The Journal *of* Immunology

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CD30/CD30 Ligand (CD153) Interaction Regulates CD4+ T Cell-Mediated Graft-versus-Host Disease[1]

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CD30, a TNFR family member, is expressed on activated CD4+ and CD8+ T cells and B cells and is a marker of Hodgkin's lymphoma; its ligand, CD30L (CD153) is expressed by activated CD4+ and CD8+ T cells, B cells, and macrophages. Signaling via CD30 can lead to proliferation or cell death. CD30-deficient (−/−) mice have impaired thymic negative selection and increased autoreactivity. Although human alloreactive T cells preferentially reside within the CD30+ T cell subset, implicating CD30 as a regulator of T cell immune responses, the role of CD30/CD153 in regulating graft-vs-host disease (GVHD) has not been reported. We used a neutralizing anti-CD153 mAb, CD30−/− donor mice, and generated CD153−/− recipient mice to analyze the effect of CD30/CD153 interaction on GVHD induction. Our data indicate that the CD30/CD153 pathway is a potent regulator of CD4+, but not CD8+, T cell-mediated GVHD. Although blocking CD30/CD153 interactions in vivo did not affect alloreactive CD4+ T cell proliferation or apoptosis, a substantial reduction in donor CD4+ T cell migration into the gastrointestinal tract was readily observed with lesser effects in other GVHD target organs. Blockade of the CD30/CD153 pathway represents a new approach for preventing CD4+ T cell-mediated GVHD. *The Journal of Immunology,* 2004, 173: 2933–2941.

U pon T cell activation, CD28 and other costimulatory receptors, including CD27, CD30, CD134, CD137, and CD154, are up-regulated. CD30, a 120-kDa type I transmembrane glycoprotein member of the TNFR family, is expressed on Reed-Sternberg cells in Hodgkin's disease (1), B cells, and mitogen-stimulated T cells (2, 3). The peak time for CD30 expression after TCR activation is 4–5 days in vitro (2), and either CD28 or IL-4R signaling are required for CD30 up-regulation (4). CD30 signals augment T cell proliferation at low levels of in vitro TCR stimulation (4, 5).

Similar to other members of the TNFR family, CD30 engagement may regulate T cell survival. For example, CD30 signaling regulates peripheral T cell responses, controlling T cell survival and down-regulating cytolytic capacity (2, 6–12). CD30 also regulates thymocyte survival. Thymic selection appears to be influenced by the level of CD30 expression. In one study, CD30-deficient (CD30−/−) mice were reported to express a negative selection defect (9), although selection was not affected in another study using a different CD30−/− mouse strain (13). Conversely, CD30-overexpressing mice had increased thymocyte apoptosis after TCR engagement (10).

CD30 ligand (CD30L, CD153) is a 40-kDa type II membrane-associated glycoprotein belonging to the TNF family (3, 5, 14, 15). CD153 is expressed on activated T cells, primarily CD4+ T cells of both Th1 and Th2 phenotype, as well as on a subset of accessory cells (2, 3, 14, 15) and B cells (16–19). In addition, CD153 can provide signals for B cell growth and differentiation (3, 15).

In vivo, CD30+ T cells are increased in Omenn's syndrome, which has features similar to graft-vs-host disease (GVHD)[5] that occurs after bone marrow transplantation (BMT) (20). These findings, as well as the known function of CD30 on controlling T cell responses, survival, and cytolytic effector cell function, prompted us to examine the role of the CD30/CD153 pathway in GVHD. Results obtained using a blocking anti-CD153 mAb, CD30−/−, and newly generated CD153−/− mice in well-characterized experimental models of allogeneic BMT indicate that the CD30/CD153 pathway is a critical regulator of CD4+ but not CD8+ T cell-mediated GVHD.

Materials and Methods

Mice

BALB/c (H-2d), B6.C-H2bm12/KhEg (termed bm12), and B6.C-H2bm1/KhEg (termed bm1) recipients were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 (termed B6) mice were purchased from the National Institutes of Health (Bethesda, MD). CD30−/− mice were generated as described (9) and further backcrossed seven generations with B6. B6 CD153−/− mice were generated as described below. Enhanced GFP (eGFP) transgenic mice, on the B6 background, were obtained from the laboratory of J.S.S., and bred at the University of Minnesota (Minneapolis, MN) (21). Donors and recipients were 8–12 wk of age.

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[5] Abbreviations used in this paper: GVHD, graft-vs-host disease; 7-AAD, 7-amino-actinomycin D; BM, bone marrow; BMT, BM transplantation; CD30L, CD30 ligand; CD95L, Fas ligand; eGFP, enhanced GFP; ES, embryonic stem cell; LN, lymph node; Neo, Neomycin; TBI, total body irradiation; TCD, T cell depleted.

Generation of CD153$^{-/-}$ mice

The targeting vector for CD153 was designed to replace a 594-bp region of the *CD153* gene including exon1 with a Neomycin (Neo) resistance gene cassette flanked by a 6.5-kb arm of 5′ and a 4.3-kb arm of 3′ CD153 genomic DNA. A Neo cassette and a thymidine kinase cassette were inserted into the pKO Scrambler V903 vector (Lexicon Genetics, Woodland, TX). The 5′ arm was amplified by PCR using the Pwo polymerase (Roche, Nutley, NJ) and the primers (sense) 5′-ATTCGGTACCATCGAATCAG TAGCATCAG-3′; and (antisense) 5′-ATTCGGTACCGTCCCACATCA CACCTTATC-3′. The 3′ arm was cloned from the pBeloBAC11 vector genomic library digested with *Bgl*II and *Sac*II. These arms were inserted into the multiple cloning sites of pKO Scrambler V903 (Fig. 1*B*). The targeting vector was electroporated into RW4 embryonic stem cells (ES) (Genome Systems, St. Louis, MO) and selected by the antibiotic, G418. Genomic DNA was extracted from selected ES colonies and analyzed by Southern blotting using 5 μg of DNA digested with *Sph*I. The probe for screening was outside the vector. Four of 89 clones were positive for homologous recombination (Fig. 1*C*). Positive ES clones were microinjected into embryos obtained from B6. Male chimeric mice were bred with B6 mice and agouti pups were selected for analysis of germline transmission. Agouti mice were intercrossed to obtain homozygotes, and tail DNA was screened by PCR. Primers for screening of tail DNA were designed to amplify genomic DNA of the replaced region, giving a 1026-bp product that is longer in the mutant gene than in the wild-type gene (Fig. 1*D*). The sequence for the screening primers was as follows: sense, 5′-ATGAG GAGAGATAAGGTGTG-3′; and antisense, 5′-CACGGATTGAGGA CATAACC-3′. CD153 deletion was confirmed in CD153$^{-/-}$ (Fig. 1*E*). Mice used in this study were F9 after backcrossing into the B6 background for further progeny.

GVHD induction

To assess CD4$^+$ vs CD8$^+$ T cell-mediated GVHD lethality, bm12 or bm1 recipients were sublethally irradiated (6.0 Gy total body irradiation (TBI)) (day −1) and given highly purified lymph node (LN) CD4$^+$ or CD8$^+$ T cells from B6 CD30$^{+/+}$ or CD30$^{-/-}$ donors on day 0 (22). Cohorts of bm12 recipients of B6 CD30$^{+/+}$CD4$^+$ T cells were given control IgG or anti-CD153 mAb as described below. In other studies, bm12 recipients were lethally irradiated on day −1, reconstituted with B6 T cell-depleted (TCD) bone marrow (BM) on day 0, and given CD4$^+$ T cells from B6 CD30$^{-/-}$, CD30$^{+/+}$, or B6 GFP donors along with the BM inocula. To determine the role of CD30/CD153 blockade on CD4$^+$ T cell-mediated GVHD induced in a fully allogeneic lethally irradiated system, B6

CD153$^{+/+}$ or B6 CD153$^{-/-}$ recipients were conditioned with 9.0 Gy TBI on day −1. BMT into B6 CD153$^{-/-}$ and B6 CD153$^{+/+}$ mice were performed 1 day later (day 0). Recipients received 6 × 10^6 BALB/c TCD BM, along with the indicated doses of CD4$^+$ T cells from donor BALB/c spleen and LN following selection using Miltenyi column magnetic bead enrichment (purity was >98% CD4$^+$ T cells; Miltenyi Biotec, Auburn, CA). Mice were weighed twice weekly and monitored daily for survival and clinical GVHD.

Anti-CD153 mAb

The rat anti-murine CD153 Ab, RM153, was purified from ascites as described (14) or generated using a hollow fiber bioreactor (National Cell Culture Center, Minneapolis, MN). Control IgG was purchased (Rockland Immunochemicals, Gilbertsville, PA). Anti-CD153 mAb and control IgG were administered at 200 μg/dose i.p. daily from days −1 to +5 then three times per week through day +21.

Analysis of serum cytokine/chemokine levels after BMT

On days 2, 4, and 6 after BMT, sera were obtained from five mice per group per time point to measure proinflammatory cytokines (IL-1β, IL-6, IL-12p70, and TNF-α), Th1/T cytokine type 2 cytokines (IL-2 and IFN-γ), Th2/Tc2 (IL-4, IL-5, IL-10, IL-13), and chemokines (JE, KC, and MIP-2) and vascular endothelial growth factor by multiplex analysis using the Luminex method (Austin, TX) and murine-specific commercial kits (sensitivity 1–5 pg/ml; R&D Systems, Minneapolis, MN). The results were interpolated from standard curves of relevant recombinant proteins (R&D Systems).

Analysis of CD4$^+$ T cell proliferation and apoptosis in vivo

Highly enriched CD4$^+$ T cell populations (>98%) from spleen and LNs of BALB/c mice were obtained by positive selection using Miltenyi bead separation columns (Miltenyi Biotech). CD4$^+$ T cells were adjusted to 2 × 10^7/ml in 2.5 μM CFDA (Molecular Probes, Eugene, OR) for 15 min at 37°C. Cells were resuspended in PBS and incubated for another 30 min at 37°C. CFDA labeled cells were washed, counted, and adjusted to appropriate concentration with TCD BM for i.v. injection into irradiated (9.0 Gy, by ^{60}Co gamma irradiator) B6 recipient mice. On day 5 after BMT, spleen cells were obtained from recipient mice and analyzed for CFDA staining. In addition, apoptosis was simultaneously measured using an annexin V-PE apoptosis detection kit (BD Pharmingen, San Diego, CA). Briefly, cells were washed twice in cold PBS, resuspended in binding buffer at a concentration of 1 × 10^6/ml and 100 μl of cells were transferred into

FIGURE 1. Generation of CD153$^{-/-}$ mice. The intron-exon structure of the mouse CD30L (*CD153*) gene and corresponding mRNA are shown (*A*). The targeting vector for CD153 was designed to replace a 594-bp region of the *CD153* gene including exon1 (*A*) with a Neo cassette and thymidine kinase cassette (*B*) as described in *Materials and Methods*. The targeting vector was electroporated into RW4 ES cells and selected by G418. *B*, Genomic DNA was extracted and analyzed by Southern blotting using a probe outside the vector. *C*, Four of 89 clones were positive for homologous recombination (HR). Positive ES clones were microinjected into embryos obtained from B6. Male chimeric mice were bred with B6 mice and agouti pups were analyzed for germline transmission. *D*, Primers were designed to amplify genomic DNA of the replaced region giving a 1026-bp product that is longer in the mutant gene than in the wild-type (WT) gene. *E*, RT PCR analysis (*lanes 1–4*) of fresh (*lanes 1* and *3*) or in vitro activated (*lanes 2* and *4*) spleen cells obtained from wild-type and CD153$^{-/-}$ (knockout) mice demonstrate expression of CD30 and CD153 in CD153$^{+/+}$ but only CD30 in CD153$^{-/-}$ mice.
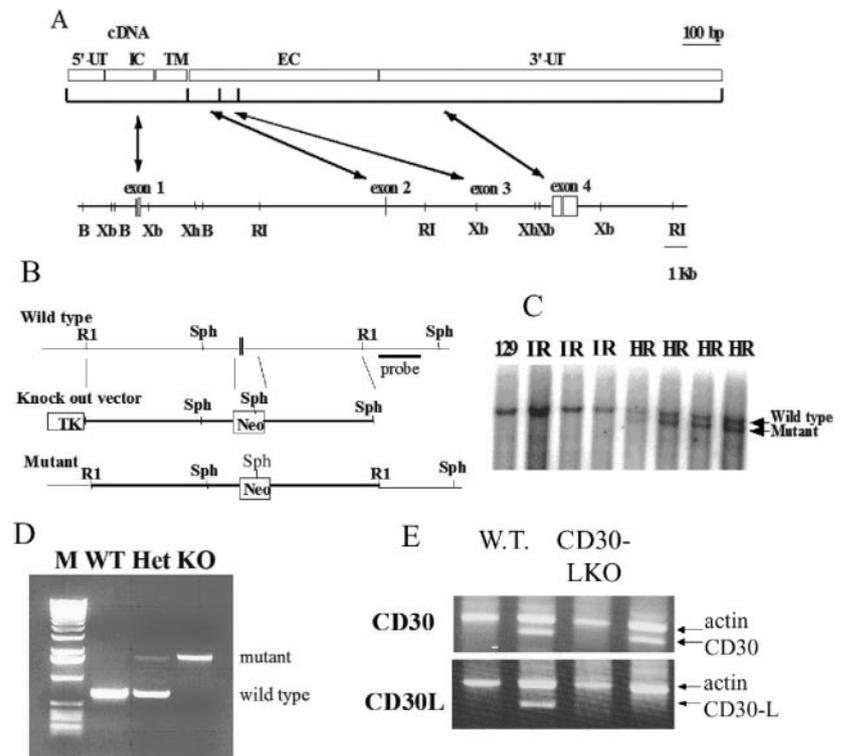
staining tubes and stained with 5 μl of annexin V-PE and 5 μl of 7-ami-noactinomycin D (7-AAD; BD Pharmingen). Cells were incubated at room temperature for 15 min in the dark, 400 μl of binding buffer was added to each tube, and cells were analyzed by flow cytometry within 1 h. Unstained and single-stained (i.e., annexin V-PE only and 7-AAD) cells were used to set compensation and quadrants for analysis.

In vivo imaging

As reported previously, images were taken with a Magnafire color camera (Optronics, Goleta, CA) mounted onto a Leica MZFLIII stereomicroscope using a GFP2-bandpass filter and a ×0.63 transfer lens (Leica Microsystems, Bannockburn, IL) (23). Zoom factors from ×3.5–10 were used. Exposure times were optimized for GVHD control mice for each organ and identical times were used for all other groups. Mice receiving allogeneic BM only (non-GFP) served as negative controls for background autofluorescence in the green channel, and as demonstrated in a previous publication, resulted in black images and are not shown in this publication to save space (23). Instead, images of syngeneic controls consisting of irradiated B6 mice transplanted with B6 BM and B6 GFP⁺ T cells are shown to distinguish homeostatic expansion from the alloantigen-induced proliferation in the GVHD control mice. Three mice per group were examined at 1 wk and 2 wk after BMT. A representative image is illustrated.

Statistical analysis

The Kaplan-Meier product-limit method was used to calculate survival rates. Differences between groups were determined using the log-rank test and were considered significant for p values ≤ 0.05.
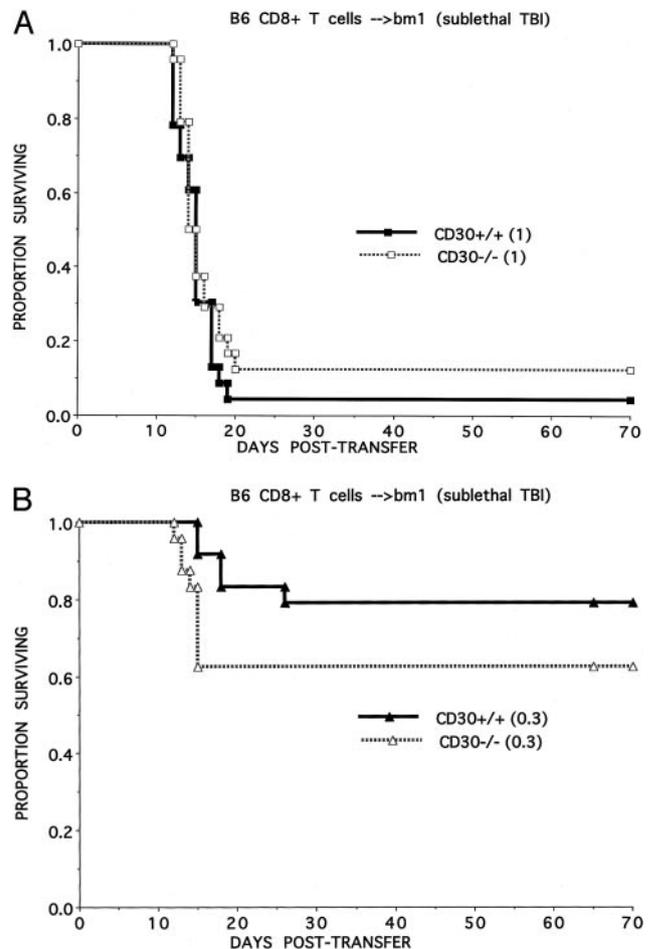
Results

The absence of CD30 expression on donor CD8⁺ T cells does not influence GVHD-induced mortality in sublethally irradiated, MHC class I-only disparate recipients

Activated CD8⁺ T cells up-regulate CD30, and CD30 engagement affects the expression of cytolytic molecules (CD95, perforin) and the homing of cytolytic effector cells (11). Therefore, we hypothesized that the CD30/CD153 pathway might be a critical regulator of CD8⁺ T cell-mediated GVHD. CD8⁺ T cells from B6 CD30⁺/⁺ vs CD30⁻/⁻ donors were infused into sublethally irradiated MHC class I-disparate bm1 recipients. No significant differences in survival rates were evident in recipients of a CD8⁺ T cell dose of 1×10^6 (Fig. 2A) or 0.3×10^6 cells (Fig. 2B), although the lower dose of CD8⁺ T cells resulted in only a 21% mortality rate. Taken together, these data indicate that the absence of CD30 on donor CD8⁺ T cells has minimal influence on GVHD.

The absence of CD30 expression on donor CD4⁺ T cells reduces GVHD-induced mortality in MHC class II only or full MHC and multiple minor Ag-disparate recipients

CD30 also is up-regulated on CD4⁺ T cells and CD30 engagement regulates proliferation, Th cytokine production, and augments alloantigen responses in vitro (2, 4, 5, 11). To determine whether the absence of the CD30/CD153 pathway would affect CD4⁺ T cell-mediated GVHD, CD4⁺ T cells from B6 CD30⁺/⁺ or B6 CD30⁻/⁻ donors were infused into sublethally irradiated MHC class II-disparate bm12 recipients. Recipients of CD30⁻/⁻ vs CD30⁺/⁺CD4⁺ T cells at a dose of 0.3×10^5 cells had a significantly different survival rate (67% vs 18%, $p = 0.0007$) (Fig. 3A). There was no survival difference at a higher CD4⁺ T cell dose (10^5 cells) (Fig. 3B), indicating that CD30 expression is less important under more aggressive GVHD conditions involving higher CD4⁺ T cell doses (Fig. 3B).

Because proinflammatory cytokines induced by heavy irradiation might affect the requirement for T cell costimulation, and to simulate BMT regimens that use full myeloablative regimens, studies were performed in bm12 recipients that were lethally irradiated and reconstituted with B6 BM cells containing B6 CD30⁺/⁺ or CD30⁻/⁻CD4⁺ T cells (1×10^6) (Fig. 4A). Recipients of CD30⁺/⁺CD4⁺ T cells had a 25% survival rate vs 88% in recip-

FIGURE 2. The absence of CD30 expression on donor CD8⁺ T cells does not reduce GVHD lethality in MHC class I-only disparate recipients. Sublethally irradiated bm1 recipients were infused with 1×10^6 CD8⁺ (*A*) or 0.3×10^6 (*B*) T cells obtained from CD30⁺/⁺ or CD30⁻/⁻ donors, as indicated. Numbers in parentheses are ×10⁶ CD8⁺ T cells per recipient. Three replicate experiments ($n = 8$ mice/group/experiment) were performed. Statistical analyses are $p > 0.05$ for comparisons of CD30⁺/⁺ and CD30⁻/⁻ results in *A* and *B*.
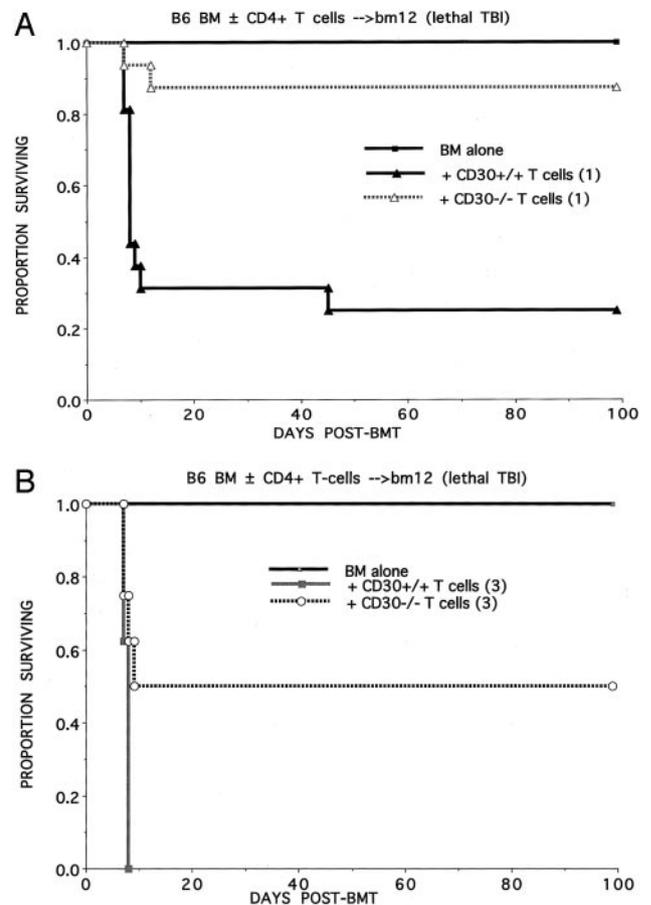
ients of CD30⁻/⁻CD4⁺ T cells ($p = 0.003$). When a 3-fold higher CD4⁺ T cell dose was infused, 50% of recipients of CD30⁻/⁻ T cells survived vs 0% of controls ($p = 0.02$) (Fig. 4B). Despite the survival advantage using CD30⁻/⁻ donor CD4⁺ T cells, there were no significant differences in the serum levels of proinflammatory cytokines (IL-1β, IL-6, IL-12p70, and TNF-α), Th1 cytokines (IL-2 and IFN-γ), Th2 cytokines (IL-5, IL-10, and IL-13), or chemokines (JE, KC, and MIP-2) in recipients of CD30⁺/⁺ vs CD30⁻/⁻CD4⁺ T cells ($n = 5$ mice/group on days 2, 4, and 6 after BMT; data not shown). Moreover, flow cytometry analysis was performed on five mice per group on day 6 after BMT to quantify the absolute number of CD4⁺ T cells present in the spleen, a major GVHD target organ. Flow cytometric analysis of splenocytes on 6 days after BMT, a time of peak donor T cell proliferation in vivo, revealed that there were no significant differences in the absolute CD4⁺ T cell number ($n = 5$/group, data not shown) in recipients of CD30⁺/⁺ or CD30⁻/⁻CD4⁺ T cells. These data indicate that the survival advantage conferred by CD30⁻/⁻ CD4⁺ T cells is not due to a reduction in donor T cell proliferation in the spleen early after BMT, nor to a reduction or skewing in cytokine production.
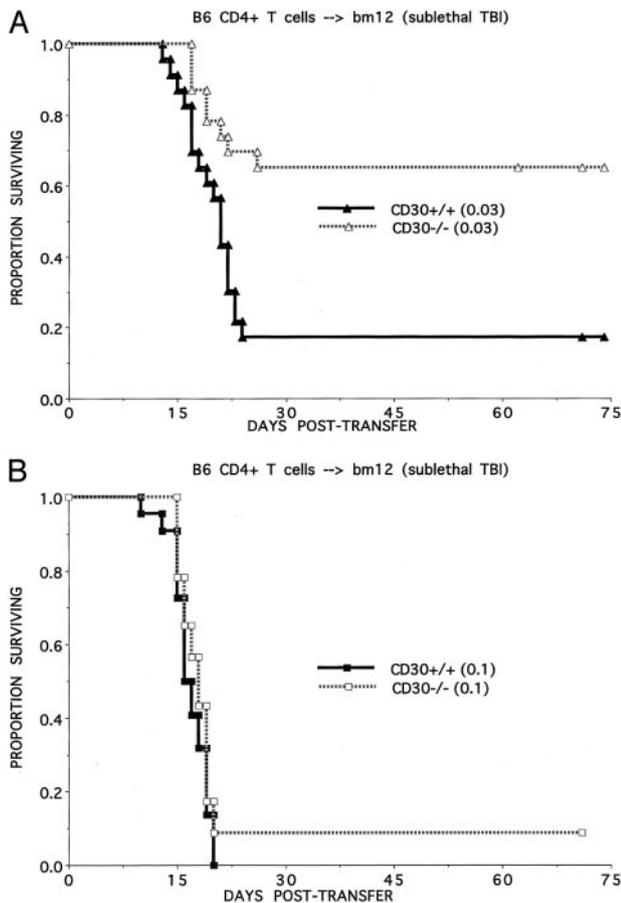
FIGURE 3. The absence of CD30 expression on donor CD4^{+} T cells reduces GVHD mortality in sublethally irradiated MHC class II-only disparate recipients, but only at lower CD4^{+} T cell doses. Sublethally irradiated bm12 recipients were infused with 0.03×10^6 (*A*) or 0.1×10^6 (*B*) CD4^{+} T cells obtained from CD30^{+/+} or CD30^{−/−} donors, as indicated. Numbers in parentheses are $\times10^6$ CD4^{+} T cells per recipient. Three replicate experiments (*n* = 8 mice/group/experiment) were performed. Statistical analyses are *p* = 0.0007 (*A*), and *p* = 0.13 (*B*).

FIGURE 4. The absence of CD30 expression on donor CD4^{+} T cells reduces GVHD mortality in lethally irradiated MHC class II-only disparate recipients. Lethally irradiated bm12 recipients were infused with B6 BM cells alone, or supplemented with 1×10^6 (*A*) or 3×10^6 (*B*) CD4^{+} T cells obtained from CD30^{+/+} or CD30^{−/−} donors, as indicated. Numbers in parentheses are $\times10^6$ CD4^{+} T cells per recipient. Two replicate experiments (*n* = 8 mice/group/experiment) were performed. Statistical analyses are *p* = 0.003 (*A*), and *p* = 0.02 (*B*).

Targeting of CD153 by a blocking mAb inhibits CD4^{+} T cell-mediated GVHD lethality

As another approach to determine whether blockade of the interaction between CD30 and CD153 would result in impaired CD4^{+} T cell-mediated GVHD, sublethally irradiated bm12 recipients were given B6 CD30^{+/+}CD4^{+} T cells and control IgG or anti-CD153 mAb. Anti-CD153 mAb abrogated GVHD-induced lethality in recipients of two different cell doses (Fig. 5*A*). A similarly high degree of efficacy in blocking GVHD by anti-CD153 mAb infusion could be seen in lethally irradiated bm12 recipients of B6 CD4^{+} T cells at a dose of 1×10^6 per mouse with anti-CD153 mAb infusion, resulting in 12% mortality as compared with 88% mortality in control IgG-treated recipients (Fig. 5*B*). In the same study, a cohort of recipients given CD30^{−/−}CD4^{+} T cells had the same survival as those given CD30^{+/+}CD4^{+} T cells and anti-CD153 mAb (data not shown). Similarly, at a higher CD4^{+} T cell dose of 3×10^6 cells, 100% of controls succumbed, compared with a mortality rate of 25% with anti-CD153 mAb (Fig. 5*C*) and 50% with B6 CD30^{−/−}CD4^{+} T cells and control IgG (*p* = 0.14 vs anti-CD153 mAb) (data not shown). Taken together, these data indicate that precluding CD30/CD153 interaction by either infusing an antagonistic anti-CD153 mAb or CD30^{−/−} results in a similar degree of inhibition of CD4^{+} T cell-mediated GVHD lethality.

CD4^{+} T cells cause less mortality when infused into heavily conditioned, fully allogeneic CD153^{−/−} vs CD153^{+/+} recipients

Because CD153 also is expressed on activated CD4^{+} T cells, it is possible that the inhibitory effect of anti-CD153 mAb on GVHD-induced lethality could be partially conferred by depleting activated CD153^{+}CD4^{+} T cells. To determine whether the absence of host CD153 expression alone would be sufficient to reduce CD4^{+} T cell-mediated GVHD lethality, B6 CD153^{−/−} mice were generated as described (Fig. 1) and used as recipients of fully allogeneic BALB/c CD4^{+} T cells. B6 CD153^{−/−} or B6 CD153^{+/+} mice were lethally irradiated (9.0 Gy TBI), and given BALB/c TCD BM along with highly purified BALB/c CD4^{+} T cells (Fig. 6). There was a significantly superior survival noted in B6 CD153^{−/−} recipients of BALB/c CD4^{+} T cells (1×10^6) as compared with wild-type control recipients (75% vs 12%) (Fig. 6*A*). In addition to the survival advantage, CD153^{−/−} recipients exhibited markedly less weight loss and had other milder GVHD symptoms (data not shown). Engraftment could be readily monitored by flow cytometry. Therefore, we used this opportunity to be certain that the lower GVHD mortality seen in these heavily irradiated recipients was not due to preferential host cell recovery. At 4 mo after BMT, the percentage of PBMC that was donor in origin ranged from 95 to 99% (B220^{+} cells), 52–91% (Mac1^{+} cells), 69–94% (NK1.1[+]
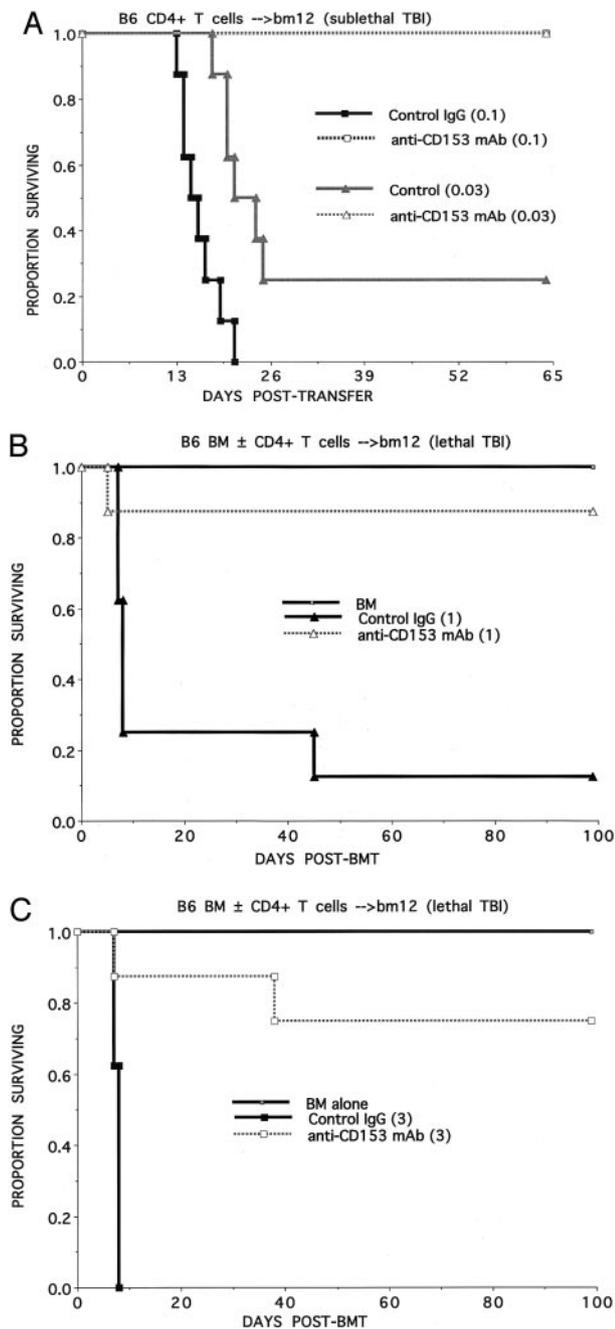
FIGURE 5. The administration of a blocking anti-CD153 mAb was highly effective in inhibiting CD4$^+$ T cell-mediated mortality in either sublethally or lethally irradiated MHC class II-only disparate recipients. *A*, Sublethally irradiated bm12 recipients were infused with 0.03×10^6 or 0.1×10^6 CD4$^+$ T cells obtained from wild-type B6 donors. Lethally irradiated bm12 recipients were infused with B6 BM alone or supplemented with 1×10^6 (*B*) or 3×10^6 (*C*) CD4$^+$ T cells obtained from wild-type B6 donors. Numbers in parentheses are $\times 10^6$ CD4$^+$ T cells per recipient. Eight mice per group per experiment were analyzed. Control IgG or anti-CD153 mAb was given as described in *Materials and Methods*. Statistical analyses are $p < 0.002$ for both cell dose comparisons (*A*), $p = 0.0045$ (*B*), and $p = 0.0018$ (*C*).

cells), 74–87% (CD4$^+$ T cells), and 40–64% (CD8$^+$ T cells) in six individual mice analyzed. These data indicated that multilineage donor engraftment had occurred (data not shown). Insufficient numbers of control CD153$^{+/+}$ mice ($n = 1$) remained at 4 mo after BMT to make statistical comparisons.
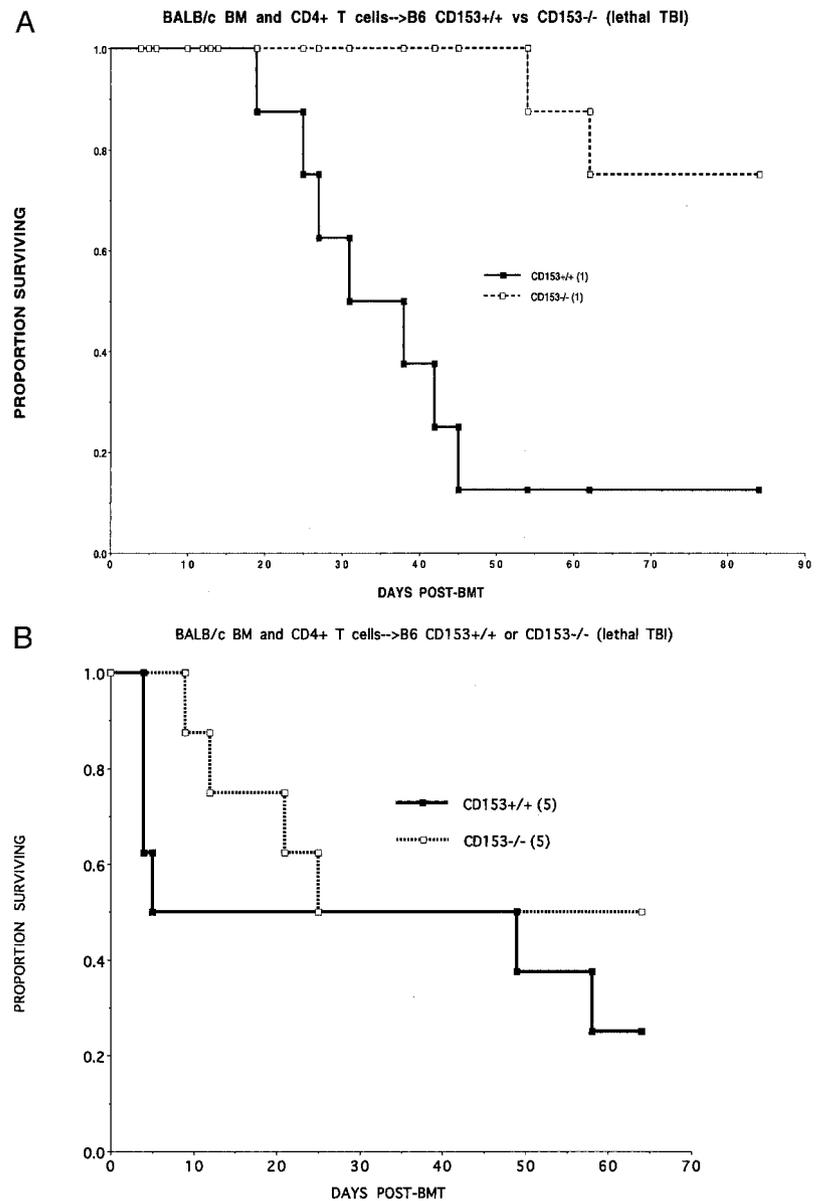
Although a GVHD protective effect was observed in CD153$^{-/-}$ vs CD153$^{+/+}$ recipients given 1×10^6 BALB/c CD4$^+$ T cells, at a higher CD4$^+$ T cell dose (5×10^6), the difference was no longer evident (50% vs 75% mortality, respectively; $p = 0.14$) (Fig. *6B*). These observations are consistent with those described above in an isolated MHC class II-only disparate system in which a survival advantage was observed under conditions of lower but not higher donor CD4$^+$ T cell infusion. Collectively, these data indicate that CD30/CD153 interaction regulates GVHD-induced lethality across a full MHC and multiple minor histocompatibility Ag disparities, which is particularly evident at low CD4$^+$ T cells doses. Therefore, both CD153-expressing host cells and donor CD30-expressing donor T cells are required for optimal CD4$^+$ T cell-mediated GVHD lethality.

Early after BMT, proliferation and apoptosis rates of CD4$^+$ T cells transferred into lethally irradiated allogeneic CD153$^{+/+}$ or CD153$^{-/-}$ recipients are comparable

One explanation for the reduced GVHD lethality observed in CD153$^{-/-}$ vs CD153$^{+/+}$ recipients of allogeneic CD4$^+$ T cells was the possibility that the infused T cells failed to proliferate or underwent a higher degree of apoptosis in the former as compared with the latter recipients. To determine optimal kinetics of proliferation in this transplant model, CFDA-labeled BALB/c CD4$^+$ T cells were transplanted into 9.0-Gy irradiated B6 CD153$^{+/+}$ recipients. Significant proliferation was initially observed on day 5 posttransplant (data not shown). The proliferation of CFDA$^+$CD4$^+$ T cells was subsequently examined in both B6 CD153$^{+/+}$ and B6 CD153$^{-/-}$ recipient spleens at day 5 (Fig. *7A*) and day 8 (Fig. *7B*) posttransplant. Donor CD4$^+$ T cells, examined from individual mice, demonstrated multiple divisions at these time points in both recipient groups. The total donor T cell numbers were calculated (total spleen cell number × percentage of CFDA$^+$CD4$^+$) and no significant differences were detected (data not shown). To investigate apoptotic levels in these donor CD4$^+$ T cells, annexin V staining was performed on recipient spleen cells at day 5 (Fig. *7C*). No significant differences in annexin V staining were detected in either CFDA-labeled (donor) or unlabeled (host) populations in both recipient groups. Thus, the reduced GVHD lethality observed in CD153$^{-/-}$ vs CD153$^{+/+}$ recipients did not appear to be due to effects on CD4$^+$ T cell proliferation, as assessed after infusing wild-type CD4$^+$ T cells infused into allogeneic CD153$^{-/-}$ recipients. In addition, the survival advantage in settings in which the CD30/CD153 pathway was not operative did not appear to be due to apoptosis of CD4$^+$ T cells that had localized to the spleen at early time points after BMT.

Blockade of CD30/CD153 interactions reduces the migration of CD4$^+$ T cells into the gastrointestinal tract and the skin

To investigate the effect of the CD30/CD153 pathway on effector T cell trafficking to GVHD target organs, in vivo imaging studies with GFP transgenic CD4$^+$ T cells were performed. For imaging studies, B6 wild-type BM was combined with purified CD4$^+$ effector T cells, obtained from B6 eGFP Tg mice, and infused into lethally irradiated bm12 mice. Based upon the survival data depicted in Fig. 5B, a CD4$^+$ T cell dose of 10^6/recipient was chosen. Cohorts of mice were given either anti-CD153 or irrelevant mAb as described above. As an additional imaging control, eGFP$^+$ effector T cells and BM were infused into lethally irradiated syngeneic B6 mice to compare normal homeostatic expansion of GFP$^+$ T cells to alloantigen-driven proliferation. bm12 mice were transplanted with BM only (no GFP$^+$ effectors) as a negative control for imaging to verify lack of autofluorescence. It was previously determined that day 7 was the first optimal time point for detecting

A

BALB/c BM and CD4+ T cells-->B6 CD153+/+ vs CD153-/- (lethal TBI)

B

BALB/c BM and CD4+ T cells-->B6 CD153+/+ or CD153-/- (lethal TBI)

FIGURE 6. Lethally irradiated CD153$^{-/-}$ recipients of fully allogeneic donor BM and CD4$^+$ T cells have a marked reduction in GVHD-induced mortality as compared with CD153$^{+/+}$ recipients at lower but not higher CD4$^+$ T cell doses. B6 CD153$^{-/-}$ or B6 CD153$^{+/+}$ recipients ($n = 8$ mice/group) were lethally irradiated and infused with BALB/c BM supplemented with BALB/c CD4$^+$ T cells at a dose of 1×10^6 (A) or 5×10^6 (B). Statistical analysis was $p = 0.0018$ (A), and $p = 0.14$ (B).

GFP$^+$ cells in this strain combination (P.A.T., unpublished data). By 1 wk after BMT, lymphoid organs (LNs, Peyer's patches, and spleen) had a similar influx of GFP$^+$ cells in irrelevant and anti-CD153 mAb-treated GVHD mice, indicating that CD30/CD153 did not appear to adversely affect homing into these organs (data not shown). A representative illustration of the mesenteric LN is shown as imaged at 1 and 2 wk after BMT (Fig. 8A). A transient decrement in GFP$^+$CD4$^+$ T cells was observed in the skin (Fig. 8B) and lung (data not shown) in anti-CD153 vs irrelevant mAb-treated recipients such that fewer GFP$^+$ cells were present in the former at 1 wk but not 2 wk after BMT. Modest reductions in GFP$^+$ cells were seen in the liver at both time points (Fig. 8C). The major effects of CD30/CD153 pathway blockade were in the extent of GFP$^+$ T cell infiltration into the gastrointestinal tract, as illustrated by images of the stomach (Fig. 8D), ileum (Fig. 8E), and colon (Fig. 8F). There was a marked and persistent decrement in GFP$^+$CD4$^+$ T cells that migrated to these sites in anti-CD153 mAb-treated recipients. Notably, the LN adjacent to the colon imaged at 1 wk after BMT reveals no substantial effect of anti-CD153 vs irrelevant mAb. These findings suggest that the improved survival in anti-CD153 mAb-treated recipients may be due to im-

paired donor CD4$^+$ T cell homing to or expansion within GVHD target organs, especially those of the gastrointestinal tract.

Discussion

In this study, we demonstrate that the signaling via the CD30/CD153 pathway is required for optimal GVHD-induced lethality by donor CD4$^+$ but not CD8$^+$ T cells. CD4$^+$ T cell-mediated GVHD lethality was inhibited in situations in which there was an isolated MHC class II-only disparity or a full MHC plus multiple minor Ag disparities. However, the influence of CD30 ligation on GVHD-induced lethality was not evident at higher CD4$^+$ T cell doses, indicating that the requirement for CD30 engagement could be obviated if the vigor of GVHD induction was increased by infusing large numbers of T cells. Collectively, these data provide new evidence that CD30 regulates alloresponses in vivo that result in a clinically relevant disease process, GVHD.

The observation that alloreactive CD4$^+$ T cell-mediated GVHD lethality was more dependent than CD8$^+$ T cells upon CD30 ligation was somewhat unexpected based upon the finding that alloantigen-activation induces CD30 on CD8$^+$ and to a lesser extent on CD4$^+$ T cells (24, 25). MHC class II-disparate CD30$^{-/-}$CD4$^+$
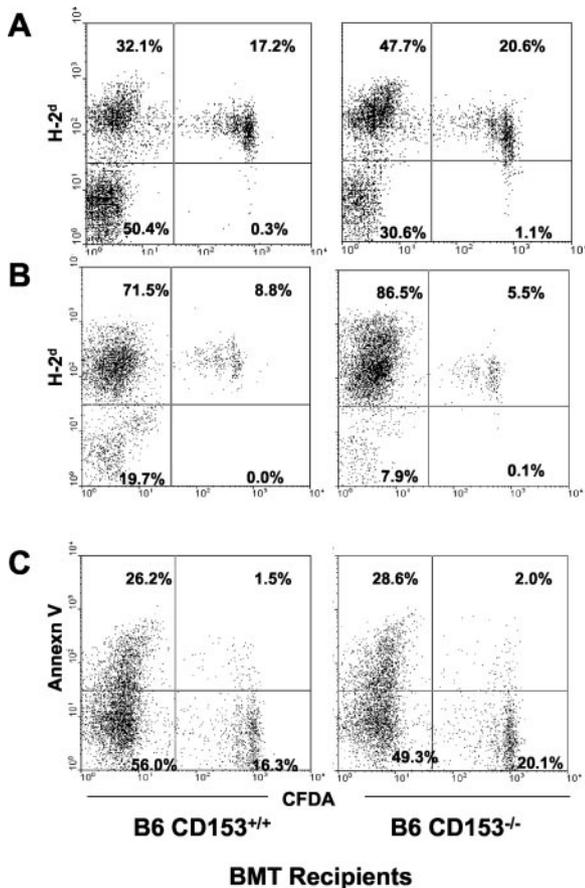
FIGURE 7. Similar proliferation and apoptotic frequencies of donor CD4$^+$ T cells transferred into lethally irradiated B6 CD153$^{+/+}$ vs B6 CD153$^{-/-}$ BMT recipients. BALB/c TCD BM and 1×10^7 CFDA-labeled CD4$^+$ T cells were transplanted into 9.0-Gy conditioned B6 CD153$^{+/+}$ or B6 CD153$^{-/-}$ recipients. Five (*A*) and 8 (*B*) days after BMT, the spleens were removed and individually assessed for donor CD4 T cell division (H-2^{d+}CFDA$^+$). Data represents results from individual mice (day 5, $n =$ 3–4/group; day 8, 2/group). No differences were detected in the percentage of H-2^{d+}CFDA$^+$ in B6 CD153$^{+/+}$ vs B6 CD153$^{-/-}$ recipients ($p > 0.5$). *C*, Annexin V analysis was performed in BMT recipients. BMT recipients were given CFDA-labeled BALB/c CD4$^+$ T cells as above. Spleen cells were removed 5 days after BMT and stained for annexin V and 7-AAD. Data shown are cells within the lymphoid gate. No differences in the percentage of CFDA$^+$ annexin V staining cells were observed between the B6 CD153$^{+/+}$ (1.1–1.5%) vs B6 CD153$^{-/-}$ (0.9–2.0%).

T cells infused into either sublethally or more aggressively conditioned lethally irradiated recipients, a setting in which there are reduced T cell costimulatory requirements, resulted in markedly lower GVHD lethality than CD30$^{+/+}$CD4$^+$ T cells. However, this benefit was largely negated by higher CD4$^+$ T cell doses. In contrast, MHC class I-only disparate CD8$^+$ T cells from CD30$^{-/-}$ or CD30$^{+/+}$ donors infused into sublethally irradiated recipients, a setting in which T cell costimulatory pathway dependency is highest, resulted in comparable survival at two distinct CD8$^+$ T cell doses. Consistent with the lack of observed survival benefits in a CD8$^+$ T cell-mediated GVHD system, we have not seen improvements in survival rates in lethally irradiated B10.BR recipients of a low (5×10^6/recipient), intermediate (15×10^6/recipient), or high (25×10^6/recipient) dose of fully MHC-disparate B6 CD30$^{-/-}$ vs CD30$^{+/+}$ splenocytes ($n = 24$–32 mice/group) (data not shown). In this GVHD system, CD8$^+$ T cells, as well as CD4$^+$ T cells, are required for optimal GVHD-induced lethality.

Using anti-CD153 mAb, we observed a profound inhibition of CD4$^+$ T cell-mediated GVHD lethality. Because CD153 is up-regulated on activated CD4$^+$ T cells, one interpretation is that the mAb cleared CD4$^+$ T cells. Therefore, to better define the role of CD153 expression on host cells, especially APCs, CD153$^{-/-}$ mice were generated to be used as recipients. Consistent with the infusion of a blocking anti-CD153 mAb, lethally irradiated CD153$^{-/-}$ recipients of fully MHC-disparate CD4$^+$ T cells had a markedly superior survival as compared with CD153$^{+/+}$ recipients. As observed with CD30$^{-/-}$ donor CD4$^+$ T cells, GVHD lethality was comparable in CD153$^{-/-}$ and CD153$^{+/+}$ recipients if high numbers of CD4$^+$ T cells were infused. Collectively, these data are most consistent with the explanation that the absence of CD30 ligation on donor CD4$^+$ T cells by CD153 expressed on host cells per se is responsible for the suboptimal GVHD lethality observed.

It is clear that CD4$^+$ T cells express CD30 following activation, supporting an important role of CD30 ligation on CD4$^+$ T cell function. Although some studies proposed CD30 as a marker for Th2 cells (20, 26), others have found that both Th1/T cytokine type 1 and Th2/T cytokine type 2 cytokines are produced upon CD30 engagement (27–29). Therefore, CD30 engagement can regulate Th cytokine production, the exact nature of which may depend upon the state of activation and/or differentiation of T cells at the time of CD30 ligation. In our studies, serum cytokine analysis did not reveal a differential effect on Th1 or Th2 cytokine production in mice receiving MHC class II-only disparate CD30$^{+/+}$ vs CD30$^{-/-}$CD4$^+$ T cells at the time points studied (days 2, 4, and 6). In addition to the effects of CD30 ligation on CD4$^+$ Th cytokine production, CD30 signaling has been shown to affect peripheral T cell responses, controlling T cell survival and down-regulating the capacity for mediating cytolysis via inhibiting Fas ligand (CD95L) and perforin expression and cytolytic effector cell trafficking (2, 6–12). Our preliminary studies have not revealed a role of CD30 signaling in initial alloreactive CD4$^+$ T cell expansion, as measured by splenic flow cytometry on day 6 after BMT with identical mean CD4$^+$ T cell numbers in recipients of either MHC class II-only disparate CD30$^{+/+}$ or CD30$^{-/-}$CD4$^+$ T cells. Because the vast majority of the recipients of CD30$^{+/+}$CD4$^+$ T cells succumbed to GVHD shortly after this flow cytometry analysis and there were no differences in the absolute number of CD4$^+$ T cells present in the spleen of recipients of either CD30$^{+/+}$ or CD30$^{-/-}$ cells, it would seem unlikely that a defect in CD4$^+$ T cell expansion per se was responsible for the lower GVHD lethality observed in recipients of CD30$^{-/-}$CD4$^+$ T cells. In our studies, strong proliferation of donor BALB/c CD4$^+$ T cells was observed beginning at 5 days after BMT in both B6 CD153$^{+/+}$ and B6 CD153$^{-/-}$ recipients, with no apparent differences in CD4$^+$ T cell numbers at this time. Because CD30 can regulate both apoptotic and anti-apoptotic genes, apoptosis of responding CD4$^+$ donor cells was also examined. No differences in the percentage of annexin V$^+$ cells were detected on donor cells in CD153$^{+/+}$ and CD153$^{-/-}$ recipients early posttransplant. These findings support the notion that early afferent GVH events posttransplant associated with the initial expansion of donor T cells in lymphoid tissue were not affected by CD153-mediated signals (11).

Rather, we favor the possibility that blocking CD30 ligation precludes donor T cell-mediated cytolysis of host cells and/or donor CD4$^+$ T effector cell trafficking into host tissues. Both the perforin (30) and CD95L pathways have been implicated in CD4$^+$ T cell-mediated GVHD (31). With respect to our studies, it is interesting that greater numbers of perforin and CD95L-deficient CD4$^+$ T cells vs cytotoxically normal CD4$^+$ T cells were required to mediate comparable GVHD lethality and weight loss in heavily irradiated MHC-disparate recipients (32). Thus, one explanation
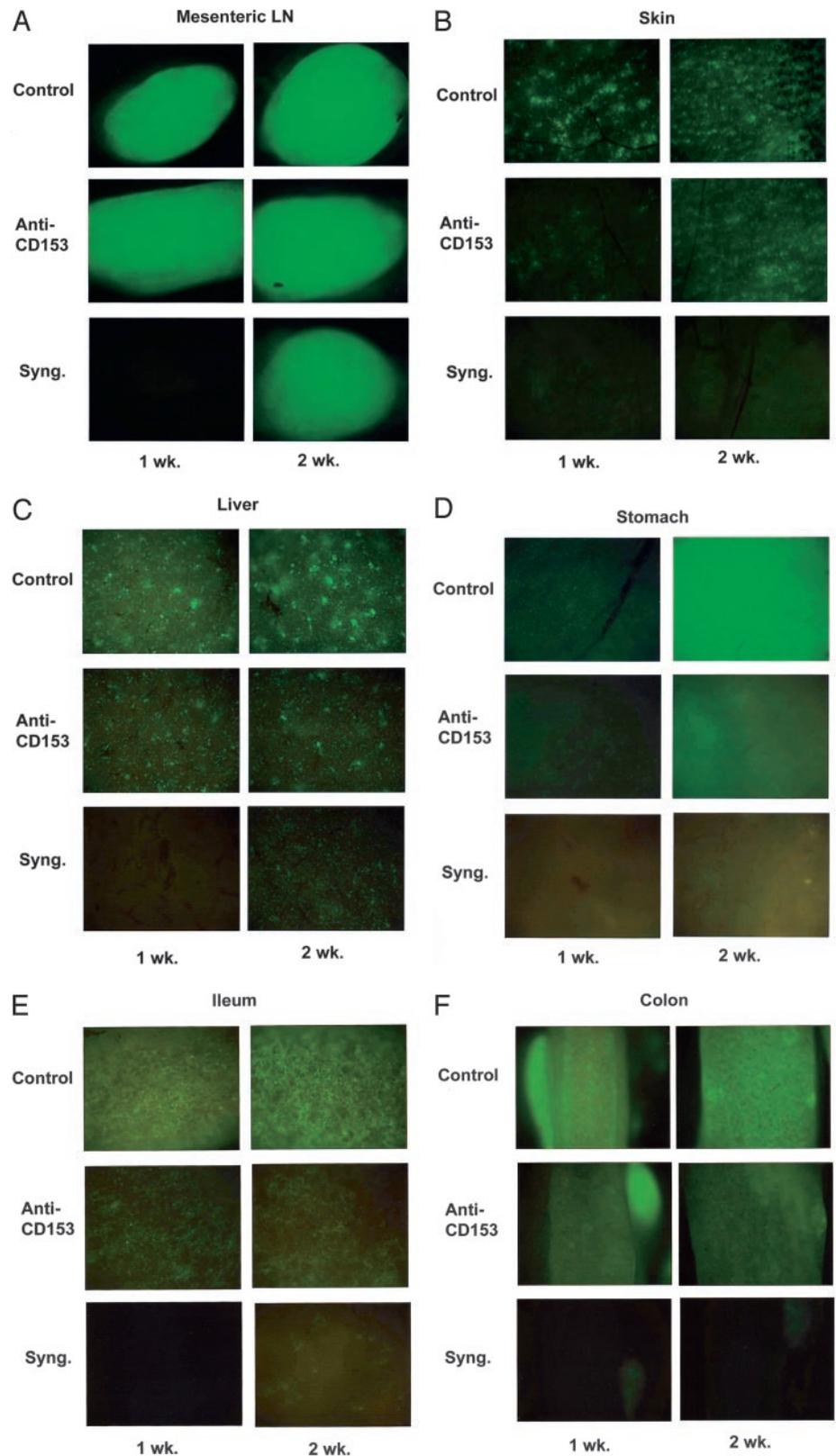
FIGURE 8. Anti-CD153 mAb infusion reduces allogeneic CD4$^+$ T cell infiltration into GVHD target organs, especially the gastrointestinal tract. B6 GFP$^+$CD4$^+$ T cells (10^6) were infused into lethally irradiated MHC class II-disparate bm12 or syngeneic recipients. Whole body imaging was performed on three mice per group at 1 and 2 wk after BMT, as indicated. A representative image from each group is shown for mesenteric LN (A), skin (B), liver (C), stomach (D), ileum (E), and colon (F). Anti-CD153 mAb did not affect GFP$^+$CD4$^+$ T cell infiltration into the mesenteric LNs (A) or other LNs such as the inguinal LN or Peyer's patches LN (data not shown). In anti-CD153 vs irrelevant mAb-treated recipients, GFP$^+$CD4$^+$ T cells were markedly reduced at both time points in the stomach (D), ileum (E), and colon (F), suggesting that the gastrointestinal tract was the major site of benefit for CD30/CD153 blockade.

for the lowered CD4$^+$ T cell-mediated GVHD observed when CD30 engagement is precluded could be down-regulation of expression of these molecules (11). In addition to regulation of cytolytic activity, CD30 signaling up-regulates CCR7 (11). Thus, an alternative mechanism for a suboptimal GVHD lethality response by CD30$^{-/-}$CD4$^+$ T cells may involve effector cell trafficking resulting in inadequate homing of CD4$^+$ T cells to secondary lymphoid organs such as intestinal Peyer's patches (33) or GVHD target organs. Although we did not observe differences in secondary lymphoid organ homing, we did observe substantial reductions in CD4$^+$ T cell migration in GVHD target organs, particularly the gastrointestinal tract, with lesser findings in the skin, liver, and lung. The gastrointestinal tract is a vital target tissue of GVHD and tissue injury can result in diarrhea and high levels of endotoxin

into the circulation. Thus, the profound reduction of GFP⁺CD4⁺ T cell infiltration into the stomach, ileum, and colon in anti-CD153 mAb-treated recipients could explain the superior survival and lower weight loss seen in settings in which CD30/CD153 interactions are blocked.

In summary, we have shown that blockade of the CD30/CD153 pathway reduces CD4⁺ T cell-mediated GVHD. We further demonstrate that these findings can be observed using one of a variety of approaches to preclude CD30/CD153 interactions including the administration of CD30⁻/⁻ donor CD4⁺ T cells, CD30⁺/⁺CD4⁺ T cells together with the infusion of a blocking anti-CD153 mAb, or use of CD153⁻/⁻ recipients. A marked effect of precluding CD30 ligation on CD4⁺ T cell-mediated GVHD mortality was observed in either sublethally or lethally irradiated recipients, although the infusion of high CD4⁺ T cell doses abrogated this survival advantage. The major effect of precluding CD30/CD153 interactions is upon inhibiting the homing of CD4⁺ T cells to the gastrointestinal tract, which was associated with increased survival and lower weight loss.

Because CD30/CD153 specific reagents are in clinical trials, future studies may be considered to prevent or treat gastrointestinal GVHD by targeting the CD30/CD153 pathway.

Acknowledgments

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