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Butyrophilin Btn2a2 Inhibits TCR Activation and Phosphatidylinositol 3-Kinase/Akt Pathway Signaling and Induces Foxp3 Expression in T Lymphocytes

Johannes U. Ammann, Anne Cooke, and John Trowsdale

The butyrophilin-related protein Btn2a2 was upregulated on murine APC including CD19⁺ B cells, CD11b⁺F4/80⁺ peritoneal macrophages, and CD11c⁺ bone marrow-derived dendritic cells after activation with LPS or Pam3CysK4, suggesting a role in modulation of T lymphocytes. Consistent with this, binding of mouse Btn2a2-Fc to CD3⁺ primary mouse T cells stimulated with anti-CD3 and anti-CD28 reduced the number of proliferating cells and entry of cells into the cell cycle. Binding of Btn2a2-Fc to anti-CD3-stimulated T cells inhibited CD3 ϵ , Zap70, and subsequent Erk1/2 activation. It also interfered with activation of the regulatory subunit of PI3K, p85, and activation of Akt in T cells stimulated with both anti-CD3 and anti-CD28. Inhibition of Akt activation by Btn2a2-Fc was, in contrast to inhibition by programmed death ligand-1-Fc, not overcome by anti-CD28 costimulation. Using Foxp3-GFP-transgenic, naive T cells, Btn2a2-Fc induced de novo expression of Foxp3 in a dose-dependent manner, and Btn2a2-Fc-induced CD4⁺CD25⁺Foxp3⁺ T cells had inhibitory properties. The data indicate an important physiological role for Btn2a2 in inhibiting T cell activation and inducing Foxp3⁺ regulatory T cells. *The Journal of Immunology*, 2013, 190: 5030–5036.

T lymphocyte activation requires two classes of signals: Ag-specific and coregulatory. B7 protein family molecules engage ligands on T cells involved in coregulation and include a number of butyrophilin-related molecules. Butyrophilin, a type I transmembrane glycoprotein, was purified from bovine milk (1). The human BTN1A1 gene mapped to the extended MHC region (2). Nearby, six related genes grouped into three families: BTN2A1, BTN2A2, BTN2A3, BTN3A1, BTN3A2, and BTN3A3 (3, 4). Genes orthologous to BTN1A1 and BTN2A2, Btn1a1 and Btn2a2, respectively, were mapped to mouse chromosome 13. Another butyrophilin-related gene, near HLA-DRA, was named BTNL-II or BTNL2, Btnl2 in mouse (5), and three other butyrophilin-like genes on chromosome 5 were named BTNL3, BTNL8, and BTNL9 (6). Other distant relatives of BTNL2 in mouse are Btnl1, Btnl5, Btnl6, Btnl7, and Btnl9 (7) and the Skint genes (8, 9).

BTN1A1 was expressed predominantly in mammary gland tissue (10, 11), although mouse Btn1a1 was detected in other tissues, including thymic epithelial cells (12). BTN2A1 and 2A2 were detected in many tissues (3, 13). Similarly, mouse Btn2a2 protein was found on the surface of nonactivated CD19⁺ B cells, CD11c⁺

dendritic cells (DC), CD11b⁺F4/80⁺ peritoneal macrophages, NK1.1⁺ NK cells, and on CD3⁺ T cells, when activated, and by immunofluorescence on thymic epithelial cells (12). Human BTN3 proteins (BTN3A1, -A2, and -A3) were detected on a variety of cells and tissues (14, 15). Mouse Btnl1 was expressed on bone marrow-derived DC, macrophages, and activated B cells (16) and at high levels in the small intestine, where its expression on enterocytes was increased after treatment with IFN- γ (17). Mouse Btnl2 was also widely expressed (5, 18, 19).

It has been suspected that butyrophilin family molecules would have a coreceptor role, with the possible exception of BTN1A1, which, through homotypic interaction, facilitates milk droplet secretion (20). However, exosomes in human breast milk, containing BTN1A1, inhibited cytokine production by PBMC and led to an expansion of CD4⁺Foxp3⁺ T cells (21). In support of a coreceptor role, mouse Btn1a1-Fc or Btn2a2-Fc fusion proteins inhibited T cell proliferation and IL-2 and IFN- γ production by CD4⁺ or CD8⁺ T cells activated with anti-CD3 or anti-CD3 and anti-CD28 (12). A dose-dependent inhibition of anti-CD3- and anti-CD28-induced T cell proliferation was also observed with plate-bound mouse Btnl2-Fc (18, 19). In addition, inhibition of IL-2 production by Btnl2-Fc was detected (19). Btnl2 engagement overcame the effects of the positive coregulatory molecule ICOS ligand on T cell proliferation and reduced secretion of cytokines such as TNF- α , GM-CSF, IL-2, IL-4, IL-6, IL-17, and IFN- γ , but not IL-10 (18). Btnl1 also affected T cell proliferation through inhibition of cell cycle entry (16). For BTN3A1, also called BTN3A, a stimulatory role in stress sensing by $\gamma\delta$ -T cells was demonstrated when bound by a specific Ab (22, 23).

In an experimental autoimmune encephalomyelitis (EAE) mouse model, a blocking anti-Btnl1 Ab led to induction of EAE after vaccination with low doses of MOG (16). The Ab led to increased Th17 cells and IL-17 cytokine levels, suggesting a protective role for Btnl1 in the pathogenesis of EAE by preventing Th17 polarization (16). Using a model system for the interaction of intraepithelial lymphocytes, it was shown that Btnl1 on enterocytes inhibited IL-6 and IFN- γ production by these cells (17).

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J.U.A., A.C., and J.T. designed research and analyzed data; J.U.A. performed research; and J.U.A. and J.T. wrote the paper.

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Abbreviations used in this article: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; iTreg, induced regulatory T cell; nTreg, naturally occurring regulatory T cell; PD-L1, programmed death ligand-1; Treg, regulatory T cell.

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We set out to investigate the function of Btn2a2 in relation to T lymphocyte regulation.

Materials and Methods

C57BL/6 mice at age 6 wk were from Harlan Laboratories (Loughborough, U.K.). C57BL/6 and NOD Foxp3-GFP transgenic mice were housed in specific pathogen-free conditions according to Home Office requirements. Experiments were approved by the Ethical Review Committee.

Cells

Mouse cells were harvested as described (12). CD3⁺ or CD4⁺ T cells were enriched by negative selection (Stem Cell Technologies, London, U.K.). When naive T cells of NOD Foxp3-GFP mice were purified, negatively enriched CD4⁺ T cells were stained with anti-CD4, anti-CD25, and anti-CD62L Abs at 1 μ g/ml (eBioscience, Hatfield, U.K.) and sorted for CD4⁺ CD25⁻ CD62L^{high} Foxp3⁻ GFP⁻ cells. CD19⁺ B cells were enriched from spleen by positive selection. Macrophages were from peritoneal lavage. Bone marrow-derived DC were prepared as described (24). CD3⁺ 2B4 cells (25) were maintained in complete RPMI 1640, 5% FCS.

Professional APCs were activated in RPMI 1640 with 10% FCS, 100 ng/ml LPS (Sigma-Aldrich), or 1 μ g/ml Pam3CysK4 for 16 h. For T cell signaling and activation, plates were coated with 1 μ g/ml anti-CD3 clone 145-2C11, 1–5 μ g/ml anti-CD28 clone 37.51 (eBioscience), 10 μ g/ml anti-human Fc (Newmarket Scientific, Kennett, U.K.), and 10 μ g/ml Fc fusion protein (12).

For signaling assays, CD3⁺ primary T cells, or 2B4 cells, were seeded at 0.5×10^6 /ml, centrifuged, and incubated at 37°C and 5% CO₂. Stimulation was stopped using ice-cold PBS. For analysis of cell cycle progression or Foxp3-GFP expression, cells were incubated in RPMI 1640 with 10% FCS, 50 μ M 2-ME, and 2 mM L-glutamine (both from Sigma-Aldrich) for 3–7 d at 37°C and 5% CO₂. Human rTGF- β 1 (TGF- β) was from R&D Systems (Abingdon, U.K.).

For Treg induction experiments, CD4⁺CD25⁻CD62L^{high}Foxp3⁻ cells were activated with 1 μ g/ml anti-CD3, 1 μ g/ml anti-CD28, 10 μ g/ml anti-human Fc, and 10 μ g/ml Btn2a2-Fc at 0.5×10^6 cells/ml for 4 d. Induced regulatory T cells (Treg; CD4⁺CD25⁺Foxp3⁺), or control cells, were sorted and mixed with nonactivated CD3⁺CD25⁻Foxp3⁻ T cells 1:1, the latter labeled with 1 μ M eFluor647 (eBioscience). Mixed cells were cultured at 1.0×10^6 /ml with 1 μ g/ml anti-CD3, 1 μ g/ml anti-CD28, and 10 μ g/ml Fc fusion protein for another 4 d. Coinhibition was evaluated by dilution of cell proliferation dye in CD8⁺ T cells by flow cytometry and secretion of IFN- γ by ELISA (eBioscience). TGF- β in supernatants was probed by biological assay (26). The TGF- β assay is sensitive to 60–100 pg/ml.

Surface expression of Btn2a2 or other molecules was detected by flow cytometry as described previously (12). Cellular DNA content was assessed by propidium iodide or Hoechst 33342 staining (both Sigma-Aldrich) of cells.

Preparation and analysis of cell lysates

Cell pellets were lysed in buffer with protease inhibitor mix and phosphatase inhibitor mix (Sigma-Aldrich) at 1:100. Cell lysates on ice were mixed vigorously before centrifugation at 15,000 rpm and 4°C for 20 min. For protein immunoprecipitation, cells were lysed at $1-4 \times 10^7$ /ml in buffer with 20 mM Tris-HCl (pH 8), 150 mM NaCl, and 1% Nonidet P-40, and then with low-stringency buffer: 10 mM HEPES (pH 7.6), 250 mM NaCl, 0.1% Nonidet P-40, and 5 mM EDTA. CD3 ϵ was precipitated with clone CD3-12 (AbD Serotec) and p85 with Ab from Cell Signaling Technology. Biotinylated anti-Zap70 Ab (eBioscience) was used at 1 μ g/ml.

Immunoprecipitates were separated in 10% SDS-PAGE and Western blotted. The following Abs were used: anti-p-Tyr (clone 4G10; Millipore), anti- β -actin-HRP (Sigma-Aldrich), and anti-CD3 ϵ (clone CD3-12; AbD Serotec); and from Cell Signaling Technology: anti-Zap70 (clone D1C10E), anti-p-Erk1/2 (D3F9), anti-Erk1/2 (clone 137F5), anti-p-p85, anti-p85 (clone 19H8), anti-p-Akt (Ser⁴⁷³; clone D9E), anti-p-Akt (Tyr³⁰⁸; clone C31E5E), anti-Akt (clone C67E7), and anti-p27kip1.

Results

Activation of APC upregulates cell-surface Btn2a2

To probe the role of Btn2a2 in regulation of T cells by professional APC, we studied surface expression on CD19⁺ B cells, bone marrow-derived CD11c⁺ DC, and CD11b⁺ F4/80⁺ peritoneal macrophages. Cells were activated for 16 h with the TLR ligands LPS, which activates TLR4, or the lipoprotein Pam3CysK4, which activates TLR1 and TLR2. Activation of cells was confirmed

by cell-surface expression of MHC class II for DC and peritoneal macrophages or CD86 for B cells. Btn2a2 expression on the surface of activated B cells, bone marrow-derived DC, and peritoneal macrophages was increased compared with nonactivated cells (Fig. 1).

Btn2a2 binding inhibits cell cycle entry of activated T cells

Btn2a2-Fc engagement reduced proliferation and metabolic activity in anti-CD3- or anti-CD3/CD28-stimulated T cells (12). This could indicate a reduced progression of T cells into the cell cycle. To investigate this, enriched CD3⁺ primary T cells were stained with cell proliferation dye and activated using 1 μ g/ml anti-CD3, 1 μ g/ml anti-CD28, and either Btn2a2-Fc or human Fc at 10 μ g/ml. Four days after stimulation, cells were analyzed for dilution of the cell proliferation dye eFluor 670. Fig. 2A shows reduced T cell proliferation in the sample activated in presence of Btn2a2-Fc compared with the control. DNA content, analyzed by propidium iodide staining, showed that fewer cells progressed through the cell cycle when stimulated with Btn2a2-Fc (Fig. 2B).

To investigate the molecular basis for this, expression of p27^{kip1}, a putative inhibitor of the transition between the G₀/G₁ phase and S phase of the cell cycle, was studied (27). CD3⁺ T cells stimulated with anti-CD3 and Btn2a2-Fc fusion protein were analyzed by Western blotting (Fig. 2C). The activation with anti-CD3 alone was chosen because it was reported that CD28 costimulation inhibits expression of p27^{kip1} (28). High levels of p27^{kip1} were found by Western blotting in T cells stimulated with anti-CD3 and Btn2a2-Fc (Fig. 2B). These results indicate that ligation of Btn2a2-Fc leads to higher p27^{kip1} levels, which reduce cell cycle entry of T cells.

Btn2a2 ligation inhibits TCR activation

To investigate whether Btn2a2-Fc coinhibits activation of T cells at the level of the TCR and CD3 complex, cells of the T cell line 2B4 were stimulated for 4 min using anti-CD3 and Btn2a2-Fc. Activation of the TCR/CD3 complex was assessed by analysis of CD3 ϵ phosphorylation (Fig. 3). After stimulation for 4 min, cells were lysed, and the molecule of interest was precipitated. As shown in Fig. 3A, interaction of Btn2a2-Fc with the T cells strongly reduced CD3 ϵ phosphorylation. The molecule

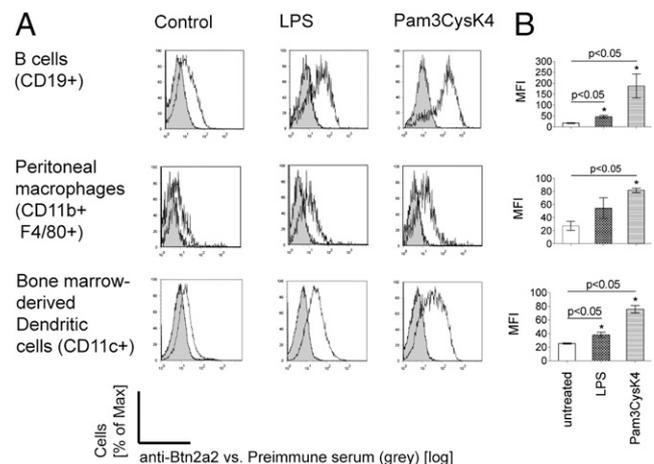


FIGURE 1. Increased cell-surface expression of Btn2a2 on APC after activation. APC: splenic B cells; bone marrow DC (20 ng/ml GM-CSF and 10 ng/ml IL-4); peritoneal macrophages enriched by adhesion to plastic. Activation was for 16 h with 100 ng/ml LPS or 1 μ g/ml Pam3CysK4. (A) Anti-Btn2a2 Ab (black outline) and preimmune serum (grey filled). Data representative of three experiments. (B) *Significance ($p < 0.05$) confirmed with paired t test. MFI, Mean fluorescence intensity.

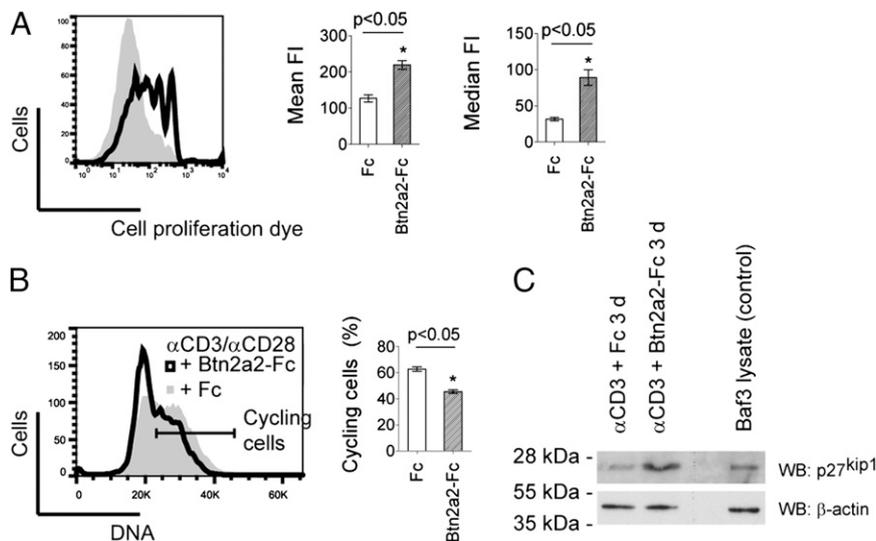


FIGURE 2. Btn2a2 inhibited entry into the cell cycle of anti-CD3⁺ and anti-CD28-activated CD3⁺ primary T cells. T cells from spleen and lymph nodes were stimulated for 4 d using 1 μg/ml anti-CD3, 1 μg/ml anti-CD28, and 10 μg/ml fusion protein. **(A)** Cell proliferation of cells 4 d after activation in Btn2A2-Fc (black outline) or Fc control (gray area) visualized with 1 μM eFluor670. **(B)** DNA content visualized using propidium iodide. Percent cycling cells (horizontal bar) calculated by subtracting the 2-fold percentage of cells in the left-hand part of the G₀/G₁ peak from 100%. **(C)** Four days after activation of cells with 1 μg/ml anti-CD3 in presence of Fc control or BtN2A2-Fc, expression of the cell cycle entry inhibitor p27^{kip1} was analyzed by Western blotting for p27^{kip1}. Total of 1 × 10⁵ cells per lane. Mouse Baf/3 cells were positive control for p27^{kip1}. Loading control: β-actin. FACS data representative of three experiments with three replicates. *Statistical significance as for Fig. 1. FI, Fluorescence intensity.

responsible for transmitting the activation signal from the TCR/CD3 complex to downstream signaling pathways is the tyrosine kinase Zap70, which is activated by tyrosine phosphorylation.

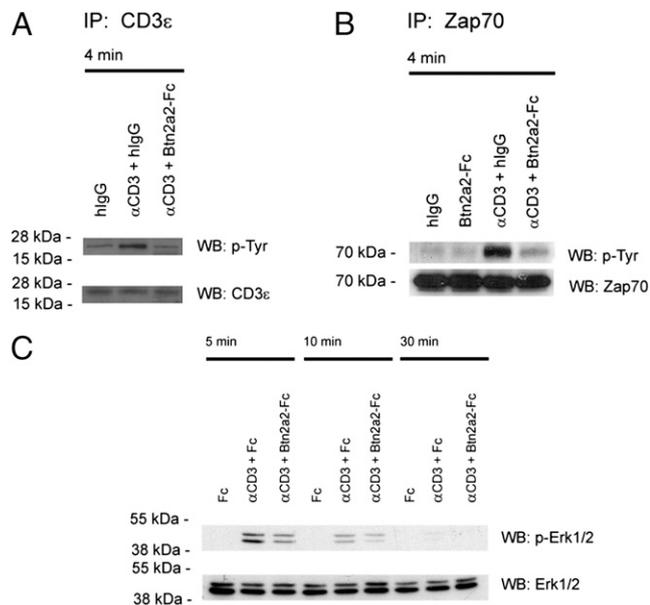


FIGURE 3. Btn2a2 coligation inhibits TCR signaling in anti-CD3-activated T cells. **(A)** 2B4 cells were stimulated for 4 min with 1 μg/ml anti-CD3 (clone 2c11) in 10 μg/ml Btn2a2-Fc or hlgG. Control: 2B4 cells stimulated with 10 μg/ml hlgG. After immunoprecipitation (IP) with anti-CD3ε (clone CD3-12) and SDS-PAGE, analysis was for tyrosine phosphorylation (4G10). **(B)** 2B4 cells stimulated 5 min with 1 μg/ml anti-CD3 in 10 μg/ml Btn2a2-Fc or hlgG. After lysis, samples were immunoprecipitated with biotinylated anti-Zap70 Ab, treated as in (A), and analyzed for phosphorylation (4G10). **(C)** CD3⁺ primary T cells were stimulated with 1 μg/ml anti-CD3 and 10 μg/ml fusion protein, treated as above, and analyzed for phosphorylation of Erk1 and Erk2 (p-Erk1/2). Ab to total Erk1/2 (Erk1/2) was used for loading. Data from more than two independent experiments. WB, Western blot.

Zap70 phosphorylation was reduced when cells were stimulated with anti-CD3 in the presence of Btn2a2-Fc (Fig. 3B).

Erk1 and Erk2, activated by tyrosine phosphorylation, are downstream targets of signals from the TCR, receptors for mitogens, growth factors, or cytokines, and critically involved in cell regulation (29). We assessed whether Btn2a2-Fc inhibition influences anti-CD3-induced Erk1/2 activation. CD3⁺ T cells were stimulated with anti-CD3 (Fig. 3C). Btn2a2 coligation inhibited anti-CD3-induced Erk1/2 activation, detected by reduced phosphorylation of Erk1/2. This occurred at different time points, indicating that the activation of Erk1/2 was significantly reduced, not merely delayed, by Btn2a2-Fc coligation (Fig. 3C).

Btn2a2 ligation inhibits PI3K and Akt signaling

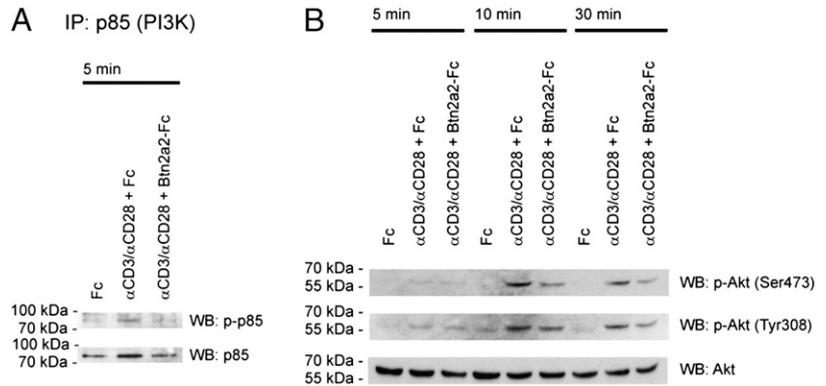
We next studied the influence of Btn2a2-Fc on the PI3K/Akt pathway. This pathway is activated by costimulatory molecules, such as CD28, ICOS, and LFA-1. In addition, it can be activated by signaling from the TCR/CD3 complex. Signaling from CD28 activates the regulatory subunit of PI3K, p85, by tyrosine phosphorylation.

Stimulation with anti-CD3 and anti-CD28 induced phosphorylation of p85 at 5 min (Fig. 4A). Conversely, phosphorylation of p85 was not detectable in the presence of Btn2a2-Fc. An increase in phosphorylation of Akt at Ser⁴⁷⁸ and Tyr³⁰⁸ was detected 10 min after stimulation with anti-CD3, anti-CD28, and Fc (Fig. 4B). A weaker increase in Akt phosphorylation was observed in the presence of Btn2a2-Fc, even after 30 min. These results indicate that Btn2a2 coligation inhibits anti-CD3- and anti-CD28-induced activation of the PI3K/Akt pathway.

Influence of anti-CD28 on T cells inhibited by Btn2a2

The extent of T cell activation depends on the balance of positive and negative coregulatory stimuli. Increasing doses of anti-CD28 overcame the inhibition of T cell proliferation by programmed death ligand-1 (PD-L1)-Fc (30). This effect, of CD28 signaling on anti-CD3-activated CD3⁺ primary T cells coinhibited with PD-L1-Fc, was reproduced by us. To compare T cell coinhibition exerted by Btn2a2-Fc with the inhibition by PD-L1-Fc, CD3⁺

FIGURE 4. Btn2a2 binding interferes with activation of the PI3K/Akt pathway in anti-CD3- and anti-CD28-activated T cells. CD3⁺ T cells stimulated with 1 μg/ml anti-CD3, 1 μg/ml anti-CD28, and 10 μg/ml fusion protein as indicated. **(A)** Samples were immunoprecipitated (IP) with anti-p85 Ab, treated as in Fig. 3, and phosphorylation analyzed using a p-p85 Ab. p85 Ab (total-p85) controlled loading. **(B)** Blotted proteins were analyzed for phosphorylation of Akt using Abs (p-Akt [Ser⁴⁷³] or p-Akt [Tyr³⁰⁸]). Ab to total Akt (Akt): sample loading. Data representative of three experiments. WB, Western blot.



T cells were stimulated with anti-CD3 and anti-CD28 along with Btn2a2-Fc. As the surface expression levels and affinities of receptors for Btn2a2 and PD-L1 may differ, we titrated the concentration of anti-CD28. Stimulated samples were analyzed by flow cytometry for expression of the T cell activation markers CD25 and CD69, and DNA content, 3 d after initial stimulation (Fig. 5A). The right panels of the figure indicate the relative inhibition values. The percentage of cells progressing through the cell cycle, expressing CD25 or CD69, increased in line with increasing anti-CD28 concentrations in presence of Btn2a2-Fc as well as the control. Hence, inhibition by Btn2a2 on anti-CD3-induced T cell activation was not overcome by anti-CD28 costimulation.

To compare inhibition of Akt activation by Btn2a2 with PD-L1, CD3⁺ T cells were stimulated with anti-CD3, with or without anti-CD28. Btn2a2-Fc, or PD-L1-Fc, reduced activation of Akt in T cells stimulated with anti-CD3 (Fig. 5B). Stronger induction of phosphorylation of Akt was observed in control cells stimulated with anti-CD3 and anti-CD28. In the presence of Btn2a2-Fc, the levels of phosphorylation of Akt at Tyr³⁰⁸ and Ser⁴⁷³ were similar to the level in cells treated with anti-CD3 and Fc control, without anti-CD28 (Fig. 5B). In the presence of PD-L1-Fc, samples stimulated with anti-CD3 and anti-CD28 exhibited more Akt phosphorylation than the sample treated with anti-CD3 and PD-L1-Fc and more than those treated with anti-CD3, anti-CD28, and Btn2a2-Fc. These data suggest that Btn2a2 is

more effective than PD-L1 at inhibiting anti-CD3- and anti-CD28-induced PI3K/Akt signaling.

Induction of Foxp3⁺ expression in naive T cells by Btn2a2

Downregulation of MAPK cascade signaling has been implicated in the development of Treg (31–33). In addition, inhibition of signaling via the PI3K/Akt pathway has been reported to control expression of the transcription factor Foxp3 (34, 35). We studied whether Btn2a2 engagement induced expression of Foxp3 in naive T lymphocytes. T cells from Foxp3-GFP-transgenic animals were negatively enriched and sorted for CD4⁺CD25⁻Foxp3⁻GFP⁻CD62L^{high} cells. The sorted naive T cells were activated for 3 d with 1 μg/ml anti-CD3, 1 μg/ml anti-CD28, plus 10 μg/ml of Fc fusion protein.

As shown in Fig. 6A, Btn2a2 coinhibition induced de novo expression of Foxp3 in naive T cells stimulated for 3 d with anti-CD3 and anti-CD28 (5.59 versus 1.29% in the Fc control). After 7 d of stimulation, 15.8% CD4⁺CD25⁺Foxp3⁺ T cells were detected in the sample stimulated in presence of Btn2a2-Fc versus 1.15% in the Fc control stimulation.

Titration of Btn2a2-Fc fusion protein indicated that the induction of Foxp3 in naive CD4⁺ T cells was dose dependent (Fig. 6C). We investigated induction of Foxp3 expression by Btn2a2-Fc in relation to induction by exogenous TGF-β. A strong induction of Foxp3⁺ expression was observed when T cells were stimulated with anti-CD3, anti-CD28, and control Fc protein, in presence of

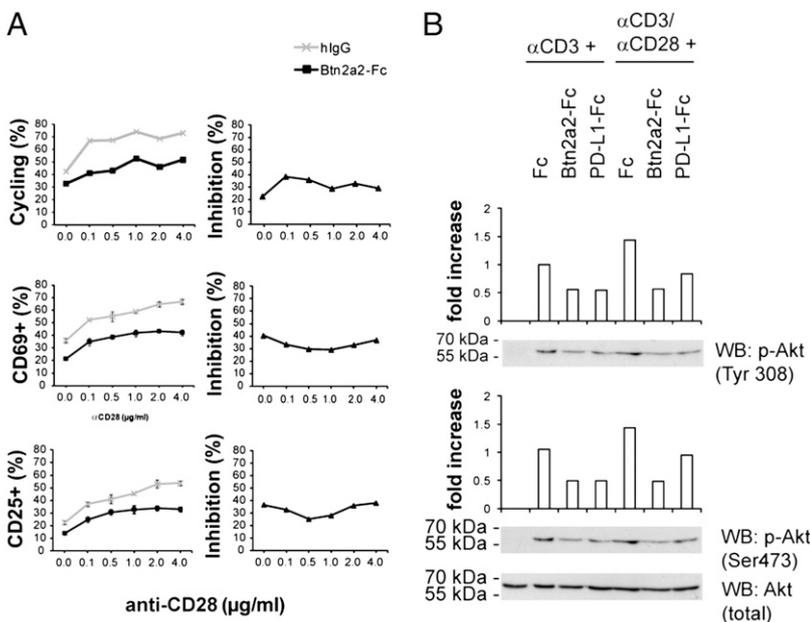
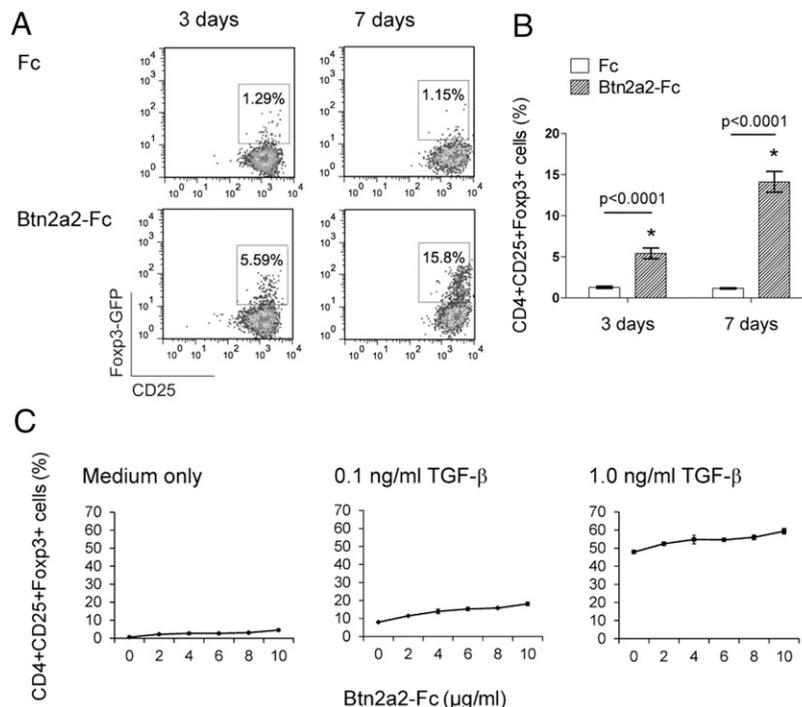


FIGURE 5. Btn2a2-mediated inhibition of anti-CD3-induced T cell activation cannot be overcome by anti-CD28 costimulation. **(A)** CD3⁺ T cells activated for 3 d with 1 μg/ml anti-CD3, either 10 μg/ml Btn2a2-Fc or hlgG, and anti-CD28. Hoechst 33342 was used for DNA cell cycle analysis and CD69 and CD25 Abs for T cell activation (left panel). Relative inhibition (right panel) was calculated by dividing percent cycling cells stimulated with anti-CD3 and Btn2a2-Fc by percent cycling cells with anti-CD3 and hlgG. The quotient was multiplied by 100% for the percent ratio. Three replicates per sample with data from three experiments. **(B)** T cells stimulated for 30 min with 1 μg/ml anti-CD3 and 10 μg/ml fusion protein or 1 μg/ml anti-CD3, 5 μg/ml anti-CD28, and 10 μg/ml fusion protein. Fusion proteins: Fc backbone alone or the Btn2a2-Fc or PD-L1-Fc. Ab for total Akt (Akt) was used for equal loading. Intensities measured were relative to stimulation with anti-CD3 and Fc. Data from three experiments. WB, Western blot.

FIGURE 6. Btn2a2-Fc binding induces de novo expression of Foxp3 in naive primary T cells. Naive T cells ($CD4^+ Foxp3^-, CD25^-, CD62L^{high}$) were sorted from enriched $CD4^+$ T cells harvested from spleen and lymph nodes of NOD Foxp3-GFP mice. **(A)** Cells were activated for 3 or 7 d with 1 $\mu\text{g/ml}$ anti-CD3, 1 $\mu\text{g/ml}$ anti-CD28, and 10 $\mu\text{g/ml}$ fusion protein and analyzed for Foxp3-GFP expression. **(B)** Statistical analysis using an ANOVA test. **(C)** Cells activated for 3 d with 1 $\mu\text{g/ml}$ anti-CD3, 1 $\mu\text{g/ml}$ anti-CD28, and 10 $\mu\text{g/ml}$ fusion protein as indicated in medium only or in medium supplemented with 0.1 or 1.0 ng/ml TGF- β . Cells shown in (A) and (B) were gated on lymphocytes, single cells, and $CD4^+$ cells. Cells in (C) also gated on $CD25^+$. Data from more than three independent experiments. Three independent samples analyzed per data set in (B) and (C). Data shown in (A) are representative of four replicates. *Significance ($p < 0.05$).



TGF- β . The induction of Foxp3 by TGF- β was further increased by Btn2a2-Fc in a dose-dependent way (Fig. 6C).

Assessment of cell-culture supernatants harvested at day 4 after activation with anti-CD3, anti-CD28, and Btn2a2-Fc or Fc, or 1 ng/ml TGF- β using a biological assay suggested that Btn2a2-Fc did not induce expression of TGF- β (Fig. 7A). However, a contribution of TGF- β induced by Btn2a2 cannot be ruled out entirely due to the detection limit of the assay.

Foxp3⁺ T cells induced by Btn2a2 ligation are functional Treg

To show that the Btn2a2-Fc induced $CD4^+CD25^+Foxp3^+$ cells are functional, Treg, we sorted for $CD4^+CD25^+Foxp3^+$ T cells (Btn2a2-induced Foxp3⁺ T cells) and incubated them with nonactivated

$CD3^+CD25^-Foxp3^-$ T cells, stained with cell proliferation dye at a 1:1 ratio, and stimulated with 1 $\mu\text{g/ml}$ anti-CD3, 1 $\mu\text{g/ml}$ anti-CD28, plus 10 $\mu\text{g/ml}$ Fc fusion protein (Fig. 7B). For the negative control arm of the experiment, T cells were activated in presence of activated $CD4^+CD25^+Foxp3^-$ T cells (control Foxp3⁻ T cells) at a 1:1 ratio. As positive control, nonactivated T cells were activated in presence of naturally occurring $CD4^+CD25^+Foxp3^+$ cells (natural Foxp3⁺), harvested, and sorted from spleen and lymph nodes of NOD Foxp3-GFP mice. After 4 d, proliferation of $CD8^+$ T cells in the samples was analyzed. $CD8^+$ T cells were chosen for analysis to exclude the possibility that proliferation of Btn2a2-induced $CD4^+Foxp3^+$, $CD4^+Foxp3^-$, or naturally occurring $CD4^+$ T cells influenced analysis. As shown in Fig. 7B, Btn2a2-induced

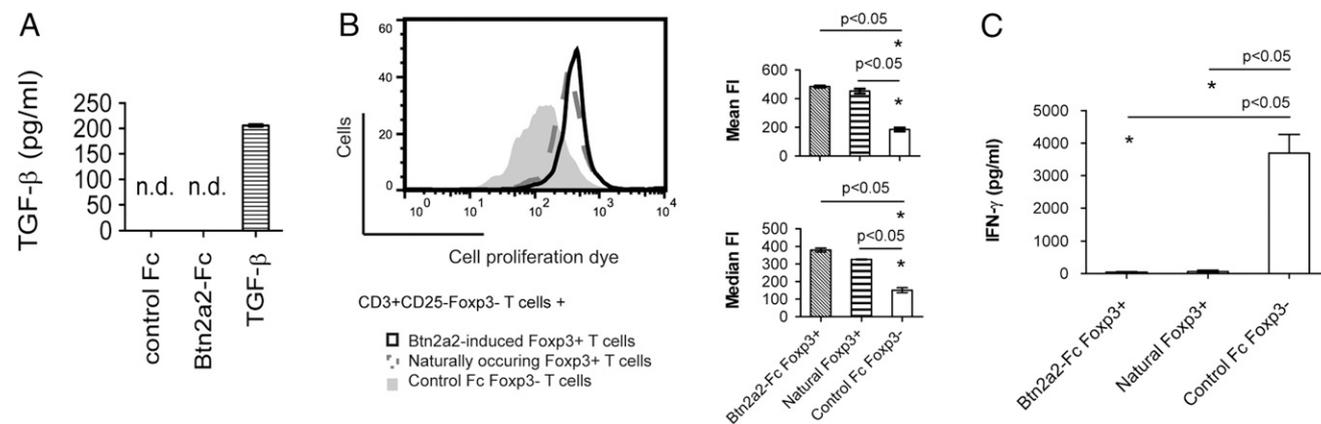


FIGURE 7. Btn2a2-Fc-induced $CD4^+CD25^+Foxp3^+$ cells inhibit T cell proliferation and do not secrete TGF- β or IFN- γ . **(A)** $CD4^+CD25^-CD62L^{high}Foxp3^-$ cells from NOD Foxp3-GFP-transgenic mice were activated for 4 d using 1 $\mu\text{g/ml}$ anti-CD3, 1 $\mu\text{g/ml}$ anti-CD28, and 10 $\mu\text{g/ml}$ Btn2a2-Fc or Fc fusion protein or 1 ng/ml TGF- β . Four days after initial stimulation, TGF- β was assayed in supernatants. **(B)** After 4 d, cells were sorted, and $CD4^+CD25^+Foxp3^+$ (Btn2a2 Foxp3⁺) or $CD4^+CD25^+Foxp3^-$ (control Foxp3⁻) T cells were incubated 1:1 with freshly harvested and sorted $CD3^+CD25^-Foxp3^-$ from the same mouse strain. $CD3^+CD25^-Foxp3^-$ T cells were stained with 1 μM eFluor. Mixed cells were activated with 1 $\mu\text{g/ml}$ anti-CD3, 1 $\mu\text{g/ml}$ anti-CD28, and 10 $\mu\text{g/ml}$ control Fc fusion protein. Positive control: $CD4^+CD25^+Foxp3^+$ (natural Foxp3⁺) from freshly harvested spleen and lymph nodes. After 4 d, proliferation of $CD8^+$ T cells analyzed by FACS. *Left panel*, Representative FACS plot. *Right panel*, Summary mean fluorescence intensity (FI) data. **(C)** Concentration of IFN- γ was measured by ELISA. Experiments were repeated three times, and three independent samples were analyzed. *Significance ($p < 0.05$) with unpaired *t* test. n.d., Not detected.

Foxp3⁺ T cells inhibited proliferation of CD8⁺ T cells with a similar efficiency as naturally occurring Foxp3⁺ T cells from NOD mice. Secretion of IFN- γ was not detected in samples containing Btn2a2 Foxp3⁺ or naturally occurring Foxp3⁺ T cells, but was detectable in the sample containing control Foxp3⁻ T cells (Fig. 7C). These results show that CD4⁺Foxp3⁺ T cells induced in presence of Btn2a2-Fc are functional Treg.

Discussion

Coregulatory molecules expressed on APC are involved in fine-tuning of T cell activation and differentiation. We found that TLR ligands such as LPS and Pam3CysK4 increased cell-surface expression of Btn2a2 on APC, consistent with a role for Btn2a2 in limiting T cell activation in inflammation.

Binding of Btn2a2 to anti-CD3- and anti-CD28-activated T cells reduced the number of proliferating cells and inhibited entry into the cell cycle. Inhibition of cell cycle entry has been noted for binding of Btl1, PD-L1, and PD-L2 fusion proteins to T cells (16, 36). In T cells coinhibited with Btn2a2-Fc, higher levels of the G₀/G₁ to S phase inhibitor p27^{kip1} were detected. High p27^{kip1} levels are considered as a hallmark of naive, as well as of anergic, T cells (37). Activation of the PI3K/Akt pathway promotes degradation of p27^{kip1} (28). The high level of p27^{kip1} in cells coinhibited by Btn2a2 is consistent with inhibition of the PI3K/Akt pathway by Btn2a2.

Little has been reported on the signaling pathways influenced by binding of butyrophilin-like molecules to T cells. Anti-CD3-treated DO11.10 T cells costimulated with BTNL2-Fc exhibited reduced activation of the transcription factor NFAT and NF- κ B (19). We show in this study that Btn2a2 inhibited anti-CD3 induced phosphorylation of CD3 ϵ , Zap70, Erk1, and Erk2. Our time-course studies indicated that this was not simply a delayed activation.

Signals transmitted by the PI3K/Akt pathway are important for full activation of T cells. Engagement of the TCR/CD3 complex activates the PI3K/Akt pathway, but stronger activation is achieved through concomitant signals from CD28 (38). In our experiments, tyrosine phosphorylation of p85, which took place 5 min after anti-CD3/CD28 stimulation of T cells, was negated by Btn2a2-Fc. Coinhibition with Btn2a2-Fc inhibited activation of Akt, a downstream target of PI3K, 10 min after activation.

Inhibition of anti-CD3-induced T cell activation by Btn2a2-Fc was not overcome by anti-CD28-induced cosimulation, as was the case with PD-L1 (30). Similarly, inhibition of Akt phosphorylation by Btn2a2-Fc was not reversed by high concentrations of anti-CD28. These findings indicate that Btn2a2 has a unique role in regulation of T cells.

Treg are central mediators of immune tolerance by suppression of various effector T cells. They are characterized by the expression of the transcription factor Foxp3 (39, 40). Mutations of Foxp3 lead to fatal autoimmune pathologies (39, 41, 42). Naturally occurring Treg (nTreg) develop in the thymus, whereas induced Treg (iTreg) develop in the periphery (43). In the presence of TGF- β , peripheral naive CD4⁺ T cells were induced to express Foxp3, thereby leading to the development of iTreg (44, 45). Inhibition of Erk1/2 or Akt signaling promotes induction of Treg. We found that Btn2a2 inhibits activation of Erk1/2 and is a potent inhibitor of Akt activation. We found a positive relationship between the concentration of Btn2a2-Fc and the frequency of CD4⁺CD25⁺Foxp3⁺ T cells induced in vitro. Btn2a2-induced CD4⁺CD25⁺Foxp3⁺ T cells inhibited proliferation of T cells and IFN- γ production.

A dose-dependent induction of Foxp3 expression by Btn2a2 was also observed in the presence of TGF- β . This cytokine is potent

for induction and maintenance of Foxp3 expression but was apparently not required for expression of Foxp3 in the thymus (46). We detected no TGF- β in supernatants containing Btn2a2-induced Foxp3⁺ T cells, possibly indicating that Foxp3 expression is induced via the inhibition of TCR and PI3K/Akt signaling pathways, although some contribution of TGF- β cannot be ruled out at this stage. We have shown that Btl1a1 and Btn2a2 are expressed on epithelial cells in the thymus (12). Hence, butyrophilin molecules could play a role in induction of nTreg cells in the thymus. In addition, Btn2a2 might contribute to T cell tolerance in the periphery, involving encounters between TLR-activated professional APC that induce iTreg. Taken together, our findings indicate that BTN-related molecules may be involved in the induction of both nTreg and iTreg.

By inducing expression of Foxp3, Btn2a2 might be involved in the suppression of Th17 cell differentiation, because Foxp3 represses expression of the IL-17 gene (40). This hypothesis is consistent with the finding that Btl1, another butyrophilin-related molecule, reduced levels of Th17 cells in an autoimmune disease animal model (16). Reduction of Th17 cell levels could be due to repression of IL-17 gene expression by Foxp3 or by induction of Treg, which suppress Th17 activation and proliferation. When expressed on enterocytes, Btl1 inhibited production of the proinflammatory cytokine IL-6 (17). Btl2 also inhibited IL-6 production (18). IL-6 inhibits Treg-mediated suppression (47), downregulates TGF- β -induced Foxp3 expression (48), induces Th17 cells in presence of TGF- β (49), and reprograms Treg to express cytokines such as IL-17 and IFN- γ (50). BTN-related molecules may therefore be a therapeutic target, to be exploited for treatment of Th17-driven autoimmune diseases.

There is increasing evidence that butyrophilin family molecules regulate T cells (12, 16, 18, 19). With Btn2a2, this may be by direct inhibition of T cell activation, or indirectly, by inducing differentiation into Foxp3⁺ Treg. Inhibition of primary T cell activation by Btn2a2 engagement was profound and not overcome by costimulation with anti-CD28. We traced the effect to inhibition of Akt activation, which was not subjugated by anti-CD28 costimulation, as it is by PD-L1 engagement. Due to the ability of BTN-related molecules to induce Foxp3 expression in T cells and to inhibit IL-6 production, they may be important regulators of the Treg-effector T balance, by shifting the equilibrium toward T cell tolerance.

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Disclosures

The authors have no financial conflicts of interest.

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