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A Crucial Role for Host APCs in the Induction of Donor CD4+CD25+ Regulatory T Cell-Mediated Suppression of Experimental Graft-versus-Host Disease

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The application of allogeneic bone marrow transplantation (BMT), which is an effective therapy against hematological diseases, has been impeded by the development of its major toxicity, graft-versus-host disease (GVHD) (1). GVHD results from immunologically mediated injury to host tissues (2, 3), and alloreactive donor T effector cells (Teffs) are essential for the induction and pathologic changes characteristic of GVHD (1, 4). Regulation of an immune response is now known to be mediated by multiple subtypes of regulatory/suppressor cells (5–7). The best-described bona fide populations of regulatory cells are CD4+CD25+ and express the forkhead/winged helix transcription factor Foxp3 (8, 9). These so-called “natural” regulatory T cells (Tregs) develop in the thymus. Several lines of evidence demonstrate that donor CD4+CD25+ natural Tregs reduce GVHD (10–15). GVHD severity depends, in part, on the balance between the donor natural CD4+CD25+Foxp3+ T cells (Tregs) and Teffs (2, 10, 16–18). Although IL-10 can contribute, very little is known about the mechanisms of Treg-mediated GVHD suppression (17). Specifically, the nature and presentation of its target Ags are not well understood.

Significant progress has been made in our understanding of the role of APCs and their interaction with alloreactive donor T cells in the induction of GVHD. Key roles for host APCs in the induction and the donor-derived APCs in the exacerbation of acute GVHD have been established (19). However, it is not known whether the host and/or donor APCs play a crucial role in regulating donor Treg-mediated GVHD suppression.

Because natural Tregs are CD4+CD25+ T cells, they recognize allo- or self-antigenic peptides presented on MHC class II molecules, and the interactions with class II are critical for their survival and activation (20). In the context of MHC-matched allogeneic BMT, donor Tregs can, therefore, respond to self- and/or minor Ags on MHC class II+ host hematopoietic cells and also on the donor bone marrow (BM)-derived cells. However, what actually transpires postallo transplant, specifically whether host or donor APCs are the key activators of Tregs and whether the Ags are alloantigens or self-Ags, are unknown. Therefore, we examined whether donor Tregs’ recognition of host MHC class II was critical for regulation of GVHD (20) and found that donor Tregs’ activation by MHC II on recipient hematopoietic APCs is necessary and sufficient for Treg-mediated suppression of GVHD.

Materials and Methods

Mice

Female C57BL/6 (B6, H-2b, CD45.2+) and BALB/c (H-2d, CD45.2+) mice were purchased from the Charles River Laboratories (Wilmington, MA).
B6-Ly5.2/Cr (B6-CD45.1, H-2b, CD45.1+) mice were purchased from the National Cancer Institute-Frederick (Rockville, MD). C3H.SW (H-2b, CD45.2+) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II-deficient (B6.129-H2-Ab-1tm1GruN12, Abb, CD45.2+) and B6-background MHC class I-deficient (B6.129-B2m1/2m1/2 N12, B2m-KO, CD45.2+) mice were purchased from Taconic Farms (Germantown, NY). B6-background IDO-deficient (B6.129-Ido1tm1Alm, IDO-KO, CD45.2+) mice were purchased from the Jackson Laboratory.

**BM chimeras**

Recipient mice were irradiated (137Cs source) with 10 or 11 Gy total body irradiation before transplant of syngeneic BM cells. Donor BM cells were harvested from femurs and tibias of donor mice, washed, and counted. BM cells (5 × 10^6) were infused into recipient mice through the tail vein. Recipient mice were maintained on acidified water (pH < 3) for 3 wk. Donor hematopoietic cell engraftment was confirmed using peripheral blood by flow cytometry 2–3 mo after transplantation. Anti-CD45.1 or anti-CD45.2 mAbs were used for identification of donor-derived WBCs. For generating MHC class II-deficient BM chimeras [Abb→B6-CD45.1], recipient mice were thymectomized 2–3 wk before transplant, as described previously (21). For generating MHC class I-deficient (B2m-KO) BM chimeras [B2m-KO→B6-CD45.1], recipient mice were treated with 200 μg anti-NK1.1 mAb (PK136) on days −2 and −1 (18).

**BM transplantation**

Host mice were irradiated (137Cs source) with 9 or 10 Gy total body irradiation 1 d before BMT. In some experiments, host mice were treated with 200 μg anti-NK1.1 mAb (PK136) for depletion of functional NK cell activity. Donor BM cells were harvested from femurs and tibias, and T cells in the BM were magnetically isolated using CD90.2-positive and a MACS LS column (both from Miltenyi Biotec, Auburn, CA). In some experiments, T cells in the BM were removed by incubation with anti-CD90.2 (HO13.4) mAb, followed by 10% v/v Low-Tox M Rabbit Complement (Accurate Chemical & Scientific, Westbury, NY). Spleen T cells were magnetically isolated by MACS LS, or LD columns (Miltenyi Biotec). A CD8 isolation kit, a CD4+CD25+ Treg isolation kit, or a pan T cell isolation kit (Miltenyi Biotec) and biotinylated anti-CD25 mAb (eBioscience, San Diego, CA) were used for CD8 T cell, CD4+CD25+ T cell, and CD4-depleted T cell isolation, respectively. Purities of isolated T cells were checked by flow cytometry. T cell numbers were determined based on cell count and purity. Synergic or allogeneic T cell-depleted BM (TCDBM) and T cells were infused through the tail vein. Host mice were housed in sterilized microisolator cages and maintained on acidified water (pH < 3) for 3 wk, as described previously. The conventional T cell dose was adjusted (greater numbers) for the MHC-mismatched model. Higher numbers of only CD8 T cells (6 × 10^6) were used along with anti-NK1.1 to diminish resistance and induce GVHD in the MHCII−/− animals; however, similar doses of Treg numbers (1 × 10^6) were used, and that was sufficient to regulate GVHD. In the minor Ag-mismatched MHC-matched model, similar doses of conventional donor T cells and Tregs were used in wild-type (WT) and MHCII−/− animals, as outlined in the figure legends. The induction of GVHD was similar between the WT and MCHII−/− recipients in this CD8 T cell-dependent model. Survival was monitored daily, and clinical GVHD was assessed weekly, as described previously. All animal studies were performed per the institutional Institutional Animal Care and Use Committee guidelines of the University of Michigan and the University of Miami Animal Care and Use Committee.

**Ex vivo priming donor T cells with host dendritic cells**

B6 BM dendritic cells (DCs) were induced with GM-CSF, as described previously (22). C3H.SW naive spleen cells and B6 DCs were cocultured in 10% FCS/RPMI 1640 supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, 0.02 mM 2-ME, and 10 mM HEPES (pH 7.75) at a 10:1 ratio (C3H.SW spleen cells 1 × 10^6/ml, B6 1 × 10^6 cells/ml) in six-well polystyrene culture plates (BD Biosciences, San Jose, CA) for 36 h. After 36 h of coculture, C3H.SW CD8+ cells were isolated by negative isolation using a CD8 isolation kit and CD11c microbeads (Miltenyi Biotec) and used for BMT.

**Abs and flow-cytometric analysis**

FITC−, PE, or allophycocyanin-conjugated mAbs to mouse CD4 (RM4-4), CD8a (53-6.7), CD90.2 (53-2.1), CD229.1 (30C7), CD45.1 (A20), and biotinylated anti-CD25 mAb were used for detecting CD4+CD25+ Treg cell populations. Data were analyzed with Accuri C6 flow cytometer (BD Biosciences). All data were shown as representative data from at least two independent experiments. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) and included the Student’s t test for comparing the means of two groups and one-way ANOVA for comparing means of multiple groups. Differences were considered significant when p < 0.05.
CD45.2 (104) were purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-FoxP3 mAb (clone FJK-16s) was purchased from eBioscience. Spleen or BM cells were incubated with anti-CD16/CD32 (2.4G2; Fc block; BD Biosciences) mAb for 15 min at 4°C in staining buffer (2% FCS containing PBS). After washing, cells were fixed with FACS Lysing Solution (BD Biosciences). The cells were resuspended with staining buffer and analyzed on a FACS Calibur (BD Biosciences). The following procedure was performed for intracellular FoxP3 staining. Fixed cells were washed with permeabilization buffer (eBioscience) and stained with PE-conjugated anti-FoxP3 mAb (FJK-16s; eBioscience) for 30 min at 4°C. The cells were washed with permeabilization buffer and staining buffer. The cells were resuspended with staining buffer and analyzed on a FACS Calibur.

Statistical analysis
The Student t test was used for the statistical analysis of in vitro data, and the Wilcoxon rank test was used to analyze survival data; p < 0.05 was considered statistically significant.

Results
Direct Ag presentation by host tissues is required for donor Treg-mediated GVHD protection

We tested the requirement for direct Ag presentation by host tissues in mitigating acute GVHD by donor-derived Tregs. We first used an MHC-matched, minor Ag-disparate C3H.SW (H-2b) → C57BL/6 (B6-H2b, CD45.1) model of CD8+ T cell-mediated GVHD (18, 23, 24). Control experiments were performed to determine the optimal dose of Tregs and Teffs (Supplemental Fig. 1). Based on these experiments, 2 × 10^5 CD8 conventional donor T cells and TCDBM from C3H.SW donors were infused into lethally irradiated WT or MHC class II−/− Abb B6 animals with or without 1 × 10^5 donor C3H.SW-derived CD4+CD25+ Tregs (ratio of 2:1). Donor Tregs reduced GVHD clinical severity and significantly improved survival in the WT B6 animals (Fig. 1A). By contrast, the clinical severity and mortality of GVHD were not improved by the Tregs in B6 MHC class II−/− Abb hosts (Fig. 1B), indicating an absolute requirement for host MHCII+ cells for Treg protection from GVHD.

To determine that a requirement for recipient MHC class II was not strain dependent or limited to MHC-matched BMT, we also examined Treg suppression in the MHC-mismatched BALB/c → B6 model of GVHD. We treated B6 WT and Abb recipients with anti-NK1.1 mAb to deplete NK cells to prevent rejection of donor BM. B6 mice were irradiated and transplanted with TCDBM and CD8 T cells with or without Tregs from BALB/c donors. Donor Tregs reduced GVHD severity and improved survival in the WT animals but not in the Abb animals (Fig. 1C, 1D).

The lack of reduction in the severity of GVHD by the donor Tregs was also confirmed by target organ (liver and colon) histopathology in the allogeneic Abb recipients (Fig. 2). Thus, data from these models demonstrate the requirement of MHC class II expression on host tissues in donor Treg-mediated GVHD protection.

MHC class II expression by host APCs is necessary and sufficient for GVHD protection

The above experiments demonstrated that a complete absence of MHC class II on all host tissues (hematopoietic and nonhematopoietic cells) prevents GVHD protection by donor Tregs. To determine whether Treg suppression of GVHD required MHC class II only on host hematopoietic cells or on host nonhematopoietic cells, we used Abb → B6 chimeras as recipients in GVHD experiments. Briefly, B6 animals were thymectomized and, upon recovery, were lethally irradiated (11 Gy) and transplanted with TCD syngeneic BM from MHC II-deficient Abb (II−/−) or WT B6 animals to generate [Abb → B6] or WT [B6 → B6] animals. Thymectomy was performed to exclude the possibility of development of Tregs from the infused BM, despite the absence of MHC II on hematopoietic cells in the periphery (25), as well as to prevent the development of autoimmune disease upon receiving BM from class II−/−-deficient animals (25, 26). Thus, the [B6 → B6] chimeras express class II Ags on all tissues as in the WT. By contrast, the [Abb → B6] animals express MHC II only on nonhematopoietic tissues and, as such, the donor CD4+CD25+ Tregs are not able to directly interact with the host hematopoietic APCs. However, the hematopoietic-derived APCs in the [B6 → B6] and the [Abb → B6] animals can activate the CD8+ Teffs by the intact expression of class I. These animals were used 3 mo later, upon complete donor engraftment, as recipients in an allogeneic BMT (18, 23). The chimeras received lethal irradiation and were injected with TCDBM from allogeneic C3H SW donors with or without allosecreactive CD8+ T cells. A cohort of the animals that received the CD8+ T cells were also injected with donor Tregs. Donor Tregs reduced GVHD severity and mortality in the allogeneic [B6 → B6] animals (Fig. 3A) but not in the [Abb → B6] animals (Fig. 3B), demonstrating that MHC II expression on host hematopoietic-derived APCs is necessary for the induction of donor Treg-mediated reduction in GVHD mortality.

Although MHC class II expression is primarily limited to APCs, during inflammatory processes, such as GVHD, MHC class II is upregulated on epithelial and endothelial cells (27). Therefore, we next examined whether expression of class II exclusively on the host hematopoietic-derived APCs is sufficient for GVHD suppression by donor Tregs. We generated BM chimeras expressing MHC class II on BM-derived APCs but lacking MHC class II on nonhematopoietic/epithelial target cells. We did this by reconstituting lethally irradiated MHC II−/− mice with B6 BM cells (B6 → MHC II−/− [Abb]). Four months later, the [B6 → Abb] chimeras were used as hosts in allogeneic BMT. Infusion of donor Tregs significantly reduced the severity and mortality from GVHD in these [B6 → Abb] animals (Fig. 3C).

Donor Tregs survive, despite the absence of MHC class II expression by host APCs

We next analyzed whether the lack of GVHD protection by the donor Tregs reflected their inability to survive in the class II−/− hosts (20). We analyzed donor Teff and Treg expansion in the host spleens on day +17 after allogeneic BMT. Tregs significantly suppressed the expansion of CD8+ Teffs in the spleens of WT hosts but not MHC
Surprisingly, donor CD4+ numbers and Treg (CD4+FoxP3+) numbers were similar in WT and Abb animals, indicating that these cells were maintained in the Abb hosts, despite their inability to prevent GVHD (Fig. 4B, 4C). Given the critical requirement of TCR/MHC interactions for the survival of naive T cells, we reasoned that the interactions between the donor Tregs and MHC class II expression on donor BM-derived cells might be sufficient for proliferation/maintenance of Tregs in Abb hosts (28).

To formally confirm this premise and because MHC class II−/− mice are available only on a B6 background, we used a syngeneic BMT model. WT B6 Tregs, along with TCDBM from Abb donors, were transplanted into WT or Abb B6 hosts following lethal irradiation. The numbers of Tregs were significantly lower only in the Abb hosts that also received Abb BM (data not shown). Host APC-derived IL-10 and IDO are not required for Treg-mediated GVHD protection

We next addressed the mechanisms that underpin the requirement of host APCs for donor Treg-mediated reduction of GVHD. IL-10 and IDO regulate GVHD, whereas APC-derived IL-10 and IDO

![FIGURE 3. Requirement of MHC class II expression on host APCs for Treg-mediated suppression of GVHD.](http://www.jimmunol.org/)

**A**, [B6→B6-CD45.1] BM chimera hosts were irradiated (9 Gy) on day −1 and infused (on day 0) with syngeneic B6 5×10⁶ BM (●, n = 10), allogeneic C3H.SW 5×10⁶ BM and 2×10⁵ CD8+ T cells (○, n = 10), or C3H.SW 5×10⁶ BM, 2×10⁵ CD8+ T cells, and 1×10⁵ CD4+CD25+ T cells (□, n = 9). Survival was monitored daily (top panel), and GVHD clinical score was monitored weekly (bottom panel). Data combined from two experiments are shown. p = 0.0089; ● versus ○ (survival). **B**, [Abb→B6-CD45.1] BM chimera hosts were irradiated (9 Gy) on day −1 and infused (on day 0) with allogeneic C3H.SW 5×10⁶ BM and 2×10⁵ CD8+ T cells (●, n = 11) or C3H.SW 5×10⁶ BM, 2×10⁵ CD8+ T cells, and 1×10⁵ CD4+CD25+ T cells (○, n = 6). Survival was monitored daily (top panel), and GVHD clinical score was monitored weekly (bottom panel). Data combined from two experiments are shown. p = 0.4331; ● versus ○ (survival). **C**, [B6-CD45.1→Abb] BM chimera hosts were irradiated (9 Gy) on day −1 and transplanted (on day 0) with allogeneic C3H.SW 5×10⁶ TCDBM and 2×10⁵ CD8+ T cells (●, n = 9) or C3H.SW 5×10⁶ TCDBM, 2×10⁵ CD8+ T cells, and 1×10⁵ CD4+CD25+ T cells (○, n = 10). Survival was monitored daily (top panel), and GVHD clinical score was monitored weekly (bottom panel). Data are combined from two similar experiments. p = 0.0139; ● versus ○ (survival).

![FIGURE 4. MHC class II expression on host cells is critical for donor Treg-mediated GVHD suppression.](http://www.jimmunol.org/)

Expansion of C3H.SW donor (CD229.1+) T cells was analyzed using day 17 spleen cells. BMT was performed using C3H.SW→B6 and C3H.SW→Abb models, as shown in Fig. 1A and 1B, and host spleen cells were harvested on day 17. The cells were counted, stained, and analyzed by flow cytometry. Absolute numbers of C3H.SW donor-derived (CD229.1+) CD8+, CD4+, and CD4+FoxP3+ T cells are shown. Data are from one of two experiments with similar results.

**A**, Absolute numbers of C3H.SW donor-derived (CD229.1+) CD8+ T cells are shown. Data are from one of two experiments with similar results. **p** < 0.05; **pp** < 0.01.
were shown to play an important role in Treg-mediated immunoregulation under certain conditions (5, 28, 29); however, their role in donor Treg-mediated suppression of GVHD is not known. Therefore, we next determined whether the production of these immunoregulatory molecules by host APCs is critical for the Tregs to suppress GVHD. We generated BM chimeras using BM from IL-10–deficient (IL-10−/−) B6 or IDO-deficient (IDO−/−) B6 mice and transferring into lethally irradiated WT B6 mice, generating (IL-10−/−B6→B6) and (IDO−/−B6→B6) animals so that BM-derived APCs would be incapable of producing IL-10 or IDO, respectively. Three months later, the chimeras were reirradiated on day −1 and injected on day 0 with TCDBM and 2 × 10^6 CD8+ T cells, with or without 1 × 10^6 CD4^+CD25^+ cells from allogeneic C3H.SW or syngeneic B6 donors. As expected, the allogeneic (IL-10−/−B6→B6) and (IDO−/−B6→B6) mice that did not receive donor Tregs showed clinical signs and died of GVHD (Fig. 5, bottom panels). By contrast, all of the allogeneic (IL-10−/−B6→B6) and (IDO−/−B6→B6) recipients that were cojected with donor Tregs survived, with minimal to no signs of GVHD (Fig. 5, bottom panels). These data collectively demonstrate that expression of IL-10 or IDO by host APCs is not critical for regulation of GVHD by donor Tregs.

**FIGURE 5.** Effect of deficiencies of immunoregulatory molecules IL-10 and IDO in host APCs on Treg-mediated suppression of GVHD. A, [B6→B6-Cd45.1] BM chimera hosts were irradiated (9 Gy) on day −1 and infused (on day 0) with syngeneic B6 5 × 10^6 BM (●, n = 13), allogeneic C3H.SW 5 × 10^6 BM and 2 × 10^6 CD8+ T cells (●, n = 14), or C3H.SW 5 × 10^6 BM, 2 × 10^6 CD8+ T cells, and 1 × 10^6 CD4^+CD25^+ T cells (○, n = 12). Survival was monitored daily (top panel). Data from three combined experiments are shown. p = 0.0006; ● versus ○. [IL-10-KO→B6-Cd45.1] BM chimera hosts were irradiated (9 Gy) on day −1 and transplanted (on day 0) with allogeneic C3H.SW 5 × 10^6 BM and 2 × 10^6 CD8+ T cells (●, n = 15) or C3H.SW 5 × 10^6 BM, 2 × 10^6 CD8+ T cells, and 1 × 10^6 CD4^+CD25^+ T cells (○, n = 14). Survival was monitored daily (bottom panel). Combined data from three similar experiments are shown. p = 0.0002; ● versus ○. B, [B6→B6-Cd45.1] BM chimera hosts were irradiated (9 Gy) on day −1 and infused (on day 0) with syngeneic B6 5 × 10^6 BM (●, n = 12), allogeneic C3H.SW 5 × 10^6 BM and 2 × 10^5 CD8+ T cells (●, n = 12), or C3H.SW 5 × 10^6 BM, 2 × 10^5 CD8+ T cells, and 1 × 10^5 CD4^+CD25^+ T cells (○, n = 12). Survival was monitored daily (top panel). Data from three similar experiments are shown. p = 0.0121; ● versus ○. [IDO-KO→B6-Cd45.1] BM chimera hosts were irradiated (9 Gy) on day −1 and transplanted (on day 0) with allogeneic C3H.SW 5 × 10^6 BM (●, n = 13) or C3H.SW 5 × 10^6 BM, 2 × 10^5 CD8+ T cells, and 1 × 10^5 CD4^+CD25^+ T cells (○, n = 14). Survival was monitored daily (bottom panel). Data from combined three similar experiments are shown. p = 0.0177; ● versus ○.

Host APCs are needed for induction of Treg-mediated suppression of GVHD caused by activated Teffs

We next analyzed whether donor Tregs protected GVHD only through regulation of donor Teff activation by the host APCs. Therefore, we next determined whether donor Tregs would mitigate GVHD initiated by already activated Teffs. To test this, we generated the [β2m−/−→B6] chimeras (as described in Materials and Methods), such that host APCs would not be able to further activate the donor CD8+ Teffs but would be able to activate donor Tregs by intact class II expression. Previous reports showed that, in these chimeras, activation of alloreactive donor T cells ex vivo by host APCs would mediate a graft versus host response (23, 31). We stimulated C3H.SW CD8+ T cells with syngeneic (C3H.SW) or allogeneic (B6) BM-derived DCs for 36–48 h in an MLR (as described in Materials and Methods). These primed CD8+ T cells were infused into lethally irradiated [β2m−/−→B6] mice along with TCDBM, with and without Tregs from C3H.SW. Ninety percent of the recipients of CD8+ T cells cultured with syngenic DCs survived without any evidence of GVHD, whereas only 40% of animals that received CD8+ T cells that had been primed ex vivo with host-type DCs that did not receive donor Tregs survived with signs of GVHD (Fig. 6). By contrast, a significantly greater number (80%) of animals that received ex vivo-primed alloreactive CD8+ T cells along with naive donor Tregs survived the observation period with minimal signs of GVHD (Fig. 6). These data demonstrate that donor Tregs, once activated by host APCs, can suppress GVHD mediated by already activated alloreactive Teffs.

**FIGURE 6.** Tregs suppress GVHD, even after Teffs are primed. [β2m-KO→B6-Cd45.1] BM chimera hosts were irradiated (9 Gy) on day −1 and infused (on day 0) with syngeneic B6 5 × 10^6 BM (●, n = 15), allogeneic C3H.SW 5 × 10^6 BM and ex vivo-primed 2 × 10^5 CD8+ T cells (●, n = 14), or C3H.SW 5 × 10^6 BM, ex vivo-primed 2 × 10^5 CD8+ T cells, and 1 × 10^5 CD4^+CD25^+ T cells (○, n = 13). Survival was monitored daily. Data from four similar experiments are shown. p = 0.0482; ● versus ○.
GVHD. Thus, these data confirm and extend previous observations on the role of host APCs in the maintenance of suppressor (regulatory) T cells that are required for tolerance in MHC-mismatched hematopoietic chimeras (33–35).

Our data provide several perspectives on donor Treg-mediated reduction in GVHD. Although MHC class II expression is primarily limited to APCs, during GVHD, MHC class II molecules aberrantly express on nonhematopoietic cells, such as epithelial and endothelial target cells (27, 36–38). Thus, the possible MHC class II-expressing cell types that are present in the BMT hosts are the host hematopoietic APCs and the host nonhematopoietic tissues. Our results suggest that class II expression on host APCs is necessary and sufficient for the induction of Treg-mediated suppression of GVHD, whereas its expression on host nonhematopoietic tissues is not obligatory. A simple reason for a crucial role of class II expression and Ag presentation by host APCs to donor Tregs, which are CD4+T cells, might be a consequence of their requirement for Treg survival. However, this was surprising for the following reasons. Our data showed that although the lack of Ag presentation by functional MHC class II on host APCs led to the loss of GVHD protection, the numbers of donor Tregs were maintained. This indicates that the lack of GVHD protection from the absence of MHC class II on host tissues was not a mere consequence of the loss of Tregs. The reason for the maintenance of donor Tregs, despite the lack of MHC class II on hosts, might be due to the presence of MHC class II on the infused donor BM and the cells derived from them. Studies are underway to determine whether donor BM-derived cells contribute to the maintenance of donor Treg numbers and GVHD protection. Nonetheless, our data demonstrate that the expression of MHC class II on donor BM cells alone is not sufficient for induction of GVHD protection. The reasons for the lack of Treg-mediated suppression by the aberrantly expressed MHC class II on nonhematopoietic cells might be because the regulation may occur in lymphoid organs and only CD62L+ Tregs might suppress conventional T cells and/or despite the expression of MHC class II by nonprofessional APCs, these cells may not express the full repertoire of costimulatory molecules and other signals that might be critical for Treg function.

This raises the question of why if the Tregs survived in the absence of host MHC class II, they did not suppress GVHD. One reason for the requirement of host APCs might be reflective of the kinetics of Treg activation after allogeneic BMT. This notion is consistent with other data that Tregs need to be given early or they are likely to be ineffective (39). Another is the nature of the Ag. Donor-derived cells can certainly indirectly present Ag (40). The nature, type, and repertoire of Ags and whether it is alloantigens or self-Ags or both that donor Tregs respond to after allogeneic BMT are not known. Our data suggest that the requirement of host APCs reflects the requirement of only allopeptides or the better ability of these cells to present the relevant Ag repertoire (alloantigens ± self-Ags) compared with donor APCs. In contrast to the requirements of donor natural Tregs, our data did not address whether the host APCs are required for GVHD protection mediated by adaptive, Ag-specific Tregs. The answer to this question may be interesting. However, such adaptive Tregs are not fully characterized in GVHD and will be pursued in future studies. Furthermore, in clinical BMT, the relevant immune-dominant Ags that drive Treg or Teff responses in GVHD are largely unknown; translational attempts are being made only with donor polyclonal natural Tregs (41). As such, our data are particularly germane to current translation efforts.

IL-10 and IDO have been implicated separately in Treg-mediated suppression of immune responses and in graft-versus-host processes (17, 30, 42–44). Our data showed that host APC-derived IL-10 or IDO is not required for donor Treg-mediated reduction of GVHD. However, our data did not specifically address the roles of these immunoregulatory proteins in the context of GVHD regulation without Tregs. It should be noted that Tregs were also shown to regulate immune responses by directly suppressing the activation of naïve T cells by APCs in the secondary lymphoid organs or in the peripheral tissues after the Teffs had been activated (45–47). Furthermore, although the TCR repertoire of the donor Tregs infused into WT and MHC II recipient animals was similar (because the donors were identical), it is possible that following BMT, the repertoire of the surviving Tregs in the WT and MCHII−/− animals may be distinct. However, the number of Tregs was not significantly different early after the transplant, suggesting that, at least at those time points, the activation status of Tregs is unlikely to be different in the WT and MCHII−/− animals. Nonetheless, future studies will carefully analyze the potential distinctions in donor Tregs’ activation and repertoire with Vβ spectratyping.

We also found that unmanipulated donor Tregs could suppress GVHD induced by preactivated effector cells in B2M−/−→B6 chimeras. Thus, Tregs need not interact specifically with host-derived APCs. Given that, in general, Tregs require activation to function, this suggests that donor APCs are also sufficient, although this does not resolve whether Tregs are recognizing minor histocompatibility Ags or self-peptides. The sufficiency of donor APCs supports the kinetic hypothesis for why host APCs are essential for Treg-mediated mitigation of GVHD. This suppression of GVHD induced by preactivated effectors also suggests that Tregs may be acting in GVHD target tissues, although it is also possible that they act on effectors’ secondary lymphoid tissues, most likely spleen, because CD62L− effectors are not recruited well to lymph nodes. Our data showed that donor Tregs can suppress Teffs after they have been activated. This suggests that once Tregs have been activated by host APCs they can suppress already activated donor Teffs, perhaps in the peripheral target tissues after allogeneic BMT. Nonetheless, it is also possible that Tregs might inhibit the activation of alloreactive T cells in the secondary lymphoid organs. Future studies will address these issues.

In summary, our findings demonstrate a critical role for host APCs in the regulation of GVHD by donor natural Tregs and suggest that augmenting host APC-mediated activation of Tregs early posttransplant will enhance Treg-mediated reduction of GVHD. Because cellular therapy for the reduction of GVHD with natural donor Tregs is being tested in phase I/II clinical trials, our data, as such, may have significant ramifications. Our data suggest that the consequence of loss of effective Ag presentation by host APCs might lead to loss of an effective use of Treg-based cell therapies in mitigating GVHD.

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**Disclosures**

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**References**


