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A Role for Intrathymic B Cells in the Generation of Natural Regulatory T Cells

Stacey N. Walters,*,† Kylie E. Webster,*,† Stephen Daley,‡ and Shane T. Grey*,†

B cells inhabit the normal human thymus, suggesting a role in T cell selection. In this study, we report that B cells can modulate thymic production of CD4$^+$ Foxp3$^+$ T cells (regulatory T cells [Tregs]). Mice with transgenic expression of BAFF (BAFF-Tg) harbor increased numbers of Helios$^+$Foxp3$^+$ thymic Tregs and, similar to some human autoimmune conditions, also exhibit increased numbers of B cells colonizing the thymus. Distinct intrathymic B cell subpopulations were identified, namely B220$^+$, IgM$^+$, CD23^{hi}, CD21^{int} cells; B220$^+$, IgM$^+$, CD23^{lo}, CD21^{lo} cells; and a population of B220$^+$, IgM$^+$, CD23^{lo}, CD21^{hi} cells. Anatomically, CD19$^+$ B cells accumulated in the thymic medulla region juxtaposed to Foxp3$^+$ T cells. These intrathymic B cells engender Tregs. Indeed, thymic Treg development was diminished in both B cell–deficient BAFF-Tg chimeras, but also B cell–deficient wild-type chimeras. B cell Ag capture and presentation are critical in vivo events for Treg development. In the absence of B cell surface MHC class II expression, thymic expansion of BAFF-Tg Tregs was lost. Further to this, expansion of Tregs did not occur in BAFF-Tg/Ig hen egg lysozyme BCR chimeras, demonstrating a requirement for Ag specificity. Thus, we present a mechanism whereby intrathymic B cells, through the provision of cognate help, contribute to the shaping of the Treg repertoire. ***The Journal of Immunology*****, 2014, 193: 170–176.**

The thymus is the central anatomical site for the differentiation and selection of T cells. Generation of productive T cells restricted to the host's MHC is directed by the strength of cognate interactions between the TCR and MHC molecules expressed by intrathymic APCs. T cells that express the transcription factor Foxp3 provide a necessary regulatory function required for the maintenance of immune homeostasis (1). The majority of regulatory T cells (Tregs) are generated within the thymus, where cognate TCR–MHC interactions directed by intrathymic APCs play a deterministic role in shaping the resulting Treg repertoire (2, 3). Among the thymic APC populations, presentation of tissue-specific Ags in an AIRE-regulated manner by medullary thymic epithelial cells (mTECs) is critical for Treg selection (1, 4). Further to this, dendritic cells (DCs), through the capture and shuttling of peripheral Ags to the thymic medulla, also play a distinct role in shaping the size and repertoire of thymic Treg cells (5). It is of interest that the thymus also contains other cell populations with APC activity including B cells, but their respective role in thymic Treg development is less clear.

Cells expressing typical B cell markers IgM, CD19, CD20, and CD22 can be found in the normal human fetal, postnatal, and adult thymus (6, 7). Human thymic B cells exhibit an activated phenotype and accumulate within the medulla, particularly around Hassall's corpuscles, but also in the perivascular and intralobular spaces (6, 8). Thymic B cells comprise a minor, but discernible, population in mice, appearing in the thymus during early fetal development and reaching a stable frequency by birth, therein maintained throughout adult life (9–11). The particular anatomical location of B cells within the thymic medulla suggests a functional role in T cell selection. Evidence that B cells can play a role in T cell selection first came to light through rodent studies examining minor lymphocyte-stimulating (Mls) Ags encoded by the M locus (12). In this case, clones reactive to specific Mls Ags, such as T cells bearing the Vβ6 TCR element reactive for Mls-1a, are deleted by B cells in the thymus (13–15). Subsequent studies extended this concept to other model Ags (16, 17) and so demonstrate that B cells can contribute to the shaping of the T cell repertoire through negative selection. Whether B cells can also participate in other thymic events such as the selection of Tregs is unclear.

In contrast to normal conditions in which B cells comprise a minor thymic population, expanded thymic B cell numbers have been reported for human subjects with autoimmune conditions such as myasthenia gravis and systemic lupus erythematosus (SLE) (8, 18–21). Similar findings have been reported for autoimmune prone MRL lpr/lpr and (NZB × NZW) F1 mice (9, 22, 23). Paradoxically, some clinical studies indicate abnormalities in Tregs in SLE that include increased Foxp3$^+$ cells (24, 25). Overexpression of BAFF, as in BAFF-transgenic (BAFF-Tg) mice, results in the expansion and extrasplenic distribution of B2 cell subsets concordant with the development of autoimmunity, sharing pathological features with SLE and Sjögren's syndrome (26, 27). BAFF-Tg mice also show an increased frequency and number of Foxp3$^+$ Treg cells, which can suppress potent T cell responses (28). Significantly, the expansion of Tregs in BAFF-Tg

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S.N.W. performed the experiments, analyzed data, and cowrote the manuscript; K.E.W. performed thymic histology and analysis; S.D. performed the Ki-67 experiments; and S.T.G. designed the study, analyzed data, and cowrote the manuscript.

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Abbreviations used in this article: BAFF-Tg, transgenic expression of BAFF; B.M., bone marrow; DC, dendritic cell; Mls, minor lymphocyte-stimulating; μMT$^{-/-}$, B cell–deficient; mTEC, medullary thymic epithelial cell; SLE, systemic lupus erythematosus; Treg, regulatory T cell; WT, wild-type.

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mice is B cell dependent. In this study, we show that in BAFF-Tg mice, B cells accumulate in the medullary region of the thymus and engender the development of thymic Tregs. Treg expansion in BAFF-Tg mice requires B cell surface MHC class II (MHC II) and an intact BCR repertoire, indicating thymic B cells provide cognate help to engender Treg development. B cells have emerged as potent immune regulators (29) in the contexts of intestinal inflammation (30, 31), autoimmunity (32, 33), and organ transplantation (28, 34). This work exposes a novel role for thymic B cell involvement in Treg development as a means to generate T cell tolerance.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from the Animal Resource Centre (Perth, Australia). B cell–deficient ($\mu MT^{-/-}$) mice were purchased from The Jackson Laboratory. BAFF-Tg mice, MHC $II^{-/-}$ mice, MD4 mice, and Foxp3-DTR mice housed under conventional barrier protection and handled in accordance with the Garvan Institute of Medical Research and St. Vincent's Hospital animal experimentation and ethics committee, which complies with the Australian code of practice for the care and use of animals for scientific purposes.

Flow cytometry

Cell suspensions of spleen 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% Na azide using the following Abs (obtained from BD Pharmingen unless otherwise stated): CD25, BrdU, IgM, B220, CD4, Foxp3 (eBioscience and BioLegend), Helios (BioLegend), CD1d, CD23, CD21 conjugated to PE, APC, FITC, PerCP, or biotin.

B cell isolation

Splenic B cells were isolated via magnetic separation using a MACS B cell isolation kit (Miltenyi Biotec) and an AUTOMACS system according to the manufacturer's instructions.

Bone marrow chimeras

Mice were sublethally irradiated with 2 doses of 600 rad with 2 h of resting between doses. Twenty-four hours later, mice were reconstituted (i.v.) with 5×10^6 bone marrow (B.M.) cells. (Isolated B cells were also injected i.v. when reconstituting with $\mu MT^{-/-}$ B.M.)

Treg proliferation

BrdU 200 μl (10 mg/ml) was injected i.p. into WT and BAFF-Tg mice five times at 12-h intervals, splenocytes were isolated, and BrdU uptake was examined by flow cytometry.

Treg survival

GFP-expressing splenocytes were injected i.v. into WT and BAFF-Tg mice. Spleens were analyzed, and GFP splenocytes were recovered by flow cytometry day 15 postinjection.

Treg conversion

($Foxp3^-$) $CD25^-CD4^+$ T cells from Foxp3-DTR were FACS sorted and injected i.v. into WT and BAFF-Tg mice, and GFP^+ ($Foxp3^+$) splenocytes were then recovered by flow cytometry day 15 postinjection.

Histology

Thymi from BAFF-Tg and age-matched C57BL/6 mice were embedded in OCT (Sakura) and frozen. Sections (6–8 μm) were cut using a Leica CM1900 cryostat (Leica Microsystems), air dried, and fixed in acetone. After blocking (30% horse serum), sections were incubated with primary Abs, followed by secondary reagents and DAPI (Invitrogen). Where required, normal rat serum was used to block remaining reactive sites prior to incubation with biotinylated reagents. B cells were detected with anti-CD19 biotin (eBioscience; eBio1D3) and streptavidin Cy3 (Jackson ImmunoResearch Laboratories) or anti-CD19–purified (eBio1D3) and anti-rat Cy2 (Jackson ImmunoResearch Laboratories); Tregs with anti-Foxp3 biotin (eBioscience; FJK-16s) and streptavidin Cy3; and mTECs with anti–MTS-10 Ab (gift from Richard Boyd, Monash University) and anti-rat Cy2 (15, 35). After final washing and mounting with fluorescent mounting medium (DakoCytomation), slides were examined with a Zeiss Axiovert 200M microscope (Carl Zeiss).

Statistics

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

Results

Increase in BAFF-Tg Tregs is not due to peripheral conditions

BAFF-Tg mice have expanded B cells that engender increased peripheral $CD4^+$ $Foxp3^+$ T cells (28). However, it is not known whether Treg expansion occurs in the periphery or centrally in the thymus. To further investigate the mechanism for increased Tregs, we examined Treg proliferation, survival, and conversion rates in the periphery. The potential for increased proliferation was assessed by determining the percentage of $BrdU^+CD4^+CD25^+$ cells in the periphery of WT and BAFF-Tg mice after five injections of 10 mg/ml BrdU at 12-h intervals. It was found that BAFF-Tg Tregs did not have increased proliferation compared with WT Tregs with the median $BrdU^+$ cell frequency being 2.0 versus 2.1%, respectively ($n \geq 5$; $p = 0.2592$) (Fig. 1A). This finding was verified by flow cytometric analysis of Ki-67 levels in BAFF-Tg and WT splenic $CD4^+Foxp3^+$ T cells, in which no difference in the frequency of Ki-67^+ Tregs was observed (data not shown).

To assess the persistence of peripheral Tregs in BAFF-Tg mice, GFP-expressing splenocytes were adoptively transferred to BAFF-Tg or WT mice, and the frequency of splenic GFP-expressing $CD25^+$ cells remaining was analyzed. In this case, no difference was found between the persistence of GFP-expressing $CD4^+$ $CD25^+$ T cells in either BAFF-Tg or WT mice, the median value being 3.1% in WT mice and 3.8% in BAFF-Tg mice ($n \geq 5$; $p = 0.8245$) (Fig. 1B). BAFF can promote B cell survival (36), and consistent with this, the survival of GFP-expressing $B220^+IgM^+$ B cells was enhanced in BAFF-Tg mice compared with WT mice, the frequency of GFP-expressing B cells being 52.2 versus 32.3%, respectively ($n = 4$; $p = 0.0246$) (Fig. 1C). Thus, BAFF is not acting as an acute trophic factor enhancing Treg persistence in the periphery.

To assess a role for increased Treg conversion in the periphery, FACS-sorted GFP^- ($Foxp3^-$) $CD25^-CD4^+$ T cells from Foxp3-DTR (37) mice were adoptively transferred into WT and BAFF-Tg mice. GFP^+ ($Foxp3^+$) splenocytes were then recovered. There was no difference between the conversion of GFP^- ($Foxp3^-$) $CD25^-CD4^+$ T cells to GFP^+ $Foxp3^+$ Tregs in WT mice versus BAFF-Tg mice, the median value being 1.1% in WT mice and 0.8% in BAFF-Tg ($n = 5$; $p = 0.2463$) (Fig. 1D). These data show that the expansion of Tregs in BAFF-Tg is not due to peripheral mechanisms, suggesting a thymic origin for the increase.

Increased thymic Tregs in BAFF-Tg mice

Helios, a member of the Ikaros transcription factor family, is preferentially expressed by thymic Tregs, such that the ratio of $Helios^+$ to $Helios^-$ Tregs in the periphery is ~3:1 in WT mice (38). Therefore, changes in the output of thymic Tregs versus the peripheral generation would be reflected in an altered ratio of peripheral $Helios^+/Helios^-$ $Foxp3^+CD4^+$ T cells. We found for BAFF-Tg mice that the ratio of peripheral $Helios^+$ Tregs/$Helios^-$ Tregs was increased from ~3:1 to ~10:1 when compared with WT mice ($n = 6$) (Fig. 2A). Analysis of thymic single-positive $CD4^+$ $Foxp3^+$ Treg frequencies revealed that BAFF-Tg mice harbored a ~2-fold increase in thymic Tregs compared with WT mice with the median being 8.5% versus 3.9% ($n = 6$; $p = 0.0017$). The increased frequency of $Foxp3^+$ cells was due to ~3.5-fold increase

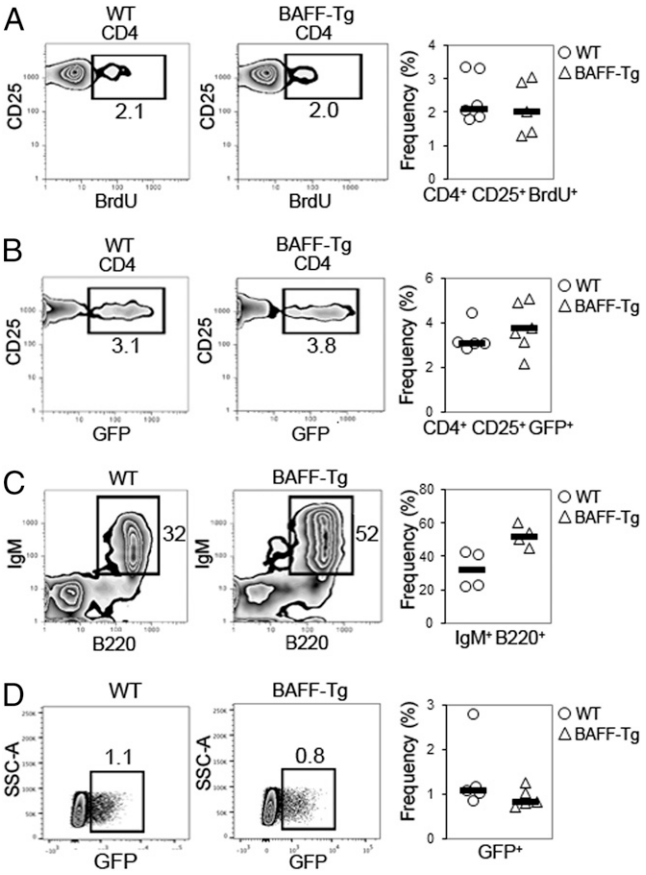

FIGURE 1. Treg expansion in BAFF-Tg mice is not due to peripheral conditions. (**A**) Representative flow cytometry plots and cumulative data of frequency of proliferating splenic $CD4^+CD25^+BrdU^+$ Treg cells in WT ($n = 6$) and BAFF-Tg mice ($n = 5$); NS, $p = 0.2592$ from three individual experiments. (**B**) Representative flow plots and cumulative data of frequency of surviving splenic $CD4^+CD25^+GFP^+$ Tregs in WT ($n = 5$) and BAFF-Tg mice ($n = 6$); NS, $p = 0.8245$ from three individual experiments. (**C**) Representative flow plots and cumulative data of frequency of surviving splenic $IgM^+B220^+GFP^+$ B cells in WT ($n = 4$) and BAFF-Tg mice *(n = 4)*; $p = 0.0246$ from two individual experiments. (**D**) Representative flow plots and cumulative data of frequency of converted GFP^+ Tregs in WT ($n = 5$) and BAFF-Tg mice ($n = 5$); NS, $p = 0.2463$ from two individual experiments.

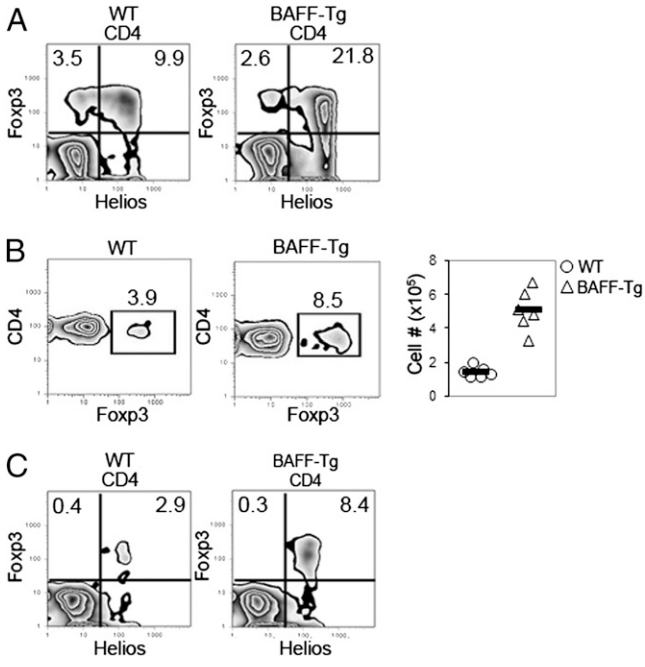

FIGURE 2. Expanded frequency and number of Thymic Tregs in BAFF-Tg mice. (**A**) Frequency of splenic $CD4^+Foxp3^+Helios^{+/-}$ lymphocytes in WT ($n = 6$) and BAFF-Tg mice ($n = 6$) from two individual experiments. (**B**) Frequency; $p = 0.0017$ and calculated numbers; $p = 0.0012$ of thymic $CD4^+$ $Foxp3^+$ Treg cells in WT ($n = 6$) and BAFF-Tg mice ($n = 6$) from three individual experiments. (**C**) Frequency of thymic $CD4^+$ $Foxp3^-$ $Helios^{+/-}$ thymocytes in WT ($n = 6$) and BAFF-Tg mice ($n = 5$) from three individual experiments.

in the absolute numbers of $Foxp3^+$ cells in BAFF-Tg mice, the median being 5.1×10^5 for BAFF-Tg compared with 1.4×10^5 for WT mice ($n = 6$; $p = 0.0012$) (Fig. 2B). To determine if the increase in intrathymic Tregs could be attributed to recirculating peripheral Tregs rather than increased thymic output, we examined the pattern of Helios expression on thymic Tregs. All thymic $Foxp3^+$ cells were $Helios^+$, both for BAFF-Tg but also WT mice, whereas there was little evidence of Helios-negative Tregs in either the WT or BAFF-Tg ($n \geq 5$) thymus. The presence of $Helios^-$ Tregs would have been indicative of recirculating Tregs (Fig. 2C). Thus, the expansion of peripheral Tregs in BAFF-Tg mice (28, 39) is due to increased thymic Tregs.

Thymic expansion of Tregs is B cell dependent

A number of mechanistic experiments were next conducted to determine if B cells play a role in the development of thymic Tregs. To establish a necessary requirement for B cells in the generation of increased thymic Tregs in BAFF-Tg mice, radiation B.M. chimeras comprising BAFF-Tg hosts transplanted with $\mu MT^{-/-}$ B.M. were generated. WT and BAFF-Tg hosts transplanted with WT B.M. were also generated as controls. The frequency of thymic $CD4^+Foxp3^+$ T cells in control B.M. chimeras at 10 wk post–B.M. transplant was 6.6% in WT B.M. → WT chimeras, whereas the frequency of thymic Tregs in WT B.M. → BAFF-Tg chimeras was increased ~3-fold to 17.7% ($n \geq 6$; $p = 0.0007$). The absolute numbers of $CD4^+Foxp3^+$ T cells were increased ~5-fold in WT B.M. → BAFF-Tg chimeras compared with WT B.M. → WT chimeras, the median number being 0.25×10^6 versus 1.28×10^6, respectively ($n \geq 6$; $p < 0.0001$) (Fig. 3A). In contrast, examination of the frequency of Tregs in $\mu MT^{-/-}$ B.M. → BAFF-Tg chimeras revealed that in the presence of BAFF, but in the absence of B cells, the frequency of thymic $CD4^+$ $Foxp3^+$ T cells was reduced by ~30% compared with WT B.M. → BAFF-Tg chimeras. Further, the frequency of Tregs in $\mu MT^{-/-}$ B.M. → BAFF-Tg chimeras was also decreased compared with WT B.M. → WT chimeras ($n \geq 4$; $p = 0.0043$) (Fig. 3A, 3B). These data show that in the absence of B cells, there is a reduction of thymic Tregs in BAFF-Tg mice and also that the presence of BAFF cannot rescue this reduction in the absence of B cells. To further test whether B cells might influence Treg numbers in WT mice, the frequency of thymic Tregs in $\mu MT^{-/-}$ mice was analyzed. It was found that the frequency of $CD4^+Foxp3^+$ T cells was reduced in $\mu MT^{-/-}$ mice compared with WT mice by ~1.7-fold, the median frequency being 2.3% for $\mu MT^{-/-}$ mice versus 3.9% in WT mice ($n \geq 4$; $p = 0.0226$). The absolute numbers of thymic $CD4^+Foxp3^+$ T cells in $\mu MT^{-/-}$ mice was also decreased; indeed, the median number of $CD4^+Foxp3^+$ T cells in $\mu MT^{-/-}$ mice was 0.6×10^5 compared with 1.4×10^5 in WT mice ($n \geq 4$; $p = 0.0095$) (Fig. 3C). These data demonstrate that the expansion of thymic Tregs is reduced in the absence of B cells in both WT and BAFF-Tg mice. Hence, B cells provide modulatory control over the homeostatic production of Tregs.

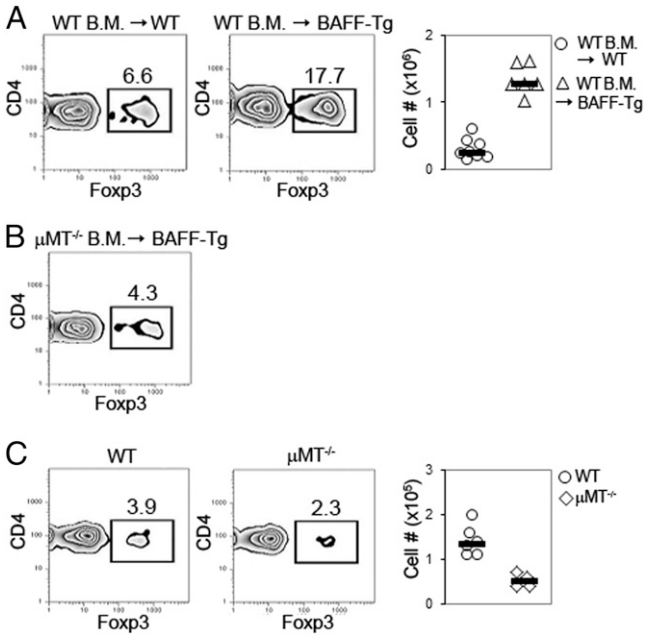

FIGURE 3. Expansion of thymic Tregs is B cell dependent. (**A**) Frequency of thymic CD4$^+$ Foxp3$^+$ Tregs in B.M. chimeras; WT B.M. → WT ($n = 8$) and WT B.M. → BAFF-Tg mice ($n = 6$); $p = 0.0007$ and calculated numbers $p < 0.0001$ from three individual experiments. (**B**) Frequency of thymic CD4$^+$Foxp3$^+$ Tregs in B.M. chimeras; $\mu MT^{-/-}$ B.M. → BAFF-Tg mice ($n = 4$) when compared with WT B.M. → WT mice ($n = 8$) (A); $p = 0.0043$ from two individual experiments. (**C**) Frequency $p = 0.0226$ and calculated numbers $p = 0.0095$ of thymic CD4$^+$Foxp3$^+$ Tregs in WT ($n = 6$) and $\mu MT^{-/-}$ mice ($n = 4$) from three individual experiments.

Characterization of resident thymic B cells in WT and BAFF-Tg mice

The B cell dependency of increased thymic Tregs in BAFF-Tg mice suggested an intrathymic role for B cells in Treg expansion. As reported (9, 40), we found that IgM$^+$B220$^+$ B cells inhabited the thymus of WT mice, albeit at low frequencies. Remarkably, BAFF-Tg mice harbored a ~7-fold increase in the frequency of resident thymic B cells compared with WT mice, the median being 2.1 versus 0.3%, respectively ($n \geq 7$; $p < 0.0001$). The increased frequency was due to an increase in the absolute numbers of resident thymic B cells in BAFF-Tg mice, an increase of ~16-fold with BAFF-Tg mice having a median of 5.2×10^4 and WT mice having a median of 0.3×10^4 ($n \geq 6$; $p < 0.0003$) (Fig. 4A). Using the markers IgM, B220, CD21, and CD23 to analyze these resident thymic B cells further, we could identify distinct intrathymic B cell subpopulations, namely B220$^+$IgM$^+$ CD23^{hi}CD21^{int} cells; B220$^+$IgM$^+$CD23^{lo}CD21^{lo} cells; and a population of B220$^+$IgM$^+$CD23^{lo}CD21^{hi} cells (Fig. 4B, 4C). Thus, the normal thymus harbors a complex niche of distinct B cell subsets, which may imply distinct roles in T cell selection.

B cells accumulate in the thymic medulla associated with mTECs and Tregs

To assess the anatomical location of the resident thymic B cells histological sections of the thymus were stained with an mAb to CD19. In the WT thymus, CD19$^+$ cells could be found scattered throughout the medulla, but less evidence for significant cortical localization could be observed (Fig. 4D). In the BAFF-Tg thymus, CD19$^+$ cells were also restricted to the thymic medulla. However, in the case of the BAFF-Tg thymus, CD19$^+$ cells exhibited a tendency to accumulate in clusters within the thymic medulla

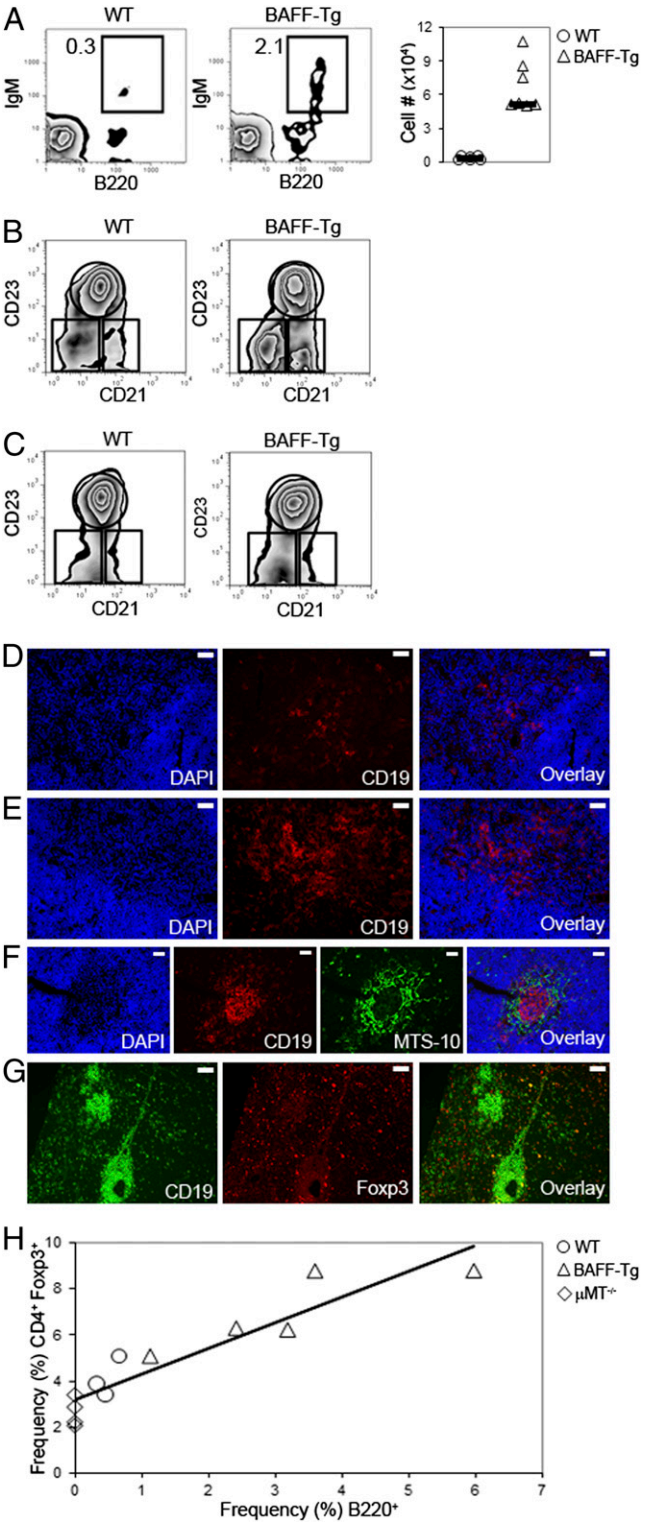

FIGURE 4. B cell populations in the thymus of BAFF-Tg mice. (**A**) Frequency; $p < 0.0001$ and calculated numbers; $p = 0.0003$ of thymic IgM$^+$ B220$^+$ B cells in WT ($n = 6$) and BAFF-Tg mice ($n = 7$) from three individual experiments. (**B**) B cell subsets in the spleen of WT and BAFF-Tg mice. (**C**) B cell subsets in the thymus of WT and BAFF-Tg mice. (**D**) Histological analysis of location of WT thymic CD19$^+$ B cells (scale bars, 100 μm). (**E**) Histological analysis of location of BAFF-Tg thymic CD19$^+$ B cells (scale bars, 100 μm). (**F**) Histological analysis of location of BAFF-Tg thymic CD19$^+$ B cells in relation to MTS-10$^+$ mTECs (scale bars, 100 μm). (**G**) Histological analysis of location of BAFF-Tg thymic CD19$^+$ B cells in relation to Foxp3$^+$ Tregs (scale bars, 200 μm). (**H**) Correlation of thymic Tregs and thymic B cells; $y = 1.1112x + 3.2019$; $r = 0.8573$.

(Fig. 4E, 4G). Using the marker MTS-10 to reveal mTECs, it could be seen that these B cell clusters inhabited anatomical locations adjacent to mTECs (Fig. 4F). Colabeling for Foxp3 and CD19$^+$ to highlight potential intrathymic B/Treg interactions revealed some CD19$^+$ cells juxtaposed to Foxp3$^+$ cells (Fig. 4G). Thus, B cells can be found inhabiting the thymic medulla in close proximity to thymic APCs and Tregs. Indeed, regression analysis comparing the numbers of B cells and Tregs in $\mu MT^{-/-}$, WT, and BAFF-Tg mice revealed a strong correlation ($r = 0.8573$) between the presence or absence of resident thymic B cells and the numbers of CD4$^+$Foxp3$^+$ Tregs (Fig. 4H).

Thymic B cells provide cognate help for the expansion of natural Tregs

Intrathymic Treg cell selection requires cognate interactions with peptide–MHC II complexes on the surface of thymic APCs (4, 41). We could show that resident thymic B cells express surface MHC II molecules, as well as the costimulatory molecules CD80 and CD86 (data not shown), indicating they have the required machinery for Treg selection. To assess whether B and T cell cognate interactions are critically involved in Treg development in BAFF-Tg mice, we generated radiation B.M. chimeras by which BAFF-Tg recipients received $\mu MT^{-/-}$ donor marrow plus an infusion of MHC $II^{-/-}$ donor B cells. The resultant mice lack MHC II on B cells but all other thymic populations remain MHC II^+, thereby eliminating the ability of B cells to present cognate peptide/MHC II complexes to CD4$^+$ T cells. The frequency of thymic CD4$^+$Foxp3$^+$ T cells in $\mu MT^{-/-}$ B.M. plus WT donor B cells → BAFF-Tg chimeras was 8.9%, whereas the frequency of thymic Tregs in $\mu MT^{-/-}$ B.M. plus MHC $II^{-/-}$ donor B cells → BAFF-Tg chimeras was reduced ~3-fold to 2.9% ($n = 5$; $p < 0.0001$) (Fig. 5A). Thus, in BAFF-Tg mice, expansion of thymic Tregs is dependent upon cognate B–T cell interactions provided by peptide/MHC II complexes. Further to this, in WT chimeras, the frequency of thymic B cells were equivalent, eliminating artifacts due to disparate thymic B cell frequencies (Fig. 5B); however, the frequency of thymic CD4$^+$Foxp3$^+$ T cells was reduced 1.4-fold to ~2.4% ($n \geq 4$; $p = 0.0141$) in WT chimeras receiving MHC $II^{-/-}$ B cells as compared with WT chimeras receiving WT B cells (Fig. 5C). These data demonstrate that B cells can influence thymic Treg development through the provision of cognate help in both BAFF-Tg, as well as WT mice.

An intact BCR repertoire is required for Treg expansion

To assess the role of Ag specificity in the expansion of Tregs in BAFF-Tg mice, radiation B.M. chimeras were generated by which BAFF-Tg were reconstituted with donor marrow from MD4-Tg mice in which B cells express the monoclonal hen egg lysozyme–specific BCR (42). The resultant mice would therefore lack an intact and diverse BCR repertoire required for Treg generation by intrathymic B cells.

The frequency of thymic CD4$^+$Foxp3$^+$ T cells in WT B.M. → BAFF-Tg chimeras was 12.9%, whereas the frequency of thymic Tregs in MD4-Tg B.M. → BAFF-Tg chimeras was reduced ~8.5-fold to 1.5% ($n = 5$; $p < 0.0001$) (Fig. 5D). Thus, in BAFF-Tg mice, expansion of thymic Tregs is dependent upon an intact BCR repertoire. Further to this, in WT chimeras, the frequency of thymic B cells was equivalent, eliminating artifacts due to disparate thymic B cell frequencies (Fig. 5E). However, the frequency of thymic CD4$^+$ Foxp3$^+$ T cells was reduced ~1.7-fold to ~1.82% ($n \geq 5$; $p = 0.0066$) in WT chimeras receiving MD4-Tg B.M. as compared with WT chimeras receiving WT B.M. (Fig. 5F). These data demonstrate that B cells can influence thymic Treg development in a process dependent upon the provision of a diverse range of BCR signaling in both BAFF-Tg as well as WT mice.

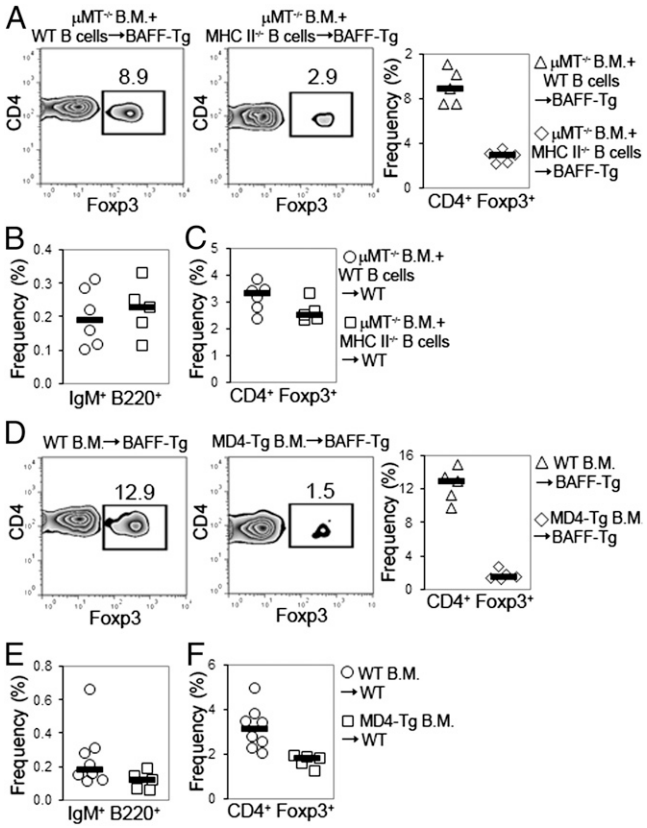

FIGURE 5. Expansion of Tregs in mice is dependent on direct interaction with B cells. (**A**) Representative flow cytometry plots and cumulative frequency of thymic CD4$^+$Foxp3$^+$ Tregs in B.M. chimeras; $\mu MT^{-/-}$ B.M. + WT donor B cells → BAFF-Tg and $\mu MT^{-/-}$ B.M. + MHC $II^{-/-}$ donor B cells → BAFF-Tg mice ($n = 5$); $p \leq 0.0001$ from two individual experiments. (**B**) Frequency of thymic B cells in B.M. chimeras; $\mu MT^{-/-}$ B.M. + WT donor B cells → WT ($n = 6$) and $\mu MT^{-/-}$ B.M. + MHC $II^{-/-}$ donor B cells → WT mice ($n = 5$); NS, $p = 0.6573$ from two individual experiments. (**C**) Frequency of thymic CD4$^+$ Foxp3$^+$ Treg cells in B.M. chimeras; $\mu MT^{-/-}$ B.M. + WT donor B cells → WT ($n = 6$) and $\mu MT^{-/-}$ B.M. + MHC $II^{-/-}$ donor B cells → WT mice ($n = 5$); $p = 0.0141$ from two individual experiments. (**D**) Representative flow plots and cumulative frequency of thymic CD4$^+$ Foxp3$^+$ Tregs in B.M. chimeras; WT B.M. → BAFF-Tg and MD4-Tg → BAFF-Tg mice ($n = 5$); $p \leq 0.0001$ from two individual experiments. (**E**) Frequency of thymic B cells in B.M. chimeras; WT B.M. → WT ($n = 8$) and MD4-Tg B.M. → WT mice ($n = 5$) are not statistically different between groups; $p = 0.1254$ from two individual experiments. (**F**) Frequency of thymic CD4$^+$Foxp3$^+$ Treg cells in B.M. chimeras; WT B.M. → WT ($n = 8$) and MD4-Tg B.M. → WT mice ($n = 5$); $p = 0.0066$ from two individual experiments.

Discussion

Our data raise important questions regarding the role of thymic B cells in the selection of the Treg repertoire under steady-state conditions. Natural Tregs develop in the thymus, most likely selected via engagement with a strong agonist peptide that would otherwise cause deletion (43). Although some studies have raised the possibility that Treg selection may begin in the cortex (44, 45), the majority favor the view that the thymic medulla is the site where Treg precursors mature after contact with intrathymic APCs (2, 4, 46). In this study, we were able to identify B cells within the thymic medulla in close proximity with mTECs and also Tregs, suggesting that thymic B cells were involved in Treg maturation.

TCR specificity has an important role in the thymic selection of Tregs (2, 46) suggesting that the nature of APCs encountered within the thymus dictate the shaping of the Treg repertoire (47). The thymus harbors a network of APCs including cortical TECs, mTECs, and DCs. Evidence indicates these cells could act coordinately to control T cell development, as deletion of MHC II from either B.M.-derived cells or thymic epithelial cells still allows Treg development (41, 48, 49). The question remains whether the diverse range of intrathymic APCs make individual and specific contributions to the development of Tregs with unique specificities (47). TECs, through the stochastic expression of tissue-specific Ags regulated by AIRE and via intrathymic autophagy (4, 50), would most likely contribute different Ags than peripheral APCs. The thymus contains a large population of plasmacytoid DCs and SIRPα DCs that are derived from the periphery (51), which consequently would be expected to capture peripheral Ag and then migrate to present peripheral Ags for Treg selection (5, 47). B cells would represent excellent candidates for the presentation of peripheral Ags to nascent T cells within the thymus. B cells express a high density of MHC II and costimulatory molecules (52) necessary for Treg development. Ab-mediated Ag capture is characterized by enhanced endocytosis and accelerated delivery to endocytic processing and MHC II loading compartments facilitating Ag-specific presentation (53). Indeed, as shown in this study, absence of B cell–expressed MHC II resulted in reduced thymic Treg output. Further, the diverse repertoire of the BCR and the specificity of Ab-mediated Ag capture would allow for the capture of important cryptic and neo-Ags that otherwise may be present in limiting quantities. Our data support a model in which B cells capture peripheral Ags through their BCR and subsequently accumulate in the thymic medulla to present these captured Ags via surface MHC II complexes to nascent Tregs and suggest a mechanism by which B cells could potentially contribute to the shaping of the Treg repertoire.

It is interesting to consider that in BAFF-Tg mice, a model of lupus and Sjrogren's syndrome (36), B cells colonize the medullary region of the thymus and exert a marked influence on the development of Tregs. This may reflect the situation whereby B cells can normally engender Tregs, but in the case of BAFF-Tg mice with increased numbers of B cells, this function is correspondingly enhanced. A characteristic feature of BAFF-Tg mice is the increased numbers of circulating B cells with a phenotype like that of splenic marginal zone B cells (54), a prominent cell type in other autoimmune prone mouse strains (55), and a subset of human subjects with Sjrogren's syndrome (27). Further to this, B cells have been shown to colonize the thymus in excessive numbers in autoimmune disease including myasthenia gravis and lupus (8, 19–21). Of interest, some clinical studies indicate abnormalities in Tregs in lupus that include increased Foxp3$^+$ cells (24, 25). These observations raise a paradox with regards the role of Tregs in autoimmune diseases. One possibility is that increased Tregs represent a feedback response to reign in B cell autoimmunity.

The thymic Tregs engendered by B cells in BAFF-Tg mice are able to suppress powerful T cell responses including rejection of pancreatic islets and skin allografts (28). Thus, BAFF-Tg mice are effectively immunologically tolerant of allografts. Recently, a role for B cells in the maintenance of tolerance to clinical organ transplants emerged. Reports from transplant trials, in which subjects maintained stable graft function after stopping immunosuppression termed operationally tolerant, displayed a B cell signature including elevated numbers of circulating B cells in the periphery (34, 56). Major features of tolerant grafts included high expression of CD20 but also increased frequencies of transitional B cells (34). B cells could facilitate graft tolerance by inducing allograft-specific Tregs across an MHC barrier (57, 58), a mechanism that may be of significance in the context of graft-versus-host disease. Though the mechanism by which B cells could provide immunological tolerance to solid-organ grafts in the clinic are as yet unidentified, our data with BAFF-Tg mice suggests that B cells can accumulate within the thymus, where they can present cognate MHC–Ag complexes to engender thymic Tregs, mechanisms previously associated with DCs (5, 41).

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Disclosures

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