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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Supplementary material

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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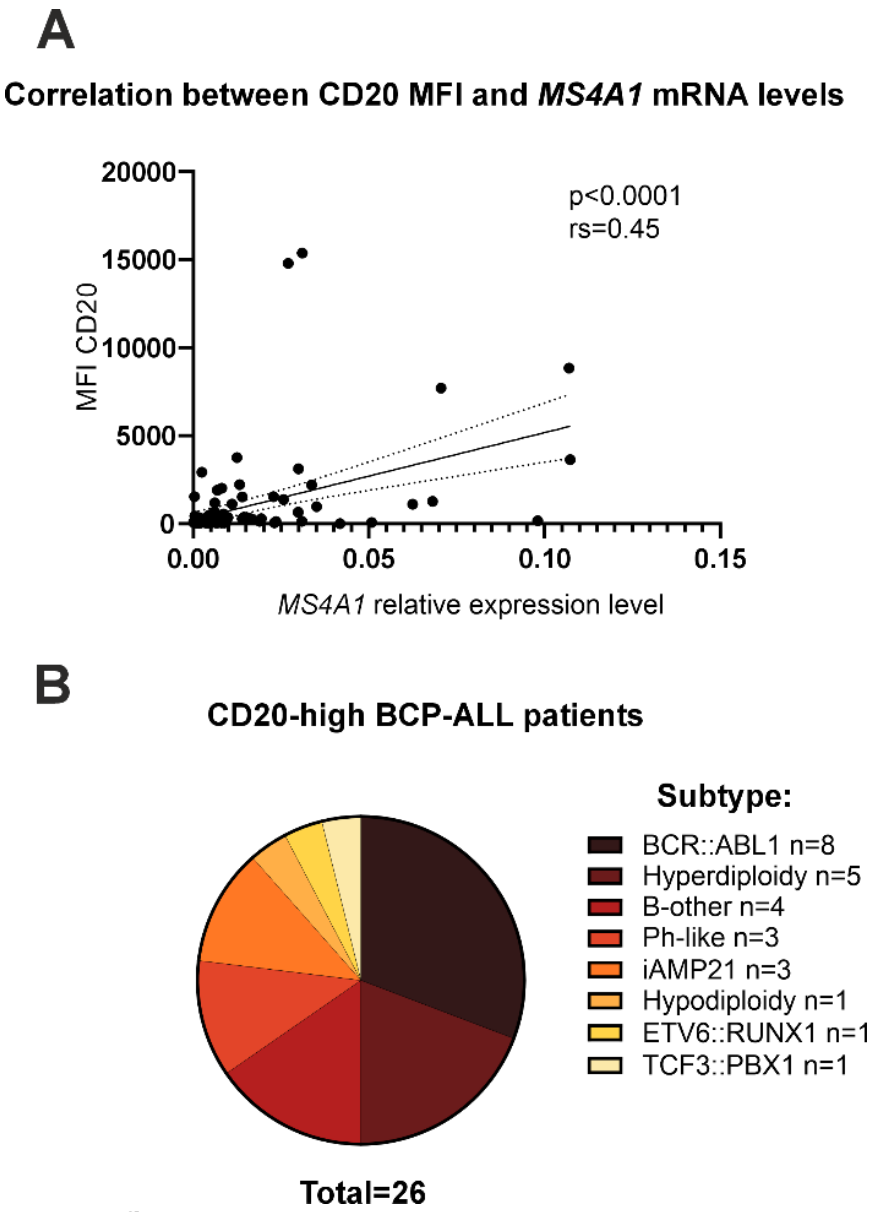
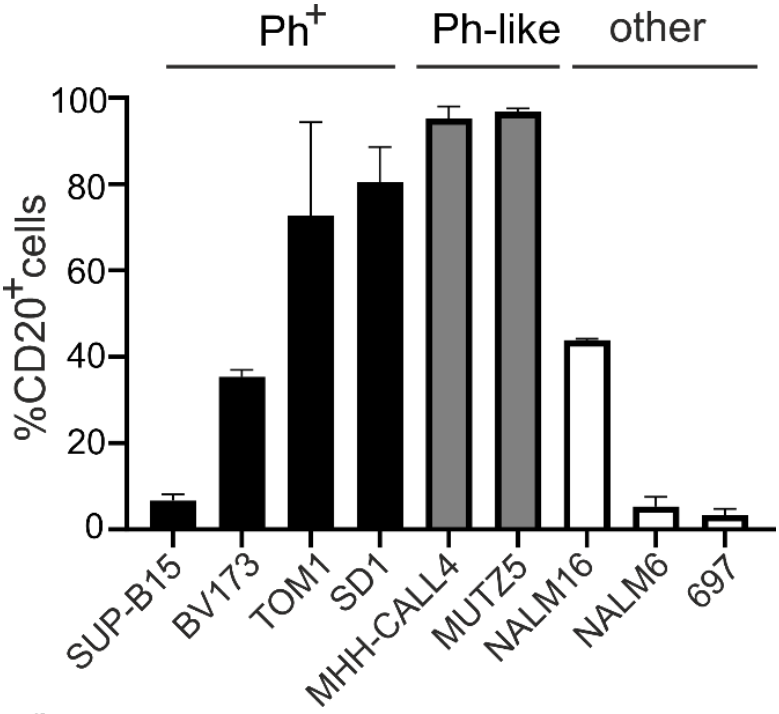
Supplementary figures



Supplementary figure 1.

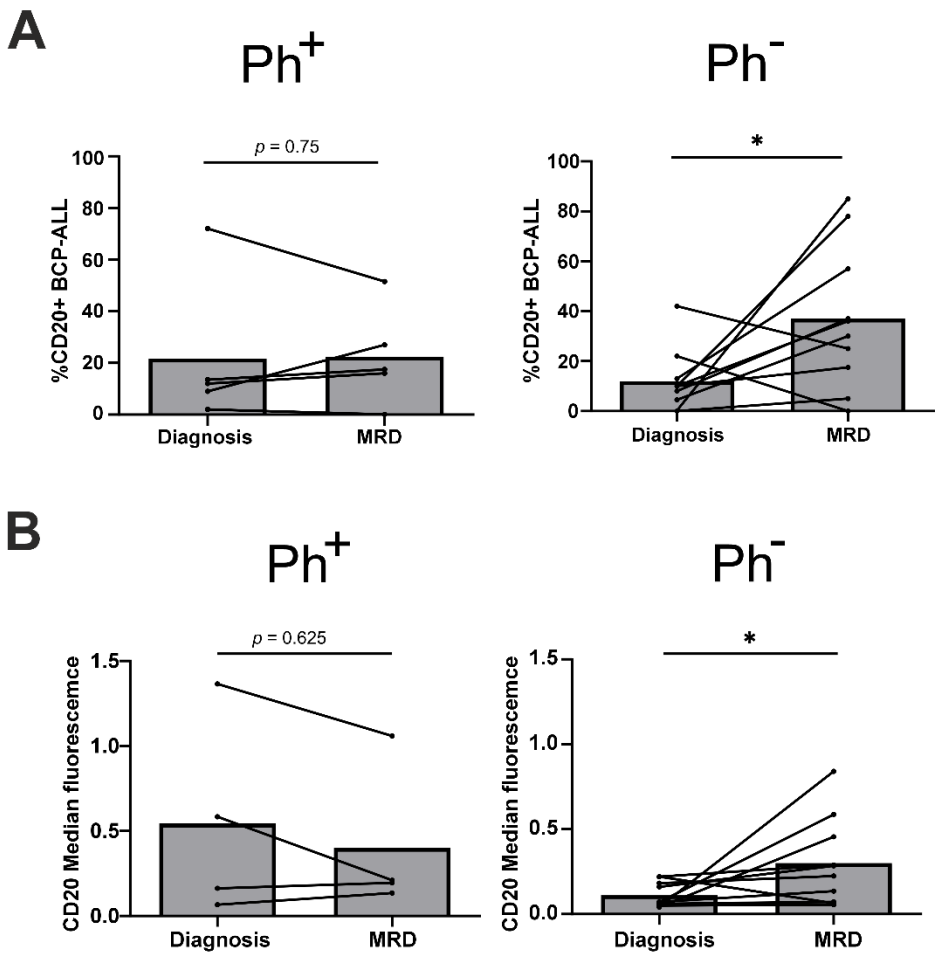
A. The Spearman correlation between *MS4A1* mRNA levels assessed by qPCR and CD20 surface protein levels assessed by flow cytometry (MFI) in the BCP-ALL primary samples (n=117 pediatric BCP-ALL patients) was analyzed using GraphPad Prism software.

B. Representation of genetic subtypes among CD20-high BCP-ALL pediatric patients. Patients with CD20 levels assessed by flow cytometry exceeding the MFI of 400 (22% of patients with the highest CD20 levels, n=26) were classified as CD20-high (BCR::ABL1 n=8, hyperdiploidy n=5, B-other n=4, Ph-like n=3, iAMP21 n=3, hypodiploidy n=1, ETV6::RUNX n=1, TCF::PBX1 n=1).



Supplementary figure 2.

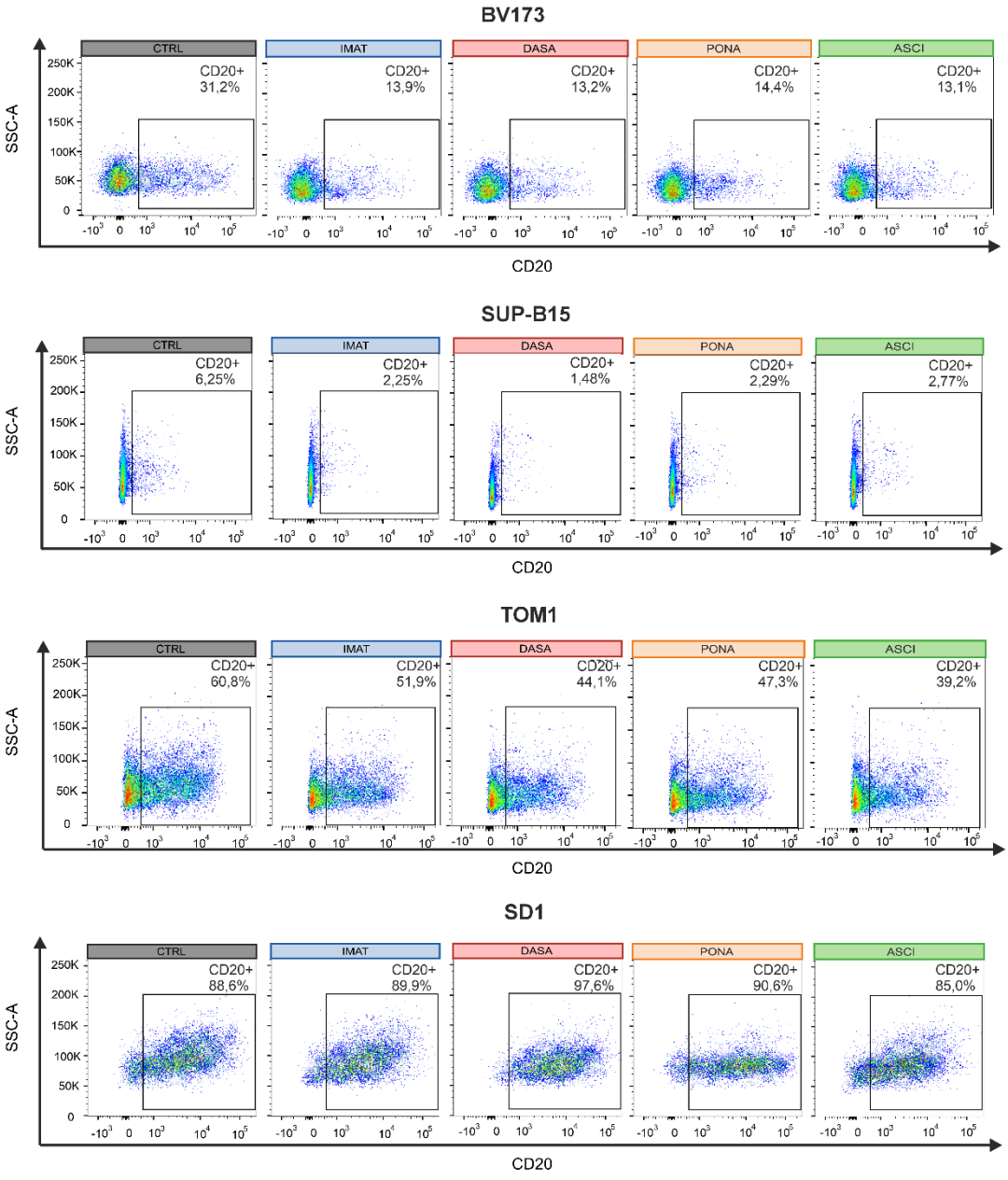
Levels of surface CD20 were assessed in untreated BCP-ALL cell lines using flow cytometry. Mean percentages form 3 or 4 independent measurements are shown.



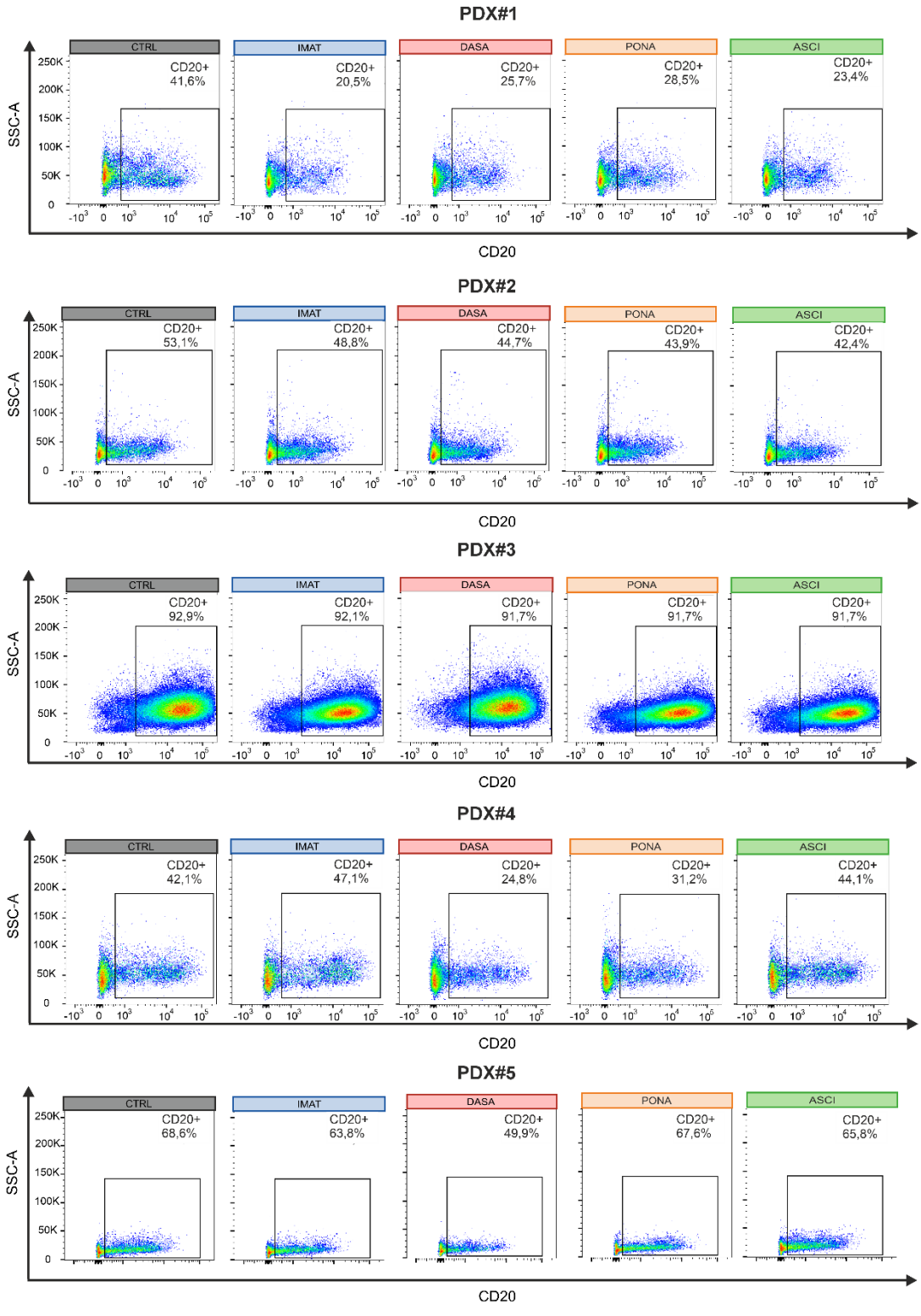
Supplementary figure 3. Changes in CD20 expression during treatment of adult BCP-ALL patients

Flow cytometry was employed to evaluate the percentages of $CD20^+$ BCP-ALL cells (**A**) and the median CD20 levels in BCP-ALL cells (**B**) in primary samples from adult BCP-ALL patients (n=15). These assessments were conducted at the time of diagnosis and during the measurable residual disease (MRD) evaluation specifically between 50 to 85 days after the initiation of induction therapy. Patients underwent treatment based on the Polish Adult Leukaemia Group (PALG) protocols PALG ALL6 or PALG ALL7. The supplementary methods provide a comprehensive list of drugs incorporated into the treatment protocols. Exclusion criteria for patients undergoing PALG ALL7 included those with 20% or more $CD20^+$ BCP-ALL cells, as these patients received RTX and the cells expressing CD20 were eliminated, as reported in [1]. *P* values were calculated using the Wilcoxon matched-pairs signed-rank test.

A


BV173
CTRL
IMAT
DASA
PONA
ASCI
CD20+ 31,2%
CD20+ 13,9%
CD20+ 13,2%
CD20+ 14,4%
CD20+ 13,1%
SSC-A
CD20
SUP-B15
CD20+ 6,25%
CD20+ 2,25%
CD20+ 1,48%
CD20+ 2,29%
CD20+ 2,77%
TOM1
CD20+ 60,8%
CD20+ 51,9%
CD20+ 44,1%
CD20+ 47,3%
CD20+ 39,2%
SD1
CD20+ 88,6%
CD20+ 89,9%
CD20+ 97,6%
CD20+ 90,6%
CD20+ 85,0%

B


PDX#1
CTRL
IMAT
DASA
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ASCI
CD20+ 41,6%
CD20+ 20,5%
CD20+ 25,7%
CD20+ 28,5%
CD20+ 23,4%
SSC-A
CD20
PDX#2
CD20+ 53,1%
CD20+ 48,8%
CD20+ 44,7%
CD20+ 43,9%
CD20+ 42,4%
PDX#3
CD20+ 92,9%
CD20+ 92,1%
CD20+ 91,7%
CD20+ 91,7%
CD20+ 91,7%
PDX#4
CD20+ 42,1%
CD20+ 47,1%
CD20+ 24,8%
CD20+ 31,2%
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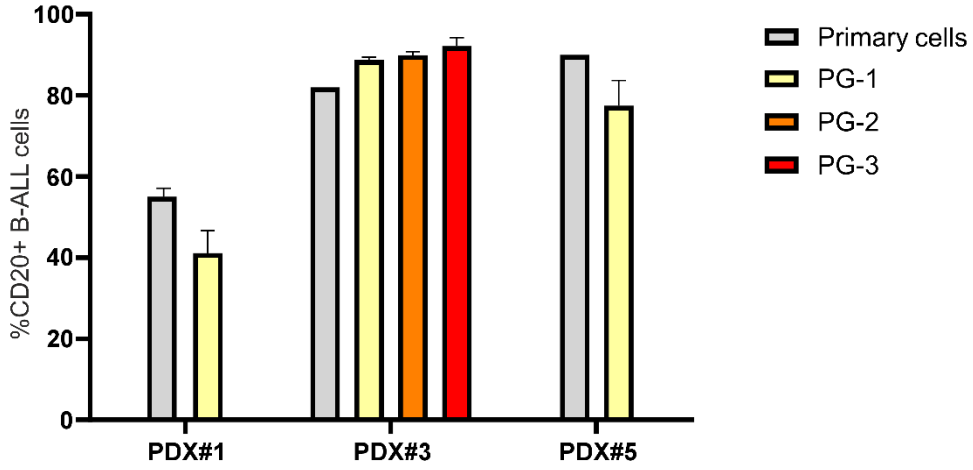
CD20+ 65,8%

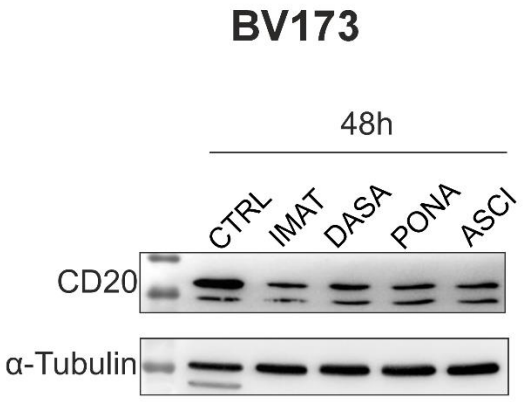
C



Supplementary figure 4. TKIs-mediated downregulation of CD20 on BCP-ALL cell lines and PDXs.

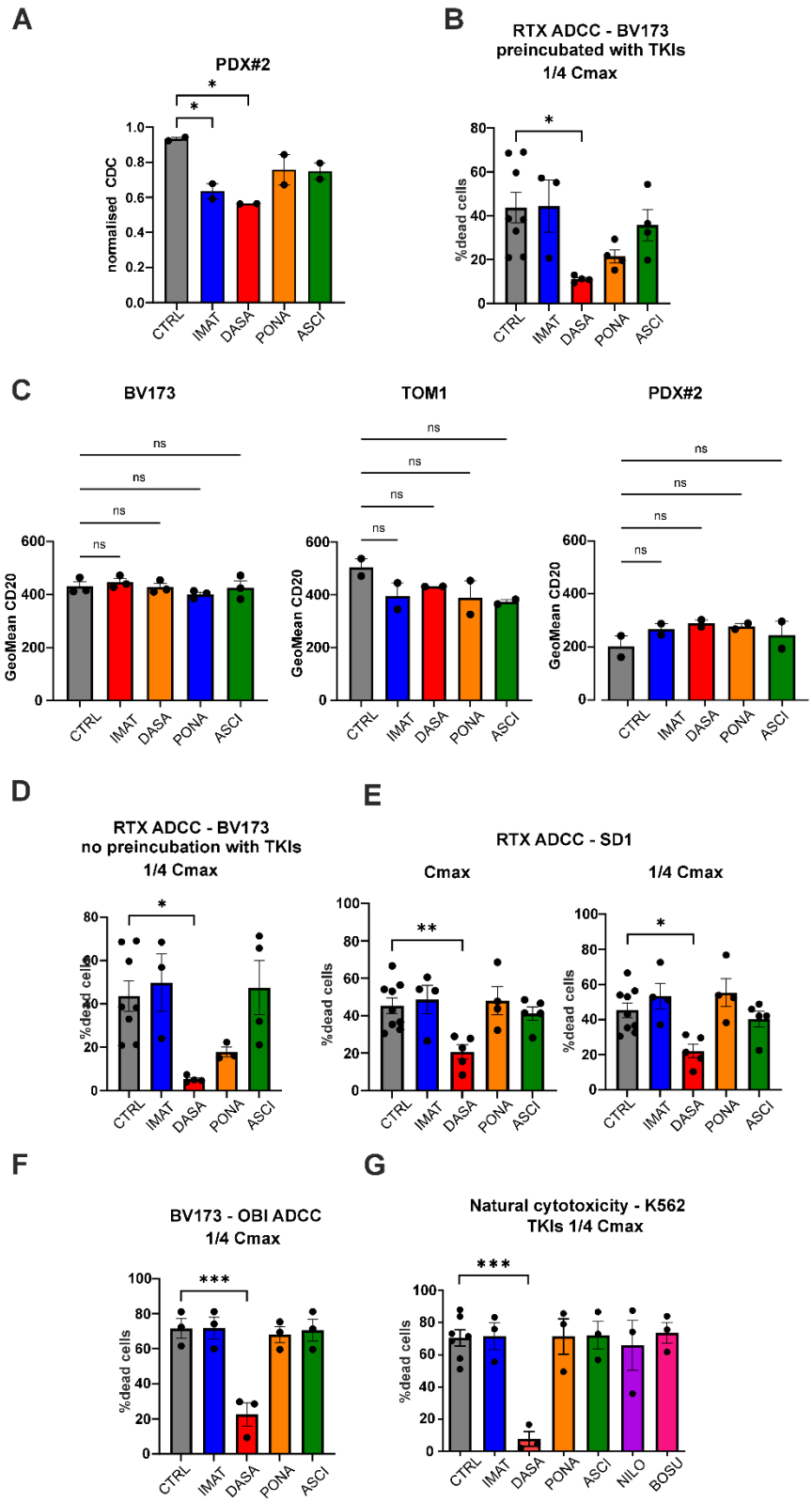
A., B. Ph^+ BCP-ALL cell lines (**A**) and PDXs (**B**) were incubated for 48 h with Cmax concentrations of TKIs *in vitro* for 48 h. After treatment, the CD20 surface protein levels on live cells were determined by flow cytometry using anti-CD20 antibody. Representative dot plots are shown.

C. Comparison of the percentage of $CD20^+$ BCP-ALL cells between primary and PDX cells. The percentage of $CD20^+$ BCP-ALL cells was determined by flow cytometry using anti-CD20 antibody. The first measurement was performed in primary cells at the time of the diagnostic assessment. The next measurements were performed after the first and subsequent generations of PDX cells in NSG mice.



Supplementary figure 5. TKI-mediated downregulation of CD20 protein.

BV173 cells were exposed to Cmax concentrations of TKIs for 48 h. The levels of CD20 protein were determined by immunoblotting. The image shows representative blots of four independent experiments.


A
PDX#2
normalised CDC
CTRL
IMAT
DASA
PONA
ASCI
B
RTX ADCC - BV173
preincubated with TKIs
1/4 Cmax
%dead cells
C
BV173
TOM1
PDX#2
GeoMean CD20
ns
D
RTX ADCC - BV173
no preincubation with TKIs
1/4 Cmax
E
RTX ADCC - SD1
Cmax
1/4 Cmax
F
BV173 - OBI ADCC
1/4 Cmax
G
Natural cytotoxicity - K562
TKIs 1/4 Cmax
NILO
BOSU

Supplementary figure 6. Effects of TKIs on CDC and ADCC mediated by anti-CD20 mAbs and the levels of CD20 in BCP-ALL cells.

A. PDX#2 BCP-ALL cells (target cells) were preincubated with Cmax concentrations of TKIs or DMSO (control) for 48 h. Next, the target cells were incubated with human serum and 20 µg/mL RTX for 1 h. Upon incubation, dead cells were stained with PI and the cytotoxicity was assessed by flow cytometry as % of PI-positive single cells. CDC was normalized to the highest technical replicate per experiment. The presented data are the means +/- SEM from two independent repeats.

B. CFSE-stained BV173 target cells were either preincubated with ¼ Cmax concentrations of TKIs or DMSO (control) for 48 h. Next, the target cells were incubated for 4 h with $CD16^+$ NK92 cells in the effector:target ratio of 2.5:1, 10 µg/mL RTX, and ¼ Cmax concentrations of TKIs or DMSO (control). Dead target cells were stained with 7AAD and assessed in flow cytometry as the percentage of CFSE and 7AAD double-positive cells. The presented data are the means +/- SEM from at least three independent repeats.

C. Ph^+ BCP-ALL cell lines and PDXs were incubated with Cmax concentrations of TKIs *in vitro* for 4 h. After treatment, the CD20 expression levels were determined by flow cytometry using an anti-CD20 antibody. The presented data are the means +/- SEM from at least two independent repeats.

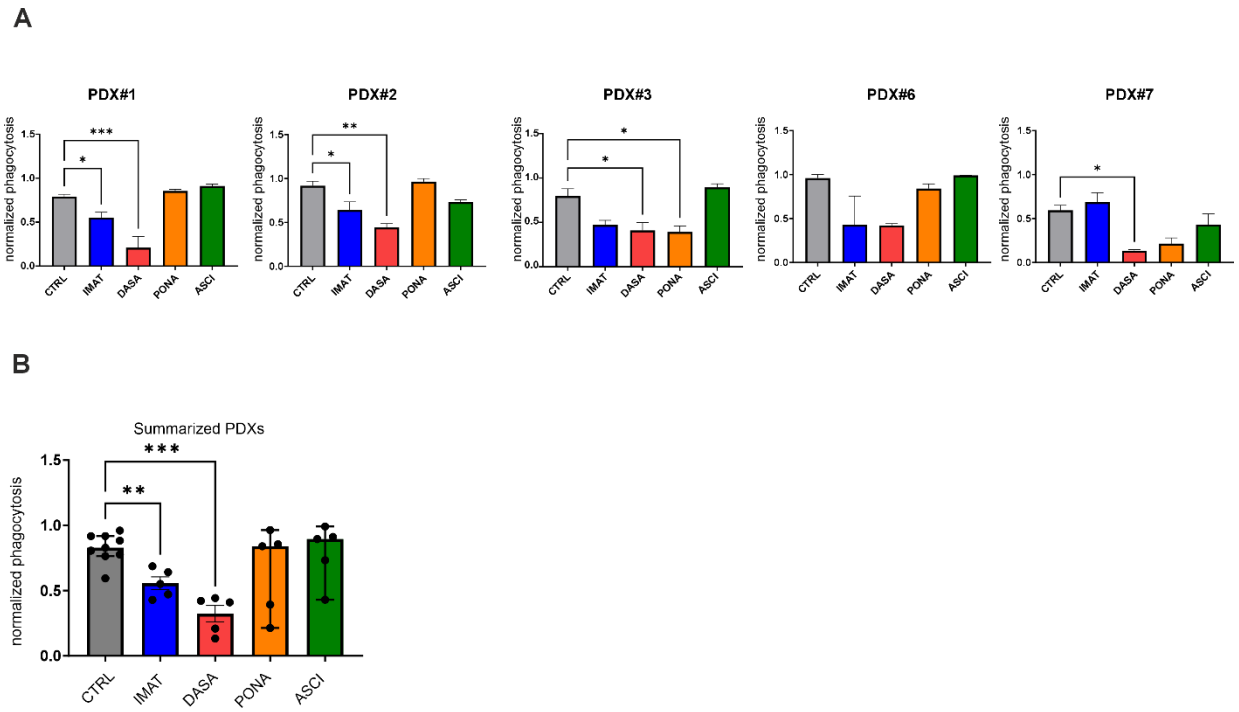
D. CFSE-stained BV173 target cells were incubated for 4 h with $CD16^+$ NK92 cells in effector:target ratio of 2.5:1, 10 µg/mL RTX and ¼ Cmax concentrations of TKIs. The presented data are the means +/- SEM of at least three independent experiments. The percentages of dead cells were assessed as described in **B.**

E. CFSE-stained SD1 target cells were incubated for 4 h with healthy donors' PBMCs in effector:target ratio of 25:1, 10 µg/mL RTX and Cmax (left panel) or ¼ Cmax (right panel) concentrations of TKIs. The presented data are the means +/- SEM of independent experiments using PBMCs from at least four donors. The percentages of dead cells were assessed as described in **B***.*

F. CFSE-stained BV173 target cells were incubated for 4 h with $CD16^+$ NK92 cells in effector:target ratio of 2.5:1, 10 µg/mL OBI and ¼ Cmax concentrations of TKIs. The presented data are the means +/- SEM of at least three independent experiments. The percentages of dead cells were assessed as described in **B.**

G. NK cells were isolated from healthy donors and incubated with ¼ Cmax TKIs and CFSE-stained K562 target cells in 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 PI double-positive cells.

The differences between groups in **A-G** were assessed by one-Way ANOVA with Dunnett's post hoc test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



Supplementary figure 7. **Effects of TKIs on RTX-mediated ADCP**.

A. Primary CD20^{+} BCP-ALL PDXs cells were stained with CFSE and opsonized with 5 µg/ml RTX. Next, the PDXs cells and primary human M1 macrophages were preincubated for 2 h with ¼ Cmax concentrations of TKIs and then co-incubated for 1 h in the presence of ¼ Cmax concentrations of TKIs. The percentage of phagocytosis was assessed by flow cytometry as the fraction of double-positive macrophages. The background phagocytosis was assessed using cetuximab. After background subtraction, phagocytosis was normalized to the highest technical replicate per donor. Differences were calculated using one-way ANOVA followed by Dunnett's multiple comparisons test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

B. Summarized results showing mean ADCP of five PDXs presented in **A** (n=5 PDXs). *P* values were calculated using Welch's ANOVA test followed by Dunnett's T3 test for multiple comparisons, **$P < 0.01$, ***$P < 0.001$.

Supplementary methods

Cell culture

Human Ph^+ BCP-ALL cell lines (SD1, BV173, TOM1, SUP-B15) were purchased from DSMZ. SD1, BV173, and SUP-B15 were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (Sigma-Aldrich). TOM1 cell line was maintained in RPMI 1640 supplemented with 20% FBS (Gibco). NK92 cell line expressing CD16 was cultured in X-Vivo 20 (Lonza) with 5% Human AB serum (Merck). BCP-ALL PDXs were incubated in StemSpan™ SFEM II medium (STEMCELL Technologies), 20% FBS (Gibco) supplemented with 20 ng/mL of recombinant IL-3 (R&D Systems), and 10 ng/mL of recombinant IL-7 (R&D Systems). Peripheral blood mononuclear cells (PBMC) were obtained by Lymphoprep™ (STEMCELL Technologies Germany GmbH) separation from buffy coats obtained from the Regional Blood Center in Warsaw. All cell lines were cultured at 37°C and 5% CO_2 and routinely tested for the presence of Mycoplasma DNA to prevent contamination.

Reagents

Chemotherapeutic drugs used for *in vitro* experiments are listed in Supplementary Table 9. L-asparaginase, methylprednisolone and imatinib mesylate were dissolved in water. All other compounds were dissolved in DMSO (Sigma Aldrich). In all experiments, control groups were treated with DMSO. Monoclonal antibodies: RTX (Riximyo, Sandoz GmbH), cetuximab (Erbitux, Eli Lilly and Company) and obinutuzumab (Gazyvaro, Roche) were a kind gift from Institute of Hematology and Transfusion Medicine.

Analysis of the *MS4A1* mRNA levels

Primary cells

Total RNA was isolated from lymphoblasts collected at diagnosis and resuspended in TRIzol solution using chloroform extraction according to the Invitrogen protocol followed by Qiamp RNAeasy mini kit (Qiagen). The concentration and purity of the nucleic acid were determined using NanoDrop Spectrophotometer-8000 (ThermoFisher Scientific). The complementary DNA (cDNA) was obtained with the use of the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). The expression level of the CD20-encoding gene *MS4A1* was assessed by q-PCR using AriaMx Real-time PCR System (Agilent). Real-time PCR for *MS4A1* (CD20) was performed according to the manufacturer's instruction using 60 ng of the cDNA, TaqMan Gene Expression Master Mix (ThermoFisher Scientific), and the TaqMan gene expression assay (Hs01096429_m1;ThermoFisher Scientific). *B2M* was used as a housekeeping gene (Hs00187842_m1;ThermoFisher Scientific). Results were analyzed with the use of the AriaMX software.

Cell lines

Total RNA was isolated from cell lines after 24 h incubation with TKIs, according to the Invitrogen protocol followed by Qiamp RNAeasy mini kit (Qiagen). The concentration and purity of the nucleic acid were determined using NanoDrop Spectrophotometer-8000 (ThermoFisher Scientific). The complementary DNA (cDNA) was obtained with the use of the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher Scientific). The levels of the *MSRA1* gene were assessed by q-PCR using LightCycler® 480 SYBR Green I Master (Roche). The reaction was performed using 12.5 ng of the cDNA. *RLP29* and *GUSB* were used as housekeeping genes. The relative expression levels of genes were calculated using LightCycler 480 Software 1.5 (Roche) as described in [2]. Primer sequences are listed in Supplementary Table 11.

Assessment of CD20 levels by flow cytometry in primary samples

Adult patients

Bone marrow or peripheral blood samples from all patients were analyzed at diagnosis and underwent a follow-up assessment (MRD) after 50-85 days. The material processing was carried out in accordance with Euro Flow guidelines. Briefly: bone marrow/peripheral blood samples were incubated with a mixture of fluorescence-labeled mAbs for 15 min at room temperature in the dark. The samples were stained with the following combination of mAbs: CD45 V500 (clone HI30), CD20 V450 (clone 2H7), CD81 FITC (clone JS-81), CD22 PE (clone S-HCL-1), CD34 Per-Cy5.5 (clone 8G12), CD19 PE-Cy7 (clone J3-119), CD10 APC (clone HI10A), CD38 APC (clone HB7) (BD Biosciences). Lysis of red blood cells was done using BD Pharm Lyse solution (BD Biosciences). Immunophenotyping at diagnosis was performed by collecting 100,000 cellular events. Leukemic cells were identified using a gate that included all $CD19^+$ cells on a CD19/SSC-A dot plot. The threshold was set according to the upper limit of the background fluorescence of lymphoid cells not expressing B-cell markers. All flow cytometric analyses were carried out on a FACS Canto II flow cytometer (BD Biosciences) at the Institute of Hematology and Transfusion Medicine in Warsaw. The instrument was aligned and calibrated daily with the use of CaliBRITE beads (BD FACSDiva CS&T IVD Beads, BD Biosciences) according to the manufacturer's instructions.

Pediatric patients

Bone marrow samples from all patients were analyzed at the time of diagnosis and underwent a follow-up assessment after 15 days of treatment (referred to as MRD15). The samples were stained with the following combination of mAbs: CD45 V500 (clone HI30), CD19 PE-Cy7 (clone J3-119), CD34 Per-Cy5.5 (clone 8G12) (BD Biosciences), and CD20-Pacific Blue (clone 2H7), (Biolegend). Bone marrow samples were incubated with a mixture of fluorescently-labeled mAbs for 15 min at room temperature in the dark. Lysis of mature red blood cells was done using BD Pharm Lyse solution (BD Bioscience). Then, the samples were washed with Cell Wash solution (BD Biosciences). Leukemic cells were identified using a gate that included all $CD19^+$ cells, with low SSC and low to dim CD45 expression. All flow cytometric analyses were carried out on a FACS Canto or FACS Lyric flow cytometer (BD Biosciences, San Jose, CA, USA) at the Department of Pediatric Hematology and Oncology, Medical University of Silesia in Zabrze.

PDXs generation

For generation of patient-derived xenografts (PDXs), primary BCP-ALL cells isolated from the bone marrow of pediatric and adult BCP-ALL patients were implanted *i.v.* to NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (The Jackson Laboratory), as previously described in [3]. All *in vivo* procedures were approved by the Local Ethics Committee of the University of Life Sciences, Warsaw, Poland and Warsaw University, Warsaw (WAW2/095/2019, 639/2018). Details of the specific PDXs are listed in Supplementary Table 7. The measurements of CD20 percentages in various PDXs and their generations are presented in Supplementary Figure 4C.

Assessment of CD20 levels after drug treatment

BCP-ALL cell lines and PDXs were incubated for 48 h in their medium with the following drugs: (IMAT - imatinib; DASA - dasatinib; PONA - ponatinib; ASCI - asciminib; ARAC - cytarabine; DEX - dexamethasone; M-PRED - methylprednisolone; DNR - daunorubicin; LASP - L-asparaginase; MTX - methotrexate; VCR - vincristine; 4-CYCL - 4-hydroperoxycyclophosphamide). The concentrations of drugs used for the immunophenotyping experiments were selected through cytotoxicity screening in order to establish EC50 doses corresponding to each cell line. The concentrations tested were not higher than Cmax concentrations according to pharmacokinetics

literature search. If a highest achievable concentration of certain drug did not reach the cytotoxic effect of EC50, Cmax concentration were used (see Suppl. Table 9). Next, the cells were rinsed in PBS/0.1% BSA, incubated for 15 minutes with human F_C block (BD Biosciences), and stained with anti-CD20 antibody at a dilution 1:20 (see Suppl. Table 10). 7-AAD (Thermo Fisher) was added before the flow cytometry analysis to exclude dead cells. The results on the heatmap are the log of the mean fluorescence (geometric mean) values normalized to control (fold over control) after the deduction of isotype control background.

Natural cytotoxicity

NK cell were isolated using EasySep™ Human NK Cell Isolation Kit Next (StemCell Technologies). K562 target cells were labeled with CellTrace™ CFSE Cell Proliferation Kit (Thermo Fisher) at a final concentration of 0.25 µM for 10 min at 37°C before the assay. Effector and target cells in the effector:target ratio of 10:1 were coincubated at 37°C for 4 h in presence of TKIs at their maximal clinically achievable concentration (Cmax) and ¼ of Cmax.

CDC assay

BCP-ALL cell lines or PDX were preincubated for 48 h with TKIs prior to the CDC assay. After 48 h, the live cells were separated from the dead using Lymphoprep™ (STEMCELL) and subjected to 10% (PDX#2) or 20% (BV173) human AB serum collected from a healthy donor and 20 µg/mL RTX for 1 h. After the incubation, the cells were stained using Propidium Iodide (PI) viability reagent and analyzed using flow cytometry. Percentages of dead cells in the samples without RTX were used as a background control.

ADCC assay

Model with target cell preincubation

BV173 cells (target cells) were stained with CFSE at a final concentration of 3.5 µM for 10 min at 37°C. Target cells were then preincubated for 48 h at 37°C with Cmax or ¼ of Cmax concentrations of TKIs. After the incubation period, live cells were separated from dead cells using Lymphoprep™ (STEMCELL) density gradient medium. Next, the cells were coincubated on a 96-well plate with NK92 cells in the effector:target ratio of 2.5:1 with or without RTX (10 µg/mL) and TKIs (Cmax, ¼ Cmax) for 4 hours. The percentage of cytotoxicity was calculated in the CFSE-positive population, using 7-AAD.

Model without target cell preincubation

BV173 targets: BV173 cells (target cells) were stained with CFSE at a final concentration of 3.5 µM for 10 min at 37°C. Next, the cells were coincubated on a 96-well plate with NK92 cells in the effector:target ratio of 2.5:1 with or without RTX (10 µg/mL) or OBI (10 µg/mL) and TKIs (Cmax, ¼ Cmax). The percentage of cytotoxicity was calculated in the CFSE-positive population using 7-AAD.

SD1 targets: Human PBMC were obtained from buffy coats as described above. SD1 cells (target cells) were labeled with CFSE (ThermoFisher) at a final concentration of 1 µM for 10 min at 37°C before the cytotoxicity assay. Target and effector cells were coincubated at 37°C for 4 h in the presence of TKIs at their maximal clinically achievable concentration (Cmax) and ¼ of Cmax, in the effector:target ratio of 25:1.

Differentiation of macrophages from peripheral blood monocytes and M1 polarization

For the generation of monocyte-derived macrophages (MDMs) from PBMC, the cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicilin/streptomycin and 1% sodium pyruvate. PBMC from healthy donors were isolated as described above. For monocyte isolation, 5×10^6 PBMCs were seeded in 100/20 mm Advanced Tissue Culture Dish (Greiner Bio-One) and allowed to adhere at 5% CO_2, 37°C for 2 h in their medium supplemented with 1% human AB serum (Sigma-Aldrich). Non-adherent cells were removed by washing the plate with medium and discarding it. Adherent cells were stored overnight at 5% CO_2, 37°C in fresh medium and subsequently supplemented with 100 ng/mL human Macrophage Colony Stimulating Factor (M-CSF)(BD Biosciences). After 48 h, the plate was washed thoroughly with medium, which was then removed and replaced with fresh medium supplemented with 100 ng/mL M-CSF. After next 48 h, the medium was removed, the cells were washed thoroughly with PBS, and covered with 5 mL of non-enzymatic cell dissociation solution Cellstripper™ (Corning) for 15 min at 37°. After dissociation and gentle scraping of the plate bottom using a sterile plunger of 1 mL BD Plastipak Syringe (BD Biosciences), cells were rinsed with PBS, counted, and seeded onto a 96-well, flat-bottom tissue culture-treated plate in their medium, supplemented with 100 ng/mL M-CSF, 10 ng/mL of IFN-γ and 2 ng/mL of LPS in the density of 100,000 cells in 100 µL per well. After 24 h of incubation at 5% CO_2, 37°C, the medium was gently removed from wells using a multi-channel pipette and replaced with fresh medium supplemented with the same concentration of differentiating factors. The cells were incubated at 5% CO_2, 37°C for an additional 24 h prior to the antibody-dependent phagocytosis (ADCP) assay.

Immunoblots

Cell extracts from BCP-ALL cell lines were lysed with RIPA buffer (CD20, α-tubulin) and immunoblotted as previously described[3]. Antibodies used are listed in Supplementary Table 10.

In vitro degranulation assay

NK cell were isolated using EasySep™ Human NK Cell Isolation Kit Next (StemCell Technologies). 100,000 NK cells and 100,000 K562 target cells were placed onto wells of 96-well plate. The cells were subsequently treated with GolgiStop™ Protein Transport Inhibitor (BD Biosciences) to inhibit the surface CD107a recycling and an anti-CD107a APC antibody was added to each well. The cells were then incubated for 4 hours in 37°C in presence of Cmax concentrations of TKIs. Samples were stained using BD Fixable Viability Stain 510 (BD Biosciences) and antibodies against CD56. The percentage of degranulation was determined within live $CD56^+$ cells, with background values subtracted. These background values were obtained from samples containing NK cells but lacking target cells.

Statistical analysis and data visualization

Statistical analyses and data visualization were performed using Prism 9.5.1 software (GraphPad Software). The type of statistical test and appropriate post-hoc corrections are described in Figure legends. *P* values were considered statistically significant when lower than 0.05 ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). The Experimental schemes of the CDC, ADCC and natural cytotoxicity assays (Figure 3), as well as ADCP assay (Figure 5A) and the whole blood *ex vivo* assay for testing the degranulation of NK cells (Figure 4B) were designed using biorender.com.

Text editing

Authors utilized GPT-3, OpenAI's large-scale language-generation model to enhance the grammatical and lexical quality of this text.

Supplementary Table 1. Drug exposure during the induction phase of the ALL-IC BFM 2009 and AEIOP BFM 2017 protocols.

ALL-IC BFM 2009 Protocol			
Drug	**Route of administration**	**Per-Day Dose**	**Days of Administration**
Induction Phase of Protocol I			
Prednisone	*p.o.*	60 mg/m^2	1-28
Vincristine	*i.v.*	1.5 mg/m^2 (max. 2 mg)	8, 15, 22, 29
Daunorubicin	*p.i.* over 1 hour	30 mg/m^2	8, 15 (22 and 29 in IR and HR patients)
L-asparaginase (L-ASP)	*p.i.* over 1 hour	5,000 IU/m^2	12, 15, 18, 21, 24, 27, 30, 33
Methotrexate	*i.t.*	Age-adjusted dose: < 1 year: 6 mg <1 -2) years: 8 mg <2 -3) years: 10 mg ≥ 3 years: 12 mg	1, 12, 33*
AIEOP- BFM 2017 Protocol			
Protocol IA-Pred.(pB-ALL patients or unknown immunophenotype), Induction Phase			
Prednisone (alternatively Prednisolone)	*p.o./i.v.*	60 mg/m^2	1-28
Vincristine	*i.v.*	1.5 mg/m^2 (max. 2 mg)	8, 15, 22, 29
Daunorubicin	*p.i.* over 1 hour	30 mg/m^2	8,15,22,29
PEG-L-ASP **	*p.i.* over 2 hours	2 500 IU/m^2 (max. 3 750 IU)	12,26
Methotrexate	*i.t.*	Age-adjusted dose: < 1 year: 6 mg <1 -2) years: 8 mg <2 -3) years: 10 mg ≥ 3 years: 12 mg	12,33***

p.i. (per infusionem) - administered through controlled continuous infusion, *p.o.* (*per os*) – administered orally, *i.v.* - administered intravenously, *i.t.* - administered by intrathecal injection

* If CNS positive, or CNS neg. but blasts in CSF, or traumatic LP: additional MTX IT on day 18/27
** In case of hypersensitivity to PEG-L-Asparaginase, Erwinia Asparaginase is given at a dosage of 20 000 IU/m2/dose p.i. (1 h) or i.m. every second day for the remaining days of scheduled Asparaginase treatment, i.e. until two weeks after the last scheduled PEG-L-Asparaginase dose in this element.
*** In case of initial CNS3 status additional intrathecal Methotrexate is given on days 19 and 26

Supplementary Table 2. Drug exposure during the induction phase of the EsPhALL2017/COGAALL1631 protocol

EsPhALL2017/COGAALL1631 Protocol			
Drug	**Route of administration**	**Per-Day Dose**	**Days of Administration**
Induction IA Part 2*			
Prednisone/Prednisolone	*p.o.*	60 mg/m^2	15-28
Vincristine	*i.v.* over 1 min	1.5 mg/m^2 (max. 2 mg)	15, 22
Daunorubicin	*i.v.* over 1-15 mins	25 mg/m^2	15, 22
Methotrexate	*i.t.*	Age-adjusted dose: <1 -2) years: 8 mg <2 -3) years: 10 mg ≥ 3 years: 12 mg	29
Imatinib	*p.o.*	340 mg/m^2 (max. 800 mg)	Daily
Induction IB			
Cyclophosphamide	*i.v.* over 30-60 mins	1000 mg/m^2	1, 28
Mercaptopurine	*p.o.*	60 mg/m^2	1-28
Cytarabine	*i.v.* over 1-39 mins or *s.c.*	75 mg/m^2	3-6, 10-13, 17-20, 24-27
Methotrexate	*i.t.*	Age-adjusted dose: <1 -2) years: 8 mg <2 -3) years: 10 mg ≥ 3 years: 12 mg	10, 24
Imatinib**	*p.o.*	340 mg/m^2 (max. 800 mg)	Daily

p.o. (*per os*) – administered orally, *i.v.* - administered intravenously, *i.t.* - administered by intrathecal injection, *s.c.* - administered subcutaneously

*Prior to BCR::ABL1 detection and qualification to EsPhALL2017/COGALL1631 protocol (10-15 days from initial diagnosis), patients were treated with induction treatment of the default front-line ALL treatment (ALL-IC BFM 2009 or AIEOP- BFM 2017).

** Patients begun imatinib once daily at the time of trial enrollment, which was no later than Day 15.

Supplementary Table 3. Drug exposure during the pretreatment and induction phases of PALG ALL6 protocol

PALG ALL6 protocol			
Drug	**Route of administration**	**Per-Day Dose**	**Days of administration**
Ph$^-$ patients			
<55 y/o			
Pretreatment			
Prednisone	*p.o.*	60 mg/m^2 (≥40 y/o – 40mg/m^2)	-7 to -1
Liposomal cytarabine	*i.t.*	50 mg	1x between -7 and -3
Induction			
Prednisone	*p.o./i.v.*	60 mg/m^2 (≥40 y/o – 40mg/m^2)	1-28
Vincristine	*i.v.*	2 mg	1, 8, 15, 22
Daunorubicin	*i.v.*	50 mg/ m^2 (≥40 y/o – 40 mg/m2)	1, 8, 15, 22
Peg-asparaginase	*i.v.*	1,000 IU/m^2	13
Liposomal cytarabine	*i.t.*	50 mg	10
≥55 y/o			
Pretreatment			
Dexamethasone	*p.o.*	10 mg/m^2	-5 to -1
Methotrexate	*i.t.*	12 mg	1x between -5 and -1
Induction			
Dexamethasone	*p.o.*	10 mg/m^2	1 to 7, 15 to 21
Vincristine	*i.v.*	2 mg	1, 8, 15, 22
Daunorubicin	*i.v.*	30 mg/m^2	1, 8, 15, 22
Peg-asparaginase	*i.v.*	1,000 IU/m^2	10
Methotrexate/Cytarabine/Dexamethasone	*i.t.*	12 mg/40 mg/4mg	12, 20, 27, 34
Ph$^+$ patients			
<55 y/o			
Pretreatment			
Prednisone	*p.o.*	60 mg/m^2 (≥40 y/o – 40mg/m^2)	-7 to -1
Liposomal cytarabine	*i.t.*	50 mg	1x between -7 and -3
Induction			
Prednisone	*p.o.*	60 mg/m^2 (≥40 y/o – 40mg/m^2)	1-28
Vincristine	*i.v.*	2 mg	1, 8, 15, 22
Daunorubicin	*i.v.*	40 mg/ m^2	1, 8, 15
Liposomal cytarabine	*i.t.*	50 mg	10
Imatinib	*p.o.*	600 mg	Daily from day 1
Dasatinib*	*p.o.*	100 – 140 mg	Daily
≥55 y/o			
Pretreatment			
Dexamethasone	*i.v.*	10 mg/m^2	-5 to -1
Methotrexate	*i.t.*	12 mg	1x between -5 and -1
Induction			
Dexamethasone	*p.o.*	10 mg/m^2	1-2, 8-11, 15-18, 22-25
Vincristine	*i.v.*	1 mg	1, 8, 15, 22
Methotrexate/Cytarabine/Dexamethasone	*i.t.*	12 mg/40 mg/4mg	12, 20, 27, 34
Imatinib	*p.o.*	600 mg	Daily from day 1
Dasatinib*	*p.o.*	100 mg	Daily

* In case of imatinib resistance or intolerance

Supplementary Table 4: Drug exposure during the pretreatment and induction phases of PALG ALL7 protocol

PALG ALL7 protocol			
Drug	**Route of administration**	**Single dose**	**Days of administration**
Ph$^-$ patients			
Pretreatment			
Dexamethasone	*p.o.*	10 mg/m^2	-5 to -1
Methotrexate/Dexamethasone	*i.t.*	15 mg/4 mg	1x between -5 and -1
Induction			
Dexamethasone	*p.o./i.v.*	40 mg (≥70 y/o – 20mg)	1-2, 8-9, 15-16, 22-23
Vincristine	*i.v.*	2 mg	1, 8, 15, 22
Daunorubicin	*i.v.*	50 mg/ m^2 (≥40 y/o – 40 mg/m2; ≥55 y/o – 30 mg/m2)	1, 8, 15, 22
Peg-asparaginase	*i.v.*	2,000 IU/m^2 (max. 3750 IU) (≥55 y/o – 1,000 IU/m^2)	20
Methotrexate/Cytarabine/Dexamethasone	*i.t.*	15 mg/40 mg/4mg	13, 27
Rituximab*	*i.v.*	375 mg/m2	1, 8
Ph$^+$ patients			
Pretreatment			
Dexamethasone	*i.v.*	12 mg/ m^2 (≥55 y/o – 10 mg/ m^2)	-5 to -1
Methotrexate/Dexamethasone	*i.t.*	15 mg/4 mg	1x between -5 and -1
Induction			
Dexamethasone	*p.o./i.v.*	40 mg (≥70 y/o – 20mg)	1-2, 8-9, 15-16, 22-23
Vincristine	*i.v.*	2 mg (≥55 y/o – 1 mg)	1, 8, 15, 22
Methotrexate/Cytarabine/Dexamethasone	*i.t.*	15 mg/40 mg/4mg	10, 24
Rituximab*	*i.v.*	375 mg/ m^2	1, 8
Imatinib	*p.o.*	600 mg	daily from day 1
Dasatinib**	*p.o.*	140 mg	daily
Ponatinib***	*p.o.*	45 mg	daily

* If CD20 is detected on ≥20% blasts
** In case of imatinib resistance or intolerance
*** In case of dasatinib resistance or detection of T315I mutation

Supplementary Table 5: Clinical characteristics of CD20-high and CD20-low patients

	CD20 high	**CD20 low**	***P value***
n	26	91	-
% of Total	22.22 %	77.78 %	
Treatment protocol			
ALL-IC 2009 (n)	3	7	-
AIEOP-BFM 2017 (n)	23	84	
Sex, n(%)			
Male	15 (57.69 %)	47 (51.65 %)	0.59 **
Female	11 (42.31 %)	44 (48.35 %)	
Age at ALL diagnosis (years)			
Median	5.23 (3.24-10.52)	5 (2.5-7.0)	0.089*
<10	18	76	
10-14	5	12	
15-18	3	3	
WBC (n/ul)			
Median	13465 (3875-36147.5)	14000 (5550-58000)	**0.038***
Unknown/ Missing	10	14	
BM blasts at diagnosis (%)			
20-39	0	0	0.16*
40-59	1	5	
60-79	1	3	
>=80	15	70	
Unknown/ Missing	9	14	
CNS involvement			
CNS1 (n)	17	68	0.50 **
CNS2 (n)	3	5	
CNS3 (n)	2	6	
Unknown/ Missing (n)	4	12	
Steroidoresistance			
Yes (n)	3	5	0.13 **
No (n)	12	63	
Unknown/ Missing (n)	11	23	
FCM-based MRD at day 15			
Positive (>10%) (n)	7	7	**0.003****
Negative (n)	12	66	
Unknown (n)	8	18	
PCR-based MRD at day 33			
Positive (n)	8	32	0.97**
Low, but positive (<10^-4)	7	25	
Negative (n)	5	21	
Unknown/ Failed (n)	6	9/4	
Risk group			
SR (n)	2	16	0.36**
MR (n)	6	33	
HR (n)	6	16	
Unknown/ Missing (n)	12	26	
Complete Remission Achieved			
Yes	15	66	0.52**
No	1	2	

Unknown/ Missing	10	23	
Death			
n	4	3	**0.02****
% of total	15.38 %	3.30 %	
Relapse			
n	4	10	0.54**
% of total	15.38 %	10.99 %	

*U-Mann Whitney test, **-Chi-Square test

Supplementary Table 6: Clinical characteristics of adult BCP-ALL patients

All adult BCP-ALL patients enrolled (n)	**72**
Sex (n)	
Male	32
Female	40
Age at ALL diagnosis (n)	
≥55	26
<55	46
Median (years)	46,5
BCR-ABL status (n)	
Ph^+	26
Ph^-	46
CD20-positivity (n)	
$CD20^+$	31
$CD20^-$	41
CNS involvement (n)	
Yes	13
No	39
Unknown/Missing	20
CR after induction (n)	
Yes	38
No	7
Unknown/Missing	27

CNS – Central Nervous System; CR – Complete Remission

Supplementary Table 7: Characteristics of patients used for generation of PDXs. Samples were collected at the time of diagnosis.

Sample ID	**BCP-ALL subtype**	**Risk group**	**Age group**	**Sex**
PDX#1	Ph^+	HR	adult	M
PDX#2	Ph^+	HR	pediatric	M
PDX#3	Ph^+ CML-LBC	HR	adult	F
PDX#4	Ph^+	HR	pediatric	M
PDX#5	Ph^+	HR	adult	M
PDX#6	Ph^+	HR	pediatric	M
PDX#7	Ph-like	HR	pediatric	M

CML LBC – Chronic Myeloid Leukemia Lymphoblastic Crisis

Supplementary Table 8: Characteristics of patients used for *ex vivo* experiments presented in Fig. 4B.

Sample ID	**Diagnosis**	**Treatment**	**Sex**
Ex vivo patient #1	BCP-ALL	DASA 140 mg QD *p.o.*	F
Ex vivo patient #2	CML	DASA 140 mg QD *p.o.*	M
Ex vivo patient #3	CML	ASCI 40 mg BID *p.o.*	M
Ex vivo patient #4	CML	DASA 100 mg QD *p.o.*	F
Ex vivo patient #5	CML	DASA 100 mg QD *p.o.*	M
Ex vivo patient #6	CML	ASCI 40 mg BID *p.o.*	M
Ex vivo patient #7	CML	IMAT 400 mg QD *p.o.*	M
Ex vivo patient #8	CML	IMAT 400 mg QD *p.o.*	F

BCP-ALL – B-Cell Precursor Acute Lymphoblastic Leukemia; CML – Chronic Myeloid Leukemia; QD (*quaque die*) – once a day; *p.o.* (*per os*) – administered orally; BID (*bis in die*) – twice a day

Supplementary Table 9. Selected drugs and concentrations for testing CD20 level changes in BCP-ALL cell lines and functional assays.

Drug	**Abbrev.**	**Concentration [nM]**				**Supplier**
		BV173	**SUP-B15**	**SD1**	**TOM1**	
cytarabine	ARAC	6.25	15	400	500	Selleckchem
dexamethasone sodium phosphate	DEX	200	150	200	200	Abcam
daunorubicin Hcl	DNR	5	10	10	5	Selleckchem
methotrexate	MTX	5	15	5	10000	Sigma Aldrich
vincristine	VCR	0.5	1	0.4	1	Selleckchem
4-hydroperoxy-cyclophosphamide	4-CYCL	120	120	120	1500	Niomech
imatinib mesylate	IMAT (Cmax)	8000	8000	8000	8000	Santa Cruz Biotechnology
	IMAT (¼ Cmax)	2000	2000	2000	2000	
dasatinib	DASA (Cmax)	160	160	160	160	Sigma Aldrich
	DASA (¼ Cmax)	40	40	40	40	
ponatinib	PONA (Cmax)	140	140	140	140	Selleckchem
	PONA (¼ Cmax)	35	35	35	35	
asciminib	ASCI (Cmax)	1600	1600	1600	1600	Selleckchem
	ASCI (¼ Cmax)	400	400	400	400	
Drug	**Abbrev.**	**Concentration [nM]**				**Supplier**
		BV173	**SUP-B15**	**SD1**	**TOM1**	
L-asparaginase	L-ASP	1	0.1	1	10	Servier IP UK Ltd
Drug	**Abbrev.**	**Concentration [nM]**				**Supplier**
		BV173	**SUP-B15**	**SD1**	**TOM1**	
methylprednisolone	M-PRED	50	50	50	50	Pfizer

Supplementary Table 10. Antibodies used in flow cytometry and immunoblotting

Antibody	**Clone**	**Catalog no.**	**Company**
Flow cytometry antibodies			
anti-hCD20 PE	L27	345793	BD Biosciences
anti-hCD3 BV421	UCHT1	562427	BD Biosciences
anti-hCD3 BV510	SK7	740202	BD Biosciences
anti-hCD107a APC	H4A3	641581	BD Biosciences
Western Blot antibodies			
anti-CD20	polyclonal	ab27093	abcam
	EP459Y	ab78237	abcam
anti-α-Tubulin	DM1A	T6199	Sigma-Aldrich

Supplementary Table 11. List of primers used for RT-PCR

Primers	Sequence
MS4A1_F	GAATGGGCTCTTCCACATTGCC
MS4A1_R	TCTCCGTTGCTGCCAGGAGT
RPL_F	CAGCTCAGGCTCCCAAAC
RPL_R	GCACCAGTCCTTCTGTCCTC
GUSB_F	GAAAATATGTGGTTGGAGAGCTCATT
GUSB_R	CGGAGTGAAGATCCCCTTTTTA

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