CD4 T Cell-Mediated Alloresistance to Fully MHC-Mismatched Allogeneic Bone Marrow Engraftment Is Dependent on CD40-CD40 Ligand Interactions, and Lasting T Cell Tolerance Is Induced by Bone Marrow Transplantation with Initial Blockade of this Pathway

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CD4 T Cell-Mediated Alloresistance to Fully MHC-Mismatched Allogeneic Bone Marrow Engraftment Is Dependent on CD40-CD40 Ligand Interactions, and Lasting T Cell Tolerance Is Induced by Bone Marrow Transplantation with Initial Blockade of this Pathway

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Costimulatory blockade can be used to promote allogeneic marrow engraftment and tolerance induction, but on its own is not 100% reliable. We sought to determine whether one or the other of the CD4 or CD8 T cell subsets of the recipient was primarily responsible for resistance to allogeneic marrow engraftment in mice receiving costimulatory blockade, and to use this information to develop a more reliable, minimal conditioning regimen for induction of mixed chimerism and transplantation tolerance. We demonstrate that a single anti-CD40 ligand mAb treatment is sufficient to completely overcome CD4 cell-mediated resistance to allogeneic marrow engraftment and rapidly induce CD4 cell tolerance, but does not reliably overcome CD8 CTL-mediated alloresistance. The data suggest that costimulation, which activates alloreactive CTL, is insufficient to activate alloreactive CD4 cells when the CD40 pathway is blocked. The addition of host CD8 T cell depletion to anti-CD40 ligand treatment reliably allows the induction of mixed chimerism and donor-specific skin graft tolerance in 3 Gy-irradiated mice receiving fully MHC-mismatched bone marrow grafts. Thus, despite the existence of multiple costimulatory pathways and pathways of APC activation, our studies demonstrate an absolute dependence on CD40-mediated events for CD4 cell-mediated rejection of allogeneic marrow. Exposure to donor bone marrow allows rapid tolerization of alloreactive CD4 cells when the CD40 pathway is blocked, leading to permanent marrow engraftment and intrathymic tolerization of T cells that develop subsequently.

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resistance to fully mismatched allogeneic marrow engraftment, and that a conditioning regimen consisting of depleting anti-CD8 mAb on day −1, a single injection of anti-CD40L mAb on day 0, and 3 Gy TBI is sufficient to reliably allow engraftment of fully allogeneic marrow and CD4 T cell tolerance in every strain combination tested. This protocol is associated with robust donor-specific tolerance to solid tissue grafted on day 1, and hence has considerable relevance to cadaveric organ transplantation.

Materials and Methods

**Mice**

Eight- to 12-week-old female C57BL/6 (B6; H-2b), B10.A (B10.A; H-2a), A.SW (H-2c), B10.BR (H-2k), BALB/c (H-2d) and B10.RIII (H-2r) mice were purchased from Frederick Cancer Research Center (Frederick, MD) or from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a specific pathogen-free microisolation environment, as previously described (13).

**Conditioning and BMT**

Age-matched (8–12 wk old) mice received 3 Gy TBI and were injected i.v. on the same day (day 0) with unseparated BM harvested from MHC-mismatched donors (8–12 wk old). Mice were injected i.p. with the indicated doses of rat IgG2b anti-mouse CD4 mAb GK1.5 and anti-mouse CD8 mAb 2.43 on day −1. Hamster anti-mouse CD40L mAb (MR1) was injected i.p. on day 0 (0.5 mg or 2 mg). Murine CTLA4Ig was injected i.p. as a single dose (0.5 mg) on day +2. The MR1 hybridoma was kindly provided to us by Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH). CTLA4Ig (14) was prepared in our laboratory from a cell line transfected with CTLA4Ig (kindly provided by Terry Strom, Beth Israel/Deaconess Hospital, Boston, MA).

**Flow cytometric analysis (FCM) of multilineage chimerism in white blood cells**

FCM of multilineage chimerism was performed as previously described (9). In brief, forward angle and 90° light scatter properties were used to distinguish lymphocytes, monocytes, and granulocytes in peripheral white blood cells. Two-color FCM was used to distinguish donor and host cells of particular lineages, and the percentage of donor cells was calculated as previously described (9), by subtracting control staining from quadrants containing donor and host cells expressing a particular lineage marker, and by dividing the net percentage of donor cells by the total net percentage of donor plus host cells of that lineage. Dead cells were excluded using propidium iodide staining. Nonspecific FcR binding was blocked by anti-mouse FcR mAb 2.4G2 (15). FITC-conjugated mAbs included anti-CD4, anti-CD8, anti-B220 (all purchased from Pharmingen, San Diego, CA), and anti-MAC1 (Caltag, San Francisco, CA). Negative control mAb HOPC1-FTTC, with no reactivity to mouse cells, was prepared in our laboratory. Biotinylated anti-H-2Dα (9) and anti-H-2Kβ (14) mAb 34-2-12, anti-H-2Kβ mAb 36-7-5 (Pharmingen) and control mAb HOPC1 were developed with PE-streptavidin.

**FCM analysis of T cell receptor Vβ usage**

PBLs were stained with FITC-conjugated anti-Vβ5.1/2, Vβ11, and Vβ8.1/2 or control mAbs vs PE-conjugated anti-CD4 mAb (all purchased from Pharmingen). Nonspecific PE-conjugated rat IgG2a (Pharmingen) served as a negative control. Two-color FCM analysis was performed on gated CD4+ cells. Splenocytes were stained with FITC-conjugated anti-Vβ5.1/2, Vβ11, and Vβ8.1/2 or control mAbs vs PE-conjugated anti-CD4 mAb (or anti-CD8 mAb; Pharmingen). Three-color FCM analysis was performed on gated host-type class I (KH95)-high, CD8-negative (CD4), or CD4-negative (CD8+) cells, and the percentage of Vβ-positive control cells in this gate was corrected for the percentage of TCRβ-high cells in the same gate, as previously described (6). Thymocytes were stained with FITC-conjugated anti-TCRβ- (Pharmingen), or anti-Vβ5.1/2, Vβ11, or Vβ8.1/2 vs BIO-conjugated KH95 (anti-Dβ, Pharmingen) developed with CyChrome-streptavidin (Pharmingen). For B10.A controls, gated 34-2-12-high cells were analyzed in a similar fashion. Background staining (as determined by nonreactive mAb HOPC-FTTC in the same gate) was subtracted from the percentage of cells staining with each anti-Vβ mAb.

**Skin grafting**

Full thickness tail skin (~1 cm2) from B10.A or B10.BR (donor-specific) and fully MHC-mismatched B10.RIII (third party) mice were grafted on the dorsal thoracic wall, sutured with 5–0 silk, bandaged, and followed by daily visual inspection. Grafts were defined as rejected when <10% of the graft remained viable.

**Mixed lymphocyte reactions (MLR)**

Splenocytes were cultured in triplicate wells containing 4 × 103 responders with 4 × 103 stimulators (30 Gy) in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 15% (v/v) controlled processed serum replacement (Sigma, St. Louis, MO), 0.09 mM nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM 2-ME, and 0.01 M HEPES buffer at 37°C in 5% CO2 for 3–4 days before they were pulsed with [3H]thymidine and harvested ~18 h later. Stimulation index was calculated by dividing mean cpm from allogeneic responses by mean cpm from anti-self (or anti-host in the case of BMT recipients) responses, which were similar to background cpm (i.e., cpm with no stimulator cell population).

**Cell-mediated lympholysis (CLL) assay**

Splenocytes from controls, BMT recipients and normal mice were resuspended in RPMI 1640 (Mediatech, Herndon, VA) containing 10% FBS (Sigma), 0.09 mM nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM 2-ME, and 0.01 M HEPES buffer. Responder and stimulator cells (30 Gy) were diluted to a concentration of 8 × 105 cells/ml, and 100 μl responder cells were cocultured with 100 μl stimulator cells per well. Cultures were set up in two rows of three replicates each, and after 5 days of incubation in 5% CO2 at 37°C, 2-fold serial dilutions were prepared from the second row of triplicates, so that cytolytic capacity could be examined at five different responder-to-target ratios. A total of 8000 51Cr-labeled, 2-day concanavalin-A-stimulated lymphoblasts were added to each well and incubated for four hours in 5% CO2 at 37°C before they were harvested. The percent of specific lysis was calculated with the following formula: percent of specific lysis = (experimental release – spontaneous release)/(maxi- mum release – spontaneous release) × 100%.

**Statistical analysis**

Statistical significance was determined with a two-tailed Student’s t test for comparison of means with unequal variances. A p value of <0.05 was considered to be statistically significant.

**Results**

Incompletely depleting doses of anti-CD4 and anti-CD8 mAbs on day −1 plus MR1 and 3 Gy TBI on day 0 permit induction of long-term multilineage chimerism

We have previously demonstrated that treatment of B6 mice with depleting doses of anti-CD4 mAb GK1.5 (1.76 mg) and anti-CD8 mAb 2.43 (1.4 mg) on day −1, in combination with anti-CD40L mAb MR1 on day 0 and 3 Gy TBI on day 0, allowed engraftment of B10.A fully MHC-mismatched marrow and acceptance of donor skin grafted in the peritransplant period (10). Because complete peripheral T cell depletion is more difficult to achieve in large animals and humans than in mice, we wished to determine whether a similar outcome could be achieved if less than fully depleting doses of anti-CD4 and anti-CD8 mAbs were given in this regimen. B6 mice were treated with various doses of these mAbs on day −1, followed on day 0 by 0.5 mg of MR1 and 3 Gy TBI, and injection of 20 × 106 unseparated BM cells from fully MHC-mismatched B10.A donors. Donor hematopoiesis was then assessed at multiple time points after BMT by FCM analysis of peripheral white blood cells.

Injection of MR1 alone (0.5 mg/mouse) to 3 Gy-irradiated mice permitted induction of lasting mixed chimerism in only two of six animals (Fig. 1, a and b). Consistent with previous results (10), administration of a standard dose of TCD mAbs (1.76 mg GK1.5 and 1.4 mg 2.43) on day −1 to mice receiving 3 Gy TBI and 0.5 mg MR1 on day 0 allowed induction of high levels of lasting, multilineage mixed chimerism in six of six mice (data not shown). Reduction of the dose of TCD mAbs to 1/4 of the standard dose still allowed lasting multilineage chimerism to be achieved in all...
mice \((n=6); \text{Fig. 1, } a \text{ and } b\). We evaluated a variety of progressively lower doses of TCD mAbs in combination with MR1 and 3 Gy TBI, and, as is shown in Fig. 1, a and b, administration of only 1/64 of the standard TCD mAb dose (0.025 mg GK1.5 and 0.02 mg 2.43) was sufficient to allow induction of lasting chimerism in six of the mice receiving 0.5 mg of MR1 and 3 Gy TBI on day 0. Similar results were obtained in a repeat experiment.

Although significant numbers (>0.3%) of T cells were not detected by FCM in the blood of mice that received the standard dose of TCD mAbs, at dilutions of one-sixteenth and more of the standard dose of TCD mAbs, T cells were readily detectable (>0.3%) in the peripheral blood by 1 wk (data not shown). Although T cell concentrations were significantly lower \((p<0.001)\) than those in mice receiving MR1 without TCD mAbs, those receiving 1/64 of the standard TCD mAb dose had substantial numbers of host T cells (range 11.81–22.63% of PBL vs 38.7% in normal B6) in their blood by 1 wk. Donor T cells were not detectable. Because it is unlikely that this level of reconstitution occurred from the thymus within 1 wk, we concluded that injection of a single dose of MR1 on day 0 reliably allowed the induction of lasting, multilineage mixed chimerism in mice receiving an incompletely depleting dose of TCD mAbs and 3 Gy TBI followed by fully MHC-mismatched allogeneic BMT.

To determine whether or not donor-specific tolerance was achieved in mice receiving BMT with the above protocols, skin grafting was performed 1 day following BMT. All mice that displayed lasting mixed chimerism, regardless of whether completely or incompletely depleting doses of TCD mAb were administered along with MR1 and 3 Gy TBI, accepted skin grafts for the duration of follow-up (>200 days, data not shown). In mice treated with MR1 and 3 Gy TBI alone, the two of six mice that showed sustained chimerism accepted donor skin grafts, whereas mice that did not develop lasting chimerism (chimerism undetectable by 6–10 wk) rejected donor skin. Mice treated with 1/4 of the standard TCD dose and 3 Gy TBI alone rejected donor skin by 47 days. Third-party (B10.RIII) skin was rejected by day 47 in all treatment groups, regardless of whether or not chimerism was induced (data not shown). The delayed third-party skin graft rejection observed in some mice (especially those receiving the higher dose of TCD mAbs in combination with MR1) reflects the temporary immunosuppressive effect of the conditioning regimen. The regimen involving 1/64 TCD mAbs, MR1, and 3 Gy TBI was only slightly immunosuppressive, with all third-party skin grafts completely rejected by 20 days posttransplant, whereas donor-specific grafts were accepted for the duration of follow-up (34 wk; data not shown).

The long-term chimeras that accepted donor skin grafts for the duration of follow-up, regardless of TCD treatment, showed tissue chimerism in the bone marrow, spleen, and thymus, and demonstrated donor-specific unresponsiveness in MLR and CML at 34 wk (data not shown). The two long-term chimeras that were prepared with 3 Gy TBI and MR1 alone also showed donor-specific unresponsiveness in MLR and CML, as well as marrow, spleen, and thymic chimerism at the time of sacrifice (34 wk) (data not shown). Thus, lasting chimerism and systemic donor-specific tolerance was reliably induced across a full MHC barrier in chimeras prepared with an incompletely depleting dose of TCD plus MR1, similar to results seen in other mixed chimeras (10). Although similar results were obtained in a minority of mice receiving MR1 without TCD mAbs, MR1 alone did not reliably allow this outcome to be achieved.

Central deletion has been established as the major mechanism for maintenance of tolerance in mixed chimeras prepared by a variety of regimens (6–8, 10, 11, 17). We examined whether or not donor-reactive T cells in PBL and thymus were deleted by assessing the usage of certain Vβ subunits within the TCR repertoire. The donor strain B10.A expresses I-E, which is required to present superantigens derived from mammary tumor virus 8 and 9 endogenous retroviruses encoded in the B6/B10 background genome (18–20). Developing thymocytes whose TCR contain Vβ5 and Vβ11 subunits, which bind to these superantigens, are deleted in I-E-positive B10.A mice, but not in B6 mice, because they do not express I-E (19, 21). The mice that received low-dose TCD mAbs plus MR1 showed profound reductions in the percentage of Vβ5+ CD4 PBL (normal B6, 2.56%; normal B10.A, 0.00%) to 0.18 ± 0.13%, and Vβ11+ CD4 PBL (normal B6, 5.07%; normal
B10.A, 0.00%) to 0.17 ± 0.14% at 8 wk post-BMT. At the time of sacrifice 34 wk post-BMT, the chimeras prepared with low-dose (1/64) TCD mAbs plus MR1 showed a profound reduction in the percentage of Vß5+ and Vß11+ mature single-positive thymocytes compared with naive mice or BMT recipients prepared with 3 Gy TBI and MR1 alone that did not develop lasting mixed chimerism (data not shown). These data suggest that central deletion of donor-reactive T cells is one of the major mechanisms maintaining tolerance in long-term chimeras prepared with 3 Gy TBI, MR1, and incompletely depleting doses of TCD mAbs.

Correlation of delayed recovery of host CD8 cells with the development of lasting chimerism in chimeras prepared with limiting doses of TCD mAbs, MR1, and 3 Gy TBI

Because 100% of BMT recipients conditioned with the incompletely depleting 1/64 dilution of the standard TCD mAbs dose in combination with MR1 and 3 Gy TBI developed lasting chimerism and tolerance, we further titrated the TCD mAbs to determine the minimal dose of TCD mAbs required to assure the development of lasting chimerism. In a single experiment, we compared the development of chimerism and tolerance in mice conditioned with MR1, 3 Gy TBI, and a dose of TCD mAbs ranging from 0.055 mg to 0.004 mg of GK1.5 and 0.044 – 0.003 mg of 2.43 (1/32 to 1/512 of standard dose). Again, all mice receiving the 1/64 dose of TCD mAbs showed lasting chimerism. However, when the dose of TCD mAbs was reduced further, results were more variable, with some animals showing lasting chimerism, and others showing only initial chimerism that declined markedly by 6–10 wk post-BMT. More than half of the mice treated with 1/128 to 1/512 of the standard TCD mAbs dose (plus MR1 and 3 Gy TBI) became lasting chimeras and specifically accepted donor skin grafted 1 day following BMT (data not shown).

We investigated the relationship between recipient T cell recovery and the development of lasting donor chimerism in these groups of mice to see whether recovery of one or the other T cell subset could predict the ultimate loss of chimerism. Substantial levels of recipient CD4 cells were measurable in the blood of most animals by 2 wk post-BMT, but there was wide variation, from ~5% to 30% CD4 cells in PBL of various animals. The higher levels of CD4 cells at this time point were not associated with a failure of chimerism by 6 wk and later post-BMT, as animals with both high and low levels of CD4 cells showed successful and unsuccessful maintenance of chimerism (data not shown). However, substantial host CD8 cell recovery, which became evident in only some animals by 6 wk post-transplant, showed a significant association with a failure to maintain chimerism by 6 wk. A lack of chimerism at this time point was seen only in mice with host CD8 cell recovery to at least 5% by 6 wk post-BMT (Fig. 2). At earlier time points, the recovery of CD8 cells was minimal in all animals, and did not predict the achievement or failure to achieve durable chimerism.

Treatment with MR1 overcomes CD4 cell-mediated, but not CD8 cell-mediated, resistance to allogeneic marrow engraftment in 3-Gy-irradiated mice

The association of early host CD8 recovery with failure of durable engraftment in mice receiving incompletely depleting doses of TCD mAbs along with MR1 and 3 Gy TBI led us to hypothesize that recipient CD8+ cells were responsible for donor marrow rejection in these mice, and to speculate that CD8 depletion alone might be sufficient to ensure the reliable achievement of chimerism and tolerance in mice receiving MR1 and 3 Gy TBI. To address this hypothesis, we evaluated marrow engraftment in mice treated with CD8 TCD mAb alone, along with MR1 and 3 Gy TBI. As is shown in Fig. 3, six of six mice treated with a depleting dose of anti-CD8 mAb (0.35 mg) plus MR1 developed high levels of durable multilineage chimerism. These mice were specifically tolerant to donor Ags, as they accepted donor skin grafted on day 1 post-BMT, while rejecting third-party skin grafted at the same time (Fig. 4). This regimen has produced similar results in many repeated (>7) experiments. In contrast, mice (n = 5) treated with anti-CD8 mAb plus MR1 and 3 Gy TBI without BMT rejected both B10.A and B10.RIII skin within 14 days (not shown). The ability of the mice that did not receive BMT to reject skin grafted on day 1 and of mice that did receive BMT to reject third-party skin illustrates the requirement for BMT for tolerance induction in this model. Skin grafted on day 1 as the only source of Ag did not induce tolerance in mice receiving this regimen.

In contrast to recipients of anti-CD8 mAb, all seven BMT mice treated with anti-CD4 mAb, MR1 and 3 Gy TBI failed to develop chimerism and rejected donor skin grafts within 15 days following BMT (Figs. 3 and 4). As expected from previous results (5), 3 Gy-irradiated mice treated with both anti-CD4 and anti-CD8 mAbs, or with either mAb alone without MR1, also failed to achieve durable chimerism or tolerance (Figs. 3 and 4). Among
animals treated with MR1 and 3 Gy TBI without any TCD mAb, only two of seven developed durable chimerism (Fig. 3) and accepted donor skin grafts (Fig. 4). Thus, anti-CD8 mAb, but not anti-CD4 mAb, greatly augmented the capacity of MR1 to overcome resistance to allogeneic marrow engraftment and permit the induction of lasting chimerism and donor-specific tolerance. These results indicate an obligate role for CD40-CD40L interactions in inducing CD4 cell-mediated rejection of allogeneic marrow, and granulocytes as determined by two-color FCM at various times post-BMT. The mice treated with anti-CD8 plus MR1 or anti-CD4, anti-CD8 plus MR1 showed high levels of lasting chimerism. Two of seven mice treated with MR1 alone became long lasting chimeras (MR1 chimeras), whereas five mice failed to achieve lasting mixed chimerism (MR1-non chimeras). Anti-CD4 or MR1, or a combination of anti-CD4 plus anti-CD8 mAbs treatment (without MR1) did not induce lasting chimerism. Results are shown as mean ± SEM in each group. Similar results were obtained in all of the lineages tested (CD4 and CD8 T cells, monocytes, B cells), and granulocytes are shown as being representative of all lineages.

FIGURE 3. Long-term multilineage chimerism in peripheral blood of mice receiving CD8-depleting mAbs plus MR1. B6 mice were treated with 3 Gy TBI and received 20 × 10^6 unseparated BM cells from fully MHC-mismatched B10.A donors. All the groups are from one experiment. a, Percentages of long-term (>20 wk) chimeras among recipients of the various mAb treatments are presented. CD4 and/or CD8-depleting mAbs (0.44 mg GK1.5 and 0.35 mg 2.43) were given on day −1, and MR1 (0.5 mg) was given on day 0. Treatment with anti-CD4 and CD8 mAbs plus MR1 (n = 7), and treatment with anti-CD8 mAb plus MR1 (n = 6) successfully induced long-term chimerism in every mouse. Treatment with anti-CD4 mAb plus MR1 (n = 7) did not induce long-term chimerism in any mice. A single injection of MR1 (n = 7) allowed induction of lasting mixed chimerism in only two of seven mice. Anti-CD4, CD8, or combination treatment (without MR1) did not induce lasting mixed chimerism in any mice. b, The mean percentage of donor cells among peripheral blood granulocytes as determined by two-color FCM at various times post-BMT. The mice treated with anti-CD8 plus MR1 or anti-CD4, anti-CD8 plus MR1 showed high levels of lasting chimerism. Two of seven mice treated with MR1 alone became long lasting chimeras (MR1 chimeras), whereas five mice failed to achieve lasting mixed chimerism (MR1-non chimeras). Anti-CD4 or MR1, or a combination of anti-CD4 plus anti-CD8 mAbs treatment (without MR1) did not induce lasting chimerism. Results are shown as mean ± SEM in each group. Similar results were obtained in all of the lineages tested (CD4 and CD8 T cells, monocytes, B cells), and granulocytes are shown as being representative of all lineages.

FIGURE 4. Skin graft tolerance in mixed chimeras prepared with 3 Gy TBI. 20 × 10^6 BMT, anti-CD8 mAb plus MR1. BMT recipients prepared with anti-CD8 mAb plus MR1 (●; n = 6), and both anti-CD4 and CD8 mAbs plus MR1 (■; n = 7) accepted donor grafts permanently, while third-party grafts were rejected by 47 days (median survival time (MST) 14 days in anti-CD8 mAb group, and MST 39 days in anti-CD4 and CD8 mAbs plus MR1 groups) postgrafting. BMT with anti-CD4 and CD8 mAb treatment alone induced slight donor skin graft prolongation (▲; MST 39 days, n = 7), but all grafts rejected by 47 days postgrafting. BMT recipients treated with anti-CD4 mAb plus MR1 rejected donor skin within 15 days (□; MST 13 days, n = 7). Among BMT recipients treated with MR1 alone, two of seven became chimeras and accepted donor skin grafts permanently (▲; MST 39 days, n = 7), and rejected third-party skin by 32 days, but nonchimeras rejected donor skin (▼; MST 25 days, n = 5). BMT recipients treated with anti-CD4 mAb (■; MST 13 days, n = 7) or anti-CD8 mAb alone (▲; MST 13 days, n = 7) did not accept donor skin. Donor (B10.A) and third-party (B10.RIII) skin was grafted 1 day post-BMT.
and a variable requirement for this pathway in permitting CD8 cell-mediated major rejection to occur.

To assess the robustness of the tolerance induced by BMT in mice treated with anti-CD8 mAb, MR1, and 3 Gy TBI, repeat skin grafting was performed \(~140\) days following BMT and primary skin grafting (\(n = 5\)). Every mouse that had accepted the primary donor skin graft (i.e., all durable mixed chimeras) also accepted the secondary donor-type skin grafts. Nonchimeric mice that did not accept primary donor skin grafts, also rejected secondary donor skin grafts within \(14\) days after grafting (data not shown). All mice in all groups rejected secondary third-party (B10.RIII) grafts within \(14\) days (data not shown). Thus, MR1 is able to reliably prevent CD4 cell-mediated rejection of donor bone marrow cells when CD8 cells are depleted, allowing the establishment of permanent donor-specific tolerance though induction of mixed chimerism.

The chimeras prepared with anti-CD8 mAb plus MR1 and 3 Gy TBI also demonstrated high levels of donor chimerism among bone marrow cells, splenic T and B cells and thymocytes when they were sacrificed 28 wk post-BMT (Table I). In contrast, the mice receiving allogeneic BMT after treatment with 3 Gy TBI and MR1 alone in which long-term peripheral blood chimerism was not observed did not show measurable chimerism in the marrow, spleen, or thymus (Table I).

To further evaluate the establishment of tolerance, CML and MLR assays were performed in mice sacrificed 13 wk post-BMT. Chimeras prepared with anti-CD8 mAb plus MR1 and 3 Gy TBI were unresponsive toward donor and host Ags, while retaining reactivity against third-party (B10.RIII) alloantigens in both CML and MLR assays (Fig. 5, \(a\) and \(b\)). In contrast, control mice treated with anti-CD8 mAb plus MR1 and 3 Gy TBI without BMT showed reactivity against B10.A and B10.RIII alloantigens in both CML and MLR assays (Fig. 5, \(a\) and \(b\)).

**Delayed deletion of donor-reactive CD4 T cells in chimeras prepared with CD8 depletion plus MR1 and 3 Gy TBI**

We have previously demonstrated that B6 mice receiving allogeneic B10.A BMT with costimulatory blockade alone (MR1 plus CTLA4Ig) show partial deletion of V\(\beta^5\) and V\(\beta^11\) peripheral blood CD4 cells as early as \(1\) wk after BMT (11). To examine whether deletion of donor-reactive T cells occurs in mice treated with CD8 depletion plus MR1, PBLs were analyzed for the presence of these V\(\beta\) subunits on their TCRs. Mice receiving BMT with CD8 depletion plus MR1 and 3 Gy TBI did not show deletion of donor-reactive V\(\beta^5\) (not shown) or V\(\beta^11\) CD4 cells at \(1\) wk post-BMT (Fig. 6). However, deletion of these V\(\beta\) was observed at subsequent time points, and progressed over time (Fig. 6). The percentages of V\(\beta^8.1/2\)-bearing CD4 cells, which do not recognize superantigens on the donor or host, were not reduced at any time point, ruling out a nonspecific deletional process. The slight increase in mean percentage of V\(\beta^8.1/2\)-bearing CD4+ cells in the mice receiving BMT with CD8 depletion plus MR1 and 3 Gy TBI may be a compensatory effect reflecting the deletion of V\(\beta^5\) and V\(\beta^11\) populations.

The chimeras that received BMT with CD8 depletion plus MR1 and 3 Gy TBI were sacrificed 28 wk post-BMT and intrathymic deletion was examined. As is shown in Table II, these chimeras showed profound deletion of V\(\beta^5\) and V\(\beta^11\) host-type CD4 single-positive thymocytes. In contrast, nonchimeric controls that received BMT with MR1 alone did not show marked deletion of these donor-reactive thymocyte subpopulations.

**Long-term chimerism and tolerance in mice conditioned with anti-CD8 mAb, MR1 and 3 Gy TBI in another strain combination including multiple minor histoincompatibilities in addition to full MHC mismatch**

To determine whether the above results could be generalized to include additional strain combinations and genetic backgrounds, we evaluated a similar treatment strategy in BALB/c (H-\(^2\)) recipients of fully MHC-mismatched plus multiple minor Ag-mismatched B10.BR (H-\(^2\)) marrow. Because BALB/c mice tend to have higher percentages of CD8 cells in the PBL than B6 mice, we treated these animals with a full standard dose of anti-CD8 mAb (1.4 mg on day \(-1\)) in combination with MR1 (2 mg on day 0). All animals receiving BMT following treatment with anti-CD8 mAb, MR1, and 3 Gy TBI in this strain combination became durable chimeras (Fig. 7, \(a\) and \(b\)) and also showed specific acceptance of donor skin grafts (Fig. 7c). Neither anti-CD8 mAb nor MR1 treatment alone (with 3 Gy TBI) was sufficient to induce durable mixed chimerism or tolerance in this strain combination (Fig. 7, \(a–c\)).

**Discussion**

Recent reports have demonstrated that administration of anti-CD40L, with or without CTLA4Ig or donor-specific blood transfusion, could induce prolonged acceptance of donor heart, skin, and islet allografts in mice (22, 23), as well as kidneys and islets in primates (24–26). However, none of these models have been associated with systemic donor-specific tolerance across MHC barriers in euthymic recipients, as measured by the stringent tests of primary donor skin graft acceptance and by in vitro assays.

### Table I. Tissue chimerism in chimeras prepared with anti-CD8 mAb plus MR1\(^a\)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percent of Donor Cells ± SD</th>
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<tr>
<td></td>
<td>BM</td>
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<tr>
<td></td>
<td>CD4⁺</td>
</tr>
<tr>
<td>NLB6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>NLB10.A</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>Chimeras ((n = 4)) (anti-CD8 plus MR1)</td>
<td>33.94 ± 15.02</td>
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<tr>
<td>Nonchimeras ((n = 3)) (MR1 alone)</td>
<td>0.28 ± 0.13</td>
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\(^a\) The percentage of donor MHC class I (34-2-12)-positive cells among bone marrow cells, thymocytes, CD4, CD8, and B220-positive splenocytes was determined by two-color FCM 28 wk post-BMT. The mice treated with 0.5 mg MR1 (day 0) alone did not show long-term chimerism in peripheral blood, and none of these mice showed donor chimerism in any of these tissues examined by FCM. Mice treated with CD8-depleting mAb (day 1) plus 0.5 mg MR1 (day 0) showed long-term peripheral blood chimerism, and showed high levels of donor chimerism in these tissues.

\(^p < 0.05\); statistically significant differences between CD8-depleting mAb plus MR1 group and MR1-treated group.
Costimulatory blockade has only been shown to lead to systemic tolerance across full MHC barriers in euthymic mice when used in combination with allogeneic BMT. This tolerance can be achieved by giving conventional marrow doses to mice treated with anti-CD40L mAb, CTLA4Ig, and 3 Gy TBI (11), or by giving very high marrow doses to mice receiving similar treatment without any host irradiation (17). In both instances, tolerance is associated with early (by 1 wk) peripheral deletion of donor-reactive CD4 cells and, in the long term, after donor hematopoietic cells have seeded the host thymus, with a central deletional mechanism of tolerance (11, 17). However, a major limitation to the clinical applicability of these strategies is that they are not successful in 100% of mice. Complete reliability in animal models would be an essential requirement before such an approach could be considered for clinical application.

Based on results obtained in the above model, we postulated that a failure of early CTL tolerance might be responsible for the inability to achieve durable chimerism in some mice receiving combined anti-CD40L mAb, CTLA4Ig, and 3 Gy TBI (11), or by giving very high marrow doses to mice receiving similar treatment without any host irradiation (17). In both instances, tolerance is associated with early (by 1 wk) peripheral deletion of donor-reactive CD4 cells and, in the long term, after donor hematopoietic cells have seeded the host thymus, with a central deletional mechanism of tolerance (11, 17). However, a major limitation to the clinical applicability of these strategies is that they are not successful in 100% of mice. Complete reliability in animal models would be an essential requirement before such an approach could be considered for clinical application.

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When CTL responses were tested in the same animals, a different pattern was observed. Some animals showed donor-specific unresponsiveness, but some did not, even though they showed chimerism by day 25. However, at later time points (day 35 or later), all chimeric animals showed donor-specific unresponsiveness. These results demonstrate that, in contrast to MLR responses, CTL responses were not uniformly tolerant to the donor in this early period, even when substantial donor chimerism was present. These results suggest that CD8 cells were variably tolerized to donor Ags by BMT with costimulatory blockade, and that this cell population may be responsible for the failure to achieve durable chimerism in a proportion of animals receiving allogeneic BMT with this regimen. Significant donor chimerism was not always measurable.

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The results presented here are consistent with previous studies, in which donor CD4+ cells exposed in vitro to host alloantigens in the presence of anti-CD40L mAb were tolerized and lost the ability to induce graft-vs-host disease (27). We demonstrate in this
Because anti-CD40L mAb is sufficient to overcome CD4 cell-mediated resistance to allogeneic marrow engraftment and allow the induction of tolerance, we surmise that the requirement for CTLA4Ig in non-CD8-depleted mice reflects the need to specifically block the CD28-B7 pathway of CD8 T cell activation, and that this pathway does not need to be independently blocked for tolerance to be induced among CD4 cells that encounter APC in the presence of anti-CD40L. It can be inferred that B7 is expressed on some APC in the presence of CD40 blockade, and that this is sufficient to activate CD8 T cells, but not CD4 T cells, to donor Ags.

Because Th appeared to be unresponsive to the donor at very early times post-BMT, the anti-donor CTL that persist in some animals probably differentiate via CD4 cell-independent pathways. CD40L is expressed mainly on activated CD4\(^+\) T cells, and not on CD8 cells (28–30). CD40-dependent activation of APC by CD40L on activated Th is a major pathway by which help is provided for CTL generation against minor histocompatibility Ags and other peptide Ags (31–33). However, anti-viral CTL responses can occur in CD40L-deficient mice (34). CD8\(^+\) CTL activation that is independent of CD40L/CD40 interactions occurs by both CD4 Th-dependent (35) and CD4 cell-independent pathways (34, 36–38) and in the absence of CD4-mediated APC conditioning (31–34, 39–41). CD4 cell-independent APC activation can occur via LPR (42–44), C3R (45), Fc\(\gamma\)R (46), and CpG oligonucleotides (45, 47–49), and these pathways are associated with the production of mediators of CD8 T cell activation, such as type I IFNs (44, 46, 50), TNF-\(\alpha\) (51), IL-12 (52–57), and IL-15 (58). Thus, we hypothesize that the failure to achieve engraftment in a proportion of animals receiving BMT with costimulatory blockade as the only immunosuppression may reflect such “bypass activation” of APCs due to exposure to microorganisms that cannot be controlled, and that this activation leads to the Th-independent activation of allo-reactive recipient CD8 cells that then reject the donor marrow and prevent tolerance induction. Such a pathway could be important, because humans are frequently exposed to microorganisms that might thereby preclude the ability to reliably use this approach to tolerance induction.

Although the CD28 pathway of costimulation both stimulates high-level IL-2 production and may provide an essential survival signal, CD28-independent T cell activation clearly occurs and is capable of causing graft rejection in mice (59, 60). Several additional costimulatory pathways have been described (reviewed in Ref. 61) that may have the capacity to compensate for the absence or blockade of CD28 signaling. Furthermore, naive CD8\(^+\) T cells with high affinity for their ligands can differentiate into cytolytic effector cells with “signal 1” without the apparent involvement of costimulatory molecules (62). Thus, there are several possible

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**Table II. Intrathymic deletion in long-term chimeras prepared with anti-CD8 mAb, MR1, and 3 Gy TBI**

<table>
<thead>
<tr>
<th>CD4 single positive thymocytes</th>
<th>V(\beta8)1/2</th>
<th>V(\beta5)1/2</th>
<th>V(\beta11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLB6</td>
<td>14.74</td>
<td>3.37</td>
<td>3.36</td>
</tr>
<tr>
<td>NLB10.A</td>
<td>17.54</td>
<td>0.60</td>
<td>0.37</td>
</tr>
<tr>
<td>Chimera (n = 4)</td>
<td>17.98 ± 1.72</td>
<td>0.97 ± 0.77(^*)</td>
<td>0.40 ± 0.15(^*)</td>
</tr>
<tr>
<td>(anti-CD8 plus MR1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonchimera (n = 3)</td>
<td>16.51 ± 1.63</td>
<td>2.4 ± 0.37</td>
<td>2.97 ± 0.27</td>
</tr>
<tr>
<td>(MR1 alone)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) The percentage of V\(\beta8\)\(^+\), V\(\beta5\)\(^+\), and V\(\beta11\)\(^+\) cells among host MHC class I high and CD8 negative (CD4 single-positive) thymocytes, respectively, was determined by three-color FCM 28 wk post-BMT. The data are presented as mean percentage ± SD.

\(^*\) \(p < 0.05\); statistically significant differences between chimeras prepared with anti-CD8 plus MR1 vs nonchimeric recipients of MR1 alone.
explanations for the inability of CTLA4Ig to reliably prevent CD8$^+$ T cell activation, and further studies will be needed to determine the role of additional costimulatory pathways in CD8$^+$ T cell responsiveness to alloantigens in the absence of CD4 cell help.

In most of our experiments (e.g., Figs. 1 and 3), a minority of animals receiving BMT with anti-CD40L alone (plus 3 Gy TBI) developed lasting mixed chimerism and donor-specific tolerance (Fig. 4). Therefore, blockade of APC activation via the CD40 pathway is sometimes sufficient to allow tolerization of allo-reactive CD8$^+$ cells in addition to CD4 cells, even without specific blockade of the CD28-B7 pathway. We hypothesize that this occurs in animals in which CD40-independent APC activation has not occurred, and in which B7 expression on APC presenting alloantigens is markedly down-regulated due to CD40 blockade. We have attempted to improve the reliability of tolerance induction with MR1 alone, 3 Gy TBI, and BMT by increasing the MR1 dose. The highest dose evaluated, 4 mg, increased the induction of chimerism, but was still much less than 100% reliable. Thus, if MR1 alone has the potential to reliably overcome CD8-mediated resistance, exceedingly high MR1 doses would be required.

It has recently been demonstrated that asialo GM1$^+$CD8$^+$ T cells are responsible for costimulatory blockade-resistant mouse skin graft rejection (63), and CD8$^+$ T cells have been shown to be responsible for costimulatory blockade-resistant rejection in intestinal (64) and skin allograft models (65). Although these studies demonstrated prolonged allograft acceptance in the presence of costimulatory blockade with CD8 cell depletion, the allografts were ultimately rejected, and donor-specific tolerance was not achieved. In a cardiac allograft model, treatment with anti-CD8 mAb plus anti-CD40L induced long-term heart graft acceptance and operational tolerance (66). However, systemic tolerance was not demonstrated in those models, and our results suggest that tolerance for MHC-mismatched skin grafts, a more stringent test of tolerance, is not achieved with anti-CD8 and MR1 without BMT. In our studies, mice receiving CD8 depletion plus anti-CD40L and 3 Gy TBI without BMT rejected fully MHC-mismatched allogeneic
skin grafts within 14 days (not shown) and did not develop tolerance in MLR and CML assays (Fig. 5). In contrast, the addition of BMT at the time of skin grafting allows skin graft tolerance (primary and secondary grafts) and MLR and CML tolerance to be observed, indicating that systemic tolerance is achieved. Unlike responses to MHC-mismatched skin allografts given without BMT, and unlike some antiviral responses (39, 65, 68), CD4 cell-mediated resistance to MHC-mismatched marrow engraftment is completely dependent on the CD40-CD40L pathway. Because CD4 cells become rapidly tolerized by donor marrow given in the presence of anti-CD40L, it is possible that their early tolerant state makes them resistant to activation by APC activated by CD40-independent pathways. Additionally, interactions between the rapidly tolerized CD4 cells and APC may render the APC tolerogenic for CD8 cells, and perhaps naive CD4 cells, that subsequently encounter donor Ag on those APC. Such transfer of tolerance to CD8 cells via an APC encountered by a tolerant CD4 cell may account for the donor-reactive CD8 cell depletion that has been seen in mice receiving donor-specific transusions and anti-CD40L mAb (65).

Central deletion of donor-reactive thymocytes is the major mechanism maintaining long-term tolerance in mixed allogeneic chimeras prepared with anti-CD40L and CTLA4Ig (11, 17). However, evidence has been obtained for early (by 1 wk post-BMT) peripheral deletion of donor-reactive CD4+ T cells in both of these models (11, 17). B6 mice receiving B10.A BMT with the new regimen described here (i.e., CD8 cell depletion plus MR1 with 3 Gy WBI) also showed long-term central deletion of donor-reactive T cells. In the periphery, these mice showed complete deletion of donor-reactive Vβ11+ CD4+ T cells by 5–8 wk post-BMT, but showed no evidence of such deletion at 1 wk post-BMT. In the same experiments, mice receiving BMT with CTLA4Hg plus MR1, without CD8 depletion, showed statistically significant deletion of this Vβ at 1 wk (data not shown), consistent with our previous results (11). The lack of Vβ11 deletion by day 7 in the mice treated with CD8 cell-depleting mAb is consistent with published data suggesting that CD8+ T cells, especially when activated, are the most efficient producers of endogenous superantigens that delete CD4 cells using this Vβ, and may transfer these superantigens to the class II+ cells that present them (69–71). The chimeric mice in our studies that were depleted of CD8+ cells showed marked deletion of Vβ11+ CD4+ T cells before the recovery of CD8+ cells, perhaps due to viral superantigens produced by other organs, such as lung, brain, gonadal tissue (70), and intestine (72). Because endogenous superantigens do not necessarily behave as transplantation Ags (73), definitive data on peripheral deletion will await ongoing studies using TCR transgenic mice.

In previous studies, we showed that costimulatory blockade with one injection of anti-CD40L or of CTLA4Hg obviates the need for thymic irradiation or repeated administration of TCD mAbs to overcome intrathymic alloresistance in mice receiving one injection of deleting anti-CD4 and anti-CD8 mAbs (10). The current demonstration that anti-CD4 mAb is not required to achieve such results (i.e., that anti-CD40L and anti-CD8 mAb alone are sufficient to allow the reliable induction of durable mixed chimerism and transplantation tolerance) is of considerable clinical relevance. The capacity of the adult human thymus to reconstitute T cells declines steadily with increasing age, so that the time to achieve T cell reconstitution after chemotherapy with or without stem cell transplantation increases with advancing age (74). The consequences of delayed thymic reconstitution are much more dramatic for CD4 cells than for CD8 cells in humans, as the latter subset recovers much more readily (75). Thus, a greater concern exists about prolonged CD4 cell than CD8 cell depletion in adult humans receiving T cell ablation in a conditioning protocol. Therefore, the observation that CD4 depletion is not required in a regimen that reliably allows the induction of lasting mixed chimerism and transplantation tolerance is highly encouraging. The low toxicity and reliability of mixed chimerism and tolerance induction across different, full MHC barriers, with or without multiple minor Ag differences, with the nontoxic regimen of CD8-depleting mAb, 3 Gy TBI, and anti-CD40L, suggests that this approach may have considerable potential for clinical application. Evaluation of similar regimens in large animal models is clearly warranted at this point.

In summary, an absence of CD40/40L signaling is not sufficient to reliably allow the induction of mixed chimerism and donor-specific tolerance in 3 Gy-irradiated mice, but does reliably overcome the CD4 cell-mediated barrier to allogeneic engraftment and allows the rapid tolerization of host CD4 cells. CD8 T cell-mediated allogeneic marrow rejection sometimes, but not always, occurs independently of the CD40-CD40L pathway.Recipient CD8 depletion overcomes this variable, but as yet poorly understood and therefore uncontrollable CD8 cell-mediated alloresistance. BMT plays a critical role in inducing long-term systemic tolerance of both CD4 and CD8 cells under blockade of the CD40-40L pathway. This reliable approach (BMT with anti-CD40L mAb and CD8-depleting mAb) to inducing donor-specific skin graft tolerance, which is considered to be the most stringent test of tolerance, warrants evaluation in large animal preclinical models, as it may have considerable clinical potential.

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