Published in final edited form as:

Transfusion. 2019 February ; 59(2): 744–753. doi:10.1111/trf.15109.

Alloimmunogenicity of an Isolated MHC Allele is Affected by the Context of MHC Mismatch in a Murine Model

Krystalyn E. Hudson^{1,2}, Andrea S.L. Wong¹, Amanda L. Richards¹, Linda M. Kapp¹, James C. Zimring^{1,2,3}

¹BloodworksNW Research Institute, Seattle, WA

²University of Washington School of Medicine, Department of Laboratory Medicine, Seattle WA

³University of Washington School of Medicine, Department of Medicine, Division of Hematology, Seattle, WA

Abstract

Background: Humoral alloimmunization to HLA can represent a barrier to solid organ transplantation, can lead to a refractory state in patients requiring platelet transfusion, and can also contribute to TRALI. While exposure to HLA mismatched cells/tissues are generally required for HLA alloimmunization, the effect of the extent of MHC mismatch between donor and recipient is poorly understood.

Study Design/Methods: A novel mouse was generated that allows the expression of a single MHCI alloantigen, K^d. Alloimmune responses to K^d were studied in C57BL/6 mice transfused with splenocytes from different donor mice, allowing the analysis of responses to K^d as an isolated alloantigen, or in the context of additional mismatched MHC molecules. Advanced tools were utilized to study responses to K^d, including TCR transgenic mice that recognize the immunodominant K^d peptide presented by C57BL/6 mice to CD4⁺ T cells.

Results/Findings: A single MHCI alloantigen mismatch is less immunogenic than when the same alloantigen is encountered in context of additional mismatched MHC alloantigens. This difference is due, at least in part, to induction of CD4⁺ helper T cells, as the effect is overcome by increasing either mature CD4⁺ T cell help through immunization or by increasing the precursor frequency of naïve CD4⁺ T cells by adoptive transfer from TCR transgenic donors.

Conclusion: These findings indicate that the immunogenicity of a single alloantigen can be affected by the context in which it is encountered, demonstrating the potential for cooperative effects between different mismatched MHC alloantigens.

Please address correspondence to: James C. Zimring, M.D., Ph.D., BloodworksNW Research Institute, 1551 Eastlake Ave E, Seattle, WA 98102 jzimring@bloodworksnw.org.

Disclosure: The authors have no conflicts of interest to report relevant to the work contained in the submitted manuscript.

Conflict of interest: The authors have no conflicts of interest to report relevant to the work contained in the submitted manuscript.

Introduction:

Human leukocyte antigens (HLA) are amongst the most immunogenic humoral alloantigens and also represent the major target for organ transplant rejection by antibodies; the presence of anti-HLA antibodies in transplant recipients correlates to rejection of multiple organ types, including heart, kidney, lung and liver^{1,2}. Preformed HLA antibodies can be a significant barrier to transplant; however, *de novo* anti-HLA formed over the course of an initial transplant can also result in graft failure³⁻⁵. Anti-HLA can also result in patients becoming refractory to transfusion of platelets (PLTs), and in extreme cases, prevent PLT transfusion therapy as a viable approach to treating thrombocytopenia, leading to morbidity and mortality from hemorrhage⁶. Finally, anti-HLA antibodies in blood donors can be responsible for Transfusion Related Acute Lung Injury (TRALI)⁷.

HLAs are epitopes that differ between genetic variants of MHC I and/or MHC II, which are the major targets recognized by CD8+ and CD4+ T cells, respectively. Primary humoral alloimmunization can occur as a result of transplant and may also have been induced by previous transplantation. Pregnancy is also a major source of anti-HLA⁸. Finally, immune responses to environmental antigens can also lead to “natural anti-HLA” that are interpreted as cross-reacting with HLA; however, in such cases effects upon rejection are typically less severe^{9,10}.

It has been well described that degree of mismatch correlates with likelihood of transplant rejection and also with the rate of alloimmunization. The observation that the more alloantigens to which one is exposed, then the more likely one is to become alloimmunized, is not surprising. However, this observation is equally consistent with at least two separate interpretations- leading to two distinct hypotheses. First, that the relative immunogenicity of each alloantigen is an independent factor, and that increased rates of alloantibodies are simply due to the aggregate risk of immunization to independent antigens. Second, that there is a cooperative effect between HLA mismatches, such that the immune response against a particular alloantigen in isolation will be less than the immune response to the very same alloantigen in the context of other alloantigens. To the best of our knowledge, these two interpretations have not been rigorously explored.

In this report we tested these hypotheses using a novel strain of mice in which the K^d alloantigen was inserted into the K^b locus of a C57BL/6 (B6) mouse. As B6 mice are of the H-2^b haplotype, K^d is a single alloantigen in this context. Immune responses of B6 mice were compared when exposed to K^d either as a single alloantigen or in the context of a full MHC mismatch. Whereas a strong anti-K^d alloantibody response was observed when a fully H-2 mismatched infusion of leukocytes was given (*i.e.* BALB/c (H-2^d) or B6.H2^d (H-2^d)), no significant humoral alloimmune response was generated upon exposure to leukocytes selectively expressing the same K^d as an isolated antigen. This lack of response to the isolated alloantigen was overcome by increasing CD4+ T cells precursor frequency, suggesting a mechanism by which increased mismatch augments immunogenicity of individual alloantigens through expanding the pool of alloantigen specific CD4+ T cells.

Materials and Methods:

Mice

C57BL/6-Tg(UBC-GFP), CMV-CRE, B6.H2^d and B6.Thy1.1 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). TCR75 mice were generously provided by Pat Bucy ¹¹ and crossed to B6.Thy1.1 at Bloodworks NW vivarium to generate TCR75 homozygous for the Thy1.1 congenic marker. The Con-K^d mice were generated by electroporating embryonic stem (ES) cells derived from a C57BL/6 mouse with a targeting construct that consisted of the inserted sequence (as described in the manuscript and depicted in Figure 1), flanked by homology arms for the K locus. Vector design and electroporation was carried out in collaboration with Ozgene Inc (Perth, AUS). as a paid vendor. In addition, a neomycin phosphotransferase (NEO) cassette was included in the targeting construct to allow for selection of ES cells that had incorporated the construct; the NEO cassette was flanked by flippase recognition target (FRT) sites, allowing its subsequent excision by crossing with flippase (FLP) transgenic mice. Cloned gene modified ES cells were injected into female mice, and chimeric coat color pups were then bred to FLP transgenic mice (B6 background) to allow removal of the NEO cassette, resulting in the final gene modification shown in Figure 1. The resulting mice were bred to wild-type B6 mice to remove the FLP transgene. All genetic changes, including homologous recombination, lack of random integration, and removal of NEO were confirmed by Southern Blot Analysis on ES cells, founder pups, and progeny of FLP breeding, respectively (data not shown). Con-K^d mice were crossed with CMV-CRE mice to remove the stop cassette and to allow constitutive expression of K^d (Con-K^d-On mice). Con-K^d-On mice were propagated by breeding to wild-type B6 mice to remove the CMV-CRE gene. All procedures were performed according to protocols approved by the Bloodworks Northwest Institution Animal Care and Use Committee (IACUC).

Leukocyte harvest and staining

Spleens were collected, mechanically disrupted, and red blood cells were lysed with RBC lysis buffer (Sigma, St. Louis MO). Leukocytes were resuspended in PBS and 5×10^6 cells were injected via tail vein. To detect anti-K^d antibodies, a flow crossmatch against antigen-expressing splenocytes was performed. Sera were collected at days 14 and 29, diluted 1:10 and incubated with an equal mix of leukocytes from ConK^d-On and C57BL/6-Tg (UBC-GFP) mice. Goat anti-mouse immunoglobulins conjugated to allophycocyanin (APC) (BD Bioscience) were used as a secondary antibody.

To assess K^d expression, PBMCs and whole blood were stained with antibodies against CD45 (Thermo Fisher Scientific, Waltham, MA), Ter119 (Thermo Fisher Scientific), CD41 (Thermo Fisher Scientific), H-2K^b (BD Bioscience, San Jose, CA) and H-2K^d (BD Bioscience). For platelet and RBC staining, APC conjugated to anti-H-2K^d antibody was amplified using the FASER kit (Miltenyi, Germany) according to manufacturer's directions. To determine K^d and K^b expression on splenocytes, spleens were harvested from C57BL/6, ConKd-On, and B6.H2^d mice. Spleens were collagenase digested (1mg/mL, Type IV, Sigma), RBC lysed, and stained with antibodies to delineate leukocyte subsets, as previously described (ref: Richards & Hudson: Transfusion paper).

Adoptive transfer of TCR75

CD4⁺ T cells were enriched from the spleens of TCR75.Thy1.1 mice using MagniSort Mouse CD4⁺ T cell enrichment kit (Thermo Fisher Scientific) according to manufacturer's guidelines. Enriched CD4⁺ T cells were labeled with 5μM of CFDA (Life Technologies, Carlsbad, CA) and 3×10⁶ cells were adoptively transferred via lateral tail vein injections into B6 recipient mice. On the next day, recipient mice were injected with 5×10⁶ donor leukocytes (by tail vein) prepared as described above. At 3 days post leukocyte infusion, spleens were harvested and TCR75 T cells identified using antibodies against CD4 and Thy1.1. Proliferation was assessed via CFSE dilution on an LSR II flow cytometer (BD Bioscience).

Immunization with K^d peptides

B6 mice were immunized with 100μg of peptide QEGPEYWEEQTQRAK (GenScript, Piscataway NJ) emulsified in complete Freund adjuvant (CFA) in one footpad and the flank. Two weeks later, mice were infused with 5×10⁶ leukocytes, as above. Sera were collected at 14 days post leukocyte infusion and cross-matched to detect anti-K^d antibody, as described above.

Statistics

Experiments presented in Figure 1F were analyzed with a one-way ANOVA with a multiple comparisons post-test, comparing each experimental group against control B6, to determine significance. Experiments presented in Figure 2 were analyzed with a repeated measures two-way ANOVA with a multiple comparisons post-test, comparing each experimental group against control B6. Figure 3 was analyzed with a two-way ANOVA with a multiple comparisons post-test, comparing each experimental group against control B6. Significance was set at p 0.05, where ***p 0.001, **p 0.01, *p 0.05 and n.s. = not significant.

Results:

Generation of a novel mouse with conditional expression of K^d under natural regulatory elements

To test the ability of a single MHCI antigen to generate an alloimmune response, we generated a novel mouse (Con-K^d) with conditional expression of K^d on a B6 background. Homologous recombination was utilized to replace the endogenous K^b gene with K^d (Figure 1A). The targeting construct also contained a stop cassette consisting of 4 tandem repeats of a poly-A tail to terminate translation and was flanked by *loxP* sites, which are recognition sequences for Cre recombinase. As such, exposure to Cre is predicted to remove the lox-stop-lox cassette from the genome and result in selective expression of K^d in tissues that express Cre. The inserted K^d and native K^b genes are 90.5% similar, and 84.3% identical at the amino acid level (Figure 1B).

Analysis of K^d expression was carried out on splenocytes by flow cytometry. B6 (H-2^b) and BALB/c (H-2^d) mice were used as controls for expression of K^b and K^d, respectively. Whereas anti-K^d reacted with BALB/c and not B6 splenocytes, no expression of K^d was detected on CD45⁺ splenocytes from the Con-K^d mouse, indicating that the stop cassette

prevented detectable expression (Figure 1C). In contrast, crossing the Con-K^d mouse with CMV-CRE transgenic mice, to delete the *loxP*-flanked stop cassette in all tissues, resulted in broad expression of K^d on CD45⁺ leukocytes. Together, these data confirm that the conditional expression cassette was working, as designed, to generate a mouse that expressed K^d only when crossed with a CRE-expressing mouse.

To assess expression patterns of the ConK^d cassette, ConK^dCMV-CRE mice were bred to achieve homozygosity at the ConK^d locus and remove the CMV-CRE gene; these mice will be referred to as “ConK^d-On” for the remainder of the manuscript. Although our knock-in approach was designed to maintain natural regulatory elements, the expression of K^d in ConK^d-On mice was nevertheless somewhat altered from the natural H-2^d locus. K^d expression on CD45⁺ leukocytes was slightly less in the ConK^d-On mice than in BALB/c mice (Figure 1D, left). Mice express MHCI on both platelets and red blood cells (RBCs) as well, albeit at significantly lower levels¹². To maximize detection in the RBC and platelet compartments, staining was amplified using Fluorescence Amplification by Sequential Employment of Reagents (FASER) Kit. K^d was detected on platelets from the ConK^d-On mice, but at significantly lower levels than on BALB/c platelets (Figure 1D, middle). RBCs from ConK^d-On mice had no detectable K^d, unlike BALB/c RBCs that had low levels of detectable antigen (Figure 1D, right).

Staining with antibodies to lineage specific markers was used to evaluate levels of K^d expression on different leukocyte subsets within the CD45⁺ population. In all cases, B6-H2^d and B6 were used as positive and negative controls, respectively. K^d levels were equivalent between ConK^d-On and B6-H2^d mice for each subset of dendritic cells analyzed (i.e. CD8a⁺ DCs, CD11b⁺ DCs, and plasmacytoid DCs (pDCs)) as well as neutrophils, B lymphocytes and red pulp macrophages (RPM) (Figure 1E). In contrast, K^d expression was slightly less on monocytes (both inflammatory and resident) and T cells. Thus, the slight decrease in K^d expression in ConK^d-On splenocytes was likely due to monocyte and T cell populations. To determine whether the genetic construct resulted in alterations in leukocyte subset percentages, frequencies of antigen presenting cells and lymphocytes were determined in B6, ConK^d-On, or B6.H2^d mice (Figure 1F). A statistically significant but subtle decrease was seen in the percentage of monocytes in ConK^d-On mice, compared to control B6. No other significant differences between ConK^d-On and B6 leukocyte subsets were observed.

Isolated expression of K^d as a single alloantigen is not sufficient to generate a humoral alloimmune response.

To assess the immunogenicity of a single alloantigen mismatch (K^d), 5 million splenocytes from ConK^d-On mice were injected intravenously into B6 mice (K^b). Humoral alloimmunization was monitored by flow cytometry-based crossmatch using ConK^d-On splenocytes as targets. Using this approach, very low levels of anti-K^d antibodies were detected in B6 mice that received ConK^d-On leukocytes, which did not achieve statistical significance (Figure 2). Moreover, this low response was not boosted or otherwise altered by a second exposure to ConK^d-On splenocytes.

The lack of observed immunogenicity of ConK^d-On splenocytes was neither due to administration of leukocytes under intrinsically non-immunogenic conditions nor failure of

the assay to detect anti-K^d, as immunization with splenocytes from BALB/c mice resulted in statistically significant anti-K^d alloantibody response. To test the hypothesis that lack of immunogenicity was due to non-H2 associated BALB/c background genes, leukocytes from B6.H2^d mice (congenic for the entire H-2^d locus) were used, which induced a strong alloimmune response (albeit weaker than BALB/c) and was increased upon a second exposure to B6.H2^d splenocytes. Thus, lack of immunogenicity of ConK^d-On splenocytes was not entirely a function of differences in non-H2 background genes. In aggregate, these findings indicate that alloantibody responses to K^d occur and are boostable when K^d is encountered with the whole H-2^d locus; however, isolated K^d expression does not result in a significant alloantibody response, even upon re-exposure.

Lack of immunogenicity of K^d as a single alloantigen is due to lack of sufficient CD4⁺ T cell precursors

One of the immunodominant epitopes of K^d responses, recognized by CD4⁺ T cells in B6 mice, has previously been mapped to QEGPEYWEEQTQRAK, and is presented by I-A^b (red box, Figure 1A). To test if lack of immunogenicity of K^d was due to an insufficient CD4⁺ T cell response, B6 mice were first immunized with QEGPEYWEEQTQRAK emulsified in CFA, followed by a subsequent infusion of B6, ConK^d-On, B6 x BALB/c F1, or BALB/c splenocytes two weeks post-immunization. After peptide/CFA immunization, a single ConK^d-On leukocyte infusion induced a strong anti-K^d alloantibody response of a similar magnitude to BALB/c leukocytes (Figure 3A). The anti-K^d alloantibody response was not an artifact of the peptide itself as no anti-K^d was detected in B6 mice that received CFA/peptide immunization followed by B6 leukocytes (Figure 3A), indicating that the QEGPEYWEEQTQRAK peptide did not recapitulate B cell epitopes present on native intact cell surface K^d. Likewise, no alloimmune response to K^d expressing leukocyte infusion was observed when pre-immunization with CFA was administered in the absence of QEGPEYWEEQTQRAK peptide, indicating that the enhancement was not a non-specific effect of inflammation (Figure 3B). Taken together, these data demonstrated that poor immunogenicity of ConK^d-On leukocytes was circumvented by immunization with a K^d-derived peptide, suggesting that ConK^d-On splenocytes are poorly immunogenic due to insufficient induction of a helper CD4⁺ T cell response.

The TCR75 mouse is transgenic for a T cell receptor (TCR) that recognizes a peptide from K^d that is presented by I-A^b. To test if the lack of humoral alloimmunization to ConK^d-On splenocytes was due to a low precursor frequency of CD4⁺ T cells, CD4⁺ T cells from TCR75 mice were enriched and labeled with CFSE to allow for proliferation assessment (Figure 4A). Three million CD4⁺ TCR75 T cells were adoptively transferred into wild-type B6 recipients followed by intravenous infusion of splenocytes from B6, ConK^d-On, B6 x BALB/c F1, and BALB/c mice. Infusion of ConK^d-On splenocytes induced a strong antibody response to K^d, if given after adoptive transfer of TCR75 (Figure 4C). No antibody binding was detected on B6 targets or in response to immunization with B6 leukocytes. As predicted, the transferred TCR75 cells showed a strong proliferative response to ConK^d-On leukocytes, as evidenced by CFSE dilution, consistent with the experimental goal of increasing functional precursor frequencies of K^d specific CD4⁺ T cells. (Figure 4B). Proliferation was antigen specific, as no CFSE dilution was observed when antigen negative

(B6) leukocytes were infused (Figure 4B). Proliferation was also observed with BALB/c leukocytes and B6xBALB/c F1, both of which were used as a positive control

Discussion:

In the current manuscript, we juxtapose the humoral immunogenicity of the same alloantigen (K^d), either as an isolated antigen, or in the context of other MHC mismatches. K^d is substantially more immunogenic when in the context of an entirely mismatched H-2 locus. A complete strain difference (BALB/c) was only slightly more immunogenic than the isolated H-2^d congenic difference; thus, while background genes outside the H-2^d locus may influence immunogenicity, the major effect appears to be transmitted through the H-2 locus. In contrast to an entire H-2 mismatch, exposure to K^d as an isolated alloantigen resulted in only a weak and insignificant alloantibody response, which did not show a secondary recall effect, as no boosting was observed upon re-exposure to K^d . Whereas the complexity of the MHC repertoire that can be presented to CD4⁺ T cells by a recipient has been shown to affect alloimmunization to MHC¹³, to the best of our knowledge, this is the first report on how mismatch of separate donor MHC genes may affect the immunogenicity of a particular donor MHC alloantigen. Analyzing the immunogenicity of HLA molecules in humans is complicated by effects that cross-reactive antigens in different recipients affect responses²⁷. The current model controls for these variables by using identical recipients.

In the model used herein, exposure to leukocytes provides both the direct pathway (*i.e.* donor APCs can present peptide from K^d on donor MHCII to stimulate recipient T cells) and also the indirect pathway (*i.e.* recipient APCs can present peptide from K^d on their MHCII to recipient T cells). As such, in the experiments using adoptive transfer of TCR75, we cannot distinguish between stimulation of TCR75 by APCs of donor or recipient origin, either of which may have processed and presented K^d on MHCII (I-A^b). The same is the case with the peptide/CFA stimulation that primes a CD4⁺ T cell response, although this latter case generates mature CD4⁺ T cell help through the indirect pathway that would be boosted by either direct or indirect pathways upon leukocyte infusion. Although we cannot distinguish direct from indirect pathways herein, it is worth noting that we have previously reported that the direct pathway is not required for alloimmunization to class I MHC alloantigens on transfused leukocytes¹⁴; a separate group has reported the opposite finding, but in a different system¹⁵.

The mechanism(s) by which K^d is more immunogenic in the context of a mismatched H-2 locus is not entirely clear; however, this difference can be overcome either through priming with a K^d peptide presented by I-A^b or by adoptive transfer of naïve T cells specific for this peptide/I-A^b complex (*i.e.* TCR75). We interpret these data to indicate that the CD4⁺ T cell precursor frequency for K^d is too low to be effectively activated by K^d as an isolated alloantigen, consistent with related observations in other systems^{16,17}. It is unclear why the whole H-2^d locus would overcome a low CD4⁺ T cell frequency and generate K^d -specific alloantibodies. Presumably it is not a change to K^d itself, but rather the availability and/or precursor frequency of CD4⁺ T cell help to other alloantigens in the H-2^d locus that are able to provide help to B cells encoding immunoglobulins specific for K^d . This could occur through a linked recognition mechanism by which B cells that consume K^d (via their BCR)

present peptides on MHCII from other alloantigens associated with the K^d that was consumed^{18,19}. As such, K^d specific B cells could get additional help from other non-K^d alloantigens; in this case, the peptides would likely be linked via physical association of cell membranes or fragments. Alternatively, if K^d specific CD4⁺ T cells are being stimulated by professional antigen presenting cells that have consumed donor leukocytes, CD4⁺ T cells specific for alloantigens other than K^d may promote expansion of the K^d specific CD4⁺ T cells, either through effects of cytokines^{20,21}, T-T cell contact^{22,23}, or through enhancing the potency of the antigen presenting cell by activation signals²⁴.

It is worth noting that the expression of K^d on splenocytes from the ConK^d-On mouse is slightly less on CD45⁺ leukocytes than it is from the natural H-2^d locus, on a few leukocyte subsets (e.g. monocytes). Thus, we cannot unequivocally rule out that the natural K^d specific CD4⁺ T cell precursor frequency is sufficient for the dose of K^d from BALB/c or B6.H2^d mice but not for the lower dose on ConK^d-On; however, we do not favor this interpretation as the level of expression of K^d on ConK^d-On does not differ between most cell types, is only slightly less than the natural H-2^d locus overall, and does not induce immunity upon re-exposure. Moreover, the ability of ConK^d-On leukocytes to stimulate proliferation of TCR75 T cells demonstrates it delivers enough antigen to drive CD4⁺ T cell expansion.

Nevertheless, the previously reported non-linear relationship of TCR75 mediated transplant rejection suggests a cooperativity even amongst CD4⁺ T cells of a single specificity¹¹. As such, there could be a threshold effect between dose of K^d from ConK^d-On and BALB/c or B6.H2^d upon that natural frequency of alloantigen specific CD4⁺ T cells. A second concern is the alternate interpretation that K^d has the same immunogenicity in all cases, but that the increased anti-K^d detected for the full H-2k mismatch is actually anti-D^d and/or anti-L^d cross-reacting with K^d. We cannot unequivocally rule this out, as cross-reactivity (by definition) has dual specificity, and as such, individual targets cannot assess this with a polyclonal antiserum. This is one potential explanation for our findings that would be more trivial. However, as above, we do not favor this explanation as both the peptide/CFA and TCR75 experiments indicate that plentiful anti-K^d specific B cells are available for a serological response, in the event that sufficient help is present.

While splenocytes are commonly used, in bulk, as an immunogen (as in this study), it is a complex mixture of leukocytes of different properties. It has been reported that different leukocyte subsets have different effects upon alloimmunization²⁵. Because the ConK^d mouse allows conditional expression of K^d, it generates the technical ability to direct K^d expression through breeding with tissue specific CRE transgenics. Although outside the scope of the current report, this capacity will allow future studies on differential effects of leukocyte subsets.

In summary, the data in this report are consistent with the interpretation that the immunogenicity of the same MHCII alloantigen is altered as a result of mismatch amongst other distinct MHC alleles. As with any model, it does not necessarily follow that the same phenomenology will be present in that being modeled (e.g. human biology). However, if such biology is present in humans, it may affect strategies for MHC mismatch in transfusion and/or transplantation. It is also worth noting that the strength of alloimmunity is also influenced by the murine strain combinations being used²⁶, and would likely also differ for

humans across different HLA barriers. Thus, a personalized/precision medicine based approach would likely have to guide such strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

This work was supported by a grant from the National Institutes of Health (NHLBI) - R01 HL114525-01A1 and also a National Blood Foundation award to K.H.

These studies were supported in part by a grant from the N.I.H; R01 HL114525-01A1

References:

1. McKenna RM, Takemoto SK, Terasaki PI. Anti-HLA antibodies after solid organ transplantation. *Transplantation*. 2000;69(3):319–326. [PubMed: 10706035]
2. Valenzuela NM, Reed EF. Antibodies in transplantation: the effects of HLA and non-HLA antibody binding and mechanisms of injury. *Methods Mol Biol*. 2013;1034:41–70. [PubMed: 23775730]
3. Premaud A, Filloux M, Gatault P, et al. An adjustable predictive score of graft survival in kidney transplant patients and the levels of risk linked to de novo donor-specific anti-HLA antibodies. *PLoS One*. 2017;12(7):e0180236.
4. Sole-Gonzalez E, Mirabet S, Brossa V, et al. De Novo Anti-HLA Antibodies After Heart Transplantation Are Associated With Adverse Events in the Long-term Follow-up of Cardiac Transplant Recipients. *Transplant Proc*. 2016;48(9):3030–3032. [PubMed: 27932139]
5. Malheiro J, Martins LS, Tafulo S, et al. Impact of de novo donor-specific anti-HLA antibodies on grafts outcomes in simultaneous pancreas-kidney transplantation. *Transpl Int*. 2016;29(2):173–183. [PubMed: 26404891]
6. Stanworth SJ, Navarrete C, Estcourt L, Marsh J. Platelet refractoriness--practical approaches and ongoing dilemmas in patient management. *Br J Haematol*. 2015;171(3):297–305. [PubMed: 26194869]
7. Peters AL, Van Stein D, Vlaar AP. Antibody-mediated transfusion-related acute lung injury; from discovery to prevention. *Br J Haematol*. 2015;170(5):597–614. [PubMed: 25921271]
8. Honger G, Fornaro I, Granado C, Tiercy JM, Hosli I, Schaub S. Frequency and determinants of pregnancy-induced child-specific sensitization. *Am J Transplant*. 2013;13(3):746–753. [PubMed: 23311303]
9. Morales-Buenrostro LE, Terasaki PI, Marino-Vazquez LA, Lee JH, El-Awar N, Alberu J. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. *Transplantation*. 2008;86(8):1111–1115. [PubMed: 18946350]
10. Sicard A, Amrouche L, Suberbielle C, et al. Outcome of kidney transplantations performed with preformed donor-specific antibodies of unknown etiology. *Am J Transplant*. 2014;14(1):193–201. [PubMed: 24224759]
11. Honjo K, Yan Xu X, Kapp JA, Bucy RP. Evidence for cooperativity in the rejection of cardiac grafts mediated by CD4 TCR Tg T cells specific for a defined allopeptide. *Am J Transplant*. 2004;4(11):1762–1768. [PubMed: 15476474]
12. Chapman LM, Aggrey AA, Field DJ, et al. Platelets present antigen in the context of MHC class I. *J Immunol*. 2012;189(2):916–923. [PubMed: 22706078]
13. Semple JW, Speck ER, Milev YP, Blanchette V, Freedman J. Indirect allorecognition of platelets by T helper cells during platelet transfusions correlates with anti-major histocompatibility complex antibody and cytotoxic T lymphocyte formation. *Blood*. 1995;86(2):805–812. [PubMed: 7606011]
14. Gilson CR, Cadwell CM, Smith NH, Hendrickson JE, Zimring JC. MHC II on transfused murine blood is not required for alloimmunization against MHC I. *Vox Sang*. 2010;99(4):369–374. [PubMed: 20546207]

15. Kao KJ, del Rosario ML. Role of class-II major histocompatibility complex (MHC)-antigen-positive donor leukocytes in transfusion-induced alloimmunization to donor class-I MHC antigens. *Blood*. 1998;92(2):690–694. [PubMed: 9657772]
16. Moon JJ, Chu HH, Pepper M, et al. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity*. 2007;27(2):203–213. [PubMed: 17707129]
17. Ford ML, Koehn BH, Wagener ME, et al. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J Exp Med*. 2007;204(2):299–309. [PubMed: 17261633]
18. Crotty S. A brief history of T cell help to B cells. *Nat Rev Immunol*. 2015;15(3):185–189. [PubMed: 25677493]
19. Snow EC, Noelle RJ, Uhr JW, Vitetta ES. Activation of antigen-enriched B cells. II. Role of linked recognition in B cell proliferation to thymus-dependent antigens. *J Immunol*. 1983;130(2):614–618. [PubMed: 6600245]
20. Malandro N, Budhu S, Kuhn NF, et al. Clonal Abundance of Tumor-Specific CD4(+) T Cells Potentiates Efficacy and Alters Susceptibility to Exhaustion. *Immunity*. 2016;44(1):179–193. [PubMed: 26789923]
21. Friedman RS, Jacobelli J, Krummel MF. Surface-bound chemokines capture and prime T cells for synapse formation. *Nat Immunol*. 2006;7(10):1101–1108. [PubMed: 16964261]
22. Ramming A, Thummler K, Schulze-Koops H, Skapenko A. Homotypic T-cell/T-cell interaction induces T-cell activation, proliferation, and differentiation. *Hum Immunol*. 2009;70(11):873–881. [PubMed: 19682527]
23. Sabatos CA, Doh J, Chakravarti S, et al. A synaptic basis for paracrine interleukin-2 signaling during homotypic T cell interaction. *Immunity*. 2008;29(2):238–248. [PubMed: 18674934]
24. Sporri R, Reis e Sousa C. Newly activated T cells promote maturation of bystander dendritic cells but not IL-12 production. *J Immunol*. 2003;171(12):6406–6413. [PubMed: 14662839]
25. Semple JW, Speck ER, Cosgrave D, Lazarus AH, Blanchette VS, Freedman J. Extreme leukoreduction of major histocompatibility complex class II positive B cells enhances allogeneic platelet immunity. *Blood*. 1999;93(2):713–720. [PubMed: 9885234]
26. Sayeh E, Aslam R, Speck ER, et al. Immune responsiveness against allogeneic platelet transfusions is determined by the recipient's major histocompatibility complex class II phenotype. *Transfusion*. 2004;44(11):1572–1578. [PubMed: 15504162]
27. Lucas DP, Leffell MS, Zachary AA. Differences in immunogenicity of HLA antigens and the impact of cross-reactivity on the humoral response. *Transplantation*. 2015;99(1):77–85. [PubMed: 25187165]

Figure. 1. Generation and characterization of the Con-K^d mouse.

A. A targeting construct encoding full length H-2K^d with a 5' transcription stop sequence (red box) flanked by *loxP* sites were used to replace the endogenous *H2-K1* gene in the B6 genome via homologous recombination. In the absence of Cre recombinase, *H2-K1* of the D haplotype is not transcribed. To generate the B6-ConK^d-on mouse, Con-K^d mice were crossed to B6.C-TgCMV-Cre mice, resulting in deletion of the stop sequence at the germline level. **B.** H-2K^d and H-2K^b sequences at the protein level are highly similar. Pair-wise sequence alignment of H-2K^b (top) and H-2K^d (bottom) was performed using EMBOS

Needle. A colon (:) indicates conservative substitutions while a period (.) indicates semi-conservative substitutions. CD4⁺ T cells from TCR transgenic mouse, TCR75, recognize the H-2K^d peptide (red box) presented in the context of I-A^b. **C.** Heterozygous Con-K^d-on mice express both H-2K^d and H-2K^b on CD45⁺ cells. **D.** Expression of H-2K^d on leukocytes (CD45⁺), platelets (CD41⁺) and red blood cells (Ter119⁺) from BALB/c, Con-K^d-on and B6 mice. **E.** Expression of H-2K^d and H-2K^b was evaluated on leukocytes from B6, Con-Kd-On, and B6.H2d mice and **F.** and frequencies of each subset was determined. For analysis, the following phenotypes were used to delineate leukocyte subsets: T cells: Thy1.2⁺CD19⁻, B cells: Thy1.2⁻CD19⁺, RPMs: Thy1.2⁻CD19⁻TER119⁻CD11^{-/lo}CD11b^{-/lo}F4/80⁺; CD8a⁺ DCs: Thy1.2⁻CD19⁻TER119⁻CD11c^{hi}CD11b⁻CD8a⁺; CD11b⁺ DCs: Thy1.2⁻CD19⁻TER119⁻CD11c^{hi}CD11b⁺CD8a⁻; pDCs: Thy1.2⁻CD19⁻TER119⁻CD11c^{int}Ly6C^{hi}; inflammatory monocytes: Thy1.2⁻CD19⁻TER119⁻CD11c^{-/lo}CD11b⁺Ly6G⁻Ly6C^{hi}CD115⁺; resident monocytes: Thy1.2⁻CD19⁻TER119⁻CD11c^{-/lo}CD11b⁺Ly6G⁻Ly6C^{lo}CD115⁻; neutrophils: Thy1.2⁻CD19⁻TER119⁻CD11c^{-/lo}CD11b⁺Ly6G⁺ and a high side scatter. For significance, *p 0.05. These experiments were performed at least 3 times with at least 3 mice per group and representative experiments are shown.

Figure 2. Con-K^d-on leukocytes have low immunogenicity.

B6 mice were infused with leukocytes from B6, *Con-K^d-on*, B6.H2^d, B6 X BALB/c F1 and BALB/c mice. Serum was collected 14 days post-infusion, followed by a second infusion of leukocytes and a second serum collection 14 days later. Sera were tested for anti-K^d antibodies by flow crossmatch using *Con-K^d-on* targets and by flow cytometry; B6 targets served as negative control. A two-way ANOVA with repeated measures was performed against B6 leukocytes and for significance **p 0.01, *p 0.05, ***p 0.001, ****p 0.0001, n.s. is not significant. This experiment was performed 3 times with 8–10 mice per group, with similar results each time. A representative experiment is shown in this figure, the results of the other two experiments are shown in supplemental figure 1.

Figure 3. Induction of specific CD4⁺ T cells via CFA/peptide immunization prior to leukocyte infusion circumvented the poor immunogenicity of Con-K^d-on leukocytes.

A. B6 mice were given peptide QEGPEYWEEQTQRAK emulsified in CFA, followed by a single infusion of leukocytes from B6, B6.K^d, B6 X BALB/c F1 and BALB/c leukocytes two weeks later. Sera were collected two weeks post leukocyte infusion and flow crossmatch was performed with *Con-K^d-on* targets to detect the presence anti-K^d antibodies. B6 targets served as negative control. **B.** As a control for non-specific inflammation effects, mice were treated with CFA only (no peptide) followed by leukocyte infusion. A two-way ANOVA with multiple comparisons post-test comparing each experimental group to B6 was utilized for analysis and for significance, ****p 0.0001. This experiment was performed 3 times with 3–4 mice per group, with similar results each time; a representative experiment is shown. The results of the other two experiments are shown in supplemental figure 2.

Figure 4. Increasing the frequency of H-2K^d specific CD4⁺ T cells prior to Con-K^d-On leukocyte infusion resulted in alloantibody response.

A. H-2K^d specific CD4⁺ T cells from TCR75 TCR transgenic mouse were isolated from the spleen, CFSE labeled, and adoptively transferred into B6 recipients one day prior to leukocyte infusion. The purity of CD4⁺ T cells isolated from TCR75 TCR transgenic mouse is typically ~85%. **B.** At three days post leukocyte infusion, subsets of the mice were sacrificed for T cell proliferation analysis. TCR75 T cells were identified as CD4⁺Thy1.1⁺ and proliferation was measured indirectly via CFSE dilution in response to leukocytes from B6, ConK^d-On, B6 X BALB/c F1 and BALB/c leukocytes. **C.** At 14 days post leukocyte infusion, sera were collected and flow crossmatch was performed using B6 or Con-K^d-on targets to detect the generation of anti-K^d antibody production. This experiment was performed 2 times with 3 mice per group, with similar results each time; a representative experiment is shown. The second iteration of the experiment is shown in supplemental figure 3.