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CD28-Specific Antibody Prevents Graft-Versus-Host Disease in Mice

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The costimulatory molecules B7-1 and B7-2 regulate T cell activation by delivering activation signals through CD28 and inhibitory signals through CTLA4. Graft-vs-host disease (GVHD) is caused by activated donor T cells. Previously, we showed that CD28-deficient donor T cells induced less-severe GVHD than wild-type donor T cells, suggesting that CD28 signals exacerbate GVHD. In this paper we demonstrate that CTLA4 signals attenuate the severity of GVHD. Targeting the CD28 receptor with a specific mAb modulates the receptor in vivo, inhibits donor T cell expansion, and prevents GVHD. CTLA4 signaling was necessary for this effect because treatment with a soluble ligand that blocks binding of B7 to both CD28 and CTLA4 did not prevent GVHD as effectively as anti-CD28 mAb. These results support the current model of T cell costimulation in which CD28 signals amplify GVHD while CTLA4 signals inhibit GVHD, providing evidence that selective targeting of CD28 might be a better therapeutic strategy for inducing immunological tolerance than blocking the ligands for both CD28 and CTLA4. The Journal of Immunology, 2000, 164: 4564–4568.

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3Abbreviation used in this paper: GVHD, graft-vs-host disease.

Materials and Methods

Mice

C57BL/6 (B6), B6.C-H2<sup>b</sup> <sup>bm12</sup> (bm12), B6.C-H2<sup>b</sup> <sup>bm2</sup> (bm1), (B6 × BALB/c)F<sub>1</sub> (CF1), BALB/c H2<sup>dm2</sup> (dm2), and B6.SJL-Ly5<sup>a</sup> Ppr<sup>c</sup> Pep3<sup>a</sup> (B6.Ly5<sup>a</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). (B6 × bm12)F<sub>1</sub> (B6 × bm1)F<sub>1</sub>, and (B6 × dm2)F<sub>1</sub> (dm2B6F<sub>1</sub>) mice were bred at the Fred Hutchinson Cancer Research Center (Seattle, WA). Founders for the 2C transgenic strain were kindly provided by Dr. Dennis Y. Loh (Nippon Roche Research Center, Kamakur-shi, Japan). Homozygous B6 CD28<sup>−/−</sup> mice were a generous gift of Dr. Craig Thompson (2). 2C CD28<sup>−/−</sup> mice were generated by crossing 2C to CD28<sup>−/−</sup>. All the mice used in this study were housed in microisolator cages.

T cell purification and transplantation

Our protocol for T cell purification and transplantation has been described in detail (19, 21). CD<sup>4</sup><sup>+</sup> and CD<sup>8</sup><sup>+</sup> T cells were purified by positive selection using a magnetic cell separation system (Miltenyi Biotech, Auburn, CA). To avoid graft rejection, F<sub>1</sub> mice were used as recipients in all experiments. (B6 × bm12)F<sub>1</sub> or (B6 × bm1)F<sub>1</sub> mice were exposed to 700 cGy from 60 Co sources at 20 cGy/min. CB6F<sub>1</sub> or dm2B6F<sub>1</sub> mice were exposed to 750 cGy. Purified CD<sup>4</sup><sup>+</sup> or CD<sup>8</sup><sup>+</sup> cells from B6 donors were injected via the tail vein into irradiated (B6 × bm12)F<sub>1</sub> or (B6 × bm1)F<sub>1</sub> recipients, respectively. In some experiments, Ly5.1-congenic recipients were utilized to distinguish donor cells from host cells. Irradiated CB6F<sub>1</sub> or dm2B6F<sub>1</sub> recipients were transplanted with 6–15 × 10<sup>6</sup> purified CD8<sup>+</sup> cells from 2C donors. Within each experiment, all recipients were injected with an identical number of 2C CD8 cells.

Preparation and administration of Abs

Both anti-CD28 (37.51) and anti-CTLA4 (9H10) are hamster IgG and were kindly provided by Dr. James Allison (University of California, Berkeley, CA). Murine CTLA4-Ig and control L6-Ig were kindly provided by Dr. Robert Peach (Bristol-Myers Squibb, Princeton, NJ). Control hamster Ig was purchased from IGN Pharmaceuticals (Aurora, OH). All the Abs, unless indicated, were injected i.p. at 100 μg/dose every other day for 14 days starting on the day of the transplant.

Flow cytometry

To detect donor CD4 or CD8 cells, splenocytes were isolated from the recipients and stained with mAbs specific for Ly5.1 (A20-1.7, mouse IgG2a; American Type Culture Collection, Manassas, VA) and CD4 (GK1.5) or CD8 (53-6.7). For detection of 2C donor cells, mAbs specific for CD8 and 2C TCR (1B2) were used. The 1B2 hybridoma was kindly provided by Dr. D. Loh (22), and FTTC-conjugated 1B2 was prepared in our laboratory. Other mAbs used in this study included: anti-B220 (RA3-6B2), anti-CD28 (37.51), anti-CD25 (7D4), anti-CTLA4 (9H10), mouse anti-hamster IgG (192.1), and isotype control Abs. Except where noted, all mAbs were conjugated with fluorescein isothiocyanate, allophycocyanin, or phycoerythrin.
mAbs used for FACS analysis were obtained from PharMingen (San Di-ego, CA). To test for CD28 modulation in vivo, freshly isolated spleno-cytes were incubated with saturating amounts of anti-CD28 mAb or normal hamster IgG for 30 min at 4°C. After washing, the cells were labeled with FITC-conjugated mouse anti-hamster IgG. Intracellular detection of CTLA4 was conducted as previously described (23). Briefly, cells were fixed with 1% paraformaldehyde, permeabilized with 0.3% saponin, and stained with anti-CTLA4 mAb followed by FITC-conjugated mouse anti-hamster IgG. We used a FACSscan with CellQuest software (Becton Dickin-son, San Jose, CA) for flow cytometric analysis.

Statistical analysis
Continuous distributions were compared by Student’s t tests. Survival dis-tributions were compared by log-rank tests. Two-sided p values <0.05 were considered significant.

Results

CTLA4 signals inhibit GVHD
To determine the effect of CTLA4 signals on the development of GVHD, we first tested whether CTLA4 blockade with a nonstimula-tory, bivalent mAb would accelerate GVHD. Sublethally irradiated (700 cGy) (B6 × bm12)F1 mice were transplanted with purified CD4+ cells from wild-type B6 mice and treated with anti-CTLA4 mAb or hamster IgG at 100 μg/dose every other day for a total of eight doses. Treatment with anti-CTLA4 mAb was shown to ac-celerate GVHD lethality (p = 0.005) (Fig. 1A).

B7:CTLA4 interaction may inhibit CD28- or TCR-driven intra-cellular signals. To distinguish between these two possibilities, experiments were performed to test the effect of B7:CTLA4 inter-action in development of GVHD induced by CD28-deficient T cells. Sublethally irradiated (B6 × bm12)F1 mice were transplanted with CD4+ cells from CD28−/− B6 mice, and the recipients were treated with CTLA4-Ig or control L6-Ig. CTLA4-Ig treatment significantly accelerated and exacerbated GVHD lethality compared with control treatment (p = 0.00008) (Fig. 1B). These results indicate that the B7:CTLA4 interaction plays a pro-ective role in the development of GVHD independent of CD28, and interference with the B7:CTLA4 interaction enhances GVHD mortality by removing regulatory controls on TCR-driven intra-cellular responses.

Anti-CD28 mAbs prevent lethal GVHD
Simultaneous blockade of B7 interaction with anti-CD28 and CTLA4 by administration of soluble CTLA4-Ig or B7-specific mAbs can partially inhibit the development of GVHD in mice (9–12). Because CD28 signals enhance GVHD (19, 20), while CTLA4 signals inhibit GVHD (Fig. 1), we reasoned that the severity of GVHD would be decreased by selectively blocking CD28 costimulation while still allowing CTLA4 engagement on donor T cells. We tested the effect of anti-CD28 mAb in preventing GVHD based on the observation that the administration of intact anti-CD28 mAb inhibits T cell expansion in vivo (24–26), even though anti-CD28 mAb amplifies T cell activation in vitro. Sublethally irradiated MHC class II incompatible (B6 × bm12) or MHC class I incompatible (B6 × bm1)F1 mice were transplanted with B6 CD4+ or CD8+ T cells, respectively. Recipients were treated with anti-CD28 mAb, CTLA4-Ig, or hamster IgG plus L6-Ig at 100 μg/dose every other day from day 0 to day 14. Irradiated controls that were not transplanted developed transient pancytopenia, but all recovered and survived longer than 100 days (Fig. 2, A and B). Recipients injected with allogeneic T cells and treated with control Abs became acutely ill with progressive weight loss, ruffled fur, and kyphosis, and all died at a median of 15 days after transplant. Both CTLA4-Ig and anti-CD28 mAb significantly improved survival as compared with control Abs (p < 0.0001), but anti-CD28 mAb was significantly more effective than CTLA4-Ig (p < 0.01).

Anti-CD28 mAb inhibits donor T cell expansion
To elucidate the mechanisms by which anti-CD28 treatment pre-vents GVHD, we tested the effects of anti-CD28 mAb on donor T cell activation and expansion. Sublethally irradiated (B6.Ly5.1 × bm12)F1 mice (five to six mice per group) were transplanted with purified CD4+ cells from B6.Ly5.2 donors and treated with anti-CD28 mAb, CTLA4-Ig, or hamster IgG plus L6-Ig. In recipients treated, respectively, with control Abs, CTLA4-Ig, and anti-CD28 mAb, the percentages of CD4+Ly5.1+ donor T cells in the blood were 18.8 ± 0.3%, 4.4 ± 1.0%, and 1.7 ± 0.4% on day 6, and 52.1 ± 12.9%, 19.1 ± 14.2%, and 7.3 ± 4.2% on day 15. These data suggest that both CTLA4-Ig and anti-CD28 mAb inhibited donor T cell expansion in vivo and that anti-CD28 mAb was significantly more effective than CTLA4-Ig (p < 0.01). In further experiments, we have tested the effect of anti-CD28 mAb on the
expansion of donor T cells in peripheral lymphoid organs. We found that anti-CD28 mAb inhibited donor T cell expansion and was superior to CTLA4-Ig (Fig. 3, upper panels). We also found that anti-CD28 mAb induced CD28 modulation on CD4 \textsuperscript{+} Ly5.1\textsuperscript{2} donor T cells, whereas control Abs and CTLA4-Ig did not have this effect (Fig. 3, lower panels). Inhibitory effects of anti-CD28 mAb on donor T cell expansion and CD28 modulation were also observed in transplantation of B6 CD8 cells into (B6.Ly5.1 \textsuperscript{3} bm1)F\textsubscript{1} (data not shown).

Treatment with anti-CD28 mAb does not inhibit expression of CD25 and CTLA4
To follow the fate and function of T cells that recognize recipient alloantigen in vivo, we have used a model in which 2C TCR transgenic T cells were transplanted into CB6F1 recipients that express the specific alloantigen L\textsuperscript{d}. In this model, 2C cells engrafted, expanded, and became effectors leading to extensive destruction of host B cells and double positive thymocytes (21). In additional experiments, we tested the effect of anti-CD28 mAb on activation of 2C cells in CB6F\textsubscript{1} recipients. Sublethally irradiated CB6F\textsubscript{1} mice were transplanted with purified CD8\textsuperscript{+} cells from 2C wild-type or 2C CD28\textsuperscript{−/−} mice and treated with anti-CD28 mAb or hamster IgG. On day 4, 2C cells in recipient spleen were analyzed for expression of CD25 and CTLA4 (Fig. 4). CTLA4 expression was induced in wild-type 2C cells and in CD28\textsuperscript{−/−} 2C cells and was not affected by anti-CD28 treatment, indicating that CD28 signals are not needed for activation-dependent expression of CTLA4. Higher level of CD25 expression was induced in wild-type 2C cells than in CD28\textsuperscript{−/−} 2C cells, and CD25 expression was not affected by anti-CD28 treatment. These results show that treatment with anti-CD28 mAb did not block early CD28 signaling that is largely required for CD25 expression.

Anti-CD28 mAb selectively inhibits expansion of alloreactive T cells and destruction of host B cells in the recipients
To determine whether anti-CD28 mAb prevents GVHD by depleting CD28\textsuperscript{+} T cells in vivo, we transplanted purified CD8\textsuperscript{+} 2C T cells into irradiated CB6F\textsubscript{1} recipients. In this experiment, we used L\textsuperscript{d} loss mutant dm2B6F\textsubscript{1} recipients as negative controls. Treatment with anti-CD28 mAb had no effect on the number of 2C cells on day 14 in dm2B6F\textsubscript{1} recipients, indicating that this mAb did not deplete resting CD28\textsuperscript{+} cells in vivo. Treatment with anti-CD28 mAb decreased the number of 2C cells in CB6F\textsubscript{1} recipients, indicating that anti-CD28 mAb interfered with expansion of donor T cells that recognize recipient alloantigens (Fig. 5A). The number of host B cells was 50-fold higher in CB6F\textsubscript{1} recipients treated with anti-CD28 mAb than in CB6F\textsubscript{1} recipients treated with control Ab, but 0.07-fold lower than in dm2B6F\textsubscript{1}, negative controls (Fig. 5B). These results indicate that GVHD was reduced in severity but not completely prevented by treatment with anti-CD28 mAb.

Discussion
In this study, we have investigated the role of CD28 and CTLA4 in the T cell response to alloantigens in vivo by using models of GVHD in sublethally irradiated mice. Under the conditions tested, donor T cells cause damage to the recipient hematopoietic system, resulting in marrow failure. We showed that anti-CD28 mAb is more effective than CTLA4-Ig in the prevention of GVHD, demonstrating that the selective hindrance of CD28 signals is a better approach to achieve transplant tolerance than B7 blockade, as predicted by other investigators (20, 24, 27). We suspect that the
protective effect of anti-CD28 mAb is the result of CD28 modulation that precludes the participation of B7:CD28 interaction in sustaining the expansion of alloreactive T cells. It is also possible that binding of the anti-CD28 mAb causes a reduction or a qualitative change in costimulatory signals by excluding CD28 from the TCR/Ag contact cap. Alternatively, anti-CD28 mAb might deliver a partial agonistic signal that leads to early termination of clonal expansion in vivo.

Anti-CD28 mAb have notably different effects on T cell responses in vitro and in vivo. In vitro, they enhance proliferation in short-term assays, but in vivo, they prevent proliferation. We have shown that anti-CD28 mAb caused nearly completed modulation of CD28 in vivo (Fig. 3). In separate experiments, we have found that anti-CD28 mAb do not cause modulation of CD28 within the same time frame in vitro (data not shown). The reason for the difference in modulation remains for further investigation, but may be related to interaction with Fc receptors, causing extensive mobilization of CD28 molecules into intracellular contact caps in vivo. We suspect that the immunosuppressive activity of anti-CD28 mAb is related to this rapid modulation of CD28 receptor from the T cell surface, as observed in a rat heart allograft model (28).

The blockade of CTLA4-signals exacerbated GVHD independently of CD28 expression on donor T cells (Fig. 1), and CTLA4 expression and function were not affected by treatment with anti-CD28 mAb (Fig. 4). Our observations were consistent with previous reports showing that B7:CTLA4 interactions have a negative regulatory role on the capacity of CD28”/−” recipients to respond to tumor Ags or alloantigens (29, 30). Thus, CTLA4 retains its ability to inhibit T cell responses and protect from acute GVHD in the absence of CD28, indicating that cross-linking of CTLA4 can directly inhibit signaling events initiated through the TCR (31, 32). Alternatively, CTLA4 might inhibit other costimulatory signals such as those transduced by inducible co-stimulator (ICOS) or CD134 (OX40). Therefore, the preservation of CTLA4-negative regulatory signals should be helpful in preventing GVHD.

Treatment with anti-CD28 mAb may lead to B cell expansion in normal mice (33), but the number of host B cells in sublethally irradiated dm2B6F1 recipients was not affected by anti-CD28 treatment (Fig. 5B). Thus, we can conclude that the increase in the number of host B cells in CB6F1 recipients treated with anti-CD28 mAb results from decreased GVHD severity. The optimal dose and schedule of anti-CD28 mAb has not been determined. It is unlikely, however, that more than 100 μg anti-CD28 mAb per dose would achieve better results, because 100 μg/dose induced maximal CD28 modulation in vivo (data not shown). Incomplete prevention of GVHD by anti-CD28 treatment was consistent with our previous observation that CD28-deficient donor T cells have some ability to induce GVHD (19). These results suggest that other costimulatory systems can participate in allogeneic responses. Recently published results have indicated that CD154:CD40 pathway plays a particularly important role in the development of the immune responses (27, 34, 35). Saito et al. (27) have shown that treatment with anti-CD154 mAb ameliorates the manifestations of GVHD induced by CD28”/−” T cells. Thus, it is reasonable to expect that the blockade of CD28 and CD154, while preserving CTLA4 function, would be an effective strategy to induce transplantation tolerance.

In summary, our findings provide evidence that selective targeting of CD28 is more immunosuppressive than targeting B7 and blocking the function of both CD28 and CTLA4. Thus, treatment with an anti-CD28 mAb or other selective CD28 inhibitors could be applied for induction of T cell tolerance in human transplantation. The use of CD28 inhibitors in combination with agents that block other costimulatory interactions such as CD154:CD40 might be required for maximum effects.

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