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The Pathogenesis of Polyomavirus-Associated Allograft Nephropathy in Mice

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Abstract

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BK virus is a fairly ubiquitous human polyomavirus that has emerged as an increasing threat in renal transplantation. Polyomavirus-associated allograft nephropathy (PVAN), caused by BK virus, has been implicated in up to 60% of graft loss. Surprisingly, much remains unknown about the pathogenesis of this condition. We recently published a mouse model of PVAN, utilizing mouse polyomavirus (MPyV) infection and kidney allograft transplantation.

Herein, we use the mouse model of PVAN to further investigate clinical variables and disease mechanism. We find that the timing, source, and initial titer of infection have a significant impact on the severity of PVAN, with acute, high titer infection of the transplant recipient producing the most profound disease. We find no correlation between PVAN and ischemia/reperfusion injury or MHC matching, despite the fact that improved MHC matching results in a decreased viral load. Intrigued by the lack of correlation between viral load and disease, we observe that splenectomized aly/aly mice (which are unable to mount a primary adaptive immune response) do not develop PVAN despite exceptionally high viral loads. This implies that PVAN is not simply a consequence of direct viral cytopathology, but instead requires an intact host immune response.

To continue to investigate the role of the host immune system on PVAN pathogenesis, we performed adoptive transfer experiments. Only the transfer of anti-allo (and not anti-viral) T-cells into acutely infected, transplanted aly/aly mice resulted in the lethal PVAN phenotype. Similarly, we transferred OVA-specific TCR transgenic CD8 T-cells (OT-I cells) into B6 mice after transplantation and MPyV infection. This transfer caused rejection and death when the targeted OT-I epitope was displayed on the transplanted kidney, but not when it was expressed by recombinant MPyV. Consistent with this data, addition of immunosuppression into the mouse model resulted in improved allograft function and survival. Taken together, these data allow us to propose a immunopathogenic mechanism for PVAN, in which a subclinical alloimmune response is boosted by the inflammatory microenvironment of the infected kidney, becoming sufficiently augmented to mediate clinical rejection. This previously unappreciated interplay between host alloimmunity and viral inflammation may have important implications for human transplantation.

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Introduction

I. An Introduction to Virology

The etymology of the word virus is from the Latin *virus*, meaning poison. Indeed, there is significant philosophical debate on whether a virus is just a dead piece of "poison," or whether it constitutes life. Proponents of viruses as living beings are quick to point out that viruses contain nucleic acid encoding genes, which are subject to evolutionary pressure and natural selection. They can also "reproduce," with the ability to create multiple copies of themselves through self-assembly. Of course, viruses do not have cellular structure and lack the ability to reproduce outside of a host cell, which is inconsistent with many definitions of life. Regardless of the point of view, it is clear that viruses are important infectious agents which have tremendous impact on global health and disease.

Historically speaking, there is evidence of viral infection throughout the course of human history. It is estimated that smallpox emerged in Africa around 10,000 BC, and there is ancient Egyptian art from ~1500 BC that depicts a priest with symptomatic evidence of poliovirus infection (1). As diverse and pathogenic viruses have evolved, so have the immune systems of potentially infected organisms. The immune system of human beings is comprised of two branches. Innate immunity allows us to respond to pathogenic infection in a non-antigen specific manner. It was inherited from our ancient ancestors and is possessed by a wide variety of organisms. Adaptive immunity, on the other hand, is only possessed by higher vertebrates, first appearing in the evolutionary line around the time of jawless fish (2). Although slower than innate immunity, adaptive immunity gives us the capability to respond to infection in a pathogen-specific manner.

It also gives us the ability to generate memory, preventing multiple infections with the same pathogen.

In modern times, discussions of virology and immunology often begin around 1800 with Edward Jenner, who successfully vaccinated a child for smallpox using the related cowpox virus. Although this underlies the modern principles of vaccination, Jenner did not possess the conceptual idea of a viral infection as distinct from other causative pathogens. Less than 100 years later, Louis Pasteur may have been the first to suggest the modern concept of a virus as a pathogen discrete from bacteria. Unable to detect a bacterial agent for rabies by microscopy, he speculated that the causative pathogen may have been too small to be seen under a light microscope (3). In the late 1800s, with the advent of the Chamberland filter (which contains pores small enough to filter out bacteria), the tobacco mosaic virus became the first successfully identified viral pathogen. It was initially studied by Dmitry Ivanovsky and then by Matrinus Beijerinck, who called the causative pathogen a "*contagium vivum fluidum*," re-introducing the word virus and giving it its modern biological definition (4).

Over the past two centuries, we have come to appreciate the diversity and ubiquity of viruses. It is estimated that there are ~10^{31} viruses on the planet, most of which are harmless bacteriophages living in the oceans (5). For obvious reasons, our studies are biased toward viruses causing diseases in animals and livestock, in plants and agriculture, and especially in humans. In modern virus classification, two systems are used in conjunction. The International Committee on Taxonomy of Viruses has established a taxonomic structure containing an Order, Family, Subfamily, Genus and Species based on viral properties. It currently contains 6 orders (6). The Baltimore

classification system is based on the mechanism of mRNA production and currently contains 7 groups (7).

Although the properties of viruses vary greatly between these groups, all virus particles (virions) contain genetic material of nucleic acid surrounded by a protective protein coat called a capsid. Many also possess a lipid envelope (derived from the host cell membrane) which surrounds the protein coat, and they may possess other translated proteins as well. Virus morphology also varies greatly. The size of most viruses is between 20 and 400 nm in diameter, and the shape varies from helical to icosohedral to more complex designs. The nucleic material may be DNA or RNA, single stranded or double stranded, and may be positive sense, negative sense, or ambisense.

II. Polyomavirus

Polyomaviridae is a family of non-enveloped viruses that infects a variety of vertebrates. Mouse polyomavirus (MPyV), first described in 1953 (8, 9), is the founding member of the family. It was named polyoma, meaning many tumors, after studies indicated that its inoculation into newborn mice induced a vast array of solid tumors (10). Each polyomavirus (PyV) contains a small, superhelical, circular double-stranded DNA genome of approximately 5 kb. The genome contains an early region encoding the non-structural small and large T antigens (and sometimes middle T antigen), as well as a late region encoding the viral capsid proteins VP1, VP2, and VP3 (and sometimes agnoprotein). The capsid proteins enclose the genome in a non-enveloped icosahedral shell. Polyomaviruses bind to over 30 different cell types. Binding depends on the interaction between VP1 and host cell glycoproteins containing terminal sialic acids (11);

α4β1 intergrin acts as a post-attachement cell receptor for MPyV (12). Once MPyV enters the cell, it is transported to the nucleus, and early transcription proceeds from a single origin of DNA. Alternative splicing of the primary RNA transcripts results in small T (sT), middle T (mT), and large T (LT) proteins (13). Although polyomaviruses have been used to study many basic eukaryotic processes, they have been extensively studied in the setting of cancer biology due to the oncogenic potential of the T proteins (14).

Generally speaking, the polyomaviruses are widely prevalent in their natural animal reservoirs and establish persistent but harmless infections in immunocompetent hosts. They also display relatively strict species specificity. Three of the most studied polyomaviruses are Simian Virus 40 (SV40) (15) and the human BK (16)and JC (17) viruses, (named for the patients from which they were first isolated). BK virus (BKV) leads to a complication primarily in kidney transplants called BK virus nephropathy; JC virus (JCV) leads to a potentially fatal CNS condition in immunocompromised patients called Progressive Multifocal Leukoencephalopathy (PML). More recently, several other human polyomaviruses have been identified. These include Karolinska Institute virus (KIV) (18) and Washington University virus (WUV) (19), named for the institutions at which they were first identified. The possible link of these viruses to human disease is still unclear. Merkel cell polyomavirus (MCV) has also been recently identified, and as its name suggests, it has been linked to Merkel cell carcinoma (MCC), an aggressive skin cancer (20).

III. BK Virus

BK virus (BKV) is a human polyomavirus, first isolated in 1970 from a Sudanese kidney transplant recipient with a uretal stricture (16). It is transmitted via respiratory or oral-enteric routes at a peak age of 2-5 years, and may present either asymptomatically or as a mild "flu-like" illness. It is now known that it utilizes gangliosides GD1b and GT1b as its cellular receptors (21). After binding to the host cell, it enters in a similar fashion to SV40, mediated by non-clathrin coated vesicles resembling caveolae (22). After the initial infection, it establishes a persistent infection in the renal tubular epithelial cells and the uroepithelium. It is extremely prevalent in the adult population, with approximately 80-90% of all adults showing seropositivity by age ten (23). Although 1-5% of all healthy adults have detectable virus in the urine, this is an asymptomatic viruria (24), and it is generally believed that there is no clinical consequence of the virus in immunocompetent adults.

The BK virus genome is closely related to the JC virus genome and is made up of three parts (25). The non-coding control region (NCCR) contains the origin of replication as well as regulatory regions. The early coding region contains the genes for small and large T antigens, which are products of alternative splicing. The T antigens in BKV are known to bind to the tumor suppressor proteins p53 and Rb and cause initiation of the host cell cycle. The late coding region contains the genes for agnoprotein and the three viral capsid proteins: VP1, VP2, and VP3. Agnoprotein has a variety of functions, and has been implicated in cell cycle checkpoints, DNA repair mechanisms, capsid assembly, and viral release. VP1, VP2, and VP3 make up the viral capsid, and, as implicated in other polyomaviruses, VP1 has an important role in binding to the receptor of the host cell (22).

IV. BK Virus Associated Allograft Nephropathy

Initially, it was found that 10-60% of kidney transplant patients excreted BK virus in the urine following transplantation. However, this was thought to be relatively benign, associated with transient graft dysfunction (22). It was not until 1995 that BKV-associated nephropathy (BKVN), or, more generally speaking, polyomavirus associated allograft nephropathy (PVAN), was first reported (26). This led to increased study and care as the full and serious consequences of BKV infection following transplantation began to be appreciated. Although BK reactivation has been indentified in patients with AIDS (27), hematological malignancies (28), recent bone marrow transplants (27), recent lung transplants (29), and recent heart transplants (30), it is primarily and increasingly associated with recent renal transplantation and the corresponding immunosuppression (31). Currently, PVAN affects up to 10% of renal transplant recipients and has been implicated in up to 60% of all graft failures (32, 33).

The clinical picture of PVAN continues to evolve. Currently, the gold standard diagnostic test is an allograft biopsy, and is indicated in transplant recipients with increasing serum creatinine levels reflecting deteriorating graft dysfunction (34). Diagnostic findings on biopsy include intranuclear viral inclusions in uroepithelial cells, often displaying polymorphic nuclei (35). Biopsy may also reveal interstitial inflammation or tubular atrophy. Early PVAN preferentially affects the renal medulla and then spreads to the cortex and proximal tubules, but PVAN lesions in the kidney can be multifocal and randomly distributed, leading to a potentially high false negative rate of 10-30% (34).

At the 2009 Banff allograft meeting, a classification schema for grading PVAN was proposed and adopted. Early PVAN (Stage A) is characterized by limited cytopathic alterations with a lack of necrosis or inflammation. Stage B (florid changes) is characterized by increased inflammation, cytopathic changes, necrosis, and early fibrosis. Finally, Stage C is characterized by late sclerosing changes, including significant tubular atrophy, interstitial fibrosis, and scarring (35, 36). Because BKV cytopathology often occurs focally, the most difficult diagnosis to exclude is acute cellular rejection (ACR). Several groups have reported histologic features that separate a diagnosis of ACR from PVAN. These include complement C4d deposition, intimal arteritis, and HLA-DR expression (37-40). Due to this difficulty and the potentially high false negative rate, it is recommended that a negative biopsy be repeated, especially in cases of "presumptive PVAN" (sustained BKV viremia despite an initial negative biopsy). To make a diagnosis of "definitive PVAN," a positive biopsy should be followed up with an ancillary technique. Most often, this technique is immunohistochemistry utilizing cross-reactive antibodies for the Large T antigen of SV40, but staining for BKV VP1 or agnoprotein has also been used (41).

It would behoove the medical community to find a way to identify PVAN earlier, before a detectable increase in serum creatinine and potentially irreversible allograft damage. Correspondingly, much study has gone into low-cost early screening for PVAN. Many potential early indicators of PVAN are relatively inconspicuous, and most are simply indirect measures of viral replication. These include viruria and viremia (42, 43), the shedding of decoy cells (uroepithelial cells in the urine with intranuclear BKV inclusion bodies) (44-46), detection of VP1 mRNA in the urine (47), and detection of

PyV aggregates in the urine by electron microscopy (38). The predictive value of these variables has been hampered by the fact that detectable virus can also be found in a minority of healthy, immunocompetent adults. Nonetheless, some progress has been made. The presence of decoy cells in the urine is low-cost and has a good negative predictive value (48); the detection of BKV loads in blood or urine by PCR is more costly, but has been shown to have good positive and negative predictive values (43, 49). However, the ways in which these tests are performed and evaluated remains non-standardized. As *Babel et al* have concluded, "a standardized, approved PCR assay that enables quantification of BKV load is urgently needed for the diagnosis and monitoring of [PVAN] (50)."

The risk factors for PVAN also remain unclear and imprecise, and conflicting reports have been published. Some risk factors relate to the donor and recipient, such as age, male gender, and white ethnicity (44). Others relate to the status of the transplant, such as viral coinfection, placement of urethral stents (51), degree of HLA mismatch (52), episodes of acute rejection, interstitial inflammation (53), and BKV antibody status (54-56). Properties of the infective strain of virus can also influence risk, such as variants in VP1 (54, 57) and sequence alterations in the NCCR (58). As Hirsch *et al* has pointed out, the central feature of these risk factors is "a disrupted balance between BKV reactivation in renal tubular epithelial cells and BKV specific cellular immune control (41)." Not surprisingly, as PVAN is often thought of as a disease of over-immunosuppression, various immunosuppressive regimens have also been identified as risk factors. While data varies greatly, some studies have implicated tacrolimus and

monoclonal or polyclonal antibody therapies as risk factors when compared to cyclosporine or mTOR inhibitor combinations (59-61).

Currently, there are no commercially available anti-viral agents with proven efficacy against BKV, although few well-designed prospective studies have been carried out (62, 63). Therefore, reduction of immunotherapy remains the cornerstone of treatment. The current thinking is that PVAN represents a disruption of balance between BKV replication and virus-specific immune surveillance. Therefore, therapy is to reduce immunosuppression and allow the host immune system to control the virus (64, 65). This must be balanced with avoiding immune-mediated rejection of the transplanted kidney. Reduction of immunosuppression is also supported by the clinical belief that PVAN incidence has risen due to the increasing potency of immunosuppressive drugs, especially tacrolimus and mycophenolate mofetil. While there is good retrospective evidence for the effectiveness of reducing immunosuppression (61, 66), it does not work for all patients (50). More importantly, the actual ways in which this reduction occurs remains largely non-standardized (63), with at least two to three successful strategies reported (60, 67, 68).

V. Immunologic Responses to BKV Infection

Given the current dogma that PVAN represents a disruption between low levels of smoldering virus replication and host immunosurveillance, examination of the host immune response to BKV infection is prudent. Indeed, several studies have correlated patient outcome with the efficacy of the patient's antiviral immune response (64, 69-72).

By understanding the innate and adaptive immune response to BKV infection, we may gain important insight into disease pathogenesis and potential avenues for treatment. Although innate immunity is an important player in mammalian antiviral defense, not much is known about its role against BKV, and some of the provided information is contradictory. For example, one group found that there was an association between the HLA-C7 allele and sustained BK viremia, suggesting that this might be explained by the activity of natural killer (NK) cells (73); another group found no role for NK cells in BKV reactivation (74). In addition, another group has correlated lower levels of dendritic cells with an increased risk of PVAN, observable both before transplantation and after PVAN diagnosis (75, 76). It has been proposed that pre-transplantation levels of dendritic cells could help with PVAN risk stratification (50).

Much more is known about the adaptive response to BKV infection. Humoral immunity is definitely present, as BKV neutralizing antibodies targeting VP1 have been identified (72). However, there is considerable debate about the protective function of these antibodies (55, 77), which may provide incomplete protection. In addition to the B cell response, BKV-specific T cells have been strongly implicated in the maintenance of latency (65). Although both CD8+ and CD4+ T cell responses have been identified (72), there is no clear pattern of immunodominance, and responses have been observed to large T, small t, VP1, VP2, and VP3 (78). Numerous studies have correlated reconstitution of the BKV-specific cellular immune response to a decrease in BK viremia (79, 80). However, other studies have indicated a potentially immunopathogenic effect for BKV specific T-cells (81), indicating that they may serve a dual function as both “friend and foe” (50). Despite the implications for adaptive immunity in the control of PVAN, there

are still too few studies correlating immune response to disease severity, and it is currently recommended that clinicians do not test for BKV-specific antibodies or BKV-specific T-cells in their PVAN patients (41).

VI. Kidney Transplantation in Mice

Kidney transplantation in mice has been well characterized and carried out for over 35 years (82), providing a powerful tool for studying human organ transplantation. However, it is important to note differences between mouse and human kidney transplantation. One important difference stems from the well-recognized hierarchy of immunogenicity of different transplanted organs in mice, namely, that kidney allografts routinely survive long-term in immunocompetent mice. In other words, HLA-mismatched kidneys in mice are accepted, even in the absence of immunosuppression (83). Although the allografts survive long term, they slowly accumulate damage; the timing and histology of the injury in this model most closely resembles chronic rejection in humans (84).

Initially, high mortality rates and a technically complex procedure limited the usefulness of this model (85, 86). However, in 1995, Zhang *et al* described a method utilizing improved microsurgical techniques to achieve a success rate of over 90% (87). Most mouse kidney transplantation performed today is similar to this published technique, although modifications have been described (88). In performing a mouse kidney transplant with concurrent removal of the native kidneys, the microsurgeon first resects the left kidney of the donor mouse with the artery, vein, and ureter intact. This kidney is placed in the right flank of the recipient, and the renal artery and vein are

anastamosed in an end to side fashion to the recipient aorta and inferior vena cava, respectively. The ureter (containing a patch of donor bladder) is anastamosed to the recipient bladder. After the donor kidney has been implanted, both native kidneys are removed. To achieve maximum success rates, donor and recipient mice between 8 and 12 weeks of age should be used (88, 89).

VII. A Mouse Model of PVAN

Despite years of clinical investigation, much remains unknown about the pathogenesis of PVAN. One of the reasons for difficulty in predicting PVAN incidence and in standardizing treatment is due to the heterogeneous backgrounds, underlying conditions, and treatment of kidney transplant patients. Another reason is the lack of information from an animal model. Polyomaviruses have a very narrow host range, so most of the currently available data on PVAN has been bound by the limitations of human studies. Recently, our group published a mouse model of PVAN utilizing acute infection with mouse polyomavirus (MPyV) and transplantation of allogeneic (MHC-mismatched) kidneys between a C3H (H-2b) mouse and a C57B/6 (H-2k) mouse. To our knowledge, this is the first published animal model of BKVN or PVAN (90).

One of the strengths of this model is that like human BKV, MPyV is a ubiquitous, asymptomatic pathogen that persists life-long in the majority of immunocompetent hosts. Furthermore, the pattern of distribution of the infected organs and tissues is similar in the two species. Finally, in both healthy mice and humans, MPyV infection results in persistent anti-viral T and B cell memory and virus-specific neutralizing antibodies (91). Nonetheless, there are some discrepancies between BKV and MPyV. One important

distinction is that while no immunodominant epitopes have been identified for BKV infection, strongly immunodominant epitopes have been identified for both CD4+ and CD8+ T cells during MPyV infection in C57B/6 mice (92, 93), as well as an immunodominant CD8+ epitope for C3H mice (94, 95). Although this difference may illustrate one of the problems in substituting one polyomavirus for another, it may also be a powerful experimental tool, allowing us to assay CD4+ and CD8+ T cell responses during mouse PVAN in a manner not possible in human patients.

In establishing this model, we found that transplantation of an allogeneic kidney and concomitant acute MPyV infection resulted in loss of the allograft, as indicated by an elevated serum creatinine and a mean survival time of approximately two weeks. This was in stark contrast to transplantation of a syngeneic kidney and concomitant acute MPyV infection, which resulted in no increase in serum creatinine (when compared to a syngeneic or allogeneic kidney transplant without infection) and long term (>60 days) survival. Additional studies in which the native kidneys were not removed suggested that MPyV preferentially replicates in the allogeneic kidney, reaching levels that are 1000 fold higher than in native kidneys or in kidney isografts. Correspondingly, more histopathologic damage is seen in the kidney allografts that in native kidneys or in kidney isografts. Finally, it was found that PVAN in mice resulted in an augmented alloreactive (anti-donor) CD8+ T cell response, but not in an increase in the anti-viral CD8+ T cell response (90).

When considering the implications of current or future findings for clinical renal transplantation, several caveats of this experimental model need to be considered. First, a general property of the mouse kidney transplant model, unlike human transplantation, is

that allogeneic kidneys are not acutely rejected by immunocompetent recipients. An additional caveat of the experimental design is that naïve recipients are acutely infected by MPyV. Since 80-90% of humans show evidence of seroconversion to BKV, transplantation into a naïve recipient (or from a naïve donor, for that matter) is rare. The only scenario where naïve recipients may be more frequent is in pediatric transplantation (96-98). Finally, recipient mice in this model are not immunosuppressed, which is in sharp contrast to the evolving immunosuppressive regimens given to human transplant recipients.

Despite these caveats, key similarities in the histologic pattern of kidney injury in this mouse model of PVAN and human BKVN, as well as the similarities in the natural history of MPyV and BKV, suggest that this model may provide insights into the pathogenesis of polyomavirus infections in the setting of renal transplantation. In addition, this model allows us to address questions that were previously difficult to explore. For example, much of the conflicting data on PVAN risk factors has not been explored in the context of an animal model. The manipulation of conditions in the mice will allow us to explore controversial variables implicated in PVAN etiology, such as cold ischemic time (52, 99, 100), the size of the initial viral inoculum (58), and the degree of MHC-mismatching (52, 101, 102). We can also explore the source of BKV in PVAN (whether reactivation comes from host reservoirs or from the transplanted donor kidney), as data from other viruses such as CMV and EBV has indicated that the source of infection can impact incidence and severity of clinical disease (103).

Finally, and perhaps most significantly, we can use this model to better understand the pathogenesis of PVAN and the interplay between the virus and the host

immune system. PVAN is only seen rarely in other instances of immunosuppression, such as AIDS, cancer, or transplantation of other organs besides the kidney. This implies that it takes both immunosuppression and allogeneic kidney transplantation to result in clinically measurable, active BKV replication. The fact that immunosuppression plays such a significant role, down to the type of immunosuppressive regimen used, also has strong implications for the host immune response in PVAN pathogenesis. Although the model does not require it, it will be interesting to see what effect (if any) immunosuppression has on our mouse model of PVAN. The vast array of immunological and genetic tools available for mice also makes it easy to manipulate the immune response of the transplant recipient. Our early data already implicate the alloimmune response in PVAN (90), but much investigative work remains to be done. By manipulation of the host immune response, we hope to better understand the mechanism of PVAN pathogenesis and the role of host immunity in clinical disease.

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Chapter II:

Adaptive Immunity Rather than Viral Cytopathology Mediates Polyomavirus-Associated Nephropathy in Mice

Abstract

Nephropathy associated with BK polyomavirus (BKV) causes kidney allograft dysfunction and failure. Understanding the pathogenesis of polyomavirus-associated allograft nephropathy (PVAN) is hampered by the species specificity of *Polyomaviridae* family members. Using a mouse polyomavirus (MPyV) kidney transplant model, we investigated clinically relevant variables that may contribute to PVAN. We found that the timing and source (i.e., donor versus recipient) of MPyV infection and the titer of the viral inoculum have significant effects on the extent of allograft injury, with acute infection of the recipient by high-titer MPyV inoculums producing the most profound PVAN. In contrast, altering the degree of MHC matching or increasing ischemia/reperfusion injury (IRI) by prolonging the cold ischemic time of the allograft did not affect the severity of PVAN. Survival correlated positively with serum creatinine levels, but not with viral loads in the kidney allograft. Using splenectomized aly/aly mice, which are unable to mount primary adaptive immune responses, we further demonstrate that persistent high viral loads in the kidney are not sufficient to cause advanced PVAN. These findings suggest that the mechanism of PVAN injury is not a direct consequence of viral cytopathology, but rather involves an interplay between viral infection and the recipient anti-donor immune response.

Introduction

Despite 15 years of intensive investigation, BKV-associated nephropathy (BKVN) remains a significant clinical problem complicating kidney transplantation (1, 2). BKV infection following renal transplantation is associated with renal dysfunction, increased cost related to viral monitoring (3), and diagnostic and treatment dilemmas. A recent analysis of national registry data indicates that the treatment of BKVN is associated with increased rates of graft loss and retransplantation (4, 5). There is emerging evidence that BKV infection may be associated with an increased risk of malignancy following transplantation, providing additional impetus for determining more effective management strategies (6, 7). Concerns about BKVN are no longer limited exclusively to renal transplantation, as there are reports of BKV-induced renal dysfunction in lung, heart, and liver transplant recipients (8-10). The absence of therapeutic anti-viral agents with proven efficacy against BKV further complicates the management of patients with BK viremia or BKVN following transplantation (11).

Much remains unknown about the pathogenesis of BKVN, including the clinical factors affecting the development and progression of BKVN. This may be at least in part due to the heterogeneous nature of transplant recipients and their post-operative management. Currently, routine monitoring for BKV post-transplant to facilitate early detection, together with a reduction in net immunosuppression to allow clearance of virus by the immune system, is the mainstay of management (12, 13). While this approach has resulted in lower rates of graft dysfunction and loss, an understanding of clinical variables predictive of outcome would allow for risk stratification and potentially improve outcomes by identifying populations at high risk of developing BKVN.

To this end, we previously described a mouse model of polomavirus-associated nephropathy in kidney allografts. We showed that acute infection by MPyV in mice bearing allogeneic kidneys resulted in severe graft injury and rapid recipient death. In contrast, uninfected recipients of renal allografts or MPyV-infected recipients of isografts survived long-term. Replication of MPyV was substantially greater in allogeneic kidneys as compared to syngeneic kidneys, and MPyV infection significantly augmented the anti-donor $CD8^+$ T cell response (14). A strength of this model is that like human BKV, MPyV is a ubiquitous, asymptomatic pathogen that persists life-long in the majority of immunocompetent hosts. Furthermore, the pattern of distribution of the infected organs and tissues is similar in the two species. Finally, in both healthy mice and humans, MPyV infection results in persistent anti-viral T and B cell memory and virus-specific neutralizing antibodies (15).

Herein we report the results of studies using this model, designed to determine how clinical variables known to be associated with other viral infections following transplantation affect the incidence and outcome of BKV nephropathy following renal transplantation. Our data indicate that the development of full-blown PVAN is dependent upon an intact adaptive immune response. This implies that viral cytopathology alone is insufficient to mediate severe allograft injury.

Materials and Methods

Animals and mouse kidney transplantation

C57BL/6 (H-2^b), C3H/HeJ (H-2^k), and B6C3F1 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Alymphoplasia (aly/aly) mice on the C57BL/6 background were originally obtained from F. Lakkis (University of Pittsburgh) and were bred and housed in specific pathogen free facilities at Emory University. $K^bD^bß_2m^{-/-}$ mice on the C57BL/6 background were obtained from R. Ahmed (Emory University). Vascularized kidney transplants were performed in 8- to 12- week-old male mice as previously described (14). Briefly, the left kidney of the donor mouse was resected with the artery, vein, and ureter attached, and placed in the right flank of the recipient. The renal artery and vein were anastomosed in an end to side fashion to the receipient aorta and inferior vena cava, respectively. The ureter, with a patch of donor bladder, was anastomosed to the recipient bladder. After implantation of the donor kidney, both receipient native kidneys were removed. Overall, surgical mortality was less than 10% and there were no significant differences in peri-operative mortality between experimental groups. All procedures were performed in accordance with the Institutional Animal Care and Use Committee of Emory University.

MPyV infection

MPyV (strain A2) was prepared as previously described (16). At 8–12 weeks of age, mice received 1.5×10^6 plaque-forming units (PFU) of MPyV subcutaneously (s.c.) in both hind footpads (17), unless noted otherwise. The construction of recombinant MPyV virus carrying the Hemagglutinin (HA) epitope tag embedded in the Large T open reading frame (MPyV-HA) has been described elsewhere (18).

Creatinine measurements

Renal function was assessed by measuring the creatinine (Cr) concentration in plasma using the modified kinetic Jaffe reaction, as previously reported (14). The baseline level of mouse serum creatinine is approximately 0.2 mg/dL, as reported by our group and others (14, 19).

Real-time PCR for MPyV DNA

Taqman real-time polymerase chain reaction (PCR) was used to quantify MPyV genome copies, as previously described (20). The assay's detection limit is 10 copies of genomic viral DNA.

Histologic evaluation

Harvested kidneys were fixed in 10% neutral-buffered formalin and paraffin-embedded. Serial 4 μm sections were stained with Harris' hematoxylin and eosin (H&E). Samples were evaluated by a nephropathologist blinded with regard to the experimental conditions (A.B.F.) for the following criteria: percentages of globally and segmentally sclerotic glomeruli, percentages of cortical inflammation and fibrosis, the presence of interstitial plasma cells and inclusions, the Banff allograft pathology classification scores (21, 22) (as shown in Figure 2); and the Banff diagnostic category (if applicable).

Immunohistochemistry

Staining for the HA epitope tag in recombinant MPyV-HA was done per the manufacturer's instructions, utilizing a primary anti-HA rabbit polyclonal IgG antibody (Acris, Herford, Germany) and a biotinylated secondary goat anti-rabbit IgG antibody

(Abcam, Cambridge, MA). To optimize staining, Target Retrieval Solution (Dako, Dako, Denmark) was used.

Statistical Analysis

For survival, significance of the difference between groups was evaluated using the *log-rank* test. For viral loads and serum Cr levels, significance of the difference was evaluated using either a two-tailed Mann-Whitney U test or the Kruskal-Wallis test and (if significant differences were found) Dunn's multiple-comparison procedure. These calculations were done using Prism statistical software (GraphPad, La Jolla, CA). A p value < 0.05 was considered significant. For histological samples, hierarchical clustering was conducted in SAS JMP version 9.0 (SAS Institute, Cary, NC).

Results

The timing and source of MPyV infection are key determinants of allograft injury

Clinical data and experience with viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) demonstrate that the timing of exposure, the origin of exposure (donor derived versus recipient derived), and pre-existing recipient immunity to the virus affect the incidence and severity of clinical disease (23). We used our MPyV kidney transplant model to address the impact of these variables on the development and progression of PVAN. To accomplish this, we investigated five different conditions, each of which involved the transplant of a kidney from a C3H donor into a B6 recipient. In two groups, the C3H donor was infected, either ~35 days (Donor Persistent) or ~9 days (Donor Acute) prior to transplantation. In another group, a naïve kidney was transplanted into a B6 recipient that had been infected ~35 days previously (Recipient Persistent). In the final two groups, MPyV was not administered until after transplantation, with the recipient receiving virus 1 day (Recipient Acute) or ~16 days (Recipient Delayed Acute) after transplantation.

Varying the source and timing of MPyV infection had a marked effect on survival and other measurable clinical variables. Consistent with previous findings (14), the Recipient Acute group with infection one day after transplantation showed 100% mortality (Figure 1A). Of the remaining groups, only the Donor Acute group had decreased survival at a rate that approached statistical significance; survival was nearly 100% in all other groups (Figure 1A). As seen in Figure 1B, recipient survival correlated closely with serum Cr, with markedly elevated levels in the Recipient Acute mice and

mildly elevated levels in the Donor Acute mice, suggesting that recipient death was due to MPyV-induced renal dysfunction. With respect to viral load, all groups other than the Recipient Persistent group had high, statistically similar viral loads (Figure 1C). Thus, viral load in the kidney was not predictive of the severity of PVAN in this model. These data indicate that only an acute infection occurring near the time of transplantation causes sufficient injury of the transplanted kidney to result in death of the recipient.

Kidney histopathology shows features of rejection and polyomavirus-associated nephritis

Samples from the various experimental groups described in Figure 1 were evaluated by a blinded nephropathologist (A.B.F.). As summarized in Figure 2A, syngeneic grafts from either MPyV-infected or uninfected mice demonstrated no histologic features of acute rejection or injury. In contrast, all of the allogeneic grafts displayed features of acute rejection ranging from borderline acute cellular rejection to Banff grades IA and IIA acute cellular rejection (Figure 2B). The dendrogram in Figure 2A shows the individual scores for each of the diagnostic categories of the Banff classification. Of note, uninfected allogeneic controls showed a level of damage consistent with previous reports at both early and late time points (19, 24-26).

To determine the impact of MPyV infection on allograft injury, we compared samples from infected mice to time-matched, uninfected allogeneic controls. Frequently observed findings included dense tubulointerstitial inflammation with occasional arteritis (Figure 2C). The tubulointerstitial inflammation was predominantly mononuclear and frequently included plasma cells. Features of chronicity, including glomerulosclerosis,

interstitial fibrosis, and tubular atrophy, were present in some cases. In addition, features suggestive of concurrent polyomavirus-associated nephritis were identified, including crescents (n = 2, Figure 2E) of the type that have been associated with polyomavirus infection (27, 28) and a rare viral inclusion (data not shown). Thus, histological examination of the samples reveals features of both rejection and PVAN.

We were also interested in directly visualizing MPyV in infected kidney allografts. Because no commercially antibody is available to detect MPyV-infected cells, we utilized a recombinant MPyV virus carrying the hemagglutinin (HA) epitope tag embedded in the Large T antigen open reading frame (MPyV-HA). The experimental design was analogous to the lethal Recipient Acute condition: a C3H kidney was transplanted into a bilaterally nephrectomized B6 mouse that was infected with MPyV-HA at day 1 post-transplantation. Mice were sacrificed shortly before death at day 14 after transplantation and immunohistochemistry was performed. As shown in Figure 2E, kidney allografts infected with MPyV-HA (but not those infected with the parental strain) showed strong nuclear staining for HA in a pattern indicating polyomavirus infection. Taken together, the histological and immunohistochemical data demonstrate a number of key similarities between PVAN in this mouse model and BKVN in humans.

Prolonged cold ischemia does not increase the severity of PVAN

Previous studies in non-transplant mouse models have shown that injury mediated by chemical toxins or by renal artery ligation increased the permissivity of kidneys for MPyV replication (29). This, together with the observation that the magnitude of PVAN was greatest in mice infected near the time of transplantation, suggested that ischemia

reperfusion injury (IRI) contributed to the pathology of PVAN. To determine the effect of prolonged IRI on PVAN, we extended the period of cold ischemia (the time the kidney was stored on ice between removal and transplantation) from 1 hour (data shown in Figure 1) to 2, 3, or 5 hours. We predicted that prolonging the period of cold ischemia would increase the magnitude of the MPyV-induced injury of the transplanted kidney as reflected by increased serum Cr, increased viral loads, and accelerated death of the recipient. Unexpectedly, no significant differences among these outcome measures were observed with cold ischemic times ranging up to 5 hours (Figure 3). We were unable to extend the cold ischemic time beyond 5 hours, as longer cold storage times resulted in death even in uninfected recipients. In addition, prolonged cold ischemic time did not affect recipient mortality or allograft function in the setting of sub-lethal injury induced by persistent MPyV infection of the donor or acute infection of the recipient by low-dose (1000 PFU per mouse) viral inoculums (data not shown). Thus, in the mouse model of PVAN, prolonged cold ischemic time does not exacerbate the severity of injury.

MPyV-associated transplant nephropathy is dependent on viral inoculum titer

For many clinically relevant viral infections, including BKV, the magnitude of viral infection is thought to be associated with disease severity (30). To examine the potential relationship between the magnitude of the initial viral infection and the development of PVAN, mice were infected with a 1,000-fold lower MPyV dose. Previous work from our group has shown that the magnitude of viral replication is nearly identical for inoculation by high and low doses of MPyV; the major difference is that the time to peak viral loads is delayed with low-dose inoculation, as is the expansion of the anti-MPyV CD8 T-cell

response (31). As shown in Figure 4A, administration of low-dose MPyV inoculum one day post-transplantation to nephrectomized B6 recipients of C3H kidneys resulted in nearly 100% survival. Serum Cr and MPyV viral loads within the kidney were also reduced relative to inoculation with high doses of MPyV (data for inoculation with high doses of MPyV are shown in Figure 1A), with the values for both of these parameters below the level of detection in half of the mice (Figure 4B). This lack of a PVAN phenotype demonstrates that the size of the initial viral inoculum is a major factor governing the magnitude of allograft injury and consequently of recipient survival in the setting of acute MPyV infection.

MHC matching does not confer protection from PVAN

The impact of MHC matching on the development and progression of BKVN in clinical kidney transplantation remains controversial; some groups report that MHC matching has a protective effect while other groups associate it with an increased risk of PVAN (32-34). We next investigated the effect of donor and recipient MHC matching on the development of PVAN in mice undergoing kidney transplantation. First, the effect of an absence of MHC class I molecules was examined by transplanting B6 $K^bD^bß_2m^{-/-}$ kidneys into B6 mice acutely infected by MPyV 1 day after transplantation. As shown in Figure 5, the lack of MHC class I molecules did not impact the development of PVAN, as reflected by comparable values for serum Cr, viral load, and histologic injury between MHC class I deficient and wild type recipients.

To directly investigate the role of MHC matching, kidneys from B6 x C3H (B6C3F1) kidneys were transplanted into B6 mice infected with MPyV 1 day after

transplantation. When compared to fully allogeneic transplants utilizing C3H donor kidneys (Figure 1), recipients of these semi-allogeneic kidneys had statistically similar survival curves, as well as similar degrees of histologic injury (Figures 6A and 6C). However, partial MHC matching did result in slightly lower viral loads. Although this difference did not achieve statistical significance ($p=0.1119$), it suggests that MHC-restricted anti-viral T cells conferred some control of MPyV infection (Figure 6B and 1C). Thus, in the mouse model of acute recipient infection with MPyV in the absence of immunosuppression, MHC matching may affect viral load but does not protect recipients from the development of PVAN.

High viral loads in the absence of adaptive immunity are not associated severe PVAN

To investigate whether MPyV infection in the absence of anti-viral and anti-donor T and B cell responses contributed to PVAN, we used alymphoplasia (aly/aly) mice, which lack lymph nodes due to defects in nuclear factor-κB-inducing kinase (35). When splenectomized, these mice lack all secondary lymphoid organs and are unable to generate a primary adaptive immune response. To directly assess the potential role of MPyV-mediated cytolysis in the development of PVAN, splenectomized and nephrectomized B6 aly/aly mice were transplanted with a B6C3F1 donor kidney and infected with MPyV 1 day after transplantation. In sharp contrast to the allograft damage and loss observed in MPyV-infected wild type B6 recipients of B6C3F1 kidneys (Figure 6), infected aly/aly mice bearing B6C3F1 kidneys exhibited 100% survival (Figure 7A). Correspondingly, most recipient mice displayed relatively low serum Cr levels and showed only focal interstitial lymphocytic infiltrates, even 60 days after transplantation

(Figures 7B and 7C). However, consistent with their inability to generate adaptive anti-viral immunity, these mice uniformly maintained extremely high viral loads in the donor kidneys, averaging approximately 10^{10} PFU /mg, 60 days after transplantation (Figure 7B). In fact, this is 2-3 logs higher than the titers observed in infected recipients of fully MHC-mismatched kidneys that failed to survive (Figures 1C and 3C). These data indicate that persistently high MPyV loads alone are not associated with increased allograft injury or loss and that the mechanism of damage and rejection in the mouse model of PVAN is dependent on an intact adaptive immune system.

Discussion

We employed a mouse model to investigate clinically relevant variables and probe the mechanism of PVAN pathogenesis. Factors associated with an increased severity of PVAN in this model included acute infection rather than chronic or persistent infection, infection of the recipient as opposed to the donor, and infection with a high-titer viral inoculum. Unexpectedly, neither prolonged cold ischemic time of the transplanted kidney nor degree of MHC matching between donor and recipient affected graft survival. Finally, our data demonstrate that in the MPyV model, a high viral burden in the transplanted kidney in the absence of an adaptive immune response does not result in severe injury of the kidney allograft.

When considering the implications of these findings for clinical renal transplantation, several caveats of this experimental model need to be considered. First, a general property of the mouse kidney transplant model is that allogeneic kidneys are not acutely rejected by immunocompetent mice. Additional caveats of the experimental design are that naïve recipients are acutely infected by MPyV and that recipient mice are not immunosuppressed. Despite these caveats, key similarities in the histologic pattern of kidney injury in this mouse model of PVAN and human BKVN, as well as the similarities in the natural history of MPyV and BKV (as discussed in the Introduction), suggest that this model may provide insights into the pathogenesis of polyomavirus infections in the setting of renal transplantation.

The finding that acute but not persistent infection was associated with PVAN suggests that pre-existing immunologic memory for polyomavirus may provide partial

protection from the development of PVAN. Seroepidemiologic studies show that BKV infection occurs in the majority of individuals during the first decade of life, with 75% of individuals demonstrating antibodies to BKV by age 10 and 83% by age 20 (36). This model most closely resembles the clinical scenario encountered by young, BKV naïve children undergoing renal transplantation with kidneys from adult donors, who then develop primary BKV infections in the early post-transplant period. Indeed, two studies found that 30% and 44% of pediatric kidney recipients were seronegative for BKV at the time of transplantation (37, 38). This raises the possibility that some cases of pediatric PVAN may result from a primary BKV infection, rather than reactivation of preexisting BKV infection. Although seronegative children undergoing renal transplantation are at increased risk for primary BKV infection and for developing PVAN (37, 39), the available data are insufficient to determine whether primary BKV infection or viral reactivation is associated with a worse outcome (40).

The observation that acute MPyV infection was associated with PVAN-mediated allograft failure suggested that factors in addition to virally-induced inflammation contributed to the pathogenesis of PVAN. Danger, as originally described by Matzinger et al., is known to augment immune responses (41). In the setting of MPyV infection, it has been reported that renal injury, either by biochemical agents or by ischemia, increased susceptibility to viral replication in the kidney (29). We thus hypothesized that ischemia/reperfusion injury would be an important clinical variable that could potentiate PVAN. Consistent with this hypothesis, an association between cold ischemic time and BKV viruria has been reported in humans (42). However, our results did not demonstrate an association between prolonged cold storage time and the development of PVAN. It

may be that our standard 1 hour cold storage time is sufficient to produce maximal injury. Consistent with our data, others have reported that cold ischemic time was not associated with BKVN in humans (32, 43).

The principle of MHC-restricted T cell recognition underlies the rationale for MHC matching as a means of optimizing the antiviral immune response in organ transplantation. However, reports of the effect of MHC matching on the development and progression of BKVN in clinical renal transplantation are contradictory (32-34, 44). Our data suggest that MHC matching improves control of MPyV infection in kidney transplants. Unexpectedly, improved viral control did not confer protection from PVAN. However, this is consistent with our observation that viral load does not correlate with the severity of PVAN, as demonstrated by the finding that MPyV infected, splenectomized aly/aly recipients of B6C3F1 kidneys survive long-term despite exceptionally high viral loads. We interpret these data as indicating that a direct viral cytopathic effect is not sufficient to cause PVAN. Rather, our data suggest that the pathogenesis of PVAN involves an interplay between viral infection and the anti-donor immune response.

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Figure Legends

Figure 1: Timing and source of infection by MPyV predict outcome. C3H donor kidneys were transplanted into B6 recipients with bilateral nephrectomy. Mice were infected with MPyV at various time points. (A) Survival. (■) Donor Persistent (DP) infection: donor infected at day -35 pre-transplantation, (▼) Donor Acute (DA) infection: donor infected at day -9 pre-transplantation, (●) Recipient Persistent (RP) infection: recipient infected at day -35 pre-transplantation, (▲) Recipient Acute (RA) infection: recipient infected at day 1 post-transplantation, (♦) Recipient Delayed Acute (RDA) infection: recipient infected at day 16 post-transplantation, (○) Uninfected: no virus given. n = 6-12. (B) Serum creatinine at days 8-10 and (C) viral load in the kidney at day 60. Dots represent individual mice. Dashed lines indicate limits of detection.

Figure 2: Histopathology demonstrates features of rejection and polyomavirus-associated nephritis. Histopathology was evaluated by a blinded nephropathologist. In syngeneic (syn) transplants, B6 kidneys were transplanted into B6 recipients with bilateral nephrectomy. In all other cases, C3H donor kidneys were transplanted into B6 recipients with bilateral nephrectomy, and mice were infected with MPyV at various time points, as described in Figure 1. Uninfected mice, denoted as "allo, no virus," served as time-matched negative controls. (A) Dendrogram demonstrating hierarchical clustering of histologic samples. The dendrogram on the right shows how closely related the cases are, and the dendrogram on the bottom shows how closely related the features (Banff scoring, e.g.) are. "Early" samples were gathered in the second week after transplantation; "late" samples were gathered at least 35 days post-transplantation. The

syngeneic transplant cases cluster together, showing no diagnostic abnormality (NDA), denoted as group 1 on the dendrogram. The remainder of experimental conditions show some abnormality, ranging from borderline to type 2 acute cellular rejection (ACR), denoted as group 2 on the dendrogram. Standard abbrieviations for Banff allograft pathology classification scores were used: tubulitis (t), intimal arteritis (v), interstitial inflammation (i), glomerulitis (g), interstitial fibrosis (ci), tubular atrophy (ct), chronic transplant glomerulopathy (cg), mesangial matrix expansion (mm) chronic vasculopathy (cv), arteriolar hyalinosis (ah), and peritubular capillaritis (ptc). The percent of the cortical interstitium occupied by inflammation, fibrosis, and tubular atrophy are designated as %i, %ci, and %ct, respectively; and the percent global and segmental glomerulosclerosis are designated as %glomGS and %glomSS, respectively. Features also given are the presence of plasma cells, inclusions, and crescents (0 = absent, 1 = present). (B) Representative sections from each of the experimental conditions, as summarized in 2A. (H&E staining, original magnification x400) (C) Arteritis, taken from Recipient Acute (RA) sample. (H&E staining, original magnification x400) (D) Segmental glomerulosclerosis with crescent, taken from Recipient Persistent (RP) sample. (H&E staining, original magnification x400) (E) Detection of MPyV-HA by immunohistochemical staining for hemagglutinin. (immunohistochemical staining with a hematoxylin counterstain, original magnification x400).

Figure 3: Prolonged cold ischemic time does not increase the magnitude of MPyV-associated renal allograft injury. C3H donor kidneys were removed, placed in ice cold saline for 1-5 hours, and then transplanted into B6 recipients with bilateral nephrectomy.

Mice were infected with MPyV on day 1 post transplantation. (A) Survival with varied ischemic time. (▼) 2 hours ischemia, (●) 3 hours ischemia, (■) 5 hours ischemia. n = 3-7 mice. (B) Serum creatinine and (C) viral load at time of death. n = 2-5. (The number of data points per group is decreased from (A) due to mouse death before samples could be obtained.) Dots represent individual mice. Dashed lines indicate limits of detection.

Figure 4: Low-dose MPyV infection is not associated with allograft loss. C3H donor kidneys were transplanted into B6 recipients with bilateral nephrectomy. Mice were infected with low dose (1.0-3.0 x 10^3 PFU) MPyV on day 1 post-transplantation. (A) Survival (n = 6 mice). (B) Serum creatinine and viral load at day 60 after transplantation. Dots represent individual mice.

Figure 5: MHC class I-deficient B6 donor kidneys are not rejected by MPyV-infected B6 recipients. Donor kidneys from either WT B6 or from $K^bD^bß_2m^{-/-}$ (Class $I^{-/-}$) B6 mice were transplanted into bilaterally nephrectomized B6 recipients. Mice were infected with MPyV on day 1 post transplantation. (A) Serum creatinine during acute infection (days 8-10 after transplantation). (B) Serum creatinine during persistent infection (days 25-41 after transplantation). (C) Viral load in kidney during persistent infection. Dots represent individual mice. Dashed lines indicate limit of detection. (D) Representative histology during persistent infection. (H&E staining, original magnification x400)

Figure 6: MHC matching does not confer protection from PVAN. C3B6F1 donor kidneys were transplanted into B6 recipients with bilateral nephrectomy. Mice were infected with MPyV on day 1 post transplantation. (A) Survival (n = 6 mice). (B) Serum creatinine and viral load at day 8 after transplantation. Dots represent individual mice. (C) Representative histology at day 8 after transplantation. (H&E staining, original magnification x400)

Figure 7: High viral load is not associated with increased loss of renal allografts. B6C3F1 donor kidneys were removed and transplanted into B6 aly/aly recipients with splenectomy and bilateral nephrectomy. Mice were infected with MPyV on day 1 post transplantation. (A) Survival (n = 5 mice). (B) Serum creatinine and viral load at day 60 after transplantation. Dots represent individual mice. (C) Representative histology at day 60 after transplantation. (H&E staining, original magnification x400)

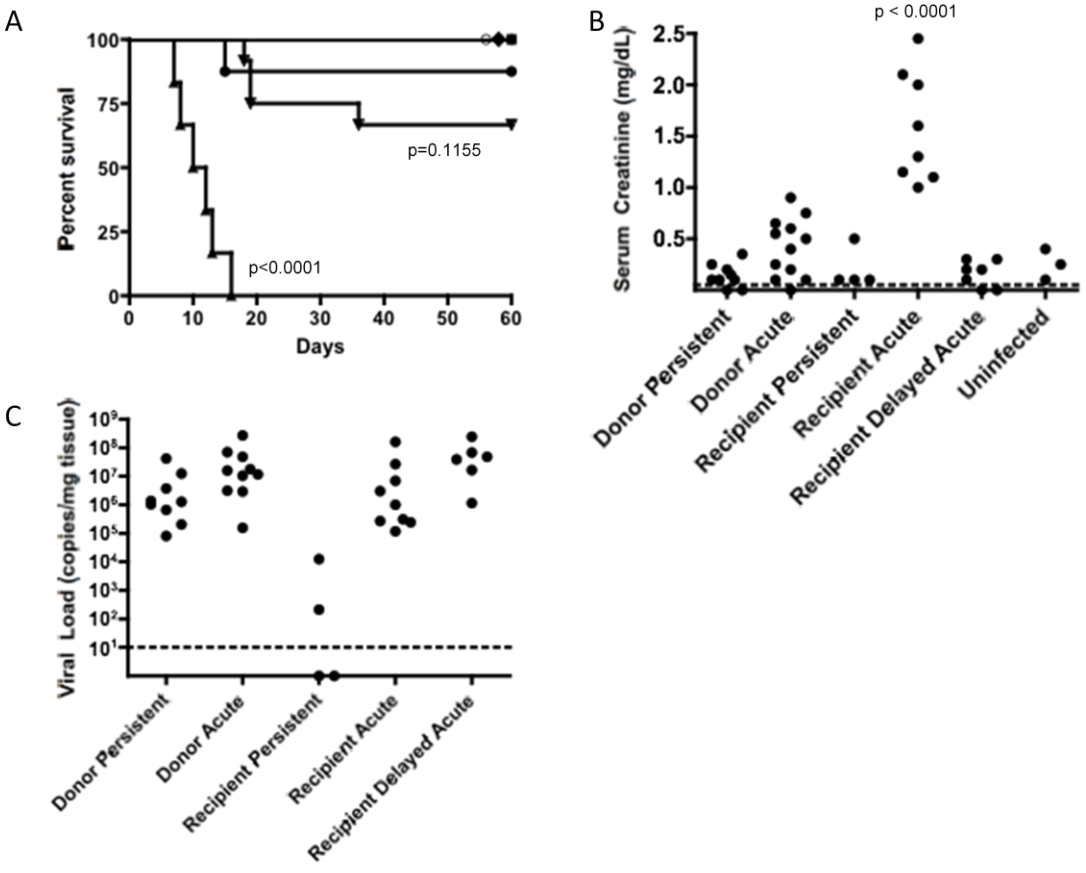
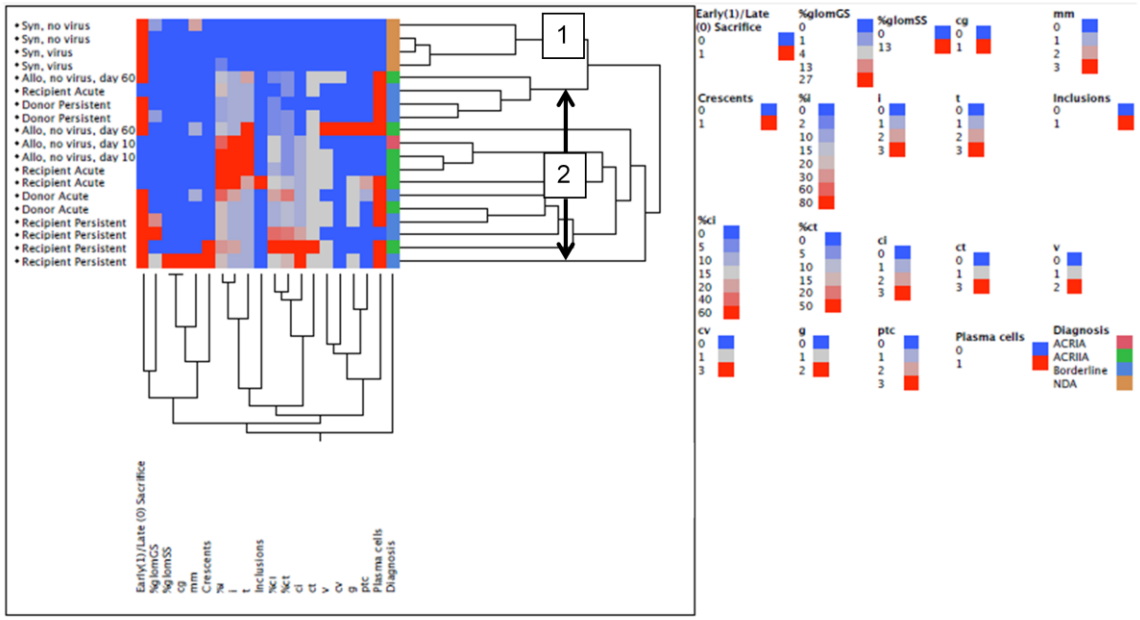

A
Percent survival
Days
p=0.1155
p<0.0001
B
Serum Creatinine (mg/dL)
p < 0.0001
Donor Persistent
Donor Acute
Recipient Persistent
Recipient Acute
Recipient Delayed Acute
Uninfected
C
Viral Load (copies/mg tissue)
Donor Persistent
Donor Acute
Recipient Persistent
Recipient Acute
Recipient Delayed Acute

Figure 1

A


Syn, no virus
Syn, no virus
Syn, virus
Syn, virus
Allo, no virus, day 60
Recipient Acute
Donor Persistent
Donor Persistent
Allo, no virus, day 60
Allo, no virus, day 10
Allo, no virus, day 10
Recipient Acute
Recipient Acute
Donor Acute
Donor Acute
Recipient Persistent
Recipient Persistent
Recipient Persistent
Recipient Persistent
1
2
Early(1)/Late (0) Sacrifice
%glomGS
%glomSS
cg
mm
Crescents
%i
i
t
Inclusions
%ci
%ct
ci
ct
v
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g
ptc
Plasma cells
Diagnosis
Early(1)/Late (0) Sacrifice
0 1
%glomGS
0 1 4 13 27
%glomSS
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Crescents
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ct
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v
0 1 2
cv
0 1 3
g
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ptc
0 1 2 3
Plasma cells
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Diagnosis
ACRIA
ACRIIA
Borderline
NDA

Figure 2

B

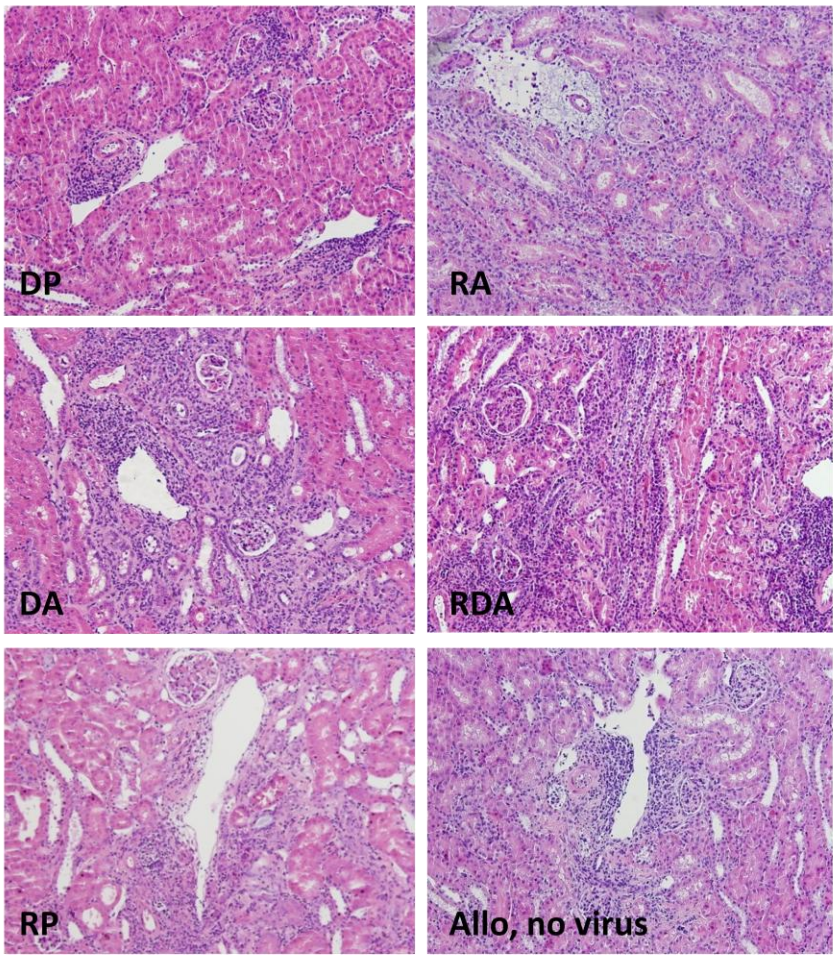

Figure 2

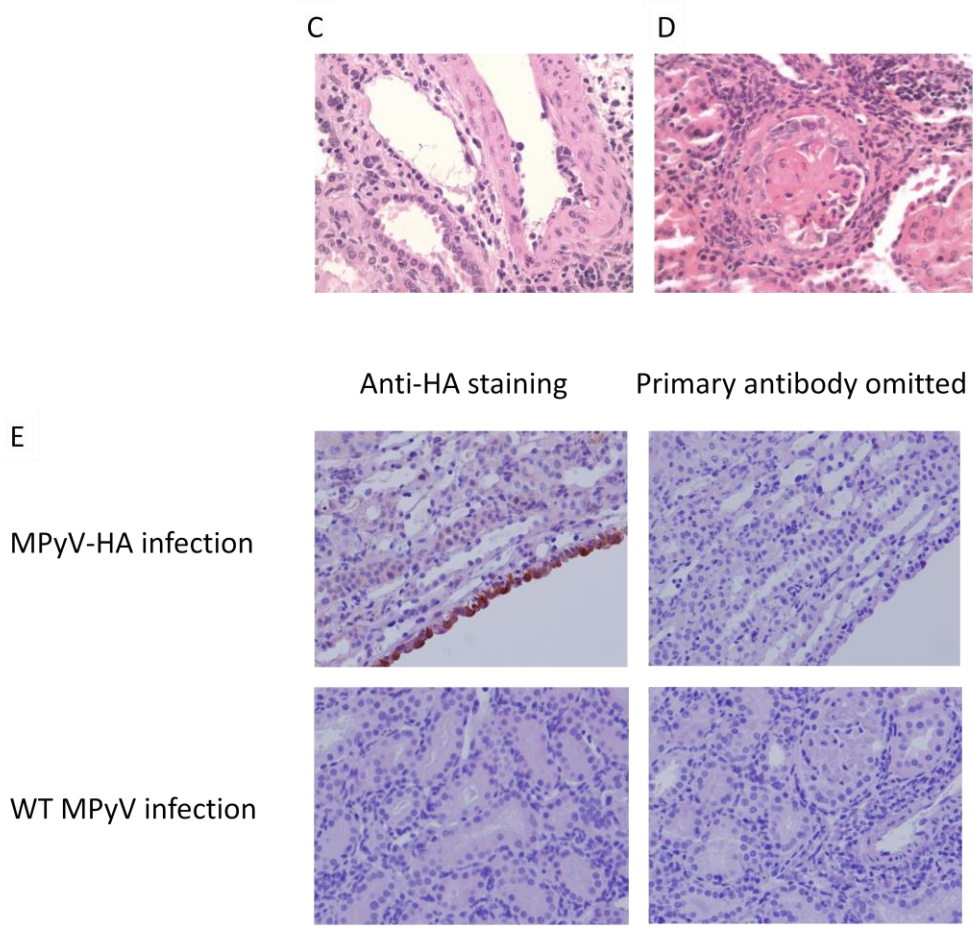
C
D
Anti-HA staining
Primary antibody omitted
E
MPyV-HA infection
WT MPyV infection

Figure 2

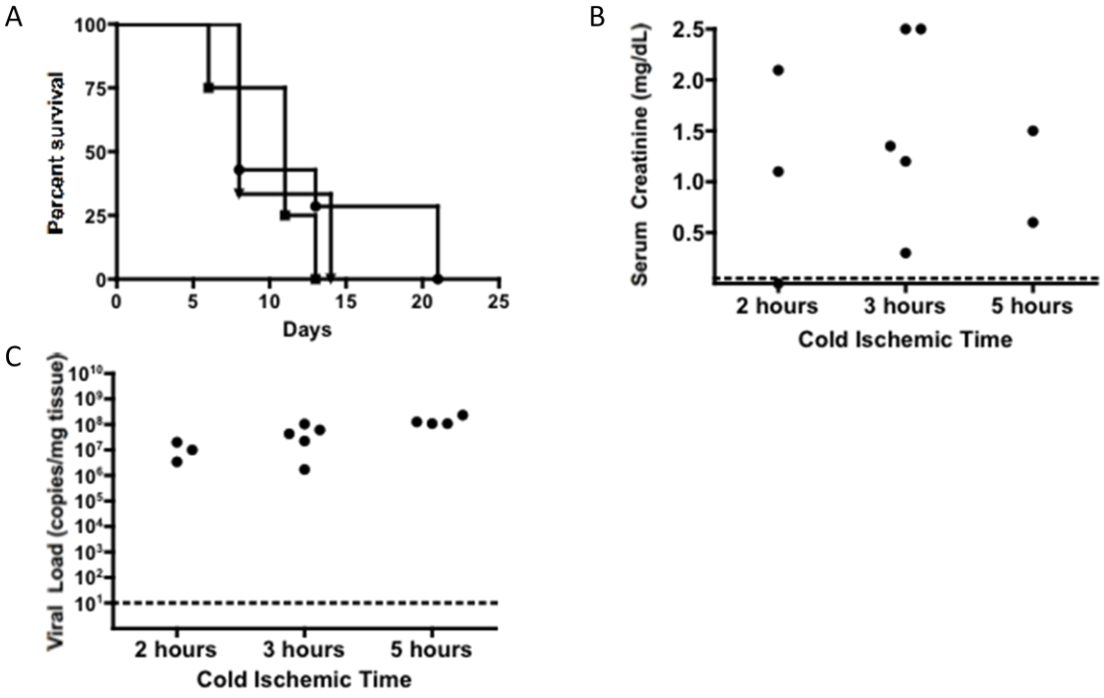
A
Percent survival
100
75
50
25
0
0
5
10
15
20
25
Days
B
Serum Creatinine (mg/dL)
2.5
2.0
1.5
1.0
0.5
2 hours
3 hours
5 hours
Cold Ischemic Time
C
Viral Load (copies/mg tissue)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
2 hours
3 hours
5 hours
Cold Ischemic Time

Figure 3

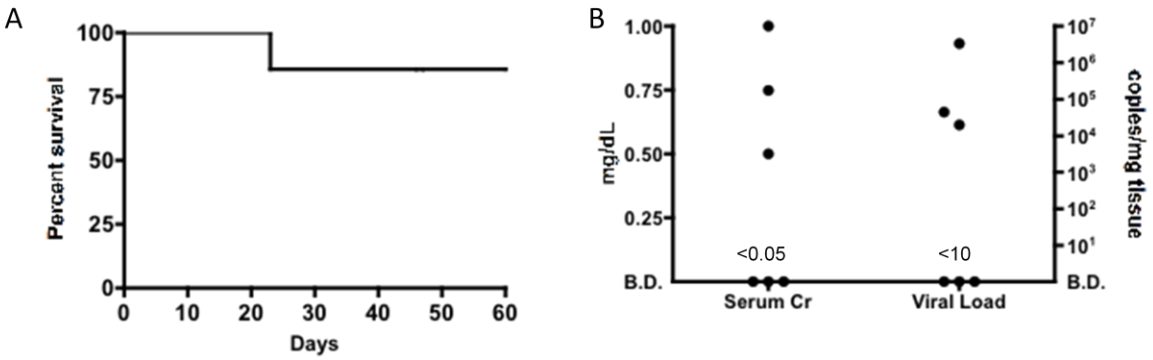
A
Percent survival
100
75
50
25
0
0
10
20
30
40
50
60
Days
B
mg/dL
1.00
0.75
0.50
0.25
B.D.
<0.05
<10
Serum Cr
Viral Load
B.D.
copies/mg tissue
10^7
10^6
10^5
10^4
10^3
10^2
10^1

Figure 4

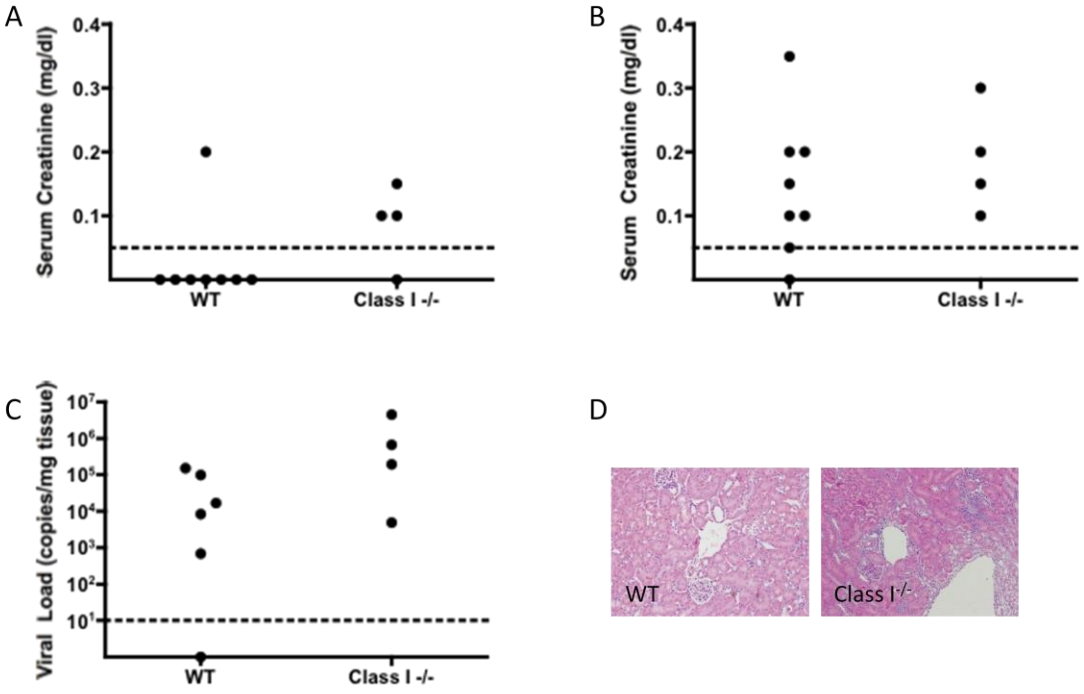
A
Serum Creatinine (mg/dl)
0.4
0.3
0.2
0.1
WT
Class I -/-
B
Serum Creatinine (mg/dl)
0.4
0.3
0.2
0.1
WT
Class I -/-
C
Viral Load (copies/mg tissue)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
WT
Class I -/-
D
WT
Class I -/-

Figure 5

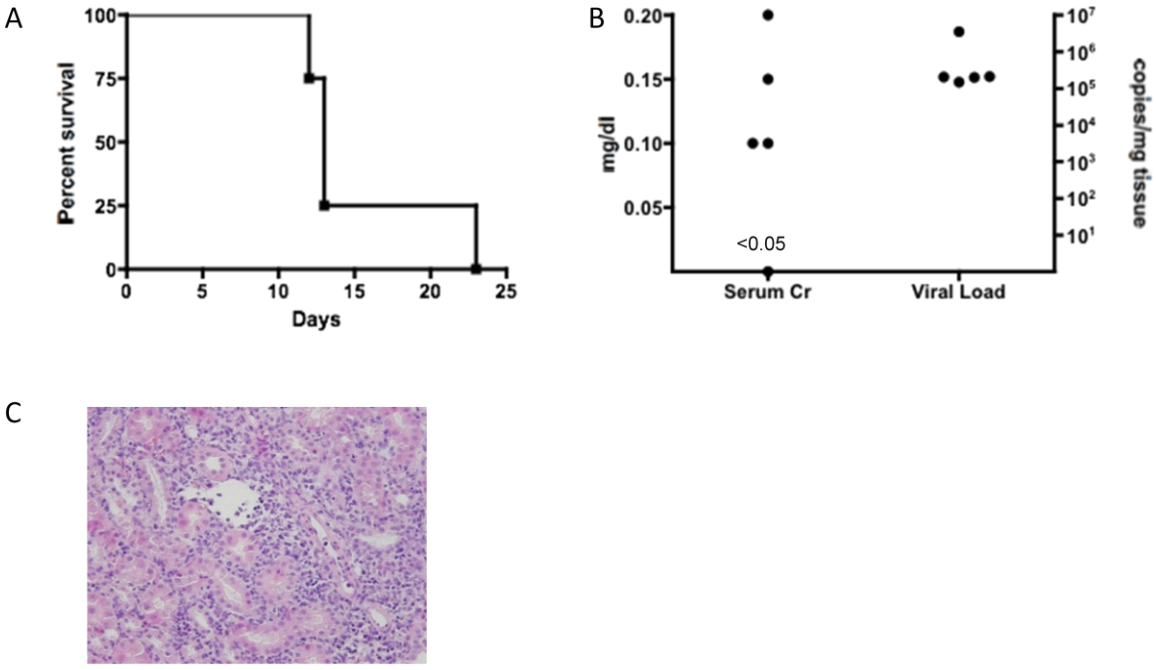
A
100
75
50
25
0
Percent survival
0
5
10
15
20
25
Days
B
0.20
0.15
0.10
0.05
mg/dl
<0.05
Serum Cr
Viral Load
copies/mg tissue
C

Figure 6

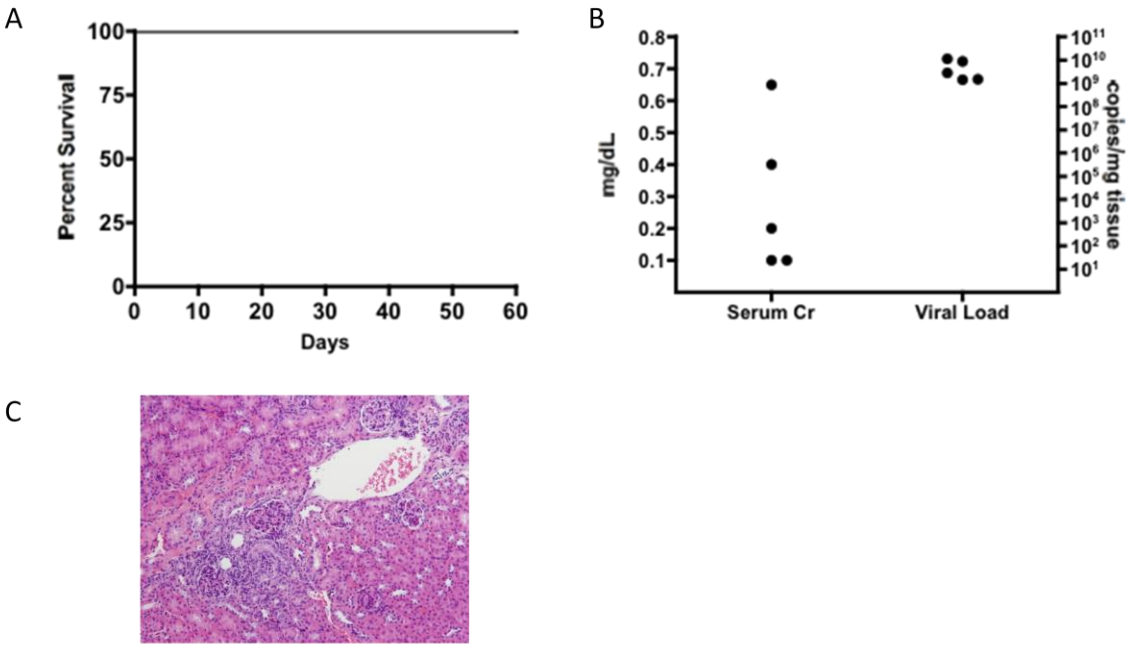
A
Percent Survival
100
75
50
25
0
0
10
20
30
40
50
60
Days
B
mg/dL
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
copies/mg tissue
Serum Cr
Viral Load
C

Figure 7

Chapter III:

Alloimmunity Promotes Allograft Loss in a Mouse Model of Polyomavirus-Associated Allograft Nephropathy

Abstract

Although PVAN is an important cause of kidney allograft dysfunction and loss, its pathogenesis is not well understood. Here, we use mouse polyomavirus (MPyV) and kidney transplantation, in conjunction with lymphoid deficient (aly/aly) and transgenic ovalbumin mouse strains, to investigate the roles of viral cytolysis, the anti-viral immune response, and alloimmunity in PVAN pathogenesis. In splenectomized aly/aly mice that are unable to generate an effective primary T-cell response, MHC-mismatched allografts are tolerated after MPyV infection, despite extremely high viral loads. Transfer of anti-allo (but not anti-viral) T-cells into these mice results in allograft rejection. We also adoptively transferred OVA-specific TCR transgenic CD8 T-cells (OT-I cells) into B6 mice after transplantation and MPyV infection. This transfer caused rejection and death when the targeted epitope was displayed on the transplanted kidney, but not when it was expressed by recombinant MPyV. Additional studies indicated that this OT-I mediated rejection does not occur if MPyV is replaced by a virus lacking kidney tropism. Moreover, immunosuppression with tacrolimus promoted allograft and mouse survival under otherwise lethal conditions. These data suggest that the predominant mechanism of allograft rejection in PVAN is an augmented allo-immune T-cell response, which may have important implications for treatment in human transplantation.

Introduction

BK virus (BKV) is a human polyomavirus first isolated in 1971 from the urine of a kidney transplant patient with a uretal stricture (1). While more than 80% of adults show evidence of early childhood exposure to BKV (2), this infection is asymptomatic in the vast majority of the immunocompetent population. Atlhough BK reactivation has been indentified in patients with AIDS (3), hematological malignancies (4), recent bone marrow transplants (3), recent lung transplants (5), and recent heart transplants (6), it is primarily and increasingly associated with recent renal transplantation and the corresponding immunosuppression (7). BKV-associated nephropathy (BKVN), or, more generally speaking, polyomavirus associated allograft nephropathy (PVAN), affects up to 10% of renal transplant recipients and has been implicated in up to 60% of all graft failures (8, 9).

Currently, there are no commercially available anti-viral agents with proven efficacy against BKV, although few well-designed prospective studies have been carried out (10, 11). Therefore, reduction of immunotherapy remains the cornerstone of treatment. The current thinking is that PVAN represents a disruption of balance between BKV replication and virus-specific immune surveillance. Therefore, therapy is to reduce immunosuppression and allow the host immune system to control the virus (12, 13). This must be balanced with avoiding immune-mediated rejection of the transplanted kidney. Reduction of immunosuppression is also supported by the clinical belief that PVAN incidence has risen due to the increasing potency of immunosuppressive drugs, especially tacrolimus and mycophenolate mofetil. While there is good retrospective evidence for the effectiveness of reducing immunosuprression (14, 15), it

does not work for all patients (16), and, more importantly, the actual ways in which this reduction occurs remains largely non-standardized (11).

Despite years of clinical investigation, much remains unknown about the pathogenesis of PVAN. One of the reasons for difficulty in predicting PVAN incidence and in standardizing treatment is due to the heterogeneous backgrounds, underlying conditions, and treatment of kidney transplant patients. Another reason is the lack of information from an animal model. Polyomaviruses have a very narrow host range, so most of the currently available data on PVAN has been bound by the limitations of human studies. Recently, we published a mouse model of PVAN, in which acute infection with mouse polyomavirus (MPyV) and transplantation of an allogeneic kidney resulted in allograft loss (17). Further studies in this model revealed that the adaptive immune response, and not viral cytolysis, was responsible for PVAN-mediated rejection (Albrecht et al, manuscript accepted).

In this study, we further investigate the adaptive immune requirements for PVAN-mediated rejection of kidney allografts in the mouse model. Using adoptive transfer of T-cells in both alymphoplasia (aly/aly) mice and in the mOVA system, we demonstrate that allogeneic T-cells, and not anti-viral T-cells, are necessary and sufficient to mediate rejection. We also find that this is not a consequence of generalized viral inflammation or acute infection. Surprisingly, but in line with this published data, immunosuppression actually provides a beneficial effect in the mouse model of PVAN. These data suggest a mechanism in which viral-induced inflammation in the kidney augments the alloimmune response, leading to PVAN-mediated rejection of the allograft. Taken together, these studies may support investigation into new therapeutic avenues for the treatment of PVAN.

Materials and Methods:

Mice and kidney transplantation

C3H/HeJ (H-2^k), C57BL/6 (H-2^b), and B6C3F1 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Alymphoplasia (aly/aly) mice on the B6 background were obtained from F. Lakkis (University of Pittsburgh). OT-I and mOVA mice on the B6 background were obtained from C. Larsen (Emory University). Kidney transplants were performed in 8- to 12- week-old male mice as previously described (Albrecht et al, manuscript accepted). There were no significant differences in peri-operative mortality between groups, and overall surgical mortality was less than 10%. All transplantation and procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Emory University.

MPyV infection

MPyV was prepared as previously described (18). At one day post-transplantation, mice received 1.5×10^6 plaque-forming units (PFU) of MPyV subcutaneously (s.c.) in both hind footpads, unless noted otherwise. The construction of recombinant MPyV virus carrying the SIINFEKL epitope embedded in the middle T open reading frame (MPyV.OVAI) has been described elsewhere (19).

Murine herpesvirus 68 (MHV-68) infection

MHV-68 was obtained from L. Kean (Emory University), prepared and administered as previously described (20). Briefly, B6 mice received 1.0×10^5 PFUs of MHV-68

intraperitoneally (i.p.) on day 1 post-transplantation.

T cell purification and transfer

For adoptive transfer experiments using anti-viral T-cells, splenocytes were harvested from C57BL/6 mice 8 days after MPyV infection. For those using anti-allo T cells, spleens or draining lymph nodes were isolated from C57BL/6 mice 14 days after administration of a C3H/HeJ skin graft. T cells were purified by positive selection using an AutoMACS (anti-CD90 (Thy1.2)-coated microbeads, Miltenyi Biotec). A total of 1×10^7 T-cells were transferred on days 0 or 1 post-transplantation.

OT-I cell transfer

Bulk splenocytes from OT-I mice were assayed for CD8 expression via flow cytometry. A total of 3.5×10^6 OT-I cells (transgenic CD8+ T cells specific for chicken ovalbumin) were transferred on days -2 or -1 pre-transplantation.

Tacrolimus administration

Tacrolimus was obtained from Astellas Pharmas US, Inc and administered as previously described (21). Briefly, tacrolimus was dissolved in 0.9% sodium chloride and administered s.c. daily at a dose of 60 ug/mouse in a final volume of 400 uL.

Creatinine measurements

To assay renal function, the creatinine (Cr) concentration in plasma was measured using the modified kinetic Jaffe reaction, as previously reported. The baseline level of mouse serum creatinine is approximately 0.2 mg/dL, as reported by our group and others (17, 22).

Real-time PCR for MPyV DNA

Taqman real-time polymerase chain reaction (real-time PCR) was used to quantify genome copies of MPyV, as previously described (23). The limit of detection is 10 copies of genomic viral DNA.

Histologic evaluation

Kidney sections were fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial 4 μm sections were stained with Harris' hematoxylin and eosin (H&E).

Statistical Analysis

For survival, significance was evaluated using the *log-rank* test. For viral loads and serum Cr levels, significance was evaluated using either a two-tailed Mann-Whitney U test or the Kruskal-Wallis test and (if applicable) Dunn's multiple-comparison procedure. These calculations were performed with Prism statistical software (GraphPad, La Jolla, CA). A p value < 0.05 was considered significant.

Results

An augmented alloimmune response, but not an augmented anti-viral response, promotes PVAN

Previous work with MPyV-infected, splenectomized alymphoplasia mice (aly/aly), which are incapable of generating an adaptive immune response, demonstrated that a high viral load alone was insufficient to cause kidney allograft rejection (Albrecht et al, manuscript accepted). This suggested that the adaptive immune system was required for kidney dysfunction and rejection in the mouse model of PVAN. To further investigate the adaptive immune requirements for PVAN-mediated rejection, we performed adoptive transfer experiments. Splenectomized aly/aly mice were transplanted with C3B6F1 kidneys and infected by MPyV on day 1 post-transplantation. On days 0 or 1 post-transplantation, we transferred anti-viral T cells (isolated from a WT B6 mouse at the peak of infection) or anti-allo T cells (isolated from another WT B6 mouse receiving a C3H skin graft).

We compared the results of these adoptive transfers to the previously published condition without adoptive transfer, in which the mice exhibited 100% survival despite extremely high viral loads (Figure 1). Interestingly, only the transfer of anti-allo T cells, and not the transfer of anti-viral T cells, caused a significant increase in PVAN-mediated rejection and death (Figure 1A). The transfer of anti-viral T cells did result in a significant decrease in viral load (indicating that the anti-viral cells were functional) with a mild increase in serum creatinine levels, indicative of kidney dysfunction, albeit non-lethal. Correspondingly, the transfer of anti-allo cells resulted in unaltered, extremely high viral loads, with increased serum creatinine (Figure 1B-C). The transfer of anti-allo cells also caused an increased degree of histologic damage

(Figure 1D). This data suggests that anti-allo T cells, and not anti-viral T-cells, are required for PVAN-mediated rejection of kidney allografts in mice.

Anti-donor OT-I cells, but not anti-viral OT-I cells, promote PVAN

In the experiments utilizing aly/aly mice, there were uncontrollable differences between the adoptively transferred populations of anti-viral and anti-allo T cells. Most notably, these populations may have differed in absolute number and activation state. To better control for variables in the transferred cell population, we performed adoptive transfer experiments utilizing OT-I cells, TCR transgenic CD8 T cells specific for chicken ovalbumin (OVA), which were isolated from naïve mice. In one set of experiments, the transferred OT-I cells recognized membrane bound ovalbumin (mOVA) on the surface of the transplanted kidney and were functionally anti-donor. In the other set of experiments, a recombinant MPyV was utilized, expressing the epitope recognized by OT-I cells in the middle T reading frame (MPyV.OVAI). In this latter experiment, the transferred OT-I cells were functionally anti-viral. Thus, while the actual physical target of the adoptively transferred OT-I cells changed in these two experiments, their number and activation state were kept constant.

In the experiment involving anti-donor OT-I cells, an mOVA kidney on the B6 background was transplanted in to a B6 mouse with bilateral nephrectomy. Mice were infected with MPyV on day 1 post-transplantation, with OT-I cells transferred on days -2 or -1 pre-transplantation. In the presence of both OT-I cells and virus (but not either one alone), there was PVAN-mediated rejection of the mOVA kidneys and subsequent mouse death (Figure 2B). Correspondingly, these mice had elevated serum creatinines and evidence of increased histologic

damage at the time of death. In agreement with previously published reports, we saw no correlation between viral load and survival.

In the experiment involving anti-viral OT-I cells, B6 kidneys were transplanted into passively immunized B6 mice, with OT-I cell transfer on days -2 or -1 pre-transplantation. Passive immunization was necessary to prevent an extra-renal cause of death, but such immunization does not prevent allograft dysfunction and rejection in the mouse model of PVAN (data not shown). On day 1 post-transplantation, mice were infected with the recombinant MPyV.OVAI. Infection with MPyV.OVAI and transfer of anti-viral OT-I cells resulted in 100% survival, low serum creatinines, and less evidence of histologic damage (Figure 3). Taken together, these data support the conclusions drawn from the aly/aly experiments; namely, that the mechanism of allograft dysfunction and rejection in the mouse model of PVAN depends on an anti-donor immune response, but not on an anti-viral one.

Kidney allograft rejection is not solely due to acute viral infection

Acute viral infections can affect local and systemic environments and result in altered pathology. In both humans and mice, it has been demonstrated the acute infections result in decreased graft survival (24-26). To demonstrate that the kidney allograft rejection we observed was not due to generalized inflammation or another consequence of acute viral infection, we substituted murine herpesvirus-68 (MHV-68) for MPyV. MHV-68, like MPyV, is endemic in mice, but lacks tropism for the kidney, initially replicating in lung tissue and then establishing latency in B cells (27). For these experiments, we transplanted a B6 mOVA kidney into a nephrectomized B6 mouse. OT-I cells were adoptively transferred ~1 day before transplantation, and mice were

infected by MHV-68 1 day post transplantation. Under these same conditions, we found that 80% of mice infected with MPyV suffered an acute death (Figure 2).

In sharp contrast to the high mortality seen with MPyV, the allograft recipient mice infected with MHV-68 exhibited 100% survival (Figure 4A). Correspondingly, these mice showed serum creatinine levels consistent with uninfected recipients (Figure 4B), and their histology displays low levels of subclinical damage (Figure 4C). While we cannot rule out the possibility that another viral infection may recapitulate the results seen with MPyV, these data indicate that a generalized acute viral infection alone is insufficient to accelerate kidney allograft loss.

Tacrolimus administration improves survival in the mouse model of PVAN

Data generated thus far suggests that the mechanism of PVAN is mediated by the alloimmune response. If this mechanism is indeed correct, we would predict that increased immunosuppression would be beneficial in this model. To test this prediction, we transplanted C3H kidneys into B6 recipients with bilateral nephrectomy. Mice were infected by MPyV on day 1 post transplantation, and given daily tacrolimus injections for 40 days post-transplantation.

In contrast to the acute rejection and death within 2 weeks seen in mice not given immunosuppression, 50% of the mice receiving tacrolimus survived past day 40 (Figure 5A). For further analysis, we divided the mice receiving tacrolimus into two groups: those that died before 40 days were labeled “short-term survival,” while those that survived to 40 days were labeled “long-term survival.” The short-term survival mice were indistinguishable from the mice receiving no treatment, indicating kidney allograft dysfunction as the cause of death. Both of these lethal groups had elevated serum creatinines (Figure 5B) and strong histologic evidence of

inflammation and infiltration (Figure 5D) at the time of death. In contrast, the long term survival mice had serum creatinines in the normal range (Figure 5B) and significantly less kidney damage by histology (Figure 5D). In support of our earlier conclusion precluding viral cytolysis as a significant cause of allograft damage in the PVAN model, all three groups had statistically similar viral loads (figure 5C).

Discussion

As polyomavirus associated allograft nephropathy continues to play a major role in renal allograft dysfunction and loss, it is crucial that we gain a better understanding of the pathogenesis of this condition. Previous work from our group established a mouse model of disease and, using that model, reported that the adaptive immune response (and not viral cytolysis alone) was essential for PVAN-mediated rejection and death. The studies reported herein further refine these findings, implicating the alloimmune response and abdicating the anti-viral response as the essential immunologic component of PVAN pathogenesis. Support for this conclusion comes from the use of alymphoplasia mice, where only transfer of anti-allo T-cells is sufficient to cause allograft rejection, as well as from the mOVA system, where OT-I cell transfer recapitulates the PVAN phenotype if and only if the cells are directed against the kidney graft. In line with predictions based on this data, immunosuppression results in increased survival and improved allograft function.

Since this model requires acute infection with MPyV, we wanted to rule out the possibility that the observed PVAN phenotype was simply a general consequence of acute infection at the time of transplantation. To investigate, we specifically chose MHV-68 because it lacked tropism for the kidney. As seen in Figure 4, infection with MHV-68, in place of MPyV, does not result in substantial allograft damage or rejection. These data suggest that circulating inflammatory molecules and other systemic changes associated with acute infection are insufficient to cause PVAN; rather, it is the local environment of the kidney that must be compromised in order to promote disease. In support of this conclusion, it has been reported that renal transplant patients with PVAN demonstrate an extremely high level of proinflammatory

transcripts in their allografts, greater in magnitude than what is normally seen in cases of acute rejection (28).

Taken together, these data allow us to propose a mechanism for the pathogenesis of PVAN (Figure 6). In order to get PVAN-mediated rejection of a transplanted kidney, we require both an acute MPyV infection and allograft transplantation. It is well established that mice tolerate an allogeneic kidney transplant in the absence of infection (29), so the unadulterated alloimmune response generated against the allograft, taken alone, is insufficient to cause rejection. Our previously published data also indicate that direct viral cytolysis alone is insufficient for irreversible graft injury in mice. This is demonstrated by the aly/aly experiment (Albrecht et al, manuscript accepted), in which no adaptive immune response results in survival despite high viral loads, and by the fact that acute MPyV infection is insufficient to cause rejection of a kidney isograft (17). With support from our MHV-68 data, we therefore conclude that the mechanism of action by which MPyV contributes to PVAN pathogenesis involves localized inflammation in the kidney. This environment causes augmentation of the alloimmune response, and it is this boosted alloimmune response that is now sufficient to reject the kidney and cause the PVAN phenotype.

We are very interested in this interplay between polyomavirus infection of the kidney and host alloimmunity. Exactly how the local kidney environment must be compromised in order to boost the alloimmune response remains an area of future study, in which gene chip analysis or gene arrays may be helpful. Based on our MHV-68 data, we believe the molecule or molecules responsible for mediating this phenotype will be acting predominantly on the local level. Correspondingly, it will be interesting to observe how the kidney-infiltrating T-cells are changed in response to the altered microenvironment of the kidney. We are currently undertaking studies

in which kidney-infiltrating T cells are isolated and phenotyped. We hypothesize two non-mutually exclusive possibilities for the pathogenic transformation of these T cells: alterations in their recruitment and number, and/or changes in their function or cytotoxicity. This interface between virus and adaptive immune system could also prove to be a useful target for therapeutic manipulation in the future, especially since there are no therapeutic agents commercially available with proven efficacy against BKV.

We are aware of the seemingly paradoxical nature of immunosuppression in our animal model of PVAN. In human care, the accepted treatment for PVAN is reduction of immunosuppression. Despite its non-standardization, there is solid retrospective evidence providing support that this is an efficacious treatment for most patients. An oft cited rationale for this treatment regimen is that it ultimately lowers the viral load (supported by our data), and this results in reduced viral cytolysis in the transplanted kidney (not supported by our data). In contrast with this clinical scenario, immunosuppression in our mouse model actually provides a beneficial effect to the host. Since no baseline immunosuppression is necessary (mice spontaneously accept kidney allografts), addition of immunosuppression to transplanted, infected mice improves kidney function and increases survival.

Using the mechanism proposed in Figure 6, the differences in altering immunosuppressive regimens between mice and humans can be explained. For a human kidney transplant patient, a decrease in immunosuppression allows the anti-BK memory response to recover, which in turn lowers the viral burden. Contrary to some presuppositions, our data does not support a mechanistic role for viral cytolysis in PVAN. Instead, our model suggests that a lower viral load fails to augment the alloimmune response, allowing symptoms of BKVN to subside. In mice in the PVAN model, there is no pre-existing memory to recover; they are naïve

with respect to MPyV. In this case, addition of immunosuppression further inhibits the adaptive immune response, including that portion directed against the allograft. This alloimmune response is now insufficient, even when boosted, to cause clinical rejection of the allogeneic kidney.

However, while this model and interpretation do suggest a rationale for why reduction of immunosuppression can be effective in humans, they do not rule out the possibility that increased immunsuppression could also be beneficial with respect to BKVN. In theory, increased immunosuppression could reduce the alloimmune response, making it insufficient (even when augmented) to reject the transplanted kidney. There are several studies which found no benefit to reducing immunosuppression in the clinic, especially in patients with chronic BKV infection or overt PVAN, and in some cases reduction of immunosuppression was even found to be detrimental (30, 31). With a lack of therapeutic options for these patients, one has to wonder whether an increase in immunosuppression would provide benefit. Of course, there are a fair number of confounding variables to consider, including the nephrotoxicity of most immunosuppressive agents, and possible co-infection with pathogens relevant to transplant recipients, such as CMV and EBV. Nonetheless, especially with the advent of Belatacept and with many new immunotherapies in the pipeline, we may want to revisit the ways in which we alter the immunosuppressive regimen in response to PVAN

In conclusion, we provide novel data from the mouse model of PVAN, indicating that a boosted alloimmune response is the primary immunologic mechanism of disease. At the minimum, these data suggest a reinterpretation of the rationale for reducing immunosuppression in the human clinical scenario; at the maximum, they suggest investigation into alternative modifications of immunosuppressive regimens. Importantly, these data also highlight a

previously undervalued target for therapeutic intervention – the interplay between viral inflammation and the host alloimmune response. By better understanding the pathogenesis of PVAN, we can continue to improve treatment and prognosis for kidney transplant patients.

Acknowledgements

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Figure Legends:

Figure 1: An augmented alloimmune response, but not an augmented anti-viral response, promotes PVAN. C3B6F1 donor kidneys were transplanted into B6 aly/aly recipients with splenectomy and bilateral nephrectomy. Mice were infected by MPyV on day 1 post transplantation. T-cells were transferred on days 0 or 1 post-transplantation. (A) Survival. (■) Anti-Viral T cells transferred, (▼) Anti-Allo T cells transferred, (●) No cells transferred (shown from previously published work (Albrecht et al, manuscript accepted) for comparison). n = 5-8. (B) Serum creatinine at day 60 or time of death and (C) viral load in kidneys at day 60 or time of death. n = 2-5. (The number of data points per group is decreased from (A) due to mouse death before samples could be obtained.) Dots represent individual mice. Dashed lines indicate limits of detection. (D) Representative histology at day 60 or time or death. While the transfer of anti-viral T cell shows expected infiltration, the transfer of anti-allo T cells shows the greatest degree of histologic damage. (H&E staining, original magnification x400)

Figure 2: Anti-donor CD8+ T-cells and MPyV are both required to cause rejection. B6 mOVA donor kidneys were transplanted into B6 recipients with bilateral nephrectomy. Mice were infected by MPyV on day 1 post transplantation. OT-I cells were transferred on days -2 or -1 pre-transplantation. (A) Survival. (■) OT-I cell transfer and MPyV infection (n=5), (▼) OT-I cell transfer alone (n=3), (●) MPyV infection alone (n=3). (B) Serum creatinine at day 30 or time of death and (C) viral load in kidneys at day 30 or time of death. . (The number of data points per group is decreased from (A) due to mouse death before samples could be obtained.) Dots represent individual mice. Dashed lines indicate limits of detection. (D) Representative

histology. OT-I cell transfer and viral infection together result in a greater degree of histologic damage than either condition alone. (H&E staining, original magnification x400)

Figure 3: Anti-viral OT-I cells and MPyV are insufficient to cause rejection. B6 donor kidneys were transplanted into B6 recipients with bilateral nephrectomy. Donors were infected 3 days before transplantation .Recipients were passively immunized with VP-1 antibody 2 and 1 days before transplantation.. OT-I cells were transferred on days 0 or 1 post-transplantation. (A) Survival. (■) OT-I cell transfer and MPyV.OVAI infection, (▼) OT-I cell transfer alone, (●) MPyV.OVAI infection alone. n = 3-4. (B) Serum creatinine and viral load in kidneys at day 21. Dots represent individual mice. Dashed lines indicate limits of detection. (D) Representative histology. OT-I cell transfer and viral infection together result in infiltration and subclinical histologic damage. (H&E staining, original magnification x400)

Figure 4: Viral infection lacking kidney tropism does not recapitulate the PVAN phenotype seen in MPyV infection. B6 mOVA donor kidneys were transplanted into B6 recipients with bilateral nephrectomy. Mice were given OT-I cells and infected on day 1 post transplantation. Mice were infected with either MHV-68 or MPyV on day 1 post-transplantation. MPyV data is from Figure 2 and is shown for comparison. (A) Survival. (●) MHV-68 infection, (▼) MPyV infection. n = 4. (B) Serum creatinine at day 30. Dots represent individual mice. Dashed line indicates limit of detection. (C) Representative histology at day 30. OT-I cell transfer and MHV-68 infection result is subclinical damage with significant infiltration. (H&E staining, original magnification x400)

Figure 5: Immunosuppression improves survival. C3H donor kidneys were transplanted into B6 recipients with bilateral nephrectomy. Mice were infected by MPyV on day 1 post transplantation. Mice were injected with 60 ug tacrolimus s.c. daily for 40 days, starting at day 0. (A) Survival. (■) Tacrolimus treatment, (▼) No treatment (shown from previously published work (Albrecht et al, manuscript accepted) for comparison). n = 8. (B) Serum creatinine and (C) viral load in kidneys. Short-term survival indicates mouse death and measurement at 11-19 days; long term survival indicates measurement at 40 days. . (The number of data points per group is decreased from (A) due to mouse death before samples could be obtained.) Dots represent individual mice. Dashed lines indicate limits of detection. (D) Representative histology. Samples from the lethal conditions (no treatment and short-term survival) show a higher degree of histologic damage than those from the non-lethal long-term survival group. (H&E staining, original magnification x400)

Figure 6: A proposed model describing the mechanism of PVAN-mediated kidney allograft rejection. As we have demonstrated, direct viral cytolysis from polyomavirus infection alone is insufficient to cause irreversible graft injury in mice. Likewise, it is well established in the literature that mice tolerate an allogeneic kidney transplant, suggesting that alloimmune-mediated injury alone is also insufficient for acute kidney rejection. However, when this alloimmune injury encounters the polyomavirus-induced inflammation of the kidney, the response is boosted and the kidney is now rejected. This model can also be used to explain the seemingly paradoxical results of altering immunosuppressive regimens in the murine model when compared to the human clinic. In humans, reduction of immunosuppression allows for recovery of the memory anti-PyV response. The recovery of this response controls the viral

infection, lowering or blocking virus-induced inflammation in the kidney. With less viral inflammation, the alloimmune response cannot be sufficiently boosted to cause clinical rejection of the graft. In contrast, mice in the PVAN model are naïve in regards to PyV exposure. Therefore, increasing immunosuppression in mice simply increases suppression of T-cells. Correspondingly, there is a reduction in the alloimmune response, which is now insufficient (even when boosted) to induce failure of the allogeneic kidney.

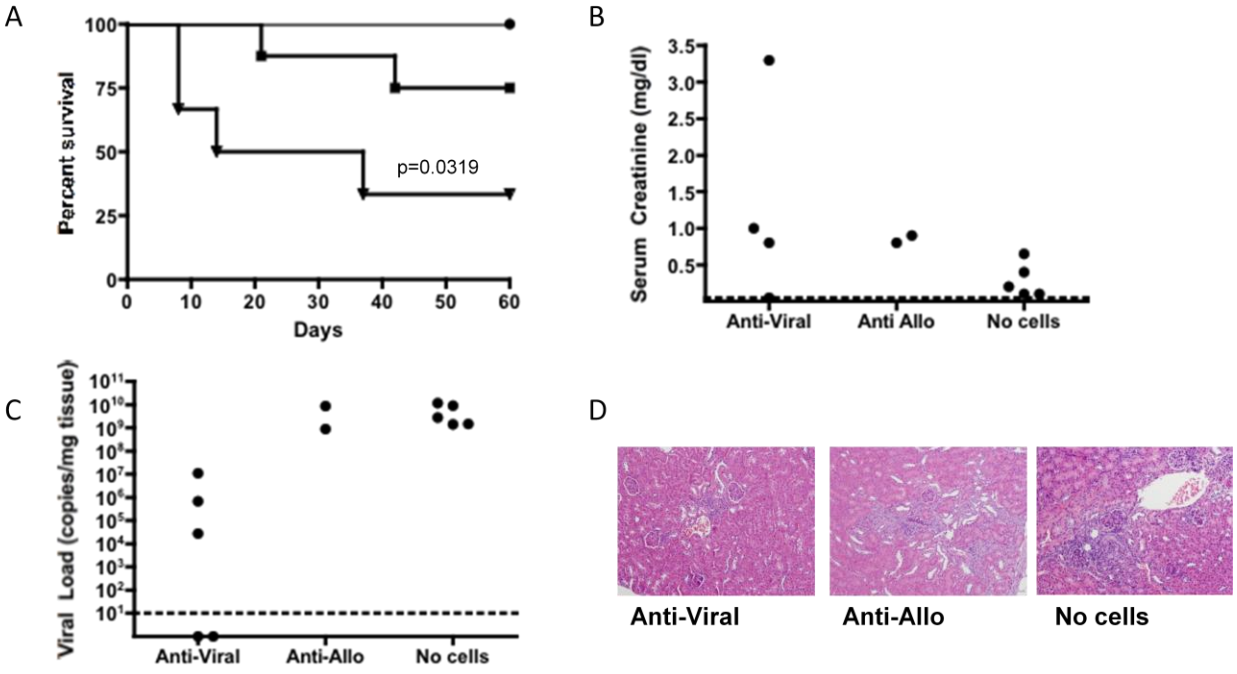
A
Percent survival
100
75
50
25
0
0
10
20
30
40
50
60
Days
p=0.0319
B
Serum Creatinine (mg/dl)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Anti-Viral
Anti Allo
No cells
C
Viral Load (copies/mg tissue)
Anti-Viral
Anti-Allo
No cells
D
Anti-Viral
Anti-Allo
No cells

Figure 1

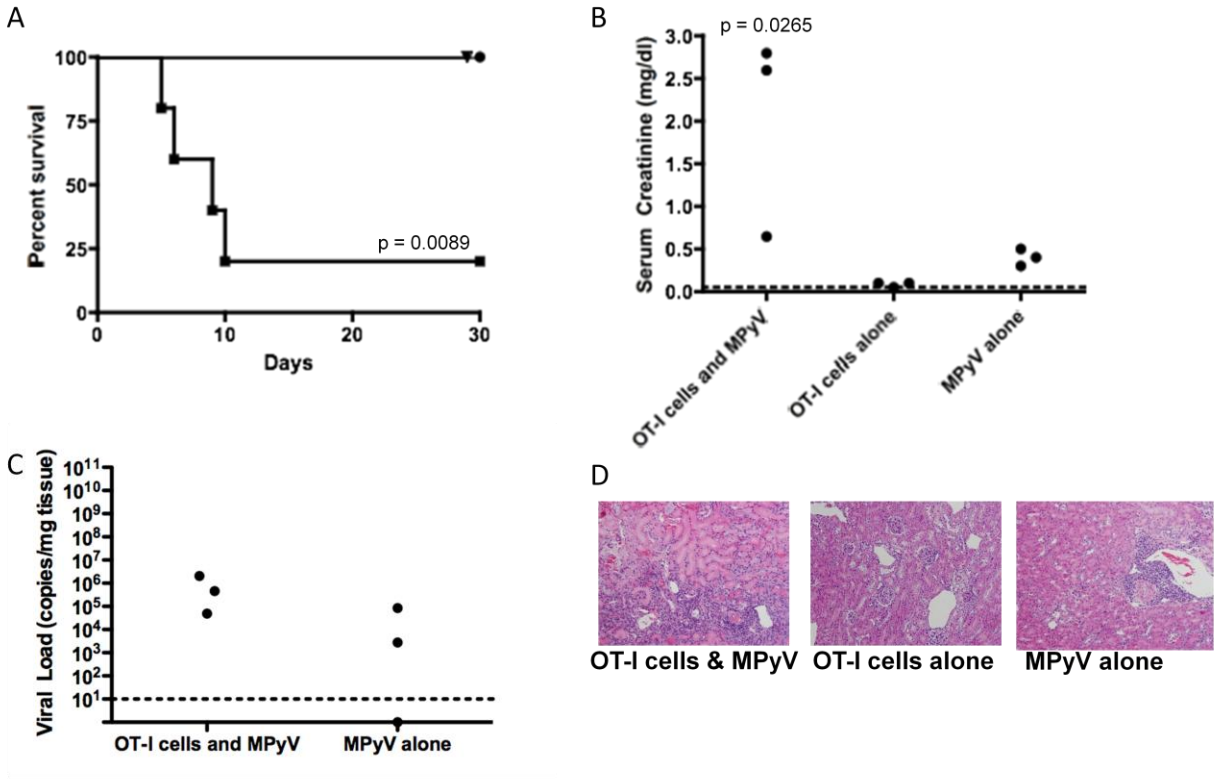
A
Percent survival
100
75
50
25
0
0
10
20
30
Days
p = 0.0089
B
p = 0.0265
Serum Creatinine (mg/dl)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
OT-I cells and MPyV
OT-I cells alone
MPyV alone
C
Viral Load (copies/mg tissue)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
OT-I cells and MPyV
MPyV alone
D
OT-I cells & MPyV
OT-I cells alone
MPyV alone

Figure 2

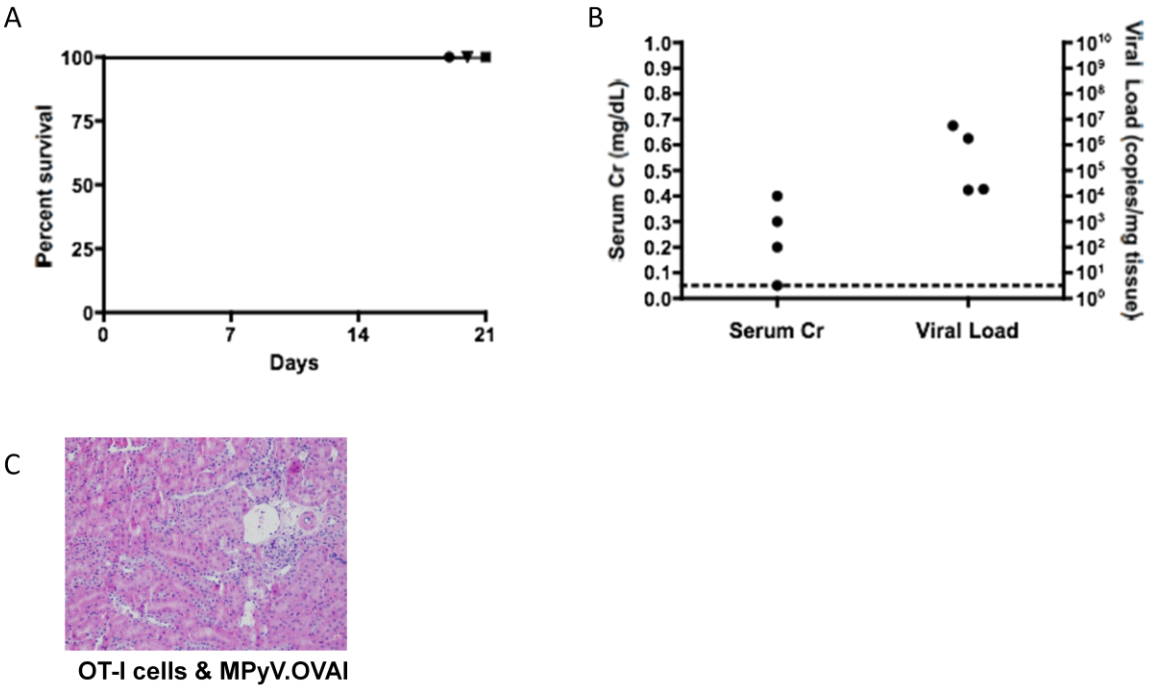
A
Percent survival
100
75
50
25
0
0
7
14
21
Days
B
Serum Cr (mg/dL)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Viral Load (copies/mg tissue)
Serum Cr
Viral Load
C
OT-I cells & MPyV.OVAI

Figure 3

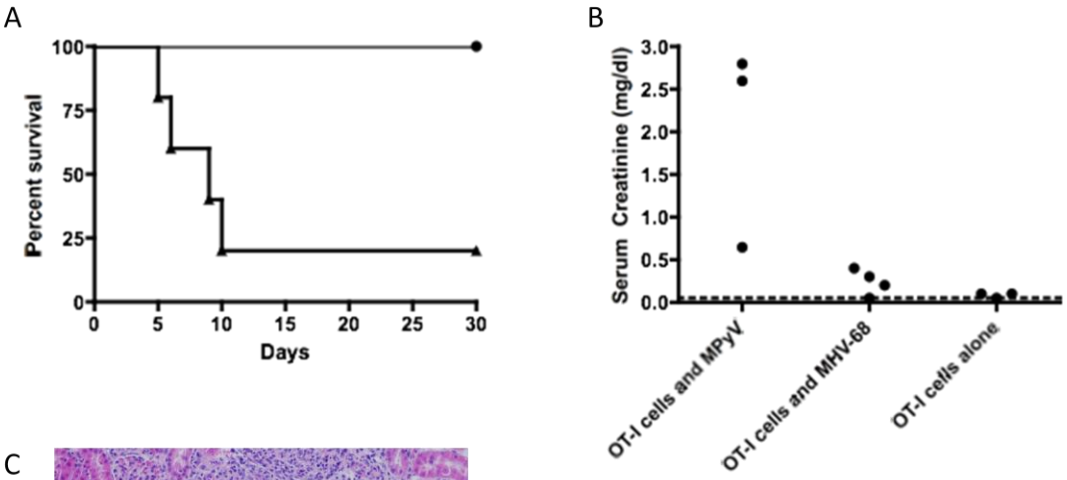
A
100
75
50
25
0
Percent survival
0
5
10
15
20
25
30
Days
B
Serum Creatinine (mg/dl)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
OT-I cells and MPyV
OT-I cells and MHV-68
OT-I cells alone
C

Figure 4

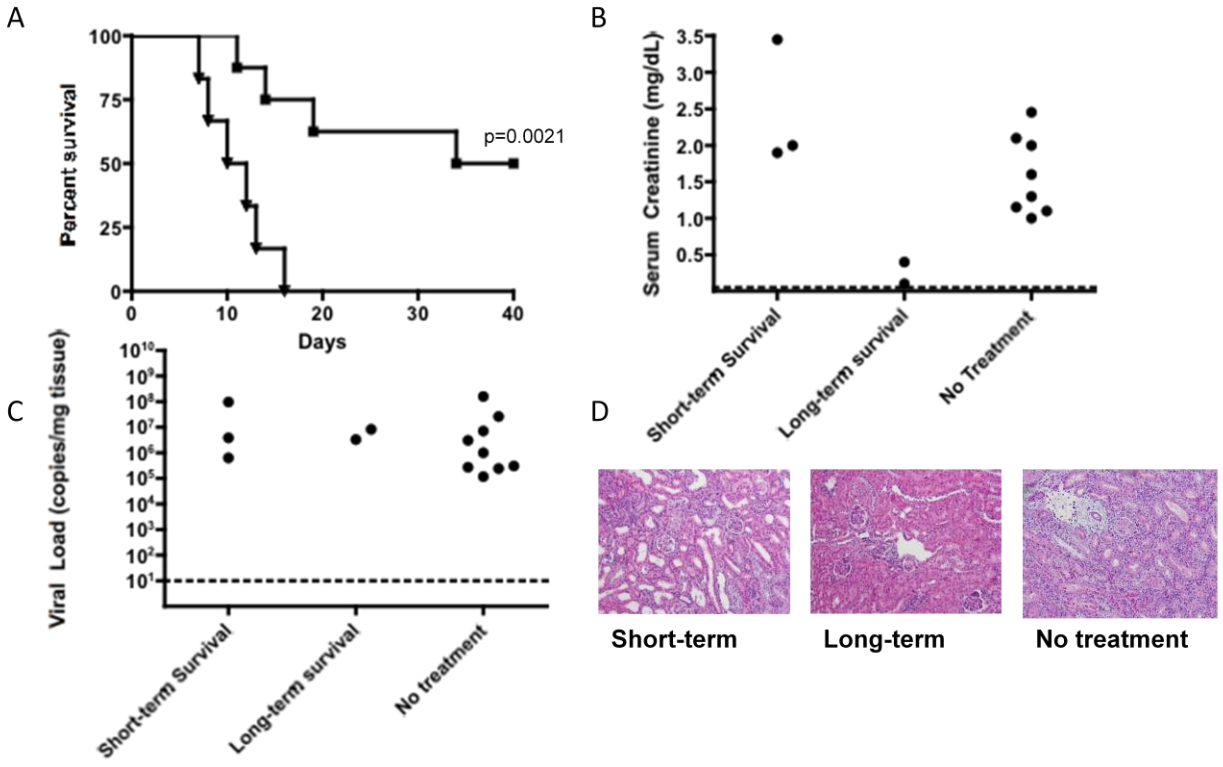
A
Percent survival
100
75
50
25
0
0
10
20
30
40
Days
p=0.0021
B
Serum Creatinine (mg/dL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Short-term Survival
Long-term survival
No Treatment
C
Viral Load (copies/mg tissue)
Short-term Survival
Long-term survival
No treatment
D
Short-term
Long-term
No treatment

Figure 5

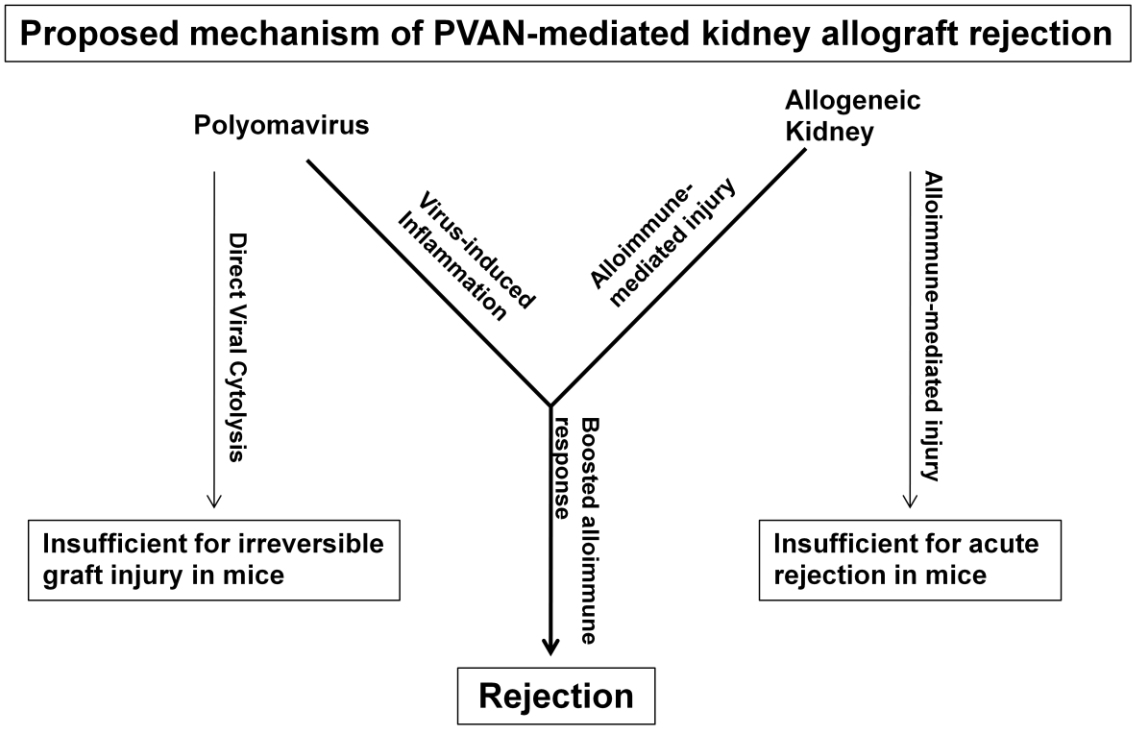
Proposed mechanism of PVAN-mediated kidney allograft rejection
Polyomavirus
Allogeneic Kidney
Direct Viral Cytolysis
Virus-induced Inflammation
Alloimmune-mediated injury
Alloimmune-mediated injury
Boosted alloimmune response
Insufficient for irreversible graft injury in mice
Insufficient for acute rejection in mice
Rejection

Figure 6

Chapter IV:

Discussion

Interest in human disease caused by polyomaviruses has increased markedly over the last 30 years. Although mouse polyomavirus (MPyV) was first discovered in 1953 (1, 2), for many years it was primarily studied due to its link to tumor formation and cancer (3). It was not until 1971 that the first two human polyomaviruses were discovered, named JC virus (JCV) and BK virus (BKV) after the initials of the first two patients in which they were identified (4, 5). Both these viruses were discovered in the context of human disease: BKV from the urine of a Sudanese patient with a ureteric stenosis, and JCV from glial cell cultures from the brain of a patient with Progressive Multifocal Leukoencephelopathy (PML). However, pathogenic events from both of these viruses were initially thought to be fairly rare. This perception began to change in the 1980s, when immunodeficiency due to AIDS caused a drastic increase in the number of PML cases (6). Later, in the mid 2000s, PML was linked to several monoclonal antibody therapies designed to inhibit T cell trafficking into the brain (7). Both of these events caused of surge of interest in PML and JCV (8). Similarly, BKV had been detected in the urine of renal transplant patients in the 1970s, 1980s, and early 1990s, but this viruria was initially thought to be associated with transient graft dysfunction at worst (9). It was not until 1995 that PVAN was first diagnosed, after a needle biopsy was taken from a renal transplant recipient suspected of acute rejection (10). In subsequent years, more cases were reported (11-14), and the threat of PVAN (perhaps fueled by more efficacious immunosuppressive agents) rekindled an interest in BKV. Finally, study of human polyomaviruses has been given a boost in recent years, with the 2007-2008 discoveries of three new human polyomaviruses. Karolinska Institute virus (KIV) and Washington University virus (WUV) were identified from patients with respiratory infections (15, 16), while Merkel cell polyomavirus (MCV) was identified in patients with Merkel cell carcinoma (MCC), an aggressive skin cancer (17). For over 35 years, JCV and BKV

were the only known human polyoma viruses; multiple sources agree that the concentrated discovery of these three new pathogens has revitalized interest in this family of viruses (18, 19).

Since PVAN was not identified in the literature until 1995, much of the research on BKV has been done over the past 15 years. Over that time, we have managed to learn enough about the virus and disease to lower the estimated incidence of graft loss from from 40-60% to 10% or less (20-22). Nonetheless, much remains unknown about the pathogenesis of PVAN. BKV is ubiquitous, and we can safely assume that the vast majority of adult transplant recipients have been exposed to the virus during childhood (23). Nonetheless, only a portion of the transplant recipients will exhibit BKV viruria (30-50%), BKV viremia (13-22%), or PVAN (1-11%), despite similar immunosuppressive regimens (24-26). Also, although PVAN has been reported in a variety of other organ transplants and conditions that require immunosuppression, (27-30), these events are rare, suggesting the importance of factors relating to the transplanted kidney (25). Several risk factors have been identified for PVAN, such as age, gender, viral coinfection, and immunosuppressive regimen, but many others remain controversial (9, 31). Even the role of BKV-specific immunity remains unclear. Humoral immunity alone is insufficient to prevent the onset of PVAN, but recent evidence indicates that antibodies may have a partially protective effect (32-34) . BKV-specific T cell immunity successfully controls viral load but may also be immunopathogenic, implicating it as "both friend and foe," (24, 35-37) . The establishment of an animal model of PVAN (38) allows us to address many of these controversies and questions from an angle that was not previously available. To our knowledge, this is not only the first animal model of PVAN, but the first animal model of any human disease caused by a polyomavirus.

The studies reported herein address a number of clinical and mechanistic questions about PVAN, while simultaneously refining the model. First, we chose to investigate clinical variables relating to disease. We found that the timing and source of infection were key factors in predicting the severity of disease, with 100% of acutely infected kidney recipients rejecting their graft. Decreasing the size of the initial viral inoculum also prevented the onset of mouse PVAN. Interestingly, we found no correlation between the degree of MHC matching or of ischemia/reperfusion injury (IRI) and the severity of disease, despite divided opinions in the literature (34, 39, 40). Most significantly, we found that viral loads in the kidney, ranging from very low (undetectable) to extremely high (up to 10^{11}), did not correlate with allograft injury or survival. This implied that the mechanism of PVAN-mediated injury is not a direct consequence of viral cytopathology, but requires interaction with an intact host immune system. These results are presented in Chapter 2.

The research outlined in Chapter 3 provide a more mechanistic route of investigation, attempting to ascertain whether the host immune system contributes to PVAN via its anti-viral or anti-allo response. These experiments take full advantage of the animal model, utilizing the genetically modified lymphoid deficient (aly/aly) and transgenic ovalbumin mouse strains. Work done in both these strains supports the same conclusion, that the predominant mechanism of allograft rejection in PVAN is an augmented alloimmune T-cell response. In line with this prediction, addition of immunosuppression into the mouse model results in improved allograft function and survival.

In interpreting this mouse model, and in the possible future extrapolation of results to the human transplant clinic, there are three caveats to consider. The first is that immunocompetent mice do not spontaneously and acutely reject allogeneic kidney transplants. The second is that

our model requires an acute infection by MPyV. The third is that recipient mice in this model are not immunosuppressed. These three caveats will now be addressed individually.

First, it has been long known that mice will spontaneously accept allogeneic kidneys (41). Interestingly, this is not true of all transplants; other organs, such as the heart and skin, require stringent MHC matching (42-44). Why kidney transplants in mice can tolerate so much more plasticity is a worthy question in itself, which may provide insight useful in the field of transplantation. Importantly, our model could be used in the future to examine this very issue, as acute infection by MPyV to an otherwise tolerated allogeneic kidney "breaks" this tolerance. By studying the various changes associated with acute MPyV infection, we may gain applicable knowledge about the tolerance of allografts. Indeed, preliminary data from our group in this animal model suggests that PD-L1 expression by tubular epithelial cells of the allograft and PD-1 expression by kidney infiltrating T cells varies per transplantation conditions (J. Albrecht and Y. Dong, data not shown). As the PD-1/PD-L1 pathway is an important negative regulator of the immune system (45) and has been implicated in tolerance of other organs (46-48), it is a distinct possibility that it is involved in the tolerance of kidney allografts. Future studies in the mouse model may reveal other molecules, pathways, and cell types involved in tolerance, with possible extrapolation to human transplantation.

The second caveat of the model system is its requirement for acute MPyV infection. In order to observe 100% rejection and death, MPyV infection must occur close to the time of transplantation. For the sake of consistency within the model, we have been infecting the recipient at day 1 post-transplantation, but we have obtained identical results when infecting the kidney donor three days prior to transplantation (J. Albrecht, unpublished observations). Most likely reflecting differences between MPyV and BKV, as well as between mouse and human

kidney transplantation, this is one area where the mouse model of PVAN differs significantly from clinical human BKVN. With the vast majority of adults exposed to BKV, most adult kidney transplants are from exposed donors to exposed recipients. Indeed, the human scenario most closely resembling the animal model is in pediatric transplantation. Two studies have found that 30% and 44% of pediatric kidney recipients were seronegative for BKV at the time of transplantation (49, 50), with the vast majority receiving a kidney from a seropositive donor. We know that these seronegative children are at increased risk for developing PVAN (49, 51), and that the course of their disease resembles acute BKV infection and not reactivation. What is not known is whether primary BKV infection or viral reactivation is associated with a worse outcome (52). Based on the fact that persistent infection in our model was associated with better renal function and survival than acute innoculation, our model may support the conclusion that primary PyV infection is associated with a worse outcome. If this conclusion can be confirmed in the clinic, it may someday lead to donor and recipient BKV screening for pediatric renal transplant patients.

The final caveat of the system is the lack of immunosuppression. Interestingly, but consistent with our other results, we found that the addition/increase in immunosuppression actually decreases the incidence of PVAN. What makes this finding most surprising, and what may prevent some clinicians from accepting this model and result, is that PVAN is largely regarded as a disease of over-immunosuppression (53). The discussion in Chapter 3 is extensively devoted to explaining this paradoxical result. What it ultimately boils down to is the difference between an acute infection (to which the mouse recipient is naïve) and a persistent smoldering infection (to which the human recipient possesses a memory T-cell response). The obvious, and perhaps most clinically interesting question, is whether an increase in

immunosuppression could ever be used as a therapy to PVAN. First of all, there are many barriers to the sheer practicality of such a proposal, such as the nephrotoxicity of most immunosuppressive agents and the co-existence of other opportunistic infections like CMV. But let us assume these barriers can be overcome. If BK virus itself is insufficient to cause PVAN (as our results with MPyV in Chapters 2 and 3 indicate), and if we assume that an increase in immunosuppression essentially eliminates the host immune response, it follows that there would be no way to mediate disease and that symptoms of PVAN would resolve. While this total immune ablation does seem highly philosophical and potentially unrealistic, the alloimmune mechanism proposed in Chapter 3 suggests that we need only selectively eliminate the alloimmune response, or spare/reintroduce the anti-viral response, in order to combat disease. One can much more easily imagine an immunosuppressive therapy that selectively targets anti-allo cells, or an adoptive transfer therapy that reintroduces anti-viral T-cells specific for BKV. With potentially new immunosuppressive agents in the pipeline, especially ones that act through novel mechanisms, it is important to think about the treatment of BKV with a scientifically informed open mind.

Over the course of these studies, we were also able to further refine and define the mouse model of PVAN as it relates to human disease. To begin with, we were able to extensively look at the histology in this model, comparing acutely infected mice (with failing allografts due to PVAN) to persistently infected mice (which routinely survive long term). A nephropathologist, blinded to the experimental conditions, found that the acutely infected mice displayed a phenotype consistent with rejection and PVAN. Features consistent with PVAN, such as crescents (54, 55) and a rare viral inclusion were also identified. In attempting to directly visualize infection by immunohistochemistry, we published what we believe to be the first

images of MPyV viral inclusions taken from a kidney in vivo. (MPyV inclusions are not easily detectable like those from BKV; the commercially available antibodies for detection do not cross-react.)

We also wanted to confirm that this phenotype was not a generalized consequence of acute viral infection. Acute infection with MHV68 indicated that a non-nephrotropic virus was unable to recapitulate the PVAN phenotype, and future work will serve to refine the model even further. For example, a follow up experiment to the MHV-68 infection should utilize a virus that is capable of infecting the kidney. Results from this experiment will indicate whether the PVAN phenotype is specific to polyomaviruses, or whether it is a general consequence of renal infection and/or inflammation.

Of course, another goal in refining the system would be to get it to closely mirror the clinical course of PVAN in humans. In this case, we would want to begin with an allogeneic kidney transplant in mice that is rejected in the absence of immunosuppression. The literature has identified some possible MHC mismatches that would result in rejection (41, 56), although changing mouse strains alters the MHC haplotypes and limits the MPyV-related tools available. In addition, we have found that C57BL/6 PD-L1$^{-/-}$ allogeneic kidneys are promptly rejected by immunocompetent C3H mice, and it is possible that kidneys from other knockout mice may be spontaneously rejected as well. If we were to then add immunosuppression on top of this framework, we would hope that it would restore tolerance. Finally, infection with MPyV would have to break the tolerance. One potential complication from this hypothetical model does arise, since the immunosuppression necessary for tolerance may prevent the MPyV from causing a phenotype. In that case, the immunosuppression would need to be titered down to a point where

it allows acceptance of an allogeneic kidney in the absence of virus, but is ineffectual in the presence of MPyV.

This mouse model of PVAN has the potential to examine a large number of questions and issues surrounding BKVN and transplantation. One area we are particularly interested in is the mechanism by which the alloimmune response is boosted in the presence of MPyV. Based on the failure of MHV68 to recapitulate the MPyV results, we believe that the anti-allo response is boosted in the microenvironment of the kidney, most likely due to local inflammatory mediators. Future experiments in this model will involve characterizing both the kidney infiltrating lymphocytes (KILs) and the kidney microenvironment. To characterize the KILs, T cells can be purified from the allograft of an acutely infected recipient and characterized by flow cytomtery. This can be compared to the KILs from the allograft of a persistently infected recipient, to the KILs from the allograft of an uninfected recipient, or to the KILs from the isograft of an acutely infected recipient. Phenotypic markers of function, exhaustion, activation, trafficking, and recruitment can be examined, as well as absolute number of cells. In addition, functional assays can be performed on the anti-allo KILs, using allo-antigen stimulated production of IFNγ, IL-2, and/or TNFα as readouts.

Although more difficult, we can also assay and characterize the microenvironment of the kidney. First of all, using established protocols, various cell types of the kidney can be identified (57, 58). Renal tubular epithelial cells (TECs) will be of special interest, since they have been implicated in both polyomavirus infection and renal transplantation (59, 60). The TECs can be characterized by immunohistochemistry or by flow cytometry for phenotypic markers or molecules associated with infection. In addition, to help identify local inflammatory mediators of the kidney, gene expression microarrays may be useful. Once potential targets have been

identified, genetic or immunogenic tools can be used to manipulate the appropriate molecules within the model of PVAN. For example, knockout mice may be used, or perhaps the protein of interest is commercially available and can be administered exogenously. The most scientifically rigorous and fulfilling experiments would identify one molecule (or lack thereof) that allows for survival of an acutely infected allograft, or, conversely, that causes rejection of an uninfected allograft (or of an acutely infected isograft).

Countless other issues can be examined in the mouse model of PVAN. We can alter the strains of transplanted mice to result in different MHC-mismatching, utilize the vast array of knockout mice available, mutate the genome of the virus, administer drugs (such as immunosuppressants or anti-virals) to transplant recipients, co-infect with other pathogens, or manipulate the recipient immune system. The greatest limiting factor for all these experiments is the time and cost associated with kidney transplantation in mice. Nonetheless, as demonstrated by the data presented herein, the mouse model of PVAN is a powerful tool with which to investigate the pathogenesis of disease. Future studies will continue to use this model, with the ultimate goal of improving clinical outcome for renal allograft recipients and for all patients undergoing transplantation.

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