Background—The interaction between CD40 on antigen-presenting cells and CD40L on T cells is critical in allograft rejection. CD154 blockade suppresses allograft rejection, but the role of this pathway in allograft vasculopathy remains obscure.

Methods and Results—A vascularized murine heterotopic cardiac transplant model was used to test whether perioperative CD154 blockade suppresses allograft vasculopathy or whether long-term CD154 blockade is required to suppress allograft vasculopathy. Perioperative CD154 blockade consisted of MR1 given on days −1, 1, and 3; long-term blockade consisted of MR1 given on days −1, 1, and 3 and continued twice weekly for 8 weeks. Allografts treated with perioperative or long-term CD154 blockade survived indefinitely. Perioperative and long-term treatment with control antibody (Ha4/8) resulted in uniform early rejection. Perioperative CD154 blockade transiently reduced early T-cell and macrophage infiltration in parallel with a transient reduction in endothelial adhesion receptor expression. Although perioperative CD154 blockade prevented allograft failure, it did not reduce allograft vasculopathy; mean neointimal cross-sectional area in perioperative MR1-treated and Ha4/8-treated recipients was 43±7% and 50±12%, respectively (P=NS). In contrast, mean neointimal cross-sectional area in long-term, MR1-treated recipients was 19±3% (P<0.001 versus perioperative MR1). Long-term CD154 blockade also suppressed endothelial E-selectin, P-selectin, and intracellular adhesion molecule 1 expression and improved graft function 3.5-fold versus control (P<0.05).

Conclusions—These data show that perioperative CD154 blockade mitigates acute rejection but long-term CD154 blockade may result in decreased allograft endothelial activation and is required to suppress allograft arteriopathy.

Key Words: transplantation ■ cell adhesion molecules ■ immunology ■ rejection

Transplant-associated coronary artery disease (TCAD)1 is the leading cause of late cardiac allograft failure. By 5 years after transplantation, TCAD develops in ≈50% of recipients.1 In TCAD, proliferation of neointimal cells concentrically narrows the coronary arterial lumen along its entire course.2 This intimal lesion is more diffuse, with less lipid than native coronary artery atherosclerosis. In humans, both cell-mediated and humoral alloimmunity contribute to the development of TCAD. The expanded intima of human allograft coronary arteries contains CD4+ and CD8+ T cells and major histocompatibility complex (MHC) class II macrophages concentrated just beneath the luminal endothelium.3 Activated allograft endothelial cells also express MHC class II molecules. These data suggest that endothelial cells may activate antigen-specific CD4+ T cells, promote endothelialitis, and contribute to TCAD. The presence of CD4+ T cells, macrophages, and activated endothelial cells in human intimal allograft lesions has created burgeoning interest in the role of costimulatory molecules in initiating or exacerbating the development of TCAD.

CD154, an important T-cell costimulatory molecule that is transiently expressed on activated CD4+ T cells, binds CD40 on classic antigen-presenting cells, endothelial cells, and other cells. Among other functions, CD154-CD40 interaction has been shown to activate endothelial cells.4 The ephemeral expression of CD154 on activated CD4+ T cells and its elemental role in the antigen-specific immune response make it an attractive target for anti-rejection therapeutics.

In addition to upregulating key costimulatory molecules, CD154-CD40 interactions may play other roles in TCAD. For example, ligation of endothelial CD40 upregulates endothelial adhesion receptors, such as CD54 (intracellular adhesion molecule [ICAM]-1) and CD106 (vascular cell adhesion molecule [VCAM]-1), which have been implicated in regulating trafficking of T cells and macrophages into TCAD lesions. We also have demonstrated recently that CD154-
mediated signals induce endothelial cells to secrete chemokines, such as monocyte chemoattractant protein-1, important in attracting leukocytes to inflammatory lesions. CD154–CD40 interactions also induce expression of inducible nitric oxide synthase and proinflammatory cytokine production.

In the murine cardiac allograft model, simultaneous, perioperative blockade of the two critical costimulatory signals, CD40–CD154 interaction (blocked with MR1, an anti-CD154 mAb) and CD28–B7 interaction (blocked with the fusion protein CTLA4-Ig), potently suppressed murine cardiac allograft rejection and delayed the development of allograft arteriopathy. Although MR1 alone suppressed lymphocytic infiltration, arteriopathy developed in 2 of 3 allografts at 2 months. Others have shown that perioperative CD154 blockade indefinitely prolongs survival of most allografts, but arteriopathy was not examined. These data led us to investigate whether long-term CD154 blockade, without other immunosuppressive therapies, might not only prevent rejection but might mitigate the late development of TCAD. To test this hypothesis, we used a heterotopic vascularized murine cardiac transplant model to investigate the role of the CD40–CD154 pathway in the pathogenesis of TCAD.

Methods

Experimental Animals

Experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee at Columbia University. Male mice 10 to 15 weeks of age and weighing 25 to 35 g were obtained from Jackson Laboratories. B10A (H-2a) mice served as donor animals and C57BL/6J (H-2b) mice as recipients. Four groups were studied; recipients assigned to the perioperative treatment groups were given either anti-murine CD154 mAb, MR1 (American hamster IgG ATCC and purified mAb provided by Biogen, Inc), or American hamster IgG control, Ha4/8 (Biogen, Inc). Perioperative groups were designated MR1(s) and Ha4/8(s), respectively. Recipients assigned to the long-term treatment groups were given either the anti-CD154 antibody MR1 or the control antibody Ha4/8 given on days −1, +1, and +3 and continued twice weekly for 8 weeks. These groups were designated MR1(c) and Ha4/8(c), respectively. In all four arms, no other immunosuppressant was given.

Heart Transplantation

The procedure for performing cardiac transplants has been previously described. In brief, after anesthesia, donor hearts were harvested and transplanted heterotopically into recipient abdomens. The donor aorta and pulmonary artery were anastomosed end-to-side to the recipient’s abdominal aorta and inferior vena cava, respectively, with warm ischemic time maintained constant at 30 minutes.

Assessment of Graft Survival

During the follow-up period, allografts were palpatated by a blinded observer every other day, and a palpation score was assigned on the basis of a 3-point scoring system, 0 to 3 (3, strong contraction, soft graft; 2, moderate contraction, firmer graft; 1, weak contraction with hard graft; and 0, no contraction). At 60 days or graft failure, the graft was explanted for further analysis.

Echocardiographic Imaging and Image Analysis

Two-dimensional echocardiographic assessment of allograft function was performed with an HP SONOS 5500 ultrasound system (Hewlett-Packard) with a 12-MHz transducer. Before echocardiographic examination, recipient mice were anesthetized again, and the dorsiolumbar region was shaved. Echocardiographic images were obtained in the prone position at a depth of 3 cm in the short- and long-axis views, with a 120-Hz frame rate. They were recorded on VHS for off-line analysis. Normally, the myocardium thickens with each contraction; by tracing the endocardial contour at both end-systole and end-diastole, this thickening can be measured, allowing the calculation of fractional area change (FAC). Short-axis images of beating hearts taken at the mid–papillary muscle level were used to measure FAC: %FAC = (EDA – ESA)/EDA · 100, where EDA = end-diastolic area and ESA = end-systolic area. This technique has been previously described to measure the function of native and transplanted murine hearts.

Histomorphometric Quantification of TCAD Area

A blinded observer performed histomorphometry of the TCAD area by using previously published methods. At 60 days after transplantation, grafts from MR1-treated recipients were harvested and fixed in 10% formalin, embedded in paraffin, and sliced into 5-μm sections with a microtome. Grafts from Ha4/8-treated recipients were harvested at graft failure (mean, 25 days). Sections were taken at one third of the distance from the base to the apex of the heart and stained with hematoxylin and eosin (for gross morphology) as well as van Gieson stain to delineate the internal elastic lamina. The images were captured with a Sony DXC-970 MD 3CCD color camera attached to a PC, and the images were processed with a Zeiss image analysis program. The intimal, medial, and luminal boundaries of all identifiable blood vessels in the section were traced, and planimetric areas were calculated by image analysis software. Percent luminal obliteration was calculated in areas not distorted by sectioning.

Immunohistochemistry

For immunohistochemical analysis of tissue sections obtained at 3 and 16 days, frozen tissue sections were stained. For immunohistochemical analysis of later time points, sections were obtained from paraffin blocks. Sections were stained with optimal concentrations of the following antibodies: rat anti-murine CD8, rat anti-murine CD4 (both from ATCC), hamster anti-murine CD3, control hamster IgG, American hamster anti-mouse CD54 (ICAM), rat anti-mouse C6DE2 (E-selectin), rat anti-mouse C6D2P (F-selectin), or rat anti-mouse CD106 (VCAM) (from Pharmingen). The primary antibodies were detected with biotinylated rabbit anti-hamster IgG or biotinylated goat anti-rat IgG. Peroxidase activity was detected with the chromogen 3-amino-9-ethylcarbazole (Vector Laboratories). Sections were counterstained with Mayer hematoxylin (Sigma).

Results

Allografts in recipients receiving monotherapy with MR1, given either perioperatively or chronically, all survived for the 60-day observation period. In contrast, grafts in recipients given the control antibody (Ha4/8) were rejected within 31 days (Figure 1, A and B). Graft function, measured by palpation, in the MR1(s) group initially declined, then stabilized, producing a mean palpation score at 60 days of 0.9 ± 0.1 (P < 0.05 compared with the Ha4/8(s) group) (Figure 1C). Similarly, in the MR1(c) group, graft function declined modestly in the first several weeks, then stabilized, producing a mean palpation score at 60 days of 2.0 ± 0.03 (Figure 1D) (P < 0.05 compared with the Ha4/8(c) group). At 60 days, MR1(c) allograft function was also better than MR1(s) allograft function (P < 0.05).

Echocardiographic assessment was performed on recipients in the MR1(c) and Ha4/8(c) groups to confirm allograft function with the use of FAC. Increased FAC correlates with better cardiac function. Echocardiograms were performed on the MR1(c)-treated recipients at days 20 to 25 and on the Ha4/8(c)-treated recipients at days 5 to 7. Echocardiography was not performed simultaneously in Ha4/8(c)-treated recipients because at day 20, almost all of these grafts had been rejected. Despite measuring FAC in the MR1(c) group 2 weeks later, MR1(c)-treated recipient’s grafts had a mean FAC 3.5-fold higher than Ha4/8(c)-treated recipient’s grafts (P < 0.05) (Figure 2, A and B).
At 60 days after transplantation, the MR1(s) and MR1(c) grafts were harvested, and sections were taken. Representative elastin-stained vessels are shown in Figure 3. Figure 3A, a representative section from an Ha4/8(c)-treated graft, shows a large neointimal lesion obliterating the lumen in an elastin-stained vessel. Figure 3B, a representative section from an MR1(s)-treated graft (day 60), also shows a neointimal lesion similar to the one seen in the Ha4/8(s)-treated section. Figure 3C, a representative section from an MR1(c)-treated allograft, shows almost no neointimal lesion. Histomorphometric analysis of planimetered vessels was performed to quantify the lesions (Figure 3D). The mean atherosclerotic area in the MR1(c) group was significantly less than that of the MR1(s) group (19/1006 yes% versus 43/1006 yes%, respectively, P<0.001). The mean atherosclerotic area of the Ha4/8(s) (not shown) and Ha4/8(c) groups did not differ from each other or the MR1(s) group. Interestingly,
although allografts in the MR1(s) group exhibited little acute graft rejection measured by transplant scores, they had a similar degree of TCAD compared with the Ha4/8-treated animals.

To examine the effect of CD154 blockade on vascular expression of adhesion receptors previously implicated in primary cardiac isograft and allograft failure, immunohistochemistry for P-selectin, ICAM-1, and E-selectin was performed on representative sections. Figure 4A shows representative sections from MR1(s)- and Ha4/8(s)-treated allografts taken at days 3 and 16 after transplantation. Qualitative analysis of these sections reveals that the MR1(s)-treated allografts exhibit less adhesion receptor expression at day 3 after transplantation. At day 16, 13 days after MR1 therapy ended, adhesion receptor expression in the MR1(s) and the control Ha4/8(s) groups was indistinguishable. MR1 therapy did not change immunohistochemical staining for VCAM-1 at 3 and 16 days after transplantation (data not shown). Figure 4B shows representative paraffin sections from MR1(c)-treated and Ha4/8(c)-treated allografts at days 30 and 60, respectively. This qualitative staining demonstrates a concordance between the absence of TCAD and the absence of adhesion receptor staining.

To establish the immunologic relevance of this observation, T-cell infiltrates were examined immunohistochemically at days 3 and 16 in frozen sections taken from MR1(s)- and Ha4/8(s)-treated recipients. T-cell infiltrates were stained for CD3 and then for CD4 and CD8 (Figure 5). Quantitative analysis for stained lymphocytes demonstrated a pattern similar to that seen for the adhesion receptors (Figure 6). The number of infiltrating T cells (CD3+) in the MR1(s)-treated group was <50% that of control animals on day 3 but by day 16 increased to nearly equal the Ha4/8(s)-treated group. CD4+ T-cell infiltration appears to account for the increasing number of T cells in the allograft between days 3 and 16. MR1 treatment did not change the number of infiltrating CD8+ T cells at either time point.

**Discussion**

The CD40-CD154 pathway initially was implicated in transplant rejection because of its pivotal role in the immune response. CD40-CD154 interactions play central roles in both cellular and humoral immune responses. Evidence suggesting the importance of this receptor pair in cardiac allograft
rejection has come from many sources. CD154 expression has been demonstrated on T cells and on endothelial cells in biopsy specimens from human cardiac allografts and correlated closely with acute rejection in these patients. In rhesus monkeys that received only perioperative CTLA4-Ig and humanized anti-CD154 (hu5c8) therapy, survival of renal allografts mismatched at the MHC I and II loci was indefinitely prolonged. In the murine model of cardiac allograft transplantation, single-dose CD154 blockade with a monoclonal antibody on the day of transplantation led to indefinite survival of 71% of the grafts in one study and 75% in another. Grafts from the MR1(s)- and MR1(c)-treated groups in the present study survived indefinitely. These studies demonstrate that anti-CD154 monotherapy can suppress acute rejection effectively.

The CD40-CD154 pathway also has been implicated in the pathogenesis of atherosclerosis. In human coronary atherosclerosis and TCAD, CD40 expression is markedly upregulated on lesional endothelial cells, smooth muscle cells, macrophages, and foam cells. In LDL receptor–deficient mice, which have a propensity for the development of atherosclerosis, treatment with anti-CD154 reduced lesional atherosclerotic progression compared with control antibody or saline treatment. CD154 expression also occurs in native coronary artery disease and TCAD lesions; however, its cellular distribution is controversial. Some investigators have shown that CD154 is widely expressed on endothelial cells, macrophages, smooth muscle cells, and T cells in native atherosclerosis. Other studies have failed to document endothelial expression of CD154. In both diseases, CD40 is expressed on cells with CD54 and CD106. Moreover, the extent of CD40, CD54, and CD106 expression correlates significantly with the extent of atherosclerosis.

Further support for the role of CD40-CD154 interactions in the immunopathogenesis of native atherosclerosis and TCAD comes from studies in mice. CD154 blockade in apolipoprotein E and LDL receptor knockout mice fed atherogenic diets reduced the size of native atherosclerotic lesions by 59% and their lipid content by 79%. Taken together, these observations make TCAD an obvious target for experimentation with anti-CD154 therapies. In a murine allograft model previously described, perioperative treatment with MR1 (250 μg on days 0, 2, 4, and 6) does not prevent TCAD, with severe neointimal proliferation developing in 2 of 3 grafts. This finding echoes our results with perioperative treatment, which also did not prevent delayed development of TCAD. Although simultaneous CD40-CD154 and CD28-B7 blockade did reduce TCAD, the utility of long-term blockade of the CD40-CD154 pathway alone to reduce cardiac transplant atherosclerosis had not been established before the work presented here.

The MR1(c)-treated recipients not only had uniform allograft survival but also had a significant absolute reduction in luminal obliteration by TCAD, indicating that long-term therapy with MR1 can reduce the development of TCAD. This is the first time this has been demonstrated with the use of CD154 blockade as monotherapy. The data do not necessarily contradict the...
Recent report showing that mice deficient in CD154 are not protected from TCAD.\textsuperscript{19} Disease progression in mice given antibodies directed against a given gene product may be quite dissimilar from disease progression in mice that are developmentally and persistently null for the same gene product, in which compensatory immune mechanisms may develop as the result of developmental absence of the gene product.

Comprehensive immunohistochemical staining of leukocyte adhesion receptors and T-cell infiltrates at early and later time points revealed an intriguing pattern. Shortly after transplantation, MR1 monotherapy reduced both leukocyte adhesion receptors and T-cell infiltrates. Without continued monotherapy, adhesion receptor expression and T-cell infiltration increased in parallel to the levels seen in the control group. Specifically, the reduction in CD4\textsuperscript{+} infiltrates but not CD8\textsuperscript{+} infiltrates followed this pattern. One potential mechanism by which CD40-CD154 blockade could inhibit T-cell transmigration is by blocking the cognate interaction between CD40 on endothelial cells and CD154 on CD4\textsuperscript{+} cells, suppressing endothelial activation. Early reductions in CD54 (ICAM-1), CD62P (P-selectin), and CD62E (E-selectin) suggest a mechanism for this decrease in migration. Absence of immunohistochemical staining of the adhesion receptors in the MR1(c) group further strengthens this conclusion. These data provide insight into how long-term MR1 monotherapy might decrease TCAD. In a similar murine model, blockade of CD54 and CD11a/CD18 (LFA-1) reduced TCAD.\textsuperscript{11,20} The authors of these reports concluded that this was probably secondary to decreased leukocyte migration.

Further support for a role of CD154-CD40 interactions in regulating leukocyte trafficking comes from in vitro studies. We and others have demonstrated that CD40 ligation induces upregulation of intercellular adhesion molecules CD54, CD106 (VCAM-1), and CD62E (E-selectin) on endothelial cells. Moreover, we recently demonstrated that CD154-CD40 interactions induce endothelial cells to secrete the chemokines monocyte chemotactic protein-1 and RANTES.\textsuperscript{4} These intercellular adhesion molecules and chemokines may also participate in TCAD development.

CD54, CD62P, and other molecules in this class are essential to leukocyte adhesion and diapedesis across the endothelium in inflammatory responses. Taken together, these studies show that CD40 blockade reduces adhesion receptor expression and leukocyte traffic during therapy. Only long-term CD154 blockade prevents TCAD, whereas short-term therapy does not. These data suggest a pivotal role in TCAD for T cells and the CD154 pathway in the posttransplantation period. The optimal length of CD154 blockade remains unknown. If therapy is interrupted 3 days after transplantation, TCAD develops. This suggests that MR1 effectively suppresses the antigenic stimulus that produces TCAD but does not induce a durable regulatory T-cell response and that the antigenic stimulus driving TCAD remains very strong in the late posttransplantation period. This contrasts with recent data obtained in the setting of bone marrow transplantation, which suggests that CD154 blockade induces a regulatory CD4\textsuperscript{+} T-cell population that promotes engraftment.\textsuperscript{21} The results of experiments in this report quantify the magnitude of the improvement in TCAD in an established murine model and shed light on understanding of the durability of CD154 blockade with respect to TCAD development.

Acknowledgments

This work was supported in part by National Institutes of Health grants HL-60900 and HL-53397 to Dr Pinsky, MR1 and H4/8 antibodies were provided by Biogen, Inc. The authors acknowledge the expert technical assistance of Isaak Aronson and Yu-Shan Zou.

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_Circulation_. 2002;105:1609-1614; originally published online March 11, 2002;
doi: 10.1161/01.CIR.0000013022.11250.30
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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