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Acquisition of Humoral Transplantation Tolerance upon De Novo Emergence of B Lymphocytes

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A major obstacle to transplantation tolerance is humoral immunity. In this paper, we demonstrate that the intrinsic developmental propensity of the B lymphocyte compartment for acquisition of self-tolerance can be harnessed to induce humoral unresponsiveness to transplanted allotrigens. In the current study, when transitional B cells developed in the presence of donor lymphoid cells, the mature B lymphocyte compartment failed to mount a donor-specific alloantibody response to an organ transplant—despite unrestricted acute T cell-mediated allograft rejection. Specifically, we generated an experimental system wherein a B6 strain B cell compartment developed de novo in the presence of F1 (B6xBALB/c) lymphoid cells and in a T cell-deficient setting. Following establishment of a steady-state B cell compartment, these B6 mice were transplanted with heterotopic cardiac allografts from allogeneic BALB/c donors. The mice were then inoculated with purified syngeneic B6 T cells. As expected, all cardiac allografts were acutely rejected. However, the B lymphocyte compartment of these mice was completely inert in its capacity to form a BALB/c-specific alloantibody response. Using an allotigens-specific Ig transgenic system, we demonstrated that this profound degree of humoral tolerance was caused by clonal deletion of alloreactive specificities from the primary B cell repertoire. Thus, de novo B cell compartment development at the time of transplantation is of critical importance in recipient repertoire “remodeling” to a humoral tolerant state. The Journal of Immunology, 2011, 186: 000–000.

Donor-specific immunological tolerance remains elusive despite current immunotherapy. Importantly, the activation of naive, alloreactive B lymphocytes perpetuates acute and chronic rejection (1) of organ transplants via the formation of donor-specific Abs (DSAs) (2) and Ag presentation to T lymphocytes (3). Thus, induction of donor-specific humoral tolerance is an important priority of transplantation research (4). B lymphocyte self-tolerance develops via clonal deletion, receptor editing, clonal anergy, and competition for survival factors (5–8). Immature B cells and their lineal descendents, the transitional (TR) B cells exiting the bone marrow (BM), are highly susceptible to clonal elimination, editing, or anergy following BCR engagement with cognate self Ag (8, 9). It is our contention that the natural propensity for clonal regulation of specificities early in B cell development can be harnessed to achieve humoral transplantation tolerance (10, 11). The data in this study demonstrate that selection during de novo emergence of the B cell compartment in the presence of allotrigens deletes alloreactive clones. This “remodeling” of the primary repertoire to an allo-tolerant state prevents the recipient immune system from mounting a DSA response.

Materials and Methods

Mice

C57BL/6 (B6), B6 scid, BALB/c, B6B12BALB/c (F1), B6Tcr−−, and B10. Cg-Tg(3-83) were purchased from The Jackson Laboratory (Bar Harbor, ME). C.B17scid mice were purchased from Charles River Laboratories (Wilmington, MA). All animal procedures were in accordance with the Animal Welfare Act.

Abs, immunohistochemical staining, BrdU labeling, and flow cytometric analysis

A total of 1–2 × 10⁶ lymphoid cells were surface stained in 96-well microtiter plates with the following Abs: PerCP–anti-CD45R (B220, RA3-6B2), allophycocyanin–anti-IgM (II/41), FITC–PE–anti-CD21/35 (7G6), FITC–mouse anti-mouse H-2D(d) (34-2-12), FITC–mouse anti-mouse H-2K(d) (SF1-1.1), PE–mouse anti-H-2K(b) (AF6-88.5), allophycocyanin–anti-CD4 (RM4-5), PerCP–anti-CD8α (53-6.7), PE–anti-CD44 (IM7), FITC–anti-CD23 (B3B4), and streptavidin–allophycocyanin/PerCP (BD Biosciences, San Jose, CA), and FITC–anti-CD22 (BD) (eBioscience, San Diego, CA). The biotinylated anti-3-83 idiotype mAb was a generous gift of Dr. David Nemazee, The Scripps Research Institute. FACs analysis was performed using a FACSCalibur (BD Biosciences, San Jose, CA) and the data were analyzed using FlowJo Software (Version 8.8.6; Tree Star, Ashland, OR). For immunohistochemical analysis, tissues were processed for routine histology with serial paraffin sections, which were stained with the biotinylated anti-3–83 idiotype mAb (1:300), PBS wash, streptavidin-HRP, DAB chromogen, and hematoxylin counterstain (K0679; Dako, Carpinteria, CA). Continuous BrdU labeling was performed with i.p. inoculation of 0.5 mg BrdU (Sigma-Aldrich). St. Louis, MO) in PBS every 12 h for 3 d. Splenocytes were stained in standard FACs buffer, washed twice with protein-free PBS, and permeabilized using Fix & Perm (Caltag Lab-
oratories, Burlingame, CA). Cells were washed, incubated with DNase (Sigma DN-25), washed, and stained with FITC–anti-BrdU (BD Biosciences) before FACS analysis.

**BM stem cell reconstitution and adoptive transfer**

B6 scid and C.B17 scid recipients received a retro-orbital injection of 5–10 × 10^6 T and B lineage-depleted BM cells. Depletions were performed on LD depletion columns (Miltenyi Biotec, Auburn, CA), using anti-B220, -CD19, -class II, -CD4, -CD8, and -CD90 microbeads (Miltenyi Biotec). At days 10–14 after BM reconstitution, adoptive transfer of 15–20 × 10^6 purified BALB/c or F1 (H-2(b/d)) lymphocytes was performed using T cell-depleted (anti-CD4, -CD8, and -CD90 microbeads) donor splenocytes. At day 88 adoptive transfer of 5–10 × 10^6 purified syngeneic T lymphocytes was performed using depleted (anti-B220, -CD19, and -class II microbeads) donor splenocytes.

**Cardiac and skin transplantation**

All organ transplants were performed with the animals under general anesthesia, as authorized by the Institutional Animal Care and Use Committee guidelines. Donor hearts were harvested for heterotopic cardiac transplantation within the abdomen. An end-to-side anastomosis of donor and recipient aortas was created, followed by an end-to-side anastomosis between the donor pulmonary artery and the recipient inferior vena cava. All anastomoses were completed with 7-0 prolene sutures. Allograft rejection was monitored by manual abdominal palpation and, following euthanasia, by gross and histological examination. During skin transplantation a round 1- to 1.5-cm-diameter full-thickness graft was harvested after removal of s.c. fat. The grafts were secured to recipient mice with absorbable suture, and evidence of rejection was tracked with regular inspection and photography.

**FIGURE 1.** Experimental strategy for de novo B cell repertoire development in the presence of alloantigen. B6scid mice underwent BALB/c allogeneic skin grafting (d-30) prior to infusion of lymphocyte-depleted B6TCR^{-/-} BM cells (d0). Experimental animals underwent infusion of purified F1 (H-2^{b/d}) B cells (d10–14), whereas control animals did not. After B cell compartment maturation to a steady state, an allogeneic BALB/c heterotic vascularized heart transplantation (d74) was performed. A total of 5–10 × 10^6 purified syngeneic B6 T cells were subsequently infused on day 88. Skin-cardiac allograft rejection (d103–110) was observed. Flow crossmatch was performed 2–12 wk after T cell infusion.

**FIGURE 2.** Kinetics of B cell compartment development. A, B220 gated lymphocytes in the peripheral blood of WT control B6 were defined as either TR B cells (IgM^+, CD21/35lo) or mature FO B cells (IgM^-, CD21/35int). B, Representative TR and FO B cell populations are shown at serial time points after i.v. infusion of B6TCR^{-/-} BM cells. C and D, The ratios of TR to FO B lymphocytes and the frequencies of B220^+ lymphocytes in control (open bars) and experimental (filled bars) mice, respectively, are shown at serial time points.

**FIGURE 3.** Presence of donor lymphoid cells during recipient B cell development. A, Control F1 (left panels) and B6 (right panels) B220^+ lymphocytes are shown for MHC class I haplotypes, Kb (top panels) and KbDd (bottom panels). B, Representative B220^+ lymphocytes from experimental mice at serial time points are gated for donor-derived F1(H-2^{b/d}) cells. C, The mean frequency of F1-derived B220^+KbDd^+ lymphocytes in peripheral blood of experimental mice. Each time point represents the average ± SD for at least five mice.
Flow crossmatch

To determine the amount of DSAs in each recipient, flow crossmatch was performed 2–12 wk after adoptive transfer of T cells. Donor splenocytes from syngeneic, allogeneic, and third-party strains were incubated separately with recipient serum at the listed dilutions for 1 h, followed by wash in FACS buffer. After incubation with recipient serum, donor lymphocytes were counterstained with anti-B220 and anti-CD3 mAbs and then incubated with FITC-conjugated rat anti-mouse IgGs: -IgG1 (A85-1), -IgG2a2b (R2-40), and -IgG3 (R40-82) (BD Biosciences). This technique permitted specific gating on the T and B lymphocyte populations to assess the degree of recipient serum IgG binding to donor-derived lymphocytes—i.e., B and T cell crossmatch. Control and experimental groups were analyzed with a Student t test comparing the fold difference in the mean fluorescence indices for anti-IgGs to allogeneic versus syngeneic splenocytes after a B and T cell crossmatch.

Results

De novo B cell repertoire development in the presence of alloantigen

B6 scid mice were transplanted with a skin allograft from BALB/c donors. These mice were subsequently inoculated with enriched hematopoietic stem cells from syngeneic B6 TCR–/– (i.e., T cell-deficient) donors (Fig. 1). Newly emerging B cells started repopulating the periphery of these mice 1–2 wk following BM inoculation (Fig. 2A–C). As expected, these B cells were B220+ cells (Fig. 2A,2B,2D) and exhibited a TR phenotype (i.e., IgM+,
CD21/35lo) (12, 13). TR B cells are the immediate precursors of follicular (FO) B cells (i.e., IgMint, CD21/35int), which are responsible for initiating a productive Ab response within the germinal center (14). Maturation from the TR to FO stages is an important tolerance checkpoint where clonal deletion eliminates “autoreactive” specificities upon binding cognate Ag (10).

Full B cell reconstitution to a steady state occurred by 8–10 wk following BM inoculation in all B6 scid mice (Fig. 2B–D). These recipients were divided into two cohorts. The experimental group received T-depleted splenocytes from F1 donors to expose the developing B cell compartment to alloantigen (Fig. 1). These splenocytes were inoculated within 10–14 d following injection...
of hematopoietic stem cells when the “deletion-susceptible” TR cells were the predominant phenotype (Fig. 2B). We predicted that development of the B6-derived B cell compartment in the presence of alloantigen (i.e., skin and F1 splenocytes) would lead to the deletion of allorreactive specificities. Notably, the F1 cells were detectable up to 10 wk after inoculation and were therefore present while the B cell repertoire achieved a steady state (Fig. 3). The control group did not receive an F1 splenocyte inoculum. As such, the B cell compartment of control mice achieved a steady state in the absence of circulating allogenic cells.

Incacity of the B cell compartment generated in the presence of alloantigen to mount a DSA response despite acute T cell-mediated rejection

Following full B cell compartment development, both control and experimental recipients underwent heterotopic cardiac transplantation from allogenic BALB/c donors. Given the absence of a T lymphocyte compartment in the recipients, these mice had an activated B cell compartment (Fig. 4A). The sera from an experimental or control recipient displayed comparable titers of IgG Ab (Fig. 4B). All control recipients mounted a donor-specific IgG response, detectable on B and T cell crossmatch by 2 wk following adoptive transfer of T cells (Fig. 5A, 5B). Importantly, despite the absence of DSA in the experimental mice, total serum IgG titers were comparable to that of control mice (Fig. 4B). Notably, prior to the adoptive transfer of T cells, neither control nor experimental mice had detectable DSA titers (data not shown). Overall, these data demonstrate that de novo generation of the B lymphocyte compartment in the presence of alloantigen abrogates its capacity for producing a donor-specific IgG response, despite ongoing T cell-mediated acute allograft rejection.

Deletion of donor-specific clones from the primary B cell compartment results from de novo repertoire emergence in the presence of alloantigen

To determine whether B cell compartment emergence in the presence of alloantigen causes clonal deletion of allorreactive specificities from the primary repertoire, we used a well-established BCR transgenic system specific for H-2^b (i.e., 3-83 BCR-Tg [7]). When the 3-83Tg is bred onto the H-2^d background, the B lymphocyte compartment consists of >95% idotype-positive (id^+ ) clones. As expected, breeding the 3-83Tg onto the H-2^b background leads to complete deletion of the id^+ specificities (7). This system has been previously used to track the in vivo fate of alloreactive B cells subject to costimulatory blockade (15, 16). We predicted that the humoral tolerance we observed in the setting of de novo B cell emergence was the result of clonal deletion, and that the 3-83Tg system would permit us to test this contention. Therefore, we inoculated BALB/c scid recipients with lymphocyte-depleted BM stem cells from 3-83Tg (H-2^d, nondeleting background) and WT BALB/c mice at a 1:1 ratio (Fig. 6A). We confirmed that by day 8 following stem cell injection, >50% of the newly emerging B lymphocytes were id^+ (Supplemental Fig. 1A). On day 13 the majority of B lymphocytes detectable in the peripheral blood exhibited the “deletion-susceptible” TR phenotype (IgM^+ , CD21/35^lo ) (Fig. 6B) (12, 13).
These mice were divided into two distinct groups. The control group received 15–20 × 10⁶ T-depleted syngeneic BALB/c (H-2b) splenocytes (Fig. 6A). The experimental group received 15–20 × 10⁶ T-depleted allogeneic F1(H-2d/b) splenocytes (Fig. 6A). The peripheral blood of these mice was serially analyzed for the presence of the id⁺, H-2b reactive B cell clones. As early as 24 h following adoptive transfer of the T cell-depleted splenocytes, the peripheral blood of experimental recipients demonstrated a dramatic reduction in the frequency of id⁺ (Fig. 6C, Supplemental Fig. 1B). An absence of the id⁺ clones was evident in the peripheral blood 2 wk following F1 splenocyte transfer (Fig. 6C, Supplemental Fig. 1B). Importantly, the adoptively transferred donor F1 cells were detectable in the peripheral blood and lymphoid organs up to 2 mo after BM reconstitution (Fig. 6D, 6E, Supplemental Fig. 1C). Cohorts of control and experimental mice were euthanized 2 mo following BM reconstitution. Despite the presence of the non-Tg BALB/c BM-derived B cells, the id⁺ alloreactive clones were absent from the B cell compartment of BM, spleen, and lymph node of the experimental mice (Figs. 6D, 6E, 7A).

Of note, a cohort of the 1:1 (3-83:WT) chimeric mice did not receive F1 cells and, instead, underwent allogeneic B6 heterotopic heart transplantation (HHT) (day 14). Interestingly, the id⁺ specificities in these mice were not deleted, indicating that the allograft alone is insufficient to drive complete clonal deletion of alloreactive specificities from the emerging B cell compartment (Fig. 7B). In addition, we note that these HHT recipients demonstrated id⁺ B cells with a predominance of a developmentally arrested phenotype (IgMloIdloCD21/35lo) and a high turnover rate (Figs. 7B, 8), reminiscent of the well-described “autoantigen-driven” anergic peripheral B cell phenotype (17).

**Discussion**

Induction of humoral tolerance to organ allografts is the likely key to abrogation of chronic rejection. We hypothesized that B lymphocyte tolerance in the setting of organ transplantation can be most effectively achieved by eliminating alloreactive clones from the primary repertoire at the time of transplantation (11). Although deletional tolerance during development is a well-established fact in B cell tolerance (7, 18, 19), its applicability to the achievement of clinical transplantation tolerance has not been systematically assessed in basic murine studies. The objective of the current study was to do so. Our results indicate that the B cell compartment’s natural developmental propensity toward clonal deletion may be recapitulated for the purpose of acquiring transplantation tolerance.

Deletional B cell tolerance was shown to be the mechanism underlying transplantation tolerance after costimulatory blockade in mice (15). Moreover, a series of recent clinical studies from the Terasaki group have suggested that clonal deletion may be associated with less need for maintenance immunosuppression (20, 21). In both these lines of investigation, donor-specific transfusion (DST) seems to be a requirement for improved survival. In addition, a series of clinical and basic studies have previously pointed to the potential utility of DST for improving long-term allograft survival (22–24). The mechanistic explanation for the beneficial effect of DST has been attributed to a state of “chimerism” (25–28). Moreover, a tolerant clonal state was achieved upon vigorous ablative therapy combined with establishment of a donor chimeric state, using BM stem cell transfusion (29, 30). The present study is in line with these important findings in that donor chimerism via a DST-like infusion of F1 lymphoid cells was required for deletional B cell tolerance to occur. We contend that the de novo repertoire development in a chimeric state, which follows ablative therapies, may be key to the robust tolerance observed (20, 21, 29, 30).

We chose to use T cell-depleted F1 lymphoid cells for two reasons. First, the F1-derived lymphocytes, serving primarily as bystanders with circulating membrane-bound alloantigens, would be immunologically inert in the shared haplotype recipients, specifically because F1 lymphoid cells do not cause a graft-versus-host response in the parent strain. Second, the F1 lymphocytes were depleted of T cells to prevent a scenario in which host B lymphocyte development would occur in the presence of homeostatically proliferating F1 T cells in the “empty” T cell niche of the reconstituting hosts.

We do not believe that the cell type within the F1 splenocyte inoculum was critical to conferring the clonal deletion and humoral tolerance phenotype. Following MACS depletion of T splenocytes, the remaining F1-derived lymphocytes were 84–88% B cells and

**FIGURE 7.** Presence of donor lymphoid cells regulates alloreactive B cell clonal deletion versus anergy. A, B lymphocytes at d56 (left column) from WT BALB/c, (3-83Tg:WT, 1:0 and 1:1) controls, and experimental BALB/c scid chimeric recipients are gated as TR (IgM⁺, CD21/35⁻), mature FO (IgM⁻, CD21/35⁻), and marginal zone B cells (IgM⁻, CD21/35⁻). B, The bottom two panels are representative of d28 in control (1:1) mice and BALB/c scid mice that received an allogeneic HHT (d14) prior to infusion of BM cells. Right panels, B lymphocytes are characterized for their expression of 3-83 idiotype and IgM at d28 and d56. These results demonstrate that the presence of the cardiac allograft alone is insufficient to drive total clonal deletion of alloreactive specificities from the primary B cell repertoire. MZB, marginal zone B cell.
<0.5% T cells, and dendritic cells and macrophages constituted the remaining 12–16% (data not shown). Our laboratory is in the process of fractionating cell types to determine if a clonal deletion advantage is conferred by one cell type versus another. In classic studies from the Nemazee, Goodnow, and Basten laboratories, among others, B cell tolerance could be conferred by MHC class I Ag expression in various peripheral tissues, such as the liver (31), but was most critically dependent upon the degree of BCR affinity for the Ag in question (18, 32, 33) and the relative competition among B cells for finite resources within that FO niche (10, 34, 35). Overall, we suspect that the circulating membrane-bound alloantigen within the F1 splenocyte inoculum was the only critical factor for deletion of the alloreactive specificities emerging within the developing B cell repertoire.

This study provides specific mechanistic evidence that de novo emergence of the B lymphocyte compartment in the presence of sufficient alloantigen (36) promotes “reertoire remodeling” to an allo-tolerant state. We extrapolate that B cell-specific ablative therapy (e.g., Rituxan and belimumab) combined with DST at the time of transplantation may be a pivotal step in achievement of humoral transplantation tolerance. Indeed, two preclinical studies in nonhuman primate transplantation models have already presented encouraging results, suggesting the efficacy of “preemptive” B cell-directed induction immunotherapy (37, 38).

It remains to be determined whether establishment of donor chimerism, using DST, combined with transient B cell-specific induction therapy followed by repertoire reconstitution, holds the key to humoral transplantation tolerance in the clinical setting—results from the Terasaki group strongly support this approach (20).

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Disclosures
The authors have no financial conflicts of interest.

References

FIGURE 8. Anergic phenotype of alloreactive B cell clones in the absence of donor-derived lymphoid cells. The presence of a cardiac allograft alone during de novo B lymphocyte emergence promotes a developmentally arrested, anergic phenotype. A and B, This figure is an expansion of the analysis presented in Fig. 7, focusing on the id+ B cells in experimental recipients of heterotopic heart transplants (i.e., no donor F1 splenocyte infusion). C, Representative histograms of IgM+id+ B cells in control (thin lines, n = 4) and HHT recipients (thick lines, n = 3) for expression of the 3-83 idiotype, IgM, CD21/35, B220, CD23, CD44, CD22, and BrdU incorporation after 3 d of continuous labeling.