4-1BB-dependent inhibition of immunosuppression by activated CD4$^+$CD25$^+$ T cells

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Abstract: 4-1BB (CD137) is a costimulatory molecule involved in the activation and survival of CD4, CD8, and natural killer cells. Although a great deal has been learned as to how 4-1BB-mediated signaling governs the immunity of conventional T cells, the functional role of 4-1BB in the context of CD4$^+$CD25$^+$ regulatory T cell (Tr) activation is largely unknown. Using 4-1BB-intact and -deficient mice, we investigated the effect of the 4-1BB/4-1BB ligand pathway on the suppressive function of Tr cells. Our data indicate that although 4-1BB is expressed on Tr cells, its contribution to their proliferation is minimal. We also showed that signaling through the 4-1BB receptor inhibited the suppressive function of Tr cells in vitro and in vivo. It is interesting that anti-4-1BB-mediated but not anti-GITR-directed inhibition was more potent when Tr cells were preactivated. Collectively, these data indicate that 4-1BB signaling is critical in Tr cell immunity. *J. Leukoc. Biol.* 75: 785–791; 2004.

Key Words: *regulatory T lymphocytes · GVHD · tolerance · suppression*

INTRODUCTION

Since their discovery, CD25$^+$ (CD4$^+$CD25$^+$) regulatory T (Tr) cells have been found to play important roles in immune function [1–3]. Upon coculture, they immunosuppress CD4$^+$CD25$^-$ and CD8$^+$ T cell function, presumably by down-regulating interleukin (IL)-2 production [2–5] by a distinctive process in that it is rendered more potent by prior activation of the CD25$^+$ cells [2]. Despite rapid progress, the molecular basis of the immunosuppression remains elusive. Some studies have suggested that IL-10 and transforming growth factor-β (TGF-β) [4] contribute to the suppressive potency of the CD25$^+$ Tr cells, but subsequent observations of IL-10 and TGF-β-deficient mice do not fully support this view [2, 6]. Suppression appears to be contact-dependent and is not mediated by IL-4 or IL-10, as CD25$^+$ Tr cells from IL-4- or IL-10-deficient mice are as effective as those from wild-type mice [2]. Recent data suggested that glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) plays some part in inhibiting CD25$^+$ Tr cell-mediated suppression [7].

4-1BB, the inducible T cell antigen (Ag) present on CD4$^+$, CD8$^+$, natural killer, and dendritic cells, provides CD28-independent costimulation of T cell activation [8–10]. 4-1BB-mediated signaling plays a critical role in preventing activation-induced cell death, promoting the rejection of cardiac allografts and skin transplants, enhancing integrin-mediated cell adhesion, increasing T cell cytolytic potential, and eradicating established tumors [8–14]. 4-1BB-deficient mice have normal T and B cell numbers but have defects in Ag-specific interferon-γ expression and cytolytic T lymphocyte (CTL) activity [15].

Although 4-1BB is constitutively expressed on CD25$^+$ Tr cells [16, 17], the consequence of this expression is largely unknown. In the present study, we analyzed its effect in systems involving CD4$^+$ T cell immunity. Using wild-type and 4-1BB-deficient mice, we showed that 4-1BB signaling is required to neutralize the suppressive function of CD25$^+$ Tr cells in vitro and in vivo and that this neutralizing action is much more potent when the CD25$^+$ Tr cells are activated. Thus, signaling through the 4-1BB receptor is critical for CD25$^+$ Tr cell immunity. The ability of 4-1BB-dependent regulatory processes to counter the suppressive effect of CD25$^+$ Tr cells in vitro and in vivo also points to a novel role for the 4-1BB receptor.

MATERIALS AND METHODS

Mice

Female mice (5–6 weeks) were used in all experiments. Wild-type C57BL/6 mice were purchased from Harlan (Indianapolis, IN) and B6.C-H2^{bm12}/KhEg (bm12) mice, from The Jackson Laboratory (Bar Harbor, ME). 4-1BB-deficient C57BL/6 mice [15] were bred and maintained under specific, pathogen-free conditions in the animal facilities of the University of Ulsan (Korea).

Reagents and antibodies (Ab)

Anti-CD3 monoclonal Ab (mAb; 145.2C11), biotin-labeled CD25 (7D4), biotin–major histocompatibility complex (MHC) II (I-A^b, AF6-120.1), Fc blocker

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(2.4G2), fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (H129.19) mAb, and isotype-control Ab were purchased from BD PharMingen (San Diego, CA). Phycoerythrin (PE)-conjugated anti-CD25 (PC61.5) and biotin–CD8a (53-6.7) were purchased from E-Biosciences (San Diego, CA). Anti-CD4, anti-CD8, as well as streptavidin-conjugated microbeads, were obtained from Miltenyi Biotec (Auburn, CA). Recombinant human (rh)IL-2 was purchased from PeproTech (Rocky Hill, NJ). Dr. Robert S. Mittler (Emory University, Atlanta, GA) kindly provided agonistic anti-4-1BB mAbs (3H3 and 3E1), and production of agonistic anti-GITR mAb, DTA-1 has been described [7].

Cell isolation

Cell populations were isolated with a VarioMACS™ magnetic cell sorter (Miltenyi Biotec), according to the manufacturer's protocols. Briefly, red blood cell-depleted splenocytes were combined with lymph node cell suspensions in phosphate-buffered saline (PBS), supplemented with 0.5% bovine serum albumin, and incubated with Fc receptor-blocking mAb 2.4G2 for 10 min at 4°C. $CD8^+$ T and MHC II^+ cells were depleted by staining with biotinylated anti-CD8 and anti-MHC II mAb and streptavidin microbead. The $CD25^+$ Tr cells were enriched by incubating the $CD8^-$MHC II^- fraction with a biotinylated anti-CD25 mAb and microbeads. $CD25^-$ T cells were isolated from the $CD8^-$MHC II^-CD25^- fraction with microbead-conjugated anti-CD4 mAb. The $CD25^+$ and $CD25^-$ populations were >90% and >96% pure, respectively. To activate the purified $CD25^+$ Tr cells, they were plated at 2×10^6/well in six-well plates with 0.5 μg/ml anti-CD3 mAb and 20 U/ml rhIL-2 for 3 days. Activated $CD25^-$ T cells were prepared by adding 0.5 μg/ml anti-CD3 mAb to the culture and incubating for 24 h. The activated cells were extensively washed with PBS and used immediately.

Cell proliferation

$CD25^+$ Tr cells (1×10^5 cells/well) and $CD25^-$ T cells (2×10^5 cells/well) were incubated with X-irradiated (20 Gy) splenocytes (5% with respect to total cells/well) for 3 days in the presence 0.5 μg/ml anti-CD3 mAb alone or in combination with 5 μg/ml 3H3 (anti-4-1BB mAb) or DTA-1 (anti-GITR mAb). rhIL-2 (10–20 U/ml) and a different number of $CD25^+$ Tr cells were used in some experiments. The cells were labeled with 1 μCi/well [^{3}H]-thymidine for the final 8 h, harvested, and counted in a liquid scintillation counter (Packard, Albertville, MN).

Flow cytometry

Naïve $CD25^-$ T cells and $CD25^+$ Tr cells were stained with PE-conjugated anti-CD25 and FITC-labeled anti-CD4 mAb after blocking with Fc receptor-blocking mAb 2.4G2 for 10 min at 4°C. Expression of 4-1BB on $CD25^+$ Tr cells was measured by staining with FITC-conjugated 3E1 mAb for 30 min at 4°C and analysis on a FACScan™ (BD Biosciences, San Jose, CA).

Induction of graft-versus-host disease (GVHD)

Recipient mice (bm12) were sublethally irradiated with 6.0 Gy (^{137}Cs) total body irradiation. After 6 h, the mice were intravenously infused with freshly purified $CD25^-$ T cells (2×10^5) or $CD25^-$ and $CD25^+$ Tr cells (2×10^5) from wild-type C57BL/6 mice. In a separate experiment, $CD25^-$ cells were prepared from 4-1BB-deficient mice and naïve $CD25^+$ Tr cells from wild-type mice and activated as described above. The naive $CD25^-$ T cells (3×10^5) were injected into bm12 recipients together with naïve or activated $CD25^+$ Tr cells (3×10^5). Where indicated, the recipient mice were injected intraperitoneally with 200 μg 3H3 or DTA-1 mAb. The mice were monitored daily for GVHD lethality.

RESULTS

The 4-1BB does not influence $CD25^+$ Tr cell proliferation

$CD25^+$ Tr cells constitutively express 4-1BB at a low level and the same increases upon activation [16]. To assess the significance of this expression, we purified $CD25^+$ Tr cells from wild-type C57BL/6 mice as described previously (**Fig. 1A**). Flow cytometric analysis confirmed constitutive- as well as activation (with anti-CD3/IL-2)-enhanced 4-1BB expression on the surface of naïve $CD25^+$ Tr cells (Fig. 1B). Having found that the $CD25^+$ Tr cells express 4-1BB, we tested whether this expression was responsible for activation signals, as it is in conventional T cells [18]. When naïve $CD25^+$ Tr cells from wild-type mice were stimulated with anti-CD3 mAb, the addition of agonistic anti-4-1BB mAb (3H3) resulted in negligible

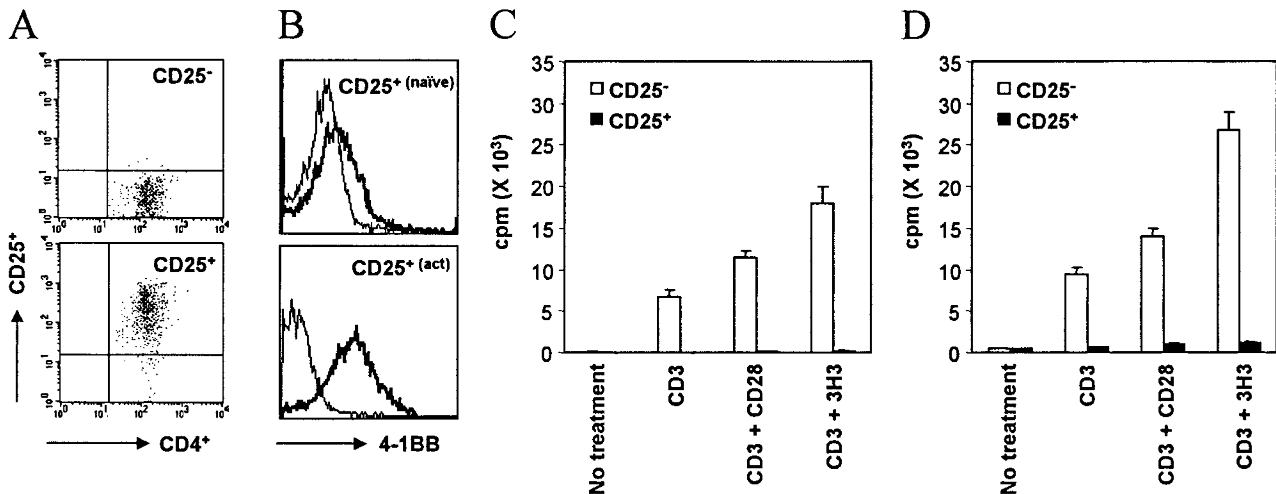

Fig. 1. $CD25^+$ Tr cells show resistance to 4-1BB-mediated signaling. (A) The purity of $CD25^-$ and $CD25^+$ cells assessed by staining with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD25 mAb. (B) 4-1BB expression on naïve and activated $CD25^+$ Tr cells. Cells were cultured in RPMI-1640 medium, supplemented with 0.5 μg/ml anti-CD3 mAb and 20 U/ml IL-2 for 3 days. They were then stained with FITC-conjugated anti-4-1BB mAb (3E1) and were analyzed with a FACScan™. (C) Freshly purified $CD25^-$ T and $CD25^+$ Tr cells were plated in 96-well culture plates at 1×10^5/well. Cells were activated with 0.5 μg/ml anti-CD3 mAb in the absence or presence of 5 μg/ml anti-CD28 or 3H3 (anti-4-1BB mAb) for 3 days. (D) $CD25^-$ T and $CD25^+$ Tr cells were activated as described in Materials and Methods. After washing cells with PBS, they were plated at 1×10^5/well and stimulated with 0.5 μg/ml anti-CD3 mAb in the absence or presence of 5 μg/ml anti-CD28 or 3H3 (anti-4-1BB mAb) for 3 days. All samples were labeled with 1 μCi [^{3}H]-thymidine for the last 8 h.

enhancement of proliferation, whereas proliferation of CD25⁻ T cells was stimulated by the addition of 3H3 mAb (Fig. 1C). If the $CD25^+$ Tr cells were contaminated with a few effector cells, and these cells would be expanded to a certain extent during the proactivation step, then the anti-4-1BB stimulation may be stimulating those activated effectors to produce IL-2 and thereby break suppression. To remove this possibility and to test the effect of 4-1BB stimulation on the activated $CD25^+$ Tr cells, $CD25^+$ Tr cells were activated with anti-CD3 mAb plus IL-2 for 3 days and $CD25^-$ cells with anti-CD3 mAb alone for 24 h, and proliferation assay was performed. Activated $CD25^+$ Tr cells showed no significant enhancement of proliferation by 3H3 mAb (Fig. 1D). Thus, signaling via the 4-1BB in $CD25^+$ Tr cells does not result in cell proliferation as it does in conventional $CD4^+$ or $CD8^+$ T cells [19].

Signal through the 4-1BB receptor is required to neutralize the suppressive effect of $CD25^+$ Tr cells in vitro

Previous results (Fig. 1B) showed that 4-1BB was expressed at a high level on rIL-2-activated $CD25^+$ Tr cells. Thus, we hypothesized that activation of $CD25^+$ Tr cells might be required to exert 4-1BB-mediated signaling. We tested whether 4-1BB-mediated signaling requires IL-2 to promote proliferation in coculture experiments. $CD25^-$ T cells were cultured with $CD25^+$ Tr cells in the presence of agonistic anti-4-1BB (3H3) mAb with and without IL-2. To establish the role of 4-1BB, $CD25^-$ T cells from 4-1BB-deficient mice were cultured with $CD25^+$ Tr cells from wild-type mice to direct the 4-1BB effects to the suppressors rather than the responders. $CD25^-$ T cell proliferation was clearly inhibited by the Tr cells, and the 3H3 mAb permitted the proliferation of the $CD25^-$ T cells in the presence of exogenous IL-2 (**Fig. 2A**). This result suggests that 4-1BB signaling, together with exogenous IL-2, can antagonize the suppression by $CD25^+$ Tr cells in a manner similar to the effect of GITR ligation, which efficiently reverses suppressive function of naïve $CD25^+$ Tr cells [16]. Similar results were obtained when $CD25^-$ T cells from 4-1BB-deficient mice were cocultured with $CD25^+$ Tr cells from wild-type mice (Fig. 2B). To determine whether 4-1BB stimulation could neutralize the suppressive activity of activated $CD25^+$ Tr cells, we prepared "activated" $CD25^+$ Tr cells by culturing them with anti-CD3 mAb and IL-2 for 3 days. When naïve $CD25^-$ T cells were cocultured with activated $CD25^+$ Tr cells, agonistic anti-4-1BB mAb (3H3) efficiently induced proliferation even in the presence of $CD25^-$ T cells from 4-1BB-deficient mice (Fig. 2, E and F) but not in the presence of naive $CD25^+$ Tr cells as previously reported (Fig. 2, C and D).

Signaling through 4-1BB attenuates the suppression of activated $CD25^+$ Tr cells

To further confirm the observed, desuppressive effect of 3H3 mAb, naïve $CD25^-$ T cells were cocultured with different ratios of $CD25^+$ T cells with control IgG, 3H3, or DTA-1 mAb. As previously reported [16], DTA-1 mAb only reversed the suppression when the activated $CD25^+$ Tr cell number is low. In contrast, the 3H3 mAb abrogated the suppression of activated $CD25^+$ Tr cells at all ratios tested (**Fig. 3A**). To test the

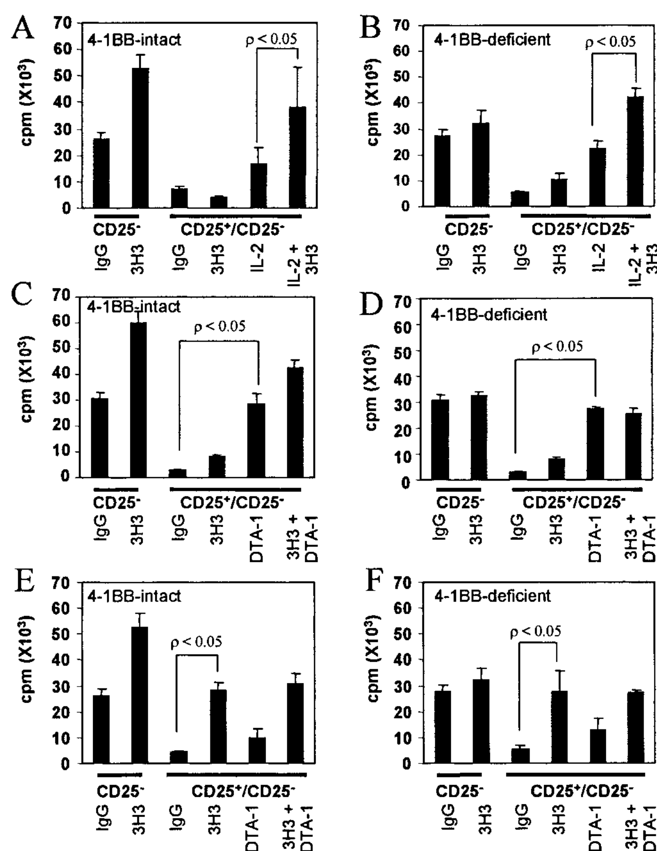

Fig. 2. Effect of 4-1BB on the suppressive activity of $CD25^+$ Tr cells. $CD25^+$ Tr cells were purified from wild-type C57BL/6 mice, and $CD25^-$ T cells, from wild-type and 4-1BB-deficient C57BL/6 mice. To exclude the effect of anti-4-1BB mAb on activated $CD25^-$ T cells, 1×10^5 wild-type $CD25^+$ Tr cells were mixed with 2×10^5 $CD25^-$ T cells from wild-type or 4-1BB-deficient mice and were stimulated with 0.5 μg/ml anti-CD3 mAb in the presence of 5 μg/ml rat immunoglobulin G (IgG), 3H3, DTA-1 mAb, and/or 10 U/ml rhIL-2. (A and B) Naive 4-1BB-intact and -deficient $CD25^-$ T cells were cocultured with naïve $CD25^+$ Tr cells in the presence of 5 μg/ml control IgG, 3H3 mAb, and/or 10 U/ml IL-2. (C and D) Naïve $CD25^+$ Tr cells were mixed with freshly isolated $CD25^-$ T cells from 4-1BB-intact and -deficient mice in the presence of 5 μg/ml control IgG, 3H3, or DTA-1 mAb. (E and F) Activated $CD25^+$ Tr cells were prepared as described previously and cocultured with naive $CD25^-$ T cells from 4-1BB-intact and -deficient mice in the presence 5 μg/ml control IgG, 3H3, or DTA-1 mAb. Proliferation was measured on the third day by labeling with 1 μCi [^{3}H]-thymidine for the last 8 h.

dose-dependent effect of 3H3 mAb, naive $CD25^-$ T cells and activated $CD25^+$ Tr cells (2:1 ratio) were cocultured with the indicated doses of 3H3 or DTA-1 mAb. Agonistic anti-4-1BB mAb (3H3) abrogated activated $CD25^+$ Tr cell-mediated suppression in a dose-dependent manner when added to cultures containing $CD25^+$ Tr cells and $CD25^-$ T cells (Fig. 3B).

It is possible to deduce from these results that $CD25^+$ Tr cells express a high level of GITR even in the resting status [7], but only a subpopulation of naïve $CD25^+$ Tr cells expressed 4-1BB (Fig. 1B). In this condition, 4-1BB stimulation could not reverse the suppression of naïve $CD25^+$ Tr cells as a result of the low level of 4-1BB, suggesting that to elicit effective desuppression, 4-1BB must be expressed at higher levels. Thus, we conclude that the suppression of activated but not "resting" $CD25^+$ Tr cells is more effectively neutralized by 4-1BB than GITR stimulation.

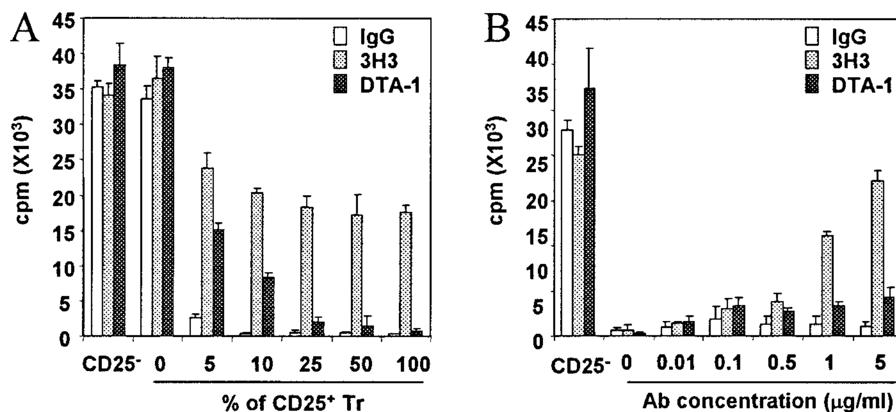

Fig. 3. Stimulation through the 4-1BB receptor reverses the suppression of activated CD25^{+} Tr cells. (A) Naïve CD25^{-} Tr cells (2×10^5) from 4-1BB-deficient mice were cocultured with the indicated number of activated CD25^{+} Tr cells and treated with 5 μg/ml control IgG, 3H3, or DTA-1 mAb. (B) Naïve CD25^{-} Tr cells (2×10^5) from 4-1BB-deficient mice and 2×10^5 of activated CD25^{+} Tr cells were mixed with the indicated concentration of control IgG, 3H3, or DTA-1 mAb. All samples were stimulated with 0.5 μg/ml anti-CD3 mAb, and proliferation was measured on the third day.

4-1BB stimulation renders CD25^{-} T cells resistant to suppression but does not fully alter the suppressive function of CD25^{+} Tr cells

4-1BB stimulation is known to enhance the proliferation, function, and survival of CD4^{+} and CD8^{+} T cells [11, 20]. We therefore tested whether CD25^{-} T cells stimulated by 3H3 mAb are resistant to suppression by CD25^{+} Tr cells. Activated CD25^{-} T cells were prepared from wild-type and 4-1BB-deficient mice as a negative control and were cultured with anti-CD3 mAb for 24 h. This activation step led to the increased expansion of 4-1BB-positive cells (>70%; data not shown). Stimulation through 4-1BB rendered activated CD25^{-} T cells more resistant to the suppression by CD25^{+} Tr cells but not activated CD25^{-} T cells from 4-1BB-deficient mice (**Fig. 4, A** and **B**). It is interesting that activated CD25^{-} T cells were poorly suppressed by "naïve" CD25^{+} Tr cells but not by activated CD25^{+} Tr cells (Fig. 4C). In a separate experiment, we tested whether 4-1BB stimulation permanently altered the suppressive activity of the CD25^{+} Tr cells, which were activated with anti-CD3 mAb and IL-2 for 3 days in the presence

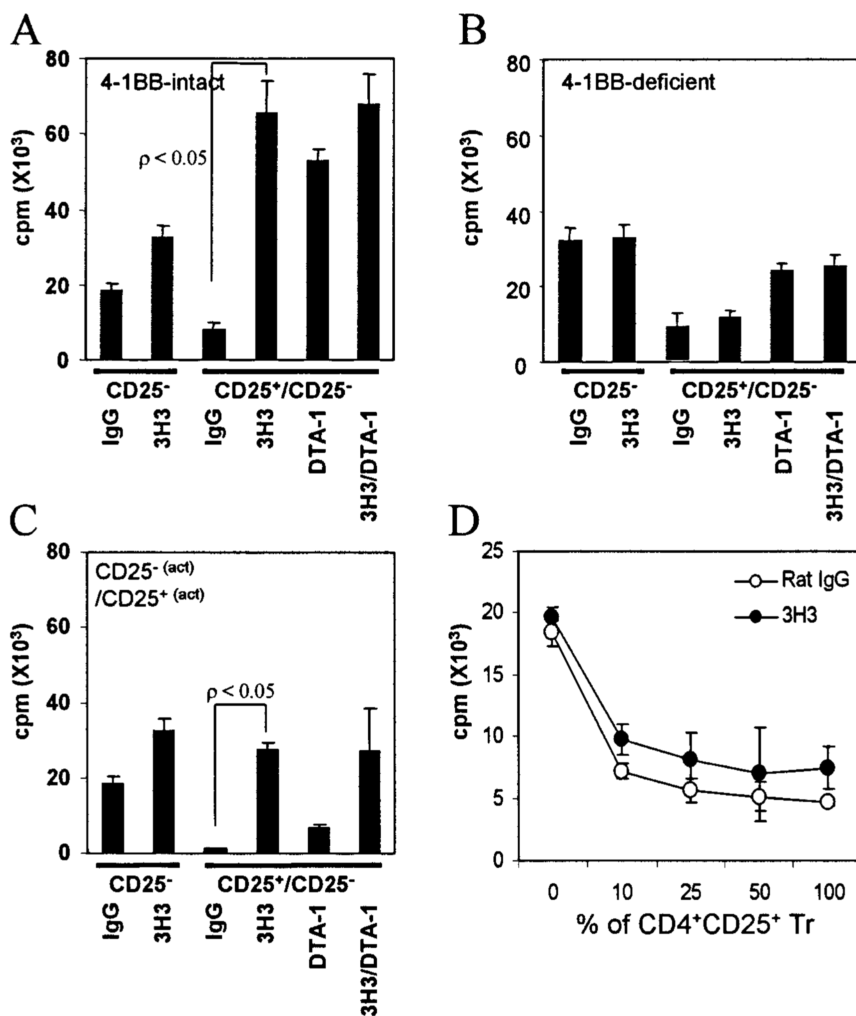

Fig. 4. Effect of signaling through 4-1BB on in vitro suppression of activated CD25^{-} T cells by CD25^{+} Tr cells. (A and B) Activated CD25^{-} T cells were prepared from 4-1BB-intact and -deficient mice as described previously. Activated CD25^{-} T cells and naïve CD25^{+} Tr cells were mixed and stimulated with 0.5 μg/ml anti-CD3 mAb in the presence of 5 μg/ml control IgG, 3H3, or DTA-1 mAb for 3 days. (C) Activated CD25^{-} T cells and CD25^{+} Tr cells were prepared from wild-type C57BL/6 mice, mixed, and stimulated with anti-CD3 mAb in the presence of 5 μg/ml control IgG, 3H3, or DTA-1 mAb for 3 days. (D) CD25^{+} Tr cells were incubated in plates with 0.5 μg/ml anti-CD3 mAb and 20 U/ml IL-2 for 3 days in the presence or absence of 3H3 (5 μg/ml) mAb. The cells were harvested, washed with PBS, and were then serially diluted and cocultured with freshly isolated CD25^{-} T cells for 3 days. Samples were labeled with 1 μCi [^{3}H]-thymidine for the last 8 h, and the extent of cellular proliferation was enumerated.

or absence of 3H3 mAb. Activated cells were extensively washed and cocultured with naïve CD25⁻ T cells. We found that they recovered their ability to suppress upon removal of the 3H3 mAb (Fig. 4D), suggesting that sustained stimulation through 4-1BB is required for the increased resistance of activated CD25⁻ T cells.

Signals through 4-1BB abrogate prolonged survival in the face of GVHD induction by transfer of $CD25^+$ Tr cells

We also tested whether 4-1BB stimulation can inhibit the suppressive activity of $CD25^+$ Tr cells in vivo. We used a system in which purified T cell subsets are introduced into MHC-disparate, sublethally irradiated recipients [21]. We transferred freshly purified $CD25^-$ T cells or $CD25^-$ and $CD25^+$ Tr cells into sublethally irradiated bm12 recipients together with control IgG, DTA-1, or 3H3 mAb. Transfer of $CD25^+$ Tr cells delayed death from GVHD, and injection of DTA-1 or 3H3 mAb abolished this delay (**Fig. 5A**). Thus, stimulation through GITR or 4-1BB neutralized the suppressive activity of $CD25^+$ Tr cells in vivo. However, 20–25% of the mice treated with 3H3 mAb survived, perhaps because of differences in the extent of activation of the $CD25^+$ Tr cells in the initial immune response. We therefore prepared activated $CD25^+$ Tr cells from wild-type C57BL/6 mice by stimulating them with anti-CD3 mAb and exogenous IL-2 and did the same with naïve $CD25^-$ T cells from 4-1BB-deficient mice to rule out the effect of 3H3 mAb on $CD25^-$ T cells, which from wild-type and 4-1BB-deficient mice, behaved only slightly differently in this GVHD system when 3×10^5 cells were transferred into bm12 mice as described previously [22]. Therefore, in the present study, we transferred the naïve or activated $CD25^+$ Tr cells with naïve $CD25^-$ T cells into sublethally irradiated bm12 mice along with 3H3 mAb, which caused accelerated death when activated rather than naïve $CD25^+$ Tr cells, which were transferred (Fig. 5B). Taken together, we concluded that stimulation through the 4-1BB receptor has a critical role in the neutralization of activated $CD25^+$ Tr cells.

DISCUSSION

In an exception to the activation-dependent expression pattern of 4-1BB on conventional T cells [8], a recent study reported that Tr cells express this receptor in a constitutive manner [16]. The significance of this expression in the regulation of Tr cells, however, has not been explained in detail.

In this study, we present evidence that 4-1BB signaling is critical for modulation of the suppressor function of activated but not resting $CD25^+$ Tr cells. By contrast, GITR stimulation regulates the activity of naïve but not activated $CD25^+$ Tr cells [7, 16]. As resting $CD25^+$ Tr cells express GITR on their surface at much higher levels than they do 4-1BB [16], it is

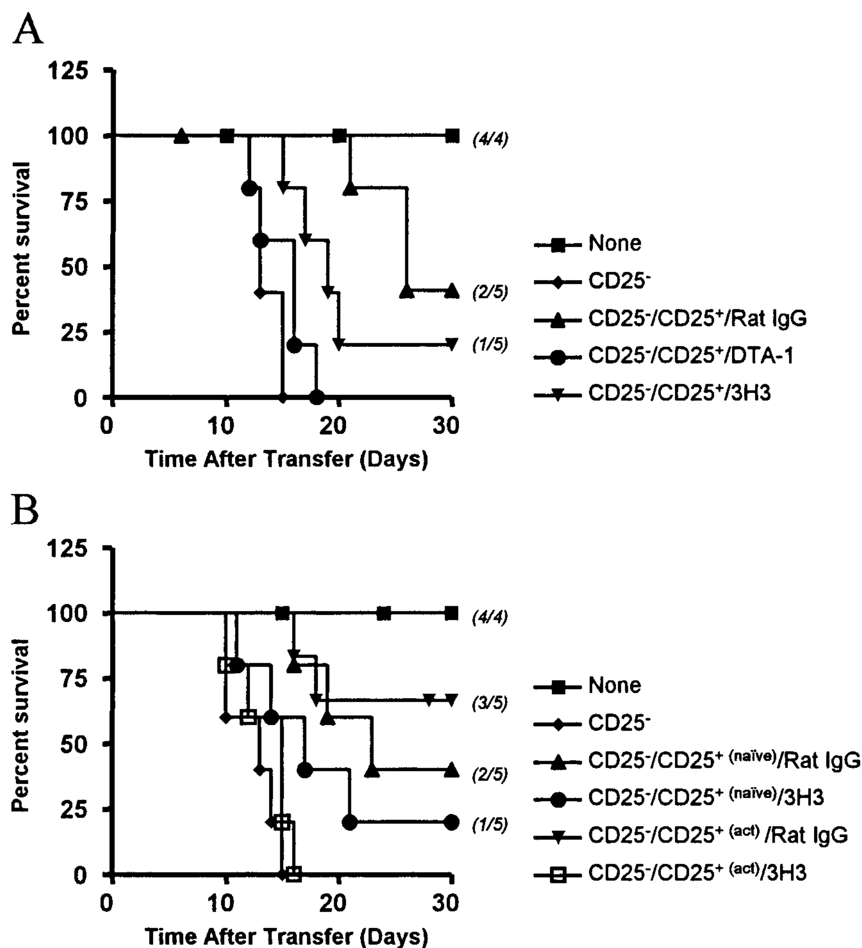

Fig. 5. The effect of signaling through the 4-1BB receptor on the protective function of $CD25^+$ Tr cells during GVHD. (A) Recipient mice (bm12) were sublethally irradiated with 6.0 Gy total body irradiation. Six hours later, 2×10^5 freshly purified $CD25^-$ and/or $CD25^+$ T cells from wild-type C57BL/6 mice were infused into the bm12 recipients by tail-vein injection together with DTA-1 or 3H3 mAb. (B) To exclude any effect of 4-1BB on the $CD25^-$ T cells, in a separate experiment, 3×10^5 freshly purified $CD25^-$ T cells from 4-1BB-deficient C57BL/6 mice were injected into bm12 recipients together with 3×10^5 naïve or activated $CD25^+$ Tr cells. The mice were monitored daily for death caused by GVHD lethality.

possible that 4-1BB must be fully expressed to efficiently antagonize suppression of $CD25^+$ Tr cell function.

We also confirmed that agonistic anti-4-1BB mAb not only neutralizes the function of activated $CD25^+$ Tr cells in vitro and in vivo but also renders 4-1BB-expressing $CD25^-$ T cells more resistant to suppression by $CD25^+$ Tr cells. In this process, the suppressor potential of the $CD25^+$ Tr cells increases, and the $CD25^-$ T cells become resistant to suppression. As a result, it may be that only activated $CD25^+$ Tr cells are able to efficiently suppress the activated $CD25^-$ T cells. In that case, 4-1BB, 4-1BBL, and GITR ligand-expressing cells, which are known to be dendritic cells (unpublished), would be important for initiation, maintenance, and fine-tuning of an optimal immune response.

Although our present experiments suggest that 4-1BB is critical for countering the suppression of $CD25^+$ Tr cell function, it is not clear how this is achieved. Further, although several other molecules are involved in the modulation of $CD25^+$ Tr cell function, including CTLA-4 [23], tumor necrosis factor-related activation-induced cytokine/receptor activator of nuclear factor-κB [24], inducible costimulator (ICOS)/ICOS ligand [25], CD40/CD40 ligand [26], and B7/CD28 [27], it remains to be determined how they regulate the suppression of $CD25^+$ Tr cells. A possible role for CTLA-4 and programmed death-1 ligand (PD-L1) in T cell–T cell regulation has recently been suggested [4, 28], but the surface molecules involved have not been clearly defined [6]. Shimizu et al. [7] report that GITR differs from CD28 or CTLA-4 in the way it attenuates suppression and does not down-regulate CTLA-4 and TGF-β expression. To test whether 4-1BB-mediated signaling of Tr cells affects PD-L1 and CTLA-4 molecules, we performed a flow cytometric analysis: Ligation of 4-1BB had no appreciable effect on these molecules (data not shown). We also tested whether 4-1BB molecules affect the function and development of $CD25^+$ Tr cells and found that 4-1BB-deficient mice showed no deficiency of Tr cells in lymphoid and nonlymphoid organs (data not shown). Moreover, the level of suppression obtained with $CD25^+$ Tr cells from 4-1BB-deficient mice was comparable with that achieved with $CD25^+$ Tr cells from wild-type mice (data not shown).

In spite of intensive study of regulatory T cells, much uncertainty remains regarding their mode of action [29]. It is important for the development of new therapeutic approaches to transplantation, autoimmune diseases, and infections to understand modulation of regulatory T cells at the cellular and molecular levels [30–33]. Our results provide novel insight into how costimulatory molecules on the surface of $CD25^+$ Tr cells modulate the immune response.

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