CD8 T-cell recognition of acquired alloantigen promotes acute allograft rejection

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Classification: Immunology and inflammation

Running title: Semi-direct allorecognition

Key words: semi-direct allorecognition, CD8 cytotoxicity, allorecognition, alloimmunity

ABSTRACT

Adaptive CD8 T cell immunity is the principal arm of the cellular alloimmune response, but its development requires help. This can be provided by CD4 T cells that recognise alloantigen 'indirectly', as self-restricted allopeptide, but this process remains unexplained, because the target epitopes for CD4 and CD8 T cell recognition are 'unlinked' on different cells (recipient and donor antigen presenting cells (APCs), respectively). Here, we test the hypothesis that the presentation of intact and processed MHC class I alloantigen by recipient dendritic cells (the 'semi-direct' pathway) allows linked help to be delivered by indirect-pathway CD4 T cells for generating destructive cytotoxic CD8 T cell alloresponses. We show that CD8 T cell-mediated rejection of murine heart allografts that lack haematopoietic APCs requires host secondary lymphoid tissue (SLT). SLT is necessary because within it, recipient dendritic cells can acquire MHC from graft parenchymal cells and simultaneously present it as intact protein to alloreactive CD8 T cells and as processed peptide alloantigen for recognition by indirect-pathway CD4 T cells. This enables delivery of essential help for generating cytotoxic CD8 T cell responses that cause rapid allograft rejection. In demonstrating the functional relevance of the semi-direct pathway to transplant rejection, our findings provide a solution to a long-standing conundrum as to why SLT is required for CD8 T cell allorecognition of graft parenchymal cells, and suggest a mechanism by which indirect-pathway CD4 T cells provide help for generating effector cytotoxic CD8 T cell alloresponses at late time points after transplantation.

SIGNIFICANCE STATEMENT

Cytotoxic CD8 T cell responses against mismatched MHC class I alloantigen are the principal arm of the cellular response against a transplanted organ. How CD4 T cells deliver essential help for development of these cytotoxic responses remains unclear. Here we show that recipient dendritic cells present acquired MHC alloantigen both as intact protein, for recognition by cytotoxic CD8 T cells, and as processed allopeptide, for recognition by helper CD4 T cells. Our findings suggest a mechanism by which help is provided for generating effector cytotoxic CD8 T cell alloresponses at late time points after transplantation, and solve a long-standing conundrum as to why host lymphoid tissue is required for CD8 T cell allorecognition of graft parenchymal cells.

INTRODUCTION

Cytotoxic CD8 T cell responses directed against MHC Class I alloantigens are one of the principal mediators of acute allograft rejection [1, 2]. Exceptionally, at very high precursor frequency, cytotoxic CD8 T cells can effect graft rejection autonomously [3], but generally, differentiation of naïve CD8 T cells to fully functional cytotoxic effector cells capable of mediating acute allograft rejection requires help from activated CD4 T cells. How such help is delivered remains unclear. Studies of conventional CD8 T cell responses against non-transplant antigens have demonstrated that CD4 T cell help does not involve cognate cell-surface interaction between the helper CD4 T cell and cytotoxic CD8 T cell, but that instead, help is delivered to an intermediary antigen presenting cell (APC), which is then licensed to prime CD8 T cells [4-6]. A critical requirement for such help is the expression of both the CD4 and CD8 T cell epitopes on the same APC, and an analogous three-cell cluster model (Figure 1A) in transplantation is only possible if help for CD8 T cells is provided by CD4 T cells that recognise intact MHC class II alloantigen on the surface of donor APC, via the so-called *direct-pathway* [7]. Murine studies have confirmed that restricting CD4 T cell help to the direct pathway generates strong cytotoxic CD8 T cell alloresponses that effect rapid allograft rejection [8].

The indirect-pathway of allorecognition, whereby CD4 T cells recognise processed alloantigen as self-restricted allopeptide [7, 9], is now viewed as being at least as important as the direct pathway for initiating and mediating allograft rejection. This partly reflects the unique ability of indirect-pathway CD4 T cells to act as helper T cells for generating sophisticated alloantibody responses [10], but it is now also clear that effective cytotoxic CD8 T cell responses can be generated when CD4 T cell help is restricted exclusively to the indirect-pathway [11]. This is surprising, because the delivery of indirect-pathway T cell help is only readily explained by postulating the unlikely formation of a cumbersome four-cell cluster, comprising CD4 and CD8 T lymphocytes and both recipient and donor APC (Figure 1b); a cluster in which there are no apparent cell surface ligands to enable physical linkage between the donor APC / recipient CD8 T cell couplet and the recipient APC / CD4 T cell couplet. This raises concerns of potentially uncontrolled CD8 T cell alloimmunity, because such

'unlinked' help could in principle be provided by concurrent exposure to any unrelated antigen.

The appreciation that many types of cells, but particularly DCs, can capture ('trogocytose') membrane fragments from other cells [12, 13] has prompted the proposal that cytotoxic CD8 T cell alloimmunity may be initiated by a 'semi-direct' pathway, whereby intact donor MHC class I alloantigen is recognised after its acquisition onto the surface of recipient DCs. In support, murine studies have detailed *in vivo* capture of membrane alloantigen by host cells [14, 15]. This phenomenon provides a possible mechanism by which the same recipient APC as re-presents intact MHC class I alloantigen could simultaneously present processed allopeptide for recognition by indirect-pathway CD4 T cells. This would enable formation of a linked three-cell cluster (Figure 1c) that obviates many of the concerns associated with the above four-cell cluster model. However, although we have recently demonstrated simultaneous presentation of intact and processed alloantigen by recipient DCs following heart transplantation [16], the contribution of trogocytosis of alloantigen to graft rejection has yet to be clarified. To have functional relevance, presentation of intact donor alloantigen by recipient APCs must, at least in certain circumstances, be more effective for triggering cytotoxic alloimmunity than conventional encounter on donor APCs, and this has not been shown. Addressing this key concern is challenging, because it requires development of an experimental model in which CD8 T cell allorecognition of target MHC class I alloantigen is limited to the surface of recipient, but not donor, APCs. One way this could perhaps be achieved is by resolving the paradox created by the seminal observations that CD8 T cells can still effect allograft rejection when target MHC class I alloantigen is expressed by graft parenchyma only [17], but that rejection of vascularised allografts does not occur in the absence of secondary lymphoid tissue [18]. Why secondary lymphoid tissue (SLT) is necessary for CD8 T cell allorecognition of graft parenchymal cells has not been explained, but one elegant and compelling solution is that alloreactive CD8 T cell activation occurs in lymphoid tissue because shed, intact MHC class I alloantigen is only presented by recipient APCs at these sites.

Here, we show that rejection of heart allografts in mice lacking haematopoietic APCs is mediated by alloreactive CD8 T cells, and that the participation of CD8 T cell is dependent upon the acquisition and presentation of intact MHC Class I alloantigen by recipient DCs within host SLT. We further demonstrate that re-presentation of intact alloantigen enables the delivery of essential help from indirect-pathway CD4 T cells. These findings provide strong functional support for the provision of indirect-pathway CD4 T cell help to alloreactive CD8 T cells via a 'linked' three-cell cluster model.

RESULTS

The requirement for secondary lymphoid tissue for differentiation of effector cytotoxic CD8 T cell alloimmune responses

Key to the proposed 'semi-direct' pathway for generating CD8 T cell alloimmunity is presentation of intact MHC Class I alloantigen by recipient APCs. In the expectation that this presentation would occur chiefly within host SLT, we first sought to determine under what circumstances lymphoid tissue was required for effector CD8 T cell activation and cardiac allograft rejection, by incorporating alymphoplastic (*aly/aly)* mice that lack lymph nodes and Peyer's patches due to a point mutation in the NF-kB-inducing kinase gene [19]. We also incorporated TCR-transgenic *Rag1*$^{-/-}$ 2C TCR (2C) mice, whose CD8 T cells recognise H-2L^{d} alloantigen [20], in the anticipation that when present at high frequency, 2C CD8 T cell activation would not require T cell help, and that this would, for the initial set of experiments, eliminate the confounding role of SLT in initiating helper CD4 T cell responses.

As previously reported [18], splenectomised *aly/aly* H-2^{b} mice did not reject BALB/c cardiac allografts (Figure 2a). However, increasing the precursor frequency of the alloreactive CD8 T cell population in the recipients by adoptive transfer of 2C CD8 T cells into the splenectomised *aly/aly* recipients restored prompt rejection of BALB/c heart grafts (median survival time (MST) = 10 days, Figure 2a). We reasoned that the rapid graft rejection mediated by 2C CD8 T cells without a requirement for SLT reflected the ability of the transferred 2C CD8 T cells to interact with professional donor APCs bearing target MHC Class I alloantigen, perhaps within the parenchyma of the heart graft. To test this, heart donors were depleted of haematopoietic APCs prior to heart graft retrieval by administering lethal irradiation (Figure s1); a strategy that resulted in depletion of all MHC class II-expressing APCs from the graft parenchyma, as demonstrated by the inability of heart grafts from irradiated mice to provoke direct-pathway CD4 T cell alloresponses (Figure 2b). Heart grafts that were depleted of haematopoietic cells were not rejected by recipients that lacked SLT, even after adoptive transfer of a 2C CD8 T cell effector population (Figure 2c). In contrast, when irradiated BALB/c grafts were transplanted into unmodified *Rag1*$^{-/-}$

2C TCR mice (that harboured SLT), rapid rejection ensued and strong IFN-γ CD8 T cells responses were elicited in the 2C CD8 T cell population (Figure 2c and 2d). The inability of the transferred 2C CD8 T cells to effect rejection in SLT-deficient recipients was not due to lack of target alloantigen within the irradiated hearts, because transfer of activated 2C CD8 T cells provoked rapid heart graft rejection (Figure 2c). Similarly, irradiated BALB/c hearts were rejected in wild-type C57BL/6 (B6) recipients (albeit at a slightly slower tempo than when transplanted into *Rag1*$^{-/-}$ 2C TCR mice [Figure 2c]), and provoked an IFN-γ CD8 T cell alloresponse within the endogenous CD8 T cell population that, although initially weaker than the response provoked by an unmodified BALB/c heart graft, was, by day 12, of similar intensity (Figure 2e). Thus, although heart allografts can be rejected in the absence of host lymphoid tissue, this is dependent upon the presence of donor haematopoietic APCs within the graft, and requires an unusually high precursor frequency of alloreactive CD8 T cells; lymphoid tissue is normally required to facilitate productive effector CD8 T cell alloresponses and for rejection of heart allografts devoid of haematopoietic APCs.

Intact MHC alloantigen is presented by host dendritic cells within SLT

We hypothesised that the requirement for lymphoid tissue to facilitate CD8 T cell mediated rejection of allografts lacking haematopoietic APCs likely reflected a critical role for SLT as the site for presentation of alloantigen (either processed or intact) by recipient DCs. This was examined by creating CD11c-DTR [21] to B6 bone-marrow chimeric recipients (CD11c-DTR-BM), in which it was possible to administer diphtheria toxin at high enough doses to induce profound depletion of recipient DCs. The CD11c-DTR-BM recipients were reconstituted with an effector 2C CD8 T cell population (not on a DTR background and therefore not susceptible to diphtheria toxin administration), and also received depleting anti-CD4 mAb, to exclude the confounding role for host DCs in activating host CD4 T cells. As expected, CD11c-DTR-BM mice that were reconstituted with 2C CD8 T cells, but that did not receive diphtheria toxin, rejected irradiated BALB/c heart grafts rapidly (MST 13 days; Figure 3a), with development of moderately strong CD8 T cell alloimmunity (Figure 3b). In contrast, depletion of the host DCs prolonged allograft survival significantly (MST 26

days, Figure 3a), albeit heart allografts were still rejected, perhaps indicating that even high doses of diphtheria toxin do not achieve complete or prolonged destruction of the recipient DC population [22, 23]. Graft rejection was dependent upon the transferred 2C CD8 T cell population, because depletion of CD4 T cells prevented, as expected [10], the generation of alloantibody (not shown) and it is notable that CD4 T cell depleted CD11c-DTR-BM mice that were otherwise unmodified neither rejected irradiated BALB/c heart grafts nor mounted IFN-γ CD8 T cell alloresponses (Figures 3a and 3b).

This necessity for host DCs to activate CD4 T cell-independent CD8 T cell alloimmunity likely reflects their role in presenting intact MHC class I alloantigen acquired from donor cells. In support, although as previously reported [16], we were unable to demonstrate acquired MHC class I alloantigen on the surface of host DCs following transplantation (not shown), DCs purified from B6 recipients of an irradiated BALB/c heart graft, provoked an IFN-γ CD8 T cell response upon transfer to secondary naïve B6 mice, albeit this response was weaker than following transfer of DCs purified from recipients of unmodified BALB/c heart allografts (figure 3c). In addition, whereas *in vitro* culture of purified naïve B6 CD8 T cells with BALB/c endothelial cells generated only minimal CD8 T cell proliferation, the response was enhanced by addition of B6 DCs to the culture (Figure 3d), implying that the DCs acquired and presented intact MHC class I alloantigen from the BALB/c endothelial cells. Hence these *in vivo* and *in vitro* data highlight a functional role for presentation of intact MHC class I alloantigen on recipient DCs in triggering cytotoxic CD8 T cell alloimmunity.

The presentation of processed alloantigen by host dendritic cells enables delivery of indirect-pathway help for generating cytototoxic CD8 T cell alloimmunity.

The major teleological advantage of presentation of intact MHC class I alloantigen by host DCs is the potential for the responding alloreactive CD8 T cells to receive linked help via a three-cell cluster from CD4 T cells that simultaneously recognise self-restricted processed alloantigen presented on the same APC (Figure 1c). Hence, although the transfer of large numbers of 2C CD8 T cells in the preceding

experiments facilitated examination of the presentation of intact alloantigen by recipient DCs, the lack of requirement for CD4 T cell help in initiating this response prevented definitive study of the three-cell cluster model. However, it is notable that irradiated BALB/c hearts that do not provoke a direct-pathway CD4 T cell response are rapidly rejected in WT B6 mice (Figure 2c), provoke strong CD8 T cell alloimmunity (Figure 2e), and that rejection in unmodified CD11c-DTR-BM recipients is CD4 T cell dependent (Figure 3a). Although these observations suggest a role for indirect-pathway CD4 T cells and alloantigen presentation by host DCs in triggering effector CD8 T cell alloimmunity, the generation of alloantibody (Figure 4a) from preservation of indirect-pathway helper CD4 T cell alloresponses [10] complicates interpretation of the contribution of CD8 T cell cytotoxicity to rejection.

To distinguish the role of alloantigen presentation by host DCs in generating cytotoxic CD8 T cell alloimmunity from that of the production of alloantibody, a further series of experiments were performed, in which irradiated BALB/c heart allografts were transplanted into CD11c-DTR BM chimeric mice as above, and with recipient humoral alloimmunity blocked by depletion of host B cells (Figure 4a, Figure s2). B cell depletion did not compromise rejection of irradiated BALB/c heart allografts by B6 mice (Fig 4b). All grafts were rejected rapidly, with rejection dependent upon host CD8 T cells (Figure 4b) and associated with development of strong IFN-γ CD8 T cell alloimmunity (Figure 4c). As above (Figures 3a), rejection was delayed by ablation of recipient DCs (Figure 4b). Critically, however, host CD4 T cells were also required to effect rapid graft loss (Figures 4b and c).

The graft rejection kinetics, coupled with the CD8 T cell ELISPOT data, strongly suggest that the cytotoxic CD8 T cell response is mediating graft rejection, but to confirm this, B cell-depleted 'GzmBCrexRosa26YFP' H-2^b mice [24] were challenged with an irradiated BALB/c heart allograft and tamoxifen administered concurrently. CD8 T cells in these recipients that differentiate into granzyme B-expressing effector cells are indelibly marked with enhanced yellow fluorescence protein (YFP). Ten days after transplantation, populations of YFP^{+ve} effector CD8 T cells were readily detectable within the spleen and within the rejecting heart allograft (Figure 4d), with a higher proportion of recovered CD8 T cells from the heart allograft expressing YFP

(Figure 4e). Similarly, because irradiated heart allografts do not provoke a direct-pathway CD4 T cell response (Figure 2b), help for cytotoxic CD8 T cells can theoretically only be provided by indirect-pathway CD4 T cells. To confirm this, *Rag2*$^{-/-}$ C57BL/6 mice were reconstituted with GzmBCrexRosa26YFP CD8 T cells and with TCR75 CD4 T cells that recognise donor H-2K^{d} alloantigen as self-restricted, processed allopeptide exclusively via the indirect pathway. Whereas irradiated BALB/c grafts were not rejected in *Rag2*$^{-/-}$ C57BL/6 recipients reconstituted with TCR75 CD4 T cells only (Figure 4f), TCR75 CD4 T cells provided help for differentiating effector cytotoxic CD8 T cell responses (Figure 4e) that were responsible for rapid allograft rejection (Figure 4f).

When coupled to the earlier observation that host DCs are essential for CD4 T-cell independent 2C CD8 T cell alloresponses (Figures 2c and d), these latter experiments provide functional support for the three-cell cluster model and presentation of intact MHC class I alloantigen by host cells for generating cytotoxic alloimmunity (Figure 1c), with linked help provided by indirect-pathway CD4 T cells.

DISCUSSION

The present studies address the important question of how CD4 T cell help is provided to alloreactive CD8 T cells for the development of destructive cytotoxic responses, and our results help to resolve a long-standing debate on the mechanism by which help is delivered by CD4 T cells with indirect allospecificity. The use of donor mice deleted of haematopoietic cells, allied to ablation of DCs or absence of host SLT enabled us to confirm that in certain circumstances, host DCs were critical for priming cytotoxic CD8 T cell responses that effected rapid allograft destruction. In doing so, our findings provide a solution to the paradox created by two earlier landmark studies that observed that SLT was required for rejection of a vascularised allograft [18], but that cytotoxic CD8 T cells could mediate allograft rejection when expression of target MHC class I alloantigen was limited to graft parenchyma [17]. Moreover, because our model was refined to exclude the contribution of alternative effector mechanisms of graft rejection, we believe our manuscript is the first to demonstrate the functional relevance of the 'semi-direct' pathway to transplant rejection.

The means by which immune cells acquire or 'trogocytose' membrane proteins from other cells are becoming better understood [12]. Most data relate to acquisition of MHC-peptide complexes from the surface of APC by responding T cells; a process dependent upon downstream signalling of the TCR [25]. The immunological consequences of such trogocytosis are still not clear and may include: increasing competition between T cells through reduction in available target MHC / peptide epitope; increased T cell activation from continued positive signalling via the internalised complexes; and regulatory T cell – T cell interaction. Trogocytosis may hold particular relevance for transplantation, because the ability to transfer allogeneic membrane proteins between donor and recipient immune cells is likely to have more profound consequences than transfer of self-proteins. Nevertheless, few studies have reported functional significance for either solid organ or haematopoietic stem cell transplantation [26, 27] and our findings thus emphasise the potentially important contribution of trogocytosis in initiating adaptive alloimmunity.

As to why host DCs were required in our experiments for generating CD4 T cell-independent, cytotoxic 2C CD8 T cell alloresponses to an irradiated heart allograft, this presumably reflects that intact MHC class I alloantigen is re-presented by host DCs in association with the necessary co-stimulatory ligands for effector differentiation of the 2C CD8 T cells. This in turn suggests that the DCs have been activated by acquisition of alloantigen. One would perhaps anticipate that DCs would only express intact alloantigen for a short period, because of their principal role as antigen processors, but retention of soluble, unprocessed antigen within DCs for subsequent re-presentation to B cells has been described [28, 29]. Whether this function is restricted to a particular DC subset is not clear. In any event, even transient presentation of intact alloantigen by the host DC may be sufficient to drive full effector activation of the alloreactive CD8 T cell fraction, because unlike CD4 T cells [30], brief engagement with the CD8 TCR may be sufficient to trigger an 'autopilot' response [31] and multiple rounds of division [32].

Although we studied a solid organ transplant model, our findings may be equally pertinent to the delivery of CD4 T cell help for development of cellular graft-versus-host responses following haematopoietic stem cell transplantation. As above, direct-pathway donor CD4 T cell responses against host will be reliant upon recognition of host MHC class II alloantigen on the surface of host professional APCs. Such recognition will not occur with ablation of host bone marrow and attainment of full haematopoietic chimerism; instead, the dominant pathway for triggering donor CD4 T cell alloresponses is likely the presentation of processed host alloantigen by donor APCs. These indirect-pathway CD4 T cells could theoretically provide 'linked' help to alloreactive donor CD8 T cells for generating cytotoxic graft versus host responses, assuming that target host intact MHC class I alloantigen is first acquired onto the surface of the same donor APC as presents processed allopeptide to the helper CD4 T cell.

It is striking that host SLT is required for 2C CD8 T cell mediated rejection of irradiated heart allografts, but not for rejection of wild-type heart grafts with an intact haematopoietic component. This presumably relates to differences in the availability of target MHC class I alloantigen; that even in the absence of SLT, the

artificially high frequency of TCR-transgenic, alloreactive CD8 T cells can interact productively with the large numbers of donor DCs normally released from a heart allograft. In contrast, much fewer host DCs are likely to acquire and re-present intact MHC class I alloantigen, and SLT may consequently be needed to facilitate their encounter with alloreactive CD8 T cells. Whether the recipient DCs capture alloantigen within the graft and then transit to the regional lymphoid tissue or whether shed alloantigen is acquired by DCs resident within SLT is not clear, and is the focus of on-going investigation.

Our experiments involving WT, non-transgenic CD8 T cell responders demonstrated that CD4 T cell help was critical for cytotoxic, effector differentiation. We have previously demonstrated simultaneous presentation of intact and processed alloantigen by the same DC [16]. Within the SLT, this dual presentation presumably allows the indirect-pathway helper CD4 T cell to license the DC for effective presentation of MHC class I alloantigen to the alloreactive CD8 T cell, that binds either simultaneously, or sequentially [4], to the same DC. It is important to acknowledge, however, that our experiments do not provide definitive proof of this three cell interaction. Our conclusion is based on the observations that host DCs are essential for both helper cell-independent CD8 T cell alloresponses against MHC class I expressed on parenchymal graft cells, and, when responder CD8 T cell numbers are limited, for generating essential indirect-pathway CD4 T cell help. Hence our experiments do not exclude the possibility that indirect-pathway CD4 T cell help is provided through interaction with one particular host DC subset, while the alloreactive CD8 T cells are activated through interaction with another. This seems unlikely, because it would again represent an unlinked four-cell cluster, and the effectiveness, and control, of CD8 T cell activation is likely to be much greater if the helper CD4 T cell and alloreactive CD8 T cell determinants are expressed on the same host DC. It would be theoretically possible to visualise this interaction using dynamic intravital microscopy [33], but such an approach would neither provide information relating to cytotoxic differentiation nor its functional relevance to graft rejection. A further outstanding question relates to the manner of indirect-pathway CD4 T cell help. It is unclear whether there is the requirement for cell-cell contact,

through CD40/CD40L interactions for example, or whether paracrine IL-2 secretion is sufficient to provide the necessary help for CD8 T cell activation.

MATERIALS AND METHODS

Animals

Wildtype (wt) C57BL/6 (B6; H-2^b) and BALB/c (H-2^d) mice were purchased from Charles River Laboratories (Harlow, UK). Bm12 mice - B6(C)-H2-Ab1bm12/KhEgJ (H-2^{bm12}) and C57BL/6 CD11c-DTR (H-2^b) mice [34] were purchased from The Jackson Laboratory (Bar Harbor, ME). ALY/NscJcl-aly/aly (aly/aly; H-2^b) mice were purchased from CLEA Inc. (Japan). TCR transgenic *Rag1*$^{-/-}$ 2C TCR (2C; H-2^b) mice [20] were gifted by Geetha Chalasani (University of Pittsburgh, PE). C57BL/6 *Rag2*$^{-/-}$ mice (H-2^b) were gifted by Prof T Rabbitts (University of Cambridge, UK). C57BL/6-Tg(K^d)RPb mice (B6.K^d; H-2^b) that express transgenic H-2K^d were gifted by Dr R.P. Bucy (University of Alabama, Birmingham, AL [35]). TCR transgenic *Rag1*$^{-/-}$ TCR75 mice (TCR75; H-2^b), specific for I-A^b restricted H-2K^d_{54-68} peptide were gifted by Prof P. Bucy (University of Alabama, Birmingham, AL [36]). GzmBCrexRosa26YFP (H-2^b) mice [24] were gifted by Prof D Fearon (University of Cambridge, UK).

Heterotopic cardiac transplantation

Vascularised cardiac allografts were transplanted intra-abdominally using the technique of Corry and colleagues[37]. Splenectomy was performed in aly/aly recipient mice prior to implantation of the donor organ. In experiments involving GzmBCrexRosa26YFP mice as recipients or transfer of purified GzmBCrexRosa26YFP CD8 T cells, 1mg tamoxifen was administered i.p. to the recipient the day before and every second day after transplant. Graft rejection was defined as cessation of palpable myocardial contraction, confirmed at explant.

Purification and adoptive transfer of leucocyte subsets

2C transgenic CD8 T cells were purified from *Rag1*$^{-/-}$ 2C TCR mice using an autoMACS Separator (Mitenyi Biotec). 'Activated' 2C TCR CD8 were obtained by purification from *Rag1*$^{-/-}$ 2C TCR mice challenged one week earlier with a BALB/c heterotopic heart graft. For adoptive transfer, 10^6 purified 2C TCR transgenic CD8 T cells were administered i.v. on the day of transplantation. In certain experiments, *Rag2*$^{-/-}$ mice were reconstituted with 10^5 TCR75 CD4 T cells and 10^6 GzmBCrexRosa26YFP CD8 T

cells, purified from respective host mice using an autoMACS Separator (Mitenyi Biotec).

Dendritic cells were purified from recipient mice spleens four days after transplantation with a heart graft and adoptively transferred into naïve secondary C57Bl/6 mice as described previously [16]. Naïve secondary mice received 3 x 10^6 cells (the approximate total recovered from four recipients) transferred i.v.

Generation of bone marrow chimeras

Chimeric mice that were CD11c-DTR$^+$ only in the haematopoietic compartment (CD11c-DTR-BM mice) were created by lethally-irradiating C57BL/6 mice (13 Gy in 2 x 6.5 Gy fractions) and reconstituting with 2 x 10^7 bone marrow cells from C57BL/6.CD11c-DTR mice. Such chimeric mice tolerate high doses of diphtheria toxin necessary to induce profound depletion of the DC fraction without developing the severe toxicity associated with those doses in standard CD11c-DTR mice [38]. Chimerism was confirmed by flow cytometric analysis of PBMCs 4 weeks following reconstitution.

Dendritic cell, T and B cell depletion.

CD11c-DTR-BM chimeric heart graft recipients were treated with i.p. 32ng/g diphtheria toxin (List Biological Laboratories Inc. (Campbell, CA) on days -3, -1 and +1 in relation to transplantation and thrice weekly thereafter. For B cell depletion, recipients were treated with 250µg i.p. depleting anti-CD20 mAb (18B12, IgG2a gifted by Cherie Butts, Biogen Idec Boston, MA) on day -7 and fortnightly thereafter. Because of concerns that CD8 T cells are resistant to treatment with depleting anti CD8 mAb [11], combined B and CD8 T cell depletion was instead achieved by reconstituting *Rag2*$^{-/-}$ C57BL/6 mice with C57Bl/6 CD4 T cells, purified using standard magnetic bead separation (Mitenyi Biotec).

Depletion of the haematopoietic cell fraction in donor mice.

Depletion of haematopoietic cells in donor BALB/c mice was achieved by lethal irradiation (13 Gy in 2 x 6.5 Gy fractions) on day -7 and treatment with 1 mg i.p. depleting anti-CD4 mAB (rat IgG2b, clone YTS 191.1; hybridoma purchased from

European Collection of Cell Cultures at the Health Protection Agency) on day -2 in relation to transplantation. Depletion was confirmed by flow cytometric analysis of serum and splenic tissue collected at procurement of the heart graft. Absence of haematopoietic cells within the heart graft was additionally confirmed by the inability of heart grafts from haematopoietic-cell depleted B6.K^{d} donor mice to trigger 'direct-pathway' CD4 T cell responses in recipient bm12 mice, as assessed by quantifying division of CFSE-labelled TCR75 CD4 T cells seven days after transfer at time of transplant. In this model, TCR75 CD4 T cells only recognise target, I-A^{b}-restricted H-2K^{d} peptide epitope on the surface of donor cells; presentation of processed, I-A^{bm12}-restricted H-2K^{d}-peptide by recipient APCs (via the 'indirect-pathway') does not provoke a response [10].

CFSE CD4 T cell proliferation and Flow cytometry

Proliferation of TCR75 CD4 T cells was determined by CFSE-labelled cell division at day 7 following adoptive transfer, as described previously [10]. FITC conjugated CD11c (clone HL3), APC conjugated CD4 (clone GK1.5), PE-Cy7 conjugated CD4 (clone GK1.5) and PE conjugated CD8 (clone 53-6.7) were purchased from BD Pharmingen (San Diego, CA). Peripheral blood and splenic single-cell suspensions were blocked with anti-mouse CD16/CD32 (clone 2.4G2) before staining with the relevant Abs and dead cell exclusion dye 7-aminoactinomycin (both from BD Pharmingen). Biotinylated Abs were detected by allophycocyanin-conjugated streptavidin (Invitrogen), and all cells were analyzed on a FACSCanto II flow cytometer with FACSDiva software (Becton Dickinson U.K., Oxford, U.K.).

Endothelial cell culture and *in vito* CD8 T cell proliferative responses

In vitro proliferative CD8 T cell responses were assayed by challenging splenic CD8 T cells with BALB/c endothelial cells or irradiated (25 Gy) BALB/c splenocytes as stimulators. Endothelial cells were obtained by extraction from suspensions of 10 to 14 day-old neonatal murine hearts by incubation with biotinylated anti-CD31 (clone MEC 13.3, BD Pharmingen); CD105 (clone MJ7/18, BioLegend) and Isolectin B4 (clone B-1205, Vector). Endothelial cells were then extracted using an autoMACS Separator and the resultant cells cultured in flasks pre-coated with 1% Gelatin in standard

growth medium (Hepes buffered DMEM with 10% FCS, 100 IU/ml penicillin-streptomycin, 2mM L-Glutamine) to which Endothelial Cell Growth Factor (E9640, Sigma-Aldrich Inc. St Louis, MO) was added. Responders and stimulators were co-cultured in a 1:1 ratio at $4x10^5$ cells/well for 6 days prior to addition of 1 μCi/well [^{3}H]thymidine for 6 hours. Cells were then harvested and [^{3}H]thymidine incorporation measured.

Assay of circulating anti-H-2K^d alloantibody and CD8 T cell ELISPOT.

Serum samples were collected from experimental mice at intervals and analysed for the presence of anti–H-2Kd IgG alloantibody by ELISA as previously described [10]. For each sample, an absorbance vs. dilution curve was plotted, and the area under the curve calculated [39] and expressed as the percentage of positive control (pooled hyperimmune) serum. CD8 T cell ELISPOT was performed as described [40].

Statistical analysis

Data were presented as mean ± SEM where appropriate. Mann-Whitney-U test was used for analysis of nonparametric data. 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.

Study approval

Mice were bred and maintained in specific-pathogen-free animal facilities and all experiments were approved by the United Kingdom Home Office under the Animal (Scientific Procedures) Act 1986. All mice were 6–10 weeks of age at the beginning of the experiments.

Sources of Funding

This work was supported by a British Heart Foundation project grant, the National Institute of Health Research Cambridge Biomedical Research Centre and the National Institute of Health Research Blood and Transplant Research Unit. SH was supported by an Academy of Medical Sciences / Wellcome Trust starter grant and the European Society for Organ Transplantation Junior Basic Science Research Grant. JA and IH were supported by Wellcome Trust Clinical Research Training Fellowships and Raymond and Beverly Sackler Scholarships.

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Figure 1: Possible mechanisms for provision of CD4 T cell help to alloreactive CD8 T cells.

Analogous to provision of CD4 T cell help for cytotoxic CD8 T cell responses against nominal protein antigen, simultaneous linked 'direct-pathway' allorecognition of MHC class I and class II alloantigen on donor antigen presenting cells (APCs) by cytotoxic CD8 and helper CD4 T cells, respectively, is considered the dominant helper mechanism **(a).** Nevertheless, indirect-pathway CD4 T cells that recognise self-restricted alloantigen following internalisation and processing by recipient APCs can also provide help; an 'unlinked' four cell-cluster model has been proposed **(b)**. Provision of linked help by indirect pathway CD4 T cells may be achieved by a three cell cluster model in which the recipient APC simultaneously presents intact and processed MHC Class I alloantigen to, respectively, the cytotoxic CD8 T cell and helper CD4 T cell **(c)**.

Figure 1

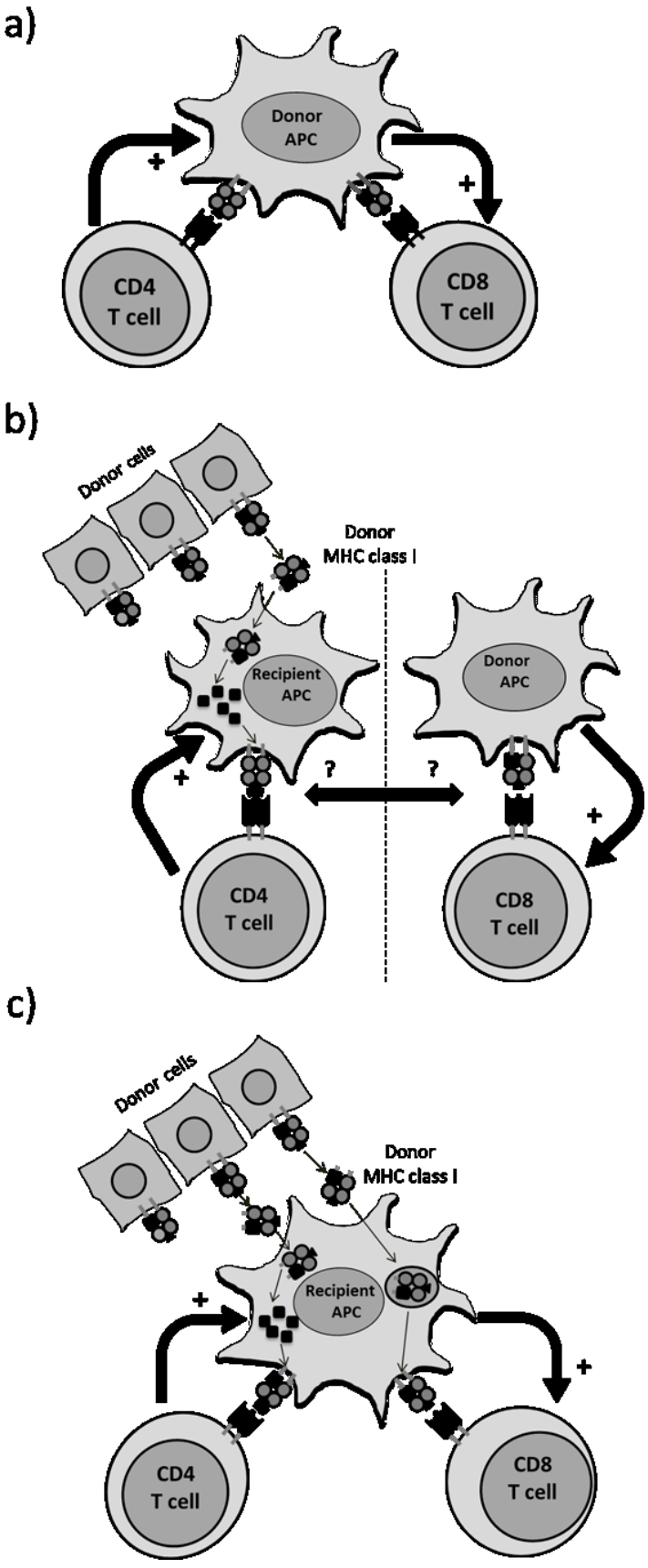

a)
Donor
APC
+
+
CD4
T cell
CD8
T cell
b)
Donor cells
Donor
MHC class I
Recipient
APC
Donor
APC
?
?
+
+
CD4
T cell
CD8
T cell
c)
Donor cells
Donor
MHC class I
Recipient
APC
+
+
CD4
T cell
CD8
T cell

Figure 2: Secondary lymphoid tissue is normally required for differentiation of effector cytotoxic CD8 T cell alloimmunity.

(a) Whereas, BALB/c heart allografts are not rejected in splenectomised, alymphoplastic (*aly/aly*) H-2^b mice, rapid rejection was restored by adoptive transfer of H-$2L^d$-reactive '2C-TCR' CD8 T cells at transplantation. **(b)** Heart allografts were depleted of haematopoietic cells by treating donor animals with a combination of lethal irradiation and anti-CD4 mAb. Depletion was confirmed by comparing proliferation of CFSE-labelled TCR75 CD4 T cells, adoptively transferred into bm12 recipients at transplant with B6.K^d heart allografts procured from either naïve or lethally-irradiated donor mice**:** lower panel – representative flow cytometric division profiles; top panel – percentage of parent cells undergoing at least one division cycle. In this model, TCR75 CD4 T cells recognise H-$2K^d$ peptide complexed to I-A^b on donor cells, but do not recognise recipient I-A^{bm12}-restricted antigen. **(c)** Kaplan Meier survival analysis of heart allografts from irradiated BALB/c donors transplanted into wild-type C57BL/6 (B6), or *Rag1*$^{-/-}$ 2C TCR, or splenectomised *aly/aly* H-2^b mice that were adoptively transferred with naïve or activated 2C TCR CD8 T cells. **(d)** Purified 2C CD8 T cells from *Rag1*$^{-/-}$ 2C TCR recipients of heart allografts from un-modified or lethally-irradiated BALB/c donors exhibit similar *in vitro* interferon-γ recall ELISPOT responses against donor (BALB/c) stimulators. **(e)** Interferon-γ recall ELISPOT assay comparison of the cytotoxic CD8 T cell alloresponse at early (day 4) and late (day 12) time points after transplantation of B6 mice with heart allografts from either naïve or lethally irradiated BALB/c donors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Kaplan Meier in a,c; Mann-Whitney test in b,d,e). Data are representative of two independent experiments resulting in at least n=4 in each group.

Figure 2

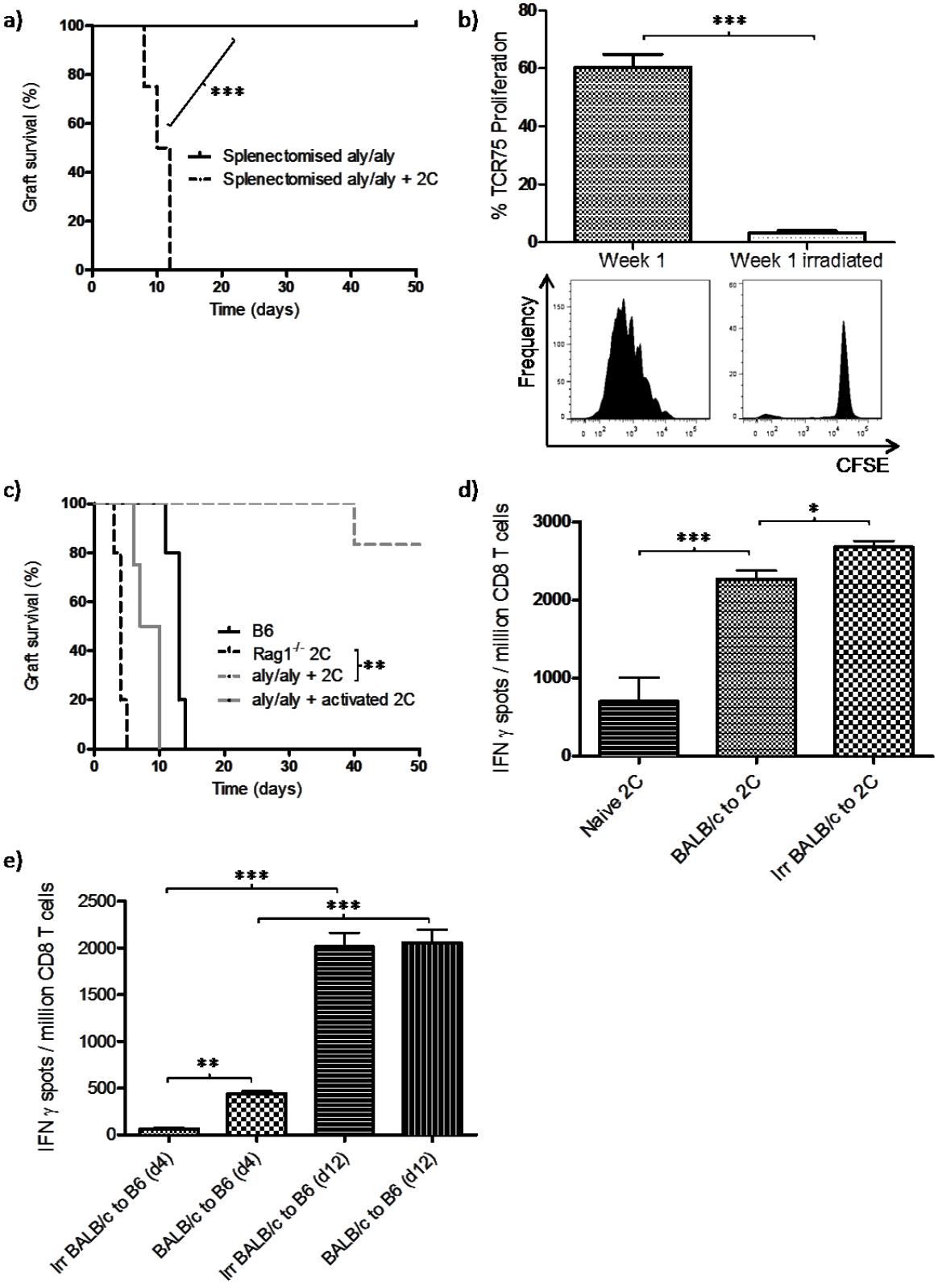

a)
Graft survival (%)
Time (days)
***
Splenectomised aly/aly
Splenectomised aly/aly + 2C
b)
% TCR75 Proliferation
***
Week 1
Week 1 irradiated
Frequency
CFSE
c)
Graft survival (%)
Time (days)
B6
Rag1-/- 2C
aly/aly + 2C
aly/aly + activated 2C
**
d)
IFN γ spots / million CD8 T cells
***
*
Naive 2C
BALB/c to 2C
Irr BALB/c to 2C
e)
IFN γ spots / million CD8 T cells
***
***
**
Irr BALB/c to B6 (d4)
BALB/c to B6 (d4)
Irr BALB/c to B6 (d12)
BALB/c to B6 (d12)

Figure 3: The generation of CD4 T-cell independent CD8 T cell alloimmunity requires host dendritic cells.

Heart allografts from lethally-irradiated BALB/c donors were transplanted into CD4 T cell depleted 'CD11c-DTR-BM' recipients. Control mice received no additional treatment, whereas experimental mice were adoptively transferred with *Rag1*$^{-/-}$ 2C CD8 T cells alone (2C CD8), or in combination with high-dose diphtheria toxin (2C CD8 +DT) to ablate host DCs. **(a)** Kapan-Meier survival analysis. **(b)** Minimal CD8 T cell alloresponses, as assayed by IFN-γ ELISPOT, were observed in CD4 T cell depleted (but otherwise unmodified) CD11c-DTR-BM recipients of irradiated BALB/c heart allografts (control). These were significantly augmented by adoptive transfer of *Rag1*$^{-/-}$ 2C CD8 T cells (2C CD8). This augmentation was dependent upon the host DC population (2C CD8 +DT; * $P < 0.05$). Included for reference are the typical cytotoxic CD8 T cell responses generated in WT C57BL/6 (B6) recipients of irradiated BALB/c grafts (WT B6). **(c)** Four days after transplantation with heart allografts from WT (WT donor) or irradiated BALB/c (irrad donor) donors, CD11c DCs were purified from B6 recipient spleens and adoptively transferred into naïve, secondary B6 hosts. Cytotoxic CD8 T cell alloimmunity in the secondary hosts was assayed 10 days after transfer as above; included as control are the responses observed in naïve B6 mice. Individual data points represent responses observed in individual mice. **(d)** The response of purified B6 CD8 T cells to *in vitro* challenge with BALB/c splenocytes (SPC) or cultured BALB/c endothelial cells (EC) was quantified by standard mixed lymphocyte thymidine-incorporation assay. Responses to endothelial cell challenge were significantly increased by addition of B6 DC to culture. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (Kaplan Meier in a; Mann-Whitney test in b,c,d). Data are representative of two independent experiments resulting in at least n=6 in each group.

Figure 3

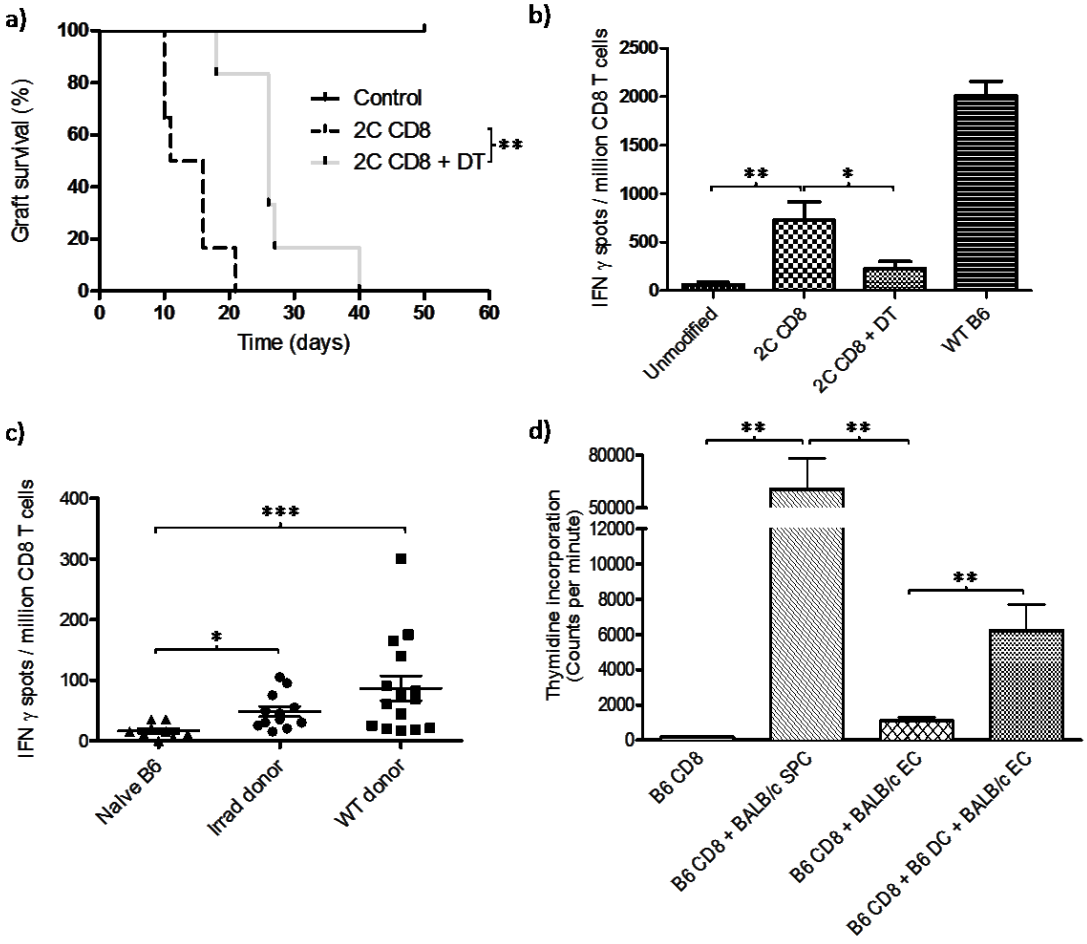
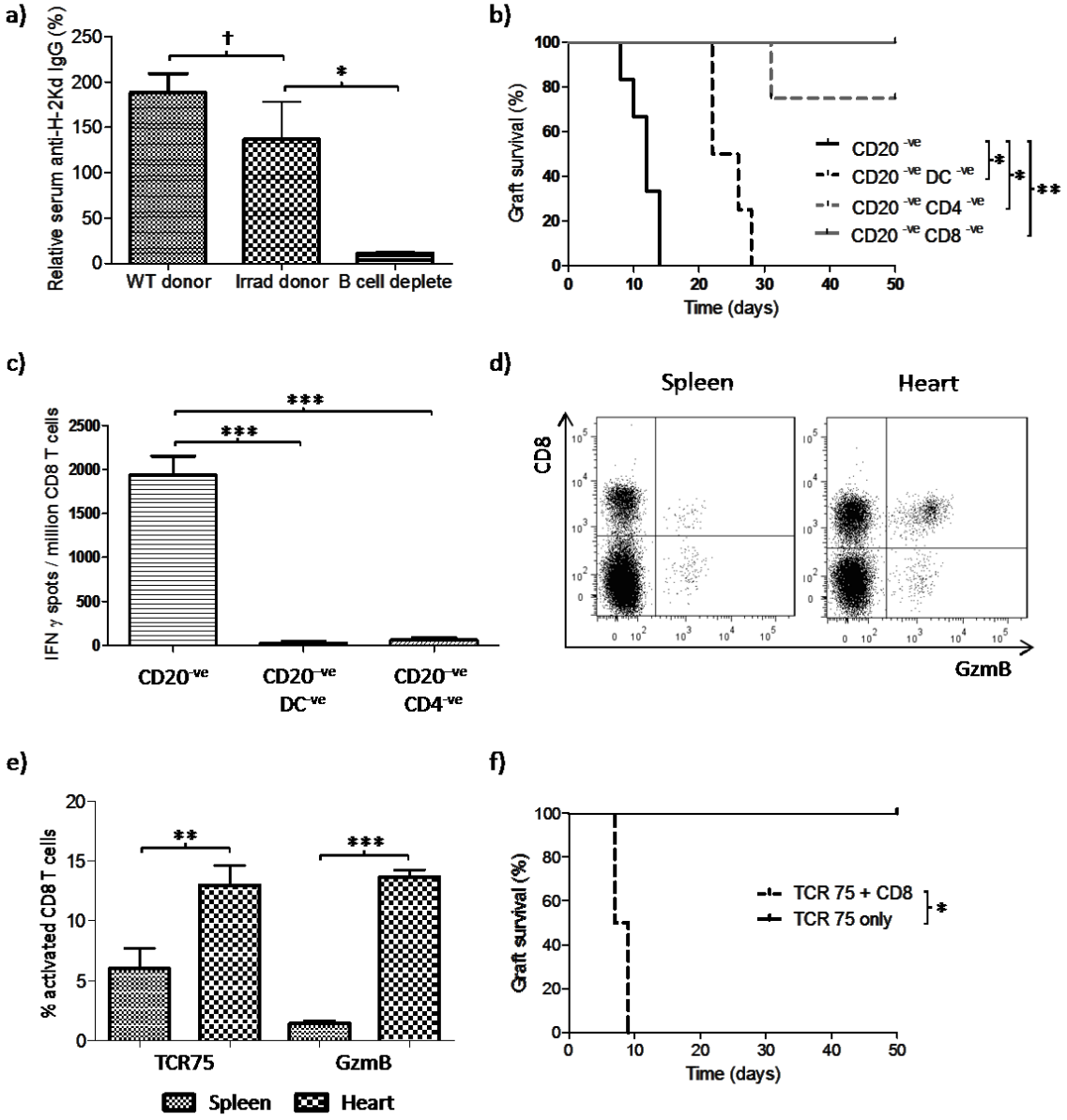

a)
Graft survival (%)
Time (days)
Control
2C CD8
2C CD8 + DT
**
b)
IFN γ spots / million CD8 T cells
Unmodified
2C CD8
2C CD8 + DT
WT B6
c)
IFN γ spots / million CD8 T cells
Naïve B6
Irrad donor
WT donor
d)
Thymidine incorporation (Counts per minute)
B6 CD8
B6 CD8 + BALB/c SPC
B6 CD8 + BALB/c EC
B6 CD8 + B6 DC + BALB/c EC

Figure 4: The generation of cytotoxic effector alloresponses within the endogenous CD8 T cell repertoire requires host DCs and help from indirect-pathway CD4 T cells.

(a) Anti-H-2K^{d} alloantibody responses in C57BL/6 (B6) recipients of heart allografts from lethally-irradiated BALB/c donors (irrBALB/c) are similar in magnitude to those generated following transplantation with unmodified BALB/c heart allografts († *P* = NS), and abrogated by B cell depletion. BALB/c heart grafts devoid of haematopoietic APCs were transplanted into B cell depleted CD11c-DTR-BM B6 recipients that were additionally depleted of either DCs (CD20^{-ve}DC^{-ve}) or CD4 T cells (CD20^{-ve}CD4^{-ve}). Combined B and CD8 T cell-deficient recipients (CD20^{-ve}CD8^{-ve}) of irradiated BALB/c heart allografts were created by reconsituting *Rag2*$^{-/-}$ B6 mice with purified B6 CD4 T cells. Control CD11c-DTR-BM B6 recipients were depleted of B cells only (CD20^{-ve}). Graft survival was assessed by Kaplan Meier analysis **(b)** and CD8 T cell alloimmunity quantified by IFN-γ ELISPOT **(c)**. To evaluate effector CD8 T cell differentiation, CD20-depleted GzmBCrexRosa26YFP H-2^{b} mice were transplanted with irradiated BALB/c grafts and tamoxifen administered concurrently. Representative flow cytometry plots **(d)** depicting granzyme B positive effector CD8 T cells within the recipient spleen and heart allograft, and expressed graphically **(e)** as percentage of purified CD8 T cell. Figure **(e)** also depicts a similar analysis for *Rag2*$^{-/-}$ C57BL/6 recipients of irradiated BALB/c heart allografts that were reconstituted with GzmBCrexRosa26YFP CD8 T cells and with indirect-pathway TCR75 helper CD4 T cells. Limiting CD4 T cell help to the indirect pathway resulted in effective CD8 T cell effector differentiation. **(f)** Whereas *Rag2*$^{-/-}$ C57BL/6 recipients reconstituted with TCR75 CD4 T cells did not reject irradiated BALB/c heart allografts, grafts were rejected promptly if an effector population of GzmBCrexRosa26YFP CD8 T cells were additionally transferred. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; (Mann-Whitney test in a,c,e; Kaplan Meier in b,f). Data are representative of n=4 in d,e,f; and two independent experiments resulting in at least n=5 in each group in a,b,c.

Figure 4


a)
Relative serum anti-H-2Kd IgG (%)
250
200
150
100
50
0
†
*
WT donor
Irrad donor
B cell deplete
b)
Graft survival (%)
100
80
60
40
20
0
0
10
20
30
40
50
Time (days)
CD20 -ve
CD20 -ve DC -ve
CD20 -ve CD4 -ve
CD20 -ve CD8 -ve
*
*
**
c)
IFN γ spots / million CD8 T cells
2500
2000
1500
1000
500
0
***
***
CD20-ve
CD20-ve DC-ve
CD20-ve CD4-ve
d)
Spleen
Heart
CD8
GzmB
e)
% activated CD8 T cells
20
15
10
5
0
**
***
TCR75
GzmB
Spleen
Heart
f)
Graft survival (%)
100
80
60
40
20
0
0
10
20
30
40
50
Time (days)
TCR 75 + CD8
TCR 75 only
*