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Measures of Viral Protective Immunity as Indicators of Alloimmunity in Renal Transplantation

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B.S.E., University of Pennsylvania, 2006

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A thesis submitted to the Faculty of the
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Abstract

Measures of Viral Protective Immunity as Indicators of Alloimmunity in Renal Transplantation
By Jonathan M. Beus

Solid organ transplantation is an important life-sustaining intervention for individuals with organ failure. Despite the significant benefits of replacing a failed organ and considerable improvements in transplantation that have occurred over the past decades, transplantation is associated with substantial morbidity due to the requirement for chronic immunosuppression. Despite the narrow therapeutic index of many immunosuppressive therapies, objective measures of therapeutic immunosuppression are lacking. One potential approach to monitoring immunosuppressive therapy is to exploit measures of viral protective immunity. Significant overlap exists in the biologic mechanisms that mediate viral protective immunity and those that effect alloimmunity. Sensitive measures of viral immunity are already available in the form of assays to detect virus-specific antibodies and polymerase chain reaction-based assays to detect circulating virus. The high sensitivity of such assays and the similarities between viral immunity and alloimmunity suggest that these tools could direct the clinical management of post-transplant immunosuppression. To explore this possibility, this thesis describes a retrospective cohort analysis of the outcomes of two independent cohorts of renal transplant recipients. An association is identified between decreased rates of acute rejection and low or high Epstein-Barr Virus (EBV) DNA load values. This pattern is interrupted at intermediate EBV-load values, likely as a result of over-aggressive changes in immunosuppression in response to viral reactivation. Additionally, patients with a specific pattern of humoral immunity to EBV demonstrate a significantly lower rate of acute rejection. This finding is consistent with similar studies in the autoimmune literature and raises the possibility of identifying a subset of patients who may benefit from lighter immunosuppressive regimens without sacrificing graft function. In summary, although future studies are likely to be required, this thesis provides evidence that measures of viral protective immunity may prove beneficial in the management of post-transplant immunosuppression.

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Introduction

For patients with end-stage organ failure, solid organ transplantation is an important life-prolonging therapy (1, 2). Despite the significant benefits of renal transplantation, kidney transplant recipients still face many challenges after transplantation, primarily related to the need for chronic immunosuppression. Immunosuppression is required to prevent the transplant recipient's immune system from attacking and destroying the "foreign" organ, but many immunosuppressive drugs both interfere with the immune system's normal response to infectious pathogens and have adverse effects unrelated to their direct effects on the immune system, such as metabolic, cardiovascular, and even nephrotoxic effects (3-7). The delicate balance between providing sufficient immunosuppression to protect the transplanted graft and minimizing drug-related adverse effects is highlighted by the fact that while advances in immunosuppressive drugs and regimens over the last several decades have significantly reduced the incidence of acute rejection episodes, long-term outcomes for renal transplant recipients have remained relatively constant (8, 9).

In light of the significant morbidities associated with and narrow therapeutic windows of many commonly used modern immunosuppressive agents, it is desirable to minimize immunosuppression while maintaining a functioning graft. At present, no objective measure of immunosuppression is available for clinical practice. Without an ongoing measure of immune functions, clinicians may not have an indication to adjust immunosuppression until clinical signs and symptoms become apparent. An objective measure of aggregate immunosuppression would allow for adjustement of non-optimal immunosuppression prior to the development of clinical harm to the patient. Several approaches to measuring aggregate immune function are in development, but have not yet achieved clinical utility (10-12). Importantly, the majority of the assays in development either rely on *ex vivo* manipulation of T cells, potentially leading to artificial measurements, or depend on the detection of substances produced during an immune response and are, thus, likely to be nonspecific for alloresponses and detectable only after the

initiation of a rejection episode.

A promising alternative approach to immune monitoring is based on the recognition that significant overlap exists between the primary mechanisms that regulate protective immunity against viral pathogens and those that play an important role in allograft rejection. In particular, the immune response to viral infections is predominated by T cell effects (13) and most transplant rejection is also primarily mediated by T cells (14). The significant overlap between viral protective immunity and alloimmunity is demonstrated by the clinical observation that viral infections including those mediated by cytomegalovirus (CMV), Epstein-Barr virus (EBV), and polyoma BK virus (BK) are significant sources of morbidity during post-transplant immunosuppression (15, 16). These findings suggest that a patient's risk of rejecting their transplanted graft at any point is inversely related to their concurrent ability to control viral infections.

The majority of common viral infections after transplantation occur as the result of increased replication of chronic infections or reactivation of latent infections. As latent (CMV and EBV) or chronic (BK) infections, these viruses are present in many healthy individuals, but are controlled at low, non-pathogenic levels by competent immune systems (17-20). In transplant recipients and other immunosuppressed individuals, suppression of the immune system allows these viruses to reactivate and/or increase replication. While this certainly leads to an increased risk of developing clinical syndromes related to viral infection, patients may experience viremia at levels that are higher than in normal, healthy patients, but not high enough to cause symptoms (sub-clinical viremia). In this case, modern polymerase chain reaction (PCR)-based assays are sensitive enough to detect low levels of viremia. Due to the relatively high sensitivity of PCR, the detection of sub-clinical viremia in a transplant patient may serve as a marker of over-immunosuppression. Importantly, the decreased risk of rejection would be indicated by the viremic episode, not the result of it.

Previous reports in lung transplant recipients suggested that patients with EBV viremia

were at decreased risk for severe rejection episodes (21). The goal of the present study was to explore whether this effect holds true in kidney transplant patients and to understand the magnitude of the reduction in acute rejection risk following viremia. To address this question, the multivariable time-to-event model known as the extended Cox regression model was chosen. This model allows for the fact that rejection episodes occur at any time post transplantation, but that not all patients experience an episode of acute rejection (right-censored data). Additionally, as a multivariable model, it allows for the adjustment for other variables such as those described in the background section, below. Finally, it allows for the variables in the model to vary with time which is important in that patients may have viremia at some points, but not at others.

Another approach to exploring the relationship between immunity to EBV and alloimmunity is by assessing the relationship between acute rejection rates and the presence of pre-transplant EBV antibodies. While it seems that viral antibodies should be fairly specific markers for viral immunity, the failure to develop antibodies to both common EBV antigens (viral capsid antigen (VCA) and Epstein-Barr nuclear antigen (EBNA)) may represent a broad measure of immune function. Previous studies have identified that patients with several autoimmune diseases including multiple sclerosis and systemic lupus erythematous have higher titers, relative to controls, of antibodies to the EBNA antigen (22, 23). This suggests that higher titers of EBNA antibodies are associated with more aggressive immune function, to the point of autoimmunity, and possibly that lower titers of EBNA antibodies might be associated with less aggressive immune function. Similarly, abnormal patterns of EBV serology (including VCA+EBNA-) confer an increased risk of both EBV- and non-EBV- associated neoplasms, suggesting that EBV serology broadly reflects immune competence (24, 25). In the present study, we ask whether EBNA serology is associated with alloimmunity by Kaplan-Meier analysis of time to acute rejection stratified by EBNA serology and by extended Cox regression models to control for variables that are already known to be associated with acute rejection.

Background

Need for Immune Monitoring and Current Approaches

At present, post-transplant renal function is primarily monitored using serum creatinine and assessment of peripheral drug levels. While elevated serum creatinine can suggest rejection, increases in serum creatinine are relatively non-specific and can be affected by a variety of factors including BK nephropathy, elevated calcineurin inhibitor levels, recurrence of the original disease necessitating the transplant, or even hypovolemia. Additionally, a rise in serum creatinine suggests that the acute rejection process has already begun to affect the kidney such that renal impairment occurs. Thus, monitoring of serum creatinine levels is informative, but leaves much to be desired in the accurate diagnosis of patients post-transplant. Therapeutic drug monitoring (TDM) is also commonly used for immunosuppressive agents including cyclosporine, rapamycin, and tacrolimus. While TDM helps to minimize toxicity, there is controversy over how well single-time point measurements of drug levels reflect the effects of the drugs on the immune system, particularly for CNIs (12, 26-28). The discordance between single-time point measurements and pharmacodynamics likely occurs through variations in drug metabolism, sensitivity to drug effects, and the immunogenicity of the donor-recipient pairing.

To circumvent the problem of varying responses to immunosuppressive medications by patients, a variety of biomarkers have been pursued as pharmacodynamic measures of immune function. One approach has been the measurement of mRNA transcripts of genes related to immune function in urine and blood. Several investigators have identified associations between acute rejection and the expression levels of genes representing lymphocyte activation or trafficking (11, 29, 30). While these results are promising, the receiver-operator characteristics of single gene measurements to-date are generally not sufficient to guide clinical decision making. Other investigations have focused on identifying global gene expression profiles from microarray analyses (31, 32). Due to the broad functions assessed by these methods, they offer the possibility of increased specificity for the discrimination of varying inflammatory causes of graft

dysfunction (such as cellular rejection, antibody-mediated rejection, and BK nephropathy). Unfortunately, the broad coverage of microarray analyses also leads to increased complexity of analysis and problems obtaining large enough sample sizes to support analyses of the large multitude of analytes. At present, broad microarray analyses have demonstrated potential, but do not appear to be ready for clinical implementation (30, 31).

Other approaches to immune monitoring have included detection of immune mediators (e.g. cytokines) in peripheral body fluids, measurement of anti-HLA antibodies in the peripheral blood, *ex vivo* measures of T-cell reactivity, and the assessment of peripheral blood cellular phenotypes (11, 30, 32-35). Elevated levels of several urine chemokines or sHLA-DR and increased quantities of sCD30 in the blood have been associated with acute rejection (11, 30, 35, 36), but these changes may not occur until a rejection process is already underway. The detection of anti-HLA antibodies, particularly those specific for donor HLA antigens is associated with poor long-term outcomes (34, 35), but this addresses just one of several possible immunologic processes that is concerning. As a potential complement to the detection of anti-HLA antibodies, T-cell reactivity has been assessed by *ex vivo* stimulation followed by detection of either IFN-γ production (ELISPOT) or metabolic activity by intracellular ATP production (iATP) (11, 32, 33). While these assays demonstrate some utility, they may be limited by the fact that their *ex vivo* stimulation represents the potential for T-cell reactivity rather than actual *in vivo* activity. Additionally, the iATP assay is limited to CD4 T-cells, thus missing the relevant effects of CD8 T-cells. Finally, additional investigations have associated higher levels of certain cellular phenotypes including regulatory T-cells (Tregs) and transitional B-cells with patients who are able to withdrawal immunosuppression without complications (11, 35). While these cellular phenotypes may help identify patients who might benefit from immunosuppressive withdrawal, it is currently unclear whether they can guide more discrete changes in immunosuppressive medications.

While investigations into biomarkers for transplantation are promising, no approach has

yet reached clinical utility (32, 34, 35). Additionally, due to the varied pathologies and etiologies in transplanted kidneys many investigators recognize that a clinically-applicable approach is likely to require the integration of information from several different approaches (10, 28, 34). EBV viral load monitoring may be an important component of such a combined approach. The promising characteristics of an immune monitoring approach based on EBV viral load include the fact that the control of EBV reactivation represents an *in vivo* immune process and that it could reflect aggregate immune function prior to the initiation of an immune response and pathology to the graft.

Current Outcomes in Renal Transplantation

Kidney transplant recipients enjoy quite good early outcomes. In the most recent data, 1-year graft survival ranged from 91% for recipients of deceased donor kidneys to 96.3% for recipients of living donor kidneys. With time, grafts continue to fail and the deceased donor and living donor cohorts diverge at later times with 5-year graft survival rates of 69.3% and 81.4%, respectively (37). The precise causes of individual cases of graft failure have been difficult to define, but include varying contributions of hypertension, recurrence of the original renal disease necessitating the transplant, drug toxicity (particularly from CNIs), infection (including BK virus), and chronic cellular and antibody mediated rejection processes (38).

Although acute rejection can occur at any time, the majority of rejection episodes occur within the first 6 months after transplantation (9). Acute rejection occurs when the recipient's immune system recognizes the transplanted organ as "foreign" based on its non-native HLA expression and initiates an immune response. While the advent of protocol (not-for-cause) biopsies has revealed that an acute rejection process can occur in the absence of clinical symptoms, most commonly, episodes of acute rejection are identified clinically by increased serum creatinine levels. When suspected clinically, acute rejection episodes are confirmed by histopathologic examination of a biopsy from the transplanted kidney. Acute rejection is typically

classified as cellular or antibody-mediated based on clinical and histopathological characteristics, although mixed episodes with components of both cellular and antibody-mediated rejection can occur (39, 40). Cellular rejection, primarily mediated by T-cells, occurs when the recipient's T-cells are activated by recognition of donor alloantigens. The activated T-cells then infiltrate and begin destroying the foreign tissue through local secretion of cytokines, induction of apoptosis, activation of humoral responses, and direct cytoxocity mediated by effectors such as perforin and granzyme (41). Histopathologically, acute cellular rejection is characterized by the infiltration of monocytes and lymphocytes into the tubulointerstitium. More severe forms of cellular rejection are progressively indicated by intimal arteritis and transmural arteritis (39, 40). As its name suggests, antibody mediated rejection is effected primarily by antibodies to HLA molecules and possibly to endothelial antigens. Once bound to their targets in the transplanted organ, the antibodies mediate damage through mechanisms including complement activation (41). Antibody mediated rejection is diagnosed histopathologically by staining for complement component C4d and by the presence of acute tubular necrosis, peritubular capillaritis, glomerulitis, thromboses, or arterial fibrinoid necrosis. Definitive diagnosis of antibody mediated rejection further requires the detection of circulating donor specific antibodies (39, 40).

The treatment for acute rejection depends primarily on the type and severity of the rejection episode. For mild cases of cellular rejection, increased doses of maintenance immunosuppression or a pulse of oral steroids is often sufficient while more severe cases may require high dose intravenous steroids or even several days of anti-thymocyte globulin infusions to deplete T cells. The treatment of antibody-mediated rejection primarily seeks to decrease circulating antibodies to the donor HLA antigens and can include plasmapheresis and/or I.V. immunoglobulin therapies. While improved detection and treatment of acute rejection make it an uncommon cause of graft failure, in the absence of treatment, acute rejection poses a significant threat to the transplanted kidney.

As described above, the primary aim of the present study was not to determine whether

EBV reactivation directly affects alloimmunity, but rather to determine whether EBV load is associated with alloimmunity. As this does not represent a causal relationship, the concept of confounding does not apply. As the goal is to use the proposed assay without respect to other patient factors, interest lies in the association between EBV and acute rejection rates without adjusting this association for additional patient factors. Nonetheless, if the association of acute rejection with EBV simply reflected an effect that was already known to be caused by a factor that is more easily measured (e.g. demographic factors), an assay based on viral load would increase cost with no added clinical value. To address this issue, the association between EBV load and acute rejection rates were assessed both with no other factors in the model and with adjustment for commonly available factors known to be associated with renal transplant outcomes.

As discussed below, model adjustments were considered for several factors including human leukocyte antigen (HLA) matching, organ donor type, induction regimen, and demographic variables, among others. The degree of HLA matching between the donor and the recipient is a measure of immunologic similarity between the donor organ and the recipient. Although there are several HLA loci, the HLA A, B, and DR loci are of primary interest in transplantation (42, 43). Each individual has two alleles for each of the HLA loci, so for each locus the number of matches between the donor and the recipient is between 0 and 2. Donor and recipient pairs with more HLA matches are more immunologically similar. As a result, the fewer the number of HLA matches, the greater is the risk of acute rejection and poor transplant outcomes (43, 44).

Another important factor that contributes to the risk of acute rejection is the source of the transplanted organ or donor type. Unlike many other solid organs, the relative redundancy of human kidneys allows for transplantation from living donors. In contrast to deceased donors, kidneys from living donors tend to be healthier given that they have not suffered the trauma of the disease process that resulted in the death of the donor and that they do not require extended

preservation times because living-donor transplants can generally be electively scheduled (45-47). Living donors can be further classified based on their relationship to the patient. Donation from family members including parents, siblings, and offspring is termed living-related donation and is beneficial in that the relationship confers increased genetic and, therefore, immunologic similarity between the donor and the recipient. Donation from living-unrelated donors frequently occurs from spouses, close friends, and in paired-donation scenarios. Living-unrelated donation maintains the benefits of a living donor, but not necessarily the benefits of genetic and immunologic similarities. Based on these factors, patients who receive organs from living donors have a lower risk of rejection than those who receive organs from deceased donors. Furthermore, the risk of rejection is slightly lower for recipients of living-related organs than for living-unrelated organs (48).

Additional factors that are known to contribute to the rate of acute rejection and/or graft failure of kidney allografts are previous history of kidney transplantation, dialysis status at the time of transplant, the type of induction medication used at transplantation, delayed graft function, recipient race, recipient age at transplant, and patient non-adherence with the prescribed medication regimen (42, 43, 45, 49-59). A significant minority of renal transplant recipients are patients who previously underwent a kidney transplant, but experienced failure of their transplanted kidney. In appropriate circumstances, these individuals can benefit from a repeat kidney transplant. As these patients have previously been exposed to a foreign kidney, there is a higher probability that they will have immunologic memory, including T cell memory and pre-existing antibodies against potential recipients. As a result, patients who have previously received kidney transplants may be at increased risk of acute rejection, although newer methods for detecting anti-HLA antibodies in these patients may have significantly decreased this effect in the recent era (52, 53). Whether or not patients require dialysis prior to transplantation and the length of time that a patient spends on dialysis prior to transplantation has also been shown to impact the

risk of acute rejection. This affect has been attributed to the possibility that chronic dialysis results in a generalized inflammatory state in patients (55, 56).

Many kidney transplant recipients receive extra immunosuppressive therapy at the time of transplantation, known as induction therapy, primarily because the risk of rejection is highest early after transplantation. The risk of rejection is, therefore, affected by both whether a patient receives induction therapy and by the type of induction therapy (50, 54, 57). In the present study, patients fell into three possible groups: did not receive induction therapy, received induction therapy with an anti-IL2-receptor antibody (basiliximab or daclizumab), or received depletional induction therapy (ATG or alemtuzumab). In the U.S., recipient race has been reported to be associated with the risk of acute rejection and graft survival, although it is poorly understood what are the relative contributions of biological effects and socio-economic status (46, 58). Some evidence suggests that older recipients, particularly those over 60 have worse graft outcomes, but may actually have lower risk of rejection (43, 49). Finally, the risk of acute rejection is significantly increased for patients who fail to abide by the prescribed immunosuppressive protocol as they often do not receive sufficient immunosuppression to prevent their immune system from responding to the transplanted kidney (51).

EBV Infection in Renal Transplant Recipients

While several chronic or latent viruses are important in transplantation and commonly measured, this initial study was focused on EBV. The primary concern with the common chronic virus polyoma BK virus is that it can infect the kidney and causes problems with kidney function unrelated to acute rejection (15). The occurrence of CMV viremia was excluded as a variable of interest because CMV is thought to have immunosuppressive effects directly as a result of the virus (60). While this complicates the analysis in that immunosuppression can cause CMV viremia and that CMV viremia can further depress the immune system, there is also some suspicion that active CMV infection can augment the immune system's response to an allograft

(foreign transplanted organ) such that the risk of rejection is increased (15). Thus, EBV likely has the most benign direct effects on kidney allografts.

Epstein-Barr virus (EBV) is a gamma-herpesvirus that infects greater than 90% of individuals by adulthood (61, 62). Like the other human herpesviruses, after primary infection with EBV, the virus establishes lifelong latency in the host. Although the virus is never completely cleared, healthy individuals rarely experience clinical disease related to the infection because the immune system maintains effective control over the virus (63). Although clinical disease is rare in healthy individuals, the initial (primary) infection with EBV can cause the mononucleosis syndrome, primarily in adolescents, and latent infection has been associated with several types of neoplasms (61). EBV is more problematic in immunosuppressed individuals, including renal transplant recipients. Patients who have not been previously exposed to EBV at the time of transplant are identified by EBV seronegativity and are at risk of infection from the donor organ or from other typical exposure routes (61, 64). EBV can also be problematic for patients with previous exposure to and, thus, latent EBV infection (EBV seropositive). While the healthy immune system typically controls EBV reactivation adequately, the chronic immunosuppression after transplantation indefinitely places transplant patients at risk of EBV reactivation (63, 65). The primary infection of or reactivation of latent EBV in transplant patients can cause the syndrome associated with infectious mononucleosis, hepatitis, pneumonitis, gastroenteritis, and hematologic pathologies (64). Importantly, uncontrolled EBV reactivation can lead to post-transplant lymphoproliferative disorder (PTLD), a condition associated with significant morbidity (62, 64, 65). PTLD is characterized by the massive proliferation of primarily B lymphocytes that cannot exit the cell cycle due to the EBV viral program (65). The large number of lymphocytes can invade and impair the functions of vital organs including the transplanted kidney (61). Additionally, the proliferation may become neoplastic if not appropriately treated (61, 64). The naiveté of patients who are seronegative to EBV means that they do not have immunological memory for EBV and when combined with the suppression of

their immune system, puts them at much greater risk of PTLD from a primary infection (61, 64, 66). Given the significant differences between primary and secondary EBV infection, the present study was limited to patients with positive serology for EBV at the time of transplantation.

As described above, due to the differences between primary EBV infection and secondary EBV infection, and the additional risk of complications with EBV conferred by a seronegative recipient receiving an organ from a seropositive donor, kidney transplant recipients are tested for latent EBV prior to transplant. Patients are not directly tested for latent EBV, but are rather tested for the presence of antibodies to known EBV antigens. Patients are typically tested for both IgM and IgG antibodies to the EBV antigen viral capsid antigen (VCA) and for IgG antibodies to Epstein-Barr nuclear antigen (EBNA). The typical pattern of EBV serology in patients with primary EBV infection is VCA IgG+, VCA IgM+, and EBNA IgG-. The acuity of the infection is indicated by the presence of IgM antibodies and EBNA antibodies may not be present for several months after primary infection (67). The classic pattern for latent EBV infection includes IgG antibodies to both VCA and EBNA, in the absence of VCA IgM antibodies. Interestingly, some individuals do not develop EBNA antibodies or lose them secondarily to immunosuppression (67). Some clinicians suggest that the absence of EBNA antibodies in the presence of VCA IgG antibodies is indeterminate for latent EBV infection (67), but the presence of VCA IgG antibodies and the understanding that not all individuals develop EBNA antibodies indicates that this pattern is also possibly suggestive of latent infection (68).

The most significant known risk factor for post-transplant EBV replication and PTLD is the transplantation of an EBV seropositive organ into an EBV seronegative recipient. The other significant risk factors are primarily related to the degree of immune suppression and include the type of induction therapy used at transplant, the type of immuosuppressive medication used, and the levels of immunosuppressive medication used (61, 69-71). Patients undergoing induction therapy with depletional agents such as alemtuzumab or anti-thymocyte globulin are at increased risk of EBV viremia relative to patients who undergo induction with an anti-IL2R agent or no

induction at all (61, 64). Additionally, some immunosuppressive agents including costimulation or adhesion blocking therapies increase the risk of EBV reactivation and PTLD (72, 73). Although several antiviral agents show some *in vitro* activity against EBV, there are presently no therapies that successfully treat *in vivo* EBV replication (74).

Methods

Study Design

This is a retrospective cohort study to determine whether EBV load is associated with rates of acute rejection. Data for this study were obtained from two different institutions: Emory University Hospital in Atlanta, GA and the NIH Clinical Research Center in Bethesda, MD. Four hundred ninety-five patients transplanted at Emory between January 1, 2005 and August 31, 2009 as well as 138 patients transplanted at the NIH between 1999 and 2006 met inclusion criteria and were included in the analysis. Data for the Emory cohort were obtained by direct export of information from the primary electronic medical record as well as from specialized transplant records. When necessary, information was obtained by manual review of medical records by J.M.B. Data for the NIH cohort were obtained by direct export from the electronic medical record. Loss to follow up in both cohorts was minimal given the specialized nature of transplant and the need to follow up at the respective transplant center for immunosuppressive management. Patients transferring their care to another transplant center were identified by review of the medical record and were censored from the data set at the time of transfer. Potential subjects with missing information for inclusion and exclusion criteria were excluded from the study. Missing values were allowed for EBV load and their treatment is addressed below.

Null Hypothesis

Primary

H_0: The hazard of acute rejection is not different for renal transplant recipients with active EBV viremia than those without active viremia.

Secondary

H_0: The hazard of acute rejection for renal transplant recipients who are EBNA seronegative at the time of transplantation is not different from that for patients who are EBNA seropositive.

Characteristics of Study Populations

For Both Emory University Cohort and NIH Clinical Research Center NIDDK Cohort

Inclusion Criteria

1) Kidney only (no simultaneous liver, pancreas, etc.) transplant at Emory University Hospital between 1/1/2005 and 8/31/2009 or at the NIH clinical center between 1999 and 2006
2) EBV seropositivity at transplant (for EBV PCR study) defined by the presence of IgG antibodies to EBNA, VCA, or both and negative IgM antibodies to VCA.

Exclusion Criteria

1) History of transplant of another organ
2) Known HIV infection

Primary Outcome Variables

The primary outcome variable is the time to the first clinically significant episode of acute rejection, censored for death, transition to another transplant center, or graft failure. In some cases, the time to first clinically significant acute cellular rejection was used as the outcome variable. For patients in the Emory University cohort, acute rejection episodes were assessed by review of all renal biopsies performed and clinical notes surrounding each biopsy. Given the subjective nature of transplant renal biopsy interpretation, pathologic and clinical diagnoses were recorded separately. Pathologic diagnoses were determined based on histopathologic features and comments recorded by the pathologist. Clinical diagnoses were determined by combining the pathologic interpretation with the managing clinicians' notes, the treatment chosen to respond to the biopsy results, and each patient's clinical course following the biopsy. Except where noted, the clinical diagnosis was used to determine whether a patient had a rejection episode. Clinical diagnoses were additionally classified by whether the episode was consistent with cellular rejection, antibody-mediated rejection, or a combination of cellular and antibody-mediated

rejection ("mixed"). In most cases, pathologic and clinical diagnoses were concordant. While incongruence between the diagnoses was rare, a common scenario leading to discordance occurred when the pathologic diagnosis was interpreted as suspicious for "Borderline" acute cellular rejection in the Banff grading system for renal allograft biopsies(75), but the patient's renal function returned to baseline without significant treatment or modification of the immunosuppressive regimen.

Patients in the NIH cohort were assessed for acute rejection episodes using a similar method of segregating pathologic and clinical diagnoses. Clinical diagnoses were determined by a panel of clinicians caring for the patient.

Covariates and Measurement

Covariates considered in both cohorts of this study included recipient age at transplant, recipient race, donor type, recipient gender, previous history of kidney transplant, HLA matching, induction therapy, and EBV viremia. Recipient age at transplant was considered as a continuous variable. The appropriate form for race in Cox regression model was determined using Martingale residuals as described below. Recipient race was obtained from the medical records of patients and, based on the demographics of both the Emory and NIH cohorts, was classified into "African American or Black", "Caucasian", and "Other". Donor type (deceased, living related, and living unrelated) was obtained from the medical record as that reported at the time of transplant. Recipient gender was obtained from medical records. Information regarding kidney transplant history was obtained from electronic medical records including pre-transplant evaluations, clinic notes, and hospital discharge summaries. HLA typing information for both transplant recipients and donors was obtained from the medical charts of patients. This was used to calculate the number of allele matches between the donor and recipient at each of the A, B, and DR loci (0-2 for each locus). The type of induction therapy used for each patient was determined by review of the hospital discharge summary and/or from medication records.

Values for EBV PCR were extracted directly from patients' electronic medical records. In cases where a quantitative value was required, values listed as "low positive" were problematic. To address this, the value was declared as one-half of the lower limit of detection of the assay. To include EBV PCR as a time-dependent variable in extended Cox regression models, it was necessary to format the data into a 'counting process' data set. In this format, individual records are created for each distinct time point for each individual. We created a row of data for each single day for each patient up until their first acute rejection episode. The EBV status for each day was summarized in several different forms including using the most recent value available, using the mean of all previous values, using the geometric mean of all previous values, and using the maximum of all previous values.

Information regarding some variables of interest was available only for one cohort or the other. Data for dialysis status at transplantation, adherence with the prescribed medication regimen, and delayed graft function were only available for the Emory cohort. Dialysis status at transplantation was determined by chart review and classified only by whether the patient was on dialysis (including both hemodialysis and peritoneal dialysis) or not. Patients were classified as potentially non-compliant if the clinician noted suspected non-adherence with follow-up or medications or if patients admitted to missing medication doses (e.g. as a result of financial difficulty). Delayed graft function was defined as requiring hemodialysis within the first week after transplantation. Data for donor age and donor gender were available only for the NIH cohort. These were directly extracted from each patient's medical record.

For the second part of the study, VCA and EBNA serology were obtained from electronic medical records. The latest recorded value prior to 7 days after the patient's transplant was used as the pre-transplant value. Patients were considered to be latently infected with EBV if they had IgG antibodies to either or both VCA and EBNA in addition to an absence of IgM antibodies to EBV antigens.

Sample Size Calculations

Sample size calculations were complicated by the fact that the primary analysis method was extended Cox regression and that the effect variable of primary interest was time-varying. The number of required events (acute rejection episodes) to detect the expected effect size is given by:

$$D = (Z_{1-\alpha} + Z_{1-\beta})^2 \, [P(1-P) \, (\log\Delta)^2]^{-1}$$

Where $Z_{1-\alpha}$ is the standard normal deviate at the desired one-sided significant level, $Z_{1-\beta}$ is the standard normal deviate of the desired power, P is the proportion of the sample assigned to the treatment group, and $\log\Delta$ is the log hazard ratio of the treatment and control groups (76). It is important to note that D is the number of events, not the total sample size, so it must be adjusted by the proportion of patients with events to obtain the total sample size. The difficulty resulting from a time-varying covariate is that P cannot be simply determined because a patient may move in and out of the treatment group over the course of time. Therefore, the sample size calculations are presented as a function of varying values for the proportion of individuals in the treatment group. Sample size calculations are based on a 95% confidence level with 80% power. Based on the event rate in our cohort, the rate of acute rejection was estimated at 30%. The presumed value for R^2 between viral load and other regression variables in the model was estimated at 0.2. As can be seen in Appendix 1, at a hazard ratio of 0.5, the number of patients available in the Emory cohort is sufficient to detect on effect of EBV viremia with as little as 15% of the patient-time in the EBV viremia group.

Analysis

All analysis was performed with SAS 9.2 for Windows and R 2.13. Cox regressions were performed using SAS PROC PHREG, the "coxph" function of the R "survival" package, and the R "Design" package. Except where noted, tests were performed at the 5% significance level.

Demographic information is presented as frequencies for categorical variables. For continuous variables, summaries are given by means, medians, and inter-quartile ranges. For analyses of time to first clinically significant acute rejection stratified by a single categorical variable, the Kaplan-Meier method was used with the log-rank test for testing of significance. The primary method of multivariable analysis was Cox regression including both proportional hazards and extended forms. The variables considered for inclusion to adjust the model for commonly known determinants of acute rejection as described above were based on literature review, subject matter knowledge, and availability of data. The approach to Cox regression modeling was based on several prominent textbooks in survival analysis (77, 78).

As described below, prior to modeling, univariate analyses were performed for all variables of potential interest. Initial models included all covariates. The first step in modeling was to assess proportional hazards for each variable in the model. This was performed by plotting the scaled Schoenfeld residuals for each variable as functions of various forms of time, including linear time, the logarithm of time, ranked time, and time in the Kaplan-Meier form. When the proportional hazards assumption is met, the slope of a regression line between the scaled Schoenfeld residuals and time will be zero. The R "cox.zph" function, available in the R "survival" package also provides a statistical test for assessing this relationship. Variables that were found to violate the proportional hazards assumption were transformed according to the obtained plots to obtain no change over time for each of the variables in the model. Proportional hazards were reassessed using the same methods to ensure that the transformations corrected violations of the proportional hazards assumption.

Following correction of non-proportional hazards, potential interaction terms between variables in the models were assessed. The interaction of primary interest was that between recipient gender and donor type. This term was explored because of the potential for women to become allosensitized to both living-related donors and living-unrelated donors through childbirth. If a living-unrelated donor is the recipient's spouse and the recipient has previously

given birth to the donor's child, it is expected that the recipient will have increased alloimmunity to the donor's tissues. Similarly, a living-related donor could be the patient's child such that the recipient has been allosensitized to the donor.

The next step in modeling was to remove unnecessary variables to refine the models. This was performed to maximize the comparability between the models for the Emory cohort and the NIH cohort. As a result, some variables in either model were retained even though they may not be significant in one model because they were important in the other model. Variables were subsequently removed based on the results of Wald Chi-square tests for parameter estimates, the effect of removing the variable on other parameter estimates, and the importance of the given parameter in the model for the other cohort. Additionally, refined models were compared with full models using the likelihood ratio test and by comparing the calculated AIC (Akaike information criterion) between models. Lower AIC values are associated with models that better fit a given data set.

Once the baseline models were established, consideration was turned to evaluating the effect of EBV load on acute rejection rates. It was determined *a priori* to evaluate the effect of EBV viral load as a logarithmic function of EBV load. This was based on the nature of viral reactivation and the observed clinical correlation between viral reactivation and clinical disease. As the functional form of the relationship between EBV load and acute rejection rates was not previously known, it was necessary to approach this subject in an exploratory manner without an *a priori* hypothesis about the form of the relationship. The EBV load data was formatted into the counting process format as noted above. Penalized smoothing splines were subsequently used in time-dependent extended Cox regression to explore the functional relationship between each parameterization of EBV load and time to first acute rejection episode (78). This approach was used with only EBV load in the model and with EBV load added to the previously established models. The functional forms for the relationship between viral load and acute rejection rates could then be inferred by inspection of plots of the fitted splines versus EBV viral load. While

these plots are the results of primary interest in this exploratory analysis, they can also be used to parameterize EBV viral load to conduct hypothesis tests, although the significance of such tests is limited by their *post hoc* nature.

Results

Development of Cox Regression Model for Time to Acute Rejection (Emory Cohort)

There were 598 patients who received kidney transplants alone at Emory University Hospital between January 1, 2005 and August 31, 2009. Of these 495 met the study criteria. The demographics of the study population are shown in Table 1. The median age at the time of transplantation was 52.0 years with a range from 18.6 to 80.6 years. Female recipients comprised 43% of the study population. Approximately equal numbers of patients were Black or African American (44.6%) and White (45.5%). Patients in the cohort were followed for a median of 1114 days. The majority (67.9%) of patients received their grafts from deceased donors. Slightly more living donors were related (17.4%) than unrelated (14.7%). Most patients (88.9%) were on dialysis at the time of transplantation. A minority of patients had previously received at least one kidney transplant (7.1%).

As described above, the primary outcome of the study was time to the first acute rejection episode. Rejection-free survival, censored for death, is shown in Figure 1. Approximately 24.0% of patients experienced at least one episode of clinically significant acute rejection within the first year after transplantation. Three year rejection free survival was 69.0%.

For the Emory University cohort, variables that were considered to potentially significant risk factors for acute rejection rates included age at transplant, number of HLA A mismatches, number of HLA B mismatches, number of HLA DR mismatches, whether or not the patient experienced delayed graft function, recipient gender, patient adherence with the prescribed regimen, whether the patient was on dialysis at the time of transplant, recipient race, previous history of renal transplant, the donor type, and the year that the transplant was performed. Except for age, all variables were treated as categorical variables.

To assess the functional form of the relationship between age and the rate of acute rejection, several approaches were used. First, a univariate Cox regression model was fit for time to first acute rejection using a penalized spline for age as the covariate of interest. The spline

terms were then plotted as shown in Appendix 2(A). Inspection of this figure indicates that age has little effect on the hazard of rejection and that if it to be included in a regression model, age can be appropriately modeled as a linear continuous variable. Although the spline appears to suggest higher hazards at the extremes of age, this is based on very few observations and is associated with wide confidence intervals. The second approach used was to examine a plot of the Martingale residuals of a Cox regression model for time to acute rejection with no covariates versus age as shown in Appendix 2(B). This plot is consistent with the first in suggesting that the appropriate functional form for age is as a continuous linear variable. When age in years is entered a Cox regression model as the only regression variable for time to acute rejection, the estimated HR (95% C.I.) for each 1-year increase in age is 0.998 (0.987-1.01) with a Wald test p-value of 0.78. Similarly to the plots in Appendix 2, this suggests that age at transplant has little impact on the rate of acute rejection in this cohort.

The effects of each categorical variable on time to first acute rejection were explored with Kaplan-Meier analysis and the log-rank test. Results of these analyses are shown in Table 2 and Figure 2. In these univariate analyses, factors that were found to be significantly associated with time to acute rejection included the number of HLA B mismatches, the number of HLA DR mismatches, the presence of delayed graft function, patient non-adherence, the race of the recipient, and the year that the transplant was performed.

As expected, increasing numbers of HLA mismatches at each locus were associated with increased rates of acute rejection (Table 2, Figure 2 (A-C)). While delayed graft function is generally more likely to be a sign of early acute rejection rather than a cause, acute rejection episodes immediately after transplantation likely represent a different etiology than those that occur later. As a result, delayed graft function was thought to be an important covariate to account for this group of rejection episodes (Figure 2 (D)). Highlighting the importance of adequate immunosuppression, patients who were identified as failing to consistently take their prescribed medications had a much higher rate of acute rejection (Figure 2 (F)). Patients on

dialysis at the time of transplantation may have had a slightly higher rate of acute rejection (Figure 2 (G)), possibly related to the increased inflammatory state induced by chronic dialysis (55, 56). Consistent with previous evidence, Black or African American patients had an increased rate of acute rejection relative to patients who were white or from other races (Figure 2 (H)) (46, 58). Progressively improving patient management over the course of the study period was associated with decreased rates of acute rejection in later years (Figure 2 (J)). Based on similarities in their survival curves, 2006 was combined with 2007 and 2008 was combined with 2009. Factors that were not significantly associated with the rate of acute rejection in univariate analyses included the number of HLA A mismatches, recipient gender, being on dialysis at the time of transplantation, the type of donor, and whether the patient had previously received a kidney transplant (Figure 2 (A, E, G, J, I)). Perhaps most surprising among these was the lack of association between donor type and the rate of acute rejection.

As described in the methods section, model development was performed in concert with the model for the NIH cohort, below. An interaction term was added between recipient gender and donor type because this term was found to affect parameter estimates important in the model for the NIH cohort. Next, proportional hazards were checked for each variable using scaled Schoenfeld residuals (78). Based on these analyses, several variables were transformed with time-dependent components. Terms were added for the product of log10(time) and recipient age, HLA B mismatches, HLA DR mismatches, non-adherence, and recipient of transplants from living-related donors. The effect of transplant year had a complex time-dependence and it was not considered necessary to obtain parameter estimates for this variable, so the Cox regression models were stratified by transplant year. The effect of delayed graft function (DGF) had a strong early time dependence until approximately 30 days post-transplant. After 30 days post transplant, the effect of DGF appear to be reasonably constant over time. To accommodate this relationship separate variables were created for DGF < 30 days after transplantation and >= 30 days after transplantation. Additionally, a term was created for the product of log10(time) and DGF < 30

days after transplantation. These transformations resulted in approximately zero correlation between scaled Schoenfeld residuals and time for every variable in the model, suggesting that model assumptions were met.

The resulting model prior to elimination of variables that either where not significant or that did not affect other parameter estimates is presented in Appendix 3. Variables that remained significant in this multivariate model included age, the time-dependent effect of age, the number of HLA DR mismatches, the time-dependent effect of HLA DR mismatches, the presence of DGF at early times, the time-dependent effect of DGF, and the time-dependent effect of non-adherence. The interpretation of these effects is made difficult by the interaction terms and time-dependent effects. A simplified interpretation follows. Older age is associated with increasing rates of rejection, but the strength of this effect decreases with later time post-transplant. HLA DR mismatches are associated with increased rates of rejection, but again the strength of this effect decreases with time. Delayed graft function only contributes to increased rates of rejection immediately after transplantation, but this effect decreases quickly. Non-adherence is not associated with increased rates of acute rejection immediately after transplantation, but is associated with increasing rates of acute rejection as the time after the transplant progresses. This is likely due to increasing rates of non-adherence over time as well as a cumulative effect of non-adherence over time. The log-likelihood for this model is -672.0. The likelihood ratio test comparing this model to a model with no parameters yields $\chi^2 = 114.4$ with 26 degrees of freedom, thus p = 4.6 x 10^-13. The AIC for this model is 1396.1 which has no real meaning in isolation, but is used to determine whether model refinements improve the fit of the model.

Based on the results of the model presented in Appendix 3 and simultaneous development of the model for the NIH cohort, the least significant parameters were sequentially removed from the model to develop a reduced model. The removal of a given parameter was considered to be acceptable if the LR test comparing the reduced model to the original full model was not less than 0.05 and the removal of the parameter did not affect the other (non EBV load)

parameter estimates by > 20%. With this approach, previous kidney transplant, recipient race, the number of HLA A mismatches, and the variable representing whether the patient was on dialysis at transplant were dropped from the model. This model is presented in Table 3. These modifications resulted in a decrease in the AIC to 1388.1. The log-likelihood for this model was -674.0. As this model has 20 degrees of freedom, relative to the full model, the -2*log(LR) was 4.02, yielding p = 0.33. The AIC suggests that the reduced model is preferable and the likelihood-ratio test indicates that the full model is not significantly better than the reduced model.

Development of Cox Regression Model for Time to Acute Rejection (NIH Cohort)

To compare results across two different cohorts, similar data were available for patients who received kidney transplants at the National Institutes of Health Clinical Center between 1999 and 2006 under intramural research programs of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). In this NIH cohort, data from 138 patients were available. Baseline demographics for this cohort are shown in Table 4. Relative to the Emory cohort, the NIH cohort had a slightly lower proportion of women (36% vs. 43%) and fewer Black or African American patients (23.9% vs. 44.6%). As the NIH cohort patients were generally transplanted in earlier years, the follow-up time for the NIH patients (median: 2062 days) was significantly longer than that for the Emory patients (median: 1114 days). The NIH cohort was also younger on average (mean: 42.7 yrs) than the Emory patients (mean: 50.3 yrs), likely due to the inclusion of adolescents in this cohort. Kidney recipients in the NIH cohort were more likely to receive transplants from living donors than in the Emory cohort (69.6% vs. 31.1%). Finally, almost all patients in the NIH cohort received some type of induction therapy (92.8%).

Kaplan-Meier analysis for time to first clinically significant acute rejection is shown in Figure 3 (A) and (B). Forty-five percent of patients experienced at least one episode of acute rejection within the first year after transplantation.

In contrast to the Emory cohort, information was not available regarding each patient's

dialysis status at transplant or their adherence with the prescribed medication regimen. Nonetheless, additional information was available concerning the age and gender of each patient's donor. As with the Emory cohort, the analysis began with univariate explorations. To assess the functional form of the relationship between age and the rate of acute rejection, the model was fit with a penalized spline and the log of the hazard plotted versus age. Additionally, the Martingale residuals from the null model were also plotted against age (not shown). As with the Emory cohort, these plots suggested that the appropriate form to enter age into the model was as a linear continuous variable. Similar analyses for donor age revealed that donor age could be entered into the model as a continuous linear variable as well (not shown).

Kaplan-Meier survival curves for single categorical variables of interest are shown in Figure 4. Summaries of these comparisons as well as the results of univariable Cox regression models are presented in Table 5. For all three HLA types assessed, increasing numbers of mismatches were associated with increased rates of acute rejection. This effect was not significant for HLA A mismatches, but was significant for HLA B mismatches ($p=0.013$) and HLA DR mismatches ($p=0.037$). The univariate analyses additionally revealed that the type of donor and the donor gender were significantly associated with acute rejection.

As described above, the model for the NIH cohort was developed in parallel with that for the Emoy cohort. All available covariates potentially associated with acute rejection were included in an initial Cox regression model including recipient age, HLA A mismatches, HLA B mismatches, HLA DR mismatches, donor type, recipient gender, recipient race, history of previous kidney transplant, donor gender, and donor age. Prior to further model refinement, proportional hazards were assessed as described previously. To correct for non-proportional hazards, several time dependent variables were entered into the model. Terms were entered for the products of log10(time) and 1 HLA DR mismatch, Caucasian race, and donor age. For 'other' race, separate terms were created for times < 180 days after transplantation and for >= 180 days after transplantation. These changes resulted in a model with approximately no correlation

between the scaled Schoenfeld residuals for each variable and time. As with the model for the Emory cohort, an interaction term was included between gender and donor type.

After establishment of the time-dependent model due to proportional hazards violations, terms that did not significantly impact either this or the Emory model were removed. Terms that were removed included HLA A mismatches, recipient gender, recipient race, renal transplant history, donor gender, and donor age. This reduced model is presented in Table 6. Relative to the full model which had 21 degrees of freedom, the -2*log(LR) chi square value was 13.8. With 11 degrees of freedom in the reduced model, this resulted in a p-value of 0.185 for the likelihood ratio test. Relative to the AIC of 655.4 in the full model, the AIC of the reduced model was 649.1. In this refined model. HLA B mismatches were associated with increased hazard of rejection. Additionally, HLA DR mismatches were associated with a slight, but non-significant increased hazard of rejection. For 1 HLA DR mismatch, this effect decreased with the log10 of time. Among female recipients, receiving a graft from a living-unrelated donor (LURD) was associated with an increased hazard of acute rejection. The HR (95% CI) for LURD among females was 8.96 (2.87 – 28.01). This effect for LURD was essentially absent for males. A similar, but non-significant effect was observed for living-related donation among females.

Exploration of the Effect of EBV Viremia on Acute Rejection Rate

While it was hypothesized that larger magnitudes of EBV viremia would be a sign of a higher degree of immunosuppression and, therefore, a lower rate of acute rejection, the functional relationship between EBV viremia and acute rejection rate was not known *a priori*. EBV viremia was included as a time-dependent covariate in the Cox regression model in one of several forms: log10(most recent EBV viral load), log10(mean EBV viral load before any given time), log10(geometric mean of EBV viral load before any given time), and log10(maximum EBV viral load before any given time). To explore the relationship between each of these parameters and

acute rejection rates, Cox regression models were run with each of these variables parameterized by penalized smoothing splines (78).

The results of fitting the various forms of EBV viral load without any other covariates in the models are presented in Figure 5 for Emory and Figure 6 for the NIH cohort. Within cohorts, the shape of the fitted splines was quite similar between the different parameterizations of EBV viral load. It was necessary to create an artificial value for observations where EBV data was not present, thus this was coded as "-1". This was done so that data could be used for patients who did not have EBV viral load data available until later post-transplant. With this method, patients did not have to have EBV viral load data available at all time points. When comparing the Emory and the NIH cohorts, several slight differences were noted. First, a wider range of EBV viral loads were present in the NIH data set such that there is a region of EBV viral load values that is plotted on the right end of the NIH fits that is not present on the Emory fits. This wider range of values results in a larger y-axis scale as well. Finally, the EBV PCR assay used for the NIH data allowed for more precise measurement of small values (i.e. < 300 copies/uL), such that the fit is more smooth for the NIH data in this region than is the fit for the Emory data. Despite these distinctions, a similar overall pattern is observed between the two cohorts. The log(hazard) is fairly constant at low values (within the error of the fit), but there is a sudden increase in the log(hazard) at intermediate values of EBV. This is then followed by a declining hazard with large EBV values. As can be seen in Figure 5, the fit for the logarithm of the geometric mean of previous values of EBV has two areas of increased hazard. The cause of this is not presently clear, but it should be recognized that the range of log(hazard) in this plot (the vertical axis) is much smaller than that of the other plots, so the apparent form of this fit is exaggerated relative to the others.

As noted, the form of the fits followed a fairly similar pattern between the cohorts and parameterizations of EBV viral load, particularly when considering the error surrounding these

fits. As a result, it was decided to use one particular parameterization for EBV going forward. The most consistent fit between the Emory cohort and the NIH cohort appeared to be that for the logarithm of the maximum previous EBV value. Additionally, this parameterization resulted in the most distinct region of increased hazard for intermediate EBV values.

Based on inspection of the fitted splines, several new variables were created to represent the logarithm of the maximum previous EBV value. One variable was created to represent non-observed EBV values. An additional variable was created to represent values in the increased hazard region between 1000 and 10,000 copies/uL. When parameterized this way, the hazard ratio in the Emory cohort comparing values in this region to those that were observed (not missing) above and below this region was 5.10 (95% CI: 1.67 – 15.63) in the unadjusted model and 4.63 (95% CI: 1.47 – 14.62) in the adjusted model. For the NIH cohort, this hazard ratio was 3.12 (95% CI: 1.36 – 7.14) for the unadjusted model and 2.57 (95% CI: 1.09 – 6.11) for the adjusted model. Therefore, adjustment of the model for these easily measureable factors does partially, but not completely, abrogate the association between EBV and acute rejection rates suggesting that EBV load is informative apart from these measures. Similarly, the fitted splines for the logarithm of the maximum previous EBV value were affected, but not substantially, by inclusion of covariates in the models as shown in Figure 7.

The fitted splines for the NIH cohort in Figure 6 did suggest that above a certain threshold of EBV values, the hazard of acute rejection significantly decreased. Based on inspection of the plot, an additional variable was created to represent max EBV values greater than 5000 in the NIH cohort. When just this term and the term representing missing EBV values were entered into a Cox regression model, the hazard ratio for values over 5000 relative to other (non-missing) values was 0.33 (95% CI: 0.08 – 1.39). When this term was added to the regression model previously described, the estimate for this HR was 0.28 (95% CI: 0.06 – 1.24). Interestingly, when this term was put in a model with all available covariates, including the type of induction therapy, the estimate was 0.17 (95% CI: 0.03 – 0.83). These findings do suggest that

perhaps there is a threshold value of EBV, above which the rate of acute rejection significantly decreases, but that the association with EBV interacts with induction regimen. Unfortunately, there do not appear to be enough events to assess this hypothesis.

Larger Values of EBV Viral Load are Associated with More Aggressive Changes in Immunosuppression

We were interested in determining whether clinicians responded to higher levels of EBV load with more aggressive decreases in immunosuppression. We therefore looked at the effect of EBV detection on tacrolimus levels in patients. We developed a linear regression model with the outcome variable calculated as the difference between the mean tacrolimus level for the 60 days following an EBV measurement and the mean tacrolimus level prior to the EBV measurement. Variables considered for inclusion as potential predictors were the mean previous tacolimus level, time since transplant, the year that the measurement was taken, and the base-10 logarithm of the EBV load. As patients could contribute multiple observations to the regression, a mixed effects model was employed to account for the correlation between observations for specific patients. The mean previous tacrolimus level could contribute to the change in tacrolimus levels in that smaller previous values might be associated with smaller changes. Time since transplant was included in the model because standard clinical practice is to decrease immunosuppressive doses with increasing time post-transplant. It was known that the standard clinical response to EBV detection at the institution changed over time, so the year of the event was considered to be a potentially important factor. Finally, the EBV viral load was the regressor of primary interest.

As seen in the plots in Figure 8, all of the variables considered do appear to be associated with the calculated change in tacrolimus. Larger previous levels of tacrolimus were associated with larger decreases, increasing time since transplant was associated with larger decreases in tacrolimus, the magnitude of the change was dependent on year, and larger values of viral load were associated with larger decreases in tacrolimus concentration. These four variables were

included in a linear mixed effects model with each variable as a fixed effect and each variable as a random effect by patient. Models were compared using model AICs and with the likelihood ratio test. Individual fixed effects and random effects were assessed with the model likelihood ratio test after elimination of the effect from the model. The final model included all four variables as fixed effects with the logarithm of EBV viral load and time since transplantation as random effects. A random intercept was also included, by patient. Potentially influential observations were assessed by calculating Cook's distances and DFBETAS (for each variable) for each patient using the R package 'influence.ME'. Preliminary cutoff values of 4/n for Cook's distance and 2/sqrt(n) for DFBETAS to suggest influential observations were based recommended thresholds (79). Several potentially influential observations were identified by these techniques, but review of the cases did not reveal data entry errors or largely unusual values for most of these. One patient (indicated by the point on the far left of the plot in Figure 8 A) was removed from the analysis as it was determined that they were not receiving tacrolimus.

The results of the mixed effects model are presented in Table 7. As shown in the table, for each 10-fold increase in viral load, the expected change in mean tacrolimus levels is -0.73 (95% CI: -1.16 to -0.30). This suggests that larger magnitude EBV viral reactivation was associated with larger decreases in immunosuppression.

While it was expected that decreasing immunosuppressive levels, including tacrolimus would be associated with increased rates of rejection, we wanted to confirm this in our population to support the hypothesis that the increased hazard of rejection at intermediate EBV values is associated with decreases in immunosuppression. Figure 9 demonstrates that declining tacrolimus levels are clearly associated with increasing hazard of acute rejection. Ideally, we would like to control for the effect of declining immunosuppression levels on the relationship between EBV viral load and the hazard of acute rejection. We used the data available for tacrolimus levels to control for this effect as fully as possible, but this will not completely account for the effect of tacrolimus and obviously cannot control for the effects of changes in other immunosuppressive

medications. As shown in Figure 9 (B), the effects of a change in tacrolimus level and the mean tacrolimus level over the preceding 60 days were controlled for with penalized spline fits for these values. Controlling for tacrolimus levels and changes in this manner resulted in a decrease in the magnitude of the increased hazard in the region of intermediate EBV values.

Pre-Transplant EBV Serology Is Associated With Immune Competence

As described in **Background**, the majority of patients are tested for antibodies to both VCA and EBNA prior to transplantation. In the Emory cohort, 493 patients the inclusion criteria and had data available for both VCA and EBNA antibodies pre-transplant. In Kaplan-Meier analysis of time to first clinically significant rejection, stratified by EBNA serology (among EBV seropositive patients), patients who were EBNA-, had a significantly decreased rate of rejection ($p=0.002$, Figure 10(A)). In the NIH cohort, 46 patients had complete EBV serology information and met the inclusion criteria. Patients with negative or equivocal (indeterminate) EBNA serology were again much less likely to experience acute rejection than those with EBNA positive serology in the NIH cohort (Figure 10(B)).

We were interested in determining whether EBNA serology might just be the effect of an association between EBNA and a known risk factor for rejection. We explored associations between EBNA and other recipient-specific variables and most were not associated with EBNA. However, we found that Black and African-American patients were significantly more likely to have EBNA antibodies (95.9%, $p < 0.001$) than Caucasians (86.6%) or patients of other races (85.7%). Additionally, patients who were on dialysis immediately prior to transplantation had a much higher frequency of EBNA antibodies (92.2%) than those who were not (78.2%, $p < 0.001$). An additional factor that was considered was a potential difference in the magnitude of calculated panel-reactive antibodies (CPRA) between EBNA negative and EBNA positive patients. CPRA is an estimate of the percentage of deceased donors that a given recipient would be incompatible with, based on the recipient's repertoire of anti-HLA antibodies (69). We

hypothesized that the presence of EBNA antibodies might be associated with a broader repertoire of anti-HLA antibodies. The distribution of CPRA values by EBNA serology is shown in Figure 11. When the median CPRA values were compared between groups using a non-parametric test, EBNA negative patients had a slightly higher median (EBNA positive median: 0, EBNA negative median: 4, $p = 0.049$). CPRA values were additionally split into quintiles to compare the groups by quintile frequencies. The fisher exact test for quintiles by group yielded a p-value of 0.27.

A Cox regression model of the Emory cohort with just EBNA serostatus as a regressor yielded a HR (95% CI) comparing EBNA negative to EBNA positive patients of 0.24 (0.09 – 0.66). When stratified by race and dialysis status, the HR (95% CI) for this comparison was still 0.28 (0.10 - 0.76). As EBNA serostatus is measured at a single time point prior to transplantation, it is conceivable that EBNA would only be related to acute rejection hazard at early times after transplantation. To assess the potential time dependence of the effect of EBNA, the scaled Schoenfeld residuals for the EBNA term were plotted as a function of various forms of time using the cox.zph function of the R survival package. As shown in Figure 12, the effect of the EBNA term appears to be constant over time.

Discussion

In this study we provide evidence for the potential utility of measures of viral immunity in monitoring post-transplant immunosuppression. This concept is derived from common clinical practice in the management of post-transplant immunosuppression in that clinically significant infection is often the only indication of over-immunosuppression. Based on this principle, it is conceivable that assessment of subclinical infection may allow clinicians to adjust immunosuppression prior to the development of clinical signs and symptoms suggestive of inadequate or excessive immunosuppression. The findings presented here do suggest that viral reactivation as measured by PCR of peripheral blood is associated with rates of acute rejection, although the precise form of this relationship remains to be determined. Additionally, we present strong evidence that acute rejection rates are related to the presence or absence of EBV EBNA antibodies prior to transplantation.

At present, the most promising approach to improve post-transplant outcomes is likely to be the optimization of immunosuppressive regimens at the individual level. While renal transplantation is associated with significant benefits for patients with end-stage renal disease, recipients still face a variety of morbidities, primarily associated with immunosuppressive medications. Efforts are underway to develop new immunosuppressive medications with fewer side effects (70), but it is unlikely that any therapy effective at preventing transplant rejection will be without the infectious complications related directly to immunosuppression. The significant overlap between mechanisms of protective immunity and those of alloimmunity makes it improbable that purely immunosuppressive therapies could target just alloimmunity with high specificity. This fundamental impediment is increasingly highlighted by evidence that, in alloantigen naïve individuals, a considerable fraction of alloimmunity appears to develop as a result of cross-reactive immunity to microbial pathogens (71, 80).

The difficulty in specifically targeting alloimmunity emphasizes the importance of developing methods to guide the optimization of transplant immunosuppression, particularly in

individual patients. As noted previously, the development of immune monitoring methods is an area of active investigation. These approaches include a wide array of targets, but we favor assessment of viral reactivation for several reasons. First, the assessment can be performed on a specimen as easily accessible as peripheral blood. Second, viral PCR provides unusually high specificity (for the virus of interest) and substantial sensitivity. Finally and most relevant, this approach provides a direct measure of *in vivo* immune function without *ex vivo* manipulation or stimulation of the immune response.

While measures of viral immunity may be a promising approach to the assessment of aggregate immune status, several features contributed to the challenge of studying these relationships. At present, EBV viral load is measured relatively infrequently, particularly in the absence of clinical signs and symptoms of systemic infection. This suggests that determinations of EBV viral load were likely biased by clinical circumstances. Patients with tenuous clinical courses or significant evidence of systemic infection were more likely to have their EBV levels measured. It is possible that the selection of patients who were sicker contributed to increased rejection rates at intermediate levels of EBV reaction. In the current study, infrequent viral load measurements, particularly in the Emory cohort, also limited our ability to obtain precise estimates of the relative hazard of acute rejection at specific levels of EBV reactivation. An additional benefit of more frequent measurements of EBV viral load would have been the ability to explore the relationship between changes in EBV viral load and the hazard of acute rejection. It is likely that a sudden decrease in EBV levels is more concerning for increased rates of acute rejection than is a persistent low or undetectable level of viral load. As the patients in the NIH cohort were enrolled in clinical research protocols, they were more frequently assessed for EBV viral load. This likely decreased the bias associated with selection of patients who were clinically suspicious for systemic infection.

This study additionally highlighted several of the difficulties inherent in time-to-event analysis. Using the time to an event as the primary outcome makes use of the additional

information available regarding timing relative to a binary outcome, but clearly adds additional complexity to the study analysis. One primary result of this was the need to choose a single defining event for each individual. In this study, the defining event was chosen as the first rejection episode as this was the earliest and most easily determined measure of alloimmunity available to us. While acute rejection is a clinically significant event, associated with morbidity for the patient, and an important component of long term graft outcome, it is not necessarily the most important outcome after transplantation. When detected early enough, most rejection episodes readily respond to treatment. As a result, patients can, and frequently do, have multiple episodes of acute rejection. One of the implications of this for the present study was that any data available after a first rejection episode was not used in analysis. Time-to-event methods are available which allow for multiple events per subject (81). While not presented here, we plan to employ such approaches in future analyses of the current hypotheses.

An additional feature of the present study that highlighted the utility of time-to-event analysis, but contributed to the complexity of analysis, was the changing values of EBV load over time. For each patient, viral load was measured at varying times and multiple values were available for some patients. A given patient might have no detectable viral load at one time point, but have a large viral load several months later. This information and the temporal relationship between each measurement and rejection episodes would have obviously been lost in a binary analysis such as logistic regression and difficult to account for in Kaplan-Meier analysis. One of the significant benefits of extended Cox regression is the ability to allow for changes in the values of regression variables over time. This type of data is fairly common in clinical research as patients often have changing clinical features or laboratory data. One difficulty in building a model with a time-dependent variable is determining the best functional form for the variable. One possibility was to enter just the most recent EBV viral load into the model, but in this case all previous values for the patient are ignored at any given time point. These previous values may provide relevant information to the model. As a result, several different forms were explored for

summarizing a given patient's EBV viral load data at any given point. As previously noted, these included just the most recent value, the maximum of all previous values, the mean of all previous values, and the geometric mean of all previous values. In the present study each of these representations yielded fairly similar results for the relationship between EBV viral load and acute rejection hazard. This is not surprising given the significant correlation between each of these forms, but does not rule out the possibility that one particular summary method would be more informative than the others if conducted on a data set with more frequent observations.

One of the primary strengths of the present study was that we did not require *a priori* knowledge regarding the relationship between EBV viral load and acute rejection rates. Our initial hypothesis was that increasingly large EBV viral load concentrations would be associated with progressively decreasing rates of acute rejection. It was also conceivable that a certain threshold EBV value existed above which acute rejection rates were all similarly decreased relative to low or non-detectable EBV viral load. Despite these hypotheses, we had no previous data to suggest the correct functional form for the relationship. To facilitate the analysis, we made use of penalized fitting splines to assess the functional form for the relationship in our particular data sets. This approach allowed us to avoid misspecification of the model. Nonetheless, it does distort the interpretation of hypothesis tests performed after inspection of the form of the fitted splines. This is because the hypotheses were not explicitly stated *a priori*, but were rather based on the relationships observed in the data.

Although the exploration of the relationship between EBV load and acute rejection rates was not based on an explicit *a priori* hypothesis, the interpretation of the fitted splines as representative of the relationship was bolstered by observing similar relationships in the two distinct cohorts. Importantly, while some of the patients in each cohort were operated on by the same transplant surgeon (A.D.K.), they were otherwise quite distinct in their characteristics. Patients in the Emory cohort were much more likely to be black or African American and receive a kidney from a deceased donor. Patients in the NIH cohort were more likely to have received

induction immunosuppressive therapy, to be enrolled in research protocols with experimental immunosuppressive therapy, and had an increased rate of acute rejection. The increased rate of acute rejection in the NIH cohort is likely the result of frequent surveillance biopsies for research purposes. These regularly planned, not-for cause biopsies are likely to detect low-grade rejection early on. Secondly, many patients in the NIH cohort were enrolled in research protocols designed to minimize immunosuppression. While patients were carefully monitored for acute rejection, these protocols may have placed patients at increased risk. Unlike the Emory cohort, we were not able to adjust for the effect of poor adherence with medication regimen in the NIH cohort. Importantly, non-adherence was expected to be less of a concern in the NIH cohort because of the motivation required to seek out and enroll in a clinical trial at the NIH.

Despite the differences between the Emory and the NIH cohorts, the relationship between EBV load and acute rejection rates followed a similar pattern in both cohorts. Low levels of EBV reactivation were associated with similar rates of acute rejection to those with non-detectable EBV. Intermediate values of EBV were associated with significant increases in the rate of acute rejection, which is likely related to aggressive reduction in immunosuppression by the clinician in response to elevated viral replication. Finally, the highest values appear to be associated with declining rates of acute rejection.

The finding of elevated rates of acute rejection at intermediate values for EBV viral load was surprising, but may partially explain conflicting findings in previous studies of the relationship between EBV reactivation and acute rejection (21, 82). As shown in this study, higher levels of viral load were associated with lower concentrations of the immunosuppressive medication tacrolimus after the level was observed. This suggests that the finding of increased rates of acute rejection for intermediate levels of viral load might be related to aggressive reduction in immunosuppression. It is clinically appropriate to reduce immunosuppression in the setting of elevated viral replication and this is, in fact, the ultimate goal of monitoring aggregate immunosuppression by viral load. This may suggest that the decreases in immunosuppression

were overzealous and that a smaller decrease might be more appropriate. Ideally, we would completely control for changes in immunosuppression, but not all patients are on equivalent immunosuppressive regimens. Additionally, we do not presently have complete data regarding medication doses or levels for each medication in all patients.

The inability to adequately control for the effect of changes in immunosuppression highlights the difficulty in performing this study in a retrospective manner. The retrospective approach was necessary due to time constraints and beneficial in that it allowed an initial exploration of the study hypothesis in a relatively economic manner. Nonetheless, we would ideally conduct this study in a prospective manner with clinicians blinded to the results of EBV analysis performed for the study. Clinicians would still assess EBV viral load when indicated clinically, but clinical management would not be impacted by values recorded for the study.

While the primary goal of the present study was to assess whether EBV viral load is related to acute rejection rates, we were also interested in other mechanisms by which viral immunity and alloimmunity might be related. In this respect, we found strong evidence that pre-transplant EBV antibodies are closely associated with acute rejection rates, even after adjusting for a number of potentially confounding variables. The second cohort of transplant recipients confirmed this association in a distinct population. It has previously been reported in the autoimmune literature that autoimmunity is associated with the presence of or higher titers of EBNA antibodies (22, 23, 83). In light of the considerable similarities between alloimmunity and autoimmunity, our findings are in agreement with these previous reports. Previous studies have suggested that EBNA antibodies may cross react with molecular targets of autoimmunity (83). In the present study, it is unlikely that the increased rates of acute rejection in patients with EBNA antibodies is the result of cross-reactivity of these antibodies with alloantigens. All patients in this study, both at Emory and at the NIH, were cross-match negative at transplantation. This means that, at transplantation, no recipient had detectable responses against their donor's alloantigens. If the EBNA antibodies present prior to transplantation were cross-reactive with alloantigens, the

cross-match would be expected to be positive. We also found no obvious association between EBNA serology and calculated plasma reactive antibodies (CPRA), a measure of the percentage of deceased donors for which the recipient would be incompatible based on the presence of anti-HLA antibodies. These observations suggest that the association between EBNA serology and acute rejection is unlikely to be directly caused by EBNA antibodies. It is possible that some underlying immune defect is present in patients who do not develop EBNA antibodies and this also decreases the potency of these patients' responses to alloantigens.

This study provides credence to the possibility of using assays of protective immunity to guide individual immunosuppressive therapy. Significant work is still required to develop these ideas into clinically applicable assays. Based on the limited data regarding EBV viral load in this study and the bias that may result from intermittent and non-scheduled viral load assessment, the next step in this work would be to conduct a prospective study with frequent, scheduled EBV assessments to which clinicians caring for patients would be blinded. As previously discussed these measures would additionally allow for assessment of the prognostic value of not only absolute viral load, but also rates of change of viral load. In addition to EBV, it also seems prudent to assess a variety of other common latent or persistent viral infections including BK, JC, CMV, HHV6, and VZV. EBV was chosen for this initial study because of its prevalence and importance in transplantation, but several other viruses have relatively high prevalence and may provide additional prognostic information.

We believe studying additional viruses will be beneficial for several reasons. Although many latent viruses have relatively high prevalence, there are still some patients who are not infected. Understanding the prognostic value of several different viral infections would allow for alternative monitoring strategies for patients with different patterns of infection. Additionally, it is possible that reactivation of certain viruses is associated with particular immune deficiencies. As a result, combining information regarding several different viruses may provide more specific prognostic information. Finally, the reactivation of some latent viruses might be more sensitive to

changes in immunosuppression than others, thus improving the sensitivity of the assay.

An additional benefit of a prospective study would be that pre-specified adverse outcomes could also be captured. Just as under-immunosuppression is associated with acute rejection, over-immunosuppression would be expected to cause increased rates of serious infections or neoplasms. It would, therefore, be desirable to explore the relationship between viral loads and these adverse outcomes.

One potential difficulty in developing measures of viral immunity for clinical use is that it is not possible to directly randomize patients to specific viral loads. Depending on the outcomes of the prospective study, one possible way to work around this limitation would be to randomize patients to targeted viral loads. The targets could be achieved by altering immunosuppressive doses. There is no guarantee that targeted levels would consistently be achieved or would be stable, but this is likely to be the preferred approach in this circumstance.

Identification of the association between EBNA serostatus and acute rejection rates is beneficial in that it may help to identify patients at decreased risk of rejection who might benefit from less intense immunosuppressive regimens. This benefit would only be limited to the small, but not infrequent, subset of patients who exhibit VCA+EBNA- serology (9.3% of patients with IgG antibodies to either or both VCA and EBNA in the Emory cohort). Better understanding of the cause of this effect could enhance our understanding of alloimmunity in general and more clearly identify specific areas of overlap between protective immunity and alloimmunity. An initial study with the potential for high yield would be to quantify the titers of EBNA antibodies. The designation of EBNA antibodies as ‘negative’ or ‘positive’ is somewhat artificial in that it actually represents a dichotomization of a continuous variable. A quantitative EBNA concentration, determined by serial dilution, is available. It is possible that the relationship between EBNA serology and acute rejection rates follows a dose-response curve.

As previously noted, we suspect that EBNA serology serves as an indirect marker of immune competence rather than as the causative agent of alloimmunity. If this is the case, then

we would expect other alterations in immune function. Microarray experiments comparing peripheral blood mRNA transcripts between EBNA negative and EBNA positive patients may reveal differences in the expression patterns of immune mediators. Additionally, several immune cellular phenotypes are known to be closely associated with alloimmunity (84-91). It is conceivable that EBNA serostatus is associated with alterations in the frequencies of important cellular phenotypes such as memory T cells, regulatory T cells, or transitional B cells among others. Therefore, a potentially important next step would be to compare the frequencies of these cellular populations between patients with known EBNA serostatus by using flow cytometry.

In conclusion, we have shown that clinical measures of viral immunity appear to be associated with alloimmunity. Further refinement of our understanding of these relationships may allow for their use in the optimization of clinical immunosuppression. Each transplant recipient and recipient-donor pair presents a unique combination of immunologic function and alloimmunity. To continue to improve outcomes for transplant recipients, objective assays of aggregate immune function are necessary to personalize immunosuppression on the individual level and even over time in a given individual.

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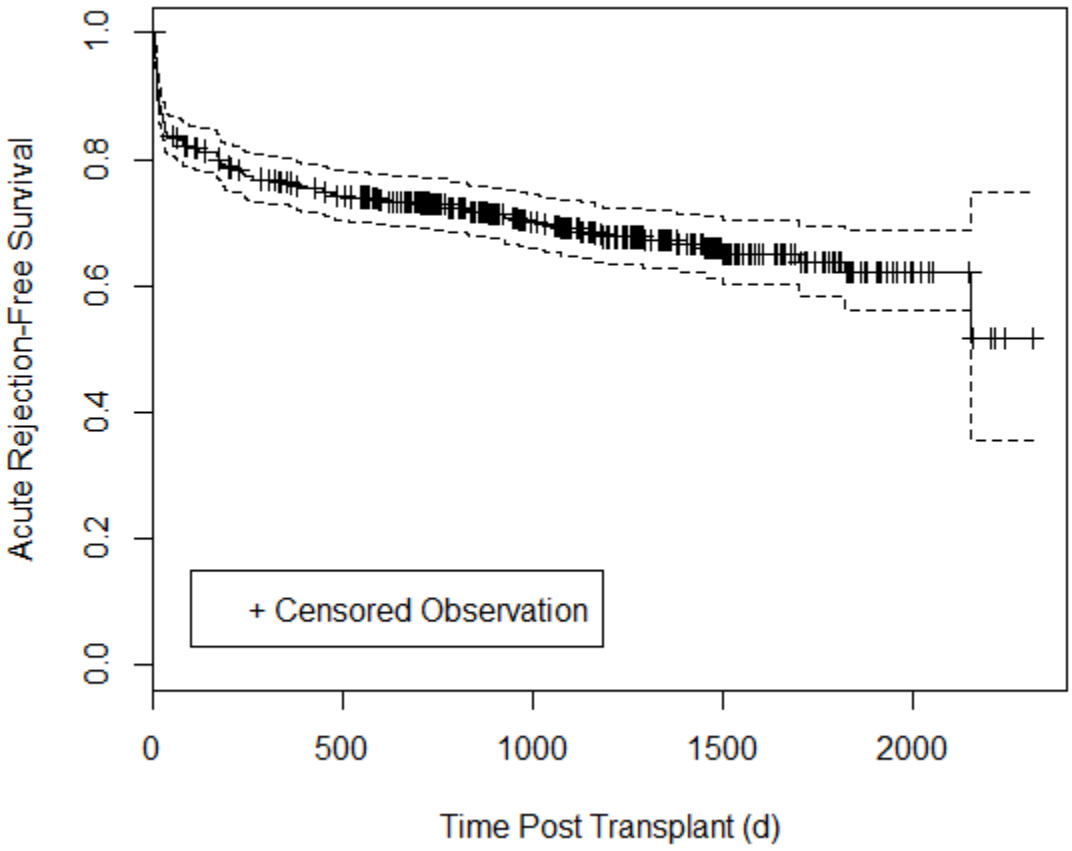
Figures

Figure 1. Time to First Rejection for Emory University Renal Transplant Recipients



The time to the first clinically significant acute rejection episode for the individuals in the Emory University cohort are summarized in this Kaplan-Meier survival curve. The dashed lines indicate the 95% confidence interval. Individuals were censored at death or graft failure. The 1-year and 3-year rejection-free survival times were 76.0% and 69.0%, respectively.

Figure 2. Univariate Kaplan-Meier Analysis of Time to Acute Rejection by Categorical Variables (Emory Cohort)

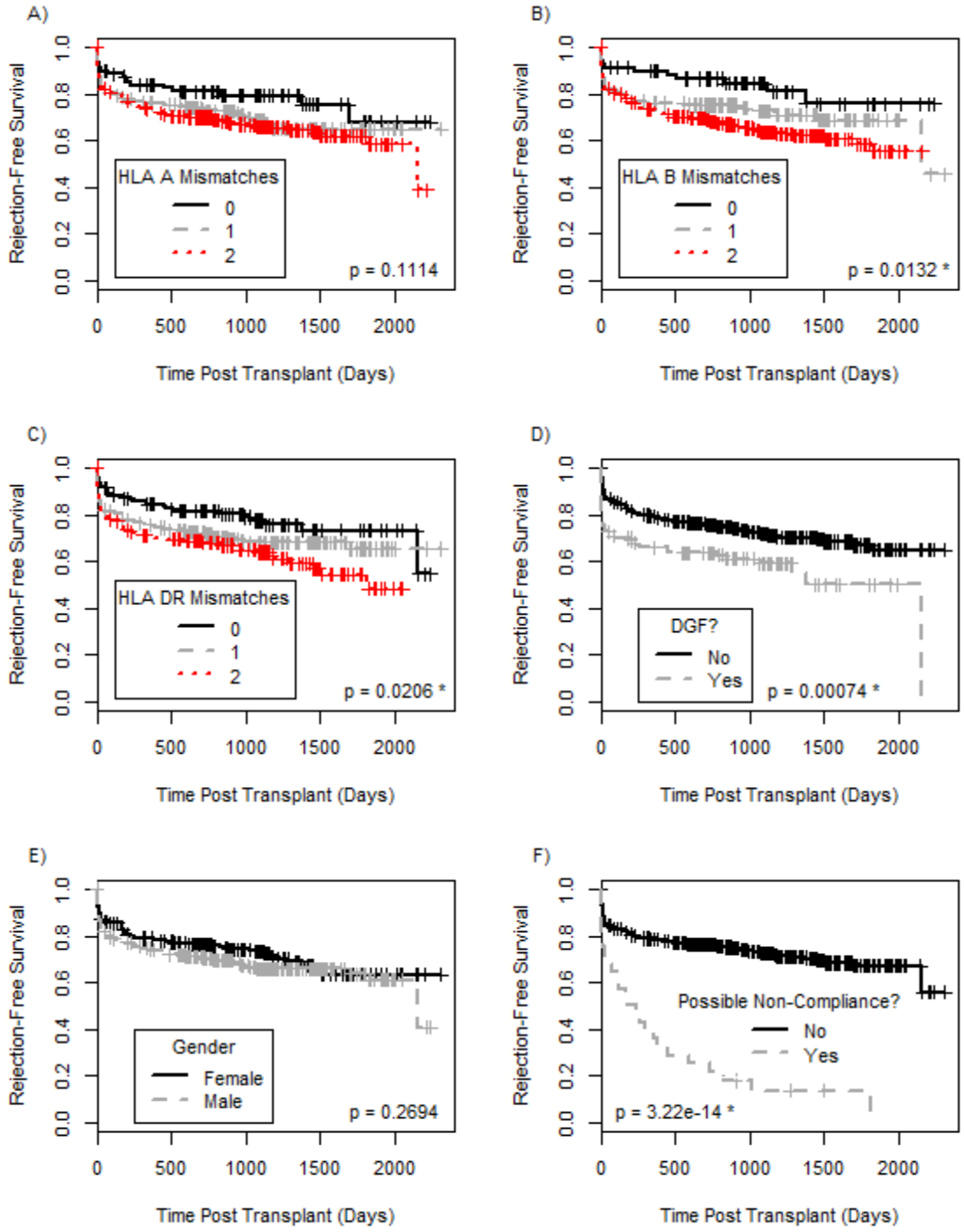
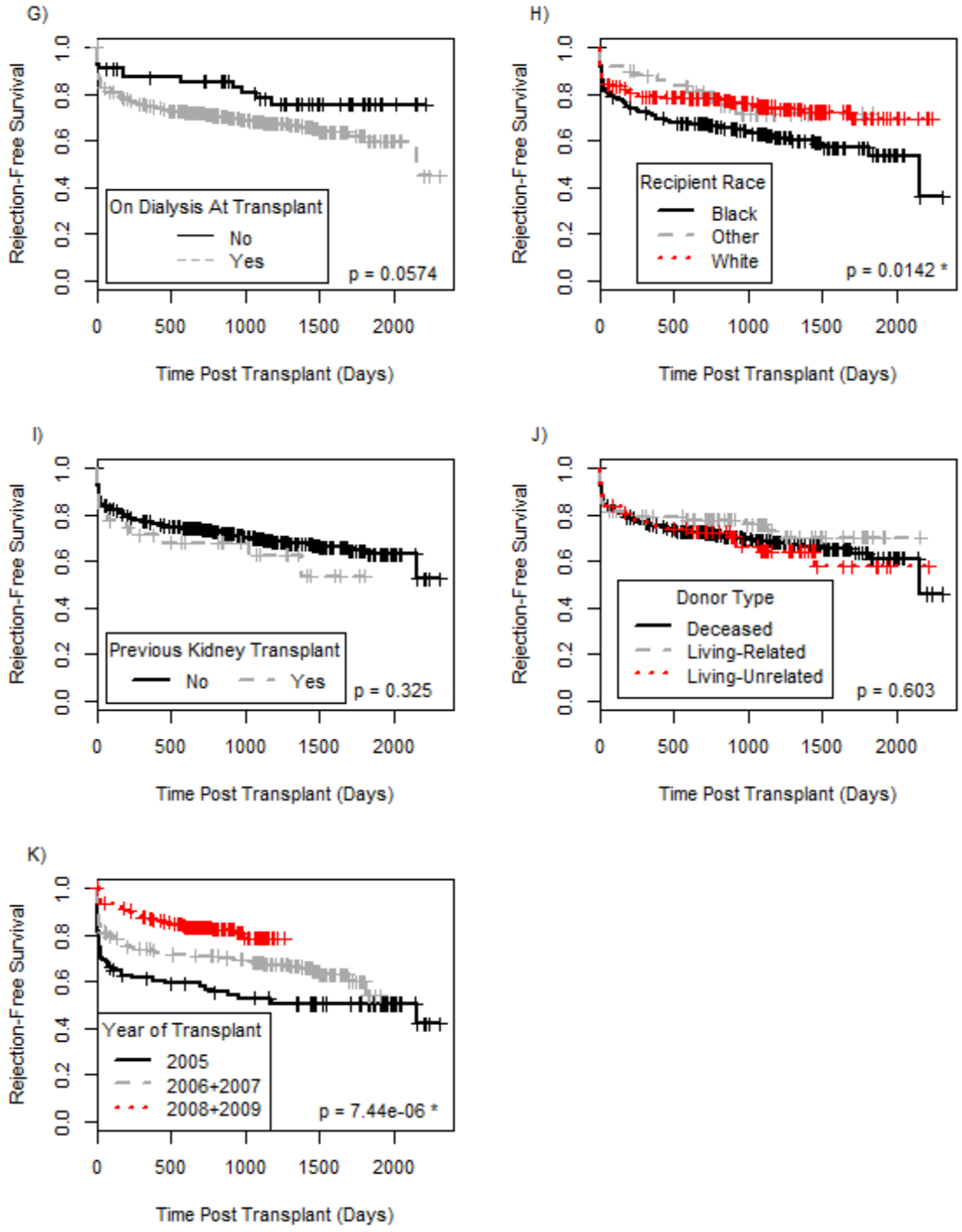


Figure 2 (cont.)

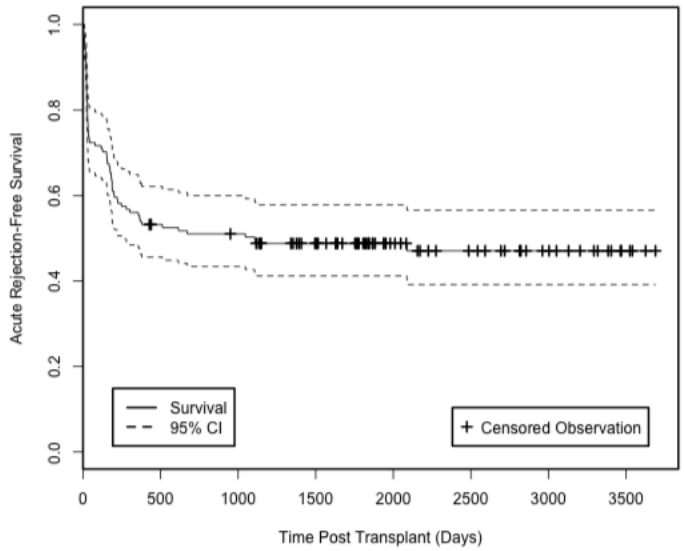


The panels in this figure show the Kaplan-Meier acute rejection-free survival curves for univariate analysis of factor variables in the Emory cohort. These curves and results correspond to the results displayed in Table 2. The variable "On Dialysis at Transplant" corresponds to whether a patient was transplanted prophylactically before needing dialyisis or was on either peritoneal- or hemo- dialysis at the time of transplantation. Previous kidney transplant refers to whether a patient had previously received another renal transplant that has since failed. Year of transplant was classified as one of "2005", "2006 or 2007", or "2008 or 2009". Some years were

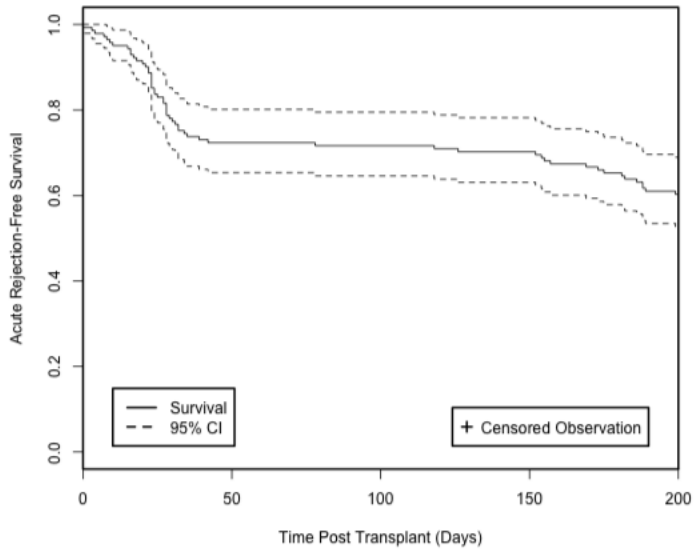
collapsed into single variables because of similarities in the outcomes for these years. As shown in Table 2, the variables that were found to significantly impact rejection rate in univariate analyses were HLA B mismatches, HLA DR mismatches, delayed graft function, non-adherence, the race of transplant recipient, and the year that the transplant was performed. Although not statistically significant, the dialysis status of the patient at transplantation was nearly associated with acute rejection rates.

Figure 3. Kaplan-Meier Curve for Time to First Clinically Significant Acute Rejection (NIH Cohort)

A)



B)



These figures show acute rejection-free survival for patients in the NIH cohort (n=138). Approximately 45% of individuals experienced at least one episode of acute rejection in the first year after transplantation. Importantly, many patients were enrolled in clinical research protocols attempting to minimize immunosuppression as quickly as possible, thus putting these patients at increased risk of rejection. Figure (B) simply expands the survival curve for the first 200 days after transplantation.

Figure 4. Effect of Single Variables on Time to First Clinically Significant Acute Rejection (NIH Cohort)

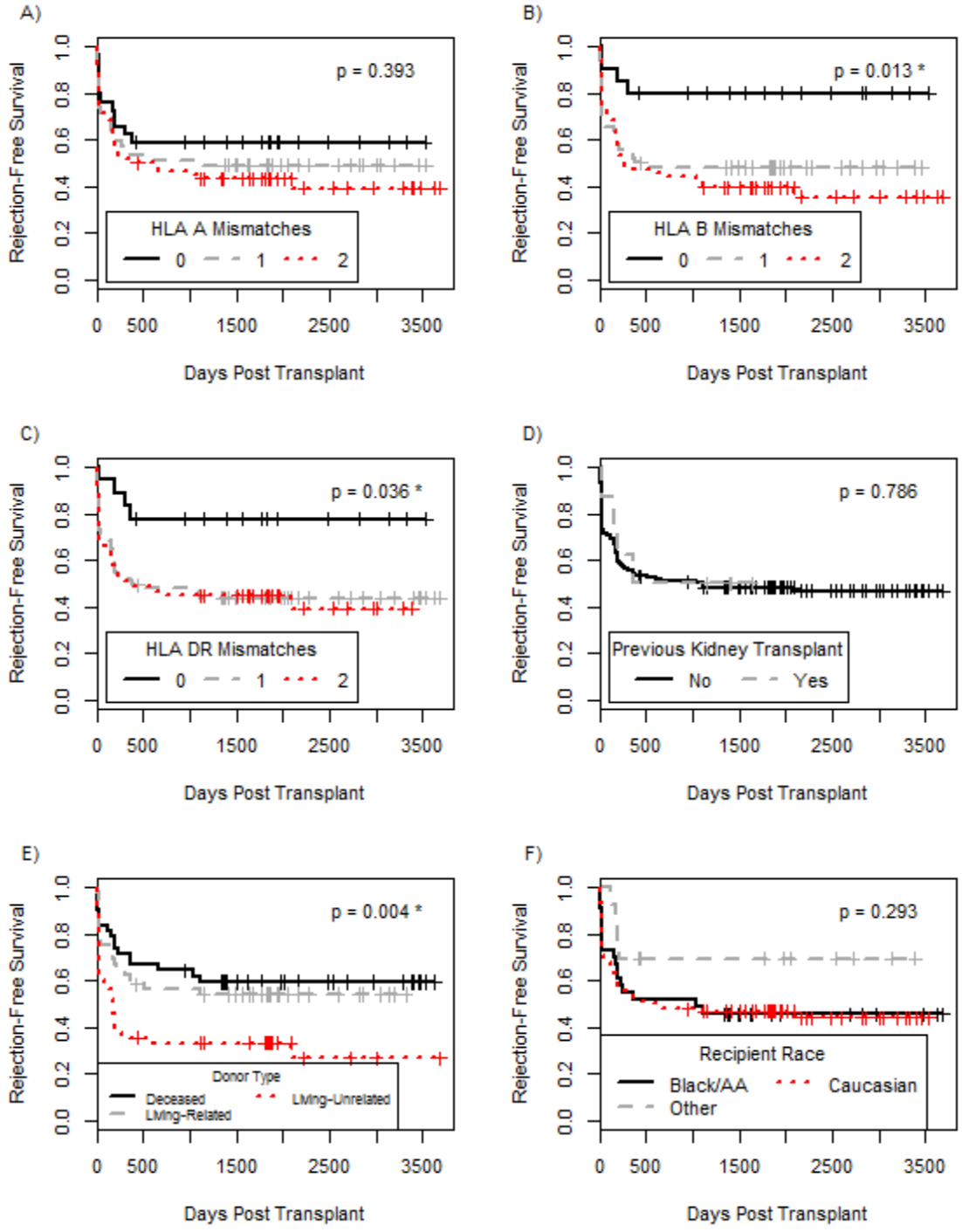
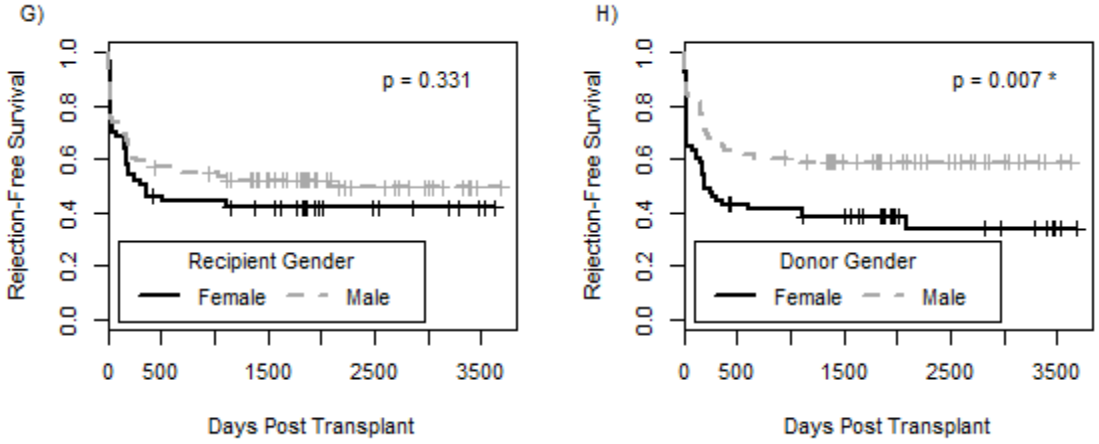
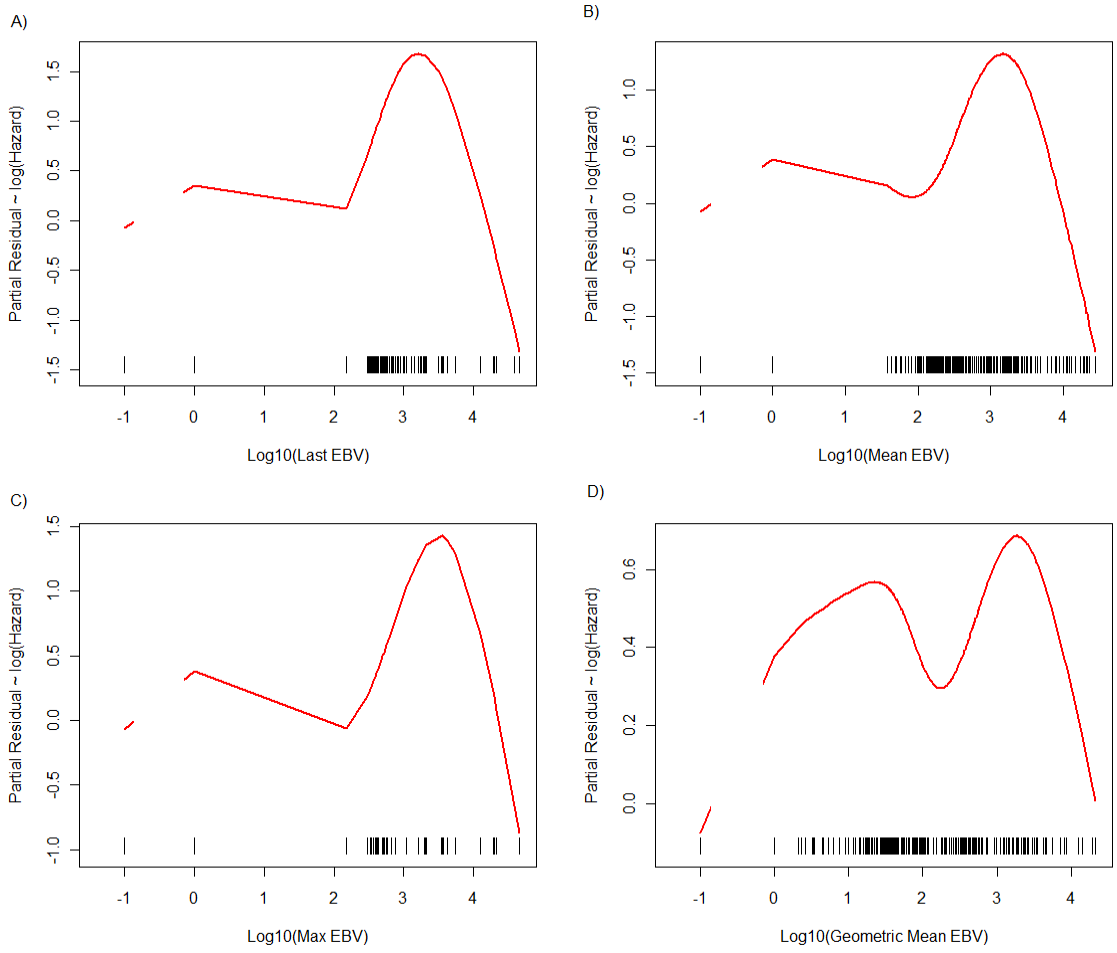


Figure 4 (cont.)



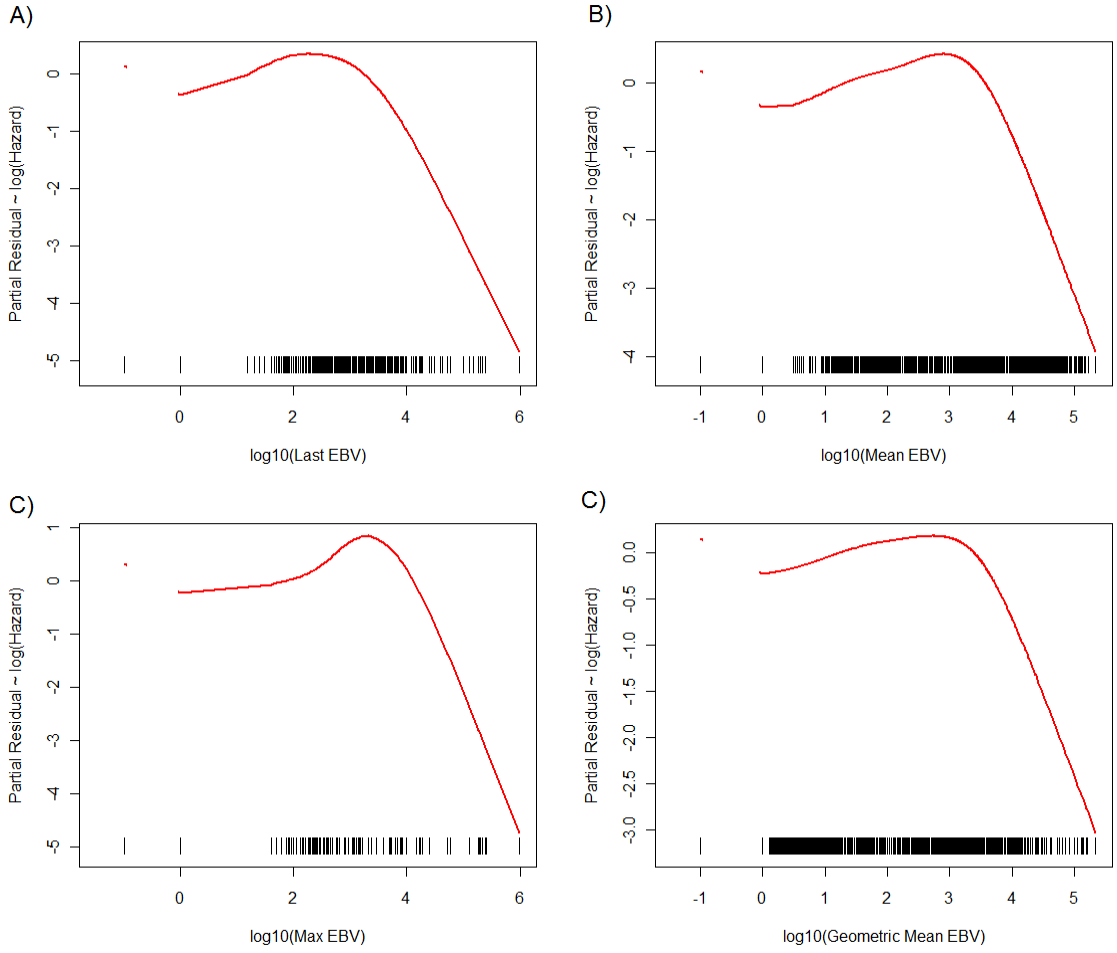
The panels in this figure show the Kaplan-Meier acute rejection-free survival curves for univarate analysis of factor variables in the Emory cohort. These curves and results correspond to the results displayed in Table 5. As shown in Table 5, variables that were found to significantly impact acute rejections rates included HLA B mismatches, HLA DR mismatches, donor type, and donor gender.
Abbreviations: HLA: Human leukocyte antigen

Figure 5. Penalized Spline Fits for the Relationship Between Various Forms of EBV Viral and the Hazard of Acute Rejection, No Other Covariates (Emory Cohort)



The panels in this figure show the fit of the relationship between the log-hazard of acute rejection and the log10 of various summarized forms of EBV viral load for the Emory cohort. The penalized spline function in the R 'survival' package was used to fit the relationships shown here. No other covariates were included in the time-dependent Cox regression models used to prepare this figure. For times where given individuals did not have EBV viral load measurements available, the EBV variables were coded as '-1'. The lines at the bottom each plot represent individual observations. The functions labeling the x-axes refer to functions applied to all EBV PCR values for a given patient available prior to a given point in time. As shown, these functions included, the most recent value (A), the mean of previous values (B), the maximum of previous values (C), and the geometric mean of previous values (D). The initial hypothesis for this study was that larger viral load values would be associated with decreased rates of acute rejection. As demonstrated here, the relationship appears to be more complex. With the exception of the geometric mean parameterization, intermediate values of EBV viral load appear to be associated with an increased hazard of acute rejection while the lower and higher extremes appear to be associated with decreased rates of acute rejection. One possible explanation for the observed shape of the fit with respect to the geometric mean parameterization is that this functional form allows for more discrimination at smaller EBV values because it is not as heavily weighted to large EBV values.

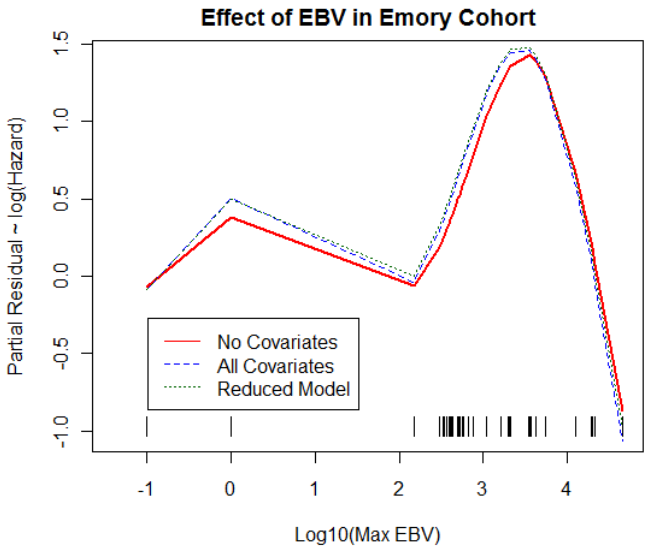
Figure 6. Penalized Spline Fit for the Relationship Between Various Forms of EBV Viral Load and the Hazard of Acute Rejection, No Other Covariates (NIH Cohort)



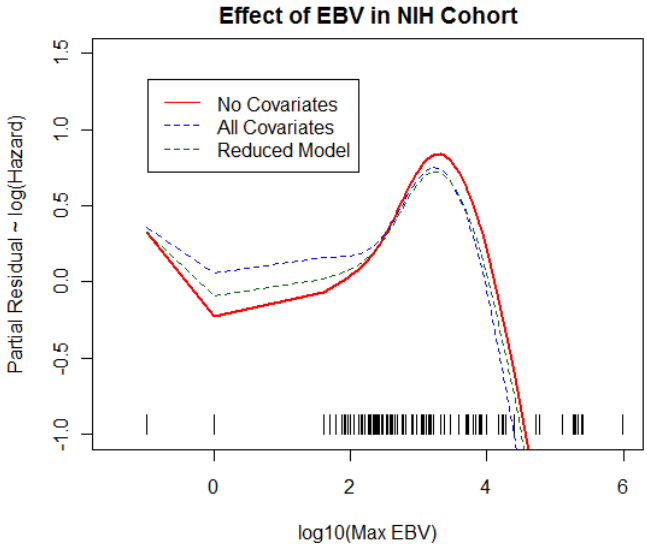
The panels in this figure show the fit of the relationship between the log-hazard of acute rejection and the log10 of various summarized forms of EBV viral load for the NIH cohort. The penalized spline function in the R 'survival' package was used to fit the relationships shown here. No other covariates were included in the time-dependent Cox regression models used to prepare this figure. For times where given individuals did not have EBV viral load measurements available, the EBV variables were coded as '-1'. The lines at the bottom each plot represent individual observations. The functions labeling the x-axes refer to functions applied to all EBV PCR values for a given patient available prior to a given point in time. As shown, these functions included, the most recent value (A), the mean of previous values (B), the maximum of previous values (C), and the geometric mean of previous values (D). The initial hypothesis for this study was that larger viral load values would be associated with decreased rates of acute rejection. As demonstrated here, the relationship appears to be more complex. As in the Emory cohort, intermediate values of EBV viral load appear to be associated with an increased hazard of acute rejection while the lower and higher extremes appear to be associated with decreased rates of acute rejection.

Figure 7. Comparison of Splines Representing the Hazard Associated with EBV Load With and Without Adjusting for Covariates Known to Be Associated with Acute Rejection

A)



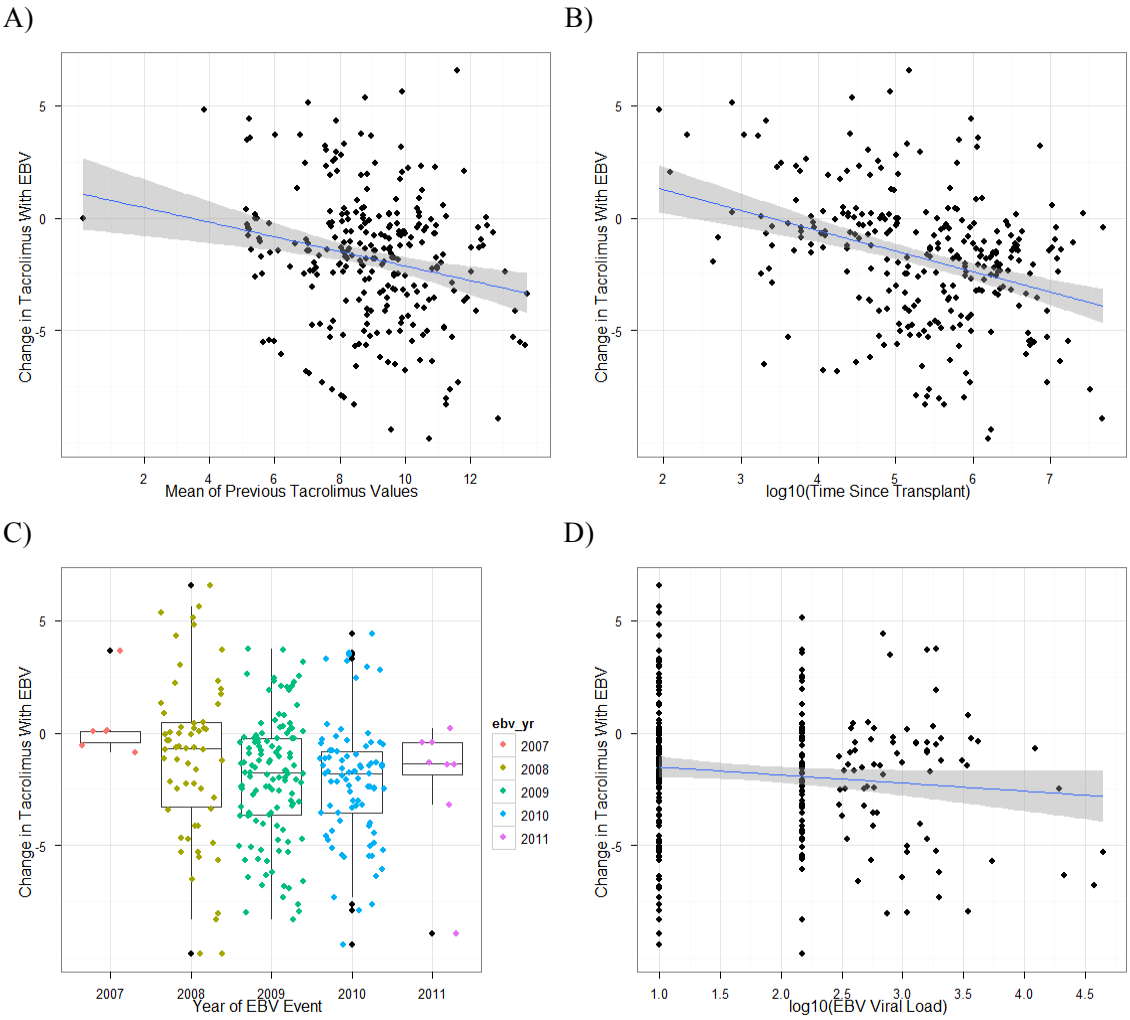
B)



The plots in this figure again show the penalized spline fit of the log-hazard for various values of EBV viral load. The y-axes have been re-scaled relative to previous figures and the plots for the Emory and the NIH cohorts are shown in a stacked arrangement to highlight the similarity in curve shapes between these two cohorts. As previously noted, intermediate values of EBV viral load appear to be associated with higher rates of acute rejection while lower and higher values for EBV viral load are associated with decreased rates of acute rejection in both cohorts. These plots additionally demonstrate the effect of adjusting for several other factors in the Cox regression models. As described in the text, the point of controlling for other factors in these models is to assess whether EBV viral load remains associated with acute rejection even after adjusting for effects that are more readily measureable and known to place patients at increased rates of

rejection. The red curves represent the effect of EBV viral load when no other variables are included in the models, the dashed blue lines show the effect of EBV viral load after adjusting for all available variables, and the dashed green lines demonstrate the effect of EBV viral load when controlling for all variables considered to be important after the development of multivariate Cox regression models (those presented in Table 3 and Table 6). In either model, the shape of the fit is mildly affected by controlling for these other variables.

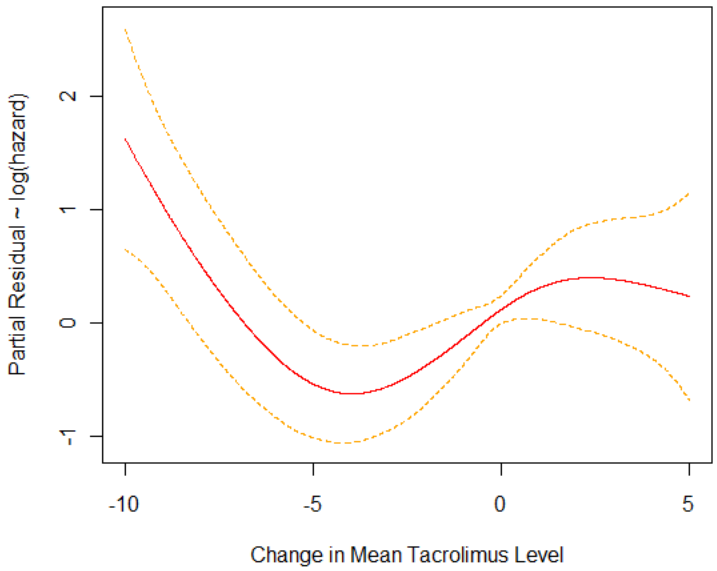
Figure 8. Assessment of Variables Affecting the Change in Tacrolimus Levels After Detection of EBV Replication (Emory Cohort)



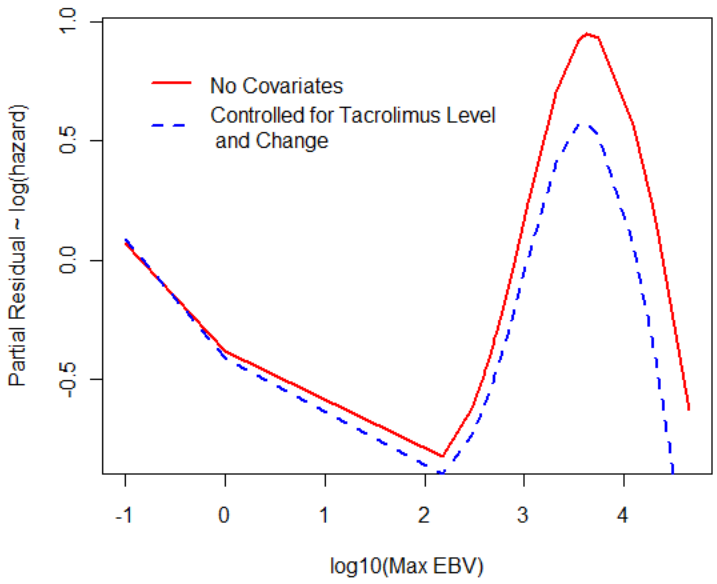
To assess the possibility that the increase in the rate of acute rejection at intermediate values of EBV viral load was associated with decreased immunosuppression, the change in serum tacrolimus levels was assessed as a function of several other factors. Each point represents the change in tacrolimus level over the 60 days following any EBV viral load determination. Panel (A) demonstrates that higher previous average tacrolimus levels are associated with larger decreases. Panel (B) shows that the longer the time since transplantation, the larger is the decrease in tacrolimus level. Panel (C) demonstrates a slight, but significant difference in the changed tacrolimus level between years in which the measurement was taken. This effect was explored because it was thought that the aggressiveness of the change in tacrolimus dose might have varied as clinical management changed over time. Panel (D) demonstrates that the detection of larger magnitude EBV viral replication was associated with larger decreases in tacrolimus levels.

Figure 9. Effect of Change in Tacrolimus Levels on Rejection Hazard

A)



B)

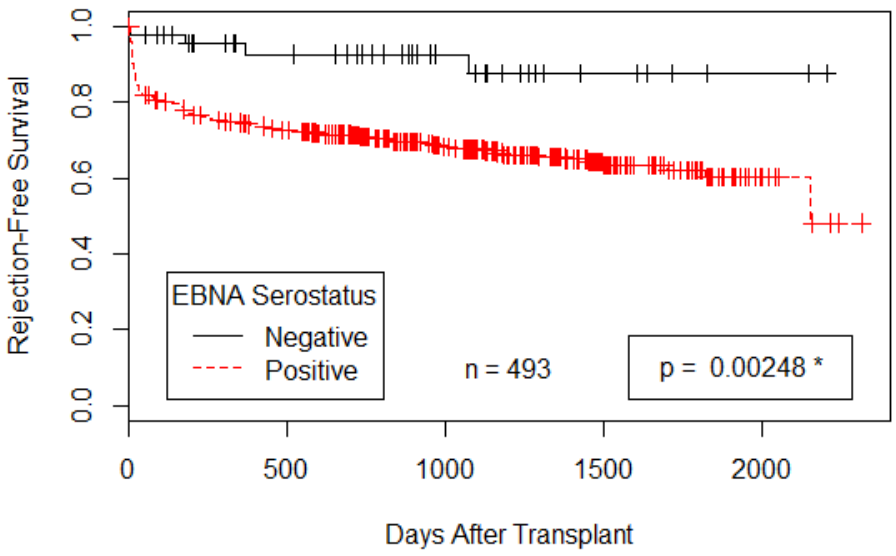


(A) To further assess the effect of changes in tacrolimus levels on the rate of acute rejection, a time-dependent Cox regression model was developed for the hazard of acute rejection as a function of the change in mean tacrolimus level for the 2 months preceding any point in time relative to the mean for tacrolimus for all times prior to the 2 month interval. The interval of changes in mean tacrolimus levels was truncated to between -10 ng/mL and +5 ng/mL because values outside of this range were associated with large error estimates. As seen from the plot in panel (A), increases in tacrolimus were associated with a slight, but stable increase in the log-hazard of acute rejection. It is likely that patients who appear to be clinically tenuous, with

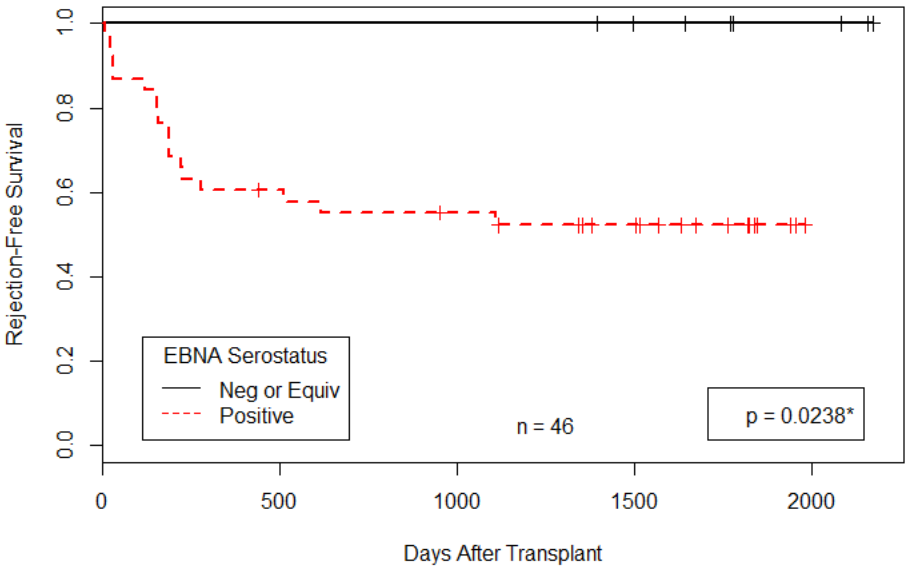
respect to their kidney function have their tacrolimus dose increased. This group is likely to have an increased rate of rejection prior to the medication change and the change in medication does not fully compensate. Similarly, patients with decreases of -4 or -5 ng/mL in magnitude experienced decreased log-hazard acute rejection, likely related to decreasing their immunosuppression in the first place. Importantly, patients with changes below -5 ng/mL experienced precipitous increases in their log-hazard of acute rejection. As expected, this confirms that large decreases in tacrolimus levels are associated with increased rates of acute rejection. Panel (B) demonstrates the effect of controlling for changes in mean tacrolimus level on the relationship between EBV viral load and acute rejection hazard. As shown, controlling for the effect of changes in immunosuppression leads to a decrease in the peak of acute rejection hazard at intermediate EBV viral load values. This is consistent with the hypothesis that the peak at intermediate values of EBV viral load is the result of aggressive decreases in immuosuppression when EBV viral reactivation is detected. Nonetheless, it is clear that controlling for changes in tacrolimus does not fully eliminate the peak. It is possible that this is a result of not being able to completely adjust for the effect of tacrolimus (i.e. time dependence) and because this model does not adjust for the effects of changes in other immunosuppressive agents.

Figure 10. Kaplan-Meier Survival Curves for Time to First Acute Rejection by EBNA-Serology

A)

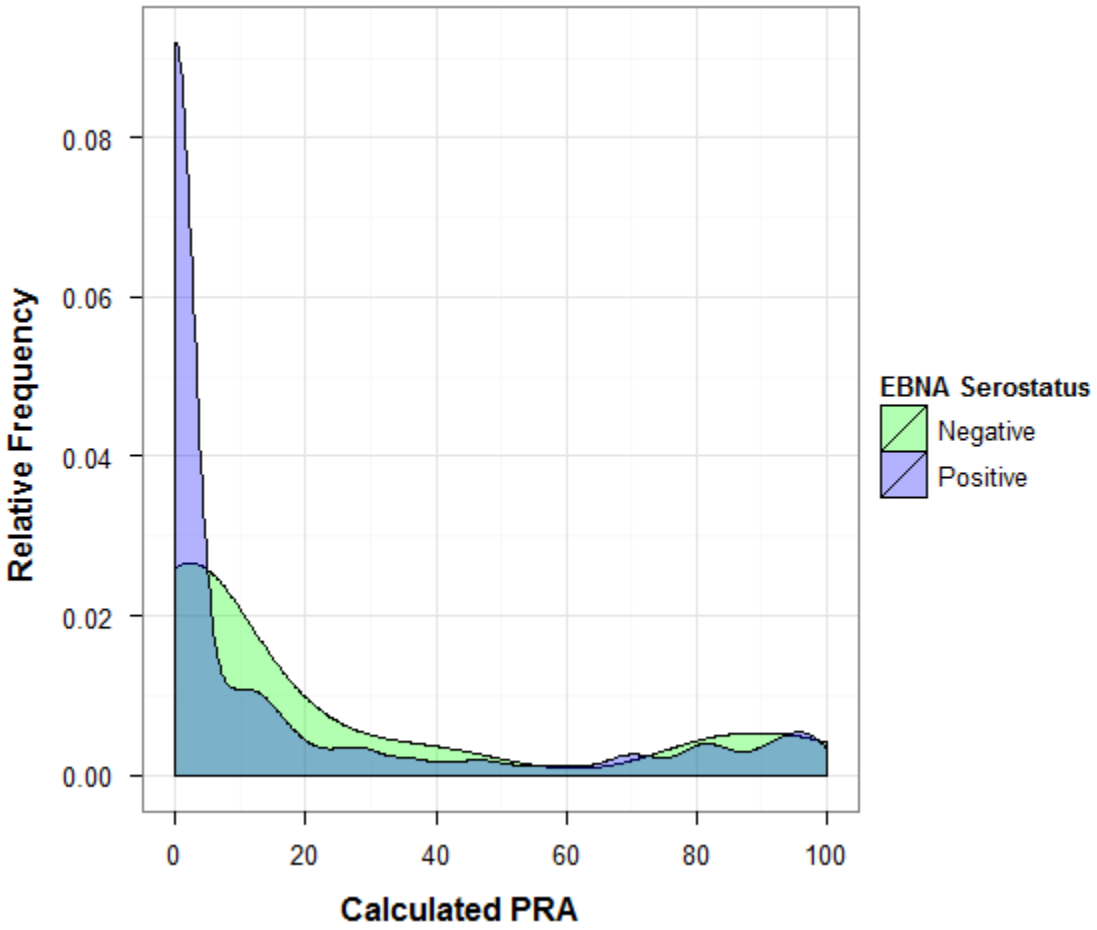


B)



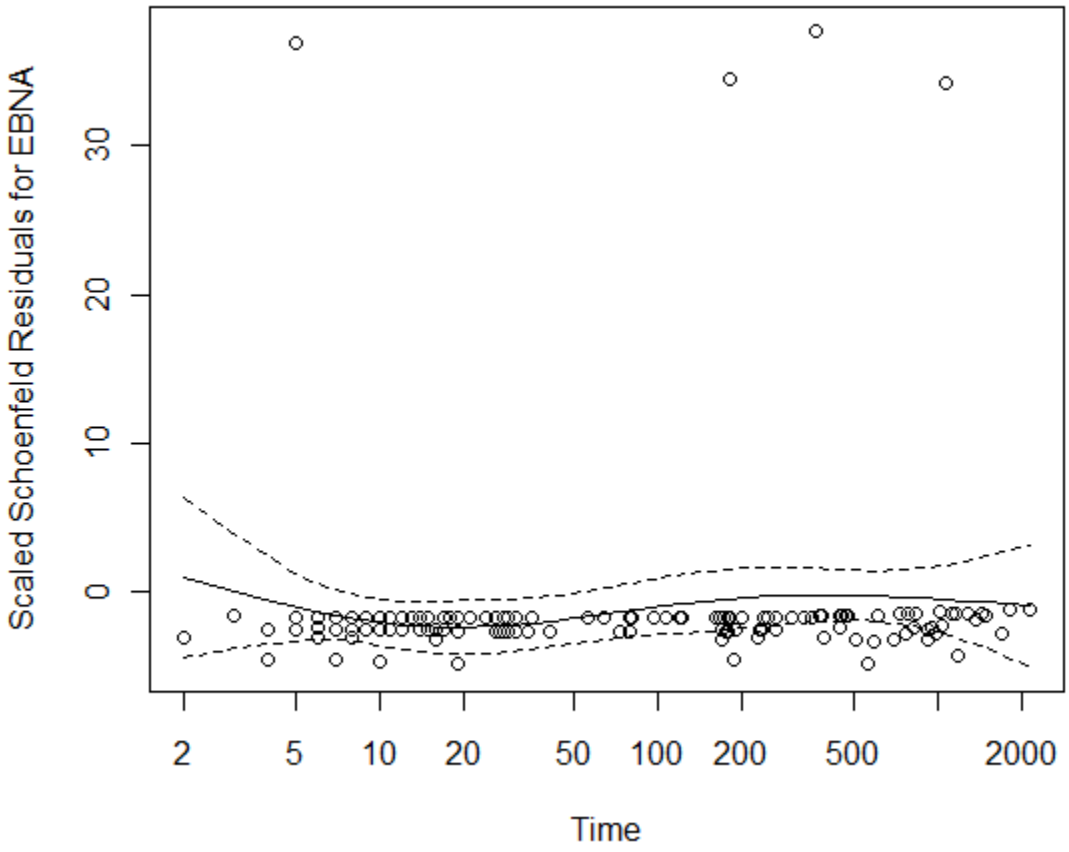
(A) Kaplan-Meier survival curves of patients in the Emory cohort with EBV latency by EBNA serostatus demonstrates that patients without EBNA antibodies at the time of transplant have significantly lower rates of acute rejection. EBNA is one of two antibodies assessed prior to transplantation to determine whether a patient has previously been infected with EBV. All patients in this figure tested positive for EBV VCA IgG antibodies, suggesting that they did have EBV latency, but did not develop EBNA antibodies. (B) The absence of EBNA antibodies is associated with decreased rates of rejection in the NIH cohort as well.

Figure 11. Comparison of the Distribution of CPRA Values by EBNA Serology



This figure suggests that the distributions of calculate panel reactive antibody (CPRA) do not differ between EBNA seronegative and EBNA seropositive patients. CPRA is a value calculated based on the presence of anti-HLA antibodies to estimate the proportion of potential deceased donor's that a given recipient would not be compatible with. Although EBNA antibodies appear to be associated with alloimmunity, this effect is not related to associated increases in anti-HLA antibodies.

Figure 12. Scaled Schoenfeld Residuals for EBNA Serostatus



Scaled Schoenfeld residuals in Cox regression models are a measure of the model error attributable to a given regression variable. Plots of Scaled Schoenfeld residuals versus time can be used to assess the time-dependence of variables in Cox regression models. The horizontal linearity in this figure suggests that the effect of EBNA antibodies on the hazard of acute rejection does not vary with time.

Tables

Table 1. Demographics of Emory University Cohort

Gender	Female Male	43% 57%
Race	Black or African American Caucasian Other	44.6% 45.5% 9.9%
Follow-up Time (d)	Median (IQR)	1114 (739 – 1488)
HLA A mismatches	0 1 2	17.8% 34.5% 47.7%
HLA B mismatches	0 1 2	13.9% 28.7% 57.4%
HLA DR mismatches	0 1 2	23.8% 43.8% 32.3%
Donor Type	Deceased Donor Living-Related Donor Living-Unrelated Donor	67.9% 17.4% 14.7%
Age at Tx (y)	Range Median (IQR) Mean	18.6-80.6 52.0 (38.8-60.8) 50.2
Induction Therapy	None Anti-IL2R antibody Anti-thymocyte globulin	25.1% 19.4% 55.6%
Previous Kidney Transplant		7.1%

For the Emory University cohort, 495 patients met the inclusion and exclusion crieteria. The demographic characteristics of these individuals are summarized in the table above. HLA mismatches refers to the number of mismatches between the recipient and donor at the indicated locus. As there are two alleles at each locus for each individual, the number of mismatches ranges between 0 and 2.Donor type refers to the source of the transplanted kidney. Living-related donors are those kidneys that come from living individuals that are biologically related to the transplant recipient (e.g. children, parents, siblings). Living-unrelated donors are those kidneys that derive from living individuals that are not biologically related to the transplant recipient (e.g. spouses, friends, paired-donations). Induction therapy is immunosuppressive therapy administered in the peri-transplant period, a period of high risk of transplant rejection. Anti-IL2R antibodies refer to agents that target the interleukin-2 receptor such as basiliximab or daclizumab. Anti-thymocyte globulin refers to polyclonal antibody preparations targeting human lymphocytes.

Table 2. Summary of Univariate Kaplan-Meier Analysis and Cox Regression For Time to First Acute Rejection

		Univariate Cox Regression				p-value for KM Log-Rank Test
		Parameter Estimate	SE	HR (95% CI)	Wald Test p-value	
Age at Transplant	1-Yr Increase in Age	-0.00161	0.0059	0.998 (0.987-1.01)	0.78	----
HLA A Mismatches	1 vs 0	0.403	0.268	1.496 (0.886 -2.528)	0.116	0.111
	2 vs 0	0.528	0.255	1.696 (1.029 – 2.795)	----	
HLA B Mismatches	1 vs 0	0.558	0.329	1.747 (0.916-3.331)	0.016*	0.013*
	2 vs 0	0.828	0.306	2.289 (1.257 – 4.168)	----	
HLA DR Mismatches	1 vs 0	0.378	0.229	1.459 (0.932 – 2.286)	0.022*	0.021*
	2 vs 0	0.636	0.233	1.889 (1.197 – 2.982)	----	
Delayed Graft Function	Yes vs. No	0.593	0.178	1.809 (1.277 – 2.564)	0.0009*	0.0007*
Gender	Male vs. Female	0.183	0.166	1.200 (0.868 – 1.661)	0.270	0.269
Non-adherence	Yes vs. No	1.524	0.220	4.588 (2.979 – 7.066)	4.7E-12	3.2E-14*
On Dialysis at Transplant	Yes vs. No	0.566	0.302	1.760 (0.975 – 3.179)	0.061	0.057
Recipient Race	Other vs. Black	-0.493	0.309	0.611 (0.334 – 1.120)	0.016*	0.014*
	White vs. Black	-0.464	0.172	0.629 (0.449 – 0.880)	----	
Previous Kidney Transplant	Yes vs. No	0.285	0.290	1.329 (0.753 – 2.348)	0.327	0.325
Donor Type	Living-Related vs. Deceased	-0.206	0.234	0.814 (0.514 – 1.288)	0.543	0.603
	Living-Unrelated vs. Deceased	0.068	0.222	1.071 (0.692 – 1.656)	---	
Year of Transplant	2006+2007 vs. 2005	-0.431	0.190	0.650 (0.448 – 0.944)	1.4E-5*	7.44E-6*
	2008+2009 vs. 2005	-1.097	0.233	0.334 (0.212 – 0.527)	----	

Prior to the development of a multivariate model, the unique effects of all factors on rejection were assessed independently. The results above include 153 acute rejection events in 495 patients. As above, HLA mismatches refers to the number of mismatched HLA alleles between the recipient and the donor at the specified loci. Delayed graft function is defined as the need for dialysis in the first week after transplantation as a result of a non-immediately functioning

transplanted kidney. Non-adherence was identified subjectively by clinician report that the patient had not been taking their medications or observation that peripheral blood levels of a monitored medication were significantly below their expected level. The variable "On Dialysis at Transplant" corresponds to whether a patient was transplanted prophylactically before needing dialyisis or was on either peritoneal- or hemo- dialysis at the time of transplantation. Previous kidney transplant refers to whether a patient had previously received another renal transplant that has since failed. Year of transplant was classified as one of "2005", "2006 or 2007", or "2008 or 2009". Some years were collapsed into single variables because of similarities in the outcomes for these years. The effect of year of transplant is likely due to improving clinical management over these time periods. Effects that were significant for single variables are highlighted in green. As shown in the table, the variables that were found to significantly impact rejection rate in univariate analyses were HLA B mismatches, HLA DR mismatches, delayed graft function, non-adherence, the race of transplant recipient, and the year that the transplant was performed. Although not statistically significant, the dialysis status of the patient at transplantation was nearly associated with acute rejection rates.

Table 3. Post-Refinement Cox Regression Model of Factors Associated with Acute Rejection (Emory Cohort)

		Parameter Estimate	SE	HR (95% CI)	Wald Test p-value
Age (y)	1-yr Increase	0.050	0.015		0.001
	(1-yr increase)*log10(time)	-0.026	0.008		0.001
HLA B Mismatches	1 vs. 0	0.160	0.383	1.173 (0.553 - 2.487)	0.677
	2 vs. 0	0.284	0.367	1.329 (0.648 - 2.726)	0.438
HLA DR Mismatches	1 vs. 0	1.389	0.606		0.022
	(1 vs. 0)*log(time)	-0.650	0.290		0.025
	2 vs. 0	1.380	0.617		0.025
	(2 vs. 0)*log(time	-0.481	0.292		0.099
Delayed Graft Function	< 30 d vs. None	5.512	1.169		2.41E-06
	(< 30 d vs. None)*log(time)	-4.302	1.166		2.25E-04
	>=30 d vs. None	0.256	0.297	1.291 (0.722 - 2.310)	0.389
Gender	Male vs. Female	-0.088	0.214		0.681
Non-adherence	Yes vs. No	0.024	0.628		0.970
	(Yes vs. No)*log(time)	0.844	0.304		0.005
Donor Type	Living-Unrelated Donor (LUT) vs. Deceased	0.460	0.383		0.230
	Living-Related Donor (LRT) vs. Deceased	1.400	0.865		0.106
	(LRT vs. Deceased)*log(time)	-0.834	0.468		0.075
Gender*Donor Type Interaction	(Male vs. Female)*(LUT vs. Deceased)	-0.364	0.473		0.441
	(Male vs. Female)*(LRT vs. Deceased)	0.629	1.077		0.559
	(Male vs. Female)*(LUT vs. Deceased)*log(time)	-0.178	0.612		0.771

This Cox regression model is based off of that in Table 2, following the removal of the variables representing previous kidney transplant, recipient race, the number of HLA A mismatches, and dialysis at the time of transplant. Again, hazard ratios and confidence intervals are not shown for variables with interaction terms because these values change depending on the desired levels for each variable. Regression variables with significant effects are highlighted in green and include age (in years), a time-dependent term for age, the number of HLA DR mismatches, a time-dependent term the number of HLA DR mismatches, time-dependent effects for delayed-graft function, and a declining effect of non-adherence with the log of time after transplant.
This model includes 495 observations with 153 acute rejection events. The likelihood ratio test for this model of 110.4 with 20 degrees of freedom yields p = 1.67 x 10^-14. The AIC for this

model is 1388.1. Abbreviations are as follows: HLA: human leukocyte antigen, MM: mismatch, DGF: delayed graft function, LUT: living-unrelated donor, LRT: living-related donor.

Table 4. Demographics of NIH Cohort

Gender	Female	36.2%
	Male	63.8%
Race	Black or African American	23.9%
	Caucasian	66.7%
	Other	9.4%
Follow-up Time (d)	Median (IQR)	2062 (1765-2993)
HLA A mismatches	0	21.0%
	1	35.5%
	2	43.5%
HLA B mismatches	0	14.5%
	1	37.7%
	2	47.8%
HLA DR mismatches	0	13.0%
	1	52.9%
	2	34.1%
Donor Type	Deceased Donor	30.4%
	Living-Related Donor	34.8%
	Living-Unrelated Donor	34.8%
Age at Transplant (Years)	Range	9.9 – 68.3
	Median (IQR)	43.4 (32.5-52.6)
	Mean	42.7
Induction Therapy	None	7.2%
	Anti-IL2R antibody	22.5%
	Alemtuzumab	27.5%
	Anti-thymocyte globulin	42.8%
Previous Kidney Transplant		5.8%

This table provides a summary of the demographic information of the 138 eligible patients in the NIH cohort. Relative to the Emory cohort, this group had fewer women, had fewer Black or African American patients, had longer follow-up, was younger, had more living donors, and were more likely to receive induction therapy. Induction therapy is typically strong immunosuppressive therapy given around the time of transplant to minimize alloimmunity to the allograft during this vulnerable time. Anti-IL2R antibodies include basiliximab and daclizumab. Alemtuzumab is a monoclonal antibody to human CD52. IQR: Inter-quartile range.

Table 5. Summaries of Univariable Analyses for Time to Acute Rejection (NIH Cohort)

		Univariate Cox Regression				p-value for KM Log-Rank Test
		Parameter Estimate	SE	HR (95% CI)	Wald Test p-value	
Age at Transplant	1-Yr Increase in Age	-0.012	0.009	0.988 (0.971 – 1.006)	0.196	----
HLA A Mismatches	1 vs 0	0.299	0.351	1.348 (0.677 – 2.684)	0.400	0.393
	2 vs 0	0.450	0.335	1.569 (0.814 – 3.023)	---	
HLA B Mismatches	1 vs 0	1.240	0.536	3.454 (1.208 – 9.877)	0.024*	0.0132*
	2 vs 0	1.429	0.524	4.173 (1.493 – 11.662)	----	
HLA DR Mismatches	1 vs 0	1.227	0.524	3.409 (1.221 – 9.524)	0.0542	0.037*
	2 vs 0	1.262	0.536	3.534 (1.236 – 10.107)	----	
Recipient Gender	Male vs. Female	-0.234	0.241	0.791 (0.494 – 1.268)	0.330	0.331
Recipient Race	Other vs. Black	-0.778	0.553	0.459 (0.155 – 1.357)	0.311	0.293
	White vs. Black	0.011	0.275	1.011 (0.590 – 1.733)	----	
Donor Type	Living-Related vs. Deceased	0.201	0.323	1.223 (0.649 – 2.303)	0.005*	0.004*
	Living-Unrelated vs. Deceased	0.873	0.300	2.395 (1.330 – 4.314)	---	
Previous Kidney Transplant	Yes vs. No	-0.138	0.515	0.871 (0.318 – 2.389)	0.789	0.786
Donor Gender	Male vs. Female	-0.642	0.243	0.526 (0.327 – 0.847)	0.008*	0.007*
Donor Age	1-Yr Increase in Age	0.009	0.009	1.009 (0.991 – 1.028)	0.309	---

As in the analysis of the Emory cohort, univariate analyses for the effects of each potential regression variable were undertaken prior to the development of multivariate models. This table presents the results of both KM analyses (for factor variables) and univariate Cox regression models. Variables that were found to be clinically significant in univariate analysis are highlighted in green and include HLA B mismatches, HLA DR mismatches, donor type, and donor gender.

Abbreviations: HLA: Human leukocyte antigen; SE: standard error; HR: hazard ratio; CI: confidence interval

Table 6. Cox Regression for Time to First Clinically Significant Acute Rejection (NIH Cohort)

		Parameter Estimate	SE	HR (95% CI)	P
Age	1-yr Increase in Age	-0.012	0.010	0.988 (0.969 - 1.007)	0.210
HLA B Mismatches	1 vs. 0	1.270	0.600	3.560 (1.098 - 11.549)	0.034
	2 vs. 0	1.236	0.597	3.441 (1.069 - 11.082)	0.038
HLA DR Mismatches	1 vs. 0	0.894	0.918		0.330
	(1 vs. 0)*log(time)	-0.207	0.381		0.590
	2 vs. 0	0.325	0.605	1.384 (0.423 - 4.534)	0.590
Donor Type	Living-Related Donor (LRT) vs. Deceased	1.020	0.619		0.100
	Living-Unrelated Donor (LUT) vs. Deceased	2.193	0.581		0.00016
Gender	Male vs. Female	0.848	0.578		0.140
Gender*Donor Type Interaction	(Male vs. Female)*(LRT vs. Deceased)	-1.238	0.719		0.085
	(Male vs. Female)*(LUT vs. Deceased)	-2.077	0.681		0.002

This table presents the Cox regression model used for examining the effect of EBV viral load. It is based on a full model corrected for violations of the proportional hazards assumption. Variables that were considered not to be required were removed to refine the model. The log(likelihood) of the model is -313.6. For the likelihood ratio test versus the empty model, this yields a chi-square value of 34.3. With 11 degrees of freedom, this results in p=0.0003. The AIC for this model is 649.1 which is improved relative to the full model.
Abbreviations: HLA: Human leukocyte antigen, MM: mismatch(es), LRD: living-related donor, LUR: living-unrelated donor.

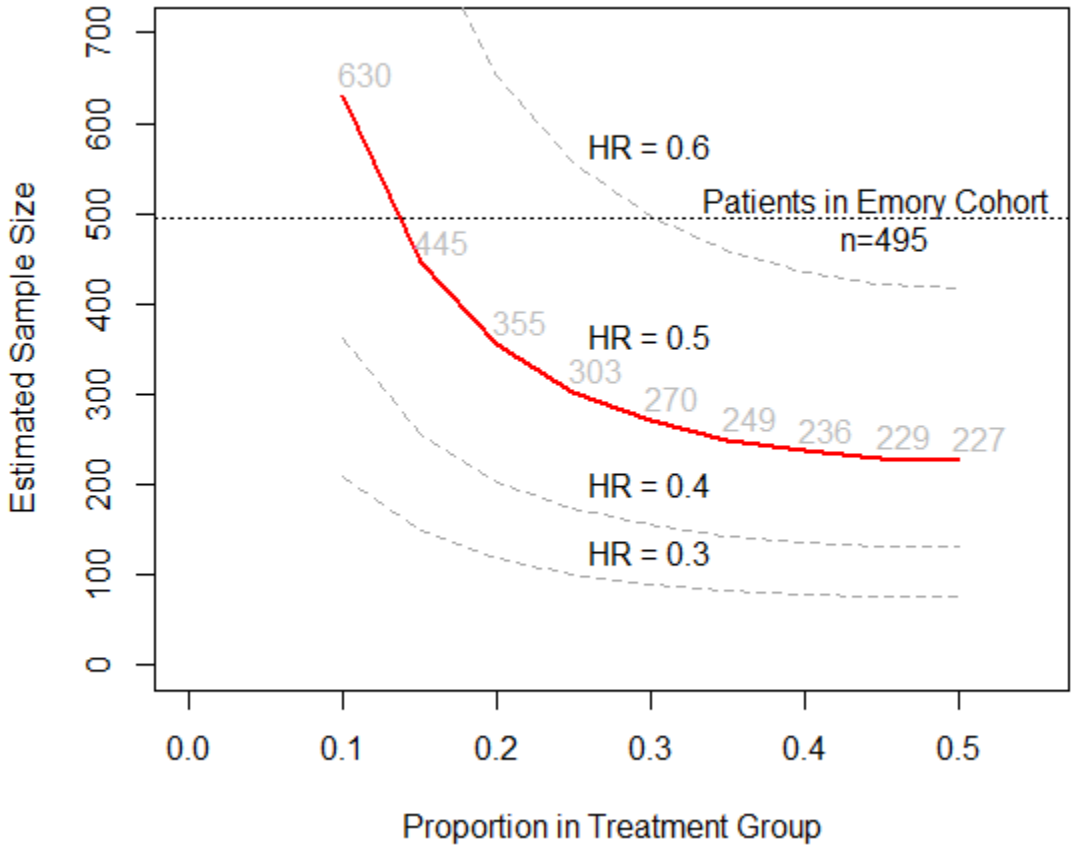
Table 7. Mixed-Effects Model for Change in Tacrolimus Level With EBV PCR

Variable		Parameter Estimate	SE	Parameter 95% CI	
Intercept		12.56	2.15	8.35	16.77
Mean Previous Tacrolimus Levels		-0.57	0.16	-0.88	-0.27
log10 (Viral Load)		-0.73	0.22	-1.16	-0.30
log10 (Days Post-Transplant)		-1.41	0.25	-1.90	-0.92
Year of Event	2008 vs. 2007	-0.79	1.16	-3.08	1.49
	2009 vs. 2007	-0.41	1.23	-2.82	1.99
	2010 vs. 2007	0.04	1.29	-2.50	2.58
	2011 vs. 2007	2.63	1.57	-0.44	5.69

To further assess the relationship between EBV viral load and the rate at which tacrolimus levels were decreased, a mixed effects model was developed. Figure 8 demonstrated that larger viral loads were associated with larger magnitude decreases in tacrolimus levels. The development of this mixed effects model was necessary to account for the correlation between observations from within the same individual. Additionally, this mixed effects models allowed for the control of the other variables that were found to significantly impact the magnitude of the change in tacrolimus level. The model included fixed effects for the mean of previous tacrolimus levels, the base-10 log of viral load (previous maximum), the base-10 log of the number of days since transplantation, and the year in which the EBV viral measurement was made. Random effects were maintained for the intercept, the base-10 log of EBV viral load, and the base-10 log of the number of days since transplantation, all by individual patient. As shown in the table, this model suggests that each 10-fold increase in EBV viral load is associated with a -0.73 (95% CI: -1.16 to -0.30) decrease in the change of tacrolimus levels. This is consistent with the hypothesis that larger magnitude EBV viral loads are associated with more aggressive decreases in immunosuppression.

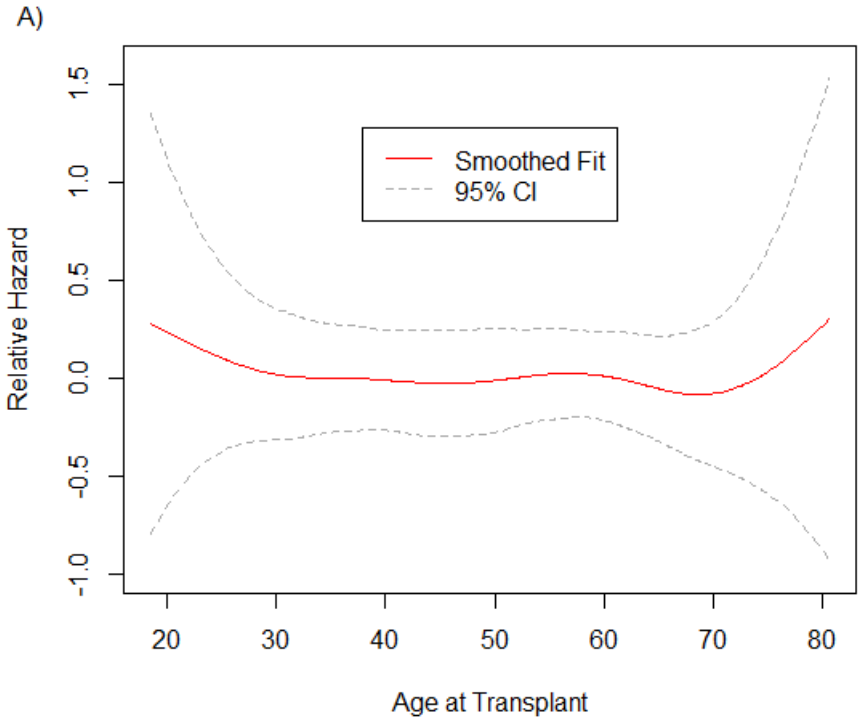
Appendix

Appendix 1. Estimated Sample Size Versus Proportion of Patients in EBV Viremia Group



This figure shows the estimated sample size for various assumptions regarding the patients in the Emory cohort. The sample sizes were estimated using methods for sample size calculations for Cox proportional hazards regression as described above (page 18). Given that individual patients could move in and out of the treatment group at various times, depending on their EBV PCR status, it was not possible to estimate an exact proportion of patients expected to be in the treatment group which is why it is allowed to vary. Additionally, the magnitude of the effect of EBV viremia on the hazard ratio was not known a priori so calculations were performed for various hazard ratios. As shown in the figure, the sample size of 495 patients available in the Emory cohort is large enough to detect an HR comparing EBV viremia to absence of EBV viremia of 0.5 with as few as 15% of patients in the EBV viremia group. The samples size calculations shown here are calculated at the 95% significance level with 80% power. The R2 value for the correlation between EBV viremia and other regression variables was estimated to be 0.2.

Appendix 2. Assessment of Functional Form for Age at Transplant



(A) To determine how best to include age at transplant in the regression model for time to acute rejection, a univariate Cox regression model was assessed with age entered into the model as a penalized-spline fit. As in this figure, the fit of the penalized spline can then be plotted against age to determine the appropriate functional form. The fit is fairly linear and horizontal except at the extremes of age. The error associated with the estimates at the extremes of age is large enough that it appears reasonable to model the impact of age on the log-hazard of acute rejection as a linear factor. (B) Similarly to the approach in (A), the plot in B shows the Martingale residuals (a measure of error) for a null Cox regression model as a function of age at transplant. This, again, suggests that the log hazard can appropriately be modeled as a linear function of age.

Appendix 3. Cox Regression Model Corrected for Non-Proportional Hazards (Emory Cohort)

		Parameter Estimate	SE	HR (95% CI)	P
Age (y)	1-yr Increase	0.050	0.015		0.001
	(1-yr increase)*log10(time)	-0.026	0.008		0.001
HLA A Mismatches	1 vs. 0	-0.307	0.357	0.736 (0.365 - 1.482)	0.390
	2 vs. 0	-0.272	0.363	0.761 (0.374 - 1.551)	0.453
HLA B Mismatches	1 vs. 0	0.233	0.445	1.262 (0.528 - 3.018)	0.600
	2 vs. 0	0.379	0.434	1.461 (0.623 - 3.424)	0.383
HLA DR Mismatches	1 vs. 0	1.478	0.615		0.016
	(1 vs. 0)*log(time)	-0.679	0.292		0.020
	2 vs. 0	1.497	0.627		0.017
	(2 vs. 0)*log(time	-0.527	0.294		0.073
Delayed Graft Function	< 30 d vs. None	5.481	1.174		3.01E-06
	(< 30 d vs. None)*log(time)	-4.311	1.168		2.24E-04
	>=30 d vs. None	0.218	0.306	1.243 (0.682 - 2.266)	0.477
Gender	Male vs. Female	-0.098	0.215		0.649
Non-adherence	Yes vs. No	-0.097	0.634		0.878
	(Yes vs. No)*log(time)	0.864	0.306		0.005
On Dialysis	Yes vs. No	0.389	0.329	1.475 (0.774 - 2.811)	0.237
Race	Other vs. Black/African American	-0.324	0.327	0.723 (0.381 - 1.373)	0.322
	Caucasian vs. Black/African American	-0.226	0.198	0.798 (0.541 - 1.175)	0.253
Donor Type	Living-Unrelated Donor (LUT) vs. Deceased	0.565	0.393		0.150
	Living-Related Donor (LRT) vs. Deceased	1.449	0.867		0.095
	(LRT vs. Deceased)*log(time)	-0.790	0.468		0.092
Previous Kidney Transplant	Yes vs. No	0.024	0.331	1.024 (0.536 -1.958)	0.943
Gender*Donor Type Interaction	(Male vs. Female)* (LUT vs. Deceased)	-0.342	0.478		0.475
	(Male vs. Female)* (LRT vs. Deceased)	0.667	1.080		0.537
	(Male vs. Female)* (LUT vs. Deceased)* log(time)	-0.196	0.611		0.749

This Cox regression model includes all variables that were considered to be readily available and potentially associated with acute rejection. The presented model is corrected for non-proportional hazards. Interaction and time-dependent terms are indicated by "*". Hazard ratios and confidence intervals are not shown for variables with interaction terms because these values change depending on the desired levels for each variable. This model includes 495 observations with 153 acute rejection events. Regression variables with significant effects are highlighted in green and include age (in years), a time-dependent term for age, the number of HLA DR mismatches, a time-dependent term the number of HLA DR mismatches, time-dependent effects for delayed-graft function, and a declining effect of non-adherence with the log of time after transplant. The likelihood ratio test for this model of 114.4 with 26 degrees of freedom yields p = 4.6 x 10^-13. The AIC for this model is 1396.1. Abbreviations are as follows: HLA: human leukocyte antigen, MM: mismatch, DGF: delayed graft function, LUT: living-unrelated donor, LRT: living-related donor.