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Syk Inhibition with Fostamatinib Leads to Transitional B Lymphocyte Depletion

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Abstract

Cell signaling initiated by the B cell receptor is critical to normal development of B lymphocytes, most notably at the transitional B cell stage. Inhibition of this signaling pathway with the syk inhibitor, fostamatinib, has produced significant efficacy in lymphoid malignancies and autoimmune conditions. Here, we demonstrate that short-term use of fostamatinib impairs B lymphocyte development at the transitional stage without affecting mature B cell populations. Additionally, IL-10 producing B cells remained relatively constant throughout the treatment period. These findings provide insight into the mechanism of action of B cell receptor inhibition in autoimmune disease. As the development of agents targeting B cell receptor signaling proceeds, monitoring for long-term consequences as well as functional evaluation of B cell subsets may further improve our understanding of this rapidly growing class of novel agents.

Keywords

B-cell antigen receptor; Signaling therapies; Transitional B cells; Fostamatinib

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Conflict of Interest

Dr. Friedberg received research support from Rigel pharmaceuticals

Author Contributions

P.M.B. designed the research, analyzed data, drafted the paper and approved the final version of the paper; C.W. performed experiments, analyzed data, assisted in drafting the paper and approved the final version of the paper; J.S.-C. performed experiments and approved the final version of the paper; J.L.K analyzed data, assisted in drafting the paper and approved the final version of the paper; A.F.R analyzed data, assisted in drafting the paper and approved the final version of the paper; J.J. and J.R. performed experiments and approved the final version of the paper; I.S. supervised the research, analyzed data, reviewed drafts and approved the final version of the paper; J.W.F planned the research, analyzed data, reviewed drafts, obtained funding for the work, and approved the final version of the paper.

1. Introduction

The protein tyrosine kinase syk (spleen tyrosine kinase), is a critical component of B cell receptor (BCR) signaling, functioning as the major downstream effector protein. Promising data have been generated using an orally administered small molecule inhibitor of syk, fostamatinib, in the treatment of lymphoid malignancies as well as autoimmune diseases such as rheumatoid arthritis and immune thrombocytopenic purpura [1–3]. As a result, ongoing studies are attempting to confirm these findings in addition to the development of new agents targeting similar molecules.

During B cell development, immature B cells are generated in the bone marrow and transit to the spleen as they progress toward immune competence. Between immature and mature populations, critical intermediates have been termed “transitional B cells”. As transitional cells relocate anatomically, mature surface markers including the BCR are gradually acquired. In addition to this phenotypic change, functional changes occur in this subpopulation of cells. In the more immature T1 subset, engagement of the BCR promotes apoptosis. Murine studies suggest that BCR stimulation in the T2 subset leads to proliferation and differentiation into more mature functional B cell subsets [4–7].

As a mediator of the BCR signal, syk plays an important role in both the maturation and survival of the B cell lineage. Syk deficient murine models demonstrate a developmental block at the transitional B cell stage and an absence of B cells in peripheral lymphoid organs [8]. These observations suggest particular importance of the BCR in the transitional stage of B cell maturation.

The effects of biological agents on IL-10 production by B cells is also of interest as this function has been ascribed to different B cell subsets including regulatory B cells with a transitional phenotype [9–10]. Moreover, IL-10 production by transitional cells may be defective in systemic lupus erythematosus (SLE) and restoration of IL-10 production by naïve B cells has been proposed to contribute to the beneficial effect of rituximab in multiple sclerosis [9, 11]. On the other hand, B cell-derived IL-10 may also exert pathogenic functions. In non-Hodgkin lymphoma, IL-10 may act as an autocrine growth factor for lymphoma cells in addition to providing immunosuppressive properties needed for disease progression [12–14]. Further, IL-10 inhibition by fostamatinib in B cell NHL has been demonstrated *in vitro*, potentially contributing to its anti-lymphoma effect [15].

We therefore investigated whether fostamatinib impacted transitional and IL-10 producing B cell populations *in vivo* in patients with lymphoid malignancies to better understand the mechanism of action of BCR inhibition and the potential long term consequences.

2. Materials and Methods

2.1. Sample procurement and cell isolation

Samples were obtained from a prospective phase 1/2 clinical trial with support from Rigel Pharmaceuticals as previously reported [2]. Eleven patients, all with relapsed or refractory B-cell lymphoid malignancies [7 diffuse large B cell lymphoma (DLBCL), 2 follicular lymphoma (FL), 2 mantle cell lymphoma (MCL)] had adequate peripheral blood mononuclear cells (PBMC) for analysis. Patients provided written informed consent according to the Declaration of Helsinki on an Institutional Review Board-approved protocol for the collection and use of samples for research purposes. Patients underwent blood draws on days 1, 29 and 57 of treatment with fostamatinib at either 200 or 250 mg daily. 8 mL of whole blood was collected into sodium heparin CPT tubes and processed within 2 hours of collection. Samples were centrifuged at 1500xg at 20°C for 30 minutes

prior to isolation and storage. Mitotracker Green extrusion (MTG) and the staining of CD19, IgD, CD27, CD38, CD24 as well as CD3/live/dead exclusion allow the identification of B cell subsets defined by the expression of IgD and CD27 and the fine discrimination of naïve and transitional B cells. Intracellular staining of IL-10 was obtained after a 5 hour stimulation with PMA and Ionomycin (500 ng/ml) and subsequent fixation / permabilization of parallel samples.

2.2. Flow cytometry analysis

After thawing, PBMCs purified through Ficoll density gradient centrifugation were first pulsed with 20 nM of MTG FM dye (Invitrogen) in complete RPMI1640 medium at 37°C for 30 min, and then washed in warm medium. Afterwards, cells were stained in PBS/0.5% BSA in the presence of 5% normal mouse serum on ice for 30 min with the following fluorochrome-labeled mouse anti-human monoclonal antibodies: PE-anti-IgD (IA6-2, BD Biosciences), PE-Alexa610-anti-CD24 (SN3, Invitrogen), PerCP-Cy5.5-anti-CD38 (HIT2, BD), Pacific Blue-anti-CD3 (SP34-2, BD), Qdot605-anti-CD27 (CLB-27/1, Invitrogen), and APC-Cy7-anti-CD19 (SJ25C1, BD). Cells were then washed with PBS and stained in PBS containing LIVE/DEAD aqua-fluorescent reactive dye (Invitrogen) on ice for 30 min. Stained cells were washed with PBS/0.5% BSA, and data were collected using the LSRII Flow Cytometer (BD) and analyzed with the FlowJo software (Tree Star). Cells were classified into naïve and transitional subsets. Lymphocytes gated through the FSC-A vs SSC-A plot were further interrogated by the ratios of Height to Width in both forward scatter and side scatter, as well as their ability to uptake the amine-reactive Aqua fluorescent dye in order to gate out cell aggregates and dead cells, respectively. Live CD19⁺CD3⁻ B cells were then selected for analysis. IgD⁺CD27⁻ B cells, which consists of naïve and transitional B cells, can be separated into MTG naïve (N) and MTG⁺ cells with the latter population including the totality of transitional B cells. The CD24/CD38 expression boundary of the MTG⁻ naïve cells was used as a gate and superimposed onto the MTG⁺ cells to identify the late T3 transitional cells. The remainder of the MTG⁺ cells was then identified as early T1/T2 transitional cells. Gating strategy based on the extrusion of MTG and expression of CD24 and CD38 was described in detail previously [16]. Identical analysis was performed, with the exception of MTG staining, for the IL-10 samples; fine determination of T1/T2 versus T3 was not possible in these samples due to the fixation protocol. The IL-10⁺ gate was determined by comparing the stained unstimulated cells to stimulated for all samples.

2.3. Statistical analysis

P-values were calculated using the non-parametric Wilcoxon signed rank test for paired samples. To account for the 8 independent hypotheses tested, we used a Bonferroni adjusted threshold for statistical significance ($p < 0.006$). Difference in the absolute number of IL10⁺ B cells were tested for significance using a nonparametric one-way analysis of variance (Kruskal-Wallis).

3. Results and Discussion

In our patients, total CD19⁺ B cells remained unaffected after 2 months of treatment with fostamatinib (figure 1A). However, the early transitional (T1/T2) cells were rapidly depleted from the peripheral blood within the first month of treatment ($p = .0029$) and continued to decline over the second month ($p = .0039$; figure 1A). There was a lesser magnitude decrease in the late transitional (T3) cells at 2 months ($p = .02$), with a concomitant increase in the naïve population ($p = .0059$). The decrease in the T1/T2 population was also observed when absolute cell counts for the B cell subsets were calculated ($p = .0039$; figure 1B). Figure 1C further demonstrates the decrease in the number of T1/T2 B cells compared to the relative

stability of CD19+ B cells and the fractions of the four core subsets defined by IgD/CD27. No association was identified between infections or clinical responses and the degree of transitional B cell depletion (data not shown).

These findings recapitulate murine models demonstrating a block in B cell development with inhibition of BCR signaling [8]. A B cell developmental block from T1 to T2 has been observed in mice lacking the cytoplasmic tail of Ig α , important for the initial signaling of the BCR [6] as well as in mice deficient in Bruton's tyrosine kinase (BTK) [4], CD45 [17], BLNK [18], PLC β [19] and BCAP [20]. Previous data has demonstrated human CD27- B cells to have a 5 month half life as compared to 1 month in the CD27+ population [21]. Further, we have previously reported a substantially higher proliferation rate for transitional as compared to naïve B cells [22]. Together, the higher proliferation rate, shorter survival and increased dependency on tonic BCR signaling may explain the depletion of the transitional cell population relative to other subsets with short term syk inhibition.

Fostamatinib has been reported to induce a transient decrease in mature B cell populations in a variety of murine models with brief as well as prolonged administration periods. [23–25] An increase in normal B cells was noted in a E μ -TCL1 model of CLL with fostamatinib treatment, similar to what we and others have observed in patients with hematologic malignancies [26]. Whether the relative lack of change in total CD19+ B cells relate to the species specific differences in half-lives of various B cell populations versus differences in chemotaxis and integrin signaling is not known.

These results provide mechanistic insights of syk inhibition in autoimmune diseases. Early B cell tolerance is enforced through checkpoints that censor the maturation of autoreactive transitional B cells. One of the main checkpoints for peripheral tolerance operates at the T2 developmental stage [27]. Given that these early checkpoints appear to be defective in autoimmune diseases such as SLE and rheumatoid arthritis [28–30], it could be postulated that dampening of enhanced BCR signaling in these diseases could contribute to the restoration of proper censoring.

Ongoing investigations of syk inhibitors are using prolonged administration schedules. A subset of T2 cells (T2-MZP) represents the precursor of murine marginal zone B cells which play critical roles in the generation of quick protective responses against blood-borne encapsulated bacteria. Whether similar developmental pathways exist in humans and whether more prolonged syk inhibition may block the development and/or survival of the human marginal zone equivalent remains to be determined. However, in this short treatment period, the IgD+CD27+ blood population, proposed by some to represent a recirculating marginal zone population, was not significantly affected and instead increased in several subjects. Given the pleiotropic effects of syk on multiple innate immune cells, it remains to be determined whether any consequences of its inhibition on B cell maturation or marginal zone defects will occur.

Figure 2A demonstrates a lack of change in total IL-10 producing B cells over the 2 month treatment period. Other than a slight decrease within the transitional compartment, IL-10+ B cell subsets were not significantly affected by syk inhibition (figure 2B).

Decreased IL-10 production has been demonstrated with syk inhibition as well as with blockade of other molecules downstream of the BCR. In SLE, serum IL-10 levels may be significantly elevated and play a pathogenic role [31]. Moreover, newly described pro-B10 cells may also be significantly increased in a fraction of SLE subjects [10]. As serum IL-10 has been demonstrated to be a poor prognostic factor in non-Hodgkin lymphoma [32], understanding the etiology of this decrease with BCR inhibition may further define the mechanism of action for this class of agents. Our results suggest that syk inhibition does not

significantly decrease the percentage and absolute numbers of peripheral IL-10+ B cells suggesting that decreased IL-10 production as a result of BCR and specifically syk inhibition may be a result of decreased production from sources other than B lymphocytes.

In conclusion, we have demonstrated that BCR signaling inhibition with fostamatinib does not diminish mature B cell populations, but does result in significant depletion of transitional B cells without decreasing total IL-10 producing B cells from the peripheral blood. Given the clinical efficacy of fostamatinib and other agents active downstream of the BCR including BTK, phosphoinositide 3-kinase and mammalian target of rapamycin [33–35] these results have key implications on clinical development of these rationally targeted agents.

Highlights

Agents targeting B cell receptor signaling have demonstrated significant efficacy.

We examined the effects of fostamatinib on normal B lymphocytes.

Short term syk inhibition does not affect mature B cell populations.

Peripheral blood transitional B cell populations are depleted with fostamatinib.

IL-10 producing B cells are unaffected with fostamatinib.

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Figure 1. Transitional B cells are depleted over the treatment period

Panel A. Frequencies of CD19+ (lymphocytes), and T1/T2 Transitional, T3 Transitional and Naive (of CD19+) at baseline (B), 1 and 2 months. Statistical significance is indicated by the bars underneath each plot. Grey bars indicate $p < 0.05$ for the comparison, and black bars indicate $p < 0.006$, the Bonferroni adjusted significance threshold. *Panel B.* Median absolute cell count for the T1/T2 transitional cell, T3 transitional cell, and naïve components of the total CD19+IgD+CD27- population at baseline, 1 month and 2 months post therapy. *Panel C.* Heatmap representation of perfect change in log2 absolute cells at 2 months vs. baseline. The relative change from baseline to 2 months in the B lymphocyte populations is depicted

according to the color scale shown; a decrease is depicted in shades of green; an increase is shown in shades of red.

Figure 2. Change in the number of IL-10 producing B cells following syk inhibition

Panel A. The average percentages and total numbers of IL-10⁺ B cells do not significantly change over the treatment period implying that B lymphocyte IL-10 production is not altered by syk inhibition. *Panel B.* Heatmap representation of IL-10 producing B cells in absolute counts. Total IL-10 producing B cells (the leftmost panel) were further divided into the five core B cell subsets based on their phenotype (subsequent five panels). In each panel, rows are individual subjects, columns are the three time points measured. Gray indicates missing data.