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Rapid Deletional Peripheral CD8 T Cell Tolerance Induced by Allogeneic Bone Marrow: Role of Donor Class II MHC and B Cells

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Mixed chimerism and donor-specific tolerance are achieved in mice receiving 3 Gy of total body irradiation and anti-CD154 mAb followed by allogeneic bone marrow (BM) transplantation. In this model, recipient CD4 cells are critically important for CD8 tolerance. To evaluate the role of CD4 cells recognizing donor MHC class II directly, we used class II-deficient donor marrow and were not able to achieve chimerism unless recipient CD8 cells were depleted, indicating that directly alloreactive CD4 cells were necessary for CD8 tolerance. To identify the MHC class II+ donor cells promoting this tolerance, we used donor BM lacking certain cell populations or used positively selected cell populations. Neither donor CD11c+ dendritic cells, B cells, T cells, nor donor-derived IL-10 were critical for chimerism induction. Purified donor B cells induced early chimerism and donor-specific cell-mediated lympholysis tolerance in both strain combinations tested. In contrast, positively selected CD11b+ monocytes/myeloid cells did not induce early chimerism in either strain combination. Donor cell preparations containing B cells were able to induce early deletion of donor-reactive TCR-transgenic 2C CD8 T cells, whereas those devoid of B cells had reduced activity. Thus, induction of stable mixed chimerism depends on the expression of MHC class II on the donor marrow, but no requisite donor cell lineage was identified. Donor BM-derived B cells induced early chimerism, donor-specific cell-mediated lympholysis tolerance, and deletion of donor-reactive CD8 T cells, whereas CD11b+ cells did not. Thus, BM-derived B cells are potent tolerogenic APCs for alloreactive CD8 cells. The Journal of Immunology, 2008, 181: 4371–4380.

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To evaluate the role of CD4 cells recognizing alloantigen directly on donor APCs for CD8 tolerance, we have now used MHC class II-deficient donor BM. The role of defined cell lineages in inducing donor-specific tolerance was addressed by either administering donor BM lacking certain cell populations to evaluate their necessity or by using positively selected BM cell fractions to test their sufficiency for induction of early peripheral CD8 T cell tolerance. The results demonstrate a strong but not requisite role for donor B cells in this tolerance induction.

Materials and Methods

Mice

Female C57BL/6 (B6; H-2b) and B10.A (H-2b) mice were purchased from the Frederick Cancer Research Center. Female B10.S (H-2s), TCRβ−/− (on C57BL/6 background; H-2b), IL-10−/− (on the C57BL/6 background; H-2b), μMT (on the C57BL/6 background; H-2b), and diphertheria toxin (DT) receptor-transgenic (DTR tg) expressing DTR under the mouse CD11c promoter; backcrossed six to seven generations onto the C57BL/6 background; H-2b) mice were purchased from The Jackson Laboratory. All mice were housed in a specific pathogen-free microisolator animal facility. 2C mice were phenotyped by FACS and DTR tg mice were analyzed for purity by flow cytometry. Mac1−/− (on the C57BL/6 background; H-2b) mice were purchased from The Jackson Laboratory. I-Aβ−/− (on the C57BL/6 background; H-2b) mice were purchased from Taconic Farms. DTR tg as well as 2C TCR tg mice (12) were bred in our laboratory. Anti-CD11c promoter; backcrossed six to seven generations onto the C57BL/6 background; H-2b), mice were purchased from The Jackson Laboratory. All mice were housed in a specific pathogen-free microisolator environment. Animal experimental protocols were reviewed and approved by the institutional committee at Massachusetts General Hospital.

Conditioning and BMT

Eight- to 12-wk-old male mice received 3 Gy of TBI from a 137Cs irradiator on day −1 with respect to BMT. Hamster anti-mouse CD154 mAb (MR1; 2 mg; produced at National Cell Culture Center) was administered i.p. on day 0 before transplantation with 20–25 × 10⁶ fully MHC-mismatched BM cells (BMCs) by tail vein injection. CD8-depleting mAb (2.43, 1.44 mg), where mentioned, was administered i.p.

Dendritic cell (DC) depletion with diphtheria toxin (DT)

DT was purchased from Sigma-Aldrich as lyophilized powder and then resuspended with sterile water at a concentration of 1 mg/ml. Depletion of DCs was performed by i.p injection of 100 ng of DT in 1 ml of PBS three times per week until day 18. Efficiency of depletion of CD11c−/− DCs was checked 1 day after the last DT injection by FACS analysis of collagenase-digested spleen and was −93%.

MACS isolation or depletion of donor cell populations

Donor cells were purified or depleted by MACS. Donor whole BM was harvested, counted, and incubated according to the manufacturer’s protocol for 15–20 min with either anti-CD11b (Mac-1−/− cells), anti-CD19, or anti-CD4/8 magnetic beads in PBS with 0.5% BSA and 2 mM EDTA. Cells were washed, resuspended, and sorted with L5 (for CD19− and Mac1−/− fractions) or CS columns (for T cell depletion; Miltenyi Biotec) according to the manufacturer’s protocol. Both positive and negative fractions were analyzed for purity by flow cytometry. Mac1−/− and CD19− cells were >97% pure. CD19 and T cell depletion was >99% effective, but Mac1 depletion was only − 60–80% effective.

Preparation of B6 mice with a traceable population of transgenic CD8 cells specific for the Ld MHC class I Ag (2C/B6 chimeras)

Mice were prepared as previously described (10). Briefly, 5 million BMCs from 2C-TCR-transgenic B6 mice in which a transgenic TCR recognizing Ld is expressed on >99% of CD8 T cells were transplanted into naive B6 mice treated with 3 Gy of TBI on the same day. The resulting mice were chimeric for the 2C TCR and are referred to as 2C/B6 mice. After 8 wk, the percentage of 2C cells among CD8+ T cells in the peripheral blood was checked 1 day after the last DT injection by FACS analysis of collagenase-digested spleen and was >99%.

FIGURE 1. Requirement for direct CD4 alloreactivity for CD8 tolerance. A, B10.S mice received a wt B6 or I-Aβ−/− BM graft after conditioning with TBI and anti-CD154. The incidence of mixed chimerism for the B cell lineage at indicated time points is shown. Chimerism was defined as ≥5% donor MHC class I-positive cells among B220+ B cells. One representative experiment of two is shown (n = 7 animals/group per experiment). B, The percentage of donor chimerism ± SEM over time is shown for B cells and CD4 T cells in peripheral blood of both groups shown in A. C, Mice were treated as described for A. In addition, they received one dose of anti-CD8 mAb. CD8 T cells were <0.5% of lymphocytes in white blood cells by 2 wk after BMT. The incidence of chimerism for the B cell lineage is shown at the indicated time points. One representative experiment of two is shown (n = 6–7 animals/group per experiment). D, The percentage of donor chimerism ± SEM over time is shown for B cells and CD4 T cells in peripheral blood of the groups shown in C. In all animals, B cell chimerism is similar to chimerism of myeloid cell lineages and CD4 T cell chimerism is similar to CD8 chimerism (unless CD8s were depleted).
A 

Donor (B6; DTR tg) 

Recipient (B10.S) 

DT 

d-1 0 3x/1 wk from d1-18 

TBI 

3 Gy 

Anti-CD154 

BMT 

DT 

B 

E 

Donor MHC class I 

C 

D 

Weeks after BMT 

% chimeric animals 

Weeks after BMT 

% donor cells 

Weeks after BMT 

% donor chimerism 

FIGURE 2. Donor CD11c<sup>+</sup> DCs are not necessary for induction of chimerism. A, Experimental design: B10.S mice received conditioning with 3 Gy of TBI and anti-CD154. In addition, they were or were not treated with DT. On day 0, they received 20×10<sup>5</sup> BMCs from DTR tg or wt B6 mice, which were either treated or not treated with DT on the day before BM harvest. B, The percentage of donor-derived cells among gated CD11c<sup>+</sup> splenocytes 1 day after the last DT injection for two individual mice receiving DTR tg BM either without (left panel) or with DT treatment (right panel). Both of these mice had similar levels of multilineage chimerism (around 15% for B cells and 30% for granulocytes). C, Incidence of donor chimerism in the B cell lineage, which is representative also for the granulocyte lineage, is shown 3 and 9 wk after BMT for mice of the four groups mentioned above (n = 7–8 animals/group; one of two similar experiments is shown). D and E, Levels of donor chimerism in the four groups mentioned above is shown for B cells (D) and CD4 T cells (E).

Flow cytometric analysis of multilineage chimerism in white blood cells

Donor chimerism after allogeneic BMT. Three-color flow cytometric analysis was used to determine multilineage chimerism. Donor-derived cells were identified by FITC-conjugated anti-MHC class I Abs (anti-H-2<sup>D</sup> mAb 34-2-12 for H-2<sup>a</sup>, anti-H-2<sup>D</sup> mAb KH95 for H-2<sup>b</sup>). The cells were counterstained with PE- or allophycocyanin-conjugated anti-CD4, -CD8, -B220 (BD Biosciences/Becton Dickinson), and -Mac1 (Caltag Laboratories) mAbs. Negative control mAbs included HOPC1-FITC (prepared in our laboratory) and rat anti-mouse IgG2a-PE or -allophycocyanin (BD Biosciences/Becton Dickinson). Propidium iodide stain was used to exclude dead cells from analysis. Chimerism was analyzed among CD4 and CD8 T cells, B cells, and granulocytes at 2, 6, and 10 wk after BMT. A lineage was defined as chimeric when ≥5% donor MHC class I-positive cells were found within the respective cell lineage.

2C deletion analysis. 2C CD8 T cells were identified by staining with the unconjugated 1B2 clonotypic mAb (13) and anti-mouse IgG1-allophycocyanin as secondary Ab and counterstained with CD8-PE. Relative deletion was quantified by dividing the percentage of 2C CD8 T cells in the spleen on day 7 by the percentage of 2C CD8 T cells present in peripheral blood before B10.A BMT.

Cell-mediated lympholysis (CML) assay

The CML assay was performed as previously described (11). Spleen cells from control and experimental mice were suspended in CML medium consisting of RPMI 1640 (Mediatech) with 10% FCS (Sigma-Aldrich), 0.09 mM nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 10 μg/ml streptomycin, 0.05 mM 2-ME, and 0.01 M HEPES buffer. Responder and stimulator cells (8×10<sup>5</sup> each; stimulators 30 Gy irradiated) were plated in triplicates in 96-well round-bottom plates.

Cells were incubated for 5 days at 37°C in 6% CO2. A series of 2-fold dilutions was then prepared, allowing for cytolytic readout at a total of five different responder:target ratios. Target cells were prepared with a 2-day incubation with Con A stimulation (2 μg/ml) followed by labeling with <sup>51</sup>Cr (1 μCi/ml). Eight thousand target cells were added to each well and incubated for 4 h at 37°C in 6% CO2. Plates were harvested by using the Titerek supernatant collection system (Skatron) and <sup>51</sup>Cr release was quantified with an automated gamma counter. Percent specific lysis was calculated as [experimental release − spontaneous release]/(total release − spontaneous release)] × 100%.

Skin grafting

Mice were shaved and anesthetized with ketamine/xylazine. Full-thickness tail skin (0.5–1.0 cm<sup>2</sup>) from donor and irrelevant third-party mice were grafted on either side of the back of recipient mice. Grafts were checked for viability on a daily base starting from day 7 after skin transplantation for ~3 wk and then were followed twice per week and later once per week until day 100. A graft was considered rejected, when <10% of the graft remained viable.

Results

CD4 T cell recognition of donor MHC class II is required for CD8 tolerance induction

To investigate the role of directly alloreactive CD4 T cells for CD8 tolerance, we transplanted either wild-type (wt) B6 or MHC class II-deficient I-A<sup>β</sup><sup>−/−</sup> (H-2<sup>b</sup>) marrow to fully MHC-mismatched B10.S (H-2<sup>a</sup>) recipients. All animals achieved durable multilineage chimerism following wt B6 BMT. In contrast, I-A<sup>β</sup><sup>−/−</sup> BM failed to engraft (Fig. 1, A and B). Depletion of CD8 T cells (Fig. 1, C and D), however, permitted stable multilineage chimerism in 100% of the animals receiving either MHC class II-positive or -negative BM with similar incidence and levels of chimerism in all lineages between the groups. Thus, directly alloreactive recipient CD4 T cells are required to tolerize CD8 T cells in this model.
**Donor BM-derived CD11c<sup>+</sup> DCs are not required for induction of stable mixed chimerism**

In an attempt to identify the tolerogenic MHC class II<sup>+</sup> donor APC, we used DTR tg donors expressing primate DTR under the mouse CD11c promoter. Treatment of DTR tg mice with DT depletes 90–95% of CD11c<sup>+</sup> DCs from the spleen, lasting for 48–72 h (14). Fig. 2A shows the experimental design. DTR tg or wt B6 donors were either treated or not treated with one dose of DT on day −1. On day 0, BMT with 2×10<sup>6</sup> BMCs from all four groups was performed. B10.S recipients of BM from DT-treated DTR tg or wt B6 donors were also treated with DT, starting on day −1 and then continued three times per week until day 18. One day after the last DT injection, one mouse in each group with comparable levels of peripheral chimerism was euthanized for FACS assessment of depletion of donor-derived CD11c<sup>+</sup> DCs in collagenase-digested spleen. As shown in Fig. 2B, gated CD11c<sup>+</sup> splenocytes demonstrated a 93% reduction in donor-derived DCs in the DT-treated compared with the untreated recipient of DTR tg BM.

Fig. 2C shows the incidence of multilineage mixed chimerism 3 and 9 wk after BMT in animals receiving DTR tg BM with or without DT treatment. Control animals receiving wt B6 BM with or without DT treatment are also shown. Although the incidence of chimerism was slightly lower in recipients of wt compared with transgenic donor BM, no difference was observed between DT-treated and untreated recipients of DTR tg or wt BM in the incidence (Fig. 2C) or levels (Fig. 2, D and E) of donor chimerism. Therefore, donor BM-derived CD11c<sup>+</sup> DCs are not necessary for induction of stable mixed chimerism in this model.

**Donor BM-derived B cells are not required for induction of chimerism and tolerance**

B cells represent the major MHC class II<sup>+</sup> cell population in the BM. To determine whether or not B cells are essential for tolerance induction with our regimen, we performed BMT using either wt B6 or B cell-deficient μ<sup>MT</sup> mice as donors in two different fully MHC-mismatched strain combinations, B6→B10.S and B6→B10.A. No difference in the incidence (Fig. 3A) or levels (Fig. 3B) of donor chimerism was seen when marrow from wt vs μ<sup>MT</sup> B6 donors was given to B10.S recipients. These mice were skin grafted with donor-type B6 and third-party B10.A skin. Long-term acceptance of donor-type skin grafts was achieved in 60–70% of the animals in both groups, whereas third-party grafts were promptly rejected, demonstrating donor-specific tolerance (Fig. 3C).

**Donor BM-derived T cells are not required for chimerism or tolerance induction using BMT with anti-CD154**

Donor T cells have been shown to promote engraftment of allogeneic marrow in humans and in a variety of mouse models. We therefore evaluated the role of donor BM-derived T cells for induction of stable mixed chimerism using the fully MHC-mismatched B6 (H-2<sup>b</sup>)→B10.S (H-2<sup>s</sup>) strain combination. Recipient mice were conditioned with 3 Gy of TBI and anti-CD154 before receiving 2×10<sup>6</sup> BMCs that were either unfractionated or ex vivo depleted of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. No difference in the incidence of long-term stable chimerism was observed (Fig. 4A), although the levels of chimerism were lower in the T cell-depleted group in the first 10 wk after BMT (Fig. 4B). Since new donor T cells are generated in the recipient thymus of chimeric mice within a few weeks and may be of importance for the maintenance of chimerism and tolerance, we next used TCRβ<sup>−/−</sup> mice, which lack αβ T cells, as BM donors in the same strain combination. Long-term stable mixed chimerism was observed in ≈80% of animals receiving TCRβ<sup>−/−</sup> BM and 100% of those receiving wt B6 BM (Fig. 4A). Levels of B cell and myeloid chimerism were comparable in both groups (Fig. 4B and data not shown). This experiment
was repeated in a different strain combination (B6→B10.A) with similar results, although an overall lower incidence of chimerism (60–70%) was seen in both the experimental and the control group (data not shown). To test for donor-specific tolerance, mice receiving either wt B6 or TCRβ−/− donor cells were grafted with tail skin from either wt B6 or third-party B10.RIII (H-2r) mice. Third-party skin was promptly rejected in both groups, whereas long-term (>100 days) acceptance of donor-derived skin grafts was observed in 60% of BMT recipients, with no difference between those receiving wt or TCRβ−/− marrow (Fig. 4C). Thus, donor BM-derived T cells are not necessary for induction of long-term stable mixed chimerism and skin graft tolerance in this model.

Donor-derived IL-10 is not required for induction of CD8 and CD4 tolerance

Because the cytokine IL-10 can be produced by multiple cell types, including T cells, B cells, and DCs and has been implicated in many tolerance models, we considered the possibility that IL-10 produced by any of these donor cell populations might play a role in tolerance induction in our model. We therefore compared the outcome of allogeneic BMT with our conditioning regimen using either wt or IL-10−/− BM donors. No difference was found between the ability of wt or IL-10−/− BM to induce multilineage chimerism (Fig. 5), indicating that donor-derived IL-10 is not required for tolerance induced by allogeneic BMT and anti-CD154.

Early donor-specific CML tolerance is achieved by BMT with 3 Gy of TBI and anti-CD154

To further analyze the ability of various cell types to induce tolerance in our model, we transplanted fractionated BMC populations. Since donor stem cells are not transplanted to all groups with this approach, long-term chimerism cannot be used as a readout of tolerance. We therefore used short-term measures of tolerance in these animals, including the level of early (day 7) splenic chimerism in the granulocyte and B cell lineages. Mice transplanted...
with our regimen were compared with a group of mice receiving the same regimen without anti-CD154. Fig. 6A shows that chimerism was undetectable by day 7 after BMT in the group not receiving anti-CD154, indicating that rejection occurs in the absence of anti-CD154, consistent with our previous results (15). We recently demonstrated in chimeras prepared with our regimen that donor-specific unresponsiveness of CTLs is induced by 2 wk after BMT (11), concomitant with deletion of donor-reactive 2C CD8 T cells (10). To evaluate tolerance at an earlier time point, we analyzed CTL responses at day 8 after BMT in the spleens of mice treated with 3 Gy of TBI and anti-CD154 followed by allogeneic BMT. Unresponsiveness to donor targets with measurable responses to third-party Ags was observed (Fig. 6B), indicating that donor-specific unresponsiveness was established by day 8. We therefore used three parameters, early chimerism, donor-specific CML responses, and deletion of donor-reactive CD8 T cells, as measures of tolerance induced by different marrow fractions. This analysis was performed in two different strain combinations: B6→B10.S and B10.A→B6 (deletion of Ld-reactive 2C cells could only be tested in the latter).

B cells but not CD11b+ cells are sufficient to achieve early chimerism

Mice received 3 Gy of TBI with anti-CD154 followed by 25 × 10⁶ whole BMCs (WBM), MACS-selected CD19+ or Mac1+ cells or MACS-depleted CD19+ or Mac1+ cells from a fully allogeneic donor strain. Percentage of donor B cells and granulocytes in spleen on day 7 after BMT is shown. Pooled data from two to three experiments per strain combination are shown. A, Recipient mice were B10.S, donor mice were B6 (n = 6 per group from two experiments). B, Recipient mice were B6 and donor mice were B10.A (n = 10–12 per group from three experiments).

Donor BM-derived B cells induce early donor-specific CTL unresponsiveness

Fig. 8 shows the results of day 7 CML assays performed using spleens from mice transplanted with the marrow cell fractions described above, the chimerism of which is shown in Fig. 7. Recipients of Mac1+ marrow are not presented because of the impurity of the inoculum. All groups that achieved donor chimerism showed unresponsiveness to the donor in CML assays. In recipients of Mac1+ cells, which did not induce chimerism in either of the following experiments.
strain combination, anti-donor CTL reactivity could only be de-
tected in the B10.A
3
B6, but not in B6
3
B10.S strain combina-
tion. In the groups receiving CD19
/H11002
 cells, no anti-donor CTL re-
sponse could be detected in either strain combination. The groups
receiving WBM, CD19
/H11002
, or Mac1
/H11002
 cells (data not shown) showed
low responses to third-party targets, suggesting the possible exis-
tence of a CD19
/H11002
Mac1
/H11002
nonspecifically suppressive cell popula-
tion in the donor marrow. Most strikingly, isolated B cells induced
donor-specific unresponsiveness in all animals, which retained
strong third-party responses, indicating that donor BM-derived B
cells, although not absolutely required, are sufficient to reliably
induce early chimerism and donor-specific CML tolerance.

Donor BM-derived B cells induce early deletion of
donor-reactive CD8 T cells

We recently described a model system to analyze deletion of do-
nor-reactive CD8 T cells by using 2C/B6 syngeneic chimeric mice
(10). B6 mice were irradiated with 3 Gy of TBI and then trans-
planted with syngeneic BMCs from TCR-transgenic syngeneic
mice expressing the alloreactive 2C TCR on CD8 T cells. This
TCR recognizes the L
4
molecule with high affinity (12). The re-
sulting 2C/B6 chimeras express the transgenic receptor on 5–30% of
their peripheral CD8 T cells, allowing analysis of deletion of a
donor-specific CD8 T cell population while retaining a polyclonal
response. Eight weeks after syngeneic 2C BMT, these mice were
then treated with 3 Gy of TBI and anti-CD154 followed by
BMT from donors either expressing (B10.A) or not expressing
(B10.S) the L
4
molecule. Our previous studies with this system

FIGURE 8. Donor B cells induce donor-specific unresponsiveness in CML assay 7 days after BMT. Recipient B10.S or B6 mice were conditioned as described in Materials and Methods, then transplanted with 25 × 10^6 whole BMCs (WBM), MACS-selected CD19
/H11001
 or Mac1
/H11001
 cells or MACS-depleted
CD19
/H11002
 from fully MHC-mismatched B6 or B10.A donor mice as indicated. Cytotoxic activity in whole splenocytes on day 7 after BMT was measured in a 51Cr release assay against donor B6 or third-party B10.RIII targets after a 5-day restimulation culture with the respective stimulators. Each line indicates mean ± SEM of percent specific lysis for two to three different experiments. Upper row, Recipient mice were B10.S, donor mice were B6 (n = 6 per group from two experiments); lower row, recipient mice were B6 and donor mice were B10.A (n = 8–12 per group from three experiments).

FIGURE 9. B cells induce early deletional CD8 T cell tolerance. Recipient B6 mice expressing a transgenic donor-specific alloreactive TCR on a fraction of recipient CD8 T cells were generated (2C/B6 chimeras; for details, see Materials and Methods). Eight weeks later, these 2C/B6 chi-
meras received conditioning with TBI and anti-CD154 and were then trans-
planted with 25 × 10^6 whole BMCs (WBM), MACS-selected CD19
/H11001
 or
Mac1
/H11001
 cells or MACS-depleted CD19
/H11002
 cells from fully MHC-mismatched
ligand-bearing B10.A donor mice (L
4
positives; □ indicating B cell-con-
taining and ○ indicating B cell-depleted grafts) or whole BMCs from
non-ligand-bearing B10.S donor mice (L
4
negative; □). The percentage of
2C
/H11001
 cells among splenic CD8 T cells was determined by FACS on day 7
after BMT and then normalized to the percentage of 2C TCR expression
among peripheral blood CD8 T cells 1 wk before BMT. Mean ± SEM are shown for five animals per group.
demonstrated deletion of donor-reactive CD8 T cells in recipients of this protocol as early as day 7 after BMT (10), consistent with results using a different transgenic TCR in a different model (16). We now analyzed the capacity of isolated BM cell fractions to induce deletion of donor-reactive 2C CD8 T cells (Fig. 9). Mice that were transplanted with cell fractions containing B cells (WBM, CD19<sup>+</sup>) showed a profound deletion of 2C cells by day 7, whereas recipients of fractions not containing B cells showed less (CD19<sup>−</sup>) or no (Mac1<sup>+</sup>) 2C deletion compared with the nonspecific control (B10.S). Thus, donor BM-derived B cells are able to induce profound, early deletional CD8 T cell tolerance after BMT with anti-CD154.

Discussion

We have investigated the role of donor MHC class II expression and individual marrow cell populations in the induction of stable mixed chimerism and tolerance using a protocol consisting of 3 Gy of TBI on day -1 and anti-CD154 mAb on day 0 followed by fully MHC-mismatched allogeneic BMT (11). We demonstrate here that MHC class II expression on donor BM-derived APCs is critical for CD8 cell tolerance. Furthermore, we found that a transplant consisting of purified B cells is sufficient to induce early donor-specific CD8 tolerance, as measured by donor chimerism, donor-specific unresponsiveness in CML assays, and deletion of donor-reactive CD8 T cells (2C/B6 model) by 7 days after BMT. Analysis of chimerism and tolerance in experiments using B cell-deficient BM (either ex vivo depleted or gene targeted) suggests that B cells are sufficient, but not required, for rapid deletional tolerance of alloreactive peripheral CD8 cells. We also demonstrate that donor BM-derived CD11c<sup>+</sup> DCs and donor-derived T cells are not necessary to establish durable chimerism and that CD11b<sup>+</sup> cells are incapable of inducing even initial chimerism or tolerance.

A number of studies have investigated the role of B cells for T cell tolerance induction to self and foreign Ags. Naive (but not memory) T and B cell tolerance to a soluble protein Ag can be induced with small resting B cells as APCs (17–19). In a cardiac allograft model, donor B cell pretreatment combined with depletion of recipient B cells by anti-IgD mAb prolonged graft survival times indefinitely (20). Injection of syngeneic B cells coated with OVA was shown to induce OVA-specific deletional CD8 T cell tolerance (21). This was recently confirmed using retrovirally transduced B cells expressing the specific Ag, which led to peripheral, but not intrathymic CD8 tolerance (22). Thus, the ability of donor B cells to rapidly induce donor-specific CD8 unresponsiveness in our model is consistent with previous findings. The kinetics of deletion in our 2C/B6 model (10) are also comparable to those in the OVA model. However, in contrast to a recent study in the heart transplant model (23), early tolerance of CD8 cells in BMT recipients was critically dependent on CD4 cell recognition of donor class II MHC molecules, whereas in the heart allograft model donor MHC class II was dispensable. Taken together, the present data suggest that presentation of both class I and class II MHC by donor B cells to recipient CD8 and CD4 cells, respectively, may facilitate the CD4–CD8 interactions required for CD8 tolerance. One study suggested that the type of B cell activation may influence their ability to tolerate CD8 T cells, as B cells activated with LPS, but not those activated with anti-CD40/anti-Ig, were tolerogenic, with surface TGF-β expression and IL-10 production as possible mediators (24). Although blockade of CD154-CD40 interactions undoubtedly plays a critical role in rendering donor B cells tolerogenic, the molecular mechanism by which B cells promote CD8 cell tolerance has not been identified in our studies. We tested the role of donor IL-10 by using IL-10 knockout donor BM and found no difference in the ability to induce tolerance compared with wt donors in our model. In a preliminary experiment, we were also unable to detect a role for TGF-β in tolerance induction (C. Gibbons and M. Sykes, unpublished data).

DCs, initially described as the most potent inducers of adaptive immune responses, have recently attracted attention as mediators of tolerance to self and foreign Ags (25). “Tolerogenic DCs” have an immature phenotype, with low expression of MHC class II and costimulatory molecules such as CD80 and CD86 (26). These cells efficiently endocytose Ag and can induce T cell anergy or T regulatory cells in vivo, depending on the experimental conditions. However, there is no prior information on the requirement for donor-derived DCs in the context of allogeneic BMT. A recent in vitro study showed that immature allogeneic DCs induced specific T cell tolerance to a subsequent challenge with immunogenic DCs. Furthermore, recipient DCs loaded with donor apoptotic cells were able to “cross-tolerize” the direct alloantigen presentation pathway (27). In some cases DCs may promote T cell tolerance by producing IDO (28), but this pathway did not appear to be involved in a mixed chimerism model related to the one examined here (29). In the context of allogeneic BMT, Kaufman et al. (30) described a facilitating cell (FC) population in the donor marrow, which is CD8<sup>−</sup>CD3<sup>+</sup>TCR<sup>+</sup> and promotes engraftment of hematopoietic stem cells without the capacity to induce GVHD. The same group later reported induction of T regulatory cells by FCs (31) and found that this population included 65% CD11c<sup>+</sup> cells resembling precursor plasmacytoid DCs (32). However, the FC population is a very small fraction of BM whose practical utility may be limited. Our study suggests that with a minimal conditioning regimen for fully mismatched allogeneic BMT, donor-derived CD11c<sup>+</sup> DCs are not necessary for induction of multilineage mixed chimerism. Moreover, since at least some of these cells should be included in the Mac1<sup>+</sup> marrow population, our data indicate that they are not effective at inducing early peripheral CD8 cell tolerance. Nevertheless, it is possible that FCs may promote the partial 2C deletion and initial chimerism seen in recipients of CD19-negative BM cells.

Macrophages have been implicated in tolerance induction to a foreign protein Ag (33) and peritoneal macrophages were reported to facilitate engraftment of allogeneic hematopoietic stem cells in rats (34). Monocytes can differentiate into DCs and therefore potentially exert similar functions as described for DCs (35). In vitro studies revealed that blockade of the B7-CD28 costimulatory pathway leads to an alternative type of macrophage activation which mediates suppression of T cell responses (36). In this study, we show that a BM-derived highly purified CD11b<sup>+</sup> (Mac1<sup>+</sup>) population was not able to induce early chimerism. One simple explanation would be that these cells, which may be short-lived, were not present in sufficient numbers by 7 days after BMT. Alternatively, the Mac1<sup>+</sup> BM fraction, which contains myeloid cells at various stages of differentiation, may not contain sufficient mature monocytes to exert tolerogenic effects (37). In addition, strong early anti-donor CML responses were seen in at least one strain combination, suggesting active rejection of these cells. Although our data suggest that donor monocytes/macrophages are insufficient to induce early chimerism and tolerance, they do not indicate whether or not this population is necessary, since MACS depletion of CD11b<sup>+</sup> cells, which represent ~40% of total BMCs, was only ~70% efficient.

GVHD induced by donor T cells is a major obstacle to the use of BMT to induce donor-specific tolerance in the HLA-matched and especially the HLA-mismatched transplant setting (38). Unfortunately, depleting T cells from the donor graft to avoid GVHD reduces the efficiency of engraftment (39, 40). In the context of
allograft tolerance, it is therefore important to know whether T cells are an important component of the donor BM for tolerance induction. Furthermore, it has been reported that activated T cells may express MHC class II (41), so that T cells could theoretically be the source of donor class II promoting tolerance in our model. However, the results of our studies using either ex vivo T cell depletion or gene-targeted T cell-deficient donor mice demonstrate that donor T cells are not necessary to induce or maintain chimerism or skin graft tolerance with our costimulatory blockade-based regimen. Umemura et al. (42) have obtained similar results using CD4−, CD8−, and CD4/8 double-deficient mice as BM donors in a protocol involving treatment with antilymphocyte serum followed by allogeneic BMT and rapamycin. In contrast, mice receiving sublethal TBI followed by T cell-depleted BMT achieved chimerism that was not accompanied by skin graft tolerance, whereas T cell-replete BMT assured skin graft tolerance (43). Another study showed that central T cell tolerance in lethally irradiated recipients can be achieved by Ag-expressing T cells in the presence of isolated class I MHC disparity (44), but this study did not address the necessity of donor T cells, which have been shown in other models to be dispensable for central tolerance induction (B. Nikolic and M. Sykes, unpublished data). Our results are consistent with those in a mixed chimerism model using busulfan and anti-CD154 conditioning, in which T cell-depleted marrow was effective in inducing tolerance (45). Taken together, these findings indicate that donor T cells are not necessary for induction or maintenance of stable mixed hematopoietic chimerism or central or peripheral tolerance in regimens where the host-vs-graft response is adequately suppressed or tolerated.

Taken together, our study demonstrates a potent effect of donor-derived B cells for induction of early CD4 and CD8 T cell tolerance in a fully allogeneic BMT model using a minimal anti-CD154-based conditioning regimen. Donor B cells were sufficient, but not required in both strain combinations tested to induce peripheral T cell tolerance. In the same model, donor-derived T cells are dispensable. Together with the finding that MHC class II expression on donor BM is critical for CD8 tolerance, the results of this study suggest that MHC class II presented by any hematopoietic cell can play this tolerance-promoting role and that no particular cell type is absolutely required.

One possible explanation for the ability of multiple sources of donor class II MHC to promote tolerance is that exosomes, which can be produced by B cells (46, 47) and DCs (48), present MHC class II in a manner that is tolerogenic to T cells. Circulating donor class II-bearing exosomes have been implicated in an oral tolerance model (49), and exogenous donor MHC-bearing exosome administration promoted graft survival in a rat heart transplantation model (50). Indeed, exosome production is induced by B cells upon interactions with CD4 T cells recognizing them (51). However, a role for soluble exosomes would not readily explain the role for CD4 cells in tolerizing CD8 cells in our model. We think that direct killing of donor-reactive CD8 cells by recipient CD4 cells is also an unlikely explanation for this requirement, as our published studies show that the early partial deletion of peripheral donor-reactive CD8 cells is similar in control and CD4-depleted mice, and that it is only after marrow rejection that donor-reactive CD8 cells appear to expand in the CD4-depleted group (10). Thus, we favor a model in which CD4-CD8 interactions involve APC populations that are conditioned by CD4 cells to be tolerogenic for CD8 cells, culminating in CD8 cell deletion. It is possible that pickup and presentation of intact donor MHC molecules by recipient APCs promotes tolerance induction in our model and that donor APC populations are not involved, consistent with the observed requirement for recipient but not donor B7 molecules in achieving tolerance (J. Kurtz, F. Raval, C. Vallot, J. Der, and M. Sykes, manuscript submitted for publication). Such pickup may occur through the production of membrane exosomes (52). Further studies in progress should help to address these possibilities.

These findings have important implications for an understanding of tolerance mechanisms and for the clinical use of hematopoietic cell transplantation to induce organ allograft tolerance, for which nontoxic regimens are required. Combined transplantation of BM and kidney can induce donor-specific tolerance and avoid long-term immunosuppression in monkeys and humans (5, 53). Donor kidneys have survived without immunosuppression for >10 years in monkeys and 8 years in patients (6, 7, 54). It is interesting to note that the monkeys and some of the patients lost initial mixed hematopoietic chimerism over weeks to months, but retained the kidney nonetheless; therefore, only transient donor chimerism seems to be necessary to achieve donor-specific tolerance when kidney transplantation and BMT are performed simultaneously. Thus, in light of our findings it would be interesting to consider a solid organ transplant combined with costimulatory blockade and BMT vigorously depleted of donor T cells, but enriched with a tolerogenic, non-self-renewing cell population such as B cells to minimize the risk of GVHD.

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Disclosures

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