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BAFF regulates activation of self-reactive T cells through B-cell dependent mechanisms and mediates protection in NOD mice

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Targeting the BAFF/APRIL system has shown to be effective in preventing T-cell dependent autoimmune disease in the NOD mouse, a spontaneous model of type 1 diabetes. In this study we generated BAFF-deficient NOD mice to examine how BAFF availability would influence T-cell responses in vivo and the development of spontaneous diabetes. BAFF-deficient NOD mice which lack mature B cells, were protected from diabetes and showed delayed rejection of an allogeneic islet graft. Diabetes protection correlated with a failure to expand pathogenic IGRP-reactive CD8$^+$ T cells, which were maintained in the periphery at correspondingly low levels. Adoptive transfer of IGRP-reactive CD8$^+$ T cells with B cells into BAFF-deficient NOD mice enhanced IGRP-reactive CD8$^+$ T-cell expansion. Furthermore, when provoked with cyclophosphamide, or transferred to a secondary lymphopenic host, the latent pool of self-reactive T cells resident in BAFF-deficient NOD mice could elicit beta cell destruction. We conclude that lack of BAFF prevents the procurement of B-cell-dependent help necessary for the emergence of destructive diabetes. Indeed, treatment of NOD mice with the BAFF-blocking compound, BR3-Fc, resulted in a delayed onset and reduced incidence of diabetes.

Keywords: BAFF · Diabetes · Islet allograft · NOD mice · Self-reactive T cells

Introduction

The TNF-ligand family molecules BAFF (B-cell activating factor also known as BLyS, TNFSF13b) and APRIL (A proliferation induced ligand, TNFSF13) have emerged as important players in the development of autoimmunity) [1]. BAFF may play a causal role in autoimmunity as mice transgenic for BAFF demonstrate increased titers of self-reactive antibodies and develop autoimmune symptoms very similar to systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS) [1, 2]. Further to this, elevated BAFF and APRIL levels have been detected in sera from human patients with rheumatoid arthritis, SLE, and SS [3–5]. The key feature of BAFF in autoimmunity may be its ability to promote the survival of autoreactive B cells when in excess [6]. Of interest, high BAFF levels can drive the expansion of marginal zone B cells [7], a B-cell subset with high self-reactivity [8] and associated with animal models of autoimmunity including type 1 diabetes (T1D) [9, 10], SLE [11], and SS [2]. BAFF and APRIL can interact with B cells via multiple receptors: B-cell maturation antigen (BCMA) [12] or transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) [12, 13], whereas BAFF can also bind to BR3 (BAFF receptor 3) (otherwise known as BAFF-R) [14]. In contrast to situations with high BAFF levels, mature B-cell development is prevented in BAFF-deficient mice [15]. The potent role for BAFF and APRIL in B-cell development makes the BAFF/APRIL system of ligands and receptors attractive therapeutic targets for the treatment of autoimmune conditions [16–19].

BAFF and APRIL are implicated in the aetiology of T1D and the BAFF/APRIL system has been suggested as a therapeutic target

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for T1D [20]. Administration of the BAFF and APRIL antagonist BCMA-Fc to NOD mice, a model of T1D, reduced the number of circulating B cells and prevented diabetes by a mechanism dependent upon regulatory Foxp3$^+$ T cells [21]. Further, administration of an anti-BAFF mAb reduced B cells and delayed the onset of diabetes, but also reversed clinical signs when administered to NOD mice with new-onset diabetes [22]. In the NOD mouse killing of pancreatic beta cells is T-cell mediated though the progression to diabetes requires B-cell help [23]. As B-cell depletion prevents diabetes [21, 24, 25] the diabetes protection afforded to NOD mice by targeting the BAFF/APRIL system [21, 22] may have been secondary to B-cell reduction. However, some studies suggest that BAFF can directly modulate T-cell activity. T cells express the BAFF receptors transmembrane activator and calcium modulator and cyclophylin ligand interactor [13, 21] and BR3 [21, 26, 27] and exogenous BAFF costimulates both human and mouse T cells [28, 29]. Given the potent protective effect of BAFF/APRIL inhibition on diabetes development, and the potential of BAFF/APRIL inhibition as a therapeutic target for T1D [20], further analysis of the mechanisms by which BAFF/APRIL support T1D are warranted. In this study we generated BAFF-deficient NOD mice to examine how BAFF availability would influence self-reactive T cells and the development of spontaneous diabetes.

Results

Immunophenotype of BAFF-deficient NOD mice

To better determine the importance and mechanism of BAFF action in T1D, we generated NOD mice that were deficient in BAFF. Immunophenotypic analysis revealed NOD.BAFF$^{-/-}$ mice exhibited a profound deficit in the frequency of IgM$^+$ B220$^+$ B cells in the spleen (Fig. 1A). This decrease related to a loss in both frequency and absolute number of mature follicular and marginal zone B cells (Fig. 1B and C). With regards to T cells, NOD.BAFF$^{-/-}$ mice showed a slightly increased frequency most likely reflecting the loss of B cells (Fig. 1D), however the absolute numbers of splenic T cells, both CD4$^+$ and CD8$^+$ subsets, in NOD.BAFF$^{-/-}$ mice was reduced as compared to littermate NOD.BAFF$^{+/+}$ mice (Fig. 1E); an observation also reported for BAFF-deficient mice on the C57BL/6 background [15]. However, there were no differences in the relative proportions of T-cell subpopulations including regulatory (Foxp3$^+$), naïve (CD44^{Lo}) and memory (CD44^{hi}) T-cell subsets between BAFF$^{-/-}$ and NOD.BAFF$^{+/+}$ mice (data not depicted). Thus, on the NOD background, the B-cell phenotype of NOD.BAFF$^{-/-}$ mice is consistent with previous results showing that BAFF is required for splenic T1 development to proceed to mature follicular and marginal zone cells [15].

NODBAFF$^{-/-}$ mice are diabetes resistant

Cohorts of both female and male NOD.BAFF$^{-/-}$ mice, as well as NOD.BAFF$^{+/-}$ and NOD.BAFF$^{+/+}$ littermates, were followed for diabetes incidence. The incidence of diabetes for female littermate NOD.BAFF$^{+/+}$ was 100% at 30 weeks of age (Fig. 2A) whereas the incidence for male NOD.BAFF$^{+/+}$ mice at 30 weeks of age was ~40% (Fig. 2B). Male and female NODBAFF$^{+/-}$ heterozygous mice presented with a similar diabetes incidence as their BAFF$^{+/+}$ littermates. In contrast, female NOD.BAFF$^{-/-}$ showed a profound and absolute resistance to diabetes when followed out to 40 weeks of age (Fig. 2A). Similarly, BAFF-deficient male NOD mice also exhibited a profound resistance to diabetes (Fig. 2B). The incidence of diabetes for both female and male NOD.BAFF$^{-/-}$ mice at 40 weeks of age was 0% (Logrank, $p < 0.0001$ BAFF$^{+/+}$ vs. BAFF$^{-/-}$ $n \geq 20$ per group).

Effect of BAFF-deficiency on the pancreatic infiltrate

We conducted histological analysis of the pancreata from the protected NOD.BAFF$^{-/-}$ mice > 12 weeks of age and compared this to age-matched NOD.BAFF$^{+/+}$ mice. Representative histology for each group is shown in Figure 3A and insulitis scores for these mice are shown in Figure 3B. While NOD.BAFF$^{-/-}$ mice did exhibit evident insulitis, both the severity and penetrance of insulitis was reduced compared to diabetic control NOD mice. Indeed the frequency of islets exhibiting heavy insulitis (grade 3 or 4, Fig. 3B) in NOD.BAFF$^{-/-}$ mice was <10% versus ~40% in NOD.BAFF$^{+/+}$ groups. Further, the proportion of severely infiltrated islets in the NOD.BAFF$^{-/-}$ mice did not increase over time (data not depicted). Thus, the severity of insulitis in NOD.BAFF$^{-/-}$ mice was at a level equivalent to that exhibited by prehyperglycaemic female NOD mice.

Delayed islet graft rejection in NOD.BAFF$^{-/-}$ mice

Because beta cell destruction and diabetes is T-cell mediated and NOD.BAFF$^{-/-}$ mice were strongly protected from spontaneous diabetes we examined their T-cell function in another model. Rejection of MHC-disparate islet grafts in NOD mice requires CD4$^+$ and CD8$^+$ T cells specific for islet autoantigens but also alloreactive T cells specific for MHC determinants [30]. Therefore, we examined the fate of BALB/c H2^d islet allografts transplanted under the renal capsule of NOD.BAFF$^{-/-}$ mice. As shown in Figure 4A, NOD.BAFF$^{+/+}$ mice promptly rejected their islet allografts; the median survival time being ~17 days. In contrast, NOD.BAFF$^{-/-}$ mice showed delayed graft rejection, with some grafts persisting for >50 days without exogenous immunosuppression (Logrank, $p < 0.01$ for female NOD.BAFF$^{+/+}$ vs. NOD.BAFF$^{-/-}$ $n \geq 7$ per group).

T-cell function of BAFF-deficient NOD mice

Because NOD.BAFF$^{-/-}$ mice showed profound protection from diabetes and delayed islet allograft rejection, we questioned whether T-cell function had been grossly impaired due to loss of BAFF. To test T-cell effector function in vivo independent of islet

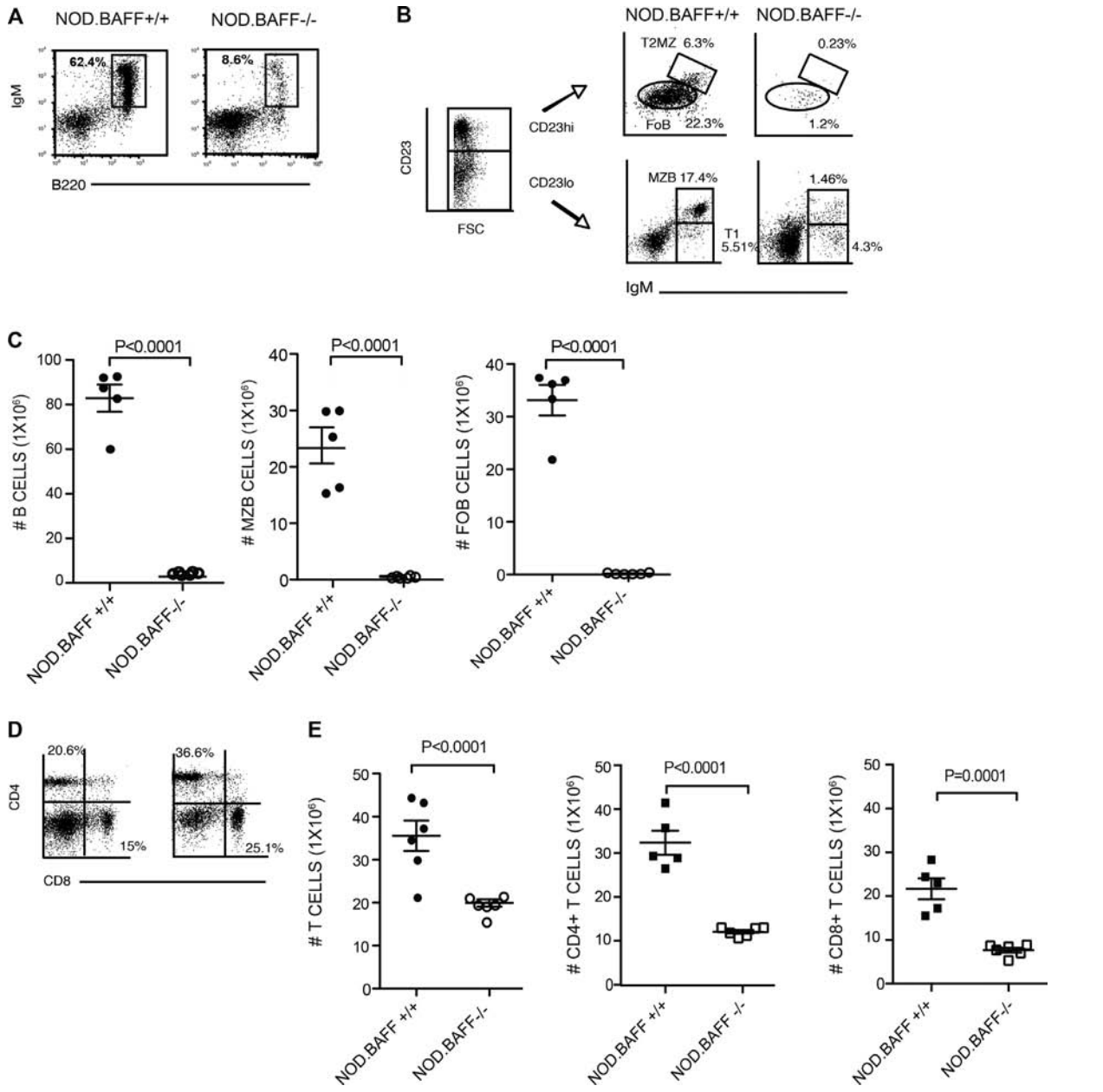

Figure 1. Immunophenotype of BAFF-deficient NOD mice. (A) Representative FACS plots illustrating frequency of IgM$^+$ B220$^+$ splenocytes in 6–8 week old littermate NOD.BAFF$^{+/+}$ and NOD.BAFF$^{-/-}$ mice, $n \geq 6$ per group. (B) Representative FACS plots illustrating frequency of splenic B-cell subsets in 6–8 week old littermate NOD.BAFF$^{+/+}$ and NOD.BAFF$^{-/-}$ mice. FOB, follicular B cell; MZB, marginal zone B cell; T2MZ, transitional type 2 cell; and T1, transitional type I cell. Numbers represent percentage of total lymphocytes. $n \geq 6$ per group. (C) Calculated absolute number of splenic, IgM$^+$ B220$^+$ B cells; FOB, and MZB cells for indicated mouse lines at 6–8 week of age from (A) and (B). Data show mean ± SD from individual mice; $n \geq 6$ per group. (D) Representative FACS plots illustrating frequency of splenic CD4$^+$ and CD8$^+$ T cells in 6–8 week old littermate NOD.BAFF$^{+/+}$ and NOD.BAFF$^{-/-}$ mice. (E) Calculated absolute number of splenic T cells as well as CD4$^+$ and CD8$^+$ T cells from (D). Data show mean ± SD from individual mice; $n \geq 6$ per group. (A–E) Data shown are representative of three independent experiments. p values calculated by the Student's t-test analysis.

destruction, we examined the ability of NOD.BAFF$^{-/-}$ mice to reject a BALB/c H2^d allogeneic skin graft. Skin allograft rejection is a classic test of T-cell effector function. As shown in Figure 4B, both NOD.BAFF$^{-/-}$ and NOD.BAFF$^{+/+}$ mice showed robust rejection of the H2^b allografts. These data demonstrate dendritic cell function and T-cell effector function was not grossly impaired in the absence of BAFF.

NOD.BAFF$^{-/-}$ mice harbor a latent pool of islet-destructive T cells

We considered that the diabetes resistance and delayed graft rejection of NOD.BAFF$^{-/-}$ mice could reflect a lack of islet-reactive T cells. Prior to the onset of overt diabetes, NOD mice present with a clinically silent mononuclear lesion surrounding the pancreatic

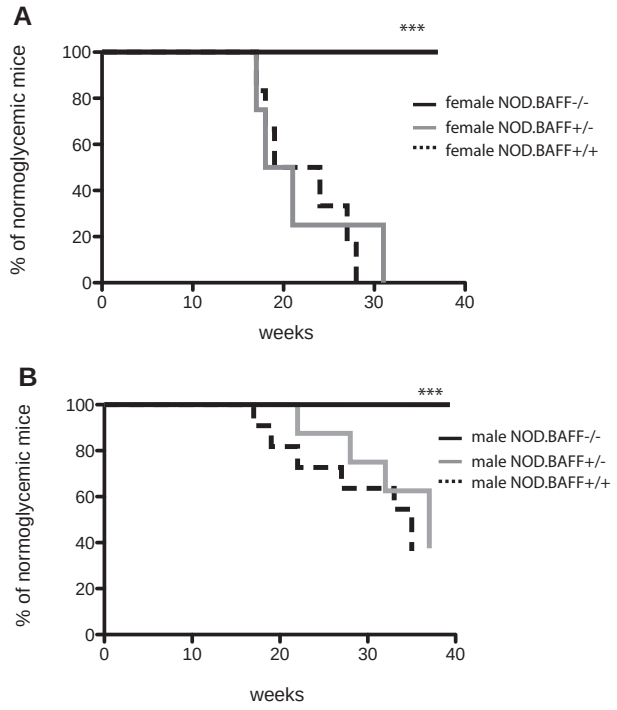

Figure 2. Diabetes incidence for NOD.BAFF$^{-/-}$ mice. (A) Diabetes incidence in cohorts of female NOD.BAFF$^{-/-}$ (solid black line), and littermate NOD.BAFF$^{+/-}$ (solid gray line) and NOD.BAFF$^{+/+}$ (broken black line) mice. $n \geq 20$ per group. (B) Diabetes incidence in cohorts of male mice. $n \geq 20$ per group. ***$p < 0.0001$ (Mantel-Cox Log-Rank analysis) for both female and male NOD.BAFF$^{+/+}$ vs. NOD.BAFF$^{-/-}$. Diabetes is indicated by a blood glucose level (BGL) >16 mM. (A and B) Data shown are representative of three independent experiments. *p* values calculated by the Student's *t*-test analysis.

islets. Administration of cyclophosphamide to NOD mice converts clinically silent insulitis to a destructive state resulting in hyperglycemia [31, 32]. We administered two injections of cyclophosphamide 2 weeks apart to NOD.BAFF$^{-/-}$, NOD.BAFF$^{+/-}$, and NOD.BAFF$^{+/+}$ mice and destruction of insulin-producing beta cells was determined by following blood glucose levels. NOD.BAFF$^{+/+}$ rapidly became hyperglycaemic as did NOD.BAFF$^{-/-}$ mice following cyclophosphamide administration (Fig. 4C) demonstrating that BAFF was not required per se for T cell-dependent beta cell killing. Significantly, the susceptibility of NOD.BAFF$^{-/-}$ mice to cyclophosphamide-induced diabetes demonstrated the existence of a functional, but latent, pool of islet-reactive T cells.

Adoptive transfer of NOD.BAFF$^{-/-}$ T cells reveals their diabetogenic potential

To further address whether there is a pool of fully functional autoreactive T cells in NOD.BAFF$^{-/-}$ mice, we adoptively transferred equal numbers of lymphocytes from female NOD or NOD.BAFF$^{-/-}$ mice into female NOD.SCID mice and monitored the development of diabetes. Figure 4D shows that the NOD.BAFF$^{-/-}$ T cells were capable of transferring diabetes at an incidence similar to that of the NOD T cells ($p = 0.0805$). We interpreted these data, together with the results of the cyclophosphamide model, to indicate that diabetes resistance in NOD.BAFF$^{-/-}$ mice does not result from a lack of islet-reactive T cells. Indeed, once provoked, or transferred to a permissive environment, T-cell acquisition of effector function proceeds normally in the absence of BAFF allowing islet destruction and onset of diabetes.

NOD.BAFF$^{-/-}$ mice fail to expand islet-specific CD8$^+$ cytotoxic T cells

The accumulation of self-reactive cytotoxic CD8$^+$ T cells (CTLs) specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) in the pancreatic lymph node (PLN) is a characteristic feature of disease progression in NOD mice [33, 34]. Therefore, we tracked the frequencies of self-reactive IGRP-specific CD8$^+$ T cells in female NOD.BAFF$^{-/-}$ and NOD.BAFF$^{+/+}$ mice (Fig. 5A). An increase in the frequency of IGRP-specific CD8$^+$ T cells from 4 to 16 weeks of age was seen in the PLN of female NOD.BAFF$^{+/+}$ mice. Compared to the PLN of 16-week old NOD.BAFF$^{+/+}$ mice, NOD.BAFF$^{-/-}$ harbored a much-reduced frequency of IGRP-specific CD8$^+$ T cells. Of interest, the frequencies of IGRP-specific CD8$^+$ T cells in the PLN of NOD.BAFF$^{-/-}$ mice were more similar to that observed for 4 week old prediabetic NOD.BAFF$^{+/+}$ mice. Further to this, we examined the frequency of IGRP-specific CD8$^+$ T cells in the insulitic lesions of the pancreatic islets. As shown in Figure 5B, NOD.BAFF$^{-/-}$ showed a reduced frequency of IGRP-specific CD8$^+$ T cells in the pancreatic islet infiltrate compared to NOD.BAFF$^{+/+}$ mice. Thus in the absence of BAFF CD8$^+$ T cells low frequencies of autoreactive CD8$^+$ T cells can be found in the pancreatic islets and PLN. The IGRP-specific CD8$^+$ T cells fail to expand in the PLN and pancreatic islets, but remain at prediabetic levels.

B-cell reconstitution restores IGRP-expansion in BAFF-deficient NOD hosts

Why was the latent pool of islet-reactive CTL unable to expand in NOD.BAFF$^{-/-}$ mice? Though CD8$^+$ T cells mediate beta cell killing, this process requires B-cell help in the form of soluble factors [35] but also direct cognate help [36]. We considered that the critical role for BAFF in driving diabetes was to support B-cell development, which in turn provided help to self-reactive T cells. To test whether the failure to expand CD8$^+$ T cells related to a lack of B cells, IGRP-specific NOD.8.3 TCR T cells were adoptively transferred to NOD.BAFF$^{+/+}$ or NOD.BAFF$^{-/-}$ mice with or without FACS-sorted NOD splenic B cells. Because B-cell development proceeds to the splenic T1 stage in the absence of BAFF (Fig. 1B), NOD.BAFF$^{-/-}$ mice will not reject transferred B cells as has been observed for NOD.μMT mice, which entirely lack peripheral B cells. After transfer, T-cell expansion was assessed by tracking tetramer-positive CD8$^+$ IGRP$^+$ cells. As shown, the presence of B cells enhanced the expansion of IGRP-specific NOD.8.3 TCR T cells in the PLN of NOD.BAFF$^{-/-}$ mice (Fig. 5B). This suggested to us that BAFF-deficiency prevents diabetes by impairing

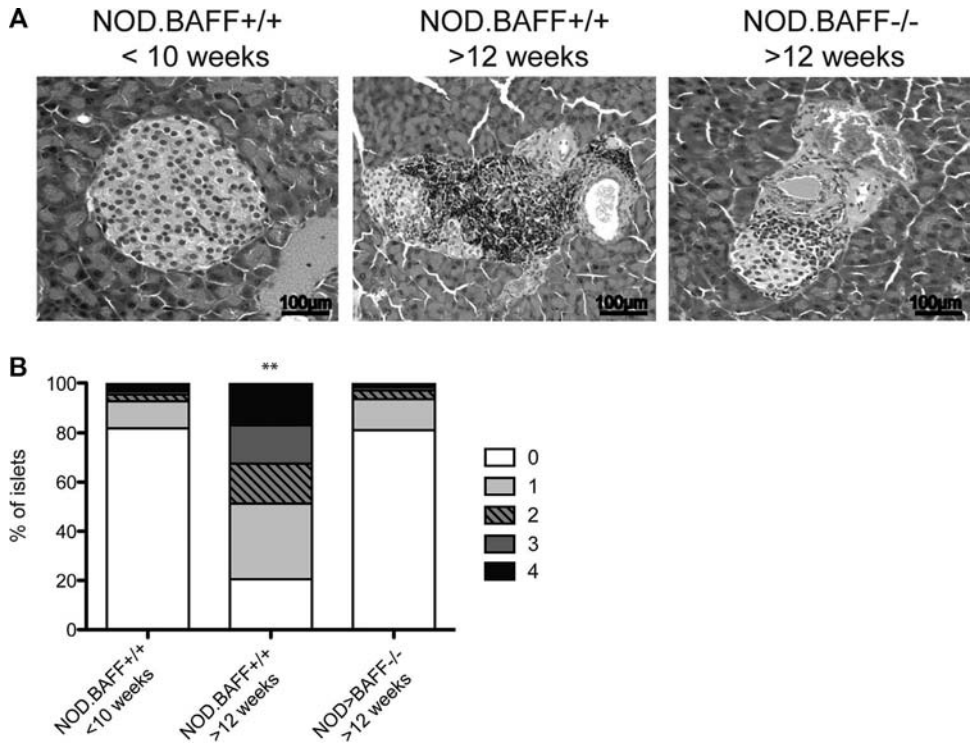

Figure 3. Insulitis scores for NOD.BAFF$^{-/-}$ mice. (A) Representative histological section of pancreas from prediabetic NOD.BAFF$^{+/+}$ mice (6 to 8 weeks old); diabetic NOD.BAFF$^{+/+}$ mice (>12 to 15 weeks old) and normoglyceamic NOD.BAFF$^{-/-}$ mice (>12–15 weeks old) Magnification (100×). Scale bar = 100 μm. (B) Female NOD.BAFF$^{+/+}$ and NOD.BAFF$^{-/-}$ mice were scored for severity of insulitis, ~50 to 80 islets were scored from 5 to 7 mice per group. Prediabetic NOD.BAFF$^{+/+}$ mice (6 to 8 weeks old); diabetic NOD.BAFF$^{+/+}$ mice (>12 to 15 weeks old) and normoglyceamic NOD.BAFF$^{-/-}$ mice (>12 to 15 weeks old). Insulitis scores for NOD.BAFF$^{-/-}$ mice were significant **($p < 0.01$). p values resulted comparing insulitis level at grade 4 between NOD.BAFF$^{+/+}$ and NOD.BAFF$^{-/-}$ mice by the Student's *t*-test analysis. (A and B) Data/Images shown are representative of three independent experiments.

expansion of cytolytic CD8$^+$ T cells secondary to a restriction in the availability of B-cell help.

Disrupting the BAFF pathway in the preclinical phase prevents diabetes onset

To test the effect of therapeutic disruption of the BAFF pathway prior to the onset of hyperglycaemia, in otherwise BAFF-sufficient mice, NOD mice were treated with 10 μg (i.p.) BR3-Fc twice weekly from 9 to 15 weeks of age (12 injections over a 6-week period); control groups were administered PBS or 10 μg (i.p.) of HuIvIg (intravenous globulin) over the same period (Fig. 6A). 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 was no significant difference in diabetes incidence between PBS and HuIvIg treated groups ($p = 0.1309$ PBS vs. HuIvIg, $n \geq 20$). In contrast, we found that NOD mice treated with BR3-Fc from 9 to 15 weeks of age showed a delayed onset and reduced incidence of diabetes (diabetes incidence 5/10 at 50 weeks of age; $p = 0.0195$, $n \geq 10$ Logrank vs. HuIvIg). BR3-Fc treatment reduced the absolute number of peripheral IgM$^+$ B220$^+$ B cells by about 80%; B-cell reduction was prominent for both follicular B and marginal zone B cells (Fig. 6B); a result consistent with the role of BAFF in promoting B-cell development after the T1 checkpoint [15].

Discussion

The major finding of this study is that targeting the BAFF system by a genetic approach can prevent diabetes and delay islet allograft rejection in the spontaneous NOD mice, thus uncovering an obligate role for BAFF in the progression to clinically overt diabetes. This provides support to studies whereby BAFF-blockade via administration of a receptor-decoy fusion protein [21], or via anti-BAFF mAb [22], protects NOD mice from diabetes. Indeed, here, utilizing soluble BR3-Fc fusion protein to block BAFF ligand interactions with its cognate receptors in vivo markedly delayed diabetes progression in female NOD mice. These studies provide preclinical proof of concept data that targeting the BAFF pathway may have therapeutic utility as a treatment for T1D [20].

There is strong evidence that beta cell destruction in the NOD mouse is T-cell mediated, and that cytotoxic CD8$^+$ T cells specific for islet-antigens play an important role in final beta cell killing [37]. NOD.BAFF$^{-/-}$ harbor a latent pool of cytotoxic CD8$^+$ T cells that unless provoked, as with cyclophosphamide treatment, or

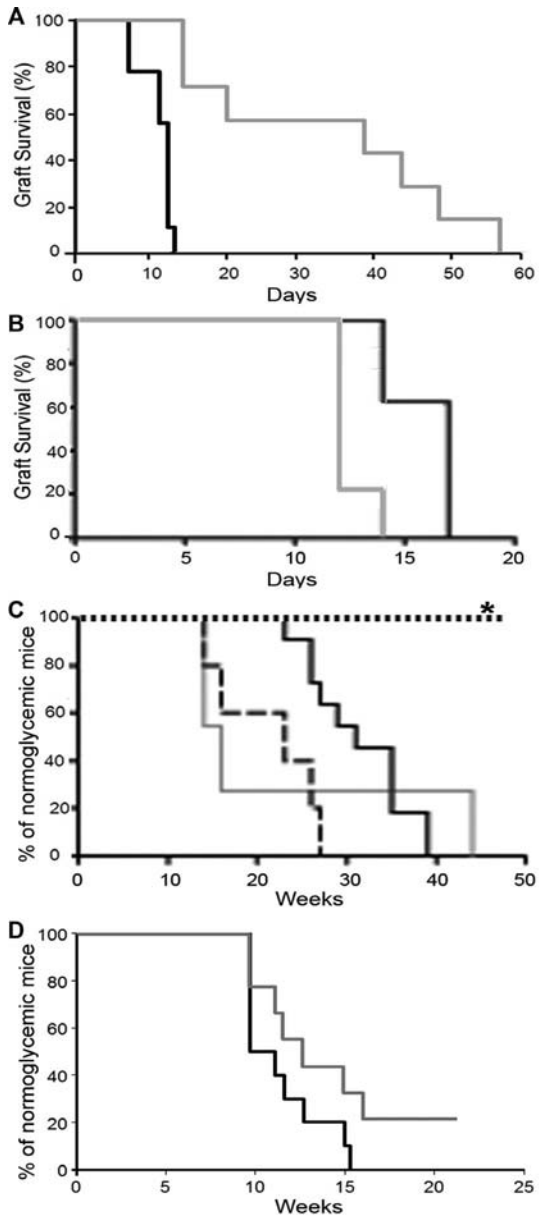

Figure 4. in vivo T-cell responses of NOD.BAFF$^{-/-}$ mice. (A) Delayed islet graft rejection in NOD.BAFF$^{-/-}$ mice. Kaplan–Meier plots showing survival of allogeneic BALB/c islet grafts transplanted into diabetic NOD.BAFF$^{-/-}$ (gray line, $n = 6$) or NOD.BAFF$^{+/+}$ (black line, $n = 8$) mouse recipients. $p < 0.01$ for NOD.BAFF$^{-/-}$ versus NOD.BAFF$^{+/+}$ (Mantel-Cox Log-Rank analysis). (B) BAFF is not required T-cell effector function per se. Kaplan–Meier cumulative plots showing survival of allogeneic BALB/c skin grafts transplanted onto NOD.BAFF$^{-/-}$ (gray line, $n = 6$) and NOD.BAFF$^{+/+}$ (black line, $n = 8$) mouse recipients. Normal T-cell mediated rejection of allogeneic skin grafts was seen for NOD.BAFF$^{-/-}$ mice. (C) Diabetes incidence following two injections of cyclophosphamide 2 weeks apart to littermate NOD.BAFF$^{+/+}$ (solid gray line, $n = 11$), NOD.BAFF$^{+/-}$ (broken black line, $n = 6$), NOD.BAFF$^{-/-}$ (solid black line, $n = 13$) mice. No difference in diabetes incidence was seen between groups. * Indicates diabetes incidence for NOD.BAFF$^{-/-}$ not administered cyclophosphamide over the same period. Diabetes is indicated by a BGL >16 mM. (D) BAFF.NOD$^{-/-}$ T cells transfer diabetes to NOD.*scid* mice. Diabetes incidence for NOD.*scid* mice receiving NOD T cells (solid black line, $n = 10$) and NOD.BAFF$^{-/-}$ T cells (solid gray line, $n = 9$). No difference in diabetes incidence was seen between groups. (A–D) Data shown are representative of three independent experiments. p values calculated by the Student's t-test analysis.

through transfer to a lymphopenic host, will remain quiescent. These data show that in the absence of BAFF self-reactive T cells fail to become sufficiently activated to mediate beta cell killing. Why was the latent pool of islet-reactive CTL unable to expand in BAFF-deficient NOD mice? Of interest the phenotype of the NOD.BAFF$^{-/-}$ mice is reminiscent of B-cell deficient NOD.μMT mice. In this case, B-cell deficient NOD mice are protected from diabetes [24], due to a failure of non-B-cell APCs to provide sufficient activation signals to drive T-cell activation [32, 36, 38]. However, B-cell deficient NOD mice do harbor a repertoire of islet reactive T cells, as indicated by evident insulitis [21, 32, 36] and the presence of islet-specific CD8$^+$ T cells in the PLN [39]. Like NOD.BAFF$^{-/-}$ mice, the latent pool of islet reactive T cells in NOD.μMT mice can be provoked to convert to destructive insulitis by the administration of cyclophosphamide [32] but also through adoptive transfer to secondary lymphopenic hosts [40]. The strong similarities between NOD.BAFF$^{-/-}$ mice and B-cell deficient NOD mice with regard to diabetes development and the latent T-cell repertoire points to the possibility that diabetes protection in NOD.BAFF$^{-/-}$ mice is secondary to B-cell deficiency. Thus we conclude that in the case of the NOD model, BAFF deficiency does not directly interfere with T-cell function, but rather impairs activation of cytolytic CD8$^+$ T cells secondary to a restriction in the availability of B-cell help [36]. Indeed, once this requirement is by-passed, T-cell activation, and acquisition of effector function proceeds normally in the absence of BAFF.

It is noteworthy that B cells are generally required for diabetes development in the spontaneous NOD model [24], whereby this is not always the case in alternative accelerated NOD-based models of diabetes [35, 41, 42]. This may reflect a dependence upon B cells as APCs in the spontaneous model, which may not be maintained under other conditions. For instance, in viral-induced NOD models a nominal beta cell self-antigen is revealed in the context of a strong immunological adjuvant and in this case DCs, not B cells [41], may be the more dominant APC. Alternatively, B-cell dependency can be bypassed where a strong activation signal is provided to self-reactive T cells. This occurs in the case where self-reactive T cells are adoptively transferred into secondary lymphopenic recipients and can undergo extensive rounds of homeostatic expansion [43], or through TCR transgenic systems that artificially expand the clone size of specific self-reactive T cells [42, 44, 45]. These examples contrast to the spontaneous NOD model whereby B-cell depletion reduces the frequency of self-reactive T cells [21, 39]. We interpret these, and other data, to indicate that B-cell help is required to sufficiently activate self-reactive T cells to the point where they can kill beta cells [37, 39]. It is also interesting to consider the B-cell requirements of differing animal models of diabetes in the context of clinical T1D. Though B-cell reduction with rituximab showed some protective efficacy for subjects with T1D [46], one case-study provided evidence that B cells are not absolutely required for the development of T1D in man [47].

Our findings may have important implications for the clinical treatment of T1D and for islet transplantation. Anti-CD20 monoclonal antibody based treatments targeting B cells are already in

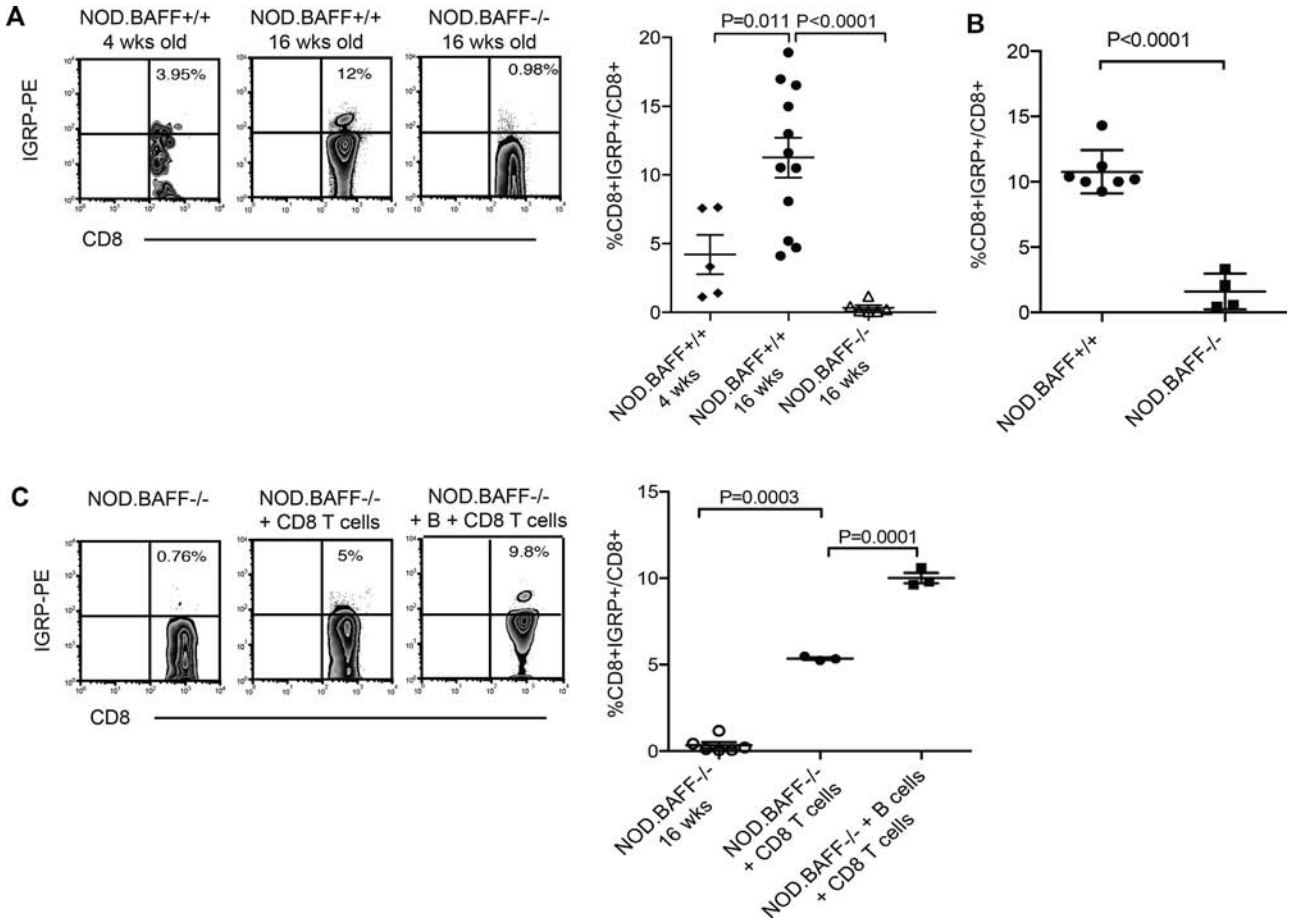

Figure 5. Reduction of self-reactive $CD8^+$ T cells in BAFF-deficient NOD mice. (A) Representative dot plots and cumulative data showing frequency of IGRP-specific $CD8^+$ T cells in pancreatic lymph nodes of 4-week-old female NOD.$BAFF^{+/+}$ and 16-week-old female NOD.$BAFF^{+/+}$ and NOD.$BAFF^{-/-}$ mice. Background was determined by TUM-tetramer staining. (B) Cumulative data showing frequency of IGRP-specific $CD8^+$ T cells in pancreatic islets of 16-week-old female NOD.$BAFF^{+/+}$ or NOD.$BAFF^{-/-}$ mice. (C) Representative dot plots and cumulative data showing frequency of IGRP-tetramer positive NOD8.3 $CD8^+$ T cells in the pancreatic lymph nodes of NOD.$BAFF^{-/-}$ recipients when transferred either alone (+ $CD8^+$ T cells) or when transferred with purified splenic IgM^+ $B220^+$ B cells (+ B cells + $CD8^+$ T cells). (A–C) Data represents mean ± SEM; $n \geq$ (A) 5, (B) 4 or (C) three mice per group; each point represents one mouse. Data shown are representative of three independent experiments. p values calculated by the Student's t-test analysis.

clinical use for the treatment of non-Hodgkin's lymphoma, and have been trialed for T1D in humans [46]. Whereas BAFF antagonists are undergoing clinical trials for autoimmune conditions including SS and SLE [19]. In preclinical models, targeting B cells by way of an anti-CD20 monoclonal antibody reduced diabetes incidence [25], whereas, treatment with the BAFF/APRIL-blocking agent BCMA-Fc [21] prevented diabetes, or as we demonstrate here, with a BAFF-blocking BR3-Fc protein, resulted in a delayed onset and reduced incidence of diabetes in NOD mice. These data would support the continuing development of BAFF/APRIL blockers for T1D [20].

Islet transplantation is emerging as a potential treatment for T1D and hypoglycemic unawareness [48]. In B-cell sufficient mice, BAFF-blockade coupled with immunosuppression in the form of rapamycin can achieve improved islet allograft survival [49]. The mechanism of action in that study included impaired $CD4^+$ T-cell activation, further suggesting that in a BAFF-deficient environment T-cell activation is impaired. Similar results were achieved in a non-human primate islet transplant model with anti-CD20 monoclonal antibody treatment [50]. We found that NOD.$BAFF^{-/-}$ mice show delayed rejection of an islet allograft, which may involve a failure to sufficiently activate pathogenic $CD8^+$ as well as $CD4^+$ T cells. Collectively these data might indicate that B cells are mechanistically involved in islet transplant rejection, but are redundant for rejection of vascular grafts [51]. Thus, the delayed islet-graft survival seen here without exogenous immunosuppression in the stringent NOD transplant model further supports BAFF as a therapeutic target for clinical islet transplantation. Depletion of B cells via targeting BAFF may be important as an alternative strategy where T-cell directed therapies run concomitant risks associated with cytokine release syndrome [52] and emergent viral infection [53], or where clinically significant CD20 negative B cells evade deletion with anti-CD20 monoclonal antibody [54].

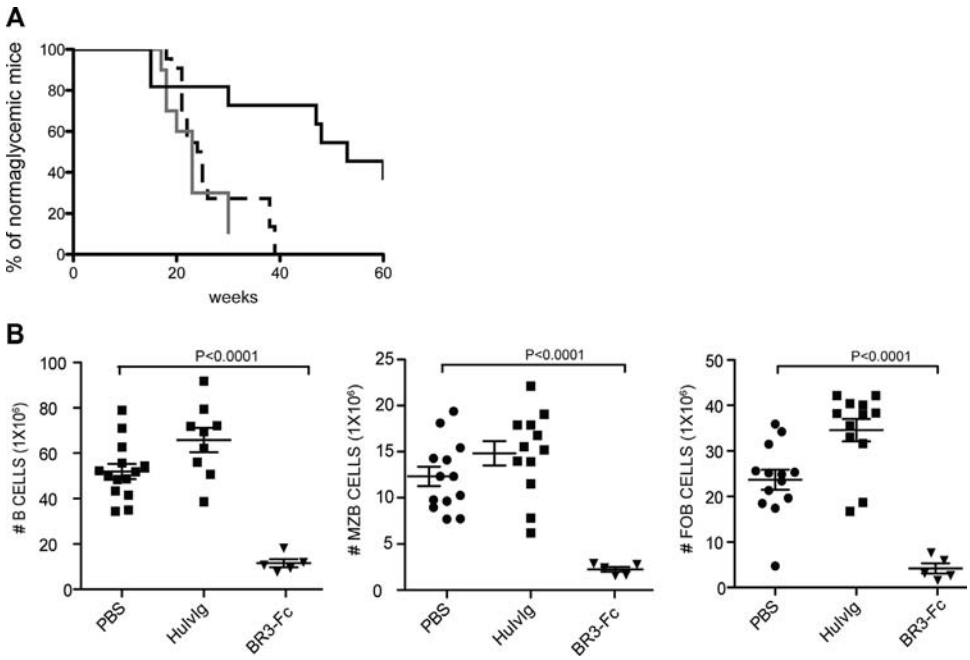

Figure 6. Administration of BR3-Fc significantly delays diabetes in NOD mice. (A) Diabetes incidence was followed for NOD mice administered BR3-Fc (black line, $n = 10$), HuIvIg (gray line, $n = 20$) and PBS (broken line, $n = 30$) from 9 to 15 weeks of age. *, $p = 0.0041$ (Mantel-Cox Log-Rank analysis) for BR3-Fc treatment vs. HuIvIg. (B) Absolute numbers of IgM^+ $B220^+$ B cells as well as MZB and FOB cells in NOD mice treated in (A) at 16 weeks of age. Data represents mean ± SEM; each point represents one mouse. (A, B) Data shown are representative of three independent experiments. *p* values calculated by the Student's *t*-test analysis.

Materials and methods

Mice

NOD/Lt (NOD) and NOD.*scid* mice were obtained from WEHI Kew, Melbourne, Australia. NOD.8.3 mice were obtained from Pere Santamaria, Julia McFarlane Diabetes Researchers Center at the University of Calgary, Alberta, and have been described [44]. C57BL/6.BAFF$^{-/-}$ mice [15] that lack TNFSF13b (BAFF) were obtained from Dr. Susan Kalled (Biogen-Idec, Boston, USA). BAFF-deficient NOD mice were generated in our facility by backcrossing C57BL/6.BAFF$^{-/-}$ mice onto the NOD background for 15 generations. N15 NOD.BAFF$^{-/-}$ mice were subsequently maintained as heterozygotes to generate littermate matched NOD.BAFF$^{+/+}$, NOD.BAFF$^{+/-}$, and NOD.BAFF$^{-/-}$ mice for analysis. The incidence of diabetes in NOD.BAFF$^{+/+}$ is identical to that of founder NOD/Lt mice, e.g. ≥70% by 30 weeks of age for NOD/Lt and NOD.BAFF$^{+/+}$ mice ($n \geq 15$ per group). All animal experiments were performed with the approval of the St. Vincent's Campus Animal Experimentation and Ethics Committee (AEEC).

Determination of diabetes

Diabetes was determined by measurement of blood glucose levels (BGLs), using an Accu-Check Advantage glucometer with Accu-Check II strips (Roche). Mice with a BGL > 16 mM on two consecutive readings were scored as diabetic.

Flow cytometric analysis

Lymphocytes were isolated from spleen, PLNs, and whole pancreas using standard techniques. Immunophenotyping mAb for T cells; CD4 (L3T4)(GK1.5), CD8a (Ly2)(53-6-7), CD44 (Pgp-1, Ly-24) (IM7), CD62 (L-selectin, LECAM-1, Ly-22) (MEL-14); B cells, [10]: IgM (11/41), B220/CD45R (RA-6B2), CD21/CD35 (7G6), CD23/Fc RII (B3B4); isotype controls: IgG1,λ; IgG1, κ, IgG2b, κ and IgG2a, κ were all from Becton and Dickinson. Foxp3 was detected with the Foxp3-staining kit (eBioscience). $IGRP_{206\text{-}214}$ (H-$2K^d$/VYLKTNVFL) and TUM (H-$2K^d$/KYQAVTTTL) tetramers were generated at the NIH-Tetramer Core Facility (Atlanta, GA, USA) with peptides from Mimotope (Australia). Flow cytometric analysis was conducted on a FACS Calibur flow cytometer (Becton and Dickinson).

B-cell depletion

To study the effect of B-cell depletion upon diabetes incidence, NOD mice were treated twice-weekly for 6 weeks from 9 weeks of age with 40 μg/mL of a fusion protein consisting of the extracellular portion of the BAFF receptor BR3 fused to the Fc domain

of human IgG (BR3-Fc) as previously described [55]. As a control mice were also treated with PBS, or 50 μg/mL of intravenous globulin (IVIg). BR3-Fc was a kind gift from Dr. S. Kalled (Biogen, IDEC, Boston).

Islet transplantation

To induce diabetes, recipient mice were injected with 180 mg/kg of streptozotocin (STZ) (Sigma, St. Louis, MO) in 10 mM citrate buffer pH 4.2. Mice with a blood glucose value ≥ 16 mM were selected as transplant recipients. On the transplant day, islets were prepared from the pancreata of donor BALB/c (H-2^d) mice, at a ratio of three pancreata per recipient, and placed under the inferior renal pole as we have described [56]. Rejection was scored as a return to hyperglycemia, based on a blood glucose level greater than 16 mM.

Skin transplantation

To prepare the graft bed, recipient mice were anesthetized with halothane (Aldrich, Milwaukee WI, USA), and a superficial, square incision revealing the panniculus carnosus was made on the left thorax. Donor tail skin (H-2^d) was prepared and secured to the graft bed with superglue (Superglue Corporation, Rancho Cucamonga, CA, USA). Rejection was assessed by daily visual inspection of the graft.

Cyclophosphamide induced diabetes

Mice were administered two-doses (200 mg/kg i.p.) of cyclophosphamide (CyP; -(Bis ((2)-chloroethyl)amino)tetrahydro-2H-1,3, 2-oxazaphosphorine 2-oxide) dissolved in PBS 2 weeks apart. Diabetes was scored as onset of hyperglycaemia, based on a BGL greater than 16 mM.

Adoptive transfer studies

A total of 5×10^6 purified T cells pooled from spleen and pancreatic lymph nodes of 12–16 week old NOD.BAFF$^{+/+}$ or NOD.BAFF$^{-/-}$ mice were transferred (i.v.) into NOD.*scid* mice. Mice were monitored for diabetes (BGL > 16 mM) for 22 weeks. For in vivo proliferation experiments, 5×10^6 splenic T cells were purified (MACS Pan-T-cell isolation kit, Miltenyi Biotec) from NOD8.3 TCR mice were then transferred with, or without purified splenic B cells (MACS Pan-B-cell isolation kit, Miltenyi Biotec), to 6 week old NOD.BAFF$^{+/+}$ or NOD.BAFF$^{-/-}$ recipients. Transferred cells were harvested 3-days post transfer for analysis by FACS.

Histopathology

Formaldehyde-fixed, paraffin-embedded pancreata sections (5 μm) were heamatoxylin and eosin stained. Insulitis was scored (100 × magnification) as follows: grade 0, no insulitis; grade 1, periinsulitis; 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 an Olympus BX51 microscope with and Olympus DP70 camera.

Statistical Analysis

Statistical significance for in vitro experiments and analysis of $CD8^+$ T-cell numbers 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 Logrank (Mantel-Cox) method with 2 degrees of freedom (SatView Software, Acton, MA). *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

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Abbreviations: **APRIL**: a proliferation-inducing ligand · **BAFF**: B-cell activating factor belonging to the TNF family · **BCMA**: B-cell maturation antigen · **BGL**: blood glucose level · **BR3**: BAFF receptor 3 · **HuIVIg**: intravenous globulin · **IGRP**: islet-specific glucose-6-phosphatase catalytic subunit-related protein · **NOD.μMT**: B-cell deficient NOD mice · **PLN**: pancreatic LN · **SLE**: systemic lupus erythematosus · **SS**: Sjogren's syndrome · **T1D**: type 1 diabetes · **TNFSF13b**: tumor necrosis factor ligand superfamily member 13b

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