Ex Vivo Expansion of CD4^+CD25^+FoxP3^+ T Regulatory Cells Based on Synergy between IL-2 and 4-1BB Signaling


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**Ex Vivo Expansion of CD4⁺CD25⁺FoxP3⁺ T Regulatory Cells Based on Synergy between IL-2 and 4-1BB Signaling**


Naturally occurring CD4⁺CD25⁺FoxP3⁺ regulatory T (Treg) cells constitute 5–10% of peripheral T cells and are critical for the establishment and maintenance of immunological tolerance to self Ags (1–4). Deficiencies in the development or function of these cells are associated with severe autoimmunity in humans and various animal models of congenital and acquired diseases (5–7). Treg cells are also involved in immune tolerance to allogeneic Ags in settings of transplantation and graft-vs-host disease (3, 8, 9) and are implicated in immune evasion mechanisms used by tumors and pathogens (10–12). These findings provide support for the central role played by these cells in regulating peripheral mechanisms of immune tolerance.

Treg cells manifest their tolerogenic effects by a complex and overlapping set of mechanisms that operate through direct cell-to-cell contact or through soluble factors. Some of these mechanisms are well established and include suppression of target T effector (Teff) cells by blocking IL-2 expression, which is necessary for Treg cell expansion and survival (13), physically eliminating Treg cells by apoptosis (14), or inhibiting Teff cell function by TGF-β (15) and IL-10 (16). Treg cells have recently been demonstrated to target NK as well as dendritic cells (DC) for functional down-regulation. Although the inhibition of NK function may operate through TGF-β-dependent mechanisms (17, 18), DC function is blocked by the CTLA-4/B7 axis (19). Treg cells, therefore, have the ability not only to modulate adaptive but also innate immune responses, and as such present an important therapeutic target for immunomodulation.

Naturally arising Treg cells are a distinct cell population derived from CD4⁺CD8⁻CD25⁺ thymocytes positively selected by interactions with MHC class II-peptide high-affinity ligands on thymic CD11c⁺ DC activated using thymic stromal lymphopoietin synthesized by Hassalls’s corpuscles (20). Although the nature and extent of the molecular mechanisms involved in the regulation of Treg cell development, function, and homeostasis remain to be fully characterized, signaling through TCR, CD28, and IL-2R is critical to some of these functions (1, 21, 22). Animals lacking molecules involved in CD28 and IL-2R signaling fail to develop Treg cells and are prone to autoimmune diseases (21, 22). In addition to CD28, Treg cells express other receptors, such as OX-40, PD1, CD27, glucocorticoid-induced TNFR (GITR), and CTLA-4, which are also implicated in the costimulation of these cells (23). Although the role of CD28 costimulation in the development and homeostasis of Treg cells is very well established, it is unclear whether signaling via other costimulatory receptors can bypass the need for CD28. A better understanding of the role of various costimulatory signals in the regulation of Treg development and effector function will be critical to the establishment of Treg-based therapeutic approaches.

The importance of signaling through TCR, CD28, and IL-2R for the development and function of Treg cells served as the basis of several recently developed ex vivo expansion protocols (24–27). Almost all of these protocols relied on the use of solid support-bound agonistic Abs to CD28 and CD3 in the presence of high-dose IL-2 (25–27). Although effective ex vivo, these approaches
faces major limitations for their application to in vivo settings due to the constitutive expression of CD28 on all T cells and the observed toxicity of anti-CD28 Ab in clinical trials (28). Expansion protocols that target costimulatory receptors that show preferential or restricted expression to Treg cells may have applicability to in vivo settings. Therefore, we sought to exploit signaling through the 4-1BB receptor as an alternative to CD28 costimulation for the expansion of Treg cells. The rationale for using 4-1BB signaling is based on the restricted constitutive expression of 4-1BB in a subpopulation of Treg cells in the naive host (29, 30). Although Teff cells show inducible expression of 4-1BB in response to antigenic stimulation (31). Therefore, signaling via 4-1BB may allow for specific expansion of Treg over Teff cells.

The costimulatory molecule 4-1BB is a TNFR family member with inducible expression on the surface of activated CD4+ and CD8+ Teff and NK cells (32). The expression of 4-1BB ligand (4-1BBL, CD137L) is also inducible on activated DC, macrophages, and B cells (32). Signaling through 4-1BB/4-1BBL was initially demonstrated to play an important role in the activation, proliferation, survival, and differentiation of CD4+ and CD8+ Teff cells (31, 32). However, a recent set of studies have shown that costimulation via 4-1BB/4-1BBL has diverse, and in some instances, opposing effects on the regulation of the immune system. For example, the use of agonistic Abs to the 4-1BB receptor in experimental models was shown on the one hand to activate the immune system leading to the eradication of established tumors (33) and viral clearance (34), and conversely to down-regulate the immune system for the blockade of autoimmunity and asthma (35, 36). Although the exact nature of the mechanisms responsible for this dichotomy is not fully understood, polarization of the immune response toward Th1 vs Th2 or other regulatory mechanisms, such as induction/expansion of Treg cells as demonstrated in this study, are some possibilities.

We chose to use 4-1BBL, instead of 4-1BB Ab, for stimulation because signals generated by agonistic Abs may qualitatively and quantitatively differ from those delivered by natural ligands (37–39). Inasmuch as native 4-1BBL does not have costimulatory function as a soluble protein (40, 41), we generated the novel form SA-4-1BBL by fusing the extracellular domains of 4-1BBL to a modified form of core streptavidin (SA), thereby allowing for the existence of oligomeric 4-1BBL proteins capable of cross-linking 4-1BB receptors. We demonstrate in this study, to our knowledge for the first time, that Treg cells up-regulated their expression of 4-1BBL in response to IL-2, and that repeated stimulation with soluble SA-4-1BBL and CD3 Ab in the presence of irradiated splenocytes resulted in rapid expansion and survival of Treg cells. Expanded Treg cells expressed membranous TGF-β and maintained up-regulated expression of both CD25 and 4-1BB throughout the 24-day culture period. Expanded Treg cells demonstrated potent suppressive activity ex vivo by inhibiting T cell proliferative responses and in vivo by preventing the rejection of allogeneic islets. Interestingly, Treg cells had no suppressive function in the physical presence of SA-4-1BBL, and this effect was completely reversible upon removal of the chimeric protein. Possible implications for the dual function of signaling via 4-1BB, vis-à-vis Treg expansion and licensing T eff cells resistant to Treg suppression in the regulation of immune responses in vivo, are discussed. The findings reported in this study show that signaling via 4-1BB using the novel soluble form SA-4-1BBL presents an effective means of expanding Treg cells ex vivo with potential therapeutic applications to various immune-based disorders.

Materials and Methods

**Mice**

BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory and maintained under specific pathogen-free conditions at the University of Louisville (Louisville, KY). This study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville, and animals were cared for in accordance to institutional and National Institutes of Health guidelines.

**T cell sorting and phenotyping**

Spleen and lymph node cells were harvested from naive BALB/c mice and processed into single cell suspension, and RBCs were lysed using ACK solution. For cell sorting, cells were stained with CD4-FITC, CD25-PE, and CD8-allophycocyanin Abs. CD4+CD25+ (Teff) and CD4+CD25- (Treg) T cells were sorted using a FACSVantage cell sorter (BD Biosciences). Sorted cells were >95% pure. Naive and expanded Treg cells were phenotyped using CD4-allophycocyanin, CD4-FITC, CD8-PE, CD8-PerCP, 4-1BBL-PE, CD25-PE, CD95-FITC, biotin-CD137, biotin-CD28, biotin-GITR, and biotin-TGF-β Abs as well as FITC-labeled avidin in flow cytometry. Isotype Abs with matched fluorochromes were used as controls. Intracellular FoxP3 staining was performed according to the manufacturer’s protocol (eBioscience).

For receptor expression assays, sorted CD4+CD25+ or CD4+CD25- T cells were cultured in 96-well plates for 2 days alone, in the presence of IL-2 (25 U/ml; Roche) and irradiated splenocytes (1 × 10^5/well), or both. After 2 days of culture, a portion of the cells cultured with IL-2 and irradiated splenocytes were harvested, washed twice with PBS, and cultured for another 2 days without IL-2 or irradiated splenocytes. After culturing, all cells were stained with anti-4-1BB Ab and analyzed by flow cytometry.

**Construction and expression of chimeric 4-1BBL protein**

Total RNA was isolated from mouse splenocytes stimulated with LPS (5 μg/ml) for 2 days and used for RT-PCR to amplify the extracellular domain of 4-1BBL (aa 104–309) with primers (sense) 5′-ATCGTATCCGGACCGAGCTTGAGGACTTAGC-3′ and (antisense) 5′-GGACTCGAGCATAGCAGCTTGAGGACTTAGC-3′. The PCR product was cloned into PCRII.TOPO vector (Invitrogen Life Technologies), and a single clone containing the accurate sequence for 4-1BBL was digested with EcoRI and XhoI and subcloned into pMT/Bip/5′HisA expression vector containing a 6xHis Tag and core SA sequence as previously described (42). Chimeric 4-1BBL (SA-4-1BBL) was expressed using the Drosophila expression system (DES; Invitrogen Life Technologies) (43), purified using Sepharose column, tested for endotoxin by Limulus amebocyte lysate kit (Charles River Breeding Laboratories), and quantified. The protein was subjected to Western Blot analysis under native and denaturing conditions using goat anti-SA Ab (Pierce) as previously described. SA control proteins were produced in our laboratory using S2 cells as described for SA-4-1BBL and previously published (42).

**Receptor binding assay**

Splenocytes were stimulated with 5 μg/ml Con A (Sigma-Aldrich) in total MLR medium (DMEM supplemented with 5% FBS, 2 mM t-glutamine, 100 μM penicillin/streptomycin, 10 mM HEPES, 100 mM MEM-sodium pyruvate (Invitrogen Life Technologies), 1.36 mM/0.027 M folic acid/ammonium, 0.137 M arginine-HCL, and 50 mM 2-ME) for 48 h. Activated or naïve CD4+ T cells were then incubated with SA-4-1BBL (200 ng/10^6 cells) or molar equivalent of SA control protein (76 ng/10^6 cells) on a rotary shaker at 4°C for 30 min. After incubation, cells were washed several times with PBS, stained with CD4-allophycocyanin, CD8-PerCP, 4-1BBL-PE Abs, and anti-SA-FITC, and analyzed by flow cytometry. Naive and SA-incubated cells were used as negative controls.

For blocking assays, 1 × 10^6 activated cells were incubated with an excessive amount (50 μg/10^6 cells) of Ab against 4-1BB (3H5) provided by Dr. R. Mittler (Emory University, Atlanta, GA) (44) for 30 min at 4°C. Cells were then washed several times with PBS and incubated with 200 ng of SA-4-1BBL for an additional 30 min. After incubation, cells were washed with PBS, stained with CD4-allophycocyanin, CD8-PerCP, 4-1BBL-PE, and SA-FITC Abs, and analyzed by flow cytometry. Resterilizing and SA-incubated cells were used as negative controls.

**T cell proliferation assay**

CD4+ T cells (5 × 10^5/well) sorted from naive BALB/c mice were cultured with 0.5 or 5 μg/ml anti-CD3 (BD Biosciences) and irradiated syngeneic splenocytes (1 × 10^5/well) in the presence of varying concentrations of soluble SA-4-1BBL or the molar equivalent of control SA protein. Cells were cultured...
ious ratios in U-bottom 96-well plates in the presence of 0.5
H11003 Ab, irradiated splenocytes (2
dilated splenocytes. Cells were maintained at a concentration of 1
H11003 SA protein. Data are mean
SA-4-1BBL. Sorted CD4
in nanograms per milliliter or equimolar amount of SA. Anti-CD3 Ab at 5
control SA protein (gray-filled histogram) and binding of SA-4-1BBL (open histogram) on CD4
labeled with CFSE (Molecular Probes) (43) and used in suppression assays
The 2
H9262 RT-PCR for FoxP3
Freshly sorted or expanded CD4
for 4 days, pulsed with [3H]thymidine during the last 16 h of the culture, and
harvested on a Tomtec Harvester 96 for quantification of incorporated radioactivity. Results were expressed as mean cpm of triplicate wells.

Ex vivo expansion of CD4+CD25+ T cells
Sorted CD4+CD25+ or CD4+CD25− T cells were cultured in 6-well plates (4–8 × 105 cells/well) and activated with 0.5 μg/ml anti-CD3, 1 μg/ml SA-4-1BBL, and 25 U/ml IL-2 in the presence of 1 × 106/ml irradiated splenocytes. Cells were maintained at a concentration of 1 × 106 cells/ml by changing or adding culture medium containing IL-2 (25 U/ml) every 3–4 days. Cells were reactivated every 10–12 days using the initial activation conditions and maintained with IL-2. Expanded cells were collected at various time points and used for functional and phenotypic analyses. In some assays, sorted CD4+CD25− T cells were cultured in 96-well plates for 3 days with indicated concentrations of anti-CD28 in the presence or absence of 1 μg/ml SA-4-1BBL. Proliferation was assessed by [3H]thymidine incorporation.

Suppression assays
Freshly sorted or expanded CD4+CD25− T cells were cocultured with a fixed number of sorted CD4+CD25− T cells (2.5 × 105 cell/well) at various ratios in U-bottom 96-well plates in the presence of 0.5 μg/ml CD3 Ab, irradiated splenocytes (2 × 105/well), and varying concentrations of soluble SA-4-1BBL or control SA protein as indicated in each experiment. In selected experiments, sorted CD4+CD25− T cells were activated with irradiated APCs and PMA/ionomycin for 2 days. Activated cells were then preincubated with a high concentration (4 μg/ml) of SA-4-1BBL for 45 min on ice, washed extensively, and used for suppression assay. To differentiate between the proliferation of CD4+CD25+ and CD4+CD25− T cells in coculture experiments, CD4+CD25+ or CD4+CD25− T cells were labeled with CFSE (Molecular Probes) (43) and used in suppression assays as described. Proliferation was assessed using flow cytometry.

RT-PCR for FoxP3
The 2 μg of total RNA isolated from freshly sorted CD4+CD25−, CD4+CD25+ T cells, or expanded Treg cells were used in RT-PCR and amplified using primers specific for FoxP3 (forward 5′-CAGCTGCTCA CAGTGCCCCTAG and 5′-CATTTGCAGCAGTGGGTAG) and for HPRT (forward 5′-GAAGTTGTGGATACGGCCACAG and 5′-GAG GGTAGGCTGGCATCTAGGCT at 33 and 27 cycles, respectively.

Allogeneic MLR
Spleen and lymph node cells from naive BALB/c mice (1 × 106/well) were cultured for 5 days in the presence of irradiated (2000 cGy) C57BL/6 splenocytes (1 × 106/well). Expanded Treg cells were added to the cultures at different responder to Treg cell ratios as indicated in each experiment. Cells were pulsed with [3H]thymidine during the last 16 h of culture.

Islet transplantation
Male BALB/c mice were rendered diabetic by a single i.v. injection of 200 mg/kg streptozotocin (Biomol) and diabetes was confirmed by two consecutive blood glucose readings higher than 300 mg/dl. One day before islet transplantation, 5–8 × 106 expanded Treg cells were transferred into each animal by i.v. injection. Donor islets were harvested from fully mismatched C57BL/6 mice and transplantation was performed as previously described (43). Transplanted animals were monitored three times weekly and rejection confirmed by two consecutive blood glucose readings over 300 mg/dl.

Statistics
Proliferation/suppression assays were analyzed using Mann-Whitney U test, whereas survival was analyzed using log-rank test in SPSS software. Values for p < 0.05 were considered significant.

Results
Generation of a chimeric 4-1BBL molecule having potent costimulatory activity as a soluble protein
Effective cross-linking of costimulatory receptors on immune cells is a prerequisite for productive signal transduction and cell activation (45). Under physiological conditions, costimulatory receptors are cross-linked by cell membrane-bound ligands (38, 46). As such, many

FIGURE 1. Construction and characterization of SA-4-1BBL protein. A, The extracellular domain of mouse 4-1BBL was cloned C-terminal to core SA in the pMT/Bip/V5-HisA vector. B, Western blot analysis of purified SA-4-1BBL protein under denaturing (lane 2) and native (lane 3) conditions. C, Binding of SA-4-1BBL to 4-1BB receptor. Naive or ConA activated BALB/c splenocytes were incubated with SA-4-1BBL (200 ng/ml) cells or equimolar amount of control SA protein (gray-filled histogram) and binding of SA-4-1BBL (open histogram) on CD4+ and CD8+ T cells was assessed by flow cytometry using anti-4-1BB. Some activated cells were incubated with anti-4-1BB to block the receptor before incubation with SA-4-1BBL. D, Stimulation of T cells with SA-4-1BBL. Sorted CD4+ T cells were stimulated using anti-CD3 Ab (0.5 μg/ml) and irradiated syngeneic splenocytes in the presence of soluble SA-4-1BBL in nanograms per milliliter or equimolar amount of SA. Anti-CD3 Ab at 5 μg/ml was used as positive control. *p < 0.05 compared with each other and control SA protein. Data are mean ± SD for C and D and are representative of three independent experiments with similar results.
costimulatory ligands have limited activity as soluble proteins due to inefficient signal transduction (40, 41, 47). Although agonistic Abs against costimulatory receptors are effective in delivering immunological signals, there is evidence suggesting that signals delivered by Abs may quantitatively and qualitatively differ from signals transduced by natural ligands (37–39). In an attempt to use signaling via 4-1BB as an alternative to CD28-mediated costimulation for the expansion of Treg cells, we constructed a novel form of 4-1BBL chimeric protein. Costimulation with SA-4-1BBL generated a vigorous and statistically significant (p < 0.05) proliferative response in CD4+ T cells that was dose-dependent and SA-4-1BBL-specific because presence of SA control protein did not result in increased proliferation (Fig. 1D). Taken together, these results demonstrate that SA-4-1BBL exists as tetramers and oligomers, binds to its receptor 4-1BB, and transduces a potent costimulatory signal in T cells.

**SA-4-1BBL renders T cells refractive to Treg suppression while driving the proliferation of both Treg and T cells**

It was recently reported that a subpopulation of naturally occurring CD4+CD25+FoxP3+ Treg cells constitutively express 4-1BB receptor (29, 30). The role of 4-1BB-mediated signaling in the function and regulation of Treg cells has been subject of two recent studies with opposing findings (29, 51). Whereas one study presented evidence for the inhibition of Treg cell suppressive function by 4-1BB signaling (29), the other demonstrated that 4-1BB could induce the proliferation of Treg cells without a major effect on their suppressive function (51). To clarify this discrepancy, we investigated the role of 4-1BB signaling in Treg cell function using...
SA-4-1BBL protein. Sorted CD4^+CD25^+ Treg cells from naive BALB/c mice markedly inhibited the proliferative response of CD4^+CD25^- Teff cells induced by CD3 stimulation (Fig. 2A).

Proliferation was restored when cultures were supplemented with 1μg/ml chimeric SA-4-1BBL, but not SA control protein used at an equimolar concentration.

To test whether the proliferative response caused by SA-4-1BBL was due to the inhibition of the suppressive function of Treg cells allowing for the restoration of CD4^+CD25^- Teff cell proliferation, or the induction of Treg cell proliferation, Teff cells were labeled with CFSE and used in coculture experiments. Costimulation with SA-4-1BBL increased the proliferation of Teff cells from 56% in control culture to 75% (Fig. 2B, top). Addition of Treg cells to Teff cell cultures markedly reduced the proliferation of Teff cells (30%). However, the proliferative response was partially restored by adding SA-4-1BBL proteins to the culture (62%). Inability to fully restore the proliferation of Teff cells in response to SA-4-1BBL costimulation in coculture experiments may be due to competition between Treg and Teff cells for SA-4-1BBL protein and/or other factors, such as IL-2 (52). In a parallel set of experiments, CFSE-labeled Treg cells were used in coculture experiments to test whether Treg cells could also proliferate in response to SA-4-1BBL stimulation. Significant Treg cell proliferation was observed in response to SA-4-1BBL in cultures containing only Treg cells (44% vs 17% for the control) as well as cultures containing Teff plus Treg cells (58% vs 28% for controls) (Fig. 2B, bottom).

To further elucidate whether SA-4-1BBL directly inhibits the suppressor function of Treg cells or renders Teff cells refractive to the inhibitory function of Treg cells or both, we performed suppression studies using either Teff or Treg cells pretreated with SA-4-1BBL. Treg and Teff cells were first activated to up-regulate the 4-1BB receptor and then incubated with a high concentration (4μg/ml) of SA-4-1BBL for 45 min on ice. After several washes to remove the free ligand, these cells were used in suppression assays. Treg cells preincubated with SA-4-1BBL suppressed the proliferation of Teff cells similar to that observed with Treg cells without preincubation (Fig. 2C). The addition of soluble SA-4-1BBL to the culture relieved the suppressive effect of Treg cells, demonstrating that 4-1BBL signaling to Teff cells license these cells to overcome suppression by Treg cells. This finding was further corroborated by the demonstration that Teff cells preincubated with SA-4-1BBL were resistant to the suppressive effect of Treg cells. The higher levels of proliferation seen in culture where SA-4-1BBL preincubated Teff cells used may be due to high levels (4μg/ml) of SA-4-1BBL used for preincubation.

4-1BB and IL-2 have synergistic effects on the proliferation of CD4^+CD25^+ Treg cells

Signals transduced through TCR (Signal 1), CD28 (Signal 2), and IL-2R (Signal 3) have been shown to play important roles in the development, homeostasis, and function of Treg cells (53). Given
our findings that signaling via 4-1BB also results in the proliferation of Treg cells, a systematic study was performed to assess the relative role of each signal to ex vivo Treg cells proliferation. Although addition of either IL-2 (25 U/ml) or SA-4-1BBL (1 µg/ml) to cocultures of sorted naive CD4⁺/CD25⁻ T cells and irradiated APCs in the presence of CD3 stimulation was sufficient to break anergy and induce Treg cell proliferation, addition of both SA-4-1BBL and IL-2 generated a greater proliferative response than that elicited by either agent alone (Fig. 3A).

To further assess the relative contribution of Signal 1, 2, and 3 on Treg cell proliferation, stimulation assays were performed in the absence of APCs. As shown in Figure 3B, provision of Signal 1 (CD3) or 2 (SA-4-1BBL) alone was insufficient to induce Treg cell proliferation whereas Signal 3 in the form of IL-2 or combination of Signal 1 and 2 resulted in minimal expansion. Signal 1 or 2 in combination with IL-2 (Signal 3) had significant proliferative effect. The effect is not limited to the amount of reagents used because similar synergistic effects on Treg cell proliferation were observed with combinations of different concentrations of 4-1BBL, IL-2, or anti-CD3 Ab (data not shown). The most dramatic effect on Treg cell proliferation was observed when all three signals were used together. These results demonstrate that there is a hierarchy in the effect of these signals on Treg cell proliferation: Signal 1 and 2 having no effect, Signal 3 via IL-2 being essential, Signal 3 with either Signal 1 or 2 being effective, and combination of the three signals resulting in the most pronounced response.

We next tested whether the costimulatory effect of 4-1BB on Treg cell proliferation can be further enhanced by CD28 signaling used by various studies for Treg expansion ex vivo (25–27). When different concentrations of anti-CD28 Ab (0.125–1 µg/ml) were used with a fixed amount of SA-4-1BBL (1 µg/ml), there was a synergistic effect on Treg cell proliferation (Fig. 3C).

**IL-2 up-regulates the expression of 4-1BB receptor on CD4⁺CD25⁻ Treg cells**

The observed synergy between IL-2 and 4-1BBL may be due to a positive feedback exerted by IL-2 on the expression of 4-1BB receptor. To test this hypothesis, sorted Treg and Teff cells were cultured in the presence or absence of IL-2 and/or irradiated APCs for 2 days. Cells were then harvested and analyzed by flow cytometry for the expression of 4-1BB (Fig. 3D). Consistent with published studies (29, 51), only 22% of freshly sorted Treg cells expressed 4-1BB, whereas none of the Teff cells scored positive for this receptor. The expression of 4-1BB was down-regulated to background levels (2%) when cells were cultured alone for 2 days. Culturing Treg cells in the presence of irradiated APCs had a minimal effect on the maintenance of 4-1BB expression on Treg cells (8%). In marked contrast, addition of IL-2 to Treg cell cultures resulted in not only the maintenance but also the moderate up-regulation (29% vs 22% for fresh cells) of 4-1BB receptor. Addition of irradiated APCs to cultures supplemented with IL-2 further up-regulated (53%) the expression of 4-1BB on Treg cells.

To provide further evidence that 4-1BB expression on Treg cells is regulated by IL-2, cells maintained in the presence of APCs plus IL-2 were extensively washed then cultured in the absence of IL-2 for 2 days. Removal of IL-2 resulted in the down-regulation of 4-1BB expression from 53% to background levels (Fig. 3D, top). Regulation of 4-1BB expression by IL-2 was specific for Treg cells as treatment of Teff cells using similar regimens resulted in minimal changes in 4-1BB expression (Fig. 3D, bottom). To our knowledge, these results are the first to demonstrate that IL-2 maintains or up-regulates the expression of 4-1BB on Treg cells and provide a mechanistic basis for the observed synergy between IL-2 and 4-1BBL on the proliferation of Treg cells ex vivo.

**Expansion of CD4⁺CD25⁺ Treg cells using SA-4-1BBL**

Effective ex vivo expansion of Treg cells has the potential to become an important therapeutic intervention for the treatment of various immune disorders. Having demonstrated that Treg cells respond to stimulation by CD3, 4-1BBL, and IL-2 in short-term cultures, we assessed the potential of this approach for their long-term expansion ex vivo. CD4⁺CD25⁺ T cells were sorted from the spleen and lymph nodes of naive BALB/c mice (Fig. 4A) and subjected to an initial round of activation with 0.5 µg/ml soluble CD3 Ab, 25 U/ml IL-2, and irradiated splenocytes, in the presence or absence of anti-CD28 and SA-4-1BBL. When different concentrations of anti-CD28 Ab (0.125 to 1 µg/ml) were used with a fixed amount of SA-4-1BBL (1 µg/ml) to cocultures of sorted naive CD4⁺CD25⁻ T cells and irradiated APCs in the presence of CD3 stimulation was sufficient to break anergy and induce Treg cell proliferation, addition of both SA-4-1BBL and IL-2 generated a greater proliferative response than that elicited by either agent alone (Fig. 3A).

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**FIGURE 4.** Ex vivo expansion of Treg cells using SA-4-1BBL. CD4⁺CD25⁺ Treg cells were sorted from the spleen and lymph nodes of naive BALB/c mice and cultured in the presence of 0.5 µg/ml soluble anti-CD3 Ab, 1 × 10⁶ irradiated syngeneic splenocytes, and 25 U/ml IL-2 with or without 1 µg/ml soluble SA-4-1BBL in 6-well plates. Every 3–4 days, cells were split with fresh medium supplemented with IL-2 and plated at a concentration of 1 × 10⁵ cells/ml. A, Flow cytometry analysis of CD4⁺CD25⁺ populations before sorting and after expansion with or without SA-4-1BBL. B, Fold expansion of naive Treg cells ex vivo for cells cultured with (□) or without (■) SA-4-1BBL. Arrows indicate secondary and tertiary activations. Results from the least three independent expansions are depicted.
as an alternative to solid support-bound immobilized anti-CD28 Ab and expansion efficiency could be enhanced by combination of these two costimulatory agonists.

**Phenotype of expanded CD4⁺CD25⁺ Treg cells**

Expanded Treg cells were characterized to assess their expression of various "classical" Treg cell surface markers using flow cytometry. Expanded Treg cells expressed CD25, CD28, GITR, Fas, CD62 ligand, and cell surface TGF-β. Importantly, all of these markers were considerably up-regulated in SA-4-1BBL expanded Treg cells as compared with those expanded without SA-4-1BBL (Figs. 4A and 5A). Expanded Treg cells also expressed the signature transcriptional factor FoxP3, as assessed by RT-PCR (Fig. 5B) as well as intracellular staining (Fig. 5C), and showed increased expression as compared with Treg cells without SA-4-1BBL stimulation. Importantly, all expanded Treg cells expressed high levels of 4-1BB, which was maintained throughout the 24-day culture period.

**Expanded CD4⁺CD25⁺ Treg cells are suppressive**

To test whether expanded Treg cells maintained their regulatory function, we performed classical CD3 stimulation-based suppression assay. Similar to naïve Treg cells, expanded cells remained anergic in response to CD3 stimulation, were capable of suppressing the polyclonal proliferation of CD4⁺ Teff cells, and this suppressive function could be inhibited by addition of 4-1BBL to the assay (Fig. 6A).

Further evidence for the suppressive function of expanded Treg cells was provided using MLR as alloantigen-driven proliferative responses. Spleen and peripheral lymph node cells from naïve BALB/c mice were used as responders to irradiated C57BL/6 splenocytes in the presence of various amounts of Treg cells. There was a potent inhibition of alloantigen-driven Teff cell proliferation by expanded Treg cells and this suppression was significant (p < 0.05) even at a 10:1 responder to Treg cell ratio (Fig. 6B). Taken together, these data demonstrate that expanded Treg cells are endowed with the classical suppressive function ascribed to naturally occurring Treg cells.

**Expanded Treg cells prevent allogeneic islet graft rejection**

To test the function of expanded Treg cells in a more physiological setting and determine whether they possess immunomodulatory function in vivo, 5–8 × 10⁶ SA-4-1BBL expanded Treg cells were transferred into chemically induced diabetic BALB/c mice one day before transplantation with fully mismatched C57BL/6 allogeneic islets. Although all control animals acutely rejected their grafts with a mean survival time of 14.3 ± 1.8 days (Fig. 7), animals...
**FIGURE 6.** Expanded Treg cells suppress polyclonal and Ag-specific proliferation of Teff cells ex vivo. A, Polyclonal (anti-CD3 Ab) suppression assays were performed using cells sorted from the spleen and peripheral lymph nodes of naive BALB/c mice and cultured alone or at 1:1 ratio for 3 days as described in Fig. 2A. Cultures were supplemented with irradiated splenocytes, anti-CD3 Ab (0.5 μg/ml) in the presence or absence of 1 μg/ml SA-4-1BBL and expanded Treg cells (Exp-Treg). B, Alloantigen suppression assays. Spleen and peripheral lymph node cells from naive BALB/c mice (stimulators) were cocultured with irradiated splenocytes from naive C57BL/6 mice (stimulators) and expanded Treg cells (Exp-Treg) at the indicated ratio for 5 days. *p < 0.05 compared with each other and controls. Data are mean ± SD and are representative of four independent experiments for A and two independent experiments for B with similar results. Treg cells used in these experiments were in their IL-2 maintenance phase following primary, secondary, or tertiary activation as described in Fig. 4B.

Receiving Treg cells showed increased survival with four of six animals not rejecting their grafts within the 86-day observation period (mean survival time = 68.7 ± 10.0 days, p < 0.05).

**FIGURE 7.** Expanded Treg cells prevent the rejection of allogeneic islets. Naïve BALB/c mice rendered diabetic by a single injection of streptozotocin were adoptively transferred with 5–8 × 10⁶ expanded Treg cells one day before transplantation with allogeneic C57BL/6 islets (●). Control animals did not receive Treg cells but were transplanted with allogeneic islets (○). Blood glucose was monitored three times weekly and rejection was confirmed by two consecutive blood glucose readings above 300 mg/dL. Survival was compared using Kaplan-Meier log-rank test (p < 0.05). Treg cells used in these experiments were in their IL-2 maintenance phase following primary, secondary, or tertiary activation as described in Fig. 4B.

**Discussion**

Naturally occurring CD4⁺CD25⁺FoxP3⁺ Treg cells have recently emerged as an important therapeutic target for the prevention or treatment of various immune-based disorders (4). The requirement of TCR, CD28, and IL-2R signaling for development and homeostasis of Treg cells (13, 54) served as the basis of a limited number of ex vivo expansion protocols. This therapeutic potential, however, has been curtailed by the lack of reliable and effective ex vivo or in vivo expansion protocols (25–27, 55). In this study, we sought to develop an alternative approach to CD28-based costimulation for the expansion of Treg cells ex vivo by targeting another costimulatory pathway, 4-1BB/4-1BBL. The rationale for costimulation via 4-1BB stems from studies demonstrating that a subpopulation of Treg cells constitutively express 4-1BB (29, 51) and signaling through 4-1BB receptor is critical for the activation, proliferation, and long-term survival of Teff cells (32). Insofar as Treg and Teff cells share some signaling pathways for activation, proliferation, and possibly maintenance, we reasoned that costimulation via 4-1BB may affect Treg cells in a similar fashion as Teff cells, and as such may provide an important means to effectively expand Treg cells ex vivo.

Many costimulatory molecules, including 4-1BBL, do not generate productive signals in their native soluble form upon binding to their receptors (40, 41, 47). However, the novel chimeric form of 4-1BBL generated in this study had potent activity on Teff as well as Treg cells when used as a soluble protein. Importantly, the chimeric protein manifested dual function on Treg cells: 1) synergized with CD3, CD28, and IL-2 stimulation to promote Treg cell expansion, and 2) rendered Teff cells refractive to suppression by naive as well as activated Treg cells when physically present in the culture medium. These findings are in conflict with a previous study demonstrating that an agonistic Ab against 4-1BB did not effect suppression in Treg/Teff coculture experiments (56). However, our findings are consistent with a more recent study reporting the direct effect of 4-1BB signaling on the suppressive function of Treg cells as well as rendering Teff cells refractive to suppression (29). Both of these effects were only observed with activated Treg and Teff cells, but not naive cells. In marked contrast, we demonstrated that 4-1BB signaling rendered Teff cells refractive to suppression by Treg cells without a significant effect on the suppressive function of Treg cells and this effect was independent of the activation status of the cells. Furthermore, this previous study reported lack of proliferation by 4-1BB signaling in Treg cells, whereas we demonstrated such a mitogenic effect. Although the source of these discrepancies is unknown, the use of an agonistic Ab to 4-1BB in this previous study vs SA-4-1BBL in our study and experimental models may provide an explanation. Consistent with this notion is another study reporting mitogenic activity of 4-1BB signaling on Treg cells using a recombinant form of 4-1BBL (51).

Unlike 4-1BB signaling that renders Teff cells refractive to the suppressive function of Treg cells, signaling through two other members of the TNF family of receptors, GITR and OX40, has been shown to act on Treg cells for the abrogation of their suppressive function (29, 56, 57). Stimulation with GITR not only suppresses the function of Treg cells (56), but also induces their proliferation (57). In contrast to GITR and 4-1BBL, signaling via OX40 has an inhibitory, but not mitogenic, effect on Treg cells. Moreover, signaling through OX40 is only effective in blocking the suppressive function of naïve, but not activated Treg cells, whereas signaling via GITR (57) is effective in inhibiting the suppressive function of both naïve and activated Treg cells. Taken together, our data and previously published studies suggest that receptors with constitutive expression on Treg cells, but inducible...
expression on Teff cells, may serve as important regulatory switches that assure productive immune responses against infections without extensive normal tissue damage under physiological conditions.

We demonstrated a hierarchy in Treg cell requirement for signaling via CD3, 4-1BB, and IL-2. Although provision of all of these three signals was required for maximal proliferation of Treg cells, signaling via IL-2R and 4-1BB showed synergy and were sufficient to drive Treg proliferation without CD3 stimulation. The synergy between IL-2R and 4-1BB appears to operate via a positive feedback mechanism in which IL-2 maintains or up-regulates the expression of 4-1BB on Treg cells. Furthermore, Treg cells costimulated with SA-4-1BBL up-regulated their expression of CD25 as compared with cells stimulated without SA-4-1BBL, suggesting a positive feedback loop between these two signals. The up-regulation of 4-1BB on Treg cells may be mediated by IL-2 induced STAT5 and c-Jun as shown for activated CD8+ T cells (58, 59). Our data suggest that the expression of 4-1BB on Treg cells is inducible, rather than constitutive, and may be maintained in vivo by endogenous IL-2 or other factors with physiological consequences.

Although the exact function of 4-1BB signaling in the regulation of Treg cells is unknown, it is tempting to speculate that this receptor system may serve as an alternate negative feedback loop to activation-induced cell death for the control of Treg cells following infections and IL-2 synthesized by Teff cells may play a critical role in this regulation. Engagement of 4-1BB on activated APCs with 4-1BB on the surface of activated T eff and Treg cells may drive the proliferation of both cell populations through its mitogenic activity while rendering Teff cells refractive to Treg suppression, thereby allowing expanded T eff cells to cope with infection. IL-2 expressed by Treg cells may further synergize with 4-1BB signaling to augment this response. This notion is consistent with a recent study demonstrating that IL-2 and IL-4 inhibited 4-1BB signaling to augment this response. This notion is consistent with a study reporting that 4-1BB mediates inhibition of Treg cell function. This notion is consistent with a study demonstrating that IL-2 and IL-4 inhibited the suppressive function of Treg cells and induced the proliferation of both Treg and Teff cells in coculture experiments (60). Once infection is cleared, Teff cells may cease to produce IL-2, which in turn will lead to the down-regulation of 4-1BB receptor on Treg and Teff cells. The lack of 4-1BB receptor and IL-2 will render Teff cells refractive to the suppressive function of Treg cells, thereby controlling the inflammatory process and limiting tissue damage.

Periodic stimulation with soluble SA-4-1BBL, anti-CD3 Ab, and a low dose of exogenous IL-2 resulted in 110-fold expansion of Treg cells within 3 wk. Expanded Treg cells were all CD25bright and a low dose of exogenous IL-2 resulted in 110-fold expansion thereby controlling the inflammatory process and limiting tissue damage. These expanded Treg cells were refractive to the suppressive function of Treg cells, its mitogenic activity while rendering Teff cells refractive to Treg suppression, thereby allowing expanded T eff cells to cope with infection. IL-2 expressed by Teff cells may further synergize with 4-1BB signaling to augment this response. This notion is consistent with a study demonstrating that IL-2 and IL-4 inhibited 4-1BB signaling to augment this response. This notion is consistent with a study reporting that 4-1BB mediates inhibition of Treg cell function. This notion is consistent with a study demonstrating that IL-2 and IL-4 inhibited the suppressive function of Treg cells and induced the proliferation of both Treg and Teff cells in coculture experiments (60). Once infection is cleared, Teff cells may cease to produce IL-2, which in turn will lead to the down-regulation of 4-1BB receptor on Treg and Teff cells. The lack of 4-1BB receptor and IL-2 will render Teff cells refractive to the suppressive function of Treg cells, thereby controlling the inflammatory process and limiting tissue damage.

In conclusion, the data presented demonstrate that costimulation via 4-1BB receptor using a soluble form of chimeric 4-1BBL represents a new and alternative approach to CD28-based stimulation (25–27, 55) for the effective expansion of Treg cells ex vivo. Combination of SA-4-1BBL and anti-CD28 Ab had a synergistic effect on Treg cell expansion in short-term cultures, suggesting that synergy between these two costimulatory pathways may result in better expansion yield than either pathway alone. However, the advantage of targeting only 4-1BB over CD28-based expansion lies in the constitutive expression of 4-1BB on a subset of Treg cells and its up-regulation by IL-2, which may provide a positive feedback loop to specifically and effectively expand Treg cells over Teff cells in vivo using SA-4-1BBL and IL-2. Sustained expression of 4-1BB on activated Treg cells may further improve the efficacy of this approach. Efforts are underway to establish such protocols in our laboratory.

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Disclosures

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