BTNL2-Ig Protein Attenuates Type 1 Diabetes In NOD Mice

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Abstract

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease in which insulin-producing β-cells are destroyed. Although butyrophilin-like 2 (BTNL2) has been shown to be a negative T cell regulator *in vitro,* its ability to inhibit T cell responses *in vivo* has not been determined. In this study, we determined the effect of a recombinant BTNL2-IgG2a Fc (rBTNL2-Ig) fusion protein on T1D development *in vivo*. We show here that *in vivo* administration of rBTNL2-Ig ameliorates T1D in NOD mice. This is associated with the ability of rBTNL2-Ig to inhibit the proliferation, activation, and inflammatory cytokine production from autoreactive T cells *in vivo*. In addition, rBTNL2-Ig treatment increases the generation of regulatory T cells. Our results suggest that targeting the BTNL2 pathway has the potential to be used in the prevention and treatment of patients with T1D.

Keywords

BTNL2; Type 1 Diabetes; autoreactive T cells; regulatory T cells

Introduction

Around 36 million people suffer from Type 1 Diabetes (T1D) worldwide according to a report from the International Diabetes Federation reported in 2015. T1D is an autoimmune disease characterized by the infiltration of lymphocytes into the islets of the pancreas followed by destruction of insulin-secreting beta-cells, leading to high blood-sugar concentrations that needs daily insulin injections. Exogenous insulin therapy is not a cure. If it is not treated properly, T1D can cause long-term complications, such as kidney disease, blindness, heart attacks, strokes and amputations.

The NOD mouse is the widely used animal model for human T1D [1]. NOD mice share many of the characteristics of this human disease. It is generally believed that T lymphocytes

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Conflict of Interest

The authors declare that they have no conflict of interest.

(T cells) play a major role in the pathogenesis of T1D. T cell activation requires at least two signals. The first signal is through the interaction between the T-cell receptor (TCR) and peptides presented on MHC molecules. The second signal requires the interaction between co-stimulatory and co-inhibitory molecules on antigen-presenting cells (APCs) and their receptors on T cells. Among the T cell co-stimulatory and co-inhibitory molecules, the B7 family is of central importance. B7 family members include B7–1, B7–2 [2], PD-L1 (B7-H1) [3], PD-L2 (B7-DC) [4], B7-H2 [also called inducible T cell co-stimulator ligand (ICOSL), B7h, B7RP-1, GL50] [5], B7-H3 [6], B7-H4 (B7x, B7S1) [7], B7-H5 (HHLA2) [8], and B7-H6 [9], etc.

Butyrophilin (BTN) and BTN-like (BTNL) molecules share sequence, structural, and functional similarity with the B7 family [10]. Therefore, BTN and BTNL molecules are considered as extended B7 family members [11]. It has been reported that BTNL2 can inhibit T cell proliferation and cytokine production *in vitro* [12, 13]. However, the ability of BTNL2 to inhibit T cell responses *in vivo* and to treat T1D has not been reported.

In this study, we determined the effect of rBTNL2-Ig fusion protein on T1D. We demonstrate here that *in vivo* administration of rBTNL2-Ig ameliorates T1D in NOD mice. This is related to its ability to inhibit T cell proliferation, activation and cytokine production *in vivo*. rBTNL2-Ig treatment also significantly increases the generation of regulatory T cells (Tregs) in NOD mice.

Methods

Mice

NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed, treated, and handled according to a protocol approved by the University of Connecticut Animal Care and Use Committee.

Production of rBTNL2-Ig

rBTNL2-Ig fusion protein that contains the extracellular domain of the mouse BTNL2 (aa27–452) and the constant region of mouse IgG2a was produced by transfecting the gene containing expression vector into HEK-293 cells. The fusion protein was purified from the expression system using Protein G Sepharose 4 Fast Flow according to the manufacturer's instructions (GE Healthcare). The endotoxin level was less than 0.01 EU/ml of 1 μg of purified rBTNL2-Ig as determined by a Limulus Amebocyte Lysate assay. Recombinant mouse IgG2a Fc protein (Control Ig) was purchased from BXCell (West Lebanon, NH).

In vitro T cell proliferation assay

T cells were purified from female NOD mice by an immunomagnetic system (Miltenyi Biote Inc., Auburn, CA), and the purity of the cells was usually >95%. T cells were stimulated with anti-CD3 antibody (Biolegend) in the presence of rBTNL2-Ig or control Ig. The cells were pulsed with [^{3}H] thymidine (1 μCi/well) (PerkinElmer, Inc., Downers Grove, IL) for12 hours before harvest, and then collected by a cell harvester (Brandel, Missouri

City, Texas). [3H] thymidine incorporation was measured by liquid scintillation spectroscopy (PerkinElmer, Inc.).

Treatment protocol

Groups of female NOD mice were injected with indicated doses of recombinant rBTNL2-Ig, or control Ig intraperitoneally (i.p.) twice weekly from 4 to 16 weeks of age. The mice were monitored for blood glucose levels by test strips (Advanced glucose meter, CVS health, USA) every week. Mice with a blood glucose measurement of greater than 250 mg/dL on two consecutive readings were considered diabetic.

Histological analysis

The pancreata were harvested from the mice, fixed with 4% paraformaldehyde and prepared for paraffin sections. The paraffin sections were stained with hematoxylin and eosin (H&E). At least 100 islets were counted and blindly scored. Double staining of insulin and CD45 was developed using primary antibodies followed by FITC- or Texas red-conjugated secondary antibody.

Isolation of islet-infiltrating cells (IICs)

Islets were isolated with collagenase IV (Gibco, USA) digestion of the pancreata, as described previously [14]. The IICs suspension was then subjected to flow cytometric analysis.

Flow cytometric analysis

Single-cell suspensions were generated from spleens as described previously [30]. Single-cell suspensions were stained with fluorochrome-conjugated antibodies against CD4, CD8, CD25, Foxp3, CD69, CD62L and CD44 (BioLegend, Inc, San Diego, CA, USA). Intracellular staining of cytokine γ-interferon (IFNγ) was performed after 4–5 hours of phorbol myristic acid (50 ng/ml, Sigma) plus ionomycin (1 µg/ml; Sigma) stimulation. Isotype-matched controls were included in all experiments. The samples were analyzed on a FACS Calibur or LSRFortessa X-20 Cell Analyzer (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR)

Statistical analysis

P values were based on the two-sided Student's t test. A confidence level above 95% (p <0.05) was determined to be significant.

Results

rBTNL2-Ig inhibits the proliferation and activation of T cells from NOD mice in vitro

It has been shown that rBTNL2-Ig can suppress T cell proliferation and cytokine production from non-diabetes-prone C57BL/6 mice [12, 13]. We determined whether rBTNL2-Ig could affect the autoreactive T-cell response. T cells were purified from female NOD mice, and stimulated with 1 µg/ml anti-CD3 antibody in the presence of graded doses of rBTNL2-Ig or control Ig protein. T cell proliferation was measured by [3H] thymidine incorporation. As

shown in Figure 1A, rBTNL2-Ig inhibited anti-CD3-induced T cell proliferation in a dose-dependent manner. Furthermore, rBTNL2-Ig inhibited the T cell proliferation across a range of anti-CD3 concentrations (Figure 1B).

We next examined whether rBTNL2-Ig affects IL-2 production from T cells *in vitro*. As shown in Figure 1C, the production of IL-2 was reduced by 64.6%, 65.4%, 68.2% and 92.6% in the presence of 2, 5, 10 and μg/mL rBTNL2-Ig, respectively (Fig. 1C). We also determined whether rBTNL2-Ig affects T cell activation. CD69 is an early T-cell activation marker. We found that rBTNL2-Ig inhibited the expression of CD69 by CD4 and CD8 T cells, and the inhibition was across a range of anti-CD3 concentrations (Figure 1D-F). Taken together, these data suggest that rBTNL2-Ig inhibits the proliferation, IL-2 production, and activation of T cells from diabetes-prone NOD mice *in vitro*.

rBTNL2-Ig treatment ameliorates T1D in NOD mice

We then assessed the effect of rBTNL2-Ig on autoimmune diabetes. To this end, female NOD mice were injected with varying doses of rBTNL2-Ig (25 or 50 μg) or control Ig (25 μg) twice weekly from 4 to 16 weeks of age. The development of diabetes was monitored over time. As shown in Figure 2, 9% and 27% of NOD mice in the control-Ig-treated group developed diabetes at 12 and 16 weeks of age, respectively. In contrast, no NOD mice in the rBTNL2-Ig-treated groups developed diabetes during this period. The results suggest that rBTNL2-Ig treatment delayed diabetes onset in NOD mice.

Beyond 16 weeks of age, the incidence of diabetes was slightly decreased in the 25 μg rBTNL2-Ig-treated group, with 83% incidence in control Ig-treated group, whereas 75% incidence in the rBTNL2-Ig-treated group at week 28. In contrast, the incidence of diabetes was significantly decreased in the 50 μg rBTNL2-Ig-treated group, with 80% vs 20% of incidence in the control Ig- vs rBTNL2-treated group at week 28. The data suggest that treatment with rBTNL2-Ig attenuates diabetes in NOD mice.

rBTNL2-Ig treatment increases the generation of Tregs and inhibits the activation of T cells in the pancreas

Studies have shown that CD4+Foxp3+ Tregs play an important role in the prevention of autoimmune diabetes. Since it has been reported that BTNL2 can increase the generation of Tregs *in vitro*[15], we analyzed the percentages of Tregs in IICs of the NOD mice. As shown in Figure 3A and B, rBTNL2-Ig treatment increased the percentages of CD4+Foxp3+ Tregs in IICs, as compared to control Ig treatment.

We next evaluated the activation of T cells in IICs, and found that rBTNL2-Ig treatment reduced CD69 expression on both CD4 and CD8 T cells (Figure 3C, D). The results suggest that rBTNL2-Ig inhibited the activation of CD4 and CD8 T cells in IICs. We also performed intracellular staining of IFN-γ. As shown in Figure 3E and F, rBTNL2-Ig treatment decreased the percentages of IFNγ–producing CD4 and CD8T cells, suggesting an inhibition of T helper 1 (Th1) and cytotoxic CD8 cell function after rBTNL2-Ig treatment.

To further confirm that rBTNL2-Ig treatment inhibits T cell activation, we analyzed the expression of CD62L and CD44 by CD4 and CD8 T cells in IICs. It has been reported that

naïve T cells express CD62L, whereas effective T cells express CD44. As shown in Figure 3G–J, rBTNL2-Ig treatment increased the percentages of CD62L+ cells, but decreased the percentages of CD44+ cells in both CD4 and CD8 T cells. Collectively, our data suggest that rBTNL2-Ig treatment increases the generation of Tregs and reduces the activation of both CD4 and CD8 T cells in IICs.

rBTNL2-Ig also increases Treg generation and suppresses T cell activation in the spleen

We next determined whether the enhanced Treg generation and reduced T cell activation also occurred in the spleen of rBTNL2-Ig-treated NOD mice. As shown in Figure 4A and B, rBTNL2-Ig treatment increased the percentage of CD4+CD25+Foxp3+ Tregs in the spleen. In addition, rBTNL2-Ig reduced the percentages of CD69+ cells (Figure 4C, D) and IFNγ-producing cells (Figure 4E, F) in splenic CD4 and CD8 T cells.

T cells can be divided into CD44−CD62L+ naïve, CD44+CD62L− effective memory, and CD44+CD62L+ central memory T cells based on the expression of CD44 and CD62L. We examined these T cell subsets in the NOD mice. As shown in Figure 4G–J, rBTNL2-Ig treatment increased the percentages of naïve T cells and decreased the percentages of effective memory and central memory CD4 and CD8 T cells. Taken together, our results suggest that rBTNL2-Ig treatment enhances the generation of Tregs and inhibits the activation and IFNγ cytokine production from T cells in the spleen.

rBTNL2-Ig reduced the inflammation infiltration of islet

We also performed histological examination with H&E and immunofluorescent staining to determine the degree of insulitis in the NOD mice at 32 weeks of age. In control Ig-treated mice, the patterns of insulitis were visible and the islets were infiltrated seriously. In contrast, in rBTNL2-Ig-treated mice, the degree of inflammation was decreased and the islets maintained nearly intact morphology (Fig. 5A). We counted the islets under a microscope and evaluated the scores. We found that the percentages of islets with low scores increased and islets with high scores decreased after rBTNL2-Ig treatment (Fig. 5B).

We then calculated the percentages of islets with inflammation infiltration and loss. As shown in Figure 5C, rBTNL2-Ig treatment significantly reduced both inflammation and loss of islets, as compared to control Ig treatment.

Discussion

We show here that *in vivo* administration of rBTNL2-Ig attenuates autoimmune diabetes in NOD mice. To the best of our knowledge, this is the first report that rBTNL2-Ig fusion protein is able to ameliorate T1D *in vivo*. The NOD mouse is the best available animal model for human T1D [1]. The development of diabetes in NOD mice is the result of an imbalance of pathogenic/activated T cells and Tregs. Our data suggest that rBTNL2-Ig inhibits pathogenic/activated T cells by affecting their proliferation, activation, and inflammatory cytokine production.

It has been reported that the putative receptor for BTNL2 is expressed on activated T cells [12, 13], suggesting that BTNL2 can directly acts on T cells. This notion is supported by our

data and those from others showing that rBTNL2-Ig inhibits the proliferation of purified T cells [12, 13]. The BTNL2 putative receptor seems to be distinct from the receptors for the known B7 family molecules because the BTNL2 protein failed to bind to these receptors [12, 13]. The nature of the BTNL2 receptor remains to be determined. It has also been shown that rBTNL2-Ig inhibits proximal TCR signals including AP-1, NFAT and NFκB in DO11.10 hybridoma cells [13]. The BTNL2-induced signal pathways in T cells also need to be further investigated.

We have, in addition, demonstrated that rBTNL2-Ig treatment significantly increased the generation of Tregs in NOD mice. Our results are consistent with previous report that BTNL2 can induce the production of Tregs *in vitro* [15]. Tregs typically express high levels of CD25 and the transcription factor Foxp3. The thymus supports the development of Tregs, generating so-called natural Tregs that populate the periphery. Since the phenotypes of the BTNL2-induced Tregs are similar to thymus-derived Tregs [15], it is possible that rBTNL2-Ig treatment enhances the development of natural Tregs in the thymus. However, the possibility that the increased production of Tregs in BTNL2-Ig-treated NOD mice is caused by enhanced production of induced Tregs in the periphery cannot be excluded.

The BTNL2 mutation has been associated with many autoimmune diseases, such as sarcoidosis, ulcerative colitis, Kawasaki disease, osteoarthritis, systemic lupus erythematosus, multiple sclerosis, Graves' disease, Crohn's disease, and rheumatoid arthritis [16]. BTNL2 is thought to play a role in limiting unwanted inflammation and enhancing the generation of Tregs. Our results showing that rBTNL2-Ig treatment attenuates T1D in mice are consistent with these results.

In conclusion, we have shown that *in vivo* administration of rBTNL2-Ig ameliorates T1D in NOD mice. Both the inhibition of pathogenic/activated T cells and generation of Tregs likely play a critical role in attenuating T1D. The ability of rBTNL2-Ig to negatively regulate autoimmune diabetes in NOD mice provides proof of principle for further investigation. More research is needed to understand the mechanisms by which BTNL2 attenuates autoimmune diabetes. Our results suggest that rBTNL2-Ig has the potential to be used in the clinical treatment of T1D.

Acknowledgments

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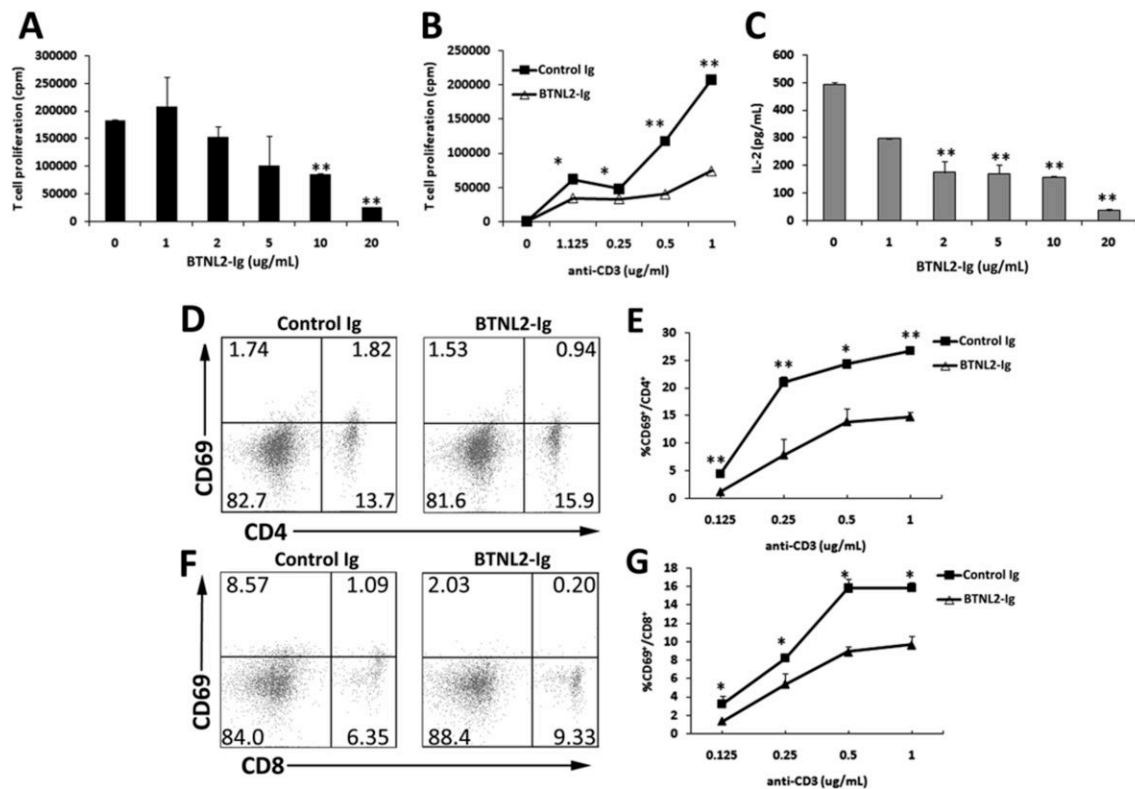
Figure 1.
rBTNL2-Ig inhibits T cell function from NOD mice *in vitro*. Purified CD3+ T cells were stimulated with (A, C) 1 μg/ml plate bound anti-CD3, or (B, D-G) varying concentrations of anti-CD3 in the presence of indicated concentrations of rBTNL2-Ig or control Ig protein for (D-G) 12 h (for early T cell marker CD69 detection), or (A-C) 72 h (for T-cell proliferation and IL-2 production). (A, B) For the T-cell proliferation assay, the cells were pulsed with 1 μCi of [3H]-thymidine 12 hours before harvest. (C) The levels of IL-2 in the supernatant were measured by an ELISA kit. (D-G) T cells were examined for CD69 expression by CD4 and CD8 T cells by flow cytometry. (D, F) Representative flow cytometric profiles and (E, G) are statistical analysis of the percentages of CD69+ cells in CD4 or CD8 T cells. Data represent three independent experiments and are expressed as means ± SEM. *P < 0.05, ** P < 0.01 vs control Ig.
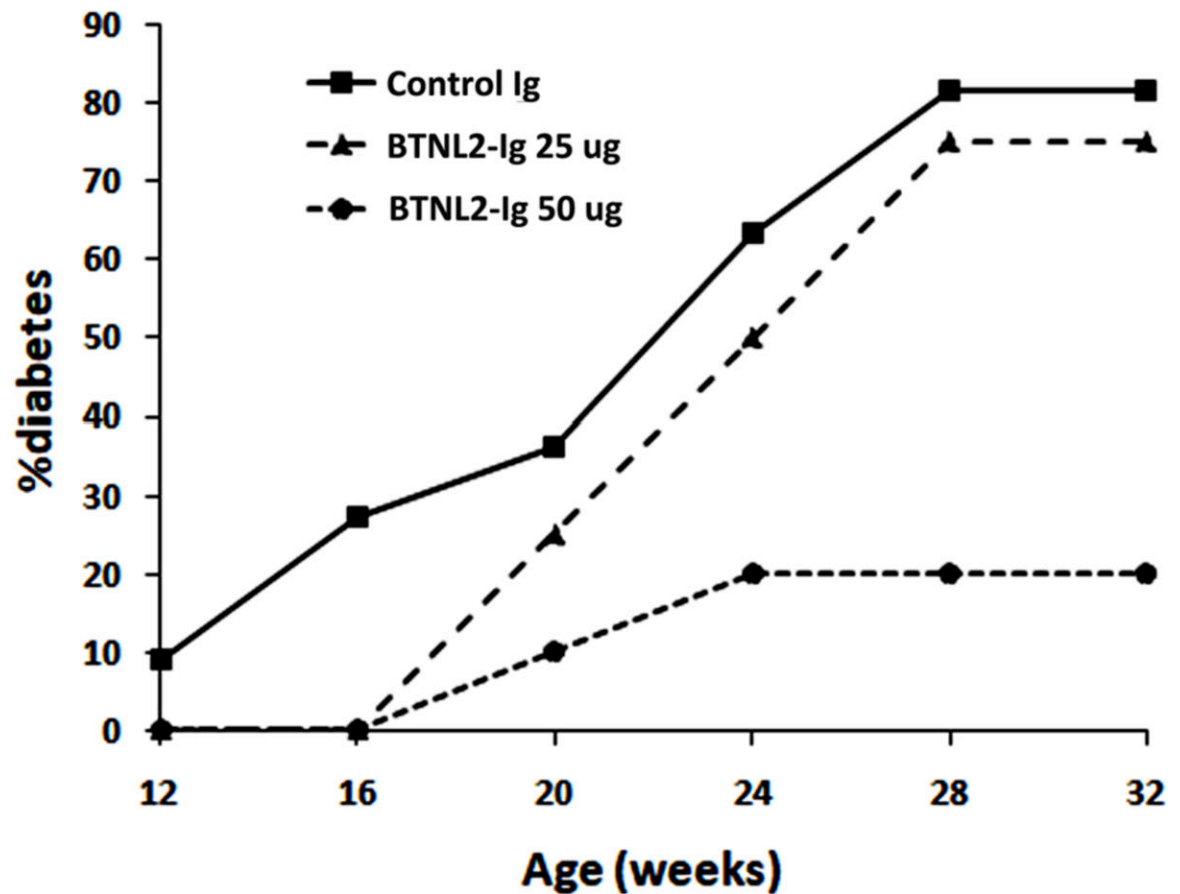
Figure 2.
rBTNL2-Ig treatment delays the onset and reduces the incidence of autoimmune diabetes. Female NOD mice were injected i.p. with rBTNL2-Ig (25 or 50 μg) or control Ig (25 μg) twice weekly from 4 to 16 weeks of age. The mice were monitored for blood glucose levels each week. Mice with blood glucose of greater than 250 mg/dL on two consecutive readings were considered diabetic. The percentages of onset of diabetes are plotted at 12, 16, 20, 24, 28 and 32 weeks of age. The data are pooled from 3 independent experiments with 4–6 mice per group.
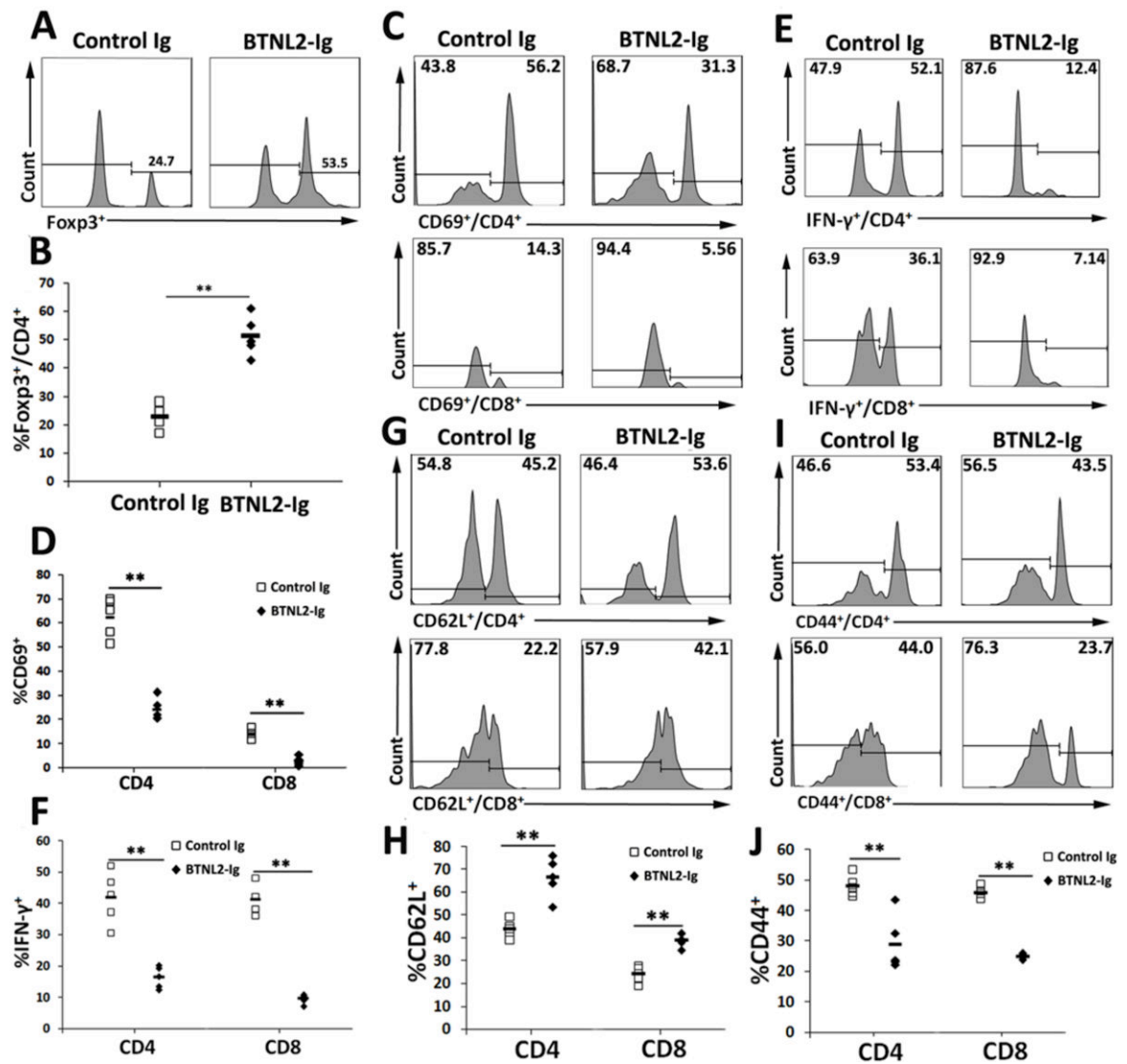
Figure 3.
rBTNL2-Ig increases the percentage of Tregs and reduces the activation of T cells in IICs. IICs were harvested from rBTNL2-Ig- or control Ig-treated NOD mice at week 32 of age. The IICs were analyzed for the percentages of (A, B) CD4$^+$Foxp3$^+$ Tregs, (C, D) CD69$^+$ cells in CD4 and CD8 T cells, (E, F) IFNγ-producing CD4 and CD8 T cells, (G, H) CD62L$^+$ cells and (I, J) CD44$^+$ in CD4 and CD8 T cells. (A, C, E, G, I) Representative flow cytometric profiles and (B, D, F, H, J) statistical analysis of means ± SEM. *P < 0.05, ** P < 0.01 vs control Ig. The data are representative of 3 independent experiments with 4–6 mice per group.
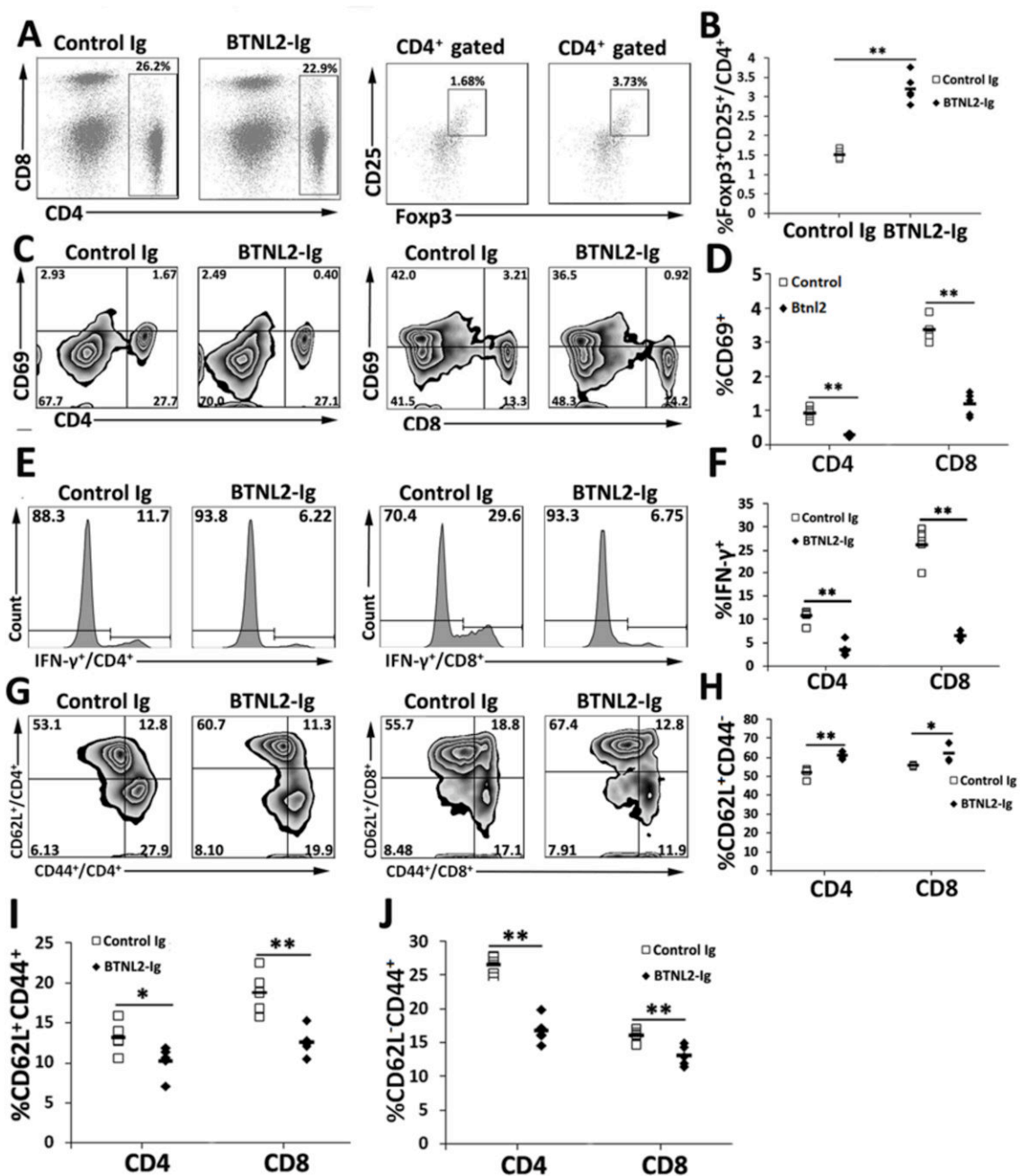
Figure 4.
rBTNL2-Ig increases the generation of Tregs and decreases the activation of T cells in the spleen. The spleens were harvested from rBTNL2-Ig or control Ig-treated NOD mice at week 32 of age. The splenocytes were analyzed for the percentages of (A, B) CD4$^+$ CD25$^+$Foxp3$^+$ Tregs, (C, D) CD69$^+$ cells in CD4 and CD8 T cells, (E, F) IFNγ-producing CD4 and CD8 T cells, and (G-J) CD44$^-$CD62L$^+$ naïve, CD44$^+$CD62L$^-$ effective memory, and CD44$^+$CD62L$^+$ central memory CD4 and CD8 T cells. (A, C, E, G, I) Representative flow cytometric profiles and (B, D, F, H, J) statistical analysis of means ± SEM. *P < 0.05,

** P < 0.01 vs control Ig. The data are representative of 3 independent experiments with 4–6 mice per group.
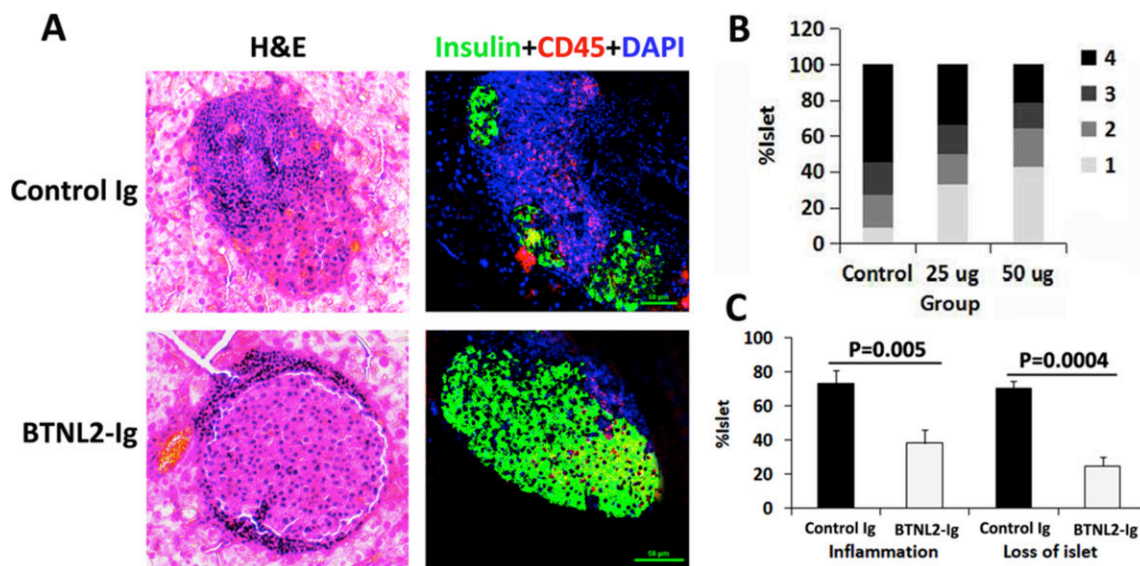
Figure 5.
rBTNL2-Ig treatment attenuates insulitis in NOD mice. Pancreata were harvested from control Ig- and rBTNL2-Ig-treated NOD mice at 32 weeks of age. Sections of pancreata were subjected to H&E staining, or immunofluorescent staining with anti-insulin and anti-CD45 antibodies. (A) Representative sections of H&E and immunofluorescent staining. (B) Insulitis scores based on H&E staining. Scores were given as follows: score 1, free of infiltrates; score 2, <25%; score 3, 25–50%, and score 4, >50% infiltrates per islet. (C) Percentages of inflammation and loss of islets calculated according to the severity of intra islet infiltrates and insulin staining, respectively. The data are representative of 3 independent experiments with 4–6 mice per group.