CD4+ CD25+ T cells control autoimmunity in the absence of B cells.

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Objective. TNF-ligand family members BAFF and APRIL can exert powerful effects upon B cell activation and development, Th1-type immune responses and autoimmunity. We examined the effect of blocking BAFF and APRIL upon the development of autoimmune diabetes.

Research Design and Methods. Female NOD mice were administered BCMA-Fc from 9-15-weeks-of-age. Diabetes incidence, islet pathology, as well as 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-type cytokines IL-7, IL-15 and IL-17. Thus T cell activation was blunted with reduced B cells. However, BCMA-Fc treated mice still harboured detectable diabetogenic T cells; suggesting regulatory mechanisms contributed to diabetes prevention. Indeed, BCMA-Fc treated mice accumulated increased CD4+ CD25+ Tregs with age. CD4+ CD25+ cells were essential for maintaining euglycaemia as their depletion abrogated BCMA-Fc-mediated protection. BCMA-Fc did not directly effect Treg homeostasis as; CD4+ CD25+ Foxp3+ T cells did not express TACI or BR3 receptors; and, CD4+ CD25+ Foxp3+ T cell frequencies were equivalent in WT, BAFF−/−, TACI−/−, BCMA−/− and BR3−/− mice. Rather, B cell depletion resulted in CD4+ CD25+ T cell-mediated protection from diabetes; as anti-CD25 mAb treatment precipitated diabetes in both diabetes resistant NOD.μMT−/− 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 pre-hyperglycaemic state.

Abbreviations:
APC, antigen presenting cell; APRIL, a proliferation induced ligand; BAFF, B cell activating factor belonging to the TNF family; BCMA, B cell maturation antigen; SPLN, spleen; PLN, pancreatic lymph node; FoB, follicular B cell; MZB, marginal zone B cell; T1, transitional type 1 cell; T2MZ, transitional type 2 cell; TACI, transmembrane activator and calcium-modulator and cyclophilin ligand interactor; Tg, Transgenic.
The members of the TNF-ligand family of molecules BAFF (B cell activating factor belonging to the TNF family, also known as BLyS, TNFSF13b) and APRIL (A proliferation induced ligand) can exert powerful effects upon B cell development, survival and function, T cell activation, Th1-type 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 cyclophillin 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-Tg mice harbour increased titres of self-reactive antibodies and develop autoimmune symptoms very similar to lupus and Sjogren’s syndrome (10; 11). Forced expression of BAFF also results in a marked expansion of marginal zone B cells (12), a B cell subset associated with autoimmune conditions including lupus (13), Sjogren’s syndrome (11) and more recently T1D (14; 15). Thus the BAFF/APRIL system can be considered a pro-inflammatory pathway, associated with the development of autoimmunity (7; 8). Indeed studies 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 T1D. This study was undertaken to test the hypothesis that targeting the BAFF/APRIL system would have multiple inhibitory effects upon the spontaneous development of autoimmune diabetes in the NOD model.

**RESEARCH DESIGN AND METHODS**

**Mice.** C57BL/6, NOD.SCID and NOD/Lt (NOD) mice were obtained from WEHI Kew, Melbourne, Australia. NOD.µMT−/− mice were kindly provided by Dr Serreze (18). BAFF−/−, BCMA−/− and TACI−/− mice were kindly provided by Dr Susan Kalled, Biogen IDEC. BR3−/− mice were a kind gift of Dr Rajewsky (19). All animal experiments were approved by the St Vincent’s Campus Animal Experimentation and Ethics Committee (AEEC).

**Diabetes incidence studies.** NOD mice were administered 150 µg of BCMA-Fc per treatment based upon 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 kindly provided by Dr Susan Kalled Biogen IDEC. Controls were treated with PBS, or 150 µg of intravenous globulin (HuIvIg). For Adoptive transfer studies, splenocytes (1x10⁷) from prediabetic 16 week-old female NOD donors or BCMA-Fc treated mice, were transferred (i.v.) into NOD.SCID recipients. Glucose levels were monitored twice weekly from 10 weeks-of-age onwards for BCMA-Fc treated mice, or starting with transfer of splenocytes; a BGL > 12.0 mmol/L on two consecutive readings was scored as diabetic.

**Phenotypic analysis of mononuclear cells.** Lymphocytes were isolated and
analysed by flow cytometry exactly as described (15). T cell subpopulations were identified as follows; CD8α (Ly2)(53-6-7), memory-effector cells CD44hi CD62Llow; Tregs, CD4+ (L3T4) (GK1.5), CD25+ (7D4) and (PC61) and Foxp3+ (Foxp3-staining kit, eBioscience). Diabetogenic T cell clones were identified based on expression of CD4 (H129.19) and CD40 (3/23) as described (21).

B cell subpopulations were identified exactly as described (15): follicular B cells (FoB), CD23hi, IgM+, CD21low, marginal zone B cells (MZB) cells, CD23low, IgMhi CD21hi, transitional type 1 cells (T1) as CD23low, IgMhi, CD21low; transitional type 2 cells (T2MZ), CD23hi, IgM+, CD21hi. Isotype controls: IgG1,λ; IgG1,κ, IgG2b,κ and IgG2a,κ. Flow cytometric analysis was conducted on a FACScalibur flow cytometer (BD Biosciences).

Cytokine analysis. Cytokine profile of sera samples was performed by Lincoplex-mouse 9 x plex cytokine kit from Linco Research Inc., following manufacturers instructions. The assays were carried out at the UNSW Inflammation Disease Unit in conjunction with Taline Hampartzoumian.

Histopathology. Formaldehyde-fixed, paraffin-embedded pancreata sections (5 µm) were heamatoxylin and eosin stained. Insulitis was scored (100x magnification) as follows: grade 0, no insulitis; grade 1, peri-insulitis; grade 2, insulitis involving < 25% islet; grade 3, insulitis involving > 25% islet; 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 200 µg of the anti-CD25 mAb PC61 (WEHI monoclonal antibody facility, Australia) fortnightly for a total of 4-injections. Control mice received 200 µg rat IgG1κ (BD Biosciences). BCMA-Fc treated NOD mice were first inoculated on the 16th week. NOD.µMT-/- 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 t-test (GraphPad Software, San Diego, CA). Diabetes incidence studies were graphed as Kaplan-Meier survival plots and analyzed using the Logrank (Mantel-Cox) method with 2-degrees of freedom (GraphPad PRISM Software, Inc). P-values represent comparison between different treatments. * (P < 0.05), **(P < 0.01), ***/(P < 0.001).

RESULTS

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

Effect of BCMA-Fc treatment upon peripheral B cell populations. Examination of peripheral lymphoid populations prior to, and at the cessation of the 9-15 week BCMA-Fc-treatment was carried out by flow cytometry. Given the well-described requirement of BAFF in the regulation of
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steady-state B cell homeostasis (1), we first examined B cell populations. As shown in Fig. 2A, all three known BAFF and APRIL receptors were expressed by NOD IgM+ B220+ B cells, suggesting 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. 2B). Further analysis conducted at the end of the 9-15 week treatment period demonstrated that BCMA-Fc treatment reduced the frequencies of mature follicular and marginal zone B cell subsets, as well as the immature T2MZ cells in the spleen and PLN (Fig. 2D). Similarly, the absolute numbers of the follicular, marginal zone and T2MZ subsets were reduced by ~80-90% (Fig. 2E). In contrast, the frequency and absolute numbers of T1 precursors in the spleen were less affected by BCMA-Fc treatment (Fig. 2D 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 upon peripheral T cell populations and Th1 cytokines. In contrast to its effect on B cell populations, administration of BCMA-Fc from 9-15 weeks-of-age did not impact upon the absolute number of peripheral T cells, or CD4+ and CD8+ T cell subsets (Fig. 3A). However the frequency of splenic CD4+ and CD8+ T cells was proportionally increased, presumably due to the decreased number of B cells (data not depicted). To determine how BCMA-Fc treatment affected the activation of effector T cell clones, we analysed 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 CD44hi CD4+ or CD44hi CD8+ T cells (data not depicted), however the frequency of CD4+ and CD8+ CD40+ T cells were reduced in both the spleen and PLN of BCMA-Fc treated mice (Fig. 3B). The reduction in frequency of pathogenic CD40+ T cells in BCMA-Fc treated mice was associated with a decrease in the circulating levels of IL-7, IL-15 and IL-17 (Fig. 3C); cytokines critical for the expansion and activation of effector T cells.

Effect of BCMA-Fc treatment upon B cell repopulation. To examine how BCMA-Fc treatment affected B cell repopulation, further analysis was carried out on long-term 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 as compared to HuIvIg treated control mice (Fig. 4, A and B). While the PLN of BCMA-Fc-treated mice harboured the similar frequencies and numbers of FoB and MZB cells as control mice, an increase in both the frequency and 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 compared this to control treated mice. Representative histology for each group is shown in Fig. 5A and insulin scores for these mice are shown in Fig. 5C. While BCMA-Fc treated NOD mice did exhibit insulitis at the 16-week time point, the severity was reduced compared to HuIvIg-treated and diabetic control NOD mice at 16-weeks. Indeed the frequency of islets
exhibiting heavy insulitis (grade 3 & 4) at 16 weeks was only ~ 20% versus ~ 45-50% in control groups. Flow cytometric analysis of the pancreatic infiltrate revealed that the frequency of FoB was markedly reduced ($P = 0.014, n \geq 5$) in the BCMA-Fc treated mice at 16-weeks (Fig. 5D). No change was observed in the ratios of CD4+ to CD8+ T cells, though their frequencies were increased (Fig. 5D), again most probably due to 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. $\geq 50$ weeks-of-age) (Fig. 5A and C). Thus while BCMA-Fc-treated mice did exhibit evident insulitis, the severity of insulitis was maintained at a level equivalent to that exhibited by pre-hyperglycaemic 8-15-week-old NOD mice.

Given that NOD mice exhibit a progressive insulitis from the pre-clinical to hyperglycaemic phase, these data suggest that BCMA-Fc treatment had not reversed the autoimmune process as determined by the persistence of a mononuclear cell pancreatic infiltrate, but had 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 that of control treated mice (Fig. 5D). 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 harboured T cells with self-reactive potential, splenocytes from normoglycemic BCMA-Fc-treated NOD mice were adoptively transferred into NOD.SCID (severe combined immunodeficient) recipients (Fig. 5E). As a positive control, other groups received splenocytes from prediabetic 16 week-old mice. We found that ~90% (7/8) NOD.SCID mice receiving these control splenocytes developed diabetes. Interestingly, ~50% (4/8) of NOD.SCID mice receiving splenocytes from BCMA-Fc-treated NOD mice did develop hyperglycaemia ($P = 0.088$, NOD splenocytes vs BCMA-Fc treated splenocytes, $n = 8$). These data demonstrate that BCMA-Fc-treated mice still harboured 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. Regulatory T cells that express the markers CD4+ and CD25+ can control the progression to overt diabetes in the NOD model (23). We analysed the frequency and number of CD4+ CD25+ T cells in the spleen as well as the PLN and pancreas of BCMA-Fc treated NOD mice at 16 weeks-of-age. As shown in Fig. 6A and 6B, compared to control-treated NOD mice, 9-15 week BCMA-Fc treated mice harboured 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. 6C and D), demonstrating they belonged to the set of regulatory T cells. Analysis of the long-term (≥ 50 weeks-of-age) BCMA-Fc protected mice revealed increased (≥2-fold) frequencies of CD4+ CD25+ T cells in both the spleen and PLN (Fig. 6E). Further, different to 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. 6E). 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 euglycaemia.

**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 IgG1κ. In preliminary experiments, we could show that administration of a single dose of PC61 (200 μg i.p.) induced a ~90% reduction in the frequency of peripheral CD4+ CD25+ Foxp3+ T cells for ~14 days (Fig. 7A). For the experiment, NOD mice were first treated with BCMA-Fc from 9-15 weeks-of-age, and then at the cessation of BCMA-Fc treatment, mice were administered the anti-CD25 mAb PC61 (200 μg i.p.) every 14-days (total of four injections), and blood glucose levels were followed. Kaplan-meier survival analysis showed that BCMA-Fc treated NOD mice remained diabetes free, whereas control PC61 treated NOD mice developed hyperglycaemia with the expected frequency (Fig. 7B). 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 ≥ 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-dependent manner. To examine whether disrupted BAFF or APRIL signalling 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 carried on a C57BL/6 background, we believe the analysis is valid as the median frequency of CD4+ CD25+ Foxp3+ T cells were similar between NOD and C57BL/6 mice (P = 0.3513, n ≥ 7); being ~9% for both strains (Fig. 8A). Also shown in Figure 8A, the frequency of peripheral CD4+ CD25+ Foxp3+ T cells in BAFF−/−, BR3−/−, BCMA−/− and TACI−/− mice were comparable to WT mice. Further, to determine whether BAFF or APRIL could engage Tregs directly, we examined TACI and BR3 expression on CD4+ CD25+ T cells. CD4+ CD25+ T cells did not express significant levels of the receptors BR3 or TACI (Fig. 8B), 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 utilised B cell deficient NOD.μMT−/− mice (18). NOD.μMT−/− mice exhibit a number of features, with regards to diabetes development reminiscent of BCMA-Fc treated mice. These include a reduced degree of insulitis (Fig. 5, B and C), and resistance to diabetes development (Fig. 8C). To assess whether CD4+ CD25+ T cells were important in maintaining euglycaemia in NOD.μMT−/− mice, ~16-week old mice were treated with PC61 (200μg i.p. each) every 14-days for a total of 4-injections and blood glucose levels were followed. This treatment precipitated diabetes in ~80% (P = 0.0184, n = 10) of NOD.μMT−/− (Fig. 8C), a dramatic result when compared to control NOD.μMT−/− mice. Therefore, targeting CD25+ regulatory populations triggered diabetes onset in B cell deficient NOD.μMT−/− mice that are otherwise resistant.
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DISCUSSION
Emerging evidence demonstrates that B cells can impact upon 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 harboured 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 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 ~9-15 weeks-of-age (15), show an increased capacity to act as APC’s 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 ~9-15 weeks-of-age, that is prior to the onset of hyperglycaemia. Thus by reducing the availability of B cells to act as APC’s, BCMA-Fc treatment curtailed B and T cell interactions during the critical 9-15 week time period, halting the progression from clinically silent insulitis to overt hyperglycaemia. 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-type T cell-derived cytokines at 16 weeks-of-age. Additional evidence to support this hypothesis comes from studies treating human CD20 expressing NOD mice 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. Regulatory T cells 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 hyperglycaemic-free state. Further, these CD4+ CD25+ T cells expressed the Treg lineage marker Foxp3 suggesting they belonged to the set of natural Tregs (36). To address the question of how BCMA-Fc might effect CD4+ CD25+ T cell homeostasis we examined Foxp3+ Treg frequencies in mice in which BAFF and APRIL signalling 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 9-15 weeks exhibited a delayed, and reduced incidence of diabetes. Together with our study 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 demonstrates 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.µMT+/- which lack B cells and are resistant to diabetes (18). Treatment of NOD.µMT+/- mice with the anti-CD25 mAb PC61 precipitated the onset of diabetes, demonstrating that the hyperglycemic free state in NOD.µMT+/- mice is also dependent upon CD25+ regulatory cells.
In this study we demonstrate that targeting the BAFF and APRIL pathway with BCMA-Fc prior to the onset of hyperglycaemia prevented diabetes in spontaneously diabetic NOD mice. We hypothesise 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 APC’s; and a second mechanism also related to decreased B cells, that induced or allowed regulatory T cells 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.

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REFERENCES


FIGURE LEGENDS

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 (grey line, n = 20) and PBS (broken line, n = 30) from 9-15 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.

Fig. 2. Effect of BCMA-Fc treatment upon peripheral B cells. A: Expression of BCMA, BR3 and TACI (black line) on IgM+ B220+ NOD splenocytes. Grey line = isotype control. Representative FACS plots are shown. B: Frequency of IgM+ B220+ cells in peripheral blood of NOD mice treated with BCMA-Fc from 9-15 weeks-of-age (open circles, n = 5) and HuIvIg control mice (grey circles, n = 5). Time indicates period post first injection. C: Gating strategy used for identification of B cell subsets. D: Representative FACS plots illustrating frequency of B cell subsets in the spleen (SPLN) and pancreatic lymph nodes (PLN) from 16 week-old NOD mice treated with PBS, HuIvIg or BCMA-Fc from 9-15-weeks-of-age. FoB, follicular B cell; T2MZ, transitional type 2 cell; MZB, marginal zone B cell; and T1, transitional type I cell. Numbers represent percentage of total lymphocytes. E: Absolute numbers, calculated from values in (D), of B cell subsets in SPLN and PLN from 16 week-old NOD mice treated with; PBS (black circles); HuIvIg (grey circles); or BCMA-Fc (open circles). Values from individual mice are shown (≥ 8 per group). Bar represents median value. * (P < 0.05), **( P < 0.01), ***( P < 0.001).

FIG. 3. Effect of BCMA-Fc on peripheral T cells and cytokines. A: Absolute number of CD4+ (upper panel) and CD8+ T cells (lower panel) from 16-week-old NOD mice treated with PBS (black circles), HuIvIg (grey circles) or BCMA-Fc (open circles), from 9-15 weeks-of-age. Values from individual mice are shown (n ≥ 8 per group). B: Representative FACS 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; grey line = isotype control, n = 7 mice per group. C: Serum cytokine levels in HuIvIg (grey circles) and BCMA-Fc (open circles) treated NOD mice at 16 weeks-of-age. Values from individual mice are shown (n ≥ 3 per group). Significant differences between sample means are indicated. Bar represents median value. * (P < 0.05), ***(P < 0.001).

FIG. 4. Effect of BCMA-Fc-treatment on B cell repopulation. A: Representative FACS plots illustrating frequency of B cell subsets from NOD mice treated with HuIvIg at 30 weeks-old or BCMA-Fc at ≥ 50-weeks-old. Numbers represent percentage of total lymphocytes. B: Absolute numbers of B cell subsets from HuIvIg (grey circles) and BCMA-Fc (open circles) treated NOD mice. Values calculated from A. Results from individual mice are shown (n ≥ 3 per group). Bar represents median value. * (P < 0.05), **(P < 0.01).

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 9-15 weeks at 16 or ≥ 50 weeks-of-age shown (upper panels). Sections from HuIvIg- and PBS-treated mice at 16 weeks are also shown (lower panels). B: Representative pancreatic sections from a NOD.µMT−/− mouse. C: Insulitis scores for treated mice, ~ 20 to 70 islets were scored from 4-7 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 between HuIvIg control mice. D: Representative FACS 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 (1x10$^7$) from (≥ 50 week-old) normoglycemic BCMA-Fc treated NOD mice (black line, $n = 8$) and prediabetic 16 weeks-of-age NOD mice (grey line, $n = 8$) were adoptively transferred (i.v.) 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.

FIG. 6. CD4+ CD25+ T cells in BCMA-FC-treated NOD mice. A: Representative FACS 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. B: Pooled data showing frequencies and calculated absolute numbers of CD25-expressing CD4 T cells of PBS (black circles), HuIvIg (grey circles) or BCMA-Fc (open circles) treated NOD mice at 16-weeks. Values from individual mice are shown ($n ≥ 7$ per group). Bar represents median value. C: Representative FACS 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 (black circles), HuIvIg (grey circles) and BCMA-Fc (open circles) 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 (grey circles) or BCMA-Fc (open circles) treated NOD mice. $n ≥ 3$ per group. Bar indicates median value. (** $P <0.01$), (*** $P <0.001$).

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 IgG1, κ). Representative FACS plot is shown ($n ≥ 4$ per group). B: Diabetes incidence was followed for NOD mice administered BCMA-Fc 9-15 weeks (solid black line, $n = 5$), PC61 (broken line, $n = 5$), or BCMA-Fc 9-15 weeks plus PC61 (solid grey line, $n = 8$). **$P = 0.001$ (Mantel-Cox Log-Rank analysis) for BCMA-Fc alone versus 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 mice (black circles) and NOD mice (open circles). Right plot; frequencies of splenic CD4+CD25+Foxp3+ T cells in BAFF−/− (black circles), BR3−/− (open squares), TACI−/− (open circles) and BCMA−/− (open triangles) mice. Values from individual mice are shown ($n ≥ 4$ 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. Grey line = isotype control. Representative FACS plots are shown. C: Diabetes incidence was followed for NOD.µMT−/− mice (black line, $n = 5$) and NOD.uMT−/− administered PC61 (solid grey line, $n = 10$). * $P = 0.0181$ (Mantel-Cox Log-Rank analysis) for control versus PC61 treatment.
Figure 1.
Figure 2.
Targeting the BAFF/APRIL pathway prevents diabetes

Figure 3.
Targeting the BAFF/APRIL pathway prevents diabetes

Figure 4a.

A

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<table>
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| CD21 |
|      |
| IgM |
| 1.9% |
| 23%  |
| 1.5% |
| 2%   |
Targeting the BAFF/APRIL pathway prevents diabetes

Figure 4b.
Figure 5

Figure 6.
Targeting the BAFF/APRIL pathway prevents diabetes
Targeting the BAFF/APRIL pathway prevents diabetes

Figure 7.
Targeting the BAFF/APRIL pathway prevents diabetes

Figure 8.

A

\[
\begin{align*}
\text{% of CD25+Foxp3+ T cells (left)} & \quad \text{% of CD25+Foxp3+ T cells (right)} \\
0 & \quad 0 \\
15 & \quad 15 \\
10 & \quad 10 \\
5 & \quad 5 \\
0 & \quad 0
\end{align*}
\]

B

- **B Cells (B220)**
- **CD4+ T cells**
- **CD25-expressing CD4 T cells**

BR3

TACI

C

\[
\begin{align*}
\text{% of Normoglycemic mice (left)} & \quad \text{Time (weeks) (right)} \\
0 & \quad 0 \\
0.2 & \quad 0 \\
0.4 & \quad 0 \\
0.6 & \quad 0 \\
0.8 & \quad 0 \\
1 & \quad 0 \\
\end{align*}
\]