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Alloantibodies Prevent the Induction of Transplantation Tolerance by Enhancing Alloreactive T cell Priming

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Abstract

Circulating alloantibodies in transplant recipients are often associated with increased antibody-mediated as well as cellular rejection. We tested the hypothesis that alloantibodies facilitate cellular rejection by functioning as opsonins to enhance T cell activation using a BALB/c to C57BL/6 heart or skin transplant model. Long-term heart and skin survival induced with anti-CD154 alone or in combination with donor-specific transfusion (DST), respectively, was abrogated by the presence of anti-K^d mAbs, and alloreactive T cell-activation as well as acute rejection was observed. The prevention of graft acceptance in the skin model was dependent on anti-K^d binding to and converting DST from tolerigenic to immunogenic. Adoptive transfer of CFSE-labeled TCR-Tg T cells into B6 recipients treated with anti-CD154/DST revealed the ability of anti-K^d to enhance the proliferation of anti-K^d-specific T cells via the indirect pathway, as well as of non-K^d reactive, recipient MHC-restricted $CD4^+$ and $CD8^+$ T cells. Thus alloantibodies with restricted specificity are able to facilitate the indirect presentation as well as the cross-presentation of a larger repertoire of ‘linked’ donor-derived antigens. These observations highlight the ability of alloantibodies to function not only in classical humoral rejection but also as opsonins that facilitate the CD40-CD154-independent activation of alloreactive T cells.

Keywords

rodent; T cells; alloantibodies; transplantation

Introduction

Tolerance induction remains elusive in larger recipients including non-human primates and humans, in spite of success in rodent models. One explanation is that pre-transplant patients have high frequencies of memory alloreactive T cells that are resistant to tolerance induction (1, 2). These allo-reactive T cells can be generated by allo-sensitization as a result of previous pregnancies, blood transfusions, or transplants. In addition, responses to pathogens often result in the sensitization of alloreactive T cells by cross-reactivity, and the cumulative lifetime exposure to pathogens can result in a high frequency of memory alloreactive T

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cells. Indeed deliberate sensitization or infection of rodent recipients, resulting in high frequencies of memory alloreactive $CD4^+$ and $CD8^+$ T cells, leads to an acquired resistance to tolerance induction (3-6). In addition, we recently reported that memory alloreactive B cells alone, or in combination with alloantibodies, prevented the acquisition tolerance in anti-CD154-treated recipients (7). Thus the presence of memory B cells and alloantibodies, or T cells, can independently resist tolerance induction.

Alloantibodies in the clinical setting are strongly correlated with the occurrence of hyperacute, acute antibody-mediated (AAMR), antibody-mediated cellular and chronic rejection (AACR) (8-11). The importance of antibodies, upstream of complement activation, in precipitating acute rejection was suggested by Wasowska *et al.* in an experimental model with B-cell deficient recipients that exhibited delayed rejection of allogeneic hearts (12). Acute rejection was restored to normal kinetics by the administration of complement-activating alloreactive mAbs, but not non-complement-activating mAbs (13, 14). The mechanistic basis for these observations were postulated to be alloantibodies binding to vascular endothelium and stimulating the local production chemokines such as monocyte chemotactic protein 1 (MCP-1) and neutrophil chemoattractant growth-related oncogene alpha (KC), which attract effector cells including macrophages, monocytes, basophils, neutrophils and T cells into the graft to mediate acute rejection. These and other similar observations provided the mechanistic basis for the clinical use of C4d deposition as a marker of antibody-deposition and antibody-mediated allograft rejection (15-18)

In addition to the established mechanisms of antibodies binding to graft endothelium to induce allograft rejection, alloantibodies may also be able to contribute to acute allograft rejection by facilitating T cell activation. In particular, we hypothesize that alloantibodies promote allospecific T cell priming through the generation of opsonized donor cells, which are more efficiently taken up and presented by antigen-presenting cells (APC). In this study, we used anti-CD154 and DST to induce long-term allogeneic skin survival and anti-H-$2K^d$ mAbs to test this hypothesis and to define the mechanism by which alloantibodies can prevent the induction of graft acceptance by anti-CD154-based therapies.

MATERIALS AND METHODS

Mice

Male and female C57BL/6 (B6; H-2^b), BALB/c (H-2^d), and C3H (H-2^K) mice, aged 7-9 weeks, were purchased from The Jackson Laboratories (Bar Harbor, ME) and The National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). $Rag^{-/-}$B6 (B6.129P372-*Rag1/Rag2*^{tm1Mnz}/J), TCRβδ$^{-/-}$ B6 (B6.129P2-*Tcrb*^{tm1Mom}Tcrd^{tm1Mom}/J), Act-OVA B6 (C57BL/6-Tg (CAG-OVA) 916Jen/J), OT-I B6 (C57BL/6-Tg (TcraTcrb) 1100Mjb/ /J), and OT-II B6 (C57BL/6-Tg (TcraTcrb) 425Cbn/J) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Female C57BL/6Ji-Kbtm1N12 mice ($K^{b-/-}$) were purchased from Taconic (Germantown, NY) at 6 weeks of age. Act-OVA BALB/c mice were a generous gift from Dr. Marisa Alegre (The University of Chicago), the TCR75 $CD4^+$ TCR Tg mice were from Dr. R. Pat Bucy (The University of Alabama at Birmingham), the K^d.B6 mice were from Dr. Sasha Chervonsky (The University of Chicago) and the $CD8^{-/-}$ B6 mice were from Dr. Yang-Xin Fu (The University of

Chicago). All animals were maintained and bred in the pathogen-free Carlson Barrier animal facility at The University of Chicago. The use of mice for these studies are summarized in Table 1 and have been reviewed and approved by The University of Chicago Institutional Animal Care and Use Committee.

Skin Transplantation

Donor tail skin was removed by carefully using sterile forceps, placed in a petri dish containing a PBS-soaked gauze and cut into $2cm^2$ square pieces. Donor tail skin was placed on the recipient bed and secured with 7-0 nylon sutures, then two ¾"x 3" adhesive band-aids were wrapped around the recipient trunk to preserve graft integrity during healing. In some experiments, 200 μl/mouse of sera from naïve or pre-sensitized B6 mice (immunized with 10^7 BALB/c spleen cells i.p. at day −14) were injected intra-venously (i.v.) into recipients on days −2, 0 and 2 post-transplantation of BALB/c skin into B6 recipients treated with anti-CD154 and DST. In other groups, 0.5mg anti-K^d IgG2a (ATCC HB159, BioXCell, West Lebanon, NH) or isotype control IgG2a (ATCC C1.18.4, BioXCell, West Lebanon, NH) mAbs were i.v., on the day of transplantation. On day seven post-transplantation, the band-aids were removed and skin grafts monitored. Rejection was defined as >90% scarring of the skin graft. In some experiments, CFSE-labeled TCR75, OTI, and OT II TCR Tg T cells ($0.25\text{-}1 \times 10^6$) were transferred by intravenous (i.v.) injection in B6 recipients, immediately post-skin transplantation. Animals were sacrificed on day five for analysis of CFSE dilution by flow cytometry.

Heterotopic Heart Transplantation

Heart transplantation was performed as previously described (19). Briefly, the donor aorta and pulmonary artery were anastomosed to the recipient abdominal aorta and inferior vena cava respectively. Graft function was monitored by abdominal palpation daily until rejection, which was defined as total cessation of contractions and was confirmed by direct visualization of the allograft.

Costimulatory Blockade Regimen

Donor splenocytes (DST) was prepared by passing whole splenocyte preparations through a sterile 0.2μm filter and resuspending in PBS, and 2×10^7 cells were injected i.v. on the day of transplantation. Anti-CD154 mAbs were purified from protein-free hybridoma medium (Invitrogen; Carlsbad, CA) using 45% ammonium sulfate precipitation and dialyzed for 48 hours in PBS. 1mg of anti-CD154 mAbs/dose/mouse was injected i.v. on days 0, 7 and 14 post-transplantation. Human CTLA4Ig (huCTLA4Ig; 0.5mg /mouse) (Bristol-Myers Squibb; New York City, NY) was administered daily, by intra-peritoneal (i.p.) injection, for 7 days post-skin transplantation.

Flow Cytometry

PE-conjugated anti-mouse Vβ8.3, PE-conjugated anti-mouseVα2, APC-conjugated anti-mouse CD4, APC-conjugated Strepavidin, APC-conjugated anti-mouse CD19, APC-conjugated anti-mouse Gr-1, APC-conjugated anti-mouse CDllc, APC-conjugated anti-mouse CDllb, PerCP-conjugated anti-mouse CD19, Biotin-conjugated anti-mouse IgM,

Biotin-conjugated anti-mouse IgG2a, Biotin-conjugated anti-mouse CD45.1, Biotin-conjugated anti-mouse Vβ5, FITC-conjugated anti-mouse IgG, Pe-Cy7-conjugated anti-mouse CD4, and Pe-Cy7-conjugated anti-mouse CD8 were purchased from BD Biosciences (San Jose, CA). Cells from were labeled on ice for 45 min with appropriate antibodies, followed by two washes in 2% FBS/PBS 0.01% Sodium Azide (FACS buffer). Cells were then resuspended in FACS buffer and analyzed on the FACScanto, or LSRII benchtop analyzers (BD Biosciences) using FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR).

IFN-γ ELISPOT

Assays were performed as previously described in detail (20). Briefly, ELISPOT plates (Millipore, Bedford, MA) were coated overnight with anti-IFN-γ mAb (BD Biosciences, San Jose, CA), and then blocked with sterile 10% FBS in PBS (PBST). Responder splenocytes were harvested from transplant recipients, and in some experiments T cells were purified by negative selection using a mouse Pan T cell Isolation kit (Miltenyi Biotec, Auburn, CA). Responders (10^6/well) were incubated with or without anti-CD3 (clone 2C11), C3H, BALB/c, or B/6 stimulators (0.4 x 10^6/well; γ-irradiated at 1200 Rads for 10 minutes), and then incubated overnight. Biotinylated anti-IFN-γ detection mAb (BD Biosciences, San Jose, CA) was added followed by horseradish peroxidase (HRP)-conjugated anti-biotin (BD Biosciences). Plates were developed, as previously described, and the resulting spots were analyzed using an Immunospot Series 1 Analyzer (Cellular Technology Ltd., Shaker Heights, OH).

Statistical Analysis

Statistical analysis to determine differences between groups were performed using the Student's t test for equal or unequal variances using Prism 4 for Macintosh (GraphPad, San Diego, CA). Kaplan Meier survival analysis was performed to determine significant differences in median graft survival between groups. A value of $p<0.05$ was considered statistically significant.

RESULTS

I. Alloantibodies elicit allograft rejection in anti-CD154 treated recipients.

Our previous findings that low-levels of circulating alloantibodies can synergize with memory B cells to mediate CD154-independent heart allograft rejection (7) prompted us to test whether a higher levels of circulating alloantibodies alone have the same effect. We observed a single dose of 0.5mg anti-K^d mAbs injected on the day of BALB/c cardiac allograft transplantation prevented the induction of long-term acceptance in anti-CD154-treated recipients (Figure 1A). Because cardiac allografts are vascularized with recipient endothelium and are therefore susceptible to antibody-mediated rejection (21), it is possible that the observations of rejection are due, at least in part to antibodies binding to the cardiac allograft. We therefore also tested whether allogeneic skin grafts are similarly affected by anti-K^d mAbs. Untreated B6 recipients rejected BALB/c skin grafts while recipients treated with anti-CD154/DST accepted their grafts long term (MST=60 days) (Figure 1B). Administration of anti-K^d mAbs, but not isotype control mAbs, to anti-CD154/DST-treated

recipients on the day of skin transplantation resulted in the acute rejection of the skin allografts (MST=12 days; p=0.0013) (22). To confirm that the rejection was not due to the presence of contaminating endotoxin (23-25), anti-K^d mAbs were denatured by heating for 10 minutes at 100°C and then injected into anti-CD154/DST-treated B6 recipients. Additionally, anti-K^d mAbs injected into B6 recipients of C3H skin grafts (H-2^k) treated with anti-CD154/C3H-DST. Under both conditions, all of the skin allografts were accepted long term (MST=60 days). These experiments demonstrate that anti-K^d mAbs can also mediate acute skin allograft rejection in anti-CD154/DST-treated recipients, based on the specific recognition/binding of H-2K^d on donor graft and/or DST, and not through non-antigen-specific effects. Finally we confirmed that the transfer of serum from pre-sensitized mice (200 μl/mouse; day −2, 0 and 2 post-transplantation) but not from naïve mice also prevented BALB/c skin graft acceptance in B6 mice treated with anti-CD154/DST (Figure 1C).

II. Alloantibody-mediated rejection is T cell dependent.

The observations that anti-K^d mAbs could override the tolerigenic effects of anti-CD154 ± DST to elicit acute graft rejection in both heart and skin models were consistent with the hypothesis that anti-K^d mAbs can function as opsonins to enhance alloreactive T cell activation and T cell-dependent rejection. Such a hypothesis would predict increased frequencies of alloreactive IFNγ-producing cells in B6 recipients treated with anti-CD154/DST and anti-K^d mAbs compared to those not receiving anti-K^d mAbs. Indeed, we observed B6 cardiac or skin recipients treated anti-CD154±DST and anti-K^d mAbs had an increased frequency of primed BALB/c-specific IFN-γ-producing cells compared to recipients that did not receive anti-K^d mAbs (Figure 2A & 2B). These data collectively support the conclusion that anti-K^d mAbs are able to override the immunosuppressive effects of anti-CD154 ± DST to facilitate the priming of allospecific T cells that mediate allograft rejection.

To test the necessity of T cells, TCR$\beta\delta^{-/-}$ mice that lack both $\alpha\beta$ and $\gamma\delta$ T cell subsets, as well as $CD8^{-/-}$ mice, were used as recipients of BALB/c skin grafts. Long-term graft survival (>80 days) was achieved in the TCR$\beta\delta^{\square/-}$ recipients receiving anti-CD154/DST and anti-K^d mAbs (p=0.0003) (Figure 2C), confirming that the rejection observed in B6 mice was T cell-dependent and that anti-K^d mAb binding to the skin allograft was not sufficient to cause acute rejection in the absence of T cells. Graft rejection was significantly delayed (MST=26 days) (p=0.0027) in $CD8^{-/-}$ recipients compared to WT B6 recipients, suggesting that both $CD4^+$ and $CD8^+$ T cells contribute to anti-K^d-mediated graft rejection. Experiments were not performed in $CD4^{-/-}$ recipients because the presence of $CD4^+$ T cells was essential for anti-CD154-mediated graft acceptance.

III. Anti-K^d mAb recognition of H-2K^d on DST is necessary and sufficient to mediate skin allograft rejection in anti-CD154/DST-treated recipients.

To gain insights into the mechanism by which anti-K^d mAbs prevent the induction of long-term skin allograft acceptance by anti-CD154/DST, we tested whether the anti-K^d mAbs recognized H-2K^d expressed on the graft or on the DST to prevent tolerance induction. To this end we generated F1 C3H x K^d.B6 (K^d.H-2^{bxk}) mice to be used as a source of

donor tail skin and DST. Positive controls were anti-K^d mAbs mediating the rejection of F1 C3H x K^d.B6 (K^d.H-2^{bxk}) skin allografts when both skin and DST were from K^d.B6 x C3H F1 (K^d.H-2^{bxk}) (Group I). Negative controls were the acceptance of C3H x B6 F1 (H-2^{bxk}) skin allografts in B6 recipients receiving anti-CD154/H-2^{bxk} DST and anti-K^d mAbs (Table II; Group II). When H-2K^d expression was confined to only the K^d·H-2^{bxk} allograft, and the H-2^{bxk} DST lacked K^d expression, the majority of grafts were accepted (MST>55 days) by recipients receiving anti-CD154/DST and anti-K^d mAbs (Group III). In contrast, when H-2K^d was expressed only on DST, but absent on allograft tissue, rejection was observed in the recipients treated with anti-CD154 and anti-K^d mAbs (Group IV). These observations demonstrate that anti-K^d recognition of H-2K^d on DST, but not on the skin graft, is necessary and sufficient to precipitate CD154-independent skin allograft rejection. To address the concern that rejection induced by the anti-K^d mAbs was due to a non-specific elimination of the DST, we increased the amount of DST administered 4-fold (Group V), which normalized for the amount of DST present after 24 hours in the spleens of B/6 recipients receiving anti-K^d mAbs compared to recipients that did not (data not shown). The kinetics of graft rejection in this group was also similar to B6 recipients receiving the normal dose of DST and anti-K^d mAbs (Group I), suggesting that accelerated clearance of DST is unlikely to be the primary cause of the loss of tolerogenicity.

IV. Anti-K^d mAbs prime K^d-specific T cells as well as T cells that recognize “Linked Antigens” on the DST

We hypothesized that the anti-K^d mAbs converted the DST from tolerigenic to immunogenic, thereby facilitating an anti-CD154-independent activation of graft-reactive T cells. To visualize the fate of graft-reactive T cells in the presence or absence of anti-K^d mAbs *in vivo*, we performed an adoptive transfer assay, initially with TCR75 $CD4^+$ T cells that specifically recognize the K^d $_{54\text{-}68}$ peptide presented on I-A^b MHC Class II molecules. 0.25 x 10^6 CFSE-labeled TCR75 T cells transferred into non-transplanted B6 recipients underwent minimal proliferation, while significant cell proliferation was observed when TCR75 T cells were injected into transplanted but untreated B6 recipients (Figure 3). TCR75 T cell proliferation was significantly reduced in anti-CD154/DST treated-B6 recipients, and anti-K^d mAbs was able to restore T cell proliferation (p=0.0067). These studies confirm that anti-K^d mAbs facilitated the anti-CD154-independent proliferation of K^d-specific T cells *in vivo*.

It is well established that during the course of an immune response to self-antigens, increased diversification of autoreactive specificities arises through a process of epitope spreading (26). To test whether anti-K^d antibodies can promote the priming of donor-reactive T cells that recognize donor antigens not recognized by the anti-K^d mAb, 0.8-1 x 10^6 CFSE-labeled, OVA-specific $CD4^+$ OT-II T cells were transferred into B6 recipients of Act-OVA BALB/c grafts. OT-II T cells injected into naïve B6 recipients had minimal T cell proliferation, while OT-II T cells displayed robust proliferation upon transfer into untreated B6 recipients of Act-OVA BALB/c grafts (Figure 4). OT-II T cell proliferation was reduced in B6 recipients treated with anti-CD154/DST, and it was restored by the addition of anti-K^d mAbs (p= 0.0015).

The observations that $CD8^+$ T cells contribute to the rejection of skin allografts in anti-CD154/DST-treated recipients receiving anti-K^d mAbs (Figure 2C) prompted us to further test whether anti-K^d mAbs can promote the presentation of exogenously derived donor antigens to $CD8^+$ T cells. To this end, 0.8-1 x 10^6 CFSE-labeled $CD8^+$ OT-I T cells were adoptively transferred into B6 recipients of Act-OVA BALB/c grafts. Minimal OT-I T cell proliferation was observed in non-transplanted B6 recipients, while robust OT-I T cell proliferation was observed in untreated B6 recipients of Act-OVA BALB/c grafts (Figure 4). Anti-CD154/DST inhibited OT-I T cell proliferation while anti-K^d mAbs were able to restore robust OT-I cell proliferation ($p=0.001$). These observations confirm the ability of anti-K^d mAbs to enhance cross-presentation of graft-derived antigens and induce the proliferation of $CD8^+$ T cells in recipients treated with anti-CD154/DST.

V. Anti-K^d mAbs facilitate the rejection of allografts lacking K^d but expressing "Linked Antigens"

The observation that anti-K^d mAbs enhanced the activation of T cells specific for donor-antigens not recognized by the anti-K^d mAbs, led us to test whether these observations are functionally significant and capable of eliciting the rejection of a second, non-K^d-expressing skin allograft. The experimental approach involved the transplantation of two adjacent allogeneic skin grafts onto B6 recipients. B6 recipients treated with anti-CD154 and anti-K^d mAbs accepted both skin grafts when the grafts and DST were from H-2^{bxk} (B6 x C3H F1) donors, and rejected both grafts when the grafts and DST were from K^d.H-2^{bxk} donors (Table III; Group I and II). When the DST was from K^d. H-2^{bxk} donors and the grafts were from K^d ·H-2^b and H-2^{bxk} donors, both grafts were both rejected (Group III). In contrast, when the grafts were from K^d ·H-2^b and H-2^{bxk} donors, and the DST was a combination of cells from both donors, only the K^d-expressing graft was rejected (Group IV). Finally when the grafts were from K^d ·H-2^{bxk} or H-2^{bxk} donors, and the DST was a combination of cells from the same two donors, only the K^d-expressing graft was rejected in recipients treated with anti-CD154 (Group V). These observations confirm that linked antigens on the DST were necessary and sufficient for the priming of non-K^d-specific T cells, and that by-stander effects generated by opsonins comprising anti-K^d mAbs and K^d ·H-2^b DST did not result in the sufficient priming of H-2^k T cell responses to cause the rejection of non-K^d- but H-2^k-expressing allogeneic skin grafts.

VI. Alloantibody-mediates B7-dependent, CD154-independent skin allograft rejection.

We hypothesized that anti-K^d generated opsonins can bind to FcγR and complement receptors on APCs, and that this interaction promoted APC maturation and induced the expression of costimulatory molecules that compensated for the blockade of the CD40-CD154 pathway. To test this hypothesis, we additionally targeted the B7-1/B7-2-CD28 pathway by using the CTLA4Ig fusion protein to block the CD28-B7 pathways (27, 28). B6 recipients of BALB/c skin grafts treated with anti-CD154/DST alone or anti-CD154/DST plus CTLA4Ig displayed long-term graft survival (>60 days; Figure 5) (29). In contrast to the rejection of skin grafts in recipients receiving anti-CD154/DST plus anti-K^d mAbs, the addition of CTLA4Ig resulted in 75% of recipients accepting their skin allografts (Figure 5). We conclude from these observations that anti-K^d mAbs generated opsonins that promoted

the B7-dependent activation of alloreactive T cells and the rejection of skin allografts in anti-CD154/DST-treated recipients.

DISCUSSION

A consensus is emerging that donor-specific antibodies (DSA) are predictive of poor graft outcome (30, 31), and the activation of complement downstream of DSA binding to graft endothelium comprise the currently accepted paradigm for the mechanistic basis of how DSA effects graft loss (32). The observations in this study challenge the limited scope of this paradigm, and demonstrate the ability of alloantibodies to function as opsonins to facilitate CD40-CD154-independent T cell-activation and the rejection of allogeneic heart and skin grafts. The activation and necessity for allospecific T cells in anti-K^d-mediated allograft rejection is supported by observations of an increased precursor frequency of donor-specific T cells secreting IFN-γ in B6 recipients treated with anti-CD154±DST and anti-K^d mAbs; a robust proliferation of allospecific T cells upon transfer into B6 recipients treated with anti-CD154/DST and anti-K^d mAbs; and the inability of anti-K^d mAbs to mediate skin allograft rejection in TCRβδ$^{-/-}$ recipients.

In the cardiac tolerance model, prevention of long-term graft survival anti-K^d mAbs was associated with an anti-CD154-independent activation of alloreactive T cells. However, it was unclear whether events downstream of anti-K^d binding to the graft endothelium also contributed to and were necessary for rejection. We therefore turned to the skin transplant model, which is less susceptible to allo-antibody-mediated rejection, and where the addition of DST is necessary for the induction of long-term graft survival. In that model, we demonstrate a critical role of both polyclonal graft-reactive antibodies in the sera of pre-sensitized mice or purified anti-K^d mAbs binding to DST to prevent long-term skin graft acceptance in anti-CD154/DST treated recipients. Indeed, if K^d expression was restricted to the skin graft, anti-K^d mAbs had minimal effect on tolerance induction whereas if K^d expression was restricted to the DST, tolerance induction was abrogated. We addressed the concern that the anti-K^d mAbs were simply inducing the rapid elimination of DST, by administered 4-fold more DST to normalize for their more rapid clearance in the presence of anti-K^d mAbs. We observed that anti-K^d mAbs were still able to prevent the induction of long-term graft survival. These observations collectively support a conclusion that anti-K^d mAbs can function as opsonins to enhance alloreactive T cell activation, in addition to their binding to donor endothelial cell binding. In the skin model, anti-K^d mAbs converted the DST from tolerigenic to being immunogenic. Studies are ongoing to visualize the fate of DST in recipients with or without circulating anti-K^d mAbs to provide a more detailed understanding of how DST can be tolerigenic or immunogenic. The ability of alloantibodies to function as opsonins to enhance allograft rejection adds to their recognized role of binding to graft endothelium to elicit humoral rejection. Indeed we speculate that the opsonic activity of alloantibodies may, in part, explain the clinical observations that DSA in the absence of C4d deposition are predictors of graft loss (33), while acknowledging that insensitivity in the detection of C4d may also be a contributory factor. The requirement of C3 for long-term graft survival prevented the use of C3$^{-/-}$ recipients to test the necessity of complement in our model of alloantibody-mediated allograft rejection. However, given that oponins contain complement, we hypothesize that the presence of complement plays an

important role in the ability of DST to become immunogenic. Future studies are underway to determine the necessity of complement in alloantibody-mediated allograft rejection.

T cell suppression in transplantation tolerance through linked-recognition is a well-recognized concept that explains infectious tolerance to third-party antigens, if these antigens are linked and presented on the same antigen presenting or donor cell that expresses the tolerized donor antigens (34, 35). We demonstrate a similar phenomenon with regards to the prevention of CD154-induced tolerance by anti-K^d mAbs, which facilitated the proliferation of not only K^d-specific TCR-75 T cells, but also of non-K^d-specific OT-II T cells recognizing OVA antigens expressed on the Act-OVA BALB/c DST and skin grafts. Further we show that the anti-K^d promoted the cross-presentation of DST from Act-OVA BALB/c donors, by demonstrating the proliferation of $CD8^+$ OT-I T cells in recipients treated with anti-CD154/DST. Based on published observations, we infer from these cross-priming observations that $CD8^+$ dendritic cells (DCs) are likely to be the antigen-presenting cell subset targeted by opsonized DST, although we cannot exclude the possibility that other DC subsets may also participate in the activation of alloreactive $CD4^+$ T cells primed by the indirect pathway (36). Again, studies to visualize the fate of opsonized and non-opsonized DST in vivo will provide insights into the APC subset involved in DST uptake and presentation to T cells.

Non-K^d graft-reactive T cells activated in the presence of anti-K^d mAbs and anti-CD154/DST have the ability to reject allografts lacking K^d, as illustrated by the series of two skin allograft transplantations. We also show that this process, which resembles “epitope-spreading’, requires linked-recognition in that the non-K^d antigens have to be co-expressed on the K^d-expressing DST. Further we demonstrate that the administration of a mixture of K^d and non-K^d-expressing third-party allogeneic DST does not result in the priming of the third-party T cells nor the rejection of third-party skin grafts. Thus signals generated by the uptake of opsonized K^d-expressing DST are unable to result in the by-stander activation of APCs presenting third-party antigens. These observations may be explained by anti-K^d mAbs binding to DST, leading to the generation of the antibody- and complement-coated DST which then bind to APCs via Fcγ R and complement receptors (37-40). This binding should lead to the activation and maturation of APCs which, when under inflammatory conditions, have been shown to undergo major phenotypic and functional modification, including the reduction of further uptake of exogenous antigen (41, 42). While we do not provide a direct demonstration of APC maturation in the presence of anti-K^d, our observations that tolerance can be induced with the combination of anti-CD154/DST and CTLA4Ig but not in the presence of anti-CD154/DST are consistent with increased expression of B7 in DCs matured in the presence of anti-K^d mAbs (42). Studies to track the cells taking up opsonized versus non-opsonized DST are ongoing, and should provide mechanistic insights. The *in vivo* blockade experiments also suggest a therapeutic strategy for the successful induction of tolerance in the presence of opsonizing DSA. Finally these observations underscore the potential ability of DSA with limited specificities to prime a larger repertoire of alloreactive T cells, which may have significant implications to the clinical scenario of transplanting sensitized recipients with a restricted repertoire of DSA. Our observations suggest that high-titer DSA to a restricted donor antigen repertoire may

be comparably detrimental as broadly reactive DSA, with regards to their ability to generate opsonins and prime a broadly reactive alloreactive T cell repertoire.

In summary, our study demonstrates that alloantibodies can function as opsonins to enhance CD154-independent T cell priming and acute allograft rejection in anti-CD154-treated recipients. In the model of skin graft rejection, where long-term graft survival is induced in the presence of DST, antibodies bind to and convert the DST from tolerigenic to immunogenic. Further donor-reactive antibodies binding to DST leads to enhanced indirect presentation of alloantigen, epitope-spreading and cross-presentation of alloantigens. Thus our study provides experimental evidence for a second mechanism by which donor-reactive antibodies can facilitate acute graft rejection, in addition to the well accepted role of mediating complement-dependent humoral- or antibody-mediated rejection.

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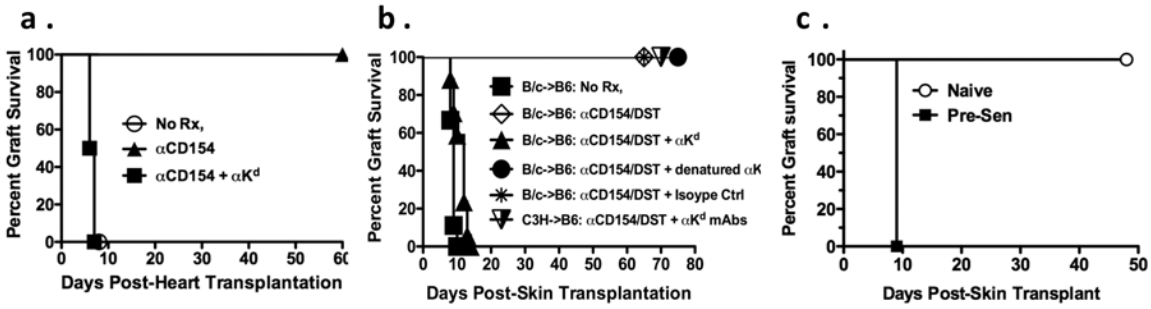

Figure 1.

Anti-K^d mAbs elicits CD154-independent cardiac and skin allograft rejection. *a.* Rejection of BALB/c heart grafts by B6 recipients treated with anti-CD154 and anti-K^d mAbs (N = 4) (closed square) compared to B6 recipients treated with anti-CD154 alone (N = 15) (closed triangle) or untreated recipients (N = 4) (open circle). *b.* Rejection of BALB/c skin grafts by B6 recipients treated with anti-K^d mAbs plus anti-CD154/DST (N = 20) (closed triangle) in comparison to long-term graft survival of BALB/c grafts by B6 recipients treated with anti-CD154/DST (N = 11) (open diamond) and rejecting untreated B6 recipients of BALB/c grafts (N = 9) (closed square). Acceptance of BALB/c grafts by B6 recipients treated with anti-CD154/DST and denatured anti-K^d mAbs (N = 5) (closed circle), or mouse IgG isotype control (N = 4) (asterisk), and C3H grafts by B/6 recipients treated with anti-K^d mAbs and anti-CD154/DST (N = 4) (half closed downward triangle). *c.* The transfer of serum from pre-sensitized mice (200 μl/mouse; day −2, 0 and 2 post-transplantation; Pre-Sen), but not from naïve mice, prevented BALB/c skin graft acceptance in B6 mice treated with anti-CD154/DST (N=4/group).

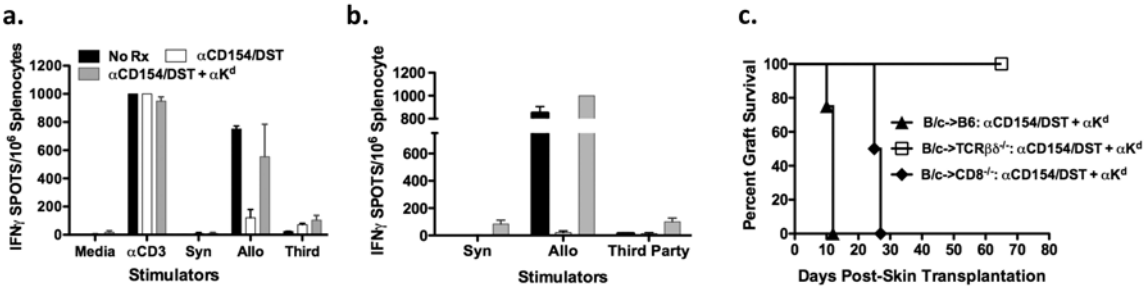

Figure 2.

Role of T cells in anti-K^d mAb-dependent graft rejection *a*. Frequencies of alloreactive IFN-γ producing cells in untreated B6 recipients (N = 3) (black), or in recipients of cardiac transplants treated with anti-CD154/DST (N = 4) (white), or anti-CD154/DST with anti-K^d (N = 4) (grey). Splenocytes were stimulated *in vitro* with irradiated B6 (Syn), BALB/c (Allo), C3H (Third-Party) stimulators, and controls were anti-CD3 or media. Data are presented as mean ± standard error. *b*. The frequency of alloreactive T cells secreting IFN-γ were measured in untreated B6 recipients (N = 3) (black), anti-CD154/DST treated B6 recipients (N = 3) (white), and anti-CD154/DST with anti-K^d mAbs (N = 4) (grey). *c*. Acceptance of BALB/c grafts by TCRβδ$^{-/-}$ recipients treated with anti-CD154/DST and anti-K^d mAbs (N = 6) (open square) compared to rejection by similarly treated B6 recipients (N = 20; p = 0.0003) (closed triangle). Comparably treated B6.CD8$^{-/-}$ recipients exhibited delayed rejection (N = 4; p = 0.0027) (closed diamond).

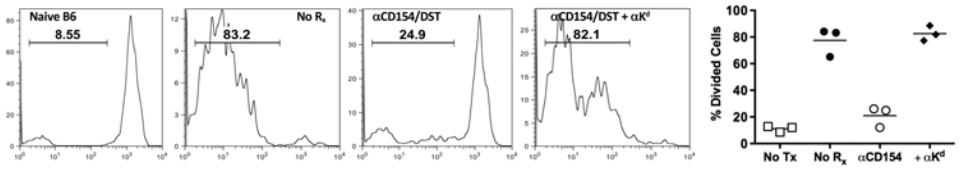

Figure 3.

Allospecific TCR75 $CD4^+$ T cells proliferate in the presence of anti-K^d mAbs. CFSE-labeled TCR75 TCR-Tg T cells (0.25×10^6) were injected i.v. at the time of skin transplantation. TCR75 T cells were harvested from the draining lymph nodes five days later and proliferation was measured by flow cytometry. Representative histogram of TCR75 T cell proliferation from untransplanted B6 recipients (naïve B6) (1st Panel), B6 recipients of BALB/c grafts without treatment (No R_x) (2nd Panel), treated with anti-CD154/DST (αCD154/DST) (3rd Panel), or with anti-CD154/DST and anti-K^d mAbs (αCD154/DST + αK^d) (4th Panel). The percentage of dividing TCR75Tg T cells from individual mice in each group are summarized (N = 3/ group). Significantly higher percentages of proliferating TCR75 Tg T cells were observed in B6 recipients treated with anti-K^d mAbs and anti-CD154/DST compared to recipients treated with anti-CD154/DST ($p = 0.0067$).

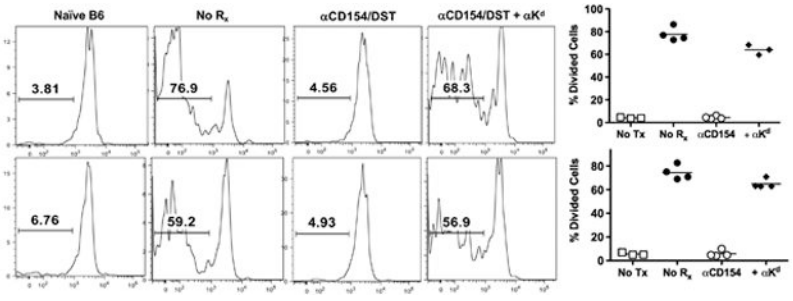

Figure 4.

Anti-K^d mAbs primes T cells specific for alloantigens not recognized by anti-K^d mAbs and enhances presentation of exogenously derived alloantigens. All animals were injected with 5uM CFSE labeled 0.8 x 10^6- 10^7 OT-II and OT-I TCR Tg T cells at the time of skin transplantation. Actin-OVA BALB/c mice were used as donors for all skin transplants. Flow cytometric analysis of OT-II (top row) and OT-I (bottom row) TCR Tg T cells was measured in the draining lymph nodes five days post-skin transplantation of untransplanted B6 recipients (1st Column), B6 recipients of BALB/c-OVA grafts without treatment (2nd Column), B6 recipients of BALB/c-Ova grafts treated with anti-CD154/DST (3rd Column), and B6 recipients of BALB/c-OVA grafts treated with anti-CD154/DST and anti-K^d mAbs (4th Column). Significantly higher percentages of proliferating cells were observed in B6 recipients treated with anti-K^d mAbs and anti-CD154/DST compared to recipients treated with anti-CD154/DST (for OT-1 $p = 0.0001$, for OT-II; $p = 0.0015$).

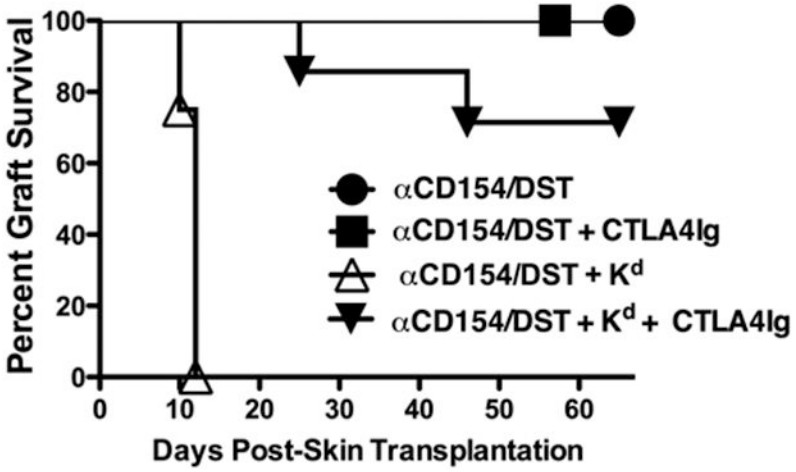

Figure 5.

CTLA4Ig overrides the effects of Anti-K^d mAbs and facilitates graft acceptance in anti-CD154/DST-treated recipients. Acceptance of BALB/c skin grafts by B6 recipients treated with anti-CD154/DST + CTLA4Ig (N =4) (closed square) anti-CD154/DST + CTLA4Ig with anti-K^d mAbs (N = 7) (closed downward triangle) but rejection by B6 recipients treated with anti-CD154/DST and anti-K^d mAbs (N = 15) (open upward triangle). B6 recipients treated with anti-CD154/DST (N = 11) (closed circle) also accepted BALB/c skin grafts.

table I

List of mice used.

Mouse Strain	Experimental Purpose	Figure/Table Used
BALB/c	Donor	Figures 1, 2,3,5
C57BL/6	Recipient	Figures 1, 2,3,4,5 Tables I, II
C3H	Donor	Figures 1 Tables I, II
TCRβδ$^{-/-}$	Recipient, test the requirement of T cells	Figure 2
CD8$^{-/-}$	Recipient, test the requirement of CD8^{+} T cells	Figure 2
K^{d}.C57BL/6 (K^{d} Tg) (K^{d}.B6)	Used for crossing to other strains	Table I, II
C3H x K^{d}.C57BL/6 F1 (K^{d}.H-2^{bxk})	Donor, test the importance of K^{d} expression on DST	Table I, II
C3H x C57BL/6 F1 (H-2^{bxk})	Donor, test the importance of K^{d} expression on DST; control	Table I, II
TCR75 TCR TG	Used CD4^{+} T cells for adoptive transfer	Figure 3
Act-Ova C57BL/6	Donor, expresses ovalbumin as a transmembrane protein under the actin promoter	Figure 4
OT-I C57BL/6	Used CD8^{+} T cells for adoptive transfer	Figure 4
OT-II C57BL/6	Used CD4^{+} T cells for adoptive transfer	Figure 4

table II

Anti-K^d mAb recognition of H-2K^d on DST is necessary and sufficient to mediate skin allograft rejection in recipients treated with anti-CD154/DST

Group	Graft	DST	Mean ± SEM
I.	K^d .H-2^{bxk}	K^d .H-2^{bxk}	11.5 ± 0.3 (N=6)
II.	H-2^{bxk}	H-2^{bxk}	60.0 ± 0.0 (N=6)
III.	K^d .H-2^{bxk}	H-2^{bxk}	52.3 ± 7.6 (N=6)
IV.	H-2^{bxk}	K^d .H-2^{bxk}	13.2 ± 0.4 (N=6)
V.	H-2^d	4X H-2^d	11.5 ± 0.5 (N=4)

table III

Anti-K^d mAbs promote epitope-spreading and cross-presentation of donor alloantigen to alloreactive T cells in vivo.

Skin Grafts	DST	MST ± SEM
I. No K^d on DST or Graft		
H-2^{bxk}	H-2^{bxk}	[a]57.7 ± 1.6 (N=6)
H-2^{bxk}		[a]57.0 ± 1.6 (N=6)
II. Linked K^d on DST and Graft		
K^d .H-2^{bxk}	K^d .H-2^{bxk}	11.5 ± 0.3 (N=6)
K^d .H-2^{bxk}		11.5 ± 0.3 (N=6)
III. Linked K^d on DST Only		
K^d	K^d .H-2^{bxk}	11.5 ± 0.5 (N=8)
H-2^{bxk}		12.8 ± 4.3 (N=8)
IV. Linked K^d on Graft Only		
K^d.H-2^{bxk}	½ K^d +	11.3 ± 0.3 (N=11)
H-2^{bxk}	½ H-2^{bxk}	[b]48.3 ± 5.4 (N=11)
V. Unlinked K^d on DST and Graft		
K^d	½ K^d +	10.8 ± 0.4 (N=6)
H-2^{bxk}	½ H-2^{bxk}	[c]53.0 ± 5.5 (N=6)