Published in final edited form as:

Am J Transplant. 2019 April ; 19(4): 998–1010. doi:10.1111/ajt.15147.

Peri-Transplant VLA-4 Blockade Inhibits Endogenous Memory CD8 T Cell Infiltration into High Risk Cardiac Allografts and CTLA-4Ig Resistant Rejection

Shoichi Iida^{1,2}, Satoshi Miyairi¹, Charles A. Su^{1,3}, Toyofumi Abe^{1,4}, Ryo Abe², Kazunari Tanabe², Nina Dvorina¹, William M. Baldwin 3rd¹, and Robert L. Fairchild^{1,3}

¹Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

²Tokyo Women's Medical University, Tokyo, Japan;

³Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH, USA

⁴Department of Urology, Osaka University School of Medicine, Osaka, Japan;

Abstract

Recipient endogenous memory CD8 T cells expressing reactivity to donor class I MHC infiltrate MHC-mismatched cardiac allografts within 24 hours after reperfusion and express effector functions mediating graft injury. The current study tested the efficacy of VLA-4 blockade to inhibit endogenous memory CD8 T cell infiltration into cardiac allografts and attenuate early post-transplant inflammation. Peri-transplant anti-VLA-4 mAb given to C57BL/6 (H-2^b) recipients of AJ (H-2^a) heart allografts completely inhibited endogenous memory CD4 and CD8 T cell infiltration with significant decreases in macrophage, but not neutrophil, infiltration into allografts subjected to either minimal or prolonged cold ischemic storage (CIS) prior to transplant, reduced intra-allograft IFN- γ -induced gene expression and prolonged survival of allografts subjected to prolonged CIS in CTLA-4Ig treated recipients. Anti-VLA-4 mAb also inhibited priming of donor-specific T cells producing IFN- γ until at least day 7 post-transplant. Peri-transplant anti-VLA plus anti-CD154 mAb treatment similarly prolonged survival of allografts subjected to minimal or increased CIS prior to transplant. Overall, these data indicate that peri-transplant anti-VLA-4 mAb inhibits early infiltration memory CD8 T cell infiltration into allografts with a marked reduction in early graft inflammation suggesting an effective strategy to attenuate negative effects of heterologous alloimmunity in recipients of higher risk grafts.

Correspondence: Robert L. Fairchild, fairchr@ccf.org.

Shoichi Iida and Satoshi Miyairi contributed equally to the manuscript and should be considered co-first authors

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

DISCLOSURE

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

Introduction

The use of calcineurin inhibitors to suppress donor-reactive T cell responses has increased solid organ transplant survival, but early post-transplant acute rejection episodes continue to occur in some patients and undermine the success of transplantation to treat end-stage organ disease (1). In addition to the high frequency of allogeneic MHC-reactive T cells in the naïve T cell repertoire, most allograft recipients have endogenous memory CD4 and CD8 T cell repertoires that contain high frequencies of donor allogeneic MHC-reactive cells (2, 3). These memory T cells are often generated during immune responses to viral and bacterial infections as well as during environmental exposure to other types of antigens (2, 4–6). In addition to their rapid response, hallmark characteristics of memory T cells include low antigen thresholds for activation and different costimulatory signaling requirements versus those required for activation of naïve T cells. These donor-reactive memory T cells present an important barrier to successful transplantation as the T cells can rapidly infiltrate allografts and mediate acute and chronic graft tissue injury. Seminal clinical studies have indicated that the presence of high numbers of donor-reactive memory T cells in the peripheral blood of kidney transplant patients prior to the transplant results in increased incidence of acute rejection episodes and poorer graft function during the first year after transplant (7, 8). These results implicate donor-reactive endogenous memory T cell mediated graft injury during the first-year post-transplant with an important impact on graft function and outcome despite the use of standard of care immunosuppression.

Studies from this laboratory have documented the rapid infiltration and activation of endogenous memory CD4 and CD8 T cells with donor-reactivity in vascularized heart allografts in a mouse model (9–11). Despite activation to express IFN- γ , perforin and granzyme B within the allograft by 24 hours post-transplant, initial studies indicated that the memory CD8 T cells were unable to mediate sufficient acute graft tissue injury to directly mediate rejection of the allografts (12). We then realized that the heart allografts were subjected to the minimal cold ischemic storage (CIS) time possible, about 30 minutes, before revascularization in the recipient and that the rapidity of the transplant might be attenuating graft ischemia reperfusion injury and optimal activation of the memory T cells within the graft. Indeed, imposition of a longer CIS prior to transplant resulted in a more robust activation of the endogenous donor-reactive memory CD4 and CD8 T cells within the allograft and a marked increase in allograft tissue necrosis by day 5 post-transplant (13, 14). In contrast to the ability of peri-transplant CTLA-4Ig to markedly prolong survival of allografts subjected to minimal CIS prior to transplant, allografts subjected to prolonged CIS prior to transplant had only a modest enhancement in survival in CTLA-4Ig treated recipients and this CTLA-4Ig resistant rejection was mediated by the infiltration and activation of the endogenous memory CD8 T cells.

Leukocyte infiltration into tissue sites of inflammation can be effectively inhibited by antibodies that block the function of molecules required for leukocyte arrest on vascular endothelial cells and infiltration into the parenchymal tissue (15–20). Studies from this and other laboratories have indicated the efficacy of anti-LFA-1 antibodies in inhibiting the infiltration of alloantigen-primed T cells, macrophages and neutrophils into allografts and prolonging allograft survival (21–25). LFA-1 is also required for assembly of the

supramolecular activation complex during T cell receptor mediated activation so anti-LFA-1 mAb is also an effective inhibitor of de novo priming of naïve donor-reactive T cells (26–30). Clinical studies indicated that in a very low number of treated patients the potent immunosuppression generated by chronic weekly administration of anti-LFA-1 mAb can lead to occurrence of progressive multifocal leukoencephalopathy (PML) in psoriasis patients and post-transplant lymphoproliferative disease (PTLD) in kidney transplant recipients prompting discontinuation of its clinical use (31, 32). VLA-4 is another integrin expressed by memory and activated primary T cells and antibody-mediated antagonism of VLA-4 inhibits T cell infiltration into tissue inflammatory sites and is currently used clinically to treat autoimmune disease (33–35). This has prompted us to test the efficacy of anti-VLA-4 mAb to inhibit endogenous memory CD4 and CD8 T cell infiltration into heart allografts subjected to minimal vs. prolonged CIS prior to transplant and the impact of this treatment on allograft survival with and without additional treatment with costimulatory blockade.

Methods

Mice

C57BL/6 (H-2^b) and A/J (H-2^a) mice were purchased from Charles River Laboratories (Wilmington, MA). Male mice, 8–10 weeks of age, were used in all experiments. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic.

Cardiac transplantation and harvest

Heterotopic, intra-abdominal cardiac transplantation was performed as developed by Corry and coworkers (36). Donor hearts were subjected to either 0.5 or 8 h of CIS in Ringer's solution before transplantation and all grafts began spontaneous contraction upon revascularization in recipients. Graft survival was monitored by abdominal palpation of the graft and rejection was confirmed by laparotomy. At the time of graft harvest, the circulatory system was drained prior to removal of the heterotopic graft, which was either immediately snap-frozen in liquid nitrogen or placed in media for digestion and preparation of single cell aliquots to analyze graft-infiltrating cells.

In vivo antibody treatment

Allograft recipients were treated with 200 µg i.p. of anti-VLA-4 mAb (rat IgG2b, clone PS/2 from Bio X Cell, West Lebanon, NH) or control rat IgG (Sigma-Aldrich, St. Louis, MO) on days –1 and 0 or on days 3 and 4. The dose of anti-VLA-4 mAb used was based on results from a limited preliminary study indicating efficacy in prolonging allograft survival. Recipient CD4 T cell were depleted by giving a 1:1 cocktail of anti-CD4 mAb YTS191 and GK1.5 (Bio X Cell), 200 µg i.p. on days –3, –2, –1 and weekly following transplant. CD4 T cell depletion was 98% as assessed by staining peripheral blood cells from treated recipients. For costimulatory blockade, 250 µg CTLA-4Ig (Bio X Cell) was given i.p. on days 0 and +1 and 400 µg anti-CD154 mAb MR-1 (Bio X Cell) was given on days 0 and +1 each with anti-VLA-4 mAb or control rat IgG.

Flow cytometry

Flow cytometric detection of graft-infiltrating cells was performed using a modification of the method published by Afanasyev and colleagues (11, 13, 37). Briefly, harvested tissues were weighed prior to 1 h incubation at 37°C in RPMI with Type II collagenase (Sigma-Aldrich). After incubation, tissue was pressed through a 40 mm filter and the collected cells were washed twice in RPMI, counted using a hemocytometer, and stained for common leukocyte phenotypic surface markers (CD45, CD4, CD8, Gr1, and F4/80) using commercially available antibodies (BD Bioscience, San Jose, CA; eBioscience, San Diego, CA). Flow cytometry was performed using a FACSCalibur (BD Biosciences) cytometer and FlowJo analysis software (Tree Star Inc., Ashland OR). The forward scatter and FL1 (CD45⁺) channels were used to gate the leukocytes in the graft tissue followed by analysis of the specific leukocyte populations. For each sample, 200,000 events were accumulated. Total numbers of each leukocyte population were calculated by: (the total number of leukocytes counted) × (% of the leukocyte population counted in the CD45⁺ cells)/100. The data are reported as number of each leukocyte population/mg graft tissue.

RNA purification and qRT-PCR

Snap-frozen grafts were crushed, homogenized, and RNA was isolated using fibrous tissue kits (Qiagen, Valencia, CA). Reverse transcription and real-time PCR were performed using commercially available reagents, probes, and a 7500 fast real-time thermocycler, all from Applied Biosystems (Foster City, CA).

ELISPOT assays

ELISPOT assays to enumerate donor-reactive T cells in the spleens of cardiac graft recipients were performed as previously detailed (10, 11). Briefly, splenic responder cells and mitomycin C-treated T cell depleted autologous or donor stimulator spleen cells were cocultured for 24 h at 37°C in serum-free HL-1 media in 96 well plates coated with anti-IFN- γ or anti-IL-2 capture mAb (BD Biosciences). To compare alloreactive CD4⁺ and CD8⁺ T cell priming, each cell population was purified from spleen cell suspensions using magnetic bead chromatography (Stem Cell Technologies) and then these purified responder cells were stimulated with T-cell depleted splenocytes. After culture, all cells were washed from the plate and biotinylated anti-IFN- γ or anti-IL-2 detecting mAb (BD Biosciences, San Jose, CA, USA) were added, followed by anti-biotin alkaline phosphatase. After development with the chromagen, the total number of spots per well was quantified using an ImmunoSpot Series 4 Analyzer (Cellular Technology Ltd., Shaker Heights, OH).

Histopathology and immunocytochemistry

A midventricular portion of the cardiac graft was embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) and methanol-fixed sections were stained with hematoxylin and eosin (H&E), Masson Trichrome, or with anti-CD4, anti-CD8, anti-Mac2 or anti-C4d. Staining antibodies were visualized using rat or rabbit on mouse HRP-Polymer Kits (Biocare Medical, Concord, CA) as secondary antibodies followed by DAB and counterstained with hematoxylin. Images were captured and analyzed with Image-Pro Plus (Media Cybernetics, Silver Springs, MD, USA).

Statistics

Graft survival between experimental groups was compared using Kaplan-Meier survival curves and Log-rank statistics. Other statistical analyses were performed using the Mann-Whitney nonparametric test to analyze differences between experimental groups. A p value < 0.05 was considered a significant difference between groups. Error bars reflect the standard error of the mean (SEM) for each test group.

Results

Antagonism of VLA-4 decreases distinct early post-transplant inflammatory events in cardiac allografts.

Reperfusion of heart allografts is quickly followed by intense infiltration of host leukocytes, including macrophages, neutrophils and donor-reactive memory CD4 and CD8 T cells into cardiac allografts within 24–48 hours following graft reperfusion and the activation of these leukocytes to express inflammatory functions within the graft (11). The requirement for VLA-4 engagement for leukocyte infiltration into heart allografts subjected to minimal CIS was tested in groups of C57BL/6 mice receiving complete MHC-mismatched A/J cardiac allografts or isografts following treatment with anti-VLA-4 mAb or with rat IgG on the day before and the day of transplant. The grafts were retrieved 48 hours after reperfusion and the number of CD8 T cells, CD4 T cells, macrophages and neutrophils infiltrating isografts (Figure 1A) and allografts (Figure 1B) was determined. Memory CD4 and CD8 T cells were present at low numbers in isografts at 48 h post-transplant but were markedly increased in allografts. Similarly, macrophage and neutrophil infiltration was increased in allografts vs. isografts. When compared to control IgG-treated allograft recipients, treatment with anti-VLA-4 mAb decreased memory CD4 and CD8 T cell infiltration into allografts to the levels observed in isografts. Macrophage infiltration into the allografts was also inhibited by peri-transplant anti-VLA-4 mAb treatment whereas neutrophil infiltration was not diminished.

In the previous experiment the cardiac allografts were transplanted as quickly after harvest from the donor to the recipient as possible, about 30 min of cold ischemic storage (CIS) time in Ringer's solution on ice while the recipient was being prepared for the transplant. We have recently reported that increasing the CIS time to 6–8 hours increases intra-allograft inflammation shortly after revascularization that includes marked increases in memory CD4 and CD8 T cell, macrophage and neutrophil numbers in the graft and the ability of peri-transplant anti-LFA-1 mAb to inhibit this infiltration and attenuate post-transplant inflammation (13). Therefore, we tested the efficacy of peri-transplant anti-VLA-4 mAb treatment on the early post-transplant inflammation in allografts subjected to the prolonged CIS prior to transplant. Similar to the impact on allografts subjected to minimal CIS, peri-transplant treatment with anti-VLA-4 mAb decreased memory CD4 and CD8 T cell and macrophage numbers in allografts subjected to prolonged CIS to baseline/isograft levels whereas effects on neutrophil infiltration were minimal when assessed 48 hours after revascularization.

The impact of anti-VLA-4 mAb mediated inhibition of early post-transplant infiltration of memory T cell and macrophage infiltration into cardiac allografts was further examined by

determining mRNA expression levels of proinflammatory genes associated with the infiltration of these leukocyte populations (Figure 2A and B). Our previous studies indicated that the increased mRNA expression of various proinflammatory cytokine genes in cardiac allografts is accompanied by concomitant increases in the cytokine protein (14). Peri-transplant anti-VLA-4 mAb markedly decreased allograft expression of genes associated with effector memory T cell and macrophage activation (IFN- γ , TNF α , perforin, granzyme B and FasL) and graft infiltration (CXCL9/Mig, CXCL10/IP-10 and CCL2/MCP-1). Consistent with the modest impact on neutrophil infiltration into the allografts, peri-transplant treatment with anti-VLA-4 mAb had little impact on the expression of the neutrophil chemoattractant CXCL1 in the allografts.

Peri-transplant anti-VLA-4 mAb treatment prolongs cardiac allograft survival

Allograft recipient treatment with anti-VLA-4 mAb on the day before and the day of transplantation resulted in a significant, but modest, prolongation in graft survival when compared to rat control IgG treated recipients, from a median survival of 7.5 days to 13.5 days (Figure 3A). This effect in prolonged allograft survival in recipients treated with peri-transplant anti-VLA-4 mAb was accompanied by decreased myocyte damage and leukocyte infiltration when assessed on day 7, the time of allograft rejection in control treated recipients (Figure 3B). Identical effects were observed for allografts subjected to prolonged CIS. Administration of additional doses of anti-VLA-4 mAb on days 7 and 14 did not extend the survival of the allografts subjected to prolonged CIS prior to transplant (S. Miyairi, data not shown).

Consistent with the modest prolongation in allograft survival, peri-transplant anti-VLA-4 mAb markedly inhibited the de novo priming of donor-reactive CD4 and CD8 T cells to IFN- γ producing cells in the recipient spleen when assessed on day 7 post-transplant, but this priming quickly recovered to the numbers observed in control treated recipients within a week (Figure 4). The anti-VLA-4 mAb treatment had a significant but modest inhibition of donor-reactive CD4 and CD8 T cells producing IL-2 when assessed on day 7 but not day 14 post-transplant. The anti-VLA-4 mAb mediated inhibition of naïve donor-reactive T cell priming was consistent with the increased expression of VLA-4 on splenic CD4 and CD8 T cells beginning on day 5 following transplant with allografts subjected to prolonged CIS (Figure S1 and data not shown). Expression of LFA-1 on these T cells also began to increase in the recipient spleen on day 5.

Anti-VLA-4 mAb inhibition of endogenous memory CD8 T cell infiltration into high risk allografts abrogates CTLA-4Ig resistant rejection

Whereas peri-transplant administration of CTLA-4Ig to recipients of heart allografts subjected to minimal CIS extends allograft survival to more than 50 days, heart allografts subjected to 8 h CIS are rejected between days 15–22 post-transplant and this CTLA-4Ig resistant rejection is mediated by endogenous memory CD8 T cell activation within the graft (13, 14). Peri-transplant administration of anti-VLA-4 mAb to block the infiltration of these endogenous memory CD8 T cells into heart allografts subjected to 8 h CIS reversed the CTLA-4Ig resistant rejection of the allografts, extending graft survival from a median graft survival of 16 to 29.5 days post-transplant (Figure 5A). Allograft rejection in recipients

treated with both anti-VLA-4 mAb and CTLA-4Ig was accompanied by the presence of high numbers of donor-reactive T cells producing IFN- γ in the recipient spleen (Figure 5B) and similar evidence of T cell mediated rejection as in the CTLA-4Ig resistant rejection with interstitial infiltrates of CD8 T cells and macrophages infiltrating the graft myocardium as well as the endothelium, media and adventitia of arteries (Figure 5C and D).

We and others have reported the ability of peri-transplant CD4 T cell depleting therapies to prolong allograft survival in mouse models by recipient treatment with CD4 T cell depleting mAb to inhibit de novo primary effector CD8 T cell development (38–40). The effectiveness of CD4 depletion on heart allograft survival is markedly reduced in allografts subjected to prolonged vs. minimal CIS, due in part to the activation of donor-reactive endogenous memory CD8 T cells (13). Therefore, mice were treated with CD4 T cell-depleting mAb and then received complete MHC-mismatched heart allografts subjected to either minimal or prolonged CIS with or without peri-transplant anti-VLA-4 mAb (Figure 6A and B). Depletion of CD4 T cells prolonged survival of all allografts subjected to minimal ischemia beyond day 65 post-transplant whereas allografts subjected to prolonged CIS began to reject by day 14 post-transplant and all were rejected by day 45. Importantly, peri-transplant treatment with anti-VLA-4 mAb extended the survival of the allografts subjected to prolonged CIS to that observed in CD4 T cell depleted recipients of allografts subjected to minimal CIS (MST 65 days), indicating that inhibition of endogenous memory CD8 T cells infiltration in allograft subjected to prolonged CIS can equilibrate graft outcomes to those observed in allografts subjected to minimal CIS.

Synergistic effects of peri-transplant VLA-4 and CD154 blockade in extending survival of allografts subjected to prolonged CIS

Finally, the impact of combining peri-transplant costimulatory blockade with anti-CD154 mAb plus VLA-4 blockade was tested. Peri-transplant anti-CD154 mAb treatment extended survival of allografts subjected to minimal CIS to day 55–75 post-transplant whereas survival of allografts subjected to prolonged CIS was only extended to day 30–45 post-transplant (Figure 7). However, peri-transplant anti-VLA-4 mAb plus anti-CD154 mAb extended survival of allografts subjected to minimal or prolonged CIS to day 80–120 post-transplant. Surviving allografts subjected to either minimal or prolonged CIS in recipients treated with anti-VLA-4 plus anti-CD154 mAb were harvested on day 100 post-transplant and prepared sections stained to detect collagen and C4d deposition (Figure B-E). At day 100 post-transplant, allografts subjected to either minimal or prolonged CIS prior to transplant to recipients treated with both anti-CD154 plus anti-VLA-4 mAb had similar and severe manifestations of acute and chronic rejection with deposition of collagen as well as similar C4d deposition in interstitial capillaries and large vessels, with obvious arteriopathy in the large vessels.

Discussion

There is a critical need to identify new strategies that can effectively inhibit ischemia-reperfusion induced graft inflammation and injury to improve graft outcomes. Previous studies from this and other laboratories reported the efficacy of anti-LFA-1 mAb in

inhibiting early leukocyte infiltration into allografts and de novo priming of donor-reactive T cell responses in mouse and non-human primate transplant models resulting in marked improvement in graft outcomes (23, 24, 41–47). Anti-LFA-1 mAb have been tested in patients with psoriasis and in kidney transplant patients and shown to be effective immunosuppressants, but a small number of treated patients developed PML or PTLN that spurred the removal of anti-LFA-1 mAb from clinical use (31, 32). The goal of the current study was to test the efficacy of blocking another integrin expressed by activated T cells and macrophages, VLA-4, on the outcome of vascularized heart allografts in a mouse model. The key findings of this study are that a short two-day peri-transplant treatment of allograft recipients with anti-VLA-4 mAb is as effective as anti-LFA-1 mAb in blocking endogenous memory CD4 and CD8 T cell infiltration into allografts that markedly decreases the intensity of early post-transplant inflammation and that this peri-transplant VLA-4 blockade can be combined with blockade of CD40-CD154 interactions to achieve long-term allograft survival. These benefits of the peri-transplant anti-VLA-4 mAb treatment were equally observed in recipients of allografts subjected to the minimal CIS prior to transplant as well as in recipients of higher risk allografts subjected to 8 h of CIS.

Several important differences were noted in the early and late post-transplant consequences of peri-transplant treatment with anti-LFA-1 vs. anti-VLA-4 mAb. Although both anti-integrin antibodies were very effective in inhibiting early endogenous memory CD4 and CD8 T cell infiltration into allografts subjected to minimal or prolonged CIS prior to transplant, peri-transplant anti-LFA-1 mAb had little effect on neutrophil and macrophage infiltration into allografts whereas peri-transplant anti-VLA-4 mAb effectively inhibited macrophage but not neutrophil infiltration (13, 47). This difference in neutrophil and macrophage graft infiltration was reflected by the decreased allograft expression of the macrophage chemoattractant CCL2 by anti-VLA-4 but not anti-LFA-1 mAb and the inability of either mAb to decrease allograft expression of the neutrophil chemoattractant CXCL1. The ability of anti-VLA-4 mAb but not anti-LFA-1 mAb to decrease allograft expression of TNF α mRNA may account for the difference in allograft CCL2 expression whereas blocking either VLA-4 or LFA-1 had no discernable effect on allograft expression of IL-6 (S. Iida, data not shown). In contrast, peri-transplant treatment with anti-LFA-1 mAb extended allograft survival from day 7–8 to 30–40 days whereas anti-VLA-4 mAb only extended survival to day 12–15 (13, 47). This difference was reflected by the ability of anti-LFA-1 mAb treatment to inhibit de novo donor-reactive CD4 and CD8 T cell priming beyond day 25 post-transplant whereas these responses returned to control levels by day 14 in anti-VLA-4 mAb treated allograft recipients. Furthermore, delaying allograft recipient treatment with anti-LFA-1 mAb to day 3 and 4 post-transplant continued to generate inhibitory effects on the alloimmune response and extended allograft survival (13, 47), whereas the immunosuppressive effects of anti-VLA-4 mAb were lost when given to recipients on these later days post-transplant (S. Iida, data not shown). We realize that various anti-LFA-1 and anti-VLA-4 mAb are likely to yield some differences on the alloimmune response and on allograft outcomes, but in conjunction with the studies of other investigators it is clear that when given peri-transplant as a single blocking agent anti-LFA-1 mAb yields superior suppression of alloimmunity.

Neutrophil infiltration and activation is an important innate immune component that increases inflammation and promotes the development of acute tissue injury following reperfusion of ischemic tissues (48–50). Following reperfusion of allografts, such early acute injury can enhance adaptive immune responses and the development of chronic tissue injury. Despite the inability of peri-transplant VLA-4 blockade to mitigate neutrophil infiltration into allografts and allograft IL-6 mRNA expression, restoration of de novo priming of donor-reactive T cells by day 14 post-transplant in anti-VLA-4 mAb conditioned allograft recipients was not influenced by neutrophil mediated inflammation as neutrophil depletion did not prolong the anti-VLA-4 mAb mediated delay in donor-reactive T cell priming or further enhance allograft survival (S. Iida, data not shown). These results suggest little impact of neutrophil activity in the higher risk allografts on the down-stream donor-reactive T cell responses when early endogenous memory CD8 T cell infiltration into allografts is blocked by anti-VLA-4 mAb.

These results highlight the importance of early donor-reactive memory T cell activation in amplifying inflammation and tissue injury following reperfusion of the higher risk allografts. Peri-transplant treatment with anti-VLA-4 mAb in recipients of higher risk cardiac allografts yielded similar extensions of survival as allografts subjected to minimal CIS. This suggests that the inhibition of early graft inflammation mediated by treatment with anti-VLA-4 mAb can mitigate the inflammatory factors that undermine optimal graft outcomes and inhibit donor-reactive immune mechanisms to achieve similar outcomes as the lower risk allografts. This is reminiscent of the considerable interest in the use of ex vivo perfusion of lung and liver grafts to attenuate early post-transplant inflammation to reverse the negative impact of prolonged cold ischemic graft storage and improve long-term graft outcomes (51–53). The current study suggests that similar results can be achieved with peri-transplant anti-VLA-4 mAb and is supported by the similar extensions in survival of allografts subjected to minimal vs. prolonged CIS prior to transplant when recipients are conditioned with peri-transplant anti-VLA-4 plus anti-CD154 mAb. In addition to the current study focusing on efficacy of peri-transplant integrin blockade in heart allografts subjected to prolonged CIS prior to transplant, the combined use of anti-LFA-1 or anti-VLA-4 mAb with anti-CD154 mAb has been shown to block memory T cell resistant rejection of skin, islet and tracheal allografts in mouse models (23, 24, 44, 45). Importantly, combined treatment with anti-LFA-1 mAb and CTLA-4Ig enhanced islet but not kidney allograft survival in more stringent non-human primate models (41, 54). Peri-transplant VLA-4 blockade did enhance the survival of the high risk cardiac allografts in CTLA-4Ig treated recipients but only modestly by 2–3 weeks before donor-reactive T cells emerge to mediate rejection.

Overall these studies indicate the efficacy of a short course of anti-VLA-4 mAb in attenuating the ischemia-reperfusion induced innate and adaptive responses within high risk allografts that had been subjected to prolonged CIS prior to transplant. The ability to attenuate this initial inflammation facilitates the potency of additional treatment strategies to achieve the long-term graft survival observed in recipients of allografts subjected to the minimal amount of CIS that can be treated to achieve optimal graft outcomes. The results suggest that short-term use of VLA-4 targeting antibodies that are already in clinical use may be effective agents for inhibiting the early post-transplant inflammatory events that undermine optimal graft outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by NIH RO1-AI04059 and PO1-AI087586 (R.L.F.). C.A.S. was supported in part by NIH TL1-24991, T32-AI089474, and the Case Western University School of Medicine MSTP.

Abbreviations:

CIS	cold ischemic storage
h	hour
IFN	interferon
LFA-1	lymphocyte function associated antigen-1
mAb	monoclonal antibody
PML	progressive multifocal leukoencephalopathy
PTLD	post-transplant lymphoproliferative disease
TNFα	tumor necrosis factor alpha
VLA-4	very late antigen-4

References

1. Lentine KL, Gheorghian A, Axelrod D, Kalsekar A, L'italien G, Schnitzler MA. The implications of acute rejection for allograft survival in contemporary U.S. kidney transplantation. *Transplantation* 2012; 94: 241–249. [PubMed: 22874796]
2. Selin LK, Brehm MA, Naumov YN, Cornberg M, Kim SK, Clute SC, et al. Memory of mice and men: CD8⁺ T-cell cross-reactivity and heterologous immunity. *Immunol Rev* 2006; 211: 164–181. [PubMed: 16824126]
3. Benichou G, Gonzalez B, Marino J, Ayasoufi K, Valujskikh A. Role of memory T cells in allograft rejection and tolerance. *Front Immunol* 2017; 8: DOI: 10.3389/fimmu.2017.00170.
4. Adams AB, Pearson TC, Larsen CP. Heterologous immunity: an overlooked barrier to tolerance. *Immunol Rev* 2003; 196: 147–160. [PubMed: 14617203]
5. Adams AB, Williams MR, Jones TR, Shirasugi N, Durham MM, Kaech SM, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* 2003; 111: 1887–1895. [PubMed: 12813024]
6. Brehm MA, Markees TG, Daniels KA, Greiner DL, Rossini AA, Welsh RM. Direct visualization of cross-reactive effector and memory allo-specific CD8 T cells generated in response to viral infections. *J Immunol* 2003; 170: 4077–4086. [PubMed: 12682237]
7. Augustine JJ, Siu DS, Clemente MJ, Schulak JA, Heeger PS, Hricik DE. Pre-transplant IFN-gamma ELISPOTs are associated with post-transplant renal function in African American renal transplant recipients. *Am J Transplant* 2005; 5: 1971–1975. [PubMed: 15996247]
8. Heeger PS, Greenspan NS, Kuhlenschmidt S, DeJelo C, Hricik DE, Schulak JA, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of post-transplant rejection episodes. *J Immunol* 1999; 163: 2267–2275. [PubMed: 10438971]

9. El-Sawy T, Miura M, Fairchild R. Early T cell response to allografts occurring prior to alloantigen priming up-regulates innate mediated inflammation and graft necrosis. *Am J Pathol* 2004; 165: 147–157. [PubMed: 15215170]
10. Schenk AD, Gorbacheva V, Rabant M, Fairchild RL, Valujskikh A. Effector functions of donor-reactive CD8 memory T cells are dependent on ICOS induced during division in cardiac grafts. *Am J Transplant* 2009; 9: 64–73. [PubMed: 18976292]
11. Schenk AD, Nozaki T, Rabant M, Valujskikh A, Fairchild RL. Donor-reactive CD8 memory T cells infiltrate cardiac allografts within 24-h posttransplant in naive recipients. *Am J Transplant* 2008; 8: 1652–1661. [PubMed: 18557725]
12. Setoguchi K, Hattori Y, Iida S, Baldwin WM, 3rd, Fairchild RL. Endogenous memory CD8 T cells are activated within cardiac allografts without mediating rejection. *Am J Transplant* 2013; 13: 2293–2307. [PubMed: 23914930]
13. Su CA, Iida S, Abe T, Fairchild RL. Endogenous memory CD8 T cells directly mediate cardiac allograft rejection. *Am J Transplant* 2014; 14: 568–579. [PubMed: 24502272]
14. Tsuda H, Su CA, Tanaka T, Ayasoufi K, Min B, Valujskikh A, et al. Allograft dendritic cell p40 homodimers activate donor-reactive memory CD8+ T cells. *JCI Insight* 2018; 3: DOI: 10.1172/jci.insight.96940.
15. Bromley SK, Mempel TR, Luster AD. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol* 2008; 9: 970–980. [PubMed: 18711434]
16. Castellani ML, Bhattacharya K, Tagen M, Kempuraj D, Perrella A, De Lutiis M, et al. Anti-chemokine therapy for inflammatory diseases. *Int J Immunopathol Pharmacol* 2007; 20: 447–453. [PubMed: 17880758]
17. Allen S, Moran N. Cell adhesion molecules: therapeutic targets for inhibition of inflammatory states. *Semin Thromb Hemost* 2015; 41: 563–571. [PubMed: 26322694]
18. Pandolfi F, Franza L, Altamura S, Mandolini C, Cianci R, Ansari A, et al. Integrins: integrating the biology and therapy of cell-cell interactions. *Clin Ther* 2017; 39: 2420–2436. [PubMed: 29203050]
19. McLean LP, Cross RK. Integrin antagonists as potential therapeutic options for the treatment of Crohn's disease. *Expert Opin Investig Drugs* 2016; 25: 263–273.
20. Nicolls MR, Gill RG. LFA-1 (CD11a) as a therapeutic target. *Am J Transplant* 2006; 6: 27–36. [PubMed: 16433753]
21. Abdi R, Smith RN, Makhlof L, Najafian N, Luster AD, Auchincloss H, et al. The role of CC chemokine receptor 5 (CCR5) in islet allograft rejection. *Diabetes* 2002; 51: 2489–2495. [PubMed: 12145162]
22. Isobe M, Yagita H, Okumura K, Ihara A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* 1992; 255: 1125–1129. [PubMed: 1347662]
23. Kitchens WH, Haridas D, Wagener ME, Song M, Ford ML. Combined costimulatory and leukocyte functional antigen-1 blockade prevents transplant rejection mediated by heterologous memory alloresponses. *Transplantation* 2012; 93: 997–1005. [PubMed: 22475765]
24. Kitchens WH, Haridas D, Wagener ME, Song M, Kirk AD, Larsen CP, et al. Integrin antagonists prevent costimulatory blockade-resistant transplant rejection by CD8+ memory T cells. *Am J Transplant* 2012; 12: 69–80. [PubMed: 21942986]
25. Lunsford KE, Koester MA, Eiring AM, Horne PH, Gao D, Bumgartner GL. Targeting LFA-1 and CD154 suppresses the in vivo activation and development of cytolytic (CD4-independent) CD8+ T cells. *J Immunol* 2005; 175: 7855–7866. [PubMed: 16339521]
26. Chou YK, Edwards DM, Weinberg AD, Vandenbark AA, Kotzin BL, Fontenot AP, et al. Activation pathways implicate anti-HLA-DP and anti-LFA-1 antibodies as lead candidates for intervention in chronic berylliosis. *J Immunol* 2005; 174: 4316–4324. [PubMed: 15778396]
27. Freiberg BA, Kupfer H, Maslanik W, Delli H, Kappler J, Zaller DM, et al. Staging and resetting T cell activation in SMACs. *Nat Immunol* 2002; 3: 911–917. [PubMed: 12244310]
28. Gorochoy G, Gross G, Waks T, Eshhar Z. Anti-leukocyte function-associated antigen-1 antibodies inhibit T-cell activation following low-avidity and adhesion-independent interactions. *Immunology* 1993; 79: 548–555. [PubMed: 8406579]

29. Rutigliano JA, Johnson TR, Hollinger TN, Fischer JE, Aung S, Graham BS. Treatment with anti-LFA-1 delays the CD8⁺ cytotoxic T-lymphocyte response and viral clearance in mice with primary respiratory syncytial virus infection. *J Virol* 2004; 78: 3014–3023. [PubMed: 14990720]
30. Springer TA, Dustin ML, Kishimoto TK, Marlin SD. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu Rev Immunol* 1987; 107 :223–252.
31. Carson KR, Focosi D, Major EO, Petrini M, Richey EA, West DP, et al. Monoclonal antibody-associated progressive multifocal leukoencephalopathy in patients treated with rituximab, natalizumab, and efalizumab: a Review from the Research on Adverse Drug Events and Reports (RADAR) Project. *Lancet Oncol* 2009; 10: 816–824. [PubMed: 19647202]
32. Vincenti F, Mendez R, Pescovitz M, Rajagopalan PR, Wlikinson AH, Butt K, et al. A phase I/II randomized open-label multicenter trial of efalizumab, a humanized anti-CD11a, anti-LFA-1 in renal transplantation. *Am J Transplant* 2007; 7: 1770–1777. [PubMed: 17564637]
33. Isobe M, Suzuki J, Yagita H, Okumura K, Yamazaki S, Nagai R, et al. Immunosuppression to cardiac allografts and soluble antigens by anti-vascular cell adhesion molecule-1 and anti-very late antigen-4 monoclonal antibodies. *J Immunol* 1994; 153: 5810–5818. [PubMed: 7527450]
34. Stegall MD, Ostrowska A, Haynes J, Karrer F, Kam I, Gill RG. Prolongation of islet allograft survival with an antibody to vascular cell adhesion molecule 1. *Surgery* 1995; 118: 366–369. [PubMed: 7543705]
35. Elices MJ, Osborn L, Takada Y, Crouse C, Lubowskyj S, Hemler ME, et al. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* 1990; 60: 577–584. [PubMed: 1689216]
36. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. *Transplantation* 1973;16:343–50. [PubMed: 4583148]
37. Afanasyeva M, Georgakopoulos D, Belardi DF, Ramsundar AC, Barin JG, Kass DA, et al. Quantitative analysis of myocardial inflammation by flow cytometry in murine autoimmune myocarditis: correlation with cardiac function. *Am J Pathol* 2004; 164: 807–815. [PubMed: 14982835]
38. Krieger NR, Yin DP, Fathman CG. CD4⁺ but not CD8⁺ cells are essential for allojection. *J Exp Med* 1996; 184: 2013–2018. [PubMed: 8920888]
39. Nozaki T, Rosenblum JM, Ishii D, Tanabe K, Fairchild RL. CD4 T cell-mediated rejection of cardiac allografts in B cell-deficient mice. *J Immunol* 2008; 181: 5257–5263. [PubMed: 18832680]
40. Wood SC, Lu G, Burrell BE, Bishop DK. Transplant acceptance following anti-CD4 versus anti-CD40L therapy: evidence for differential maintenance of graft-reactive T cells. *Am J Transplant* 2008; 8: 2037–2048. [PubMed: 18828767]
41. Anderson DJ, Lo DJ, Leopardi F, Song M, Turgeon NA, Strobert EA, et al. Anti-leukocyte function-associated antigen 1 therapy in a nonhuman primate renal transplant model of costimulatory blockade-resistant rejection. *Am J Transplant* 2016; 16: 1456–1464. [PubMed: 26602755]
42. Badell IR, Russell MC, Thompson PW, Turner AP, Weaver TA, Robertson JM, et al. LFA-1-specific therapy prolongs allograft survival in rhesus monkeys. *J Clin Invest* 2010; 120: 4520–4531. [PubMed: 21099108]
43. Beus JM, Hashmi SS, Selvaraj SA, Duan D, Stempora LL, Monday SA, et al. Heterologous immunity triggered by a single, latent virus in *Mus musculus*: combined costimulation- and adhesion-blockade decrease rejection. *PLoS One* 2013; 8: e71221. [PubMed: 23940724]
44. Murakawa T, Kerklo MM, Zamora MR, Wei Y, Gill RG, Henson PM, et al. Simultaneous LFA-1 and CD40 ligand antagonism prevents airway remodeling in orthotopic airway transplantation: implications for the role of respiratory epithelium as a modulator of fibrosis. *J Immunol* 2005; 174: 3869–3879. [PubMed: 15778341]
45. Nicolls MR, Coulombe M, Beilke J, Gelhaus HC, Gill RG. CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J Immunol* 2002; 169: 4831–4839. [PubMed: 12391193]

46. Nicolls MR, Coulombe M, Yang H, Bolwerk A, Gill RG. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN- γ or IL-4. *J Immunol* 2000; 164: 3627–3634. [PubMed: 10725719]
47. Setoguchi K, Schenk AD, Ishii D, Hattori Y, Baldwin WM III, Tanabe K, et al. LFA-1 antagonism inhibits early infiltration of endogenous memory CD8 T cells into cardiac allografts and donor-reactive T cell priming. *Am J Transplant* 2011; 11: 923–935. [PubMed: 21466654]
48. El-Sawy T, Belperio JA, Strieter RM, Remick DG, Fairchild RL. Inhibition of polymorphonuclear leukocyte-mediated graft damage synergizes with short-term costimulatory blockade to prevent cardiac allograft rejection. *Circulation* 2005; 112: 320–331. [PubMed: 15998678]
49. Kreisel D, Sugimoto S, Tietjens J, Zhu J, Yamamoto S, Krupnick AS, et al. Bcl3 prevents acute inflammatory lung injury in mice by restraining emergency granulopoiesis. *J Clin Invest* 2011; 121: 265–276. [PubMed: 21157041]
50. Kreisel D, Sugimoto S, Zhu J, Nava R, Li W, Okazaki M, et al. Emergency granulopoiesis promotes neutrophil-dendritic cell encounters that prevent mouse lung allograft acceptance. *J Clin Invest* 2011; 118: 6172–6182.
51. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. *Nature* 2018;557:50–6. [PubMed: 29670285]
52. Quintini C, Martins PN, Shah S, Killackey M, Reed A, Guarrera J, et al. Implementing an innovated preservation technology: The American Society of Transplant Surgeons' (ASTS) Standards Committee White Paper on Ex Situ Liver Machine Perfusion. *Am J Transplant* 2018;18:In press.
53. Yeung JC, Zamel R, LKlement W, Bai XH, Machuca TN, Waddell TK, et al. Towards donor lung recovery-gene expression changes during ex vivo lung perfusion of human lungs. *Am J Transplant* 2018; 18: 1518–1526. [PubMed: 29446226]
54. Turgeon NA, Avila JG, Cano JA, Hutchinson JJ, Badell IR, Page AJ, et al. Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet cell transplantation. *Am J Transplant* 2010; 10: 2082–2091. [PubMed: 20883542]

Isografts

Allografts

Figure 1: Anti-VLA-4 mAb inhibits memory CD4 and CD8 T cell infiltration into high risk cardiac allografts.

Groups of 3–5 C57BL/6 mice received (A) C57BL/6 isografts or (B) complete MHC mismatched A/J cardiac allografts subjected to 0.5 or 8 h cold ischemic storage (CIS) prior to transplantation and were treated with 200 μ g rat IgG or anti-VLA-4 mAb on days –1 and 0. Grafts were harvested 48 h after transplantation, weighed, and digested to prepare single cell suspensions. Aliquots of graft infiltrating cells were stained with anti-CD45 mAb and either anti-CD4, anti-CD8, anti-F4/80 or anti-Ly-6G mAb, and then analyzed and quantified by flow cytometry. *p < 0.05 versus IgG treated recipients.; n.s. not significantly different.

A

B

Figure 2. Intra-graft cytokine and chemokine mRNA expression in cardiac allografts from untreated vs. anti-VLA-4 mAb treated recipients

(A) Groups of 3–5 C57BL/6 mice received complete MHC mismatched A/J cardiac allografts subjected to 0.5 or 8 h cold ischemic storage (CIS) prior to transplantation and were treated with 200 μ g rat IgG or anti-VLA-4 mAb on days –1 and 0. Grafts were harvested 48 h after transplant, whole cell RNA was isolated from graft homogenates, and quantitative real-time PCR was used to measure expression levels of mRNA encoding the indicated cytokines. Data represent mean mRNA expression level $\pm$ SEM. * p < 0.05. (B) Groups of 4–5 C57BL/6 mice received C57BL/6 isografts or complete MHC mismatched A/J cardiac allografts subjected to 8 h cold ischemic storage (CIS) prior to transplantation and were treated with 200 μ g rat IgG or anti-VLA-4 mAb on days –1 and 0. Grafts were harvested 48 h after transplant, whole cell RNA was isolated from graft homogenates and from the native heart of isograft recipients, and quantitative real-time PCR was used to measure expression levels of mRNA encoding the indicated chemokines. Data indicate individual expression of mRNA for each test chemokine with the bar indicating the mean mRNA expression level for each group. * p < 0.05; n.s., not significantly different.

Figure 3. Peri-transplant treatment with anti-VLA-4 mAb prolongs cardiac allograft survival. (A) Groups of 3–5 C57BL/6 mice received (A) C57BL/6 isografts or (B) complete MHC mismatched A/J cardiac allografts subjected to 0.5 or 8 h cold ischemic storage (CIS) prior to transplantation and were treated with 200 μ g rat IgG or anti-VLA-4 mAb on days –1 and 0. Graft survival was monitored daily by abdominal palpation and allograft rejection was confirmed visually in all recipients by laparotomy. * $p < 0.05$ vs. allograft survival in anti-VLA-4 mAb treated vs. untreated recipients. (B) Representative images depicting the differences in mononuclear cell infiltration into allografts at the time the grafts are rejected in control treated recipients. Grafts were harvested from each group and prepared sections stained with hematoxylin and eosin. Magnification, 200 $\times$.

Figure 4: Peri-transplant anti-VLA-4 mAb inhibits naïve donor-reactive CD8 and CD4 T cell development to IFN- γ or IL-2 producing cells.

Groups of 4 C57BL/6 mice received C57BL/6 isografts or complete MHC mismatched A/J cardiac allografts subjected to 0.5 or 8 h cold ischemic storage (CIS) prior to transplantation and were treated with 200 μ g rat IgG or anti-VLA-4 mAb on days -1 and 0. On day 7 or 14 post-transplant, aliquots of unseparated spleen cells, enriched CD4 or enriched CD8 T cells from the recipient spleen were cultured overnight with syngeneic or donor A/J stimulator cells on anti-IFN- γ mAb or anti-IL-2 mAb coated filters. Numbers of donor-reactive T cells producing IFN- γ were determined by ELISPOT. Numbers of spots in co-cultures with syngeneic stimulators were always < 20 spots/ 5×10^5 stimulator cells. The data shown are representative of two independent experiments. *p < 0.05 versus control treated recipients; n.s. not significantly different.

Figure 5: Peri-transplant VLA-4 blockade disrupts CTLA-4Ig-resistant rejection of high risk cardiac allografts.

Groups of 4–5 C57BL/6 mice received complete MHC mismatched A/J cardiac allografts subjected to 8 h cold ischemic storage (CIS) prior to transplantation and were treated with 200 μ g rat IgG or anti-VLA-4 mAb on days –1 and 0 and with 250 μ g CTLA-4Ig on days 0 and 1. (A) Graft survival was monitored daily by abdominal palpation and allograft rejection was confirmed visually in all recipients by laparotomy. * $p < 0.05$ vs. allograft survival in CTLA-4Ig conditioned recipients without anti-VLA-4 mAb. (B) At the time of allograft rejection in anti-VLA-4 mAb plus CTLA-4 Ig conditioned recipients, recipient spleens were harvested and cell aliquots were tested by ELISPOT assay for numbers of cells producing IFN- γ following overnight culture with recipient vs. allograft donor stimulator cells. (C and D) Allografts were harvested at the time of rejection in recipients treated with (C) CTLA-4Ig or (D) CTLA-4Ig plus anti-VLA-4 mAb and prepared sections were stained to detect CD4 and CD8 T cells and macrophages (Mac2⁺ cells). Magnification 200 $\times$.

Figure 6: Peri-transplant anti-VLA-4 mAb rescues the enhanced survival of high risk cardiac allografts in recipients depleted of CD4 T cells.

Groups of 4–5 C57BL/6 mice received complete MHC mismatched A/J cardiac allografts subjected to (A) 0.5 h or (B) 8 h cold ischemic storage (CIS) prior to transplantation and were treated with 200 μ g control rat IgG or anti-CD4 mAb on days –3, –2, –1 prior to transplant and weekly thereafter to deplete CD4 T cells. The indicated recipient groups were also treated with 200 μ g anti-VLA-4 mAb on days –1 and 0. Graft survival was monitored daily by abdominal palpation and allograft rejection was confirmed visually in all recipients by laparotomy. For A: * $p < 0.05$ vs. allograft survival in recipients without CD4 T cell depletion. For B: * $p < 0.05$ vs. allograft survival in recipients without CD4 T cell depletion and vs. allograft survival in recipients with CD4 T cell depletion without anti-VLA-4 mAb treatment.

Figure 7: Peri-transplant anti-VLA-4 mAb enhances CD154-mediated costimulatory blockade to increase survival of high risk cardiac allografts.

Groups of 4–5 C57BL/6 mice received complete MHC mismatched A/J cardiac allografts subjected to 0.5 or 8 h cold ischemic storage (CIS) prior to transplantation and were treated with 400 μ g anti-CD154 mAb MR-1 on days 0 and +1 with or without 200 μ g anti-VLA-4 mAb on days –1 and 0. (A) Graft survival was monitored daily by abdominal palpation and allograft rejection was confirmed visually in all recipients by laparotomy. $**p < 0.01$ vs. allograft survival in recipients without anti-VLA-4 mAb. (B–E) Recipients of allografts subjected to (B and C) minimal or (D and E) prolonged CIS and treated with anti-VLA-4 plus anti-CD154 mAb were sacrificed at the time of graft rejection and the grafts harvested for histologic evaluation by staining with: (B and D) Masson's trichrome or antibody to detect (C and E) C4d deposition. Manifestations of similar acute and chronic injury are observed in allografts subjected to minimal or prolonged CIS prior to transplantation and

recipient treatment with anti-CD154 plus anti-VLA-4 mAb. Interstitial mononuclear cell infiltrates are evident in trichrome stains (B and D). Immunohistology of adjacent sections demonstrates diffuse linear deposits of C4d on capillaries (C and E). Arteries with extensive neointimal formation are evident in all panels as indicated by white arrows that mark the internal elastic lamina.