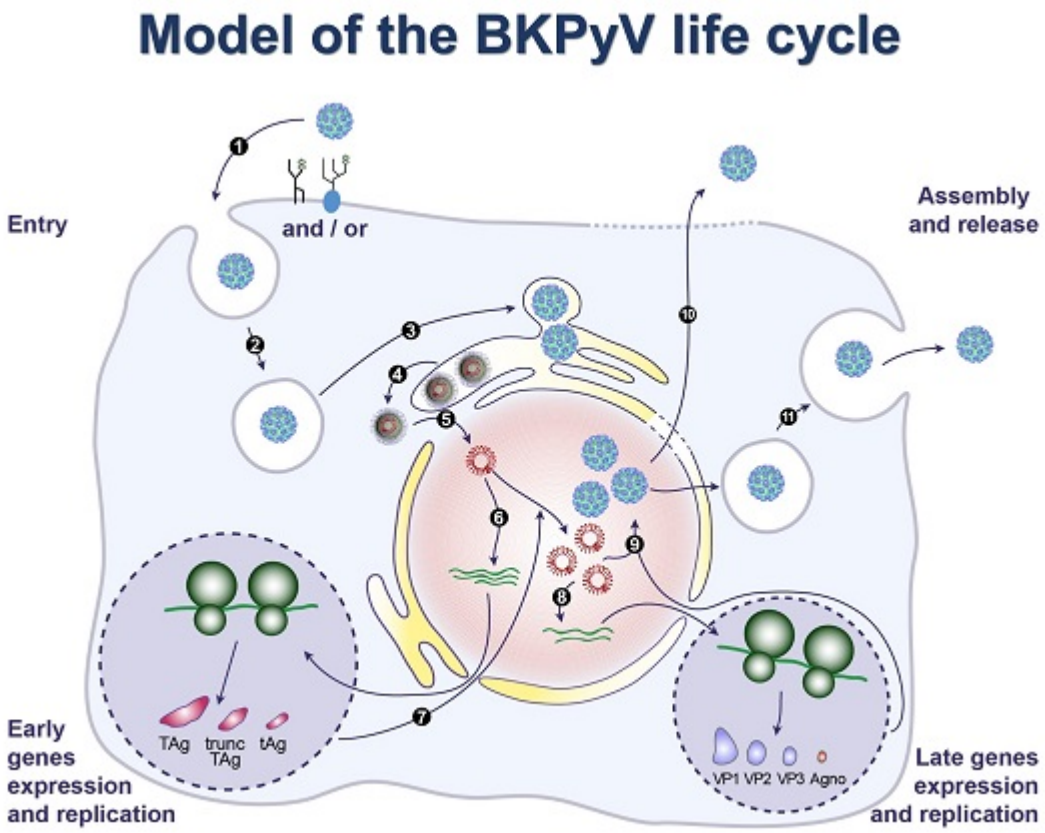


Figure 1.5. BKV replication cycle

Entry, both early and late gene expression and virus particle assembly and release. 1. BKV virion attachment to the ganglioside receptors at the cell surface. 2. Host cell entry through a caveola-mediated endocytosis. 3. 10 h post-infection the virus transfers from the late endosomes to the endoplasmic reticulum (ER). 4. Partial capsid uncoating in ER. 5. Viral genome transport into the nucleus via the nuclear pore complex using VP2/VP3 NLS and the importin α/β1 import pathway. 6. Approximately 24 h post-infection, early gene expression begins. 7. Translocation of the early proteins into the nucleus for DNA replication. 8. Late gene expression. 9. Translocation of late proteins into the nucleus for assembly. 10. Virions release from infected cells after cell lysis. 11. Some virions may be released into the extracellular environment through a non-lytic egress that depends on the cellular secretion pathway. [Adapted from (Helle et al., 2017)

1.2 Epidemiology and transmission

The discovery of BK virus was serendipitous. Unexpectedly, a large number of papovavirus particles were found in a patient's urine sample that had been collected more than three months post-transplant, and many inclusion–bearing cells had been seen, which confirmed the presence of papovavirus. Later, inoculation of the virus into a culture of secondary monkey fibroblasts provided excellent confirmation of a cytopathic effect and resulting in the identification of virus particles on day 19 (S. D. Gardner et al., 1971).

BK virus is an endemic, non-seasonal, ubiquitous virus which is not related to the socio-economic status of the individual. Primary infection results in either a subclinical infection or sometimes associates with mild flu-like symptoms; after primary infection in immunocompetent subjects, the virus persists and establishes latency. It is unknown whether the virus is truly latent or just demonstrates low levels of gene expression.

Based on age at time of antibody seroconversion, the virus is found to be mainly acquired and transmitted between children; a study found that the virus was transmitted more in children who attended nurseries in comparison to those who stayed at home (Mantyjarvi, Meurman, Vihma, & Berglund, 1973). The primary route of BKV transmission is via direct contact (mostly in children) (Goudsmit, Dillen, Van Strien, & Van der Noordaa, 1982). Additionally, other routes were proposed, such as sexually through semen, urine in immunocompromised and pregnant women due to virus replication in the urinary tract and the faecal–oral route (Jiang, Abend, Johnson, & Imperiale, 2009). Congenital transmission is also predicted as BK virus IgM antibodies were found in cord blood samples, brain and kidney samplings of an aborted foetus and urine of a one-month old baby, which suggests reactivation or primary infection of BK virus during pregnancy (Taguchi, Nagaki, Saito, Haruyama, & Iwasaki, 1975). Blood-borne transmission of BKV was also indicated and it is worth mentioning that soiled water is the first suspect in BK virus infection (Jiang et al., 2009; Siguier, Sellier, & Bergmann, 2012).

BKV was found globally, in both developed and developing countries, as confirmed from many epidemiological studies (Wendy A Knowles, 2001; Ocampo, Rosso, Pacheco, & Villegas, 2017), except in some populations where the prevalence is 0% which means complete absence of BK virus in remote areas such as some aboriginal tribes in Brazil, Malaysia and India (W. A. Knowles, 2006).

1.2.1 *Epidemiology in immunocompetent subjects*

Overall BK virus seroprevalence within the human population reaches up to 90%. However, the number of seropositive individuals starts to decrease slightly after the age of 40 years (Kean et al., 2009; W. A. Knowles et al., 2003). In the general population, BK seroprevalence (IgG) approaches 90% in children from 5-9 years

old, whilst in infants less than two years old antibodies are acquired from the mother. Infection gradually drops with age to 70-80% in adults (40-60) years, which may explain the lack of ongoing antigenic stimulation in most of the adults. Asymptomatic urinary shedding of BKV occurs in up to 62% of healthy BKV-seropositive individuals (H. H. Hirsch & Steiger, 2003; Ling et al., 2003; Sundsfjord et al., 1999), yet the reason behind the shedding is not well understood, unless it could be a consequence of periodic viral reactivation.

Anti-virus IgM antibodies were detected in children aged 2-5 years old, but it is rare to detect IgM in adult samples; some studies reported IgM antibodies in 19% of adult sera. A possible explanation of these rare findings may be differences in the sensitivity and specificity of the methods used in antibody detection. However, it is hard to confirm whether the IgM is a result of primary BK infection or re-infection (W. A. Knowles, 2006).

Generally, there is no difference in BKV seroprevalence in relation to gender. The importance of antibody prevalence as a part of BK virus diagnosis lies in its stability. BK antibody levels could remain stable for more than 11 years, in other words, long-lived memory B cells which make it a reliable and reasonable parameter in PyV disease and infection (Antonsson et al., 2010). Understanding the humoral immune response against BKV is an essential tool in choosing the best candidate for transplant donation.

1.2.2 *Epidemiology in immunocompromised patients*

BKV was first isolated from urine of a kidney transplant recipient who was suffering from ureteral fibrosis and obstruction after transplant. Subsequently, four years after discovery of the virus, the virus antigen was detected utilising an indirect immunofluorescence assay with urine of 3 patients who received chemotherapy to treat their malignancies in 1975 (Reese, Reissig, Daniel, & Shah, 1975).

As mentioned previously, asymptomatic BKV shedding in urine of healthy individuals reaches up to 62%. However, in patients with supressed immunity such as post kidney transplant recipients, latent virus reactivation could lead to viremia and significant renal and urinary tract pathology, nephropathy, ureteral stenosis and cystitis.

The prevalence of BK viruria in renal transplant recipients has been found to be between 30–57% (Adrian Egli et al., 2009). Even though the viruria does not necessary lead to the development of BKV disease it causes viremia in 5-30% of renal transplant recipients and the frequency of BKV nephropathy is reported to be 1–10%, mainly in recipients with sustained viremia or high viral loads and whom are diagnosed at an average of 24 weeks post-transplant (H. H. Hirsch & Steiger, 2003).

1.3 Pathophysiology

In healthy individuals, it is believed that BKV infects the respiratory tract in childhood, then goes latent in the urinary tract (uro-epithelial cells; (W. A. Knowles et al., 2003). However, following any immunosuppression or absence of immune surveillance, the virus activates and replicates in the distal tubule epithelial cells, which may lead to inflammation, tissue damage and unfolding of the tubular basement membrane. This tissue necrosis event leads to viral spread in intratubular spaces and peritubular capillaries then to adjacent cells, resulting in active infection and progression of BKN and ureteric stenosis (Fig 1.6). Moreover, Zeng and colleagues proposed that some miRNAs from virus infected cells, such as tubular epithelial cells, may regulate the BKV life cycle and help in host immune evasion (Zeng et al., 2019). Asymptomatic urinary shedding was detected in 7% of immunocompetent subjects, with a median viral load of 4.64 log copies/mL (A. Egli et al., 2009), while it is rare to observe virus DNA in healthy donors' plasma.

After kidney transplantation, due to the induction of immunosuppression therapy and reduction of immunity as a consequence, 30% of recipients were found to shed virus, with 1-10% of those developing BKN and 50-80% of the BKN patients losing their grafted kidney within two years post viral detection (Yi, Knight, & Lunsford, 2017) (Figure 1.6). BKVAN has been increasingly detected for the past 10–15 years in post kidney transplant recipients. Moreover, the virus is known to cause haemorrhagic cystitis, pneumonia, meningoencephalitis and retinitis. Furthermore, it has been reported to cause reno-urinary neoplasms (Alexiev et al., 2013; Papadimitriou et al., 2016).

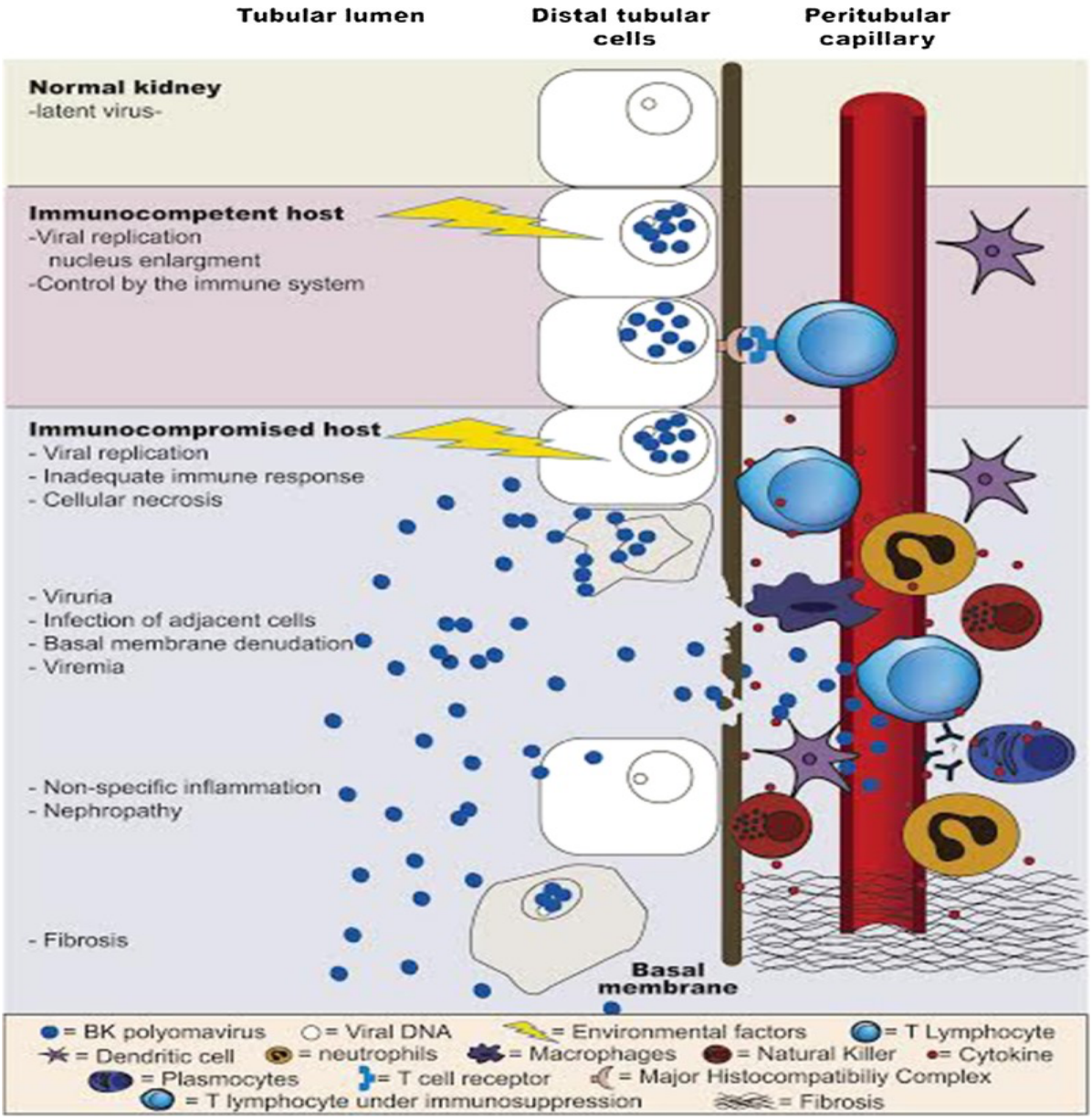


Figure 1.6. BKV virus disease progression

Development of BKV disease from latency in immunocompetent hosts (top) to reactivation and BKPyAN in immunocompromised subjects (bottom) adopted from (Lamarche et al., 2016).

1.4 Immune responses to viral infections

It is well known that most vertebrates have developed mechanisms to induce immune responses targeted against foreign antigen and this immunity is completely dependent on discriminating self from non-self-proteins (Janeway Jr, 1992; Medzhitov & Janeway Jr, 2000). After any invasion by microorganisms such

as viruses, immune recognition goes through two separate strategies: the stimulation of innate as well as adaptive immune responses. The innate immune response is considered as the first line of defence against microorganisms, which produce an immediate non-antigen-specific response but without immunological memory, whereas the adaptive immune response is more specific and delayed. It induces antigenic specific responses against the invading pathogen with development of immunological memory (Janeway, 2001).

1.4.1 Innate immune defences

The body's innate immunity starts before the microorganism invades the internal system. Anatomic barriers like skin, mucosa or surfaces within the body, such as endothelial cells and basement membranes, are the first obstacles that prevent viruses from penetrating the body (Janeway Jr & Medzhitov, 2002). Additionally, in the gastrointestinal tract, some viruses can be digested and inactivated by gastric acid, bile salts and enzymes (Weiss, 1985; Veloso Alves Pereira, 2015). Furthermore, most body fluids and tissues contain an array of viral inhibitors that prevent viral attachment or inhibit virus infection (Pfaender et al., 2013).

In most viral infections, the immunocompetent host takes the opportunity to attack the virus before reaching the cells. However, if the virus manages to defeat the above obstacles and penetrates cells, it will be recognised by the immune system. That consists of a complex network of cells and soluble molecules that are induced in response to non-self-antigen recognition. Various soluble molecules, e.g. pentraxins, lectins, ficolins and also some non-immunological molecules participating in inhibitory functions against microbes like transferrin and lactoferrin (Kariminik, Yaghobi, & Dabiri, 2016). More importantly, type 1 interferons are considered as key molecules in the innate defence response against viruses, due to their strong anti-viral activities by activating gene expression of many antiviral proteins such as; TNF-alpha, Fas/FasL, caspase-4, caspase-8, dsRNA activated protein kinase (PKR), death activating protein kinases (DAP kinase; (Chawla-Sarkar et al., 2003). Also, it activates natural killer (NK) cells and dendritic cells (DCs) that initiate the adaptive immune response and give the infected cell a transitory resistance phenotype (Le Bon & Tough, 2002). Furthermore, innate immunity against viruses involves another component called

intrinsic immunity; it is an interferon (INF)-independent antiviral response that is mediated by expressed cellular proteins (so-called intrinsic host restriction factors), these factors act immediately to control viral gene expression (Boutell & Everett, 2013).

Generally, the main cellular players of innate immunity targeting viral infections are monocytes, DCs, NK (the most important), neutrophils, eosinophils, basophils and mast cells. These cells are responsible for expression of a set of germlines encoded pattern recognition receptors (PRRs); these receptors are able to recognize conserved molecular patterns associated with viruses and trigger an immediate inflammatory response by production of pro-inflammatory cytokines and chemokines (Janeway Jr & Medzhitov, 2002; Kawai & Akira, 2007; Medzhitov, 2007).

DCs are antigen-presenting cells, and the main function of these cells is antigen processing and presentation at the cell surface where they act as a vehicle linking the innate and the adaptive immune responses. In post kidney transplant and immunosuppressed patients, DC levels in the peripheral blood drop significantly with greater reduction in plasmacytoid DCs. This drop may last up to three months after transplantation but due to the immunosuppression the deficiency could last longer in recipients (Hesselink et al., 2005). Therefore, any reduction in DC populations in peripheral blood will lead to reduction in virus antigen presentation and lack of activated specific T-cells being induced against the virus which may result in increased virus replication. As a result, Drake and colleagues, using a mouse model, found that elevation of DC numbers is directly proportional to the enhancement of virus specific CD8+ T cells (Drake et al., 2001). Despite the mechanism of DC activation after BKV infections not being completely understood, previous studies suggested the important role of DCs in induction of the adaptive immune response and generation of T-cells specific to BKV (G. R. Ambalathingal et al., 2017).

NK cells are another crucial component of innate immunity during viral infections. The role of NK cells and their activation mechanism are not yet well understood. NK function is mainly controlled by a balance of activating and inhibitory signals provided by killer-cell immunoglobulin-like receptors (KIRs) through the interaction with specific Human Leucocyte Antigen (HLA)-class I ligands; the

reduction of HLA expression is believed to increase NK cell activation. In addition, in antibody-dependent cellular cytotoxicity (ADCC) NK Receptors FcγRIIIA and/or FcγRIIIC bind to the Fc portions of antibodies bound to target cells, which leads to NK activation (Rouse & Mueller, 2019). Further, some cytokines and other receptors such as the NKG2 family may enhance NK cell activation and signalling.

Furthermore, it has been proposed that some non-classical HLA molecules, such as HLA-G have an inhibitory effect on NK cells at the maternal/fetal interface by protecting the foetus from attack by NK cells of the mother (Carosella, Favier, Rouas-Freiss, Moreau, & LeMaoult, 2008). However, other reports found that the inhibitory effect of HLA-G was due to T-cells and not NK cells (Van der Meer et al., 2007). In some viral infections such as CMV, the virus produces some proteins that target HLA class I molecules and prevent antigen presentation on the infected cells. However, some of these virus proteins may differentiate between classical and non- classical HLA molecules, while others effect both types. Studying the effect of HLA-G on evasion of anti-viral immunity is essential to understanding its effect on viral reactivation.

Trydzenskaya et al. highlighted the role of KIRs in controlling BK infection. Patients with BKVN were found to have a lower number of KIRs compared with controls (Trydzenskaya et al., 2013). The same group also reported a significant drop in KIR3DS1 of NK cells in patients with BKAN and in BK-reactivated patients when comparing to controls group (Trydzenskaya et al., 2013). Interestingly, viruses have developed many strategies to evade recognition by NK cells (Figure 1.7), such as BKV miRNA that mediates downregulation of the NKG2D ligand. Also, a correlation has been observed between KIR genotypes and CMV infection in post kidney transplant recipients. A combination of activating receptor (KIR2DS2) and inhibitory receptor (KIR2DL3) was observed to be associated with protection against CMV viremia (van Duin, 2014). A different study reported a significant reduction of CMV viremia in kidney transplant recipients with KIR B haplotypes containing multiple activating KIRs (Gonzalez, 2014).

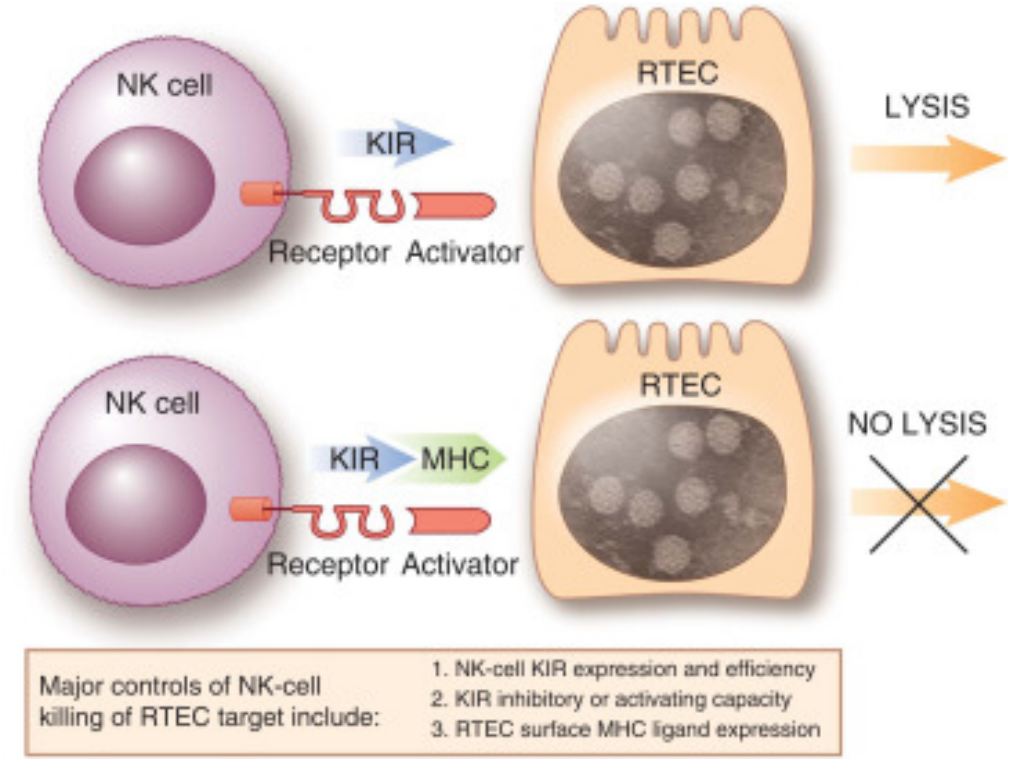


Figure 1.7. BKV mechanism in evading NK cell activity

The virus inhibits the expression of activator molecules on the virus infected cell, also encourages it to produce transforming growth factor–beta (TGF-β), which suppresses NK cell function and promotes virus resistance (Acott, 2013).

1.4.2 *Adaptive responses*

The initiation of adaptive immune responses is triggered by DCs. Briefly, DCs carry receptors on their surface that recognize many pathogens, then engulf pathogens and digest them within the cell. When a DC takes up a pathogen, the cell becomes activated and migrates to the lymph node to present the virus antigen to lymphocytes.

Adaptive immune responses act against both virus particles and virus infected cells via either: humoral immunity (antibodies), which mainly work via binding to epitopes expressed by native proteins at the surface of free virions and block viral attachment or penetration of target cells. Additionally, this can result in viral lysis via complement opsonisation by Fc receptor-bearing cells that mediate antibody-

dependent cellular cytotoxicity (ADCC). Alternatively, they can neutralize virus particles and prevent host cell entry, or by forming antibody–antigen complexes and stimulate macrophages (phagocytosis) or reduce the virus particles by viral agglutination (Figure 1.8). However, it has been found that the humoral immune response is not capable of protecting against progression of BK virus nephropathy, as patients with anti-BKV levels similar to healthy individuals still develop BKN (P. Comoli, Binggeli, Ginevri, & Hirsch, 2006).

The alternative means of the adaptive immune response in controlling infection is a cytotoxic mechanism that works through eliminating virus infected cells; when the virus enters the host cell, it expresses the virus antigen on its membrane even before viral assembly, which means destruction of those cells is essential to avoid viral replication. The presented antigens are recognised and attacked either by cytotoxicity mechanisms mediated by CD8+ lymphocytes or humoral mechanisms mediated by ADCC, phagocytosis or complement activation. Even though CD8+ T cells and NK cells are both able to kill virus-infected cells, they utilise different activation and signalling mechanisms; unlike CD8+ T cells, NK cells express an array of activation and inhibition receptors to respond to changes caused by any infection. Normally, NK cell activation is inhibited by increased expression of MHC-I molecules on target cells, that interact with NK cell inhibitory receptors and including killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs) (Rouse & Mueller, 2019; van Erp, van Kampen, van Kasteren, & de Wit, 2019).

Finally, CD4+ T cells are key to mediating both humoral and cell-mediated responses against viruses, providing help for antibody production by B cells and the activation of CD8+ cytotoxic T cell responses targeting virus infected cells (Janeway & Medzhitov, 2002).

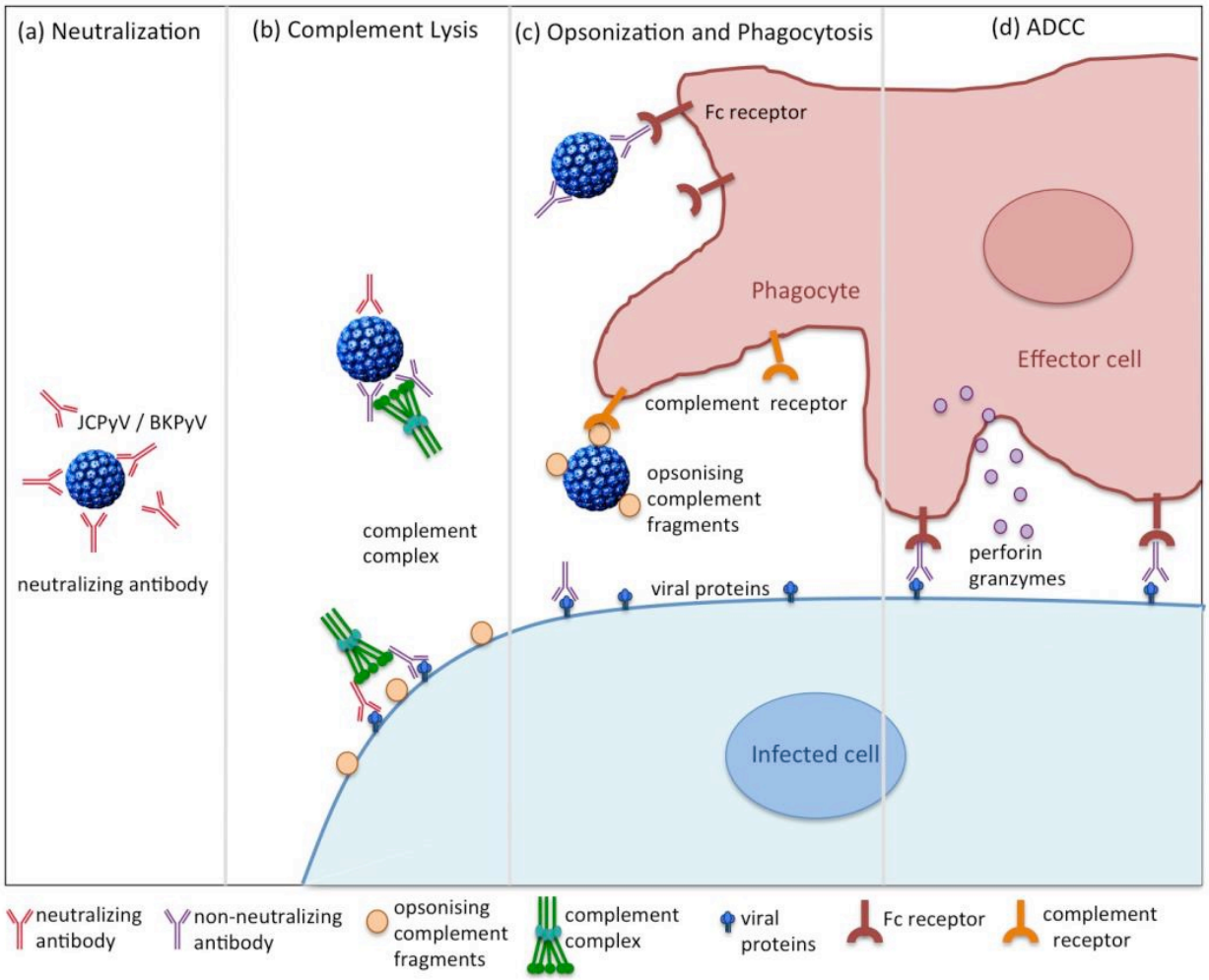


Figure 1.8.Humoral response

Different mechanisms of *Host humoral immune response in controlling virus infection (Kardas, 2015).*

Adaptive immune responses to BK

1.4.2.1 Humoral immune defence

Adaptive humoral immune responses are initiated immediately after exposure to BKV antigens. More than 80% of the population in early age (20-29) carry BK antibodies in their blood, and 71% of people aged 50-59 are seropositive for BKV (Adrian Egli et al., 2009). Commonly, during viral infection, the antibodies neutralize the virus particles by binding to the surface proteins of the virus and prevent it from interacting with the cell surface receptors that enable viral entry.

In BKV infections, some virus genotypes are able to evade antibodies due to mutations in the virus receptors, for example, antibodies raised against type I viruses are not able to bind to type II viruses (Pastrana et al., 2013). Hence, we cannot completely rely on the recipient's seropositivity against BK virus reactivation, based on a study in paediatric kidney transplants, seronegative recipients were at risk of BK reactivation and BKPyAN. However, being seropositive for BK virus does not necessarily give full protection from post–transplant reactivation or BKPyAN but, surprisingly, it has been observed that high levels of anti BK antibodies are correlated with low CD8+ responses and high levels of viremia. Furthermore, Leboeuf et al evaluated and compared BK virus IgG levels in viremic patients and non-viremic controls at different periods following transplantation; no significant difference was observed among the study groups (Leboeuf et al., 2017b). Also, in the same study, antibody levels were evaluated in patients who were viremic and those who cleared the virus; again no significant difference was detected which weakens the correlation between antibody levels and virus clearance and suggests that anti-BK antibodies are only a marker of past and recent exposure to the virus (Leboeuf et al., 2017a). However, BKV specific humoral immune responses play a significant role in neutralizing virus, even though they are unable to resolve viral latency and persistence. It is worth mentioning when studying BKV humoral responses there is no standard serological assay for measuring anti-BK antibodies. Also, sero-positivity does not necessarily mean the development of anti-BK T-cell memory and these factors highlight the importance of better understanding the mechanisms that provide specific stable cellular defences against the virus (P. Comoli et al., 2004).

1.4.2.2 Cellular defence

Cellular immunity is strongly believed to play an important role in BK virus clearance and control of BKVN in KTR and normal individuals. Drummond and associates illustrated the importance of cellular immune responses in inhibiting BK virus reactivation in healthy volunteers (H. H. Hirsch, 2005). Further, a study comparing post renal transplant patients with or without viruria, and healthy individuals, found that KTRs had lower levels of BK specific T-cell responses in comparison to healthy subjects, which could be predicted due to the induction of immunosuppression. Moreover, a considerable amount of literature has been published describing links or a correlation between specific cellular immune responses and the control of BK virus replication; low levels of BKV-specific T-cells, which produce INF-γ, correlate with progression to BKV nephropathy, while elevation of these cell populations correlates with resolution of nephropathy (S. Hariharan et al., 2005; Koralnik, Du Pasquier, & Letvin, 2001; Prosser, Orentas, Jurgens, Cohen, & Hariharan, 2008). The global cellular immune response to BK virus in KTR individuals was measured using a Cylex ImmuKnow Test (Cylex, Columbia, MD, USA) where the mean±standard deviation of the adenosine triphosphate (ATP) was 102.9±58.6 ng/ml, in patients with viremia versus 227.2±146.4 ng/ml in viruric recipients, and 231.8±150.8 ng/ml in those neither aviremic nor viruric. This also leads to the conclusion that a reduction in immune cell function is correlated with viral replication (Batal et al., 2008).

Impaired BK-specific cellular immune responses may allow for nephropathy disease progression. This has led to the assumption that regular monitoring of BK specific T-cell responses prior to and following transplantation is considered a sensitive and crucial marker in identifying patients with a higher risk of experiencing BK reactivation. Pre-transplant and post-transplant monitoring were illustrated by Schachtner et al. When they compared two groups of KTR individuals with and without BK replication, using an INF-γ ELIspot assay to measure T-cell responses, they found that individuals with lower BK specific T-cell responses were more likely to develop virus reactivation and to have remarkable low level of INF-γ, CD4+, CD8+ and CD3+ T-cells, whereas, recipients with a higher or more stable levels of specific immune cells inhibited virus replication (Schachtner, Stein, Babel, & Reinke, 2015).

Many studies have aimed to identify and characterise the BK virus specific CD4+ and CD8+ T-cells directed against virus proteins which provide protection against disease reactivation. A recent report indicated that T-cell responses mounted against BKV are predominantly CD4 T-cells and these are able to control viral replication in the absence of CD8+ T-cells, in other words it is CD4 T-cell restricted (Schachtner et al., 2011). In 2014, Weist et al. described a mechanism behind CD4 T-cell recognition of BKV on antigen presenting cells; they found that the perforin–granzyme pathway is the mechanism behind CD4+ T-cell mediated killing. Additionally, CD4+ T-cells were found to up-regulate pro-inflammatory cytokines such as IFN-γ and TNF-α while down-regulating the expression of CD107a that is expressed during cytokine secretion (Weist et al., 2014) (Alter, Malenfant, & Altfeld, 2004). BKV-specific T-cell immunity and its role in viral clearance was investigated in HSCT patients; specific $CD4^+$ and $CD8^+$ T-cell recovery were associated with successful viral clearance (Apiwattanakul et al., 2017). Additionally, KT patients with absence of BKV specific T-cell immunity pre-transplant, tend to develop BKV DNAemia post-transplant (Schachtner et al., 2015).

In another context, Binggeli et al compared Tcell responses to the two main BK virus proteins, in healthy controls and KTR individuals with BKV viremia (Binggeli et al., 2007). There were lower responses to LTag compared to VP-1 Ag and it was also observed that VP-1 preferentially stimulated CD4+ T-cells while LTag stimulated CD8+ T-cells. Cellular responses to the viral LT protein were more frequent than responses to VP protein, plus the population of T-cells that specifically secreted INF-γ was remarkably higher in patient with viral clearance compared to controls. Surprisingly, effector memory T-cells were observed in patients with BKV nephropathy, indicating that BKV antigen expression can affect or control T-cell proliferation and expansion. In contrast, a prospective study found that large T-antigen specific immunity but not immunity to VP1 after transplantation can protect patients from development of viremia. In this regard, many studies have confirmed that LT Ag appears early in the virus life cycle, making it a target of the cellular immune response in comparison to the VP protein; (van Aalderen et al, 2012). To conclude, the VP protein has been shown to be more immunogenic and stronger than the LT protein, whilst LT is more abundant and appears earlier than VP. In 2018, Blazquez-Navarro et al found that

VP triggered immune responses were mainly associated with reducing the viral load, while LT triggered responses were associated with acceleration of infected cell death (Blazquez-Navarro et al., 2018).

Moreover, a number of studies have observed that BKV mediated T-cell responses are mediated by cytokines such as INF-γ, tumour necrosis factor (TNF), and granzyme B (associated with CD107 expressing T-cells) after stimulation with BKV VP1 and LTag proteins. Commonly, specific cellular immune responses to BK virus are measured by T-cell production of INF-γ and other cytokines using flow cytometry and multiplex analysis of PBMCs stimulated with BKV peptide mix.

In KTR individuals the expression of cytokine expression declines as a result of the initiation of immunosuppression (IS) therapy aimed at reducing allograft rejection. Findings from Mueller and colleagues on studying post-KTR individuals with BKPyAN, demonstrated an expansion of T-cells showing a specific production of IL-2, INF-γ and TNF-α, using an ELISPOT assay and monitoring *in vitro* expanded PBMCs. The results showed responses to all BK virus capsid proteins except agnoprotein (large T, small t, VP1, VP2, and VP3) and interestingly these individuals showed greater numbers of CD4+ T-cells compared with an asymptomatic group (Mueller et al., 2011), whilst the T-cells that are supposed to produce these three cytokines were found more frequent in asymptomatic patients or those without virus reactivation. Mueller et al suggested that the strong T-cell reactivation in BKPyAN patients could be due to exhaustion of these cells and loss of their capacity for cytokine production (Mueller et al., 2011).

Most T-cell studies regarding BKV infection have focused on studying *in vitro* PBMC expansion/mixed lymphocyte peptide culture (MLPC); stimulating the immune cells using all the five BKV antigenic peptides (15mPs) for stimulating (IFN-γ), which elicited CD4+ T-cell responses even though CD8+ cells are believed to be the core of the cytotoxic cellular response; their epitopes have been studied less than CD4+ T-cells, and mainly those that are restricted to HLA*0201. Now, the direction being taken is to be able to detect even low numbers of BKV–specific T-cells and identification of T-cell epitopes, which are restricted to specific HLAs. In 2016 a study utilised bioinformatics to predict 9mer epitopes in the early viral gene (EVGR) presented by 14 HLAs that are common in North America and Europe; thirty nine epitopes were confirmed experimentally using (INF-γ)

enzyme-linked immune-spot assays, where several early studies characterized BKV-specific CD8 epitopes restricted to HLA A1, A2, A3, A24, B7 and B8 (Y. Chen et al., 2008; Cioni, Leboeuf, Comoli, Ginevri, & Hirsch, 2016; Li et al., 2006; Provenzano et al., 2006; Ramaswami et al., 2009; Schneidawind et al., 2010). Moreover, the same groups found that some BKV epitopes had significant homology with JCV; the same study and others suggested that previous exposure to JCV may lead to cross protection from BKV disease (G. R. Ambalathingal et al., 2017). Also, a study proved the higher sensitivity of the mixed peptide pool method compared to the single pool. This strong level of homology raises the possibility of development of immune monitoring tools that may apply to both BKV and JCV.

Furthermore, as mentioned, during viral infections, the immune system protects the host by activating innate and adaptive immune responses, including IFN secretion, cytolysis by NK cells as well as both specific cellular and humoral immunity to fight and control virus replication (Geraghty, 1987). However, most viruses develop strategies to evade host immune recognition and facilitate virus replication; one of these tactics is modulating expression of non-classical human leucocyte antigen (HLA) class I molecules. Several viruses were found to inhibit MHC molecule restricted antigen presentation to immune cells, by down regulation of both major histocompatibility complex (MHC) class-l and -ll molecules (Amiot, Vu, & Samson, 2014a). Other viruses modulate the expression of non-classical HLA class-I antigen (HLA-G) to prevent immune recognition (Shiroishi et al., 2006). HLA-G modulation in virus infection was proposed in several virus infections such as Rabies, HIV-1, HSV, Epstein-Barr virus and influenza virus (H.-X. Chen et al., 2011; Gazit et al., 2007; Lozano et al., 2002; Yan, Lin, Chen, & Chen, 2009) as induction of HLA-G molecules suppresses the function of several immune cells such as T-cells, NK-cells and antigen presenting cells (Shiroishi et al., 2006). Previous work in our laboratory has shown that cytomegalovirus is capable of inducing HLA-G expression on T cells and other leucocytes (Albayati et al, 2017).

Thus, the role of immunosuppression in permitting BKV virus replication is well known and the focus should be directed to better understanding host immunity to BKV. Current approaches, after identification of remarkable numbers of BKV-specific T-cell epitopes, should focus on expanding the HLA restriction alleles

based on ethnic groups, which will be a useful monitoring tool for BKV disease especially for regions other than North America and Europe (G. R. Ambalathingal et al., 2017).

1.5 Diagnosis

Since BKVN has no current treatment, the most important approach in BKV disease management is to facilitate an earlier and regular viral diagnosis and management strategy and to intervene before progression to disease. Due to the lack of standardized guidelines for BKV detection, it has been suggested to initiate an international reference standard to improve BKV diagnosis and detection (Greer, Forman, & Valsamakis, 2015). A prospective study found that BKVN is one of the early complications following kidney transplantation and most of the cases appear just months post-transplant. Further, a French prospective study followed renal transplant recipients for 12 months; reported that BKV was detected in 10.9% of the patients and the median time to viral load detection was 90 (23-214) days (Almeras et al., 2011). Similar data were found in a different prospective study of 32 patients, (Thakur, Arora, Nada, Minz, & Joshi, 2011) which reported that the greatest incidence of BKV viral load was in the first month post-transplant (Figure 1.9). Moreover, a different cohort study made the same findings; after 18 month of post-transplant patient follow up, they noted that the incidence of viruria and viremia peaked during the third month after transplant with 28% and 31% of patients, respectively (Koukoulaki et al., 2009). Based on the earlier literature, BKV screening in post-renal transplant patients within the first year and not after 24 months is highly recommended. Urine and plasma (PCR) appeared to be the most practical methods in detecting BKV in healthy people and BKV reactivation in post renal transplant patients. BK viruria precedes viremia by approximately four weeks, while that precedes BKAN and renal dysfunction by a median of eight weeks. However, renal biopsy and urine cytology are the main tools in diagnosing and confirming BKVN.

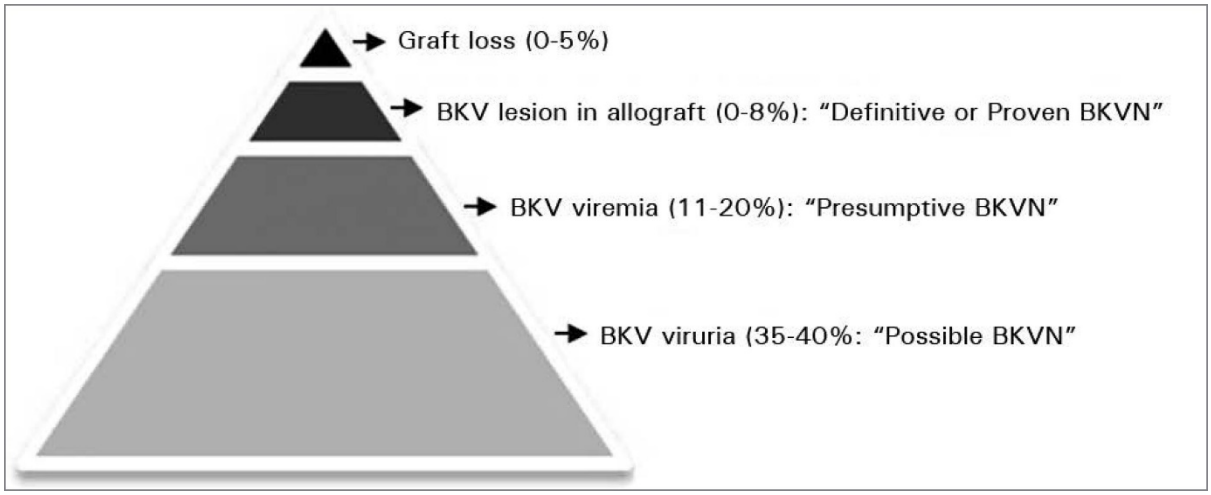


***Figure 1.9.* BK virus events in post renal transplant recipients.**

(Rafael Brandão Varella et al., 2014)

1.5.1 Plasma

Detecting BKV in plasma using real–time PCR is the preferred method at most transplant centres, due to its high sensitivity and specificity in BKPyAN diagnosis; the sensitivity reached up to 100% and specificity is approximately 90%, with a positive predictive value (PPV) of 50% and negative predictive value (NPV) of 100%, even though the final viral load cut-off for BKVN has not been established yet but some reports have suggested that BK viral load more than 4 log copies/ml is significantly associated with BKVN on biopsy (H. H. Hirsch et al., 2005); (Ding et al., 2002; H. H. Hirsch et al., 2002; H. H. Hirsch & Steiger, 2003).

1.5.2 *Urine*

Urine analysis is considered the most common non-invasive method of BKV detection. It can be screened for cytology (decoy dells): cells with enlarged nucleus containing a single large basophilic intra-nuclear inclusion. Or quantitative estimation of BKV DNA using real time-PCR assays which are superior to cytology regarding sensitivity and specificity. When comparing the two techniques, urine PCR has 100% sensitivity and 78% specificity compared to 25% sensitivity and a specificity of 84% through visualising decoy cells. Thus, urine viral load assessment in BKV screening is an essential and conventional tool in detection of BKV viruria, since viruria may proceed viremia (Pinto et al., 2013) and that viruria

with viral loads more than 10^7/ml is considered a risk factor for BKVN, even though the urine test only provides information limited to the presence of the virus DNA in the urothelium tissue.

Other types of urine analysis have been proposed, such as: detecting BKV VP1 mRNA levels as a way of measuring viral replication (Ding et al., 2002); and measuring BKV aggregates in urine and the Haufen method that is used to describe cast–like three dimensional BKV aggregates. However, all these approaches either need further validation or are expensive or not suitable for routine lab testing as they require electron microscopy (Singh et al., 2009) Ambalathingal et al., 2017).

1.5.3 *Biopsy*

Renal biopsies are still the gold standard in diagnosis and confirmation of BKVN, especially for patients with high viral load more than 4 $\log_{10}$ copies/ml, to exclude other renal underlying diseases and drug toxicity. Multiple biopsies from medulla tissue should be performed as the virus is not present uniformly within the tissue and to avoid sampling error.

Electron microscopy (EM) of allograft biopsies is considered one the best definitive tools in diagnosis and confirmation of BKPyAN. It is preferred over histology as it not only aids differentiation between acute rejection and BKVN, but also provides for a differential diagnosis of other viral infections within allografts such as adenovirus, Herpes Simplex virus, Epstein–Barr virus and CMV. EM is able to measure the dimension of the viral inclusions, which are typically between 30-50 nm in diameter and appears as crystalloid structures, whereas the herpes family and adenovirus inclusions are remarkably bigger; about 120-150 nm and 70-90 nm, respectively (Bardak et al., 2016). But as mentioned before electron microscopy is not suitable for routine labs.

Histologically, BKV disease has been classified into three grades to allow for a comparative analysis. Tubular atrophy and interstitial fibrosis are considered the most crucial signs of poor outcome and signs for immediate reduction of immunosuppression therapy (Masutani et al., 2012; Sar et al., 2011; R. Sharma et al., 2016).

1.6 Factors influencing BKV reactivation after kidney transplant

It is well known that the main factor affecting BKV reactivation and progression of BKPyAN in renal transplant recipients is immunosuppressive therapy (H. Hirsch, Yakhontova, Lu, & Manzetti, 2016; H. H. Hirsch, Drachenberg, Steiger, & Ramos, 2006; H. H. Hirsch & Steiger, 2003), in addition to active tubular lesions from surgical trauma which could increase the possibility of BK virus reactivation. Briefly, KTR individuals receive two types of immunosuppressive drugs: induction medication that is administered immediately after the transplant to prevent the acute rejection risk, such as monoclonal or polyclonal antibodies that attack host immune cells (thymoglobulin/ATG) or attack CD25-expressing cells (anti-CD25), and maintenance therapy, such as cyclosporine and corticosteroids that block T cell communication. These are in addition tacrolimus that reduce immune responses to the transplanted organ. Hirsch et al observed a strong association between tacrolimus administration and risk of developing BK viremia when compared with cyclosporine (Hirsch & Randhawa, 2013). Also, there is a correlation between risk of developing BK viremia and corticosteroid administration. A combination of three different suppressant agents also appears to associate with greater risk of BK reactivation (Borni-Duval et al., 2013). But the risk of BK viremia appears to be less common in people who are receiving mTOR inhibitors (sirolimus or everolimus), maybe because they are considered less potent than the calcineurin inhibitors (Jacobi et al., 2013; Suwelack, Malyar, Koch, Sester, & Sommerer, 2012).

Apart from immunosuppressive drugs, many other factors are believed to influence BKV disease. Shenagari et al, claimed that the most predictive factor for developing BK viruria in post-transplant recipients is undergoing pre-transplant dialysis treatment. Additionally, donor and recipient sero-status can play a role in development of BKVAN. When the donor has anti-BK antibodies and the recipient is seronegative that may increase the risk of developing BK viremia and even acute rejection when compared to seronegative donors and recipients, presumably because the recipient is BKV negative and hence has no memory cells or antibodies specific for BKV (Shenagari et al., 2017). Furthermore, the difference in anti-BK titre between the donors and the recipients may be a risk for reactivation (Wunderink et al., 2017). Older age and male gender of recipients are also

suggested to be associated with virus reactivation, in addition to higher level of HLA mismatch, ischemic injury, prior graft loss and prior acute rejection (D. L. Bohl et al., 2008; D. L. Bohl et al., 2005; H. H. Hirsch et al., 2002); (Leboeuf et al., 2017b) (Table 1.1).

Table 1.1. Factors that influence BKV disease.

Adopted and modified from (Pham, Schaenman, & Pham, 2014).

Virus related	Donor related	Recipient related
Virus genotype	Mechanical or ischaemic reperfusion injury	Low or absent cytotoxic T lymphocytes
Mutation in non-coding control region	Kidney from deceased donor absence of HLA-C7	Male
	BKV seropositive donor	Elderly people
	High BK- specific antibody titres (marking of higher recent BKV exposure and possibly graft load)	White and Asian ethnicity
	Older age (defined as older than 60 years)	Cytomegalovirus infection
	Female	Delayed graft function
	African American ethnicity	Diabetes mellitus
		Post –transplant early BKV reactivation of donor origin
		BKV seronegative
		ABO-mismatch
		Human lymphocyte antigen mismatch including absence of matching HLA-B 44 and HLA- DR15
		Steroid maintenance
		Lymphocyte depleting agents
		Immunosuppressive therapy
		Acute rejection episodes

1.7 Management and intervention

Currently, the keystone strategy in preventing BK reactivation would be early and regular monitoring of BKV replication combined with reducing, stopping or modifying the immunosuppressive therapy regimen with care to avoid immune restoration and rejection. Unfortunately, there are only a few controlled trials in the management of BK viremia and BKVN (Brennan et al., 2005; Johnston et al., 2010).

The recommended approach in controlling BKV infection in renal transplantation is the gradual reduction of immunosuppression, which comes with the risk of acute rejection. According to the Kidney Disease Improving Global Outcomes (KDIGO) recommendations, transplant recipients should be screened monthly for the first three to six months after transplant, then every three months for the first year of the recovery. Unless there is unexplained increases in serum creatinine levels after resolving of acute rejection, then the test should be performed more frequently (Lamarche et al., 2016); (Figure 1.10). As mentioned earlier, the most useful screening tool for BK viremia or viruria is quantification of virus DNA using real time-PCR assays. Additionally, renal biopsies should be performed if the viral load exceeds 10,000 copies/ml, to track any histological changes that are associated with BKPyAN.

A study suggested that fluctuations in the immunosuppressant levels correlated with co-existence of BKVN and acute rejection, indicating more clinical care and monitoring during drug administration, particularly for patients showing either acute rejection or BKVN and particularly both (van Aalderen, Heutinck, Huisman, & ten Berge, 2012). Even though there are no perfect approaches in reducing immunosuppression, many approaches have been suggested. It has been shown that administration of Calcineurin inhibitor (CNI) based therapies are not associated with BK viremia after transplant, whereas it is known to be one of the major risks of developing BKVAN and nephrotoxicity (Alméras et al., 2008; Matas, 2011).

There is no strong evidence of any antiviral drugs with clinical efficacy against BKV, even though many studies demonstrated the anti-BKV effect of agents such as leflunomide, cidofovir, fluoroquinolones and cyclosporine, in treating BKVN. In the most common experience, the approach has been to compare the antiviral

effects with decreases in immunosuppression. But most of the cases have come from uncontrolled observational studies, which makes it difficult to consider therapeutic efficacy (Burgos et al., 2006; Josephson et al., 2006; Song et al., 2016; Wadei et al., 2006).

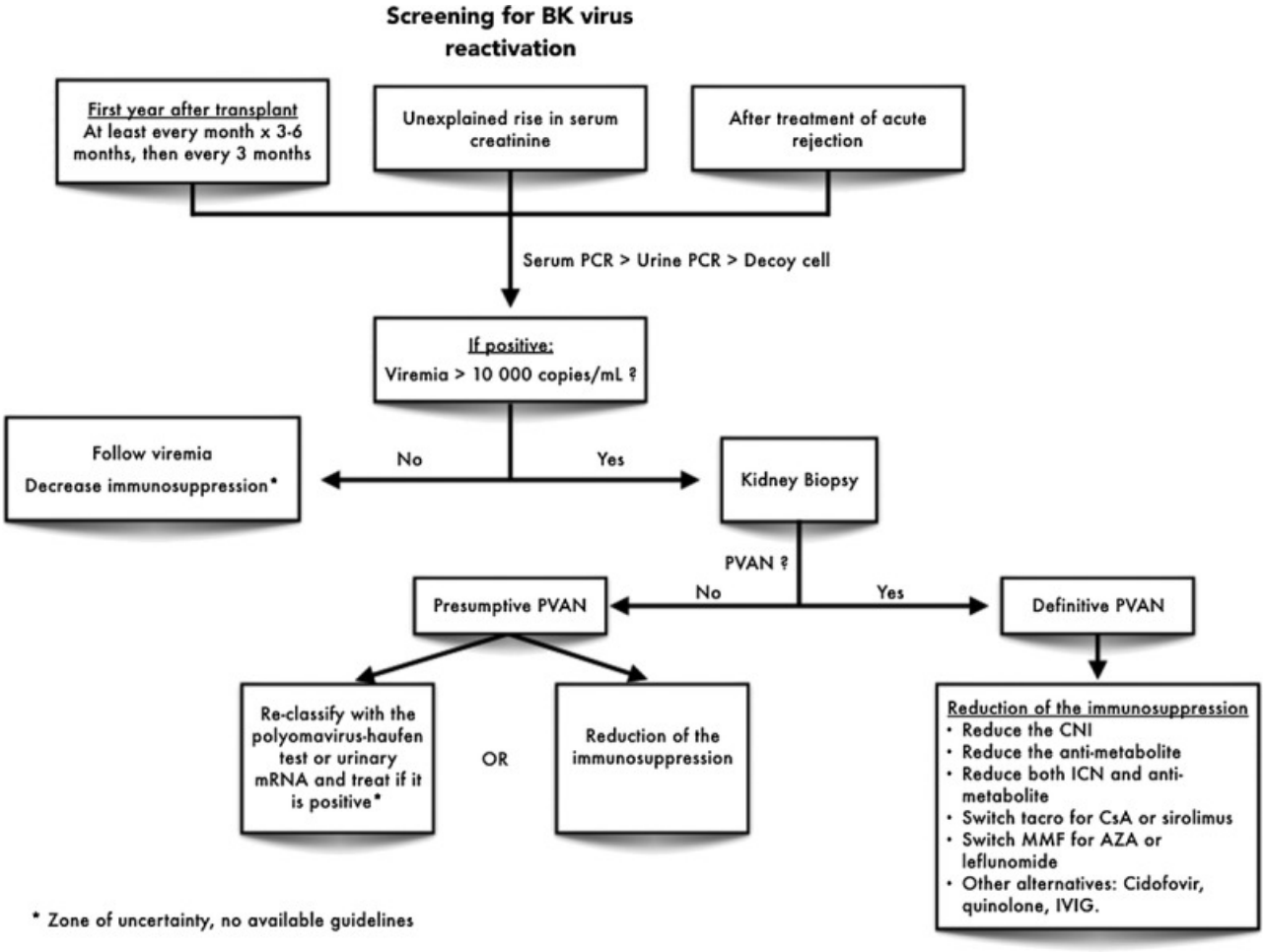


Figure 1.10. Clinical algorithm for screening BKV reactivation

From Lamarche et al. (2016).

1.7.1 *Intravenous immunoglobulin (IVIG)*

This is one of the proposed options in treating BKV nephropathy. As in other viral infections, immunoglobulin contains antibodies that neutralize the virus particles and prevent host cell entry. Based on *in vitro* incubation of BKV with IVIG for two hours before infection, 90% of the viral DNA was diminished whilst, if the IVIG was given directly to cells before the 2 hours, the percentage was significantly less. In contrast, a different study observed no benefit and even worsened long term effects because of the high dose of IVIG (Halim et al., 2016). Many case reports and case series studies have demonstrated the effect of IVIG in BKV control, but no control studies have been reported (Sener et al., 2006; Wadei et al., 2006) (Almeras et al., 2011).

1.7.2 *Fluoroquinolone*

In vitro, Fluoroquinolone administration was proposed as a potential agent in controlling BKV reactivation. It inhibits LT antigen helicase activity and reduces LT antigen expression (M. C. Sharma et al., 2006). Based on a trial when Fluoroquinolone was administered as antibiotic prophylaxis during the kidney transplant only a few of the patients who received Fluoroquinolone developed viremia (Wojciechowski et al., 2012). However, after three months of treatment in KTR individuals it failed to reduce the viral load and prevent viremia and caused bacterial resistance.

1.7.3 *Adoptive immunotherapy*

Cellular immune therapy refers to ex-vivo transfer of BK-reactive T-cells (educated) in order to restore antiviral immunity in immunocompromised individuals. The approach has been proved to be effective and safe in treating or preventing BK virus reactivation. It was developed in the early 1990s by Riddell to treat a patient suffering from Epstein Barr virus (EBV) complications following a haematopoietic stem cell transplant (Riddell & Greenberg, 1990); (Mani, Jin, & Schmitt, 2014). Over the past decades, many chronic virus–associated diseases have been treated using T-cell adaptive transfer, such as with CMV. In 2017, Tzannous et al used adoptively transferred virus-specific T cells from third party donors that recognize five common viruses: BKV, EBV, CMV, and HHV-6 and adenovirus in 38 patients. The aim was to evaluate cure rates for patients and where clinical improvement was demonstrated in 92% of patient treated for BKV (Tzannou et al., 2017). A recent study on renal recipients with persistent BKV

replication and BKPyVN found that adoptive T- cell immunotherapy may influence BKV replication and reduce virus load (Jahan et al.). Furthermore, identifying immunodominant peptides would be another interesting immunotherapeutic approach for treating transplant recipients. The characterization of viral immunogenic antigens or T-cell immunodominant epitopes is essential for the successful generation of virus–specific T-cells and peptides for vaccination. Several research groups have successfully defined persistent virus epitopes via *in vitro* expansion of virus specific T cells, using synthetic viral peptides, however, there are only very limited studies regarding BKV immuno-dominant epitopes. In 2003, Comoli and his group were the first who produced BK-specific T-cell lines from renal transplant patients with viremia; they were able to produce active T-cells against BK infected cells *in vitro* (P. Comoli et al., 2003). Furthermore, a different group succeeded in producing 15 lines of BK specific T-cells, but the cell expansion was limited and 20% of NK cells was found in the final product (Blyth et al., 2011). Clinically, an experiment where 11 HSCT recipients were treated with either single or multiple viral infections with a donor derived multivirus (Epstein-Barr virus, adenovirus, cytomegalovirus, BK virus, and human herpesvirus 6) specific T-cell line. Five of seven patients were completely cleared from BKV viremia without any significant side effects; in addition, a role for the PD1/PDL1-L2 receptor on BKV infected cells was proposed in controlling viral reactivation as when expressed by virus infected cells, prevening T-cell activation and inhibition of the receptors which results in restoration of T-cells and their expansion (Dekeyser, Francois, Beaudreuil, & Durrbach, 2015). Recently, a clinical trial succeeded in treating 86% solid organ and stem cell transplant recipients with BKV viremia using virus specific cells that were isolated from donors and/or unrelated donor (third party) blood (Nelson et al., 2020). Even though immunotherapy solutions in management of BKV disease are still in the early stages of development cellular immunotherapy should be considered at least for patients with higher risk of developing post-transplant complications.

1.8 Thesis outlines

The aim of the thesis is to gain more understanding of the humoral and cellular immune responses to BKV in healthy subjects and in transplant recipients. Additionally, to gain better insights of pre-transplant and post-transplant risk factors for BKV infection, including pre-transplant anti BKV levels and virus genotypes involved.

So, in the first place we hypothesized that the presence of BKV specific cellular immunity pre-transplant may influence the severity of the infection post-transplant. Secondly, we investigated whether BKV infection will increase HLA-G expression that promotes viral evasion. Further, we tested the hypothesis that BKV sero-reactivity is correlated with preceding viremia and risk of BKV infection in patients positive for some HLA alleles. Finally, we tested if some particular BKV genotypes were associated with deployment of BKV viremia and progression of disease.

The current study was conducted to primarily evaluate the following aspects:

- investigate cellular immune responses in renal transplant recipients with different virus replication status, to overlapping 15 mer peptide pools representing all virus antigens, specifically, interferon γ secreting cells, and comparing to healthy controls, despite the low pathological potential.
- Measure immunoglobulin G responses to BKV VP1 antigen using an ELISA assay to evaluate the presence of specific immunity and identify previous infection in renal transplant recipients.
- Measure cell surface HLA-G in healthy and renal transplant recipients to explore its importance in viral evasion of immunity
- Measure soluble plasma HLA-G (sHLA-G) in healthy and renal transplant receipts.
- Evaluate BKV IgG antibody quantitatively using a standard ELISA with recombinant antigen and not cross reacting with other polyomaviruses
- Study the association between the recipients' HLA allele status and development of BKV viremia.
- Investigate BKV genotyping using only 100bp of the BKV VP1 gene.

- Investigate the prevalence of BKV genotypes in renal transplant recipients and study the association of particular genotypes to the development of viremia or BKVN disease

CHAPTER TWO

2 Materials and methods

2.1 Study population and sampling

2.1.1 Healthy subjects

Healthy volunteers from the Institute of Infection and Global Health, Ronald Ross Building, University of Liverpool, were recruited through an advertisement in the department monthly magazine. They were identified as healthy subjects and were recruited to donate urine and later blood samples after signing a written informed consent. Project ethical approval was granted by the Interventional Ethics Committee (Ref: UoL001481). 71 urine samples were collected in 20 ml sterile containers and stored at -20ºC until the time of the experiment. Subsequently, blood samples were collected from each urine positive donor under aseptic conditions in 10 ml heparin vials and then processed using density gradient separation for PBMC isolation. DNA was extracted from the urine samples and separated plasma was stored at -20ºC.

2.1.2 Renal transplant patients

From mid-2018 kidney transplant recipients (KTR) from the Royal Liverpool and Broadgreen University Hospital were recruited and divided into four different groups as follows: a) BKV negative, never qPCR positive (12 patients), b) BKV positive, currently plasma qPCR positive (8 patients), c) historically BKV positive patients who are currently negative but were plasma qPCR positive in the past (13 patients), and d) pre- transplant (2 patients). All patients were treated with standard immunosuppressive therapy and consented to donate 10-40ml of blood and 20ml of urine, collected by Hospital research nurses. The specimens were transferred to the lab for DNA extraction, PBMC and plasma isolation. Urine and plasma samples were stored at -20ºC. Ethical approval was obtained from Central Bristol NRES committee (ID: IRAS 229214; PR 17/SW/0220).

Furthermore, pre-transplant samples, comprising archived frozen serum samples, were collected from the Royal Liverpool University Hospital Virology Laboratory. Four different samples were used from each KTR: pre-transplant, pre-viremia, during the viremia and post-viremia. Also, the same samples were used for BKV genotyping.

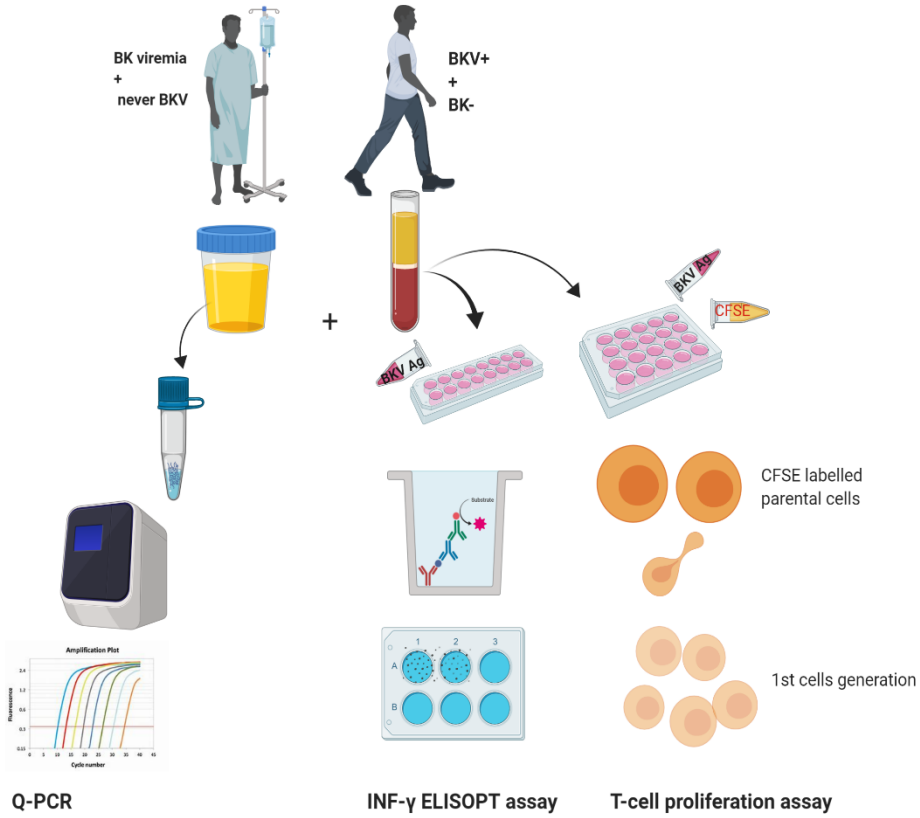


Figure 2.1. Sampling and methods used in chapter 3

Measuring Cellular immune responses to BKV in healthy individuals and KTRs, urine and blood samples were collected and ELISPOT, PCR and T-cell proliferation assays were performed

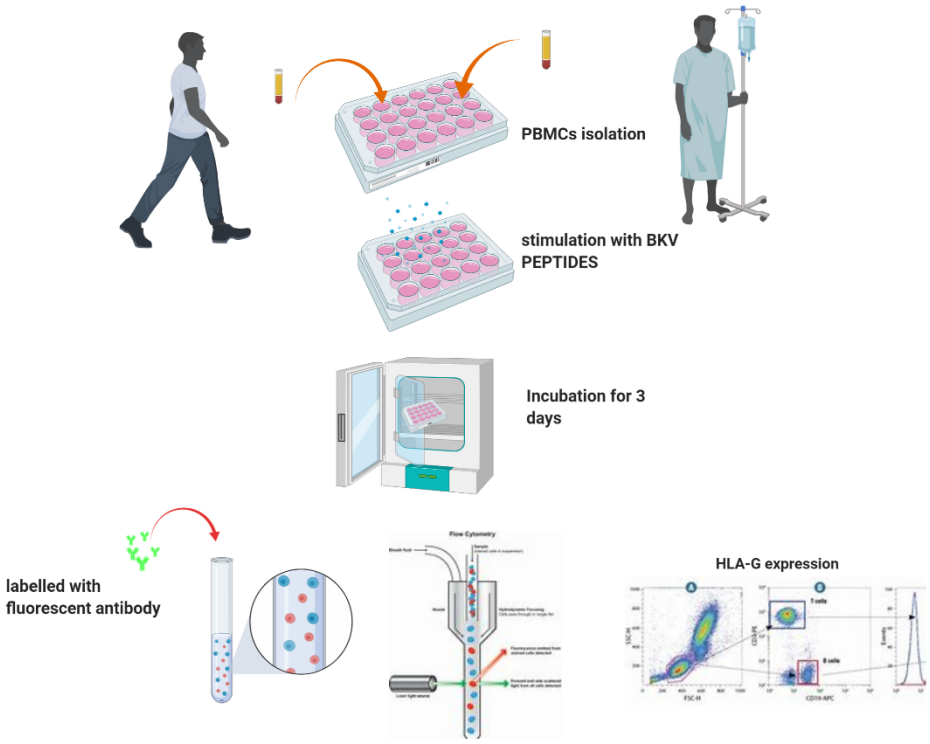


Figure 2.2.sampling and methods used in chapter 4

Blood samples were used to investigate Human Leucocyte Antigen – G (HLA-G) expression in BKV infected individuals using flow cytometry.

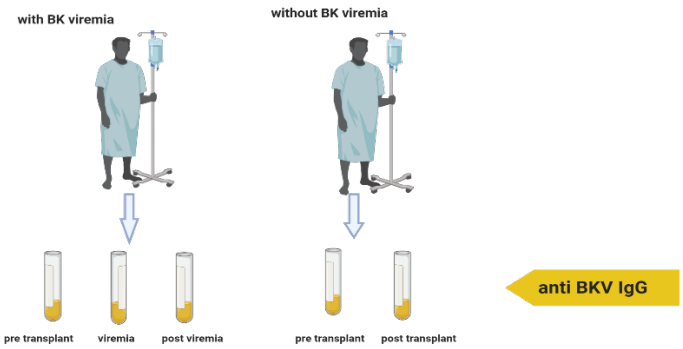


Figure 2.3. Sampling and methods used in chapter 5

Levels of anti BKV IgG in KTRs were studied by collecting three different urine samples from patients: pre-viremia, viremia and post-viremia

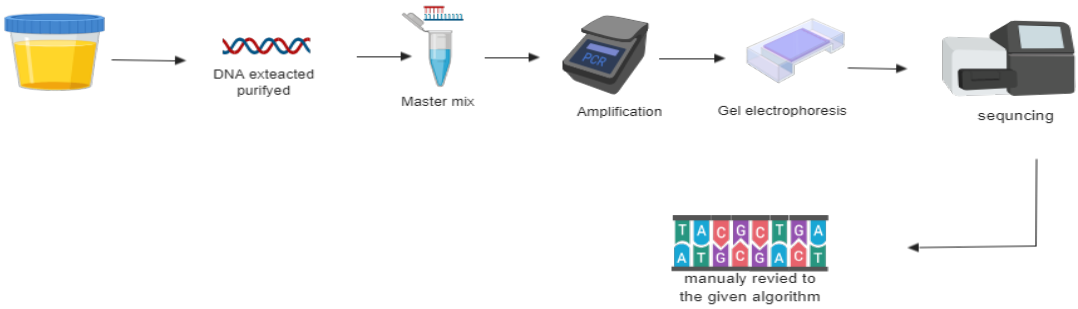


***Figure 2.4. Methods used in chapter** 6*

To identify BKV genotypes among KTRs with BKV viremia; urine samples were collected from patients with BKV viremia, then nested PCR and Sanger sequencing were performed. Later, the obtained sequences were retrieved and compared between the samples

2.2 Stocks and reagent preparation

All chemicals and reagents used were obtained from either *Sigma Aldrich, UK* or *Thermo Fisher, UK*, while most laboratory disposables, tips and plastic ware were purchased from *Starlab, UK* unless otherwise stated.

1X PBS

Two and a half PBS tablets were dissolved in 500 mL distilled water, autoclaved (for sterile work) and stored at room temperature before use**.**

Tissue culture medium

RPMI 1640 Medium containing L-glutamine by Life Technologies Ltd., UK, was treated with 10% (v/v) FBS and 100U penicillin / 0.1 mg/ml streptomycin (both from Sigma, UK) stored at 4°C.

3 M Sodium acetate (CH_3COONa) solution, pH 5.2

24.6 g of sodium acetate powder was completely dissolved in 70 mL of distilled water, then the solution topped up with glacial acetic acid to the required pH, filtered and stored at RT.

FACS buffer

FACS buffer was prepared by making up 1X PBS to 0.5% (v/v) FBS, 2 mM EDTA prepared freshly every time.

TAE buffer x1

The tris base was dissolved in 750 ml of deionized water, then acetic acid, EDTA and water were added to adjust the volume to 1 L. Then the stock solution was diluted 1:10 before use.

CFSE stain

To prepare a 5 mM stock of CFSE stain (Invitrogen C34554); 18 µl of DMSO was added to CSFE cell trace lyophilized vial mixed well then aliquoted into 3 µl in an

Eppendorf tube and stored at -20°C. On the day of the experiment, the CFSE working solution was prepared at 1:2000 concentration; 3 µL of CFSE to 6 mL 1X PBS.

2.3 Cellular assays

2.3.1 PBMC separation

Fresh blood (35-40 ml) was collected from donors in heparin tubes (green). First, all the separation process was undertaken in a biosafety cabinet II (MAT-2 CLASS II, Thermo Fisher Scientific Inc. USA). The cabinet was cleaned with 70% ethanol, then the samples were aliquoted into two 50 ml sterile Falcon tubes (*Fisher Scientific Inc. USA)*. 1/2 volume of Ficoll-Paque Plus solution (Sigma-Aldrich Ltd, UK) was added then the blood sample was pipetted carefully over it, using an automated pipette without disturbing the Ficoll layer. The Falcon tube was then covered and balanced then placed in the centrifuge *(Sorvall, Thermo Fisher Scientific Inc., USA*) for 25-30 min at 400 g with brake off and 1 Acceleration setting at 22°C. Following the previous step, the tube containing the sample was removed from the centrifuge carefully using a rack to avoid disturbance of the layers, buffy coat and plasma were transferred by pipette, each to a new 15 ml sterile tube. Then, the cells were washed twice by filling the tube with sterile PBS and centrifuged for 5 min at 400 g with acceleration setting 5 and brake 5 at 22°C, then the pellet was re-suspended between the two washes. After the second wash, cells were re-suspended in complete warm RPMI 1640 medium plus 2 mM L-glutamine (with 10% FBS and 1% Penicillin-Streptomycin), then counted and adjusted to reach the required volume for both surface labelling and ELISPOT experiments.

2.3.2 Preparation of BK Virus peptides

A peptide pool of 170 peptides (15 amino acids in length with overlaps of 11 amino acids chemically synthesized, analysed and purified by LC-MS) of BKV early large T antigen, small-T antigen, VP1, and VP2 were purchased from JPT Peptide Technologies Inc., UK, as a lyophilized powder. The powder was dissolved in DMSO

(Sigma-Aldrich Inc., UK), diluted with PBS to create stock solutions (25 mg/ml). The peptide stocks were aliquoted and stored at -20° C.

2.3.3 Enzyme-linked immune-spot assay (ELISPOT)

This test is known to be one of the fastest growing and most sensitive tools in biomedical research for studying cytokine secretion at the single–cell level, in addition to its crucial role in vaccine development, transplantation studies, virus research (such as HIV-1), cancer research and allergy research.

In this assay, the cells were cultured on a pre-coated surface with a specific capture antibody, in the presence or absence of stimuli. Then cell-secreted proteins in response to the stimuli such as cytokines were captured by the specific antibodies on the coated surface. After incubation for an appropriate period, cells were washed, and the detection antibody was added to the wells containing secreted cytokines. These detection antibodies were either biotinylated and followed by a streptavidin-enzyme conjugate, or the antibody was directly conjugated to an enzyme. Lastly, the precipitating substrate was added to develop visible spots on the wells. Each spot corresponds to an individual cytokine-secreting cell.

Procedure

In this study INF-γ secreting cells were evaluated, using a *Human IFN-γ ELISpot PLUS (ALP)* kit from Mabtech AB (Sweden), following the procedure given:

A-Preparation of the ELISPOT plate:

1- Wash the required number of strips with 200 µl/well sterile PBS. And keep the unused strips in the provided foil.

2- Then, condition the plate with 200 µl/well complete RPMI 1640 plus 2 mM L-glutamine media (with 10% FBS and 1% Penicillin-Streptomycin), for 30 min at RT.

B-Incubation of the cells in the plate (sterile conditions):

1-Discard the medium and add the stimuli followed by 100 µl of cell suspension. Alternatively, mix the cell suspension with stimuli in separate tubes and add to the wells.

2-Use the mAb CD3-2 as positive control at dilution of 1:1000.

3- Cover and incubate the plate in a 37ºC humidified incubator with 5% CO_2 for 24-48 hours without moving the plate.

4- Wrap the plate with aluminium foil to avoid evaporation.

C-Detection of the spots:

1- Discard the cell suspension and wash the wells four times with PBS 200 µl/well and dry the plate against a blue paper towel.
2- Dilute the detection antibody to 1 µg/ml in PBS. Then Add 100 µl/well of the diluted detection Ab and incubate it for 2 hours at RT.
3- Wash the plate four times and dry it well against a blue paper towel.
4- Add 100 µl/well of streptavidin (ALP) after 1:1000 dilution and incubate for 1 hour at RT.
5- Wash the plate four times with PBS.
6- Filter the ready to use substrate using a 0.45 µm filter, then add 100 µl/well; the spots should appear within 5-20 min.
7- Stop colour development by washing the plate with distilled or tap water, leave it to dry then count the spots using an ELISPOT reader (Autoimmun Diagnostika GmbH, Germany).

2.3.4 HLA-G expression

PBMC culture with BKV peptides

A 48 well plate was designed for culture of PBMC with the BKV peptide pool, divided into three parts: three days, five days and seven days. Each part was divided into two other parts; stimulated with 1 µg/ml peptide mix and non-stimulated control wells. Then the plate was incubated at 37ºC with 5% CO_2 until the time of acquisition.

2.3.4.1 Surface staining and labelling

After incubation, cells were mixed by pipette and 300 µL was aspirated into several 1.5 mL labelled Eppendorf tubes to start antibody labelling.

2.3.4.2 Antibody labelling

The freshly extracted PBMCs from healthy donor were cultured with the BKV peptide pool and then labelled with monoclonal antibodies (mAbs) for each antigen expressed by different subset of T lymphocytes. The selected Abs were based on optimal matching of three colours (FITC, PE, and APC) that are suitable for the BD FACSCANTO II flow cytometer machine (BD Biosciences, Oxford).

2.3.4.3 Flow cytometry

The assay is based on labelling cell cultures with fluorescent Abs tagged with fluorochromes that emit light of a defined wavelength when exposed to a special laser with known wavelength. Inside the machine, many detectors are employed to determine scatter patterns of labelled cells; these are converted into forward (FSC) and side scatter (SSC). FSC is for determining cell size whereas SSC depends on the cell's granularity, which leads to the ability to differentiate different cell populations in some circumstances.

Procedure

1. Aliquot 300 µl of fresh or cultured PBMCs in all your tubes
2. Dispense 3 µl of anti-human HLA-G APC Monoclonal antibody (MEM-G/9; Thermo Fisher Scientific Inc., USA) in tubes except negative control and isotype control.
3. Add 3 µl of anti-human CD3 FITC (Bio Legend Inc. USA) to the required tubes.
4. Load 3 µl of anti-human CD4 PE, anti-human-CD8 PE, anti-human CD56 PE, anti-human-CD14 PE and anti-human-CD19 PE (all from Bio Legend Inc., USA) into the correspond tubes (Table 2.1).
5. Aliquot 3 µl of mouse IgG1 Isotype control APC (Thermo Fisher Scientific Inc. USA) in isotype labelled tube.
6. Vortex and incubate at 4ºC for 30 min or at RT for 20 min in the dark.
7. After the incubation, fill the tubes with 2 ml of PBS and spin at 400 g for 8 min, discard the supernatant and re-suspend the pellets.

Table 2.1 Monoclonal Ab used for HLA-G phenotyping.

Cell type	Antibodies	Label	Company	Host	volume
T-helper	Anti-CD3+, CD4+, HLA-G	FITC+PE+APC	BD	Mouse	3µl
T-cytotoxic	Anti-CD3+, CD8+, HLA-G	FITC+PE+APC	BD	Mouse	3µl
NK cells	Anti-CD3+, CD56+, HLA-G	FITC+PE+APC	BD	Mouse	3µl
Monocytes	Anit-CD14+, HLA-DR+, HLA-G	FITC+PE+APC	BD	Mouse	3µl
B cells	Anti-CD19+, HLA-G	PE+APC	BD	Mouse	3µl
Isotype	Mouse IgG1	APC	BD	Mouse	3µl
Negative control	-	-			

Cells were assessed using a BD FACS CANTO II flow cytometer. Later, samples were analysed with Flow JO software provided by (FlowJo, BD LLC. USA), cells were gated for lymphocytes and monocytes, then each gate was moved to a new graph with a scale of cell labels to define each type of cell.

2.4 BKV ELISA

Both healthy subjects and patient participants were tested for anti BKV-IgG. A semi-quantitative Sandwich enzyme-linked immunosorbent assay (ELISA) commercial kit was used. The kit was provided by *VIDIA (Czech Republic).*

Sample preparation

Stored plasma samples were thawed at RT for 30 min before the experiment, then centrifuged for 5 s at maximum speed and transferred to the assay well within 15 min.

Procedure

1. serum samples were Diluted 101 times in dilution buffer (eg: 5 µl of serum to 500 µl of dilution buffer, each sample in duplicates.
2. Then,100 µl of standards and diluted samples incubate for 30 min at RT.
3. After incubation, wash the wells four times with 250 µl of wash buffer.
4. Mix the bottle of anti-IgG conjugate and add 100 µl into each well, then incubate for 30 min at room temperature.
5. wash again as step 3
6. Pipette 100 µl of TMB-O substrate into each well and incubate for 10 min at RT in the dark.
7. Stop the reaction by adding 100 µl of stop solution.
8. Measure the absorption at 450 nm with microplate reader within 10 min of adding the stop solution.

2.5 Soluble HLA-G ELISA

Plasma samples from healthy subjects and post KTR individuals from Liverpool Royal Hospital with or without detected BKV DNA, were tested for s HLA-G by Sandwich ELISA. The commercial kit was provided by Exbio (Prague, Czech Republic) and was used for quantitative measurement of s HLA-G. Briefly, the principle of the test is that calibrators and samples were incubated in a pre-coated well with anti sHLA-G antibodies for 20 hours. Then the wells were washed, labelled with monoclonal anti-human β2-microglobulin, and incubated for 60 min. Following another wash, substrate solution (TMB) was added to react with the remaining conjugate. Finally, the reaction was terminated by adding acidic solution to stop the reaction and measure the absorbance. The absorbance was directly proportional to the s HLA-G concentration.

All the reagents were brought to RT 30 min before the experiment.

Sample preparation

Stored frozen (-20ºC) EDTA plasma samples were thawed just prior to the assay. Subsequently, the samples were diluted eight times in dilution buffer 1 provided and mixed well.

Reagent preparation

S HLA-G lyophilized master calibrator was reconstituted in 700µl of distilled water, left to dissolve for at least 15 min at RT to obtain a stock solution of 625 units/ml concentration. Then, five serial 2-fold dilutions were performed from the stock. Working conjugate solution was prepared of one part of concentrate conjugate solution and 99 parts of conjugate diluent. Then, wash concentrate solution was prepared by diluting one part of the concentrate wash solution and 9 parts of distilled water.

Assay procedure

1-Add 100 µl of calibrators, diluted samples and dilution buffer into the microtiter plate wells.

2- Seal the plate and incubate at 2-8ºC for a maximum of 20 hours without shaking.

3-Wash the wells five time with the diluted wash solution.

4-Dispense 100 µl of conjugate working solution into each well.

5- Incubate the plate for 1 hour at RT, then place it on an orbital shaker at 300 rpm.

6- Wash the wells 5 times with the diluted wash solution.

7-Pipette 100 µl of substrate solution and cover the plate with aluminium foil to avoid light exposure.

8-Incubate the plate for 25-30 min at RT without shaking.

9-Stop the reaction by adding 100 µl of stop solution.

10-Read the absorbance of the plate by microplate reader at 450 nm within 5 min of adding the stop solution.

2.6 Molecular assays

2.6.1 DNA extraction

Viral nucleic acid was extracted using a QIAamp DSP Virus Kit (Qiagen Inc, Germany). The tubes have a QIAamp silica-based membrane, this membrane binds to the nucleic acids from lysed samples, while the rest of the lysate is rapidly removed by vacuum pressure. Then, the bound nucleic acids in the membrane are washed to remove contaminants and then eluted in a volume of 20 µl or 60 µl.

Sample and Reagent preparation

Preparing Protease enzyme solution:

The entire contents of the vial containing 4.4 ml Protease Solvent was added to the lyophilized Protease vial and mixed carefully by inverting the vial several times, to avoid foaming.

Lysis Buffer Preparation:

Lysis buffer was prepared by adding carrier RNA to an internal control; the purpose of this mixture was to improve viral DNA binding to the column membrane. Carrier RNA solution was prepared by adding 310 µl of Elution Buffer (AVE) to a tube of 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl. Then, the volume of Lysis Buffer -carrier RNA mix needed per sample was calculated.by selecting the number of samples to be processed and provided lysis buffer volume and apply it to the following equation: n x 0.55 ml = y ml

y ml x 11.2 µl/ml = z µl

Where: n = number of samples to be processed y = lysis buffer volume, z = carrier RNA/elution buffer (AVE) volume to add to lysis buffer.

Wash Buffer 1 (AW1) preparation

Using a measuring cylinder, 25 ml of ethanol (96–100%) was added to the 19 ml concentrated Wash Buffer 1 (AW1) bottle and mixed well before use.

Wash Buffer 2 (AW2) preparation

30 ml of ethanol (96–100%) was added to 13 ml concentrated Wash Buffer 2 (AW2) bottle and mixed well before the procedure.

Procedure:

1. Add 75 µl of the Protease solution into each lysis tube.

2. Pipette 500 µl plasma or serum to the lysis tube. Then Add 500 µl of prepared lysis buffer (containing 11.2 µg/ml carrier RNA).

3. Incubate at 56°C in a heating plate for 15 min.

4. Centrifuge at full speed for ≥5 s

5. Open the tube with care.

6. Pipette 600 µl ethanol (96–100%) into the lysis tube, c, Incubate for 5 min at RT (15–25°C).

7. Centrifuge at full speed for ≥5 s.

8. Carefully load all the lysate into the Column Extender without touching the column tube membrane or wetting the rim.

9. Turn on the vacuum pump to draw in all the lysate, then open the valve and release the vacuum pressure.

10. Pipette 600 µl wash buffer 1 to the column tube.

11. Load 750 µl wash buffer 2 to the column tube without wetting the rim

12. Carefully without wetting the rim add 750 µl of ice cooled ethanol (96–100%) to the column tube.

13. Insert the column tube into a new dry wash tube, and centrifuge for 1 min at full speed to dry the membrane completely; discard the filtrate.

14. Place the column tube in a new wash tube and incubate for 3 min at 56°C with open lid to dry out any moisture.

17. pipette 20 µl to 60 µl of Elution Buffer onto the centre of the membrane.

18. Secure the lid and incubate for ≥3 min at room temperature (15–25°C).

19. After the incubation, spin for 1 min at full speed (approximately 20,000 x g, or 14,000 rpm),

2.6.2 Nucleic acid amplification (QPCR)

DNA amplification and quantitation were performed by quantitative real time PCR using *artus BK Virus PCR Kits CE (Qiagen)*. The kit was designed for ready to use of BKV detection system by DNA amplification on Rotor-Gene instrument. The kits provide reagents; master mix and primers were designed for specific amplification of a 274 bp region of the BK virus genome.

Procedure:

1. Prepare and place the required number of PCR tubes in the cooling block.

2. Prepare the master-mix according to the following (per sample): The reaction mix typically contains all the components needed for PCR except the sample.

3. Add 10 µl of the prepared master mix into each of the PCR tubes. Then add 15 µl of the sample DNA to the mixture.

4. Add 15 µl of each of the four quantification standards, as a positive control and 15 µl of water as a negative control.

5. Close and insert the tubes into the Rotor-Gene instrument and secure it using the security ring.

6. Create the following temperature profile:

45 three step cycles: 15 s at 95ºC, 30 s at 65ºC and 20 s at 72ºC.

For DNA quantitation a standard curve was generated of the four standards.

Table 2.2. QPCR master mix preparation.

BK Virus RG Master	7 µl
BK Virus RG Mg-Sol	3 µl
BK Virus RG IC	1.5 µl
Total volume	11.5 µl

2.6.3 BKV genotyping

After identifying BKV positive donors, their samples were genotyped to identify and estimate the prevalence of BKV genotypes in post kidney transplant recipients. The genotyping protocol was designed based on amplification of a 100-bp region of theVP1 gene of BKV as described in (Morel et al., 2017); the primers were designed for nested PCR by (OligoPerfect™ Designer, Thermo Fisher Scientific Inc. Web Tools):

F (5`-GGTYATTGGAATAACTAGYATGC) and

R (5`-TCCAARTAGGCCTTATGRTCAG). The same method used previously for DNA extraction was applied.

2.6.3.1 Nested PCR

Primers:

Primer design and preparation

The outer primer for the first PCR was frwBKVP1 (5'CCCCAACCAAAAGAAAAGGAGAGTGTCAC3') and revBKVP1 (5' GTCTTTCTGTGCCCCATCAAACACCCTA3') and 2 frwBKVP1in2 (5'GGAGGAGTAGAAGTTCTAGAAGTTAAAACT3') and 2 revBKVP1in2 (5-GGAAATTGGGTAAGGATTCTTTACAGATCT3'). The inner primer sequences were obtained from (Morel et al., 2017) and designed in SIGMA while the outer pair were designed by our lab and produced by Sigma. All the primers were obtained

as lyophilized powder that were prepared based on the technical sheet. The amplification master mix was prepared based on the protocol given in Table 2.2.

Table 2.3 BKV genotyping (PCR mix preparation).

Reagent	Stock Conc.	U	Provider	µl/reaction	Final Conc.	U	Total Master Mix
qPCR Buffer w/o $MgCl_2$ **(from kit: 10966034)**	10	X	Invitrogen	5	1.0	X	40
$MgCl_2$ **(from kit: 10966034)**	50	mM	Invitrogen	1.5	1.5	mM	12
Molecular Grade H_2O	/	/	Invitrogen	35.3	/	/	282.4
dNTP **(18427088)**	10	mM	Invitrogen	1	0.2	mM	8
Taq **(10966034)**	/	/	Invitrogen	0.2	2	U/rxn	1.6
Forward primer:	10000	nM	IDT	1	200	nM	8
Reverse primer:	10000	nM	IDT	1	200	nM	8
Master Mix Input:				45.0			360
DNA Input:				5.0			40
Total (µl):				50.0			400

Table 2.4. First PCR cycler profile.

	Stage	Temperature (°C)	Time	Cycles
Reaction details	Initial Denaturation	95	04:00	
	Denaturation	95	00:20	45 cycles
	Annealing	55	00:20	
	Elongation	72	01:00	
	Elongation	72	10:00	

Table 2.5. Second PCR cycler profile.

	Stage	Temperature (°C)	Time	Cycles
Reaction details	Initial Denaturation	95	02:00	
	Denaturation	95	00:30	35 cycles
	Annealing	55	00:30	
	Elongation	72	01:00	
	Elongation	72	10:00	

2.6.3.2 Gel visualization

60 ml of TBE buffer was added to 1.2 g of 2% agarose powder gel, mixed well, heated in a microwave for 2.5 min and allowed to cool down. Then 6 µl of SYBR safe gel stain was added to the gel, mixed and poured into the gel chamber and left to solidify. The gel tank was filled with 1x TBE buffer. 7 µl of sample, negative control and appropriate electrophoresis ladder were added to the wells. Finally, the gel was run for 45 min at 100V before visualizing the band.

2.6.3.3 Measuring DNA concentration

For estimating the amplified DNA concentration, a *Qubit dsDNA HS Assay Kit (Thermo Fisher)* was used as following:

1.Dilute the dsDNA HS reagent in dsDNA HS buffer1: 200. Prepare enough working solution for all samples and standards.

2. Pipette 190 µL of Qubit working solution to each of the standard tubes. Then add 10 µL of each standard to the appropriate tube, mix and vortex for 2-3 s.

3. Pipette working solution into sample tubes to obtain a final volume of 200 µL in each tube.

4. Add each sample to a tube containing the working solution, then vortex for 2–3 seconds.

5. Incubate for 2 min at RT.

6. Read standard and sample concentrations on the instrument screen.

DNA purification

For DNA purification a *PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher)* was used as follows:

1. Add the gel piece containing the DNA fragment into the Spin Column inside a wash tube.

2. Spin the tube for 1 min at >10,000 × g, then discard the filtrate and insert the Spin Column into a new wash tube.

3. Pipette 500–700 µL Wash Buffer 1 containing ethanol to the Spin Column.

4. Centrifuge for 1 min at >10,000 × g, then discard the filtrate and insert the Spin Column into a new Wash Tube.

5. Centrifuge again at maximum speed for 2–3 min to dry out any remaining Wash Buffer and ethanol.

6. Place the Spin Column into an Elution Tube.

7. Add 50 µL Elution Buffer to the Spin Column with avoiding the rim of the tube.
8. Incubate for 1 minute at room temperature.

9. Spin the tube at >10,000 × g for 1 min.

10. Store the purified DNA at -20ºC for short term storage.

2.6.3.4 Sequencing

The purified PCR products were sent to the company for Sanger sequencing (Source Biosciences Inc. UK). The samples were packed with both forward and reverse primers frwBKVP1 (5'CCCCAACCAAAAGAAAAGGAGAGTGTCAC3') and revBKVP1 (5' GTCTTTCTGTGCCCCATCAAACACCCTA3') and sent to the company after online submission. The sequencing data was referred electronically via E-mail and the forward and reverse sequences were aligned and accessed with software that allows (BLAST) comparison with standard sequences.

2.7 Statistical analysis

Data in this thesis were mostly analysed by Prism Graph Pad version.6, MEGA, FLOW JO and SPSS software. One-way ANOVA and paired and unpaired t tests were used for analysis and comparison between the tested groups.

CHAPTER THREE

3 Immune responses against BK virus in healthy immunocompetent subjects and post kidney transplant recipients.

Introduction

BK virus (BKV) is a member of the polyomavirus family that was discovered in 1971 (S. D. Gardner et al., 1971). The virus is an emerging opportunistic infectious agent that could co-evolve with its host and persist without any specific symptoms, as shown by its high distribution and low mortality rate. Despite its high seroprevalence only 19% of healthy donors shed BK virus in their urine after primary infection, which is mainly sub-clinical; the virus persists in the urinary and renal epithelium and establishes latency.

In immunocompromised hosts, the virus can reactivate in the uro-epithelial tissue and cause viruria and viremia. The main cause of the immune dysfunction that leads to BKV reactivation is the initiation of immunosuppression therapy after transplantation in order to prevent post-transplant cell injury that ultimately leads to graft rejection.

Currently, due to lack of effective anti-viral drugs for BKV, the keystone strategy in preventing BK reactivation is reducing the immunosuppressive therapy regimen while taking care to avoid acute rejection. Routinely, the recommended approach in most transplant centres after detecting BK viremia, is gradual reduction in immunosuppressive drugs in order to restore the immune defences against BKV, which comes with a risk of acute graft rejection. Unfortunately, only a few controlled trials have investigated management of BK viremia and BKV nephropathy (BKVN); (Hardinger, Koch, Bohl, Storch, & Brennan, 2010a; Puneet Sood et al., 2012). Moreover, adoptive T-cell transfer is one of the successful methods for reconstitution of the immune response against the virus. The method has shown effective clinical results with many chronic viruses such as CMV, EBV, adenovirus, and JCV. However, the issue with BKV is the lack of characterized T-

cell epitopes. This would also require HLA matching of transferred T-cells from the donor and recipient.

Cellular immunity plays an important role in BK virus clearance and controlling of BKVN in KT individuals. A considerable amount of literature has been published on the presence of a link or correlation between the specific cellular immune response and controlling BKV replication (P Comoli, Hirsch, & Ginevri, 2008; Dekeyser et al., 2015); the main debate is whether this mechanism depends on CD4 T-cells only or both CD4+ and CD8+ T-cells, which are directed to recognise endogenously expressed viral proteins in the context of MHC-class-I molecules.

In common, most of the BKV studies are based on *in vitro* PBMC expansion/mixed lymphocyte peptide culture (MLPC); stimulating the immune cells using five BKV antigenic peptides so as to be able to detect even low number of BKV–specific T-cells. A previous study identified the high sensitivity of the mixed pool method compared to using single pools (Trydzenskaya et al., 2011). Also, a recent study has used bioinformatics to predict 9mer peptide epitopes in the early viral gene (EVGR) presented by 14 HLA class-I molecules that are commonly found in North America and Europe; thirty-nine epitopes were confirmed experimentally using (INF-γ) enzyme-linked immune-spot assays, where early studies characterized BKV specific CD8 epitopes restricted to HLA A1, A2, A3, A24, B7 and B8 (G. Ambalathingal et al., 2017; Cioni et al., 2016). Moreover, the same group found that some BKV epitopes had significant homology with JCV; the same study and others suggested that a previous exposure to JCV may lead to cross-protection from BKV disease. This strong level of homology raises the possibility of development of immune monitoring tools that may apply to both BKV and JCV. Current approaches after identification of remarkable numbers of BKV- specific T-cell epitopes should focus on expanding the HLA restriction alleles based on ethnic groups, which will be a useful monitoring tool for BKV disease, especially for regions other than North America and Europe (G. Ambalathingal et al., 2017).

As previously mentioned the most useful approach in BKV management in most transplant centres is restoring virus specific immunity and avoiding the risk of graft rejection by reducing the immune suppression regimen (Smith et al., 1998). Many previous studies demonstrated the importance of virus specific immunity in

recovery from polyomavirus infection (Leboeuf et al., 2017b; Schachtner et al., 2011).

Generation of the humoral immune response to BKV could be a crucial tool in prediction of BKV viremia and the timeline of virus elimination post-transplant. However, most of the literature that studied BKV virus humoral response was based on a hemagglutination inhibition assay (Coleman, Gardner, & Field, 1973; De Stasio et al., 1979; DeStasio et al., 1980; S. Gardner, MacKenzie, Smith, & Porter, 1984; S. D. Gardner et al., 1971; HOGAN, BORDEN, McBain, Padgett, & Walker, 1980; W. A. Knowles et al., 2003; Shah, Daniel, Madden, & Stagno, 1980; Stoian, Hozoc, Iosipenco, Nastac, & Melencu, 1983) while some recent studies used ELISA assays to measure anti BKV titres. One of these studied by Bohl et al showed that donors' pre-transplant antibody titres could predict duration of post-transplant viruria (D. Bohl, Ryschkewitsch, Major, Storch, & Brennan, 2005) and Hariharan et al reported a transitory correlation between elimination of BKV and development of immunoglobulin G (IgG) antibodies in recipient with biopsy proven BKV nephropathy (Sundaram Hariharan et al., 2005).

The studies of this chapter were investigating cellular immune responses in renal transplant recipients with different status of virus replication, to an overlapping 15 mer peptide pool from all virus antigens, specifically, interferon γ secreting cells, as well as in healthy subjects as a comparison, despite the low pathological potential. Additionally, monitoring immunoglobulin G responses to BKV VP1 antigen, using an ELISA assay, enables for the evaluation of the presence of specific antibody immunity which could be an indication of previous infection in the renal transplant recipients.

3.1 Study populations

3.1.1 Healthy subjects

Healthy volunteers aged from 18-74, both male and female who are members of the Department of Clinical Infection, Microbiology & Immunology, Ronald Ross building, were identified through an advertisement in the department monthly magazine. They were recruited to donate a urine sample after obtaining written informed consent. Project ethical approval was granted by the University of Liverpool Interventional Ethics Committee. 71 urine samples were collected in 20 ml sterile containers and stored at -20ºC until the time of the experiment. Blood samples were collected from participants who tested positive for virus DNA and also from control urine negative subjects.

3.1.2 Renal Transplant Patients

Patients from the Renal Transplant Unit in the Royal Liverpool University Hospital were recruited and consented to donate 40 ml fresh blood and 20 ml urine sample. The blood samples were collected into heparin tubes then centrifuged for 30 min to separate PBMC and plasma, while the urine was stored at -20ºC for later PCR.

3.2 BKV screening

A total of 71 urine samples from healthy subjects, were tested for BKV Ab, then the presence of BK virus DNA in BKV IgG positives, using PCR (Fig 3.1). Thirteen (18.3%) were found to have been infected with BKV via the presence of serum BKV antibodies (Table 3.1). Four (30.7%) of them were urine BKV PCR positive, whilst the remaining 9 (69%) were found to be BKV PCR negative, with a median age of 48 and four of them were male volunteers without any known pathological problems. However, one of the male subjects is on monthly anti-TNF therapy for an arthritic condition.

Regarding the renal transplant patients, the samples obtained were already confirmed positive or negative for BKV; the sample were categorised based on presence of BKV in the patient blood: BKV positive (n=9), BKV negative (n=12), pre-transplant (n=2) and BKV positive historical (n=10; Table 3.2).

Table 3.1. Baseline characteristics of healthy subjects.

	BKV IgG +	**BKV IgG (-)**
	n=13	n=58
Age, mean of years (range)	48 (40-64)	34 (18-74)
Male sex%	11(84%)	40 (68.9%)
Pathological conditions	1	0
BKV DNA+(PCR)	4 (30.7%)	0

Table 3.2. Baseline characteristics of renal transplant patients.

Characteristics	**Total**	**BKV+**	**BKV (-)**	**BKV historical**	**Pre-transplant**	**P value**
	n=33	**n=9**	**n=12**	**n=10**	**n=2**	
Age (mean)	53.7	55.6	60	58.3	41	NS
Gender; male (%)	26	9(100)	7(58)	8(80)	2(100)	NS
Serum creatinine (mean)	219	197	136	158	385	NS
Re-transplant (%)	1	1 (11)				
Underlying diseases						
Polycystic kidney	7	3(33.3)	3(25)	1(10)		NS
Obstructive uropathy	2		2(16.6)			NS
Glomerulonephritis	5	4(44.4)		1(10)		NS
Other	17	2(22.2)	7(58.3)	8(80)		NS
Rejection	1	1(11)				

Quantitation data for Cycling A. Green

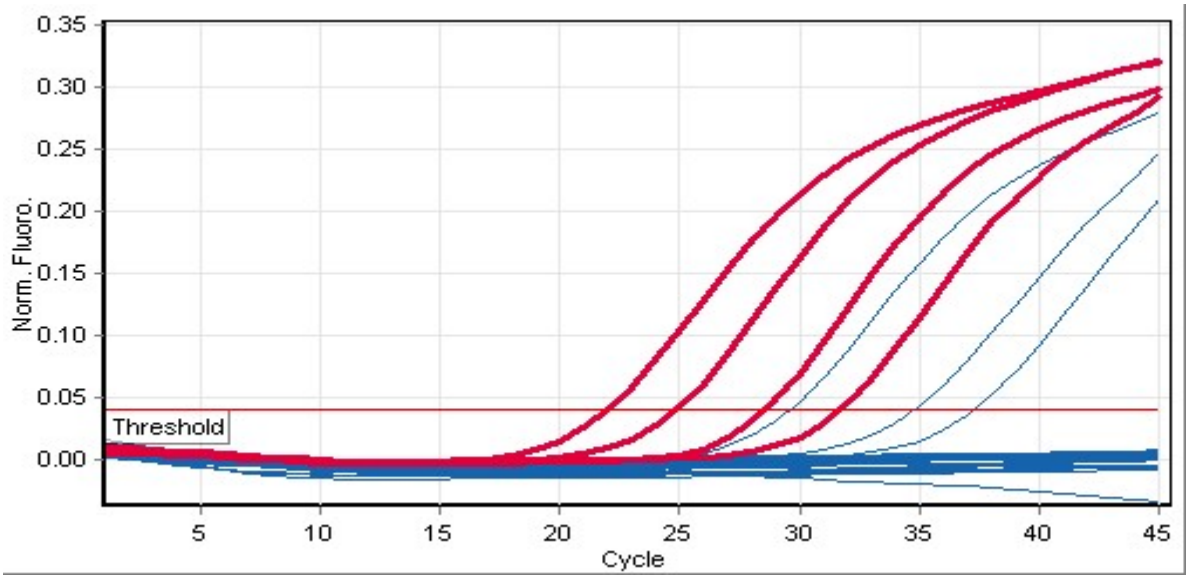


Figure 3.1.BKV qPCR plot

Illustrates the amplification of BKV DNA from healthy subjects' urine samples. The red lines represent the PCR standards, the blue lines represent the samples, and the black line is the negative control.

3.3 Healthy subjects

3.3.1 BK virus antigen specific cellular responses in healthy controls.

This study aimed to measure BKV specific cellular immunity and test the association between cellular and humoral immune responses to BK virus that could be a crucial tool in understanding the impact of BK virus reactivation post kidney transplant. A small but well-defined sample of healthy individuals was recruited and categorized into two groups: BK+ urine and BK- urine

Initially, the range of the cellular response was measured by isolating PBMCs from whole blood samples, then cells were stimulated and incubated with BK virus immunogenic peptide pools. INF-γ production was then measured using an ELISPOT assay (Fig 3.3). Participants who were positive for urine BK DNA demonstrated significantly higher cellular responses compared to the BK urine

negative group (p<0.004). The number of spots in the negative control (cut-off) was subtracted from all the samples (Fig 3.2).

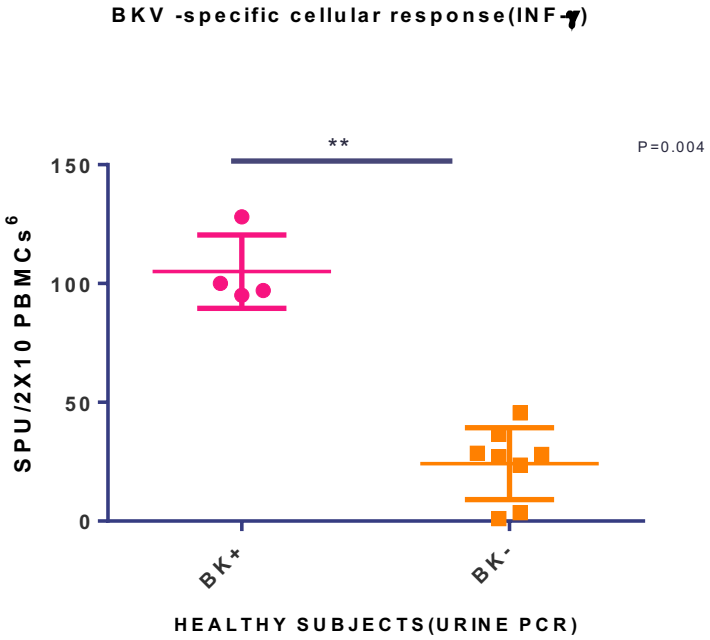
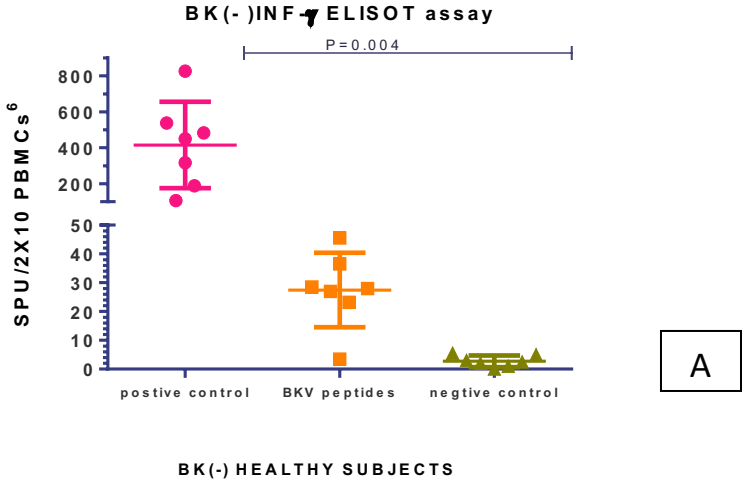
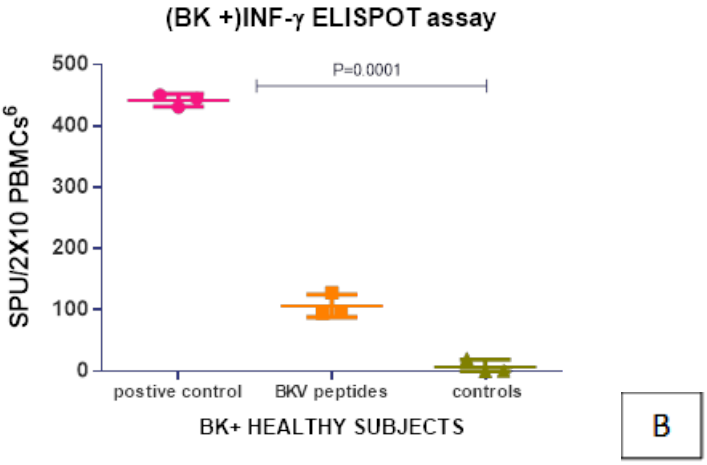


Figure 3.2. Cellular responses to BKV peptides

BKV specific INF-γ secreting cells using Enzyme-linked immune-spot (ELISPOT) responses to BKV after stimulation with BK overlapping peptide pools. The responses to overlapping peptide pools were measured by an interferon (IFN)-γ–ELISPOT assay. Responses are demonstrated in healthy people within the two categories: BK+ subjects with virus DNA in urine and anti BKV IgG, and BK- subjects with negative urine and anti BKV IgG. Points represent individual subjects, bars are means ± standard error of the mean, student t-test *was used to compare the means.*





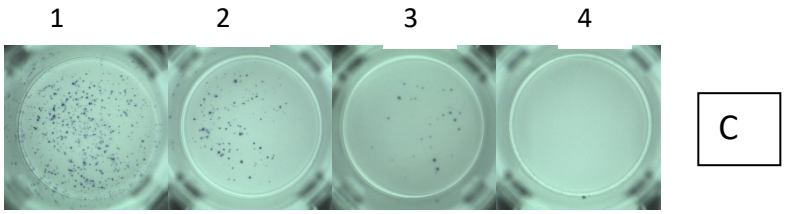


Figure 3.3 Enzyme-linked immune-spot (ELISPOT) assay

Elispot assays of samples of BKV + and BKV - subjects including negative and positive controls. Isolated PBMCs were stimulated with anti-CD3 antibody (positive control), unstimulated cells (negative control) and BKV- peptide stimulated cells. An ELISPOT assay in immunocompetent subjects with urine BKV, B immunocompetent subjects with negative urine BKV, bars indicate mean ± SD values, and the statistical significance was assessed using one-way ANOVA test (P < 0.0001). C. representative example of 1- positive control, 2- BKV positive subjects, 3- BKV negative subjects and 4- background wells (these data include the same data as in Fig 3.2.

3.3.2 BK virus antigen specific humoral responses in healthy controls.

BK virus-specific IgG was measured in 13 plasma samples from healthy participants: 4 with detected BKV in urine and 9 without, using a qualitative ELISA kit. Seropositive status was assigned if the optical density (OPD) of the ELISA was over the cut-off value. After testing the serological status (seropositive or seronegative), the antibody titres in the seropositive donors were estimated semi-quantitatively. Our data showed that BKV IgG concentration was slightly higher in subjects with negative BK DNA urine mean 0.48±0.03 in comparison to positive urine with mean =0.4±0.2 (P=0.3776).

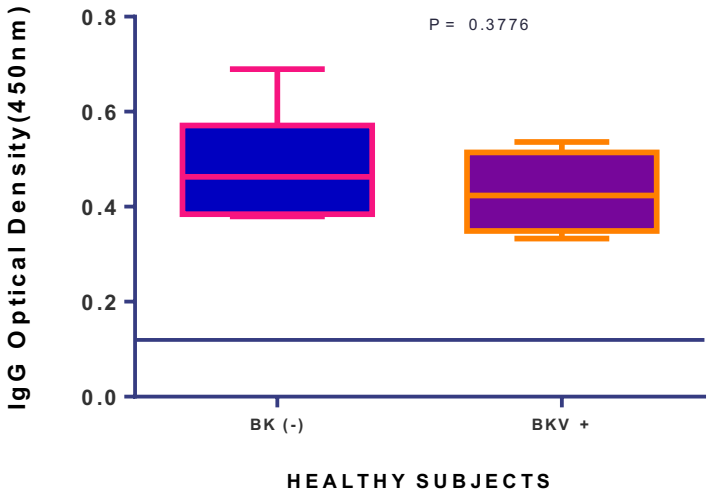


Figure 3.4 . BKV serum IgG levels

BKV IgG OD (450nm) in relation to BK in urine. Plasma samples from healthy donors with BKV positive (n=4) and negative (n=9) urine were tested for BKV IgG antibody using a sandwich ELISA. Box and whisker plots show 10-90 percentile and antibody levels are expressed as OD at (450nm). The statistical significance was assessed using student's t-test (P= 0.3776).

3.3.3 Correlation between humoral and cellular anti-BK responses.

The levels of cellular and humoral immune responses to BK virus in healthy subjects were compared. There was a slight but not statistically significant

negative correlation between levels of cellular immunity and humoral immunity in both participant categories (p=0.4; Fig. 3.5).

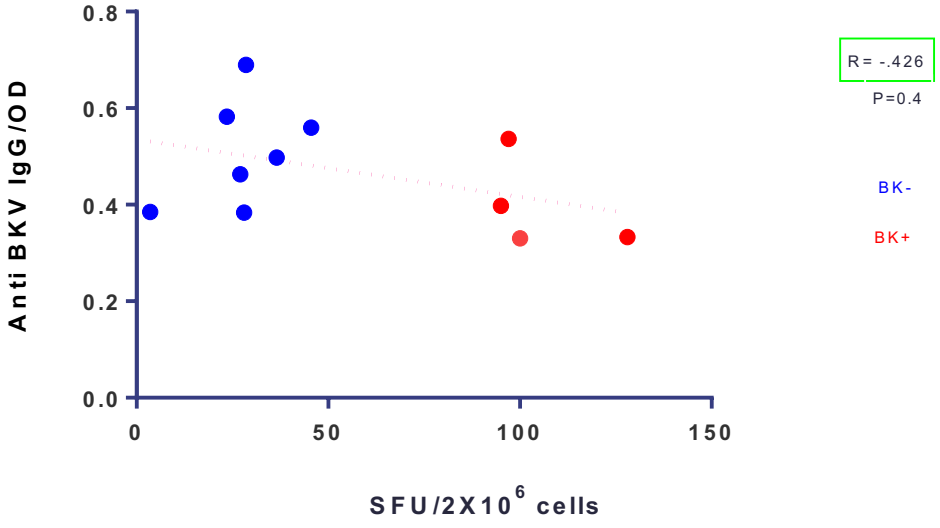


Figure 3.5 Correlation between BKV specific cellular immunity and anti BKV IgG in healthy subjects with or without BKV.

The plot shows correlation between number of BKV specific INF-γ secreting cells and levels of anti –BKV antibody in BKV+ and BKV - immunocompetent donors. The optical density shows a slight but not significant negative correlation with increasing number of INF-γ secreting cells. The p value and r were calculated using Spearman's correlation.

3.4 Renal transplant Patients

3.4.1 BK virus antigen specific cellular responses in renal patients.

Cellular immune responses were measured in PBMCs from different groups of kidney transplant recipients; pre-transplant, post-transplant who were chosen as:

never had BKV, BKV+ and patients with resolved BKV infection. ELISPOT assays were performed to assess BKV specific T-cells after stimulating the cells with four BKV peptide pools. BKV specific immunity SFU was lowest in recipients with active BKV infection among the patient groups. Recipients who never experienced BKV replication after transplant showed higher specific cellular responses to BKV than BKV+ recipients (P<0.005). The patient group with historical BKV infection showed cellular immunity against BKV peptides that was higher than the BKV+ group but without reaching statistical significance (P<0.1). However, pre-transplant kidney recipients demonstrated a difference between specific cellular immunity against BKV in comparison to post-transplant individuals; P<.001 in comparison to BK+ and P< 0.03 in comparison to the BK- group (Fig. 3.6).

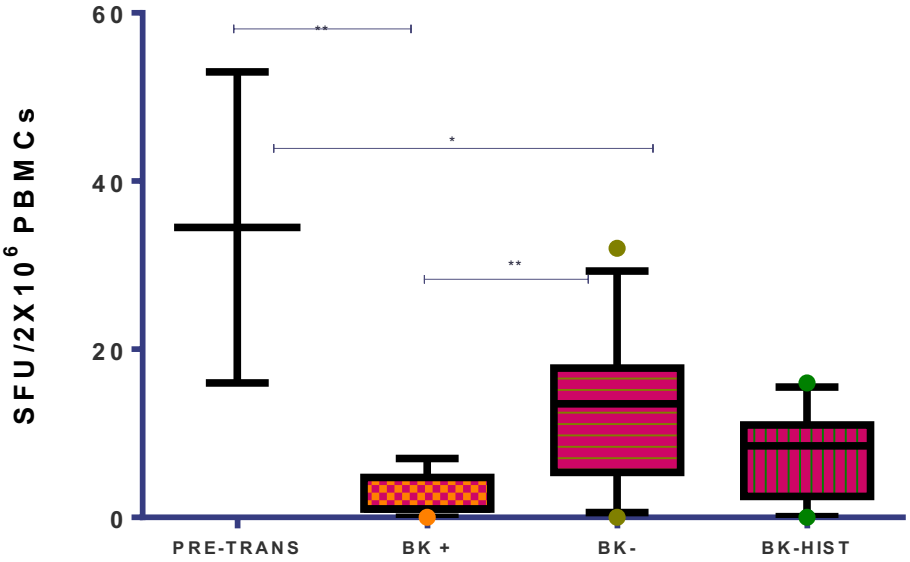


Figure 3.6.IFN-γ response of PBMC of KT patients

2 pre-transplant patients, 9 BKV positive patients, 12 BKV negative recipients and 10 historical positive for BKV.

*Patients were categorized into four different groups based on BKV infection, stimulated with the BKV peptide pool and compared for SFU by ELISPOT assay. Unpaired t test was used to compare between the patient groups. * p< 0.05, ** p<0.005*

Next, the levels of anti-BKV antibody were assessed among the different patient groups; serum anti-BKV was found to be significantly higher in the post-transplant patients in comparison to the pre-transplant group (P<0.005). In BKV +

recipients, the observed anti-BKV levels were found to be significantly higher than in the pre-transplant group ($p<0.0001$) (Fig. 3.7). Similar results were found for the BKV-group and patients who resolved BKV after transplant (data not shown). Moreover, in post-transplant groups, statistical differences in anti-BKV levels were observed between patients with historical BKV infection and patients without reported BKV infection after transplant ($p<0.005$), whilst no difference in the anti-BKV levels were seen between BK+ and BKV- groups (Fig. 3.7). Interestingly, there seems to be a group of 5 BK- patients with very low antibody levels, with 4 in the pre-transplant group and one in the BKV (+) group.

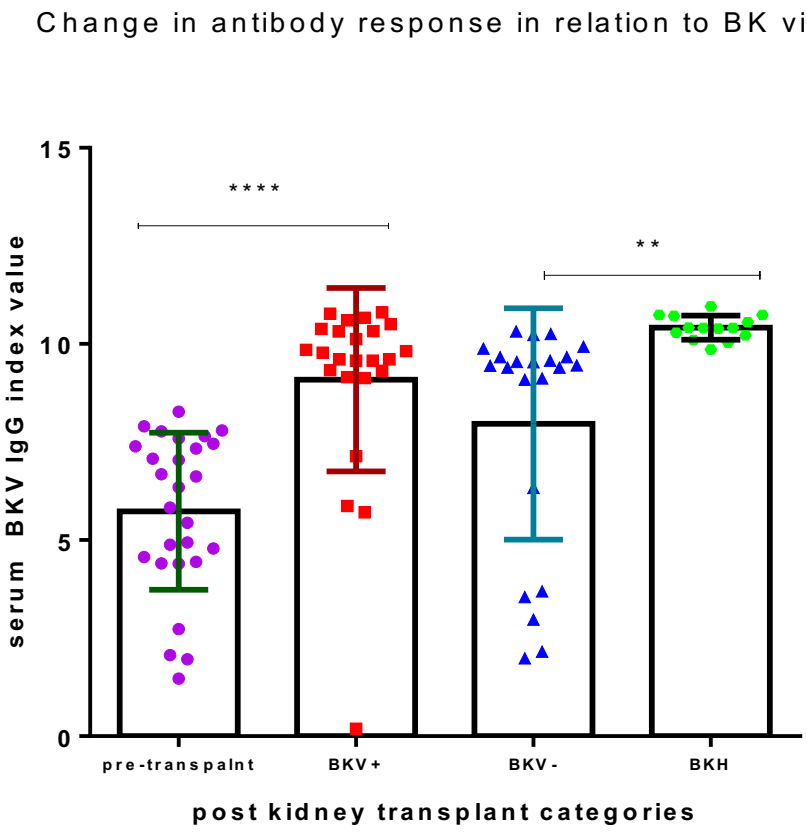


Figure 3.7.BK polyomavirus antibody response

*BKV specific IgG was measured in groups of renal transplant recipients: pre-transplant and post-transplant with positive, negative BKV and recipients with historical BKV infection. An unpaired t test was used to compare between the patient groups (** P<0.005 and *** P<0.00005). BKH = patients with a historical episode of BK viremia.*

3.4.2 Correlation between BKV IgG level and urine viral load

The relationship between BKV viral load and level of anti BKV IgG was investigated in some patients with active BKV virus, either viruria or viremia (n=12). A strong negative correlation was observed between the presence of virus DNA in the urine and the level of immunoglobulin G (r=-0.7, p<0.0005; Fig. 3.8).

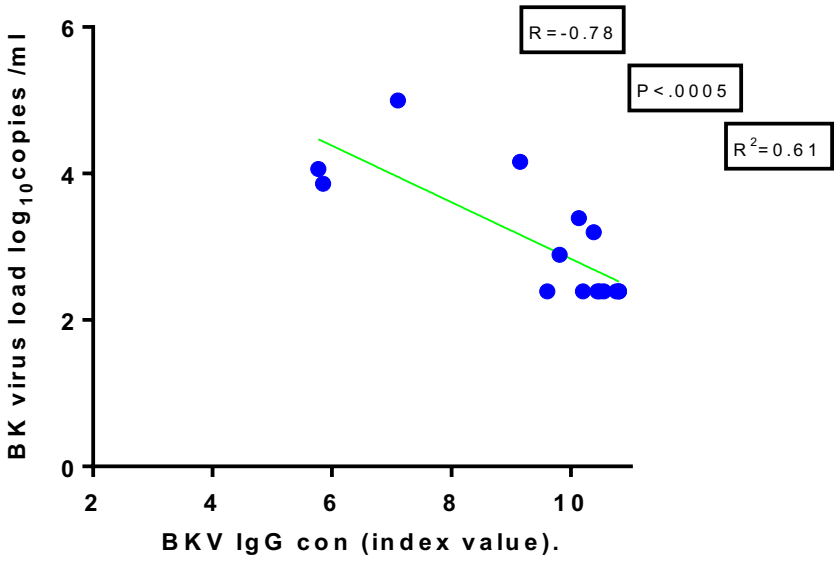


Figure 3.8. Correlation between BKV IgG level and urine viral load

Plot of anti-BKV IgG against BKV viral load where only samples from recipients with active BKV viremia or viruria were compared. The optical density shows a negative correlation with increasing viral load (P<0.0005).

3.5 Discussion

Controlling BKV reactivation is believed to be via the presence of T-cell dependent immune responses (P. Comoli et al., 2006; Lukacher, Moser, Hadley, & Altman, 1999); therefore quantification of T-cell responses and level of humoral response to virus would be of importance in guiding the levels of administered immunosuppressive therapies in BKV-positive patients and assessment of BKV specific cellular immunity and the level of anti BKV IgG in healthy donors using urine BK positivity as a marker for presence of virus.

Here, the presence of BKV specific cellular immunity was assessed and evaluated by measuring T-lymphocyte IFN-γ secretion using an ELISPOT assay and humoral immunity using a semi quantitative ELISA. Immune responses were analysed in healthy control subjects who were found to be either positive or negative for BKV in their urine as a measure of infectivity and who are deemed immune competent. Additionally, the same responses were tested in different categories of renal transplant recipients based on detection of BKV DNA.

The data showed a clear presence of BKV specific T-cell immune responses in healthy subjects with BKV detected in urine in comparison to healthy subjects with no virus detected, who had significantly lower levels. Dekeyser in a similar study utilising BKV seropositive healthy subjects reported that BKV specific immune responses could be detected in 71% of seropositive individuals, with 91% of the response mediated by CD4+ T-cells with only 31% mediated by CD8+ T-cells (Dekeyser et al., 2015). Moreover, the same observation was reported by others (Abate et al., 2010; Chanouzas, Small, Borrows, & Ball, 2018) when studying CMV specific cellular immune responses in uninfected patients with or without immunosuppression, which is applicable to our data as the T-cells have not previously encountered virus antigen if the subjects had always been BKV (-). However, it remains possible that our urine BKV (-) subjects had previously become infected with BKV and either eliminated the virus or it remained dormant, as we tested the urine as a marker for infection.

The specific anti-BKV IgG levels among the above same two groups were similar. Interestingly, a negative correlation was reported between the presence of BKV specific cellular immune responses and anti-BKV IgG levels. The presence of BKV specific T-cell responses could be evidence of efficient control of virus replication

due to efficient immune surveillance in immune competent subjects who encountered the virus. As mentioned above, detection of similar levels of anti-BKV IgG in both groups could be due to previous elimination or latency of the virus in the urine of negative individuals as seroprevalence in the healthy population reaches up to 87% (A. Egli et al., 2009).

In renal transplant recipients, the pathophysiology of BKV reactivation is still unclear and many factors have been suggested to influence the reactivation post-transplant, either host dependent, virus dependent or exterior modulator dependent such as immunosuppression (H. H. Hirsch & Steiger, 2003).

Our results indicated that patients with persistent or increasing BKV load (viremia) showed significantly lower BKV specific cellular immune responses compared to the other tested groups which could be a result of impaired immunity due to immunosuppression, agrees with the work of Comoli et al who showed that patients with ongoing viremia demonstrated the lowest response (P. Comoli et al., 2006) and another very recent study (Bae et al., 2020a). This may be because the actively viremic patients have not had long enough to generate a strong cellular response to BKV in the presence of ongoing immunosuppression. As the historical group showed a good cellular response in comparison to the viremia group which suggests a relationship between recoveries of cellular immunity in parallel with resolution of viremia. Moreover, results were consistent with restoration of cellular immunity in response to reduction of immune suppression as patients with resolved viremia showed high cellular responses. Also, it is worth taking into account other factors that may influence cellular immunity such as age, sex and prescribed medications.

Next, regarding anti-BKV IgG levels among the patient groups, humoral responses were detected in pre-transplant groups; however, significantly higher levels were observed in post-transplant groups: viremia patients, BKV negative patients and the group who had resolved virus infection regardless of the immunosuppression induction.

However, in both the pre-transplant and BKV- groups there was a small subset of around 20% of patients with very low specific antibody levels. These may never previously have been exposed to BKV. Similarly, there were several patients in the viremia group showing low levels of anti-BKV antibodies. One possibility is that

these were initially BKV (-) patients who had received a kidney from a BKV (+) donor and had not yet produced a secondary antibody response due to receiving immunosuppressive therapy. It would be of interest to test anti-BKV IgM levels in these patients as an indication of whether they have made a primary immune response to the virus. Specific anti-BKV secretory IgA levels would also be relevant as BKV infects the urothelium which is part of the mucosal immune system.

In these investigations of both cellular and humoral immunity, it was observed that there is a relationship between anti-BKV IgG levels, urine BKV DNA and specific cellular immunity. It is proposed that the presence of virus, as detected by viral DNA, leads to induction of an anti-BKV IgG response during immunosuppressive therapy treatment (absence of cellular immunity) but this humoral response doesn't seem to protect from virus reactivation and progression of BKV viremia. However, with the reduction of immune suppression cellular immunity is restored as supported by previous findings (Hammer et al., 2006; Sundaram Hariharan et al., 2005; H. H. Hirsch et al., 2006).

CHAPTER FOUR

4 Human leucocyte G expression after BKV infection

Introduction

In the early 1970s BK polyomavirus was initially identified from kidney transplant recipients (KTR); the virus causes asymptomatic infection in early age with a seroprevalence up to 90% (Adrian Egli et al., 2009). Following primary infection, the virus establishes latency in the uroepithelial tissue. In immunocompromised patients the virus replicates and causes BKPyAN in 1-10% of recipients (Egli et al., 2007; H. H. Hirsch & Steiger, 2003).

In this chapter, the hypothesis that HLA-G expression and soluble HLA-G both have a role to play in BKV immune evasion was tested. So, the expression of cell surface HLA-G and soluble plasma HLA-G (sHLA-G) were evaluated in PBMCs from healthy individuals with known BKV status and KTRs with BKV viremia to a BKV peptide pool. The participant urine samples were tested for BKV DNA using quantitative real time PCR (qPCR) and blood samples were collected from health subjects who were urine positive and KTRs with positive plasma, then PBMC isolated and stimulated with BKV peptides for three days before phenotyping the cells.

4.1 Study Samples

4.1.1 Healthy Subjects

Healthy volunteers aged from 18-74, both male and female who work in the Department of Clinical Infection, Microbiology and Immunology were identified through advertisement in the department monthly magazine. They were recruited to donate urine samples after giving written informed consent. Project ethical approval was granted by The University of Liverpool Interventional Ethics Committee. 80 urine samples were collected in 20 ml sterile container and stored at -20ºC until the time of the experiment. Blood samples were subsequently collected from participants whose urine samples tested positive for BK virus DNA.

4.1.2 Renal Transplant Patients

Patients from the Renal Transplant Unit in the Royal Liverpool University Hospital were consented to donate 40 ml fresh blood samples, the samples were collected in heparin tubes then centrifuged for 25-30 min to separate PBMC and plasma.

4.2 BKV screening

Out of the 71 urine samples from healthy subjects that were tested, 5 (7%) tested positive for BKV DNA with variable viral loads and 75 (93.7%) were negative (Fig. 4.1).

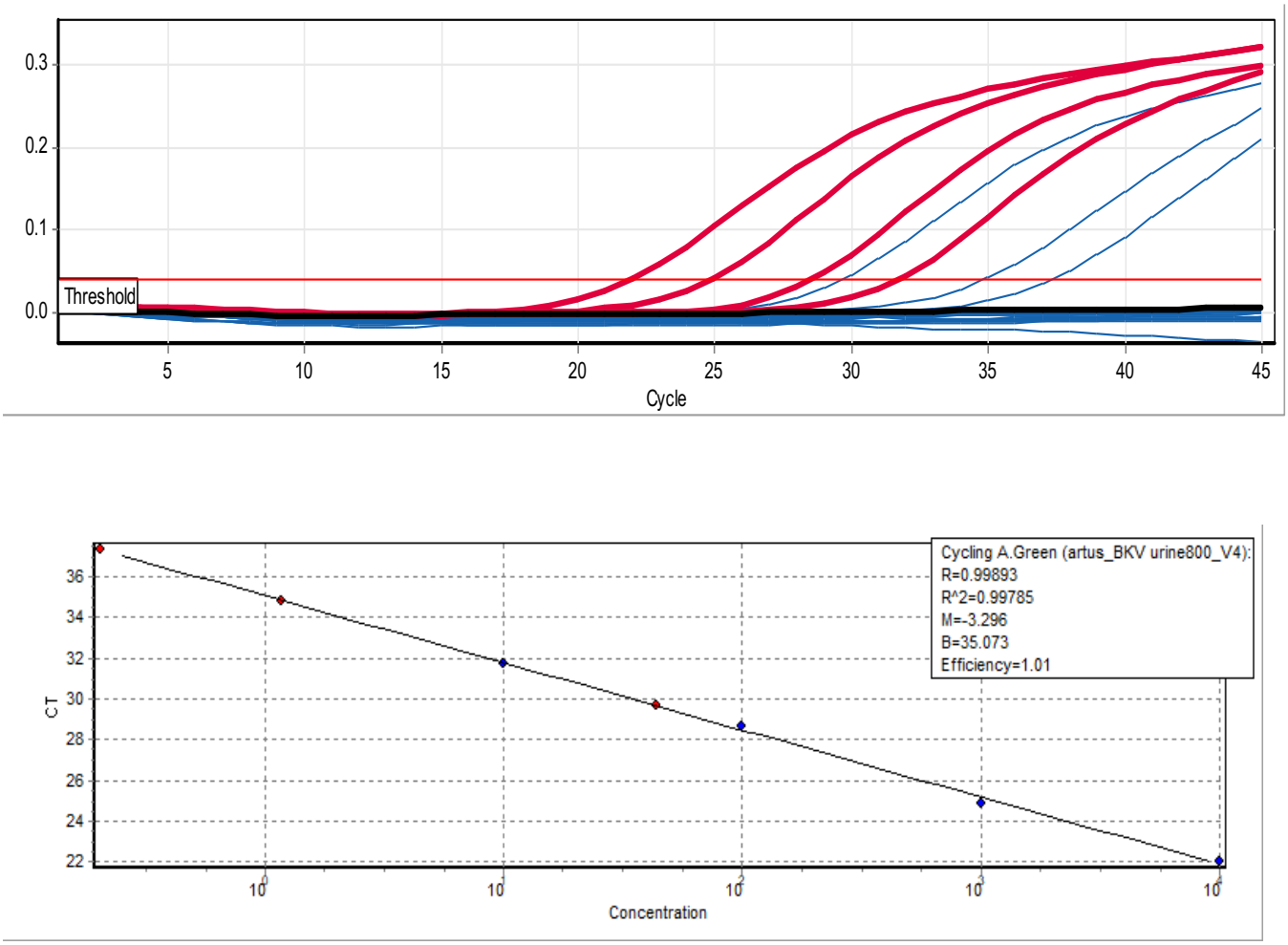


Figure 4.1. Representative RT-qPCR amplification plot

Fluorescent signal against the number of the cycles and generated standard curve by CT value vs the virus load concentration. Includes the amplified sample in the blue line, 4 quantitation standards in the red lines and negative control black line. The lower figure illustrates the standard curve that was generated from the CT of the four BKV standards with known BKV DNA concentration.

4.3 Anti HLA-G antibody validation utilising the JEG-3 cell line -

The monoclonal anti-HLA-G antibody used in the experiment was highly specific for HLA-G with no cross reactivity found with the other HLA molecules. This specific antibody has been validated using the choriocarcinoma cell line (JEG-3) known to express HLA-G (Yie, Li, Li, Xiao, & Librach, 2005). Cells were incubated in culture media for 30 min with either an isotype control antibody, the anti-HLA-G antibody or unlabelled. The cells were then acquired by Accuri C6 flow cytometry and analysed. The percentage of cells expressing HLA-G was found to be 87.47% while the background expression on the unlabelled cells was 0.68% (Fig. 4.2). The validation was performed by our laboratory and we used the same batch of the tested anti-HLA-G antibody as previously described (Fig. 4.2) (Z. Albayati et al., 2017).

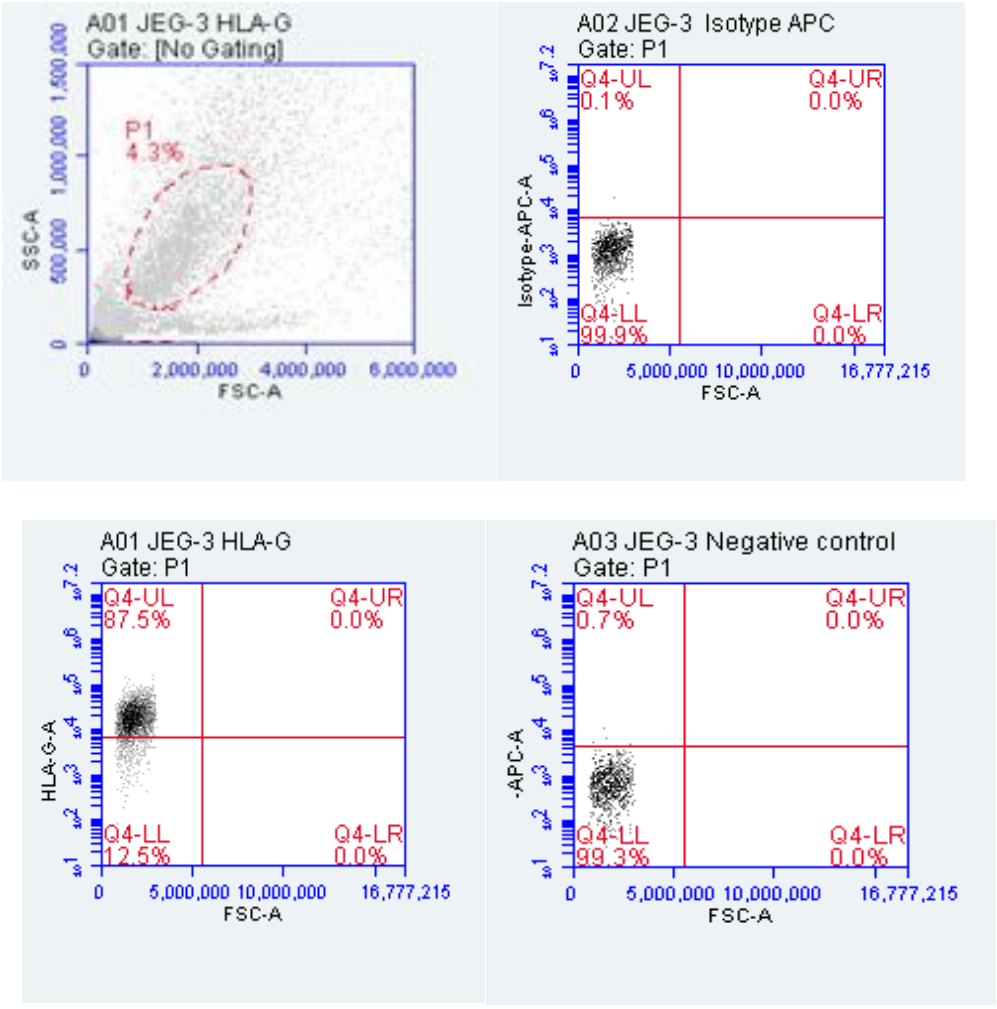


Figure 4.2. Flow cytometry plot of JEG-3 carcinoma cells

The cells were labelled with anti HLA-G antibody; the plots in order: gated cells, side against forward scatter, cells labelled with isotype control antibody, cells labelled with anti HLA-G antibody and unlabelled calls used as a negative control.

4.4 HLA-G expression in healthy subjects

4.4.1 HLA-G expression in BKV (+) healthy subjects (resting cells)

Surface HLA-G expression on lymphocyte subsets and monocytes of immunocompetent participants with BKV DNA detected in urine was as follows: the population that showed the highest expression was monocytes with 14.8%±15.5, followed by CD3+CD56+ cells with 9.3%±4.7, then 7.16%±4.2 for CD19+ (B-cells), whilst the cells that showed the lowest percentage were CD3+CD4+ and CD3+CD8+ T-cells with expression levels of 2.6% ±2.11 and 3.3% ±2.7 respectively (Fig. 4.3).

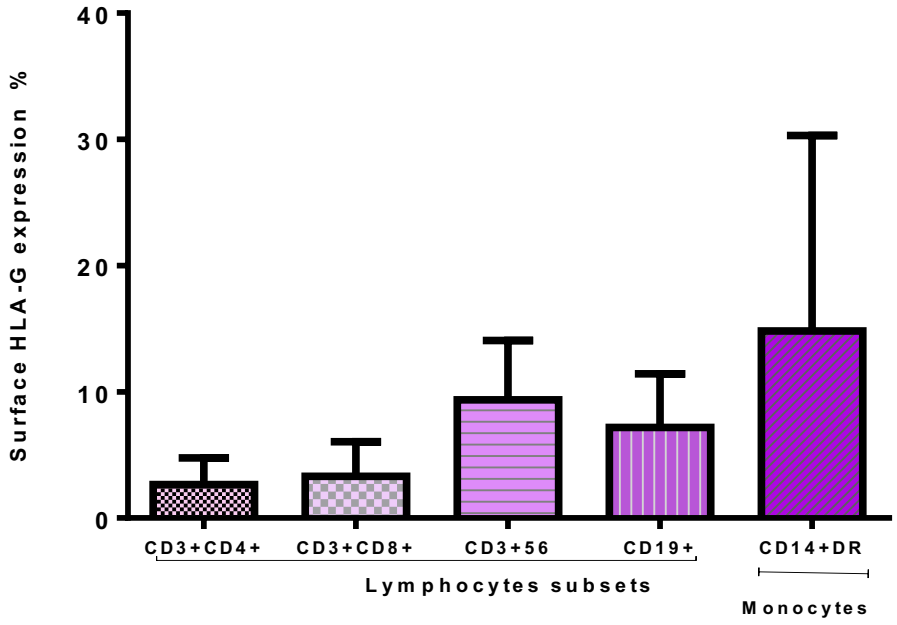


Figure 4.3. Mean % of cells expressing surface HLA-G on resting PBMC.

Cells were isolated from immunocompetent subjects with BKV (+) urine n=4.

4.4.2 HLA-G expression in BKV (+) healthy subjects PBMCs after culture with BKV peptide pool.

After a 3-day incubation of isolated cells with BKV peptides, expression of HLA-G on the surface of the monocyte population was the highest among the PBMCs populations with 11.9%±3.7 followed by CD3+CD56+ T-cells with 8.55%±8.5, then CD19+ B-cells with 6.6%±2.7. CD3+CD8+ T cells showed the lowest expression 3.7%±3.3 followed by CD3+CD4+ T-cells with 3.8%±3.7 (Fig 4.4).

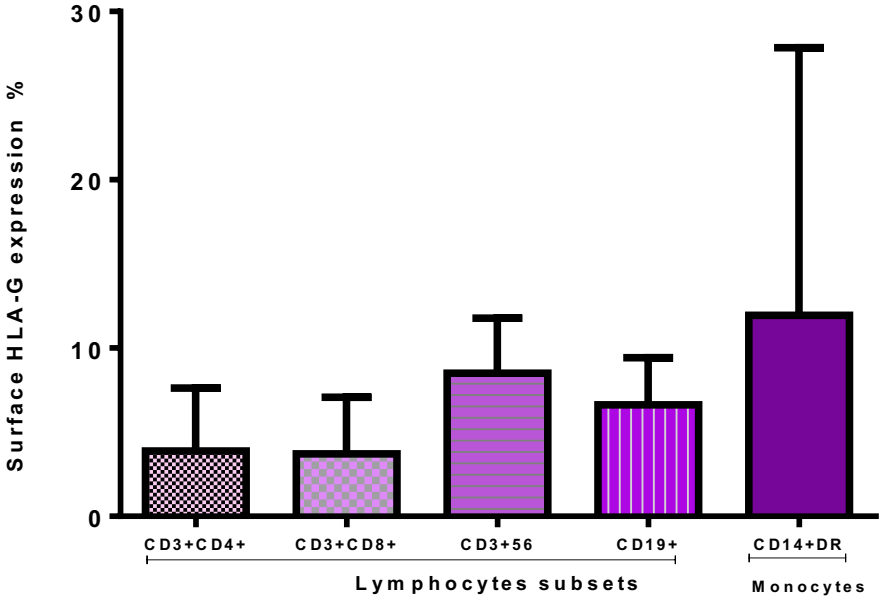


Figure 4.4. HLA-G expression chart of PBMCs after culture with BKV peptide pool, each bar represents the expression on individual subsets.

4.4.3 HLA-G expression in resting and cultured PBMCs from BKV (+) subjects

After three days incubation of cells with BKV peptides, the expression of HLA-G was compared in the cultured cells and resting cells to evaluate the effect of the BKV peptide pool on the various cell populations. It was observed that the proportion of surface HLA-G+ cells increased in some of the lymphocyte populations such as CD3+CD4+ and CD3+CD8+ cells but this was not significantly different ($p=0.06$ and $p=0.08$, respectively). Other populations showed no difference in HLA-G expression before and after culture ($p=0.8$, $p=0.86$ and $p=0.83$ for CD3+CD56+, CD19+ and CD14+ DR+ cells, respectively) (Fig. 4.5).

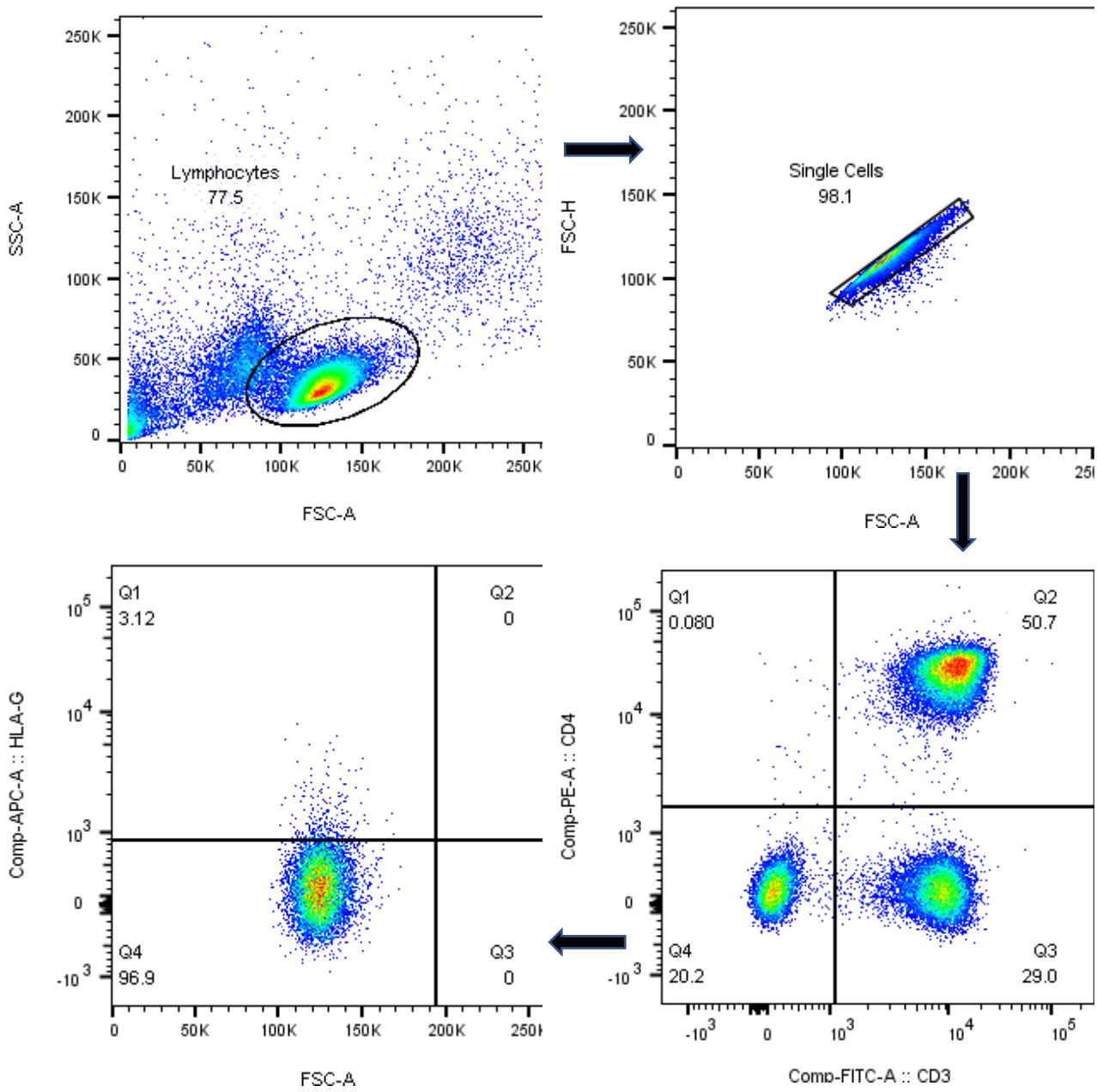


Figure 4.5. Flow cytometry gating strategy

Initially, FSC vs SSC gating was used to identify the distinct cell types (lymphocytes). Then, A forward scatter height (FSC-H) vs. forward scatter area (FSC-A) density plot used to exclude doublets. After that, two parameter density plots were generated with two axis that represent particular markers within our sample to identify CD3+CD4+ cell populations. Finally, from the identified CD3+CD4+ population, HLA-G + cells were represented against FSC-A.

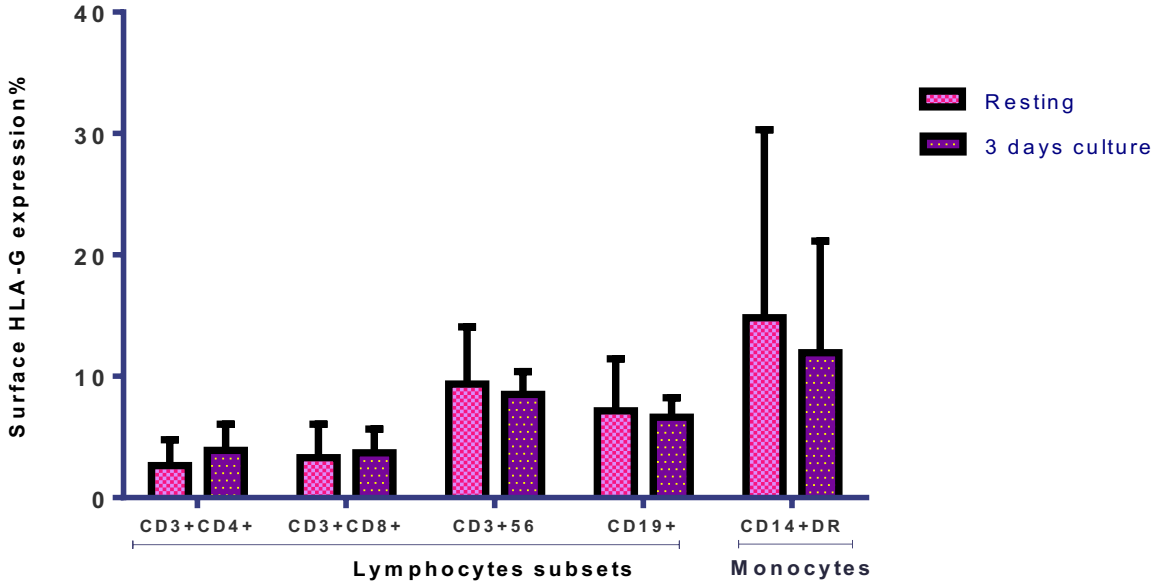


Figure 4.6. Comparison of surface HLA-G expression on resting PBMCS with and without culture with BKV peptides,

in healthy subjects (n = 4) with detected BKV in urine. P values were calculated using paired multiple t-tests

4.4.4 Expression of HLA-G on resting PBMCs from BKV (-) subjects.

Likewise, the expression of HLA-G was evaluated in healthy subjects who were found to be urine negative for BKV DNA. CD14+DR cells showed the highest levels of expression of HLA-G with mean of 29.9%±14 (P<.0001), whilst the other populations showed relatively lower expression; CD3+CD4+ (2.76%±3.3), CD3+CD8+ (2.40%±2.00), CD3+CD56+ (2.36%±3.6) and CD19+ (3.86%±2.3) (Fig. 4.7.

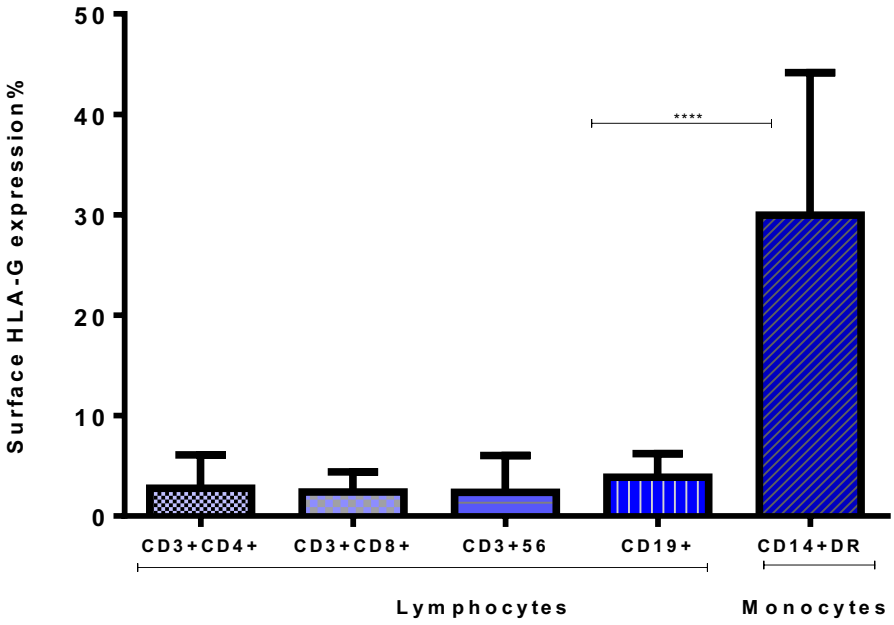


Figure 4.7. HLA-G expression on resting PBMCs isolated from healthy subjects with BKV - urine. One-way ANOVA was used to compare between means.

4.4.5 Expression of HLA-G on peptide-stimulated PBMCs from BKV (-) subjects

As performed previously with the positive subjects, PBMCs from the BK (-) group were stimulated with BKV peptides to assess any changes in the expression of HLA-G on the cell surface. Generally, the cell subsets show different levels of expression; a greater proportion of CD14+DR cells expressed HLA-G (27%±22.3) whilst CD3+CD4+ expressed the lowest (3.4%±3.3). Mean percentage of other cell types expressing HLA-G were: 10.4%±12, 6, 5%±8.6, and 4.6%±5.8 for CD3+CD56+, CD3+CD8 and CD19+ cells, respectively. The difference between CD14+DR+ cells and the other cell populations was statistically significant ($p<0.05$) (Fig. 4.8).

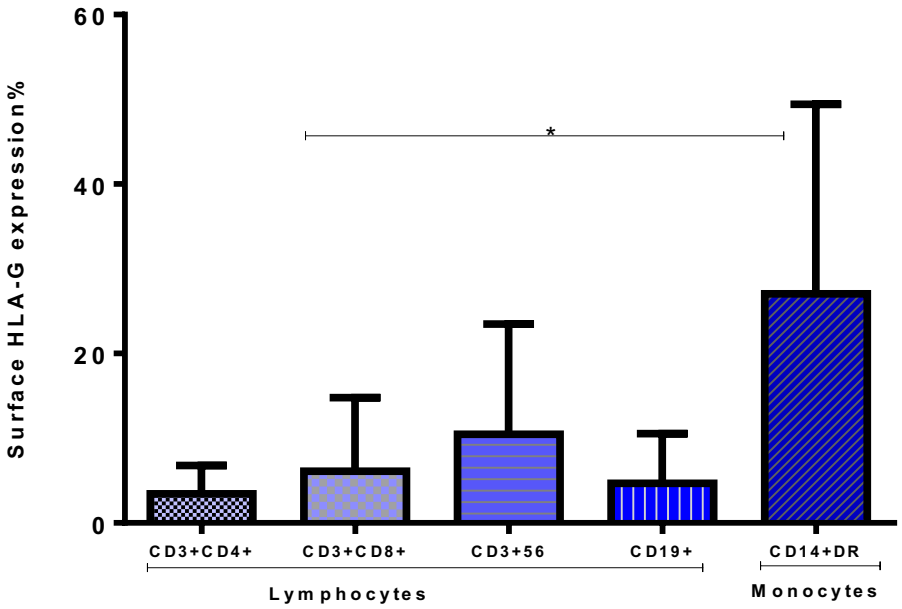


Figure 4.8. HLA-G expression on PBMCs isolated from healthy BKV (-) subjects and cultured with BKV peptide.

One-way ANOVA test was performed to compare the means.

4.4.6 Comparison of HLA-G expression in resting PBMCs from urine BK (-) healthy subjects and after culture with BKV peptides

Here, HLA-G expression before and after BKV peptide stimulation was compared in PBMC isolated from urine BKV (-) individuals. A slight upregulation of HLA-G expression was observed after incubation with virus peptides in most of the cell populations, including CD3+CD56+ and CD3+CD8+ cells. However, the differences were not statistically significant after testing using a standard paired t-test (Fig. 4.9).

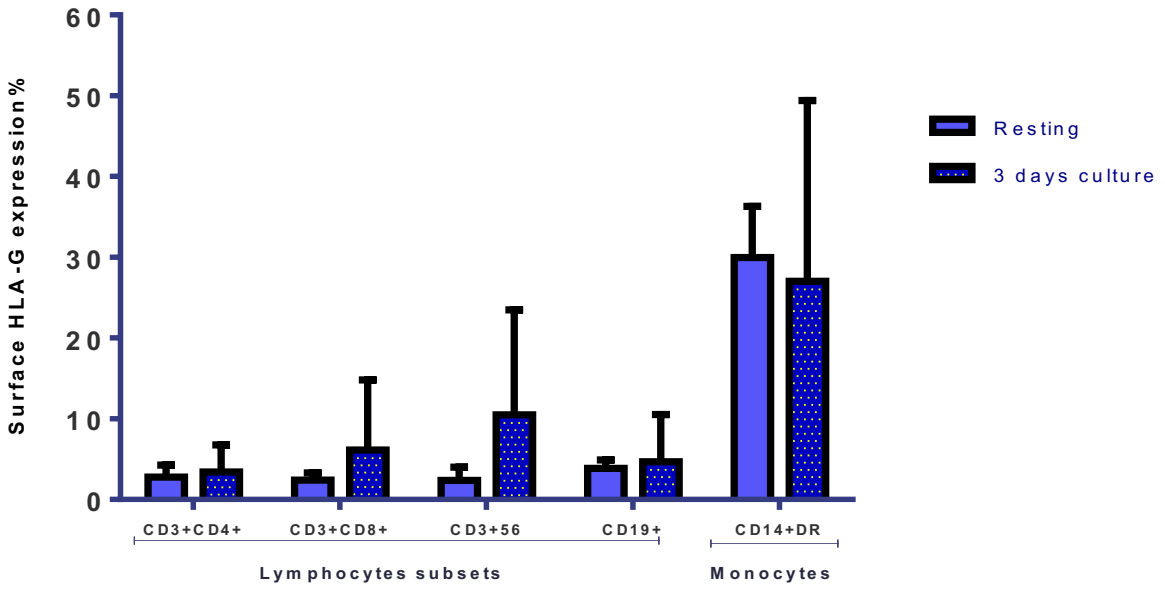


Figure 4.9. Comparison of surface HLA-G expression on resting PBMC and after culture with BKV peptide*, in healthy subjects who tested BKV (-) in their urine sample.*

4.4.7 HLA-G expression by resting PBMCs in urine BKV (-) and BKV (+) subjects

After assessing HLA-G expression in BKV (+) and BKV (-) subjects, the two groups were compared for HLA-G expression regarding resting cells. Some subpopulations from subjects with BKV (+) urine exhibited higher HLA-G expression levels in comparison to the BKV (-) group, such as CD3+CD8+, CD3+CD56+ and CD19+ cells. However, some populations showed no difference in expression levels, including CD3+CD4+ cells. Interestingly, monocytes from

BKV (-) subjects expressed more HLA-G than those from BKV (+) subjects (Fig. 4.10). However, none of these differences reached statistical significance.

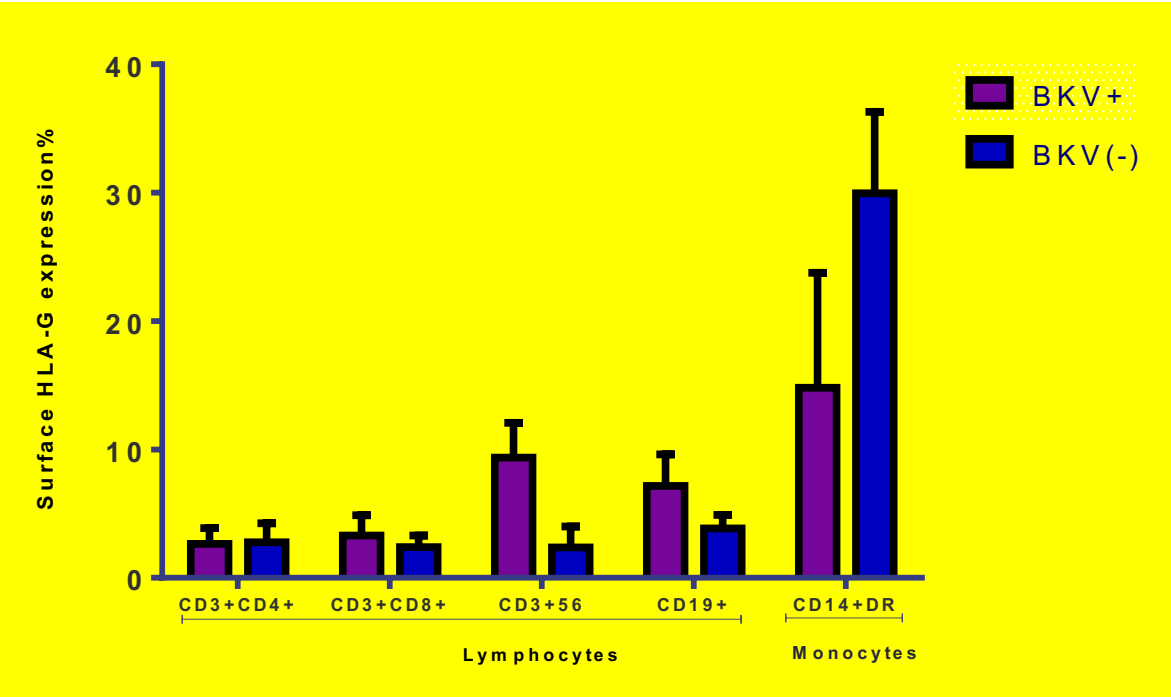


Figure 4.10. HLA-G expression on resting PBMCs subpopulations from healthy subjects with BKV + and BKV- urine.

Paired t-test was used to calculate the statistical significance.

4.4.8 HLA-G expression by PBMCs in BKV (-) and BKV (+) subjects (after culture with BKV peptides

On the third day of incubation with BKV peptides, HLA-G expression was assessed in two tested groups. The percentage of HLA-G+ cells is illustrated in Figure 4.11 Some subpopulations from the urine BKV (-) group showed an elevation of HLA-G expression in comparison to the BKV (+) group: CD3+CD8+, CD3+CD56+, and CD14+DR cells. However, T-helper lymphocytes and B-cells showed no difference in HLA-G expression between the two groups.

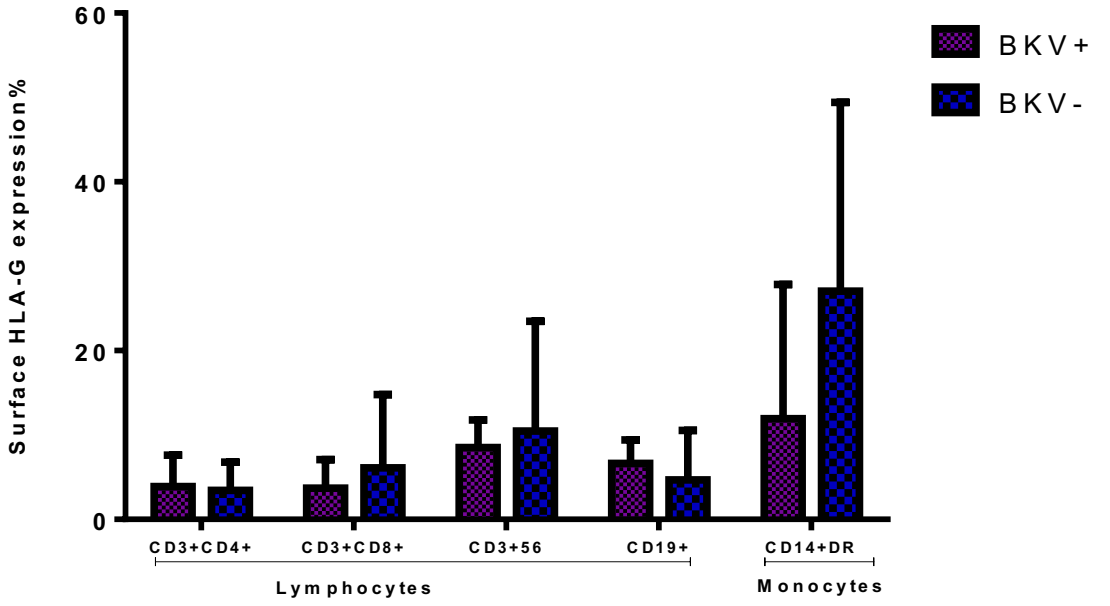


Figure 4.11. HLA-G expression on PBMC subpopulations from with BKV + and BKV- urine after culture with BKV peptides.

Paired t-test was used to calculate the statistical significance (P>0.05).

4.5 HLA-G expression in KTR recipients

After investigating HLA-G expression in immunocompetent subjects with BKV infection, we went on to study the same expression on post renal transplant recipient with BKV infection, as the main difference between healthy and patient groups is administration of immune suppressants and consequently attenuation of the normal immune response.

Four patients with confirmed post-transplant BKV viremia and three recipients who never tested positive for BKV infection after transplantation were evaluated for HLA-G cell surface expression. As with the healthy subjects, PBMCs were cultured

with BKV peptides for three days and HLA-G expression was compared in the resting cells and the cultured cells (Fig. 4.12).

4.5.1 HLA-G expression on resting PBMCs from KTR with BKV viremia

HLA-G cell surface expression in transplant patients was found to be significantly higher than in healthy subjects. However, the expression was found to be variable among lymphocyte and monocyte subpopulations. CD3+CD56+, CD14+DR and CD3+CD4+ cells showed higher expression, 68%±12, 55%±7.4 and 52%±21,4, respectively, whilst CD3+CD8+ and CD19+ cell HLA-G expression was found to be 38%±8.3 and 48%±9.2, respectively (p=0.06 using one-way ANOVA test).

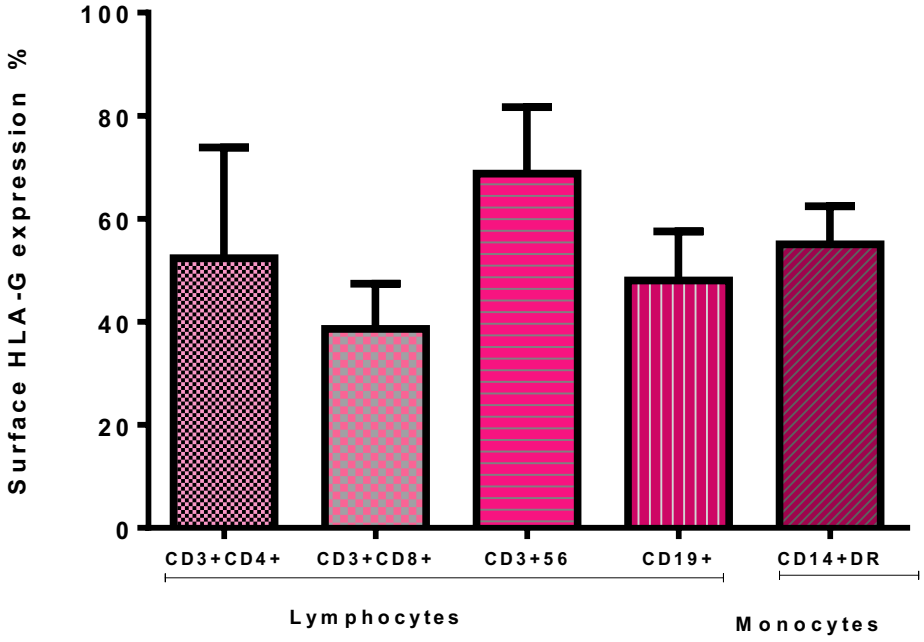


Figure 4.12. HLA-G expression on resting PBMCs from renal transplant recipients with BKV viremia.

A one-way ANOVA was used to compare between means (p>0.05).

4.5.2 Comparison of HLA-G expression in resting cells of healthy donors and transplant patients.

Here, HLA-G surface expression was compared between healthy subjects with BKV DNA and renal recipients with BKV viremia, in various subsets of lymphocytes and monocytes. Most lymphocytes subsets show that transplant patients with BKV viremia express higher levels of HLA-G compared to healthy individuals; CD3+CD4+ p=0.011, CD3+CD8+ p=0.001, CD3+56 p=0.0006, CD19+ p=0.001 and CD14+DR p<0.005.

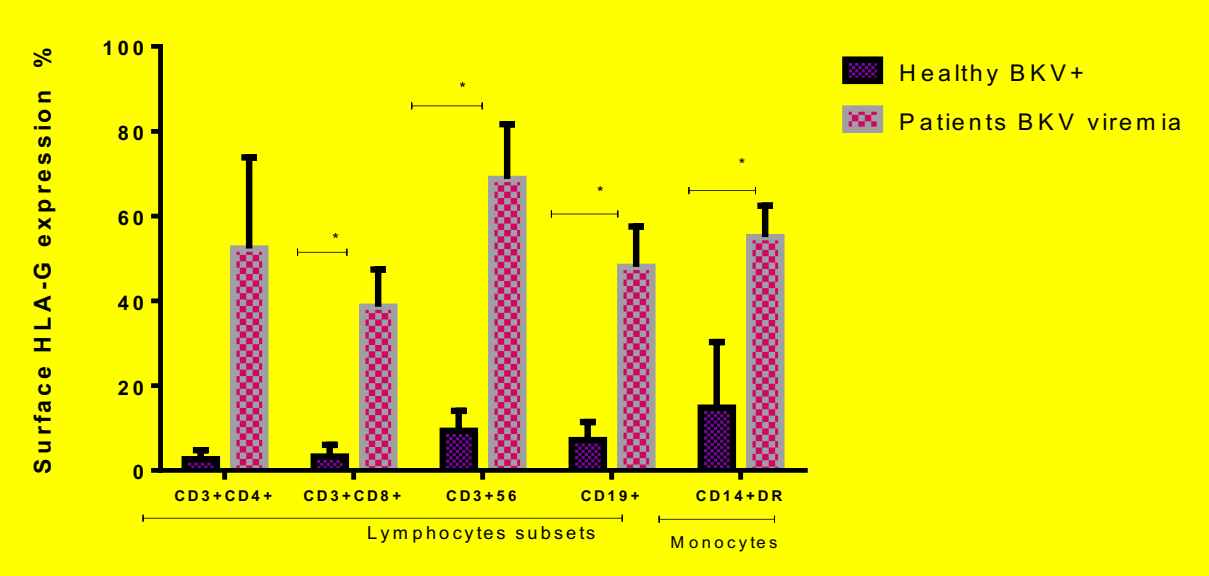


Figure 4.13. Comparison of Surface HLA-G expression in healthy BK+ donors and renal recipients with BKV viremia.

Multiple t –test was used to compare the mean of surface HLA-G levels in lymphocyte subsets and monocytes.

4.5.3 HLA-G expression in PBMCs cultured with BKV peptides.

After three days culture of the patient's lymphocyte subsets with BKV peptides to measure HLA-G expression on monocytes and some lymphocyte subsets; HLA-G expression was generally found to decrease in comparison to the resting cells; the highest expression was reported on CD3+CD56+ sub-populations with 47%±28.9 followed by CD14+ 43%±3.1, then CD3+CD8+, CD19+ and CD3+CD4+ with 35%±13.7, 30%±15.5 and 27%±11.3, respectively (P= 0.4)

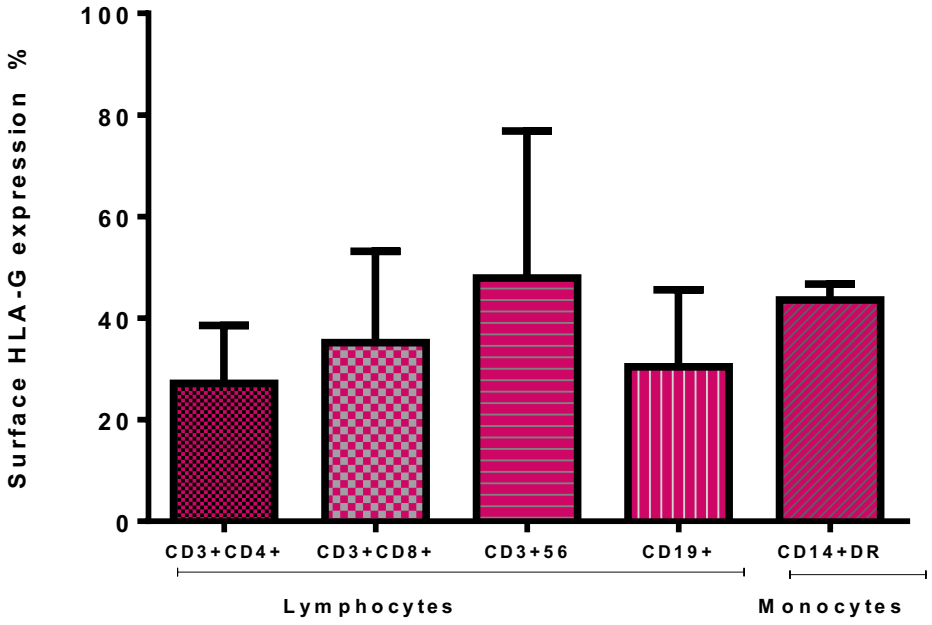


Figure 4.14 HLA-G expression on PBMCs from renal transplant recipients with BKV viremia, cultured with BKV peptides.

One-way ANOVA test was performed to compare the means (P= 0.4).

4.5.4 Comparison of HLA-G expression on resting and cultured PBMCs from KTR individuals with BKV viremia.

Next, here we compared the expression between resting and cultured cells to identify whether HLA-G expression patterns could be altered on specific cell types in KTR patients presenting with BKV viremia. Interestingly, in patients presenting with BKV viremia we could identify a decrease in HLA-G expression after the incubation with BKV peptides even though this elevation was not significant except for monocyte population (p=<0.05. Fig 4.15).

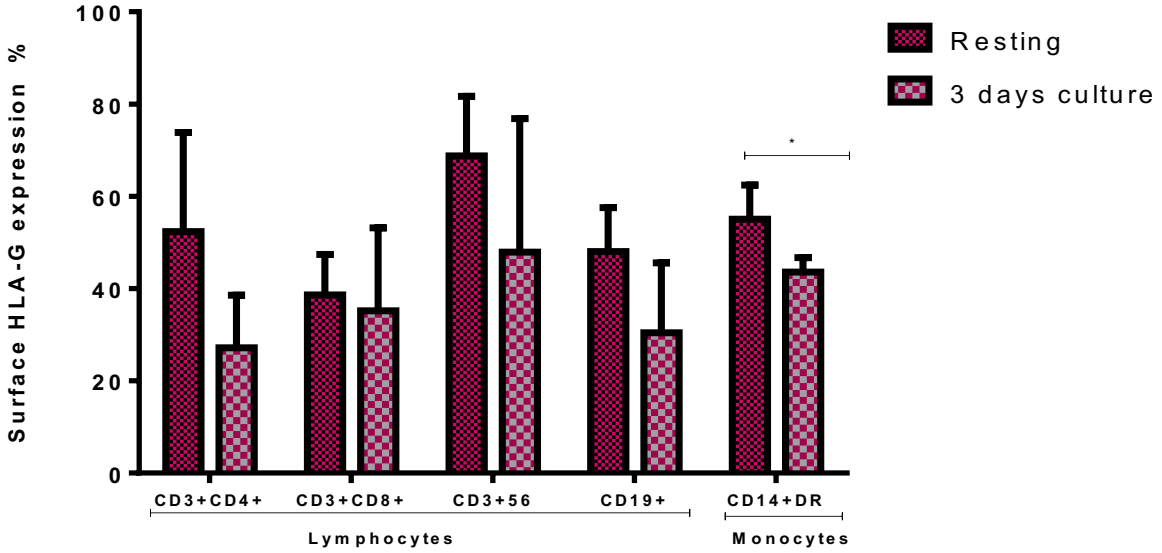


Figure 4.15. Mean proportions of HLA-G+ cells in subpopulations of PBMCs resting and post culture with BKV peptides.

A paired multiple t-test was used (P<0.05).*

4.5.5 HLA-G expression in resting cells of KTR individuals who never demonstrated post-transplant BKV.

Similar to the viremia recipients, HLA-G was evaluated on monocytes and lymphocyte surfaces of PBMCs isolated from recipients who never had a BKV episode. Monocytes had the highest proportion of positive cells among all

leucocyte sub-populations with 51%±6.0 CD3+CD56+ and CD19+ cells showed approximately the same levels of HLA-G expression, 32%±3.6 and 31%±2.5 respectively. CD3+CD4+ and CD3+CD8+ were found to be the lowest with 13%±3 and 11%±2.3, respectively (P<0.0001; Fig 4.15).

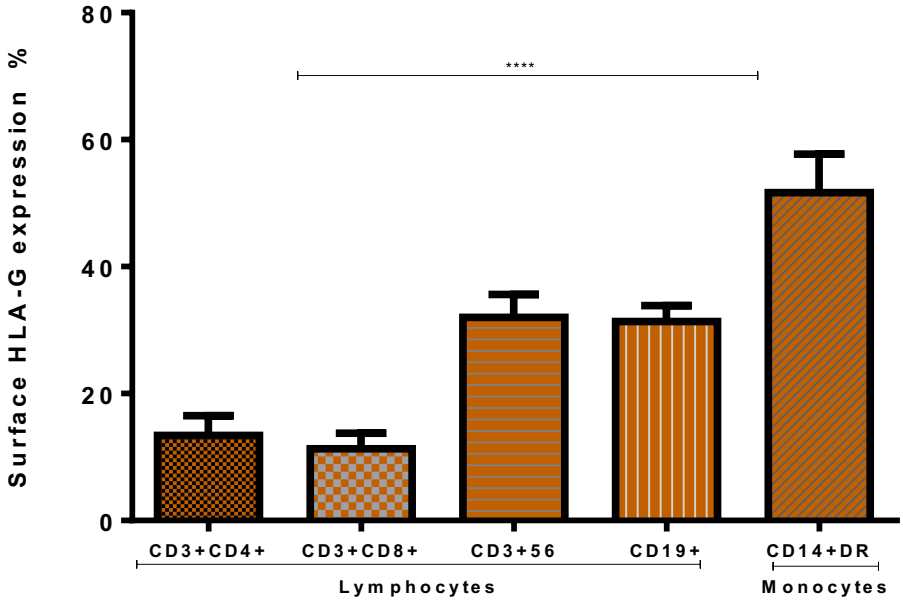


Figure 4.16. HLA-G expression on resting PBMCs isolated from renal transplant recipients who never had a BKV episode after transplantation.

*A one-way ANOVA was used to compare between means (**** P<0.0001).*

4.5.6 HLA-G expression in PBMC from KTRs cultured with BKV peptides who never demonstrated post-transplant BKV

Cell sub-populations from recipients who never demonstrated BKV infection after transplantation showed variable HLA-G expression patterns after culture with BK peptides; monocytes had the highest with mean expression of 61%±4.2, followed

by CD3+CD56+ and CD19+ cells with relatively similar expression of 37%±2.4 and 35%±4.2 respectively. The lowest expression was reported for CD3+CD8+ and CD3+CD4+ sub-populations with means of 14%±0.49 and 12%±0.7, respectively ($p<0.0001$; Fig 4.16).

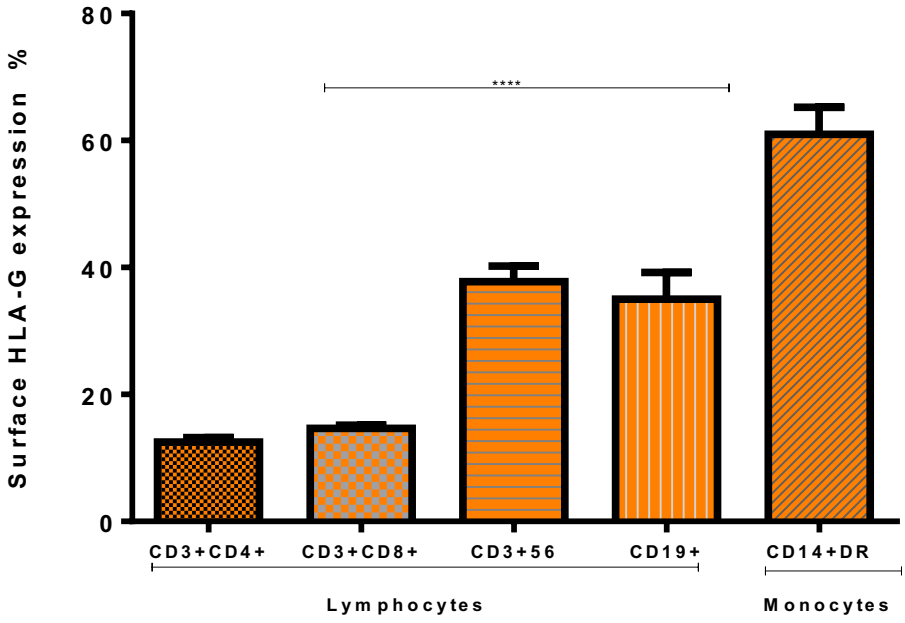


Figure 4.17. Histogram of mean %HLA-G+ cells in PBMC from KTRs who never a BKV episode had cultured with BKV peptides.

P value was calculated using one-way ANOVA test ($p<0.0001$).

4.5.7 Comparison of HLA-G expression on resting and cultured PBMC with BKV peptides in KTR individuals who never had post-transplant BKV

After comparison of HLA-G expression pre- and post-stimulation with BKV peptide, a slight upregulation of expression after induction with the virus antigens was observed in most of the PBMC sub-populations. However, no statistically significant difference was observed after using a paired t-test (Fig. 4.17). So, among the never had BKV recipients the HLA-G expression was not altered with incubation with BKV peptides

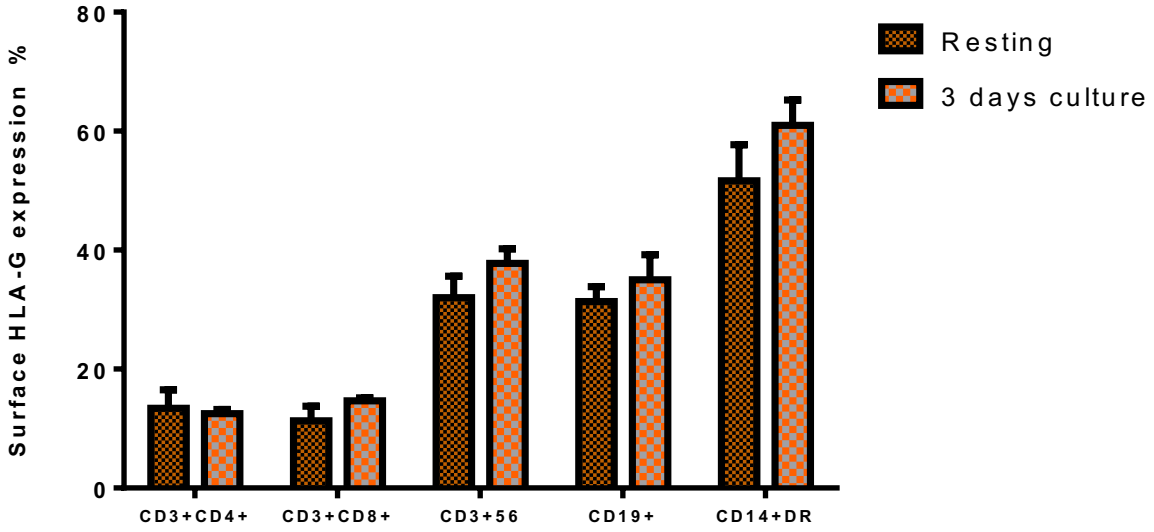


Figure 4.18 Comparison of surface HLA-G expression on resting PBMC and after culture with BKV peptides, in renal transplant recipients negative for a BKV episode.

4.5.8 HLA-G expression on resting PBMCs in KTR individuals with or without BKV viremia

After comparison of HLA-G expression between KTRs who never developed BKV viremia, we then studied two groups of renal transplant recipients, with BKV

viremia and without, to identify the influence of the BKV viremia on HLA-G expression. In general, the viremia group demonstrated a higher percentage of HLA-G-expressing cells in comparison to the control group. CD3+CD8+ T-cells showed a significant difference between the two groups of recipients ($p<0.001$) and the same was found for the CD3+CD56+ population ($p<0.001$). Other cell populations such as CD3+CD4+ and CD19+ also showed a smaller but still significant difference in HLA-G expression patterns ($p<0.05$). However, the expression on monocytes did not differ between the tested groups (Fig. 4.18).

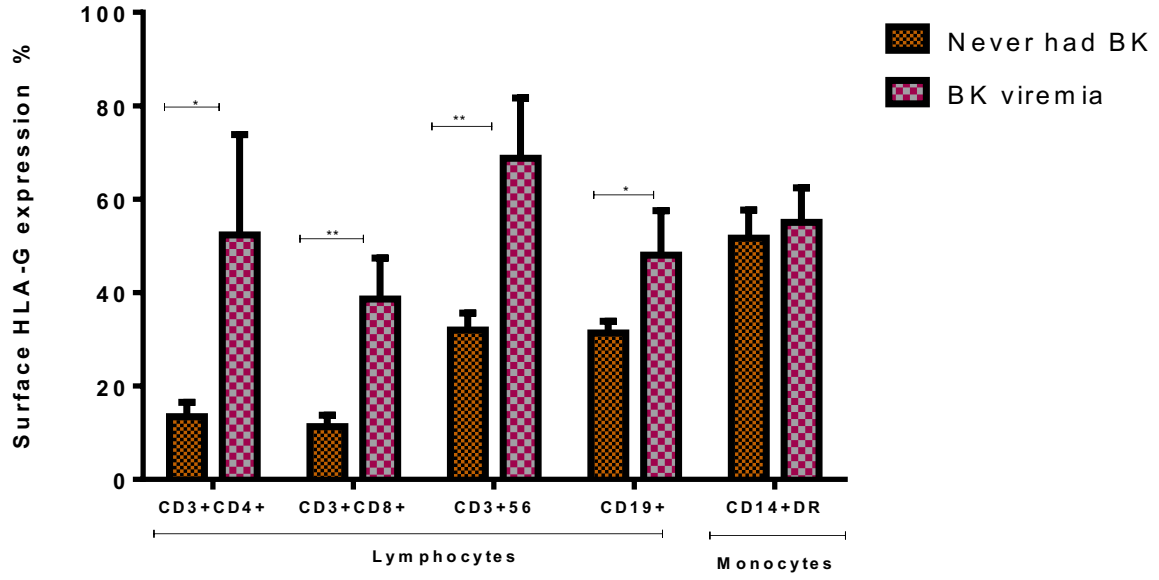


Figure 4.19. Histogram illustrating the HLA-G expression in resting PBMC.

PBMC isolated from two groups of renal transplants recipients: with BKV viremia and without. Paired multiple t test was used to compare between the tested groups (P<0.05; ** P<0.001).*

4.5.9 HLA-G expression on PBMC cultured with BKV peptides in KTR individuals with or without BKV viremia.

Like the resting cells the cultured cells showed differences in HLA-G expression between recipients with BKV viremia and the control group; patients with BKV viremia expressed more HLA-G in most of the PBMC population than the never

had group. Interestingly, higher proportions of all tested lymphocyte sub-populations were found to express HLA-G in the viremia group after culture with BKV peptides, while monocyte populations showed the opposite.

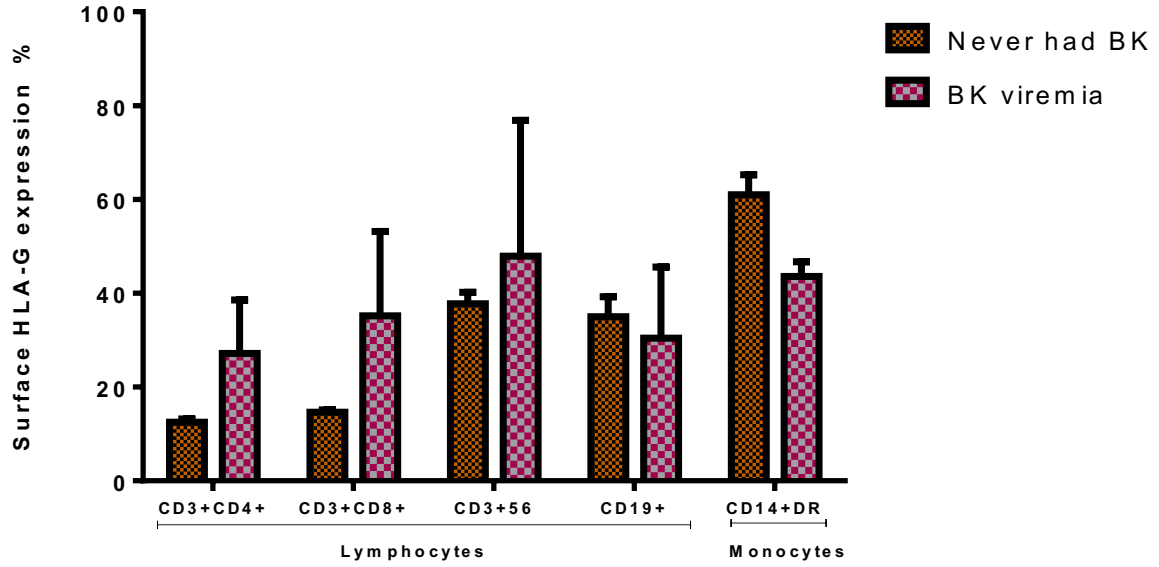


Figure 4.20 Histogram illustrating the HLA-G expression in PBMC from KTRs cultured with BKV peptides

PBMC were isolated from two groups of renal transplants recipients: with BKV viremia and without. Paired multiple t test was used to compare between the tested groups. p=0.1 CD3+CD4+, p=0.2 CD3+CD8+, p=0.66 CD3+CD56+, p=0.71 CD19+ and p=0.007 DR+CD14+ cells.

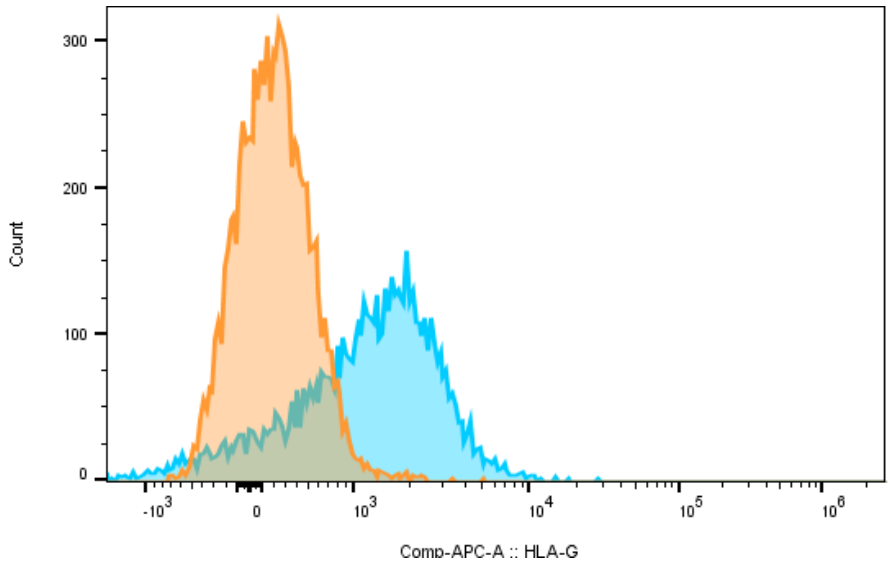


Figure 4.21. Flow cytometry overlay Histogram illustration of HLA-G expression on CD3+CD4+ T lymphocytes.

Healthy donor with BKV shedding shown in orange and renal recipient WITH BKV viremia in blue.

Table 4.1. Summary of significant differences in HLA-G expression in the tested groups and the cell subpopulations.

Figure#	Change	Significance	Study group	Cell population
3		no	Healthy BK+ (resting)	
4		no	Healthy BK+ (cultured)	
5	Increase	no	Healthy BK+ (resting /cultured)	
6		yes	Healthy-BKV- (resting)	CD14+DR
7		Yes	Healthy-BKV- (cultured)	CD14+DR
8	Increase	no	Healthy-BKV- (resting/cultured)	
9	Increase (BKV+)	no	Healthy BKV+/BKV-(resting)	
10	Increase (BKV-)	no	Healthy BKV+/BKV-(cultured)	
12		no	Patient BK+(resting)	
13		no	Patient BK+(cultured)	
14	Increase (resting)	no	Patient BK+ (resting/cultured	
15		Yes	Patients-BK-(resting)	CD14+DR
16		Yes	Patients-BK-(cultured)	CD14+DR
17	Increase	no	Patients-BK- (resting/cultured	
18	Increase	Yes	Patients BKV+/BKV- (resting	CD3+CD4+
19	Increase	no	Patients BKV+/BKV-(cultured)	

4.6 Soluble HLA-G

4.6.1 Soluble HLA-G titres in healthy subjects

Here, we tested the influence of BKV on plasma soluble HLA-G concentrations, so our observations may contribute more understanding of the role these molecules contribute in BKV infection. Using a specific ELISA kit, plasma soluble HLA-G (sHLA-G) was measured from healthy subjects' plasma with BKV (+) or BKV (-) tested urine. Median plasma sHLA-G titres in individuals with BKV (+) urine was 64.7 U/ml whilst it was slightly higher 68.752U/ml in the BKV negative group with no statistically significant difference between the two groups ($p=0.6$; Fig. 4.22).

4.6.2 Soluble HLA-G titres in KTR individuals

After evaluation of cell surface HLA-G expression, we investigated the expression plasma soluble HLA-G in the same study groups: healthy subjects and post renal transplant recipients.

As in the healthy subjects, plasma (sHLA-G) was measured in renal transplant recipients with BKV viremia (n=4) and recipients who never had viremia (n=5). Median plasma (sHLA-G) titre in viremia recipients was found to be 192 U/ml whilst in the BKV (-) group was found to be 214.3 U/ml, although this did not prove to be significantly different (Fig. 4.23).

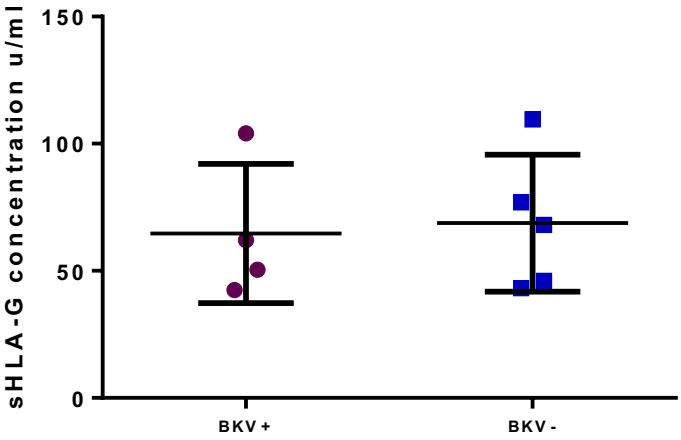


Figure 4.22 Levels of sHLA-G in healthy subjects with (n=4) and without (n=5) urinary BKV.

The middle line represents the median. No statistically significant difference was found after a Mann–Whitney U test was performed.

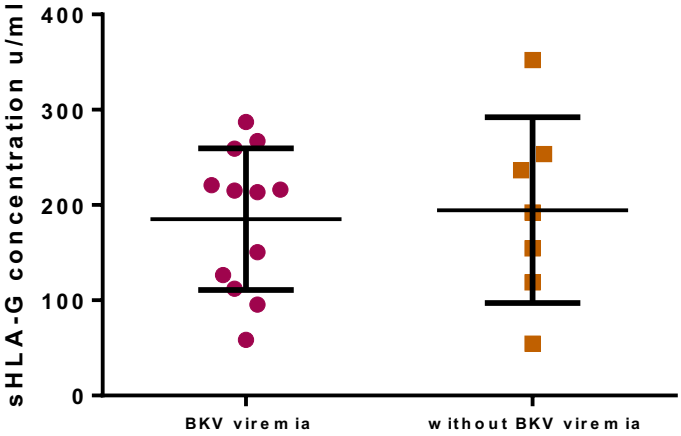


Figure 4.23. Levels of sHLA-G in renal transplant recipients with BKV viremia (n=12) and without (n=7).

Patients without BKV viremia show similar levels of sHLA-G in comparison to viremia patients and where the middle line represents the median. No statistically significant difference was found when a Mann–Whitney U test was performed.

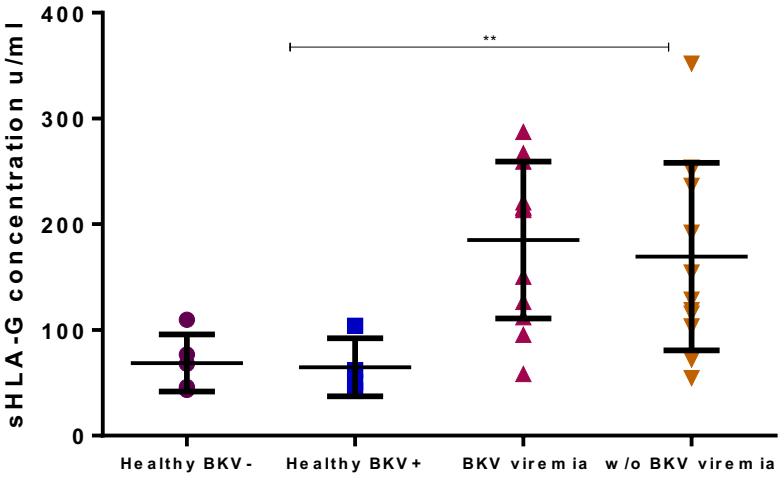


Figure 4.24. Comparison of plasma sHLA-G levels between healthy controls and renal transplant patients with or without BKV viremia.

The graph above demonstrates the difference in sHLA-G levels in healthy donors and renal transplant recipients as; patients show a significant increase in the plasma HLA-G in comparison to the healthy donors. The middle line represents the median. A one-way ANOVA test was performed (P<0. 005); n=28.

4.7 Discussion

It is well known that the immune system has efficient mechanisms of eliminating viral infections and that some viruses have developed several strategies to evade host immune responses. The HLA-G molecule has been reported to influence viral escape from immunity (H.-X. Chen et al., 2011; Gazit et al., 2007). This molecule aids viruses in immune evasion and protecting allografts using two proposed mechanisms; either: inhibition of NK and CD8+ T-cells cytolytic function, or suppression of CD4+ T-cell allo-proliferative responses, that can both be responsible for graft rejection (Rouas-Freiss, LeMaoult, Moreau, Dausset, & Carosella, 2003). Here, the expression of cell surface HLA-G and plasma soluble HLA-G (sHLA-G) was investigated in groups of healthy subjects with and without BKV in their urine and renal transplant patients with BKV viremia as well as those who never demonstrated BKV infection.

Our findings show that there was no significant difference in HLA-G cell surface protein expression between healthy subjects with BKV detected in their urine, and

urine BKV (-) subjects, except for the monocyte population that demonstrated higher levels of expression of HLA-G among all PBMCs populations in both healthy groups. Regarding PBMC cultures stimulated with BKV peptides, HLA-G expression was shown to be slightly higher after incubation with BKV peptides in BKV (-) individuals. Furthermore, even though there was no significant difference, BKV (+) subjects were found to have a higher proportion of HLA-G+ cells than the negative subjects. *In vitro*, Albayati et al showed that HLA-G surface expression increases on several leucocyte subsets with *in vitro* immune stimulation after 7 days of incubation with CMV AD169 protein extract (Zaid Albayati et al., 2018) This observation agrees with a study describing the role of HLA-G in HIV infection, where it has been suggested that monocytes of HIV-1 infected patients express higher levels of HLA-G than a control uninfected group (Lozano et al., 2002). Also, an additional study supports our finding that CD4+ HLA-G+ and CD8+HLA-G+ cells could be found in the peripheral blood of healthy subjects in low numbers during inflammation (Feger et al., 2007). Further studies have also illustrated the role of HLA-G upregulation in controlling host immune responses following viral infection (Cordero et al., 2009; da Silva et al., 2014). Even though our findings do not show a significant difference in HLA-G cell surface protein expression on other lymphocyte populations, it remains a possibility that the slight upregulation observed may become statistically significant when increasing the sample size.

In the field of solid organ transplantation, HLA-G plays a vital role in modulation of induced immune responses. It may play a two-fold role; induction of HLA-G after transplantation has been shown to increase graft survival by down-regulating host alloimmune cells but on the other hand it may help viral immune evasion (Lazarte, Adamson, Tumiati, & Delgado, 2018). In reviewing the literature, many studies have investigated the role of HLA-G in the context of viral infections, and many studied its importance after solid organ transplantation (Créput et al., 2003; Creput et al., 2003; Rouas-Freiss, Naji, Durrbach, & Carosella, 2007). Regarding transplantation, most studies have discussed the role HLA-G expression can play on graft survival. One study revealed that in transplant recipients infected with CMV, those carrying genotypic restrictions relating to lower HLA-G expression patterns had a higher incidence of acute organ rejection (Z.-K. Jin et al., 2012).

Here we investigated the influence of HLA-G induction on BKV viremia patients after renal transplant. It has been observed that viremia patients demonstrated a

significant up-regulation of HLA-G cell surface expression in comparison to those individuals who never demonstrated BKV infection, either in resting cells or after culture with BKV peptide; particularly CD8+ cytotoxic T-cells and CD56+ T-cell populations. Similar to the healthy subjects, the highest expression of HLA-G+ among all subpopulations was reported on the monocyte fraction, which would potentially be able to suppress the function of cells expressing ligands for HLA-G. Also, no significant difference was observed with or without incubation with BKV peptides. These findings are consistent with those previously described (Z.-K. Jin et al., 2012) with regards to a role of HLA-G in CMV infected kidney recipients. It is possible that whole or live virus particles would be required to stimulate an increase in HLA-G expression *in vitro* rather than just the peptide pool used here.

Moreover, our data reported no significant differences in plasma sHLA-G concentrations between BKV (+) and BKV (-) healthy subjects. Additionally, no difference was observed between viremia renal transplant patients and those without BKV viremia. However, in comparison of plasma sHLA-G between healthy subjects and renal transplant recipients a remarkable difference was observed between the two groups as the concentration was significantly higher in the transplant patients. The findings could be explained by the fact that transplant recipients were treated with immunosuppressive drugs after transplantation, in addition to BKV viremia the combination of the two factors could consequently lead to the up-regulation of sHLA-G as previously proposed (Akhter et al., 2012; Levitsky et al., 2009) (Zaid Albayati et al., 2018). Any role of HLA-G in suppressing immune responses to BKV or, in transplant patients, preventing rejection, would be dependent upon cells expressing any of the ligands for HLA-G.

In conclusion, based on our knowledge this is the first clinical report describing the impact of cell surface and soluble HLA-G expression patterns regarding active BKV infection in renal transplant recipients. Our study demonstrates that higher proportions of monocytes express HLA-G than other lymphocyte subsets. Furthermore, patients with active BKV infection showed dramatic increases in HLA-G expression in comparison to recipients who did not encounter infection post-transplantation. And finally, no difference was observed in plasma sHLA-G levels among the tested groups.

CHAPTER FIVE

5 BK virus antibody titres in post renal transplant recipients

Introduction

BK Polyomavirus infection is an asymptomatic infection that is mainly acquired at an early age; the virus seroprevalence reaches up to 90% within the healthy population and decreases with age. After primary infection the virus establishes latency in urothelium and tubular cells which is associated with the long-term presence of anti-BKV IgG in the serum and transitory excretion of the virus in 7-55% of healthy subjects' urine (H. H. Hirsch & Randhawa, 2013; R. Sharma et al., 2016).

In the absence of proper immune surveillance, some virus infections, including BKPyV, may establish reinfection. In solid organ transplantation, induction of immunosuppressive therapy is essential to prevent rejection and graft loss, however, it enhances conditions for opportunistic viruses such as polyomaviruses and herpesviruses to replicate in the absence of an optimal immune response. Reactivation of the virus after transplantation without proper immunity leads to BKV viremia and progression of BK disease.

BKV may cause haemorrhagic cystitis in hematopoietic stem cell transplant recipients and BKV-associated nephropathy (BKVAN) in kidney transplant recipients. However, as yet, no standard method is available to predict BKV infection after transplantation and the most effective treatment to-date is periodic monitoring of the recipient blood for BKV DNA and reduction of immunosuppressive therapy when viremia is detected (Hardinger, Koch, Bohl, Storch, & Brennan, 2010b; H. H. Hirsch et al., 2005).

In renal transplant recipients, the prevalence of detected BKV DNA from urine has been reported to be 30–57%, slightly higher than in healthy subjects (Adrian Egli et al., 2009). Whereas the frequency of BKV detection in blood ranges from 15-30% among renal transplant recipients, only 1-10% of these patients were reported to develop BKVAN and graft rejection (H. H. Hirsch & Steiger, 2003; P. Sood et al., 2012). Currently, in most transplantation departments, diagnosis of

BKV disease and identification of recipients at risk mainly rely on detecting virus DNA in blood, as the presence of BKV viruria does not necessarily mean development of BKV disease unless virus load exceeds $1x10^4$ copies/ml (H. H. Hirsch & Steiger, 2003).

Many factors have been suggested to increase risks of BKV viremia and BKVAN; either pre-transplant factors such as immunosuppressive therapy regime, type of transplant, age, sex, ethnicity, HLA mismatch, acute rejection episodes, underlying diseases and pre-transplant organ cold and warm ischemic time (D. L. Bohl et al., 2008; D. L. Bohl et al., 2005; H. H. Hirsch et al., 2006). Or post-transplant factors such as tacrolimus treatment and anti-rejection drug treatments (H. H. Hirsch et al., 2002).

Several studies have suggested an association between pre-transplant anti-BKV sero-status and the recipient's infection status (D. L. Bohl et al., 2008; S. Hariharan et al., 2005; Randhawa et al., 2008). However, the risk of high or low pre-transplant anti-BKV levels (titres) has not been clearly determined.

Here, we hypothesise that recipient's pre-transplant anti-BKV IgG levels and identification of some recipient HLA types may help in a patients' risk stratification and pre-transplant assessment. We therefore aimed to 1-Measure anti BKV antibody levels before and after transplantation to test prior exposure and changes in antibody responses in relation to any subsequent development of viremia. 2-Compare the incidence of BK viremia in post renal transplant patients expressing different HLA types and with different levels of pre–transplant anti-BK titres.

In this chapter, pre-transplant serum samples from renal transplant recipients had been collected prospectively. The tested groups were identified as recipients with confirmed post-transplant BKV viremia and recipients with BKVAN. Three different samples were collected from each recipient: pre-transplant, during the onset of BKV viremia and post-viremia, preferably more than one year from time of transplant. IgG antibody levels were evaluated quantitatively using standard ELISA with recombinant antigen not cross reacting with other polyomaviruses such as JC and Merkel cell polyomavirus.

5.1 Subject samples

Demographic baseline data were notably similar among the study groups: viremic >1000 copies/ml and the never had viremia group. From a total of 38 renal transplant recipients, 18 (47.0%) developed viremia after transplantation with an average virus load of $5.3x10^3$ copies/ml and peak viremia of $10x10^4$ copies/ml within the first year of transplantation, 15 (39.4%) never had detectable BKV DNA from time of transplant until the end of the study and 5 (13.0%) detected with sustained low virus load. Only three (16.6%) cases from the viremia group who developed BKVAN, and 6 cases of acute rejection were found; half of it were due to complications of BKVAN and the other half due to other cases (Table 5.1). No significant difference was observed between the study groups in relation to age, underlying diseases, immunosuppression therapy and sex.

Table 5.1. Patients' characteristics

		Recipients =38				**Viremia=18**		
		Non-viremia	Viremia	low virus	P-value	No BKPyVAN	BKPyVAN	p-value
No. patients (%)		15(39.5)	18(47.4)	5(13)		15(83.3)	3(16.6)	
Patient's sex (male)		8(53.3)	12(66.6)	2(40)	0.2	10(66.6)	2(66.6)	NS
Age (mean±/-SD)		58.17±14.7	56±13.7	59.6±12.6	0.8	57±11	46.6±5	NS
First graft (%)		11(73.3)	17(100)	4(80)	NS	15(100)	3(100)	NS
Underlying condition (%)								
Obstructive (%) urology		3(20)	0	1(20)	NS			NS
Hypertension		2(13)	6(35)	2(40)	NS	6(40)	0	NS
Polycystic kidney		4(26.6)	1(5.8)	0	NS	1(6.6)	0	NS
Other		6(40.0)	11(58.8)	2	NS	8(60)	3	NS
Induction immune suppression (%)								
Campath		70.9	71.5	40	NS	12(80)	3(100)	
Simulect		29.1	28.5	60	NS	3(20)	0	
Maintenance suppression (%)								
MMF+Advagraf		4(16)	2(28)	1(20)	NS	1(6.6)	1(30)	
Azathioprine		4(16)	4(22)	0	NS	3(20)	1(30)	

MMF+prograf		3(12.5)	2(11)	1(20)	NS	2(13)	0	
Advagraf (%)		2(8.3)	2(28)	1(20)	NS	5(30)	1(30)	
MMF		6(25)	3(42)	2(40)	NS	3(20)	0	
MMF+TAC (%)		5(20)	1(5)	0		1(6.6)	0	
HLA mismatch mean±SD								
A		0.88±.58	0.88±.78	1±.7	NS	0.92±.37	1	
B		0.77±.54	0.77±.83	0.4±.54	NS	0.84±.3	0.66±.033	
DR		0.66±.68	0.44±.72	0.6±.54	NS	0.92±.57	0.66±33	0.4
A+B+DR		2.2±1.4	2.1±1.9	2.4±1.5	NS	2.7±1.9	2.3±1.33	
Acute rejection episode (%)		3(12.5)	3(16.6)				1(33)	
post operation period(months)			3.5	10				

NS; no significance differences between the recipient's groups. MMF; mycophenolate mofetil therapy. TAC; tacrolimus. The data are shown as mean± standard deviation or percentage. The P-value was calculated using student`s *t* test, one-way ANOVA, chi- Square and Fisher`s exact test. P-value <0.05 considered significant.

5.2 Specific anti-BKV antibody response

To investigate prevalence of pre-transplant anti-BKV IgG and the association between pre-transplant anti-BKV levels and development of BK viremia and BKPyAN, a sandwich ELISA test was used to measure anti-BK-IgG. Our data indicated that 10 (66.6%) of the never had viremia group were anti-BKV IgG positive and up to 17 (94.0%) of recipients who developed BK viremia were anti-BKV IgG positive, whereas all the patients who developed BKVAN were anti-BKV IgG positive (Table 5.2).

Pre-transplant mean anti-BKV IgG levels in recipients with viremia were found to be lower in comparison to those who never had BKV viremia: 6.87±0.32, and 7.704±0.31 respectively, whilst the mean in patients who developed BKVAN was found to be 7.5±0.94 (p=0.14; Fig. 5.1).

Post-transplant mean anti-BKV IgG levels in recipients with BK viremia was significantly higher in comparison to the never had viremia group 10.21±0.5 vs. 4.627±2.9. The mean was also higher in patients who developed BKVAN in comparison to the never had group 10.50±0.23 p<0.05 (Fig. 5.2).

Table 5.2. Comparison of pre- and post-transplant BKV Ab titre in renal transplant recipients with viremia and patients who never had viremia.

BKV status	**Pre-transplant seroprevalence (%)**	**pre-transplant Ab titre**	**post operation >1 year**	**P-value**
Never BKV	66.6	7.704 ± 0.31	4.627±2.9	0.7
BKV viremia	94.4	6.87± 0.32	10.21±0.5	<0.05
BVPyAN	100	7.5±0.94	10.50±0.23	<0.04
		P=0.14		

P-value was calculated using paired student`s *t* test, and p-value <0.05 was considered significant.

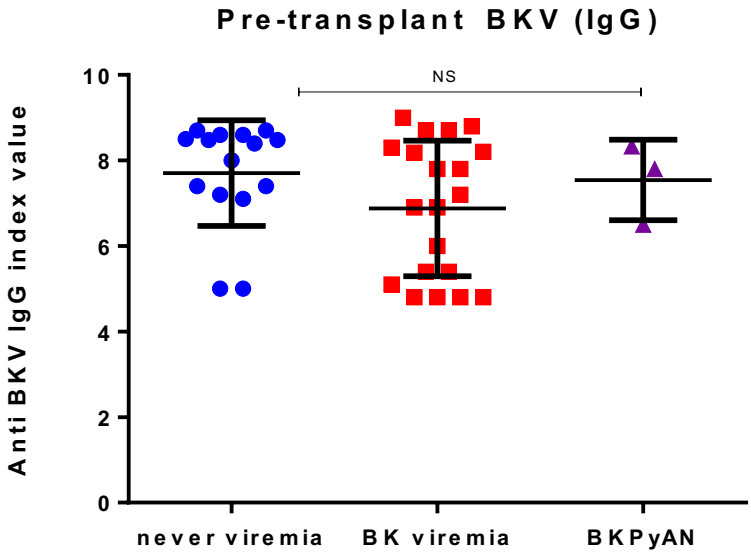


Figure 5.1. Mean titres of pre-transplant anti-BKV IgG

Antibody in pre-transplant recipients who developed BKV viremia following transplant. One-way ANOVA test was used to compare the mean antibody titres between the groups (P=0.14), N=38.

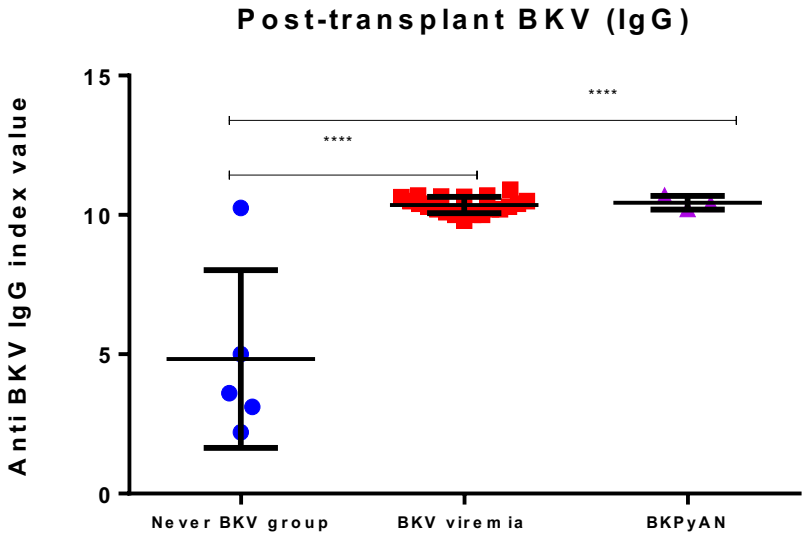


Figure 5.2 Mean titres of post-transplant anti-BKV IgG

Antibody levels in post-transplant recipients who developed BKV viremia after the transplant. One-way ANOVA test was used to compare the mean antibody titres between the groups (P<0.05), N=28, while p<.0005 between never BKV group and BKV viremia group and p<.0005 between never BKV group and BKPyAN group using a t-test.

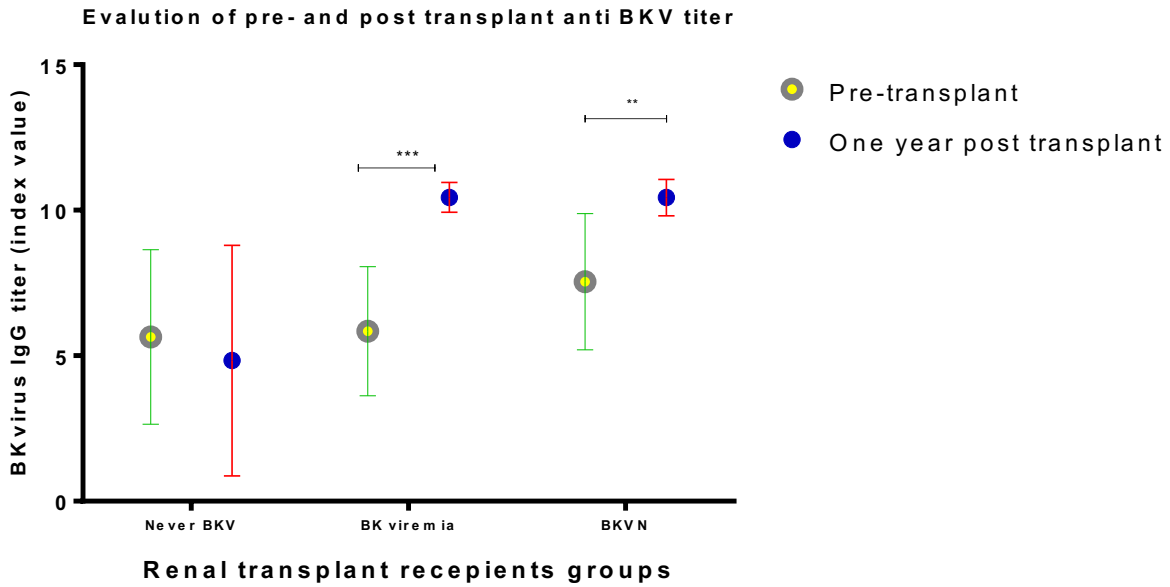


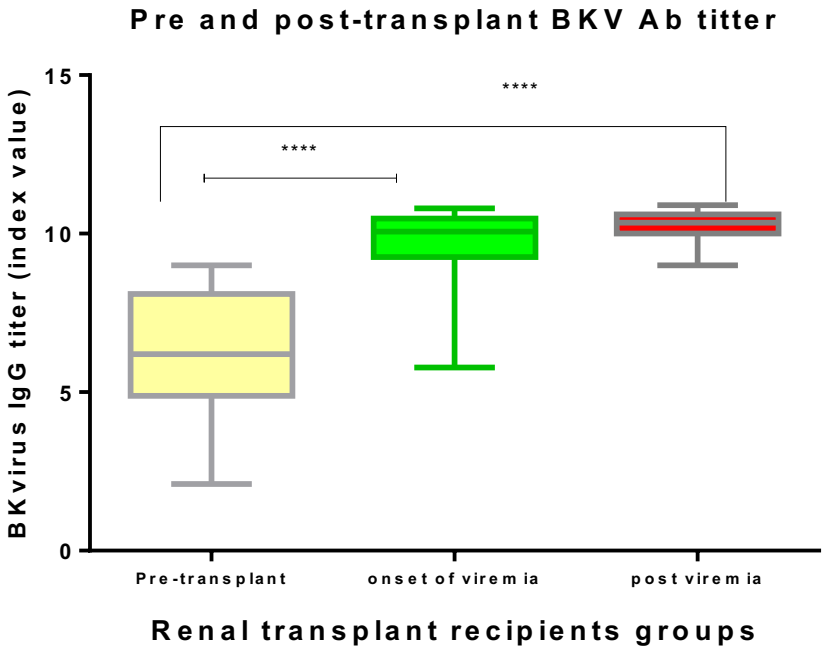
Figure 5.3. Pre- and post-transplant anti BKV titres.

In three different groups of renal transplant recipients; never had BKV viremia, with BKV viremia and recipients with BKPYAN. Multiple t-test was used to compare the means: p< 0.0005 for viremia patients, p<0.006 for recipients with BKPYAN and p<0.6 in never BKV group.

5.3 Pre-transplant and post-transplant follow up

To monitor the level and change in anti-BKV IgG levels in recipients who developed BKV viremia after transplantation, levels of anti-BKV specific antibody in each patient were measured at three different time points: pre-transplant, onset of BKV viremia and after virus clearance. No significant change was observed in mean pre-transplant antibody levels 4.980±2.70 and one-year post-transplant antibody levels were 4.627±2.90, in never had BKV recipients with calculated P-value of 0.77 (Fig 5.4B)

However, in the viremia group the observed mean pre-transplant antibody level was found to be 6.24, then remarkably increased to 9.421 during BK viremia, with CI difference of (-4.494 to -1.857) and with a calculated $p<0.00001$ between the two groups. Comparing the level from the onset of viremia and after virus clearance, post-viremia mean antibody was found to be elevated to 10.21 with CI difference of (-2.104 to 0.5324) and a calculated $p=0.8$ (Fig. 5.4A). Despite the virus clearance from recipient plasma after viremia the antibody titres remained high and even closer to the viremia status ($p=0.83$). In contrast with the never BKV group the mean antibody titre was slightly lower than the pre-transplant level ($p=0.7$; Fig. 5.4B).



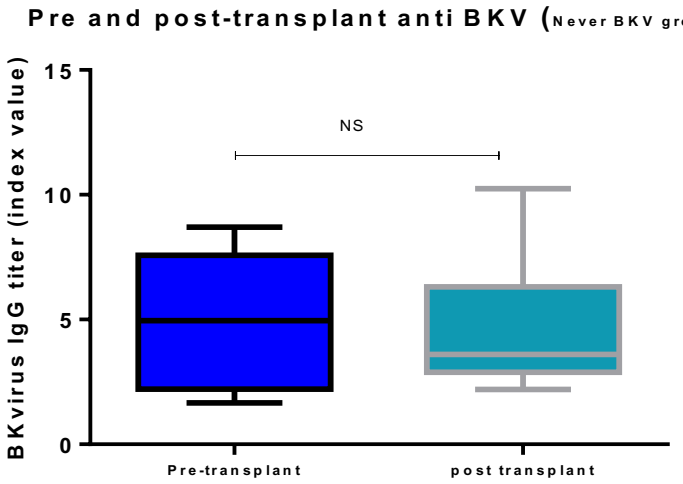


Figure 5.4. BKV antibody titre change in recipients

A *Patients with post-transplant BKV viremia.* ***B*** *BKV antibody titre changes in recipients who never developed BKV viremia after transplantation. For the post-transplant BKV viremia group, the antibody was measured prior to the transplant, at the onset of the viremia and after virus clearance; each box represents the interquartile range. P-value calculated using t- test. ****=* ***p****< 0.0001*

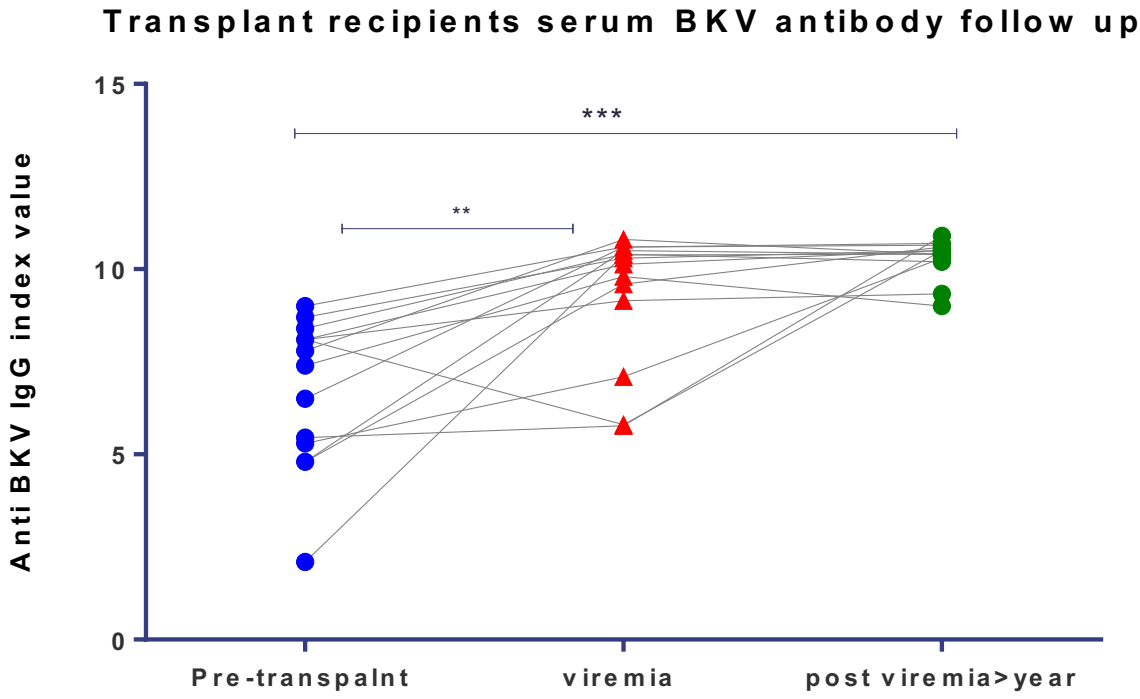


Figure 5.5. Difference in BKV IgG antibody titres.

Among pre- transplant patients, onset of viremia and one-year post in each individual recipient who developed BKV viremia. Blue circular points represent mean antibody titres pre-transplant, red triangular points at the onset of the viremia and green circular points shows antibody titres after one year of the transplant. Mean Ab titres differed significantly ($p<0.001$) in the BKV viremia group, and ($p<0.0001$) in post viremia group.

5.4 Risk of individual Human leukocyte antigen (HLA) alleles in transplant patients developing BKV DNAemia and viremia

Major histocompatibility complex (MHC) contains the genetic loci that are responsible of foreign tissue or organ rejection, and HLA molecules are the human MHC. Class I HLA are expressed on most nucleated cells, however class II is only expressed on B lymphocytes, antigen-presenting cells (monocytes, macrophages, and dendritic cells), and activated T lymphocytes (Choo, 2007). HLA molecules

play a role in the immune cells antigen identification, particularly natural killer cells (NK) and cytotoxic T-cell activation, however the exact role of HLA alleles in BKV infection and reactivation is still unclear. Here, we determined the risk of some HLA alleles in development of BKV viremia.

As described above, a cohort of post renal transplant recipient were recruited for evaluation of pre-transplant potential risks of BKV viremia, frequency of variant HLA alleles was investigated in two patient categories; kidney transplant recipient (KTR) individuals with BKV viremia and KTR individuals without BKV viremia, patients' HLA- A, B and DR data were collected from the hospital database. Alleles present in the patient groups at high frequency were chosen for more detailed analysis. The data showed that some HLA alleles were highly frequent in recipients with BKV viremia, including HLA-A1, HLA-A2, HLA-B44, HLA-DR1, HLA-DR4 and HLA-DR15 with others more frequent in non-viremic recipients, including HLA-B8 and HLA-DR13.

A total of 17 recipients who were positive for the HLA-B44 allele; 14 (82%) of them developed BKV viremia in comparison to three without viremia (p=0.02). Furthermore, 11 (91%) recipients of the 12 positives for the HLA-DR15 allele developed BKV viremia over only one without viremia (p=0.01). Conversely, 5 (71%) of patients were positive for the HLA-B8 allele and 4 (80%) of HLA-DR13 positive individuals demonstrated no virus replication (p=0.2 and p=0.15, respectively (Table 5.3).

To study the association between the recipients' HLA allele status and development of BKV viremia, a Kaplan-Meier survival curve was generated to evaluate the risk of BKV viremia after a two-year period following transplantation. HLA-B44 and HLA-DR15 alleles were found to be significantly associated with the risk of developing BKV viremia (p=0.001 and p=0.02 respectively; Fig. 5.6 and 5.7).

Furthermore, a multivariable (Cox regression) analysis was performed to identify risk factors that may be associated with the development of BKV viremia, factors such as age, sex and MHC class-I and -II were tested (Table 5.4). HLA-B44 and HLA-DR15 showed significant association with viremia (HR: 0.296, 95%CI: 0.11-0.73, P=0.009) and (HR: 44, 95%:0.18-1.08 P= 0.07). Male gender showed a trend towards an increased risk of viremia (HR: 0.4, 95% CI: 0.63-1.12 and

p=0.08). However, recipient's HLA-A2 status, age or HLA mismatch was not associated with the risk of developing BKV viremia.

Table 5.3. Frequency of HLA alleles in transplant recipients in relation to BK viremia.

The table illustrates the frequency of selected HLA alleles in two groups of transplant recipients: with BKV viremia or without.

	BKV viremia N=23 (%)	**No viremia N=15 (%)**	**P value**
HLA-A1 (%)	6(26)	6(40)	0.7
A2	6(26)	5(33)	0.8
HLA-B8	2(8.6)	5(33)	0.2
B44	14(60)	3(20)	0.02*
B51	1(4.3)	1(6.6)	1
HLA-DR1	6(26)	2(13)	0.6
DR3	2(8.6)	2(13)	1
DR4	7(30)	2(13)	0.3
DR7	2(8.6)	4(26)	0.37
DR11	1(4.3)	2(13)	0.5
DR13	1(4.3)	4(26)	0.15
DR15	11(47)	1(6.6)	0.01*

All P-values correspond to the HLA in the corresponding row, SPSS software was used to calculate the p values.

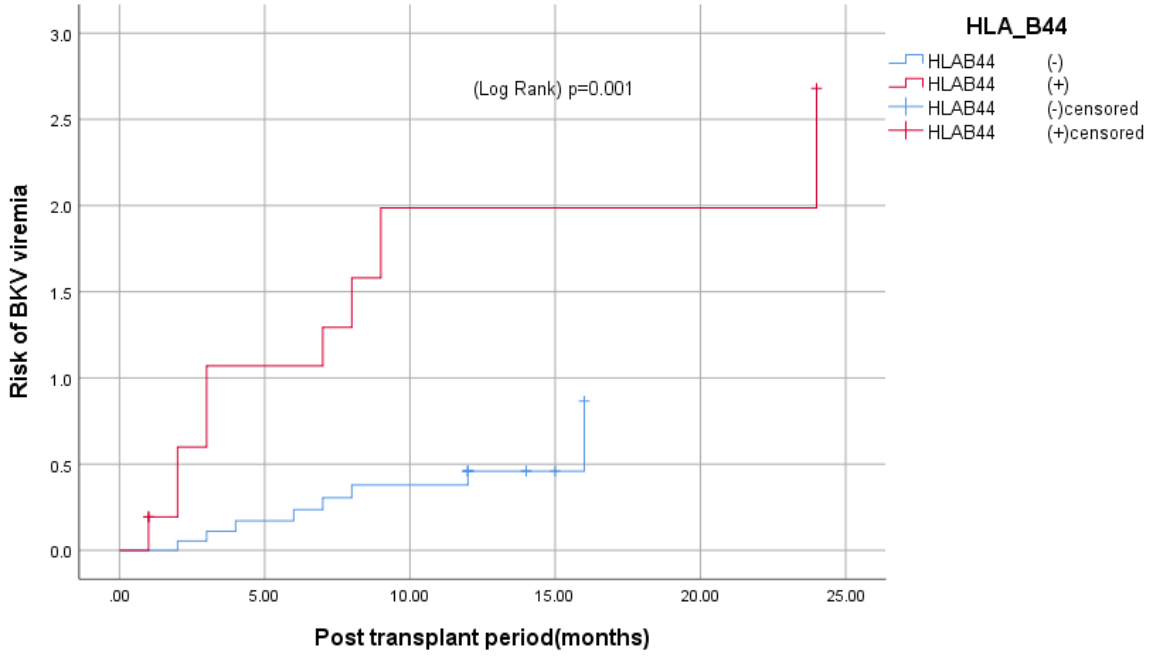


Figure 5.6. Risk of BKV viremia

The risk in the first two years post transplantation based on HLA-B44 status. Kaplan-Meier survival curves were generated for 17 renal recipients with HLA-B44 and 20 without HLA-B44. Tick marks represent censored recipients. The p-value was calculated using a log-rank (Mantel-Cox) test.

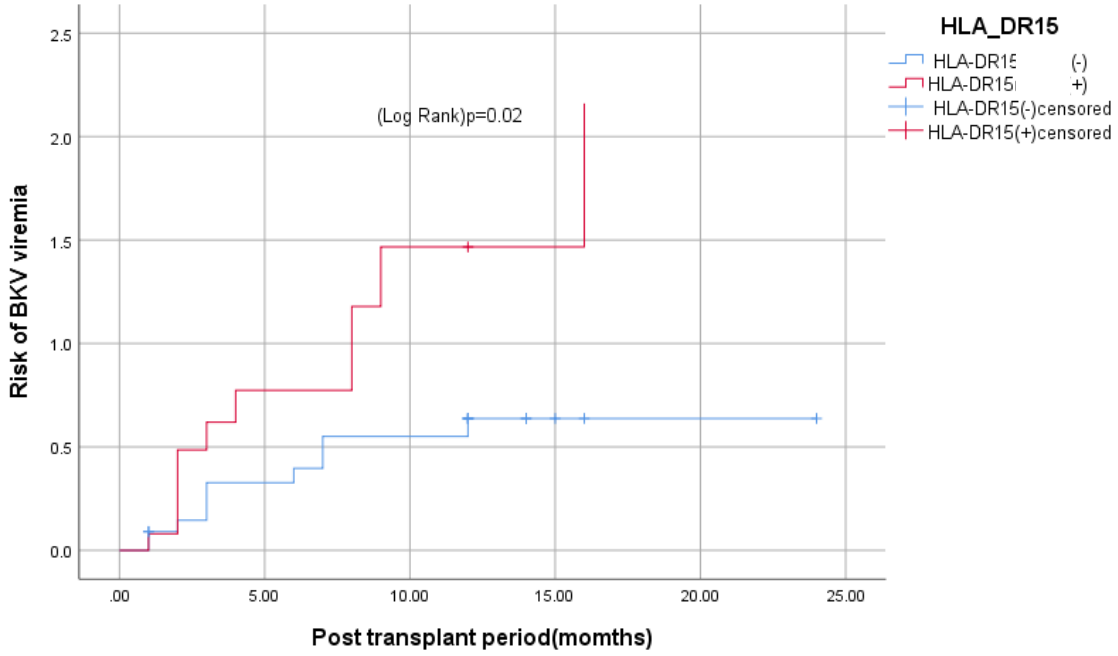


Figure 5.7. Risk of BKV viremia

The risk in the first two years post transplantation based on HLA-DR 15 status. Kaplan-Meier survival curves were generated for 13 renal recipients with HLA-DR15 and 24 without HLA-DR15. Tick marks represent censored recipients. The p-value was calculated using a long-rank (Mantel-Cox) test

Table 5.4. Cox regression analysis of multivariant factors that associate with development of BKV viremia.

	HR	**95% CI**	**P- value**
Age	0.46	.009-0.15	0.6
Gender	0.42	0.63-1.12	0.08
HLA-B44	0.296	0.11-0.73	.009
HLA-DR15	0.44	0.18-1.08	0.07
HLA-A2		0.51-3.20	0.59
HLA-mismatch			0.28
A	2.9	0.6-13.7	0.177
B	2.3	0.06-27	0.489
DR	0.18	0.22-1.5	0.115

HR: hazard ratio, CI: confidence interval.

5.5 Discussion

BKV viremia and progression of BKV nephropathy are crucial problems that could jeopardize the success of renal transplantation, as no functional antivirus therapy is yet available to prevent virus reactivation. The current effective treatment strategy is based on reduction of administering immunosuppressive therapies to retrieve immunity with care to avoid acute graft rejection (Borni-Duval et al., 2013; H. H. Hirsch et al., 2006). Around 15-30% of the recipients developed BKV viremia and 1-10% developed BKVN and loss of the graft (H. H. Hirsch & Randhawa, 2013). However, BKV reactivation outcomes could be avoided with predictions based around the recipients' pre-transplant risk factors as no BKV viremia risk factors have yet been identified.

In this chapter, we investigated pre-transplant anti-BKV IgG antibody levels in relation to development of BKV viremia in the recipients within a period of two years post-transplant. Additionally, we studied the role of some HLA molecules in associating with pathogenesis of BKV reactivation, leading to disease. Finding a significant positive correlation between pre-transplant anti-BKV levels, HLA alleles and development of BKV viremia would be useful in predicting development of viremia.

The first observation was that 94.0% of seropositive pre-transplant recipients who developed BKV viremia did so within the first year of transplantation. Additionally, no significant difference was observed in mean pre-transplant anti-BKV IgG titres in recipients who developed BKV viremia, BKPyAN and the control group. (H. H. Hirsch et al., 2002). Bohl et al concluded that pre-transplant seropositivity may not prevent BKV reactivation and viremia but may ease the severity of the infection (D. L. Bohl et al., 2008).

Secondly, we observed that during the onset of BKV viremia, specific immunity was generated to BKV that resulted in an increase in anti-BKV IgG levels about two-fold in comparison to pre-transplant levels; this stayed constant even after virus clearance and reduction in treatment with immunosuppressive therapies. It is worth noting that this increase in the antibody immune response was generated despite the weak immune status of recipients after transplantation. Consequently, this observation suggests that a specific anti-BKV IgG humoral response is strong

and not directly affected by changes in treatment with immunosuppressive drugs but could be affected by an increase in virus reactivation.

After clearance of viremia, anti-BKV IgG levels unexpectedly increased and remained stable for more than a year post clearance in the viremia and BKPyAN groups, while it tended to decrease in the control group who never experienced viremia one-year following post-transplant. This could be explained by the effect of immune suppressive therapy because it is proposed to reduce the humoral immune response (D. L. Bohl et al., 2008; Keven et al., 2003; Smith et al., 1998)

Finally, we undertook a short retrospective investigation into linking HLA alleles to risk factors of BKV pathogenesis in renal transplant recipients. HLA molecules are known to play an important role in immune activation after infection. In most viral infections, HLA molecules present virus peptides to T-cell receptors, which activate CD8+ specific cytotoxic cell responses that lead to specific killing of virus infected cells. Furthermore, CD4+ T-cells assist in the generation of effector CD8+ T-cells and activation of B-cells that produce virus specific antibodies. Alternatively, NK cells may kill virus-infected cells that have altered or down-regulated cell surface class I HLA molecules.

In transplantation, HLA -A, B, DR are known as major transplantation antigens. A match between the donor and the recipient at these loci increases the likelihood of graft survival, and the presence of reactive anti-HLA antibodies in recipients against donor lymphocytes is a major obstacle to kidney transplantation (Gebel, Bray, & Nickerson, 2003) .

We noted that some alleles were frequent in the recipient population studied, such as HLA-A2, HLA-B44 and HLA-DR15. Several studies have suggested that some HLA alleles may promote development of BKV viremia in renal transplant recipients. Teutsch et al observed that recipients who were HLA-A28 and HLA-A68 positive showed a higher risk of developing viremia (Teutsch et al., 2015). However, HLA-A9 and HLA-A*02 alleles in the donor and the recipient were reported to be associated with a significant increase of development of BKyVAN (Teutsch et al., 2015). Also, a study by Bohl et al found that the lack of HLA-C7 in the donor was associated with a higher risk of developing sustained viremia, that was explained by HLA-C7 involvement BKV cellular response and antigen presentation (D. L. Bohl et al., 2005).

In the present work, we observed that the presence of HLA-B44 and HLA-DR15 was significantly associated with an increased risk of viremia among the recipients. However, in an earlier study by Masutani, in 2013, it was observed that HLA-A2, HLA-B44 and HLA-DR15 in recipients were associated with lower risk of BKV viremia (Masutani, Ninomiya, & Randhawa, 2013). A possible explanation for these results may be that rejection resulting from an HLA mismatch leads to an increase in the prescribed dose of immunosuppressive drugs and hence reduction of the response to BKV, allowing for viral reactivation. Another possibility is that the ongoing rejection response indirectly results in virus reactivation. Another explanation would be in the typing methodology; in our study we used the molecular based typing known to be more specific than the serological typing whilst the other study used the serological typing method (Bozón et al., 1997). The drawback to serological typing is that HLA-A specificities known to be found mainly in non-Caucasian populations are not identified. Another possibility is that there were differences in the incidence of variant BKV virus strains in the different studies (Yu et al., 1997).

An inherent increased risk in the development of BKyVAN in patients who were HLA-B44 or DR15 may also be because those alleles are not as efficient at presenting BK peptides to CD8+ or CD4+ T cells, respectively. This could be studied further by investigating theoretical binding affinities of immunodominant BK peptides to these HLA molecules.

CHAPTER SIX

6 BKV genotypes and its influence on development of BKV viremia in kidney transplant recipients.

Introduction

BKV infection is an asymptomatic virus infection that is mainly acquired in early age. The virus establishes latency in the urothelium with the transient presence of virus particles in the urine. 15-85% of the population excrete BKV in their urine, while up to 90% of the healthy population are found to be seropositive (W. A. Knowles et al., 2003). In the context of immunosuppression, or in case of insufficient immunity, latent BKV can reactivate in the kidney causing BKV viremia, renal stenosis and BKV disease in renal transplant recipients. BKPyVN or BKV disease is one of the serious problems that threaten 1-10% of renal transplant recipients and which can lead to the loss of the transplanted graft (H. H. Hirsch & Randhawa, 2013). Currently, the initial strategy in treating BKV reactivation in most renal transplant centres is periodic screening for virus DNA in recipient blood to monitor for elevation in viral load, and thereby reduce immune suppressant therapy in cases of confirmed BKV viremia.

Based on the variable region that encodes for the VP1 protein, BKV has been previously classified into four different genotypes; genotype I, II, III, and IV (L. Jin, 2001). Further, based on the DNA sequence variation several other subtypes and subgroups were identified; Ia, Ib1, Ib2, Ic, IVa1, IVa2, IVb1, IVb2, IVc1, and IVc2 (Nishimoto et al., 2007; Zhong et al., 2009). BKV subtypes showed different geographical distributions; for example genotype I was found to be the most prevalent genotype worldwide (80%), followed by IV (15%) in Europe and Asia (Ikegaya et al., 2006; Zhong et al., 2009), while genotypes II and III were rarely identified (Ikegaya et al., 2006; Takasaka et al., 2004). A better understanding of virus subtypes and their prevalence in different regions is needed for distinguishing between the various subtypes and the relatedness to disease. Furthermore, this will provide information regarding quality of the diagnostic assays to be used in different regional settings.

Many risk factors have been proposed to play a role in the development of BKPyVN, either donor related, recipient related or virus-related. Yet very little is known regarding BKV genotypes and the associated risks for disease progression within post-renal transplant patients. Here, we investigated the prevalence of BKV genotypes in renal transplant recipients with BK viremia and the association of particular genotypes to the development of viremia or BKVN disease using a new simple BKV genotyping method.

6.1 Patient demographics

Thirty-two patients who underwent a kidney transplant 2017-2020 and had experienced BKV viremia at least once post-transplant, in the Renal Transplant Department at the University Royal Hospital Liverpool were included in the study. Patients' demographic characteristics are illustrated (Table 6.1). All patients were under maintenance immunosuppressive therapy receiving either Mycophenolate mofetil or Azathioprine in addition to Tacrolimus. Mean patient age was 55.8 years with 22 males and 10 females included. Underlying diseases diagnosed were polycystic kidney (n=3), obstructive urology (n=3), hypertension (n=5) and other (n=21).

Table 6.1 Demographic data of recipients who developed BKV viremia after transplant.

Patients' characteristic	Values
Age means	55.8± 15
Sex(male)%	68
Underlying diseases%	
Polycystic kidney	9.3
Obstructive urology	9.3
Hypertension	15.6
Other	65.8
BKPyVN%	6.2
Viremia%	56.2

6.2 BKV genotyping in viremic renal transplant recipients

In total 32 renal transplant recipients who developed BKV viremia within the first year of transplant were studied, urine or serum samples with confirmed BKV viremia were collected retrospectively. BKV DNA was isolated, and 28 samples were successfully amplified using a nested PCR (Fig. 6.1). The amplified DNA was subsequently sequenced using Sanger sequencing methodology. The analysis revealed that 6 (21.4%) of samples were genotype I-b1, and 22 (78.5%) were genotype I-b2 and none were identified with genotypes II, III or IV (Table 6.2). Amplification of four samples failed probably due to low viral loads in the samples.

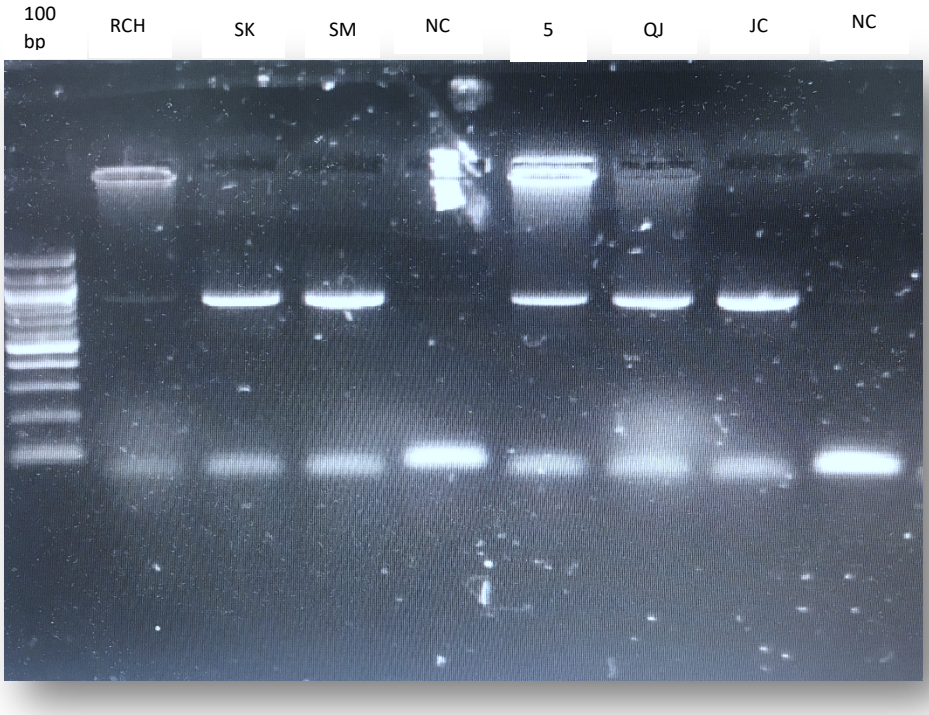


Figure 6.1. Gel electrophoresis

Showing amplification of the VP1 gene of BKV DNA using nested PCR, where the bands illustrate amplified DNA from recipients' samples, 100bp ladder and negative controls.

Table 6.2. BKV genotypes in urine and plasma of recipients with viremia after transplant n=32.

The table shows each sample`s ID, viral load log10 copies /ml, amplification result, type of sample and genotype.

Sample#	**Viral load Log10 copies/ml**	**PCR**	**Sample type**	**Genotype**	
				Algorithm	Phylogenetic tree
1	3.17	pos	plasma	Ib-2	Ib-2
2	2.87	pos	plasma	Ib-2	Ib-2
3	3.4	pos	plasma	Ib-2	Ib-2
4	3.39	pos	plasma	Ib-2	Ib-2
5	4	pos	plasma	Ib-1	Ib-1
6	3.8	pos	plasma	Ib-1	Ib-1
7	5	pos	plasma	Ib-2	Ib-2
8	2.39	pos	plasma	Ib-2	Ib-2
9	2.39	pos	plasma	Ib-2	Ib-2
10	2.39	pos	plasma	Ib-1	Ib-2
11	2.39	pos	plasma	Ib-2	Ib-2
12	3.8	pos	urine	Ib-2	Ib-2
13	4.5	pos	urine	Ib-1	Ib-1
14	4.8	pos	urine	Ib-2	Ib-2
15	2.3	pos	plasma	Ib-2	Ib-2
16	3.9	pos	urine	Ib-2	Ib-2
17	4.9	pos	urine	Ib-2	Ib-2
18	5.9	pos	urine	Ib-2	Ib-2
19	8.9	pos	urine	Ib-1	Ib-1
20	6.1	pos	urine	Ib-2	Ib-2
21	3.47	pos	urine	Ib-2	Ib-2
22	3.9	pos	urine	Ib-2	Ib-2
23	5.6	pos	urine	Ib-1	Ib-1
24	2.3	pos	plasma	Ib-2	Ib-2
25	3.2	pos	plasma	Ib-1	Ib-1
26	2.3	pos	plasma	Ib-2	Ib-2
27	2.3	pos	plasma	Ib-2	Ib-2
28	2.3	pos	plasma	Ib-2	Ib-2

6.3 Viremia versus genotype

The association between peak viral load and BKV subtypes was analysed; mean BKV viral load from plasma samples for each genotype was as follows: genotype Ib-1 was 3.6 $\log_{10}$ copies/ml ± 0.24, range from (3.2-4 $\log_{10}$ copies /ml) and mean 2.7 $\log_{10}$ copies /ml ± 0.2, range from (2.3-5 $\log_{10}$ copies /ml) for genotype Ib-2; p=0.07 for difference between the two genotypes. Mean viral load from urine samples was 6.2 $\log_{10}$ copies /ml ± 0.9 and ranged from (4.5-8.9) for Ib-1 genotype and 4.4 $\log_{10}$ copies /ml ± 0.3, range from (3.8-6.1 $\log_{10}$ copies /ml) for Ib-2 genotype; p=0.055 for difference between the two groups as shown (Fig. 6.2). Only two of the viremic patients developed BKPyVN with a mean BKV viral load of 2.3 $\log_{10}$ copies /ml, both of them were genotype lb 2. Among our tested group, only two patients developed BKPyVN and both were Ib-2 genotype.

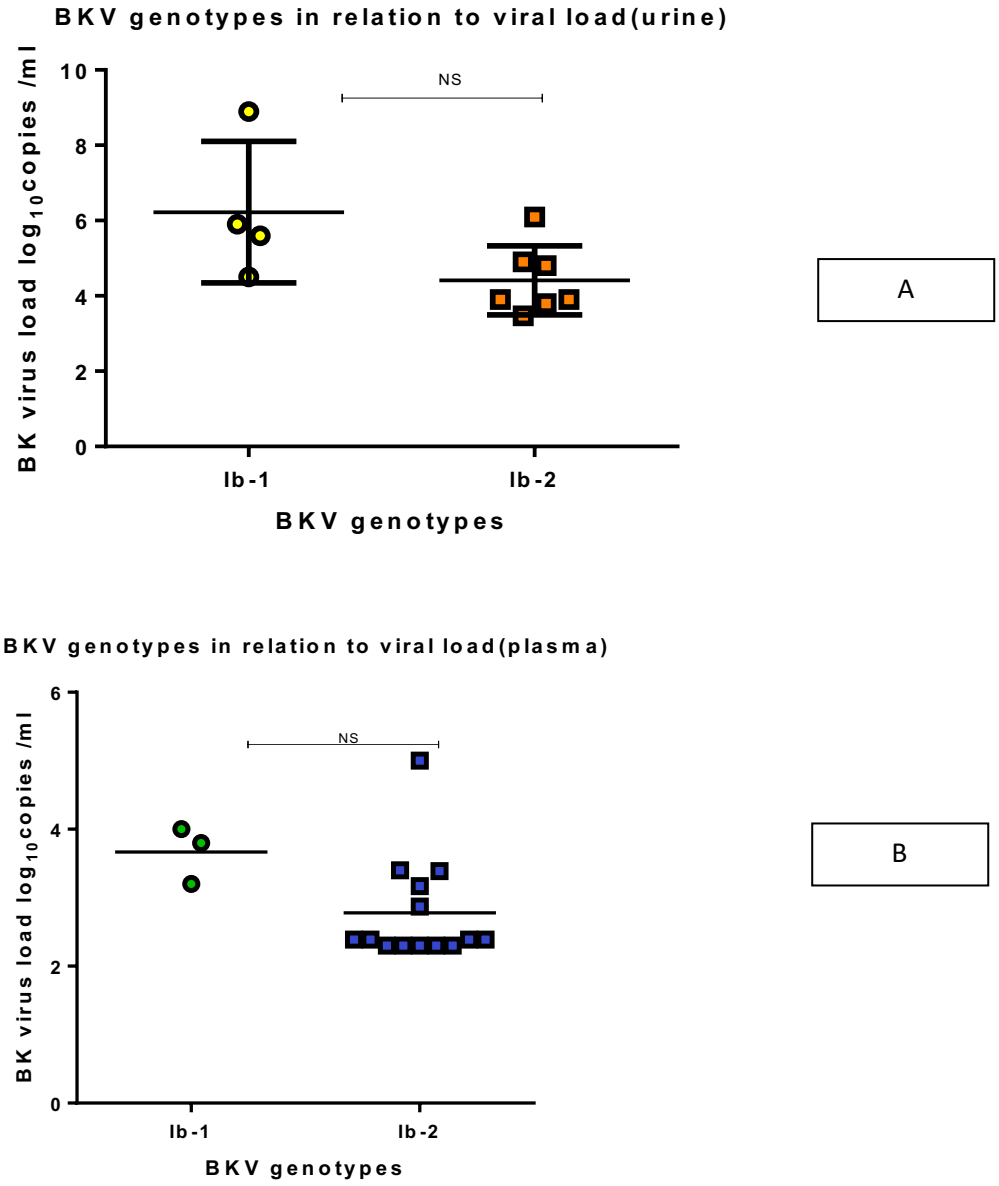


Figure 6.2. Renal transplant recipient`s BKV genotypes

*Genotypes in relation to viral load (log10 copies/ml) in urine **A** and plasma **B** samples. Horizontal bar represents mean viral load between the tested groups, p value was calculated using student's t-test*

Table 6.3.BKV genotypes and occurrence of BKV infection and disease.

P value was calculated using student's t -test or chi-square test

Variables	**Genotype Ib-1**	**Genotype Ib-2**	**P-value**
Age **(mean)**	54.4±14	56.7±15.3	0.7
Sex (%)	6(19.3)	25(80.6)	0.5
Male	3(50)	18(72)	
Female	3(50)	7(28)	
HLA-mismatch	2.57±1.9	2.7±1.4	0.9
Asymptomatic BKV ***(Log_{10}copies/ml)*** **(viruria)**	6.2 ± 0.9	4.4 ± 0.3	0.055
BKviremia ***(Log_{10}copies/ml)***	3.6±.24	2.7±.02	0.07
BKPyVN	0	2	NS

6.4 Validation of BKV algorithm and BKV phylogenetic tree.

Performing BKV subtyping was mainly based on a phylogenetic tree analysis of the VP1 fragment, nucleotides (1564-2215) and the Large T antigen nucleotides (3021-3715). However, in this study we compared the previous method with a new simple and reliable strategy for BKV genotyping, where the method is based on amplification of only a 100bp segment of BKV VP1 from nucleotides 1977-2076 (BK Typing and Group region (BKTGR (Morel et al., 2017). Following Sanger sequencing of the purified amplicon the sequences were manually reviewed and applied to the given new algorithm.

All 28 sequences were aligned along with 40 complete BKV sequences retrieved from GenBank. A phylogenetic tree was constructed in order to cluster the strains based on BKV subtypes (Fig. 6.3). Our unknown sequences were 6 sample under lb-1 subtype and 22 samples under lb-2 subtype. The retrieved reference sequences were as follows: la (n=7), lV (n=7), Ib-1 (n=6), lc (n=6), lb-2 (n=5),

lll (n=5) and ll (n=4) subtypes. Next, we determined our sequences' subtypes based on the 100bp BKTGR scheme (Fig 6.4 and 6.5). Comparing the two methods, a 98% (67 sample) similarity of the subtypes was obtained. Only one sample`s subtype differed between the two methods.

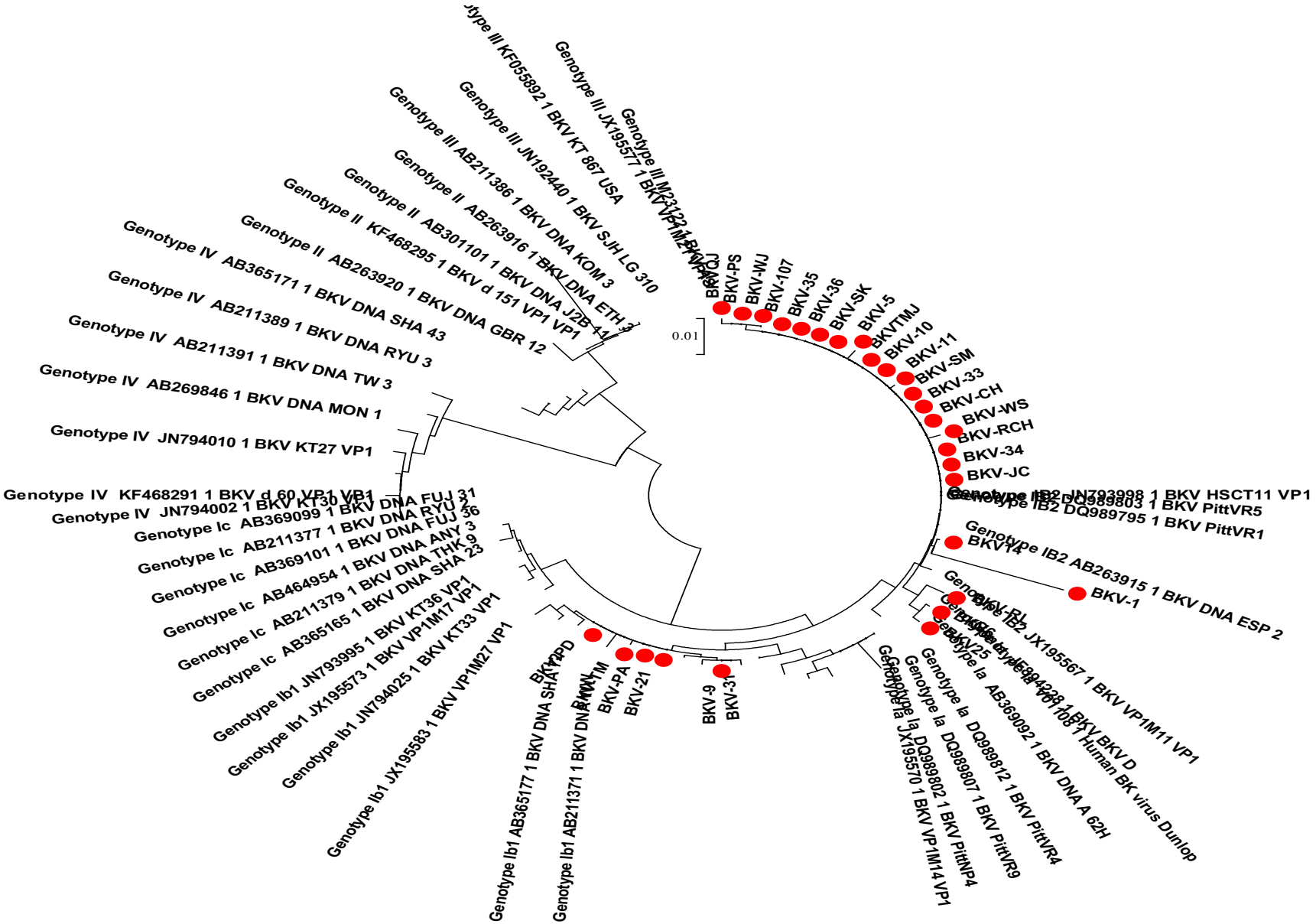

Genotype III KF055892 1 BKV KT 867 USA
Genotype III JN192440 1 BKV SJH LG 310
Genotype III AB211386 1 BKV DNA KOM 3
Genotype II AB263916 1 BKV DNA ETH 3
Genotype II AB301101 1 BKV DNA J2B 41
Genotype II KF468295 1 BKV d 151 VP1 VP1
Genotype II AB263920 1 BKV DNA GBR 12
Genotype IV AB365171 1 BKV DNA SHA 43
Genotype IV AB211389 1 BKV DNA RYU 3
Genotype IV AB211391 1 BKV DNA TW 3
Genotype IV AB269846 1 BKV DNA MON 1
Genotype IV JN794010 1 BKV KT27 VP1
Genotype IV KF468291 1 BKV d 60 VP1 VP1
Genotype IV JN794002 1 BKV KT30 VP1
Genotype Ic AB369099 1 BKV DNA FUJ 31
Genotype Ic AB211377 1 BKV DNA RYU 2
Genotype Ic AB369101 1 BKV DNA FUJ 36
Genotype Ic AB464954 1 BKV DNA ANY 3
Genotype Ic AB211379 1 BKV DNA THK 9
Genotype Ic AB365165 1 BKV DNA SHA 23
Genotype Ib1 JN793995 1 BKV KT36 VP1
Genotype Ib1 JX195573 1 BKV VP1M17 VP1
Genotype Ib1 JN794025 1 BKV KT33 VP1
Genotype Ib1 JX195583 1 BKV VP1M27 VP1
0.01
BKV-PS
BKV-WJ
BKV-107
BKV-35
BKV-36
BKV-SK
BKV-5
BKVTMJ
BKV-10
BKV-11
BKV-SM
BKV-33
BKV-CH
BKV-WS
BKV-RCH
BKV-34
BKV-JC
Genotype IB2 JN793998 1 BKV HSCT11 VP1
Genotype IB2 DQ989803 1 BKV PittVR5
Genotype IB2 DQ989795 1 BKV PittVR1
BKV-14
Genotype IB2 AB263915 1 BKV DNA ESP 2
BKV-1
Genotype IB2 JX195567 1 BKV VP1M11 VP1
Genotype Ia AB369092 1 BKV DNA A 62H
Genotype Ia DQ989812 1 BKV PittVR4
Genotype Ia DQ989807 1 BKV PittVR9
Genotype Ia DQ989802 1 BKV PittNP4
Genotype Ia JX195570 1 BKV VP1M14 VP1
BKV-9
BKV-31
BKV-21
BKV-PA
Genotype Ib1 AB211371 1 BKV DNA
Genotype Ib1 AB365177 1 BKV DNA SHA

Figure 6.3. Maximum likelihood Phylogenetic analysis tree

In red dots 28 renal transplant recipients with BKV infection with 40 BKV VP1 complete reference sequences retrieved from GenBank.

Figure 6.4. Data sequences alignment results.

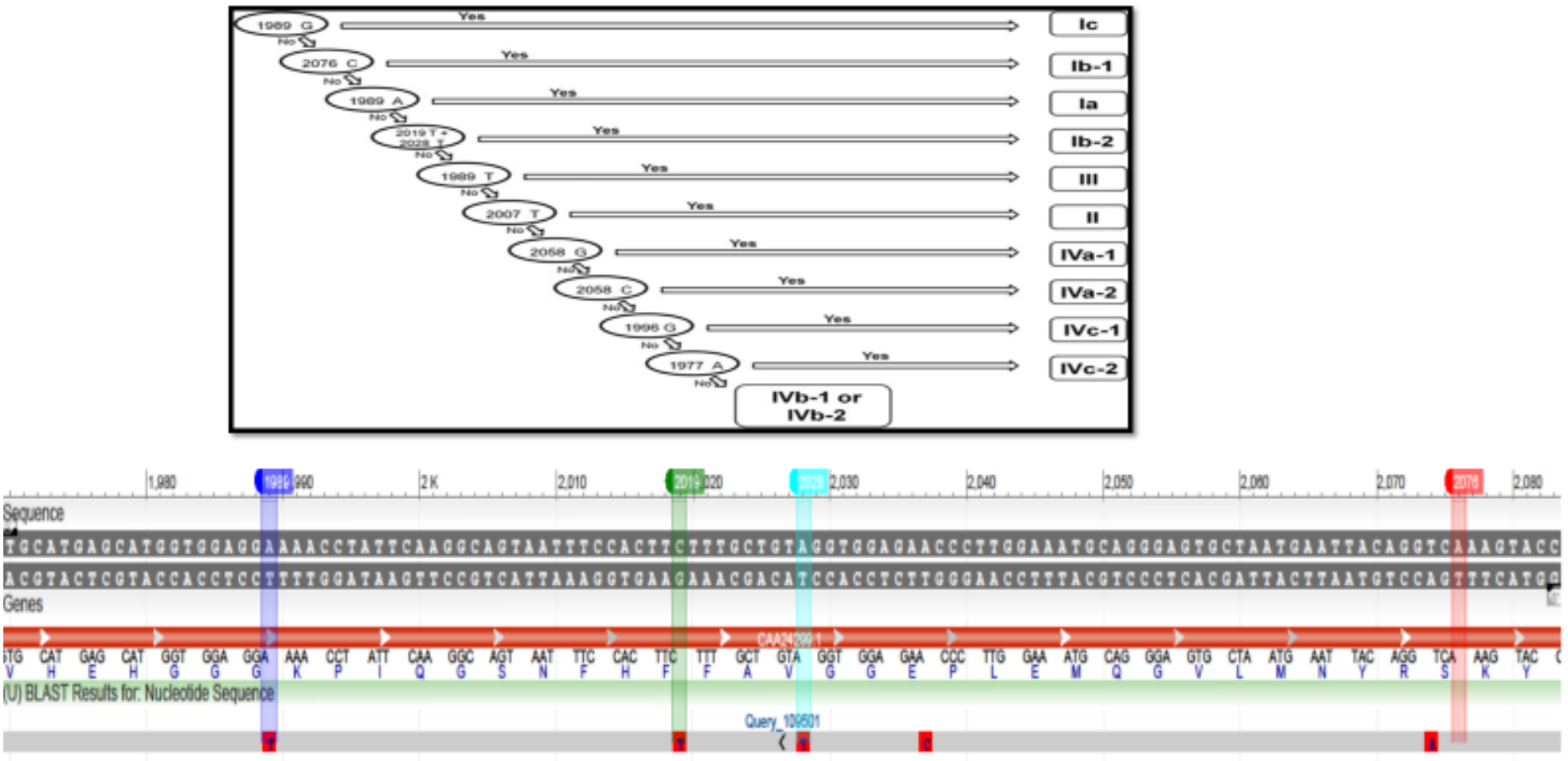


Figure 6.5. BKV subtyping algorithm scheme based on detected mutations in the BKTGR of VP1 gene.

6.5 Discussion

Here we aimed to investigate any associations between BKV genotypes and development of BKV viremia or BKVAN in renal transplant recipients. The samples were sequenced and aligned with BKV complete genome sequences obtained from the literature to evaluate genetic diversity using a genotyping method based on amplification of only 100bp from the BKV VP1 gene.

We reported the prevalence of BKV genotypes in a cohort of renal transplant recipients. Our data show that most of the viremic renal transplant recipient were infected with BKV genotype I with the highest proportion (78.5%) categorised under subtype I-b2 and substantially fewer categorised under subtype I-b1 (21.3 %). Also, it is implied that there is no association between BKV genotype and development of BKV viremia in renal transplant recipients.

These findings are found to be in concordance with a previous study showing the distribution of BKV genotypes in other European populations; subtype Ib-2 was most frequently detected in European and American populations, while Ic subtype was predominantly identified in the Asian population. The subtype IV distribution was variable among Asian, American and European populations (Zheng et al., 2007) (Ikegaya et al., 2006; Zhong et al., 2009). No other genotype was detected in our study and could be explained by the fact that our participants were from the Liverpool area, where 91% of population are of Caucasian European ethnicity. Additionally, the limited sample size may have impacted on the findings. Also, we should keep in mind our study group were patients who experienced BKV viremia at least once post-transplant, as some other excluded patients who had low viral loads could be infected with other genotypes.

Some virus genotypes, such as CMV gp1 subtype, have been reported to influence the course of disease after renal transplant (E. Nogueira, Ozaki, Tomiyama, Camara, & Granato, 2009). Additionally, HCV virus genotype 1 has been correlated with clinical outcome of disease in immunocompromised patients (Núñez & Soriano, 2005). Our data suggest no specific association between BKV genotype and intensity or development of BKV viremia in renal transplant recipients. Furthermore, the two reported patients who developed BKPyAN were from genotype Ib-2. The same observation was reported for relation of BKV infection in 385 renal transplant recipients (H. F. Wunderink et al., 2019). This finding

provides further support to the hypothesis that progressive BKV disease after transplant is not associated with specific genotype, but the strength of cellular immunity induced against the virus (H. F. Wunderink et al., 2019).

However, a study by Varella et al, proposed specific association between BKV subtypes and BKV viruria, as recipients infected with the Ia genotype exhibited higher viral load over others infected with Ib genotype (Baksh et al., 2001; R. B. Varella et al., 2018; Yogo et al., 2009). Recently, Korth demonstrated a remarkable correlation (P=0.007) between BK genotype II and IV compared to genotype 1 and III (Korth et al., 2019). The point here is that in our cohort group we were not able to detect genotype Ia which weakens the comparison between the two findings. Moreover, other studies suggested that some genotypes have different viral replication kinetics compared to others, for example subtype I has been shown to replicate more efficiently in cell culture than other tested subtypes and subtype Ib-1 replication shows lower viral loads when compared to subtype Ib-2 (Drew, Walsh, Laoi, & Crowley, 2012; Nukuzuma et al., 2006; Tremolada et al., 2010). This leads us to the concept that each genotype has diverse biological properties that may influence virus resistance and reactivation.

Until recently, BKV genotyping has been based on phylogenetic analysis, and availability of a simple subtyping method provides clear information regarding association with BKV disease. For BKV genotyping, initially, our obtained sequence subtypes were identified according to generating a phylogenetic tree. Here we validated the identified subtypes using a new simplified method, based on a 100bp fragment (BKTGR). After the comparison between the phylogenetic analyses using the new algorithm, our data demonstrates 98% concordance. Only one sample demonstrated a difference in predicted genotypes between the two methods. The possible explanation of this discordance that the new algorithm failed to differentiate subtypes in samples with mixed infection (Morel et al., 2017).

Several strategies were proposed for BKV subtyping; a study developed a BKV subtyping method based on high resolution melting analysis (Matsuda, Qazi, & Iwaki, 2011), but which failed to distinguish between strains with the same polymorphism. Further, Gard et.al proposed a real time PCR technique for BKV subtyping in bone marrow recipients, based on amplification of the virus VP1 gene where the method manged to discriminate the subtypes but not the sub-groups

as the used assay was unable to differentiate between strains with same amount of SNPs (Gard, Niesters, & Riezebos-Brilman, 2015).

The most notable limitation of this study is the small sample size due to discounting follow up of some of the renal recipients in the main hospital. Additionally, we were not able to detect all BKV genotypes such as the lV subtype, which could be due to variations in the geographical distribution of genotypes with low frequencies locally. Larger regional studies could be performed with patients from different geographical areas to confirm our findings, however, from the results presented it is apparent that the genotype Ib-2 is the most prevalent in the Liverpool area.

To conclude, our data reveals no significant differences between viral genotype and viral loads. No association was found between BKV genotype and disease progression in renal transplant recipients. Larger and prospective studies will be required to gain a better understanding and evaluation of BKV subtypes with the risks of developing BKV viremia and BKVAN.

CHAPTER SEVEN

7 Discussion

Even though BKV infection is normally an asymptomatic infection in immunocompetent subjects it can lead to serious complications and even graft loss in renal transplant recipients due to the administration of immunosuppressive treatments, and lack of antiviral treatments. Therefore, better understanding of the host immune responses controlling the virus and the risk factors that influence BKV disease progression will be invaluable when aiming to avoid disease progression in post-transplant recipients.

In this thesis we aimed to understand the host immune responses that are induced against BKV infection in healthy populations and renal transplant patients. Our aims were to study both cellular and humoral immune responses induced against BKV in different study categories, such as healthy individuals with or without detected virus and renal transplant recipients with or without post-transplant viremia. A further aim was to link specific HLA genotypes with disease outcome and severity to identify host restriction factors that control disease.

As an initial objective of our project, we evaluated the cellular immune response against BKV peptides using an ELISPOT assay to measure INF-γ secreting T-cells in both study groups. Further, to measure the impact of BKV infection on the immune cells, induction of both sHLA-G and cell surface HLA-G expression following infection were studied. Flow cytometry was used for cell phenotyping and measurement of cell surface HLA-G protein expression levels. The proportions of HLA-G+ cells were estimated in various PBMC subpopulations including CD3+CD4+, CD3+CD8, CD19, CD14+HLA-DR+ and CD3+CD56+ cells, along with isotype control as cut off and unstimulated cells as negative controls. The third part of this thesis was evaluating humoral immune responses in renal transplant patient; pre-transplant anti BKV IgG, serostatus and activity were compared to post transplant in cases with or without development of BKV viremia. Lastly, BKV virus genotype prevalence in renal transplant patients were demonstrated and their relation to development of BKV viremia was investigated using a new simple genotyping method.

7.1 BKV specific IFN-γ immune responses and antibody responses in healthy subjects and renal transplant recipients.

In order to evaluate post-transplant immune responses against BKV viremia and the mechanism of virus clearance we measured BKV specific IFN-γ secreting cell responses using an ELISPOT assay in a cohort of healthy subjects and kidney transplant recipients with various statuses of BKV.

BKV shedding in urine of healthy individuals and renal transplant recipients was measured as our first objective in BKV screening, due to its high sensitivity and early detection compared to that expected in plasma. However, detection of virus DNA in urine doesn't necessarily reflect BKV infection and induction of immune responses. Individuals who become infected with BKV may not completely eliminate virus but may continue to shed virus in the absence of any pathological effects. Therefore, BKV serological status was further tested, as an indicator of previous BKV infection. Those individuals who tested positive for BKV IgG, were tested for BKV DNA in urine. Those individuals identified with positive antibody responses would be expected to also harbour CD4 T cell responses which are required to induce antibody responses. The comparison of cellular immune responses was therefore based on BKV positive serological status (IgG) and not only DNA excretion in urine. Given this, it would have been relevant to have tested individuals who were anti BKV IgG (+) for cellular responses even though they did not show viral shedding in urine.

After comparison of virus cellular immune responses in healthy subjects and renal transplant patients, our data indicated that healthy subjects had a higher cellular response than patients, irrespective of BKV status. Regarding the patient groups with different BKV status; T-cell response results were highest in the group who never had BKV, followed by the historical BKV (+) group. Here, high responses could suggest strong BKV-specific immunity in the negative group and recovery of BKV-specific immunity after BKV replication in the historical patients. It is possible that patients who were BK negative had previously encountered the virus but either eliminated it completely or reduced it to undetectable levels; this would

be consistent with their strong immune response. On the other hand, low level of cellular immune responses in the BK viremia group can implicate impaired BKV-specific immunity at the time of viremia, which is more likely due to the immunosuppressive therapies that transplant individuals receive in order to dampen immune responses and prevent acute graft rejection, however the cellular response was restored.

Next, we measured anti-BKV IgG levels in the same groups. In the healthy group no significant differences were observed between anti BKV IgG (+), urine (+) and anti BKV IgG (-) urine (-) subjects. Again, this may be because some apparently virus negative subjects had reduced virus secretion into urine to undetectable levels. In contrast, patient groups showed significant differences based on BKV status. A significant difference was found between pre- and post-transplant patients who developed viremia. Also, anti BKV IgG levels were significantly higher in patients who cleared the virus than those who never experienced infection. Interestingly, a significant negative correlation was observed between BKV viral load and anti-BKV IgG levels as well as elevation of the IgG levels post-transplant. This has implications regarding the role BKV specific humoral immune responses play in controlling viral replication as the increase in the negative group was not significantly different from that observed pre-transplant.

A caveat with interpreting the anti-BKV antibody results is that modification of a semi-quantitative test was utilised. It was therefore difficult to determine a clear cut-off point between anti-BKV antibody +ve and -ve subjects. This contrasts with the experience of CMV in our laboratory which always showed a clear discrimination between antibody +ve and -ve sera (Albayati et al, 2018). This may result from CMV stimulating stronger antibody responses than those induced in BKV infected individuals.

We demonstrated that the BKV-ELISPOT assay may facilitate in predicting the progression of BKV infection; BK virus specific IFN-γ immune response was higher in patients who cleared BKV infection post-transplant. Also, the results were significantly lower in patients who showed development of BK viremia. As samples were taken at time of viremia, it is likely that sufficient time had not elapsed for an effective immune response to be mounted. Our study is limited with a relatively small sample size and was single centred. Also, donor information such as

serostatus and neutralizing antibody was not available, which is believed to correlate with post-transplant viremia (Abend et al., 2017; Bae et al., 2020b; Solis et al., 2018). Future studies on the current topic are therefore recommended with larger sample sizes and from multiple transplant centres.

7.2 Induction of human leucocyte antigen G (HLA-G) in response to BKV peptide stimulation in healthy subjects and renal transplant recipients.

Here, we hypothesized that BKV infection is influencing HLA-G expression on some lymphocyte subsets and modulating cells' action as BK virus escape strategy to evade immune responses. It has been suggested that CMV infection upregulates HLA-G molecules during infection which can inhibit NK cell activation, critical in restricting viral spread (Wilkinson et al., 2008). More recently, our group has shown that CMV can upregulate HLA-G expression on a range of leucocyte subsets, predominantly CD56+ T-cells and B-cells (Albayati et al., 2018).

We demonstrated HLA-G expression on groups of PBMCs isolated from healthy subjects with or without BKV and renal transplant recipients with viremia or who never had viremia. It was observed that culturing the cells of BKV negative healthy subject with BKV peptides slightly increased the expression of HLA-G, especially on monocyte populations in the healthy group. These results reflect findings of previous studies where it was found that low levels of HLA-G+ cells are to be found in healthy subjects (Lozano et al., 2002; Feger et al., 2007). This was unexpected as HLA-G is mainly limited to the feto-maternal interface and it plays a role in preventing maternal immune reactions against foetal tissues (Rebmann, da Silva Nardi, Wagner, & Horn, 2014). Our results demonstrate that culturing PBMCs with BKV peptides induced a slight induction of HLA-G on monocyte populations of both BKV (+) and BKV (-) healthy subjects. However, other lymphocyte subsets such as CD3+CD4+ and CD3+CD8+ cells showed very low levels of expression.

In renal transplant patients, a slight up-regulation of HLA-G expression was observed in CD3+CD8+ and CD3+CD56+ T-cells and monocyte populations,

which is in agreement with previous findings which reported low levels of expression of HLA-G on CD8+ T-cells in peripheral PBMCs from renal allograft recipients (Lu et al., 2011; Xiao et al., 2013). Finally, after measuring induction of HLA-G in healthy subjects and renal recipients with BKV virus viremia, it was clear that patients with BKV virus viremia showed higher percentages of HLA-G on all the lymphocytes subsets. However, it is important to note that upregulation of HLA-G could be due to the influence of immunosuppression as all our patients are under immunosuppressive therapy administration, which is known to have an indirect effect on HLA-G via increasing cytokine production such as IL-12, IL-10, and IL-4 by activated T-helper cells (Daniel et al., 2005). In future work a larger follow up study should be conducted with renal transplant recipients in different stages of BKV viremia. Also investigating the influence of immunosuppressive therapy on cultured lymphocytes with BKV peptides *in vitro* would likely be revealing. Further, additional approaches must be used to overcome the limitation of flow cytometry data analysis, through using multicolour flow cytometry to increase the information from each cell sample.

7.3 sHLA-G concentrations in healthy subjects and renal transplant recipients.

The presence of sHLA-G in serum of either liver or kidney transplant recipients has raised the hypothesis that its presence has an association with immune suppression and preventing acute graft rejection (Lila et al., 2002). The role of sHLA-G in virus infection is apparent with its ability to bind KIR2DL4 on PBMCs, especially NK cells, and produce pro-inflammatory and angiogenic cytokines (Van der Meer et al., 2007) as well as induce apoptosis in CD8+ lymphocytes and NK cells and thereby inhibit T-cell responses (Fournel et al., 2000).

Our data revealed that no significant difference was observed in plasma sHLA-G levels in healthy subjects with BKV Ab, either with BKV DNA or without. The same observation was reported in the patient groups as no difference was found between patients with BKV viremia and those who never had viremia. However, sHLA-G levels were found to be higher in renal transplant patients in comparison to the healthy subjects. This difference could be due to induction of

immunosuppressive therapy but also does not exclude the influence of BKV on inducing immune evasion as most of our viremic patients were undergoing a reduction in their immunosuppressive therapy regime in order to prevent virus replication through increasing induced immunity.

7.4 BKV pre- and post-transplant IgG responses in renal transplant receipts

Evaluating risk factors that may influence BKV reactivation and development of viremia could lead to an early diagnosis of patients at risk for BKPyAN, and consequently preventive treatment, that may improve disease outcome (Buehrig et al., 2003; H. H. Hirsch et al., 2002). Pre-transplant and pre-viremia evaluation of BKV humoral immune responses would help in understanding whether high anti-BKV titres pre-transplant or during viremia could protect from or prevent BKV disease development and if recipients with high risk could be monitored closely with appropriate intervention.

Our data showed that pre-transplant anti BKV levels were similar in patients with BK viremia and those who never had BKV viremia, which concurs with previous studies which found that pre-transplant seropositivity did not prevent BK viremia or was even considered as a risk factor for BKPyAN (D. L. Bohl et al., 2008; H. H. Hirsch et al., 2002). Another study demonstrated elevation of BKV viremia in seronegative patients (Sood et al., 2013). This raises the question as to whether serum anti-BKV IgG could effectively protect against BKV infection in the urothelium and whether local levels of specific secretory IgA or IgM may be more relevant. This would be difficult to investigate directly, but specific antibody levels in secretory fluids such as nasal or lachrymal secretions may be more relevant, representing secretions at other mucosal sites.

After following up evaluation of three different samples from each patient during different periods of BKV reactivation; pre-transplant, viremia and post viremia, we found that levels of virus-specific IgG antibodies were relatively low pre-transplant, however, they significantly increased with development of the viremia in parallel with the reduction of the immunosuppression therapy. The levels of humoral immune responses remained high even after the clearance of viremia. Interestingly, the viruria persistence after elimination of the virus from plasma in

some patients may be a result of a slower or weaker virus-specific secretory antibody response in these individuals. On the other hand, in patients who never experienced an episode of BKV viremia after transplant the level of anti-BKV IgG responses were found to be steady pre- and post-transplant. Our data confirmed the observations of Pastrana et al. that patients receiving immunosuppressive therapies can still develop efficient humoral immune responses (Pastrana et al., 2013).

The above findings could be limited by the relatively low number of evaluated patients. Also, unavailability of a standardized, fully quantitative serological assay for anti-BKV IgG may have led to some variability within the assays and less reliable quantification of differences in antibody titres. Furthermore, the ELISA test used was against VP1 antigen only, but it is unknown if antibodies against other BKV antigens would yield the same results. Additionally, pre-transplant seropositivity does not necessarily mean development of BKV specific memory T-cells that are necessary to control BKV reactivation, it could be result of a patient's contact with the virus previously (P. Comoli et al., 2004).

7.5 Risk of BK Infection in HLA-A02, B44 and DR15–positive Kidney Transplant Recipients.

MHC molecules play an important role in antigen presentation to immune cells and in the generation of T-cell responses. Briefly, after viral infection, viral peptides are presented by MHC class-II and interact with CD4+ cells to generate T-helper cells that aid in the generation of cell mediated immunity by the activation of CD8+ cells and activation of B cells for virus specific antibody production. MHC class-I presentation of antigen activates CD8+ T-cells to produce virus specific cellular response by generating cytotoxic T-cells which can eliminate virally infected cells expressing the antigen as well as T cell memory (Rouse & Mueller, 2019). So, identification of alleles of MHC molecules that influence progression of BKV infection may help in predicting BKV reactivation risks in KTR individuals prior to transplantation and predict development of viremia after transplant.

Here we investigated the effect of some HLA alleles in the development of BKV viremia in kidney transplant recipients. Certain alleles were found to be associated with clearance of some virus infections such as HIV-1 and HCV (Mina et al., 2015;

Rao, Hoof, van Baarle, Keşmir, & Textor, 2015). We evaluated some common HLA alleles such as HLA-A02, HLA-B44 and HLA-DR 15 and their association with BKV viremia in the recipients. It was observed that the presence of HLA-B44 and HLA-DR15 were significantly associated with increased risk of viremia among the recipients which differs from previous findings who demonstrated the influence of particular HLA alleles in reducing the risk of BKV viremia (Masutani et al., 2013; Herman F Wunderink et al., 2019). A possible explanation for this contradiction is the difference in the HLA typing methods used to determine genotypes among the transplantation centres. Additionally, an understanding of the importance of the clinical role of HLA alleles in influencing virus infections is not clear and the need to initiate studies with uniform viral load screening strategies in viremic patients, without interference of immunosuppressive therapy, would be highly informative. Furthermore, although, this study is one of the few on the impact of HLA alleles on BKV viremia in KTR; our study is limited with regards to its retrospective nature that it is based in a single centre and considering that the sample size was relatively small, particularly in the BKV+ control group. These findings need to be confirmed by larger prospective studies involving multiple centres.

7.6 Association of BKV genotypes in development of BK viremia

Finally, we aimed to investigate the BKV genotype prevalence in renal transplant patients and to evaluate whether BKV genotypes could associate with development of BKV viremia in post renal transplant recipients retrospectively. Our data indicated that all our tested groups were infected with BKV subtype I; 78.5% of the tested group were infected with subtype I-b2, while 21.4% were infected with subtype I-b1, the two most common BKV subtypes in the UK, although there is some geographical variation with other subtype predominating in different parts of the world.

No significant correlation was observed between the genotypes and BKV viremia, which contrasts with previous literature reporting an association between viral subtypes and development of disease after transplant, with the first two studies reporting on CMV subtypes (Eliana Nogueira, Ozaki, Tomiyama, Câmara, & Granato, 2009; Sarcinella, Mazzulli, Willey, & Humar, 2002; R. B. Varella et al.,

2018). This inconsistency could be due to variability of the viral load quantification between transplant centres, such as variability of using different controls to measure copy number and different primers and probe assays that are highly important in detection of BKV genotypes.

BKV subtyping and subgrouping were based mainly on phylogenetic analysis which is less discriminating than when the analysis is based on the 327bp region used in some BKV-typing studies (Trofe-Clark et al., 2013). However, our genotyping method was based on analysis of only 100bp of the VP1 gene to permit discrimination between subtypes and subgroups (Morel et al., 2017).

An important future recommendation would be the adoption of a genotyping method that is simple and accessible to routine laboratories, to better understand virus related factors that lead to BKV disease progression. The notable limitation of this study is the small sample size; due to limited follow up in the renal transplant recipients in the hospital which may have restricted us being able to detect the other BKV genotypes, such as subtype IV, although this could also be due to variant geographical distributions of these genotypes. Furthermore, the low numbers of samples obtained prevented us from repeating the amplification in some samples.

7.7 Conclusion

In this work it has been shown that immunocompetent subjects with BKV detected in their urine were able to mount specific BKV cellular immune responses, which were deficient in KTR individuals who developed viremia, but these immune responses were found to be restored after resolving viremia. This leads to the conclusion that control of BKV replication in KTR individuals is highly correlated with the generation of BKV-specific cellular immune responses.

HLA-G expression has been suggested to have a negative role in virus evasion from the development of host immune responses and in cancer development (Amiot, Vu, & Samson, 2014b), but also it was found to be beneficial such as in aseptic shock (Monneret et al., 2007). The role of this molecule in modulating immunity requires further investigation as it is still not clear whether it is related to virus or the host response. Better understanding this association would be

highly beneficial in utilising HLA-G parameters as a therapeutic marker when treating BKV infection in KTR individuals, Also, we showed that IgG responses to BKV infection was stable in healthy subjects, whilst in BKV (+) transplant patients was mainly influenced by virus replication (viremia). However, using pre-transplant anti-BKV IgG levels as a biomarker for predicting BKV infection is not sufficient, and a patient's sero-reactivity should be considered when seeking a more accurate prediction of outcome.

Finally, BKV genotype Ib-2 was found to be the most dominant genotype among our patients with no direct influence of specific genotype being associated with increased risks of viremia or development of BKV disease.

7.8 Future directions

BK virus infection has become one of the most recognizable obstacles in solid organ transplant, especially kidney transplant and in hematopoietic stem cell transplant recipients due to influence of immunosuppression regimens. Despite the current detection and screening methods after transplantation, an extra focus on the pre-transplant factors must establish, for example, a pre-transplant algorithm or risk assessment that may help deciding the best approach in treating the recipients after transplantation. By studying some pre-transplantation risk factor such as donors seropositivity, donor-recipient HLA mismatching, and other virus-related factors like serotypes and genomic mutations and measuring humoral immunity against the other BKV genotypes for better prediction and early elimination of the virus replication before the disease progression.

As there is no specific antiviral treatment for BK viremia, there is an urgent need of randomized multicentre clinical trials that investigates the reasons of activation of the virus in some patients but not others, and the immune response toward the virus replication and disease progression. Also, there is a crucial need of testing and evaluating some anti-viral combination therapies that may help in elimination of the virus without the need of reducing immunosuppression.

8 References

Abate, D., Saldan, A., Fiscon, M., Cofano, S., Paciolla, A., Furian, L., . . . Mengoli, C. (2010). Evaluation of cytomegalovirus (CMV)-specific T cell immune reconstitution revealed that baseline antiviral immunity, prophylaxis, or preemptive therapy but not antithymocyte globulin treatment contribute to CMV-specific T cell reconstitution in kidney transplant recipients. *The Journal of infectious diseases, 202*(4), 585-594.

Abend, J. R., Changala, M., Sathe, A., Casey, F., Kistler, A., Chandran, S., . . . Wojciechowski, D. (2017). Correlation of BK virus neutralizing serostatus with the incidence of BK viremia in kidney transplant recipients. *Transplantation, 101*(6), 1495-1505.

Acott, P. D. (2013). Natural killer cell response to BK virus infection in polyoma virus–associated nephropathy of renal transplant recipients. *Kidney International, 84*(2), 233-235. doi:https://doi.org/10.1038/ki.2013.148

Akhter, A., Das, V., Naik, S., Faridi, R. M., Pandey, A., & Agrawal, S. (2012). Upregulation of HLA-G in JEG-3 cells by dexamethasone and hydrocortisone. *Archives of gynecology and obstetrics, 285*(1), 7-14.

Alalawi, F., Kossi, M., Jenkins, J., & Halawa, A. (2020). BK Virus Infection in Adult Renal Transplant Recipients; Risk Factors and their Impact on Allograft Survival. 2020. doi:10.15761/TiT.1000278

Albayati, Z., Alyami, A., Alomar, S., Middleton, D., Bonnett, L., Aleem, S., . . . Christmas, S. E. (2017). The Influence of Cytomegalovirus on Expression of HLA-G and its Ligand KIR2DL4 by Human Peripheral Blood Leucocyte Subsets. *Scandinavian Journal of Immunology, 86*(5), 396-407. doi:10.1111/sji.12594

Albayati, Z., Heyworth, S., Moberly, J., Middleton, D., Flanagan, B., Hammad, A., & Christmas, S. (2018). Changes in HLA-G Expression by Leucocyte Subsets Following Renal Transplantation and the Effect of Immunosuppressive Drugs on Leucocyte HLA-G Expression in Vitro. *OBM Transplantation, 2*, 1-1. doi:10.21926/obm.transplant.1804032

Alexiev, B. A., Randhawa, P., Vazquez Martul, E., Zeng, G., Luo, C., Ramos, E., . . . Papadimitriou, J. C. (2013). BK virus-associated urinary bladder carcinoma in transplant recipients: report of 2 cases, review of the literature, and proposed pathogenetic model. *Hum Pathol, 44*(5), 908-917. doi:10.1016/j.humpath.2012.09.019

Alméras, C., Foulongne, V., Garrigue, V., Szwarc, I., Vetromile, F., Segondy, M., & Mourad, G. (2008). Does reduction in immunosuppression in viremic patients prevent BK virus nephropathy in de novo renal transplant recipients? A prospective study. *Transplantation, 85*(8), 1099-1104. doi:10.1097/TP.0b013e31816a33d4

Almeras, C., Vetromile, F., Garrigue, V., Szwarc, I., Foulongne, V., & Mourad, G. (2011). Monthly screening for BK viremia is an effective strategy to prevent BK virus nephropathy in renal transplant recipients. *Transpl Infect Dis, 13*(2), 101-108. doi:10.1111/j.1399-3062.2011.00619.x

Alter, G., Malenfant, J. M., & Altfeld, M. (2004). CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods, 294*(1-2), 15-22. doi:10.1016/j.jim.2004.08.008

Ambalathingal, G., Francis, R., Smyth, M., Smith, C., & Khanna, R. (2017). BK Polyomavirus: Clinical Aspects, Immune Regulation, and Emerging Therapies. *Clinical Microbiology Reviews, 30*, 503-528. doi:10.1128/CMR.00074-16

Ambalathingal, G. R., Francis, R. S., Smyth, M. J., Smith, C., & Khanna, R. (2017). BK Polyomavirus: Clinical Aspects, Immune Regulation, and Emerging Therapies. *Clin Microbiol Rev, 30*(2), 503-528. doi:10.1128/cmr.00074-16

Amiot, L., Vu, N., & Samson, M. (2014a). Immunomodulatory properties of HLA-G in infectious diseases. *Journal of immunology research, 2014*.

Amiot, L., Vu, N., & Samson, M. (2014b). Immunomodulatory properties of HLA-G in infectious diseases. *Journal of immunology research, 2014*, 298569-298569. doi:10.1155/2014/298569

Antonsson, A., Green, A. C., Mallitt, K. A., O'Rourke, P. K., Pawlita, M., Waterboer, T., & Neale, R. E. (2010). Prevalence and stability of antibodies to the BK and JC polyomaviruses: a long-term longitudinal study of Australians. *J Gen Virol, 91*(Pt 7), 1849-1853. doi:10.1099/vir.0.020115-0

Apiwattanakul, N., Hongeng, S., Anurathapan, U., Pakakasama, S., Srisala, S., Techasaensiri, C., & Andersson, B. S. (2017). Viral-specific T-cell response in hemorrhagic cystitis after haploidentical donor stem cell transplantation. *Transplant Infectious Disease, 19*(6), e12775.

Bae, H., Na, D., Chang, J., Park, K., Min, J., Ko, E., . . . Oh, E. (2020a). Usefulness of BK virus-specific interferon-γ enzyme-linked immunospot assay for predicting the outcome of BK virus infection in kidney transplant recipients. *Korean J. Intern. Med*.

Bae, H., Na, D. H., Chang, J.-Y., Park, K. H., Min, J. W., Ko, E. J., . . . Oh, E.-J. (2020b). Usefulness of BK virus-specific interferon-纬 enzyme-linked immunospot assay for predicting the outcome of BK virus infection in kidney transplant recipients. *The Korean Journal of Internal Medicine*.

Baez, C. F., Brandão Varella, R., Villani, S., & Delbue, S. (2017). Human polyomaviruses: the battle of large and small tumor antigens. *Virology: research and treatment, 8*, 1178122X17744785.

Baksh, F. K., Finkelstein, S. D., Swalsky, P. A., Stoner, G. L., Ryschkewitsch, C. F., & Randhawa, P. (2001). Molecular genotyping of BK and JC viruses in human polyomavirus-associated interstitial nephritis after renal transplantation. *Am J Kidney Dis, 38*(2), 354-365. doi:10.1053/ajkd.2001.26101

Barth, H., Solis, M., Lepiller, Q., Sueur, C., Soulier, E., Caillard, S., . . . Fafi-Kremer, S. (2017). 45 years after the discovery of human polyomaviruses BK and JC: Time to speed up the understanding of associated diseases and treatment approaches. *Crit Rev Microbiol, 43*(2), 178-195. doi:10.1080/1040841x.2016.1189873

Batal, I., Zeevi, A., Heider, A., Girnita, A., Basu, A., Tan, H., . . . Randhawa, P. (2008). Measurements of global cell-mediated immunity in renal transplant recipients with BK virus reactivation. *Am J Clin Pathol, 129*(4), 587-591. doi:10.1309/23ygpb1e758eccfp

Binggeli, S., Egli, A., Schaub, S., Binet, I., Mayr, M., Steiger, J., & Hirsch, H. H. (2007). Polyomavirus BK-specific cellular immune response to VP1 and large T-antigen in kidney transplant recipients. *Am J Transplant, 7*(5), 1131-1139. doi:10.1111/j.1600-6143.2007.01754.x

Blazquez-Navarro, A., Schachtner, T., Stervbo, U., Sefrin, A., Stein, M., Westhoff, T. H., . . . Or-Guil, M. (2018). Differential T cell response against BK virus regulatory and structural antigens: A viral dynamics modelling approach. *PLoS computational biology, 14*(5), e1005998-e1005998. doi:10.1371/journal.pcbi.1005998

Blyth, E., Clancy, L., Simms, R., Gaundar, S., O'Connell, P., Micklethwaite, K., & Gottlieb, D. J. (2011). BK virus-specific T cells for use in cellular therapy show specificity to multiple antigens and polyfunctional cytokine responses. *Transplantation, 92*(10), 1077-1084. doi:10.1097/TP.0b013e31823328c0

Bohl, D., Ryschkewitsch, C., Major, E., Storch, G., & Brennan, D. (2005). BK VIRUS ANTIBODY TITERS MARKEDLY INCREASE WITH VIREMIA: Abstract# 463. *American Journal of Transplantation Supplement, 5*.

Bohl, D. L., Brennan, D. C., Ryschkewitsch, C., Gaudreault-Keener, M., Major, E. O., & Storch, G. A. (2008). BK virus antibody titers and intensity of infections after renal transplantation. *J Clin Virol, 43*(2), 184-189. doi:10.1016/j.jcv.2008.06.009

Bohl, D. L., Storch, G. A., Ryschkewitsch, C., Gaudreault-Keener, M., Schnitzler, M. A., Major, E. O., & Brennan, D. C. (2005). Donor origin of BK virus in renal transplantation and role of HLA C7 in susceptibility to sustained BK viremia. *Am J Transplant, 5*(9), 2213-2221. doi:10.1111/j.1600-6143.2005.01000.x

Borni-Duval, C., Caillard, S., Olagne, J., Perrin, P., Braun-Parvez, L., Heibel, F., & Moulin, B. (2013). Risk factors for BK virus infection in the era of therapeutic drug monitoring. *Transplantation, 95*(12), 1498-1505. doi:10.1097/TP.0b013e3182921995

Boutell, C., & Everett, R. D. (2013). Regulation of alphaherpesvirus infections by the ICP0 family of proteins. *Journal of General Virology, 94*(3), 465-481.

Bozón, M. V., Delgado, J. C., Selvakumar, A., Clavijo, O. P., Salazar, M., Ohashi, M., . . . Yunis, E. J. (1997). Error rate for HLA-B antigen assignment by serology: implications for proficiency testing and utilization of DNA-based typing methods. *Tissue Antigens, 50*(4), 387-394. doi:10.1111/j.1399-0039.1997.tb02892.x

Brennan, D. C., Agha, I., Bohl, D. L., Schnitzler, M. A., Hardinger, K. L., Lockwood, M., . . . Storch, G. A. (2005). Incidence of BK with tacrolimus versus cyclosporine and impact of preemptive immunosuppression reduction. *Am J Transplant, 5*(3), 582-594. doi:10.1111/j.1600-6143.2005.00742.x

Bridges, B., Donegan, S., & Badros, A. (2006). Cidofovir bladder instillation for the treatment of BK hemorrhagic cystitis after allogeneic stem cell transplantation. *American journal of hematology, 81*(7), 535-537.

Buehrig, C. K., Lager, D. J., Stegall, M. D., Kreps, M. A., Kremers, W. K., Gloor, J. M., . . . Larson, T. S. (2003). Influence of surveillance renal allograft biopsy on diagnosis and prognosis of polyomavirus-associated nephropathy. *Kidney international, 64*(2), 665-673.

Burgos, D., Lopez, V., Cabello, M., Sola, E., Gutierrez, C., García, I., . . . Molina, M. G. (2006). *Polyomavirus BK nephropathy: The effect of an early diagnosis on renal function or graft loss.* Paper presented at the Transplantation proceedings.

Carosella, E. D., Favier, B., Rouas-Freiss, N., Moreau, P., & LeMaoult, J. (2008). Beyond the increasing complexity of the immunomodulatory HLA-G molecule. *Blood, 111*(10), 4862-4870.

Chanouzas, D., Small, A., Borrows, R., & Ball, S. (2018). Assessment of the T-SPOT. CMV interferon-γ release assay in renal transplant recipients: A single center cohort study. *PloS one, 13*(3).

Chawla-Sarkar, M., Lindner, D. J., Liu, Y.-F., Williams, B., Sen, G. C., Silverman, R. H., & Borden, E. C. (2003). Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis, 8*(3), 237-249.

Chen, H.-X., Chen, B.-G., Shi, W.-W., Zhen, R., Xu, D.-P., Lin, A., & Yan, W.-H. (2011). Induction of cell surface human leukocyte antigen–G expression in pandemic H1N1 2009 and seasonal H1N1 influenza virus–infected patients. *Human immunology, 72*(2), 159-165.

Chen, Y., Trofe, J., Gordon, J., Autissier, P., Woodle, E. S., & Koralnik, I. J. (2008). BKV and JCV large T antigen-specific CD8+ T cell response in HLA A*0201+ kidney transplant recipients with polyomavirus nephropathy and patients with progressive multifocal leukoencephalopathy. *J Clin Virol, 42*(2), 198-202. doi:10.1016/j.jcv.2008.01.005

Choo, S. Y. (2007). The HLA system: genetics, immunology, clinical testing, and clinical implications. *Yonsei medical journal, 48*(1), 11-23. doi:10.3349/ymj.2007.48.1.11

Cioni, M., Leboeuf, C., Comoli, P., Ginevri, F., & Hirsch, H. H. (2016). Characterization of Immunodominant BK Polyomavirus 9mer Epitope T Cell Responses. *Am J Transplant, 16*(4), 1193-1206. doi:10.1111/ajt.13598

Coleman, D., Gardner, S., & Field, A. (1973). Human polyomavirus infection in renal allograft recipients. *Br Med J, 3*(5876), 371-375.

Comoli, P., Azzi, A., Maccario, R., Basso, S., Botti, G., Basile, G., . . . Ginevri, F. (2004). Polyomavirus BK-specific immunity after kidney transplantation. *Transplantation, 78*(8), 1229-1232.

Comoli, P., Basso, S., Azzi, A., Moretta, A., De Santis, R., Del Galdo, F., . . . Ginevri, F. (2003). Dendritic cells pulsed with polyomavirus BK antigen induce ex vivo polyoma BK virus-specific cytotoxic

T-cell lines in seropositive healthy individuals and renal transplant recipients. *J Am Soc Nephrol, 14*(12), 3197-3204.
Comoli, P., Binggeli, S., Ginevri, F., & Hirsch, H. H. (2006). Polyomavirus-associated nephropathy: update on BK virus-specific immunity. *Transpl Infect Dis, 8*(2), 86-94. doi:10.1111/j.1399-3062.2006.00167.x
Comoli, P., Hirsch, H. H., & Ginevri, F. (2008). Cellular immune responses to BK virus. *Current Opinion in Organ Transplantation, 13*(6), 569-574. doi:10.1097/MOT.0b013e3283186b93
Cordero, E., Veit, T., Da Silva, M., Jacques, S., Silla, L., & Chies, J. (2009). HLA-G polymorphism influences the susceptibility to HCV infection in sickle cell disease patients. *Tissue Antigens, 74*(4), 308-313.
Créput, C., Durrbach, A., Menier, C., Guettier, C., Samuel, D., Dausset, J., . . . Rouas-Freiss, N. (2003). Human leukocyte antigen-G (HLA-G) expression in biliary epithelial cells is associated with allograft acceptance in liver-kidney transplantation. *Journal of hepatology, 39*(4), 587-594.
Creput, C., Le Friec, G., Bahri, R., Amiot, L., Charpentier, B., Carosella, E., . . . Durrbach, A. (2003). Detection of HLA-G in serum and graft biopsy associated with fewer acute rejections following combined liver–kidney transplantation: possible implications for monitoring patients. *Human immunology, 64*(11), 1033-1038.
da Silva, G. K., Vianna, P., Veit, T. D., Crovella, S., Catamo, E., Cordero, E. A. A., . . . Kuhmmer, R. (2014). Influence of HLA-G polymorphisms in human immunodeficiency virus infection and hepatitis C virus co-infection in Brazilian and Italian individuals. *Infection, Genetics and Evolution, 21*, 418-423.
Daniel, V., Naujokat, C., Sadeghi, M., Wiesel, M., Hergesell, O., & Opelz, G. (2005). Association of Circulating Interleukin (IL)-12–and IL-10–Producing Dendritic Cells with Time Posttransplant, Dose of Immunosuppression, and Plasma Cytokines in Renal-Transplant Recipients. *Transplantation, 79*(11), 1498-1506.
De Stasio, A., Orsi, N., Pana, A., Seganti, L., Sinibaldi, S., & Valenti, P. (1979). The importance of pre-treatment of human sera for the titration of hemagglutination-inhibiting antibodies towards BK virus. *Annali Sclavo; rivista di microbiologia e di immunologia, 21*(3), 249.
Dekeyser, M., Francois, H., Beaudreuil, S., & Durrbach, A. (2015). Polyomavirus-Specific Cellular Immunity: From BK-Virus-Specific Cellular Immunity to BK-Virus-Associated Nephropathy? *Front Immunol, 6*, 307. doi:10.3389/fimmu.2015.00307
DeStasio, A., Mastromarino, P., Pana, A., Seganti, L., Sinibaldi, L., Valenti, P., & Orsi, N. (1980). CHARACTERIZATION OF A GLYCOPROTEIN INHIBITOR PRESENT IN HUMAN-SERUM AND ACTIVE TOWARDS BK-VIRUS HEMAGGLUTINATION. *Microbiologica, 3*(3), 293-305.
Ding, R., Medeiros, M., Dadhania, D., Muthukumar, T., Kracker, D., Kong, J. M., . . . Suthanthiran, M. (2002). Noninvasive diagnosis of BK virus nephritis by measurement of messenger RNA for BK virus VP1 in urine. *Transplantation, 74*(7), 987-994. doi:10.1097/01.tp.0000032151.07990.1e
Drake, D. R., 3rd, Shawver, M. L., Hadley, A., Butz, E., Maliszewski, C., & Lukacher, A. E. (2001). Induction of polyomavirus-specific CD8(+) T lymphocytes by distinct dendritic cell subpopulations. *J Virol, 75*(1), 544-547. doi:10.1128/jvi.75.1.544-547.2001
Drew, R. J., Walsh, A., Laoi, B. N., & Crowley, B. (2012). Phylogenetic analysis of the complete genome of 11 BKV isolates obtained from allogenic stem cell transplant recipients in Ireland. *J Med Virol, 84*(7), 1037-1048. doi:10.1002/jmv.23240
Dugan, A. S. (2008). *The interactions between BK virus and host cell receptors*: Brown University.
Egli, A., Binggeli, S., Bodaghi, S., Dumoulin, A., Funk, G. A., Khanna, N., . . . Hirsch, H. H. (2007). Cytomegalovirus and polyomavirus BK posttransplant. *Nephrology Dialysis Transplantation, 22*(suppl_8), viii72-viii82. doi:10.1093/ndt/gfm648
Egli, A., Infanti, L., Dumoulin, A., Buser, A., Samaridis, J., Stebler, C., . . . Hirsch, H. H. (2009). Prevalence of Polyomavirus BK and JC Infection and Replication in 400 Healthy Blood Donors. *The Journal of Infectious Diseases, 199*(6), 837-846. doi:10.1086/597126

Egli, A., Infanti, L., Dumoulin, A., Buser, A., Samaridis, J., Stebler, C., . . . Hirsch, H. H. (2009). Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. *J Infect Dis, 199*(6), 837-846. doi:10.1086/597126

Feger, U., Tolosa, E., Huang, Y. H., Waschbisch, A., Biedermann, T., Melms, A., & Wiendl, H. (2007). HLA-G expression defines a novel regulatory T-cell subset present in human peripheral blood and sites of inflammation. *Blood, 110*(2), 568-577. doi:10.1182/blood-2006-11-057125

Feltkamp, M. C., Kazem, S., van der Meijden, E., Lauber, C., & Gorbalenya, A. E. (2013). From Stockholm to Malawi: recent developments in studying human polyomaviruses. *Journal of General Virology, 94*(3), 482-496.

Ganguly, N., Clough, L., Dubois, L., Mcguirk, J., Abhyankar, S., Aljitawi, O., . . . Ganguly, S. (2010). Low-dose cidofovir in the treatment of symptomatic BK virus infection in patients undergoing allogeneic hematopoietic stem cell transplantation: A retrospective analysis of an algorithmic approach. *Transplant Infectious Disease, 12*(5), 406-411.

Gard, L., Niesters, H. G., & Riezebos-Brilman, A. (2015). A real time genotyping PCR assay for polyomavirus BK. *J Virol Methods, 221*, 51-56. doi:10.1016/j.jviromet.2015.04.024

Gardner, S., MacKenzie, E., Smith, C., & Porter, A. (1984). Prospective study of the human polyomaviruses BK and JC and cytomegalovirus in renal transplant recipients. *Journal of clinical pathology, 37*(5), 578-586.

Gardner, S. D., Field, A. M., Coleman, D. V., & Hulme, B. (1971). New human papovavirus (B.K.) isolated from urine after renal transplantation. *Lancet, 1*(7712), 1253-1257.

Gazit, E., Sherf, M., Balbin, E., Muratov, A., Goldstein, I., & Loewenthal, R. (2007). HLA-G expression is induced in Epstein-Barr virus–transformed B-cell lines by culture conditions. *Human immunology, 68*(6), 463-468.

Gebel, H. M., Bray, R. A., & Nickerson, P. (2003). Pre-transplant assessment of donor-reactive, HLA-specific antibodies in renal transplantation: contraindication vs. risk. *Am J Transplant, 3*(12), 1488-1500. doi:10.1046/j.1600-6135.2003.00273.x

Gheit, T., Dutta, S., Oliver, J., Robitaille, A., Hampras, S., Combes, J.-D., . . . Cherpelis, B. (2017). Isolation and characterization of a novel putative human polyomavirus. *Virology, 506*, 45-54.

Goudsmit, J., Dillen, P. W. v., Van Strien, A., & Van der Noordaa, J. (1982). The role of BK virus in acute respiratory tract disease and the presence of BKV DNA in tonsils. *Journal of medical virology, 10*(2), 91-99.

Greer, A. E., Forman, M. S., & Valsamakis, A. (2015). Comparison of BKV quantification using a single automated nucleic acid extraction platform and 3 real-time PCR assays. *Diagn Microbiol Infect Dis, 82*(4), 297-302. doi:10.1016/j.diagmicrobio.2015.04.006

Gross, L. (1953). A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. *Proceedings of the Society for Experimental Biology and Medicine, 83*(2), 414-421.

Halim, M. A., Al-Otaibi, T., Gheith, O., Mosaad, A., Zakaria, Z., Said, T., . . . Nampoory, N. M. (2016). Long-Term Follow-Up of Active Treatment Versus Minimization of Immunosuppressive Agents in Patients With BK Virus-Associated Nephropathy After Kidney Transplant. *Exp Clin Transplant, 14*(1), 58-65.

Hammer, M., Brestrich, G., Andree, H., Engelmann, E., Rosenberger, C., Tillmann, H., . . . Volk, H. D. (2006). HLA type-independent method to monitor polyoma BK virus-specific CD4+ and CD8+ T-Cell immunity. *American Journal of Transplantation, 6*(3), 625-631.

Hardinger, K. L., Koch, M. J., Bohl, D. J., Storch, G. A., & Brennan, D. C. (2010a). BK-virus and the impact of pre-emptive immunosuppression reduction: 5-year results. *Am J Transplant, 10*(2), 407-415. doi:10.1111/j.1600-6143.2009.02952.x

Hardinger, K. L., Koch, M. J., Bohl, D. J., Storch, G. A., & Brennan, D. C. (2010b). BK-virus and the impact of pre-emptive immunosuppression reduction: 5-year results. *American journal of transplantation : official journal of the American Society of Transplantation and the*

American Society of Transplant Surgeons, 10(2), 407-415. doi:10.1111/j.1600-6143.2009.02952.x

Hariharan, S., Cohen, E. P., Vasudev, B., Orentas, R., Viscidi, R. P., Kakela, J., & DuChateau, B. (2005). BK virus-specific antibodies and BKV DNA in renal transplant recipients with BKV nephritis. *Am J Transplant, 5*(11), 2719-2724. doi:10.1111/j.1600-6143.2005.01080.x

Hariharan, S., Cohen, E. P., Vasudev, B., Orentas, R., Viscidi, R. P., Kakela, J., & DuChateau, B. (2005). BK virus-specific antibodies and BKV DNA in renal transplant recipients with BKV nephritis. *American journal of transplantation, 5*(11), 2719-2724.

Helle, F., Brochot, E., Handala, L., Martin, E., Castelain, S., Francois, C., & Duverlie, G. (2017). Biology of the BKPyV: An Update. *Viruses, 9*(11). doi:10.3390/v9110327

Hesselink, D. A., Vaessen, L. M., Hop, W. C., Schoordijk, W., Ijzermans, J. N., Baan, C. C., & Weimar, W. (2005). The effects of renal transplantation on circulating dendritic cells. *Clin Exp Immunol, 140*(2), 384-393. doi:10.1111/j.1365-2249.2005.02755.x

Hirsch, H., Yakhontova, K., Lu, M., & Manzetti, J. (2016). BK polyomavirus replication in renal tubular epithelial cells is inhibited by sirolimus, but activated by tacrolimus through a pathway involving FKBP-12. *American Journal of Transplantation, 16*(3), 821-832.

Hirsch, H. H. (2005). BK virus: opportunity makes a pathogen. *Clin Infect Dis, 41*(3), 354-360. doi:10.1086/431488

Hirsch, H. H., Brennan, D. C., Drachenberg, C. B., Ginevri, F., Gordon, J., Limaye, A. P., . . . Trofe, J. (2005). Polyomavirus-associated nephropathy in renal transplantation: interdisciplinary analyses and recommendations. *Transplantation, 79*(10), 1277-1286.

Hirsch, H. H., Drachenberg, C. B., Steiger, J., & Ramos, E. (2006). Polyomavirus-associated nephropathy in renal transplantation: critical issues of screening and management. *Adv Exp Med Biol, 577*, 160-173. doi:10.1007/0-387-32957-9_11

Hirsch, H. H., Knowles, W., Dickenmann, M., Passweg, J., Klimkait, T., Mihatsch, M. J., & Steiger, J. (2002). Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med, 347*(7), 488-496. doi:10.1056/NEJMoa020439

Hirsch, H. H., & Randhawa, P. (2013). BK polyomavirus in solid organ transplantation. *Am J Transplant, 13 Suppl 4*, 179-188. doi:10.1111/ajt.12110

Hirsch, H. H., & Steiger, J. (2003). Polyomavirus BK. *Lancet Infect Dis, 3*(10), 611-623. doi:10.1016/s1473-3099(03)00770-9

HOGAN, T. F., BORDEN, E. C., McBain, J., Padgett, B., & Walker, D. (1980). Human polyomavirus infections with JC virus and BK virus in renal transplant patients. *Annals of Internal Medicine, 92*(3), 373-378.

Hurdiss D , L., Morgan E , L., Thompson R , F., Prescott E , L., Panou M , M., Macdonald, A., & Ranson N , A. (2016). New Structural Insights into the Genome and Minor Capsid Proteins of BK Polyomavirus using Cryo-Electron Microscopy. *Structure, 24*(4), 528-536. doi:10.1016/j.str.2016.02.008

Ikegaya, H., Saukko, P. J., Tertti, R., Metsarinne, K. P., Carr, M. J., Crowley, B., . . . Yogo, Y. (2006). Identification of a genomic subgroup of BK polyomavirus spread in European populations. *J Gen Virol, 87*(Pt 11), 3201-3208. doi:10.1099/vir.0.82266-0

Jacobi, J., Prignitz, A., Büttner, M., Korn, K., Weidemann, A., Hilgers, K. F., . . . Wullich, B. (2013). BK viremia and polyomavirus nephropathy in 352 kidney transplants; risk factors and potential role of mTOR inhibition. *BMC nephrology, 14*(1), 207.

Jahan, S., Scuderi, C., Francis, L., Neller, M. A., Rehan, S., Crooks, P., . . . John, G. T. T-cell adoptive immunotherapy for BK nephropathy in renal transplantation. *Transplant Infectious Disease, n/a*(n/a), e13399. doi:10.1111/tid.13399

Janeway, C. A. (2001). How the immune system works to protect the host from infection: a personal view. *Proceedings of the National Academy of Sciences, 98*(13), 7461-7468.

Janeway Jr, C. A. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunology today, 13*(1), 11-16.

Janeway Jr, C. A., & Medzhitov, R. (2002). Innate immune recognition. *Annual review of immunology, 20*(1), 197-216.
Jiang, M., Abend, J. R., Johnson, S. F., & Imperiale, M. J. (2009). The role of polyomaviruses in human disease. *Virology, 384*(2), 266-273. doi:10.1016/j.virol.2008.09.027
Jin, L. (2001). Molecular Methods for Identification and Genotyping of BK Virus. In L. Raptis (Ed.), *SV40 Protocols* (pp. 33-48). Totowa, NJ: Humana Press.
Jin, Z.-K., Xu, C.-X., Tian, P.-X., Xue, W.-J., Ding, X.-M., Zheng, J., . . . Lin, Y. (2012). Impact of HLA-G 14-bp polymorphism on acute rejection and cytomegalovirus infection in kidney transplant recipients from northwestern China. *Transplant Immunology, 27*(2), 69-74. doi:https://doi.org/10.1016/j.trim.2012.06.008
Johnston, O., Jaswal, D., Gill, J. S., Doucette, S., Fergusson, D. A., & Knoll, G. A. (2010). Treatment of Polyomavirus Infection in Kidney Transplant Recipients: A Systematic Review. *Transplantation, 89*(9), 1057-1070. doi:10.1097/TP.0b013e3181d0e15e
Josephson, M. A., Gillen, D., Javaid, B., Kadambi, P., Meehan, S., Foster, P., . . . Atwood, W. (2006). Treatment of renal allograft polyoma BK virus infection with leflunomide. *Transplantation, 81*(5), 704-710.
Kardas, P. (2015). *JC and BK polyomavirus-like particles as targets of innate and adaptive humoral immunity.* University_of_Basel,
Kariminik, A., Yaghobi, R., & Dabiri, S. (2016). Innate Immunity and BK Virus: Prospective Strategies. *Viral Immunol, 29*(2), 74-82. doi:10.1089/vim.2015.0099
Kawai, T., & Akira, S. (2007). Signaling to NF-κB by Toll-like receptors. *Trends in molecular medicine, 13*(11), 460-469.
Kean, J. M., Rao, S., Wang, M., & Garcea, R. L. (2009). Seroepidemiology of human polyomaviruses. *PLoS Pathog, 5*(3), e1000363. doi:10.1371/journal.ppat.1000363
Keven, K., Sahin, M., Kutlay, S., Sengul, S., Erturk, S., Ersoz, S., & Erbay, B. (2003). Immunoglobulin deficiency in kidney allograft recipients: comparative effects of mycophenolate mofetil and azathioprine. *Transplant Infectious Disease, 5*(4), 181-186. doi:10.1111/j.1399-3062.2003.00035.x
Khalili, K., & Stoner, G. L. (2004). *Human polyomaviruses: molecular and clinical perspectives*: John Wiley & Sons.
Knowles, W. A. (2001). The epidemiology of BK virus and the occurrence of antigenic and genomic subtypes. *Human polyomaviruses: molecular and clinical perspectives*, 527-559.
Knowles, W. A. (2006). Discovery and epidemiology of the human polyomaviruses BK virus (BKV) and JC virus (JCV). *Adv Exp Med Biol, 577*, 19-45. doi:10.1007/0-387-32957-9_2
Knowles, W. A., Pipkin, P., Andrews, N., Vyse, A., Minor, P., Brown, D. W., & Miller, E. (2003). Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40. *J Med Virol, 71*(1), 115-123. doi:10.1002/jmv.10450
Koralnik, I. J., Du Pasquier, R. A., & Letvin, N. L. (2001). JC virus-specific cytotoxic T lymphocytes in individuals with progressive multifocal leukoencephalopathy. *J Virol, 75*(7), 3483-3487. doi:10.1128/jvi.75.7.3483-3487.2001
Korth, J., Anastasiou, O. E., Bräsen, J. H., Brinkhoff, A., Lehmann, U., Kribben, A., . . . Widera, M. (2019). The detection of BKPyV genotypes II and IV after renal transplantation as a simple tool for risk assessment for PyVAN and transplant outcome already at early stages of BKPyV reactivation. *Journal of Clinical Virology, 113*, 14-19. doi:https://doi.org/10.1016/j.jcv.2019.02.002
Korup, S., Rietscher, J., Calvignac-Spencer, S., Trusch, F., Hofmann, J., Moens, U., . . . Ehlers, B. (2013). Identification of a novel human polyomavirus in organs of the gastrointestinal tract. *PloS one, 8*(3), e58021.
Koukoulaki, M., Grispou, E., Pistolas, D., Balaska, K., Apostolou, T., Anagnostopoulou, M., . . . Drakopoulos, S. (2009). Prospective monitoring of BK virus replication in renal transplant recipients. *Transpl Infect Dis, 11*(1), 1-10. doi:10.1111/j.1399-3062.2008.00342.x

Krumbholz, A., Bininda-Emonds, O. R., Wutzler, P., & Zell, R. (2009). Phylogenetics, evolution, and medical importance of polyomaviruses. *Infection, Genetics and Evolution, 9*(5), 784-799.

Lamarche, C., Orio, J., Collette, S., Senécal, L., Hébert, M.-J., Renoult, É., . . . Delisle, J.-S. (2016). BK Polyomavirus and the Transplanted Kidney: Immunopathology and Therapeutic Approaches. *Transplantation, 100*(11), 2276-2287. doi:10.1097/TP.0000000000001333

Lazarte, J., Adamson, M. B., Tumiati, L. C., & Delgado, D. H. (2018). 10-Year Experience with HLA-G in Heart Transplantation. *Hum Immunol, 79*(8), 587-593. doi:10.1016/j.humimm.2018.05.003

Le Bon, A., & Tough, D. F. (2002). Links between innate and adaptive immunity via type I interferon. *Current opinion in immunology, 14*(4), 432-436.

Leboeuf, C., Wilk, S., Achermann, R., Binet, I., Golshayan, D., Hadaya, K., . . . Hirsch, H. H. (2017a). BK Polyomavirus-Specific 9mer CD8 T Cell Responses Correlate With Clearance of BK Viremia in Kidney Transplant Recipients: First Report From the Swiss Transplant Cohort Study. *Am J Transplant, 17*(10), 2591-2600. doi:10.1111/ajt.14282

Leboeuf, C., Wilk, S., Achermann, R., Binet, I., Golshayan, D., Hadaya, K., . . . Hirsch, H. H. (2017b). BK Polyomavirus-Specific 9mer CD8 T Cell Responses Correlate With Clearance of BK Viremia in Kidney Transplant Recipients: First Report From the Swiss Transplant Cohort Study. *Am J Transplant*. doi:10.1111/ajt.14282

Levitsky, J., Miller, J., Wang, E., Rosen, A., Flaa, C., Abecassis, M., . . . Tambur, A. (2009). Immunoregulatory profiles in liver transplant recipients on different immunosuppressive agents. *Human immunology, 70*(3), 146-150.

Li, J., Melenhorst, J., Hensel, N., Rezvani, K., Sconocchia, G., Kilical, Y., . . . Barrett, A. J. (2006). T-cell responses to peptide fragments of the BK virus T antigen: implications for cross-reactivity of immune response to JC virus. *J Gen Virol, 87*(Pt 10), 2951-2960. doi:10.1099/vir.0.82094-0

Liddington, R. C., Yan, Y., Moulai, J., Sahli, R., Benjamin, T. L., & Harrison, S. C. (1991). Structure of simian virus 40 at 3.8-A resolution. *Nature, 354*(6351), 278-284. doi:10.1038/354278a0

Lila, N., Amrein, C., Guillemain, R., Chevalier, P., Latremouille, C., Fabiani, J.-N., . . . Carpentier, A. (2002). Human leukocyte antigen-G expression after heart transplantation is associated with a reduced incidence of rejection. *Circulation, 105*(16), 1949-1954.

Lim, E. S., Reyes, A., Antonio, M., Saha, D., Ikumapayi, U. N., Adeyemi, M., . . . Wang, D. (2013). Discovery of STL polyomavirus, a polyomavirus of ancestral recombinant origin that encodes a unique T antigen by alternative splicing. *Virology, 436*(2), 295-303. doi:10.1016/j.virol.2012.12.005

Ling, P. D., Lednicky, J. A., Keitel, W. A., Poston, D. G., White, Z. S., Peng, R., . . . Rooney, C. M. (2003). The dynamics of herpesvirus and polyomavirus reactivation and shedding in healthy adults: a 14-month longitudinal study. *The Journal of Infectious Diseases, 187*(10), 1571-1580.

Lozano, J. M., González, R., Kindelán, J. M., Rouas-Freiss, N., Caballos, R., Dausset, J., . . . Peña, J. (2002). Monocytes and T lymphocytes in HIV-1-positive patients express HLA-G molecule. *Aids, 16*(3), 347-351.

Lu, N., Zhang, Y., Zou, X., Yang, X., Tian, J., Zhen, J., . . . Shi, W. (2011). HLA-G on peripheral blood CD4+ T lymphocytes: a potential predictor for acute renal rejection. *Transplant International, 24*(11), 1103-1111.

Lukacher, A. E., Moser, J. M., Hadley, A., & Altman, J. D. (1999). Visualization of polyoma virus-specific CD8+ T cells in vivo during infection and tumor rejection. *The Journal of Immunology, 163*(6), 3369-3378.

Mani, J., Jin, N., & Schmitt, M. (2014). Cellular immunotherapy for patients with reactivation of JC and BK polyomaviruses after transplantation. *Cytotherapy, 16*(10), 1325-1335. doi:10.1016/j.jcyt.2014.04.003

Mantyjarvi, R. A., Meurman, O. H., Vihma, L., & Berglund, B. (1973). A human papovavirus (B.K.), biological properties and seroepidemiology. *Ann Clin Res, 5*(5), 283-287.

Masutani, K., Ninomiya, T., & Randhawa, P. (2013). HLA-A2, HLA-B44 and HLA-DR15 are associated with lower risk of BK viremia. *Nephrol Dial Transplant, 28*(12), 3119-3126. doi:10.1093/ndt/gft298

Masutani, K., Shapiro, R., Basu, A., Tan, H., Wijkstrom, M., & Randhawa, P. (2012). The Banff 2009 Working Proposal for polyomavirus nephropathy: a critical evaluation of its utility as a determinant of clinical outcome. *Am J Transplant, 12*(4), 907-918. doi:10.1111/j.1600-6143.2012.03993.x

Matas, A. J. (2011). Chronic progressive calcineurin nephrotoxicity: an overstated concept. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons, 11*(4), 687-692. doi:10.1111/j.1600-6143.2011.03505.x

Matsuda, Y., Qazi, Y., & Iwaki, Y. (2011). A rapid and efficient method BK polyomavirus genotyping by high-resolution melting analysis. *J Med Virol, 83*(12), 2128-2134. doi:10.1002/jmv.22239

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature, 449*(7164), 819-826.

Medzhitov, R., & Janeway Jr, C. (2000). Innate immunity. *New England Journal of Medicine, 343*(5), 338-344.

Mina, M. M., Luciani, F., Cameron, B., Bull, R. A., Beard, M. R., Booth, D., & Lloyd, A. R. (2015). Resistance to hepatitis C virus: potential genetic and immunological determinants. *The Lancet Infectious Diseases, 15*(4), 451-460.

Mishra, N., Pereira, M., Rhodes, R. H., An, P., Pipas, J. M., Jain, K., . . . Lipkin, W. I. (2014). Identification of a novel polyomavirus in a pancreatic transplant recipient with retinal blindness and vasculitic myopathy. *The Journal of infectious diseases, 210*(10), 1595-1599.

Monneret, G., Voirin, N., Krawice-Radanne, I., Bohé, J., Lepape, A., Rouas-Freiss, N., & Carosella, E. D. (2007). Soluble human leukocyte antigen-G5 in septic shock: marked and persisting elevation as a predictor of survival. *Critical care medicine, 35*(8), 1942-1947.

Morel, V., Martin, E., Francois, C., Helle, F., Faucher, J., Mourez, T., . . . Brochot, E. (2017). A simple and reliable strategy for BK virus subtyping and subgrouping. *J Clin Microbiol*. doi:10.1128/jcm.01180-16

Mueller, K., Schachtner, T., Sattler, A., Meier, S., Friedrich, P., Trydzenskaya, H., . . . Babel, N. (2011). BK-VP3 as a new target of cellular immunity in BK virus infection. *Transplantation, 91*(1), 100-107.

Nelson, A. S., Heyenbruch, D., Rubinstein, J. D., Sabulski, A., Jodele, S., Thomas, S., . . . Grimley, M. S. (2020). Virus-specific T-cell therapy to treat BK polyomavirus infection in bone marrow and solid organ transplant recipients. *Blood Advances, 4*(22), 5745-5754. doi:10.1182/bloodadvances.2020003073

Nishimoto, Y., Zheng, H. Y., Zhong, S., Ikegaya, H., Chen, Q., Sugimoto, C., . . . Yogo, Y. (2007). An Asian origin for subtype IVBK virus based on phylogenetic analysis. *Journal of Molecular Evolution, 65*(1), 103-111. doi:10.1007/s00239-006-0269-6

Nogueira, E., Ozaki, K. S., Tomiyama, H., Camara, N. O., & Granato, C. F. (2009). Clinical correlations of human cytomegalovirus strains and viral load in kidney transplant recipients. *Int Immunopharmacol, 9*(1), 26-31. doi:10.1016/j.intimp.2008.08.020

Nogueira, E., Ozaki, K. S., Tomiyama, H., Câmara, N. O. S., & Granato, C. F. H. (2009). Clinical correlations of human cytomegalovirus strains and viral load in kidney transplant recipients. *International immunopharmacology, 9*(1), 26-31.

Nukuzuma, S., Takasaka, T., Zheng, H. Y., Zhong, S., Chen, Q., Kitamura, T., & Yogo, Y. (2006). Subtype I BK polyomavirus strains grow more efficiently in human renal epithelial cells than subtype IV strains. *J Gen Virol, 87*(Pt 7), 1893-1901. doi:10.1099/vir.0.81698-0

Núñez, M., & Soriano, V. (2005). Hepatitis C Virus (HCV) Genotypes and Disease Progression in HIV/HCV-Coinfected Patients. *The Journal of Infectious Diseases, 191*(1), 1-1. doi:10.1086/426515

Ocampo, L. Á., Rosso, F., Pacheco, R., & Villegas, A. (2017). Epidemiology of polyomavirus BK (BKV) and the emergent African variant in kidney and bone marrow transplant recipients in the Fundacion Valle del Lili in Cali, Colombia. *Diagnostic Microbiology and Infectious Disease, 88*(3), 236-240. doi:https://doi.org/10.1016/j.diagmicrobio.2017.01.025

Olsen, G. H., Hirsch, H. H., & Rinaldo, C. H. (2009). Functional analysis of polyomavirus BK non-coding control region quasispecies from kidney transplant recipients. *Journal of medical virology, 81*(11), 1959-1967.

Papadimitriou, J. C., Randhawa, P., Rinaldo, C. H., Drachenberg, C. B., Alexiev, B., & Hirsch, H. H. (2016). BK Polyomavirus Infection and Renourinary Tumorigenesis. *Am J Transplant, 16*(2), 398-406. doi:10.1111/ajt.13550

Pastrana, D. V., Ray, U., Magaldi, T. G., Schowalter, R. M., Cuburu, N., & Buck, C. B. (2013). BK polyomavirus genotypes represent distinct serotypes with distinct entry tropism. *J Virol, 87*(18), 10105-10113. doi:10.1128/jvi.01189-13

Pfaender, S., Heyden, J., Friesland, M., Ciesek, S., Ejaz, A., Steinmann, J., . . . Tsiavaliaris, G. (2013). Inactivation of hepatitis C virus infectivity by human breast milk. *The Journal of Infectious Diseases, 208*(12), 1943-1952.

Pham, P. T., Schaenman, J., & Pham, P. C. (2014). BK virus infection following kidney transplantation: an overview of risk factors, screening strategies, and therapeutic interventions. *Curr Opin Organ Transplant, 19*(4), 401-412. doi:10.1097/mot.0000000000000101

Pinto, G. G., Poloni, J. A., Carneiro, L. C., Baethgen, L. F., Barth, A. L., & Pasqualotto, A. C. (2013). Evaluation of different urine protocols and DNA extraction methods for quantitative detection of BK viruria in kidney transplant patients. *J Virol Methods, 188*(1-2), 94-96. doi:10.1016/j.jviromet.2012.12.006

Prosser, S. E., Orentas, R. J., Jurgens, L., Cohen, E. P., & Hariharan, S. (2008). Recovery of BK virus large T-antigen-specific cellular immune response correlates with resolution of bk virus nephritis. *Transplantation, 85*(2), 185-192. doi:10.1097/TP.0b013e31815fef56

Provenzano, M., Bracci, L., Wyler, S., Hudolin, T., Sais, G., Gosert, R., . . . Spagnoli, G. C. (2006). Characterization of highly frequent epitope-specific CD45RA+/CCR7+/- T lymphocyte responses against p53-binding domains of the human polyomavirus BK large tumor antigen in HLA-A*0201+ BKV-seropositive donors. *J Transl Med, 4*, 47. doi:10.1186/1479-5876-4-47

Ramaswami, B., Popescu, I., Macedo, C., Metes, D., Bueno, M., Zeevi, A., . . . Randhawa, P. S. (2009). HLA-A01-, -A03-, and -A024-binding nanomeric epitopes in polyomavirus BK large T antigen. *Hum Immunol, 70*(9), 722-728. doi:10.1016/j.humimm.2009.05.003

Randhawa, P., Bohl, D., Brennan, D., Ruppert, K., Ramaswami, B., Storch, G., . . . Viscidi, R. (2008). Longitudinal analysis of levels of immunoglobulins against BK virus capsid proteins in kidney transplant recipients. *Clin. Vaccine Immunol., 15*(10), 1564-1571.

Rao, X., Hoof, I., van Baarle, D., Keşmir, C., & Textor, J. (2015). HLA preferences for conserved epitopes: a potential mechanism for hepatitis C clearance. *Frontiers in Immunology, 6*, 552.

Rebmann, V., da Silva Nardi, F., Wagner, B., & Horn, P. A. (2014). HLA-G as a tolerogenic molecule in transplantation and pregnancy. *Journal of immunology research, 2014*.

Reese, J. M., Reissig, M., Daniel, R. W., & Shah, K. V. (1975). Occurrence of BK virus and BK virus-specific antibodies in the urine of patients receiving chemotherapy for malignancy. *Infection and immunity, 11*(6), 1375-1381.

Reploeg, M. D., Storch, G. A., & Clifford, D. B. (2001). BK virus: a clinical review. *Clinical Infectious Diseases, 33*(2), 191-202.

Riddell, S. R., & Greenberg, P. D. (1990). The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. *Journal of immunological methods, 128*(2), 189-201.

Rinaldo, C. H., Tylden, G. D., & Sharma, B. N. (2013). The human polyomavirus BK (BKP yV): virological background and clinical implications. *Apmis, 121*(8), 728-745.

Rouas-Freiss, N., LeMaoult, J., Moreau, P., Dausset, J., & Carosella, E. D. (2003). HLA-G in Transplantation: A Relevant Molecule for Inhibition of Graft Rejection? *American Journal of Transplantation, 3*(1), 11-16. doi:10.1034/j.1600-6143.2003.30103.x

Rouas-Freiss, N., Naji, A., Durrbach, A., & Carosella, E. D. (2007). Tolerogenic functions of human leukocyte antigen G: from pregnancy to organ and cell transplantation. *Transplantation, 84*(1), S21-S25.

Rouse, B. T., & Mueller, S. N. (2019). 25 - Host Defenses to Viruses. In R. R. Rich, T. A. Fleisher, W. T. Shearer, H. W. Schroeder, A. J. Frew, & C. M. Weyand (Eds.), *Clinical Immunology (Fifth Edition)* (pp. 365-374.e361). London: Content Repository Only!

Sar, A., Worawichawong, S., Benediktsson, H., Zhang, J., Yilmaz, S., & Trpkov, K. (2011). Interobserver agreement for Polyomavirus nephropathy grading in renal allografts using the working proposal from the 10th Banff Conference on Allograft Pathology. *Hum Pathol, 42*(12), 2018-2024. doi:10.1016/j.humpath.2011.03.008

Sarcinella, L., Mazzulli, T., Willey, B., & Humar, A. (2002). Cytomegalovirus glycoprotein B genotype does not correlate with outcomes in liver transplant patients. *Journal of clinical virology, 24*(1-2), 99-105.

Schachtner, T., Muller, K., Stein, M., Diezemann, C., Sefrin, A., Babel, N., & Reinke, P. (2011). BK virus-specific immunity kinetics: a predictor of recovery from polyomavirus BK-associated nephropathy. *Am J Transplant, 11*(11), 2443-2452. doi:10.1111/j.1600-6143.2011.03693.x

Schachtner, T., Stein, M., Babel, N., & Reinke, P. (2015). The Loss of BKV-specific Immunity From Pretransplantation to Posttransplantation Identifies Kidney Transplant Recipients at Increased Risk of BKV Replication. *Am J Transplant, 15*(8), 2159-2169. doi:10.1111/ajt.13252

Schneidawind, D., Schmitt, A., Wiesneth, M., Mertens, T., Bunjes, D., Freund, M., & Schmitt, M. (2010). Polyomavirus BK-specific CD8+ T cell responses in patients after allogeneic stem cell transplant. *Leuk Lymphoma, 51*(6), 1055-1062. doi:10.3109/10428191003746323

Schwarz, A., Linnenweber-Held, S., Heim, A., Framke, T., Haller, H., & Schmitt, C. (2016). Viral origin, clinical course, and renal outcomes in patients with BK virus infection after living-donor renal transplantation. *Transplantation, 100*(4), 844-853.

Sener, A., House, A. A., Jevnikar, A. M., Boudville, N., McAlister, V. C., Muirhead, N., . . . Luke, P. P. (2006). Intravenous immunoglobulin as a treatment for BK virus associated nephropathy: one-year follow-up of renal allograft recipients. *Transplantation, 81*(1), 117-120. doi:10.1097/01.tp.0000181096.14257.c2

Shah, K., Daniel, R., Madden, D., & Stagno, S. (1980). Serological investigation of BK papovavirus infection in pregnant women and their offspring. *Infection and immunity, 30*(1), 29-35.

Sharma, M. C., Zhou, W., Martinez, J., Krymskaya, L., Srivastava, T., Haq, W., . . . Lacey, S. F. (2006). Cross-reactive CTL recognizing two HLA-A*02-restricted epitopes within the BK virus and JC virus VP1 polypeptides are frequent in immunocompetent individuals. *Virology, 350*(1), 128-136. doi:10.1016/j.virol.2006.02.040

Sharma, R., Tzetzo, S., Patel, S., Zachariah, M., Sharma, S., & Melendy, T. (2016). BK Virus in Kidney Transplant: Current Concepts, Recent Advances, and Future Directions. *Exp Clin Transplant, 14*(4), 377-384. doi:10.6002/ect.2016.0030

Shenagari, M., Monfared, A., Eghtedari, H., Pourkazemi, A., Hasandokht, T., Khosravi, M., & Asharfkhani, B. (2017). BK virus replication in renal transplant recipients: Analysis of potential risk factors may contribute in reactivation. *Journal of Clinical Virology, 96*, 7-11.

Shiroishi, M., Kuroki, K., Rasubala, L., Tsumoto, K., Kumagai, I., Kurimoto, E., . . . Maenaka, K. (2006). Structural basis for recognition of the nonclassical MHC molecule HLA-G by the leukocyte Ig-like receptor B2 (LILRB2/LIR2/ILT4/CD85d). *Proceedings of the National Academy of Sciences, 103*(44), 16412-16417.

Shishido-Hara, Y., Ichinose, S., Higuchi, K., Hara, Y., & Yasui, K. (2004). Major and minor capsid proteins of human polyomavirus JC cooperatively accumulate to nuclear domain 10 for assembly into virions. *J Virol, 78*(18), 9890-9903.

Siguier, M., Sellier, P., & Bergmann, J. F. (2012). BK-virus infections: a literature review. *Med Mal Infect, 42*(5), 181-187. doi:10.1016/j.medmal.2012.04.011

Singh, H. K., Andreoni, K. A., Madden, V., True, K., Detwiler, R., Weck, K., & Nickeleit, V. (2009). Presence of urinary Haufen accurately predicts polyomavirus nephropathy. *J Am Soc Nephrol, 20*(2), 416-427. doi:10.1681/asn.2008010117

Smith, K. G., Isbel, N. M., Catton, M. G., Leydon, J. A., Becker, G. J., & Walker, R. G. (1998). Suppression of the humoral immune response by mycophenolate mofetil. *Nephrol Dial Transplant, 13*(1), 160-164. doi:10.1093/ndt/13.1.160

Snydman, D. R., Kwak, E. J., Vilchez, R. A., Randhawa, P., Shapiro, R., Butel, J. S., & Kusne, S. (2002). Pathogenesis and management of polyomavirus infection in transplant recipients. *Clinical infectious diseases, 35*(9), 1081-1087.

Solis, M., Velay, A., Porcher, R., Domingo-Calap, P., Soulier, E., Joly, M., . . . Bahram, S. (2018). Neutralizing Antibody–Mediated Response and Risk of BK Virus–Associated Nephropathy. *Journal of the American Society of Nephrology, 29*(1), 326-334.

Song, T.-R., Rao, Z.-S., Qiu, Y., Liu, J.-P., Huang, Z.-L., Wang, X.-D., & Lin, T. (2016). Fluoroquinolone prophylaxis in preventing BK polyomavirus infection after renal transplant: A systematic review and meta-analysis. *The Kaohsiung Journal of Medical Sciences, 32*(3), 152-159. doi:https://doi.org/10.1016/j.kjms.2016.01.004

Sood, P., Senanayake, S., Sujeet, K., Medipalli, R., Van-Why, S. K., Cronin, D. C., . . . Hariharan, S. (2013). Donor and recipient BKV-specific IgG antibody and posttransplantation BKV infection: a prospective single-center study. *Transplantation, 95*(6), 896-902. doi:10.1097/TP.0b013e318282ba83

Sood, P., Senanayake, S., Sujeet, K., Medipalli, R., Zhu, Y. R., Johnson, C. P., & Hariharan, S. (2012). Management and outcome of BK viremia in renal transplant recipients: a prospective single-center study. *Transplantation, 94*(8), 814-821. doi:10.1097/TP.0b013e31826690c6

Sood, P., Senanayake, S., Sujeet, K., Medipalli, R., Zhu, Y. R., Johnson, C. P., & Hariharan, S. (2012). Management and Outcome of BK Viremia in Renal Transplant Recipients: A Prospective Single-Center Study. *Transplantation, 94*(8), 814-821. doi:10.1097/TP.0b013e31826690c6

Stoian, M., Hozoc, M., Iosipenco, M., Nastac, E., & Melencu, M. (1983). Serum antibodies to papova viruses (BK and SV 40) in subjects from the area with Balkan endemic nephropathy. *Virologie, 34*(2), 113.

Sundsfjord, A., Osei, A., Rosenqvist, H., Van Ghelue, M., Silsand, Y., Haga, H.-J., . . . Moens, U. (1999). BK and JC viruses in patients with systemic lupus erythematosus: prevalent and persistent BK viruria, sequence stability of the viral regulatory regions, and nondetectable viremia. *The Journal of Infectious Diseases, 180*(1), 1-9.

Suwelack, B., Malyar, V., Koch, M., Sester, M., & Sommerer, C. (2012). The influence of immunosuppressive agents on BK virus risk following kidney transplantation, and implications for choice of regimen. *Transplantation reviews, 26*(3), 201-211.

Taguchi, F., Nagaki, D., Saito, M., Haruyama, C., & Iwasaki, K. (1975). Transplacental transmission of BK virus in human. *Jpn J Microbiol, 19*(5), 395-398.

Takasaka, T., Goya, N., Tokumoto, T., Tanabe, K., Toma, H., Ogawa, Y., . . . Yogo, Y. (2004). Subtypes of BK virus prevalent in Japan and variation in their transcriptional control region. *J Gen Virol, 85*(Pt 10), 2821-2827. doi:10.1099/vir.0.80363-0

Teutsch, K., Schweitzer, F., Knops, E., Kaiser, R., Pfister, H., Verheyen, J., . . . Di Cristanziano, V. (2015). Early identification of renal transplant recipients with high risk of polyomavirus-associated nephropathy. *Medical Microbiology and Immunology, 204*(6), 657-664. doi:10.1007/s00430-015-0398-x

Thakur, R., Arora, S., Nada, R., Minz, M., & Joshi, K. (2011). Prospective monitoring of BK virus reactivation in renal transplant recipients in North India. *Transpl Infect Dis, 13*(6), 575-583. doi:10.1111/j.1399-3062.2011.00634.x

Tremolada, S., Akan, S., Otte, J., Khalili, K., Ferrante, P., Chaudhury, P. R., . . . Gordon, J. (2010). Rare subtypes of BK virus are viable and frequently detected in renal transplant recipients with BK virus-associated nephropathy. *Virology, 404*(2), 312-318. doi:10.1016/j.virol.2010.05.012

Trofe-Clark, J., Sparkes, T., Gentile, C., Van Deerlin, V., Sawinski, D., & Bloom, R. (2013). BK virus genotype variance and discordant BK viremia PCR assay results. *American Journal of Transplantation, 13*(4), 1112-1113.

Trydzenskaya, H., Juerchott, K., Lachmann, N., Kotsch, K., Kunert, K., Weist, B., . . . Babel, N. (2013). The genetic predisposition of natural killer cell to BK virus-associated nephropathy in renal transplant patients. *Kidney Int, 84*(2), 359-365. doi:10.1038/ki.2013.59

Tzannou, I., Papadopoulou, A., Naik, S., Leung, K., Martinez, C. A., Ramos, C. A., . . . Omer, B. (2017). Off-the-Shelf Virus-Specific T Cells to Treat BK Virus, Human Herpesvirus 6, Cytomegalovirus, Epstein-Barr Virus, and Adenovirus Infections After Allogeneic Hematopoietic Stem-Cell Transplantation. *J Clin Oncol*, Jco2017730655. doi:10.1200/jco.2017.73.0655

van Aalderen, M. C., Heutinck, K. M., Huisman, C., & ten Berge, I. J. (2012). BK virus infection in transplant recipients: clinical manifestations, treatment options and the immune response. *Neth J Med, 70*(4), 172-183.

Van der Meer, A., Lukassen, H., Van Cranenbroek, B., Weiss, E., Braat, D., Van Lierop, M., & Joosten, I. (2007). Soluble HLA-G promotes Th1-type cytokine production by cytokine-activated uterine and peripheral natural killer cells. *Molecular Human Reproduction, 13*(2), 123-133.

van Erp, E. A., van Kampen, M. R., van Kasteren, P. B., & de Wit, J. (2019). Viral Infection of Human Natural Killer Cells. *Viruses, 11*(3), 243. doi:10.3390/v11030243

Varella, R. B., Almeida, J. R., Lopes, P. d. F., Matos, J. P. S. d., Menezes, P., & Lugon, J. R. (2014). BK polyomavirus in Kidney transplant recipients: screening, monitoring and clinical management. *Jornal Brasileiro de Nefrologia, 36*, 529-534.

Varella, R. B., Zalona, A. C. J., Diaz, N. C., Zalis, M. G., & Santoro-Lopes, G. (2018). BK polyomavirus genotypes Ia and Ib1 exhibit different biological properties in renal transplant recipients. *Virus Res, 243*, 65-68. doi:10.1016/j.virusres.2017.10.018

Wadei, H., Rule, A. D., Lewin, M., Mahale, A., Khamash, H., Schwab, T., . . . Lager, D. (2006). Kidney transplant function and histological clearance of virus following diagnosis of polyomavirus-associated nephropathy (PVAN). *American Journal of Transplantation, 6*(5p1), 1025-1032.

Weist, B. J., Schmueck, M., Fuehrer, H., Sattler, A., Reinke, P., & Babel, N. (2014). The role of CD4(+) T cells in BKV-specific T cell immunity. *Med Microbiol Immunol, 203*(6), 395-408. doi:10.1007/s00430-014-0348-z

Wilkinson, G. W., Tomasec, P., Stanton, R. J., Armstrong, M., Prod'homme, V., Aicheler, R., . . . Llewellyn-Lacey, S. (2008). Modulation of natural killer cells by human cytomegalovirus. *Journal of clinical virology, 41*(3), 206-212.

Wojciechowski, D., Chanda, R., Chandran, S., Lee, B., Webber, A., Macaraig, M., . . . Vincenti, F. (2012). Ciprofloxacin prophylaxis in kidney transplant recipients reduces BK virus infection at 3 months but not at 1 year. *Transplantation, 94*(11), 1117-1123. doi:10.1097/TP.0b013e31826ec74e

Wunderink, H. F., De Brouwer, C. S., Gard, L., De Fijter, J. W., Kroes, A. C. M., Rotmans, J. I., & Feltkamp, M. C. W. (2019). Source and Relevance of the BK Polyomavirus Genotype for Infection After Kidney Transplantation. *Open Forum Infect Dis, 6*(3), ofz078. doi:10.1093/ofid/ofz078

Wunderink, H. F., Haasnoot, G. W., de Brouwer, C. S., van Zwet, E. W., Kroes, A. C., de Fijter, J. W., . . . Feltkamp, M. C. (2019). Reduced Risk of BK Polyomavirus Infection in HLA-B51–positive Kidney Transplant Recipients. *Transplantation, 103*(3), 604-612.

Wunderink, H. F., van der Meijden, E., van der Blij-de Brouwer, C. S., Mallat, M. J., Haasnoot, G. W., van Zwet, E. W., . . . Feltkamp, M. C. (2017). Pretransplantation Donor-Recipient Pair Seroreactivity Against BK Polyomavirus Predicts Viremia and Nephropathy After Kidney Transplantation. *Am J Transplant, 17*(1), 161-172. doi:10.1111/ajt.13880

Xiao, L., Zhou, W., Shi, B., Feng, K., He, X., Wei, Y., . . . Xiao, W. (2013). HLA-G expression in the peripheral blood of live kidney transplant recipients. *Chinese medical journal, 126*(14), 2652-2655.

Yan, W.-H., Lin, A., Chen, B.-G., & Chen, S.-Y. (2009). Induction of both membrane-bound and soluble HLA-G expression in active human cytomegalovirus infection. *The Journal of Infectious Diseases, 200*(5), 820-826.

Yi, S. G., Knight, R. J., & Lunsford, K. E. (2017). BK virus as a mediator of graft dysfunction following kidney transplantation. *Curr Opin Organ Transplant*. doi:10.1097/mot.0000000000000429

Yie, S.-m., Li, L.-h., Li, G.-m., Xiao, R., & Librach, C. L. (2005). Progesterone enhances HLA-G gene expression in JEG-3 choriocarcinoma cells and human cytotrophoblasts in vitro. *Human Reproduction, 21*(1), 46-51. doi:10.1093/humrep/dei305

Yogo, Y., Sugimoto, C., Zhong, S., & Homma, Y. (2009). Evolution of the BK polyomavirus: epidemiological, anthropological and clinical implications. *Rev Med Virol, 19*(4), 185-199. doi:10.1002/rmv.613

Yu, N., Ohashi, M., Alosco, S., Granja, C., Salazar, M., Hegland, J., & Yunis, E. (1997). Accurate typing of HLA-A antigens and analysis of serological deficiencies. *Tissue Antigens, 50*(4), 380-386. doi:10.1111/j.1399-0039.1997.tb02891.x

Zaman, R. A., Ettenger, R. B., Cheam, H., Malekzadeh, M. H., & Tsai, E. W. (2014). A novel treatment regimen for BK viremia. *Transplantation, 97*(11), 1166-1171. doi:10.1097/01.TP.0000441825.72639.4f

Zeng, G., Wang, Z., Huang, Y., Abedin, Z., Liu, Y., & Randhawa, P. (2019). Cellular and viral miRNA expression in polyomavirus BK infection. *Transplant Infectious Disease, 21*(5), e13159.

Zheng, H. Y., Nishimoto, Y., Chen, Q., Hasegawa, M., Zhong, S., Ikegaya, H., . . . Yogo, Y. (2007). Relationships between BK virus lineages and human populations. *Microbes Infect, 9*(2), 204-213. doi:10.1016/j.micinf.2006.11.008

Zhong, S., Randhawa, P. S., Ikegaya, H., Chen, Q., Zheng, H.-Y., Suzuki, M., . . . Yogo, Y. (2009). Distribution patterns of BK polyomavirus (BKV) subtypes and subgroups in American, European and Asian populations suggest co-migration of BKV and the human race. *Journal of General Virology, 90*(1), 144-152. doi:https://doi.org/10.1099/vir.0.83611-0