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B Cell and T Cell Dissimilarities in BAFF-Deficient versus BR3-Deficient C57BL/6 Mice

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BAFF is a potent B cell survival and differentiation factor with three receptors, TACI, BCMA, and BR3. B cells are greatly reduced in BAFF-deficient mice, and among mice deficient in a single BAFF receptor, B cell reduction is characteristic only of BR3-deficient mice. Nevertheless, there may be important differences between BR3-deficient mice, in which interactions between BAFF and only BR3 are abrogated, and BAFF-deficient mice, in which interactions between BAFF and all its receptors are abrogated. We demonstrate that: 1) the numbers of $CD19^+$ cells in C57BL/6 (B6).*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice diverge as the mice age; 2) the distribution of B cell subsets significantly differ between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice regardless of age or sex; 3) the relationships of $CD3^+$ and $CD4^+$ cells to B cells vastly differ between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice as a function of age and sex; 4) the numbers and percentages of $CD4^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+$ are greater in B6.*Baff*$^{-/-}$ mice than in B6.*Br3*$^{-/-}$ mice; and 5) for any given number of $CD19^+$ cells or $CD4^+$ cells, percentages of $Foxp3^+$ cells and $CD4^+CD25^+Foxp3^+$ cells are lower in B6.*Br3*$^{-/-}$ mice than in B6.*Baff*$^{-/-}$ mice, with proliferation of these cells being greater, and survival being lesser, in B6.*Br3*$^{-/-}$ mice than in B6.*Baff*$^{-/-}$ mice. Collectively, these observations raise the possibility that interactions between TACI and/or BCMA and BAFF modulate expression of B cell subsets and $Foxp3^+$ cells and may help explain prior enigmatic observations of autoimmunity and autoimmune disease in mice despite the absence of functional engagement of BR3 by BAFF. ***The Journal of Immunology*****, 2022, 209: 2133–2140.**

The B cell-activating factor of the TNF family (BAFF) (also known as BLyS), a 285-aa member of the TNF superfamily, is best known for its ability to promote B cell survival and differentiation (1–4). B cells are greatly reduced in BAFF-deficient mice (5, 6) and in mice and humans treated with a BAFF antagonist (7–11).

BAFF has three receptors: BCMA, TACI, and BR3 (12–15). TACI and BR3 are each broadly expressed on mature B cells, whereas BCMA expression is largely limited to terminally differentiated plasmablasts/plasma cells (16–19).

Single deficiency of the individual BAFF receptors in C57BL/6 (B6) mice yields markedly divergent phenotypes. B6.*Bcma*$^{-/-}$ mice display a near-normal phenotype, with normal numbers of lymphocytes and lymphocyte subsets, normal in vitro function of these cells, and no in vivo immunodeficiency other than a relative decrease in Ag-specific long-lived Ig-secreting plasma cells following immunization (6, 20, 21). B6.*Taci*$^{-/-}$ mice harbor increased numbers of B cells, and these mice as they age may develop elevated circulating titers of autoantibodies (22–24). In contrast, B6.*Br3*$^{-/-}$ mice resemble B6.*Baff*$^{-/-}$ mice, at least superficially, with marked reductions in spleen B cells, mature recirculating B cells, and baseline and Ag-induced serum IgG levels (25, 26). This is not surprising, because BR3 has been ascribed an indispensable role in BAFF-driven B cell survival (25–27).

The reported similar phenotypes of B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice notwithstanding, BR3 deficiency is not identical to BAFF deficiency. In a BR3-deficient state, BAFF interactions with its other receptors, TACI and BCMA, are not abrogated, whereas in a BAFF-deficient state, interactions between BAFF and all its receptors are abrogated. In this report, we document that striking differences exist between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice in B cell subsets, $Foxp3^+$ cells, and relationships between B cells and $CD3^+$ and $CD4^+$ cells. We speculate that at least some of these differences may play important roles in the development of, or protection from, autoimmunity and autoimmune disease.

Materials and Methods

General

All reported studies were approved by the University of Southern California Institutional Animal Care and Use Committee.

Mice

All mice used in this study bore the identical *Foxp3-gfp* knock-in. B6 mice bearing a *Foxp3-gfp* knock-in (28) were intercrossed with B6.*Baff*$^{-/-}$ mice (6) and B6.*Br3*$^{-/-}$ mice (26) to yield the respective mice bearing the *Foxp3-gfp* knock-in.

Genotyping was monitored by PCR. Because the *Foxp3* gene is located on the X chromosome, male mice were hemizygous for the *Foxp3-gfp* knock-in, whereas females were bred to homozygosity for the *Foxp3-gfp* knock-in. All mice were housed in the same specific pathogen-free room. Mice 2 to 3 mo of age were designated as "young," and mice ≥6 months of age were designated as "old."

Cell surface staining

Spleen mononuclear cells were stained with fluorochrome-conjugated mAb-specific for CD3, CD4, CD8, CD19, CD21, CD23, and CD25 (BioLegend or BD Pharmingen) and analyzed by FACS. B cells were defined as $CD19^+$, follicular (FO) B cells as $CD19^+CD21^+CD23^+$, marginal zone (MZ) B cells as $CD19^+CD21^{hi}CD23^{lo}$, and age-associated B cells (ABCs) as $CD19^+CD21^-CD23^-$.

In vivo proliferation of $Foxp3^+$ cells

Spleen mononuclear cells were surface stained for CD4, fixed and permeabilized, and stained with allophycocyanin-conjugated anti–Ki-67 mAb. Control samples were identically treated, substituting Armenian hamster IgG isotype

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; ABC, age-associated B cell; B6, C57BL/6; FO, follicular; MZ, marginal zone; WT, wild-type.

control mAb for the anti–Ki-67 mAb (BioLegend). Cells were gated on $CD4^+Foxp3^+$ cells and analyzed for Ki-67 expression.

In vivo survival of Foxp3$^+$ cells

Spleen mononuclear cells were stained for CD4 and stained with the APC Annexin V Apoptosis Detection Kit with 7-aminoactinomycin D (7-AAD; BioLegend) according to the manufacturer's instructions. Cells were gated on $CD4^+Foxp3^+$ cells and analyzed for Annexin V binding and 7-AAD inclusion.

Statistical analysis

All analyses were performed using SigmaPlot software (SYSTAT). Parametric testing was performed by the unpaired Student *t* test. When the data were normally distributed but the equal variance test was not satisfied, the Welch *t* test was performed. When the data were not normally distributed, nonparametric testing was performed by the Mann–Whitney rank sum test. Because the residuals were not normally distributed and/or the variances were not constant, correlations were calculated using Spearman rank order correlation.

Results

*B cell subsets in B6 wild-type, B6.*Baff$^{-/-}$*, and B6.*Br3$^{-/-}$ *mice*

Because sex and age can affect the numbers and percentages of individual lymphocyte populations (28), we analyzed cohorts of young male, young female, old male, and old female B6 wild-type (WT), B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice. Among B6 WT mice, B ($CD19^+$) cells were numerically greater in male mice than in female mice for each age group (young and old) and were numerically greater in young mice than in old mice for each sex group (male and female), although statistical significance was not achieved for any comparison ($p \geq 0.189$). Among B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice, $CD19^+$ cells were numerically greater in female mice than in male mice, with differences achieving statistical significance in old B6.*Baff*$^{-/-}$ mice ($p < 0.001$) (Table I). B cells were also numerically greater in young B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice than in the corresponding old mice, with the differences being statistically significant in male B6.*Baff*$^{-/-}$ mice ($p = 0.006$), male B6.*Br3*$^{-/-}$ mice ($p < 0.001$), and female B6.*Br3*$^{-/-}$ mice ($p < 0.001$).

Consistent with previous reports (5, 6, 14, 15), B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice each harbored markedly fewer numbers (≥74% reduction) and percentages (≥58% reduction) of $CD19^+$ cells than did B6 WT mice, regardless of the age or sex of the mice ($p < 0.001$ for each comparison) (Figs. 1, 2A, 2B). The marked reductions in $CD19^+$ cells in both B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice notwithstanding, their B cell profiles diverged. $CD19^+$ cell numbers were modestly lower in B6.*Br3*$^{-/-}$ mice than in B6.*Baff*$^{-/-}$ mice, with the differences between the two achieving statistical significance in old male and old female mice (Table I). Dramatically and unexpectedly, the reductions in B cells were not equal across discrete B cell subpopulations. Whereas FO and MZ B cells as percentages of B cells were lower in B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice than in B6 WT mice, percentages of ABCs were higher, and the B cell subset distributions were remarkably similar in all age/sex cohorts (Figs. 1, 2C–E). Moreover, numbers of FO B cells and ABCs differed between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice in all age/sex cohorts, with FO B cells being greater in B6.*Br3*$^{-/-}$ mice and ABCs being greater in B6.*Baff*$^{-/-}$ mice. Furthermore, numbers of MZ B cells were greater in young male, young female, and old female B6.*Br3*$^{-/-}$ mice than in the corresponding B6.*Baff*$^{-/-}$ mice (Table I).

*$CD3^+$ and $CD4^+$ cells in B6 WT, B6.*Baff$^{-/-}$*, and B6.*Br3$^{-/-}$ *mice*

It is well known that $CD4^+$ T cells play a central role in facilitating and promoting B cell activation, proliferation, and differentiation. Accordingly, we assessed not only B cells and B cell subsets, but also assessed $CD3^+$ and $CD4^+$ cells and their associations with B cells and B cell subsets.

In B6 WT mice, numbers of $CD3^+$ cells were similar across all age/sex cohorts ($p \geq 0.385$ for each comparison). Young male and female mice each harbored modestly greater (29.0–38.8%) numbers of $CD4^+$ cells than did the corresponding old mice ($p \leq 0.043$), whereas no differences were appreciated between males and females among young or old mice ($p \geq 0.266$). In each age/sex cohort, numbers of $CD3^+$ and $CD4^+$ cells strongly correlated with numbers of $CD19^+$ cells ($r \geq 0.714$; $p \leq 0.002$ for each comparison). Numbers of $CD3^+$ and $CD4^+$ cells also strongly correlated with numbers of FO B cells and MZ B cells in each age/sex cohort, whereas correlations with ABCs were not as strong, although they remained statistically significant in young females, old males, and old females but not in young males (Table II).

Numbers of $CD3^+$ and $CD4^+$ cells in young and old female B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice were each similar to those in the corresponding B6 WT mice ($p \geq 0.070$ for each comparison), whereas young and old male B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice harbored fewer numbers of $CD3^+$ and $CD4^+$ cells than did the corresponding B6 WT mice ($p \leq 0.020$ for each comparison). In addition, modest numerical differences in $CD3^+$ and $CD4^+$ cells between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice were noted in all age/sex cohorts, with the differences achieving statistical significance in old females (Table I).

*Relationships between $CD3^+$ and $CD4^+$ cells on one hand and B cells and B cell subsets on the other hand in B6 WT, B6.*Baff$^{-/-}$*, and B6.*Br3$^{-/-}$ *mice*

Numbers of $CD3^+$ and $CD4^+$ cells each strongly correlated in both B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice with numbers of $CD19^+$ cells in young males, young females, and old males, whereas the correlations were uniformly weaker in old females, with statistical significance being achieved only between $CD19^+$ cells and $CD3^+$ cells in B6.*Br3*$^{-/-}$ mice (Table II). In sharp contrast, the relationships in B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice between $CD3^+$ and $CD4^+$ cells, on one hand, and FO B cells, MZ B cells, and ABCs, on the other hand, frequently diverged. In B6.*Baff*$^{-/-}$ mice, $CD3^+$ and $CD4^+$ cells correlated significantly with FO and MZ B cells in young female, but not young male, mice, whereas in B6.*Br3*$^{-/-}$ mice, the correlations were significant in young male, but not young female, mice (Table II). Moreover, $CD3^+$ and $CD4^+$ cells correlated significantly with FO B cells in old female B6.*Baff*$^{-/-}$ mice but not in old female B6.*Br3*$^{-/-}$ mice. Strikingly, $CD3^+$ and $CD4^+$ cells in young male, young female, and old male B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice strongly correlated with ABCs, but $CD3^+$ and $CD4^+$ cells failed to correlate with ABCs old female B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice (Table II).

*Foxp3$^+$ cells in B6 WT, B6.*Baff$^{-/-}$*, and B6.*Br3$^{-/-}$ *mice*

Pronounced differences between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice in $Foxp3^+$ cells were also observed. Whereas no statistically significant differences in percentages or numbers of $Foxp3^+$ cells were appreciated across young female B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice ($p \geq 0.155$ for all comparisons), statistically significant differences emerged as B6.*Br3*$^{-/-}$ females aged, with the percentage and number of $Foxp3^+$ cells in old female B6.*Br3*$^{-/-}$ mice being lower not only than those in old female B6 WT mice, but also lower than those in old female B6.*Baff*$^{-/-}$ mice as well ($p < 0.001$ for all comparisons) (Table III). Whereas the percentages of $Foxp3^+$ cells increased with age in B6 WT and B6.*Baff*$^{-/-}$ females ($p < 0.001$ for each comparison), the percentage of $Foxp3^+$ cells did not increase with age in B6.*Br3*$^{-/-}$ females but actually *decreased* numerically with age ($p = 0.315$).

In male mice, differences were already apparent at a young age. The number of $Foxp3^+$ cells in young male B6.*Baff*$^{-/-}$ mice and

Table I. Spleen cell populations in B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice

Age/Sex	Genotype	Cell Population					
		CD3$^+$	CD4$^+$	CD19$^+$	FO B Cells	MZ B Cells	ABCs
Young							
Male	WT (n = 25)	21.7 (± 8.84)	11.8 (± 4.89)	31.5 (24.1–39.9)	21.8 (± 9.75)	1.52 (0.880–1.83)	2.56 (1.94–3.43)
	Baff$^{-/-}$ (n = 21)	15.9 (± 6.71)	8.70 (± 3.43)	5.40 (3.95–8.20)	0.129 (± 0.187)	0.010 (0.010–0.025)	3.84 (2.71–6.96)
	Br3$^{-/-}$ (n = 21)	12.5 (± 4.82)	6.84 (± 2.99)	4.30 (2.55–6.60)	0.786 (± 0.535)	0.070 (0.020–0.130)	2.05 (1.37–3.06)
	p value, WT versus *Baff*$^{-/-}$	**0.020**	**0.018**	**<0.001**	**<0.001**	**<0.001**	**0.002**
	p value, WT versus *Br3*$^{-/-}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.223
	p value, *Baff*$^{-/-}$ versus *Br3*$^{-/-}$	0.070	0.069	0.070	**<0.001**	**0.013**	**<0.001**
Young							
Female	WT (n = 20)	22.4 (± 12.0)	12.1 (± 6.23)	26.3 (15.7–35.8)	18.8 (9.83–25.1)	1.13 (0.760–1.60)	2.26 (1.38–2.72)
	Baff$^{-/-}$ (n = 25)	20.5 (± 7.69)	10.8 (± 4.11)	6.90 (5.50–9.10)	0.100 (0.050–0.350)	0.010 (0.010–0.055)	4.96 (3.97–6.63)
	Br3$^{-/-}$ (n = 21)	19.3 (± 8.93)	9.29 (± 3.86)	5.90 (3.45–8.85)	0.700 (0.350–1.25)	0.060 (0.020–0.115)	3.05 (1.65–5.68)
	p value, WT versus *Baff*$^{-/-}$	0.973	0.749	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	p value, WT versus *Br3*$^{-/-}$	0.549	0.148	**<0.001**	**<0.001**	**<0.001**	0.095
	p value, *Baff*$^{-/-}$ versus *Br3*$^{-/-}$	0.646	0.195	0.168	**<0.001**	**0.007**	**0.016**
Old							
Male	WT (n = 16)	18.2 (16.1–21.9)	9.05 (7.73–10.8)	30.5 (23.1–36.1)	23.6 (17.3–27.9)	1.52 (1.14–1.73)	1.65 (0.975–2.36)
	Baff$^{-/-}$ (n = 20)	11.4 (9.03–14.9)	5.70 (4.50–8.10)	3.15 (2.63–4.80)	0.000 (0.000–0.175)	0.000 (0.000–0.010)	2.08 (1.50–3.91)
	Br3$^{-/-}$ (n = 19)	10.7 (6.50–14.0)	4.50 (3.10–6.60)	2.10 (1.30–2.80)	0.200 (0.100–0.400)	0.000 (0.000–0.006)	0.780 (0.520–1.16)
	p value, WT versus *Baff*$^{-/-}$	**<0.001**	**0.003**	**<0.001**	**<0.001**	**<0.001**	**0.050**
	p value, WT versus *Br3*$^{-/-}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.006**
	p value, *Baff*$^{-/-}$ versus *Br3*$^{-/-}$	0.354	0.084	**0.003**	**0.005**	0.743	**<0.001**
Old							
Female	WT (n = 31)	18.9 (± 6.51)	8.76 (± 3.37)	24.9 (17.4–31.9)	18.1 (13.3–22.9)	1.95 (± 0.897)	1.44 (0.710–2.30)
	Baff$^{-/-}$ (n = 21)	19.2 (± 5.77)	9.97 (± 3.35)	5.90 (4.25–7.70)	0.100 (0.050–0.155)	0.0167 (± 0.030)	4.02 (3.00–5.52)
	Br3$^{-/-}$ (n = 26)	16.0 (± 4.93)	7.39 (± 2.57)	2.40 (1.98–2.83)	0.400 (0.300–0.600)	0.0623 (± 0.045)	1.32 (0.865–1.44)
	p value, WT versus *Baff*$^{-/-}$	0.864	0.165	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	p value, WT versus *Br3*$^{-/-}$	0.070	0.166	**<0.001**	**<0.001**	**<0.001**	0.603
	p value, *Baff*$^{-/-}$ versus *Br3*$^{-/-}$	**0.044**	**0.004**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Results are expressed as means (± SD) when the normality test passed. Otherwise, results are expressed as medians (25th–75th percentiles). Spleen cell counts are in millions. p values ≤0.05 are in boldface.

both the percentage and number of Foxp3$^+$ cells in young male B6.*Br3*$^{-/-}$ mice were significantly lower than those in the corresponding young male B6 WT mice ($p \leq 0.010$ for all comparisons), and these relationships persisted as the mice aged and became old males ($p \leq 0.010$ for all comparisons). As with old female mice, the percentages and numbers of Foxp3$^+$ cells in young and old male B6.*Br3*$^{-/-}$ mice were significantly lower than those in the corresponding B6.*Baff*$^{-/-}$ mice (Table III), and, as with females, the percentages of Foxp3$^+$ cells significantly increased with age in B6 WT and B6.*Baff*$^{-/-}$ males ($p < 0.001$ for each comparison) but failed to do so in B6.*Br3*$^{-/-}$ males ($p = 0.085$).

CD4$^+$*CD25*$^+$*Foxp3*$^+$ *cells in B6 WT, B6.*Baff$^{-/-}$*, and B6.*Br3$^{-/-}$ *mice*

Although expression of Foxp3 is a hallmark of regulatory T cells in mice (29–31), CD4$^+$CD25$^-$Foxp3$^+$ cells may display in vivo plasticity and convert to proinflammatory Th17 cells (32). Accordingly, we also assessed percentages and numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells in our mice.

In young males, percentages and numbers of these cells were each significantly lower in B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice than in corresponding B6 WT mice ($p \leq 0.004$), whereas no significant differences were appreciated between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice (Table III). In contrast, no significant differences were detected in percentages and numbers of Foxp3$^+$ cells across young female B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice ($p \geq 0.144$). In old mice, however, percentages and numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells in both male and female B6.*Br3*$^{-/-}$ mice not only were significantly lower than those in B6 WT mice ($p \leq 0.002$), but were also significantly lower than those in B6.*Baff*$^{-/-}$ mice (Table III). As is the case for all Foxp3$^+$ cells, percentages of CD4$^+$CD25$^+$Foxp3$^+$ cells significantly increased in male and female B6 WT and B6.*Baff*$^{-/-}$ mice ($p < 0.001$ for each comparison) but failed to do so in male B6.*Br3*$^{-/-}$ mice ($p = 0.498$). Strikingly, the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells significantly *decreased* in female B6.*Br3*$^{-/-}$ mice ($p = 0.047$).

Although many of the Foxp3$^+$ cell–related analyses failed to significantly differ between young B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice, they all did significantly differ in both old males and old females. Accordingly, we combined old male and old female mice and assessed the relationships between percentages of Foxp3$^+$ cells and numbers of CD19$^+$ and CD4$^+$ cells. In old B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice, increasing numbers of CD19$^+$ cells associated with increasing percentages of Foxp3$^+$ cells (Fig. 3A, 3B, left). Strikingly, for any given number of CD19$^+$ cells harbored by the mice, percentages of Foxp3$^+$ cells were lower in B6.*Br3*$^{-/-}$ mice than in B6.*Baff*$^{-/-}$ mice. Analyses with percentages of CD4$^+$CD25$^+$Foxp3$^+$ cells revealed the same relationships (Fig. 3A, 3B, right).

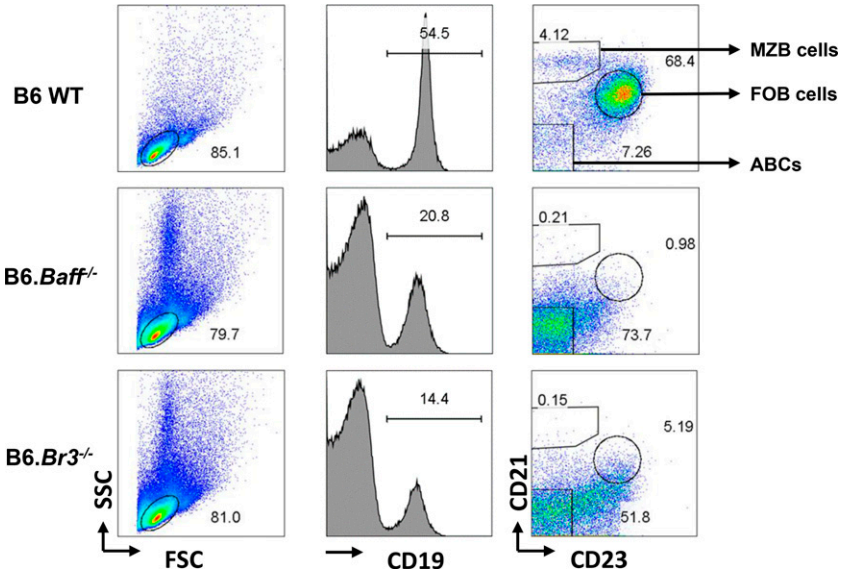

FIGURE 1. Identification of FO B cells, MZ B cells, and ABCs in B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice. Spleen cells from representative B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice were stained for surface CD19, CD21, and CD23. Cells were gated on the lymphocyte population (closed ovals in the plots in the left panel) and analyzed for CD19 expression (middle panel). Cells were gated on the CD19$^+$ population and analyzed for CD21 and CD23 expression (right panel). CD19$^+$CD21$^+$CD23$^+$ cells were taken as FO B cells, CD19$^+$CD21$^+$CD23^{lo} cells were taken as MZ B cells, and CD19$^+$CD21$^-$CD23$^-$ cells were taken as ABCs. Numbers in the tracings indicate the percentages of analyzed cells within the indicated zones.

The dichotomy between old B6.*Baff*$^{-/-}$ mice and old B6.*Br3*$^{-/-}$ mice in their relationships between percentages of Foxp3$^+$ cells and CD4$^+$ cells was even more dramatic than in those between percentages of Foxp3$^+$ cells and CD19$^+$ cells. In old B6 WT mice, percentages of Foxp3$^+$ cells steadily increased with increasing number of CD4$^+$ cells. In old B6.*Baff*$^{-/-}$ mice, percentages of Foxp3$^+$ cells did not change with increasing number of CD4$^+$ cells. In old B6.*Br3*$^{-/-}$ mice, percentages of Foxp3$^+$ cells steadily *declined* with an increasing number of CD4$^+$ cells (Fig. 3C, 3D, left). Analyses with percentages of CD4$^+$CD25$^+$Foxp3$^+$ cells revealed the same divergent relationships (Fig. 3C, 3D, right).

In vivo proliferation and survival of Foxp3$^+$ *cells in old B6 WT, B6.*Baff$^{-/-}$*, and B6.*Br3$^{-/-}$ mice

To explore the basis for the difference in numbers and percentages of Foxp3$^+$ cells between old B6.*Baff*$^{-/-}$ mice and old B6.*Br3*$^{-/-}$ mice, we first assessed expression of Ki-67, a marker of recent cell division, in Foxp3$^+$ cells. Whereas the percentages of Ki-67$^+$ Foxp3$^+$ cells were similar in old B6 WT and old B6.*Baff*$^{-/-}$ mice ($p = 0.738$), the percentage of Ki-67$^+$Foxp3$^+$ cells in old B6.*Br3*$^{-/-}$ mice was significantly greater than that in corresponding B6 WT ($p = 0.003$) and B6.*Baff*$^{-/-}$ mice ($p = 0.026$) (Fig. 3E). Thus, the relative reductions in numbers and percentages of Foxp3$^+$ cells in old B6.*Baff*$^{-/-}$ mice cannot be attributed to attenuated proliferation.

We then assessed whether decreased survival of Foxp3$^+$ cells in old B6.*Br3*$^{-/-}$ mice could have been contributory. To that end, we assessed the absence of Annexin V expression by, and the exclusion of 7-AAD from, Foxp3$^+$ cells. Compared to survival of Foxp3$^+$ cells in old B6 WT mice, survival of these cells was reduced in old B6.*Baff*$^{-/-}$ mice ($p < 0.001$) and even further reduced in old B6.*Br3*$^{-/-}$ mice ($p = 0.002$ in comparison with old B6.*Baff*$^{-/-}$ mice) (Fig. 3F), suggesting that the reductions in numbers and percentages of Foxp3$^+$ cells in old B6.*Br3*$^{-/-}$ mice may be attributable, at least in part, to reduced survival of these cells.

Discussion

Although previous studies revealed similar numbers of B cells in B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice and similar reduced levels of circulating Ig and Ag-induced IgG responses (25, 26), these studies were likely limited to young adult mice (given that the ages of the mice were not reported and that the "default" age of experimental mice is young adult). Thus, one could have prematurely concluded that the immune cell phenotypes of B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice are highly similar. Whereas our results confirm that numbers of B (CD19$^+$) cells in young male and young female B6.*Baff*$^{-/-}$ mice are similar to those in corresponding B6.*Br3*$^{-/-}$ mice, many important differences between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice emerged.

First, the similarity in numbers of CD19$^+$ cells did not persist as the mice aged. By the time the mice were ≥6 months of age, both male and female B6.*Br3*$^{-/-}$ mice harbored significantly fewer CD19$^+$ cells than did the corresponding B6.*Baff*$^{-/-}$ mice.

Second, even in young mice, the distribution of B cell subsets significantly differed between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice. Regardless of age or sex, percentages and numbers of FO B cells were greater in B6.*Br3*$^{-/-}$ mice than in corresponding B6.*Baff*$^{-/-}$ mice, whereas percentages and numbers of ABC were greater in B6.*Baff*$^{-/-}$ mice than in corresponding B6.*Br3*$^{-/-}$ mice. These observations raise the possibility that BAFF, which is plentiful in B6.*Br3*$^{-/-}$ mice, interacts with the BAFF receptors TACI and/or BCMA on non-ABC B cells and modulate their survival and/or expansion. Studies with mice doubly deficient in BR3 and TACI and doubly deficient in BR3 and BCMA will help address this possibility.

Third, although numbers of CD3$^+$ and CD4$^+$ cells did not generally differ between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice (with old females being the exception, in which the differences between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice, albeit small, were statistically significant), the relationships of these cells to B cells vastly differed between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice as a function of age and sex. In young females, numbers of FO B cells and MZ B cells correlated significantly with numbers of CD3$^+$ and CD4$^+$ cells in B6.*Baff*$^{-/-}$ mice but not in B6.*Br3*$^{-/-}$ mice, whereas numbers of FO B cells correlated significantly with numbers of CD3$^+$ and CD4$^+$ cells in old female B6.*Br3*$^{-/-}$ mice but not in old female B6.*Br3*$^{-/-}$ mice. In young males, FO B and MZ B cells correlated significantly with numbers of CD3$^+$ and CD4$^+$ cells in B6.*Br3*$^{-/-}$ mice but not in B6.*Baff*$^{-/-}$ mice, whereas in old males, MZ B cells in both B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice correlated significantly with numbers of CD3$^+$ and CD4$^+$ cells. It is not known whether discrete B cell subsets exert differential effects on T cell expansion. Depending on the in vivo context (e.g., BAFF-deficient versus BAFF-sufficient, male versus female, young versus old), different B cell subsets may differentially facilitate expansion of T cells or T cell subsets. Elucidation of the mechanisms that underpin these age- and sex-based differences and

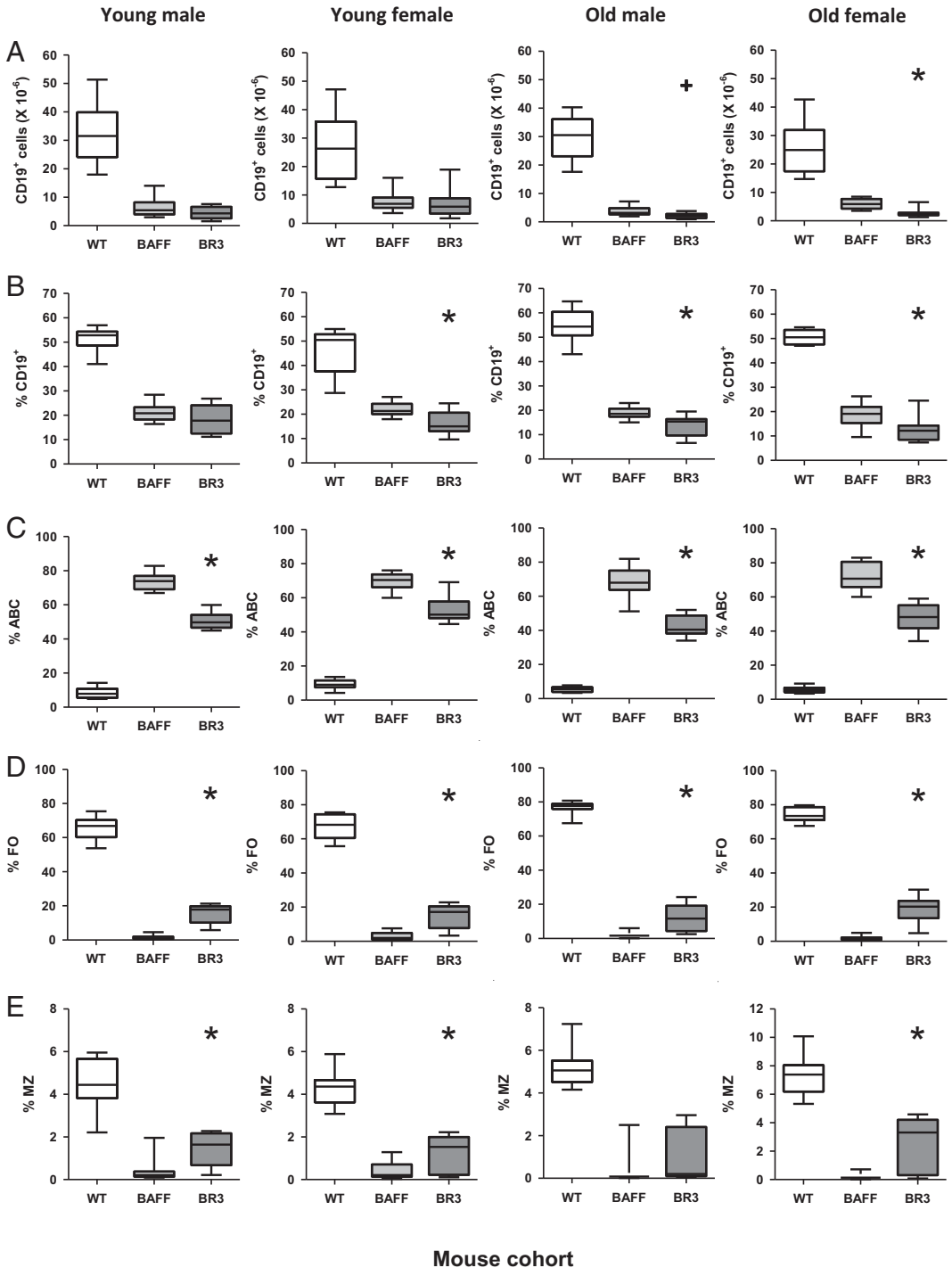

FIGURE 2. B cells and B cell subsets in B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice. Spleen cells from young male (25 B6 WT, 21 B6.*Baff*$^{-/-}$, and 21 B6.*Br3*$^{-/-}$), young female (20 B6 WT, 25 B6.*Baff*$^{-/-}$, and 21 B6.*Br3*$^{-/-}$), old male (16 B6 WT, 20 B6.*Baff*$^{-/-}$, and 19 B6.*Br3*$^{-/-}$), and old female (31 B6 WT, 21 B6.*Baff*$^{-/-}$, and 26 B6.*Br3*$^{-/-}$) mice were stained for surface CD19, CD21, and CD23 and analyzed for number of CD19$^+$ cells (**A**), percentage of CD19$^+$ cells (of mononuclear cells) (**B**), percentage of ABCs (of CD19$^+$ cells) (**C**), percentage of FO B cells (of CD19$^+$ cells) (**D**), and percentage of MZ B cells (of CD19$^+$ cells) (**E**). Results are plotted as box plots. The lines inside the boxes indicate the medians; the outer borders of the boxes indicate the 25th and 75th percentiles; and the bars extending from the boxes indicate the 10th and 90th percentiles. BAFF, B6.*Baff*$^{-/-}$; BR3, B6.*Br3*$^{-/-}$; WT, B6 WT. All B6 WT values are significantly different from the corresponding B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ values. *$p < 0.001$ (B6.*Baff*$^{-/-}$ versus B6.*Br3*$^{-/-}$); $^+p = 0.003$ (B6.*Baff*$^{-/-}$ versus B6.*Br3*$^{-/-}$).

the biological ramifications of these differences will require additional experimentation.

Fourth, the similar numbers of total CD4$^+$ cells in B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice notwithstanding, numbers and percentages of CD4$^+$Foxp3$^+$ cells differed between the two mouse lines, with values being greater in B6.*Baff*$^{-/-}$ mice than in B6.*Br3*$^{-/-}$ mice. Whereas these differences were relatively modest in young mice (and not statistically significant in young females), the differences were striking by the time the mice reached ≥6 months of age. The differences cannot be attributed solely to differential expression of CD25$^-$CD4$^+$Foxp3$^+$ cells, because there were significant differences between old B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice in the numbers and percentages of CD25$^+$CD4$^+$Foxp3$^+$ cells as well.

Table II. Correlations between CD3$^+$ and CD4$^+$ cells and B cell subsets in the spleens of B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice

		Young Male		Young Female		Old Male		Old Female	
Mouse	Subset	CD3$^+$	CD4$^+$	CD3$^+$	CD4$^+$	CD3$^+$	CD4$^+$	CD3$^+$	CD4$^+$
B6 WT	FO B	0.888	0.872	0.892	0.899	0.684	0.727	0.918	0.881
		<0.001	**<0.001**	**<0.001**	**<0.001**	**0.003**	**0.001**	**<0.001**	**<0.001**
	MZ B	0.901	0.879	0.877	0.889	0.670	0.578	0.747	0.698
		<0.001	**<0.001**	**<0.001**	**<0.001**	**0.004**	**0.018**	**<0.001**	**<0.001**
	ABC	0.245	0.258	0.471	0.508	0.515	0.619	0.802	0.762
		0.235	0.209	**0.036**	**0.022**	**0.040**	**0.011**	**<0.001**	**<0.001**
B6.*Baff*$^{-/-}$	FO B	0.349	0.425	0.520	0.594	0.557	0.568	0.014	0.055
		0.120	0.054	**0.008**	**0.002**	**0.011**	**0.009**	0.948	0.810
	MZ B	0.187	0.285	0.660	0.667	0.612	0.611	0.439	0.453
		0.411	0.208	**<0.001**	**<0.001**	**0.004**	**0.004**	**0.046**	**0.039**
	ABC	0.800	0.863	0.841	0.837	0.865	0.858	0.412	0.386
		<0.001	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.063	0.083
B6.*Br3*$^{-/-}$	FO B	0.633	0.642	0.234	0.333	0.423	0.532	0.609	0.713
		0.002	**0.002**	0.303	0.137	0.070	**0.019**	**<0.001**	**<0.001**
	MZ B	0.637	0.621	−0.016	0.098	0.555	0.669	0.539	0.661
		0.002	**0.003**	0.944	0.670	**0.014**	**0.002**	**0.005**	**<0.001**
	ABC	0.697	0.712	0.869	0.853	0.728	0.708	0.174	−0.018
		<0.001	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.391	0.931

Results in each cell are the correlation coefficient (top) and p value (bottom). The mice analyzed are identical to those in Fig. 2. p values ≤0.05 are in boldface.

Fifth, for any given number of CD19$^+$ cells harbored by the mice, percentages of Foxp3$^+$ cells and CD4$^+$CD25$^+$Foxp3$^+$ cells were lower in B6.*Br3*$^{-/-}$ mice than in B6.*Baff*$^{-/-}$ mice. For CD4$^+$ cells, the relationships with percentages of Foxp3$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ cells more starkly differed between old B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice, with percentages of Foxp3$^+$ or CD4$^+$CD25$^+$Foxp3$^+$ cells not changing in response to increasing numbers of CD4$^+$ cells in the former but actually decreasing in the latter. Moreover, in vivo proliferation and survival of these cells differed between old B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice, with proliferation being greater, but survival being lesser, in B6.*Br3*$^{-/-}$ mice than in B6.*Baff*$^{-/-}$ mice. Collectively, these observations raise the possibility that interactions between TACI and/or BCMA and BAFF, which circulates at supranormal levels in B6.*Br3*$^{-/-}$ mice, modulate expression of Foxp3$^+$ (and CD4$^+$CD25$^+$ Foxp3$^+$) cells. Studies with mice doubly deficient in BR3 and TACI and doubly deficient in BR3 and BCMA will help elucidate the underlying mechanisms.

This difference in Foxp3$^+$ cells may help explain heretofore enigmatic observations. Whereas A/WySnJ mice bear a mutated *Br3* gene and display deficiencies in B cells and Ab responses similar to those in B6.*Baff*$^{-/-}$ or B6.*Br3*$^{-/-}$ mice (6, 14, 15), A/WySnJ mice develop elevated serum titers of IgG anti-dsDNA Abs and clinically overt glomerulonephritis as they age (33). Because the mutant BR3 is surface-expressed and binds BAFF (14), circulating BAFF levels in these mice are not significantly different from those in congenic A/J mice that express WT BR3 and do not develop autoimmune features (33). Levels of Foxp3$^+$ cells were not assessed in these mice, so it may be that in an A/J genetic background, selective

Table III. Spleen Foxp3$^+$ cells in B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice

		CD4$^+$ Cell Population			
Age/Sex	Genotype	Percentage of Foxp3$^+$	No. of Foxp3$^+$	Percentage of CD25$^+$Foxp3$^+$	No. of CD25$^+$Foxp3$^+$
Young male	WT (n = 25)	11.6 (11.3–11.9)	1.39 (0.970–1.69)	10.7 (10.1–11.1)	1.24 (0.875–1.52)
	Baff$^{-/-}$ (n = 21)	11.3 (10.4–12.1)	0.910 (0.720–1.13)	10.0 (8.80–10.8)	0.800 (0.615–0.930)
	Br3$^{-/-}$ (n = 21)	10.6 (9.54–11.0)	0.680 (0.460–1.02)	9.50 (8.70–10.1)	0.590 (0.400–0.870)
	p value, WT versus *Baff*$^{-/-}$	0.247	**0.010**	**0.005**	**0.004**
	p value, WT versus *Br3*$^{-/-}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	p value, *Baff*$^{-/-}$ versus *Br3*$^{-/-}$	**0.019**	**0.042**	0.352	0.059
Young female	WT (n = 20)	11.3 (± 0.880)	1.40 (± 0.756)	10.2 (± 0.730)	1.24 (± 0.650)
	Baff$^{-/-}$ (n = 25)	11.6 (± 1.41)	1.30 (± 0.580)	10.1 (± 0.908)	1.10 (± 0.475)
	Br3$^{-/-}$ (n = 21)	11.3 (± 1.34)	1.08 (± 0.484)	10.6 (± 1.34)	0.976 (± 0.442)
	p value, WT versus *Baff*$^{-/-}$	0.346	0.732	0.503	0.553
	p value, WT versus *Br3*$^{-/-}$	0.973	0.155	0.473	0.175
	p value, *Baff*$^{-/-}$ versus *Br3*$^{-/-}$	0.420	0.179	0.144	0.356
Old male	WT (n = 16)	13.2 (12.7–15.2)	1.24 (1.11–1.43)	11.2 (10.9–12.6)	1.05 (0.890–1.20)
	Baff$^{-/-}$ (n = 20)	14.2 (13.1–15.2)	0.840 (0.640–1.32)	11.4 (11.0–11.8)	0.625 (0.502–1.09)
	Br3$^{-/-}$ (n = 19)	11.5 (10.1–13.0)	0.530 (0.350–0.750)	10.1 (8.40–11.2)	0.460 (0.290–0.640)
	p value, WT versus *Baff*$^{-/-}$	0.533	**0.010**	0.348	**0.008**
	p value, WT versus *Br3*$^{-/-}$	**<0.001**	**<0.001**	**0.002**	**<0.001**
	p value, *Baff*$^{-/-}$ versus *Br3*$^{-/-}$	**<0.001**	**0.006**	**0.003**	**0.018**
Old female	WT n = 31)	14.1 (13.0–15.5)	1.15 (0.860–1.62)	11.6 (± 0.955)	0.940 (0.730–1.23)
	Baff$^{-/-}$ (n = 21)	15.4 (12.8–18.1)	1.38 (1.16–1.78)	12.3 (± 2.03)	1.12 (0.950–1.36)
	Br3$^{-/-}$ (n = 26)	10.8 (9.76–11.9)	0.775 (0.603–0.935)	9.55 (± 1.89)	0.650 (0.522–0.783)
	p value, WT versus *Baff*$^{-/-}$	0.119	0.055	0.198	0.070
	p value, WT versus *Br3*$^{-/-}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	p value, *Baff*$^{-/-}$ versus *Br3*$^{-/-}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Results are expressed as means (± SD) when the normality test passed. Otherwise, results are expressed as medians (25th–75th percentiles). Spleen cell counts are in millions. p values ≤0.05 are in boldface.

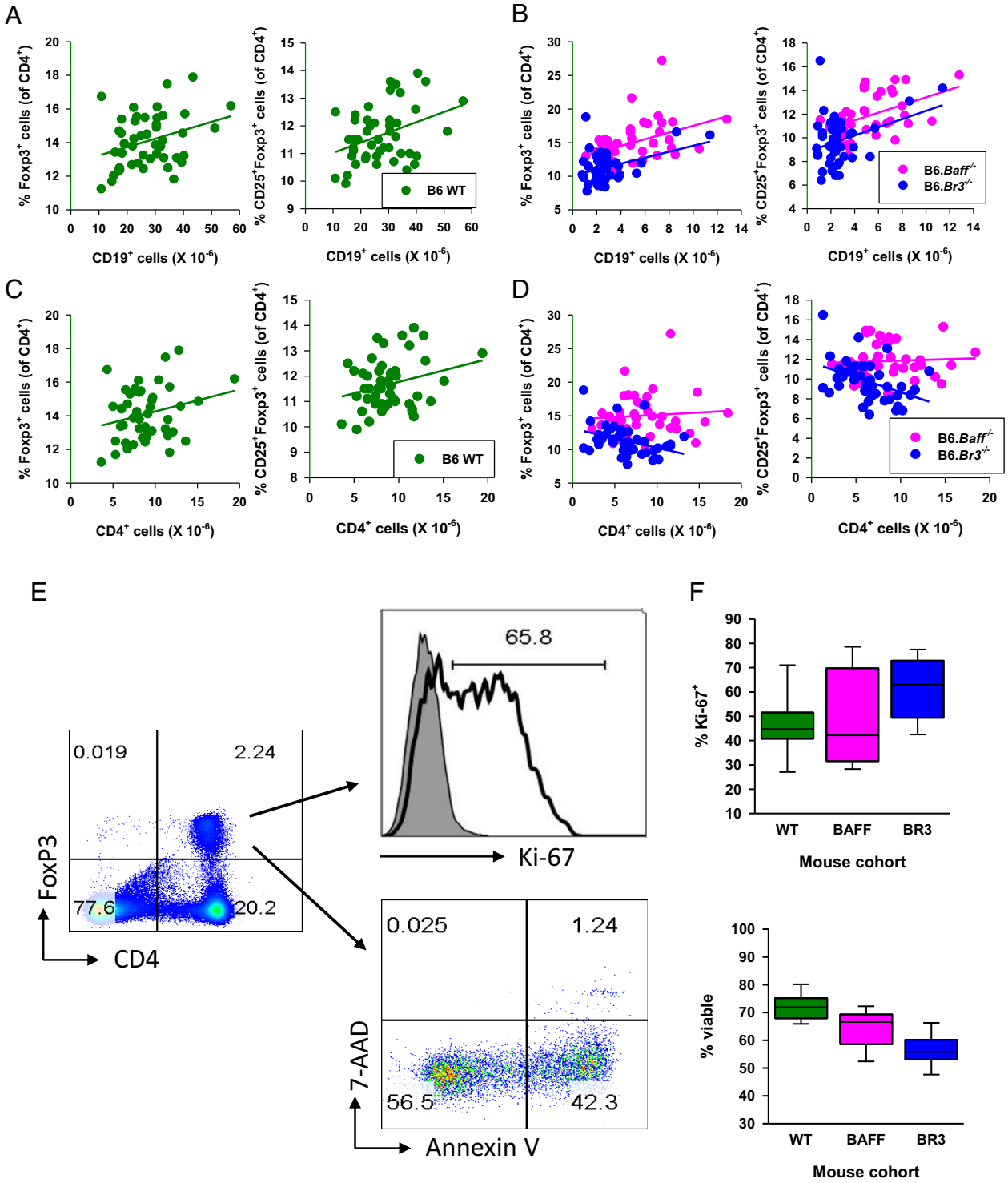

FIGURE 3. Relationships between percentages of Foxp3$^+$ cells and numbers of CD19$^+$ and CD4$^+$ cells in old B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice. Foxp3$^+$ cells as percentages of CD4$^+$ cells (left panel) and CD25$^+$Foxp3$^+$ cells as percentages of CD4$^+$ cells (right panel) versus CD19$^+$ cells (**A** and **C**) and CD4$^+$ cells (**B** and **D**) are plotted for the 47 old B6 WT (green), 41 old B6.*Baff*$^{-/-}$ (pink), and 45 old B6.*Br3*$^{-/-}$ (blue) mice reported in Fig. 2. Circles represent the individual mice, and the lines represent the linear regressions. Spleen cells from the indicated old mice were gated on the CD4$^+$Foxp3$^+$ population (**E**, left) and analyzed for Ki-67 expression (E, top right) and for Annexin V expression and 7-AAD exclusion (E, bottom right). The solid line indicates Ki-67 staining, the gray-filled area indicates staining with the isotype control mAb (E, top right), and viable cells are those in the left bottom quadrant (E, bottom right). A representative sample is shown. (**F**) Results from the 21 old B6 WT, 16 old B6.*Baff*$^{-/-}$, and 34 old B6.*Br3*$^{-/-}$ mice tested are plotted as in Fig. 2.

abrogation of BR3-mediated signaling leads to a reduction in Foxp3$^+$ cells and thereby facilitates expression of an underlying and otherwise dormant autoimmune diathesis.

In a related vein, disruption of the *Br3* gene in systemic lupus erythematosus–prone NZM 2328 mice leads to marked reductions in B cells but has no discernable effect on development of renal immunopathology or clinical disease (34). Foxp3$^+$ cells were not analyzed in this study, so it is possible that reductions in B cells are counterbalanced by reductions in Foxp3$^+$ cells, resulting in no net protective effect on development of disease. Detailed analyses of the

effects of BR3 deficiency on Foxp3$^+$ cells and their relationships to B cells and B cell subsets in autoimmune-prone hosts is warranted.

In addition to the many phenotypic differences between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice, several phenotypic differences were consistently observed between males and females. In both young and old B6 WT, B6.*Baff*$^{-/-}$, and B6.*Br3*$^{-/-}$ mice, CD19$^+$ cells were numerically greater in females than in corresponding males. This cannot be attributed to testosterone-driven lower expression of BAFF in males than in females (35), because a dichotomous level of CD19$^+$ cells was observed even in B6.*Baff*$^{-/-}$ mice, in which neither males nor females express any BAFF. This difference between males and females also extended to numbers and percentages of Foxp3$^+$ and CD4$^+$CD25$^+$ Foxp3$^+$ cells in each of the mouse lines. Because only lymphocytes from the spleen were evaluated in our study, it remains to be established whether the observed phenotypic differences between B6.*Baff*$^{-/-}$ and B6.*Br3*$^{-/-}$ mice also extends to lymphocytes from other lymphoid tissues. In addition, whether the sex-based differences in CD19$^+$ and Foxp3$^+$ cells are replicated in autoimmune-prone hosts and how such differences, if present, affect development of autoimmunity and autoimmune disease needs to be investigated.

Disclosures

The authors have no financial conflicts of interest.

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