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Unlinked Memory Helper Responses Promote Long-Lasting Humoral Alloimmunity


Essential help for long-lived alloantibody responses is theoretically provided only by CD4 T cells that recognize target alloantigen, processed and presented by the allospecific B cell. We demonstrate that in an allosresponse to multiple MHC disparities, cognate help for class-switched alloantibody may also be provided by CD4 T cells specific for a second “helper” alloantigen. This response was much shorter-lived than when help was provided conventionally, by Th cell recognition of target alloantigen. Nevertheless, long-lasting humoral alloimmunity developed when T cell memory against the helper alloantigen was first generated. Costimulatory blockade abrogated alloantibody produced through naïve Th cell recognition of target alloantigen but, crucially, blockade was ineffective when help was provided by memory responses to the accessory helper alloantigen. These results suggest that memory Th cell responses against previously encountered graft alloantigen may be the dominant mechanism for providing help to generate new specificities of alloantibody in transplant patients receiving immunosuppression. *The Journal of Immunology*, 2012, 189: 000–000.

A lllograft rejection has long been considered the culmination of cellular alloimmunity, but recent clinical studies indicated that alloantibody directed against mismatched donor MHC Ags may be as least as important in effecting the rejection of kidney (1, 2) and heart allografts (3, 4). Two main histological variants of alloantibody-mediated rejection are described (5), with rodent studies confirming that alloantibody can contribute to acute graft rejection (6) and to progression of chronic transplant arteriopathy (TA) (7, 8). As with conventional protein Ags, help from CD4 T cells is essential for generating alloantibody against MHC alloantigens (6, 9, 10), but the complexities of CD4 T cell allorecognition have prevented elucidation of how precisely this help is provided. CD4 T cells recognize alloantigen through two distinct pathways (11–14). In the direct pathway, which is unique to transplantation, alloantigen is recognized intact on the surface of donor APCs, whereas in the indirect pathway, alloantigen is processed and presented as peptide fragments by recipient APCs for self-restricted recognition. One study suggested that alloreactive B cells can receive help from direct-pathway CD4 T cells (15), but this has remained controversial (16), and we demonstrated recently that only indirect-pathway CD4 T cells act as T follicular helper (Tfh) cells for generating long-lasting, class-switched alloantibody (17).

The provision of help by indirect-pathway CD4 T cells conforms to the fundamental tenet, first proposed by Noelle and Snow (18), that B cell presentation of internalized, processed target Ag that enables linked cognate interaction with the CD4 TCR is essential for effective delivery of help. According to this tenet, alloreactive direct-pathway CD4 T cells (that cannot interact with the MHC class II/peptide complex of the allospecific B cell) are unable to provide help; additionally, help is restricted to indirect-pathway CD4 T cells that recognize the same target alloantigen that binds the BCR. In support, recent analysis of the humoral response to vaccinia virus emphasized that exquisite T–B cell epitope linkage also pertains to protein subunits of complex pathogens (19). Nevertheless, Hunziker et al. (20) highlighted that presentation of processed lymphocytic choriomeningitis virus by nonviral specific B cells can derive help for induction of bystander hypergammaglobulinemia, although the mechanism responsible was not assessed. This raises the possibility that, in the context of a complex immune response targeted typically against multiple disparate alloantigens, indirect-pathway CD4 T cells specific for one alloantigen may provide B cell help for production of alloantibody directed against a different graft alloantigen.

Confirmation of this mechanism would have important consequences for our understanding of the helper arm of humoral alloimmunity, because it represents an “unlinked” or dissociated system whereby Th cell activation occurs independently of the alloreactive B cell response. Hence, it would be possible for memory CD4 T cells generated from prior exposure to one helper alloantigen to provide help to naïve B cells responding to a second, newly encountered target alloantigen; help that may be quantitatively and qualitatively different from that delivered conventionally by those naïve CD4 T cells specific for the target alloantigen. To formally examine this concept of dissociated help, we developed murine heart transplant models that incorporated monoclonal populations of alloreactive T and B lymphocytes to limit helper CD4 T cell allorecognition to a single alloantigen distinct from the B cell target.

Materials and Methods

**Animals**

*Tcrbd*−/− B6 mice (H-2b) (21) and H-2b MHC class II-deficient mice (22) were purchased from The Jackson Laboratory (Bar Harbor, ME). *Tcrbd*−/− mice were crossed with MHC class II-deficient mice to create mice that...
were TCR and MHC class II deficient. TCR-transgenic RAG2−/− TEa mice (H-2d), specific for I-Ab−restricted I-EK52−68 peptide (23), were gifted by Prof. A. Rudensky (University of Washington, Seattle, WA). TCR-transgenic RAG2−/− Mar mice (H-2d), specific for I-Ab−restricted H-Y peptide (24), were gifted by Dr. D. Scott (Imperial College, London, U.K.).

**Flow cytometry**

FITC-conjugated anti-mouse CD3 (clone 145-2C11), FITC-conjugated anti-mouse CD44 (clone IM7), FITC-conjugated anti-mouse I-Ab (clone AF6-120.1), PE-conjugated anti-mouse CD19 (clone 1D3), PE-conjugated anti-mouse TCR Vβ6 (clone RR4-7), PE-conjugated anti-mouse I-Ek (clone 14-4S), biotinylated anti-mouse H-2Kd (clone SFI-1.1), biotinylated anti-mouse CD62L (clone MEL-14), and biotinylated and allophycocyanin-conjugated anti-mouse CD4 (clone GK1.5) were purchased from BD Pharminogen (San Diego, CA). HEL protein was biotinylated using the ImmunoProbe biotinylation kit (Sigma). Peripheral blood (depleted of erythrocytes by incubating with 0.17 M NH4Cl red cell lysis buffer) and splenic single-cell suspensions were blocked with anti-mouse CD16/CD32 (clone 2.4G2) before staining with the relevant Abs and dead cell exclusion dye 7-aminomucycarin (both from BD Pharminogen). Biotinylated Abs were detected by allophycocyanin-conjugated streptavidin (Invitrogen) or PE-Cy7-conjugated streptavidin (BD Pharminogen), and all cells were analyzed on a FACSCanto II flow cytometer with FACSDiva software (Becton Dickinson U.K.; Oxford, U.K.).

**CFSE cell proliferation**

Single-cell suspensions of splenocytes obtained from Mar or TEa mice were stained with 5 mM CFSE (Invitrogen) in the dark for 5 min and then were rested for 5% T cells or PE-Cy7-conjugated streptavidin (BD Pharminogen), and all cells were analyzed on a FACS Canto II flow cytometer with FACSDiva software (Becton Dickinson U.K.; Oxford, U.K.).

**Results**

Help for class-switched anti–MHC class I alloantibody can be provided by CD4 T cells that recognize unrelated alloantigen

The ability of CD4 T cells that recognize additional graft alloantigens to provide help for anti–MHC class I alloantibody production was examined using a model similar to that used recently to assess allo specific T cells (17). C57BL/6 TCRβ−/− mice, which lack all T cells yet retain relatively normal B cell homeostasis and compartmentalization (34), were challenged with BALB/c (H-2d) heart allografts. Helper function was provided by adoptive transfer of monoclonal populations of TCR-transgenic CD4 T cells. Thus, as described previously (17), TCRβ−/− mice reconstituted with Rag1−/− TCR75 CD4 T cells specific for I-Aβ−restricted H-2Kd52−68 allopeptide, which provide classical, linked helper recognition of target MHC class I alloantigen (Fig. 1A), mounted a robust anti–H-2Kd IgG alloantibody response (Fig. 1B) that was even stronger than that observed in wild-type B6 recipients (Fig. 1B); we reported recently that this is associated with 7-aminomucycarin (both from BD Pharminogen).
To exclude the possibility that activation of the transferred CD4 T cells simply provided bystander help to the H-2Kd–specific B cells, female Tcrbd<sup>2−2</sup> mice were reconstituted with Mar CD4 T cells and challenged simultaneously with male B6 splenocytes from Rag2<sup>2−2</sup> mice (to provide a source of male Ag without contaminating T and B cells) and a female BALB/c heart graft. No anti–H-2Kd IgG alloantibody was generated (Fig. 1D), suggesting that help provided through T cell recognition of additional alloantigen requires coexpression of the B cell target and helper Ag on the allograft.

The provision of help through recognition of additional graft alloantigens was not a facet of homeostatic proliferation of the helper CD4 T cell population following transfer into an empty niche (35), because Mar CD4 T cells did not undergo division upon transfer into naive female Tcrbd<sup>2−2</sup> mice (Fig. 1E). In contrast, robust proliferation was observed following challenge with a male B6 heart graft (Fig. 1E).

**FIGURE 1.** H-2Kd–specific B cells can receive effective help from CD4 T cells that recognize unrelated antigen. (A) Diagrammatic representation of classical linked help provided to H-2Kd–specific B cells by H-2Kd peptide–specific TCR75 CD4 T cells. (B) Serum levels (mean ± SD) of anti–H-2Kd IgG alloantibody, after challenge with BALB/c heart allografts, at day 14 in B6 (n = 3), Tcrbd<sup>2−2</sup> (n = 3), and Tcrbd<sup>2−2</sup> recipients adoptively transferred with 10<sup>7</sup> splenocytes from Rag1<sup>2−2</sup> TCR75 mice (monoclonal CD4 T cells specific for I-A<sup>β</sup>–restricted H-2Kd peptide) (n = 3). (C) Diagrammatic representation of possible unlinked help provided to H-2Kd–specific B cells by either TEa CD4 T cells (specific for self-restricted I-E peptide) or Mar CD4 T cells (specific for self-restricted H-Y peptide). (D) Serum levels of anti–H-2Kd IgG alloantibody, following transplantation of male or female BALB/c heart allografts into Tcrbd<sup>2−2</sup> recipients adoptively transferred with 10<sup>7</sup> splenocytes from Rag2<sup>2−2</sup> TEa or Mar mice as a source of monoclonal CD4 T cells. One group of Mar CD4 T cell–constituted Tcrbd<sup>2−2</sup> recipients of a female BALB/c graft were immunized additionally with 10<sup>7</sup> male B6 Rag2<sup>2−2</sup> splenocytes to provide unlinked activation of the Mar CD4 T cells. Data are the values for each individual mouse, with mean values indicated. (E) Representative flow cytometry plots (of two independent experiments) of CFSE-labeled Mar CD4 T cells 7 d after adoptive transfer into unchallenged B6 and Tcrbd<sup>2−2</sup> mice or Tcrbd<sup>2−2</sup> mice that received a male B6 heart graft. The indicated levels of cell division refer to the percentage division of the parent population, as calculated using FlowJo software. *p ≤ 0.01, two-tailed unpaired t test.

CD4<sup>+</sup> T cells that recognize additional alloantigen interact with B cell MHC class II

Given the requirement for cognate interaction between the B and T helper lymphocytes in generating class-switched Ab to conventional protein Ag, we hypothesized that help from CD4 T cells specific for additional alloantigen would depend similarly upon presentation of their appropriate allopeptide epitope by the MHC class I–specific B cell. To test this, bone marrow chimeric mice were created in which the B cells lacked MHC class II expression (expression on non-B cell APCs was maintained) and were T cell deficient (B.CII<sup>−−</sup>, Fig. 2A). Although lacking MHC class II expression on B cells, CD4 T cell alloimmunity in these mice was not impaired, because adoptively transferred TEa T cells responded to challenge with H-2<sup>d</sup> alloantigen with comparable vigor to control B.CII<sup>++</sup> chimeric mice that retained B cell MHC class II expression (Fig. 2B). This mirrors our recent finding that division of TCR75 CD4 T cells is comparable in control and experimental bone...
marginal chimeras (17). Despite this, when reconstituted with Mar CD4 T cells, female B.CII−/− recipients of male BALB/c heart grafts failed to mount an anti–H-2Kd IgG alloantibody response (Fig. 2C); in contrast, modestly strong responses developed in TEa CD4 T cell–reconstituted B.CII+/+ recipients simultaneously challenged with 10^7 BALB/c splenocytes. Division in similarly challenged Tcrbd−/− mice is shown for comparison. The indicated levels of cell division were calculated using FlowJo software.

**FIGURE 2.** CD4 T cells that recognize additional helper alloantigen interact with MHC class II expressed on allospecific B cells. (A) Representative flow cytometric plots depicting CD19 and I-Ab staining of live peripheral blood cells from T cell–deficient, female bone marrow chimeric mice (B.CII−/−) (right panel), highlighting that MHC class II was not expressed on B cells (upper right quadrant) but was maintained in a proportion of non-B cell APCs (upper left quadrant). Control bone marrow chimeric mice (B.CII+/+) are shown for comparison (left panel). (B) Representative plots of Tcrbd CD4 T cell division in chimeric B.CII−/− or B.CII+/− mice following i.v. challenge with 10^7 BALB/c splenocytes. Division in similarly challenged Tcrbd−/− mice is shown for comparison. The indicated levels of cell division were calculated using FlowJo software. (C) Serum anti–H-2Kd IgG alloantibody following challenge of Mar CD4 T cell–reconstituted (using 10^7 splenocytes from Rag2−/− Mar mice, as Fig. 1) B.CII−/− and B.CII+/− mice with a male BALB/c heart allograft. Anti–H-2Kd alloantibody responses to a female BALB/c heart allograft in Mar CD4 T cell–reconstituted B.CII−/− recipients simultaneously challenged i.v. with male APCs are also depicted. Data are the values for each individual mouse, with mean values indicated. The p values were calculated using a two-tailed unpaired t test. Values from naive mice are shown for comparison.

All specific B cells present acquired helper alloantigen as processed allopeptide

We hypothesized that the above requirement for recognition of B cell MHC class II expression and processing of additional, adjacent alloantigens on the donor cell by the class I–specific B cell. This was examined by creating tetra-parental H-2b mosaic mice, whose cells expressed either I-E or H-2Kd on their surface but not both (Fig. 3A). Hearts from these mice were transplanted into TEa-reconstituted Tcrbd−/− recipients, in the expectation that recipient H-2Kd−specific B cells would be unable to simultaneously acquire donor I-E Ag and, thus, would not receive help from TEa CD4 T cells for production of anti–MHC class I H-2Kd alloantibody (Fig. 3B). Compared with the response to BALB/c heart allografts, in which I-E and H-2Kd alloantigens are coexpressed on donor cells, anti–H-2Kd alloantibody production following transplantation with mosaic I-E/Kd hearts was reduced (Fig. 3C). Nevertheless, the mosaic hearts provoked a weak response that was greater than background observed in naive mice. This might be explained by the observation that confinement of H-2Kd and I-E expression to different cells in the mosaic mice is not absolute; a small percentage (4.1 ± 0.75%, mean ± SD) of PBMCs in mosaic mice express both H-2Kd and I-E (Fig. 3A), demonstrating the percentage of I-E– and H-2Kd–expressing cells from mosaic I-E/Kd heart allografts express either I-E or H-2Kd but not both. Presentation of I-E peptide by recipient H-2Kd–specific B cells is not absolute; a small percentage (4.1 ± 0.75%, mean ± SD) of PBMCs in mosaic mice express both H-2Kd and I-E (Fig. 3A), demonstrating the percentage of I-E– and H-2Kd–expressing cells from mosaic I-E/Kd heart allografts express either I-E or H-2Kd but not both. Presentation of I-E peptide by recipient H-2Kd–specific B cells is not expected. (C) Serum anti–H-2Kd IgG alloantibody in Tcrbd CD4 T cell–reconstituted Tcrbd−/− recipients, following challenge with BALB/c (n = 6) or mosaic (n = 5) heart allografts. Data represent the values for each individual mouse, with mean values indicated. The p values were calculated using a two-tailed unpaired t test. Values from naive mice (n = 8) are shown for comparison.
prematurely reflecting exchange of membrane proteins through
trogocytosis (36) prior to heart graft procurement. Unfortunately,
this exchange prevents definitive interpretation of the results ob-
tained using mosaic mice as donors.

One possible explanation for how allospecific B cells acquire
graft alloantigen other than that recognized by their BCR is that
small numbers of B cells express a second BCR specific for ad-
ditional alloantigen, either because of incomplete receptor editing
(37) or because of bystander acquisition of the second receptor
from activated B cells (38). The possibility of dual-receptor ex-
pression was examined using T cell–deficient mice that contained
only a single, monoclonal population of B cells, which were
created by crossing SWHEL.BCR transgenic mice onto a Rag2−/−
background (Fig. 4A). SWHEL mice were originally created using
homologous recombination to target a HEL-specific IgH V region
gene to the endogenous IgH locus (28); therefore, the BCR trans-
fectional anti-HEL B cell population can class-switch. However,
gene to the endogenous IgH locus (28); therefore, the BCR trans-

homologous recombination to target a HEL-specific IgH V region
within which the transgenic HEL-specific B cell population is
mature B cells express a single HEL-specific BCR (Fig. 4A).
SWHEL mice were also crossed onto a Rag2−/− background; such
mice lack T cells but maintain a polyclonal population of B cells,
within which the transgenic HEL-specific B cell population is
nested (Fig. 4A). Notably, reconstitution of SWHEL.Rag2−/− mice
with a helper population of TCR75 CD4 T cells and challenge
with membrane HEL Ag expressing B6 (B6.mHEL) × B6.Kd F1
heart grafts provoked an anti-HEL IgG Ab response that was
comparable to similarly treated SWHEL.Tcerbd−/− mice (Fig. 4B).

To examine the presentation of processed helper alloantigen by
the allospecific B cell, HEL-specific B cells were purified from
SWHEL.Rag2−/− mice 4 d after challenge with either a B6.mHEL ×
B6.I-E F1 heart or a B6.mHEL heart allograft and adoptively
transferred into Tcerbd−/− mice. The primary SWHEL.Rag2−/−
recipients did not develop an anti-HEL alloantibody response
data not shown), reflecting the absence of a helper T cell pop-
ulation; the helper arm was instead provided in the secondary
Tcerbd−/− mice by adoptive transfer of CD4 T cells from Rag2−/−
TEa mice, primed 5 d previously by immunization with I-Eα52-68
peptide. Anti-HEL IgG alloantibody was detectable in the sec-
ondary Tcerbd−/− mice that received SWHEL B cells from SWHEL.
Rag2−/− recipients of B6.mHEL × B6.I-E F1 heart allografts;
however, crucially, transfer of SWHEL B cells from recipients
challenged with a B6.mHEL heart, which could not have en-
countered helper I-E alloantigen, did not provoke a response (Fig.
4C). Thus, these experiments provide definitive confirmation that
B cells with a single receptor specific exclusively for one allo-
antigen can receive effective help from CD4 T cells that recognize
exclusively another graft alloantigen and that this help is depen-
dent upon B cell presentation of the helper Ag (Fig. 4D).

FIGURE 4. Allospecific B cells present acquired helper alloantigen as processed allopeptide. (A) Representative flow cytometric graphs of gated live
lymphocytes from SWHEL.BCR–transgenic mice crossed onto the Rag2−/− (SWHEL.Rag2−/−) or Tcerbd−/− (SWHEL.Tcerbd−/−) background. Both strains
lack CD3 T cells (left panel; analysis of SWHEL mouse included for comparison); although only a select population of SWHEL.Tcerbd−/− B cells binds HEL,
all B cells in SWHEL.Rag2−/− mice are HEL specific (middle panel and right panel, which shows percentages from individual mice). (B) Serum anti-HEL
IgG Ab responses following transplantation of B6.mHEL × B6.Kd F1 heart allografts into Tcer75 CD4 T cell–reconstituted SWHEL.Tcerbd−/− mice or
similarly reconstituted SWHEL.Rag2−/− mice. Circulating anti-HEL IgG Ab in Tcerbd−/− mice following adoptive transfer of TEa CD4 T cells, purified
from primed Rag2−/− TEa mice, and splenic B cells, purified from SWHEL.Rag2−/− mice that had received either a B6.mHEL heart graft or a B6.mHEL ×
B6.I-E F1 heart graft 4 d earlier. The p values were calculated using a two-tailed unpaired t test. (D) Diagrammatic representation of unlinked help provided
by TEa CD4 T cells to HEL-specific SWHEL B cells that present I-E peptide following challenge with a B6.mHEL × B6.I-E F1 (mHEL.I-E) heart allograft.

B6.mHEL x B6.I-E F1 graft

B6.mHEL × B6.I-E F1 graft

B6.mHEL x B6.I-E F1 graft
Memory helper CD4 T cell recognition of additional alloantigen

The above models, wherein the Ags for Th cell recognition are dissociated from those acting as B cell targets, theoretically enable memory T cell responses from prior alloantigen exposure to provide help to naive B cells that recognize additional, newly encountered, graft alloantigen. To examine how help provided from recognition of additional alloantigen differs according to memory status, Mar CD4 T cell–reconstituted female Tcrbd−/− mice were challenged with a male B6 skin graft and further challenged 6 wk later with a male BALB/c heart graft, and the anti–H-2Kd alloantibody response was quantified. In this model, help for alloantibody production can only be provided by Ag-experienced Mar CD4 T cells, whereas the H-2Kd–specific B cells remain in a naive state until a heart allograft is introduced. Male skin grafting resulted in marked expansion of the Mar CD4 T cells and acquisition of memory phenotype (Fig. 5A); notably, the anti–H-2Kd alloantibody response to the BALB/c heart allograft was markedly different from that observed in control mice challenged with a female B6 skin graft: circulating alloantibody was readily detectable at termination of the experiment on day 50 (Fig. 5B) and was associated with the presence of long-lived plasma cells within bone marrow (Fig. 5C).

Memory CD4 T cell responses differ characteristically from naive responses in their reduced requirements for costimulation, greater proliferation, and secretion of a different cytokine spectrum (39, 40). We hypothesized that, in clinical transplantation, the principal pathway for providing help for alloantibody responses against newly encountered alloantigen—naive Th cell recognition of the processed target Ag—is likely inhibited by concurrent immunosuppression administration; instead, memory CD4 T cell responses against additional graft alloantigen becomes the dominant mechanism for delivery of help. This was tested by comparing the impact of blocking CD40–CD40L signaling on the alloantibody response to a heart allograft in recipients with memory and naive helper CD4 T cell populations. Blockade was achieved by administering anti-CD154 mAb (41), a protocol that abrogated the robust anti–H-2Kd alloantibody response to BALB/c heart allografts generated when help was provided conventionally, by TCR75 T cells (Fig. 5D). When help was provided by naive Mar CD4 T cells, anti-CD154 mAb treatment was similarly effective at blocking anti–H-2Kd alloantibody responses; however,

**FIGURE 5.** Memory CD4 T cell recognition of additional helper alloantigen results in long-lived alloantibody production that is costimulation independent. (A) Number of circulating Mar CD4 T cells, as a percentage of total lymphocytes in Tcrbd−/− mice reconstituted with 10^7 splenocytes from Rag2−/− Mar mice, 6 wk after receiving either female (naive Mar, n = 7) or male (memory Mar, n = 4) B6 skin grafts (left panel, mean ± SD; naive B6 mice are included as comparison). Representative (of three mice/group) flow cytometry dot plots of CD44 and CD62L staining on live splenic CD4 T cells from the same groups. Values indicate numbers of CD44^hiCD62L^low memory CD4 T cells within gated area, expressed as the percentage of total live CD4 population. (B) Serum levels of anti–H-2Kd IgG alloantibody, following transplantation of male BALB/c heart allografts, into Tcrbd−/− recipients reconstituted with naive or memory Mar CD4 T cells, as described above, with or without anti-CD154 mAb treatment at the time of transplant. Data are values for each individual mouse, with mean values indicated. (C) Number of H-2Kd–specific IgG-secreting Ab-secreting cells (ASCs) per 10^6 plated cells within spleen and bone marrow 50 d after transplantation of male BALB/c heart allografts into Tcrbd−/− recipients, reconstituted as above with naive or memory Mar CD4 T cells, with or without anti-CD154 mAb treatment at the time of transplant. Values for naive Tcrbd−/− mice are included for comparison. Data represent a minimum of three mice/group (mean ± SD). (D) Serum levels of anti–H-2Kd IgG alloantibody in TCR75–CD4 T cell–reconstituted Tcrbd−/− recipients that received a BALB/c heart allograft with (n = 5) or without (n = 5) concurrent treatment with anti-CD154 mAb. Data are the values for each individual mouse, with mean values indicated. Values for the TCR75 group that did not receive anti-CD154 treatment were published previously (17). *p < 0.05, **p < 0.005, two-tailed unpaired t test. ns, Not significant.
in contrast, it failed to inhibit the development of the long-lasting responses aided by memory Mar CD4 T cells (Fig. 5B), with seeding of H-2Kd-specific, long-lived plasma cells to the bone marrow preserved (Fig. 5C) but reduced.

Recognition of additional helper alloantigen contributes to TA

Alloantibody responses that develop in human heart transplant recipients are associated with early graft loss (3, 4), and although BALB/c heart grafts in the Mar T cell–reconstituted recipients did not reject acutely, the contribution of alloantibody to chronic graft failure was assessed by quantifying the severity of TA. In the absence of a humoral arm, indirect-pathway Mar CD4 T cells can autonomously effect development of modest TA in male allografts, because their adoptive transfer into T and B cell–deficient Rag2−/− recipients provoked more severe TA than observed in similarly reconstituted Tcrbd−/− recipients of female hearts, even when the Mar population in the latter was activated by simultaneous immunization with male B6 splenocytes (Fig. 6A). TA was slightly greater in the Mar-reconstituted Tcrbd−/− recipients of male BALB/c heart grafts than in Mar-reconstituted Rag2−/− recipients (Fig. 6A), although this difference was not statistically significant. Thus, transient alloantibody responses promoted by unlinked, naive Th cell responses to a second alloantigen do not appear to contribute to TA development. In contrast, when long-acting alloantibody responses were generated by providing help via memory Mar CD4 T cells, TA was much more severe (Fig. 6A). Furthermore, although anti-CD154 treatment ameliorated arteriopathy development in recipients with a naive Mar helper population (58.2 ± 19.2% versus 21.9 ± 11.5% mean luminal stenosis ± SD, p = 0.013), the same treatment had less impact on the memory group (90.4 ± 4.7% versus 73.4 ± 10.0% mean luminal stenosis ± SD, p = 0.057, Fig. 6A). The heightened TA in the memory group is unlikely to simply reflect greater activation of an effector Mar T cell population that mediates vasculopathy development autonomously, because memory T cell generation in Rag2−/− recipients did not alter the severity of arteriopathy (38.6 ± 15.0% versus 43.0 ± 15.1% mean luminal stenosis ± SD, p = 0.720) (Fig. 6A). In support, endothelial C4d complement deposition was readily detectable in allografts in which memory CD4 T cells generated long-lasting alloantibody, irrespective of the administration of costimulation blockade (Fig. 6B). In contrast, C4d deposition was not detectable in Rag2−/− recipients that did not develop alloantibody or in mice with short-lived alloantibody responses triggered by naive Mar CD4 T cell help (Fig. 6B). Thus, these experiments demonstrate that memory indirect-pathway CD4 T cells generate more severe TA, most likely as a consequence of their ability to promote long-lasting alloantibody responses, and that costimulation blockade fails to prevent this damage.

Discussion

The experiments were designed to determine the alloantigen specificity of CD4 T cells that provide help for alloantibody production. We demonstrated that CD4 T cells specific for one
alloantigen can provide help to B cells whose allospecificity is for a different alloantigen expressed by cells within the graft. Moreover, CD4 T cell memory responses against additional graft alloantigens are able to provide costimulation-independent B cell help for new specificities of alloantibody that can contribute to graft failure. These findings might be clinically relevant, because the problems associated with long-lasting humoral alloimmunity pose one of the major challenges for clinical transplantation. “Sensitized” patients on the waiting list for kidney transplantation have preformed alloantibody that often results in either excessively long waiting times or precludes transplantation entirely. In addition, the development of donor-specific alloantibodies after organ transplantation is associated with early graft failure. Alloantibody responses often develop in transplant recipients who are seemingly adequately immunosuppressed with stable initial graft function, indicating that their development is relatively resistant to conventional immunosuppressive treatment. Our demonstration that, memory CD4 T cells that recognize one graft alloantigen can deliver CD40-independent help to naïve alloreactive B cells that recognize another, may explain how these alloantibody responses occur.

Our findings appear to contravene the maxim of “linked recognition,” whereby, as exemplified by recent analysis of humoral responses to complex viral Ag (19), help for T-dependent Ab responses is restricted to CD4 T cells specific for the same Ag that is recognized by the BCR. However, it has long been appreciated, as demonstrated readily by haptenated systems, that Th cell epitopes are not necessarily derived from the same region of target Ag as the B cell epitope; indeed, shared helper recognition has been proposed as the mechanism responsible for simultaneous development of autoantibody against several different, yet structurally complexed, autoantigens (42). Nevertheless, our work differs, in that although the inability of Mar CD4 T cells to provide help for alloantibody responses against a female heart allo-graft (despite simultaneous challenge with male Ag) suggests a necessity for coexpression of the target and helper alloantigen on the same cell, the nature of this linkage is much less obvious and appears simply a facet of tethering within a common membrane (either surface or intracellular). The possibility that recognition of minor histocompatibility Ags could provide help for alloantibody responses was suggested two decades ago (43); however, this concept was largely ignored and only became verifiable more recently, with the availability of monoclonal populations of allo-specific B and T helper cells.

With regard to how T cell help is provided by recognition of additional graft alloantigen, our experiments highlighting the importance of MHC class II–restricted presentation indicate that cognate interaction with the CD4 TCR is still a fundamental requirement. Although the additional helper alloantigen could theoretically be acquired through non-Ag–specific mechanisms that operate in professional APCs, these pathways are generally ineffective in B cells. It is also unlikely that B cells express the relevant helper determinant following capture of MHC class II/allopeptide complexes from other APCs that have already processed alloantigen, because no alloantibody was produced in the bone marrow chimeric recipients that lacked MHC class II selectively on B cells. Instead, the requirement for coexpression of the target and helper alloantigen on the same donor cell suggests that BCR-mediated internalization is responsible. If so, the experiments incorporating B cell–transgenic recipients, in which all B cells express a single specificity of BCR, confirm that acquisition of helper Ag occurs through BCR recognition of target alloantigen and not, for example, through capture of a second BCR specific for the helper Ag (38). Presumably, as target Ag is internalized via the BCR, fragments of donor cell (most likely the surrounding membrane portions) that contain additional helper alloantigen are also captured. Real-time visualization of B cell synapse formation revealed that spreading and then contraction of the BCR concentrates target Ag to the synapse, while simultaneously excluding other membrane proteins (44), perhaps indicating that nonselective acquisition is unlikely. Nevertheless, Suzuki et al. (45) recently demonstrated effective acquisition of follicular dendritic cell surface protein by B cells as they acquire their target Ag; similarly, CD8 T cells can acquire additional membrane proteins from target cells during TCR-mediated internalization of MHC class I complex (46).

Long-lasting humoral immunity is a defining trait of the germinal center response, help for which is limited to a select, highly specialized subset of CD4 T$_{FH}$ cells (47, 48). The durable alloantibody responses generated when T cell help is provided through conventional recognition of target alloantigen are similarly characterized by germinal center formation and establishment of a bone marrow plasma cell niche (17); we further confirmed that indirect-pathway CD4 T cells that recognize target alloantigen acquire a signature T$_{FH}$ cell phenotype (17). In comparison, help provided through naïve recognition of additional “helper” alloantigen is less effective, with the transient Ab production and absence of bone marrow plasma cell deposition suggesting that short-lived extrafollicular, and not germinal center, foci are responsible. Nevertheless, the dissociated nature of our model, in which the target for Th cell recognition is distinct from that for alloreactive B cell recognition, theoretically enables cellular memory responses against one alloantigen to provide help to naïve B cells responding to another; our experiments highlight that, in this situation, the alloantibody response against newly encountered MHC class I alloantigen differs markedly. The ability of H-Y–specific memory CD4 T cells to provide help for production of long-lasting anti–MHC class I alloantibody and for formation of a bone marrow plasma cell niche suggests that the memory Mar CD4 T cells perform T$_{FH}$ cell function for development of germinal center responses. However, the outcomes of extrafollicular and germinal center responses may differ less distinctly than thought previously (49), and formal assessment of secondary follicle development, which was not performed in this study, will be the subject of future studies.

Despite the reported requirement for CD40/CD154 signaling in germinal center formation (50), its blockade did not prevent memory cellular responses against additional helper alloantigen from mediating the development of durable alloantibody responses. Presumably, CD40 costimulation is not essential for productive interaction between the B and helper T lymphocyte, but is instead necessary for initiating T cell activation upon cognate encounter with dendritic cells; once activated, CD40-independent B cell help that results in long-lasting alloantibody production is possible. In support, agonistic CD28 Ab promotes germinal center development in CD154-deficient mice (51), and additional ligands, such as complement C4B-binding protein (52), were noted to signal through the B cell CD40 complex. In contrast, the robust and long-lived humoral alloresponses that were mediated by conventional, naïve helper recognition of target alloantigen were completely inhibited by anti-CD154 treatment; although unproven, this suggests that memory recognition of additional alloantigen may be the dominant pathway for developing new specificities of alloantibody in human transplant patients. This ability of memory helper CD4 T cells to provide costimulation blockade–resistant help to naïve B cells with a different Ag specificity may hold wider relevance. For example, in patients with humoral autoimmune disease, unlinked helper recognition...
**Disclosures**

The authors have no financial conflicts of interest.

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