Modulation of Target Antigen Density Improves CAR T-cell Functionality and Persistence

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Abstract

Purpose: Chimeric antigen receptor T-cell (CART) therapy targeting CD22 induces remission in 70% of patients with relapsed/refractory acute lymphoblastic leukemia (ALL). However, the majority of post-CD22 CART remissions are short and associated with reduction in CD22 expression. We evaluate the implications of low antigen density on the activity of CD22 CART and propose mechanisms to overcome antigen escape.

Experimental Design: Using ALL cell lines with variable CD22 expression, we evaluate the cytokine profile, cytotoxicity, and in vitro CART functionality in the setting of low CD22 expression. We develop a high-affinity CD22 chimeric antigen receptor (CAR) as an approach to improve CAR sensitivity. We also assess Bryostatin1, a therapeutically relevant agent, to upregulate CD22 and improve CAR functionality.

Results: We demonstrate that low CD22 expression negatively impacts in vitro and in vivo CD22 CART functionality and impairs in vivo CART persistence. Moreover, low antigen expression on leukemic cells increases naïve phenotype of persisting CART. Increasing CART affinity does not improve response to low-antigen leukemia. Bryostatin1 upregulates CD22 on leukemia and lymphoma cell lines for 1 week following single-dose exposure, and improves CART functionality and in vivo persistence. While Bryostatin1 attenuates IFNγ production by CART, overall in vitro and in vivo CART cytoxicity is not adversely affected. Finally, administration of Bryostatin1 with CD22 CART results in longer duration of in vivo response.

Conclusions: We demonstrate that target antigen modulation is a promising strategy to improve CD22 CAR efficacy and remission durability in patients with leukemia and lymphoma.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Introduction

While overall survival for pediatric B-cell acute lymphoblastic leukemia (ALL) treated with risk-adapted, multi-agent chemotherapeutic regimens is greater than 85% at 5 years, patients who relapse have limited therapeutic options offering high likelihood of cure (1, 2). For these patients, chimeric antigen receptor T cells (CART) targeting CD19 can induce remissions in a high percentage of patients and is potentially curative (3–5). However, longer follow-up data has now shown that a portion of the patients achieving remission will subsequently relapse with either poor CART persistence or loss of the targeted CD19 epitope (5–8). We developed a chimeric antigen receptor (CAR) targeting CD22 (9), an alternative clinically validated ALL antigen (10), and demonstrated a 70% remission induction rate at biologically active doses, including activity in patients relapsing with CD19 CART-resistant ALL (11). Similar to CD19 CART, remission durability is reduced in a substantial number of patients achieving remission after CD22 CART due to poor CART persistence or altered target antigen expression (11). However, in contrast to most resistant leukemias relapsing after CD19 CAR or blinatumomab, relapse after the CD22 CART typically occurs with sustained, but diminished, cell surface CD22 expression on the leukemia (11).

As experience with CART therapy expands, thresholds of antigen expression required to activate CARs have been identified in preclinical models (12–15). At lower target antigen site densities, CARs are less functional, producing less cytokines such as IFNγ or IL2, despite augmentation with costimulatory molecules or alterations of CART binding affinity (13). Importantly, this threshold for activation is relatively high compared with that required for activation through the T-cell receptor (16–18). However, the functional ramifications of this site density limitation are not fully understood and the implications of site density on in vivo CART cell behavior have not been evaluated. We have extensively studied CD22 site density on a variety of B-cell malignancies, as well as normal B cells (19). While normal B cell CD22 site density...
values are approximately $10^4$ molecules/cell (19), mean ALL CD22 site density is a log lower, with a wide range in expression, from $10^{-2}$–$10^4$ (20). In addition, specific ALL subtypes such as MLL-rearranged ALL have even lower CD22 site density (20). These characteristics of CD22 make it a clinically relevant model of reduced antigen expression to evaluate the effects of site density on CART efficacy.

With the goal of developing approaches to overcome immune escape, we systematically evaluated the impact of CD22 site density on CART function. Our data demonstrates reduced CART activity, shortened CART persistence, and altered CART phenotype following in vitro exposure to leukemia with lower antigen site density. We identified a therapeutically feasible approach to enhance CART efficacy through increasing site density using Bryostatin1, a natural product originally isolated from the marine bryozoan *Bugula neritina* previously shown to modulate Protein Kinase C (PKC; ref. 21). We found that Bryostatin1, previously shown to upregulate CD22 expression in chronic lymphocytic leukemia (CLL; ref. 22), increases CD22 expression on pre-B ALL and diffuse large B-cell lymphoma (DLBCL), and improves antileukemia CART response, memory T-cell formation, and durability of response. This is the first report to validate target antigen modulation as a clinically feasible and relevant approach to enhance the efficacy and durability of CART therapy.

**Materials and Methods**

**Clinical trial and patient data**

Patients screened for enrollment on CD19 CART (NCT02028455) or CD22 CART (NCT02315612) trials at the NCI were evaluated for site density of both CD19 and CD22 antigens on the surface of leukemic blasts, measured using flow cytometric methods as described previously, particularly relevant to CD22 detection (REF. PMID: 20872890). Both protocols were approved by the NCI Institutional Review Board and the NIH Recombinant DNA Advisory Committee and were in accordance with U.S. Common Rule. All subjects provided written informed consent, or parental permission with minor assent was obtained when appropriate. Patients treated on the CD22 CART trial also had serial evaluations for CD22 at the time of enrollment and at relapse. In addition, 4 CD22 CART trial patients were evaluated for CAR T-cell expansion over time by flow cytometry.
Nalm6 xenograft and PDX in vivo studies
CD22 CAR functionality and antileukemic efficacy were evaluated in vivo in Nalm6 xenograft or PDX models with NOD.Cg-Pkdcsid1Il2rgtm1Wjl/SzJ (NSG) mice (The Jackson Laboratory and bred in-house) ages 6–10 weeks. Mice received $1 \times 10^6$ GFP–Luciferase–tumor cells (Nalm6, Nalm6 CRISPR variant, or PDX) intravenously on day 0. On day 3, mice received CD22–transduced T cells or mock-transduced T cells intravenously at the indicated quantity. To monitor leukemia burden, luciferin-D (Caliper Life Sciences) was injected into mice intraperitoneally and imaged 4 minutes later using In Vivo Imaging System (IVIS) Technology (Caliper Life Sciences). Bioluminescent signal flux (luminescence) for mice was measured using Living Image Version 4.1 Software (Caliper Life Science). All animal studies were conducted in accordance with an approved animal protocol (PB-027-2-M) with the NCI and University of Colorado Denver Animal Care and Use Committees.

Bryostatin1 treatment
For all in vitro experiments and tumor/CAR pretreatment prior to intravenous injection, Bryostatin1 was administered to cells at a concentration of 1 ng/mL (1 nmol/L). DMSO vehicle control was administered to control-treated cells at an equal volume. Prior to coculture or intravenous injection, Bryostatin1- and DMSO–treated cells were washed three times in sterile PBS. For all coculture or intravenous injection, Bryostatin1- and DMSO–administered to control-treated cells at an equal volume. Prior to experiments, Bryostatin1 was diluted in PBS and administered via intraperitoneal injection at 40 μg/kg (1 μg/25 g mouse). For control-treated groups, an equal volume of DMSO was diluted in PBS and administered in the same manner.

RNA sequencing and data analysis
Sixteen RNA sequencing (RNA-seq) samples were pooled and sequenced on one NextSeq high output run using Illumina TrueSeq Poly A RNA kit v3 with paired end sequencing. All samples had greater than 30 million reads pass filter reads with a base call quality of above 95% of bases with Q30 and above. Reads of the samples were trimmed for adapters and low-quality bases using Trimmomatic software before alignment with the reference genome Human-hg19 and the annotated transcripts using STAR. The mapping quality statistics were calculated using Picard software and library complexity was measured in terms of unique fragments in the mapped reads using Picard MarkDuplicate utility. The Partek Flow informatics pipeline was used to generate fold change and differentially expressed gene data, as well as for data visualization. Gene set enrichment analysis (GSEA) of the differentially expressed genes was performed as described previously and using standard parameters.

Flow cytometry
FACS analysis of cell surface CAR and protein expression was performed using an LSR II Fortessa Flow Cytometer (BD Biosciences). CD22 CAR was detected by incubation with 22-Fc (R&D Systems), followed by incubation with human IgG-specific PE-F (ab)2 (Thermo Fisher Scientific). The following human mAbs were used for detection of cell surface proteins: CD22-APC, CD22-PE, CD19-Pacific Blue, CD45-PerCP/Cy5.5, CD3-APC/Cy7, PDI-PE/Cy7, LAG3-APC, TIM3-Pacific Blue, CD8-APC, CD8-PE/Cy7, CD45RA-APC, CD45RO-PE/Cy7, CCR7-Pacific Blue, CD4-Pacific Blue, and CD69-APC (all from BioLegend). CD22 site density was determined using Quantibrite-PE Beads (BD Biosciences) using methods described previously (PMID: 20872890). Dead cells were identified using eFluor 506 fixable viability dye (Thermo Fisher Scientific). GFP–expressing leukemia was identified through the FITC channel.

In vitro cytokine and leukemia clearance assays
Cytokine production assays. CAR or mock T cells were cocultured with tumor cells in RPMI at a 1:1 effector-to-target ratio in 96-well plates and incubated at 37°C for 20 hours. Plates were spun at 1,200 RPM for 6 minutes to pellet the cells, and cytokine concentrations in the culture supernatants were measured using IL2 ELISA (R&D systems), IFNγ ELISA (R&D systems), or Granzyme B ELISA (Thermo Fisher Scientific) kits according to the manufacturer’s directions.

Leukemia clearance assays. $1 \times 10^5$ CAR or mock T cells were cocultured with tumor cells in RPMI at a 1:1 effector to target ratio in 96-well plates. After initiation of coculture, plates were incubated in an IncuCyte ZOOM and imaged for green object count. CAR functionality and antileukemic efficacy were evaluated in vitro in Nalm6 xenograft or PDX models with NOD.Cg-Pkdcsid1Il2rgtm1Wjl/SzJ (NSG) mice (The Jackson Laboratory and bred in-house) ages 6–10 weeks. Mice received $1 \times 10^6$ GFP–Luciferase–tumor cells (Nalm6, Nalm6 CRISPR variant, or PDX) intravenously on day 0. On day 3, mice received CD22–transduced T cells or mock-transduced T cells intravenously at the indicated quantity. The luciferase signal was monitored using Living Image Version 4.1 Software (Caliper Life Science). All animal studies were conducted in accordance with an approved animal protocol (PB-027-2-M) with the NCI and University of Colorado Denver Animal Care and Use Committees

Results
Decreased CD22 site density on pre-B-cell ALL impacts CD22-directed CART functionality
At baseline, CD22 site density on ALL blasts is significantly lower than CD19 site density (23). We have previously shown that leukemic blasts from patients with refractory ALL treated with CD22 CART therapy demonstrated a significant decline in CD22 site density at relapse compared with pretreatment (11), and we now validate these findings that 8 of 20 CD22-CAR–treated patients relapsed with a 50% reduction of CD22 expression (Fig. 1A; median site density pre-CAR 2,714, post-CAR median 1,435). Patients with lower initial blast CD22 site density tended to have decreased CD22 CAR expansion (Fig. 1B). Moreover, the patients with lower initial CD22 site densities were only able to maintain stabilization of disease (SD), whereas patients with higher initial site density achieved minimal residual disease (MRD)–negative complete response (CR; Fig. 1B). Collectively, these observations suggest that the lower and dynamic expression of CD22, as compared with CD19, impacts CART activity.

To determine whether CD22 site density directly impacts CART function, we generated Nalm6 ALL cell lines with varying expressional density at relapse compared with pretreatment (11), and we now validate these findings that 8 of 20 CD22-CAR–treated patients relapsed with a 50% reduction of CD22 expression (Fig. 1A; median site density pre-CAR 2,714, post-CAR median 1,435). Patients with lower initial blast CD22 site density tended to have decreased CD22 CAR expansion (Fig. 1B). Moreover, the patients with lower initial CD22 site densities were only able to maintain stabilization of disease (SD), whereas patients with higher initial site density achieved minimal residual disease (MRD)–negative complete response (CR; Fig. 1B). Collectively, these observations suggest that the lower and dynamic expression of CD22, as compared with CD19, impacts CART activity.
Figure 1.
CD22 expression decreases following CD22 CART, and decreased CD22 expression attenuates CART expansion and persistence. A, Patient samples pre- and post-CD22 CART therapy were evaluated for CD22 expression using Quantibrite-PE bead evaluation. Statistics were performed using Wilcoxon test. B, Four separate CD22 CART patient samples, two at dose level 1 and two at dose level 2, were evaluated for site density using Quantibrite-PE analysis and CD22 CAR using flow cytometry. CR, MRD-negative complete response; SD, stable disease. C, A total of 1 $\times$ 10^5 tumor cells were cocultured with 1 $\times$ 10^5 CD22 CAR cells from CD22 CART patient samples for 18 hours. Supernatant was evaluated by Meso Scale Multiplex proinflammatory cytokine panel. Statistics were calculated using unpaired t-test (***, $P < 0.0001$; ****, $P < 0.0002$; **, $P < 0.002$). D, Kaplan-Meier curves comparing CD22^- with CD22^lo and Nalm6 leukemia-bearing mice with different CART doses. E, Peripheral blood was collected from mice at interval timepoints and assessed for CAR expansion using flow cytometry and analyzed on Fortessa flow machine. F, CAR expansion was evaluated from bone marrow of mice 16 days after tumor injection. Cells were stained for flow cytometry and analyzed on Fortessa flow machine. Statistics were calculated using Mann–Whitney test (***, $P < 0.0001$; *, $P < 0.03$).
pattern of diminished production of multiple T-cell cytokines by patient-derived CD22 CART products in response to low-CD22-expressing cell lines (Supplementary Fig. S1C).

Site density affects CD22 CART activation, efficacy, expansion, and phenotype

While low CD22 site expression did not affect CART-mediated eradication of ALL in vitro, in vivo clearance of CD22hi ALL by CD22 CART was impaired compared with clearance of parental Nalm6, as shown previously (11). Moreover, eradication of CD22lo ALL was not rescued by increased dose of CD22 CART (Fig. 1D). Importantly, diminished ability for CD22 CART to clear CD22lo ALL was associated with poor early expansion of CART (Fig. 1E), consistent with our observations in patients treated with the CD22 CART (Fig. 1B). Finally, CD22 CARTs were significantly reduced in bone marrow of CD22lo leukemia–bearing mice at day 16 after CAR injection as compared with parental Nalm6-bearing mice (Fig. 1F). Together, these findings demonstrate that CD22 CARTs are less effective at clearing CD22lo ALL due to ineffective CAR expansion.

We next assessed the impact of site density on the activation of CART during initial expansion. PD1 expression on CART was reduced by 30% after 24 hours of in vitro coculture with CD22lo ALL compared with parental Nalm6 (Fig. 2A). Following 8 days of in vitro coculture, CD22lo-exposed CARTs had a higher percentage of naïve cells (CCR7+ and CD45RA+) compared with Nalm6-exposed CARTs (Fig. 2B). When evaluated in vivo, there was no difference in CAR PD1 expression at day 16, and by day 30 there was only a modest decrease in PD1 expression on persisting CD22 CART in the CD22lo leukemia group as compared with the Nalm6-bearing group (Fig. 2C). In vivo analysis of CART phenotype 16 days after injection showed a marked change in the CCR7 and CD45RA profile and a trend toward more naïve cells following CD22lo leukemia exposure compared with Nalm6 exposure (Fig. 2D). This combined in vitro and in vivo data suggests that, in the presence of low antigen density, CART may not be able to convert to a memory phenotype, thereby resulting in decreased durability of response in vivo.

Increased CAR affinity does not improve CAR sensitivity to low site density leukemia

The CD22-specific scFv (m971) used in the construction of the CD22 CART has a relatively low binding affinity (9, 24). Thus, we modified the anti-CD22 scFv (m971:17) to generate a higher affinity CD22 CART (CD22V1 CART; Supplementary Fig. S2A) with improved binding at lower antigen densities, while maintaining the same 41BB costimulatory domain. However, this scFv modification did not enhance in vitro cytokine production or cytotoxicity (Fig. 3A and B). Furthermore, the CD22V1 CART did not improve clearance of CD22lo leukemia cells in xenografts (Fig. 3C). To evaluate the cause of this lack of enhanced activity in vitro, T cells expressing the CD22V1 CART were evaluated for persistence, activation, exhaustion, and memory phenotype both in vitro and in vivo. One day after coculture with CD22lo leukemia, the CD22V1 CART had similar PD1 expression as the original CAR (Supplementary Fig. S2B). However, both on day 1 and day 8 of in vitro coculture, a higher percentage of CD22V1 CART remained naïve compared with the original CD22 CART (Fig. 3D). CD22V1 CART persisted in the presence of CD22lo leukemia in vitro at day 16 (Fig. 3E), with similar expression of PD1, TIM3, and LAG3 compared with the original CAR (Supplementary Fig. S2C). Similar to the in vitro findings, a higher percentage of CD22V1 CART remained naïve as compared with the original CD22 CART when responding to both CD22lo and parental Nalm6 leukemia (Fig. 3F; Supplementary Fig. S2E). Finally, a population of EMRA CART (CCR7+, CD45RA+) consistent with terminal differentiation, emerged in the CD22V1 CART, that was not present in the original CD22 CART and that was more pronounced when targeting CD22lo leukemia (Fig. 3F; Supplementary Fig. S2F).

Bryostatin1 increases CD22 antigen expression on ALL and DLBCL cell lines

We hypothesized that CART activity could be improved through a drug-mediated increase in antigen expression. Bryostatin1 has been shown to increase CD22 on CLL (22). Thus, we tested the impact of Bryostatin1 on CD22 expression in two pre-B ALL cell lines and two DLBCL cell lines. All four cell lines demonstrated an increase in CD22 expression upon exposure to Bryostatin1 at 1 nmol/L in vitro (Fig. 4A). Bryostatin1 did not alter CD19 expression on ALL cell lines, but a modest increase was observed on DLBCL cell lines (Fig. 4A). Interestingly, increased CD22 expression persisted and, in fact, increased at 1 week following removal of Bryostatin1 (Fig. 4B). Furthermore, a single dose of Bryostatin1 administered intraperitoneally increased CD22 mean fluorescence intensity (MFI) on Nalm6 for 1 week, followed by a return to baseline expression 12 days post-drug administration (Fig. 4C). Finally, we confirmed that Bryostatin1 upregulated CD22 on a CD22-lo relapse patient-derived ALL xenograft (Fig. 4D).

Bryostatin1-mediated CD22 upregulation may occur via mechanisms other than increased CD22 gene expression mediated by PKC inhibition

Consistent with previous reports in CLL (22), PKCβII protein levels decreased in ALL cells upon exposure to Bryostatin1 (Supplementary Fig. S3A), suggesting a similar PKC-driven mechanism of CD22 upregulation. However, neither the PKCβII inhibitor, Enzastaurin, nor a broad PKC inhibitor, Staurosporine, was able to upregulate CD22 (Supplementary Fig. S3B). To further interrogate potential mechanisms of CD22 upregulation, we performed RNA-seq analysis on two Bryostatin1-treated and -untreated MLL-rearranged ALL cell lines [KOPN8 (MLL-MLT1 fusion oncogene) and SEM (MLL-AFF1 fusion oncogene)], both of which show robust upregulation of CD22 with Bryostatin1 exposure (Supplementary Fig. S4A). There were marked differences between the two cell lines in the magnitude of the transcriptional response to Bryostatin1 (Fig. 5A). While both of these cell lines represent MLL-rearranged tumors, KOPN8 demonstrated 1,524 genes with a greater than 2-fold change at 24 hours, whereas only one gene demonstrated a 2-fold change with Bryostatin1 exposure in SEM, indicating variability in leukemic response to Bryostatin1. Nonetheless, by lowering the threshold to a fold change of 1.5, a common set of genes could be found to be altered in both SEM and KOPN8, including several cell surface proteins such as CD82, FLT1, FGFR1, and TL10 (Supplementary Fig. S4B), suggesting potential specificity of Bryostatin1 to cell surface molecules. Interestingly, neither cell line demonstrated changes in the CD22 mRNA over the course of Bryostatin1 treatment (Fig. 5B). Using the 756 genes with 2-fold increased expression versus control in KOPN8 and GSEA we found significant enrichment in genes known as intrinsic components of the plasma membrane (Supplementary Table S1). Finally, Bryostatin1-treated cells contained less vacuolated CD22 at 24 hours after exposure compared with DMSO controls suggesting that the increase in CD22 surface expression
mediated by Bryostatin1 may involve changes in membrane trafficking (Supplementary Fig. S4C).

In vitro CD22 CART functionality, but not CD19 CART, is enhanced following Bryostatin1 priming of leukemia cells

We next sought to determine whether Bryostatin1-mediated increase in CD22 site density on leukemic cells could enhance the functionality of CD22 CART. Bryostatin1 pretreatment of both B-ALL and DLBCL cell lines significantly enhanced in vitro production of IL2, IFNγ, and Granzyme B by the CD22 CAR (Fig. 5C–E). Conversely, the CD19 CAR did not show improved Granzyme B production with the leukemia exposure to Bryostatin1 (Supplementary Fig. S5A). However, Bryostatin1 did not reduce efficacy of the CD19 CAR in vivo (Supplementary Fig. S5B).

Exposure of CART to Bryostatin1 attenuates cytokine production but does not affect in vitro or in vivo cytotoxicity

Bryostatin1 modulates PKC isoforms which play an important role in T-cell receptor signaling pathways (25, 26).
Figure 3. A total of 1 x 10^5 tumor cells were cocultured with 1 x 10^5 mock, CD22, or CD22^{V1} CAR and assessed for IFNγ and IL2 cytokines by ELISA from cell culture supernatants [statistics were calculated using paired t test (*, P < 0.0332)]. A, Annexin V staining was assessed over time using IncuCyte ZOOM (B). These data are representative of two separate experiments and were consistent across two different effector-to-target ratios. C, NSG mice were injected with 1 x 10^6 GPF-positive CD22neg, CD22lo, or Nalm6 tumor cells on day 0. On day 3, 5 x 10^6 CD22 or CD22^{V1} CAR were injected for treatment. Mice were imaged using IVIS technology and luciferin-D intraperitoneal injections. Luminescence quantification is shown on the right. These data are representative of two separate experiments. D, CD22 or CD22^{V1} CART was coincubated with tumor cells. On days 1 and 8, CAR was harvested, stained for flow cytometry, and analyzed on Fortessa flow machine. Statistics were calculated using unpaired t test (*, P < 0.0332). E and F, Mice were injected with CD22^{lo} or Nalm6 leukemia on day 0. A total of 5 x 10^6 CD22 CAR T cells were administered on day 3, and mice were sacrificed on day 16. Bone marrow cells were stained for flow cytometry and analyzed on Fortessa flow machine. Statistics were calculated using unpaired t test (****, P < 0.0001; ***, P < 0.0332).
Bryostatin1 upregulates CD22, but not CD19, and CD22 increased expression is durable for 1 week after drug exposure. A, Four cell lines were coincubated with 1 nmol/L Bryostatin1 for 24 hours and analyzed using flow cytometry 1 day after Bryostatin1 exposure. Site density was analyzed through use of standardized Quantibrite-PE beads. Statistics were calculated using unpaired t test (**, P < 0.0002; ***, P < 0.0021; ****, P < 0.0332). B, Cell lines were coincubated with 1 nmol/L Bryostatin1 for 24 hours, washed, and analyzed using flow cytometry at 1 and 7 days after Bryostatin1 exposure. MFI fold change = CD22 MFI\textit{Bryostatin1}/CD22 MFI\textit{DMSO}. C, NSG mice were injected with 1 × 10⁶ GFP-positive Nalm6 leukemia cells on day 0. Bryostatin1 was administered at 0.8 mg/kg on day 3. Mice were sacrificed 7 and 12 days after Bryostatin1 injection, and CD22 was evaluated through flow cytometry. MFI fold change = CD22 MFI\textit{Bryostatin1}/CD22 MFI\textit{DMSO}. D, CD22-low relapse PDX cell line was cultured in vitro with 1 nmol/L Bryostatin1 for 24 hours and measured for CD22 expression using CD22 antibody.
RNA-seq analysis shows no substantive difference in CD22 expression; Bryostatin1-mediated increased CD22 augments CART potency; and CART exposure to Bryostatin1 decreases IFNγ production but increases Granzyme B production, and does not adversely affect tumor clearance in vitro or in vivo. A and B, KOPN8 or SEM cell lines were exposed to 1 nmol/L Bryostatin1 for 16, 24, 48, or 72 hours (h). RNA was extracted and analyzed by RNA-seq. Cell lines were coincubated with 1 nmol/L of Bryostatin1 for 24 hours. Then 1 × 10^5 target tumor cells were cocultured with 1 × 10^5 CD22 CAR for 16 hours. IL2 (C), IFNγ (D), and granzyme B (E) were measured by ELISA from cell culture supernatants. Statistics were calculated using unpaired t-test (***, P < 0.0001; **, P < 0.0021; *, P < 0.0332). CD22 CART cells were coincubated with 1 nmol/L of Bryostatin1 for 24 hours and washed. Then 1 × 10^5 target tumor cells were cocultured with 1 × 10^5 CD22 CAR for 18 hours. IFNγ (F) and granzyme B (G) were measured by ELISA from cell culture supernatants. Statistics were calculated using unpaired t test (***, P < 0.0001; **, P < 0.0021; *, P < 0.0332). H, Mock or CD22 CAR T cells were coincubated at 1:1 effector-to-target ratio with either CD22neg, CD22lo, or Nalm6 cells. Cell death was monitored by loss of GFP-positive cells using IncuCyte ZOOM. I, NSG mice were injected with CD22neg, CD22lo, or Nalm6 on day 0, CD22 CAR T cells on day 3, and then were given either DMSO control or Bryostatin1 at 40 μg/kg once weekly for 2 weeks. Leukemia progression was monitored using IVIS technology and luciferin-D intraperitoneal injections.

Figure 5.
Target Antigen Density Modulation Affects CAR Functionality
the ultimate goal of utilizing Bryostatin1 in combination with CAR therapy, we evaluated the direct impact of Bryostatin1 on CD22 CAR functionality, independent of its effects on leukemic site density. CD22 CART pretreated with Bryostatin1 produced attenuated levels of IFNγ when subsequently cocultured with ALL or DLBCL cell lines relative to DMSO control pretreated CD22 CAR, but produced significantly increased levels of Granzyme B (Fig. 5F and G). In vitro cytotoxicity kinetics of Bryostatin1-exposed CART was similar to DMSO-exposed CART (Fig. 5H). To evaluate the combined effect of Bryostatin1 on leukemic CD22 site density and CART functionality we repeated functional assays using Bryostatin1-treated leukemia with Bryostatin1-exposed CART. Attenuated IFNγ production by CART-exposed cells was restored (Fig. 5F) and Granzyme B production was augmented (Fig. 5G) in the presence of Bryostatin1 pretreated leukemia cells. Finally, we evaluated Bryostatin1 effects on in vivo CAR activity against CD22lo leukemia, which does not modulate CD22 expression following Bryostatin1 exposure (Supplementary Fig. S4D). Bryostatin1 did not impact the ability of the CD22 CAR to attenuate progression of the CD22lo leukemia or CD22 CAR ability to clear the Nalm6 leukemia in vivo (Fig. 5I; Supplementary Fig. S6A). Together, these findings demonstrate that exposing CART to Bryostatin1 affects IFNγ and Granzyme B production, but does not negatively impact overall CART functionality in vivo.

**Bryostatin1 affects CART persistence and memory phenotype, and improves durability of leukemic response**

Finally, we evaluated the impact of Bryostatin1-mediated increase in CD22 site density on in vivo CAR persistence and functionality using the parental Nalm6, which does not express sufficient CD22 to maximize cytokine response (Supplementary Fig. S1) and does modulate CD22 expression in response to Bryostatin1 (Fig. 4). We tested the administration of Bryostatin1 as a “priming therapy” prior to CART infusion. Mice were injected with Bryostatin1 pretreated tumor cells, followed by CART injection. Seven days after CART injection, T-cell phenotype was evaluated. Within the CD8+ T-cell population, we found significant cumulative enrichment of central memory (CCR7+, CD45RA⁻) and effector memory (CCR7−, CD45RA−) cells, and fewer naïve cells, in the spleens of mice that received Bryostatin1-pretreated Nalm6 (Fig. 6A). With Bryostatin1 exposure, CART demonstrated similar activation without evidence of exhaustion, as evidenced by stable PD1 expression, without increase in TIM3 or LAG3 (Supplementary Fig. S5A). At 30 days, mice treated with Bryostatin1 after CART injection had slightly increased CD22 CART in bone marrow (Fig. 6B). We then tested Bryostatin1 administration for 2 weeks after subcurative dose of CD22 CART infusion in Nalm6 (Fig. 6C; Supplementary Fig. S6B) and SEM (Fig. 6C; Supplementary Fig. S6C), demonstrating that Bryostatin1 improved durability of remission, extending beyond cessation of Bryostatin1. Finally, we evaluated the effects of Bryostatin1 administration following CD22 CART infusion the CD22-low relapse PDX model, demonstrating improved ability to clear leukemia (Fig. 6D; Supplementary Fig. S6D). Collectively, these results demonstrate that Bryostatin1 is effective as both a priming therapy to increase antigen expression prior to CAR T-cell infusion and as a potential rescue following emergence of CD22 CAR-resistant leukemia.

**Discussion**

CAR-T therapy has provided patients with relapsed or refractory pre-B-cell ALL a potentially curative therapeutic option, but remission duration is shortened in a high percentage of patients due to changes in target antigen expression. Furthermore, recent studies have begun to identify limitations in CAR activity in the setting of low antigen site density (11–15, 27–33). Our recent clinical experience with a CD22 CAR supports low antigen density as a mechanism of escape from CAR-targeted therapy (11). Here, we evaluate the characteristics of CAR failure in the setting of low site density, focusing on the effects on CAR function and persistence. Moreover, we provide the first preclinical evidence to support the use of drug-mediated antigen modulation to overcome such limitations. Our data establishes two important findings: (i) low site density on tumor cells results in significant changes in the persistence and phenotype of CART, and (ii) drug-mediated increase in target antigen site density improves CAR T-cell functionality and length of remission.

We first assessed the effects of site density on short-term CART functionality, and observed a reduction in cytokine production as target antigen expression declines, consistent with previous studies (12, 15, 32, 33). We further demonstrate that the CD22 CAR delayed in vivo progression of, but failed to clear, low site density leukemia. Although activation and exhaustion markers, including PD1, TIM3, and LAG3, were not significantly different on CART exposed to low site density leukemia, lower site density leukemia induced a significant decrease in numbers of persisting CART, with a more naïve memory phenotype, suggesting diminished T-cell expansion and conversion to memory phenotype. Whether this finding represents a recruitment of less CAR-expressing T cells into the expansion pool or less potent activation of the same percentage of CAR-expressing T cells will require further testing. This is the first clear evidence that site density not only affects short-term activity of CART, but also affects long-term persistence, with implications for the durability of CART-induced remissions. However, as these findings were in a xenograft model, it is impossible to ascertain the exact implications for CAR persistence in an immunocompetent host, in which nonleukemic expression of CAR antigen could impact sustained expansion of the CART. Thus, these findings require confirmation in immunocompetent models or in patients.

We next proceeded to evaluate potential strategies to overcome the limitations on cellular therapy imposed by low antigen density, through either improving CAR functionality or augmenting tumor sensitivity. We attempted to improve CAR functionality through enhancing the affinity of the CD22 CAR, thereby improving sensitivity to low site density. Although binding affinity can improve the efficacy of soluble antibody-based therapeutics (34), our data does not demonstrate improvement in CAR functionality with increased scFv affinity. Interestingly, while CD22V1 CAR had a larger naïve T-cell population, especially against CD22lo leukemia, it also demonstrated a more pronounced terminally differentiated effector memory population (CCR7+, CD45RA−), suggesting that when the T cell is activated by the CD22V1 CAR, it may become terminally differentiated rather than converting to a memory phenotype. While this data is supported by experience with some CARs (35), the complexity of individual CAR constructs may preclude the ability to generalize these findings (28, 36).
Bryostatin1 treatment pre-CAR infusion alters T-cell phenotype without T-cell exhaustion, and post-CAR infusion improves durability of remission in vivo. A, Nalm6 cells were exposed to 1 nmol/L Bryostatin1 for 24 hours, then injected into mice on day 0. CD22 CAR T cells were administered on day 3, and mice were sacrificed on day 10. Bone marrow cells were stained for flow cytometry and analyzed on Fortessa flow machine. Statistics were calculated using unpaired t test (*, P < 0.0332). B, NSG mice were injected with Nalm6 on day 0, CD22 CART on day 3, and then were given either DMSO control or Bryostatin1 at 40 mg/kg once weekly for 2 weeks. Mice were sacrificed 30 days after tumor injection. Cells were stained for flow cytometry and analyzed on Fortessa flow machine. C, NSG mice were injected with 1/10^6 GPF-positive Nalm6 or SEM tumor cells on day 0. On day 3, either 3/10^6 (Nalm6) or 2/10^6 (SEM) mock or CD22 CAR were injected for treatment. Mice were given 40 μg/kg of Bryostatin1 or DMSO once weekly for 2 weeks. Mice were imaged using IVIS technology and luciferin-D intraperitoneal injections. D, NSG mice were injected with 1 × 10^6 GPF-positive PDX tumor cells on day 0. On day 42, 3 × 10^6 Mock or CD22 CAR were injected for treatment. Mice were given 40 μg/kg of Bryostatin1 or DMSO once weekly for 2 weeks starting on day 45. Mice were imaged using IVIS technology and luciferin-D intraperitoneal injections.
To improve tumor sensitivity, we found that Bryostatin1 upregulated CD22 expression in ALL and DLBCL cell lines, as well as in a CD22-low relapse PDX, thereby improving CART cytokine production and memory phenotype. As a modulator of PKC, Bryostatin1 has variable effects on PKC protein levels based on exposure parameters (37–41). In CLL cell lines, Bryostatin1-mediated upregulation of CD22 correlated with decreased PKC (22). We found that Bryostatin1 was associated with decreased PKCβII, but PKC inhibitors did not provide the same upregulation of CD22 as Bryostatin1. We also found that CD22 mRNA levels were not significantly altered in Bryostatin1-treated ALL cell lines and that multiple cell surface molecules were upregulated. These findings suggest that Bryostatin1 has a much more complex mechanism of upregulation of CD22, but further evaluation will be needed to elucidate the exact mechanism of CD22 upregulation.

We demonstrate that CAR functionality can be indirectly improved via Bryostatin1-mediated increase in CD22 expression. However, Bryostatin1 has been previously implicated in directly affecting T-cell function (25, 26). Although Bryostatin1 attenuated IFNγ production by CD22 CART, Granzyme B production was increased and there was ultimately no adverse effect on in vitro or in vivo cytotoxicity. The net effect of Bryostatin1 on CART functionality and antigen expression level resulted in overall augmented cytokine production and cytotoxicity, and improved durability of response. This indicates that Bryostatin1 could be administered during CART expansion or to rescue patients following post-CART relapse resulting from reduced antigen expression. However, we also postulate that Bryostatin1 could be used to prime tumors prior to CART therapy based on our observation that site density affects CAR T-cell persistence.

While combination therapies are the cornerstone of oncologic treatments, CART has not yet been combined with other drugs with the intent of augmenting sensitivity to low antigen levels. Prior preclinical studies have shown improved CAR activity with drug modulation (14, 42); however, to our knowledge, our findings are the first to describe how drug-induced increased antigen expression can improve CAR T-cell activity. Bryostatin1 has been used in clinical trials for oncologic patients with more than 1,400 individuals treated, including one phase I pediatric clinical trial. The pediatric phase I study showed the drug to be well-tolerated, but recommended increasing the dose administration in subsequent trials (43). While adult phase I and II studies have established the MTD, the measured effect was tumor response and PKC inhibition rather than surface protein expression. Our data supports the concept of using Bryostatin1 to modulate target antigen density in combination with a targeted immunotherapy such as CART, but clinical experience is needed in pediatric patients specifically characterizing CD22 antigen expression as a biomarker to establish Bryostatin1 dosing.

Taken together, our observations contribute to an understanding of the complexity in developing effective CAR therapies in tumors where lower expression is a characteristic of the targeted antigen. Because CD22 expression has been targeted successfully by immunotherapy and is likely comparable with many other antigens currently being evaluated (12, 28), CD22 represents a broadly applicable and clinically relevant model antigen. As such, the effects of antigen expression on CAR persistence and phenotype described here may be pertinent to many other potential target antigens. Finally, our findings with Bryostatin1 support the initiation of trials testing whether drug-mediated upregulation of antigen expression can improve efficacy and remission durability following targeted immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Target Antigen Density Modulation Affects CAR Functionality


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