Diversity of the CD4 T Cell Alloresponse: The Short and the Long of It

Highlights

- Direct-pathway CD4 T cell alloresponses are extremely short lived.
- Indirect pathway responses vary markedly according to target alloantigen.
- In chronic rejection, MHC class I alloantigen is continually processed and presented.
- The kinetics of the alloreactive CD4 T cell response are altered profoundly.

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In Brief

The T cell allore cognition pathways governing rejection of organ allografts are poorly understood. Using a model of chronic heart graft rejection, Ali et al. show that continual presentation of MHC class I allopeptide drives late division in the responding alloreactive CD4 T cell population, resulting in a markedly augmented maintenance phase.
Diversity of the CD4 T Cell Alloresponse: The Short and the Long of It

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SUMMARY

MHC alloantigen is recognized by two pathways: “directly,” intact on donor cells, or “indirectly,” as self-restricted allopeptide. The duration of each pathway, and its relative contribution to allograft vasculopathy, remain unclear. Using a murine model of chronic allograft rejection, we report that direct-pathway CD4 T cell alloresponses, as well as indirect-pathway responses against MHC class II alloantigens, are curtailed by rapid elimination of donor hematopoietic antigen-presenting cells. In contrast, persistent presentation of epitope resulted in continual division and less-profound contraction of the class I allopeptide-specific CD4 T cell population, with approximately 10,000-fold more cells persisting than following acute allograft rejection. This expanded population nevertheless displayed sub-optimal anamnestic responses and was unable to provide co-stimulation-independent help for generating alloantibody. Indirect-pathway CD4 T cell responses are heterogeneous. Appreciation that responses against particular alloantigens dominate at late time points will likely inform development of strategies aimed at improving transplant outcomes.

INTRODUCTION

Chronic rejection, leading to late graft loss, remains the major challenge for solid organ transplantation. T cells play a critical role in the development of chronic rejection (Ali et al., 2013; Libby and Pober, 2001), but it is not clear whether the early T cell response following transplantation is sufficient to mediate chronic rejection or, as seems more likely, persistent alloantigen-driven T cell responses are required over a longer period of time.

CD4 T cells recognize alloantigen through two distinct pathways. In the “direct pathway,” alloreactive T cells recognize intact donor MHC molecules presented on the surface of donor antigen-presenting cells (APCs), whereas in the “indirect pathway,” T cells recognize major, and minor, histocompatibility antigens that have been acquired by recipient APCs, processed and presented as self-MHC-restricted peptides (Ali et al., 2013; Jiang et al., 2004). The relative contribution of these pathways to chronic graft rejection remains unclear (Benichou, 1999; Gould and Auchincloss, 1999; Nadazdin et al., 2011). It has generally been assumed that direct-pathway CD4 T cell alloresponses are short lived due to rapid destruction of donor APCs following transplantation. Consequently, chronic rejection is considered to be largely mediated by indirect-pathway CD4 T cell responses (Baker et al., 2001; Ciubotariu et al., 1998; Haynes et al., 2012; Hornick et al., 2000; Safinia et al., 2010). However, late direct-pathway responses have been reported in primate studies (Nadazdin et al., 2011), possibly reflecting upregulated expression of MHC class II on allograft endothelium. Similarly, the indirect CD4 T cell allorecognition pathway is generally regarded as a single entity but is instead presumably a culmination of multiple responses against potentially every disparate alloantigen expressed by the graft. Given that these antigens are likely to be expressed at different concentrations in the graft and, in the case of MHC class II, predominantly expressed on the hematopoietic components of the graft, it is plausible that the duration and strength of indirect-pathway responses differ depending on the target alloantigen. This concept has yet to be examined definitively.

Here, we show in a murine model of chronic allograft rejection that direct-pathway CD4 T cell responses are short lived but also that indirect-pathway responses are heterogeneous and vary markedly according to target antigen. Whereas those directed against MHC II allopeptide decline rapidly after transplant, the persistent presentation of immunogenic target epitope provokes continued division of MHC class I allopeptide-specific CD4 T cells and results in a markedly augmented late maintenance phase. Anamnestic function in this expanded population is nevertheless sub-optimal. The implications of our findings to late graft rejection are discussed.

RESULTS

Experimental Approach and Characterization of Transplant Model

To examine the CD4 T cell allorecognition pathways active at early and late time points after transplantation, a donor strain (bm12.Kd.IE) was created that differed from the C57BL/6 recipient strain at the I-A^bm12 and I-E^d MHC class II and H-2K^d MHC
class I loci (Figure 1A), enabling direct and indirect CD4 T cell recipient alloresponses to be assessed by adoptive transfer of populations of TCR-transgenic CD4 T cells with precise specificity for alloantigen. Following transplantation of male bm12.Kd.IE hearts into female C57BL/6 recipients, direct-pathway CD4 T cell alloimmunity to be assessed by adoptive transfer of CFSE-labeled, TCR-transgenic ABM CD4 T cells. (B) I-A\(^{\alpha}\)-restricted indirect pathway CD4 T cell responses against MHC class I H-2K\(^{d}\), MHC class II I-E, and minor H-Y dby antigen could be similarly assessed by transfer of TCR-transgenic TCR75, Te6a, and Marilyn CD4 T cells, respectively (Figure 1B). (C and D) Whereas syngeneic C57BL/6 heart grafts survive indefinitely, bm12.Kd.IE heart allografts were not rejected acutely (Figure 1C) but showed progressive allograft vasculopathy (Figure 1D), with rejection characterized by development of germinal center (GC) anti-class I (H-2K\(^{d}\)) and anti-class II (I-E) alloantibody and anti-nuclear autoantibody responses (Figures 1E–1H).

**Heterogeneity of Indirect-Pathway CD4 T Cell Responses**

Responses of the transferred allospecific CD4 T cell clones to the heart allograft were quantified by carboxyfluorescein succinimidyl ester (CFSE) division profiles (Figure S2) but with the modification that CD4 T cell clones were transferred early, at transplantation, or late on day 28 after transplant (to measure CD4 T cell alloresponses during the first and fifth week, respectively). We reasoned that division of the transferred CD4 T cells would be contingent upon expression of, and stimulation by, the appropriate alloantigen epitope (Obst et al., 2005). In the first week after heart transplantation, robust indirect-pathway CD4 T cell responses were detected against H-2K\(^{d}\) MHC class I alloantigen, MHC class II I-E alloantigen, and minor male H-Y alloantigen were assessed by division of adoptively transferred TCR75, Te6a, and Marilyn CD4 T cells, respectively (Figure 1B): these
were preserved, albeit the anti-H-Y response was slightly attenuated. In contrast, the anti-I-E response was undetectable (Figure 2A).

Indirect Alloresponses against MHC Class II Alloantigen Are Limited by Antigen Availability

Further experiments revealed that the anti-I-E indirect-pathway response decayed by week 2 (Figure 2B). To test the hypothesis that this reflected elimination of donor hematopoietic cells (the likely major source of MHC class II alloantigen), anti-MHC class II indirect-pathway responses were evaluated following transplantation with bm12.kd.IE hearts from donors that had either been subject to lethal irradiation or depleted of the dendritic cell (DC) fraction. Both approaches resulted in marked attenuation of the early (week 1) anti-class II indirect-pathway CD4 T cell response (Figure 2C). In contrast, adoptive transfer of donor DCs into C57BL/6 recipients of a bm12.Kd.IE heart allograft at a late time point (day 28) after transplantation partially restored the late TEs CD4 T cell response (Figure 2D). Parallel experiments, in which DCs from recipients of bm12.kd.IE heart grafts were stained with “YAe” clonotypic antibody, confirmed that expression of I-E peptide epitope for TEs CD4 T cell recognition mirrored the presence and survival of the donor hematopoietic fraction (Figure 2E). As expected, recipient hematopoietic cells were also required for TEs CD4 T cell responses, reflecting their role as professional APCs for I-A<sup>b</sup>-restricted alloantigen presentation (Figure 2C). Additional experiments incorporating T- and B-cell-deficient Rag2<sup>−/−</sup> mice as recipients of bm12.kd.IE heart allografts surprisingly revealed that the rapid destruction of donor hematopoietic cells by C57BL6/6 recipients was not a consequence of innate NK cell recognition but was instead mediated by C57BL/6’s adaptive alloresponses (Figure S3).

Although MHC class II is generally not thought to be expressed by resting murine endothelium, activation-induced upregulation, as typically occurs within the endothelium of rejecting allografts, has been described (Hasegawa et al., 1998; Milton and Fabre, 1985). In support, I-E MHC class II was detectable on cultured bm12.Kd.IE endothelial cells and was evident on immunohistochemical staining of bm12.Kd.IE heart allografts explanted at 1 week (Figure 3).

The rapid decline of the anti-MHC class II indirect-pathway CD4 T cell response is therefore surprising but may reflect transient expression of MHC class II on graft parenchymal cells; memory TEs CD4 T cells (Figure S4), which would be expected to respond to target epitope even when encountered in the absence of costimulatory ligands, proliferated more robustly than naive cells when transferred into C57BL/6 recipients of bm12.Kd.IE heart allografts at the time of transplant but failed to proliferate when transferred at late time points (Figure 2D).

Persistent Indirect-Pathway CD4 T Cell Activation from Graft Parenchymal Expression of MHC Class I

Unlike anti-MHC class II indirect-pathway responses, donor DC depletion or donor irradiation made little impact on CD4 T cell allorecognition of H-2K<sup>d</sup> allopeptide (Figure 4A). To confirm that late anti-MHC class I indirect-pathway CD4 T cell responses were due to recognition of MHC class I alloantigen on graft parenchyma, bm12.Kd.IE bone marrow chimeric mice were created in which H-2<sup>Kd</sup> expression was restricted to the hematopoietic lineage, whereas the graft parenchyma expressed the I-A<sup>bm12</sup> and I-E alloantigens (Figure 4B). Heart grafts from these mice provoked only a transient anti-MHC class I indirect-pathway response (Figure 4C). Similarly, BALB/c heart allografts, which are rejected within 7 days in C57BL/6 recipients and which presumably do not shed alloantigen thereafter, did not elicit indirect-pathway responses against MHC class I at late time points (Figure 4D). Moreover, treatment of C57BL/6 recipients with anti-CD154 mAb at transplant with a BALB/c heart allograft, a protocol that prevents acute graft rejection but nevertheless results in chronic graft damage (Figure S5; Larsen et al., 1996), restored late TCR75 CD4 T cell responses, whereas indirect-pathway responses against MHC class II antigen were still only short lived (Figure 4D).

Direct-Pathway CD4 T Cell Responses Are Short Lived and Curtailed by Innate and Adaptive Alloimmunity

The longevity of direct-pathway CD4 T cell allorecognition was assessed in C57BL/6 recipients of bm12.Kd.IE heart grafts by adoptive transfer of I-A<sup>bm12</sup> reactive ABM CD4 T cells. As for anti-MHC class II indirect-pathway CD4 T cells, direct
allorecognition was short lived (Figure 5A) and prolonged upon transplantation of bm12.Kd.IE heart grafts into C57BL/6""
Late Allopeptide Presentation Shapes the Dynamics of the Endogenous CD4 T Cell Alloresponse

The confirmation, from the above adoptive transfer experiments of TCR75 CD4 T cells, that MHC class I allopeptide epitope is expressed at late time points in recipients of chronically rejecting heart allografts raises the question how this late presentation influences dynamics of the response of the endogenous alloreactive CD4 T cell population. To address this, H-2Kd-peptide-specific host T cell responses were mapped by labeling splenocytes from transplanted mice with Kd-specific tetramers using a similar approach to that described for CD4 T cell responses against conventional protein antigen (Tubo et al., 2013). Following transplantation with a bm12.Kd.IE cardiac allograft, H-2Kd allopeptide-specific CD4 T cells underwent typical expansion, contraction, and memory phases (Figure 6A), but in comparison to challenge with a BALB/c heart allograft (Figure 6A), the expansion phase was much more marked and approximately 10,000-fold more cells persisted into the memory phase. Similar levels of H-2Kd alloantigen are expressed on BALB/c and bm12.Kd.IE cardiomyocytes (Figure S6); hence, the increased number of H-2Kd-specific CD4 T cells at late time points following challenge with a bm12.Kd.IE heart allograft is presumably due to ongoing allopeptide presentation.

In support, a less-profound contraction in the allospecific CD4 T cell population occurred in C57BL/6 recipients of a BALB/c heart allograft that received anti-CD154 antibody at transplantation (Figure 6B), and this late expansion in recipients of chronically rejecting heart allografts correlated with continued division of the H-2Kd allopeptide-specific CD4 T cell population (Figure 6C).

Antigen persistence is associated with CD4 T cell exhaustion and defective anamnestic responses (Crawford et al., 2014). Surface phenotyping of the Kd-specific tetramerpos CD4 T cell population revealed that the percentage of antigen-experienced CD44hi CD4 T cells was comparable 5 weeks after transplantation with a BALB/c or bm12.Kd.IE heart allograft but that the antigen-experienced CD4 T cells in recipients of a bm12.Kd.IE heart allograft were predominantly CD44hiCCR7hiCD62Lhi effector memory, whereas those in BALB/c heart-grafted recipients were more skewed toward CD44hiCCR7hiCD62Llo central memory (Figure 6D). To assess the functional consequences of this difference, T-cell-deficient TcrbΔΔ C57BL/6 mice were reconstituted with CD4 T cells purified from recipients of bm12.Kd.IE or BALB/c heart grafts and the reconstituted TcrbΔΔ mice then challenged with a C57BL/6 donor heart that expressed H-2Kd as a transgene (B6.Kd; Honjo et al., 2004) while simultaneously receiving anti-CD154 mAb. We reasoned that only established H-2Kd-specific memory CD4 T cells would be able to provide co-stimulation-independent help for development of anti-H-2Kd alloantibody responses against the B6.Kd graft (Conlon et al., 2012a). Whereas anti-H-2Kd alloantibody was not observed in TcrbΔΔ recipients reconstituted with CD4 T cells from bm12.Kd.IE grafted mice, weak but consistent anti-H-2Kd alloantibody responses developed in TcrbΔΔ recipients reconstituted with CD4 T cells from BALB/c-grafted mice (Figure 6E), despite the vastly reduced number of H-2Kd allopeptide-specific CD4 T cells present in this latter group at late time points after transplantation (Figure 6B). Thus, chronic allopeptide presentation drives continual division of the responding alloreactive CD4 T cell population, with persistence of a greatly expanded population but with functional evidence of altered memory development.

DISCUSSION

Transplantation is unique in that MHC alloantigen can be recognized by host T cells by at least two distinct pathways. By modifying the standard technique of transfer of CFSE-labeled TCR-transgenic CD4 T cells, our experiments confirm that direct-pathway CD4 T cell allorecognition is short lived and limited by innate and/or adaptive alloimmune-mediated elimination of donor APCs. We also detail that indirect-pathway responses to chronically rejecting allografts are heterogeneous and vary according to target alloantigen, with responses against MHC class II peptides also limited by rapid destruction of donor hematopoietic cells.

Fundamental to our experimental approach was the design of a transplant model wherein direct and indirect T cell alloresponses could be assessed simultaneously and in which proliferation of a particular alloreactive T cell population when transferred at transplantation acted as control for the same population transferred at later time points. Thus, interpretation of results should not be confounded by potential differences in TCR affinity between the various TCR-transgenic clones; such concerns are further obviated by the development of additional models that enabled H-2Kd-allopeptide-specific TCR75 CD4 T cells to act either exclusively via the indirect pathway or exclusively via the direct pathway. Additionally, to reconcile observations obtained from adoptive transfer of TCR-transgenic CD4 T cells with events occurring within the endogenous alloreactive CD4 T cell population, we used synthetic MHC class II I-Aβ7/10-allopeptide tetrameric complexes to assess host CD4 T cell responses against the...
same dominant H-2Kd epitope as recognized by TCR75 CD4 T cells. This approach has been detailed recently for responses against conventional protein antigen (Tubo et al., 2013), but our studies revealed that the alloreactive CD4 T cell responses in the chronic and acute rejection models differ profoundly. In chronic rejection, less-profound contraction of the alloreactive CD4 T cell population occurred, with a markedly augmented maintenance phase. This presumably reflects continual cell division from failure to clear target allopeptide epitope, but whether this was due to ongoing cell cycling of the original alloreactive population or to late recruitment of naive alloreactive CD4 T cells (Lin et al., 2010) was not assessed.

Our findings raise the question whether the continued division and expansion of class I allopeptide-specific CD4 T cells contributes to progression of allograft vasculopathy. Our experiments were not designed specifically to address this, and it is plausible that chronically stimulated CD4 T cells are unable to mediate allograft rejection, as suggested by our data demonstrating that some aspects of allospecific CD4 T cell memory are inhibited by the continual presence of target epitope. Against this, preservation of limited anti-viral function has been described for exhausted CD4 T cells (Yi et al., 2009), and similarly, chronic salmonella infection is controlled by late CD4 T cell responses (Nelson et al., 2013). Notwithstanding, an intriguing possibility raised by our findings is that in “tolerant” recipients, the class I allopeptide-specific CD4 T cell population will be either static or dividing only minimally. Although the technical challenges are daunting, one could envisage that, in the future, tetramer characterization of the division profile of the responding alloreactive CD4 T cell population may be used clinically to identify patients at risk of chronic rejection or those in whom immunosuppression can be safely discontinued.

What then are the implications of our findings for clinical transplantation? It has been suggested that expression of MHC class II is different within human organ allografts, in that human endothelial cells upregulate MHC class II in response to IFN-γ, albeit levels are still lower than for MHC class I (Piotti et al., 2014; Rose, 1998). However, it is also clear, as we demonstrated in our model, that murine endothelium can express MHC class II, and although this expression is unable to activate direct-pathway CD4 T cell responses (Kreisel et al., 2004), it is essential for those responses to then autonomously effect graft rejection (Grazia et al., 2004). Hence, available evidence suggests that the parenchymal components of murine and human organ allografts express MHC class II. Cultured human endothelial cells can activate CD4 T cell in vitro (Adams et al., 1992; Hughes et al., 1990), although this has not been a consistent finding (Ma and Pober, 1998). Whether the parenchymal expression of MHC class II alloantigen on human allografts can drive direct-pathway CD4 T cell activation has not been tested and seems unlikely, because clinical studies report this response to be short lived (Baker et al., 2001). This further suggests that, despite concurrent administration of immunosuppression, donor hematopoietic cells are rapidly eliminated in human recipients, and it is notable that the T cell response against MHC class II alloantigen was similarly brief in our BALB/c to C57BL/6 heart transplant model, in which acute rejection was avoided by concurrent administration of anti-CD154 antibody.

Irrespective of inter-species discrepancies in immune structure and function, our principal finding that murine indirect-pathway CD4 T cell responses vary considerably according to target alloantigen is likely to be relevant to human transplantation, albeit there may be differences in the alloantigen targeted or in the constancy of expression of its epitope. Understanding the relative timing and contribution of direct and indirect CD4 T cell alloresponses to allograft rejection has become all the more important following the recent introduction of allogeneic-specific regulatory T cell (T-reg cell) therapy to clinical transplantation (Brunstein et al., 2011; Di Ianni et al., 2011; Newell et al., 2011; Trzonkowski et al., 2009; Wood et al., 2012). Our results suggest that, for a particular recipient, the success of allogeneic-specific T-reg cell therapy will be governed by whether target epitope is expressed concurrently and provide an explanation for why, in certain animal models, T-reg cells with indirect allosepecificity are more effective than those with direct allosepecificity in preventing chronic rejection (Joffre et al., 2008; Tsang et al., 2008). T-reg cells with direct allosepecificity are likely to be effective only if injected in the immediate post-transplant period. Indirect-pathway T-reg cells hold greater potential for administration at later time points after transplantation as a means of controlling development of chronic rejection, but the success of such an approach will

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**Figure 5. Direct-Pathway CD4 T Cell Responses Are Short Lived and Curtailed by Innate and Adaptive Alloimmunity**

(A) Direct-pathway CD4 T cell responses were assessed in C57BL/6 recipients of bm12.Kd.IE cardiac allografts by quantifying division of I-Abm12-reactive ABM CD4 T cells, adoptively transferred at transplantation or 5 weeks later. Division of ABM CD4 T cells following transfer into naive C57BL/6 recipients is shown for comparison.

(B) Direct-pathway responses were prolonged in Rag2–/– recipients of an unmodified bm12.Kd.IE heart allograft.

(C) Direct-pathway CD4 T cell alloresponses were also assessed in bm12 recipients of a BALB/c × C57BL/6 F1 (CB6F1) heart allograft by adoptive transfer of TCR75 CD4 T cells. In this strain combination, TCR75 CD4 T cells do not recognize recipient I-Abm12-restricted H-2Kb allopeptide but respond to H-2Kd peptide presented by I-A^d^ on donor cells.

(D) TCR75 CD4 T cell direct-pathway responses are short lived and significantly downregulated by lethal irradiation of the donor or depletion of either donor B cells or DCs.

(E) Syngeneic C57BL/6 or allogeneic CB6F1 CD4 T cells were injected into either T- and B-cell-deficient C57BL/6 Rag2–/– mice (left panel) or Rag2L2rg mice that additionally lack NK cells (right panel) and the presence of circulating injected cells (expressed as percentage of PBMCs) assessed by flow cytometry.

(F) Direct-pathway responses were assessed as in (B) by quantifying division of TCR75 CD4 T cells that were adoptively transferred at the time of, or 1 week after (week 2), transplantation of CB6F1 heart grafts into wild-type, NK cell-depleted, or T-cell-deficient bm12 recipients.

*p < 0.05, **p < 0.01, and ***p < 0.001 (Mann-Whitney test in A, B, D, and F). Data are representative of two independent experiments resulting in n = 5 in each group (A, B, D, and F; mean and SEM of n = 5 mice per group) and one experiment with n = 3 in each group (E).
require consideration of which indirect-pathway CD4 T cell responses are active.

**EXPERIMENTAL PROCEDURES**

**Animals**

Wild-type (WT) C57BL/6 (B6; H-2b) and BALB/c (H-2d) and CB6F1 (C57BL/6 x Balb/c F1) were purchased from Charles River Laboratories. Bm12 mice - B6(C)-H2-Ab1bm12/KdEq (H-2bm12m12), B6.CD11c-DTR (H-2b », B6.FVB-Tg), BALB/c.CD11c-DTR (H-2d » C.FVB-Tg), and Tcrbd » B6 (H-2bm12) mice (Jung et al., 2002) were purchased from The Jackson Laboratory. BALB/c.CD11c-DTR mice were crossed with B6 to obtain CB6F1 (H-2bm12) mice expressing the DTR gene (CB6F1.CD11c-DTR). Tcrbd » B6 mice were crossed with bm12 mice to create bm12.Tcdrbd » T-cell-deficient mice. C57BL/6 Rag2 » (H-2bm12) were gifted by Prof. T. Rabbitts (University of Cambridge). Rag2L2rg (Song et al., 2010); H-2b Rag2 » lacking the IL-2 receptor γ chain; NK deficient was kindly gifted by Dr. Francesco Colucci (University of Cambridge). C57BL/6 mice that lack expression of I-A^b but express surface I-E antigen on APCs due to I-E^a gene incorporation (ABOIE) were gifted by Prof. C. Benoist (Joslin Diabetes Center; Le Meur et al., 1985). C57BL/6-Tg(Kd)RPb mice (B6.Kd; Honjo et al., 2000) expressing surface I-E antigen on APCs due to I-E^a gene incorporation were gifted by Prof. T. Rabbitts (University of Washington; Grubin et al., 1997); Rag1 »/I-E, and H-2K^d, but not I-A^d. For TCR transgenic animals, Rag2 »/TEa mice (H-2b; TEa), specific for I-A^a-restricted I-E^k/DT peptide, were gifted by Prof. A. Rudensky (University of Washington; Grubin et al., 1997); Rag1 »/TCR75 mice (H-2c; TCR75), specific for I-A^a-restricted H-2K^d/DT peptide, were gifted by Prof. P. Bucy (University of Alabama; Honjo et al., 2004); Rag2 »/ABM mice (H-2b; ABM), I-A^ab12-restricted, were gifted by Dr. T. Crompton (Imperial College; Backstrom et al., 1999); and Rag1 »/Rag2 »/Lm7 mice (H-2c; Mar), specific for the I-A^a-restricted H-2K^d/DT peptide, were gifted by Dr. Di Scott (Imperial College; Lantz et al., 2000). Mice were bred and maintained in specific-pathogen-free animal facilities, and all experiments were approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

**Generation of Bone Marrow Chimeras**

To create chimeric mice that were CD11c-DTR † transgenic only in bone marrow cells, C57BL/6 mice were lethally irradiated (2 × 6.5 Gy) and reconstituted with 2 × 10^7 bone marrow cells from C57BL/6.CD11c-DTR mice. The DCs in these mice remained susceptible to diphtheria toxin (DT) treatment, but the mice were significantly more resistant to overall toxicity (Sopoulos and Jung, 2008). Bm12.Kd.IE mice with expression of class I H-2K^d-restricted hematopoietic compartment were generated by lethal irradiation of bm12.IE mice and reconstituted with 2 × 10^7 bone marrow cells from bm12.Kd.IE mice. Chimerism was confirmed by flow cytometric analysis of PBMCs at least 4 weeks following reconstitution.

**Skin and Heterotopic Cardiac Transplantation**

Full-thickness tail skin was sutured as 1 cm² grafts onto the recipient's back. Vascularized cardiac allografts were transplanted intra-abdominally using the technique of Corry and colleagues (Corry et al., 1973). Heart graft rejection was defined as cessation of palpable myocardial contraction, confirmed at explant for histology. In certain experiments, recipients of BALB/c heart allografts were injected i.p. with 500 µg anti-CD154 mAb (clone MR-1; BE0017-1; Bio X Cell) on days -2, 0, 2, and 4 in relation to transplantation, a protocol that prevents acute allograft rejection but that results in development of chronic allograft vasculopathy.

**In Vivo Depletion and Transfer of Donor and Recipient Leukocyte Subsets**

Hematopoietic cells were depleted as previously described (I.G.H., J.M.A., S.J.F. Harper, E. Wlodek, M.C.N., M.S.Q., R.M., K.S.-P., E.M.B., J.A.B., M.R. Clatworthy, T.M.C., and G.J.P., unpublished data) by lethal irradiation (2 × 6.5 Gy) of donor mice 7 days before heart allograft procurement. B6.CD11c-DTR, BALB/c.CD11c-DTR, and CB6F1.CD11c-DTR donors and C57BL/6 (BL/6.CD11c-DTR) chimeric heart graft recipients were treated with i.p. 30 mg/m² DT (List Biological Laboratories) on days -5, -3, and -1 (days 0) or thrice weekly in recipients. To deplete CD4 T cells, mice were treated with 1 mg i.p. anti-CD4-depleting mAb (rat IgG2b; clone YTS 191.1; biotyldrom from European Collection of Cell Cultures; Health Protection Agency; Porton Down U.) on days -5, -3, and +1 relative to transplantation and weekly thereafter. To deplete B cells, mice were treated with 250 µg i.p. depleting anti-CD19 mAb (18B12; IgG2a gifted by Cherie Butts, Biogen Idec) on day -7 and, for recipients, fortnightly thereafter. To deplete NK cells, mice were treated with 0.5 mg i.p. depleting anti-NK1.1 mAb (mouse 1G7G2b; clone PK13-1; biotyldrom from ATCC; LGG Standards Partnership) on days -2, 0, and +2 and weekly thereafter. Cell depletion was confirmed by flow cytometry.

In certain experiments, Rag2 »/Rag2L2rg mice were adoptively transferred i.v. 1 × 10^7 donor CD4 T cells (purified with anti-CD4 mouse IgM MicroBeads; Miltenyi Biotec) or 1 × 10^7 donor B cells (purified with anti-mouse CD19 MicroBeads) using an autoMACS Separator (Miltenyi Biotec).

In another experiment, 1 × 10^5 memory CD4 T cells were purified from recipients of bm12.Kd.IE or BALB/c heart grafts 6 weeks after transplant using magnetic bead separation as above and transferred into naive C57BL/6.Bm12.Kd.IE mice that were subsequently challenged with B6.Kd cardiac allografts.

**Quantification of Circulating Antibodies**

 Serum samples were collected from experimental mice at intervals and analyzed for the presence of anti-H-2K^d IgG alloantibody by ELISA as previously described (Conlon et al., 2012b). In anti-I-E alloantibody quantified by
flow cytometry, briefly, serum was serially diluted and incubated with ABOIE bone-marrow-derived DCs (BMDGs) (prepared as previously described; Siva- ganesh et al., 2013); bound alloantibody was detected with FITC-conjugated anti-mouse IgG mAb (STAR 70; Serotec) and analyzed. The geometric mean-channel fluorescence was plotted against dilution, and the AUC was calculated as for H-2Kb alloantibody.

Circulating alloantibody levels were determined by HEp-2 indirect immuno-fluorescence as previously described (Callaghan et al., 2012).

Mouse Heart Homogenization and Endothelial Cell Culture

Mouse hearts were finely digested, digested with collagenase (1 mg/ml collagenase A [Roche], 1 mg/ml DNase1 [Roche], and 2% FCS [Sigma-Aldrich] in DMEM) and passed through a 40-μm nylon strainer to yield a single-cell suspension.

For endothelial cell culture, 10- to 14-day neonatal hearts were homogenized as above and endothelial cells isolated by incubating with biotin-conjugated antibodies against CD31 (clone MEC 13.3; BD Pharmingen), CD105 (clone MJ7/18; BioLegend), and isobolin B4 (clone B-1205; Vector) followed by anti-biotin MicroBeads and purified using an autoMACS Separator (both Miltenyi Biotec). Endothelial cells were cultured overnight in growth medium (HEPES-buffered DMEM with 10% FCS, 100 IU/ml penicillin-streptomycin, and 2 mM L-glutamine; Life Technologies) in tissue culture flasks (Nunc; Thermo Scientific) pre-coated with 1% gelatin (Sigma-Aldrich) in PBS. Non-adherent cells were removed and endothelial cell growth factor (E9640; Sigma-Aldrich) added to the culture medium. Cells were passaged by incubation with trypsin-EDTA solution (Life Technologies) at 37°C until cells detached.

Histopathology and Immunohistochemistry

Splenic GCs were identified by double-labeling 7 μm cryostat sections with rat anti-mouse B220 (clone RA3-6B2; BD Pharmingen) detected with Cy3-conjugated goat anti-rat IgG and FITC-conjugated rat anti-mouse I-Aβ (clone AF6-120.1; BD Pharmingen). Sections were counterstained with 1% gelatin (Sigma-Aldrich) in PBS. Non-adherent cells were removed and endothelial cell growth factor (E9640; Sigma-Aldrich) added to the culture medium. Cells were passaged by incubation with trypsin-EDTA solution (Life Technologies) at 37°C until cells detached.

Flow Cytometry

For tetramer labeling, spleen single-cell suspensions in FACS buffer were blocked with anti-CD16/CD32 and incubated with PE-conjugated MHC class II I-Aβ tetramers presenting MHC class I Kβ peptide 54-68 (QEGPEWEEGT QRAK), kindly gifted by the NH Core Tetramer facility. After 1 hr at 37°C, cells were washed and tetramer-bound cells were enriched using anti-PE microbeads (Moon et al., 2007; Tubo et al., 2013; Miltenyi Biotech) and an Automacs separator. Additional cell surface antigens were then labeled for flow cytometry as above, and antigen-specific CD4 T cells were identified as tetramer- and CD4-positive but negative for CD19, CD11c, CD11b, and CD8. Antibody-specific CD4 T cells were enumerated after MACS separation by Trucount analysis according to manufacturer’s instructions (BD Biosciences).

CFSE CD4 T Cell Proliferation

Proliferation of splenocytes from TCR75, TEa, ABM, and Mar mice were determined by CFSE-labeled cell division at day 7 following adoptive transfer as previously described (Conlon et al., 2012b) using PE-Cy7-conjugated anti-CD4 plus APC-Cy7-conjugated anti-CD90.1/Ty1.1 (congenic marker) to identify TCR75, PE-conjugated anti-I-Vi8 to identify ABM, biotin-conjugated Vs2 and PE-conjugated anti-I-Vi6 to identify Tea, and PE-conjugated anti-I-Vi8.3 to identify Mar cells. CFSE-labeled splenocytes were transferred either early, at transplantation, or late on day 28 after transplant (to measure CD4 T cell alloresponses during the first and fifth week, respectively). In one experiment, proliferation of TEa splenocytes was examined late after transfer of 5 × 106 donor bm12.Kd.IE BMDGs (generated as described above). Proliferation was quantified using FlowJo (Treestar) as the percentage of cells from the original parent population that had undergone division.

Statistical Analysis

Data were presented as mean ± SEM where appropriate. Mann-Whitney U test was used for analysis of nonparametric data. Two-way ANOVA was employed for comparison of anti-H-2Kb, anti-I-, and anti-nuclear antibody levels. Graft survival was depicted using Kaplan-Meier analysis and groups compared by log rank (Mantel-Cox) testing. Analysis was conducted using GraphPad 4 (Graph-Pad Software). Values of p < 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.099.

AUTHOR CONTRIBUTIONS

Conceptualization, J.M.A., J.A.B., and G.J.P.; Investigation, J.M.A., M.C.N., T.M.C., M.S.Q., I.G.H., R.M., and K.S.-P.; Designed, Developed, and Produced Essential Reagents: R.W. and M.C.N.; Writing – Original Draft, J.M.A. and G.J.P.; Writing – Review & Editing, J.M.A., M.C.N., T.M.C., M.S.Q., I.G.H., R.M., K.S.-P., E.M.B., and J.A.B.

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