DONOR CD4 T CELLS CONTRIBUTE TO CARDIAC ALLOGRAFT VASCULOPATHY BY PROVIDING HELP FOR AUTOANTIBODY PRODUCTION.

Win: Autoantibody and allograft vasculopathy.

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³ Abbreviations: CAV, cardiac allograft vasculopathy.

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ABSTRACT

Background

The development of autoantibody following heart transplantation is increasingly associated with poor graft outcome, but what triggers its development and whether it has a direct causative role in graft rejection is not clear. Here, we study the development of anti-nuclear autoantibody in an established mouse model of heart allograft vasculopathy.

Methods and Results

Humoral vascular changes, including endothelial complement staining, were present in bm12 heart grafts, explanted 50 days after transplantation. Alloantibody was not detectable, but long-lasting autoantibody responses developed in C57BL/6 recipients from the third week following transplantation. No autoantibody was generated if donor CD4 T cells were depleted prior to heart graft retrieval or in recipients that lacked B cell MHC class II expression, indicating that humoral autoimmunity is a consequence of donor CD4 T cell allore cognition of the MHC class II complex on recipient autoreactive B cells. An effector role for autoantibody in graft rejection was confirmed by abrogation of humoral vascular rejection, and attenuation of vasculopathy, in B cell-deficient recipients and by development of vascular obliteration and accelerated rejection in recipients primed for autoantibody prior to transplantation.

Conclusions

Passenger CD4 T cells within heart transplants can contribute to allograft vasculopathy by providing help to recipient B cells for autoantibody generation.

Keywords: allograft vasculopathy; autoantibody; graft-versus-host-disease; allore cognition.
INTRODUCTION

Despite improvements in immunosuppressive therapy, the long-term clinical success of heart transplantation is limited by the development of chronic allograft vasculopathy (CAV) \(^1,2\). Therapeutic strategies that specifically prevent the development of CAV may prove more successful at prolonging graft survival, but their design is hampered by a lack of understanding of the mechanisms responsible for its initiation and progression.

CAV manifests as diffuse vascular intimal hyperplastic lesions, consisting primarily of smooth muscle cells, macrophages, fibroblasts, leukocytes, as well as extracellular matrix formation, which affect the vessels in a concentric fashion \(^3\). Adventitial scarring may additionally cause a fixed restrictive element around the vessel and contribute to the luminal loss \(^4\). A unifying concept has now emerged for how disparate risk factors such as ischaemia-reperfusion injury, infection and metabolic abnormalities such as hypercholesterolemia, glucose intolerance and hypertension promote the progression of CAV. Interferon-\(\gamma\) (IFN-\(\gamma\)), in part through induction of inducible nitric oxide synthetase, is a critical determinant linking impairment in smooth muscle contractility following endothelial injury with later structural changes in CAV \(^5\).

Although its aetiology is multi-factorial, most studies have highlighted that CAV is a culmination of adaptive immune effector responses that are triggered by T cell recognition of alloantigen CAV \(^1,6\); either ‘directly’, as intact alloantigen on the surface of donor APC or ‘indirectly’ as self-restricted allopeptide following processing by host APC \(^7\). T cell responses directed against self-antigens have, however, also become increasingly associated with allograft rejection \(^8-11\). Although often directed against graft-specific antigens, it is unclear whether these autoimmune responses can contribute to graft rejection, because self-restricted autoreactive T cells are unable to bind to peptide autoantigens expressed in the context of donor MHC by graft cells. They may instead provide help
for autoantibody, which, by binding to exposed autoantigen on graft endothelial cells, provides a putative mechanism for stimulating vasculopathy $^{12,13}$. In support, autoantibody responses have been reported following transplantation in rodent models $^{10,14}$ and are associated with early graft failure in human transplant recipients $^{13,15,16}$. A causal link between autoantibody and CAV has, however, not been established definitively.

It is also unclear why transplantation should trigger autoimmunity. Animal studies suggest that T cell alloreognition is required $^{10}$ and that indirect pathway responses alone are sufficient $^{17}$. Most plausibly, host antigen presenting cells (APCs) that have been activated following interaction with allopeptide-specific CD4 T cells break T cell tolerance to autoantigens by co-presenting self-peptides in a stimulatory fashion $^{18}$. Activated autoreactive T cells may then provide help for generating autoantibody. However, the only study to address simultaneously the kinetics of T and B cell reactivity to self and allo-antigens following heart transplantation reported that although autoantibody against cardiac myosin developed after the T cell alloimmune response, it preceded anti-myosin T cell autoimmunity $^{19}$.

Here we study the development of autoantibody in a mouse model of chronic cardiac allograft vasculopathy, to determine how T cell help is provided to autoreactive B cells and to clarify the role of autoantibody in allograft rejection. We report the unexpected finding that donor CD4 T cells, present within the heart graft, provide help to recipient B cells for the generation of autoantibody; autoantibody that in turn contributes to CAV development.
MATERIALS AND METHODS

Animals

B6.H-2^bm12 (bm12), C57BL/6 (H-2^b) [WT (B6); MHC class II-deficient (MHCII^-/-, 20); recombinase-activating gene-2 knockout (RAG2^-/-, 21); μ-chain knock-out (μMT, 22)] mice were bred in-house. CBA/Ca (H-2^k) mice were purchased from Charles River Laboratories, UK. Bm12 were crossed to RAG2^-/- to obtain H-2^bm12 RAG2^-/- mice (bm12RAG2^-/-). Animals were maintained in specific-pathogen-free conditions and all experiments approved by the UK Home Office under the Animal (Scientific Procedures) Act 1986.

Heterotopic cardiac transplantation

Cardiac allografts were transplanted intra-abdominally and rejection defined as complete cessation of palpable myocardial contraction. Grafts were excised at predetermined time points after transplantation, and either stored at -80°C or fixed in 10% buffered formalin. In some experiments, donor mice were lethally irradiated (13Gy) 24 hours before organ retrieval or were injected i.p. with 0.5mg of depleting anti-CD4 mAb (YTS 191.1) on the sixth and fifth day prior to retrieval.

Generation of bone-marrow chimeras

To generate chimeric BCII^+/+ mice, RAG2^-/- mice were sublethally irradiated (2Gy) and 20 hours later reconstituted with 2 x 10^7 BMCs obtained from MHCII^-/- B6 mice, depleted of T cells using anti-CD90 microbeads and Automacs machine separation (Miltenyi Biotec, UK). Control chimeric BCII^+/+ were generated by reconstituting sublethally irradiated RAG2^-/- mice with WT B6 BMCs. Chimerism was confirmed by flow cytometric analysis of peripheral blood lymphocytes 8 weeks after reconstitution.

Recipient immunisation

A 20-mer peptide, corresponding to the disparate, hypervariable α-region of the β-chain of the I-
A^{bm12} antigen (EYWNSQPEFLEQKRAELDTV), was synthesized by standard Fmoc chemistry (Immune Systems, UK; peptide purity 90%). Mice were immunised subcutaneously with 50 μg peptide, emulsified in CFA.

Autoantibody was induced by intraperitoneal injection of 5 x 10^6 splenic bm12 CD4 T cells, purified by Automacs separation, by first eliminating MHC class II positive cells with anti-MHC class II microbeads and then positively selecting CD4 T cells using anti-CD4 microbeads. CD4 T cell purities, assessed by flow cytometry, were > 99%, with no detectable I-A^{bm12}. In certain experiments, purified bm12 CD4 T cells were lysed by three cycles of freeze-thawing.

Autoantibody was passively transferred by intravenous injection of 200 μL heat-inactivated serum (pooled from B6 recipients of bm12 heart grafts) three times weekly for four weeks after heart grafting.

Autoantibody and anti-bm12 alloantibody and determination.

Antinuclear autoantibody responses were determined by HEp-2 indirect immunofluorescence (23, The Binding Site Ltd, UK), by incubation dilutions of test sera on slides coated with HEp-2 cells and detecting bound autoantibody with FITC-conjugated anti-mouse IgG mAb (STAR 70; Serotec). For each slide, five random photomicrographs were taken and scored according to immunofluorescence intensity (1-5, by an observer blinded to the study groups (EMB)).

Alloantibody responses were assayed using flow cytometry, by adding serial dilutions of test sera to 5 x 10^5 bm12 or B6 BM DCs (24) and detecting bound alloantibody with FITC-conjugated anti-mouse IgG mAb (STAR 70). Results were expressed as geometric mean-channel fluorescence against serum dilution. Serum from CBA/Ca (H-2^k) animals immunised with 1.5 x 10^7 bm12 splenocytes emulsified in CFA was used as positive control.
Histopathological examination

Hearts were paraffin-mounted and stained with haematoxylin and eosin (H&E) or van Gieson (EVG). The severity of vasculopathy was scored by a cardiac histopathologist (MG), blinded to the study groups, as follows: 0, no vascular damage; 1, <25% vascular occlusion; 2, 25-50% occlusion; 3, >50% occlusion but with residual lumen; and 4, no identifiable lumen.

Complement C4d deposition was assessed on cryostat sections by an avidin-biotin-peroxidase technique (Vector Laboratories, Inc., USA), using unconjugated rat anti-mouse C4 mAb (16D2; Abcam Inc., USA), and rabbit anti-rat IgG (Abcam) as secondary. 16D2 anti-mouse C4 mAb binds to C4, C4b and C4d, but because C4 is not cell-bound and C4b is short-lived with an in vitro half-life measured in minutes, positive staining represents C4d deposition. Sections were visualised using chromagen 3, 3’-diaminobenzidine (Sigma-Aldrich) and counterstained with Harris’ hematoxylin (BDH, UK). Allograft IgG deposition was similarly detected using biotinylated rabbit anti-mouse IgG (STAR 11B, Serotec).

Statistical analysis

Graft survival was depicted using Kaplan-Meier analysis and groups compared by log-rank. Comparison between groups for severity of vasculopathy and autoantibody scores was performed by Mann-Whitney U test as they were non-Gaussian in distribution (GraphPad Prism, GraftPad Software, Inc. USA).
RESULTS

The development of CAV in MHC class II-mismatched bm12 heart grafts.

The immunological mechanisms responsible for the development of CAV were studied in a well-established mouse model of chronic heart graft rejection \(^{26-28}\), in which the donor bm12 strain differs from the recipient B6 by only 3 amino acid residues in the MHC class II I-A antigen. Bm12 heart allografts were rejected slowly by B6 recipients (Figure 1A), and along with patchy inflammatory infiltrates (Figure 1B) developed progressive intimal thickening and luminal narrowing (Figure 1C), morphologically resembling human cardiac allograft vasculopathy. Syngeneic grafts and bm12 grafts transplanted into MHC class II-deficient (MHCII\(^{-/-}\)) recipients survived indefinitely with no evidence of CAV or parenchymal damage (Figure 1).

Rejecting bm12 heart grafts showed additional histological features (Figure 2) considered pathognomic of humoral vascular rejection \(^{29}\). In 13 of 16 bm12 hearts examined, vascular fibrinoid necrosis and/or vascular inflammation was present and associated with C4d complement and IgG endothelial deposition. These changes were not evident in syngeneic grafts, nor in bm12 hearts transplanted into either MHCII\(^{-/-}\) mice or B cell-deficient recipients. IgG deposition within bm12 hearts grafted into B6 recipients have been noted previously \(^{28}\).

Autoantibody, but not alloantibody, develops following heart transplantation

B6 recipients of bm12 heart allografts did not develop detectable circulating IgG alloantibody (Figure 3A). This likely reflects absence of a conformational epitope on the I-A\(^{bm12}\) antigen for B cell recognition, rather than a defect in the provision of T cell help for alloreactive B cells, because B6 mice, immunised with (CBA/Ca x bm12) F1 splenocytes (to provide additional 3\(^{rd}\) party H-2\(^{k}\)
alloantigens as a source for helper T cell activation) developed alloantibody directed against CBA/Ca, but not bm12 antigens (Figure 3B).

Bm12 heart grafts instead elicited strong IgG anti-nuclear autoantibody (ANA) responses in B6 recipients that were detectable from week 3 onwards, were still present 16 weeks after transplantation and were associated with long-lasting germinal-centre B cell follicles in recipients’ spleens (Figure 4). Autoantibody was not generated in MHCII−/− recipients or in recipients of syngeneic grafts. Distinctive patterns of HEp-2 staining have been ascribed to differing specificities of autoantibody 30 and although patterns were consistent for individual animals, they varied between animals within groups (Figure 4D), suggesting that the autoantibody response targets multiple autoantigens. Nevertheless, autoantibody was not due to polyclonal, non-specific B cell activation; responses against irrelevant OVA protein or 3rd party H-2Kd alloantigen did not develop (not shown).

**T cell help for autoantibody production is provided by donor CD4 T cells within the heart graft.**

The absence of isotype-switched autoantibody in MHCII−/− recipients suggests a T cell-dependent response. To examine the suggested role of indirect pathway T cells in providing help for autoantibody production 17, 19, B6 mice were immunised with a synthetic bm12 allopeptide (incorporating the disparate amino-acid residues) fourteen days prior to transplantation. Heart grafts in peptide-primed animals were rejected more rapidly (Figure 5A), highlighting both the immunogenicity of the peptide and its contribution via indirect-pathway recognition to bm12 heart graft rejection. Predictably, given the above finding of a lack of B cell epitope on the I-A bm12 alloantigen, peptide priming did not provoke alloantibody (not shown). More surprisingly, however, the autoantibody response was not augmented (Figure 5B), suggesting that indirect pathway T cells do not provide help for its development.
We hypothesized that help is instead provided by passenger bm12 CD4 T cells that migrate from the heart graft after transplantation; as precedent, injection of bm12 splenocytes into B6 mice instigates graft-versus-host responses that generate ANA. B6 mice were therefore transplanted with bm12 heart grafts that lacked CD4 T cells. Three approaches were used. Bm12 RAG2-deficient (bm12RAG2−/−) mice, that lacked T and B lymphocytes, were created and WT bm12 donors were depleted of CD4 T cells, by treating with either 13Gy lethal irradiation or anti-CD4 mAb. Autoantibody production was abrogated in all three groups (Figure 5C), confirming a critical requirement for donor CD4 T cells in providing help to recipient autoreactive B cells.

**Help for autoantibody production is provided by cognate interaction between donor CD4 T cells and recipient B cells.**

To confirm that donor CD4 T cells provide help for autoantibody production through direct alloreognition of MHC class II on autoreactive B cells, and not through soluble factors released following interaction with recipient DCs and macrophages, B6 BM chimeric mice were created that lacked MHC class II expression specifically on B cells, but had otherwise normal APCs (BCII−/− mice, Figure 6A). In contrast to control BCII++ animals, autoantibody was not detectable in BCII−/− recipients of bm12 heart allografts (Figure 6B), consistent with a requirement for cognate interaction between the TCR of donor CD4 T cells and MHC class II on recipient autoreactive B cells.

**An effector role for autoantibody in the development of CAV.**

Given the histological findings demonstrating antibody-mediated vascular damage, we sought to verify that autoantibody played an effector role in allograft rejection. Compared to WT controls, heart grafts from donors either deficient or depleted of CD4 T cells developed less severe vasculopathy (although this only reached statistical significance in the irradiated group) and did
not demonstrate endothelial complement deposition (Figures 7A and B). Similarly, C4d endothelial staining was not detectable in hearts transplanted into BCII\(^{-/-}\) mice, but was present in allografts from control BCII\(^{+/+}\) recipients (Figure 6C). Rejection times of heart grafts from anti-CD4 mAb treated and bm12RAG2\(^{-/-}\) donors were, however, comparable to controls, and surprisingly, hearts from irradiated donors were rejected more rapidly (Figure 7C). Notably, allografts from bm12RAG2\(^{-/-}\) and anti-CD4 mAb treated donors contained similar lymphocyte infiltrates as seen in WT hearts (Figure 7D). These infiltrates are typical of acute cellular responses; their presence suggests that conventional recipient T cell alloimmunity can effect graft rejection in the absence of a humoral autoimmune response. In comparison, marked fibrosis, but minimal lymphocytic infiltration, was present in the irradiated hearts suggesting that radiation damage was responsible for their rapid failure.

An effector role for autoantibody is further suggested by marked attenuation of vasculopathy in bm12 hearts transplanted into B cell-deficient _MT_ animals, with survival of all hearts until explant at day 50 (Figure 8). However, although passive transfer of autoantibody to _MT_ recipients was associated with focal endothelial C4d staining, neither vasculopathy nor rejection was restored (Figure 8).

We thought it possible that passive transfer was unable to re-create the long-lasting and progressive levels of autoantibody that are present in WT recipients, so as an alternative approach, bm12 hearts were transplanted into B6 recipients already primed for autoantibody by transfer of bm12 CD4 T cells two weeks previously (Figure 8D). All heart grafts developed severe vasculopathy, with vascular obliteration, and were rejected rapidly (Figure 8). Because the injected CD4 T cells were highly purified and do not express MHC class II, CD4 T cell administration is unlikely to have sensitised against the disparate I-A\(^{bm12}\) alloantigen. In support, immunisation with
lysed, rather than whole, bm12 CD4 T cells, did not accelerate bm12 heart graft rejection, and did not augment the severity of vasculopathy (Figure 8). Similarly, bm12 heart grafts transplanted into CD4 T cell-immunised μMT recipients survived indefinitely, without developing vasculopathy, and allografts in recipients primed for indirect-pathway responses by I-A<sup>bm12</sup>-peptide-immunisation had vasculopathy comparable in severity to WT controls, rather than the vascular obliteration associated with administration of whole bm12 CD4 T cells (Figure 8B). Thus the accelerated rejection in the recipients that received donor CD4 T cells is the consequence of priming for autoantibody, and not T cell alloimmunity.
DISCUSSION

The results of this study provide important new insights into the development of autoantibody following transplantation and its contribution to allograft vasculopathy. Two major findings emerged: help for autoantibody production is provided through donor CD4 T cell allore cognition of MHC class II on recipient B cells; and autoantibody contributes to the development of vasculopathy. Our results thus highlight the novel concept that passenger donor CD4 T cells within a cardiac allograft can contribute to its rejection.

Autoimmune responses are increasingly described in recipients of solid organ transplants, but their contribution to graft damage is unclear, because although associated with poor outcome, this may simply indicate non-pathogenic bystander activation that is triggered by a particularly aggressive (and consequently damaging) alloimmune response. Rodent studies have highlighted the contribution of T cell autoimmunity to graft rejection, but the only convincing evidence of an effector role for autoantibody is the beneficial response to the depletion of autoantibody in renal transplant patients with vascular rejection associated with anti-angiotensin receptor autoantibodies.

The demonstration of a requirement for donor CD4 T cells in initiating humoral autoimmunity enabled us to distinguish the contribution of autoantibody to CAV from that of host alloimmunity. The histological features, particularly complement deposition on allograft endothelium, are pathognomonic of humoral vascular rejection, which, given the absence of these features in μMT recipients and our experiments highlighting the lack of anti-bm12 alloantibody responses, are presumably triggered by autoantibody binding. Notably, complement deposition is absent in allografts from CD4 T cell deficient-donors and is restored in μMT recipients by passive transfer of autoantibody. An effector role for autoantibody is confirmed by the early rejection and
development of severe vasculopathy in bm12 hearts transplanted into animals primed for humoral autoimmunity by transfer of donor CD4 T cells. Allosensitisation caused by residual I-A<sup>bmi12</sup>-expressing APCs within the transferred T cells is highly unlikely; severe CAV did not occur in either donor T cell-injected μMT recipients or WT recipients immunised with either bm12-peptide or lysed bm12 CD4 T cells.

Not all of the experiments undertaken here demonstrated a role for autoantibody in the development of CAV. Passive transfer of autoantibody did not restore CAV in B cell deficient recipients, but as noted, the amount administered was likely insufficient for the development of CAV, albeit enough to provoke endothelial complement deposition. A similar explanation was provided for the recent observation that passive transfer of anti-MHC class I alloantibody to B-cell deficient recipients triggered allograft endothelial cell signaling, but did not restore CAV<sup>12</sup>. More notably, the severity of CAV, although reduced in heart grafts from CD4 T cell-depleted or bm12RAG2<sup>−/−</sup> donors, was not statistically different from WT grafts. As explanation, autoantibody likely complements conventional alloimmune responses; all heart grafts (including those from CD4 T cell-deficient donors) contain lymphocytic infiltrates typical of an acute cellular response and vasculopathy still develops in hearts transplanted into B cell-deficient recipients. A reconciliatory interpretation of our results is that the effector role of autoantibody is largely masked in unmodified recipients by concurrent strong alloimmune responses, but becomes apparent upon augmentation by injection of donor T cells.

Our findings add to the wider body of work emphasizing the importance of alloantibody in chronic allograft rejection<sup>35-37</sup>, and although the mechanisms responsible are unclear, binding to target alloantigens on graft endothelial cells is thought to trigger CAV development. A difficulty in postulating a similar role for autoantibody is that the target autoantigens are intracellular. The
association between autoantibody and endothelial complement deposition in our experiments suggests, however, that target autoantigens are translocated to the surface of allograft endothelial cells, presumably as a stress response\textsuperscript{38,39} to transplantation. This requirement for additional stress may explain why autoimmune responses that develop following heart transplantation do not cause vasculopathy in the recipient’s native heart (our own data and\textsuperscript{10,40}). Similarly, the presence of autoantibody in recipients of heart allografts did not result in overt autoimmune disease, such as glomerulonephritis, perhaps because, in the absence of alloimmune-mediated inflammation, it takes longer (generally greater than 10 weeks\textsuperscript{31}) than the time-scale of our experiments for autoantibody to effect damage of native organs.

The novel donor CD4 T cell-dependent mechanism described here for effecting allograft damage constitutes an unusual manifestation of graft-versus-host recognition and is presumably a consequence of the minimal antigen disparity between the bm12 and B6 mouse strains that permits extended survival of donor lymphocyte populations by circumventing host defenses such as NK cell cytolysis. Our findings therefore have immediate relevance to other murine models of CAV that rely on limited donor-recipient antigen disparity to avoid acute rejection\textsuperscript{41-44}. A more searching question is the extent to which similar donor lymphocyte-driven responses are responsible for causing vasculopathy in human recipients of organ transplants? No study has as yet examined the role of passenger donor lymphocytes in the development of transplant-induced autoantibody. Their involvement is supported, at least theoretically, both by the recent demonstration that appreciable numbers of peripheral T cells migrate through non-lymphoid organs, such as the heart\textsuperscript{45-47}, and by historical transplant studies that document donor leukocyte ‘micro-chimerism’ in human transplant recipients\textsuperscript{48}. This micro-chimerism includes populations
of donor T cells\textsuperscript{49,50}, whose presence is strikingly manifest in the rare, but usually devastating, acute GVH disease that can occur after solid organ transplantation.

In conclusion, we report the novel finding that donor CD4 T cells within heart allografts contribute to the development of CAV by providing help to recipient B cells for autoantibody production. To what extent donor lymphocytes contribute to autoantibody development in human heart transplant recipients is currently speculative. Nevertheless, their potential involvement raises the possibility that depleting lymphocytes in transplant organs prior to engraftment will reduce the severity of CAV and improve long-term graft survival.
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DISCLOSURES

None.

REFERENCES


Figure 1. MHC class II-mismatched heart grafts are rejected slowly and develop allograft vasculopathy.

(A) Kaplan-Meier survival curves of cardiac grafts. (B) H&E-stained paraffin sections of day 50 bm12 heart allografts, depicting cellular inflammatory infiltrates with accompanying areas of myocyte necrosis and replacement fibrosis (i). Infiltrates consist of (ii) plasma cells (arrowed), lymphocytes and neutrophils. Syngeneic B6 heart grafts (iii), and bm12 grafts in MHCII–/– B6 recipients (iv), remain disease free. (C) EVG-stained paraffin sections of bm12 heart allografts in WT recipients, demonstrating an initially cellular vasculopathy (day 28), that is later more fibrotic (day 50). Syngeneic B6 grafts or bm12 hearts transplanted in MHCII–/– mice do not develop vasculopathy.
Figure 2. Humoral vascular responses are evident in bm12 heart allografts.

(A) H&E-stained paraffin sections of day 50 bm12 heart allografts reveal (i) vascular fibrinoid necrosis and (ii) vascular inflammatory infiltrates (arrowed). Cryostat immunohistochemical staining of day 50 heart allografts in WT recipients demonstrates IgG (B) and C4d (C) vascular deposition, not present in syngeneic grafts or bm12 grafts transplanted into either MHCII−− or μMT recipients.
Figure 3. B6 recipients of bm12 heart grafts do not develop alloantibody.

(A) Anti-bm12 alloantibody responses in WT B6 recipients of bm12 heart grafts. Depicted is the analysis of day 50 sera, but no alloantibody was detected at earlier time points (not shown). (B) Immunisation of B6 mice (n=3) with (bm12 x CBA/Ca) F1 splenocytes generates alloantibody (mean ± SEM) against CBA/Ca target cells, but not against bm12 targets.
Figure 4. Anti-nuclear autoantibody develops following bm12 heart grafting.

(A) Representative images of indirect immunofluorescent staining of HEp-2 cells with weekly sera samples from B6 recipients of either bm12 heart grafts (WT) or syngeneic grafts, or MHCII−/− recipients of bm12 hearts. (B) Graphical depiction (mean ± SEM) of fluorescence intensity (grade 1-5) for each group. *, p < 0.05 c.f. either recipients of syngeneic hearts or allografted MHCII−/− mice. (C) H&E-stained paraffin sections depicting germinal centre secondary follicles within the spleens of recipients of bm12 allografts, but not in recipients of syngeneic grafts. (D) Representative photomicrographs of HEp-2 staining from different B6 recipients of day 50 bm12 heart allografts revealing distinct staining patterns: i) coarse-speckled; ii) nuclear-homogenous; iii) cytoplasmic and nuclear homogenous.
Figure 5. T cell help for autoantibody production.

(A) B6 mice were immunised with synthetic bm12 peptide in CFA. Kaplan-Meier survival analysis: *, p < 0.05 c.f. WT recipients. (B) IgG autoantibody responses (mean ± SEM) to a bm12 heart graft in peptide-primed recipients compared to non-immunised WT recipients or recipients immunised with CFA only. (C) IgG autoantibody responses (mean ± SEM) following transplantation with hearts from un-manipulated WT bm12 donors (—, n=6) or bm12 donors either genetically-deficient in CD4 T cells (bm12RAG2−/−, n=4)) or depleted of CD4 T cells ((anti-CD4 mAb treated, ↓, n=6)); lethally irradiated (ρ, n=4)) *, p < 0.05 for WT compared to all three groups of CD4 T cell-deficient donors.
Figure 6. Cognate interaction between donor CD4 T cells and recipient B cells is essential for autoantibody production.

(A) Flow cytometric analysis of peripheral blood from B6 RAG2 \(^{-/-}\) mice reconstituted with either WT BMC (BCII \(^{+/+}\)), or MHCII \(^{-/-}\) BMC (BCII \(^{-/-}\)), sampled eight weeks after reconstitution. MHC class II is not expressed on B cells in BCII \(^{-/-}\) mice (red box), but is expressed on other APC subsets (black box). (B) Representative images from HEp-2 fluorescent staining with sera sampled weekly after bm12 heart transplantation, and depicted graphically according to mean ± SEM of fluorescence intensity (grade 1-5). Autoantibody responses in unmodified WT recipients are shown as comparison. *, \(p < 0.05\) when compared to BCII \(^{+/+}\). (C) Representative photomicrographs of C4d complement staining, depicting endothelial complement deposition in bm12 hearts transplanted into control BCII \(^{+/+}\) recipients, but not in hearts transplanted into BCII \(^{-/-}\) recipients.
Figure 7. Survival and vasculopathy development in cardiac allografts from CD4 T cell-deficient donors.

(A) Severity of allograft vasculopathy from: WT donors (n=17); from anti-CD4 mAb-treated donors (Depleted, n=6); from lethally irradiated donors (Irradiated, n=4); and from bm12RAG2−/− donors (n=4), graded (0-4) and plotted as mean ± SEM. *, p < 0.05. (B) Representative photomicrographs of C4d complement staining, depicting endothelial complement deposition in cardiac allografts from WT bm12 donors, but not in allografts from CD4 T cell-deficient donors. (C) Kaplan-Meier survival curves of bm12 cardiac allografts. *, p < 0.05 when compared to survival of WT donors. (D) Photomicrographs of day 50 heart allografts depicting fibrosis in grafts from: (i) irradiated donors; and cellular infiltrates in grafts from (ii) anti-CD4 mAb-treated donors and (iii) bm12RAG2−/− donors.
Figure 8. Autoantibody contributes to the development of allograft vasculopathy.

(A). Kaplan-Meier survival curves of bm12 cardiac allografts in: WT (n=17); μMT (n=8); and μMT mice passively transferred with autoantibody following transplantation (AT μMT, n=4). Bm12 hearts were also transplanted into WT (Preformed, n=5, MST=35days) or μMT (Challenged μMT, n=4) mice that were injected i.p. with bm12 CD4 T cells two weeks prior to transplantation or into WT injected with lysed bm12 CD4 T cells (Lysate-primed, n=4). *, p < 0.01, when compared to WT recipients, μMT recipients and challenged μMT recipients. (B) Allografts were harvested at day 50 and the severity of vasculopathy (0-4) depicted as mean ± SEM. Included is the vasculopathy score for bm12 allografts in bm12 peptide-immunized mice (P-primed). *, p < 0.05. (C) Representative photomicrographs depicting focal endothelial complement deposition in bm12 hearts transplanted into μM1 recipients that received autoantibody i.v. after transplantation (AT μMT), but not in hearts transplanted into μMT recipients. (D) Representative indirect immunofluorescent staining of HEp-2 cells with sera sampled weekly after bm12 heart transplantation into: μMT recipients; WT recipients; and WT recipients with preformed autoantibody.
FIGURE 1

A

![Graph showing graft survival over days post-transplantation.](image)

B

![Images of tissue samples.](image)

C

![Additional images of tissue samples.](image)
FIGURE 2

A

B

WT

Syngeneic

IgG

MHCIi$^{-/-}$

$\mu$MT

IgG

WT

Syngeneic

C4d

MHCIi$^{-/-}$

$\mu$MT

C4d
FIGURE 3

A

Anti-bm12 IgG AlloAb (Geo-mean fluorescence)

WT (n=4)
Syngeneic (n=4)
Naive serum (n=2)
Positive control

Log$_3$ Reciprocal Serum Dilution

B

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IgG AlloAb (Geo-mean fluorescence)

CBA/Ca target cells
bm12 target cells

Log$_3$ Reciprocal Serum Dilution
FIGURE 4

A

Week 1  Week 2  Week 3  Week 4  Week 5  Week 6  Week 7  Week 16

WT

MHCII\(^{-/-}\)

Syngeneic

B

Week Post-Transplantation

0  1  2  3  4  5  6  7  16

IgG AutoAb (1-5+)

WT (n=6)  MHCII\(^{-/-}\) (n=5)  Syngeneic (n=5)

C

ALLOGRAFT  SYNGENEIC

D

(i)  (ii)  (iii)
**FIGURE 5**

**A**

Graft Survival (%)

- WT (MST=95 days)
- CFA-primed (MST=80 days)
- Peptide-primed (MST=35 days) *

Day Post-Transplantation

**B**

IgG AutoAb (1-5+)

- WT (n=6)
- Peptide-primed (n=7)
- CFA-primed (n=6)

Week Post-Transplantation

**C**

IgG AutoAb (1-5+)

- Weeks Post-Transplantation
FIGURE 6

A

B

C

Week Post-Transplantation

IgG AutoAb (1-5+)

WT

MHC Class II

CD19

BCII^{++/+}

BCII^{+-/-}

BCII^{++/+}

BCII^{+-/-}

Circulation Heart Failure

Wk1  Wk2  Wk3  Wk4  Wk5  Wk6  Wk7

50μm

50μm
FIGURE 7

A

B

C

D
FIGURE 8

A

Graph Survival (%)

Day Post-Transplantation

- mMT
- Challenged mMT
- WT
- AT mMT
- Lysate primed
- Preformed

B

Vascularity Score

- L.A.
- mMT
- AT mMT
- Lysate primed
- Preformed
- Challenged mMT
- Peptide primed

C

D

Wk0  Wk1  Wk2  Wk3  Wk4  Wk5  Wk6  Wk7

- mMT
- WT
- Preformed
Donor CD4 T cells contribute to cardiac allograft vasculopathy by providing help for autoantibody production.

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