Increased CD4$^+$Foxp3$^+$ T Cells in BAFF-Transgenic Mice Suppress T Cell Effector Responses

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The cytokine B cell activation factor of the TNF family (BAFF) is considered to perform a proinflammatory function. This paradigm is particularly true for B cell-dependent immune responses; however the exact role for BAFF in regulating T cell immunity is ill-defined. To directly assess the effect of BAFF upon T cells, we analyzed T cell-dependent immune responses in BAFF-transgenic (Tg) mice. We found that T cell responses in BAFF-Tg mice are profoundly compromised, as indicated by their acceptance of islet allografts and delayed skin graft rejection. However, purified BAFF-Tg effector T cells could reject islet allografts with a normal kinetic, suggesting that the altered response did not relate to a defect in T cell function per se. Rather, we found that BAFF-Tg mice harbored an increased number of peripheral CD4$^+$Foxp3$^+$ T cells. A large proportion of the BAFF-expanded CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs) were CD62L^{low}CD103^{high} and ICAM-1^{high}, a phenotype consistent with an ability to home to inflammatory sites and prevent T cell effector responses. Indeed, depletion of the endogenous BAFF-Tg Tregs allowed allograft rejection to proceed, demonstrating that the increased Tregs were responsible for preventing alloimmunity. The ability of BAFF to promote Treg expansion was not T cell intrinsic, as Tregs did not express high levels of BAFF receptor 3, nor did excessive BAFF trigger NF-κB2 processing in Tregs. In contrast, we found that BAFF engendered Treg expansion through an indirect, B cell-dependent mechanism. Thus, under certain conditions, BAFF can play a surprising anti-inflammatory role in T cell biology by promoting the expansion of Treg cells. *The Journal of Immunology,* **2009, 182: 793–801.**

Members of the TNF family and their cognate receptors collectively play a central role in regulating normal immune function. The B cell activation factor of the TNF family (BAFF[2] also known as Blys/TALL1), was first identified within expressed sequence tag databases based upon its homology with TNF (for reviews and references therein, see Ref. 1). The major described function to date for BAFF is as an essential B cell survival and activation factor, which can effect B cell tolerance when overexpressed (1–3). The observation that T cells express the BAFF receptors TACI (4) and BAFF receptor 3 (BR3) (5, 6) suggests that BAFF may also be involved in the regulation of T cell responses. This concept is supported by in vitro studies showing that addition of exogenous BAFF costimulates both human and mouse T cells (7, 8). A potential role for BAFF in regulating T cell activation and function is also supported by in vivo studies. BAFF-transgenic (Tg) mice exhibit exaggerated T-dependent Ag responses, enhanced delayed-type hypersensitivity (DTH) responses, and have an expanded CD4$^+$CD62L^{low}, CD44^{high} memory population (9). Conversely, loss of BAFF signaling impairs the development of Ag-specific T cell responses (6). Together, these data support the concept that BAFF promotes Th1 polarization and has a proinflammatory function with regard to primary T cell responses.

Despite this evidence, a number of caveats bring into question the exact role of BAFF in the regulation of T cell-dependent immunity. Indeed, given the exaggerated T cell responses elicited in BAFF-Tg mice, it is somewhat surprising that BAFF-deficient mice were shown to have normal numbers of T cells and could mount normal immune responses, including rejection of an allograft, suggesting that BAFF is not required per se for T cell function (3, 6, 9). Furthermore, B cell-deficient BAFF-Tg mice no longer exhibit the expanded CD44^{high} memory T cell population or exaggerated DTH responses, suggesting these T cell phenotypes are secondary to B cell hyperactivation (9). In addition, residual signaling can occur through the mutant BR3 receptor expressed by A/WySnJ mice, suggesting the altered T cell function of these mice may relate to an effect of aberrant vs loss of receptor signaling (10). These caveats prompted us to examine the role of BAFF in the regulation of T cell immune responses. We found that T cell effector responses in BAFF-Tg mice are profoundly immunocompromised, as indicated by their acceptance of tissue allografts. The mechanism for their impaired T cell immunity related to an expanded number of peripheral CD4$^+$Foxp3$^+$ regulatory T cells (Tregs). Thus, BAFF has a complex, dichotomous role in immunity mediated by the differential regulation of T and B cell-dependent immune responses.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from the Animal Resource Center (Perth, Australia). BAFF-Tg mice (11) were a gift from Dr. S. Kalled (Biogen IDEC) and B cell-deficient μMT$^{-/-}$ mice (Jax Labs) were

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[2] Abbreviations used in this paper: BAFF, B cell-activating factor of the TNF family; BR3 (also known as BAFFR), BAFF receptor 3; DTH, delayed-type hypersensitivity; Treg, regulatory T cell; Tg, transgenic; mBSA, mouse BSA; WT, wild type; MST, mean survival time; GITR, glucocorticoid-induced TNFR.

bred and housed in our facility. All procedures and experiments conducted complied with institutional Animal Ethics Committee guidelines.

Islet transplantation

To induce diabetes, recipient mice (H-2^b) were injected with 180 mg/kg streptozotocin (Sigma-Chemical) in 10 mM citrate buffer (pH 4.2) and blood glucose levels were determined using a Glucometer Dex (Bayer). Mice with a blood glucose value ≥360 mg/dl and a body weight of 20–25 g were selected as transplant recipients. On the transplant day, islets were prepared from the pancreata of donor (H-2^d) mice at a ratio of three pancreata per recipient. For the transplant, the kidney was accessed by a left flank incision and brought into the wound by gentle blunt dissection. A small nick was made in the kidney capsule at the inferior renal pole and the islets were deposited through the nick toward the superior pole of the kidney. Blood glucose levels were analyzed on postoperative days 1, 2, and 5 and then daily until rejection. Nephrectomy was performed at postoperative day 100.

Immunohistochemical analysis of islet grafts

The position of the graft area on the kidney was marked with India ink (Eberhard Faber) and the kidney was fixed in 60% methanol/10% acetic acid with 30% chloroform for 24 h. The tissue was processed and embedded in paraffin using standard protocols. The entire graft area was serially cut and contiguous regions (spanning ~75 μM) were examined for general morphology by H&E.

Skin transplantation

To prepare the graft bed, recipient mice (H-2^b) were anesthetized with 2.5% avertine (200 μl/100 g; Sigma-Aldrich) 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).

Adoptive transfer experiments

$RAG^{-/-}$ mice were transplanted with an islet graft. Splenocytes were prepared by mechanical disruption with frosted glass slides (Menzel-Glaser) in 1× PBS. Single-cell suspensions were obtained by filtering through a 70-μm nylon strainer (Falcon,; BD Biosciences). Erythrocytes were removed by osmotic lysis with sterile RBC lysis solution. T cells were isolated using a FACSVantage SE Cell Sorter (with FACSDiva Option; BD Biosciences), running BD FACSDiva software version 4.1.2 (BD Biosciences), and an excitation laser line: blue diode (488 nm) and HeNe (633 nm); nozzle size: 70 μm; temperature: 4°C. Splenocytes (2×10^7) and T cells (2×10^6) were adoptively transferred via tail vein injection.

Mixed lymphocyte reaction

MLRs were performed at a standard concentration of 2×10^5 stimulator and responder cells per well in 96-well plates. Stimulator cells were inactivated before being seeded using gamma irradiation (250 rad). Optimal assay duration corresponding to maximal proliferation was 4–5 days.

In vitro T cell assays

For in vitro T cell proliferative responses, splenic T cells were purified using a MACS Pan-T cell isolation kit (Miltenyi Biotec). T cells were then cultured in 0.2 ml of standard culture medium at a density of 2×10^5 cells/well in 96-well plates (Costar) and stimulated with anti-CD3 (0.1–10 μg/ml) or anti-CD3 (0.1–10 μg/ml) plus anti-CD28 (2 μg/ml) for 72 h. Cells were pulsed with ^{3}H for the last 6 h and data represent proliferation (cpm) of triplicate cultures.

DTH responses

Mice were immunized with mouse BSA (mBSA) in CFA (12) and then challenged 7 days later with a s.c. injection of mBSA; the generation of a DTH response was determined by analysis of footpad swelling 8 and 24 after challenge.

Flow cytometry

Cell suspensions of spleen and lymph nodes and thymus were prepared according to standard protocols and stained for FACS analysis in PBS containing 0.5% BSA, 2 mM EDTA, and 0.02% sodium azide using the following Abs (obtained from eBioscience, unless otherwise stated): PE-, PE-Cy5.5-, or Alexa Fluor 405-conjugated anti-CD4 (RM4-5; eBioscience and Caltag Laboratories, respectively), FITC-, allophycocyanin- or biotin-conjugated anti-CD25 (PC61.5), FITC- or allophycocyanin-Cy7-conjugated anti-CD8 (53-6.7; eBioscience and BD Biosciences, respectively), FITC- or allophycocyanin-conjugated anti-CD44 (IM7), FITC- or biotin-conjugated anti-CD122 (TM-β1; BD Biosciences), PE- or allophycocyanin-conjugated anti-Foxp3 (FJK-16s), FITC-conjugated anti-CD54 (YN1/1.7.4), anti-CD38 (90), anti-CD86 (GL1), anti-CD80 (16-10A1), PE-conjugated anti-CD152 (CTLA-4, UC10-4B9), anti-CD62L (MEL-14), anti-CD45RB (16A; BD Biosciences), anti-PD1 (J43), anti-ICOS (7E.17G9; BD Biosciences), anti-CD127 (A7R34), anti-CCR5 (HM-CCR5(7A4)), biotin-conjugated anti-CD103 (2E7), anti-CD69 H1.2F3), anti-glucocorticoid-induced TNFR (GITR; DTA-1), anti-CD27 (LG.3A10), biotin-conjugated chicken anti-TGFβ (R&D Systems), FITC-rat IgG2aκ, FITC-rat IgG2bκ, PE-rat IgG2aκ, PE-rat IgG2bκ, biotin-rat IgG2a, PE-Ar Ham IgG1, biotin-conjugated chicken IgY (IgG) (Jackson Immuno-Research Laboratories), and Pacific Blue-conjugated streptavidin (Molecular Probes). Allophycocyanin-anti- and PE-anti-mouse/rat Foxp3 staining sets were purchased from eBioscience, and protocol was followed according to the manufacturer's instructions. Samples were acquired on a FACScan (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

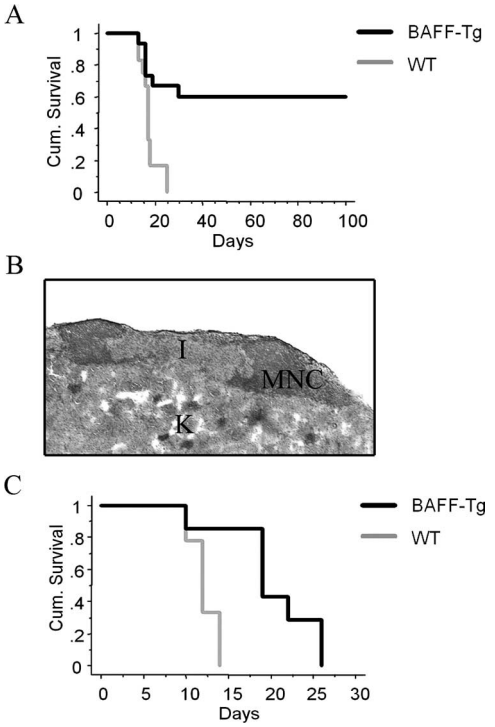

FIGURE 1. BAFF-Tg mice are tolerant of allografts. *A*, Allogeneic islet graft survival in BAFF-Tg ($n = 15$) vs WT mice ($n = 12$; $p = 0.001$). *B*, Photomicrograph of representative histological section through graft-containing portion of the kidney harvested from a BAFF-Tg mouse at 100 days after transplantation. Section was stained with H&E. I, Islet graft; MNC, mononuclear cell accumulation; K, kidney parenchyma. *C*, Allogeneic skin graft survival in BAFF-Tg ($n = 7$) vs WT ($n = 9$; $p = 0.0058$).

Depletion of Tregs

For in vivo depletion of $CD4^+CD25^+$ T cells, mice were injected with purified rat anti-mouse CD25 IgG1 mAb (PC61; BioExpress) i.p. (250 μg). The efficacy of CD25 depletion was confirmed by flow cytometry. In vitro-depleted $CD4^+CD25^+$ T cells were isolated using a FACSVantage SE Cell Sorter (with FACSDiva Option; BD Biosciences), running BD FACSDiva software version 4.1.2 (BD Biosciences), and an excitation laser line: blue diode (488 nm) and HeNe (633 nm); nozzle size: 70 μm; temperature: 4°C.

Suppression assay

$CD4^+CD25^-$ effector T cells and $CD4^+CD25^+$ T cells were isolated using a FACSVantage SE Cell Sorter (with FACSDiva Option; BD Biosciences), running BD FACSDiva software version 4.1.2 (BD Biosciences), and an excitation laser line: blue diode (488 nm) and HeNe (633 nm); nozzle size: 70 μm; temperature: 4°C. $CD4^+CD25^-$ effector T cells (2×10^5) were

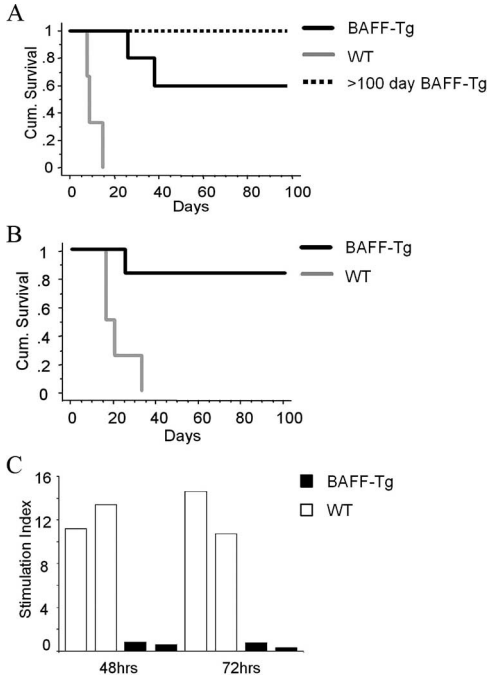

FIGURE 2. Allograft tolerance is T cell dependent. *A*, RAG$^{-/-}$ mice received an islet allograft and 7–14 days later were reconstituted with BAFF-Tg ($n = 5$) or WT splenocytes ($n = 3$; $p = 0.0101$). Splenocytes from BAFF-Tg mice that were tolerant of their allografts (≥100-day survival) were transferred to RAG$^{-/-}$ recipients transplanted with an allogeneic islet graft ($n = 4$; $p = 0.0042$). *B*, RAG$^{-/-}$ mice received an islet allograft and 7–14 days later were reconstituted with BAFF-Tg ($n = 6$) or WT T cells ($n = 5$; $p = 0.0025$). *C*, MLR response of T cells from BAFF-Tg and WT mice stimulated for 48 and 72 h with BALB/c splenocytes (stimulators). Data shown are representative of five experiments conducted.

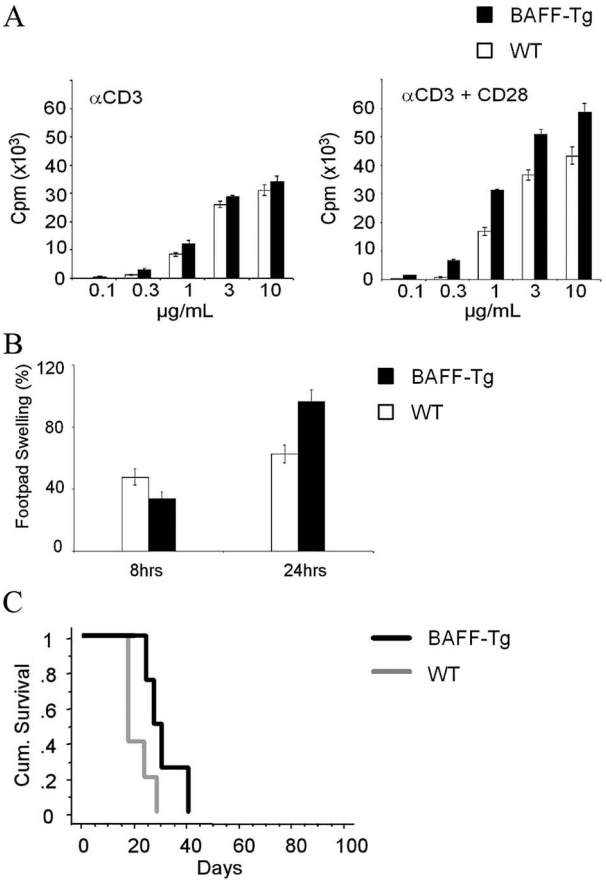

FIGURE 3. BAFF-Tg T cells are not anergic. *A*, Proliferation assays (cpm) of T cells from BAFF-Tg and WT mice stimulated with anti-CD3 or anti-CD3 plus anti-CD28 for 72 h. Data represent mean ± SEM from one of three independent experiments conducted. Proliferative responses are not statistically different between groups. *B*, In vivo T cell proliferative response (DTH). BAFF-Tg ($n = 5$) and WT ($n = 5$) mice were immunized with mBSA in CFA and challenged 7 days later with mBSA. Footpad swelling was determined 8 and 24 h after challenge. Data represent mean ± SEM footpad swelling. Proliferative responses are not statistically different between groups. *C*, RAG$^{-/-}$ mice received an islet allograft and 7–14 days later were reconstituted with BAFF-Tg ($n = 5$) or WT CD4$^+$CD25$^-$ effector T cells ($n = 5$). Rejection responses are not statistically different between groups.

stimulated with anti-CD3 (1 μg/ml) and cocultured with increasing numbers of CD4$^+$CD25$^+$ T cells. Cells were pulsed with [^{3}H]thymidine for the last 6 h. ^{3}H Incorporation (cpm) was assayed at 72 h.

Immunoblotting

Cell populations were isolated by cell sorting on FACSVantage or FACSAria cell sorters (BD Biosciences). Whole-cell lysates were prepared with radioimmunoprecipitation assay buffer (25 mM HEPES, 250 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 100 μg/ml AEBSF, 10 μg/ml leupeptin, 1 mM sodium pervanadate, and 10 mM sodium fluoride). All lysates were subjected to PAGE on 4–12% gradient NuPAGE Bis-Tris gels (Invitrogen) in an XCell SureLock Mini-Cell apparatus (Invitrogen) and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore) with an XCell II Blot module (Invitrogen). Membranes were blocked and Abs were diluted in 5% BSA/0.2% Tween 20/TBS.

Bone marrow chimeras

Mice were sublethally irradiated with 950 rad and 24 h later reconstituted with 5×10^6 bone marrow cells.

Statistics

Statistical analysis of T cell subsets was performed using the Student *t* test on Instat (version 2.01) software (GraphPad Software). Graft survival was plotted as Kaplan-Meier curves and analyzed using the log rank test on Statview software (SAS Institute).

Results

BAFF-Tg mice have an altered alloimmune response favoring allograft tolerance

To determine the role of BAFF in regulating T cell immune responses, we examined the ability of BAFF-Tg mice to reject an islet allograft. The rejection of an allograft is dependent upon the T cell response against donor MHC determinants presented by host and donor APCs, and as such it is an excellent model to study T cell activation and function. BAFF-Tg mice express full-length murine BAFF under the control of the liver-specific α1 antitrypsin promoter and the apolipo-protein E enhancer (11). This results in high circulating levels of BAFF in the serum; indeed, the average BAFF concentration was found to be ~700–1100 ng/ml in BAFF-Tg mice vs ~ 4–5 ng/ml in C57BL/6 (WT) mice as determined by ELISA ($p < 0.05$, $n \geq 5$/group).

Full mismatched BALB/c (H-2^d) islet allografts were transplanted into two groups of (H-2^b) diabetic recipients: WT and BAFF-Tg mice (Fig. 1*A*). WT mice rejected the graft with a mean survival time (MST) of 20 ± 3.8 days ($n = 12$). In marked contrast, the majority (~60%, $n = 15$) of BAFF-Tg mice (H-2^b) failed to acutely reject their islet allografts, but rather exhibited a dramatic and prolonged allograft survival for ≥100 days ($p = 0.001$). The functionality of long-term grafts was tested by reappearance

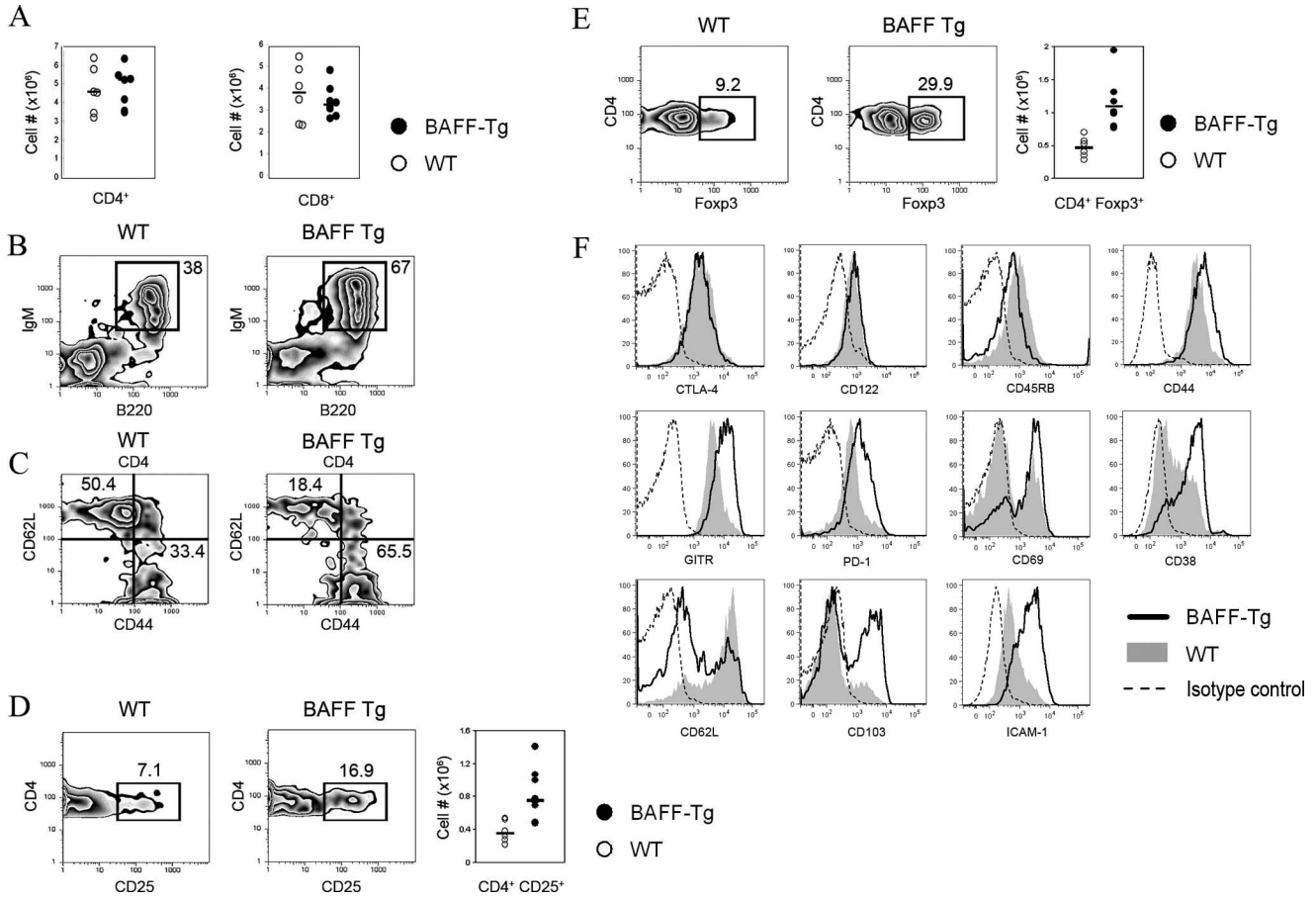

FIGURE 4. Expanded frequency and number of Treg in BAFF-Tg mice. *A*, Cell numbers $CD4^+$ (*left panel*) and $CD8^+$ (*right panel*) T cells from BAFF-Tg and WT mice. Calculated absolute numbers are not statistically different between groups. *B*, BAFF-Tg mice have an increased frequency of mature B cells in the periphery compared with WT mice. *C*, BAFF-Tg mice exhibited an altered ratio of naive and memory T cells in the periphery compared with WT mice. *D*, Frequency ($p = 0.0004$) and calculated absolute numbers ($p = 0.029$) of splenic $CD4^+CD25^+$ T cells from WT and BAFF-Tg mice. *E*, Frequency ($p = 0.0009$) and calculated absolute numbers ($p = 0.029$) of splenic $CD4^+Foxp3^+$ T cells from WT and BAFF-Tg mice. *F*, Flow cytometric analysis of Treg markers on WT and BAFF-Tg $CD25^+Foxp3^+$ T cells.

of hyperglycemia after graft removal. Histological analysis of surviving grafts revealed normal islet morphology (Fig. 1*B*); however, there was the presence of a marked accumulation of mononuclear cells surrounding, but not infiltrating, the islet graft without evidence of overt rejection.

Skin transplantation represents an even more stringent allograft model than islet transplantation. Therefore, we next examined the ability of BAFF-Tg mice to reject H-2^d skin grafts (Fig. 1*C*). We found that WT recipients rejected H-2^d skin allografts with a MST ~12 days. Akin to their altered response to islet allografts, BAFF-Tg mice exhibited a significant delay in the rejection of a skin allograft exhibiting a MST of ~20 days ($n = 7$; $p = 0.0058$). Thus, different than the well-defined proinflammatory effects of BAFF on B cell activation, excessive BAFF alters the T cell-dependent alloimmune response, favoring long-term survival of an islet allograft and delayed rejection of a skin allograft.

The altered alloantigen response in BAFF-Tg mice is T cell intrinsic

Failure to reject islet allografts was shown to be cell autonomous (Fig. 2*A*), as adoptive transfer of BAFF-Tg splenocytes (2×10^7) into H-2^b $RAG^{-/-}$ recipients, which had previously received a H-2^d islet allograft, resulted in ~60% ($n = 5$; $p = 0.0101$) long-term graft acceptance. This phenotype was transferable, as adoptive transfer of splenocytes from BAFF-Tg mice that had accepted their grafts for 100 days resulted in 100% graft acceptance ($n = 4$; $p = 0.0042$). In contrast, all $RAG^{-/-}$ mice receiving splenocytes from WT donors rapidly rejected their allografts (Fig. 2*B*). Transfer of FACS-purified T cells (2×10^6 in total) from WT mice was sufficient to facilitate rapid rejection of the islet allograft. However, T cells from BAFF-Tg mice were unable to reject their allografts; ~80% of $RAG^{-/-}$ recipients receiving BAFF-Tg T cells accepted their allograft for >100 days ($n \geq 5$; $p = 0.0025$). We then examined the ability of purified BAFF-Tg T cells to respond to H-2^d alloantigen in a MLR (Fig. 2*C*). In contrast to T cells from WT mice, T cells from BAFF-Tg did not mount a detectable MLR response to H-2^d BALB/c stimulators. Collectively, these data demonstrate that the inability of BAFF-Tg mice to reject an allograft relates to a T cell intrinsic change in alloresponsiveness.

Proliferative responses of BAFF-Tg T cells

The mechanisms underlying the changed ability of BAFF-Tg mice to mount an allograft or MLR response were investigated. We examined the in vitro proliferative responses of purified splenic T cells isolated from BAFF-Tg mice (Fig. 3*A*). In contrast to their poor alloresponses in a MLR, we found that purified BAFF-Tg T cells exhibited an equivalent proliferative response to anti-CD3 or anti-CD3 plus anti-CD28 as compared with T cells isolated from WT mice. We next examined the ability of BAFF-Tg T cells to proliferate in response to antigenic challenge in vivo by looking at

DTH responses (Fig. 3*B*). We found, that like WT mice, BAFF-Tg mice could mount a rapid and robust DTH response. Together these experiments demonstrate that BAFF-Tg T cells can respond normally to TCR stimulation, that they are not simply anergic, and that they do not harbor any gross abnormalities with regard to T cell function.

BAFF-Tg effector T cells can mediate allograft destruction

To test the competency of BAFF-Tg effector T cells to mediate allograft destruction, $RAG^{-/-}$ mice that had previously been transplanted with an allogeneic islet graft were adoptively transferred with 2×10^6 BAFF-Tg or WT $CD4^+$ $CD25^-$ effector T cells (Fig. 3*C*). WT effector T cells promptly rejected islet allografts. BAFF-Tg $CD4^+CD25^-$ effector T cells were just as effective as WT effector T cells in mediating allograft rejection; indeed, all mice receiving BAFF-Tg $CD4^+CD25^-$ effector T cells rapidly rejected their allografts ($n = 5$; $p = 0.8984$), demonstrating that BAFF-Tg effector T cells are functionally capable of rejecting an allograft. Thus, the inability of BAFF-Tg mice to reject allografts is not due to a relative lack or deficiency in effector T cells.

Increased numbers of peripheral Foxp3$^+$ T cells in BAFF-Tg mice

To elucidate the mechanism by which BAFF modulates T cell function, we examined the peripheral T cell compartment of BAFF-Tg mice (Fig. 4*A*). There was no alteration in the absolute numbers of peripheral $CD4^+$ ($n \geq 6$; $p = 0.8362$) and $CD8^+$ ($n \geq 6$; $p = 0.8085$) T cells in BAFF-Tg mice as compared with WT mice. However, identical to previous reports (6, 13) (Fig. 4, *B* and *C*), we did find that BAFF-Tg mice exhibited an increased frequency of mature B cells and an altered ratio of naive and memory T cells in the periphery.

Strikingly, our analysis of splenic T cell populations (Fig. 4*D*) revealed that BAFF-Tg mice harbored a ~2.5-fold increase ($n \geq 6$; $p = 0.0004$) in the frequency of $CD4^+CD25^+$ T cells as compared with WT mice, the median being ~17% in BAFF-Tg mice, as compared with only ~7% in WT mice. This increase was also reflected in the absolute numbers, as the number of $CD4^+CD25^+$ T cells was ~2-fold higher in BAFF-Tg mice ($n \geq 6$; $p = 0.029$) as compared with WT mice, the median number being $\sim 0.77 \times 10^6$ vs $\sim 0.35 \times 10^6$, respectively. Thus, BAFF-Tg mice have an expanded number of T cells with a surface phenotype reminiscent of Tregs.

Tregs can be identified by their expression of the T regulatory lineage specification factor Foxp3 (12). We found (Fig. 4*E*) that the frequency of splenic Foxp3 expressing T cells in BAFF-Tg mice was increased ~3-fold ($n \geq 6$; $p = 0.0009$), the median being ~30% in BAFF-Tg mice vs ~9% in WT mice. Similarly, the absolute number of Foxp3$^+$ T cells was increased ~3-fold ($n \geq 6$; $p = 0.028$) from a median of $\sim 0.46 \times 10^6$ in WT mice to $\sim 1.2 \times 10^6$ in BAFF-Tg mice.

Phenotypic profile of BAFF-Tg Tregs

We next compared the expression of a panel of cell surface molecules associated with Treg function on $CD25^+Foxp3^+$ T cells from BAFF-Tg vs WT mice (Fig. 4*F*). We found that BAFF-Tg $CD25^+FoxP3^+$ T cells expressed molecules consistent with a Treg phenotype, such as CTLA4, GITR, CD122, and $CD45RB^{low}$. In addition to this, BAFF-Tg $CD25^+Foxp3^+$ T cells showed marked up-regulation of many functionally important molecules. Compared with WT cells, a greater frequency showed high levels of expression of ICAM-1, CD38, CD103, GITR, PD-1, and CD44, indicative of an increased state of activation. Consistent with this interpretation, the frequency of $CD62L^{low}CD69^{high}CD25^+$ Foxp3$^+$ T cells in the BAFF-Tg mice were greatly increased compared with WT mice.

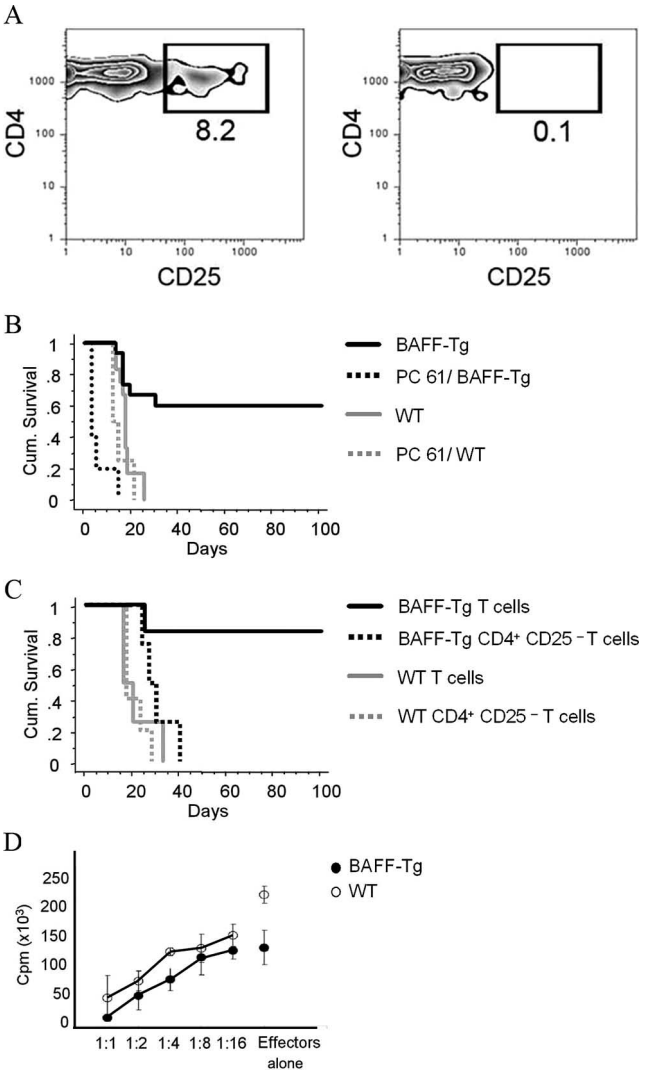

FIGURE 5. BAFF-expanded Treg control alloreactivity. *A*, Elimination of peripheral $CD4^+CD25^+$ T cells after administration of the anti-CD25 mAb PC61. *B*, Allogeneic islet graft survival; in BAFF-Tg mice ($n = 5$) vs BAFF-Tg mice treated with PC61 ($n = 5$; $p = 0.0001$). Allogeneic islet graft survival; in WT mice ($n = 5$) vs WT mice treated with PC61 ($n = 5$) are not statistically different. *C*, Islet allograft survival in $RAG^{-/-}$ mice reconstituted with BAFF-Tg T cells ($n = 6$) vs $CD4^+CD25^+$-depleted BAFF-Tg T cells ($n = 4$; $p = 0.0167$) or WT T cells ($n = 4$) vs $CD4^+CD25^+$-depleted WT T cells ($n = 4$) are not statistically different. *D*, $CD4^+CD25^+$ T cells from BAFF-Tg or WT mice were incubated in increasing ratios with a fixed number of anti-CD3-treated $CD4^+CD25^-$ T cells plus irradiated splenocytes as APCs for 72 h. Cultures were pulsed for the last 6 h and ^3H incorporation (cpm) was assayed. Data represent mean ± SEM of triplicate cultures from one of three independent experiments conducted.

Elimination of CD25$^+$ T cells restores normal allograft responses to BAFF-Tg mice

The significance and function of the expanded Treg population in BAFF-Tg mice was examined in vivo. To achieve this, BAFF-Tg mice were administered the anti-CD25 mAb PC61 (250 μg i.p.) to deplete $CD25^+$ cells (Fig. 5*A*) and graft survival was followed (Fig. 5*B*). We found that ~60% of control untreated BAFF-Tg mice demonstrated long-term survival of their islet allografts. In

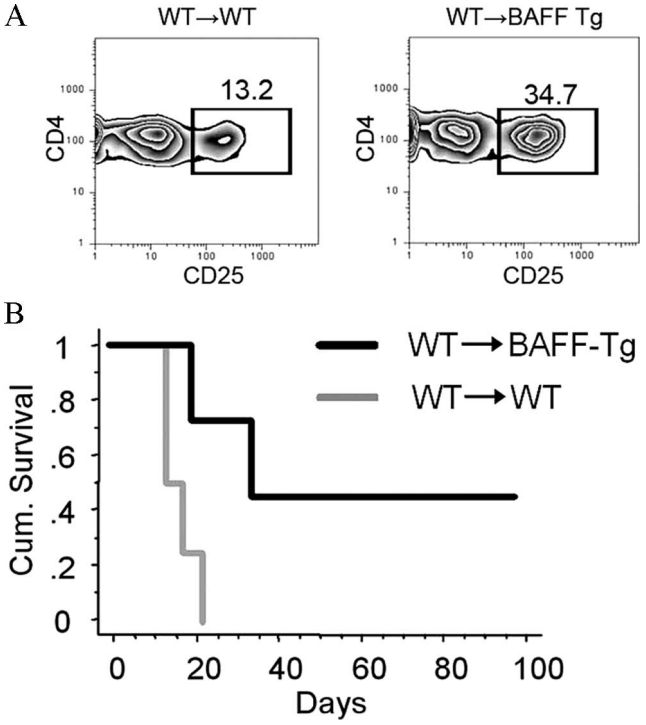

FIGURE 6. Kinetics of Treg expansion in BAFF-Tg mice. *A*, Frequency of splenic $CD4^+CD25^+$ Treg in bone marrow chimeras; WT→WT ($n = 4$) vs WT→BAFF-Tg ($n = 4$; $p = 0.0009$). *B*, Allogeneic islet graft survival in WT→WT ($n = 4$) compared with WT→BAFF-Tg ($n = 4$) mice ($p = 0.007$).

contrast, all of the PC61-treated, BAFF-Tg mice promptly rejected their allografts ($n = 5$; $p = 0.0001$) with a kinetic similar to that observed for WT mice with or without PC61 treatment. These data indicate that the expanded number of $CD4^+CD25^+$ cells harbored by BAFF-Tg mice can prevent allograft rejection.

As an alternative approach, 2×10^6 BAFF-Tg T cells with or without their endogenous $CD4^+CD25^+$ T cells were adoptively transferred into $RAG^{-/-}$ mice that had previously been transplanted with an allogeneic islet graft (Fig. 5*C*). In this experiment, ~80% of the $RAG^{-/-}$ mice receiving BAFF-Tg T cells replete with their endogenous $CD4^+CD25^+$ T cells accepted their graft. In contrast, all of the $RAG^{-/-}$ mice receiving $CD4^+CD25^+$-depleted BAFF-Tg T cells promptly rejected their allografts ($n \geq 4$; $p = 0.0167$). As expected, all $RAG^{-/-}$ recipients receiving WT T cells, with or without their endogenous $CD4^+CD25^+$ T cells, rejected their allograft ($n = 4$; $p = 0.8984$). Thus, we demonstrate that the underlying mechanism for the impaired T cell-dependant immunity is a direct result of the suppressive activity of $CD4^+CD25^+$ T cells in the periphery.

In vitro suppressor function of BAFF-Tg $CD4^+CD25^+$ T cells

We next addressed whether tolerance to allografts in BAFF-Tg mice related purely to the increased numbers of endogenous Treg or whether excessive BAFF enhanced Treg suppressive function on a per cell basis. We compared the ability of purified $CD4^+CD25^+$ T cells from BAFF-Tg or WT mice to inhibit anti-CD3-stimulated WT $CD4^+CD25^-$ T cell proliferation in vitro (Fig. 5*D*). We found that the suppressive activity of BAFF-Tg $CD4^+CD25^+$ T cells was equivalent to that of WT cells. This result demonstrates that Treg from BAFF-Tg mice are not more potent than WT

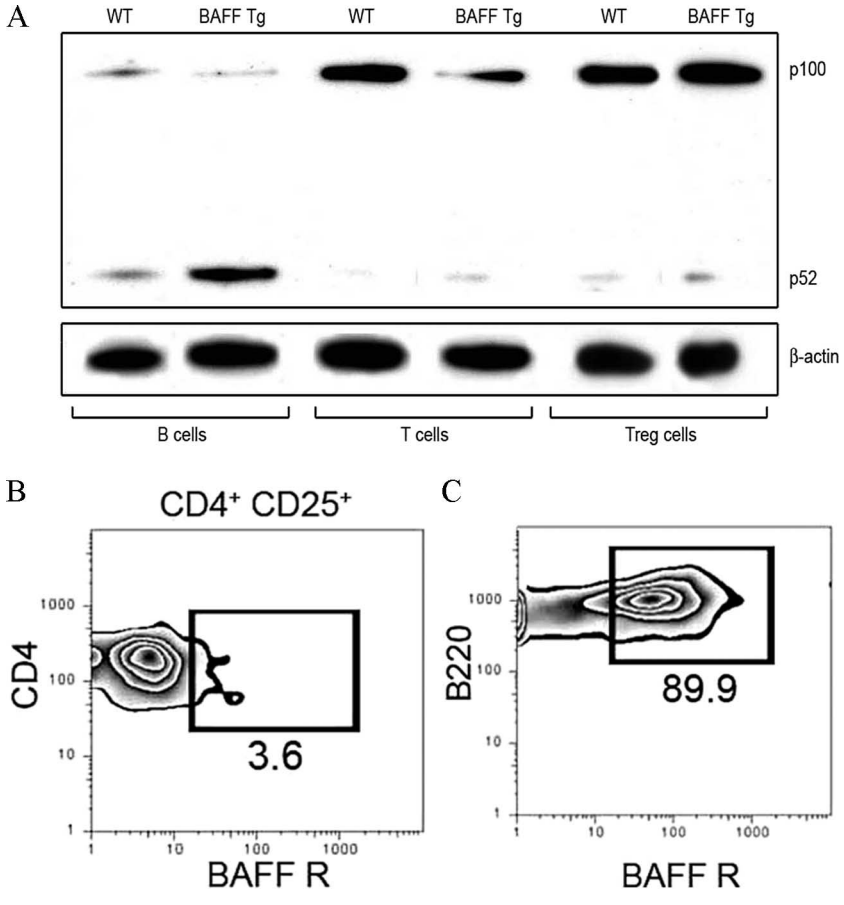

FIGURE 7. BAFF does not signal NF-κB2 activation in T cells. *A*, Immunoblot analysis of purified splenic B cells, T cells, and Treg from WT and BAFF-Tg mice. *B*, Frequency of BR3 on splenic $CD4^+CD25^+$ T cells in WT mice. *C*, Frequency of BR3 on $B220^+$ B cells in WT mice.

Treg in vitro and indicate that the impaired T cell-dependent immunity observed for BAFF-Tg mice is most likely directly related to the increased frequency and number of endogenous Treg.

Kinetics of Treg expansion in BAFF-Tg mice

To directly assess how exogenous BAFF would affect Tregs in vivo, C57BL/6 mice were treated with commercially available recombinant BAFF. Administration of BAFF (10 μg/mouse i.v. daily for 7 days; $n = 4$) had no effect on Treg homeostasis (data not depicted). These data suggested that prolonged exposure to high levels of BAFF was necessary to engender the increase in Tregs. To test this, we generated radiation bone marrow chimeras in which BAFF-Tg mice were reconstituted with WT bone marrow (Fig. 6*A*). Analysis of the frequency of $CD4^+CD25^+$ T cells in WT→BAFF-Tg chimeric mice revealed a ~2.5-fold ($n = 4$; $p = 0.0009$) expansion of Tregs as compared with WT→WT mice, the median being ~34% vs ~13%, respectively. To test the function of these expanded $CD4^+CD25^+$ T cells, the WT→BAFF-Tg and WT→WT mice were transplanted with a H-2^d islet allograft (Fig. 6*B*). We found that WT→WT mice exhibited a vigorous alloimmune response; however, ~50% of W→BAFF-Tg mice failed to reject their allografts but rather exhibited long-term graft acceptance ($n = 4$; $p = 0.007$). These data indicate that BAFF can engender increased Treg numbers; however, this effect requires chronic exposure to BAFF.

Expansion of Tregs in BAFF Tg mice is not T cell intrinsic

The BAFF receptor BR3 can be expressed by T cells under certain circumstances (9, 10). Ligation of BR3 by BAFF activates NF-κB2 by triggering the processing of p100 to p52 (13). Thus, if BAFF was acting directly upon Treg in vivo, we reasoned that this event should be evidenced by an increase in NF-κB2 p100 processing (Fig. 7*A*). In control experiments where we examined NF-κB2 activation by Western blot analysis in FACS-sorted B cells from either WT or BAFF-Tg mice, we observed a significant increase in the processing of NF-κB2 p100 in BAFF-Tg B cells, as indicated by the increased presence of the p52 component of NF-κB2 as compared with WT B cells. These data are consistent with the concept that BAFF promotes B cell survival via activation of NF-κB2 (13). To determine whether BAFF was activating NF-κB2 in vivo in Tregs, FACS-sorted T cells and Tregs from WT or BAFF-Tg mice were analyzed for NF-κB2 p100-p52 processing. In contrast to B cells from WT or BAFF-Tg mice, there was no evidence of NF-κB2 activation in purified T cells and more importantly no evidence of NF-κB2 activation in Tregs isolated from either BAFF-Tg or WT mice.

We also examined BAFF receptor expression on Tregs by flow cytometry (Fig. 7*B*). Flow cytometric analysis revealed only low expression levels of the BAFF receptor BR3 on $CD4^+CD25^+$ T cells; in contrast, the majority of splenic $B220^+$ B cells were $BR3^+$. We interpret these data to indicate no correlation between BAFF-dependent expansion of Tregs, signaling through NF-κB2 and the density of expression of the BR3 receptor on Treg.

B cell-dependent expansion of Tregs in BAFF-Tg mice

BAFF is a critical regulator of B cells (1). Because our data did not support a T cell intrinsic mechanism for the expansion of Tregs, we next questioned the role of B cells in the BAFF expansion of Tregs. Accordingly, we first generated radiation bone marrow chimeras in which WT or BAFF-Tg mice were reconstituted with bone marrow from either WT or $\mu MT^{-/-}$ B cell-deficient donors; approximately 10 wk after reconstitution, the frequency of Tregs in each group was examined (Fig. 8*A*). We found that the frequency

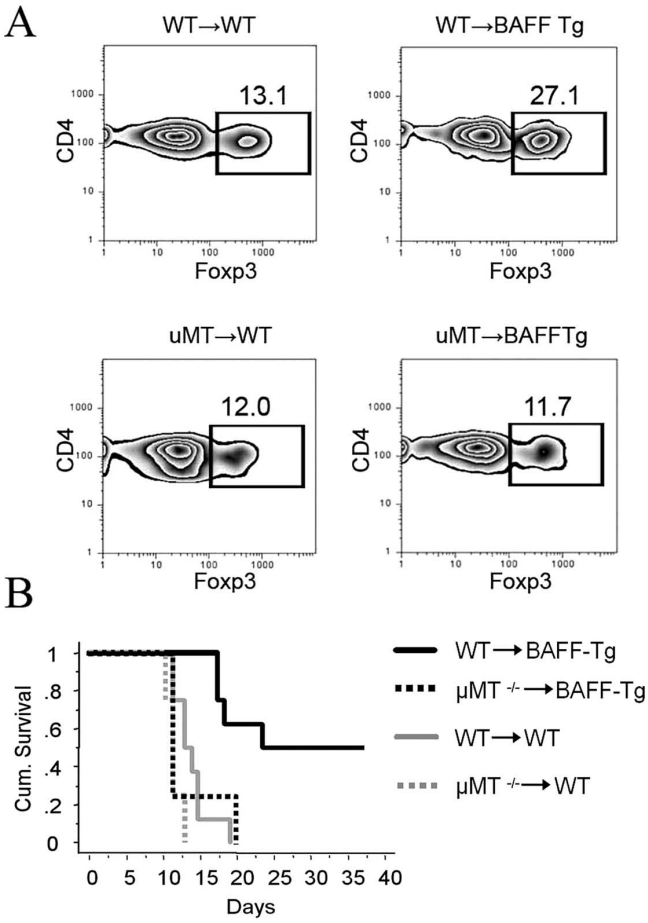

FIGURE 8. BAFF expansion of Treg is B cell dependent. *A*, Frequency of splenic $CD4^+Foxp3^+$ Treg in bone marrow chimeras: WT→WT ($n = 8$) vs WT→BAFF-Tg ($n = 8$; $p = 0.0007$), $\mu MT^{-/-}$→WT ($n = 4$; $p = 0.3688$), $\mu MT^{-/-}$→BAFF-Tg ($n = 4$; $p = 0.1592$). *B*, Allogeneic islet graft survival in WT→WT ($n = 8$) compared with WT→BAFF-Tg ($n = 8$) mice, $\mu MT^{-/-}$→WT ($n = 4$) mice, $\mu MT^{-/-}$→BAFF-Tg ($n = 4$). Only WT→BAFF-Tg mice are statistically significant ($p = 0.007$).

of splenic $CD4^+Foxp3^+$ T cells in WT→WT chimeras was ~13%, whereas the frequency of Tregs in WT→BAFF Tg chimeras was increased ~3- to ~27% ($n = 8$; $p = 0.0007$). In contrast, examination of the frequency of Tregs in $\mu MT^{-/-}$→BAFF Tg chimeras revealed that in the absence of B cells BAFF was not able to expand Tregs. Indeed, the frequency of Treg in $\mu MT^{-/-}$→BAFF Tg chimeras ($n = 4$) was similar to both WT→WT and $\mu MT^{-/-}$→ WT chimeras. Moreover, we found that the ability of these chimeras to reject an allograft correlated with the frequency of Tregs (Fig. 8*B*). That is, although ~50% of WT→BAFF Tg chimeras accepted their islet allografts ($n = 4$; $p = 0.007$), all of the WT→WT, $\mu MT^{-/-}$→WT, and, importantly, the $\mu MT^{-/-}$→BAFF Tg chimeras acutely rejected their islet allografts. These data demonstrate that the increase in Tregs in BAFF-Tg mice is B cell dependent.

Discussion

Our results provide novel and surprising insights into the role of BAFF in regulating T cell immune responses. The major finding of our study is that under specific conditions, BAFF can play a powerful anti-inflammatory role. Indeed BAFF-Tg mice accept islet allografts and exhibit delayed rejection of skin allografts. This conclusion contrasts with the concept of BAFF performing a proinflammatory role, particularly for B cell (7, 14, 15), but also T

cell-dependent immune reactions (6, 16). A proinflammatory function for BAFF in T cell responses has also been inferred from studies on BAFF-Tg mice that harbor an expanded $CD4^+CD62L^{low}$ $CD44^{high}$ activated memory T cell compartment and exhibit exaggerated DTH responses (9, 11). Although these phenotypes are consistent with BAFF playing a Th1-promoting role for T cells, this evidence suffers a number of important caveats. The most significant being that B cell deficiency normalizes the exaggerated T cell phenotype of BAFF-Tg mice (9), indicating that these phenotypes may be secondary to B cell hyperactivation. In comparison, by focusing our study on T cell-dependent, but B cell-independent immune effector responses such as allograft rejection (17), we were able to reveal a novel anti-inflammatory function for BAFF in T cell biology.

The mechanism for the impaired T cell responses of BAFF-Tg mice relates to the unexpected finding that these mice harbor an expanded number of $CD4^+Foxp3^+$ T cells circulating in the periphery. These BAFF-expanded $Foxp3^+$ T cells express a number of features, which mark them as belonging to the subset of naturally occurring Tregs. These features include expression of Foxp3, high expression of GITR, CD38, PD-1, and CD44 (12, 18–20). In vitro suppression assays functionally confirmed their status as bona fide T suppressor cells. Significantly, in vivo elimination of these cells restored normal T effector responses to alloantigens in BAFF-Tg mice, demonstrating that the anti-inflammatory phenotype of BAFF-Tg mice was dependent upon these endogenous Tregs.

The normal suppressive activity of BAFF-Tg Tregs exhibited in vitro indicated that their ability to inhibit T cell effector function related to their increased number, as opposed to an enhanced function. However, the BAFF-expanded $CD25^+Foxp3^+$ Tregs exhibited a cell surface phenotype consistent with an increased activation status and an enhanced ability to home to inflamed sites. For instance, $CD62L^{low}$ but $CD103^{high}$ Tregs are capable of preferentially localizing to inflamed sites where they suppress the T cell effector response (21, 22). BAFF-Tg $Foxp3^+CD25^+$ Tregs express lower levels of CD62L and high levels of CD103 as well as the inflammatory cellular adhesion protein ICAM-1. This phenotype is consistent with the capability of BAFF-Tg Tregs to suppress allograft rejection in vivo. Some data suggest that Tregs are capable of preventing the priming of naive T cells, an event that would require their presence in lymphoid tissues (23). Interestingly, BAFF-Tg Tregs exhibited a poor capacity to prevent such T cell activation, as shown by the normal DTH responses of BAFF-Tg mice, suggesting that the dominant Tregs in BAFF-Tg mice is representative of an activated type Tregs typical of those recruited to sites of inflammation.

Although we are the first to identify that BAFF can play a role in Treg development, the concept that BAFF can play an important role in lymphoid lineage development is not new. Indeed, BAFF is required for B cell maturation in the spleen, such that $BAFF^{-/-}$ mice are devoid of mature B cells (3), conversely BAFF-Tg mice display an exaggerated number of mature B cells (14). The ability of BAFF to drive B cell development relates to an intrinsic effect of BAFF upon B cells, mediated principally by the BAFF receptor BR3 (10). Ligation of BR3 by BAFF triggers processing of NFκB2 p100 to p52, allowing p52 to complex with RelB and translocate to the nucleus to activate target genes (13). BAFF-dependent activation of the NF-κB2 pathway can provide a survival signal through the induction of Bcl family genes that rescue B cells from deletion (2, 10, 24). We initially postulated that BAFF promoted the expansion of Tregs through a similar mechanism that is by triggering NF-κB2 activation through BAFF-BR3 interactions. However, we found that BR3 was only weakly expressed on Tregs, and no evidence for NF-κB2 processing was observed in FACS-sorted Tregs from WT or BAFF-Tg mice. Collectively, these data indicate that the mechanism by which BAFF expands Treg is not through a T cell intrinsic mechanism.

Forced overexpression of BAFF such as via a transgenic approach results in a marked expansion of B cells (11, 14). BAFF-expanded B cells generate high titers of autoantibodies, are hyperreactive to mitogenic stimulation, and show an increased APC capacity (2, 14, 25). By generating radiation chimera B cell-deficient BAFF-Tg mice, we could show, perhaps paradoxically, that B cells were also necessary for the expansion of Tregs that suppress T cell-dependent immunity. Thus, although excessive BAFF can exacerbate B cell-dependent inflammatory conditions on one hand, we now demonstrate that BAFF can simultaneously promote the expansion of potent Tregs through a mechanism also dependent upon B cells. Our major finding is that, under certain conditions, BAFF can play a paradoxical anti-inflammatory role with regard to T cell biology. We demonstrate that BAFF-activated B cells can directly control T cell-dependent immunity by promoting the expansion of $Foxp3^+$ Tregs, which in turn suppress T cell-mediated effector functions such as allograft rejection.

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Disclosures

The authors have no financial conflict of interest.

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