Analysis of the Effects of the Bruton’s tyrosine kinase (Btk) Inhibitor Ibrutinib on Monocyte Fcγ Receptor (FcγR) Function*

Li Ren1, Amanda Campbell2, Huiqing Fang3, Shalini Gautam3, Saranya Elavazhagan3, Kevin Fatechand3, Payal Mehta4, Andrew Stiff5, Brenda F. Reader5, Xiaokui Mo5, John C. Byrd5, William E. Carson III5, Jonathan P. Butchar5,1, and Susheela Tridandapani3,2

From the 4Key Laboratory for Molecular Enzymology and Engineering, Ministry of Education, Jilin University, Changchun 130000, China and the 5Department of Internal Medicine and 6Center for Biostatistics, Ohio State University, Columbus, Ohio 43210

The irreversible Bruton’s tyrosine kinase (Btk) inhibitor ibrutinib has shown efficacy against B-cell tumors such as chronic lymphocytic leukemia and B-cell non-Hodgkin lymphoma. Fcγ receptors (FcγR) on immune cells such as macrophages play an important role in tumor-specific antibody-mediated immune responses, but many such responses involve Btk. Here we tested the effects of ibrutinib on FcγR-mediated activities in monocytes. We found that ibrutinib did not affect monocyte FcγR-mediated phagocytosis, even at concentrations higher than those achieved physiologically, but suppressed FcγR-mediated cytokine production. We confirmed these findings in macrophages from Xid mice in which Btk signaling is defective. Because calcium flux is a major event downstream of Btk, we tested whether it was involved in phagocytosis. The results showed that blocking intracellular calcium flux decreased FcγR-mediated cytokine production but not phagocytosis. To verify this, we measured activation of the GTPase Rac, which is responsible for actin polymerization. Results showed that ibrutinib did not inhibit Rac activation, nor did the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester. We next asked whether the effect of ibrutinib on monocyte FcγR-mediated cytokine production could be rescued by IFNγ priming because NK cells produce IFNγ in response to antibody therapy. Pretreatment of monocytes with IFNγ abrogated the effects of ibrutinib on FcγR-mediated cytokine production, suggesting that IFNγ priming could overcome this Btk inhibition. Furthermore, in monocyte–natural killer cell co-cultures, ibrutinib did not inhibit FcγR-mediated cytokine production despite doing so in single cultures. These results suggest that combining ibrutinib with monoclonal antibody therapy could enhance chronic lymphocytic leukemia cell killing without affecting macrophage effector function.

Fcγ receptors (FcγRs)3 are critical for antibody-mediated responses because they provide for initial contact of the effector cell to the opsonized target cell and subsequently activate effector signaling pathways that lead to target destruction (1). Activation of FcγR also elicits cytokine production by monocytes, which serves to activate other immune effectors, such as natural killer (NK) cells (2–8). This involves the activation of Src kinases and Syk and then branches to other mediators (9). One such mediator downstream of Syk is Bruton’s tyrosine kinase (Btk), which activates the PLCγ/calcium signaling pathway (10) and interacts with multiple proteins and pathways, leading to pleiotropic cellular responses (11, 12). Btk is a member of the Tec family of kinases, which are expressed in mammals as well as other organisms such as Drosophila (13).

Btk is well known as a downstream mediator of the B cell receptor (14). This makes it a candidate therapeutic target for autoantibody diseases as well as for B cell lymphomas in which B cell receptor signaling is overactive. Indeed, the Btk inhibitor ibrutinib (PCI-32765) showed efficacy in murine models of arthritis and lupus and in canines with spontaneous B cell non-Hodgkin lymphoma (15). Presently, ibrutinib is approved for clinical marketing by the Food and Drug Administration for the treatment of relapsed mantle cell lymphoma, Waldenström macroglobulinemia and chronic lymphocytic leukemia (http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm432240.htm).

Ibrutinib binds irreversibly to Btk and blocks its kinase activity (15, 16). It is also capable of blocking activation of the related Tec kinase Itk (17). Because numerous receptors utilize Btk (and Itk), ibrutinib can dampen the responses of immune cells to activating stimuli. For example, ibrutinib can inhibit degranulation and antibody-dependent cellular cytotoxicity and IFNγ production by NK cells (18) and block FcγR-mediated cytokine

Received for publication, August 21, 2015, and in revised form, November 19, 2015 Published, JBC Papers in Press, December 1, 2015, DOI 10.1074/jbc.M115.687251

* This work was supported by National Institutes of Health Grants P01-CA095426 and R01 CA162411 (to S. T. and J. C. B.), R01 CA177292 and R01 CA183444 (to J. C. B.), and T32 HL007946 (to B. F. R.) and by Ohio State University College of Medicine McWhinney Bridge Fund 244749 (to J. P. B.). The authors declare that they have no conflicts of interest with the content of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

† To whom correspondence may be addressed: Div. of Hematology, Ohio State University Wexner Medical Center, 437 W. 12th Ave., Columbus, OH 43210. Tel.: 614-247-6768; Fax: 614-247-4575; E-mail: butchar.2@osu.edu.

§ To whom correspondence may be addressed: Div. of Hematology, Ohio State University Wexner Medical Center, 437 W. 12th Ave., Columbus, OH 43210. Tel.: 614-247-6768; Fax: 614-247-4575; E-mail: tridandapani.2@osu.edu.

‡ The abbreviations used are: Fcγ-R, Fcγ receptor; NK, natural killer; Btk, Bruton’s tyrosine kinase; PLC, phospholipase c; XID, X chromosome-linked immune-deficient; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBMC, peripheral blood monocyte; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N''-tetraacetic acid tetrakis(acetoxymethyl ester).
Ibrutinib Effects on Monocyte Function

production in monocytes/macrophages (19). However, the effects of ibrutinib on monocyte/macrophage FcγR have not been fully elucidated, especially within the context of neighboring immune cells.

Here we examined the effects of ibrutinib on monocyte/macrophage FcγR signaling. We found that, although the drug blocked FcγR-mediated cytokine production, phagocytic ability was unaffected. Further inquiry revealed that this was because ibrutinib did not inhibit Rac activation downstream of FcγR. Interestingly, we also found that priming with IFNγ prevented the inhibition of FcγR function by ibrutinib. In co-cultures of monocytes and NK cells, the endogenous production of IL-12 and IFNγ following incubation with opsonized tumor cells was sufficient to overcome the negative effects of ibrutinib. Neutralizing either IL-12 or IFNγ reduced the levels of both and of TNFα. Therefore, although ibrutinib can block Btk (and Itk) in isolated cultures, proinflammatory intercellular communication is sufficient to overcome its inhibitory effects on monocytes and NK cells. These results suggest that ibrutinib may not be detrimental to patients undergoing antibody therapy and may explain early clinical results showing an enhanced effect when CD20 antibodies such as rituximab or ofatumumab are added to ibrutinib in chronic lymphocytic leukemia (20, 21).

Experimental Procedures

Antibodies and Reagents—Ibrutinib and BAPTA-AM were purchased from Selleck Chemicals (Houston, TX). Recombinant human TNFα (used at 50 ng/ml) and anti-TNFα (used at 5 μg/ml) were purchased from R&D Systems (Minneapolis, MN). TRIlzo® was purchased from Invitrogen. Reverse transcriptase, random hexamers, and SYBR Green PCR mix were purchased from Applied Biosystems (Foster City, CA). Anti-pBtk, anti-pPLCγ2 (Tyr-753 and Tyr-1217), anti-SyK, anti-pErK, anti-pAKT, and anti-pNF-κB for Western blotting were purchased from Cell Signaling Technology (Beverly, MA). Anti-Rac2 was obtained from Abcam (Cambridge, MA). Anti-PLCγ2, anti-Btk, and anti-Syk were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against actin and GAPDH as well as goat and mouse HRP-conjugated secondary antibodies were purchased from Abcam (Cambridge, MA). Anti-Rac was purchased from Santa Cruz Biotechnology (Beverly, MA). Rabbit HRP-conjugated secondary antibody was purchased from Cell Signaling Technology. Human and mouse ChromPure whole-molecule IgG were bought from Jackson ImmunoResearch Laboratories (West Grove, PA).

Western Blotting—Western blots were done as described previously (22). Briefly, cells were lysed in TN1 buffer (50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM Na2P2O7, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na3VO4, and 10 μg/ml each aprotinin and leupeptin). Protein-matched lysates were boiled in Laemmli sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with the antibody of interest, and developed by Pierce ECL 2 Western blotting substrate (Thermo Scientific, Rockford, IL). Densitometry was done using ImageJ (National Institutes of Health).

ELISA Measurement of Cytokine Production—96-well plates were coated with 9 μg/ml human or mouse IgG diluted in PBS overnight at 4 °C. The plates were washed twice with sterile PBS, and cells were added for the incubation periods. Supernatants were collected and centrifuged at 16,000 × g to clear cells and debris and then analyzed for cytokines using sandwich ELISAs (R&D Systems) according to the protocols of the manufacturer.

Peripheral Blood Monocyte Isolation—Human peripheral blood monocytes (PBMs) were isolated from deidentified source leukocyte packs via Ficol centrifugation (Mediatech, Manassas, VA), followed by CD14-positive selection using MACS® (Miltenyi Biotec, Inc., Cambridge, MA). PBMs were resuspended in RPMI 1640 medium containing 10% heat-inactivated FBS (HyClone, Logan, UT), penicillin/streptomycin, and t-glutamate (Invitrogen). The purity of monocytes obtained was >97%, as determined by flow cytometry with CD14 antibody.

Preparation of Heat-aggregated IgG—Human or mouse heat-aggregated IgG was prepared according to methods described previously (23). In brief, whole human or mouse IgG at a concentration of 350 μg/ml in incomplete RPMI medium was incubated at 62 °C in a water bath for 30 min and immediately placed on ice.

Culture of Murine Bone Marrow Macrophages—Wild-type and BTK mutant homozygous mice (XID) were housed and euthanized according to institution-approved animal care and use protocols. Bone marrow-derived macrophages were generated as described previously (24). Briefly, mouse bone marrow cells were flushed, cultured in RPMI medium containing 10% fetal bovine serum, and incubated on plates for 1 week with 20 ng/ml recombinant mouse M-CSF (R&D Systems) and 10 μg/ml polymyxin B, both replenished on days 3 and 5. Cells were harvested for use on day 7.

MTS Assay—The MTS cell proliferation assay kit was purchased from Promega Corp. (Madison, WI), and the assays were done according to the instructions of the manufacturer. Cells were pretreated with ibrutinib at the indicated concentrations and then incubated in 96-well plates for 24 h. Following addition of CellTiter 96® Aqueous One solution reagent and incubation, absorbance at 490 nm was measured using a standard plate reader.

Rac Activity Assay—The Rac1/Cdc42 activation assay kit was purchased from EMD Millipore Corp. (Billerica, MA), and the assay was done in accordance with the provided protocol. Cells were pretreated with ibrutinib or BAPTA-AM and stimulated with the heat-aggregated IgG for the indicated times. Cells were lysed in MLB buffer containing 10 mM Na2VO4 and 10 μg/ml each of aprotinin and leupeptin. Protein-matched cell lysates were incubated with GST-PAK1-PBD beads for 1 h at 4 °C with gentle rocking. GTPγS/GDP positive and negative controls were also prepared. After 1 h of incubation, beads were washed with MLB buffer and then boiled in 2X Laemmli sample buffer (60 mM Tris, (pH 6.8), 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 1% 2-mercaptoethanol) for 5 min. Western blot analyses were done to measure Rac.

Phagocytosis Assays—Phagocytosis assays were performed as described previously (25). Briefly, sheep red blood cells (Colorado Serum Co., Denver, CO) were fluorescently labeled with PKH-26 (Sigma), opsonized with anti-sheep red blood cell antibody (Sigma), and washed to remove excess antibody. These were then added to the effector cells for the indicated
times at 37 °C. Following hypotonic lysis of non-phagocytosed sheep red blood cells, effector cells were placed onto slides, and scoring of phagocytosis was done via fluorescence microscopy in a blinded manner. The phagocytic index was defined as the total number of sheep red blood cells ingested by 100 effector cells.

Co-cultures of Monocytes and NK Cells—Human NK cells and PBMs were isolated from leukopacks purchased from the Red Cross (n = 3). PBMs were isolated via adherence to Teflon-coated plates, and NK cells were isolated using Rosette-Sep (Siemens SAS, Paris, France) as described previously (26). The breast cancer cell line SKBR3 was incubated for 1 h at 37 °C with 100 µg/ml Herceptin and then washed and added to 96-well plates. Monocytes and NK cells were pretreated for 4 h with or without 1 µM ibrutinib, washed, and then plated in isolation or in co-culture into wells with or without the Herceptin-opsonized SKBR3 tumor cells. Neutralizing antibodies against either human IFNγ (5 µg/ml) or human IL-12 (10 µg/ml) (R&D Systems) were added to the appropriate wells. Cultures were incubated for 24 h, and then cleared supernatants were collected and analyzed for IL-12 and IFNγ by ELISA.

Statistical Analyses—For the experiments that involved placing the cells of each donor across multiple conditions, data were analyzed by using analysis of variance with repeated measures for phospho-Btk, phospho-PLCγ2, and phosphorylated Syk. Membranes were reprobed for β-actin to verify equivalent loading. Results showed that ibrutinib blocked IgG-mediated phosphorylation of Btk and PLCγ2 but not of upstream Syk (Fig. 1A).

Next we examined the effect of ibrutinib on FcγR-mediated cytokine production by pretreating cells with or without ibrutinib and then incubating them with or without immobilized IgG for 24 h. Cleared supernatants were assayed for TNFα production by ELISA, and the results showed that ibrutinib blocked FcγR-mediated cytokine production (Fig. 1B), in agreement with Chang et al. (19). As part of a later experiment (Fig. 8), we also measured IL-12 as a cytokine known to not require Btk signaling.

Following this, we pretreated monocytes with or without ibrutinib and then tested the effect of ibrutinib on phagocytosis. Results showed that ibrutinib did not significantly affect the phagocytic ability of monocytes (Fig. 1C). As controls for the efficacy and relative specificity of ibrutinib, we also tested the effects of ibrutinib on TLR4- and TLR8-mediated cytokine production. The results showed that it did not inhibit TNFα production following LPS treatment but significantly inhibited TNFα following treatment with the TLR8 agonist CL075 (data not shown). This is in agreement with previous studies showing that, although Btk is involved with TLR4- and TLR8-mediated cellular activation (33, 34), it is not required for TLR4-mediated TNFα production (35).

Higher Concentrations of Ibrutinib Do Not Affect Monocyte FcγR-mediated Phagocytosis—The disparity between the ability of ibrutinib to inhibit FcγR-mediated cytokine production and its inability to block FcγR-mediated phagocytosis raised the possibility that a greater concentration of ibrutinib would be required to block phagocytosis. To test this, we pretreated monocytes with 0, 1, 5, or 10 µM ibrutinib and then treated them for 15 min with heat-aggregated IgG and measured phosphoproteins as shown in Fig. 1A. The results showed that...
Ibrutinib Effects on Monocyte Function

**FIGURE 2. The effect of ibrutinib concentration on viability and FcγR function.** PBMs were isolated and pretreated with 1, 5, or 10 μM ibrutinib (IB) or left untreated (UT). A, pretreated PBMs were incubated for 15 min with 350 μg/ml heat-aggregated whole human IgG (∆IgG) or left untreated and then lysed and analyzed via Western blot for phosphorylated Btk, phosphorylated PLCγ2, and phosphorylated Syk. Membranes were reprobed for β-actin to verify equivalent loading (n = 3, a representative blot is shown). B, pretreated PBMs were incubated overnight, and then cell survival was measured using an MTS assay (n = 4). C, pretreated PBMs were incubated for 24 h in 96-well plates precoated without (PBS) or with 10 μg/ml whole human IgG. Cleared supernatants were collected and analyzed by ELISA for TNFα (n = 3). D, pretreated PBMs were assayed for phagocytic ability as described under “Experimental Procedures” (n = 4). Error bars represent mean ± S.D.

the lowest concentration completely inhibited phosphorylation of Btk and PLCγ2 but that no concentration inhibited the phosphorylation of upstream Syk (Fig. 2A).

We then pretreated monocytes with ibrutinib, incubated the cells for 24 h, and then performed an MTS proliferation assay as a measure of cell survival. As shown in Fig. 2B, none of the ibrutinib concentrations affected survival. Following this, we tested the effects of these increasing ibrutinib concentrations on FcγR-mediated cytokine production and phagocytosis. Although FcγR-mediated cytokine was blocked even by the lowest concentration of ibrutinib (Fig. 2C), not even the highest concentration significantly affected phagocytosis (Fig. 2D).

**Macrophages from Xid Mice Show Defects in FcγR-mediated Cytokine Production but Not in Phagocytosis**—Xid (CBA/N) mice show an intrinsic defect in B cell development (36) and have a cytokine-to-thymidine mutation at position 219. This results in an arginine-to-cysteine change within the N-terminal region of Btk (37, 38), which contains the pleckstrin homology region that interacts with protein kinase C (39). It has been shown previously that peritoneal macrophages from Xid mice do not show defects in their ability to ingest heat-killed Escherichia coli (32). Regarding FcγR-mediated phagocytosis, the xid gene was able to rescue the decreased ability of peritoneal macrophages from autoimmune-prone NZB mice to ingest opsonized sheep red blood cells (40). Here we examined the FcγR responses of Xid mouse bone marrow-derived macrophages (BMMs) to compare them with the results seen with human monocytes.

Upon stimulation with heat-aggregated IgG, BMMs from Xid mice showed significantly reduced phosphorylation of Btk and the downstream PLCγ2 (Fig. 3A). Similarly, Xid BMMs showed reduced FcγR-mediated TNFα production (Fig. 3B). However, FcγR-mediated phagocytosis was not different between wild-type and Xid mice (Fig. 3C). These results suggest that FcγR responses of macrophages from these Btk-defective mice closely resemble the responses seen in ibrutinib-treated human PBMs in that cytokine production, but not phagocytosis, is affected.

**Intracellular Calcium Flux Is Required for FcγR-mediated Cytokine Production but Not for Phagocytosis**—One major signaling event downstream of Btk that is blocked by ibrutinib and other Btk inhibitors is calcium flux (19, 28). Here we tested the effects of ibrutinib within the context of calcium signaling and actin polymerization. To begin, we pretreated monocytes with or without the calcium chelator BAPTA-AM for 30 min and then treated the cells for 15 min with heat-aggregated IgG. Western blot analyses showed that BAPTA-AM had no effect on the phosphorylation of Btk or PLCγ2, as expected (Fig. 4A).

To then determine the effect of calcium chelation on FcγR-mediated cytokine release, we pretreated monocytes with or without ibrutinib or BAPTA-AM and then incubated the cells on immobilized IgG for 24 h. ELISAs with cleared supernatants showed that both ibrutinib and BAPTA-AM significantly reduced FcγR-mediated cytokine production (Fig. 4B), indicating that inhibitor treatment was effective. Next, we pretreated monocytes for 30 min with or without BAPTA-AM or ibrutinib and performed phagocytosis assays. The results showed that neither BAPTA-AM nor ibrutinib could significantly reduce FcγR-mediated phagocytosis (Fig. 4C), despite their effect on FcγR-mediated cytokine production. These results suggest
that, although calcium flux may be required for FcγR-mediated cytokine production, the effects of ibrutinib on calcium signaling do not affect FcγR-mediated phagocytosis.

Rac is a GTPase that is responsible for actin polymerization following FcγR activation and subsequent phagocytosis. To determine whether ibrutinib or downstream Ca2+ mobilization had an effect on Rac, we pretreated monocytes with or without ibrutinib for 30 min, followed by treatment with heat-aggregated IgG for 5 min. We then performed a Rac activation assay and found that neither ibrutinib (Fig. 5A) nor chelation of Ca2+ (Fig. 5B) affected FcγR-mediated Rac activation. Collectively, these results suggest that the Btk inhibitor ibrutinib blocks FcγR-mediated cytokine production, likely by its effect on calcium signaling, but this has little to no effect on Rac activation. Therefore, ibrutinib shows minimal effects on monocyte-mediated phagocytosis. This is summarized in Fig. 5C and is in agreement with Patel et al. (41), in whose work Vav could activate Rac independently of Ca2+.

**IFNγ Rescues FcγR-mediated Cytokine Production following Ibrutinib Treatment**—It has been well established that priming of monocytes with IFNγ can markedly enhance FcγR-mediated cytokine production as well as other functions, such as phagocytosis and reactive oxygen species production. This is important in the context of antibody therapy because NK cells are known to secrete substantial quantities of IFNγ upon engaging an antibody-coated target. This led us to test whether IFNγ pretreatment could overcome the effects of ibrutinib and whether the IFNγ produced by NK cells could act in a paracrine fashion on monocytes to combat the inhibition by ibrutinib. To begin, we pretreated monocytes overnight with or without IFNγ, treated them with ibrutinib or left them untreated, and then incubated them for 24 h in 96-well plates with or without immobilized IgG. The results from the ELISAs showed that ibrutinib blocked FcγR-mediated cytokine production as in the figures above but that pretreatment with IFNγ rescued this inhibition (Fig. 5D).

We then proceeded to test the effects of ibrutinib on downstream mediators of FcγR activation to determine which were affected and which might be rescued by IFNγ. Ibrutinib has been shown to inhibit the activation of NF-κB, Erk, and Akt (42–44), so we tested whether IFNγ priming could rescue their phosphorylation following FcγR activation in ibrutinib-pretreated monocytes. To begin, we incubated PBMs with ibruti-
nib for 1 h and then treated them with heat-aggregated IgG to activate the FcγR or left them untreated. Protein lysates were collected to measure the phosphorylation of Btk itself, along with NF-κB, Akt, and Erk. As shown in Fig. 6, pretreatment with ibrutinib led to significant reductions in the phosphorylation of all four proteins. Next, to test whether IFNγ could prevent this block of phosphorylation, we primed monocytes with or without IFNγ overnight, treated them with ibrutinib for 1 h, and stimulated the cells with heat-aggregated IgG or left them untreated. The results showed that IFNγ did not block the activity of ibrutinib against Btk, NF-κB, or Akt (Fig. 7, A–C, respectively) because significant decreases in FcγR-mediated phosphorylation were still seen with ibrutinib. However, IFNγ prevented the ibrutinib-mediated reduction in FcγR-mediated Erk phosphorylation (Fig. 7D, p = 0.259). These results suggest that IFNγ can rescue Erk pathway activation downstream of FcγR.

Because IFNγ could reverse the suppression of FcγR-mediated cytokine production that was seen with ibrutinib, we tested whether measurable IFNγ would be produced when NK cells and monocytes were co-cultured in the presence of antibody-coated target cells. Simultaneously, because it has been shown in macrophages from Xid mice that Btk deficiency leads to enhanced IL-12 production (45), we also wished to determine whether IgG-stimulated monocytes would produce IL-12 even after ibrutinib treatment. This IL-12, if produced, might serve to further activate the IgG-stimulated NK cells.

To examine this, PBMs and autologous NK cells were treated for 4 h with ibrutinib or left untreated and then incubated either separately or in co-culture. Each single and co-culture incubation was done for 24 h with or without Herceptin-coated SKBR3 breast cancer cells (FcγR-activating stimuli), and co-culture wells were also treated with neutralizing antibodies against IL-12 or IFNγ or left untreated. Following these incubations, cleared supernatants were collected and analyzed by ELISA for TNFα. * p < 0.05 versus untreated cells. Error bars represent mean ± S.D.
Ibrutinib Effects on Monocyte Function

FEBRUARY 5, 2016 • VOLUME 291 • NUMBER 6

JOURNAL OF BIOLOGICAL CHEMISTRY 3049

Ibrutinib Effects on Monocyte Function

Ibrutinib was significantly higher in co-cultures than in single cultures, with the exception of monocyte TNFα versus co-culture with TNFα. This increase was likely due to the intercellular communication between monocytes and NK cells because the neutralizing antibodies against IL-12 and IFNγ each reduced the levels of the other (Fig. 8, A and B, respectively). Importantly, ibrutinib had little to no effect on FcγR-mediated cytokine production in these co-cultures. The levels of IL-12 and TNFα were unchanged, whereas IFNγ showed a small but statistically significant decrease. Because ibrutinib did not reduce the levels of TNFα in co-cultures but neutralizing antibody against IFNγ did, it appears as though the level of IFNγ that was produced in co-cultures was still high enough to rescue monocytes from the effects of ibrutinib. Collectively, these results suggest that ibrutinib may not inhibit FcγR activity in heterogeneous immune cell populations, such as in patients undergoing treatment.

Discussion

Ibrutinib is approved for the treatment of relapsed mantle cell lymphoma, Waldenström macroglobulinemia, and chronic lymphocytic leukemia. Because of the potential negative influence on NK cells published by our group and others (18, 29), its role in combination therapy with monoclonal antibodies such as rituximab has been questioned. However, NK-cell mediated antibody-dependent cellular cytotoxicity is only one mechanism by which therapeutic antibodies deplete tumor cells. In

![Figure 6. Ibrutinib blocks FcγR-mediated phosphorylation of Btk, NF-κB, Akt, and Erk. A–D. PBM were isolated and treated for 60 min with (+) 1 μM ibrutinib (IB) or left untreated (UT, −), washed, and incubated for 15 min either without (−) or with (+) 350 μg/ml heat-aggregated whole human IgG (ΔIgG). Western blot analyses were done to measure the phosphorylation levels of Btk (A), NF-κB (B), Akt (C), and Erk (D). The bottom blots in each top panel show loading controls. Representative blots are shown. Bottom panels, densitometric analyses of blots, plotting the ratios of phosphoproteins to loading controls for pBtk (A, n = 8 donors), pNF-κB (B, n = 3 donors), pAkt (C, n = 6 donors), and pErk (D, n = 5 donors). *, p < 0.05. Error bars show mean ± S.D.

![Figure 7. Ibrutinib rescues FcγR-mediated Erk signaling. A–D. PBM were isolated and primed overnight with (+) 10 ng/ml recombinant human IFNγ or left untreated (−). Cells were then treated for 60 min with 1 μM ibrutinib (IB) or left untreated, washed, and then incubated for 15 min with 350 μg/ml heat-aggregated whole human IgG (ΔIgG) or left untreated. Western blot analyses were done to measure levels of pBtk (A), pNF-κB (B), pAkt (C), and pErk (D). The bottom blots in each top panel show loading controls. Representative blots are shown. Bottom panels, densitometric analyses of blots, plotting the ratios of phosphoproteins to loading controls for pBtk (A, n = 10 donors), pNF-κB (B, n = 3 donors), pAkt (C, n = 4 donors), and pErk (D, n = 5 donors). Error bars show mean ± S.D. ns, no statistical significance; *, p < 0.05.

A

IB

ΔIgG

IFNγ

pBtk

Btk

B

IB

ΔIgG

IFNγ

pNF-κB

β actin

C

IB

ΔIgG

IFNγ

pAkt

β actin

D

IB

ΔIgG

IFNγ

pErk

β actin

ns

FEBRUARY 5, 2016 • VOLUME 291 • NUMBER 6

JOURNAL OF BIOLOGICAL CHEMISTRY 3049

A

IB

ΔIgG

IFNγ

pBtk

Btk

B

IB

ΔIgG

IFNγ

pNF-κB

β actin

C

IB

ΔIgG

IFNγ

pAkt

β actin

D

IB

ΔIgG

IFNγ

pErk

β actin

ns
Ibrutinib Effects on Monocyte Function

![Graphs showing cytokine production in monocytes](image)

Addition to direct killing and complement-mediated killing, monocye- and macrophage-mediated, antibody-dependent phagocytosis represents another relevant mechanism of tumor elimination. Here we examined in detail the effects of ibrutinib on monocye FcγR function, which has also been shown to be important for antibody-mediated tumor clearance. The results demonstrated that ibrutinib did not adversely affect the phagocytic ability of monocyes despite its ability to block FcγR-mediated TNFα production by monocyes. We also tested the effects of the more selective Btk inhibitor CGI-1746 (28) and found that it was equal in its ability to block FcγR-mediated cytokine production in monocyes (data not shown). This is in contrast to the findings with NK cells, where ibrutinib, but not CGI-1746, blocked NK-cell antibody-dependent cellular cytotoxicity because activated NK cells express Itk, which is not inhibited by CGI-1746 (18). Our results regarding TNFα pro-

duction are in agreement with others both for primary human and murine cells. For example, Horwood et al. (46) have shown that TNFα mRNA was reduced in mononuclear cells from Xid mice following LPS treatment. Chang et al. (19) have shown that ibrutinib reduced FcγR-induced TNFα, IL-1β, and IL-6 in primary monocyes.

The lack of ibrutinib effect on monocyte/macrophage-mediated phagocytosis is in agreement with Mangla et al. (32), who found that phagocytosis of heat-killed fluoresceinated E. coli was not affected in macrophages from Xid mice. This lack of effect is likely not due to insufficient levels of ibrutinib because we tested with levels of 1 and 10 μM, and we saw effective blocking of Btk phosphorylation even at 1 μM. In addition, it has been shown that as little as 1–10 nM is sufficient to block FcγR-mediated cytokine production in monocyes and THP-1 cells (19), which suggests that Btk kinase activity (and, most notably, its downstream Ca²⁺ mobilization) is not required for phagocytosis. Indeed, it appears more likely that the lack of ibrutinib effect on phagocytosis is due to the non-dependence of phagocytosis on Ca²⁺ mobilization (47–50). Ca²⁺ mobilization is a major downstream event following Btk activation, and both genetic and pharmacologic reduction of Btk activity reduce the mobilization of Ca²⁺. For example, mast cells of Xid mice are defective in Ca²⁺ mobilization (51), as are B cells, primary monocyes, and THP-1 cells treated with ibrutinib (19).

Our results with human PBMs did not show a consistent increase in FcγR-mediated IL-12 production, but, perhaps as importantly, we did not observe any decrease. It has been shown previously by Mukhopadhyay et al. (52) that Xid mice displayed a macrophage-dependent skewing toward Th1 characteristics that led to a delay in the clearance of filarial infection. Follow-up work by the same group has shown that the decreased nitric oxide induction in Btk-deficient macrophages permitted higher levels of IL-12 production (45, 53). Therefore, in the context of multiple immune cells, it is possible, and even perhaps likely, that ibrutinib treatment would not block the production of IL-12. This, in turn, might serve to elicit IFNγ from neighboring NK cells. The NK cells would produce biologically relevant (albeit perhaps small) amounts of IFNγ, which, in turn, would work toward ameliorating the effects of ibrutinib on monocyes. Along with this, ibrutinib has already been shown to promote Th1 responses by inhibiting Itk in Th2 cells (17), which could serve as an additional source of IFNγ for the monocyes.

We also found that ibrutinib could not block TNFα production in monocyes primed with IFNγ (Fig. 5D) or in co-cultures of monocyes and NK cells (Fig. 8C). Therefore, within a mixed cell setting, it is likely that FcγR-activated monocyes can still produce not only IL-12 (discussed above) but also other chemokines/cytokines that could serve to activate neighboring cells.

When taken collectively, these data suggest that, although ibrutinib can effectively inhibit proinflammatory responses in isolated cells, its ability to do so within the whole organism is limited. This may explain why early clinical results showed an enhanced effect when adding rituximab or ofatumumab to ibrutinib in treatment for chronic lymphocytic leukemia (20, 21). Anti-CD20 antibodies such as rituximab and obinutuzumab are key components of therapy for diseases such as...
chronic lymphocytic leukemia or non-Hodgkin lymphoma, and these results suggest a potential mechanism by which their effectiveness would not be compromised by concurrent treatment with a Btk inhibitor.

Author Contributions—J. P. B. and S. T. designed the study and wrote the manuscript. L. R., A. C., S. G., H. F., E. K., F. P., M. A., S., and B. F. R. performed experiments and acquired data for the figures. X. M., J. C. B., W. E. C., J. P. B., and S. T. analyzed and interpreted the data. J. C. B. and W. E. C. performed critical revisions of the manuscript. All authors reviewed and approved the final version of this manuscript.

References

1. Hogarth, P. M., and Pietersz, G. A. (2012) Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. Nat. Rev. Drug Discov. 11, 311–331

2. Debets, J. M., Van de Winkel, J. G., Ceuppens, J. L., Dieteren, I. E., and Buurman, W. A. (1990) Cross-linking of both Fc γ R and Fc γ RII induces secretion of tumor necrosis factor by human monocytes, requiring high affinity Fc-Fc γ R interactions: functional activation of Fc γ RII by treatment with proteases or neuraminidase. J. Immunol. 144, 1304–1310

3. Roda, J. M., Joshi, T., Butchar, J. P., McAlees, J. W., Lehman, A., Tridandapani, S., and Carson, W. E., 3rd. (2007) The activation of natural killer cell effector functions by cetuximab-coated, epidermal growth factor receptor positive tumor cells is enhanced by cytokines. Clin. Cancer Res. 13, 6419–6428

4. Kruttmann, J., Kirnbauer, R., Köck, A., Schwarz, T., Schöpf, E., May, L. T., Sehgal, P. B., and Luger, T. A. (1990) Cross-linking Fc receptors on monocytes triggers IL-6 production: role in anti-CD3-induced T cell activation. J. Immunol. 145, 1337–1342

5. Scholl, P. R., Ahern, D., and Geha, R. S. (1992) Protein tyrosine phosphorylation induced via the IgG receptors Fc γ R and Fc γ RII in the human mononuclear cell line THP-1. J. Immunol. 149, 1751–1757

6. Cao, X., Wei, G., Fang, H., Guo, J., Weinstein, M., and Cao, J. (2004) The inositol 3-phosphatase PTEN negatively regulates Fc γ R signaling, but supports Toll-like receptor 4 signaling in murine peritoneal macrophages. J. Immunol. 172, 4851–4857

7. Ganesan, L. P., Joshi, T., Fang, H., Kutala, V. K., Rada, J., Trotta, R., Lehman, A., Kuppusamy, P., Byrd, J. C., Carson, W. E., Caligiuri, M. A., and Tridandapani, S. (2006) FCyR-induced production of superoxide and inflammatory cytokines is differentially regulated by SHIP through its influence on PI3K and/or Ras/Erk pathways. Blood 108, 718–725

8. Shah, P., Fatechand, K., Patel, H., Fang, H., Justinianno, S. E., Mo, X., Jarijoua, D., Tridandapani, S., and Butchar, J. P. (2013) Toll-like receptor 2 ligands regulate monocyte FcγR expression and function. J. Biol. Chem. 288, 12345–12352

9. Ravetch, J. V., and Bolland, S. (2001) IgG Fc receptors. Annu. Rev. Immunol. 19, 275–290

10. Kurosaki, T., and Tsukada, S. (2000) BLNK: connecting Syk and Btk to the B cell receptor. Discov. Med. 1, 436–446

11. Khan, W. N. (2009) B cell receptor and BAFF receptor signaling regulation and B. F. R. performed experiments and acquired data for the figures. X. M., J. C. B., W. E. C., J. P. B., and S. T. analyzed and interpreted the data. J. C. B. and W. E. C. performed critical revisions of the manuscript. All authors reviewed and approved the final version of this manuscript.

Author Contributions—J. P. B. and S. T. designed the study and wrote the manuscript. L. R., A. C., S. G., H. F., E. K., F. P., M. A., S., and B. F. R. performed experiments and acquired data for the figures. X. M., J. C. B., W. E. C., J. P. B., and S. T. analyzed and interpreted the data. J. C. B. and W. E. C. performed critical revisions of the manuscript. All authors reviewed and approved the final version of this manuscript.

References

1. Hogarth, P. M., and Pietersz, G. A. (2012) Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. Nat. Rev. Drug Discov. 11, 311–331

2. Debets, J. M., Van de Winkel, J. G., Ceuppens, J. L., Dieteren, I. E., and Buurman, W. A. (1990) Cross-linking of both Fc γ R and Fc γ RII induces secretion of tumor necrosis factor by human monocytes, requiring high affinity Fc-Fc γ R interactions: functional activation of Fc γ RII by treatment with proteases or neuraminidase. J. Immunol. 144, 1304–1310

3. Roda, J. M., Joshi, T., Butchar, J. P., McAlees, J. W., Lehman, A., Tridandapani, S., and Carson, W. E., 3rd. (2007) The activation of natural killer cell effector functions by cetuximab-coated, epidermal growth factor receptor positive tumor cells is enhanced by cytokines. Clin. Cancer Res. 13, 6419–6428

4. Kruttmann, J., Kirnbauer, R., Köck, A., Schwarz, T., Schöpf, E., May, L. T., Sehgal, P. B., and Luger, T. A. (1990) Cross-linking Fc receptors on monocytes triggers IL-6 production: role in anti-CD3-induced T cell activation. J. Immunol. 145, 1337–1342

5. Scholl, P. R., Ahern, D., and Geha, R. S. (1992) Protein tyrosine phosphorylation induced via the IgG receptors Fc γ R and Fc γ RII in the human mononuclear cell line THP-1. J. Immunol. 149, 1751–1757

6. Cao, X., Wei, G., Fang, H., Guo, J., Weinstein, M., and Cao, J. (2004) The inositol 3-phosphatase PTEN negatively regulates Fc γ R signaling, but supports Toll-like receptor 4 signaling in murine peritoneal macrophages. J. Immunol. 172, 4851–4857

7. Ganesan, L. P., Joshi, T., Fang, H., Kutala, V. K., Rada, J., Trotta, R., Lehman, A., Kuppusamy, P., Byrd, J. C., Carson, W. E., Caligiuri, M. A., and Tridandapani, S. (2006) FCyR-induced production of superoxide and inflammatory cytokines is differentially regulated by SHIP through its influence on PI3K and/or Ras/Erk pathways. Blood 108, 718–725

8. Shah, P., Fatechand, K., Patel, H., Fang, H., Justinianno, S. E., Mo, X., Jarijoua, D., Tridandapani, S., and Butchar, J. P. (2013) Toll-like receptor 2 ligands regulate monocyte FcγR expression and function. J. Biol. Chem. 288, 12345–12352

9. Ravetch, J. V., and Bolland, S. (2001) IgG Fc receptors. Annu. Rev. Immunol. 19, 275–290

10. Kurosaki, T., and Tsukada, S. (2000) BLNK: connecting Syk and Btk to the B cell receptor. Discov. Med. 1, 436–446

11. Khan, W. N. (2009) B cell receptor and BAFF receptor signaling regulation and B. F. R. performed experiments and acquired data for the figures. X. M., J. C. B., W. E. C., J. P. B., and S. T. analyzed and interpreted the data. J. C. B. and W. E. C. performed critical revisions of the manuscript. All authors reviewed and approved the final version of this manuscript.

Author Contributions—J. P. B. and S. T. designed the study and wrote the manuscript. L. R., A. C., S. G., H. F., E. K., F. P., M. A., S., and B. F. R. performed experiments and acquired data for the figures. X. M., J. C. B., W. E. C., J. P. B., and S. T. analyzed and interpreted the data. J. C. B. and W. E. C. performed critical revisions of the manuscript. All authors reviewed and approved the final version of this manuscript.
Ibrutinib Effects on Monocyte Function

28. Di Paolo, J. A., Huang, T., Balazs, M., Barbosa, J., Barck, K. H., Bravo, B. J., Carano, R. A., Darrow, J., Davies, D. R., DeForge, L. E., Diehl, L., Ferrando, R., Galiano, S. L., Giannetti, A. M., Gribling, P., Hurez, V., Hymowitz, S. G., Jones, R., Kropf, J. E., Lee, W. P., Maciejewski, P. M., Mitchell, S. A., Rong, H., Staker, B. L., Whitney, J. A., Yeh, S., Young, W. B., Yu, C., Zhang, J., Reif, K., and Currie, K. S. (2011) Specific Btk inhibition suppresses B cell- and myeloid cell-mediated arthritis. Nat. Chem. Biol. 7, 41–50

29. Da Rott, F., Engelberts, P. J., Taylor, R. P., Breij, E. C., Gritti, G., Rambaldi, A., Introna, M., Parren, P. W., Beurskens, F. J., and Golay, J. (2015) Ibrutinib interferes with the cell-mediated anti-tumor activities of therapeutic CD20 antibodies: implications for combination therapy. Haematologica 100, 77–86

30. Borg, M., Belén Almejín, M., Podaza, E., Colado, A., Fernández Grecco, H., Cabrejo, M., Bezars, R. F., Giordano, M., and Gambarle, R. (2015) Ibrutinib impairs the phagocytosis of rituximab-coated leukemic cells from chronic lymphocytic leukemia patients by human macrophages. Haematologica 100, e140–142

31. Jongstra-Bilen, J., Puig Cano, A., Hasija, M., Xiao, H., Smith, C. I., and Smith, C. I., and Jongstra-Bilen, J. (2005) Bruton's tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of human monocytes. J. Immunol. 181, 288–298

32. Mangla, A., Khare, A., Vineeth, V., Panday, N. N., Mukhopadhyay, A., Ravindran, B., Bal, V., George, A., and Rath, S. (2004) Pleiotropic consequences of Bruton tyrosine kinase deficiency in myeloid lineages lead to poor inflammatory responses. Blood 104, 1191–1197

33. Doyle, S. L., Jefferies, C. A., Feighery, C., and O’Neill, L. A. (2007) Signaling by Toll-like receptors 8 and 9 requires Bruton’s tyrosine kinase. J. Biol. Chem. 282, 36953–36960

34. Doyle, S. L., Jefferies, C. A., and O’Neill, L. A. (2005) Bruton’s tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of p65 on serine 536 during NFκB activation by lipopolysaccharide. J. Biol. Chem. 280, 23496–23501

35. Pérez de Diego, R., López-Granados, E., Pozo, M., Rodríguez, C., Sabina, P., Ferreira, A., Fontan, G., García-Rodríguez, M. C., and Alemany, S. (2006) Bruton’s tyrosine kinase is not essential for LPS-induced activation of human monocytes. J. Allergy Clin. Immunol. 117, 1462–1469

36. Scher, I., Steinberg, A. D., Berning, A. K., and Paul, W. E. (1975) X-linked B-lymphocyte immune defect in CBA/N mice: II: studies of the mechanisms underlying the immune defect. J. Exp. Med. 142, 637–650

37. Thomas, J. D., Siders, P., Smith, C. I., Vorechovsky, I., Chapman, V., and Paul, W. E. (1993) Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science 261, 355–358

38. Rawlings, D. J., Safran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., and Copeland, N. G. (1993) Mutation of unique region of Bruton tyrosine kinase in immunodeficient XID mice. Science 261, 358–361

39. Yao, L., Kawakami, Y., and Kawakami, T. (1994) The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. Proc. Natl. Acad. Sci. U.S.A. 91, 9715–9719

40. Russell, P. J., and Steinberg, A. D. (1983) Studies of peritoneal macrophage function in mice with systemic lupus erythematosus: depressed phagocytosis of opsonized sheep erythrocytes in vitro. Clin. Immunol. Immunopathol. 27, 387–402

41. Patel, J. C., Hall, A., and Caron, E. (2002) Vav regulates activation of Rac but not Cdc42 during FcyR-mediated phagocytosis. Mol. Biol. Cell 13, 1215–1226

42. Herman, S. E., Mustafa, R. Z., Gryf, J. A., Pittaluga, S., Chang, S., Chang, B., Farooqui, M., and Wiestner, A. (2014) Ibrutinib inhibits BCR and NF-κB signaling and reduces tumor proliferation in tissue-resident cells of patients with CLL. Blood 123, 3286–3295

43. Dasmahapatra, G., Patel, H., Dent, P., Fisher, R. I., Friedberg, J., and Grant, S. (2013) The Bruton tyrosine kinase (BTK) inhibitor PCI-32765 synergistically increases proteasome inhibitor activity in diffuse large-B cell lymphoma (DLBCL) and mantle cell lymphoma (MCL) cells sensitive or resistant to bortezomib. Br. J. Haematol. 161, 43–56

44. Herman, S. E., Gordon, A. L., Hertlein, E., Ramanunni, A., Zhang, X., Jäglowski, S., Flynn, J., Jones, I., Blum, K. A., Bugy, J. J., Handy, A., Johnson, A. J., and Byrd, J. C. (2011) Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. Blood 117, 6287–6296

45. Mukhopadhyay, S., George, A., Bal, V., Ravindran, B., and Rath, S. (1999) Bruton’s tyrosine kinase deficiency in macrophages inhibits nitric oxide generation leading to enhancement of IL-12 induction. J. Immunol. 163, 1786–1792

46. Horwood, N. J., Mahon, T., McDaid, J. P., Campbell, J., mano, H., Brennan, F. M., Webster, D., and Foxwell, B. M. (2003) Bruton’s tyrosine kinase is required for lipopolysaccharide-induced tumor necrosis factor α production. J. Exp. Med. 197, 1603–1611

47. McNeil, P. L., Swanson, J. A., Wright, S. D., Silverstein, S. C., and Taylor, D. L. (1986) Fc-receptor-mediated phagocytosis occurs in macrophages without an increase in average [Ca2+]i. J. Cell Biol. 102, 1586–1592

48. Di Virgilio, F., Meyer, B. C., Greenberg, S., and Silverstein, S. C. (1988) Fc-receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca2+ levels. J. Cell Biol. 106, 657–666

49. Lennartz, M. R., Lefkowith, J. B., Bromley, F. A., and Brown, E. J. (1993) Immunoglobulin G-mediated phagocytosis activates a calcium-independent, phosphatidylyethanolamine-specific phospholipase. J. Leukocyte Biol. 54, 389–398

50. Karimi, K., and Lennartz, M. R. (1995) Protein kinase C activation precedes arachidonic acid release during IgG-mediated phagocytosis. J. Immunol. 155, 5786–5794

51. Setoguchi, R., Kinashi, T., Sagara, H., Hirosawa, K., and Takatsu, K. (1998) Defective degranulation and calcium mobilization of bone-marrow derived mast cells from Xid and Btk-deficient mice. Immunol. Lett. 64, 109–118

52. Mukhopadhyay, S., Sahoo, P. K., George, A., Bal, V., Rath, S., and Ravindran, B. (1999) Delayed clearance of filarial infection and enhanced Th1 immunity due to modulation of macrophage APC functions in xid mice. J. Immunol. 163, 875–883

53. Mukhopadhyay, S., Mohanty, M., Mangla, A., George, A., Bal, V., Rath, S., and Ravindran, B. (2002) Macrophage effector functions controlled by Bruton’s tyrosine kinase are more crucial than the cytokine balance of T cell responses for microfilarial clearance. J. Immunol. 168, 2914–2921