Tyrosine kinase inhibitors impair B cell immune responses in CML through off-target inhibition of kinases important for cell signaling

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Our results call for close monitoring of patients on TKI to assess the long-term impact of impaired B cell function

Abstract
Tyrosine kinase inhibitors (TKI) have significant off-target multi-kinase inhibitory effects. We aimed to study the impact of TKIs on the in vivo B-cell response to vaccination. Cellular and humoral responses to influenza and pneumococcal vaccines were evaluated in 51 chronic phase CML patients on imatinib, or second-line dasatinib and nilotinib and 24 controls. Following vaccination, CML patients on TKI had significant impairment of IgM humoral response to pneumococcus compared to controls (IgM titer 79.0 vs. 200 U/ml, p=0.0006), associated with significantly lower frequencies of peripheral blood IgM memory B cells. To elucidate whether CML itself or treatment with TKI was responsible for the impaired humoral response, we assessed memory B-cell subsets in paired samples collected before and after imatinib therapy. Treatment with imatinib was associated with significant reductions in IgM memory B-cells. In vitro co-incubation of B-cells with plasma from CML patients on TKI or with imatinib, dasatinib or nilotinib induced significant and dose-dependent inhibition of Bruton's tyrosine kinase and indirectly its downstream substrate, phospholipase-C-γ2, both important in B cell signaling and survival. These data indicate that TKI, through off-target inhibition of kinases important in B-cell signaling, reduce memory B-cell frequencies and induce significant impairment of B-cell responses in CML.
Introduction

The tyrosine kinase inhibitors (TKIs) imatinib, nilotinib and dasatinib are remarkably effective as single-agent therapy for chronic myeloid leukemia (CML) in chronic phase (CP). To date very few in vivo human studies have addressed the long-term impact of these molecular-targeted drugs on the immune function. Data from in vitro and animal studies have documented seemingly contradictory effects of imatinib on the immune response, ranging from impaired antigen-specific T-cell responses to reversal of T-cell tolerance and potentiation of anti-tumor immune responses. The limited in vitro data available with second-generation TKIs nilotinib (Novartis) and dasatinib (Bristol-Myers Squibb) show impaired antigen-specific T-cell responses; however recent studies report rapid mobilization and expansion of BCR-ABL−negative lymphoid cells in dasatinib-treated patients. Few studies have examined the impact of TKIs on B cell responses to antigen in vivo, although hypogammaglobulinemia has been reported in CML patients treated with imatinib. A recent murine study reported that imatinib may directly impair class-switch recombination following B cell activation through downregulation of activation-induced cytidine deaminase. To date, no studies have examined the impact of first and second-generation TKIs on B cell responses to vaccination in patients with CML.

We hypothesized that TKI may interfere with vaccine-induced cellular and humoral immune responses in CML patients on TKI through their off-target multi-kinase inhibitory effects. We characterized T and B cell responses to vaccination against influenza and pneumococcus in CP-CML patients receiving imatinib, dasatinib and nilotinib and healthy controls. We found that the B cell response to pneumococcal vaccine is significantly impaired in CML patients, associated with loss of memory B cell subsets. Furthermore, we showed that all 3 TKIs suppress an important kinase in B cell receptor signaling (BCR) and survival,
namely Bruton's tyrosine kinase (Btk), and indirectly its downstream substrate phospholipase C (PLC)-γ2 in a dose-dependent manner. Our findings suggest that TKIs may interfere with B-cell activation and induction of humoral immune responses in vivo through their off-target multi-kinase inhibitory effects.

**Design and methods**

**Patients and controls**

Fifty-one CP-CML patients in complete cytogenetic response (CCyR) on standard-dose imatinib (n=26), dasatinib (n=13) or nilotinib (n=12) and 24 adult controls were recruited in this study during two influenza seasons (2008 and 2009). Patient characteristics are summarized in Table-1. All patients were on second-line therapy with dasatinib or nilotinib and had failed previous therapy with imatinib (supplementary Table 1). Healthy controls were recruited amongst hospital staff. The median age for CML patients was 52 years and for controls 41 years (p=0.10). All patients and controls were vaccinated against influenza (Influenza vaccine Ph. Eur. 2008/2009, or 2009/2010, CSL biotherapies, Germany) and the pandemic influenza A (H1N1) in 2009.24 Forty-five of 51 CML patients and 12/24 healthy controls were concomitantly immunized with the 23-valent polysaccharide pneumococcal (PPS) vaccine (Pneumovax-II; Sanofi Pasteur MSD, UK). Only patients and controls who had not received a pneumococcal vaccine within the previous 5 years were re-immunized.

Peripheral blood mononuclear cells (PBMCs) and serum samples were collected from all patients and donors prior to vaccination and responses were assessed at 4 weeks and at 2–3 months post-immunization. All patients and adult controls gave informed written consent and the local institutional ethics board approved the study protocol.
Determination of influenza specific CD8+ and CD4+ T-cell responses by flow cytometry

Multi-parameter flow-cytometry was employed to assess immunological T-cell responses to influenza virus both quantitatively by GILGFVFTL/HLA-201 (FluMP) pentamer staining, and qualitatively using intracellular cytokine (IC) assay for TNF-α, IFN-γ, IL-2 and the cytotoxicity marker CD107a as described previously.25

Cells were acquired on FACS Calibur™ (BD Biosciences, Oxford UK) and data were analyzed using FlowJo™ software (TreeStar, San Carlos, CA).

Determination of pneumococcal serum titers for IgM and IgG

Serum titers of pneumococcal IgM and IgG antibodies were determined using ELISA as previously described.26

Determination of plasma levels of imatinib, dasatinib and nilotinib

TKI and metabolite plasma detection was based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) using TurboFlow sample preparation as previously described. 27

B cell phenotyping

PBMC were stained with PE-cyanin7 (PC7) conjugated anti-CD19 (Coulter Immunotech High Wycombe, UK), PE-conjugated anti-human IgD (Southern Biotechnology Associates, Birmingham, USA), APC-conjugated anti-human IgM (The Jackson Laboratory, Bar Harbor, USA) and FITC-conjugated anti-CD27 (DakoCytomation, Glostrup Denmark).

Intracellular phospho-specific flow cytometric assay

We assessed the impact of therapeutic doses of imatinib, dasatinib and nilotinib on Btk phosphorylation using intracellular phosphor-specific flow cytometric assay, following co-
incubation of B cells from CML patients on TKI therapy (imatinib, n=3, nilotinib n=4 and dasatinib n=3) with autologous serum. Briefly, following stimulation with H2O2 for 15 mins at 37°C, PBMC were fixed and stained with PE-conjugated anti-phosphorylated Btk (pBtk-PE) and APC-conjugated anti-CD19 (both BD Biosciences, San Jose, CA). Data were acquired on the FACSCalibur™ and FlowJo was used for analysis.

To assess the impact of TKI on normal B cells, PBMC from healthy controls were cultured in the presence or absence of increasing concentrations of TKIs namely, 1-50 μM of imatinib, 1-25 μM of nilotinib and 1-100 nM of dasatinib (all, LC Laboratories, Woburn, MA) for 2 hours. PBMCs were then stimulated with goat anti-human IgG and IgM F(ab’)2 (10 μg/ml) for 20 mins at 37°C. Cells were stained with PE-conjugated anti-phosphorylated Btk (pBtk-PE) or PE-conjugated anti-phosphorylated PLC-γ2 (pPLC-γ2-PE), APC-conjugated anti-CD19 (BD Biosciences, San Jose, CA), PerCP-conjugated anti-human IgM (BD Biosciences, Oxford UK) and FITC-conjugated anti-CD27 (DakoCytomation, Glostrup Denmark).

**Statistical analysis**

Fisher’s exact test was used to compare proportions. Continuous variables were compared using the Mann-Whitney test or the Kruskal Wallis test. Paired samples were compared using the Wilcoxon signed rank. Multivariate analysis was performed using a logistic regression model. All reported p values are two-sided. Analyses were performed using SPSS version 17.
Results

Vaccination with influenza A induces CD8+ and CD4+ T-cell responses in patients on TKI and healthy controls

The induction of T-cell responses to Flu vaccination was assessed directly ex-vivo by flow-cytometric enumeration of antigen-specific CD8+ and CD4+ T-lymphocytes using IC assay for IFN-γ and TNF-α. A T-cell response was defined to be Flu-specific if at least one cytokine was detected following in vitro antigen-stimulation. Before vaccination, T-cell responses against Flu could be detected in 21/51 (41.2%) patients on TKI and 12/24 (50%) controls (p=0.15), indicating the presence of pre-existing memory T-cells to Flu in patients with CML on TKI and in healthy controls (Figure-1A).

Following vaccination, flu-specific T-cells were induced in 24/51 (47.0%) patients on TKI (median 0.15% TNF-α+CD3+T-cells, range 0.05–0.64%) and 15/24 (62.5%) healthy controls (median 0.40% TNF-α+CD3+ T-cells, range 0.12–2.0%), p=0.16 (Supplementary Tables 2A-B), Figure-1B.

In 12 HLA-A*0201+ CML patients (including 3 imatinib, 6 dasatinib and 3 nilotinib-treated patients), and 4 HLA-A*0201+ controls we also confirmed the presence of circulating Flu-specific memory CD8+ T-cells by HLA-A2/FluMP pentamer staining. An increase of at least 2-fold Flu-specific CD8+ T-cells was detected in 2/4 controls and 5/12 patients (median 0.44% of total CD8+ T-cells, range 0.1-1.51%). All patients and donors with detectable Flu-specific CD8+ T-cells by pentamer analysis also had detectable responses by IC staining, indicating that HLA-A2/FluMP CD8+ T-cells are functional (data not shown). An example of a patient on dasatinib with a robust CD8+ T-cell response (Patient-33) to influenza vaccination is presented in Figure-1C.
We next evaluated the functional quality of the influenza vaccine-induced T-cell response by flow-cytometric analysis of markers related to T-helper cell-1 (TNF-α, IFN-γ and IL-2) and degranulation/cytotoxicity (CD107a) in individuals with a positive vaccine-induced T cell response (responders) and in whom sufficient numbers of cells were available for analysis, i.e. 9/24 ‘responders’ in the CML and 7/15 ‘responders’ in the healthy control groups. We found no significant differences in the quality of the T-cell response to influenza vaccine in the two groups as summarized in Supplementary Table 2C.

**CML patients on TKI achieve lower pneumococcal IgM titers after vaccination**

Forty five CML patients on TKI and 12 healthy controls were vaccinated against the polysaccharide pneumococcal (PPS) vaccine (Pneumovax II) and all could be evaluated for response. Prior to vaccination, the median pneumococcal IgG levels were 123 U/ml in CML patients compared to 71.5 U/ml in controls (p=0.3); 16/45 patients and 3/12 controls had pre-vaccination pneumococcal IgG levels >200 U/ml (p=0.7). In contrast, the pre-vaccine pneumococcal IgM levels were significantly lower in CML patients on TKI (median 15, range 3-72 U/ml) compared to healthy controls (median 38, range 13-78 U/ml), p=0.002.

We assessed the humoral response to PPS vaccine by measuring pneumococcal IgM levels 4 weeks following vaccination. Eleven of 12 (92%) of controls had a positive IgM humoral response (defined as a 4-fold rise in serum IgM titer or IgM >200 U/ml post-vaccination) compared to only 18/45 (40%) of CML patients on TKI (p=0.002). Moreover, pneumococcal IgM titers achieved at 4 weeks were significantly lower in CML patients on TKI compared to controls (median, 79 U/ml, range 5-200 vs. 200 U/ml, range 58-200, p=0.0006; Figures-2A and 2B), supporting the notion that CML patients on TKI have an impaired IgM response to
vaccination. Of note we only found a weak correlation between the pre- and post-vaccination IgM levels in CML patients on TKI ($R^2=0.17$).

We also assessed humoral responses to the vaccine in patients and controls without prior evidence of pneumococcal infection or immunization (defined as IgG <200 U/ml prior to vaccination). Of 24/45 evaluable patients, 6/24 (25%) failed to mount both an IgM and IgG response compared to 0/9 healthy controls ($p=0.15$), suggesting the presence of global B cell memory impairment in a proportion of CML patient on TKI. The characteristics of the 6 CML patients who failed to mount an IgM and IgG response to PPS vaccine are presented in Table-1B. We found no significant differences in the underlying characteristics (including age, Sokal score, spleen size, duration and response to TKI therapy) between the 6/24 patients who failed to mount a humoral response compared to the 18/24 responders.

We did not find significant differences in the post-vaccine humoral response rates (7/19, 4/12 and 7/14, $p=0.87$) or pneumococcal IgM serum titers (median 75, 80 and 69 U/ml, $p=0.7$) in patients treated with imatinib, nilotinib or dasatinib, respectively (Figure-2C); although our study was not specifically designed to look at differences in the 3 treatment groups.

**IgM memory B cells are markedly reduced in CML patients who do not mount a pneumococcal IgM response after vaccination**

To further elucidate the mechanisms underlying the impaired humoral immune response to PPS in patients with CML on TKI, we determined the percentages of IgM memory B cells (CD19+ CD27+ IgMhigh IgD+/lo) and switched memory B cells (CD19+ CD27+ IgM- IgD-) before and 4 weeks following immunization and correlated these with pneumococcal IgM and IgG levels respectively. We had sufficient samples to perform this analysis in 39 patients and
10 healthy controls. Of note the percentage of IgM memory and switched memory B cells did not differ significantly before and after immunization (data not shown).

CML patients were stratified based on their 4 week pneumococcal IgM response into 2 groups of ‘vaccine responders’ and ‘vaccine non-responders’ (Figure-2B). We found no significant difference in fold-increase in anti-pneumococcal IgM response in CML responders and healthy controls (p=0.5). In contrast, CML patients who failed to mount a pneumococcal IgM response had significantly lower IgM memory B cell frequencies at vaccination compared to patients who mounted a positive pneumococcal IgM response (median 6.5% vs. 17.6%, p=0.005) and compared to healthy controls (median 6.5% vs. 12.8%, p=0.02) (Figure-3A). Furthermore, in patients with CML, we found a significant correlation between IgM memory B cell frequencies at vaccination and the post-vaccine pneumococcal IgM titer ($R^2=0.36$, p<0.0001), Figure-3B. Interestingly, the IgM memory B cell frequency for the one healthy donor who failed to mount a positive IgM pneumococcal vaccine response was within the normal range (14.6%).

Impaired IgM responses to vaccination with PPS vaccines have been reported in the elderly. To exclude an impact of age on the pneumococcal humoral response in our slightly older CML patient population compared to controls, we performed univariate and multivariate analyses including age, Sokal score, spleen size and IgM memory B cell frequencies. On univariate and multivariate analyses the IgM memory B cell frequency at vaccination was the only independent predictor for a positive IgM humoral response (p=0.006).

The gating strategy employed for the analysis of the B cell phenotype in vaccine responders and non-responders is shown in Supplementary Figure-1. Of note we found no significant
difference in CD19+ B cell frequencies between responders and non-responders (p=0.92, data not shown).

In line with the normal pre-vaccine pneumococcal IgG titers in CML, we found no significant differences in the frequencies of switched memory B cells between CML patients on TKI and controls before vaccination (median 13.2% vs. 8.9%, p=0.30, data not shown); however, the frequencies of switched memory B cells were significantly lower in the 6 patients who failed to mount an appropriate IgM and IgG response compared to the 33 patients who had an appropriate IgM or IgG response to the pneumococcal vaccine (median, 6.3% vs. 13.7%, p=0.0031, Figure-3C and Table-1B).

**Treatment with imatinib is associated with a significant decrease in the frequencies of IgM memory and class switched memory B cells**

To investigate whether the loss of IgM memory B cell subset in CML patients is related to CML itself or to treatment with TKIs, we studied B cell subsets in paired samples collected from 15 CP-CML patients at diagnosis and once CCyR was achieved on imatinib. The patient characteristics are summarized in Table-2. Only patients on imatinib were studied, as paired samples from diagnosis and following therapy were not available for patients on dasatinib and nilotinib.

No significant differences were found in the frequencies of IgM memory and switched memory B cells in CML patients at diagnosis (i.e. prior to initiation of imatinib) (n=15) compared to healthy controls (n=10) (median 12.6% vs. 12.8%, p=0.85, and 14.1% vs. 8.9%, p=0.21 respectively). However, we found a significant reduction in IgM memory B cell frequencies in CP-CML patients following treatment with imatinib compared to diagnosis (median 6.5%, range 2.5-21.0% at CCyR, vs. 12.6%, range 3.9-33.5% at diagnosis, p=0.003),
Figure-3D. Similarly, there was a significant reduction in the frequencies of class switched memory B cells following treatment with imatinib compared to diagnosis (median 7.48%, range 2.3-22.3% at CCyR, vs. 14.1% range 6.1-28.0% at diagnosis, p= 0.001), Figure-3E, indicating that TKI are responsible for the lower frequencies of memory B cells in CML. FACS plots from two representative patients are presented in Figure-3F.

**Plasma from CML patients on TKI co-incubated with autologous B cells inhibits Btk phosphorylation**

To understand the molecular basis through which TKIs inhibit B cell activation, we co-incubated plasma from 3 vaccinated CML patients on imatinib (Patients 5, 8 and 12), 4 on nilotinib (Patients 15, 17 and 20) and 3 on dasatinib (Patients 27, 28 and 29) with autologous B cells, and assessed their impact on Btk phosphorylation by phosphoflow analysis on gated CD19+ B cells. Imatinib, dasatinib and nilotinib levels in CML plasma samples used for these studies were within therapeutic range (Supplementary Table-3).

We noted significant inhibition in the mean fluorescence intensity (MFI) and percentage Btk phosphorylation in B cells of patients treated with imatinib (median inhibition 50%, range 35-53%), nilotinib (median inhibition 65%, range 61-68%) and dasatinib (median inhibition 83%, range 81-86%), (Figure-4).

**Imatinib, dasatinib and nilotinib inhibit Btk and PLC-γ2 phosphorylation in a dose-dependent manner**

We also assessed the impact of increasing doses of imatinib, dasatinib and nilotinib on the phosphorylation of Btk and its downstream signaling molecule PLC-γ2, in healthy donor B cells. Untreated normal B cells showed little evidence of phosphorylated PLC-γ2 (0.21%, range 0.03-0.84%) and pBtk (0.74%, range 0.62-0.81%) (Figure-5A, right and left top panel
respectively). Upon stimulation with goat anti-human IgG and IgM F(ab’)2, CD19+ B cells responded by expressing increased levels of pBtk (57.4%, range 57.2-57.9%) and pPLC-γ2 (57.0%, range 56.9-58.8%) (Figure-5A, lower panel). We then investigated the impact of TKIs on Btk and PLC-γ2 phosphorylation in gated CD19+ B cells derived from healthy donors. Co-incubation with TKIs for 2-14 hours did not impair B cell viability (Fig 5B). Imatinib inhibited phosphorylation of Btk (IC50= 8.4µM) and PLC-γ2 (IC50= 9.16µM) in gated CD19+ B cells in a dose-dependent manner (Figure-5C). We next determined the impact of nilotinib and dasatinib on pBtk and pPLC-γ2. Similarly, nilotinib and dasatinib dose-dependently inhibited pBtk (IC50=6.1 µM and IC50=21.9 nM respectively) and pPLC-γ2 (IC50=9.66 µM and IC50=22.7 nM respectively) (Figure-5C, left panel). Each experiment was performed a minimum of 3 times.

Collectively, our data provide clear evidence that all 3 TKIs can suppress B cell activation through their off-target kinase inhibition.

**Imatinib, dasatinib and nilotinib inhibit Btk and PLC-γ2 within the memory B cell subset**

To assess if TKIs can inhibit B cell activation in memory B cell subsets, we examined the impact of these drugs on phosphorylation of Btk and PLC-γ2 by phosphoflow analysis on gated naïve B cells (CD19+CD27-), IgM memory B cells (CD19+ CD27+ IgMhigh IgD+/lo) and switched memory B cells (CD19+ CD27+ IgM- IgD-). With all 3 TKIs tested, we showed a reduction in levels of pBtk in gated CD19+ B cells as assessed by quantitation of MFI on phosphoflow analysis. Furthermore, inhibition of phosphorylation could be demonstrated in the memory B cell subsets as shown in Figure-6.
Discussion

In this study we show that CP-CML patients treated with imatinib, dasatinib or nilotinib have significant impairment in their B cell response to pneumococcal polysaccharide vaccine. The impaired humoral response to the vaccine was associated with loss of memory B cell subsets following treatment with TKIs. Although in patients on second-line dasatinib and nilotinib a prior effect of imatinib on B cell function cannot be excluded, our in vitro data confirmed that all 3 TKIs are capable of dose-dependently suppressing one or more kinases important in BCR signaling, survival and memory formation, as demonstrated by reduced phosphorylation of Btk and indirectly its substrate PLC-γ2, providing a possible mechanism for TKI-induced B cell impairment.

In contrast, we did not find a significant difference in the memory T-cell response to influenza vaccine in patients with CML on TKI and healthy controls. Although a number of studies have shown that TKIs inhibit T cell proliferation in vitro,4-6 our data are in keeping with previous reports of successful induction of T-cell responses to viral and tumor antigen vaccines in patients with CML on TKIs.24;30-33 The discrepancy in the results of in vitro and in vivo studies may be partly related to the differential impact of TKIs on regulatory T cells in vivo,8 which in turn may facilitate the induction of a successful effector T cell response. Our study is however limited by the small sample size and it is possible that with a larger cohort of patients and controls, small differences in the T-cell response to vaccination could be detected. It is also possible that TKI may interfere with the induction of naïve T cell responses, not tested here, or may impact on T cell responses to a weaker cognate antigen.

We found a strong correlation between pneumococcal IgM vaccine response and IgM memory B cell frequencies; 60% of patients with CP-CML on TKI had significantly impaired
pneumococcal IgM antibody responses to vaccination, associated in almost all cases with a significant loss of IgM memory B cells. In comparison effective pneumococcal IgM responses following vaccination were seen in nearly all controls and in 18/45 CML patients on TKI, associated with normal IgM memory B cell frequencies. Impaired IgM responses to vaccination in association with significant reduction in IgM memory B cells has been reported in a number of conditions including CVID, HIV, congenital asplenia, as well as in the elderly and children under the age of 2 years. The importance of IgM memory B cells in host protection against pneumococcal infection has been studied most extensively in CVID patients; in these patients a strong correlation was shown between IgM memory B cell frequencies and the incidence of encapsulated bacterial infection.\textsuperscript{34,35} Similarly in HIV-positive individuals, loss of memory B cells correlated with a decrease in the pneumococcal IgM response.\textsuperscript{26} It is however not clear whether CML patients on TKI have more pneumococcal infection compared to the normal population. Whereas earlier studies suggested an increased infection rate in dasatinib treated CML patients,\textsuperscript{36,37} larger prospective studies have failed to confirm these results.\textsuperscript{2} Patients with CML-CP with low IgM memory B cells may derive some protection against infections from prior immune memory or cross-protection from other immune subsets. It is possible that patients with CML-CP on TKI who do not mount adequate responses to PPS vaccine may respond to vaccination with the conjugated pneumococcal vaccine, which obviously would need testing in similar settings.\textsuperscript{38} In our study we also investigated the impact of other factors including age, gender, spleen size and Sokal score on the pneumococcal humoral response. On univariate and multivariate analyses, IgM memory B cell frequency remained the only significant predictive factor for a vaccine-induced pneumococcal IgM response.
IgM memory B cells recognize T-independent (TI) antigens such as PPS by virtue of a prediversified surface IgM and can respond immediately to antigen without T-cell help.\textsuperscript{39,40} Although an IgM response is believed to be the hallmark for PPS vaccine, switched memory B lymphocytes may also be involved in the anti-PPS Ab response. Studies in SCID mice transplanted with human B lymphocytes showed that both IgM memory and switched memory B lymphocytes are involved in the anti-polysaccharide immune response.\textsuperscript{41} In our study a small number of CML patients (6/24 evaluable) failed to mount both an IgM and IgG response to the vaccine; the poor humoral response was associated with significantly lower switched and IgM memory B cell frequencies, supporting a role for switched memory B cells in the anti-PPS humoral response. These data suggest that TKIs may affect both T-dependent and T-independent B cell activation signals in vivo, resulting in global B cell dysfunction in.

The impaired humoral response and loss of B cell subsets seen in our study could be a consequence of CML itself or a direct effect of TKI treatment. B cell progenitors are part of the leukemic clone in a subset of CML patients\textsuperscript{42} and it is therefore possible that TKIs may block BCR-ABL in Ph+ B cell lymphoid cells, thereby inducing B cell immune deficiency. Conversely, Ph+ B cells may be hypo- or dysfunctional and as a result fail to mount an effective humoral response to a pathogenic antigen. However, our data do not support these scenarios as we observed a significant decrease in the frequencies of memory B cell subsets at CCyR, when a state of minimal residual disease was achieved, compared to diagnosis. Instead, our results favor a direct quantitative and qualitative effect of TKI on B cells. We found a significant reduction in IgM memory and switched memory B cells following treatment with imatinib, suggesting that TKIs might interfere with the production and maintenance of B cell memory. These data may provide a possible explanation for recent reports of graft-versus-host disease (GVHD) response to imatinib, despite a lack of
correlation with PDGF receptor phosphorylation, and further support the use of TKI in B cell mediated immune disorders such as rheumatoid arthritis or chronic GVHD.

Polysaccharides stimulate B cells via cross-linking of multiple antigen receptors, resulting in activation of Btk, a critical enzyme in the TI-2 signaling cascade. Btk and its downstream substrate PLC-\(\gamma\)2 are involved in BCR signaling, survival and memory formation. We hypothesized that through their off-target kinase inhibition, TKIs may impair the intracellular phosphorylation of Btk and indirectly inhibit its downstream substrate PLC-\(\gamma\)2, resulting in impaired IgM responses to vaccination and a decrease in the memory B cell compartment. We found that co-incubation of plasma from CML patients on TKI with autologous B cells resulted in significant inhibition of Btk phosphorylation. Plasma from CML patients on dasatinib induced more profound suppression of Btk kinase activity compared to plasma from patients on imatinib or nilotinib, suggesting that dasatinib may have more potent off-target Btk inhibitory activity compared to the more specific BCR-ABL inhibitors such as imatinib or nilotinib. These findings support previous work showing dasatinib to be a strong inhibitor of Btk phosphorylation. Therapeutic concentrations of imatinib, dasatinib and nilotinib were also shown to inhibit Btk phosphorylation in activated B cells from healthy controls. Finally, we showed that all 3 TKI suppress Btk activity in memory B cell subsets, known to be critical to B cell memory development and class switching. Our study was not designed to address the underlying reasons for differential responses to pneumococcal vaccine in CML patients on TKI. A possible explanation could be variations in serum TKI levels due to inter-individual differences in drug metabolism or adherence that may in turn impact the in vivo B cell response to the vaccine.
In conclusion, treatment with TKIs is associated with loss of memory B cell subsets and impaired humoral immune responses to PPS vaccine, likely driven by the off-target kinase inhibitory activity of these drugs. Our results call for close monitoring of patients on TKI to assess the long-term impact of impaired B cell function on immune surveillance and susceptibility to infection and cancer. The inhibitory effect of imatinib, dasatinib and nilotinib on memory B-cell expansion and antibody production provides further rationale for studies of selective TKIs in the treatment of autoimmune diseases and cGVHD.

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Authorship
KR, HL PK and DM designed the study and research
HL, KR, DM, PK, and AK analyzed data
HL, KR and DM wrote the manuscript
HL, MH, AK, AS, SB, RJF and TS performed the experiments
DM, JA, DrM, and HL recruited patients
HL, AB, IG, KS and SA collected the clinical samples
MH, AK, AA, KS, DrM, NC, SM, AC, LF, JG, KS, EJS commented on the manuscript

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Reference List


Figure and table legend

**Figure 1: T cell responses to Influenza A vaccination in patients with CML on TKI and healthy controls.** PBMC collected before and 2-3 months post-vaccination were thawed and stimulated for 24 h with or without seasonal influenza vaccine at a final concentration of 1.5 µg/mL of hemagglutinin antigens or with PMA (50ng/ml) and ionomycin (2ug/ml, Sigma Aldrich) (positive control) for 19 h at 37°C. Brefeldin A (10 µg/mL) (Sigma Aldrich, Gillingham, UK) was added alone or with monensin (0.7µl/mL) (BD/Pharmingen, San Diego, CA) and the degranulation marker CD107a-FITC (BD/Pharmingen, San Diego, CA). PBMCs were washed and stained with anti-CD3 and anti-CD8 antibodies, fixed/permeablized (all BD Biosciences, Oxford UK) and stained with anti–IFN-γ, anti–TNF-α anti-IL-2 antibodies (all BD/Pharmingen, San Diego, CA). Data acquisition was performed using FACSCaliburTM (BD Biosciences, Oxford UK) and a minimum of 300,000 events were acquired. The threshold of positivity for cytokines and CD107a was set in order to minimize non-specific staining in non-stimulated cells (negative control). Following vaccination, a response was considered positive if there was a minimum of 0.10% Flu-specific TNF-α or INF-γ-producing T-cells and the percentage of antigen-specific TNF-α or INF-γ-producing T-cells was 2-fold or higher compared to pre-vaccination level. (A) Examples of pre-existing CD8+ and CD4+ T cell responses to influenza before vaccination in patients on TKI and a healthy control. (B) Examples of T cell responses to influenza A vaccination in patients on TKI using intracellular cytokine assay. (C) Detection of influenza-specific CD8+ T cells using an HLA-A2 restricted GILGFVFTL (FluMP) pentamer: the FACS plot from a CML patient on dasatinib showing a robust CD8+ T cell response to influenza vaccination is presented. Unstimulated PBMC from HLA-A0201 patients and healthy controls were stained with HLA-A0201/GILGFVFTL (FluMP) Pro5™ MHC I Pentamer (Pro-immune, Oxford UK) conjugated to APC, and co-
stained with anti-CD8-FITC (Pro-immune, Oxford UK), and anti-CD3-PerCP (BD Biosciences, Oxford UK).

**Figure 2: Pneumococcal IgM response following vaccination.** (A) Pneumococcal IgM titers are presented at 4 weeks following vaccination in healthy controls and CML patients on TKI. A positive IgM pneumococcal response was defined as a 4-fold rise in serum IgM titers or an IgM titer > 200 U/ml 4 weeks post-immunization irrespective of the pre-immunization titer. (B) The pneumococcal IgM response is presented before and 4 weeks after vaccination in responders (black lines) and non-responders (dashed lines) for healthy controls and CML patients on TKI. (C) The post-immunization pneumococcal IgM titers are presented for CML patients on imatinib, nilotinib and dasatinib. Bars represent medians with interquartile range.

**Figure 3: Relationship between memory B-cell subsets and pneumococcal humoral response.** PBMC were incubated with PE-cyanin 7 (PC7) conjugated anti-CD19 (Coulter Immunotech High Wycombe, UK), PE-conjugated anti-human IgD (Southern Biotechnology Associates, Birmingham, USA), APC-conjugated anti-human IgM (The Jackson Laboratory, Bar Harbor USA) and FITC-conjugated anti-CD27 (DakoCytomation, Glostrup Denmark). Cells were then washed and acquired on FACSCalibur™ (BD Biosciences, Oxford UK). A minimum of 5000 events were acquired on the B cell gate and the results are expressed as a percentage of CD19 events. FlowJo software (TreeStar, San Carlos, CA) was used for data analysis. Since IgM memory B cells express both IgD and IgM, co-expression of either IgD or IgM together with CD27 was used to define this subset. IgM memory B cells (CD19+ CD27+ IgMhigh IgD+/lo) and switched memory B cells (CD19+ CD27+ IgM- IgD-) were calculated using a modified Piqueras classification.²⁸
(A) Patients who fail to mount a pneumococcal IgM response have significantly lower frequencies of IgM memory B cells compared to responders and healthy controls. (B) Scatter plot evaluating the association between pneumococcal IgM titers and IgM memory B cell frequencies in CML patients. Samples were correlated using Spearman rank correlation test. (C) Frequencies of class switched memory B-cells in the 33 patients who achieved a post-immunization IgG > 200 U/ml compared to the 6 patients who failed to mount a positive pneumococcal IgM and IgG response; bars represent medians with interquartile range. A positive IgM Pneumovax II response was defined as a 4-fold rise in serum IgM titers or an IgM titer >200 U/ml 4 weeks post-immunization irrespective of the pre-immunization titer. A positive IgG response was defined as a 2-fold rise in serum IgG titer or an IgG titer >200 U/ml at 1 or 3 months. (D) IgM memory B cell frequencies at diagnosis (prior to initiation of imatinib) and once CCyR was achieved on imatinib. (E) Class switched memory B cell frequencies at diagnosis (prior to initiation of imatinib) and once CCyR was achieved on imatinib. (F) B-cell phenotype of a CML patient who developed a positive pneumococcal IgM response (Patient A) compared to a non-responder (Patient B).

Figure 4: Inhibition of Btk phosphorylation in CD19+ B cells from CML patients on TKI co-incubated with autologous plasma. Cryopreserved PBMCs from CML patients on TKI (imatinib, n=3, nilotinib n=3 and dasatinib n=3) were thawed, washed and co-cultured with autologous plasma or RPMI/10%FCS overnight. PBMCs were then stimulated with 5mL of 50 mM of H2O2 for 15 minutes at 37°C. The stimulation was terminated by the addition of 5 mL pre-warmed Cytofix Buffer (BD Biosciences, San Jose, CA) at 37°C for 12 minutes. Cells were fixed permabilized and stained with PE-conjugated anti-phosphorylated Btk (pBtk-PE) (BD Biosciences, San Jose, CA) and APC-conjugated anti-CD19 (BD Biosciences, San...
Jose, CA). Data acquisition was performed on the FACSCalibur™ and FlowJo software was used for analysis. Mean fluorescence index (MFI) of Btk phosphorylation following incubation with autologous plasma in gated CD19+ B cells from representative CML patients on imatinib, dasatinib or nilotinib is presented (right panel).

**Figure 5: Btk and PLC-γ2 phosphorylation inhibition by imatinib, dasatinib and nilotinib.** (5A) To assess the impact of TKI on normal B cells, PBMCs from healthy controls were isolated and cultured in the presence or absence of increasing concentrations of TKIs namely, 1-50 μM of imatinib (LC Laboratories, Woburn, MA), 1-50 μM of nilotinib (LC Laboratories, Woburn, MA) or 1-100 nM of dasatinib (LC Laboratories, Woburn, MA) for 2 hours. PBMCs were then stimulated with goat anti-human IgG and IgM F(ab')2 (0.5 mg/ml solution) at a final concentration of 10 μg/ml for 20 mins and cells were stained with PE-conjugated anti-phosphorylated Btk (pBtk-PE) or PE-conjugated anti-phosphorylated PLC-γ2 (pPLC-γ2-PE), APC-conjugated anti-CD19 (BD Biosciences, San Jose, CA), PerCP-conjugated anti-human IgM (BD Biosciences, Oxford UK) and FITC-conjugated anti-CD27 (DakoCytomation, Glostrup Denmark). Cells were gated on lymphocytes: the panels on the top depict the unstimulated negative control and on the bottom anti-IgG and IgM-induced phosphorylation of Btk (left) and PLC-γ2 (right). (5B) PBMCs were co-incubated with imatinib (10 μM), nilotinib (10 μM) and dasatinib (100 nM) for 14 hours and the viability of CD19+ B cells was assessed by staining with FITC conjugated Annexin and APC-conjugated anti-CD19 (both BD Biosciences, San Jose, CA).

(5C) Curve fit (linear regression) of TKI doses plotted against the percentage of Btk phosphorylation inhibition induced by each of the three TKI, imatinib, nilotinib and dasatinib (each experiment was performed a minimum of 3 times). The Y bar represents the percentage of gated population in which phosphorylated Btk or PLC-γ2 are detected.
**Figure 6: Btk and PLC-γ2 phosphorylation inhibition in B-cell subsets.** (6A) Btk phosphorylation in B cell subsets cultured in the presence or absence of 10 μM of imatinib, 100 nM of dasatinib or 10 μM of nilotinib for 2 hours and stimulated with 10 μg/ml of anti-human IgG and IgM F(ab’)2 for 20 minutes. Effect of the TKI on pBtk inhibition is shown in gated IgM memory B cell, switched memory B cell and naive B cell subsets. Each experiment was performed a minimum of 3 times. (6B) MFI of BTK phosphorylation in gated CD19+ B cells from a representative healthy donor following incubation with 10 μM of imatinib, 100 nM of dasatinib or 10 μM of nilotinib.

**Table 1: Patient characteristics** (A) The characteristics of 51 CML-CP patients on TKI and 24 healthy controls in this study are presented. (B) The characteristics of the 6 patients who failed to mount an appropriate IgM and IgG response to pneumococcal vaccination are presented.

**Table 2: Characteristics of the 15 CP-CML patients whose B cell subsets were analyzed at diagnosis (i.e. prior to initiating imatinib therapy) and once CCyR was achieved on imatinib are presented.** *Duration of treatment with imatinib when remission sample was collected.
1A

Negative control

Flu antigen stimulation

Imatinib (patient 1)

Dasatinib (patient 27)

Nilotinib (patient 15)

Healthy Control (HC 2)
1B

Pre-vaccination | Week 4 post-V | Week 12 post-V

Imatinib (patient 2)

Dasatinib (patient 35)

Nilotinib (patient 17)

1C

Pre-vaccination | 4 weeks post vaccination | 9 weeks post Vaccination

HLA A0201 influenza–specific pentamer, APC
2A

![Graph comparing Pneumococcal IgM at 4 weeks (U/ml) between Controls and CML patients with p = .0006.]

2B

![Graph showing the progression of Pneumococcal IgM (U/ml) over time for Healthy controls and CML patients.]

Healthy controls  CML patients
Pneumococcal IgM at 4 weeks (U/ml)

- Controls
- Imatinib
- Nilotinib
- Dasatinib

Statistics:
- p = 0.0007
- p = 0.0047
- p = 0.026
- p = n.s.
3A

![Graph showing IgM memory B cell subset (%)](image)

- Controls
- CML patients, IgM response
- CML patients, no IgM response

Significance levels:
- p = .02
- p = .005
- p = .2

3B

![Graph showing IgM memory B cell (%) vs. Pneumococcal IgM (U/ml)](image)

IgM memory B cell (%) vs. Pneumococcal IgM (U/ml)

0 - 40

0 - 300
3C

**Switched memory B cell (%)**

- **IgG>200 after vaccination**
- **No IgG or IgM response**

**p = .0031**

3D

**IgM memory B cell subset (%)**

- **At diagnosis**
- **at CCyR**

**p = 0.003**
3E

Class switched memory B cell subset (%)

At diagnosis  at CCyR

p = 0.001

3F

Before Imatinib

IgM PerCP Cy5.5

Patient A

Patient B

After Imatinib

CD27 PE

12.8  14.1

13.5  5.19

12.6

17.1

5.46
5A

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Table 1A

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### Table 1B

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Tyrosine kinase inhibitors impair B cell immune responses in CML through off-target inhibition of kinases important for cell signaling


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