BIOLOGY

Roles of CD28, CTLA4, and Inducible Costimulator in Acute Graft-versus-Host Disease in Mice

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T cells deficient for CD28 have reduced ability to expand and survive, but still cause graft-versus-host disease (GVHD). Inducible costimulator (ICOS), a member of the CD28 family, is expressed on antigen-activated T cells and plays unique roles in T cell activation and effector function. We hypothesized that ICOS contributes to the development of GVHD in the absence of B7:CD28/CTLA4 costimulation. In this study, we evaluated the roles of CD28, CTLA4, and ICOS in the pathogenesis of acute GVHD after myeloablative allogeneic bone marrow transplantation. Unexpectedly, we found that blocking CD28 and CTLA4 signals using the clinically relevant reagent CTLA4-Ig increases the severity of GVHD mediated by $CD4^+$ T cells, and that such treatment does not add any benefit to the blockade of ICOS. In contrast, selectively blocking CD28 and ICOS, but not CTLA4, prevents GVHD more effectively than blocking either CD28 or ICOS alone. Taken together, these results indicate that CD28 and ICOS are synergistic in promoting GVHD, whereas the CTLA4 signal is required for T cell tolerance regardless of ICOS signaling. Thus, blocking CD28 and ICOS while sparing CTLA4 represents a promising approach for abrogating pathogenic T cell responses after allogeneic bone marrow transplantation.

Biol Blood Marrow Transplant 17: 962-969 (2011)

KEY WORDS: T cell, Costimulation, BMT, GVHD

INTRODUCTION

Graft-versus-host disease (GVHD) remains the major complication of allogenic hematopoietic cell transplantation (HCT), producing high morbidity and mortality [1]. GVHD is initiated by mature donor T cells that recognize disparate histocompatibility antigens of the recipient. An efficient T cell response requires costimulatory signals delivered by antigen-presenting cells (APCs) in addition to signals delivered through the T cell receptor after recognition of a specific antigen [2]. CD28 has been well characterized and is the most effective costimulatory molecule expressed by naïve and activated T cells. Costimulation through CD28 regulates multiple aspects of T cell function, including cytokine secretion, proliferation, and cell survival [3,4]. By using CD28-deficient mice, we and others [5,6] have found that CD28 costimulation plays an important role in the development of GVHD, although T cell activation and GVHD can still proceed in the absence of CD28. Furthermore, T cell responses to high-affinity or high-abundance antigens, often present in transplant recipients, are far less dependent on CD28 costimulation than are T cell responses to low-affinity or low-abundance antigens [7-9]. This makes it difficult to induce transplantation tolerance by blocking the CD28 signal alone.

CTLA4, another member of the CD28 family, competes with CD28 binding to the same ligands (B7.1 and B7.2; B7 hereinafter) and delivers an inhibitory signal to T cell activation [10]. Inducible costimulator (ICOS), the third member of the CD28 family [11], is expressed on T cell surface after activation and plays unique roles in T cell activation and

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Financial disclosure: See Acknowledgments on page 968.

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Received January 7, 2011; accepted March 14, 2011

1083-8791/$36.00
doi:10.1016/j.bbmt.2011.01.018

differentiation [12,13], germinal center formation, and immunoglobulin class switching [14,15]. ICOS ligand B7h is constitutively expressed at low levels on APCs and is up-regulated by tumor necrosis factor (TNF)-α or lipopolysaccharides [16,17]. Additional studies have suggested that CD28 and ICOS play distinct roles in T cell differentiation, with the CD28 signal responsible for T cell activation and the ICOS signal responsible for certain effector functions [18-21].

In cardiac transplantation models, blockade of B7h:ICOS interaction produced a modest but significant prolongation of graft survival [20,22]. Efficiency was increased with delayed blockade as opposed to early blockade, indicating an effect on primed T cells [23]. Furthermore, the coblockade of B7:CD28/CTLA4 and ICOS ligand:ICOS pathways was significantly more effective in prolonging graft survival than the blocking of either alone [22,24]. The role of ICOS in GVHD is complex. ICOS blockade was found to exacerbate acute GVHD but to inhibit chronic GVHD in a nonirradiated parent-into-F1 model [25]; however, recent studies indicated that ICOS blockade ameliorated GVHD in myeloablative bone marrow transplantation (BMT) models mediated by $CD4^+$ and $CD8^+$ T cells [26,27], with distinct effects in $CD4^+$ versus $CD8^+$ T cells in one model of single major histocompatibility complex (MHC) antigen disparity [28]. In this study, we tested the hypothesis that ICOS might play a significant role in the development of GVHD in the absence of B7:CD28/CTLA4 binding. We found that selectively blocking B7:CD28 and ICOS ligand:ICOS while sparing B7:CTLA4 interactions most effectively prevented acute GVHD.

MATERIALS AND METHODS

Mice

ICOS-deficient mice on a C57BL/6 (B6) background were kindly provided by Dr Chen Dong (M.D. Anderson Cancer Center, Houston, TX) [12,29]. CD28/ICOS-deficient mice on a B6 background were kindly provided by Dr Tak Mak (Ontario Cancer Institute, Toronto, Canada). B6, B6.*C-H2*^{bm12} (bm12), B6.*C-H2*^{bm1} (bm1), CD28-deficient, and B6.SJL-*Ly5*^{a} *Ptprc*^{a} *Pep3*^{b} (B6.Ly5.1) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). (B6.Ly5.1 $\times$ bm12)F1 mice were bred at the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

T Cell Purification and Transplantation

Our protocol for T cell purification using a magnetic cell separation system has been described previously [6,28]. The purity of T cells used for transplantation ranged from 91% to 97%. In nonmyeloablative transplantation models, recipient mice (B6.bm1) were exposed to 600 cGy of total-body irradiation (TBI) at 120 cGy/min, a dose range that is immunosuppressive but not lethal for this strain of mice. Purified $CD8^+$ T cells from different donors on a B6 background were suspended in phosphate-buffered saline and injected via the tail vein into 7- to 8-week-old irradiated recipients within 24 hours after irradiation. In myeloablative models, (B6 $\times$ bm12)F1 mice were exposed to 1100-1200 cGy of TBI, and BALB/c mice were exposed to 800-900 cGy of TBI. T cell–depleted (TCD) bone marrow (BM) cells alone or in combination with purified $Thy1.2^+$ cells from indicated donors were injected via the tail vein within 24 hours after irradiation. Recipient mice were monitored every other day for clinical signs of GVHD (eg, ruffled fur, hunched back, lethargy, diarrhea) and for mortality.

Administration of Antibodies

Murine CTLA4-Ig and control L6-Ig (kindly provided by Robert Peach, Bristol-Myers Squibb, Princeton, NJ) were injected i.p. at 100 μg/mouse every other day for 14 days starting on day 0, as described previously [30]. Anti-ICOS monoclonal antibody (mAb; hybridoma 7E.17G9.G1, rIgG2b, produced at National Cell Culture, Minneapolis, MN) or irrelevant rat IgG was injected i.p. at 200 μg/day from day 0 to day 5, then 3 times weekly up to day 28 after BMT, as described previously [27].

Immunofluorescence Analysis

Two-, 3-, or 4-color flow cytometry was performed to measure the expression of surface molecules and intracellular cytokines according to standard techniques. Fluorescein isothiocyanate–labeled anti-CD4, biotin-labeled anti-Fas ligand (FasL), phycoerythrin-labeled anti-CD4, anti-interferon (IFN)-γ, anti–TNF-α, anti-IgG isotype control, and Cy-Chrome–labeled anti-CD4 were purchased from BD Pharmingen (San Diego, CA). Phycoerythrin-labeled anti-IgG2a was purchased from Caltag (Burlingame, CA). Biotin-labeled anti-Ly5.1 mAb was prepared in our laboratory. Biotinylated antibodies were detected with streptavidin–Cy-Chrome or streptavidin-APC. The level of FasL expression is presented as mean fluorescence index (MFI), which equals the mean fluorescence intensity of cells stained with a specific mAb divided by the mean fluorescence intensity of cells stained with isotype control.

Cytokine and Histopathologic Analysis

Blood samples were obtained from BMT recipients at the specified times, and cytokine analysis was

performed using a cytometric bead array kit as described previously [28]. Histopathology findings in small intestine, liver, and skin were assessed by an expert pathologist (C.L.) using coded samples as described previously [31].

Statistical Analysis

For comparison of recipient survival among groups in GVHD experiments, the log-rank test was used to determine the statistical significance. The Student *t* test was used to compare the engraftment and expansion of donor T cells.

RESULTS

Blocking Ligands of CD28 and CTLA4 Exacerbated GVHD Induced by $CD4^+$ T Cells after Allogeneic BMT

It is generally believed that CD28 and ICOS deliver positive costimulation to T cell responses, whereas CTLA4 delivers negative costimulation. This concept predicts that coblockade of CD28 and ICOS with sparing of CTLA4 should lead to good control of T cell alloresponses in transplantation; however, this has not been proven in the context of allogeneic BMT. To address the roles of CD28, ICOS, and CTLA4 in the development of GVHD, we first examined the effect of blocking CD28 and CTLA4 in the presence or absence of ICOS costimulation after myeloablative BMT in mice. CTLA4-Ig was used to block B7 as an effective CD28 and CTLA4 antagonist [32]. The B6→bm12 BMT model was initially used only with MHC II incompatibility between donor and recipient. $CD4^+$ cells were purified from wild-type (WT) or $ICOS^{-/-}$ B6 donors and injected into lethally irradiated bm12 mice. These recipients were divided into 2 groups and treated with L6-Ig or CTLA4-Ig. Consistent with results of previous studies by our group and others [26-28], $ICOS^{-/-}$ $CD4^+$ T cells induced significantly delayed GVHD (Figure 1A). Surprisingly, under these conditions, treatment with CTLA4-Ig actually accelerated the GVHD caused by WT $CD4^+$ T cells in bm12 recipients compared with controls ($P < .01$; Figure 1A); however, the treatment had no effect on GVHD induced by $ICOS^{-/-}$ T cells. To confirm this result, we used a fully MHC-mismatched B6→BALB/c BMT model, and found that treatment with CTLA4-Ig also accelerated the GVHD induced by $CD4^+$ donor T cells ($P < .05$; Figure 1B). These data differ from those reported by other studies demonstrating that blocking B7:CD28/CTLA4 interactions results in a reduction of GVHD, rather than acceleration [33-37]. The major difference between our current study and the previous studies is that GVHD was induced only by $CD4^+$ T cells in our study, whereas GVHD was induced by both $CD4^+$ and $CD8^+$ T cells in the other studies. In our study, negative regulation through CTLA4 dominated the positive regulation through CD28 on $CD4^+$ T cells, which is consistent with 2 recent reports indicating that B7 plays an essential role in tolerance on alloreactive $CD4^+$ T cells in MHC II–mismatched transplantation models [38,39]. The dominant negative role of CTLA4 over the positive role of CD28 on $CD4^+$ T cells might be attributed to the down-regulation of immune responses through B7:CTLA4 ligation on effector T cells via T cell–T cell or T cell–T regulatory cell interactions [40,41].

To gain insight into the underlying mechanisms, we measured T cell activation and expansion in the myeloablative B6→bm12 BMT model. In 6-day T cell transfer experiments, the absolute numbers of WT donor T cells ($CD4^+Ly5.1^-$) were $4.8 \pm 2.1 \times 10^5$/spleen in the recipients treated with L6-Ig and $6.7 \pm 2.0 \times 10^5$/spleen in those treated with CTLA4-Ig. The absolute numbers of $ICOS^{-/-}$ donor T cells were $8.1 \pm 0.4 \times 10^5$/spleen in the recipients treated with L6-Ig and $3.5 \pm 1.2 \times 10^5$/spleen in those treated with CTLA4-Ig. There was no significant difference ($P > .05$) between any 2 groups, indicating that the CD28, CTLA4, and/or ICOS signals had no significant effect on the early expansion of donor CD4 T cells. These data are in agreement with the previous findings of our group and others showing that blocking ICOS had no effect on T cell proliferation [26,28]. Given that blockade of CD28 reduces T cell proliferation, we reasoned that additional CTLA4 blockade would reverse the effect of CD28 blockade, and thus the combinational blockade of CD28, CTLA4, and ICOS had no significant impact on $CD4^+$ T cell proliferation in vivo.

We also measured the expression of IFN-γ, TNF-α, and FasL, each of which plays an important role in the induction of GVHD by donor $CD4^+$ cells. Six days after BMT, we evaluated donor T cells in recipient spleen for the intracellular expression of IFN-γ and TNF-α (% positive cells) and surface expression of FasL (MFI) (Figure 1C). In separate experiments, we assessed how Th1/Th2 cytokines were affected by the blockade of CD28, ICOS, or both by measuring interleukin (IL)-2, IL-4, IL-5, IFN-γ, and TNF-α in recipient sera 14 days after BMT (Figure 1D). On day 6, we found that treatment with CTLA4-Ig increased expression of IFN-γ, TNF-α, and FasL on WT T cells compared with treatment with L6-Ig ($P < .05$ for each effector molecule) (Figure 1C). Our data support the idea that coblockade of CD28 and CTLA4 accelerated GVHD induced by $CD4^+$ T cells (Figure 1A and B). Absence of ICOS ($ICOS^{-/-}$ T cells) had little or no effect on the expression of these effector molecules at the single cell level on day 6 (Figure 1C), but significantly suppressed production of TNF-α and IFN-γ, but not of IL-5, in recipient sera on day 14 (Figure 1D). These results

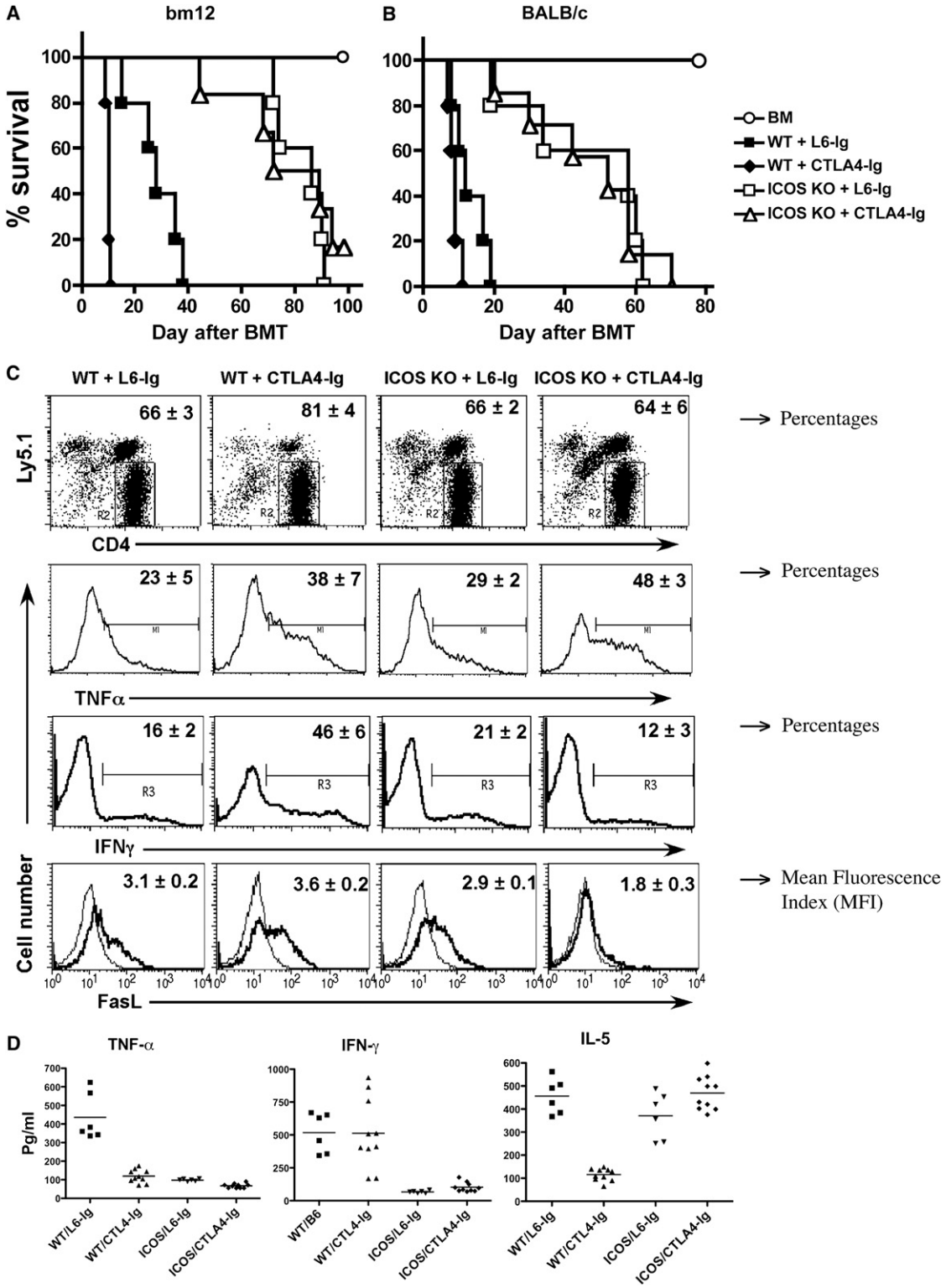

Figure 1. Roles of CD28, CTLA4, and ICOS in GVHD induced by CD4 T cells in a myeloablative BMT model. Lethally irradiated (B6.Ly5.1 × bm12)F1 mice (A) or BALB/c mice (B) underwent transplantation with TCD-BM alone or TCD-BM plus purified $CD4^+$ cells at 1×10^6/mouse from WT or $ICOS^{-/-}$ B6 donors. L6-Ig or CTLA4-Ig was injected i.p. at 100 μg/mouse every other day, for a total of 8 doses. Data were obtained for 1 experiment in each model, and 5 or 6 mice were included in each group. (C) BMT was set up as in (A), and recipient spleen was collected at 6 days after transplantation. Splenocytes were stained individually for surface expression of FasL and intercellular expression of IFN-γ and TNF-α, in combination with surface expression of CD4 and Ly5.1. The expression of surface FasL (MFI) and intracellular IFN-γ or TNF-α (% positive) are shown on gated $CD4^+/Ly5.1^-$ donor cells. The thin lines represent cells stained with isotype control mAb, and the thick lines represent specific mAbs for FasL. The results represent 2 replicate experiments. (D) BMT was set up as in (A), with peripheral blood collected from each recipient on day 14. The levels of TNF-α, IFN-γ, IL-5, IL-2, and IL-4 in the recipient serum were measured as described in Materials and Methods. IL-2 and IL-4 were below detectable levels (data not shown). The data were pooled from 2 replicate experiments, and each data point represents a cytokine concentration in one individual mouse.

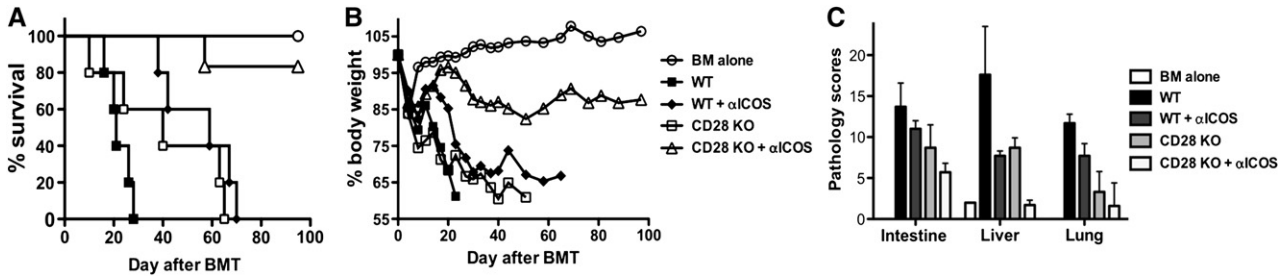

Figure 2. Roles of CD28 and ICOS in the development of GVHD induced by CD4+ T cells in a myeloablative BMT model. Lethally irradiated BALB/c mice underwent transplantation with TCD-BM alone or with TCD-BM plus 2×10^6 $CD4^+$ T cells from WT or CD28 KO B6 donors. A group of recipients with WT or CD28 KO cells were treated with anti-ICOS mAb or irrelevant control, as described in Materials and Methods. Recipient survival (A), weight loss (B), and pathology scores (C) are shown. The data are from one experiment with 5 or 6 recipients in each group; a similar outcome was observed in another experiment in which T cell dose and anti-ICOS treatment differed somewhat.

confirmed the previous findings by us and others [26-28], indicating that the decreased production of Th1 cytokines likely contributed to the reduced ability of $ICOS^{-/-}$ $CD4^+$ T cells to cause GVHD. CTLA4-Ig treatment on $ICOS^{-/-}$ T cells significantly decreased IFN-γ ($P < .05$) and FasL ($P < .01$), but significantly increased TNF-α ($P < .01$) compared with control treatment on day 6. Furthermore, the cytokine profile on day 14 was similar in the recipients of $ICOS^{-/-}$ T cells treated with CTLA4-Ig and those treated with L6-Ig (Figure 1D). Taken together, these data might explain why treatment with CTLA4-Ig did not further reduce GVHD induced by $ICOS^{-/-}$ $CD4^+$ T cells (Figure 1A and B).

Blocking ICOS (Anti-ICOS) and CD28 (Knockout) While Sparing CTLA4 Prevents GVHD Mediated by $CD4^+$ T Cells

Our previous work showed that the CTLA4 signal plays a protective role in GVHD development [5,30]. Thus, we hypothesized that blocking ICOS and CD28 while sparing CTLA4 would ameliorate GVHD under conditions in which the absence of CD28 alone was ineffective in preventing lethality. To test this hypothesis, we used $CD28^{-/-}$ B6 mice as donors and gave BALB/c recipients BM supplemented with $CD4^+$ T cells and then treated with antagonistic anti-ICOS mAb to block ICOS. Using this strategy, we found that blockade of ICOS ($P = .002$), but not the absence of CD28 alone ($P = .10$), significantly delayed GVHD lethality in recipients (Figure 2A). However, blockade of ICOS and the absence of CD28 were able to prevent GVHD lethality in >80% of recipients and significantly reduce weight loss more effectively than either blockade of ICOS alone ($P = .02$) or the absence of CD28 alone ($P = .009$) (Figure 2A and B). Furthermore, blockade of ICOS and the absence of CD28 significantly improved pathology scores in intestine, liver, and lung tissues compared with intact costimulation, blockade of ICOS, or the absence of CD28 alone (Figure 2C). We therefore concluded that CD28 and ICOS contribute synergistically to the development of GVHD induced by CD4 T cells.

Roles of CD28, CTLA4, and ICOS in T Cell Expansion and Cytokine Production

To elucidate the mechanisms by which simultaneous blockade of CD28 and ICOS prevent GVHD, we measured the expansion of donor $CD4^+$ T cells in recipient spleens. $CD4^+$ T cells were purified from WT or CD28-deficient B6 mice and transferred together with TCD-BM from B6 $Ly5.1^+$ donors into irradiated BALB/c recipients. Donor T cells were identified as $CD4^+H2^{b+}Ly5.1^-$ in recipient spleens at 6 days after transplantation. The absolute number of donor $CD4^+$ cells was an average of $8.2 \pm 1.8 \times 10^5$ per mouse for WT cells and $6.9 \pm 1.6 \times 10^5$ per mouse for CD28 knockout (KO) cells ($P = .60$), indicating that both WT and CD28 KO $CD4^+$ T cells had a similar potential to expand in vivo. Treatment with anti-ICOS mAb actually increased the expansion of WT donor $CD4^+$ T cells ($P = .05$; Figure 3A); however, treatment with anti-ICOS reduced the expansion of CD28 KO cells, because the absolute number of CD28 KO cells was significantly lower than that of WT cells and CD28 KO cells with control treatment ($P < .05$; Figure 3A). These results indicate that $CD4^+$ T cell expansion depends on both CD28 and ICOS.

Th1 cytokines (ie, IFN-γ and TNF-α) play a critical role in GVHD induced by $CD4^+$ T cells [42,43]. We investigated how serum cytokines are affected by the blockade of CD28, ICOS, or both by measuring IL-2, IL-4, IL-17, IFN-γ, and TNF-α in recipient serum at 18 days after BMT (Figure 3B). At this time point, the levels of IL-2, IL-4, and IL-17 were very low or undetectable, and IFN-γ was detectable but not significantly different among the groups (data not shown). The absence of CD28 or blockade of ICOS alone reduced TNF-α production, but this reduction was not significant ($P > .05$; Figure 3B); however, the absence of CD28 and blockade of ICOS significantly reduced TNF-α production compared with either factor alone ($P = .01$ in both cases; Figure 3B). Taken

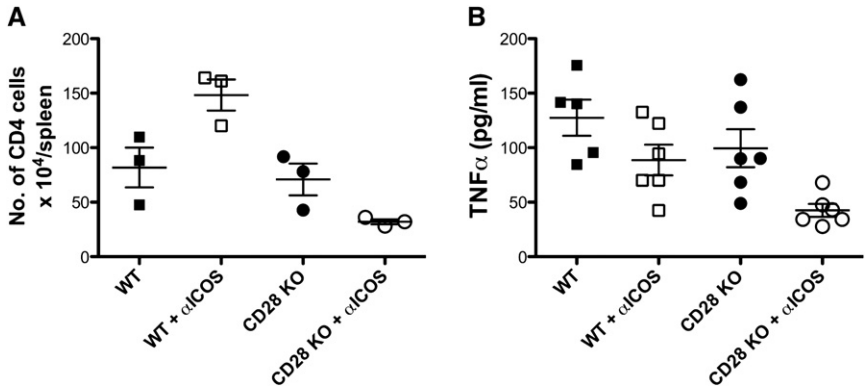

Figure 3. Roles of CD28, CTLA4, and ICOS in GVHD induced by $CD4^+$ T cells. Lethally irradiated BALB/c mice underwent transplantation with TCD-BM from normal B6 $Ly5.1^+$ mice or TCD-BM plus purified $CD4^+$ cells from WT or $CD28^{-/-}$ B6 donors. Half of the recipients were also treated with anti-ICOS or control mAb. (A) Six days after BMT, recipient spleen was collected and stained for expression of CD4, Ly5.1, and $H2^b$. Data are the absolute number of donor T cells ($CD4^+Ly5.1^-$ $H2^{b+}$) in individual mice (n = 3 in each group), representing 1 of 2 replicate experiments in similar settings. (B) In separate experiments as described in (A), recipient peripheral blood samples were collected at 3 weeks after BMT. The TNF-α level in recipient serum is shown in individual mice (n = 5 or 6 per group), and the data were pooled from 2 replicate experiments.

together, these data indicate that blocking CD28 and ICOS, while sparing CTLA4 resulted in reduction of T cell expansion and TNF-α production during development of GVHD induced by donor $CD4^+$ T cells.

Roles of CD28, CTLA4, and ICOS in GVHD Mediated by $CD8^+$ or $CD4^+$ Plus $CD8^+$ T Cells

Because clinical HCT typically includes $CD8^+$ T cells, we investigated whether the absence of CD28 and/or ICOS expression on donor $CD8^+$ T cells would influence GVHD lethality when WT, CD28 KO, ICOS KO, or CD28/ICOS double-KO (DKO) mice were used as the source of donor $CD8^+$ T cells (Figure 4A). Cohorts of MHC class I–disparate bm1 mice were sublethally irradiated and given purified $CD8^+$ T cells from one of the aforementioned donor strains. Whereas donor $CD8^+$ T cells from WT and ICOS KO mice had comparable survival, recipients of CD28-deficient $CD8^+$ T cells had significantly prolonged survival ($P < .01$). However, $CD8^+$ T cells from DKO mice did not further prolong survival. In previous studies using the same model system, CD25-depleted $CD8^+$ T cells from ICOS KO mice resulted in a significantly reduced GVHD lethality rate [27]. Whether the difference between these 2 studies is related to the use of a CD25-depleted versus CD25-replete T cell graft is unknown. Nonetheless, our findings indicate that the absence of ICOS did not have a major effect on GVHD lethality in this $CD8^+$ T cell–mediated GVHD lethality model.

Because clinical HCT grafts contain both $CD4^+$ and $CD8^+$ T cells, we performed studies using B6 WT, CD28 KO, ICOS KO, and DKO mice as sources of donor $CD4^+$ and $CD8^+$ T cells injected into lethally irradiated BALB/c recipients with both MHC and minor histocompatibility antigen differences. As shown in Figure 4B, $CD28^{-/-}$ or $ICOS^{-/-}$ T cells induced significantly less GVHD compared with WT T cells ($P < .001$). There was no difference in recipient survival between $CD28^{-/-}$ and $ICOS^{-/-}$ cells

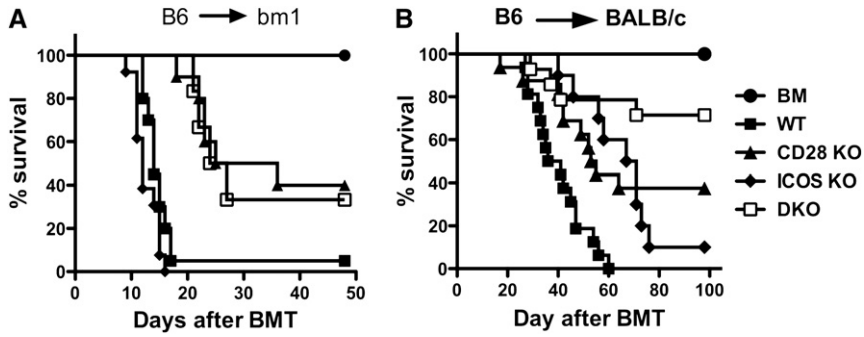

Figure 4. Roles of CD28 and ICOS on GVHD induced by $CD8^+$ T cells alone or by both $CD4^+$ and $CD8^+$ T cells. (A) B6 bm1 mice were sublethally irradiated and underwent transplantation with 1×10^6 purified $CD8^+$ cells per recipient from WT, CD28 KO, ICOS KO, and DKO B6 donors. Recipient survival is shown, and the data are from 2 replicate experiments with 6-15 recipients per group ($P < .01$, CD28 KO vs WT). (B) Lethally irradiated BALB/c mice underwent transplantation with TCD-BM alone or TCD-BM plus 1-2 $\times$ 10^6 T cells (CD4 and CD8) from WT, CD28 KO, ICOS KO, and DKO B6 donors. Recipient survival is shown, and the data are pooled from 3 replicate experiments with 11-16 mice per group ($P < .001$, CD28 KO vs WT; $P < .001$, ICOS KO vs WT; $P = .70$, CD28 vs ICOS; $P = .06$, DKO vs CD28 KO; $P = .01$, DKO vs ICOS KO).

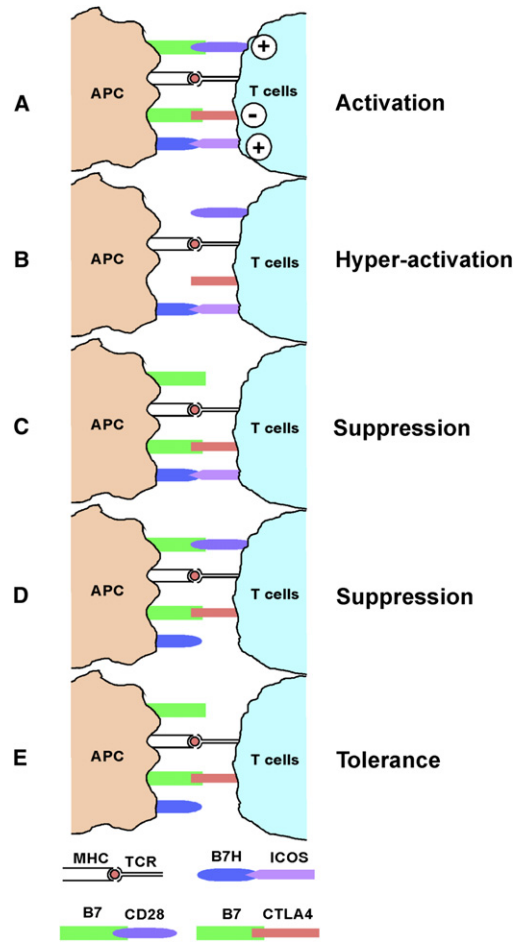

Figure 5. Roles of CD28 and ICOS on GVHD induced exclusively by $CD4^+$ T cells or driven primarily by $CD4^+$ cells and facilitated by $CD8^+$ T cells. (A) CD28 and ICOS signaling positively regulated T cell responses to alloantigens and supported GVHD development in an additive or synergistic manner, whereas CTLA4 was a negative regulator. (B) Blockade of B7 (both CTLA4 and CD28) resulted in hyperactivation of the allogeneic T cell response. (C and D) Blockade of CD28 or ICOS resulted in suppression. (E) Blockade of both CD28 and ICOS with sparing of CTLA4 led to T cell tolerance.

(P = .70). Moreover, DKO T cells induced less GVHD than $CD28^{-/-}$ (P = .06) or $ICOS^{-/-}$ (P = .01) T cells. Thus, in a $CD4^+$ T cell–driven and $CD8^+$ T cell–facilitated model system that more closely simulates clinical allogeneic HCT, the absence of both CD28 and ICOS provided the highest GVHD protective effects.

DISCUSSION

Using murine BMT models, which are representative of clinical allogeneic HCT settings, we found that CD28 and ICOS signaling positively regulated T cell responses to alloantigens and supported GVHD development in an additive or synergistic manner, whereas CTLA4 was a negative regulator (Figure 5A). In the situation where GVHD is exclusively induced by $CD4^+$ T cells or driven primarily by $CD4^+$ T cells and facilitated through $CD8^+$ T cells, blockade of B7 (both CTLA4 and CD28) results in hyperactivation of the allogeneic T cell response (Figure 5B), blockade of CD28 or ICOS results in suppression (Figure 5C and D), and blockade of both CD28 and ICOS with sparing of CTLA4 leads to T cell tolerance (Figure 5E).

An elegant in vitro study by Nurieva et al. [44] showed that in the absence of positive costimulation mediated by CD28 and ICOS, negative costimulatory molecules including CTLA4 and PD-1 actively instruct T cells to develop into tolerant cells, characterized by inactivation of intrinsic signaling and transcriptional programs. The current study extends those in vitro findings in clinically relevant models of GVHD and shows that T cell immunity and tolerance are determined by the combination of costimulatory signals. Our study also provides direct evidence to support the blocking of CD28 and ICOS signals with sparing of CTLA4 signals as an effective approach to prevent GVHD through manipulation of the CD28 family of costimulatory molecules in vivo. More selective CD28 blockade, rather than a B7 blockade (eg, belatacept and nonactivating CD28-specific antibodies), has been produced [45,46], and a fully humanized antibody against human ICOS has been generated [47]. These reagents can be used in the translation of our research finding into clinical practice in allogeneic HCT.

ACKNOWLEDGMENTS

We thank Dr Claudio Anasetti for helpful discussions of this project, Dr Yaming Liang for technical assistance, Dr Chen Dong for the ICOS KO mice, and Dr Tak Mak for the CD28/ICOS DKO mice. We are grateful for the technical assistance provided by the staff of the Flow Cytometry and Mouse Core Facility at the H. Lee Moffitt Cancer Center.

Financial disclosure: This work was supported by National Institutes of Health grants CA118116 and CA143812 (to X.-Z.Y.) and 2R01 L56067, AI34495, and P01 AI 056299 (to B.R.) and Canadian Institutes of Health Research grant MOP 84544 (to W.-K.S.). The authors have no conflicts of interest to disclose.

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