Role of the Fc Region in CD70-Specific Antibody Effects on Cardiac Transplant Survival

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Background. The role of the CD70-specific antibody and the mechanisms by which it extends transplant survival are not known.

Methods. Fully major histocompatibility complex-mismatched heterotopic heart transplantation (BALB/c to C57BL/6) was performed. Treated mice received intraperitoneal injections of wild-type (WT) CD70-specific antibody (FR70) or IgG_1 or IgG_{2a} chimeric antibodies on days 0, 2, 4, and 6 posttransplantation.

Results. WT FR70 antibody significantly extended heart transplant survival to 19 days compared with untreated mice (median survival time [MST]=10 days). Graft survival using the nondepleting IgG_1 antibody was significantly shorter (MST=14 days), whereas the survival using depleting IgG_{2a} antibody (MST=18) was similar to that using WT FR70. The FR70 and IgG_{2a} antibodies demonstrated a greater efficiency of fixing mouse complement over the IgG_1 variant in vitro. $CD4^+$ and $CD8^+$ T-cell graft infiltration was reduced with treatment; however, this was most pronounced with WT FR70 and IgG_{2a} antibody therapy compared with the IgG_1 chimeric variant. Circulating donor-specific IgG alloantibodies were initially reduced with WT FR70 treatment (day 8 posttransplantation) but increased at days 15 and 20 posttransplantation to the level detected in untreated controls.

Conclusion. We conclude that WT (FR70) and the IgG_{2a} depleting variant of CD70-specific antibody reduce graft infiltrating $CD4^+$ and $CD8^+$ T cells, transiently reduce serum alloantibody levels, and extend graft survival. In contrast, the nondepleting IgG_1 variant of this antibody showed lower efficacy. These data suggest that a depleting mechanism of action and not merely costimulation blockade plays a substantial role in the therapeutic effects of CD70-specific antibody.

Keywords: CD70, Heart transplantation, Antibody therapy, Fc region.

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Organ rejection is a pressing problem in the clinical transplant setting. Currently used immunosuppressive agents such as cyclosporine A and mycophenolate mofetil are associated with significant side effects such as increased susceptibility to infection and malignancies. The therapeutic use of antibodies targeting T lymphocytes is an approach being tested and applied in many autoimmune disease models (*1*), in the treatment of cancers (*2*), and in the prevention of transplant rejection (*3*).

The CD27-CD70 pathway has recently emerged as a potential target to treat alloimmune responses, cancers, and inflammatory diseases. The restricted expression of CD70 on normal cells makes it a good therapeutic target for monoclonal antibody-based therapies. CD70 is a member of the tumor necrosis factor (TNF) family and is expressed aberrantly in a number of carcinomas and on activated lymphocytes in inflammatory and alloimmune settings (*4*, *5*). Its expression is tightly regulated (*6*) and it is expressed transiently on T-cell activation. Conversely, CD27 is expressed constitutively on

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both naive and memory T cells and also on subsets on B cells, NK cells, and hemopoietic progenitor cells (*7*).

CD27-deficient mice produce a smaller number of effector $CD4^+$ and $CD8^+$ T cells compared with wild-type (WT) mice, which show that the CD27-CD70 pathway is important for the effector T-cell response (*8*). CD27-mediated signals are also important for optimal quality of the memory $CD8^+$ T-cell pool by preventing the apoptosis of $CD8^+$ T cells in a primary response (*9*). Also, an increased presence of $CD4^+CD70^+$ (activated) T cells in the periphery in rheumatoid arthritis (*10*) and systemic lupus erythematosis (*11*) indicates that increased CD70 expression might serve as a useful marker of abnormal T-cell activity in these disease models and controlled T-cell activation is important to prevent pathogenesis.

Therapeutic antibodies require only intermittent administration due to their long half-life and prolonged effects. These characteristics and their specificity of action minimize treatment side effects. Indeed, the therapeutic effect of CD70-specific antibody has been shown in animal models of autoimmune encephalomyelitis (*12*) and cardiac allograft rejection (*13*); however, its precise mechanism of action has not been investigated. Previously, we have tested a combination of antibodies, including anti-CD70, anti-CD8, and anti-CD154 in transplant models. This approach, which we termed multihit therapy, showed striking effects in challenging presensitized skin (*14*) and cardiac transplant models (*15*). This is a significant finding because no other short-term treatment has been able to contain the responses of primed T-cell responders (*16*, *17*).

In this article, we discuss the therapeutic approach of targeting the CD70 antigen to improve allograft survival. Although the efficacy of WT FR70 (CD70-specific rat IgG_{2b} antibody) as part of an antibody combination therapy has been shown in our presensitized skin and heart transplant models, it is imperative that the mode of action of FR70 as a single therapeutic agent in the transplant setting is determined.

To understand the contribution of the Fc region of FR70 to its therapeutic efficacy, variants of WT FR70 comprising identical Fab regions but different Fc regions were exploited. The Fc regions of these chimeric proteins were mouse IgG_{2a} and mouse IgG_1.

RESULTS

Effects of Treatment on Graft Survival in Naive Recipients

To determine the effects of treatment with CD70-specific antibody (FR70), we transplanted recipient C57BL/6 ($H\text{-}2^b$) mice with fully major histocompatibility complex-mismatched BALB/c (H-2d) vascularized cardiac grafts and treated them with FR70 (500 μg) on the day of transplantation and 250 μg/dose on days 2, 4, and 6 posttransplantation. Significant prolongation of graft survival was seen in the treated group (median survival time [MST]=19 days), whereas the untreated controls had an MST of 10 days ($P<0.0001$; Fig. 1A).

We also tested the effects of two mouse chimeric antibodies composed of $mIgG_1$ or $mIgG_{2a}$ Fc and the Fab regions of the WT FR70 antibody. IgG_1 or IgG_{2a} antibodies were administered as for the FR70 antibody and abdominal palpitation was performed regularly to determine the time of graft rejection.

Treatment with the IgG_{2a} antibody prolonged graft survival to 18 days when compared with untreated controls that had a MST of 10 days ($P=0.004$; Fig. 1A). This was not significantly different to the survival of FR70-treated animals. IgG_1 also improved graft survival significantly to MST=14 days in comparison with untreated controls ($P=0.004$); however, this antibody was not as effective as FR70 and IgG_{2a} in delaying graft rejection ($P=0.0001$).

To interpret the survival data, it was important to determine whether the WT FR70 antibody and the $mIgG_1$ and $mIgG_{2a}$ antibodies exhibit similar binding efficacies to their target molecule CD70. P815-CD70 transfectants were preblocked by incubation in the presence of FR70 antibody or the chimeric IgG_1 or IgG_{2a} antibodies. Residual CD70 expression was then detected by FITC-conjugated FR70 antibody staining, and flow cytometric analysis confirmed that all three antibodies bind CD70 in a similar manner (Fig. 1B). In addition, the ability of FR70 and the chimeric antibodies to fix mouse complement was determined by culturing P815-CD70 transfectants in the presence of C57BL/6 normal mouse serum with FR70 or the chimeric IgG_1 or IgG_{2a} antibodies. The deposition of C1q was subsequently investigated by antibody staining and flow cytometric analysis. This assay clearly revealed a greater efficiency of IgG_{2a} to fix mouse complement over IgG_1 in vitro (Fig. 1C). These data suggest that FR70 and the IgG_{2a} antibodies may be more effective at mediating cell depletion than the IgG_1 antibody which may be relatively nondepleting in terms of complement-dependent effector mechanisms. Furthermore, these findings suggest that some of the effector mechanisms of FR70 are mediated by the Fc region such as those involving complement fixation and not merely the blockade of the CD27-CD70 costimulatory pathway.

Intragraft T Cells

Histological analysis of grafts harvested at different time points after transplantation showed that the number of infiltrating $CD4^+$ and $CD8^+$ T cells remained relatively high in untreated recipients, while treatment with FR70 seemed to reduce the infiltration of $CD8^+$ T cells into the graft at day 8 (Fig. 2). $CD4^+$ T-cell infiltration was also reduced compared with untreated mice; however, this did not reach statistical significance. IgG_1 antibody treatment, however, did not prevent the influx of $CD4^+$ or $CD8^+$ T cells at day 8 and showed similar levels of infiltration as the untreated controls. IgG_{2a} treatment showed similar numbers of graft-infiltrating $CD4^+$ T cells as with FR70 at day 8 posttransplantation, while infiltration of $CD8^+$ T cells was intermediate to that after FR70 and IgG_1 antibody therapy.

Further immunohistochemical staining carried out on grafts harvested at day 15 posttransplantation showed that FR70 treatment maintained significantly lower levels of $CD4^+$ and $CD8^+$ T cells in comparison with IgG_1 antibody-treated animals (Table 1). At day 20 posttransplantation, there was no further increase in $CD4^+$ and $CD8^+$ T cells in FR70-treated recipients, and the level of $CD4^+$ T-cell infiltration was similar to that of IgG_{2a}-treated recipients. No data were available for the $mIgG_1$-treated mice at this time point

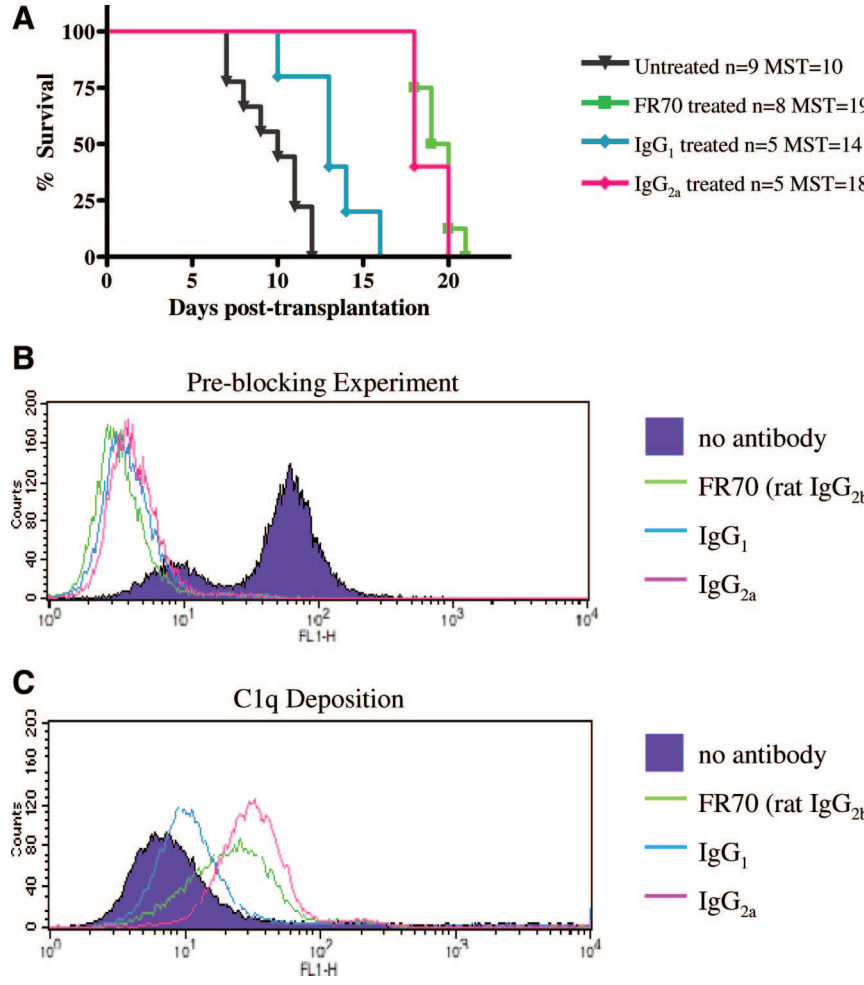

FIGURE 1. Cardiac allograft survival is prolonged after treatment with CD70-specific antibody in naive heart transplant recipients. (A) Recipient C57BL/6 (H-2^b) mice received heterotopic, non-life supporting fully vascularized heart grafts from BALB/c (H-2^d) donors. They were treated with wild-type CD70-specific antibody (FR70) or chimeric IgG_1 or IgG_{2a} antibodies 500 μg administered intraperitoneally on the day of transplantation (day 0) and 250 μg/dose on days 2, 4, and 6 posttransplantation. (B) Preblocking of P815-CD70 transfectants confirms that the FR70 antibody, IgG_1, and IgG_{2a} chimeric antibodies bind CD70 equivalently. P815-CD70 transfectants were preblocked by incubation in the presence of FR70 antibody (*green line*) or the chimeric FR70 antibodies IgG_1 (*blue line*) or IgG_{2a} (*pink line*). Residual CD70 expression was then detected using a FITC-conjugated FR70 antibody followed by flow cytometric analysis. Control cells were pretreated with PBS alone, and subsequent antibody staining shows the level of CD70 expression (*filled purple peak*). (C) FR70 and IgG_{2a} chimeric antibody fix mouse complement more efficiently than the IgG_1 chimera in vitro. P815-CD70 transfectants were cultured in the presence of C57BL/6 normal mouse serum (2%) with FR70 (*green line*) or the chimeric IgG_1 (*blue line*) or IgG_{2a} (*pink line*) antibody. Cells were subsequently analyzed for deposition of C1q using FITC-conjugated antisera and flow cytometric analysis.

because the grafts were rejected (MST=14, Fig. 1A). These observations suggest that the depletion of CD70 expressing cells using FR70 and IgG_{2a} antibody delays cellular infiltration and ultimately preserves graft function for longer when compared with antibodies that simply block the CD27-CD70 costimulatory pathway.

Donor-Specific Antibody Levels

Sera from recipients were obtained from tail vein blood at different time points after transplantation and analyzed for the presence of donor (BALB/c)-specific IgG antibodies. Figure 3(A) shows background levels of allo-specific IgG serum from representative naive recipients before transplantation (day 0) with geometric mean fluorescent intensities (MFI) of 21 and 18. After transplantation at day 8, significant levels of donor-specific antibodies were detected in untreated mice (MFI=1053). In contrast, WT FR70 treatment resulted in lower levels of donor-specific IgG (MFI=644 at day 8, for the representative recipient shown) in the sera derived from peripheral blood. However, a gradual increase in level was seen on days 15 and 20 posttransplantation, which seemed to correlate with graft rejection for this group (MST=19 days; Fig. 1A). The effect of treatment with IgG_1 or IgG_{2a} antibody was similar to that of FR70 at the time points tested before graft rejection. **SDC 2** (see **Figure,** http://links.lww.com/TP/A540) shows the increase in IgG alloantibodies for individual mice in each treatment group at different time points after transplantation.

$CD25^+Foxp3^+$ T Cells

To examine the effects of CD70-specific antibody therapy on the numbers of $CD4^+$ $CD25^+Foxp3^+$ T cells, spleens were harvested from treated and control heart transplant recipients. Splenocytes were isolated and analyzed for the presence of $CD4^+CD25^+Foxp3^+$ T cells by antibody staining and flow cytometric analysis. On day 8 posttransplantation, the percentage of $CD4^+$ T cells with $CD25^+Foxp3^+$ phenotype was significantly higher in the untreated group (24.4%) compared with the FR70 treatment group (11.6%, $P=0.02$; Fig. 4). Moreover, the numbers of $CD4^+CD25^+Foxp3^+$ T cells, which may be regulatory in nature, remained low in the treated group on days 15 and 20 posttransplantation. Treatment with IgG_1 or IgG_{2a} chimeric antibodies resulted in a similar decline in the percentage of splenic $CD4^+CD25^+Foxp3^+$ cells as that seen with FR70 therapy at all time points tested after transplantation. **SDC 3** (see **Figure,** http://links.lww.com/TP/A541) shows the percent of $CD4^+CD25^+Foxp3^+$ T cells present in the spleens of individual mice from each treatment group. These data show that splenic Tregs do not correlate with graft outcome.

To determine the effects of CD70-specific therapy on the numbers of graft-infiltrating $Foxp3^+$ cells, frozen sections of cardiac allografts were stained with a Foxp3-specific anti-

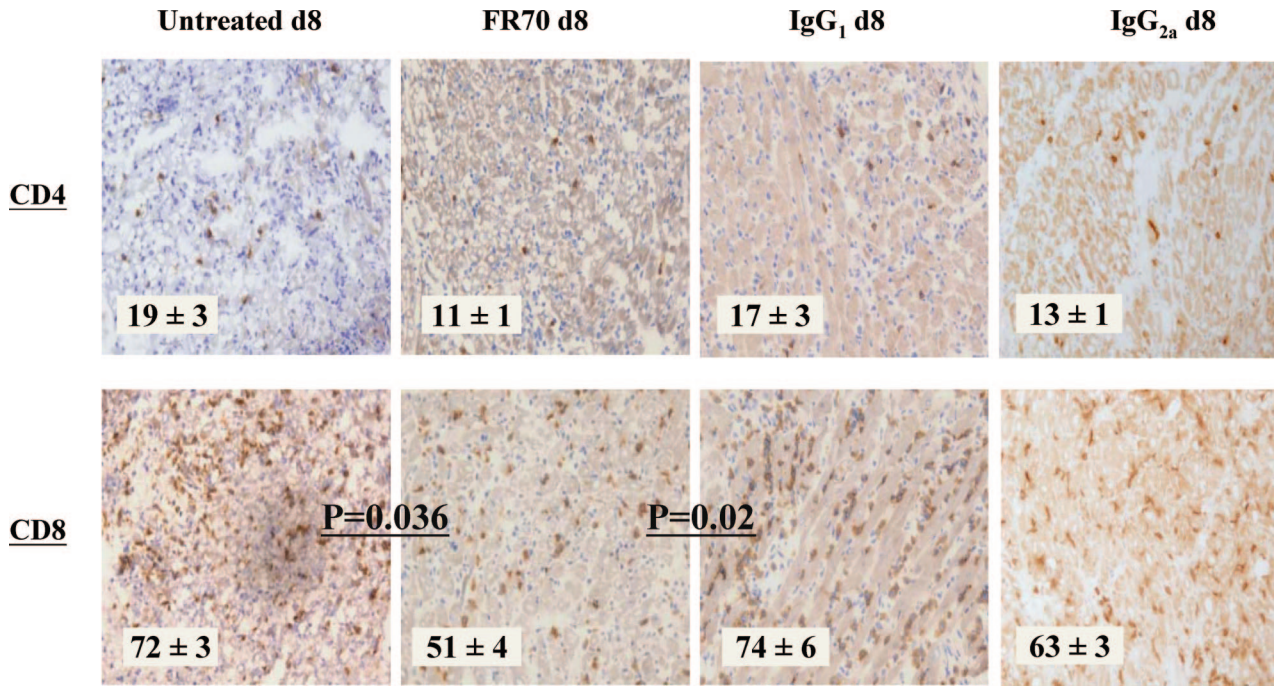

FIGURE 2. Graft-infiltrating T cells are reduced after FR70 antibody therapy. Immunohistochemical staining was carried out on snap-frozen sections of heart grafts using CD4- and CD8-specific antibodies and was developed using the diamino-benzedine system. Each brown precipitate identifies a single $CD4^+$ or $CD8^+$ T cell (magnification ×200).

TABLE 1. Numbers of graft infiltrating $CD4^+$ and $CD8^+$ T cells

	Untreated	FR70 WT			IgG_1 variant		IgG_{2a} variant		
	d8 (n=5)	d8 (n=4)	d15 (n=3)	d18–20 (n=5)	d8 (n=3)	d13–15 (n=4)	d8 (n=4)	d15 (n=3)	d18–20 (n=4)
CD4	23[a]	8	14	10	12	25	12	14	15
	15	11	13	10	19	29	15	21	13
	11	11	13	31	20	14	14	11	13
	30	13		18		26	11		20
	15			11					
CD4 mean	19[b]	11	13	16	17	24	13	15	15
CD8	76	43	39	23	65	43	71	58	35
	62	56	32	36	86	51	62	59	46
	72	43	27	32	72	42	61	52	63
	71	60		35		40	57		55
	77			64					
CD8 mean	72	51	33	38	74	44	63	56	50

[a] Number of infiltrating $CD4^+$ or $CD8^+$ T cells in the graft of each individual mouse at specific time points posttransplantation in each treatment group.
[b] The mean number of infiltrating T cells for each group.

body, developed with the diamino-benzedine system, and the positive cells were enumerated using light microscopy. No effect was seen on the numbers of graft-infiltrating $Foxp3^+$ cells because both untreated mice and those receiving FR70, IgG_1, or IgG_{2a} antibodies showed a mean of less than 3 $Foxp3^+$ T cells/field of vision at all time points tested after transplantation (n=3–5 mice in each group, data not shown).

DISCUSSION

The primary function of the TNF superfamily of molecules is to regulate cell survival. Blocking the interactions of these molecules with their ligands minimizes the proinflammatory effects of immune cells and reduces autoimmune and inflammatory diseases. The CD27-CD70 pathway is known to enhance IFN-γ, IL-2, and TNF-α production (*18*, *19*) by initiating Th1 differentiation of $CD4^+$ T cells. It is also known to upregulate antiapoptotic molecules such as Bcl-XL to promote survival of effector T cells (*20*).

Using a fully major histocompatibility complex-mismatched, naive heart transplant model, we have shown that an antibody specific for CD70 (FR70) given as a short therapy significantly extends allograft survival. The num-

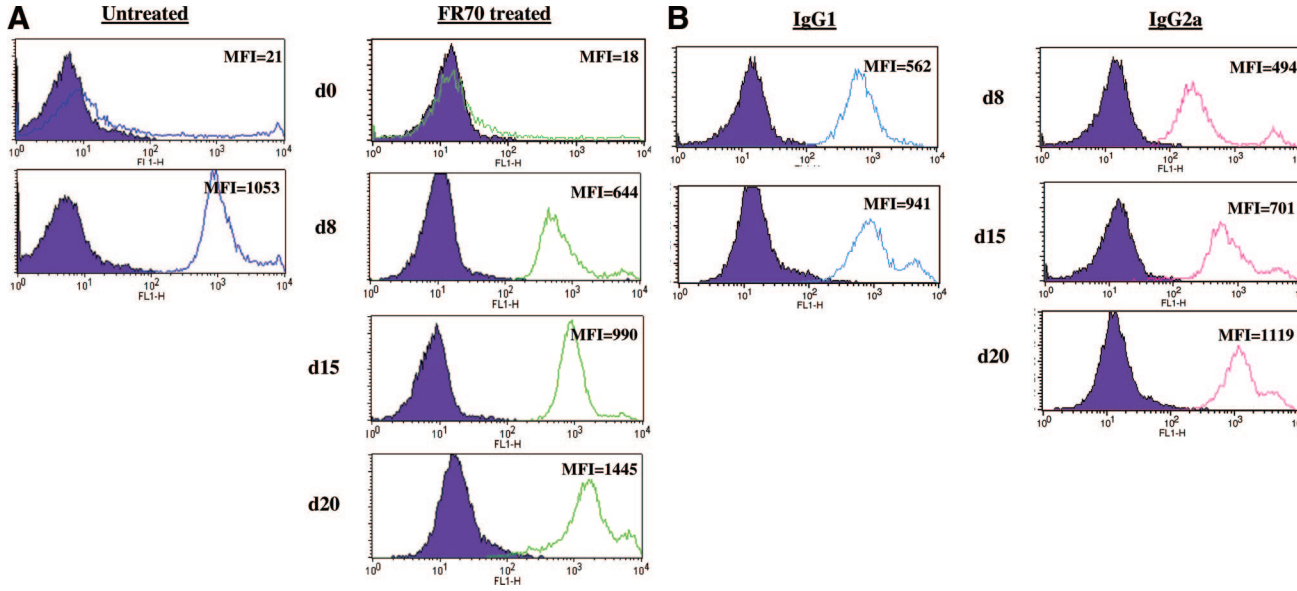

FIGURE 3. FR70 and the chimeric IgG_1 and IgG_{2a} antibody therapies affect the level of donor-specific IgG alloantibodies in the peripheral blood. Recipient serum was analyzed for the presence of IgG alloantibody using donor splenic target cells and FITC-labeled goat anti-mouse IgG antibody. Each peak is from a representative mouse from each group (n=4–9). Geometric mean fluorescent intensity (MFI) is shown for each peak. (A) FR70-treated animals showed marginally lower levels of IgG antibodies at day 8 posttransplantation compared with the untreated control animals. However, a gradual increase in IgG was seen at days 15 and 20 which corresponded with graft rejection. (B) The effect of treatment with IgG_1 and IgG_{2a} antibodies on donor-specific IgG alloantibodies in peripheral blood was similar to that of FR70.

ber of graft-infiltrating $CD4^+$ and $CD8^+$ T cells (day 8 posttransplantation) declined with FR70 treatment and these low numbers were maintained with this therapy at days 15 and 20 posttransplantation (Table 1). Furthermore, FR70 reduced the level of donor-specific IgG antibody in the periphery at day 8; however, it was not able to maintain this and an increase in antibody titers was seen at days 15 and 20, which corresponded to graft rejection (MST=19 days).

We also determined the contribution of the Fc region of the FR70 antibody reagent by exploiting variants composed of the Fab region of the native rat IgG2b FR70 antibody and mouse IgG_1 or IgG_{2a} Fc. The mouse chimeras had identical Fab regions to the WT FR70 antibody but differed in the Fc region. Both chimeric antibodies were able to extend graft survival however; the nondepleting mIgG1 protein resulted in only moderate prolongation of graft survival compared with untreated controls. These data support a contribution of both the blocking of the CD27-CD70 pathway and depletion of CD70 expressing cells to the efficacy of CD70-specific antibody and improved graft survival. It was confirmed that FR70 and the chimeras IgG_1 and IgG_{2a} can block CD70 with equal binding efficacy. In addition, FR70 and IgG_{2a} were able

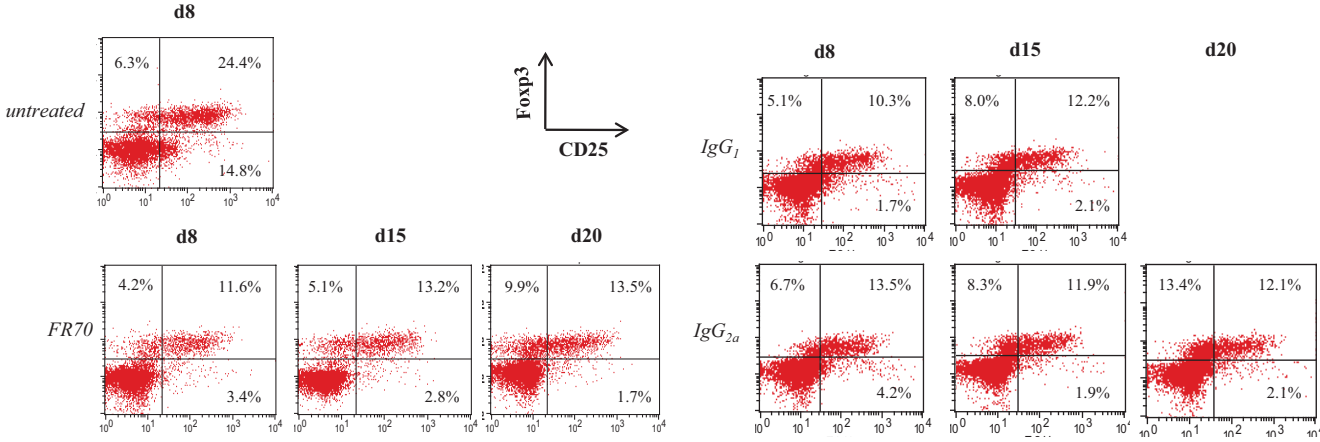

FIGURE 4. Splenic $CD4^+CD25^+$ $Foxp3^+$ Tregs in treated recipients and untreated controls are unaltered by therapy. The percent of splenic $CD4^+CD25^+Foxp3^+$ T regulatory cells (Tregs) in both control and treated mice was determined by antibody staining and flow cytometric analysis. Plots are shown from a representative mouse from each group (n=3–6). The effects of treatment with IgG_1 and IgG_{2a} are also shown. No day 20 data were generated for the IgG_1-treated group due to rejection (median survival time [MST]=14 days).

to fix mouse complement more efficiently than IgG_1, suggesting that FR70 and $mIgG_{2a}$ work at least in part by complement-dependent depletion of target cells, while the dominant mechanism of action of $mIgG_1$ may not be the depletion of target cells. It is well known that high titers of donor-specific antibodies are a significant risk factor in the development of transplant-associated cardiac allograft vasculopathy and chronic rejection (*21–23*) and very few existing therapies show dramatic effects in lowering them (*24*). For instance, CD20-specific antibody, rituximab, is used in the transplant clinic. It successfully removes B cells from the periphery but has to be used in conjunction with antibody removal procedures to maintain minimal circulating alloantibody levels (*25*). Despite inadequate knowledge of the mechanisms responsible for alloantibody-mediated damage to the graft, a decrease in their titer is likely to improve long-term graft survival.

Indeed, the initial drop in IgG alloantibodies at day 8 after treatment is favorable for graft survival however; the return of allo-specific IgG in the peripheral blood by days 15 and 20 is likely to contribute to graft rejection. Moreover, because CD70 is expressed on activated graft-specific $CD4^+$ T cells, CD70-specific therapy could affect alloantibody production by the removal of T-cell help. It has been shown that in addition to binding CD70 expressed by activated T cells, the FR70 antibody binds activated B cells and dendritic cells by virtue of their transient expression of CD70 (*26*). Therefore, there may be direct effects of this treatment on activated B cells that serve as important antigen-presenting cells. Further research is required to determine the indirect effects of CD70-specific antibody on T cells due to its effect on these antigen-presenting cell populations.

In this study, we investigated the presence of regulatory T cells ($CD4^+CD25^+Foxp3^+$ Tregs) in the spleens of recipient mice at various time points after transplantation. Regulatory T cells are believed to play a crucial role in controlling the immune response (*27*), including the recipient's response against transplanted allogeneic tissue (*28*, *29*). Interestingly, the number of splenic Tregs was significantly higher in the untreated transplanted group at day 8 (24.4% of $CD4^+$ $CD25^+$ T cells expressed this phenotype) when compared with FR70-treated recipients (11.6%). Antibody staining showed that this reduced number of double-positive $CD25^+Foxp3^+$ T cells was maintained at days 15 and 20 post-transplantation with a simultaneous increase in $CD25^-Foxp3^+$ T cells at day 20. This may suggest the downregulation of surface CD25 rather than the depletion of $CD25^+$ cells; however, further experiments must be carried out to investigate this possibility. Antibody therapy did not have any effect on graft-infiltrating $Foxp3^+$ cells, which remained low at all time points tested. These data suggest that the presence of Tregs does not correlate with graft outcome.

It has been shown that the CD27-CD70 pathway is required for $CD8^+$ T-cell activation but $CD4^+$ T-cell activation is not affected by blocking this pathway using anti-CD70 antibody (*13*). Our data demonstrate the importance of the CD27-CD70 costimulatory pathway in alloimmune responses because both the depletion of activated T cells expressing CD70 and the blockade of CD70 lead to increased graft survival. We suggest that the depletion of activated T cells is an important mechanism for improving graft survival and the prevention of further T-cell expansion and survival may be an alternative mechanism for the therapeutic effects of CD70-specific antibodies. Further work must be carried out to confirm these proposals. Indeed, CD70-specific antibody, as a monotherapy, has modest effects in achieving graft survival and may be increasingly effective when used in combination with other therapeutic reagents as shown previously (*15*). Early clinical trials with therapeutic anti-CD70 antibodies have been carried out to assess use of this antibody for the treatment of lymphomas (*30*) and cancers (*31*).

We conclude that CD70-specific antibody can significantly prolong graft survival in naive heart transplant recipients and reduce the infiltration of $CD4^+$ and $CD8^+$ T cells into the allograft, thus reducing tissue damage and improving graft survival. Although the IgG_1 variant maintained an ability to prolong graft survival, the Fc region-mediated depleting mechanism of action seems to play a substantial role in the effects of CD70-specific antibody therapy.

MATERIALS AND METHODS

Antibody Therapy

WT CD70-specific antibody (FR70) was purchased from BioXcell (New Hampshire) and was used at 500 μg/dose at day 0 and 250 μg/dose on days 2, 4, and 6 posttransplantation. For the production of IgG_1 and IgG_{2a} chimeric antibodies, FR70 variable regions were sequenced using standard procedures. The FR70 VK region was amplified from the representative sequencing clone by polymerase chain reaction (PCR) to contain an Asc I site and a portion of the osteonectin signal sequence at the 5′-end, and a portion of the murine kappa constant region and an Mfe I site at the 3′-end. The PCR product was purified, cloned into pCR4Blunt-TOPO, and sequence-verified. The FR70 VK plasmid was digested with Asc I and Mfe I, and the VK fragment was then cloned into an expression vector containing the osteonectin signal sequence and the murine kappa constant region to produce the FR70-mKappa expression plasmid.

The FR70 VH region was amplified from the representative sequencing clone by PCR to contain an Afe I site and a portion of the osteonectin signal sequence at the 5′-end, and a portion of the murine IgG1 or IgG2a heavy chain constant region and an Nhe I cloning site at the 3′-end. The PCR products were purified, cloned into pCR4Blunt-TOPO, and sequence-verified. The FR70 VH plasmids were digested with Afe I and Nhe I, and the VH fragments were cloned into an expression vector containing the osteonectin signal sequence and the murine IgG1 or murine IgG2a constant region to produce FR70-mIgG1 or FR70-mIgG2a.

CHO-S cells were transfected with the FR70-mIgG1 plus FR70-mKappa plasmids or the FR70-mIgG2a plus FR70-mKappa plasmids using DMRIE-C (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Stable clones were selected and screened for expression using a murine gamma/murine kappa sandwich ELISA. These chimeric antibodies were also used at the same dose as the WT FR70 antibody.

Mice

BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) at 6 to 8 weeks of age were supplied by Harlan, UK. All mice were housed under standard conditions, and all procedures were carried out in compliance with United Kingdom Home Office regulations.

Heart Transplant Model

In brief, heterotopic, non-life supporting fully vascularized heart transplantation into the abdomen was performed from BALB/c to C57BL/6 mice using previously described methodology (*32*). Graft rejection was classified as a loss of detectable heart beat on palpation.

Detection of Donor-Specific (Alloreactive) Antibodies

Serum collected from the tail vein of C57BL/6 recipients was analyzed for the presence of donor BALB/c-specific IgG antibodies. BALB/c splenocytes were incubated with PBS containing 2% BSA and 5% normal goat serum and rat anti-mouse CD16/CD32 Fc block (0.5 μg/10^6 cells) for 20 min on ice to prevent nonspecific binding. Hamster anti-mouse CD3-PE (1 μg/10^6 cells) was then added directly to the cells to enable subsequent gating on $CD3^+$ T cells. After a further 20-min incubation, cells were washed, and recipient or control (naive syngeneic) serum was added (5 μL/well, dilution 1:10) for 20 min. This was followed by another wash step and the addition of goat anti-mouse IgG-FITC (Sigma, UK) for 20 min on ice. Cells were washed twice in PBS/2% BSA, resuspended, and analyzed using a BD FACScan flow cytometer and CellQuest software.

Foxp3 Staining of Splenocytes

Recipient splenocytes were harvested and blocked with 10% normal mouse serum and rat anti-mouse CD16/CD32—Fc block (0.5 μg/10^6 cells) in PBS/2% FCS for 10 min on ice. CD4-PE-Cy5 and CD25-PE (BD Biosciences, Oxford, UK) were added to cells for 30 min on ice followed by a wash with PBS/2%BSA and incubation for 1 hr with fixation/permeabilization buffer (eBioscience, Hatfield, UK). Permeabilization buffer was used to wash cells and anti-Foxp3-FITC antibody (eBioscience) or FITC-conjugated IgG_{2a} isotype control was then added and incubated in the dark at 4°C for 30 min. Cells were washed with Permeabilization Buffer and resuspended in PBS/2% FCS before flow cytometric analysis using a BD FACScan and CellQuest software.

FR70 Preblocking

P815-CD70 transfectants (5×10^5/well) were cultured in 96-flat well plates and preblocked by incubation with FR70 antibody or the chimeric FR70 antibodies IgG_1 or IgG_{2a} (10 μg/2×10^5 cells) for 20 min on ice. Cells were washed twice with PBS/2%FCS (200 μL/well). Residual CD70 expression was then detected by incubation with the FITC-conjugated FR70 antibody (10 μg/well; BD Bioscience) for a further 15 min on ice. Cells were washed twice in PBS (200 μL/well) and fixed in 1% paraformaldehyde (200 μL/well) before flow cytometric analysis using an FACS Calibur and Cellquest software (BD).

Complement Fixation Assay

P815-CD70 transfectants (2×10^5 cells/well) were cultured in 96-flat well plates in Roswell Park Memorial Institute medium containing freshly isolated C57BL/6 normal mouse serum (2%) and the FR70 or chimeric IgG_1 or IgG_{2a} antibody (10 μg/mL). Control cells were cultured in the absence of antibody. After incubation for 2 hr at 37°C in 5%CO_2, cells were harvested and analyzed for C1q deposition. Cells were preblocked in PBS/2%BSA for 10 min on ice. A FITC-conjugated rabbit antibody to human C1q (1 μg/stain, DAKO, which cross-reacts with mouse antigens) was then added to the cells and left for 20 min on ice. Cells were washed twice in PBS/2%BSA (200 μL/well) and fixed in 1% paraformaldehyde (200 μL/well) before flow cytometric analysis using a FACS Calibur flow cytometer and Cellquest software (BD).

Detection of $CD4^+$ and $CD8^+$ T Cells in Frozen Heart Sections

Transverse sections of heart grafts were snap-frozen in liquid nitrogen, sectioned at 5 μm using a −20°C cryostat, and left to air dry overnight. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS for 10 min. Sections were washed with PBS and incubated for 30 min with Block Solution (PBS/0.05% Tween-20, 3% bovine serum albumin [BSA] and 10% normal goat serum). Purified rat anti-mouse CD4 or CD8 (BD Biosciences) diluted 1:25 in Block Solution was added and kept in a humidified chamber for 1 hr. After washing, biotinylated polyclonal anti-rat Ig (BD Biosciences) diluted 1:100 in PBS was added for 1 hr and then incubated with avidin and biotinylated horseradish peroxidase Complex system (Vector Laboratories, Peterborough, UK) for 40 min and finally diamino-benzedine. On the appearance of brown precipitates, sections were washed with distilled water, counter stained with hematoxylin, dehydrated, cleared in xylene, and mounted in nonaqueous mounting medium. Ten fields of vision at ×400 magnification were observed and positive cells were enumerated by a blinded investigator using a Light microscope and LUCIA software (version 4.6 Nikon).

Statistical Analysis

Survival data were analyzed using the Kaplan-Meier method, and the log-rank test was used to verify the significance of the difference in survival between groups. For all other data, statistical analysis was performed with an unpaired Student *t* test. A value of *P* less than or equal to 0.05 was considered statistically significant.

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