Journal of Pharmacological and Toxicological Methods 71 (2015) 61–67

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Original article

Development and pharmacological validation of novel methods of B cell activation in rat whole blood

John Shin [a,*,1], Marianne Spatz [b,1], Philip Brandish [a], Francois Gervais [b], Jie Zhang-Hoover [c], Erica Leccese [c], Alexandra Hicks [a]

[a] Department of Immunology, Oncology & IMR, Merck Research Laboratories, 33 Avenue Louis Pasteur, Boston, MA 02115, USA
[b] Department of Genetics & Pharmacogenomics, Merck Research Laboratories, 33 Avenue Louis Pasteur, Boston, MA 02115, USA
[c] Department of In Vivo Pharmacology, Merck Research Laboratories, 33 Avenue Louis Pasteur, Boston, MA 02115, USA

ARTICLE INFO

Article history:
Received 11 November 2014
Accepted 20 December 2014
Available online 26 December 2014

Keywords:
B cell
BTK
Dextran-coupled anti-IgD
Methods
PI3Kδ
Rat
Synergy
SYK
TLR9
Whole blood

ABSTRACT

Introduction: Whole blood functional assays are pharmacologically relevant in the drug discovery process to evaluate potency in a relevant biological matrix, to support establishment of PK/PD relationships and to aid in human dose predictions. However development of B cell activation assays by BCR ligation in rat whole blood has not been previously described. The aim of the present study was to develop novel methods of B cell activation in rat whole blood. **Methods:** B cell activation in rat whole blood was evaluated by measuring CD86 up-regulation via flow cytometry. Rat B cells in whole blood were stimulated with dextran-coupled anti-IgD or a combination of anti-IgD and TLR9 agonist. BTK, SYK, and PI3Kδ inhibitors were added to rat whole blood prior to activation with dextran-coupled anti-IgD or anti-IgD and TLR9 agonist combination for pharmacological validation of the assay. **Results:** Both methods of stimulation in rat whole blood evoked robust B cell activation in a uni-modal fashion. Highly selective inhibitors of BTK, SYK, and PI3Kδ dose-dependently attenuated B cell activity evoked by both dextran-coupled anti-IgD and combined anti-IgD and TLR9 agonist. Compound potencies and rank order determined by the two assays were comparable. **Discussion:** Two novel methods were developed to stimulate B cells in rat whole blood, that have the potential to be used to support drug discovery efforts in the therapeutic targeting of B cells. Furthermore, we pharmacologically validated these whole blood assays using highly selective inhibitors of BTK, SYK, and PI3Kδ, signaling kinases which are downstream of the B cell receptor.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

There is a large body of evidence supporting a pathological role of aberrant B cells in autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematous (SLE) and multiple sclerosis (MS; Edwards et al., 2004; Hauser et al., 2008; Navarra et al., 2011). B cells have been suggested to contribute to the pathology of autoimmune diseases due to their many biological functions, including presentation of antigens, co-stimulation of autoreactive T cells and production of autoantibodies (Takemura, Klimiuk, Braun, Goronzy, & Weyand, 2001; Wither, Roy, & Brennan, 2000; Zhao et al., 2008). Indeed, pharmacological modulation of B cell function with therapeutic antibodies such as rituximab, belimumab and epratuzumab has demonstrated clinical efficacy in autoimmune diseases, highlighting the pathological role of this cell type (Dörner et al., 2006; Edwards et al., 2004; Navarra et al., 2011).

Novel mechanisms of targeting B cells are also being pursued clinically. The B cell receptor (BCR) is a transmembrane protein which upon antigen binding evokes B cell activation, proliferation and differentiation (Dal Porto et al., 2004). Spleen tyrosine kinase (SYK), Bruton's tyrosine kinase (BTK) and phosphatidylinositol-4,5-bisphosphate 3-kinase delta (PI3Kδ) are signaling kinases downstream of the BCR (Puri, Di Paolo, & Gold, 2013). Inhibitors of these targets are in preclinical and clinical development for a variety of diseases, including the SYK inhibitor fostamatinib, which has shown clinical efficacy in RA, and the BTK and PI3Kδ inhibitors Ibrutinib and Idelalisib respectively, with positive therapeutic benefits in chronic lymphocytic leukemia (CLL; Weinblatt et al., 2010; Byrd et al., 2013; Brown et al., 2014).

Whole blood functional assays are pharmacologically relevant and important in the drug discovery process to evaluate potency in a relevant biological matrix, to support establishment of PK/PD relationships and to aid in human dose predictions. BCR-mediated B cell stimulation

* Corresponding author at: Departments of Immunology, Oncology & IMR, Merck Research Laboratories, 33 Avenue Louis Pasteur, Boston, MA 02115, USA. Tel.: +1 617 992 2096; fax: +1 617 992 2486.
E-mail address: john.shin@merck.com (J. Shin).
[1] Authors contributed equally to this work.

http://dx.doi.org/10.1016/j.vascn.2014.12.006
1056-8719/© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

assays have been reported in human and mouse whole blood with the use of anti-BCR antibodies to cross-link the receptor. However development of BCR-mediated B cell activation assays in rat whole blood has been problematic. The exact reason for this is unclear, however the reported higher levels of endogenous immunoglobulins in rat serum compared to human or mouse serum, resulting in leaching of the exogenous anti-BCR antibodies, is likely a key contributing factor (Finkelman et al., 1980; Plebani et al., 1983; Rousseaux & Bazin, 1979; Van Vollenhoven et al., 1989). As rat is a commonly used preclinical species to evaluate drug efficacy and safety, we sought to overcome these issues and develop a BCR-mediated B cell stimulation assay in rat whole blood. We leveraged two different methods of B cell stimulation, utilizing dextran-coupled anti-IgD and combined use of anti-IgD and a TLR9 agonist. With both approaches, we were able to show robust activation of rat B cells in whole blood. Furthermore, we demonstrated that highly selective inhibitors of BTK, SYK, and PI3kδ showed inhibitory pharmacologic activity in both these assays. Our studies have identified two novel methods of BCR-mediated B cell activation in rat whole blood to support drug discovery efforts in the therapeutic targeting of this cell type and have expanded on the biology previously reported in human and mouse B cells of BCR downstream kinase signaling pathways and BCR/TLR synergistic effects in the rat.

2. Materials and methods

2.1. Materials

ACK lysing buffer was purchased from Lonza, and DMEM culture media and heat inactivated fetal calf serum (FCS) were from Life Technologies. Anti-rat IgD (clone Mard-3) was purchased from AbD Serotec. Dextran-coupled anti-IgD, consisting of approximately 15 anti-IgD antibodies (Mard-3) per dextran molecule, was conjugated by Fina BioSolutions. TLR9 ligands ODN2006 (5′-tcgtcgttttgtcgttttgtcgtt-3′) and ODN2395 (5′-tcgtcgttttcggcgc:gcgccg-3′) which are synthetic DNA molecules containing unmethylated CpG motifs, were purchased from InvivoGen. Fluorescently labeled anti CD86 (clone 24F), CD45R (clone HIS24), and CD3 (clone 1F4) were purchased from Becton Dickinson, while labeled CD45RA (clone OX33) was purchased from eBioscience. Anti-CD32/FC block (clone D34-485), mouse IgG1κ (clone MOPC21), BD FACS lysing solution, and BD cytofix were purchased from Becton Dickinson. Ibrutinib (BTK inhibitor) and Idelalisib (PI3Kδ inhibitor) were purchased from Cayman Chemical. Propriety BTK and SYK inhibitors were synthesized at Merck as previously described (De Man et al., 2013; Moy et al., 2013).

2.2. Animals and maintenance

Female Lewis rats (8–10 weeks, 150–175 g, Harlan Laboratories) were pair-housed in standard ventilated cages in a 12 h light/dark cycle and temperature-controlled environment (72 °F), relative humidity: 30%–70%. Water and pelleted-chow were provided ad libitum. All studies were performed in accordance with the National Institute of Health Guide to the Care and Use of Laboratory Animals and the Animal Welfare Association for the Assessment and Laboratory Animal Care Program. The procedures were approved by the Merck Institutional Animal Care and Use Committee.

2.2.1. Rat blood and spleen collection

Female Lewis rats were anesthetized by isoflurane inhalation. Blood was carefully collected by cardiac puncture, using an 18G needle and a syringe pre-coated with sodium heparin. The blood was transferred into a 15 mL tube containing 100 units of sodium heparin and used immediately. For spleen collection, female Lewis rats were euthanized by carbon dioxide inhalation, and the spleen was removed. Each spleen was placed in a 50 mL tube containing DMEM media, and used immediately thereafter.

2.3. Rat splenic B cell assay

The spleen from female Lewis rat was physically passed through a 70 μM nylon mesh directly into the media to prepare a single cell suspension. After centrifugation, two volumes of ACK lysing buffer were added to the cell pellet for 1 min to lyse the red blood cells. The splenocytes were then re-suspended in media, washed, and plated in a 96 well tissue culture plate. Each well contained 1 million splenocytes in 180 μL volume of DMEM media containing 5% FCS. For compound studies, 180 nL compound or DMSO control was added to each well using the ECHO 520 liquid dispenser then incubated for 1 h at 37 °C. The splenocytes were then stimulated with 3.12 μg/mL anti-IgD (Mard-3) for 20 h, in a 37 °C humidified incubator. After incubation, cells were washed twice with PBS/0.2% BSA solution, and then centrifuged. The cell pellet was re-suspended with 10 μg/mL FC block and incubated for 15 min on ice. Fluorescently labeled CD45R, CD3, and CD86 antibodies were subsequently added at 1.8 μg/mL for an additional 45 min on ice. The cells were fixed in cold BD cytofix for 10 min on ice, washed, re-suspended with PBS/0.2% BSA solution, and analyzed by flow cytometry. B cells were gated on $CD45R^+$ and $CD3^-$ cells from the cells with lymphoid scatter profile, and CD86 staining was measured as Mean Fluorescence Intensity (MFI) or percent positive cells. All flow cytometry analyses were performed using FlowJo version 10 software.

2.4. Rat whole blood B cell assay

135 μL of rat whole blood was plated in each well of a 96 well tissue culture plate. For compound studies, 135 nL inhibitor or DMSO control was added to each well using the ECHO 520 liquid dispenser and then incubated for 1 h at 37 °C, prior to the activation step. BCR and TLR agonists used were anti-IgD, dextran-coupled anti-IgD, ODN2395 and ODN2006. Corresponding isotype controls used were IgG1κ and amino-functionalized dextran. Agonist and relevant controls were directly added to the whole blood and incubated for 20 h in a 37 °C humidified incubator. After incubation, fluorescently labeled CD3, CD45R or CD45RA, and CD86 antibodies were directly added at 1.8 μg/mL for 45 min on ice. Red blood cells were lysed and the cells were fixed in one step using 20 volumes of BD lysing solution for 15 min at room temperature. The cells were then washed twice and re-suspended in PBS/0.2% BSA solution, and analyzed by flow cytometry. B cells were gated on $CD3^-$ and $CD45R^+$ or $CD45RA^+$ cells from the cells with lymphoid scatter profile, and CD86 staining was measured as Mean Fluorescence Intensity (MFI) or percent positive cells. All flow cytometry analyses were performed using FlowJo version 10 software.

2.5. Statistical analysis

All statistical analyses and curve fitting were performed using GraphPad Prism 6 software. Statistical significance was determined using one-way and two-way Anova followed by Tukey's post-test. A p-value < 0.05 was considered as statistically significant. Nonlinear regression analysis was used to determine concentration response curves and compound IC_{50} values.

3. Results

3.1. Activation of rat splenic B cells and attenuation with SYK, BTK, and PI3Kδ inhibitors

Preliminary studies in rat splenic B cells were performed to validate the use of CD86 as a marker of B cell activation. Following 20 h of stimulation with 3.12 μg/mL anti-IgD, the splenic B cell population displayed a uni-modal shift of 6.7 fold increase in CD86 Mean Fluorescence Intensity (MFI) or 61% $CD86^+$ B cells relative to the isotype control (Fig. 1A). Anti-IgD induced CD86 expression

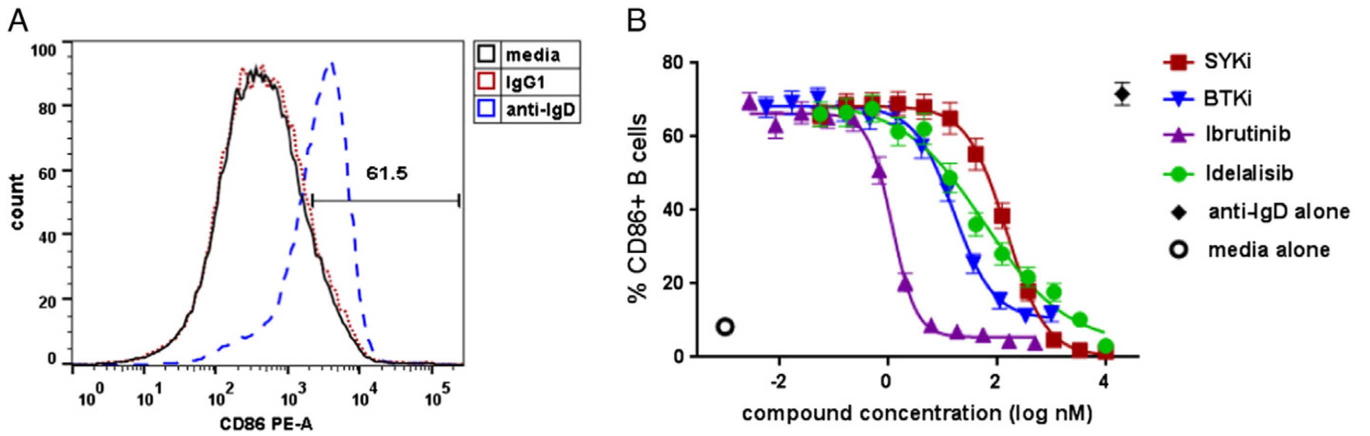

Fig. 1. (A) Representative flow cytometry histogram of CD86 up-regulation on rat splenic B cells stimulated with anti-IgD (dashed line) versus IgG1κ isotype control (dotted line). (B) Anti-IgD induced CD86 expression on rat splenic B cells in the presence of SYK, BTK, and PI3Kδ inhibitors, as measured by flow cytometry. Data are mean ± SD from two replicate animals.

was attenuated in a dose dependent manner with inhibitors of SYK (Merck proprietary SYKi), BTK (Ibrutinib and Merck proprietary BTKi) and PI3Kδ (Idelalisib; Fig. 1B).

3.2. Activation of rat B cells with dextran-coupled anti-IgD in whole blood

We next stimulated B cells in whole blood with anti-IgD or dextran-coupled anti-IgD, for 20 h. Both agonists produced concentration dependent up-regulation of CD86 on B cells with a greater degree of response observed with dextran-coupled anti-IgD versus anti-IgD alone (Fig. 2A). Maximal absolute percentages of $CD86^{+}$ B-cells were 25% following 3.75 μg/mL anti-IgD compared with 62% following 10 μg/mL dextran-coupled anti-IgD. Amino-functionalized dextran and IgG1κ isotype control had no effects (Fig. 2B). As submaximal concentration of agonist is commonly used to study the effects of compounds in in vitro functional assays, we used the effective concentration of 95% (EC_{95}) of 5 μg/mL dextran-coupled anti-IgD for subsequent studies to test kinase inhibitors.

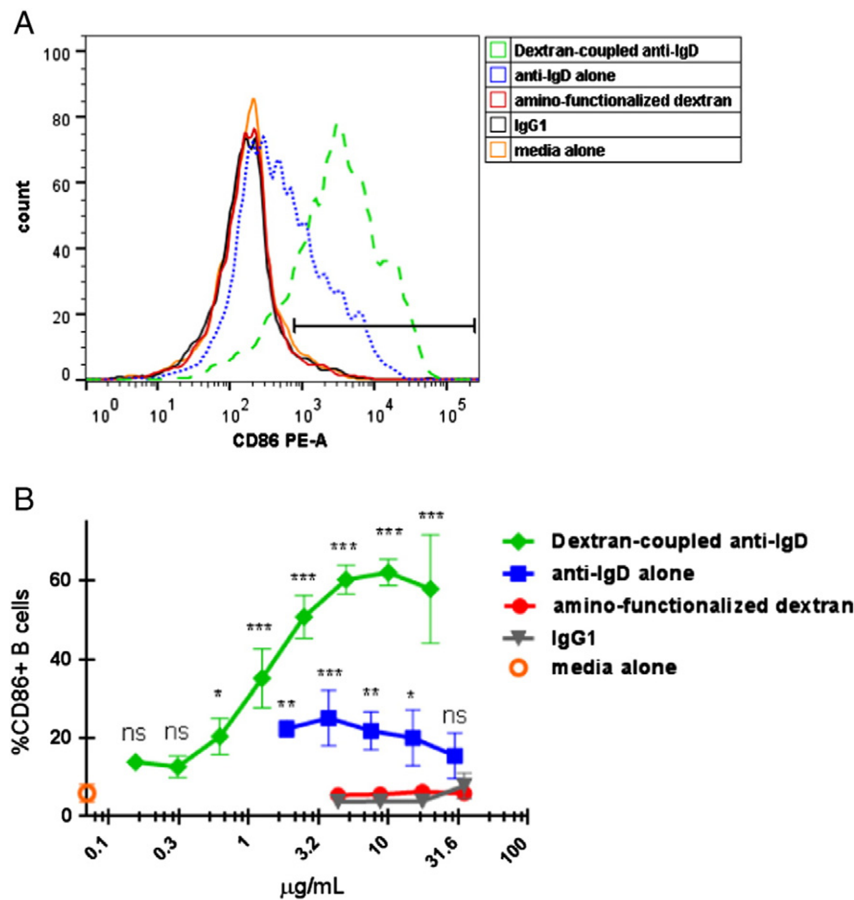

Fig. 2. (A) Representative flow cytometry histogram of CD86 up-regulation on rat B cells in whole blood stimulated with 5 μg/mL dextran-coupled anti-IgD (dashed line) or 3.75 μg/mL anti-IgD (dotted line). (B) Dose–response of CD86 up-regulation on rat B cells in whole blood stimulated with dextran-coupled anti-IgD (diamonds) or unconjugated anti-IgD (squares), as measured by flow cytometry. Data are mean ± SD from three replicate animals. *p < 0.05, **p < 0.01, ***p < 0.001, compared to media alone (one-way Anova).

3.3. Activation of rat B cells with anti-IgD and TLR9 co-stimulation in whole blood

We next combined anti-IgD to activate BCR with TLR9 stimulation (ODN2006 and ODN2395) based on previously published synergies in human and mouse B cells. The TLR ligands ODN2006 and ODN2395 specifically target TLR9 and activate B cells in similar fashion (Vollmer et al., 2004). In our initial study, rat whole blood was stimulated with a concentration of each TLR9 agonist in the presence and absence of 15 μg/mL anti-IgD. Anti-IgD or TLR9 stimulation alone produced modest up-regulation of CD86 and increases in the percentages of $CD86^+$ B cells in whole blood (approximately 20–40%) above the unstimulated sample (Fig. 3A, B & C). Co-stimulation with anti-IgD and TLR9 agonists resulted in a greater increase in the percentage of $CD86^+$ cells in whole blood compared to either agonist alone (Fig. 3A, B & C). Although co-stimulation with 2.5 μM ODN2006 and 15 μg/mL anti-IgD resulted in the greatest degree of B cell activation, co-stimulation with 2.5 μM ODN2395 and 15 μg/mL anti-IgD produced greater synergy in B cell activation. This combination of ODN2395 and anti-IgD resulted in a 10 fold increase in CD86 MFI (48% $CD86^+$ B cells) above ODN2395 alone and 4 fold increase in CD86 MFI (26% $CD86^+$ B cells) above anti-IgD alone (Fig. 3C). Therefore, ODN2395 co-stimulation was chosen over ODN2006 for further optimization and use in subsequent studies. Anti-IgD and ODN2395 concentrations were then cross titrated in rat whole blood, to determine the optimal combination that resulted in the greatest synergistic response. Concentration-dependent synergistic effects were observed with ODN2395 at each concentration of anti-IgD; altering the concentration of anti-IgD did not result in further activation of B cells (Fig. 3D). The combination of 3.75 μg/mL anti-IgD and 2.2 μM ODN2395 resulted in the greatest degree of effect compared to comparable concentrations of ODN2395 stimulation alone. These concentrations of combined agonists were used in subsequent studies to test kinase inhibitors.

3.4. Pharmacological validation of rat B cell stimulation by both methods in whole blood

Treatment with highly selective inhibitors of SYK (Merck proprietary SYKi), BTK (Ibrutinib and Merck proprietary BTKi) and PI3Kδ (Idelalisib) over a range of concentrations resulted in dose-dependent inhibition of B cell CD86 expression by both methods of stimulation (Figs. 4A & B). Whole blood potencies for these four compounds had the same rank order and similar potencies with both stimulation methods (Table 1).

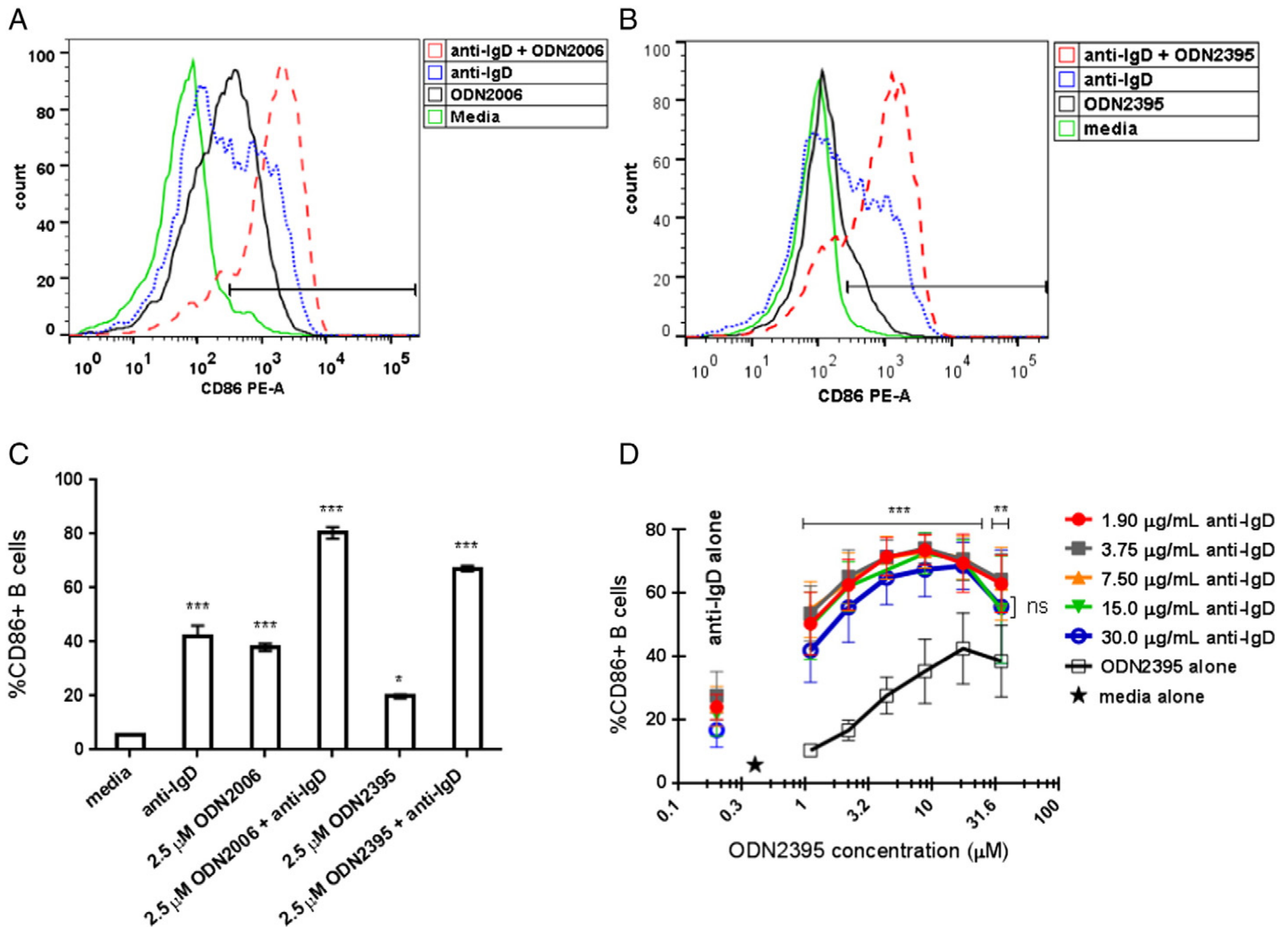

Fig. 3. (A) and (B) Representative flow cytometry histogram of CD86 up-regulation on rat B cells in whole blood stimulated with 2.5 μM ODN2006 and 2.5 μM ODN2395 in the presence and absence of 15 μg/mL anti-IgD (solid black and dashed lines, respectively). (C) Percentages of CD86 expression on rat B cells in whole blood stimulated with 2.5 μM ODN2006 and 2.5 μM ODN2395 in the presence and absence of 15 μg/mL anti-IgD. Data are mean ± SD of two replicate animals. *$p < 0.05$, ***$p < 0.001$, compared to media alone (one-way Anova). (D) Percentages of CD86 expression on rat B cells in whole blood following dose-dependent cross titration of anti-IgD and ODN2395. Data are mean ± SD from four replicate animals. **$p < 0.01$, ***$p < 0.001$ for ODN2395 and anti-IgD combinations compared to ODN2395 alone (two-way Anova).

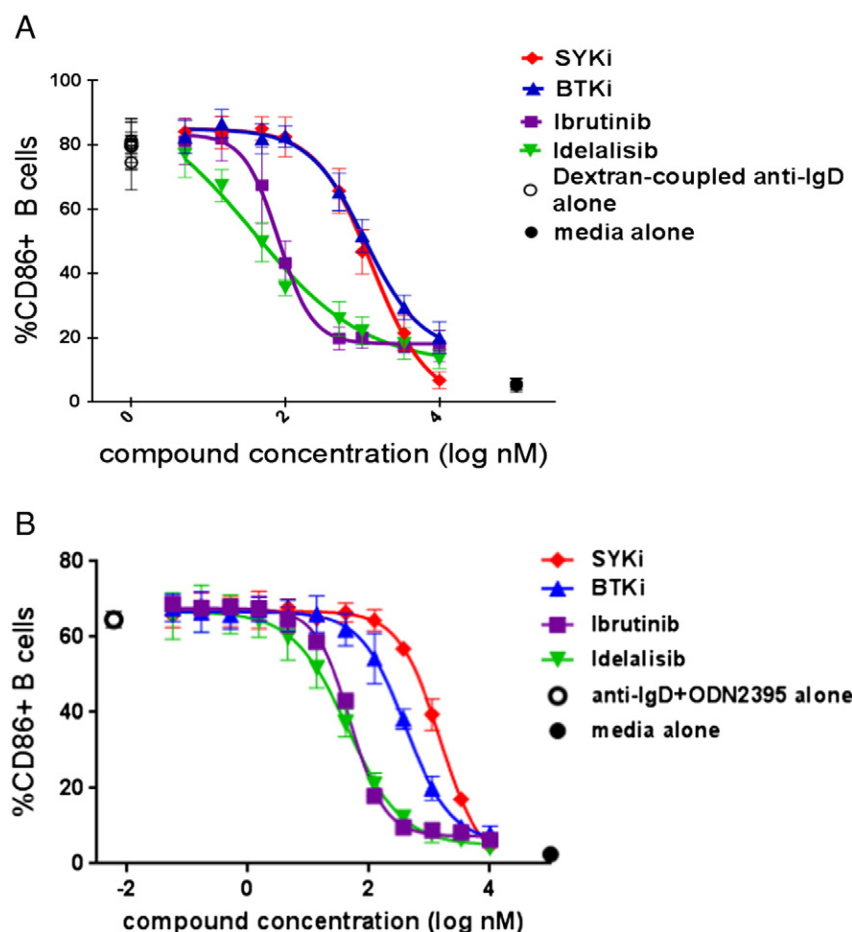

Fig. 4. Effects of SYK, BTK, and PI3Kδ inhibitors on percentages of CD86 expression on rat B cells in whole blood following stimulation with (A) dextran-coupled anti-IgD and (B) anti-IgD and ODN2395 combination. Data are mean ± SD of four replicate animals.

4. Discussion

Whole blood functional assays are pharmacologically relevant in the drug discovery process to evaluate potency in a biologically relevant matrix, to support the establishment of PK/PD relationships and to aid in human dose predictions. In the case of activation and measurement of immune cell activity to support the development of drugs for inflammatory and autoimmune diseases, methods of B cell activation have been reported in both human and mouse whole blood (Xu et al., 2012). However, methods of quantification of B cell activity, requiring both stimulation and detection of cellular activation in rat whole blood remain to be established. Historically, it has been a challenge to stimulate B cells in rat whole blood using soluble anti-IgM or anti-IgD to evoke BCR ligation likely as a result of the reported higher levels of endogenous immunoglobulins in rat serum compared to human or mouse serum resulting in leaching of the exogenous anti-BCR antibodies (Finkelman et al., 1980; Plebani et al., 1983; Rousseaux & Bazin, 1979; Van Vollenhoven et al., 1989). As rat is a highly relevant preclinical efficacy and safety species in drug discovery, we sought to overcome these issues and develop two different methods of B cell stimulation, using dextran-coupled anti-IgD or combined use of anti-IgD and a TLR9 agonist, in rat whole blood. Furthermore, we validated these assays pharmacologically with the use of highly selective inhibitors of BTK, SYK, and PI3Kδ, downstream BCR signaling kinases. These rat BCR-mediated B cell stimulation assays in whole blood represent novel methods to aid in the drug discovery process in the development of B cell therapies for the potential treatment of inflammatory and autoimmune diseases.

Cross-linking of B-cell receptors by anti-Ig conjugated to high molecular weight dextran to mimic antigen-stimulation has previously been demonstrated to greatly enhance B cell activation in purified human and murine B cells in vitro (Brunswick et al., 1988; Rehe et al., 1990). Compared with soluble anti-Ig antibodies, whose use is limited by requirement for high molar concentrations that mediate the rapid modulation of surface Ig, dextran-conjugated anti-Ig antibodies have been shown to evoke greater B cell activation and proliferation. We adopted this method to rat whole blood, and in similarity to what have been reported in purified human and murine B cells in vitro, observed enhanced B cell activation, as measured by the percentage of $CD86^+$ B cells, with dextran-coupled anti-IgD when compared to unconjugated anti-IgD. To our knowledge, this is the first report on the use of dextran-coupled anti-IgD in a whole blood system. Unconjugated anti-IgD produced minimal increases in the percentage of $CD86^+$ B cells in whole blood, despite the use of high concentrations. The reason for this remains unclear, however, we propose that high levels of endogenous serum immunoglobulins in rat whole blood can leach the

Table 1
Compound potencies of SYK, BTK and PI3Kδ inhibitors on B cell activation in rat whole blood evoked by dextran-coupled anti-IgD or anti-IgD in combination with ODN2395. Data are mean ± SD in nanomolar of four replicate animals.

Inhibitor	Dextran-coupled anti-IgD (N = 4)	Anti-IgD + ODN2395 (N = 4)
SYKi	1102 ± 152	1304 ± 84
BTKi	1024 ± 23	403 ± 13
Ibrutinib	86 ± 9	49 ± 4
Idelalisib	81 ± 10	44 ± 4

unconjugated anti-BCR agonist, leaving less available to fully occupy the BCR. Partial occupancy is not sufficient to reach the threshold for full B cell activation and therefore, we observed suboptimal B cell activation in whole blood despite stimulating with high concentrations of anti-IgD (Finkelman et al., 1980; Plebani et al., 1983; Rousseaux & Bazin, 1979; Van Vollenhoven et al., 1989). Compared with dextran-coupled anti-IgD, dextran alone did not have effects on B cell activation, suggesting that the carrier molecule was not contributing to the stimulus. These data are consistent with the lack of contribution of dextran in activation of human and murine B cells (Brunswick et al., 1988; Rehe et al., 1990). The enhanced stimulatory capacity of dextran-coupled anti-IgD has been proposed to be due to enhanced cross-linking of the BCR due to the presentation of a multivalent array of anti-Ig molecules on a large carrier molecule (Rehe et al., 1990). In further support of this mechanism, we utilized highly selective pharmacological inhibitors of the kinases, SYK (SYKi; Moy et al., 2013), BTK (Ibrutinib and a proprietary Merck compound, BTKi; Byrd et al., 2013; De Man et al., 2013) and PI3Kδ (Idelalisib; Brown et al., 2014), that mediate downstream signaling following cross-linking of the BCR. Dextran-coupled anti-IgD-evoked B cell activation in rat whole blood was inhibited by each of the kinase inhibitors providing evidence for BCR engagement as the mechanism mediating response to this stimulus.

In addition to the established role of the BCR in B cell function, Toll-like receptors (TLR), a group of proteins critical to innate immune responses, are expressed in B cells and play a biological role. In this respect, several groups have recently shown that TLR9, which mediates biological responses to single stranded unmethylated CpG motifs, synergizes with BCR to amplify B cell activation in purified human and murine B cells (Iwata et al., 2012; Leadbetter et al., 2002). This synergy has been proposed to play a potential pathological role in SLE where autoreactive B cells are activated by self-derived DNA, resulting in auto-antibody production (Christensen et al., 2005; Leadbetter et al., 2002; Viglianti et al., 2003; Wen et al., 2013). Upon BCR ligation, the BCR and TLR9 translocate to the auto-phagosome-like compartment allowing for proximal BCR bound DNA antigen presentation to TLR9. B cells are subsequently hyper-activated with increased MAPK and NF-κB signaling, B cell proliferation and pro-inflammatory cytokine production (Chaturvedi, Dorward, & Pierce, 2008; Yi, Yoon, & Krieg, 2003; Iwata et al., 2012). In the present studies, we leveraged this reported synergistic biology to evoke B cell activation in rat whole blood. Expanding on previously published data in purified human and murine B cells, synergy was observed with BCR and TLR9 co-stimulation in rat whole blood. Our data show that partial occupancy of the BCR is evident with either anti-IgD or TLR9 stimulation as a proportion of B cells are not activated following administration of these agonists alone. Co-stimulation with combined anti-IgD and TLR9 agonism enables those B cells with partial BCR occupancy to reach the threshold for full activation, resulting in robust B cell stimulation in whole blood as measured by increases in CD86 expression. To our knowledge, this is the first report of synergistic effects of BCR and TLR9 co-stimulation in rat B cells and in a whole blood system. It has previously been demonstrated that the synergistic signaling between BCR and TLR9 stimulation in purified human and murine B cells is dependent on the downstream signaling kinases, SYK and BTK (Iwata et al., 2012; Kenny et al., 2013). Using highly selective inhibitors of SYK, BTK and PI3Kδ, we confirmed that the BCR and TLR9 synergistic response in rat B cells is dependent on SYK and BTK and further show a novel role for PI3Kδ in this signaling interaction, which has not been previously reported.

In summary, two novel methods were developed to stimulate B cells in rat whole blood, namely utilizing dextran-coupled anti-IgD and combined use of anti-IgD and a TLR9 agonist, that have the potential to be used to support drug discovery efforts in the therapeutic targeting of B cells. Furthermore, we pharmacologically validated this whole blood assay using highly selective inhibitors of BTK, SYK, and PI3Kδ, signaling kinases that are downstream of the BCR. These studies have thus expanded on the biology previously reported in human and mouse B cells of BCR downstream kinase signaling and BCR/TLR synergistic effects in rat B cells, and support these kinases as potential therapeutic targets in autoimmune diseases.

Acknowledgments

This research was wholly funded by Merck & Co. Inc.

References

Brown, J.R., Byrd, J.C., Coutre, S.E., Benson, D.M., Flinn, I.W., Wagner-Johnston, N.D., et al. (2014). Idelalisib, an inhibitor of phosphatidylinositol 3-kinase p110d, for relapsed/refractory chronic lymphocytic leukemia. *Blood*, *123*(22), 3390–3397.

Brunswick, M., Finkelman, F.D., Highet, P.F., Inman, J.K., Dintzis, H.M., & Mond, J.J. (1988). Picogram quantities of anti-Ig antibodies coupled to dextran induce B cell proliferation. *The Journal of Immunology*, *140*(10), 3364–3372.

Byrd, J.C., Furman, R.R., Coutre, S.E., Flinn, I.W., Burger, J.A., Blum, K.A., et al. (2013). Targeting BTK with Ibrutinib in relapsed chronic lymphocytic leukemia. *The New England Journal of Medicine*, *369*, 32–42.

Chaturvedi, A., Dorward, D., & Pierce, S.K. (2008). The B cell receptor governs the subcellular location of Toll-like receptor 9 leading to hyperresponses to DNA-containing antigens. *Immunity*, *28*, 799–809.

Christensen, S.R., Kashgarian, M., Alexopoulou, L., Flavell, R.A., Akira, S., & Shlomchik, M.J. (2005). Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *The Journal of Experimental Medicine*, *202*(2), 321–331.

Dal Porto, J.M., Gauld, S.B., Merrell, K.T., Mills, D., Pugh-Bernard, A.E., & Cambier, J. (2004). B cell antigen receptor signaling 101. *Molecular Immunology*, *41*, 599–613.

De Man, A.P.A., Sterrenburg, J., Raaijmakers, H.C.A., Kaptein, A., Oubrie, A., Rewinkel, J.B.M., et al. (2013). *Btk inhibitors. WO2013010380.*

Dörner, T., Kaufmann, J., Wegener, W.A., Teoh, N., Goldenberg, D.M., & Burmester, G.R. (2006). Initial clinical trial of epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus. *Arthritis Research & Therapy*, *8*(3).

Edwards, J.C.W., Szczepanski, L., Szechinski, J., Filipowicz-Sosnowska, A., Emery, P., Close, D.R., et al. (2004). Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *The New England Journal of Medicine*, *350*, 2572–2581.

Finkelman, F.D., Woods, V.L., Wilburn, S.B., Mond, J.J., Stein, K.E., Berning, A., et al. (1980). Augmentation of in vitro humoral immune responses in the mouse by an antibody to IgD. *The Journal of Experimental Medicine*, *152*, 493–506.

Hauser, S.L., Waubant, E., Arnold, D.L., Vollmer, T., Antel, J., Fox, R.J., et al. (2008). B-cell depletion with rituximab in relapsing–remitting multiple sclerosis. *The New England Journal of Medicine*, *358*, 676–688.

Iwata, S., Yamaoka, K., Niiro, H., Nakano, K., Wang, S., Akashi, K., et al. (2012). Amplification of Toll-like receptor-mediated signaling through spleen tyrosine kinase in human B-cell activation. *The Journal of Allergy and Clinical Immunology*, *129*(6), 1595–1601.

Kenny, E.F., Quinn, S.R., Doyle, S.L., Vink, P.M., Van Eenennaam, H., & O'Neill, L.A.J. (2013). Bruton's tyrosine kinase mediates the synergistic signalling between TLR9 and the B cell receptor by regulating calcium and calmodulin. *PloS One*, *8*(8), 1–14.

Leadbetter, E.A., Rifkin, I.R., Hohlbaum, A.M., Beaudette, B.C., Shlomchik, M.J., & Marshak-Rothstein, A. (2002). Chromatin–IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature*, *416*, 603–607.

Moy, L.Y., Jia, Y., Caniga, M., Lieber, G., Gil, M., Fernandez, X., et al. (2013). Inhibition of spleen tyrosine kinase attenuates allergen-mediated airway constriction. *American Journal of Respiratory Cell and Molecular Biology*, *49*(6), 1085–1092.

Navarra, S.V., Guzmán, R.M., Gallacher, A.E., Hall, S., Levy, R.A., Jimenez, R.E., et al. (2011). Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: A randomised, placebo-controlled, phase 3 trial. *Lancet*, *377*, 721–731.

Plebani, A., Mira, E., Mevio, E., Monafo, V., Notarangelo, L.D., Avanzini, A., et al. (1983). IgM and IgD concentrations in the serum and secretions of children with selective IgA deficiency. *Clinical and Experimental Immunology*, *53*, 689–696.

Puri, K.D., Di Paolo, J.A., & Gold, M.R. (2013). B-cell receptor signaling inhibitors for treatment of autoimmune inflammatory diseases and B-cell malignancies. *International Reviews of Immunology*, *32*, 397–427.

Rehe, G.T., Katona, I.M., Brunswick, M., Wahl, L.M., June, C.H., & Mond, J.J. (1990). Activation of human B lymphocytes by nanogram concentrations of anti-IgM–dextran conjugates. *European Journal of Immunology*, *20*, 1837–1842.

Rousseaux, J., & Bazin, H. (1979). Rat immunoglobulins. *Veterinary Immunology and Immunopathology*, *1*, 61–78.

Takemura, S., Klimiuk, P.A., Braun, A., Goronzy, J.J., & Weyand, C.M. (2001). T cell activation in rheumatoid synovium is B cell dependent. *The Journal of Immunology*, *167*, 4710–4718.

Van Vollenhoven, R.F., Swenson, C.D., Soriano, A., Goidl, E.A., Coico, R.F., Jeanette Thorbecke, G., et al. (1989). Serum IgD levels in mice: Effect of strain, age and autoimmune disease. *Journal of Autoimmunity*, *2*, 259–267.

Viglianti, G.A., Lau, C.M., Hanley, T.M., Miko, B.A., Shlomchik, M.J., & Marshak-Rothstein, A. (2003). Activation of autoreactive B cells by CpG dsDNA. *Immunity*, *19*, 837–847.

Vollmer, J., Weeratna, R., Payette, P., Jurk, M., Schetter, C., Laucht, M., et al. (2004). Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *European Journal of Immunology*, *34*, 251–262.

Weinblatt, M.E., Kavanaugh, A., Genovese, M.C., Musser, T.K., Grossbard, E.B., & Magilavy, D.B. (2010). An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. *The New England Journal of Medicine*, *363*, 1303–1312.

Wen, Z., Xu, L., Chen, X., Xu, W., Yin, Z., Gao, X., et al. (2013). Autoantibody induction by DNA-containing immune complexes requires HMGB1 with the TLR2/MicroRNA-155 pathway. *The Journal of Immunology*, *190*, 5411–5422.

Wither, J.E., Roy, V., & Brennan, L.A. (2000). Activated B cells express increased levels of costimulatory molecules in young autoimmune NZB and (NZB 3 NZW)F mice. *Clinical Immunology*, *94*(1), 51–63.

Xu, D., Kim, Y., Postelnek, J., Vu, M.D., Hu, D.Q., Liao, C., et al. (2012). RN486, a selective Bruton's tyrosine kinase inhibitor, abrogates immune hypersensitivity responses and arthritis in rodents. *The Journal of Pharmacology and Experimental Therapeutics*, *341*(1), 90–103.

Yi, A., Yoon, J., & Krieg, A.M. (2003). Convergence of CpG DNA- and BCR mediated signals at the c-Jun N-terminal kinase and NF-kB activation pathways: Regulation by mitogen-activated protein kinases. *International Immunology*, *15*(5), 577–591.

Zhao, X., Okeke, N.L., Sharpe, O., Batliwalla, F.M., Lee, A.T., Ho, P.P., et al. (2008). Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. *Arthritis Research & Therapy*, *10*(4).