Augmentation of recipient adaptive alloimmunity by donor passenger lymphocytes within the transplant.


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Abstract:

Chronic rejection of solid organ allografts remains the major cause of transplant failure. Donor-derived tissue-resident lymphocytes are transferred to the recipient during transplantation, but their impact on alloimmunity is unknown. Using mouse cardiac transplant models, we show that graft-versus-host recognition by passenger donor CD4 T-cells markedly augment recipient cellular and humoral alloimmunity, resulting in more severe allograft vasculopathy and early graft failure. This augmentation is enhanced when donors were pre-sensitised to the recipient, is dependent upon avoidance of host NK cell recognition, and is due, in part, to provision of cognate help for allospecific B-cells from donor CD4 T-cells recognising B-cell MHC class II in a peptide-degenerate manner. Passenger donor lymphocytes may therefore influence recipient alloimmune responses profoundly and represent a therapeutic target in solid organ transplantation.
Main text:

Introduction

Solid organ transplantation provides an effective therapy for patients with kidney, liver, heart, and pulmonary failure. Long-term graft survival is limited by adaptive alloimmune responses directed against transplant (typically allogeneic MHC) antigens, that are expressed within the organ and on endothelial cell surfaces and that interface with circulating recipient immune cells. In addition, it is now appreciated that a substantial number of memory T cells reside within non-lymphoid tissues (Mueller et al., 2013, Shin and Iwasaki, 2013, Sathaliyawala et al., 2013). Solid organ allografts may therefore deliver “passenger” donor lymphocytes to the recipient after transplantation. Currently, little is known about whether passenger lymphocytes remain in the allograft or reach recipient secondary lymphoid organs, or how long they survive, given that their likely recognition by NK cells might be expected to ensure rapid elimination. However, the precise role of NK cells in solid organ transplantation remains unclear (Gill, 2010, Hadad et al., 2014, van der Touw and Bromberg, 2010, Hidalgo et al., 2010), and early transplant studies indicate that circulating donor lymphocytes are often detectable in human transplant recipients, albeit in small numbers (Starzl et al., 1992a). Their presence may manifest as devastating, acute graft-versus-host (GVH) disease (Sharma et al., 2012), or as passenger lymphocyte syndrome, in which haemolysis is triggered by donor B cell recognition of mismatched ABO blood group antigens in the recipient (Nadarajah et al., 2013). Thus the impact of passenger lymphocytes on the recipient immune response to the allograft has still to be clarified (Turner et al., 2014).

We have recently shown that, in a murine heart transplant model with an isolated MHC class II-mismatch [B6(C)-H2bm12/KhEgJ (bm12) to C57BL/6 (B6)], passenger bm12 CD4 T cell
recognition of I-A^b MHC class II on host B cells triggers the production of anti-nuclear autoantibody, which causes allograft vasculopathy (Motallebzadeh et al., 2012, Win et al., 2009). **GVH recognition by passenger lymphocytes may also possibly contribute to graft rejection through other mechanisms. For example, activation of host DCs and macrophages following recognition of surface MHC class II by donor CD4 T cells, could prompt more vigorous host alloimmunity from more effective processing and presentation of graft alloantigen as self-restricted peptide fragments.**

To examine the possibility that passenger donor lymphocytes augment conventional host alloimmunity, we developed a murine transplant model incorporating a new bm12-derivative donor strain that expresses additional MHC class I and class II alloantigens to act as targets for conventional cellular and humoral allore cognition (Ali et al., 2016). Here we describe that in this model, heart allografts provoke autoantibody production in B6 recipients as a consequence of GVH recognition by passenger donor CD4 T cells. We show that even though donor CD4 T cells survive for only a few days after heart transplantation, their survival provokes a marked and long-lasting augmentation of cellular and humoral alloimmunity, and results in early allograft rejection. This augmentation is, however, prevented in completely-mismatched strain combinations by rapid NK cell killing of donor lymphocytes. These data have important clinical implications, suggesting that partial MHC mis-match between donor and recipient to promote NK cells responses against passenger lymphocytes may reduce alloimmune responses.
Results

*Heart allografts with isolated MHC class I and class II disparities provoke allo- and auto-antibody responses*

Human organs procured for transplantation, including kidney, liver and heart, contain significant populations of effector and effector-memory CD4 and CD8 T lymphocytes (Figure s1). We therefore sought to examine the impact of these passenger donor lymphocytes on recipient adaptive alloimmune response. To address this question, we developed a novel mouse strain that expressed multiple MHC alloantigens, sufficient to stimulate cellular and humoral alloimmunity, in addition to provoking humoral autoimmunity. A series of backcrosses were performed between bm12, B6.K<sup>d</sup> (Honjo et al., 2004b) and B6.I-E (Conlon et al., 2012a) strains to derive the ‘bm12.K<sup>d</sup>.IE’ strain, which differs from the B6 recipient strain at the classical MHC class I K and class II A and E loci (H-2<sup>b</sup>, K<sup>bd</sup>, A<sup>bm12</sup>, E, D<sup>b</sup>; Figures 1a, s2).

As expected, when bm12.K<sup>d</sup>.IE hearts allografts were transplanted into B6 recipients, the additional MHC class I H-2K<sup>d</sup> and class II I-E mismatched alloantigens provoked strong alloimmune responses, with production of long-lasting alloantibody to both antigens (Figure 1b). Recipients also developed antinuclear autoantibody (Figure 1c), that was comparable in magnitude to the responses previously observed in B6 recipients of bm12 heart allografts (Win et al., 2009, Motallebzadeh et al., 2012). These antibody responses were associated with C4d complement deposition on heart graft endothelium (Figure 1d), which was not evident in syngeneic heart transplants, suggesting a humoral component to the allograft vasculopathy that developed within allografts by day 100 (Figure 1e).
Graft versus host allorecognition provokes recipient humoral autoimmunity

To determine whether, as in the bm12 to B6 model, autoantibody production in B6 recipients of bm12.K^d.IE heart allografts was due to donor CD4 T cell allorecognition of recipient I-A^b MHC class II (Callaghan et al., 2012, Win et al., 2009), bm12.K^d.IE donor mice were treated with depleting anti-CD4 mAb prior to sacrifice. This resulted in profound depletion of circulating and tissue resident CD4 T cell compartments by the time of heart allograft procurement (Figures 2a and 2b). Anti-CD4 antibody was not carried over to the recipient (Figure 2c), but nevertheless, donor treatment with anti-CD4 mAb abrogated the recipient autoantibody response (Figure 2d), confirming that passenger CD4 T cells within the bm12.K^d.IE donor heart are responsible for initiating recipient humoral autoimmunity.

Despite the development of humoral autoimmunity, no overt autoimmune disease was observed in kidney, liver, skin or native heart in B6 recipients up to 100 days after transplantation with a bm12.K^d.IE heart allograft (Figure s3).

Augmentation of conventional alloimmunity by graft-versus-host allorecognition

The ability, through specific targeting of the donor CD4 T cell population, to independently manipulate recipient autoimmune and alloimmune responses, provided a means to examine whether GVH allorecognition augments host alloimmunity. Comparison of recipient cellular and humoral alloimmune responses in recipients of unmodified and CD4 T cell-depleted bm12.K^d.IE heart allografts revealed that alloantibody responses against the H-2K^d alloantigen were substantially reduced in recipients of CD4 T cell-depleted allografts (Figure 3a and s4). Responses against the disparate donor MHC class II alloantigen were similarly ameliorated (Figure 3b). Allo- and auto- antibody responses were restored in recipients of
CD4 T cell-depleted bm12.K^d.IE heart allografts by adoptive transfer of purified donor CD4 T cells at transplantation (Figures 2d & 3a).

The disparate H-2k^d alloantigen might be expected to act as a target for recognition by recipient cytotoxic CD8 T cells (Harper et al., 2015), but whereas B6 recipients of fully MHC-mismatched BALB/c heart allografts generated robust CD8 T cell responses, the response in recipients of unmodified bm12.K^d.IE heart allografts was weak and transient (Figure 3c). Nevertheless, cytotoxic CD8 T cell responses were barely detectable in recipients of CD4 T cell-depleted bm12.K^d.IE heart transplant recipients at any time-point (Figure 3c). Helper CD4 T cell alloresponses were also examined in the recipient groups, by evaluating proliferation of TCR75 CD4 T cells that were adoptively-transferred five weeks after the heart transplant. TCR75 CD4 T cells recognise K^d alloantigen via the ‘indirect pathway’ (Ali et al., 2013) as self, I-A^b-restricted, but not donor I-A^bm12-restricted, allopeptide (Honjo et al., 2004a, Conlon et al., 2012b) and in recipients of CD4 T cell-replete heart grafts, marked TCR75 T cell proliferation was observed, indicating on-going presentation of immunogenic K^d allopeptide epitope. In contrast, TCR75 T cell responses in recipients of CD4 T cell depleted hearts were approximately 50% weaker (Figure 3d).

_Graft-versus-host allore cognition contributes to allograft rejection_

The marked reduction in the alloimmune response to CD4 T cell-depleted bm12.K^d.IE heart allografts ameliorated graft rejection, in that vasculopathy was minimal in heart allografts from CD4 T cell-depleted donors and comparable to that observed in syngeneic heart transplants (Figure 4a). In addition, all heart transplants from CD4 T cell-depleted donors were beating strongly at day 50 (Figure 4b). Adoptive transfer of donor CD4 T cells at time of
transplant to recipients of CD4 T cell-depleted bm12.K\(^d\).IE heart allografts restored the
development of allograft vasculopathy (Figure 4a).

In contrast to human organs where memory T cell populations dominate (Fig s1), the CD4 T
cell compartment in mice housed in specific-pathogen-free conditions is maintained in a
largely naïve state. We therefore sought to examine how memory CD4 T cells within an
allograft might influence host alloimmunity, by priming Bm12.K\(^d\).IE donors with a B6 skin
graft six weeks prior to procurement of the heart allograft to generate resident memory T
cells. Heart allografts from such donors were rejected more rapidly by B6 recipients than
heart grafts from naïve donors, and triggered augmented auto- and allo- antibody responses
(Figure 4a-d).

**Recipient T and B cells are essential mediators of the accelerated rejection triggered by
early graft-versus-host recognition**

Amplification of the recipient alloreactive T-B lymphocyte axis is likely the principal
mechanism by which donor CD4 T cell GVH recognition triggers accelerated graft rejection,
because host germinal centre alloantibody responses were much less established in
recipients of T-cell depleted, than T cell-replete, heart allografts (Figure 4e), as was
complement C4d deposition on allograft endothelium (Figure 1d). Similarly, heart grafts
were not rejected, and developed only minimal vasculopathy, when transplanted into either
B cell-depleted (Figure s5) or T cell deficient Tcrbd\(^{-/-}\) recipients (Figures 4a and 4b). To
examine whether the augmentation in recipient CD4 T cell responses triggered by donor
GVH recognition was dependent upon host B cell immunity, B cell-depleted recipients were
transplanted with either CD4 T cell-replete or deficient bm12.K\(^d\).IE heart grafts, and
proliferation of transferred TCR75 T cells examined as above. For recipients of CD4 T cell-
replete heart grafts, proliferation of adoptively-transferred TCR75 T cells was substantially less in B cell depleted than in untreated recipients (Figure 3e), and approximated that observed in untreated recipients of CD4 T cell-depleted bm12.Kd.IE heart allografts. Furthermore, unlike B cell-replete recipients, proliferation of transferred TCR75 CD4 T cells in B cell-depleted recipients was not influenced by depletion of donor CD4 T cells (Figure 3e). The role of recipient B cells in GVH-mediated augmentation of recipient T cell alloreactivity does not simply reflect function as the major cell population expressing target I-A\textsuperscript{b} for optimal GVH activation of donor CD4 T cells, because the latter still divided readily in B cell-depleted donors (Figure 4f), such that no CFSE staining was detectable in the sub-population of alloreactive bm12.Kd.IE CD4 T cells as early as three days after transfer (Figure 4f).

Although transplantation of bm12.Kd.IE hearts into Tcrbd\textsuperscript{-/-} recipients prompted autoantibody and weak alloantibody responses (Figure 4g and 4h), there was no associated germinal centre activity (Figure 4e). Thus the requirement for host CD4 T cells in bm12.Kd.IE heart graft rejection appears to reflect provision of essential help for development of sophisticated host humoral alloimmunity, a function not provided by donor CD4 T cells - indeed, transferred donor CD4 T cells are rapidly killed by adaptive alloimmune recognition in B6 hosts, because whereas bm12.Kd.IE CD4 T cells were readily identified seven days after transfer into Rag-2\textsuperscript{-/-} hosts, they were undetectable following transfer into WT B6 hosts (Figure 4i). Hence it is unlikely that the donor CD4 T cells survive long enough to contribute directly to the progression of AV. Their effect appears to be mediated principally through a relatively short-lived interaction with host B cells, but prolonged augmentation of humoral alloimmunity is dependent upon additional help from host CD4 T cells.
Peptide-independent recognition of the B cell MHC class II complex by donor CD4 T cells promotes plasma cell differentiation, but requires concurrent BCR ligation

In considering how donor CD4 T cells amplify humoral alloimmunity, transfer of bm12 CD4 T cells into B6 hosts prompted up-regulation of MHC Class II expression on mature B cells (Figure 5a), in keeping with global activation from recognition of all allogeneic MHC class II complexes on their surface. Yet only a limited repertoire of antibody directed against nuclear self-antigen was produced (not shown). To examine the hypothesis that plasma cell differentiation requires BCR ligation, in addition to cognate interaction between the MHC class II complex and donor CD4 T cell, Tcrbd−/− B6 mice were challenged with purified bm12 CD4 T cells and immunised with ovalbumin protein. In this situation, CD4 T cell help for humoral responses can only be provided by the transferred donor CD4 T cells. Control Tcrbd−/− mice received bm12 CD4 T cells only. As expected, mice in both groups developed antinuclear autoantibody, but anti-ovalbumin IgG responses were only detectable in the group immunised with ovalbumin (Figures 5b and 5c). Similarly, challenge of Tcrbd−/− B6 mice with CD4 T cells from bm12 mice that expressed transgenic H-2Kd antigen (bm12.Kd) provoked autoantibody, but also strong anti-Kd IgG alloantibody, which was not observed in Tcrbd−/− B6 mice challenged with bm12 CD4 T cells (Figures 5d and 5e). Bm12.Kd CD4 T cells are selected against reactivity to self (I-A\textsuperscript{bm12})-restricted Kd peptide, and are unable to provide help to Kd-specific bm12 B cells for generating anti-H-2Kd antibody (Figures 5f and 5g). Thus, their provision of help for generating anti-H-2Kd antibody in B6 hosts reflects peptide-degenerate direct pathway allore cognition of I-A\textsuperscript{b} MHC class II on H-2Kd-specific B6 B cells that, in concert with simultaneous BCR ligation, provokes class-switched alloantibody. These alloantibody responses presumably explain why bm12.Kd.IE CD4 T cells
are undetectable within a week of transfer into B6 Tcrbd\textsuperscript{-} mice, but survive long-term in Rag-2\textsuperscript{-} mice (figure 4i). In summary, despite being tolerant to H-2K\textsuperscript{d} antigen on the bm12 background, bm12.K\textsuperscript{d} CD4 T cells provoke anti-K\textsuperscript{d} alloantibody when transferred into B6 hosts; this alloantibody results in rapid destruction of the bm12.K\textsuperscript{d} CD4 T cells.

**NK cell allorecognition is essential for preventing GVH-mediated amplification of the host adaptive alloimmune response.**

Whether the amplification of host humoral immunity by GVH recognition is an intrinsic component of the alloresponse, or is dependent on the degree of MHC mismatch between donor and recipient, has not been addressed. Given that donor bm12.K\textsuperscript{d}.IE CD4 T cells survive long-term in Rag-2\textsuperscript{-} B6 hosts (Figure 4i), we examined whether innate immune evasion, and specifically, lack of NK cell allorecognition of donor lymphocytes, was critical in triggering autoantibody generation. In this regard, CD4 T cells purified from the completely-mismatched BALB/c donor strain did not survive when injected into B6 Rag-2\textsuperscript{-} hosts and did not provoke humoral auto- or allo-immunity upon injection into B6 Tcrbd\textsuperscript{-} mice (Figures 6a-c). This contrasts with long-term survival and development of strong IgG allo- and auto-antibody when purified CD4 T cells from the less-mismatched strains were injected (Figure 6a-c). Furthermore, depletion of NK cells by administration of anti-NK1.1 antibody resulted, in the B6 Rag-2\textsuperscript{-} recipients, in long-term survival of transferred BALB/c CD4 T cells, and, in Tcrbd\textsuperscript{-} recipients, provoked class-switched auto and allo-antibody responses (Figures 6b-d) that were even stronger than those observed upon administration of CD4 T cells from the less mismatched donor strains. It should be noted that NK T cells, which also express NK1.1, do not develop in Tcrbd\textsuperscript{-} mice (Figure 6e); these experiments therefore serve as an apposite control that the administered anti-NK1.1 antibody is acting
principally upon NK cells. Irrespective of GVH recognition, injection of BALB/c CD4 T cells into wild-type, immunocompetent B6 mice would be expected to provoke alloantibody, but it was notable that autoantibody was only produced if host NK cells were depleted simultaneously (Figure 6e), confirming that elimination of the transferred donor CD4 T cell population, by either host cytotoxic CD8 T cell or alloantibody responses, does not occur quickly enough to obviate a GVH response, and that NK cell allorecognition is instead essential for its prevention.

**Host NK cell allorecognition prevents donor passenger lymphocytes from triggering accelerated rejection of completely MHC-mismatched allografts.**

These observations suggest that GVH mediated amplification of host alloimmune effector responses is normally prevented in MHC-mismatched transplant models by host NK cell alloreactivity. The role of NK cells in rejection of completely-mismatched BALB/c hearts by B6 recipients was therefore examined. However, in this model of acute rejection, unmodified B6 recipients reject BALB/c heart allografts within days and it seemed unlikely that host NK cell depletion would influence such a robust rejection response. Instead, a further model of chronic alloantibody-mediated allograft vasculopathy was developed, in which B6 Tcrbd−/− recipients of BALB/c heart allografts are reconstituted at transplantation with B6 TCR75 CD4 T cells, but at limiting numbers (10^3 per mouse), such that rejection occurs slowly and is mediated by anti-H-2k^d germinal centre alloantibody responses, with help provided by differentiation of the transferred TCR75 T cells to follicular helper T cells (Figure 7a, manuscript submitted). In marked contrast to the gradually evolving anti-K^d alloantibody responses observed in NK-cell replete recipients, responses in the NK cell-depleted recipients were stronger (Figure 7b), and the heart grafts were rejected within the
first week (Figure 7c). Autoantibody was also generated in the NK-cell depleted recipients (Figure 7d), confirming the development of graft versus host responses mediated by donor BALB/c CD4 T cells. Critically, autoantibody generation, the augmented alloantibody response and rapid allograft rejection were ameliorated in NK cell-depleted recipients by depletion of CD4 T cells from the BALB/c donor prior to heart graft procurement (Figures 7b-d).

Finally, to test the relevance of our findings to a model in which graft rejection is prevented by administration of immunosuppression, as occurs routinely in clinical practice, heart allografts from BALB/c donor mice that had been challenged six weeks earlier with a B6 skin graft were transplanted into B6 recipients that were treated with anti-CD154 monoclonal antibody at transplantation. In B6 recipients of heart grafts from unmodified donors, this protocol results in long-term allograft survival (Larsen et al., 1996, Ali et al., 2016), without development of autoantibody (Figure 7e), but in recipients of heart allografts from challenged donors (that contained memory passenger CD4 T cells), depletion of NK cells at transplantation resulted in development of anti-nuclear autoantibody and more pronounced splenic germinal centre activity (Figures 7e & f). Despite the robust germinal centre response, anti H-2Kd alloantibody responses were not observed (not shown).

Depletion of CD4 T cells in the donor prior to heart allograft procurement abrogated the autoantibody response (Figures 7e & f).
Discussion

Although the presence of donor lymphocytes in the circulation of recipients of solid organ allografts was first demonstrated over two decades ago (Starzl et al., 1992a, Starzl et al., 1992b), the extent to which they impact upon recipient alloimmunity has remained unclear. Clarification of the contribution of passenger donor lymphocytes to graft rejection has become more pertinent with the realisation that non-lymphoid tissue contains substantial populations of either resident or circulating memory T lymphocytes, and their presence has been described within all solid organs currently transplanted in humans (Casey et al., 2012, Sathaliyawala et al., 2013). Here, we used a combination of donor CD4 T cell depletion and adoptive transfer of donor CD4 T cells, in conjunction with transplantation of heart allografts from primed donors, to demonstrate that graft-versus-host allorecognition by donor CD4 T cells augments recipient alloimmunity, and that this augmentation is more pronounced for allografts procured from donors sensitised against recipient MHC. Our findings thus reveal an unreported mechanism by which donor lymphocytes may influence graft rejection, and suggests that their impact may be more important than previously considered.

Pivotal to this augmentation of host alloimmunity is the ability of donor CD4 T cells to recognise host MHC class II via the ‘direct-pathway’ (Ali et al., 2013). This provides an unusual form of ‘peptide-degenerate’ help, reflecting the unique nature of direct-pathway allorecognition (Macdonald et al., 2009, Ali et al., 2013), in which the precursor frequency of CD4 T cells that respond to a particular MHC class II alloantigen is 100 to 1000 fold greater than for the response against conventional, self-restricted peptide antigen, because all MHC class II alloantigen complexes are recognised as foreign, irrespective of bound peptide. This
results in activation of all recipient B cells, but we detail for the first time that differentiation to an IgG antibody secreting plasma cell is dependent upon simultaneous B cell receptor ligation. Thus although donor CD4 T cells can provide help to recipient B cells in an antigen-independent fashion, antigen-specificity is maintained through the requirement for B cell receptor ligation. This atypical help does not, however, completely replicate conventional cognate help provided by CD4 T cells with self-restricted specificity for peptide derived from target antigen, because although GVH recognition by donor CD4 T cells could trigger auto- and allo- antibody responses, these were not sustained in the absence of a recipient CD4 T cell population, and allograft rejection did not occur. Our work therefore details a previously unreported interaction between donor and recipient T and B lymphocytes as depicted in Figure 8.

Passenger lymphocytes only augmented recipient alloimmunity if they were not eliminated rapidly by recipient NK cells, and our findings therefore also highlight a novel mechanism by which NK cells inhibit graft rejection. The contribution of NK cell alloresponses to allograft rejection is still debated (Gill, 2010, Hadad et al., 2014, van der Touw and Bromberg, 2010). It has been suggested that host NK cells promote allograft rejection (Maier et al., 2001, Uehara et al., 2005, Kroemer et al., 2008), either through the destruction of opsonised donor cells, or perhaps through regulation of T cell immunity (Maier et al., 2001). Our results suggest the converse; that a major function of NK cells is inhibition of destructive cellular and humoral alloimmunity that is triggered by passenger CD4 T cell GVH recognition. This accords with several studies reporting a key role for NK cells in allograft tolerance (Beilke et al., 2005, Yu et al., 2006). The mechanisms by which NK cells promote tolerance in these studies have not been firmly established, but inhibition of recipient alloimmune
responses through killing of donor DCs may be important (Yu et al., 2006); in support Laffont has reported that NK cell-mediated destruction of donor DCs down-regulates CD4 T cell alloimmunity (Laffont et al., 2008). The different mechanism highlighted by our study - the killing of passenger lymphocytes - may be more clinically relevant, because irrespective of NK cell allorecognition, adaptive alloimmune recognition would be expected to result in prompt destruction of donor DCs; indeed in the Laffont study, CD8α−/− mice were studied to obviate rapid killing by cytotoxic T lymphocytes (Laffont et al., 2008). Our results reveal that the critical window for passenger donor CD4 T cells to augment host alloimmunity is within the first few days after transplant, and that evasion of NK cell-mediated killing is essential for this effect. Notably, donor CD4 T cells prompt host adaptive responses that engender their own rapid destruction, but the delay in development of these responses, at most a few days when compared to NK cell recognition, is sufficient for GVH recognition to occur.

What are the implications of our study for clinical solid organ transplantation? One could argue, on the basis of the derived nature of the bm12.Kd.IE donor strain and the lack of requirement for administration of immunosuppression that the clinical relevance is limited. Similarly, it is perhaps surprising that passenger donor lymphocytes were present in sufficiently large numbers within heart allografts to provoke such a marked augmentation in the host’s alloimmune response. Against this, CD4 T cells could be readily detected in all human organs that were sampled that have been procured for transplantation but not used. In addition, to counter concerns regarding the wider applicability of the bm12.Kd.IE model, we employed an additional model of chronic allograft vasculopathy using completely mismatched BALB/c donor and B6 recipient strains. This model enabled clarification of the crucial role of host NK cells in killing donor haematopoietic cells, but nevertheless, the
potential for passenger donor lymphocytes to augment host alloimmunity was again observed. In addition, our experiments in which acute rejection of a MHC-mismatched heart was prevented by concurrent administration of immunosuppression revealed that memory donor CD4 T cells were able to provide co-stimulation independent help to naïve recipient B cells for production of a GC autoantibody response.

With regards the seemingly large numbers of donor lymphocytes contained within our murine heart allografts, our experiments were not able to distinguish whether these were truly resident within the parenchyma or trapped within the microcirculation of the heart allograft, and it is possible that different procurement and storage techniques used in clinical transplantation denude a heart allograft of most of its passenger lymphocyte populations. Against this, deliberate flushing of our mouse hearts via the coronary arteries at explant (as typically occurs in clinical heart allograft procurement) did not make any appreciable difference to the numbers of CD4 T cells subsequently found within the heart allograft (not shown). In any event, it is important to stress that the heart allograft model was used as a means of delineating the precise mechanism by which donor CD4 T cells influence the host's response to an allograft. In this respect, whereas the impact of passenger lymphocytes in clinical cardiac transplantation may be limited, lung, small bowel and composite tissue allografts will almost certainly transfer large numbers of donor lymphocytes that originate from organised lymphoid tissue contained within the allograft. Transplant outcomes for these organs are poorer than for other organs, and it is notable, for example, that a recent report of chronic face allograft rejection described the development of autoimmune, sclerodema-type features consistent with skin manifestations of chronic GVH (Petruzzo et al., 2015). One might therefore predict that transplant outcomes would be
particularly poor for individuals that receive such organs from donors matched for killer cell immunoglobulin-like receptor (KIR) recognition (which occurs in approximately 50% of kidney transplant pairings (van Bergen et al., 2011)), because the avoidance of immediate host NK cell detection would enable passenger donor lymphocytes to potentiate host alloimmunity. However, the impact of NK cell alloreactivity in transplant outcomes remains uncertain (Tran et al., 2013, van Bergen et al., 2011), possibly because studies to date have avoided the confounding impact of HLA mismatches on allograft survival by including only HLA-matched donor – recipient combinations, whereas our findings suggest that KIR-ligand matching would compromise transplant outcomes when donor and recipient are mismatched additionally at the HLA class II loci.

We think it likely that the ability of donor CD4 T cells to provide peptide-independent help to host B cells has implications beyond solid organ transplantation. In haematopoietic stem cell transplantation, an association between chronic GVH disease and humoral immunity is increasingly recognised (Nakasone et al., 2015, Dubovsky et al., 2014, Shimabukuro-Vornhagen et al., 2009, Svegliati et al., 2007). Our findings suggest that this may relate to a chimeric state in which the co-existence of populations of donor and recipient T and B lymphocytes tends to provoke destructive alloantibody responses. Persistence of a mixed chimeric state additionally implies that reciprocal NK cell tolerance to donor and host had been achieved (Narni-Mancinelli et al., 2013), which may be particularly relevant to strategies for haematological malignancy that use less toxic, non-myeloablative conditioning to initially establish mixed haematopoietic chimerism, and then later convert to full donor chimerism by infusion of donor lymphocytes (Chang and Huang, 2013). Our findings suggest that inhibition of host NK cell alloresponses may enable GVH recognition by CD4 T cells
within the subsequent donor infusion to provide promiscuous help for antibody production from residual host B cells that are concurrently engaging target antigen. This may explain recent reports documenting the development of humoral immunity against tumour antigen following establishment of mixed haematopoietic chimerism (Bellucci et al., 2004, Kremer et al., 2014). Similarly, the presence of donor CD4 T cells within donor lymphocyte infusions has recently been associated with loss of donor mixed haematopoietic chimerism (Kim et al., 2004, Hock et al., 2014), but rather than this being a bystander consequence of the general inflammatory milieu created by the GVH response (Hock et al., 2014), our results suggest that the loss may instead be due to cognate recognition of MHC class II on the surface of recipient alloreactive B cells by donor CD4 T cells.

In summary, we demonstrate an unexpected role for donor passenger CD4 T cells within allografts in the provision of help to recipient B cells for generating humoral responses directed against the transplant. Passenger donor lymphocytes may therefore influence recipient alloimmune responses more profoundly than previously considered and represent a therapeutic target in solid organ transplantation.
Materials and Methods

Animals

B6 (H-2b) and BALB/c mice (H-2d) were purchased from Charles River Laboratories (Margate, UK).

Bm12 mice (B6(C)-H2-Ab1bm12/KhEgJ [H-2bm12]) and T cell receptor-deficient mice (H-2b, Tcrbd⁻/⁻) B6.129P2-Tcrb<sup>tm1Mom</sup>Tcrr<sup>tm1Mom</sup>/J were purchased from the Jackson Laboratory (Bar Harbor, ME).

Tcrbd⁻/⁻ mice were backcrossed onto bm12 to create Tcrbd⁻/⁻ bm12 mice. C57BL/6 Rag-2⁻/⁻ mice (H-2b) were gifted by Prof T. Rabbitts (Laboratory of Molecular Biology, Cambridge, UK). TCR-transgenic Rag-1⁻/⁻ TCR75 mice (H-2b), specific for I-A<sup>b</sup>–restricted H-2K<sup>d</sup><sub>54-68</sub> peptide (Honjo et al., 2004a) and C57BL/6-Tg(K<sup>d</sup>)RPb (B6.K<sup>d</sup>) mice, which express the full sequence of H-2K<sup>d</sup> (Honjo et al., 2004b), were gifted by Prof. P. Bucy (University of Alabama, Birmingham, AL). B6.K<sup>d</sup> mice were backcrossed onto a bm12 background to create bm12.K<sup>d</sup> mice. B6 mice that lack I-A<sup>b</sup>, but express I-Eα (B6.I-E) (Conlon et al., 2012a), were gifted by Prof. C. Benoist (Joslin Diabetes Center, Boston, MA). The F<sub>1</sub> offspring of Bm12.K<sup>d</sup> and B6.I-E mice were bm12.K<sup>d</sup>.IE. All animals were maintained in specific pathogen–free facilities, and experiments were approved by the UK Home Office Animal (Scientific Procedures) Act 1986.

Heterotopic cardiac transplantation

Fully vascularized cardiac allografts were transplanted intra-abdominally (Conlon et al., 2012b).

Rejection, defined as cessation of palpable myocardial contraction, was confirmed at explant. Grafts were excised at predetermined time points after transplantation and stored at -80°C or fixed in 10% buffered formalin. In certain experiments heart allografts were retrieved from donor mice challenged with a recipient strain skin allograft 6 weeks earlier, or recipients were additionally injected i.p. with 500μg anti-CD154 mAb (clone MR-1; BE0017-1; Bio X Cell West Lebanon, NH, US) on days -2 and 0 in relation to transplantation; a protocol that prevents acute allograft rejection but that results in development of chronic allograft vasculopathy.
**Dendritic cell purification and culture**

Bone marrow–derived dendritic cells (BMDCs) were prepared as described previously (Curry et al., 2007). Briefly, BM was flushed from femurs and tibias with HBSS (Invitrogen). Cells were disaggregated by passing through a 40-μm mesh, and BM cells cultured in six-well plates at 3×10^6/ml in 6 ml complete medium (RPMI 1640, 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine; all Invitrogen), supplemented with murine GM-CSF (Peprotech) at 20 ng/ml and recombinant murine IL-4 (PeproTech) at 10 ng/ml. Cells were maintained by replacing half the culture medium with fresh medium on alternate days. Nonadherent cells were discarded on day 4 and DCs used on day 8 for flow cytometric analysis.

**Assessment of recipient humoral immunity**

- **Autoantibody quantification**

Antinuclear autoantibody responses were determined by HEp-2 indirect immunofluorescence (The Binding Site, Birmingham, UK) as described previously (Callaghan et al., 2012), by incubating test sera on slides coated with HEp-2 cells and detecting bound antibody with FITC-conjugated goat anti-mouse IgG (STAR 70; Serotec, Oxford, UK). For each test serum, photomicrographs were taken, and the intensity of staining was determined by integrated morphometric analysis using MetaMorph software. The fluorescence value was then derived by comparison with a standard curve, obtained for each assay by serial dilutions of a pooled hyperimmune serum that was assigned an arbitrary value of 1000 fluorescence units.

- **Assay of circulating anti-MHC class II I-E and anti-BALB/c alloantibody**

Sera were collected from experimental animals weekly and analysed for anti-I-E alloantibody (at week four in the case of BALB/c alloantibody) by flow cytometric detection of binding to target cells. Briefly, target B6.I-E and BALB/c BMDCs were first blocked with anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen, San Diego, CA, USA) and then incubated with serial dilutions (three-fold) of
heat-inactivated test serum for 30 minutes. Bound alloantibody was detected with FITC-conjugated goat anti-mouse IgG (STAR 70; Serotec, Oxfordshire, UK) and cells analysed by flow cytometry. For each sample, the geometric mean-channel fluorescence was obtained, and plotted against dilution, and the AUC was then calculated as a percentage of the AUC of a standard of pooled hyperimmune sera.

- **Determining circulating anti–H-2K\(^d\) alloantibody and anti-Ovalbumin antibody**

Serum samples were collected from experimental animals weekly and analyzed for the presence of anti–H-2K\(^d\) IgG alloantibody by ELISA. In brief, 96-well ELISA plates (Immulon 4HBX; Thermo, Milford, MA) were coated with recombinant conformational H-2K\(^d\) at 5 µg/ml in Na\(_2\)CO\(_3\)–NaHCO\(_3\) buffer (pH 9.6). Plates were blocked with 1% Marvel dried skimmed milk powder (Premier International Foods, Cambridge, UK), tripling serial dilutions of test sera added and bound IgG antibody detected by incubating with biotinylated rabbit F(ab')\(_2\) anti-mouse IgG (STAR11B; AbD Serotec, Oxford, UK) and ExtrAvidin Peroxidase conjugate (Sigma, Poole, UK). Sure Blue substrate (KPL, Gaithersburg, MD) was then added, the reaction stopped by the addition of 0.2 M H\(_2\)SO\(_4\), and the absorbance (OD 450 nm) measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK). For each sample, an absorbance versus dilution curve was plotted, and the area under the curve (AUC) was calculated (42). The AUC of an experimental sample was expressed as the percentage of positive control (pooled hyperimmune) serum.

In certain experiments mice were additionally immunized with Ovalbumin protein 100 µg in IFA subcutaneously. Anti-Ovalbumin antibody was assayed in a similar fashion, performed on test sera 4 weeks after immunization, using an OVA-specific ELISA.

**CD8 T cell IFN-γ ELISPOT**

CD8 T cell ELISPOT was performed as described (Sivaganesh et al., 2013). Briefly, purified CD8 T cells were mixed with irradiated BALB/c stimulator splenocytes and added to Multiscreen HTS filtration
system plates (Millipore, Billerica, MA) that had been coated with anti-mouse IFN-γ (BD Pharmingen) in 0.1M bicarbonate buffer, pH9.6. Plates were incubated at 37°C and 5% CO₂ for 20 h, and after washing, spots were developed with biotinylated rat anti-mouse IFN-γ (BD Pharmingen) followed by streptavidin-HRP and the substrate, H₂O₂ together with the 3-amino-9-ethylcarbazole (AEC) colour indicator. Plates were read (Autoimmun Diagnostika, Straßberg, Germany) and data expressed as spot counts per 10⁶ responder CD8 T cells for each well.

**Flow cytometry**

APC-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-mouse CD19 (1D3), PE-conjugated anti-mouse CD90.1/Thy1.1 (clone OX-7), PE–Cy7– conjugated anti-mouse CD4 (clone L3T4), PE-conjugated anti-mouse H-2Kd (SF1-1.1) and FITC-conjugated anti-mouse I-Aβ (clone AF6-120.11) were purchased from BD Pharmingen. Peripheral blood (depleted of erythrocytes by incubating with 0.17 M NH₄Cl red cell lysis buffer) and splenic single-cell suspensions were blocked with anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen), before staining with the relevant Abs and dead cell exclusion dye 7-aminoactinomycin D (7-AAD; BD Pharmingen). All cells were analyzed on a FACSCanto II flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA).

**T cell proliferation assay**

Single-cell suspensions of splenocytes obtained from TCR75 mice were stained with 5 μM CFSE (Invitrogen, Molecular Probes, Paisley, UK) in the dark for 5 min and then quenched with 5%FCS/PBS. CFSE-stained splenocytes (2x10⁶ to 5 x10⁶) were injected i.v. into recipient mice and spleens harvested 4 or 7 d later; flow cytometry was performed using allophycocyanin-conjugated anti-CD4 plus PE-conjugated anti-CD90.1/Thy1.1 to identify TCR75 T cells. Proliferation of WT BM12.Kd.IE CD4 T cells in B6 hosts was assessed similarly, by analysis of CSFE-staining three days after transfer of 5x10⁶ cells with the caveat that in contrast to transfer of a monoclonal population analysis was restricted to the relatively small (~5%) alloreactive population nested within a large
wildtype repertoire that did not undergo proliferation. Proliferation was quantified using FlowJo™ (Treestar, Oregon).

**Histology, immunohistochemistry and immunofluorescence**

Formalin-fixed hearts were paraffin-mounted and stained using haematoxylin and eosin and Weigert’s Elastin van Gieson (EVG) method to delineate the internal elastic lamina and the severity of AV assessed morphometrically as reported previously (Motallebzadeh et al., 2012). Complement C4d deposition was assessed on 7µm cryostat sections of donor heart allografts explanted after 50 days by an avidin-biotin-peroxidase technique (Vector Laboratories, Inc., USA), using unconjugated rat anti-mouse C4 mAb (16D2; Abcam Inc., USA), as described previously (Win et al., 2009). Germinal centres (GC) were quantified on 7µm cryostat sections of recipient spleens harvested 50 days following transplant by immunofluorescence staining of B220+ B cells using rat anti-mouse B220 (clone RA3-6B2, BD Pharmingen, San Diego, CA) detected with Cy3-conjugated goat anti-rat IgG (clone 112-165-143, Jackson ImmunoResearch Laboratories, West Grove, PA) and peanut agglutinin (PNA)+ GC B cells using FITC-conjugated PNA (Vector Laboratories, Peterborough, U.K.) as described previously (Conlon et al., 2012b). Numbers of PNA+ GC were expressed as a percentage of total (B220+) lymphoid follicles.

**In vivo depletion and transfer of donor and recipient lymphocyte subsets**

Donor mice were injected i.p. with 2 x 1.0 mg doses of depleting anti-CD4 mAb (YTS 191.1; hybridoma from the European Collection of Animal Cell Cultures) six and one day before heart graft procurement. Depletion of CD4 T cells (typically >99%) was confirmed by flow cytometric analysis of peripheral blood. To confirm cardiac parenchymal CD4 T cell depletion, donor hearts were homogenised following incubation with collagenase digestion buffer as previously described (Sivaganesh et al., 2013), with a single-cell suspension prepared by filtration through a 40-µm nylon cell strainer. CD4 T cells were quantified by flow cytometry, with a mean of 5137 CD4 T cells identified in an untreated donor heart.
In certain experiments recipients of CD4 T cell deplete allografts were adoptively transferred i.v. 1x10^7 donor CD4 T cells (purified with anti-mouse CD4 MicroBeads (Mitenyi Biotec, Bergisch Gladbach, Germany) using an autoMACS Separator (Mitenyi Biotec).

Depletion of the mature recipient B cell population was achieved by i.p. injection of 250μg depleting anti-CD20 mAb (18B12; gifted by Cherie Butts at Biogen Idec Boston MA, US) seven days prior to and 14 days after transplantation (Ueki et al., 2011). Depletion of B cells was confirmed by flow cytometry of PBLs the day before heart transplantation.

Depletion of the recipient NK cell population was achieved by i.p. injection of 500μg depleting anti-NK1.1 (PK136; hybridoma from the European Collection of Animal Cell Cultures) two and one day prior to transplant or cell transfer and three times weekly thereafter. Depletion of NK cells was confirmed by flow cytometry of PBMCs the day before transplantation/transfer.

Adoptive transfer studies of purified C57BL/6, BALB/c, bm12.K(6), bm12.K(6).IE CD4 T cells into C57BL/6, Tcrbd(−) and Tcrbd(−).bm12 mice were performed with i.v. injection of 1x10^7 cells purified as above.

**Statistics**

Mann-Whitney U test was used for analysis of nonparametric data. Two-way ANOVA was employed for comparison of intensity of HEp-2 fluorescence scores and anti-H-2K^d^ antibody levels. Graft survival was depicted using Kaplan-Meier analysis and groups compared by log-rank (Mantel-Cox) testing. Analysis was conducted using GraphPad 4 (Graph- Pad Software, San Diego, CA, USA). Values of \( P < 0.05 \) were considered significant.
Author contributions

Designed the experiments: IGH, JMA, JAB, MRC, TMC, GJP
Conducted the experiments: IGH, JMA, SJFH, EW, JA, MCN, MSQ, RM, KSP
Designed, developed and produced essential reagents: MCN
Wrote the first draft of the paper: IGH, JMA, GJP
Reviewed and edited the manuscript, approving the final version: IGH, JMA, SJFH, EW, JA, MCN, MSQ, RM, KSP, EMB, JAB, MRC, TMC, GJP
References and notes


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Figure Legends

Fig. 1. Heart allografts with isolated MHC class I and class II disparities provoke allo- and auto- antibody responses.

A series of backcrosses were performed to generate the ‘bm12.Kd.IE’ donor strain that differs from C57BL/6 recipient mice at the I-A locus and mismatched H-2Kd and I-E loci (a). In contrast to syngeneic heart transplants, bm12.Kd.IE heart allografts triggered robust and durable IgG anti-Kd and anti-I-E alloantibody (b) and anti-nuclear autoantibody (c) responses. These were associated with complement C4d deposition on allograft endothelium, which was not observed in allografts transplanted into T cell deficient Tcrbd−/− recipients (Scale bars, 100 μm) (d), and with development of progressive allograft vasculopathy (e). *P < 0.05, **P < 0.01 and ***P < 0.001 (2-way ANOVA in b,c, Mann-Whitney test in e). Data are representative of two independent experiments (b-e; mean and s.e.m. of n = 7 mice per group in b,c,e or n=4 mice per group in d).
Fig. 2. Passenger CD4 T lymphocytes within bm12.K^d.IE heart allografts trigger recipient anti-nuclear humoral autoimmunity.

Treatment of donor bm12.K^d.IE mice with anti-CD4 mAb resulted in depletion of CD4 T cells in the circulation (a) and within the heart (b) by the time of procurement of the heart graft six days later with flow cytometric analysis of PBMC and heart allograft homogenate. The recipient splenic CD4 T cell population was unaltered by the donor treatment (c), indicating that antibody was not carried-over to recipients. Depleting the donor CD4 T cell compartment (CD4 deplete bm12.K^d.IE) abrogated recipient IgG antinuclear autoantibody responses; these were restored by adoptively-transferring purified donor CD4 T cells to recipients at the time of transplant (d). *P < 0.05, **P < 0.01 and ***P < 0.001 (2-way ANOVA in d). Data are representative of one experiment (d; mean and s.e.m. of n = 7 mice per group) or two independent experiments (a-c; n = 6 mice per group).
Fig. 3. Graft-versus host recognition by passenger donor CD4 T cells within the heart allograft augments conventional host alloimmunity

Anti-K^d IgG alloantibody responses in C57BL/6 (B6) recipients of bm12.K^d.IE heart allografts from CD4 T cell depleted donors (CD4 deplete bm12.K^d.IE) were significantly attenuated, and restored by adoptive transfer of purified donor CD4 T cells to recipients at the time of transplant (a). Anti-I-E IgG responses were similarly abrogated (b). Cytotoxic CD8 T cell alloresponses in B6 recipients of unmodified bm12.K^d.IE heart allografts were much weaker than those observed in B6 recipients of BALB/c heart grafts, but nevertheless, significantly greater than those generated in recipients of CD4-T cell depleted bm12.K^d.IE heart allografts (c). Indirect-pathway CD4 T cell responses, detected by quantifying proliferation of CFSE-labelled, K^d-allopeptide-specific ‘TCR75’ CD4 T cells transferred four weeks after transplant, expressed as a percentage of parent population divided (boxes) (d), were similarly reduced in B6 recipients of CD4 T cell-depleted bm12.K^d.IE heart allografts (e). Donor CD4 T cell-mediated augmentation of host indirect-pathway CD4 T cell responses was not observed in B cell depleted B6 recipients (e). *P < 0.05, **P < 0.01 and ***P < 0.001 (2-way ANOVA in a,b, and Mann-Whitney test in c,e). Data are representative of one experiment (c-e; mean and s.e.m. of n = 6 mice per group in c,e or n = 6 mice per group in d) or two independent experiments (a,b; mean and s.e.m. of n = 6 mice per group).
Fig. 4. Graft-versus host recognition by passenger donor CD4 T cells accelerates heart allograft rejection, but is dependent upon the host T-B cell axis.

Compared to C57BL/6 (B6) recipients of T cell-replete bm12.K\textsuperscript{d}.IE heart allografts, allograft vasculopathy was significantly less severe in allografts from T cell-depleted donors (a), with all hearts beating strongly at harvest (b); vasculopathy was restored by adoptive transfer of purified donor CD4 T cells at transplant (CD4 reconstitution) (a). Conversely, transplantation of B6 recipients with heart allografts from bm12.K\textsuperscript{d}.IE donors challenged with a B6 skin graft (challenged donor) provoked stronger anti-nuclear IgG autoantibody (c) and anti-K\textsuperscript{d} IgG alloantibody (d) responses, with heart grafts rejected more rapidly (b). Allograft rejection is dependent upon host T and B cells (a), with the kinetics of rejection and the development of allograft vasculopathy in the different experimental groups mirroring recipient splenic germinal centre activity (e). Host B cells are not required for optimal graft-versus-host activation of donor CD4 T cells, because upon transfer of CFSE-labelled bm12.K\textsuperscript{d}.IE CD4 T cells, the alloreactive fraction (f – boxed) divided similarly robustly in wild-type and B cell-depleted B6 recipients (f). Compared to wild-type B6 recipients, transplantation of bm12.K\textsuperscript{d}.IE allografts into T cell-deficient Tcrbd\textsuperscript{-/-} B6 recipients provoked similar antinuclear IgG autoantibody responses (g), but weak and transient anti-K\textsuperscript{d} IgG alloantibody with higher levels of alloantibody than observed in control naïve serum achieved at week 1 (inset panel) (h), without development of splenic germinal centre activity (e). Whereas adoptively-transferred bm12.K\textsuperscript{d}.IE CD4 T cells are readily detectable 7 days after transfer into Rag-2\textsuperscript{-/-} B6 mice, they were undetectable after transfer into wild-type B6 or Tcrbd\textsuperscript{-/-} mice (i). *P < 0.05, **P < 0.01 and ***P < 0.001 (Mann-Whitney test in a,e,h (inset box) [comparisons in a and e are to the bm12.K\textsuperscript{d}.IE group], Log Rank (Mantel-Cox) test in b and 2-way ANOVA in c,d,g,h). Data are representative of one experiment (a-f,i; mean and s.e.m. of n = 4 mice per group in a-f or n = 3 mice per group in i) or two independent experiments (g,h; mean and s.e.m. of n = 4 mice per group).
Fig. 5. Peptide-independent recognition of the B cell MHC class II complex by donor CD4 T cells promotes plasma cell differentiation, but requires concurrent BCR ligation

Seven days after transfer of bm12 CD4 T cells into C57BL/6 (B6) hosts, flow cytometric analysis of the splenic CD19<sup>+</sup> B cell compartment demonstrates global upregulation of MHC class II expression, not evident upon transfer of syngeneic C57BL/6 CD4 T cells (a). Whereas intravenous transfer of Tcrbd<sup>−/−</sup> B6 mice with bm12 CD4 T cells provoked antinuclear IgG autoantibody (b), only those mice simultaneously immunised subcutaneously with ovalbumin (OVA) protein developed anti-OVA IgG (c). Similarly, intravenous transfer of Tcrbd<sup>−/−</sup> B6 mice with either purified bm12 CD4 T cells or bm12 CD4 T cells that expressed H-<sup>2</sup>K<sup>d</sup> transgene (bm12.K<sup>d</sup>) provoked antinuclear IgG autoantibody (d), but anti-K<sup>d</sup> IgG alloantibody was only generated in Tcrbd<sup>−/−</sup> B6 mice that received bm12.K<sup>d</sup> CD4 T cells (e). Adoptive transfer of purified bm12.K<sup>d</sup> CD4 T cells into T cell deficient Tcrbd<sup>−/−</sup> bm12 recipients of a BALB/c heart allograft confirmed that bm12 and bm12.K<sup>d</sup> CD4 T cells can provide help for generating humoral alloimmunity, as determined by flow cytometric detection of bound test sera to target BALB/c BMDCs (f), but that bm12.K<sup>d</sup> CD4 T cells are tolerant to self (I-<sup>A</sup><sub>bm12</sub>)-restricted H-2<sup>K</sup><sup>d</sup> peptide and do not provide help for generating anti-K<sup>d</sup> IgG alloantibody (g). *<i>P</i> < 0.05, **<i>P</i> < 0.01 and ***<i>P</i> < 0.001 (Mann Whitney test in c,f and 2-way ANOVA in e,g). Data are representative of one experiment (b-g; mean and s.e.m. of n = 4 mice per group) or three independent experiments (a; n = 6 mice per group).
Fig. 6. NK cell allore cognition is essential for preventing GVH-mediated amplification of the host adaptive alloimmune response.

Flow cytometric analysis of the peripheral blood mononuclear cell fraction demonstrates that whereas C57BL/6, bm12 and bm12.K\textsuperscript{d} CD4 T cells survive long-term following adoptive transfer into Rag-2\textsuperscript{-/-} mice, BALB/c CD4 T cells are rapidly undetectable (a). Analysis of sera four weeks after transfer reveals that unlike transfer of bm12.K\textsuperscript{d} CD4 T cells, transfer of BALB/c CD4 T cells to Tcrbd\textsuperscript{-/-} C57BL/6 mice does not provoke anti-K\textsuperscript{d} IgG alloantibody (b) or antinuclear IgG autoantibody (c). In contrast, circulating BALB/c CD4 T cells are detectable two weeks after transfer into C57BL/6 Rag-2\textsuperscript{-/-} mice depleted of NK cells by administration of anti-NK1.1 mAb (d), and their transfer into NK cell-depleted Tcrbd\textsuperscript{-/-} C57BL/6 mice provokes anti-K\textsuperscript{d} IgG alloantibody (b) and antinuclear autoantibody (c). Anti-nuclear autoantibody responses are not generated by adoptive transfer of BALB/c CD4 T cells into wild-type C57BL/6 mice unless NK cells are first depleted (e). *\(P < 0.05\), **\(P < 0.01\) and ***\(P < 0.001\) (2-way ANOVA in a,e and Mann Whitney test in b,c,d). Data are representative of two experiments (mean and s.e.m. of n = 4 mice per experimental group).
Fig. 7. Host NK cell alloreactivity is critical for preventing donor passenger lymphocytes from augmenting host adaptive alloimmunity.

A model of alloantibody-mediated allograft vasculopathy was developed in which $10^3$ TCR75 CD4 T cells are transferred into C57BL/6 Tcrbd−/− mice at transplant with a BALB/c heart allograft (a). In contrast to reconstituted Tcrbd−/− (CD4+ve Tcrbd−/−) recipients, depletion of NK cells in the reconstituted Tcrbd−/− recipients (NK cell−ve host) results in more rapid allograft rejection (b), stronger anti-$\kappa^d$ IgG alloantibody responses (c), and the generation of anti-nuclear autoantibody (d). The impacts of recipient NK cell-depletion were ameliorated by depleting CD4 T cells in donor mice (CD4−ve donor NK cell−ve host) prior to heart allograft procurement (b-d). Analysis of allograft vasculopathy was not performed due to the rapid rejection of the NK cell-deplete recipient group.

Acute rejection of BALB/c heart allografts was prevented by administration of anti-CD154 mAb at transplant to C57BL/6 recipients. (e) Anti-nuclear IgG autoantibody responses five weeks after transplant in recipients of heart allografts from unmodified BALB/c donors or from BALB/c donors challenged with a C57BL/6 heart allograft six weeks earlier or from similarly-primed BALB/c donors that were depleted of CD4 T cells prior to heart allograft procurement. An additional group of recipients of allografts from primed donors were depleted of NK cells at transplant. (f). Corresponding splenic germinal centre activity in the above recipient groups five weeks after transplantation.

*P < 0.05, **P < 0.01 and ***P < 0.001 (Log Rank (Mantel-Cox) test in a, 2-way ANOVA in b,c). Data are representative of two independent experiments (b-d; mean and s.e.m. of n = 5 mice per group b,d, or n=5 mice per group c).
Fig. 8. Proposed model for augmentation of host adaptive alloimmunity by passenger lymphocytes.

NK cell allore cognition normally results in rapid destruction of donor passenger lymphocytes within solid organ allografts (1). If NK cell allore cognition is avoided, ‘peptide-degenerate’ graft-versus-host recognition by donor CD4 T cells can activate all recipient B cells (2), but class-switched antibody secretion is dependent upon simultaneous ligation of BCR with target antigen (3). Activated B cells drive enhanced activation of host CD4 T cells with indirect allospecificity (4), which provide reciprocal help for development of germinal centre alloantibody responses (5), presumably reflecting unique T follicular helper cell function of host CD4 T cells in providing cognate, allopeptide-specific help (5). This results in long-term augmentation of humoral alloimmunity (6), with more rapid progression of allograft vasculopathy and early allograft failure (7). Enhanced activation of indirect-pathway CD4 T cells may also contribute to allograft rejection through the provision of help for generating heightened host CD8 T cell cytotoxic alloresponses (8).