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Original Article

Efficiency of Inotuzumab Ozogamicin in Relapsed/Refractory B-Cell Acute Lymphoblastic Leukemia in Relation to CD22 Expression

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Abstract

Introduction B-cell acute lymphoblastic leukemia (B-ALL) is characterized by the proliferation of immature B lymphoid blasts causing bone marrow failure. Inotuzumab ozogamicin (InO), an anti-CD22 targeted therapy, is an effective treatment option for relapsed/refractory (R/R) B-ALL.

Objectives The primary aim was to determine the efficiency of InO in relation to CD22 expression in leukemic cells. The other objectives were to study the efficiency of InO in different cytogenetically defined B-ALL groups and to examine the post-InO CD22 modulation in residual leukemic cells.

Materials and Methods This retrospective study was done in a cohort of R/R B-ALL patients treated with InO. The CD22 expression was studied by normalized median fluorescent intensity (nMFI), percentage of leukemic blasts positive for CD22, and also the pattern of CD22 expression with flow cytometry.

Results A total of 21 R/R B-ALL cases treated with InO were enrolled in the study. Measurable residual disease (MRD) negativity was achieved in 17 cases with InO. The MRD-negative group showed a diverse CD22 expression patterns. We found that InO was effective in leukemic cells weakly expressing CD22. No considerable difference in CD22 nMFI and its percentage was noted among the post-InO MRD positive and negative cohorts. MRD negativity was achieved in all Philadelphia chromosome-positive (Ph+) R/R B-ALL cases. All four post-InO MRD+ cases showed downmodulation of CD22.

Conclusion InO is effective in treating R/R B-ALL, including in cases with weak CD22 expression, and can serve as a bridge to hematopoietic stem cell transplant.

Keywords
- inotuzumab
- relapsed B-ALL
- CD22
- flow cytometry
- measurable residual disease

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Introduction

B-cell acute lymphoblastic leukemia (B-ALL) is a hematologic malignancy characterized by the proliferation of immature B-lymphoid blasts in the bone marrow, causing cytopenia with or without leukocytosis.[1] The improved aggressive chemotherapeutic regimen achieves complete response (CR) in 80 to 90% of patients. However, some patients continue to have poor results even with substantial developments in chemotherapy, which mandates the need for studying new therapeutic approaches.[2,3] A promising treatment option for B-ALL is immunotherapy, which targets particular antigens on B cells.[4]

CD22 is a transmembrane glycoprotein associated with B cells, comprised of immunoglobulin domains and N-linked glycosylation sites. It is expressed in most cases of B-ALL and serves as a feasible target for immunotherapies intended to treat B-cell malignancies.[5] The development of targeted monoclonal antibodies such as inotuzumab ozogamicin (InO) marks a notable advancement in the management of relapsed and refractory B-ALL (R/R B-ALL).[6] By specifically targeting cell surface antigen CD22, it offers a more precise and less toxic alternative to traditional cytotoxic chemotherapy.

The efficacy of InO, particularly in treating R/R B-cell precursor ALL (BCP-ALL), highlights the potential of targeted approaches in cancer treatment.[7] Patients treated with InO achieved considerably high rates of measurable residual disease (MRD) negativity, progression-free survival, and 2-year overall survival (OS).[8] InO enhances treatment effectiveness and also reduces the risk of treatment-related side effects, which is especially important for older adults who may be more susceptible to adverse reactions.[9,10] InO is also reported to be effective in R/R B-ALL cases with extramedullary disease.[11] InO functions by binding to CD22-positive B cells, delivering a cytotoxic-compound ozogamicin calicheamicin directly to the malignant cells, thereby inducing cell death.[7,9] However, its efficacy in patients with varying CD22 expression profiles is still an area of active research.

Flow cytometry (FCM) is routinely used in diagnostic immunophenotyping of leukemias and lymphomas. It is also indispensable for studying the expression of cellular markers like CD19 and CD22 for initiating targeted therapy through drugs like blinatumomab and InO, respectively.[12] It is also a rapid and effective tool for the detection of MRD in B-ALL with a sensitivity of 1×10^{-5}.[13,14] MRD negativity is an independent and key prognostic marker in B-ALL cases treated with InO therapy.[15] Understanding how CD22 antigen expression in leukemic cells influences treatment response to InO shall improve the outcomes for patients with R/R B-ALL.

This study aims to evaluate the efficiency of InO in patients with R/R B-ALL in relation to CD22 expression in leukemic cells. To achieve this, patients were segregated into two categories based on the post-InO MRD status by FCM:

(1) those who achieved negative MRD after InO, and
(2) those with positive MRD after InO. The CD22 expression in leukemic blasts and burden of disease before inotuzumab were compared between these groups.

We also aimed to study the efficiency of InO in different cytogenetically defined B-ALL groups and wished to assess the post-InO CD22 modulation in residual leukemic cells.

Materials and Methods

Study Design and Setting

This retrospective study was done at Apollo Cancer Center, Chennai, Tamil Nadu, India, in a cohort of R/R B-ALL patients treated with InO. All pediatric and adult patients who had recurrence of disease after achieving a period of remission (relapse) and those who were positive for residual disease at two successive time points (refractory) treated with InO in the last 5 years were included.

We retrospectively recruited all the patients treated with InO for this study from July 2019 to June 2024. A total of 23 patients with R/R B-ALL cases treated with InO were identified. However, after necessary exclusions, a total of 21 patients were enrolled in the study.

Inclusion and Exclusion Criteria

Twenty-one patients with R/R B-ALL treated with InO were included in this study. A patient started on InO elsewhere and referred to our center for allogenic hematopoietic stem cell transplant (HSCT) after assessing the MRD status was excluded. This case was not included in the study population as the details of pre-InO were not available. Similarly, one patient that had taken prior blinatumomab and InO without an in-between MRD assessment was also excluded.

Flow Cytometric Analysis and Assessment of CD22 Expression

Diagnostic immunophenotyping and MRD assessments were done by multiparametric FCM using a 10-color Navios Ex flow cytometer (Beckman-Coulter, Inc., California, United States). The quality check for fluidics, optical alignments, and detectors was done daily with Flow-Check Pro and Flow-Set Pro fluorospheres as per the manufacturer's recommendations. The samples were processed by a bulk lyse, stain, and wash protocol. A minimum of 50,000 events per tube were acquired at diagnosis and relapse. The first pull bone marrow sample was preferred, and 1.6 million events were minimum acquired for MRD assessment. The sensitivity of our B-ALL MRD assay is 0.002%, which was established with limit of blank, limit of detection, and lower limit of quantification assays. More details on sample processing and analysis of MRD in B-ALL using CD19 as the gating marker is described in our earlier published article.[16] The antibodies (Tube 1) used for studying B lymphoid blasts are shown in ►**Table 1**. We used anti-CD22 tagged with phycoerythrin (PE)-Texas Red Excitation-Conjugated Dye (ECD) from Beckman Coulter Life Sciences (clone: SJ10.1H11). We also analyzed cytoplasmic CD22 expression in cases with leukemic blasts $> 1\%$ before initiating InO. MRD was reported as negative if the residual disease was $< 0.01\%$.

We analyzed the FCM files of BCP-ALL cases treated with InO at time points of diagnosis, pre-InO and post-InO. We also studied CD22 expression in leukemic blasts at diagnosis, pre-InO and post-InO therapy time points.

Table 1 Antibodies in Tube 1 of acute leukemia panel

KO	BV421	FITC	PE	ECD	PC5.5	PC7	APC	APC700	APC750
CD45	CD73	CD38	CD58	CD22	CD10	CD34	CD19	CD123	CD20

Abbreviations: APC, allophycocyanin; BV, brilliant violet; ECD, Excitation-Conjugated Dye; FITC, fluorescein isothiocyanate; KO, Krome Orange; PC, Phycoerythrin-cyanine; PE, phycoerythrin.

Gating Strategy

Analysis was done on Kaluza software (version 2.1, Beckman-Coulter, Inc.). After ensuring stable acquisition with time plots, the dead cells and debris were eliminated with the forward scatter (FSC) versus side scatter plot, and singlets were selected with FSC height versus FSC area. From the viable singlets, events positive for CD19 were gated. The CD19-positive B cell events were studied with all combinations of markers for leukemia-associated immunophenotype and different from the normal pattern. The CD22 expression in leukemic blasts was studied by utilizing normalized median fluorescent intensity (nMFI), percentage of CD22 positive blast, and the pattern of CD22 expression (dim, medium, heterogeneous, and bright). We studied the nMFI of CD22 in which the CD22 MFI of the leukemic blasts is normalized to the CD22 MFI of the T lymphocytes in the following way:

$$\text{nMFI of CD22 of leukemic blasts} = \frac{\text{CD22 MFI of leukemic blasts}}{\text{CD22 MFI of T lymphocytes (negative control)}}$$

The percentage of CD22-positive blasts was studied by setting a negative threshold using T lymphocytes.

Cytogenetics

Conventional karyotyping was done from standardized unstimulated/stimulated cultures by Colcemid (HiMedia Laboratories, Maharashtra, India) incubation and G banding. Interphase fluorescence in situ hybridization (FISH) was done with Vysis FISH probes (Abbott, Illinois, United States) for t(9;22), t(12;21), t(1;19), t(v;11q21), and *TP53* deletion. Next-generation sequencing was done using a custom capture kit (133 genes). The deoxyribonucleic acid and ribonucleic acid libraries are sequenced at $>250\times$ coverage on validated Illumina (Illumina, Inc., San Diego, California, United States) sequencing platforms. Nonsynonymous and splice site variants found in the coding region were identified using the Lo Freq (version 2) variant caller (Genome Institute of Singapore, Singapore).

Primary and Secondary Outcome

Primary outcome: Remission after InO therapy, refractory to InO therapy, and relapse after InO therapy. *Secondary outcome*: Immunomodulation of CD22 antigen expression after InO therapy.

Statistical Analysis

The post-InO MRD negative and positive groups were compared for CD22 nMFI in blasts, the percentage of CD22 expression in blasts, and the burden of disease pre-InO with an unpaired *t*-test.

Ethical Approval

The study was approved by the institutional ethics committee (IEC-BMR, Apollo Hospitals, Chennai; application no.: ASH-C-S-014/06-24) on June 27, 2024. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Results

Out of the total 21 patients enrolled for the study, there were 12 male patients and 9 female patients. Fourteen cases were aged <18 years, and 7 cases were aged >18 years of age (median 22.6). Of the final 21 cases, 9 cases were Philadelphia chromosome (Ph) positive, two cases were Ph-like (*JAK2::GOLGA4* and *EBF1::PDGFRB*), two cases showed *TP53* deletion, two cases had *KMT2A* rearrangement, and remaining cases had no definite cytogenetic abnormalities (►**Fig. 1**).

Diagnostic baseline immunophenotyping results were available for 14 patients whose diagnoses were performed in our laboratory and unavailable for seven refractory cases of patients who were referred to our center for further management. Out of the 14 cases, 6 cases showed dim CD22 expression, 5 cases showed heterogeneous CD22 expression, and 1 case showed medium CD22 expression. Two cases were negative for CD22 (►**Fig. 1**). The CD22 expression in residual/relapsed leukemic cells was studied before starting InO (pre-InO) in all 21 cases. Cytoplasmic CD22 staining showed no significant difference, neither in the percentage nor in the intensity, as compared with surface CD22 expression. Most cases had one cycle of InO and was used as a bridging therapy to allogenic HSCT. InO was given on day 1, 8, and 15. Post-InO MRD assessment by multiparametric FCM was done on day 28. We compared the CD22 expression intensity in leukemic blasts at diagnosis and pre-InO time points in the post-InO MRD-negative and MRD-positive groups.

Out of the 21 patients, 17 were negative for residual disease and 4 were positive for residual disease post-InO treatment. All pediatric cases were MRD-negative post-InO therapy except one case that was resistant to InO. Three MRD-positive cases were in patients aged >18 years of age. The MRD-negative group showed a diverse CD22 expression before InO therapy (pre-InO). Nine cases showed dim expression, six cases showed heterogeneous expression, and one case showed medium CD22 expression (►**Fig. 2**). One case was negative for CD22 expression. None of the cases showed bright CD22 expression. The median leukemic CD22

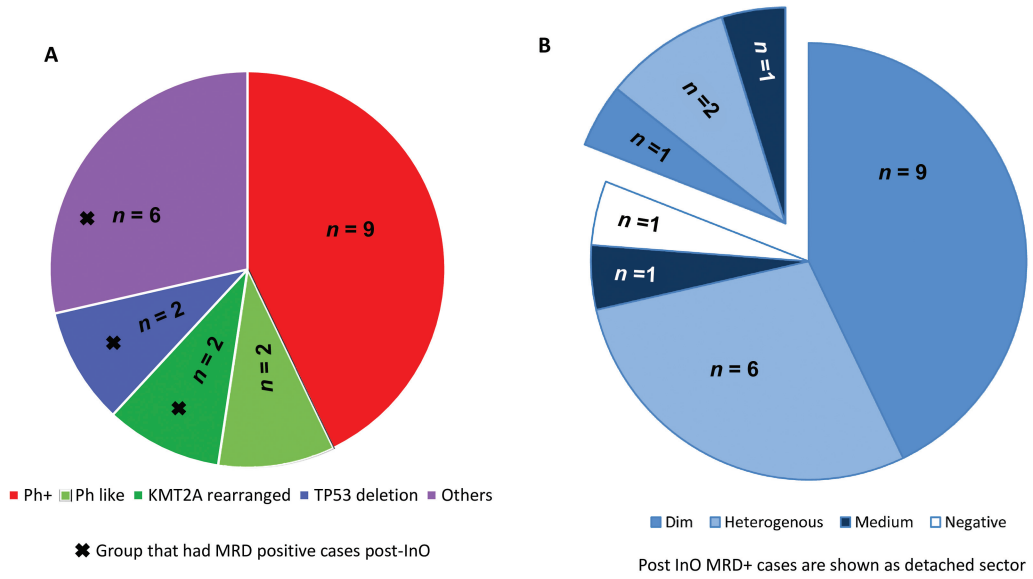

Fig. 1 (A) Cytogenetic abnormality in relapsed/refractory (R/R) B-cell acute lymphoblastic leukemia (B-ALL) treated with inotuzumab. (B) Intensity of CD22 in cases prior inotuzumab therapy.

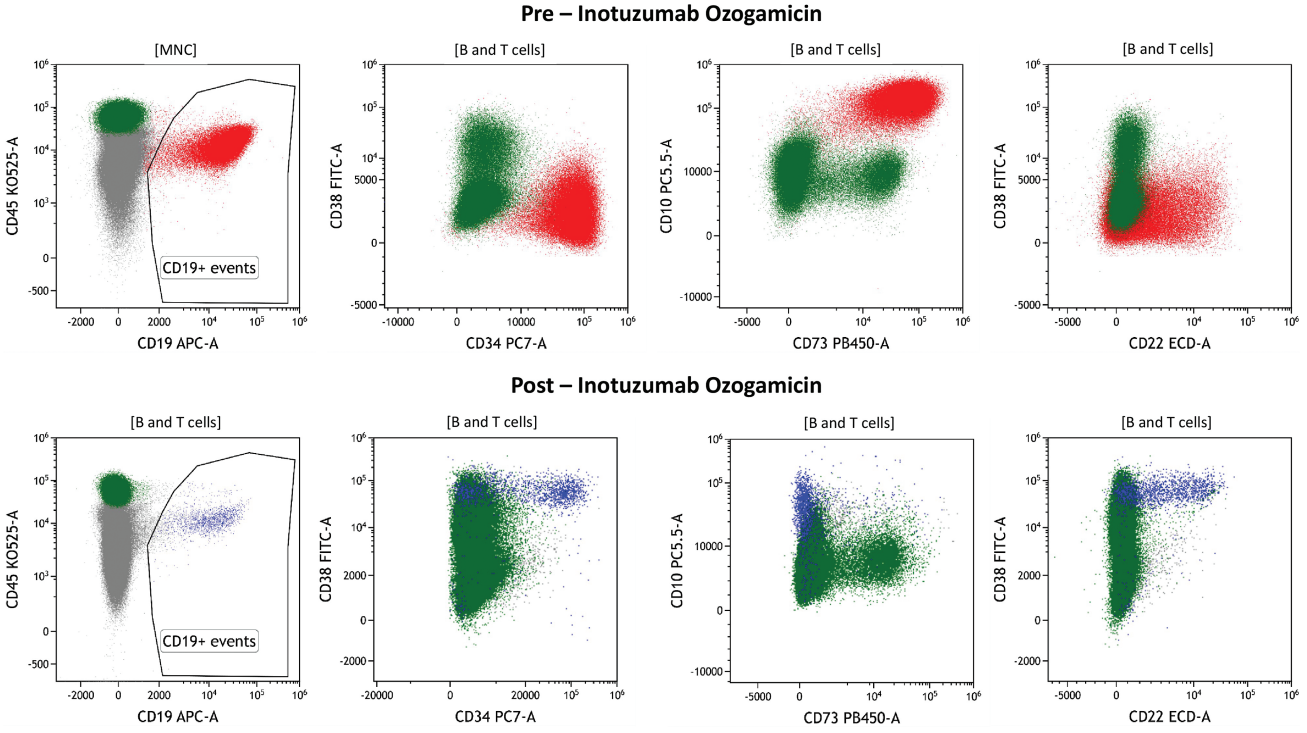

Fig. 2 Efficiency of inotuzumab ozogamicin (InO) in clearing high burden of disease in a case of refractory B-cell acute lymphoblastic leukemia (B-ALL) that had weak (dim-negative) CD22 before starting InO. Post-InO measurable residual disease (MRD) was negative. Red: leukemic events; green: normal T cells; blue: hematogones and mature B cells.

nMFI and percentage of CD22 expression in the post-InO MRD-negative group were 28.2 and 63.4%, respectively. Eight cases had a similar CD22 expression pattern as noted at baseline. One case that had CD22-negative leukemic blasts at diagnosis showed residual disease after standard chemotherapy. This case showed upmodulation (heterogeneous) of CD22 expression and was MRD-negative after InO. One case with residual disease of 0.01% after standard chemotherapy was negative for CD22 but responded to InO (►**Fig. 3**).

Of the four cases that were MRD-positive, one showed dim, two cases showed heterogeneous, and one case showed medium CD22 expression before starting InO. The median leukemic CD22 nMFI and percentage of CD22 expression in the post-InO MRD-positive group was 30.6 and 87.4%, respectively. All post-InO MRD-positive cases had baseline immunophenotype done elsewhere and referred to our center. Two cases had *KMT2A* rearrangement, one case had *TP53* deletion, and the other case had no defined cytogenetic

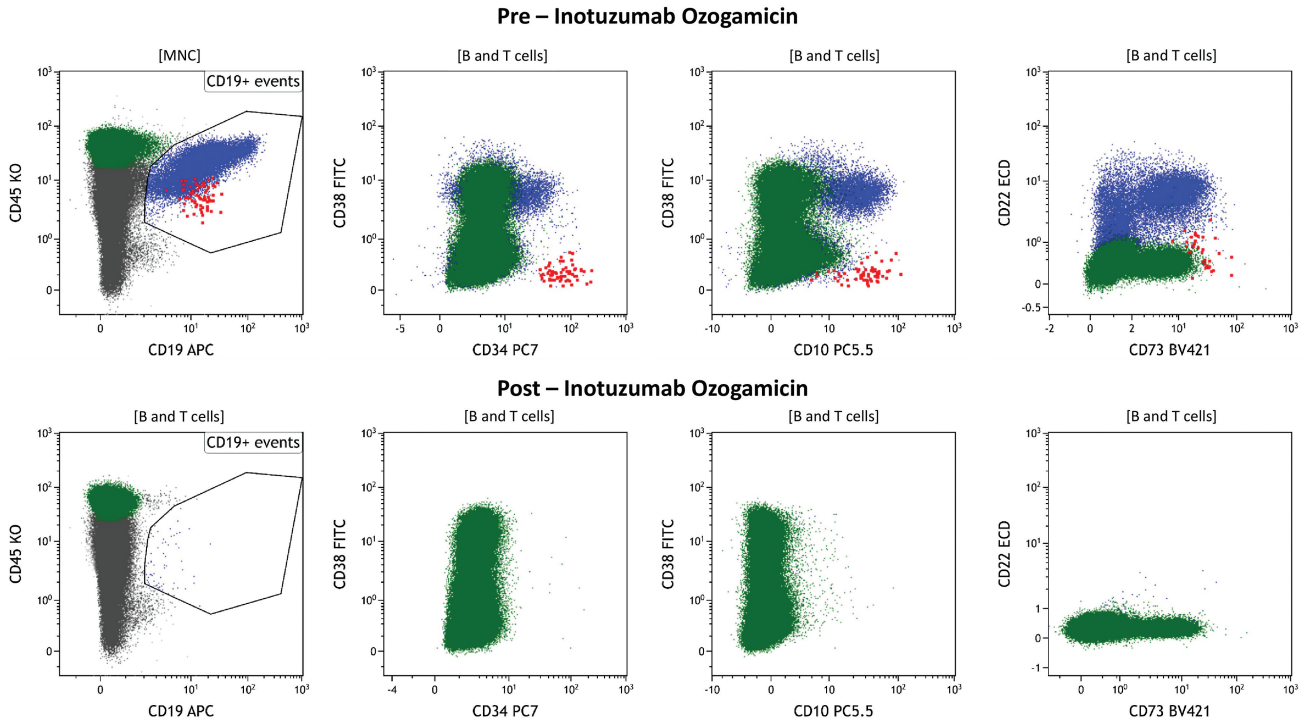

Fig. 3 Efficiency of inotuzumab ozogamicin (InO) in a case of B-cell acute lymphoblastic leukemia (B-ALL) with persistent residual disease planned for matched sibling hematopoietic stem cell transplant (HSCT). Pre-InO measurable residual disease (MRD) assessment showed low residual disease at 0.01%, which was negative for CD22. Post-one cycle of InO MRD was negative. Red: leukemic events; green: normal T cells; blue: hematogones and mature B cells.

abnormality. We also compared the pre-InO CD22 expression by percentage and nMFI in leukemic blasts between the MRD-negative and MRD-positive groups. There was no significant difference in percentage ($p = 0.18$) and nMFI ($p = 0.8$) of CD22 expression in leukemic blasts between both groups (►**Table 2**).

Negative MRD was achieved even in cases that had blasts > 10% before starting InO (►**Fig. 2**). It was also effective in nine cases that had a petite residual disease of 1 to 0.01%. InO also decreased the disease by 2 to 3 logs in the MRD-positive group. Only one case that had refractory MRD of 0.21% was completely resistant to InO (post-InO MRD was 0.93%). This case had no defined cytogenetic defect and showed pre-InO MRD with dim CD22 expression (84% positive for CD22) and was expected to respond to InO. Cases that had residual disease post-InO ($n = 4$) showed a decrease in nMFI and

Table 2 Comparison of CD22 expression in post-inotuzumab MRD negative and positive cohort

	Post-inotuzumab MRD-negative cases ($n = 17$)	Post-inotuzumab MRD-positive cases ($n = 4$)	Two-tailed *p*-value
Normalized MFI of CD22 in leukemic blasts before inotuzumab ozogamicin	28.2 ± 21.4	30.6 ± 10.9	0.8
% of CD22 in leukemic blasts before inotuzumab ozogamicin	63.4 ± 31.6	87.4 ± 9.1	0.18
Pattern of CD22 expression in leukemic blasts before inotuzumab ozogamicin	Negative: 01 Dim: 09 Heterogeneous: 06 Medium: 01 Bright: 00	Negative: 00 Dim: 01 Heterogeneous: 02 Medium: 01 Bright: 00	NA
Burden of disease before inotuzumab ozogamicin	Mean blast % = 23.8 > 10% = 06 > 1% to < 10% = 06 > 0.1% to < 1% = 02 > 0.01 to < 0.1% = 03 > 0.001 to < 0.01% = 00	Mean blast % = 37.3 > 10% = 02 > 1% to < 10% = 01 > 0.1% to < 1% = 01 > 0.01 to < 0.1% = 00 > 0.001 to < 0.01% = 00	0.44

Abbreviations: MFI, median fluorescent intensity; MRD, measurable residual disease.

Table 3 Comparison of CD22 expression before and after inotuzumab therapy in the MRD-positive cohort

Post-inotuzumab MRD-positive group ($n = 4$)				
	Pre-inotuzumab		Post-inotuzumab	
	CD22 nMFI	CD22 (%)	CD22 nMFI	CD22 (%)
Case 1	41.63	92.0	13.6	34.1
Case 2	20.57	73.1	4.2	7.7
Case 3	33.67	86.5	11.9	27.2
Case 4	16.20	67.7	13.4	51.6

Abbreviations: nMFI, normalized median fluorescent intensity; MRD, measurable residual disease.

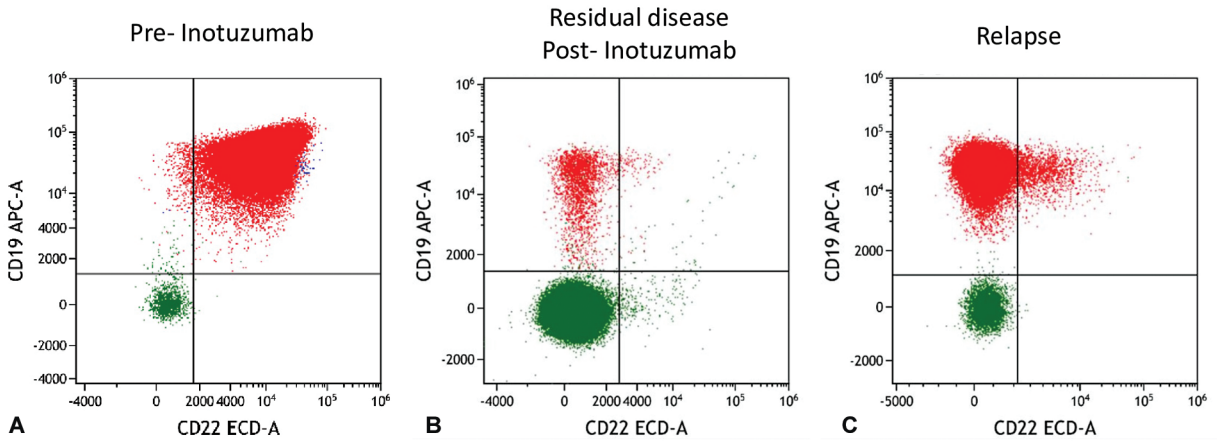

Fig. 4 Downmodulation of CD22 in a case of relapsed/refractory (R/R) B-cell acute lymphoblastic leukemia (B-ALL) that was measurable residual disease (MRD)-positive post-inotuzumab ozogamicin (InO) therapy. (**A**) CD19/CD22 dot plot before InO therapy. (**B**) CD19/CD22 dot plot after InO showing MRD at 0.13%. (**C**) CD19/CD22 dot plot of the same case at relapse. Red: leukemic events; green: normal T cells.

percentage of leukemic events positive for CD22, indicating downmodulation of CD22 in residual leukemic cells post-InO therapy (►**Table 3**). This is similar to other immunotherapy escape mechanisms. CD22-negative relapse was noted in a case post-InO therapy (►**Fig. 4**).

Discussion

FCM-based immunophenotyping is widely used to study the expression of antigens that can be targeted by immunotherapy. An optimal expression of the target antigen in leukemic cells is considered the prerequisite for starting immunotherapy. Previous studies have shown that the efficacy of the targeted therapy correlates with the expression intensity of the target antigen.[12,17] CD22 is expressed in leukemic cells of most of the B-ALL cases, but the intensity of expression is weak as compared to normal B cells.[18] Anti-CD22 therapy has proven to be an effective option in achieving remission in R/R B-ALL.[19,20] Hence, the efficacy of InO in R/R B-ALL with attenuated CD22 expression is uncertain. We studied the efficiency of InO in R/R B-ALL patients in relation to CD22 expression in blast cells. To assess the expression of CD22 in leukemic cells, we have used percentage expression, nMFI, and intensity patterns based on internal positive and negative controls. Few studies have measured the CD22 expression by molecules of equivalent soluble fluorochrome (MESF) and antibody-bound per cell (ABC) by quantiBRITE beads (BD Biosciences, New Jersey, United States).[18] However, the indicators we used were more practical and reflected the real-world experience.

We found that InO was effective in leukemic cells expressing weak CD22, and no significant difference in baseline CD22 nMFI and percentage expression was noted among the post-InO MRD positive and negative cohorts. Hence, leukemic cases with weak CD22 expression can attain MRD negativity and should not be abstained from InO therapy. O'Brien et al found no significant difference in CD22 percentage expression at baseline among cases with and without residual disease. However, they found differences in CD22 ABC in both groups.[21] Furthermore, one of our cases with CD22 expression of 84% was resistant to InO. Kantarjian et al have reported that half of the nonresponders to InO had CD22 > 90%. Hence, other factors, like the sensitivity of the leukemic cells to calicheamicin, also play a role in the response and resistance to InO, and not just CD22 expression.[2,8]

We had two cases of *KMT2A* rearranged B-ALL, which were MRD-positive post-InO therapy. CD22-negative B-ALL cases have been largely described in *KMT2A*-rearranged B-ALL cases.[8,21] Hence, these patients have been reported as resistant to InO. O'Brien et al reported low CD22 percentage expression and ABC in all *KMT2A*-rearranged B-ALL cases.[21]

The INO-VATE trial showed decreased CD22 MESF in patients with *KMT2A* rearrangement, and no significant difference was found in their OS with InO irrespective of CD22 expression. Seven of their 19 *KMT2A* cases showed CD22 expression of $>90\%$.[8] The reason for resistance to InO in *KMT2A* rearranged B-ALL is not clearly understood. Though lack of CD22 expression is attributed as a reason, our *KMT2A* rearranged cases showed dim CD22 (73%) and medium (92%) CD22 expression. Lineage switch may be another reason for refractoriness in B-ALL.[2]

InO was also found very successful in achieving CR in Ph-positive B-ALL cases as compared to Standard chemotherapy (SC).[2,6,8] All Ph-positive cases achieved MRD negativity after InO therapy in our study group, keeping in consensus with earlier studies. The efficiency of InO in Ph-like B-ALL cases is yet to be studied as the INO-VATE trial did not include these cases. In contrast, two cases of Ph-like B-ALL in our cohort responded to InO. The trial also reported that *TP53*-mutated ALL cases were not resistant to InO. We had two cases of B-ALL with *TP53* deletion, and one was positive and the other was MRD-negative after InO therapy. Hence, molecular indicators of poor response to SC are not applicable to InO except in *KMT2A*-rearranged B-ALL cases.

Though patients with CD22 expression $>90\%$ have a better response to InO, it is not restricted to R/R B-ALL patients with decreased CD22 expression. Even in patients with CD22 expression $<90\%$, InO has a better response rate as compared to standard chemotherapy (►**Figs. 2** and **3**). The in vitro trial (NC701564784) showed that three out of five cases with negative or low CD22 expression responded to InO.[8] They also observed good early response, but the long-term efficacy of InO as a single agent is poor in weakly expressed CD22 cases. It is an effective salvage regimen and serves as a bridge to HSCT.[22] It is better to use a combination chemotherapy than a monotherapy in weakly expressed CD22 cases.[23] This is also applicable to *KMT2A*-rearranged and cases of R/R B-ALL with adverse cytogenetics and high peripheral absolute blast counts ($>1\times10^9$/L).[24]

The killing potency of InO in CD22-negative/dim leukemic cells is less understood. The suitable explanation is based on the density of CD22 molecules on leukemic cells. The ability of therapeutic antibodies to deliver cytotoxic hits even in very low CD22-expressing leukemic cells is insufficient to give a positive signal by FCM. The other hypothesis on the killing potency of InO in CD22-negative/dim leukemic cells is the transport of CD22 molecules from cytoplasm to surface upon activation.[25] "Do the therapeutic and diagnostic antibodies attach to distinct CD22 molecule epitopes?" or "Is it restricted to particular cell membrane microdomains?" are questions that require considerable research.[2,26–28] The clone and brightness of tagged fluorochrome used for studying CD22 should be judiciously selected for immunophenotyping. Bright fluorochromes like PE, allophycocyanin, and brilliant violet dyes can be of good choice.

Many authors have reported downmodulation of CD22 expression after InO treatment.[8,21,29,30] Ryland et al demonstrated a homozygous truncating mutation in the exon 4 of *CD22* after 59 days of starting InO therapy as a mechanism of resistance in a case of R/R B-ALL that relapsed after an initial response.[31] All three cases, which were MRD-positive post-InO, showed downmodulation of CD22. INO-VATE trial phase 1/2 identified four patients with complete CD22 negativity after InO treatment. This downmodulation of target antigen in leukemic cells is a well-known escape mechanism following immunotherapy.[32] Very less literatures are available on the role of InO in R/R B-ALL with weak CD22 expression and modulation of CD22 antigen in leukemic cells post-InO therapy. Our findings suggest that InO is effective in leukemic cells expressing weak CD22 and these cases should not be abstained from InO therapy. This may be true for other monoclonal antibody therapies like blinatumomab used in acute leukemias. However, more research is needed in the future to identify the threshold of minimum CD22 expression in leukemic blasts that correlates with the potency of InO therapy. The limitation of this study is the smaller sample size, as many R/R BCP-ALL patients could not afford InO therapy due to financial constraints.

Conclusion

InO has a better overall response rate compared to standard salvage chemotherapy in R/R B-ALL. CD22 expression intensity alone may not predict InO response, as patients with both high and low CD22 levels responded similarly. InO is a highly effective salvage regimen and serves as a bridge to HSCT in R/R B-ALL cases even if CD22 is weakly expressed. However, the long-term outcome with only InO in such cases appears bleak.

Data Availability Statement

Data will be available from the corresponding author upon request.

Authors' Contributions

Conception and design: T.R.R. Provision of study materials or patients: All authors. Collection and assembly of data: T.R.R., E.S., and B.M. Data analysis and interpretation: T.R.R. Manuscript writing: T.R.R. Final approval of manuscript: All authors.

Patient Consent

Waiver of consent document was approved by the Institutional Ethics Committee.

Funding

None.

Conflict of Interest

None declared.

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