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Minireview

Nonhuman Primate Transplant Models Finally Evolve: Detailed Immunogenetic Analysis Creates New Models and Strengthens the Old

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Nonhuman primate (NHP) models play a critical role in the translation of novel therapies for transplantation to the clinic. However, although MHC disparity significantly affects the outcome of transplantation, until recently, experiments using NHP models were performed without the ability to rigorously control the degree of MHC disparity in transplant cohorts. In this review, we discuss several key technical breakthroughs in the field, which have finally enabled detailed immunogenetic data to be incorporated into NHP transplantation studies. These advances have created a new gold-standard for NHP transplantation research, which incorporates detailed information regarding the degree of relatedness and the degree of MHC haplotype disparity between transplant pairs and the precise MHC alleles that both donors and recipients express. The adoption of this new standard promises to increase the rigor of NHP transplantation studies and to ensure that these experiments are optimally translatable to patient care.

Key words: MHC, nonhuman primate, transplant

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Although standard-of-care immunosuppression results in significant short-term success after solid organ transplant (SOT) and hematopoietic stem cell transplantation (HSCT), these potentially life-saving treatments continue to face long-term challenges: chronic rejection and graft loss for SOT, acute and chronic graft-versus-host disease (GvHD) and donor hematopoietic graft rejection for HSCT, and on- and off-target toxicities of long-term immunosuppression for both. Thus, there is a critical unmet need in both fields to develop novel, selective immunomodulatory strategies and ultimately, to induce immune tolerance after transplantation. To make progress toward this challenging goal, clinically relevant translational models are needed. For the past 2 decades, studies using nonhuman primates (NHP) and in particular, rhesus macaques (RM) and cynomolgus macaques, have been critical to advances made in the field of SOT, leading to insights into the mechanism of action of immunomodulatory strategies and guiding the clinical implementation of several new agents (1–5). However, despite the critical role that NHP studies play in bridging basic and clinical research, until very recently, they have been undertaken with a significant disadvantage in comparison to other model systems and clinical trials. Although studies in rodents, canines and in patients always include detailed knowledge of both the degree of relatedness and the degree of major histocompatibility (MHC) matching between transplant pairs, NHP transplantation studies have usually been performed with minimal information regarding animal pedigree or MHC genetics. Given the critical impact that both MHC- and minor histocompatibility-antigenic disparity makes on transplantation, ignorance of these relationships represented a significant "wild-card" in the interpretation of NHP studies of SOT and minimized the usefulness of NHP models for preclinical studies of HSCT and GvHD in settings other than parent-to-offspring haploidentical transplants. In this review, we will discuss the state of the field with respect to MHC immunogenetics and highlight paradigm-changing advances that promise to fundamentally improve the rigor with which primate transplantation studies are performed and to open important new avenues for NHP translational research.

The macaque MHC: duplicated genes, expanded expression profiles

The functional similarities between human and NHP (in particular, RM and cynomolgus macaque) immune systems are well documented and are evidenced by the fact that both diagnostic and therapeutic agents, designed for use in patients, are much more often cross-reactive with NHP targets than with either murine or canine counterparts (1–16). This represents a significant advantage for NHP models, in terms of both the rapidity and the rigor with which

experimental conclusions can be translated to the clinic. However, although functional homology is high between macaques and humans, significant genetic disparity exists between the two species, especially in the MHC, which is structurally much more complex in macaque species (17–26). This complexity has, historically, made comprehensive analysis of the MHC in these experimental animals difficult to perform, leading to experimentation that usually proceeded without a complete description of the degree of MHC disparity between transplant pairs.

Although macaque and human evolution diverged relatively recently (25 million years ago; Ref. 22) and thus, the two species share significant overall sequence homology (90–94%; Ref. 22), one of the most structurally divergent areas of the genome is the MHC. As described in detail by Daza-Vamenta et al. (18), one of the most important distinctions between the two MHC regions is size: although the human MHC spans approximately 3.7 megabases (Mb) on human chromosome 6, the RM MHC is much larger, comprising 5.3 Mb on the rhesus chromosome 4 (the macaque homologue of human chromosome 6; Ref. 27). The increased size of the RM MHC region is caused by significant genetic duplication and reshuffling, encompassing both the macaque MHC Class I A and B genes (referred to as "mamu-A" and "mamu-B", RM lack a structural homolog of human HLA-C) and to a lesser extent, the Class II genes. The extent of the expansion and duplication is most striking in the Mamu-B region, in which a massive increase in size has occurred, from 100 kb in humans to over 1.3 Mb in its RM paralogue (18,22). The result is that rather than having only one HLA-A, HLA-B and HLA-C gene per chromosome (as occurs in humans), the RM MHC contains up to four mamu-A genes per chromosome and, remarkably, as many as 14 full-length mamu-B genes per chromosome. Thus, a heterozygous RM could theoretically possess 8 Mamu-A and 28 Mamu-B genes, compared to a heterozygous human, who would have only two HLA-A, two HLA-B and two HLA-C genes per cell.

The increased complexity of RM MHC genetics is not isolated to chromosomal structure, but rather, results in parallel complexity of MHC expression. Thus, using cDNA cloning strategies, complex transcriptional patterns for the macaque MHC have been documented, including the existence of up to 7 mamu-A transcripts and up to 18 mamu-B transcripts per cell for some haplotypes (24,25,28). Importantly, these studies also demonstrated that the expression of RM MHC RNAs is not codominant, as it is in humans. Rather, "major" and "minor" cDNAs were identified, suggesting transcriptional control of MHC class I gene expression and resulting in a hierarchal expression pattern for the macaque MHC (25). Given the complex, hierarchical expression pattern of macaque MHC cDNAs, allele-specific DNA-based MHC typing is not sufficient to predict the MHC expression pattern for these animals. This results in a major barrier to MHC typing in macaques, because traditional cDNA-cloning-based approaches to gene expression analysis have been both costly and complicated to perform on large transplant cohorts. Furthermore, reagents for interrogating cell-surface expression of RM MHC molecules are not available.

The evolution of macaque MHC typing: microsatellite and pyrosequencing breakthroughs

Recently, two technical advances have occurred in the field that have fundamentally improved the ability to MHC-type macaques. The first major development was the application of DNA microsatellite-based typing to rhesus pedigree analysis and MHC haplotyping. This advance capitalized on the identification of multiple panels of DNA microsatellite probes, capable of discerning the inheritance patterns of both autosomal and sex chromosomes between generations of macaques, such that accurate pedigrees could be drawn to depict the complex familial relationships present in macaque breeding groups (18–22,26,29). This analysis has now been used to ascertain mating patterns and paternity in multiple colonies throughout the world and confirms macaque harem mating, in which a small number of males mate with multiple females within a colony to produce offspring. Thus, as shown in example in Figure 1, in the RM breeding colony sponsored by the National Institute for Allergy and Infectious Diseases (NIAID), a single sire (animal ID#S1 is shown in the Figure) will often mate with as many as 20 females, producing 10–20 offspring a year, all related as half-siblings through the patriline. In 2005, Penedo et al. further described eight MHC-linked microsatellites, that could be used to rapidly and inexpensively define the inheritance of complete MHC haplotypes in a previously pedigreed colony (30). Although, this analysis does not identify individual MHC alleles, when combined with pedigree analysis, it identifies the origin of each MHC haplotype within a family group, thus permitting the determination of the degree of MHC haplotype sharing within a colony and between potential transplant pairs. Our group has collaborated with other scientists in the NIAID- and NIDDK-sponsored Nonhuman Primate Transplant Tolerance Cooperative Study Group (NHPCSG) to apply microsatellite-based pedigree analysis and MHC haplotyping to NIAID NHP colonies, which were established to support NHPCSG research. We also devised a strategy to grahpically depict MHC haplotype sharing within a coloy (Figure 1), to facilitate transplant choices for NHPCSG researchers. This work is most complete for the RM colonies, but is also underway for both the Mauritian-origin and Indonesian cynomolgus macaques. Thus, in Figure 1, MHC haplotypes are depicted for each animal in the RM breeding group using color-coded symbols that are overlaid onto the pedigree chart. These symbols enable an investigator to rapidly determine both the degree of relatedness and the degree of MHC haplotype sharing between full- and half-siblings in the colony, significantly facilitating transplant choice based on both of these critical parameters.

The second major advance has been the application of pyrosequencing to NHP MHC typing. Thus, in 2009,

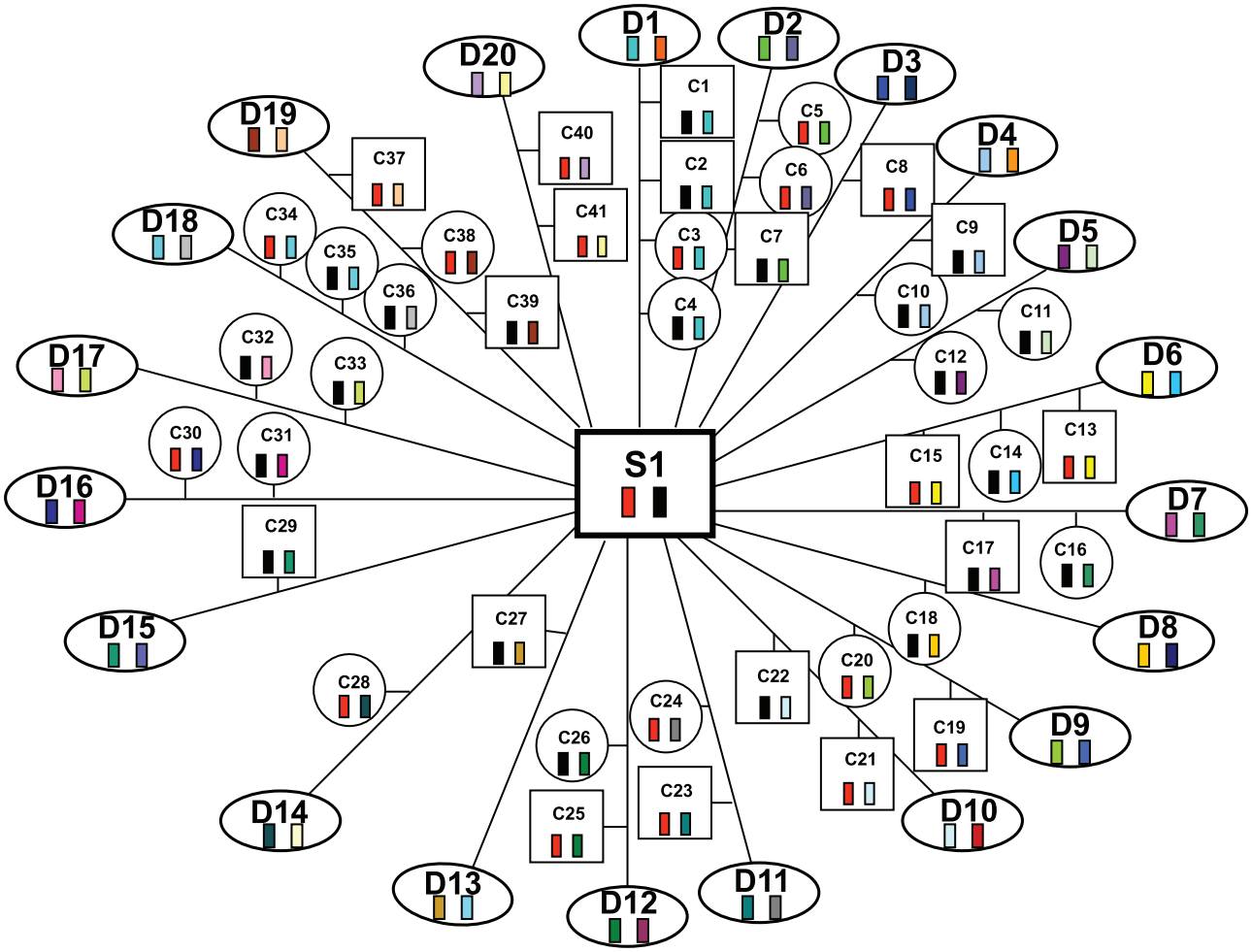

Figure 1: Pedigree and MHC Inheritance Map for S1, his mating pairs and his offspring. The sire, S1, is depicted at the center of the circular pedigree. Each dam with whom, he mated is depicted on the edge of the circle, with a linear connection to S1. Offspring from each of the mating pairs are depicted emanating from these connections. Circles indicate female offspring and dams. Squares indicate male offspring and sires. MHC haplotypes are indicated by color-coded bars associated with each animal. S1's MHC haplotypes are indicated as either a red or a black bar. MHC haplotypes for each of the dams are indicated by unique color-coded bars. The inheritance of an MHC haplotype from the sire or dam is indicated by the color of the bars for each of the offspring.

Wiseman et al. reported a technological breakthrough in the typing of RM expressed MHC alleles, by the application of massively parallel pyrosequencing of cDNA-PCR amplicons (31). The introduction of MHC pyrosequencing has had two major impacts on the field: First, it has enabled rapid, comprehensive analysis of MHC expression for large numbers of experimental animals, in a multiplexed fashion. Second, it has provided an even more detailed description of macaque MHC expression patterns than previously possible, including an exponential increase in the discovery of novel MHC Class I alleles. Pyrosequencing has also confirmed the MHC expression complexity that was originally determined through cDNA cloning. Thus, Wiseman et al. documented the expression of four mamu-A alleles and nine mamu-B alleles from a single haplotype, confirmed the hierarchical MHC expression patterns originally observed through cDNA cloning techniques (25) and showed that these expression patterns, even of the least abundant transcripts, were heritable. Although pyrosequencing remains a relatively costly technique, its advent is already changing the field of NHP transplantation, because researchers can now determine the complete MHC type for any experimental animal and use this information during transplant planning and during the interpretation of experimental outcomes (32–34). Importantly, given the requirement for specialized expertise to accurately perform pyrosequence analysis, pyrosequence-based MHC typing can be merged with DNA-based microsatellite analysis,

such that, for any "family trio" (consisting of a sire, dam and one of their offspring), once MHC expression patterns are mapped to microsatellite-defined haplotypes, high-throughput microsatellite typing can subsequently be used to infer the complete MHC expression pattern for all of their subsequent progeny.

A new era of defined MHC disparity in NHP transplants studies

Given the importance of MHC disparity to transplant outcome, the NIAID has supported a major effort to capitalize on the new technical advances, to fundamentally improve the rigor of transplant studies using NHP models. This effort has included the creation of RM directed-breeding groups for which exhaustive microsatellite- and pryosequencing-based MHC typing is performed, with efforts underway to extend this level of detailed typing to the NIAID cynomolgus colonies. As a result of this initiative, a critical new tool is emerging for the NHPCSG transplant tolerance researchers: an NHP colony for which the degree of information available about the relatedness of potential transplant pairs and their degree of MHC disparity will closely match what is available clinically. Our group has created a similar colony at the Yerkes National Primate Research Center, designed to allow all NHP transplant studies (including those not funded through the NIAID program) to proceed in the setting of detailed MHC-based transplant planning.

For HSCT, the availability of the Yerkes Primate Center MHC-defined RM colony has enabled the creation of a new translational model to study one of the most devastating clinical complications of transplantation and GvHD. The creation of the recently published RM GvHD model (35) was impossible without this colony, given its focus on sibling and half-sibling MHC haplo-identical transplant pairs, rather than parent-offspring pairs. The creation of a model that utilizes MHC-defined sibling HSCT donors has an important advantage over parent-offspring transplant models, because parent-offspring models are significantly more challenging to sustain, given their continued reliance on breeding-age sires and dams as transplant donors. The results described in Miller et al. have established the immunologic natural history of NHP GvHD and describe the first examination of the ability of T-cell costimulation blockade to reduce its grave clinical consequences, including gastrointestinal injury, liver dysfunction and early death (35). The RM GvHD model is expected to offer several unique advantages for rapid clinical translation of novel therapeutics compared with existing murine models of GVHD. Thus, despite the ability of rodent models to address mechanistic questions in a high-throughput fashion, clinical conclusions drawn from these studies are often limited by the fact that rodents are in-bred and housed in specific pathogen-free conditions and are well documented to have a much more easily controlled alloresponse than either primates or patients. In addition, the rodent response to transplant conditioning-induced tissue injury and the expression pattern of key immune molecules, are significantly different from patients. These distinctions decrease the predictive power of studies performed only in rodent models and underscore the importance of the creation of an MHC-defined primate model of GVHD. Indeed, the studies described in Miller et al. (35) were designed, in part, to provide preclinical data for the first clinical trial of CTLA4Ig for *in vivo* GvHD prevention, which is currently being conducted at Emory University (http://clinicaltrials.gov/ct2/show/NCT01012492).

For SOT, the first studies are starting to emerge using the new MHC-defined primate resources and are underscoring the importance MHC disparity in NHP transplantation outcomes (32–34,36,37). This includes work published by our group in 2010, in which we described the results of an experiment in which the degree of alloproliferation (as measured through CFSE-MLR analysis) was mapped to the degree of MHC disparity between potential transplant pairs (33). Four cohorts were compared: (i) two MHC-haplotype matched pairs; (ii) one MHC-haplotype matched pairs; (iii) autologous controls (with responder and stimulator cells derived from the same animal) and (iv) a cohort of animals for whom haplotype information was not available at the time of the CFSE-MLR. It is this last cohort that most closely models the transplant experiments that have been historically performed in NHP, in which neither detailed pedigree nor MHC haplotype information was available. As shown in Figures 2 and 3 (reproduced from Larsen et al.; Ref. 33), potential transplant pairs matched at both MHC haplotypes displayed minimal pretransplant alloproliferation ($1.1 \pm 1\%$), not statistically different than autologous controls ($1.3 \pm 1.5\%$), which is similar to what has been shown in human MLR studies (38). As expected, pairs matched at just one MHC haplotype displayed significantly more alloproliferation ($8.4 + 8.5\%$) than either the two MHC-haplotype matched pairs or the autologous controls ($p < 0.01$). The percentage of cells that had proliferated in the MLR cohort with unknown MHC disparity ($17 \pm 14.9\%$) was higher than for either the one or two MHC-haplotype matched pairs ($p < 0.01$). However, the proliferation measured in this untyped cohort was not uniform, with these MLR pairs displaying high variance (Figure 3) and with some animals demonstrating surprisingly low levels of alloproliferation. This led us to hypothesize that some pairs included in this group serendipitously possessed significant degrees of relatedness or MHC similarity. *Post hoc* pyrosequencing analysis of one of the low-proliferating pairs confirmed that although these two animals had no known familial relationship and were obtained from two different primate colonies, they shared an MHC haplotype and a resultant high degree of MHC similarity (33). These results underscore the fact that serendipitously high levels of MHC similarity, even between animals from different colonies, can occur and point to an important confounder that must be acknowledged for all primate transplant studies that are performed without knowledge of MHC sharing:

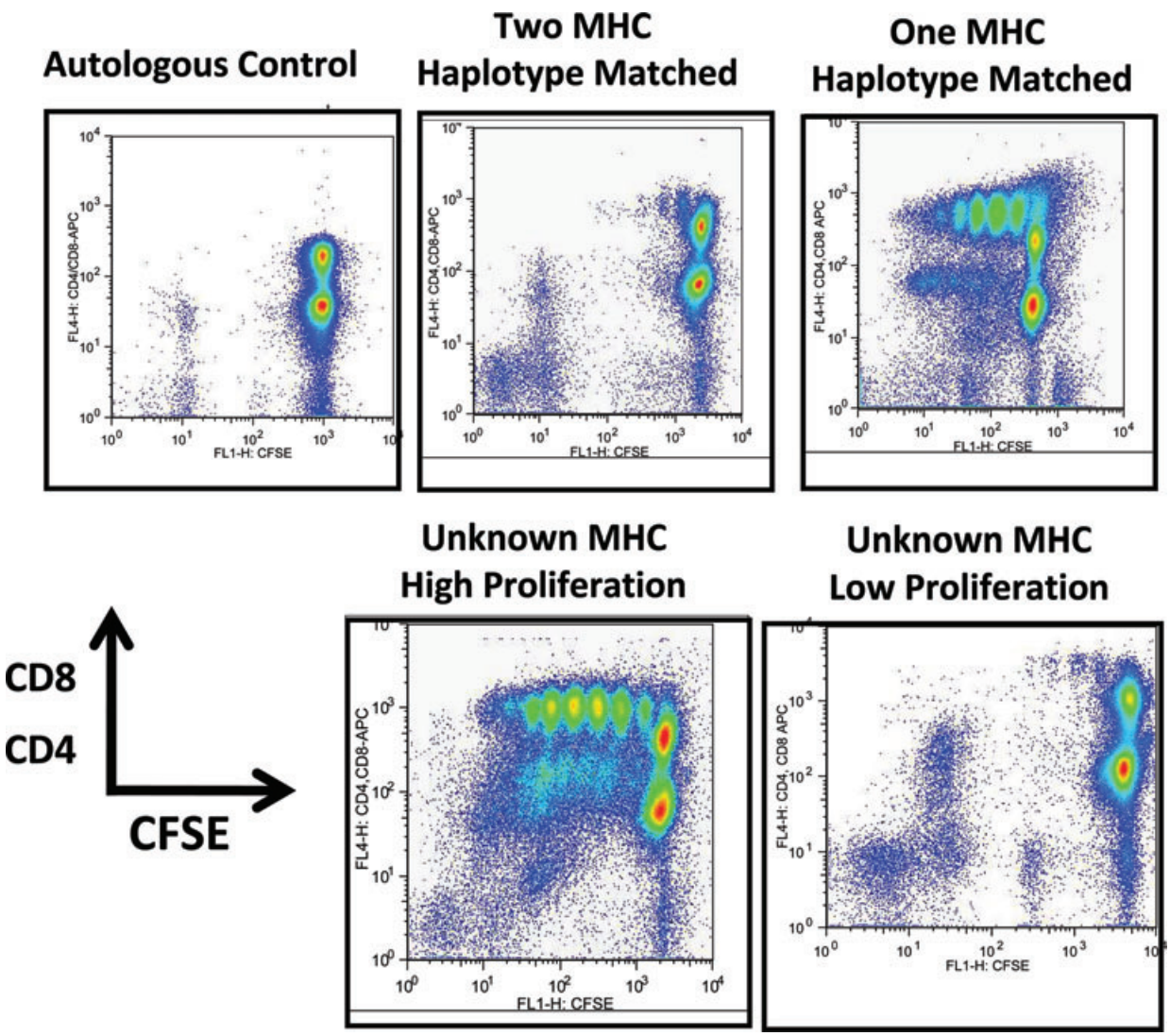

Figure 2: CFSE MLR analysis reveals increasing alloproliferation with increasing MHC disparity. This figure shows CFSE fluorescence for both CD4+ and CD8+ cells after a 5-day MLR culture. Shown (left to right, top row) are a representative autologous control, a two MHC-haplotype matched pair and a one MHC-haplotype matched pair. The bottom row shows a representative pair with unknown MHC disparity and high proliferation (left) and a representative pair with unknown MHC disparity and low proliferation (right).

because alloreactivity is affected by MHC disparity, transplant success or failure may not be able to be attributed solely to the immunosuppressive strategy if MHC matching is not also taken into account. Indeed, our group has documented that in RM mixed-chimerism-induction experiments, increased MHC matching is directly related to the length of donor engraftment (33). The correlation of MHC disparity to NHP transplant outcome will likely extend to solid organ transplantation as well. Thus, as shown in Figure 4, although renal transplant rejection occurred rapidly after withdrawal of sirolimus therapy when transplant pairs were matched at only one MHC haplotype, a two MHC-haplotype matched pair has demonstrated long-term allograft acceptance (>420 days) in the absence of any immunosuppression. These results suggest that unknown MHC disparity could significantly confound the interpretation of the results of transplantation experiments and that pedigreed and MHC-defined colonies should become the gold-standard for NHP SOT studies—allowing for the most clarity during the investigation of novel immunosuppressive regimens, without the confounder of unknown degrees of familial relatedness or MHC matching between transplant pairs.

The future: a level MHC playing field

The introduction of microsatellite- and pyrosequencing-based MHC analysis, along with the commitment by the NIAID and by the NHP research community to improve the rigor of primate transplantation experiments, has resulted in a significant leap forward for primate models of

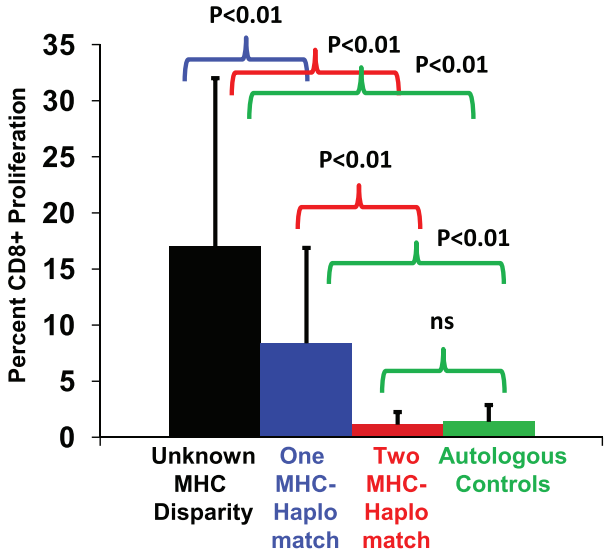

Figure 3: The amount of CD8± T cell alloproliferation correlated with the degree of MHC disparity. CFSE-MLR analysis was performed on two –MHC-haplotype matched pairs (n = 8), one MHC-haplotype matched pairs (n = 48), autologous controls (n = 61) and animals for whom MHC disparity Information was not available (n = 99). The percent of CD8+ T cells remaining at the end of the 5-day MLR incubation period that had undergone at least one round of cell division (percentage proliferation) was then determined using the FloJo flow cytometry analysis program. Shown are the average percentage proliferation and the standard deviation for all four groups. Statistical significance was determined by ANOVA analysis of the log-transformed data followed by a posthoc Tukey HST test to determine significant differences for pair-wise comparisons.

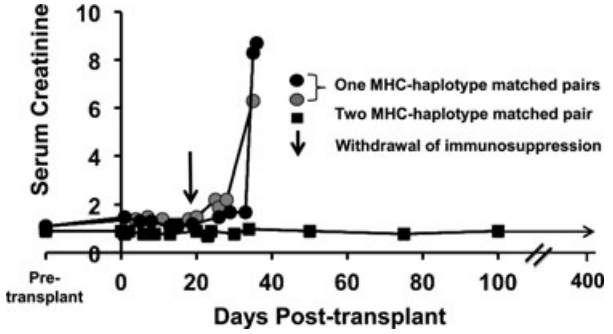

Figure 4: Prolonged renal allograft acceptance after transplant between a two MHC-haplotype matched pair. One MHC-haplotype matched transplants demonstrated rejection of donor kidneys within 2 weeks of withdrawal of sirolimus immunosuppression. In contrast, a two MHC-haplotype matched pair has had prolonged renal allograft acceptance (> 1 year posttransplant) despite withdrawal of sirolimus immunosuppression. Circles indicate one MHC-haplotype matched pairs. Square indicate two MHC-haplotype matched pair. Arrow indicate date of withdrawal of sirolimus.

both SOT and HSCT. Although controlling for MHC disparity may not be universally sufficient for tolerance-induction, it will assuredly make interpretation of all primate transplant experiments more rigorous and reproducible. The issue of a level playing field is critical: eliminating the confounder of variable MHC matching promises to significantly increase the certainty with which predictions of therapeutic success or failure can be made from primate experiments. Experiments to test novel tolerance-induction strategies for SOT using NHP models can now be planned on the basis of three key immunogenetic criteria: the degree of relatedness, the degree of MHC haplotype disparity between transplant pairs and the precise MHC alleles that both donors and recipients express. This expanded level of detail does present a new challenge for the NHP research community. Whereas previously, transplant planning decisions were relatively simple and were usually based only on basic demographic data and limited DNA-based MHC typing, the advent of detailed pedigree information and complete MHC analysis now creates a decision-tree for primate transplant experiments whose complexity rivals clinical transplant planning. At the recent NHPCSG steering committee meeting (February 2011, Washington, DC), a new web-based application, "Immunogenetic Management Software", which was created by members of the NHP transplant community, was described (39). This tool enables both the visualization and management of the complex immunogenetic datasets now required for NHP transplant planning and promises to streamline the information-management workflow that will be required for NHP transplantation experiments.

The last several years have witnessed a rapid evolution of our ability to perform detailed immunogenetic analysis for NHP transplantation experiments, which have raised the bar for the degree of specificity required for transplant planning and data interpretation. These advances promise to increase the rigor of this critical translational model and help ensure that conclusions made with NHP are optimally translatable to patients undergoing both solid organ and hematopoietic stem cell transplantation.

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Disclosure

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

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