$CD4^+CD25^+$ T-Cells Control Autoimmunity in the Absence of B-Cells

Eliana Mariño, Jeanette Villanueva, Stacey Walters, David Liuwantara, Fabienne Mackay, and Shane T. Grey

OBJECTIVE—Tumor necrosis factor ligand family members B-cell–activating factor (BAFF) and a proliferation-inducing ligand (APRIL) can exert powerful effects on B-cell activation and development, type 1 T-helper cell (Th1) immune responses, and autoimmunity. We examined the effect of blocking BAFF and APRIL on the development of autoimmune diabetes.

RESEARCH DESIGN AND METHODS—Female NOD mice were administered B-cell maturation antigen (BCMA)-Fc from 9 to 15 weeks of age. Diabetes incidence, islet pathology, and T- and B-cell populations were examined.

RESULTS—BCMA-Fc treatment reduced the severity of insulitis and prevented diabetes development in NOD mice. BCMA-Fc–treated mice showed reduced follicular, marginal-zone, and T2MZ B-cells. B-cell reduction was accompanied by decreased frequencies of pathogenic $CD4^+CD40^+$ T-cells and reduced Th1 cytokines IL-7, IL-15, and IL-17. Thus, T-cell activation was blunted with reduced B-cells. However, BCMA-Fc–treated mice still harbored detectable diabetogenic T-cells, suggesting that regulatory mechanisms contributed to diabetes prevention. Indeed, BCMA-Fc–treated mice accumulated increased $CD4^+CD25^+$ regulatory T-cells (Tregs) with age. $CD4^+CD25^+$ cells were essential for maintaining euglycemia because their depletion abrogated BCMA-Fc–mediated protection. BCMA-Fc did not directly affect Treg homeostasis given that $CD4^+CD25^+Foxp3^+$ T-cells did not express TACI or BR3 receptors and that $CD4^+CD25^+Foxp3^+$ T-cell frequencies were equivalent in wild-type, $BAFF^{-/-}$, $TACI^{-/-}$, $BCMA^{-/-}$, and $BR3^{-/-}$ mice. Rather, B-cell depletion resulted in $CD4^+CD25^+$ T-cell–mediated protection from diabetes because anti-CD25 monoclonal antibody treatment precipitated diabetes in both diabetes-resistant NOD.$\mu MT^{-/-}$ and BCMA-Fc–treated mice.

CONCLUSIONS—BAFF/APRIL blockade prevents diabetes. BCMA-Fc reduces B-cells, subsequently blunting autoimmune activity and allowing endogenous regulatory mechanisms to preserve a prehyperglycemic state. ***Diabetes*** **58:1568–1577, 2009**

From the Immunology and Inflammation Program, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia.
Corresponding author: Shane T. Grey, s.grey@garvan.org.au.
Received 31 October 2008 and accepted 16 March 2009.
Published ahead of print at http://diabetes.diabetesjournals.org on 31 March 2009. DOI: 10.2337/db08-1504.
© 2009 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See http://creativecommons.org/licenses/by-nc-nd/3.0/ for details.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

See accompanying commentary, p. 1479.

The members of the tumor necrosis factor (TNF) ligand family of molecules B-cell–activating factor (BAFF) (also known as BLyS, TNFSF13b) and a proliferation-inducing ligand (APRIL) can exert powerful effects on B-cell development, survival, and function; T-cell activation; and type 1 T-helper cell (Th1) immune responses and autoimmunity (1). BAFF exists as both a soluble and a membrane bound molecule and is expressed by a wide range of inflammatory-activated cells, including monocytes, macrophages, dendritic cells, and T-cells (2). In contrast, APRIL is processed intracellularly and exerts its function as a soluble protein. BAFF and APRIL can bind to one of two receptors: B-cell maturation antigen (BCMA) (3) or transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI) (3,4), whereas BAFF can also bind to BR3 (otherwise known as BAFF-R) (5). These receptors are found on a wide range of B-cell subsets including immature, transitional, mature, memory, and germinal center B-cells, as well as on plasma cells (2). Further, activated T-cells can express the receptors BR3 and TACI (4,6).

BAFF has emerged as an important player in the development of autoimmunity. Elevated BAFF and APRIL levels have been detected in sera from human patients with rheumatoid arthritis, lupus, and Sjogren's syndrome (7–9). Moreover, BAFF-transgenic mice harbor increased titers of self-reactive antibodies and develop autoimmune symptoms very similar to those of lupus and Sjogren's syndrome (10,11). Forced expression of BAFF also results in a marked expansion of marginal-zone B-cells (MZBs) (12)—a B-cell subset associated with autoimmune conditions including lupus (13), Sjogren's syndrome (11), and, more recently, type 1 diabetes (14,15). Thus, the BAFF/APRIL system can be considered a proinflammatory pathway associated with the development of autoimmunity (7,8). Indeed, studies designed to explore the therapeutic potential of BAFF pathway blockers for the treatment of autoimmune conditions are underway (16,17). This background makes targeting the BAFF/APRIL system a potential therapeutic candidate for the treatment of type 1 diabetes. This study was undertaken to test the hypothesis that targeting the BAFF/APRIL system would have multiple inhibitory effects on the spontaneous development of autoimmune diabetes in the NOD model.

RESEARCH DESIGN AND METHODS

C57BL/6, NOD.SCID, and NOD/Lt (NOD) mice were obtained from The Walter and Eliza Hall Institute of Medical Research (WEHI) Kew, Melbourne, Australia. NOD.$\mu MT^{-/-}$ mice were provided by Dr. Serreze (18). $BAFF^{-/-}$, $BCMA^{-/-}$, and $TACI^{-/-}$ mice were provided by Dr. Susan Kalled (Biogen Idec). $BR3^{-/-}$ mice were a gift from Dr. Rajewsky (19). All animal experiments

were approved by the St. Vincent's Campus Animal Experimentation and Ethics Committee.

Diabetes incidence studies. NOD mice were administered BCMA-Fc (150 μg per treatment) based on previous studies (20). BCMA-Fc is a fusion protein—the extracellular portion of BCMA fused to the Fc domain of human IgG. BCMA-Fc was provided by Dr. Susan Kalled (Biogen Idec). Controls were treated with PBS or intravenous globulin (HuIvIg) (150 μg). For adoptive transfer studies, splenocytes (1×10^7) from pre-diabetic 16-week-old female NOD donors or BCMA-Fc–treated mice were transferred intravenously into NOD.SCID recipients. Glucose levels were monitored twice weekly from 10 weeks of age onward for BCMA-Fc–treated mice or starting with transfer of splenocytes; a blood glucose level >12.0 mmol/l on two consecutive readings was scored as indicative of diabetes.

Phenotypic analysis of mononuclear cells. Lymphocytes were isolated and analyzed by flow cytometry exactly as previously described (15). T-cell subpopulations were identified as follows: CD8a (Ly2)(53-6-7) and memory-effector cells $CD44^{high}CD62L^{low}$ and regulatory T-cells (Tregs) $CD4^+$ (L3T4) (GK1.5), $CD25^+$ (7D4) and (PC61), and $Foxp3^+$ (Foxp3-staining kit; eBioscience, San Diego, CA). Diabetogenic T-cell clones were identified based on expression of CD4 (H129.19) and CD40 (3/23) as previously described (21). B-cell subpopulations were identified exactly as previously described (15): follicular B-cells (FoB) ($CD23^{high}$, IgM^+, and $CD21^{int}$), MZBs ($CD23^{low}$, IgM^{high}, and $CD21^{high}$), transitional type 1 (T1) cells ($CD23^{low}$, IgM^{high}, and $CD21^{low}$), and transitional type 2 (T2MZ) cells ($CD23^{high}$, IgM^+, and $CD21^{high}$). Isotype controls included IgG_1, λ; IgG_1, κ; IgG_{2b}, κ; and IgG_{2a}, κ. Flow cytometric analysis was conducted on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA).

Cytokine analysis. Cytokine profile of sera samples was performed with a LINCOplex mouse 9-plex cytokine kit from Linco Research (St. Charles, MO), following the manufacturer's instructions. The assays were carried out at The University of New South Wales Inflammation Disease Unit in conjunction with Taline Hampartzoumian.

Histopathology. Formaldehyde-fixed, paraffin-embedded pancreata sections (5 μm) were hematoxylin and eosin stained. Insulitis was scored (100 × magnification) as follows: grade 0, no insulitis; grade 1, peri-insulitis; grade 2, insulitis involving <25% islet; grade 3, insulitis involving >25% islet; and grade 4, insulitis involving >75% and/or complete islet infiltration. Photos were taken using a Leica DC300 camera on a Leica DMRB microscope.

Anti-CD25 antibody treatment. Mice were administered the anti-CD25 monoclonal antibody (mAb) PC61 (200 μg) (The Walter and Eliza Hall Institute of Medical Research [WEHI] mAb facility, Melbourne, Australia) fortnightly for a total of four injections. Control mice received 200 μg rat IgG_1κ (BD Biosciences). BCMA-Fc–treated NOD mice were first inoculated on the 16th week. $NOD.\mu MT^{-/-}$ mice were administered PC61 beginning at ~16 weeks of age. The frequency of $CD25^+$ T-cells was determined by analysis of $CD4^+CD25^+$ (mAb 7D4) $Foxp3^+$ cells. Diabetes incidence was followed as described above.

Statistical analysis. Statistical significance for mononuclear cell analysis was determined by calculating *P* values using the Student's *t* test (GraphPad Software, San Diego, CA). Diabetes incidence studies were graphed as Kaplan-Meier survival plots and analyzed using the Mantel-Cox log-rank method with 2 degrees of freedom (GraphPad Prism; GraphPad Software). *P* values represent comparison between different treatments as indicated in the figure legends.

RESULTS

Disrupting the BAFF/APRIL pathway in the preclinical phase prevents diabetes onset. To test the effect of disrupting the BAFF/APRIL pathway before the onset of hyperglycemia, we injected NOD mice with 150 μg BCMA-Fc intraperitoneally (i.p.) twice weekly from 9 to 15 weeks of age (12 injections over a 6-week period); control groups were administered PBS or HuIvIg (150 μg i.p.) over the same period (Fig. 1). We found that all NOD mice treated with PBS or HuIvIg from 9 to 15 weeks of age developed diabetes with the expected high frequencies. There were no significant differences in diabetes incidence between PBS- and HuIvIg-treated groups ($P = 0.1309$; $n \geq 10$). In contrast, we found that NOD mice treated with BCMA-Fc from 9 to 15 weeks of age were completely protected from diabetes (diabetes incidence 0 of 10 at 50 weeks of age; $P = 0.0041$, $n \geq 10$, log-rank vs. HuIvIg).

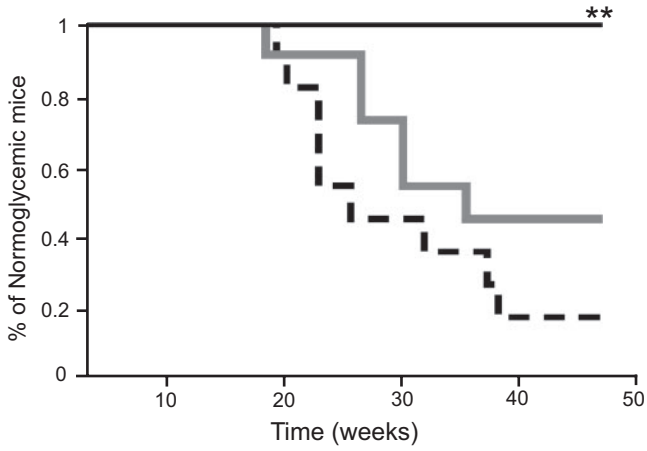

FIG. 1. Administration of BCMA-Fc prevents diabetes in NOD mice. Diabetes incidence was followed for NOD mice administered BCMA-Fc (black line) ($n = 10$), HuIvIg (gray line) ($n = 20$), and PBS (broken line) ($n = 30$) from 9 to 15 weeks of age. **$P = 0.0041$ (Mantel-Cox log-rank analysis) for BCMA-Fc treatment vs. HuIvIg; $P < 0.0001$ for BCMA-Fc treatment vs. PBS.

Effect of BCMA-Fc treatment on peripheral B-cell populations. Examination of peripheral lymphoid populations before and at the cessation of the 9- to 15-week BCMA-Fc treatment was carried out by flow cytometry. Given the well-described requirement of BAFF in the regulation of steady-state B-cell homeostasis (1), we first examined B-cell populations. As shown in Fig. 2*A*, all three known BAFF and APRIL receptors were expressed by NOD IgM^+ $B220^+$ B-cells, suggesting that NOD B-cells would be sensitive to BAFF/APRIL blockade. Tracking IgM^+ $B220^+$ cells in the blood during the course of BCMA-Fc and HuIvIg treatment revealed a steady reduction in the peripheral B-cell frequency during the BCMA-Fc treatment period, reaching a nadir at ~4 weeks (Fig. 2*B*). Further analysis conducted at the end of the 9- to 15-week treatment period demonstrated that BCMA-Fc treatment reduced the frequencies of mature follicular and MZB subsets, as well as the immature T2MZ cells in the spleen and pancreatic lymph node (PLN) (Fig. 2*D*). Similarly, the absolute numbers of the follicular, marginal-zone, and T2MZ subsets were reduced by ~80–90% (Fig. 2*E*). In contrast, the frequency and absolute numbers of T1 precursors in the spleen were less affected by BCMA-Fc treatment (Fig. 2*D* and *E*), a result consistent with the role of BAFF in promoting B-cell development after the T1 checkpoint (22).

Effect of BCMA-Fc treatment on peripheral T-cell populations and Th1 cytokines. In contrast to its effect on B-cell populations, administration of BCMA-Fc from 9 to 15 weeks of age did not impact the absolute number of peripheral T-cells or $CD4^+$ and $CD8^+$ T-cell subsets (Fig. 3*A*). However, the frequency of splenic $CD4^+$ and $CD8^+$ T-cells was proportionally increased, presumably as a result of the decreased number of B-cells (data not depicted). To determine how BCMA-Fc treatment affected the activation of effector T-cell clones, we analyzed the expression of CD44 and CD40 on peripheral CD4 and CD8 T-cell populations. CD44 is expressed by activated T-cells, whereas CD40 has been identified as a marker for highly diabetogenic T-cell clones (21). BCMA-Fc treatment did not alter the frequency of $CD44^{high}CD4^+$ or $CD44^{high}CD8^+$ T-cells (data not depicted); however, the frequency of $CD4^+$ and $CD8^+CD40^+$ T-cells was reduced in both the spleen and PLN of BCMA-Fc–treated mice (Fig. 3*B*). The reduction in frequency of pathogenic $CD40^+$ T-cells in BCMA-Fc–treated mice was associated with a decrease in

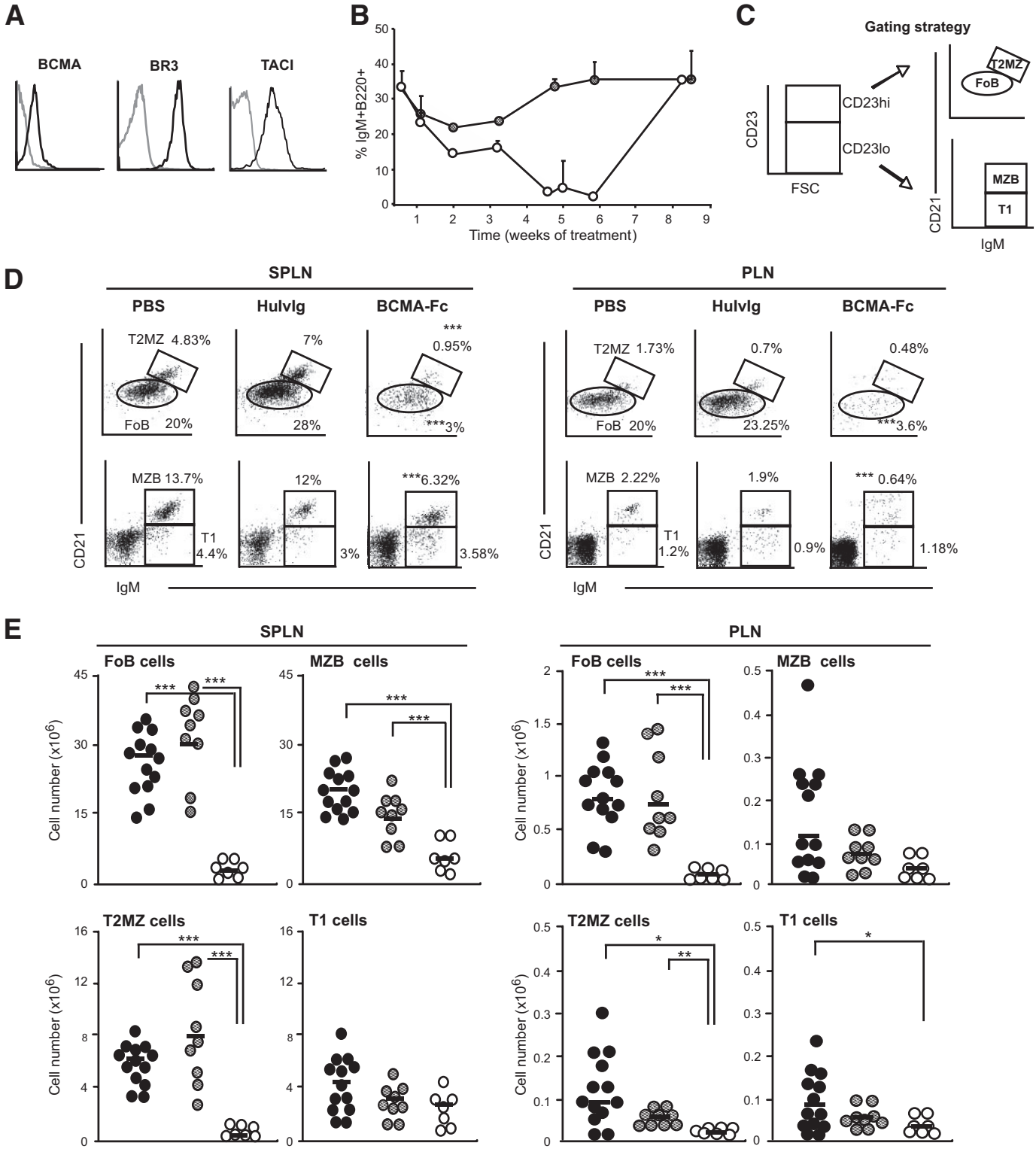

FIG. 2. Effect of BCMA-Fc treatment on peripheral B-cells. *A*: Expression of BCMA, BR3, and TACI (black line) on IgM$^+$B220$^+$ NOD splenocytes. Gray line, isotype control. Representative fluorescence-activated cell sorting plots are shown. *B*: Frequency of IgM$^+$B220$^+$ cells in peripheral blood of NOD mice treated with BCMA-Fc from 9 to 15 weeks of age (○) ($n = 5$) and HuIvIg control mice (▨) ($n = 5$). Time indicates period post–first injection. *C*: Gating strategy used for identification of B-cell subsets. *D*: Representative fluorescence-activated cell sorting plots illustrating frequency of B-cell subsets in the spleen (SPLN) and PLN from 16-week-old NOD mice treated with PBS, HuIvIg, or BCMA-Fc from 9 to 15 weeks of age. Numbers represent percentage of total lymphocytes. *E*: Absolute numbers, calculated from values in *D*, of B-cell subsets in the spleen and PLN from 16-week-old NOD mice treated with PBS (●), HuIvIg (▨), or BCMA-Fc (○). Values from individual mice are shown ($n \geq 8$ per group). The bar represents median value. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. FSC, forward light scatter.

the circulating levels of interleukin (IL)-7, IL-15, and IL-17 (Fig. 3*C*), cytokines critical for the expansion and activation of effector T-cells.

Effect of BCMA-Fc treatment on B-cell repopulation. To examine how BCMA-Fc treatment effected B-cell repopulation, further analysis was carried out in long-term

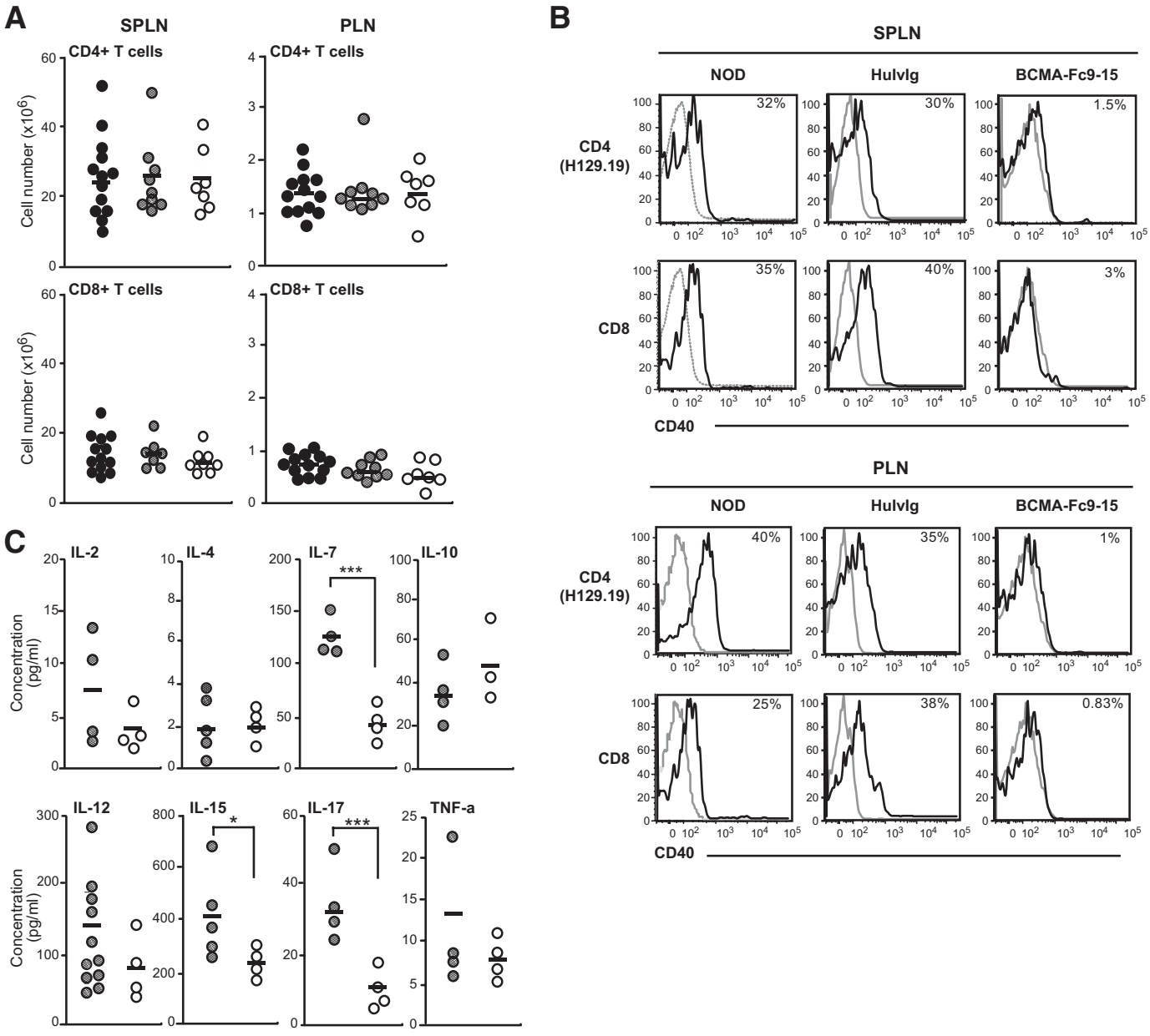

FIG. 3. Effect of BCMA-Fc on peripheral T-cells and cytokines. *A*: Absolute number of CD4$^+$ (*upper panel*) and CD8$^+$ (*lower panel*) T-cells from 16-week-old NOD mice treated with PBS (●), HuIvIg (▨), or BCMA-Fc (○) from 9 to 15 weeks of age. Values from individual mice are shown ($n \geq 8$ per group). *B*: Representative fluorescence-activated cell sorting plots illustrating frequency of CD4$^+$CD40$^+$ (*upper panel*) and CD8$^+$CD40$^+$ (*lower panel*) T-cells from treated mice at 16 weeks of age. Black line, CD40; gray line, isotype control ($n = 7$ per group). *C*: Serum cytokine levels in HuIvIg- (▨) and BCMA-Fc– (○) treated NOD mice at 16 weeks of age. Values from individual mice are shown ($n \geq 3$ per group). Significant differences between sample means are indicated. Bar represents median value. *$P < 0.05$ and ***$P < 0.001$. SPLN, spleen.

surviving (≥50 weeks of age) BCMA-Fc–treated mice. BCMA-Fc–protected mice exhibited normal frequencies and absolute numbers of FoB, MZB, T2MZ, and T1 B-cell subsets in the periphery compared with those in HuIvIg-treated control mice (Fig. 4*A* and *B*). Although the PLN of BCMA-Fc–treated mice harbored frequencies and numbers of FoB and MZB cells similar to those of control mice, an increase in both the frequency and the number of T2MZ and T1 cells was observed (Fig. 4*A* and *B*). These data demonstrate that BCMA-Fc–treated NOD mice could repopulate their mature B-cell pool. These data also demonstrate that BCMA-Fc treatment provides long-term protection from diabetes in NOD mice despite the return of B-cell populations.

Effect of BCMA-Fc treatment on the pancreatic infiltrate. We conducted histological analysis of the pancreata from the BCMA-Fc–protected NOD mice at 16 and ≥50 weeks of age and a comparison with treated control mice. Representative histology for each group is shown in Fig. 5*A*, and insulitis scores for these mice are shown in Fig. 5*C*. Although BCMA-Fc–treated NOD mice did exhibit insulitis at the 16-week time point, the severity was reduced compared with that in HuIvIg-treated and diabetic control NOD mice at 16 weeks. Indeed, the frequency of islets exhibiting heavy insulitis (grades 3 and 4) at 16 weeks was only ~20 vs. ~45–50% in control groups. Flow cytometric analysis of the pancreatic infiltrate revealed that the frequency of FoB was markedly reduced ($P =$

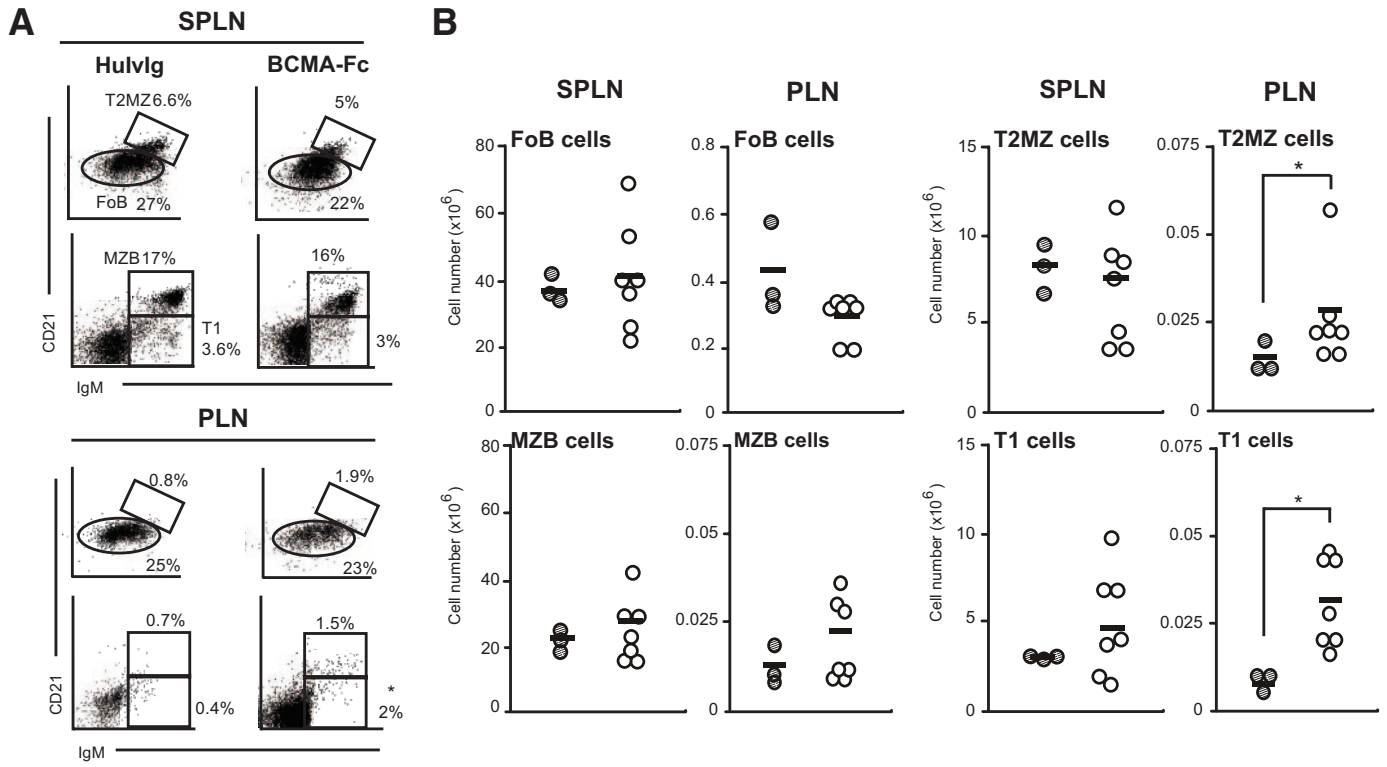

FIG. 4. Effect of BCMA-Fc treatment on B-cell repopulation. *A*: **Representative fluorescence-activated cell sorting plots illustrating frequency of B-cell subsets from NOD mice treated with HuIvIg at 30 weeks of age or BCMA-Fc at ≥50 weeks of age. Numbers represent percentage of total lymphocytes.** *B*: **Absolute numbers of B-cell subsets from HuIvIg- (▨) and BCMA-Fc– (○) treated NOD mice. Values calculated from** *A*. **Results from individual mice are shown ($n \geq 3$ per group). Bar represents median value. *$P < 0.05$ and **$P < 0.01$. SPLN, spleen.**

0.014; $n \geq 5$) in the BCMA-Fc–treated mice at 16 weeks (Fig. 5*D*). No changes were observed in the ratios of $CD4^+$ to $CD8^+$ T-cells, though their frequencies were increased (Fig. 5*D*), again, most probably as a result of the decrease in FoB.

The proportion of severely infiltrated islets in the BCMA-Fc–treated mice did not increase over time, as evidenced by analysis of the long-term–protected mice (e.g., ≥50-week-old mice) (Fig. 5*A* and *C*). Thus, although BCMA-Fc–treated mice did exhibit evident insulitis, the severity of insulitis was maintained at a level equivalent to that exhibited by prehyperglycemic 8- to 15-week-old NOD mice.

Given that NOD mice exhibit a progressive insulitis from the preclinical to hyperglycemic phase, these data suggest that BCMA-Fc treatment has not reversed the autoimmune process as determined by the persistence of a mononuclear cell pancreatic infiltrate but, rather, halted the progression to fulminant diabetes. Thus, we questioned whether this related to a change in the nature of the insulitic lesion or active regulation. Analysis of the B- and T-cell subsets infiltrating the pancreas in the protected BCMA-Fc–treated mice showed that the frequencies of infiltrating B- and T-cell subsets were similar to those of treated control mice (Fig. 5*D*). Thus, in long-term–protected mice, B-cells are not prohibited from forming a part of the insulitic lesion. To test whether the BCMA-Fc–protected NOD mice still harbored T-cells with self-reactive potential, splenocytes from normoglycemic BCMA-Fc–treated NOD mice were adoptively transferred into NOD.SCID recipients (Fig. 5*E*). As a positive control, other groups received splenocytes from pre-diabetic 16-week-old mice. We found that ~90% (7 of 8) NOD.SCID mice receiving these control splenocytes developed diabetes. Interestingly, ~50% (4 of 8) of NOD.SCID mice receiving splenocytes from BCMA-Fc–treated NOD mice did develop hyperglycemia ($P = 0.088$, NOD vs. BCMA-Fc–treated splenocytes; $n = 8$). These data demonstrate that BCMA-Fc–treated mice still harbored T-cells with self-reactive potential, but these T-cells are unable to precipitate diabetes in their BCMA-Fc–treated hosts.

Time-dependent accumulation of $CD4^+CD25^+$ T-cells in BCMA-Fc–treated NOD mice. The long-term protection afforded by BCMA-Fc treatment despite the persistence of self-reactive T-cells prompted us to investigate possible regulatory mechanisms. Tregs that express the markers $CD4^+$ and $CD25^+$ can control the progression to overt diabetes in the NOD model (23). We analyzed the frequency and number of $CD4^+CD25^+$ T-cells in the spleen, PLN, and pancreas of BCMA-Fc–treated NOD mice at 16 weeks of age. As shown in Fig. 6*A* and *B*, compared with treated control NOD mice, 9- to 15-week BCMA-Fc–treated mice harbored an increased frequency of splenic $CD4^+CD25^+$ T-cells at 16 weeks of age. The majority (>90%) of these $CD4^+CD25^+$ T-cells were also $Foxp3^+$ (Fig. 6*C* and *D*), demonstrating that they belonged to the set of Tregs. Analysis of the long-term (≥50 weeks of age) BCMA-Fc–protected mice revealed increased (twofold or more) frequencies of $CD4^+CD25^+$ T-cells in both the spleen and PLN (Fig. 6*E*). Further, in contrast with the 16-week time point, the increased frequency of Tregs was also reflected as an increase in the absolute numbers of $CD4^+CD25^+$ T-cells in the spleen and PLN (Fig. 6*E*). Thus, BCMA-Fc–treated mice showed an accumulation of Treg numbers over time. The increased number of Tregs was associated with a halting of the autoimmune attack and permanent euglycemia.

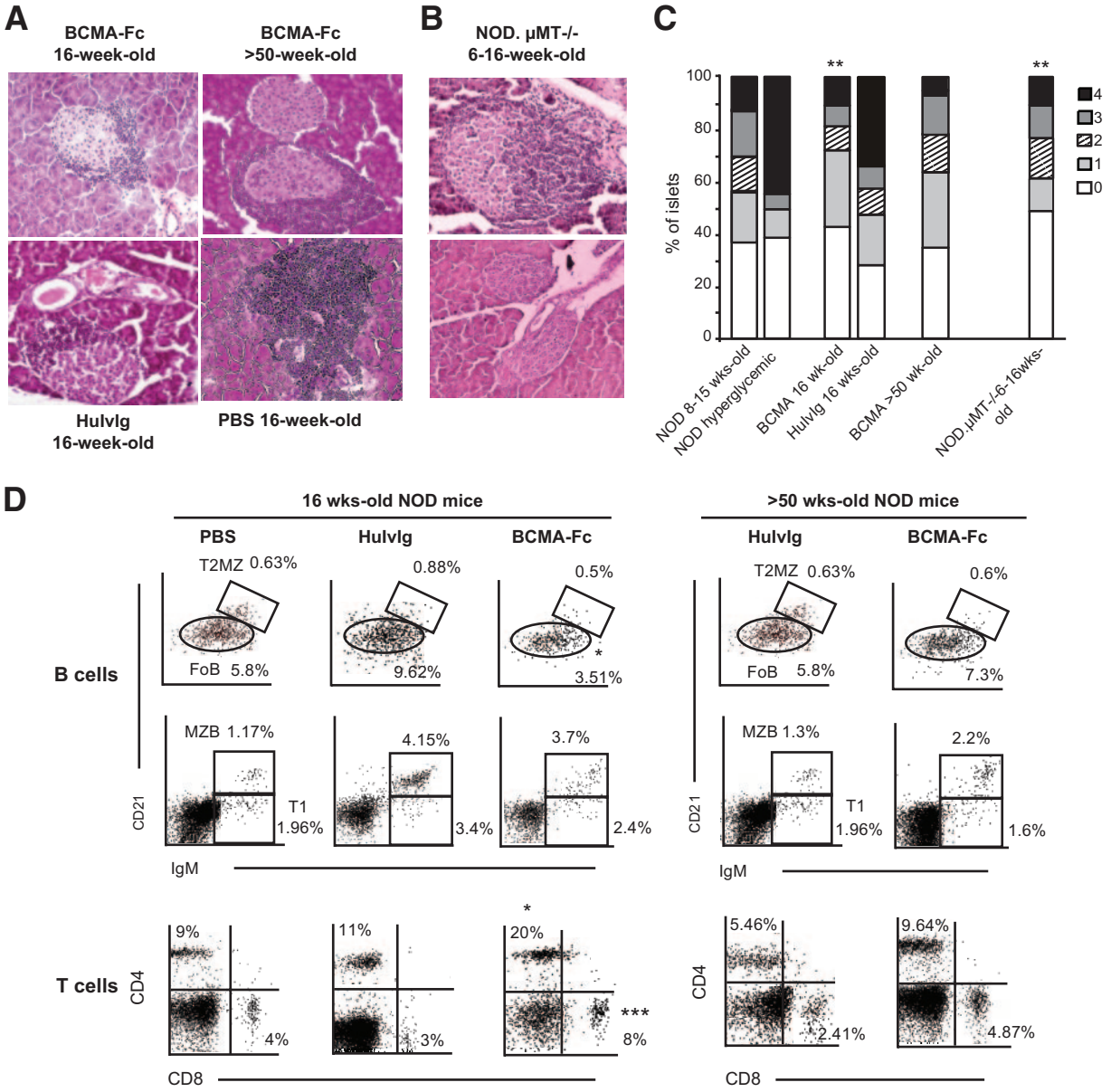

FIG. 5. Histology and insulitis scores of BCMA-Fc–treated NOD mice. *A*: Representative histological section of pancreas from normoglycemic mice treated with BCMA-Fc from 9 to 15 weeks at 16 or ≥50 weeks of age is shown (*upper panels*). Sections from HuIvIg- and PBS-treated mice at 16 weeks are also shown (*lower panels*). *B*: Representative pancreatic sections from an NOD.μMT$^{-/-}$ mouse. *C*: Insulitis scores for treated mice; ~20–70 islets were scored from four to seven mice per group. Differences in insulitis scores for BCMA-Fc–treated mice at 16 and ≥50 weeks were significant ($P < 0.01$). *P* values resulted comparing insulitis level at grade 4 among HuIvIg control mice. *D*: Representative fluorescence-activated cell sorting plots illustrating frequency of pancreatic B-cell (*upper panel*) and T-cell (*lower panel*) subsets in treated NOD mice at 16 and ≥50 weeks of age ($n > 7$ per group). *E*: Splenocytes (1×10^7) from ≥50-week-old normoglycemic BCMA-Fc–treated NOD mice (black line) ($n = 8$) and pre-diabetic 16-week-old NOD mice (gray line) ($n = 8$) were adoptively transferred (intravenously) to NOD.SCID recipients. Diabetes incidence was then followed over time. $P = 0.08$ (Mantel-Cox log-rank analysis) for BCMA-Fc–treated vs. NOD islets. (A high-quality digital representation of this figure is available in the online issue.)**

BCMA-Fc–mediated protection from diabetes requires the presence of CD4$^+$CD25$^+$ T-cells. We next determined whether CD4$^+$CD25$^+$ T-cells were required for BCMA-Fc–mediated protection from diabetes. To achieve this, mice were administered the anti-CD25 mAb PC61. Control mice received PBS or rat IgG$_1$κ. In preliminary experiments, we could show that administration of a single dose of PC61 (200 μg i.p.) induced an

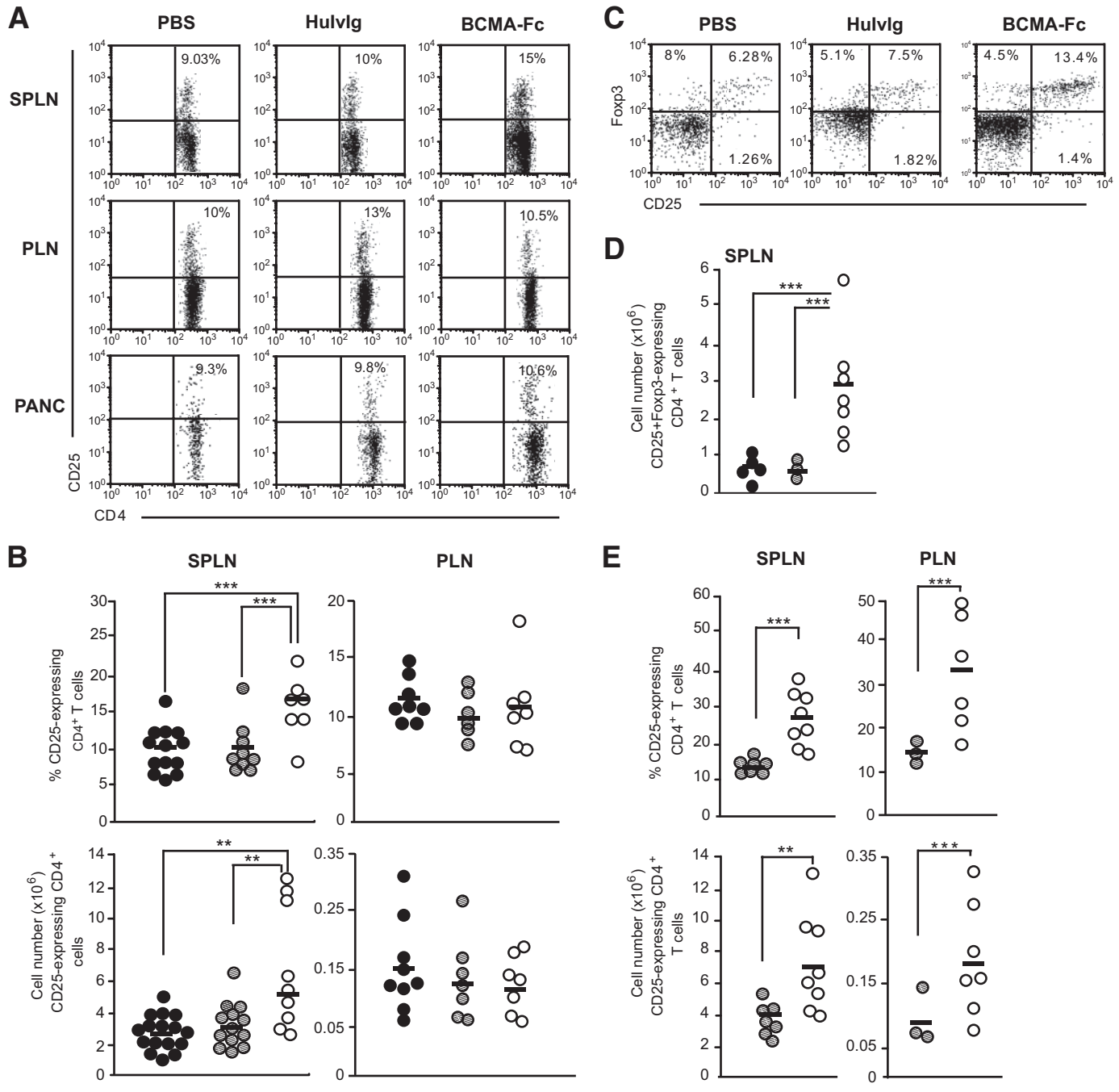

FIG. 6. $CD4^+CD25^+$ T-cells in BCMA-FC–treated NOD mice. *A*: Representative fluorescence-activated cell sorting plot illustrating frequency of CD25-expressing $CD4^+$ T-cells from 16-week-old PBS-, HuIvIg-, and BCMA-Fc–treated NOD mice ($n > 7$ per group) is shown. *B*: Pooled data showing frequencies and calculated absolute numbers of CD25-expressing CD4 T-cells of PBS- (●), HuIvIg- (▨), or BCMA-Fc– (○) treated NOD mice at 16 weeks. Values from individual mice are shown ($n \geq 7$ per group). Bar represents median value. *C*: Representative fluorescence-activated cell sorting plots illustrating frequency of $CD4^+CD25^+Foxp3^+$ cells from 16-week-old PBS-, HuIvIg-, and BCMA-Fc–treated NOD mice. *D*: Absolute numbers, calculated from *C*, of $CD4^+CD25^+Foxp3^+$ cells from PBS- (●), HuIvIg- (▨), and BCMA-Fc– (○) treated NOD mice. Values from individual mice are shown. Bar represents median value. *E*: Cumulative data showing frequency and calculated absolute numbers of CD25-expressing CD4 T-cells from HuIvIg- (▨) or BCMA-Fc– (○) treated NOD mice ($n \geq 3$ per group). Bar indicates median value. $^{**}P < 0.01$ and $^{***}P < 0.001$. PANC, pancreas; SPLN, spleen.

~90% reduction in the frequency of peripheral $CD4^+CD25^+Foxp3^+$ T-cells for ~14 days (Fig. 7*A*). For the experiment, NOD mice were first treated with BCMA-Fc from 9 to 15 weeks of age; at the cessation of BCMA-Fc treatment, mice were administered the anti-CD25 mAb PC61 (200 μg i.p.) every 14 days (a total of four injections), and blood glucose levels were monitored. Kaplan-Meier survival analysis showed that BCMA-Fc–treated NOD mice remained diabetes free, whereas control PC61-treated NOD mice developed hyperglycemia with the expected frequency (Fig. 7*B*). In contrast, subsequent administration of PC61 after BCMA-Fc treatment precipitated diabetes in 100% of mice ($P = 0.001$ BCMA-Fc vs. BCMA-Fc plus PC61; $n \geq 5$).

Blocking the BAFF/APRIL pathway indirectly modulates $CD4^+CD25^+Foxp3^+$ T-cells. We next focused on determining the mechanism by which BCMA-Fc treatment would effect protection in a $CD4^+CD25^+$ T-cell–depen-

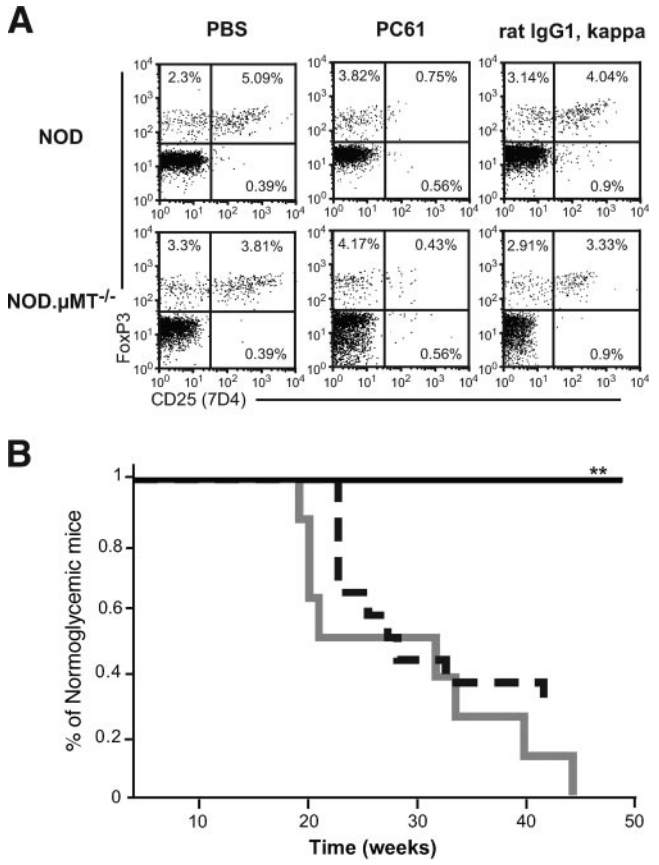

FIG. 7. CD25$^+$ T-cells are required for BCMA-Fc–mediated protection from diabetes. *A*: Frequency of CD4-expressing CD25$^+$ (mAb 7D4) Foxp3 T-cells in peripheral blood of 16-week-old NOD (*upper panel*) and NOD.μMT$^{-/-}$ (*lower panel*) mice treated with PC61 or isotype control (rat IgG$_1$, κ). Representative fluorescence-activated cell sorting plot is shown ($n \geq 4$ per group). *B*: Diabetes incidence was followed for NOD mice administered BCMA-Fc from 9 to 15 weeks of age (solid black line) ($n = 5$), PC61 (broken line) ($n = 5$), or BCMA-Fc from 9 to 15 weeks of age plus PC61 (solid gray line) ($n = 8$). **$P = 0.001$ (Mantel-Cox log-rank analysis) for BCMA-Fc alone vs. BCMA-Fc plus PC61 treatment.

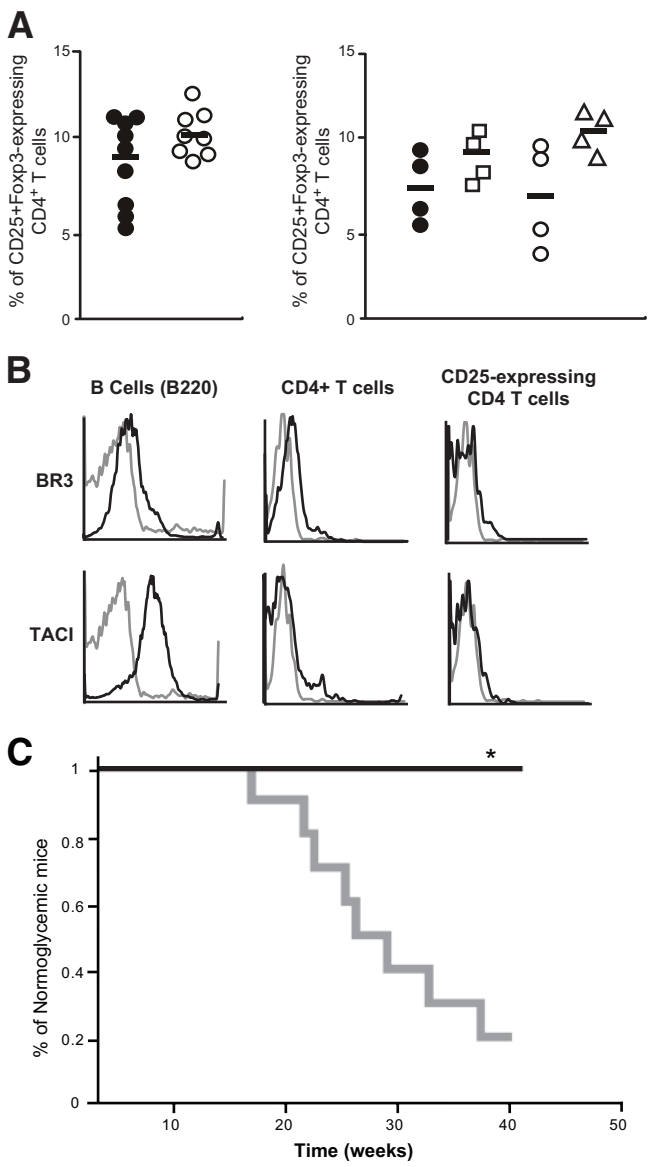

FIG. 8. CD25+ T-cells are required for diabetes resistance in B-cell–deficient NOD mice. *A*: Left plot: frequencies of splenic CD4$^+$CD25$^+$Foxp3$^+$ T-cells in C57BL/6 (●) and NOD (○) mice. Right plot: frequencies of splenic CD4$^+$CD25$^+$Foxp3$^+$ T-cells in BAFF$^{-/-}$ (●), BR3$^{-/-}$ (□), TACI$^{-/-}$ (○), and BCMA$^{-/-}$ (△) mice. Values from individual mice are shown ($n \geq 4$ per group). Bar represents median value. Differences are not significant ($P > 0.05$). *B*: Expression of BR3 and TACI (black line) on C57BL/6 splenic, B220$^+$ B-cells, CD4$^+$ T-cells, and CD25-expressing CD4$^+$ T-cells. Gray line, isotype control. Representative fluorescence-activated cell sorting plots are shown. *C*: Diabetes incidence was followed for NOD.μMT$^{-/-}$ mice (black line) ($n = 5$) and NOD.μMT$^{-/-}$ mice administered PC61 (solid gray line) ($n = 10$). *$P = 0.0181$ (Mantel-Cox log-rank analysis) for control vs. PC61 treatment.

dent manner. To examine whether disrupted BAFF or APRIL signaling would engender an accumulation of CD4$^+$CD25$^+$Foxp3$^+$ T-cell numbers over time, we examined the frequency of CD4$^+$CD25$^+$Foxp3$^+$ T-cells in mice deficient for BAFF or the BAFF and APRIL receptors BCMA and TACI, as well as the BAFF receptor BR3. Though these mutations were on a C57BL/6 background, we believe that the analysis is valid given that the median frequency of CD4$^+$CD25$^+$Foxp3$^+$ T-cells was similar between NOD and C57BL/6 mice ($P = 0.3513$; $n \geq 7$): ~9% for both strains (Fig. 8*A*). Also shown in Fig. 8*A*, the frequency of peripheral CD4$^+$CD25$^+$Foxp3$^+$ T-cells in BAFF$^{-/-}$, BR3$^{-/-}$, BCMA$^{-/-}$, and TACI$^{-/-}$ mice was comparable with that in wild-type mice. Further, to determine whether BAFF or APRIL could engage Tregs directly, we examined TACI and BR3 expression in CD4$^+$CD25$^+$ T-cells. CD4$^+$CD25$^+$ T-cells did not express significant levels of the receptors BR3 or TACI (Fig. 8*B*) in contrast to splenic B-cells. These data indicate that it is unlikely that targeting the BAFF/APRIL system resulted in increased CD4$^+$CD25$^+$Foxp3$^+$ T-cells via a direct mechanism.

CD4$^+$CD25$^+$ T-cells reign in destructive T-cells in B-cell–deficient NOD mice. B-cell subpopulations were severely reduced by BCMA-Fc treatment. To explore the relationship between a reduction in B-cells and CD4$^+$CD25$^+$ T-cells, we used B-cell–deficient NOD.μMT$^{-/-}$ mice (18). NOD.μMT$^{-/-}$ mice exhibit a number of features, with regard to diabetes development, reminiscent of those in BCMA-Fc–treated mice. These include a reduced degree of insulitis (Fig. 5*B* and *C*) and resistance to diabetes development (Fig. 8*C*). To assess whether CD4$^+$CD25$^+$ T-cells were important in maintaining euglycemia in NOD.μMT$^{-/-}$mice, ~16-week-old mice were treated with PC61 (200 μg i.p. each) every 14 days for a total of four injections and blood glucose levels were followed. This treatment reduced the frequency of peripheral

$CD4^+CD25^+Foxp3^+$ T-cells as observed for B-cell–sufficient NOD mice (Fig. 7*A*). Treatment with PC61 precipitated diabetes in ~80% ($P = 0.0184$; $n = 10$) of NOD.$\mu MT^{-/-}$ mice (Fig. 8*C*)—a dramatic result when compared with data in control NOD.$\mu MT^{-/-}$ mice. Therefore, targeting $CD25^+$ regulatory populations triggered diabetes onset in B-cell–deficient NOD.$\mu MT^{-/-}$ mice that are otherwise resistant.

DISCUSSION

Emerging evidence demonstrates that B-cells can impact multiple stages in the pathogenesis of autoimmune diabetes (24). BAFF and APRIL play critical roles in supporting B-cell survival (1), such that BCMA-Fc–treated mice harbored a much reduced B-cell pool, providing one potential mechanism by which BCMA-Fc prevented diabetes in NOD mice. BAFF-activated B-cells show an enhanced antigen-presenting cell (APC) capacity (25), and in vivo BAFF-activated B-cells facilitate heightened Th1 T-cell responses (26). In the NOD model, B-cells undergo a marked expansion from ~9 to 15 weeks of age (15), show an increased capacity to act as APCs during this time (15,27), and can present captured autoantigen to self-reactive T-cells (15,28,29). The APC function of B-cells is essential for the activation (30) and expansion (31) of the $CD4^+$ T-cell repertoire from ~9 to 15 weeks of age, i.e., before the onset of hyperglycemia. Thus, by reducing the availability of B-cells to act as APCs, BCMA-Fc treatment curtailed B- and T-cell interactions during the critical 9- to 15-week time period, halting the progression from clinically silent insulitis to overt hyperglycemia. This concept is consistent with our observation that BCMA-Fc–treated mice exhibited a reduced proportion of B-cells in association with a diminished frequency of $CD4^+CD40^+$ diabetogenic T-cells and a reduced level of Th1 T-cell–derived cytokines at 16 weeks of age. Additional evidence to support this hypothesis comes from studies in which NOD mice expressing human CD20 have been treated with an anti-CD20 mAb (32); in this case, reducing B-cells from 9 weeks of age delays diabetes onset. Depletion of B-cells with anti-CD20 impairs activation of adaptive and autoreactive $CD4^+$ T-cell responses (32,33), further demonstrating the required role for B-cells in the activation of self-reactive $CD4^+$ T-cells (30,34).

Our studies uncover an additional and perhaps unexpected mechanism by which targeting the BAFF and APRIL system can prevent diabetes, namely through increasing $CD4^+CD25^+$ T-cells. Tregs expressing $CD25^+$ and Foxp3 can control the development of autoimmune diabetes (35), and we found that $CD4^+CD25^+$ T-cells were required for the continued maintenance of a hyperglycemia-free state. Further, these $CD4^+CD25^+$ T-cells expressed the Treg lineage marker Foxp3, suggesting that they belonged to the set of natural Tregs (36). To address the question of how BCMA-Fc might affect $CD4^+CD25^+$ T-cell homeostasis, we examined $Foxp3^+$ Treg frequencies in mice in which BAFF and APRIL signaling were disrupted. These data demonstrated that loss of BAFF or APRIL did not increase the frequency of $Foxp3^+$ Tregs per se, suggesting that BCMA-Fc altered Treg homeostasis through an indirect mechanism. Of interest, an increased frequency of Tregs was demonstrated in two recent studies in which NOD mice were treated with the B-cell depleting agents: anti-CD20 or anti-CD22 mAb, respectively (32,37). Both studies also reported that NOD mice treated between 9 and 15 weeks exhibited a delayed and reduced incidence of diabetes. Together with our study results, these data indicate that reducing B-cells in autoimmune NOD mice is associated with an increased frequency of cells with a Treg phenotype. Significantly, our present data demonstrate that in the absence of B-cells, endogenous Tregs control the progression of autoimmunity in NOD mice. This conclusion is further supported by our analysis of NOD.$\mu MT^{-/-}$ mice that lack B-cells and are resistant to diabetes (18). Treatment of NOD.$\mu MT^{-/-}$ mice with the anti-CD25 mAb PC61 precipitated the onset of diabetes, demonstrating that the hyperglycemia-free state in NOD.$\mu MT^{-/-}$ mice is also dependent on $CD25^+$ regulatory cells.

These are, to our knowledge, the first results to show that the resistance of B-cell–deficient NOD mice to diabetes can be due to the activity of Tregs. However, they are consistent with emerging results from other models of autoimmune disease. Indeed, NOD.H-2h4 mice develop spontaneous autoimmune thyroiditis, whereas B-cell–deficient NOD.H-2h4 mice are protected from autoimmunity (38). Treatment of B-cell–deficient NOD.H-2h4 with PC61 induced the rapid onset of autoimmunity (39), similar to the results obtained in NOD.$\mu MT^{-/-}$ mice, suggesting a common mechanism whereby the absence of B-cells allows a regulatory pathway to prevent autoimmunity. A number of possibilities exist to explain this intriguing relationship between B-cells and Tregs. As an example, Olson et al. (40) using a model of Crohn's disease demonstrated that B-cells could block Treg activity in a glucocorticoid-induced tumor necrosis factor receptor (GITR)-dependent manner. Thus, activated NOD B-cells may directly suppress the activity of Tregs through mechanisms including expression of GITR ligand (40). Alternatively, because the number of B-cells increases as disease progresses (15), the opportunity for T-cells to engage activating APCs (e.g., B-cells) (30,31) versus a tolerizing APC (e.g., macrophages or dendritic cells) (41) may decrease. In this scenario, BCMA-Fc treatment could be protective via two potential mechanisms. First, BCMA-Fc, by reducing B-cells, may alter the balance in the production of effector cells versus Tregs, leading to an accumulation of $CD4^+CD25^+$ Tregs. Second, without B-cells to expand up self-reactive T-cell clones, endogenous regulatory cells may simply be able to reign in the present autoreactive T-cell pool, preventing the progression to hyperglycemia. These hypotheses should be explored in further studies.

In this study, we demonstrate that targeting the BAFF and APRIL pathway with BCMA-Fc before the onset of hyperglycemia prevented diabetes in spontaneously diabetic NOD mice. We hypothesize that the major action achieved by blocking BAFF and APRIL was a reduction in peripheral B-cells, thereby limiting their involvement in diabetes pathogenesis. This prevented diabetes progression through two mechanisms: a dampening of T-cell autoimmune activity and subsequent elaboration of cytokines, most likely achieved by decreasing the availability of B-cells to act as APCs; a second mechanism also related to decreased B-cells, which induced or allowed Tregs to reign in the autoreactive potential of diabetogenic T-cells. This second mechanism highlights a novel pathway by which targeting B-cells may provide resistance to autoimmunity.

ACKNOWLEDGMENTS

This study was supported by a grant from the Juvenile Diabetes Research Foundation International (5-2005-1132) to S.T.G. E.M. is supported by a National Health and

Medical Research Council Dora Lush Fellowship. S.T.G. is supported by a BioFirst Award from the Office for Science and Medical Research, NSW.

No potential conflicts of interest relevant to this article were reported.

We greatly appreciate the technical assistance of Eric Schmied from the Biological Testing Facility at Garvan.

REFERENCES

1. Mackay F, Sierro F, Grey ST, Gordon TP. The BAFF/APRIL system: an important player in systemic rheumatic diseases. Curr Dir Autoimmun 2005;8:243–265
2. Mackay F, Kalled SL. TNF ligands and receptors in autoimmunity: an update. Curr Opin Immunol 2002;14:783–790
3. Gross JA, Johnston J, Mudri S, Enselman R, Dillon SR, Madden K, Xu W, Parrish-Novak J, Foster D, Lofton-Day C, Moore M, Littau A, Grossman A, Haugen H, Foley K, Blumberg H, Harrison K, Kindsvogel W, Clegg CH. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. Nature 2000;404:995–999
4. von Bulow GU, Bram RJ. NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. Science 1997;278:138–141
5. Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, Hession C, Schneider P, Sizing ID, Mullen C, Strauch K, Zafari M, Benjamin CD, Tschopp J, Browning JL, Ambrose C. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science 2001;293:2108–2111
6. Ng LG, Sutherland AP, Newton R, Qian F, Cachero TG, Scott ML, Thompson JS, Wheway J, Chtanova T, Groom J, Sutton IJ, Xin C, Tangye SG, Kalled SL, Mackay F, Mackay CR. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. J Immunol 2004;173:807–817
7. Cheema GS, Roschke V, Hilbert DM, Stohl W. Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. Arthritis Rheum 2001;44:1313–1319
8. Stohl W. B lymphocyte stimulator protein levels in systemic lupus erythematosus and other diseases. Curr Rheumatol Rep 2002;4:345–350
9. Tan SM, Xu D, Roschke V, Perry JW, Arkfeld DG, Ehresmann GR, Migone TS, Hilbert DM, Stohl W. Local production of B lymphocyte stimulator protein and APRIL in arthritic joints of patients with inflammatory arthritis. Arthritis Rheum 2003;48:982–992
10. Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, Tschopp J, Browning JL. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J Exp Med 1999;190:1697–1710
11. Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, Tschopp J, Cachero TG, Batten M, Wheway J, Mauri D, Cavill D, Gordon TP, Mackay CR, Mackay F. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. J Clin Invest 2002;109:59–68
12. Batten M, Groom J, Cachero TG, Qian F, Schneider P, Tschopp J, Browning JL, Mackay F. BAFF mediates survival of peripheral immature B lymphocytes. J Exp Med 2000;192:1453–1466
13. Wellmann U, Werner A, Winkler TH. Altered selection processes of B lymphocytes in autoimmune NZB/W mice, despite intact central tolerance against DNA. Eur J Immunol 2001;31:2800–2810
14. Rolf J, Motta V, Duarte N, Lundholm M, Berntman E, Bergman ML, Sorokin L, Cardell SL, Holmberg D. The enlarged population of marginal zone/CD1d(high) B lymphocytes in nonobese diabetic mice maps to diabetes susceptibility region Idd11. J Immunol 2005;174:4821–4827
15. Mariño E, Batten M, Groom J, Walters S, Liuwantara D, Mackay F, Grey ST. Marginal-zone B-cells of nonobese diabetic mice expand with diabetes onset, invade the pancreatic lymph nodes, and present autoantigen to diabetogenic T-cells. Diabetes 2008;57:395–404
16. Ramanujam M, Davidson A. The current status of targeting BAFF/BLyS for autoimmune diseases. Arthritis Res Ther 2004;6:197–202
17. Ding C, Foote S, Jones G. B-cell-targeted therapy for systemic lupus erythematosus: an update. Bio Drugs 2008;22:239–249
18. Serreze DV, Chapman HD, Varnum DS, Hanson MS, Reifsnyder PC, Richard SD, Fleming SA, Leiter EH, Shultz LD. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. J Exp Med 1996;184:2049–2053
19. Sasaki Y, Casola S, Kutok JL, Rajewsky K, Schmidt-Supprian M. TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. J Immunol 2004;173:2245–2252
20. Pelletier M, Thompson JS, Qian F, Bixler SA, Gong D, Cachero T, Gilbride K, Day E, Zafari M, Benjamin C, Gorelik L, Whitty A, Kalled SL, Ambrose C, Hsu YM. Comparison of soluble decoy IgG fusion proteins of BAFF-R and BCMA as antagonists for BAFF. J Biol Chem 2003;278:33127–33133
21. Wagner DH Jr, Vaitaitis G, Sanderson R, Poulin M, Dobbs C, Haskins K. Expression of CD40 identifies a unique pathogenic T cell population in type 1 diabetes. Proc Natl Acad Sci U S A 2002;99:3782–3787
22. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, Frew E, Scott ML. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. Science 2001;293:2111–2114
23. Chatenoud L, Salomon B, Bluestone JA. Suppressor T cells: they're back and critical for regulation of autoimmunity! Immunol Rev 2001;182:149–163
24. Silveira PA, Grey ST. B cells in the spotlight: innocent bystanders or major players in the pathogenesis of type 1 diabetes. Trends Endocrinol Metab 2006;17:128–135
25. Yang M, Hase H, Legarda-Addison D, Varughese L, Seed B, Ting AT. B cell maturation antigen, the receptor for a proliferation-inducing ligand and B cell-activating factor of the TNF family, induces antigen presentation in B cells. J Immunol 2005;175:2814–2824
26. Sutherland AP, Ng LG, Fletcher CA, Shum B, Newton RA, Grey ST, Rolph MS, Mackay F, Mackay CR. BAFF augments certain Th1-associated inflammatory responses. J Immunol 2005;174:5537–5544
27. Wheat W, Kupfer R, Gutches DG, Rayat GR, Beilke J, Scheinman RI, Wegmann DR. Increased NF-kappa B activity in B cells and bone marrow-derived dendritic cells from NOD mice. Eur J Immunol 2004;34:1395–1404
28. Serreze DV, Fleming SA, Chapman HD, Richard SD, Leiter EH, Tisch RM. B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Immunol 1998;161:3912–3918
29. Noorchashm H, Lieu YK, Noorchashm N, Rostami SY, Greeley SA, Schlachterman A, Song HK, Noto LE, Jevnikar AM, Barker CF, Naji A. I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet beta cells of nonobese diabetic mice. J Immunol 1999;163:743–750
30. Greeley SA, Moore DJ, Noorchashm H, Noto LE, Rostami SY, Schlachterman A, Song HK, Koeberlein B, Barker CF, Naji A. Impaired activation of islet-reactive CD4 T cells in pancreatic lymph nodes of B cell-deficient nonobese diabetic mice. J Immunol 2001;167:4351–4357
31. Tian J, Zekzer D, Lu Y, Dang H, Kaufman DL. B cells are crucial for determinant spreading of T cell autoimmunity among beta cell antigens in diabetes-prone nonobese diabetic mice. J Immunol 2006;176:2654–2661
32. Hu CY, Rodriguez-Pinto D, Du W, Ahuja A, Henegariu O, Wong FS, Shlomchik MJ, Wen L. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest 2007;117:3857–3867
33. Bouaziz JD, Yanaba K, Venturi GM, Wang Y, Tisch RM, Poe JC, Tedder TF. Therapeutic B cell depletion impairs adaptive and autoreactive CD4+ T cell activation in mice. Proc Natl Acad Sci U S A 2007;104:20878–20883
34. Falcone M, Lee J, Patstone G, Yeung B, Sarvetnick N. B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice. J Immunol 1998;161:1163–1168
35. Tang Q, Adams JY, Penaranda C, Melli K, Piaggio E, Sgouroudis E, Piccirillo CA, Salomon BL, Bluestone JA. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. Immunity 2008;28:687–697
36. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 2005;22:329–341
37. Fiorina P, Vergani A, Dada S, Jurewicz M, Wong M, Law K, Wu E, Tian Z, Abdi R, Guleria I, Rodig S, Dunussi-Joannopoulos K, Bluestone J, Sayegh MH. Targeting CD22 reprograms B-cells and reverses autoimmune diabetes. Diabetes 2008;57:3013–3024
38. Yu S, Dunn R, Kehry MR, Braley-Mullen H. B cell depletion inhibits spontaneous autoimmune thyroiditis in NOD.H-2h4 mice. J Immunol 2008;180:7706–7713
39. Yu S, Maiti PK, Dyson M, Jain R, Braley-Mullen H. B cell-deficient NOD.H-2h4 mice have CD4+CD25+ T regulatory cells that inhibit the development of spontaneous autoimmune thyroiditis. J Exp Med 2006;203:349–358
40. Olson TS, Bamias G, Naganuma M, Rivera-Nieves J, Burcin TL, Ross W, Morris MA, Pizarro TT, Ernst PB, Cominelli F, Ley K. Expanded B cell population blocks regulatory T cells and exacerbates ileitis in a murine model of Crohn disease. J Clin Invest 2004;114:389–398
41. King C, Davies J, Mueller R, Lee MS, Krahl T, Yeung B, O'Connor E, Sarvetnick N. TGF-beta1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. Immunity 1998;8:601–613