Mechanisms of early peripheral CD4 T-cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: evidence for anergy and deletion but not regulatory cells

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Anti-CD154 (CD40L) monoclonal antibody (mAb) plus bone marrow transplantation (BMT) in mice receiving CD8 cell-depleting mAb leads to long-term mixed hematopoietic chimerism and systemic donor-specific tolerance through peripheral and central deletional mechanisms. However, CD4+ T-cell tolerance is demonstrable in vitro and in vivo rapidly following BMT, before deletion of donor-reactive CD4 cells is complete, suggesting the involvement of other mechanisms. We examined these mechanisms in more detail. Spot enzyme-linked immunosor-}

Introduction

The combination of costimulatory blockade with bone marrow transplantation (BMT) allows the induction of high levels of long-term stable donor hematopoietic chimerism across full major histocompatibility complex (MHC) and minor histoincompatibility barriers.1-4 When recipient CD8+ T cells are depleted, mixed chimerism can be reliably established in mice receiving only 3 Gy total body irradiation (TBI) and one injection of anti-CD154 monoclonal antibody (mAb).5 Peripheral CD4+ T cells are rapidly tolerated by donor marrow when CD40/CD154 interactions are blocked.6 This tolerization protocol is reliably successful in even the most "resistant" recipient strains1,2,5 and leads to systemic tolerance, as measurable in vitro and by the most stringent in vivo test, skin allografting. In these models, donor antigen presented by skin grafts does not play a requisite role in tolerance induction and does not itself induce tolerance, because durable mixed chimerism and systemic tolerance are achieved regardless of whether skin is grafted early, and third-party skin is rejected even when grafted at the time of conditioning.1-2,5

In studies of BMT with costimulatory blockade, thymus-independent early deletion of donor-reactive CD4 T cells in the peripheral lymphoid tissues has been demonstrated.1,6,7 Both activation-induced cell death and "passive cell death" initially contribute to this peripheral CD4 cell deletion,8 but only passive cell death may be critical for tolerance induction.9 However, these studies of CD4 cell deletion in recipients of bone marrow (BM) transplants with anti-CD154 mAb relied on the analysis of T cells with T-cell receptor (TCR) Vβ subunits that recognize endogenous superantigens presented by donor MHC as a surrogate for alloreactive T cells. Because endogenous superantigens are not classical transplantation antigens and their tissue distribution is distinct, superantigen-reactive T cells may not represent all peripheral alloreactive T cells. Furthermore, Vβ analyses have shown that, although this deletion begins within the first few weeks following BMT, it takes several months to complete itself. In contrast, donor skin grafted 1 day after BMT is specifically accepted, whereas third-party grafts are rejected, demonstrating that the specific tolerance to donor antigens develops rapidly. Consistently, donor-specific tolerance is detectable in mixed lymphocyte reactions within 8 days following BMT.6 Therefore, other, nondeletional mechanisms may play a role in initial CD4 cell tolerance induction by BMT with anti-CD154.

Cell populations with immune regulatory properties may play a role in the induction of tolerance with the use of costimulatory blockade. In vitro, the combination of anti-CD154 mAb with suboptimal levels of anti-CD3 stimulates production of interleukin 10 (IL-10), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) by CD4+ T cells, followed by apoptosis.10 CD4+ T cells tolerated to alloantigens ex vivo with anti-CD154 include a
CD4+CD25+ T-cell population that down-modulates both in vitro and in vivo alloresponses. CD4+ regulatory cells have been implicated in models of linked suppression and “infectious tolerance” involving anti-CD154 and anti-CD8 mAbs in a minor antigen-mismatched skin graft model.

We have further evaluated the mechanisms involved in the early induction and the long-term maintenance of donor-specific tolerance in mixed chimeras prepared with anti-CD154. In a TCR transgenic model, we demonstrate that specific peripheral deletion of alloreactive T cells occurs in recipients of BM transplants with anti-CD154 over a period of 4 weeks following BMT. In contrast to regimens not involving BMT, no evidence implicated either cytokine deviation or regulatory cell populations in tolerance induction or maintenance. The early mechanisms of CD4+ cell tolerance in recipients of BM transplants and anti-CD154 may involve anergy followed by peripheral deletion. Similar to other mixed hematopoietic chimera models, the robust, long-term, donor-specific tolerance in these recipients is ultimately maintained through central deletion. These observations are consistent with the concept that cell populations that down-regulate T-cell responses are not maintained in an environment in which effective deletion of T cells with that specificity occurs.

Materials and methods

Animals

Female C57BL/6 (B6; H-2b), B10.A (H-2a), B10.RIII (H-2r), A.SW (H-2s), A/J (H-2a), C57BL/6-JF-γ−/−, BALB/c-IFN-γ−/−, C57BL/10, C57BL/10-AND transgenic (tg), C57BL/6 (CD45.1), B6-Rag1−/−, B6-SCID, and B10.BR mice were purchased from Frederick Cancer Research Center (Frederick, MD), The Jackson Laboratory (Bar Harbor, ME), or Taconic (Germantown, NY). Mice were housed in a specific pathogen-free microisolator environment, as described.

Conditioning and BMT

Age-matched (7-14 weeks old) recipient mice were treated with a nonmyeloablative protocol as previously described, using a non-myeloablative dose of TBI (3 Gy) followed by injection of 15 to 25 × 10^6 bone marrow cells (BMCs) as described. Antimouse CD154 mAb (MR1; 0.5 mg or 2.0 mg) was administered on day 0, and anti-CD8 mAb (2.43; 1.44 mg/mouse) was administered on day −1 as described.

Flow cytometric analysis of multilineage chimerism in white blood cells

Flow cytometric (FCM) analysis of multilineage chimerism in peripheral white blood cells (WBCs) was performed as previously described, using fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-CD8, anti-B220 (PharMingen, San Diego, CA), and anti-Mac1 (Caltag, San Francisco, CA) with biotinylated anti-H-2Dd mAb 34-2-12 plus phycoerythrin-coupled anti-CD4, anti-B220 (PharMingen, San Diego, CA), and anti-Mac1 (Caltag, San Francisco, CA) were coated with a capture mAb in sterile PBS overnight. Anti-IFN-γ (R4-6A2),–IL-4, and Vβ11 and Vβ11D T cells were isolated from the spleens of C57BL/10 AND TCR transgenic mice and injected intravenously into C57BL/10 mice. Ninety-six well spot enzyme-linked immunosorbent (ELISPOT) plates (R & D Systems, Minneapolis, MN) was added as at various concentrations.

Results

Deletion of alloreactive CD4+ T cells following BMT and anti-CD154

Allogeneic BMT with costimulatory blockade induces peripheral deletion of CD4+ T cells recognizing endogenous superantigens (SAgs) presented by donor MHC. However, as CD8+ cells play a critical role in producing the Mtv determinants required to delete Vβ3+ and Vβ11+ T cells, CD8+ cell depletion precludes the ability to examine early (week 1) Vβ5+ and Vβ11+ deletion following BMT with anti-CD154 and anti-CD8 mAb treatment. Thus, to further assess early deletion of donor-reactive CD4+ T cells in mice receiving this protocol, we used AND TCR transgenic mice. The AND TCR recognizes a pigeon cytochrome c peptide in the context of I-Ek+, is positively selected on I-Ak+, and crossreacts with an I-Aa+ alloantigen.

Mixed lymphocyte reaction (MLR) assay

MLR reactions were performed as described by using serum-free medium and triplicate wells containing 4 × 10^5 responder cells with 4 × 10^5 stimulators (irradiated with 3000 cGy). Recombinant human IL-2 (rhIL-2; R & D Systems, Minneapolis, MN) was added at as various concentrations.

ELISPOT assay

Ninety-six-well spotted enzyme-linked immunosorbent (ELISPOT) plates (Polytronics, Rockland, MA) were coated with a capture mAb in sterile phosphate-buffered saline (PBS) overnight. Anti-IFN-γ (R4-6A2),–IL-4,–IL-5 (TFRK-4) capture mAbs were used at 4, 2, and 5 mg/mL, respectively (PharMingen). The plates were washed twice with sterile PBS, blocked for 1.5 hours with PBS containing 1% bovine serum albumin (BSA), then washed 3 times. Responder cells were added to wells containing irradiated stimulator cells and incubated for 42 hours for IFN-γ and IL-4, and 48 hours for IL-5. The plates were washed 3 times with PBS, then 4 times with PBS containing 0.025% Tween (PBST). Biotinylated antilymphokine detection mAbs (IL-2, JES6-5H4; IFN-γ, XMG1.2; IL-4, BV4D1-D11; IL-5, TFRK-5) were added at 2 μg/mL (PharMingen) either for 5 hours at room temperature or overnight at 4°C. After washing 3 times with PBST, avidin-horseadish peroxidase (1:2000) was added for 1.5 hours. Four washes with PBS were performed before addition of the developing solution (800 μL 3-amino 9-ethylcarbazole [AEC; Sigma], 10 mg dissolved in 1 mL dimethylformamide in 24 mL of 0.1 M sodium acetate, pH 5.0, catalyzed by 12 μL H2O2). Spots were counted and analyzed on a computer-assisted ELISPOT image analyzer (CTL, Cleveland, OH).

Skin grafting

Full-thickness tail skin (∼1.0 cm²) was grafted and monitored as described.

Adoptive transfer of AND tg CD4+ T cells

CD4+ T cells were positively selected from C57BL/10-AND tg spleens using magnetic beads (MACS; Miltenyi Biotech, Auburn, CA) per the manufacturer’s protocol. The purity of the Vα11 Vβ3+ cell population determined by FCM was more than 90%. Cells (5 × 10^6) were injected intravenously into recipient C57BL/10 mice in sterile PBS. One week later, these mice and control B10 mice received anti-CD8, 3 Gy TBI, and anti-CD154 with either B10.A BMCs (irrelevant; B10-AND + B10.A BM T), A.SW BMCs (recognized by AND TCR; B10-AND + A.SW BM T), or sterile PBS (B10-AND).

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received anti-CD8 mAbs, 3 Gy TBI, anti-CD154 mAbs, and BM transplants from either B10.A (H-2\(^b\); not recognized by the AND TCR) or A.SW mice (H-2\(^b\); IA\(^b\) recognized by the AND TCR) to induce mixed chimerism. Additional groups of C57BL/10 (B10) mice received B10.A or A.SW BMCs without AND CD4 T cells. Chimerism and AND\textsuperscript{CD4\textsuperscript{T}} T cells within the peripheral blood were followed.

All BM transplant recipients developed long-term multilineage chimerism at similar levels. One week after BMT, B10-AND recipients of A.SW (n = 7) or B10.A (n = 4) marrow showed 14.91 ± 2.33% and 15.15 ± 1.51% B-cell chimerism, respectively (data not shown). Because an anticoniotypic mAb is not available for the AND transgenic TCR, transgenic cells were identified on the basis of CD4 expression with both V\alpha\textsubscript{11} and V\beta\textsubscript{3}. This V\alpha\textsubscript{11}/V\beta\textsubscript{3} combination is expressed on less than 0.2% of normal B10 or A.SW CD4 cells and was increased to 2% to 3% in adoptive recipients of AND cells (Figure 1). At 1 week after BMT, the percentage of AND (V\alpha\textsubscript{11}/V\beta\textsubscript{3}) CD4\textsuperscript{T} T cells in the blood was significantly decreased in mice that received A.SW BMCs compared with those receiving B10.A BMCs or no BM transplant (Figure 1). In recipients of A.SW BMT, the percentage of V\alpha\textsubscript{11}/V\beta\textsubscript{3} cells within the CD4 T-cell population declined further by 2 weeks after BMT and, by 4 weeks after BMT, was indistinguishable from that in B10 control mice that received A.SW BMT without AND cells. In contrast, B10-AND mice that received non-ligand-bearing B10.A BMCs had similar percentages of AND CD4 T cells in PBLs at 1, 2, and 4 weeks after BMT compared with control mice (control mice not shown at individual time points). The decreased percentage of AND CD4 T cells in both groups over time probably reflected dilution by recent thymic emigrants (evidenced by the appearance of donor CD4\textsuperscript{T} T cells in the PBLs; data not shown). Even at 4 weeks after BMT, however, AND CD4 T cells were still detectable in the blood of recipients of non-ligand-bearing B10.A BMC. Thus, by 4 weeks after BMT, almost complete deletion of these donor-reactive CD4 T cells had occurred only in recipients of ligand-bearing A.SW marrow.

**Addition of exogenous IL-2 is unable to restore in vitro response to donor antigens**

In the above-mentioned experiments, we did not detect expansion of the AND CD4\textsuperscript{T} T cells prior to their subsequent deletion, similar to previous results assessing deletion of donor-reactive CD4 T cells expressing V\beta\textsubscript{5} and V\beta\textsubscript{11}.

These data suggest that allospecific CD4 T cells do not undergo significant expansion prior to deletion. We previously demonstrated donor-specific tolerance of IL-2-producing cells by day 4 after BMT in mixed chimeras prepared with this regimen.\textsuperscript{9} This tolerance of IL-2–producing cells and lack of expansion of allospecific CD4 T cells suggested that the combination of BMT and anti-CD154 might induce anergy prior to deletion. In classical anergy, exogenous IL-2 restores the proliferative T-cell response to antigen.\textsuperscript{22} As donor-specific tolerance is established within 1 week after BMT (as measured in MLR\textsuperscript{B}), we assessed whether exogenous IL-2 could restore the response of recipient T cells to donor antigens at this time. As shown in Figure 2, the addition of IL-2 at concentrations of 2 U/mL or 5 U/mL did not restore the proliferation of splenic T cells from 1-week chimeras in response to stimulation with B10.A donor antigens, whereas responses to third party were maintained. Therefore, specific tolerance was not overcome by exogenous IL-2. At higher concentrations of IL-2 (10 and 20 U/mL), background proliferation was increased and precluded detection of specific responses to donor and third-party alloantigens in all groups (not shown). Similar results were obtained at 2 and 5 weeks after BMT (not shown). Thus, anergy in early chimeras was not IL-2 reversible.

**Lack of a role for IFN-\gamma or cytokine deletion in the establishment of mixed chimerism and tolerance using anti-CD154**

In several models using costimulatory blockade without BMT, IFN-\gamma has been shown to play a critical role in graft prolongation.\textsuperscript{23,24} To assess the role of IFN-\gamma in the induction of peripheral CD4\textsuperscript{T} T-cell tolerance and mixed chimerism in mice receiving BM transplants with anti-CD154, we compared IFN-\gamma\textsuperscript{−/−} and wild-type (WT) B6 mice. To ensure the complete absence of IFN-\gamma-producing cells by day 4 after BMT in mixed chimeras prepared with anti-CD154, we compared IFN-\gamma\textsuperscript{−/−} B6 recipients. As shown in Figure 3A, all IFN-\gamma\textsuperscript{−/−} mice that received our protocol developed long-lasting, multilineage chimerism (8 of 8), similar to WT control mice receiving the same treatment (7 of 8). Chimerism levels were similar in both groups (Figure 3B, and data not shown). The lack of long-term chimerism in any of the IFN-\gamma\textsuperscript{−/−} recipients that received anti-CD8, TBI, and BM (without anti-CD154) demonstrates that BMC rejection by CD4\textsuperscript{T} T cells did not require IFN-\gamma. Both WT and IFN-\gamma\textsuperscript{−/−} chimeras accepted donor skin grafted 14 weeks after BMT for more than 100 days, while rejecting third-party grafts within 18 days (data not shown). At the time of killing (28 weeks after BMT), all chimeras demonstrated donor-specific MLR nonresponsiveness (data not shown). No significant difference was observed in the percentages of V\beta\textsubscript{11} and V\beta\textsubscript{5} CD4\textsuperscript{T} T cells (data not shown) in PBLs of WT or IFN-\gamma\textsuperscript{−/−} mixed chimeras at 3, 9, or 21 weeks after BMT, and these levels were ultimately similar to those in control Balb/c-IFN-\gamma\textsuperscript{−/−} mice. In IFN-\gamma\textsuperscript{−/−} mice that received anti-CD8 mAb and BM transplants without anti-CD154, no deletion of these V\beta T cells was observed. Thus, peripheral CD4\textsuperscript{T} T-cell tolerance induced with BMT and anti-CD154, including deletion, does not require IFN-\gamma.

**Figure 1. Deletion of alloreactive AND CD4\textsuperscript{T} T cells in mice receiving A.SW BMT with anti-CD154.** AND transgenic CD4\textsuperscript{T} T cells (5 × 10\(^5\)) were adoptively transferred into B10 mice (B10-AND). These mice and control B10 mice were treated with anti-CD8, 3 Gy TBI, and anti-CD154 with either B10.A BMCs (irrelevant; B10-AND + B10.A BMC), A.SW BMCs (recognized by AND TCR; B10-AND + A.SW BMT), or sterile PBS (B10-AND). The percentage of V\alpha\textsubscript{11}/V\beta\textsubscript{3} cells in the CD4\textsuperscript{T} PBL population was assessed by 3-color FC, and the mean (plus SD) for each respective group is shown. B10-AND mice that received B10.A BMCs had significantly higher percentages (P < 0.05) of AND CD4\textsuperscript{T} T cells at 1, 2, and 4 weeks after BMT compared with B10-AND mice that received A.SW BMCs. No significant difference was seen between mice that received irrelevant B10.A BMCs and mice that received no BMCs (individual time points not shown for these controls), whereas B10-AND mice that received A.SW BMCs showed significant deletion by 1 week after BMT. By 4 weeks after BMT, B10-AND mice that received A.SW BMCs had levels of V\alpha\textsubscript{11}/V\beta\textsubscript{3} CD4\textsuperscript{T} T cells that were not significantly different from those in B10 mice that had not received AND CD4\textsuperscript{T} T cells (that received A.SW BMCs), or from those in control B10 or control A.SW mice. * These bars represent the average of weeks 1, 2, and 4 in each group. n.s. indicates not significant.
To determine whether Th2 cytokine deviation might contribute to early CD4+ T-cell tolerance, we performed ELISPOT analysis on splenocytes from animals killed at 2, 4, 8, 15, and 36 days after BMT to compare IFN-γ and IL-4 production (Tables 1-2). CD8+ cell-depleted B6 recipients of anti-CD154, 3 Gy TBI, and B10.A BM transplants were compared with recipients of conditioning without BMT and to recipients of BMT, anti-CD8, and 3 Gy TBI without anti-CD154. On day 4 and day 8 after BMT, the numbers of IFN-γ-producing cells in spleens of BM transplant recipients were similar in response to donor and third-party antigens. By day 15, the IFN-γ response to donor was undetectable (Table 1) in mice that received the full regimen, whereas responses to third-party but not recipient antigens were present. In contrast to the tolerance achieved with anti-CD154 and BMT, a sensitized antidonor IFN-γ response was observed at 8, 15, and 36 days in recipients of BMT without anti-CD154. Thus, specific tolerance of IFN-γ-producing cells was evident by 15 days after BMT in recipients of BM transplants with anti-CD154.

Mixed chimerism was not associated with IL-4 production in response to donor antigens (Table 2). By day 4, the antidonor IL-4 response was significantly lower than that to third-party antigens in mixed chimeras. Higher antidonor IL-4 responses were seen on day 4 in a conditioned control, and in a mouse that received BMT without anti-CD154 (Table 2). By day 8, 3 of 3 mixed chimeras showed a specific lack of IL-4 production to the donor compared with third-party antigens, and the antidonor responses were markedly lower than that of the conditioned control animal. BMT without anti-CD154 led to a sensitized antidonor IL-4 response by this time (Table 2). Thus, tolerance of IL-4-producing cells was achieved in mixed chimeras between 4 and 8 days after BMT. A specific lack of an IL-5 response to donor antigens was also observed in mixed chimeras by day 15 (data not shown), further ruling out a Th2 shift.

Similar results were obtained in additional experiments involving ELISPOT analyses of early (1-3 weeks) and long-term chimeras (data not shown). Thus, both Th1- and Th2-producing cytokine responses were rapidly and specifically tolerated in mixed allogeneic chimeras, and increased Th2 cytokine production cannot be implicated in tolerance induction.

**Lack of evidence for a role for regulatory/suppressive mechanisms in the maintenance of tolerance**

To investigate whether regulatory cells maintained tolerance in mixed chimeras, we injected 30 × 10^6 naive host-type (B6) splenocytes 17 weeks after BMT into chimeras prepared with anti-CD8 mAb, anti-CD154, and 3 Gy TBI. In mixed chimeras prepared with lethal TBI, administration of 20 to 30 × 10^6 nontolerant host-type spleen cells rejects donor BMCs and breaks tolerance. In contrast, in models involving suppressive mechanisms, tolerance is resistant to higher doses of nontolerant host T cells. As shown in Figure 4, mixed chimeras prepared with anti-CD154, anti-CD8, and 3 Gy TBI lost measurable chimerism within 3 weeks following infusion of naive spleen cells (AdoptTrans 1-3). Control chimeras remained stable. Donor-reactive Vβ5+ and Vβ11+ CD4+ T cells appeared in the blood of recipients of spleenocyte infusions after chimerism disappeared, and these animals rejected donor skin grafts (data not shown). When nontolerant B6 splenocytes were infused at earlier time points (< 4 weeks after BMT) to mixed chimeras prepared with anti-CD8, anti-CD154, and 3 Gy TBI, a clear effect was not demonstrated (data not shown). The variability may have reflected persistent circulating anti-CD154 mAb at this time (as detectable by ELISA; data not shown). Overall, these data suggest that the maintenance of tolerance in chimeras prepared with anti-CD154 does not involve a powerful suppressive mechanism.
The above-mentioned studies could not rule out the possibility that a regulatory cell population might contribute to initial tolerance induction prior to the deletion of donor-reactive CD4 T cells. Because anti-CD154 mAb is still present in the sera of these mice at 4 weeks after BMT (data not shown), the variable ability to break tolerance with the transfer of naive splenocytes might reflect the influence of this circulating mAb or the activity of a regulatory cell population. To distinguish between these possibilities, we removed the tolerant splenocytes from the mAb-containing environment early following BMT. Immunoincompetent recipient mice (B6-SCID or B6-Rag1<sup>−/−</sup>) were grafted with both donor and third-party skin grafts and then reconstituted 8 days later with spleen cells from either tolerant chimeric recipients 2 weeks after BMT, nontolerant controls, or a combination of both. In the first experiment (shown in Figure 5) B6 (CD45.2) mice received anti-CD8 mAb, 3 Gy TBI, and anti-CD154, followed by B10.A BMT. Two weeks later, chimeric mice (determined by FCM analysis of WBCs prior to killing, data not shown) and B6 (CD45.1) mice that had received only 3 Gy TBI and CD8-depleting mAb (without anti-CD154 or BMT) were killed, and their splenocytes were harvested. FCM analysis showed that, among splenocytes from mixed chimeras at this point. Controls received only spleen cells (containing 5 × 10<sup>6</sup> CD4<sup>+</sup> T cells) from chimeric mice (tolerant) or 5 × 10<sup>6</sup> CD4<sup>+</sup> T cells from the B6 (CD45.1) mice (naive). Two different mixtures containing different ratios of tolerant and naive CD4<sup>+</sup> T cells were injected into additional groups of B6-SCID mice: One group received a 1:1 ratio of tolerant to naive (5 × 10<sup>6</sup> of each) CD4<sup>+</sup> splenocytes, and the other received a 3:1 ratio (15 × 10<sup>6</sup> tolerant to 5 × 10<sup>6</sup> naive CD4<sup>+</sup> splenocytes). As shown in Figure 5, all SCID mice that received CD4<sup>+</sup> splenocytes, regardless of origin, rejected fully MHC-mismatched third-party grafts within 19 days, whereas SCID controls accepted both donor and third-party grafts indefinitely. Recipients of naive CD4<sup>+</sup> T cells, with or without an equal or 3-fold greater number of tolerant CD4<sup>+</sup> T cells, rejected donor-type skin within 28 days. In contrast, mice that received only tolerant CD4<sup>+</sup> T cells accepted donor skin grafts for more than 60 days. Thus, recently tolerized CD4<sup>+</sup> T cells did not suppress the rejection of donor-type skin grafts by naive, nontolerant CD4<sup>+</sup> T cells. Similar results were obtained in a repeat experiment involving adoptive transfer into B6 Rag1<sup>−/−</sup> recipients (data not shown).

After 60 days, mice that received only splenocytes from chimeric animals slowly rejected the donor grafts (Figure 5). No donor cells were detectable in WBCs of these adoptive recipients 6 weeks after transfer. In addition to confirming the incomplete deletion of donor-reactive CD4 cells in chimeras 2 weeks after BMT, this result demonstrates that donor antigens expressed on skin grafts are insufficient to maintain the tolerance of CD4 cells obtained from mixed chimeras at this point.

To assess whether BMT with anti-CD154 might induce regulatory cells that mediate “linked suppression,”<sup>12</sup> we prepared mixed chimeras and grafted them on day +1 after BMT with donor-type (B10.A) and third-party skin grafts that were either fully MHC- and minor antigen–mismatched to the BMC donor strain (A.SW), MHC-matched to the donor but expressing minor histocompatibility differences (A/J), or MHC class II and minor antigen matched to the donor but class I mismatched (B10.BR) (Table 3). As shown in Figure 6 and Table 3, all chimeras accepted donor skin more than 100 days, while eventually rejecting the various third-party grafts. Fully mismatched A.SW grafts were rejected quickly, within 20 days. Grafts sharing the entire donor MHC, but mismatched at multiple minor histocompatibility loci, were all rejected within 55 days. Four of 5 chimeras rejected grafts sharing donor MHC class II but mismatched for class I within 80 days (B10.BR). Thus,

### Table 1. Splenic IFN-γ ELISPOT assay in mice receiving BM transplant with anti-CD8, anti-CD40L, and 3 Gy TBI

<table>
<thead>
<tr>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 36</th>
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<tr>
<td>chimera 1</td>
<td>chimera 2</td>
<td>chimera 3</td>
<td></td>
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<tr>
<td>nil B6</td>
<td>B10.A</td>
<td>3rd party</td>
<td>nil B6</td>
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<tr>
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<td>88</td>
<td>87</td>
<td>0</td>
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<td>Conditioned control</td>
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<td>BMT with no MR1</td>
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<td>ND</td>
<td>ND</td>
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<td>chimera 1</td>
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</tr>
<tr>
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</tr>
<tr>
<td>chimera 3</td>
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<td>67</td>
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</table>

Each value represents the mean number of spots (2 wells) obtained with 1 × 10<sup>6</sup> initial splenocytes. Chimeras received 3 Gy TBI, anti-CD8 mAb, MR1, and BMT; conditioned controls received TBI, anti-CD8, MR1; BMT with no MR1 received TBI, anti-CD8, and BMT. ND indicates not done.

### Table 2. Splenic IL-4 ELISPOT assay in mice receiving BM transplant with anti-CD8, anti-CD40L, and 3 Gy TBI

<table>
<thead>
<tr>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 36</th>
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<tr>
<td>chimera 1</td>
<td>chimera 2</td>
<td>chimera 3</td>
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<tr>
<td>nil B6</td>
<td>B10.A</td>
<td>3rd party</td>
<td>nil B6</td>
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<td>22</td>
<td>252</td>
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<td>Conditioned control</td>
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<tr>
<td>BMT with no MR1</td>
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<tr>
<td>chimera 3</td>
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</table>

Each value represents the mean number of spots (2 wells) obtained with 1 × 10<sup>6</sup> initial splenocytes. Chimeras received 3 Gy TBI, anti-CD8 mAb, MR1, and BMT; conditioned controls received TBI, anti-CD8, MR1; BMT with no MR1 received TBI, anti-CD8, and BMT. ND indicates not done.
linked suppression did not induce tolerance to skin grafted immediately after BMT. Because tolerance mediated by regulatory cells may take several months to develop after transplantation, similarly prepared mixed chimeras \( (n = 4) \) received skin grafts 13 weeks after BMT from B10.A donors. The mixed chimeras all accepted the B10.A skin grafts indefinitely (>100 days), while rapidly rejecting B10.BR grafts (days 15, 19, 27, and 30). Thus, BMT with anti-CD154 does not induce a cell population that tolerizes T cells with other specificities encountering their antigenic ligands on antigen-presenting cells (APCs) that also express donor antigens.

**Discussion**

The combination of anti-CD154 mAb and BMT permits the rapid establishment of specific CD4 T-cell tolerance to the donor, as demonstrated by the acceptance of donor-type skin grafted 1 day after BMT and by donor-specific MLR nonresponsiveness by 1 week after BMT \( (n = 4) \) (Tables 1-2). However, analyses of donor-reactive \( V_{\beta} \)- and \( V_{\beta}11 \)-bearing CD4 \(^+ \) T cells in these experiments showed no measurable deletion of these cells before 2 weeks after BMT, suggesting that donor-specific tolerance develops before completion of deletion of donor-reactive T cells. However, the superantigen-mediated deletion of CD4 \(^+ \) T cells depends, to some extent, on the presence of CD8 \(^+ \) T cells, which are depleted in this model, and may not necessarily reflect the deletion of conventional alloreactive CD4 \(^+ \) T cells. In the present studies, we demonstrate significant deletion of alloreactive AND TCR transgenic CD4 \(^+ \) T cells during the first week after BMT in mice treated with anti-CD8 and anti-CD154, but show that the deletion is not complete until 3 to 4 weeks after BMT. Therefore, mechanisms in addition to deletion must play a role in establishing initial peripheral donor-specific CD4 T-cell tolerance.

In various models, costimulatory blockade may induce T-cell anergy, Th2 deviation, suppression, or apoptosis. The failure to observe early expansion of donor-specific CD4 T cells in these (Figure 1) or our previous studies using \( V_{\beta} \) markers suggests that anergy may be induced within this population prior to their deletion. However, the addition of exogenous IL-2 was unable to restore the proliferative response of recipient CD4 T cells cocultured with donor antigens (Figure 2). Although classical anergy can be reversed by the addition of IL-2, another form of anergy has been identified that cannot be reversed by IL-2 and is induced by a signal through cytolytic T lymphocyte-associated antigen 4 (CTLA-4). Further studies in our model support this mechanism, as the blockade of CTLA-4/B7 interactions at the time of BMT with anti-CD154 significantly impairs the establishment of mixed chimerism and tolerance (J.K. et al, manuscript in preparation).

Whereas IFN-\( \gamma \) has been shown to be crucial for the long-term survival of cardiac allografts when anti-CD154 is combined with CTLA-4-immunoglobulin (CTLA-4-Ig) without BMT, and of skin allografts when anti-CD154 is combined with donor-specific transfusion (DST) in thymectomized mice, the absence of IFN-\( \gamma \) did not impair the establishment of CD4 \(^+ \) T-cell tolerance or

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**Figure 4.** Administration of \( 3 \times 10^6 \) naive B6 splenocytes 17 weeks after BMT to mixed chimeras prepared with anti-CD8, 3 Gy TBI, and anti-CD154 leads to loss of chimerism. Mixed chimeras were prepared with anti-CD8, 3 Gy TBI, anti-CD154, and B10.A BMDCs. Percentage of donor granulocytes in peripheral WBCs are shown for individual animals. Similar profiles of donor chimerism were observed in CD4, CD8, B-cell, and monocyte lineages.

**Figure 5.** Failure of tolerized CD4 \(^+ \) spleen cells to prevent the rejection of donor skin grafts by naive host-type CD4 \(^+ \) spleen cells. B6-SCID recipients were grafted with donor-specific (B10.A; panel A) and third-party (A.SW; panel B) tail skin grafts 8 days prior to the adoptive transfer of spleen cells. Day 0 is the day of adoptive transfer. All mice that received naive B6 splenocytes rejected B10.A skin within 28 days, regardless of whether spleen cells from chimeric mice were coadministered (recipients of the 3:1 ratio \( n = 5 \)) had a slight, but significant, prolongation compared with recipients of the 1:1 ratio \( n = 4 \) and naive cells alone \( n = 2 \). Mice that received splenocytes only from chimeric mice accepted donor skin grafts for more than 60 days but rejected them by day 77 (\( n = 3 \)). All recipients of spleen cells (regardless of origin) rejected third-party grafts within 19 days. SCID mice that received no spleen cells accepted both donor and third-party grafts indefinitely (\( n = 3 \)). Chm and naive B6 indicate recipients of either spleen cells from chimeric or nonchimeric mice, respectively. 3:1 and 1:1 indicate the ratio of tolerant to naive CD4 cells given to recipients of both tolerized and naive splenocytes in combination.

**Figure 6.** Survival of donor and various third-party primary skin grafts on mice receiving anti-CD8, 3 Gy TBI, BM transplants, and anti-CD154. Recipient mice were grafted 1 day after BMT with both donor and third-party skin grafts. All chimeric mice accepted donor skin grafts for more than 100 days (B10.A). Mice that received fully MHC- and minor antigen–mismatched grafts (A.SW) rejected these grafts within 20 days (\( n = 3 \)). Chimeras that received grafts that shared donor MHC and were mismatched for minor histocompatibility antigens ([A] were all rejected by day 55 (\( n = 7 \)). MHC class I–mismatched grafts (B10.BR) sharing donor MHC class II were rejected by 4 of 5 chimeras within 80 days.
Th2 cytokine deviation has been described in regimens using costimulatory blockade,\textsuperscript{32} and it has been reported that CD154 blockade is less effective in a predominantly Th1 environment.\textsuperscript{39} We have previously shown that by day 4 after BMT, animals receiving our BMT regimen already show specific tolerance of IL-2–producing cells to the donor.\textsuperscript{3} We now examined the possible role of cytokine deviation in the induction of tolerance in this model. Our ELISpot analyses showed no increase in donor-reactive IFN-γ, IL-4, or IL-5–producing cells in mixed chimeras compared with controls at any time. In fact, donor-specific tolerance of IFN-γ–producing splenocytes was evident by 15 days after BMT in mixed chimeras and was achieved more rapidly for the Th2 cytokines, providing further evidence that host alloreactive CD4 T cells are rapidly rendered completely nonresponsive to the donor following BMT with anti-CD154. Moreover, these results argue that cytokine deviation does not play a major role in the mechanism of early CD4+ T-cell tolerance.

A body of literature has implicated CD25\textsuperscript{+}CD45RB\textsuperscript{+}CD4+ regulatory T cells in the maintenance of peripheral tolerance to organ-specific self-antigens.\textsuperscript{30,31} Regulatory T cells have also been implicated in various models using anti-CD154 for tolerance induction. CD4+ T cells stimulated with alloantigens ex vivo in the presence of anti-CD154 included a population of CD4+CD25+ T cells that down-modulated alloresponses in vitro and in vivo.\textsuperscript{11} CD4+ T-cell–dependent regulatory mechanisms have been implicated in models of linked suppression and infectious tolerance using anti-CD154 and anti-CD8 mAbs in a minor antigen-mismatched skin graft model.\textsuperscript{12,13}

We assessed the potential role of regulatory mechanisms in the development of early CD4+ T-cell tolerance in recipients of BM transplants with anti-CD154. In long-term mixed chimeras prepared with anti-CD8 and anti-CD154 mAbs and 3 Gy TBI, the administration of 30 \( \times 10^6 \) naive host-type cells led to the rapid loss of chimerism and rejection of donor skin grafts, suggesting that potent suppression of antidonor responses is not a major mechanism maintaining tolerance in these chimeras. However, these studies did not rule out the possibility that suppression contributes to initial CD4 tolerance. This was difficult to assess by infusing nontolerant recipient lymphocytes at early time points, because persisting anti-CD154 mAb and donor BMC-derived cells might tolerate these cells through the same mechanisms as those affecting pre-existing host T cells. Therefore, we adoptively transferred recently tolerated lymphocytes (2 weeks after BMT) into immunodeficient mice to test their potential to suppress rejection by naive CD4 cells. These recently tolerized CD4+ T cells did not markedly suppress the rejection of donor-type skin grafts by naive, nontolerant CD4+ T cells, even when they were administered in a 3:1 tolerant/naive ratio. Although it might be argued that homeostatic proliferation of infused T cells in immunodeficient adoptive recipients precludes the ability to detect suppression in this assay,\textsuperscript{42} adoptive transfer to T-cell–deficient mice has been used successfully to demonstrate the presence of regulatory cells in mice receiving allografts with costimulatory blockade and other mAbs.\textsuperscript{43–46}

The rapid rejection of third-party skin grafts by adoptive recipients of tolerant chimeric CD4+ T cells alone demonstrates that the marked donor skin prolongation is not due to generalized hyporesponsiveness. However, recipients of tolerant spleen cells eventually rejected donor skin. This rejection may be the result of loss of donor chimerism, as no detectable WBC chimerism (from cotransferred donor spleen cells from chimeras) was observed at 6 weeks after infusion. These donor hematopoietic cells may have been rejected by natural killer (NK) cells in immunodeficient mice. The result suggests that donor skin is an insufficient source of donor antigen to maintain tolerance and highlights the critical role of hematopoietic chimerism in maintaining tolerance of peripheral CD4 T cells in this model. Furthermore, these data also demonstrate that the early mechanisms involved in peripheral CD4+ T-cell tolerance are reversible and that donor-reactive CD4+ T cells that are not deleted by 2 weeks after BMT regain the ability to reject donor grafts in the absence of donor chimerism and/or persisting anti-CD154 mAb. A requirement for persisting chimerism to maintain tolerance in this pre-existing CD4 cell population is consistent with anergy as a mechanism of tolerance before deletion occurs, as other studies have shown dependence on antigen persistence for the maintenance of anergy.\textsuperscript{47–49}

In a minor histocompatibility antigen–mismatched skin graft model, anti-CD154 can induce a state of linked suppression, in which tolerized CD4+ T cells induce tolerance to other antigens expressed on skin grafts sharing the same antigens to which the CD4+ T cells were tolerized.\textsuperscript{12} Our studies involving early and later grafting of third-party skin expressing both donor and third-party alloantigens on the same cells provides no evidence for linked suppression in this model. The observation that donor MHC class I–or minor histocompatibility antigen–mismatched skin grafted 1 day after BMT was rejected more slowly than the fully MHC-mismatched third-party grafts may be explained by the prolonged (> 4 weeks) peripheral depletion of CD8 T cells observed because of treatment with anti-CD8 mAb, although temporary suppression of alloreactive CD8 T cells by recipient CD4 T cells cannot be ruled out. Studies performed in long-term B10.A to B6 mixed chimeras, in which B10.12 skin grafts were rapidly rejected, demonstrate an absence of linked suppression, consistent with the absence of major suppressive mechanisms in these animals.

The combination of costimulatory blockade and BMT induces a robust state of life-long donor-specific tolerance. We have now further defined the mechanisms of specific tolerance induced among peripheral CD4+ T cells. Similar to other models of mixed chimerism, the long-term maintenance of donor-specific tolerance relies on intrathymic deletion,\textsuperscript{5} and no strong peripheral regulatory mechanisms appear to be involved. Furthermore, through the use of an alloreactive transgenic T-cell population, we demonstrated that the deletion of alloreactive CD4+ T cells is complete by about 4 weeks after BMT. In contrast to other regimens that include costimulatory blockade, we could find no evidence for a major role for a suppressive/regulatory mechanism or cytokine deviation in either the long-term maintenance or early induction of donor-specific tolerance. Collectively, our results are consistent with a tolerance mechanism whereby donor-reactive CD4 T cells are initially anergized by donor hematopoietic cells in the presence of

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**Table 3. The genetic loci of the strains of mice used for the experiment in Figure 6**

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References


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Mechanisms of early peripheral CD4 T-cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: evidence for anergy and deletion but not regulatory cells

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