

Butyrophilin-like 2 Modulates B7 Costimulation To Induce Foxp3 Expression and Regulatory T Cell Development in Mature T Cells

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Naive T cell activation involves at least two signals from an APC, one through the TCR via interaction with peptide–MHC complexes and a second through ligation of CD28 with B7 ligands. Following activation, T cells upregulate a host of other membrane-bound costimulatory molecules that can either promote or inhibit further T cell maturation and proliferation. In some cases, it is necessary to attenuate T cell activation to prevent deleterious inflammation, and inhibitory members of the B7/butyrophilin family of ligands have evolved to balance the strong stimuli the activating B7 ligands confer. Human genetic association and *in vitro* studies have implicated one such ligand, BTNL2, in controlling inflammation at mucosal surfaces. In this study, we show that recombinant mouse BTNL2 modifies B7/CD28 signaling to promote expression of Foxp3, a transcription factor necessary for regulatory T cell (Treg) development and function. BTNL2 blocks Akt-mediated inactivation of Foxo1, a transcription factor necessary for Foxp3 expression. Immunophenotyping and gene profiling reveal that BTNL2-induced Treg share many properties with natural Treg, and *in vivo* they suppress enteritis induced by mouse effector T cells. These findings describe a mechanism by which environmental Ag-specific Tregs may be induced by APC expressing specific modulators of costimulatory signals. *The Journal of Immunology*, 2013, 190: 2027–2035.

Regulatory T cells (Treg), identified by their requisite transcription factor Foxp3, are a subset of CD4 T cells that have become central to our understanding of immune tolerance. Foxp3 is induced in thymic Treg precursors, generating so-called natural Treg (nTreg) that populate peripheral lymphoid and nonlymphoid organs and chronically suppress deleterious inflammatory responses against self tissues, environmental Ags, and commensal microorganisms. Foxp3 can also be induced in naive CD4 cells under appropriate conditions *in vitro* (1, 2) and *in vivo* (3, 4), generating induced Treg (iTreg). Although Treg specific for peripheral neo-Ag can originate from the pre-existing repertoire of nTreg, it has recently become clear that colonic Treg specific for commensal bacteria arise from conversion of naive CD4 T cells into iTreg (5). Several signaling pathways contribute to Foxp3 induction, including those downstream of the TCR (6, 7), the CD28 coreceptor (8), and the receptors for TGF β and IL-2 (9–11). Elucidating how each of these pathways acts on the *Foxp3*

promoter and discovery of novel factors that promote iTreg development are areas of intense investigation in Treg biology.

Important modulation of TCR-mediated T cell activation is provided by the CD28 family of receptors, in which CD28 and ICOS potentiate T cell proliferation and differentiation and CTLA-4, PD-1, and BTLA attenuate these responses. It has become evident in recent years that this regulation of TCR signaling is critical not only for effector T cell function, but also for Treg development (12, 13). CD28 signaling in the thymus directly contributes to Foxp3 induction (8). Diabetes-prone NOD mice deficient for CD28 or the CD28 ligands, B7-1 and B7-2, develop exacerbated diabetes resulting from decreased numbers of Treg (14). More recent work has suggested a role for another B7 family member, the PD-1 ligand PD-L1, in Foxp3 induction in peripheral naive T cells both *in vivo* and *in vitro* (15). Whereas high concentrations of TGF- β are well known to induce Foxp3 expression in CD3/CD28-stimulated T cells (1), recombinant PD-L1 coimmobilized with anti-CD3 and anti-CD28 was found to synergize with suboptimal quantities of TGF- β to generate suppressive Foxp3⁺ iTreg. Another recent report demonstrated PD-L1-mediated conversion of human Th1 cells into iTreg, suggesting that this biology is conserved between mice and humans (16). The mechanism by which inhibitory costimulatory molecules participate in Treg differentiation remains to be defined; however, a role for reduced activity of the PI3K/Akt/mTOR pathway is supported (15).

Several other inhibitory B7 or B7-like molecules have been identified to date, all of which suppress T cell activation through unknown receptors. These include B7H3, B7H4, B7S3, and members of the butyrophilin family of B7-related ligands (17–20). Butyrophilin-like 2 (BTNL2) is one inhibitory ligand of particular interest due to its strong genetic association with human autoimmune and inflammatory disease (21–24). BTNL2 is strongly expressed in epithelial cells of the small intestine, and expression

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Abbreviations used in this article: BTNL2, butyrophilin-like 2; iTreg, induced regulatory T cell; KO, knockout; nTreg, natural regulatory T cell; Treg, regulatory T cell.

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is upregulated in mouse models of inflammatory bowel disease (22). Because of its expression in the gut and its functional activity in limiting T cell activation, BTNL2 is thought to play a role in limiting unwanted inflammation directed against commensal bacteria or dietary Ag.

In this work, we demonstrate that BTNL2 costimulation during T cell activation promotes *de novo* Foxp3 expression and the development of suppressive iTreg. Our studies provide new insight into how opposing activating and inhibitory signals generated by the B7/butyrophilin family of ligands can be integrated to dictate T cell differentiation and promote dominant tolerance.

Materials and Methods

Animals

Six- to 12-wk-old female C57BL/6 (Taconic), C57BL/6.SJL (The Jackson Laboratory), and C57BL/6 RAG2 knockout (KO) (Taconic) mice were housed under specific pathogen-free conditions at the Amgen animal facility. Studies were conducted in accordance with National Institutes of Health guidelines for the proper use of animals in research and Amgen Institutional Animal Care and Use Committee–approved protocols.

Proteins and plate coating for T cell stimulations

Various reagents were purchased from commercial sources, including anti-mouse CD3 (clone 145-2C11; BD Biosciences), murine rB7-1.Fc (R&D Systems, carrier-free), murine rB7-2.Fc (R&D Systems, CF), and purified human IgG (Sigma-Aldrich). Murine rBTNL2.Fc was prepared at Amgen, as previously described (22). Proteins were immobilized to flat-bottom 96-well plates (Costar, Corning 3595) in 100 μ l PBS overnight. Stimulation coating contained 22 μ g/ml total protein, consisting of 2 μ g/ml anti-CD3 and 20 μ g/ml rB7/rBTNL2/human IgG. Equivalent m.w. ratios were plated at 2 molecules of anti-CD3/10 molecules of rBTNL2.Fc/2.5 molecules of rB7.Fc, and equal loading achieved by offsetting with the proper amount of human IgG. Before adding cells to coated plates, unbound protein was washed off twice with 200 μ l PBS.

CD4 T cell isolation

Single-cell suspensions were prepared from spleens and lymph nodes from female C57BL/6 mice (6–8 wk old; Taconic). CD4 cells were purified using the EasySep mouse CD4 T cell enrichment kit (Stem Cell Technologies). In some experiments, Treg were depleted first (biotin anti-mouse CD25, eBioscience [PC61.5]; anti-biotin beads and AutoMACS separation, Miltenyi Biotec), followed by CD4-positive selection (mouse CD4 beads; Miltenyi Biotec). For all 96-well stimulations, 100,000 purified CD4 T cells were added per well.

In vitro stimulations

CD4 cells were cultured in a final volume of 200 μ l complete RPMI 1640 medium containing 10% FBS (HyClone), 1 mM sodium pyruvate (Invitrogen), 100 μ M nonessential amino acids (Invitrogen), 0.1% 2-ME (Invitrogen), 100 U/ml penicillin (Invitrogen), and 2 mM L-glutamine (Invitrogen). After adding 100,000 purified CD4 to wells, another 100 μ l containing media alone or media supplemented with human rTGF- β (R&D Systems) and mouse rIL-2 (R&D Systems) was added, bringing the final concentration to 0.36 ng/ml rTGF- β and 10 ng/ml rIL-2. Cultures were carried out for 3–5 d, depending on the experiment. After harvesting 100 μ l culture supernatant for cytokine analysis (Milliplex MAP mouse cytokine/chemokine premixed 22-plex), proliferation was measured by [³H]thymidine incorporation, adding 1 μ Ci/well during the last 8 h of culture on day 3, before harvesting cells and measuring cpm on Topcount (Perkin Elmer). Total cell numbers in culture were enumerated by harvesting CD4 cells from individual stimulation conditions in triplicate. Beckman Coulter's Vicell automated cell viability analyzer counted total viable cells/ml and measured percent viability of individual stimulation conditions.

CD4 T cell global gene expression analysis

Twelve-well tissue culture plates (Falcon) were coated with the conditions indicated above. Plates were coated overnight and washed twice before adding cells. Purified splenic C57BL/6 CD4 cells were isolated, as described above, and plated at 1 million per well. Cells were immediately spun down at 750 rpm to initiate uniform exposure to immobilized protein stimulation. At the chosen time points, CD4 cells were pipetted off,

transferred to Eppendorf tubes, and spun, and cell pellets were lysed in RNeasy lysis buffer (Qiagen). Total RNA was isolated with Qiagen RNeasy kit, quantitated, and submitted for Affymetrix microarray analysis. Fifty nanograms of total RNA were amplified on an ArrayPlex (Beckman Coulter) using the Ovation RNA Amplification System V2 and WB reagent (Nugen). Of the amplified cDNA, 4.4 μ g was labeled using the FL Ovation cDNA Biotin Module V2 (Nugen), according to the manufacturer's recommendations. Half of the labeled cDNA was hybridized onto Affymetrix mouse HT_MG-430_PM arrays (Affymetrix) using a GeneTitan (Affymetrix), according to Affymetrix technical protocols. Microarray data can be accessed through the Gene Expression Omnibus repository (GSE42385, <http://www.ncbi.nlm.nih.gov/gds>). Data were analyzed in Rosetta Resolver for gene signatures, and in Biobase for Transfac analysis to identify transcription factor signatures overrepresented in the datasets. The Biobase F-match algorithm was applied to an input list that included all upregulated genes at the 4-h time point with a *p* value threshold of 0.05 and fold change values >1.3. The resultant output matrices were further pruned by a matched promoter *p* value of 0.005.

Signaling Western blots

Six-well tissue culture plates (Falcon) were coated with anti-CD3, rB7, rBTNL2, and human IgG at molecular ratios indicated in T cell stimulation methods above. For 6-well plates, 3.33 ml PBS with protein was incubated overnight before washing wells twice with PBS and adding cells. CD4 cells were purified, as described above, and 10 million cells were added per well. Plates were immediately spun down at 750 rpm to initiate uniform exposure to immobilized protein stimulation, and plates were moved to a 37° incubator. Two minutes prior to the chosen time point, plates were spun at 1200 rpm for 1.5 min. Media was aspirated off wells, plates moved onto ice, and 500 μ l cell lysis buffer (Clontech) containing protease and phosphatase inhibitors (Pierce) was added to wells. Plates were incubated on ice for 30 min before lysates were transferred to Eppendorf tubes for clearing by centrifugation. For Western blotting, reduced lysates containing equal cell equivalents were loaded onto 4–20% SDS-PAGE electrophoresis gels, and resolved proteins were transferred onto nitrocellulose membranes using an iBlot Dry Blotting System (Invitrogen). Membranes were probed with the Abs rabbit anti-phospho-Foxo1 (Thr²⁴)/Foxo3a (Thr³²), rabbit anti-phospho-Foxo1 (Ser²⁵⁶), rabbit anti-Foxo1 (C29H4), rabbit anti-pan-actin, rabbit anti-phospho-Akt (Ser⁴⁷³) (D9E), and rabbit anti-pan-Akt (11E7) from Cell Signaling Technology using the manufacturer's suggested concentrations and protocols. Membranes were developed using Amersham ECL Western blotting reagents (GE Healthcare).

FACS analysis

Purified CD4 cells harvested from plate stimulations were spun and resuspended in FACS buffer containing PBS with 1% BSA and 0.1% sodium azide. Surface staining was performed with PE anti-mouse CD25 (PC61; BD Biosciences), PE anti-mouse CD62L (MEL-14; BD Biosciences), PE anti-mouse CD39 (24DM51; eBioscience), and PE anti-mouse GARP (YG1C86; eBioscience). Cells were incubated with Abs at 5 μ g/ml for 30 min on ice, followed by two washes with FACS buffer. Subsequent intracellular staining was performed with the Foxp3 intracellular staining kit (eBioscience), according to manufacturer's recommendation. Allophycocyanin or PE anti-mouse Foxp3 (FJK-169; eBioscience) was incubated with cells for 30 min at 2.5 μ g/ml on ice. Following intracellular staining, cells were washed twice and fixed with Cytotfix (BD Biosciences) before reading on FACSCalibur (BD Biosciences).

In vivo suppression assay

Twelve-well tissue culture plates were coated overnight with 1 ml PBS containing 2 μ g/ml anti-CD3 (2C11; BD Biosciences) and an equivalent of 20 μ g/ml human IgG. For the anti-CD3 plus rB7-2.Fc stimulation, this was 3 μ g/ml rB7-2.Fc plus 15.5 μ g/ml human IgG, and for anti-CD3 plus rB7-2.Fc plus rBTNL2.Fc, this was 3 μ g/ml rB7-2.Fc plus 11 μ g/ml rBTNL2.Fc plus 4.5 μ g/ml human IgG. Cells were isolated from C57BL/6 (CD45.2⁺) spleen and lymph nodes, CD25⁺ cells removed by positive selection (CD25 beads; Miltenyi Biotec), and remaining CD4 cells purified by positive selection (CD4 beads; Miltenyi Biotec). One million CD4 cells were added to 12-well plates, and cells were cultured in 1 ml media containing 0.36 ng/ml rTGF- β and 10 ng/ml murine rIL-2. On day 4, 0.5 ml exhausted media was removed from wells, and 1 ml fresh media with rTGF- β and rIL-2 at the same concentrations as above were added to cultures. On day 5, all cells were harvested out of 12-well plates, counted on Vicell, and stained for CD4, CD25, and Foxp3, as above. Remaining cells from each stimulation were prepared for adoptive transfer by adjusting volume to 20 million cells/ml. Fresh, control nTreg suppressor

cells were prepared by CD4/CD25-positive selection (Miltenyi Biotec) of cells isolated from C57BL/6 lymph nodes. Unactivated effector CD4 T cells were prepared from spleen and lymph nodes from congenic C57BL/6 SJL mice (CD45.1⁺) by depleting CD25⁺ cells first and then positively selecting CD4 cells. A 1:1 ratio of suppressor/effector cells in 200 μ l PBS was injected i.v. (1 million suppressors, 1 million effectors) into C57BL/6 RAG2 KO hosts. Animal weights were monitored twice weekly for 2 mo, and host animals were euthanized on day 58, harvesting spleen, mesenteric lymph node, and peripheral lymph node (cervical + inguinal) as well as the entire small intestine and colon for histology. Cells were isolated from spleen and lymph nodes and quantitated on Vicell, and remaining cells were stained for CD4, CD8, CD45.2, and CD25. Two sections each of duodenum, jejunum, ileum, proximal, and distal colon were collected and fixed in 10% neutral buffered formalin for 24 h. Tissues were subsequently dehydrated through graded alcohols and xylene and embedded in paraffin. Tissues were sectioned at 5 μ m, deparaffinized, stained with H&E, and coverslipped. Sections were examined by a pathologist and assigned a score reflecting the degree of epithelial hyperplasia and inflammation according to the following criteria: grade 0 = no change over normal; grade 0.5 = minimal inflammation, no hyperplasia; grade 1.0 = mild inflammation, no hyperplasia; grade 1.5 = mild inflammation, minimal hyperplasia; grade 2.0 = mild/moderated inflammation, minimal hyperplasia; grade 2.5 = mild/moderate inflammation, mild hyperplasia; grade 3.0 = moderate inflammation, mild/moderate hyperplasia; grade 3.5 = moderate inflammation, moderate hyperplasia; grade 4.0 = moderated/marked inflammation, moderate hyperplasia; grade 4.5 = marked inflammation, moderate/marked hyperplasia, \pm ulceration; grade 5.0 = marked inflammation, marked hyperplasia, ulceration. The inflammation scores assigned to the tissues in each pair were averaged and the average plotted. Statistical differences between groups were determined by using the Kruskal Wallis test with Dunn's multiple comparisons.

Results

BTNL2 inhibits cytokine production and Akt and Foxo phosphorylation, and promotes Foxp3 expression

A method often employed to assess costimulatory molecule function is T cell stimulation with beads or surfaces coated with anti-CD3 and coimmobilized costimulatory proteins. Our group and others have previously published that immobilized rBTNL2.Fc and anti-CD3 severely limit the ability of mouse primary T cells to become activated, proliferate, and produce inflammatory cytokines (21, 22, 25). A second signal in the form of anti-CD28 has been shown to partially restore T cell activation in the presence of BTNL2 (21, 25), and inhibition by BTNL2 can be further overcome by exogenous IL-2 (21, 25). We investigated the ability of immobilized B7 ligands, in the form of rB7-1.Fc or rB7-2.Fc, to activate primary murine T cells in the presence of rBTNL2. Fc. Fig. 1A shows typical results from these experiments. T cell proliferation was induced with anti-CD3 alone, augmented with immobilized rB7, and inhibited with rBTNL2. Coimmobilization of rB7 and rBTNL2 at the ratios described reversed BTNL2 inhibitory activity, restoring T cell proliferation.

Although rB7 stimulation reversed the proliferative block induced by rBTNL2, we hypothesized that rBTNL2 may modulate other components of rB7 costimulation and alter the phenotype of the activated T cells. Indeed, interrogation of multiple cytokines revealed that rBTNL2 still delivered a suppressive signal in the presence of B7 costimulation, resulting in greatly reduced secretion of IL-2, IL-13, IL-17, IFN- γ , and several other cytokines (Fig. 1B). In a pilot experiment designed to examine the effects of rBTNL2 on global gene expression induced by anti-CD3 alone, we observed that rBTNL2 not only suppressed the transcription of the majority of anti-CD3-induced genes, but also modulated a small set of genes characterized as targets of the Foxo1 and Foxo3 transcription factors (Supplemental Fig. 1). As Foxo transcription factors are known to be inactivated by Akt phosphorylation, we examined the phosphorylation status of Akt and Foxo1 in similar T cell stimulation conditions. Whereas anti-CD3/rB7-2 stimulation resulted in strong Akt phosphorylation at Ser⁴⁷³, inclusion of

rBTNL2 inhibited Akt phosphorylation, resulting in phospho-Ser⁴⁷³ levels similar to that found in unstimulated T cells (Fig. 2A). A more striking effect of rBTNL2 on this pathway was observed by monitoring Foxo1 phosphorylation at the Akt target residues Thr²⁴ and Ser²⁵⁶. In this study, inclusion of rBTNL2 with anti-CD3/rB7-2 stimulation resulted in complete inhibition of Foxo1 phosphorylation at these sites (Fig. 2B). Together, these results show that BTNL2 stimulation in the context of CD3/CD28 ligation selectively inhibits Akt signaling and cytokine production, while leaving pathways responsible for T cell proliferation intact.

Although the importance for Foxo transcription factors in maintaining cellular senescence and survival has been appreciated for several years, a role in promoting Foxp3 expression and Treg development has only recently been described (26–29). Foxo1 and Foxo3a were found to cooperate with TGF- β -induced signals to promote Foxp3 transcription and iTreg differentiation. To examine whether BTNL2 would also synergize with TGF- β to induce Foxp3 expression, CD4⁺ T cells were stimulated in the presence of low concentrations of TGF- β . Because BTNL2 inhibits IL-2 production (Fig. 1B) (21, 22), and because IL-2 is an important Treg growth factor, exogenous IL-2 was included to more directly assess the effects of rBTNL2 on iTreg differentiation. When T cells were stimulated with anti-CD3 alone or with rB7-1 or rB7-2, considerable T cell expansion was observed by day 4 (Fig. 3A; from 0.3 million to \sim 1.5 million CD4⁺ T cells). Inclusion of rBTNL2 in the stimulation conditions inhibited expansion only in the absence of CD28 ligands, similar to results shown in Fig. 1A, where proliferation was monitored by [³H]dT incorporation. Negligible total numbers of Foxp3⁺ cells were recovered from any of these six stimulation conditions (Fig. 3A). When exogenous IL-2 was added to the cultures, very similar results were obtained (Fig. 3B). Addition of a suboptimal concentration of TGF- β induced <20% Foxp3⁺ cells in conditions without rBTNL2. In the presence of rBTNL2, however, more than half of the cells expressed Foxp3 (Fig. 3C). Whereas the combination of TGF- β and rBTNL2 limited overall T cell expansion (Fig. 3C), addition of IL-2 restored growth without altering the percentage of Foxp3⁺ cells (Fig. 3D). In contrast, addition of IL-2 to cultures stimulated with anti-CD3 and TGF- β alone resulted in twice the number of Foxp3⁺ T cells without increasing the total number of TGF- β -induced Foxp3⁺ cells. Very similar results were obtained when CD25-depleted CD4 cells were used as the starting population, indicating the large number of Foxp3⁺ cells recovered after stimulation with rBTNL2 did not result from rapid expansion of pre-existing Treg (Supplemental Fig. 2). These data show that rBTNL2 can function in concert with B7 costimulation and TGF- β to induce Foxp3 expression without impairing a T cell's proliferative potential.

Foxp3 induction in naive T cells is a coordinated event in which the strength of TCR and CD28 signals is integrated with the surrounding cytokine and metabolic milieu. Decreasing TCR/CD28 agonism levels are known to support Foxp3 induction in the presence of TGF- β (30). Because BTNL2 is a relatively large protein, and the receptor for BTNL2 on T cells has not been identified, it was important to demonstrate that recombinant BTNL2 protein did not decrease anti-CD3-driven TCR agonism through nonspecific steric hindrance and resulting in upregulated Foxp3 expression. To address this issue, we chose two control proteins that are known to bind receptors on CD4 T cells and have the same predicted m.w. as rBTNL2.Fc: rICAM.Fc and anti-CD132. These 150-kDa control proteins were coimmobilized in the same manner as rBTNL2 with anti-CD3 and rB7-2, and Foxp3 percentages and total numbers were evaluated. Supplemental Fig. 3 shows that even though molecular equivalents of human IgG,

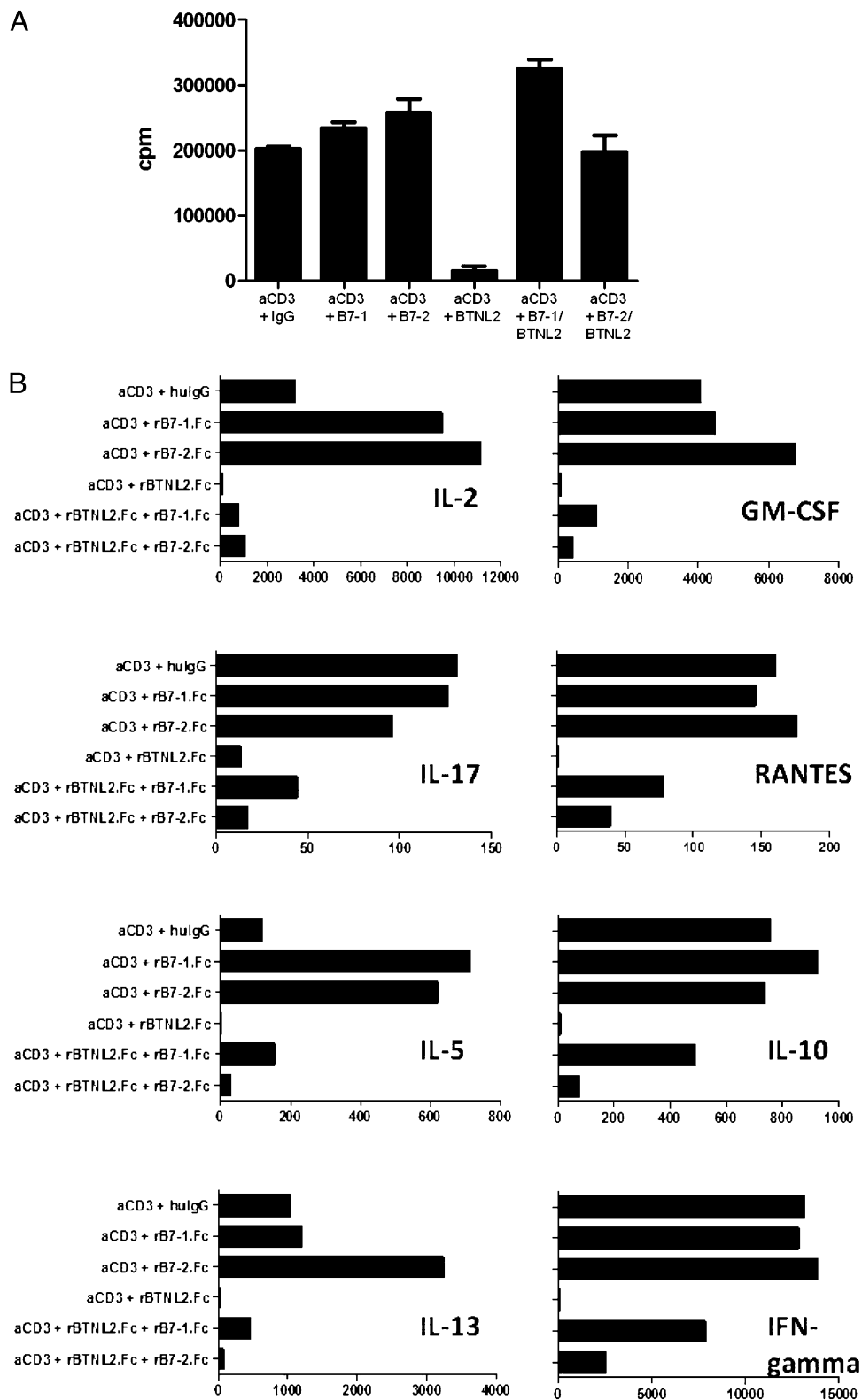
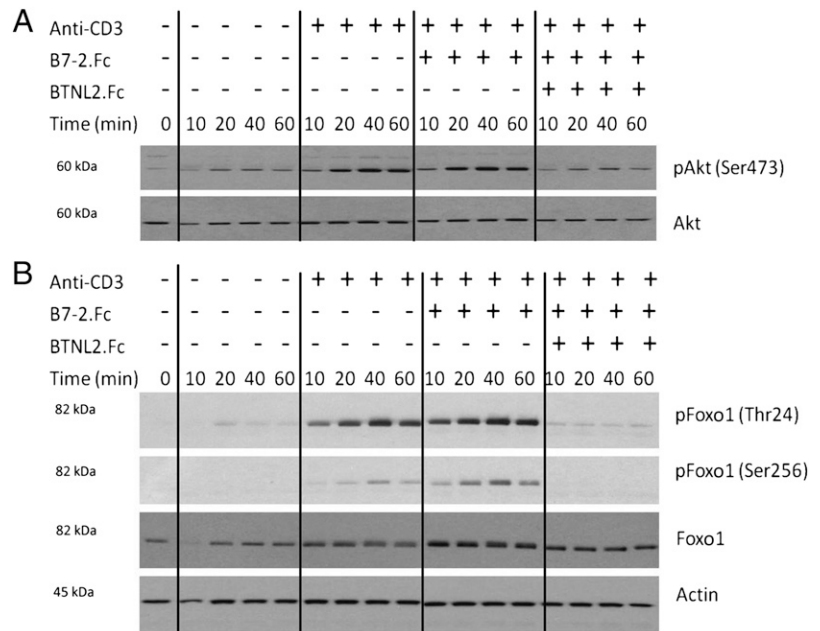


FIGURE 1. BTNL2 inhibits CD4 T cell cytokine production without impacting proliferation in the context of B7 costimulation. Purified mouse CD4 T cells were stimulated with immobilized reagents, as shown. **(A)** Proliferation was measured by pulsing cultures with [³H]thymidine on day 3. Error bars represent SEM of triplicate wells. **(B)** Cytokines were analyzed in supernatants harvested prior to measuring proliferation on day 3. Data shown are representative of at least two experiments.

rICAM, and anti-CD132 were plated at the same anti-CD3/rB7 ratios as rBTNL2, only CD4 cultures containing rBTNL2 induced Foxp3 percentages above 8% (BTNL2 34%, IgG 8%, rICAM 1%, anti-CD132 6%). Additionally, the BTNL2/B7 cultures resulted in

>2-fold expansion of total Foxp3⁺ CD4 T cell numbers compared with the control stimulations. Another method employed to investigate BTNL2's specific effects on Foxp3 induction was to alter the anti-CD3/B7 stimulation levels in the presence or absence of

FIGURE 2. BTNL2 inhibits Akt-dependent Foxo1 phosphorylation. Purified mouse CD4 T cells were stimulated with immobilized reagents, as shown. **(A)** CD4 T cell total and phosphorylated Akt levels were measured by Western blot analysis at 0, 10, 20, 40, and 60 min of stimulation with the indicated immobilized proteins. **(B)** CD4 T cell total and phosphorylated Foxo1 levels were measured by Western blot analysis at 0, 10, 20, 40, and 60 min of stimulation with the indicated immobilized proteins. Data shown are representative of at least two experiments.



BTNL2. Variable concentrations of both anti-CD3 and rB7 were plated \pm rBTNL2 and compared with the baseline conditions identified in Fig. 3 (Supplemental Fig. 4). Inclusion of BTNL2 increased Foxp3 percentages at all anti-CD3 and rB7 stimulation conditions. Total Foxp3⁺ CD4 numbers were also increased compared with the same stimulations without rBTNL2. The only exception was the lower anti-CD3 concentration of 0.5 μ g/ml, which still had the higher percentage of Foxp3⁺ cells in the presence of BTNL2, but did not generate greater total Foxp3⁺ cell numbers, indicating that the BTNL2 inhibitory effect did not allow for CD4 T cell expansion at lower TCR agonism strength as would be expected from our previous publication (22).

Foxp3⁺ CD4 T cells induced with BTNL2/B7 possess characteristics of nTreg

To further characterize the Foxp3⁺ cells generated with rBTNL2/rB7 in the presence of exogenous TGF- β and IL-2, we examined the surface markers of cells generated from these stimulation

conditions. Some markers characteristic of nTreg were strongly upregulated in rBTNL2/rB7-2-stimulated cells (Fig. 4). The most dramatic example was CD62L, the gene for which (*Sell*) is a target of Foxo1 transcriptional activation. Whereas activation of cells with anti-CD3 or anti-CD3 plus rB7-2 downregulated CD62L surface expression, addition of rBTNL2 promoted maintenance of CD62L expression on \sim 50% of the cells. High CD62L expression was observed in both Foxp3⁺ and Foxp3⁻ populations, suggesting Akt inhibition and Foxo1 activation were not dependent on Foxp3 expression.

CD39, an ectonucleotidase that converts AMP to adenosine at the cell surface, has been reported to be a marker, as well as a functionally suppressive molecule, on both human and mouse Treg (31). GARP is a surface receptor that binds latent TGF- β and has been shown to be a specific marker for activated Treg (32, 33). The genes for both of these markers are positively regulated by Foxp3 (34, 35). Both CD39 and GARP were more highly expressed on Foxp3⁺ cells in all stimulation conditions; however,

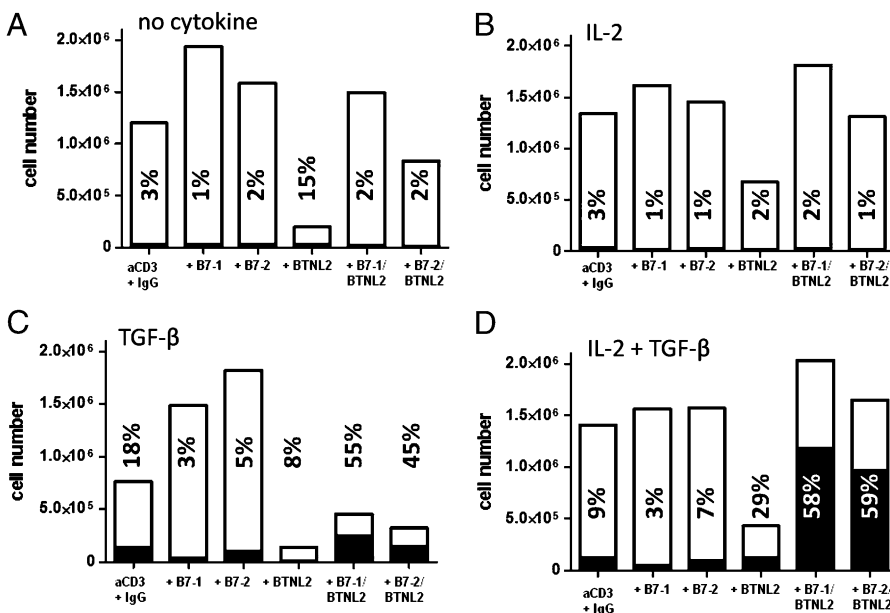


FIGURE 3. BTNL2 induces Foxp3⁺ expression without inhibiting proliferative potential. Purified CD4 T cells were stimulated with the indicated conditions. On day 4, T cells were harvested, counted, and characterized by flow cytometry. Individual culture conditions were supplemented with **(A)** no cytokine, **(B)** 10 ng/ml IL-2, **(C)** 0.36 ng/ml TGF- β , or **(D)** 10 ng/ml IL-2 and 0.36 ng/ml TGF- β . Open bars represent total cell numbers, and the shaded region is the number of Foxp3⁺ cells within that population. The CD4⁺CD25⁺Foxp3⁺ cell percentage is shown numerically. Data shown are representative of three experiments.

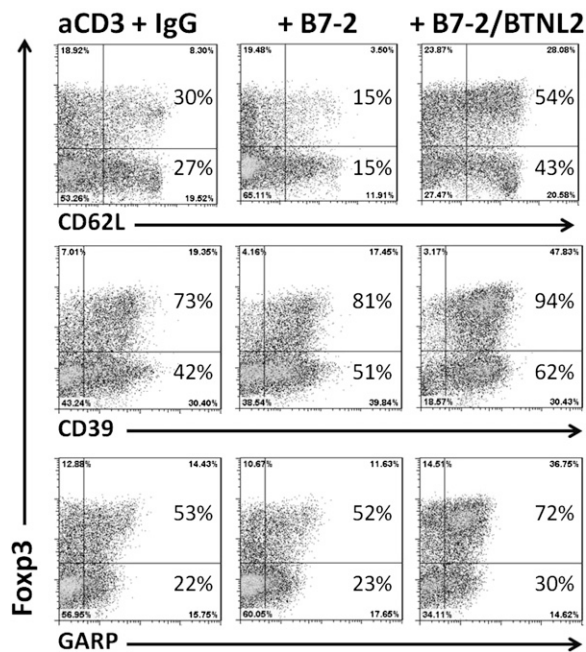


FIGURE 4. BTNL2 induces Foxp3⁺ CD4 T cells expressing Treg surface markers. Purified CD4 T cells were stimulated with the indicated conditions and supplemented with IL-2 and TGF- β . On day 4, T cells were characterized by flow cytometry. The percentages shown in the larger font were calculated as the percentage of cells expressing the indicated surface marker among either Foxp3⁺ or Foxp3⁻ cells. Data shown are representative of two experiments.

BTNL2 further upregulated their expression on both Foxp3⁺ and Foxp3⁻ cells, suggesting that Foxp3 and BTNL2 may independently promote their expression (Fig. 4). These data show BTNL2 can induce specific surface T cell markers that are shared with nTreg.

To further examine the effects of BTNL2 stimulation on T cell phenotype, we compared global gene expression profiles of CD25-depleted CD4 T cells stimulated with anti-CD3 and rB7-2 with or without rBTNL2. Cultures were also supplemented with IL-2 and low concentration TGF- β . Several genes characteristic of nTreg and TGF- β -induced iTreg were found to be similarly upregulated or downregulated in BTNL2-induced iTreg (Fig. 5A). These included proteins thought to mediate Treg suppressor function, such as Entpd1/CD39, Nt5e/CD73, Fgl2, and Lgals3. BTNL2-regulated genes were also examined for enrichment of Gene Ontology annotations (Fig. 5B). Genes for cytokines and chemokines were found to be particularly overrepresented, consistent with the capacity for BTNL2 to alter T cell effector function. In particular, several chemokines were found to be upregulated, whereas multiple proinflammatory cytokines were downregulated (Fig. 5C). Regulators of apoptosis and proliferation were also significantly enriched among genes regulated by BTNL2. In aggregate, the gene expression profile of BTNL2-induced iTreg resembles that of nTreg, but also exhibits unique regulation of expression of diverse immune effector molecules.

CD4 cells activated in the presence of rBTNL2 and rB7-2 control effector T cell expansion and enteritis when cotransferred into RAG2-deficient hosts

We next assessed whether BTNL2-induced iTreg possessed suppressor activity. Because of a predicted role for BTNL2 in intestinal immune tolerance, we were interested in using an in vivo system in which intestinal inflammation could be induced. Adoptive transfer of effector T cell populations into immunodeficient

hosts is a common approach for inducing enteritis/colitis (36). Suppression of inflammation induced by transfer of congenically marked CD25⁻CD4⁺ effector cells into RAG2-deficient hosts was assessed for three separate test suppressor populations, as follows: 1) purified CD25⁺CD4⁺ nTreg (88% Foxp3⁺); 2) CD25⁺CD4⁺ cells stimulated with anti-CD3/rB7-2 (2% Foxp3⁺); or 3) CD25⁺CD4⁺ cells stimulated with anti-CD3/rB7-2/rBTNL2 (48% Foxp3⁺). The stimulation conditions for generating the latter two suppressor populations also included IL-2 and low-dose TGF- β . Following transfer of 1 million suppressor cells and 1 million effector cells, host body weight and stool were monitored for signs of enteritis over a 2-mo period. Fig. 6A shows that only animals receiving suppressor cells preactivated with anti-CD3 and rB7-2 did not continue to gain weight. One animal from this same group was euthanized before the end of the study due to extensive weight loss and external signs of enteritis (data not shown). Although inflammation of the colon can be achieved with T cell adoptive transfer into immunodeficient hosts, we observed the greatest amount of inflammation in the small intestine, with the highest level of disease in the animals that received suppressor cells activated with anti-CD3 and rB7-2. Fig. 6B shows histopathology scores from different regions of the small intestine from each study group. Across all three sections of small intestine, the nTreg suppressor cells significantly limited inflammation compared with those activated with anti-CD3 and rB7-2, whereas the suppressor cells activated with anti-CD3, rB7-2, and rBTNL2 also limited inflammation in the duodenum and jejunum when compared with the same group.

With this T cell transfer model it is also possible to track the number of effector and suppressor cells in different immune compartments to assess the suppressor cells' ability to limit effector cell homeostatic expansion. Two months after T cell transfer, CD45.2⁻ effector T cell expansion was evaluated in spleen, peripheral lymph nodes (cervical and inguinal), and mesenteric lymph nodes. The highest number of CD45.2⁻ effector cells was recovered from animals receiving anti-CD3/rB7-2 suppressor cells (Fig. 6C). Effector T cell expansion was most efficiently limited by nTreg in all three compartments, and anti-CD3/rB7-2/rBTNL2 suppressor cells lowered the recovered number of effector cells compared with those activated with anti-CD3 and rB7-2. In the mesenteric lymph nodes, BTNL2-induced suppressor cells limited effector CD4 numbers with statistical significance, limiting effector numbers similar to nTreg. These data are consistent with the small intestine inflammation scores and demonstrate that BTNL2/B7/TGF- β -mediated Foxp3 induction can lead to suppressive CD4 T cells capable of limiting the expansion and effector functions of pathogenic T cells.

Discussion

Thymic selection of T cells results in a diverse repertoire of TCRs that is precisely tuned to detect the presence of foreign peptide sequences presented by self-MHC. If the novel peptide derives from a pathogenic infectious agent, it is incumbent on the responding T cell to translate such a stimulus into a vigorous proliferative response and differentiation program to generate a clonal population of effector T cells capable of combating the infection. To help activate such a response, T cells employ a second signal in the form of CD28, brought into close proximity to MHC/TCR interactions by its ligands, B7-1 or B7-2, present on the APC. CD28 activates several signaling molecules necessary for proliferation and differentiation, such as PI3K, Akt, NF- κ B, and MAPK. In some cases, however, such responses may be deleterious to the host. For example, if the pathogen load is high or the infection is in a vital organ, an unchecked immune response may be fatal. In addition, proinflammatory responses by T cells encountering

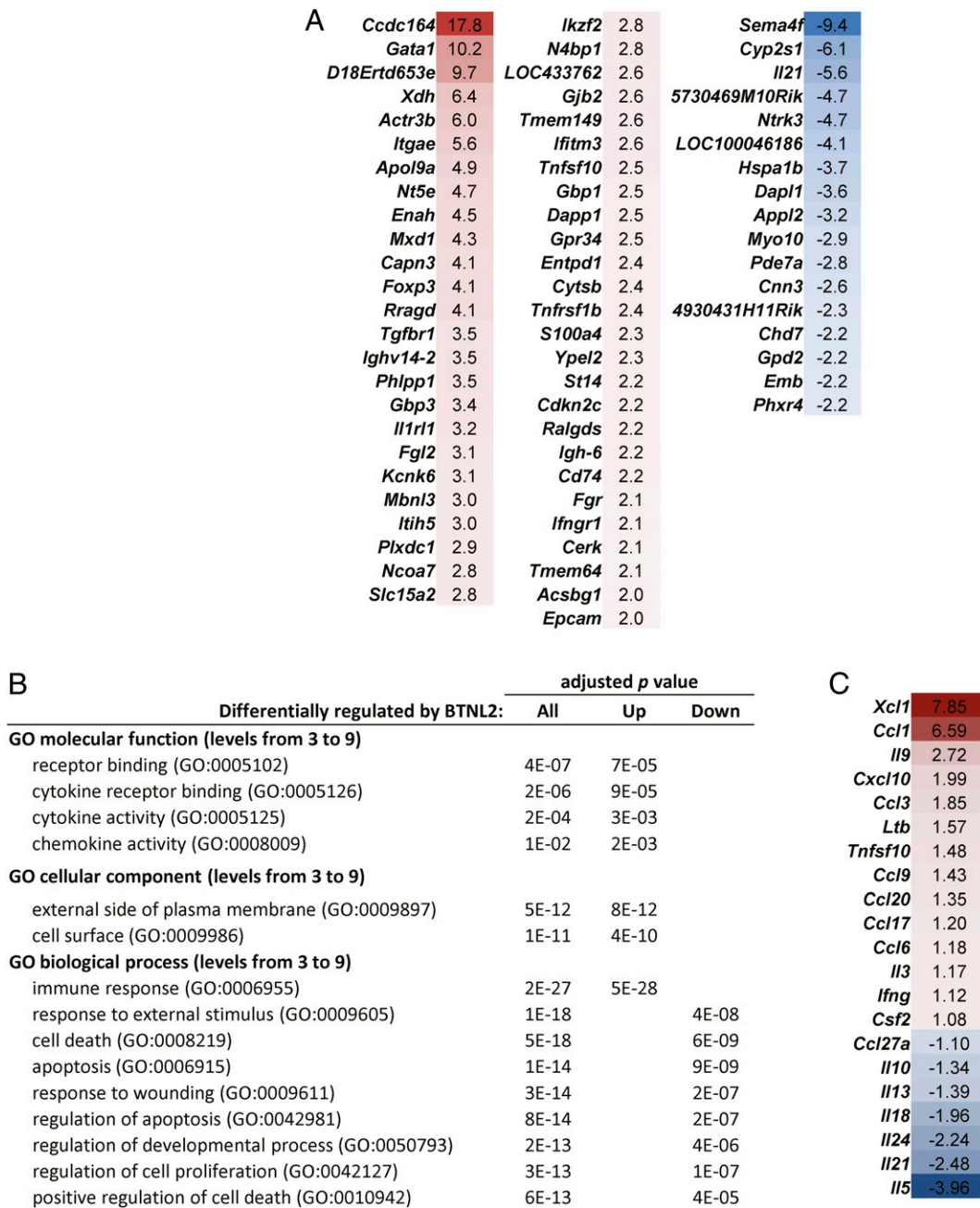


FIGURE 5. BTNL2 induces a nTreg gene signature in CD4 T cells. CD25-depleted CD4 T cells were stimulated with anti-CD3 and rB7-2.Fc in the presence or absence of rBTNL2.Fc for 3 d in cultures supplemented with IL-2 and TGF- β . Each condition was stimulated and processed independently in duplicate. RNA was isolated and assessed for gene expression by Affymetrix cDNA arrays. **(A)** Data were analyzed for genes upregulated or downregulated in the presence of BTNL2, and selected genes characteristic of nTreg and TGF- β -induced iTreg are shown along with fold changes. **(B)** All genes regulated by BTNL2 ($p < 0.01$) were examined for enrichment of Gene Ontology annotations using FatiGO. **(C)** Chemokines and cytokines regulated by BTNL2.

novel tissue-restricted self-Ags, food, benign environmental Ags, or Ags from commensal bacteria would also lead to undesirable tissue damage and pathology.

The importance in regulating the strength of TCR/CD28-activating stimuli is reflected by the large number of proteins that are capable of attenuating this pathway. These include the CTLA-4 and PD-1 coreceptors, as well as a growing list of B7-related ligands that suppress T cell function similar to PD-1 ligands, but whose receptors have yet to be identified. Furthermore, recent data suggest that the activities of these costimulatory molecules are more complex than originally imagined. For example, Butte et al. (37, 38) found an unexpected interaction be-

tween B7-1 and PD-L1. Two recent publications also identify PD-L1 as the first inhibitory ligand to directly support Foxp3 expression and Treg differentiation (15, 16).

In this study, we show that BTNL2, a previously described inhibitory ligand of the butyrophilin/B7 family, can also induce Foxp3 expression and drive iTreg formation. We observe a role for BTNL2 in tempering the B7 signal, resulting in reduced proliferation and decreased production of inflammatory cytokines. We show that BTNL2 inhibits Akt phosphorylation and preserves Foxo1 activity, which is normally not operative in B7-activated CD4 cells, and was recently reported to support Foxp3 expression (27). Consistent with these reports, we found that BTNL2

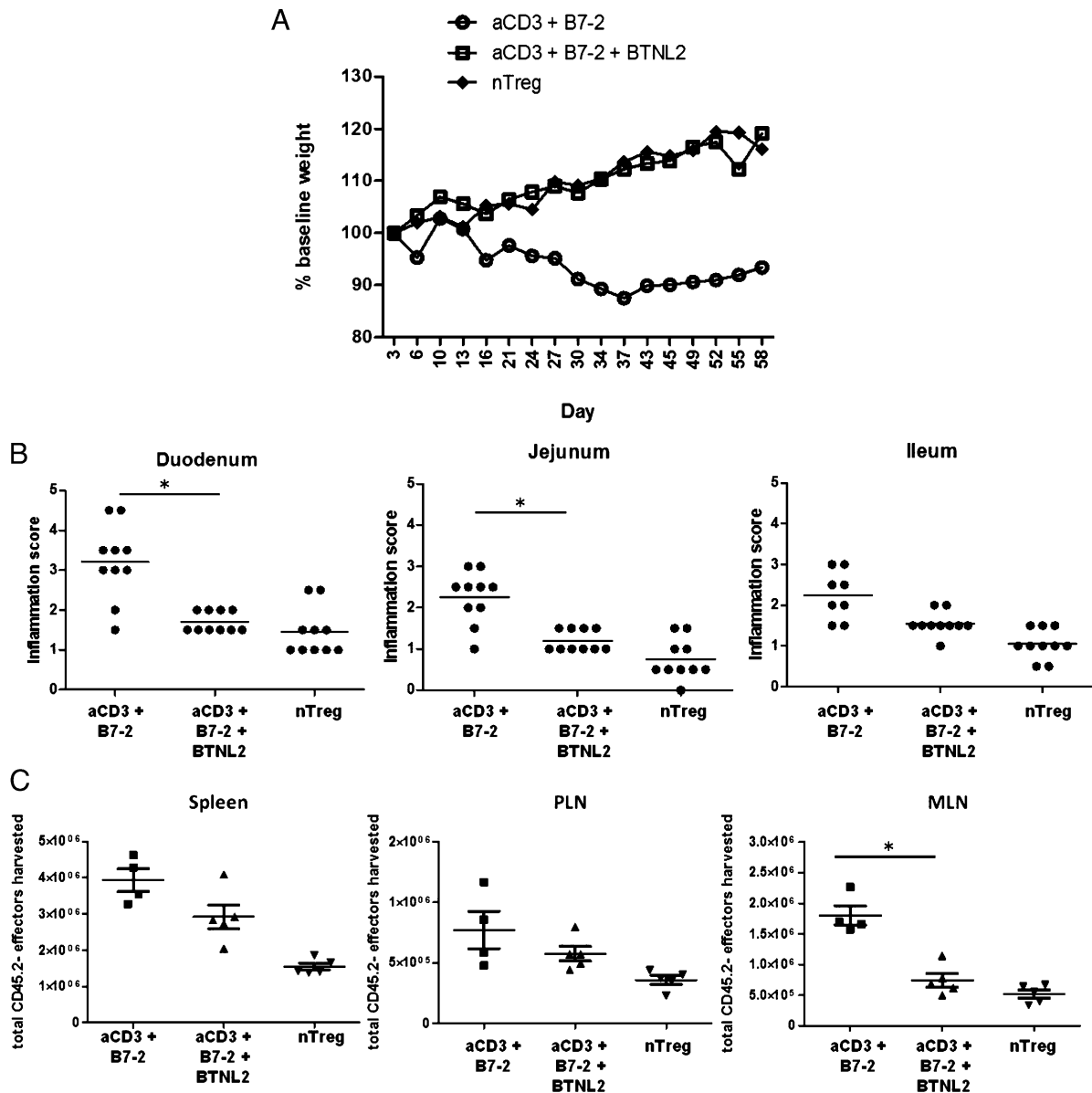


FIGURE 6. CD4 T cells activated in the presence of BTNL2 prevent enteritis when adoptively transferred with effector T cells into immunodeficient hosts. CD25-depleted CD4 T cells were activated with anti-CD3, rB7-2.Fc, TGF- β , and IL-2 for 5 d in the presence or absence of rBTNL2.Fc. One million cells from the stimulations were harvested and adoptively transferred with one million freshly isolated, congenic, CD25-depleted CD4 T effector cells into RAG2 KO hosts ($n = 5$ for each condition). A third group of freshly purified nTreg was also cotransferred with the same ratio and number of effector T cells. Animals were monitored for 58 d before being sacrificed for measurement of gut inflammation and effector T cell expansion in various immune compartments. **(A)** Average group animal weights expressed as percentage of the average baseline weight on day 3. **(B)** Duodenum, jejunum, and ileum inflammation was histologically graded. $*p \leq 0.05$. **(C)** Total congenic effector cell numbers were quantitated from spleen, peripheral, and mesenteric lymph nodes harvested on day 58. Statistical significance between the indicated groups was determined by Student t test. $*p \leq 0.001$.

synergizes with a limiting concentration of TGF- β to promote Foxp3 expression and a T cell differentiation program that shares several features with that of nTreg. Lastly, BTNL2-generated Foxp3⁺ cells were found to be suppressive *in vivo*, indicating that a sustainable Treg program was induced.

Reduced activity of Akt and its associated signaling complex mTOR has emerged as a central component of Treg development and function. Originally thought to be an important driver of T cell proliferation and metabolism, Akt/mTOR signaling was recently found to also be important for proinflammatory cytokine production, but not absolutely required for T cell proliferation (39). Consistent with this report, BTNL2 was found to reduce Akt signaling and cytokine production without impairing IL-2-induced proliferation. One potential mechanism by which BTNL2

may reduce Akt function is through Phlpp1, an Akt phosphatase recently found to be important for human and mouse Foxp3 expression (40). Phlpp1 is upregulated in mouse and human nTreg (35, 40), and we report in this work that BTNL2-induced iTreg also upregulate Phlpp1 (Fig. 5A). Identification of a putative BTNL2 receptor should allow for better understanding of how BTNL2 influences T cell differentiation.

An unexpected finding from analysis of BTNL2-affected transcription was a unique cytokine/chemokine expression profile, in which genes for several chemokines were upregulated, whereas genes for several cytokines were repressed (Fig. 5C). The chemokines expressed by BTNL2-induced iTreg are known to recruit multiple immune cell types, including T cells, B cells, NK cells, monocytes/macrophages, and dendritic cells. If this phenotype is

recapitulated in vivo following T cell encounter of cognate Ag in the context of BTNL2-expressing APC (e.g., in the small intestine), then one function for the newly generated iTreg might be to recruit immune cells to further evaluate the source of novel Ag while maintaining an immunosuppressive environment. Such processes may play an important role in establishing dominant tolerance to benign environmental Ags.

Originally shown to be an inhibitor of T cell activation, we have characterized BTNL2 as a modulator of T cell differentiation, joining PD-L1 as an inducer of Foxp3 expression and iTreg formation. The specific conditions responsible for iTreg formation are critical for controlling inflammation at mucosal sites (41), underscoring the important role such ligands may play in maintaining tolerance to environmental Ags. The continued refinement of our understanding of how the B7/butyrphilin family modulates T cell costimulation and induces different types of iTreg should enable the conceptualization of therapeutics that promote tolerance in autoimmune patients.

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Disclosures

All authors are employees of Amgen.

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