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Opposing Effects of ICOS on Graft-versus-Host Disease Mediated by CD4 and CD8 T Cells[1]

Xue-Zhong Yu,[2]*‡ Yaming Liang,* Roza I. Nurieva,§ Fei Guo,* Claudio Anasetti,*‡ and Chen Dong§

ICOS, a CD28 family member expressed on activated CD4$^+$ and CD8$^+$ T cells, plays important roles in T cell activation and effector function. Here we studied the role of ICOS in graft-vs-host disease (GVHD) mediated by CD4$^+$ or CD8$^+$ T cells in allogeneic bone marrow transplantation. In comparison of wild-type and ICOS-deficient T cells, we found that recipients of ICOS$^{-/-}$ CD4$^+$ T cells exhibited significantly less GVHD morbidity and delayed mortality. ICOS$^{-/-}$ CD4$^+$ T cells had no defect in expansion, but expressed significantly less Fas ligand and produced significantly lower levels of IFN-γ and TNF-α. Thus, ICOS$^{-/-}$ CD4$^+$ T cells were impaired in effector functions that lead to GVHD. In contrast, recipients of ICOS$^{-/-}$ CD8$^+$ T cells exhibited significantly enhanced GVHD morbidity and accelerated mortality. In the absence of ICOS signaling, either using ICOS-deficient donors or ICOS ligand-deficient recipients, the levels of expansion and Tc1 cytokine production of CD8$^+$ T cells were significantly increased. The level of expansion was inversely correlated with the level of apoptosis, suggesting that increased ability of ICOS$^{-/-}$ CD8$^+$ T cells to induce GVHD resulted from the enhanced survival and expansion of those cells. Our findings indicate that ICOS has paradoxical effects on the regulation of alloreactive CD4$^+$ and CD8$^+$ T cells in GVHD. ***The Journal of Immunology,*** **2006, 176: 7394–7401.**

The CD28 family members play a major role in T cell-mediated immune responses (1). CD28, a receptor of the B7 molecules CD80 and CD86, amplifies TCR signals and positively regulates T cell activation. CTLA4, which also binds both B7 molecules but with higher affinity, competes with the CD28 receptor and inhibits TCR signals and thus negatively regulates T cell activation (2). ICOS, the third member of the CD28 family, is expressed on the T cell surface after activation (3), and has unique roles in T cell activation and differentiation (4, 5), germinal center formation, and Ig class switching (6, 7). ICOS ligand, B7h, is constitutively expressed at low levels on APCs and is up-regulated by TNF-α or LPS (8, 9). Recent reports have suggested that CD28 and ICOS play distinct roles in T cell differentiation, the CD28 signal being responsible for T cell activation, and the ICOS signal for certain effector functions (10–13).

Because ICOS functions as an important regulatory molecule for T cell responses, intense efforts have been made to study its role in autoimmune diseases, graft rejection, and graft-vs-host disease (GVHD),[3] which are primarily mediated by T cells. However, ICOS has been shown to have inconsistent roles in different disease models. In experimental autoimmune encephalomyelitis (EAE), ICOS blockade during the effector phase abrogated the disease, but blockade during priming phase exacerbated the disease (10). Although ICOS$^{-/-}$ mice exhibited extreme sensitivity to experimental autoimmune encephalomyelitis (14), ICOS gene deletion led to complete resistance to collagen-induced arthritis in mice (15). Blocking ICOS signals with its specific Abs prolonged survival in cardiac allograft (16), but not in islet allograft models (17).

GVHD remains the major complication of allogeneic hemopoietic cell transplantation, resulting in high morbidity and mortality (18). GVHD is initiated by mature donor T cells that recognize disparate histocompatibility Ags of the recipient. In experimental models of GVHD, one report demonstrated that ICOS blockade inhibited Th2-mediated chronic GVHD, but exacerbated Th1-mediated acute GVHD (19). More recently, Taylor et al. (20) found that ICOS blockade, achieved with ICOS$^{-/-}$ mice or anti-ICOS mAb administration, resulted in significant inhibition of GVHD by reducing the number of alloantigen-specific effector cells. Results from a separate group indicated that ICOS blockade reduced GVHD morbidity and mortality by skewing toward Th2 differentiation, without affecting T cell activation, proliferation, cytotoxicity, and target organ infiltration (21). It is noticeable that these two recent reports demonstrated different mechanisms, inhibition of alloreactive T cells vs facilitation of Th2 differentiation, to explain the immunosuppressive effects of ICOS blockade.

Inconsistent outcomes after ICOS blockade in various situations may reflect the complexity of immune regulation by ICOS and the context of its action. We considered the possibility that ICOS may play distinct roles on CD4$^+$ vs CD8$^+$ T cells. In an attempt to test this hypothesis, we investigated the effects of ICOS on either population separately using allogeneic bone marrow (BM) transplantation (BMT) models for GVHD. In this study, we report that the ICOS signal inhibits GVHD initiated by CD4$^+$ alloreactive T cells, while it appears to facilitate GVHD initiated by CD8$^+$ alloreactive T cells.

Materials and Methods

Mice

C57BL/6J (B6), C3H, B6.*C-H2^{bm1}* (bm1), B6.*C-H2^{bm12}* (bm12), B6.C-H2^{bm3}/KhEgJ (B6.bm3), and B6 CD28-deficient mice were purchased

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[3] Abbreviations used in this paper: GVHD, graft-vs-host disease; BM, bone marrow; BMT, BM transplantation; Tg, transgenic; WT, wild type; TCD, T-cell depleted; FasL, Fas ligand.

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from The Jackson Laboratory. BABL/c, (BALB/c × B6)F_1 and C3H mice were purchased from National Cancer Institute (Bethesda, MD). (B6 × bm12)F_1 and (B6 × bm3)F_1 mice were bred in our animal facility. ICOS-deficient mice on B6 or DBA1 background were previously described (4, 22). The B7h knockout strain was previously generated as described elsewhere (23). Founders of 2C TCR transgenic (Tg) mice were provided by D. Loh (Nippon Roche Research Center, Kamakurshi, Japan). CD28- and ICOS-deficient 2C mice were bred in our animal facility. Mice used in this study were housed in microisolator cages at H. Lee Moffitt Cancer Center & Research Institute (Tampa, FL). 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 and transplantation has been described in detail (24, 25). Briefly, $CD4^+$ or $CD8^+$ T cells were purified by positive selection with a magnetic cell separation system (Miltenyi Biotec). The purity of $CD4^+$ or $CD8^+$ cells used for transplantation ranged from 93 to 97%. In nonmyeloablative transplantation models, recipient mice were exposed to 550 cGy at a dose rate of 120 cGy/min, a dose that is immunosuppressive but not lethal. Purified $CD4^+$ or $CD8^+$ cells from B6 wild-type (WT), CD28, or ICOS knockout donors were suspended in PBS and injected via the tail vein into 7- to 8-wk-old-irradiated B6 bm12 or bm1 recipients, respectively. In myeloablative models, bm12 mice were exposed to 1000–1100 cGy and BALB/c mice to 800–900 cGy of irradiation. T cell-depleted (TCD) BM cells alone or in combination with purified $CD4^+$ or $CD8^+$ cells from indicated donors were injected via the tail vein to recipients within 24 h after irradiation. Recipient mice were monitored every other day for clinical signs of GVHD, such as ruffled fur, hunched back, inactive or diarrhea, and mortality. Animals judged to be moribund were sacrificed and counted as GVHD lethality. Experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Cell culture and proliferation assay in vitro

$CD4^+$ or $CD8^+$ cells were purified from WT, $ICOS^{-/-}$, or $CD28^{-/-}$ B6 mice, and were used as responder cells. Splenocytes from BALB/c mice were depleted T cells, irradiated at 2000 cGy and used as stimulators. T responder cells were cultured at concentrations indicated with stimulators at 5×10^5 cells/well in 96-well, round-bottom plates in complete RPMI 1640 containing 10% FBS. Each reaction was run in triplicate, and $CD4^+$ or $CD8^+$ T cells were stimulated for 5 or 3 days, respectively. [^{3}H]TdR incorporation was measured during the last 8 h of incubation.

CFSE labeling and immunofluorescence analysis

For measurement of proliferative responses in vivo, T cells were purified from donor mice and labeled with CFSE (Molecular Probes) as described previously (26). CFSE-labeled cells at $6–10 \times 10^6$/mouse were then transferred via tail vein into previously irradiated allogeneic recipients.

Two-, three-, or four-color flow cytometry was performed to measure the expression of surface molecules and intracellular cytokines according to standard techniques. Analysis was performed by using a FACScan or FACSCalibur and CellQuest software (BD Biosciences). All of the fluorescence conjugated-Abs were purchased from BD Pharmingen, except for the biotin-labeled Ab specific for 2C TCR (1B2), which was prepared in our laboratory.

Cytokine analysis

Blood samples were obtained on day 6 after transplantation, and cytokine analysis was performed as described previously (27). Briefly, IFN-γ, TNF-α, IL-5, and MCP-1 were measured in recipient serum using a cytometric bead array kit according to the manufacturer's instructions (BD Biosciences).

Statistical analysis

The log-rank test was used to detect statistical differences in recipient survival in GVHD experiments. Student's *t* test was used to compare percentages or numbers of donor T cells.

Results

ICOS regulates GVHD mediated by $CD4^+$ T cells

To determine the role of ICOS in $CD4^+$ T cell responses to alloantigens, initial experiments were conducted to compare the ability of ICOS-deficient and WT T cells to induce GVHD in sublethally irradiated allogeneic recipients. Under this condition, donor T cells caused damage to the recipient hemopoietic system, resulting in marrow failure. At 1×10^5 cells/mouse, ICOS-deficient T cells induced death of bm12 recipients with a significant delay compared with WT T cells ($p = 0.01$), but 3×10^5 ICOS-deficient cells had an equivalent effect as 1×10^5 WT cells ($p = 0.9$). CD28-deficient cells were even less capable to induce GVHD than ICOS-deficient cells ($p = 0.02$). These data suggest that ICOS costimulation enhances the severity of GVHD mediated by $CD4^+$ T cells, but less potently than CD28 costimulation.

In the clinical hemopoietic cell transplantation settings, GVHD typically refers to the epithelial damage induced by donor T cells in major or minor histocompatibility complex-mismatched recipients that are lethally irradiated and reconstituted with marrow plus peripheral T cells from the donor. Thus, the role of ICOS was next evaluated in myeloablated recipients, where GVHD lethality is induced through epithelial damage. B6 bm12 mice were lethally irradiated and infused with BM plus purified $CD4^+$ T cells from either WT or ICOS-deficient B6 mice. GVHD lethality of recipients transplanted with ICOS-deficient cells was delayed compared with that with WT cells ($p = 0.002$; Fig. 1, *A* and *B*). These results were reproduced in another well-characterized BMT model (28), where BALB/c mice were used as recipients (Fig. 1, *C* and *D*). In a separate experiment, GVHD target organs, such as liver, spleen, and small intestine, were harvested from recipients 12 days after transplantation. Pathologic evaluation revealed severe injury in the intestine of recipients transplanted with WT donor T cells (Fig. 1*E*, *middle*), including massive lymphocyte infiltration and the architectural disruption. In contrast, the intestine of the recipients with $ICOS^{-/-}$ donor T cells (Fig. 1*E*, *bottom*) had only minor injury, and its architecture was similar to that of the recipients with BM cells alone (Fig. 1*E*, *top*). Collectively, these data demonstrated that costimulation via ICOS enhances GVHD pathology mediated by $CD4^+$ T cells.

ICOS does not affect cell division and expansion of $CD4^+$ T cells in response to alloantigens

To elucidate the mechanisms by which blockade of ICOS reduced GVHD, we first tested the role of ICOS in T cell response to alloantigen in vitro. $CD4^+$ T cells were purified from WT, $ICOS^{-/-}$, or $CD28^{-/-}$ mice and stimulated with APCs from BALB/c mice. In response to alloantigen, $ICOS^{-/-}$ cells proliferated as well as WT cells, but $CD28^{-/-}$ cells hardly proliferated (Fig. 2*A*).

To test the effects of ICOS on T cell response to alloantigen in vivo, $CD4^+$ T cells from WT, CD28- or ICOS-deficient B6 mice were transferred into irradiated BALB/c recipients, and T cell expansion was measured 4 days after cell transfer in recipient spleens (Fig. 2*B*). We found that whereas the expansion of CD28-deficient cells was significantly reduced as compared with that of WT cells ($p = 0.016$), there was no significant difference between ICOS-deficient and WT cells ($p = 0.172$). It is possible that the level of expansion might be different in the late phase of alloresponse, therefore we did a time-course experiment to compare the expansion of WT and ICOS-deficient $CD4^+$ cells in response to alloantigen. At 4 and 8 days after cell transfer, the total numbers of splenocytes of in BALB/c recipients that transferred with WT and $ICOS^{-/-}$ T cells were the same (Fig. 2*C*), and the numbers of WT and $ICOS^{-/-}$ T cells were also not significantly different (Fig. 2*D*; $p > 0.05$). At 12 days after cell transfer, total numbers of splenocytes in BALB/c recipients of $ICOS^{-/-}$ cells were significantly greater than that of WT cells ($p = 0.003$), suggesting that immune reconstitution was better in the recipient of $ICOS^{-/-}$ cells compared with that of WT cells. At this time point, $ICOS^{-/-}$ T cells were actually more than WT T cells in recipient spleen, but the difference was not significant ($p = 0.08$).

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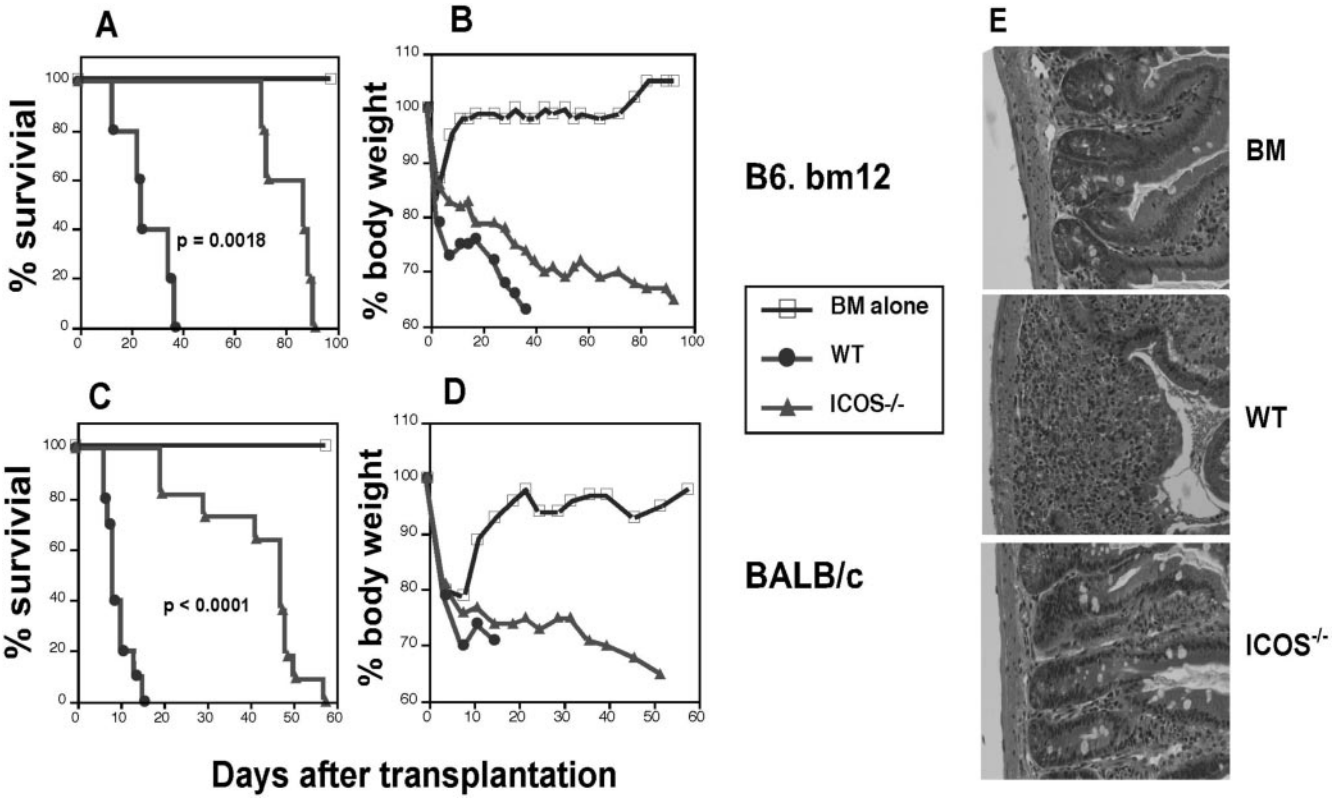

FIGURE 1. Role of ICOS in GVHD mediated by $CD4^+$ T cells in myeloablative BMT models. *A* and *B*, Lethally irradiated (1100 cGy) B6. bm12 mice were transplanted with TCD-BM alone or TCD-BM plus 2×10^6 $CD4^+$ T cells from WT or ICOS-deficient B6 donors. Five recipients were used in each group. *C* and *D*, Lethally irradiated (800–900 cGy) BALB/c mice were transplanted with TCD-BM alone or TCD-BM plus 2×10^6 $CD4^+$ T cells from WT or $ICOS^{-/-}$ B6 donors. Ten to 12 recipients were used in each group, and data are derived from two combined experiments. *E*, A separate experiment was set up as described in *C* and *D*, but the recipients were sacrificed on day 12. Liver, spleen, and intestine were harvested, and formalin-preserved organs were paraffin-embedded, sectioned, and H&E stained. The pictures show the structure of small intestine from recipients transplanted with BM alone (*top*), or BM plus WT (*middle*) or $ICOS^{-/-}$ CD4 T cells (*bottom*), and the data represent 1 of 4 mice in each group.

To compare T cell response to a different alloantigen, purified $CD4^+$ T cells from WT or $ICOS^{-/-}$ B6 mice were labeled with CFSE and injected into irradiated (B6.Ly5.1 × bm12)F_1 recipients. Donor T cells were identified as $CD4^+$/$Ly5.1^-$ (Fig. 3*A*). There was no significant difference in absolute numbers of WT and ICOS-deficient T cells in recipient spleens on day 4 ($p > 0.5$) (Fig. 3*B*). Collectively, these results indicate that ICOS does not play an essential role in cell expansion of $CD4^+$ alloreactive T cells in vivo.

ICOS enhances effector functions of $CD4^+$ T cells

Fas ligand (FasL) expression and secretion of Th1 cytokines (i.e., IFN-γ and TNF-α) are primary effector functions for $CD4^+$ T cells in inducing GVHD (29, 30). To further investigate the mechanisms by which ICOS-blockade ameliorates GVHD, we compared surface expression of FasL on $CD4^+$ T cells from WT or ICOS-deficient donors and found that $ICOS^{-/-}$ cells expressed significantly lower levels of FasL than WT cells ($p < 0.02$) (Fig. 4*A*). Cytokines and chemokines were measured in serum (B6. Ly5.1 × bm12)F_1 recipients on day 6 after transplantation. In the absence of ICOS, donor T cells produced very little MCP-1 and virtually no IFN-γ and TNF-α (Fig. 4*B*).

Cytokines were also measured in the serum of BALB/c recipients 12 days after transplantation with B6 donors (Table I). The levels of TNF-α and IFN-γ were significantly higher in the serum from BALB/c recipients of WT than that of $ICOS^{-/-}$ $CD4^+$ T cells. Interestingly, the level of IL-5 was higher in the serum from BALB/c recipients of $ICOS^{-/-}$ than that of WT cells, although not significant ($p = 0.1$). Collectively, these results suggest that GVHD amelioration may result from decreased effector functions of ICOS-deficient T cells.

ICOS-deficient $CD8^+$ T cells induce more severe GVHD than their WT counterparts

To determine the role of ICOS signaling on the CD8 T cell response to alloantigens, B6 → BALB/c BMT model was used because it was previously established (28). We compared the pathogenicity of WT vs ICOS-deficient $CD8^+$ T cells in the induction of GVHD. $CD8^+$ T cells from CD28-deficient mice were used as an additional control. As in the previous reports from us and others (24, 31), CD28-deficient cells caused less GVHD than WT cells ($p = 0.0003$) (Fig. 5*A*). In contrast, GVHD was slightly accelerated in the recipients of ICOS-deficient cells as compared with the recipients of WT cells ($p = 0.02$) (Fig. 5*A*). By using a lower cell dose (3×10^5/mouse), the difference in GVHD lethality between the recipients of WT vs ICOS-deficient cells became more obvious ($p = 0.0005$) (Fig. 5*B*). These results indicate that the ICOS signal inhibits GVHD development mediated by CD8 effector cells in sublethally irradiated recipients.

To extend the study to a myeloablative BMT setting, we compared the pathogenicity of $CD8^+$ T cells from WT or ICOS-deficient B6 donors in lethally irradiated BALB/c recipients. Recipients of BM alone survived long-term without signs of GVHD. As expected, CD28-deficient T cells caused less GVHD than WT T cells ($p = 0.05$) (Fig. 5*C*). In contrast, GVHD was accelerated in the recipients of ICOS-deficient cells as compared with the recipients of WT cells ($p = 0.03$) (Fig. 5*C*). These results indicate that

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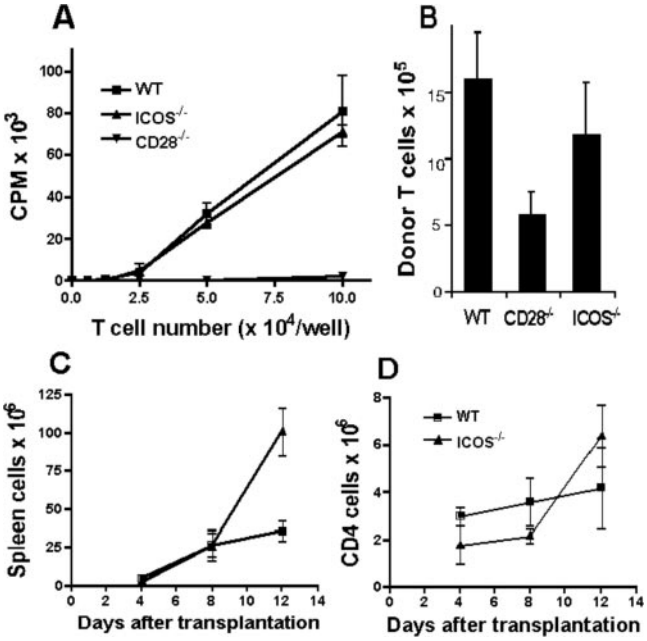

FIGURE 2. ICOS does not affect on CD4 T cell proliferation and expansion. *A*, Purified $CD4^+$ cells from WT, $ICOS^{-/-}$, or $CD28^{-/-}$ mice were stimulated with previously irradiated (2000 cGy), TCD-splenocytes from BALB/c mice. Cells were cultured for 5 days, and cell proliferation was measured by [^{3}H]TdR incorporation during the last 8 h of incubation. Data represent mean ± 1 SD cpm of triplicate wells at each cell concentration indicated. *B*, Purified $CD4^+$ cells from WT, $ICOS^{-/-}$, or $CD28^{-/-}$ B6 donors were transplanted into irradiated (900 cGy) BALB/c recipients at 4×10^6 cells/mouse. Recipient spleens were harvested at 84 h after the transplant and tested separately from three mice in each group. Splenocytes were counted and stained for expression of CD4 and $H2^b$, and absolute number of donor $CD4^+$ cells was calculated by total number of spleen cells × percentage of $CD4^+H2b^+$ cells. The data represent the average ± 1 SD of absolute number of donor $CD4^+$ T cells per spleen in BALB/c recipient. *C* and *D*, A separate experiment was set up as described in *B*, but recipient spleens were harvested 4, 8, and 12 days after cell transfer. Splenocytes were counted and strained with Abs specific for CD4 and $H2^b$ and with annexin V. The data represent the average ± 1 SD of total spleen cells (*C*) and absolute number of viable donor $CD4^+$ T cells ($CD4^+H2^{b+}$Annexin V^-; *D*) per spleen in BALB/c recipient (3–4 mice per group).

the ICOS signal inhibits GVHD development mediated by $CD8^+$ effector cells in myeloablative BMT.

ICOS decreases expansion of $CD8^+$ T cells in response to alloantigen in vivo

To determine the role of ICOS signaling on the CD8 T cell response to alloantigens, we measured cell division and expansion of 2C TCR Tg cells in response to the specific alloantigen K^{bm3} (intermediate avidity) or L^d (high avidity) in vivo (32–34). $CD8^+$ T cells were purified from WT or ICOS-deficient 2C mice, labeled with CFSE, and injected into irradiated (B6 × bm3)F_1 recipients. Four days after cell transfer, donor T cells were identified as $1B2^+$ in recipient spleens (Fig. 6*A*, *left panels*). WT 2C cells comprised 43 ± 10% of total spleen cells, whereas $ICOS^{-/-}$ 2C cells made up 57 ± 11% of total spleen cells. The proportion of annexin V^+ cells was higher in WT 2C cells than on $ICOS^{-/-}$ 2C cells (Fig. 6, *A* and *C*). The absolute number of $ICOS^{-/-}$ 2C cells ($10 \pm 4 \times 10^5$/spleen) was 3-fold higher than that of WT 2C cells ($3 \pm 2 \times 10^5$/spleen) (Fig. 6*D*), a marginally significant difference ($p = 0.06$). These results suggest that 2C ICOS-deficient cells had a lower level of apoptosis and a higher level of expansion than 2C WT cells in response to intermediate affinity alloantigen (K^{bm3}) in vivo.

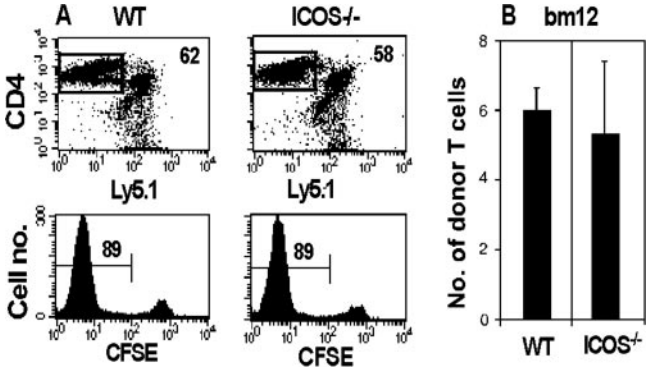

FIGURE 3. The effect of ICOS on $CD4^+$ T cell expansion and division. CFSE-labeled $CD4^+$ cells from WT or ICOS-deficient B6 donors were transplanted into irradiated (1000 cGy) (B6.Ly5.1 × bm12)F_1 recipients at 9×10^6 cells/mouse. Recipient spleens were harvested at 84 h after the transplant and tested separately from three mice in each group. *A*, *Upper panels* show the phenotype of splenocytes from each group of recipients, and the numbers indicate the percentages of donor cells ($CD4^+$/$Ly5.1^-$) in recipient spleens. *Lower panels* show the CSFE profile on gated $CD4^+$/$Ly5.1^-$ donor cells, and the numbers indicate the percentages of donor cells containing low levels of CSFE, reflecting the proportion of cells that divided rapidly in the recipient. *B*, The average ± 1 SD of absolute number of donor $CD4^+$ T cells per spleen is shown in (B6.Ly5.1 × bm12)F_1 recipient.

We next compared the rate of apoptosis and expansion of 2C cells in response to high-affinity alloantigen (L^d) in vivo. Purified $CD8^+$ 2C WT or ICOS-deficient cells were transferred into irradiated $CB6F_1$ recipients. Four days after cell transfer, WT 2C cells comprised 32 ± 21% of total spleen cells, whereas $ICOS^{-/-}$ 2C cells made up 78 ± 10% of total spleen cells (Fig. 6*B*). The proportion of annexin V^+ cells was higher on WT 2C cells than on $ICOS^{-/-}$ 2C cells (Fig. 6, *B* and *C*) ($p = 0.05$). The absolute number of ICOS-deficient 2C cells ($25 \pm 3 \times 10^5$/spleen) was 10-fold higher than that of WT 2C cells ($2.6 \pm 2 \times 10^5$/spleen) (Fig. 6*D*), which was statistically significant ($p = 0.0007$). To evaluate activation of 2C cells in vivo, serum was collected from each recipient on day 4 posttransplantation, and inflammatory cytokines were measured. 2C $ICOS^{-/-}$ cells produced significantly more TNF-α than 2C WT cells in bm3 recipients ($p = 0.015$) and $CB6F_1$ recipients ($p = 0.008$) (Fig. 6*E*). Similarly, 2C $ICOS^{-/-}$ cells produced significantly more IFN-γ than 2C WT cells in bm3 recipients ($p = 0.03$) and $CB6F_1$ recipients ($p = 0.04$) (Fig. 6*E*). These results indicate that ICOS played an inhibitory role in regulating 2C response to alloantigens in vivo.

To exclude the possibility that the inhibitory effect of ICOS on T cell expansion is a unique feature of 2C TCR-Tg cells, we compared cell division and expansion of polyclonal $CD8^+$ cells with or without ICOS in response to alloantigen in vivo. We first measured T cell proliferation in response to alloantigen in vitro. $CD8^+$ T cells were purified from WT, $ICOS^{-/-}$, or $CD28^{-/-}$ mice and stimulated with APCs from BALB/c mice. In response to alloantigen, $ICOS^{-/-}$ cells proliferated as well as WT cells at lower cell concentrations, but significantly better than WT cells at the concentrations of 5×10^4/well ($p = 0.007$) or 10×10^4/well ($p = 0.05$). As expected, $CD28^{-/-}$ cells hardly proliferated at any cell concentration (Fig. 7*A*). These results suggested that ICOS might be a negative regulator for CD8 T cell response to alloantigen.

To measure CD8 responses to alloantigens in vivo, $CD8^+$ T cells were purified from WT, CD28- or ICOS-deficient B6 donors ($H2^b$), labeled with CFSE, and injected into irradiated BALB/c recipients ($H2^d$). Four days after cell transfer, donor T cells were identified as $CD8^+$/$H2^{b+}$ in recipient spleens (Fig. 7*B*). Based on

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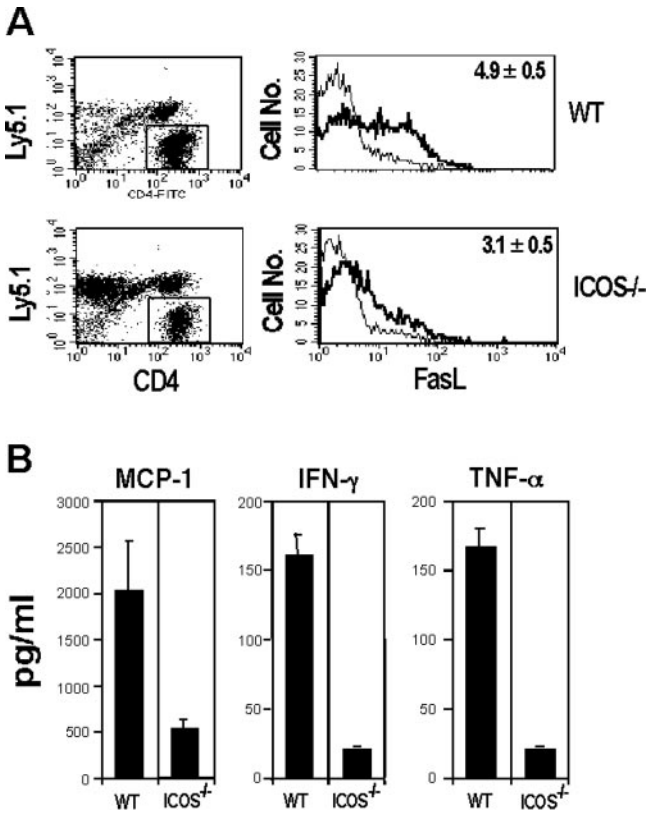

FIGURE 4. The effect of ICOS on effector functions of $CD4^+$ T cells. Purified $CD4^+$ cells from WT or ICOS-deficient B6 donors were transplanted into irradiated (1000 cGy) (B6.Ly5.1 × bm12)F_1 recipients at 5×10^6 cells/mouse. Recipients at 3–5 mice per group were sacrificed, and spleen and blood were collected 6 days after transplantation. *A*, Splenocytes were stained for surface expression of FasL in combination with CD4 and Ly5.1 expression. The FasL expression is shown on gated $CD4^+$/$Ly5.1^-$ donor cells, and the numbers indicate the mean fluorescence index ± 1 SD. The thin lines represent cells stained with isotype control mAb, whereas thick lines represent staining with specific mAb. *B*, MCP-1, IFN-γ, and TNF-α were measured in the recipient serum as described in *Materials and Methods.* The results represent three (*A*) or two (*B*) replicate experiments.

the CFSE-profile, $CFSE^{low}$ cells are fast dividing cells reflecting alloantigen-driven proliferation; whereas $CFSE^{high}$ cells are slowly dividing cells reflecting homeostatic proliferation. As shown in Fig. 7*B*, ICOS-deficient cells divided faster, while CD28-deficient cells divided more slowly, than WT cells. The absolute number of CD28-deficient cells was an average of $0.6 \pm 0.2 \times 10^5$/spleen, significantly lower than $2.5 \pm 1.1 \times 10^5$ of WT cells ($p = 0.022$). In contrast, the absolute number of ICOS-deficient cells was an average of $24.1 \pm 1.2 \times 10^5$ ($p = 0.003$) (Fig. 7*C*). These results suggest that the ICOS signal inhibits, whereas CD28 signal enhances, $CD8^+$ T cell division and expansion in response to alloantigen in vivo.

To further substantiate the role of ICOS on CD8 cell expansion in response to alloantigens, we used B7H knockout mice on a B6 background (23) as recipients. $CD8^+$ T cells from C3H mice ($H2^k$) were labeled with CFSE and injected into irradiated B6 WT or B7H knockout recipients ($H2^b$). B7 knockout recipients were used as additional controls without CD28. Four days after cell transfer, the absolute number of donor $CD8^+$ T cells was significantly lower in the spleen of B7-decifient recipient cells than that of WT cells ($p = 0.02$). In contrast, the absolute number of donor $CD8^+$ T cells was significantly higher in the spleen of B7H-decifient recipient than that of WT recipient ($p = 0.05$) (Fig. 8). These results indicate that the CD28 signal enhances, whereas the ICOS signal inhibits, expansion of $CD8^+$ T cells in response to specific alloantigen in vivo.

Discussion

Our study provides evidence that ICOS costimulation plays a distinct role in the regulation of $CD4^+$ vs $CD8^+$ T cells in response to alloantigens. The absence of ICOS costimulation resulted in a significant amelioration of GVHD mediated by $CD4^+$ alloreactive T cells. ICOS deficiency in $CD4^+$ T cells had no effect on their expansion in vivo, but their effector function was markedly impaired by a decrease in FasL expression and IFN-γ and TNF-α production. In sharp contrast, ICOS deficiency resulted in acceleration of GVHD lethality mediated by $CD8^+$ alloreactive T cells. ICOS blockade, achieved either through the use of ICOS- or B7h-deficient mice, significantly increased in vivo activation and expansion of $CD8^+$ alloreactive T cells.

Inflammatory cytokines and direct antihost cytotoxicity are the main pathogenic factors in the induction of GVHD. Because FasL expression is a major cytotoxic mediator for $CD4^+$ T cells (29, 30), we examined the role of ICOS costimulation on FasL expression. Our results highlight the fact that ICOS promotes up-regulation of TCR-driven FasL expression on $CD4^+$ T cells. ICOS deficiency also resulted in a significant decrease in the levels of MCP-1, IFN-γ, and TNF-α produced by $CD4^+$ alloreactive T cells. Decreased production of these inflammatory chemokine and cytokines was associated with ameliorated GVHD. These results support the established concept that Th1 inflammatory cytokines play an important role in the pathogenesis of GVHD. Our results are consistent with recent reports by others that ICOS blockade reduces GVHD by skewing toward Th2 differentiation (19, 21).

We unexpectedly observed that ICOS deficiency resulted in accelerated GVHD mediated by $CD8^+$ alloreactive T cells. By transferring 2C TCR Tg cells into irradiated recipients that express high (L^d) or intermediate (K^{bm3}) alloantigens, the expansion level of ICOS-deficient 2C cells was significantly higher than that of WT 2C cells (Fig. 6). These results were extended to polyclonal $CD8^+$ T cells in allogeneic recipients (Fig. 7) and further confirmed by using B7H-deficient mice as recipients (Fig. 8). Furthermore, 2C cells without ICOS secrete higher levels of inflammatory cytokines, i.e., IFN-γ and TNF-α, compared with 2C WT cells. Taken together, these

Table I. *Cytoines in serum of BALB/c recipients on day 12 (pg/ml)*[a]

	BM Alone ($n = 3$)	BM + WT ($n = 4$)	BM + $ICOS^{-/-}$ ($n = 4$)	*p* Values		
				BM vs WT	BM vs $ICOS^{-/-}$	WT vs $ICOS^{-/-}$
TNF-α	17 ± 5	190 ± 64	42 ± 14	0.006	0.05	0.004
IFN-γ	6 ± 4	266 ± 129	8 ± 8	0.019	0.81	0.007
IL-5	46 ± 33	72 ± 33	111 ± 27	0.345	0.03	0.121

[a] This is the same experiment described in Fig. 1*E*.

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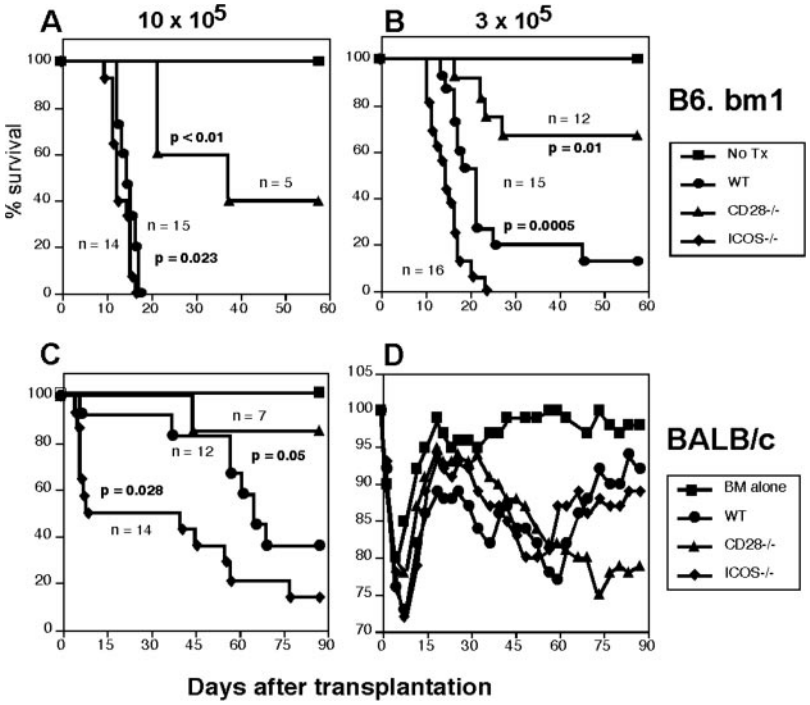

FIGURE 5. Role of ICOS in GVHD mediated by $CD8^+$ T cells. *A* and *B*, $CD8^+$ cells were purified from WT, ICOS- or CD28-deficient B6 donors and were transferred at the doses indicated into irradiated (550 cGy) bm1 recipients. A group of irradiated bm1 mice were injected with PBS alone as control without GVHD. Data were pooled from 2 to 3 replicate experiments, and the numbers next to the lines are the numbers of recipients used in each group. *C* and *D*, Lethally irradiated (800–900 cGy) BALB/c mice were transplanted with TCD-BM alone or TCD-BM plus 2×10^6 $CD8^+$ T cells from WT, $ICOS^{-/-}$, or $CD28^{-/-}$ B6 donors. The numbers next to the lines are the numbers of recipients used in each group, and data are derived from two combined experiments.

data provide compelling evidence that ICOS costimulation negatively regulates $CD8^+$ T cells in response to alloantigens.

Functional studies of ICOS have primarily focused on $CD4^+$ T cells, and limited studies of ICOS on $CD8^+$ T cells presented somewhat contradictory results. Taylor et al. (20) reported that blockage of ICOS also down-regulated CD8-mediated alloresponses and GVHD, which was inconsistent with the results in our current study. We speculated that the genetic background of ICOS mutant mice or perhaps carryover of anti-CD25 mAb used to deplete $CD25^+$ cells might be accountable for the decreased GVHD in bm1 recipients from $ICOS^{-/-}$ $CD8^+$ T cells in the experiment by Taylor et al. (20). When 2C cells were used as the donor cells, Taylor et al. (20) transplanted 2C cells together with CD4 TCR Tg cells and used specific mAb to block ICOS, whereas we used ICOS-deficient 2C cells and evaluated this population only. It is possible that the presence of alloreactive CD4 T cells might reverse the negative regulation of ICOS on 2C response, and/or engagement rather than blockage of ICOS with the specific mAb could not be formally excluded. Inconsistent with our data, Ogawa et al. (19) showed that ICOS blockade with a specific mAb significantly accelerated expansion of alloreactive $CD8^+$ T cells in a parent-into-F_1 GVHD model. In antiviral immunity, ICOS-deficient and WT $CD8^+$

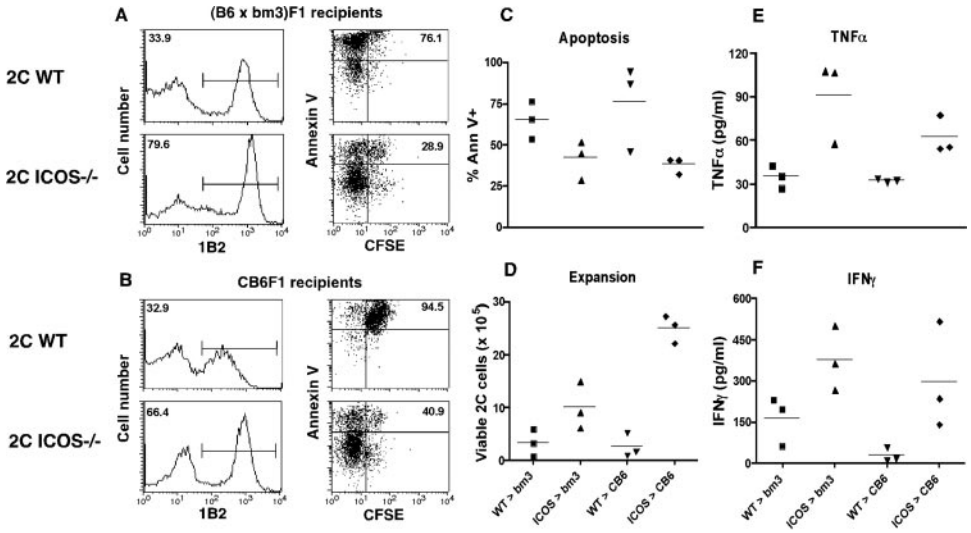

FIGURE 6. The role of ICOS in 2C cell expansion and division. CFSE-labeled $CD8^+$ cells from WT or ICOS-deficient 2C TCR-Tg donors were transplanted into irradiated (1200 cGy) (B6 × bm3)F_1 (*A*) or CB6F_1 (*B*) recipients at 6×10^6 cells/mouse. Recipient spleens were harvested at 82 h after transplantation and tested separately from three mice in each group. *Left panels* show the phenotype of recipient spleen cells, and the numbers indicate the mean percentage of $1B2^+$ cells in total splenocytes. *Right panels* show the profile of CFSE and annexin V, and the numbers indicate the mean percentage of annexin V^+ among gated $1B2^+$ cells. *C*, The percentage of annexin V^+ cells among gated $1B2^+$ cells in each recipient spleen is shown. *D*, The absolute number of $1B2^+$/Annexin V^- cells in each recipient spleen is shown. Serum was collected from each recipient on day 4 after cell transfer, and cytokine levels in the serum were measured. The levels of TNF-α (*E*) and IFN-γ (*F*) were shown in the serum of each recipient.

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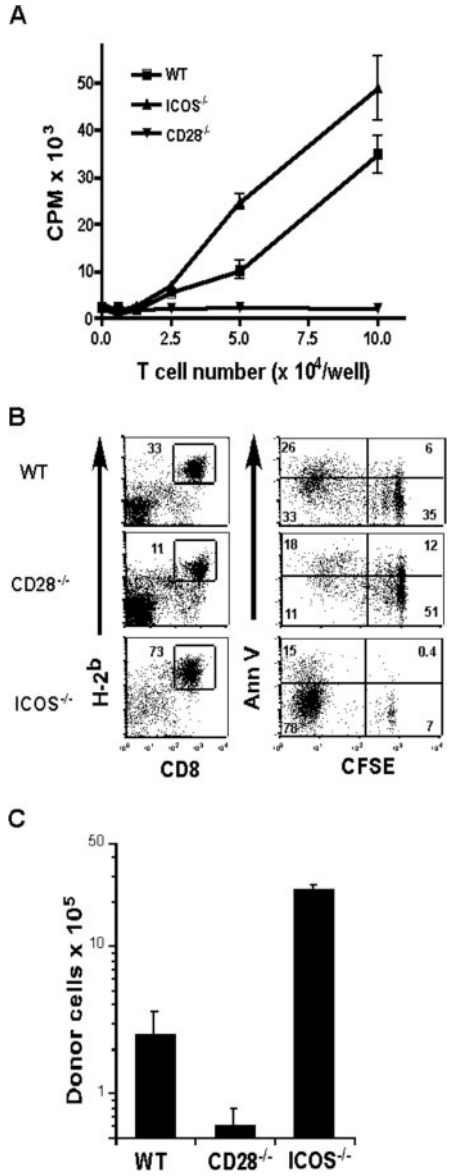

FIGURE 7. The effect of ICOS on expansion and division of polyclonal $CD8^+$ T cells. *A*, Purified $CD8^+$ cells from WT, $ICOS^{-/-}$ or $CD28^{-/-}$ mice were stimulated with previously irradiated (2000 cGy), TCD splenocytes from BALB/c mice. Cells were cultured for 3 days, and cell proliferation was measured by [^{3}H]TdR incorporation during the last 8 h of incubation. Data represent mean ± 1 SD cpm of triplicate wells at each cell concentration indicated. *B*, CFSE-labeled $CD8^+$ cells from WT, CD28- or ICOS-deficient B6 donors were transplanted into irradiated (900 cGy) BALB/c recipients at 6×10^6 cells/mouse. Recipient spleens were harvested at 88 h after the transplant and tested separately from three mice in each group. *Left panels* show the phenotype of splenocytes from each group of recipients, and the numbers indicate the percentages of donor cells ($CD8^+/H2^{b+}$) in recipient spleens. *Right panels* show the CFSE and annexin V profile on gated donor cells, and the numbers indicate the percentage of donor cells in each quadrant. *C*, The average ± 1 SD of absolute donor $CD4^+$ T cells per recipient spleen is shown. The results represent two replicate experiments in each panel.

T cells responded to a low dose of influenza virus equivalently. However, ICOS-deficient $CD8^+$ T cells had significantly greater expansion capacity and CTL activity than WT counterparts in response to a high dose of influenza virus (35). However, ICOS costimulation has been shown to enhance antitumor activity mediated by $CD8^+$ effector cells (36, 37).

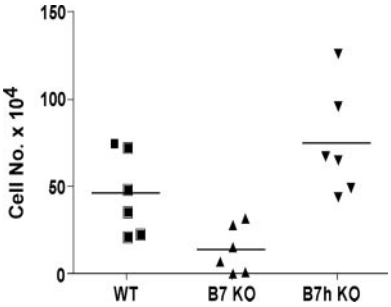

FIGURE 8. The effect of ICOS costimulation on expansion and division of polyclonal $CD8^+$ T cells. $CD8^+$ T cells from C3H donors were transplanted into irradiated (1200 cGy) B6 WT, B7H- or B7-deficient recipients at 7×10^6 cells/mouse. Recipient spleens were harvested 4 days after the transplantation, and splenocytes from each recipient were tested separately for their phenotype. The data present the absolute number of donor cells ($CD4^+H2^{k+}$) in each recipient, and the data are pooled from two replicate experiments.

The reason for these conflicting results is currently unclear, but it is possible that the contribution of ICOS costimulation to CD8 T cell responses may be critically influenced by the nature of immune response, such as the strength of the TCR signal, involvement of innate immunity, and CD28 costimulation. In our current study as well as the study by Ogawa et al. (19), $CD8^+$ T cells recognized MHC-mismatched alloantigens and received CD28 costimulation. Similarly, $CD8^+$ T cells recognized viral Ag and also received CD28 costimulation during experimental infection with influenza (35). Under those situations where the TCR signal is strong, innate immunity is likely to be involved, and the CD28 signal is present, additional ICOS signaling may restrain the response likely by enhancing activation-induced cell death. In contrast, in studies where $CD8^+$ T cells were tested to respond to weak tumor Ags in the absence of innate immunity and CD28 costimulation, the ICOS signal enhanced antitumor activity of $CD8^+$ T cells (36, 37).

The fact that ICOS costimulation has paradoxical effects on $CD4^+$ and $CD8^+$ T cells is indeed puzzling. Like ICOS, glucocorticoid-induced TNF receptor or IL-18 also have opposite effects on $CD4^+$ and $CD8^+$ T cells (38, 39). The underlying mechanism why $CD4^+$ and $CD8^+$ T cells respond differently to ICOS costimulation requires further investigation. We hypothesize that the sensitivity of these two subsets to ICOS-ligation attributes to the distinct effects. By stimulating purified T cells with beads coated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs, or anti-CD3 plus anti-ICOS mAbs, a recent study revealed that whereas CD3/CD28 beads expanded $CD4^+$ and $CD8^+$ T cells similarly, CD3/ICOS beads predominantly expanded $CD8^+$ T cells. Furthermore, only $CD8^+$ T cells could express Bcl-X_L and CD122 (IL-2Rβ) after CD3/ICOS cross-linking (40). Because $CD8^+$ T cells have a higher sensitivity in response to ICOS and subsequently to IL-2 than $CD4^+$ T cells, $CD8^+$ but not $CD4^+$ T cells may be prone to activation-induced cell death, especially when the TCR signal is strong and CD28 costimulation is present. Our data support this theory, because more $CD8^+$ T cells were undergoing apoptosis in allogeneic recipients in the presence than the absence of ICOS (Figs. 6 and 7). FasL expression on WT or $ICOS^{-/-}$ $CD8^+$ T cells was not different (data not shown), suggesting that FasL is not a primary molecule that mediates apoptosis of $CD8^+$ T cells in vivo.

We believe that the overall effect of in vivo ICOS costimulation needs to be reconsidered because ICOS can have differential effects on $CD4^+$ and $CD8^+$ effector T cells. Our data suggest that manipulation of ICOS stimulation holds therapeutic promise for

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the separation of $CD4^+$ vs $CD8^+$-mediated immune processes. Because the exact role of ICOS stimulation on human $CD4^+$ and $CD8^+$ T cell subsets needs to be determined, great caution should be exercised before applying ICOS blockade in general to the clinical situation in which both T cell subsets play important roles.

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Disclosures

The authors have no financial conflict of interest.

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