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Donor B Cells in Transplants Augment Clonal Expansion and Survival of Pathogenic CD4+ T Cells That Mediate Autoimmune-like Chronic Graft-versus-Host Disease

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We reported that both donor CD4+ T and B cells in transplants were required for induction of an autoimmune-like chronic graft-versus-host disease (cGVHD) in a murine model of DBA/2 donor to BALB/c recipient, but mechanisms whereby donor B cells augment cGVHD pathogenesis remain unknown. In this study, we report that, although donor B cells have little impact on acute GVHD severity, they play an important role in augmenting the persistence of tissue damage in the acute and chronic GVHD overlapping target organs (i.e., skin and lung); they also markedly augment damage in a prototypical cGVHD target organ, the salivary gland. During GVHD pathogenesis, donor B cells are activated by donor CD4+ T cells to upregulate MHC II and costimulatory molecules. Acting as efficient APCs, donor B cells augment donor CD4+ T clonal expansion, autoreactivity, IL-7R expression, and survival. These qualitative changes markedly augment donor CD4+ T cells’ capacity in mediating autoimmune-like GVHD, so that they mediate disease in the absence of donor B cells in secondary recipients. Therefore, a major mechanism whereby donor B cells augment cGVHD is through augmenting the clonal expansion, differentiation, and survival of pathogenic CD4+ T cells. The Journal of Immunology, 2012, 189: 222–233.

Graft-versus-host disease (GVHD) can be divided into acute GVHD (aGVHD) and chronic GVHD (cGVHD). aGVHD is characterized by T cell infiltration in target organ tissues (i.e., gut, liver, lung, and skin); cGVHD shares characteristics with systemic autoimmune diseases, such as scleroderma and lupus-like syndrome, including elevated serum levels of IgG autoantibodies, sclerodermatous skin tissue damage, and systemic tissue collagen deposition (1–7). The target organ tissues of aGVHD and cGVHD often overlap, such as in the lung and skin, but some target organs (i.e., salivary gland) are mostly unique to cGVHD (1–4). Over the past three decades, there has been little progress in prevention and treatment of cGVHD, due in part to the poor understanding of cGVHD pathogenesis (1). It is clear that aGVHD is mediated by alloreactive donor T cells (8), but it is still unclear whether cGVHD is mediated by the same T cells that mediate aGVHD, although most cGVHD is subsequent to aGVHD (1, 9).

Ag presentation is known to play a key role in both aGVHD and cGVHD pathogenesis. Host APCs were reported to initiate aGVHD, and both donor and host APCs are required for mediating maximal cGVHD (10–14). In autoimmune diseases such as lupus, activated B cells have been shown to be very potent APCs in expanding autoreactive T cells and mediating epitope spreading (15, 16). B cells produce autoantibodies in cGVHD patients, leading to the hypothesis that donor B cells play a role in cGVHD pathogenesis (17, 18). Indeed, the administration of B cell-depleting anti-CD20 could ameliorate cGVHD in some patients (19–22). In addition, donor B cells were shown to augment priming of T cells that recognize minor Ags (23), and alloantibodies were recently shown to augment cGVHD pathogenesis in a MHC-mismatched murine model (18), but the role of Ag presentation of B cells in cGVHD pathogenesis remains unclear.

To clarify the role of donor B cells in GVHD pathogenesis, we used a murine cGVHD model of MHC-matched DBA/2 donor to BALB/c recipient (7, 24, 25). In this model, although CD8+ T cells have no discernible effect (24), both donor B and CD4+ T cells are required for disease pathogenesis, offering an opportunity to understand the ways in which donor B cells alter disease progression. We observed that donor B cells in transplants had little impact on aGVHD severity, but did markedly augment cGVHD. Donor B cells in transplants mediated the initial clonal expansion of donor autoreactive CD4+ T cells, augmented their differentiation into the Th2 subset, increased their expression of IL-7Rα, and decreased their apoptosis. Subsequently, these T cells expanded in GVHD target tissues and mediated persistent tissue damage. We also found that after interacting with donor B cells, these donor CD4+ T cells were capable of mediating cGVHD in secondary...
recipients in the absence of donor B cells. These studies indicate that donor B cells in transplant play a critical APC role in regulating initial expansion, differentiation, and survival of pathogenic CD4+ T cells that mediate cGVHD pathogenesis.

Materials and Methods

Mice

DBA/2 and BALB/c mice were purchased from the National Cancer Institute animal production program (Frederick, MD). Rag2<−/− BALB/c mice were purchased from Tacdon Farms (Germantown, NY). Luciferase transgenic (Luc+) DBA/2 mice were backcrossed from Luc+ FVB/N mice that were established by the C. Contag laboratory (26) for at least 10 generations. Mice were maintained in a pathogen-free room in the City of Hope Animal Resource Center (Duarte, CA). All animal protocols were approved by the City of Hope Institutional Animal Care and Use Committee.

Induction and assessment of GVHD

Mice were irradiated 6–8 h prior to hematopoietic cell transplantation (HCT) using a [137Cs] source at a dose of 800 cGy. Recipients were injected with T and B cell-depleted (TBCD) donor bone marrow (BM) cells and a dose of splenocytes containing 5 × 10^6 CD4+ cells, including CD25+ splenocytes (SPL, ∼40 × 10^5) and CD25+ B220+ splenocytes (B220+SPL, ∼20 × 10^5). Depletion was achieved using biotin-conjugated Abs and streptavidin-conjugated magnetic beads, and then passed twice through an AutoMACS cell sorter (Miltenyi Biotec). Purity of depletion was >99%. For experiments using positive selection, CD4+conjugated beads were used. Purity of the CD4+ fraction exceeded 95%. For adoptive transfer experiments, as CD25 becomes expressed on both conventional and regulatory T cells, we used CD103−CD4+ T cells sorted first by depleting CD103 via biotinylated Ab, then enriched CD4+ T cells using anti-CD4 beads. CD4+ T cell purity exceeded 95%. The assessment and scoring of clinical cutaneous GVHD were described in our previous publication (25). In brief, the evaluation was based on the area of alopecia as follows: 0.5, skin ulceration but no hair loss; 3, skin ulcers with alopecia <1 cm² in area; 2, skin ulcer with alopecia 1–3 cm²; 3, skin ulcer with alopecia >15% body area; and 4, skin ulcer with alopecia >30% body area.

Abs, flow cytometry analysis, and cell sorting

FITC CD5.1 (H11-86.1), allophycocyanin CD62L (MEL-14), PE Cy7-Sca-1 (D7), PE CD40 (3/23), PE CD80 (16-10A1), PE CD86 (GL1), PE CD40L (MR1), PE CD28 (37.51), and PE streptavidin were purchased from BD Pharmingen (San Diego, CA). PE CD45 (RM4-5), PE CD44 (IM7), eFluor780 TCRB (H57-597), allophycocyanin IFN-γ (XMG1.2), PE IL-4 (11B11), PE IL-5 (TRFK5), PE IL-13 (eBio13A), PE IL-17A (eBio17B), allophycocyanin Foxp3 (FJK-16S), AlexaFlour780 B220 (RA3-6B2), PE OX40 (OX-86), and biotin CD25 (eBio7D4) were purchased from eBioscience (San Diego, CA). Biotin-PNA was purchased from Vector Laboratories (Burlingame, CA). Aqua fluorescent reactive dye for viability analysis was purchased from Invitrogen (Carlsbad, CA). Flow cytometric data were analyzed with FlowJo software (Tree Star, Ashland, OR), as described in our previous publications (24, 25, 27).

Proliferation assays

Proliferating CD4+ T cells were measured, as previously reported (25). Briefly, sorted CD4+ T cells (2 × 10^6) were incubated with irradiated (50 Gy) donor bone marrow (BM) cells and a dose of splenocytes containing 5 × 10^6 CD4+ cells, including CD25+ splenocytes (SPL, ∼40 × 10^5) and CD25+ B220+ splenocytes (B220+SPL, ∼20 × 10^5). Depletion was achieved using biotin-conjugated Abs and streptavidin-conjugated magnetic beads, and then passed twice through an AutoMACS cell sorter (Miltenyi Biotec). Purity of depletion was >99%. For experiments using positive selection, CD4+conjugated beads were used. Purity of the CD4+ fraction exceeded 95%. For adoptive transfer experiments, as CD25 becomes expressed on both conventional and regulatory T cells, we used CD103−CD4+ T cells sorted first by depleting CD103 via biotinylated Ab, then enriched CD4+ T cells using anti-CD4 beads. CD4+ T cell purity exceeded 95%. The assessment and scoring of clinical cutaneous GVHD were described in our previous publication (25). In brief, the evaluation was based on the area of alopecia as follows: 0.5, skin ulceration but no hair loss; 3, skin ulcers with alopecia <1 cm² in area; 2, skin ulcer with alopecia 1–3 cm²; 3, skin ulcer with alopecia >15% body area; and 4, skin ulcer with alopecia >30% body area.

Tissue collection for cellular analysis

Mice were killed using CO2 asphyxiation, and their spleens, skin, and lungs were collected for lymphocyte analysis. Spleens were washed using a 70-μm filter. Skin samples were cut into small (<0.5 cm²) pieces, and digested with 5 mg/ml collagenase A (Sigma, Aldrich, Carlsbad, CA) and 5 mg/ml hyaluronidase (Sigma-Aldrich) in complete RPMI 1640 media on a shaker at 37°C for 2 h, then treated with PBS containing 2% BSA and EDTA, and mashed through a 70-μm filter, and lymphocytes were separated using Lympholyte-M (Burlington, Ontario, Canada). Lung samples were thoroughly flushed of blood by injection of 5–10 ml PBS into the heart and collected in 2% BSA with heparin. Lung samples were then infused with collagenase and DNase (Worthington, Lakewood, NJ), and lung media for 2 h, then treated with PBS containing 2% BSA and EDTA, and mashed through a 70-μm filter, and lymphocytes were separated using Lympholyte-M (Burlington).

Histopathology

Tissue specimens were fixed in formalin before embedding in paraffin blocks, cut, and stained with H&E. Slides were examined at original magnification ×200–400 and visualized with an Olympus and a Pixera (600CL) cooled charge-coupled device camera (Pixera, Los Gatos, CA). Tissue damage was blindly assessed on a scoring system described previously (25). In brief, skin tissue GVHD was scored on the basis of tissue damage in the epidermis, dermis, and loss of s.c. fat; the maximum score is 9. Lung tissue was blindly evaluated on a scoring system accounting for perivascular inflammation and infiltration, and peribronchial infiltration and inflammation; the maximum score is 15. Salivary gland tissue GVHD was scored on mononuclear cell infiltration and structural disruption, as described previously (25), with a maximum score of 8. Briefly, the degree of inflammatory infiltrates was graded, as follows: grade 1, 1–5 foci of mononuclear cells were seen (>20 cells per focus); grade 2, >5 foci of mononuclear cells were seen but without significant parenchymal destruction; grade 3, multiple confluent foci were seen, with moderate degeneration of parenchymal tissue; grade 4, extensive parenchymal destruction of the gland with mononuclear cells and extensive parenchymal destruction were seen. Structural and follicular disruption was graded from 0 to 4, with 0 indicating normal structure, and scores of 1–4 indicating minor, moderate, major, and total disruption of normal structure, respectively. Colon GVHD histopathology was evaluated for increased mononuclear cell infiltration and morphological aberrations (e.g., hyperplasia and crypt loss), with a maximum score of 10.

TCR spectratyping

TCR spectratyping was performed, as previously reported (25). Briefly, paired groups of experimental mice were sacrificed 15 d after HCT, concurrently with healthy DBA/2 control mice. CD4+ T cells were sorted using CD4 beads (Miltenyi Biotec), with purity >98%, and lysed in TRIzol. RNA was purified, and cDNA was generated and amplified in a series of semiquantitative PCR assays using a library of murine Vβ primers (26). We then compared the relative area under each peak in the maximum score is 15. Salivary gland tissue GVHD was scored on mononuclear cell infiltration and structural disruption, as described previously (25), with a maximum score of 8. Briefly, the degree of inflammatory infiltrates was graded, as follows: grade 1, 1–5 foci of mononuclear cells were seen (>20 cells per focus); grade 2, >5 foci of mononuclear cells were seen but without significant parenchymal destruction; grade 3, multiple confluent foci were seen, with moderate degeneration of parenchymal tissue; grade 4, extensive parenchymal destruction of the gland with mononuclear cells and extensive parenchymal destruction were seen. Structural and follicular disruption was graded from 0 to 4, with 0 indicating normal structure, and scores of 1–4 indicating minor, moderate, major, and total disruption of normal structure, respectively. Colon GVHD histopathology was evaluated for increased mononuclear cell infiltration and morphological aberrations (e.g., hyperplasia and crypt loss), with a maximum score of 10.

Statistical analysis

Clinical cutaneous damage scoring and survival in different groups were compared by using the log-rank test (Prism, version 5.0; GraphPad Software, San Diego, CA). Comparison of two means was analyzed using an unpaired two-tailed Student t test. Comparison between curves was made by two-way ANOVA. Increases in skewing for TCR spectratyping were compared using a χ^2 test from a 2 × 2 contingency table.

Results

Donor B cells in transplants facilitated the persistence of cGVHD tissue damage in the lung and skin and initiated tissue damage in the salivary gland

We reported that, in an autoimmune-like cGVHD model using DBA/2 donors and BALB/c recipients, donor CD4+ T and B cells were both required to mediate disease pathogenesis (24). It is not yet clear how donor B cells in transplants contribute to cGVHD pathogenesis. We first determined how donor B cells in transplants influenced the course of autoimmune-like cGVHD histopathology development. Because the skin, lung, and salivary gland were...
reported to be among the most common cGVHD targets (3, 4, 29, 30), we kinetically compared tissue damage in those organs. As T regulatory cells can suppress cGVHD induction in this model, especially in the skin (25), sublethally total body irradiation (TBI)-conditioned BALB/c recipients were injected with CD25+ cell-depleted spleen cells (SPL, $\sim 40 \times 10^6$) or CD25+ and B220+ cell-depleted spleen cells (B220-SP, $\sim 20 \times 10^6$), which contained an equivalent number of CD4+ T cells ($5 \times 10^6$) and other non-B cells. Chimerism and histopathology scores were compared 5, 10, 15, 25, and 40 d post-HCT. Donor B cells had no impact on kinetics of chimerism development, and all recipients became nearly complete chimeras by day 15 after HCT (Supplemental Fig. 1A).

Skin tissue damage was evaluated by epidermal hyperplasia, dermal infiltration, hair follicle loss, and dermal fat loss. As shown in Fig. 1A and 1B, 5–15 d post-HCT, severe dermal infiltration was observed; by 15 d post-HCT, epidermal hyperplasia occurred. There was no significant difference between recipients given SPL with B cells or B220-SP without B cells in this early acute phase of GVHD. Interestingly, thereafter, the inflammation in skin tissue of the recipients without donor B cells rapidly subsided and became nearly normal by 40 d post-HCT. In contrast, in the recipients with donor B cells, epidermal hyperplasia, dermal infiltration, hair follicle loss, and dermal fat loss persisted, although there was some reduction in dermal infiltration at later time points. Additionally, hair follicles gradually disappeared. Thus, the overall GVHD histopathology score was 6-fold higher than that of recipients without donor B cells ($p < 0.01$), which was comparable to the recipients given T cell-depleted spleen cells by 40 d post-HCT (Supplemental Fig. 1C).

Furthermore, 40 d after HCT, the skin tissues of recipients with donor B cells showed severe collagen deposition as compared with recipients without donor B cells (Supplemental Fig. 1B). These results indicate that donor B cells in transplants augment cGVHD skin pathogenesis.

Lung tissue was evaluated by examining perivascular and peribronchiolar infiltration. As shown in Fig. 1C and 1D, in the recipients given no donor B cells, there was only mild infiltration in the lung tissue through the whole period from 5 to 40 d post-HCT. In contrast, in the recipients given donor B cells, although infiltration in lung tissue was mild 5–10 d after HCT, the infiltration increased with time. By 40 d post-HCT, the overall lung

![FIGURE 1.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)
tissue histopathology score was 4-fold higher than that of the recipients given no donor B cells \((p < 0.01)\), which was comparable to the recipients given T cell-depleted spleen (Supplemental Fig. 1C). These results indicate that donor B cells in transplants augment cGVHD lung tissue damage.

Salivary gland tissue damage was evaluated by examining mononuclear cell infiltration, disruption of normal duct structure, and loss of serous/mucinous fluids. As shown in Fig. 1E and 1F, in both groups, there was little tissue damage in the salivary gland 5–15 d post-HCT. In contrast, the salivary gland tissue damage in the recipients receiving donor B cells gradually increased 15–40 d post-HCT and became very severe by day 40, but the salivary gland of the recipients receiving no donor B cells exhibited little damage. The tissue damage score in the former was 5-fold greater than the latter \((p < 0.01)\), which was comparable to the recipients given T cell-depleted spleen (Supplemental Fig. 1C). These results indicate that donor B cells in transplants significantly augment induction of salivary gland tissue damage.

We also studied whether donor B cells augmented aGVHD pathogenesis by examining the targets more classically involved in aGVHD, such as colon. We found that the histopathology of colon of the recipients given SPL or B220\(^{-}\)−SPL cells was comparable at 15 and 40 d post-HCT (Supplemental Fig. 1D), suggesting that donor B cells in transplants do not have significant impact on aGVHD pathogenesis.

Taken together, the kinetic study of histopathology shows that donor B cells have little impact on aGVHD pathogenesis, but significantly increase persistent tissue damage in overlapping aGVHD and cGVHD target organs such as skin and lung; donor B cells are also critical for damaging cGVHD-specific target tissue salivary gland.

Donor CD4\(^{+}\) T cells in transplants augmented donor B cell activation and upregulation of costimulatory molecule expression in cGVHD recipients

Next, we dissected how donor CD4\(^{+}\) T and B cells in transplants interacted during cGVHD pathogenesis. First, we evaluated the impact of donor CD4\(^{+}\) T cells on donor B cell activation. Because DBA/2 donor cells also induced cGVHD in Rag-2\(^{-/-}\) BALB/c recipients, and the severity appeared to be comparable to cGVHD in wild-type (WT) BALB/c (Supplemental Fig. 2), we used Rag-2\(^{-/-}\) BALB/c recipients for the analysis to avoid any potential contamination by host B cells. Accordingly, sublethally irradiated Rag-2\(^{-/-}\) BALB/c recipients were injected with either DBA/2 donor spleen (SPL) or CD4\(^{+}\) T-depleted spleen (CD4\(^{-}\)-SPL) cells. Fifteen days post-HCT, donor B cell expression of costimulatory molecules (i.e., CD40, CD80, and CD86) and MHC II was compared, using healthy DBA/2 donor B cells as controls. At this time point, few de novo developed B cells were present, so we specifically evaluated the injected B cell population. We found that, although donor B cells showed little upregulation of those markers in recipients given no donor CD4\(^{+}\) T cells as compared with donor B cells before HCT, donor B cells markedly upregulated those markers in the presence of donor CD4\(^{+}\) T cells \((p < 0.01, \text{Fig. 2})\). These results indicate that donor CD4\(^{+}\) T cells in transplant play an important role in the initial activation of donor B cells.

Donor B cells in transplants mediated initial clonal expansion of the pathogenic autoreactive donor-type CD4\(^{+}\) T cells from the transplant

We recently reported that the alloimmune response was required for initiating clonal expansion of pathogenic donor CD4\(^{+}\) T cells in transplants that possess both donor and host reactivity in the autoimmune-like cGVHD recipients (25). Because activated B cells were proposed to be strong APCs in mediating autoreactive CD4\(^{+}\) T clonal expansion and epitope spreading in autoimmune mice (16, 31), and because donor B cells were activated early after HCT and depletion of donor B cells prevented induction of the disease (24), we evaluated the impact of donor B cells in transplants on clonal expansion within donor CD4\(^{+}\) T cell V\(\beta\) families. Eighty-seven of 176 total V\(\beta\) families were observed in 8 recipients given SPL cells, but only 57 of 176 in 8 recipients given B220\(^{-}\)-SPL cells exhibited skewing \((p < 0.01, \text{Fig. 3A, 3B})\). We also found that 13

![FIGURE 2. Donor B cells upregulated expression of costimulatory molecules upon interaction with donor CD4\(^{+}\) T cells in autoimmune-like GVHD recipients. Rag-2\(^{-/-}\) BALB/c recipients were irradiated (800 cGy, TBI) and transplanted with SPL or CD4\(^{-}\)-SPL cells. Fifteen days post-HCT, mice were sacrificed, and donor-type B220\(^{-}\)-CD19\(^{-}\) splenic B cells were analyzed for expression levels of costimulatory molecules and MHC II, as compared with DBA/2 donor splenic B cells before transplantation \((n = 8 \text{ from two independent experiments})\). Donor DBA/2 splenic B cells were assigned a relative mean fluorescence intensity value of 1 in each experiment and compared with mean fluorescence intensity of experimental mice in each experiment. Representative histograms and mean fluorescence intensity comparison summaries are shown. The \(p\) values indicate an increase in the SPL group compared with the CD4\(^{-}\)-SPL group. (A) CD40 expression levels were compared, \(p < 0.001\). (B) CD80 expression levels were compared, \(p < 0.001\). (C) CD86 expression levels were compared, \(p < 0.001\). (D) MHC II expression levels were compared, \(p < 0.001\).]
of 22 Vβ families in the former and only 8 of 22 Vβ families in the latter are skewed. These results indicate that B cells can augment clonal expansion of CD4+ T cell Vβ families.

It was of interest that donor B cells in transplants augmented clonal expansion in donor T cell Vβ families that should have been deleted by Mtv-mediated negative selection in the thymus of DBA/2 mice, including Vβ7, 8.1, 11, and 17 (33), indicating that under GVHD conditions, donor B cells are able to efficiently augment clonal expansion of residual autoreactive donor T cells in the periphery. Consistent with this hypothesis, the donor-type CD4+ T cells from recipients given donor B cells proliferated ~5-fold more in response to stimulation by syngeneic donor-type DCs and proliferated ~2-fold more in response to stimulation by allogeneic host-type DCs, as compared with the donor-type CD4+ T cells from recipients given no donor B cells (p < 0.01, Fig. 3C). These results indicate that donor B cells in transplants play an important role in augmenting the clonal expansion of cGVHD-pathogenic donor CD4+ T cells with both donor and host reactivity.

Donor B cells in transplants augmented donor CD4+ T cell IL-7Ra expression and reduced donor CD4+ T cell apoptosis in GVHD target tissues

B cells could potentially augment the survival of activated CD4+ T cells (34). We tested whether the persistent GVHD target tissue damage in the presence of donor B cells from the transplant was associated with reduced apoptosis of donor CD4+ T cells. As we had observed other qualitative changes 15 d post-HCT, at that same time point, CD4+ T cells in spleen, skin, and lung of recipients given SPL or B220–SPL cells were measured for apoptosis via annexin V staining. Indeed, CD4+ T cell apoptosis was significantly decreased in the spleen, skin, and lung of the recipients given donor B cells as compared with that of the recipients given no donor B cells, as judged by a significant reduction of annexin V staining (p < 0.01, Fig. 4A).

We then investigated how B cells provided donor CD4+ T cells with a survival advantage. Because PD-1 interaction with its ligand B7-1/H1 (PD-L1) induces activated T cell apoptosis (35–37), and because IL-7/IL-7Ra signaling augments effector T cell survival (38), besides augmenting naive T cell expansion, we compared the expression levels of PD-1 and IL-7Ra by donor CD4+ T cells from spleen, skin, and lung. We found almost all infiltrating T cells in the skin and lung were CD44highCD62Llow effector cells in both recipients with or without donor B cells (Supplemental Fig. 3A); however, there was little difference in PD-1 expression by donor CD4+ T cells (Supplemental Fig. 3B). B7-H1 expression by infiltrating macrophages and neutrophils was also similar (Supplemental Fig. 3C), suggesting that the PD-1/B7-H1 axis was not critical to their survival. Interestingly, donor effector CD4+ T cells from skin and lung tissues, especially the skin, of recipients receiving donor B cells expressed markedly more IL-7Ra, as compared with recipients receiving no donor B cells (p < 0.01, Fig. 4B). The IL-7Ra-high donor CD4+ T cells also had less apoptosis, as indicated by reduced annexin V staining (p < 0.01, Fig. 4C). These results indicate that the presence of donor B cells in transplants leads to increased donor CD4+ T cell effector cell expression of IL-7Ra expression compared with recipients not receiving B cells, subsequently reducing apoptosis of infiltrating activated donor CD4+ T cells in GVHD target tissues.

Donor B cells in transplant augmented donor CD4+ T cell differentiation into proinflammatory Th2 cells

Proinflammatory TNF-α–producing Th1, Th2, and Th17 have all been shown to mediate GVHD under different circumstances (39–41). Next, we tested whether donor B cells in transplants impact on donor CD4+ T cell differentiation into Th1, Th2, and Th17. Accordingly, 5, 10, 15, 25, and 40 d post-HCT, the donor CD4+ T cell cytokine profile in the spleen, skin, and lung of recipients with or without donor B cells was measured by intracellular staining. The IFN-γ–producing Th1 cells were the major Th subset in all tissues, and the percentage of Th1 cells in the recipients with or without donor B cells was not significantly different in all tissues at all time points (p > 0.1, Fig. 5A, Supplemental Fig. 4A).

IL-17–producing Th17 cells were rare in all cases, and largely similar between recipients with or without donor B cells, although somewhat enriched in the lung of recipients without donor B cells as compared with recipients with donor B cells (p > 0.1, Fig. 5B, Supplemental Fig. 4B). This change was not correlated to lung cGVHD pathogenesis, as described in Fig. 1.

In contrast, the percentage of IL-4–producing Th2 cells was significantly increased in recipients with donor B cells as compared with recipients without donor B cells, especially 40 d post-HCT (p < 0.01, Fig. 5C, Supplemental Fig. 4C), which correlated

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Donor B cells mediate clonal expansion of donor-type CD4+ T cells with both donor and host reactivity. BALB/c recipients were irradiated (800 cGy) and transplanted with SPL or B220–SPL cells. (A) Fifteen days post-HCT, recipients were sacrificed and the splenic CD4+ T cells were harvested and evaluated for clonal expansion via observations of skewed TCR spectra as compared with donor DBA/2 CD4+ T spectra before HCT. Example unskewed or skewed spectra are shown, with skewed peaks filled with black (n = 8 for experimental samples, n = 4 for controls). (B) Summary of TCR spectratype skewing: 22 Vβ in each mouse were analyzed; there were 87 of 176 total Vβ skewed in 8 recipients given SPL cells versus 57 of 176 total Vβ skewed in 8 recipients given B220–SPL cells, p < 0.01. +, Indicates ≥3 of 8 recipients exhibited skewing for that Vβ. (C) Fifteen days post-HCT, recipients were sacrificed and the splenic donor-type CD4+ T cells were harvested and stimulated with irradiated DBA/2 or BALB/c dendritic cells and evaluated for their proliferation using [3H]-TdR incorporation assay. Stimulating index is shown (n = 5, p < 0.01 for both).
Donor B cells increased expression of IL-7Ra, but decreased apoptosis on donor CD4+ T cells. BALB/c recipients were irradiated (800 cGy) and transplanted with SPL or B220-SPL cells. Fifteen days post-HCT, donor CD4+ T cells from the spleen, skin, and lung were evaluated for apoptosis by annexin V staining and for expression of IL-7Ra, evaluating positive cells by contour or histogram shoulder. One representative is shown from two to three replicate experiments, n = 8–12. (A) Donor CD4+ T cells are shown in histograms of annexin V staining. A higher percentage of annexin V+ CD4+ T cells was observed in the B220-SPL group in all organs (spleen, 23.6 ± 1.9 versus 17.9 ± 0.9, p < 0.05; skin, 28.7 ± 3.7 versus 12.9 ± 1.3, p < 0.01; lung, 26.8 ± 5.1 versus 14.0 ± 2.2, p < 0.05). (B) Donor CD4+ T cells are shown in histograms of IL-7Ra expression. A higher percentage of IL-7Raexp+ CD4+ T cells was observed in the SPL group in all organs (spleen, 37.2 ± 5.5 versus 21.3 ± 3.7, p < 0.05; skin, 61.62 ± 3.8 versus 34.9 ± 3.2, p < 0.001; lung, 48.5 ± 2.8 versus 29.4 ± 1.5, p < 0.001). (C) Spleen-, skin-, and lung-infiltrating donor CD4+ T cells from SPL recipients were gated on IL-7Raexp or IL-7Rahop populations and then shown in histograms of annexin V staining. The percentage of annexin V+ cells determined with contours/shoulders that show difference between the two comparing groups, IL-7Raexp group versus IL-7Rahop group, are spleen, 28.8 ± 3.3 versus 16.5 ± 1.5, p < 0.01; skin, 14.1 ± 1.8 versus 8.9 ± 0.6, p < 0.05; lung, 14.7 ± 1.0 versus 11.4 ± 0.6, p < 0.05.

with increased signs of cGVHD, as described in Fig. 1. Additionally, the Th2 cells were largely TNF-α–producing cells, although the percentage of TNF-α+ cells was similar in recipients with or without donor B cells (p < 0.1, Supplemental Fig. 4D). We previously demonstrated that TNF-α-producing Th2 cells were important mediators of lung GVHD (39). It is interesting that their presence in this model is also correlated with increased skin and salivary gland damage. These results indicate that donor B cells in transplants mainly augment proinflammatory Th2 differentiation in the autoimmune-like cGVHD recipients.

Donor B cells in transplants augmented the expansion of donor-type CD4+ T cells in GVHD target tissues and augmented the development of GVHD

Next, we asked whether these qualitative changes would lead to quantitative changes in the donor CD4+ T cell population during cGVHD pathogenesis. Therefore, we used in vivo bioluminescent imaging (BLI) to visualize the expansion of the injected donor-type CD4+ T cells in recipients given transplants with or without donor B cells. Sorted CD25−CD4+ T cells (5 × 106) from luciferase-transgenic (luc+) DBA/2 donors were coinjected with TBCD-SPL cells (5 × 106) with or without donor B220+ cells (10 × 106) into sublethally irradiated BALB/c recipients. Thereafter, in vivo BLI was performed 5, 10, 15, 20, 25, 30, and 40 d post-HCT to measure donor CD4+ T expansion. We found that, although there was no significant difference in donor CD4+ T expansion in the presence or absence of donor B cells by 10 d post-HCT, donor CD4+ T expansion in the presence of donor B cells was significantly more vigorous by 15–20 d after HCT, as reflected by the in vivo BLI intensity; the donor CD4+ T expansion persisted for >40 d in surviving recipients (Fig. 6A, 6B). Correspondingly, the cutaneous GVHD score in the latter gradually increased and reached a severity level that was ~10-fold higher in recipients receiving donor B cells than in recipients receiving no donor B cells by 40 d post-HCT (p < 0.01, Fig. 6B, 6C).

To further identify the location of donor CD4+ T cell expansion, we kinetically numerated the infiltrating donor CD4+ T cells and total B cells in the spleen, skin, and lung at 5, 10, 15, 25, and 40 d after HCT. There was no difference in the yield of donor CD4+ T cells in the spleen of recipients with or without donor B cells over the course (Fig. 6D). However, the donor CD4+ T cell yield was significantly higher in the skin and lung tissues of the recipients with donor B cells as compared with recipients without donor B cells 15–40 d post-HCT (p < 0.01, Fig. 6E, 6F). This is consistent with stronger BLI and more severe clinical GVHD in the recipients with donor B cells, as shown in Fig. 6B and 6C.

The changes of B cell percentage and yield were different from that of CD4+ T cells. As shown in Supplemental Fig. 4E, there were few (<0.8% or 0.1 × 106) B220+ B cells in the spleen of recipients without injection of donor B cells by 5–15 d after HCT, but donor B cells gradually increased 25 d after HCT; the percentage reached ~43%, and the yield reached ~3 × 106 by 40 d after HCT. In contrast, there were significantly more B cells in the spleen of recipients with injection of donor B cells 5–15 d after HCT; the percentage reached ~18%, and the yield...
reached $\sim1.5 \times 10^6$ ($p < 0.01$). However, the percentage and yield significantly declined 25 d after HCT. The percentage was only $\sim2.4\%$, and the yield was $\sim0.2 \times 10^6$ by 40 d after HCT ($p < 0.01$). We also observed little B cell infiltration ($<0.5\%$) in the skin and lung tissues in either group 5–40 d after HCT (data not shown). These results suggest that the interaction between injected donor CD4$^+$ T and B cells mainly takes place early after HCT in the lymphoid tissues.

Additionally, we observed that the persistence of inflammation in the skin tissues of recipients was associated with an increase of donor CD4$^+$ T cell infiltration in the skin tissues. Five to 15 d after HCT, skin infiltration consisted of mainly CD11b$^+$Gr-1$^+$ macrophages and neutrophils, which was markedly reduced in both groups by 25 d post-HCT (Supplemental Fig. 4F). However, the presence of donor B cells in transplant resulted in a sharp increase of donor CD4$^+$ T cells in the tissue by 15 d after HCT, and the declination of donor CD4$^+$ T cells in the skin tissue was much slower than the decline of macrophages and neutrophils (Fig. 6E, Supplemental Fig. 4F) and was associated with significantly stronger GVHD. These results indicate that donor B cells in transplants augment donor CD4$^+$ T cell infiltration and expansion in GVHD target tissues (i.e., skin) and support the persistence of tissue inflammation.

Sorted donor-type CD4$^+$ T cells from primary recipients with or without donor B cells in transplant showed a marked difference in cGVHD-inducing capacity

Next, we tested the cGVHD-inducing capacity of donor-type CD4$^+$ T cells with or without prior interaction with donor B cells in transplants in the adoptive recipients. As we observed few donor B cells late after HCT in SPL recipients, but still observed strong GVHD (Fig. 1, Supplemental Fig. 4E), we hypothesized that the continued presence of B cells may not be necessary after the initial expansion of pathogenic CD4$^+$ T cells. Accordingly, 15 d post-HCT, sorted donor-type CD4$^+$ T cells ($5 \times 10^6$, purity $>98\%$) from spleens of primary BALB/c recipients with or without donor B cells were injected into sublethally irradiated secondary BALB/c donors. We observed that donor-type CD4$^+$ T cells from primary recipients with donor B cells (B-interacted CD4$^+$ T) induced cutaneous GVHD starting 20 d after cell transfer, reaching a plateau 30 d after cell transfer. In contrast, donor-type CD4$^+$ T cells from primary recipients without donor B cells (non-B–interacted CD4$^+$ T) induced little cutaneous GVHD ($p < 0.01$, Fig. 7A).

Forty days after cell transfer, histopathology of the lung, skin, and salivary gland tissues was evaluated. We found that B-interacted CD4$^+$ T cells induced significantly more tissue dam-
age, especially in the skin and salivary gland, compared with the non-B–interacted CD4+ T cells (Fig. 7B). The histopathology score difference between the two groups varied from ∼2- to 4-fold (p < 0.01, Fig. 7C). Whereas the histopathology score of skin and salivary gland was comparable to that of primary recipients on day 40 after HCT, there was an apparent decrease in the lung in the secondary recipients (Figs. 1, 7C). The latter observation is consistent with a recent report that Abs may be important for lung cGVHD (18), as no B cells or plasma cells were transferred.

We also evaluated the total donor CD4+ T yield and the percentage of Th1, Th2, and Th17 subsets in the spleen, lung, and skin of recipients. The yield of donor-type CD4+ T cells in the spleen and lung of recipients given B-interacted or non-B–interacted CD4+ T cells was not significantly different (p > 0.1). The skin yield was >6-fold higher in the B-interacted CD4+ T recipients (p < 0.01, Fig. 7D). A plurality of CD4+ T cells was Th1 in both groups. The percentage of Th1 cells in the spleen and skin in recipients given B-interacted CD4+ T cells was 3- to 5-fold higher than that in recipients given non-B–interacted CD4+ T cells (p < 0.01, Fig. 7E), but the difference in the lung was not significant (p > 0.1, Fig. 7E). The percentage of Th2 cells and Th17 cells in the spleen, lung, and skin in recipients given B-interacted CD4+ T cells was all significantly higher than in the tissues of recipients given non-B–interacted CD4+ T cells (p < 0.01, Fig. 7F, 7G). These results further support our notion that donor B cells in transplants can augment the generation and expansion of pathogenic donor CD4+ T subsets early after HCT.

Discussion
We have demonstrated that, in a murine autoimmune-like cGVHD model of DBA/2 donor and BALB/c recipient, donor B cells augmented the persistence of GVHD tissue damage in overlapping target organs of aGVHD and cGVHD such as the lung and skin; donor B cells also augmented tissue damage in exclusive cGVHD target organs such as the salivary gland. The mechanisms whereby donor B cells in transplants contribute to pathogenesis of autoimmune-like cGVHD include the following: 1) augmenting the clonal expansion of the residual autoreactive donor CD4+ T cells in transplants; 2) augmenting donor CD4+ T differentiation into proinflammatory Th2 cells; and 3) augmenting donor CD4+ T cell expression of IL-7Rα, survival, and expansion in GVHD target tissues.

We previously showed that, in the autoimmune-like cGVHD model of DBA/2 donor and BALB/c recipient, tissue damage occurred in several target organs, including the skin, lung, and salivary gland by late post-HCT (25). However, the kinetics of disease pathogenesis of these target organs remains unknown. The skin and

![FIGURE 6. Donor B cells in transplants augmented donor-type CD4+ T cell expansion in GVHD target tissues. (A–C) BALB/c mice were irradiated (800 cGy) and injected with 5 × 10^6 CD4+CD25− splenocytes from luciferase transgenic (luc+) DBA/2 mice and WT DBA/2 TBCD SPL (5 × 10^6), with or without WT DBA/2 B220+ B cells (10 × 10^6), then monitored for expansion of donor CD4+ T cells and signs of GVHD. (A) CD4+ T cell expansion in mice was evaluated with BLI. One representative BLI pattern is shown per group per time point (n = 8 from two replicate experiments). (B) BLI intensity in terms of photons/s. A summary curve (mean ± SE) is shown. Mice receiving B cells had increased luminescence (two-way ANOVA, p < 0.01). (C) Recipients of luc+CD4+ T cells with or without B cells were evaluated after HCT for GVHD-related skin damage and hair loss. Recipients of B cells had increased clinical cutaneous scores (two-way ANOVA, p < 0.01). (D–F) BALB/c mice were irradiated (800 cGy) and injected with either SPL or B220−SPL and sacrificed 5, 10, 15, 25, and 40 d post-HCT, and their tissues were harvested and evaluated for the presence of infiltrating donor CD5.1+CD4+ T cells. Mean ± SE is shown at each time point, n = 4–8 from 2–3 replicate experiments. (D) Donor CD4+ T cell yield in the spleen was evaluated, and was not significantly different between SPL and B220−SPL groups (two-way ANOVA, p > 0.1). (E) Donor CD4+ T cell yield in the skin was evaluated. A higher yield was observed in SPL recipients (two-way ANOVA, p < 0.01). (F) Donor CD4+ T cell yield in the lung was evaluated. A higher yield was observed in SPL recipients (two-way ANOVA, p < 0.01).]
lung are target organs for both aGVHD and cGVHD (1–4). We found that, in the skin, donor B cells in transplant had no impact on the severity of dermal infiltration during the early acute phase. The early infiltrating cells were primarily macrophages and neutrophils, and these cells were incapable of persisting as time went on. However, donor B cells had marked impact on the severity of skin tissue damage late after HCT, which manifested with epidermal hyperplasia, loss of s.c. fat, loss of hair follicles, and dermal collagen deposition. This persistent skin tissue damage was strongly associated with the infiltration of donor CD4+ T cells, which was markedly augmented by donor B cells in transplants. Similarly, the increased donor-type CD4+ T cell infiltration in the lung and salivary gland tissues of recipients receiving donor B cells was associated with the GVHD tissue damage in those targets late after HCT. Therefore, donor B cells in transplants augment donor CD4+ T infiltration and cGVHD tissue pathogenesis in the aGVHD and cGVHD overlapping target organs skin and lung, as well as in the prototypical cGVHD target organ salivary gland. This augmentation may result from donor B cell expansion of autoreactive T cells that recognize Ags expressed by target tissues.

FIGURE 7. Donor CD4+ T cells from primary recipients receiving donor B cells in transplants mediate cGVHD in secondary recipients. BALB/c mice were irradiated and given either SPL or B220−SPL cells. Fifteen days post-HCT, recipients were sacrificed and their spleens and peripheral lymph nodes were harvested, and donor CD4+ T cells were sorted from them. To ensure equal doses of effector CD4+ T cells, CD103− T cells were depleted. CD103−CD4+ T cells (5 × 106) from the spleen and lymph node of primary recipients along with TBCD BM (2.5 × 106) from healthy DBA/2 donors were injected into sublethally irradiated BALB/c recipients, and the recipients were monitored for GVHD development. (A) Secondary recipients were kinetically evaluated for cutaneous cGVHD. Increased cutaneous damage was observed in secondary recipients of CD4+ T cells from primary recipients given donor B cells (B-Int-CD4) as compared with recipients of CD4+ T cells from primary recipients given no donor B cell (B-Non-Int-CD4) recipients (two-way ANOVA, p < 0.01, n = 8 from two replicate experiments). (B) and (C) Forty days post-HCT, secondary recipients were sacrificed, and their lung, skin, and salivary glands were evaluated histopathologically. Arrows indicate signs of infiltration or damage (original magnification ×200). (C) Mean ± SE of histopathology scores are shown (n = 4–6 from two replicate experiments). Increased damage was observed in recipients of B-Int-CD4 as compared with B-Non-Int-CD4 (p < 0.05 for lung, p < 0.01 for skin and salivary). (D–G) Forty days post-HCT, recipients were monitored for GVHD development. (D) Yield of donor CD4+ T cells. No significant difference was found in the spleen or lung (p > 0.05), but significantly more CD4+ T cells infiltrated the skin of recipients of B-Int-CD4 as compared with B-Non-Int-CD4 (p < 0.01). (E) Percentage of IFN-γ+ cells among donor CD4+ T cells. A higher percentage of IFN-γ+ cells was observed in recipients of B-Int-CD4 in the spleen and skin (p < 0.01), but not the lung (p > 0.05). (F) Percentage of IL-4+ cells among donor CD4+ T cells. A higher percentage of IL-4+ cells was observed in recipients of B-Int-CD4 in the spleen and skin (p < 0.01), as well as the lung (p < 0.05). (G) Percentage of IL-17+ cells among donor CD4+ T cells. A higher percentage of IL-17+ cells was observed in recipients of B-Int-CD4 in the spleen, lung, and skin (p < 0.01).
We have recently reported that donor-type CD4+ T cell clones are expanded in tumors during alloimmune response (25). In the current studies, donor B cells were found to mediate the clonal expansion of these pathogenic CD4+ T cell clones. We have also shown that donor and host reactivity, as judged by TCR spectratyping and MLR. Our finding that additional families of Vβ clones are expanded in SPL recipients as compared with B220-SPL recipients may also explain why GVHD target organs experience additional CD4+ T cell infiltration in the former group, as they may correspond to clones that mediate organ-specific damage. Unfortunately, we found it too technically challenging to acquire adequate tissue-infiltrating cells from patients with cGVHD, and these T cells derived from donor CD4+ T cells in cGVHD recipients, as de novo-developed cells are not present. Our studies address how mature B cells in transplants and de novo-developed donor B cells influence the activation and expansion of de novo-developed autoreactive T cells in cGVHD recipients.

The qualitative impact of donor B cells in transplants on donor CD4+ T cell clonal differentiation, ability of survival and expansion, as well as ability to mediate cGVHD was confirmed by observations in the secondary adoptive recipients. Whereas donor CD4+ T cells from the primary recipients given no donor B cells expanded weakly and induced only a little tissue damage in the adoptive recipients, the donor CD4+ T cells from the primary recipients given donor B cells induced severe tissue damage in the skin and salivary gland in association with expansion of donor CD4+ T subsets in the tissues. Interestingly, increased percentages of Th1, Th2, and Th17 cells were observed in the secondary recipients given CD4+ T cells exposed to B cells in primary recipients, whereas in those primary recipients, only Th2 cells were significantly expanded. This discrepancy is not yet fully understood. Our most recent preliminary data showed that MHC-II knockout donor B cells failed to augment cGVHD, and IL-4 knockout donor B cells still partially augmented cGVHD (J. Young and D. Zeng, unpublished observations). Furthermore, our data demonstrate that donor B cells augmented donor CD4+ T cell expression of IL-7Rα and increased their survival. These data suggest that donor B cells, acting as APCs, not only augment Th2 differentiation, but also improve the survival capacity of other Th subsets. Therefore, besides Th2 cells, Th1 and Th17 cells from primary recipients given donor B cells could also display superior expansion in secondary recipients that have TBI-induced lymphopenia. This observation may be of clinical importance, as it demonstrates that early B cell interaction may provide donor CD4+ T cells with the means to rapidly re-expand from low numbers, as after withdrawal from immunosuppression.

In summary, we propose that donor B cells in transplants augment the pathogenesis of cGVHD in multiple steps. Allorreactive donor CD4+ T cells are first activated by professional host APCs, which then activate autoreactive donor B cells that present alloantigens. Upon activation, these autoreactive B cells then work as potent APCs to mediate initial activation and clonal expansion of the autoreactive CD4+ T cells as well as augment differentiation of Th2 cells. The activated autoreactive B cells also augment the pathogenic CD4+ T cells that possess both allo- and autoreactivity with increased expression of IL-7Rα, leading to their increased survival in GVHD target tissues. Subsequently, the infiltrating pathogenic CD4+ T cells mediate the transition from cGVHD to cGVHD tissue damage in the overlapping target organs such as the skin and lung, as well as initiate tissue damage in cGVHD prototypical organs such as the salivary gland. Additionally, Abs from donor B cells may also augment some cGVHD tissue damage, especially in the lung, as suggested previously (18).

Our observations provide important insights into human cGVHD pathogenesis. It is known that administration of B cell-depleting anti-CD20 mAb (rituximab) is effective in treating cGVHD in some patients, but others have only a partial response or no response (50, 51). The ineffectiveness of anti-CD20 treatment may be due to the fact that cGVHD is mainly mediated by effector T and B cells, and effector lymphocytes are less sensitive to depletion by Ab treatment (50, 51). However, we raise another important possibility, as follows: once pathogenic CD4+ T cells are generated and expanded by donor B cells, depleting the donor B cells may no longer have a significant impact on the pathogenic activity of the CD4+ T cells, as we observed that purified donor CD4+ T cells from primary recipients with donor B cells were able to induce cGVHD in adoptive recipients, although CD4+ T cells from the recipients without donor B cells could not. Our studies...
indicate that removal of donor B cells from transplants before HCT or early administration of depleting anti-CD20 prior to pathogenic CD4+ T cell clonal expansion or prior to GVHD onset may be more effective in preventing cGVHD than in treating cGVHD after disease onset.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure Legends

Supplementary Figure 1. BALB/c recipients rapidly became complete chimeras, and while T cells were required for induction of cGVHD in all organs, B cells augmented cGVHD but not aGVHD. BALB/c mice were given 800 cGy irradiation and transplanted with either SPL or B220⁻-SPL. A. 5, 10, 15, 25, and 40d post-HCT, mice were sacrificed and their splenocytes were analyzed for the presence of donor T cells using CD5.1⁺TCRβ⁺ to identify donor T cells. Mean±SE is shown at each time point for two replicate experiments (n=4). No difference was observed between the two groups (p>0.1). B. 40d post-HCT, SPL and B220⁻-SPL recipient mice were sacrificed and their skin harvested and stained with trichrome to examine collagen deposition (n=4 from 2 replicate experiments). One representative per group is shown. C. 40d post-HCT, T cell-depleted recipient mice were sacrificed and their skin, lung, salivary glands, and colon were harvested and stained with H&E (n = 4 from 2 replicate experiments). One representative per group is shown. D. 15d and 40d post-HCT, SPL, B220⁻-SPL, and TCD SPL recipient mice were sacrificed and their colon harvested and stained with H&E (n = 4 from 2 replicate experiments). One representative per group is shown. GVHD tissue damage score is shown as mean ± SE. No difference between groups were observed (p>0.1).

Supplementary Figure 2. Donor but not host CD4⁺ T and B cells were required for induction of autoimmune-like GVHD. Wild-type or Rag-2⁻/⁻ BALB/c mice were irradiated (800 cGy) and transplanted with SPL, CD4⁻-SPL, or B220⁻-SPL cells from DBA/2 donors and monitored for clinical signs of GVHD and survival for up to 100 days post-HCT. At the peak GVHD time point (~d40), skin, salivary gland, and lung histopathology was evaluated. Data is
combined from 2-3 replicate experiments (n=8-12). A. Clinical cutaneous GVHD score. SPL score was higher than B220⁻SPL score in both groups (p<0.01) B. Survival curve of recipients. SPL recipients exhibited higher mortality than other groups (p<0.01) C. Histopathology score of skin, salivary gland, and lung 40d post-HCT (n=4-6). SPL score was higher than B220⁻SPL and CD4⁻SPL score in all groups (p<0.01)

Supplementary Figure 3. Donor B cells in transplants did not affect PD-1 expression on donor effector CD4⁺ T cells nor B7-H1 expression on donor macrophages. BALB/c mice were given 800 cGy irradiation and transplanted with either SPL or B220⁻SPL. 15d post-HCT, mice were sacrificed and their spleen, skin, and lungs were harvested. n=8 from 2 replicate experiments. A. Donor CD4⁺CD5.1⁺ T cells from SPL recipients were evaluated for expression of CD44 and CD62L. One representative is shown, with mean±SE for the CD44hiCD62Llo effector memory and CD44hiCD62Lhi central memory subsets shown. B. Infiltrating donor CD4⁺ T cells were evaluated for expression of PD-1. No difference in MFI was detected between the two groups (p>0.1). C. Gated B220⁻TCRβ⁻CD11b⁻Gr-1⁺ macrophages/neutrophils were evaluated for B7-H1 expression. No difference in MFI was detected between the two groups (p>0.1).

Supplementary Figure 4. Presence of donor B cells in the spleen augmented proinflammatory Th2 differentiation, and did not affect macrophage/neutrophil infiltration of the skin. BALB/c mice were irradiated and transplanted with either SPL or B220⁻SPL. 40d post-HCT, mice were sacrificed and their spleen-, skin-, and lung-infiltrating donor CD4⁺ T cells were evaluated for cytokine expression (n=6-8 from four replicate
A. The percentage of IFNγ+ cells was evaluated. One representative pattern per organ per group is shown. B. The percentage of IL-4+ cells was evaluated. One representative pattern per organ per group is shown. C. The percentage of IL-17+ cells was evaluated. One representative pattern per organ per group is shown. D. Skin-infiltrating CD4+ T cells were gated on the IL-4+ population and then evaluated for TNF-α production. One representative pattern per group is shown. E. 5, 10, 15, 25, and 40 days post-HCT, mice were sacrificed and their spleens were harvested and analyzed for percentage and yield of B220+ B cells. Mean ± SE of yield at the indicated time points is shown. The percentage of B220+ cells among splenic mononuclear cells was measured. One representative staining pattern 15d and 40d post-HCT for each group are shown for 4 replicate experiments. F. 5, 10, 15, 25, and 40d post-HCT, mice were sacrificed and their skin was harvested and analyzed for yield of gated B220−TCRβ−CD11b+/Gr-1+ macrophages/neutrophils. Mean ± SE is shown for each time point. n= 4-8 from 3-4 replicate experiments. No difference was observed between the two groups (p>0.1).