Germinal center humoral autoimmunity independently mediates progression of allograft vasculopathy

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The authors have declared that no conflict of interest exists.

Abbreviations

GC - germinal center
GVH - graft-versus-host
TAA – Transplant Associated Autoimmunity
T_{FH} - T follicular helper cell
WT - wild-type

Abstract (220 words)

The development of humoral autoimmunity following organ transplantation is increasingly recognised, but of uncertain significance. We examine whether autoimmunity contributes independently to allograft rejection.

In a MHC class II-mismatched murine model of chronic humoral rejection, we report that effector antinuclear autoantibody responses were initiated upon graft-versus-host allorecognition of recipient B cells by ***donor*** CD4 T-cells transferred within heart allografts. Consequently, grafts were rejected more rapidly, and with markedly augmented autoantibody responses, upon transplantation of hearts from donors previously primed against recipient. Nevertheless, rejection was dependent upon ***recipient*** T follicular helper (T_{FH}) cell differentiation and provision of cognate (peptide-specific) help for maintenance as long-lived GC reactions, which diversified to encompass responses against vimentin autoantigen. Heart grafts transplanted into stable donor/ recipient mixed haematopoietic chimeras, or from parental strain donors into F1 recipients (neither of which can trigger host adaptive alloimmune responses), nevertheless provoked GC autoimmunity and were rejected chronically, with rejection similarly dependent upon host T_{FH} cell differentiation.

Thus, autoantibody responses contribute independently of host adaptive alloimmunity to graft rejection, but require host T_{FH} cell differentiation to maintain long-lived GC responses. The demonstration that one population of helper CD4 T-cells initiates humoral autoimmunity, but that a second population of T_{FH} cells is required for its maintenance as a GC reaction, has important implications for how autoimmune-related phenomena manifest.

1. Introduction

Chronic rejection, whereby transplanted organs suffer gradual deterioration in function, is the major cause of failure of solid organ transplants. Although allograft rejection is generally considered to be mediated by adaptive immune recognition of graft alloantigen, an association with host autoimmunity is increasingly emphasised [1-3]. These autoimmune responses appear to be a direct consequence of the transplant; albeit the steps that lead to their development and their contribution to graft rejection remain poorly understood. Possibly the strongest evidence clinically of an effector role for transplant-associated-autoimmunity (TAA) is the association between T-cell autoimmunity against collagen type V protein and early development of bronchiolitis obliterans syndrome in human lung transplant recipients [4]. Nevertheless, several key questions relating to TAA remain unanswered. It is not known whether autoimmunity contributes to graft rejection independently or whether its principal impact is via augmentation of conventional alloimmune responses directed against the graft [5]. Similarly, although the Burlingham study reported cellular autoimmunity [4], a role for autoantibody in rejection has also been reported [6-8], albeit the relative contribution of B cell autoimmunity has yet to be clarified. In this regard, although humoral autoimmune disease was traditionally thought to be mediated by germinal center (GC) responses [9], extrafollicular foci that also result in long-lived and mutated autoantibody production have been described [10], and it is not clear whether transplant-associated humoral autoimmunity is driven principally by extrafollicular or GC responses. This is important because GC autoimmune responses are now known to require help from a specialised T follicular helper (T_{FH}) subset [11], raising the possibility of developing strategies that target this subset in anticipation of improvements in allograft survival.

The MHC class II-mismatched [B6(C)-H2bm12/KhEgJ (bm12) to C57BL/6 (B6)] mouse strain combination is one of the most widely studied murine models of heart allograft vasculopathy [12-16]. In addition, autoimmune responses triggered by transfer of donor bm12 lymphocytes has been used for study of lupus-like autoimmune disease in B6 recipients [17]. Our work in this model has described a previously unappreciated role for passenger donor CD4 T-cells within the bm12 heart graft in triggering anti-nuclear autoantibody responses in the B6 recipient. These passenger lymphocytes are immunocompetent, possibly resident within the parenchyma as resident memory T-cells [5, 18], and provide help via 'peptide-degenerate' graft-versus-host (GVH) allorecognition of MHC class II complexes on the surface of recipient B cells [5, 6, 19]. Here we use this critical role of donor bm12 CD4 T-cells in initiating autoantibody production in B6 recipients to study the contribution of humoral autoimmunity to allograft vasculopathy independently of conventional alloimmunity, and to clarify the role of host CD4 T-cells in maintaining autoantibody responses. We demonstrate that although the key step in initiating humoral autoimmunity is GVH-allorecognition by donor CD4 T-cells, its maintenance as a GC response is dependent upon the provision of help from recipient-derived T_{FH} CD4 T-cells, suggesting that donor T-cells can 'license' endogenous T_{FH} cells to support GC autoimmunity. We further show that the GC response is essential for mediating progression of allograft vasculopathy and can do so independently of conventional host alloimmune recognition.

2. Materials and Methods and materials

2.1. Animals

C57BL/6J (H-2^b; B6) and BALB/c (H-2^d) and F1 BALB/c x C57BL/6 (H-2^b.H-2^d) mice were purchased from Charles River Laboratories (Margate, UK). Bm12 mice (B6(C)-H2-Ab1bm12/KhEgJ [H-2bm12]), H-2^b T-cell receptor-deficient mice (*Tcrbd*$^{-/-}$ (B6.129P2-*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom}/J) [20]), H-2^b B cell-deficient mice (µMT (B6.129S2-*Ighm*^{tm1Cgn}/J) [21]) , and H-2DM-deficient mice (B6.129S2-*H2-DMa*^{tm1Doi}/J [22]) were purchased from the Jackson Laboratory (Bar Harbor, ME). CD45.1 B6 (H-2^b) mice were gifted from Prof. K Smith (University of Cambridge, UK). C57BL/6 *Rag2*$^{-/-}$ mice (H-2^b) were gifted by Prof. T. Rabbitts (University of Cambridge). *Sh2d1a*$^{-/-}$ B6 mice (H-2^b) [23] were gifted by Dr S. Crotty (University of California, La Jolla, California). *Sh2d1a*$^{-/-}$ B6 mice were crossed with bm12 mice to create *Sh2d1a*$^{-/-}$ bm12 mice, *Sh2d1a*$^{-/-}$ B6 mice were crossed with F1 BALB/c x C57BL/6 (H-2^b.H2^d) to create *Sh2d1a*$^{-/-}$ F1 BALB/c x C57BL/6 mice (*Sh2d1a*$^{-/-}$ CB6F1), and bm12.K^d mice were created in-house [5]. All animals were maintained in specific pathogen–free facilities, and experiments were approved by the UK Home Office Animal (Scientific Procedures) Act 1986.

2.2. Skin and Heterotopic cardiac transplantation

Full thickness donor tail skin was sutured as 1 cm^2 grafts onto the recipient's back. Vascularized cardiac allografts were transplanted intra-abdominally, as described previously [24]. Rejection, defined as cessation of palpable myocardial contraction, was confirmed at explant. In certain experiments, stable mixed haematopoietic bone marrow chimeric recipients were created using a protocol modified from Sykes [25], by conditioning congenic CD45.1B6 mice with anti-CD8 mAb (1.5 mg on days -1, 0, 1, 7), anti-CD4 mAb (1mg, days -6,-1), anti-CD154 mAb (2mg on day 0) and 4 Gy TBI (day 0), followed by intravenous injection with 10^7 CD45.2bm12 bone marrow cells. Donor CD4 T-cell depletion was achieved as described previously [5].

B cell depletion in certain recipient groups was achieved by administering depleting anti-CD20 mAb (250µg i.p. 18B12, IgG2a gifted by Cherie Butts, Biogen Idec Boston, MA) on days 25, 35 and 45 after transplant, with depletion confirmed by flow cytometric analysis of peripheral blood [26]. NK cells were depleted with anti-NK1.1 antibody, as described previously [5]. In some experiments, recipients were reconstituted with column-purified CD4 T-cells (described in section 2.1 in [27]).

The severity of cardiac allograft vasculopathy was assessed on elastin van Gieson (EVG)-stained paraffin sections by morphometric analysis [12]. All elastin-positive vessels in each section were evaluated, with approximately 10 vessels/heart analysed.

2.3. Quantification of humoral autoantibody responses

Antinuclear autoantibody responses in recipient sera were determined by HEp-2 indirect immunofluorescence (The Binding Site, Birmingham, UK) as described previously [12]. Anti-vimentin autoantibody responses were quantified by vimentin-specific ELISA, using a modified protocol from Mahesh et al [28]. The deposition of long-lived plasma cells with specificity for dsDNA was quantified by performing ELISPOT assay on purified spleen and bone marrow cells as described previously [19]. Flow cytometry characterization of T_{FH} cells and GC B cells was performed as described previously [11] and in section 2.3 in [27].

2.4. Histopathology, immunohistology and confocal imaging

7µm recipient spleen and explanted donor heart cryostat sections were air-dried and fixed in acetone. The development of GCs within the recipient's spleen was determined by staining with B220 and GL7, and GC activity compared by calculating the proportion of activated $GL7^{+ve}$ follicles against total B220 B cell follicles, and by measuring GC area. The GC area was ascertained by measuring the area occupied by $GL7^{+ve}$ $B220^{+ve}$ B cells per follicle (using CellR 2.6 software (Olymous Imaging Solutions, Germany)), and is presented as percentage GC area per

follicle (section 2.2 in [27]). Primary mAbs specific for the following mouse epitopes were used for immunohistochemical/fluorescent staining: C4d (clone 16-D2 Abcam, Cambridge, UK); and IgG-FITC (BD Pharmingen, San Diego, CA, USA). Splenic GCs were identified by double-labeling sections with rat anti-mouse B220-APC (clone RA3-6B2) and rat anti mouse GL7-FITC (both BD Pharmingen). CD4 T-cells within GCs were identified with rat anti-mouse CD4-biotin (BD Pharmingen) & Streptavidin Alexa Fluor-555 (Invitrogen, Life Technologies, Carlsbad, CA, USA). Similarly, CD45.1 or CD45.2 positive cells were identified with rat anti-mouse CD45.1 biotin (BD Pharmingen, San Diego, CA, USA) or CD45.2 biotin (BD Pharmingen, San Diego, CA, USA) and streptavidin Alexa Flour 555 (Invitrogen, Life Technologies, Carlsbad, CA, USA). Confocal images were captured using a Zeiss Laser Scanning Microscope 700 (LSM 700) (Carl Zeiss AG). Images were photographed using ZEN 2011 (Carl Zeiss AG) imaging software.

2.5. In vitro endothelial cell migration assays

The ability of sera from transplanted recipients to induce in vitro proliferation / migration of bm12 cultured endothelial cells was assessed using a 'scratch-wound' assay (detailed in section 2.5 in [27]).

2.6. Statistics

Data were presented as mean ± S.D. where appropriate. Mann Whitney tests were used for analysis of non-parametric data. Two-way ANOVA was employed for comparison of antinuclear and anti-vimentin autoantibody responses. 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.

3. Results

3.1. Progression of Allograft Vasculopathy is dependent upon both donor and recipient CD4 T-cells

To examine the relative contribution of donor 'passenger' CD4 T-cells and host CD4 T-cells to progression of allograft vasculopathy in the bm12 to B6 murine heart transplant model, we used wild-type (WT) mice and T-cell receptor-deficient mice (that lack all TCR-αβ and TCR-γδ CD4 and CD8 T-cells (*Tcrbd*$^{-/-}$)) as recipients of either CD4 T-cell-replete or CD4 T-cell-depleted bm12 heart allografts. To assess whether observed differences in outcomes between WT and *Tcrbd*$^{-/-}$ recipients related to deficiency in specific T-cell populations, in certain experiments, *Tcrbd*$^{-/-}$ recipients were reconstituted with purified syngeneic B6 CD4 T-cells on the day of transplant (Figure 1a). This reconstitution restored splenic follicular architecture (Figure 1 in [27]. As expected from our previous studies [5, 6, 19] robust, class-switched anti-nuclear autoantibody responses were observed when CD4 T-cell help was provided exclusively by the donor CD4 T-cell fraction, mirroring those seen in WT donor / recipient strains (Figure 1b). No autoantibody was observed when B cell help could only be provided by host CD4 T-cells (Figure 1b). Our previous studies have suggested a role for autoantibody in graft rejection [6, 19], as highlighted by the augmented autoantibody responses and rapid heart graft rejection observed (Figure 2a,b and c in [27]) following transplantation with bm12 hearts that contained memory CD4 T-cells with specificity for the B6 recipient (by priming with a B6 skin graft seven weeks before procurement of the heart allograft [5]). Nevertheless, CD4 T-cell-replete bm12 heart allografts transplanted into *Tcrbd*$^{-/-}$ recipients remained disease free, and instead, allograft vasculopathy only developed when both donor and recipient were CD4 T-cell replete (Figure 1c), culminating in allograft rejection (Figure 1d).

3.2. Host T_{FH} CD4 T-cells are required for maintenance of germinal center autoantibody responses

We postulated that the requirement for host CD4 T-cells in progression of allograft vasculopathy may reflect a role in maintaining the autoantibody response triggered by donor CD4 T-cell GVH allorecognition. In this respect, seven weeks post transplantation, splenic GC activity was not detectable in *Tcrbd*$^{-/-}$ recipients of WT bm12 heart allografts, but was readily evident in WT recipients of WT (CD4 T-cell-replete) bm12 heart grafts or in *Tcrbd*$^{-/-}$ recipient mice that had been reconstituted with recipient CD4 T-cells, with the majority of B cell follicles exhibiting GL7^{+} GCs with characteristic light / dark zone configuration (Figures 2a, 2b and Figure 3 in[27]). Confocal immunofluorescence microscopy further revealed the presence of CD4 T-cells within the GC follicles (Figure 2c), consistent anatomically with the T_{FH} cell subset. To examine further the suggestion from these results that the recipient CD4 T-cell population was providing help as T_{FH} cells for GC maintenance, CD45.1 B6 mice were challenged with congenic CD45.2 bm12 heart grafts, and confocal imaging was performed 7 weeks after transplantation to determine whether the GC-resident CD4 T-cells were of donor (CD45.2) or recipient (CD45.1) origin. Although CD4 T-cells were detectable within GCs, donor-derived cells were not identified (Figure 2c), suggesting that the follicular CD4 T-cells were predominantly of recipient-origin. However, it was not possible to confirm this with CD45.1 staining, because simultaneous binding to surrounding endogenous (recipient) CD45.1^{pos} B cells compromised image clarity (not shown). Instead, splenic GC responses were assessed seven weeks after transplanting CD45.2 bm12 hearts into CD45.2 *Tcrbd*$^{-/-}$ B6 recipients that had been reconstituted at transplant with a population of CD45.1 B6 CD4 T-cells. Using this approach, populations of the transferred congenically-labelled B6 CD4 T-cells were readily evident within the GC (Figure 2d). Flow cytometric characterisation of the CXCR5^{hi}PD1^{hi}BCL6^{hi} T_{FH} cell subset following challenge of CD45.1 B6 mice with CD45.2 bm12 CD4 T-cells similarly showed the presence of a small

population of recipient CD4 T-cells with T_{FH} cell surface phenotype (Figure 4a in [27]). B cells with Ki67^{+ve}Bcl-6^{+ve} GC phenotype were also readily detectable in B6 mice that were challenged with purified bm12 CD4 T cells (Figure 4c in [27]).

To definitively address the role of recipient T_{FH} cells in maintaining GC humoral autoimmunity, WT bm12 hearts were transplanted into *Sh2d1a*$^{-/-}$ B6 mice. T-cells in these mice lack SLAM-associated protein (SAP) and are unable to undergo productive interaction with B cells for differentiation of T_{FH} cells and development of GC humoral immunity [29]. As expected, transplantation into *Sh2d1a*$^{-/-}$ recipients provoked robust anti-nuclear autoantibody responses, that equalled or exceeded those observed in WT recipients (Figure 3a). Splenic GC activity was, however, not detectable when assessed seven weeks after transplant (Figure 3b). Critically, in the absence of the late GC responses, heart allograft survival was prolonged (Figure 3c), with evidence of only minimal allograft vasculopathy, similar in severity to that observed in B cell deficient (μMT) B6 recipients (Figure 3d). WT B6 recipients that were instead challenged with *Sh2d1a*$^{-/-}$ bm12 heart grafts developed GC autoantibody responses that were comparable to the response observed following challenge with WT bm12 heart grafts (Figure 3a). The severity of allograft vasculopathy in the WT and *Sh2d1a*$^{-/-}$ heart grafts were also similar (Figure 3d), as was the tempo of heart graft rejection (Figure 3c). Thus, although donor CD4 T-cell engagement with recipient B cells is essential for triggering recipient humoral autoimmunity, this interaction does not require SAP signalling. Moreover, in the absence of donor SAP signalling, long-lived GC responses still ensue, with essential T_{FH} cell function provided by the recipient CD4 T-cell subset.

To further examine the role of the recipient CD4 T-cell population in maintaining humoral GC autoimmunity, WT B6 mice were challenged intravenously with purified CD4 T-cells from 'bm12.K^{d}' mice (that express additional transgenic H-2K^{d} alloantigen). Four weeks later, CD4 T-

cells were purified from these challenged mice and transferred into secondary naïve B6 recipients. We have recently reported that, as a consequence of the mismatched I-A^{bm12} antigen, bm12.K^d CD4 T-cells provoke anti-nuclear autoantibody responses in B6 recipients, but are killed within the first week after transfer by host adaptive immune recognition of the H-$2K^d$ alloantigen [5]. Hence, the CD4 T-cell population adoptively transferred to the secondary recipient at 4 weeks will contain host B6, but not donor bm12.K^d, CD4 T-cells. Nevertheless, transfer of these cells resulted in development of GC humoral autoimmunity in the secondary recipients (Figures 3e and 3f), confirming that host CD4 T-cells are not only responsible for maintaining the GC response, but that they can trigger humoral autoimmunity upon transfer into naïve mice.

The above results highlight that at late time points the transferred bm12 CD4 T-cell population is not responsible for maintaining GC humoral autoimmunity. Flow characterisation of transferred bm12 CD4 T-cells at earlier time points (day 23) did suggest acquisition of T_{FH} cell surface phenotype (Figure 4b in [27]), but should be interpreted with caution, because the precise population was difficult to define due to widespread expression of PD-1 (possibly as a consequence of the robust GVH response), and because T_{FH} cell surface phenotype does not necessarily relate to T_{FH} cell function [29]. Of note, splenic GC activity was not detectable upon transfer of purified bm12 CD4 T-cells into *Tcrbd*$^{-/-}$ B6 recipients, and only partially restored by lentiviral transduction of the bm12 CD4 T-cell population with Bcl-6 (the master transcription factor for T_{FH} cell differentiation [30]), with a dampened autoantibody profile (Figure 5a and 5b in [27]). A transient and low-level GC response was similarly observed upon transfer of bm12 CD4 T-cells into *Sh2d1a*$^{-/-}$ recipients (Figure 5b in [27]). Late GC activity in the experimental groups corresponded with the presence of detectable anti-dsDNA IgG-producing plasma cells in

the spleen and bone marrow 7 weeks after challenge (Figure 5c in [27]). Taken together these results suggest that transferred bm12 CD4 T-cells can provide limited, but transient, T_{FH} cell function for development of GC humoral autoimmunity.

3.3. Germinal center autoantibody responses are associated with intermolecular epitope diversification

As reported previously [6], the autoantibody responses generated in WT B6 recipients of CD4-T-cell replete bm12 heart allografts were associated with endothelial complement deposition (Figure 4a). In contrast, in the absence of a late GC response and despite development of anti-nuclear autoantibody, complement deposition was not observed in CD4 T-cell-replete allografts transplanted into either *Sh2d1a*$^{-/-}$ or *Tcrbd*$^{-/-}$ recipients (Figure 4a). To examine whether GC-mediated maturation or diversification of the autoantibody response is required for progression of allograft vasculopathy, sera obtained at week 7 from the various recipient groups were tested for its ability to induce migration / proliferation of cultured bm12 endothelial cells across a scratch-wound. This functional assay was chosen because it may mimic migration of endothelial cells in response to antibody-mediated damage [31]. As shown in Figure 4b, whereas sera from WT recipients of CD4 T-cell replete heart allografts provoked vigorous endothelial cell proliferation / migration, *in vitro* endothelial cell responses were not observed with sera of either *Sh2d1a*$^{-/-}$ or *Tcrbd*$^{-/-}$ recipients. Similar patterns of endothelial migration were observed when column-purified serum immunoglobulin from the transplanted mice was added, with the negative fraction from column purification not eliciting endothelial cell responses (Figure 6 in[27]).

We hypothesised that the requirement for GC humoral immunity in progression of allograft vasculopathy reflected diversification of the autoantibody response to target additional, and potentially pathogenic, autoantigens. To test this, sera from the transplanted groups were

tested for the presence of autoantibody directed against vimentin self-antigen (Figure 4c), chosen as target because of the reported association with cardiac allograft vasculopathy [28, 32]. Anti-vimentin autoantibody responses were not detectable in any group at week seven after transplant, but were detectable by week 15 in approximately half of WT recipients of CD4 T-cell-replete bm12 heart grafts (Figure 4c). Similar late anti-vimentin responses were detectable in other recipient groups in which GC autoimmunity had developed (either WT recipients of *Sh2d1a*$^{-/-}$ bm12 heart grafts or *Tcrbd*$^{-/-}$ recipients reconstituted with WT B6 CD4 T-cells; Figure 4c). Compared to control serum from naïve mice, these late responses were associated with increased levels of circulating IgG (Figure 5d in [27]). In contrast, late anti-vimentin responses were not generated in *Sh2d1a*$^{-/-}$ or *Tcrbd*$^{-/-}$ recipients of WT bm12 heart allografts (Figure 4c).

We have recently described that the interaction between donor CD4 T-cells and recipient B cells that triggers humoral autoimmunity occurs via an unusual form of direct-pathway GVH allorecognition, in which MHC class II complexes on recipient B cells are recognised in a 'peptide-degenerate' fashion [5]. In contrast, it seemed likely that peptide-specific B cell presentation to recipient-derived T_{FH} cells would be required to sustain the autoreactive GC reaction observed in WT recipients. To test this, bone marrow chimeric mice were created (Figure 5a), in which, as a consequence of a selective defect in B cell *H2-DMa* expression (B.DM$^{-/-}$ mice), B cells were unable to exchange CLIP (Class II-associated invariant chain peptide) and were crippled in their capacity to present endogenous peptide [22]. B.DM$^{-/-}$ mice challenged with WT bm12 heart grafts generated early anti-nuclear autoantibody, above background levels observed in recipients of syngeneic B6 grafts (Figure 5b), confirming the peptide degenerate nature of the help donor CD4 T-cells provide to recipient B cells. In contrast to WT recipients,

however, there was no late augmentation in autoantibody production (Figure 5b), and in keeping, GC reactions were not evident at week 7 (Figure 5c). In the absence of the GC response, diversification of the B cell response to encompass anti-vimentin reactivity was not observed (Figure 5d), and allograft survival was prolonged (Figure 5e).

3.4. Late B cell depletion prevents epitope diversification and improves *allograft survival.*

To further examine the contribution of the late GC humoral autoimmune response to progression of allograft vasculopathy, WT recipients of CD4 T-cell-replete bm12 heart grafts were treated with depleting anti-CD20 monoclonal antibody, commencing three weeks after transplant and continued for a further three weeks [24]. We reasoned that such treatment would deplete immature, mature and established GC B cells, but would be ineffective against autoreactive plasma cells, such as those that are resident in the bone marrow [33]. In support, late treatment with anti-CD20 did not influence deposition of long-lived plasma cells specific for double-stranded DNA (dsDNA) into the bone-marrow (Figure 6a), and in keeping, the kinetics and magnitude of the anti-nuclear autoantibody response did not differ from untreated recipients (Figure 6b). Splenic GC activity was, however, reduced in treated recipients at 7 weeks after transplant (Figure 6c) and this was associated with abrogation of the late anti-vimentin antibody response (Figure 6d). All heart allografts survived in anti-CD20 treated recipients until termination of experiment (day 100), with only minimal vasculopathy development (Figure 6e). Nevertheless, it was notable that these allografts were beating appreciably less strongly at explant, and that marked cellular infiltrates were present within the allografts and were associated with fibrotic scarring (Figure 6f).

3.5. Germinal center autoantibody responses independently mediate progression of allograft vasculopathy

It seemed likely that the requirement for host T-cells to mediate rejection of bm12 heart allografts (Figures 1c and 1d) reflected their role as T_{FH} cells for providing help for GC humoral autoimmunity (Figures 3c and 3d), particularly when one considers, as previously reported [6], that B6 recipients of bm12 heart allografts do not develop alloantibody responses against the mismatched I-A^{bm12} MHC class II antigen (not shown). However, we wished to exclude a possible contribution to allograft rejection from conventional recipient CD4 T-cell allorecognition of the mismatched I-A^{bm12} alloantigen (either via the direct or indirect pathways [34]). To do so, we used an approach modified from Sykes [25] to create 'mixed chimeric' mice, in which B6 recipients stably harboured a congenic population of bm12 bone marrow (Chm.bm12/B6 mice, Figure 7 in [27]). We postulated that in these mice, the persistence of the chimeric state would signify ongoing adaptive tolerance towards the mismatched MHC class II alloantigen, but that naïve bm12 passenger CD4 T-cells within a subsequent allograft would still provide help to autoreactive B6 B cells for generating humoral autoimmunity. In support, challenge of Chm.bm12/B6 mice with a bm12 heart graft provoked similar anti-nuclear autoantibody responses as observed in WT B6 recipients (Figure 7a). Germinal center responses were also generated (Figure 7b), and late anti-vimentin autoantibody responses were above background (Figure 7c). Critically, heart allografts developed severe vasculopathy, with widespread complement deposition (Figures 7d & f). The kinetics of heart graft rejection were also similar to those observed in WT B6 recipients (Figures 7e).

As we have reported previously [5], despite the development of GC humoral autoimmunity in recipient mice, obvious autoimmune disease manifestations were not generally apparent within the timeframe of our experiments. This raises the question why the autoantibody generated in

our recipients provokes allograft vasculopathy, while apparently preserving the native arterial system. Notably, syngeneic B6 heart grafts that were transplanted into B6 recipient mice in which humoral autoimmunity was simultaneously provoked ((Figure 7a) by transfer of purified bm12 CD4 T-cells) survived indefinitely, without developing vasculopathy (Figure 7d and 7e). These experiments suggest that ischaemia –reperfusion injury is not itself sufficient to induce susceptibility in the syngeneic transplant to autoantibody-mediated damage. This is considered in detail in the discussion.

The above results suggest that despite the tolerant state to the mismatched I-A^{bm12} alloantigen that was present at transplant, the humoral autoimmune response triggered in the chimeric recipients by passenger donor CD4 T-cells results in progressive allograft vasculopathy and eventual allograft failure. They do not, however, exclude the possibility that the development of humoral autoimmunity in turn breaks allotolerance in the chimeric recipients, and that the adaptive alloimmune responses generated against the MHC class II I-A^{bm12} alloantigen contribute to allograft rejection. To examine humoral autoimmunity independently of alloimmunity, we chose to examine rejection of parental strain heart allografts by F1 hybrid offspring [35], reasoning that GVH allorecognition by passenger donor cells would stimulate recipient GC autoimmunity, but that adaptive alloimmune recognition of donor parental antigens would not be possible in the F1 recipient. As shown in figure 8, transplantation of BALB/c heart grafts into F1 BALB/c x C57BL/6 (CB6F1) recipients provoked GC anti-nuclear autoantibody responses, and these responses were associated with intra-allograft endothelial complement deposition and development of allograft vasculopathy. As anticipated, these features were dependent upon the transfer of donor strain CD4 T-cells from the heart allograft (Figures 8a-d), but as with the bm12 model, transplantation into *Sh2d1a*$^{-/-}$ SAP-deficient F1

recipients abrogated the GC response and heart grafts developed only minimal vasculopathy (Figures 8b & 8c). Alloantibody responses against MHC class I H-2K^d antigen were not generated (not shown), suggesting that self-tolerance to the BALB/c H-2^d antigens was maintained in the F1 recipient. Notably, however, although GC autoantibody responses were generated in NK-cell depleted recipients (Figures 8a and 8b), allografts in these mice remained disease free and without complement deposition (Figure 8d), suggesting a critical role for NK cells in either mediating alloantibody mediated graft damage or in triggering innate immune 'missing-self' recognition of the parental BALB/c strain.

In summary, these experiments provide strong support that humoral autoimmunity can independently mediate the progression of allograft vasculopathy, and highlight a critical contribution from host T_{FH} cells in providing help for maintaining GC autoantibody responses.

4. Discussion

Although the development of autoimmunity following solid organ transplantation is increasingly recognised, exactly how it contributes to graft rejection remains unclear. Previous studies have reported that graft-specific autoimmune responses influence allograft rejection in close concert with adaptive alloimmune responses directed against mismatched MHC alloantigen; either by precipitating / augmenting alloimmunity [5, 36] or by being triggered by ongoing alloimmune-mediated graft damage [37]. This close association makes it difficult to distinguish a direct effector role for TAA from it simply being a marker for a more aggressive, and damaging, alloimmune response. Here we extend our previous investigations into the bm12 to B6 model of heart allograft vasculopathy, and show that although host alloantibody responses are triggered by GVH recognition by passenger CD4 T-cells, the provision of help from recipient-derived T_{FH} cells is essential for maintenance of GC humoral autoimmunity. This GC response is a major

determinant of graft outcome (Figure 8e). By replicating these observations in an experimental model in which host adaptive alloimmunity against the donor is not possible, we provide strong evidence of an independent causative role for graft specific autoimmunity in chronic allograft rejection.

The bm12 to B6 murine strain combination has been one of the most studied models of chronic allograft rejection, and it is perhaps surprising that the role of autoantibody has not been more widely demonstrated. Nevertheless our previous publications [5, 6, 19], and the experimental data here continue to emphasise the contribution of humoral immunity to graft rejection. Most striking perhaps, is the robust GC response evident within recipient spleen seven weeks after transplant, with almost all follicles exhibiting GC reactions. This is supported by the reduction in the severity of allograft vasculopathy and improvement in survival when bm12 heart allografts are transplanted into *Sh2d1a*$^{-/-}$ recipients. Aside from a relatively narrow defects in NK T-cell responses and in the ability to form GC responses, *Sh2d1a*$^{-/-}$ mice are thought to have essentially normal CD4 T and B cell function. Hence, in the absence of demonstrable alloantibody against the mismatched I-A^{bm12} alloantigen, it is difficult to conclude other than GC autoantibody responses are important mediators of allograft vasculopathy, with essential help provided by recipient T_{FH} cells. This proposed contribution from humoral autoimmunity provides an explanation for why minimally-mismatched bm12 allografts (three amino acids only) appear to provoke such a robust and long-lasting response in the B6 recipient, particularly when one considers that the direct-pathway CD4 T-cell response against the disparate I-A^{bm12} MHC class II alloantigen is likely to be very short-lived [24]. Autoantibody is, however, unlikely to be the sole mediator of allograft rejection, in that allograft damage and cellular infiltrates were observed in allografts transplanted into B cell-depleted recipients (Figure 6f). In this group, allograft

vasculpathy was much less pronounced, possibly suggesting that humoral autoimmunity mediates graft damage principally via an effect on endothelium, whereas cellular alloimmunity impacts upon the graft parenchyma [38].

The demonstration that bm12 heart transplants trigger GC autoimmunity in B6 recipients raises several, as yet unanswered questions: why is the donor bm12 CD4 T-cell population relatively incapable of providing T_{FH} cell support for maintaining the GC response; what is the nature of antigen recognition by the recipient T_{FH} population; and precisely how does the GC response contributes to allograft rejection. The requirement for recipient-derived T_{FH} cells is possibly a consequence of the unusual nature of direct-pathway allorecognition , in which multiple T clones (precursor frequency of ~5 – 10% [39]) respond to the allogeneic MHC / peptide complex, but may do so with relatively low affinity. For CD4 T-cell responses against conventional antigen, the T_{FH} subset is possibly confined to those clones with highest affinity [40], and hence, the low-affinity interaction between donor CD4 T-cells and host B cells may limit the ability of the former to undergo T_{FH} differentiation. This would be in keeping with development of GC reactivity following transduction of the bm12 CD4 T-cell population with Bcl-6. It is notable, however, that the autoantibody response in this experiment was not sustained, and alternatively, it is possible that GC maintenance requires replenishment of the cell T_{FH} subset by continual differentiation from naïve cells. This has not been described previously, but in support, recent work by Shulman has demonstrated that newly-differentiated T_{FH} cells regularly migrate into existing secondary follicles [41], and our preliminary analysis suggests that at early time points (two weeks after transplant) bm12 T_{FH} cells are detectable within the follicle; their absence at late time points may reflect a limited life-span.

The differentiation of recipient T_{FH} cells following transplantation is presumably driven by antigen presentation by B cells that have been activated following interaction with donor CD4 T-cells. B cells are capable of 'soliciting' their own help [42], and hence, it is likely that these CD4 T-cells are responding to self-restricted peptide autoantigen that is presented by the activated B cell. This would imply that a diverse repertoire of autoreactive host CD4 T-cells are required for T_{FH} cell differentiation and ultimately, for graft rejection, and would provide an explanation why in the bm12 to B6 model, a monoclonal population of host, direct-pathway CD4 T-cells are unable to mediate heart allograft rejection [43].

The observed graft endothelial complement deposition and *in vitro* endothelial responses to purified serum immunoglobulin suggest that the GC principally effects vasculopathy development by influencing the 'quality' of autoantibody produced. This may relate to the GC's ability to produce mutated plasma cells that secrete high affinity autoantibody via somatic hypermutation, coupled with the deposition of these plasma cells as long-lived progeny in the bone marrow (Figure 6a). Alternatively, the demonstration of late anti-vimentin responses raises the possibility that epitope diversification to encompass additional target autoantigens may also be important for mediating graft rejection. However, although we chose to study the anti-vimentin response because of the previously-reported association with graft rejection, our data do not demonstrate that this response is specifically involved in progression of allograft vasculopathy; indeed, not all recipients developed anti-vimentin autoantibody and in those that did, the response often occurs after graft rejection. Nevertheless, vimentin is a type III intermediate filament (IF) protein that is expressed ubiquitously in mesenchymal cells and has important functions in endothelial adhesion [3]. Hence its targeting by humoral autoimmunity in potentiating allograft vasculopathy is credible, and has been suggested in clinical

transplantation [44]. Notwithstanding, a number of other autoantigens have been proposed as targets for TAA; including cardiac myosin [45], perlecan [36], and collagen V protein [7]. Our ongoing studies aim to characterise the GC autoimmune response in greater detail, and will examine which, if any, of these are important for vasculopathy progression, as well as attempting to identify novel pathogenic target autoantigens.

The development of allograft vasculopathy in parental heart grafts transplanted into F1 hybrid recipients provides the strongest evidence for an effector role for humoral autoimmunity in mediating graft specific injury, independently of conventional adaptive alloimmune responses. As in the MHC-class II-mismatched model, the development of humoral autoimmunity was dependent upon parental strain passenger CD4 T-cells that were transferred within the heart allograft, and the amelioration of allograft vasculopathy in the F1 SAP-deficient recipients was equally striking (Figure 8). Although we have recently detailed that passenger CD4 T-cells can provide help to host alloreactive B cells for generating alloantibody against MHC alloantigen expressed on donor cells [5], alloantibody was not generated in the F1 recipients, most likely because, as membrane proteins, the H-2^b and H-2^d MHC antigens provoke robust, central deletional B cell tolerance.

Given the apparent robust GC responses that are generated in recipient mice, it is surprising that autoimmune disease manifestations are not observed routinely in the recipients' native organs [5, 6, 19], particularly when one considers that administration of bm12 splenocytes is used, in B6 recipients, as a model for systemic lupus erythematosus (SLE) [17]. We do see occasional skin and gut manifestations, consistent with autoimmune disease, in long-term surviving recipients of bm12 heart transplants (> 6 months), and interestingly, of the recipient group that developed early, augmented autoantibody responses and rapid allograft rejection

following challenge with allografts from primed bm12 donors, 2 of 5 recipients had to be culled early due to the development of similar features (Figure 2 in [27]). Thus, it appears that the levels of autoantibody generally generated in our transplant recipients do not reach a threshold for provoking systemic autoimmune disease, and it is likely that the number of purified bm12 CD4 T-cells typically transferred in the SLE studies are substantially greater than are contained within a bm12 heart allograft. Of note, Zhou et al. have recently described the development of native arterial disease in B6 recipients of bm12 heart grafts, but only in atherosclerosis-prone (apoE^{-ve}) mice [46]. In Zhou's study, autoantibody responses were not assessed, but our findings suggest they would have developed, and as such, likely contributed to the arteriopathy described in native vessels. This would be consistent with a two-hit hypothesis, whereby the potential for autoantibody to effect arterial disease is greatly enhanced by concurrent damage that possibly unmasks previously sequestered target autoantigen [1].

Such a hypothesis may explain why vasculopathy is preferentially observed in the heart allografts in our experiments – that the transplant process results in damage to the allograft that is then potentiated by concurrent autoimmunity. If so, however, then the obvious candidate for such injury; antigen non-specific ischaemia reperfusion injury; does not appear to be responsible, because syngeneic heart grafts did not develop vasculopathy when transplanted into syngeneic recipients that had been simultaneously primed for humoral autoimmunity. A possible alternative is that allograft damage is initiated by innate immune recognition of non-self alloantigen. This is a recently described concept, and remains poorly understood [47], but it is now evident that monocytes [48] and NK cells[49] , can respond to allogeneic determinants, such as the recently-described polymorphisms in signal regulatory protein α [SRPα [50]].

As reported previously [35], parental strain allografts did not develop vasculopathy when transplanted into NK cell-depleted F1 recipients. The requirement for NK cells in rejection of parental strain allografts most likely reflects Fc-receptor mediated recognition of bound autoantibody on graft endothelium. Although the mechanism remains unclear, these antibody-dependent T-cell-mediated cytotoxic responses may synergise with, or be partially dependent upon, simultaneous complement activation, and it is notable that complement deposition was not observed within allografts explanted from NK-cell depleted recipients.

What then are the implications of our findings? The bm12 to B6 model remains one of the most widely studied experimental models of chronic rejection, and our results clearly have immediate relevance to the interpretation of transplant data obtained in this model, particularly that data suggesting donor-related factors modulate rejection [15]. More widely, several murine models of chronic rejection rely on minimal MHC-mismatches between donor and recipient to avoid acute graft rejection [51], and this may permit survival of passenger donor CD4 T-cells long enough to trigger recipient humoral autoimmunity. The demonstration of humoral vascular rejection in these models would therefore warrant analysis for circulating autoantibody.

With regards clinical transplantation, the role of donor CD4 T-cells in triggering recipient humoral autoimmunity has obvious parallels with the 'passenger lymphocyte syndrome' that is described in recipients of solid organ allografts, and which is increasingly emphasised [18]. To what extent the autoimmune phenomena observed in human transplant patients relates to transfer of passenger CD4 T-cells with the allograft is, however, not known, and requires further study. Notwithstanding, our experiments were designed to clarify an effector role for GC autoimmunity independently of conventional adaptive alloimmunity, and our results suggest that irrespective of the mechanisms responsible for its initiation, the development of humoral

autoimmunity in human transplant patients is likely to have a deleterious impact on transplant outcome. This may be particularly relevant to strategies that aim to promote transplant tolerance by achieving mixed haematopoietic chimerism [52], because our experiments with bone marrow chimeric recipients highlight the potential for triggering autoantibody responses even when tolerance to alloantigen is maintained. Notably, humoral autoimmunity, determined as in our studies by HEp2 indirect immunofluorescence, has been recently demonstrated in mixed chimeric kidney transplant recipients [53].

Our findings may have implications beyond transplantation. The experiments incorporating the SAP deficient bm12 CD4 T-cells illustrate that a population of CD4 T-cells that are fundamentally incapable of T_{FH} differentiation are nevertheless the key trigger for generating long-lasting GC humoral autoimmunity, with the bm12.K^d transfer experiments confirming that late help is provided by T_{FH} differentiation from naïve host CD4 T-cells. This suggests that in clinical practice, the T_{FH} cells responsible for maintaining a pathogenic GC autoantibody response may not be the same helper T-cell subset as responsible for its initiation. By such a mechanism, it would be possible for short-lived CD4 T-cells responding to, for example, a viral challenge to trigger autoimmunity, but for that population to be no longer evident once the disease is established.

Conclusion

In conclusion, this work demonstrates that autoantibody responses contribute to allograft rejection independently of recipient adaptive alloimmunity, but that this requires development of germinal centre reactions, with help provided by differentiation of recipient T follicular helper cells.

5. Acknowledgements

This work was supported by a British Heart Foundation Clinical Research Training Fellowship to MSQ. MC was supported by the Agency for Science Technology and Research (A*STAR), Singapore. JA was supported by a grant from King Saud University, Kingdom of Saudi Arabia. JMA was supported by a Wellcome Trust Clinical Research Training Fellowship and Raymond and Beverly Sackler Scholarship. RM was supported by a European Society of Organ Transplantation Junior Basic Science Grant. MAL is supported by the Bioscience and Biotechnology Research Council. The authors acknowledge support from the National Institute of Health Research Cambridge Biomedical Research Centre and the NIHR Blood and Transplant Research Unit in Organ Donation and Transplantation at the University of Cambridge in collaboration with Newcastle University and in partnership with NHS Blood and Transplant (NHSBT).

Author contributions: MSQ, RZM, GJP designed the study and wrote the manuscript. MSQ, RMZ, JA, MC, JAM, CD, TMC, ML conducted experiments. MSQ analysed the all data. MG assessed allograft vasculopathy.

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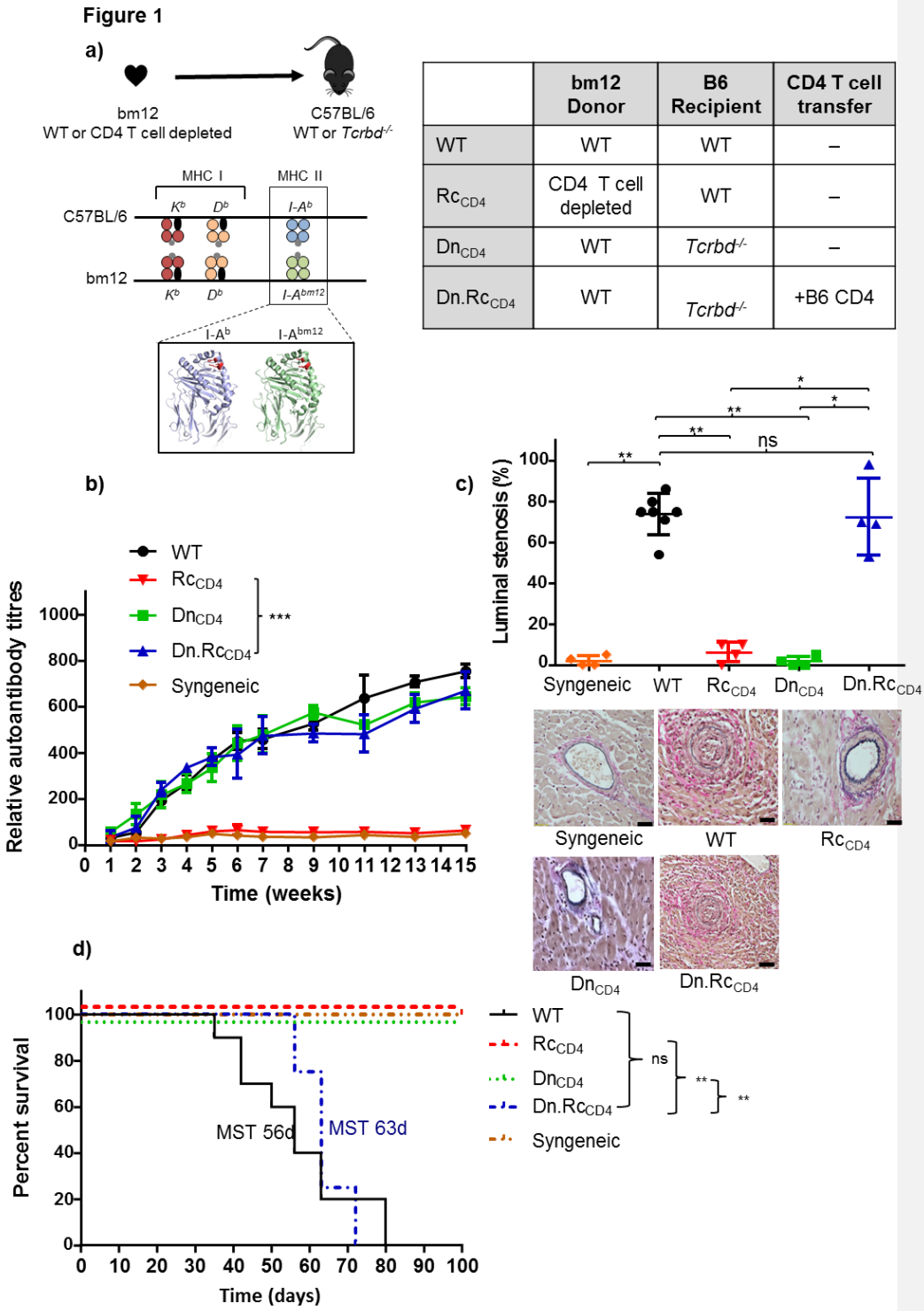
Figure 1



	bm12 Donor	B6 Recipient	CD4 T cell transfer
WT	WT	WT	–
Rc_{CD4}	CD4 T cell depleted	WT	–
Dn_{CD4}	WT	*Tcrbd*$^{-/-}$	–
$Dn.Rc_{CD4}$	WT	*Tcrbd*$^{-/-}$	+B6 CD4

Figure 1. Donor and recipient CD4 T-cell populations are required for autoantibody-mediated allograft vasculopathy.

(a) MHC class-II disparate heart grafts from wild-type (WT) or CD4 T-cell depleted bm12 donors were transplanted into WT or T-cell deficient *Tcrbd*$^{-/-}$ C57BL/6 (B6) recipients: group nomenclature depicted in table. The bm12 differs from the B6 by a three amino acid substitution in the third hypervariable region of the β chain of I-A MHC class-II molecule (illustrated). **(b)** Following transplantation, recipient autoantibody responses were measured weekly and expressed relative to positive hyperimmune control serum (1000 units). Autoantibody development was dependent upon transfer of donor bm12 CD4 T-cells, but independent of recipient CD4 T-cells. **(c)** At explant, the severity of allograft vasculopathy was determined morphometrically (histogram), with representative photomicrographs of EVG stained paraffin sections (scale bars 50μm). Severe vasculopathy (fibroproliferative arterial intimal thickening) was only observed in groups in which donor and recipient CD4 T-cell subsets were present. **(d)** Kaplan-Meier curves depicting allograft survival.

Data represent mean and SD of n = 4-10 mice per group with discrete data-points in **c** depicting samples from individual animals. ns – not significant: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Figure 2

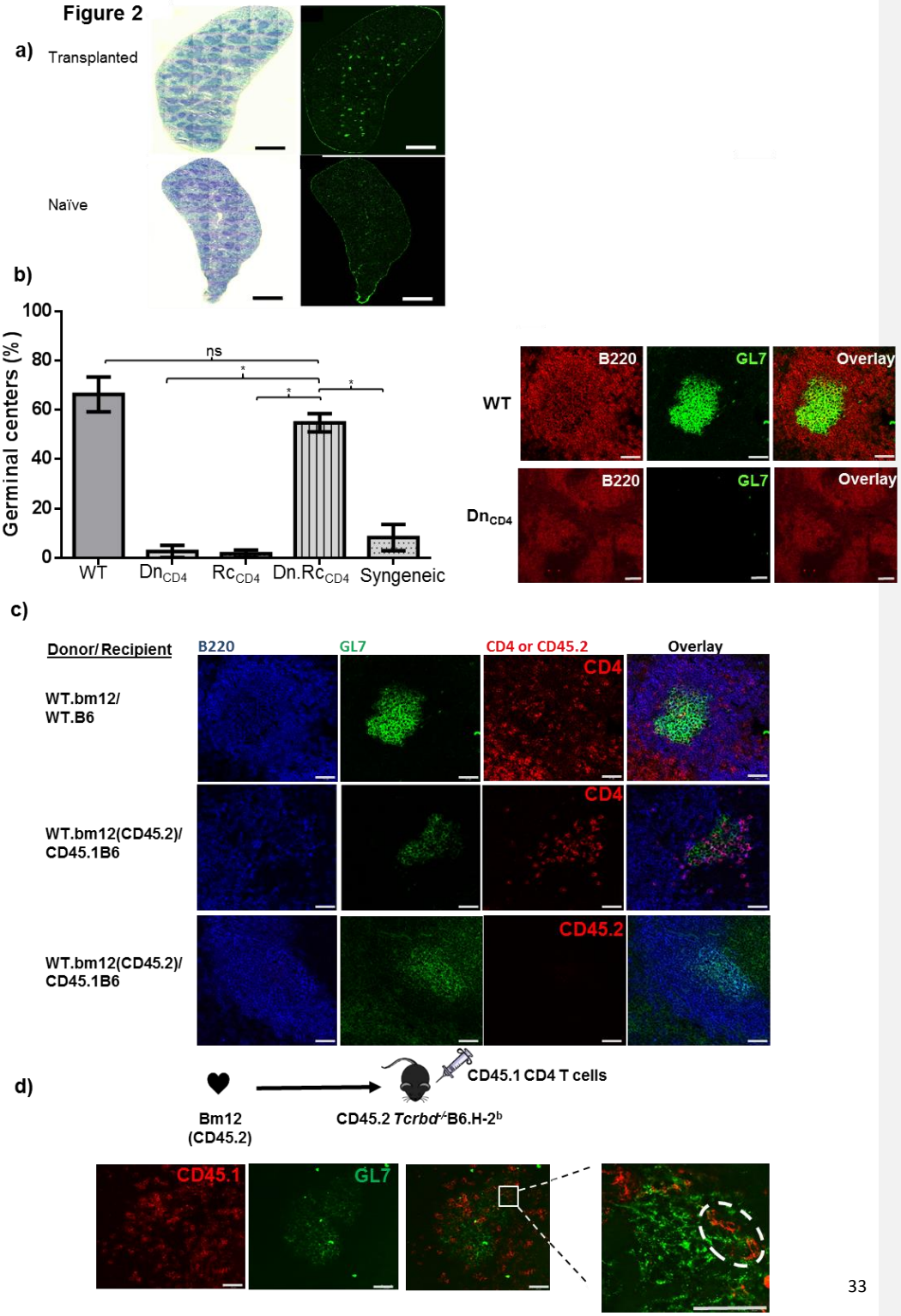

a)
Transplanted
Naïve
b)
Germinal centers (%)
100
80
60
40
20
0
ns
*
*
*
WT
Dn$_{CD4}$
Rc$_{CD4}$
Dn.Rc$_{CD4}$
Syngeneic
B220
GL7
Overlay
WT
Dn$_{CD4}$
c)
Donor/ Recipient
B220
GL7
CD4 or CD45.2
Overlay
WT.bm12/
WT.B6
CD4
WT.bm12(CD45.2)/
CD45.1B6
CD4
WT.bm12(CD45.2)/
CD45.1B6
CD45.2
d)
CD45.1 CD4 T cells
Bm12
(CD45.2)
CD45.2 Tcrbd$^{-/-}$B6.H-2^{b}
CD45.1
GL7

Figure 2: Recipient T follicular helper CD4 T-cells are required for maintaining recipient germinal center autoantibody responses.

(a) WT B6 recipients splenic section of bm12 allografts demonstrating widespread GC activity at week 7, compared to naïve mice, light microscopy of H&E-stained paraffin sections (left) and by immunofluorescence labelling of $GL7^{+ve}$ (green) secondary follicles (right) **(b)** Frequency of splenic GCs (histogram), in the various recipient groups as designated in Figure 1. Images depict representative confocal images of $GL7^{pos}$ GCs in WT B6 (top row) and *Tcrbd*$^{-/-}$ B6 (bottom row) recipients of bm12 heart allografts. *$P < 0.05$, (n=5). **(c)** Confocal immunofluorescence of splenic sections of WT B6 recipients of bm12 heart allografts at week seven post-transplantation (top row) confirmed presence of CD4 T-cells (red) within $GL7^{pos}$ (green) GC foci in B-cell follicle (blue). Following transplantation of CD45.2 bm12 heart allografts into congenic CD45.1 B6 recipients, CD4 T-cells were readily evident (middle row) within GCs, but $CD45.2^{+ve}$ donor CD4 T-cells were not detectable (lower row). **(d)** Seven weeks after transplantation of CD45.2 bm12 hearts into CD45.2 *Trcbd*$^{-/-}$ B6, a population of congenic (CD45.1) ***recipient*** B6 CD4 T-cells (transferred at transplantation) was readily evident within the GC (far right – expanded image with CD45.1 CD4 T-cell population circled. Data are representative of 4 mice per group (a) and of two independent experiments (b & c). Image scale bars; 200μm for a, 40μm for b, c and d.

Figure 3

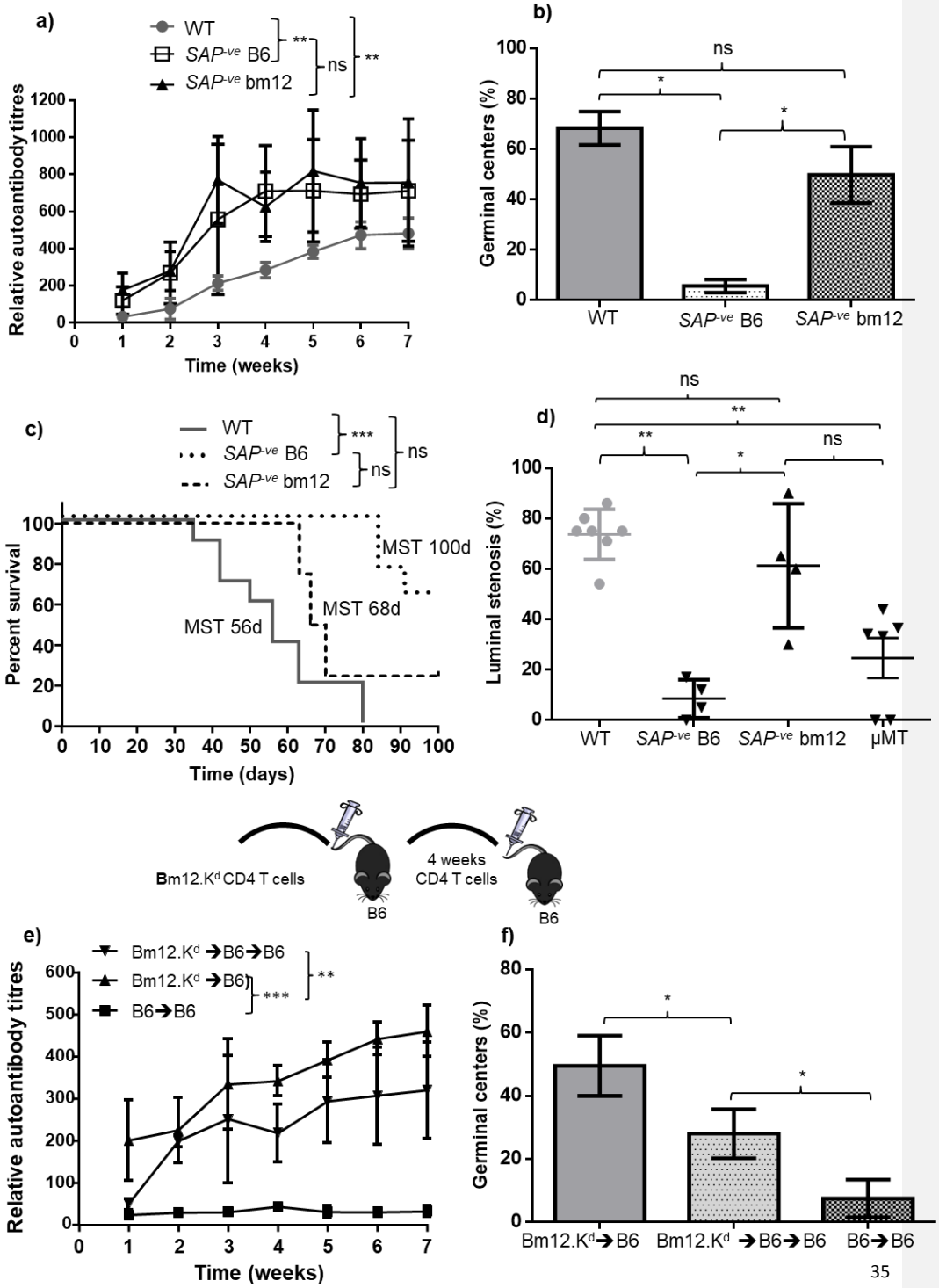

a)
WT
SAP-ve B6
SAP-ve bm12
**
ns
**
Relative autoantibody titres
Time (weeks)
b)
ns
*
*
Germinal centers (%)
WT
SAP-ve B6
SAP-ve bm12
c)
WT
SAP-ve B6
SAP-ve bm12
***
ns
ns
MST 100d
MST 68d
MST 56d
Percent survival
Time (days)
d)
ns
**
**
*
ns
Luminal stenosis (%)
WT
SAP-ve B6
SAP-ve bm12
µMT
Bm12.Kd CD4 T cells
B6
4 weeks
CD4 T cells
B6
e)
Bm12.Kd →B6→B6
Bm12.Kd →B6)
B6→B6
**
***
Relative autoantibody titres
Time (weeks)
f)
*
*
Germinal centers (%)
Bm12.Kd→B6
Bm12.Kd →B6→B6
B6→B6

Figure 3: Germinal Center autoantibody responses mediate progression of allograft vasculopathy.

Wild-type (WT) or SAP-deficient (*Sh2d1a*$^{-/-}$) bm12 heart allografts were transplanted into WT or SAP-deficient (*Sh2d1a*$^{-/-}$) C57BL/6 (B6) recipients (WT bm12 to WT B6 (WT); WT bm12 to *Sh2d1a*$^{-/-}$B6 (SAP^{-ve} B6); *Sh2d1a*$^{-/-}$bm12 to WT B6 (SAP^{-ve} bm12). Antinuclear autoantibody responses **(a)**, splenic germinal center activity **(b)**, graft survival **(c)**, and allograft vasculopathy **(d)** are depicted as described for Figures 1 & 2. Figure **(d)** also depicts the severity of vasculopathy in heart allografts transplanted from WT bm12 mice into B cell deficient μMT recipients.**(e & f)** Purified bm12.K^{d} CD4 T-cells were transferred into naïve B6 recipients, and four weeks later, CD4 T-cells were purified from these challenged mice and transferred into secondary naïve B6 recipients (Bm12.K^{d} ➔B6➔B6). Autoantibody responses **(e)** and germinal center activity at week 7 **(f)** are shown, compared to responses observed in B6 mice challenged with purified bm12.K^{d} CD4 T-cells (Bm12.K^{d}➔B6) or purified syngeneic B6 CD4 T-cells (B6➔B6).

Data represent mean and SD of n = 4-10 mice per group with discrete data-points in **d** depicting samples from individual animals. ns – not significant: *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 4

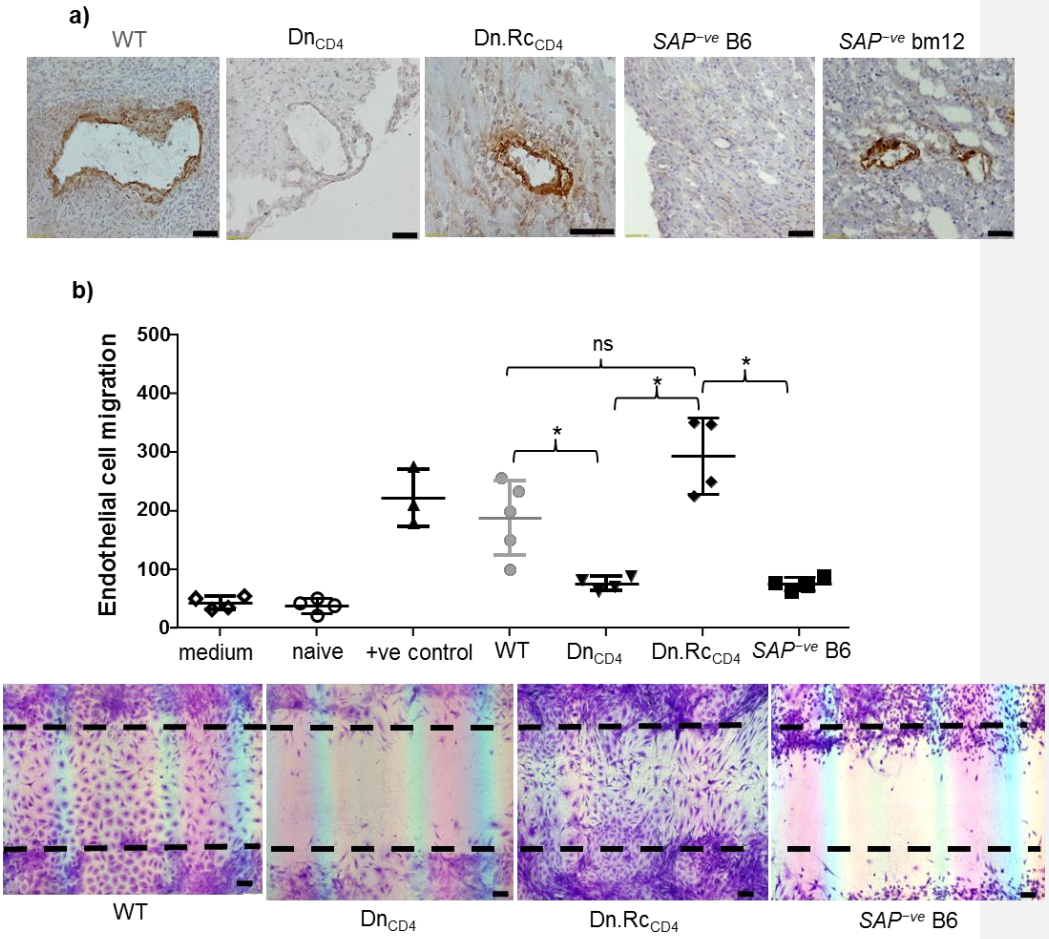

a)
WT
Dn$_{CD4}$
Dn.Rc$_{CD4}$
SAP^{-ve} B6
SAP^{-ve} bm12
b)
Endothelial cell migration
500
400
300
200
100
0
ns
*
*
*
medium
naive
+ve control
WT
Dn$_{CD4}$
Dn.Rc$_{CD4}$
SAP^{-ve} B6
WT
Dn$_{CD4}$
Dn.Rc$_{CD4}$
SAP^{-ve} B6
c)
Relative anti-vimentin IgG
1000
800
600
400
200
0
**
ns
ns
ns
*
ns
*
Week
7
15
WT
Rc$_{CD4}$
Dn$_{CD4}$
Dn.Rc$_{CD4}$
SAP^{-ve} B6
SAP^{-ve} bm12

Figure 4: Germinal center autoantibody responses trigger endothelial cell responses and are associated with intermolecular epitope diversification

(a). Immunohistochemical staining of explanted allografts reveals endothelial complement C4d deposition in donor-recipient combinations in which late GC activity was observed (WT, $Dn.RC_{CD4}$, SAP^{-ve} bm12: group designations as in Figure 1 and 3), but not in combinations in which late GC activity was absent (Dn_{CD4} and SAP^{-ve} B6). **(b)** Endothelial C4d complement deposition correlated with the ability of week 7 sera from transplanted recipients to induce in vitro proliferation / migration of bm12 cultured endothelial cells in 'scratch-wound' assay (y-axis - number of cells encroaching into scratch). Representative photomicrographs of migration into the scratch wound are included. As positive control, commercial anti-H-$2D^b$ mAb was added to endothelial cells. **(c)** Anti-vimentin IgG autoantibody responses in recipient groups were determined at weeks 7 and 15 after transplantation by indirect ELISA. Late anti-vimentin responses developed in those recipient groups in which GC activity was observed at week 7.

Data represent mean and SD of n = 4-8 mice per group, with discrete data-points in **b** & **c** depicting samples from individual animals. ns – not significant: $*P < 0.05$, $**P < 0.01$. Image scale bars are 50µm.

Figure 5

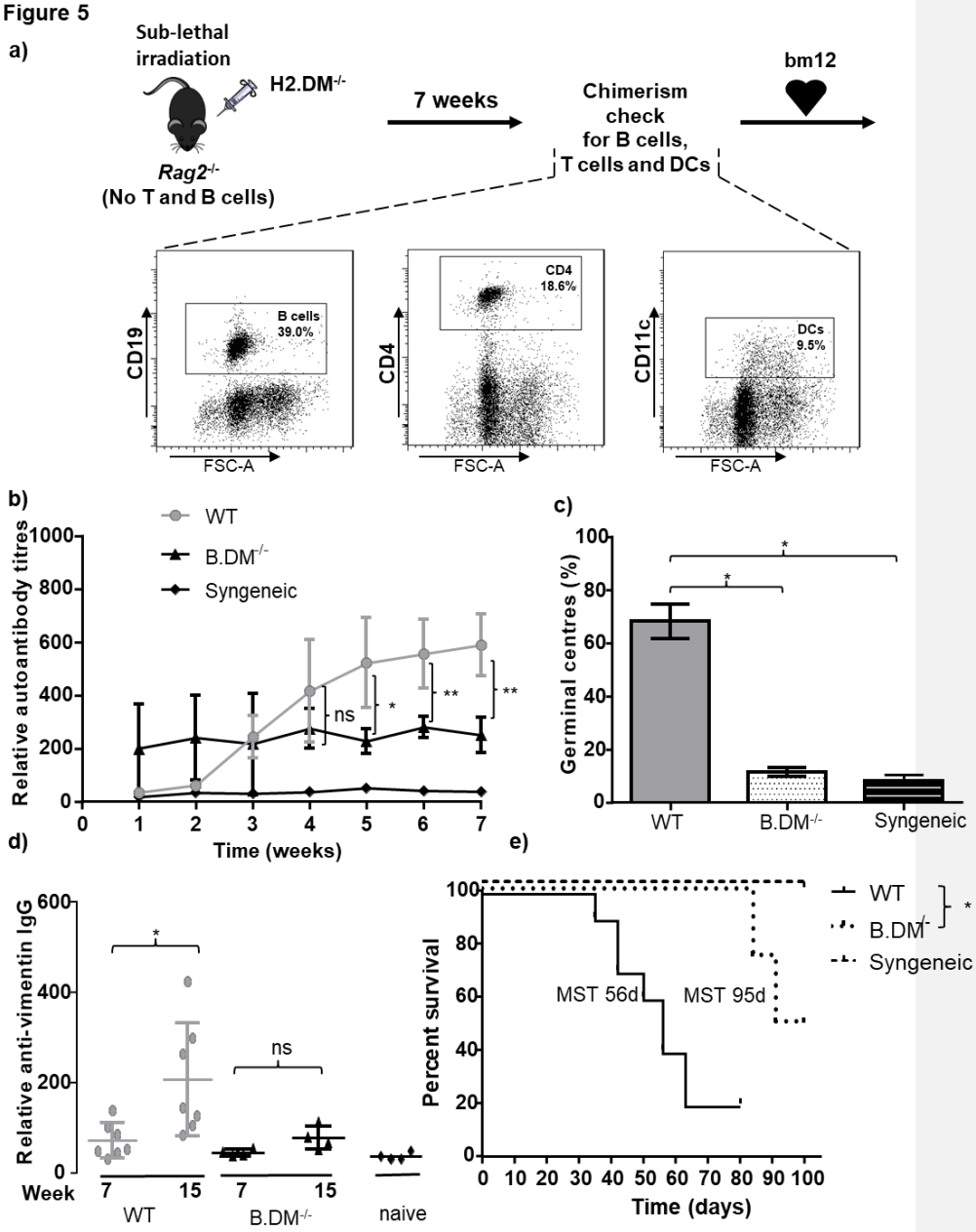

a)
Sub-lethal irradiation
H2.DM-/-
Rag2-/-
(No T and B cells)
7 weeks
Chimerism check for B cells, T cells and DCs
bm12
CD19
B cells 39.0%
FSC-A
CD4
CD4 18.6%
FSC-A
CD11c
DCs 9.5%
FSC-A
b)
WT
B.DM-/-
Syngeneic
Relative autoantibody titres
Time (weeks)
ns
*
**
**
c)
Germinal centres (%)
WT
B.DM-/-
Syngeneic
d)
Relative anti-vimentin IgG
Week
7
15
7
15
WT
B.DM-/-
naive
ns
e)
Percent survival
MST 56d
MST 95d
Time (days)
WT
B.DM-/-
Syngeneic
*

Figure 5: B cell antigen presentation is required for germinal center autoimmunity

(a) H-2^b mice with an isolated defect in B-cell antigen presentation (B.$DM^{-/-}$) were created by adoptive transfer of bone marrow from *H-2DM* genetically-deficient mice into sub-lethally irradiated *Rag2*$^{-/-}$ mice. Flow cytometry plots, gating on live lymphocyte fraction (left and middle) and macrophage / dendritic cell fraction (right), demonstrating that the resultant B-$DM^{-/-}$ mice have restored expression of $CD19^{+ve}$ B cells and $CD4^{+ve}$CD4 T-cells (gated numbers represent percentage of live lymphocytes). Whereas all B-cells will be defective for H-2DM expression, other antigen presenting cell subsets will partially originate from the H-$2DM^{+/+}$ *Rag2*$^{-/-}$ bone marrow fraction and hence are capable of relatively normal antigen presentation. Following transplantation of B-$DM^{-/-}$ mice with bm12 heart allografts, antinuclear autoantibody responses **(b)**, germinal center activity **(c)**, anti-vimentin autoantibody responses **(d),** and allograft survival **(e)** were assessed as described for Figures 3 and 4 and compared to responses observed in wild-type (WT) B6 and recipients of syngeneic (B6) transplants.

Data represent mean and SD of n = 4-10 mice per group, with discrete data-points in **d** depicting samples from individual animals. $^{*}P < 0.05$, $^{**}P < 0.01$ (2-tailed t-test for individual time-points in **b**)

Figure 6

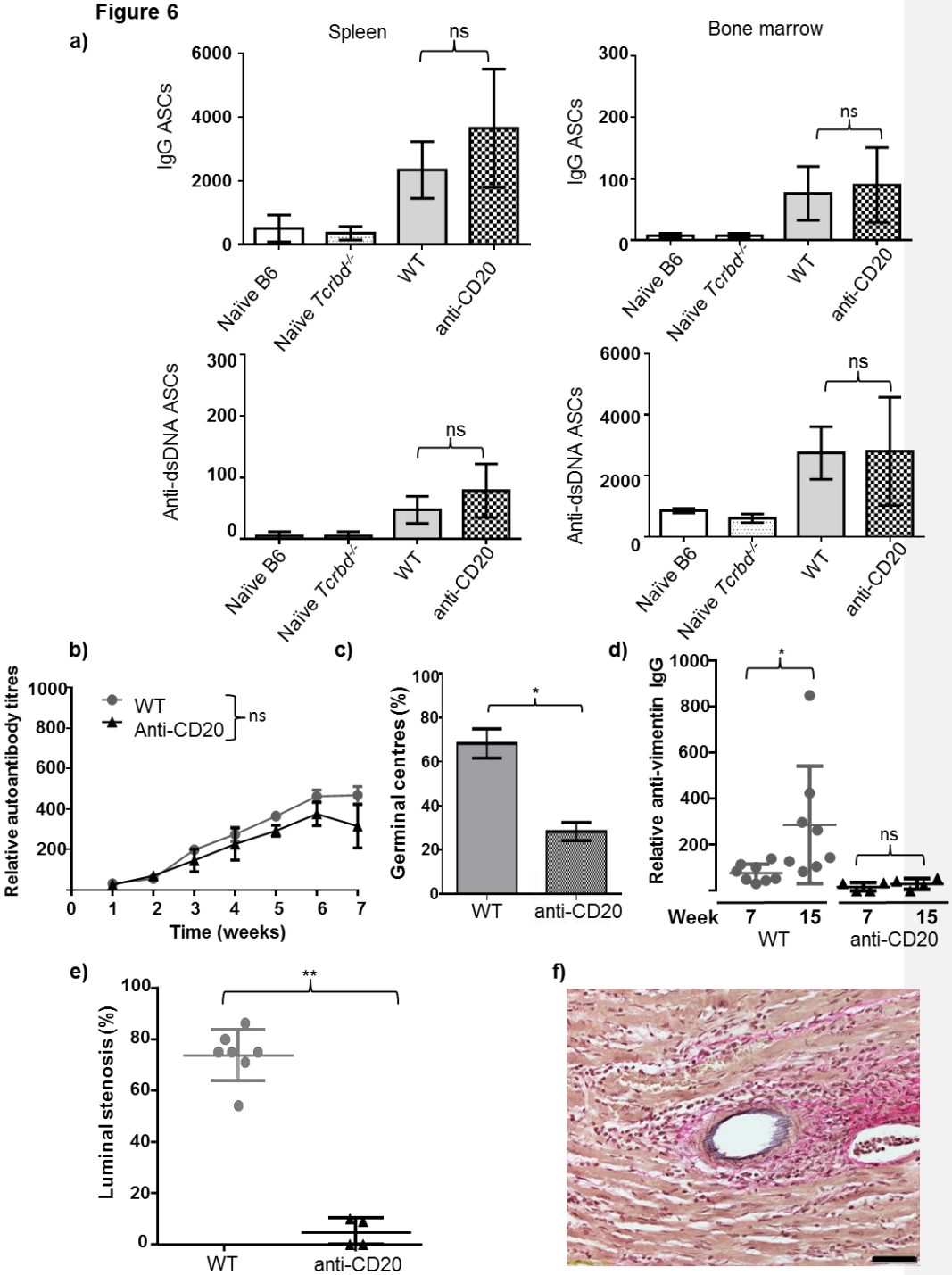

a)
Spleen
Bone marrow
IgG ASCs
Anti-dsDNA ASCs
ns
Naïve B6
Naïve Tcrbd-/-
WT
anti-CD20
b)
Relative autoantibody titres
WT
Anti-CD20
Time (weeks)
c)
Germinal centres (%)
d)
Relative anti-vimentin IgG
Week
e)
Luminal stenosis (%)
f)

Figure 6: Late B cell depletion prevents epitope diversification and improves allograft survival

C57BL/6 (B6) recipients were treated with depleting anti-CD20 on days 25, 35 and 45 after transplantation with bm12 heart allografts. Seven weeks after transplantation, IgG-secreting (top row) and anti-double-stranded DNA (dsDNA, bottom row) plasma cells were enumerated in spleen (left) and bone marrow (right) by standard ELISPOT assay and expressed as antibody secreting cells (ASCs)/ million cells plated. Shown for comparison are responses in WT B6 recipients of WT bm12 donors (wild-type) and in naïve, untreated wild-type and *Tcrbd*$^{-/-}$ B6 mice **(a)**. Anti-nuclear IgG autoantibody responses in anti-CD20 treated recipients were similar to those observed in untreated recipients **(b)**, whereas frequency of splenic GCs seven weeks after transplant were reduced **(c)**. This reduction was associated with amelioration of the late anti-vimentin autoantibody response **(d)** and with reduction in the severity of allograft vasculopathy as assessed at explant at seven weeks **(e)**. Elastin van Gieson staining of allografts at explant revealed widespread foci of cellular infiltration (**f)** (scale bars 50μm).

Data represent mean and SD of n = 4-8 mice per group, with discrete data-points in **d & e** depicting samples from individual animals. *$P < 0.05$.

Figure 7

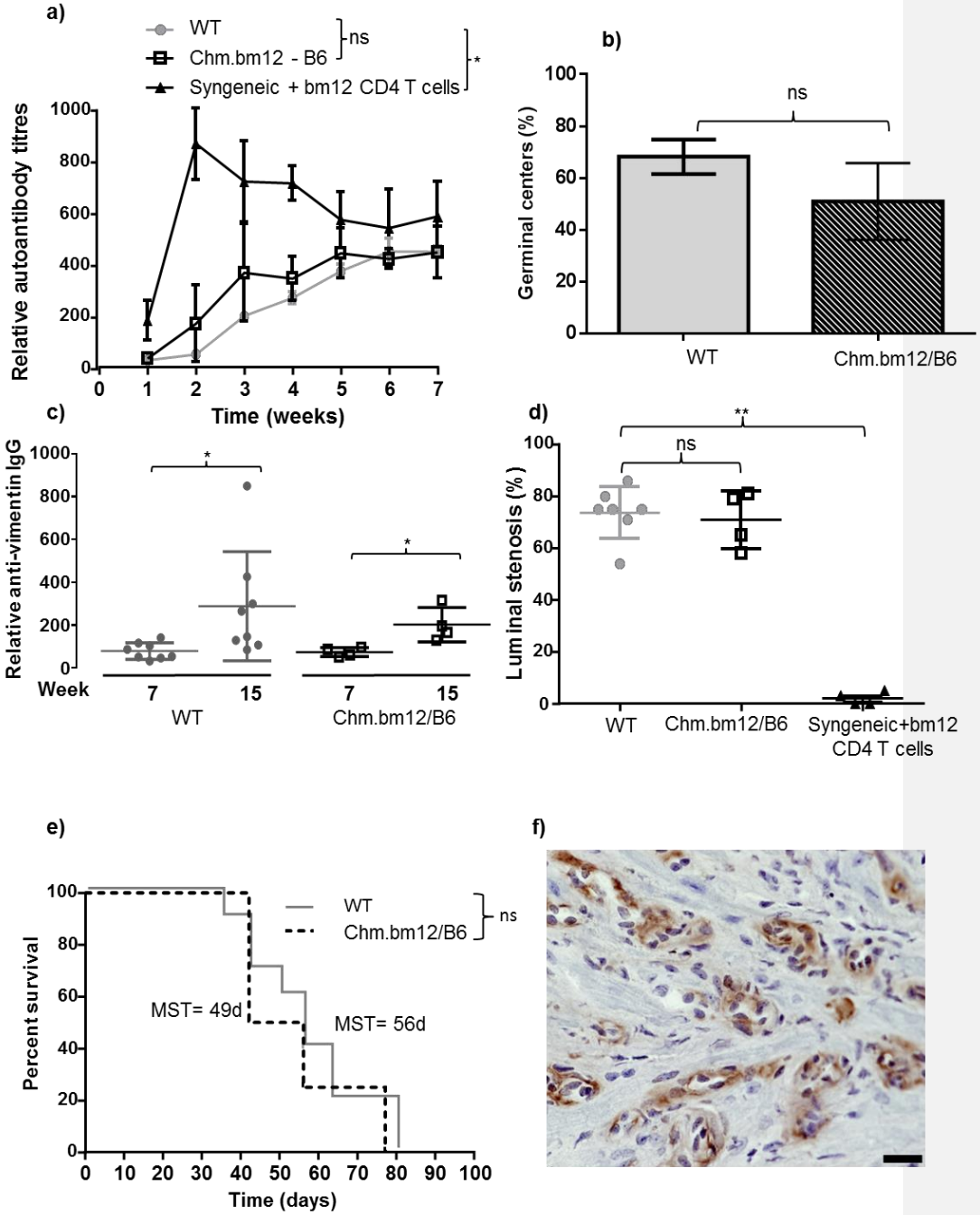

a)
WT
Chm.bm12 - B6
Syngeneic + bm12 CD4 T cells
ns
*
Relative autoantibody titres
Time (weeks)
b)
Germinal centers (%)
ns
WT
Chm.bm12/B6
c)
Relative anti-vimentin IgG
Week
7
15
WT
Chm.bm12/B6
d)
Luminal stenosis (%)
ns
**
WT
Chm.bm12/B6
Syngeneic+bm12 CD4 T cells
e)
Percent survival
WT
Chm.bm12/B6
ns
MST= 49d
MST= 56d
Time (days)
f)

Figure 7: Donor:recipient bone-marrow chimeric mice reject donor strain heart allografts

Bm12 to C57BL/6 bone marrow chimeric mice were created as described in the Methods section (Chm.bm12/B6) and 7 weeks later, upon flow cytometric confirmation of stable haematopoietic chimerism, further challenged with a bm12 heart allograft. Anti-nuclear IgG autoantibody responses **(a)**, week 7 splenic germinal center activity **(b)**, anti-vimentin IgG autoantibody responses **(c)**, allograft vasculopathy of week 7 explanted allografts **(d)**, and allograft survival **(e)** was assessed as described in Figures (1, 3 & 4) and the results compared to those achieved in wild-type C57BL/6 recipients of a bm12 heart allograft (WT.B6) and to C57BL/6 recipients transplanted with a syngeneic C57BL/6 heart allografts and challenged intravenously with purified bm12 CD4 T-cells and (syngeneic+bm12). At explant at 7 weeks, cryostat immunohistochemistry confirmed widespread endothelial C4d complement deposition **(f)**; scale bar 20µm.

Data represent mean and SD of n = 4-10 mice per group, with discrete data-points in **c & d** depicting samples from individual animals**.** *$P < 0.05$.

Figure 8

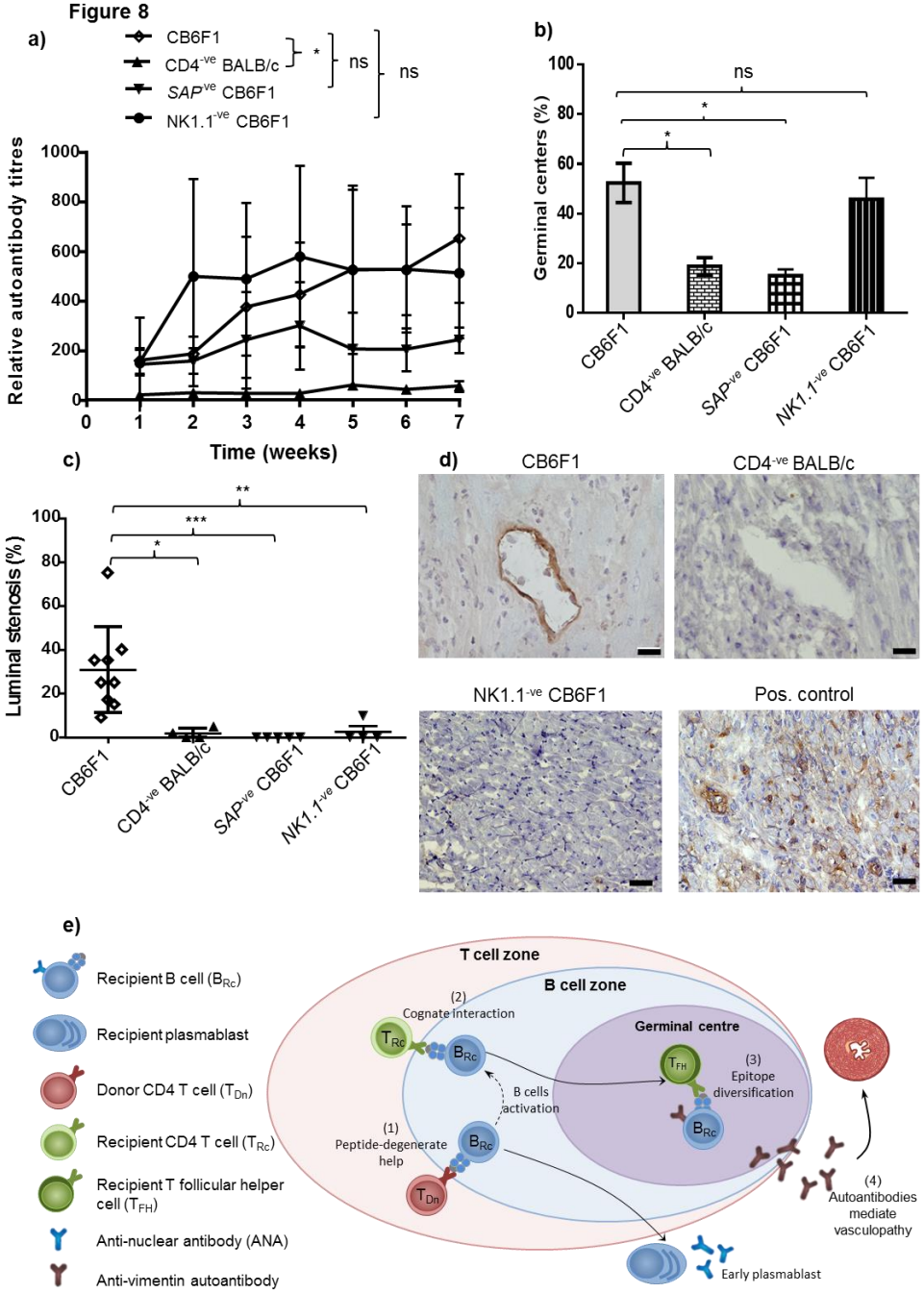

a)
CB6F1
CD4-ve BALB/c
SAPve CB6F1
NK1.1-ve CB6F1
*
ns
ns
Relative autoantibody titres
Time (weeks)
b)
Germinal centers (%)
ns
*
*
CB6F1
CD4-ve BALB/c
SAPve CB6F1
NK1.1-ve CB6F1
c)
Luminal stenosis (%)
**
***
*
d)
CB6F1
CD4-ve BALB/c
NK1.1-ve CB6F1
Pos. control
e)
Recipient B cell (BRc)
Recipient plasmablast
Donor CD4 T cell (TDn)
Recipient CD4 T cell (TRc)
Recipient T follicular helper cell (TFH)
Anti-nuclear antibody (ANA)
Anti-vimentin autoantibody
T cell zone
B cell zone
Germinal centre
(1) Peptide-degenerate help
(2) Cognate interaction
B cells activation
(3) Epitope diversification
(4) Autoantibodies mediate vasculopathy
Early plasmablast

Figure 8: Germinal centers autoantibody responses independently mediate progression of allograft vasculopathy

Wild-type BALB/c heart allografts were transplanted into either unmodified BALB/c x C57BL/6 F1 (CB6F1) mice or CB6F1 mice genetically deficient in expression of SAP (SAP^{-ve} CB6F1)) or CB6F1 mice depleted of NK T-cells ($NK1.1^{-ve}$ CB6F1). Heart grafts from CD4 T-cell-depleted BALB/c donors were transplanted into unmodified CB6F1 recipients ($CD4^{-ve}$ BALB/c). Following transplantation, anti-nuclear IgG autoantibody **(a)**, week 7 splenic GC activity **(b)**, and allograft vasculopathy at week 7 **(c)** were assessed as described in Figures 1 & 2. Cryostat immunohistochemistry of heart allografts at week 7 revealed widespread endothelial C4d complement deposition in CB6F1 recipients, but not in $CD4^{-ve}$ BALB/c nor in $NK1.1^{-ve}$ CB6F1 recipients **(d):** scale bar 20µm.

(e) Proposed model for interaction between donor CD4 T-cells and recipient B and T-cells for initiation and maintenance of germinal center autoimmunity. Passenger donor CD4 T-cells within the heart allograft provide 'peptide-degenerate' help to host B cells for production of anti-nuclear IgG autoantibody (1). Subsequent 'cognate' interaction between activated B cells and recipient T follicular helper (T_{FH}) cells results in long-lasting GC responses (2), which leads to inter-molecular epitope diversification, to encompass, along with other targets, responses against vimentin autoantigen (3). This diversification is associated with development of antibody-mediated allograft vasculopathy and eventual graft failure (4).

Data represent mean and SD of n = 4-9 mice per group, with discrete data-points in **c** depicting samples from individual animals**.** ns – not significant: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.