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TLR Agonists Prevent the Establishment of Allogeneic Hematopoietic Chimerism in Mice Treated with Costimulation Blockade

David M. Miller,* Thomas B. Thornley,* Todd Pearson,* Annie J. Kruger,* Masahiro Yamazaki,* Leonard D. Shultz,¶ Raymond M. Welsh,‡ Michael A. Brehm,‡ Aldo A. Rossini,*§ and Dale L. Greiner,‡§

Activation of TLR4 by administration of LPS shortens the survival of skin allografts in mice treated with costimulation blockade through a CD8 T cell-dependent, MyD88-dependent, and type I IFN receptor-dependent pathway. The effect of TLR activation on the establishment of allogeneic hematopoietic chimerism in mice treated with costimulation blockade is not known. Using a costimulation blockade protocol based on a donor-specific transfusion (DST) and a short course of anti-CD154 mAb, we show that LPS administration at the time of DST matures host alloantigen-presenting dendritic cells, prevents the establishment of mixed allogeneic hematopoietic chimerism, and shortens survival of donor-specific skin allografts. LPS mediates its effects via a mechanism that involves both CD4+ and CD8+ T cells and results from signaling through either the MyD88 or the type I IFN receptor pathways. We also document that timing of LPS administration is critical, as injection of LPS 24 h before treatment with DST and anti-CD154 mAb does not prevent hematopoietic engraftment but administration the day after bone marrow transplantation does. We conclude that TLR4 activation prevents the induction of mixed allogeneic hematopoietic chimerism through type I IFN receptor and MyD88-dependent signaling, which leads to the up-regulation of costimulatory molecules on host APCs and the control of alloreactive T cells. These data suggest that distinct but overlapping cellular and molecular mechanisms control the ability of TLR agonists to block tolerance induction to hematopoietic and skin allografts in mice treated with costimulation blockade.

host T cells and can be mediated through both MyD88 and type I IFN receptor signaling pathways.

Materials and Methods

Animals

BALB/c (H2b) and C57BL/6 (H2b) mice were obtained from Charles River Laboratories or The Jackson Laboratory. C57BL/10ScSnJ (H2b) and C57BL/6.SDBlb/c (H2d) mice were produced by crossing C57BL/10ScSnJ (H2b) and C57BL/6.SDBlb/c (H2d). MyD88 and bred at the University of Massachusetts Medical School. C57BL/6 (H2b) or C57BL/6.129S2-Ifnar1tm1At mice was determined by dual-labeling with Abs to H2-Kb and H2-Kd as graft was necrotic.

Full-thickness skin grafts 1–2 cm in diameter were obtained from the tibiae, filtered through sterile 70-% nylon mesh (BD Biosciences), and stored at −20°C. Mice were injected i.p. with 0.5 mg of lipopolysaccharide and dye in a volume of 0.5 ml of D-PBS. Recombinant mouse IFN-β was obtained from PBL IFN Source and injected i.p. at the indicated dose on the day of DST and the first injection of anti-CD154 mAb.

Preparation and injection of TLR agonists and recombinant IFN-β

LPS from Escherichia coli 0111:B4 (Sigma-Aldrich) was purified as previously described (26), except that phenol–PBS phase separation was conducted at 2000 × g for 30 min to accommodate larger volumes. Purified LPS was suspended in Dulbecco’s PBS (D-PBS) and stored at 4°C until used, with an assumed 10% loss during purification (26). Poly(I:C) (Sigma-Aldrich) was dissolved in D-PBS at a concentration of 1 mg/ml. Stock was filtered through 0.45-μm sterile nylon mesh (BD Biosciences) and stored at −20°C until used. PAM3Cys (EMC Microcollections) was reconstituted in PBS and was stored at −20°C. Mice were injected i.p. with 2 mg of LPS and 0.5 mg of poly(I:C) (10 mg/ml) or LPS and 5 mg of PAM3Cys (10 mg/ml) and 5 mg of PAM3Cys (10 mg/ml).

Statistical methods

Statistical analyses were made using GraphPad Prism Software (version 4.0). Comparisons of these two means used unpaired t tests. Comparisons of two means used unpaired t tests without assuming equal variance. Skin allograft survival curves were generated by the Kaplan-Meier method and compared by the
log-rank test. Duration of allograft survival is presented as the median. Values of \( p < 0.05 \) were considered statistically significant.

**Results**

**TLR agonists administered at the time of DST and anti-CD154 mAb prevent the establishment of allogeneic hematopoietic chimerism and shorten skin allograft survival**

We first investigated the hypothesis that TLR agonists given during tolerance induction with DST and anti-CD154 mAb would prevent the establishment of allogeneic hematopoietic chimerism. To test this, C57BL/6 mice were injected with our standard costimulation blockade protocol consisting of injection of BALB/c DST and anti-CD154 mAb on day \(-7\) relative to bone marrow and skin transplantation. Groups 5, 6, 7, 8, 11, and 12 were given three doses of 0.5 mg of anti-CD8 mAb i.p. on days \(-10\), \(-9\), and \(-8\) relative to bone marrow and skin transplantation. Groups 9, 10, 11, and 12 were given three doses of 0.5 mg of anti-CD4 mAb i.p. on days \(-10\), \(-9\), and \(-8\) relative to bone marrow and skin transplantation. Hematopoietic chimerism was defined as \( > 0.10\% \) donor origin PBMCs 8 wk after bone marrow injection. The percentage of donor origin PBMCs is the mean \( \pm \) SD percentage of the levels observed in chimeric mice. For groups with no chimeric mice, we used the limit of detection \(<0.10\%\) to indicate a lack of chimerism.

**All alloantibodies are not generated following the administration of LPS during costimulation blockade**

A mechanism to prevent the establishment of allogeneic chimerism by LPS could be the production of alloreactive Abs. B lymphocytes express the LPS receptor TLR4 and can be activated directly by LPS to produce Abs (6). Given that alloantibodies are known to induce allograft rejection (28), we investigated whether alloantibodies were produced in mice treated with costimulation blockade and given LPS. C57BL/6 mice were left untreated, primed with BALB/c DST, or given BALB/c DST, anti-CD154 mAb, and grafted with BALB/c bone marrow without or with LPS treatment. As shown in Fig. 1A, completely untreated (naïve) mice had background levels of circulating alloantibodies, whereas mice primed with BALB/c DST developed high levels of alloantibodies. In contrast, mice treated with anti-CD154 mAb at the time of DST injection developed only low levels of alloantibodies, even in LPS-treated mice that had rejected BALB/c bone marrow grafts. These data show that the ability of LPS to prevent the establishment of hematopoietic chimerism is not due to alloantibody production.

**Cell populations required for LPS-mediated effects in mice treated with DST and anti-CD154 mAb**

Based on the absence of alloantibodies in mice treated with DST, anti-CD154 mAb, and LPS, we next investigated the cell populations that are responsible for LPS-mediated effects on the establishment of hematopoietic chimerism and skin allograft survival. **NK cells.** NK cells are known to be a barrier to the establishment of hematopoietic chimerism (29–31). To test the role of NK cells, we depleted C57BL/6 mice of NK cells and treated them with our standard costimulation blockade protocol without or with coinjection of LPS at the time of DST. In the absence of LPS, the majority of NK-cell-depleted mice became chimeric (11 of 13) and chimeric mice exhibited permanent skin allograft survival (Table I, group 3). In contrast, mice depleted of NK cells and treated with LPS on day \(-7\) failed to become chimeric and skin allograft survival was short (Table I, group 4). These data indicate that host NK cells, although a barrier to hematopoietic
chimerism, are not solely responsible for the detrimental effects of LPS on the establishment of chimerism.

**CD8⁺ cells.** We have observed that TLR agonists impair the deletion of alloreactive CD8⁺ T cells in mice treated with DST and anti-CD154 mAb and that host CD8⁺ T cells are required for LPS to shorten skin allograft survival in these mice (4). To determine whether CD8 T cells are also required for LPS to prevent the establishment of chimerism, C57BL/6.CD8α⁻/⁻ mice were treated with our standard costimulation blockade protocol without or with injection of LPS at the time of DST. As expected, hematopoietic chimerism and prolonged skin allograft survival was observed in the majority of C57BL/6.CD8α⁻/⁻ mice treated with DST and anti-CD154 mAb (Table I, group 13). Surprisingly, LPS was able to prevent chimerism in C57BL/6.CD8α⁻/⁻ mice, and skin survival was short (Table I, group 14).

Given this surprising result, we further tested the role of CD8⁺ T cells using wild-type C57BL/6 mice depleted of CD8⁺ T cells using an anti-CD8α mAb. C57BL/6 mice were depleted of CD8⁺ cells and treated with our standard costimulation blockade protocol without or with LPS at the time of DST injection. C57BL/6 mice depleted of CD8⁺ cells became chimeric and exhibited prolonged skin allograft survival (Table I, group 5). Again, mice depleted of CD8⁺ T cells treated with LPS on day −7 failed to become chimeric and skin allograft survival was short (Table I, group 6). These data indicate that host CD8⁺ T cells are not required for LPS to prevent the establishment of hematopoietic chimerism in mice treated with costimulation blockade.

**CD8⁺ plus NK cells.** Although the detrimental effect of LPS was not dependent on host NK cells or CD8⁺ cells, it was possible that either population on its own could prevent the establishment of hematopoietic chimerism and shorten skin allograft survival. To test this, mice were depleted of both NK and CD8⁺ cells and treated with DST and anti-CD154 mAb without or with LPS at the time of DST injection. In the absence of LPS treatment, mice developed chimerism and exhibited prolonged skin allograft survival (Table I, group 7). In contrast, coinjection of LPS at the time of DST in mice depleted of both CD8 and NK cells completely prevented chimerism and shortened skin allograft survival (Table I, group 8).

**CD4 plus CD8 cells.** We next tested the possibility that host CD4⁺ T cells were capable of mediating the rejection of BALB/c allografts. However, we have previously shown and confirmed here (Table I, group 9) that CD4⁺ T cells are required for the establishment of hematopoietic chimerism in mice treated with DST and anti-CD154 mAb, presumably as a requirement for tolerization of alloreactive CD8⁺ T cells (27). As expected, CD4⁺-depleted mice treated with costimulation blockade and treated with LPS failed to become chimeric and exhibited short skin allograft survival (Table I, group 10). Therefore, we depleted both CD4⁺ and CD8⁺ cells and then treated the mice with our standard costimulation blockade protocol. Mice treated with both anti-CD4 and anti-CD8 mAb developed chimerism and exhibited permanent skin graft survival (Table I, group 11). Chimerism and prolonged skin graft survival were also observed in CD4 and CD8-depleted mice given LPS at the time of DST (Table I, group 12). These data suggest that LPS can mediate its detrimental effects through either alloreactive CD4⁺ or CD8⁺ T cells.

**LPS administration leads to the generation of effector/memory alloreactive CD8⁺ T cells**

Given that the removal of host CD8⁺ T cells is required for the establishment of chimerism, we next investigated whether effector/memory CD8⁺ T cells were generated in mice treated with LPS, costimulation blockade, and transplanted with allogeneic bone marrow. C57BL/6 mice treated with costimulation blockade and transplanted with BALB/c bone marrow exhibited very low levels of BALB/c-reactive IFN-γ-producing CD8⁺ CD11ahigh T cells 2 wk after bone marrow transplantation. In contrast, mice given LPS injection at the time of DST developed high levels of IFN-γ-producing CD8⁺ CD11ahigh T cells (Fig. 1, B and C).

**LPS up-regulates costimulatory molecules on cells in the DST in mice treated with anti-CD154 mAb**

We have previously shown that anti-CD154 mAb prevents up-regulation of CD80 on APCs in the DST (32). To investigate the activation status of cells in the DST following administration of LPS treatment, spleen cells from C57BL/6 mice were analyzed by

### Table II. TLR4, Type I IFN, and MyD88 are important in the LPS-mediated prevention of costimulation blockade-induced hematopoietic chimerism

<table>
<thead>
<tr>
<th>Group</th>
<th>Host</th>
<th>TLR Agonist</th>
<th>Chimerism Frequency</th>
<th>Donor Origin PBMCs at 8 wk (%)</th>
<th>MST of Skin Grafts (days)</th>
<th>MST of Skin Grafts in Nonchimeric Mice (days)</th>
<th>MST of Skin Grafts in Chimeric Mice (days)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>C57BL/6</td>
<td>Poly(I:C)</td>
<td>0/20 (0%)</td>
<td>&lt;0.10</td>
<td>9*</td>
<td>9</td>
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<td>2</td>
<td>C57BL/6</td>
<td>PAM</td>
<td>0/3 (6%)</td>
<td>1.76 ± 0.34</td>
<td>125</td>
<td>70</td>
<td>&gt;176</td>
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<td>3</td>
<td>C57BL/10</td>
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<td>3/7 (42.5%)</td>
<td>&lt;0.10</td>
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<td>11</td>
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<tr>
<td>4</td>
<td>C57BL/10</td>
<td>LPS</td>
<td>0/12 (0%)</td>
<td>2.8 ± 0.4</td>
<td>176</td>
<td>18</td>
<td>&gt;195</td>
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<td>5</td>
<td>C57BL/10.TLR4⁻/⁻</td>
<td>None</td>
<td>10/13 (76.9%)</td>
<td>1.94 ± 1.19</td>
<td>&gt;195</td>
<td>10</td>
<td>&gt;195</td>
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<tr>
<td>6</td>
<td>C57BL/10.TLR4⁻/⁻</td>
<td>LPS</td>
<td>9/10 (90.0%)</td>
<td>&lt;0.10</td>
<td>11**</td>
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<td>C57BL/6.MyD88⁻/⁻</td>
<td>None</td>
<td>4/5 (80.0%)</td>
<td>2.8 ± 0.4</td>
<td>176</td>
<td>18</td>
<td>&gt;195</td>
</tr>
<tr>
<td>8</td>
<td>C57BL/6.MyD88⁻/⁻</td>
<td>LPS</td>
<td>0/9 (0.0%)</td>
<td>&lt;0.10</td>
<td>11**</td>
<td>11</td>
<td>N/A</td>
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<tr>
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<td>C57BL/6.MyD88⁻/⁻</td>
<td>None</td>
<td>13/15 (87.0%)</td>
<td>2.32 ± 2.14</td>
<td>&gt;218</td>
<td>28</td>
<td>&gt;218</td>
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<td>LPS</td>
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<td>80*</td>
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<td>&gt;218</td>
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<tr>
<td>11</td>
<td>C57BL/6.MyD88⁻/⁻</td>
<td>Poly(I:C)</td>
<td>5/12 (41.7%)</td>
<td>2.22 ± 0.97</td>
<td>84</td>
<td>16</td>
<td>&gt;218</td>
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<tr>
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<td>PAM</td>
<td>0/4 (0.0%)</td>
<td>&lt;0.10</td>
<td>10º</td>
<td>10</td>
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</tr>
<tr>
<td>13</td>
<td>C57BL/6.MyD88⁻/⁻</td>
<td>None</td>
<td>4/5 (80.0%)</td>
<td>4.13 ± 0.98</td>
<td>&gt;140</td>
<td>50</td>
<td>&gt;140</td>
</tr>
<tr>
<td>14</td>
<td>C57BL/6.MyD88⁻/⁻</td>
<td>LPS</td>
<td>5/8 (62.5%)</td>
<td>7.37 ± 1.08</td>
<td>&gt;140</td>
<td>57</td>
<td>&gt;140</td>
</tr>
</tbody>
</table>

* All mice were treated with a BALB/c DST, bone marrow, and anti-CD154 mAb according to our standard protocol without or with an i.p. injection of 100 μg of LPS, 50 μg of poly(I:C), or 100 μg of Pam3Cys. Groups 1–6 and 9–16 also received a BALB/c skin graft on day 0, the day of bone marrow transplant. Hematopoietic chimerism was defined as >0.10% donor origin PBMCs 8 wk after bone marrow injection. The percentage of donor origin PBMCs is the mean ± SD percentage of the levels observed in chimeric mice. For groups with no chimeric mice, we used the limit of detection (<0.10) to indicate a lack of chimerism. Differences were evaluated using a two-tailed Student’s t test. Data are representative of at least three independent experiments. Group Host TLR Agonist Chimerism Frequency Donor Origin PBMCs at 8 wk (%) MST of Skin Grafts (days) MST of Skin Grafts in Nonchimeric Mice (days) MST of Skin Grafts in Chimeric Mice (days)
flow cytometry 15 h after treatment with BALB/c DST, anti-CD154 mAb, without or with LPS injected at the time of DST. Gating on donor (H2-Kb−2-Kd+) cells (Fig. 2A), the DST of mice treated with anti-CD154 mAb and LPS had a modest, but statistically significant increase in the expression of CD80 (MFI = 9.6 ± 0.8, n = 4) compared with mice treated only with anti-CD154 (MFI = 8.3 ± 0.4; n = 4; p = 0.025; Fig. 2B). Expression of CD86 was enhanced 2-fold on the DST of mice treated with anti-CD154 mAb and LPS (MFI = 36.0 ± 1.1, n = 4) compared with mice treated only with anti-CD154 mAb.
These data suggest that LPS increases the expression of costimulatory molecules on cells in the DST. LPS up-regulates expression of costimulatory molecules on host alloantigen-presenting dendritic cells (DCs)

Although LPS treatment appears to increase expression of CD80/86 on cells in the DST, we have observed that CD80/86 expression on the DST is not required to rescue alloreactive CD8 T cells from costimulation blockade-induced deletion in the presence of LPS (18). Therefore, we next examined the effect of LPS on host APC maturation. C57BL/6 mice were injected with CFSE-labeled BALB/c DST and treated with anti-CD154 mAb without or with injection of LPS at the time of DST. Host DCs that have phagocytosed the cells in the DST can be detected by gating on H2-Kb−2-Kd− cells that are seen in A. These cells represent cells in the BALB/c DST. The right panels are histograms that summarize the MFI + 1 SD of CD80 (B) and CD86 (C) expression. Data contain four mice per group. #, p = 0.025; *, p < 0.0001.

**FIGURE 2.** LPS administration induces up-regulation of costimulatory molecules on cells in the DST in mice treated with costimulation blockade. C57BL/6 mice were injected with 10^6 BALB/c DST and 0.5 mg of anti-CD154 mAb without or with an i.p. injection of LPS. Fifteen hours later, splenocytes were recovered and stained with Abs to H2-Kb (host), H2-Kd (donor), and CD80 or CD86 and analyzed by flow cytometry. A, Representative flow cytometry dot plots showing host H2-Kb and donor H2-Kd staining. A group of nontransplanted mice were used as negative controls for H2-Kd staining. B and C, Left panels show a representative histogram of the MFI of CD80 (B) and CD86 (C) expression on the H2-Kb+/−2-Kd− cells that are seen in A. These cells represent cells in the BALB/c DST. The right panels are histograms that summarize the MFI + 1 SD of CD80 (B) and CD86 (C) expression. Data contain four mice per group. #, p = 0.025; *, p < 0.0001.
we observed that essentially all of the alloantigen-containing host DCs were CD8α⁺. These APCs have been shown to be presenting alloantigen by the indirect Ag presentation pathway (33). Gating on H2-Kb⁺, CD8α⁺, CD11c⁺, and CD8β, and analyzed by flow cytometry. For each panel, the left most dot plot shows the cells that were considered “viable” based on Live/Dead blue staining. The dot plot second from the left shows the cells that were considered of host origin based on staining with H-2Kb. The dot plot second from the right shows the cells that were considered DCs based on staining with CD11c. The dot plot on the right shows DCs that have engulfed CFSE⁺ DST (the alloantigen-presenting DCs or “apDCs”). A, Representative flow cytometry dot plots of the input cell, i.e., CFSE-labeled BALB/c splenocytes, before injection. B, Representative flow cytometry dot plots of splenocytes from an un.injected C57BL/6 mouse. These splenocytes do not contain a CFSE⁺ population. C, Representative flow cytometry dot plots of splenocytes from a C57BL/6 mouse injected with CFSE-labeled BALB/c DST. This panel demonstrates that the spleen of animals injected with CFSE-labeled DST contain a population of host DCs that have phagocytosed CFSE-labeled DST. D, A “back gate” of the CFSE⁺CD11c⁺H2-Kb⁺ cells from C (i.e., host DCs that have phagocytosed DST). This panel indicates that the host alloantigen-presenting DCs are found in the mid-to-upper right of the Host Cell Gate (second panel from the left). That population is only present in the spleen of animals transfused with DST.

FIGURE 3. Gating scheme for in vivo tracking of phagocytosed DST. C57BL/6 mice were injected with 10 x 10⁶ CFSE-labeled BALB/c DST and 0.5 mg of anti-CD154 mAb without or with an i.p. injection of LPS. Sixteen hours later, splenocytes were harvested, stained with Live/Dead blue, Abs to H2-Kb (host), CD8α, CD11c, and CD8β, and analyzed by flow cytometry. For each panel, the dot plot on the left shows the cells that were considered “viable” based on Live/Dead blue staining. The dot plot second from the left shows the cells that were considered of host origin based on staining with H-2Kb. The dot plot second from the right shows the cells that were considered DCs based on staining with CD11c. The dot plot on the right shows DCs that have engulfed CFSE⁺ DST (the alloantigen-presenting DCs or “apDCs”). A, Representative flow cytometry dot plots of the input cell, i.e., CFSE-labeled BALB/c splenocytes, before injection. B, Representative flow cytometry dot plots of splenocytes from an un injected C57BL/6 mouse. These splenocytes do not contain a CFSE⁺ population. C, Representative flow cytometry dot plots of splenocytes from a C57BL/6 mouse injected with CFSE-labeled BALB/c DST. This panel demonstrates that the spleen of animals injected with CFSE-labeled DST contain a population of host DCs that have phagocytosed CFSE-labeled DST. D, A “back gate” of the CFSE⁺CD11c⁺H2-Kb⁺ cells from C (i.e., host DCs that have phagocytosed DST). This panel indicates that the host alloantigen-presenting DCs are found in the mid-to-upper right of the Host Cell Gate (second panel from the left). That population is only present in the spleen of animals transfused with DST.

marked up-regulation of MHC class I (MFI = 92.104 ± 6.419; n = 4) and both CD80 (MFI = 2.461 ± 371; n = 3) and CD86 (MFI = 5.032 ± 1.210; n = 3) compared with non-LPS-treated controls (H-2Kb MFI = 54.565 ± 8.207; n = 4, p < 0.001, Fig. 4A; CD80
MFI = 966 ± 61; n = 3; p < 0.01, Fig. 4B; CD86 MFI = 1,547 ± 76; n = 3; p < 0.01, Fig. 4C). These data demonstrate that LPS increases the maturation state of host DCs that have phagocytosed alloantigen.

**TLR4 expression is required on host cells for the effects of LPS on the establishment of hematopoietic chimerism in mice treated with costimulation blockade**

Previously, we have shown that LPS administration mediates its effects on skin allograft survival in mice treated with costimulation by engaging TLR4 on cells of the host (4). Therefore, to test whether engagement of TLR4 on host cells was also required for LPS to prevent the induction of hematopoietic chimerism, C57BL/10 and C56BL/10 mice were treated with BALB/c DST, anti-CD154 mAb, and transplanted with BALB/c bone marrow and skin allografts without or with injection of LPS at the time of costimulation blockade.

Treatment of wild-type C57BL/10 mice with costimulation blockade led to chimerism (three of seven) and prolonged skin graft survival (median survival time (MST) = 125 days; Table II, group 3), which were both prevented by administration of LPS (0 of 12 became chimeric; skin graft survival MST = 11 days; p < 0.05; Table II, group 4). In contrast, C57BL/10.TLR4<sup>−/−</sup> mice treated with DST and anti-CD154 mAb in the absence (10 of 13) or presence (9 of 10) of LPS also became chimeric (p = NS; Table II, groups 5 and 6) and exhibited prolonged skin graft survival (MST >195 days for LPS-treated mice vs MST = 176 days for control mice; p = NS; Table II, groups 5 and 6). These data suggest that TLR4 expression on host cells is required for the effects of LPS on the establishment of hematopoietic chimerism in mice treated with costimulation blockade.

**The effect of LPS on the establishment of hematopoietic chimerism in mice treated with costimulation blockade is not dependent through the MyD88-dependent pathway**

LPS can signal through two distinct pathways following ligation of TLR4, the MyD88-Toll/IL-1 receptor domain-containing adaptor protein (TIRAP) pathway, and the TRIF-related adaptor molecule (TRAM)-Toll/IL-1 receptor domain-containing adaptor protein inducing IFN-β (TRIF) pathway (7, 9). Therefore, we next examined whether the MyD88-TIRAP pathway was required for LPS to prevent the establishment of hematopoietic chimerism.

To test this, B6.MyD88<sup>−/−</sup> mice were treated with our standard costimulation blockade protocol without or with an injection of LPS at the time of DST and transplanted with BALB/c bone marrow. B6.MyD88<sup>−/−</sup> mice treated with DST and anti-CD154 mAb became chimeric (four of five); however, mice treated with LPS did not (zero of nine, p = 0.0015; Table II, groups 7 and 8). These data indicate that signaling through the MyD88 adaptor molecule is not required for the effects of LPS on the establishment of hematopoietic chimerism in mice treated with costimulation blockade.

**LPS treatment induces the production of type I IFN, which is sufficient to prevent the establishment of hematopoietic chimerism in mice treated with costimulation blockade**

Because the MyD88-TIRAP pathway was not required for LPS to prevent chimerism, we hypothesized that the MyD88-independent TRAM-TRIF pathway may be important. TLR4 signaling through the TRAM-TRIF pathway leads to the up-regulation of type I IFNs and it has been hypothesized that TLR4-dependent induction of type I IFN is entirely mediated through a MyD88-independent pathway (9). We first quantified the levels of IFN-α/β in the serum of wild-type C57BL/6 mice treated with costimulation blockade at various times after LPS administration using a standard IFN-α/β bioassay (25). IFN-α/β was not detected in untreated mice and only low levels were detected in mice treated with DST and...
anti-CD154 mAb at 8 h (5.0 ± 3.9 U/ml; Fig. 5). These low levels dropped to nearly undetectable levels 24 h after treatment (0.8 ± 2.1 U/ml). In contrast, mice treated with LPS had 3-fold higher levels of IFN-α/β at 8 h after treatment with DST, anti-CD154 mAb, and LPS (15.4 ± 6.0 U/ml, p < 0.05; Fig. 5). We also observed that levels of IFN-α/β at 24 h in mice treated with costimulation blockade and LPS were comparable to those in mice treated with DST and anti-CD154 mAb at 8 h (p = NS; Fig. 5). IFN-α/β was also detected at 24 h in the serum of B6/MyD88−/− mice treated with DST, anti-CD154 mAb, and LPS but not in B6. MyD88−/− mice treated with only DST and anti-CD154 mAb (22.5 ± 12 and 2.5 ± 2.9 U/ml, respectively, p < 0.05, n = 4), suggesting that LPS will induce IFN-α/β production independently of MyD88 signaling.

We next investigated whether administration of recombinant mouse IFN-β would recapitulate the effects of LPS administration. To test this, we transplanted C57BL/6 mice with BALB/c bone marrow and skin grafts using our standard costimulation blockade protocol without (group 1) or with 5.0 × 10⁴ U (group 2) or 7.5 × 10⁴ U (group 3) of IFN-β on day −7, the day of DST. Only one of three mice treated with 5.0 × 10⁴ U of IFN-β became chimeric, but two of three mice did exhibit prolonged skin graft survival (MST = 252 days; Table III). Mice treated with 7.5 × 10⁴ U of IFN-β on day −7 uniformly failed to become chimeric (zero of four; p < 0.05 vs group 1) and skin survival was short (MST = 36 days, p < 0.05 vs group 1; Table III). These data suggest that IFN-β by itself is sufficient to recapitulate the effects of LPS on the establishment of chimerism and prolongation of skin allograft survival when given at the time of tolerance induction.

**The TLR4−type I IFN pathways are important for LPS-mediated up-regulation of costimulatory molecules on host alloantigen-presenting DCs and for priming of host alloreactive CD8⁺ T cells**

Type I IFN has been reported to enhance DC maturation (34) and is important in the priming of naive T cells (35). We next hypothesized that induction of type I IFN by TLR4 activation may be critical for the up-regulation of costimulatory molecules on host alloantigen-presenting cells. To test this, wild-type C57BL/6 and B6. IFNAR1−/− mice were injected with CFSE-labeled BALB/c splenocytes and treated with anti-CD154 mAb without or with LPS injection. Fifteen hours later, spleen cells were recovered and analyzed for the expression of MHC class I and CD86. Alloantigen-presenting DCs from mice lacking the type I IFN receptor exhibited a significantly lower expression of...
MHC class I (H2-K\textsuperscript{b} MFI = 69,793 ± 7,852, p < 0.01; Fig. 6A) and the costimulatory molecule CD86 (CD86 MFI = 4,650 ± 1,066, p < 0.01; Fig. 6B) after injection of LPS compared with alloantigen-presenting DCs from wild-type mice (92,104 ± 6,419 and 6,487 ± 829, respectively). The expression of both molecules, however, was higher in B6.IFNAR\textsuperscript{−/−} mice treated with LPS when compared with their non-LPS-treated controls (p < 0.05), indicating that other factors may be involved in the maturation of host alloantigen-presenting DCs.

We next hypothesized that decreased DC activation in the mice lacking the type I IFN receptor may prevent the generation of alloreactive CD8\textsuperscript{+} effector/memory T cells. To test this, we performed intracellular flow cytometry for IFN-\gamma on CD8\textsuperscript{+} splenocytes isolated from C57BL/6 and B6.IFNAR\textsuperscript{−/−} mice 1 wk after treatment with our standard costimulation blockades and transplantation of BALB/c bone marrow without or with LPS. Interestingly, we observed that mice lacking the type I IFN receptor did not develop effector/memory CD8\textsuperscript{+} T cells when exposed to LPS, whereas wild-type C57BL/6 mice did (Fig. 6C). These data suggest that type I IFNs are required for DC induction of alloreactive CD8\textsuperscript{+} effector/memory T cells.

Type I IFN signaling is important for the effects of LPS on the establishment of hematopoietic chimerism in mice treated with costimulation blockades.

The observation that type I IFN was critical for the full maturation of host alloantigen-presenting DCs and for the priming of alloreactive CD8\textsuperscript{+} T cells prompted us to hypothesize that signaling through the type I IFN receptor would be required for the effects of LPS on chimerism in mice treated with costimulation blockades. To test this, B6.IFNAR\textsuperscript{−/−} mice were treated with DST and anti-CD154 mAb and transplanted with BALB/c bone marrow and skin without or with injection of LPS at the time of DST administration. As expected, B6.IFNAR\textsuperscript{−/−} mice not treated with LPS became chimeric (13 of 15) and chimeric mice exhibited permanent skin graft survival (MST = >218 days; Table II, group 9). Interestingly, some B6.IFNAR\textsuperscript{−/−} mice treated with LPS became chimeric (6 of 17; p = 0.0005 vs wild-type animals treated with LPS), and chimeric mice again exhibited permanent skin graft survival (MST = >218 days; Table II, group 10). However, the percentage of B6.IFNAR\textsuperscript{−/−} mice treated with LPS that became chimeric was lower than that achieved in B6.IFNAR\textsuperscript{−/−} not treated with LPS (p = 0.008; Table II, groups 9 and 10). These data suggest that although signaling through the type I IFN receptor is important, other factors may be involved.

In support of this interpretation, B6.IFNAR\textsuperscript{−/−} mice were also partially protected from the effects of poly(I:C), as some of the mice conditioned with costimulation blockades and transplanted with BALB/c bone marrow and skin became chimeric (5 of 12; p < 0.01 vs B6.IFNAR\textsuperscript{−/−} not treated with poly(I:C)) and chimeric mice also exhibited permanent skin graft survival (MST = >218 days; Table II, group 11). Interestingly, mice deficient in signaling through the type I IFN receptor were not protected from the effects of Pam3Cys (zero of four chimeric, MST = 10; Table II, group 12). These data indicate that signaling through the type I IFN receptor is important for the ability of some, but not all, TLR agonists to prevent the establishment of hematopoietic chimerism in mice treated with costimulation blockades.

<table>
<thead>
<tr>
<th>Group</th>
<th>TLR Agonist</th>
<th>Chimerism Frequency (%)</th>
<th>Donor Origin PBMCs at 8 wk (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>6/7 (85.7%)</td>
<td>3.20 ± 1.84</td>
</tr>
<tr>
<td>2</td>
<td>LPS (given on day −8)</td>
<td>6/7 (85.7%)</td>
<td>4.93 ± 2.04</td>
</tr>
<tr>
<td>3</td>
<td>LPS (given on day −7)</td>
<td>0/7 (0%)</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>4</td>
<td>LPS (given on day +1)</td>
<td>0/7 (0%)</td>
<td>&lt;0.10</td>
</tr>
</tbody>
</table>

\(^{a}\) C57BL/6 mice were treated with BALB/c DST, anti-CD154 mAb, and transplanted with BALB/c bone marrow according to our standard protocol without (group 1) or with an i.p. injection of 100 μg of LPS 24 h before DST and anti-CD154 mAb (group 2), on the day of DST and anti-CD154 mAb (group 3) or 24 h after bone marrow transplantation (group 4). Hematopoietic chimerism was defined as 0.10% donor origin PBMCs 8 wk after bone marrow injection. Percent donor origin bone marrow cells is the mean ± SD chimerism levels in chimeric mice. For groups with no chimeric mice, we used the limit of detection (<0.10) to indicate a lack of chimerism. Data are pooled from two independent experiments.

\(^{b}\) p = 0.0012 vs groups 1 and 2.

Type I IFN signaling and MyD88 act synergistically to mediate the effects of LPS on the establishment of hematopoietic chimerism in mice treated with costimulation blockades.

The observation that B6.IFNAR\textsuperscript{−/−} mice treated with LPS became chimeric at a frequency that was significantly lower than B6.IFNAR\textsuperscript{−/−} mice not treated with LPS (Table II) led us to conclude that other molecules in the TLR4 pathway were important. We hypothesized that in the absence of IFN-\alpha/\beta signaling downstream mediators of the TLR4→MyD88 axis might be sufficient to prevent the establishment of hematopoietic chimerism. To test this, we treated B6.MyD88\textsuperscript{−/−}/IFNAR\textsuperscript{−/−} mice with our standard costimulation blockades and transplanted them with BALB/c bone marrow and skin. The majority of the B6.MyD88\textsuperscript{−/−}/IFNAR\textsuperscript{−/−} mice transplanted in the absence of LPS developed hematopoietic chimerism (five of seven) and exhibited prolonged skin graft survival (MST = >140 days; Table II, group 13). Similar to TLR4\textsuperscript{−/−} mice, B6.MyD88\textsuperscript{−/−}/IFNAR\textsuperscript{−/−} mice treated with LPS also developed hematopoietic chimerism (five of eight; p < 0.001 vs Table I, group 2; p = NS vs Table II, group 13) and displayed prolonged skin allograft survival (MST = >140 days; Table II, group 14). Importantly, in the absence of both MyD88 and IFNAR1, mice not conditioned with costimulation blockade did not become chimeric (zero of three) and they exhibited short skin allografts survival (MST = 11 days). These data suggest that signaling through the type I IFN receptor and mediators downstream of MyD88 are important for LPS-mediated effects in mice treated with costimulation blockade.

Timing of LPS administration influences its ability to prevent the establishment of hematopoietic chimerism in mice treated with costimulation blockades.

To determine the kinetic relationship between administration of TLR agonists and bone marrow transplantation, four cohorts of C57BL/6 mice were treated with DST, anti-CD154 mAb, and given BALB/c bone marrow. One cohort was not treated further (group 1), one cohort was given LPS on day −8 (group 2, 24 h before DST and anti-CD154 mAb), another cohort was given LPS on day −7 (group 3), and the last cohort was given LPS on day +1 relative to bone marrow transplantation on day 0 (group 4). As expected, mice not treated with LPS became chimeric (six of seven), while those given LPS on day −7 did not (zero of seven; Table IV, groups 1 and 3, respectively). Mice treated with LPS on day −8 (group 2) became chimeric at the same frequency (six of
seven) as mice not treated with LPS (group 1). Mice treated the
day after bone marrow transplantation did not become chimeric
(zero of seven; Table IV, group 4). These data indicate that the
effect of TLR activation on the establishment of hematopoietic
chimerism in mice treated with costimulation blockade is depend-
ent on the timing of administration of the TLR agonist.

Discussion
In this report, we have shown that TLR activation at the time of
costimulation blockade prevents the establishment of mixed allo-
geneic hematopoietic chimerism and shortens skin allograft sur-
vival. Investigation of the cellular mechanisms responsible for this
effect revealed that both CD4 and CD8 T cells, but not NK cells,
were involved and that LPS administration matured host alloanti-
gen presenting DCs, leading to the generation of alloreactive ef-
tactor/memory CD8 T cells. Investigation of the molecular mech-
isms revealed that LPS effects could be mediated through either
the MyD88 pathway or the type I IFN receptor pathway.

Our working hypothesis for costimulation blockade-induced
graft tolerance is that conditioning with a transfusion of donor cells
in the presence of anti-CD154 mAb leads to a state of hypore-
sponsiveness in the donor-specific host alloreactive T cell pool
before the transplantation of the allograft. This state of donor-
specific hyporesponsiveness is thought to be due to an early abort-
tive expansion that leads to apoptosis and deletion of the majority
of the alloreactive T cell pool while rendering the remaining host
alloreactive T cells nonresponsive (36–39). This process could be
initiated by the presentation of allopeptides via the direct or indi-
rect Ag presentation pathways (40). The direct presentation hy-
pothesis postulates that in the presence of anti-CD154 mAb, rec-
ognition of immature APCs in the DST leads to host T cell hyporesponsiveness. Work from our laboratory supports this hy-
pothesis, as we have shown that failure to up-regulate the costimu-
latory molecule CD80 on cells in the DST was associated with
costimulation blockade-induced tolerance to islet allografts (32).
In other studies using a transgenic model in which B cells of the
DST expressed CD80 under the control of the IgM promoter, it
was shown that recipients of allogeneic islets could not be toler-
ized when cells of the DST expressed high levels of CD80. In
contrast, others have provided evidence for a role of indirect al-
loantigen presentation in tolerance induction (40, 41). This hypo-
thesis is based on the observation that cells of the DST are quickly
rendered apoptotic and phagocytosed by immature host DCs. Co-
administration of anti-CD154 mAb prevents the maturation of the
host alloantigen-presenting DCs and, consequently, alloreactive T
cells are tolerized by a mechanism that closely mimics those used
to maintain peripheral self-tolerance (42). TLR activation at the
time of DST transfer could be interfering with either, or both, of
these mechanisms.

We observed that APCs in the DST up-regulated the costimu-
latory molecules CD80 and CD86 in mice treated with costimula-
tion blockade plus LPS. Therefore, it is possible that TLR activa-
tion increases the immunogenicity of the DST, leading to
alloreactive T cell priming and rescue from costimulation block-
ade-induced apoptosis. TLR-mediated maturation of APCs within
the DST could result from direct engagement of TLRs expressed
on the donor cells or through the secretion of cytokines by TLR-
stimulated host cells. Our results show that hematopoietic chimer-
ism and long-term skin allograft survival were achieved in the
presence of costimulation blockade and LPS only when the host
was deficient in TLR4. This would suggest that activation of APCs
within the DST by direct ligand-receptor interaction is not suffi-
cient to prevent tolerance induction. Moreover, maturation of
APCs in the DST is likely not required for the effects of LPS on
naive allospecific T cells, as we have shown that LPS still pre-
vented the deletion of alloreactive CD8+ T cells in mice treated
with costimulation blockade even when the DST was obtained
from CD80/86 knockout donors (18). Our results, however, do not
exclude the possibility that the production of cytokines by LPS-
stimulated host cells could activate APCs in the skin allograft,
which may contribute to the rejection process. Taken together, our
data suggest that 1) there are alternative costimulatory molecules
expressed by APCs in the DST that prime donor-specific alloreact-
ive CD8 T cells or 2) Ag presentation by host APCs matured by
direct TLR stimulation is playing an important role in preventing
the deletion of alloreactive CD8 T cells.

To address the effects of TLR activation on host APCs present-
ing donor alloantigen, we used a CFSE-labeling system that
allowed us to examine the phenotype of host DCs that had engulfed
the transferred cells in the DST. We found that activation of TLR4
on the day of DST administration led to a marked up-regulation
of MHC class I and costimulatory molecules within 24 h on host DCs
that had phagocytosed CFSE-labeled DST. Therefore, we specu-
late that TLR activation on the day of tolerance induction signifi-
cantly alters the context in which allopeptides are presented to
the alloreactive T cell compartment. Instead of mimicking the mech-
anism of cross-tolerance to self-Ags and inducing transplantation
tolerance, TLR-licensed APCs deliver an immunogenic signal
that leads to the generation of cytotoxic effector/memory alloreactive
CD8 T cells and graft rejection.

Data from experiments investigating the molecular mechanism
by which LPS prevents the establishment of chimerism in mice
with costimulation blockade also supports a role for host
APCs. We observed that signaling through TLR4 and induction of
IFNa/β were more important than signaling through MyD88.
These data support the in vitro experiments by Hoebe et al. (34)
showing that LPS-induced expression of costimulatory molecules
on APCs was independent of MyD88 signaling and solely depen-
dent on TLR4 signaling and the induction of type I IFN. Support-
ing this interpretation, we observed that host alloantigen-present-
ing DCs isolated from LPS-treated animals lacking the type I IFN
receptor expressed lower levels of costimulatory molecules com-
pared with similarly treated wild-type controls. Based on these
data, we hypothesize that mice deficient in the type I IFN receptor
are more resistant to the effects of LPS due to the incomplete
maturation of their alloantigen-presenting DCs. Decreased DC
maturation also correlates with the impaired ability of these mice
to generate alloreactive CD8+ T cells.

Interestingly, we observed that although mice deficient in the
type I IFN receptor became chimeric when exposed to LPS, the
frequency of chimerism was lower than that achieved in type I IFN
receptor knockout mice not given LPS. This suggested that an
additional as yet unidentified mediator(s) might exist. We further
confirmed that these factors were MyD88 dependent. One potential
MyD88-dependent candidate is IL-6. We have previously shown
that Tregs are critical for the induction of hematopoietic chimerism
(27). IL-6 has been shown to prevent the induction of Tregs, gen-
erate proinflammatory Th17 cells (43), and render effector T cells
refractory to Treg suppression (44). Therefore, induction of both
IL-6 and type I IFN by LPS may contribute synergistically to pre-
vent hematopoietic chimerism through the generation of effector T
cells and the disruption of Tregs, a possibility our laboratory is
currently investigating.

We also observed that Pam3CSKys but not poly(I:C) prevented
the establishment of hematopoietic chimerism in naive mice
with TLR4 receptor knockout mice. This observation is perhaps not surprising, since
TLR1/2 signaling by Pam3Cys does not induce type I IFN production; however, it highlights the fact that overlapping, but distinct, immune responses are triggered during an infection, and these may act synergistically to affect tolerance induction in a clinical setting. For example, direct triggering of TLR2, but not TLR4, on Tregs has been shown to decrease the ability of regulatory cells to suppress the activity of effector T cells (45, 46). Differential effects on regulatory cells could explain why TLR agonists like LPS and poly(I:C) depend on type I IFN for their full effects on chimerism induction, whereas Pam3Cys does not.

It has long been appreciated that different tissues vary in their susceptibility to tolerance induction by costimulation blockade. For example, survival of cardiac and islet allografts can be significantly prolonged with anti-CD154 mAb monotherapy, whereas skin allografts cannot (47–49). Data also suggest that the mechanisms by which TLR agonists shorten allograft survival in mice treated with costimulation blockade may differ between tissues as well (4, 5). For example, LPS-shortened skin allograft survival in mice treated with costimulation blockade was solely dependent on host CD8+ cells (4), while CpG-mediated rejection of cardiac allografts was solely dependent on host CD4+ T cells (5). In this study, we show that neither population is the sole mediator of the effects of LPS, as either subset is sufficient to prevent the establishment of hematopoietic chimerism. Furthermore, we have previously shown that skin allograft survival could be prolonged with costimulation blockade when LPS was coadministered on the day of DST and anti-CD154 mAb if the host was deficient in either MyD88 or the type I IFN receptor (18). This indicates that a synergy between the MyD88-dependent and the MyD88-independent pathways are required to shorten skin allograft survival. In contrast, activation of either signaling pathway impairs the establishment of hematopoietic chimerism.

Reasons for these differences are not known, but could include differential susceptibility of the graft to effectors such as NK cells, which are potent killers of allogeneic hematopoietic cells but are not effectively cytolytic to solid organ allografts (15). It could also reflect the fact that hematopoietic grafts, unlike solid organ grafts, must not only evade the host’s immune system, but also home to specific niches in the recipient’s bone marrow in order for long-term hematopoietic chimerism to develop. Recent work has shown that migration of hematopoietic stem cells is affected by fluctuations in soluble mediators such as CXCL12, which can be modulated by stressors such as LPS (50).

Finally, we observed that the effects of LPS on the establishment of chimerism are temporally dependent. Administration of LPS on the day of DST and anti-CD154 mAb administration uniformly prevented the establishment of chimerism. In contrast, LPS injected 1 day before the initiation of tolerance induction did not. We speculate that treatment with LPS on the day before DST and anti-CD154 mAb administration does not prevent chimerism because the levels of proinflammatory cytokines such as type I IFN subside by the time that alloantigen (DST) is injected. Therefore, host DCs, or APCs in the DST, are not triggered to mature and present alloantigen in an immunogenic context and, consequently, naive alloreactive T cells are tolerized. Interestingly, injection of LPS the day after bone marrow transplantation prevents the establishment of chimerism. Given that alloreactive CD8+ T cells are largely deleted within the first several days of tolerance induction (38), it is likely that TLR4 activation 1 day after transplantation prevents chimerism independent of its effects on the deletion of alloreactive T cells. This is supported by preliminary observations in mice treated with costimulation blockade and transplanted simultaneously with allogeneic bone marrow and skin and injected 1 day later with LPS. In these mice, hematopoietic chimerism is prevented, but skin allografts are not acutely rejected (data not shown). This suggests that mechanisms that control the establishment of hematopoietic chimerism are not the same as those that control solid organ graft survival when LPS is administered after transplantation. One possible difference is that NK cells express various TLRs and can be stimulated directly by TLR agonists (51, 52). Thus, it is possible that NK cells may be acting as a barrier to hematopoietic engraftment when LPS is administered at this later time point.

In summary, we have demonstrated that the establishment of hematopoietic chimerism in mice treated with costimulation blockade can be prevented by the administration of TLR agonists. We recognize that the use of individual TLR agonists, such as LPS, poly(I:C), or Pam3Cys, does not mimic all aspects of an actual infection. However, this approach does permit the identification of mechanisms involved in a specific aspect (i.e., TLR engagement or dsRNA pathway activation) of a more complex infection (i.e., viral, bacterial). By dissecting the individual components of the innate immune response, we can more clearly identify the mechanisms by which an infection impairs transplantation tolerance. In this investigation, we found that the ability of LPS to prevent chimerism is dependent on the time of administration and is mediated by CD4+ and CD8+ lymphocytes when injected on the first day of tolerance induction. LPS appears to mediate this effect by enhancing the maturation of alloantigen-presenting DCs, a process dependent on the production of type I IFNs and MyD88-dependent factors. These findings highlight the complex interactions between host immunity and environmental perturbations during the establishment of hematopoietic chimerism and tolerance. This is particularly relevant because data from preclinical (1–3, 53) and clinical studies (54, 55) have demonstrated the ability of concurrent bone marrow transplantation to induce prolonged survival of allogeneic solid organs in the absence of maintenance immunosuppressive therapy. Therefore, understanding the mechanisms that underlie disruption of hematopoietic chimerism may yield novel strategies that circumvent this barrier, and lead to more effective transplantation regimens.

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Disclosures
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References