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Ligation of 4-1BB (CDw137) Regulates Graft- Versus-Host Disease, Graft- Versus-Leukemia, and Graft Rejection in Allogeneic Bone Marrow Transplant Recipients

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4-1BB is expressed on activated CD4+ and CD8+ T cells; its ligand, 4-1BB ligand is expressed on APCs. Despite expression on both T cell subpopulations, 4-1BB has been reported to predominantly affect CD8+ T cell responses. By quantifying graft-vs-host disease alloresponses in vivo, we demonstrate that both CD4+ and CD8+ T cell-mediated alloresponses are regulated by 4-1BB/4-1BB ligand interactions to approximately the same extent. 4-1BB receptor-facilitated CD4+ T cell-mediated alloresponses were partly CD28 independent. In two distinct marrow graft rejection systems, host CD8+ and CD4+ T cells each separately contributed to host anti-donor T cell-mediated allograft rejection. α4-1BB mAb increased the graft-vs-leukemia effect of a suboptimal number of donor splenocytes given later post bone marrow transplantation by bolstering allogeneic responses resulting in leukemia elimination. In summary, 4-1BB ligation is a potent regulator of CD4+ and CD8+ T cell-mediated allogeneic responses in vivo. Modifying the ligation of 4-1BB represents a new approach to altering the graft-vs-host disease and graft-vs-leukemia effects of allogeneic T cells post bone marrow transplantation. The Journal of Immunology, 2001, 166: 3174–3183.

Despite the expression of 4-1BB on both CD4+ and CD8+ T cells, the majority of data available to date has suggested that CD8+ T cells are affected to a greater extent by agonistic anti-4-1BB mAbs than CD4+ T cells (16–19). In vivo CTL generation against both alloantigen (17) and tumor Ag-bearing targets (20) can be enhanced by the administration of a mitogenic anti-4-1BB mAb infusion. Results obtained with anti-4-1BB mAb infusion into tumor-bearing mice indicated that both CD4+ and CD8+ T cells were required for an optimal anti-tumor immune response, indicating that 4-1BB receptor signaling augmented T helper support of CD8+ CTL responses (20).

This study was conducted to determine the effect of 4-1BB receptor/4-1BBL interaction on regulating alloresponses in vivo. Therefore, we analyzed three types of clinically relevant in vivo alloresponses: graft-vs-host disease (GVHD), allogeneic bone marrow (BM) graft rejection, and graft-vs-leukemia (GVL). For this purpose, we used mice with a deletion in either 4-1BB or 4-1BBL and a mitogenic anti-4-1BB mAb. GVHD is caused by alloantigen-activated donor T cells that expand in vivo, migrate into GVHD target organs, and cause tissue destruction (reviewed in Refs. 21 and 22). In situations in which both CD4+ and CD8+ T cells can recognize host alloantigenic disparities, CD8+ T cells with CTL activity are thought to be driven to expand by CD4+ T cells that provide helper cytokines, which support CD8+ T cell growth. BM graft rejection occurs as a result of a host anti-donor reaction in which host T cells recognize alloantigens present on donor BM cells. GVL involves the recognition of leukemia-associated Ags by donor-immune effector cells. In many instances, donor T cells expand and mediate the elimination of leukemia cells, which express host type alloantigens. CD4+ or CD8+ alloreactive T cells are involved in each of these three immune responses that are critical to determining the long-term outcome of BM transplantation (BMT) recipients. Because CD28/B7 interactions are potent T cell costimulatory molecule pathways that are required for optimal GVHD (23–33) and GVL responses (34), we further sought to determine whether the effects of anti-4-1BB mAb were dependent upon amplification of CD28/B7 signals. Our data
indicate that 4-1BB/4-1BBL interactions regulate both CD4+ and CD8+ T cell-mediated alloresponses in vivo. These data have potentially important clinical ramifications.

Materials and Methods

Mice

B10.BR/SgSnl (H-2b), C57BL/6 (H-2b), CD4-deficient B6 (CD28−/−), C58m11 (termcd b11; CD45.2), and C57b2m12 (termcd b12; CD45.2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-CD45.1 congenic mice were purchased from the National Institutes of Health (Bethesda, MD). B6 and BALB/c (H-2d) 4-1BB receptors deutational (−/−) mice were generated as described (35), backcrossed nine generations, and bred at the University of Minnesota. 4-1BBL−/− mice were generated as described (36) and backcrossed for five generations to B6. Mice were bred and housed in a specific pathogen-free facility in microisolator cages. Donors and recipients were used at 8–10 wk of age.

Monoclonal Abs preparation

Anti-4-1BB mAb, clone 1AH2, rat IgG1 (6), was generated as ascites fluid and purified by ammonium sulfate precipitation followed by dialysis. This anti-4-1BB mAb was found to be mitogenic based on studies in which in vitro proliferation consistently was enhanced when CD4+ T cells were exposed to microspheres coated with suboptimal amounts of anti-CD3ε along with either anti-4-1BB or irrelevant control mAb (data not shown). Control rat IgG was purchased from Rockland Laboratories (Gilbertsville, PA). mAb injections were given i.p. according to doses and schedules described below.

GVHD generation

To determine the effect of 4-1BB/4-1BBL interactions on the GVHD capacity of CD4+ or CD8− T cells, we used a system in which purified T cell subsets are given to MHC-disparate, sublethally irradiated recipients as initially described by Sprent (37). This system permits highly accurate quantification of the degree of GVHD responses as related to T cell dose. For this purpose, MHC class II (b2m2) or class I (b1m) disparate recipients were irradiated with 6.0 Gy total body irradiation (TBI) on day 0 from a 137Cesium source at a dose rate of 85 cGy/min (29, 33). Four to six hours after TBI, sublethally irradiated b2m12 recipients were given purified lymph node (LN) CD4+ T cells at doses of 0.3–3 × 105 per recipient. To measure CD8− T cell GVHD lethality, b1m recipients were injected with CD8− T cells at doses of 1.0 or 3.0 × 105 per recipient. Recipients were given irrelevant or anti-4-1BB mAb (200 µg/dose) i.p. from days −1 to +5 then thrice weekly through day +21 or +28 post transfer, as indicated. To measure the effect of 4-1BBL deficiency on CD4+ or CD8− T cell mediated GVHD lethality, b1m CD4+ T cells or b1m CD8− T cells were infused into sublethally irradiated B6 4-1BBL−/− mice.

To purify LN cells, single cell suspensions of axillary, mesenteric, and inguinal LN cells were obtained (as a source of GVHD-causing effector cells), depleted of NK cells, and enriched for CD4+ or CD8− T cells by depletions with anti-CD8 (hybridoma 2.43, rat IgG2b; provided by David Sachs, Massachusetts General Hospital, Charlestown, MA) or anti-CD4 (hybridoma GK1.5, rat IgG2b; provided by Frank Fitch, University of Chicago, Chicago, IL), respectively. Rat mAb-coated T cells were passaged through a goat anti-mouse and goat anti-rat Ig-coated column (Biotex, Edmonton, Canada). The final composition of T cells in the donor graft was examined for evidence of GVHD and tumor by necropsy (34). Flow cytometry

Flow cytometry

The T cell, B cell, and granulocyte/macrophage constituency of splenocytes, peripheral blood cells, or TDL effluent was measured using mAb directed toward CD4 or CD8, CD19, and Mac1, respectively. All fluorochrome-labeled mAb, unless indicated, were obtained from Pharmingen (San Diego, CA). For mAb recipients of B6-CD45.1 CD8− T cells, splenocyte suspensions were monitored with or with CD45.2 (clone 104-2, rat IgG2b) and or CD45.1 (clone A20-1.7, rat IgG2a), both provided by U. Hammarling (Memorial Sloan Kettering Cancer Center, New York, NY). Cells were first incubated with 2.4G2 to block Fc receptors, and then incubated with optimal concentration of fluorochrome-labeled mAb for 45 min at 4°C. Cells were then washed three times and resuspended by two- or three-color flow cytometry using FITC-, PE-, or biotin (along with SA-PerCP)-conjugated mAb purchased from PharMingen or Becton Dickinson (Mountain View, CA). Irrelevant mAb control values were subtracted from values obtained with relevant mAbs. All results were obtained using a FACSCalibur (Becton Dickinson). Forward- and side-scatter settings were gated to exclude red cells and debris, and 1 × 104 cells were analyzed for each determination.

In vitro MLR assessment

For quantifying MLR responses, CD4+ LN T cells were mixed with irradiated (30 Gy) TCD splenocyte stimulators from host strain mice as described (33). Cells were suspended in MLR medium consisting of Dulbecco’s minimal essential medium (BioWhittaker, Walkersville, MD), 10% FCS (HyClone), 2-ME (5 × 10−6 M) (Sigma, St. Louis, MO), 10 mM HEPES buffer, 1 mM sodium pyruvate (Life Technologies), and amino acid supplements (1.5 mM l-glutamine, l-arginine, and l-asparagine) (Sigma), antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml; Sigma). For microtiter well analysis, 103 cell responders and 104 irradiated stimulators were plated in replicates of six into 96-well round-bottom (Costar) plates and placed at 37°C and 10% CO2 for 2–8 days. In some wells, as indicated, anti-4-1BB mAb was added at a final concentration of 50 µg/ml. Bulk cultures were established with 0.5 × 106 responders and 0.5 × 106 irradiated stimulators/ml and cultured under the same conditions as used for microtiter wells. Supernatant was collected from bulk cultures on the days indicated for ELISA analysis of cytokines. Cell recovery was quantified on day 8. Flow cytometry was performed at the end of the culture to analyze responder cells for expression of activation Ags (CD25, CD80, Thoracic duct cannulation

For thoracic duct lymphocyte (TDL) isolation, cannulae were inserted into the thoracic duct of recipients at the time of peak proliferation (day 6) post-BMT. TDL were collected over a period of 18 h (29, 33). Irrelevant or anti-4-1BB mAb was given at a dose of 200 µg daily i.p. from days −1 to +6 post-BMT.

GVU studies

Our GVU induction procedure has been described in detail (34). In brief, B6 mice undergoing BMT for GVU assessment were conditioned with 800 cGy irradiation from an x-ray source on day −1 and infused with 8 × 105 B10.BR or 20 × 106 BALB/c TCD BCM cells on day 0. Some mice, as indicated, were administered splenocytes from B10.BR, BALB/c, or BALB/c 4-1BB−/− donors i.v. at the indicated doses on day 21 post-BMT. On day 28 post-BMT, mice were challenged with an MHC class I+ acute myeloid leukemia line, C1498 (34). C1498 (American Type Culture Collection, Manassas, VA), originally derived as a spontaneous tumor line from a B6 mouse, was grown in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% heat-inactivated FBS (HyClone, Ogden, UT), 2 mmol/L L-glutamine, 50 mmol/L 2-ME, and penicillin/streptomycin. C1498 cells were given via the i.v. route at various cell doses ranging from 2 × 106 to 2 × 107 cells/mouse. In some experiments, cohorts of mice were given either irrelevant mAb or anti-4-1BB mAb at 200 µg/dose beginning 1 day before splenocyte infusion or 1 day before tumor infusion in mice that do not receive splenocytes. mAb is continued daily for 1 wk and then two times per week for three additional weeks. All available animals were examined for evidence of GVHD and tumor by necropsy (34).

Engraftment studies

B6-CD45.1 mice were irradiated with 5.0 or 5.5 Gy TBI, as indicated, by x-ray on day −1 and then given b1m or b12 TCD BM (5 × 105) cells (33, 38). Recipients were given anti-4-1BB or irrelevant mAb (200 µg/dose) i.p. daily from days −1 to +6 then twice weekly through day 14. Peripheral blood cells were phenotyped wk post-BMT.

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CD86, CD177, CD62 ligand; all were obtained from PharMingen), forward- and side-scatter properties, and signs of early apoptosis (7-aminoactinomycin D). To measure restimulation responses, day 8 bulk MLR cultures were extensively washed free of mAb and plated at a concentration of 0.3 × 10^6/well along with 10^5/well of irradiated splenocyte stimulators. Bulk cultures were established to obtain supernatants for cytokine analysis using 0.15 × 10^6/ml responders and 0.5 × 10^6/ml irradiated stimulators. Microtiter wells were pulsed with titrated thymidine (1 μCi) for 18 h before harvesting and counted in the absence of scintillation fluid on a beta plate reader. Δcpm were calculated by subtracting the syngeneic proliferative response from the allogeneic proliferative response.

The frequency of proliferating T lymphocyte precursor (pPTL) was quantitated. Eight 3-fold serial dilutions of responder CD4^+ T cells were plated at 30 replicates in 96-well round-bottom plates and incubated for 7 days with irradiated (30 Gy), non-TCID spenic stimulators in the absence of IL-2. Anti-4-1BB mAb was added to the primary LDA at a final concentration of 50 μg/ml. Wells were pulsed with titrated thymidine for 18 h before harvesting. Wells were scored positive if their cpm exceeded the average cpm plus 3 SDs of the stimulators plated without responders. Using Poisson distribution statistics according to the method of Tasswell and with the aid of a computer program, the likelihood of a single hit was confirmed and a frequency estimate was calculated.

For CTL analysis, CTL killing was assessed by the JAM assay (39). To generate targets, splenocytes were activated with Con A (2 μg/ml) for 3 days in MLR medium as described above. Con A-activated splenocyte targets were labeled overnight with [3H]thymidine (4 μCi/ml) in 24-well plates containing 2 × 10^6 cells in a 2-ml volume. TDL effectors were spun at 500 rpm (Beckman T3-6; Beckman Coulter, Fullerton, CA) for 5 min with targets (10^6 targets/well) at E:T ratios of 12.5:1–0.8:1 in the above described medium and then incubated together for a period of 4 h. Cell pellets were harvested and then counted as described above for the MLR culture. The percent specific lysis was based upon the retention of [3H]thymidine in the experimental groups as compared with targets not exposed to effector cells (25).

Quantitation of cytokine levels by ELISA

Murine cytokine levels (IL-2, IL-4, IL-10, TNF-α, IL-13, IFN-γ) in the supernatants of MLR cultures were quantitated by ELISA (R&D Systems, Minneapolis, MN). Sensitivity of the assay was between 1 and 10 pg/ml for each assay. A standard curve using recombinant protein was generated with each assay.

Statistical analyses

Group comparisons of continuous data were made by Student’s t test. Survival data were analyzed by lifetable methods using the Mantel-Peto-Cox statistic. The actuarial survival plot with a total of eight mice per group is shown. On the x-axis are the days post-BMT and on the y-axis is the proportion of mice surviving. Anti-4-1BB mAb-treated recipients of 10^5 CD8^+ T cells had a significantly (p = 0.0015) lower survival rate than controls, comparable (p = 0.14) to control recipients of a 3-fold higher cell dose.

Results

4-1BB/4-1BBL interaction augments GVHD-induced lethality mediated by either CD4^+ or CD8^+ donor T cells

Because 4-1BB is a known CD8^+ T cell survival factor (16), we first determined whether anti-4-1BB mAb infusion would augment CD8^+ T cell-mediated GVH lethality. Sublethally irradiated bm12 recipients were given a sublethal number of highly purified B6 MHC class I disparate CD8^+ T cells (10^4). Mice that received a mitogenic anti-4-1BB mAb had a 75% lethality rate that was significantly higher than the 0% observed in controls (Fig. 1). Weight curves reflected a greater degree of weight loss in the mAb-treated group, which was evident as early as day 7 and reached a maximum difference on day 19 post transfer (data not shown). The 75% lethality rate in mAb-treated recipients given 10^5 T cells was not statistically different from the 90% lethality rate observed in controls receiving a 3-fold higher CD8^+ T cell number (Fig. 1).

Signaling via 4-1BB has been reported to be a weak agonist for CD4^+ T cells (e.g., Ref. 17). To determine whether CD4^+ T cell-mediated GVHD could be affected by anti-4-1BB mAb, we infused a nonlethal dose of highly purified CD4^+ T cells (0.3 × 10^6) along with donor BM into lethally irradiated MHC class II-disparate bm12 recipients (Fig. 2A). In contrast to the 0% lethality by day 99 post-BMT in the irrelevant mAb-treated control group, 100% of the anti-4-1BB mAb-treated group died by day 10 post-BMT. To confirm that 4-1BB/4-1BBL interaction was essential for optimal CD4^+ T cell-mediated lethality, we performed experiments in which donor CD4^+ T cells obtained from B6 4-1BB^-/- mice were infused into sublethally irradiated bm12 recipients. Data pooled for two replicate experiments with similar results are shown in Fig. 2B. As compared with recipients of B6 wild-type CD4^+ T cells, recipients of 4-1BB^-/- CD4^+ T cells had a >3-fold reduction in GVH lethality based on the observation that mice receiving 10^5 4-1BB^-/- CD4^+ T cells had a 45% lethality rate vs a 94% lethality rate in recipients of as few as 3 × 10^5 wild-type cells.

As a further confirmation of the regulatory role of 4-1BB/4-1BBL interaction on CD4^+ T cell-mediated GVH lethality, we used B6 4-1BBL^-/- or wild-type control mice as recipients of bm12 MHC class II-disparate CD4^+ T cells (Fig. 2C). As observed with 4-1BB^-/- donor T cells, there was a >3-fold reduction in GVH lethality in 4-1BBL^-/- recipients vs controls, which was most evident by comparing the survival curves of 4-1BBL recipients given 3 × 10^5 T cells as compared with wild-type control recipients given 10^5 T cells. Under these adoptive transfer conditions, recipients experience multigain system GVH-induced damage as well as BM destruction due to the reaction of donor T cells against host hemopoietic progenitor cells (29, 33). A convenient measurement of GVH-induced injury is the quantification of peripheral blood hematocrit values, which can be sequentially analyzed without sacrificing recipients (29, 33). As compared with wild-type controls, day 14 hematocrit values obtained from 4-1BBL^-/- recipients of 3 × 10^5 CD4^+ T cells indicated a reduction in GVH-mediated BM destruction (17 ± 6 vs 23 ± 5%; p = 0.067). We conclude that 4-1BB regulates CD4^+ T cell-mediated GVH responses to approximately the same extent as CD8^+ T cell responses in sublethally irradiated recipients.

To directly examine the effect of anti-4-1BB mAb on CD4^+ T cell responses, in vitro studies were performed using B6 CD4^+ T cells and TCD, irradiated bm12 stimulators. MLR results indicated that anti-4-1BB mAb did not increase proliferation or cell recovery as compared with controls (data not shown). Activation Ag expression (CD25, CD69, CD80, CD86, CD62 ligand) on CD4^+ T
revealed a higher survival rate as compared with wild-type controls. Dose titration studies investigated. In A, B6 TCD BM supplemented with highly purified B6 CD4+ T cells (0.3 × 10^6/recipient) were infused into lethally irradiated bm12 recipients. Cohorts of mice were given either irrelevant or anti-4-1BB mAb, as indicated, according to doses and schedules listed in Materials and Methods. The actuarial survival plot with a total of five mice per group is shown. On the x-axis are the days post-BMT and on the y-axis is the proportion of mice surviving. Anti-4-1BB mAb-treated recipients had a significantly (p = 0.0015) lower survival rate than controls. In B, sublethally irradiated bm12 recipients were given wild-type or 4-1BB receptor −/− purified CD4+ T cells at the doses indicated. Two experiments were performed with similar results; data from 16 mice per group have been pooled. With the exception of the highest cell dose studied (3 × 10^6), in which there was only a trend (p = 0.078) toward longer survival in recipients given −/− vs control cells, the remaining comparisons revealed that recipients of 4-1BB receptor −/− CD4+ T cells had a significantly (p < 0.0001) higher survival rate vs controls. Comparisons between the groups revealed that the GVHD induction was reduced by ≥3-fold when −/− vs wild-type cells were infused. In C, sublethally irradiated 4-1BB BL−/− B6 recipients were given highly purified bm12 CD4+ T cells at the indicated cell dose. 4-1BB BL−/− recipients had a significantly (p ≤ 0.005) higher survival rate as compared with wild-type controls. Dose titration studies revealed a >3-fold reduction in GVHD lethality in −/− recipients vs wild-type controls.

FIGURE 2. GVHD mediated by highly purified CD4+ T cells infused into MHC class II only disparate recipients is regulated by 4-1BB/4-1BBL interactions. To determine the effects of 4-1BB/4-1BBL interaction on CD4+ T cell-mediated GVHD, three distinct GVHD systems were investigated. In A, B6 TCD BM supplemented with highly purified B6 CD4+ T cells (0.3 × 10^6/recipient) were infused into lethally irradiated bm12 recipients. Cohorts of mice were given either irrelevant or anti-4-1BB mAb, as indicated, according to doses and schedules listed in Materials and Methods. The actuarial survival plot with a total of five mice per group is shown. On the x-axis are the days post-BMT and on the y-axis is the proportion of mice surviving. Anti-4-1BB mAb-treated recipients had a significantly (p = 0.0015) lower survival rate than controls. In B, sublethally irradiated bm12 recipients were given wild-type or 4-1BB receptor −/− purified CD4+ T cells at the doses indicated. Two experiments were performed with similar results; data from 16 mice per group have been pooled. With the exception of the highest cell dose studied (3 × 10^6), in which there was only a trend (p = 0.078) toward longer survival in recipients given −/− vs control cells, the remaining comparisons revealed that recipients of 4-1BB receptor −/− CD4+ T cells had a significantly (p < 0.0001) higher survival rate vs controls. Comparisons between the groups revealed that the GVHD induction was reduced by ≥3-fold when −/− vs wild-type cells were infused. In C, sublethally irradiated 4-1BB BL−/− B6 recipients were given highly purified bm12 CD4+ T cells at the indicated cell dose. 4-1BB BL−/− recipients had a significantly (p ≤ 0.005) higher survival rate as compared with wild-type controls. Dose titration studies revealed a >3-fold reduction in GVHD lethality in −/− recipients vs wild-type controls.

FIGURE 3. Anti-4-1BB mAb does not increase the proliferation response of CD4+ T cells exposed to MHC class II disparate cells in vivo. Microtiter well MLR cultures were performed using TDL. Results are expressed as mean values ± 1 SD of data obtained from four individual mice per group. Days in culture are listed on the x-axis and mean cpm (obtained without scintillant amplification) values ± 1 SD (shown by error bars) are shown on the y-axis. In each instance, cpm for MLR cultures using syngeneic stimulators was ≥50. Anti-4-1BB mAb exposure did not increase MLR proliferation to Ag re-exposure as assessed via in vitro restimulation cultures.

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observed with anti-4-1BB mAb infusion in vivo is not due to support of T cell proliferation or expansion, although we cannot exclude an effect of anti-4-1BB mAb on functioning as a survival factor by supporting late T cell expansion in vivo.

Clinical BMT are performed with donor inocula containing both CD4+ and CD8+ T cells and typically involve heavy pre-BMT conditioning therapy resulting in the release of proinflammatory cytokines that may drive or facilitate GVHD-induced tissue injury (22, 41). To more closely simulate the clinical situation, we analyzed the role of 4-1BB in heavily irradiated, B6 (H2b) recipients of MHC class I plus II disparate B10.BR (H2k) BM and donor CD4+ and CD8+ T cell-containing splenocytes (Fig. 4). In this strain combination, both CD4+ and CD8+ T cells are required for optimal GVH-induced lethality (26). Recipients of 4-1BB−/− splenocytes at two different doses had significantly higher survival rates. Moreover, recipients of a 3-fold higher number of 4-1BB−/− vs wild-type splenocytes (15 × 10⁶ vs 5 × 10⁶, respectively) had a statistical trend (p = 0.079) toward a higher survival rate, consistent with a ~3-fold reduction of GVHD-induced lethality. At both splenocyte doses, weight curves were reflective of survival outcome (data not shown). Thus, 4-1BB/4-1BBL interactions are an important regulator of CD4+ and CD8+ T cell-mediated GVHD in heavily irradiated recipients.

Anti-4-1BB mAb infusion induces the generation of donor T cells with anti-host CTL activity without affecting overall donor T cell expansion in vivo

We next sought to obtain information as to the mechanism by which GVHD was regulated by 4-1BB/4-1BBL in lethally irradiated B10.BR recipients of B6 donor splenocytes. To ensure adequate numbers of cells to be available, we infused wild-type rather than 4-1BB−/− splenocytes (containing a total of 3.6 × 10⁶ CD4+ T cells and 2.3 × 10⁶ CD8+ T cells) as a source of donor cells and then treated mice with either anti-4-1BB or irrelevant mAb from days −1 to +6 post-BMT. At that time, thoracic duct cannulation was performed as a means of analyzing alloreactive donor T cells early post-BMT. Day 6 post-BMT is the time of maximal in vivo donor T cell expansion in response to the allogeneic recipient (32), paralleling the results of in vitro MLR assays. The total number of donor T cells (n = 4/group) produced per day (mean ± 1 SD = 46 ± 8 vs 34 ± 6 × 10⁶, irrelevant vs anti-4-1BB mAb, respectively; p = 0.16), the total number of CD4+ T cells (12 ± 3 vs 12 ± 3 × 10⁶, respectively; p = 0.84) and the total number of CD8+ T cells (35 ± 7 vs 24 ± 4 × 10⁶, respectively; p = 0.10) were not significantly different between the groups. The lack of effect of anti-4-1BB mAb on donor T cell expansion was reproduced in a second experiment (data not shown). CTL killing against irradiated splenic host-stimulating allogeneic cells at E:T ratios of ≈6.25:1 was significantly increased in the anti-4-1BB mAb-treated group despite the fact that there was a lower proportion of CD8+ T cells compared with the controls (data not shown). However, with E:T ratios of 12.5:1, CTL killing was comparable in both groups (data not shown). These data would indicate that although anti-4-1BB mAb does not augment to any great extent either CD4+ or CD8+ T cell proliferation in vivo under these conditions, donor anti-host-reactive CTLs are modestly increased by anti-4-1BB mAb treatment.

The accelerated GVHD lethality mediated by anti-4-1BB mAb-stimulated CD4+ T cells is partially CD28 independent

Ligation of 4-1BB can provide a T cell-costimulatory signal to either CD28+ or CD28− T cells (e.g., Refs. 9 and 42). For CD28− T cells, 4-1BB signaling can lower the threshold of CD28 signaling required to sustain T cell proliferation and IL-2 production. Because CD28/B7 and 4-1BB/4-1BBL can act independently or synergistically in inducing different types of T cell responses, we

Table 1. ELISA analysis of cytokine levels in supernatants from TDL obtained from anti-4-1BB mAb-treated recipients of MHC class II disparate CD4+ T cells

<table>
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<tr>
<th>Day</th>
<th>IL-2</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-4</th>
<th>IL-10</th>
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</table>

* TDL were obtained from lethally irradiated bm12 recipients of T cell-depleted BM plus purified B6 CD4+, T cells (10⁶/recipient). Irrelevant or anti-4-1BB mAb (200 μg/dose) was given i.p. from day −1 until the day of cannulation. TDLs obtained on days 6–7 post-BMT were cultured with irradiated, T cell-depleted bm12 splenic stimulators. Mean values from four mice per group that were individually analyzed are listed. Supernatants were harvested on the days indicated. Values are in picograms per milliliter and sensitivity is 1–10 pg/ml.
asked whether the augmented CD4+ T cell-mediated GVHD responses observed with anti-4-1BB mAb could function in a CD28-independent fashion in vivo. Lethally irradiated bm12 were reconstituted with B6 TCD BM supplemented with purified CD4+ T cells (1 or 3 × 10^6) from CD8-/- donors. Recipients of 10^6 CD4+CD28+/- T cells were given either irrelevant or anti-4-1BB mAb. Anti-4-1BB mAb accelerated GVHD lethality such that 100% of recipients of anti-4-1BB mAb vs 60% of control recipients died with GVHD in the first 2.5 mo post-BMT (p = 0.079). Recipients of 3 × 10^6 CD4+CD28+/- T cells all died by day 12 post-BMT. Thus, the degree of GVHD acceleration was <3-fold (3 × 10^6 T cells vs 10^6 T cells and anti-4-1BB mAb, p = 0.068). Because recipients of 0.3 × 10^6 CD4+CD28+/- T cells had no lethality, and those that received anti-4-1BB mAb all died within 10 days post-BMT (Fig. 2A), these data collectively indicate that 4-1BB mAb-induced GVHD can occur in a CD28-independent fashion although CD28/B7 interaction increases the function of 4-1BB mAb in inducing GVHD.

4-1BB/4-1BBL interaction augments allogeneic BM rejection mediated by either CD4+ or CD8+ donor T cells

The data presented above clearly indicate that anti-4-1BB mAb accelerates GVHD induction by either CD8+ or CD4+ donor T cells that encounter MHC class I or II Ags, which are distributed throughout the host microenvironment. To determine whether 4-1BB signaling regulates host CD4+ or CD8+ T cells that can recognize MHC Ags present on donor BM cells, we tested anti-4-1BB mAb administration for effects on alloengraftment. Anti-4-1BB mAb administration augmented host anti-donor T cell-mediated graft rejection by either T cell subtype. In a host CD8+ T cell-mediated graft rejection system (38), as compared with controls, mice that received MHC class I only disparate cells and anti-4-1BB mAb had a significantly higher proportion of host cells present in the periphery (10 ± 0% vs 40 ± 9%, respectively; p < 0.005) early post-BMT (6 wk post-BMT) (data not shown). These differences persisted when mice were analyzed later post-BMT (4 mo post-BMT) and were noted to involve all lineages examined (Table II). In a host anti-donor CD4+ T cell-mediated graft rejection system (38), as compared with controls, mice that received MHC class II only disparate cells and anti-4-1BB mAb had a significantly higher proportion of host cells present in the periphery (26 ± 2 vs 52 ± 2%, respectively; p < 0.005) at 6 wk post-BMT (Table II). As observed for the CD8+ T cell-mediated graft rejection system, additional phenotyping analysis at 111 days post-BMT revealed that chimerism was stable and further indicated that anti-4-1BB mAb-treated recipients had significantly higher proportions of host CD4+, CD8+, CD19+, and Mac1+ cells, reflective of an effect of mAb on facilitating rejection of allogeneic donor stem cells (Table II). These data are consistent with the GVHD results, demonstrating a role for both CD8+ and, interestingly, CD4+ T cells in regulating allosponses in vivo.

Anti-4-1BB mAb augments the anti-AML response of delayed lymphocyte infusion (DLI) by stimulating an allogeneic anti-tumor response

For patients that relapse post-BMT, DLI are used as a means of achieving remission by providing a potent anti-leukemia effect via recognition of alloantigens present on leukemia cells. Anti-4-1BB mAb augments the GVHD effect of donor spleen cells infused into lethally irradiated allogeneic recipients on day 0 of BMT. Because the GVHD response is markedly diminished when donor spleen cells are given later post-BMT (e.g., Refs. 34, 43), we performed studies to determine whether anti-4-1BB mAb would facilitate a GVL effect without augmenting GVHD lethality. B6 recipients were lethally irradiated and reconstituted with B6 donor TCD BM. At 3 wk post-BMT donor spleen cells were infused. One week later mice were killed with B6 AML cells. A low donor splenocyte dose (5 × 10^6) was chosen to minimize GVH potential, which would be achieved in the irrelevant mAb-treated control groups. Control-treated recipients given DLI all died within 3 mo post-BMT (Fig. 5A) with AML (Fig. 5B). In contrast, anti-4-1BB mAb-treated recipients of DLI had a 70% survival rate at >6 mo post-BMT (p = 0.0054 vs irrelevant mAb controls) (Fig. 5A). Ninety to 100% of recipients given AML cells with or without DLI died with AML occurrence unless anti-4-1BB mAb also was given (Fig. 5B). In the latter instance, AML was found in only 30% of recipients (p = 0.0012 vs irrelevant mAb control). Thus, the combination of low dose DLI and anti-4-1BB mAb resulted in >2 log_{10} reduction in AML-associated lethality.

To more accurately assess the possible risk-benefit ratio of DLI under the cover of anti-4-1BB mAb, we used a higher DLI dose (25 × 10^6 splenocytes), which should accentuate the GVHD potential of DLI. Recipients given donor splenocytes had a 70% survival rate at 9 mo post-BMT vs only 10% of recipients given no donor spleen cells and 100-fold fewer AML cells (Fig. 6, A and B). Recipients given the same donor splenocyte and high AML dose inocula along with anti-4-1BB mAb had only a 20% long-term survival rate (p = 0.014 vs irrelevant mAb-treated controls). Anti-4-1BB mAb-treated mice given donor splenocytes and a 10-fold lower AML cell dose also had a significantly lower survival rate compared with irrelevant mAb-treated controls (p = 0.0012) (data not shown). The higher mortality rate of anti-4-1BB mAb-treated recipients was not due to a higher AML burden. None of the DLI-treated recipients of anti-4-1BB mAb had evidence of AML when examined at autopsy as compared with 30% of recipients of irrelevant mAb (p = 0.11), indicating that anti-4-1BB mAb stimulated

<table>
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<tr>
<th>Group</th>
<th>Day</th>
<th>No.</th>
<th>Donor</th>
<th>Host</th>
<th>Donor</th>
<th>Host</th>
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<td>14</td>
<td>97 (0)</td>
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<td>1 (0)</td>
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<td>46 (1)</td>
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<td>34 (10)^*</td>
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<td>11 (3)^*</td>
<td>4 (1)^*</td>
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<td>12</td>
<td>38 (9)^*</td>
<td>64 (9)^*</td>
<td>17 (3)^*</td>
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<td>8 (3)^*</td>
<td>32 (6)^*</td>
<td>9 (3)^*</td>
<td>10 (3)^*</td>
</tr>
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* B6-CD45.1 recipients were sublethally irradiated (5.5 Gy (bm12) or 5.0 Gy (bm1) TBI) on day −1 and then given 10^7 TCD BM from bm1 or bm12 donors and either irrelevant IgG or anti-4-1BB mAb as described in Materials and Methods. On the indicated day post-BMT, all surviving recipients were phenotyped to determine the donor or host origin of CD4+ or CD8+ T cells, CD19+ B cells, and Mac1+ myeloid cells. Values shown are mean percentages. The number (No.) of mice H2 phenotyped per group by two-color flow cytometry is listed. *, p < 0.05 vs controls; ^, p = 0.056.
While it is unclear from the above studies whether the GVL effect is dependent upon facilitating DLI-mediated alloresponses that have cross-reactivity against Ags expressed on AML cells, it is clear that anti-4-1BB mAb does increase DLI-GVHD. However, it is possible that anti-4-1BB mAb would also augment the antitumor responses of DLI by mechanism(s) other than to increase DLI-GVH responses. Results by others have shown that anti-4-1BB mAb increases anti-tumor immune responses.

A GVL effect albeit at the expense of GVHD-induced lethality (Fig. 6B). Examination of the weight curves is supportive of the latter conclusion. The effect of anti-4-1BB mAb on augmenting DLI-mediated GVHD was also observed in B6 recipients given AML cells and high doses of donor splenocytes ($25 \times 10^6$) (data not shown). To avoid any contribution of AML cells to the mortality process observed with DLI and anti-4-1BB mAb, studies were performed in which high dose DLI ($50 \times 10^6$ splenocytes) was administered to recipients not challenged with AML cells (Fig. 6C). Recipients given anti-4-1BB mAb had a significantly ($p = 0.0003$) lower survival rate vs irrelevant mAb controls. The degree of protection against AML recurrence was $2 \log_{10}$ in anti-4-1BB mAb-treated recipients.

Although it is unclear from the above studies whether the GVL effect is dependent upon facilitating DLI-mediated alloresponses that have cross-reactivity against Ags expressed on AML cells, it is clear that anti-4-1BB mAb does increase DLI-GVHD. However, it is possible that anti-4-1BB mAb would also augment the antitumor responses of DLI by mechanism(s) other than to increase DLI-GVH responses. Results by others have shown that anti-4-1BB mAb increases anti-tumor immune responses. A means of avoiding DLI-mediated GVHD that is augmented by anti-4-1BB mAb would be to determine whether anti-4-1BB mAb could augment the post-BMT anti-AML response of donor T cells educated
in the host thymus, which should be tolerant to host alloantigens. Therefore, anti-4-1BB mAb was administered at the same doses and schedule to recipients not given DLI. Anti-4-1BB mAb did not provide any anti-AML effect in allogeneic BMT recipients treated with anti-4-1BB mAb and challenged with AML cells (2 × 10^6) (data not shown). Subsequent studies performed in non-BMT mice also revealed no anti-AML effect of anti-4-1BB mAb in mice given as few as 10^5 cells (data not shown). Based on these data, we hypothesize that the anti-AML effect of anti-4-1BB mAb in DLI-treated recipients is most likely due to an augmented alloresponse rather than an AML-specific immune response.

Discussion

Several major findings can be derived from our study. Both CD8

+ and CD4

+ T cell donor anti-host-mediated GVHD and host anti-
donor-mediated graft rejection can be regulated by 4-1BB/4-1BBL interaction. The accelerated CD4

+ T cell-mediated GVHD lethality observed with anti-4-1BB mAb infusion was partly CD28 independent. Anti-4-1BB mAb infusion increased the anti-AML GVL responses of DLI resulting in higher survival rates in recipients given lower dose DLI. The GVL effect likely required allogeneic DLI infusion as no benefits from anti-4-1BB mAb were observed in post-BMT recipients given no DLI or in non-BMT controls. Under conditions in which the GVH response of high dose DLI was substantial, anti-4-1BB mAb further increased the alloreactivity, resulting in a lower survival rate.

We have shown that 4-1BB/4-1BBL interaction affects alloreactive CD4

+ T cell responses by several different approaches that used anti-CD4

+ mAb, 4-1BB receptor +/− donor CD4

+ T cells, or 4-1BBL +/− recipients of donor CD4

+ T cells. Both CD8

+ and CD4

+ T cell-mediated GVHD responses were affected by 4-1BB/4-1BBL ligation. Based upon these data, we have shown that CD4

+ T cells were affected to approximately the same extent as CD8

+ T cells. The generation of donor CD4

+ T cells with anti-host CTL capacity that was facilitated by anti-4-1BB mAb administration was directly demonstrated by isolation of T cells obtained from allogeneic recipients. Our previous studies demonstrating that perforin-deficient donor T cells have a reduced GVHD lethality capacity in vivo indicate that CTL generation is important for T cell-mediated GVHD (44). Interestingly, in this study, we found that neither CD4

+ nor CD8

+ T cell expansion, as measured by thoracic duct cunnation, in vivo was increased by anti-4-1BB mAb infusion. Thus, our results would suggest that other studies demonstrating that anti-4-1BB mAb is more potent in stimulating CD8

+ vs CD4

+ T cells may be due to the particular anti-4-1BB mAb clone used in their studies (17, 18). Our studies extend those of Shuford and colleagues (17), who have shown that anti-4-1BB mAb infusion supported alloreactive CD8

+ CTL generation in nonirradiated F1 recipients of parental spleen cells, which was associated with host splenic involuion and CD8

+ but not CD4

+ T cell expansion.

Previous studies have shown using a panel of mAbs that the most potent anti-4-1BB mitogenic mAbs tested were more effective in stimulating CD8

+ vs CD4

+ T cells under conditions of suboptimal CD3e stimulation. These investigators showed that there were qualitative and quantitative differences in tyrosine phosphorylation patterns between anti-4-1BB mAb-stimulated CD8

+ vs CD4

+ T cells (17). Anti-4-1BB mAb administration has been found to rescue the superantigen-mediated deletion of CD8

+ T cells to a greater degree than CD4

+ T cells (16). Tan et al., using 4-1BBL +/− mice, have shown that 4-1BBL +/− mice had 2- to 3-fold lower CD8

+ T cell expansion rates in response to lymphocytic choriomeningitis virus (LCMV) without effects on virus-spe-
cific CD4

+ T helper responses indicating a preferential effect of 4-1BBL on supporting CD8

+ vs CD4

+ T cell expansion in vivo (18). Therefore, we had anticipated that anti-4-1BB mAb might have pronounced effects on CD8

+ vs CD4

+ T cell-mediated GVHD. However, several studies have shown that 4-1BB/4-1BBL binding stimulates CD4

+ T cell responses (12, 16–18, 42, 45, 46). Moreover, the initial characterization of 4-1BB receptor by Kwon and Weissman demonstrated mRNA to be present in CTL and T helper cell lines (5) and the 4-1BB receptor is expressed on both activated CD4

+ and CD8

+ T cells (6). Additional evidence that CD4

+ T cells can be affected by 4-1BB can be derived from studies by Mittler and colleagues, who have shown that anti-4-1BB mAb suppresses in vivo humoral responses to T cell-dependent Ags by a process that did not directly inhibit either CD8

+ T cells or B cells but did presumably inhibit CD4

+ T cells (47). Optimal anti-4-1BB mAb-induced anti-tumor immune responses were dependent on the presence of both CD8

+ and CD4

+ T cells, indicating that CD4

+ T cells were enhanced by anti-4-1BB mAb treatment (20). Other studies have reported that 4-1BB can augment in vitro CD4

+ T cell proliferation, cell cycle progression, and IL-2 secretion, and suppress cell death (46). Our data extend these observations by directly demonstrating that naive CD4

+ T cells are regulated by 4-1BB/4-1BBL interactions in vivo under clinically relevant conditions. MLR studies with the particular anti-4-1BB mAb clone (1A1H2) used in our studies did not reveal any evidence of enhancement of alloresponses in vitro although with suboptimal (but not optimal) engagement of the CD3e chain we were able to demonstrate that the anti-4-1BB mAb we used was indeed mitogenic. The lack of enhancement of proliferative responses to alloantigen would suggest that the mechanism(s) involved in the augmentation of GVHD responses in vivo by the binding of 4-1BB to its ligand is not due to an increase in the early expansion phase of alloreactive CD4

+ T cells that may already be maximally driven by the recognition of host alloantigen-bearing cells. Rather, we favor the possibility that 4-1BB/4-1BBL interaction functions either to increase donor anti-host CTL-mediated tissue injury or to sustain CD4

+ T cells past the point of peak in vivo expansion. 4-1BB costimulation has been shown to preferentially support Th1 cell cytokine production while suppressing Th2 cytokine production (45). Others have shown that the enhancement of Tc1 cytokine production is observed only with CD8

+ T cells (17), whereas Th1 or Th2 cytokine production in Th1 or Th2 clones was not affected by 4-1BB binding (46). Our data with alloactivated CD4

+ T cells demonstrate that neither Th1 nor Th2 cytokine production is substantially affected by anti-4-1BB mAb exposure, consistent with data in CD4

+ T cell clones (47). Therefore, we do not favor as an explanation that the augmentation of CD4

+ T cell-mediated GVHD by 4-1BB ligation is due to a shift in the cytokine profile to Th1 cytokines. Regardless of the mechanism(s) responsible for GVHD augmentation, the fact that both CD4

+ and CD8

+ donor T cells are regulated by 4-1BB/4-1BBL interactions have significant implications for the clinical GVHD setting as both T cell types are present in the donor BM inoculum.

Results by our group and others (23–33) have shown that optimal GVHD generation requires CD28/B7 interaction. These studies demonstrate that anti-4-1BB mAb-induced CD4

+ T cell-mediated GVHD acceleration can occur albeit to a lesser extent with CD28 +/− donor T cells. These data would indicate that GVHD inhibition would be more effectively achieved by blocking both CD28/B7 and 4-1BB/4-1BBL pathways. Our findings that 4-1BB mAb-induced GVHD acceleration was less potent when infusing CD28 +/− vs wild-type T cells are consistent with investigators.
who have shown that 4-1BB receptor signaling can enhance proliferation of activated T cells that are dependent on T cell costimulation but are incapable of being costimulated through the CD28/CTLA4:B7 pathway (9, 12, 14, 36, 42, 45).

Although Melero et al. have shown that anti-4-1BB mAb was a potent inducer of CTL generation resulting in the rejection of established, poorly immunogenic tumors (20), we were unable to observe any benefit of anti-4-1BB mAb in bolstering an immune response to systemically administered AML cells unless alloreactive DLI were given. Our previous studies have shown that CD8+ T cells are the cytolytic effectors for the MHC class I+ II- AML cells used in this study. CD4+ T cells may provide help for this response. The fact that anti-4-1BB mAb treatment in either non-BMT mice or in allogeneic BMT recipients not given DLI failed to result in an observable anti-AML effect may be due to the fact that the immune response to AML cells under these conditions is weak and, in the absence of priming, anti-4-1BB mAb may not be expected to further increase the response. Alternatively, it is possible that the differences of our results as compared with Merlero et al. are related to the particular anti-4-1BB mAb clone used. Nonetheless, because anti-4-1BB mAb-treated recipients of allogeneic DLI can mount a vigorous anti-AML effect, it is likely that anti-4-1BB mAb augments alloreactivity of DLI, which is capable of eliminating AML cells via recognition of alloantigens present on the AML cells. The GVH response of DLI may be particularly amenable to an augmented GVL response under the cover of anti-4-1BB receptor expression, thereby further amplifying the mitogenic effect of anti-4-1BB mAb. Anti-4-1BB mAb infusion may be particularly beneficial in settings in which the baseline alloreactive response is suboptimal in terms of eliminating AML cells in vivo and may be far less critical if alloreactive T cells are potent mediators of GVL responses. For example, we have found that B6 recipients of BALB/c 4-1BB-/- DLI are still capable of 2 log10 elimination of AML cells in vivo (our unpublished observations). Conversely, anti-4-1BB mAb may be inadequate in supporting a GVL effect in situations in which the alloreactive response is profoundly suboptimal as is the case with allogeneic BMT recipients of TCD donor grafts that are not given DLI. Because anti-4-1BB mAb facilitates the alloreactive potential of DLI, recipients receiving both DLI and anti-4-1BB mAb would be at risk for greater GVHD-induced complications. Nonetheless, there are situations in which DLI are not especially efficacious in providing long-term disease-free survival for patients with leukemia relapsing post-BMT (e.g., acute lymphoblastic leukemia or AML) (48). In those patients, the increased risk of GVH-associated complications may be offset by the possibility of superior long-term survival due to the generation of a more potent GVL effect.

In summary, we have shown that 4-1BB/4-1BBL interactions regulate both the CD4+ and CD8+ T cell-mediated GVHD response of donor T cells given at the time of BMT or later post-BMT. In the latter instance, the GVL response of DLI could be sufficiently improved so as to translate into a superior long-term survival rate. The host anti-donor T cell-mediated BM allograft rejection mediated by both CD4+ and CD8+ T cells was shown to be dependent in part on 4-1BB/4-1BBL interactions. The fact that the 4-1BB receptor costimulatory pathway had such a pronounced effect on CD4+ T cell responses was somewhat unexpected. Furthermore, we have shown that the augmented responses of CD4+ T cells to anti-4-1BB mAb were due to effects on CTL generation and not due to either a preferential induction of Th1 or Th2 cytokine production or to increased proliferation. The increased in vivo GVHD responses of alloreactive CD4+ T cells exposed to anti-4-1BB mAb were only partly CD28 dependent. Thus, all three major allogeneic responses (GVHD; graft rejection; GVL), which are critical in determining the long-term outcome of allogeneic BMT, were affected by 4-1BB/4-1BBL interactions. Our data suggest that manipulation of the 4-1BB/4-1BBL costimulatory pathway alone or in conjunction with the CD28/CTLA4:B7 pathway could represent an important therapeutic modality for improving the outcome of allogeneic BMT.

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References


