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Modeling the Clinical Phenotype of BTK Inhibition in the Mature Murine Immune System

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Inhibitors of Bruton’s tyrosine kinase (BTK) possess much promise for the treatment of oncologic and autoimmune indications. However, our current knowledge of the role of BTK in immune competence has been gathered in the context of genetic inactivation of btk in both mice and man. Using the novel BTK inhibitor PF-303, we model the clinical phenotype of BTK inhibition by systematically examining the impact of PF-303 on the mature immune system in mice. We implicate BTK in tonic BCR signaling, demonstrate dependence of the T3 B cell subset and IgM surface expression on BTK activity, and find that B1 cells survive and function independently of BTK. Although BTK inhibition does not impact humoral memory survival, Ag-driven clonal expansion of memory B cells and Ab-secreting cell generation are inhibited. These data define the role of BTK in the mature immune system and mechanistically predict the clinical phenotype of chronic BTK inhibition. The Journal of Immunology, 2014, 193: 000–000.

B cell receptor–mediated signaling processes serve as checkpoints permitting the continuation of B cell development along with the culling of cells into particular B cell subset fates; ultimately, and with the appropriate secondary stimuli, B cells are activated to differentiate further into effector, memory, and potentially regulatory populations (1, 2). The role of BCR signals in B cell fate at numerous junctures in development predict that clinical pharmacologic inhibition of BCR signaling will impact various B cell parameters in both mouse models and in human patients.

Bruton’s tyrosine kinase (BTK) is required for B cell development, survival, proliferation, and differentiation (3–7). This has been demonstrated largely through studies from X-linked agammaglobulinemia (XLA) patients, xid mice, and btk-deficient mice (4, 5, 8–11). In a btk-null setting, profound B cell developmental defects occur. These result in peripheral B cells presenting at low frequencies and in an immature state and containing a perturbed BCR repertoire; in addition, the B1 cell lineage is absent (4, 5, 12, 13). In particular, XLA patients display a profound defect in B cell development and peripheral B cell accumulation, leading to severely impaired humoral immunity that is characterized by diminished serum Ig levels and abnormal splenic architecture (14). Resulting from the near ablation of B cells in these patients and the consequential hypogammaglobulinemia, patients typically manifest with recurrent bacterial infections. The xid strain of mice develop a less dramatic phenotype than XLA patients as xid B cells cells can successfully develop into immature B cells. Defects are observed in xid mice in naive mature follicular (FO) B cell frequency, B1 cell development, and serum Ig levels (4, 5, 15–17).

The obligate role of BTK in transmitting activating signals downstream of the BCR has generated much effort to pharmacologically target BTK in B cell–driven maladies. Indeed, in preclinical models, BTK inhibitors result in therapeutic efficacy in oncologic and autoimmune settings, including models of rheumatoid arthritis and systemic lupus erythematosus (SLE) (18–24). In the clinic, promising results with the BTK inhibitor ibrutinib have been observed in relapsed chronic lymphocytic leukemia and relapsed or refractory mantle cell lymphoma (25, 26). However, the role of BTK in B cell development and in generating and sustaining a fulminate humoral immune response has been defined largely in the absence of a functional btk gene, either through the use of xid- or btk-knockout mice (4, 5, 27–29), or by characterizing B cells from, and the overall immune competence of, XLA patients (30–32). A systematic study of how BTK inactivation in an otherwise wild-type setting impacts the mature immune system has not been performed.

Knowledge of how BTK inhibition impacts the wild-type B cell pool and humoral immune responses will predict the immune competence of patients undergoing long-term BTK inhibition and may predict, mechanistically, the B cell–driven maladies that are sensitive to BTK inhibition. In this study, the novel BTK inhibitor PF-303 was used to explore the impact of BTK inhibition in both acute and chronic settings on the mature immune system in mice.
Materials and Methods

Mice and immunizations

All procedures using animals were in accordance with regulations and established guidelines and were approved by the Pfizer Institutional Animal Care and Use Committee. CBA/CaJ, CBA/CaHN-Btk<sup>−/−</sup> (sid), B6.SJL-<sup>Pepc<sup>−<sup>/BoyJ (CD45.1) C57BL/6J, BALB/cJ, OT2 (B6. Cg-TacraTercb425CbnJ), and B1-<sup>−<sup>/ (Cby.129P2(B6)Igh < tm1Mnz > J) were bred in the laboratory (Bar Harbor, ME); C57BL/6 RAG2<sup>−/−</sup> mice were obtained from Taconic (Hudson, NY). All mice were used between the ages of 8 and 12 wk. The following immunogens were used: NP-83 (25 μg), NP<sub>2</sub>-CGG (25 μg), NP<sub>2</sub>-CGG (25 μg), and NP-Ficoll (100 μg) (all from Biosearch Technologies); PE (10 μg); Chromoaphore; alurn (100 μl); Thermo Fisher Scientific), and ODN1826 (25 μg; InvivoGen). For in vivo B cell activation, 200 μl anti-CD40 (clone 3H10; BioLegend) was used. 100 μl of PBMCs in RPMI 1640 containing 10% FCS were added to a 10 cm dish and incubated at 37˚C in 5% CO₂ for 7 days. On day 4, NP-LPS (25 μg) was added to the culture with 200 μl of media. On day 7, the B cells were harvested and used for subsequent experiments.

Transcriptional profiling

Total RNA from ex vivo–harvested B cells was purified using the RNeasy Mini Kit (QIAGEN), as per the manufacturer’s protocols. Following the manufacturer’s recommendations, 150 ng total RNA from each sample was used to prepare biotinylated cRNA for analysis on the Mouse Genome 130 1.0 expression arrays (Affymetrix). Sample generation, processing, labeling, and fragmenting were performed using the GeneChip 3 IVT Express Kit (Affymetrix). A GeneChip Hybridization, Wash, and Stain Kit was used for hybridization to the mouse arrays on a Fluidics Station 450 (both from Affymetrix). Finally, arrays were scanned using a GeneChip Scanner 3000 7G (Affymetrix) with auto loader, following the standard protocol. Gene expression profiling data were extracted using Affy (Bioconductor Microarray Expressions (MAS 5.0) software and used for subsequent statistical analyses. Analyses were performed using GeneData Expressionist. Only transcripts that were called as present in 80% of the samples and expressed at an average signal level >20 signal units in any one group were used for further analysis. A total of 20,028 qualifiers met these criteria and were carried forward in the analysis. Data QC identified one strong outlier sample in the “PBS, vehicle” group that was subsequently removed from further analysis. All microarray data are available in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE56483.

Flow cytometry

Single-cell suspensions from spleens were generated using Miltenyi Biotec C-tubes with GentleMACS, as per the manufacturer’s instructions. Single-cell suspensions from spleen were treated with RBC lysis buffer (Sigma) and washed with FACS buffer (PBS with 1% FCS). Cells were counted on a Guava ViaCount (Millipore). Cells were incubated with anti-CD16/32 (Fc block; clone 2.4G2, BD Pharmingen), washed, and stained with primary Abs diluted in PBS for 30 min at 4˚C. Abs used in these studies included CD11b-PerCP Cy5.5, CD3-PerCP Cy5.5, B220–Alexa Fluor 700, CD38–Alexa Fluor 647, and CD45–allophycocyanin Cy7 (all from BioLegend); B220–V450, Gr1-PerCP Cy5.5, CD68–PE, IgD–V450, IgG1–FITC, CD4–PE, CD3–FITC, IgG2a–FITC, and IgG2b–PE (all from BD Pharmingen); CD3–PE, B220–allophycocyanin Cy7, IgM–allophycocyanin, IgM–eFluor 650, and CD21/35–allophycocyanin Cy7 (all from eBioscience); and PNA–FITC (Vector). PE was used to detect PE-specific B cells at a concentration of 0.5 μg/ml. BrdU incorporation was detected using an anti-BrdU–Alexa Fluor 488 Ab and intracellular stain protocol, according to the manufacturer’s instructions (eBioscience). To detect IgD<sup>+</sup> B cells by FACS, spleenocytes were depleted of IgD<sup>+</sup> cells using anti-IgD–biotin–streptavidin magnetic beads and negative selection over a magnetized column (Miltenyi Biotec). OT2 T cells were stained with 5 μM CFSE (Sigma) for 10 min at 37˚C in RPMI 1640, quenched with RPMI 1640 + 5% FBS, and washed. Cells also were stained with LIVE/DEAD Fixable (Invitrogen), per the manufacturer’s instructions. Between 200,000 and 2,000,000 live events were counted using a Fortessa flow cytometer (Becton Dickinson) and analyzed used FlowJo software (TreeStar). All samples were gated first on “live” (Aqua LIVE/DEAD<sup>+</sup>) singlets. In some experiments, assessment of BWC populations was quantified using a CELL-DYN (Abbott) prior to further analysis.

BAFFR-Fc

An expression construct for the soluble BAFFR-Fc was generated by fusing a DNA sequence encoding an Ig signal peptide, the extracellular domain of BAFFR to a human IgG1 heavy chain and using the qScript One-Step Fast qRT-PCR Kit, ROX (Quanta Bio). The human IgG1 heavy chain was used to generate IgG1-specific B cells at a concentration of 0.5 μg/ml. BrdU incorporation was detected using an anti-BrdU–Alexa Fluor 488 Ab and intracellular stain protocol, according to the manufacturer’s instructions (eBioscience). To detect IgD<sup>+</sup> B cells by FACS, spleenocytes were depleted of IgD<sup>+</sup> cells using antibody-Fc-specific magnetic beads, and negative selection over a magnetized column (Miltenyi Biotec). OT2 T cells were stained with 5 μM CFSE (Sigma) for 10 min at 37˚C in RPMI 1640, quenched with RPMI 1640 + 5% FBS, and washed. Cells also were stained with LIVE/DEAD Fixable (AquA LIVE/DEAD<sup>+</sup>) (Invitrogen), per the manufacturer’s instructions. Between 200,000 and 2,000,000 live events were counted using a Fortessa flow cytometer (Becton Dickinson) and analyzed used FlowJo software (TreeStar). All samples were gated first on “live” (Aqua LIVE/DEAD<sup>+</sup>) singlets. In some experiments, assessment of BWC populations was quantified using a CELL-DYN (Abbott) prior to further analysis.

Adoptive transfers

For T2/T3 adoptive transfers, a two-step purification method was used to purify T2 and T3 B cells. CD23<sup>−</sup> cells were purified from the spleens of 20 C57BL/6J mice by staining with CD93–PE, following by positive selection using anti-PE magnetic MicroBeads and MACS LS columns (both from Miltenyi Biotec). Following the manufacturer’s instructions. T2 and T3 cells were sorted to purity on a FACs Aria II. Between 1 and 3 × 10<sup>6</sup> cells of each subset were obtained per sort. For B1 B cell adoptive transfers, peritoneal cavity contents from C57Bl/6J mice were obtained by peritoneal lavage. Cells were washed with cold PBS/0.5% BSA/2 mM EDTA and transferred into RAG2<sup>−/−</sup> mice at a 1:1 donor/host ratio. In all
adoptive-transfer experiments, cells were transferred i.v. in a volume of 200 μl ice-cold PBS. When recipient mice would be receiving PF-303 for the duration of the experiment, they were dosed with inhibitor by mouth 2 h prior to adoptive transfer of cells. For memory B cell adoptive transfers, spleens were harvested from BALB/c mice that were immunized 90 d prior with PE/alum and crushed into single-cell suspensions. CD138+ cells were depleted by incubating the cells with CD138-PE Ab (BD Pharmingen), followed by anti-PE MicroBeads (Miltenyi Biotec), and flowing the cells through a magnetic column. CD138+ cell frequency was reduced from 0.42 and 0.342% to 0.0657 and 0.0215% of total cells, respectively, in two experiments. The flow-through was adoptively transferred at 40–60 × 10^6 cells/host. Eighteen hours later, mice were dosed with either vehicle or 10 mg/kg PF-303 and challenged 2 h later with 10 μg PE in PBS. Serum Ab titers were assessed 96 h after challenge.

In vitro assays

For the IgM-internalization assay, B cells were purified by negative depletion (B Cell Isolation Kit; Miltenyi Biotec) and kept on ice. Cells were incubated with 10 μM PF-303 or fostamatinib for 30 min on ice, with inhibitor kept in solution for the duration of the experiment. Cells were incubated with 10 μg/ml goat anti-IgM Fab-biotin or F(ab')2-biotin on ice. An aliquot of unlabeled cells was set aside as a negative control. B cells were plated at a density of 100,000 cells/well in a 96-well plate and suspended in media prewarmed to 37°C. At the indicated time points, B cells were removed in triplicate at a volume of 100 μl and placed in a 96-well plate containing 100 μl 4% formaldehyde on ice. Subsequently, plates were washed five times with PBS, and IgM expression levels were detected using SA-FITC by FACS. Data are plotted as geometric mean fluorescent intensity (GMFI) relative to T = 0 GMFI. Where indicated, mice were pretreated for either 2 h or 5 d with 30 mg/kg PF-303, quaque die dosing. For B cell/OT2 coculture experiments, B cells were incubated first with 10 μg/ml F(ab')2-biotin (Jackson ImmunoResearch), washed in cold PBS, and incubated with the OVA Ag-delivery reagent (Miltenyi Biotec). ISQ peptide was added at a concentration of 10 μM where indicated. CFSE-stained OT2 cells (50,000) were added to OVA-targeted B cells (50,000) and incubated for 72 h in a round-bottom 96-well plate in a volume of 200 μl.

FIGURE 1. Impact of BTK inhibition on primary Ag-driven Ab responses. (A) Structure of PF-303. (B) Assessment of PF-303 in an in vivo BCR-mediated B cell activation assay. Mice were gavaged with the indicated dose and injected i.p. 2 h later with either anti-IgD anti-sera or anti-CD40 Ab. The expression levels of CD86 on spleen B220+ B cells were quantified by FACS 18 h later. (C) In vivo IC_{50} of PF-303 correlates with plasma concentrations of PF-303 quantified at the time of anti-IgD injection and suppression of CD86. C57BL/6 mice (D–F), BALB/c mice (G), or B1-8 hi mice (H) were gavaged with 10 mg/kg of PF-303 or vehicle and immunized i.p. 2 h later with the indicated immunogen. Mice were provided vehicle or compound quaque die dosing, and the levels of anti-NP or anti-PE IgM or IgG were quantified on day 7. Representative data from n = 2 (E and H) and n = 4 (D, F, and G) experiments. Results of a Student t test are shown as comparisons between the indicated experimental groups: *p < 0.01, ***p < 0.0001. ns, not significant.
Results

**Impact of BTK inhibition on primary Ag-driven Ab responses**

The compound (R)-5-amino-3-(4-(4-chlorophenoxy)phenyl)-1-(1-cyanopiperidin-3-yl)-1H-pyrazole-4-carboxamide (PF-303; structure shown in Fig. 1A) is a potent, orally bioavailable BTK inhibitor. The cyanamide moiety in PF-303 forms a covalent, but reversible, adduct between BTK through modification of a cysteine residue (Cys481) proximal to the ATP-binding pocket. Enzymatic assays demonstrated high potency (IC$_{50}$ = 0.64 nM; Kinact/ KI = 143,900 M$^{-1}$s$^{-1}$) toward BTK; in comparison with irreversible covalent BTK inhibitors, which exhibit Koff$^{1/2}$ = 24 h, PF-303 exhibited a Koff$^{1/2}$ = 5 h. Upon evaluation of the selectivity of PF-303 toward other kinases containing a conserved cysteine residue homologous to Cys481, comparable potency toward BMX and TEC was demonstrated; however, high selectivity was achieved for other members, including ITK and JAK3 (>10,000-fold selective). High kinase selectivity also was seen for the closely related Src family kinases, including LYN, SRC, and LCK (2000–7000-fold selective). Potency in inhibiting anti-IgM F(ab')$_2$-driven B cell proliferation in vitro was observed in mouse primary B cell assays with an IC$_{50}$ of 2 nM (data not shown).

A short-term in vivo model in which naive B cells are activated by injection of agonistic anti-IgD was first used to assess the impact of PF-303 on BCR-mediated B cell activation. Of note, xid mice were reported to be hyporesponsive to this method of in vivo B cell activation (33). In vivo treatment of mice with PF-303 2 h prior to anti-IgD injection prevents, in a dose-dependent manner, anti-IgD–driven CD86 induction by B cells, yet it does not impact CD40-mediated CD86 induction, as assessed by ex vivo analysis (Fig. 1B). The impact of PF-303 in Ags eliciting T-independent (TI) type I or type II or T-dependent (TD) Ab responses were assessed next. PF-303 prevents Ab responses to the TI type II Ag NP-Ficoll (Fig. 1D) but does not impact responses to the TI type I Ag NP-LPS (Fig. 1E) while minimally impacting responses to the TD Ags NP-CGG and PE (Fig. 1F, 1G). These data are in agreement with studies using xid- and BTK-knockout mice (4, 29, 34, 35).

By using the V$_{H}$B1-8hi BCR-transgenic mouse (36), we assessed the impact of PF-303 on TD Ab responses elicited in response to either high- or low-affinity cognate BCR/Ag interactions.

![FIGURE 2. Impact of PF-303 on the B cell transcriptome and implication of BTK in tonic BCR signaling.](http://www.jimmunol.org/)

(A) Experimental outline. (B) Principal component analysis showing the relatedness in expression patterns between the four experimental cohorts. (C) K-means clustering of 6495 BCR-dependent transcripts (ANOVA; p < 0.001; false discovery rate < 0.0031) identified four main clusters. The number of transcripts aligning to each of these clusters is shown. Venn diagrams show overlap between BCR-dependent and BTK-dependent transcripts (D), BCR-dependent versus BTK-dependent transcripts in quiescent cells (E), and BTK-dependent transcripts in activated versus quiescent B cells (F). The numbers of transcripts and gene symbols of representative transcripts associated with each group are depicted. (G) Transcripts induced by BCR signaling were analyzed for their BTK dependence by QRT-PCR. Experimental setup is the same as in (A); data were compiled from three independent experiments.
BTK inhibition mildly impacted responses driven by low-affinity interactions, whereas high-affinity responses were significantly reduced (Fig. 1H). These data indicate that BTK inhibition effectively inhibits BCR-mediated B cell activation and responses to TI type II Ags and TD Ab responses driven by high-affinity Ag/BCR encounters.

Impact of PF-303 on the B cell transcriptome and implication of BTK in tonic BCR signaling

We next investigated the impact of PF-303 treatment on the in vivo B cell transcriptome. Mice were dosed with PF-303, injected with agonistic anti-IgD 2 h later, and B220+CD23+ B cells were isolated and transcriptionally profiled after an additional 2 h (Fig. 2A). Pharmacokinetic and pharmacodynamic modeling demonstrated that BTK was fully occupied by PF-303 for the duration of the experiment (data not shown). Principal component analysis of profiled B220+CD23+ B cells indicated a substantial impact of PF-303 on both anti-IgD-treated and untreated groups (Fig. 2B). ANOVA analysis of the four treatment groups identified 6495 transcripts that were modulated by anti-IgD treatment (p < 0.001); these transcripts were deemed BCR dependent. Of the BCR-dependent transcripts, K-Means clustering was used to identify four clusters (Fig. 2C), with BTK-independent or -dependent transcripts additionally identified whose expression either increased or decreased during anti-IgD treatment. ANOVA analysis also identified 2463 BTK-dependent transcripts modulated by PF-303 treatment from the four treatment groups (p < 0.001). Demonstrating that BTK modulates BCR-driven signals in this setting, a large majority (78%) of BTK-dependent transcripts also were BCR dependent (Fig. 2D). In quiescent B cells, PF-303 treatment modulated 537 transcripts (p < 0.01, Student t test) (Fig. 2E, 2F); of these transcripts, the majority (70%) also were BCR dependent (Fig. 2E), indicating that PF-303 is modulating transcripts downstream of tonic BCR signals in quiescent B cells. Of the transcripts modulated by PF-303 in quiescent and anti-IgD-activated B cells, 231 transcripts were found to overlap (Fig. 2F), with 43% of BTK-dependent transcripts in quiescent B cells similarly BTK dependent in the setting of acute anti-IgD-mediated BCR activation. Finally, targets previously demonstrated to be induced early after BCR signaling were examined by QRT-PCR analysis (Fig. 2G) (37, 38), with anti-IgD-driven induction of c-Myc, Bcl-xL, CCL3, EGR1, CD98HC, EB12, EGR2, and IRF4 transcripts determined to be dependent on BTK activity. Collectively, these data identify BTK-dependent transcripts downstream of acute and tonic BCR signals.

BTK inhibition suppresses cognate B cell–mediated activation of T cells and germinal center initiation and progression

The data presented thus far demonstrate that PF-303 inhibits BCR signaling and negatively impacts various Ag-driven Ab responses.

**FIGURE 3.** BTK inhibition inhibits cognate B cell–mediated activation of T cells and GC initiation and progression. Impact of BTK on BCR-mediated Ag internalization. (A) B cells from wild-type or xid mice were bound with biotin anti-IgM F(ab')2 fragments. In the presence of the indicated inhibitors during culture at 37°C and as a function of time, the rate of internalization of IgM in comparison with 4°C controls was assessed by quantifying the rate of loss of biotin anti-IgM from the cell surface by FACS (% internalization in comparison with T = 0). (B) To assess the ability of B cells to present Ag to cognate OT2 CD4 T cells, OVA was targeted to the BCR using anti-IgM F(ab')2 fragments in the presence of PF-303 (blue) or vehicle (red). The OVA 232–339 peptide was used as a positive control, as indicated. Proliferation was measured by CFSE dye dilution. (C) C57BL/6 mice were gavaged with 10 mg/kg of PF-303 or vehicle and were injected i.p. with SRBCs 2 h later. Mice were dosed every day, and splenic GC B cell frequencies were assessed by FACS on day 7. (D) Mice were immunized with SRBCs i.p. and, starting on day 7 postimmunization, were dosed every day with 10 mg/kg PF-303 or vehicle, as indicated. Staggered cohorts were assessed 1 and 2 d after the initiation of dosing, with splenic GC B cell frequencies assessed by FACS. (E) Peyer’s patches from mice treated every day for 14 d with 10 mg/kg of PF-303 or vehicle were assessed for GC B cell frequencies by FACS. Dump gate = CD3, CD11b, and GR1. For graphs in (C)–(E), each symbol represents an individual mouse. The results of a Student t test are shown. Data are representative of n = 2–4 independent experiments.
We next further examined the impact of BTK inhibition on TD immune responses. First, we observed that, in vitro, PF-303 and the SYK inhibitor fostamatinib did not impact the BCR internalization rate by B cells following acute BCR cross-linking by F(ab')₂ fragments (Fig. 3A). However, when OVA Ag was targeted to a F(ab')₂–cross-linked BCR and presented by B cells to cognate OT2 CD4 T cells, we observed that PF-303 suppressed OT2 T cell proliferation, as assessed by CFSE dye dilution (Fig. 3B). This suppression could be largely overcome by the exogenous addition of the OVA₃₂₃₋₃₃₉ peptide. These data indicate that BTK is required for fulminate B cell–mediated cognate activation of CD4 T cells in vitro (Fig. 3B).

The xid mouse, in response to TD Ags, forms a diminished, but not eliminated, intrafollicular germinal center (GC) reaction capable of somatic hypermutation and memory B cell generation (27). In vivo, PF-303 led to a complete suppression of GC (defined as B220⁺Dump⁺CD38loPNA⁺) onset in mice immunized with SRBCs 7 d prior (Fig. 3C). PF-303 also was capable of eliminating already-established SRBC-elicited GCs within 48 h of PF-303 treatment initiation (Fig. 3D). When assessing the impact of chronically dosed PF-303 on endogenous gut-associated GCs, we observed that 14 d of dosing led to a significant reduction in the frequency of Peyer’s patch GC B cells (Fig. 3E). Together, these data demonstrate that BTK inhibition suppresses cognate B cell–mediated activation of CD4 T cells, prevents the generation of GCs, and drives the dissolution of established GCs.

Chronic BTK inhibition perturbs peripheral B cell subsets and abrogates the T3 B cell subset

Given the paucity of mature B cells in xid and XLA hosts, we examined the impact of chronic PF-303 dosing on B cell subset frequencies. After 14 d of treatment with a dose of PF-303 providing full occupancy of BTK over the entire dosing period, B220⁺ B cells were decreased in the spleen and lymph nodes but not in the peripheral blood (Fig. 4A, lymph node data not depicted). Serum BAFF levels were increased in PF-303–treated cohorts (Fig. 4B). Upon further dissection of the B220⁺ population, a significant decrease in number was observed in the mature naive FO and marginal zone B cell subsets (Fig. 4C, 4D). The most dramatic impact of PF-303 was the dose-dependent ablation of the T3 B cell subset in all organs examined (Fig. 4C–E); notably, the T3 B cell subset

FIGURE 4. Chronic BTK inhibition perturbs peripheral B cell subsets. CBA/CaJ mice were treated with vehicle or 1 or 5 mg/kg of PF-303, twice a day, for 14 d. (A) Percentage of B220⁺Dump⁺ cells was assessed by FACS. (B) Serum BAFF levels in the indicated cohorts. (C) FACS performed on spleens; the percentage of each B cell subset is indicated. The number of each B cell subset in the spleen (D) and the percentage of each B cell subset in the peripheral blood (E). In (A), (B), (D), and (E), each symbol represents an individual mouse, with the horizontal line representing the mean. The percentage change in means between treatment groups are depicted in some comparisons. *p < 0.01, **p < 0.001, ***p < 0.001, Student t test. PB, peripheral blood; Spl, spleen.
was the only subset modulated by PF-303 in the peripheral blood (Fig. 4E). The kinetics of T3 B cell frequency in the peripheral blood were examined in a pulse-chase experiment, with T3 B cell abrogation detectable as early as 4 d after the initiation of dosing (Supplemental Fig. 1A). Importantly, and indicating that a 14-d dosing period may be an appropriate length of time to assess the impact of chronic dosing of BTK inhibitors, longer dosing periods do not amplify changes observed upon chronic PF-303 dosing, including decreased T3 B cell frequency and total and FO B cell subset frequencies in the peripheral blood (Supplemental Fig. 1). These data show that chronic BTK inhibition reduces the total numbers of various B cell subsets in secondary lymphoid organs but not the peripheral blood, and it abrogates the presence of the T3 B cell subset.

The abrogation of the T3 B cell subset is consistent with the absence of this subset in xid mice (39). However, it is not known whether BTK is required for the development of the T3 B cell subset or for its survival and persistence. We next determined how BTK function impacts the T3 B cell subset. We FACS sorted and adoptively transferred CD45.2+ T2 (defined as B220+Dump CD93+IgMhiCD23+) or CD45.2+ T3 (defined as B220+Dump CD93+IgMloCD23+) B cell subsets into CD45.1+ recipients; after 4 d of vehicle or PF-303 treatment, the developmental destination of the cells was determined by FACS. T2 cells transferred into vehicle-treated mice remained as T2 B cells (14% of transferred cells) or differentiated further into T3 B cells (15%) or FO B cells (52%) (Fig. 5A). When T2 B cells were transferred into PF-303–treated hosts, the cells were instead blocked from differentiating further into T3 B cells (7.5%) and preferentially remained T2 cells (10.7%) or differentiated into FO cells (64%) (Fig. 5B). Strikingly, upon adoptive transfer of T3 cells, there was no difference in the differentiation profiles between either vehicle- or PF-303–treated hosts (Fig. 5C, 5D). Importantly, and serving as an internal control, a reduction in the CD45.1+ host T3 B cell population was observed in both PF-303–dosed cohorts compared with vehicle treatment (Fig. 5C, 5D). These data indicate that ablation of the T3 B cell subset by chronic dosing of PF-303 is the result of a developmental block imparted by BTK inhibition in the differentiation of T2 B cells into T3 B cells.

**Chronic BTK inhibition leads to increased levels of surface IgM expression by B cells and reduced IgM internalization rates**

In our chronic dosing studies, we observed that B cells from mice treated with PF-303 consistently displayed increased surface IgM expression levels compared with vehicle controls, with this increase occurring in a dose-dependent manner (Fig. 6A, 6B). Interestingly, IgD and IgG1 surface expression levels were impacted either mildly or not at all (Fig. 6C, 6D). Indeed, data collected across nine in vivo experiments using multiple doses and assessing B cells harvested from various organs demonstrated a consistent dose-dependent pattern of heightened IgM expression driven by PF-303 administration, with minor modulation of surface IgD levels (Fig. 6E). The kinetics of IgM and IgD surface expression modulation by peripheral blood B cells were examined in a long-term pulse-chase experiment, with heightened IgM expression detectable as early as 2 d after the initiation of dosing (Supplemental Fig. 1B). The expression levels of surface CD19, CD22, B220, and CD79b were also assessed, with a slight, yet significant, decrease in CD22 and B220 expression and an increase in the levels of CD79b (Fig. 6F). The ability of naive B cells to express both IgM and IgD isotypes results from an alternative splice reaction of premRNA transcripts encoding the VDJ region together with both the Igh-6 and Igh-5 C region genes. As such, transcript levels derived from both the Igh-6 and Igh-5 genes were assessed. The PF-303–driven increase in IgM surface expression levels was not represented at the mRNA level, because transcripts...
derived from both the *Igh*-6 and *Igh*-5 genes in purified B cells from chronic PF-303– versus vehicle-treated mice were unchanged (Fig. 6G). Because this suggests that a posttranscriptional regulatory mechanism is driving heightened IgM expression levels, we assessed the impact of chronic PF-303 treatment on IgM turnover. B cells were harvested from mice dosed with vehicle or PF-303 either 5 d prior to sacrifice (chronic treatment) or 2 h prior to sacrifice (acute treatment), as well as from *xid* mice. B cells from *xid* mice and mice chronically dosed with 30 mg/kg PF-303 2 h prior to sacrifice (“acute”), and from *xid* mice were tested for their ability to internalize anti-IgM Fab or F(ab')2 fragments as a function of time by FACS (percentage of internalization in comparison with T = 0). Data are representative of *n* = 2 independent experiments; compiled data are shown in (E). 

### Chronic BTK inhibition does not impact the frequency or function of B1 cells, implicating BTK as required only for B1 cell development

B1 cells are purveyors of protective and innate serological IgM and are absent in *xid* and BTK-null mice (4, 13). The temporal use of a BTK inhibitor provided an opportunity to test, in wild-type mice, whether BTK is required for the development of B1 cells or for their survival and/or function. Surprisingly, chronic treatment of mice with PF-303 revealed the sustained presence of both B1a and B1b subsets (Fig. 7A) in the peritoneal cavity. After the adoptive transfer of B1 cells into RAG2−/− recipients and treatment with vehicle or PF-303 for 14 d, two populations of IgMhi cells in both treatment cohorts were present at equivalent frequencies in the peritoneal cavities of recipient mice: a IgMhi B220intCD5+CD11b+ population consistent with a B1a phenotype and a IgM hiB220+CD5−CD11b+ population consistent with a B1b phenotype (Fig. 7B, 7C). Indicating PF-303 engagement of BTK, heightened IgM expression by transferred B1 cells was observed in the PF-303 cohort (Fig. 7D). The ability of transferred B1 cells to produce phosphorylcholine-reactive Ab was equivalent in both vehicle- and PF-303–treated RAG2−/− groups receiving B1 cells (Fig. 7E); in addition, treatment of wild-type mice with PF-303 did not impact anti-PC IgM titer (Fig. 7F). Although it is possible that the duration of chronic BTK inhibition required to impact B1 cells may exceed the 14 d time-frame assessed in these studies and that levels of PF-303 in the peritoneal cavity differ from plasma levels of PF-303, these data collectively indicate that B1 cells require BTK for their development but not persistence or function.
BTK inhibition does not impact IgG⁺ memory B cell or bone marrow–resident ASC survival

To test the impact of chronic BTK inhibition on memory B cell survival, mice immunized with the immunogen PE were treated with PF-303 and/or BAFFR-Fc, together with the corresponding vehicle and mouse Ig controls, for 14 consecutive days. Although B220⁻Dump² cells were depleted upon treatment with BAFFR-Fc alone or with PF-303 (Fig. 8A, 8B), no impact was observed on PE-specific isotype-switched (defined as B220⁺Dump²PE⁺CD38⁻IgM⁻IgD⁻) and IgG1⁺ memory B cells (defined as B220⁺Dump²PE⁺CD38⁻IgM⁺IgD⁻IgG1+) (Fig. 8C, 8D). Nonisotype-switched PE-specific memory B cells (defined as B220⁺Dump²PE⁺CD38⁺IgM⁺IgD⁺) were depleted by BAFFR-Fc treatment (Fig. 8E), indicating that IgM⁺ memory B cells require BAFF for survival. Memory B cells were still functional after a course of BTK-inhibitor treatment; dosing with PF-303 and/or BAFFR-Fc for 14 d, followed by a 5-d compound wash-out period, had no impact on anti-PE recall responses mounted after PE rechallenge (Fig. 8F, 8G). We also examined the impact of long-term dosing of PF-303 on serum Ab titers. After 2 wk of dosing, a minor, dose-dependent impact was observed on polyclonal IgG2a and IgG3 titers (Fig. 9A). Importantly, polyclonal IgG⁺ ASC numbers in the bone marrow remained unchanged between the vehicle and PF-303 cohorts (Fig. 9B). Mice containing an established pool of bone marrow–resident ASCs elicited by priming and boosting with the Ag NP₁⁹-CGG were treated with either PF-303 or vehicle for 33 d, with an additional 25 d wash-out period (Fig. 9C). Anti-NP IgG titers did not change in vehicle- or PF-303–treated cohorts for the duration of the experiment; in addition, NP-specific ASCs in the bone marrow did not differ between these two cohorts (Fig. 9D). Collectively, these data demonstrate that chronic BTK inhibition does not impact memory B cell or bone marrow–resident ASC survival or function.
BTK inhibition during Ag rechallenge suppresses memory cell clonal expansion and magnitude of the Ab burst

Because previous studies in \textit{xid} mice suggested that BTK is not required for memory B cell reactivation and differentiation into ASCs (27), the impact of PF-303 on memory B cell rechallenge was tested next. Mice with established PE-specific memory were treated with PF-303 compound or vehicle and rechallenged with PE; simultaneously, BrdU was added to the drinking water. Four days later, memory B cell proliferation was quantified by BrdU incorporation (40). Isotype-switched B220\(^+\)CD38\(^+\)PE\(^+\)IgD\(^-\) memory B cells (A and B), PE-specific isotype-switched and PE-specific IgG1\(^+\) memory B cells (A, C, and D), and PE-specific nonisotype-switched memory B cells (A and E) present in the spleen after the indicated treatment. To test the functionality of the memory B cells, mice were treated with the indicated compounds for 14 d, and compounds were allowed to wash-out for an additional 5 d after the final dose. (F and G) Mice were rechallenged with PE, and the serum anti-PE IgM and IgG titers were quantified 4 d later. Each symbol indicates an individual mouse. **\(p < 0.001\), Student \(t\) test. AUs, arbitrary units.

**FIGURE 8.** BTK inhibition does not affect the persistence or function of IgG1\(^+\) memory B cells. (A) BALB/c mice were immunized with PE/alum/CpG i.p. and rested for 90 d. Mice were gavaged with either vehicle or 5 mg/kg PF-303 twice daily and/or injected with 100 \(\mu\)g mouse Fc (mFc) or BAFFR-Fc every day for 14 d. Subsequently, both total B cell and memory B cell frequencies were assessed by FACS. Dump gate = CD3, CD11b, and GR1. Frequencies of B220\(^+\) Dump\(^-\) B cells (A and B), PE-specific isotype-switched and PE-specific IgG1\(^+\) memory B cells (A, C, and D), and PE-specific nonisotype-switched memory B cells (A and E) present in the spleen after the indicated treatment. To test the functionality of the memory B cells, mice were treated with the indicated compounds for 14 d, and compounds were allowed to wash-out for an additional 5 d after the final dose. (F and G) Mice were rechallenged with PE, and the serum anti-PE IgM and IgG titers were quantified 4 d later. Each symbol indicates an individual mouse. **\(p < 0.001\), Student \(t\) test. AUs, arbitrary units.
cells had incorporated BrdU in both treatment cohorts, indicating that memory B cells had entered the cell cycle, irrespective of BTK inhibition by PF-303 (Fig. 10A). However, upon quantification of B220<sup>-dump</sup> CD38<sup>+</sup>PE<sup>-</sup>Brdu<sup>+</sup> cells, a significant reduction in numbers was observed in the PF-303 group compared with vehicle controls (Fig. 10B). In addition, a significant reduction in serum anti-PE IgG titers was observed in the PF-303–treated group in comparison with vehicle controls (Fig. 10C). To test for an impact of BTK blockade on memory B cell reactivation, BALB/c mice were immunized with PE/alum/CpG i.p. and rested for 90 d. Mice were treated every day with either vehicle or 10 mg/kg PF-303 and rechallenged with an i.p. injection of PE in PBS 2 h after dosing. The PE–specific B cell response was analyzed 4 d later. (A) Percentage of B220<sup>-</sup>Dump<sup>-</sup>CD38<sup>+</sup>PE<sup>-</sup>Brdu<sup>+</sup> cells incorporating BrdU on day 4. (B) Total numbers of B220<sup>-</sup>Dump<sup>-</sup>CD38<sup>+</sup>PE<sup>-</sup>Brdu<sup>+</sup> cells/spleen. (C and D) Anti-PE IgM and IgG titers present 4 d after PE rechallenge in the indicated treatment groups. (E and F) Whole splenocytes from BALB/c mice immunized 90 d prior with PE/alum were depleted of CD138<sup>+</sup> cells and transferred into naive BALB/c recipient hosts. The next day, hosts were treated with either vehicle or PF-303 2 h prior to PE or PBS injection as indicated, and dosed every day with 10 mg/kg of PF-303 for the duration of the experiment. Anti-PE serum Ab titers were analyzed 5 d later. Each symbol represents an individual mouse. Data are representative of n = 2 experiments. **p < 0.001, ***p < 0.0001. AU, arbitrary unit; ns, not significant.

**Discussion**

These experiments model how pharmacological BTK inhibition, as opposed to genetic inactivation of the btk gene, will impact humoral immune parameters and mature immune responses in a clinical setting. Our results predict that modulation of tonic signaling, depletion of immature and anergic T3-like B cells, and an increase in IgM surface expression and serum BAFF levels will occur in the clinic. In addition, fine mapping of the types of B cell responses modulated by BTK inhibition clarifies the immune competence of treated patients by suggesting that both innate and adaptive Ig titers will not be impacted, and it suggests the potential usefulness of BTK inhibition in the treatment of both oncologic and autoimmune etiologies driven by GC and memory B cell subsets.

Our assessment of how BTK inhibition modulates the naive B cell transcriptome identified BTK-dependent and -independent transcripts downstream of the BCR. Although the majority of anti-IgD–modulated transcripts were BTK dependent, BTK inhibition had an incomplete effect in returning perturbed transcripts to baseline levels. In addition, a number of anti-IgD–dependent, BTK-independent transcripts were identified; these microarray studies demonstrate that BTK inhibition does not shut down BCR signaling entirely. Importantly, these experiments implicated BTK function in tonic BCR signaling, with tonic signals demonstrated to be necessary for B cell survival (41–43). Interestingly, we did not see similar degrees of B cell depletion observed with PF-303 treatment as was achieved with genetic deletion of BCR components or SYK (41, 43, 44); this may be due to the presence of the BCR-dependent, BTK-independent pathways unveiled in our microarray experiments.

While investigating the impact of chronic BTK inhibition on B cell subset homeostasis, we demonstrated increased serum BAFF levels and depletion of B cells in the secondary lymphoid organs but not the peripheral blood. The increase in serum BAFF levels is expected, given the established inverse correlation of BAFF levels with peripheral B cell frequencies. The differential B cell depletion...
in the peripheral blood versus the secondary lymphoid organs may be the consequence of the established role for BTK in mediating B cell homing to the chemokine CXCL13 (45). Interestingly, ibritinib was reported to induce the purging of malignant cells from the lymphoid tissues into the peripheral blood (46). Importantly, these data indicate that assessment of B cell frequencies in the peripheral blood of BTK inhibitor–treated patients may not reflect the extent of B cell depletion within the secondary lymphoid organs. We also observed that BTK inhibition impacts a developmental block and prevents replenishment of the T3 B cell subset. Prior studies in xid mice observed a defect in the T3 B cell subset (39). Importantly, this population is enriched for anergized B cells (47); because this state is maintained by chronic binding of self-Ag to the BCR, BTK inhibition likely impedes the BCR signals required for anergy induction. Our observation that chronic BTK inhibition drives heightened expression of IgM (but not other isotypes) on the surface of B cells by, in part, reducing IgM turnover is likely linked to the impact of BTK inhibition on T3 B cells and suggests that chronic BTK signals function as a rheostat modulating IgM expression levels (but not other isotypes), with an inverse correlation between BTK activity and surface IgM expression. The BTK-dependent pathways that modulate IgM surface expression remain unclear. Both collagen-induced arthritis and SLE preclinical mouse models show that chronic dosing of both the PF-303 and PF-06250112 chemical probes deplete the T3 B cell subset and increase the surface expression of IgM by FO B cells (M.J. Benson and A.L. Rankin, unpublished observations). This indicates that the phenotypes described in this study are not limited to wild-type, healthy mice or to the PF-303 compound. Whether a similar phenotype is observed in the clinic upon ibritinib treatment warrants investigation.

These studies provide a predictive overview of how a patient undergoing BTK inhibition may respond to various antigenic insults. The impact of PF-303 on primary Ab responses largely mimics the described xid phenotype, with BTK inhibition inhibiting T-independent type II, but not type I, responses and incompletely modulating T-dependent Ab responses. Importantly, for indications in which GC structures have been implicated in disease etiology, such as primary Sjogren’s syndrome or SLE, we demonstrate that BTK inhibition prevents the onset and continuations of established GC B cell responses, including in Peyer’s patches. Our in vitro studies implicate a role for PF-303 in driving impaired B cell–driven cognate CD4 T cell activation, a novel mechanism that may contribute to the prevention and dissolution of GC responses in vivo. In our studies, we demonstrate that, although memory B cells enter the cell cycle in response to secondary Ag encounter, PF-303 significantly suppressed memory B cell clonal expansion and the ASC burst. Given the relapsing and remitting nature of autoimmunity and the association of peripheral CD27+ memory B cells in both SLE and rheumatoid arthritis, perturbation of memory B cells by BTK inhibition is desirable. Interestingly, these results diverge from published data, obtained using xid mice, showing that memory B cell reactivation and the resulting ASC response are independent of BTK activity (27). This discordance may be a consequence of these studies being performed in a wild-type setting and without the potentially compensatory mechanisms at play in the xid strain or from the PF-303 compound engaging TEC and BMX to a similar degree as BTK. Indeed, functional redundancy between BTK and TEC downstream of the BCR was reported in the murine system (48). Because of the protective nature of serum Ab titers and their essential function in combating infection, we assessed how chronic BTK inhibition impacted B1 B cells, bone marrow–resident ASCs, and memory B cell survival. We conclude that the persistence of these subsets is independent of BTK function. We also tested whether dual inhibition of BTK and BAFF impacted memory B cell survival. The signals driving memory B cell survival remain obscure given the independence of this population from BAFF survival signals (49, 50). We hypothesized that concomitant BTK and BAFF blockade might uncover a redundancy between BAFFR and the BCR in their ability to transmit survival signals. We observed that BTK inhibition in the presence of BAFF antagonism did not impact IgG+ memory B cell survival, but it did deplete IgM+ memory B cells.

To summarize, these data predict the clinical course of BTK inhibition and help to clarify its clinical utility, both in the context of oncology settings, in which BTK inhibition is already used, and in the context of autoimmune diseases, in which the next generation of BTK inhibitors likely will be of use.

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