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⁺ T regulatory (Treg) cells require three distinct signals transduced via TCR, CD28, and IL-2R for their development and maintenance. These requirements served as the basis for several recently developed ex vivo expansion protocols that relied on the use of solid support-bound Abs to CD3 and CD28 in the presence of high dose IL-2. We report in this study that Treg cells up-regulate the expression of inducible costimulatory receptor 4-1BB in response to IL-2, and stimulation using this receptor via a novel form of 4-1BB ligand (4-1BBL) fused to a modified form of core streptavidin (SA-4-1BBL) was effective in expanding these cells up to 110-fold within 3 wk. Expanded cells up-regulated CD25, 4-1BB, and membranous TGF-β, suppressed T cell proliferation, and prevented the rejection of allogeneic islets upon adoptive transfer into graft recipients. Importantly, SA-4-1BBL rendered CD4⁺CD25⁻ T effector cells refractive to suppression by Treg cells. This dual function of signaling via 4-1BB, vis-à-vis Treg cell expansion and licensing T effector cells resistant to Treg cell suppression, as well as the up-regulation of 4-1BB by IL-2 may serve as important regulatory mechanisms for immune homeostasis following antigenic challenge. Stimulation using a soluble form of SA-4-1BBL represents a novel approach to expand Treg cells with potential therapeutic applications in autoimmune and transplantation.


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3 Abbreviations used in this paper: Treg, regulatory T; Teff, effector T; DC, dendritic cell; 4-1BBL, 4-1BB ligand; SA, streptavidin; GITR, glucocorticoid-induced TNFR.

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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, this expression is down-regulated shortly after activation (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 4-1BBL-PE Abs 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-a-vis Treg expansion and licensing Teff 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 naïve 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. naïve 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 × 10⁵/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 104–359) with primers (sense) 5’-ATCGAATTCCT CACCGAGCTCTGCGCAAGCG-3’ and (antisense) 5’-GGACTCTGAG CATACGGAGGTTAGAAGCG-3’. The PCR product was cloned into PCRII.1TOPO vector (Invitrogen Life Technologies), and a single clone containing the accurate sequence for 4-1BBL was digested with EcoRI and XhoI and subcloned into pMTB/BiP/V5-HisA 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 l-glutamine, 100 U/ml penicillin/streptomycin, 10 mM HEPES, 100 mM MEM-sodium pyruvate (Invitrogen Life Technologies), 1.36 mM/0.027 M folic acid/asparagine, 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/10⁶ cells) or molar equivalent of SA control protein (76 ng/10⁶ 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. Naïve and SA-incubated cells were used as negative controls.

For blocking assays, 1 × 10⁶ activated cells were incubated with an excessive amount (50 μg/10⁶ cells) of Ab against 4-1BB (3H3) provided by Dr. R. Mitter (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. Resting and SA-incubated cells were used as negative controls.

#### T cell proliferation assay

CD4+ T cells (5 × 10⁵/well) sorted from naïve BALB/c mice were cultured with 0.5 or 5 μg/ml anti-CD3 (BD Biosciences) and irradiated syngeneic splenocytes (1 × 10⁵/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
\(/\text{H}9262\) [\(3\text{H}\)]thymidine incorporation. Ab, irradiated splenocytes (2
\(/\text{H}11003\) /\(/\text{H}11003\) /\(/\text{H}11006\) 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
CD4
/\(/\text{H}9262\) Freshly sorted or expanded CD4
activation conditions and maintained with IL-2. Expanded cells were col-
every 3–4 days. Cells were reactivated every 10–12 days using the initial
Ex vivo expansion of CD4
CD25
T cells
Sorted CD4
CD25
T cells were cultured in 6-well plates (4–8 \times 10^{5} \text{cells/well}) and activated with 0.5 \(\mu\)g/ml anti-CD3, 1
\(/\text{H}11001\) g/ml SA-4-1BBL and 25 U/ml IL-2 in the presence of 1 \(\times 10^{6}\) 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
un the different responder to Treg cell ratios as indicated in each experiment. Cells were pulsed with \([\text{H}]\)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'^ T cells or CD4'^CD25'^ T cells were cultured in 6-well plates (4–8 \times 10^{5} \text{cells/well}) and activated with 0.5 \(\mu\)g/ml anti-CD3, 1
\(/\text{H}11001\) g/ml SA-4-1BBL, and 25 U/ml IL-2 in the presence of 1 \(\times 10^{6}\) 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.

**Suppression assays**

Freshly sorted or expanded CD4'^CD25'^ T cells were cocultured with a fixed number of sorted CD4'^CD25'^ T cells (2.5 \times 10^{5} \text{cells/well}) at various ratios in U-bottom 96-well plates in the presence of 0.5 \(\mu\)g/ml CD3 Ab, irradiated splenocytes (2 \times 10^{5} \text{cells/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 \(\mu\)g/ml) of SA-4-1BBL for 45

**RT-PCR for FoxP3**

The 2 \(\mu\)g 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'-CAGCTGCCTA CAGTGCCCCCTAG and 5'-CATTTGCCAGCAGTGGGTAG) and for HPRT (forward 5'-GAAGGTGTGATACAGCCCGAC and 5'-GAG GGTAGGCTGGAATGCAGTACG at 33 and 27 cycles, respectively.

**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 \times 10^{5} 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
For each histogram, the percentage of dividing cells is shown for the suppression assay as described above. BALB/c mice and cultured alone or at 1:1 ratio for 3 days. Cultures were stimulated with irradiated spleenocytes, anti-CD3 Ab (0.5 μg/ml), with or without of SA-4-1BBL (1 μg/ml) or equimolar control SA protein. B, CFSE assay to assess the proliferation of Treg and Teff cells. Teff (top panels) and Treg (bottom panels) cells were labeled with CFSE and used in suppression assay as described above. Percentage of dividing cells is shown for each histogram. C, Activated Treg and Teff cells were preincubated with SA-4-1BBL (4 μg/ml) for 45 min on ice, washed extensively, and then used in coculture suppression assays as described in A. *, p < 0.05 compared with each other. Data are mean ± SD and are representative of three independent experiments with similar results.

The extracellular portion of mouse 4-1BBL (aa 104–309) required for binding and signal transduction through 4-1BB was cloned C-terminal to SA to ensure proper folding of the protein because 4-1BBL is a type II protein (Fig. 1A) (49). The chimeric gene was subcloned in frame with the secretion signal of Drosophila in a metal-inducible expression vector and the protein was expressed in S2 insect cells. SA-4-1BBL proteins existed as tetramers and oligomers, and bind biotin, and 2) our observations that several SA chimeric molecules generated in our laboratory have potent immunological activities as compared with their native counterparts (42, 43, 48).

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FIGURE 2. Signaling via 4-1BB receptor inhibits the suppressive function of Treg cells and drives the proliferation of Treg and Teff cells. A, CD4+CD25+ Teff and CD4+CD25+ Treg cells were sorted from the spleen and peripheral lymph nodes of naive BALB/c mice and cultured alone or at 1:1 ratio for 3 days. Cultures were supplemented with irradiated spleenocytes, anti-CD3 Ab (0.5 μg/ml), with or without of SA-4-1BBL (1 μg/ml) or equimolar control SA protein. B, CFSE assay to assess the proliferation of Treg and Teff cells. Teff (top panels) and Treg (bottom panels) cells were labeled with CFSE and used in suppression assay as described above. Percentage of dividing cells is shown for each histogram. C, Activated Treg and Teff cells were preincubated with SA-4-1BBL (4 μg/ml) for 45 min on ice, washed extensively, and then used in coculture suppression assays as described in A. *, p < 0.05 compared with each other. Data are mean ± SD and are representative of three independent experiments with similar results.

binding was specific for 4-1BB because blocking of the receptor with an Ab against 4-1BB before incubation with SA-4-1BBL prevented binding (Fig. 1C, right).

To test the function of SA-4-1BBL, CD4+ T cells sorted from BALB/c mice were stimulated for 4 days with a suboptimal concentration of CD3 Ab in the presence of varying concentrations of soluble SA-4-1BBL or equimolar concentrations of SA control 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 Teff cells refractive to Treg suppression while driving the proliferation of both Treg and Teff 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 4-1BBL 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

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7298 EXPANSION OF Treg CELLS USING 4-1BBL

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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 1 μg/ml chimeric SA-4-1BBL, but not SA control protein used at an equimolar concentration.

To test whether the proliferative effect 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 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 (4 μg/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 (4 μg/ml) of SA-4-1BBL used for preincubation.

4-1BBL 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.
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 naive 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 naive 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, $5 - 8 \times 10^6$ 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

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**FIGURE 5.** Phenotypic characterization of expanded Treg cells. A. Expression of cell surface markers important for Treg cell function were analyzed on cells expanded with or without SA-4-1BBL. Percentage of positive cells and mean fluorescence intensity (MFI) values are shown on the corner of histograms for each marker. Isotype controls are depicted (filled histograms). B. RT-PCR showing the expression of FoxP3 by expanded Treg cells. F, FoxP3; H, HPRT; M, marker; Teff, CD4+CD25-; Treg, CD4+CD25+; Exp-Treg, expanded Treg. C. Intracellular staining showing the level of intracellular FoxP3 expression by Treg cells expanded with (dashed line histogram) or without (solid line histogram) SA-4-1BBL. Isotype control for FoxP3 was used as control (filled line histogram). Mean fluorescence intensity values shown for FoxP3. Data are representative of three independent experiments 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. Naive 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 (C). Control animals did not receive Treg cells but were transplanted with allogeneic islets (○). Blood glucose was monitored three 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 suppressed the 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 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).
expression on Teff cells, may serve as important regulatory switches that assure productive immune responses against infections without excessive normal tissue damage under physiological conditions.

We demonstrated a hierarchy in Treg cell requirement for signaling via CD3, 4-1BB, and IL-2R. 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 Teff cells following infections and IL-2 synthesize 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 Teff 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 Teff 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 recent study demonstrating that IL-2 and IL-4 inhibited 4-1BB signaling to augment this response. This notion is consistent with a recent 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 expressed higher levels of CD28, 4-1BB, GITR, Fas, CD62 ligand, membranous TGF-β, and intracellular Foxp3 as compared with cells expanded without SA-4-1BBL. Membranous TGF-β may play a role in 4-1BBL-mediated inhibition of Treg cell function. This notion is consistent with a study reporting that 4-1BB signaling abolishes TGF-β-mediated suppression of human CD8+ T cell differentiation by modulating the TGF-β-mediated phosphorylation of Smad2 (61). Importantly, expanded Treg cells suppressed Teff cell proliferation in response to alloantigens or CD3 stimulation and prevented the rejection of allogeneic islets in 67% of graft recipients in adoptive transfer experiments. The rejection of 33% of the grafts may be due to the inefficiency of the cell dose used for adoptive transfer or other unknown factors. These observations are consistent with a recent study demonstrating that the transfer of Treg cells, expanded from total CD4+ cells using rapamycin, Abs to CD3 and CD28, and exogenous IL-2, into diabetic graft recipients resulted in the survival of 80% of islet allografts (24).

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 subpopulation 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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