Asciminib stands out as the superior tyrosine kinase inhibitor to combine with anti-CD20 monoclonal antibodies for the treatment of CD20$^+$ Philadelphia-positive B-cell precursor acute lymphoblastic leukemia in preclinical models

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Abstract

Philadelphia chromosome-positive B-cell precursor acute lymphoblastic leukemia (Ph$^+$ BCP-ALL) is a high-risk subtype of acute lymphoblastic leukemia characterized by the presence of the *BCR::ABL1* fusion gene. Tyrosine kinase inhibitors (TKI) combined with chemotherapy are established as the first-line treatment. Additionally, rituximab, an anti-CD20 monoclonal antibody is administered to adult BCP-ALL patients with ≥20% CD20$^+$ blasts. In this study, we observed a marked prevalence of CD20 expression in patients diagnosed with Ph$^+$ BCP-ALL, indicating a potential widespread clinical application of rituximab in combination with TKI. Consequently, we examined the influence of TKI on the antitumor effectiveness of anti-CD20 monoclonal antibodies by evaluating levels of CD20 on the cell surface and conducting *in vitro* functional assays. All tested TKI were found to uniformly downregulate CD20 on leukemic cells, diminishing the efficacy of rituximab-mediated complement-dependent cytotoxicity. Interestingly, these TKI displayed varied effects on natural killer (NK) cell-mediated antibody-dependent cytotoxicity and macrophage phagocytic function. While asciminib demonstrated no inhibition of effector cell functions, dasatinib notably suppressed the anti-CD20-monoclonal antibody-mediated NK cell cytotoxicity and macrophage phagocytosis of BCP-ALL cells. Dasatinib and ponatinib also decreased NK cell degranulation *in vitro*. Importantly, oral administration of dasatinib, but not asciminib, compromised NK cell activity in patients' blood, as determined by an *ex vivo* degranulation assay. Our results indicate that asciminib might be preferred over other TKI for combination therapy with anti-CD20 monoclonal antibodies.

Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a genetically heterogeneous malignancy characterized by an uncontrolled proliferation of immature B cells. In recent years, there has been notable progress in diagnosing and treating BCP-ALL, thanks to advanced sequencing techniques and the emergence of new immunotherapies. This has led to substantial improvements in treatment outcomes for the majority of children with BCP-ALL. However, due to a higher occurrence of adverse genetic subtypes in adult BCP-ALL, over 40% of adults experience relapses,

which are often refractory to current treatments.[1,2] One of the most prevalent aggressive subtypes of BCP-ALL is characterized by the presence of the breakpoint cluster region-Abelson (*BCR::ABL1*) translocation, also known as the Philadelphia chromosome (Ph⁺ BCP-ALL). This subtype is treated with tyrosine kinase inhibitors (TKI) specifically targeting the ATP-binding site of ABL1. Originally developed for chronic myelogenous leukemia (CML), TKI include the first-generation drug imatinib, second-generation ones such as dasatinib and nilotinib, which more effectively inhibit BCR::ABL1-mutated variants, as well as the third-generation ponatinib which addresses the T315I mutation in the *ABL1* gene.[3] In addition, in 2021, a novel TKI, asciminib, was approved for the treatment of CML. Asciminib is an allosteric inhibitor that binds to the myristoyl pocket, effectively inhibiting BCR::ABL1 activity, even in the presence of the T315I mutation.[4] While monotherapy with a TKI is typically effective in CML, patients with Ph⁺ BCP-ALL require treatment with a combination of a TKI with classical chemotherapeutics and, despite this, a substantial fraction of patients experience disease recurrence.[5] Therefore, efforts are underway to actively seek further optimizations in treatment protocols to improve patients' outcomes.

One of the strategies in this pursuit is the addition of anti-CD20 monoclonal antibodies to chemotherapy regimens and TKI treatment. A phase III trial conducted in Ph⁻ BCP-ALL patients expressing CD20 revealed the significant benefit of adding rituximab, an anti-CD20 monoclonal antibody, to chemotherapy.[6] These findings influenced contemporary treatment protocols, such that rituximab is now integrated into all phases of treatment for adult BCP-ALL patients exhibiting at least 20% CD20⁺ lymphoblasts.[7] Consequently, CD20⁺ Ph⁺ BCP-ALL patients receive combination therapy comprising chemotherapy, TKI, and rituximab.[8]

Anti-CD20 monoclonal antibodies kill target tumor cells through several mechanisms, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), as well as direct induction of cell death.[9,10] Importantly, the presence of CD20 on the surface of tumor cells is a prerequisite for all these mechanisms to function effectively.

CD20 is a B-cell-specific antigen whose expression begins at the pre-B cell stage and persists into mature B cells.[11-14] At diagnosis, CD20 expression is observed in up to 50% of BCP-ALL cases, typically at moderate levels.[12,15] Some chemotherapeutic agents used in BCP-ALL treatment regimens have been demonstrated to modify CD20 expression in lymphoid cells.[16,17] Corticosteroids, essential drugs given in the induction phase of the therapy, were shown to upregulate CD20 *in vitro* and are considered a key factor contributing to the CD20 upregulation observed in BCP-ALL patients after the induction phase.[18] Conversely, certain TKI have been found to significantly decrease CD20 protein levels in B-cell lymphoma cells.[19] Moreover, dasatinib has been shown to inhibit immune effector cytotoxicity by targeting SRC family kinases, which play a pivotal role in transmitting activating signals within immune effector cells necessary for ADCC.[19-23] Despite the ongoing clinical use of combination therapy involving rituximab, chemotherapy, and TKI in CD20⁺ Ph⁺ BCP-ALL patients, comprehensive preclinical investigations into the impact of different TKI on the performance of anti-CD20 monoclonal antibodies are currently lacking.

In this study, we found that CD20 is more frequently present in Ph⁺ BCP-ALL than in other subtypes; therefore, in adult patients a TKI is commonly administered in conjunction with an anti-CD20 monoclonal antibody. Hence, we investigated the impact of first-, second-, and third-generation TKI, along with the novel, allosteric TKI asciminib, on the expression levels of CD20 on Ph⁺ BCP-ALL cells and the function of effector immune cells essential for monoclonal antibody activity. While all tested TKI reduced CD20 expression on the surface of BCP-ALL cells and diminished rituximab-mediated CDC, their effects on ADCC and ADCP varied. Our findings revealed that among the four TKI examined, asciminib had the most favorable profile. These results remained consistent across all *in vitro* functional assays and were further validated through *ex vivo* assays, which reflect the actual microenvironment found in the peripheral blood of leukemic patients.

Methods

B-cell precursor acute lymphoblastic leukemia patients

Adult patients were diagnosed and treated according to Polish Adult Leukemia Group guidelines and pediatric patients according to the modified AIEOP-BFM 2017 protocol (NCT03643276) or EsPhALL2017/COG AALL1631 (EudraCT 2017-000705-20), and as described by Urbańska *et al.*[24] The primary sample collection protocol was approved by the Bioethics Committee of the Institute of Hematology and Transfusion Medicine in Warsaw (52/2019, 24/2022) and the Bioethics Committee of the Medical University of Lodz (RNN/208/20/KE). Informed written consent was obtained from all participants and/or their parents. Mononuclear cells from the bone marrow were isolated and processed within 24 hours of collection of the sample.

Antibody-dependent cell-mediated phagocytosis assay

CD20⁺ BCP-ALL patient-derived xenograft cells (PDX), prestained with CFSE (ThermoFisher Scientific) and opsonized with 5 μg/mL rituximab or 5 μg/mL cetuximab (an anti-EGFR monoclonal antibody acting as a background control) as well as M1 macrophages differentiated from healthy donors' monocytes were separately preincubated for 2 hours at 37°C with the maximal serum concentration (Cmax) and ¼ Cmax of the various TKI. BCP-ALL cells were then transferred to the corresponding macrophage wells

and co-incubated with macrophages for 1 hour. The macrophages were subsequently stained with CD11b-APC-Cy7 antibody and analyzed by flow cytometry. Immunophagocytosis was assessed as the percentage of CFSE⁺ CD11b⁺ cells in each sample. Next, for each rituximab-incubated sample, the background (cetuximab-mediated phagocytosis) was subtracted, and the percentage of phagocytosis was normalized to the highest technical replicate per macrophage donor.

Ex vivo degranulation assay

We enrolled patients with Ph⁺ BCP-ALL or CML treated at the Institute of Hematology and Transfusion Medicine in Warsaw as well as the Department of Hematology, Transplantation and Internal Medicine, Central Clinical Hospital University Clinical Center, Medical University of Warsaw. Patients' material was obtained following informed written consent and approval of the Bioethics Committee of the Institute of Hematology and Transfusion Medicine in Warsaw (52/2019 and 24/2022). Peripheral blood samples were collected into VACUETTE® Blood Collection Tubes coated with heparin at two timepoints: before TKI administration and 1 hour after oral administration of imatinib (400 mg), dasatinib (100 mg or 140 mg) or asciminib (2 x 40 mg). In three patients, additional samples were also collected 24 hours after dasatinib administration and 12 hours after administration of the second dose of asciminib, before the delivery of a subsequent dose. A total of 150 µL of blood per replicate was pipetted onto a well plate with 400,000 K562 target cells. Next, anti-CD107a APC antibody with GolgiStop™ Protein Transport Inhibitor (BD Biosciences San Jose, CA, USA) were added to each well. The cells were then incubated for 4 hours at 37°C. Next, red blood cells were lysed using BD Lysing Buffer (BD Biosciences). The cells were stained using BD Fixable Viability Stain 510 (BD Biosciences) and antibodies against CD56 and CD3. The percentage of degranulation was calculated in live CD56⁺CD3⁻ cells after subtraction of background - samples with blood but without K562 target cells.

Results

Cases of Philadelphia chromosome-positive B-cell precursor acute lymphoblastic leukemia commonly exhibit CD20

To compare CD20 expression in different genetic subtypes of BCP-ALL, we evaluated the *MS4A1* mRNA levels in primary BCP-ALL cells by real-time quantitative polymerase chain reaction. In the analyzed cohort of 130 pediatric and adult patients, we observed the highest levels of *MS4A1* mRNA in the Ph⁺ subtype (Figure 1A). As the clinical decision about the inclusion of rituximab into the treatment protocol for adult BCP-ALL patients is based on the percentage of the CD20⁺ subpopulation detected by flow cytometry, we analyzed the percentages of CD20⁺ lymphoblasts in flow cytometry data collected at BCP-ALL diagnosis. In the Ph⁺ group, the median percentage of CD20⁺ cells was significantly higher than that in all other subtypes combined into the Ph⁻ group. Notably, it exceeded the predefined CD20-positivity cutoff value of 20% (Figure 1B). As shown in Figure 1C, the majority of patients diagnosed with Ph⁺ BCP-ALL were classified as CD20⁺. We also observed a moderate but significant correlation between *MS4A1* mRNA and surface CD20 protein levels (*Online Supplementary Figure S1A*). In addition to Ph⁺, hyperdiploid, B-other, iAMP21, and Ph-like genetic subtypes were most often represented in the group of pediatric BCP-ALL patients with the highest CD20 levels (*Online Supplementary Figure S1B*; the clinical characteristics of pediatric BCP-ALL patients are summarized in *Online Supplementary Table S5*). Accordingly, CD20 expression in BCP-ALL cell lines corresponded with observations made on primary cells (*Online Supplementary Figure S2*). These results suggest that due to the higher frequency of CD20 positivity in Ph⁺ BCP-ALL patients, combination therapy with anti-CD20 monoclonal antibodies along with TKI and chemotherapeutics is often utilized in adult BCP-ALL treatment and may also be considered for the treatment of pediatric Ph⁺ BCP-ALL.

Tyrosine kinase inhibitors promote the downregulation of CD20 in Philadelphia chromosome-positive B-cell precursor acute lymphoblastic leukemia

CD20 levels may affect the efficacy of anti-CD20 monoclonal antibodies against hematologic malignancies.[25-27] Considering the frequent occurrence of the CD20⁺ phenotype in Ph⁺ BCP-ALL at diagnosis, we investigated how the drugs commonly used in the treatment of Ph⁺ BCP-ALL affect CD20 levels. First, we compared the levels of CD20 at the time of diagnosis and after 15 days of the induction phase of chemotherapy (MRD15), which is the first timepoint of minimal residual disease assessment in pediatric BCP-ALL patients. The administered induction treatment regimen (characterized in detail in *Online Supplementary Tables S1* and *S2*) consisted of drugs including corticosteroids, L-asparaginase, vincristine, daunorubicin, methotrexate, and – in the case of Ph⁺ BCP-ALL – imatinib. While in the unselected cohort of BCP-ALL patients the CD20 levels were significantly higher at MRD15 than in the corresponding samples assessed at diagnosis (Figure 2A), this was not the case in the group of Ph⁺ BCP-ALL patients (Figure 2B). Similar effects were observed in the cohort of adult patients (*Online Supplementary Figure S3*) who received induction treatment regimens consisting of the aforementioned drugs and liposomal cytarabine, as presented in detail in *Online Supplementary Tables S3* and *S4*. Next, we analyzed the levels of CD20 protein in Ph⁺ BCP-ALL cells incubated *in vitro* for 48 hours with drugs used in the treatment of this subtype of leukemia. Our findings revealed a consistent decrease in CD20 levels following exposure to all four TKI

in three out of four tested cell lines (Figure 2C, upper panel; *Online Supplementary Figure S4A*). Additionally, when Ph⁺ BCP-ALL PDX were incubated with dasatinib, we observed a reduction in CD20 levels in all five PDX and in three out of five PDX after treatment with any of the TKI (Figure 2C, lower panel; *Online Supplementary Figure S4B*). To further explore the mechanism of CD20 downregulation by TKI, we assessed their impact on *MS4A1* mRNA levels in BCP-ALL cells. All tested TKI significantly reduced *MS4A1* mRNA levels in BV173 cells, indicating transcriptional downregulation (Figure 2D). Likewise, we found a decrease in CD20 protein amounts in lysates of BV173 cells incubated with all tested TKI (*Online Supplementary Figure S5*). In contrast, several chemotherapeutic drugs, such as L-asparaginase, daunorubicin, vincristine, and cytarabine, exhibited a contrasting effect, leading to an upregulation of CD20 levels (Figure 2E). Overall, these findings suggest that drugs used in the treatment of Ph⁺ BCP-ALL differentially affect CD20 levels, highlighting potential implications for Ph⁺ BCP-ALL treatment with anti-CD20 monoclonal antibodies.

Philadelphia chromosome-positive B-cell precursor acute lymphoblastic leukemia cells preincubated with tyrosine kinase inhibitors exhibit reduced sensitivity to complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity

Given the prominent CD20 downregulation by TKI, we next examined how these drugs affect the antitumor efficacy of the anti-CD20 monoclonal antibody rituximab. To this end, we preincubated Ph⁺ BCP-ALL cells with TKI for 48

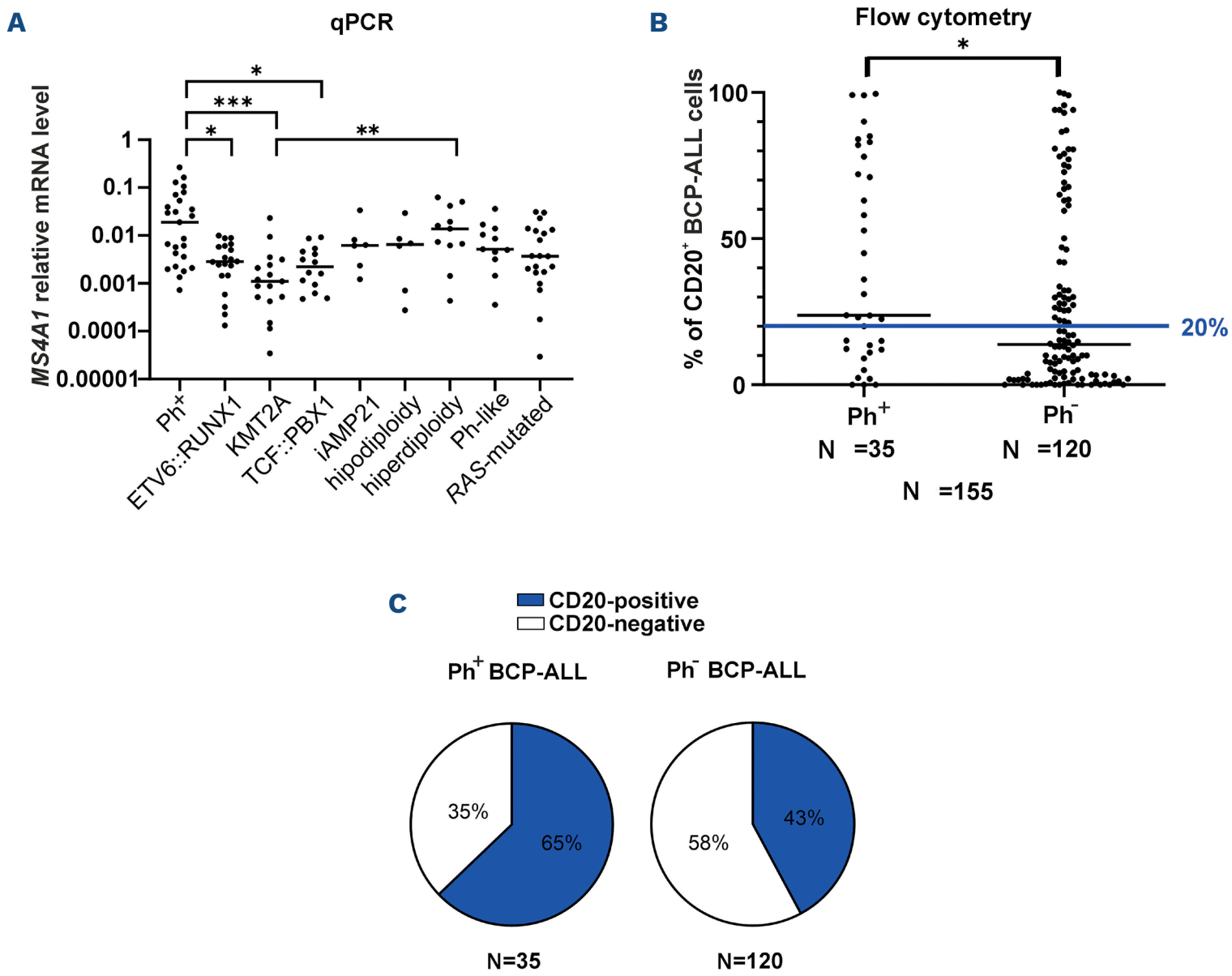

Figure 1. Philadelphia-positive B-cell precursor acute lymphoblastic leukemia blasts more frequently show CD20 positivity. (A) The median levels of *MS4A1* mRNA relative to the *B2M* housekeeping gene were determined by real-time quantitative polymerase chain reaction (qPCR) analysis of bone marrow-derived B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells isolated from pediatric and adult patients with BCP-ALL (N=130), representing defined genetic subtypes of the disease. Each dot corresponds to an individual sample. *P* values were calculated using the Kruskal-Wallis test followed by Dunn multiple comparisons. **P*<0.05, ***P*<0.01, ****P*<0.001. (B) The levels of surface CD20 were assessed by flow cytometry in BCP-ALL cells obtained from pediatric and Philadelphia chromosome-positive (Ph⁺) (N=35) and Philadelphia chromosome-negative (Ph⁻) (N=120) BCP-ALL patients at diagnosis, and the median percentage of CD20⁺ cells was calculated for each group. The clinical threshold for including rituximab in BCP-ALL treatment protocols (20% CD20⁺ blasts) is displayed as a blue line. *P* values were calculated using the Mann-Whitney test, **P*<0.05. (C) Diagrams showing the percentages of CD20⁺ and CD20⁻ patients in the Ph⁺ and Ph⁻ subgroups of BCP-ALL, assessed for the same cohort as described in (B). Patients with at least 20% CD20⁺ lymphoblasts are considered CD20-positive. **P*<0.05, ***P*<0.01, ****P*<0.001.

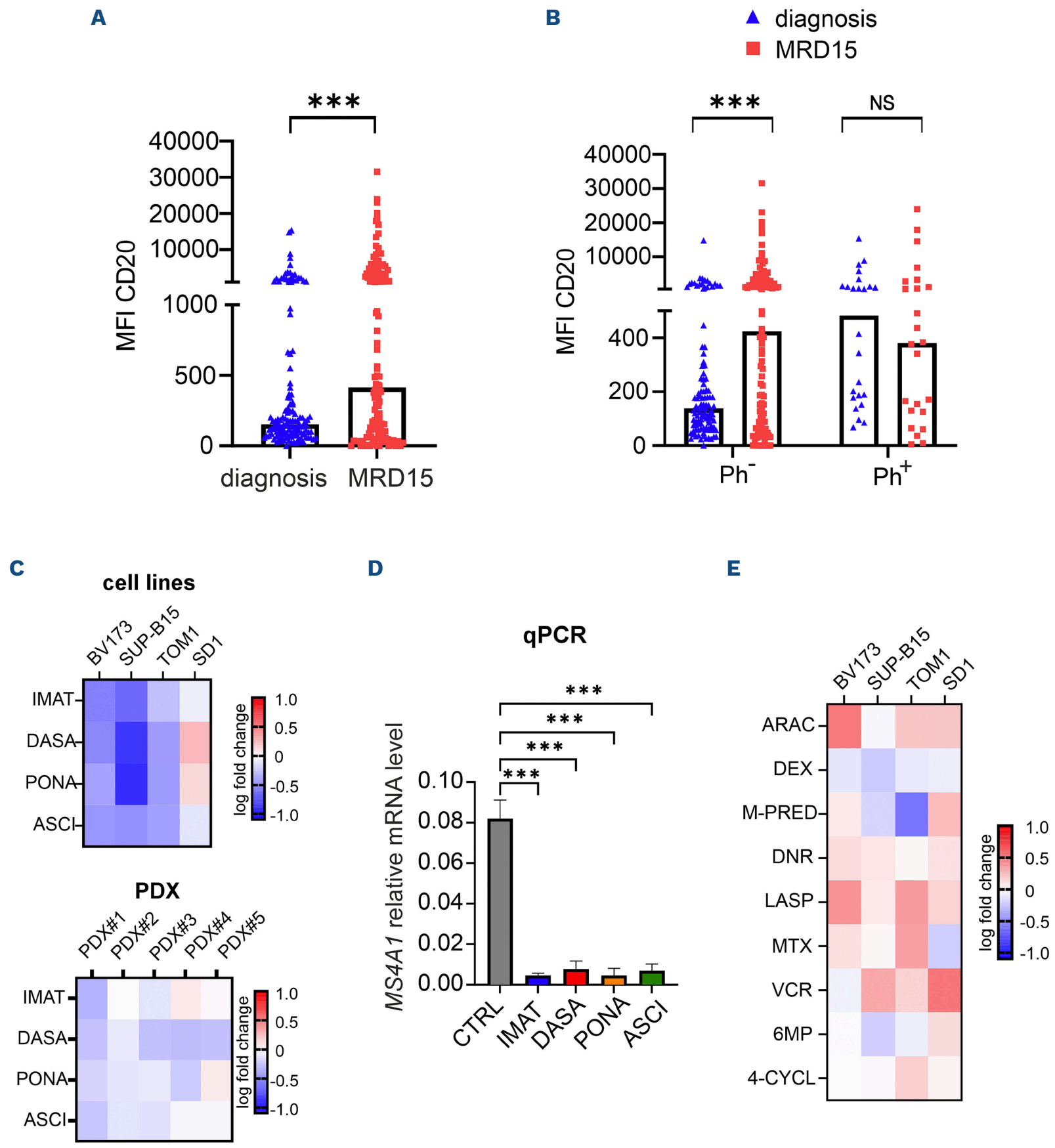

Figure 2. Tyrosine kinase inhibitors downregulate CD20 in Philadelphia-positive B-cell precursor acute lymphoblastic leukemia cells. (A) Median levels of surface CD20 were assessed in primary samples obtained from pediatric patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (N=130) at the time of diagnosis and during the assessment of measurable residual disease (MRD), on day 15 after starting induction therapy (MRD15). Patients were treated according to the modified AIEOP-BFM 2017 or Es-PhALL2017/COG AALL1631 protocol containing 2 weeks of exposure to steroids (dexamethasone or prednisone), one dose of L-asparaginase, one dose of vincristine, one dose of daunorubicin, as well as one intrathecal injection of methotrexate. Philadelphia chromosome-positive (Ph⁺) BCP-ALL patients additionally received 0-3 doses of imatinib. The *P* value was calculated using the Wilcoxon matched-pairs signed-rank test, ***$P<0.001$. (B) The differences in median CD20 levels at diagnosis and at the MRD15 assessment were compared separately for Ph⁺ (N=24) and Philadelphia chromosome-negative (Ph⁻) (N=106) pediatric patients. The *P* value was calculated using the Wilcoxon matched-pairs rank test, ***$P<0.001$. (C) Ph⁺ BCP-ALL cell lines and patient-derived xenograft cells were incubated with maximum serum concentrations (Cmax) of tyrosine kinase inhibitors (TKI) *in vitro* for 48 h. After treatment, the CD20 expression levels were determined by flow cytometry using an anti-CD20 antibody. The heatmap presents the log-fold change of the CD20 fluorescence intensity levels (geometric mean) in drug-treated cells relative to untreated controls. (D) BV173 cells were incubated with TKI at Cmax for 24 h followed by RNA isolation and quantitative polymerase chain reaction analysis. The graph shows the mean mRNA levels of *MS4A1* relative to reference genes (*RPL29, GUSB*) from three independent experiments. The *P* values were calculated using a one-way analysis of variance test followed by the Dunnett test for multiple comparisons, ***$P<0.001$. (E) Ph⁺ BCP-ALL cell lines were incubated for 48 h with drugs commonly used in Ph⁺ BCP-ALL chemotherapy protocols.

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The concentrations of the drugs were determined by cytotoxicity tests and selected to be half maximal effective concentration (EC_{50}) or Cmax, if the EC50 was not achieved. Details of the concentrations of drugs are described in the *Online Supplementary Materials*. Changes in the levels of CD20 were assessed and presented as described in (C). MFI: mean fluorescence intensity; IMAT: imatinib; DASA: dasatinib; PONA: ponatinib; ASCI: asciminib; PDX: patient-derived xenograft; qPCR: quantitative polymerase chain reaction; CTRL: control; ARAC: cytarabine; DEX: dexamethasone; M-PRED: methyl-prednisolone; DNR: daunorubicin; LASP: L-asparaginase; MTX: methotrexate; VCR: vincristine; 6MP: 6-mercaptopurine; 4-CYCLO: 4-hydroxycyclosphosphamide.

hours and evaluated their sensitivity to rituximab-mediated effector mechanisms (Figure 3A). Firstly, we assessed rituximab-mediated CDC, which relies heavily on CD20 levels.[28,29] Accordingly, all tested TKI significantly decreased the serum-induced lysis of rituximab-opsonized BV173 cells (Figure 3B). A similar reduction in rituximab-mediated CDC was observed on PDX cells (*Online Supplementary Figure S6A*), but it reached statistical significance only for imatinib and dasatinib.

In subsequent steps, we tested the effects of TKI on rituximab-mediated ADCC. In these experiments, BV173 cells were preincubated with Cmax and ¼ Cmax concentrations of TKI, and then co-incubated with a CD16-expressing NK92 cell line in the presence of TKI for 4 hours to evaluate ADCC. At Cmax concentrations, dasatinib and ponatinib significantly reduced the rituximab-mediated cytotoxicity of NK92 cells (Figure 3C), whereas at ¼ Cmax concentrations, only dasatinib did (*Online Supplementary Figure S6B*). No reduction of cytotoxicity was observed in imatinib- and asciminib-treated samples (Figure 3C, *Online Supplementary Figure S6B*). In summary, these results demonstrate that TKI induce substantial CD20 downregulation in Ph⁺ BCP-ALL cells and reduce their susceptibility to rituximab-mediated CDC. However, despite similar reductions in CD20 levels mediated by all TKI, only dasatinib and ponatinib diminished rituximab-mediated ADCC.

Cytotoxic activity of effector cells is preserved in the presence of asciminib and imatinib

Based on the above results we hypothesized that the inhibitory effect of selected TKI on ADCC may be predominated by their impact on the effector cells rather than by their effects on CD20 reduction on target tumor cells. To address this hypothesis, we tested the influence of TKI on ADCC in another variant, when the leukemic cells were co-incubated for 4 hours with cytotoxic effector cells, anti-CD20 monoclonal antibodies, and TKI, without any preincubation of target cells with TKI (Figure 3D). Importantly, after 4 hours of incubation, the influence of TKI on CD20 levels in target cells was minimal (*Online Supplementary Figure S6C*). Interestingly, results from co-incubation (Figure 3E, left panel; *Online Supplementary Figure S6D, E*) and pre-incubation (Figure 3C) variants were very consistent, thus suggesting that the inhibitory effects of dasatinib and ponatinib on ADCC are mediated mainly by blocking effectors, rather than by downregulating CD20 on leukemic cells. To further delve into these findings, we employed another Ph⁺ BCP-ALL cell line, SD1, in which TKI do not downregulate CD20 (Figure 2C), and used peripheral blood mononuclear cells isolated from healthy donors as the effectors. In this setting, only dasatinib significantly reduced the cytotoxicity of immune effectors, at both Cmax and ¼ Cmax TKI concentrations (*Online Supplementary Figure S6E*). The prominent inhibitory effect of dasatinib was also observed in the ADCC test using obinutuzumab, an anti-CD20 monoclonal antibody engineered to increase the efficacy of ADCC (Figure 3E, right panel; *Online Supplementary Figure S6F*). In all ADCC tests presented above, imatinib and asciminib did not show any significant reduction in cytotoxicity, regardless of whether they were tested with or without pre-incubation (Figure 3C, E; *Online Supplementary Figure S6B, D, E*). Finally, we compared the effects of all tested TKI on NK cell cytotoxicity without the addition of antibodies, using K562 as targets and NK cells isolated from healthy donors as effectors (Figure 3F). We found that natural cytotoxicity was almost completely abolished in the presence of dasatinib at both tested doses, Cmax and ¼ Cmax, and partially reduced with the Cmax doses of ponatinib and bosutinib (Figure 3G; *Online Supplementary Figure S6G*). Neither imatinib nor asciminib influenced the natural cytotoxicity of NK cells (Figure 3G; *Online Supplementary Figure S6G*). In summary, these results demonstrate that TKI have varying effects on ADCC mediated by anti-CD20 monoclonal antibodies, with a significant reduction in cytotoxicity largely attributed to their impact on immune effector cells rather than CD20 downregulation on leukemic cells. Notably, asciminib and imatinib did not reduce the efficacy of ADCC mediated by anti-CD20 monoclonal antibodies in any of the settings tested.

Asciminib does not impair *ex vivo* NK cell degranulation

In previous experiments, we showed that some TKI, especially dasatinib, at concentrations achievable in serum after oral administration, can negatively affect the effector functions of NK cells. To further investigate the effects of TKI on NK cell function, we performed a degranulation assay using primary NK cells isolated from healthy donors primed for degranulation with K562 cells. We found complete suppression of NK cell degranulation following dasatinib treatment, while there was partial suppression following exposure to ponatinib (Figure 4A). To further verify our results, we tested the degranulation capacity of NK cells in blood collected from CML and Ph⁺ BCP-ALL patients who received imatinib, dasatinib or asciminib. Blood samples were collected before drug administration, 1 hour, and 24 hours after oral administration of

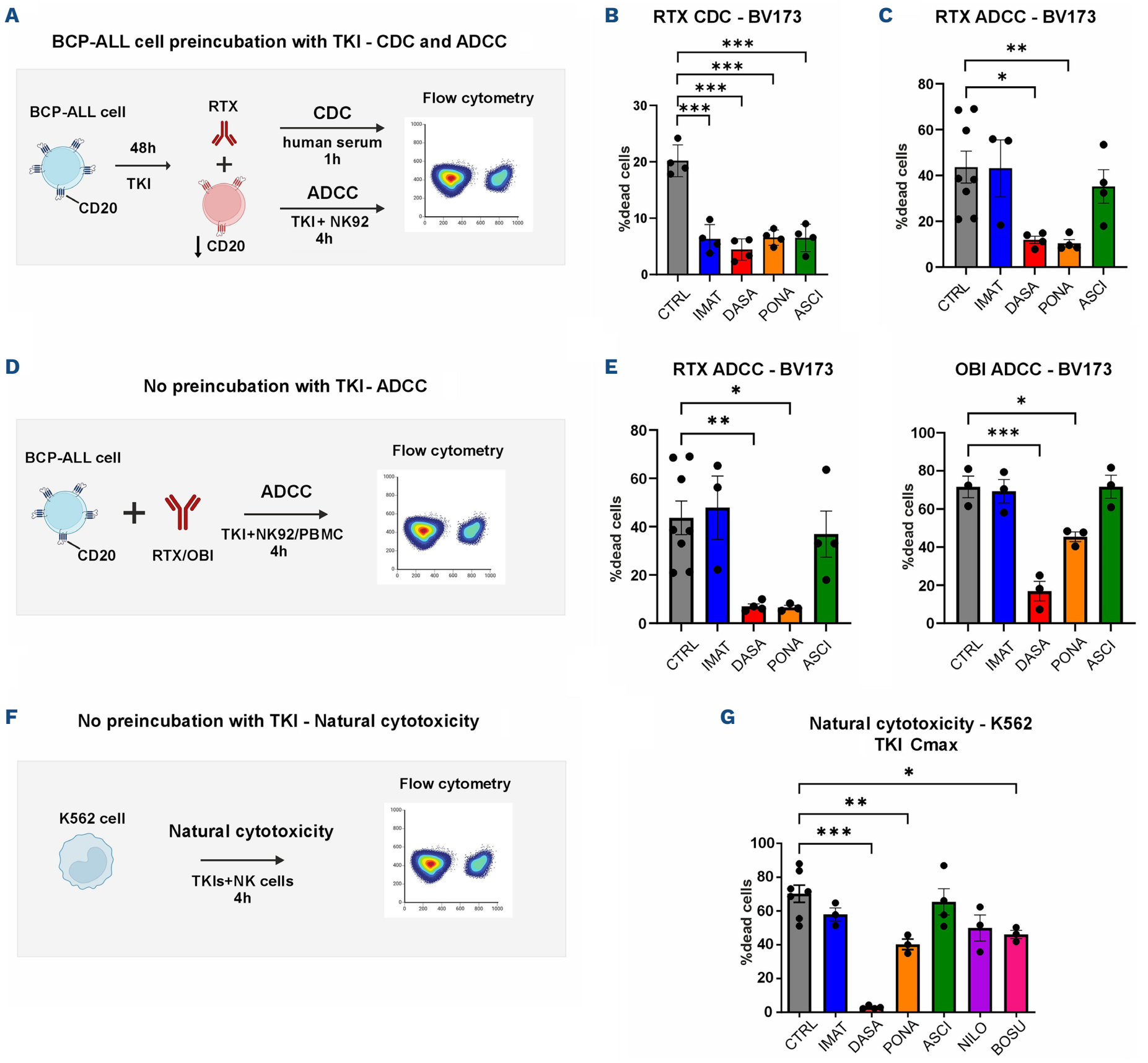

Figure 3. Effects of tyrosine kinase inhibitors on antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity mediated by anti-CD20 monoclonal antibodies. (A) Experimental scheme of the preincubation experiments. BV173 target cells were preincubated with maximum serum concentrations (Cmax) of tyrosine kinase inhibitors (TKI) or dimethylsulfoxide (DMSO, control) for 48 h. To assess complement-dependent cytotoxicity, target cells were incubated with human serum and rituximab for 1 h. To assess antibody-dependent cellular cytotoxicity, the preincubated and CFSE-stained target cells were incubated for 4 h with CD16⁺ NK92 cells, rituximab, and Cmax concentrations of TKI or DMSO (control). The percentage of dead cells was assessed by flow cytometry. (B) After 48 h incubation with Cmax concentration of TKI, BV173 cells were incubated with human serum and 20 μg/mL rituximab. Next, dead cells were stained with propidium iodide and assessed in flow cytometry. Background cytotoxicity (dead cells in the group with serum, but without rituximab) was deducted from the corresponding groups. The presented data are the means ± standard error of mean (SEM) from at least four independent repeats. (C) After 48 h incubation with Cmax concentrations of TKI, CFSE-stained BV173 cells were incubated for 4 h with CD16⁺ NK92 cells in an effector:target ratio of 2.5:1, 10 μg/mL rituximab, and Cmax concentrations of TKI or DMSO (control). Upon incubation cells were stained with 7AAD and assessed by flow cytometry as the percentage of CFSE and 7AAD double-positive cells. The presented data are the means ± SEM from at least four independent repeats. (D) Experimental plan without preincubation of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells with TKI. CFSE-stained BCP-ALL cells were incubated for 4 h with effector cells, anti-CD20 monoclonal antibodies, and Cmax concentrations of TKI or DMSO (control) and the viability of the BCP-ALL cells was assessed by flow cytometry. (E) CFSE-stained BV173 target cells were incubated for 4 h with CD16⁺ NK92 cells in an effector:target ratio of 2.5:1, Cmax concentrations of TKI and 10 μg/mL rituximab (left panel) or 10 μg/mL obinutuzumab (right panel). The

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presented data are the means ± SEM of at least three independent experiments. The percentages of dead cells were assessed as described in (C). (F) Experimental scheme of the natural cytotoxicity experiments. NK cells were isolated from healthy donors and incubated with TKI and CFSE-stained K562 target cells in an effector:target ratio of 10:1 for 4 h without the addition of antibodies. The percentage of dead cells was assessed by flow cytometry as the percentage of CFSE and propidium iodide double-positive cells. (G) After 4 h incubation with NK cells and Cmax concentrations of TKI, K562 cells were stained with propidium iodide and analyzed by flow cytometry as described in (F). The differences between groups in (B), (C), (E) and (G) were assessed by one-way analysis of variance with a Dunnett *post-hoc* test, **P*<0.05, ***P*<0.01, ****P*<0.001. CDC: complement-dependent cytotoxicity; ADCC: antibody-dependent cellular cytotoxicity; RTX: rituximab; CTRL: control; IMAT: imatinib; DASA: dasatinib; PONA: ponatinib; ASCI: asciminib; OBI: obinutuzumab; NILO: nilotinib; BOSU: bosutinib.

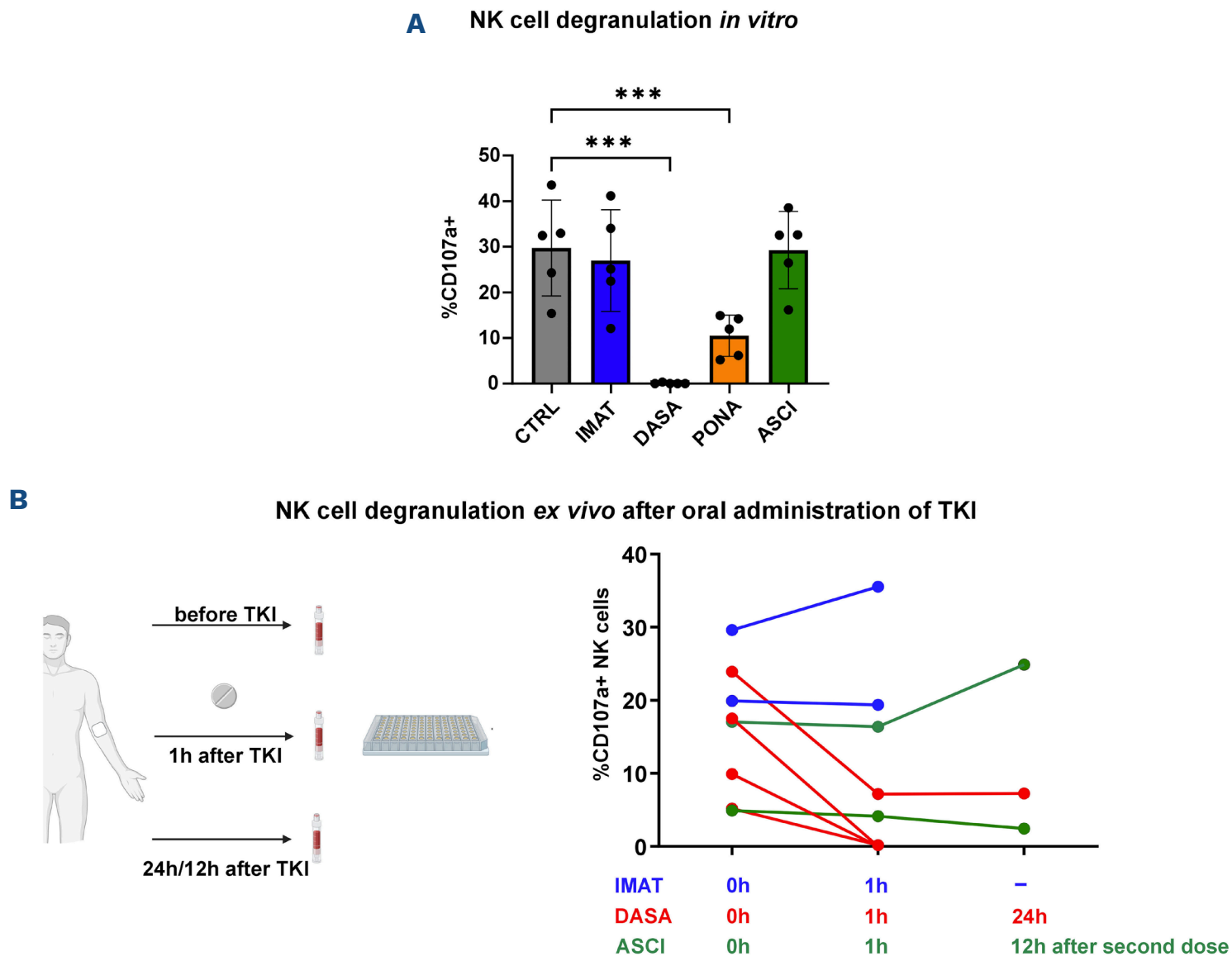
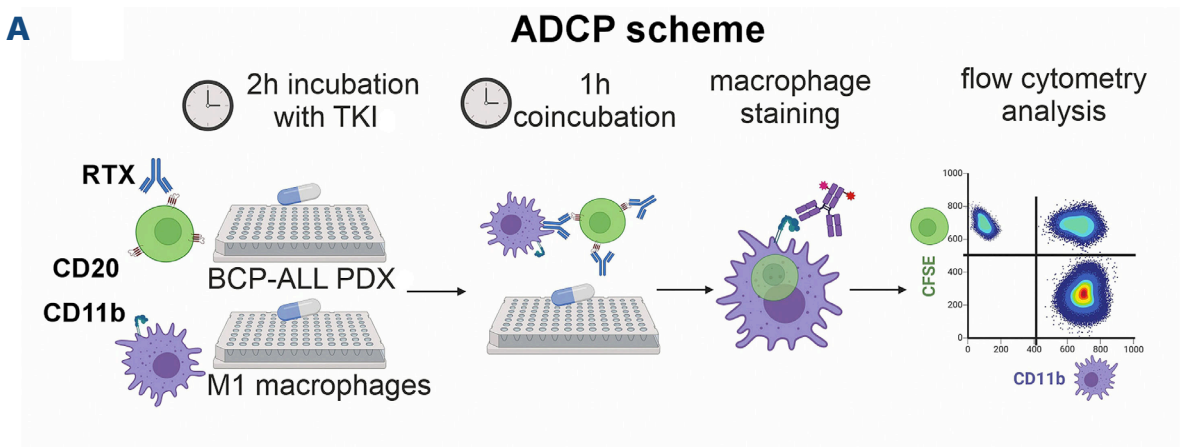


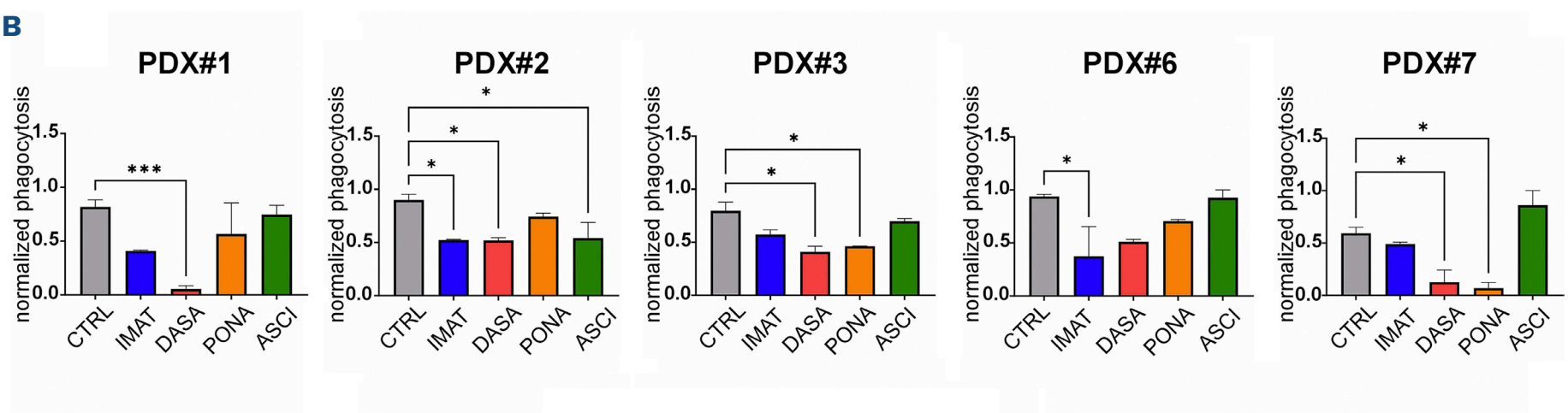
Figure 4. Effects of tyrosine kinase inhibitors on degranulation of natural killer cells *in vitro* and *ex vivo*. (A) Natural killer (NK) cells isolated from healthy donors were incubated for 4 h with K562 cells and with maximum serum concentrations (Cmax) of tyrosine kinase inhibitors in an effector:target ratio of 1:1. Degranulation was evaluated by flow cytometry as the percentage of CD107a⁺ NK cells, with background (NK cells incubated without targets) deducted. *P* values were calculated using one-way analysis of variance followed by a Dunnett multiple comparisons test, ****P*<0.001. (B) Left panel. Experimental scheme of the whole blood *ex vivo* assay for testing the degranulation of NK cells. The blood was collected before (0 h), 1 h, and 24 h after oral administration of TKI to patients with Philadelphia-positive B-cell precursor acute lymphoblastic leukemia (Ph⁺ BCP-ALL) or chronic myelogenous leukemia (CML). Next, 150 µL of whole blood were co-incubated with 400,000 K562 target cells for 4 h. Degranulation was evaluated by flow cytometry as the percentage of CD107a⁺ NK cells after subtraction of background - samples with blood but without K562 target cells. Right panel. After 4 h incubation with K562 cells and red blood cell lysis, patients' cells were stained and analyzed by flow cytometry as described above. Patients underwent initial therapy (first dose) with specific tyrosine kinase inhibitors in the case of dasatinib and asciminib, while those prescribed imatinib received subsequent doses in the regimen. CTRL: control; IMAT: imatinib; DASA: dasatinib; PONA: ponatinib; ASCI: asciminib; TKI: tyrosine kinase inhibitors.

the standard dose of the TKI, and no additional TKI was added to the assay (Figure 4B, left panel). We observed inhibition of NK cell degranulation in all blood samples collected 1 hour or 1 day after administration of dasatinib (Figure 4B, right panel). In contrast, NK cell activity was not reduced in blood samples collected at 1 hour or 24 hours after administration of asciminib nor at 1 hour after administration of imatinib (Figure 4B, right panel). These *ex vivo* results confirm that asciminib and imatinib do not interfere with NK cell function at concentrations achievable in patients' blood, while dasatinib exhibits negative effects.

Asciminib does not interfere with rituximab-mediated antibody-dependent cell-mediated phagocytosis

Next, we investigated the effects of TKI on ADCP, another immune effector mechanism relevant to the antitumor activity of rituximab.[27,30] To assess the function of macrophages, we performed an *in vitro* phagocytosis assay using Ph⁺ CD20⁺ BCP-ALL PDX as targets and human primary M1 macrophages differentiated from the primary human monocytes as effectors. Rituximab-opsonized targets and effectors were separately preincubated for 2 hours with two different concentrations of TKI, and then mixed to enable ADCP for 1 hour (Figure 5A). As shown in Figure 5B, rituximab-ADCP was reduced in the presence of Cmax dasatinib in four out of five tested PDX. Reductions of ADCP were also observed in the presence of ponatinib and imatinib in two out of five PDX. Asciminib at Cmax, but not at ¼ Cmax, reduced ADCP of one PDX tested (*Online Supplementary Figure S7A*). In the pooled experimental data from all tested PDX, we observed a reduction of rituximab-ADCP when the assay was performed in the presence of Cmax or ¼ Cmax





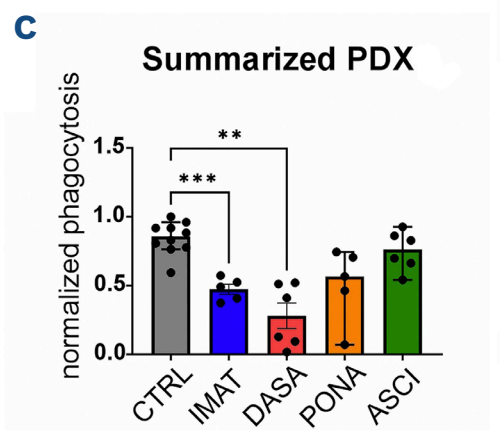


Figure 5. Effects of tyrosine kinase inhibitors on rituximab-mediated antibody-dependent cellular phagocytosis. (A) Experimental scheme for the antibody-dependent cellular phagocytosis (ADCP) assay (described in detail in the *Methods* section). Briefly, primary CD20⁺ B-cell precursor acute lymphoblastic leukemia patient-derived xenograft (PDX) cells were stained with CFSE and opsonized with 5 μg/mL rituximab. Next, the PDX cells as well as primary human M1 macrophages were separately preincubated for 2 h with maximum serum concentrations (Cmax) of tyrosine kinase inhibitors (TKI) and then co-incubated for 1 h in the presence of Cmax concentrations of TKI. The percentage of phagocytosis was assessed by flow cytometry, as the fraction of double-positive macrophages. The background phagocytosis was assessed using cetuximab. (B) After background subtraction, phagocytosis was normalized to that of the highest technical replicate per donor. *P* values were calculated using one-way analysis of variance (ANOVA) followed by a Dunnett test for multiple comparisons, **P*<0.05, ****P*<0.001. (C) Summarized results of the experiments from Figure (B) (5 PDX). *P* values were calculated using the Welch ANOVA test followed by Dunnett T3 test for multiple comparisons, ***P*<0.01, ****P*<0.001. A detailed description of the experimental setup can be found in the *Online Supplementary Methods*. ADCP: antibody-dependent cellular phagocytosis; RTX: rituximab; BCP-ALL: B-cell precursor acute lymphoblastic leukemia; CTRL: control; IMAT: imatinib; DASA: dasatinib; PONA: ponatinib; ASCI: asciminib.

concentrations of imatinib, dasatinib, and ponatinib; however, this reached statistical significance only for imatinib and dasatinib (Figure 5C, *Online Supplementary Figure S7B*). These results indicate that dasatinib and imatinib block the engulfment of rituximab-opsonized CD20⁺ Ph⁺ BCP-ALL PDX cells to the greatest extent, ponatinib to a lesser extent, while asciminib does not significantly interfere with rituximab-mediated ADCP.

In summary, although all tested TKI decreased rituximab-mediated CDC, their effects on effector cells differed, with asciminib exhibiting the most favorable profile. A summary of the effects of TKI on anti-CD20 monoclonal antibody-mediated cytotoxicity is presented in Figure 6.

Discussion

The incorporation of rituximab into chemotherapy has markedly enhanced treatment outcomes in adult Ph⁻ CD20⁺ BCP-ALL patients,[6] facilitating its inclusion in treatment protocols. Furthermore, an ongoing clinical trial will compare the efficacy of obinutuzumab against rituximab in adult BCP-ALL patients (clinicaltrials.gov: NCT04920968). Preliminary trials also reveal the potential effectiveness of anti-CD20 therapy in pediatric cohorts, suggesting an opportunity for enlarging the pool of eligible patients.[31,32] Considering that anti-CD20 monoclonal antibodies are commonly combined with other drugs, it is essential to analyze the reciprocal influences on efficacy. Our study reveals frequent CD20 expression among Ph⁺ BCP-ALL patients, implying that combined use of TKI and rituximab could be of value. TKI play a critical role in the treatment of Ph⁺ BCP-ALL and are administered in all phases of treatment. Imatinib and dasatinib serve as frontline therapies, while ponatinib is utilized in cases in which a T315I kinase domain mutation is detected. Asciminib, an allosteric inhibitor, is not yet approved for the treatment of Ph⁺ BCP-ALL. Our studies reveal substantial TKI-induced decreases in CD20 levels and interference of selected TKI with critical effector cell functions, thereby potentially affecting treatment effectiveness in Ph⁺ BCP-ALL patients and implying treatment refinements are necessary.

Rituximab is commonly used in adult BCP-ALL therapy worldwide, but its efficacy in the Ph⁺ subgroup of CD20⁺ BCP-ALL patients was mainly assessed in combination with imatinib.[33,34] A recent analysis of 119 adult patients in Mexico suggested a potential improvement in overall survival among CD20⁺ patients treated with rituximab and TKI (mostly imatinib).[33] Additionally, a Korean phase II multicenter study found that rituximab-containing regimens with imatinib were superior in CD20⁺ Ph⁺ patients than historical treatments without rituximab.[34] There is a lack of clinical evaluation of the efficacy of other TKI in combination with anti-CD20 monoclonal antibodies. Although nowadays bispecific antibodies and adoptive cellular therapies are emerging treatments for relapsed and refractory BCP-ALL patients, their high costs limit accessibility in many countries. Therefore, rituximab or its biosimilars could serve as cost-effective treatments for CD20⁺ Ph⁺ BCP-ALL, potentially reducing disease relapse rates.

Our results suggest variability among TKI in their suitability for combination with anti-CD20 antibodies. While all TKI downregulated CD20 levels similarly in both BCP-ALL cell lines and PDX and diminished rituximab-mediated CDC, dasatinib also substantially inhibited NK cell cytotoxic activity and macrophage phagocytic function at various concentrations in both *in vitro* and *ex vivo* settings. We also noted inhibitory effects of ponatinib at Cmax on ADCC. These results correspond with those in the literature, pinpointing the inhibitory influence of dasatinib and ponatinib on NK

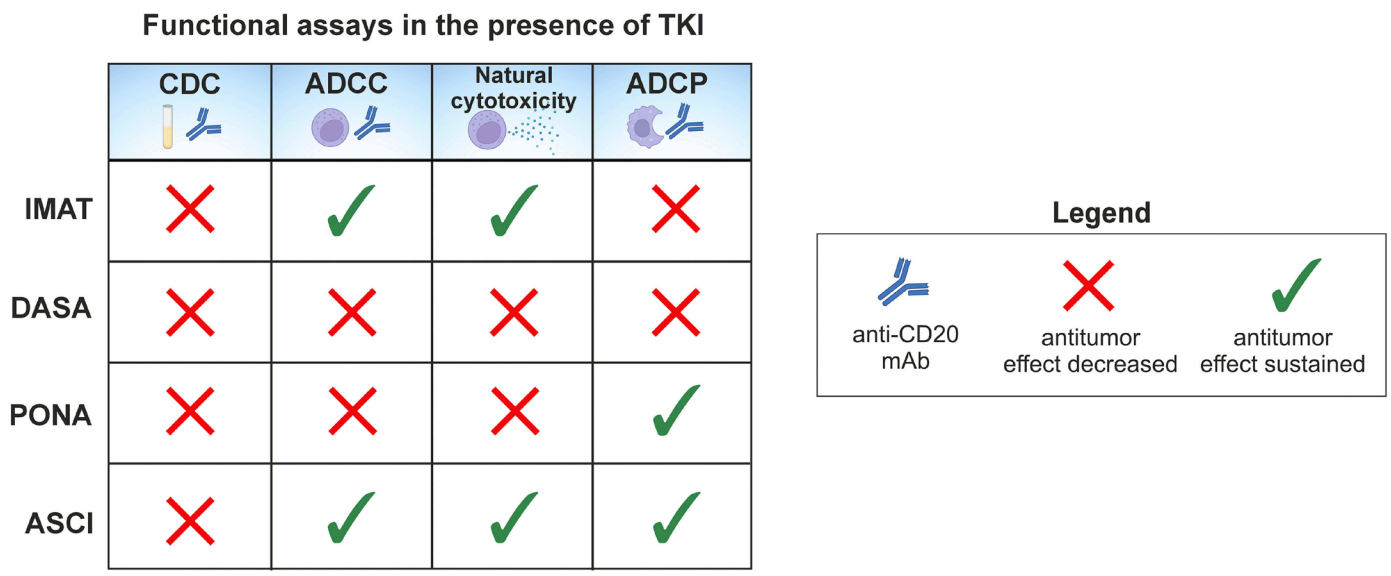


Figure 6. A summary of the effects of some tyrosine kinase inhibitors in the different functional assays. The table presents a comparison of four tyrosine kinase inhibitors, summarizing the results from Figures 3-5, indicating whether antitumor efficacy of immune effector mechanisms is reduced or maintained in each functional assay. TKI: tyrosine kinase inhibitors; CDC: complement-dependent cytotoxicity; ADCC: antibody-dependent cellular cytotoxicity; ADCP: antibody-dependent cellular phagocytosis. IMAT: imatinib; DASA: dasatinib; PONA: ponatinib; ASCI: asciminib; mAb: monoclonal antibody.

cells and ADCC ascribed to their broader suppressive activity on critical SRC kinases.[35,36] Our investigations further underscore the adverse implications of dasatinib, and to a lesser degree, imatinib on macrophage phagocytic capabilities. To the best of our knowledge, this is the first report showing the effects of TKI on human immunophagocytosis. Additional research is needed to delineate the precise mechanisms underlying these inhibitory effects. Contrary to other TKI, asciminib did not compromise any effector cell-dependent mechanisms of cytotoxicity of anti-CD20 monoclonal antibodies *in vitro*. This was confirmed by the stable NK cell degranulation observed in the blood of patients treated with asciminib. Given dasatinib's substantial negative impact on essential effector mechanisms pivotal for the efficacy of rituximab, its potential to undermine antibody efficacy in patients is considerable. Consequently, TKI selection for combination with anti-CD20 monoclonal antibodies warrants meticulous consideration, with alternatives such as asciminib potentially offering more potent combination strategies. Combining different generations of TKI should also be considered, as the combination of ponatinib with asciminib has shown promise in treating highly resistant CML and Ph⁺ BCP-ALL.[37,38]

The inhibitory effects of TKI on cytotoxic effector cells were previously tested in the context of T-cell engaging bispecific antibodies, revealing a reduction in the antitumor efficacy of blinatumomab when it was used with dasatinib and ponatinib.[39] Despite these preclinical findings, recent clinical trials have shown promising therapeutic outcomes of dasatinib or ponatinib combined with blinatumomab,[40,41] highlighting the limitations of preclinical studies in predicting clinical outcome. However, it is important to note significant differences in the mechanisms of antitumor activity between blinatumomab and rituximab. While blinatumomab targets tumor cells exclusively by engaging T cells via the CD19-CD3 interaction, rituximab activates complement, NK cells, and macrophages after binding to CD20. In our study, we observed negative effects of dasatinib and, to a lesser extent, ponatinib both on diminishing the level of a target antigen CD20 and inhibiting various types of effector cells. Therefore, we firmly believe that our preclinical data could have implications for the clinical efficacy of combinations involving rituximab.

Our research also underscores the potential importance of the selection of anti-CD20 monoclonal antibodies in therapeutic strategies for Ph⁺ BCP-ALL. Currently, only rituximab, which functions via CDC, ADCC, and ADCP mechanisms,[9,26] is employed in the treatment of BCP-ALL. Obinutuzumab, primarily facilitating ADCC,[10] is currently under clinical investigation. Our findings illustrate that all TKI reduce CD20 mRNA, total protein, and cell surface protein levels in the majority of BCP-ALL cell lines and PDX. Importantly, during the MRD assessment, we noted a subtle decline in CD20 levels in Ph⁺ BCP-ALL blasts relative to the levels at diagnosis, in contrast to the elevation observed in the Ph⁻ BCP-ALL group of patients. Given that the principal distinction in the multidrug combination therapy between Ph⁺ and Ph⁻ groups is the presence of a TKI (primarily imatinib) in the former group, it is plausible that the noted CD20 reduction is linked to the inclusion of the TKI in the treatment protocol. It is pertinent to note that the reduction of CD20 by TKI is possibly mediated by the on-target inhibition of BCR::ABL1, making it challenging to eliminate. Interestingly, this significant, albeit partial, CD20 reduction, does not negatively influence effector mechanisms aside from CDC. This finding aligns with those of previous studies indicating a specific dependency of CDC on CD20 levels, unlike ADCC, which maintains efficacy at reduced CD20 levels.[29] In the light of these findings, our results hint at the potential superiority of obinutuzumab as an anti-CD20 monoclonal antibody in conjunction with TKI, especially those not adversely affecting ADCC, such as imatinib and asciminib.

In summary, our study underscores the crucial role of precise TKI selection when these drugs are used in combination with anti-CD20 monoclonal antibodies for the treatment of CD20⁺ Ph⁺ BCP-ALL. Considering the downregulation of CD20 by all tested TKI, the use of obinutuzumab, which potently eliminates CD20⁺ cells independently of CDC, may be beneficial for CD20⁺ Ph⁺ BCP-ALL patients. In terms of TKI selection, we propose asciminib as a prime candidate for combination therapy, given its minimal impact on immune effector cell function. Our results may have a broader impact, given the ever-increasing use of immunotherapies involving immune effector cells, which are also being tested in clinical trials in combination with dasatinib and other TKI.[42]

Disclosures

EP has received consulting fees from KCR and honoraria from Astellas Pharma, Servier, Amgen, and Novartis for lectures and consulting - all unrelated to the submitted work. The other authors have no competing financial interests to declare.

Contributions

KD conceived the study and was responsible for investigations, methodology, data analysis, visualization, and writing and editing the manuscript. AD, MJ, and ZU were responsible for investigation, methodology, and visualization, and contributed to writing the manuscript. AP collected patients' sample, analzyed and interpreted data, and conceived the manuscript. MW conceived the study, and wrote and edited the study. KF and ML contributed to the conceptualization, investigation, methodology, and manuscript writing. EP collected patients' samples and performed investigations. LS and BP acquired patients' data, analyzed the data and contributed to writing the manuscript. JH and JJ performed investigations. BK acquired patients' data and analyzed their data. ELM, WM, and TS provided clinical and patients' data, and revised the manuscript. MF was responsible for

conceptualization, data analysis and interpretation, resources, supervision, funding acquisition, visualization, project administration, writing and editing. All authors have read and accepted the final version of the manuscript.

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Data-sharing statement

Original data used in this study are available from the corresponding author upon request.

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