Immunotherapy-resistant acute lymphoblastic leukemia cells exhibit reduced CD19 and CD22 expression and BTK pathway dependency

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J Clin Invest. 2024. https://doi.org/10.1172/JCI175199.

Graphical abstract

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Key Words: CD19, CD22, Leukemia, CAR-T Therapy, BTK

Conflicts of Interest: AV: received research funding from Prelude, BMS, GSK, Incyte, Medpacto, Curis and Eli Lilly and is a scientific advisor for Stelexis, Novartis, Acceleron and Celgene and receives honoraria from Stelexis and Janssen and holds equity in Stelexis and Throws Exception.
NNS: supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research and the Warren Grant Magnuson Clinical Center (ZIA BC 011823, N. Shah), receives research funding from Lentigen, VOR Bio and CARGO therapeutics and has participated in Advisory Boards (no honoraria) for Sobi, Allogene, invoX and VOR. JDB: Receives research funding from Merck, Genentech, AstraZeneca, Celldex, Seagen ADC Therapeutics, Epizyme. US: received research funding from GlaxoSmithKline, Bayer Healthcare,
Aileron Therapeutics, Novartis, has received compensation for consultancy services and for serving on scientific advisory boards from GlaxoSmithKline, Bayer Healthcare, Novartis, Celgene, Aileron Therapeutics, Stelexis Therapeutics, Pieris Pharmaceuticals, Trillium Therapeutics, Vor Biopharma, Roche, Pfizer, and has equity ownership in and is serving on the board of directors of Stelexis Therapeutics. **AS:** received research funding from Kymera Therapeutics, advisory board fees from Gilead Sciences, Rigel Pharmaceuticals and Kymera Therapeutics, consultancy fees from Janssen Pharmaceuticals and honoraria from NACE & PeerView. **SKB:** received consulting honoraria from Acrotech, Affimed, Kyowa Kirin, Seagen and is on the data safety monitoring board for Janssen. **SP:** received research funding from Amgen, Celgene/BMS Corporation, and Caribou and sits on the advisory board of Grail.
Abstract

While therapies targeting CD19 by antibodies, CAR-T cells and T cell engagers have improved the response rates in B-cell malignancies; the emergence of resistant cell populations with low CD19 expression can lead to relapsed disease. We developed an in vitro model of adaptive resistance facilitated by chronic exposure of leukemia cells to a CD19-immunotoxin. Single-cell (sc) RNAseq showed increase in transcriptionally distinct CD19\textsuperscript{low} populations in resistant cells. Mass cytometry demonstrated that CD22 was also decreased in these CD19\textsuperscript{low} resistant cells. ATAC-seq showed decreased chromatin accessibility at promoters of both CD19 and CD22 in the resistant cell populations. Combined loss of both CD19 and CD22 antigens was validated in samples from pediatric and young adult patients with B-ALL that relapsed after CD19 CAR-T targeted therapy. Functionally, resistant cells were characterized by slower growth and lower basal levels of MEK activation. CD19\textsuperscript{low} resistant cells exhibited preserved B cell receptor signaling and were more sensitive to both BTK and MEK inhibition. These data demonstrate that resistance to CD19 immunotherapies can result in decreased expression of both CD19 and CD22 and can result in dependency on BTK pathways.
Introduction

While much progress has been made in the treatment of B-cell acute lymphoblastic leukemia (B-ALL), relapsed and refractory disease is an ongoing problem. The development of CD19 CAR-T cell therapy and other CD19 immunotherapies has dramatically improved the response rates of B-ALL (1). Unfortunately, the emergence of resistant cell populations with low expression of CD19 account for approximately 30-40% of patients with relapsed ALL after CAR-T treatment (1-3). Loss of surface CD19 on malignant cells leads to reduced efficacy from CD19 targeted CAR-Ts as well as bispecific antibodies (4, 5). Understanding how resistance occurs as well as the cellular signaling that takes place in the resistant populations is critical in targeting relapsed disease after CAR-T treatment.

A potential strategy to treat CD19 resistant B-ALL patients is to evaluate therapeutics targeting the CD22 B-cell antigen (6, 7). Whether these two surface receptors are co-regulated in malignant cells has not yet been fully elucidated. Another potential therapeutic target in B-cell malignancies is the B-cell receptor (BCR) pathway. Bruton tyrosine kinase (BTK) is associated with BCR and BTK inhibitors have been approved for use in CLL and Mantle cell lymphomas but have not been rigorously evaluated in resistant B-ALL (8). While CD19 and BCR independently regulate proliferative signaling pathways, downregulation of each one has been shown to upregulate or maintain expression of the other (9, 10). We developed an in vitro model of adaptive resistance to CD19 immunotherapies and then used it to determine whether BTK signaling would be preserved in CD19 resistant B-ALL cells. We show that inhibiting this pathway could be an avenue for CD19-targeted immunotherapy resistant cell populations.
Results

Long term exposure to CD19 immunotoxin leads to development of resistant B-ALL cell lines

To generate B-ALL cell lines resistant to αCD19 based immunotherapy, parental cells of NALM-6 and REH were grown in the presence of HD37-dgRTA immunotoxin (11) at increasing concentrations over 30 days (Fig 1A). The immunotoxin is an anti-CD19 HD37 antibody clone that is conjugated to recombinant ricin B chain dgRTA (11). Once the resistant cells were generated, they were compared to the parental cells of their respective cell line for sensitivity against the CD19 immunotoxin (Fig 1B and C). The resistant NALM-6 and REH cell lines had a significantly increased IC50 when treated with the CD19 immunotoxin compared to the parental cells (Fig 1B,C). Cell growth was also observed under basal conditions, where resistant cells had a slower growth rate than parental cells (Fig 1D).

B-ALL cells resistant to CD19 immunotherapy have reduced CD19 expression and a distinct transcriptomic profile

Single cell RNA sequencing was performed to compare expression differences between NALM-6 resistant and parental cell lines (Fig 2A). A total of 6,611 NALM-6 parental and 4,606 NALM-6 resistant cells were analyzed and revealed that the resistant and parental cells form distinct clusters that can be further separated into CD19 high and low populations based on transcriptomic profiles (Fig 2B). We observed that parental cells had a relatively smaller population of CD19low expressing cells, that were found to be expanded in the resistant cells. Interestingly, NALM-6 resistant cell types also showed decreased CD22 expression when compared to parental (Fig 2C). To characterize global gene expression differences in resistant
NALM-6 cells, differentially expressed genes in dominant populations of parental and resistant cells were analyzed (Fig 2D). Notably, the expression of CD19 and CD22 was found to be significantly decreased in the resistant population. Genes regulating CD19 were also examined and revealed an increase in SOX4 as well as a decrease in CTNNBL1 and CD81, which are CD19 activators in the resistant cells (12). When analyzing the pathways in resistant cells, we observed that the BCR network was predicted to significantly affected by transcriptomic changes (Fig 2E). Importantly, BTK expression was maintained in resistant CD19low cells. Overall, gene expression changes significantly affected T and B lymphatic pathways as well as hematological cancer networks (Supplementary Fig 2, Supplementary Table 2).

Finally, chromatin accessibility and correlation to gene expression was determined in REH parental and resistant cells using ATACseq and bulk RNAseq. We observed that chromatin accessibility at both CD19 and CD22 promoter regions was decreased in resistant cells and correlated with reduced gene expression (Fig 2F). Chromatin accessibility and expression for BTK were maintained in the REH resistant cells (Fig 2F). When overall chromatin accessibility changes were analyzed via ingenuity pathway analysis, significant changes were observed in gene networks related to cancer (Supplementary Table 3).

**CD19 and CD22 protein expression are both decreased in resistant cell lines as well as clinical relapses after CD19 targeted therapy**

To determine CD19 and CD22 protein expression, FACS analysis was performed on both NALM-6 and REH cells (Fig 3A,B). Both NALM-6 and REH parental cells expressed high levels of CD19 and
CD22 that was found to be decreased in the resistant cells. After observing decreased expression of both CD19 and CD22 in our model of adaptive resistance, we wanted to examine their expression in pediatric ALL patients that were treated across a host of CD19 targeted therapies and relapsed with CD19 negative/dim disease and were screened for enrollment or treated on a trial at the NCI. We examined the status of both CD19 and CD22 in a cohort of 11 patients that relapsed after CD19 targeted therapy (9 received CD19-targeted CAR T-cells [2 in the context of a CD19/22 combinatorial CAR T-cell construct (13), ClinicalTrials.gov Identifiers: NCT01593696 and NCT03448393] and 2 received blinatumomab). Six of these patients have been previously partially described (6). In this cohort we observed that in addition to loss/diminution of CD19 expression (Fig 3C), downregulation of CD22 expression occurred in all patients upon relapse or non-response (Fig 3D).

**BTK expression is preserved in resistant CD19\textsuperscript{low} cells**

When comparing single cell expression profiles of NALM-6 parental and resistant cells, BTK expression was not downregulated in resistant cells (Fig 4A,B). To validate these trends with proteomics, mass cytometry (CyTOF) was used to determine the levels of CD19 and other B-cell signaling proteins in NALM-6 parental and resistant cells (Fig 4C and D). Consistent with the earlier transcriptional data, CD19 protein levels are reduced in resistant cells. B-cell receptor associated pPLC\textgammad and pCREB expression were also examined and demonstrated only a slight decrease in pPLC\textgammad expression in resistant cells while pCREB expression was maintained (Fig 4E-G). Finally, we obtained pre- and post- samples from a patient with B-cell lymphoma that was treated with CD19 CAR-T and relapsed after a response. Comparative immunohistochemical
examination of B-cell lymphoma samples at baseline and at relapse revealed a decrease in CD19 staining intensity in resistant cells with increase in BTK intensity (Fig 4H). These data taken together suggest that while CD19 is decreased in resistant cases, B-cell receptor associated BTK expression is preserved.

**B-cell receptor (BCR) dependence in αCD19 resistant B-ALL cells**

Since CD19 and BCR/BTK signaling are both proliferative pathways for B-cell malignancies, we next wanted to determine whether BCR signaling is playing a functional role in CD19 resistant B-ALL. Malignant B cells (Raji) with and without CRISPR aided CD19 knockout were used and loss of CD19 was confirmed by immunoblotting (Fig 5A). Cells with CD19 loss displayed activated/phosphorylated ERK MAPK, though the level of activation was reduced compared to CD19 WT cells (Fig 5C). Raji CD19 KO cells also showed loss of both CD19 and CD22 cell surface expression when compared to WT cells (Supplementary Fig 1). BTK inhibitor, Ibrutinib, led to decrease in activated/phosphorylated ERK in both parenteral and resistant cells, demonstrating a functioning BTK to be upstream of proliferative MAPK signaling. MEK inhibitor (Trametinib) was used as a positive control. Immunoblots with NALM-6 parental and resistant cells were similar to the results seen in the Raji KO cells and demonstrated reduced CD19 and a functional BTK in resistant cells (Fig 5B, D). Notably, NALM-6 resistant cells had an increased phospho-BTK/total BTK ratio when compared to parental cells (Fig 5D). Next, NALM-6 cells were then treated with MEK and BTK inhibitors and IC_{50}s were compared between parental and resistant cells (Fig 5E,F). We observed that the resistant cells were more sensitive to each inhibitor than the parental cells. The same trend was observed in REH parental and resistant cells as well as Raji WT and CD19 KO
cells treated with the same MEK and BTK inhibitors (Supplementary Fig 3). We also observed that combination treatment of the immunotoxin and BTK inhibitor on NALM-6 parental cells led to additive effects on reduced cell viability (Supplementary Fig 4).

To confirm that the MEK pathway is a downstream survival pathway in CD19 resistant patient cells, we evaluated cells obtained from resistant B-ALL patients. One patient was resistant to the HD37-dgRTA CD19 immunotoxin (ClinicalTrials.gov Identifier: NCT00450944) (Fig 5G) and the other was resistant to Blinatumomab (Fig 5H), a BiTE antibody that targets CD19 expressing cells with cytotoxic T-cells (14). The resistant cells were treated with MEK inhibitor Trametinib and Methotrexate; both cells were insensitive to Methotrexate while retaining sensitivity to MEK inhibition.

Based on our results, we propose that both CD19 and BCR are working to promote leukemic cell proliferation via MEK activation in parental cells. When CD19 expression is lost, BCR is working alone to sustain proliferation. This results in slower leukemic cell proliferation and increased relative sensitivity to BTK and ERK inhibition (Fig 5I).
Discussion

Resistance to CD19 targeting therapies is emerging as major clinical challenge in patients with B-ALL. A sizable number of relapses occur upon downregulation of the CD19 antigen, thus reducing the efficacy of CD19 targeting CART therapies, immunotoxins and bispecific antibodies. Consideration of other avenues of treatment is necessary for these patients; specifically, targeting growth pathways that are functionally active in CD19 resistant populations. To explore such pathways, we developed an in vitro model of adaptive resistance based on chronic exposure to a CD19 immunotoxin. We observed that even in B-ALL cell lines, there exists a small pool of CD19\textsuperscript{low} cells, that expands in resistant cells and proliferates only when CD19 is targeted. Additionally, these cells have reduced expression of previously defined CD19 activator genes (12) including \textit{CD81} and \textit{CTNNBL1} and elevated expression of known inhibitors of CD19 including \textit{SOX4}. Furthermore, chromatin accessibility at the CD19 promoter is reduced upon resistance, pointing to transcriptional downregulation of CD19 expression that causes adaptive resistance to CD19 targeting immunotoxin.

We observed that BTK expression was maintained in resistant cells, and is functionally relevant in activating downstream proliferative MEK pathways. CD19 and BCR have parallel pathways that promote cell proliferation however, these two receptors have been previously shown to affect each other’s signaling pathways (15). Specifically in mantle cell lymphoma cells, ROR1/CD19 complex was shown to effectively replace BCR-BTK signaling and promote cell proliferation (16). Alternatively, BCR signaling was shown to be enhanced in CD19 deficient primary B-cells (17). The relationship between CD19 and BCR has not yet been established in CD19 resistant B-ALL. Here
we demonstrate that BCR signaling remains intact in CD19 low populations and exploiting that pathway through BTK or MEK inhibition effectively inhibits growth of CD19 resistant cells.

Interestingly, our data showed that CD22 expression decreases along with CD19 expression in resistant cells. Both CD19 and CD22 are B cell associated antigens that emerge during pre-B cell development. It is possible that selective pressure of the CD19 immunotoxin leads to adaptive emergence of developmentally earlier stage of cells that lack both antigens. In fact, patients that have been shown to lose CD19 during resistance have also been shown to be positive for stem/progenitor CD34 and CD123 markers, suggesting regression to a developmentally earlier stem-like state (18). Strikingly, this reduction of CD22 in addition to CD19 was observed in set of pediatric patient samples that were resistant to CD19-targeted immunotherapies. While some subsets may have preservation of CD22 expression (19), our data along with others reporting on patient cohorts where downregulation of CD22 (6, 20) with emergence of CD19 low/negative populations is seen, is of translational importance. Collectively, while CD22 immunotherapies as well as bi-specific CAR-T therapies, targeting both CD19 and CD22 have been used in an attempt prevent or treat resistant populations, (21) the concurrent down regulation of CD22 may make CD22 targeting challenging in these cases.

Taken together, we have developed a novel in vitro model of resistance that can be used to determine transcriptional and signaling alterations that occur during this process. Our data suggests that inhibition of the BCR and MEK pathways in CD19 resistant B-ALL patients could be a therapeutic advantageous avenue for treatment and supports further testing in clinical trials.
Methods

Cell Lines and Human Samples

NALM6 and REH parental cell lines were purchased from the American Type Culture Collection and were cultured according to the manufacturer’s instruction in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin. Raji WT and CD19 KO (CRISPR/Cas9) cell lines were generated at Mount Sinai Icahn School of Medicine. All cell lines were tested for mycoplasma with a mycoplasma detection kit (InvivoGen). NALM-6 and REH parental cells were exposed to HD37-dgRTA anti-CD19 immunotoxin (11) at IC$_{10}$ (5x10^{-13}M) for 7 days. Dose was escalated according to Fig 1A for a total of 30 days of treatment. Once resistant cells were established and in culture, they were treated every 2-3 days with immunotoxin at the IC$_{75}$ dose to maintain resistance. Patient samples that were used for this study were obtained with written informed consent under approval of the Albert Einstein College of Medicine IRB.

Viability Assays

10,000 cells were plated in triplicate in 100µl of media (immunotoxin was included in media for resistant cell lines) for 72 hours. CellTiter-Blue reagent (Promega) was added to each well as per manufacturer’s instruction (20µl reagent /100µl media). Cells were incubated at 37°C for 1 hour and then read by FLUOstar Omega plate reader (BMG LABTECH). Statistical analysis of viability assays will be performed using GraphPad Prism software as nonlinear regression inhibitor concentration vs. normalized response on a variable slope. Inhibitory concentration values within a 95% confidence interval will be generated as well as standard deviation.
**Single-cell RNA Sequencing**

ScRNA sequencing was performed at Mount Sinai Icahn School of Medicine. NALM-6 Parental (NALM6) and NALM-6 Resistant (NALM6-R) cell lines were sequenced on the 10xGenomics chromium platform using 3’ v2 chemistry and the raw outputs generated by running the 10x Genomics Cell Ranger software. These outputs were merged, also using the Cell Ranger Software, to allow for joint analysis of the cell lines using the 10x Genomics Loupe browser software. Total cells analyzed were 11,217. Additional bioinformatic analysis was performed using R software.

**Bulk RNA Sequencing**

Total RNA was isolated using Qiagen RNeasy kit and quality was analyzed via Agilent 2100 Bioanalyzer. Libraries were generated and read by Illumina Hiseq 2000 as previously described in Bhattacharyya et al. (22). Sequencing was performed by Epigenomics Shared Facility at the Albert Einstein College of Medicine Center for Epigenomics.

**ATAC Sequencing**

A suspension of 50,000 cells from REH parental and REH resistant cell lines were harvested and resuspended in cold lysis buffer. Lysates were incubated with transposon reaction mixture at 37°C. ATAC-seq was performed as previously described in Bhattacharyya et al. (22). Sequencing was performed by Epigenomics Shared Facility at the Albert Einstein College of Medicine Center for Epigenomics.
**Flow Cytometry**

Flow cytometry on cell lines was performed using the Flow Cytometry Core at the Albert Einstein College of Medicine. Analysis was performed using the following antibodies: CD19 (MHCD1928; Thermo Scientific), CD22 (302506; Biolegend), Zombie NIR (423105; BioLegend) and IgG1 (25471480; Thermo Scientific).

Flow cytometric analysis was uniformly performed on all patient specimens by the NCI Flow Cytometry team. Each patient had at least 2 assessment timepoints available for analysis. NCI specimens were processed within 12 hours of collection and stained with a panel of antibodies as previously described. Red cell lysis of whole blood lysis was performed using ammonium chloride prior to staining for 30 minutes at room temperature with a panel (Table S1) of antibodies (antibody concentration according to manufacturer’s recommendations). At least 1 million cells were acquired per tube using an 8-color multiparametric approach on a 3-laser FACS Canto II (BD Biosciences, San Jose, CA) with DiVa 6.1.1 software and analyzed by FCS Express software (DeNovo Software, Los Angeles, CA). B-ALL cells were identified and distinguished from both mature B-cells and precursor B-cells/hematogones based upon expression patterns of multiple antigens that included, but were not limited to, CD19, CD10, CD20, CD34, CD38, CD45, CD22 and CD24. We utilized a previously published flow cytometry strategy specifically designed for disease detection in patients who have received CD19-targeted therapy. Inherently CD19 negative cells within the specimen, including T-cells and monocytes, were used as an internal negative control (see below).
Quantification of CD19 and CD22 Expression in Patient Samples

The antibody bound per cell (ABC) was determined as previously described (23) for anti-CD19 (clone SJ25C1) and anti-CD22 (clone S-HCL-1) (BD Biosciences, San Jose, CA) on leukemic blasts. This was done using saturating concentrations of antibody and the BD Biosciences QuantiBRITE system (QuantiBRITE standard beads and QuantiCALC software) for fluorescence quantitation. The ABC value represents the mean value of the maximum capacity of each cell to bind the antibody. QuantiBRITE PE beads are pre-calibrated standard beads containing known numbers of PE molecules bound per bead. QuantiBRITE beads were acquired on a FACSCantoTMII (BD Biosciences, San Jose, CA) on the same day at the same instrument settings as the individual patient specimens. A standard curve comparing the geometric mean of fluorescence to known PE content of the Quanti-BRITE beads was constructed using QuantiCALC software. The regression analysis, slope, intercept, and correlation coefficient were determined. By gating based upon immunophenotype using other antibodies in the panel specific for the abnormal lymphoblasts, the data from abnormal B-lymphoblasts was isolated and the geometric mean fluorescence of CD19 and CD22 staining determined. The ABC values were generated from the measured geometric mean fluorescence of the gated cells using the QuantiBRITE standard curve. T-cells served as internal negative controls for both CD19 and CD22 staining. The negative ABC range was used to confirm positivity versus negativity; blasts with CD19 or CD22 staining less than or equal to that of T cells were considered negative for CD19 or CD22 (25).
**Immunoblotting**

Total protein lysates were obtained from 1-2 million cells by lysing the samples (1% NP-40 lysis buffer 20 mmol/l Tris-HCl, pH 7.5; 1 mmol/l EDTA; 150 mmol/l NaCl; (1% NP-40), containing phosphatase inhibitor cocktails 2 and 3 and protease inhibitors (Sigma-Aldrich)) for 30–45 min at 4°C. Cells in treated conditions were either treated with Ibrutinib (Selleck Chemicals) or Trametinib (Selleck Chemicals) for 1 hour. Equal amount of protein was prepared by calculating protein concentration using Bradford reagent (Bio-Rad) and 40 μg of protein was resolved on 10–12% SDS-PAGE followed by transferring to PVDF membrane (EMD-Millipore). Western blot analysis was performed with the following antibodies: ERK (4695; Cell Signaling Technology), phospho-ERK (4377; Cell Signaling Technology), BTK (8547S; Cell Signaling Technology), phosphor-BTK (87141S; Cell Signaling Technology), CD19 (90176S; Cell Signaling Technology), and b-actin (sc1615 HRP; Santa-Cruz Biotechnology). Ratio analysis was done using BioRad Image Lab software.

**Mass Cytometry (CyTOF)**

Antibodies were either purchased pre-conjugated from Fluidigm or purchased, purified, and conjugated in-house using MaxPar X8 Polymer Kits (Fluidigm) according to the manufacturer’s instructions (Supplementary Table 1). Cells were washed with cell staining buffer (PBS with 0.2% BSA and 0.02% NaN3), labeled with Rh103 intercalator (Fluidigm) as a viability dye and stained with cell surface antibodies for 30 minutes on ice. Then cells were fixed with 1.6% formaldehyde for 10 minutes and permeabilized with ice-cold methanol and further stained with antibodies against intracellular phosphoprotein targets for 30 minutes on ice. The CyTOF antibodies are
listed in Supplementary Table. The samples were then washed and incubated in 0.125 nM iridium intercalator (Fluidigm) diluted in PBS containing 2% formaldehyde for 30 minutes and stored at 4°C until acquisition. Immediately prior to acquisition, samples were washed once with PBS, once with deionized water, and then resuspended at a concentration of 1 million cells per milliliter in deionized water containing a 1/20 dilution of EQ 4 Element Beads (Fluidigm). The samples were acquired on a CyTOF (Fluidigm) equipped with a SuperSampler fluidics system (Victorian Airships) at an event rate of <500 events per second. After acquisition, the data were normalized using the bead-based normalization algorithm in the CyTOF software (Fluidigm). Bar codes were deconvoluted using the Fluidigm de-bar-coding software. The data were gated to exclude residual normalization beads, debris, dead cells, and doublets for subsequent clustering and high dimensional analyses. Normalized and de-barcoded data were uploaded to Cytobank(26) for final analysis.

**Statistics**

Statistical analyses were performed using GraphPad Prism 8.4.3 for macOS. Data represent mean ± standard deviation. Significance was defined as P value < 0.05.

**Study Approval**

Human samples were obtained from Albert Einstein College of Medicine (2005-536) and the NCI. All patients or guardians gave informed consent for study participation and/or sample collection. Pediatric patient samples were collected as part of the NCI clinical trials NCT01593696 and NCT03448393.
**Sex as a Biological Variable**

Both male and female patients were included in the study, however, sex was not considered as a biological variable.

**Data Availability**

Sequences from scRNA-seq experiments have been deposited in the NCBI’s BioProject database (PRJNA1073528). ATAC-seq and RNA-seq sequences have been deposited into NCBI’s Gene Expression Omnibus database (GEO GSE255305 and GSE255306 respectively). All supporting data of the figures and supplementary data are provided in the Supporting Data Values file.
**Author Contributions**

SA: Writing manuscript, designing and conducting experiments and data analyses, revisions.

OG, VP, CP, BY, HW, SS, SGM, VL, CS, SA, MS: designing and conducting experiments and data analyses

DM, SP: designing and conducting single cell experiments

AS, YW, BA, SKB, MJ, MG, IM, AS, WM, US: designing and conducting experiments with primary samples, providing patient samples

KP: Bioinformatic analysis

JB, NS, AV: Writing manuscript, analyzing data, providing reagents

**Acknowledgements**

We thank the Albert Einstein College of Medicine Flow Cytometry and Epigenomics Core Facilities.
References


Figure 1: Long term exposure to CD19 immunotoxin leads to resistant ALL cell lines.
A: Schema for generation of ALL cells resistant to CD19 immunotoxin (IT, HD37 antibody clone conjugated to recombinant ricin B chain dgRTA), created with BioRender.com. B,C: NALM-6 and REH cell lines that were made resistant have a significantly higher IC50 against CD19-Immunotoxin (n=4). D: Resistant cell lines have a slower growth rate (n=3). Student’s T-test was used for analysis at each time point.
Figure 2: ALL cells resistant to CD19 immunotherapy have reduced CD19 and CD22 expression and distinct transcriptomic profile.

A: scRNASeq shows parental and resistant lines are transcriptionally distinct. B,C: Resistant cells have expanded population with decreased CD19 and CD22 expression. D: Genes that are differentially expressed in scRNA seq between resistant and parental NALM-6 cells. E: Ingenuity network analysis for N6 scRNAseq. F: ATACseq and bulk RNAseq on REH parental and resistant cells showing decrease in chromatin accessibility and expression in CD19 and CD22 and maintenance of accessibility and expression in BTK.
Figure 3: Loss of CD19 expression in CD19 CAR-T treated pediatric ALL patients corresponds with a loss of CD22 expression.

A,B: Reduced CD19 and CD22 expression on resistant cells seen by FACS analysis. Gating is based on isotype control. C,D: CD19 and CD22 antigen expression was observed in a cohort of pediatric ALL patient cells (n=11) pre-treatment and post relapse after CD19 CAR-T therapy. Significant reduction of CD22 in CD19\textsuperscript{low} patients post-relapse is seen. Wilcoxon signed-rank test was used for analysis.
Figure 4: BTK signaling is active in CD19 low resistant cells.

A: single cell RNA-Seq (scRNA-Seq) analysis of parental and resistant NALM-6 cells shows BTK expression is maintained in resistant cases. B: Ratio of high to low expression cells for CD19 and BTK shows reduced CD19 and maintained BTK expression in resistant cases. C,D: CyToF analysis for parental and resistant cells show reduced CD19 protein expression in resistant cells. E-G: Reduced CD19, slightly reduced p-PLCy and preserved pCREB protein expression is seen in resistant NALM6 cells. H: Immunohistology from a lymphoma patient pre and post CD19 CAR-T treatment stained for CD19 and BTK. HE and CD19 images are at 40X magnification, BTK images are 20X and 40X magnification.
Figure 5: Cells resistant to CD19 immunotherapy exhibit dependency on B cell receptor and MEK signaling.

A, B: Immunoblotting shows that Ibrutinib can inhibit phospho-BTK in resistant cells. Resistant cells have preserved ERK activation.

C: Phospho-ERK/total ERK ratio in Raji cells shows that pERK is maintained in Raji CD19 KO cells.

D: Phospho-BTK/total BTK ratio in Nalm-6 cells shows maintained pBTK in NALM-6 resistant cells and ibrutinib effectively inhibits phosphorylation.

E, F: Resistant NALM6 cells are more sensitive to MEK and BTK inhibition (n=4).

G: ALL patient resistant to CD19 immunotoxin is sensitive to MEK inhibitor while resistant to methotrexate (n=3).

H: ALL patient resistant to Blinatumomab is sensitive to MEK inhibitor while resistant to methotrexate (n=3).

I: Proposed model, created with BioRender.com