

The 4-1BB Costimulation Augments the Proliferation of CD4⁺CD25⁺ Regulatory T Cells¹

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The thymus-derived CD4⁺CD25⁺ T cells belong to a subset of regulatory T cells potentially capable of suppressing the proliferation of pathogenic effector T cells. Intriguingly, these suppressor cells are themselves anergic, proliferating poorly to mitogenic stimulation in culture. In this study, we find that the 4-1BB costimulator receptor, best known for promoting the proliferation and survival of CD8⁺ T cells, also induces the proliferation of the CD4⁺CD25⁺ regulatory T cells both in culture and in vivo. The proliferating CD4⁺CD25⁺ T cells produce no detectable IL-2, suggesting that 4-1BB costimulation of these cells does not involve IL-2 production. The 4-1BB-expanded CD4⁺CD25⁺ T cells are functional, as they remain suppressive to other T cells in coculture. These results support the notion that the peripheral expansion of the CD4⁺CD25⁺ T cells is controlled in part by costimulation. *The Journal of Immunology*, 2004, 173: 2428–2434.

The immune system operates on the principle of checks and balances. For T cells, there is evidence that their activities may be controlled in part by subsets of T cells specialized in regulation. The thymus-derived CD4⁺CD25⁺ T cells belong to one of these subsets. Unlike conventional T cells functioning as effector cells during an immune reaction, the CD4⁺CD25⁺ T cells act to suppress the activation of conventional T cells, and in so doing, actively maintain the immunologic tolerance in the periphery (reviewed in Refs. 1–3). Recent studies indicate that CD4⁺CD25⁺ T cells may play important roles in the prevention of certain auto- and alloimmune diseases (4–11).

Given the importance of these regulatory cells, it is critical to understand the mechanisms controlling their activation and expansion in the periphery. Intriguingly, freshly isolated CD4⁺CD25⁺ T cells are anergic, proliferating poorly to TCR stimulation in culture. How these cells expand in vivo in response to physiological stimuli has yet to be determined. Lately, it has been shown that the CD4⁺CD25⁺ T cells carrying a transgenic TCR can undergo clonal expansion in vivo in response to immunization with a specific Ag, and that the dynamics of the regulatory cell population depend largely on local environment (12–14). Aside from Ag presentation, costimulation by activated dendritic cells (DC)³ is likely to be a key factor affecting the dynamics, as it has been shown that CD4⁺CD25⁺ T cells can be expanded in vivo with bone marrow-derived, Ag-presenting DC, in a manner that is dependent on B7 expression (14). Interestingly, deficiency in B7 reduces, but not entirely eliminates, the potency of DC, suggesting that additional costimulators other than B7 may also play a role (14). Such pos-

sibility is consistent with a previous observation that the development of IL-10-secreting respiratory regulatory T cells depends on ICOS, not B7 (15).

To date, most of our understanding of the role of costimulation has come from studies of conventional types of T cells; the role of costimulation in the regulatory T cells is less explored. Previously, using a protein transfer approach, we established the existence of the costimulation threshold and the interplay between costimulators and coinhibitors during the course of T cell activation in vitro (16, 17). Moreover, our investigation led to the development of the combinatorial costimulator protein transfer strategy for cancer vaccination in vivo (18, 19). Whether the regulatory T cells can respond to specific costimulation is of particular interest to us, because it is now conceived that regulatory T cells may play a significant role in the control of antitumor immunity.

Our investigation of the role of costimulation in the expansion of regulatory T cells started with the costimulator receptor 4-1BB. The 4-1BB is a membrane receptor protein of the TNFR superfamily. Like CD28, 4-1BB is a potent costimulator, known to promote CD4⁺ and CD8⁺ T cell activation and survival (reviewed in Ref. 20). Unlike CD28, which competes for B7 with another receptor, CTLA-4 (21), 4-1BB binds monogamously to a single ligand, 4-1BBL, thereby allowing more straightforward determination. Importantly, 4-1BB gene expression is up-regulated in CD4⁺CD25⁺ T cells (22, 23). Our study shows that freshly isolated CD4⁺CD25⁺ T cells constitutively display the 4-1BB receptor protein at the cell surface, and that the signaling of this receptor by 4-1BBL can significantly augment the proliferation of these T cells in culture and in vivo. These results support the notion that the peripheral expansion of the CD4⁺CD25⁺ T cells is controlled in part by costimulation.

Materials and Methods

Mice, peptide, and cell line

BALB/c and DO11.10 (BALB/c-TgN(DO11.10)10Loh) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were maintained in a pathogen-free facility and used in accordance with institutional guidelines for animal care. The OVA_{323–339} Ag peptide was synthesized by Princeton Biomolecules (Langhorne, PA). The PRO-IAd cell line was a gift from J. Miller of the University of Chicago (Chicago, IL) and maintained, as previously described (24).

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³ Abbreviations used in this paper: DC, dendritic cell; 7-AAD, 7-aminoactinomycin D; LN, lymph node.

Antibodies

PE-conjugated hamster anti-mouse 4-1BB mAb (17B5), PE-conjugated rat anti-mouse 4-1BBL mAb (TKS-1), and isotype controls were purchased from eBioscience (San Diego, CA). Nonconjugated 4-1BB-blocking mAb (17B5) was also purchased from eBioscience and dialyzed in PBS before use. PE-conjugated goat anti-human Fc γ was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rat anti-mouse CD3 mAb (TK3) was purchased from Serotec (Oxford, U.K.). FITC-conjugated rat anti-mouse 4-1BB mAb (1AH2), FITC-conjugated rat anti-mouse CD4 mAb (GK1.5), allophycocyanin-conjugated rat anti-mouse CD4 mAb (RM4-5), biotin-conjugated rat anti-mouse CD25 mAb (7D4), PE- or allophycocyanin-conjugated streptavidin, rat anti-mouse CD16/CD32 mAb (2.4G2), and various isotype-matched controls were purchased from BD Biosciences (San Jose, CA). PE-conjugated mouse anti-DO11.10 mAb (KJ1-26) was purchased from Caltag Laboratories (Burlingame, CA).

T cell purification

CD4⁺ T cells were prepared from the spleens or lymph nodes (LNs) of BALB/c or DO11.10 mice and enriched via negative selection by magnetic cell sorting (Miltenyi Biotec, Auburn, CA), as per manufacturer's protocols. The cells were blocked with anti-mouse CD16/CD32 mAb, immunostained with FITC-conjugated anti-mouse CD4 mAb and biotin-conjugated anti-mouse CD25 mAb/PE-conjugated streptavidin, and then sorted by a FACSDiVa cell sorter (BD Biosciences). CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were separately collected; purity of the preparations was typically >98%. For some experiments, the CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were purified by magnetic cell sorting, using the CD4⁺CD25⁺ regulatory T cell isolation kit from Miltenyi Biotec, as per manufacturer's protocol. To obtain highly purified cells, the MS column step was repeated three times before the CD4⁺CD25⁺ cells were finally collected. The purity of such cell preparations was typically >98%, whereas the yield was reduced by ~40%.

Cell painting

PRO-IAd artificial accessory cells were inactivated by incubation at 37°C for 1 h with 100 μ g/ml mitomycin C (Sigma-Aldrich, St. Louis, MO), at 2×10^6 cells/ml in DMEM/2% FCS. The cells were thoroughly washed and incubated at 37°C for 30 min with palmitated protein A (16), at 30 μ g/10⁷ cells/ml in DMEM/0.1% BSA. The cells were washed and incubated at 4°C for 30 min with Fc γ 1-derivatized mouse 4-1BBL (4-1BBL-Fc) (18) or Fc γ 1-derivatized human CD28 (CD28-Fc) (16) at 30 μ g/10⁷ cells/ml in DMEM/0.1% BSA. The cells were thoroughly washed and suspended in RPMI 1164/10% FCS/15 mM HEPES/50 μ M 2-ME (R10 medium).

Proliferation assay

A total of $0.25\text{--}1 \times 10^5$ T cells/well was cultured in R10 medium in U-bottom 96-well plates in duplicates or triplicates, along with $2.5\text{--}5 \times 10^4$ PRO-IAd cells/well painted with 4-1BBL-Fc or 4 μ g of soluble 4-1BBL-Fc (per well). For Ab blocking, anti-4-1BB mAb (17B5) and isotype-matched control were each used at 5 μ g/well. For assays involving the DO11.10 TCR transgenic T cells, the OVA₃₂₃₋₃₃₉ peptide was added to the wells at 0.1–100 μ g/ml, in conjunction with the PRO-IAd cells. For assays involving BALB/c T cells, rat anti-mouse CD3 mAb (TK3) was used as a polyclonal mitogen. The mAb was diluted to 1–8 μ g/ml in PBS and bound to U-bottom 96-well plates (50 μ l/well) at 37°C for at least 6 h before the cells were plated. The cultures were pulsed at 48 h with [³H]thymidine (1 μ Ci/well) and harvested at 64 h with a Tomtec MACH III cell harvester (Hamden, CT). [³H]Thymidine incorporation was analyzed with a Micro-Beta liquid scintillation counter (Wallac, Turku, Finland).

Adoptive transfer

CD4⁺CD25⁺ T cells were purified from the spleens of DO11.10 mice, as described earlier, washed three times in PBS, thoroughly resuspended at 2.5×10^6 cells/ml in PBS, and then stained with CFSE (Molecular Probes, Eugene, OR) at 1 μ M for 5 min at room temperature. The reaction was terminated by adding FCS to 10%, followed by two washes with DMEM/10% FCS/50 μ M 2-ME. For costimulator treatment, CFSE-stained cells were resuspended in DMEM/0.1% BSA/50 μ M 2-ME and incubated at 4°C for 20 min with 4-1BBL-Fc or human IgG (30 μ g of protein/1 $\times 10^7$ cells/ml). The cells were washed twice in DMEM and injected into the hind leg footpads of two or more BALB/c mice ($2.5\text{--}5 \times 10^5$ cells in 30 μ l of DMEM per footpad). The 4-1BBL-Fc-treated cells were injected at the right footpads; human IgG (control Fc)-treated cells, left footpads. To prime the injected donor cells, 30 min before cell transfer, the OVA₃₂₃₋₃₃₉ peptide (0.08 μ g in 30 μ l of PBS per footpad) was injected into the right

and left footpads of the recipient mice. The recipient mice were maintained in the dark for 4 days and then sacrificed. Cell suspensions were prepared from the right (4-1BBL-Fc-treated) and left (human IgG-treated) popliteal LNs; cells from each treatment group were pooled. The pooled cells were then immunostained for CD4 and the DO11.10 TCR with allophycocyanin-conjugated anti-mouse CD4 mAb and PE-conjugated anti-DO11.10 mAb, respectively, in the presence of anti-mouse CD16/CD32 mAb as Fc blocker. Immediately before FACS analysis, the cells were additionally stained for DNA with 7-aminoactinomycin D (7-AAD; Molecular Probes). Four-color FACS was performed on a FACSCalibur (BD Biosciences). The donor cells were gated as a DO11.10 TCR and CD4 double-positive population, and the viability (7-AAD negativity) and cell division (CFSE decrement) of this gated population were analyzed with the CellQuest software (BD Biosciences).

Coadoptive transfer

CFSE-labeled DO11.10 CD4⁺CD25⁺ T cells, prepared as described before, were premixed with Ag-pulsed and 4-1BBL-Fc-painted PRO-IAd cells and injected in the form of a cell mixture into the footpad of BALB/c mice. For preparation of the PRO-IAd cells for coadoptive transfer, the cells were inactivated with mitomycin C, as described before, and incubated with the OVA₃₂₃₋₃₃₉ peptide (80 μ M) at 37°C for 1 h, at 10^7 cells/ml in DMEM/0.1% BSA. Palmitated protein A was then added to the cells at 30 μ g/ml, and the incubation was continued for another 50 min at 37°C, followed by 10-min incubation at 45°C. The cells were washed and incubated at 4°C for 20 min with 4-1BBL-Fc or, as control, human IgG, at 30 μ g/10⁷ cells/ml in DMEM/0.1% BSA. The cells were washed twice in DMEM and combined with the T cells at 1:1 ratio. Footpad injection (two or more mice per experiment) and FACS analysis were performed essentially as described before, with the mixed cells containing the human IgG-painted PRO-IAd cells injected ($0.5\text{--}1 \times 10^6$ total cells per foot) into the left footpads (control) and an equal number of mixed cells containing the 4-1BBL-Fc-painted PRO-IAd cells injected into the right footpads (test).

Suppression assay

DO11.10 CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were stimulated to proliferation with mitomycin C-inactivated, 4-1BBL-Fc-painted PRO-IAd cells and OVA₃₂₃₋₃₃₉ (10 μ g/ml) in U-bottom 96-well plates and R10 medium. The cells were harvested 3 days later and thoroughly washed in R10 medium. Live cells (T cells) were counted and reseeded as suppressor (0.025×10^6 cells/well) in U-bottom 96-well plates in R10 medium, together with freshly isolated DO11.10 CD4⁺CD25⁻ T cells as responder (0.05×10^6 cells/well), DO11.10 accessory cells as stimulator (0.05×10^6 cells/well), and OVA₃₂₃₋₃₃₉ (0.1 μ g/ml). The accessory cells were obtained from bulk spleen cells depleted of CD4⁺ and CD8⁺ cells by magnetic cell sorting (Miltenyi Biotec) and chemically inactivated with mitomycin C before use. The proliferation of the responder T cells was determined by the [³H]thymidine incorporation method described before.

Results

Signaling of 4-1BB augmented the proliferation of CD4⁺CD25⁺ T cells

Previously, Gavin et al. (22) and McHugh et al. (23) detected constitutive expression of the 4-1BB gene in mouse CD4⁺CD25⁺ T cells using DNA microarray. To confirm the actual presence of the 4-1BB protein on the cell surface, we isolated spleen cells from normal BALB/c mice and their congenics carrying a rearranged TCR transgene (DO11.10), and immunostained the cells with hamster anti-mouse 4-1BB Ab (17B5), together with Abs specific for CD4 and CD25. Using multicolor flow cytometry, we were able to identify and gate on CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells and examine the 4-1BB-specific stains simultaneously in both subsets (Fig. 1A). The CD4⁺CD25⁻ subset served as an internal control, because naive CD4⁺CD25⁻ T cells are known to express little 4-1BB (20). Indeed, in the freshly isolated spleen cells of BALB/c or DO11.10 origin, 4-1BB was detected only in the CD4⁺CD25⁺ subset, but not in the CD4⁺CD25⁻ subset (Fig. 1, B and C). However, the 4-1BB stain in the CD4⁺CD25⁺ subset was rather weak, suggesting a low-level presence of the 4-1BB protein on the cell surface. This observation was confirmed with the use of a second anti-4-1BB mAb (1AH2) of different species origin (rat) (data not

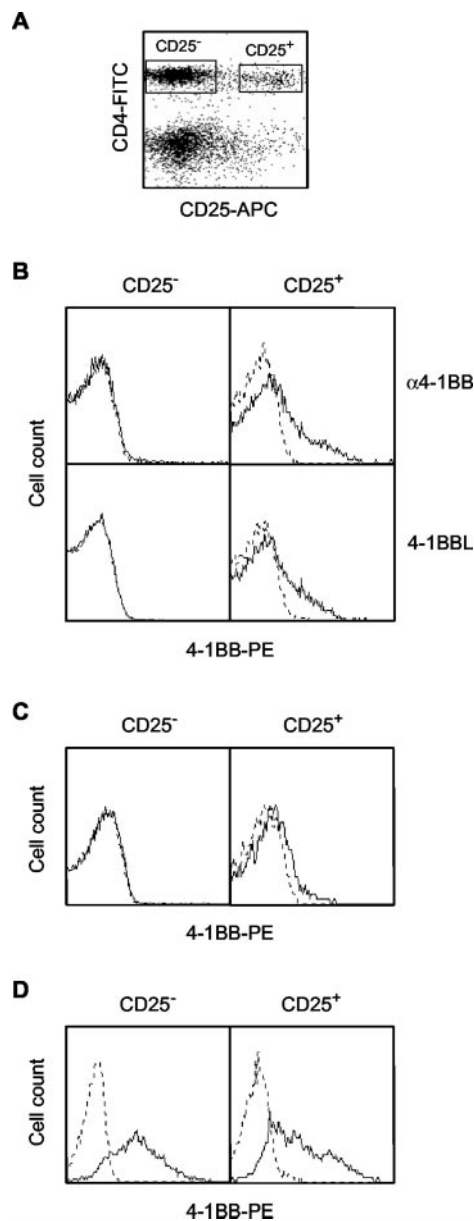


FIGURE 1. The 4-1BB receptor was present on CD4⁺CD25⁺ T cells. *A–C*, Freshly isolated bulk spleen cells were depleted of MHC II⁺ cells by magnet cell sorting (Miltenyi Biotec) and stained with biotin-conjugated anti-CD25 mAb/allophycocyanin-conjugated streptavidin, FITC-conjugated anti-CD4 mAb, and a specific reagent for 4-1BB (indicated below), in the presence of anti-CD16/CD32 mAb as Fc blocker. The stained cells were analyzed for 4-1BB expression by multicolor flow cytometry. *A*, CD4⁺CD25[−] and CD4⁺CD25⁺ cells were identified and gated. *B*, Comparative analysis of 4-1BB-specific signals from the two gated cell populations of BALB/c origin. The cells were stained with either PE-conjugated anti-4-1BB mAb (17B5, *top*) or 4-1BBL-Fc/PE-conjugated goat anti-human Fcγ Ab (*bottom*). *C*, Similar analysis of 4-1BB-specific signals in gated cells of DO11.10 origin. The cells were stained with PE-conjugated anti-4-1BB mAb. *D*, The 4-1BB staining of activated BALB/c CD4⁺CD25[−] and CD4⁺CD25⁺ T cells. Purified BALB/c splenic CD4⁺CD25⁺ and CD4⁺CD25[−] T cells were stimulated, respectively, in culture for 2 days with plate-bound anti-CD3 mAb (TK3, bound at 8 μg/ml) and murine IL-2 (100 U/ml). The cells were harvested, stained with PE-conjugated anti-4-1BB mAb, and analyzed by flow cytometry. Solid line, 4-1BB-specific staining; dashed line, isotype-matched control staining. Data in each panel are representative of two or three independent experiments.

shown), and with the use of a ligand of 4-1BB, 4-1BBL-Fc (18), as the 4-1BB-specific staining reagent (Fig. 1*B*).

To investigate whether the level of 4-1BB expression in the CD4⁺CD25⁺ T cells changes after T cell activation, splenic CD4⁺CD25⁺ T cells were isolated and stimulated for 2 days in culture with anti-CD3 mAb and IL-2. The cells so activated were again stained with anti-4-1BB mAb. Flow cytometric analysis of these cells detected considerably stronger staining than what had been detected in the freshly isolated same cells (Fig. 1*D*), suggesting that the presence of 4-1BB on the cell surface is up-regulated after cell activation.

To determine whether the presence of 4-1BB on CD4⁺CD25⁺ T cells is functionally significant, we next probed the cells with 4-1BBL-Fc. To that end, 4-1BBL-Fc was painted (16, 17) onto an artificial accessory cell line, PRO-IAd (24) (Fig. 2*A*). Splenic CD4⁺CD25⁺ T cells isolated from BALB/c mice were stimulated in culture with plate-bound anti-CD3 mAb, in combination with the PRO-IAd cells. As expected, the CD4⁺CD25⁺ T cells displayed a typical anergic phenotype and proliferated poorly in response to anti-CD3 stimulation alone (Fig. 2*B*). However, they proliferated actively when costimulated by PRO-IAd cells painted with 4-1BBL-Fc, but not by that with a control Fc protein (CD28-Fc), indicating that the cells were sensitive to 4-1BBL costimulation. The costimulation was blocked by anti-4-1BB mAb, but not by a control mAb, confirming that it was specifically mediated by the 4-1BB receptor. These results establish that costimulation via 4-1BB can potently drive the CD4⁺CD25⁺ T cells to proliferation. As expected, 4-1BBL-Fc also costimulated the proliferation of

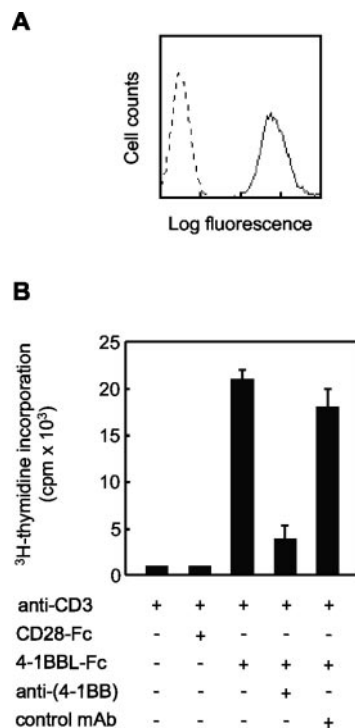


FIGURE 2. Signaling of 4-1BB augmented CD4⁺CD25⁺ T cell proliferation. *A*, PRO-IAd cells, nonpainted (dashed line) or painted with 30 μg/ml 4-1BBL-Fc (solid line), were stained with PE-conjugated anti-mouse 4-1BBL mAb and analyzed by flow cytometry. *B*, CD4⁺CD25⁺ T cells were stimulated with plate-bound anti-CD3 mAb (bound at 8 μg/ml) and PRO-IAd cells, either nonpainted or painted with indicated Fc protein, in the absence or presence of anti-4-1BB-blocking mAb (5 μg/well). The cells were pulsed with 1 μCi of [³H]thymidine per well at 48 h and harvested at 64 h; [³H]thymidine incorporation was then determined. Data are representative of three independent experiments.

CD4⁺CD25⁺ T cells isolated from LNs with similar efficiency (data not shown).

To probe the effect of 4-1BB engagement on Ag-specific proliferation, the PRO-IAd cells, which neoexpress the MHC II molecules I-A^d on the cell surface (24), were further tested as APCs for CD4⁺CD25⁺ T cells bearing the DO11.10 transgenic TCR. When painted with 4-1BBL-Fc and loaded with the cognate OVA_{323–339} peptide Ag, the artificial APC vigorously stimulated the proliferation of the Ag-specific CD4⁺CD25⁺ T cells over a range of Ag doses (Fig. 3). In contrast, the same APC showed only marginal stimulatory activity when painted with a control Fc-containing protein (human IgG). This result confirms that 4-1BB costimulation can promote Ag-specific proliferation of CD4⁺CD25⁺ T cells.

The 4-1BB binds 4-1BB ligand with high affinity (25, 26). Although both exist predominantly as membrane proteins, the high affinity interaction between the two allows the receptor to be triggered by soluble forms of 4-1BB ligand as well, as has been observed in conventional T cells (27). To determine whether this may also be the case for CD4⁺CD25⁺ regulatory T cells, we applied soluble 4-1BBL-Fc directly to cell proliferation assays, without invoking artificial accessory cells to present the ligand. The proliferation of CD4⁺CD25⁺ T cells over a range of anti-CD3 stimulation was consistently augmented by the addition of soluble 4-1BBL-Fc (Fig. 4), to the level comparable with that by 4-1BBL-Fc-painted PRO-IAd cells (Fig. 2B), suggesting that the 4-1BB receptor on these cells can be triggered efficiently by the soluble ligand. This result also confirms in a clear-cut manner the augmenting effect of 4-1BB costimulation, because it derives from a minimal set of pure reagents (FACS-purified CD4⁺CD25⁺ T cells, anti-CD3 mAb, and 4-1BBL-Fc).

As shown early in Fig. 1B, CD4⁺CD25⁺ T cells contacted with soluble 4-1BBL-Fc retained the ligand on the cell surface even after the cells had been repeatedly washed. Hence, it is likely that the cells can be continuously costimulated by the ligand once bound with the ligand, a property that can be useful for probing the effect of 4-1BB costimulation on these cells in vivo. To that end, an adoptive transfer experiment was performed in which the Ag-specific proliferation of CD4⁺CD25⁺ T cells in vivo was analyzed in response to their prior contact with soluble 4-1BBL-Fc in vitro. Thus, TCR transgenic CD4⁺CD25⁺ donor T cells were isolated from the spleens of DO11.10 mice, labeled with CFSE to allow subsequent analysis of cell division by FACS (28), and incubated with 4-1BBL-Fc at 4°C to allow binding of the protein to the

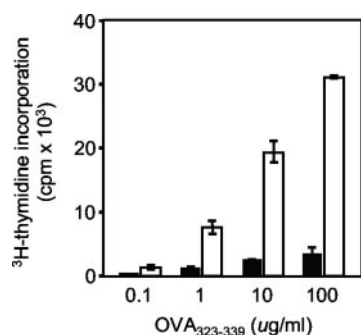


FIGURE 3. The 4-1BB costimulation augmented Ag-specific proliferation of DO11.10 CD4⁺CD25⁺ T cells. DO11.10 splenic CD4⁺CD25⁺ T cells were stimulated with indicated concentrations of the OVA_{323–339} peptide Ag, presented by the PRO-IAd artificial APC that were generated by painting with 4-1BBL-Fc (□) or human IgG as control Fc protein (■). Cell proliferation was determined, as described in Fig. 2. Data are representative of two independent experiments.

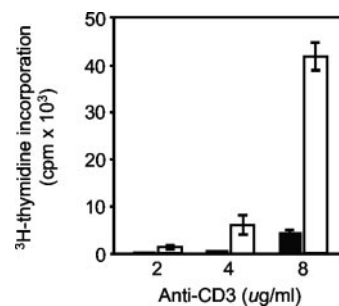


FIGURE 4. Soluble 4-1BB ligand costimulated CD4⁺CD25⁺ T cell proliferation. CD4⁺CD25⁺ T cells were stimulated with indicated concentrations of plate-bound anti-CD3 mAb, either alone (■) or in combination with 4 μg (per well) of soluble 4-1BBL-Fc (□). Cell proliferation was determined, as described in Fig. 2. Data are representative of three independent experiments.

4-1BB receptor on the cell surface; unbound ligand was subsequently removed by washing. The treated cells were then injected into the footpads of syngeneic BALB/c mice, at $\sim 2.5 \times 10^5$ cells per footpad. As control, an equal number of mock-treated T cells (incubated with human IgG as control Fc) were injected into the contralateral footpads of the same animals. To provide specific Ag stimulation to the donor cells, 30 min before cell transfusion, the recipient mice were also injected at the footpad with OVA_{323–339}. Four days after priming, draining LNs (popliteal) were isolated from the recipient mice and pooled from each group separately (test or control). Total cellular content of each group of pooled LNs was recovered and analyzed in its entirety by FACS to assure that all LN cells were accounted for. The injected donor cells were usually recovered from the draining LN at the rate of $\sim 0.1\%$.

Preliminary experiments established that the proliferation of donor CD4⁺CD25⁺ T cells in recipient animals was Ag dose dependent (data not shown), consistent with previous reports by others (12–14). Accordingly, the recipient animals were injected with a suboptimal dose of Ag (0.08 μg of peptide per footpad) to assure that the effect of costimulation would be most obvious.

The 4-1BBL-Fc augmented in vivo expansion of the donor cell population under this Ag-limiting condition. Four-color FACS showed that the counts of donor cells, identifiable by their CD4⁺KJ1–26⁺ (DO11.10 TCR-specific) status, were increased by an average (three experiments) of 2-fold in the test group relative to that in the control group (Fig. 5, A and B). CFSE analysis further showed that in both groups the donor cell population was divided into two major fractions: the dividing-cell fraction (with incrementally decreased CFSE stain; *lower left quadrant*) and nondividing-cell fraction (with highest CFSE stain; *lower right quadrant*) (Fig. 5, C and D). Although the counts of nondividing cells were relatively even across the test and control groups, that of dividing cells were increased by an average (three experiments) of 2.5-fold in the test group. Taken together, the analyses indicated that the donor cell population in the test group was expanded primarily by augmented cell proliferation.

To further demonstrate such augmented proliferation in a setting more closely simulating the physiological priming of T cells, a coadoptive transfer experiment was performed in which the donor T cells were transfused together with PRO-IAd cells as artificial APC. The PRO-IAd cells used were preloaded with OVA_{323–339} and painted with 4-1BBL-Fc. The resulting artificial APC were capable of stimulating the proliferation of DO11.10 CD4⁺CD25⁺ T cells, as demonstrated earlier in vitro (Fig. 3). In parallel, the same PRO-IAd cells loaded with Ag, but painted with human IgG, were used as control. Footpad injection and FACS analysis were

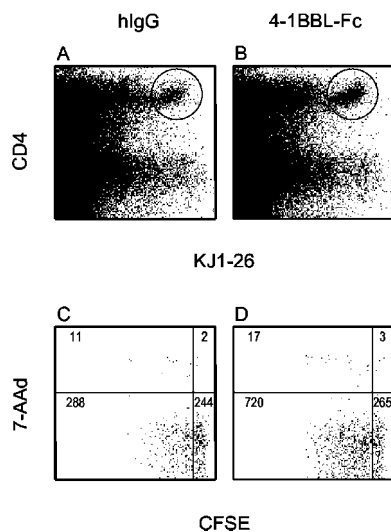


FIGURE 5. The 4-1BB ligand-coated $CD4^+CD25^+$ T cells exhibited enhanced proliferative activity in vivo. CFSE-labeled DO11.10 TCR transgenic $CD4^+CD25^+$ T cells (99% pure), treated either with a human IgG control Fc protein (A and C) or with 4-1BBL-Fc (B and D), were injected contralaterally into the left and right footpad, respectively, of syngeneic BALB/c mice (*Materials and Methods*). At day 4, cells were recovered from the draining (popliteal) LNs and stained with allophycocyanin-conjugated anti-CD4 mAb (RM4-5), PE-conjugated anti-DO11.10 mAb (KJ1-26), and 7-AAD. The cells were analyzed by four-color flow cytometry. A and B, Donor cell population (CD4 and KJ1-26 double positive, circled) was identified and gated on as a small fraction of total $CD4^+$ T cells. C and D, Proliferation (decrement of CFSE fluorescence) and death (7-AAD-positive stain) of the gated cells were analyzed by quadrant plots. Cell counts in each quadrant are indicated. Data are representative of three independent experiments. The increased counts of proliferating cells (*lower left quadrant*) produced in the 4-1BBL-Fc-treated groups (D), as compared with the counts produced in the control-treated groups (C), are statistically significant by one-tailed Student's *t* test ($p < 0.01$).

performed essentially as described before, except that the donor T cells were preformulated with the artificial APC (1:1) and administered as a cell mixture. The result showed that the donor T cells cotransferred with the 4-1BBL-Fc-painted APC proliferated more vigorously as compared with those cotransferred with the control APC (Fig. 6). Again, on average (four experiments), there was a 4-fold increase in the counts of proliferating donor T cells that was directly attributable to the presence of 4-1BBL-Fc on APC. This result agrees well with the earlier result obtained from the 4-1BBL-Fc-coated donor T cells.

In conclusion, the results from both the in vitro and in vivo experiments consistently indicate that 4-1BB costimulation promotes the proliferation of the $CD4^+CD25^+$ T cells.

The 4-1BB costimulation did not lead to IL-2 production

Activated conventional T cells usually secrete IL-2, which in turn initiates an autocrine loop for the continued expansion and survival of the proliferating population. The 4-1BB is known to promote IL-2 production in both $CD4^+$ and $CD8^+$ T cells. Particularly in the $CD4^+$ T cells, 4-1BB alone is capable of inducing IL-2 production, independently of the CD28 costimulatory pathway (29, 30). To determine whether 4-1BB could likewise promote IL-2 production in the $CD4^+CD25^+$ regulatory T cells, we stimulated freshly isolated mouse spleen $CD4^+CD25^+$ T cells with anti-CD3 mAb and soluble 4-1BBL-Fc. Forty-eight hours after stimulation, a portion of conditioned medium was taken from the culture and analyzed for IL-2, as well as a panel of other cytokines (IL-4, IL-5,

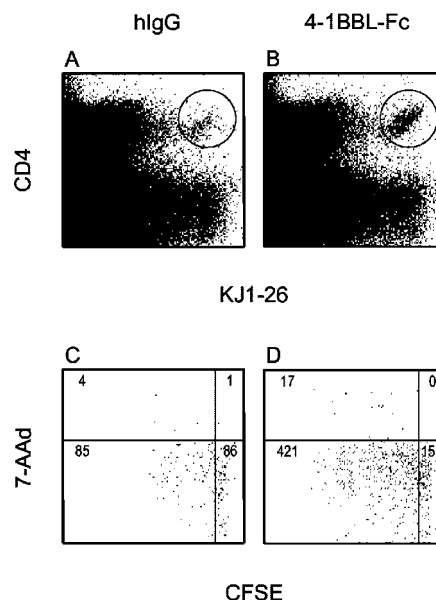


FIGURE 6. $CD4^+CD25^+$ T cells proliferated more vigorously in vivo in response to artificial APC presenting 4-1BBL-Fc. CFSE-labeled DO11.10 TCR transgenic $CD4^+CD25^+$ T cells (98% pure) were premixed with PRO-IAAd cells painted with human IgG (A and C) or the same PRO-IAAd cells painted with 4-1BBL-Fc (B and D), and the two different cell mixtures were injected contralaterally into the left and right footpads, respectively, of BALB/c mice. At day 4, donor cells were recovered and analyzed by four-color flow cytometry, as described in Fig. 5. Similarly, donor cell population (CD4 and KJ1-26 double positive, circled) was gated on (A and B), and proliferation (decrement of CFSE fluorescence) and death (7-AAD-positive stain) of the gated cells were analyzed by quadrant plots (C and D). Cell counts are indicated for each quadrant. Data are representative of four independent experiments. The increased counts of proliferating cells (*lower left quadrant*) produced with the 4-1BBL-Fc-painted APC (D), as compared with the counts produced with the control APC (C), are statistically significant by one-tailed Student's *t* test ($p < 0.025$).

IFN- γ , and TNF- α). To directly correlate cytokine secretion with cell proliferation, the rest of the same culture was also pulsed with [^3H]thymidine and assayed for proliferation. As depicted in Fig. 7, the proliferation of the $CD4^+CD25^+$ or $CD4^+CD25^-$ (control) T cells was 4-1BBL-Fc dependent. IL-2 secretion was evident in the proliferating $CD4^+CD25^-$ T cell cultures, as expected. In contrast, no IL-2 was detected in the $CD4^+CD25^+$ T cell cultures that proliferated nearly as actively as the $CD4^+CD25^-$ T cell cultures. Nonetheless, the proliferating $CD4^+CD25^+$ T cells did secrete increased amounts of IFN- γ and TNF- α , albeit at considerably lower levels than that by the $CD4^+CD25^-$ T cells. IL-4 and IL-5 were present only at marginal levels in both the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell cultures and were not significantly increased upon proliferation (data not shown). These results indicate that 4-1BB costimulation can increase the production of IFN- γ and TNF- α , but not IL-2, in $CD4^+CD25^+$ T cells. This finding is consistent with the general observation that $CD4^+CD25^+$ regulatory T cells, including those expanded under anti-CD28 costimulation (31, 32), do not produce IL-2 (reviewed in Ref. 2).

$CD4^+CD25^+$ T cells remained suppressive after 4-1BB-augmented proliferation

The $CD4^+CD25^+$ regulatory T cells are known to be able to suppress the proliferation of conventional T cells, which can be demonstrated by the well-established coculture assay (33). Such activity is generally limited toward weakly, but not strongly, stimulated

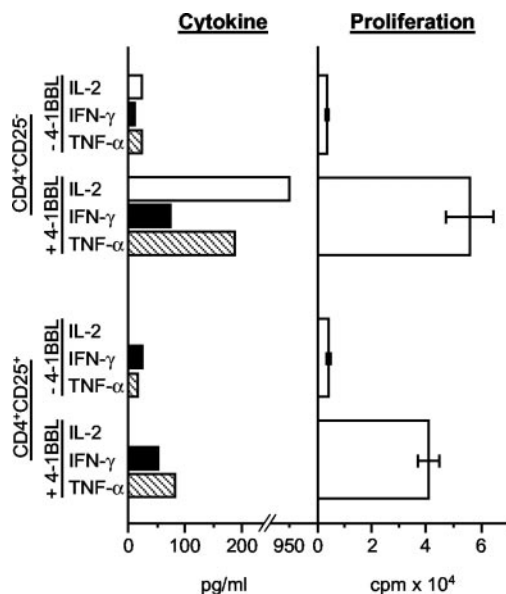


FIGURE 7. The 4-1BB-costimulated CD4⁺CD25⁺ T cells did not produce IL-2. CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells (in 200 μ l) were stimulated (in duplicate) with plate-bound anti-CD3 (bounded at 8 μ g/ml for CD4⁺CD25⁺ cells and 4 μ g/ml for CD4⁺CD25⁻ cells), with or without soluble 4-1BBL-Fc (4 μ g/well). At 48 h, 50 μ l of conditioned medium was taken from each of the duplicate wells and combined, and cytokine content in the medium was analyzed with the Cytometric Bead Array (BD Biosciences). In parallel, the remaining cultures were pulsed with [³H]thymidine (1 μ Ci/well), and the proliferation of the cytokine-producing T cells was assessed, as described in Fig. 2. Data are representative of two independent experiments.

T cells (33–36), with physiological implication that it might function primarily to raise the threshold of autoimmune reactions (35). Having established the augmenting effect of 4-1BB costimulation on cell proliferation, we sought to determine whether the CD4⁺CD25⁺ T cells remained suppressive after proliferation.

To that end, DO11.10 CD4⁺CD25⁺ T cells were first costimulated to proliferation with 4-1BBL-Fc-painted PRO-IAd cells and a high dose of OVA_{323–339} (10 μ g/ml; Fig. 3). Three days later, the proliferating cells were harvested, and their suppressive activity was subsequently assessed by the coculture assay against DO11.10 CD4⁺CD25⁻ T cells, in the presence of spleen-derived accessory cells and a low dose of OVA_{323–339} (0.1 μ g/ml). As depicted in Fig. 8, the CD4⁺CD25⁺ T cells, which ceased to proliferate under this latter condition, were able to inhibit the proliferation of the CD4⁺CD25⁻ T cells, indicating that the CD4⁺CD25⁺ T cells remained suppressive after proliferating under 4-1BB costimulation. In contrast, DO11.10 CD4⁺CD25⁻ T cells pre-expanded under the identical condition during the same experiment (CD25⁻ suppressor) did not exhibit such suppressive activity, suggesting that 4-1BB costimulation could not confer the suppressive activity to conventional T cells.

Discussion

Despite early observations that CD4⁺CD25⁺ T cells proliferate poorly to anti-CD3 stimulation, several latest reports suggest that these cells are not truly anergic (12–14). In this study, we show that the proliferation of CD4⁺CD25⁺ T cells can be significantly augmented in vitro and in vivo by 4-1BB costimulation. Furthermore, the expanded CD4⁺CD25⁺ T cells maintained their suppressive activity. Hence, 4-1BB may play a role in the expansion of functional CD4⁺CD25⁺ T cells.

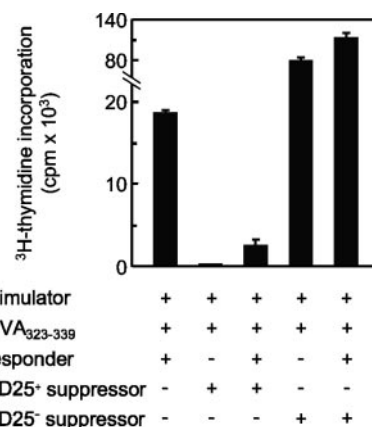


FIGURE 8. The 4-1BB-costimulated CD4⁺CD25⁺ T cells remained suppressive. DO11.10 CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells that had proliferated under 4-1BB costimulation for 3 days were harvested and tested as suppressor in coculture, together with freshly isolated DO11.10 CD4⁺CD25⁻ T cells as responder, mitomycin C-treated DO11.10 spleen cells as stimulator, and the OVA_{323–339} peptide as Ag (0.1 μ g/ml). The proliferation of the responder was determined by the [³H]thymidine incorporation method described in Fig. 2. Data are representative of two independent experiments.

The 4-1BB is known to promote the proliferation and survival of conventional T cells, especially CD8⁺ T cells. The fact that it also induces the proliferation of CD4⁺CD25⁺ T cells raises the possibility that the expansion of the regulatory cells may be tied with that of conventional T cells. Such linkage makes sense from the immune regulation standpoint, because it would offer a simple feedback loop for dynamically tuning the size of the regulatory cell population, in connection with its potential targets. Should this be correct, it may have a direct implication in certain cancer vaccination or treatment strategies that are based on enforced expression of costimulators, particularly those involving 4-1BB (18, 37–39). Although these strategies have met with various degrees of success in the past, it would be more advantageous to consider how to selectively costimulate effector T cells without expanding the regulatory T cells, as the rise of the latter would potentially compromise the therapeutic outcome.

The 4-1BB promotes the proliferation of CD4⁺CD25⁺ T cells without adversely affecting the suppressive activity of the cells. One may speculate that by allowing the CD4⁺CD25⁺ T cell population to expand, 4-1BB may contribute indirectly to increased levels of overall suppressive activity overtime. In vivo experiments are currently in progress to assess this possibility. If confirmed, this finding may have potential therapeutic implications, because the expansion of functional regulatory T cells can be beneficial in controlling harmful autoimmune and alloimmune reactions. Of note, there have been other means for expanding CD4⁺CD25⁺ T cells, such as Ag immunization (12, 13), infusion of Ag-presenting DC (14), and various other ex vivo and in vivo methods (9, 32, 40, 41). Together, these methods have now presented a range of options potentially useful for therapeutic applications.

Under suboptimal Ag stimulation, the one-time binding of 4-1BBL-Fc to CD4⁺CD25⁺ T cells induces a 2.5-fold increase in cell proliferation in vivo. A 4-fold increase was seen when the costimulator was presented by an artificial APC line. These results are significant, as they are in line with the therapeutic expectations. To put this in perspective, in the NOD mice, the susceptibility to autoimmune diabetes is linked with the lowered CD4⁺CD25⁺ T cell count, by a factor of <2-fold (1.5-fold as compared with other stains) (5). Further studies are needed to assess the potential of

4-1BBL-Fc as a therapeutic modifier for CD4⁺CD25⁺ T cells in animal models. In this regard, it is perhaps more beneficial to consider invoking additional costimulators in combination with 4-1BBL-Fc. As these costimulators may engage several additional receptors on the CD4⁺CD25⁺ T cells, such as ICOS (15), OX40 (22, 23), and CD28 and/or CTLA-4, additional costimulatory pathways may be accessed that, together, lead to a far greater effect. In fact, ICOS (15), OX40 (42), and CD28 (5, 43) have each been implicated in the development or homeostasis of the CD4⁺CD25⁺ regulatory T cells. It will be interesting to test whether multiple costimulators can indeed elicit stronger functional outputs from the CD4⁺CD25⁺ T cells when combined.

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