The Journal of Immunology

Germinal Center Alloantibody Responses Are Mediated Exclusively by Indirect-Pathway CD4 T Follicular Helper Cells

Thomas M. Conlon, Kourosh Saeb-Parsy, Jennifer L. Cole, Reza Motallebzadeh, M. Saeed Qureshi, Sylvia Rehakova, Margaret C. Negus, Chris J. Callaghan, Eleanor M. Bolton, J. Andrew Bradley and Gavin J. Pettigrew

J Immunol 2012; 188:2643-2652; Prepublished online 8 February 2012;
doi: 10.4049/jimmunol.1102830
http://www.jimmunol.org/content/188/6/2643

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Germinal Center Alloantibody Responses Are Mediated Exclusively by Indirect-Pathway CD4 T Follicular Helper Cells

Thomas M. Conlon, Kourosh Saeb-Parsy, Jennifer L. Cole, Reza Motallebzadeh, M. Saeed Qureshi, Sylvia Rehakova, Margaret C. Negus, Chris J. Callaghan, Eleanor M. Bolton, J. Andrew Bradley, and Gavin J. Pettigrew

The durable alloantibody responses that develop in organ transplant patients indicate long-lived plasma cell output from T-dependent germinal centers (GCs), but which of the two pathways of CD4 T cell allorecognition is responsible for generating allospecific T follicular helper cells remains unclear. This was addressed by reconstituting T cell-deficient mice with monoclonal populations of TCR-transgenic CD4 T cells that recognized alloantigen only as conformationally intact protein (direct pathway) or only as self-restricted allopeptide (indirect pathway) and then assessing the alloantibody response to a heart graft. Recipients reconstituted with indirect-pathway CD4 T cells developed long-lasting IgG alloantibody responses, with splenic GCs and allospecific bone marrow plasma cells readily detectable 50 d after heart transplantation. Differentiation of the transferred CD4 T cells into T follicular helper cells was confirmed by follicular localization and by acquisition of signature phenotype. In contrast, IgG alloantibody was not detectable in recipient mice reconstituted with direct-pathway CD4 T cells. Neither prolongation of the response by preventing NK cell killing of donor dendritic cells nor prior immunization to develop CD4 T cell memory altered the inability of the direct pathway to provide allospecific B cell help. CD4 T cell help for GC alloantibody responses is provided exclusively via the indirect-allorecognition pathway. ***The Journal of Immunology*****, 2012, 188: 2643–2652.**

Cellular immunity has long been regarded as the principal contributor to allograft rejection, but recent clinical data suggest that the humoral arm may be at least as important, in that the presence of donor-specific Ab either before transplantation or that develops afterward is now clearly associated with failure of kidney (1–5) and heart (6–8) allografts. As with conventional protein Ags, the development of effective alloantibody is critically dependent upon the provision of help from CD4 T cells (9–13); interventions that target CD4 T cells may thus disable both the cellular and humoral responses normally responsible for graft rejection. Although modern immunosuppressive agents effectively block cellular alloimmune responses, they act nonspecifically and risk life-threatening infection and cancer development. Ag-specific approaches that obviate these concerns by disabling only those T cells responsible for providing help to allospecific B cells remain frustratingly unrealized, and their development is hampered by limited understanding of the interactions between alloreactive T and B lymphocytes that underpin alloantibody production.

Transplantation is unusual because CD4 T cells can recognize alloantigen through two distinct pathways (14–17): in the direct pathway, which is unique to transplantation, alloantigen is recognized as intact protein on the surface of donor APCs; whereas in the indirect pathway, which is akin to recognition of conventional protein Ag, alloantigen is first processed by recipient APCs and then presented as peptide fragments in the context of host MHC class II. Which of these two pathways of alloreactive CD4 T cell activation is responsible for providing help for alloantibody production remains controversial (18, 19), not least because the humoral alloimmune response is complex and composed of several anatomically distinct components. Thus, simple assay of serum alloantibody may fail to reveal subtle yet important differences in how the helper CD4 T cell allorecognition pathway affects the various constituent arms. In this respect, the germinal center (GC) response requires special consideration (reviewed in Ref. 20) because a recently described population of highly specialized T follicular helper (T_{FH}) cells is critical to its development (21–24) and because its output—long-lived plasma cells (LLPCs) and memory B cells with high affinity for alloantigen—is likely to hold most relevance for clinical transplantation.

For nontransplant Ags, landmark studies in the 1980s highlighted the requirement for B cells to act as APCs and present processed peptide derived from their internalized target Ag for "cognate" self-restricted interaction with the TCR of Ag-specific helper CD4 T cells (25). This suggests that only indirect-pathway CD4 T cells can provide help to allospecific B cells because, unlike direct-pathway CD4 T cells, they can interact in a similar cognate fashion with the allopeptide presented by the B cell (see Fig. 1A). In support, Auchincloss and colleagues (18) have demonstrated that mice, in which absence of peripheral MHC

Department of Surgery, University of Cambridge, Cambridge CB2 0QQ, United Kingdom.

Received for publication September 30, 2011. Accepted for publication January 11, 2012.

This work was supported by a British Heart Foundation project grant and by the National Institute for Health Research Cambridge Biomedical Research Centre. K.S.-P. and C.J.C. were supported by the Academy of Medical Sciences. R.M. was supported by a clinical research fellowship from The Wellcome Trust and the Raymond and Beverly Sackler Scholarship.

Address correspondence and reprint requests to Mr. Gavin J. Pettigrew, University of Cambridge, Box 202, Level E9, Addenbrookes Hospital, Cambridge CB2 0QQ, U.K. E-mail address: gjp25@cam.ac.uk

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; DC, dendritic cell; GC, germinal center; LLPC, long-lived plasma cell; PNA, peanut agglutinin; T_{FH}, T follicular helper; WT, wild-type.

class II expression restricted CD4 T cell allorecognition to the direct pathway exclusively, mounted only limited IgM alloantibody responses to skin allografts; IgG isotype-switching did not occur. However, in that study, the lack of MHC class II expression in recipient mice may have more wide-ranging effects than simply limiting available pathways of allorecognition. Of chief concern, tonic MHC class II signaling, which is noncognate and peptide-independent, is required for optimal CD4 T cell survival, homeostatic proliferation, and reactivity (26, 27); similarly, MHC class II signaling provides essential proliferative responses in B cells (28, 29). Host MHC class II expression may thus be required for optimal activation of direct-pathway CD4 T cells and, in particular, for the ability of the latter to provide noncognate help to allospecific B cells. In addition, because the direct pathway lasts only briefly [due to rapid NK cell-mediated lysis of donor dendritic cells (DCs) (30)], Auchincloss' findings from a skin graft model may not reflect delivery of help for more rapidly evolving alloantibody responses to vascularized allografts. This concern also applies to a more recent study (31), which used adoptive transfer of monoclonal populations of TCR-transgenic CD4 T cells into recipient mice that expressed MHC class II normally to conclude similarly that only indirect-pathway CD4 T cells were capable of providing help for IgG-switched alloantibody responses against skin grafts. However, in the model adopted, hen egg lysozyme was used as a surrogate alloantigen, and the results may therefore not reflect responses against MHC alloantigens.

That direct-pathway CD4 T cells may provide help to allospecific B cells through noncognate interactions is supported by early in vitro studies demonstrating the provision of cytokine-mediated, contact-independent help for Ab responses against conventional protein Ags (reviewed in Refs. 25, 32). Although physical linkage between the allospecific B and Th cell is not possible, close proximity is presumably required and is possible through simultaneous interaction with a donor APC that expresses both the B cell target alloantigen and the allo-MHC class II determinant for CD4 T cell recognition. This "three-cell cluster model" (see Fig. 1B) was first suggested by Fabre and colleagues (19), who noted that blockade of donor MHC class II alloantigen abrogated anti-MHC class I alloantibody responses to a rat heart graft.

Assuming the paradox created by Fabre's and Auchincloss' work reflects the differences in the experimental systems examined, its resolution requires retesting using a vascularized allograft model that limits T cell activation to a particular allorecognition pathway but maintains normal recipient MHC class II expression. In this study, this is achieved by adoptive transfer of monoclonal populations of TCR-transgenic CD4 T cells, specific for MHC alloantigen via exclusively the direct or exclusively the indirect pathway, into T cell-deficient, but B cell-replete, murine recipients of heart allografts. This allows a definitive assessment of how the CD4 T cell allorecognition pathway influences alloantibody production; an assessment that includes a detailed examination of the potential for naive and memory CD4 T cells to provide help for LLPC production after GC development and the role of NK cells in curtailing direct-pathway help through rapid killing of donor DCs.

Materials and Methods

Animals

$TCR^{-/-}$ mice B6.129P2-*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom}/J ($H\text{-}2^b$) (33), MHC class $II^{-/-}$ mice B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J ($H\text{-}2^b$) (34), μMT B cell-deficient mice B6.129S2-*Ighm*^{tm1Cgn}/J ($H\text{-}2^b$) (35), and bm12 mice B6(C)-*H2-Ab1*^{bm12}/KhEgJ ($H\text{-}2^{bm12}$) were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 $RAG2^{-/-}$ mice ($H\text{-}2^b$) were a gift of Prof. T. Rabbitts (Laboratory of Molecular Biology, Cambridge, U.K.). TCR-transgenic $RAG1^{-/-}$ TCR75 mice ($H\text{-}2^b$), specific for $I\text{-}A^b$–restricted $H\text{-}2K^d_{54–68}$ peptide (36), were a gift of Prof. P. Bucy (University of Alabama, Birmingham, AL). $I\text{-}A^{bm12}$–specific TCR-transgenic $RAG2^{-/-}$ ABM mice ($H\text{-}2^b$) (37) were a gift of Dr. T. Crompton (Imperial College, London, U.K.). C57BL/6 ($H\text{-}2^b$, B6), BALB/c ($H\text{-}2^d$), and (BALB/c × B6) F1 mice were purchased from Charles River Laboratories (Margate, U.K.). All animals were maintained in specific pathogen-free facilities, and all experiments were approved by the United Kingdom Home Office under the Animal (Scientific Procedures) Act 1986.

Generation of bone marrow chimeras

To create mice that lacked MHC class II expression only on their B cells ($BCII^{-/-}$), $RAG2^{-/-}$ mice were sublethally irradiated (2 Gy) and reconstituted with 2×10^7 bone marrow cells obtained from MHC class $II^{-/-}$ mice. The B lymphocyte compartment in these mice is derived solely from donor MHC class $II^{-/-}$ bone marrow, whereas other APC lineages are formed from both donor and recipient bone marrow and are thus a heterogeneous population of MHC class II positive and negative cells. Control chimeric mice ($BCII^{+/+}$) were created by reconstituting sublethally irradiated $RAG2^{-/-}$ mice with 2×10^7 B6 bone marrow cells. B cell-replete ABM mice ($TCR^{-/-}$.ABM) were created by reconstituting sublethally irradiated $TCR^{-/-}$ mice with 2×10^7 bone marrow cells from $RAG2^{-/-}$ ABM mice. Chimerism was confirmed by flow cytometric analysis of PBMCs at least 4 wk after reconstitution.

Skin and heterotopic heart transplantation

Full-thickness tail skin was sutured as 1-cm^2 grafts onto the recipient's back. Vascularized cardiac allografts were transplanted intra-abdominally by the technique of Corry and colleagues (38) and rejection, defined as cessation of palpable myocardial contraction, confirmed at explant. T cell reconstitution was performed by i.v. injection with 10^7 splenocytes from TCR75 or ABM mice the following day.

NK and CD4 T cell depletion

Recipient $TCR^{-/-}$.ABM chimeric mice were treated with 0.5 mg anti-NK1.1–depleting mAb (mouse IgG2a, clone PK136; hybridoma purchased from ATCC-LGC Standards Partnership, Middlesex, U.K.) on days −2, 0, and +2 in relation to transplantation and weekly thereafter (39). Donor (BALB/c × bm12) F1 mice were treated on days −5 and −3 prior to removal of the hearts with 1 mg anti-CD4–depleting mAb (rat IgG2b, clone YTS191.1.1.2; hybridoma purchased from European Collection of Cell Cultures at the Health Protection Agency, Porton Down, U.K.).

Immunohistochemistry and immunofluorescence

Splenic 7 μm cryostat sections were stained for the presence of: $B220^{+ve}$ B cells using rat anti-mouse B220 (clone RA3-6B2, BD Pharmingen, San Diego, CA) directly conjugated with FITC or detected with Cy3-conjugated goat anti-rat IgG (clone 112-165-143, Jackson ImmunoResearch Laboratories, West Grove, PA); peanut agglutinin $(PNA)^{+ve}$ GC B cells using FITC-conjugated PNA (Vector Laboratories, Peterborough, U.K.); and $CD3^{+ve}$ T cells using PE-conjugated rat anti-mouse CD3 (clone 145-2C11, BD Pharmingen, San Diego, CA). Sections were counterstained with 20% Harris' hematoxylin (Sigma-Aldrich, Poole, U.K.) and viewed using an IX81 microscope with a ×20 0.70 UplanApo objective lens (Olympus, Tokyo, Japan). Images were photographed using an ORCA-ER digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) and acquired with Cell^R 2.6 software (Olympus Soft Imaging Solutions, Münster, Germany).

Generation of recombinant soluble H-2K^d

Recombinant soluble $H\text{-}2K^d$ was produced using the method previously described for rat MHC class I (40). In brief, pET-22b(+) expression plasmids containing the DNA sequences encoding either aa 1–280 of the $H\text{-}2K^d$ H chain (α1, α2, and α3 extracellular domains) or the murine β2-microglobulin (a gift of Prof. P. Lehner, University of Cambridge) were transformed into *Escherichia coli* BL21 (DE3) strain bacteria (Novagen, Merck, U.K.) and grown in LB broth (Invitrogen, Paisley, U.K.). Recombinant $H\text{-}2K^d$ H chain or β2-microglobulin was extracted from inclusion bodies released from *E. coli* pellets by chemical lysis. Soluble $H\text{-}2K^d$ molecules were generated by refolding the purified H chain and β2-microglobulin around a synthetic peptide (TYQRTRALV) (ISL, Paignton, U.K.) using the dilution method of Garboczi et al. (41). Finally, fast protein liquid chromatography purification of the refold mixture was performed (AKTA FPLC; Amersham Biosciences, Buckinghamshire,

U.K.), and the appropriate fraction was collected, pooled, filter sterilized, and stored in aliquots at 4°C.

Determining circulating anti–H-2K^d alloantibody

Serum samples were collected from experimental animals weekly and analyzed for the presence of anti–H-2K^d IgG alloantibody by ELISA. In brief, 96-well ELISA plates (Immulon 4HBX; Thermo, Milford, MA) were coated with recombinant conformational H-2K^d at 5 μg/ml in Na_2CO_3–$NaHCO_3$ buffer (pH 9.6). Plates were blocked with 1% Marvel dried skimmed milk powder (Premier International Foods, Cambridge, U.K.), tripling serial dilutions of test sera added and bound IgG Abs detected by incubating with biotinylated rabbit F(ab′)$_2$ anti-mouse IgG (STAR11B; AbD Serotec, Oxford, U.K.) and ExtrAvidin Peroxidase conjugate (Sigma, Poole, U.K.). Sure Blue substrate (KPL, Gaithersburg, MD) was then added, the reaction stopped by the addition of 0.2 M H_2SO_4, and the absorption at 450 nm measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, U.K.). For each sample, an absorbance versus dilution curve was plotted, and the area under the curve was calculated (42). The area under the curve of an experimental sample was expressed as the percentage of positive control (pooled hyperimmune) serum.

B cell ELISPOT assay

Single anti–H-2K^d IgG Ab-secreting cells were detected by ELISPOT assay. Briefly, single-cell suspensions from spleen and bone marrow were added onto 96-well MultiScreen Filter Plates (Millipore, Billerica, MA) that were previously coated with recombinant conformational H-2K^d at 2 μg/ml in Na_2CO_3–$NaHCO_3$ buffer (pH 9.6) and blocked with 10% BSA (Sigma). After incubation at 37°C, 5% CO_2 for 20 h, bound IgG Abs were detected by incubating with biotinylated rabbit F(ab′)$_2$ anti-mouse IgG (STAR11B; AbD Serotec) and ExtrAvidin Peroxidase conjugate (Sigma). Spots were developed using 3-amino-9-ethyl-carbazole solution (Sigma) and read on an AID Elispot Reader version 3.5 (Autoimmun Diagnostika, Strasburg, Germany). Data were expressed as mean number of responders per 10^6 cells (±SEM).

Autoantibody generation and detection

ABM CD4 T cells (2×10^6) purified by autoMACS separation using anti-CD4 microbeads (Miltenyi Biotec, Surrey, U.K.) were injected i.v. into bm12 mice. Mice were bled weekly and circulating autoantibody levels determined by HEp-2 indirect immunofluorescence (The Binding Site, Birmingham, U.K.). Serum was incubated on slides coated with HEp-2 cells and bound Ab detected with FITC-conjugated anti-mouse IgG (STAR 70; AbD Serotec, Oxford, U.K.). For each serum, photomicrographs were taken and the intensity of staining determined by integrated morphometric analysis using MetaMorph software (Molecular Devices, Downingtown, PA). The fluorescence value was then determined relative to a standard curve of serial diluted pooled hyperimmune serum that was assigned an arbitrary value of 1000 fluorescence units.

Flow cytometry

FITC-conjugated anti-mouse CD4 (clone GK1.5), FITC-conjugated anti-mouse CD11c (clone HL3), FITC-conjugated anti-mouse CD44 (clone IM7), PE-conjugated anti-mouse CD19 (clone 1D3), PE-conjugated anti-mouse CD90.1/Thy1.1 (clone OX-7), PE-conjugated anti-mouse CD279/PD1 (clone J43), PE-conjugated anti-mouse TCR Vβ8 (clone F23.1), allophycocyanin-conjugated anti-mouse CXCR5 (clone 2G8), PE–Cy7–conjugated anti-mouse CD4 (clone L3T4), biotinylated anti-mouse I-A^b (clone 25-9-17), biotinylated anti-mouse CD4 (clone GK1.5), biotinylated anti-mouse CD49b/pan-NK (clone DX-5), and biotinylated anti-mouse CD90.1/Thy1.1 (clone OX-7) were purchased from BD Pharmingen. Peripheral blood (depleted of erythrocytes by incubating with 0.17 M NH_4Cl red cell lysis buffer) and splenic single-cell suspensions were blocked with anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen), before staining with the relevant Abs and dead cell exclusion dye 7-aminoactinomycin D (7-AAD; BD Pharmingen). Biotinylated Abs were detected by allophycocyanin-conjugated streptavidin (Invitrogen) or allophycocyanin–Cy7–conjugated streptavidin (BD Pharmingen) and all cells analyzed on a FACSCanto II flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA).

CFSE cell proliferation

Single-cell suspensions of splenocytes obtained from TCR75 or ABM mice were stained with 5 mM CFSE (Invitrogen, Molecular Probes, Paisley, U.K.) in the dark for 5 min and then quenched with PBS plus 5% FCS. CFSE-stained splenocytes (2×10^6 to 5×10^6) were injected i.v. into recipient mice on the day of grafting, spleens harvested 4 or 7 d later, and flow cytometry performed using allophycocyanin-conjugated anti-CD4 plus PE-conjugated anti-CD90.1/Thy1.1 to identify TCR75 T cells and PE-conjugated anti- TCR Vβ8 to identify ABM cells.

Statistical analysis

Alloantibody responses were compared using the Mann–Whitney *U* test, and $p < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA).

Results

Absence of MHC class II expression on B cells abrogates IgG alloantibody responses

To examine which of the two pathways of CD4 T cell allorecognition were responsible for providing help for alloantibody production (Fig. 1), we first sought to clarify whether B cells lacking MHC class II expression, that can isotype switch in vitro (43), are capable of undergoing switching in vivo. Bone marrow chimeric mice were created with a defect in MHC class II expression limited only to B cells (BCII$^{-/-}$); MHC class II expression was maintained in the host non-B cell APC and, importantly, CD4 T cell numbers were normal (Fig. 2A). BCII$^{-/-}$ mice did not produce class-switched IgG alloantibody when challenged with a BALB/c heart allograft, whereas in contrast, control BCII$^{+/+}$ chimeric mice, whose B cells expressed MHC class II normally (Fig. 2A), developed anti–H-2K^d IgG responses similar to those seen in naive wild-type (WT) recipients (Fig. 2B). The absence of alloantibody in the BCII$^{-/-}$ mice was not due to an inability to mount indirect-pathway CD4 T cell responses, because although only a small proportion of the non-B cell APCs in the BCII$^{-/-}$ mice express MHC class II (Fig. 2A; those derived from host bone marrow), TCR-transgenic TCR75 CD4 T cells (that recognize donor H-2K^d Ag as self-restricted allopeptide) nevertheless underwent robust division when adoptively transferred into BCII$^{-/-}$ recipients the day after transplantation (Fig. 2C). This

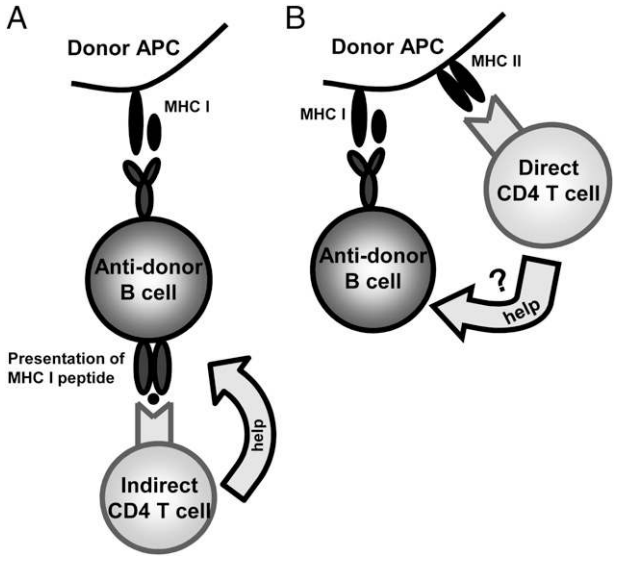

FIGURE 1. Schematic representation of the provision of help by indirect- and direct-pathway CD4 T cells to allospecific B cells. (**A**) Analogous to conventional protein Ag, indirect-pathway CD4 T cells interact cognately with allopeptide presented by B cells after internalization of their target Ag. (**B**) Donor APCs that express both B cell target alloantigen and the allo-MHC class II determinant for CD4 T cell recognition enable formation of a three-cell cluster that may provide noncognate help to allospecific B cells from direct-pathway CD4 T cells.

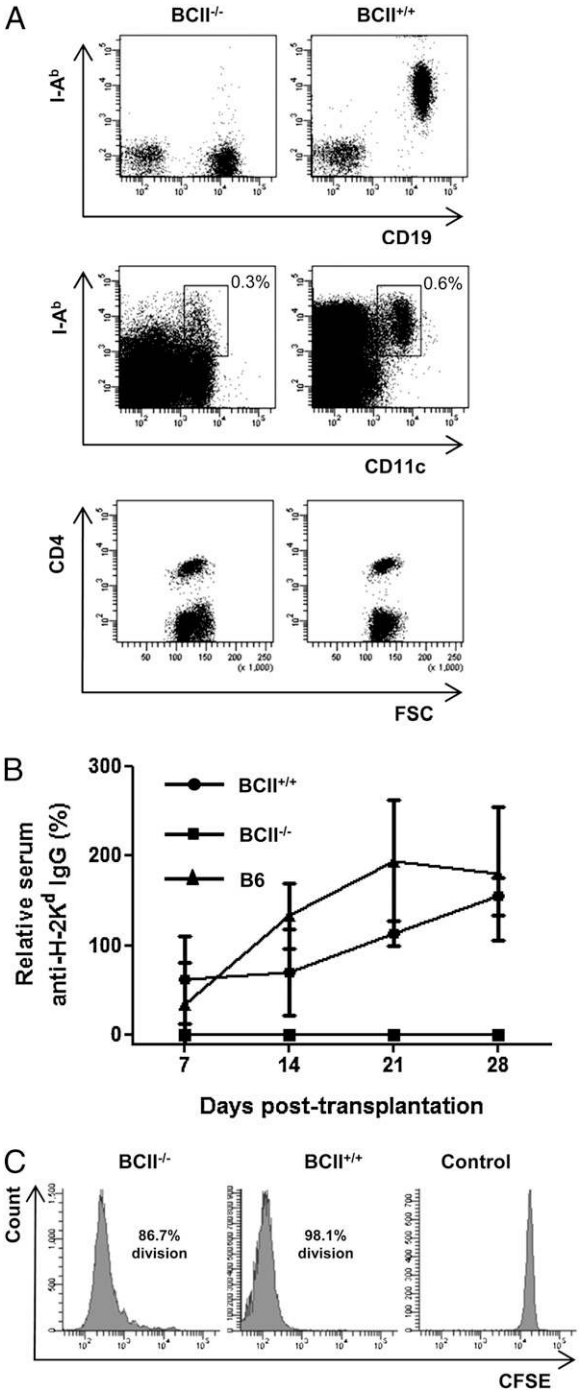

FIGURE 2. MHC class II expression on B cells is necessary for class-switched alloantibody production. (**A**) Chimeric mice lacking MHC class II expression on B cells ($BCII^{-/-}$) were created by reconstituting sublethally irradiated $RAG2^{-/-}$ mice with bone marrow from MHC class $II^{-/-}$ donors. Four weeks later, flow cytometric analysis of peripheral blood (*upper* and *lower rows*) or splenocytes (*middle row*) from chimeric mice or control chimeric mice expressing MHC class II on B cells ($BCII^{+/+}$) was performed, gating on live (7-AAD) lymphocytes. B cells (CD19) from $BCII^{-/-}$ mice lack I-A^b MHC class II expression (*upper row*), whereas I-A^b expression is maintained on host bone marrow-derived $CDIIc^+$ DCs (*middle row*). CD4 T cell numbers are comparable in both groups (*lower row*). (**B**) Serum levels of anti–H-$2K^d$ IgG alloantibody after challenge with BALB/c heart allograft. The alloantibody response in similarly grafted WT B6 mice is shown as a comparison. Data representing a minimum of three mice per group are presented as mean values ± SEM. (**C**) Representative flow cytometry plots (of two independent experiments) of CFSE-labeled TCR75 (Thy 1.1^+) CD4 T cells 4 d after adoptive transfer into $BCII^{-/-}$ or $BCII^{+/+}$ mice that received a BALB/c heart graft. The indicated levels of cell division were calculated using FlowJo software. A control plot after adoptive transfer of CFSE TCR75 CD4 T cells into $BCII^{-/-}$ recipients of a B6 syngeneic heart is shown for comparison.

response was comparable with that observed in $BCII^{+/+}$ recipients (Fig. 2C). In support of a critical role for B cell MHC class II expression in the production of class-switched Ab, we have previously demonstrated that $BCII^{-/-}$ mice do not develop IgG autoantibody responses upon induction of graft-versus-host disease (44).

These experiments demonstrate that a definitive comparison of the abilities of direct- and indirect-pathway CD4 T cells to provide help for alloantibody production requires a model in which B cell MHC class II expression is maintained. Subsequent experiments thus used $TCR^{-/-}$ mice—that despite lacking all T cells retain relatively normal B cell homeostasis and compartmentalization (45)—as recipients of heart allografts; helper function was provided by adoptive transfer of monoclonal populations of TCR-transgenic CD4 T cells.

Indirect-pathway CD4 T cells provide help for class-switched alloantibody responses

To examine the ability of indirect-pathway CD4 T cells to provide help for alloantibody production, $TCR^{-/-}$ recipients of BALB/c heart allografts were reconstituted with 5×10^6 splenocytes from $RAG1^{-/-}$ TCR75 mice. These contain a monoclonal population of CD4 T cells specific for one of the dominant peptide epitopes (H-$2K^d_{54–68}$) recognized after processing of H-$2K^d$ alloantigen in the B6 strain (36, 46, 47); an epitope that H-$2K^d$–specific B cells would be expected to present upon internalization of target alloantigen. In contrast to nonreconstituted $TCR^{-/-}$ recipients, those reconstituted with TCR75 T cells developed strong anti–H-$2K^d$ IgG alloantibody responses to a BALB/c heart graft, which were even greater than those observed in WT recipients (Fig. 3A).

Rejection kinetics were also markedly different in the two groups, in that heart grafts were rejected within 8 d in the reconstituted $TCR^{-/-}$ recipients but continued to beat strongly in the nonreconstituted recipients until termination of the experiment at day 50 (Fig. 3B). Allograft rejection in the recipients reconstituted with TCR75 splenocytes was not simply a consequence of activation of the transferred CD4 T cells, because despite comparably strong division of TCR75 T cells upon transfer into either WT or B cell-deficient (μMT) B6 recipients of BALB/c heart allografts (Fig 3C), B cell-deficient $RAG1^{-/-}$ TCR75 mice do not reject BALB/c heart allografts (Fig. 3B). These results suggest instead that indirect-pathway CD4 T cells are unable to mediate acute heart allograft rejection when acting as autonomous cellular effectors but can do so as a helper population for alloantibody production.

To examine whether the transferred indirect-pathway CD4 T cells mediated development of a GC alloantibody response, histological and immunohistochemical analysis was performed on recipient spleens harvested 50 d after heart transplantation. More than half of the follicles had undergone secondary maturation to become PNA^+ (48) GCs, and notably, transferred TCR75 T cells were detectable within the follicles (Fig. 3D). In addition, ~3-fold greater anti–H-$2K^d$ plasma cells were present within the bone marrow than within the spleen (Fig. 3E), consistent with LLPC output from a GC response (49).

Given the recent identification of a select and highly specialized subset of CD4 T_{FH} cells as pivotal for providing essential helper

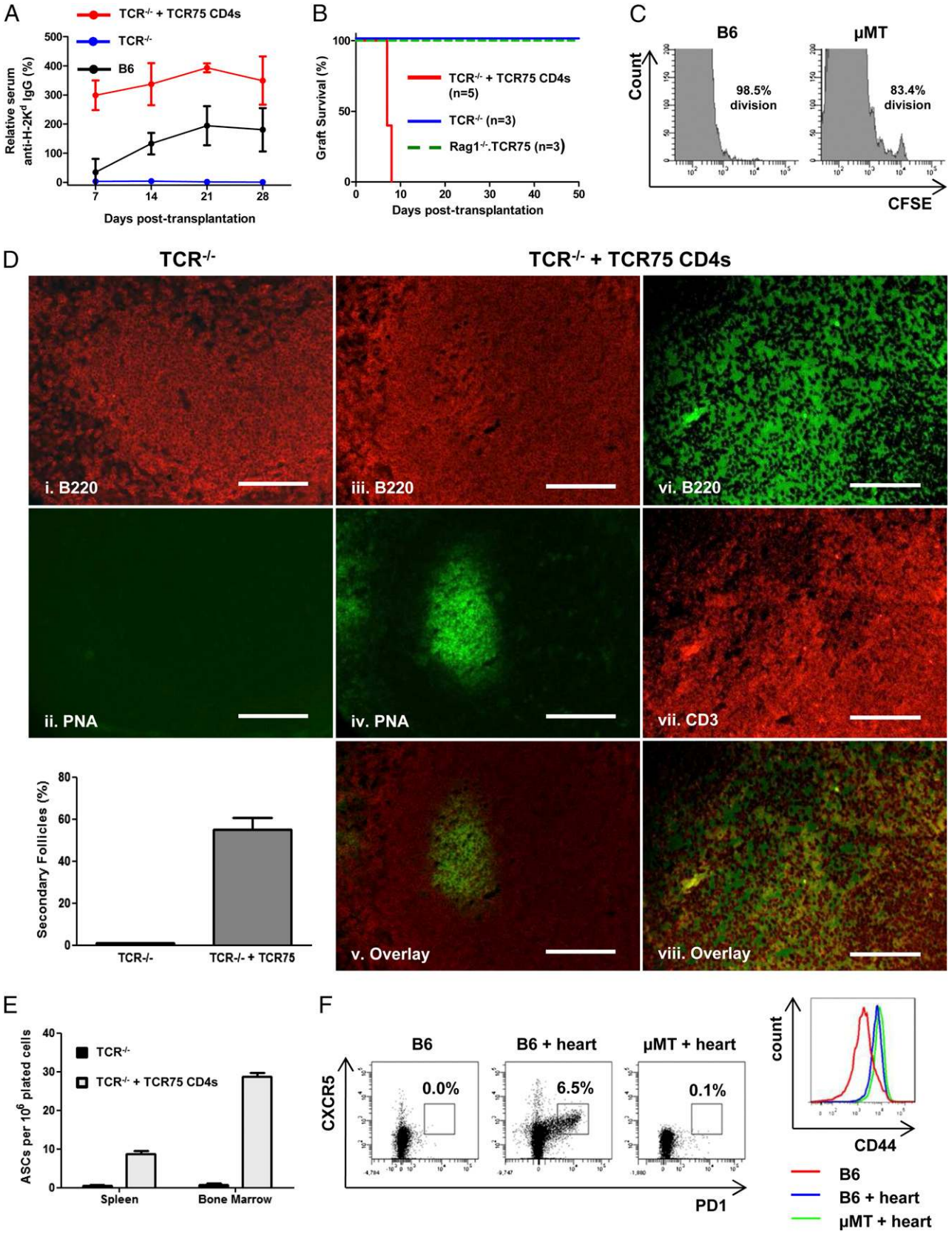

FIGURE 3. Indirect-pathway CD4 T cells provide help for GC alloantibody responses. (**A**) Serum anti–H-2K^d IgG alloantibody responses to a BALB/c heart allograft in B6, TCR$^{-/-}$, or TCR$^{-/-}$ mice adoptively transferred with H-2K^d peptide-specific TCR75 splenocytes. Data representing a minimum of three mice per group are expressed as mean ± SEM. (**B**) Kaplan–Meier survival curves of BALB/c hearts transplanted into TCR$^{-/-}$ (n = 3), TCR$^{-/-}$ adoptively transferred with TCR75 splenocytes (n = 5), or TCR75 mice (n = 3). (**C**) Representative flow cytometry histogram plots (of two independent experiments) of CFSE-labeled TCR75 CD4 T cells 4 d after adoptive transfer into B6 or µMT recipients of BALB/c heart allografts. The indicated levels of cell division were calculated using FlowJo software. (**D**) Representative photomicrographs (of two independent experiments) of immunofluorescence staining of splenic sections from TCR$^{-/-}$ mice (**i**, **ii**) and TCR$^{-/-}$ mice adoptively transferred with TCR75 splenocytes (**iii**–**viii**) (*Figure legend continues*)

signals for GC development (50–52), we sought to confirm whether the transferred TCR75 T cells acquire T_{FH} characteristics. One of the key attributes of the T_{FH} cell is that only precursors with high affinity for target Ag are selected; these presumably outcompete those Ag-specific cells of lower affinity. For these experiments, congenically labeled TCR75 T cells were therefore transferred into WT, rather than $TCR^{-/-}$, recipients of BALB/c heart grafts, as this permitted competition with the endogenous B6 population for differentiation to T_{FH} status. As shown in Fig. 3F, ~6% of the transferred TCR75 T cells acquired the $CD4^+$ $CD44^{high}$ $CXCR5^{high}$ $PD1^{high}$ (53) signature-phenotype of the T_{FH} subset. This population did not develop upon transfer either to naive B6 mice or, more notably, to transplanted μMT mice (Fig. 3F), despite upregulation of activation marker CD44 in the latter comparable with that in WT recipients (Fig. 3F). This accords with the recent demonstration that B cell Ag presentation is required for differentiation into T_{FH} status (54).

Naive direct-pathway CD4 T cells are unable to provide help to allospecific B cells

A similar adoptive-transfer model into $TCR^{-/-}$ recipients was developed to examine the role of direct-pathway CD4 T cell help in alloantibody production. $TCR^{-/-}$ mice were engrafted with BALB/c × bm12 F1 hearts and reconstituted the next day with 10^7 splenocytes from TCR-transgenic $RAG2^{-/-}$ ABM mice. These mice contain a monoclonal population of CD4 T cells that recognize I-A^{bm12} alloantigen of the F1 donors exclusively via the direct pathway. Although the transferred transgenic CD4 T cells could potentially provide help via the three-cell cluster model for development of anti-MHC class I alloantibody (Fig. 4A), anti–H-$2K^d$ IgG alloantibody was not detectable at any time point after heart transplantation (Fig. 4B). However all hearts were still beating 50 d after transplant, and thus to exclude the possibility that reduced viability caused by the transfer of ABM CD4 T cells contributed to the failure to develop humoral alloimmunity, the experiments were repeated using $TCR^{-/-}$.ABM bone marrow chimeric recipients in which the ABM CD4 T cell population was self-renewing from hematopoietic precursors (Fig. 4C). Again, alloantibody did not develop (Fig. 4B). The failure of ABM CD4 T cells to provide help for alloantibody was not due to their inability to mount a functional allospecific response, because ABM CD4 T cells underwent robust division after adoptive transfer into $TCR^{-/-}$ recipients of BALB/c × bm12 F1 heart allografts (Fig 4D); $TCR^{-/-}$.ABM bone marrow chimeric mice rejected bm12 skin grafts rapidly (MST 10 d, $n = 4$); and ABM CD4 T cells provided help through graft-versus-host recognition of I-A^{bm12} alloantigen for autoantibody production (44, 55) upon transfer into naive bm12 recipients (Fig. 4E).

Because inherent differences in the activation-induced cytokine profiles of the ABM and TCR75 T cells may influence their ability to provide B cell help, a further experimental model was devised that incorporated TCR75 T cells but limited their response to the direct pathway. TCR75 CD4 T cells were adoptively transferred into $TCR^{-/-}$ mice that had been crossed onto a bm12 background ($TCR^{-/-}$.bm12). TCR75 CD4 T cells do not respond to processed H-$2K^d$ when restricted on the MHC class II I-A^{bm12} Ag (Fig. 4F) and thus when transferred into $TCR^{-/-}$.bm12 recipients of a BALB/c × B6 F1 heart can only recognize target I-A^b/$K^d_{54–68}$ epitope directly on donor APCs (Fig. 4F); indirect recognition is not possible. When restricted in this fashion, adoptively transferred TCR75 T cells were unable to provide help for alloantibody production (Fig. 4G).

Neither prevention of donor DC killing nor memory reactivation enable direct-pathway CD4 T cells to provide allospecific B cell help

Although the above experiments suggest strongly that direct-pathway CD4 T cells are unable to provide help to alloreactive B cells, we thought it important to examine two further considerations before the three-cell cluster model could be refuted definitively: that rapid termination of the direct-pathway response, rather than an intrinsic defect in its helper potential, prevented alloantibody formation; and that direct-pathway memory CD4 T cells may differ from naive cells in their ability to provide B cell help. NK cells kill donor DCs within days of transplantation (30, 56, 57), and to obviate this killing, $TCR^{-/-}$.ABM chimeric mice were depleted of NK cells by treatment with anti-NK1.1 Ab prior to and after transplantation with BALB/c × bm12 F1 heart grafts. In addition, to prevent graft-versus-host recognition from triggering recipient humoral immunity (44, 55), donor CD4 T cells were depleted by administering anti-CD4 Ab several days before heart graft removal (Fig. 5A). However, despite effective depletion of recipient NK cells (Fig. 5B), anti–H-$2K^d$ IgG alloantibody was not detected (Fig. 5C).

Memory CD4 T cell responses differ from those of naive in costimulation requirements for activation, the degree of proliferation, and in the spectrum of cytokines secreted (58–60). To assess whether memory direct-pathway CD4 T cells could provide help for alloantibody production, $TCR^{-/-}$.ABM chimeric mice were primed with a bm12 skin graft 6 wk before challenge with a BALB/c × bm12 F1 heart graft. In principle, the MHC class II-mismatched skin graft provides target alloantigen for memory ABM CD4 T cell development, but not for anti-MHC class I alloantibody responses; the H-$2K^d$–allospecific B cell population remains Ag-inexperienced until second challenge with the heart graft. However, despite the presence of direct-pathway CD4 T cell memory, as evident by accelerated rejection of a second skin graft 6 wk later (Fig. 5D), no anti–H-$2K^d$ alloantibody responses were detectable (Fig. 5E).

Discussion

The results of this study address the long-standing controversy regarding the relative roles of the direct and indirect CD4 T cell allorecognition pathways in the alloantibody response to organ transplants. We show that only indirect-pathway CD4 T cells can differentiate into T_{FH} cells for production of LLPCs from GC

harvested 49 d after BALB/c heart transplantation. (i), (iii), and (vi) depict B220 B cell staining; (ii) and (iv) depict PNA staining; and (vii) depicts CD3 staining. (v) represents an overlay of (iii) and (iv), and (viii) represents an overlay of (vi) and (vii). Of note, PNA^+ GCs developed in $TCR^{-/-}$ recipients reconstituted with TCR75 splenocytes (iv), but not in $TCR^{-/-}$ mice, and adoptively transferred TCR75 T cells were detectable within B cell follicles (vii). Scale bar, 100 μm. The chart in the *bottom left corner* in (D) depicts the mean (±SEM) of PNA^+ secondary follicles as a percentage of total B cell follicles. (**E**) Number of H-$2K^d$–specific IgG-secreting Ab-secreting cells (ASCs) per 10^6 plated cells within spleen and bone marrow of $TCR^{-/-}$ and TCR75-reconstituted $TCR^{-/-}$ recipients 49 d after challenge with BALB/c heart allografts. Data representing a minimum of three mice per group are expressed as mean ± SEM. (**F**) Flow cytometry analysis, gating on live 7-AAD^-, $CD4^+$, Thy 1.1^+ TCR75 cells, reveals differentiation of TCR75 CD4 T cells into $PD1^{high}$, $CXCR5^{high}$ T_{FH} phenotype, 11 d after transfer into B6 recipients of BALB/c heart allografts, but not upon transfer into either naive B6 mice or μMT recipients of BALB/c heart allografts. CD44 fluorescence intensity of the gated TCR75 population is also depicted for each group.

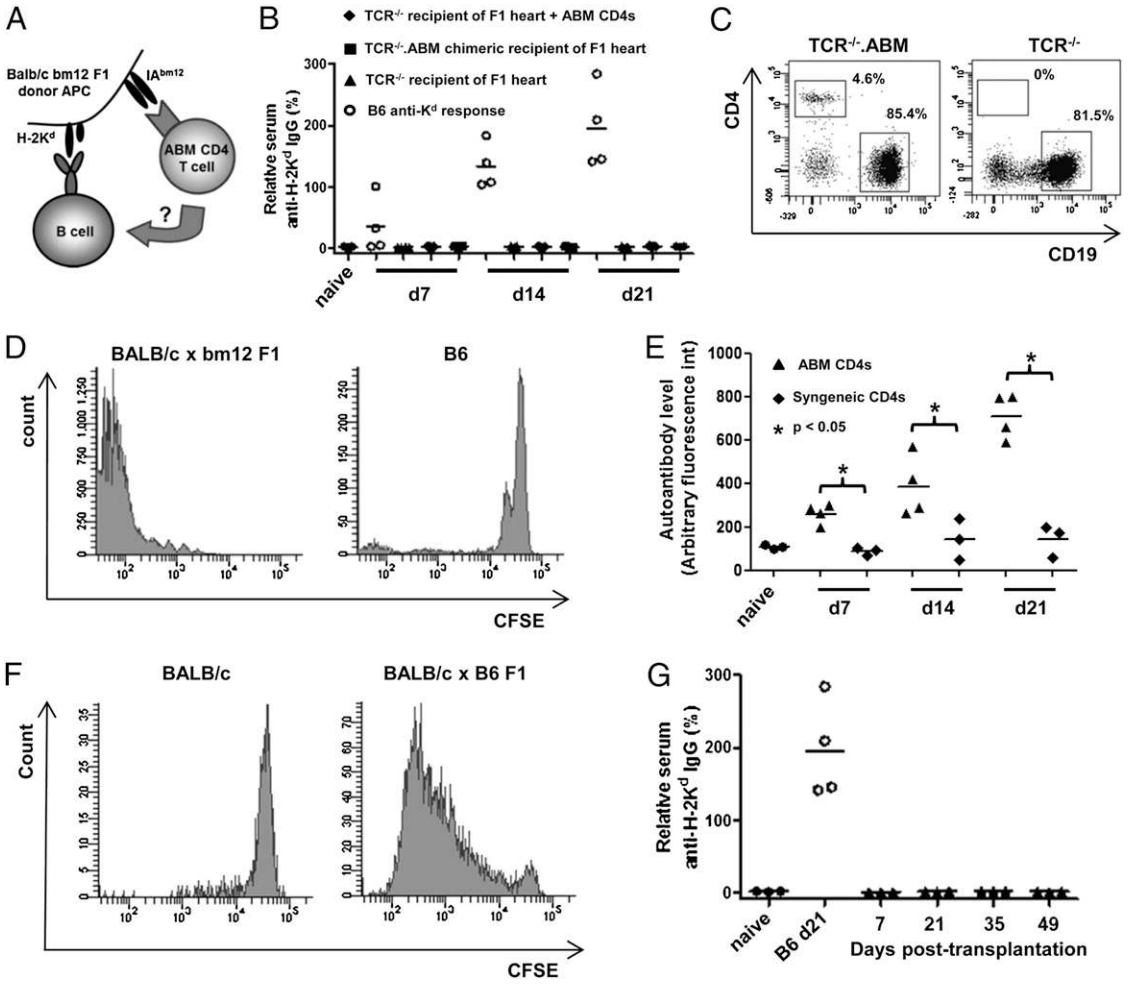

FIGURE 4. Direct-pathway CD4 T cells cannot provide help for class-switched alloantibody responses. (**A**) Schematic representation of a three-cell cluster among (BALB/c × bm12) F1 donor APCs, anti–H-2K^d–specific B cells, and direct-pathway ABM CD4 T cells. (**B**) Anti–H-2K^d IgG alloantibody response to a (BALB/c × bm12) F1 heart transplant in TCR$^{-/-}$ mice (n = 5), TCR$^{-/-}$ mice reconstituted with ABM splenocytes (n = 5), and TCR$^{-/-}$.ABM bone marrow chimeric mice (n = 3). Values from ungrafted naive mice (n = 7) and B6 recipients of BALB/c hearts (n = 4) are shown for comparison. (**C**) Representative flow cytometry analysis of peripheral blood from TCR$^{-/-}$.ABM bone marrow chimeric mice 4 wk after creation. A representative plot of naive TCR$^{-/-}$ mice is shown as a comparison. (**D**) Representative flow cytometry histogram plots (of three independent experiments) of CFSE-labeled ABM CD4 T cells 7 d after adoptive transfer into TCR$^{-/-}$ recipients of (BALB/c × bm12) F1 heart allografts. The response after a syngeneic B6 heart graft is shown for comparison. (**E**) Autoantibody responses calculated from Hep-2 indirect immunofluorescence staining after the adoptive transfer of either 2 × 10^6 ABM (n = 4) or syngeneic (n = 3) CD4 T cells into WT bm12 mice. Values from naive mice are shown as a comparison (n = 3). (**F**) Representative flow cytometry histograms (of two independent experiments), gating on CD4$^+$, Thy1.1$^+$, live (7-AAD$^-$), CFSE-labeled TCR75 CD4 T cells, 4 d after adoptive transfer into bm12.TCR$^{-/-}$ mice challenged at transfer with either a BALB/c or (BALB/c × B6) F1 heart graft. (**G**) Anti–H-2K^d IgG alloantibody responses in bm12.TCR$^{-/-}$ mice, reconstituted with TCR75 splenocytes and challenged with (BALB/c × B6) F1 heart grafts (n = 3). Values from naive mice (n = 3) and day 21 sera from B6 recipients of BALB/c hearts (n = 4) are shown for comparison.

alloantibody responses. In contrast, direct-pathway CD4 T cells are unable to provide help for class-switched alloantibody responses, even when CD4 T cell memory has been established by prior immunization or when the duration of the direct-pathway responses is extended by depletion of recipient NK cells.

The impact of humoral alloimmunity on clinical transplant outcome is increasingly emphasized, and the development of anti-HLA alloantibody after transplantation is now clearly associated with early graft failure (1, 8). In addition, approximately one-third of patients on the waiting list for a kidney transplant have detectable levels of serum alloantibody that has developed either after pregnancy or from previous transplant or blood transfusion. These "sensitized" patients frequently wait excessively long, and sometimes indefinitely, for a transplant. The mechanisms by which the anti-HLA alloantibody continues to be produced many years after the initiating challenge have received surprisingly little attention, but such durable responses most likely reflect seeding of LLPCs to the bone marrow and thus indicate prior GC activity. GCs are a characteristic, albeit not exclusive (61), feature of thymic-dependent responses, and the characterization of the T_{FH} cell as a phenotypically distinct population of CD4 T cells that is uniquely capable of providing help to GC B cells has only become apparent over the past decade (21–23). Restricting help to a highly select and limited helper population (52) may be a critical feature in developing the necessary competitive environment to ensure preferential selection of high-affinity B cells and thus drive affinity maturation (62). Allospecific T_{FH} cells have not been detailed previously, but such is their critical role in production of high-affinity and long-lasting alloantibody that their characterization may hold important clinical potential, not least because their distinct phenotype may enable development of specific strategies that deplete T_{FH} cells in the expectation that graft survival is improved.

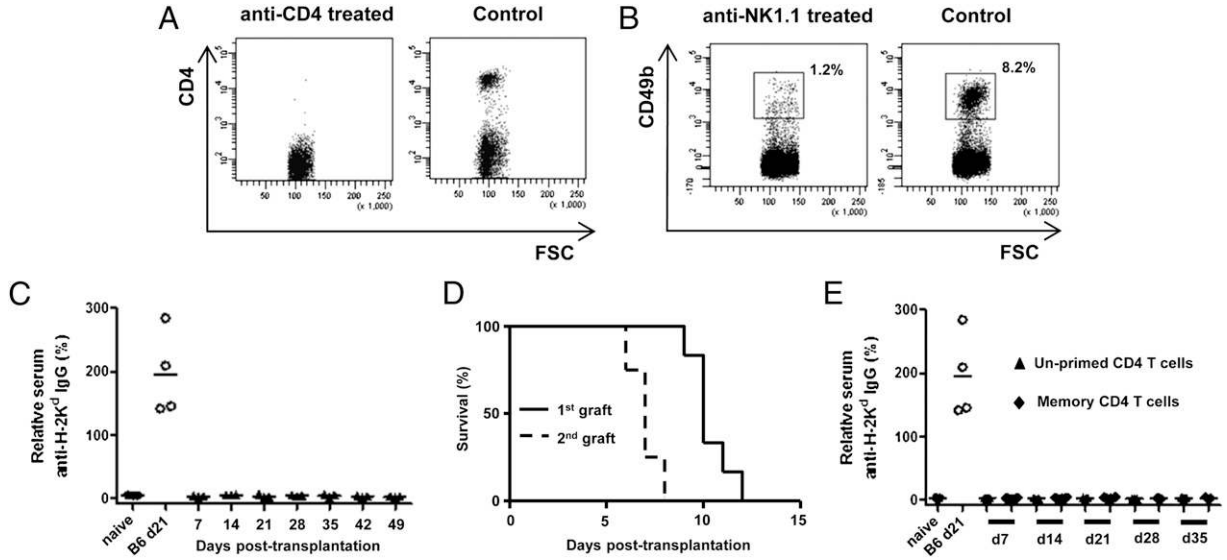

FIGURE 5. Neither prolongation of direct-pathway responses nor memory generation influences humoral alloimmunity. (**A**) Representative flow cytometry dot plots of peripheral blood from (BALB/c × bm12) F1 at heart graft procurement after CD4 T cell depletion compared with control, untreated mice. (**B**) Representative flow cytometry dot plot of splenic NK cells (CD49b) in TCR$^{-/-}$ mice 7 d after treatment with 0.5 mg anti-NK1.1 Ab compared with control untreated TCR$^{-/-}$ mice. (**C**) Serum levels of anti–H-2K^d IgG alloantibody in TCR$^{-/-}$.ABM chimeric mice depleted of NK cells and challenged with a (BALB/c × bm12) F1 heart transplant ($n = 3$). Values from control, naive mice ($n = 3$) and day 21 sera from B6 recipients of BALB/c hearts ($n = 4$) are shown for comparison. (**D**) Kaplan–Meier survival curves of first ("1st graft," $n = 6$) and second ("2nd graft," 6 wk after first, $n = 4$) bm12 skin grafts in TCR$^{-/-}$.ABM bone marrow chimeric recipients. (**E**) Anti–H-2K^d IgG alloantibody responses to a (BALB/c × bm12) F1 heart allograft in TCR$^{-/-}$.ABM chimeric mice (unprimed CD4 T cells, $n = 3$) and TCR$^{-/-}$.ABM chimeric mice primed with a bm12 skin graft 6 wk previously (memory CD4 T cells, $n = 6$). Values from naive mice ($n = 7$) and day 21 sera from B6 recipients of BALB/c hearts ($n = 4$) are shown for comparison.

Although previous studies have highlighted the ability of indirect-pathway CD4 T cells to provide help for class-switched alloantibody responses (11, 12, 18, 31), this does not necessarily indicate GC activity, because isotype switching can occur at extrafollicular foci. Thus, the differentiation of the transferred TCR75 T cells into a CD44^{high} CXCR5^{high} PD1^{high} signature phenotype, allied to their anatomical location within the follicle and the presence of allospecific LLPCs in the bone marrow, provide the first definitive evidence to our knowledge of indirect-pathway T_{FH} CD4 T cells. Notably, the T_{FH} subset did not develop upon transfer into B cell-deficient mice, supporting the concept that cognate interaction with the B cell delivers unique signals to Th cells to guide differentiation and migration to the follicle (54). This may also explain why only a relatively small percentage of the transferred TCR75 T cells acquired T_{FH} status; our ongoing studies indicate that the entire population divides several times after transfer, and thus the majority that did not acquire T_{FH} phenotype presumably made contact with non-B cell APCs.

Although previous publications, including our own, have concluded that direct-pathway CD4 T cells cannot provide help for class-switched alloantibody responses (18, 31, 63), we believed it important to reexamine this concept, because as discussed earlier and as suggested by Fabre's findings (19, 64), these studies may obscure a role for direct-pathway help either because the B cell compartment lacked MHC class II expression (18) or because nonvascularized skin graft models were studied (31). Of more significance, definitive rebuttal of direct-pathway help for alloantibody production requires specific examination of the memory CD4 T cell subset. A major consideration of direct-pathway help is that unlike the indirect pathway, it theoretically enables recognition of the Th cell epitope to be dissociated from that of the allospecific B cell epitope. For example, direct-pathway CD4 T cells target the allogeneic MHC class II on the surface of donor APCs, whereas the B cell may target a different alloantigen, typically the MHC class I, on the surface of the same cell. Hence, it would be possible for direct-pathway CD4 T cells, which have developed memory from prior challenge with target MHC class II alloantigen, to provide help to naive B cells recognizing an additional, but previously unencountered, MHC class I alloantigen on the graft. Given the resistance of memory cellular responses to costimulation blockade (58), such memory direct-pathway CD4 T cells could potentially provide effective help when naive indirect-pathway T cells are inhibited by adjuvant immunosuppression.

It was similarly necessary to exclude NK cell recognition of donor DCs, rather than any inherent defects in the helper function of direct-pathway CD4 T cells, as the reason for the inability of the adoptively transferred ABM T cells to promote alloantibody production, because although NK cells limit the duration of direct-pathway responses by rapidly killing donor DCs (30, 57), their contribution to graft rejection (65–67) raises the possibility of developing strategies that improve graft survival by depleting NK cells. Thus, despite confirming that ABM CD4 T cells can prime humoral autoimmunity through graft-versus-host allorecognition of B cell MHC class II, we have been unable to demonstrate an ability to function as direct-pathway helper cells for alloantibody production, regardless of either the duration or the degree of Ag experience of the direct-pathway response.

In summary, we provide conclusive evidence that indirect-pathway CD4 T cell responses deliver help for sophisticated GC responses that result in the production of long-lasting alloantibody, whereas the direct pathway is entirely unable to provide helper function for isotype switching. Targeting the indirect pathway, for example by administering allopeptide-specific regulatory T cells (13), may thus hold clinical potential for preventing alloantibody-mediated graft damage.

Disclosures

The authors have no financial conflicts of interest.

References

1. Hidalgo, L. G., P. M. Campbell, B. Sis, G. Einecke, M. Mengel, J. Chang, J. Sellares, J. Reeve, and P. F. Halloran. 2009. De novo donor-specific antibody at the time of kidney transplant biopsy associates with microvascular pathology and late graft failure. *Am. J. Transplant.* 9: 2532–2541.
2. Gaston, R. S., J. M. Cecka, B. L. Kasiske, A. M. Fieberg, R. Leduc, F. C. Cosio, S. Gourishankar, J. Grande, P. Halloran, L. Hunsicker, et al. 2010. Evidence for antibody-mediated injury as a major determinant of late kidney allograft failure. *Transplantation* 90: 68–74.
3. Einecke, G., B. Sis, J. Reeve, M. Mengel, P. M. Campbell, L. G. Hidalgo, B. Kaplan, and P. F. Halloran. 2009. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. *Am. J. Transplant.* 9: 2520–2531.
4. Lachmann, N., P. I. Terasaki, K. Budde, L. Liefeldt, A. Kahl, P. Reinke, J. Pratschke, B. Rudolph, D. Schmidt, A. Salama, and C. Schönemann. 2009. Anti-human leukocyte antigen and donor-specific antibodies detected by luminex posttransplant serve as biomarkers for chronic rejection of renal allografts. *Transplantation* 87: 1505–1513.
5. Lee, P. C., L. Zhu, P. I. Terasaki, and M. J. Everly. 2009. HLA-specific antibodies developed in the first year posttransplant are predictive of chronic rejection and renal graft loss. *Transplantation* 88: 568–574.
6. Tambur, A. R., S. V. Pamboukian, M. R. Costanzo, N. D. Herrera, S. Dunlap, M. Montpetit, and A. Heroux. 2005. The presence of HLA-directed antibodies after heart transplantation is associated with poor allograft outcome. *Transplantation* 80: 1019–1025.
7. Smith, J. D., N. R. Banner, I. M. Hamour, M. Ozawa, A. Goh, D. Robinson, P. I. Terasaki, and M. L. Rose. 2011. De novo donor HLA-specific antibodies after heart transplantation are an independent predictor of poor patient survival. *Am. J. Transplant.* 11: 312–319.
8. Smith, J. D., I. M. Hamour, N. R. Banner, and M. L. Rose. 2007. C4d fixing, luminex binding antibodies - a new tool for prediction of graft failure after heart transplantation. *Am. J. Transplant.* 7: 2809–2815.
9. Gracie, J. A., E. M. Bolton, C. Porteous, and J. A. Bradley. 1990. T cell requirements for the rejection of renal allografts bearing an isolated class I MHC disparity. *J. Exp. Med.* 172: 1547–1557.
10. Bradley, J. A., A. M. Mowat, and E. M. Bolton. 1992. Processed MHC class I alloantigen as the stimulus for CD4+ T-cell dependent antibody-mediated graft rejection. *Immunol. Today* 13: 434–438.
11. Pettigrew, G. J., E. Lovegrove, J. A. Bradley, J. Maclean, and E. M. Bolton. 1998. Indirect T cell allorecognition and alloantibody-mediated rejection of MHC class I-disparate heart grafts. *J. Immunol.* 161: 1292–1298.
12. Lovegrove, E., G. J. Pettigrew, E. M. Bolton, and J. A. Bradley. 2001. Epitope mapping of the indirect T cell response to allogeneic class I MHC: sequences shared by donor and recipient MHC may prime T cells that provide help for alloantibody production. *J. Immunol.* 167: 4338–4344.
13. Callaghan, C. J., F. J. Rouhani, M. C. Negus, A. J. Curry, E. M. Bolton, J. A. Bradley, and G. J. Pettigrew. 2007. Abrogation of antibody-mediated allograft rejection by regulatory CD4 T cells with indirect allospecificity. *J. Immunol.* 178: 2221–2228.
14. Jiang, S., O. Herrera, and R. I. Lechler. 2004. New spectrum of allorecognition pathways: implications for graft rejection and transplantation tolerance. *Curr. Opin. Immunol.* 16: 550–557.
15. Bolton, E. M., J. A. Bradley, and G. J. Pettigrew. 2008. Indirect allorecognition: not simple but effective. *Transplantation* 85: 667–669.
16. Gould, D. S., and H. Auchincloss, Jr. 1999. Direct and indirect recognition: the role of MHC antigens in graft rejection. *Immunol. Today* 20: 77–82.
17. Benichou, G. 1999. Direct and indirect antigen recognition: the pathways to allograft immune rejection. *Front. Biosci.* 4: D476–D480.
18. Steele, D. J., T. M. Laufer, S. T. Smiley, Y. Ando, M. J. Grusby, L. H. Glimcher, and H. Auchincloss, Jr. 1996. Two levels of help for B cell alloantibody production. *J. Exp. Med.* 183: 699–703.
19. Kelly, C. M., A. M. Benham, G. J. Sawyer, R. Dalchau, and J. W. Fabre. 1996. A three-cell cluster hypothesis for noncognate T-B collaboration via direct T cell recognition of allogeneic dendritic cells. *Transplantation* 61: 1094–1099.
20. MacLennan, I. C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12: 117–139.
21. Vinuesa, C. G., M. A. Linterman, C. C. Goodnow, and K. L. Randall. 2010. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunol. Rev.* 237: 72–89.
22. Linterman, M. A., and C. G. Vinuesa. 2010. Signals that influence T follicular helper cell differentiation and function. *Semin. Immunopathol.* 32: 183–196.
23. McHeyzer-Williams, L. J., N. Pelletier, L. Mark, N. Fazilleau, and M. G. McHeyzer-Williams. 2009. Follicular helper T cells as cognate regulators of B cell immunity. *Curr. Opin. Immunol.* 21: 266–273.
24. King, C. 2009. New insights into the differentiation and function of T follicular helper cells. *Nat. Rev. Immunol.* 9: 757–766.
25. Noelle, R. J., and E. C. Snow. 1990. Cognate interactions between helper T cells and B cells. *Immunol. Today* 11: 361–368.
26. Grandjean, I., L. Duban, E. A. Bonney, E. Corcuff, J. P. Di Santo, P. Matzinger, and O. Lantz. 2003. Are major histocompatibility complex molecules involved in the survival of naive CD4+ T cells? *J. Exp. Med.* 198: 1089–1102.
27. De Riva, A., C. Bourgeois, G. Kassiotis, and B. Stockinger. 2007. Noncognate interaction with MHC class II molecules is essential for maintenance of T cell metabolism to establish optimal memory CD4 T cell function. *J. Immunol.* 178: 5488–5495.
28. Mills, D. M., J. C. Stolpa, and J. C. Cambier. 2004. Cognate B cell signaling via MHC class II: differential regulation of B cell antigen receptor and MHC class II/Ig-alpha beta signaling by CD22. *J. Immunol.* 172: 195–201.
29. Cambier, J. C., and K. R. Lehmann. 1989. Ia-mediated signal transduction leads to proliferation of primed B lymphocytes. *J. Exp. Med.* 170: 877–886.
30. Garrod, K. R., F. C. Liu, L. E. Forrest, I. Parker, S. M. Kang, and M. D. Cahalan. 2010. NK cell patrolling and elimination of donor-derived dendritic cells favor indirect alloreactivity. *J. Immunol.* 184: 2329–2336.
31. Sauvé, D., M. Baratin, C. Leduc, K. Bonin, and C. Daniel. 2004. Alloantibody production is regulated by CD4+ T cells' alloreactive pathway, rather than precursor frequency or Th1/Th2 differentiation. *Am. J. Transplant.* 4: 1237–1245.
32. Noelle, R. J., and E. C. Snow. 1991. T helper cell-dependent B cell activation. *FASEB J.* 5: 2770–2776.
33. Mombaerts, P., A. R. Clarke, M. A. Rudnicki, J. Iacomini, S. Itohara, J. J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M. L. Hooper, et al. 1992. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* 360: 225–231.
34. Madsen, L., N. Labrecque, J. Engberg, A. Dierich, A. Svejgaard, C. Benoist, D. Mathis, and L. Fugger. 1999. Mice lacking all conventional MHC class II genes. *Proc. Natl. Acad. Sci. USA* 96: 10338–10343.
35. Kitamura, D., J. Roes, R. Kühn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 350: 423–426.
36. Honjo, K., X. Xu, and R. P. Bucy. 2004. CD4+ T-cell receptor transgenic T cells alone can reject vascularized heart transplants through the indirect pathway of alloantigen recognition. *Transplantation* 77: 452–455.
37. Bäckström, B. T., U. Müller, B. Hausmann, and E. Palmer. 1998. Positive selection through a motif in the alphabeta T cell receptor. *Science* 281: 835–838.
38. Corry, R. J., H. J. Winn, and P. S. Russell. 1973. Heart transplantation in congenic strains of mice. *Transplant. Proc.* 5: 733–735.
39. Yu, G., X. Xu, M. D. Vu, E. D. Kilpatrick, and X. C. Li. 2006. NK cells promote transplant tolerance by killing donor antigen-presenting cells. *J. Exp. Med.* 203: 1851–1858.
40. Stevens, J., K. H. Wiesmüller, P. J. Barker, P. Walden, G. W. Butcher, and E. Joly. 1998. Efficient generation of major histocompatibility complex class I-peptide complexes using synthetic peptide libraries. *J. Biol. Chem.* 273: 2874–2884.
41. Garboczi, D. N., D. T. Hung, and D. C. Wiley. 1992. HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc. Natl. Acad. Sci. USA* 89: 3429–3433.
42. Holmes, B. J., A. C. Richards, M. Awwad, L. S. Copeman, M. L. McLaughlin, E. Cozzi, H. J. Schuurman, and H. F. Davies. 2002. Anti-pig antibody levels in naïve baboons and cynomolgus monkeys. *Xenotransplantation* 9: 135–147.
43. Markowitz, J. S., P. R. Rogers, M. J. Grusby, D. C. Parker, and L. H. Glimcher. 1993. B lymphocyte development and activation independent of MHC class II expression. *J. Immunol.* 150: 1223–1233.
44. Win, T. S., S. Rehakova, M. C. Negus, K. Saeb-Parsy, M. Goddard, T. M. Conlon, E. M. Bolton, J. A. Bradley, and G. J. Pettigrew. 2009. Donor CD4 T cells contribute to cardiac allograft vasculopathy by providing help for autoantibody production. *Circ. Heart Fail.* 2: 361–369.
45. Mombaerts, P., E. Mizoguchi, H. G. Ljunggren, J. Iacomini, H. Ishikawa, L. Wang, M. J. Grusby, L. H. Glimcher, H. J. Winn, A. K. Bhan, et al. 1994. Peripheral lymphoid development and function in TCR mutant mice. *Int. Immunol.* 6: 1061–1070.
46. Honjo, K., X. Y. Xu, and R. P. Bucy. 2000. Heterogeneity of T cell clones specific for a single indirect alloantigenic epitope (I-Ab/H-2Kd54-68) that mediate transplant rejection. *Transplantation* 70: 1516–1524.
47. Honjo, K., X. Yan Xu, J. A. Kapp, and R. P. Bucy. 2004. Evidence for cooperativity in the rejection of cardiac grafts mediated by CD4 TCR Tg T cells specific for a defined allopeptide. *Am. J. Transplant.* 4: 1762–1768.
48. Rose, M. L., M. S. Birbeck, V. J. Wallis, J. A. Forrester, and A. J. Davies. 1980. Peanut lectin binding properties of germinal centres of mouse lymphoid tissue. *Nature* 284: 364–366.
49. Manz, R. A., A. Thiel, and A. Radbruch. 1997. Lifetime of plasma cells in the bone marrow. *Nature* 388: 133–134.
50. King, C., S. G. Tangye, and C. R. Mackay. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu. Rev. Immunol.* 26: 741–766.
51. Fazilleau, N., L. Mark, L. J. McHeyzer-Williams, and M. G. McHeyzer-Williams. 2009. Follicular helper T cells: lineage and location. *Immunity* 30: 324–335.
52. Fazilleau, N., L. J. McHeyzer-Williams, H. Rosen, and M. G. McHeyzer-Williams. 2009. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat. Immunol.* 10: 375–384.
53. Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325: 1006–1010.
54. Poholek, A. C., K. Hansen, S. G. Hernandez, D. Eto, A. Chandele, J. S. Weinstein, X. Dong, J. M. Odegard, S. M. Kaech, A. L. Dent, et al. 2010. In vivo regulation of Bcl6 and T follicular helper cell development. *J. Immunol.* 185: 313–326.
55. Motallebzadeh, R., S. Rehakova, T. M. Conlon, T. S. Win, C. J. Callaghan, M. Goddard, E. M. Bolton, N. H. Ruddle, J. A. Bradley, and G. J. Pettigrew. 2012. Blocking lymphotoxin signaling abrogates the development of ectopic

lymphoid tissue within cardiac allografts and inhibits effector antibody responses. *FASEB J.* 26: 51–62.
56. Laffont, S., C. Seillet, J. Ortaldo, J. D. Coudert, and J. C. Guéry. 2008. Natural killer cells recruited into lymph nodes inhibit alloreactive T-cell activation through perforin-mediated killing of donor allogeneic dendritic cells. *Blood* 112: 661–671.
57. Celli, S., M. L. Albert, and P. Bousso. 2011. Visualizing the innate and adaptive immune responses underlying allograft rejection by two-photon microscopy. *Nat. Med.* 17: 744–749.
58. London, C. A., M. P. Lodge, and A. K. Abbas. 2000. Functional responses and costimulator dependence of memory CD4+ T cells. *J. Immunol.* 164: 265–272.
59. MacLeod, M. K., E. T. Clambey, J. W. Kappler, and P. Marrack. 2009. CD4 memory T cells: what are they and what can they do? *Semin. Immunol.* 21: 53–61.
60. Stockinger, B., C. Bourgeois, and G. Kassiotis. 2006. CD4+ memory T cells: functional differentiation and homeostasis. *Immunol. Rev.* 211: 39–48.
61. de Vinuesa, C. G., M. C. Cook, J. Ball, M. Drew, Y. Sunners, M. Cascalho, M. Wabl, G. G. Klaus, and I. C. MacLennan. 2000. Germinal centers without T cells. *J. Exp. Med.* 191: 485–494.
62. Victora, G. D., T. A. Schwickert, D. R. Fooksman, A. O. Kamphorst, M. Meyer-Hermann, M. L. Dustin, and M. C. Nussenzweig. 2010. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* 143: 592–605.
63. Taylor, A. L., S. L. Negus, M. Negus, E. M. Bolton, J. A. Bradley, and G. J. Pettigrew. 2007. Pathways of helper CD4 T cell allorecognition in generating alloantibody and CD8 T cell alloimmunity. *Transplantation* 83: 931–937.
64. Fabre, J. W. 1996. The role of polymorphic donor peptides in allograft recognition and rejection. *Immunol. Rev.* 154: 21–43.
65. Maier, S., C. Tertilt, N. Chambron, K. Gerauer, N. Hüser, C. D. Heidecke, and K. Pfeffer. 2001. Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28$^{-/-}$ mice. *Nat. Med.* 7: 557–562.
66. McNerney, M. E., K. M. Lee, P. Zhou, L. Molinero, M. Mashayekhi, D. Guzior, H. Sattar, S. Kuppireddi, C. R. Wang, V. Kumar, and M. L. Alegre. 2006. Role of natural killer cell subsets in cardiac allograft rejection. *Am. J. Transplant.* 6: 505–513.
67. Kroemer, A., X. Xiao, N. Degauque, K. Edtinger, H. Wei, G. Demirci, and X. C. Li. 2008. The innate NK cells, allograft rejection, and a key role for IL-15. *J. Immunol.* 180: 7818–7826.