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

Audrea M. Burns and Anita S. Chong

Circulating alloantibodies in transplant recipients are often associated with increased Ab-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-Kd 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-Kd binding to and converting DST from tolerigenic to immunogenic. Adoptive transfer of CFSE-labeled TCR-transgenic T cell activation as well as acute rejection was observed. The prevention of graft acceptance in the skin model was dependent on specific T cells via the indirect pathway as well as of non–Kd-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 Ags. 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. The Journal of Immunology, 2011, 186: 000–000.

Tolerance induction remains elusive in larger recipients including nonhuman primates and humans, despite success in rodent models. One explanation is that pretransplant patients have high frequencies of memory alloreactive T cells that are resistant to tolerance induction (1, 2). These alloreactive T cells can be generated by allosensitization 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 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 Ab-mediated, Ab-mediated cellular and chronic rejection (8–11). The importance of Abs, upstream of complement activation, in precipitating acute rejection was suggested by Wasowska et al. (12) in an experimental model with B cell-deficient recipients that exhibited delayed rejection of allogeneic hearts. 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 was postulated to be alloantibodies binding to graft vascular endothelium and stimulating the local production chemokines such as monocyte chemotactic protein-1 and neutrophil chemoattractant growth-related oncogene α (keratinocyte chemoattractant), 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 Ab deposition and Ab-mediated allograft rejection (15–18).

In addition to the established mechanisms of Abs 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 APCs. In this study, we used anti-CD154 and donor-specific transfusion (DST) to induce long-term allogeneic skin survival and anti–H-2Kd 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-2b), BALB/c (H-2d), and C3H (H-2Kk) mice, aged 7–9 wk, were purchased from The Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). Rag2−/− (B6.129P2-Ptgtrom1Mind-Tcrd−/−/Tcr3−/−), OT-I B6 (C57BL/6-Tg [Tcr5/2] 1100Mbj/J), and OT-II B6 (C57BL/6-Tg [Tcr5/2] 425Cbm/J) mice were purchased from The Jackson Laboratory. Female C57BL/6-J-Kbtm1N12 mice (Kb−/−) were purchased from Taconic Farms (Germantown, NY) at 6 wk of age. Act-OVA BALB/c mice were a gift from Dr. M. Alegre (University of Chicago, Chicago, IL), the TCR75 CD4+ TCR transgenic (Tg) mice were from Dr. R. P. Bucy (University of Alabama, Birmingham, AL), the KdB6 mice were from Dr. A. Chervonsky (University of Chicago), and the CD8−/− B6 mice were from Dr. Y.-X. Fu (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 I 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 2-cm² square pieces. Donor tail skin was placed on the prepared flank of recipient and secured with 7-0 nylon sutures then two 3/4” × 3/4” adhesive band-aids were wrapped around the recipient trunk to preserve graft integrity during healing. In some experiments, 200 µL/mouse sera from naive or pre-sensitized B6 mice (immunized with 10⁷ BALB/c spleen cells i.p. at day −14) were injected i.v. into B6 recipients on days −2, 0, and 2 post-transplantation of BALB/c skin. These B6 recipients were then treated with anti-CD145 and DST to induce graft acceptance. In other groups, 0.1 mg anti-Kd IgG2a (ATCC HB159; Bio X Cell, West Lebanon, NH) or isotype control IgG2a (ATCC C1.18.4; Bio X Cell) mAbs were injected i.v. on the day of transplantation. On day 7 posttransplantation, the band-aids were removed, and skin grafts were 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 (0.25–1 × 10⁶) were transferred by i.v. injection in B6 recipients, immediately post-skin transplantation. Animals were sacrificed on day 5 for analysis of CFSE dilution by flow cytometry.

Heterotopic heart transplantation

Heart transplantation was performed as described previously (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) were prepared by passing whole splenocyte preparations through a sterile 0.2-µm filter and resuspending in PBS, and 2 × 10⁶ cells were injected i.v. on the day of transplantation. Anti-CD145 mAbs were purified from protein-free hybridoma medium (Invitrogen, Carlsbad, CA) using 45% ammonium sulfate precipitation and dialyzed for 3 days. Anti-Kd mAbs were purified from protein-free hybridoma medium (Invitrogen, Carlsbad, CA) using 45% ammonium sulfate precipitation and dialyzed for 3 days. Anti-Kd mAbs were purified from protein-free hybridoma medium (Invitrogen, Carlsbad, CA) using 45% ammonium sulfate precipitation and dialyzed for 3 days. Anti-Kd mAbs were purified from protein-free hybridoma medium (Invitrogen, Carlsbad, CA) using 45% ammonium sulfate precipitation and dialyzed for 3 days.

Flow cytometry

PE-conjugated anti-mouse Vβ8.3, PE-conjugated anti-mouse Vα2, allophycocyanin-conjugated anti-mouse CD4, allophycocyanin-conjugated streptavidin, allophycocyanin-conjugated anti-mouse CD19, allophycocyanin-conjugated anti-mouse CD11c, allophycocyanin-conjugated anti-mouse CD11b, 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 Abs, 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 FACS Canto II or LSRII benchtop analyzers (BD Biosciences) using FlowJo flow cytometry analysis software (Tree Star, 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) 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 (10⁶/well) were incubated with or without anti-CD3 (clone 2C11), C3H. BALB/c, or B6 stimulators (0.4 × 10⁶/well; gamma irradiated at 1200 rad for 10 min) and then incubated overnight. Biotinylated anti–IFN-γ detection mAb (BD Biosciences) was added, followed by HRP-conjugated anti-biotin mAb (BD Biosciences). Plates were developed, as previously described, and the resulting spots were analyzed using an Immunospot Series 1 Analyzer (Cellular Technology, Shaker Heights, OH).

Statistical analysis

Statistical analysis to determine differences between groups were performed using the Student 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; p < 0.05 was considered statistically significant.

Results

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 higher levels of circulating alloantibodies alone had the same effect. The list of donor and recipient mouse strains are listed in Table I. We observed that a single dose of 0.5 mg anti-Kd mAbs injected on the day of BALB/c cardiac allograft transplantation prevented the induction of long-term acceptance in anti–CD154-treated recipients (Fig. 1A). Because cardiac allografts are vascularized with recipient endothelium and are therefore susceptible to Ab-mediated rejection (21), it is possible that the observations of rejection are due, at least in part, to Abs binding to the cardiac allograft. We therefore also tested whether allogeneic skin grafts are similarly affected by anti-Kd mAbs. Untreated B6 recipients rejected BALB/c skin grafts, whereas recipients treated with anti-CD154/DST accepted their

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<td>Recipient, test the requirement of T cells</td>
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<td>CD8&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>K&lt;sup&gt;d&lt;/sup&gt;,C57BL/6 (K&lt;sup&gt;d&lt;/sup&gt; Tg) (K&lt;sup&gt;d&lt;/sup&gt;B6)</td>
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<td>OT-I C57BL/6</td>
<td>Used CD8&lt;sup&gt;+&lt;/sup&gt; T cells for adoptive transfer</td>
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<tr>
<td>OT-II C57BL/6</td>
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grafts long term (mean graft survival time [MST] = 60 d) (Fig. 1B). Administration of anti-Kd 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 d; p = 0.0013) (22). To confirm that the rejection was not due to the presence of contaminating endotoxin (23–25), anti-Kd mAbs were denatured by heating for 10 min at 100°C and then injected into anti–CD154/DST-treated B6 recipients. In addition, anti-Kd mAbs injected into B6 recipients of C3H skin grafts (H-2b) treated with anti–CD154/C3H-DST. Under both conditions, all of the skin allografts were accepted long term (MST = 60 d). These experiments demonstrate that anti-Kd mAbs can also mediate acute skin allograft rejection in anti–CD154/DST-treated recipients, based on the specific recognition/binding of H-2Kd on donor graft and/or DST and not through non–Ag-specific effects. Finally, we confirmed that the transfer of serum from presensitized mice (200 μl/mouse; days −2, 0, and 2 posttransplantation) but not from naive mice also prevented BALB/c skin graft acceptance in B6 mice treated with anti-CD154/DST (Fig. 1C).

Alloantibody-mediated rejection is T cell dependent

The observations that anti-Kd mAbs could override the tolerogenic effects of anti-CD154 with or without DST to elicit acute graft rejection in both heart and skin models were consistent with the hypothesis that anti-Kd 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 IFN-γ–producing cells in B6 recipients treated with anti-CD154/DST and anti-Kd mAbs compared with those not receiving anti-Kd mAbs. Indeed, we observed B6 cardiac or skin recipients treated with anti–CD154 with or without DST and anti-Kd mAbs had an increased frequency of primed BALB/c-specific IFN-γ–producing cells compared with recipients that did not receive anti-Kd mAbs (Fig. 2A, 2B). These data collectively support the conclusion that anti-Kd mAbs are able to override the immunosuppressive effects of anti-CD154 with or without DST to facilitate the priming of allospecific T cells that mediate allograft rejection.

To test the necessity of T cells, TCRβδ−/− mice that lack both αβ and γδ T cell subsets, as well as CD8−/− mice, were used as recipients of BALB/c skin grafts (Table I). Long-term graft survival (>80 d) was achieved in the TCRβδ−/− recipients receiving anti-CD154/DST and anti-Kd mAbs (p = 0.0003) (Fig. 2C), confirming that the rejection observed in B6 mice was T cell dependent and that anti-Kd 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 d) (p = 0.0027) in CD8−/− recipients compared with WT B6 recipients, suggesting that both CD4+ and CD8+ T cells contribute to anti–Kd-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.

Anti-Kd mAb recognition of H-2Kd 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-Kd mAbs prevent the induction of long-term skin allograft acceptance by anti–CD154/DST, we tested whether the anti-Kd mAbs recognized H-2Kd expressed on the graft or on the DST to prevent tolerance induction. To this end, we generated F1 C3H × Kd.B6 (Kd.H-2bk) mice to be used as a source of donor tail skin and DST. Positive controls were anti-Kd mAbs mediating the rejection of F1, C3H × Kd.B6 (Kd.H-2bk) skin allografts when both skin and DST were absent on allograft tissue, rejection was observed in the recipients treated with anti-CD154 and anti-Kd mAbs (group IV). These observations demonstrate that anti-Kd recognition of H-2Kd 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-Kd mAbs was due to a nonspecific 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 h in the spleens of B6 recipients receiving anti-Kd mAbs compared with 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-Kd mAbs (group I), suggesting that accelerated clearance of DST is unlikely to be the primary cause of the loss of tolerogenicity.

**FIGURE 1.** Anti-Kd 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-Kd mAbs (n = 4) (closed square) compared with 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-Kd mAbs plus anti-CD154/DST (n = 20) (closed square) in comparison with 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-Kd mAbs (n = 5) (closed circle), or mouse IgG isotype control (n = 4) (*), and C3H grafts by B6 recipients treated with anti-Kd mAbs and anti-CD154/DST (n = 4) (half closed downward triangle). C. The transfer of serum from Pre-Sen mice (200 μl/mouse; day −2, 0 and 2 posttransplantation), but not from naive mice, prevented BALB/c skin graft acceptance in B6 mice treated with anti-CD154/DST (n = 4/group). Pre-Sen, presensitized.
Anti-K\textsuperscript{d} mAbs prime K\textsuperscript{d}-specific T cells as well as T cells that recognize “linked Ags” on the DST

We hypothesized that the anti-K\textsuperscript{d} mAbs converted the DST from tolerogenic 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\textsuperscript{d} mAbs in vivo, we performed an adoptive transfer assay, initially with TCR75 CD4\textsuperscript{+} T cells that specifically recognize the K\textsuperscript{d} 54–68 peptide presented on I\textsuperscript{A\textsuperscript{d}} MHC class II molecules. A total of 0.25 × 10\textsuperscript{6} CFSE-labeled TCR75 T cells transferred into nontransplanted B6 recipients underwent minimal proliferation, whereas significant cell proliferation was observed when TCR75 T cells were injected into transplanted but untreated B6 recipients (Fig. 3). TCR75 T cell proliferation was significantly reduced in anti–CD154/DST-treated B6 recipients, and anti-K\textsuperscript{d} mAbs were able to restore T cell proliferation (p = 0.0067). These studies confirm that anti-K\textsuperscript{d} mAbs facilitated the anti–CD154-independent proliferation of K\textsuperscript{d}-specific T cells in vivo.

It is well-established that during the course of an immune response to self-Ags, increased diversification of autoreactive specificities arises through a process of epitope spreading (26). To test whether anti-K\textsuperscript{d} Ab can promote the priming of donor-reactive T cells that recognize donor Ags not recognized by the anti-K\textsuperscript{d} mAb, 0.8–1 × 10\textsuperscript{6} CFSE-labeled, OVA-specific CD4\textsuperscript{+} 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, whereas OT-II T cells displayed robust proliferation upon transfer into untreated B6 recipients of Act-OVA BALB/c grafts (Fig. 4). Anti-CD154/DST inhibited OT-I T cell proliferation, whereas anti-K\textsuperscript{d} mAbs were able to restore robust OT-I cell proliferation (p = 0.001). These observations confirm the ability of anti-K\textsuperscript{d} mAbs to enhance cross-presentation of graft-derived Ags and to induce the proliferation of CD8\textsuperscript{+} T cells in recipients treated with anti-CD154/DST.

Anti-K\textsuperscript{d} mAbs facilitate the rejection of allografts lacking K\textsuperscript{d} but expressing linked Ags

The observation that anti-K\textsuperscript{d} mAbs enhanced the activation of T cells specific for donor Ags not recognized by the anti-K\textsuperscript{d} mAbs led us to test whether these observations are functionally significant and capable of eliciting the rejection of a second, non–K\textsuperscript{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\textsuperscript{d} mAbs accepted both skin grafts when the grafts and DST were from H-2\textsuperscript{bxk} (B6 × C3H F\textsubscript{1}) donors and rejected both grafts when the grafts and DST were from K\textsuperscript{d} H-2\textsuperscript{bxk} donors (groups I and II) (Table III). When the DST was from K\textsuperscript{d} H-2\textsuperscript{bxk} donors and the grafts were from K\textsuperscript{d} H-2\textsuperscript{b} and H-2\textsuperscript{bxk} donors, both grafts were rejected (group III). In contrast, when the grafts were from K\textsuperscript{d} H-2\textsuperscript{b} and H-2\textsuperscript{bxk} donors, and the DST was a combination of cells from both donors, only the K\textsuperscript{d}-expressing graft was rejected (group IV). Finally, when the grafts were from K\textsuperscript{d} H-2\textsuperscript{bxk} or H-2\textsuperscript{bxk} donors, and the DST was a combination of cells from the same two donors, only the K\textsuperscript{d}-expressing graft was rejected (group V).

We hypothesized that anti-K\textsuperscript{d} generated opsonins can bind to FcγRs and complement receptors on APCs and that this interac-

Table II. Anti-K\textsuperscript{d} mAb recognition of H-2K\textsuperscript{d} on DST is necessary and sufficient to mediate skin allograft rejection in recipients treated with anti-CD154/DST

<table>
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<th>Group</th>
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<td>I</td>
<td>K\textsuperscript{d} H-2\textsuperscript{bxk}</td>
<td>K\textsuperscript{d} H-2\textsuperscript{bxk}</td>
<td>11.5 ± 0.3 (n = 6)</td>
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<tr>
<td>II</td>
<td>H-2\textsuperscript{bxk}</td>
<td>H-2\textsuperscript{bxk}</td>
<td>60.0 ± 0.0 (n = 6)</td>
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<td>III</td>
<td>K\textsuperscript{d} H-2\textsuperscript{bxk}</td>
<td>H-2\textsuperscript{bxk}</td>
<td>52.3 ± 7.6 (n = 6)</td>
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<td>IV</td>
<td>H-2\textsuperscript{bxk}</td>
<td>K\textsuperscript{d} H-2\textsuperscript{bxk}</td>
<td>13.2 ± 0.4 (n = 6)</td>
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<tr>
<td>V</td>
<td>H-2\textsuperscript{d}</td>
<td>4X H-2\textsuperscript{d}</td>
<td>11.5 ± 0.5 (n = 4)</td>
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Anti-K\textsuperscript{d} mAbs can promote the presentation of exogenously derived donor Ags to CD8\textsuperscript{+} T cells. To this end, 0.8–1 × 10\textsuperscript{6} CFSE-labeled CD8\textsuperscript{+} 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 nontransplanted B6 recipients, whereas robust OT-I T cell proliferation was observed in untreated B6 recipients of Act-OVA BALB/c grafts (Fig. 4). Anti-CD154/DST inhibited OT-I T cell proliferation, whereas anti-K\textsuperscript{d} mAbs were able to restore robust OT-I cell proliferation (p = 0.001). These observations confirm the ability of anti-K\textsuperscript{d} mAbs to enhance cross-presentation of graft-derived Ags and to induce the proliferation of CD8\textsuperscript{+} T cells in recipients treated with anti-CD154/DST.

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

We hypothesized that anti-K\textsuperscript{d} generated opsonins can bind to FcγRs and complement receptors on APCs and that this interac-

FIGURE 2. Role of T cells in anti-K\textsuperscript{d} mAb-dependent graft rejection. A, Frequencies of alloreactive IFN-γ-producing cells in untreated B6 recipients (n = 5) (black), or in recipients of cardiac transplants treated with anti-CD154/DST (n = 4) (white), or anti-CD154/DST with anti-K\textsuperscript{d} (n = 4) (gray). 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 ± SE. 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\textsuperscript{d} mAbs (n = 4) (gray). C, Acceptance of BALB/c grafts by TCR88/− recipients treated with anti-CD154/DST and anti-K\textsuperscript{d} mAbs (n = 6, 13) compared with rejection by similarly treated B6 recipients (n = 20; p = 0.0003, ▲). Comparably treated B6, CD8\textsuperscript{−/−} recipients exhibited delayed rejection (n = 4; p = 0.0027, ♦).
tion promoted APC maturation and induced the expression of co-stimulatory 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 d; Fig. 5) (29). In contrast to the rejection of skin grafts in recipients receiving anti-CD154/DST plus anti-Kd mAbs, the addition of CTLA4Ig resulted in 75% of recipients accepting their skin allografts (Fig. 5). We conclude from these observations that anti-Kd 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 Abs (DSAs) are predictive of poor graft outcome (30, 31), and the activation of complement downstream of DSA binding to graft endothelium comprises 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-Kd-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 with or without DST and anti-Kd mAbs; a robust proliferation of allospecific T cells upon transfer into B6 recipients treated with anti-CD154/DST and anti-Kd mAbs; and the inability of anti-Kd mAbs to mediate skin allograft rejection in TCRßβ−/− recipients.

In the cardiac tolerance model, prevention of long-term graft survival anti-Kd mAbs was associated with an anti-CD154-independent activation of alloreactive T cells. However, it was unclear whether events downstream of anti-Kd 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 alloantibody-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 Abs in the sera of presensitized mice or purified anti-Kd mAbs binding to DST to prevent long-term skin graft acceptance in anti-CD154/DST-treated recipients. Indeed, if Kd expression was restricted to the skin graft, anti-Kd mAbs had minimal effect on tolerance induction, whereas if Kd expression was restricted to the DST, tolerance induction was abrogated. We add-
pressed the concern that the anti-Kd mAbs were simply inducing the rapid elimination of DST by administering 4-fold more DST to normalize for their more rapid clearance in the presence of anti-Kd mAbs. We observed that anti-Kd mAbs were still able to prevent the induction of long-term graft survival. These observations collectively support a conclusion that anti-Kd 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-Kd mAbs converted the DST from tolerogenic to being immunogenic. Studies are ongoing to visualize the fate of opsonized and nonopsonized DST in vivo will allow us to test the prevention of CD154-induced tolerance by anti-Kd mAbs, which facilitated the proliferation of not only Kd-specific TCR75 T cells but also of non–Kd-specific OT-II T cells recognizing OVA Ags expressed on the Act-OVA BALB/c DST and skin grafts. Furthermore, we show that the anti-Kd 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. On the basis of published observations, we infer from these cross-priming observations that CD8+ dendritic cells (DCs) are likely to be the APC 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 nonopsonized DST in vivo will provide insights into the APC subset involved in DST uptake and presentation to T cells.

Non-Kd graft-reactive T cells activated in the presence of anti-Kd mAbs and anti-CD154/DST have the ability to reject allografts lacking Kd, 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-Kd Ags have to be coexpressed on the Kd-expressing DST. Furthermore, we demonstrate that the administration of a mixture of Kd and non–Kd-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 Kd-expressing DST are unable to result in the bystander activation of APCs presenting third-party Ags. These observations may be explained by anti-Kd mAbs binding to DST, leading to the generation of Ab- and complement-coated DST, which then bind to APCs via FcyR and complement receptors (37–40). This binding 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 reduc-

### Table III. Anti-Kd mAbs promote epitope-spreading and cross-presentation of donor alloantigen to alloreactive T cells in vivo

<table>
<thead>
<tr>
<th>Skin Grafts</th>
<th>DST</th>
<th>MST ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. No Kd on DST or graft</td>
<td>H-2bck</td>
<td>57.7 ± 1.6 (n = 6)*</td>
</tr>
<tr>
<td>H-2bck</td>
<td>57.0 ± 1.6 (n = 6)*</td>
<td></td>
</tr>
<tr>
<td>II. Linked Kd on DST and graft</td>
<td>Kd H-2bck</td>
<td>11.5 ± 0.3 (n = 6)</td>
</tr>
<tr>
<td>Kd H-2bck</td>
<td>11.5 ± 0.3 (n = 6)</td>
<td></td>
</tr>
<tr>
<td>III. Linked Kd on DST only</td>
<td>Kd H-2bck</td>
<td>11.5 ± 0.5 (n = 8)</td>
</tr>
<tr>
<td>Kd H-2bck</td>
<td>12.8 ± 4.3 (n = 8)</td>
<td></td>
</tr>
<tr>
<td>IV. Linked Kd on graft only</td>
<td>1/2 Kd + H-2bck</td>
<td>11.3 ± 0.3 (n = 11)</td>
</tr>
<tr>
<td>1/2 H-2bck</td>
<td>48.3 ± 5.4 (n = 11)*</td>
<td></td>
</tr>
<tr>
<td>V. Unlinked Kd on DST and graft</td>
<td>1/2 Kd + H-2bck</td>
<td>10.8 ± 0.4 (n = 6)</td>
</tr>
<tr>
<td>H-2bck</td>
<td>53.0 ± 5.5 (n = 6)*</td>
<td></td>
</tr>
</tbody>
</table>

*In four of six recipients, skin grafts were accepted for >60 d.

bIn 5 of 11 recipients, skin grafts were accepted for >60 d.

In three of six recipients, skin grafts were accepted for >60 d.

#### FIGURE 5. CTLA4Ig overrides the effects of anti-Kd 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) (●) anti-CD154/DST + CTLA4Ig with anti-Kd mAbs (n = 7) (●) but rejection by B6 recipients treated with anti-CD154/DST and anti-Kd mAbs (n = 15) (△). B6 recipients treated with anti-CD154/DST (n = 11) (●) also accepted BALB/c skin grafts.
tion of further uptake of exogenous Ag (41, 42). Although we do not provide a direct demonstration of APC maturation in the presence of anti-K, 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 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 DSAs. Finally, these observations underscore the potential ability of DSA with limited specificities to prime a larger repertoire of alloreactive T cells, and may have significant implications for the clinical scenario of transplanting sensitized recipients with a restricted repertoire of DSA. Our observations suggest that high-titer DSA to a restricted donor Ag 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 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, Abs bind to and convert the DST from tolerogenic to immunogenic. Further donor-reactive Abs binding to DST lead 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 Abs can facilitate acute graft rejection, in addition to the well-accepted role of mediating complement-dependent humoral- or Ab-mediated rejection.

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Disclosures
The authors have no financial conflicts of interest.

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