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The importance of regulatory T cells in immune tolerance is illustrated by the human immune dysregulatory disorder IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), caused by a lack of regulatory T cells due to decreased or absent expression of Foxp3. Although the majority of work on regulatory T cells has focused on their ability to suppress T cell responses, the development of significant autoantibody titers in patients with IPEX suggests that regulatory T cells also contribute to the suppression of autoreactive B cells. Using a murine model, deficient in the expression of Foxp3, we show that B cell development is significantly altered in the absence of regulatory T cells. Furthermore, we identify a loss of B cell anergy as a likely mechanism to explain the production of autoantibodies that occurs in the absence of regulatory T cells. Our results suggest that regulatory T cells, by either direct or indirect mechanisms, modulate B cell development and anergy. The Journal of Immunology, 2010, 185: 000–000.

Antigenic diversity is a key feature of B cell biology and enhances the ability of the adaptive immune system to respond to the plethora of pathogens present in the environment. An unwanted consequence of the pressure to produce this repertoire is the generation of B cells with Ag receptor specificity for autoantigens. Estimates predict as many as 50–75% of all newly produced B cells are autoreactive (1, 2), and are regulated by tolerance mechanisms, including receptor editing (3), clonal deletion (4), and anergy (5). Failure of one or more of these mechanisms may result in the development of autoimmunity, with anergic B cells representing a particularly pathogenic precursor population due to the reversible nature of anergy (6–8).

Our understanding of B cell anergy has predominately focused at the cell-intrinsic level, and multiple signaling proteins have been identified that are essential for this process (reviewed in Ref. 9). Although a number of studies highlight the ability of Th cell-derived signals to break B cell anergy (8, 10–13), it is only recently that other extrinsic factors have been identified that also break B cell anergy. These factors include signals generated by innate immune stimuli, such as complement (14), TLR signals (15), or soluble factors secreted by other immune cells, including dendritic cells (16).

In humans, mutations in the Foxp3 gene result in the development of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX), a complex immune disorder that presents with significant autoimmunity and autoantibodies against multiple autoantigens (17). The development of autoantibodies in IPEX patients suggests that regulatory T cells may represent a key, yet undefined, regulator of autoreactive B cells. Consistent with this hypothesis, a spontaneous mutation in Foxp3 in mice (Scurfy mice) results in similar autoimmune symptoms, including high titers of autoantibody (18, 19).

Further evidence for a role of regulatory T cells in modulating B cell function, including autoantibody production, has been reported both in vitro and in vivo. These include the ability of regulatory T cells to directly kill B cells (20, 21) or suppress the generation of autoantibodies both in vitro and in vivo (13, 22–25). Despite this apparent link between regulatory T cells and autoantibody production, it is surprising that no studies have directly addressed whether the absence of regulatory T cells modifies B cell development or B cell anergy. To address these questions, we used a murine model of IPEX that contains a mutation in Foxp3 (termed Foxp3−/− mice), homologous to a human mutation, resulting in a premature stop codon, and fails to express Foxp3 protein (26). In this study, we report that Foxp3−/− mice exhibited significantly altered B cell development and a failure in B cell anergy.

Materials and Methods

Mice, cell suspensions, and adoptive transfer

Forkhead box P3 (Foxp3)–sufficient (Foxp3−/−) and deficient (Foxp3−/−) mice, on a C57BL/6 background (26), were maintained at the Biological Resource Center of the Medical College of Wisconsin. Procedures were performed under approval from the Institution Animal Use and Care Committee, and all animals were used at 28 d of age, unless otherwise stated. Single-cell suspensions were generated from bone marrow (femur), spleen, and lymph node (inguinal and brachial combined) by gentle homogenization in IMDM plus 2% FCS, followed by RBC lysis. Analysis of cells from the peritoneal cavity (PEC) was performed by filling the PEC with IMDM plus 2% FCS, gentle massage, and recovery of media. Experiments using the adoptive transfer of regulatory T cells used Foxp3−/− mice on a BALB/c background. Foxp3−/− mice were adoptively transferred at birth with 1×10⁷ regulatory T cells (CD4⁺Foxp3−) and 4×10⁶ conventional T cells (CD4⁺Foxp3−) isolated from Foxp3−/− mice aged >6 wk. Serum was collected at 18 d of age.

IL-7 in vitro BM culture

Femurs from control and experimental mice were dissected, and a single-cell suspension was generated. RBCs were lysed, and remaining cells were resuspended at 5×10⁷ cells/ml. A total of 6 ml of bone marrow suspension was added per 60-mm petri dish in culture media (IMDM, 10% FCS, 1 mM sodium pyruvate [Life Technologies, Carlsbad, CA], 50 µg/ml gentamicin [Life Technologies], 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM...
L-glutamine [Life Technologies], 50 μM 2-ME, and 4% IL-7). Flow cytometry was performed on bone marrow cells at day 0 or after 6 d of culture.

**Flow cytometry and apoptosis assay**

Cell suspensions were resuspended in FACS buffer (PBS + 2% FCS + 0.05% sodium azide) at 10^6 cells/ml. A total of 1 x 10^6 cells was prestained with Fc block (24G2), then incubated with an optimal amount of Ab conjugate (eFluor450, FITC, PE, Tricolor, Alexa647, allophycocyanin, or biotin). Biotin-conjugated Abs were visualized with streptavidin-PE (eBioscience, San Diego, CA). Abs against the following molecules were used in all experiments: B220 (RA3-6B2), CD5 (53-7.3), CD11b (M1/70), CD43 (S7), CD86 (GL-1), CD80 (16-10A1), CD23 (B3B4), CD93 (AA4.1), GL-7 (GL7; all from BD Biosciences, San Jose, CA), and IgM (b-7-6) and IgD (JA12.5) (produced in house). For the measurement of apoptosis, cells were resuspended in PBS at 10^6 cells/ml, and 1 x 10^6 cells were incubated with 2.5 nM DiOC6 (Invitrogen, Carlsbad, CA) for 30 min at 37˚C, cooled to 4˚C, and then stained with appropriate Ab conjugate. Data acquisition was performed on a BD FACS Calibur or LSR II and analyzed using FlowJo software (Tree Star, Ashland, OR).

**ELISA and HEp-2 analysis**

To detect autoantibodies with DNA reactivity, 96-well ELISA plates (Corning Costar, Lowell, MA) were precoated with methyl-BSA (5 mg/ml; Sigma-Aldrich, St. Louis, MO), followed by dsDNA from *Escherichia coli* (12.5 μg/ml; Sigma-Aldrich). Plates were blocked with 3% BSA/0.1% gelatin prior to serum sample addition (diluted in PBS over a range of 1/40–1/2500). DNA-specific autoantibodies were then detected using anti-mouse IgG (H + L) HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) and tetramethylbenzidine Single Solution substrate (Invitrogen). Substrate/Enzyme reaction was stopped using 1 N HCl, and colorimetric product was read at 450 nm. Anti-arsonate Abs were detected, as previously described (27).

Total serum IgM and IgG levels were measured by coating 96-well ELISA plates (Corning Costar) with 10 μg/ml of either goat anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories). Plates were blocked with 1% BSA prior to sample addition (diluted in PBS at 1/10,000). Standard curves were generated by serial dilution of purified IgM (Sigma-Aldrich) or IgG (Southern Biotechnology Associates, Birmingham, AL). Isotype-specific Abs were then detected using either goat anti-mouse IgM or IgG HRP (Jackson ImmunoResearch Laboratories) and tetramethylbenzidine Single Solution substrate (Invitrogen). Substrate/Enzyme reaction was stopped using 1 N HCl, and colorimetric product was read at 450 nm. Standard curves were calculated, and serum concentrations were extrapolated.

Polyreactivity of serum autoantibodies was determined using HEp-2 slides (Antibodies Incorporated, Davis, CA). Experiments were carried out using the manufacturer’s recommended protocol using serum diluted in PBS over a range of 1/40–1/320. Slides were analyzed using a fluorescence microscope (Zeiss [Oberkochen, Germany] Axiosimager).
Z1 microscope with AxioCam HRc camera). Images were taken at a total original magnification of $\times630$.

**Histology**

Tissue was fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned, and stained (H&E) using a histology core facility. Sections were analyzed on a Nikon Eclipse light microscope fitted with a Photometrics CoolsnapES camera at $\times40$ or $\times100$ (total) original magnification.

**Fluorescence microscopy**

Tissue was embedded in tissue-Tec OCT compound (Sakura Finetek, Torrance, CA), snap frozen using liquid nitrogen, and then stored at $-80^\circ$C. Sections (5–8 μM) were mounted onto microscope slides, fixed for 10 min.
in ice-cold acetone, rehydrated in PBS, and then blocked for 30 min in PBS/0.5% BSA. Sections were then stained with Abs specific for B220 or CD3, conjugated to PE or Alexa488, respectively. Sections were then washed in PBS/0.05% Tween 20, semidried, covered with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA), and sealed with a glass coverslip. Images were acquired using a Zeiss Axioimager Z1 fluorescence microscope with a Nuance multispectral imaging system at x50 or x100 (total) original magnification.

ELISPOT
MultiScreen 96-well filtration plates (Millipore, Bedford, MA) were precoated with methyl-BSA (5 μg/ml; Sigma-Aldrich), followed by dsDNA from E. coli (12.5 μg/ml; Sigma-Aldrich). Plates were blocked with 3% BSA/0.1% gelatin, then incubated with cells in IMDM/5% FCS at predetermined concentrations for 4 h at 37°C. Cells were removed using PBS plus 50 mM EDTA, followed by multiple washes with PBS plus Tween 0.1%. Wells were incubated with anti-mouse IgG (H + L) biotin for 2 h at room temperature in blocking buffer, washed, then incubated with ExtrA-Avidin-Alkaline Phosphatase (Sigma-Aldrich) diluted in blocking buffer for 1 h at room temperature. Spots were developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT. Spots were read using an ImmunoSpot (Cellular Technology, Shaker Heights, OH) and ImmunoSpot 4 software.

Immunoblotting
Splenic B cells were purified by positive selection of B220+ cells using MACS beads (Miltenyi Biotec, Auburn, CA) following RBC lysis. B cells (>95% purity) were resuspended in IMDM at 10 × 10^6/ml and warmed to 37°C for 15 min. Following stimulation, cells were lysed in 1% Nonidet P-40 lysis buffer (150 mM sodium chloride [NaCl], 10 mM Tris [pH 7.5], 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSE, 10 mM sodium fluoride [NaF], 0.4 mM disodium EDTA, 1 mM aprotinin, 1 mM α-1-antitrypsin, and 1 mM leupeptin). Lysates were kept on ice for 10 min before centrifugation at 10,000 rpm for 10 min at 4°C. Supernatants were mixed with NaDodSO4 (SDS) reducing buffer and boiled for 5–10 min. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and visualized using Abs against phospho-ERK-MAPK and actin (Cell Signaling Technology, Beverly, MA, and Santa Cruz Biotechnology, Santa Cruz, CA, respectively). Abs were detected using Alexa680- or IR800-conjugated secondary Abs (Invitrogen and LI-COR, Lincoln, NE, respectively). Bands were visualized using a LI-COR Odyssey.

Statistical analysis
Statistical analysis of groups was performed using an unpaired Student t test for unpaired data (Prism version 5.0; GraphPad, San Diego, CA). Differences were considered significant when p < 0.05.

Results
Altered B cell development in the bone marrow of Foxp3-deficient mice
To determine whether the absence of regulatory T cells affects early B cell development, we analyzed bone marrow cells from wild-type (Foxp3+) or Foxp3-deficient (Foxp3−) mice. Total bone marrow cell counts (Fig. 1A, left panel) and the proportion of B220+ cells (Fig. 1A, middle panel) were decreased in Foxp3− mice compared with wild-type mice, which resulted in a significant decrease in total B cell numbers (Fig. 1A, right panel). We next determined whether B cell development was affected by the absence of Foxp3 by enumerating pro-B, pre-B, immature, and mature/recirculating B cell populations by flow cytometry. As shown in Fig. 1B, Foxp3− mice exhibited a substantial reduction in the proportions of pro-B/pre-B, immature, and mature/recirculating B cells as a percentage of the total bone marrow population. To further delineate differences between the pro-B and pre-B cell subsets, we performed an additional gating strategy to separate these populations, gating on IgM− cells and then using B220 and CD43. We observed an increase in the proportion of pro-B cells in Foxp3− mice, with a corresponding decrease in pre-B cells (Fig. 1C). Our data also showed the expansion of an IgM B220 CD43+ population in the bone marrow of Foxp3− mice.

Due to alterations in the proportion of non-B cell subsets that may have skewed our subset analysis, we performed additional analysis focusing on only B220+ cells. We found a significant increase in the proportion of pro-B cells, a decrease in pre-B cells, but no change in immature or mature/recirculating populations (Fig. 1D, left panel). However, based on absolute numbers, we found all B cell subsets to be significantly decreased in number in Foxp3− bone marrow compared with wild-type bone marrow (Fig. 1D, right panel).

Although Foxp3 is not expressed in B cells (28, 29) (Supplemental Fig. 1), suggesting the defect observed in B cell development was B cell extrinsic, we performed IL-7–driven bone marrow cultures to examine the proliferative response of bone marrow B cells from Foxp3− mice ex vivo. Our studies indicated that, although starting at lower overall numbers, bone marrow B cells from Foxp3− mice expand normally in response to IL-7 (Supplemental Fig. 2A, B). These data suggest that B cells from Foxp3− mice have no intrinsic defect in their ability to expand in response to cues essential for B cell development. Collectively, these data demonstrated that the loss of Foxp3 had a wide-reaching effect on B cell development in the bone marrow and suggested this is due to a B cell-extrinsic mechanism.

Altered B cell development in the spleen of Foxp3-deficient mice
To determine whether altered B cell development in the bone marrow of Foxp3− mice extended to the periphery, we investigated the cellular composition of the spleen. Consistent with previous findings, total splenic cell numbers were increased in Foxp3− mice (Fig. 2A, left panel) (26). However, reflected by the significant decrease in the proportion of B220+ cells, splenic B cell numbers were decreased in Foxp3− mice compared with wild-type mice (Fig. 2A, right panel). B cells were further analyzed by gating on B220+CD11b+ (B-1a B cells) and B220+CD11b− (B-2 B cells). Both B-1a B cells (B220+CD11b+) and B-2 (B220+CD11b−) subsets are shown on the left panels. Histograms on the right show the breakdown of B-1a B cells into B-1a (CD5+) and B-1b (CD5−) B cells. Data were generated from two independent experiments using a minimum of five Foxp3+ or Foxp3− mice.
numbers were reduced 3- to 4-fold in Foxp3⁻ mice compared with wild-type mice (Fig. 2A, middle and right panels).

To determine whether the observed alterations in B cell numbers accompanied changes in peripheral B cell subsets, we stained splenocytes from wild-type or Foxp3⁻ mice with Abs specific for IgM and IgD. Immature/Transitional/Marginal zone subsets were broadly identified based on a staining pattern of surface IgMhighIgDlow, whereas mature/follicular B cells exhibited a normal population of B cells, either in total number or subset distribution. We expanded these studies to investigate whether B cells in the lymph nodes of Foxp3⁻ mice were also decreased in number. Interestingly, although lymph nodes from Foxp3⁻ mice were enlarged and exhibited decreased proportions of B cells, B cell numbers are not significantly altered (Fig. 3A). B cell subset analysis in the lymph node also failed to show significant changes in B cell subset proportions (Fig. 3B, 3C). The explanation for this observation remains unclear, although we postulate, due to the significant inflammation associated with Foxp3 deficiency, that B cells may preferentially home to draining lymph nodes associated with this response.

**Differential loss of B-1 B cells in the absence of Foxp3**

To investigate whether B-1 B cells were also affected by the absence of Foxp3, we determined the frequency and absolute number of B1a and B1b B cells in spleen and peritoneal cavities. Our studies indicated that the proportion of splenic B-1a cells in Foxp3⁻ mice was...
significantly reduced (Fig. 4A, Table I). Interestingly, B-1 subsets that inhabit the peritoneal cavity were differentially regulated. In this location, the proportion and absolute number of B-1a B cells were decreased in Foxp3− mice (Fig. 4B, Table I). However, in contrast, B-1b B cells were found in normal proportions and increased numbers (Fig. 4B, Table I). These data suggested that B-1b B cells develop in a manner independent of the influence of Foxp3 deficiency.

Elevated apoptosis and activation of B cells from Foxp3-deficient mice

Our results suggested that there were both a significant reduction in the number of immature B cells that arise and exit the bone marrow of Foxp3− mice and mature B cells that populate the spleen. To investigate whether the reduction on splenic B cell numbers was caused by increased apoptosis, we used a dye that measures mitochondrial membrane potential (ΔΨm). A reduction in ΔΨm correlates with an early stage of programmed cell death (31). We found a significant decrease in the proportion of splenic B cells from Foxp3− mice with a normal ΔΨm (Fig. 5A, left and right panels). In parallel experiments, we investigated the activation status of B cells from the spleens of Foxp3− mice and showed that the majority of B cells from these mice exhibited significant cell activation, evident by an increase in the expression of CD80 and CD86 (Fig. 5B). These data suggested that splenic B cells from Foxp3− mice receive uncharacterized signals that promote their activation and increase their apoptosis.

Altered splenic architecture in the absence of Foxp3

Due to the observed defects in splenic B cell development and B cell apoptosis in Foxp3− mice, we investigated whether the anatomical locale of B cells within the splenic architecture was altered in Foxp3− mice. Using H&E staining, we observed a substantial loss of normal splenic architecture, with a clear loss in the distinction between red and white pulp areas (Fig. 6A). The loss of white pulp prompted us to examine whether normal B cell follicles, and T cell zones, were formed in the spleens of Foxp3− mice. Our results indicated a substantial change in both the frequency and architecture of splenic B cells.
of the B cell follicular areas of Foxp3− mice. Not only were follicular areas reduced in number, they were smaller in size and failed to exist alongside distinct T cell zones. Indeed, traditional T cell zones could not be detected; T cells were instead dispersed throughout the spleen (Fig. 6B).

Previous studies show that Foxp3− mice on the C57BL/6 background exhibit an increase in Ig class switching, evident by an increase in serum levels of IgG1 and IgE (26). Based on these observations, one would predict an increase in the presence of germinal centers in Foxp3− mice. To investigate whether Foxp3−

FIGURE 7. Presence of autoantibodies in Foxp3− mice. A, Serum titers of anti-DNA autoantibodies by ELISA in either Foxp3+ or Foxp3− mice. Each ○ or ● represents the mean ± SEM from n = 8 mice; ***p < 0.0001; **p < 0.0066; *p < 0.0185. B, Representative images using HEp-2 cells to detect polyreactive autoantibodies. Serum (diluted 1/160) from Foxp3+ or Foxp3− mice (showing nuclear or perinuclear staining) was incubated on HEp-2 slides, and bound autoantibody was detected using a fluorescently conjugated Ab specific for total mouse Ig (n = 4 Foxp3+ and n = 4 Foxp3− in two experiments). ***p < 0.0039. Scores (using serum diluted 1/40–1/320) indicate intensity of staining and were scored in a blinded fashion (original magnification ×630). C, Anti-DNA ASCs in Foxp3+ or Foxp3− bone marrow or spleen normalized to the total number of B cells/well. Values represent mean ± SEM of Foxp3+ (n = 6) or Foxp3− (n = 5) for two experiments. D, Detection of anti-DNA autoantibodies by ELISA in either Foxp3+ (n = 4), Foxp3− (n = 4), or Foxp3− mice adoptively transferred with 1 × 10^6 regulatory T cells and 4 × 10^6 CD4+Foxp3− cells (n = 4). Each symbol represents the mean ± SEM from n = 8 mice; ***p < 0.0001; **p < 0.0049; *p < 0.0164.
mice exhibit an increase in germinal center B cells, we stained splenocytes with the germinal center marker GL7. Our results indicated a 10-fold increase in the proportion of GL7-expressing B cells in Foxp3−/− mice (Fig. 6C). Based on total B cell numbers in Foxp3−/− mice, our studies indicate 1 in 8 of all B cells in Foxp3−/− mice express GL7, as opposed to only 1 in 625 in Foxp3+ mice. Further analysis identified that a substantial proportion of CD19+ GL7+ B cells in Foxp3−/− mice was also IgG+, a phenotype expected for germinal center B cells (Fig. 6C). Similar results were obtained in the lymph nodes from Foxp3−/− mice (data not shown). These observations paralleled significant increases in the concentration of serum IgM and IgG (Fig. 6D), similar to previous findings (26), and confirm that significant B cell dysregulation occurs in the absence of Foxp3.

These data suggested that despite substantial defects in the global architecture of the spleen, including aberrant B cell follicular areas, substantial numbers of germinal center B cells, with evidence of class switching and hypergammaglobulinemia, developed in Foxp3−/− mice.

**Autoantibody production in Foxp3-deficient mice**

Due to the observed alterations in B cell development and the evolution of autoimmunity in IPEX patients, we next determined whether autoantibodies were present in Foxp3−/− mice and enumerated the frequency of autoreactive B cells actively secreting autoantibody in wild-type compared with Foxp3−/− mice.

Our results indicated that Foxp3−/− mice produce significant amounts of Abs specific against DNA, with increased levels of anti-DNA autoantibodies of total, IgM, and IgG isotypes over a range of serum dilutions (Fig. 7A). Furthermore, the autoantibody repertoire in Foxp3−/− mice was diverse, evident by the cytoplasmic, perinuclear, and nuclear staining of HEp-2 cells (Fig. 7B, left panel). Furthermore, these results were significant down to serum titers of ∼1/320 (Fig 7B, right panel), indicating the presence of significant autoantibody titers.

Whereas the presence of serum autoantibody indicates the conversion of autoreactive B cells into Ab-secreting cells (ASCs), it fails to generate a quantitative measure of how many autoreactive B cells are actively secreting autoantibody at any moment in time. Therefore, we performed an ELISPOT assay to enumerate the frequency of anti-DNA–specific ASCs in Foxp3−/− mice compared with controls. Our results indicated that spleen and lymph node from Foxp3−/− mice contained an average of 2232 and 5107 anti-DNA ASCs per million total B cells, respectively, as opposed to only 6 and 15 anti-DNA ASCs per million total B cells in Foxp3+ mice (Fig. 7C). Data obtained from bone marrow, although trending toward an increase in the number of anti-DNA ASCs in Foxp3−/− mice, were not statistically significant (Fig. 7C). Additional experiments showed that the adoptive transfer of regulatory T cells, alongside naive Th cells as a source of inducible regulatory T cells, at birth, was sufficient to reverse the generation of anti-DNA autoantibodies in Foxp3−/− mice (Fig. 7D).

These data suggested that B cell tolerance was abrogated in Foxp3−/− mice, allowing autoreactive B cells to differentiate into ASCs and secrete autoantibody. Furthermore, the reversal of autoantibody production in Foxp3−/− mice, by the provision of regulatory T cells, confirmed that a B cell-extrinsic process initiates this phenomenon.

**Failure of B cell anergy as a mechanism for autoantibody production**

Next, we investigated whether the presence of autoantibodies in Foxp3−/− mice paralleled a loss in B cell anergy. To perform these experiments, we bred the Ars/A1 model of B cell anergy onto the Foxp3−/− background (27). Low surface expression of IgM is a common feature of anergic B cells, and an increase in expression of surface IgM is associated with a loss of B cell anergy (1, 7, 32, 33). We therefore investigated whether the absence of Foxp3 would lead to an increase in surface IgM expression on Ars/A1 B cells. As expected, Ars/A1 B cells from Foxp3−/− mice exhibited low levels of surface IgM relative to wild-type B cells from Foxp3+ mice. In contrast, Ars/A1 B cells from Foxp3−/− mice expressed high levels of surface IgM compared with Ars/A1 B cells from Foxp3+ mice consistent with a loss of B cell anergy (Fig. 8A, left panel).

Despite exhibiting a defect in the ability to upregulate activation markers, such as CD80 and CD86, following ex vivo aggregation of their BCRs, anergic B cells frequently express elevated basal levels of these activation markers (6, 11, 34, 35). We showed that Ars/A1 B cells, as previously reported, express high basal levels of CD80 and CD86 relative to wild-type B cells (Fig. 8A, middle and right panels).
panels). In contrast, Ars/A1 B cells isolated from Foxp3− mice expressed substantially increased levels of both CD80 and CD86 (Fig. 8A, middle and right panels). This suggested B cells, including those of anergic origin, undergo significant activation in the absence of Foxp3. Anergic B cells exhibit attenuated signaling mediated by BCR. This includes elevated basal ERK–MAPK that fails to be induced further upon aggregation of the BCR (6, 27). We showed that Ars/A1 B cells that have developed in the absence of Foxp3 regain their ability to induce ERK–MAPK phosphorylation following aggregation of the BCR (Fig. 8B). Interestingly, Foxp3 deficiency failed to restore basal ERK–MAPK levels of Ars/A1 B cells to control levels; indeed, B cells from Foxp3-deficient mice exhibit enhanced levels of basal ERK–MAPK phosphorylation.

In addition to surface phenotype and signaling profiles, the attenuated secretion of Ab by anergic B cells provides an overall indicator of the strength of B cell anergy. Therefore, we examined whether arsonate-specific Abs would be present in the serum of Ars/A1 mice deficient in Foxp3. Arsonate-specific Abs were not observed in serum from wild-type.Foxp3 mice and at low levels in serum from Ars/A1.Foxp3 and wild-type.Foxp3 (Fig. 8C). In contrast, serum from Ars/A1.Foxp3 mice contains significant titers of anti-arsonate Ab, suggesting a loss of B cell anergy has occurred. Additional studies have been performed confirming the specificity of this assay for anti-arsonate Abs, and no cross-reactivity was observed using serum from Ars/A1.Foxp3 mice with nitrophenyl, OVA, or BSA (data not shown). These data suggested that B cell anergy is inappropriately regulated in the absence of Foxp3.

**Discussion**

Our data suggested that the absence of regulatory T cells, due to Foxp3 deficiency, resulted in markedly abnormal B cell development in the bone marrow and spleen. These defects are likely B cell extrinsic, as Foxp3 is not expressed in B cells. The absence of regulatory T cells also led to a loss of B cell anergy, which provides an explanation for the robust production of autoantibodies in IPEX patients and Scurvy mice. However, it remains to be determined whether the loss of B cell anergy is the result of direct, or indirect, interactions between regulatory T cells and autoreactive B cells. A direct interaction is supported by studies showing regulatory T cells can suppress in vitro autobody secretion or class switch recombination (25, 36). An indirect interaction is supported by evidence that Th cells, resistant to the suppressive effect of regulatory T cells, contribute to the production of autoantibodies in a model of lupus (37). Therefore, we postulate that inappropriately T cell help, prompted by the loss of T regulatory cells, may be a contributing factor in the loss of B cell anergy. This is supported by observations by Cook et al. and Seo et al. (10, 13), showing altered responses of autoreactive B cells in the presence of T cell help.

The observed alterations in B cell development pose a number of interesting questions, particularly based on the observed changes in the bone marrow. Although our data would suggest a significant decrease in the output of B cells from the bone marrow, an observation supported by a similar reduction in B cell numbers in the spleen, Foxp3− mice are nonetheless hyperimmunoglobulinemic (26). Additional data suggest that B cell numbers are normal in both inguinal and brachial lymph nodes (Fig. 3), suggesting alterations in peripheral B cell numbers may partly reflect migration/homing processes, possibly to sites of pre-existing inflammation due to the lack of regulatory T cells. This still fails, however, to explain the severe reduction in bone marrow B cell numbers, although our data would suggest B cells from Foxp3− mice exhibit no inherent defect in IL-7–mediated expansion. We postulate that Foxp3− bone marrow may be deficient in a B cell progenitor population. An alternative hypothesis for the observed defects in early B cell development can be drawn from studies by Nakamura et al. (38). In these studies, SHIP-deficient mice were shown to exhibit similar defects in early B cell development to those observed in our Foxp3-deficient mice. The defect in SHIP-deficient mice was attributed to the overproduction of IL-6, suppressing B cell development. Interestingly, IL-6 levels are elevated in Foxp3-deficient mice (26), so it is possible a similar process is occurring. Therefore, an increase in IL-6, or another soluble factor, caused by the loss of Foxp3, may contribute to suppression of early B cell development.

Our data also highlight a number of other interesting findings. First, the expansion of an IgM+ B220+ CD43+ in the bone marrow of Foxp3− mice (Fig. 1C) may represent a previously described NK progenitor population (39). Second, we also observed a significant increase in the proportion of CD19+ GL7+ cells in Foxp3− mice (Fig. 6C). These cells most likely represent T cells or NKT cells as they are largely CD3−Gr-1− and are highly activated as they express high levels of CD69 (data not shown). Lastly, the presence of low titers of Ars-specific Abs in wild-type Foxp3− serum suggests the global activation of B cells on this background (Fig. 8C), data consistent with the hyperimmunoglobulinemia previously observed in this model (26).

In summary, our data show that B cell development and anergy are significantly altered in the absence of regulatory T cells. The loss of B cell anergy suggests that regulatory T cells may represent a key modulator of autoreactive B cells in a range of autoimmune disorders and not just IPEX. Indeed, alterations in regulatory T cell numbers or function have been reported in numerous autoimmune conditions, including systemic lupus erythematosus, type 1 diabetes, and multiple sclerosis (reviewed in Refs. 40 and 41). Targeting or enhancing this interaction could be exploited for the treatment of autoimmune disorders.

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**Disclosures**

The authors have no financial conflicts of interests.

**References**

REGULATORY T CELLS AND B CELL ANERGY


Supplemental figure 1. **B220⁺ cells fail to express Foxp3.** Representative flow cytometric analysis of bone marrow and spleen from Foxp3⁺eGFP mice. Numbers show percentage of Foxp3⁺ cells within the lymphocyte gate (cells were also stained with antibodies specific for CD4 or B220). The expression of Foxp3 was determined by the expression of eGFP. Data is representative of 2 independent experiments using a total of 6 Foxp3⁺eGFP mice.

Supplemental figure 2. **Normal expansion of bone marrow B cells from Foxp3 mice in *in vitro* IL-7 driven bone marrow cultures.** (A) Left panel; mean number of B cells in each B cell subset (using staining criteria used in Fig. 1B) from bone marrow cultures (3 x 10⁶ total bone marrow cells) at day 0. Right panel; mean number of B cells in each B cell subset (using staining criteria used in Fig. 1B) from same bone marrow cultures at day 6. Each panel shows mean ± S.E.M. of 5 independent cultures, each generated using bone-marrow from an individual mouse (Foxp3⁺ and Foxp3⁻). (B) Fold increase in total B cell (B220⁺) numbers after day 6 of bone marrow IL-7 culture (mean ± S.E.M. of data obtained from 5 independent bone marrow cultures using 5 different mice (Foxp3⁺ and Foxp3⁻).

Supplemental figure 3. **Phenotypic analysis of B cell subsets in the spleens of Foxp3⁻ mice.** Splenocytes from Foxp3⁺ or Foxp3⁻ cells were stained with antibodies specific for B220, CD93, CD23 and IgM. Cells in the lymphocyte gate were gated into 2 populations; B220⁻CD93⁻ and B220⁻CD93⁺. These gates were further sub-gated using CD23 and IgM. B220⁺CD93⁺IgM⁺CD23⁻ (T1), B220⁺CD93⁺IgM⁺CD23⁺ (T2), B220⁺CD93⁺IgMloCD23⁺ (T13/An1), B220⁺CD93⁻IgMloCD23⁺ (mature/follicular), B220⁺CD93⁻IgMhiCD23⁻ (marginal zone).