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Intermittent Antibody-based Combination Therapy Removes Allo-antibodies and Achieves Indefinite Heart Transplant Survival in Pre-sensitised Recipients

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Abstract

Background—It is well established that primed/memory T cells play a critical role in heart transplant rejection. This contributes to the challenges faced in the transplant clinic since current treatments which are efficient in controlling naïve T cell alloresponses have limited efficacy on primed T cell responders.

Methods—Fully MHC mismatched heart transplantation was performed from BALB/c to C57BL/6 mice pre-sensitised with BALB/c splenocytes 14 days pre-transplantation. A combination therapy comprising CD70-, CD154- and CD8-specific antibodies was administered at day 0 and 4 post-transplantation with Rapamycin on days 0–4.

Results—The antibody combination therapy extended heart transplant survival in pre-sensitised recipients from MST 8 days (median survival time) to MST 78 days. A decrease in the number of splenic IFN- γ secreting cells measured by ELISpot assay was seen in the treated group compared to the untreated controls. However, graft-infiltrating CD8⁺ and CD4⁺ T cells persisted despite treatment and the number of intra-graft CD4⁺ T cells increased at day 30 post-transplantation. When an additional “rescue therapy” comprising the same antibodies was re-administered at days 30, 60 and 90 post-transplantation, T cell infiltration was reduced and indefinite graft survival was observed. Furthermore, rescue therapy resulted in gradual decrease in titre and, by day 90 post-transplantation the complete loss of the pre-existing, donor-specific antibodies.

Conclusion—We conclude that our antibody combination therapy extends allograft survival in pre-sensitised recipients. When combined with intermittent antibody-mediated rescue therapy, this results in indefinite allograft survival and a loss of the pre-existing, donor-specific antibodies from the circulation.

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Keywords

pre-sensitised; heart; transplantation; antibody; T cell

Introduction

It is increasingly evident that primed/memory T cells play a major role in the immune response of a transplant recipient to allogeneic tissue (1) (2) (3) (4). Transplant patients who have had a previous transplant and thus have pre-sensitised (primed) immune cells have a higher risk of rejecting their grafts. Furthermore, a proportion of memory T cells generated in response to an infection can cross-react with the allogeneic graft and cause rejection (5) (6). Notably, such primed cross-reactive T cells are resistant to most treatments which are effective in controlling naive T cell responders (1) (3). Thus primed alloreactive T cells can destroy transplanted tissue despite therapy and represent a major obstacle for a successful long-term outcome in transplantation (7) (8). The development of new treatments, effective in the control of memory T cells is a pressing therapeutic challenge.

Therapeutic antibodies require only intermittent administration due to their long half-life and prolonged effects. These characteristics and their specificity of action minimise treatment side effects. We have developed a novel therapeutic strategy based on the premise that by increasing the quantity of antibody bound to selected target T cells a threshold will be reached that will result in their efficient depletion. For this purpose, we have used a CD8-specific antibody at a sub-optimal dose, which as a mono-therapy has only modest depleting effects on CD8⁺ T cells (8). However, when used in combination with other antibody reagents, the CD8-specific antibody functions to increase the total quantity of antibody bound to the target cells to a level necessary for their Fc-mediated depletion. Importantly, this approach, which we termed multi-hit therapy (MHT), showed striking effects in a challenging pre-sensitised skin transplant model (8). This is a significant finding since no other short-term treatment has been able to contain the responses of primed T cell responders (3) (7).

Although the efficacy of the multi-hit therapy has been demonstrated in a pre-sensitised skin transplant model (8), it is imperative that the effects of this therapy in a pre-sensitised, MHC mismatched allogeneic model are determined. Unlike skin grafts, heart grafts are vascularised immediately, resulting in the rapid response of the immune system, which is particularly aggressive in pre-sensitised recipients. Furthermore, the presence of pre-existing graft-specific antibodies are an additional challenge as these play a prominent role in acute and chronic rejection (9) (10). The pre-sensitised heart transplant model provides a unique opportunity to study the effects of novel therapies in a clinically relevant setting.

MATERIALS AND METHODS

Antibody Combination Therapy

Anti-mouse CD154-specific antibody MR1 and CD8-specific antibody YTS169.4.2.1 (BioXcell, USA) were used at 250µg/dose and 5µg/dose respectively. CD70-specific antibody FR70 (Hideo Yagita and BioXcell, USA) was used at 500µg/dose. Immediately prior to use antibodies were pooled and administered together intra-peritoneally (i.p.). Rapamycin (LC Laboratories, USA) prepared as a suspension in carboxymethylcellulose (CMC; Sigma, UK) was used at 60µg/dose i.p.

Pre-sensitised heart transplant model

BALB/c (H-2^d), C57BL/6 (H-2^b) and CBA females at 6-8 weeks of age were supplied by Harlan UK Ltd. Animals were housed under standard conditions and all procedures were carried out in accordance with United Kingdom Home Office regulations. Recipient C57BL/6 (H-2^b) mice were primed with 10⁷ donor BALB/c splenocytes i.p. 2 weeks prior to transplantation. Heterotopic, non-life supporting fully vascularised heart transplantation into the abdomen was performed using previously described methodology (Niimi, 2001). Rejection was defined as complete loss of palpable heart beat.

Detection of donor-specific (alloreactive) antibodies

Serum collected from the tail vein of heart transplant recipients (C57BL/6) was analysed for the presence of donor (BALB/c)-specific IgG antibodies. BALB/c splenocytes (donor strain) were incubated with PBS/2%BSA/5% normal goat serum and rat anti-mouse CD16/CD32 - Fc block (0.5 µg/10⁶ cells) for 20 minutes on ice to prevent non-specific binding. Hamster anti-mouse CD3-PE (BD, clone 145-2C11; 1µg/10⁶ cells) was used to gate on CD3⁺ T cells. After washing in PBS/2%BSA, recipient or control (naive syngeneic) serum was added (5 µl/well, dilution 1:10) for 20 minutes followed by another wash and the addition of goat anti-mouse IgG-FITC (Sigma, UK) for 20 minutes on ice. Cells were washed twice in PBS/2%BSA, resuspended and analysed using a BD FACScan flow cytometer and CellQuest software.

ELISpot assay

96 well Multiscreen-IP (Millipore) plates were coated with 5µg/ml rat anti-mouse IFN-γ capture antibody (R&D Systems, UK) in sterile PBS and left overnight at 4°C. Remaining binding sites were blocked by the addition of 100 µl/well PBS/1%BSA for 2 h at room temperature (RT). After washing with PBS, 2×10⁵/well recipient C57BL/6 splenocytes were cultured for 48 h with 2×10⁵ irradiated (2000 rad) donor BALB/c splenocytes. Concanavalin A type IV (Sigma, UK) was added to separate cell cultures (5 µg/ml) as a positive control. After 48 h, plates were washed with PBS/Tween and biotinylated rat-anti-mouse IFN-γ was added for 2 h at RT. Plates were washed and then incubated with streptavidin alkaline phosphatase for 1 hour at RT. After a final wash, cytokine bound to the plate was visualised by the addition of BCIP/NBT solution (Sigma). Spots from triplicate wells were enumerated using an ELISpot plate reader.

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/10⁶ cells) in 2%FCS for 10 minutes. Splenocytes were incubated with CD4-PE-Cy5 and CD25-PE (BD Biosciences, UK) for 30 minutes on ice followed by a wash with PBS/2%BSA and incubation with Fixation/Permeabilisation buffer (eBioscience, USA) for 1 h. Cells were washed once using Permeabilisation buffer and incubated with anti-Foxp3 FITC antibody (eBioscience) or FITC-conjugated IgG_{2a} isotype control (BD Biosciences) in Permeabilization Buffer at 4°C for 30 minutes in the dark. Cells were then washed with Permeabilisation Buffer and resuspended in PBS/2%FCS before flow cytometric analysis as described in figure legends (BD FACScan with CellQuest software).

Preparation of frozen tissue sections

Transversely-cut heart tissue was snap frozen in liquid nitrogen and sectioned at 5µm using a -20°C cryostat microtome. Sections were transferred to glass microscope slides and allowed to air dry for 30 minutes before immersing in ice-cold acetone for fixation for 10 minutes.

Detection of CD4⁺, CD8⁺ T cells in frozen heart sections

Endogenous peroxidase activity was blocked using 0.3% H₂O₂ in PBS for 10 minutes at RT. Sections were washed with PBS and non-specific binding sites blocked for 30 minutes at RT with Block Solution (PBS/0.05% Tween-20 containing 3% bovine serum albumin (BSA) and 10% normal goat serum). Purified rat-anti-mouse CD4 or purified rat-anti-mouse CD8 antibody (BD Biosciences, UK) diluted 1:25 in Block Solution was added and slides were kept in a humidified chamber for 1 h. After washing, biotinylated polyclonal anti-rat Ig 1:100 in PBS (BD Biosciences) was added for 1 h then incubated with Avidin and Biotinylated horseradish peroxidase Complex system (Vector Laboratories, USA) for 40 minutes followed by incubation with diaminobenzidine (DAB). Upon appearance of brown precipitates sections were washed in double distilled water. Sections were counter stained with haematoxylin, dehydrated, cleared in xylene and mounted using non-aqueous mounting medium. Ten fields of vision at x 400 magnification were observed and positive cells were enumerated by a blinded investigator using a Light microscope and LUCIA software (version 4.6 Nikon).

Preparation of formalin-fixed, paraffin wax-embedded sections

Transverse-sections of heart tissue were harvested into 4% formalin before being processed for paraffin wax embedding. Once embedded, tissue sections were cut at 2µm using a sledge microtome (Leitz, Germany). Cut sections were collected onto standard microscope slides and allowed to air dry before placing in a 65°C chamber to soften the wax. Prior to staining, sections were dewaxed in Xylene and rehydrated by immersing in an ethanol gradient (100%, 90%, 70%) and finally in water.

Detection of Foxp3 cells in paraffin wax-embedded tissue sections

Hydrated paraffin wax sections were boiled in preheated Antigen Retrieval Buffer (10mM sodium citrate, 0.05% Tween-20, pH6.0 in distilled water) for 10 minutes. After cooling for 10 minutes, slides were transferred to PBS and stained using purified rat-anti-mouse Foxp3 antibody (eBioscience, USA) 1:100 as primary antibody and staining was completed by following the protocol described previously for detection of CD4⁺ and CD8⁺ T cells in frozen sections.

Elastin-Van Geison stain

Hydrated wax sections were incubated in Miller's Elastin stain (Raymond A Lamb) for 2 h at RT. After washing for 2 minutes in distilled water, slides were differentiated in methanol for 10-20 seconds, washed again in distilled water and placed in Van Geison stain (picric acid, 1% acid fuchsin) for 5 minutes. Sections were rapidly rinsed in distilled water, dehydrated through an ethanol gradient (70%, 90% and 100%), cleared in Xylene and mounted in DPX mounting medium (VWR, UK). Slides were analysed using standard light microscopy. Elastin fibres appeared black, collagen pink and cytoplasm, muscle and red blood cells yellow. Microscope software (Lucia version 4.6) was used to measure the internal and external diameters of vessels to calculate percentage occlusion. A minimum of 10 vessels were measured per allograft and at each time point n=3-5 mice were sacrificed for analysis. Statistical analysis is therefore shown for a minimum of 30 vessels at each time point.

Statistical analysis

Survival data were analysed using the Kaplan-Meier method with the log-rank test used to verify the significance of the difference in survival between groups. For all other data statistical analysis in each independent experiment was performed with an unpaired Student's t-test. A value of P < 0.05 was considered statistically significant.

RESULTS

Effects of Treatment on Graft Survival in Pre-sensitised Recipients

Recipient mice, C57BL/6 (H-2^b) were pre-sensitised with fully MHC mismatched BALB/c (H-2^d) spleen cells 2 weeks prior to transplantation with BALB/c hearts. A multi-hit therapy (MHT) consisting of CD154- (MR1), CD70- (FR70) and CD8-specific (YTS) antibodies was administered i.p. on Day 0 (transplantation) and Day +4. Rapamycin was injected i.p. on Days 0-4. Control, untreated mice were given PBS. The combination therapy (MHT) resulted in a striking prolongation of graft survival (median survival time - MST = 78 days, n=8) compared to untreated controls (MST = 8 days, n=9), which is a highly significant result ($p<0.0001$) particularly for pre-sensitised recipients of fully allogeneic grafts (Figure 1A).

We investigated the contribution of the anti-CD70 antibody component of the combination therapy to treatment efficacy by omitting anti-CD70 Ab (FR70) from the combination, while MR1, YTS and rapamycin were administered using the standard dosing protocol. Prolonged graft survival was maintained (MST = 87 days, n=4 vs MST = 8 days, n=4 in untreated controls) in the absence of anti-CD70 antibody, however, histological analysis showed extensive damage to the transplanted heart tissue in this treatment group compared with MHT group (data not shown). These data indicate that the full combination therapy is necessary to preserve optimal graft structure and function.

Frequency of IFN- γ producing donor-specific T cells

Graft-specific T cell responders can be enumerated ex vivo using ELISpot assay where each spot in a well is representative of a single cytokine secreting cell. This is particularly informative in the pre-sensitised setting where the number of cytokine secreting cells is high before treatment administration (Figure 1B).

Our combination therapy (MHT) resulted in a significant decrease ($p<0.05$) in IFN- γ producing T cells at days 10, 20 and 30 post-transplantation compared to untreated controls (Figure 1B). Positive control wells (cells stimulated with Concanavalin A) had >50 spots/well, whereas the response to third party stimulators (CBA splenocytes) was <10 spots/well (data not shown).

Evidence for priming of animals is indicated by the presence of high titre alloantibody prior to transplantation (MFI 452, Figure 4) compared with naïve mice (MFI = 21, data not shown). To further confirm that the mice were primed ELISpot assays were carried out using splenocytes from sensitised and naïve untreated non-transplanted animals to determine the T cell response to donor antigen during 48 h culture. Sensitised animals showed a significantly higher IFN- γ response with approximately 34spots/ 2×10^5 splenocytes compared with <5spots/ 2×10^5 splenocytes for naïve control animals (data not shown).

Intra-graft T cells

Histological analysis of grafts harvested at various time points post-transplantation showed that the number of graft-infiltrating CD4⁺ and CD8⁺ T cells remained relatively high in treated recipients and was very similar to that of untreated controls at day 10 (Figure 2). Furthermore, at day 30 post-transplantation the number of CD4⁺ T cells increased significantly ($p<0.05$) relative to day 10 levels (Figure 2). Although this correlated with a significant increase ($p<0.05$) in Foxp3⁺ T cells (Tregs), graft rejection in treated recipients occurred (MST = 78 days). The latter observation suggests that the intra-graft immune response is dominated by effector T cells.

Rescue therapy

MHT therapy prolonged cardiac graft survival in treated pre-sensitised recipients, however, rejection did eventually occur and a “rescue therapy” was therefore introduced. This rescue therapy comprised the antibody combination alone (without rapamycin) administered i.p. on days 30 and 60 post-transplantation. Interestingly, this therapeutic approach resulted in indefinite survival (>110 days) of 8 out of 9 grafts (Figure 3A). We compared immuno-histochemical staining for CD4⁺, CD8⁺ and Foxp3⁺ T cells at day 60 (30 days after 1st rescue treatment) and day 90 (30 days post 2nd rescue treatment) of grafts from recipients that were treated with rescue therapy vs. standard MHT treatment. As shown in Figure 3B, rescue therapy resulted in a decrease in the number of infiltrating CD4⁺ and CD8⁺ T cells compared to the number detected in standard treatment group, although the difference did not reach statistical significance. However, rescue therapy appeared to preserve gross tissue structure in paraffin-embedded sections (data not shown). Interestingly, the number of Foxp3⁺ Tregs increased marginally in the rescue therapy group (Figure 3B).

CD25⁺/Foxp3⁺ T cells

Pre-sensitised heart transplant recipients, treated and controls, were sacrificed at various time points post-transplantation. Spleens were harvested and splenocytes isolated and analysed for the presence of CD4⁺/CD25⁺/Foxp3⁺ Tregs by antibody staining and flow cytometric analysis. On day 10 post-transplantation the percentage of Tregs with CD25⁺/Foxp3⁺ phenotype were similar (16.7-22.2%, the dot-plot for one mouse representative of each group is shown) in both treated and control mice (Figure 3C). Interestingly, the numbers of Tregs decreased progressively in the treated group as seen at day 20 and 30 post-transplantation. Subsequently, in the MHT treated group the percent of Treg in the CD4 gate increased at day 60, which coincided with the occurrence of graft loss in this group. In contrast, the numbers of CD4⁺/CD25⁺/Foxp3⁺ Tregs remained low at d60 in the group that received rescue therapy compared to those treated in the absence of rescue treatment ($p < 0.05$) (Figure 3C). Table 1 shows the data for each individual mouse in the untreated and treated groups at each time point analysed and the mean percentage of splenic CD4⁺CD25⁺Foxp3⁺ Tregs at each time point.

Donor-specific antibody levels

Sera from pre-sensitised recipients were obtained from tail bleed samples at various time points post-transplantation and analysed for the presence of donor-specific antibodies. High levels of donor-specific IgG antibodies were detected pre-transplantation indicating efficient pre-sensitisation (Figure 4A). Following transplantation (day 10) the level of donor-specific antibodies increased further in untreated mice. In contrast, MHT treatment resulted in a decrease in the level of IgG detected in the sera derived from peripheral blood (Figure 4A). Allospecific IgG antibody was measured in serum serially diluted at 1:10, 1:50 and 1:250. In control, untreated mice, IgG alloantibody titre was > 1:250 however in treated animals the titre was 1:10 as no IgG alloantibody was detected at dilutions greater than this (data not shown).

This decrease in antibody level was more pronounced on day 30. However, on day 60 antibody levels increased again (Figure 4B). Notably, rescue therapy administered at d30 and d60 resulted in a reduction of donor-specific IgG in the sera harvested on day 90 post-transplantation (Figure 4B). The high levels of donor-specific antibody ultimately correlated with graft rejection.

Occlusion of intra-graft blood vessels

Histological analysis of heart allografts using Elastin–van Gieson’s stain showed that in pre-sensitised, untreated recipients the majority of blood vessels were either completely occluded or showed intimal thickening by day 10 post-transplantation (Figure 5A) compared with untransplanted BALB/c heart tissue. In contrast, administration of MHT treatment (standard short-term protocol) prevented severe vessel occlusion with most vessels showing only mild changes. However, therapy did not prevent an increase in the percent of intra-graft blood vessels showing intimal thickening and occlusion over time (Figure 5B). The addition of rescue therapy resulted in a reduction of moderately occluded vessels with concomitant preservation of normal, healthy looking blood vessels. Nevertheless, the rescue therapy was unable to reduce the percent of severely occluded vessels (Figure 5B).

DISCUSSION

In a pre-sensitised cardiac transplant model we have shown that a short-term antibody based combination therapy (CD154-, CD70-, CD8-specific Abs plus rapamycin) significantly extends allograft survival. However, this therapy failed to prevent T cells infiltrating the graft and their numbers increased over time. This gradual increase in graft-infiltrating T cells was accompanied by delayed graft loss, thus an intermittent rescue therapy comprising our combination of antibodies (without rapamycin) was administered. Importantly, this rescue therapy reduced intra-graft T cell infiltration and achieved indefinite allograft survival.

In this study we have clearly shown that our antibody-based combination therapy can be used successfully at the later stages post-transplantation, as an intermittent, rescue therapy to extend graft survival and remove circulating allo-antibodies. These findings have been obtained using a highly stringent, fully MHC mismatched, pre-sensitised heart transplant model. This extends the potential use of antibody-based therapies which are currently used in the clinic early post-transplantation, as a part of induction therapy.

We have based the protocol of the intermittent, rescue therapy on the histological analysis of the graft tissue, more specifically the observation that the number of intra-graft CD4⁺ T cells increases approximately 20-30 days post-transplantation. This coincides with the approximate in vivo half-life of an IgG antibody in mouse (11) and man (12). Thus the data suggest a requirement for the continued presence of therapeutic antibodies in the circulation to maintain graft survival. Furthermore, our data suggest that in the pre-sensitised recipient a short-term therapy which appears highly effective in ensuring prolonged allograft survival in the absence of continuous immunosuppression fails to achieve transplant tolerance. Consequently, maintenance (rescue) therapy is needed to maintain graft survival but this can be intermittent if an antibody-based therapy is used as the key component rather than continuous non-selective immunosuppression.

The presence of high titre donor-specific antibodies (alloantibodies) is a significant risk factor for the development of transplant-associated cardiac allograft vasculopathy (TxCAV – chronic rejection) (9) (10) (13). Although the precise mechanisms responsible for alloantibody-mediated damage of the graft remain unclear, the removal of alloantibodies, or decrease in their titre, is likely to improve long-term transplant survival. Nevertheless, few existing therapies have effects on the levels of circulating allo-antibodies (14) (15) (16). This includes therapies that target B cells, such as anti-CD20 (Rituximab) Ab, which successfully removes B cells but on its own does not change the level of donor-specific antibodies (17). Our findings are intriguing as they demonstrate the possibility of removing alloantibodies from the circulation using an antibody combination therapy that is selective and should not affect the function of the immune system as a whole.

CD154-specific antibody blocks the interaction between CD154 on activated T cells and CD40 on antigen presenting cells, such as B cells (18) (19). We have previously shown that this delays isotype switching (20). Furthermore, as both CD154 and CD70 molecules are expressed on the surface of the activated graft-specific T cells, our combination therapy could exert its effects on alloantibodies via removal of T cell help. However, this mechanism does not explain removal of pre-formed circulating antibodies. As activated B cells express CD70 on their surface, it is feasible that CD70-specific antibody targets activated B cells directly. This could stop further production of graft-specific antibodies, whereas pre-existing antibodies could be adsorbed by the graft and thus removed from the circulation.

The receptor, CD27 is constitutively expressed on naïve T cells, B cells and NK cells while its ligand CD70 is expressed on both activated T and B cells. In terms of B cell development and differentiation the CD27/CD70 pathway is important in stimulating B cell activation, plasma cell formation and antibody production (24). Thus disrupting this pathway using an anti-CD70 antibody could have direct consequential effects on antibody-producing B cell differentiation in addition to its effects on activated T cells. The reduction in IgG titres could be explained by two potential mechanisms. Circulating antibody may be sequestered into the graft and not replenished by activated B cells/short-lived plasmablasts. This could be due to the direct removal of activated B cells and subsequent plasmablasts due to their depletion upon transient expression of CD70. The therapy may also indirectly affect T-dependent B cell responses due to the effects of therapy on activated CD4⁺ T cells. Although plasma cells are not targeted by MHT, these long-lived cells express CD27 and the CD27/CD70 pathway may play a critical role in their function and survival. Further work must be carried out to investigate these possibilities.

Despite the impressive ability of the intermittent antibody therapy to control rejection and achieve indefinite graft survival, we observed persistent infiltration of the graft with CD4⁺ and CD8⁺ T cells. This was accompanied by a slow but progressive narrowing of the intra-graft blood vessels and ultimately vessel occlusion similar to chronic rejection or transplant coronary artery disease (TxCAD) in clinical transplantation.

We have shown that the absence of donor-specific antibody in the circulation in long term surviving grafts receiving Rescue Therapy did not prevent the development of CAV. This may reflect an antibody-independent process in our model or sequestration of alloantibody in the graft has removed it from the circulation and contributed to the development of CAV. Also, once the process of CAV was established, removing T cells from the graft with Rescue Therapy did not reverse the process and the development of CAV was progressive. No significant reduction was seen in the degree of CAV at day 60 with and without Rescue Therapy and it was observed at day 90-110 CAV. Therefore, CAV is irreversible and MHT does not reduce it but delays its progression.

Regulatory T cells (Tregs) are thought to play a crucial role in controlling the immune response (21), including the recipient's response against transplanted allogeneic tissue (22) (23). In this study we have investigated the presence of intra-graft Foxp3⁺ T cells and Tregs (CD4⁺CD25⁺Foxp3⁺ T cells) in the spleens of recipient mice at various time points post-transplantation. Pre-sensitised recipient mice had a relatively high proportion (approximately 20% of CD4⁺ T cells) of splenic Tregs at the time of transplantation. Interestingly, transplanted recipients treated with combination therapy showed a moderate decrease in this population, which remained static in control, untreated mice. This could suggest that the combination therapy does not result in an increase in splenic Tregs and that prolonged allograft survival is not directly linked with changes in the Treg population.

Analysis of the intra-graft Treg population revealed few Tregs at day 10 post-transplantation in both treated and untreated recipients. The combination therapy resulted in an increase in Tregs at day 30 post-transplantation. However, at later time points (days 60-100) the numbers of intra-graft Tregs in recipients treated with the standard short-term MHT and those treated with MHT and intermittent rescue therapy were similar. There was no correlation between graft survival and the numbers of intra-graft Tregs detected. Nevertheless, in this study we have not analysed the regulatory capacity of the Tregs present and it is possible that therapy causes a change in Tregs function rather than number and this contributes to prolonged allograft survival in pre-sensitised heart transplant recipients.

To assess the importance of Rapamycin to the MHT treatment, experiments were carried out in the absence of Rapamycin. Graft survival in the absence of Rapamycin with the antibody combination therapy resulted in a median survival time of 9 days (n=12 mice) which was not significantly different from untreated controls (MST = 8 days, n=9). This clearly demonstrates the importance of Rapamycin to the MHT. It is given on days 0-4 post-transplantation and is thought to be critical to restrict expansion of the activated responder T cell pool to a magnitude that can be controlled by MHT to prevent allograft rejection.

This study has therefore identified an antibody-based combination therapy that, when used with intermittent rescue therapy, results in indefinite cardiac graft survival in pre-sensitised recipients.

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REFERENCES

1. Adams AB, Williams MA, Jones TR, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest.* 2003; 111(12):1887. [PubMed: 12813024]
2. Lakkis FG, Sayegh MH. Memory T cells: a hurdle to immunologic tolerance. *J Am Soc Nephrol.* 2003; 14(9):2402. [PubMed: 12937320]
3. Valujskikh A, Li XC. Frontiers in nephrology: T cell memory as a barrier to transplant tolerance. *J Am Soc Nephrol.* 2007; 18(8):2252. [PubMed: 17634436]
4. Macedo C, Orkis EA, Popescu I, et al. Contribution of Naive and Memory T-Cell Populations to the Human Alloimmune Response. *Am J Transplant.* 2009
5. Adams AB, Pearson TC, Larsen CP. Heterologous immunity: an overlooked barrier to tolerance. *Immunol Rev.* 2003; 196:147. [PubMed: 14617203]
6. Koehn B, Gangappa S, Miller JD, Ahmed R, Larsen CP. Patients, pathogens, and protective immunity: the relevance of virus-induced alloreactivity in transplantation. *J Immunol.* 2006; 176(5): 2691. [PubMed: 16493023]
7. Wu Z, Wang Y, Gao F, Shen X, Zhai Y, Kupiec-Weglinski JW. Critical role of CD4 help in CD154 blockade-resistant memory CD8 T cell activation and allograft rejection in sensitized recipients. *J Immunol.* 2008; 181(2):1096. [PubMed: 18606661]
8. Greenlaw RE, Gardner NJ, Farrar CA, et al. An antibody combination that targets activated T cells extends graft survival in sensitized recipients. *Am J Transplant.* 2008; 8(11):2272. [PubMed: 18785958]
9. Smith JD, Hamour IM, Banner NR, Rose ML. C4d fixing, luminex binding antibodies - a new tool for prediction of graft failure after heart transplantation. *Am J Transplant.* 2007; 7(12):2809. [PubMed: 17908268]

10. Rose ML, Smith JD. Clinical relevance of complement-fixing antibodies in cardiac transplantation. *Hum Immunol.* 2009; 70(8):605. [PubMed: 19375471]
11. Iwakoshi NN, Markees TG, Turgeon N, et al. Skin allograft maintenance in a new synchimeric model system of tolerance. *J Immunol.* 2001; 167(11):6623. [PubMed: 11714833]
12. Vincenti F, Kirkman R, Light S, et al. Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. *N Engl J Med.* 1998; 338(3):161. [PubMed: 9428817]
13. Wehner J, Morrell CN, Reynolds T, Rodriguez ER, Baldwin WM 3rd. Antibody and complement in transplant vasculopathy. *Circ Res.* 2007; 100(2):191. [PubMed: 17272820]
14. Jurcevic S, Dunn MJ, Crisp S, et al. A new enzyme-linked immunosorbent assay to measure anti-endothelial antibodies after cardiac transplantation demonstrates greater inhibition of antibody formation by tacrolimus compared with cyclosporine. *Transplantation.* 1998; 65(9):1197. [PubMed: 9603168]
15. Mancini D, Pinney S, Burkhoff D, et al. Use of rapamycin slows progression of cardiac transplantation vasculopathy. *Circulation.* 2003; 108(1):48. [PubMed: 12742978]
16. Deuse T, Hoyt G, Koyanagi T, Robbins RC, Schrepfer S. Prevention and inhibition but not reversion of chronic allograft vasculopathy by FK778. *Transplantation.* 2008; 85(6):870. [PubMed: 18360270]
17. Pescovitz MD. Rituximab, an anti-cd20 monoclonal antibody: history and mechanism of action. *Am J Transplant.* 2006; 6(5 Pt 1):859. [PubMed: 16611321]
18. Grewal IS, Flavell RA. The CD40 ligand. At the center of the immune universe? *Immunol Res.* 1997; 16(1):59. [PubMed: 9048208]
19. Howland KC, Ausubel LJ, London CA, Abbas AK. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *J Immunol.* 2000; 164(9):4465. [PubMed: 10779746]
20. Monk NJ, Hargreaves RE, Marsh JE, et al. Fc-dependent depletion of activated T cells occurs through CD40L-specific antibody rather than costimulation blockade. *Nat Med.* 2003; 9(10):1275. [PubMed: 14502279]
21. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol.* 2009; 10(7):689. [PubMed: 19536194]
22. Walsh PT, Taylor DK, Turka LA. Tregs and transplantation tolerance. *J Clin Invest.* 2004; 114(10):1398. [PubMed: 15545987]
23. Waldmann H, Adams E, Fairchild P, Cobbold S. Regulation and privilege in transplantation tolerance. *J Clin Immunol.* 2008; 28(6):716. [PubMed: 18777090]
24. Li XC, Rothstein DM, Sayegh MH. Costimulatory pathways in transplantation: challenges and new developments. *Immunol Reviews.* 2009; 229:271.

Figure 1.

Combination therapy extends survival of fully MHC mismatched heart transplants in pre-sensitised recipients.

A. Recipient C57BL/6 (H-2^b) mice were primed with 10^7 BALB/c (H-2^d) splenocytes i.p. 14 days prior to heterotopic, non-life supporting fully vascularised heart transplantation. Antibody therapy CD154-, CD70-, CD8-specific Abs (MR1 250 μ g/dose, FR70 500 μ g/dose, YTS 5 μ g/dose, respectively) was given on the day of transplantation (day 0) and 4 days later (day 4). Rapamycin (200 μ g/dose) was administered i.p. for 5 days consecutively (days 0-4). Cumulative data of two independent experiments with median survival time - MST is shown. The p-value for the treated vs. the untreated group is $p < 0.0001$.

B. ELISpot assays showing the number of IFN- γ producing, alloreactive T cells. Recipient C57BL/6 splenocytes were cultured with donor BALB/c splenocytes for 48 h before detection of IFN- γ by standard ELISpot assay. Each bar represents the average from 3-4 recipients from two independent experiments. The p-value is shown for comparison between untreated and treated groups. Images below the graph show the number of IFN- γ spots in a representative well where each spot corresponds to one cytokine producing cell.

Figure 2.

Graft-infiltrating T cells persist despite combination therapy.

Immuno-histochemical staining was carried out on snap-frozen sections of heart grafts using CD4- and CD8-specific antibodies. The staining was developed using the DAB system.

Brown precipitate identifies a CD4⁺ or CD8⁺ T cell. Images are at x200 magnification. The p-value is shown for comparison between treated groups at day 10 and day 30.

Figure 3B

Figure 3.

The intermittent combination therapy leads to indefinite heart allograft survival and reduced T cell infiltration.

A. Recipient C57BL/6 (H-2^b) mice were pre-sensitised, transplanted and treated as described in Figure 1 legend. Additional antibody combination treatment (rescue) was administered on days 30, 60 and 90 post-transplantation. Cumulative data of two experiments with MST is shown. The p-value for comparison between untreated and treated groups is $p < 0.0001$.

B. T cell infiltration ($CD4^{+}$ and $CD8^{+}$ T cells and $Foxp3^{+}$ Tregs) of the graft was visualised by staining with specific antibodies and development using the DAB system. Positive cells for each population are seen as brown precipitate. Images are at x200 magnification.

C. $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs in treated recipients and untreated controls. 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. Data shown are for one representative individual from each group (value closest to the mean for that group, $n=3-5$ at each time point) of two independent experiments.

Figure 4.

IgG alloantibodies in treated and untreated heart allograft recipients.

A. Recipient serum was analysed for the presence of IgG alloantibody using donor splenic target cells and FITC-labelled goat anti-mouse IgG antibody. Each peak is generated from individual recipients from two independent experiments ($n = 6$). High levels of IgG were detected pre-transplantation indicating successful priming of the recipient mice. The geometric mean fluorescent intensity (MFI) is shown for each peak. Control filled peaks indicate donor BALB/c splenocytes stained with anti-IgG-FITC antibody in the absence of recipient serum.

B. IgG alloantibodies were analysed at Day 60 and 90 in heart allograft recipients treated with standard short-term therapy (MHT) and MHT with intermittent rescue therapy. Each peak is representative of $n = 4-6$ recipients per group from two experiments.

Figure 5.

Blood vessel occlusion within the heart allograft is reduced in treated recipients.

A. Cardiac vessel occlusion in heart allografts was demonstrated using Elastin–van Gieson’s staining. Representative sections showing the appearance of a healthy vessel, from an untreated transplant recipient and tissue day 10 post-transplantation with therapy are shown.

B. Percentages of cardiac blood vessels affected by intimal thickening/occlusion were calculated in untreated controls and treated allograft recipients. A minimum of 30 vessels were analysed at each time point at x400 magnification by a blinded investigator.

TABLE 1

Percentage of splenic CD4⁺CD25⁺Foxp3⁺ T Cells in each individual C57/BL6 recipient at different time points in treated and untreated groups

	Untreated d10	MHT d10	MHT d20	MHT d30	MHT d60	MHT+R d60
% CD4 ⁺ CD25 ⁺ Foxp3 ⁺ T Cells in spleen	16.7	17.2	12.8	7.9	12.9	10.9
	18.9	19.0	13.6	11.5	16.3	11.9
	19.2	21.5	15.9	13.4	18.3	12.2
	22.2		18.1		20.1	12.5
						15.8
Mean ^a	19.3	19.2	15.1	10.9	16.9	12.7

^a mean percentage of CD4⁺CD25⁺Foxp3⁺ T Cells of each group