Multiomic Profiling of Central Nervous System Leukemia Identifies mRNA Translation as a Therapeutic Target

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ABSTRACT

Central nervous system (CNS) dissemination of B-precursor acute lymphoblastic leukemia (B-ALL) has poor prognosis and remains a therapeutic challenge. Here we performed targeted DNA sequencing as well as transcriptional and proteomic profiling of paired leukemia-infiltrating cells in the bone marrow (BM) and CNS of xenografts. Genes governing mRNA translation were upregulated in CNS leukemia, and subclonal genetic profiling confirmed this in both BM-concordant and BM-discordant CNS mutational populations. CNS leukemia cells were exquisitely sensitive to the translation inhibitor omacetaxine mepesuccinate, which reduced xenograft leptomeningeal disease burden. Proteomics demonstrated greater abundance of secreted proteins in CNS-infiltrating cells, including complement component 3 (C3), and drug targeting of C3 influenced CNS disease in xenografts. CNS-infiltrating cells also exhibited selection for stemness traits and metabolic reprogramming. Overall, our study identifies targeting of mRNA translation as a potential therapeutic approach for B-ALL leptomeningeal disease.

SIGNIFICANCE: Cancer metastases are often driven by distinct subclones with unique biological properties. Here we show that in B-ALL CNS disease, the leptomeningeal environment selects for cells with unique functional dependencies. Pharmacologic inhibition of mRNA translation signaling treats CNS disease and offers a new therapeutic approach for this condition.

INTRODUCTION

Early successes with chemotherapy for B-precursor acute lymphoblastic leukemia (B-ALL) were almost uniformly complicated by relapses in the leptomeninges of the central nervous system (CNS; refs. 1–3). The CNS was deemed a sanctuary site for relapse with sparing of resident leukemic cells due to immune privilege and poor blood–brain barrier penetration of systemic chemotherapies. This led to the implementation of universal CNS-directed therapy with craniospinal irradiation and/or intrathecal chemotherapy—treatments that are associated with long-term effects on neurocognitive function (1). Despite this aggressive treatment strategy, 30% of relapsed B-ALL patients have CNS involvement (4, 5). CNS relapse has a poor prognosis due in part to a lack of effective targeted therapies for CNS leukemia (2, 4–6). Thus, there is a great need for new therapies to treat CNS dissemination in B-ALL.

B-ALL leptomeningeal disease can be recapitulated through xenotransplantation of primary B-ALL patient samples into immunodeficient mice (7–10). Patient-derived xenografts are tractable models for interrogating B-ALL clonal dynamics and therapeutic responses. Like the majority of human patients prior to the introduction of CNS-directed therapies, many mice bearing B-ALL xenografts develop CNS disease (7–10). Human B-ALL samples exhibit intratumoral heterogeneity and are composed of genetically and functionally distinct subclones, though the impact of such diversity on CNS engraftment is unclear (7, 11, 12). Although several prior studies did not find evidence of subclonal selection in the leukemic cells in the CNS (8–10), we recently reported subclonal differences between select pairs of bone marrow (BM) and CNS in xenografts (7). Functional studies have to date largely focused on mechanisms of B-ALL trafficking to the subarachnoid space, a process dependent on signaling through VEGFA (8, 13), IL7 (14), IL15 (15), and ITGA6 (16). However, knowledge of the factors governing B-ALL survival and proliferation within the CNS remains limited.

In this study, we used the B-ALL xenograft model to identify novel requirements for leukemic CNS involvement. Using RNA sequencing (RNA-seq), we identified mRNA translation as a requirement for B-ALL disease in the CNS irrespective of clonal selection and validated this pharmacologically
in xenografts with the mRNA translation inhibitor omacetaxine mepesuccinate (OMA). Proteomic analysis suggested increased production of complement component 3 (C3) in CNS xenografts, which we demonstrated to be a key determinant of CNS disease.

RESULTS

Clonal Composition of BM and CNS Blasts in B-ALL Xenografts

We previously carried out xenografting of paired diagnosis and relapse samples from a cohort of pediatric and adult B-ALL patients (7). The diagnosis and relapse samples of 10 patients (7 diagnosis samples and 10 relapse samples) generated significant CNS disease in xenografts (Supplementary Fig. S1A–S1G; Supplementary Table S1). Leukemic blasts were isolated from dissociated CNS material using Percoll density gradient centrifugation and used for our genomic, transcriptomic, and proteomic studies. CNS cells retained the ability to engraft secondary xenografts in the BM, spleen, and CNS following intraperitoneal injection as demonstrated for three primary KMT2A-rearranged samples (Supplementary Fig. S1H–S1J). We used targeted DNA sequencing described in our previous study (7) to gain further insight into the genetic subclonal identity and clonal dynamics in the BM and CNS of primary xenografts of all 10 patient samples (Supplementary Table S2; ref. 7). We used Pairtree (17), the computational tool reported in ref. 10, to quantify the relative prevalence of mutational populations composed of leukemic variants with similar variant allele frequencies in the BM (composed of cells from bilateral femurs and tibias) and CNS of a given recipient mouse for 106 xenografts. Using Bayes factors, we compared a “concordant” model in which leukemic blasts of individual mice shared mutational population frequencies between BM and CNS with a “discordant” model in which the BM and CNS mutational populations could have different frequencies (Fig. 1A–C). We deemed BM and CNS blasts to be genetically discordant if the discordant model was at least 100 times as likely as the concordant model, reflecting the consistently different frequencies of mutational populations between paired BM and CNS samples from individual mice. For diagnosis samples, we found that BM and CNS grafts showed little variation and were almost uniformly concordant, with only 15% demonstrating discordance (6/40 mice; Fig. 1A, C, and D; Supplementary Fig. S2A–S2J). By contrast, 42 of 66 xenografts (64%) generated from relapse samples showed discordance in mutational populations present in BM and CNS of individual mice (Fig. 1B, C, E, and F; Supplementary Fig. S2A–S2J). In the relapse sample of patient 11, as was the case for 7 of 10 patients (14 of 17 samples), no recurrent expansion of any one mutational population was detected in the BM or CNS of multiple mice (Fig. 1E; Supplementary Fig. S2B–S2J). On the contrary, grafts generated from three samples (patient 1 relapse, patient 12 relapse, and patient 13 relapse) exhibited evidence of enrichment for one or more mutational populations in two or more xenografts in BM and/or CNS xenografts (Fig. 1F; Supplementary Fig. S2B and S2J). For example, CNS xenografts derived from the patient 12 relapse sample displayed enrichment for population 5 compared with BM in six xenografts, whereas BM grafts showed greater subclonal diversity with enrichment of populations 6 and 7 (Fig. 1F and G). These data suggest that in many cases, particularly at diagnosis, there is considerable genetic discordance between subclonal diversity of BM and CNS. However, there are also patient samples where genetic discordance can be found between the mutational populations present in the BM and CNS of individual mice, and in some patients, tissue-specific selection for individual subclones occurs; this is more prevalent in relapse samples.

Leukemia Cells that Disseminate to the CNS Are Transcriptionally Distinct from Cells in the BM Irrespective of Subclonal Composition

To define the transcriptional landscape of leukemia cells present in the CNS, we performed RNA-seq on 43 pairs of matched BM (cells from bilateral femurs and tibias) and CNS obtained from xenografts from 6 B-ALL patients (patient 6 n = 2 xenografts, patient 7 n = 10, patient 11 n = 9, patient 12 n = 18, patient 13 n = 1, and patient 15 n = 3) with high burden of CNS disease. Xenograft leukemia cells were grouped principally based on the patient from which they were derived (Fig. 2A). However, CNS sites were highly transcriptionally divergent from matched BM sites based on differential gene expression, multidimensional scaling, and unsupervised hierarchical clustering, with 381 CNS-upregulated and 1,064 BM-upregulated genes with greater than twofold change and a false discovery rate (FDR) < 0.05 (Fig. 2B; Supplementary...
Figure 2. BM and CNS blasts are transcriptionally distinct. A, Multidimensional scaling of RNA-seq gene counts from matched BM [cells from bilateral femurs and tibias; circles] and CNS (triangles) xenografts derived from patient 6 (n = 2 mice), patient 7 (n = 10 mice), patient 11 (n = 9 mice), patient 12 (n = 18 mice), patient 13 (n = 1 mouse), and patient 15 (n = 3 mice). B, Unsupervised hierarchical clustering of matched BM and CNS xenografts based on the normalized gene counts per million (cpm) of the top 100 CNS-upregulated genes by FDR. C, Normalized counts per million for genes indicated in graph title are shown for n = 43 BM and n = 43 CNS xenografts. Line, mean; ****, FDR < 0.001 corrected for multiple hypothesis testing in edgeR. D and E, Expression of the top 100 CNS-upregulated genes from Fig. 1C in the BM and CNS of samples that were also analyzed by targeted sequencing and reported to be genetically concordant (D; n = 21) and discordant (E; n = 28) xenografts from patients 7, 11, 12, and 13, diagnosis and relapse, with samples grouped by unsupervised hierarchical clustering with expression intensity scaled by gene.
Fig. S3A–S3H). Multiple genes previously associated with CNS dissemination including SCD, VEGFA, ITGA6, and ZAP70 were also upregulated in CNS grafts in our cohort (Fig. 2C; refs. 8, 13, 14, 16, 18, 19). This analysis revealed that CNS blast samples showed a common subset of genes that are significantly upregulated (Fig. 2B and C). Notably, the genes defining the CNS-derived blasts were upregulated in both genetically concordant and discordant CNS blasts (Fig. 2D and E). These data indicate that in B-ALL xenografts, cells in the CNS microenvironment have a distinct transcriptional phenotype compared with those isolated from the BM irrespective of subclonal composition. This may arise from the unique selection pressures in the leptomeningeal microenvironment (20, 21).

**CNS-Disseminated Blasts Transcriptionally and Phenotypically Resemble Therapy-Resistant Cells**

As described in our previous study (10), pathway analysis is more sensitive than simple gene-expression comparisons to find consistent differences between patient samples with widely diverse genetic drivers. Thus, we extended our gene-expression analysis to more broadly investigate the transcriptomic pathways driving the growth of leukemia cells in the CNS across all xenografts. We used gene set enrichment analysis (GSEA; of the data shown in Fig. 2A) for unbiased detection of pathways differentially expressed between blasts in BM and CNS (22). Network analysis grouping differentially expressed gene sets by pathway revealed the top CNS-enriched processes to be the proteasome, mRNA translation initiation and elongation, EGFR signaling, FGF signaling, glycolysis, endoplasmic reticulum (ER) transport, vesicle secretion, and mRNA splicing (Fig. 3A). Relative to matched CNS blasts, BM blasts were enriched for processes including DNA replication and DNA repair (Fig. 3A).

Prior studies have shown that treatment-resistant B-ALL xenografts and high-risk diagnosis or relapse B-ALL patient samples express hematopoietic stem cell (HSC) genes and undergo metabolic rewiring that is linked to a stress-tolerant state (7, 23–25). We found similar upregulation of HSC as well as acute myeloid leukemia (AML) stem cell (LSC) gene sets in CNS blasts (Fig. 3B). Furthermore, pathways associated with chemotherapy resistance were upregulated in both concordant and discordant CNS blasts (Fig. 3B). CNS blasts also showed evidence of the metabolic rewiring associated with chemotherapy tolerance and subsequent B-ALL relapse (7), with significant enrichment of gene sets related to mitochondrial translation, oxidative phosphorylation, and mammalian target of rapamycin (mTOR) signaling (Fig. 3B). In keeping with transcriptional upregulation of oxidative phosphorylation and mitochondrial translation gene sets, we performed functional analysis on primary KMT2A-rearranged xenograft cells using flow cytometry and Seahorse XF analyzer. Evaluation of mitochondrial membrane potential and mitochondrial mass between BM and CNS cells using tetramethylrhodamine ethyl ester (TMRE) and MitoTracker Green demonstrated greater mitochondrial membrane potential per unit mass in CNS blasts than in BM blasts from two of three patient samples (Supplementary Fig. S4A–S4C). Moreover, measuring oxygen consumption rate (OCR) in CNS blasts revealed a remarkable increase in basal mitochondrial respiration, proton leak, and a trend toward greater mitochondrial ATP production (Fig. 3C–F; Supplementary Fig. S4D). We also demonstrated an elevated maximal mitochondrial respiration along with greater mitochondrial spare capacity in CNS blasts over BM blasts (Fig. 3G; Supplementary Fig. S4E). This suggests that CNS blasts have extra mitochondrial reserve capacity available in order to produce more energy or mitochondrial ROS if needed. Overall, CNS blasts have a transcriptional profile and metabolic phenotype that resemble chemotherapy-resistant subclones, and converge on a stress-tolerant state (7, 23–25). Although our study focused on a static analysis of blasts present in the CNS and was not designed to investigate whether these distinct functional properties specifically impact the trafficking, survival, or growth of blasts in the leptomeningeal space, our data argue that improving treatment of CNS disease will require targeting of molecular pathways driving these essential processes.

**OMA Inhibits mRNA Translation and Impairs CNS Engraftment**

To provide proof of principle that the unique properties we uncovered represent critical dependencies for B-ALL CNS involvement, we focused on mRNA translation as a targetable process that drives CNS disease because mRNA translation and ribosomal biogenesis were the second most highly enriched biological process in CNS blasts (Fig. 4A). Hierarchical clustering and differential expression analysis confirmed upregulation of the leading edge genes from the Reactome “Translation” gene set described in Fig. 4A in the CNS blasts from all six patients (Supplementary Fig. S5A–S5F).

To test whether CNS-disseminated B-ALL blasts may be preferentially sensitive to perturbations in protein synthesis, we assessed the effects of in vivo treatment of xenografts with OMA. OMA is an inhibitor of mRNA translation that blocks the ribosome A site and has good blood–brain barrier penetration to the CNS (26). OMA is currently used to treat chronic myelogenous leukemia, is being trialed in AML, and showed activity in a preclinical model of breast cancer metastasis, but was not effective in an early-phase clinical trial against lymphoid leukemias (27–30). Intraperitoneal injection of OMA decreased the rate of mRNA translation in CNS blasts (Fig. 4B and C). Label-free liquid chromatography–mass spectrometry (LC-MS) analysis of CNS blasts from saline- and OMA-treated mice revealed a widespread decrease in protein abundance, with significant downregulation of proteins governing mitosis, negative regulation of B-cell apoptosis, and RNA catabolism (Fig. 4D). In mice with established B-ALL engraftment treated for 4 days (patients 6, 7, and 12) or 3 days (patient 15 due to high pretreatment leukemic morbidity), OMA significantly decreased B-ALL disease burden in the CNS and spleen, with a trend toward a smaller magnitude reduction in BM engraftment that reached significance in xenografts from one patient sample (Fig. 4E; Supplementary Fig. S5G and S5H). Our data strongly suggest that B-ALL CNS disease is critically dependent on mRNA translation.

**Posttranscriptional Upregulation of Secretory Machinery and Complement Cascade in CNS Blasts**

To determine the mechanism by which differences in mRNA translation of CNS blasts might promote B-ALL leptomeningeal disease, we first focused on translation rates. Because
**Figure 3.** CNS xenografts transcriptionally and metabolically resemble therapy-resistant cells. **A,** Cytoscape map of GSEA-identified differentially enriched gene sets upregulated in CNS and BM xenografts with the top 10 differentially regulated pathways labeled using AutoAnnotate. Node size is proportional to FDRq value of enrichment, and green edges indicate gene overlap between nodes. **B,** GSEA results showing normalized enrichment scores in CNS versus BM xenograft transcriptomes for gene sets previously associated with B-ALL chemotherapy resistance and clinical relapse in all profiled xenografts (n = 43), concordant xenografts (n = 21), and discordant xenografts (n = 28). *, FDRq score < 0.05. **C,** Pooled analysis of OCR measured by Seahorse XFe 96 analyzer with additions of oligomycin A (Oligo), carbonyl cyanide-4-phenylhydrazone (FCCP), antimycin A (Anti A), and rotenone (Rot) from BM and CNS KMT2A-rearranged xenografts (n = 3 mice patient 12 and n = 3 mice patient 15, n = 15 replicate wells). Basal respiration (**D**), ATP-linked respiration (**E**), proton leak respiration (**F**), and maximal (Max.) OCR across BM (**G**) and CNS (**G**) xenografts. Bars, mean ± SE; P values derived from two-sided unpaired Student t test (**D–G**), with *, P < 0.05; ***, P < 0.01; ns, not significant.
Figure 4. Blocking mRNA translation as a therapeutic strategy for CNS disease. A, Waveplot from GSEA in Fig. 2A showing the Reactome “Translation” gene set, the third most highly enriched gene set in CNS blasts (positively correlated). Normalized enrichment score (NES) = 7.66; FDRq < 0.001. B, B-ALL xenografts generated by intrafemoral injection of BM blasts (patients 6 and 7) or CNS blasts (patients 12 and 15) were monitored for leukemic engraftment and then treated with 1 mg/kg OMA daily for 4 days or 3 days if significant leukemic morbidity was observed (3 days patient 15; 4 days patients 6, 7, and 12) prior to analysis. C, Translation rate in CNS blasts of saline or OMA-treated patient 12 xenografts measured by O-propargyl-puromycin (OPP) incorporation (n = 5 mice). MFI, mean fluorescence intensity. D, Pathways significantly downregulated in proteomic analysis of CNS blasts in OMA-treated xenografts were identified by ClueGO analysis with Markov Cluster Algorithm (MCL) clustering, displayed as a Cytoscape enrichment map of nodes colored by gene ontology process, with size representing significance and edges showing connection between proteins in different nodes. E, Human B-ALL engraftment in tissues of NSG xenografts treated as in B, with human CD19+CD45+ cell counts normalized to saline-treated controls from the day of sacrifice (n = 5 mice per patient for vehicle; n = 5 mice per patient 6, 7, 12, and 4 mice for patient 15 for OMA). In plots, bars represent mean ± SE; two-sided unpaired t test, with *, P < 0.05; **, P < 0.01; ns, not significant.
increased mRNA translation rates are found in a number of metastatic tumors (31–33), we measured incorporation of the amino acid analogue O-propargyl-puromycin (OPP) in BM and CNS. There was no common trend toward altered CNS translation rate across xenografts from three patients, all of whom had KMT2A-rearranged leukemia (Fig. 5A). We then performed LC-MS on matched BM and CNS derived from these B-ALL patients to identify whole-proteome differences between the sites. Networks of proteins related to synaptic vesicles, secretory granules, neuronal components, and extracellular matrix were identified in CNS, with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis demonstrating protein enrichment of previously described mediators of CNS disease, including VEGF signaling and glycolysis (Fig. 5B; Supplementary Fig. S6A; Supplementary Table S3; refs. 8, 13).

Because CNS blasts did not consistently differ from BM blasts in their rate of global protein synthesis, we reasoned that CNS-engrafting cells may depend on preferential translation of a specific subset of mRNAs, a phenomenon that occurs in HSCs and embryonic stem cells (34, 35). We searched for pathways in CNS blast-upregulated proteins whose encoding mRNAs were not differentially expressed between BM and CNS sites. The alternative complement signaling cascade was among the most highly upregulated proteome pathways in non–differentially expressed mRNAs (Fig. 5C). Notably, complement signaling was also the most highly upregulated pathway in CNS blasts among all secreted proteins (Supplementary Fig. S6B) and was enriched across CNS samples from xenografts of all three patients studied regardless of mRNA transcript level (Supplementary Fig. S6A).

**Complement Component 3 Promotes Leptomeningeal Disease**

To validate that the complement signaling pathway was functionally relevant, we focused on complement C3. Following activation of the lectin, classic, or alternative pathways, cleavage of C3 triggers opsonization, cell lysis, or local inflammation depending on the cleavage product and cellular milieu (36). C3 convertase is a serine protease that cleaves C3 into the inflammatory anaphylotoxin C3a and C3b (36). C3 production by metastatic breast and lung carcinoma cells promotes survival in the leptomeningeal space by paracrine signaling through C3a (37). Our proteomic data showed that C3 was more abundant in CNS blasts (Supplementary Table S3). To test the role of C3a signaling in CNS disease, we treated B-ALL primary KMT2A-rearranged xenografts derived from a pediatric (patient 12) and an adult (patient 15) sample with SB 290157, a small-molecule inhibitor of the G protein–coupled C3a receptor shown to be effective in xenograft models of breast and lung carcinoma (ref. 37; Fig. 5D). Mice were treated from the week after cell transplantation until endpoint. SB 290157 decreased leptomeningeal B-ALL engraftment by an average of 50% in CNS xenografts (P = 0.10 patient 12, P = 0.01 patient 15, n = 7 mice DMSO, n = 7 mice SB 290157 per patient) but had no effect on BM or spleen engraftment (Fig. 5E; Supplementary Fig. S6C).

Next, we tested the effect of a C3a receptor agonist on the secondary transplants of BM from KMT2A-rearranged B-ALL xenografts derived from two pediatric (patients 11 and 12) and one adult (patient 15) sample (Fig. 5F). C3a receptor activation from the time of cell transplantation to endpoint produced a consistent increase in CNS engraftment across recipients, though this only reached statistical significance in xenografts from patient 11 (patient 11 P = 0.004, patient 12 P = 0.37, patient 15 P = 0.081) without impacting BM or spleen engraftment (Fig. 5G; Supplementary Fig. S6D).

To distinguish between the possibility of complement C3a mediating B-ALL dissemination to or invasion of the leptomeninges from C3a-mediated survival within the CNS, we treated mice with established xenografts with SB 290157, C3a receptor agonist, or vehicle immediately prior to endpoint (Fig. 5H). Unlike OMA, treatment of established B-ALL xenografts with SB 290157 or C3a receptor agonist did not appreciably alter disease burden or cell cycling, or induce apoptosis (Fig. 5H–K; Supplementary Fig. S6E). Similarly, dose–response studies of both SB 290157 and the C3a receptor agonist showed no effect on the t(4;11) B-ALL cell line RS4;11 in vitro (Supplementary Fig. S6F). These results suggest that posttranscriptional upregulation of C3 by B-ALL increases CNS disease in vivo by promoting dissemination to rather than survival within the leptomeninges.

**DISCUSSION**

Here, we have used genomic and proteomic profiling to describe the clonality and biology of B-ALL CNS xenografts...
and uncovered novel dependencies for CNS involvement in B-ALL. By combining transcriptomic and proteomic profiling in a preclinical xenograft model, we identified altered mRNA translation as a novel regulator and therapeutic vulnerability of CNS disease. Broadly, CNS blasts showed evidence of transcriptional and metabolic rewiring associated with chemotheraphy resistance that distinguished them from BM blasts. We also observed recurrent clonal discordance in BM and CNS engraftment in multiple samples from a large patient cohort, with evidence of tissue-specific selection. Despite frequent clonal discordance in populations engrafting the BM and CNS in relapse xenografts, transcriptional and metabolic rewiring was observed in CNS blasts irrespective of clonal selection, suggestive of CNS site-induced changes.

Although the ability to carry out long-term clonal propagation is thought to be a common property among individual B-ALL cells that make up a tumor and the underlying stemness programs are proposed to be plastic in B-ALL, there is clear evidence that B-ALL chemotherapy resistance in vitro and propensity to relapse clinically correlates with a distinct HSC signature expression (7, 24). Additionally, we previously showed activation of mitochondrial translation, oxidative phosphorylation, and mTOR in chemotherapy-resistant, relapse-fated B-ALL (7). Our transcriptomic and metabolic profiling indicated that similar pathways arise in CNS blasts, suggesting that the leptomeningeal environment selects for blasts with enhanced stemness and stress-resistant properties. We hypothesize that these induced cellular properties might relate to their ability to contribute to relapse. The enhanced mitochondrial spare capacity of CNS blasts may reflect an adaptation to the leptomeningeal niche that confers greater physiologic reserve against the stress of chemotherapy (38). Thus, although the CNS is considered a sanctuary site for disease relapse due to immune privilege and poor drug penetration, CNS blasts’ inherent or induced transcriptional and metabolic properties may correlate with a greater ability to withstand chemotherapy. This could contribute to their ability to originate relapse disease and warrants further investigation.

The translation of mRNA to synthesize protein is a fundamental cellular process that is a common driver of malignant growth and metastatic spread across multiple cancers (31–33, 39, 40). Indeed, depletion of L-asparaginase in the serum and, to a lesser extent in the cerebrospinal fluid (CSF), is a cornerstone of B-ALL therapy that works in part through translational inhibition; translational reprogramming can mediate L-asparaginase resistance in some cancer models (41–43). Many translation inhibitors are thus actively being pursued as antineoplastics, including OMA in tyrosine kinase inhibitor–resistant chronic myeloid leukemia and current clinical trials for AML (30, 40). Intriguingly, KMT2A-rearranged AML shows epigenetic upregulation of ribosomal genes by the KMT2A fusion protein and corresponding sensitivity to OMA in vitro (44). Differential activation of ribosomal machinery was identified as a source of intratumoral heterogeneity in single-cell RNA-seq analysis of primary B-ALL, where it correlated with expression of HSC genes (45). We demonstrate a similar upregulation of stem cell genes, ribosomal components, and translation factors in B-ALL CNS blasts. We found that B-ALL disease in the CNS is exquisitely sensitive to translation inhibition with OMA. OMA rapidly reduced levels of the mitotic machinery and B-cell antiapoptotic proteins within the leukemic xenografts, suggesting that their constant production is required to survive and divide in the leptomeningeal space. Interestingly, OMA prolonged survival in a Bcr–Abl-driven murine model of B-ALL, and although CNS disease burden was not studied, it suggests that all blasts are sensitive to some level of mRNA translation inhibition (46). Nevertheless, this dependency on translation was highly contextual because BM blasts were much less sensitive than CNS blasts in our study. Splenic engraftment was also substantially reduced with OMA, though the mechanisms underlying this and any similarities between spleen- and CNS-engrafting blasts remain outside the scope of the current study.

Complement signaling is a proinflammatory mediator of innate and adaptive immunity and thus can both promote and restrict malignant growth depending on context (47). The C3a receptor is present on choroid plexus epithelial and brain endothelial cells, and C3 signaling has been associated with increased blood–brain barrier permeability (48, 49). C3 drives leptomeningeal metastasis of breast and lung adenocarcinoma by permeabilizing the blood–CSF barrier, helping metastatic cells adapt to the nutrient-poor subarachnoid space (37). Moreover, melanoma patients with leptomeningeal disease have elevated C3 in CSF, and although serum levels of C3 are elevated compared with healthy controls at diagnosis in B-ALL, we are not aware of prior links to CNS disease (50, 51). The increase or decrease in leptomeningeal engraftment of B-ALL xenografts with respective C3a receptor activation or inhibition reveals a novel, C3a-dependent mechanism for B-ALL CNS involvement. The lack of pharmacokinetic data on CNS penetration of these compounds is a limitation of the study. Our data suggest convergent evolution on C3a signaling as a driver of leptomeningeal metastasis across cancers that could have broader clinical implications given the devastating consequences of brain involvement for many cancer types. Intriguingly, targeting C3a signaling was highly contextual. When modulated from engraftment to endpoint, C3a signaling altered B-ALL CNS disease burden in vivo, but administration of C3a receptor agonist or antagonist to mice with established grafts did not alter CNS disease burden, CNS blast cell cycling, or apoptosis. Similarly, targeting the C3a receptor did not affect B-ALL growth in vitro. These results suggest that altered translation of secreted proteins, including C3, may promote CNS disease through non–cell-autonomous mechanisms such as increasing trafficking or invasion of the leptomeninges. Conversely, proteomic profiling of OMA-treated blasts demonstrated that cells residing within the leptomeningeal space require continuous production of cell-cycle machinery and antiapoptotic factors to survive.

Overall, our findings show promise for translation to the clinic for patients with B-ALL CNS disease. As OMA has been clinically approved as Synribo (Teva Pharmaceuticals), there is a clear pathway for rapid repurposing and clinical testing in B-ALL based on our findings along with the known ability of Synribo to penetrate the CNS following subcutaneous dosing in patients. Although the effects of the drug were most pronounced on extramedullary disease, there was a
trend toward a treatment effect on BM-engrafted cells. Clinical study would be required to determine whether relapsed B-ALL patients might benefit most from OMA as a single-agent therapy or as part of combination treatment. Safe and effective doses have not yet been established for pediatric patients, and although young adults with B-ALL may be eligible for trials with adult dosing, early-phase clinical testing would be required prior to Synrho’s application to pediatric B-ALL. Targeting CNS disease by inhibiting mRNA translation represents a novel therapeutic strategy for B-ALL that may have potential to improve outcomes for patients with relapsed disease.

METHODS

Patient Samples

Primary leukemia cells were obtained upon clinical presentation at diagnosis and relapse from five adult and six pediatric B-ALL patients under protocols approved by the Research Ethics Board of the University Health Network (adult samples) or the St. Jude Institutional Review Board (pediatric samples). All patients or families provided informed written consent. Samples were viably frozen and stored at −150°C prior to retrospective selection based on availability.

Xenograft Generation and CNS Blast Purification

All animal experiments were performed with approval from the University Health Network Animal Resource Centre Review Board. Patient samples were depleted of T cells by cell sorting on a FACSAria II (BD Biosciences) for CD19+CD45− leukemic blasts using the following antibodies: anti-CD19 PE (BD, RRID:AB_2868805), anti-CD3 FITC (BD, RRID:AB_2811220), anti-CD3 APC (Beckman Coulter, clone UCHT1), anti-CD3 APC (BD, RRID:AB_2868745), or anti-CD45 FITC (BD, RRID: AB_400360) on a BD Biosciences LSRII or Celesta Cytometer. Viable BM and spleen cells were counted using a Vicell Cell Counter (Beckman Coulter), and viable CNS cells were counted using trypan blue staining and a hemocytometer. Human engraftment was analyzed using human-specific antibodies: anti-CD45 (APC, BD, Clone UCHT1), CD33 (PE-Cy7, BD, RRID: AB_399961; APC, BD, Clone R113), CD34 (APC-Cy7, BD, Clone 581), CD3 (APC, BD, Clone UCHT1), CD38 (PE, BD, RRID: AB_2868824), and CD44 (PE, BD, RRID: AB_394000; APC, BD, RRID: AB_400360) on a BD LSRII Cytometer (BD Biosciences). All animal experiments were performed with approval from the Association for Cancer Research.

RNA-Seq and Pathway Analysis

Total RNA was extracted from 1–2 × 10^6 human purified primary xenograft BM or CNS (>85% human engraftment) using TRIzol and ethanol precipitation as previously described (52). Libraries were prepared using TruSeq Stranded Total RNA Library Ribo-Zero Gold Prep Kits (Illumina) and were sequenced at The Centre for Applied Genomics (TCAG; SickKids, Toronto, Canada) with an HiSeq 4000 Sequencer (Illumina) or HiSeq 2500 Sequencer (patient 15 samples; Illumina) with an average of 3.192 × 10^6 reads per sample and an average 84.3% uniquely mapping reads. Reads were trimmed with Curtadapt and subjected to FastQC quality control analysis. RNA-seq reads were aligned against GRCh38 using STAR 2.4.2b with default parameters (53). Genes were annotated with gencode v23. Counts were obtained using HTSeq v0.7.2 with the gene_name set as the ID attribute (54). Differentially expressed genes were identified using edgeR (version 3.16.5; ref. 55). Pathway analysis was performed using preranked GSEA (version 2.0) run in classic mode with a custom group of gene sets (Bader Lab, http://download.baderlab.org/EM_Genesets/, December 1, 2019 version; ref. 22). Cytoscape v3.6.1 was used to visualize differentially enriched gene sets that were grouped using a Markov Cluster Algorithm (MCL) and annotated using AutoAnnotate v1.2 plugin (56–58).

Metabolic Profiling

Fluorescent dye staining for mitochondrial content was performed on primary xenograft cells by incubation at 37°C with 1 μmol/L MitoTracker Green (M7514) and 1 μmol/L TMRE (T668) according to the manufacturer’s instructions (Thermo Fisher) and then analyzed using a BD LSRII Cytometer (BD Biosciences). All animal experiments were performed with approval from the Association for Cancer Research. Mitochondrial respiratory profiles were performed using the XF96 extracellular flux analyzer (Seahorse Bioscience). Prior to respiration analysis, dead cells were removed from BM and CNS xenografts with a magnetic dead cell–depletion kit (Miltenyi Biotec). A total of 5 × 10^5 BM and CNS cells in 180 μL of XFe media (Seahorse Bioscience), supplemented with 11 mmol/L glucose, 2 mmol/L glutamine, and 1 mmol/L pyruvate (pH 7.4), were seeded onto XFe96 plates (102416-100, Agilent Technologies) and incubated at 37°C, in CO2 free incubator, for 1 hour. OCR and extracellular acidification rate were then evaluated using an XF96 extracellular flux analyzer (Seahorse Bioscience). To measure ATP-linked OCR, maximal respiration, and mitochondrial-dependent basal OCR and proton leak, 1 μmol/L oligomycin A, 0.5 μmol/L carbonyl cyanide-4-phenylhydrazone (FCCP), and 1 μmol/L antimycin A and 1 μmol/L rotenone were added, respectively.

Targeted DNA Sequencing and Analysis

Human engrafted cells from BM and CNS tissue (purified for human cells as above) were subjected to targeted sequencing of their patient-identified variants as previously described (7). Briefly, DNA libraries were prepared using 250 to 500 ng of DNA and the NEXTflex DNA Barcodes (BioScientific) with NEXTflex Library Prep Kit (BioScientific) and sequenced on an HiSeq 2500 sequencer. Mutual clustering to determine population frequencies and temporal ordering of mutations to infer their evolutionary relationships was performed using Pairtree as previously described (refs. 7, 17; Supplementary Methods). To determine genetic
concordance of mutational populations between CNS blasts and BM blasts of individual xenografts, we compared a concordant model of the data with a discordant model of the data using Bayes factors (see Supplementary Methods).

Clone trees were built using the Pairtree algorithm, which is published at https://github.com/morrislab/pairtree. Code for running Pairtree on the data associated with this study, and for computing the probability of discordance between paired tissue samples using Pairtree output, is available at https://github.com/morrislab/cns-ball-discordance/.

Xenograft Drug Assays

CNS cells from primary mice (patients 12 and 15) or BM cells from tertiary mice (patients 6 and 7) were transplanted intraperitoneally into sublethally irradiated NSG mice and monitored for signs of CNS disease (domed heads, poor eye grooming). After confirmation of leukemic engraftment by flow cytometry of peripheral blood, or at the earliest sign of CNS disease, mice were randomized to receive saline vehicle or 1 mg/kg OMA (Sigma-Aldrich) by daily intraperitoneal injection for 4 days—patients 6, 7, and 12 (patient 6 relapse, patient 7 diagnosis, and patient 12 diagnosis)—or 3 days—patient 15 prior to sacrifice. Mice with patient 12 and patient 15 xenografts were symptomatic. The number of days of treatment was determined based on the physical condition of control mice.

For C3a receptor inhibition assays, NSG mice were sublethally irradiated and transplanted intraperitoneally with T-cell–depleted primary patient B-ALL cells from diagnosis sample (n = 2, patient 12 or 15) as described above and subsequently treated with 1% DMSO in PBS or 10 mg/kg SB 290157 (EMD Millipore) dissolved in DMSO and resuspended in PBS at 1% v/v by intraperitoneal injection twice weekly until they displayed evidence of CNS disease.

C3a receptor agonist assays were performed by transplanting sublethally irradiated NSG mice intraperitoneally with 1.5 × 10^5 BM cells from three primary B-ALL xenografts (patient 11 diagnosis, patient 12 diagnosis, and patient 15 diagnosis), and treating intraperitoneally mice with the C3a receptor agonist CAS 944997-60-8 (VDM Bio) dissolved in 5% DMSO, 45% PEG-200, and 50% 0.9% saline vehicle until they displayed evidence of CNS disease (patient 15) or until there was evidence of substantial peripheral blood engraftment detected by flow cytometry (patients 11 and 12).

For C3a receptor inhibitor or agonist treatment of established xenografts based on peripheral blood flow cytometry, NSG mice were sublethally irradiated and transplanted intraperitoneally with 7.5 × 10^5 primary B-ALL BM xenograft cells from patient 12 or 15. Eight weeks after engraftment, mice were administered 10 mg/kg SB 290157 (EMD Millipore), C3a receptor agonist CAS 944997-60-8 (VDM Bio), or vehicle solvent 5% DMSO, 45% PEG-200, and 50% 0.9% saline for three daily doses prior to analysis 36 hours posttreatment.

Group sizes were determined by balancing sample size for statistical analysis and experimental feasibility for xenograft models through our experience with the model systems. Animals were randomized to their experimental groups after leukemic transplantation and were euthanized at prespecified endpoints of hunching, poor self-care, or domed heads in OMA and SB 291057 trials. Investigators were not blinded to treatment groups. Mice treated with the C3a receptor agonist or vehicle were sacrificed after confirming leukemic engraftment with >30% human peripheral blood leukocytes, a threshold that predates the onset of CNS symptoms in our model. Investigators were not blinded to study groups. One mouse randomized to be treated with OMA died of unknown causes prior to treatment, and was excluded from analysis.

In all drug treatments, CNS engraftment in drug treatment groups was normalized to the average of simultaneously vehicle-treated mice from the same patient samples by expressing drug-treated engraftment as a percentage of the control mean allowing engraftment comparison across experiment replicates and patient samples [percent control engraftment = {([number of CD19^+CD45^-] cells extracted from tissue of treated mouse)/[average number of CD19^+CD45^- cells extracted from tissue in vehicle controls from same experiment]) × 100}].

Translation Assay, Protein Quantification, and Pathway Analysis

Nascent protein formation was measured through incorporation of OPP according to the manufacturer’s instructions (Invitrogen) by 1 × 10^5 freshly isolated, unsorted BM or CNS blasts incubated for 30 minutes at 37°C in Dulbecco’s modified Eagle medium with 5% FBS and quantified on a BD LSRII Cytometer (BD Biosciences) from xenograft patient samples with greater than 90% human BM and CNS engraftment. A total of 1 × 10^5 cells from BM and CNS taken from primary xenografts (patient 11 diagnosis n = 4, patient 12 diagnosis n = 3, patient 15 diagnosis n = 5) or secondary xenografts (saline-treated CNS patient 12 diagnosis and patient 15 diagnosis n = 5, and OMA-treated patient 12 diagnosis n = 4, patient 15 diagnosis n = 5) were prepared for mass spectrometry as previously described (Supplementary Table S2; ref. 59). Label-free peptide quantitation yielded protein intensities that were analyzed using MaxQuant and significantly differentially abundant peptides were identified using label-free quantification–adjusted iBAQ spectral counts in edgeR (OMA treatment) or Proteome Discoverer 2.2 (BM vs. CNS blasts; Thermo Fisher Scientific; ref. 60). Functional enrichment analysis of differentially abundant peptides was performed using STRING version 10.5 and visualized with Cytoscape v3.6.1 with MCL clustering (ClusterMaker2 version 1.3), with annotation by ClueGO 2.5.3 (61–63).

Cell-Cycle and Apoptosis Analysis

Purified CNS blasts were stained with anti-human CD45 (APC, BD clone 2D1, RRID: AB_400555), fixed with Cytofix/Cytoperm (BD Biosciences, RRID: AB_2869008), and then stained with anti-Ki-67 (FITC, BD clone B56, RRID: AB_396302) and anti–active caspase-3 (PE, BD clone C92–605, RRID: AB_393906). Prior to analysis on a BD Celesta Flow Cytometer (BD Biosciences), cells were stained with Hoechst 33342 Dye (Thermo Fisher). Cells negative for Ki-67 with 2n DNA content per Hoechst staining were considered in phase G0, Ki-67° and 2n to 4n DNA content were S–G2. M.

Leukemia In Vitro Culture

RS4;11 cells (RRID: CVCL_0093) were obtained from the American Type Culture Collection and cultured in RPMI-1640 media supplemented with 10% FBS. Confirmation testing was not performed. Cells were cultured in media containing a dilution series of DMSO vehicle, SB 290157 (EMD Millipore), or C3a receptor antagonist (VDM Bio) for 72 hours prior to quantification of live cells per well based on exclusion of SYTOX Blue viability dye (Thermo Fisher) as per the manufacturer’s instructions on a BD LSRII cytometer (BD Biosciences).

Graphs and Statistical Analysis

Prism version 8 (GraphPad) was used for statistical analyses unless otherwise described. Plots were made using Prism, gplots, or ggplot2 in the R programming environment (64, 65).

Data and Materials Availability

Targeted DNA-sequencing data are available in ref. 7. RNA-seq data have been submitted to the European Genome-phenome Archive.
with the following data accession numbers: EGA study number EGA500001005647 and EGA dataset EGA000010008183. LC-MS data have been submitted to the ProteomeXchange Consortium with data set identifier PXD022411.

Authors' Disclosures

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Authors' Contributions

R.J. Vanner: Conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing. S.M. Dobson: Conceptualization, formal analysis, investigation, methodology, writing—review and editing. O.I. Gan: Investigation, writing—review and editing. E.M. Schoof: Data curation, investigation, writing—review and editing. J.A. Kennedy: Data curation, investigation, writing—review and editing. M. Chan-Seng-Yue: Data curation, investigation, writing—review and editing. J.E. Dick: Conceptualization, resources, supervision, funding acquisition, methodology, writing—review and editing.

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REFERENCES


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