LINKING CELLULAR METABOLISM TO HEMATOLOGICAL MALIGNANCIES

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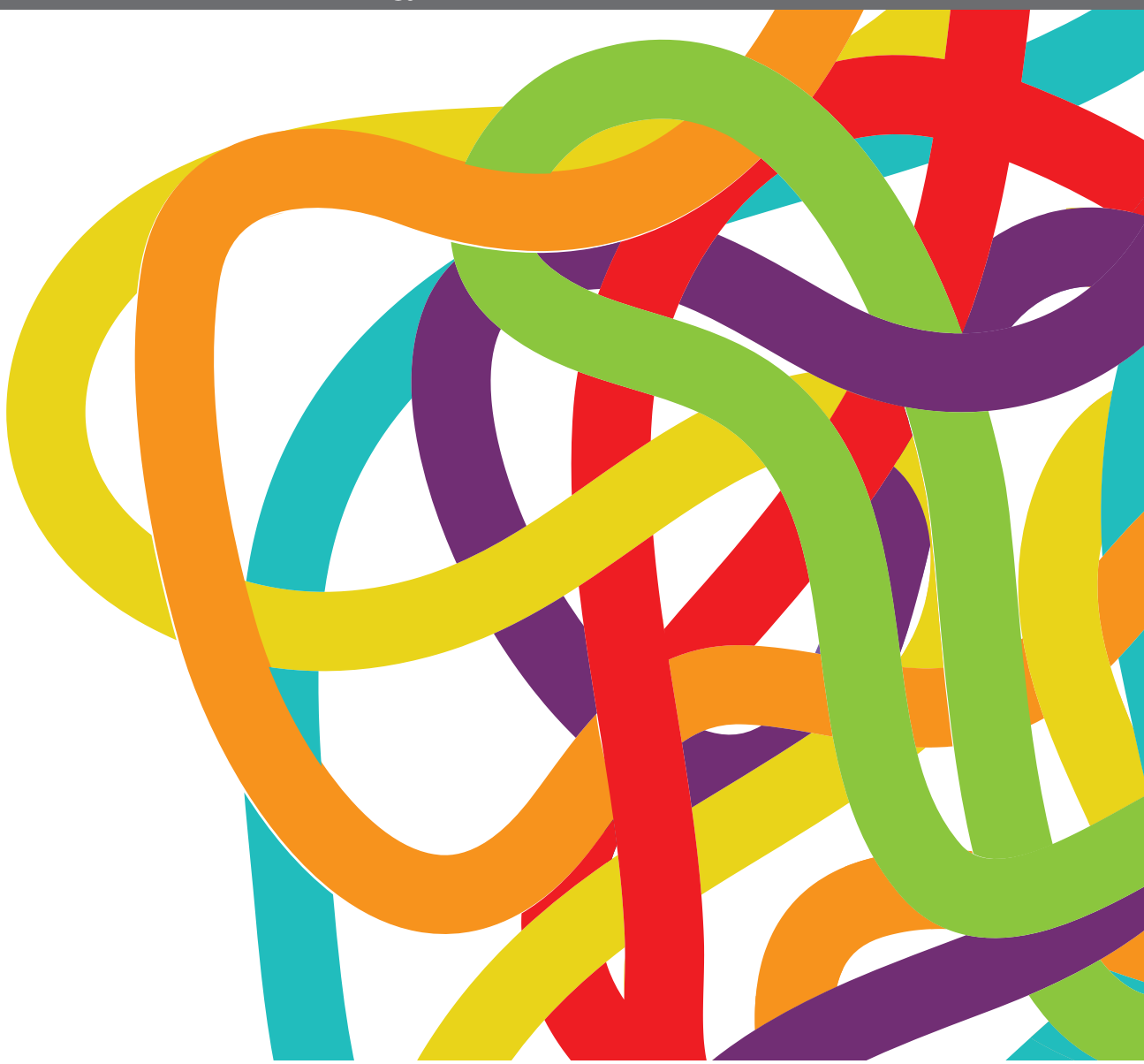

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LINKING CELLULAR METABOLISM TO HEMATOLOGICAL MALIGNANCIES

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Editorial: Linking cellular metabolism to hematological malignancies

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Editorial on the Research Topic
Linking cellular metabolism to hematological malignancies

Convincing evidence has revealed that metabolic reprogramming orchestrates tumor initiation and progression, immune evasion, and drug resistance. Targeting metabolic vulnerabilities of tumors has remarkable advantages as a therapeutic strategy that exerts prominent antitumor effects, without affecting normal cell physiology. Indeed, the current treatment options based on metabolic reprogramming present impressive curative effects in solid tumors and hematological malignancies. A better understanding of tumor metabolic remodeling and immunometabolism will deepen the insights into disease mechanisms and promote the development of promising therapeutic strategies. This Research Topic intends to highlight the current understanding of the role of cellular metabolism in hematological malignancies, with the purpose of identifying new therapeutic targets and rational metabolic therapies alone or in combination with other regimens.

The mechanism underlying metabolic reprogramming

Arginine plays a multifaceted role in numerous crucial biological processes and exerts a significant impact on carcinogenesis and immune response. Arginine auxotrophic tumors, including many hematological malignancies, lose the ability to endogenously synthesize arginine; thus, arginine depletion therapy can serve as a promising antitumor therapeutic approach. Arginase is a prevalent arginine-depleting agent in clinical practice

that converts arginine into ornithine. Du et al. illustrate the arginine metabolism pathway and summarize the current state of arginase application in the treatment of hematological malignancies. The combination of acupuncture and bortezomib is a promising therapeutic strategy to benefit multiple myeloma (MM) model mice, as outlined by Ke et al. The synergistic effects of acupuncture and bortezomib on the survival of MM mice might depend on ornithine-mediated metabolism, which is implicated in arginine and proline metabolic pathways. Furthermore, the external data from the Gene Expression Omnibus (GEO) database revealed that higher ornithine decarboxylase 1 (ODC1) expression was significantly associated with inferior prognosis in MM patients.

Emerging metabolic pathways provide new clues for identifying potential drivers of pathogenesis in hematological malignancies. As a novel characterized modality of regulatory cell death, ferroptosis has been demonstrated to be related to multiple metabolic pathways. Lan et al. summarize the recent progress in the regulatory mechanism of ferroptosis and elucidate the roles of ferroptosis in hematological malignancies. The potential of ferroptosis as a therapeutic target in hematological malignancies is further discussed.

Recent studies have revealed metabolic differences between hematopoietic and leukemic stem cells. Patel et al. depict the unique metabolic adaptations seen in leukemic stem cells (LSCs). They also highlight the advances and limitations of metabolic analysis to decipher the metabolic adaptability of LSCs. Cell number limitation and metabolic heterogeneity represent the major challenges to understanding metabolic differences in the cells of interest. Single-cell metabolic technologies provide an excellent opportunity to dissect cellular states at unprecedented depth. Zuo et al. introduce single-cell metabolomics techniques, providing clear navigation to select appropriate approaches for metabolic analysis.

Immunometabolism in the tumor microenvironment

To date, increasing evidence indicates that immune cells can alter metabolic programs to sustain their unique phenotype and function in order to cope with environmental changes. Liu et al. explore the metabolic abnormalities of tumor-associated macrophages in CD5-positive non-MYC/BCL2 double expressor lymphoma ($CD5^+$ non-DE DLBCL). They found enhanced lipid metabolism in $CD5^+$ non-DE DLBCL, accompanied by an increased M2 proportion. Targeting dysregulated lipid metabolism with metformin was able to significantly decrease M2 proportion and dampen fatty acid transporter (CD36) expression *in vitro*, indicating the clinical rationale of metformin for therapeutic treatment in $CD5^+$ non-DE DLBCL. Metabolic reprogramming existing in the tumor microenvironment (TME) can also influence the function of immune cells. Ehlers et al. found that activated natural killer (NK) cells can maintain cytotoxic capacity against tumor cells in a reduced glucose environment *in vitro*, as found in bone marrow of multiple myeloma (MM) patients, suggesting the therapeutic potential of activated NK cells for the treatment of MM.

Emerging therapeutic strategies and drug resistance

Increasing efforts are being made to develop novel antitumor strategies for hematological malignancies. Lv et al. focus on treatment advances in lymphoproliferative diseases driven by Epstein Barr virus (EBV-LPDs). The traditional treatment approaches for EBV-LPDs are summarized in this review, including chemotherapy, radiotherapy, and hematopoietic stem cell transplantation. They then highlight research advances regarding alternative treatment modalities based on pathological mechanisms, such as immunotherapy, gene therapy, and epigenetic therapy. In addition, proteasome inhibitors, selective nuclear export protein inhibitors, and JAK inhibitors have been proposed as potential therapeutic options for EBV-LPDs. Zhang et al. briefly summarize the current standard treatment for classical Hodgkin lymphoma (cHL). Meanwhile, they survey the latest clinical trials of novel therapeutic agents with the potential to overcome relapsed or refractory cHL, providing promising directions for future research in this area. Glucose metabolism not only affects tumor cell growth but also contributes to shaping the immunosuppressive microenvironment, ultimately promoting immune evasion. Targeting glucose metabolism to remodel the TME is a rational strategy to enhance the efficacy of cancer immunotherapy. Liu et al. review the advances in targeting glucose metabolism combined with cancer immunotherapy.

Drug resistance remains a major obstacle in the clinical management of solid tumors and hematological malignancies. Elucidating the mechanisms of drug resistance is essential to tackle this issue. To explore the potential mechanism of resistance to arsenic trioxide (ATO) in chronic myeloid leukemia (CML), Wang et al. utilized gene chip to profile the metabolic characteristics of ATO-resistant CML cells (K-562) and identified UNC13B as potentially responsible for ATO resistance. UNC13B knockdown remarkably inhibited proliferation and stimulated apoptosis of K-562 cells. Western blot further revealed that UNC13B might modulate both apoptosis and mitochondrial fusion by regulating MAP3K7,

CDK4, and PINK1, rendering resistance to ATO. Zhuang et al. provide a comprehensive overview of the molecular mechanisms of resistance to isocitrate dehydrogenase (IDH) inhibitors in acute myeloid leukemia (AML). A deeper understanding of the resistance mechanisms will provide rationales for novel therapeutic strategies targeting mutant IDH1/2. Several studies have demonstrated the effectiveness of homoharringtonine (HHT) for the treatment of AML. Zhang et al. profile transcriptome changes in AML cells treated with HHT and identify key metabolic pathways that may contribute to resistance to HHT in AML cells.

Collectively, the original research and review articles in this Research Topic cover a series of meaningful findings of metabolic reprogramming in hematological malignancies, providing new insights into the mechanisms underlying cellular metabolism in hematological malignancies. Moreover, several potential effective targets for cancer treatment have been proposed, and further validation is warranted.

Author contributions

XH drafted the editorial. MKK, ZZ, and JY edited the editorial. XH and HS finalized the editorial. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Activated Natural Killer Cells Withstand the Relatively Low Glucose Concentrations Found in the Bone Marrow of Multiple Myeloma Patients

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Infusion of *ex vivo* expanded and cytokine-activated natural killer (NK) cells is a promising alternative way to treat multiple myeloma (MM). However, the tumor microenvironment (TME) may suppress their function. While reduced glucose availability is a TME hallmark of many solid tumors, glucose levels within the TME of hematological malignancies residing in the bone marrow (BM) remain unknown. Here, we measured glucose levels in the BM of MM patients and tested the effect of different glucose levels on NK cells. BM glucose levels were measured using a biochemical analyzer. Compared to the normal range of blood glucose, BM glucose levels were lower in 6 of 9 patients (479-1231 mg/L; mean=731.8 mg/L). The effect of different glucose levels on NK cell cytotoxicity was tested in 4-hour cytotoxicity assays with tumor cells. 500 mg/L glucose (representing low range of MM BM) during the 4-hour cytotoxicity assay did not negatively affect cytotoxicity of activated NK cells, while higher glucose concentrations (4000 mg/L) diminished NK cell cytotoxicity. Since clinical application of NK cell therapy might require *ex vivo* expansion, expanded NK cells were exposed to a range of glucose concentrations from 500-4000 mg/L for a longer period (4 days). This did not reduce cytotoxicity or IFN-γ secretion nor affected their phenotypic profile. In summary, low glucose concentrations, as found in BM of MM patients, by itself did not compromise the anti-tumor potential of IL-2 activated NK cells *in vitro*. Although follow up studies in models with a more complex TME would be relevant, our data suggest that highly activated NK cells could be used to target tumors with a reduced glucose environment.

Keywords: NK cells, multiple myeloma, immunotherapy, tumor microenvironment, glucose

INTRODUCTION

In the last decade, considerable effort has been put in the development of NK cell-based immunotherapy to treat cancer patients due to the clinical potential and good safety profile of NK cells. Multiple clinical trials using either autologous or allogeneic NK cells in various types of hematological- and solid cancers have demonstrated that NK cells could exert anti-tumor responses in patients without significant toxicity (1–3). Mostly, *ex vivo* expanded and cytokine-activated NK cells are used to create highly cytotoxic NK cells. Nonetheless, despite these initially hopeful clinical outcomes, the therapeutic efficacy of NK cell-based immunotherapy could be improved by increasing NK-cell numbers, enhancing NK-cell activation, improving NK-cell tumor-targeting capacity, and improving *in vivo* NK-cell persistence (1).

The expansion and persistence of NK cells *in vivo* has been demonstrated to be positively correlated with the clearance of leukemic cells in patients receiving adoptive NK cell therapy (4). However, the microenvironment of tumor cells could be unfavorable and even suppressive for NK cells allowing tumor cells to escape the NK-cell antitumor response. To be able to survive in the tumor microenvironment (TME), NK cells require cytokines such as IL-2 or IL-15 that can be produced by several cell types present in the TME, but the available amount might not be enough (5, 6). The presence of other cytokines such as transforming growth factor-beta (TGF-ß) and IL-10 in the TME may play a role in the suppression of IL-2 production (7). NK cell antitumor capacity can be hindered by TME factors such as myeloid derived suppressor cells (8), hypoxia (9, 10), or factors released by the tumor cells such as prostaglandin E2, TGF-ß, IL-10, reactive oxygen species, and arginase (11–13). Additionally, the metabolic microenvironment of tumor cells could inhibit the antitumor response of immune cells such as cytotoxic T cells and NK cells (14). To sustain their growth and survival, tumor cells frequently undergo metabolic reprogramming, allowing the enhancement of glucose uptake and metabolism. This process takes place not only within a hypoxic region but also in the area where sufficient oxygen is available, a phenomenon known as aerobic glycolysis or "the Warburg effect" (15). Aerobic glycolysis is favorable for proliferating cells since it can provide both bioenergetics and biosynthesis requirements better than oxidative phosphorylation (OxPhos) (14). Due to high rates of glycolysis, the glucose supply in the tumor microenvironment can be limited. In solid tumors, glucose availability is inversely correlated with the distance from the capillaries to the tumor and glucose levels as low as 20 mg/L have been reported for colon cancer with great intratumoral variability (16, 17). To our knowledge, there is not much known about glucose levels within the microenvironment of hematological cancers.

Aerobic glycolysis appeared to be not only advantageous for tumor cells. Immune cells, such as cytotoxic T cells, have been shown to require a switch to aerobic glycolysis to exert their effector function (18). Since both tumor cells and T cells are glycolytic, metabolic competition can occur within the tumor microenvironment. The glycolytic activity of the tumor cells can cause depletion of extracellular glucose thereby limiting the availability of glucose to T cells (19). In mice, it has been demonstrated that this metabolic competition hindered T-cell metabolism resulting in a defective IFN-γ production which is crucial for antitumor response (20, 21). In NK cells, aerobic glycolysis has also been shown to be important for a potent NK-cell effector function. In mice, resting NK cells preferred OxPhos for their metabolism while highly activated NK cells enhanced especially glycolysis and to a lower extend OxPhos (22–24). In humans, NK cells upregulated both glycolysis and OxPhos upon cytokine stimulation with IL-2 or IL-12/15 (25). Additionally, CD56 bright NK cells were found to be metabolically more active than CD56 dim NK cells (25). The same group also showed that elevated levels of OxPhos were essential for NK cell effector cytotoxicity and IFN-γ production.

Our group focuses on the development and refinement of NK-cell based immunotherapy to treat patients with cancer, especially multiple myeloma (MM) as there is no cure available to date for MM. We envision to inject a high number of highly activated NK cells to patients with MM. Glucose levels in the MM microenvironment remain unknown but may be important in controlling the anti-MM response of NK cells as NK cells use glucose as primary source of energy (26). We, therefore, aimed to explore the possible consequences of MM metabolic microenvironment on the antitumor potential of activated and expanded NK cells. First, we investigated the glucose levels present in the microenvironment of patients having active MM to define the relevant *in vitro* experimental conditions. Second, based on these results, we performed 4-hour cytotoxicity assays *in vitro* to study the effect of short-term exposure to different glucose concentrations on NK cell cytotoxicity against tumor cells. Third, we studied the influence of longer exposure to the different glucose concentrations on expanded NK cells to evaluate whether NK cell effector functions could be optimized by adapting glucose levels during expansion. The results from this current study give us a better understanding whether *in vivo* glucose concentrations should be a concern for the NK-cell based immunotherapy and whether eventually an intervention might be needed to improve the therapy.

MATERIALS AND METHODS

Glucose Measurement

Leftover fresh BM samples were obtained from MM patients with active disease. The use of leftover material from clinical procedures did not require ethical approval in the Netherlands under the Dutch Code for Proper Secondary Use of Human Tissue. None of the patients objected to the use of leftover material. When feasible, samples were measured directly as a whole BM harvest. Otherwise, samples were centrifuged with speed 1170 g for 15 minutes at 4° Celsius, followed by harvesting of the "plasma" fraction which was stored in -20° Celsius before the glucose measurement was performed using YSI biochemical analyzer (Salm en Kipp, BV). Freezing the samples had minor influence on the glucose concentration, similar to variation between duplicates. Samples were measured in duplicate and the average per sample was reported.

Cell Lines and Culture Media

The K562 cell line, purchased from ATCC, was cultured in IMDM (Gibco) and supplemented with 10% fetal calf serum (FCS) (Greiner Bio-One International, GmbH), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) (1% Pen/Strep). The RPMI-8226 cell line, purchased from DSMZ, was cultured in standard RPMI-1640 medium. Standard RPMI-1640 medium refers to RPMI-1640 medium containing 2000 mg/L glucose (Cat. 11554516, ThermoFisher) and was supplemented with 10% FCS and 1% Pen/Strep for all cultures. Glucose-free RPMI-1640 medium (Cat. 11560406, ThermoFisher) was supplemented with D-(+)-Glucose (Sigma) as indicated in the individual figures and with 10% FCS and 1% Pen/Strep. All cell culture experiments were performed at 37° C in an incubator with 5% CO_2 and 21% O_2 (Sanyo MCO-20AIC, Sanyo Electric Co, Japan).

NK Cell Culture and NK Cell Expansion

NK cells were isolated from anonymous buffy coats (Sanquin blood bank, Maastricht). The use of buffy coats does not need ethical approval in the Netherlands under the Dutch Code for Proper Secondary Use of Human Tissue. Peripheral blood mononuclear cells were isolated from the buffy coats by density centrifugation using Lymphoprep (Axis-Shield). Subsequently, NK cells were isolated using the NK cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec, GmbH). For experiments in **Figure 2**, NK cells were activated overnight with 1000 U/mL recombinant human IL-2 (Proleukin, Novartis) either in standard RPMI-1640 medium containing 2000 mg/L glucose (Gibco, **Figure 2A**) or in glucose-free RPMI-1640 medium (Gibco) supplemented with the glucose concentration indicated in the **Figure 2B** (named 'culture condition'). For experiments in **Figures 3** and **4**, NK cells were expanded from CD3-depleted PBMCs in the presence of 1000 U/mL IL-2 in alpha-medium (Biochrom, GmbH) supplemented with 10% human serum (Milan Analytica, AG), 2mM L-GlutaMax (Gibco), 1.3 g/L sodium bicarbonate (Biochrom), 2000 mg/L glucose (Sigma), and 0.5% Gentamycin-Sulphate (Gibco). The cells were expanded with IL-2 for 16-22 days during which the NK cells expanded 10- to 20-fold. After expansion, the NK cells were subsequently cultured for 4 days in glucose-free RPMI-1640 medium supplemented with the glucose concentration indicated in the figure and with 1000 U/mL IL-2 (culture condition), followed by cytotoxicity assays and ELISA assays.

Labeling of Tumor Cells Lines for Cytotoxicity Assays

One day prior to the cytotoxicity assay, 2 x 10^6 cells/ml K562 cells or RPMI-8226 cells were labeled with 3 µl Vybrant CM-DiI Cell-Labeling solution (Thermo Fisher) in PBS according to the manufacturer's instruction. After adding CM-DiI to the cell suspension, cells were incubated for 5 minutes at 37°C followed by 15 minutes at 4°C in the fridge. After the last incubation, cells were washed 2 times with PBS and centrifuged (280g for 8 minutes at room temperature). K562 or RPMI-8226 cells were then resuspended in IMDM or standard RPMI-1640 medium, respectively, supplemented with 10% FCS and 1% Penicillin/Streptomycin and incubated overnight at 37°C.

Cytotoxicity Assay

On the day of the assay, the DiI-labeled tumor cells were harvested and $2x10^4$ cells per well were plated in a 96-wells plate for the cytotoxicity assay. NK cells were harvested, washed and co-cultured with the tumor cells in 1:1 Effector : Target (E:T) ratio for 4 hours in the presence of different glucose concentrations (named 'killing condition') as indicated in the figures. Specific cytotoxicity was calculated as follows: (% dead tumor cells - % spontaneous tumor cell death)/(100% - % spontaneous tumor cell death) x100.

Staining and Flow Cytometry

After a 4-hour cytotoxicity assay, cells were washed with PBS (Gibco) and stained for dead cells using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Molecular Probes™) for 30 minutes on ice in the dark. Cells were further washed with PBS buffer (PBS, 1% FCS) and fixed with 1% paraformaldehyde in PBS solution. For NK cell phenotyping, expanded NK cells were harvested after culturing in either 500, 2000, or 4000 mg/L glucose for 4 days. Subsequently, NK cells were washed in PBS and first stained for dead cells using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Molecular Probes™) for 30 minutes on ice in the dark, and subsequently stained for the following surface markers for 30 minutes on ice and in the dark: CD3-APC-Vio770, -VioBlue or -PerCP-Vio700; CD56-PE-Vio770 or -APC-Vio770; KIR2DL2/3-PE; KIR3DL1-PerCP; NKG2A-APC; KIR2DL1-FITC; NKp30-PE-Vio770; NKp44-VioBright; NKp46-PE; NKG2D-APC; PD1-VioBright; DNAM1-PE; CD96-PE-Vio770; NKG2C-APC; LAG3-VioBlue; TIM3-APC; TIGIT-PE. FMO controls were stained for Live/Dead, CD3 and CD56. All antibodies were purchased from Miltenyi Biotech. All flow cytometric analyses were performed with BD FACS Canto II. Data were analyzed with FlowJo 10.1r5 64-bit software.

ELISA Assay

Supernatants of the cytotoxicity assays were collected and analyzed for IFN-γ levels using the PeliKine compact™ ELISA kit (Sanquin). The samples were thawed at room temperature and diluted 1:2 before use. ELISA assays were performed according to the manufacturer's instructions.

Statistical Analysis

All statistical analysis was performed with GraphPad Prism 8.3 (GraphPad Software Inc, San Diego, CA, USA) using non-parametric paired t-test (Wilcoxon matched pairs test) for figure 2A or 2-way ANOVA when comparing multiple groups. * indicates a *p-value* of <0.05, ** indicates a *p-value* of <0.01.

RESULTS

Glucose Concentrations in the BM of MM Patients Are on Average Lower Than Normal Blood Glucose Levels

To get an indication of glucose levels in the bone marrow (BM), glucose levels were measured in BM samples from MM patients. The glucose concentration in the BM of MM patients ranged

between 479 to 1231 mg/L (mean = 731.8 mg/L, SD = 247.6). Compared to normal glucose levels in peripheral blood, which are < 1100 mg/L (<6.1 mmol/L) fasting or <1400 mg/L (<7.8 mmol/L) random (27), 6 of 9 MM patients had lower glucose levels with the lowest concentration reported here being twice as low as normal blood glucose levels (**Figure 1**).

Relatively Low Glucose Concentrations Present During Killing Do Not Affect NK Cell Killing Capacity While High Glucose Reduced Killing Efficacy

Glucose has been described to be an important fuel for NK cells. Therefore, we tested the effect of the glucose levels as observed in BM of MM patients, the glucose levels in normal blood, and the glucose levels used in culture media on the cytotoxic capacity of NK cells derived from healthy donors. We activated NK cells overnight with 1000 U/mL IL-2 in standard RPMI-1640 medium containing 2000 mg/L glucose and then used the NK cells in a 4-hour cytotoxicity assay against K562 cells at 500, 1000, 2000, or 4000 mg/L glucose. In the presence of 1000 mg/L, which is representative for the glucose concentration in blood, NK cells killed 19% of K562 cells on average. The presence of 500 mg/L glucose (representing the low range of MM BM) during the cytotoxicity assay did not negatively affect the killing capacity of IL-2 activated NK cells as compared to conditions with 1000 mg/L, representing blood glucose, or 2000 mg/L, which is present in standard culture media (**Figure 2A**). Higher glucose concentration of 4000 mg/L, as used in high glucose culture media, reduced the cytotoxicity of NK cells to 5.5% on average as compared to 500 mg/L, 1000 mg/L and 2000 mg/L glucose (p= 0.06) (**Figure 2A**).

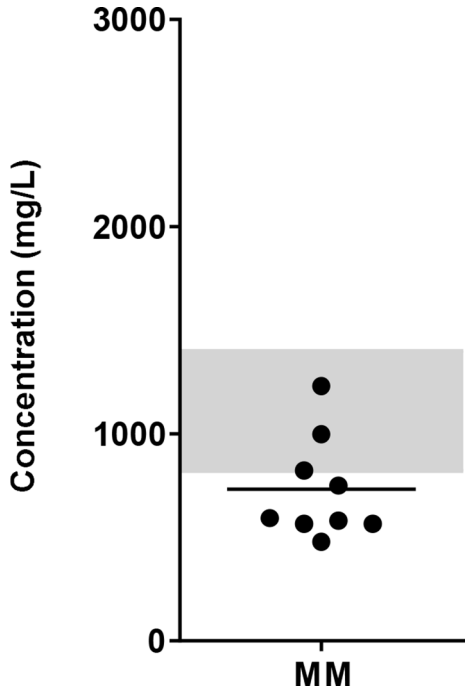

FIGURE 1 | Glucose concentration in the BM of MM patients. BM samples from newly diagnosed MM patients were collected and glucose concentrations were determined using biochemical analyzer (YSI). Grey bar indicates the reference value range for normal fasting blood glucose (4.4 – 6.1 mmol/L or 820 – 1100 mg/L up to 1400 mg/L postprandial). n = 9 subjects.

Because NK cells could be exposed for a longer period to the BM glucose levels while traveling through the BM, we investigated whether overnight exposure to different glucose concentrations affected NK cell cytotoxicity. The exposure to 500 mg/L up to 4000 mg/L glucose during overnight incubation (named 'culture condition') did not result in a lower cytotoxicity against K562 cells regardless of the glucose concentrations present during the killing process (named 'killing condition') (**Figure 2B**). After observing a reduction in NK cell-mediated killing with 4000 mg/L glucose in **Figure 2A**, we included an even higher glucose concentration of 8000 mg/L for overnight culture. In the presence of this extremely high glucose, NK cell cytotoxicity was reduced to less than 5% in all killing conditions (**Figure 2B**). This reduction in NK cell cytotoxicity was unlikely due to high osmolarity caused by the high glucose levels as we did not see a reduction in NK cell cytotoxicity when NK cells were cultured in the presence of 1000 mg/L glucose and 7000 mg/L Mannitol (**Supplementary Figure S1**).

In summary, these results showed that the presence of 500 mg/L glucose, representative for the rather low glucose concentration in BM of MM patients, during the process of killing or during overnight incubation did not reduce the NK cell tumor-killing capacity in our *in vitro* setting. Quite the opposite, the presence of higher levels glucose concentration during killing or overnight activation (4000 and 8000 mg/L) diminished NK cell cytotoxic capacity.

Expanded NK Cells Exposed to Relatively Low Glucose Levels Remain Effective Against Tumor Cells

Clinical application of NK cells might require *ex vivo* NK cell expansion to reach the large numbers of NK cells needed for NK cell infusions. As this expansion may also metabolically change the cells, we investigated how the killing capacity of expanded NK cells was affected by short-term exposure (4 hours, killing condition) or long-term exposure (4 days, culture condition) to glucose levels ranging between 500 mg/L and 4000 mg/L.

With 2000 mg/L glucose present during the cytotoxicity assay, expanded NK cells killed on average 49.5% K562 cells. RPMI-8226 cells, a MM cell line, were more resistant to NK cell-mediated killing than K562 cells and 26.6% RPMI-8226 cells were killed on average at 2000 mg/L glucose. Compared to this condition with 2000 mg/L glucose, the average cytotoxic potential of expanded NK cells against K562 and RPMI-8226 cells was not altered when NK cells were exposed to 500 mg/L or 4000 mg/L glucose during the killing process (**Figures 3A, B**). This result indicated that short exposure to varying glucose concentration did not influence the cytotoxic potential of expanded NK cells and differed from the freshly isolated NK cells that seemed to have reduced cytotoxicity with 4000 mg/L glucose during killing (**Figure 2**).

Next, we examined the effect of a four-day exposure to the different glucose concentrations on NK cell cytotoxicity.

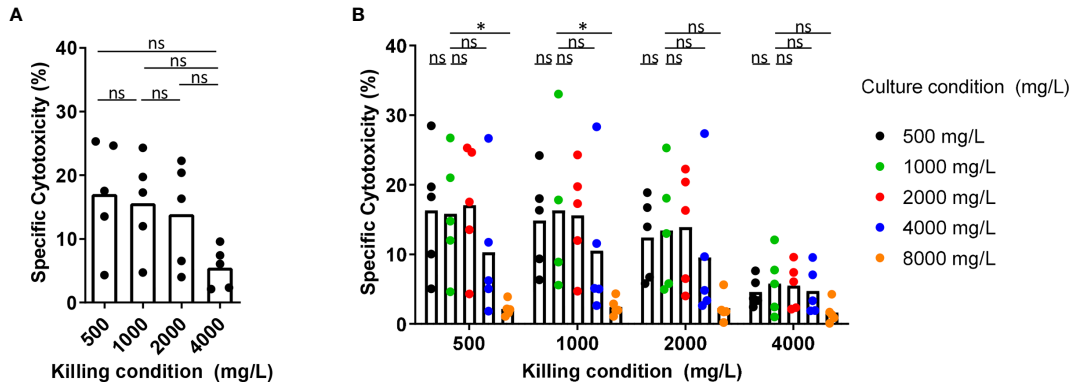

FIGURE 2 | Short-term exposure to lower glucose concentrations does not reduce NK cell cytotoxicity while higher glucose levels reduce NK cell cytotoxicity. **(A)** NK cells were overnight activated with IL-2 in standard RPMI-1640 medium with 2000 mg/L glucose, followed by a 4h cytotoxicity assay with K562 cells at 1:1 E:T ratio in different glucose concentrations (killing condition). **(B)** NK cells were cultured in different glucose concentrations overnight (culture condition) in presence of IL-2, followed by a 4h cytotoxicity assay with K562 cells at 1:1 E:T ratio in different glucose concentrations (killing condition). Tumor cells killed by NK cells are denoted as percentage specific cytotoxicity. Bars show the average of n=5 donors in 3 independent experiments. Each dot represents the average of a technical duplicate. *p < 0.05. ns, not significant.

Compared to the culture condition of 2000 mg/L glucose, the average cytotoxicity of both K562 and RPMI-8226 was around 10% lower when NK cells were cultured in 500 mg/L glucose, however this did not reach significance. The average cytotoxicity of NK cells cultured in 4000 mg/L was not different from the culture condition with 2000 mg/L (**Figures 3A, B**). The NK cell viability remained the same in all conditions when NK cells were cultured in 500, 2000, or 4000 mg/L for 4 days (**Supplementary Figure S2A**). Despite some donor variation, expanded NK cells of all donors maintained their cytotoxic capacity largely independent of the glucose concentration present during 4-day culture.

Besides production of cytotoxic granules, NK cells are known for their secretion of inflammatory cytokines such as IFN-γ and TNFα. NK cell-derived IFN-γ has multiple functions including support of antigen presentation and induction of a Th1 response, which is important for polarizing an adaptive immune response against tumor cells (28). To investigate if the secretion of IFN-γ was influenced by the different glucose levels, the supernatants of NK- and tumor cell co-cultures were analyzed for IFN-γ secretion by ELISA. Cultured in the normal culture condition with 2000 mg/L glucose, expanded NK cells secreted on average 329 pg/mL IFN-γ with K562 cells as target cells and 23 pg/mL with RPMI-8226 as target cells, showing that K562 induced a much more potent IFN-γ response in NK cells than RPMI-8226 cells (**Figures 3C, D**). Without target cells, expanded NK cells did not secrete IFN-γ (**Supplementary Figure S2B**). The average level of IFN-γ production was comparable for all the tested glucose levels during the short-term co-culture with tumor cells.

Compared to the 4-day culture condition with 2000 mg/L glucose, the average IFN-γ secretion remained constant when the expanded NK cells were exposed to 500 mg/L or 4000 mg/L for 4 days. This was found for both target cell lines K562 and RPMI-8226. These data showed that a potent IFN-γ response, comparable to the amount secreted with 2000 mg/L glucose, was still observed independent of the glucose concentration during short- or long-term culture.

During *ex vivo* expansion, NK cells frequently alter their phenotype resulting in high NKG2A expression and low expression of killer-cell immunoglobulin-like receptors (KIRs). To test if the NK cell phenotype changed by culturing in low or high glucose during the 4-day culture period, NK cells were stained for several surface markers. The gating strategy is shown in **Supplementary Figure S3**. The activating receptors NKp30, NKp46, DNAM1 and NKG2C are constitutively expressed on NK cells with NKG2C being expressed on a subset of NK cells. NKp44 and CD96 expression are induced upon activation (29). After expansion of NK cells in the presence of IL-2, the NK cells had a highly activated phenotype and expressed all seven activating receptors (**Figures 4A, B**). With 86%, the majority of expanded NK cells was NKG2A positive and on average 10-18% of the NK cells expressed one or multiple KIR receptors (**Figures 4C, D**). Moreover, extensive cytokine activation of NK cells can lead to expression of exhaustion markers. The NK cells expressed TIM3 and TIGIT but only low levels of PD1 and LAG3 after expansion with IL-2 (**Figures 4E, F**). Expression of all investigated surface molecules was irrespective of the glucose concentrations present during culture, resulting in NK cells with a rather activating phenotypic profile.

In summary, the effector functions and the phenotypic profile of expanded NK cells were not influenced by variation in glucose levels during the killing process or during culture. Follow up studies that take multiple other TME factors and nutrient state into account, could be useful to further determine the impact of glucose on NK cells.

DISCUSSION

To date, not much is known about glucose concentrations in the BM. As glucose is the primary fuel for NK cells, we aimed to gain more insight on the effect of glucose levels in the TME of MM on overnight cytokine-activated or *ex vivo* expanded NK cells and

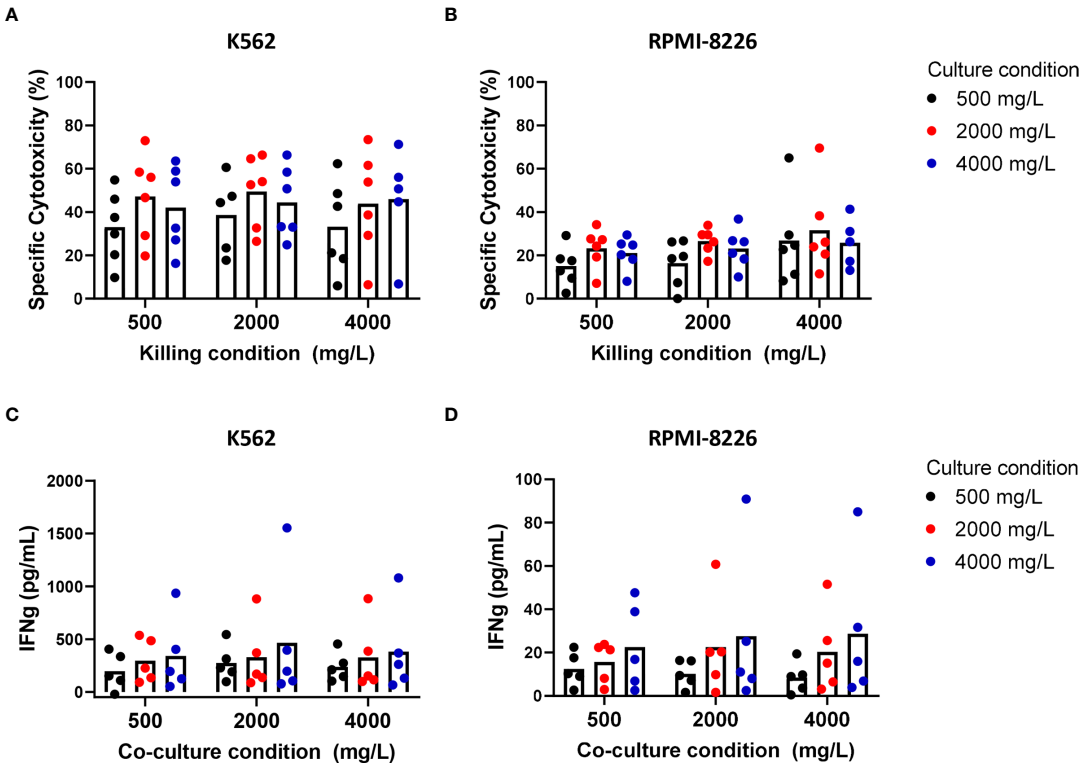

FIGURE 3 | Short-term and long-term exposure to low or high glucose levels do not reduce effector functions of expanded NK cells. NK cells were expanded with 2000 mg/L glucose and subsequently cultured in different glucose concentrations for 4 days (culture condition) followed by a 4h cytotoxicity assay (killing condition) with K562 cells or RPMI8226 cells at 1:1 E:T ratio in different glucose concentrations. Tumor cells killed by NK cells are denoted as percentage specific cytotoxicity **(A, B)**. After the co-culture with tumor cells, supernatants were collected and analyzed for secretion levels of IFNγ by ELISA **(C, D)**. Bars show the average of n=5-6 donors in individual experiments. For none of the conditions a p-value <0.05 was observed.

their antitumor response. First, we showed that glucose concentrations in the BM of MM patients with active disease were, in most cases, lower than normal blood glucose levels. Since we included only 9 patients in our study, our observation should be confirmed in a larger cohort. With this new insight on glucose concentrations in BM, we tested the effect of different glucose levels on freshly isolated- or expanded NK cells *in vitro*. As lowest glucose concentration we chose 500 mg/L since this represents the lower limit of BM glucose concentrations in MM patients. We observed that short-term exposure to 500 mg/L glucose did not have a detrimental effect on the killing capacity of overnight-activated NK cells. This may be because glucose was not completely depleted. 500 mg/L glucose is half of the normal concentration found in blood, where NK cells are known to be potent killers. Moreover, the majority of NK cells in the assays were CD56 dim NK cells. Both human NK cell subsets are functionally different and have been shown to possess different metabolic requirements as well: CD56 bright NK cells, which are the main cytokine producers have been shown to have a higher rate of glucose uptake and they appear to be metabolically more active than CD56 dim NK cells upon cytokine stimulation (25). Therefore, CD56 bright peripheral blood NK cells may be more likely to suffer more from the restricted glucose in the environment than the CD56 dim subset. In addition, during activation, NK cells are able to perform metabolic reprogramming and upregulate both glycolysis and OxPhos (30). Since we used high dose IL-2 activated NK cells, these cells could have become more or less independent on the availability of glucose. Unlike T cells that are more dependent on glucose availability to become activated, NK cells might be the better tumor cell killers in the area where low glucose concentrations are located. Moreover, to fully conclude whether this would also be true for solid tumors, the glucose levels should be reduced even further as glucose levels in solid tumors can be much lower than 500 mg/L (17). In addition, it would be interesting to further evaluate whether our findings are also true in a situation where not only glucose is reduced, but also other nutrients are limited.

Interestingly, high glucose concentrations of 4000 mg/L and even more obvious with 8000 mg/L glucose did result in a reduction of NK cell cytotoxicity in our study. This observation was highly unlikely due to high molarity since we did not observe the effect with mannitol. Our finding was in line with a previous study on unactivated human NK cells showing that short-term exposure of NK cells to 8000 mg/L glucose led to inhibition of NK cell cytotoxicity, probably due to an increase in intracellular calcium ion concentration to such high levels that it inhibited cytotoxic efficiency (31, 32).

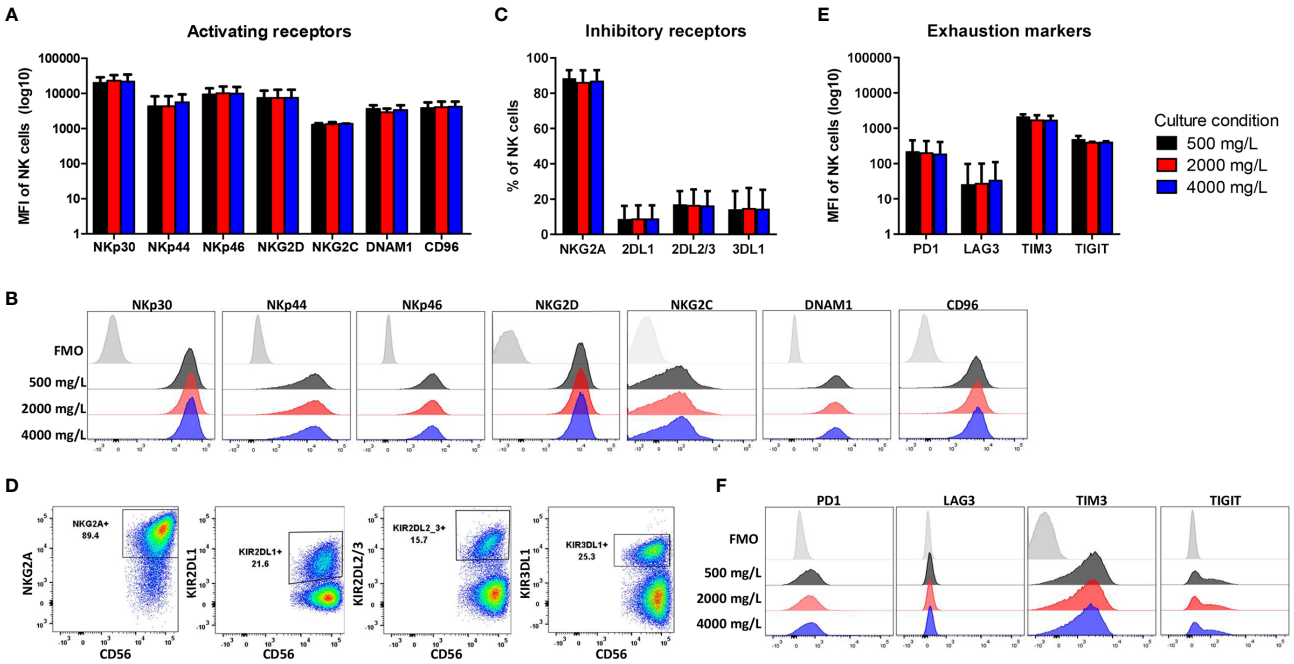

FIGURE 4 | Phenotype profile of expanded NK cells is not altered by long-term exposure to low or high glucose levels. NK cells were expanded with 2000 mg/L glucose and subsequently cultured in different glucose concentrations for 4 days (culture condition). NK cells were stained with antibodies against activating receptors **(A, B)**, inhibitory receptors **(C, D)** and exhaustion markers **(E, F)** and analyzed by flow cytometry. Bar graphs **(A, C, E)** depict the average expression of n=2 donors for NKG2C, TIM3, TIGIT and n=5 donors for all other markers, error bars indicate SD. FMO values were subtracted from MFI expression values. Representative histograms **(B, F)** or dot plots **(D)** of one donor are shown with the FMO of one of the three glucose concentrations as FMOs overlapped tightly (**Supplementary Figure S3**).

Many clinical protocols, aiming at NK cell infusion as a mean for cancer immunotherapy, will require infusion of extremely high numbers of NK cells, which necessitates *ex vivo* NK cell expansion. We therefore anticipated that this expansion period could provide an opportunity to either prime NK cells for the metabolic conditions in the tumor or enhance their function or persistence by interfering with their metabolic programming. For these experiments, we again chose 500 mg/L glucose as low, 2000 mg/L as normal, and 4000 mg/L as high glucose concentration, representing the *in vivo* BM concentrations in MM patients, glucose concentrations in standard-, and in high glucose culture media, respectively. Our data with IL-2 expanded NK cells implied that a period of acclimatization to a higher glucose concentration did not result in an altered NK cell cytotoxic capacity. Additionally, a period of acclimatization to a lower glucose level for 4 days after expansion did not reduce NK cell-mediated killing. For murine tumor-infiltrating T cells, it has been shown that inhibition of glycolysis during the *ex vivo* expansion could prime T cells towards enhanced persistence and overall anti-tumor response upon transfer into tumor-bearing immunodeficient mice (33). In human NK cells expanded with membrane-bound IL-21 K562 feeder cells, highly functional licensed NK cells used both glycolysis and OxPhos and had a greater glycolytic capacity than less functional, unlicensed NK cells that relied on OxPhos alone (34). Inhibition of NK cell cytotoxicity in highly functional NK cells was only observed after vigorous glucose deprivation and the use of metabolic inhibitors overnight (34). This provides the functional NK cells with a greater flexibility to generate energy and indicates that the cytotoxic functions can be independent of the available glucose levels. We also found that the IFN-γ response of the expanded NK cells during a 4h cytotoxicity assay was largely unaffected by the glucose concentrations that we tested. A previous study using murine NK cells showed that inhibition of glycolysis reduced IFN-γ production by NK cells when triggered by engagement of activating receptors while cytokine (IL-12/IL-18)-induced IFN-γ production remained unaffected by inhibition of glycolysis (23). Similar to murine NK cells, human cytokine-activated NK cells continued to produce IFN-γ when exposed to short-term glucose deprivation of 4 hours (35) or long-term low glucose levels (as low as 0.01 mM) for two days (36). However, CD56bright NK cells showed a defective IFN-γ production when the glycolysis rate was limited for a period of 18 hours (25) underlining the difference between CD56dim and CD56bright NK cells.

In our study, we observed a rather small reduction in glucose availability in the BM of MM patients as compared to normal peripheral blood values but we did not determine availability of other nutrients in the MM patients. In our *in vitro* assays, we reduced the amount of glucose, while other nutrients could contribute to NK cell efficacy as well. Glucose has been described as the key metabolic fuel for NK cells [summarized in (37)], but further research would be required to determine if a small reduction in glucose availability might have more impact in

combination with other nutrient deficiencies, such as glutamine, or other TME factors that may suppress NK cell effector function or alter NK cell metabolism. While NK cells do not use glutamine as fuel for OxPhos, glutamine deficiency can impair NK cell functions due to the loss of the transcription factor cMYC (26). Alternatively, the combination with TGF-β, frequently present in the MM TME (38), may enhance the effect of limited glucose as TGF-β has been shown to reduce the NK cells level of OxPhos and glycolysis (39). Hypoxia is another TME factor that can limit NK cell functions. We have previously shown that IL-2 activated NK cells retain their cytotoxic capacity when exposed to hypoxia (9). However, a shift towards glycolysis is expected when oxygen is low and it is therefore important to study the effects of varying glucose concentrations on activated NK cells in models resembling a complex TME including factors like hypoxia and altered levels of nutrients other than glucose.

Even though we show that activated and expanded NK cells can cope with a low glucose environment, combination therapy should be considered to achieve better NK cell efficacy in MM patients. We have previously described several strategies how the NK cell potency could be enhanced e.g. by the combination with monoclonal antibodies such as Daratumumab (40). Other options could include targeting of the TME to create a less NK cell suppressive TME. In MM, inhibitors of mechanistic target of rapamycin (mTOR) are tested to target the metabolism of MM cells and its TME cells that overexpress mTOR (41, 42). Dual inhibitors targeting mTORC1/mTORC2 could potentially be an interesting drug to slow tumor cell growth in combination with NK cell transfer to kill tumor cells. However, mTOR inhibitors can also suppress immune cells (22, 43) and combination therapy approaches should be tested in carefully designed studies as timing of the drugs and NK cell infusion may be very important. Alternatively, it would be relevant to explore in further studies whether manipulating NK cell metabolism during *ex vivo* expansion could be used to potentiate NK cell effector function in the MM TME.

In conclusion, our current findings showed that exposure to a relatively low glucose concentration, as found in the BM of MM patients, for either short-term or long-term culture did not have a detrimental effect on the NK cell cytotoxic capacity against tumor cells in our *in vitro* setup. Our data suggests that IL-2 activated and expanded NK cells could be well suited to function in a tumor environment where glucose availability is limited. Although this is positive news for NK-cell based immunotherapy, future studies are needed to investigate if these observations also hold true for the *in vivo* situation in patients with MM or other cancers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization, NM and FE. Methodology, NM, FE, and TO. Formal analysis, NM and FE. Investigation, NM, FE, and TO. Resources, NM, GB. Data curation, NM and FE. Writing—original draft preparation, NM and FE. Writing, review and editing, TO, LW, and GB. Supervision, LW and GB. Project administration, NM and FE. Funding acquisition, LW and GB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.622896/full#supplementary-material

REFERENCES

1. Fang F, Xiao W, Tian Z. NK Cell-Based Immunotherapy for Cancer. *Semin Immunol* (2017) 31:37–54. doi: 10.1016/j.smim.2017.07.009
2. Liang S, Xu K, Niu L, Wang X, Liang Y, Zhang M, et al. Comparison of Autogeneic and Allogeneic Natural Killer Cells Immunotherapy on the Clinical Outcome of Recurrent Breast Cancer. *OncoTargets Ther* (2017) 10:4273. doi: 10.2147/OTT.S139986
3. Veluchamy JP, Kok N, van der Vliet HJ, Verheul HM, de Gruijl TD, Spanholtz J. The Rise of Allogeneic Natural Killer Cells as a Platform for Cancer Immunotherapy: Recent Innovations and Future Developments. *Front Immunol* (2017) 8:631. doi: 10.3389/fimmu.2017.00631
4. Bachanova V, Miller JS. NK Cells in Therapy of Cancer. *Crit Rev™ Oncogenesis* (2014) 19(1-2):133–41. doi: 10.1615/critrevoncog.2014011091
5. Larsen SK, Gao Y, Basse PH. NK Cells in the Tumor Microenvironment. *Crit Rev™ Oncogenesis* (2014) 19(1-2):91–105. doi: 10.1615/critrevoncog.2014011142
6. Chiu J, Ernst DM, Keating A. Acquired Natural Killer Cell Dysfunction in the Tumor Microenvironment of Classic Hodgkin Lymphoma. *Front Immunol* (2018) 9:267. doi: 10.3389/fimmu.2018.00267

7. Mocellin S, Wang E, Marincola FM. Cytokines and Immune Response in the Tumor Microenvironment. *J Immunother* (2001) 24(5):392–407. doi: 10.1097/00002371-200109000-00002
8. Jewett A, Tseng H-C. Tumor Induced Inactivation of Natural Killer Cell Cytotoxic Function; Implication in Growth, Expansion and Differentiation of Cancer Stem Cells. *J Cancer* (2011) 2:443. doi: 10.7150/jca.2.443
9. Sarkar S, Germeraad WT, Rouschop KM, Steeghs EM, van Gelder M, Bos GM, et al. Hypoxia Induced Impairment of NK Cell Cytotoxicity Against Multiple Myeloma Can Be Overcome by IL-2 Activation of the NK Cells. *PloS One* (2013) 8(5):e64835. doi: 10.1371/journal.pone.0064835
10. Balsamo M, Manzini C, Pietra G, Raggi F, Blengio F, Mingari MC, et al. Hypoxia Downregulates the Expression of Activating Receptors Involved in NK-Cell-Mediated Target Cell Killing Without Affecting ADCC. *Eur J Immunol* (2013) 43(10):2756–64. doi: 10.1002/eji.201343448
11. Baginska J, Viry E, Paggetti J, Medves S, Berchem G, Moussay E, et al. The Critical Role of the Tumor Microenvironment in Shaping Natural Killer Cell-Mediated Anti-Tumor Immunity. *Front Immunol* (2013) 4:490. doi: 10.3389/fimmu.2013.00490
12. Pietra G, Manzini C, Rivara S, Vitale M, Cantoni C, Petretto A, et al. Melanoma Cells Inhibit Natural Killer Cell Function by Modulating the Expression of Activating Receptors and Cytolytic Activity. *Cancer Res* (2012) 72(6):1407–15. doi: 10.1158/0008-5472.CAN-11-2544
13. Vitale M, Cantoni C, Pietra G, Mingari MC, Moretta L. Effect of Tumor Cells and Tumor Microenvironment on NK-Cell Function. *Eur J Immunol* (2014) 44(6):1582–92. doi: 10.1002/eji.201344272
14. Kroemer G, Pouyssegur J. Tumor Cell Metabolism: Cancer's Achilles' Heel. *Cancer Cell* (2008) 13(6):472–82. doi: 10.1016/j.ccr.2008.05.005
15. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. *Cell Metab* (2008) 7(1):11–20. doi: 10.1016/j.cmet.2007.10.002
16. Hu X, Chao M, Wu H. Central Role of Lactate and Proton in Cancer Cell Resistance to Glucose Deprivation and Its Clinical Translation. *Signal Transduction Targeted Ther* (2017) 2(1):1–8. doi: 10.1038/sigtrans.2016.47
17. Hirayama A, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, et al. Quantitative Metabolome Profiling of Colon and Stomach Cancer Microenvironment by Capillary Electrophoresis Time-of-Flight Mass Spectrometry. *Cancer Res* (2009) 69(11):4918–25. doi: 10.1158/0008-5472.CAN-08-4806
18. Pearce EL, Pearce EJ. Metabolic Pathways in Immune Cell Activation and Quiescence. *Immunity* (2013) 38(4):633–43. doi: 10.1016/j.immuni.2013.04.005
19. Sukumar M, Roychoudhuri R, Restifo NP. Nutrient Competition: A New Axis of Tumor Immunosuppression. *Cell* (2015) 162(6):1206–8. doi: 10.1016/j.cell.2015.08.064
20. Chang C-H, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic Competition in the Tumor Microenvironment is a Driver of Cancer Progression. *Cell* (2015) 162(6):1229–41. doi: 10.1016/j.cell.2015.08.016
21. Ho P-C, Bihuniak JD, Macintyre AN, Staron M, Liu X, Amezquita R, et al. Phosphoenolpyruvate is a Metabolic Checkpoint of Anti-Tumor T Cell Responses. *Cell* (2015) 162(6):1217–28. doi: 10.1016/j.cell.2015.08.012
22. Marçais A, Cherfils-Vicini J, Viant C, Degouve S, Viel S, Fenis A, et al. The Metabolic Checkpoint Kinase Mtor Is Essential for IL-15 Signaling During the Development and Activation of NK Cells. *Nat Immunol* (2014) 15(8):749. doi: 10.1038/ni.2936
23. Keppel MP, Saucier N, Mah AY, Vogel TP, Cooper MA. Activation-Specific Metabolic Requirements for NK Cell IFN-Γ Production. *J Immunol* (2015) 194(4):1954–62. doi: 10.4049/jimmunol.1402099
24. Donnelly RP, Loftus RM, Keating SE, Liou KT, Biron CA, Gardiner CM, et al. Mtorc1-Dependent Metabolic Reprogramming Is a Prerequisite for NK Cell Effector Function. *J Immunol* (2014) 193(9):4477–84. doi: 10.4049/jimmunol.1401558
25. Keating SE, Zaiatz-Bittencourt V, Loftus RM, Keane C, Brennan K, Finlay DK, et al. Metabolic Reprogramming Supports IFN-Γ Production by CD56 bright NK Cells. *J Immunol* (2016) 196(6):2552–60. doi: 10.4049/jimmunol.1501783
26. O'Brien KL, Finlay DK. Immunometabolism and Natural Killer Cell Responses. *Nat Rev Immunol* (2019) 19(5):282–90. doi: 10.1038/s41577-019-0139-2
27. World Health Organization. Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia. (2006). Available at: https://www.who.int/iris/bitstream/handle/10665/43588/9241594934_eng.pdf
28. Mailliard RB, Son Y-I, Redlinger R, Coates PT, Giermasz A, Morel PA, et al. Dendritic Cells Mediate NK Cell Help for Th1 and CTL Responses: Two-Signal Requirement for the Induction of NK Cell Helper Function. *J Immunol* (2003) 171(5):2366–73. doi: 10.4049/jimmunol.171.5.2366
29. Sivori S, Vacca P, Del Zotto G, Munari E, Mingari MC, Moretta L. Human NK Cells: Surface Receptors, Inhibitory Checkpoints, and Translational Applications. *Cell Mol Immunol* (2019) 16(5):430–41. doi: 10.1038/s41423-019-0206-4
30. Gardiner CM, Finlay DK. What Fuels Natural Killers? Metabolism and NK Cell Responses. *Front Immunol* (2017) 8:367. doi: 10.3389/fimmu.2017.00367
31. Whalen MM. Inhibition of Human Natural Killer Cell Function in Vitro by Glucose Concentrations Seen in Poorly Controlled Diabetes. *Cell Physiol Biochem* (1997) 7(1):53–60. doi: 10.1159/000154852
32. Zhou X, Friedmann KS, Lyrmann H, Zhou Y, Schoppmeyer R, Knörck A, et al. A Calcium Optimum for Cytotoxic T Lymphocyte and Natural Killer Cell Cytotoxicity. *J Physiol* (2018) 596(14):2681–98. doi: 10.1113/JP274964
33. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting Glycolytic Metabolism Enhances CD8+ T Cell Memory and Antitumor Function. *J Clin Invest* (2013) 123(10):4479–88. doi: 10.1172/JCI69589
34. Schafer JR, Salzillo TC, Chakravarti N, Kararoudi MN, Trikha P, Foltz JA, et al. Education-Dependent Activation of Glycolysis Promotes the Cytolytic Potency of Licensed Human Natural Killer Cells. *J Allergy Clin Immunol* (2019) 143(1):346–58. doi: 10.1016/j.jaci.2018.06.047
35. Velásquez SY, Himmelhan BS, Kassner N, Coulibaly A, Schulte J, Brohm K, et al. Innate Cytokine Induced Early Release of Ifnγ and CC Chemokines From Hypoxic Human NK Cells is Independent of Glucose. *Cells* (2020) 9(3):734. doi: 10.3390/cells9030734
36. Presnell SR, Spear HK, Durham J, Riddle T, Applegate A, Lutz CT. Differential Fuel Requirements of Human NK Cells and Human CD8 T Cells: Glutamine Regulates Glucose Uptake in Strongly Activated CD8 T Cells. *ImmunoHorizons* (2020) 4(5):231–44. doi: 10.4049/immunohorizons.2000020
37. Gardiner CM. NK Cell Metabolism. *J Leukocyte Biol* (2019) 105(6):1235–42. doi: 10.1002/JLB.MR0718-260R
38. Pittari G, Vago L, Festuccia M, Bonini C, Mudawi D, Giaccone L, et al. Restoring Natural Killer Cell Immunity Against Multiple Myeloma in the Era of New Drugs. *Front Immunol* (2017) 8:1444. doi: 10.3389/fimmu.2017.01444
39. Zaiatz-Bittencourt V, Finlay DK, Gardiner CM. Canonical TGF-B Signaling Pathway Represses Human NK Cell Metabolism. *J Immunol* (2018) 200(12):3934–41. doi: 10.4049/jimmunol.1701461
40. Mahaweni NM, Bos GM, Mitsiades CS, Tilanus MG, Wieten L. Daratumumab Augments Alloreactive Natural Killer Cell Cytotoxicity Towards CD38+ Multiple Myeloma Cell Lines in a Biochemical Context Mimicking Tumour Microenvironment Conditions. *Cancer Immunol Immunother* (2018) 67(6):861–72. doi: 10.1007/s00262-018-2140-1
41. Lamanuzzi A, Saltarella I, Desantis V, Frassanito MA, Leone P, Racanelli V, et al. Inhibition of Mtor Complex 2 Restrains Tumor Angiogenesis in Multiple Myeloma. *Oncotarget* (2018) 9(29):20563. doi: 10.18632/oncotarget.25003
42. Ramakrishnan V, Kumar S. PI3K/AKT/Mtor Pathway in Multiple Myeloma: From Basic Biology to Clinical Promise. *Leukemia Lymphoma* (2018) 59(11):2524–34. doi: 10.1080/10428194.2017.1421760
43. Wang F, Meng M, Mo B, Yang Y, Ji Y, Huang P, et al. Crosstalks Between Mtorc1 and Mtorc2 Variagate Cytokine Signaling to Control NK Maturation and Effector Function. *Nat Commun* (2018) 9(1):1–17. doi: 10.1038/s41467-018-07277-9

Conflict of Interest: GB is Chief Executive Officer/Chief Medical Officer/Co-founder of CiMaas, BV, Maastricht, The Netherlands. CiMaas is producing an *ex vivo* expanded NK cell product that will be used to treat myeloma patients.

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Acupuncture Synergized With Bortezomib Improves Survival of Multiple Myeloma Mice *via* Decreasing Metabolic Ornithine

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Multiple myeloma (MM) is a hematological malignancy worldwide in urgent need for novel therapeutic strategies. Since Velcade (bortezomib) was approved for the treatment of relapsed/refractory MM in 2003, we have seen considerable improvement in extending MM patient survival. However, most patients are fraught with high recurrence rate and incurability. Acupuncture is known for alleviating patient symptoms and improving the quality of life, but it is not well investigated in MM, especially in combination with bortezomib. In this study, we employed LC-MS and UHPLC-MS together with bioinformatics methods to test serum samples from 5TMM3VT MM murine model mice with four different treatments [control (C) group, bortezomib (V) treatment group, acupuncture (A) group, and combined (VA) group]. MM mice in group VA had longer survival time than mice in group A or group V. Joint pathway analysis indicated the underlying arginine and proline metabolism pathway among the 32 significantly decreased metabolites in group VA. CCK-8 assay and *in vivo* experiments validated that ornithine, the metabolite of arginine, promoted MM cell proliferation. In addition, gene expression omnibus (GEO) database analysis suggested that MM patients with higher ornithine decarboxylase 1 (ODC1) expression were evidently associated with poor overall survival. In summary, this study demonstrates the synergistic effects of acupuncture and bortezomib on extending the survival of MM model mice and provides potential therapeutic targets in the treatment of MM.

Keywords: ornithine, metabolomics, multiple myeloma, acupuncture, ODC1

Abbreviations: 3D, Three-dimensional; A, Acupuncture; APEX, the Assessment of Proteasome Inhibition for Extending Remissions; C, Control; CCK-8, Cell counting kit-8; ESI, Electron spray ionization; FC, Fold Change; GEO, Gene expression omnibus; GEP, Gene expression profiling; IMWG, International myeloma working group; IS, Internal standards; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS, Chromatography–mass spectrometry; MGUS, Monoclonal gammopathy of undetermined significance; MS, Mass spectrometric; NCCN, National comprehensive cancer network; NP, Normal plasma; ODC1, Ornithine decarboxylase 1; OPLS-DA, Partial Least Squares Discrimination Analysis; OS, Overall survival; PCA, Principal component analysis; rpm, Rotations per minute; RT, Retention time; SMM, Smoldering myeloma; TIC, Total ion current; TT2, Total therapy 2; V, Bortezomib; VA, Acupuncture combined with bortezomib; VIP, Variable importance in the projection.

INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy with clonal proliferation of abnormal plasma cells in the bone marrow (1, 2). According to the statement of international myeloma working group (IMWG), there were nearly 159,985 new MM patients diagnosed annually worldwide. About 1% of patients with monoclonal gammopathy of undetermined significance (MGUS) progressed to MM every year (3, 4). In 2003, Velcade (bortezomib for injection) was approved by the US Food and Drug Administration for the treatment of relapsed/refractory MM as the first proteasome inhibitor (5). The latest clinical MM therapy is a new immunomodulatory therapy using chimeric antigen receptor T cells, bispecific T cell conjugates, and immune checkpoint inhibitors (6). Although the therapeutic armamentarium for MM has continued to evolve (7–14), MM still possesses the characteristics of high relapse and incurability. It is necessary to explore more effective therapies to improve MM prognosis significantly.

In the national comprehensive cancer network (NCCN) (15), adult cancer pain clinical guidelines recommend acupuncture as a comprehensive treatment option in combination with pharmacologic interventions. Nowadays, acupuncture, especially serving as a non-drug alternative to control symptoms, has become a popular adjuvant therapy in cancer treatment (16, 17). Many clinical cases reported that the combination of acupuncture and medicine not only delayed the disease progress (18), reduced the dosage (19), and minimized the side effects of the drug (20), but also relieved the pain caused by the diseases (21, 22). Therefore, the combined application of acupuncture and bortezomib in MM might have broad prospect in alleviating patient symptoms and improving the quality of life.

Since Warburg and Cori demonstrated that cancer cells increased glucose uptake and the fermentation of glucose into lactic acid to promote cellular growth, survival, and proliferation (termed "Warburg effect") in the 1920s (23), metabolic reprogramming was deemed as one of the main hallmarks and adaptive phenotypes exploited by tumor cells during all the tumor growth and metastatic progression (24), such as abnormal glucose metabolism in colorectal cancer (25), acute myeloid leukemia accompanied by abnormal glycolysis (26), lipid metabolism disorders in hepatocellular carcinoma (27), and cytochrome P450-derived arachidonic acid metabolism in pheochromocytoma (28). Modern metabolomics techniques utilize nuclear magnetic resonance or chromatography mass spectrometry to detect differential metabolites in serum and analysis of metabolic profiles.

To explore the effects of acupuncture combined with bortezomib (VA) in MM and the underlying potential mechanism, we performed chromatography–mass spectrometry (LC-MS) and UHPLC-MS together with bioinformatics, joint pathway analysis, and gene expression profiling (GEP) analysis to determine the metabolomics in MM serum samples.

METHODS

Chemicals and Reagents

The 5TMM3VT murine myeloma cells were donated by Professor Wen Zhou from the State Key Laboratory of Experimental Hematology, Department of Hematology, Xiangya Hospital, Central South University. Velcade (bortezomib for injection) was purchased from Hansoh pharma (H20173307, Jiangsu, China). Acetonitrile and methanol were purchased from Merck, Millipore Ltd (1.00030.4008, 1.06007.4008, Carrigtwohill, Ireland). Formic acid, ethyl acetate, trifluoroacetic acid, ammonium acetate, and L-arginine hydrochloride were purchased from Macklin Biochemical Co., Ltd (F809712, E809174, T818778, A801000, L800291, Shanghai, China). The 2-chloro-L-phenylalanine and dansyl chloride were purchased from Yuanye Bio-Technology Co., Ltd (B25643, S19248, Shanghai, China). The 1,4-Butane-1,1,2,2,3,3,4,4-d8-diamine was purchased from Toronto Research Chemicals (D416027, Canada). Chloroform, sodium carbonate, sodium bicarbonate, and acetone were purchased from Lingfeng Chemical Reagent Co. (Shanghai, China). Ornithine analytical standard was purchased from Solarbio (SO8470, Beijing, China). RPMI-1640 medium without arginine was purchased from Sigma-Aldrich (R1780, USA). Certified fetal bovine serum, RPMI 1640 medium, and dialyzed fetal bovine serum were purchased from Biological Industries (05-065-1A, 04-002-1A, 04-011-1b, Kibbutz Beit Haemek, Israel). Cell counting kit (CCK-8) was purchased from Yeasen Biotechnology Co., Ltd. (40203ES76, Shanghai, China).

Animal Protocols

All animal procedures were conducted in accordance with government-published recommendations for the Care and Use of Laboratory Animals and approved by the Institutional Ethics Review Boards of Nanjing University of Chinese Medicine. The experimental mice (C57BL/KaLwRij, 6–8 weeks, 18–20 g) were purchased from the institute of model animals of Nanjing University. The experimental mice were housed in the SPF laboratory animal center of Nanjing University of Chinese Medicine with 15–25°C ambient temperature and free access to food and water. After 1 week of adaptive feeding, animal experiments were started. 5TMM3VT murine myeloma cells (1×10^6) were injected *via* the tail vein into 6-week-old C57BL/KaLwRij mice. The mice were divided into four groups as follows: model control (C, only modeling, n=10) group, bortezomib (V, n=10) treatment group, acupuncture (A, n=9) treatment group, and VA (n=8) treatment group. One day later, mice in three treatment groups were treated by different methods, containing intraperitoneal injection of 1.2 mg/ml V twice a week or/and electroacupuncture (Model SDZ-II, Suzhou Medical Appliance Factory, Suzhou, China) stimulation of Hegu (29) and Zusanli (30, 31) points (2/100 Hz, 2 mA) three times a week until all the mice were dead.

Serum Sample Collection

Blood was taken from tail vein on Tuesday and Wednesday during the experimental period. Blood samples of week 4–6 were mixed in the clean Eppendorf tubes, stored on ice for 2 h, and centrifuged (5,000 rpm, 10 min) at 4°C. The blood was collected for separation of serum. Subsequently, the supernatants were transferred to clean Eppendorf tubes and stored at −80°C.

Sample Preparation for LC-MS Analysis

All serum samples were thawed on the ice. An aliquot of 45 μl serum sample was precipitated by adding 135 μl acetonitrile containing internal standards (IS) [2-Chloro-L-Phenylalanine (plasma sample-acetonitrile: IS=2,000:1)], vortex for 30 s, sonicating for 10 min at 4°C, and then stayed for 3 h on ice. Precipitated protein was removed by centrifugation (13,000 rpm, 10 min) at 4°C. Subsequently, 153 μl supernatant was transferred to glass inserts of LC-MS vials and stored at −80°C for LC-MS analysis.

LC-MS Analysis

The LC-300AD LC system (Shimadzu, Japan) coupled to a Triple TOF^{TM}5600 mass spectrometer (AB SCIEX, USA) and operated in full scan mode was used for untargeted analysis of serum samples. Each sample was run in duplicate in electron spray ionization$^{+/-}$ (ESI$^{+/-}$) modes. An aliquot of 3 μl extracted plasma sample was injected onto an ACQUITY UPLC HSS T3 C18 (2.1 × 100 mm, 1.8 μm) column (Waters, USA) operating at 40°C. The auto-sampler was conditioned at 4°C. Untargeted metabolomics were detected as described in a previous study (6).

Raw data files from LC-MS were converted by Analyst®TF 1.7 software and imported into Markview software to match the peaks and the metabolites identified by mass spectral database of Dalian Institute of Chemical Physics, Chinese Academy of Sciences. Mass-to-charge ratio difference less than 0.001 was regarded as the same substance. Then the dataset of normalized peak height intensity, retention time (RT), metabolites names, and sample numbers were analyzed by SIMCA 14.1 software. SIMCA 14.1 conducted a multivariate statistical analysis of the principal component analysis (PCA) and orthogonal partial least-squares discrimination analysis (OPLS-DA) and permutations. The metabolites with P value < 0.05 and variable importance in the projection (VIP) of >1.0 were considered as statistically significant metabolites. The metabolic joint pathway analysis was carried out on the website visualization tools of MetaboAnalyst 5.0 (32).

Sample Preparation for UHPLC-MS Analysis

Five serum samples were randomly selected from group C and group VA, respectively, and prepared for UHPLC-MS analysis. All serum samples were thawed on the ice. An aliquot of 50 μl serum sample was precipitated by adding 5 μl 1,4-Butane-1,1,2,2,3,3,4,4-d8-diamine and 167 μl methanol, vortex for 1 min, then adding 334 μl chloroform and vortex again for 1 min. Supernatants were collected by centrifugation (15,000 rpm, 10 min) at 4°C. Then 100 μl sodium bicarbonate-sodium bicarbonate buffer (pH=9) and 50 μl dansyl chloride solution (dissolved in acetone) were added to the supernatant (33), and stayed for 1 h at room temperature in dark area. Subsequently, the organic phase was extracted with acetic ether twice. Notably, trifluoroacetic acid was added before the second extraction. Finally, the organic phase was transferred to fresh tube and dried in solvent evaporator (Genevac, UK) at 45°C for 2 h. The residue was reconstituted in 100 μl of a mixture of 0.2 mol/L ammonium acetate/acetonitrile (3:7, vol/vol) for UHPLC-MS analysis.

UHPLC-MS Analysis

Waters iClass UHPLC system (Waters, USA) coupled with a Triple Quad^{TM} 6500+ (AB SCIEX, USA) was applied for targeted metabolomics analysis. An aliquot of 1 μl sample solution was injected onto Ultimate XB-AQ chromatographic column (100 mm × 2.1 mm, 3 μm) maintained at 40°C. The auto-sampler was conditioned at 4°C. For carrying out analysis, the mobile phase was composed of A (0.1% formic acid in water) and B (acetonitrile acidified by 0.1% formic acid) with different concentration gradient. The flow rate was 0.4 ml/min. Mass spectrometric (MS) parameters were applied as follows: ionization temperature 450°C, ion-source gas 1 pressure 55 psi, ion-source gas 2 pressure 55 psi, curtain gas pressure 40 psi, and ion-source voltage 5,500 V.

Cell Proliferation Assay

Cell growth was evaluated by using CCK8 assay according to the method described in the literature (34). Cells were cultured for 24 h with dialyzed fetal bovine serum and RPMI-1640 medium without arginine, then seeded at a density of 1,500 cells/well in 96-well plates. MM cells were cultured with different concentrations of arginine for 24 and 72 h. And 10 μl CCK8 was added to each well for 3 h before detection. The absorbance was measured at A450 nm with a microplate plate reader (Thermo Fisher Scientific, Inc., USA).

Statistics Analysis

Survival analyses were made by using the Kaplan Meier method. Statistical analyses were performed by using GraphPad Prism 8 software. The statistical results were conducted with Log-rank (Mantel-Cox), and value of P< 0.05 was regarded as a significant difference (*P<0.05, **P<0.01, ***P<0.001).

RESULTS

Efficacy Evaluation of VA Treatment in 5TMM3VT Myeloma Mice

The MM mouse model was established by tail vein injection of 5TMM3VT murine myeloma cells (**Figure 1A**) and subjected to four different groups: only modeling (group C), bortezomib treatment (group V), acupuncture treatment (group A), and combination therapy (group VA). As **Figure 1B** shows, the survival rates of myeloma mice in group V (median time of 73.5 days) and A (median time of 67 days) were evidently improved compared with the group C (median survival time of 47 days). Intriguingly, the median survival time of the myeloma mice treated with VA significantly prolonged to 79 days, and in the sixth week after modeling, the survival curves began to show significant differences between the group VA and the group C (**Figure 1C**).

Serum Metabolic Profiling Reveals Significant Differences Among MM Mice in Different Treatment Groups

Serum was collected from the myeloma mice in each group, which was used to examine the characteristics of metabolites by LC-MS. The results showed that the peak patterns of total ion current (TIC) obtained in ESI^{+} (**Figures 2A–D**) and ESI^{-} (**Figures 2E–H**) modes

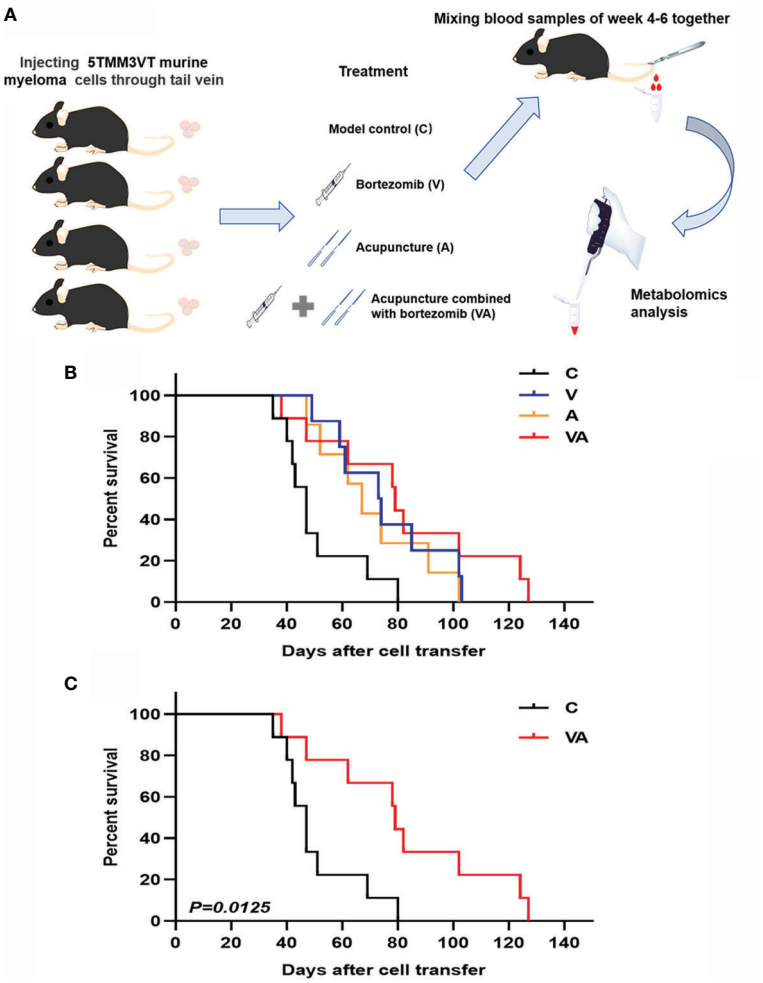

FIGURE 1 | Efficacy evaluation of VA treatment in 5TMM3VT myeloma mice. **(A)** Animal model and blood collection. **(B)** Survival curve of group C, V, A, and VA. **(C)** Survival curve of group C *vs* VA.

were distinctly different. The serum metabolites of the MM mice in each group were well separated under the same detection mode. Within 5~13 min of the injection, there were significant differences between group C and groups A, V, or VA.

The principal component analysis (PCA) was used to reflect the degree of dispersion among the four groups. Differences and changes in metabolic profiles of MM mouse serum from each group were evaluated by PCA in ESI^+ (**Figure 3A**) and ESI^- (**Figure 3B**) modes. The results displayed a significant separation of serum samples from mice in the four groups with good clustering of samples within groups (**Figures 3A, B**), as well as the three-dimensional (3D) scatter plot (**Figures 3C, D**).

The orthogonal partial least-squares discrimination analysis (OPLS-DA) model of serum metabolomics from myeloma mice showed the significant differences in group V, A, or VA compared with group C in both ESI^+ (**Figures 4A, C, E**) and ESI^- (**Figures 4G, I, K**) modes. In addition, all the permutation test results indicated that the fitted model was reliable (**Figures 4B, D, F, H, J, L**). The differential metabolites that satisfied the criterion (VIP >1.0 and P value <0.05) were considered as significantly different substances. There were 97 different substances in the serum of group V compared with group C, including 64 upregulated and 33 downregulated substances (**Figures 4A, B, G, H**). There were 151 different serum substances between group A and group C, with 113 upregulated and 28 downregulated substances (**Figures 4C, D, I, J**). Importantly, we found 174 different substances in the serum of group VA in comparison with group C, including 102 upregulated and 72 downregulated substances (**Figures 4E, F, K, L**).

Ornithine Acts as a Therapeutic Target of VA Treatment in MM Mice

To narrow down the potential therapeutic targets, all the significantly different substances from each comparison groups were collected to plot Venn diagrams. Excluding the intersection, there were 20 upregulated (**Figure 5A** and **Table 1**) and 32 downregulated (**Figure 5B** and **Table 2**) distinct metabolites in the serum of group VA. Subsequently, MetaboAnalyst 5.0 was

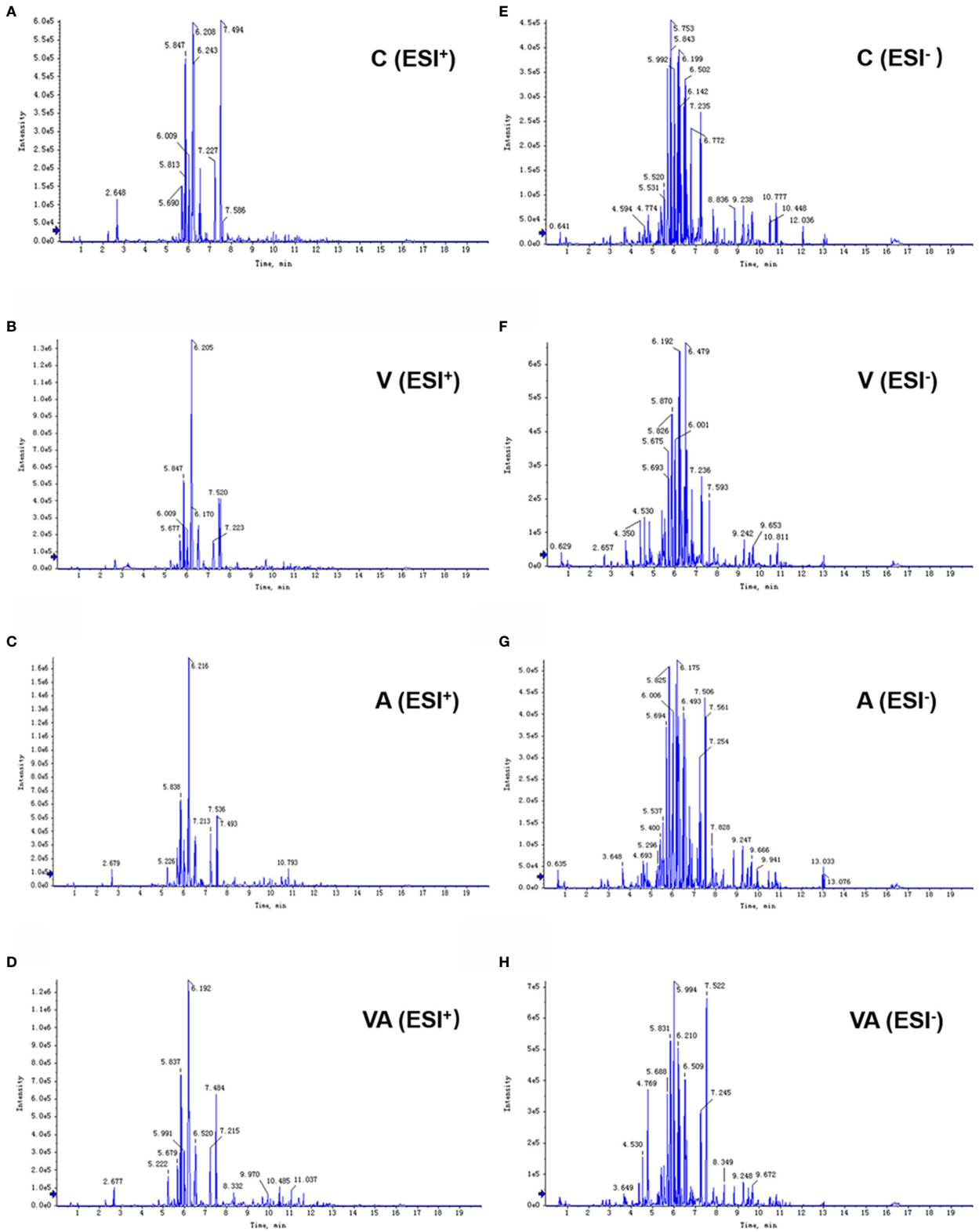

FIGURE 2 | Typical chromatograms of TIC in serum samples. TIC of group C **(A)**, V **(B)**, A **(C)**, and VA **(D)** in ESI^+ mode. TIC of group C **(E)**, V **(F)**, A **(G)**, and VA **(H)** in ESI^- mode.

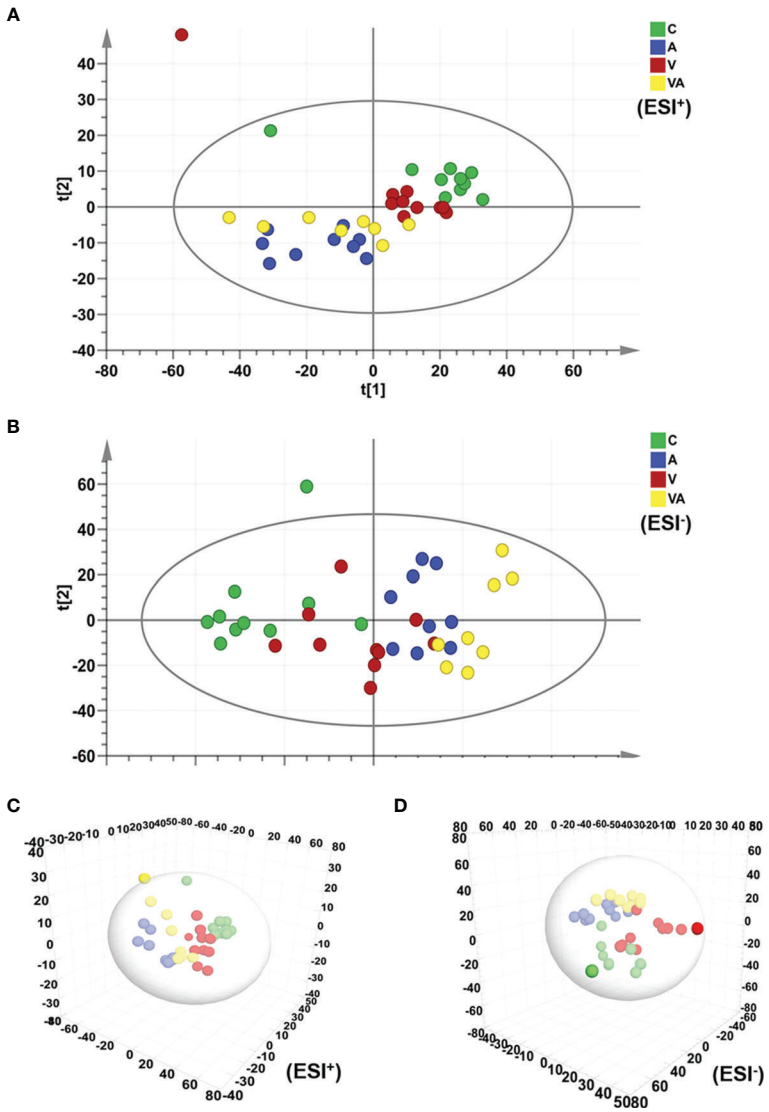

FIGURE 3 | PCA score plot based on the data of ESI$^{+/-}$ modes. **(A)** PCA score plot of all groups in ESI$^+$ mode. **(B)** PCA score plot of all groups in ESI$^-$ mode. **(C)** 3D scatter plot of all groups in ESI$^+$ mode. **(D)** 3D scatter plot of all groups in ESI$^-$ mode.

used to analyze the joint pathways of differential metabolites in ESI$^+$ (**Figure 5C**) and ESI$^-$ (**Figure 5D**) modes, respectively. In **Figure 5D**, the main pathway of arginine and proline metabolism was involved in the serum of group VA with impact 0.20964 (−log (P)=4.6259). Ornithine and arginine were major metabolites in arginine and proline metabolism pathway.

Cluster analysis and heatmap showed that both ornithine (median of m/z=133.1057307) and arginine (median of m/z=347.2197103) were significantly decreased in the serum of group VA compared with group C (**Figure 5E**). To a large degree, these data illustrated that VA treatment inhibited arginine and proline metabolism pathway, thus causing arginine and ornithine reduction. Additionally, ornithine was also involved in the regulation of the glutathione metabolic pathway (−log(P)=41.4122) in **Figure 5D**. These results suggested that ornithine might be a therapeutic target of VA treatment in MM.

VA Treatment Decreases Ornithine Concentration in the Serum of MM Mice

To further prove the above data, we conducted targeted metabolomics to detect ornithine concentration in the serum of MM mice. The chromatogram revealed a characteristic peak of ornithine standard at 3.76 min after injection (**Figure 6A**). In **Figure 6B**, according to the linear standard curve (r=0.99796), ornithine content in the serum samples of group VA (Average concentration of 7,333.33 ng/ml) was decreased by 73.36% compared with the group C (Average concentration of

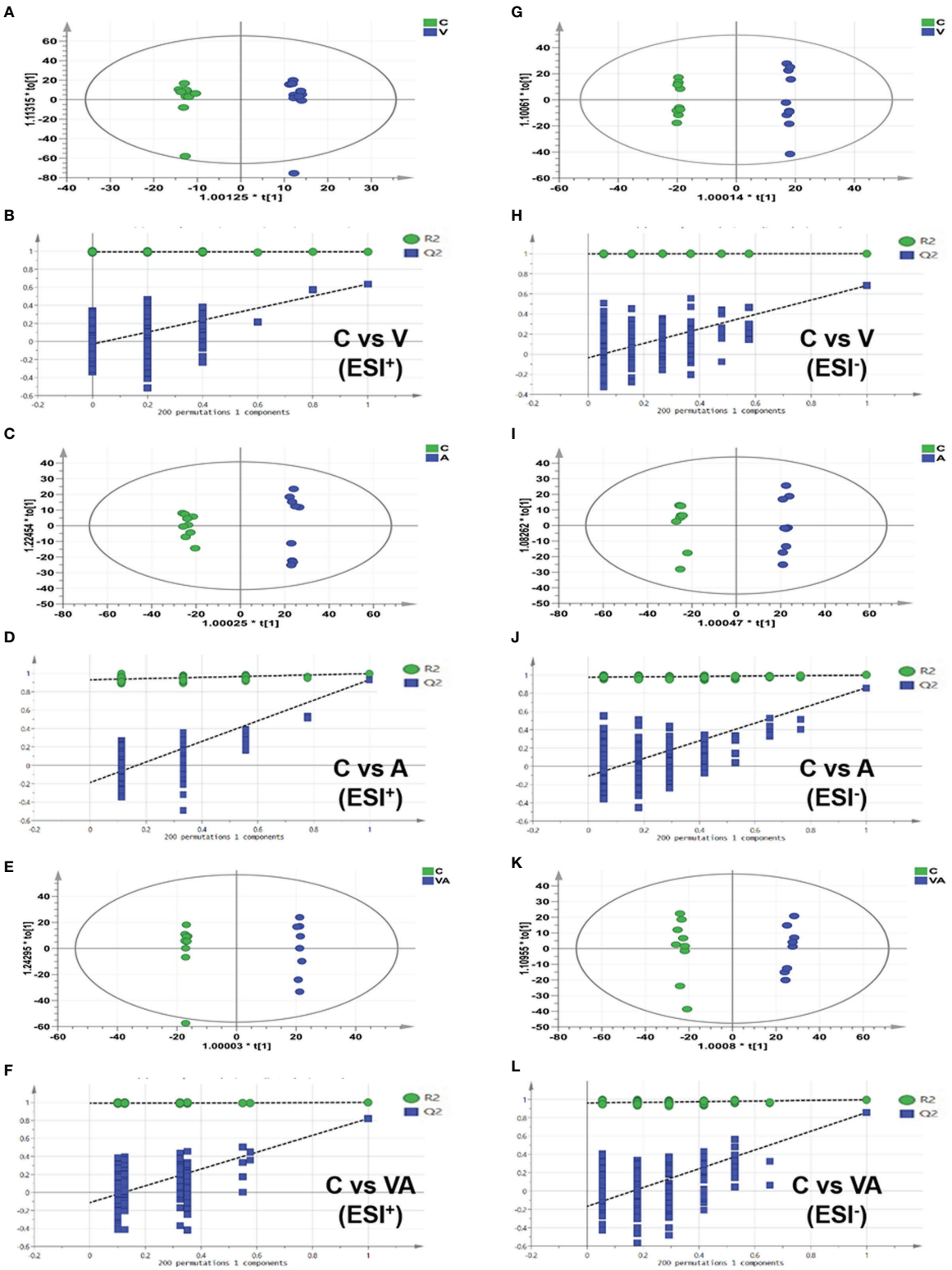

FIGURE 4 | OPLS-DA score plot based on the data of $ESI^{+/-}$ modes and validations of OPLS-DA models by 200 permutation tests. In ESI^{+} mode: **(A, B)** group C *vs* group V, **(C, D)** group C *vs* group A, **(E, F)** group C *vs* group VA. In ESI^{-} mode: **(G, H)** group C *vs* group V, **(I, J)** group C *vs* group A, **(K, L)** group C *vs* group VA.

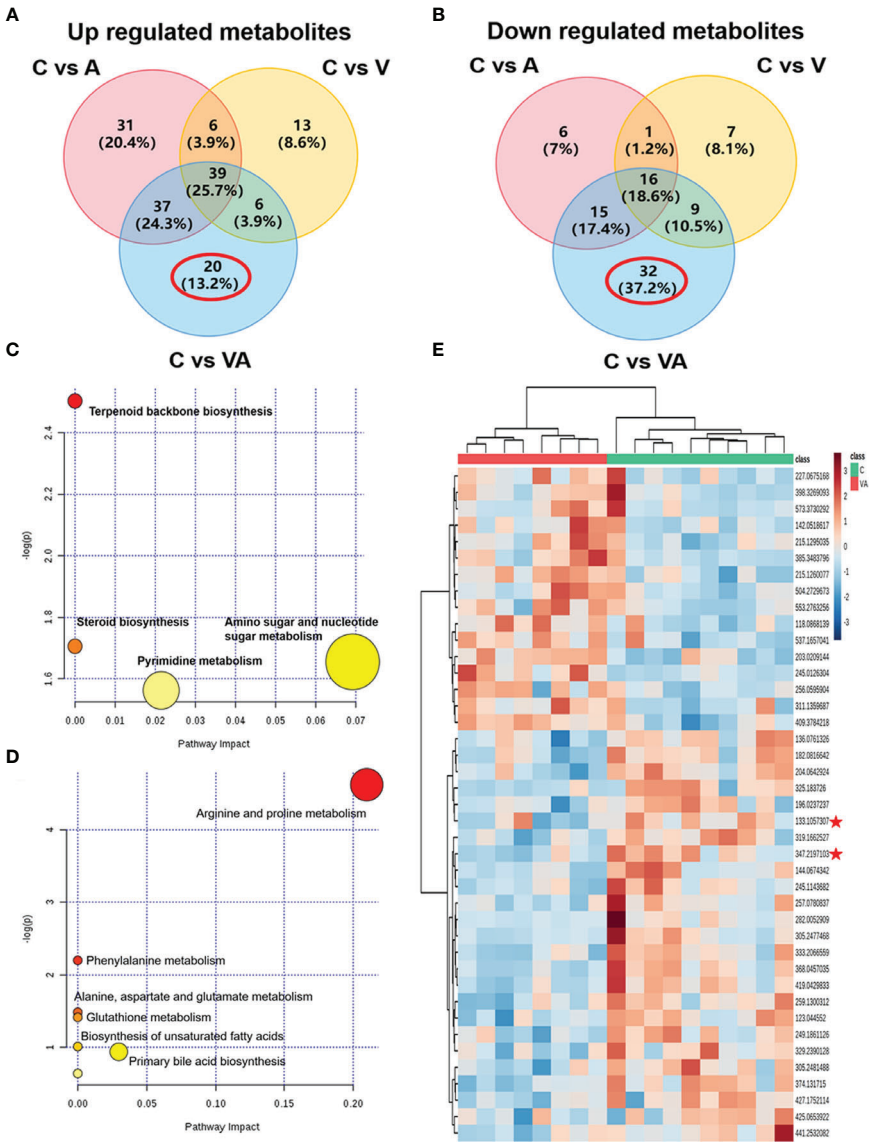

FIGURE 5 | Ornithine is a therapeutic target of VA treatment in MM mice. **(A)** Venn diagram displaying the 20 upregulated distinct metabolites in the serum of Group VA. **(B)** Venn diagram displaying the 32 downregulated distinct metabolites in the serum of Group VA. **(C)** Summary of joint pathway analysis in group VA with MetaboAnalyst 5.0. **(D)** Summary of joint pathway analysis in group VA with MetaboAnalyst 5.0. **(E)** Heatmap showing arginine and ornithine were downregulated metabolites in group VA.

1,953.33 ng/ml) (**Figure 6C**); however, it didn't reach statistical difference due to the relatively small sample size in each group and large individual variation. In agreement with previous results of untargeted metabolomics, these data confirmed that VA treatment decreased the level of serum ornithine.

Arginine and Its Metabolite Promote MM Cell Proliferation

Arginine is a semi-essential amino acid that can be metabolized into ornithine, which is a non-essential amino acid (**Figure 6D**). We further assessed the effect of supplying extra arginine on MM cell proliferation by using CCK8 assay. As shown in **Figures 7A–D**, the viability of human ARP1, H929, OCI and mouse 5TMM3VT cells was significantly increased upon serial concentration of arginine (5 nM~5 μM) treatment for 72 h, suggesting that VA treatment could regulate arginine and its metabolites to promote MM cell proliferation.

Elevated Ornithine Decarboxylase 1 Expression Is Associated With Poor Prognosis in MM

To gain further insights into the deregulated ornithine, we also explored the relationship between ODC1 known as the coding

TABLE 1 | Partially* distinct upregulated substances in group VA.

Name	m/z	*P*	FC	VIP
2'-Deoxyuridine	227.0675	0.0091	1.8167	1.3109
4-Cholesten-3-One	385.3484	0.0093	1.8663	1.2221
Calcifediol	398.3269	0.0057	1.689	1.295
D-Desthiobiotin	215.126	0.0013	1.2113	1.3391
Dimethylallyl pyrophosphate	245.0126	0.0017	2.7971	1.4729
D-Norvaline	118.0868	0.0128	1.6716	1.0571
Hypoxanthine-9-β-D-arabinofuranoside	537.1657	0.0104	1.6909	1.1158
L-Altrose	203.0234	<0.0001	2.262	1.5535
Lasalocid	573.373	0.0474	1.9948	1.2608
Leucine enkephalin amide	553.2763	0.0159	1.821	1.3428
N-Acetyl-D-glucosamine	256.0596	0.0118	1.1686	1.2742
N-Methyl-L-glutamic acid	142.0519	0.0179	2.0681	1.1724
Nα-Acetyl-L-arginine	215.1295	0.018	1.3949	1.3476
Taurolithocholic acid	504.273	0.0242	1.6907	1.355
α-Amyrin	409.3784	0.0006	1.6457	1.3498

**This table didn't list five exogenous compounds, namely, dihydrocapsaicin, benzoic acid, Apramycin, sulfa quinazoline (sulfaquinaoxaline), equol.*

gene encoding ornithine decarboxylase (**Figure 6D**) and the prognosis of MM patients. GEP analysis showed that increased ODC1 expression was associated with poor overall survival (OS) in MM patients (TT2, GSE2658) (p=0.0002; **Figure 7E**). This result was also verified in the APEX phase III clinical trial with relapsed MM patients (p=0.0009; **Figure 7F**). Furthermore, analyses of two gene expression omnibus (GEO) databases, GSE5900 (p<0.0001; **Figure 7G**) and GSE6477 (p=0.0350; **Figure 7H**), demonstrated that ODC1 mRNA was significantly increased in MM patients compared with smoldering myeloma (SMM), monoclonal gammopathy of undetermined significance (MGUS), and normal plasma (NP).

DISCUSSION

Many clinical cases have shown the certain advantages of acupuncture and medicine combination in the treatment of pain (18, 19, 21, 22). Acupuncture can increase the number of white blood cells and alleviate leukopenia induced by radiotherapy (35).

TABLE 2 | Partially* distinct downregulated substances in group VA.

Name	m/z	*P*	FC	VIP
13-Cis-Acitretin	325.1837	0.0033	0.7025	1.4494
2'-Deoxyguanosine-5'-monophosphate	368.0447	0.0017	0.3811	1.5304
2-Phenylacetamide	136.0766	0.0145	0.7426	1.0975
4-Guanidinobutyric acid	144.0681	0.0133	0.3947	1.222
4-Hydroxybenzaldehyde	123.0449	0.0056	0.5706	1.1689
5-Methyluridine	257.0788	0.0245	0.7067	1.1576
7-Ketodeoxycholic acid	441.2529	0.0499	0.6459	1.206
7z,10z,13z-Hexadecatrienoic Acid	249.1856	0.0152	0.7752	1.2389
All-cis-4,7,10,13,16-docosapentaenoic acid	329.2332	0.0403	0.8578	1.0992
Arginine	347.2198	0.0056	0.897	1.3782
Boc-β-cyano-L-alanine	427.1765	0.029	0.7437	1.1536
Cis-8,11,14-Eicosatrienoic acid	305.2477	0.0188	0.8018	1.2626
D-(+)-Octopine	245.1151	0.0209	0.5288	1.1965
Dl-Tyrosine	182.0812	0.01	0.635	1.1161
Glycochenodeoxycholic acid	484.2898	0.0037	0.2768	1.4485
Haloperidol	374.1322	0.0049	0.6947	1.419
Indole-3-pyruvic acid	204.0638	0.0064	0.5987	1.1311
L-Cysteine-glutathione gisulfide	425.0654	0.0291	0.7847	1.087
Mesterolone	305.248	0.013	0.4361	1.0414
Mucic acid	419.0485	0.0018	0.3993	1.5564
N-Acetylaspartate	196.0235	0.0252	0.7582	1.2739
O-Phospho-L-Tyrosine	282.0055	0.0094	0.5057	1.3553
Ornithine	133.1055	0.0333	0.6108	1.0133
Phe-Phe	311.1348	0.0135	0.8143	1.3764
Prostaglandin B2	333.2069	0.006	0.6439	1.385
β-Zearalenol	319.1662	0.0075	0.6886	1.3195
γ-Glu-Leu	259.1303	0.0166	0.5956	1.2689

**This table didn't list five exogenous compounds, namely, diazepam, diltiazem diacetate, diflunisal, oxfendazole, diflunisal.*

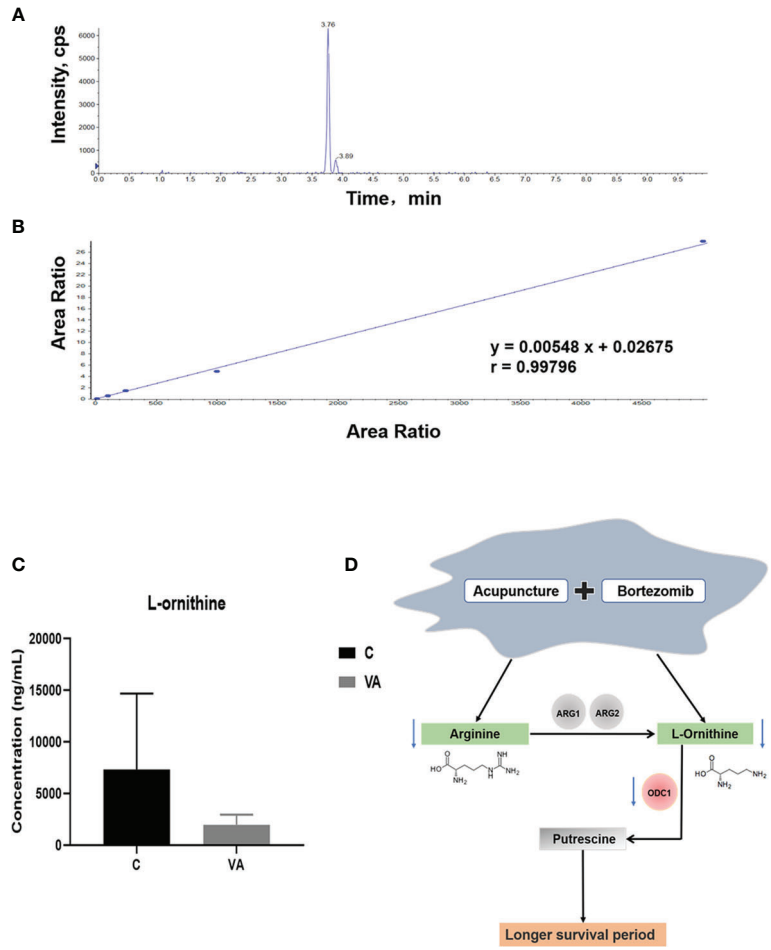

FIGURE 6 | Ornithine concentration is decreased in serum samples of group VA. **(A)** Characteristic chromatographic peak of ornithine standard. **(B)** Standard curve of ornithine in targeted metabolomics. **(C)** Ornithine concentration in serum samples of group C and VA. **(D)** The possible mechanism involved in VA treatment.

It was reported that acupuncture could reduce lymphedema and improve dyspnea symptoms in breast cancer patients (36, 37). In addition, acupuncture inhibits inflammation (38) and relieves symptoms caused by cancer *via* modulating vasomotion and stimulating the vagus nerve to modulate visceral inflammatory responses (39). Some clinical reports demonstrate that the acupuncture can be applied for symptom reduction in myeloma patients, including chemotherapy-induced peripheral neuropathy (40–42). However, there are few studies using metabolomics technology to find the therapeutic targets of MM. Our study first explored the mechanism of VA treatment for MM from the synergistic effect of VA treatment on MM and verified by *in vivo* experiment with metabolomics technology (**Figures 1A–C**).

We obtained the characteristics of metabolites in serum of MM model mice with either bortezomib or acupuncture or both treatments (**Figures 2A–H**, **3A–D**, **4A–L**). There were only 20 significantly upregulated metabolites (**Figure 5A**) and 32 significantly downregulated metabolites (**Figures 5B, E**) in group VA compared with control. Among them, ornithine as a unique downregulated metabolite in group VA was involved in the arginine and proline metabolic pathway, which showed the highest influence value (**Figures 5C, D**). Moreover, ornithine also participated in the regulation of glutathione metabolic pathway (43, 44), which may play a role during VA treatment. Therefore, it was suggested that ornithine might be a promising biomarker of VA therapy for MM (**Figures 6A–C**).

Arginine serving as a semi-essential amino acid possesses a significant impact on carcinogenesis and tumor biology (45), and it is mostly metabolized to ornithine by arginase (46, 47). Arginine metabolism is considered to be an important regulator in controlling immune response (48, 49), inhibiting antitumor immune response (50, 51), and promoting tumor development (34, 52). Ornithine is decarboxylated by ODC1 to produce putrescine, which is the rate-limiting step in polyamine biosynthesis (53, 54). Combined with cellular proliferation results (**Figures 7A–D**), we speculate that inhibiting arginine-ornithine metabolism can reduce ornithine content, thus decrease polyamine biosynthesis.

Last but not least, our data revealed that high ODC1 expression was significantly associated with poor prognosis in

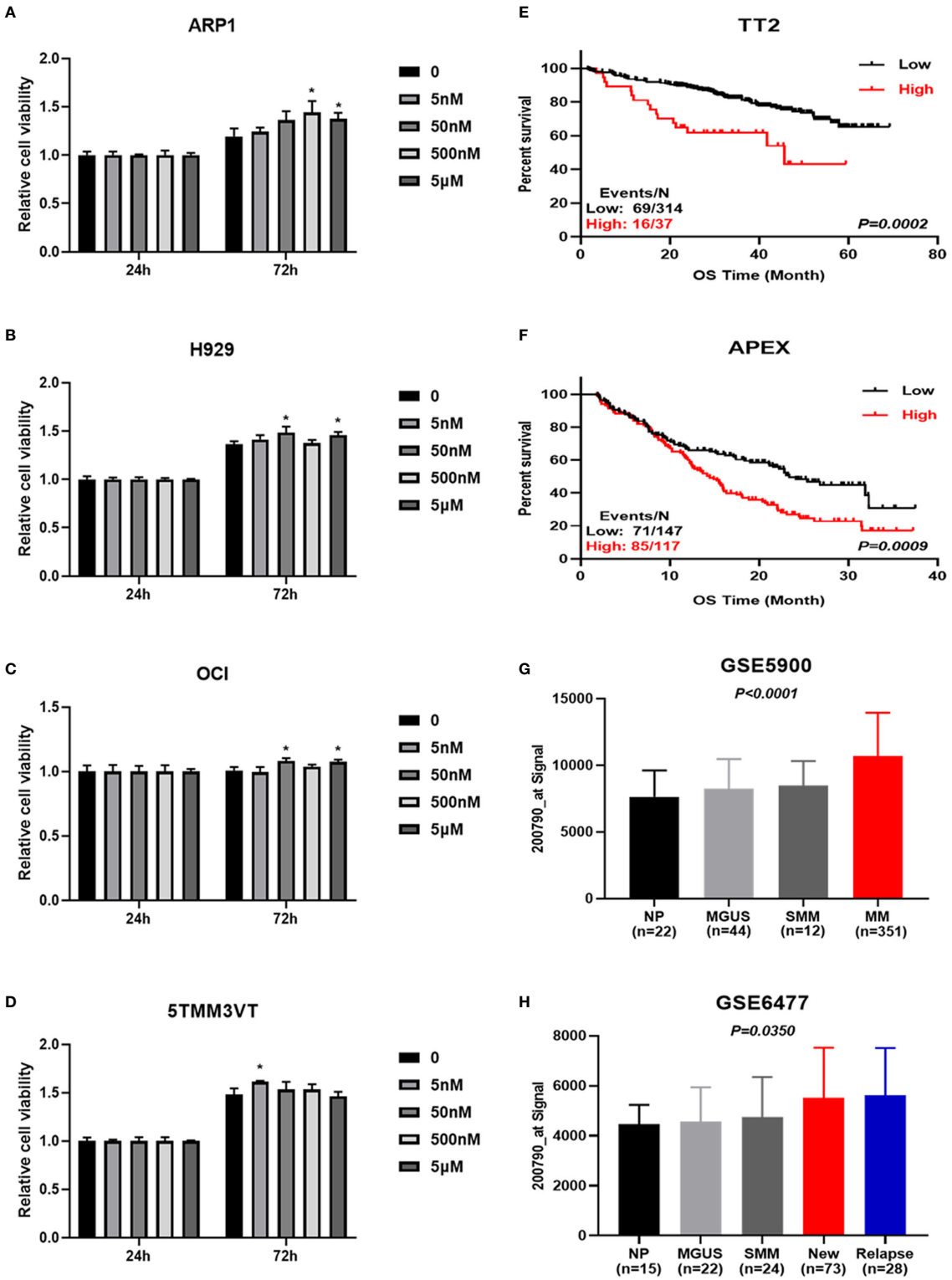

FIGURE 7 | Increased ODC1 expression is associated with poor prognosis in MM. **(A–D)** Arginine and its metabolite promoted ARP1, H929, OCI, and 5TMM3VT cell proliferation. *$P < 0.05$. **(E, F)** High ODC1 expression in MM patients was correlated with poor OS in TT2 cohort, and APEX phase III clinical trial by log-rank test. **(G, H)** The mRNA level of ODC1 from NP, MGUS, SMM, and MM was significantly increased in MM samples by ordinary one-way ANOVA test.

MM patients (**Figures 7E–H**). In fact, ODC1 is the exclusive gene encoding the rate-limiting enzyme of the polyamine biosynthesis pathway, which catalyzes ornithine to polyamines. Mounting studies reported that ODC1 expression was increased in many cancers, such as esophageal carcinoma (55), colorectal cancer (56), hepatocellular carcinoma (57), neuroblastoma (58), and ovarian cancer (59). Bianchi-Smiraglia A et al. (60) demonstrated that aryl hydrocarbon receptor (AHR) positively regulated intracellular polyamine production *via* direct transcriptional activation of ODC1 and AZIN1 genes, which inhibited the aryl hydrocarbon receptor/polyamine biosynthesis axis to suppress MM progression. Taken together, it may be concluded that combination of acupuncture and bortezomib can decrease ornithine and reduce ODC1 to prolong the survival time of MM. However, more work is needed to further validate the therapeutic effect of targeting arginine-ornithine metabolism and interfering ODC1 expression by using RNAi or difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase (61), to improve the effect of MM treatment.

In summary, our study demonstrates that combination of acupuncture and bortezomib has synergistic effects in the treatment of MM, which prolongs survival time of MM mice *via* decreasing ornithine. Targeting ornithine-mediated metabolism may be a promising way to benefit MM patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors. The data presented in the study are deposited in the Metabolights repository, accession number MTBLS3487.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Ethics Review Boards of Nanjing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

YY, CG, and BX designed the project, analyzed the data, and edited the manuscript. MK and JQ drafted the manuscript. MK, JQ, FH, XYL, HW, and XL performed the experimental work and analyzed the data. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. International Myeloma Working G. Criteria for the Classification of Monoclonal Gammopathies, Multiple Myeloma and Related Disorders: A Report of the International Myeloma Working Group. *Br J Haematol* (2003) 121(5):749–57. doi: 10.1046/j.1365-2141.2003.04355.x
2. Kourelis TV, Kumar SK, Gertz MA, Lacy MQ, Buadi FK, Hayman SR, et al. Coexistent Multiple Myeloma or Increased Bone Marrow Plasma Cells Define Equally High-Risk Populations in Patients With Immunoglobulin Light Chain Amyloidosis. *J Clin Oncol* (2013) 31(34):4319–24. doi: 10.1200/JCO.2013.50.8499
3. Joshua DE, Bryant C, Dix C, Gibson J, Ho J. Biology and Therapy of Multiple Myeloma. *Med J Aust* (2019) 210(8):375–80. doi: 10.5694/mja2.50129
4. Minnie SA, Hill GR. Immunotherapy of Multiple Myeloma. *J Clin Invest* (2020) 130(4):1565–75. doi: 10.1172/JCI129205
5. Blimark CH, Turesson I, Genell A, Ahlberg L, Bjorkstrand B, Carlson K, et al. Outcome and Survival of Myeloma Patients Diagnosed 2008-2015. Real-World Data on 4904 Patients From the Swedish Myeloma Registry. *Haematologica* (2018) 103(3):506–13. doi: 10.3324/haematol.2017.178103
6. Ke M, Kang L, Wang L, Yang S, Wang Y, Liu H, et al. CAR-T Therapy Alters Synthesis of Platelet-Activating Factor in Multiple Myeloma Patients. *J Hematol Oncol* (2021) 14(1):90. doi: 10.1186/s13045-021-01101-6
7. Chen Y, Li R, Zhu Y, Zhong S, Qian J, Yang D, et al. Dihydroartemisinin Induces Growth Arrest and Overcomes Dexamethasone Resistance in Multiple Myeloma. *Front Oncol* (2020) 10:767. doi: 10.3389/fonc.2020.00767
8. Hou J, Qian J, Li Z, Gong A, Zhong S, Qiao L, et al. Bioactive Compounds From Abelmoschus Manihot L. Alleviate the Progression of Multiple Myeloma in Mouse Model and Improve Bone Marrow Microenvironment. *Onco Targets Ther* (2020) 13:959–73. doi: 10.2147/OTT.S235944
9. Qiao L, Guo M, Qian J, Xu B, Gu C, Yang Y. Research Advances on Acupuncture Analgesia. *Am J Chin Med* (2020) 48(2):245–58. doi: 10.1142/S0192415X20500135
10. Chen Y, Li R, Zhu Y, Zhong S, Qian J, Yang D, et al. Corrigendum: Dihydroartemisinin Induces Growth Arrest and Overcomes Dexamethasone Resistance in Multiple Myeloma. *Front Oncol* (2021) 11:736373. doi: 10.3389/fonc.2021.736373
11. Dou R, Qian J, Wu W, Zhang Y, Yuan Y, Guo M, et al. Suppression of Steroid 5alpha-Reductase Type I Promotes Cellular Apoptosis and Autophagy *via* PI3K/Akt/mTOR Pathway in Multiple Myeloma. *Cell Death Dis* (2021) 12 (2):206. doi: 10.1038/s41419-021-03510-4
12. Gu C, Lu T, Wang W, Shao M, Wei R, Guo M, et al. RFWD2 Induces Cellular Proliferation and Selective Proteasome Inhibitor Resistance by Mediating P27 Ubiquitination in Multiple Myeloma. *Leukemia* (2021) 35(6):1803–7. doi: 10.1038/s41375-020-01033-z
13. Gu C, Wang W, Tang X, Xu T, Zhang Y, Guo M, et al. CHEK1 and Circchek1_246aa Evoke Chromosomal Instability and Induce Bone Lesion Formation in Multiple Myeloma. *Mol Cancer* (2021) 20(1):84. doi: 10.1186/s12943-021-01380-0
14. Guo M, Ding P, Zhu Z, Fan L, Zhou Y, Yang S, et al. Targeting RFWD2 as an Effective Strategy to Inhibit Cellular Proliferation and Overcome Drug

Resistance to Proteasome Inhibitor in Multiple Myeloma. *Front Cell Dev Biol* (2021) 9:675939. doi: 10.3389/fcell.2021.675939
15. Swarm RA, Paice JA, Anghelescu DL, Are M, Bruce JY, Buga S, et al. Adult Cancer Pain, Version 3.2019, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw* (2019) 17(8):977–1007. doi: 10.6004/jnccn.2019.0038
16. Bao T, Seidman AD, Piulson L, Vertosick E, Chen X, Vickers AJ, et al. A Phase IIA Trial of Acupuncture to Reduce Chemotherapy-Induced Peripheral Neuropathy Severity During Neoadjuvant or Adjuvant Weekly Paclitaxel Chemotherapy in Breast Cancer Patients. *Eur J Cancer* (2018) 101:12–9. doi: 10.1016/j.ejca.2018.06.008
17. Hershman DL, Unger JM, Greenlee H, Capodice JL, Lew DL, Darke AK, et al. Effect of Acupuncture *vs* Sham Acupuncture or Waitlist Control on Joint Pain Related to Aromatase Inhibitors Among Women With Early-Stage Breast Cancer: A Randomized Clinical Trial. *JAMA* (2018) 320(2):167–76. doi: 10.1001/jama.2018.8907
18. Mi WL, Mao-Ying QL, Liu Q, Wang XW, Wang YQ, Wu GC. Synergistic Anti-Hyperalgesia of Electroacupuncture and Low Dose of Celecoxib in Monoarthritic Rats: Involvement of the Cyclooxygenase Activity in the Spinal Cord. *Brain Res Bull* (2008) 77(2-3):98–104. doi: 10.1016/j.brainresbull.2008.04.008
19. Chiu HY, Hsieh YJ, Tsai PS. Systematic Review and Meta-Analysis of Acupuncture to Reduce Cancer-Related Pain. *Eur J Cancer Care (Engl)* (2017) 26(2):e12457. doi: 10.1111/ecc.12457
20. Seo SY, Lee KB, Shin JS, Lee J, Kim MR, Ha IH, et al. Effectiveness of Acupuncture and Electroacupuncture for Chronic Neck Pain: A Systematic Review and Meta-Analysis. *Am J Chin Med* (2017) 45(8):1573–95. doi: 10.1142/S0192415X17500859
21. Lin JG, Lo MW, Wen YR, Hsieh CL, Tsai SK, Sun WZ. The Effect of High and Low Frequency Electroacupuncture in Pain After Lower Abdominal Surgery. *Pain* (2002) 99(3):509–14. doi: 10.1016/S0304-3959(02)00261-0
22. Mavrommatis CI, Argyra E, Vadalouka A, Vasilakos DG. Acupuncture as an Adjunctive Therapy to Pharmacological Treatment in Patients With Chronic Pain Due to Osteoarthritis of the Knee: A 3-Armed, Randomized, Placebo-Controlled Trial. *Pain* (2012) 153(8):1720–6. doi: 10.1016/j.pain.2012.05.005
23. Liberti MV, Locasale JW. Correction to: 'The Warburg Effect: How Does it Benefit Cancer Cells?': [Trends in Biochemical Sciences, 41 (2016) 211]. *Trends Biochem Sci* (2016) 41(3):287. doi: 10.1016/j.tibs.2016.01.004
24. Faubert B, Solmonson A, DeBerardinis RJ. Metabolic Reprogramming and Cancer Progression. *Science* (2020) 368(6487):eaaw5473. doi: 10.1126/science.aaw5473
25. Lin Y, Ma C, Bezabeh T, Wang Z, Liang J, Huang Y, et al. (1) H NMR-Based Metabolomics Reveal Overlapping Discriminatory Metabolites and Metabolic Pathway Disturbances Between Colorectal Tumor Tissues and Fecal Samples. *Int J Cancer* (2019) 145(6):1679–89. doi: 10.1002/ijc.32190
26. Robinson AJ, Hopkins GL, Rastogi N, Hodges M, Doyle M, Davies S, et al. Reactive Oxygen Species Drive Proliferation in Acute Myeloid Leukemia *via* the Glycolytic Regulator PFKFB3. *Cancer Res* (2020) 80(5):937–49. doi: 10.1158/0008-5472.CAN-19-1920
27. Li Z, Guan M, Lin Y, Cui X, Zhang Y, Zhao Z, et al. Aberrant Lipid Metabolism in Hepatocellular Carcinoma Revealed by Liver Lipidomics. *Int J Mol Sci* (2017) 18(12):2550. doi: 10.3390/ijms18122550
28. Colombero C, Cardenas S, Venara M, Martin A, Pennisi P, Barontini M, et al. Cytochrome 450 Metabolites of Arachidonic Acid (20-HETE, 11,12-EET and 14,15-EET) Promote Pheochromocytoma Cell Growth and Tumor Associated Angiogenesis. *Biochimie* (2020) 171-172:147–57. doi: 10.1016/j.biochi.2020.02.014
29. Lee YC, Lin CH, Hung SY, Chung HY, Luo ST, MacDonald I, et al. Manual Acupuncture Relieves Bile Acid-Induced Itch in Mice: The Role of Microglia and TNF-Alpha. *Int J Med Sci* (2018) 15(9):953–60. doi: 10.7150/ijms.24146
30. Zhao H, Dong F, Li Y, Ren X, Xia Z, Wang Y, et al. Inhibiting ATG5 Mediated Autophagy to Regulate Endoplasmic Reticulum Stress and CD4(+) T Lymphocyte Differentiation: Mechanisms of Acupuncture's Effects on Asthma. *BioMed Pharmacother* (2021) 142:112045. doi: 10.1016/j.biopha.2021.112045
31. Zou Y, Bhat OM, Yuan X, Li G, Huang D, Guo Y, et al. Release and Actions of Inflammatory Exosomes in Pulmonary Emphysema: Potential Therapeutic Target of Acupuncture. *J Inflamm Res* (2021) 14:3501–21. doi: 10.2147/JIR.S312385
32. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, et al. MetaboAnalyst 4.0: Towards More Transparent and Integrative Metabolomics Analysis. *Nucleic Acids Res* (2018) 46(W1):W486–94. doi: 10.1093/nar/gky310
33. Fernandez-Garcia JC, Delpino-Rius A, Samarra I, Castellano-Castillo D, Munoz-Garach A, Bernal-Lopez MR, et al. Type 2 Diabetes Is Associated With a Different Pattern of Serum Polyamines: A Case(-)Control Study From the PREDIMED-Plus Trial. *J Clin Med* (2019) 8(1):71. doi: 10.3390/jcm8010071
34. Poillet-Perez L, Xie X, Zhan L, Yang Y, Sharp DW, Hu ZS, et al. Autophagy Maintains Tumour Growth Through Circulating Arginine. *Nature* (2018) 563(7732):569–73. doi: 10.1038/s41586-018-0697-7
35. Chao LF, Zhang AL, Liu HE, Cheng MH, Lam HB, Lo SK. The Efficacy of Acupoint Stimulation for the Management of Therapy-Related Adverse Events in Patients With Breast Cancer: A Systematic Review. *Breast Cancer Res Treat* (2009) 118(2):255–67. doi: 10.1007/s10549-009-0533-8
36. Lyman GH, Greenlee H, Bohlke K, Bao T, DeMichele AM, Deng GE, et al. Integrative Therapies During and After Breast Cancer Treatment: ASCO Endorsement of the SIO Clinical Practice Guideline. *J Clin Oncol* (2018) 36(25):2647–55. doi: 10.1200/JCO.2018.79.2721
37. Wang C, Yang M, Fan Y, Pei X. Moxibustion as a Therapy for Breast Cancer-Related Lymphedema in Female Adults: A Preliminary Randomized Controlled Trial. *Integr Cancer Ther* (2019) 18:1534735419866919. doi: 10.1177/1534735419866919
38. Lim HD, Kim MH, Lee CY, Namgung U. Anti-Inflammatory Effects of Acupuncture Stimulation *via* the Vagus Nerve. *PloS One* (2016) 11(3):e0151882. doi: 10.1371/journal.pone.0151882
39. O'Regan D, Filshie J. Acupuncture and Cancer. *Auton Neurosci* (2010) 157(1-2):96–100. doi: 10.1016/j.autneu.2010.05.001
40. Bao T, Goloubeva O, Pelser C, Porter N, Primrose J, Hester L, et al. A Pilot Study of Acupuncture in Treating Bortezomib-Induced Peripheral Neuropathy in Patients With Multiple Myeloma. *Integr Cancer Ther* (2014) 13(5):396–404. doi: 10.1177/1534735414534729
41. Garcia MK, Cohen L, Guo Y, Zhou Y, You B, Chiang J, et al. Electroacupuncture for Thalidomide/Bortezomib-Induced Peripheral Neuropathy in Multiple Myeloma: A Feasibility Study. *J Hematol Oncol* (2014) 7:41. doi: 10.1186/1756-8722-7-41
42. Han X, Wang L, Shi H, Zheng G, He J, Wu W, et al. Acupuncture Combined With Methylcobalamin for the Treatment of Chemotherapy-Induced Peripheral Neuropathy in Patients With Multiple Myeloma. *BMC Cancer* (2017) 17(1):40. doi: 10.1186/s12885-016-3037-z
43. Yao W, Li H, Liu Q, Gao Y, Dai J, Bao B, et al. Cellular Metabolomics Revealed the Cytoprotection of Amentoflavone, a Natural Compound, in Lipopolysaccharide-Induced Injury of Human Umbilical Vein Endothelial Cells. *Int J Mol Sci* (2016) 17(9):1514. doi: 10.3390/ijms17091514
44. Gan C, Huang X, Wu Y, Zhan J, Zhang X, Liu Q, et al. Untargeted Metabolomics Study and Pro-Apoptotic Properties of B-Norcholesteryl Benzimidazole Compounds in Ovarian Cancer SKOV3 Cells. *J Steroid Biochem Mol Biol* (2020) 202:105709. doi: 10.1016/j.jsbmb.2020.105709
45. Kus K, Kij A, Zakrzewska A, Jasztal A, Stojak M, Walczak M, et al. Alterations in Arginine and Energy Metabolism, Structural and Signalling Lipids in Metastatic Breast Cancer in Mice Detected in Plasma by Targeted Metabolomics and Lipidomics. *Breast Cancer Res* (2018) 20(1):148. doi: 10.1186/s13058-018-1075-y
46. Du T, Han J. Arginine Metabolism and Its Potential in Treatment of Colorectal Cancer. *Front Cell Dev Biol* (2021) 9:658861. doi: 10.3389/fcell.2021.658861
47. Bednarz-Misa I, Fleszar MG, Fortuna P, Lewandowski L, Mierzchala-Pasierb M, Diakowska D, et al. Altered L-Arginine Metabolic Pathways in Gastric Cancer: Potential Therapeutic Targets and Biomarkers. *Biomolecules* (2021) 11(8):1086. doi: 10.3390/biom11081086
48. Lemos H, Huang L, Prendergast GC, Mellor AL. Immune Control by Amino Acid Catabolism During Tumorigenesis and Therapy. *Nat Rev Cancer* (2019) 19(3):162–75. doi: 10.1038/s41568-019-0106-z
49. Grzywa TM, Sosnowska A, Matryba P, Rydzynska Z, Jasinski M, Nowis D, et al. Myeloid Cell-Derived Arginase in Cancer Immune Response. *Front Immunol* (2020) 11:938. doi: 10.3389/fimmu.2020.00938

50. Steggerda SM, Bennett MK, Chen J, Emberley E, Huang T, Janes JR, et al. Inhibition of Arginase by CB-1158 Blocks Myeloid Cell-Mediated Immune Suppression in the Tumor Microenvironment. *J Immunother Cancer* (2017) 5 (1):101. doi: 10.1186/s40425-017-0308-4
51. Miret JJ, Kirschmeier P, Koyama S, Zhu M, Li YY, Naito Y, et al. Suppression of Myeloid Cell Arginase Activity Leads to Therapeutic Response in a NSCLC Mouse Model by Activating Anti-Tumor Immunity. *J Immunother Cancer* (2019) 7(1):32. doi: 10.1186/s40425-019-0504-5
52. Song X, Wan X, Huang T, Zeng C, Sastry N, Wu B, et al. SRSF3-Regulated RNA Alternative Splicing Promotes Glioblastoma Tumorigenicity by Affecting Multiple Cellular Processes. *Cancer Res* (2019) 79(20):5288–301. doi: 10.1158/0008-5472.CAN-19-1504
53. Khan A, Gamble LD, Upton DH, Ung C, Yu DMT, Ehteda A, et al. Dual Targeting of Polyamine Synthesis and Uptake in Diffuse Intrinsic Pontine Gliomas. *Nat Commun* (2021) 12(1):971. doi: 10.1038/s41467-021-20896-z
54. Gamble LD, Purgato S, Henderson MJ, Di Giacomo S, Russell AJ, Pigini P, et al. A G316A Polymorphism in the Ornithine Decarboxylase Gene Promoter Modulates MYCN-Driven Childhood Neuroblastoma. *Cancers (Basel)* (2021) 13(8):1807. doi: 10.3390/cancers13081807
55. Yan W, Shih JH, Rodriguez-Canales J, Tangrea MA, Ylaya K, Hipp J, et al. Identification of Unique Expression Signatures and Therapeutic Targets in Esophageal Squamous Cell Carcinoma. *BMC Res Notes* (2012) 5:73. doi: 10.1186/1756-0500-5-73
56. Zhang C, Aldrees M, Arif M, Li X, Mardinoglu A, Aziz MA. Elucidating the Reprograming of Colorectal Cancer Metabolism Using Genome-Scale Metabolic Modeling. *Front Oncol* (2019) 9:681. doi: 10.3389/fonc.2019.00681
57. Ye Z, Zeng Z, Shen Y, Yang Q, Chen D, Chen Z, et al. ODC1 Promotes Proliferation and Mobility *via* the AKT/GSK3beta/beta-Catenin Pathway and Modulation of Acidotic Microenvironment in Human Hepatocellular Carcinoma. *Onco Targets Ther* (2019) 12:4081–92. doi: 10.2147/OTT.S198341
58. Gamble LD, Purgato S, Murray J, Xiao L, Yu DMT, Hanssen KM, et al. Inhibition of Polyamine Synthesis and Uptake Reduces Tumor Progression and Prolongs Survival in Mouse Models of Neuroblastoma. *Sci Transl Med* (2019) 11(477):eaau1099. doi: 10.1126/scitranslmed.aau1099
59. Guo T, Li B, Gu C, Chen X, Han M, Liu X, et al. PGC-1alpha Inhibits Polyamine Metabolism in Cyclin E1-Driven Ovarian Cancer. *Cancer Med* (2019) 8(18):7754–61. doi: 10.1002/cam4.2637
60. Bianchi-Smiraglia A, Bagati A, Fink EE, Affronti HC, Lipchick BC, Moparthy S, et al. Inhibition of the Aryl Hydrocarbon Receptor/Polyamine Biosynthesis Axis Suppresses Multiple Myeloma. *J Clin Invest* (2018) 128(10):4682–96. doi: 10.1172/JCI70712
61. He W, Roh E, Yao K, Liu K, Meng X, Liu F, et al. Targeting Ornithine Decarboxylase (ODC) Inhibits Esophageal Squamous Cell Carcinoma Progression. *NPJ Precis Oncol* (2017) 1(1):13. doi: 10.1038/s41698-017-0014-1

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REVIEW
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Ferroptosis: Redox Imbalance and Hematological Tumorigenesis

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Ferroptosis is a novel characterized form of cell death featured with iron-dependent lipid peroxidation, which is distinct from any known programmed cell death in the biological processes and morphological characteristics. Recent evidence points out that ferroptosis is correlated with numerous metabolic pathways, including iron homeostasis, lipid metabolism, and redox homeostasis, associating with the occurrence and treatment of hematological malignancies, such as multiple myeloma, leukemia, and lymphoma. Nowadays, utilizing ferroptosis as the target to prevent and treat hematological malignancies has become an active and challenging topic of research, and the regulatory network and physiological function of ferroptosis also need to be further elucidated. This review will summarize the recent progress in the molecular regulation of ferroptosis and the physiological roles and therapeutic potential of ferroptosis as the target in hematological malignancies.

Keywords: ferroptosis, redox, hematologic malignancies, lipid metabolism, iron homeostasis

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INTRODUCTION

Death is an irreversible regulation process in the living cells, and different ways of death relate to the distinct physiological functions. Cell death is divided into programmed cell death and cell necrosis. Programmed cell death is an actively induced and tightly controlled process of cell suicide in response to various signal stimuli. In contrast, cell necrosis is an acute, spontaneous and passive death caused by unrepairable stress under pathological conditions, such as physical, chemical, hypoxia, or insufficient energy (mainly ATP) (1–6). When the cell is necrotic, the integrity of the cytoplasmic membrane is destroyed, and the barrier function to Na^+, Ca^{2+}, and water will be lost. Water flowing into the cell could lead to cytoplasmic swelling and nucleus pyknosis, eventually leading to cell rupture (7). The most canonical way of programmed cell death is apoptosis, which relates to cell contraction, chromatin condensation, and the formation of apoptotic bodies. With the continuous advancement of research on the manner of cell death, it has been found that in addition to apoptosis, programmed cell death also comprises autophagy, programmed necrosis, pyroptosis, and ferroptosis. Ferroptosis is a new form of cell death discovered by Stockwell in searching for small molecules targeted at RAS protein mutations related to cancer. Its morphological characteristics are different from any known form of cell death, as shown in **Figure 1** (8). Iron-dependent cell membrane lipid peroxidation will lead to ferroptosis. At this time, the cell's mitochondrial membrane density will increase, while the mitochondrial cristae will decrease or disappear, and the mitochondrial outer membrane will rupture, but the nucleus will remain normal (9, 10). Since ferroptosis was defined as a new form of cell death in 2012, more and more researchers

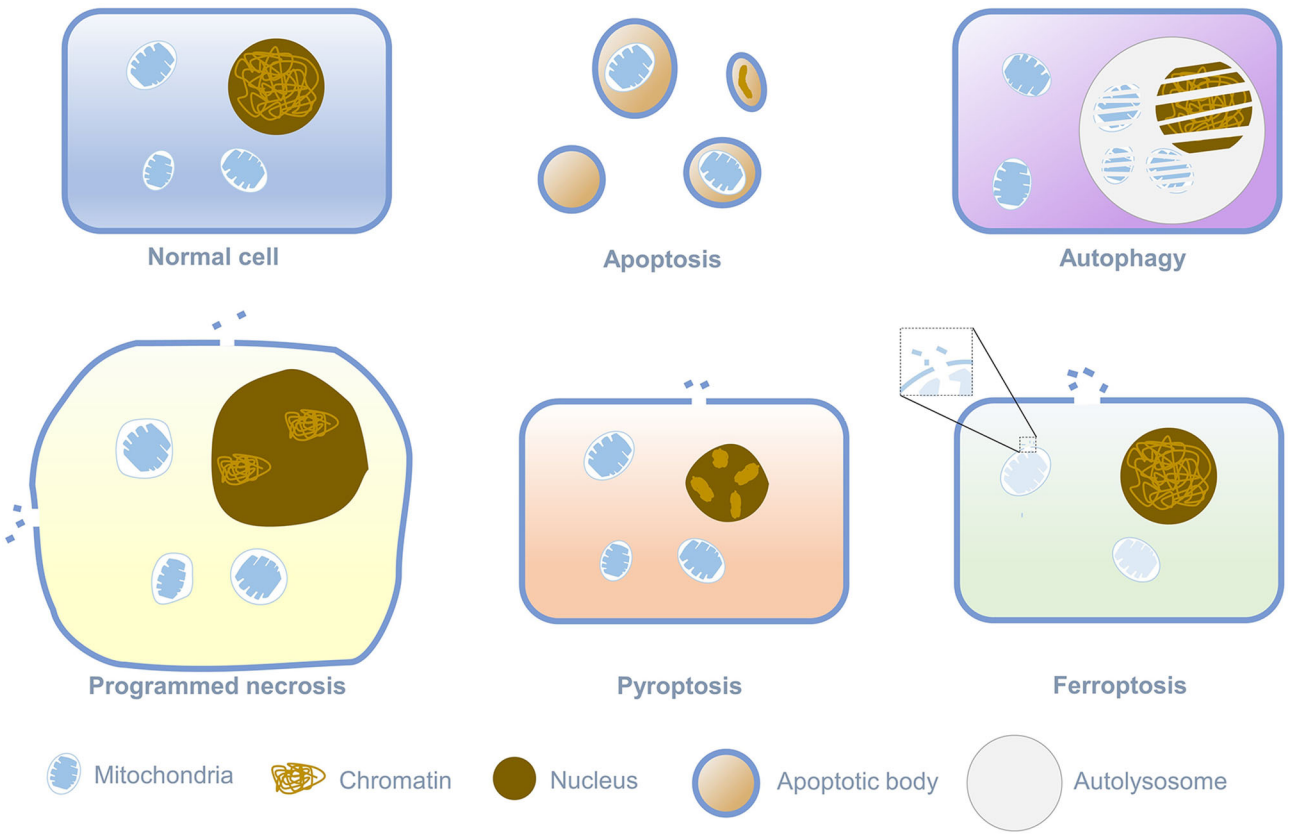

FIGURE 1 | Morphological Features of different programmed cell death.

have garnered significant attention on ferroptosis and continuously identified the correlation of ferroptosis with cancer and tumor immunity. This review will summarize recent progress on the regulatory mechanism of ferroptosis and the pathological manifestations related to ferroptosis and propose potential treatment strategies.

MECHANISM OF FERROPTOSIS

Lipid Peroxidation Leads to Ferroptosis

The most apparent feature of ferroptosis in cells is lipid peroxidation. In the process of ferroptosis, the initial and essential step is that ACSL4, as a member of the acyl-CoA synthase long-chain family (ACSL) family, specifically catalyzes polyunsaturated fatty acid (PUFA), such as arachidonoyl (AA) and adrenoyl moieties (AdA), to form a long-chain acyl-CoA, namely PUFA-CoA (11). Downregulation of ACSL4 expression or pharmacological inhibition of ACSL4 activity (thiazolidinediones or triacsin C, etc.) can prevent ferroptosis (12–14). Lysophosphatidylcholine acyltransferase 3 (LPCTA3) then selectively uses phosphatidylethanolamine (PE) or phosphatidylcholine (PC) located on the endoplasmic reticulum as the receptor for acylated PUFA to generate PUFA-PE or PUFA-PC (15). Lipids with unsaturated fatty acids are typical peroxidation targets because carbon-carbon double bonds are susceptible to reactive oxygen species (ROS). According to different peroxidation mechanisms, lipid peroxidation can be divided into enzymatic and non-enzymatic types. It is currently considered that the enzyme pathway is mainly accomplished by lipoxygenase (LOX), which is a class of dioxygenases containing non-heme iron and can directly catalyze the peroxidation of PUFA-PE (13, 16, 17). Six LOX species have been identified in the human genome and referred to as 5-LOX, 12-LOX, 15-LOX-1, 15-LOX-2, 12R-LOX, and eLOX3 according to their oxidation positions on the arachidonic acid carbon chain (11, 18). When LOX is overexpressed, cells appear to be sensitive to ferroptosis, whereas inhibiting LOX activity, in turn, protects cells from RSL3-induced ferroptosis (19). The enzymatic lipid peroxidation is the reaction where enzymes specially select and catalyze substrates to generate the products. In non-enzymatic lipid peroxidation, free and unstable ferrous ions react with hydrogen peroxide to generate ferric ions and strongly oxidizing hydroxyl radicals (OH.). Hydroxyl radicals abstract the first hydrogen in the PUFA and form resonantly stable carbon-centered lipid radical (PUFA-R.), which can react with molecular oxygen to form lipid hydroperoxyl radical (PUFA-ROO.). Another hydrogen can be extracted by lipid peroxidation

radical from the adjacent unsaturated fat chain, leading to the formation of lipid peroxides (PUFA-ROOH) and new resonant carbon center radicals. In this cycle, the chain reaction continues to proceed and generates new lipid peroxides until the concentration of PUFA-ROO is high enough for two PUFA-ROO to contact each other so that a new bond is formed (20–22). Lipid peroxidation can generate unstable hydroperoxyl groups in PUFA and promote oxidative truncation of PUFA-ROOH, creating electrophiles such as aldehydes and Michael receptors. The electrophilic products then attack proteins on the cell membrane, causing plasma membrane rupture and cell death (23, 24).

As a natural fat-soluble antioxidant, α-tocopherol can disrupt the chain reaction in lipid peroxidation and inhibit ferroptosis, owing to its high affinity for unpaired electrons (25, 26). Liproxstatin-1 and ferrostatin-1, two ferroptosis inhibitors identified by high throughput screening, have the characteristics of free radical-trapping antioxidants (RTA), and hence preventing ferroptosis by scavenging free radicals (8, 27, 28). Recent studies have demonstrated that cytochrome P450 oxidoreductase (POR) and cytochrome B5 reductase 1 (CYB5R1) can transfer electrons from NAD(P)H to downstream proteins such as cytochrome P450 (CYP), which incorrectly transfer electrons to molecular oxygen to generate hydrogen peroxide. The Fenton reaction between hydrogen peroxide and ferrous ions can induce ferroptosis (29). When intracellular expression of POR or CYB5R1 was downregulated, the H_2O_2 content was reduced, while the cell survival rate was remarkably increased (29, 30).

Lipid Peroxidation Defense System

As lipid supports the structure of the cell membrane or organelle membrane, lipid peroxidation can significantly change the physical properties of the lipid bilayer. As a marker and necessary prerequisite of ferroptosis, the accumulation of lipid peroxidation is regulated by various redox systems in cells (**Figure 2**) (21).

GPX4-GSH System

As a negative regulator for ferroptosis, GPX4 utilizes two reduced glutathione molecules (γ-glutamyl-cysteinyl-glycine, GSH) as the electron donor to reduce lipid peroxides (such as AA-OOH) into corresponding alcohols (AA-OH) and produce a molecule of oxidized glutathione (GSSG), reducing lipid peroxidation and preventing ferroptosis (31, 32).

The production of GSH in cells is inseparable from the system Xc^-, a heterodimer composed of SLC3A2 and SLC7A11, which can execute the antiport of cystine and glutamate on the cell membrane, namely takes one molecule of cystine into the cell and releases one molecule of glutamate from the cell (33). Cystine transported into the cell is rapidly reduced to cysteine, which is involved in GSH synthesis and other physiological reactions. The γ-glutamylcysteine synthase (γ-GCS) performs the first and rate-limiting step in the process of GSH synthesis: synthesis of L-γ-glutamylcysteine from L-cysteine and L-glutamate in the presence of ATP, while GSH synthase catalyzes the synthesis of GSH from glycine and γ-glutamylcysteine (34, 35). After erastin treatment, ferroptosis-inducing agents56 (FIN56) can inhibit GPX4 and cells exhibit susceptibility to ferroptosis. When GPX4 is overexpressed, FIN56-induced ferroptosis would be impeded (36). Similarly, dihydroartemisinin (DHA) can also promote ferroptosis in glioblastoma through targeted downregulation of GPX4 and accumulation of lipid peroxidation (37). Albeit GPX4-deficient hematopoietic stem cells are prone to suffer from ferroptosis *in vitro*, GPX4-deficient mice still retain general function, owing to the antioxidant function of lipophilic vitamin E. Moreover, GPX4 combined with vitamin E can also prevent hepatocellular disease (38, 39).

FSP1-CoQ_{10} System

FSP1-CoQ_{10} system can antagonize ferroptosis in GPX4-GSH system-independent mechanism. Doll and Bersuker's teams almost simultaneously demonstrated that FSP1 was strongly associated with ferroptosis (40, 41). FSP1 mainly contains two distinct structural domains, the N-terminal short hydrophobic region and FAD-dependent NAD(P)H redox region, and participates in ferroptosis through reduction of ubiquinone (also known as coenzyme Q_{10}, CoQ_{10}) and formation of panthenol ($CoQ_{10}H_2$), which is a reduced form of the fat-soluble antioxidant ubiquinone and can trap and reduce lipid peroxidation radicals (42). In addition, the overexpression of FSP1 in most tumor cells, and the treatment with the inhibitor of FSP1 (iFSP1), can make cells sensitive to RSL3 (43). Meanwhile, Bersuker et al. identified that FSP1 was a negative regulator of ferroptosis in screening for the synthesis of lethal CRISPR-Cas9, and the localization of FSP1 to the plasma membrane by myristoylation was necessary for its inhibition of ferroptosis (31). Subsequent studies demonstrated that FSP1 plays a role in membrane repair and resistance to ferroptosis in the panthenol-independent and ESCRT-III-dependent manner (44). Knockdown of FSP1 inhibits the expression of ESCRT-III subunits (CHMP5 and CHMP6), but the exact mechanism remains unclear. Furthermore, mice with knockout of the FSP1 gene did not show any abnormalities before at least one-year-old (45). In conclusion, promoting ferroptosis in tumor cells *via* targeting inhibition of FSP1would be a potential strategy for cancer therapy. Consistently, the development of FSP1 inhibitors is also of great significance for clinical research.

GCH1-BH4 System

Human GCH1 is a 270 kD complex composed of five dimers that catalyzes the conversion of guanosine triphosphate (GTP) to dihydroneopterin triphosphate (H2NTP), which is the first and rate-limiting step in the biosynthesis of BH4 (46). H2NTP was then transformed into BH4 by 6-pyruvoyl tetrahydrobiopterin synthase (PTS) and sepiapterin reductase (SPR). Kraft found that GCH1 was related to ferroptosis through CRISPR-Cas9 overexpression screening. GCH1 prevents the peroxidation of phosphatidylcholine with two polyunsaturated fatty acid chains to prevent ferroptosis through the antioxidant action of BH4, and BH4 may also be involved in the pathway of FSP1-CoQ_{10} as a biosynthetic precursor of CoQ_{10} (47). Dihydrofolate reductase

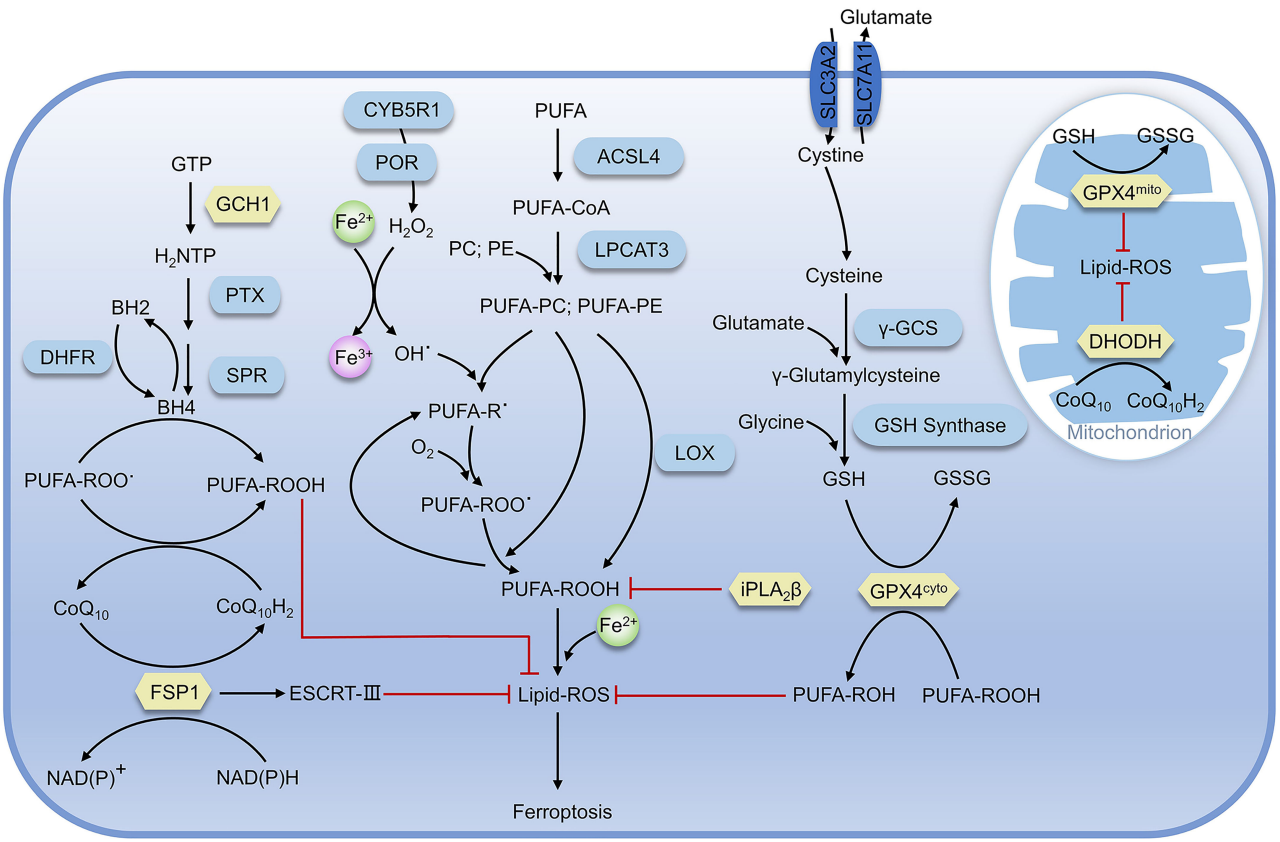

FIGURE 2 | Regulatory pathways of ferroptosis. The proteins in yellow are key defense systems; the red line represents inhibiting effect (as in **Figure 4**).

(DHFR) participates in the regulation of ferroptosis by regenerating oxidized BH4 and the combination of inhibition of DHFR by methotrexate (MTX) with inhibition of GPX4 by RSL3 can promote ferroptosis (48, 49). In summary, BH4 is the core element of this system for ferroptosis resistance. In addition to GCH1 and DHFR, there are other factors mediating ferroptosis through BH4, and the imbalance of BH4 levels may be associated with the occurrence of ferroptosis-related diseases.

iPLA$_2$β

Ca^{2+}-independent phospholipase A_2β (iPLA$_2$β) can specifically hydrolyze sn-2 acyl bonds of phospholipids, which has been recently identified as a regulator of ferroptosis. When ferroptosis occurs, the characteristic product of lipid peroxidation, 15-hydroperoxy-arachidonoyl-phosphatidylethanolamine (15-HpETE-PE), is hydrolyzed by iPLA$_2$β, which impedes subsequent ferroptosis-related effects. Decreased iPLA$_2$β expression in cells was significantly more sensitive to RSL3-induced ferroptosis and showed higher ferroptosis markers associated with PE (50). The interaction of iPLA$_2$β with various membrane substrates was simulated by computational modeling, and it was found that 15-HpETE-PE was more exposed to the membrane surface, close to the catalytic site (50). When the R747W mutation occurred in the catalytic domain, the interaction with the membrane was diminished, leading to a decline in the catalytic capacity of 15-HpETE-PE and the inhibition of ferroptosis in cells. It is demonstrated that the reduced enzyme activity may be linked to neurological diseases, including Parkinson's disease in particular. Besides, iPLA2β was further identified as a regulator of p53-mediated ferroptosis, independent of GPX4 and FSP1, inhibiting ferroptosis by eliminating AlOX12-catalyzed lipid peroxidation. Notably, like FSP1, the lack of iPLA2β has no impact on cell function or tissue development, suggesting that it may be a potential target for inducing ferroptosis in tumor cells (51).

DHODH

Mitochondria is the indispensable organelle in eukaryotes where oxidative phosphorylation, energy generation, and important other functions such as signal transmission and energy metabolism occur. Mao et al. recently demonstrated that dihydroorotate dehydrogenase (DHODH) mediated the regeneration of panthenol in mitochondria to restore peroxide-damaged mitochondrial lipids and inhibition of ferroptosis by DHODH was independent of DHODH's function in pyrimidine synthesis (52). DHODH can also produce panthenol to repair peroxide lipids, but DHODH targets mitochondrial membrane lipids instead of cytoplasmic lipids. GPX4 is subdivided into

cytoplasmic and mitochondrial types, which are referred to as $GPX4^{cyto}$ and $GPX4^{mito}$, respectively. $GPX4^{mito}$ and DHODH protect the mitochondria against oxidative damage independent of $GPX4^{cyto}$ and FSP1. Mao also demonstrated that Brequinar, an inhibitor of DHODH, impeded the proliferation of tumor cells with low GPX4 expression. In addition, combined use of ferroptosis inducers could inhibit the growth of tumor cells with high GPX4 expression, implying a new approach for cancer treatment (52).

METABOLIC REGULATION OF FE^{2+}

Iron is a vital transition metal with redox activity in the body, which has implications in biological processes such as oxidative phosphorylation, DNA synthesis, and cell signaling (53, 54). Excessive or inadequate iron levels can lead to the loss of protein function, abnormalities in intracellular signaling, as well as out-of-control of metabolic networks, thus interfering with normal physiological processes (55). Ferrous ions are reported to be involved in the Fenton reaction and promote lipid peroxidation when ferroptosis occurs. In addition, reactive oxygen species (ROS) that trigger the Fenton reaction are also related to iron (56).

In a natural evolution, organisms have evolved multiple regulatory pathways of iron homeostasis. Most of the ferric ions in nutrients are absorbed by the brush border of duodenal cells, reduced to ferrous ions by duodenal cytochrome b (Dcytb), then transported to intestinal cells by Divalent metal ion Transporter 1 (DMT1) (57). In intestinal cells, a part of ferrous ion is fixed by ferritin, while the other parts are utilized by the ferroportin (FPN, also known as SLC40A1) and transported into the blood. Ferrous ions are oxidized to ferric ions in the blood by Hephaestin (HEPH) or Ceruloplasmin (CP) (53, 58). Transferrin (Tf) can bind two ferric ions and enter into cells through endocytosis after forming a complex with transferrin receptor (TfR). In the acidic environment of the Endosome, ferric ions are dissociated from Tf and reduced to ferrous ions by a six-transmembrane epithelial antigen of the prostate 3 (STEAP3), and then are transported to the cytoplasm by DMT1, while Tf and TfR can be recycled for the next transfer (57, 59, 60). Ferric ions entering the cytoplasm can function in the various physiological processes or stay in ferritin.

Increased TfR expression was found in erastin-induced ferroptosis and p53 induced ferroptosis, suggesting that TfR is positively associated with ferroptosis (61, 62). In addition, it has been found that knockdown of FPN in neuroblastoma promotes ROS-dependent ferroptosis, and the downregulation of FPN expression leads to ferroptosis in the hippocampal area of rats and other diabetic cognitive dysfunction (63, 64). The storage of iron ions in cells is undertaken by ferritin, which is composed of a heavy chain (FTH) and a light chain (FTL) and forms a "labile iron pool (LIP)" of 12 or 24 polymers through the weak interaction. The storage pool can store more than 4500 ferrous ions. Ferritins from different species are distinct in ferritin size, amino acid sequence, iron access channel, and iron-binding site. However, from the aspects of shape and structure, they are all "pools" formed by orderly arranged helixes (65, 66). The iron storage pools control the concentration of free ferrous ions in the cytoplasm, which determines whether it acts as a beneficial cofactor or as a toxic-free radical catalyst in the cell and is key for the fate of the cell (**Figure 3**).

Nuclear Receptor Coactivator-4 (NCOA4) mediates the degradation of ferritin in an autophagy-dependent pathway where iron is isolated from ferritin and the accumulated free ferrous ions in the cytoplasm promote erastin-induced ferroptosis (67). Nuclear factor-erythroid 2-related factor 2 (NRF2) regulates the expression of FPN1, FTH, and FTL at the transcriptional level and promotes the stability of the intracellular iron environment (68). TfR and ferritin translation regulation at the transcriptional level in a cell operates through IRE-IRP. Iron regulatory proteins (IRPs) can be classified into IRP1 and IRP2. IRP1 can be converted between apo-form and holo-form, wherein the former can bind mRNA, and the latter has aconitase activity and is the main active form of IRP1 (69, 70). IRP2 is widespread in mammals and is the main executor of IRE-IRP iron regulation (69, 71). When the intracellular iron concentration is inadequate, IRP will bind to the 3' untranslated regions of mRNA (UTR) of TfR with a high affinity and specific manner to enhance the stability of the mRNA and the expression level of TfR, resulting in increased iron transport into the cell (72). When intracellular iron is abundant, IRP binds to the 5' UTR of ferritin and TfR, preventing their translation. IRP is at the control of intracellular iron level and H_2O_2, oxygen concentration, and oxidative stress signal (72, 73).

REGULATORY MECHANISMS ASSOCIATED WITH FERROPTOSIS

Effect of Ether Phospholipid on Ferroptosis

Unlike typical fatty acids, which are connected to the glycerol framework *via* two ester bonds, the sn-1 position of ether phospholipids (ePLs) is linked to the aliphatic chain *via* the ether bond, while the position of sn-2 is linked to the polyunsaturated fat chain by an ester bond (74, 75). Carbons adjacent to ether bonds can be bonded in two ways: the first is a carbon-carbon single bond to form an alkyl ether; another is a carbon-carbon double bond for the formation of vinyl ethers (also known as acetal phospholipids). Zou et al. found that peroxisome composition was associated with ferroptosis using genome-wide CRISPR-Cas9 suppressor screens, in combination with the protein network database STRING and the pathway analysis algorithm they developed. Furthermore, they found utilizing lipidomics that polyunsaturated ether phospholipids (ePLs) generated by the peroxisome pathway act as substrates of lipid peroxidation to induce ferroptosis (76). When the expression of ether phospholipid-related synthases Alkyldihydroxyacetonephosphate synthase (AGPS) and Fatty acyl-CoA reductase 1 (FAR1) in peroxisome was inhibited, the

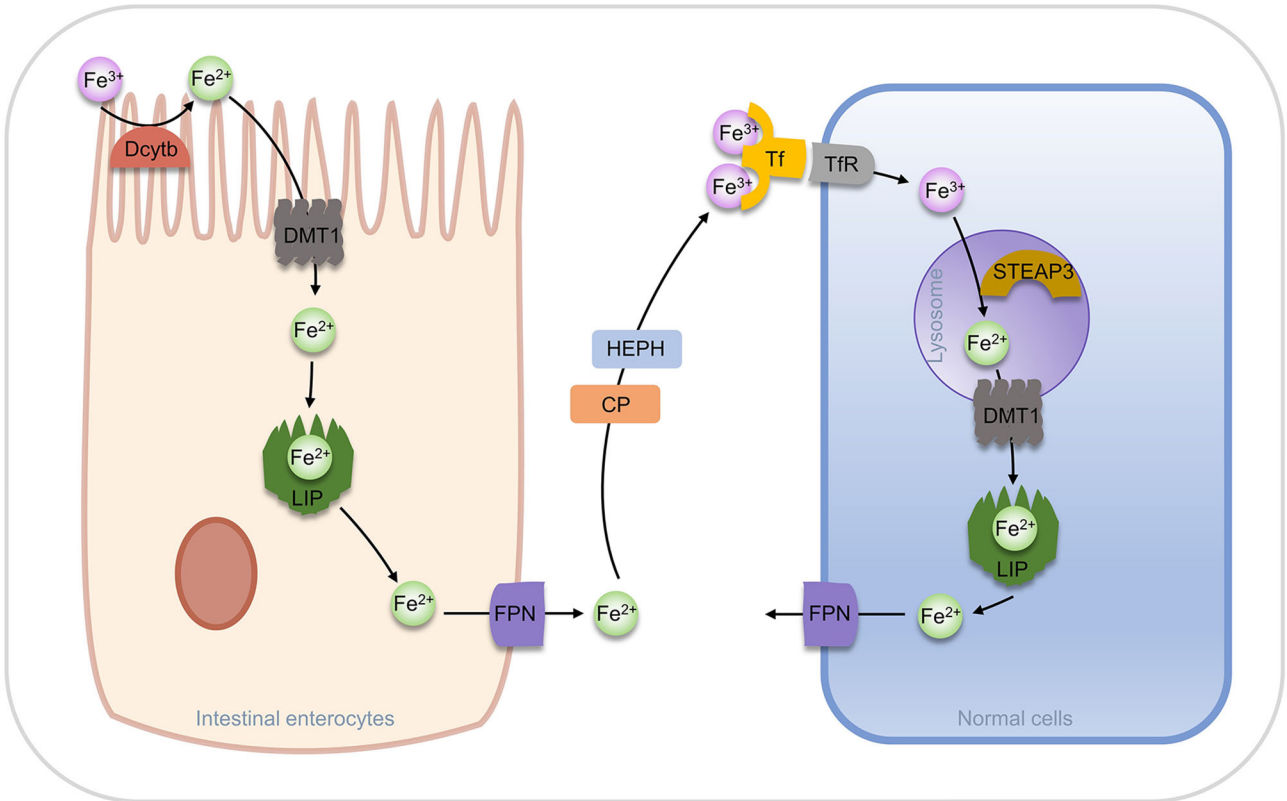

FIGURE 3 | Regulation of iron transport *in vivo*.

cells would exhibit the resistance to ferroptosis until they were re-expressed. In another study, Cui et al. established the regulatory pathway of ferroptosis involving FAR1 as FAR1-ether lipid-TMEM189 (77). FAR1 promotes ferroptosis by reducing fatty acids to generate fatty alcohols necessary for synthesizing alkyl ether lipids and acetal phospholipids (77). Cui found that acetal phospholipids generated by plasmanylethanolamine desaturase (transmembrane protein 189, TMEM189) inhibited the expression of FAR1 and subsequent ferroptosis (78). Cui had characterized the inhibitory effect of TMEM189 on ferroptosis in different cancer cells. In contrast, Zou claimed that TMEM189 was not involved in ferroptosis regulation. The two contrary results may be explained by different cancer cell lines used in the experiment that expressed discrepant levels of TMEM189. This indicates that the level of TMEM189 protein in different cancer cells results in differences in the regulatory network between cancer cells. Further studies are needed to determine whether the differences in TMEM189 protein levels among varying cancer cell lines are regulated at the gene level, transcription level, or post-translational modification level, which may also contribute to elucidating the role of TMEM189 in ferroptosis.

UPS

The ubiquitin-proteasome system (UPS) is closely linked to ferroptosis through targeting protein for degradation. The tumor suppressor BRCA1-associated protein 1 (BAP1), a member of the UCH family of deubiquitinase, is negatively associated with many tumors (79–81). BAP1 acts on system Xc^- and cleaves the mono-ubiquitin from lysine 119 of histone 2A (H2AK119Ub) in the SLC7A11 gene, thereby inhibiting the transcription of SLC7A11 and resulting in reduced cystine transport, decreased GSH production, lipid peroxide accumulation, and ultimately promoting the occurrence of ferroptosis (82, 83). Further research has demonstrated that the polycomb repressive complex 1 (PRC1), as the main E3 ubiquitin ligase of H2AUb, can enhance the binding of H2AUb to the SLC7A11 promoter and synergistically inhibit the expression of SLC7A11 with BAP1 (84). In addition, mono-ubiquitination of lysine 120 of histone 2B (H2BK120Ub) activates SLC7A11 expression, while p53 reduces H2BK120Ub level *via* promoting nuclear translocation of the deubiquitinase USP7, and finally promoting the occurrence of ferroptosis (85). Studies have shown that SLC7A11 is also positively regulated by deubiquitinase, and OTUB1, a deubiquitinase, can interact directly with SLC7A11 and repress ferroptosis by stabilizing SLC7A11. When the OTUB1 was inactivated, SLC7A11 levels diminished, and ferroptosis was promoted. It was also found that the cluster of differentiation-44 (CD44) could enhance the interaction of OTUB1 with SLC7A11 and prevent ferroptosis (86).

It has been demonstrated that deubiquitinase USP35 is overexpressed in human lung cancer tissues and cell lines.

Meanwhile, knockdown of USP35 can promote the degradation of FPN in lung cancer cells and reduce iron exportability that makes cancer cells sensible to ferroptosis, enhancing the chemotherapy effect on lung cancer cells (87). USP11 can stabilize NRF2 by deubiquitination, and USP11 inactivation can promote NRF2 degradation, making cells prone to ferroptosis and reproduction repressed (**Figure 4**) (88).

AMPK

AMPK is an AMP-dependent protein kinase that regulates the generation and consumption of ATP to maintain intracellular energy homeostasis. Under energy stress, AMPK is activated and inhibits the physiological processes that consume energy directly or indirectly. Gan et al. recently identified a new mechanism on ferroptosis inhibition in which under energy-deficient conditions, activation of AMPK mediated phosphorylation of acetyl-CoA carboxylase (ACC) and ACC inactivation inhibited the conversion of acetyl-CoA to malonyl-CoA that can generate unsaturated fatty acids and participate in lipid peroxidation. Inhibiting the synthesis of unsaturated fatty acids would ultimately prevent ferroptosis (89–91). Beclin1, the first autophagy-associated protein discovered in mammals, acts as the core of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex to promote the nucleation of autophagosomes (92). Song et al. found that Beclin1 induced ferroptosis independently of the PtdIns3K complex and could form Beclin1-SLC7A11 complex when phosphorylated at Ser90/93/96 by AMPK. This process directly inhibited the cystine transport function of system Xc^-, leading to the accumulation of lipid peroxidation and ferroptosis increase (91, 93, 94). Overexpression of Beclin1 in cancer cells promoted the effect of system Xc^- inhibitors on ferroptosis and also promoted the effect of ferroptosis on anticancer *in vivo* (94). To sum up, AMPK has positive and negative regulatory roles in ferroptosis, so better understanding and accurately judging of the roles of AMPK may be of great but the far-reaching significance for target ferroptosis to treat tumors.

ROLE OF FERROPTOSIS IN HEMATOLOGIC MALIGNANCIES TREATMENT

Leukemia

Leukemia is a malignant disease arising from the unlimited proliferation of hematopoietic stem cells, as malignant clones

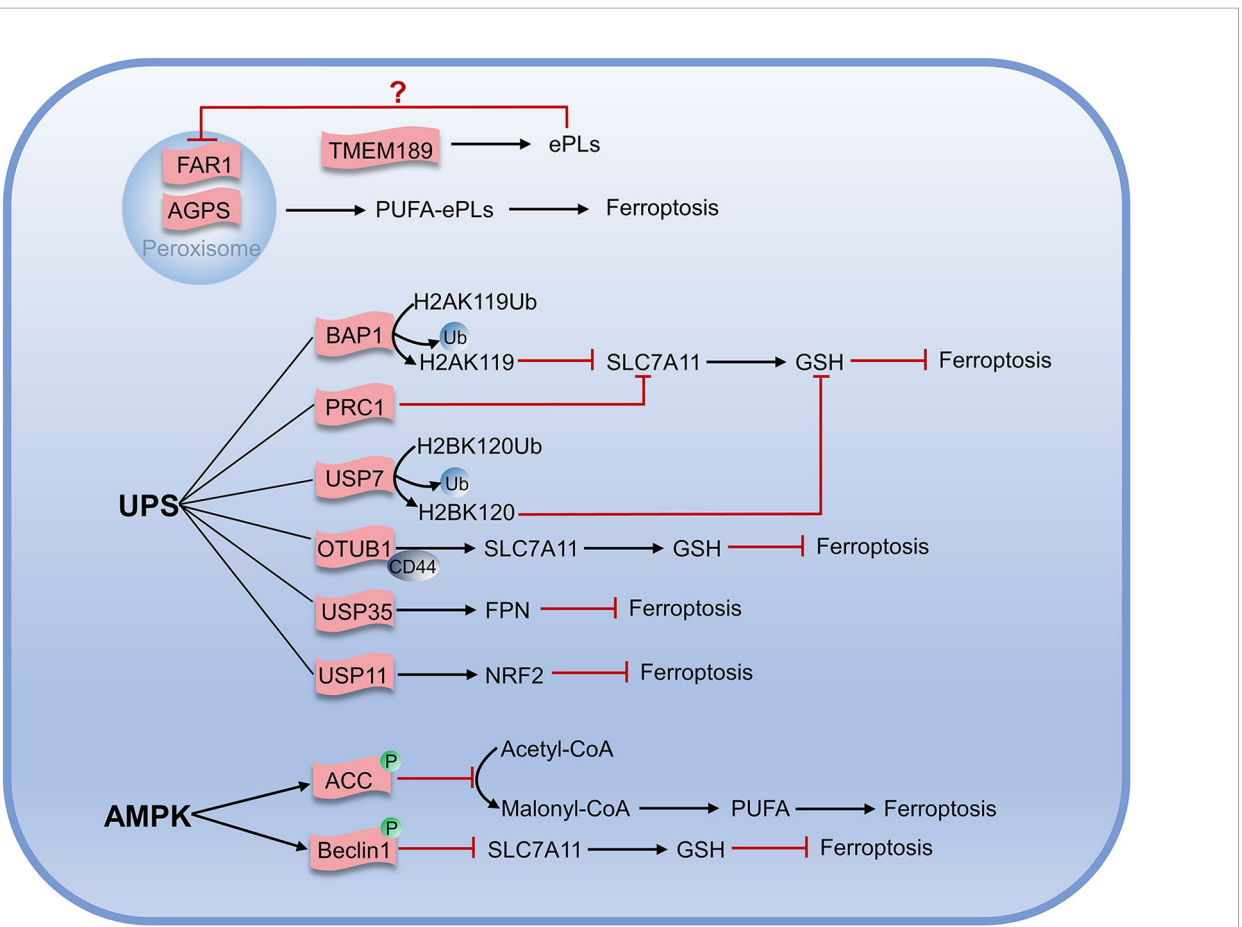

FIGURE 4 | Metabolic regulation associated with ferroptosis.

of cells could hinder the normal function of hematopoietic cells and affect the development of non-hematopoietic cells. Compared with other tumor cells, leukemia cells display higher transferrin expression and iron content, which causes the accumulation of ROS in leukemia cells more easily and ferroptosis occurs after cells undergo irreparable peroxidative damage. Thereby, promoting ferroptosis *via* further enhancing the iron content in leukemia cells seems to become a feasible strategy for leukemia treatment, which is also one of the hotspots in this field (95–97).

It has been found that Dihydroartemisinin (DHA), a derivative of the natural drug artemisinin, can induce ferroptosis in acute myeloid leukemia (AML) cells, owing to the capability of activating the phosphorylation of AMPK. Subsequently, AMPK inhibits the mTOR pathway and promotes autophagy, leading to autophagy-dependent degradation of FTH protein and the release of large amounts of free iron, which ultimately induces ferroptosis in AML cells (98). RSL3, as a small molecule inhibitor targeting GPX4, can also trigger a variety of programmed cell deaths, including ferroptosis in AML cells, and enhances the tumor suppressor effect of first-line chemotherapy drugs (cytarabine and adriamycin) on AML cells (99). Similarly, Balasubramanian et al. found that NRF2 may be a new target for the treatment of AML. Using Brusatol, an inhibitor of NRF2, could reduce the ability of NRF2 to eliminate ROS and increase the sensitivity of cytarabine and daunorubicin to AML (100). Ferroptosis induced by iron loading in leukemia cells exert a therapeutic effect, while the occurrence and poor prognosis of leukemia have been identified to be related to intracellular iron accumulation, such as inhibition of red blood cell differentiation when the iron is excessive (101). Therefore, much effort was focused on reducing of the amount of iron in cells. For example, iron chelating agents (DFO and DFX) have been used clinically to reduce the intracellular iron load (102). There are currently relatively few studies on treating leukemia with ferroptosis, and more experiments are required to elucidate the relationship between ferroptosis and leukemia treatment.

Lymphoma

Diffuse large B-cell lymphoma (DLBCL) is one of the most frequent lymphatic malignancies, with the highest incidence in the non-Hodgkin lymphoma (NHL) family (103, 104). Since Cystine is a crucial negative regulator in the ferroptosis system, the lack of cystine in cells lead to the inhibition of GSH production, which ultimately gives rise to redox imbalance and ferroptosis. Therefore, the inability of lymphocytes to synthesize cystine has been regarded as a breakthrough point for the treatment of lymphoma. At an early stage, Gout et al. revealed that sulfasalazine could be used as a system Xc^- inhibitor to significantly reduce the growth of DLBCL in the abdominal cavity of rats (105). Stockwell et al. optimized imidazole-ketone-erastin (IKE) with stronger metabolic stability and water solubility, based on canonical system Xc^- inhibitor erastin. IKE could inhibit the growth of DLBCL in mice with more potent therapeutic effects through nanoparticle delivery (106). What's more, Stockwell et al. indicated that inhibition of GPX4 activity also promoted the death of DLBCL cell lines (36). Consistently, GPX4 can reduce lipid peroxides and inhibit ferroptosis, and its overexpression has been demonstrated to be associated with poor prognosis in DLBCL patients (107).

Multiple Myeloma

Multiple myeloma (MM) is a malignant proliferative disease in plasma cells, accounting for 10% of hematological malignancies (108). MM is associated with the accumulation of atypical plasma cells in bone marrow, abnormal production of monoclonal immunoglobulin, and increased serum calcium levels (109). GPX4 and SLC7A11 are key regulators of ferroptosis and are highly expressed in MM cells. A novel immunosuppressant Fingolimod (FTY720), can promote ferroptosis by reducing GPX4 and SLC7A11 mRNA and protein levels in U66 cells, enhancing ferroptosis and autophagy through the PP2A/AMPK pathway (110). In MM cells, high proteasome activity is the determinant for degrading misfolded immunoglobulin to ensure expected survival. As a proteasome inhibitor, Bortezomib has been clinically used to treat MM patients (111). However, the autophagy process activated by the accumulation of misfolded immunoglobulin in cells exhibits the resistance of MM against Bortezomib (112). Studies have shown that iron exposure can reduce the activity of the proteasome, hence increasing the efficacy of Bortezomib and Carfilzomib (the second generation of proteasome inhibitors used for MM therapy) in MM cells and leading to severe MM cell death by promoting ferroptosis (113). In order to overcome the drug resistance of Bortezomib, docosahexaenoic acid or eicosapentaenoic acid in combination with Bortezomib were used to enhance the sensitivity of MM cells to Bortezomib (**Table 1**) (114). Specifically, docosahexaenoic acid and eicosapentaenoic acid can modulate the redox balance in MM cells by reducing the content of GSH in MM cells, thus improving the therapeutic effect of Bortezomib. These combined therapeutic results provide a novel theoretical basis and therapeutic schedule for overcoming MM resistance to Bortezomib and further benefit clinical treatment.

TABLE 1 | Inducers or inhibitors of ferroptosis in hematologic malignancies.

Cancer	Inhibitors/Inducers	Targeted sites
Leukemia	DHA	AMPK
	RSL3	GPX4
	Brusatol	NRF2
	DFO; DFX	Fe2+
Lymphoma	Sulfasalazine	system Xc-
	IKE	system Xc-
	RSL3	GPX4
Multiple myeloma	FTY720	GPX4; SLC7A11
	Bortezomib; Carfilzomib	Proteasome
	Docosahexaenoic acid; Eicosapentaenoic acid	GSH

TABLE 2 | Molecular compounds that regulate ferroptosis.

	Molecular compound	Targeted sites	Function
Inhibitor	α-Tocopherol	PUFA-ROO·	Blocks the lipid peroxidation caused by Fenton reaction
	Vitamin E	PUFA-ROO·	Blocks the lipid peroxidation caused by Fenton reaction
	Liproxstatin-1	PUFA-ROO·	Blocks the lipid peroxidation caused by Fenton reaction
	Ferrostatin-1	PUFA-ROO·	Blocks the lipid peroxidation caused by Fenton reaction
	DFO	Fe^{2+}	Consumption of iron
	DFX	Fe^{2+}	Consumption of iron
	CoQ_{10}	Lipid peroxidation	Repairs lipid peroxide
Inducer	Erastin	system Xc^-	Prevents cystine from entering cells
	IKE	system Xc^-	Prevents cystine from entering cells
	Sulfasalazine	system Xc^-	Prevents cystine from entering cells
	Sorafenib	system Xc^-	Prevents cystine from entering cells
	FIN56	GPX4	Induces GPX4 degradation
	RSL3	GPX4	Covalently inhibits GPX4, leading to accumulation of lipid peroxides
	iFSP1	FSP1	Consumption of CoQ10 leads to a decrease in GPX4 activity
	MTX	DHFR	Inhibits DHFR activity and reduce BH4 production
	Docosahexaenoic acid	GSH	Consumption of GSH
	Eicosapentaenoic acid	GSH	Consumption of GSH
	Brequinar	DHODH	Decreases DHODH activity and resultes in accumulation of mitochondrial peroxide lipids

CONCLUSION AND PERSPECTIVE

In recent years, with the deep research on ferroptosis, several pathways other than GPX4-GSH have been identified to modulate lipid peroxidation directly. Meanwhile, large amounts of experiments have also been conducted to elucidate the roles of ferroptosis in hematological malignancies. However, many questions in the ferroptosis field remain to be addressed. For example, to what extent does lipid peroxidation lead to cell rupture and ferroptosis? Are other lipid peroxidation regulatory pathways associated with GPX4-GSH synergistic or merely complementary to GPX4? How can ferroptosis be used as a target of cancer therapy further (In **Table 2** we summarize the inhibitors or inducers associated with ferroptosis)? How to avoid the side effects of ferroptosis-related drugs (e.g., the toxic side effects of increased iron intake in leukemia treatment)? In conclusion, as a new modality of regulatory cell death, ferroptosis brings new possibilities for cancer treatment. However, further research is required for elaborating the mechanism of ferroptosis and its implications in cancer, which is of great scientific significance.

AUTHOR CONTRIBUTIONS

FW and HL conceptualized the study. FW oversaw the literature review involved in all aspects of designing and writing the manuscript. FW and HL performed the literature review. FW, ZM, and HL wrote the manuscript and designed the figures. ZZ, YG, and ZM provided input on the discussion of various sections. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Szabó C. Mechanisms of Cell Necrosis. *Crit Care Med* (2005) 33(12):4–5. doi: 10.1097/01.CCM.0000187002.88999.CF
2. Padanilam BJ. Cell Death Induced by Acute Renal Injury: A Perspective on the Contributions of Apoptosis and Necrosis. *Am J Physiol-Renal Physiol* (2003) 284(4):F608–F27. doi: 10.1152/ajprenal.00284.2002
3. Ranganath RM, Rao Nagashree N. Role of Programmed Cell Death in Development. *Int Rev Cytol* (2001) 202:159–242. Academic Press. doi: 10.1016/S0074-7696(01)02005-8
4. Hirschhorn T, Stockwell BR. The Development of the Concept of Ferroptosis. *Free Radical Biol Med* (2019) 133:130–43. doi: 10.1016/j.freeradbiomed.2018.09.043
5. Sen S. Programmed Cell Death: Concept, Mechanism and Control. *Biol Rev* (1992) 67(3):287–319. doi: 10.1111/j.1469-185X.1992.tb00727.x
6. Daneva A, Gao Z, Van Durme M, Nowack MK. Functions and Regulation of Programmed Cell Death in Plant Development. *Annu Rev Cell Dev Biol* (2016) 32(1):441–68. doi: 10.1146/annurev-cellbio-111315-124915
7. Barros LF, Hermosilla T, Castro J. Necrotic Volume Increase and the Early Physiology of Necrosis. *Comp Biochem Physiol Part A: Mol Integr Physiol* (2001) 130(3):401–9. doi: 10.1016/S1095-6433(01)00438-X
8. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al. Ferroptosis: An Iron-Dependent Form of Nonapoptotic Cell Death. *Cell* (2012) 149(5):1060–72. doi: 10.1016/j.cell.2012.03.042
9. Jiang X, Stockwell BR, Conrad M. Ferroptosis: Mechanisms, Biology and Role in Disease. *Nat Rev Mol Cell Biol* (2021) 22(4):266–82. doi: 10.1038/s41580-020-00324-8
10. Xie Y, Hou W, Song X, Yu Y, Huang J, Sun X, et al. Ferroptosis: Process and Function. *Cell Death Differ* (2016) 23(3):369–79. doi: 10.1038/cdd.2015.158
11. Yang WS, Kim KJ, Gaschler MM, Patel M, Shchepinov MS, Stockwell BR. Peroxidation of Polyunsaturated Fatty Acids by Lipoxygenases Drives Ferroptosis. *Proc Natl Acad Sci USA* (2016) 113(34):E4966–E75. doi: 10.1073/pnas.1603244113
12. D'Herde K, Krysko DV. Oxidized PEs Trigger Death. *Nat Chem Biol* (2017) 13(1):4–5. doi: 10.1038/nchembio.2261

13. Kagan VE, Mao G, Qu F, Angeli JPF, Doll S, Croix CS, et al. Oxidized Arachidonic and Adrenic PEs Navigate Cells to Ferroptosis. *Nat Chem Biol* (2017) 13(1):81–90. doi: 10.1038/nchembio.2238
14. D'Herde K, Krysko DA-O. Ferroptosis: Oxidized PEs Trigger Death. *Nat Chem Biol* (2017) 13(1):4–5. doi: 10.1038/nchembio.2261
15. Hishikawa D, Shindou H, Kobayashi S, Nakanishi H, Taguchi R, Shimizu T. Discovery of a Lysophospholipid Acyltransferase Family Essential for Membrane Asymmetry and Diversity. *Proc Natl Acad Sci USA* (2008) 105 (8):2830–5. doi: 10.1073/pnas.0712245105
16. Kuhn H, Banthiya S, van Leyen K. Mammalian Lipoxygenases and Their Biological Relevance. *Biochim Biophys Acta* (2015) 1851(4):308–30. doi: 10.1016/j.bbalip.2014.10.002
17. Plount Price ML, Jorgensen WL. Analysis of Binding Affinities for Celecoxib Analogues With COX-1 and COX-2 From Combined Docking and Monte Carlo Simulations and Insight Into the COX-2/COX-1 Selectivity. *J Am Chem Soc* (2000) 122(39):9455–66. doi: 10.1021/ja001018c
18. Brash AR. Lipoxygenases: Occurrence, Functions, Catalysis, and Acquisition of Substrate *. *J Biol Chem* (1999) 274(34):23679–82. doi: 10.1074/jbc.274.34.23679
19. Shah R, Shchepinov MS, Pratt DA. Resolving the Role of Lipoxygenases in the Initiation and Execution of Ferroptosis. *ACS Cent Sci* (2018) 4(3):387–96. doi: 10.1021/acscentsci.7b00589
20. Clemente SM, Martínez-Costa OH, Monsalve M, Samhan-Arias AK. Targeting Lipid Peroxidation for Cancer Treatment. *Molecules* (2020) 25 (21):5144. doi: 10.3390/molecules25215144
21. Gaschler MM, Stockwell BR. Lipid Peroxidation in Cell Death. *Biochem Biophys Res Commun* (2017) 482(3):419–25. doi: 10.1016/j.bbrc.2016.10.086
22. Hassannia B, Vandenabeele P, Vanden Berghe T. Targeting Ferroptosis to Iron Out Cancer. *Cancer Cell* (2019) 35(6):830–49. doi: 10.1016/j.ccell.2019.04.002
23. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* (2017) 171(2):273–85. doi: 10.1016/j.cell.2017.09.021
24. Kagan VE, Tyurina YY, Sun WY, Vlasova II, Dar H, Tyurin VA, et al. Redox Phospholipidomics of Enzymatically Generated Oxygenated Phospholipids as Specific Signals of Programmed Cell Death. *Free Radical Biol Med* (2020) 147:231–41. doi: 10.1016/j.freeradbiomed.2019.12.028
25. Traber MG, Atkinson J. Vitamin E, Antioxidant and Nothing More. *Free Radic Biol Med* (2007) 43(1):4–15. doi: 10.1016/j.freeradbiomed.2007.03.024
26. Kajarabille N, Latunde-Dada GO. Programmed Cell-Death by Ferroptosis: Antioxidants as Mitigators. *Int J Mol Sci* (2019) 20(19):4968. doi: 10.3390/ijms20194968
27. Friedmann Angeli JP, Schneider M, Proneth B, Tyurina YY, Tyurin VA, Hammond VJ, et al. Inactivation of the Ferroptosis Regulator Gpx4 Triggers Acute Renal Failure in Mice. *Nat Cell Biol* (2014) 16(12):1180–91. doi: 10.1038/ncb3064
28. Shah R, Margison K, Pratt DA. The Potency of Diarylamine Radical-Trapping Antioxidants as Inhibitors of Ferroptosis Underscores the Role of Autoxidation in the Mechanism of Cell Death. *ACS Chem Biol* (2017) 12 (10):2538–45. doi: 10.1021/acschembio.7b00730
29. Koppula P, Zhuang L, Gan B. Cytochrome P450 Reductase (POR) as a Ferroptosis Fuel. *Protein Cell* (2021) 12(9):675–9. doi: 10.1007/s13238-021-00823-0
30. Yan B, Ai Y, Sun Q, Ma Y, Cao Y, Wang J, et al. Membrane Damage During Ferroptosis Is Caused by Oxidation of Phospholipids Catalyzed by the Oxidoreductases POR and CYB5R1. *Mol Cell* (2021) 81(2):355–69.e10. doi: 10.1016/j.molcel.2020.11.024
31. Bersuker K, Hendricks JM, Li Z, Magtanong L, Ford B, Tang PH, et al. The CoQ Oxidoreductase FSP1 Acts Parallel to GPX4 to Inhibit Ferroptosis. *Nature* (2019) 575(7784):688–92. doi: 10.1038/s41586-019-1705-2
32. Seibt TM, Proneth B, Conrad M. Role of GPX4 in Ferroptosis and Its Pharmacological Implication. *Free Radical Biol Med* (2019) 133:144–52. doi: 10.1016/j.freeradbiomed.2018.09.014
33. Conrad M, Sato H. The Oxidative Stress-Inducible Cystine/Glutamate Antiporter, System X (C) (-) : Cystine Supplier and Beyond. *Amino Acids* (2012) 42(1):231–46. doi: 10.1007/s00726-011-0867-5
34. Meister A. [3] Glutathione Biosynthesis and Its Inhibition. *Methods Enzymol* (1995) 252:26–30. Academic Press. doi: 10.1016/0076-6879(95)52005-8
35. Lu SC. Glutathione Synthesis. *Biochim Biophys Acta* (2013) 1830(5):3143–53. doi: 10.1016/j.bbagen.2012.09.008
36. Yang Wan S, SriRamaratnam R, Welsch Matthew E, Shimada K, Skouta R, Viswanathan Vasanthi S, et al. Regulation of Ferroptotic Cancer Cell Death by GPX4. *Cell* (2014) 156(1):317–31.
37. Yi R, Wang H, Deng C, Wang X, Yao L, Niu W, et al. Dihydroartemisinin Initiates Ferroptosis in Glioblastoma Through GPX4 Inhibition. *Biosci Rep* (2020) 40(6):BSR20193314. doi: 10.1042/BSR20193314
38. Hu Q, Zhang Y, Lou H, Ou Z, Liu J, Duan W, et al. GPX4 and Vitamin E Cooperatively Protect Hematopoietic Stem and Progenitor Cells From Lipid Peroxidation and Ferroptosis. *Cell Death Dis* (2021) 12(7):706–. doi: 10.1038/s41419-021-04008-9
39. Carlson BA, Tobe R, Yefremova E, Tsuji PA, Hoffmann VJ, Schweizer U, et al. Glutathione Peroxidase 4 and Vitamin E Cooperatively Prevent Hepatocellular Degeneration. *Redox Biol* (2016) 9:22–31. doi: 10.1016/j.redox.2016.05.003
40. Wu M, Xu L-G, Li X, Zhai Z, Shu H-B. AMID, an Apoptosis-Inducing Factor-Homologous Mitochondrion-Associated Protein, Induces Caspase-Independent Apoptosis *. *J Biol Chem* (2002) 277(28):25617–23. doi: 10.1074/jbc.M202285200
41. Ohiro Y, Garkavtsev I, Kobayashi S, Sreekumar KR, Nantz R, Higashikubo BT, et al. A Novel P53-Inducible Apoptogenic Gene, PRG3, Encodes a Homologue of the Apoptosis-Inducing Factor (AIF). *FEBS Lett* (2002) 524 (1-3):163–71. doi: 10.1016/S0014-5793(02)03049-1
42. Elguindy MM, Nakamaru-Ogiso E. Apoptosis-Inducing Factor (AIF) and Its Family Member Protein, AMID, Are Rotenone-Sensitive NADH: Ubiquinone Oxidoreductases (NDH-2). *J Biol Chem* (2015) 290 (34):20815–26. doi: 10.1074/jbc.M115.641498
43. Doll S, Freitas FP, Shah R, Aldrovandi M, da Silva MC, Ingold I, et al. FSP1 Is a Glutathione-Independent Ferroptosis Suppressor. *Nature* (2019) 575 (7784):693–8. doi: 10.1038/s41586-019-1707-0
44. Dai E, Zhang W, Cong D, Kang R, Wang J, Tang D. AIFM2 Blocks Ferroptosis Independent of Ubiquinol Metabolism. *Biochem Biophys Res Commun* (2020) 523(4):966–71. doi: 10.1016/j.bbrc.2020.01.066
45. Mei J, Webb S, Zhang B, Shu HB. The P53-Inducible Apoptotic Protein AMID Is Not Required for Normal Development and Tumor Suppression. *Oncogene* (2006) 25(6):849–56. doi: 10.1038/sj.onc.1209121
46. Ebenhoch R, Bauer M, Reinert D, Kersting A, Huber S, Schmid A, et al. Biophysical and Structural Investigation of the Regulation of Human GTP Cyclohydrolase I by Its Regulatory Protein GFRP. *J Struct Biol* (2021) 213 (1):107691. doi: 10.1016/j.jsb.2020.107691
47. Kraft VAN, Bezjian CT, Pfeiffer S, Ringelstetter L, Müller C, Zandkarimi F, et al. GTP Cyclohydrolase 1/Tetrahydrobiopterin Counteract Ferroptosis Through Lipid Remodeling. *ACS Cent Sci* (2020) 6(1):41–53. doi: 10.1021/acscentsci.9b01063
48. Soula M, Weber RA, Zilka O, Alwaseem H, La K, Yen F, et al. Metabolic Determinants of Cancer Cell Sensitivity to Canonical Ferroptosis Inducers. *Nat Chem Biol* (2020) 16(12):1351–60. doi: 10.1038/s41589-020-0613-y
49. Crabtree MJ, Channon KM. Synthesis and Recycling of Tetrahydrobiopterin in Endothelial Function and Vascular Disease. *Nitric Oxide* (2011) 25(2):81–8. doi: 10.1016/j.niox.2011.04.004
50. Sun W-Y, Tyurin VA, Mikulska-Ruminska K, Shrivastava IH, Anthonymuthu TS, Zhai Y-J, et al. Phospholipase Ipla2β Averts Ferroptosis by Eliminating a Redox Lipid Death Signal. *Nat Chem Biol* (2021) 17(4):465–76. doi: 10.1038/s41589-020-00734-x
51. Chen D, Chu B, Yang X, Liu Z, Jin Y, Kon N, et al. Ipla2β-Mediated Lipid Detoxification Controls P53-Driven Ferroptosis Independent of GPX4. *Nat Commun* (2021) 12(1):3644–. doi: 10.1038/s41467-021-23902-6
52. Mao C, Liu X, Zhang Y, Lei G, Yan Y, Lee H, et al. DHODH-Mediated Ferroptosis Defence Is a Targetable Vulnerability in Cancer. *Nature* (2021) 593(7860):586–90. doi: 10.1038/s41586-021-03539-7
53. Chen Y, Fan Z, Yang Y, Gu C. Iron Metabolism and Its Contribution to Cancer (Review). *Int J Oncol* (2019) 54(4):1143–54. doi: 10.3892/ijo.2019.4720
54. Li Y, Huang X, Wang J, Huang R, Wan D. Regulation of Iron Homeostasis and Related Diseases. *Mediators Inflamm* (2020) 2020:6062094–. doi: 10.1155/2020/6062094

55. Li J, Cao F, Yin H-l, Huang Z-j, Lin Z-t, Mao N, et al. Ferroptosis: Past, Present and Future. *Cell Death Dis* (2020) 11(2):88. doi: 10.1038/s41419-020-2298-2
56. Stockwell BR, Jiang X. The Chemistry and Biology of Ferroptosis. *Cell Chem Biol* (2020) 27(4):365–75. doi: 10.1016/j.chembiol.2020.03.013
57. Fleming MD, Romano MA, Su MA, Garrick LM, Garrick MD, Andrews NC. Nramp2 Is Mutated in the Anemic Belgrade (B) Rat: Evidence of a Role for Nramp2 in Endosomal Iron Transport. *Proc Natl Acad Sci USA* (1998) 95 (3):1148–53. doi: 10.1073/pnas.95.3.1148
58. Fuqua BK, Lu Y, Darshan D, Frazer DM, Wilkins SJ, Wolkow N, et al. The Multicopper Ferroxidase Hephaestin Enhances Intestinal Iron Absorption in Mice. *PloS One* (2014) 9(6):e98792–e. doi: 10.1371/journal.pone.0098792
59. Frazer DM, Anderson GJ. The Regulation of Iron Transport. *BioFactors* (2014) 40(2):206–14. doi: 10.1002/biof.1148
60. Torti SV, Torti FM. Iron and Cancer: More Ore to be Mined. *Nat Rev Cancer* (2013) 13(5):342–55. doi: 10.1038/nrc3495
61. Park E, Chung SW. ROS-Mediated Autophagy Increases Intracellular Iron Levels and Ferroptosis by Ferritin and Transferrin Receptor Regulation. *Cell Death Dis* (2019) 10(11):822–. doi: 10.1038/s41419-019-2064-5
62. Tang L-J, Zhou Y-J, Xiong X-M, Li N-S, Zhang J-J, Luo X-J, et al. Ubiquitin-Specific Protease 7 Promotes Ferroptosis *via* Activation of the P53/TfR1 Pathway in the Rat Hearts After Ischemia/Reperfusion. *Free Radical Biol Med* (2021) 162:339–52. doi: 10.1016/j.freeradbiomed.2020.10.307
63. Geng N, Shi BJ, Li SL, Zhong ZY, Li YC, Xua WL, et al. Knockdown of Ferroportin Accelerates Erastin-Induced Ferroptosis in Neuroblastoma Cells. *Eur Rev Med Pharmacol Sci* (2018) 22(12):3826–36. doi: 10.26355/eurrev_201806_15267
64. Hao L, Mi J, Song L, Guo Y, Li Y, Yin Y, et al. SLC40A1 Mediates Ferroptosis and Cognitive Dysfunction in Type 1 Diabetes. *Neuroscience* (2021) 463:216–26. doi: 10.1016/j.neuroscience.2021.03.009
65. Theil EC. Ferritin Protein Nanocages-the Story. *Nanotechnol Percept* (2012) 8(1):7–16. doi: 10.4024/N03TH12A.ntp.08.01
66. Tosha T, Behera RK, Ng H-L, Bhattasali O, Alber T, Theil EC. Ferritin Protein Nanocage Ion Channels: Gating by N-Terminal Extensions. *J Biol Chem* (2012) 287(16):13016–25. doi: 10.1074/jbc.M111.332734
67. Gryzik M, Asperti M, Denardo A, Arosio P, Poli M. NCOA4-Mediated Ferritinophagy Promotes Ferroptosis Induced by Erastin, But Not by RSL3 in HeLa Cells. *Biochim Biophys Acta (BBA) - Mol Cell Res* (2021) 1868 (2):118913. doi: 10.1016/j.bbamcr.2020.118913
68. Kerins MJ, Ooi A. The Roles of NRF2 in Modulating Cellular Iron Homeostasis. *Antioxidants Redox Signaling* (2018) 29(17):1756–73. doi: 10.1089/ars.2017.7176
69. Rouault TA. The Role of Iron Regulatory Proteins in Mammalian Iron Homeostasis and Disease. *Nat Chem Biol* (2006) 2(8):406–14. doi: 10.1038/nchembio807
70. Hernández-Gallardo AK, Missirlis F. Cellular Iron Sensing and Regulation: Nuclear IRP1 Extends a Classic Paradigm. *Biochim Biophys Acta (BBA) - Mol Cell Res* (2020) 1867(7):118705.
71. Meyron-Holtz EG, Ghosh MC, Iwai K, LaVaute T, Brazzolotto X, Berger UV, et al. Genetic Ablations of Iron Regulatory Proteins 1 and 2 Reveal Why Iron Regulatory Protein 2 Dominates Iron Homeostasis. *EMBO J* (2004) 23 (2):386–95. doi: 10.1038/sj.emboj.7600041
72. Cairo G, Recalcati S, Pietrangelo A, Minotti G. The Iron Regulatory Proteins: Targets and Modulators of Free Radical Reactions and Oxidative. *Free Radic Biol Med* (2002) 32(12):1237–43. doi: 10.1016/s0891-5849(02)00825-0
73. Popovic Z, Templeton Douglas M. Interaction of Iron Regulatory Protein-1 (IRP-1) With ATP/ADP Maintains a non-IRE-Binding State. *Biochem J* (2010) 430(2):315–24. doi: 10.1042/BJ20100111
74. Dean JM, Lodhi IJ. Structural and Functional Roles of Ether Lipids. *Protein Cell* (2018) 9(2):196–206. doi: 10.1007/s13238-017-0423-5
75. Lee H, Zhuang L, Gan B. Ether Phospholipids Govern Ferroptosis. *J Genet Genomics* (2021) 48(7):517–9. doi: 10.1016/j.jgg.2021.05.003
76. Zou Y, Henry WS, Ricq EL, Graham ET, Phadnis VV, Maretich P, et al. Plasticity of Ether Lipids Promotes Ferroptosis Susceptibility and Evasion. *Nature* (2020) 585(7826):603–8. doi: 10.1038/s41586-020-2732-8
77. Cui W, Liu D, Gu W, Chu B. Peroxisome-Driven Ether-Linked Phospholipids Biosynthesis Is Essential for Ferroptosis. *Cell Death Differ* (2021) 28(8):2536–51. doi: 10.1038/s41418-021-00769-0
78. Werner ER, Keller MA, Sailer S, Lackner K, Koch J, Hermann M, et al. The TMEM189 Gene Encodes Plasmanylethanolamine Desaturase Which Introduces the Characteristic Vinyl Ether Double Bond Into Plasmalogens. *Proc Natl Acad Sci USA* (2020) 117(14):7792–8. doi: 10.1073/pnas.1917461117
79. Asada S, Goyama S, Inoue D, Shikata S, Takeda R, Fukushima T, et al. Mutant ASXL1 Cooperates With BAP1 to Promote Myeloid Leukaemogenesis. *Nat Commun* (2018) 9(1):2733. doi: 10.1038/s41467-018-05085-9
80. Dey A, Seshasayee D, Noubade R, French DM, Liu J, Chaurushiya MS, et al. Loss of the Tumor Suppressor BAP1 Causes Myeloid Transformation. *Science* (2012) 337(6101):1541–6. doi: 10.1126/science.1221711
81. Bai J, Chen Z, Chen C, Zhang M, Zhang Y, Song J, et al. Reducing Hyperactivated BAP1 Attenuates Mutant ASXL1-Driven Myeloid Malignancies in Human Haematopoietic Cells. *Cancer Lett* (2021) 519:78–90. doi: 10.1016/j.canlet.2021.06.019
82. Zhang Y, Shi J, Liu X, Feng L, Gong Z, Koppula P, et al. BAP1 Links Metabolic Regulation of Ferroptosis to Tumour Suppression. *Nat Cell Biol* (2018) 20(10):1181–92. doi: 10.1038/s41556-018-0178-0
83. Zhang Y, Zhuang L, Gan B. BAP1 Suppresses Tumor Development by Inducing Ferroptosis Upon SLC7A11 Repression. *Mol Cell Oncol* (2018) 6 (1):1536845–. doi: 10.1080/23723556.2018.1536845
84. Zhang Y, Koppula P, Gan B. Regulation of H2A Ubiquitination and SLC7A11 Expression by BAP1 and PRC1. *Cell Cycle* (2019) 18(8):773–83. doi: 10.1080/15384101.2019.1597506
85. Wang Y, Yang L, Zhang X, Cui W, Liu Y, Sun Q-R, et al. Epigenetic Regulation of Ferroptosis by H2B Monoubiquitination and P53. *EMBO Rep* (2019) 20(7):e47563–e. doi: 10.15252/embr.201847563
86. Liu T, Jiang L, Tavana O, Gu W. The Deubiquitylase OTUB1 Mediates Ferroptosis *via* Stabilization of SLC7A11. *Cancer Res* (2019) 79(8):1913–24. doi: 10.1158/0008-5472.CAN-18-3037
87. Tang Z, Jiang W, Mao M, Zhao J, Chen J, Cheng N. Deubiquitinase USP35 Modulates Ferroptosis in Lung Cancer *via* Targeting Ferroportin. *Clin Transl Med* (2021) 11(4):e390–e. doi: 10.1002/ctm2.390
88. Meng C, Zhan J, Chen D, Shao G, Zhang H, Gu W, et al. The Deubiquitinase USP11 Regulates Cell Proliferation and Ferroptotic Cell Death *via* Stabilization of NRF2 USP11 Deubiquitinates and Stabilizes NRF2. *Oncogene* (2021) 40(9):1706–20. doi: 10.1038/s41388-021-01660-5
89. Lee H, Zandkarimi F, Zhang Y, Meena JK, Kim J, Zhuang L, et al. Energy-Stress-Mediated AMPK Activation Inhibits Ferroptosis. *Nat Cell Biol* (2020) 22(2):225–34. doi: 10.1038/s41556-020-0461-8
90. Lee H, Zhuang L, Gan B. Energy Stress Inhibits Ferroptosis *via* AMPK. *Mol Cell Oncol* (2020) 7(4):1761242–. doi: 10.1080/23723556.2020.1761242
91. Wei X, Yi X, Zhu X-H, Jiang D-S. Posttranslational Modifications in Ferroptosis. *Oxid Med Cell Longev* (2020) 2020:8832043–. doi: 10.1155/2020/8832043
92. Funderburk SF, Wang QJ, Yue Z. The Beclin 1-VPS34 Complex–at the Crossroads of Autophagy and Beyond. *Trends Cell Biol* (2010) 20(6):355–62. doi: 10.1016/j.tcb.2010.03.002
93. Kang R, Zhu S, Zeh HJ, Klionsky DJ, Tang D. BECN1 Is a New Driver of Ferroptosis. *Autophagy* (2018) 14(12):2173–5. doi: 10.1080/15548627.2018.1513758
94. Song X, Zhu S, Chen P, Hou W, Wen Q, Liu J, et al. AMPK-Mediated BECN1 Phosphorylation Promotes Ferroptosis by Directly Blocking System X(c)(-) Activity. *Curr Biol* (2018) 28(15):2388–99.e5. doi: 10.1016/j.cub.2018.05.094
95. Grignano E, Birsen R, Chapuis N, Bouscary D. From Iron Chelation to Overload as a Therapeutic Strategy to Induce Ferroptosis in Leukemic Cells. *Front Oncol* (2020) 10:1859. doi: 10.3389/fonc.2020.586530
96. Wang F, Lv H, Zhao B, Zhou L, Wang S, Luo J, et al. Iron and Leukemia: New Insights for Future Treatments. *J Exp Clin Cancer Res* (2019) 38 (1):406–. doi: 10.1186/s13046-019-1397-3
97. Hunter RL, Bennett B, Towns M, Vogler WR. Transferrin in Disease II: Defects in the Regulation of Transferrin Saturation With Iron Contribute to Susceptibility to Infection. *Am J Clin Pathol* (1984) 81(6):748–53. doi: 10.1093/ajcp/81.6.748
98. Du J, Wang T, Li Y, Zhou Y, Wang X, Yu X, et al. DHA Inhibits Proliferation and Induces Ferroptosis of Leukemia Cells Through Autophagy Dependent

Degradation of Ferritin. *Free Radical Biol Med* (2019) 131:356–69. doi: 10.1016/j.freeradbiomed.2018.12.011
99. Yu Y, Xie Y, Cao L, Yang L, Yang M, Lotze MT, et al. The Ferroptosis Inducer Erastin Enhances Sensitivity of Acute Myeloid Leukemia Cells to Chemotherapeutic Agents. *Mol Cell Oncol* (2015) 2(4):e1054549–e. doi: 10.1080/23723556.2015.1054549
100. Karathedath S, Rajamani BM, Musheer Aalam SM, Abraham A, Varatharajan S, Krishnamurthy P, et al. Role of NF-E2 Related Factor 2 (Nrf2) on Chemotherapy Resistance in Acute Myeloid Leukemia (AML) and the Effect of Pharmacological Inhibition of Nrf2. *PloS One* (2017) 12(5): e0177227–e. doi: 10.1371/journal.pone.0177227
101. Taoka K, Kumano K, Nakamura F, Hosoi M, Goyama S, Imai Y, et al. The Effect of Iron Overload and Chelation on Erythroid Differentiation. *Int J Hematol* (2012) 95(2):149–59. doi: 10.1007/s12185-011-0988-3
102. Neufeld EJ. Oral Chelators Deferasirox and Deferiprone for Transfusional Iron Overload in Thalassemia Major: New Data, New Questions. *Blood* (2006) 107(9):3436–41. doi: 10.1182/blood-2006-02-002394
103. Tilly H, Gomes da Silva M, Vitolo U, Jack A, Meignan M, Lopez-Guillermo A, et al. Diffuse Large B-Cell Lymphoma (DLBCL): ESMO Clinical Practice Guidelines for Diagnosis, Treatment and Follow-Up. *Ann Oncol* (2015) 26 (Suppl 5):v116–25. doi: 10.1093/annonc/mdv304
104. Martelli M, Ferreri AJ, Agostinelli C, Di Rocco A, Pfreundschuh M, Pileri SA. Diffuse Large B-Cell Lymphoma. *Crit Rev Oncol Hematol* (2013) 87(2):146–71. doi: 10.1016/j.critrevonc.2012.12.009
105. Gout PW, Buckley AR, Simms CR, Bruchovsky N. Sulfasalazine, a Potent Suppressor of Lymphoma Growth by Inhibition of the Xc– Cystine Transporter: A New Action for an Old Drug. *Leukemia* (2001) 15 (10):1633–40. doi: 10.1038/sj.leu.2402238
106. Zhang Y, Tan H, Daniels JD, Zandkarimi F, Liu H, Brown LM, et al. Imidazole Ketone Erastin Induces Ferroptosis and Slows Tumor Growth in a Mouse Lymphoma Model. *Cell Chem Biol* (2019) 26(5):623–33.e9. doi: 10.1016/j.chembiol.2019.01.008
107. Kinowaki Y, Kurata M, Ishibashi S, Ikeda M, Tatsuzawa A, Yamamoto M, et al. Glutathione Peroxidase 4 Overexpression Inhibits ROS-Induced Cell Death in Diffuse Large B-Cell Lymphoma. *Lab Invest* (2018) 98(5):609–19. doi: 10.1038/s41374-017-0008-1
108. Kyle RA, Rajkumar SV. Multiple Myeloma. *Blood* (2008) 111(6):2962–72. doi: 10.1182/blood-2007-10-078022
109. Joshua DE, Bryant C, Dix C, Gibson J, Ho J. Biology and Therapy of Multiple Myeloma. *Med J Aust* (2019) 210(8):375–80. doi: 10.5694/mja2.50129
110. Zhong Y, Tian F, Ma H, Wang H, Yang W, Liu Z, et al. FTY720 Induces Ferroptosis and Autophagy *via* PP2A/AMPK Pathway in Multiple Myeloma Cells. *Life Sci* (2020) 260:118077. doi: 10.1016/j.lfs.2020.118077
111. Meister S, Schubert U, Neubert K, Herrmann K, Burger R, Gramatzki M, et al. Extensive Immunoglobulin Production Sensitizes Myeloma Cells for Proteasome Inhibition. *Cancer Res* (2007) 67(4):1783–92. doi: 10.1158/0008-5472.CAN-06-2258
112. Cengiz Seval G, Beksac M. The Safety of Bortezomib for the Treatment of Multiple Myeloma. *Expert Opin Drug Saf* (2018) 17(9):953–62. doi: 10.1080/14740338.2018.1513487
113. Bordini J, Morisi F, Cerruti F, Cascio P, Camaschella C, Ghia P, et al. Iron Causes Lipid Oxidation and Inhibits Proteasome Function in Multiple Myeloma Cells: A Proof of Concept for Novel Combination Therapies. *Cancers (Basel)* (2020) 12(4):970. doi: 10.3390/cancers12040970
114. Chen J, Zaal EA, Berkers CR, Ruijtenbeek R, Garssen J, Redegeld FA. Omega-3 Fatty Acids DHA and EPA Reduce Bortezomib Resistance in Multiple Myeloma Cells by Promoting Glutathione Degradation. *Cells* (2021) 10 (9):2287. doi: 10.3390/cells10092287

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REVIEW
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Treatment Advances in EBV Related Lymphoproliferative Diseases

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Epstein Barr virus (EBV) can affect 90% of the human population. It can invade B lymphocytes, T lymphocytes and natural killer cells of the host and remain in the host for life. The long latency and reactivation of EBV can cause malignant transformation, leading to various lymphoproliferative diseases (LPDs), including EBV-related B-cell lymphoproliferative diseases (EBV-B-LPDs) (for example, Burkitt lymphoma (BL), classic Hodgkin's lymphoma (cHL), and posttransplantation and HIV-related lymphoproliferative diseases) and EBV-related T-cell lymphoproliferative diseases (EBV-T/NK-LPDs) (for example, extranodal nasal type natural killer/T-cell lymphoma (ENKTCL), aggressive NK cell leukaemia (ANKL), and peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS). EBV-LPDs are heterogeneous with different clinical features and prognoses. The treatment of EBV-LPDs is usually similar to that of EBV-negative lymphoma with the same histology and can include chemotherapy, radiotherapy, and hematopoietic stem cell transplant (HSCT). However, problems such as serious toxicity and drug resistance worsen the survival prognosis of patients. EBV expresses a variety of viral and lytic proteins that regulate cell cycle and death processes and promote the survival of tumour cells. Based on these characteristics, a series of treatment strategies for EBV in related malignant tumours have been developed, such as monoclonal antibodies, immune checkpoint inhibitors, cytotoxic T lymphocytes (CTLs) and epigenetic therapy. These new individualized therapies can produce highly specific killing effects on tumour cells, and nontumour cells can be protected from toxicity. This paper will focus on the latest progress in the treatment of EBV-LPDs based on pathological mechanisms.

Keywords: barr virus, lymphoproliferative disorders, lymphoma, therapy, advances

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BACKGROUND

Epstein Barr virus (EBV) is classified as a gamma-1 herpesvirus, and people are generally susceptible to this virus. Patients with EBV infection are usually asymptomatic, and the development of symptomatic disease is associated with delayed primary infection, leading to infectious mononucleosis in young people. In addition, EBV infection is associated with various EBV-related malignancies, such as nasopharyngeal carcinoma (NPC), gastric cancer subtypes, and several lymphoproliferative diseases (LPDs), especially B-cell and T-cell lymphomas (1, 2). It causes about

200000 new cancer cases worldwide every year. In cancer cells, EBV usually remains latent to escape human immune surveillance but can switch from a latent to a lytic cycle to infect new cells in response to physiological stimuli (3, 4). There are three groups of viral proteins expressed by EBV during a latent infection: (1) the Epstein Barr virus nuclear antigen (EBNA) family, which includes EBNA1–EBNA4, within which EBNA3 encompasses EBNA3a, EBNA3b and EBNA3c; (2) the late membrane protein (LMP) group, which includes LMP1 and LMP2; and (3) EBV-encoded RNA (EBER). These viral proteins follow four latent expression patterns, and different host cells express different viral proteins (3) (**Figure 1**). In cells infected with latent EBV, the immediate early (IE) proteins BZLF1 (Zta) and BRLF1 (Rta) are key for mediating transformation, but the two promoters that control Zta and Rta gene transcription are inactive (4). Upon induction by stimulants that activate B cells, such as calcium ionophores, phorbol esters and histone deacetylase (HDAC) inhibitors, these promoters are activated, resulting in the expression of immediate early lytic proteins, followed by the production of early lytic proteins (BMRF1, BALF1, BHRF1, BSLF1, etc.) and the initiation of viral DNA replication (9, 10). At the same time, early lytic proteins and DNA replication trigger the expression of late lytic proteins (VCA-p18, gp350/220, MCP, gH/gL, etc.), which eventually leads to the production of infectious virus particles such as BLLF1 and BFRF3 (4, 9). More than 70 viral proteins can be produced in the entire process (11) (**Figure 2**).

There is increasing evidence that virus latent and lytic proteins can maintain the proliferation and survival of EBV-positive cancer cells by influencing cellular mechanisms regulating the cell cycle, apoptosis and immune recognition of host cells. Research to identify therapeutics for EBV has produced many surprising results. The specific killing effect not only improves the curative effect but also greatly reduces toxicity and side effects. Therefore, in addition to chemotherapy, regulatory immunosuppressive therapy and allogeneic hematopoietic stem cell transplant (allo-HSCT), different EBV-based treatment strategies, such as immunotherapy, gene therapy and epigenetic therapy, are research hotspots. This paper will summarize EBV-LPD treatment methods and discuss research advances regarding these new schemes.

EBV-ASSOCIATED B-CELL LYMPHOPROLIFERATIVE DISORDERS

Chemotherapy

EBV was initially detected by Epstein MA et al. in isolated and cultured cells from a BL child in Uganda (12). BL is a highly proliferative B-cell non-Hodgkin lymphoma (NHL) that can be divided into three different variants: endemic (African) BL, sporadic BL and immunodeficiency-related BL (13). EBV can be detected in almost all cases of endemic BL and up to 40% of cases of immunodeficiency-related BL (14). Various intensive treatment schemes have shown great activity in patients with BL. Previous research found that CODOX-M/IVAC +/- R (cyclophosphamide, vincristine, doxorubicin, methotrexate, ifosfamide, etoposide, and cytarabine with or without rituximab), CALGB (Cancer and Leukemia Group B), Hyper-CVAD +/- R (cyclophosphamide, vincristine, doxorubicin, and dexamethasone with or without rituximab), and dose-adjusted (DA) R-EPOCH (rituximab, etoposide, prednisone, vincristine, cyclophosphamide, and

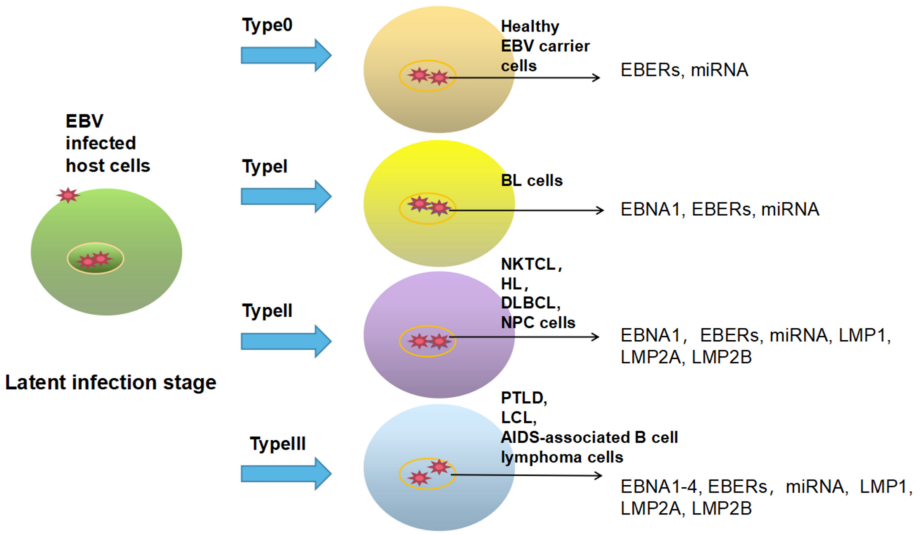

FIGURE 1 | EBV expresses different viral proteins in different host cells during a latent infection. Latent type 0: EBER and miRNA are expressed in the nucleus of host cells, mainly in EBV carriers in good health. Latent type I: EBNA1, EBER and miRNA are commonly expressed in patients with Burkitt lymphoma (BL) (5). Latent type II: EBNA1, EBER, miRNA, LMP1, LMP2A, and LMP2B are generally expressed in patients with HL, nasal NK/T cell lymphoma (NKTCL), NPC and diffuse large B-cell lymphoma (DLBCL) (6, 7). Latent type III: EBNA1–4, EBER, miRNA, LMP1, LMP2A and LMP2B can be expressed in posttransplant lymphoproliferative disease (PTLD), lymphoblastic cell line (LCL) and AIDS-associated B-cell lymphoma (8).

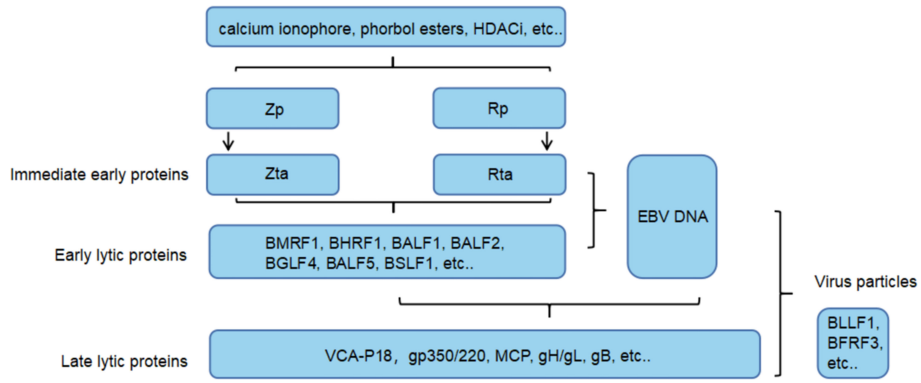

FIGURE 2 | EBV lytic cycle reactivation. After a variety of stimulants activate the EBV Z/R promoters (Zp and Rp), the two immediate early proteins reciprocally activate expression and drive the EBV lytic cycle. In this process, a series of lytic proteins are produced to regulate gene expression and induce DNA replication. Finally, the virus particles are packaged by structural proteins and released through exocytosis.

doxorubicin) achieved significant results. The best results showed that patients can achieve 95% event-free survival (EFS) and 100% overall survival (OS) (15–20) (**Table 1**). The low-dose adjusted R-EPOCH regimen represents major progress, allowing the treatment of elderly and human immunodeficiency virus (HIV)-positive patients and solving the problem of limited application of a high-intensity regimen due to severe toxicity in these patient groups. However, for patients with endemic diseases, treatment schemes are usually limited by cost, and different low-income and middle-income countries approve different schemes. For example, the OS rate of patients in sub-Saharan Africa is between 51% and 67%, which is much worse than that in resource rich countries (38). Therefore, those successful studies cannot represent the heterogeneous population. Meanwhile, chemotherapy is not sufficient to block disease activity and eradicate infected cells. There is a lack of further salvage treatment for patients with recurrent or refractory BL, and there is an urgent need to develop more effective treatments.

TABLE 1 | Summary of available therapies in EBV-B-LPD.

Therapy			Patients characteristics	Efficacy	Reference
Chemotherapy		CODOX-M/IVAC+/-R	BL	2-year EFS: 65%, 2-year OS: 73%; 3-year PFS: 74%, 3-year OS: 77%	(15, 16)
		GALGB+/-R	BL	3-year EFS: 74%, 2-year OS: 78%; 3-year EFS、OS: 45-54%	(18, 19)
		HyperCVAD +/-R	BL	3-year EFS: 80%, 3-year OS: 89%	(17)
		(DA)R-EPOCH	BL	EFS: 95%, OS: 100%	(20)
Immunotherapy	Monoclonal antibody	R-CHOP	PTLD/DLBCL	First-line treatment	–
		Rituximab	PTLD	It is related to the elimination of PTLD related mortality	(21)
		BV	PTLD/DLBCL	The patient had no disease progression 3.5 years after PTLD.	(22)
		Daradaratumumab	PTLD	A rapid decrease in EBV viral load was observed	(23)
	Checkpoint inhibitors	Nivolumab	HL	ORR: 66%	(24)
			HL	ORR: 89%, CR:59%	(25)
		Pembrolizumab	HL	ORR: 69%, CR: 22.4%	(26)
		Sintilimab	HL	ORR: 80%	(27)
		Tislelizumab	HL	CR: 62.9%, ORR: 87.1%	(28)
		Camrelizumab	HL	CR: 28%, ORR:76%	(29)
		Nivolumab + BV	HL	CR: 67%, ORR:85%	(30)
			HL	OS: 98%	(31)
			HL	It is effective and well tolerated in the elderly	(32)
		Nivolumab	PTLD	The child received CR	(33)
		Nivolumab		The woman received CR	(34)
	CTL	EBV-CTL	PTLD	CR: 84.6%	(35)
		EBV-CTL line bank	PTLD	The 6 months effective rate was 52%	(36)
		CMD-003	R/R lymphoma and PTLD	It has been granted fast track by FDA	(3)
		EBV-CTL and LMP-2-CTL	cHL	The patients were well tolerated and sustained clinical responses were observed.	(37)

Immunotherapy

Monoclonal antibodies

EBV is detected in 25–50% of Hodgkin's lymphoma (HL) cases in the United States and Europe (39, 40). In immunosuppressed individuals, EBV-encoded LMP-1 contributes to NF-κB pathway activation and induces an antiapoptotic phenotype in Reed–Sternberg (RS) cells in HL (41). CD30, a transmembrane protein belonging to the tumour necrosis factor receptor family, is specifically expressed in normally activated (rather than static) B and T cells and NK cells. The CD30-targeted antibody brentuximab vedotin (BV) showed significant antitumour activity in recurrent or refractory (R/R) HL and anaplastic large cell lymphoma (ALCL), leading to its accelerated approval by the FDA (42). The results of a preliminary phase II study showed moderate efficacy of SGN-30 in HL patients with all levels of CD30 expression (43). In addition, continuous treatment with MDX-060, a human anti-CD30 immunoglobulin G1 monoclonal antibody, was well tolerated in phase I and II clinical trials. However, there has been no study on the effect of CD30 mAbs on EBV load.

In 2003, EBV+ diffuse large B cell lymphoma (DLBCL) was first described as a unique entity in elderly patients, and *in situ* hybridization showed that this disease was related to EBV (44). In 2008, the World Health Organization (WHO) defined the temporary entity of DLBCL based on a large number of studies conducted in Asian populations and named it "EBV positive DLBCL of the elderly". After that, several groups studied the association between EBV and DLBCL in children and young people, and proved that EBV positive was also detected in these age groups (45–50), and some of which showing a larger morphological spectrum and better survival rate (51). Therefore, the term "elderly" was replaced with "not otherwise specified" (EBV + DLBCL, NOS) in the 2016 classification and is no longer considered provisional (52). Sarah Park et al. evaluated EBER expression by *in situ* hybridization in 380 samples from DLBCL patients to evaluate the significance of EBV positivity on the survival and prognosis of DLBCL patients. These researchers found that patients with EBER-positive DLBCL showed more rapid clinical deterioration and worse survival rates and treatment responses (53). CD20 is a nonglycosylated pan B-cell transmembrane phosphoprotein with a molecular weight of 35 KD that is expressed on the surface of most mature B cells. At present, rituximab, which targets CD20, combined with chemotherapy is the first-line treatment for EBV+ DLBCL. Different reactions to R-CHOP (rituximab, etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin) have been reported worldwide, but no prospective comparative study has been conducted (54). CD30 is expressed in some posttransplant lymphoproliferative diseases (PTLDs) induced by EBV infection, including DLBCL. Brentuximab vedotin (BV), a CD30-directed antibody–toxin conjugate, represents an attractive treatment. Thomas Mika et al. reported the first case of long-term control/cure of highly invasive EBV-DLBCL by combining BV and adoptive EBV-specific T cell therapy. The patient had no disease progression 3.5 years after PTLD. However, the long-term efficacy of BV monotherapy in the treatment of CD30+ DLBCL caused by PTLD has not been determined (22). Clinical trials of BV combined with other therapies are ongoing (**Table 3**). Other therapies were mainly studied in the background of PTLD, which has extensive effects on EBV+ DLBCL.

Reactivation of EBV after bone marrow transplantation usually leads to a LPD that does not respond well to standard treatment and is usually fatal. As early as 1969, the incidence rate of LPDs in solid organ and bone marrow transplant recipients was very high, between 0.5% and 17% (79, 80). EBV infection affects approximately 60–80% of patients with PTLD, including 100% of patients with early-onset PTLD (81). Associated EBV infection and CD20+ B cell proliferation were observed in 90% of cases. Therefore, in addition to stopping immunosuppressive drugs, monoclonal anti-CD20 antibodies (such as rituximab) can be used in standard treatment (82, 83). W J F M van der Velden et al. noted that pre-emptive treatment, which is defined as rituximab administration to patients with a symptomatic EBV infection, is related to the elimination of PTLD-related mortality (21). At present, there is no standardized rescue treatment. If there is no response to anti-CD20 treatment, patient prognosis is very poor. Patrick-Pascal Strunz et al. reported the first case of combined treatment with a CD38 antibody (daratumumab) and EBV-specific cytotoxic T lymphocytes for EBV+ rituximab-refractory PTLD. The flow cytometry results of a sample from the 55-year-old male patient showed the loss of CD20 and continuously high CD38 expression in the homogeneous B cell population. Therefore, after the administration of EBV-specific cytotoxic T lymphocytes to this patient, daratumumab was added, and a rapid decrease in EBV viral load was observed. However, the examination results showed early recurrence of PTLD after 2 weeks (23). The role of CD38-targeted immunotherapy in the treatment of rituximab-refractory CD38+ PTLD needs to be further explored. In addition, the CD30 antibody mentioned above is also used to treat PTLD.

Checkpoint Inhibitors

Programmed death ligand 1 (PD-L1) is an immunomodulatory molecule expressed by antigen-presenting cells that selectively binds to the PD-1 receptor on T cells to inhibit T cell immune function. Both RS cells of cHL and malignant B cells of PTLD express PD-L1, and this expression is promoted by EBV. Therefore, these diseases share this immune escape mechanism (35, 36, 84) (**Figure 3**). Nivolumab and pembrolizumab, humanized anti-PD-1 monoclonal antibodies, have been proven to be effective in the treatment of patients with R/R cHL. In 2016, Anas Younes and his team found that the overall response rate (ORR) for nivolumab was 66% with acceptable safety in cHL patients who failed autologous stem cell transplant (ASCT) (24). In 2017, a phase II study involving 221 R/R HL patients showed that the ORR for pembrolizumab was 69%, and the complete remission (CR) rate was 22.4% (26). Subsequent extended follow-up reported that the median PFS was 14 months (85). Nivolumab and pembrolizumab were approved by the FDA in 2016 and began to be used in the treatment of cHL in R/R patients in 2017. In addition, pembrolizumab can be included in consolidation treatment after ASCT. Philippe Armand et al. conducted the first immune checkpoint blocking study for

consolidation therapy in patients with R/R cHL and proved that PD-1 blockade with pembrolizumab after ASCT had acceptable safety and a significant progression-free survival (PFS) benefit (86). A large number of combined therapy studies including PD-1 have emerged (**Table 1**). The combined use of BV-nivolumab is a promising scheme. In a single arm phase I/II study, BV-nivolumab was applied to patients before ASCT, achieving a high CR of 67% and an ORR of 85% (30, 87). Among patients with high-risk R/R HL, consolidation after HCT with BV-nivolumab resulted in an estimated 18 month PFS and OS of 95% and 98% in all 59 patients, respectively (31). In addition, the combination of the two drugs is effective and well tolerated in newly treated elderly patients with HL (32). There are also a variety of new PD-1 inhibitors, such as sintilimab, tirizumab and camerezumab, which are under development. Their ORR is about 80% and CR rate is 30 – 60% (27–29) (**Table 1**). These studies excluded patients previously treated with PD-1. In the future, it will be interesting to assess the response of patients previously exposed to checkpoint inhibitors to new PD-1 inhibitors. Given the known efficacy in cHL, the use of immune checkpoint inhibitors in PTLD is promising (88). It is reported that after all conventional treatments failed and significant toxicity occurred in a child with PTLD, the salvage treatment with nivolumab achieved CR (33). Similarly, this result was also observed after application of nivolumab in a woman with cHL-like PTLD (34). At present, a phase I clinical trial to explore the efficacy of nivolumab and EBV-specific T cells in patients with R/R EBV-positive lymphoma, including PTLD, is completed, but no results were released (NCT02973113).

CTLs

Immunotherapy strategies to restore virus-specific immunity are an attractive alternative to antiviral therapy. EBV-specific CTLs of human leukocyte antigens (HLA)-matched donors or autologous lymphocytes can be activated and expanded *in vitro* and then injected into the recipient, where they can restore cellular immunity after EBV infection and eradicate EBV-infected cells. The most common adverse reaction is graft-versus-host disease (GVHD). Compared with adoptive therapy with monoclonal antibodies, CTLs can actively migrate through the microvascular wall to reach isolated tumour cells and undergo self-expansion. CTLs can kill tumour cells through cytotoxic effectors and have advantages in biological distribution and antitumour activity (89). In some initial single-centre experiments, EBV-CTLs were used to treat viral reactivation after bone marrow transplantation and achieved clear results (90, 91). In 1994, Ekaterina Doublovina et al. reported that five patients with EBV-associated lymphoma achieved sustained clinical remission after receiving peripheral blood mononuclear cells (PBMCs) from EBV-seropositive transplant donors (92). Subsequently, C M Rooney et al. prepared the first EBV CTL line *in vitro* from donor leukocytes and infused these cells into ten lymphoma patients after allogeneic transplantation to reconstruct EBV-specific immunity; the cells were found to persist for ten weeks *in vivo* without GVHD (93). The team infused EBV-CTLs in 114 HSCT patients at three different centres over a 12-year period. The CR rate reached 84.6% in 13 patients with PTLD and proved that the duration of functional CTLs was up to 9 years. The other patients who received preventive treatment did not develop PTLD [42]. The main disadvantages of generating EBV-CTLs for specific patients are that it is expensive and time-consuming; it takes three to four months to generate a suitable CTL system. Tanzina Haque et al. generated allogeneic virus-specific CTL line banks from normal donors, overcoming the main limitations. It was found that the higher the HLA match between patients and CTLs was, the better the response. In this phase II clinical trial, the 6-month response rate of 33 patients with PTLD who failed conventional treatment was 52% (94). In addition, the autologous T cell therapy CMD-003 has been approved by the FDA for the treatment of R/R lymphoma and PTLD (3) (**Table 1**). Due to cost constraints and technical difficulties, EBV-specific CTLs are always used after the patient's condition has deteriorated, and this treatment strategy is not currently being used in the clinic. A multicentre, open label, single arm, phase III study was completed in March 2021. In the context of allogeneic haematopoietic cell transplantation, the efficacy and safety of an allogeneic T cell immunotherapy called tabelecleucel in the treatment of EBV+ PTLD were evaluated. The results have not been published (NCT03392142). Other relevant clinical trials are still in progress (**Table 3**).

RS cells in EBV-positive cHL can downregulate immune-dominant EBV nuclear antigens such as EBNA3A, EBNA3B and EBNA3C so that most CTLs against potential antigens lose their effect. However, the latent proteins LMP-1 and LMP-2 expressed by tumour cells in approximately 40% of patients with Hodgkin's or non-Hodgkin's lymphoma then become the immunotherapeutic targets of CTLs (95) (**Figure 3**). M A Roskrow et al. conducted a number of phase I clinical trials in patients with cHL using polyclonal EBV-CTLs and CTLs rich in precursors targeting LMP-2 (37). A large number of EBV-specific T cells were obtained from 9 patients with advanced Hodgkin's disease and successfully expanded slowly *in vitro*. After these cells were injected into patients with multiple relapses, the activity of EBV-specific CTLs increased, which enhanced the immune response to EBV for more than 13 weeks. In addition, it was found that these CTLs could recognize LMP2A expressed by tumour cells (**Table 1**).

Gene Therapy

The transcription factor EBNA2, a latent viral gene expressed by malignant cells latently infected with EBV, is a transactivator of virus and gene expression (96). M Franken et al. showed that use of the BamHI C promoter (CP) of EBNA2 in B-cell lymphoma can regulate the expression of the suicide gene and can selectively enhance the sensitivity of EBNA2-expressing cells to ganciclovir. These results provide *in vivo* and *in vitro* support for gene therapy based on the molecular basis of tumour development (96). Jian Hua Li et al. reported the feasibility of adenovirus-mediated wild-type p53 (Ad5CMV-p53) gene therapy for NPC. The adenovirus vector regulates transgene expression through the EBV replication initiation site, which can accurately transmit p53 to EBV-positive cancer cells and induce apoptosis. This approach shows effective cytotoxicity that can be enhanced by ionizing radiation (97). The team then further demonstrated that the results of EBV-targeted gene therapy could be replicated in mouse tumour models, which is expected to support the

translation of this strategy into the clinic for NPC patients (98). At present, there is little research on gene therapy in EBV-LPDs, although there is much space for further exploration.

EBV-ASSOCIATED T- AND NK-CELL LYMPHOPROLIFERATIVE DISORDERS

Chemotherapy

Epstein Barr virus (EBV) can cause B-cell lymphoma and is also found in some T or NK cell lymphoid tumours, such as extranodal NK/T-cell lymphoma (ENKTCL), invasive NK cell leukaemia (ANKL) and chronic active Epstein Barr virus infection (CAEBV). The WHO classification of haematopoietic and lymphoid tumours was revised in 2017 (WHO 2017), and CAEBV is defined as an T cell or NK cell tumour (99). The infected cells in CAEBV are activated and clonally proliferate with the characteristics of inflammation and malignancy. Affected patients experience fatal progression after lymphoma or HLH, so treatment needs to be started before this occurs. Chemotherapy can significantly reduce disease activity and the burden of residual EBV-infected T cells, but it is usually impossible to significantly reduce EBV-DNA load before haematopoietic cell transplantation. One of the main goals of chemotherapy is to control disease and reduce the risk of transplantation-related complications. Akihisa Sawada and others selected the combination of steroids, etoposide and cyclosporine or cytotoxic chemotherapy according to the treatment of lymphoma and then performed HSCT. The OS of 79 patients with CAEBV and related diseases reached 87% (100).

The prototype of EBV-driven lymphoma is extranodal NK/T cell lymphoma of the nasal type. Due to the high expression level of P-glycoprotein in NK lymphoma cells, chemotherapy, such as CHOP- and doxorubicin-based regimens, is largely ineffective. However, the lack of asparagine synthase makes ENKTCL sensitive to L-asparaginase. DDGP (cisplatin, dexamethasone, gemcitabine and pegaspargase) was recommended as the first-line chemotherapy for NK/T-cell lymphoma in the National Comprehensive Cancer Network (NCCN) guidelines in 2020. Other schemes based on L-asparaginase, such as SMILE (dexamethasone, methotrexate, ifosfamide, asparaginase, and etoposide), also showed good efficacy; however, this regimen is highly toxic and can even lead to death (55). DDGP, P-Gemox and AspaMetDex are less toxic alternatives to SMILE (56, 57) (**Table 2**).

TABLE 2 | Summary of available therapies in EBV-T/NK-LPD.

		Therapy	Patients characteristics	Efficacy	Reference
Chemotherapy		Steroids, etoposide and cyclosporine or cytotoxic chemotherapy + HSCT	CAEBV	OS: 87%	(31)
		DDGP	NKTCL	First-line treatment.	-
		SMILE regimen	NKTCL	ORR: 79%, CR: 45%	(55)
		P-Gemox	NKTCL	ORR: 80%, CR: 51.4%	(56)
		AspaMetDex	NKTCL	ORR:77.8%	(57)
		CHOP	PTCL-NOS	Usually incurable.	–
Allo-HSCT			EBV-T/NK-LPD	It is a method to cure EBV-T/NK-LPD.	(58)
Immunotherapy	Monoclonal antibody	Daratumumab	NKTCL	ORR:35.7%	(59)
				ORR:25%	(60)
		BV	NKTCL	2 patients received CR.	(61, 62)
	Checkpoint inhibitors	Pembrolizumab	NKTCL	5 of the 7 R/R patients achieved CR.	(63)
			NKTCL	2 patients received CR 2 patients received PR.	(64)
		Nivolumab	NKTCL	3 patients with R/R NKTCL achieved clinical response.	(65)
		Sintilimab	NKTCL	ORR:68%	(66)
		Avelumab	NKTCL	CR:24%, ORR:38%	(67)
		Geptanolimab	PTCL	CR:14.6%, ORR:40.4%	(68)
		PRN371	NKTCL	It can inhibit tumor growth in NKTCL xenograft model.	(69)
		Tofacitinib	NKTCL	It can inhibit JAK3 activity *in vivo* and *in vitro*.	(70)
	CTL	LMP-CTL	NKTCL	CR was 2-6 years, and 8 survived for at least 2 years.	(71)
		LMP-CTL	NKTCL	OS: 100%, PFS:90%	(72)
Epigenetic therapy	HDAC Inhibitors	SAHA	PTCL and CTCL	It can inhibit tumor growth and metastasis in NKTCL xenograft model.	(73)
		Romidepsin	PTCL and CTCL	It can induce complete and lasting response.	(74)
		Chidamide	PTCL	It has significant single drug activity and controllable toxicity	(75)
Other approaches	Proteasome inhibitor	Bortezomib+ CHOP	NKTCL	ORR: 61.5%	(76)
		Bortezomib	CTCL and PTCL	ORR: 67%	(77)
		Bortezomib	NKTCL	ORR: 42.8%	(78)

TABLE 3 | Clinical trials of therapy for EBV LPDs.

	Intervention/treatment	Phase	Tumour type	ClinicalTrials.gov Identifier
CD30 monoclonal antibody	MDX-1401	Phase 1	R/R HL	NCT00634452
	BV	Phase 2	cHL, PTCL	NCT03947255
	BV/BV + bendamostine/BV+ dacarbazine/BV+nivolumab	Phase 2	HL, PTCL	NCT01716806
	BV/BV + nivolumab	Phase 2	R/R HL, NHL	NCT01703949
	BV + nivolumab	Phase 2	R/R HL	NCT04561206
	BV	Phase 2	R/R HL	NCT01508312
	BV + chemotherapy	Phase 1 and phase 2	Stage II-IV HIV associated HL	NCT01771107
	BV + irutinib	Phase 2	R/R HL	NCT02744612
	BV + nivolumab	Phase 2	R/R HL	NCT03057795
	BV + chemotherapy	Phase 2	Stage II-IV elderly HL	NCT01476410
	BV + chemotherapy	Phase 3	Stage IIB/IIIB-IVB adolescent HL	NCT02166463
	BV + chemotherapy	Phase 1 and phase 2	R/R DLBCL	NCT03356054
	BV + lenalidomide + rituximab	Phase 3	R/R DLBCL	NCT04404283
	BV	/	R/R PTCL	NCT04213209
	BV	Phase2	PTCL	NCT03947255
CTL	Rituximab + LMP-CTL	Phase 2	child PTLD	NCT02900976
	Tabelecleucel	Phase 3	PTLD	NCT03394365
	Tabelecleucel	Phase 2	EBV related diseases	NCT04554914
PD-1 inhibitor	Nivolumab + ifosfamide, + carboplatin+ etoposide	Phase2	R/R HL	NCT03016871
	Nivolumab+radiotherapy	Phase2	cHL	NCT03480334
	Camrelizumab +/— decitabine	Phase2	HL	NCT03250962
	Camrelizumab + GEMOX	Phase2	R/R HL	NCT04239170
	Sintilimab + RCHOP	Phase2	EBV+DLBCL	NCT04181489
	Tislelizumab + zanubrutinib	Phase2	EBV+DLBCL	NCT04705129
	Tislelizumab + dexamethasone, azacytidine + pegaspargase	Phase2	NKTCL	NCT04899414
	Tislelizumab + (azacytidine + lenalidomide)/(etoposide, pegaspargase)	/	NKTCL	NCT05058755
	Tislelizumab	Phase2	NKTCL/PTCL	NCT03493451
	Avelumab	Phase2	PTCL	NCT03046953
	PD-1 blocking antibody+ chidamide + lenalidomide + gemcitabine	Phase 4	PTCL	NCT04040491
	PD-1 antibody+ HDAC inhibitor	Phase 2	PTCL	NCT04512534
	Geptanolimab (GB226)	Phase 2	PTCL	NCT03502629
	Sintilimab + chidamide+ azacidine	Phase 2	PTCL	NCT04052659
	Nivolumab + cabiralizumab	Phase 2	PTCL	NCT03927105
HDAC inhibitor	Romidepsin + pralatrexate+durvalumab +5-azacitidine	Phase 1 and phase 2	R/R PTCL	NCT03161223
	HDAC inhibitor+PD-1 antibody	Phase 2	PTCL	NCT04512534
	Romidepsin + lenalidomide	Phase 2	PTCL	NCT02232516
	Romidepsin + Ixazomib	Phase 1 and phase 2	R/R PTCL	NCT03547700
	Romidepsin + lenalidomide+ CC-486 (5-azacitidine) + dexamethasone	Phase 1	PTCL. Etc.	NCT04447027
	Azacytidine + romidepsin + belinostat + pralatrexate + gemcitabine	Phase 2	PTCL	NCT04747236

EBV+ PTCL-NOS is a highly heterogeneous mature post-thymic T cell tumour. CHOP is the most commonly used first-line treatment for systemic PTCL, but this disease is usually incurable. When EBV-related LPD is pathological grade 3 and the patient has severe systemic symptoms, chemotherapy can be considered to control the disease condition but cannot improve patient prognosis (101).

Allo-HSCT

Numerous studies at home and abroad have proven that allo-HSCT can cure EBV-T/NK-LPD. In 2000, K Kawa et al. first reported the cure of a patient with CAEBV by allogeneic bone marrow transplantation, which also eradicated EBV-infected peripheral T cells and natural killer cells (102). A series of studies have proven an obvious survival benefit for patients treated with HSCT (103, 104). However, the prognosis of patients with active disease at allo-HSCT is worse than that of patients without active disease (105). Arai et al. found that 4 of the 5 patients with active disease at the time of HSCT experienced transplantation failure or recipient cell recovery. These findings suggest the importance of disease control before HSCT. Recently, Ichiro Yonese et al. evaluated 100 patients with newly diagnosed EBV-T/NK-LPD in Japan from January 2003 to March 2016 and found that the 3-year overall survival rates of

chemotherapy alone, allo-HSCT after chemotherapy and allo-HSCT alone were 0%, 65% and 82%, respectively (58). Therefore, chemotherapy has not solved the problem of disease activity before transplantation. Although allo-HSCT can cure patients, the risk of complications after transplantation is high, and it is generally reserved for use in severe cases.

Immunotherapy

Monoclonal Antibodies

To date, several monoclonal antibodies against human CD38 have been successfully developed, such as daratumumab, isatuximab (SAR650984) and MOR202 (59). Wang L and colleagues demonstrated that 95% of NK/T cell lymphoma (NKTCL) cases were CD38 positive and half had high CD38 expression, which was significantly correlated with adverse results, indicating the potential role of CD38 as a therapeutic target in ENKTCL (106). A subsequent case report described an Asian woman with R/R NKTCL who achieved CR with daratumumab (107). At the 2018 American Society of Haematology (ASH) meeting, preliminary results reported a good remission rate with daratumumab for the treatment of patients with R/R NKTCL, and the ORR was 35.7% (59). The latest phase 2 study of R/R NKTCL included 32 Asian patients, and the ORR reached 25% after the application of single drug daratumumab. All patients were well tolerated, but none reached CR and the response duration was short (60). This may indicate that daratumumab alone is insufficient to treat patients with invasive characteristics, especially those with poor prognosis. Whether daretouximab can be used in combination with drugs for the treatment of NKTCL remains to be further studied. The CD30 drug–antibody conjugate brentuximab vedotin is effective in NKTCL because CD30 was reported by Feng Y et al. to be expressed in approximately 50% of 622 patients (108). To date, no clinical trials have been conducted specifically for recurrent/refractory NKTCL, but two patients were reported to achieve CR after BV treatment. Hee Kyung Kim et al. reported a case of an R/R NKTCL male patient with skin lesions who received brentuximab vedotin monotherapy and achieved long-term CR after 4 cycles. Subsequently, Li Mei Poon et al. treated ENKTCL that relapsed after chemotherapy with a combination of brentuximab vedotin with bendamustine and achieved CR (61, 62) (**Table 2**).

Checkpoint Inhibitors

EBV-driven latent membrane protein LMP1 is overexpressed to activate the NF-κB/MAPK and JAK/STAT signalling pathways, which leads to high PD-L1 expression. Based on the involvement of these signalling pathways, anti-PD-1 antibodies and JAK1/2/3 inhibitors have been the subject of specific drug research and applications (59) (**Figure 3**). Anti-PD-1 antibodies such as pembrolizumab and nivolumab can disrupt the interaction between PD-L1 and PD-1, thereby restoring the antitumour activity of activated T cells (109). Kwong et al. Have confirmed the efficacy of pembrolizumab in NKTCL patients (63); five of the seven patients with advanced disease achieved a CR. Immunohistochemical staining of residual lesions showed only a few CD56+EBER+ cells in infiltrating lymphocytes. This finding is consistent with the hypothesis that pembrolizumab

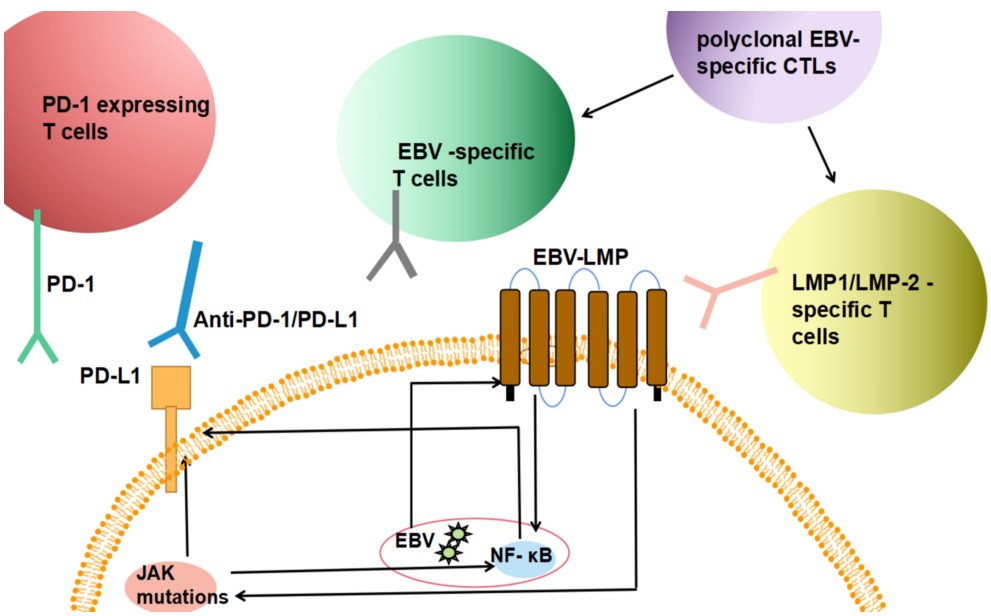

FIGURE 3 | Summary of the immunotherapeutic mechanism of EBV infected tumor cells. EBV infected tumor cells express PD-L1, and binding with PD-1-expressing T cells in microenvironment can inhibit immune killing function; EBV drives the overexpression of latent membrane protein LMP and the activates NF- κB/MAPK and JAK/STAT signaling pathways lead to high expression of PD-L1. Therefore, after the infusion of polyclonal EBV specific CTLs, the increased frequency of EBV and LMP specific T cells and the application of PD-1/PD-1/L1 drugs can reduce immunosuppression and kill tumour cells.

treatment allows T cells to recognize and kill EBV-infected NK/T lymphoma cells. Xin Li et al. applied pembrolizumab in seven patients with R/R ENKL after extensive pretreatment. Two had CR and two had PR. The total response rate was 57% (64). In an independent study, three patients with R/R NKTCL also achieved a clinical response with low-dose nivolumab (65). In addition to these two popular drugs, some other PD-1/PD-L1 studies have preliminary results. In a multicenter, single arm, phase 2 clinical trial, among 28 R/R NKTCL patients, 75% achieved ORR after continuous use of sindilimab for 24 months (66). The PD-L1 inhibitor avelumab also showed monodrug activity in some patients with R/R NKTCL. In the open label phase 2 study, Seok Jin Kim et al. reported a CR of 24% (5/21) and an ORR of 38% (8/21) (67). More relevant prospective studies are still in progress (**Table 3**). PRN371 is a small molecule selective JAK3 inhibitor. Nairismägi ML and his colleagues conducted a preclinical evaluation of PRN371 and found that it significantly inhibited tumour growth in an NKTCL xenotransplantation model carrying a JAK3 activating mutation, which is consistent with the *in vitro* results (69). In addition, tofacitinib can significantly inhibit JAK3 activity *in vivo* and *in vitro*, but its clinical application in cancer treatment is limited by pan-JAK inhibitory activity. Shotaro Ando et al. found that treatment of EBV-positive T and NK cell lines with tofacitinib reduced phosphorylated STAT5 levels, inhibited proliferation, induced G1 phase arrest and reduced EBV LMP1 and EBNA1 expression (70). In addition, Erika Onozawa et al. determined that the JAK1/2 inhibitor ruxolitinib can inhibit the phosphorylation of STAT3, thereby reducing the survival rate of EBV-positive cells and cytokine production in CAEBV patients (110) (**Table 2**).

PD-1 expression can be detected in 30–60% of PTCL/NOS cases (111). In a single arm multicenter phase 2 study completed in China, all R/R PTCL patients received at least one dose of geptanolimab. Of the 89 patients with FAS, 40.4% achieved ORR, and 14.6% achieved CR. Patients with PD-L1 expression ≥ 50% benefited more from treatment (68). A large number of trials of PD-1 inhibitors alone or in combination with other drugs in PTCL are still ongoing (**Table 3**).

CTLs

Greater than 90% of cases of natural killer (NK)/T cell NHL, nasal type, are related to latent type II EBV. Tumour cells can express the weakly immunogenic EBV antigens LMP1, LMP2 and EBNA1. The stimulation of cytotoxic T lymphocytes (CTLs) targeting LMP1 and LMP2 showed efficacy in EBV+ NKTCL. In a clinical trial of LMP-CTLs in 52 EBV-associated lymphomas in the United States, 5 of 11 NKTCL patients received CTLs as consolidation treatment after primary radiotherapy or autologous stem cell transplantation. CR was achieved for 2–6 years, 8 patients survived for at least 2 years, and the longest period of CR was 6 years. In half of the patients, EBV levels could not be detected in plasma during CR (71). One patient with refractory NKTCL after autologous stem cell transplantation also remained in CR for 2 years after CTL infusion. Cho and colleagues studied LMP-CTL treatment in 8 patients with local disease and 2 patients with advanced NKTCL. All patients achieved CR within 4 years, with OS and PFS rates of 100% and 90%, respectively (72). However, since the 5-year survival rate of radiotherapy and chemotherapy can be as high as 90%, it is not clear whether patients with early disease truly benefit from CTL infusion. In conclusion, these trials represent significant advances in NKTCL and EBV-associated lymphoma. The role of CTL treatment in maintaining local disease after first-line treatment remains to be clarified. Longer follow-up and larger studies are needed to confirm these results. At present, there is no phase III clinical trial to guide clinical treatment.

Epigenetic Therapy

Histone Deacetylase Inhibitors

Reactivation of the EBV lytic cycle can enable cytotoxic antiviral drugs to achieve a specific killing effect on EBV-positive cells. These types of therapy include chemical lytic inducers and nucleoside analogue antiviral prodrugs. Studies have shown that HDAC inhibitors can reactivate the lytic cycle and lead to enhanced apoptosis of NPC and gastric cancer cells (112–114). HDAC inhibitors are divided into three categories according to their chemical structure: hydroxamate, cyclic peptide and benzamide. Suberoylanilide hydroxamic acid (SAHA) induces apoptosis and/or cell cycle arrest in some T and NK cell lines. SAHA also inhibited tumour progression and metastasis in a mouse xenograft model, demonstrating that it can inhibit EBV-related T and NK cell lymphoma (73). The cyclic peptide romidepsin has been studied by Bertrand Coiffier et al., who been found that romidepsin monotherapy can induce a complete and lasting response in patients with recurrent or refractory PTCL of all major subtypes, regardless of the number or type of previous treatments, and has controllable toxicity (74). At present, SAHA and romidepsin have been approved by the FDA for the treatment of many types of malignant tumours, such as peripheral and cutaneous T-cell lymphomas (115). Both drugs can induce an EBV lysis cycle at acceptable concentrations in patient plasma (113, 116). Chidamide is a novel oral benzamide HDAC inhibitor. In a phase II study, Y. Shi et al. evaluated the efficacy and safety of chidamide in recurrent or refractory peripheral T-cell lymphoma (PTCL) in a Chinese population and found that it has significant single-agent activity and controllable toxicity (75). Seventy-nine patients were enrolled, and an ORR of 28% was reported; 14% of the patients achieved a CR/CRu, and the OS was 21.4 months. Among these patients, the effects were more pronounced and the response was more durable in AITL patients. Most adverse events were limited to grades 1–2. Based on the results of this critical trial, CFDA approved chidamide for this indication in December 2014. However, it should be noted that HDAC, especially romidepsin, may lead to serious adverse events based on the mechanism of EBV cleavage recirculation activation. In an open label prospective pilot study, SJ Kim et al. found that three of the five NKTCL patients treated with romidepsin had a rapid increase in EBV DNA titer in their blood and an increase in liver enzymes and bilirubin levels (117). Therefore, patients with NKTCL should avoid using romidepsin, but patients with recurrent refractory diseases without other

treatment options can consider the combination of two or more research drugs with strong biological principles. Joo Hyun Kim et al. conducted high-throughput screening of FDA approved drugs and tested them in EBV positive cell lines. It was found that phosphodiesterase 5 (PDE5) inhibitors, such as sildenafil, seem to be non-toxic and effective inhibitors of romidepsin induced EBV reactivation (118). A large number of clinical trials of HDAC inhibitors combined with other drugs are ongoing (**Table 3**).

Other Approaches

Proteasome Inhibitors

Proteasome inhibitors have been shown to inhibit cell growth and promote cell death in a variety of cancers. Bortezomib is the first proteasome inhibitor approved by the FDA in 2003 for the treatment of multiple myeloma (MM) and mantle cell lymphoma (119). The manipulation of normal ubiquitin proteasome system function by EBV is vital for virus replication and the survival of virus-infected cells. EBNA-1, LMP-2A and LMP-1 inhibit proteasome-mediated degradation to maintain virus latency, while bortezomib can reactivate the EBV lytic cycle in EBV-related BL cells (120). Induction of the EBV lytic cycle can activate the radioisotope [^{125}I]2'-fluoro-2'-deoxy-β-D-5-iodouracil-arabinofuranoside, selectively inhibiting the growth of BL xenografts in severe combined immunodeficiency (SCID) mice (121). Granato M et al. found that bortezomib activates endoplasmic reticulum (ER) stress and that C/EBP-β, C-Jun N-terminal kinase (JNK) and autophagy mediate the transformation of a latent to a lytic EBV infection. It is more difficult to induce the EBV lysis cycle in lymphoid cells than in epithelial cells, and this effect is limited to BL cells (122). In addition to reinducing the lysis cycle, bortezomib can affect the apoptosis of EBV-related malignant tumour cells. The latent EBV membrane protein LMP-1 has been shown to activate the NF-κB pathway in BL and NPC, and this pathway has strong tumorigenic activity and may contribute to resistance to apoptosis inducers (123, 124). Bortezomib can protect the inhibitory protein IκBα and block NF-κB activation, leading to malignant cell apoptosis (125–128). It has been reported that bortezomib can induce the apoptosis of NK lymphoma/leukaemia cells, and this result was verified *in vivo* (76, 77, 129). In a phase I study, bortezomib combined with CHOP was administered to patients with advanced invasive T-cell lymphoma, and the ORR was 61.5%. Pier Luigi Zinzani et al. obtained a 67% ORR in patients with cutaneous T-cell lymphoma and invasive T/NK cell lymphoma treated with bortezomib monotherapy. In a study involving 6 NKTCL patients, the ORR after treatment with the B-GIFOX (bortezomib, gemcitabine, oxaliplatin and ifosfamide) regimen was 42.8% (78).

Selective Nuclear Export Protein Inhibitors

XPO1 is a nuclear export protein responsible for transporting biological macromolecules from the nucleus, including tumour suppressor proteins (TSPs) (e.g. p53, p73, FOXO3a, and IκB), growth regulators (e.g., glucocorticoid receptors), and oncogenic proteins (e.g., mRNA, c-myc, cyclin D, Bcl-2, and Bcl-6). In healthy cells, this process is strictly regulated to maintain an appropriate balance between cell growth and apoptosis (130). Studies have found that high levels of XPO1 are associated with poor clinical prognosis of multiple myeloma, DLBCL, glioblastoma and other diseases (130–132). As a linker protein, the EBV protein SM is involved in the nuclear output of mRNA encoding the soluble EBV gene. XPO1 inhibitors can covalently bind to XPO1 active sites and inhibit the output of corresponding mRNAs to the cytoplasm for translation, thereby effectively inhibiting EBV replication and virus transmission (133). Based on the mechanism of action of XPO1 inhibitors, they have broad application prospects in EBV-LPD and are under clinical investigation. A double-blind, placebo-controlled, phase I study of ATG-527 (KPT-335, verdinexor) for the treatment of CAEBV is ongoing; the aim of this study is to evaluate the safety, tolerability, pharmacokinetics and overall treatment response of different dose levels of ATG-527 in patients with CAEBV.

Exosomes

Exosomes are small membrane-bound vesicles secreted by cells. These extracellular vesicles (EVs) carry a wide range of molecules and affect intercellular communication, which contributes to the pathogenesis of various diseases and infections (134, 135). There is considerable evidence that EBV-related exosomes specifically package a variety of viral components that may promote EBV infection (such as LMP-1, lmp-2a, EBER, viral RNA, and miRNA) (136–139), which may help EBV establish the surrounding tumour microenvironment to promote tumour growth and survival. In addition, Keryer-Bibens C et al. found that galectin-9-containing exosomes inhibited EBV-specific T cell proliferation and induced apoptosis (136). Therefore, blocking these exosomes can restore immune surveillance while resisting tumours. Exosomes have been designated as an effective target for cancer treatment, but they need to be further explored.

CONCLUSION

In general, there has been great progress in our understanding and treatment of EBV-LPDs in recent years from traditional chemotherapy, changes in immunosuppressants and HSCT to immunotherapy, gene therapy and epigenetic therapy stemming from discoveries of signaling pathways and virus latency and lysis cycles; together, this improved understanding will lead to the development of better treatment options with fewer side effects. Among such potential treatments, proteasome inhibitors, HDAC inhibitors and JAK inhibitors have been tested *in vitro* and *in vivo* using xenotransplantation models. The effects of rituximab, nivolumab, pembrolizumab, daratumumab, bortezomib and CTLs have been confirmed in clinical trials. However, we must appreciate that most of the research results are limited to specific groups. For heterogeneous populations of EBV-LPDs, immunotherapy targets, checkpoint inhibitors, CTLs, small molecule targets and even gene therapy are still

elusive. We hope that as the data and science related to these methods mature, they will provide alternative treatments as both monotherapy and combination therapy.

AUTHOR CONTRIBUTIONS

KL and TY design, analysis and draft the manuscript. MY, ZC: supervision, funding acquisition, writing–review and editing. YZ: methodology, and writing–review and editing. FL: design, supervision, funding acquisition, methodology, project administration, writing–review and editing. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Kimura H, Fujiwara S. Overview of EBV-Associated T/NK-Cell Lymphoproliferative Diseases. *Front Pediatr* (2018) 6:417:417. doi: 10.3389/fped.2018.00417
2. Hui KF, Chan TF, Yang W, Shen JJ, Lam KP, Kwok H, et al. High Risk Epstein-Barr Virus Variants Characterized by Distinct Polymorphisms in the EBER Locus are Strongly Associated With Nasopharyngeal Carcinoma. *Int J Cancer* (2019) 144(12):3031–42. doi: 10.1002/ijc.32049
3. Hui KF, Yiu SPT, Tam KP, Chiang AKS. Viral-Targeted Strategies Against EBV-Associated Lymphoproliferative Diseases. *Front Oncol* (2019) 9:81:81. doi: 10.3389/fonc.2019.00081
4. Israel BF, Kenney SC. Virally Targeted Therapies for EBV-Associated Malignancies. *Oncogene* (2003) 22(33):5122–30. doi: 10.1038/sj.onc.1206548
5. Kis LL, Takahara M, Nagy N, Klein G, Klein E. Cytokine Mediated Induction of the Major Epstein-Barr Virus (EBV)-Encoded Transforming Protein, LMP-1. *Immunol Lett* (2006) 104(1-2):83–8. doi: 10.1016/j.imlet.2005.11.003
6. Rowe M, Kelly GL, Bell AI, Rickinson AB. Burkitt's Lymphoma: The Rosetta Stone Deciphering Epstein-Barr Virus Biology. *Semin Cancer Biol* (2009) 19 (6):377–88. doi: 10.1016/j.semcancer.2009.07.004
7. Kis LL, Takahara M, Nagy N, Klein G, Klein E. IL-10 can Induce the Expression of EBV-Encoded Latent Membrane Protein-1 (LMP-1) in the Absence of EBNA-2 in B Lymphocytes and in Burkitt Lymphoma- and NK Lymphoma-Derived Cell Lines. *Blood* (2006) 107(7):2928–35. doi: 10.1182/blood-2005-06-2569
8. Carbone A, Gloghini A, Dotti G. EBV-Associated Lymphoproliferative Disorders: Classification and Treatment. *Oncol* (2008) 13(5):577–85. doi: 10.1634/theoncologist.2008-0036
9. Kieff E, Rickinson AB. Epstein Barr virus and its replication. In: Fields BN, Knipe DM, Howley P, et al., editors. *Fields Virology*. Philadelphia, PA: Lippincott–Raven (2001). p. 2511–75.
10. Mainou BA, Everly DN, Raab-Traub N. Unique Signaling Properties of CTAR1 in LMP1-Mediated Transformation. *J Virol* (2007) 81(18):45–. doi: 10.1128/JVI.01001-07
11. Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr Virus and the Origins of Associated Lymphomas. *New Engl J Med* (2004) 350(13):1328–37. doi: 10.1056/NEJMra032015
12. Epstein M, Achong B, Barr Y. Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. *Lancet* (1964) 1(7335):702–3. doi: 10.1016/s0140-6736(64)91524-7
13. Jacobson C, Lacasce A. How I Treat Burkitt Lymphoma in Adults. *Blood* (2014) 124(19):2913–20. doi: 10.1182/blood-2014-06-538504
14. Molyneux EM, Rochford R, Griffin B, Newton R, Bailey S. Burkitt's Lymphoma. *Lancet* (2012) 379(9822):1234–44. doi: 10.1016/S0140-6736(11)61177-X
15. Magrath I, Adde M, Shad A, Venzon D, Seibel N, Gootenberg J, et al. Adults and Children With Small non-Cleaved-Cell Lymphoma Have a Similar Excellent Outcome When Treated With the Same Chemotherapy Regimen. *J Clin Oncol* (1996) 14(3):925–34. doi: 10.1200/jco.1996.14.3.925
16. Barnes JA, Lacasce AS, Feng Y, Toomey CE, Neuberg D, Michaelson JS, et al. Evaluation of the Addition of Rituximab to CODOX-M/IVAC for Burkitt's Lymphoma: A Retrospective Analysis. *Ann Oncol Off J Eur Soc Med Oncol* (2011) 22(8):1859–64. doi: 10.1093/annonc/mdq677
17. Thomas DA, Faderl S, O'Brien S, Bueso-Ramos C, Cortes J, Garcia-Manero G, et al. Chemoimmunotherapy With Hyper-CVAD Plus Rituximab for the Treatment of Adult Burkitt and Burkitt-Type Lymphoma or Acute Lymphoblastic Leukemia. *Cancer* (2006) 106(7):1569–80. doi: 10.1002/cncr.21776
18. Rizzieri DA, Johnson JL, Byrd JC, Lozanski G, Blum KA, Powell BL, et al. Improved Efficacy Using Rituximab and Brief Duration, High Intensity Chemotherapy With Filgrastim Support for Burkitt or Aggressive Lymphomas: Cancer and Leukemia Group B Study 10 002. *Br J Haematol* (2014) 165(1):102–11. doi: 10.1111/bjh.12736
19. Rizzieri DA, Johnson JL, Niedzwiecki D, Lee EJ, Vardiman JW, Powell BL, et al. Intensive Chemotherapy With and Without Cranial Radiation for Burkitt Leukemia and Lymphoma: Final Results of Cancer and Leukemia Group B Study 9251. *Cancer* (2010) 100(7):1438–48. doi: 10.1002/cncr.20143
20. Dunleavy K, Pittaluga S, Shovlin M, Steinberg SM, Cole D, Grant C, et al. Low-Intensity Therapy in Adults With Burkitt's Lymphoma. *N Engl J Med* (2013) 369(20):1915–25. doi: 10.1056/NEJMoa1308392
21. van der Velden WJFM, Mori T, Stevens W, Haan AD, Stelma FF, Blijlevens N, et al. Reduced PTLD-Related Mortality in Patients Experiencing EBV Infection Following Allo-SCT After the Introduction of a Protocol Incorporating Pre-Emptive Rituximab. *Bone Marrow Transplant* (2013) 48 (11):1465–71. doi: 10.1038/bmt.2013.84
22. Mika T, Strate K, Ladigan S, Aigner C, Schroers R. Refractory Epstein-Barr Virus (EBV)-Related Post-Transplant Lymphoproliferative Disease: Cure by Combined Brentuximab Vedotin and Allogeneic EBV-Specific T-Lymphocytes. *Front Med* (2019) 6:295. doi: 10.3389/fmed.2019.00295
23. Strunz PP, Schmalzing M, Heidemeier A, Rasche L, Kortüm KM. Response to Daratumumab in Rituximab-Resistant EBV-Associated PTLD Following Allogenic Stem Cell Transplantation From an EBV Seronegative Donor. *Leukemia Lymphoma* (2019) 60(14):1–4. doi: 10.1080/10428194.2019.1636981
24. Younes A, Santoro A, Shipp M, Zinzani PL, Timmerman JM, Ansell S, et al. Nivolumab for Classical Hodgkin's Lymphoma After Failure of Both Autologous Stem-Cell Transplantation and Brentuximab Vedotin: A Multicentre, Multicohort, Single-Arm Phase 2 Trial. *Lancet Oncol* (2016) 17(9):1283–94. doi: 10.1016/S1470-2045(16)30167-X
25. Herrera AF, Chen RW, Palmer J, Tsai NC, Lee HJ. PET-Adapted Nivolumab or Nivolumab Plus ICE As First Salvage Therapy in Relapsed or Refractory Hodgkin Lymphoma. *Blood* (2019) 134(Supplement_1):239–. doi: 10.1182/blood-2019-123162
26. Chen R, Zinzani PL, Fanale MA, Armand P, Moskowitz CH. Phase II Study of the Efficacy and Safety of Pembrolizumab for Relapsed/Refractory Classic Hodgkin Lymphoma. *J Clin Oncol* (2017) 35(19):2125–32. doi: 10.1200/JCO.2016.72.1316
27. Pys A, Phs C, Pys D, Pwj E, Pxs F, Pwq G, et al. Safety and Activity of Sintilimab in Patients With Relapsed or Refractory Classical Hodgkin

Lymphoma (ORIENT-1): A Multicentre, Single-Arm, Phase 2 Trial. *Lancet Haematol* (2019) 6(1):e12–e9. doi: 10.1016/S2352-3026(18)30192-3
28. Song Y, Gao Q, Zhang H, Fan L, Zhou J, Zou D, et al. Treatment of Relapsed or Refractory Classical Hodgkin Lymphoma With the Anti-PD-1, Tislelizumab: Results of a Phase 2, Single-Arm, Multicenter Study. *Leukemia* (2020) 34(2):533–42. doi: 10.1038/s41375-019-0545-2
29. Song Y, Wu J, Chen X, Lin TY, Single-Arm ZJA. Multicenter, Phase II Study of Camrelizumab in Relapsed or Refractory Classical Hodgkin Lymphoma. *Clin Cancer Res* (2019) 25(18):7363–9. doi: 10.1158/1078-0432.CCR-19-1680
30. Moskowitz AJ, Advani RH, Bartlett NL, Vose JM, Herrera AF. Brentuximab Vedotin and Nivolumab for Relapsed or Refractory Classic Hodgkin Lymphoma: Long-Term Follow-Up Results From the Single-Arm Phase 1/2 Study. *Blood* (2019) 134(Supplement_1):238–. doi: 10.1182/blood-2019-122576
31. Herrera AF, Chen L, Nieto Y, Holmberg L, Feldman TA. Consolidation With Nivolumab and Brentuximab Vedotin After Autologous Hematopoietic Cell Transplantation in Patients With High-Risk Hodgkin Lymphoma. *Blood* (2020) 136(Supplement 1):19–20. doi: 10.1182/blood-2020-136384
32. Cheson BD, Bartlett NL, LaPlant B, Lee HJ, Advani RJ, Christian B, et al. Brentuximab Vedotin Plus Nivolumab as First-Line Therapy in Older or Chemotherapy-Ineligible Patients With Hodgkin Lymphoma (ACCRU): A Multicentre, Single-Arm, Phase 2 Trial. (2020) 7(11):e808–15. doi: 10.1016/S2352-3026(20)30275-1
33. Kassa C, Reményi P, Sinkó J, Kállay K, Kertész G, Kriván G. Successful Nivolumab Therapy in an Allogeneic Stem Cell Transplant Child With Post-Transplant Lymphoproliferative Disorder. *Pediatr Transplantation* (2018) 22(8):e13302. doi: 10.1111/petr.13302
34. Gunes AK, Demir I, Pehlivan M. Classical Hodgkin Lymphoma-Like Post-Transplant Lymphoproliferative Disease After Allogeneic Stem Cell Transplantation for Primary Myelofibrosis is Successfully Treated With Nivolumab: A Case Report. *J Oncol Pharm Pract* (2021) 27(2):509–12. doi: 10.1177/1078155220946462
35. Chen BJ, Chapuy B, Ouyang J, Sun HH, Roemer M, Xu ML, et al. PD-L1 Expression Is Characteristic of a Subset of Aggressive B-Cell Lymphomas and Virus-Associated Malignancies. *Clin Cancer Res* (2013) 19(13):3462–73. doi: 10.1158/1078-0432.CCR-13-0855
36. Ouyang J, Juszczynski P, Rodig SJ, Green MR, Shipp MA. Viral Induction and Targeted Inhibition of Galectin-1 in EBV+ Posttransplant Lymphoproliferative Disorders. *Blood* (2009) 117(16):4315–22. doi: 10.1182/blood-2010-11-320481
37. Roskrow MA, Suzuki N, Gan YJ, Sixbey JW, Rooney CM. Epstein-Barr Virus (EBV)-Specific Cytotoxic T Lymphocytes for the Treatment of Patients With EBV-Positive Relapsed Hodgkin's Disease. *Blood* (1998) 91(8):2925–34. doi: 10.1182/blood.V91.8.2925.2925_2925_2934
38. Gopal S, Gross TG. How I Treat Burkitt Lymphoma in Children, Adolescents, and Young Adults in Sub-Saharan Africa. *Blood* (2018) 132 (3):254–63. doi: 10.1182/blood-2018-04-844472
39. Wu TC, Mann RB, Charache P, Hayward SD, Staal S, Lambe BC, et al. Detection of EBV Gene Expression in Reed-Sternberg Cells of Hodgkin's Disease. *Int J Cancer* (2010) 46(5):801–4. doi: 10.1002/ijc.2910460509
40. Pallesen G, Hamilton-Dutoit SJ, Rowe M, Young LS. Expression of Epstein-Barr Virus Latent Gene Products in Tumour Cells of Hodgkin's Disease. *Lancet* (1991) 337(8737):320. doi: 10.1016/0140-6736(91)90943-J
41. Carbone A, Gloghini A, Larocca LM, Antinori A, Falini B, Tirelli U, et al. Human Immunodeficiency Virus-Associated Hodgkin's Disease Derives From Post-Germinal Center B Cells. *Blood* (1999) 93(7):2319–26. doi: 10.1182/blood.V93.7.2319
42. Song Y, Guo Y, Huang H, Li W, Ke X, Feng J, et al. Phase II Single-Arm Study of Brentuximab Vedotin in Chinese Patients With Relapsed/Refractory Classical Hodgkin Lymphoma or Systemic Anaplastic Large Cell Lymphoma. *Expert Rev Hematol* (2021) 14(9):867–75. doi: 10.1080/17474086.2021.1942831
43. Bartlett N, Younes A, Carabasi M, Forero A, Rosenblatt J, Leonard J, et al. A Phase 1 Multidose Study of SGN-30 Immunotherapy in Patients With Refractory or Recurrent CD30+ Hematologic Malignancies. *Blood* (2008) 111(4):1848–54. doi: 10.1182/blood-2007-07-099317
44. Oyama T, Ichimura K, Suzuki R, Suzumiya J, Ohshima K, Yatabe Y, et al. Senile EBV+ B-Cell Lymphoproliferative Disorders: A Clinicopathologic Study of 22 Patients. *Am J Surg Pathol* (2003) 27(1):16–26. doi: 10.1097/00000478-200301000-00003
45. Nan FF, Zhang L, Li L, Li X, Sun ZC, Zhang XD, et al. Clinical Features and Survival Impact of EBV-Positive Diffuse Large B-Cell Lymphoma With Different Age Cutoffs. *Eur Rev Med Pharmacol Sci* (2020) 24(17):8947–56. doi: 10.26355/eurrev_202001_22836
46. Lu TX, Liang JH, Miao Y, Fan L, Wang L, Qu XY, et al. Epstein-Barr Virus Positive Diffuse Large B-Cell Lymphoma Predict Poor Outcome, Regardless of the Age. *Sci Rep* (2015) 5(1):12168. doi: 10.1038/srep12168
47. Beltran BE, Morales D, Qui?Ones P, Medeiros LJ, Miranda RN, Castillo JJ. EBV-Positive Diffuse Large B-Cell Lymphoma in Young Immunocompetent Individuals. *Clin Lymphoma Myeloma Leukemia* (2011) 11(6):512–6. doi: 10.1016/j.clml.2011.07.003
48. Cohen M, Narbaitz M, Metrebian F, Matteo ED, Preciado MV, Chabay PA. Epstein-Barr Virus-Positive Diffuse Large B-Cell Lymphoma Association is Not Only Restricted to Elderly Patients. *Int J Cancer* (2015) 135(12):2816–24. doi: 10.1002/ijc.28942
49. Cohen M, Matteo ED, Narbaitz M, Carreño FA, Preciado MV, Chabay PA. Epstein-Barr Virus Presence in Pediatric Diffuse Large B-Cell Lymphoma Reveals a Particular Association and Latency Patterns: Analysis of Viral Role in Tumor Microenvironment. *Int J Cancer* (2012) 132(7):1572–80. doi: 10.1002/ijc.27845
50. Uccini S, Al-Jadiry MF, Scarpino S, Ferraro D, Alsaadawi AR, Al-Darraji AF, et al. Epstein-Barr Virus–Positive Diffuse Large B-Cell Lymphoma in Children: A Disease Reminiscent of Epstein-Barr Virus–Positive Diffuse Large B-Cell Lymphoma of the Elderly. *Hum Pathol* (2015) 46(5):716–24. doi: 10.1016/j.humpath.2015.01.011
51. Hong JY, Yoon DH, Suh C, Huh J, Do IG, Sohn I, et al. EBV-Positive Diffuse Large B-Cell Lymphoma in Young Adults: Is This a Distinct Disease Entity? *Ann Oncol* (2015) 26(3):548–55. doi: 10.1093/annonc/mdu556
52. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 Revision of the World Health Organization Classification of Lymphoid Neoplasms. *Blood* (2016) 127(20):2375–90. doi: 10.1182/blood-2016-01-643569
53. Park S, Lee J, Ko YH, Han A, Jun HJ, Lee SC. The Impact of Epstein-Barr Virus Status on Clinical Outcome in Diffuse Large B-Cell Lymphoma. *Blood* (2007) 110(3):972–8. doi: 10.1182/blood-2007-01-067769
54. Crombie JL, Lacasce AS. Epstein Barr Virus Associated B-Cell Lymphomas and Iatrogenic Lymphoproliferative Disorders. *Front Oncol* (2019) 9:109–. doi: 10.3389/fonc.2019.00109
55. Yamaguchi M, Kwong YL, Kim WS, Maeda Y, Suzuki R. Phase II Study of SMILE Chemotherapy for Newly Diagnosed Stage IV, Relapsed, or Refractory Extranodal Natural Killer (Nk)/T-Cell Lymphoma, Nasal Type: The NK-Cell Tumor Study Group Study. *J Clin Oncol* (2011) 29(33):4410–6. doi: 10.1200/JCO.2011.35.6287
56. Li X, Cui Y, Sun Z, Zhang L, Li L, Wang X, et al. DDGP Versus SMILE in Newly Diagnosed Advanced Natural Killer/T-Cell Lymphoma: A Randomized Controlled, Multicenter, Open-Label Study in China. *Clin Cancer Res* (2016), 5223–8. doi: 10.1158/1078-0432.CCR-16-0153
57. Wang JH, Wang L, Liu CC, Xia ZJ, Huang HQ, Lin TY, et al. Efficacy of Combined Gemcitabine, Oxaliplatin and Pegaspargase (P-Gemox Regimen) in Patients With Newly Diagnosed Advancedstage or Relapsed/Refractory Extranodal NK/T-Cell Lymphoma. *Oncotarget* (2016) 7(20). doi: 10.18632/oncotarget.8647
58. Yonese I, Sakashita C, Imadome KI, Kobayashi T, Yamamoto M, Sawada A, et al. Nationwide Survey of Systemic Chronic Active EBV Infection in Japan in Accordance With the New WHO Classification. *Blood Adv* (2020) 4 (13):2918–26. doi: 10.1182/bloodadvances.2020001451
59. Lv K, Li X, Yu H, Chen X, Wu X. Selection of New Immunotherapy Targets for NK/T Cell Lymphoma. *Am J Trans Res* (2020) 12(11):7034–47.
60. Huang H, Zhu J, Yao M, Kim TM, Yoon DH, Cho SG, et al. Daratumumab Monotherapy for Patients With Relapsed or Refractory Natural Killer/T-Cell Lymphoma, Nasal Type: An Open-Label, Single-Arm, Multicenter, Phase 2 Study. *J Hematol Oncol* (2021) 14(1):25. doi: 10.1186/s13045-020-01020-y

61. Kyung KH, Mi MS, Hoon MJ, Eun PJ, Seonggyu B, Seog KW. Complete Remission in CD30-Positive Refractory Extranodal NK/T-Cell Lymphoma With Brentuximab Vedotin. *Blood Res* (2015) 50(4):254–6. doi: 10.5045/br.2015.50.4.254
62. Poon LM, Kwong YL. Complete Remission of Refractory Disseminated NK/T Cell Lymphoma With Brentuximab Vedotin and Bendamustine. *Ann Hematol* (2016) 95(5):847–9. doi: 10.1007/s00277-016-2627-9
63. Jaccard A, Hermine O. A Major Turning Point in NK/T-Cell Lymphoma? *Blood J Am Soc Hematol* (2017). doi: 10.1182/blood-2017-03-769075
64. Li X, Cheng Y, Zhang M, Yan J, Li L, Fu X, et al. Activity of Pembrolizumab in Relapsed/Refractory NK/T-Cell Lymphoma. *J Hematol Oncol* (2018) 11 (1):15. doi: 10.1186/s13045-018-0559-7
65. Chan T, Li J, Loong F, Khong PL, Tse E, Kwong YL. PD1 Blockade With Low-Dose Nivolumab in NK/T Cell Lymphoma Failing L-Asparaginase: Efficacy and Safety. *Ann Hematol* (2017). doi: 10.1007/s00277-017-3127-2
66. Tao R, Fan L, Song Y, Hu Y, Zhang W, Wang Y, et al. Sintilimab for Relapsed/Refractory Extranodal NK/T Cell Lymphoma: A Multicenter, Single-Arm, Phase 2 Trial (ORIENT-4). *Signal Transduction Targeted Ther* (2021) 6(1):365. doi: 10.1038/s41392-021-00768-0
67. Kim SJ, Lim JQ, Laurensia Y, Cho J, Yoon SE, Lee JY, et al. Avelumab for the Treatment of Relapsed or Refractory Extranodal NK/T-Cell Lymphoma: An Open-Label Phase 2 Study. *Blood* (2020) 136(24):2754–63. doi: 10.1182/blood.2020007247
68. Shi Y, Wu J, Wang Z, Zhang L, Wang Z, Zhang M, et al. Efficacy and Safety of Geptanolimab (GB226) for Relapsed or Refractory Peripheral T Cell Lymphoma: An Open-Label Phase 2 Study (Gxplore-002). *J Hematol Oncol* (2021) 14(1):12. doi: 10.1186/s13045-021-01033-1
69. Nairisma?Gi ML, Gerritsen ME, Li ZM, Wijaya GC, Chia B, Laurensia Y, et al. Oncogenic Activation of JAK3-STAT Signaling Confers Clinical Sensitivity to PRN371, a Novel Selective and Potent JAK3 Inhibitor, in Natural Killer/T-Cell Lymphoma. *Leukemia* (2018). doi: 10.1038/s41375-017-0004-x
70. Ando S, Kawada JI, Watanabe T, Suzuki M, Kimura H. Tofacitinib Induces G1 Cell-Cycle Arrest and Inhibits Tumor Growth in Epstein-Barr Virus-Associated T and Natural Killer Cell Lymphoma Cells. *Oncotarget* (2016) 7 (47):76793–805. doi: 10.18632/oncotarget.12529
71. Bollard CM, Gottschalk S, Torrano V, Diouf O, Rooney CM. Sustained Complete Responses in Patients With Lymphoma Receiving Autologous Cytotoxic T Lymphocytes Targeting Epstein-Barr Virus Latent Membrane Proteins. *J Clin Oncol* (2013) 32(8):798. doi: 10.1200/JCO.2013.51.5304
72. Cho SG, Kim N, Sohn HJ, Lee SK, Oh ST, Lee HJ, et al. Long-Term Outcome of Extranodal Nk/T Cell Lymphoma Patients Treated With Postremission Therapy Using EBV LMP1 and LMP2a-Specific CTLs. *Mol Ther* (2015) (8):1401–9. doi: 10.1038/mt.2015.91
73. Siddiquey MNA, Nakagawa H, Iwata S, Kanazawa T, Suzuki M, Imadome KI, et al. Anti-Tumor Effects of Suberoylanilide Hydroxamic Acid on Epstein–Barr Virus-Associated T Cell and Natural Killer Cell Lymphoma. *Cancer Sci* (2014) 105(6):713–22. doi: 10.1111/cas.12418
74. Coiffier B, Pro B, Prince HM, Foss F, Sokol L. Results From a Pivotal, Open-Label, Phase II Study of Romidepsin in Relapsed or Refractory Peripheral T-Cell Lymphoma After Prior Systemic Therapy. *J Clin Oncol Off J Am Soc Clin Oncol* (2012) 30(6):631. doi: 10.1200/JCO.2011.37.4223
75. Shi Y, Dong M, Hong X, Zhang W, Feng J, Zhu J, et al. Results From a Multicenter, Open-Label, Pivotal Phase II Study of Chidamide in Relapsed or Refractory Peripheral T-Cell Lymphoma. *Ann Oncol* (2015) 26(8):1766. doi: 10.1093/annonc/mdv237
76. Lee S, Kang B-Y, Ryoo H. Phase I Study of Proteasome Inhibitor Bortezomib Plus CHOP in Patients With Advanced, Aggressive T-Cell or NK/T-Cell Lymphoma. *Ann Oncol* (2008). doi: 10.1093/annonc/mdn431
77. Zinzani PL, Musuraca G, Tani M, Stefoni V, Baccarani M. Phase II Trial of Proteasome Inhibitor Bortezomib in Patients With Relapsed or Refractory Cutaneous T-Cell Lymphoma. *J Clin Oncol* (2007) 25(27):4293–7. doi: 10.1200/JCO.2007.11.4207
78. Farid M, Yau YW, Tay K, Quek R, Tao M, Koo GC, et al. A Promising New Regimen for the Treatment of Advanced Extranodal NK/T Cell Lymphoma. *Acta Oncol* (2011) 50(4):589–90. doi: 10.3109/0284186X.2010.516272
79. McKhann CF. Primary Malignancy in Patients Undergoing Immunosuppression for Renal Transplantation. *Transplantation* (1969) 8 (2):209–12 doi: 10.1097/00007890-196908000-00033
80. Penn I, Hammond W, Brettschneider L, Starzl TE. Malignant Lymphomas in Transplantation Patients. *Transplant Proc* (1969) 1(1):106–12.
81. Thompson MP. Epstein-Barr Virus and Cancer. *Clin Cancer Res* (2004) 10 (3):803–21. doi: 10.1158/1078-0432.CCR-0670-3
82. Dolcetti R. B Lymphocytes and Epstein-Barr Virus: The Lesson of Post-Transplant Lymphoproliferative Disorders. *Autoimmun Rev* (2008) 7(2):96–101. doi: 10.1016/j.autrev.2007.02.012
83. Llaurador G, Mclaughlin L, Wistinghausen B. Management of Post-Transplant Lymphoproliferative Disorders. *Curr Opin Pediatr* (2017) 29. doi: 10.1097/MOP.0000000000000445
84. Green MR, Rodig S, Juszczynski P, Ouyang J, Shipp MA. Constitutive AP-1 Activity and EBV Infection Induce PD-L1 in Hodgkin Lymphomas and Posttransplant Lymphoproliferative Disorders: Implications for Targeted Therapy. *Clin Cancer Res* (2012) 18(6):1611–8. doi: 10.1158/1078-0432.CCR-11-1942
85. Chen R, Zinzani P, Lee H, Armand P, Johnson N, Brice P, et al. Pembrolizumab in Relapsed or Refractory Hodgkin Lymphoma: 2-Year Follow-Up of KEYNOTE-087. *Blood* (2019) 134(14):1144–53. doi: 10.1182/blood.2019000324
86. Armand P, Chen YB, Redd RA, Joyce RM, Bsat J, Jeter E, et al. PD-1 Blockade With Pembrolizumab for Classical Hodgkin Lymphoma After Autologous Stem Cell Transplantation. *Blood* (2019) 134(1):22–9. doi: 10.1182/blood.2019000215
87. Herrera AF, Moskowitz AJ, Bartlett NL, Vose JM, Ramchandren R, Feldman TA, et al. Interim Results of Brentuximab Vedotin in Combination With Nivolumab in Patients With Relapsed or Refractory Hodgkin Lymphoma. *Blood* (2018) 131(11):1183–94. doi: 10.1182/blood-2017-10-811224
88. Armand P. Immune Checkpoint Blockade in Hematologic Malignancies. *Blood* (2015) 125(22):3393–400. doi: 10.1182/blood-2015-02-567453
89. Foster AE, Rooney CM. Improving T Cell Therapy for Cancer. *Expert Opin Biol Ther* (2006) 6(3):215–29. doi: 10.1517/14712598.6.3.215
90. Riddell, Watanabe K, Goodrich J, Li C, Agha M, Greenberg P. Restoration of Viral Immunity in Immunodeficient Humans by the Adoptive Transfer of T Cell Clones. *Science* (1992) 257(5067):238–41. doi: 10.1126/science.1352912
91. Heslop HE, Brenner MK, Rooney CM. Donor T Cells to Treat EBV-Associated Lymphoma. *N Engl J Med* (1994) 331(10):679–80. doi: 10.1056/NEJM199409083311017
92. Papadopoulos EB, Ladanyi M, Emanuel D, Mackinnon S, Boulad F, Carabasi MH, et al. Infusions of Donor Leukocytes to Treat Epstein-Barr Virus-Associated Lymphoproliferative Disorders After Allogeneic Bone Marrow Transplantation. *N Engl J Med* (1994) 330(17):1185–91. doi: 10.1056/NEJM199404283301703
93. Doubrovina E, Oflaz-Sozmen B, Prockop SE, Kernan NA, Abramson S, Teruya-Feldstein J, et al. Adoptive Immunotherapy With Unselected or EBV-Specific T Cells for Biopsy-Proven EBV+ Lymphomas After Allogeneic Hematopoietic Cell Transplantation. *Blood* (2012) 119(11):2644. doi: 10.1182/blood-2011-08-371971
94. Haque T, Wilkie GM, Jones MM, Higgins CD, Crawford DH. Allogeneic Cytotoxic T-Cell Therapy for EBV-Positive Posttransplantation Lymphoproliferative Disease: Results of a Phase 2 Multicenter Clinical Trial. *Blood* (2007) 110(4):1123–31. doi: 10.1182/blood-2006-12-063008
95. Poppema S, Potters M, Visser L, van den Berg AM. Immune Escape Mechanisms in Hodgkin's Disease. *Ann Oncol* (1998) 9(suppl 5):S21–S4. doi: 10.1093/annonc/9.suppl_5.S21
96. Franken M, Estabrooks A, Cavacini L, Sherburne B, Scadden DT. Epstein-Barr Virus-Driven Gene Therapy for EBV-Related Lymphomas. *Nat Med* (1997) 2(12):1379–82. doi: 10.1038/nm1296-1379
97. Li JH, Huang D, Sun BF, Zhang X, Liu FF. Efficacy of Ionizing Radiation Combined With Adenoviral P53 Therapy in EBV-Positive Nasopharyngeal Carcinoma. *Int J Cancer* (2015) 87(4):606–10. doi: 10.1002/1097-0215(20000815)87:4<606::AID-IJC23>3.0.CO;2-O
98. Li JH, Chia M, Shi W, Ngo D, Strathdee CA, Huang D, et al. Tumor-Targeted Gene Therapy for Nasopharyngeal Carcinoma. *Cancer Res* (2002) 62(1):171–8.

99. Arai A. Chronic Active Epstein–Barr Virus Infection: The Elucidation of the Pathophysiology and the Development of Therapeutic Methods. *Microorganisms* (2021) 9(1):180. doi: 10.3390/microorganisms9010180
100. Sawada A, Inoue M, Kawa K. How We Treat Chronic Active Epstein-Barr Virus Infection. *Int J Hematol* (2017) 105(4):406–18. doi: 10.1007/s12185-017-2192-6
101. Cohen J. Characterization and Treatment of Chronic Active Epstein-Barr Virus Disease: A 28-Year Experience in the United States. *Blood* (2011) 117 (22):5835–49. doi: 10.1182/blood-2010-11-316745
102. Takayuki O, Yoshikazu H, Arai H, Inoue M, Kawa K. Blood Stem-Cell Transplantation for Chronic Active Epstein-Barr Virus With Lymphoproliferation. *Lancet* (2000) 356(9225):223–4. doi: 10.1016/S0140-6736(00)02488-0
103. Kawa K, Sawada A, Sato M, Okamura T, Sakata N, Kondo O, et al. Excellent Outcome of Allogeneic Hematopoietic SCT With Reduced-Intensity Conditioning for the Treatment of Chronic Active EBV Infection. *Bone Marrow Transplantation* (2011) 46(1):77–83. doi: 10.1038/bmt.2010.122
104. Kimura H, Ito Y, Kawabe S, Gotoh K, Nakamura S. EBV-Associated T/NK-Cell Lymphoproliferative Diseases in Nonimmunocompromised Hosts: Prospective Analysis of 108 Cases. *Blood* (2011) 119(3):673–86. doi: 10.1182/blood-2011-10-381921
105. Arai A, Sakashita C, Hirose C, Imadome KI, Yamamoto M, Jinta M, et al. Hematopoietic Stem Cell Transplantation for Adults With EBV-Positive T- or NK-Cell Lymphoproliferative Disorders: Efficacy and Predictive Markers. *Bone Marrow Transplantation* (2016) 51(6):879–82. doi: 10.1038/bmt.2016.3
106. Wang L, Wang H, Li PF, Lu Y, Xia ZJ, Huang HQ, et al. CD38 Expression Predicts Poor Prognosis and Might be a Potential Therapy Target in Extranodal NK/T Cell Lymphoma, Nasal Type. *Ann Hematol* (2015) 94 (8):1381–8. doi: 10.1007/s00277-015-2359-2
107. Hari P, Raj R, Olteanu H. Targeting CD38 in Refractory Extranodal Natural Killer Cell-T-Cell Lymphoma. *New Engl J Med* (2016) 375(15):1501–2. doi: 10.1056/NEJMc1605684
108. Feng Y, Rao H, Lei Y, Huang Y, Fang W, Yu Z, et al. CD30 Expression in Extranodal Natural Killer/T-Cell Lymphoma, Nasal Type Among 622 Cases of Mature T-Cell and Natural Killer-Cell Lymphoma at a Single Institution in South China. *Chin J Cancer* (2017) 36(006):258–66. doi: 10.1186/s40880-017-0212-9
109. Reiss KA, Forde PM, Brahmer JR. Harnessing the Power of the Immune System *via* Blockade of PD-1 and PD-L1: A Promising New Anticancer Strategy. *Immunotherapy* (2014) 6(4):459–75. doi: 10.2217/imt.14.9
110. Onozawa E, Shibayama H, Takada H, Imadome KI, Aoki S, Yoshimori M, et al. STAT3 is Constitutively Activated in Chronic Active Epstein-Barr Virus Infection and can be a Therapeutic Target. *Oncotarget* (2018) 9 (57):31077–89. doi: 10.18632/oncotarget.25780
111. Krishnan C, Warnke RA, Arber DA, Natkunam Y. PD-1 Expression in T-Cell Lymphomas and Reactive Lymphoid Entities: Potential Overlap in Staining Patterns Between Lymphoma and Viral Lymphadenitis. *Am J Surg Pathol* (2010) 34(2):178. doi: 10.1097/PAS.0b013e3181cc7e79
112. Hui KF, Chiang A. Suberoylanilide Hydroxamic Acid Induces Viral Lytic Cycle in Epstein-Barr Virus-Positive Epithelial Malignancies and Mediates Enhanced Cell Death. *Int J Cancer* (2010) 126(10):2479–89. doi: 10.1002/ijc.24945
113. Hui KF, Ho DN, Tsang CM, Middeldorp JM, Tsao G, Chiang A. Activation of Lytic Cycle of Epstein-Barr Virus by Suberoylanilide Hydroxamic Acid Leads to Apoptosis and Tumor Growth Suppression of Nasopharyngeal Carcinoma. *Int J Cancer* (2012) 131(8):1930–40. doi: 10.1002/ijc.27439
114. Kawanishi M. Epstein-Barr Virus Induces Fragmentation of Chromosomal DNA During Lytic Infection. *J Virol* (1993) 67(12):7654–8. doi: 10.1128/jvi.67.12.7654-7658.1993
115. Harrison SJ, Bishton M, Bates SE, Grant S, Piekarz RL, Johnstone RW, et al. A Focus on the Preclinical Development and Clinical Status of the Histone Deacetylase Inhibitor, Romidepsin (Depsipeptide, Istodax()). *Epigenomics* (2012) 4(5):571–89. doi: 10.2217/epi.12.52
116. Hui FK, Chiang SAK, Cheung LAK, Choi CK, Yeung PL, Middeldorp JM. Inhibition of Class I Histone Deacetylases by Romidepsin Potently Induces Epstein-Barr Virus Lytic Cycle and Mediates Enhanced Cell Death With Ganciclovir. *Int J Cancer* (2016) 138(1):125–36. doi: 10.1002/ijc.29698
117. Kim SJ, Kim JH, Ki CS, Ko YH, Kim JS, Kim WS. Epstein-Barr Virus Reactivation in Extranodal Natural Killer/T-Cell Lymphoma Patients: A Previously Unrecognized Serious Adverse Event in a Pilot Study With Romidepsin. *Ann Oncol Off J Eur Soc Med Oncol* (2016) 27(3):508–13. doi: 10.1093/annonc/mdv596
118. Kim JH, Kim WS, Park C. Sildenafil Prevents HDACi-Induced Epstein-Barr Virus Reactivation Through the PKG Pathway in NK/T Cell Lymphoma; Potential Implications for HDACi-Mediated Fatal Complications. *Antiviral Res* (2021) 189:105063. doi: 10.1016/j.antiviral.2021.105063
119. Kane RC. United States Food and Drug Administration Approval Summary: Bortezomib for the Treatment of Progressive Multiple Myeloma After One Prior Therapy. *Clin Cancer Res* (2006) 12(10):2955. doi: 10.1158/1078-0432.CCR-06-0170
120. Shirley CM, Chen J, Shamay M, Li H, Ambinder RF. Bortezomib Induction of C/Ebpβ Mediates Epstein-Barr Virus Lytic Activation in Burkitt Lymphoma. *Blood* (2011) 117(23):6297–303. doi: 10.1182/blood-2011-01-332379
121. Fu DX, Tanhehco Y, Chen J, Foss CA, Fox JJ, Chong JM, et al. Bortezomib-Induced Enzyme-Targeted Radiation Therapy in Herpesvirus-Associated Tumors. *Nat Med* (2008) 14(10):1118. doi: 10.1038/nm.1864
122. Granato M, Romeo MA, Tiano MS, Santarelli R, Gonnella R, Gilardini Montani MS, et al. Bortezomib Promotes KHSV and EBV Lytic Cycle by Activating JNK and Autophagy. *Sci Rep* (2017) 7(1):13052. doi: 10.1038/s41598-017-13533-7
123. Vaysberg M, Hatton O, Lambert SL, Snow AL, Wong B, Krams SM, et al. Tumor-Derived Variants of Epstein-Barr Virus Latent Membrane Protein 1 Induce Sustained Erk Activation and C-Fos. *J Biol Chem* (2008) 283 (52):36573–85. doi: 10.1074/jbc.M802968200
124. Morris MA, Dawson CW, Young LS. Role of the Epstein-Barr Virus-Encoded Latent Membrane Protein-1, LMP1, in the Pathogenesis of Nasopharyngeal Carcinoma. *Future Oncol* (2009) 5(6):811–25. doi: 10.2217/fon.09.53
125. Adams J. Proteasome Inhibition: A Novel Approach to Cancer Therapy. *Trends Mol Med* (2002) 8(4 Suppl):S49–54. doi: 10.1016/S1471-4914(02)02315-8
126. Zhang XM, Lin H, Chen C, Chen DM. Inhibition of Ubiquitin-Proteasome Pathway Activates a Caspase-3-Like Protease and Induces Bcl-2 Cleavage in Human M-07e Leukaemic Cells. *Biochem J* (1999) 340(Pt 1):127–33. doi: 10.1042/bj3400127
127. Huynh M, Pak C, Markovina S, Callander NS, Chng KS, Wuerzberger-Davis SM, et al. Hyaluronan and Proteoglycan Link Protein 1 (HAPLN1) Activates Bortezomib-Resistant NF-Kappa B Activity and Increases Drug Resistance in Multiple Myeloma. *J Biol Chem* (2018) 293(7):2452–65. doi: 10.1074/jbc.RA117.000667
128. Fu DX, Tanhehco YC, Chen J, Foss CA, Fox JJ, Lemas V, et al. Virus-Associated Tumor Imaging by Induction of Viral Gene Expression. *Clin Cancer Res* (2007) 13(5):1453–8. doi: 10.1158/1078-0432.CCR-06-2295
129. Shen L, Au W, Guo T, Wong K, Wong M, Tsuchiyama J, et al. Proteasome Inhibitor Bortezomib-Induced Apoptosis in Natural Killer (NK)-Cell Leukemia and Lymphoma: An In Vitro and *In Vivo* Preclinical Evaluation. *Blood* (2007) 110(1):469–70. doi: 10.1182/blood-2007-02-072900
130. Tai YT, Landesman Y, Acharya C, Calle Y, Zhong MY, Cea M, et al. CRM1 Inhibition Induces Tumor Cell Cytotoxicity and Impairs Osteoclastogenesis in Multiple Myeloma: Molecular Mechanisms and Therapeutic Implications. *Leukemia* (2014) 28(1):155–65. doi: 10.1038/leu.2013.115
131. Bertucci F, Finetti P, Birnbaum D. XPO1, Therapeutic And Prognostic Target in Sarcomas. *Oncoscience* (2016) 3(5-6):143–4. doi: 10.18632/oncoscience.304
132. Liu X, Chong Y, Tu Y, Liu N, Yue C, Qi Z, et al. CRM1/XPO1 is Associated With Clinical Outcome in Glioma and Represents a Therapeutic Target by Perturbing Multiple Core Pathways. *J Hematol Oncol* (2016) 9(1):108. doi: 10.1186/s13045-016-0338-2
133. Boyle SM, Ruvolo V, Gupta AK, Swaminathan S. Association With the Cellular Export Receptor CRM 1 Mediates Function and Intracellular Localization of Epstein-Barr Virus SM Protein, a Regulator of Gene Expression. *J Virol* (1999) 73(8):6872–81. doi: 10.1128/jvi.73.8.6872-6881.1999

134. Sin-Yeang T, Che NA, Ali SA, Soo-Beng KA. Exosomes in Human Immunodeficiency Virus Type I Pathogenesis: Threat or Opportunity? *Adv Virol* (2016) 2016:9852494. doi: 10.1155/2016/9852494
135. Milane L, Singh A, Mattheolabakis G, Suresh M, Amiji MM. Exosome Mediated Communication Within the Tumor Microenvironment. *J Controlled Release* (2015) 219:278–94. doi: 10.1016/j.jconrel.2015.06.029
136. Keryer-Bibens C, Pioche-Durieu C, Villemant C, Souquère S, Nishi N, Hirashima M, et al. Exosomes Released by EBV-Infected Nasopharyngeal Carcinoma Cells Convey the Viral Latent Membrane Protein 1 and the Immunomodulatory Protein Galectin 9. *BMC Cancer* (2006) 6(1):283. 6, 1 (2006-12-08). doi: 10.1186/1471-2407-6-283
137. Meckes DG. Exosomal Communication Goes Viral. *J Virol* (2015) 89 (10):5200–3. doi: 10.1128/JVI.02470-14
138. Aga M, Bentz G, Raffa L, Torrisi MR, Kondo S, Wakisaka N, et al. Exosomal Hif1α Supports Invasive Potential of Nasopharyngeal Carcinoma-Associated LMP1-Positive Exosomes. *Oncogene* (2014) 33(37):4613–22. doi: 10.1038/onc.2014.66
139. Mrizak D, Martin N, Barjon C, Jimenez-Pailhes AS, Mustapha R, Niki T, et al. Effect of Nasopharyngeal Carcinoma-Derived Exosomes on Human Regulatory T Cells. *JNCI J Natl Cancer Institute* (2015) 107(1):dju363–dju. doi: 10.1093/jnci/dju363

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UNC13B Promote Arsenic Trioxide Resistance in Chronic Lymphoid Leukemia Through Mitochondria Quality Control

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In clinical practice, arsenic trioxide can be used to treat a subset of R/R CML patients, but resistance tends to reappear quickly. We designed an experiment to study arsenic trioxide resistance in K-562 cells. Previously, we identified the *UNC13B* gene as potentially responsible for arsenic trioxide resistance in K-562 cells *via* gene chip screening followed by high-content screening. We aimed to investigate the role and mechanism of the *UNC13B* gene in K-562 cells, an arsenic trioxide-resistant chronic myeloid leukemia cell line. *In vitro* lentiviral vector-mediated *UNC13B* siRNA transfection was performed on K-562 cells. The roles of *UNC13B* in cell proliferation, apoptosis and cell cycle pathways, and colony formation were analyzed by CCK-8 assay, fluorescence-activated cell sorting, and soft agar culture, respectively. Gene chip screening was used to define the possible downstream pathways of *UNC13B*. Western blot was performed to further validate the possible genes mediated by *UNC13B* for arsenic trioxide resistance in patients with chronic myeloid leukemia. *UNC13B* downregulation significantly inhibited growth, promoted apoptosis, decreased colony formation, reduced the duration of the G1 phase, and increased the duration of the S phase of K-562 cells. Western blot results confirmed that *UNC13B* may modulate the apoptosis and proliferation of arsenic trioxide-resistant chronic myeloid leukemia cells through the mediation of MAP3K7, CDK4, and PINK1. *UNC13B* is a potential therapeutic target for patients with arsenic trioxide-resistant chronic myeloid leukemia.

Keywords: arsenic trioxide, drug resistance, *UNC13B*, mitochondria, chronic myeloid leukemia

INTRODUCTION

Chronic myeloid leukemia (CML) is a malignant tumor resulting from a clonal proliferation of bone marrow hematopoietic stem cells, accounting for 15% of adult leukemias (1). According to clinical trials, an estimated 20–30% of patients fail to achieve an expected therapeutic effect with the use of tyrosine kinase inhibitors (TKIs) or develop resistance to TKIs after preliminary efficacy is obtained

(1). Furthermore, most patients with CML have a high probability of developing blast crisis (2). New-generation TKIs have an increased potency in the treatment of CML; however, the use of these agents fails to prevent disease progression in some patients (3). TKI in combination with X regimen is an option for patients with CML; X drugs include interferon, cytarabine, homoharringtonine, and arsenic trioxide (ATO) (4).

ATO use has been approved for the treatment of patients with primary, relapsed, and refractory acute promyelocytic leukemia. Wang et al. reported that ATO inhibits the Wnt/β-catenin signaling pathway by downregulating the expression of CD44 on the surface of K-562 cells, which ultimately inhibits cell proliferation and prevents tumorigenesis (5). ATO resistance decreases treatment efficacy in patients with leukemia.

To explore the potential mechanism of ATO resistance, we previously developed an ATO-resistant CML cell line (K-562) cells; moreover, we identified that *UNC13B* was highly expressed in such cells *via* gene chip screening. We hypothesized that this gene may be related to ATO resistance in K-562 cells.

MATERIALS AND METHODS

Target Gene Screening

Previously, we identified ATO-resistant K-562 cells (ATCC, Virginia, USA) by culturing K-562 cells with varied concentrations of ATO. The surviving cells were passaged and repeatedly treated with ATO until a stable ATO-resistant cell line was achieved. To evaluate the effect of the target gene on ATO resistance, we isolated and analyzed ATO-resistant K-562 cell RNAs using GeneChip PrimeView Human Gene Expression Assay (Thermo Fisher Scientific, Waltham, MA). From hundreds of highly expressed genes, 20 genes were selected and downregulated for high-content screening *via* the CCK-8 assay. *UNC13B* was identified as a target gene for further analysis in the current study.

Cell Culture and Transfection

K-562 cells were cultured in an RPMI 1640 medium containing 15% fetal bovine serum at 37°C and an incubator atmosphere of 5% CO_2. Based on the nucleotide sequence of *UNC13B* in the GenBank database (GenBank: NM 020313.2), the ATO-resistant K-562 cells were transfected with *UNC13B* shRNA (shUNC13B) using a lentivirus vector. Cells were also transfected with non-target shRNA as control (shCtrl). Gene expression was screened 24 h after transfection.

Reverse Transcription-PCR

Reverse Transcription-PCR was used to analyze *UNC13B* expression levels of the transfected cells after *UNC13B* knockdown, following a previously reported procedure (6). The total RNA was extracted *via* the TRIzol method (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA. The amplification reaction was performed with an ABI 7500 system (Thermo Fisher Scientific). GAPDH was used as an internal reference. Some samples were processed according to the instruction of the 3' IVT Plus kit (Thermo Fisher Scientific) for the evaluation of the genes on a chip.

CCK-8 Assay

After various treatments, cells were cultured in a 96-well plate for 24, 48, and 72 h. At each time point, 10 μL of CCK-8 was added into the wells and cultured with the cells in the incubator for 4 h. Finally, the optical density at 490 nm was determined *via* a microplate reader (iMARK; Bio-Rad, Hercules, CA).

Flow Cytometry

Apoptosis and cell cycle analysis were performed by flow cytometry. For cell apoptosis evaluation, annexin-APC was used to stain the cells prior to the test according to the manufacturer's instruction (BD Biosciences, Franklin Lakes, NJ). Propidium iodide was used to stain the cells for cell cycle examination. The cells were analyzed in a BD FACSCelesta flow cytometer (BD Biosciences). Fluorescence-activated cell sorting was performed to analyze target cell ratios.

Colony Formation Assay

K-562 cells were collected and suspended with a density of 500 cells/mL using RPMI 1640 medium with 20% fetal bovine serum. We used 9 mL of suspended cells and 1 mL of 3% low-melting-point agarose solution to prepare a cell agarose suspension, which was seeded into a 6-well plate with 3 mL into each well in triplicate. The cells were placed in a refrigerator at 4°C for 10 min and transferred to a cell incubator for culture. After 18 days of culture, the cells were stained with a 0.05% crystal violet staining solution and then observed under a microscope.

Ingenuity Pathway Analysis

The RNA of the *UNC13B* knockdown ATO-resistant K-562 cells was tested using Ingenuity pathway analysis (IPA; Qiagen, Hilden, Germany) and the 3' IVT Plus kit (Thermo Fisher Scientific) for downstream gene screening. A gene interaction network was built with IPA, and genes related with the growth and apoptosis of tumor cells were identified; some of these genes were selected for Western blot analysis.

Western Blotting

The cells were collected and disrupted by sonication, total protein was extracted, protein concentrations were determined using a bicinchoninic acid quantitative kit (Beyotime Biotechnology, China), and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% non-fat dry milk at 4°C for 6 h. Mouse anti-human UNC13B monoclonal antibody (ab97664, abcam) was added to the membrane and incubated overnight at 4°C. Mouse or goat anti-human IgG secondary antibody was then incubated for 2 h. The contents of UNC13B, MAP3K7 (ab109526, abcam), CDK4

(#12790, CST), and PINK1 (ab23707, abcam) proteins on PVDF membranes were detected by chemiluminescence.

Statistical Analysis

Data are presented as mean ± standard deviation. Student's t-test was used to assess the differences between two groups. Statistical significance was established at $p < 0.05$.

RESULTS

UNC13B Was Positively Correlated With K-562 Cell Proliferation

UNC13B (hMUNC13, MUNC13-2, UNC13, Unc13h2) codes UNC-13 homolog B (UNC13B), was a target of diacylglycerol second messenger pathway. UNC13B is primarily located in vesicles, and promotes exocytosis by promoting vesicle maturation. Since our pervious results showed significantly elevated levels of UNC13B in ATO-tolerant cell lines, we first investigated the relationship between UNC13B and cell survival in this study. We transfected UNC13B shRNA (shUNC13B) with a lentivirus vector to analyze the association between UNC13B and cell proliferation. After shUNC13B transfection, *UNC13B* expression in the K-562 cells significantly decreased compared to that of the control (**Figures 1A–C**). UNC13B levels were further identified in clinical samples with ATO-resistant. The results showed that UNC13B levels were significantly increased in ATO-resistant sample compared to para-cancer tissues (**Figure 1D**). CCK-8 analysis showed that *UNC13B* knockdown had a greater inhibitory effect on the growth of ATO-resistant K-562 cells than that of control cells during the 5-day culture period (**Figure 2**).

UNC13B Downregulation Promotes Cell Apoptosis

Further, we analyzed the reasons for the increase in live cell number, verified the apoptosis, viability and cell cycle after transfection. Flow cytometry analysis indicated a significant increase in the apoptosis ratio of ATO-resistant K-562 cells after *UNC13B* knockdown. Furthermore, the colony formation assay showed that the size of the formed tumors in the shUNC13B group was much smaller than those in the control group (**Figure 3**). Moreover, flow cytometry analysis showed a decrease and an increase in the number of G1 and S phase cells, respectively, among the ATO-resistant K-562 cells, albeit no significant change in the number of G2/M phase cells (**Figure 4**). These results suggest that the down-regulation of UNC13B leads to apoptosis and affects cell viability.

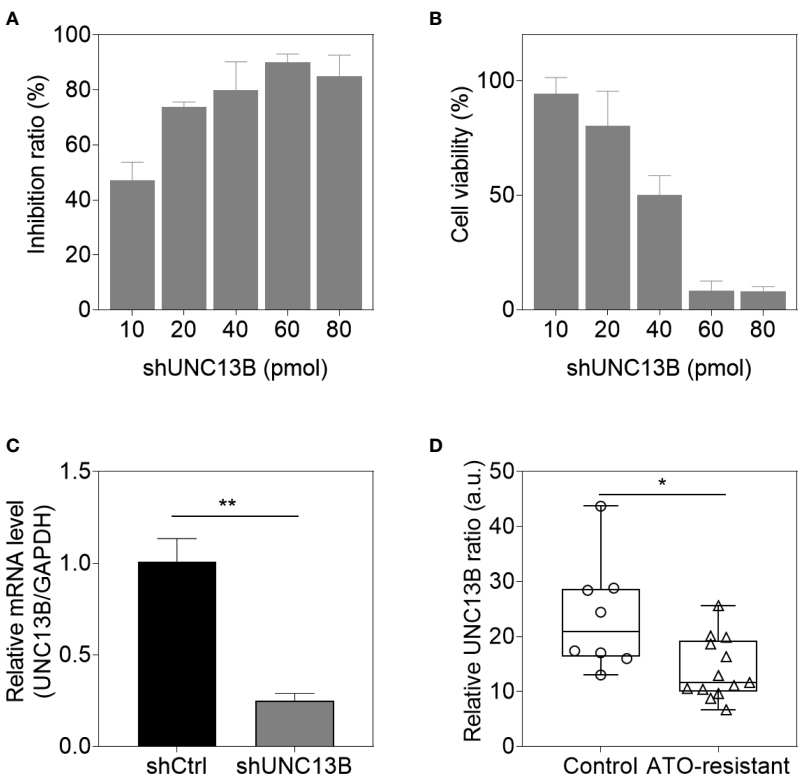

FIGURE 1 | *UNC13B* expression of arsenic trioxide (ATO)-resistant K-562 cells after *UNC13B* knockdown. Cell viability **(A)** and transfection efficiency **(B)** were identified 48 h after transfection in the range of 10 pmol to 80 pmol shUNC13B. **(C)** The cells were analyzed 3 days post-knockdown *via* quantitative PCR. **(D)** Transcriptional levels of UNC13B were identified in ATO-tolerance clinical samples (n=13), control group referred to paracancer tissues (n=8). *$p < 0.05$, **$p < 0.01$.

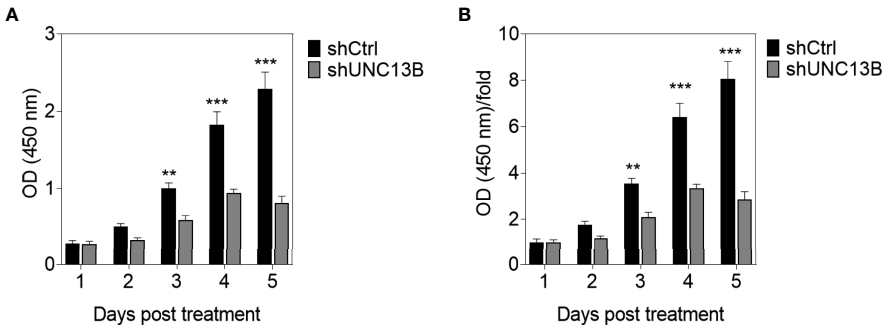

FIGURE 2 | CCK-8 evaluation of the influence of *UNC13B* knockdown on the proliferation of arsenic trioxide (ATO)-resistant K-562 cells. **(A)** Optical density (OD) value of the cells measured at 450 nm and tested *via* CCK-8 assay in different timepoints. **(B)** OD/fold of **(A)**. $^{**}p < 0.01$, $^{***}p < 0.001$.

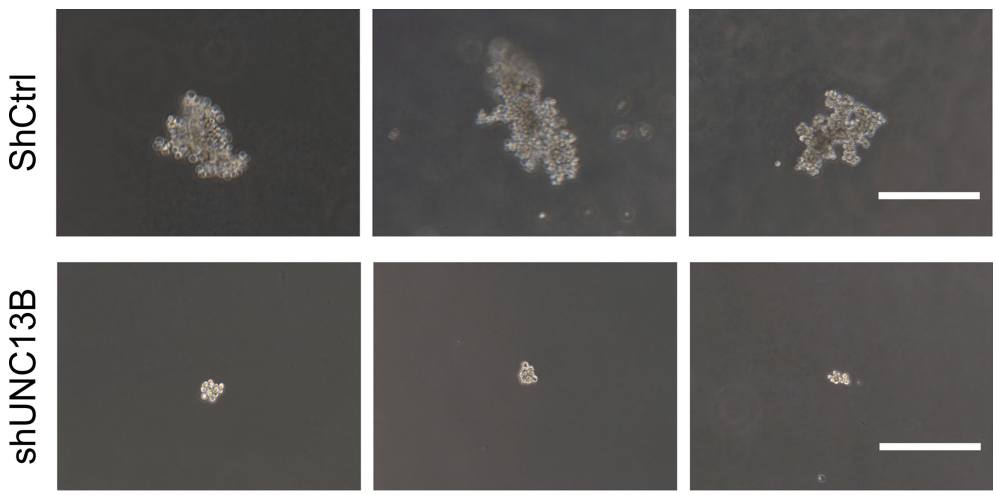

FIGURE 3 | Soft agarose colony formation of arsenic trioxide (ATO)-resistant K-562 cells after *UNC13B* knockdown. Three representative images of each group. Scale bar: 500 μm.

UNC13B Is Involved in Mitochondrial Fusion Regulation

We analyzed downstream genes involved in UNC13B, in the gene interaction network, approximately 20 genes showed reduced expression after *UNC13B* knockdown (**Figure 5**). IPA demonstrated that *UNC13B* may affect downstream genes and the proliferation and apoptosis of K-562 cells through an interaction with NRAS and SRSF1, genes with significant differences included PINK1 (mitochondrial fusion), PPPICB (chromosome structure, cell cycle),QKI (RNA binding protein), MAPKAPK2 (DNA damage), NFYA (DNA binding), MAP3K20 (cell cycle, signaling), CDK4 (cell cycle G1 phase), PML (apoptosis), VTI1A (protein transport) (**Figure 5**). According to the results of **Figure 4**, we excluded genes related to cell cycle, we further evaluated genes *via* western blot and found that *UNC13B* knockdown induced an obvious upregulation of MAP3K7, CDK4, and PINK1 proteins (**Figure 6**). Above results indicated that UNC13B regulated both apoptosis and mitochondrial fusion, and further promote cell tolerance to ATO.

DISCUSSION

Most patients with CML improve considerably with TKI treatment; however, CML cannot be cured using TKI (7), because TKIs cannot completely eliminate leukemia stem cells from the bone marrow, which is the root cause of relapse in patients with chronic-phase CML after remission. After more than 20 years of observing the effects of TKIs, it has been clinically found that TKIs do not have a good therapeutic effect on patients with CML in the accelerated phase or during blast crisis. Moreover, some patients respond slowly to TKI treatment,

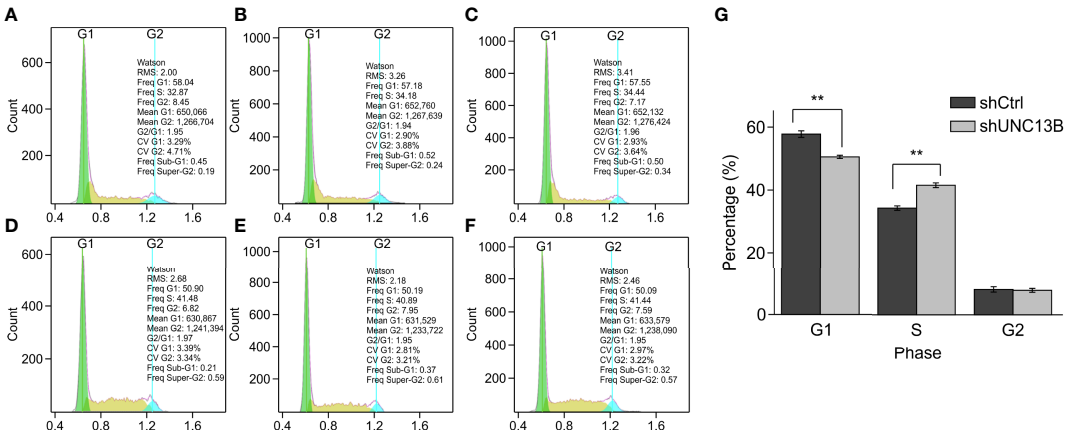

FIGURE 4 | Cell cycle analysis by fluorescence-activated cell sorting (FACS) of arsenic trioxide (ATO)-resistant K-562 cells after *UNC13B* knockdown. **(A–C)** FACS analysis of the shCtrl group. **(D–F)** FACS analysis of the shUNC13B group. **(G)** Quantification of the G1, S, and G2 phases of the shCtrl and shUNC13B groups. $^{**}p < 0.01$.

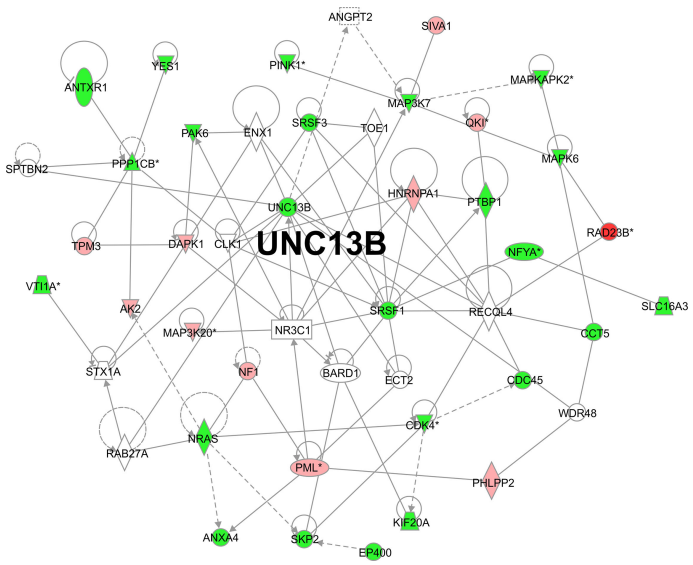

FIGURE 5 | Gene interaction network based on ingenuity pathway analysis. *p<0.05.

whereas others exhibit primary drug resistance. Studies have shown that the abovementioned phenomenon may result from tumor-related gene mutations (8), novel *BCR-ABL* transcripts (9), *BCR-ABL* fusion mutations (such as T315I) (10), and long-term medication history (11). Hematopoietic stem cell transplantation (HSCT) remains the only radical cure for CML; nonetheless, based on the relatively slow progress of CML and the relatively high risk of allograft HSCT (allo-HSCT), allo-HSCT is not recommended as the first choice for CML treatment. TKIs are recognized as an early CML treatment method; however, they can only prolong life rather than cure the disease. Once the patient undergoes a sudden change (for example, a T315I mutation), the optimal transplant period is missed, thereby resulting in an extremely poor prognosis.

By the 19th century, Fowler's solution, or ATO, was used in the treatment of CML. Before the advent of chemotherapeutics, ATO was the gold standard for CML treatment. A 2-week ATO treatment course can achieve complete hematological remission in 74% of patients with chronic-phase CML (12, 13). Due to its unique mechanism of action, ATO plays a unique role in CML treatment, even in the era of TKIs (14). ATO reportedly destroys CML cells by reducing *PML* expression and kills CML tumor stem cells (15). Therefore, ATO has excellent application prospects in the eradication of CML, thereby bringing new

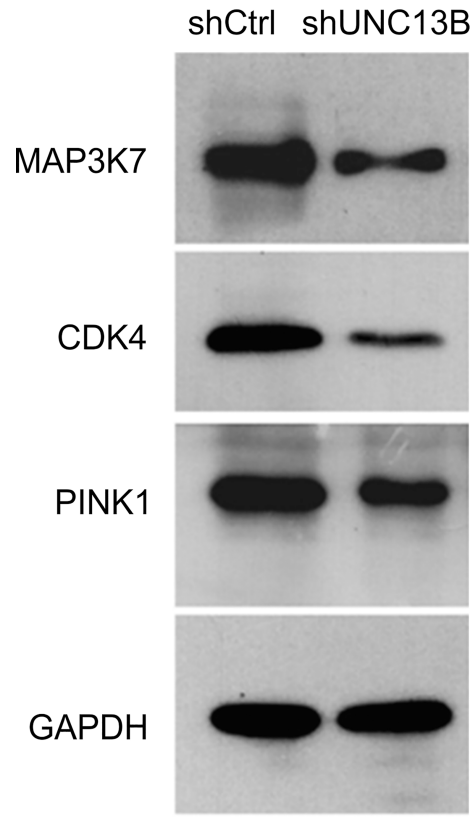

FIGURE 6 | Western blotting analysis of key proteins mediated by *UNC13B*.

treatment opportunities for patients with CML who could not benefit from HSCT. Additionally, two clinical studies have shown that 74% of patients who undergo ATO treatment for CML attain complete remission (12, 13).

ATO has been used in combination with other drugs in a variety of cancers, such as T-cell acute lymphoblastic leukemia (16), myelodysplastic syndromes (17), idiopathic thrombocytopenic purpura (18), acute myeloid leukemia (19), and multiple myeloma (20); moreover, it has been used in patients with both newly diagnosed and refractory or relapsed cancers. ATO shows a certain degree of curative effect, and so our group has been examining ATO resistance in leukemia. In the early stage of the current study, *UNC13B* expression was found to be increased in tumor cells of ATO-resistant patients (data not shown). Through *in vitro* experiments, it was found that *UNC13B* expression was significantly increased in ATO-resistant K-562 cells; hence, we speculate that *UNC13B* is an important gene for ATO resistance.

We found that the growth ability of CML K-562 cells was significantly inhibited after downregulation of *UNC13B*. To clarify the underlying mechanism, we investigated changes in relevant regulatory molecules in K-562 cells.

NRAS (neuroblastoma RAS viral oncogene homolog) belongs to the RAS guanosine triphosphatase gene family. Acting as a molecular switch, NRAS is located on the cell membrane surface and can transfer extracellular signals to the nucleus to regulate cell proliferation, differentiation, and apoptosis (21). Patients with acute myeloid leukemia and *NRAS* mutations have a low overall complete remission rate of approximately 10% (22).

SR proteins are responsible for regulating constitutive and alternative splicing in genes and may shuttle between the nucleus and cytoplasm. *SRSF1* (serine/arginine-rich splicing factor 1), a typical SR protein family member, is localized on chromosome 17q23 and contains an N-terminal RRM domain and a C-terminal RS domain. The RRM domain is responsible for mediating RNA binding and determining the substrate specificity of SR proteins. The RS domain is mainly involved in protein-protein interactions and regulates the formation of SRSF1-ESE complexes *via* phosphorylation and dephosphorylation of serine residues. ESE binding to SRSF1 can activate and inhibit splicing. SRSF1 regulates alternative splicing by recognizing and binding to ESEs (23). *SRSF1* is a key target of Myc, which directly or indirectly binds to the *SRSF1* promoter region and activates its transcription (24).

Furthermore, it has been found that dinaciclib, a CDK1/2 inhibitor, produces cytotoxicity by inhibiting the expression of *CHD1* and *MAP3K7* (a recently discovered protein), and prevents the proliferation of mouse prostate cells (25). CDK4 is one of eight cyclin-dependent kinases, all of which can bind to d-type cyclins after mitotic signal activation to form CDK4-cyclin complexes, thereby promoting reverse transcription and playing a role in the process of cell proliferation (26). In tumor cells, CDK4/6 hyperactivation results in genomic and chromosomal instability with uncontrolled cell proliferation, which ultimately leads to abnormal cell cycle regulation (27). PINK1 promotes tumor survival, protects cancer cells from different cytotoxic agents (28), and exerts its biological function through oxidative stress (29). In the present study, we found that the downregulation of *UNC13B* could in turn downregulate MAP3K7, CDK4, and PINK1 in K-562 cells; hence, *UNC13B* might directly or indirectly promote the biological activity of ATO-resistant CML cells by affecting the expression of the abovementioned proteins.

PTEN induced kinase 1(PINK1) is mainly located in mitochondria, and its function is to inhibit mitochondrial dysfunction (30). Mitochondria are the site of oxidative metabolism in eukaryotes, where sugars, fats and amino acids eventually oxidize and release energy. At the same time, mitochondria can also store calcium ions and act synergistically with structures such as endoplasmic reticulum and extracellular matrix to control the dynamic balance of calcium ion concentration in cells.

Previous reports have shown that PINK1 is essential for mitochondrial quality control (30). PINK1 inhibition leads to mitochondrial excessive fusion, and further improve the efficiency of oxidative phosphorylation (31). In our results, the expression level of PINK1 was also significantly decreased with the inhibition of UNC13B. This suggests that the quality control of mitochondria will be significantly affected during UNC13B down-regulation, and the accompanying metabolic disorders will further affect the cell energy metabolism.

Due to insufficient experimental funds, this experiment did not conduct gene chip experiments, nor did it detect the entire series of proteins in major signaling pathways.

We found that *UNC13B* downregulation may in turn downregulate the contents of MAP3K7, CDK4, and PINK1 in K-652 cells. Moreover, *UNC13B* may directly or indirectly promote the biological activities of ATO-resistant CML cells by affecting the abovementioned proteins, the underlying mechanism may involve mitochondrial quality control. These findings provide potential benefits for the treatment of patients with CML. Furthermore, our study may provide a treatment strategy for the clinical treatment of non-ATO-resistant CML. Although the findings of this study are very promising, they should be verified *via* further studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

X-BW performed the experiments, prepared the figures and wrote the manuscript. BL, Y-BY, and L-HY collected and analyzed data. L-PY and XX initiated and directed the whole research. X-BW, L-HY and L-PY contributed to the manuscript equally. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. O'Brien S, Berman E, Borghaei H, Deangelo DJ, Devetten MP, Devine S, et al. NCCN Clinical Practice Guidelines in Oncology: Chronic Myelogenous Leukemia. *J Natl Compr Cancer Network JNCCN.* (2009) 7(9):984–1023. doi: 10.6004/jnccn.2009.0065
2. Melo JV, Barnes DJ. Chronic Myeloid Leukaemia as a Model of Disease Evolution in Human Cancer. *Nat Rev Cancer* (2007) 7(6):441–53. doi: 10.1038/nrc2147
3. Kantarjian H, Shah NP, Hochhaus A, Cortes J, Shah S, Ayala M, et al. Dasatinib Versus Imatinib in Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia. *New Engl J Med* (2010) 362(24):2260–70. doi: 10.1056/NEJMoa1002315
4. Wang W, Lv FF, Du Y, Li N, Chen Y, Chen L. The Effect of Nilotinib Plus Arsenic Trioxide on the Proliferation and Differentiation of Primary Leukemic Cells From Patients With Chronic Myoloid Leukemia in Blast Crisis. *Cancer Cell Int* (2015) 15:10. doi: 10.1186/s12935-015-0158-4
5. Wang Y, Yang J, Li J, Wang RC, Yuan J, Li Y, et al. Effect of Arsenic Trioxide on K562 Cell Proliferation and Its Mechanism. *Zhongguo shi yan xue ye xue za zhi.* (2017) 25(1):90–3. doi: 10.7534/j.issn.1009-2137.2017.01.015
6. Ye Y, Xu X, Zhang M, Qiu D, Bai X, Wang J, et al. Low-Dose Arsenic Trioxide Combined With Aclacinomycin A Synergistically Enhances the Cytotoxic Effect on Human Acute Myelogenous Leukemia Cell Lines by Induction of Apoptosis. *Leuk Lymphoma* (2015) 56(11):3159–67. doi: 10.3109/10428194.2015.1011155
7. Bower H, Bjorkholm M, Dickman PW, Hoglund M, Lambert PC, Andersson TM. Life Expectancy of Patients With Chronic Myeloid Leukemia Approaches the Life Expectancy of the General Population. *J Clin* (2016) 34(24):2851–7. doi: 10.1200/JCO.2015.66.2866
8. Mustjoki S. Non-Ph Variants in CML: Guilty Drivers? *Blood* (2018) 132(9):880–1. doi: 10.1182/blood-2018-07-862698
9. Giustacchini A, Thongjuea S, Barkas N, Woll PS, Povinelli BJ, Booth CAG, et al. Single-Cell Transcriptomics Uncovers Distinct Molecular Signatures of Stem Cells in Chronic Myeloid Leukemia. *Nat Med* (2017) 23(6):692–702. doi: 10.1038/nm.4336
10. Strand V, Goncalves J, Hickling TP, Jones HE, Marshall L, Isaacs JD. Immunogenicity of Biosimilars for Rheumatic Diseases, Plaque Psoriasis, and Inflammatory Bowel Disease: A Review From Clinical Trials and Regulatory Documents. *BioDrugs Clin Immunother Biopharm Gene Ther* (2020) 34(1):27–37. doi: 10.1007/s40259-019-00394-x
11. Yu G, Chen F, Yin C, Liu Q, Sun J, Xuan L, et al. Upfront Treatment With the First and Second-Generation Tyrosine Kinase Inhibitors in Ph-Positive Acute Lymphoblastic Leukemia. *Oncotarget* (2017) 8(63):107022–32. doi: 10.18632/oncotarget.22206
12. Hoonjan M, Jadhav V, Bhatt P. Arsenic Trioxide: Insights Into its Evolution to an Anticancer Agent. *J Biol inorganic Chem JBIC Publ Soc Biol Inorganic Chem* (2018) 23(3):313–29. doi: 10.1007/s00775-018-1537-9
13. Chen S, Song Z, Zhang A. Small-Molecule Immuno-Oncology Therapy: Advances, Challenges and New Directions. *Curr topics med Chem* (2019) 19(3):180–5. doi: 10.2174/1568026619666190308131805
14. Ji K, Mayernik L, Moin K, Sloane BF. Acidosis and Proteolysis in the Tumor Microenvironment. *Cancer Metastasis Rev* (2019) 38(1-2):103–12. doi: 10.1007/s10555-019-09796-3
15. Baccarani M, Abruzzese E, Accurso V, Albano F, Annunziata M, Barulli S, et al. Managing Chronic Myeloid Leukemia for Treatment-Free Remission: A Proposal From the GIMEMA CML WP. *Blood Advances* (2019) 3(24):4280–90. doi: 10.1182/bloodadvances.2019000865
16. Kouhpaikar H, Sadeghian MH, Rafatpanah H, Kazemi M, Iranshahi M, Delbari Z, et al. Synergy Between Parthenolide and Arsenic Trioxide in Adult T-Cell Leukemia/Lymphoma Cells *In Vitro*. *Iranian J Basic Med Sci* (2020) 23(5):616–22. doi: 10.22038/ijbms.2020.40650.9610
17. Valenzuela M, Glorieux C, Stockis J, Sid B, Sandoval JM, Felipe KB, et al. Retinoic Acid Synergizes ATO-Mediated Cytotoxicity by Precluding Nrf2 Activity in AML Cells. *Br J Cancer* (2014) 111(5):874–82. doi: 10.1038/bjc.2014.380
18. Huang L, Liu Z, Jiang H, Li L, Fu R. Decitabine Shows Synergistic Effects With Arsenic Trioxide Against Myelodysplastic Syndrome Cells *via* Endoplasmic Reticulum Stress-Related Apoptosis. *J Invest Med* (2019) 67(7):1067–75. doi: 10.1136/jim-2018-000953
19. Huang QS, Liu Y, Wang JB, Peng J, Hou M, Liu H, et al. All-Trans Retinoic Acid Plus High-Dose Dexamethasone as First-Line Treatment for Patients With Newly Diagnosed Immune Thrombocytopenia: A Multicentre, Open-Label, Randomised, Controlled, Phase 2 Trial. *Lancet Haematol* (2021) 8(10):e688–e99. doi: 10.1016/S2352-3026(21)00240-4
20. Cholujova D, Bujnakova Z, Dutkova E, Hideshima T, Groen RW, Mitsiades CS, et al. Realgar Nanoparticles Versus ATO Arsenic Compounds Induce *In Vitro* and *In Vivo* Activity Against Multiple Myeloma. *Br J Haematol* (2017) 179(5):756–71. doi: 10.1111/bjh.14974
21. Cuiffo B, Ren R. Palmitoylation of Oncogenic NRAS Is Essential for Leukemogenesis. *Blood* (2010) 115(17):3598–605. doi: 10.1182/blood-2009-03-213876

22. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS Mutations in AML: A Study of 2502 Patients. *Blood* (2006) 107(10):3847–53. doi: 10.1182/blood-2005-08-3522
23. Caceres JF, Screaton GR, Krainer AR. A Specific Subset of SR Proteins Shuttles Continuously Between the Nucleus and the Cytoplasm. *Genes Dev* (1998) 12 (1):55–66. doi: 10.1101/gad.12.1.55
24. Mao DY, Watson JD, Yan PS, Barsyte-Lovejoy D, Khosravi F, Wong WW, et al. Analysis of Myc Bound Loci Identified by CpG Island Arrays Shows That Max is Essential for Myc-Dependent Repression. *Curr Biol* (2003) 13(10):882–6. doi: 10.1016/s0960-9822(03)00297-5
25. Washino S, Rider LC, Romero L, Jillson LK, Affandi T, Ohm AM, et al. Loss of MAP3K7 Sensitizes Prostate Cancer Cells to CDK1/2 Inhibition and DNA Damage by Disrupting Homologous Recombination. *Mol Cancer Res MCR.* (2019) 17(10):1985–98. doi: 10.1158/1541-7786.MCR-18-1335
26. Hamilton E, Infante JR. Targeting CDK4/6 in Patients With Cancer. *Cancer Treat Rev* (2016) 45:129–38. doi: 10.1016/j.ctrv.2016.03.002
27. Liu M, Liu H, Chen J. Mechanisms of the CDK4/6 Inhibitor Palbociclib (PD 0332991) and its Future Application in Cancer Treatment (Review). *Oncol Rep* (2018) 39(3):901–11. doi: 10.3892/or.2018.6221
28. Lazarou M, Sliter DA, Kane LA, Sarraf SA, Wang C, Burman JL, et al. The Ubiquitin Kinase PINK1 Recruits Autophagy Receptors to Induce Mitophagy. *Nature* (2015) 524(7565):309–14. doi: 10.1038/nature14893
29. Pridgeon JW, Olzmann JA, Chin LS, Li L. PINK1 Protects Against Oxidative Stress by Phosphorylating Mitochondrial Chaperone TRAP1. *PloS Biol* (2007) 5(7):e172. doi: 10.1371/journal.pbio.0050172
30. Yang Y, Ouyang Y, Yang L, Beal MF, McQuibban A, Vogel H, et al. Pink1 Regulates Mitochondrial Dynamics Through Interaction With the Fission/ Fusion Machinery. *Proc Natl Acad Sci USA* (2008) 105(19):7070–5. doi: 10.1073/pnas.0711845105
31. Chen Y, Dorn GW2nd. PINK1-Phosphorylated Mitofusin 2 is a Parkin Receptor for Culling Damaged Mitochondria. *Science* (2013) 340 (6131):471–5. doi: 10.1126/science.1231031

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REVIEW
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Deciphering Metabolic Adaptability of Leukemic Stem Cells

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Therapeutic targeting of leukemic stem cells is widely studied to control leukemia. An emerging approach gaining popularity is altering metabolism as a potential therapeutic opportunity. Studies have been carried out on hematopoietic and leukemic stem cells to identify vulnerable pathways without impacting the non-transformed, healthy counterparts. While many metabolic studies have been conducted using stem cells, most have been carried out *in vitro* or on a larger population of progenitor cells due to challenges imposed by the low frequency of stem cells found *in vivo*. This creates artifacts in the studies carried out, making it difficult to interpret and correlate the findings to stem cells directly. This review discusses the metabolic difference seen between hematopoietic stem cells and leukemic stem cells across different leukemic models. Moreover, we also shed light on the advancements of metabolic techniques and current limitations and areas for additional research of the field to study stem cell metabolism.

Keywords: hematopoietic stem cells, metabolism, metabolic techniques, leukemic stem cells (LSCs), leukemia

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INTRODUCTION

Cancer cells metabolically reprogram to thrive in a stressed environment, lead to disease progression and even drug resistance. This is considered as metabolic adaptability of cancer cells. They can be metabolically flexible (ability to use different substrates as a source of energy) and/or metabolically plastic (ability to process a substrate through different pathways). Although metabolic adaptability is beneficial for the cancer cells, it can also serve as a limiting factor for their survival and hence making them vulnerable to inhibitors. This review will focus on such metabolic adaptations seen in leukemic stem cells compared to healthy counter parts as well as advancement and limitation in the field (1).

Like hematopoietic stem cells (HSCs), leukemic stem cells (LSCs) are quiescent, have self-renewal potential and the ability to engraft. Additionally, LSCs are also defined by their leukemogenic potential. To eradicate LSCs, researchers are exploring the field of metabolism to find metabolic differences between LSCs and their healthy counterparts. In leukemia, transformed cells acquire metabolic changes, which could be oncogene driven in addition to environmental or stress cues to aid survival. Not only do LSCs in the bone marrow metabolize differently from non-transformed cells, but they also have a different metabolic profile from the bulk of the disease. Furthermore, LSCs are a heterogeneous population and, in some cases, rare cells, posing a limitation to study. However, with an increase in the number of studies related to metabolism, new techniques

have been developed to overcome the shortcomings of older methods. In this review, we focus on the metabolic difference between hematopoietic and leukemic stem cells, the new techniques developed and the challenges in studying and metabolically targeting leukemic stem cells.

METABOLISM OF HEMATOPOIETIC STEM CELLS

HSCs are found in a hypoxic environment of the bone marrow (2). HIF-1α (Hypoxia Inducible Factor) is a hypoxic sensor stabilized under low oxygen concentration (3). Among other genes, HIF-1α induces expression of lactate dehydrogenase (LDH) and pyruvate dehydrogenase kinase (PDK) gene family, which are essential regulators of anerobic glycolysis (4). Inhibition or deletion of HIF-1α, PDK2/4, and LDHA leads to increased proliferation and exhaustion of the HSCs (4–6). However, inhibition of pyruvate kinase (PKM2), another glycolysis rate-limiting enzyme, has no impact on the regular function of HSCs unless they are stressed by serial transplant (4). On top of glucose metabolism, fatty acid desaturation (FADS) and oxidation (FAO) are also essential for the maintenance, proliferation, and differentiation of HSCs (7–9). An active FAO leads to asymmetric cell division of HSCs giving rise to downstream progenitors, while inhibition of FAO leads to symmetric cell division, promoting self-renewal (8).

Owing to the glycolytic nature of HSCs, compared to the hematopoietic progenitors and mature hematopoietic cells, they have reduced mitochondrial respiratory capacity, turnover rate, and mitochondrial activity but a high mitochondrial mass (10, 11). Inhibition of mitochondrial carrier homolog 2 (MTCH2), a regulator of mitochondrial activity, leads to an increase in mitochondrial size, ATP production, and reactive oxygen species (ROS), leading to a shift from glycolysis to oxidative phosphorylation (OxPhos) that triggers the entry of HSCs into cell cycle (12). This subsequently causes a functional decline of HSCs, marked by the accumulation of dysfunctional mitochondria due to asymmetric cell division and loss of Dynamin-related protein 1 (Drp1) function, a key regulator of mitochondrial fission (13). On the contrary, mitochondrial fusion protein, mitofusin2 contributes to maintaining lymphoid potential in HSCs (14). Moreover, inhibiting fumarate hydratase, a critical enzyme of the citric acid cycle (CAC), reduces the long-term repopulating potential of HSCs (15). On the contrary, inhibiting mitochondrial phosphatase, Ptpmt1, impairs HSC differentiation by activating 5' Adenosine Monophosphate-activated Protein Kinase (AMPK), aiding in the maintenance of self-renewal potential (16, 17). Individual metabolites also play an important role in maintaining of HSCs (18); however, significantly more work needs to be done to understand their role in HSC function.

Most of the data collected above were done with young HSCs, however, opposed to young HSCs, aged HSCs have increased autophagy, dysfunctional chaperone-mediated autophagy, metabolic activity, myeloid bias with reduced engraftment, and hematopoiesis potential, as well as accumulation of dysfunctional mitochondria (9, 13, 19). However, increasing the mitochondrial membrane potential using mitochondrially targeted coenzyme-Q10 (Mitoquinol, MitoQ) or pharmacological activation of chaperon mediating autophagy reverses the aging HSC phenotype (9, 20). This indicates that the right balance of

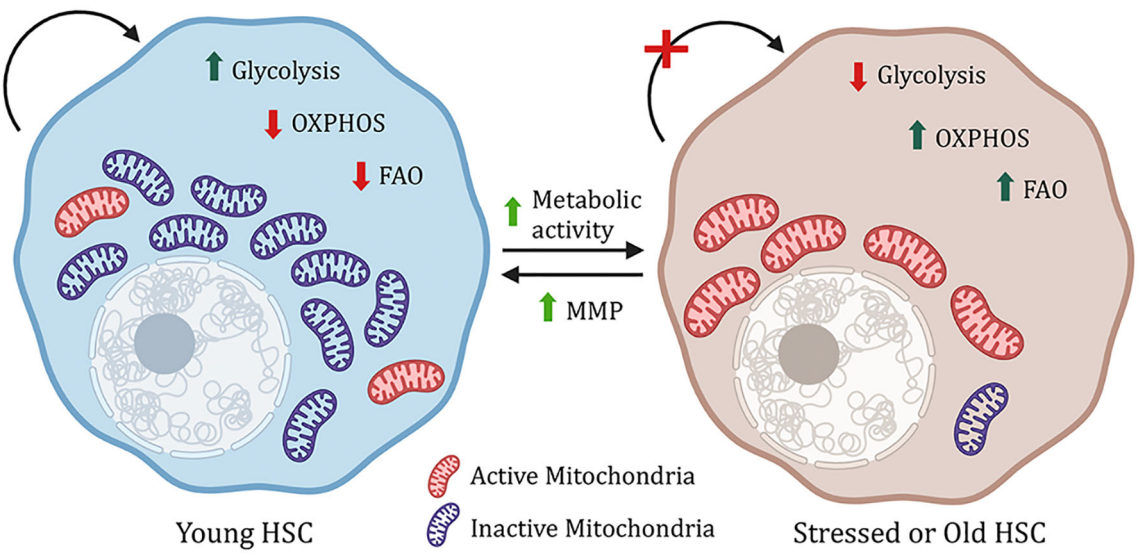

FIGURE 1 | Metabolic regulation of HSCs. Young hematopoitic stem cells have a higher mitochondrial mass, but the mitochondria are inactive, and the cells rely on glycolysis. However, under stress, increase in metabolic activity, OXPHOS (oxidative phosphorylation) and FAO (fatty acid oxidation) lead to impaired stem cell function. This phenomenon can be reversed by increasing mitochondrial membrane potential (MMP).

anerobic glycolysis and OxPhos is essential for maintaining healthy HSCs (**Figure 1**).

METABOLISM IN MYELOID LEUKEMIA

Acute Myeloid Leukemia (AML)

AML is mostly a clonal disorder commonly seen in the aged population and comprises 62% of all leukemia-related deaths (21). It is genetically heterogeneous and complex leukemia with multiple known mutations (NPM1, CEBPa, DNMT3A, Flt3-ITD, IDH, TET2, ASXL, SF3B1) and translocations (AML-ETO, MLL-AF9) (22). Patients usually have more than one of these cytogenetic abnormalities, and based on the combination, a patient's prognosis can be predicted (23, 24). AML LSCs are metabolically classified as cells with low levels of ROS. LSCs with less ROS are quiescent, have a self-renewal potential, and resist drug treatment (25). These cells are dependent on mitochondrial function as well as mitophagy for their survival. Inhibition of mitochondrial translation, mitochondrial chaperonin, CLPB or even mitophagy regulator, FIS1 (Mitochondrial Fission 1 protein) and its upstream target AMPK leads to loss of LSC self-renewal potential, myeloid differentiation, and cell cycle arrest and ultimately cell death even for the resistant AML LSCs (26–28).

Dependency of AML LSCs on mitochondrial function is associated with decreased levels of mitochondrial reactive oxygen species and concomitantly increased reliance on OxPhos rather than glycolysis as in normal HSCs to meet the cell's energy demand (25). Indeed, instead of relying on incomplete glucose oxidation through the Embden-Meyerhof-Parnas glycolytic pathway, AML LSCs depend on substrate oxidation – especially fatty acid and amino acid catabolism – in mitochondria. One potential reason could be increased expression of pyruvate dehydrogenase kinase (PDK) observed in AML patients, which inhibits glycolysis by phosphorylating and inactivating the enzyme pyruvate dehydrogenase (29, 30). Inhibiting amino acid metabolism and FAO – especially very-long-chain, polyunsaturated fatty acids - along with the traditional therapy eradicates the sensitive and resistant AML LSCs, respectively (31–36). More specifically, non-essential amino acid cysteine, which forms an integral part of glutathione synthase functionality as the rate-limiting substrate for glutathione biosynthesis, maintains the redox balance in AML and prevents oxidative stress (**Figure 2**). Inhibition of cysteine or depletion of glutathione impairs the activity of electron complex II, subsequently inhibiting OxPhos, eradicating the AML LSCs (37, 38). Glutamine is another amino acid that feeds into the CAC and regulates OxPhos. Inhibition of glutaminase, a critical enzyme for glutamine metabolism, has also been found to eliminate AML LSCs (39–41). Glutaminolysis also fuels the synthesis of glutathione – the main soluble antioxidant metabolite - by providing one of the rate-limiting substrates along with cysteine – glutamate (42–44). Regulation of glutathione synthesis by a mechanism of cysteine depletion or inhibition of glutaminolysis is in part controlled by sirtuin 5 (SIRT5) and is a targetable metabolic vulnerability in AML (38, 45). Glutathione synthesis and recycling are also

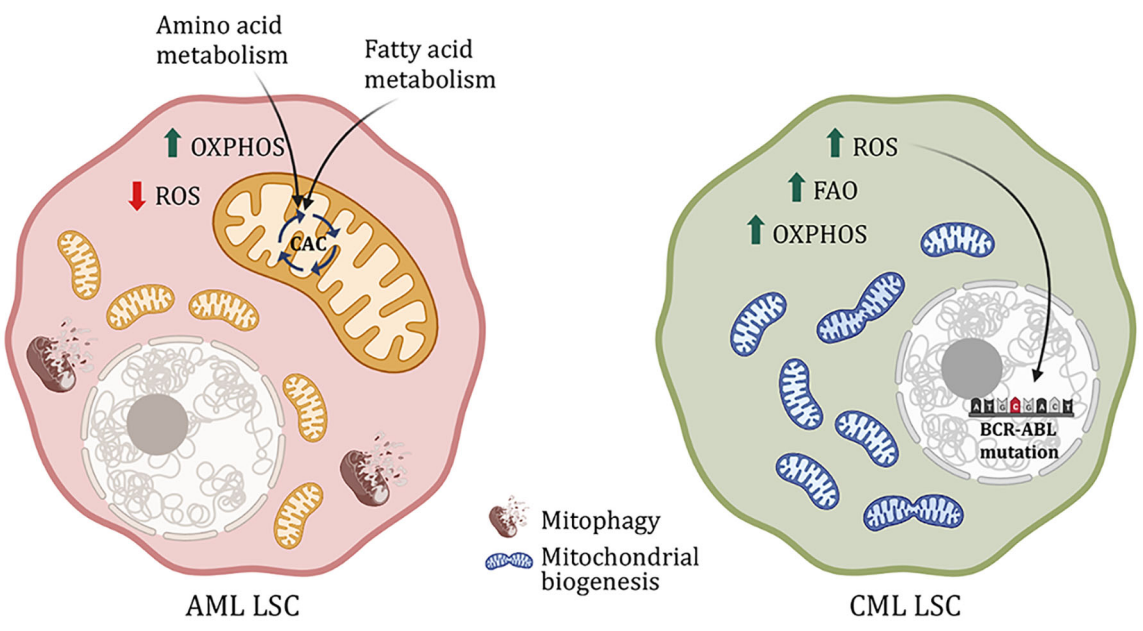

FIGURE 2 | Metabolic difference between AML and CML LSCs. AML LSCs are reliant on OxPhos and yet have low ROS level controlled by mitophagy. Moreover, AML LSCs use amino acid and fatty acid metabolism to fuel the citric acid cycle. CML LSCs on the other hand exhibit increased mitochondrial biogenesis, FAO, OXPHOS and ROS. ROS leads to DNA instability and subsequently. Causes mutation in the BCR-ABL oncogene, rendering the cells resistant to drug treatment.

influenced by the kinase ataxia telangiectasia mutated (ATM) through transcriptional control of glucose 6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the pentose phosphate pathway (PPP) (46). PPP generates reducing equivalent (the reduced form of nicotinamide adenine dinucleotide phosphate - NADPH) essential to the recycling of oxidized glutathione, and rate-limiting cofactors for anabolic reactions, like fatty acid and cholesterol synthesis. The availability of NADPH, and its non-phosphorylated precursor NAD (critical for metabolic reactions in glycolysis and CAC/OxPhos), is also constrained by NAD synthesis, which can be pharmacologically targeted in AML (43). Both ATM and G6PD are targetable to sensitize AML cells to chemotherapy (47). Another alternate glucose metabolism pathway, the hexosamine biosynthesis pathway (HBP), is also upregulated in AML patient samples. Inhibition of glutamine fructose-6-phosphate amidotransferase (GFAT), a rate-limiting enzyme of HBP, induces differentiation and apoptosis of AML cells and eliminates the tumor burden not only from the bone marrow but also the peripheral blood in AML xenograft mouse models (48). Although extensive studies have been done to understand metabolism in AML and drug-resistant AML, it is still unknown whether these metabolic changes are common across all AML sub-types or whether the oncogene plays a role in deciding the metabolic fate of the AML cells, both the blast and LSCs.

Chronic Myeloid Leukemia (CML)

CML is more prevalent in the geriatric population. Due to a single translocation in phenotypic HSCs, it forms BCR-ABL, a constitutively active tyrosine kinase. Unlike normal HSCs, CML cells are highly dependent on OxPhos regulated by sirtuin 1 (SIRT1)-mediated activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) (49–51). SIRT1 is a NAD-dependent histone deacetylase that deacetylates and activates PGC1α, a regulator of mitochondrial biogenesis, both of which are found upregulated in CML. Dual treatment with SIRT1 and tyrosine kinase inhibitors (TKI) reduces CML cell proliferation, mitochondrial gene suppression and subsequent cell death in transgenic CML mouse model (51). Additionally, as opposed to AML, in CML, an increase in OxPhos also increases ROS production, causing DNA damage and genomic instability. This leads to mutations in the BCR-ABL oncogene, eventually causing oncogene-dependent resistance to TKI (**Figure 2**) (52–54). With TKI treatment, CML stem and progenitor cells accumulate lipids as well as nucleic acids with an increase in FAO (55). Moreover, metabolic stress created by TKI activates AMPK, suppressing which reduces disease progression (56). AMPK regulates energy homeostasis and leads to glucose and fatty acid uptake upon activation. And in the case of BCR-ABL-independent TKI resistance, glycolytic genes are upregulated along with increased glucose uptake, lactate production, and reduced oxygen consumption (50, 57–59). Although, this metabolic phenotype is partly similar to normal HSCs, the difference between HSCs and resistant CML LSCs is the dependency on the glycolytic pathway. Inhibition of PKM2 and LDHA, important enzymes for aerobic glycolysis, reduces disease progression and improves survival of CML mice, without impacting the surrounding HSCs (4, 59, 60). On the other hand, in advanced blast crisis CML, the stem cells have increased branched-chain amino acid metabolism (61). Additionally, these cells also upregulate CD36 fatty acid transporter and utilize gonadal adipose tissue lipolysis to fuel fatty acid metabolism as a source of energy as well as evade chemotherapy (62). Hence, targeting mitochondrial metabolic pathways in the CML stem and progenitor cells along with TKI treatment increases cell death and improves survival (49, 58, 63).

METABOLISM IN LYMPHOID LEUKEMIA

Acute Lymphocytic Leukemia (ALL)

ALL is the most common childhood leukemia marked by accumulation of immature B-cells (B-ALL) or T-cells (T-ALL). Chromosomal abnormalities like BCR-ABL translocation in the lymphoid progenitor lead to B-ALL, while T-ALL occurs due to gain-of-function Notch1 mutation in the lymphoid progenitor cells (64). B-ALL CD34+ cells from human BM have an upregulation of genes regulating glycolysis like glucose transporters *Glut1*, *Glut4*, and *Ldh*, with downregulation of CAC and FAO-related genes like *Idh3b*, *Sdhc*, *Fh*, and *Mdh* (65). They have increased glucose consumption and lactate production compared to the non-transformed human CD34+ cells indicating utilization of aerobic glycolysis in the former (65, 66). Additionally, increased glucose consumption in B-ALL cells and glutamine metabolism are linked to therapy resistance (67–69). Inhibiting glycolysis and glucose uptake reduced nucleotide and amino acid metabolism, decreased leukemogenesis and proliferation, increased apoptosis of B-ALL cells and sensitized these cells to glucocorticoid treatment (66, 68). Moreover, glucose is utilized not only for glycolysis but also as a starting point for PPP to reduce oxidative stress. B-cell genes *Pax5* and *Ikzf1* usually repress *G6pd*, a rate-limiting enzyme of PPP, hence reducing PPP activity (**Figure 3**). However, in B-ALL, the enzyme PP2A (Protein Phosphatase 2) switches glucose utilization from glycolysis to PPP while glycolysis is predominant HSCs. Inhibiting PP2A as well as G6PD or activating *Pax5* and *Ikzf* leads to reduced PPP activity sensitizing B-ALL cells to treatment (70). This target's the unique vulnerability of B-ALL cells without impacting the non-transformed cells. Glucose utilization is important for the survival of B-ALL cells, whereas NOTCH1 activation in T-ALL leads to a metabolic switch from glycolysis to glutaminolysis (71, 72). The reduced glycolysis in T-ALL cells compared to normal T-cells can be attributed to Notch1-mediated AMPK activation (73). Moreover, inhibition of Notch1 signaling also leads to accumulation of glutamine and increased activity of complex 1 in the mitochondrial electron transport chain, conferring resistance to treatment (**Figure 3**) (72). Inhibition of glutamine synthesis as well as loss of AMPK signaling along with Notch1 inhibition sensitizes T-ALL cells towards apoptosis (72, 73). In addition to glucose and glutamine metabolism, targeting one

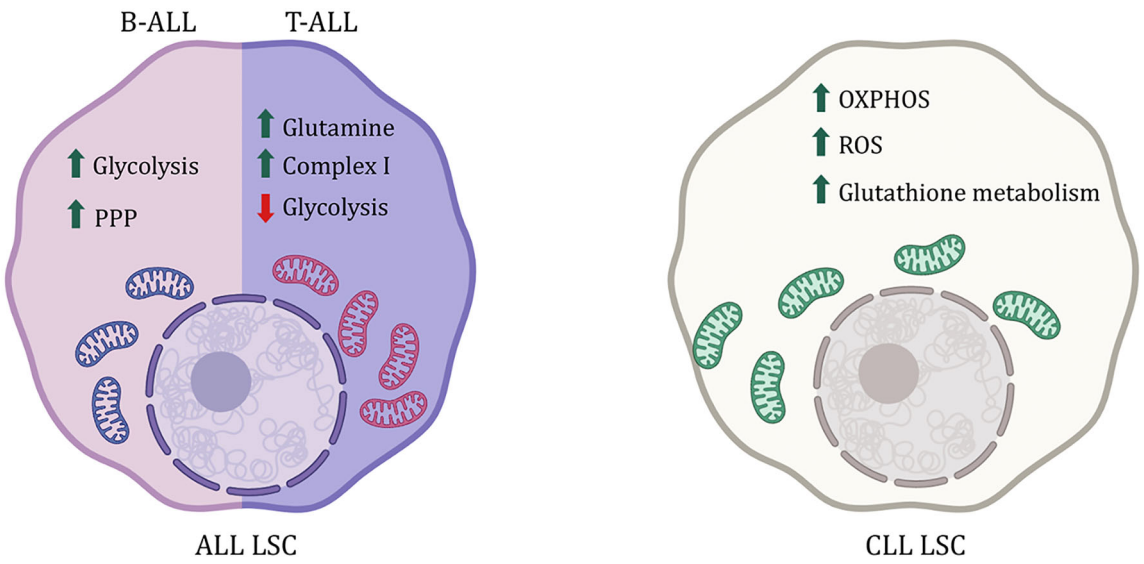

FIGURE 3 | Metabolic difference between ALL and CLL LSCs. B-ALL LSCs are reliant on Pentose phosphate pathway (PPP) and glycolysis where as T-ALL LSCs rely more on glutamine metabolism. CLL on the other had sees an increase in ROS and is reliant on OXPHOS. Increase in glutathione metabolism is also reported as an antioxidant to the high amount of ROS.

carbon metabolism through the inhibition of serine hydroxymethyltransferase (SHMT) interferes with the supply of NADPH and one-carbon pools for proliferation has shown promise as a therapeutic strategy in treating T-ALL. In line with these findings, the amount of dietary folate has been shown to modulate metabolism in hematopoietic cells (74, 75). This signifies that depending on the type of ALL cells rely on different metabolic pathways.

Chronic Lymphocytic Leukemia (CLL)

CLL like CML is found mainly in older adults marked by mutated mature CD5+ B-cells or memory B-cells in BM, blood, and even lymph nodes (76). Resting B-cells and memory B-cells are more glycolytic than activated B-cells (77). However, in CLL, the mutated B-cells rely on OxPhos over glycolysis and have reduced glucose uptake unlike normal HSCs (78, 79). CLL lymphocytes have an increase in ROS plus mitochondrial respiration and also have an active antioxidant activity *via* glutathione metabolism, protecting CLL cells from chemotherapy (**Figure 3**) (80, 81). In line with this, CLL lymphocytes also have overexpressed glutamine dehydrogenase, which plays a role in glutathione synthesis, and abolishing the glutathione-mediated protection mechanism leads to apoptosis of CLL cells (81, 82). Moreover, poor prognosis in CLL is marked by an accumulation of lipids, particularly ceramide and lipoprotein lipases, indicating active lipid metabolism, making them susceptible to FAO inhibitors, even in cases of treatment resistance (83–87).

Overall, one can affirm that metabolism is plastic, and different leukemia types have various metabolic vulnerabilities. Moreover, metabolism also changes with drug treatment. Understanding the mechanisms of metabolic change in leukemia, and following drug treatment, is important to identify target pathways without impacting the normal cells.

LIMITATIONS AND ADVANCEMENTS

Cell Number

Cell number limitation is one of the major impediments to understanding metabolic differences in the cells of interest, such as LSC. Due to the need for millions of cells for metabolic assays, the studies are usually conducted with a broader pool of cells or cell lines instead of the specific cells of interest in a complex model. Furthermore, metabolic differences due to phenotypic cellular heterogeneity, even within genetically homogeneous cell populations should be considered. All of this makes it challenging to have conclusive results for the cell of interest. This highlights the need to improve metabolic techniques to study smaller numbers of cells. One such advancement has been in metabolic profiles using ultra-high pressure liquid chromatography-mass spectrometry (UHPLC-MS). A high-throughput UHPLC-MS method developed recently can profile metabolites from as few as 10,000 cells (59, 88–90). The technique involves the use of a larger pool of cells from the same tissue and of heavy labeled standard spike-in to call peaks while analyzing the few cell samples (59, 88). Additional methods are being developed to afford the quantitation of small molecule metabolites from hundreds of cells at the expense of the breadth of the coverage of multiple metabolic pathways (18, 91). More targeted, single-cell metabolomics approaches have also been proposed, which leverage CyTOF (cytometry of time of flight)-

or capillary electrophoresis (CE)-based MS techniques for rapidly detecting a subset of metabolites (92). This technique allows to determine metabolite abundance in a specific population pool where cell number is a limitation, for instance, HSCs and LSCs (9, 31, 89, 93). Global metabolic profiling at steady state provides a snapshot view of the abundance of metabolites in cells. However, it fails to determine whether metabolites accumulate because of increased production or reduced usage. Thus, to infer the activity of a specific metabolic pathway, UHPLC-MS needs to be accompanied by other metabolic assays.

In Vivo Metabolic Flux

To understand the metabolic pathway activity over time and in different conditions, metabolic flux analysis provides more detailed information about substrate preference and consumption rate for catabolic or anabolic purposes. Metabolic tracing with stable isotope-labeled substrates has been widely utilized but more in cell lines which are usually not representative of the complexity of the cells within their environment. *In vivo* metabolic flux analysis (especially with radiolabeled or fluorescent tracers), on the other hand, proves efficient in such cases (94, 95). The most widely used methods to administer heavy carbon labeled tracers in a mouse are by gastric gavage or bolus injection *via* a tail vein or continuous infusion *via* cannulation (96). These administration methods can achieve a high plasma concentration of the tracer; however, they also cause stress to the animal due to anesthesia or animal handling, defeating the purpose of studying metabolism at steady state (97). To overcome this limitation and enable long-term tracer administration, tracers can also be delivered through water (97). Although this technique reduces stress in mice, facilitating studies at steady state, it fails to consider the variability in the diet among individual mice and hence the variability of the amount of tracer administered. Additionally, the possible metabolism of tracer as it circulates through the hepatic portal system and before it reaches the organ of interest must be taken into consideration. Even though metabolic flux assays provide more information than global metabolic profiles, the limitation to these methods is the time window available to harvest the tissue and cells of interest without losing the cellular tracing. For this reason, researchers flash-freeze the whole organ soon after harvesting. But this limits the study of the metabolic flux to an organ as a whole instead of individual cell types within the organ. The field needs a better technique to understand metabolic fluxes at the cellular level, and this is one of the reasons why *in vivo* metabolic tracing of bone marrow has not been carried out until very recently (98).

Metabolic Heterogeneity

Metabolic heterogeneity between tissues has been widely studied; however, it is yet uncovered between cells within the same tissue and between tumors (96). One major impediment in analyzing metabolites at a single cell level is the sensitivity of metabolite detection. Metabolite reporter using an alkyne tagged 'Click' chemistry is a potential solution to overcome this limitation (99). Using different alkyl groups can aid in multiplexing different samples and facilitate the use of multiple tracers in the same experiment, thus saving cost and time (99). Moreover, the enhanced signal of alkyne detection by MS helps determine metabolites at a single-cell level (99). However, it becomes difficult to identify one cell especially with overlapping peaks (99). In the current era of single-cell analysis, a computational model for studying metabolic phenotypes in the tumor microenvironment at a single cell level has also been developed (100), along with methods for imaging single-cell behavior in various microenvironments (101). They combine global metabolic gene expression with dimensional reductionists model and clustering algorithm to determine the metabolic gene expression profile of individual cells in head and neck tumors and melanoma (100). Although this technique considers heterogeneity and depicts metabolic profiles of different cell types, gene expression cannot always be correlated to metabolic activity. Further metabolic enzyme-based assays should be carried out to validate the computational analysis.

Some of the widely used techniques to determine heterogeneity based on mitochondrial mass and activity involve electron microscopy and confocal imaging using fluorescent markers like mitotrackers for mitochondrial abundance in each cell, TMRE for determination of mitochondrial membrane potential, or even fluorescent labeling of metabolic enzymes. However, these *ex vivo* fluorescence markers do not yield reproducible information. The results vary with changes in concentration, temperature, oxygen concentration, and cell count, pointing towards the importance of detailed experimental descriptions in publications. One recent imaging technique developed to measure mitophagy *in vivo* is based on a transgenic mouse model expressing the pH-sensitive fluorescent protein mt-Kiema localized to the mitochondria (102). Upon mitophagy, the pH of the mitochondria drops, changing the color of the mt-Kiema protein (102). Crossing these mice to transgenic leukemic mice would help expand our understanding of mitophagy in normal HSCs versus LSCs with or without drug treatment. This model can also be used for time-lapse studies as well as live-cell imaging. Another technique to study metabolic heterogeneity, called fluorescence-activated mitochondria sorting (FAMS), is based on flow cytometry. Using this technique, mitochondria from different types of tissues can be isolated based on their membrane potential, mitochondrial protein markers and (103).

Currently, no technique alone can be used to understand the metabolic complexity of a heterogeneous cell population. Therefore, it is important to use different metabolic assays to address the same question before making a conclusive statement.

Drug Targeting

Understanding metabolic differences in tumor versus normal cells has recently seen significant advancements. Many drugs have been repurposed as metabolic inhibitors or activators, like tigecycline (a known antibiotic) which can inhibit mitochondrial protein translation leading to inhibition of OxPhos (49, 104). Another drug repurposed is metformin (clinically used as an anti-diabetic) which is being used as an AMPK activator, currently in phase II clinical trials for CLL (ClinicalTrials.gov: NCT01750567) (105, 106).

However, most of these drugs are non-specific or have multiple targets; for instance, a widely used AMPK inhibitor, dorsomorphin, can also inhibit VEGF and BMP-SMAD signaling (107), while shikonin, a drug used for PKM2 inhibition, can also inhibit STAT3, Fak, Src, cMyc and PI3K-AKT signaling pathways and cause cellular apoptosis *via* activation of c-JUN-N-terminal kinase (JNK) (108–111). Another example would be mubritinib, a known inhibitor of ERRB2, that can localize to the mitochondria and inhibit complex I of the electron complex chain (112). New metabolic inhibitors are being developed, for instance, IACS-010750, an inhibitor of elector complex chain complex 1, a drug currently under phase I clinical trials for AML (ClinicalTrials.gov: NCT02882321) (113). To keep up with the metabolic discoveries, inhibitors with more specificity need to be developed to reduce side effects and off-target impacts and provide better therapeutic options.

CONCLUSION

Metabolism can still be considered a developing field of cellular biology. No single technique can or should be used to address questions regarding stem cell metabolism. With the advent of single-cell technologies for transcriptomic and epigenetics, science has moved from bulk cellular analysis to understanding cellular heterogeneity within the same population, which needs to be adapted even in metabolism. Moreover, the changes in metabolism are not unique to stem cells, highlighting the need for studies to be carried out with appropriate controls to enhance LSC specificity and reduce side effects of metabolic inhibitors on HSCs. These metabolic differences would represent a condition of LSC-specific vulnerability that could potentially be targeted.

AUTHOR CONTRIBUTIONS

SP outlined and wrote the manuscript; TN and AD provided intellectual help with manuscript writing; RW supervised and assisted with manuscript writing. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. Fendt SM, Frezza C, Erez A. Targeting Metabolic Plasticity and Flexibility Dynamics for Cancer Therapy. *Cancer Discov* (2020) 10(12):1797–807. doi: 10.1158/2159-8290.CD-20-0844
2. Cipolleschi MG, Dello Sbarba P, Olivotto M. The Role of Hypoxia in the Maintenance of Hematopoietic Stem Cells. *Blood* (1993) 82(7):2031–7. doi: 10.1182/blood.V82.7.2031.2031
3. Simsek T, Kocabas F, Zheng J, Deberardinis RJ, Mahmoud AI, Olson EN, et al. The Distinct Metabolic Profile of Hematopoietic Stem Cells Reflects Their Location in a Hypoxic Niche. *Cell Stem Cell* (2010) 7(3):380–90. doi: 10.1016/j.stem.2010.07.011
4. Wang YH, Israelsen WJ, Lee D, Yu VWC, Jeanson NT, Clish CB, et al. Cell-State-Specific Metabolic Dependency in Hematopoiesis and Leukemogenesis. *Cell* (2014) 158(6):1309–23. doi: 10.1016/j.cell.2014.07.048
5. Takubo K, Nagamatsu G, Kobayashi CI, Nakamura-Ishizu A, Kobayashi H, Ikeda E, et al. Regulation of Glycolysis by Pdk Functions as a Metabolic Checkpoint for Cell Cycle Quiescence in Hematopoietic Stem Cells. *Cell Stem Cell* (2013) 12(1):49–61. doi: 10.1016/j.stem.2012.10.011
6. Takubo K, Goda N, Yamada W, Iriuchishima H, Ikeda E, Kubota Y, et al. Regulation of the HIF-1alpha Level Is Essential for Hematopoietic Stem Cells. *Cell Stem Cell* (2010) 7(3):391–402. doi: 10.1016/j.stem.2010.06.020
7. Kobayashi H, Morikawa T, Okinaga A, Hamano F, Hashidate-Yoshida T, Watanuki S, et al. Environmental Optimization Enables Maintenance of Quiescent Hematopoietic Stem Cells Ex Vivo. *Cell Rep* (2019) 28(1):145–58.e9. doi: 10.1016/j.celrep.2019.06.008
8. Ito K, Carracedo A, Weiss D, Arai F, Ala U, Avigan DE, et al. A PML-PPAR-Delta Pathway for Fatty Acid Oxidation Regulates Hematopoietic Stem Cell Maintenance. *Nat Med* (2012) 18(9):1350–8. doi: 10.1038/nm.2882
9. Dong S, Wang Q, YR K, Diaz A, Tasset I, Kaushik S, et al. Chaperone-Mediated Autophagy Sustains Haematopoietic Stem-Cell Function. *Nature* (2021) 591(7848):117–23. doi: 10.1038/s41586-020-03129-z
10. Vannini N, Girotra M, Naveiras O, Nikitin G, Campos V, Giger S, et al. Specification of Haematopoietic Stem Cell Fate via Modulation of Mitochondrial Activity. *Nat Commun* (2016) 7:13125. doi: 10.1038/ncomms13125
11. de Almeida MJ, Luchsinger LL, Corrigan DJ, Williams LJ, Snoeck HW. Dye-Independent Methods Reveal Elevated Mitochondrial Mass in Hematopoietic Stem Cells. *Cell Stem Cell* (2017) 21(6):725–9.e4. doi: 10.1016/j.stem.2017.11.002
12. Maryanovich M, Zaltsman Y, Ruggiero A, Goldman A, Shachnai L, Zaidman SL, et al. An MTCH2 Pathway Repressing Mitochondria Metabolism Regulates Haematopoietic Stem Cell Fate. *Nat Commun* (2015) 6:7901. doi: 10.1038/ncomms8901
13. Hinge A, He J, Bartram J, Javier J, Xu J, Fjellman E, et al. Asymmetrically Segregated Mitochondria Provide Cellular Memory of Hematopoietic Stem Cell Replicative History and Drive HSC Attrition. *Cell Stem Cell* (2020) 26(3):420–30.e6. doi: 10.1016/j.stem.2020.01.016
14. Luchsinger LL, de Almeida MJ, Corrigan DJ, Mumau M, Snoeck HW. Mitofusin 2 Maintains Haematopoietic Stem Cells With Extensive Lymphoid Potential. *Nature* (2016) 529(7587):528–31. doi: 10.1038/nature16500
15. Guitart AV, Panagopoulou TI, Villacreces A, Vukovic M, Sepulveda C, Allen L, et al. Fumarate Hydratase Is a Critical Metabolic Regulator of Hematopoietic Stem Cell Functions. *J Exp Med* (2017) 214(3):719–35. doi: 10.1084/jem.20161087
16. Liu X, Zheng H, Yu WM, Cooper TM, Bunting KD, Qu CK. Maintenance of Mouse Hematopoietic Stem Cells Ex Vivo by Reprogramming Cellular Metabolism. *Blood* (2015) 125(10):1562–5. doi: 10.1182/blood-2014-04-568949
17. Yu WM, Liu X, Shen J, Jovanovic O, Pohl EE, Gerson SL, et al. Metabolic Regulation by the Mitochondrial Phosphatase PTPMT1 Is Required for Hematopoietic Stem Cell Differentiation. *Cell Stem Cell* (2013) 12(1):62–74. doi: 10.1016/j.stem.2012.11.022

18. Agathocleous M, Meacham CE, Burgess RJ, Piskounova E, Zhao Z, Crane GM, et al. Ascorbate Regulates Haematopoietic Stem Cell Function and Leukaemogenesis. *Nature* (2017) 549(7673):476–81. doi: 10.1038/nature23876
19. Ho TT, Warr MR, Adelman ER, Lansinger OM, Flach J, Verovskaya EV, et al. Autophagy Maintains the Metabolism and Function of Young and Old Stem Cells. *Nature* (2017) 543(7644):205–10. doi: 10.1038/nature21388
20. Mansell E, Sigurdsson V, Deltcheva E, Brown J, James C, Miharada K, et al. Mitochondrial Potentiation Ameliorates Age-Related Heterogeneity in Hematopoietic Stem Cell Function. *Cell Stem Cell* (2021) 28(2):241–56.e6. doi: 10.1016/j.stem.2020.09.018
21. Shallis RM, Wang R, Davidoff A, Ma X, Zeidan AM. Epidemiology of Acute Myeloid Leukemia: Recent Progress and Enduring Challenges. *Blood Rev* (2019) 36:70–87. doi: 10.1016/j.blre.2019.04.005
22. Saultz JN, Garzon R. Acute Myeloid Leukemia: A Concise Review. *J Clin Med* (2016) 5(3):33–50. doi: 10.3390/jcm5030033
23. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *New Engl J Med* (2016) 374(23):2209–21. doi: 10.1056/NEJMoa1516192
24. Dohner H, Dolnik A, Tang L, Seymour JF, Minden MD, Stone RM, et al. Cytogenetics and Gene Mutations Influence Survival in Older Patients With Acute Myeloid Leukemia Treated With Azacitidine or Conventional Care. *Leukemia* (2018) 32(12):2546–57. doi: 10.1038/s41375-018-0257-z
25. Lagadinou ED, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, et al. BCL-2 Inhibition Targets Oxidative Phosphorylation and Selectively Eradicates Quiescent Human Leukemia Stem Cells. *Cell Stem Cell* (2013) 12 (3):329–41. doi: 10.1016/j.stem.2012.12.013
26. Pei S, Minhajuddin M, Adane B, Khan N, Stevens BM, Mack SC, et al. AMPK/FIS1-Mediated Mitophagy Is Required for Self-Renewal of Human AML Stem Cells. *Cell Stem Cell* (2018) 23(1):86–100.e6. doi: 10.1016/j.stem.2018.05.021
27. Sharon D, Cathelin S, Mirali S, Di Trani JM, Yanofsky DJ, Keon KA, et al. Inhibition of Mitochondrial Translation Overcomes Venetoclax Resistance in AML Through Activation of the Integrated Stress Response. *Sci Transl Med* (2019) 11(516):15. doi: 10.1126/scitranslmed.aax2863
28. Chen X, Glytsou C, Zhou H, Narang S, Reyna DE, Lopez A, et al. Targeting Mitochondrial Structure Sensitizes Acute Myeloid Leukemia to Venetoclax Treatment. *Cancer Discov* (2019) 9(7):890–909. doi: 10.1158/2159-8290.CD-19-0117
29. Cui L, Cheng Z, Liu Y, Dai Y, Pang Y, Jiao Y, et al. Overexpression of PDK2 and PDK3 Reflects Poor Prognosis in Acute Myeloid Leukemia. *Cancer Gene Ther* (2020) 27(1-2):15–21. doi: 10.1038/s41417-018-0071-9
30. Zabkiewicz J, Pearn L, Hills RK, Morgan RG, Tonks A, Burnett AK, et al. The PDK1 Master Kinase is Over-Expressed in Acute Myeloid Leukemia and Promotes PKC-Mediated Survival of Leukemic Blasts. *Haematologica* (2014) 99(5):858–64. doi: 10.3324/haematol.2013.096487
31. Jones CL, Stevens BM, D'Alessandro A, Reisz JA, Culp-Hill R, Nemkov T, et al. Inhibition of Amino Acid Metabolism Selectively Targets Human Leukemia Stem Cells. *Cancer Cell* (2018) 34(5):724–40.e4. doi: 10.1016/j.ccell.2018.10.005
32. Farge T, Saland E, de Toni F, Aroua N, Hosseini M, Perry R, et al. Chemotherapy-Resistant Human Acute Myeloid Leukemia Cells Are Not Enriched for Leukemic Stem Cells But Require Oxidative Metabolism. *Cancer Discov* (2017) 7(7):716–35. doi: 10.1158/2159-8290.CD-16-0441
33. Samudio I, Harmancey R, Fiegl M, Kantarjian H, Konopleva M, Korchin B, et al. Pharmacologic Inhibition of Fatty Acid Oxidation Sensitizes Human Leukemia Cells to Apoptosis Induction. *J Clin Invest* (2010) 120(1):142–56. doi: 10.1172/JCI38942
34. Stevens BM, Jones CL, Pollyea DA, Culp-Hill R, D'Alessandro A, Winters A, et al. Fatty Acid Metabolism Underlies Venetoclax Resistance in Acute Myeloid Leukemia Stem Cells. *Nat Cancer* (2020) 1:1176–87. doi: 10.1038/s43018-020-00126-z
35. Tcheng M, Roma A, Ahmed N, Smith RW, Jayanth P, Minden MD, et al. Very Long Chain Fatty Acid Metabolism Is Required in Acute Myeloid Leukemia. *Blood* (2021) 137(25):3518–32. doi: 10.1182/blood.2020008551
36. Subedi AL,Q, Sharon D, Cathelin S, Xu C, Voisin V, Bader GD, et al. Nicotinamide Phosphoribosyltransferase Inhibitors Selectively Induce Apoptosis of AML Stem Cells by Disrupting Lipid Homeostasis. *Cell Stem Cell* (2020) 28(10):1851–67.e8. doi: 10.2139/ssrn.3707266
37. Pei S, Minhajuddin M, Callahan KP, Balys M, Ashton JM, Neering SJ, et al. Targeting Aberrant Glutathione Metabolism to Eradicate Human Acute Myelogenous Leukemia Cells. *J Biol Chem* (2013) 288(47):33542–58. doi: 10.1074/jbc.M113.511170
38. Jones CL, Stevens BM, D'Alessandro A, Culp-Hill R, Reisz JA, Pei S, et al. Cysteine Depletion Targets Leukemia Stem Cells Through Inhibition of Electron Transport Complex II. *Blood* (2019) 134(4):389–94. doi: 10.1182/blood.2019898114
39. Jacque N, Ronchetti AM, Larrue C, Meunier G, Birsen R, Willems L, et al. Targeting Glutaminolysis has Antileukemic Activity in Acute Myeloid Leukemia and Synergizes With BCL-2 Inhibition. *Blood* (2015) 126 (11):1346–56. doi: 10.1182/blood-2015-01-621870
40. Matre P, Velez J, Jacamo R, Qi Y, Su X, Cai T, et al. Inhibiting Glutaminase in Acute Myeloid Leukemia: Metabolic Dependency of Selected AML Subtypes. *Oncotarget* (2016) 7(48):79722–35. doi: 10.18632/oncotarget.12944
41. van Gastel N, Spinelli JB, Sharda A, Schajnovitz A, Baryawno N, Rhee C, et al. Induction of a Timed Metabolic Collapse to Overcome Cancer Chemoresistance. *Cell Metab* (2020) 32(3):391–403.e6. doi: 10.1016/j.cmet.2020.07.009
42. Gregory MA, Nemkov T, Reisz JA, Zaberezhnyy V, Hansen KC, D'Alessandro A, et al. Glutaminase Inhibition Improves FLT3 Inhibitor Therapy for Acute Myeloid Leukemia. *Exp Hematol* (2018) 58:52–8. doi: 10.1016/j.exphem.2017.09.007
43. Jones CL, Stevens BM, Pollyea DA, Culp-Hill R, Reisz JA, Nemkov T, et al. Nicotinamide Metabolism Mediates Resistance to Venetoclax in Relapsed Acute Myeloid Leukemia Stem Cells. *Cell Stem Cell* (2020) 27(5):748–64.e4. doi: 10.1016/j.stem.2020.07.021
44. Gallipoli P, Giotopoulos G, Tzelepis K, Costa ASH, Vohra S, Medina-Perez P, et al. Glutaminolysis Is a Metabolic Dependency in FLT3(ITD) Acute Myeloid Leukemia Unmasked by FLT3 Tyrosine Kinase Inhibition. *Blood* (2018) 131(15):1639–53. doi: 10.1182/blood-2017-12-820035
45. Yan D, Franzini A, Pomicter AD, Halverson BJ, Antelope O, Mason CC, et al. Sirt5 Is a Druggable Metabolic Vulnerability in Acute Myeloid Leukemia. *Blood Cancer Discov* (2021) 2(3):266–87. doi: 10.1158/2643-3230.BCD-20-0168
46. Zhang Y, Lee JH, Paull TT, Gehrke S, D'Alessandro A, Dou Q, et al. Mitochondrial Redox Sensing by the Kinase ATM Maintains Cellular Antioxidant Capacity. *Sci Signal* (2018) 11(538):11. doi: 10.1126/scisignal.aaq0702
47. Gregory MA, D'Alessandro A, Alvarez-Calderon F, Kim J, Nemkov T, Adane B, et al. ATM/G6PD-Driven Redox Metabolism Promotes FLT3 Inhibitor Resistance in Acute Myeloid Leukemia. *Proc Natl Acad Sci USA* (2016) 113(43):E6669–E78. doi: 10.1073/pnas.1603876113
48. Asthana A, Ramakrishnan P, Vicioso Y, Zhang K, Parameswaran R. Hexosamine Biosynthetic Pathway Inhibition Leads to AML Cell Differentiation and Cell Death. *Mol Cancer Ther* (2018) 17(10):2226–37. doi: 10.1158/1535-7163.MCT-18-0426
49. Kuntz EM, Baquero P, Michie AM, Dunn K, Tardito S, Holyoake TL, et al. Targeting Mitochondrial Oxidative Phosphorylation Eradicates Therapy-Resistant Chronic Myeloid Leukemia Stem Cells. *Nat Med* (2017) 23 (10):1234–40. doi: 10.1038/nm.4399
50. Flis K, Irvine D, Copland M, Bhatia R, Skorski T. Chronic Myeloid Leukemia Stem Cells Display Alterations in Expression of Genes Involved in Oxidative Phosphorylation. *Leukemia Lymphoma* (2012) 53(12):2474–8. doi: 10.3109/10428194.2012.696313
51. Abraham A, Qiu S, Chacko BK, Li H, Paterson A, He J, et al. SIRT1 Regulates Metabolism and Leukemogenic Potential in CML Stem Cells. *J Clin Invest* (2019) 129(7):2685–701. doi: 10.1172/JCI127080
52. Nieborowska-Skorska M, Kopinski PK, Ray R, Hoser G, Ngaba D, Flis S, et al. Rac2-MRC-cIII-Generated ROS Cause Genomic Instability in Chronic Myeloid Leukemia Stem Cells and Primitive Progenitors. *Blood* (2012) 119 (18):4253–63. doi: 10.1182/blood-2011-10-385658
53. Koptyra M, Falinski R, Nowicki MO, Stoklosa T, Majsterek I, Nieborowska-Skorska M, et al. BCR/ABL Kinase Induces Self-Mutagenesis via Reactive

Oxygen Species to Encode Imatinib Resistance. *Blood* (2006) 108(1):319–27. doi: 10.1182/blood-2005-07-2815
54. Sattler M, Verma S, Shrikhande G, Byrne CH, Pride YB, Winkler T, et al. The BCR/ABL Tyrosine Kinase Induces Production of Reactive Oxygen Species in Hematopoietic Cells. *J Biol Chem* (2000) 275(32):24273–8. doi: 10.1074/jbc.M002094200
55. Boren J, Cascante M, Marin S, Comin-Anduix B, Centelles JJ, Lim S, et al. Gleevec (STI571) Influences Metabolic Enzyme Activities and Glucose Carbon Flow Toward Nucleic Acid and Fatty Acid Synthesis in Myeloid Tumor Cells. *J Biol Chem* (2001) 276(41):37747–53. doi: 10.1074/jbc.M105796200
56. Saito Y, Chapple RH, Lin A, Kitano A, Nakada D. AMPK Protects Leukemia-Initiating Cells in Myeloid Leukemias From Metabolic Stress in the Bone Marrow. *Cell Stem Cell* (2015) 17(5):585–96. doi: 10.1016/j.stem.2015.08.019
57. Kominsky DJ, Klawitter J, Brown JL, Boros LG, Melo JV, Eckhardt SG, et al. Abnormalities in Glucose Uptake and Metabolism in Imatinib-Resistant Human BCR-ABL-Positive Cells. *Clin Cancer Res* (2009) 15(10):3442–50. doi: 10.1158/1078-0432.CCR-08-3291
58. Kluza J, Jendoubi M, Ballot C, Dammak A, Jonneaux A, Idziorek T, et al. Exploiting Mitochondrial Dysfunction for Effective Elimination of Imatinib-Resistant Leukemic Cells. *PLoS One* (2011) 6(7):e21924. doi: 10.1371/journal.pone.0021924
59. Patel SB, Nemkov T, Stefanoni D, Benavides GA, Bassal MA, Crown BL, et al. Metabolic Alterations Mediated by STAT3 Promotes Drug Persistence in CML. *Leukemia* (2021) 35(12):3371–82. doi: 10.1038/s41375-021-01315-0
60. Gao P, Shen S, Li X, Liu D, Meng Y, Liu Y, et al. Dihydroartemisinin Inhibits the Proliferation of Leukemia Cells K562 by Suppressing PKM2 and GLUT1 Mediated Aerobic Glycolysis. *Drug Des Devel Ther* (2020) 14:2091–100. doi: 10.2147/DDDT.S248872
61. Hattori A, Tsunoda M, Konuma T, Kobayashi M, Nagy T, Glushka J, et al. Cancer Progression by Reprogrammed BCAA Metabolism in Myeloid Leukaemia. *Nature* (2017) 545(7655):500–4. doi: 10.1038/nature22314
62. Ye H, Adane B, Khan N, Sullivan T, Minhajuddin M, Gasparetto M, et al. Leukemic Stem Cells Evade Chemotherapy by Metabolic Adaptation to an Adipose Tissue Niche. *Cell Stem Cell* (2016) 19(1):23–37. doi: 10.1016/j.stem.2016.06.001
63. Alvarez-Calderon F, Gregory MA, Pham-Danis C, DeRyckere D, Stevens BM, Zaberezhnyy V, et al. Tyrosine Kinase Inhibition in Leukemia Induces an Altered Metabolic State Sensitive to Mitochondrial Perturbations. *Clin Cancer Res* (2015) 21(6):1360–72. doi: 10.1158/1078-0432.CCR-14-2146
64. Garcia EG, Iyer S, Garcia SP, Loontiens S, Sadreyev RI, Speleman F, et al. Cell of Origin Dictates Aggression and Stem Cell Number in Acute Lymphoblastic Leukemia. *Leukemia* (2018) 32(8):1860–5. doi: 10.1038/s41375-018-0130-0
65. Boag JM, Beesley AH, Firth MJ, Freitas JR, Ford J, Hoffmann K, et al. Altered Glucose Metabolism in Childhood Pre-B Acute Lymphoblastic Leukaemia. *Leukemia* (2006) 20(10):1731–7. doi: 10.1038/sj.leu.2404365
66. Liu T, Kishton RJ, Macintyre AN, Gerriets VA, Xiang H, Liu X, et al. Glucose Transporter 1-Mediated Glucose Uptake is Limiting for B-Cell Acute Lymphoblastic Leukemia Anabolic Metabolism and Resistance to Apoptosis. *Cell Death Dis* (2014) 5:e1470. doi: 10.1038/cddis.2014.431
67. Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM, et al. Gene-Expression Patterns in Drug-Resistant Acute Lymphoblastic Leukemia Cells and Response to Treatment. *New Engl J Med* (2004) 351(6):533–42. doi: 10.1056/NEJMoa033513
68. Hulleman E, Kazemier KM, Holleman A, VanderWeele DJ, Rudin CM, Broekhuis MJ, et al. Inhibition of Glycolysis Modulates Prednisolone Resistance in Acute Lymphoblastic Leukemia Cells. *Blood* (2009) 113(9):2014–21. doi: 10.1182/blood-2008-05-157842
69. Dyczynski M, Vesterlund M, Bjorklund AC, Zachariadis V, Janssen J, Gallart-Ayala H, et al. Metabolic Reprogramming of Acute Lymphoblastic Leukemia Cells in Response to Glucocorticoid Treatment. *Cell Death Dis* (2018) 9(9):846. doi: 10.1038/s41419-018-0625-7
70. Xiao G, Chan LN, Klemm L, Braas D, Chen Z, Geng H, et al. B-Cell-Specific Diversion of Glucose Carbon Utilization Reveals a Unique Vulnerability in B Cell Malignancies. *Cell* (2018) 173(2):470–84.e18. doi: 10.1016/j.cell.2018.02.048
71. Nguyen TL, Nokin MJ, Teres S, Tome M, Bodineau C, Galmar O, et al. Downregulation of Glutamine Synthetase, Not Glutaminolysis, is Responsible for Glutamine Addiction in Notch1-Driven Acute Lymphoblastic Leukemia. *Mol Oncol* (2020) 15(5):1412–31. doi: 10.1002/1878-0261.12877
72. Herranz D, Ambesi-Impiombato A, Sudderth J, Sanchez-Martin M, Belver L, Tosello V, et al. Metabolic Reprogramming Induces Resistance to Anti-NOTCH1 Therapies in T Cell Acute Lymphoblastic Leukemia. *Nat Med* (2015) 21(10):1182–9. doi: 10.1038/nm.3955
73. Kishton RJ, Barnes CE, Nichols AG, Cohen S, Gerriets VA, Siska PJ, et al. AMPK Is Essential to Balance Glycolysis and Mitochondrial Metabolism to Control T-ALL Cell Stress and Survival. *Cell Metab* (2016) 23(4):649–62. doi: 10.1016/j.cmet.2016.03.008
74. Henry CJ, Nemkov T, Casas-Selves M, Bilousova G, Zaberezhnyy V, Higa KC, et al. Folate Dietary Insufficiency and Folic Acid Supplementation Similarly Impair Metabolism and Compromise Hematopoiesis. *Haematologica* (2017) 102(12):1985–94. doi: 10.3324/haematol.2017.171074
75. Garcia-Canaveras JC, Lancho O, Ducker GS, Ghergurovich JM, Xu X, da Silva-Diz V, et al. SHMT Inhibition Is Effective and Synergizes With Methotrexate in T-Cell Acute Lymphoblastic Leukemia. *Leukemia* (2021) 35(2):377–88. doi: 10.1038/s41375-020-0845-6
76. Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, Durig J, et al. Cellular Origin and Pathophysiology of Chronic Lymphocytic Leukemia. *J Exp Med* (2012) 209(12):2183–98. doi: 10.1084/jem.20120833
77. Garcia-Manteiga JM, Mari S, Godejohann M, Spraul M, Napoli C, Cenci S, et al. Metabolomics of B to Plasma Cell Differentiation. *J Proteome Res* (2011) 10(9):4165–76. doi: 10.1021/pr200328f
78. Vangapandu HV, Havranek O, Ayres ML, Kaipparettu BA, Balakrishnan K, Wierda WG, et al. B-Cell Receptor Signaling Regulates Metabolism in Chronic Lymphocytic Leukemia. *Mol Cancer Res* (2017) 15(12):1692–703. doi: 10.1158/1541-7786.MCR-17-0026
79. Galicia-Vazquez G, Smith S, Aloyz R. Del11q-Positive CLL Lymphocytes Exhibit Altered Glutamine Metabolism and Differential Response to GLS1 and Glucose Metabolism Inhibition. *Blood Cancer J* (2018) 8(1):13. doi: 10.1038/s41408-017-0039-2
80. Jitschin R, Hofmann AD, Bruns H, Giessl A, Bricks J, Berger J, et al. Mitochondrial Metabolism Contributes to Oxidative Stress and Reveals Therapeutic Targets in Chronic Lymphocytic Leukemia. *Blood* (2014) 123(17):2663–72. doi: 10.1182/blood-2013-10-532200
81. Zhang W, Trachootham D, Liu J, Chen G, Pelicano H, Garcia-Prieto C, et al. Stromal Control of Cystine Metabolism Promotes Cancer Cell Survival in Chronic Lymphocytic Leukaemia. *Nat Cell Biol* (2012) 14(3):276–86. doi: 10.1038/ncb2432
82. Mayer RL, Schwarzmeier JD, Gerner MC, Bileck A, Mader JC, Meier-Menches SM, et al. Proteomics and Metabolomics Identify Molecular Mechanisms of Aging Potentially Predisposing for Chronic Lymphocytic Leukemia. *Mol Cell Proteom* (2018) 17(2):290–303. doi: 10.1074/mcp.RA117.000425
83. Thurgood LA, Dwyer ES, Lower KM, Chataway TK, Kuss BJ. Altered Expression of Metabolic Pathways in CLL Detected by Unlabelled Quantitative Mass Spectrometry Analysis. *Br J Haematol* (2019) 185(1):65–78. doi: 10.1111/bjh.15751
84. Liu PP, Liu J, Jiang WQ, Carew JS, Ogasawara MA, Pelicano H, et al. Elimination of Chronic Lymphocytic Leukemia Cells in Stromal Microenvironment by Targeting CPT With an Antiangina Drug Perhexiline. *Oncogene* (2016) 35(43):5663–73. doi: 10.1038/onc.2016.103
85. Nikitin EA, Malakho SG, Biderman BV, Baranova AV, Lorie YY, Shevelev AY, et al. Expression Level of Lipoprotein Lipase and Dystrophin Genes Predict Survival in B-Cell Chronic Lymphocytic Leukemia. *Leukemia Lymphoma* (2007) 48(5):912–22. doi: 10.1080/10428190701245112
86. Spaner DE, Lee E, Shi Y, Wen F, Li Y, Tung S, et al. PPAR-Alpha Is a Therapeutic Target for Chronic Lymphocytic Leukemia. *Leukemia* (2013) 27(5):1090–9. doi: 10.1038/leu.2012.329
87. Huang C, Tu Y, Freter CE. Fludarabine-Resistance Associates With Ceramide Metabolism and Leukemia Stem Cell Development in Chronic

Lymphocytic Leukemia. *Oncotarget* (2018) 9(69):33124–37. doi: 10.18632/oncotarget.26043
88. Nemkov T, Hansen KC, D'Alessandro A. A Three-Minute Method for High-Throughput Quantitative Metabolomics and Quantitative Tracing Experiments of Central Carbon and Nitrogen Pathways. *Rapid Commun Mass Spectromet: RCM* (2017) 31(8):663–73. doi: 10.1002/rcm.7834
89. DeVilbiss AW, Zhao Z, Martin-Sandoval MS, Ubellacker JM, Tasdogan A, Agathocleous M, et al. Metabolomic Profiling of Rare Cell Populations Isolated by Flow Cytometry From Tissues. *Elife* (2021) 10:23. doi: 10.7554/eLife.61980
90. Nemkov T, Reisz JA, Gehrke S, Hansen KC, D'Alessandro A. High-Throughput Metabolomics: Isocratic and Gradient Mass Spectrometry-Based Methods. *Methods Mol Biol* (2019) 1978:13–26. doi: 10.1007/978-1-4939-9236-2_2
91. Luo X, Li L. Metabolomics of Small Numbers of Cells: Metabolomic Profiling of 100, 1000, and 10000 Human Breast Cancer Cells. *Anal Chem* (2017) 89(21):11664–71. doi: 10.1021/acs.analchem.7b03100
92. Duncan KD, Fyrestam J, Lanekoff I. Advances in Mass Spectrometry Based Single-Cell Metabolomics. *Analyst* (2019) 144(3):782–93. doi: 10.1039/C8AN01581C
93. Adane B, Ye H, Khan N, Pei S, Minhajuddin M, Stevens BM, et al. The Hematopoietic Oxidase NOX2 Regulates Self-Renewal of Leukemic Stem Cells. *Cell Rep* (2019) 27(1):238–54.e6. doi: 10.1016/j.celrep.2019.03.009
94. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, et al. Glucose Feeds the TCA Cycle via Circulating Lactate. *Nature* (2017) 551(7678):115–8. doi: 10.1038/nature24057
95. Reisz JA, D'Alessandro A. Measurement of Metabolic Fluxes Using Stable Isotope Tracers in Whole Animals and Human Patients. *Curr Opin Clin Nutr Metab Care* (2017) 20(5):366–74. doi: 10.1097/MCO.0000000000000393
96. Neinast MD, Jang C, Hui S, Murashige DS, Chu Q, Morscher RJ, et al. Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. *Cell Metab* (2019) 29(2):417–29.e4. doi: 10.1016/j.cmet.2018.10.013
97. Sun RC, Fan TW, Deng P, Higashi RM, Lane AN, Le AT, et al. Noninvasive Liquid Diet Delivery of Stable Isotopes Into Mouse Models for Deep Metabolic Network Tracing. *Nat Commun* (2017) 8(1):1646. doi: 10.1038/s41467-017-01518-z
98. Jun S, Mahesula S, Mathews TP, Martin-Sandoval MS, Zhao Z, Piskounova E, et al. The Requirement for Pyruvate Dehydrogenase in Leukemogenesis Depends on Cell Lineage. *Cell Metab* (2021) 33(9):1777–92.e8. doi: 10.1016/j.cmet.2021.07.016
99. Thiele C, Wunderling K, Leyendecker P. Multiplexed and Single Cell Tracing of Lipid Metabolism. *Nat Methods* (2019) 16(11):1123–30. doi: 10.1038/s41592-019-0593-6
100. Xiao Z, Dai Z, Locasale JW. Metabolic Landscape of the Tumor Microenvironment at Single Cell Resolution. *Nat Commun* (2019) 10(1):3763. doi: 10.1038/s41467-019-11738-0
101. Carlson P, Dasgupta A, Grzelak CA, Kim J, Barrett A, Coleman IM, et al. Targeting the Perivascular Niche Sensitizes Disseminated Tumour Cells to Chemotherapy. *Nat Cell Biol* (2019) 21(2):238–50. doi: 10.1038/s41556-018-0267-0
102. Sun N, Malide D, Liu J, Rovira II, Combs CA, Finkel T. A Fluorescence-Based Imaging Method to Measure In Vitro and In Vivo Mitophagy Using Mt-Keima. *Nat Protoc* (2017) 12(8):1576–87. doi: 10.1038/nprot.2017.060
103. MacDonald JA, Bothun AM, Annis SN, Sheehan H, Ray S, Gao Y, et al. A Nanoscale, Multi-Parametric Flow Cytometry-Based Platform to Study Mitochondrial Heterogeneity and Mitochondrial DNA Dynamics. *Commun Biol* (2019) 2:258. doi: 10.1038/s42003-019-0513-4
104. Skrtic M, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, et al. Inhibition of Mitochondrial Translation as a Therapeutic Strategy for Human Acute Myeloid Leukemia. *Cancer Cell* (2011) 20(5):674–88. doi: 10.1016/j.ccr.2011.10.015
105. Bruno S, Ledda B, Tenca C, Ravera S, Orengo AM, Mazzarello AN, et al. Metformin Inhibits Cell Cycle Progression of B-Cell Chronic Lymphocytic Leukemia Cells. *Oncotarget* (2015) 6(26):22624–40. doi: 10.18632/oncotarget.4168
106. Vakana E, Altman JK, Glaser H, Donato NJ, Platanias LC. Antileukemic Effects of AMPK Activators on BCR-ABL-Expressing Cells. *Blood* (2011) 118(24):6399–402. doi: 10.1182/blood-2011-01-332783
107. Dasgupta B, Seibel W. Compound C/Dorsomorphin: Its Use and Misuse as an AMPK Inhibitor. *Methods Mol Biol* (2018) 1732:195–202. doi: 10.1007/978-1-4939-7598-3_12
108. Mao X, Yu CR, Li WH, Li WX. Induction of Apoptosis by Shikonin Through a ROS/JNK-Mediated Process in Bcr/Abl-Positive Chronic Myelogenous Leukemia (CML) Cells. *Cell Res* (2008) 18(8):879–88. doi: 10.1038/cr.2008.86
109. Thakur R, Trivedi R, Rastogi N, Singh M, Mishra DP. Inhibition of STAT3, FAK and Src Mediated Signaling Reduces Cancer Stem Cell Load, Tumorigenic Potential and Metastasis in Breast Cancer. *Sci Rep* (2015) 5:10194. doi: 10.1038/srep10194
110. Ni F, Huang X, Chen Z, Qian W, Tong X. Shikonin Exerts Antitumor Activity in Burkitt's Lymphoma by Inhibiting C-MYC and PI3K/AKT/mTOR Pathway and Acts Synergistically With Doxorubicin. *Sci Rep* (2018) 8(1):3317. doi: 10.1038/s41598-018-21570-z
111. Zhao X, Zhu Y, Hu J, Jiang L, Li L, Jia S, et al. Shikonin Inhibits Tumor Growth in Mice by Suppressing Pyruvate Kinase M2-Mediated Aerobic Glycolysis. *Sci Rep* (2018) 8(1):14517. doi: 10.1038/s41598-018-31615-y
112. Baccelli I, Gareau Y, Lehnertz B, Gingras S, Spinella JF, Corneau S, et al. Mubritinib Targets the Electron Transport Chain Complex I and Reveals the Landscape of OXPHOS Dependency in Acute Myeloid Leukemia. *Cancer Cell* (2019) 36(1):84–99.e8. doi: 10.1016/j.ccell.2019.06.003
113. Molina JR, Sun Y, Protopopova M, Gera S, Bandi M, Bristow C, et al. An Inhibitor of Oxidative Phosphorylation Exploits Cancer Vulnerability. *Nat Med* (2018) 24(7):1036–46. doi: 10.1038/s41591-018-0052-4

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The Metabolic Signature of AML Cells Treated With Homoharringtonine

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Acute myeloid leukemia (AML) is a hematologic malignancy. The overall prognosis is poor and therapeutic strategies still need to be improved. Studies have found that abnormalities in metabolisms promote the survival of AML cells. In recent years, an increasing number of studies have reported the effectiveness of a protein synthesis inhibitor, homoharringtonine (HHT), for the treatment of AML. In this study, we demonstrated that HHT effectively inhibited AML cells, especially MV4-11, a cell line representing human AML carrying the poor prognostic marker FLT3-ITD. We analyzed the transcriptome of MV4-11 cells treated with HHT, and identified the affected metabolic pathways including the choline metabolism process. In addition, we generated a line of MV4-11 cells that were resistant to HHT. The transcriptome analysis showed that the resistant mechanism was closely related to the ether lipid metabolism pathway. The key genes involved in these processes were *AL162417.1*, *PLA2G2D*, and *LPCAT2* by multiple intergroup comparison and Venn analysis. In conclusion, we found that the treatment of HHT significantly changed metabolic signatures of AML cells, which may contribute to the precise clinical use of HHT and the development of novel strategies to treat HHT-resistant AML.

Keywords: homoharringtonine, AML, ether lipid metabolism, RNA-seq, choline metabolism

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy featured by a proliferation of myeloblasts that are unable to undergo normal differentiation (1). It is the most prevalent form of leukemia in adults and the second major type of acute leukemia in children (2, 3). The disease progresses rapidly, with a survival of only a few weeks to a few months if untreated. In the United States, the 5-year overall survival rate for adults with relapsed/refractory AML (excluding APL) is approximately 10% (4).

Homoharringtonine (HHT) is a plant alkaloid that inhibits protein synthesis and has anti-tumor properties. In October 2012, HHT was approval by the U.S. Food and Drug Administration (FDA) for the treatment of chronic or accelerated CML after failure treatment of 2 or more tyrosine kinase inhibitors (5, 6). HHT has been used in China for more than 40 years to treat AML (7). A multicenter

phase 3 trial found that the HAA (homoharringtonine-cytarabine-aclarubicin) regimen could be used as an alternative induction therapy for untreated AML, particularly in those patients with favorable and intermediate cytogenetics (8). Previous studies have found multiple mechanisms of HHT in treating AML, including targeting FLT3 pathway, antioxidant defense, SP1/TET1/5hmC pathway, TRAIL and p53 pathway, NF-κB pathway and the expression of myosin-9 and eIF4E (9–15). The mechanisms of resistance to HHT in AML include the reactivation of PI3K/AKT signaling pathway and the overexpression of MDR1, MRP1, P170-glycoprotein, MCL-1, and MAP4K1 (16–20). In addition, an increasing number of studies have reported synergistic effects of HHT combined with FLT3 inhibitors for the treatment of AML with FLT3-ITD (9, 21, 22). Recent research has shown that the treatment of FLT3 inhibitor gilteritinib leads to reprogramming of glycolipid metabolism in the tumor microenvironment, resulting in early and late drug resistance (23). Also, HHT in combination with glutaminase inhibitor CB-839 exacerbates AML mitochondrial reactive oxygen species and apoptosis (24). Venetoclax is a potent and selective oral inhibitor of BCL-2, a key regulator of mitochondrial energy metabolism that plays an important role in the survival of AML stem cells (25, 26). A study has shown a strong synergistic effect of venetoclax and HHT in diffuse large B-cell lymphoma *in vitro* and *in vivo* (27). Although a major progress has been made, the detailed mechanisms of effectiveness and resistance in HHT-based therapy in AML still need to be explored. And, the mechanisms of HHT metabolism-related studies in AML have not been reported.

Deregulating cellular metabolism is a hallmark of cancer (28). In AML, knockdown of ANRIL leads to decreased glucose uptake and inhibits survival of leukemia cells (29). Inhibition of glycolysis attenuates the proliferation of AML cells and enhances the cytotoxicity of Ara-C (30). Also, acetyl-CoA carboxylase 1(ACC1) was found to be the rate-limiting enzyme for fatty acid synthesis, and upregulation of ACC1 protein expression has the potential to be an effective strategy for the treatment of AML (31). Therefore, metabolic abnormalities in AML are highly involved in disease progression and resistance to the treatment, which need to be further investigated. In this study, we explored the change in transcriptome of HHT-treated AML cells and identified key metabolism pathways that may affect sensitivity and resistance of AML cells to HHT.

MATERIALS AND METHODS

Cell Culture

Human AML cell lines MOLM-13 and MV4-11 were obtained from DSMZ (Braunschweig, Germany) and ATCC (VA, USA). The cells were maintained in RPMI 1640 (Hyclone, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Gemini, USA), 100 U/ml penicillin, and 100μg/ml streptomycin (Hyclone, Thermo Fisher Scientific, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay and Flow Cytometry

For the cell viability assay, 6×10^4 cells were plated in each well in a 96-well plate with complete RPMI 1640 supplemented with 10% FBS. The plate was incubated for 24 hours before detection. Cell viability was measured by the cell counting kit-8 (CCK-8, Solaribio, Beijing, China). For the apoptotic assay, cells were seeded in 24-well plates at 0.75×10^6 cells/well and incubated for 18 hours and subjected to flow cytometry. The Annexin V-FITC Apoptosis Detection Kit was obtained from DOJINDO (Japan) (AD10-50). Flow cytometric analysis was performed by a Cytoflex flow cytometer (Beckman, USA).

Transcriptome Analysis

We obtained the resistant MV4-11 strain (R) by gradually increasing the concentration of HHT and kept the cells in culture with 10 nM for about 10 months. After obtaining the resistant strain, we cultured the resistant strain off-drug for about 3 months to obtain the detoxified strain (O). Together with the parental MV4-11 cells (N), R and O cells were cultured with or without 10 nM HHT for 6 hours. The samples were designated N0 and N6 for parent cells without or with HHT, O0 and O6 for off-drug cells without or with HHT, and R0 and R6 for resistant R cells with or without HHT, respectively. Cells were collected in lyophilization tubes and frozen in liquid nitrogen for 10 minutes. The isolation of RNA and next-generation of sequencing were performed by Genedenovo Biotechnology Co., Ltd (Guangzhou, China). Raw data and normalized gene expression data are deposited in the sequence read archive database under accession numbers PRJNA832421. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed by Omicshare tools (https://www.omicshare.com/tools/). Gene Set Enrichment Analysis (GESA) was performed by using software GSEA and MSigDB (32).

Statistics

Data visualizing and statistical analysis were performed using GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA). Differences between experimental groups were analyzed using unpaired Student t test. p value < 0.05 was considered significant.

RESULTS

HHT Significantly Induced Apoptosis of MV4-11 and MOLM13

In the cell viability assay, we found that MV4-11 and MOLM13 were sensitive to the treatment of HHT, with IC_{50} of 7.92 nM and 12.98 nM, respectively (**Figure 1A**). Flow cytometry showed that HHT induced significant apoptosis in MV4-11 and MOLM13 (**Figure 1B**). To explore the resistant mechanism in HHT-treated AML cells, we established MV4-11 that resisted to HHT by gradually increasing the concentration of HHT in the medium. The cell viability assay showed that MV4-11 resistant strain and off-drug strain were less sensitive to the treatment of HHT, compared with the original MV4-11 (**Figure 1C**).

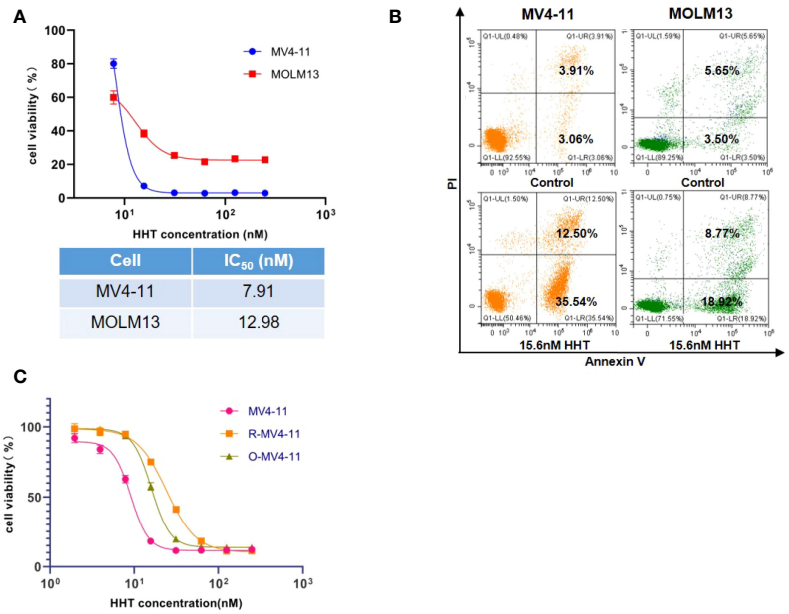

FIGURE 1 | AML cell lines were sensitive to HHT and the establishment of HHT-resistant cells. MV4-11, MOLM13 cells were treated with different concentrations of HHT for 24 hours, and cell viability was detected by CCK-8 **(A)**. AML cells were treated without or with 15.6 nM HHT for 18 hours, and analyzed by flow cytometry **(B)**. Cells were treated with different concentrations of HHT for 24 hours. The cell viability of MV4-11, MV4-11 resistant strain (R-MV4-11), and off-drug strain (O-MV4-11) was detected by CCK-8 **(C)**.

Choline Metabolism Was Associated With HHT Treatment in AML

We performed transcriptome analysis in 3 strains of MV4-11 cells treated by HHT or out of HHT, including the original strain (N0 and N6), the HHT-resistant strain (R0 and R6), and the off-drug strain (O0 and O6). The genetic heat map analysis and principal component analysis revealed a large inter-group variability and a small intra-group variability (**Figures 2A, B**),

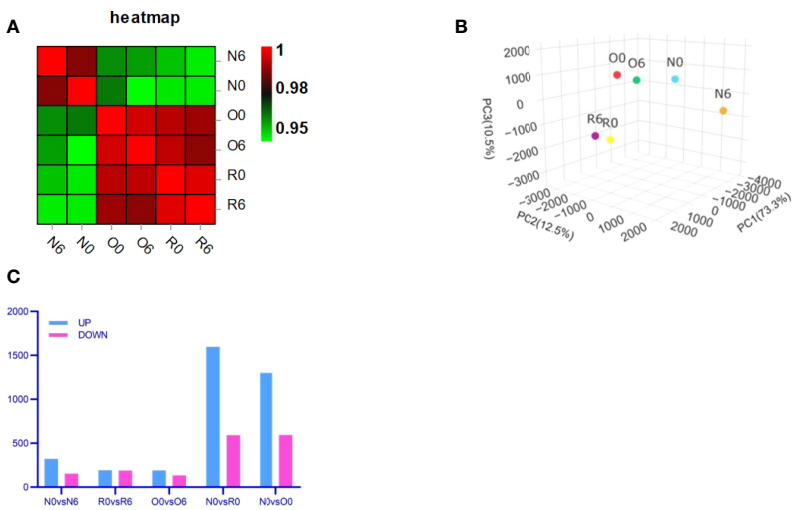

FIGURE 2 | Transcriptome analysis showed an ideal cell line modeling. Correlation heat map **(A)** and Principal Component Analysis (PCA) **(B)** were used to assess intra- and inter-group differences in the samples. Bar charts showed the number of significantly different genes between groups (FDR < 0.05, multiple of difference greater than or equal to 2) **(C)**.

which was in accordance with the number of up- and down-regulated genes in each group (**Figure 2C**). These data indicate an ideal cell line modeling in our transcriptome analysis.

In groups of N0 vs N6, O0 vs O6, and R0 vs R6, genes in metabolism processes were found to be significantly enriched by GO enrichment analysis (**Figures 3A–C**). To clarify the specific metabolic pathways, we performed KEGG enrichment analysis and found that genes in the choline metabolism pathway were significantly enriched after short-term intervention with HHT or out of HHT (N0 vs N6, p = 0.01; O0 vs O6, p = 0.02; R0 vs R6, p = 0.001) (**Figures 3D–F**). In contrast, this metabolic pathway was not significantly enriched in groups of long-term intervention of HHT (**Figures 4C, D**). Therefore, we speculated that the choline metabolism pathway is involved in the anti-leukemia effect of HHT in AML.

Ether Lipid Metabolism Was Associated With the Resistance to HHT in AML

In groups of N0 vs R0 and N0 vs O0, GO enrichment analysis revealed that genes in metabolism-related biological processes were significantly enriched (**Figures 4A, B**). To clarify specific metabolic pathways, we performed KEGG enrichment analysis and found that the genes in ether lipid metabolism pathway were significantly enriched after long-term HHT intervention (N0 vs R0, p= 0.048) (**Figure 4C**). In the off-drug group, genes in this pathway were also significantly enriched (N0 vs O0, p = 0.02) (**Figure 4D**). In contrast, this metabolic pathway was not significantly enriched in HHT short-term intervention groups (N0 vs N6, p = 0.30; O0 vs O6, p = 0.51; R0 vs R6, p = 0.25). Therefore, we conclude that the ether lipid metabolism pathway is involved in promoting the development of resistance to HHT in AML cells. Interestingly, in the N0 vs O0 group, "arginine and proline metabolism" (p = 0.037), "alanine, aspartate and glutamate metabolism" (p = 0.044), and "glycerophospholipid metabolism" (p = 0.045) were also significantly enriched, and these pathways may contribute to the re-sensitization of O0 strain to HHT.

AL162417.1, PLA2G2D, and LPCAT2 Were Key Genes in the Metabolic Signature of HHT-Treated AML Cells

By using KEGG enrichment analysis, we identified the core set of genes associated with the treatment of HHT in AML cells. Drug efficacy gene of HHT was *AL162417.1* (**Figures 5A, C**). The key genes of resistance to HHT were *PLA2G2D*, *LPCAT2*, *UGT8*, *CHPT1*, and *GDPD1* (**Figure 5B**).

Since the fluctuation of drug resistance genes should not be significant changed after short-term administration or withdrawal of HHT, *CHPT1*, *UGT8*, and *GDPD1* were excluded (**Figure S1A**). The key drug resistance-related genes were identified as *PLA2G2D* and *LPCAT2* (**Figure 5D**). In addition, we found that *PLA2G2D* and *LPCAT2* were significantly enriched in the group of N0 vs O0 by GSEA (**Figure S1B**), which further validated *PLA2G2D* and *LPCAT2* as key HHT-resistance genes in AML cells.

DISCUSSION

Tumorigenesis and progression require metabolic reprogramming of cancer cells (33, 34), and understanding this process is important for tumor diagnosis, treatment, and

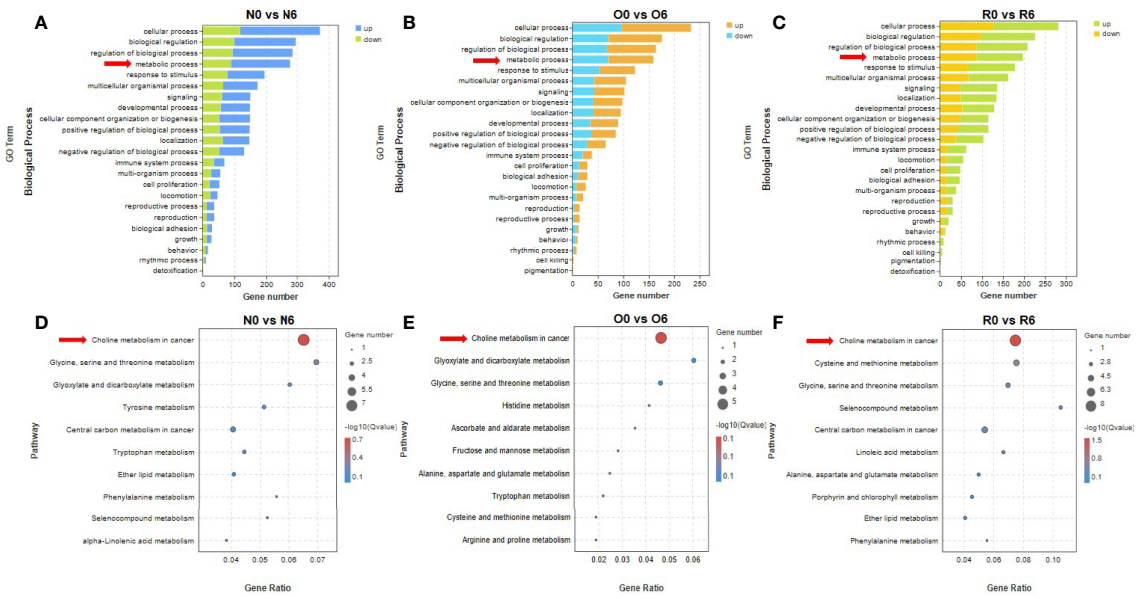

FIGURE 3 | The drug efficacy mechanism of HHT was related to choline metabolism. Biological processes significantly affected in different cell lines with 10 nM HHT treatment for 6 hours **(A, B)** or HHT withdraw for 6 hours in the resistant line were found by GO enrichment analysis **(C)**. HHT was found to significantly affect metabolism-related signaling pathways by KEGG enrichment analysis **(D–F)**.

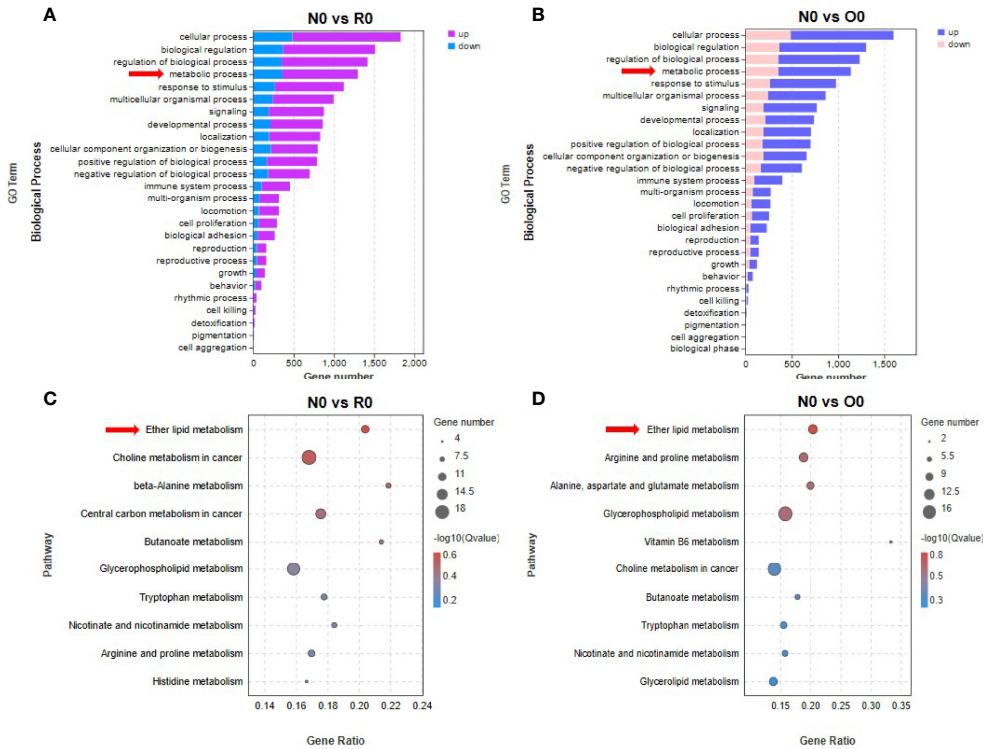

FIGURE 4 | Ether lipid metabolism was associated with the mechanism of HHT resistance. By GO enrichment analysis, several biological processes were found to be significantly affected in long-term HHT-treated cell lines **(A, B)**. Significant effects of HHT on metabolism-related signaling pathways were found by KEGG enrichment analysis **(C, D)**.

prognosis. Abnormalities in choline metabolism have been reported in a variety of tumors. Several studies have reported that C-choline PET/CT can detect biochemical recurrent prostate cancer recurrence and survival (35, 36). In Glioblastomas cell lines U87MG and GBM5, choline increases tumor cell proliferation and anti-apoptosis by activating α7- and α9-containing nicotinic receptors and promoting AKT and ERK phosphorylation (37). The increased choline uptake was reported in endometrial cancer cell lines compared to normal endometrial stromal cells (38). CHPT1 drives cytidine diphosphate-choline (CDP-Cho) to generate phosphatidylcholine (PtdCho). Knockdown of CHPT1 inhibits the growth and proliferation of breast cancer cells, and *in vivo* experiments have shown that knockdown of CHPT1 inhibits the early metastasis of breast cancer cells (39). Genomic pooling analysis of colorectal cancer gut microbes shows that the choline trimethylamine-lyase gene is overexpressed in colorectal cancer (40). It is reported that in some types of tumors, the upregulation of choline metabolic pathway may lead to proliferation and anti-apoptosis of tumor cells. Wang, Musharraf et al. found significant alterations in choline metabolism in AML patients (41, 42). Besides, increased phosphorylcholine (PCho) was found to be a common feature of all observed AML cell lines (43). However, the specific pathological significance of abnormal choline metabolism in AML has not been revealed. In this study, we found that the drug efficacy mechanism of HHT is closely related to the choline metabolism, and further identified key genes in the pathway. Note that *AL162417.1* is a novel protein-coding gene that is a homolog of *RALGDS*, which activates CHK (44), and promotes the production of PCho. Therefore, *AL162417.1* could serve as a potential target of HHT in treating AML cells. The current findings on this gene are limited, which deserves further in-depth studies.

Ether lipids play a role in membrane fusion, participate in cellular differentiation and signaling, and act as endogenous antioxidants (45). Ether lipid levels have been shown to elevate in tumors. The enzyme alkylglycer-onephosphate synthase (AGPS) is upregulated in several types of aggressive human cancer cells and primary tumors, and knockdown of AGPS in cancer cells decreases survival of tumor cells, cancer invasiveness, and tumor growth [24]. In this study, we found that HHT resistance mechanism is closely related to the ether lipid metabolism pathway, and further identified key genes as *PLA2G2D* and *LPCAT2*. *PLA2G2D* encodes a secreted member of the phospholipase A2 family, which produces lysophospholipids and free fatty acid and involves in ether lipid metabolism. Studies show that oxidative stress may induce the expression of *PLA2G2D* in mouse and human monocyte-derived macrophages through lipid peroxidation. The high expression of *PLA2G2D* increases viral infection, and mice lacking *PLAG2D* are protected from COVID-19

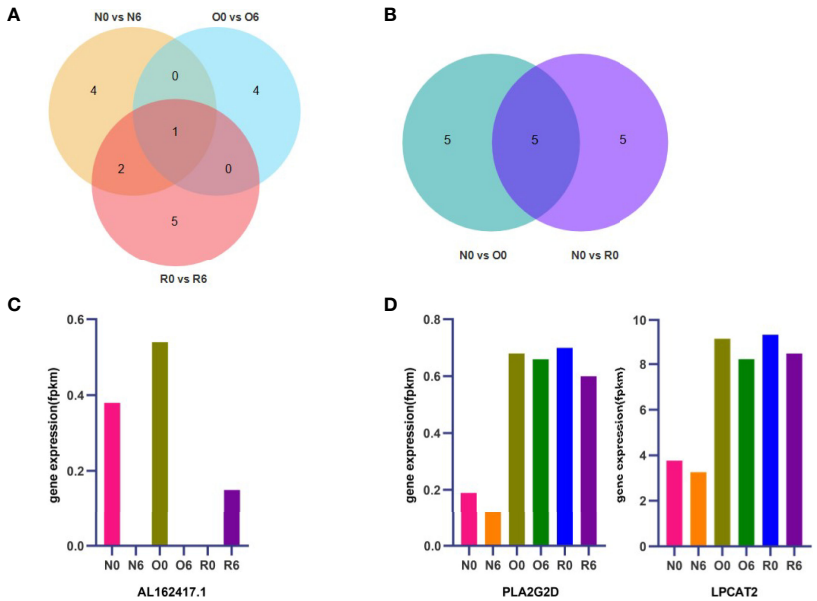

FIGURE 5 | *AL162417.1*, *PLA2G2D*, and *LPCAT2* are candidate genes for drug efficacy and resistance. The core genes of drug efficacy and resistance related pathways identified by KEGG enrichment analysis and the shared key genes were found by Venn diagram **(A, B)**. Expression of key genes for drug efficacy and resistance were detected by RNA-seq **(C, D)**.

invasion (46, 47). Besides, it has been reported that upregulation of *PLA2G2D* could be a potential biomarker for cancer immunotherapy (48). *LPCAT2* is involved in ether lipid metabolism and is a member of the family of enzymes encoding lysophospholipid acyltransferases. The expression level of *LPCAT2* is positively correlated with aggressive prostate cancer (49). Importantly, *LPCAT2* mediated lipid droplet production has been shown to promote resistance to chemotherapy in colorectal cancer (50). Therefore, *LPCAT2* is more promising candidate genes for HHT resistance. Furthermore, by ether lipids metabolism KEGG signaling pathway diagram (**Figure S2**), we found that PLA2G2D and LPCAT2 are involved in the conversion between lysoPAF and plasmanylcholine, but overall promote lysoPAF to plasmanylcholine production.

In summary, our study found that the mechanism of HHT efficacy in AML is related to choline metabolism, and the key candidate gene was *AL162417.1*. The resistance mechanism is related to ether lipid metabolism, and the core resistance candidate genes are *PLA2G2D* and *LPCAT2*. This study provides a new perspective for the rational clinical use of HHT and the development of new HHT-resistant treatment strategies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YP, CC, and YC conceived the project. YLZ, NL, ZC and HW performed the experiments. QZ, DZ, JH, YG and YMZ analyzed data. YLZ, ZC, and HW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.931527/full#supplementary-material

REFERENCES

1. Newell LF, Cook RJ. Advances in Acute Myeloid Leukemia. *BMJ* (2021) 375: n2026. doi: 10.1136/bmj.n2026
2. Burd A, Levine RL, Ruppert AS, Mims AS, Borate U, Stein EM, et al. Precision Medicine Treatment in Acute Myeloid Leukemia Using Prospective Genomic Profiling: Feasibility and Preliminary Efficacy of the Beat Aml Master Trial. *Nat Med* (2020) 26(12):1852–8. doi: 10.1038/s41591-020-1089-8
3. Carter JL, Hege K, Yang J, Kalpage HA, Su Y, Edwards H, et al. Targeting Multiple Signaling Pathways: The New Approach to Acute Myeloid Leukemia Therapy. *Signal Transduct Target Ther* (2020) 5(1):288. doi: 10.1038/s41392-020-00361-x
4. DeWolf S, Tallman MS. How I Treat Relapsed or Refractory Aml. *Blood* (2020) 136(9):1023–32. doi: 10.1182/blood.2019001982
5. Cortes J, Lipton JH, Rea D, Digumarti R, Chuah C, Nanda N, et al. Phase 2 Study of Subcutaneous Omacetaxine Mepesuccinate After Tki Failure in Patients With Chronic-Phase Cml With T315i Mutation. *Blood* (2012) 120 (13):2573–80. doi: 10.1182/blood-2012-03-415307
6. Lu S, Wang J. Homoharringtonine and Omacetaxine for Myeloid Hematological Malignancies. *J Hematol Oncol* (2014) 7:2. doi: 10.1186/1756-8722-7-2
7. Harringtonine Research Collaborative Group. Preliminary Clinical Evaluation of Harringtonine Alkaloids in the Treatment of Acute Leukemia. *Zhejiang Tumor Newslett* (1976) (01):56–62.
8. Jin J, Wang JX, Chen FF, Wu DP, Hu J, Zhou JF, et al. Homoharringtonine-Based Induction Regimens for Patients With De-Novo Acute Myeloid Leukaemia: A Multicentre, Open-Label, Randomised, Controlled Phase 3 Trial. *Lancet Oncol* (2013) 14(7):599–608. doi: 10.1016/S1470-2045(13)70152-9
9. Lam SS, Ho ES, He BL, Wong WW, Cher CY, Ng NK, et al. Homoharringtonine (Omacetaxine Mepesuccinate) as an Adjunct for Flt3-Itd Acute Myeloid Leukemia. *Sci Transl Med* (2016) 8(359):359ra129. doi: 10.1126/scitranslmed.aaf3735
10. Zhang JJ, Geng HY, Liu L, Zhang H. Synergistic Cytotoxicity of Homoharringtonine and Etoposide in Acute Myeloid Leukemia Cells Involves Disrupted Antioxidant Defense. *Cancer Manage Res* (2019) 11:1023–32. doi: 10.2147/cmar.S187597
11. Li C, Dong L, Su R, Bi Y, Qing Y, Deng X, et al. Homoharringtonine Exhibits Potent Anti-Tumor Effect and Modulates DNA Epigenome in Acute Myeloid Leukemia by Targeting Sp1/Tet1/5hmc. *Haematologica* (2020) 105(1):148–60. doi: 10.3324/haematol.2018.208835
12. Cao H, Cheng Y, You L, Qian J, Qian W. Homoharringtonine and Saha Synergistically Enhance Apoptosis in Human Acute Myeloid Leukemia Cells Through Upregulation of Trail and Death Receptors. *Mol Med Rep* (2013) 7 (6):1838–44. doi: 10.3892/mmr.2013.1440
13. Tan M, Zhang Q, Yuan X, Chen Y, Wu Y. Synergistic Killing Effects of Homoharringtonine and Arsenic Trioxide on Acute Myeloid Leukemia Stem Cells and the Underlying Mechanisms. *J Exp Clin Cancer Res* (2019) 38 (1):308. doi: 10.1186/s13046-019-1295-8
14. Zhang T, Shen S, Zhu Z, Lu S, Yin X, Zheng J, et al. Homoharringtonine Binds to and Increases Myosin-9 in Myeloid Leukaemia. *Br J Pharmacol* (2016) 173 (1):212–21. doi: 10.1111/bph.13359
15. Chen XJ, Zhang WN, Chen B, Xi WD, Lu Y, Huang JY, et al. Homoharringtonine Deregulates Myc Transcriptional Expression by Directly Binding Nf-Kappab Repressing Factor. *Proc Natl Acad Sci U.S.A.* (2019) 116(6):2220–5. doi: 10.1073/pnas.1818539116
16. Wang L, You LS, Ni WM, Ma QL, Tong Y, Mao LP, et al. Beta-Catenin and Akt Are Promising Targets for Combination Therapy in Acute Myeloid Leukemia. *Leuk Res* (2013) 37(10):1329–40. doi: 10.1016/j.leukres.2013.06.023
17. Zhang W, Lu Y, Zhen T, Chen X, Zhang M, Liu P, et al. Homoharringtonine Synergy With Oridonin in Treatment of T (8, 21) Acute Myeloid Leukemia. *Front Med* (2019) 13(3):388–97. doi: 10.1007/s11684-018-0624-1
18. Zhou DC, Ramond S, Viguie F, Faussat AM, Zittoun R, Marie JP. Sequential Emergence of Mrp- and Mdr1-Gene Over-Expression as Well as Mdr1-Gene Translocation in Homoharringtonine-Selected K562 Human Leukemia Cell Lines. *Int J Cancer* (1996) 65(3):365–71. doi: 10.1002/(SICI)1097-0215(19960126)65:3<365::AID-IJC15>3.0.CO;2-9
19. Chen P, Zhan W, Wang B, You P, Jin Q, Hou D, et al. Homoharringtonine Potentiates the Antileukemic Activity of Arsenic Trioxide Against Acute Myeloid Leukemia Cells. *Exp Cell Res* (2019) 376(2):114–23. doi: 10.1016/j.yexcr.2019.02.008
20. Russo D, Michelutti A, Melli C, Damiani D, Michieli MG, Candoni A, et al. Mdr-Related P170-Glycoprotein Modulates Cytotoxic Activity of Homoharringtonine. *Leukemia* (1995) 9(3):513–6.
21. Wang F, Huang J, Guo T, Zheng Y, Zhang L, Zhang D, et al. Homoharringtonine Synergizes With Quizartinib in Flt3-Itd Acute Myeloid Leukemia by Targeting Flt3-Akt-C-Myc Pathway. *Biochem Pharmacol* (2021) 188:114538. doi: 10.1016/j.bcp.2021.114538
22. Zhang C, Lam SSY, Leung GMK, Tsui SP, Yang N, Ng NKL, et al. Sorafenib and Omacetaxine Mepesuccinate as a Safe and Effective Treatment for Acute Myeloid Leukemia Carrying Internal Tandem Duplication of Fms-Like Tyrosine Kinase 3. *Cancer* (2020) 126(2):344–53. doi: 10.1002/cncr.32534
23. Joshi SK, Nechiporuk T, Bottomly D, Piehowski PD, Reisz JA, Pittsenbarger J, et al. The Aml Microenvironment Catalyzes a Stepwise Evolution to Gilteritinib Resistance. *Cancer Cell* (2021) 39(7):999–1014.e8. doi: 10.1016/j.ccell.2021.06.003
24. Gregory MA, Nemkov T, Park HJ, Zaberezhnyy V, Gehrke S, Adane B, et al. Targeting Glutamine Metabolism and Redox State for Leukemia Therapy. *Clin Cancer Res* (2019) 25(13):4079–90. doi: 10.1158/1078-0432.CCR-18-3223
25. Short NJ, Konopleva M, Kadia TM, Borthakur G, Ravandi F, DiNardo CD, et al. Advances in the Treatment of Acute Myeloid Leukemia: New Drugs and New Challenges. *Cancer Discovery* (2020) 10(4):506–25. doi: 10.1158/2159-8290.CD-19-1011
26. Lagadinou ED, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, et al. Bcl-2 Inhibition Targets Oxidative Phosphorylation and Selectively Eradicates Quiescent Human Leukemia Stem Cells. *Cell Stem Cell* (2013) 12 (3):329–41. doi: 10.1016/j.stem.2012.12.013
27. Klanova M, Andera L, Brazina J, Svadlenka J, Benesova S, Soukup J, et al. Targeting of Bcl2 Family Proteins With Abt-199 and Homoharringtonine Reveals Bcl2- and Mcl1-Dependent Subgroups of Diffuse Large B-Cell Lymphoma. *Clin Cancer Res* (2016) 22(5):1138–49. doi: 10.1158/1078-0432.CCR-15-1191
28. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discovery* (2022) 12(1):31–46. doi: 10.1158/2159-8290.CD-21-1059
29. Sun LY, Li XJ, Sun YM, Huang W, Fang K, Han C, et al. Lncrna Anril Regulates Aml Development Through Modulating the Glucose Metabolism Pathway of Adipor1/Ampk/Sirt1. *Mol Cancer* (2018) 17(1):127. doi: 10.1186/s12943-018-0879-9
30. Chen WL, Wang JH, Zhao AH, Xu X, Wang YH, Chen TL, et al. A Distinct Glucose Metabolism Signature of Acute Myeloid Leukemia With Prognostic Value. *Blood* (2014) 124(10):1645–54. doi: 10.1182/blood-2014-02-554204
31. Ito H, Nakamae I, Kato J, Yoneda-Kato N. Stabilization of Fatty Acid Synthesis Enzyme Acetyl-Coa Carboxylase 1 Suppresses Acute Myeloid Leukemia Development. *J Clin Invest* (2021) 131(12):e141529. doi: 10.1172/jci141529
32. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles. *Proc Natl Acad Sci U.S.A.* (2005) 102(43):15545–50. doi: 10.1073/pnas.0506580102
33. Martinez-Reyes I, Chandel NS. Cancer Metabolism: Looking Forward. *Nat Rev Cancer* (2021) 21(10):669–80. doi: 10.1038/s41568-021-00378-6
34. Pedroza-Torres A, Romero-Cordoba SL, Justo-Garrido M, Salido-Guadarrama I, Rodriguez-Bautista R, Montano S, et al. Micrornas in Tumor Cell Metabolism: Roles and Therapeutic Opportunities. *Front Oncol* (2019) 9:1404. doi: 10.3389/fonc.2019.01404
35. Michaud L, Touijer KA, Mauguen A, Zelefsky MJ, Morris MJ, Lyashschenko SK, et al. C-Choline Pet/Ct in Recurrent Prostate Cancer: Retrospective Analysis in a Large U.S. Patient Series. *J Nucl Med* (2020) 61(6):827–33. doi: 10.2967/jnumed.119.233098
36. Giovacchini G, Guglielmo P, Mapelli P, Incerti E, Gajate AMS, Giovannini E, et al. C-Choline Pet/Ct Predicts Survival in Prostate Cancer Patients With Psa < 1 Ng/Ml. *Eur J Nucl Med Mol Imaging* (2019) 46(4):921–9. doi: 10.1007/s00259-018-4253-3
37. Pucci S, Fasoli F, Moretti M, Benfante R, Di Lascio S, Viani P, et al. Choline and Nicotine Increase Glioblastoma Cell Proliferation by Binding and Activating Alpha7- and Alpha9- Containing Nicotinic Receptors. *Pharmacol Res* (2021) 163:105336. doi: 10.1016/j.phrs.2020.105336

38. Trousil S, Lee P, Pinato DJ, Ellis JK, Dina R, Aboagye EO, et al. Alterations of Choline Phospholipid Metabolism in Endometrial Cancer Are Caused by Choline Kinase Alpha Overexpression and a Hyperactivated Deacylation Pathway. *Cancer Res* (2014) 74(23):6867–77. doi: 10.1158/0008-5472.CAN-13-2409
39. Jia M, Andreassen T, Jensen L, Bathen TF, Sinha I, Gao H, et al. Estrogen Receptor Alpha Promotes Breast Cancer by Reprogramming Choline Metabolism. *Cancer Res* (2016) 76(19):5634–46. doi: 10.1158/0008-5472.CAN-15-2910
40. Thomas AM, Manghi P, Asnicar F, Pasolli E, Armanini F, Zolfo M, et al. Metagenomic Analysis of Colorectal Cancer Datasets Identifies Cross-Cohort Microbial Diagnostic Signatures and a Link With Choline Degradation. *Nat Med* (2019) 25(4):667–78. doi: 10.1038/s41591-019-0405-7
41. Wang Y, Zhang L, Chen WL, Wang JH, Li N, Li JM, et al. Rapid Diagnosis and Prognosis of *De Novo* Acute Myeloid Leukemia by Serum Metabonomic Analysis. *J Proteome Res* (2013) 12(10):4393–401. doi: 10.1021/pr400403p
42. Musharraf SG, Siddiqui AJ, Shamsi T, Choudhary MI, Rahman AU. Serum Metabonomics of Acute Leukemia Using Nuclear Magnetic Resonance Spectroscopy. *Sci Rep* (2016) 6:30693. doi: 10.1038/srep30693
43. Lo Presti C, Fauvelle F, Mondet J, Mossuz P. The Differential Activation of Metabolic Pathways in Leukemic Cells Depending on Their Genotype and Micro-Environmental Stress. *Metabolomics* (2020) 16(1):13. doi: 10.1007/s11306-020-1633-z
44. Gallego-Ortega D, Ramirez de Molina A, Ramos MA, Valdes-Mora F, Barderas MG, Sarmentero-Estrada J, et al. Differential Role of Human Choline Kinase Alpha and Beta Enzymes in Lipid Metabolism: Implications in Cancer Onset and Treatment. *PloS One* (2009) 4(11):e7819. doi: 10.1371/journal.pone.0007819
45. Dean JM, Lodhi IJ. Structural and Functional Roles of Ether Lipids. *Protein Cell* (2018) 9(2):196–206. doi: 10.1007/s13238-017-0423-5
46. Vijay R, Hua X, Meyerholz DK, Miki Y, Yamamoto K, Gelb M, et al. Critical Role of Phospholipase A2 Group Iid in Age-Related Susceptibility to Severe Acute Respiratory Syndrome-Cov Infection. *J Exp Med* (2015) 212(11):1851–68. doi: 10.1084/jem.20150632
47. Wong LR, Zheng J, Wilhelmsen K, Li K, Ortiz ME, Schnicker NJ, et al. Eicosanoid Signaling Blockade Protects Middle-Aged Mice From Severe Covid-19. *Nature* (2022) 605(7908):146–51. doi: 10.1038/s41586-022-04630-3
48. Liu H, Xu R, Gao C, Zhu T, Liu L, Yang Y, et al. Metabolic Molecule Pla2g2d Is a Potential Prognostic Biomarker Correlating With Immune Cell Infiltration and the Expression of Immune Checkpoint Genes in Cervical Squamous Cell Carcinoma. *Front Oncol* (2021) 11:755668. doi: 10.3389/fonc.2021.755668
49. Williams KA, Lee M, Hu Y, Andreas J, Patel SJ, Zhang S, et al. A Systems Genetics Approach Identifies Cxcl14, Itgax, and Lpcat2 as Novel Aggressive Prostate Cancer Susceptibility Genes. *PloS Genet* (2014) 10(11):e1004809. doi: 10.1371/journal.pgen.1004809
50. Cotte AK, Aires V, Fredon M, Limagne E, Derangere V, Thibaudin M, et al. Lysophosphatidylcholine Acyltransferase 2-Mediated Lipid Droplet Production Supports Colorectal Cancer Chemoresistance. *Nat Commun* (2018) 9(1):322. doi: 10.1038/s41467-017-02732-5

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REVIEW
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Arginase: Mechanisms and Clinical Application in Hematologic Malignancy

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Compared to normal tissues and cells, the metabolic patterns of tumor illnesses are more complex, and there are hallmarks of metabolic reprogramming in energy metabolism, lipid metabolism, and amino acid metabolism. When tumor cells are in a state of fast growth, they are susceptible to food shortage, resulting in growth suppression. Using this metabolic sensitivity of tumor cells to construct amino acid consumption therapy does not harm the function of normal cells, which is the focus of metabolic therapy research at the moment. As a non-essential amino acid, arginine is involved in numerous crucial biological processes, including the signaling system, cell proliferation, and material metabolism. Rapidly dividing tumor cells are more likely to be deficient in arginine; hence, utilizing arginase to consume arginine can suppress tumor growth. Due to the absence of arginine succinate synthase, arginine succinate lyase, and ornithine carbamoyl transferase in some blood tumors, arginases may be employed to treat blood tumors. By investigating the mechanism of arginase treatment and the mechanism of drug resistance in greater depth, arginase treatment becomes more successful in hematological cancers and a new anti-cancer agent in clinical practice.

Keywords: arginase, hematologic malignancy, mechanisms, clinical application, therapy

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INTRODUCTION

As a heterogeneous disease, the metabolic patterns of tumors differ significantly from normal tissues (1). Metabolic reprogramming of tumor cells refers to the metabolic changes caused by gene mutations and structural changes in tumor cells, such as enhanced glycolysis exemplified by the 'Warburg effect,' increased glucose uptake and consumption, increased amino acid uptake and catabolism such as glutamine, and increased lipid and protein synthesis. These metabolic alterations will contribute to the malignant proliferation of tumor cells and the adaptation to an unfavorable living environment (2–6). The distinction between tumor cell and normal cell metabolic pathways encourages the development of antitumor therapies based on metabolic pathways (7). Metabolic reprogramming allows tumor cells to thrive in the microenvironment of nutritional deficiency and hypoxia, but there are also metabolic vulnerabilities. Compared to normal cells, tumor cells are dependent on exogenous nutrients, especially amino acids (8, 9). The metabolic reliance of tumor

cells on exogenous amino acids has become the theoretical foundation for the creation of amino acid deprivation therapy (9).

Arginine is a multifunctional amino acid, involved in many important biological functions in the body, including cell proliferation, signal transduction, muscle growth, nerve transmission, etc., involved in protein synthesis, nucleotide synthesis, urea cycle and other important metabolic pathways, and as a prerequisite for the synthesis of many molecular substances such as citrulline, nitric oxide, glutamic acid, proline biosynthesis (10, 11). Arginine is also classified as a semi-essential amino acid in human body. Under normal physiological conditions, there are endogenous and exogenous sources of arginine, that is, the production of arginine from the starting through ornithine cycle and the uptake of extracellular arginine by cells through membrane protein transporters (12). The key enzymes for arginine synthesis and metabolism mainly include arginine succinate synthase (ASS), arginine succinate lyase (ASL) and ornithine carbamoyl transferase (OCT) (**Figure 1**). The low expression of these key enzymes makes it impossible for many malignant tumors to synthesize arginine endogenously and become arginine deficient tumor cells, including many hematological malignancies (13). Arginine auxotrophic tumors can be treated by arginine-depleting enzymes without interfering with normal cell physiology, thus becoming a potential antitumor strategy (14).

As an enzyme that depletes arginine, arginase has become a crucial component of arginine depletion treatment. It has been shown to have an anti-tumor impact on hematological malignancies, mostly by triggering autophagy, apoptosis, oxidative stress, and even interfering with the progression of the cell cycle to induce tumor cell death. As a result, this introduction focuses on the current state of arginase application in hematological cancers, associated research mechanisms, and its potential as a treatment for hematological tumors.

ARGININE NUTRITIONAL DEFICIENCIES AND DEPRIVATION THERAPY

The fundamental aspect of tumor cells is metabolic reprogramming, which is to adapt to their rapid multiplication by modifying their metabolic processes, as well as the survival milieu of hypoxia and nutritional shortage (15). During the process of metabolic reprogramming, it is also susceptible to dietary deficiencies, particularly for the growth of essential

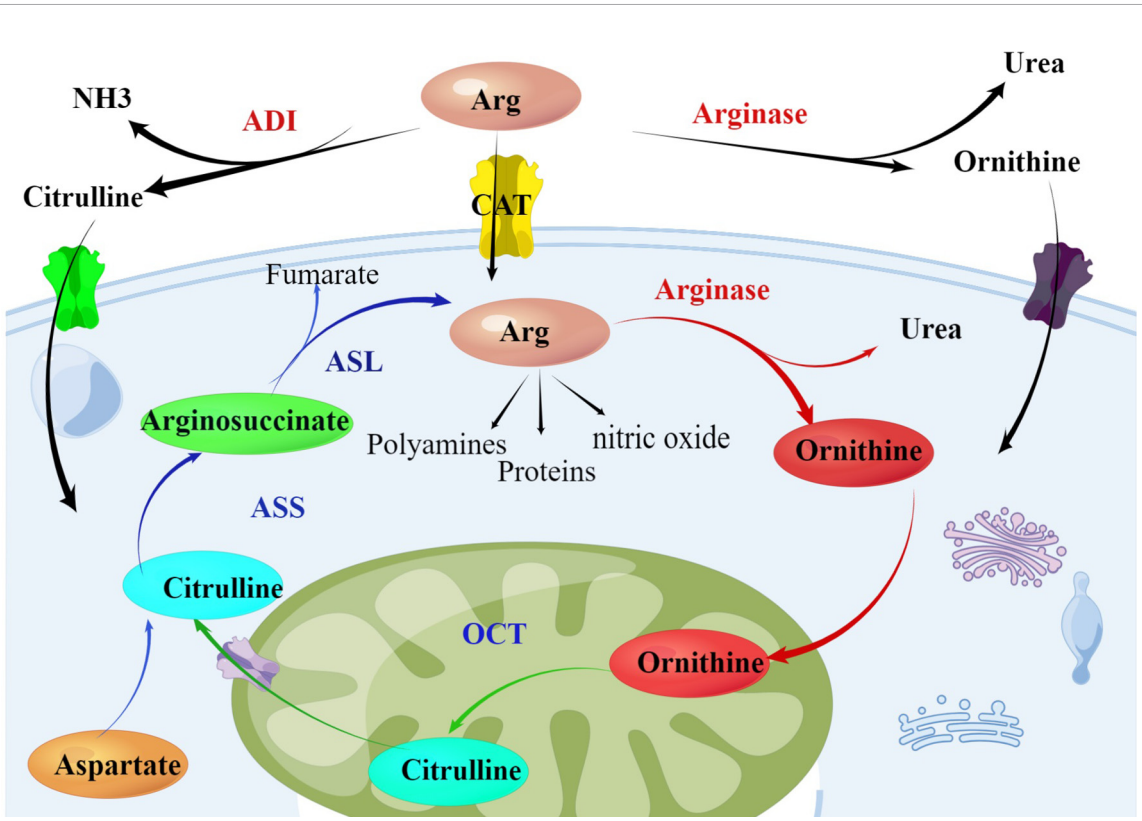

FIGURE 1 | Arginine metabolism within the urea cycle: Arginase catalyzes the hydrolysis of arginine into ornithine and OCT catalyzes the synthesis of citrulline from ornithine. Aspartate and citrulline are condensed by the enzymatic activity of ASS and the resulting intermediate arginosuccinate is then split by ASL, producing again arginine. Tumor cells lacking the enzyme ASS rely on exogenous supply of arginine. The endogenous sources of arginine can be used as a potential therapeutic intervention targets in treating cancer. Arg, Arginine; ADI, arginine deiminase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; OCT, ornithine carbamoyltransferase.

nutrients like amino acids (16). Currently, amino acid deprivation therapy for amino acid nutritional deficit has become a promising anti-tumor therapy and has yielded positive outcomes in numerous tumor treatments. The concept of amino acid deprivation was developed in the therapy of tumors, and L-asparaginase was launched more than 50 years ago for the treatment of juvenile leukemia. L-asparaginase is currently one of the most important medications for the combination chemotherapy of juvenile acute lymphoblastic leukemia, which enhances the remission rate of this disease (17, 18). As a result of the efficacy of asparaginase in the therapy of leukemia, there are numerous metabolic enzymes that investigate amino acid nutritional deficits, including enzymes that target arginine metabolism, glutamine, and cysteine.

As a semi-essential amino acid, arginine participates in numerous cellular biological processes, transforms into other amino acids (such as glutamine, proline, etc.), and creates proteins, urea, and agmatine (19).On a biological level, the primary sources of arginine in plasma are dietary digestion and absorption and other protein modification, whereas intestinal-kidney axis arginine synthesis contributes for just 5-15 percent. In the *de novo* synthesis of arginine, ornithine and carbamoyl phosphate are catalyzed by OCT to produce citrulline, which is subsequently catalyzed by ASS1 and ASL to produce L-arginine. The rate-limiting enzymes of endogenous arginine synthesis are ASS1 and ASL, which are typically expressed in normal cells and unaffected by arginine consumption (19, 20).

Due to the low or non-expression of essential enzymes in arginine endogenous synthesis, especially ASS1, ASL, and OCT, in numerous cancers, tumor cells cannot ordinarily generate endogenous arginine and are highly dependent on exogenous arginine; these tumors are referred to as arginine-auxotrophic. Studies reveal that more than 70 percent of cancers, including melanoma, hepatocellular carcinoma, renal cell carcinoma, glioblastoma, and prostate cancer, as well as a variety of hematological tumors, have arginine shortage (21–23). Cai Y et al. investigated the expression level of the ASS1 gene in various malignancies using the UALCAN database and determined that the expression level of ASS1 in acute myeloid leukemia (AML) was the lowest of all cancers, thereby making AML a typical arginine auxotrophic tumor (24). In addition to Hodgkin's lymphoma and non-Hodgkin's lymphoma, low expression of ASS1 was observed in additional solid hematological malignancies (22).

Current studies on the genetic mechanism of low expression of ASS1 gene have confirmed that methylation of CPG regions within the ASS1 gene promoter may be a common epigenetic mechanism of ASS1 gene silencing expression (23), or may include other mechanisms such as inhibition of ASS1 promoter by hypoxia inducible factor HIF-1α. Through the study of glioblastoma cases, Syed N et al. found that the CpG island of ASS1 with methylation was up-regulated in the sensitivity to arginine deprivation therapy, further verifying that the expression of ASS1 was affected by the methylation of gene promoter (25). In the study of melanoma cells by Tsai WB et al., it was found that the regulation of ASS1 expression was caused by the interaction between the negative regulator HIF-1α and the positive transcription regulator c-Myc and Sp4. HIF-1α combined with the E-box site at the promoter of ASS1 gene could induce the silence of ASS1 expression and cause arginine consumption sensitivity (26). These related studies further induced epigenetic mechanisms by exploring the expression of ASS1 and ASL genes, thus providing ideas for the development of new treatments.

Arginine depletion therapy for arginine-auxotrophic tumors by arginine depletion enzyme (ADE) paves the way for a new anti-tumor treatment. In scientific research of arginine metabolism, the primary degrading enzymes are arginine decarboxylase (ADC), arginine deiminase (ADI), and arginase. ADI is mostly generated from prokaryotes, and ornithine cannot be synthesized *via* the ADI route as a source of energy for their growth. Mycoplasma-derived ADI is the first antitumor medication to be documented. Even though it has some therapeutic effects, it is easily detected and inactivated by the immune system because of its immunogenicity. So ADI must be bioengineered into PEG-ADI in clinical settings to minimize its immunogenicity (27). ADC, an additional arginine-depriving enzyme, is mostly expressed in mammals. Due to its cytotoxicity to healthy normal cells, it is not employed as a cancer treatment at this time. In contrast to these two enzymes, the arginases currently under investigation are derived from humans and lack immunogenicity and high toxicity. Currently, it has been demonstrated that they have potential therapeutic benefits on a variety of solid and hematological malignancies (11, 28).

ARGINASE AND ITS APPLICATION IN HEMATOLOGICAL MALIGNANCY

Arginase is a manganese-dependent metal ion hydrolase that catalyzes arginine metabolism to generate non-toxic products ornithine and urea. Human tissues express two distinct kinds of arginase, arginase I and arginase II, which are encoded by two distinct genes on separate human chromosomes (29). Human arginase I is mostly found in the cytoplasm of liver cells and is primarily responsible for the consumption of excess ammonia in human tissues *via* the urea cycle, whereas human arginase II is a mitochondrial enzyme with high expression e.g.in kidney tissue. Arginase II is primarily involved in the creation of nitric oxide and polyamines, as opposed to the arginine urea cycle (30, 31).

In the initial investigation, the arginase was purified from animal liver; however, due to the high dose need of purified arginase treatment, further research and application are limited (32). Human arginase was PEGylated to produce recombinant PEGylated arginase (rhArg-peg), and the catalytic activity of arginase was enhanced by substituting cobalt ions for manganese ions. Its half-life and activity time were lengthened, and its immunogenicity was diminished (33, 34). RhArg has therapeutic potential for malignancies lacking OCT or ASS expression. According to studies, arginine deiminase is

ineffective in tumors expressing ASS, mostly because arginine can be synthesized from citrulline *via* ASS and ASL (33, 35).

Although arginase therapy in hematological malignancies has not yet reached the approved clinical stage, many studies have confirmed the potential therapeutic activity of arginase in hematological malignancies. Francis Mussai et al. confirmed that pegylated recombinant human arginase (BCT-100) led to rapid consumption of arginine inside and outside cells, thereby inducing cell cycle arrest and cell death. They further revealed that it is because of defective expression of ASS and OCT in AML cells (36). In addition to the therapeutic effect in AML cells, BCT-100 treatment can block the expression of cyclin D3 in cells, thereby inducing apoptosis of T- acute lymphoblastic leukemia (T-ALL) cells *in vitro* and *in vivo*, reflecting the potential therapeutic effect of BCT-100 on T-cell malignancies by consuming arginine (28). In addition, Carmela De Santo et al. showed that BCT-100 combined with dexamethasone had obvious cytotoxicity on acute lymphoblastic leukemia (ALL) cells. Although ALL cells up-regulated the expression of arginine transporter CAT-1 and endogenous ASS or OCT, it still could not prevent the cytotoxicity of BCT-100 (37). The combination of BCT-100 and cytarabine has been effective in the treatment of AML and ALL *in vivo* and *in vitro*, but drug resistance easily develops, so that further research is needed. In hematological solid tumors, Zeng X et al. demonstrated that recombinant human arginase can inhibit the growth of non-Hodgkin's lymphoma (NHL) cells by arginine deprivation, which may play an anti-tumor role by cell cycle arrest and caspase-dependent apoptosis (38).

EFFECT OF ARGINASE TREATMENT ON HEMATOLOGICAL MALIGNANCY

Using recombinant arginase to treat nutritionally deficient hematological cancers is expected to trigger cell death, which may be mediated by autophagy, cell cycle arrest, or oxidative stress. After treatment with recombinant human arginase, studies have demonstrated that apoptosis results in cell death in ALL, but AML exhibits caspase-independent and non-apoptotic cell death (39). In the research of the influence of BCT-100 on the cell cycle and proliferation, Francis Mussai et al. discovered that the G0/G1 phase of AML cells treated with BCT-100 remained unchanged. Compared to untreated cells, the expression of cyclin A was substantially elevated, whereas the expression of cyclins B and E was lowered, resulting in cell cycle arrest, necrosis, and death (36).

The increased generation of ROS is the cause of tumor cell death produced by BCT-100. Studies demonstrate that BCT-100 therapy further induces ROS generation in bladder cancer cells, blocking phosphorylation of AKT and mTOR and triggering autophagy and death of tumor cells (39). S. Xu et al. also discovered that rhArg slows tumor cell proliferation *via* autophagy, cell cycle arrest, and oxidative stress in the research on hepatocellular carcinoma and breast cancer (40).

STUDY ON THE RESISTANCE MECHANISM OF ARGINASE

In arginase-treated cells, the expression of ASS1 is restored and up-regulated, allowing arginine-deprived tumor cells to recover endogenous arginine synthesis. c-MYC is a positive regulator of ASS1 expression, and its activation is the primary cause of ASS1 reexpression (41). Arginine deprivation enhances ROS generation in the tumor microenvironment, which in turn activates Gas6/Axl to initiate the PI3K/Akt/GSK3B pathway, resulting in c-Myc oncogene expression upregulation (42). By replacing the negative regulator HIF-1a of the ASS1 gene promoter and competitively occupying the E box of the promoter, up-regulated c-Myc restores and up-regulates the expression of the ASS1 gene, thereby restoring the endogenous synthesis of arginine (43).

Dietary restriction can trigger autophagy in cells. Through autophagy, cells can recruit damaged proteins and defective organelles into autophagosomes for digestion and breakdown misfolded proteins or organelles by binding to lysosomes, thereby supplying the energy and materials necessary for cell survival. Also present in arginase treatment is autophagy. Short-term arginine deprivation therapy decreases ATP and NO levels, induces endoplasmic reticulum stress by activating the mTOR pathway, promotes cell autophagy, and nourishes cells with critical nutrients. In the absence of arginine for an extended period of time, cancer cells can activate the Bax-mediated apoptotic pathway or re-express the ASS gene to produce resistance to arginases (44, 45). Zeng X et al. confirmed that recombinant human arginase activated autophagy in lymphoma cells, human promyelocytic leukemia cells, and human T-ALL cells, which served a protective function (38). In practice, the combination of autophagy inhibitors (such as 3-methyladenine and chloroquine) can augment the anti-tumor activity of arginase and induce apoptosis in tumor cells (46).

In addition to the aforementioned potential resistance mechanisms, arginase therapy is associated with the activation of several metabolic pathways. During the development of arginase resistance, proteomics reveals that the cellular metabolism shifts from glutamine dependence to glucose dependence (47, 48). Several investigations have demonstrated that once the arginase consumes arginine, cell growth becomes dependent on aspartic acid. Aspartic acid is the precursor of pyrimidine nucleotides and can be used as the substrate for arginine synthesis by ASS. Therefore, arginase therapy should be combined with different metabolic inhibition treatments to achieve better therapeutic effects, which requires additional research (48).

CONCLUSION

As a semi-essential amino acid in the human body, arginine participates in a variety of vital biological processes. By arginine-degrading enzymes, arginine depletion therapy can

limit the growth of nutritionally deficient tumors. Hematological cancers, including AML, ALL and various lymphomas, have been shown to respond favorably to this treatment. Through recombinant engineering, arginase has been transformed into pegylated recombinant human arginase, which has a longer half-life, and less adverse effects. Currently, the application of arginase to hematological cancers has entered the clinical research phase, and additional investigation is required to confirm the therapeutic benefit. Drug resistance mechanisms of arginase therapy is still in its exploratory phase, and more study is required to clarify it. To develop more effective treatment options for hematological cancers based on arginase, more in-depth discussion and study is required.

AUTHOR CONTRIBUTIONS

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REFERENCES

1. Judge A, Dodd MS. Metabolism. *Essays Biochem* (2020) 64:607–47. doi: 10.1042/EBC20190041
2. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
3. Callao V, Montoya E. Toxohormone-Like Factor From Microorganisms With Impaired Respiration. *Science* (1961) 134:2041–2. doi: 10.1126/science.134.3495.2041
4. Lunt SY, Vander Heiden MG. Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation. *Annu Rev Cell Dev Biol* (2011) 27:441–64. doi: 10.1146/annurev-cellbio-092910-154237
5. Xia L, Oyang L, Lin J, Tan S, Han Y, Wu N, et al. The Cancer Metabolic Reprogramming and Immune Response. *Mol Cancer* (2021) 20:28. doi: 10.1186/s12943-021-01316-8
6. Sun L, Song L, Wan Q, Wu G, Li X, Wang Y, et al. Cmyc-Mediated Activation of Serine Biosynthesis Pathway is Critical for Cancer Progression Under Nutrient Deprivation Conditions. *Cell Res* (2015) 25:429–44. doi: 10.1038/cr.2015.33
7. Zhang Y, Yang JM. Altered Energy Metabolism in Cancer: A Unique Opportunity for Therapeutic Intervention. *Cancer Biol Ther* (2013) 14:81–9. doi: 10.4161/cbt.22958
8. Icard P, Lincet H. A Global View of the Biochemical Pathways Involved in the Regulation of the Metabolism of Cancer Cells. *Biochim Biophys Acta* (2012) 1826:423–33. doi: 10.1016/j.bbcan.2012.07.001
9. Scott L, Lamb J, Smith S, Wheatley DN. Single Amino Acid (Arginine) Deprivation: Rapid and Selective Death of Cultured Transformed and Malignant Cells. *Br J Cancer* (2000) 83:800–10. doi: 10.1054/bjoc.2000.1353
10. Oberkersch RE, Santoro MM. Role of Amino Acid Metabolism in Angiogenesis. *Vascul Pharmacol* (2019) 112:17–23. doi: 10.1016/j.vph.2018.11.001
11. Feun LG, Kuo MT, Savaraj N. Arginine Deprivation in Cancer Therapy. *Curr Opin Clin Nutr Metab Care* (2015) 18:78–82. doi: 10.1097/MCO.0000000000000122
12. Jahani M, Noroznezhad F, Mansouri K. Arginine: Challenges and Opportunities of This Two-Faced Molecule in Cancer Therapy. *BioMed Pharmacother* (2018) 102:594–601. doi: 10.1016/j.biopha.2018.02.109
13. Caldwell RB, Toque HA, Narayanan SP, Caldwell RW. Arginase: An Old Enzyme With New Tricks. *Trends Pharmacol Sci* (2015) 36:395–405. doi: 10.1016/j.tips.2015.03.006
14. Lukey MJ, Katt WP, Cerione RA. Targeting Amino Acid Metabolism for Cancer Therapy. *Drug Discov Today* (2017) 22:796–804. doi: 10.1016/j.drudis.2016.12.003
15. Boroughs LK, DeBerardinis RJ. Metabolic Pathways Promoting Cancer Cell Survival and Growth. *Nat Cell Biol* (2015) 17:351–9. doi: 10.1038/ncb3124
16. Garcia-Bermudez J, Williams RT, Guarecuco R, Birsoy K. Targeting Extracellular Nutrient Dependencies of Cancer Cells. *Mol Metab* (2020) 33:67–82. doi: 10.1016/j.molmet.2019.11.011
17. Jaffe N, Traggis D, Das L, Frauenberger G, Hann HW, Kim BS, et al. Favorable Remission Induction Rate With Twice Weekly Doses of L-Asparaginase. *Cancer Res* (1973) 33:1–4.
18. Richards NG, Kilberg MS. Asparagine Synthetase Chemotherapy. *Annu Rev Biochem* (2006) 75:629–54. doi: 10.1146/annurev.biochem.75.103004.142520
19. Fultang L, Vardon A, De Santo C, Mussai F. Molecular Basis and Current Strategies of Therapeutic Arginine Depletion for Cancer. *Int J Cancer* (2016) 139:501–9. doi: 10.1002/ijc.30051
20. Husson A, Brasse-Lagnel C, Fairand A, Renouf S, Lavoinne A. Argininosuccinate Synthetase From the Urea Cycle to the Citrulline-NO Cycle. *Eur J Biochem* (2003) 270:1887–99. doi: 10.1046/j.1432-1033.2003.03559.x
21. Ascierto PA, Scala S, Castello G, Daponte A, Simeone E, Ottaiano A, et al. Pegylated Arginine Deiminase Treatment of Patients With Metastatic Melanoma: Results From Phase I and II Studies. *J Clin Oncol* (2005) 23:7660–8. doi: 10.1200/JCO.2005.02.0933
22. Delage B, Luong P, Maharaj L, O'Riain C, Syed N, Crook T, et al. Promoter Methylation of Argininosuccinate Synthetase-1 Sensitises Lymphomas to Arginine Deiminase Treatment, Autophagy and Caspase-Dependent Apoptosis. *Cell Death Dis* (2012) 3:e342. doi: 10.1038/cddis.2012.83
23. Qiu F, Huang J, Sui M. Targeting Arginine Metabolism Pathway to Treat Arginine-Dependent Cancers. *Cancer Lett* (2015) 364:1–7. doi: 10.1016/j.canlet.2015.04.020
24. Cai Y, Chow JPH, Leung YO, Lu X, Yuen CH, Lee WL, et al. NEI-01-Induced Arginine Deprivation Has Potent Activity Against Acute Myeloid Leukemia Cells Both *In Vitro* and In Vivo. *Mol Cancer Ther* (2021) 20:2218–27. doi: 10.1158/1535-7163.MCT-21-0120
25. Syed N, Langer J, Janczar K, Singh P, Lo Nigro C, Lattanzio L, et al. Epigenetic Status of Argininosuccinate Synthetase and Argininosuccinate Lyase Modulates Autophagy and Cell Death in Glioblastoma. *Cell Death Dis* (2013) 4:e458. doi: 10.1038/cddis.2012.197
26. Tsai WB, Aiba I, Lee SY, Feun L, Savaraj N, Kuo MT. Resistance to Arginine Deiminase Treatment in Melanoma Cells is Associated With Induced Argininosuccinate Synthetase Expression Involving C-Myc/HIF-1alpha/Sp4. *Mol Cancer Ther* (2009) 8:3223–33. doi: 10.1158/1535-7163.MCT-09-0794
27. Veronese FM, Mero A. The Impact of PEGylation on Biological Therapies. *BioDrugs* (2008) 22:315–29. doi: 10.2165/00063030-200822050-00004
28. Hernandez CP, Morrow K, Lopez-Barcons LA, Zabaleta J, Sierra R, Velasco C, et al. Pegylated Arginase I: A Potential Therapeutic Approach in T-ALL. *Blood* (2010) 115:5214–21. doi: 10.1182/blood-2009-12-258822
29. Vockley JG, Jenkinson CP, Shukla H, Kern RM, Grody WW, Cederbaum SD. Cloning and Characterization of the Human Type II Arginase Gene. *Genomics* (1996) 38:118–23. doi: 10.1006/geno.1996.0606
30. Krebs HA, Hems R, Lund P. Accumulation of Amino Acids by the Perfused Rat Liver in the Presence of Ethanol. *Biochem J* (1973) 134:697–705. doi: 10.1042/bj1340697
31. Morris SM Jr, Bhamidipati D, Kepka-Lenhart D. Human Type II Arginase: Sequence Analysis and Tissue-Specific Expression. *Gene* (1997) 193:157–61. doi: 10.1016/S0378-1119(97)00099-1

32. Bach SJ, Hawkins RA, Swaine D. A Short Method for the Purification of Arginase From Ox Liver. *Biochem J* (1963) 89:263–5. doi: 10.1042/bj0890263
33. Cheng PN, Lam TL, Lam WM, Tsui SM, Cheng AW, Lo WH, et al. Pegylated Recombinant Human Arginase (Rharg-Peg5,000mw) Inhibits the *In Vitro* and *In Vivo* Proliferation of Human Hepatocellular Carcinoma Through Arginine Depletion. *Cancer Res* (2007) 67:309–17. doi: 10.1158/0008-5472.CAN-06-1945
34. Stone EM, Glazer ES, Chantranupong L, Cherukuri P, Breece RM, Tierney DL, et al. Replacing Mn(2+) With Co(2+) in Human Arginase I Enhances Cytotoxicity Toward L-Arginine Auxotrophic Cancer Cell Lines. *ACS Chem Biol* (2010) 5:333–42. doi: 10.1021/cb900267j
35. Yoon JK, Frankel AE, Feun LG, Ekmekcioglu S, Kim KB. Arginine Deprivation Therapy for Malignant Melanoma. *Clin Pharmacol* (2013) 5:11–9. doi: 10.2147/CPAA.S37350
36. Mussai F, Egan S, Higginbotham-Jones J, Perry T, Beggs A, Odintsova E, et al. Arginine Dependence of Acute Myeloid Leukemia Blast Proliferation: A Novel Therapeutic Target. *Blood* (2015) 125:2386–96. doi: 10.1182/blood-2014-09-600643
37. De Santo C, Booth S, Vardon A, Cousins A, Tubb V, Perry T, et al. The Arginine Metabolome in Acute Lymphoblastic Leukemia can be Targeted by the Pegylated-Recombinant Arginase I BCT-100. *Int J Cancer* (2018) 142:1490–502. doi: 10.1002/ijc.31170
38. Zeng X, Li Y, Fan J, Zhao H, Xian Z, Sun Y, et al. Recombinant Human Arginase Induced Caspase-Dependent Apoptosis and Autophagy in non-Hodgkin's Lymphoma Cells. *Cell Death Dis* (2013) 4:e840. doi: 10.1038/cddis.2013.359
39. Zhao Z, Zhang P, Li W, Wang D, Ke C, Liu Y, et al. Pegylated Recombinant Human Arginase 1 Induces Autophagy and Apoptosis *via* the ROS-Activated AKT/mTOR Pathway in Bladder Cancer Cells. *Oxid Med Cell Longev* (2021) 2021:5510663. doi: 10.1155/2021/5510663
40. Xu S, Lam SK, Cheng PN, Ho JC. Recombinant Human Arginase Induces Apoptosis Through Oxidative Stress and Cell Cycle Arrest in Small Cell Lung Cancer. *Cancer Sci* (2018) 109:3471–82. doi: 10.1111/cas.13782
41. Long Y, Tsai WB, Wangpaichitr M, Tsukamoto T, Savaraj N, Feun LG, et al. Arginine Deiminase Resistance in Melanoma Cells is Associated With Metabolic Reprogramming, Glucose Dependence, and Glutamine Addiction. *Mol Cancer Ther* (2013) 12:2581–90. doi: 10.1158/1535-7163.MCT-13-0302
42. Kuo MT, Long Y, Tsai WB, Li YY, Chen HHW, Feun LG, et al. Collaboration Between RSK-EphA2 and Gas6-Axl RTK Signaling in Arginine Starvation Response That Confers Resistance to EGFR Inhibitors. *Transl Oncol* (2020) 13:355–64. doi: 10.1016/j.tranon.2019.12.003
43. Roeksomtawin S, Navasumrit P, Waraprasit S, Parnlob V, Sricharunrat T, Bhudhisawasdi V, et al. Decreased Argininosuccinate Synthetase Expression in Thai Patients With Cholangiocarcinoma and the Effects of ADI-PEG20 Treatment in CCA Cell Lines. *Oncol Lett* (2018) 16:1529–38. doi: 10.3892/ol.2018.8807
44. Chen CL, Hsu SC, Ann DK, Yen Y, Kung HJ. Arginine Signaling and Cancer Metabolism. *Cancers (Basel)* (2021) 13(14):3541. doi: 10.3390/cancers13143541
45. Changou CA, Chen YR, Xing L, Yen Y, Chuang FY, Cheng RH, et al. Arginine Starvation-Associated Atypical Cellular Death Involves Mitochondrial Dysfunction, Nuclear DNA Leakage, and Chromatin Autophagy. *Proc Natl Acad Sci USA* (2014) 111:14147–52. doi: 10.1073/pnas.1404171111
46. Li Y, Zeng X, Wang S, Fan J, Wang Z, Song P, et al. Blocking Autophagy Enhanced Leukemia Cell Death Induced by Recombinant Human Arginase. *Tumour Biol* (2016) 37:6627–35. doi: 10.1007/s13277-015-4253-x
47. Brashears CB, Barlin M, Ehrhardt WR, Rathore R, Schultze M, Tzeng SC, et al. Systems Level Profiling of Arginine Starvation Reveals MYC and ERK Adaptive Metabolic Reprogramming. *Cell Death Dis* (2020) 11:662. doi: 10.1038/s41419-020-02899-8
48. Rabinovich S, Adler L, Yizhak K, Sarver A, Silberman A, Agron S, et al. Diversion of aspartate in ASS1-deficient tumours fosters *de novo* pyrimidine synthesis. *Nature* (2015) 527:379–83. doi: 10.1038/nature15529

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REVIEW
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The Molecular Mechanisms of Resistance to IDH Inhibitors in Acute Myeloid Leukemia

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Gain-of-function mutations of isocitrate dehydrogenases 1/2 (IDH1/2) play crucial roles in the development and progression of acute myeloid leukemia (AML), which provide promising therapeutic targets. Two small molecular inhibitors, ivosidenib and enasidenib have been approved for the treatment of IDH1- and IDH2-mutant AML, respectively. Although these inhibitors benefit patients with AML clinically, drug resistance still occurs and have become a major problem for targeted therapies of IDH-mutant AML. A number of up-to-date studies have demonstrated molecular mechanisms of resistance, providing rationales of novel therapeutic strategies targeting mutant IDH1/2. In this review, we discuss mechanisms of resistance to ivosidenib and enasidenib in patients with AML.

Keywords: cancer metabolism, IDH1, IDH2, acute myeloid leukemia, drug resistance

INTRODUCTION

Isocitrate dehydrogenases 1/2 (IDH1/2) are metabolic enzymes catalyzing the oxidative decarboxylation of isocitrate to α-KG and reducing NAD(P)+ to NAD(P)H in the tricarboxylic acid (TCA) cycle (1). In human cells, IDH1 localizes to peroxisome and cytoplasm (2, 3) and IDH2 localizes to mitochondria (4). IDH1 plays a prominent role in glucose sensing (5) and lipid metabolism (6), while IDH2 is involved in regulating oxidative respiration. Thus, both IDH1 and IDH2 are thought to play key roles in cellular metabolisms. Also, the activity of IDH1/2 confers protection from oxidative damage, since NADPH is involved in reducing glutathione by glutathione reductase and α-KG is implicated as a potent antioxidant (7).

Mutations to IDH1/2 are important events in several types of cancers, including acute myeloid leukemia (AML), glioma, angio-immunoblastic T-cell lymphoma, chondrosarcoma, intrahepatic cholangiocarcinoma, and so on (8–11). In AML, IDH1/2 mutations were found in 16~33% patients, with R132H accounting for over 93% of IDH1 variants and R140Q/R172K being predominant in IDH2 variants (12, 13). These mutations gain a neomorphic catalytic function that converts α-KG to the oncometabolite R-2-hydroxyglutarate (2-HG) (14). The accumulating 2-HG, acting as a competitive inhibitor of α-KG, occupies the catalytic sites of multiple α-KG-dependent dioxygenases to inhibit their catalytic activity competitively. The potential targets of 2-HG inhibition that have acquired significant attention mainly include JmjC domain-containing

histone demethylases (JmjC KDMs), tet methylcytosine dioxygenase 2 (TET2), and prolyl hydroxylases (PHD). JmjC KDMs and TET2 regulate gene expression by participating in histone and DNA demethylation, respectively, which contributes to the blockade of cell differentiation and the development of cancers. In addition, PHDs, the regulatory proteins catalyzing degradation of hypoxia-inducible factor 1α (HIF-1α), also can be inhibited by 2-HG that promotes oncogenesis and tumor progression (15).

Multiple studies have investigated the impact of IDH1/2 mutations on the prognosis of AML, with inconclusive results. Generally, IDH1 mutations are associated with an inferior outcome and IDH2 mutations are associated with a relatively favorable prognosis in AML (12, 16–19). In AML, mutant IDH1/2 serves as a poor prognostic factor in cytogenetically normal (CN)-AML with mutant NPM1 without FLT3-ITD (12). Several studies analyzed the specific clinical characteristics of AML patients with IDH1/2 mutations, and reported that IDH1/2 mutations are associated with old age, low WBC, high platelets, normal cytogenetics, and mutant NPM1 (12).

The discovery of IDH mutations in cancers promotes the rapid development of targeted inhibitors. Enasidenib (AG-221) and ivosidenib (AG-120) are inhibitors approved by FDA for the treatment of refractory or relapsed R/R AML with IDH2 or IDH1 mutations (20). Vorasidenib is a potent, oral, brain-penetrant dual inhibitor targeting both IDH1 and IDH2 mutants, which is undergoing a phase III INDIGO study (NCT04164901) in patients with residual or recurrent grade II glioma (21). A first-in-human phase I study (NCT02492737) of vorasidenib demonstrated well tolerability and preliminary antitumor activity in patients with low-grade gliomas (22).

Enasidenib and ivosidenib are first-in-class small molecule inhibitors targeting mutant IDH2 or IDH1. Biochemical and cellular analyses showed that ivosidenib was a highly selective inhibitor of IDH1, with no inhibition to IDH2 at micromolar concentrations. Preclinical data demonstrated that the treatment of ivosidenib significantly decreases the level of 2-HG in tumor models and promotes differentiation of primary human AML blast cells (23). Notably, ivosidenib and enasidenib demonstrated excellent clinical efficacy in IDH1/2-mutated R/R AML patients, with overall response rates (ORRs) of 41.6% and 40.3%, respectively, total CR rates of 21.6% and 19.3%, respectively, and median overall survival (mOS) of 8.8 months and 9.3 months, respectively. The median event-free survival (mEFS) duration for enasidenib-treated AML patients was 6.4 months. Vorasidenib showed preliminary antitumor activity in recurrent or progressive non-enhancing IDH1/2-mutated low-grade glioma with an objective response rate of 18% and a median progression-free survival of 36.8 months.

Although enasidenib or ivosidenib have remarkable advantages as therapeutic agents in the treatment of R/R AML, drug-resistance inevitably occurs, rendering disease progression. Some AML patients with IDH mutations have no response to monotherapy with mIDH inhibitors, and some patients relapsed with elevated circulating levels of 2-HG and acquired resistance to IDH-targeted therapies. The mechanism of drug-resistance is complex. In this review, we discuss the mechanisms of resistance to enasidenib or ivosidenib and potential strategies to overcome these mechanisms (**Figure 1**).

SECONDARY IDH MUTATIONS

Allosteric inhibitors of IDH mutations exhibit similar binding characteristics, which was validated by using structure modeling. enasidenib was confirmed to bind to an allosteric site of mIDH2 within the homodimer interface, which stabilized the open conformation of mutant enzyme and suppressed production of 2-HG. Hydrogen bonds and hydrophobic interactions anchor enasidenib binding with Q316 residues of mIDH2 (24). In addition, interactions between enasidenib and other surrounding residuals including D312, W164, V294, V297, L298, V315, I319, and L320 also contribute to high inhibitory of potency (24). A recent case report showed two patients with gain resistance to enasidenib relapsed for secondary IDH2 mutations including Q316E and I319M. The structure modeling revealed that Q316 mutation led to diminished hydrogen bonds between enasidenib and IDH2, whereas I319M mutation led to steric hindrance for the bulky side chain. Another study described an AML patient with initial IDH1-R132H mutation who had a clinical response to ivosidenib, followed by relapse. Subsequent sequencing showed emergence of a secondary IDH1-S280F known as the cause of drug-resistance. The structure modeling confirmed that S280F mutation created steric hindrance between ivosidenib and mIDH1 dimer interface. These studies provide direct evidence for the resistance mechanism of second-site mutations, such as Q316E and I319M in IDH2 mutations and S280F in IDH1 mutations, that result in therapeutic resistance.

LEUKEMIA STEMNESS

Accumulating evidence has confirmed that cancer derives from cancer stem cells, a population of self-renewal cells that contribute to resistance to multiple therapies (25). Multipronged genomic analysis reveals the promoters of genes related to transcriptional regulation of leukemia stemness exhibit significant hypermethylation, which is closely related to primary resistance to IDH inhibitors (26). The molecular drivers of hypermethylated phenotype including FOXC1, CD99 and DNMT3A are identified as critical regulators of leukemia stemness. Additionally, targeting sequencing indicated co-occurring mutations of transcription factors related to hematopoietic differentiation including RUNX1, CEBPA and GATA2, are also associated with significantly worse response to IDH inhibitors (IDHi). Multi-logistic regression analysis showed that increased stemness is one of the mechanisms of IDHi primary resistance, and the score of stemness can be used as a potential predictive biomarker for IDH inhibitor response (26).

The Wnt/β-catenin signaling pathway, key components of the cascade for maintaining cancer cell stemness, participates in diverse physiological processes including proliferation,

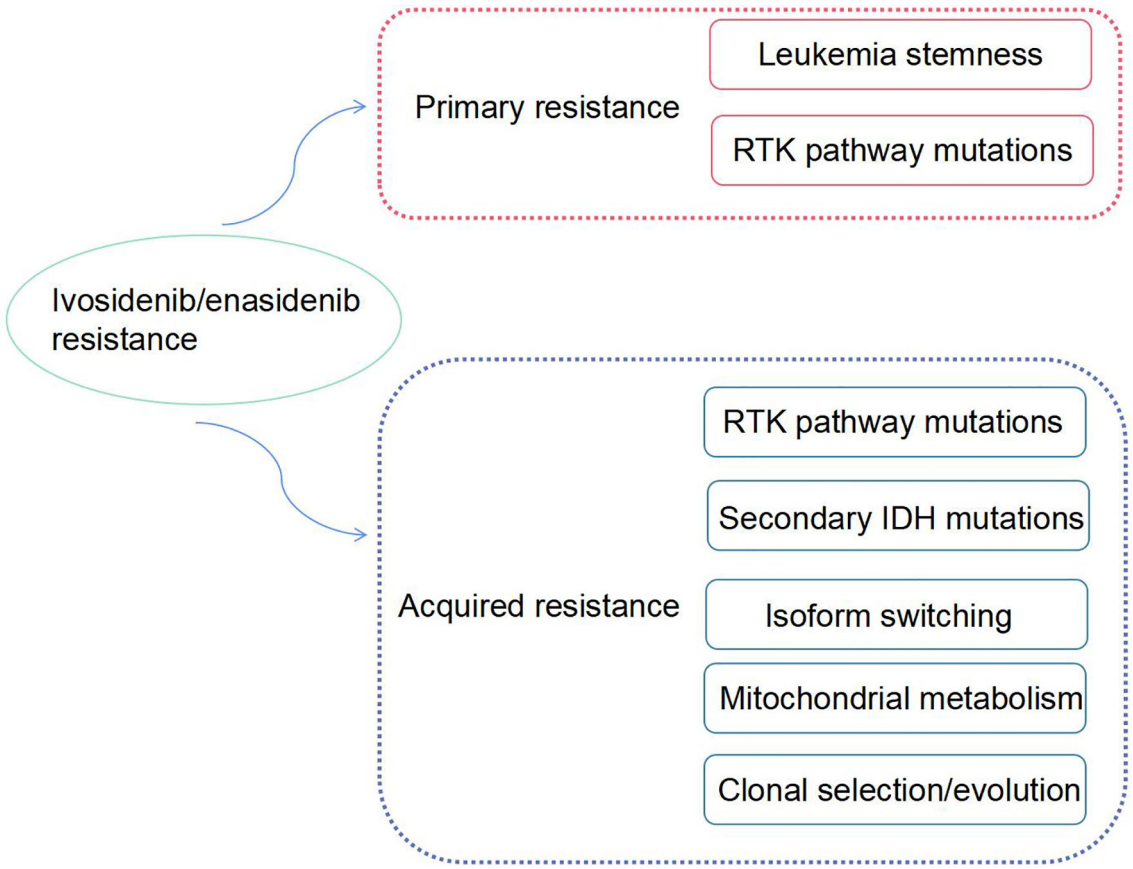

FIGURE 1 | The mechanisms of ivosidenib/enasidenib resistance in AML.

differentiation, apoptosis, invasion, migration, and tissue homeostasis, and its deregulation is closely related to initiation and progression of tumors (27–29). Whereas, 2-HG induces the hypermethylated inhibitory signals of Wnt, which lead to the improvement of stemness (30). Accumulating 2-HG resulted from IDH mutations improves leukemia stemness to block cell differentiation, which induces primary resistance to IDH inhibitors.

ISOFORM SWITCHING

Both IDH1 and IDH2 mutations have been reported in cancers, whereas usually only one mutation is identified in a certain cancer (31, 32). An AML patient with IDH mutation is usually treated by one type of small molecular inhibitor, either enasidenib or ivosidenib, to block disease progression. However, Harding et al. described four patients identifying IDH mutation isoform switching, either from mutant IDH1 to mutant IDH2 or vice versa (33).

Importantly, in this case report, two R/R AML patients with initial IDH1-R132C mutation achieved durable remissions with therapy of ivosidenib, but leukemia cells recurred with emergence of neomorphic mutation IDH2-R140Q. The third patient who suffered from treatment-refractory intrahepatic cholangiocarcinoma with IDH1-R132C obtained a sustained partial response to ivosidenib. The disease progressed subsequently with emergence of a new IDH2-R172V mutation. The fourth case, an R/R AML patient with initial IDH2-R140Q mutation achieved a durable remission with enasidenib, but disease progression occured with emergence of a new IDH1-R132C mutation which was sensitive to combined blockade IDH1/2 by vorasidenib. The isoform switching from IDH1 mutations to IDH2 mutations or vice versa were accompanied with elevated levels of 2-HG and disease progression. Therefore, isoform switching of IDH mutations is identified as one of the mechanisms of acquired resistance to IDH-targeted inhibitors. However, the precise frequency of isoform switching of IDH mutation remain unclear, which is essential to be determined in studies with large populations.

RTK PATHWAY MUTATIONS

Choe et al. performed a comprehensive genomic analysis in a large population of R/R AML patients carrying IDH mutations who were treated by ivosidenib, and confirmed that RTK

pathway mutations are associated with primary resistance to ivosidenib. They performed co-occurring mutation profiling in these patients by NGS and found that baseline mutations of the individual RTK pathway genes including NRAS and PTPN11, and in the grouped RTK pathway genes including NRAS, KRAS, PTPN11, KIT, and FLT3, were implicated with an importantly lower likelihood of achievement of CR or CRh as a best response to ivosidenib (34). These results are consistent with previous work, in which NRAS mutation is associated with a worse response to enasidenib in R/R AML patients with mIDH2 (35). Therefore, co-occurring RTK pathway mutation is one of the mechanisms of primary resistance to mIDH inhibitors.

Additionally, Choe et al. observed emergence of RTK pathway mutations in about 35% relapsed cases achieving CR or CRh after monotherapy of ivosidenib, suggesting RTK pathway mutations are also associated with acquired resistance to ivosidenib (34). The biological processes that explain why RTK pathway mutations are implicated in both primary and acquired resistance to mIDH inhibitors are unclear. Several hypotheses were raised. First, it may be that the proliferative and prosurvival effects of RTK pathway activation are sufficiently strong oncogenic signals to reduce dependency on 2-HG. Second, it is possible that RTK pathway-activating mutations contribute to a differentiation block that remains enforced after initiation of ivosidenib treatment. A third hypothesis is that IDH1/2 mutations result in activation of some components of RTK signaling, which would not be reversed by ivosidenib in cases with co-occurring RTK pathway mutations.

MITOCHONDRIAL METABOLISM

Metabolic adaptations derived from changes of energy and intermediary metabolism in cancer cells are thought to meet biosynthetic and energetic requirements for proliferation (36). IHD1/2 play crucial roles in cell metabolism including Krebs cycle, cytosolic and mitochondrial redox, (oxidative phosphorylation) OxPHOS, and anabolism such as lipid biosynthesis. A better understanding of contributions of IDH mutations to metabolism and metabolic homeostasis may promote promising therapeutic strategies. Recently, several studies demonstrated that cancer cells carrying IDH mutations display some metabolic specificities, especially enhanced mitochondrial oxidative metabolism compared with wild-type cancer cells, and these cells tend to show vulnerability to mitochondrial inhibition (37–43).

Stuani et al. found AML patients with IDH mutations exhibited an enhanced mitochondrial oxidative metabolism which supports resistance to IDH mutation inhibitors. They performed multi-omics and functional approaches to investigate the mechanism of resistance. While IDH1 mutant inhibitor reduced 2-HG oncometabolite and CEBPα methylation, it failed to reverse (fatty acid β-oxidation) FAO and OxPHOS. OxPHOS, as a master regulator of mitochondrial biogenesis, and biosynthesis or degradation of FA, activation of PGC1α (peroxisome proliferator-activated receptor-γ coactivator-1) was not reversed after the inhibition of mIDH1. Importantly, FAO is a crucial biochemical process for sustaining OxPHOS and mitochondrial function in AML cells. Analysis of transcriptomic data from four clinical trials in 10 resistant patients demonstrated that genes associated with high OxPHOS function are enriched. Collectively, the OxPHOS phenotype was also confirmed as a nongenetic mechanism of IDHi resistance.

CLONAL SELECTION/EVOLUTION

Accumulating emergence of somatic mutations in cancers promote the development of clonal heterogeneity (44). AML has been firmly established as a highly dynamic oligoclonal disease by using single-cell DNA sequencing approaches (45, 46). A good understanding of evolution of clonal heterogeneity is helpful to make a precise investigation of mechanisms of drug resistance.

Quek et al. studied the clonal basis of response and acquired resistance to enasidenib treatment (47). An analysis of paired diagnosis/relapse samples did not identify second site mutations in IDH2 at relapse. Instead, relapse arose by clonal evolution, or selection, of terminal or ancestral clones, highlighting multiple bypass pathways that could potentially be targeted to restore differentiation arrest. The increased variant allele frequency (VAF) of colony stimulating factor 3 receptor (CSF3R), (FMS-like tyrosine kinase 3) FLT3, and Cbl proto-oncogene (CBL), were identified as potential risks for acquired resistance. Relapse is also associated with concurrent mutations in U2 small nuclear RNA auxiliary factor 1 (U2AF1) and hematopoietic transcription factors, including RUNX1, BCL6 corepressor like 1 (BCORL1), GATA2, and BAF chromatin remodeling complex subunit BCL11A (BCL11A). The deletion of all or part of chromosome 7 is a risk factor for relapse after enasidenib treatment. Less reported variations in other genes including nuclear factor kappa B subunit 1 (NFKB1), DEAD-box helicase 1 (DDX1), microtubule associated scaffold protein 1 (MTUS1), DEAH-box helicase 15 (DHX15), and DEAF1 transcription factor (DEAF1), contribute to clonal evolution related to relapse. Mutations in DHX15 (R222G) and DDX1 (G699A) are notable due to their vital role in altering RNA splicing.

CONCLUSION

In summary, this review concludes the resistance mechanisms of approved mIDH1/2 inhibitors and the increased understanding of drug-resistance mechanism would promote the development of corresponding strategies. To better investigate resistance mechanisms, development of novel effective approaches for detecting gene mutations is extremely important, such as next generation sequencing (NGS) and single-cell RNA sequencing (scRNA-seq). Future strategies should pay attention to the development of rational combination therapies with mIDH inhibitors or agents which can overcome the resistance to improve the response duration. The emerging biological mechanisms and clinical insights into these issues will provide guidance for rational treatment in the future.

AUTHOR CONTRIBUTIONS

(I) Conception and design: YC, CC, and YP; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: None; (V) Data analysis and interpretation: None; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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REFERENCES

1. Reitman ZJ, Yan H. Isocitrate Dehydrogenase 1 and 2 Mutations in Cancer: Alterations at a Crossroads of Cellular Metabolism. *J Natl Cancer Institute* (2010) 102(13):932–41. doi: 10.1093/jnci/djq187
2. Geisbrecht BV, Gould SJ. The Human PICD Gene Encodes a Cytoplasmic and Peroxisomal NADP(+)-Dependent Isocitrate Dehydrogenase. *J Biol Chem* (1999) 274(43):30527–33. doi: 10.1074/jbc.274.43.30527
3. Lee SM, Koh HJ, Park DC, Song BJ, Huh TL, Park JW. Cytosolic NADP (+)-Dependent Isocitrate Dehydrogenase Status Modulates Oxidative Damage to Cells. *Free Radical Biol Med* (2002) 32(11):1185–96. doi: 10.1016/S0891-5849(02)00815-8
4. Nekrutenko A, Hillis DM, Patton JC, Bradley RD, Baker RJ. Cytosolic Isocitrate Dehydrogenase in Humans, Mice, and Voles and Phylogenetic Analysis of the Enzyme Family. *Mol Biol Evol* (1998) 15(12):1674–84. doi: 10.1093/oxfordjournals.molbev.a025894
5. Ronnebaum SM, Ilkayeva O, Burgess SC, Joseph JW, Lu D, Stevens RD, et al. A Pyruvate Cycling Pathway Involving Cytosolic NADP-Dependent Isocitrate Dehydrogenase Regulates Glucose-Stimulated Insulin Secretion. *J Biol Chem* (2006) 281(41):30593–602. doi: 10.1074/jbc.M511908200
6. Shechter I, Dai P, Huo L, Guan G. IDH1 Gene Transcription is Sterol Regulated and Activated by SREBP-1a and SREBP-2 in Human Hepatoma HepG2 Cells: Evidence That IDH1 may Regulate Lipogenesis in Hepatic Cells. *J Lipid Res* (2003) 44(11):2169–80. doi: 10.1194/jlr.M300285-JLR200
7. Qiao S, Lu W, Glorieux C, Li J, Zeng P, Meng N, et al. Wild-Type IDH2 Protects Nuclear DNA From Oxidative Damage and is a Potential Therapeutic Target in Colorectal Cancer. *Oncogene* (2021) 40(39):5880–92. doi: 10.1038/s41388-021-01968-2
8. Middeke JM, Metzeler KH, Röllig C, Krämer M, Eckardt JN, Stasik S, et al. Differential Impact of IDH1/2 Mutational Subclasses on Outcome in Adult AML: Results From a Large Multicenter Study. *Blood Adv* (2022) 6(5):1394–405. doi: 10.1182/bloodadvances.2021004934
9. Eckel-Passow JE, Lachance DH, Molinaro AM, Walsh KM, Decker PA, Sicotte H, et al. Glioma Groups Based on 1p/19q, IDH, and TERT Promoter Mutations in Tumors. *New Engl J Med* (2015) 372(26):2499–508. doi: 10.1056/NEJMoa1407279
10. Zhu GG, Nafa K, Agaram N, Zehir A, Benayed R, Sadowska J, et al. Genomic Profiling Identifies Association of IDH1/IDH2 Mutation With Longer Relapse-Free and Metastasis-Free Survival in High-Grade Chondrosarcoma. *Clin Cancer Res* (2020) 26(2):419–27. doi: 10.1158/1078-0432.CCR-18-4212
11. Farshidfar F, Zheng S, Gingras MC, Newton Y, Shih J, Robertson AG, et al. Integrative Genomic Analysis of Cholangiocarcinoma Identifies Distinct IDH-Mutant Molecular Profiles. *Cell Rep* (2017) 18(11):2780–94. doi: 10.1016/j.celrep.2017.02.033
12. Paschka P, Schlenk RF, Gaidzik VI, Habdank M, Krönke J, Bullinger L, et al. IDH1 and IDH2 Mutations are Frequent Genetic Alterations in Acute Myeloid Leukemia and Confer Adverse Prognosis in Cytogenetically Normal Acute Myeloid Leukemia With NPM1 Mutation Without FLT3 Internal Tandem Duplication. *J Clin Oncol* (2010) 28(22):3636–43. doi: 10.1200/JCO.2010.28.3762
13. Marcucci G, Maharry K, Wu YZ, Radmacher MD, Mrózek K, Margeson D, et al. IDH1 and IDH2 Gene Mutations Identify Novel Molecular Subsets Within *De Novo* Cytogenetically Normal Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study. *J Clin Oncol* (2010) 28(14):2348–55. doi: 10.1200/JCO.2009.27.3730
14. Clark O, Yen K, Mellinghoff IK. Molecular Pathways: Isocitrate Dehydrogenase Mutations in Cancer. *Clin Cancer Res* (2016) 22(8):1837–42. doi: 10.1158/1078-0432.CCR-13-1333
15. Loenarz C, Schofield CJ. Expanding Chemical Biology of 2-Oxoglutarate Oxygenases. *Nat Chem Biol* (2008) 4(3):152–6. doi: 10.1038/nchembio0308-152
16. Im AP, Sehgal AR, Carroll MP, Smith BD, Tefferi A, Johnson DE, et al. DNMT3A and IDH Mutations in Acute Myeloid Leukemia and Other Myeloid Malignancies: Associations With Prognosis and Potential Treatment Strategies. *Leukemia* (2014) 28(9):1774–83. doi: 10.1038/leu.2014.124
17. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *New Engl J Med* (2016) 374(23):2209–21. doi: 10.1056/NEJMoa1516192
18. Patel JP, Gönen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, et al. Prognostic Relevance of Integrated Genetic Profiling in Acute Myeloid Leukemia. *New Engl J Med* (2012) 366(12):1079–89. doi: 10.1056/NEJMoa1112304
19. Green CL, Evans CM, Zhao L, Hills RK, Burnett AK, Linch DC, et al. The Prognostic Significance of IDH2 Mutations in AML Depends on the Location of the Mutation. *Blood* (2011) 118(2):409–12. doi: 10.1182/blood-2010-12-322479
20. Arora S, Balasubramaniam S, Zhang W, Zhang L, Sridhara R, Spillman D, et al. FDA Approval Summary: Pembrolizumab Plus Lenvatinib for Endometrial Carcinoma, a Collaborative International Review Under Project Orbis. *Clin Cancer Res* (2020) 26(19):5062–7. doi: 10.1158/1078-0432.CCR-19-3979
21. Mellinghoff IK, Bent MJVD, Clarke JL, Maher EA, Peters KB, Touat M, et al. INDIGO: A Global, Randomized, Double-Blind, Phase III Study of Vorasidenib (VOR; AG-881) vs Placebo in Patients (Pts) With Residual or Recurrent Grade II Glioma With an Isocitrate Dehydrogenase 1/2 (IDH1/2) Mutation. *J Clin Oncol* (2020) 38(15_suppl):TPS2574–TPS. doi: 10.1200/JCO.2020.38.15_suppl.TPS2574
22. Mellinghoff IK, Penas-Prado M, Peters KB, Burris HA3rd, Maher EA, Janku F, et al. Vorasidenib, a Dual Inhibitor of Mutant IDH1/2, in Recurrent or Progressive Glioma; Results of a First-In-Human Phase I Trial. *Clin Cancer Res* (2021) 27(16):4491–9. doi: 10.1158/1078-0432.CCR-21-0611
23. Popovici-Muller J, Lemieux RM, Artin E, Saunders JO, Salituro FG, Travins J, et al. Discovery of AG-120 (Ivosidenib): A First-In-Class Mutant IDH1 Inhibitor for the Treatment of IDH1 Mutant Cancers. *ACS medicinal Chem Lett* (2018) 9(4):300–5. doi: 10.1021/acsmedchemlett.7b00421
24. Yen K, Travins J, Wang F, David MD, Artin E, Straley K, et al. AG-221, a First-In-Class Therapy Targeting Acute Myeloid Leukemia Harboring Oncogenic IDH2 Mutations. *Cancer Discovery* (2017) 7(5):478–93. doi: 10.1158/2159-8290.CD-16-1034
25. Najafi M, Mortezaee K, Majidpoor J. Cancer Stem Cell (CSC) Resistance Drivers. *Life Sci* (2019) 234:116781. doi: 10.1016/j.lfs.2019.116781
26. Wang F, Morita K, DiNardo CD, Furudate K, Tanaka T, Yan Y, et al. Leukemia Stemness and Co-Occurring Mutations Drive Resistance to IDH Inhibitors in Acute Myeloid Leukemia. *Nat Commun* (2021) 12(1):2607. doi: 10.1038/s41467-021-22874-x
27. Salik B, Yi H, Hassan N, Santiappillai N, Vick B, Connerty P, et al. Targeting RSPO3-LGR4 Signaling for Leukemia Stem Cell Eradication in Acute Myeloid Leukemia. *Cancer Cell* (2020) 38(2):263–78.e6. doi: 10.1016/j.ccell.2020.05.014
28. Soleas JP, D'Arcangelo E, Huang L, Karoubi G, Nostro MC, McGuigan AP, et al. Assembly of Lung Progenitors Into Developmentally-Inspired Geometry Drives Differentiation *via* Cellular Tension. *Biomaterials* (2020) 254:120128. doi: 10.1016/j.biomaterials.2020.120128
29. Choi BR, Cave C, Na CH, Sockanathan S. GDE2-Dependent Activation of Canonical Wnt Signaling in Neurons Regulates Oligodendrocyte Maturation. *Cell Rep* (2020) 31(5):107540. doi: 10.1016/j.celrep.2020.107540

30. Zhan T, Rindtorff N, Boutros M. Wnt Signaling in Cancer. *Oncogene* (2017) 36(11):1461–73. doi: 10.1038/onc.2016.304
31. Hartmann C, Meyer J, Balss J, Capper D, Mueller W, Christians A, et al. Type and Frequency of IDH1 and IDH2 Mutations are Related to Astrocytic and Oligodendroglial Differentiation and Age: A Study of 1,010 Diffuse Gliomas. *Acta neuropathologica* (2009) 118(4):469–74. doi: 10.1007/s00401-009-0561-9
32. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 Mutations Result in a Hypermethylation Phenotype, Disrupt TET2 Function, and Impair Hematopoietic Differentiation. *Cancer Cell* (2010) 18(6):553–67. doi: 10.1016/j.ccr.2010.11.015
33. Harding JJ, Lowery MA, Shih AH, Schvartzman JM, Hou S, Famulare C, et al. Isoform Switching as a Mechanism of Acquired Resistance to Mutant Isocitrate Dehydrogenase Inhibition. *Cancer Discovery* (2018) 8(12):1540–7. doi: 10.1158/2159-8290.CD-18-0877
34. Choe S, Wang H, DiNardo CD, Stein EM, de Botton S, Roboz GJ, et al. Molecular Mechanisms Mediating Relapse Following Ivosidenib Monotherapy in IDH1-Mutant Relapsed or Refractory AML. *Blood Adv* (2020) 4(9):1894–905. doi: 10.1182/bloodadvances.2020001503
35. Amatangelo MD, Quek L, Shih A, Stein EM, Roshal M, David MD, et al. Enasidenib Induces Acute Myeloid Leukemia Cell Differentiation to Promote Clinical Response. *Blood* (2017) 130(6):732–41. doi: 10.1182/blood-2017-04-779447
36. Alvarez-Calderon F, Gregory MA, Pham-Danis C, DeRyckere D, Stevens BM, Zaberezhnyy V, et al. Tyrosine Kinase Inhibition in Leukemia Induces an Altered Metabolic State Sensitive to Mitochondrial Perturbations. *Clin Cancer Res* (2015) 21(6):1360–72. doi: 10.1158/1078-0432.CCR-14-2146
37. Tateishi K, Wakimoto H, Iafrate AJ, Tanaka S, Loebel F, Lelic N, et al. Extreme Vulnerability of IDH1 Mutant Cancers to NAD+ Depletion. *Cancer Cell* (2015) 28(6):773–84. doi: 10.1016/j.ccell.2015.11.006
38. Thiele I, Swainston N, Fleming RM, Hoppe A, Sahoo S, Aurich MK, et al. A Community-Driven Global Reconstruction of Human Metabolism. *Nat Biotechnol* (2013) 31(5):419–25. doi: 10.1038/nbt.2488
39. Turcan S, Makarov V, Taranda J, Wang Y, Fabius AWM, Wu W, et al. Mutant-IDH1-Dependent Chromatin State Reprogramming, Reversibility, and Persistence. *Nat Genet* (2018) 50(1):62–72. doi: 10.1038/s41588-017-0001-z
40. Izquierdo-Garcia JL, Cai LM, Chaumeil MM, Eriksson P, Robinson AE, Pieper RO, et al. Glioma Cells With the IDH1 Mutation Modulate Metabolic Fractional Flux Through Pyruvate Carboxylase. *PloS One* (2014) 9(9): e108289. doi: 10.1371/journal.pone.0108289
41. Chan SM, Thomas D, Corces-Zimmerman MR, Xavy S, Rastogi S, Hong WJ, et al. Isocitrate Dehydrogenase 1 and 2 Mutations Induce BCL-2 Dependence in Acute Myeloid Leukemia. *Nat Med* (2015) 21(2):178–84. doi: 10.1038/nm.3788
42. Baccelli I, Gareau Y, Lehnertz B, Gingras S, Spinella JF, Corneau S, et al. Mubritinib Targets the Electron Transport Chain Complex I and Reveals the Landscape of OXPHOS Dependency in Acute Myeloid Leukemia. *Cancer Cell* (2019) 36(1):84–99.e8. doi: 10.1016/j.ccell.2019.06.003
43. Grassian AR, Parker SJ, Davidson SM, Divakaruni AS, Green CR, Zhang X, et al. IDH1 Mutations Alter Citric Acid Cycle Metabolism and Increase Dependence on Oxidative Mitochondrial Metabolism. *Cancer Res* (2014) 74 (12):3317–31. doi: 10.1158/0008-5472.CAN-14-0772-T
44. Dagogo-Jack I, Shaw AT. Tumour Heterogeneity and Resistance to Cancer Therapies. *Nat Rev Clin Oncol* (2018) 15(2):81–94. doi: 10.1038/nrclinonc.2017.166
45. Miles LA, Bowman RL, Merlinsky TR, Csete IS, Ooi AT, Durruthy-Durruthy R, et al. Single-Cell Mutation Analysis of Clonal Evolution in Myeloid Malignancies. *Nature* (2020) 587(7834):477–82. doi: 10.1038/s41586-020-2864-x
46. Morita K, Wang F, Jahn K, Hu T, Tanaka T, Sasaki Y, et al. Clonal Evolution of Acute Myeloid Leukemia Revealed by High-Throughput Single-Cell Genomics. *Nat Commun* (2020) 11(1):5327. doi: 10.1038/s41467-020-19119-8
47. Quek L, David MD, Kennedy A, et al. Clonal Heterogeneity of Acute Myeloid Leukemia Treated With the IDH2 Inhibitor Enasidenib. *Nat Med* (2018) 24 (8):1167–77. doi: 10.1038/s41591-018-0115-6

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Single-Cell Metabolomics in Hematopoiesis and Hematological Malignancies

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Aberrant metabolism contributes to tumor initiation, progression, metastasis, and drug resistance. Metabolic dysregulation has emerged as a hallmark of several hematologic malignancies. Decoding the molecular mechanism underlying metabolic rewiring in hematological malignancies would provide promising avenues for novel therapeutic interventions. Single-cell metabolic analysis can directly offer a meaningful readout of the cellular phenotype, allowing us to comprehensively dissect cellular states and access biological information unobtainable from bulk analysis. In this review, we first highlight the unique metabolic properties of hematologic malignancies and underscore potential metabolic vulnerabilities. We then emphasize the emerging single-cell metabolomics techniques, aiming to provide a guide to interrogating metabolism at single-cell resolution. Furthermore, we summarize recent studies demonstrating the power of single-cell metabolomics to uncover the roles of metabolic rewiring in tumor biology, cellular heterogeneity, immunometabolism, and therapeutic resistance. Meanwhile, we describe a practical view of the potential applications of single-cell metabolomics in hematopoiesis and hematological malignancies. Finally, we present the challenges and perspectives of single-cell metabolomics development.

Keywords: single-cell metabolomics, metabolic reprogramming, hematopoiesis, hematological malignancies, glucose metabolism, amino acids metabolism, lipid-related metabolism

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INTRODUCTION

Metabolism consists of a series of biochemical reactions that occur within a living organism to maintain life. As genetic or non-genetic alterations, tumor cells rewire metabolic pathways to adapt to their rapid growth and proliferation. Metabolic reprogramming of cancer cells is now deemed one of the hallmarks of cancer (1). Increasing evidence suggests that dysregulated cell metabolism facilitates tumor initiation, progression, metastasis, and drug resistance. The metabolic alterations of cancer cells are mainly reflected in the increase in glucose and glutamine uptake and fatty acid metabolism, which are crucial for promoting the rapid synthesis of nucleotides, proteins, and lipids, meeting energy requirements and maintaining redox homeostasis (2–4). Moreover, metabolic alterations also modulate cell signaling pathways and post-translational modifications (PTMs) (5). Metabolites can serve as signaling molecules that directly affect both pro-inflammatory and anti-inflammatory outcomes (6–8). Emerging evidence suggests that metabolic regulation of PTMs on

DNA and histones impacts gene expression (9, 10). Furthermore, metabolic enzymes have been reported to have 'moonlighting' functions as RNA-binding proteins (11). Metabolic alterations in cancer are triggered by various mechanisms that instigate signaling pathways and regulate the expression of metabolism-related genes (3).

Alterations in metabolic processes vary from cancer to cancer, as nutrient availability, oncogenic activation, proliferative state or microenvironment are spatially and temporally heterogeneous. As a result, each type of cancer cell has distinct needs in terms of energy and biomass production (12–14). When analyzing the metabolism of different hematological malignancies, the heterogeneity between them should also be considered.

A growing number of studies regard cancer as a kind of metabolic diseases, as do hematological malignancies. Hematological malignancies can be classified as leukemia, myeloma, and lymphoma and are often deadly. The most common hematological malignancies include acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM), Hodgkin lymphoma (HL), and non-Hodgkin lymphoma (NHL). Aberrant metabolism and metabolic reprogramming play important roles in the pathogenesis of hematologic disorders. The metabolic characteristics of leukemia cells are usually different from those of their normal counterparts, manifested by increased glycolysis, glutaminolysis, and lipogenesis. Metabolic differences provide new therapeutic targets to overcome hematological malignancies. Moreover, metabolic reprogramming contributes to an immunosuppressive microenvironment, increasing the probability of resistance to anticancer therapies. Recurrence and refractory always exist in patients with hematologic malignancies, and long-term overall survival remains unsatisfactory. Newer and more sophisticated therapeutic approaches are imperative. Metabolic therapies alone or in combination with other treatment regimens, such as immunotherapy, targeted therapy, and chemotherapy, bring new opportunities for patients with hematologic malignancies.

Metabolomics provides the best view of biological phenotypes by profiling changes in endogenous metabolites. Insights into the role of metabolic reprogramming in tumor biology have largely been accomplished by bulk metabolic analysis techniques. However, bulk analyses neglect intratumoral heterogeneity, so the mechanisms underlying critical disease events of hematological malignancies remain obscure, such as treatment resistance and clonal evolution. Propelled by a set of recent technological advances in single-cell metabolomics, new insights into tumor metabolism are rapidly emerging, which are often not available on other omics layers (**Figure 1**). Single-cell metabolomics technologies will provide an understanding of hematological malignancies at unprecedented depth and reveal new insights into the pathogenesis of hematologic malignancies.

In this review, we summarize the unique metabolic characteristics of hematologic malignancies. Then, we illustrate the research progress of single-cell metabolomics technology. Applications and potential of single-cell metabolomics in hematopoiesis and hematologic malignancies are discussed. Finally, we present the challenges and perspectives of single-cell metabolomics development. This review will provide a clear navigation of numerous single-cell metabolomics technologies and strategies.

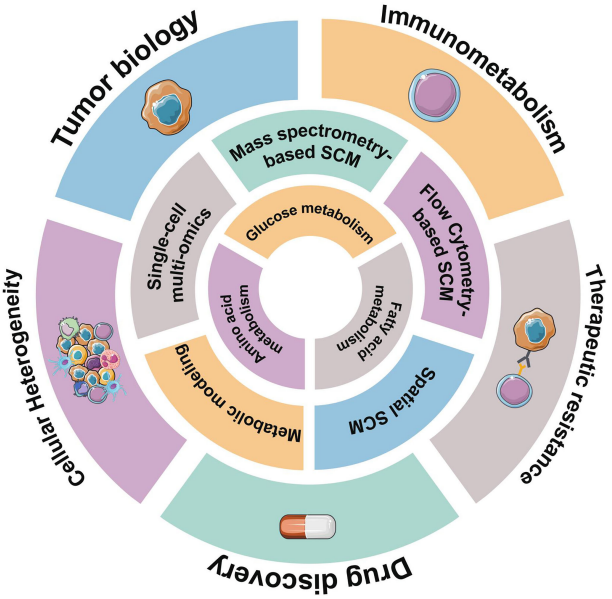

FIGURE 1 | Overview of representative single-cell metabolomics methods for metabolic profiling. Hematological malignancies have abnormal metabolic characteristics, reflected in glucose metabolism, amino acids metabolism, and lipid-related metabolism. Some emerging single-cell metabolomics (SCM) techniques can help us better understand metabolic signatures at the single-cell level and provide unprecedented insights into hematological malignancies.

UNIQUE METABOLIC PROPERTIES OF HEMATOLOGIC MALIGNANCIES

Glucose Metabolism

The most common metabolic alteration in cancer is aerobic glycolysis. In the presence of oxygen, normal cells will take up glucose for respiration and continue with oxidative phosphorylation (OXPHOS), whereas some tumor cells are more likely to take glucose for glycolysis to rapidly produce ATP, anabolic intermediates and lactate (1), which is known as the Warburg effect. Lactate can promote tumor cell growth and metastasis by stimulating angiogenesis and acidifying the tumor microenvironment, and also cause local inflammatory responses (15–17). A high level of glucose consumption is a conserved characteristic of most hematological malignancies (**Figure 2**). The PI3K-AKT/mTOR signaling pathway activates the expression of downstream glycolytic genes, including *GLUT1, HK2, PFKFB3, LDHA*, *PKM2* and suppressors of the tricarboxylic acid (TCA) cycle such as *PDK* (4), resulting in a shift in glucose utilization (**Figure 3**). Competitive glucose metabolism as a target boosts the emergence of novel therapeutic approaches for hematologic malignancies.

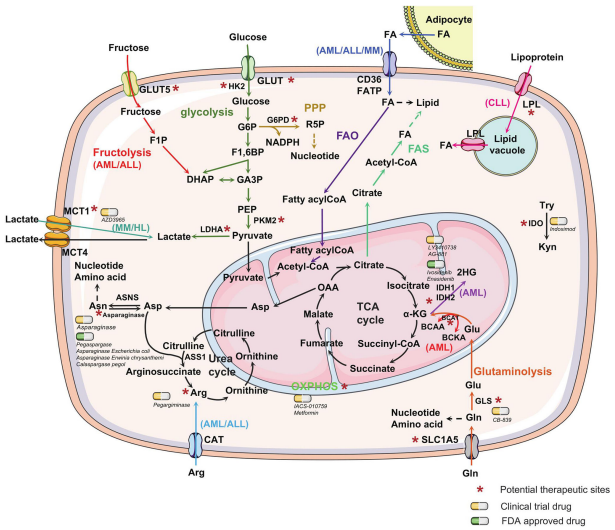

FIGURE 2 | Metabolic alterations in hematological malignancies. Metabolic alterations in hematological malignancies are mainly reflected in the increase in glucose and glutamine uptake and fatty acid metabolism to promote the rapid synthesis of nucleotides, proteins, and lipids. Different metabolic pathways are marked with distinct colors. Unique metabolic characteristics exhibited in one or a few types of hematological malignancies are labeled. Some special enzymes or reaction processes in the metabolic process, which can be used as potential therapeutic targets, are marked with an asterisk. The yellow capsules represent the drugs in clinical trials, whereas the green capsules represent FDA-approved drugs targeting the metabolic process of hematological malignancies. The dashed line with arrow represents anabolism. GLUT, glucose transporter; HK2, hexokinase2; G6P, glucose-6-phosphate; G6PD, Glucose-6-phosphate dehydrogenase; R5P, ribose-5-phosphate; PPP, pentose phosphate pathway; NADPH, nicotinamide adenine dinucleotide phosphate; F1,6BP, fructose-1,6-biphosphate; GA3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; F1P, fructose-1-phosphate; PEP, phosphoenolpyruvate; PKM2, pyruvate kinase M2; FA, fatty acid; FATP, fatty acid transport protein; OAA, oxaloacetate; α-KG, α-ketoglutarate; Asp, Asparagine; Asn, asparagine; ASNS, asparagine synthetase, ASS1, arginine succinate synthase-1; Arg, arginine; CAT, cationic amino acid transporters; BCAA, branched-chain amino acid; BCKA, branched-chain Keto acid; IDH, isocitrate dehydrogenases; 2HG, 2-hydroxyglutarate; Glu, Glutamate; Gln, glutamine; GLS, glutaminase; Try, tryptophan; Kyn, kynurenine; IDO, indoleamine 2,3-dioxygenase, LPL, lipoprotein lipase; MCT, monocarboxylate transporter; LDHA, lactate dehydrogenase A; FAO, fatty acid oxidation; FAS, fatty acid synthesis; TCA, tricarboxylic acid cycle.

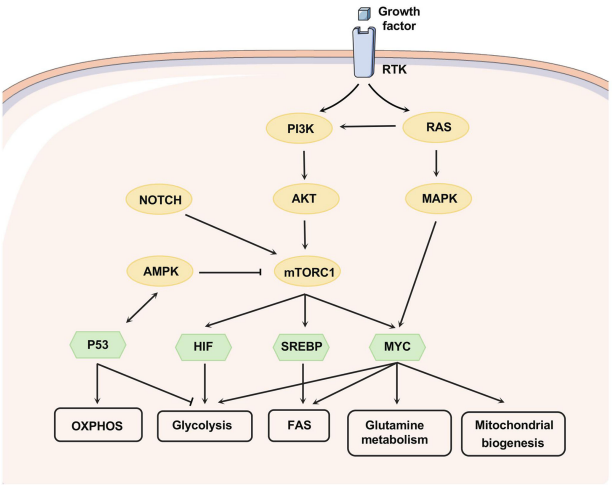

FIGURE 3 | Signaling pathways that regulate metabolism. Growth factors affect metabolism by activating RTKs, and the PI3K/AKT axis can be activated as downstream of RTK and RAS. mTORC1, downstream of PI3K/AKT, can be activated upon AKT-mediated phosphorylation or suppressed through AMPK-mediated phosphorylation. NOTCH1 signaling promotes the activation of PI3K-AKT-mTOR signaling. PI3K-AKT-mTOR signaling can alter metabolism by regulating multiple transcription factors, nutrient transporters, and phosphorylating metabolic enzymes. HIF, MYC, and SREBP-1 are downstream transcription factors of mTORC1 that promote glycolysis, glutamine metabolism, fatty acid synthesis, and mitochondrial biogenesis. MYC can collaborate with HIF to enhance the expression of genes involved in glucose uptake and glycolysis, including LDHA, pyruvate dehydrogenase kinase-1 (PDK-1), and HK-2. Apart from this, MYC targets function to enhance mitochondrial biogenesis and function, especially glutamine metabolism. MYC can induce glutamine transporters expression (e.g. SLC7A5 and SLC1A5) and increase the levels of glutaminase. SREBP as a downstream effector of mTORC1 can induce the expression of several key enzymes related to fatty acid and sterol biosynthesis, such as ATP citrate lyase (ACLY) and fatty acid synthase. Moreover, SREBP interacts with MYC to regulate lipogenesis and then promote tumorigenesis. P53 forms a positive feedback loop with AMPK to repress glycolytic activity and promote OXPHOS and PPP. Phosphoserine STAT3 binds to and activates the promoter of LPL.

Cellular glucose uptake is mediated by transmembrane glucose transporters (GLUTs), and most hematological malignancies, such as AML, CML, B-ALL and MM, take up abundant glucose through overexpressed GLUT1. Studies have demonstrated GLUT1 as a therapeutic target for hematological malignancies (**Figure 2**) (18–22). MM cells also exhibit an unexpected dependence on GLUT4, GLUT8 and GLUT11. Myeloma cells exhibit reliance on GLUT4 for basal glucose consumption, maintenance of apoptotic effector Mcl-1 expression, growth, and survival, while GLUT8 and GLUT11 are required for proliferation and viability in myeloma (23). Likewise, 60% of CLL cells overexpressed GLUT4, facilitating glucose transport (24). The human immunodeficiency virus (HIV) protease inhibitor ritonavir has an off-target inhibitory effect on GLUT4 expression in CLL and MM, resulting in the reduction of cell viability (23, 24). Notably, GLUT1 is not increased in CLL cells, and CLL cells seem not to follow the Warburg effect (25, 26). Distinct signatures of glucose metabolism have been found in AML patients, which demonstrated prognostic value in cytogenetically normal AML patients (27). Internal tandem duplication (ITD) mutation in the Fms-like tyrosine kinase 3 gene (FLT3/ITD) causes a significant increase in aerobic glycolysis through AKT-mediated upregulation of mitochondrial hexokinase 2 (HK2) and renders leukemia cells highly dependent on glycolysis (28). The combination of the FLT3 tyrosine kinase inhibitor sorafenib and glycolytic inhibitor 2-deoxy-d-glucose (2-DG) enhanced cytotoxicity in AML (28).

Pyruvate kinase (PKM2) and lactate dehydrogenase A (LDHA) play important roles in the initiation, maintenance, and progression of CML and AML in mice, and deletion of PKM2 or LDHA results in significantly prolonged disease latency (29). LDHA inhibitor oxamate suppressed proliferation and induced apoptosis in T-ALL

cell lines and primary T-ALL cells through the c-Myc-ROS and PI3K/AKT/GSK3β signaling pathways (29). In the myeloma microenvironment, lactate is also produced by stromal cells and then enters myeloma cells *via* monocarboxylate transporter 1 (MCT1). Lactate contributes to the survival of MM cells in autocrine or paracrine manners (30). Treatment with α-cyano-4-hydroxy cinnamate (CHC), a known inhibitor of MCT, dose-dependently induced cell death in MM cell lines and primary MM cells (31). HL cells are prone to undergo OXPHOS within mitochondria. Some non-cancer cells, such as tumor-associated macrophages (TAMs) with high glycolysis, could promote tumor growth in HL (32). HL tumor cells have high mitochondrial metabolism, high expression of MCT1, and uptake and utilization of lactate released from TAMs.

Leukemia cells show a marked dependence on the pentose phosphate pathway (PPP), which is a branch of glycolysis, to generate ribose-5-phosphate (R5P) for nucleic acid synthesis and NADPH for biosynthetic reactions and oxidative balance (**Figure 2**). In AML serum samples, the PPP intermediate D-ribose phosphate was reduced (27). Critical PPP genes were upregulated in 61% of patients with AML (33). Glucose-6-phosphate dehydrogenase (G6PD) is the first enzyme in the PPP pathway, whose inhibitor 6-aminonicotinamide (6AN) induces cytotoxicity against AML cells *in vitro* and *in vivo* (34). In addition to PPP, fructolysis is an alternative strategy to provide carbon intermediates for the glycolytic pathway in AML (35). AML cells can compensate for low glucose levels by upregulating fructose transporter GLUT5 (36). GLUT5 is upregulated in Philadelphia chromosome-positive ALL (Ph^+ ALL), leading to imatinib resistance, thus targeting GLUT5 might be promising in Ph^+ ALL patients (37, 38).

Pyruvate and some other fuel sources, such as glutamine, can enter the mitochondrial TCA cycle and undergo OXPHOS, which is an important reaction in mitochondria. Although aerobic glycolysis is the main metabolic mode of hematological malignancies, some of them also increase OXPHOS to gain energy and anabolic precursors. The examined untreated CLL patients exhibit a metabolic signature of oxidative stress (25), and OXPHOS is a predominant pathway in CLL for energy production (39). CLL cells have an increased mitochondrial number and mass, displaying heightened mitochondrial respiration, elevated levels of reactive oxygen species (ROS), and enhanced antioxidant capacity (25, 40–42). Metformin inhibits mitochondrial complex I, inducing apoptosis of quiescent CLL cells and inhibiting cell cycle entry (43). Activated 5' AMP-activated kinase (AMPK) inhibits mammalian target of rapamycin complex 1 (mTORC1) while promoting oxidative metabolism and mitochondrial complex I activity, resulting in a decreased level of aerobic glycolysis in T-ALL cells (44). AML patient samples display an increased mitochondrial mass without a concomitant increase in respiratory chain complexes activity, which makes AML cells seem more susceptible to oxidative stress (45). Increased glycolysis and inefficient OXPHOS in AML patients may contribute to drug resistance (46). However, in different AML cell lines, NB4 cells tend to undergo glycolysis, while THP-1 cells are recognized to be dependent on OXPHOS (47). THP-1 cells are resistant to 2-DG treatment, while NB4 cells are sensitive to 2-DG treatment; the difference is that AMPK responds differently to 2-DG (47).

Isocitrate dehydrogenase (IDH) catalyzes the decarboxylation of isocitrate to α-KG, however, mutant IDH1 and IDH2 reduce α-KG to 2-hydroxyglutarate (2HG), which could alter the epigenetic landscape of leukemic progenitors (48, 49). IDH mutations are prone to occur in AML (50, 51), and the FDA-approved drugs ivosidenib and enasidenib have been identified as small molecules, targeting IDH1 and IDH2 in AML, respectively. Furthermore, vorasidenib (AG-881) and LY3410738 are under investigation in phase I trials for the treatment of AML patients with IDH1 and/or IDH2 mutation (52, 53).

Amino Acids Metabolism

Apart from glucose, cancer cells rely heavily on glutamine to obtain the necessary energy and building blocks to survive and proliferate. Glutamine serves as a carbon source for the replenishment of TCA cycle intermediates and a nitrogen source for the biosynthesis of nucleotides and amino acids. Glutamine is converted to glutamate by glutaminase (GLS). Almost all hematological malignancies depend on glutamine metabolism, and targeting glutamine metabolism has been proven to have therapeutic potential in the treatment of hematological malignancies (40, 54–57). For example, the glutaminase inhibitor CB-839 inhibits glutathione (GSH) production, induces mitochondrial reactive oxygen species (mitoROS) and causes apoptosis in AML and ALL (57). Knockdown of the glutamine transporter SLC1A5 inhibits glutamine uptake, induces apoptosis and suppresses tumor formation in a mouse AML xenotransplantation model (58). Stable SLC1A5 downregulation by a lentiviral approach inhibited human myeloma cell line growth *in vitro* and in a murine model (59). Activating mutations in NOTCH1 are common in T-ALL, and inhibition of NOTCH1 signaling in T-ALL drives a metabolic crisis, with prominent inhibition of glutaminolysis and promotes autophagy (60).

In addition, AML and ALL show dependence on arginine, and most AML and ALL cells lack arginine succinate synthase-1 (ASS1) and/or ornithine transcarbamylase (OTC), relying on extracellular arginine availability (61–63). AML constitutively expresses the cationic amino acid transporters CAT-1 and CAT-2B for arginine uptake, while ALL expresses CAT-1 in the absence of CAT-2A or CAT-2B (62, 63). BCT-100, a pegylated human recombinant arginase, leads to a rapid arginine depletion and could serve as a novel therapeutic agent for AML and ALL cells (62, 63). Arginine metabolism is significantly enriched in MM patients, promoting the urea cycle, and the elevated levels of urea, creatinine, and uric acid in plasma may be related to impaired renal function and damaged toxin excretion during the progression of MM (64, 65).

Indoleamine 2,3-dioxygenase (IDO) is an immunomodulatory enzyme that facilitates tryptophan catabolism into the immunoregulatory metabolite kynurenine (Kyn) (66). IDO and Kyn can manipulate the immunosuppressive tumor microenvironment by affecting T-cell maturation and proliferation and inducing differentiation into T regulatory cells (67). AML patients were shown

to express IDO, and high IDO expression and elevated levels of Kyn were correlated with poor clinical outcomes in AML patients (68, 69). Inhibition of IDO expression can disrupt immune tolerance as an AML treatment option (68). Although individual CML patients differed in their rates of IDO production, the present data indicate that CML should be added to malignancies with higher IDO activity (70). CLL cells also express an active IDO enzyme and produce high levels of Kyn, which plays a role in the survival and drug resistance of leukemic cells (71). Another study demonstrates that the levels of serum Kyn and Trp are useful for predicting the prognosis of individual HL patients (72).

Asparagine is essential for DNA synthesis, RNA synthesis, protein metabolism, and survival of leukemic cells, however, ALL cells lack asparagine synthetase (ASNS). ALL cells are auxotrophic for asparagine and highly sensitive to asparaginase treatment (73, 74). Asparaginase depletes the source of asparagine for leukemic cells, leading to the death of leukemic cells, and the antileukemia effect has been shown in clinical treatment of ALL (75–77). Four drugs asparaginase *Erwinia chrysanthemi*, asparaginase *Escherichia coli*, calaspargase pegol, and pegaspargase, have been approved by the FDA to treat ALL, whereas pegaspargase was also feasible in higher-stage NHL (**Figure 2**).

Branched-chain amino acids (BCAAs) include leucine, isoleucine and valine, and branched-chain amino acids transaminases 1 (BCAT1) transfers α-amino groups from BCAAs to α-ketoglutarate (αKG) to produce glutamate and their respective branched chain ketoacids (BCKAs). BCAT1 is significantly overexpressed in AML leukemia stem cells (LSCs), resulting in enhanced α-KG amination and thus lowered intracellular levels of α-KG (78). BCAT1 is also aberrantly activated in CML, and blocking BCAT1 gene expression or enzymatic activity induces cellular differentiation and impairs the propagation of blast crisis CML (79).

Lipid-Related Metabolism

Fatty acids (FAs) are key synthetic raw materials for cell membranes and important energy reserves. The oxidation and synthesis of FAs were shown to contribute to cancer growth. In CLL, STAT3 is constitutively activated, which also activates LPL transcription, resulting in elevated intracellular lipoprotein lipase levels (80). STAT3 also activates the fatty acid translocase CD36 and facilitates FAs uptake in CLL cells (81). LPL induces cellular uptake of lipoproteins, prompts the hydrolysis of triglycerides into free fatty acids (FFAs) and shifts CLL cell metabolism toward utilization of FFAs (82, 83). FFAs bind to proliferator-activated receptor (PPAR)-α as ligands, and the FFA-PPARα complex functions as a transcription factor to activate OXPHOS genes (84). The B-cell receptor (BCR) inhibitor ibrutinib could reduce LPL mRNA and protein levels and inhibit FFAs metabolism in CLL cells (85). Perhexiline inhibits carnitine palmitoyltransferases (CPT), thereby suppressing fatty acid transport into mitochondria and leading to massive CLL cell death (86).

Adipocytes could support cancer cells through the provision of FAs. ALL cells stimulate adipocytes lipolysis and take up FFAs released by adipocytes for OXPHOS (87). Adipocyte-derived FFAs can alleviate the dependence of ALL cells on *de novo* lipogenesis and reverse the cytotoxicity of pharmacological acetyl-CoA carboxylase (ACC) inhibition. In addition, the unsaturated fatty acid oleic acid protects ALL cells from modest concentrations of chemotherapy (87). Obesity was associated with worse outcomes and increased relapse rates in patients older than 10 years at ALL diagnosis (88). In addition to promoting fatty acid metabolism, MM cells induce lipolysis in bone marrow (BM) adipocytes and then take up the released FFAs through fatty acid transporter proteins (FATP), leading to growth (89). AML cells are also supplied free fatty acids from BM adipocytes, and utilize fatty acid oxidation (FAO) to generate energy (90).

Understanding the metabolic patterns of hematological malignancies will help us to better develop treatment plans for their metabolic changes. Metabolomics has rapidly begun to expand the research scope of genomics, transcriptomics, and proteomics. The comprehensive metabolic profiles offer a functional readout of cellular state, which sits closest in proximity to clinical phenotype. Interrogating metabolic rewiring of hematological malignancies at the single-celll resolution might help to elucidate the underlying causes of metabolic dysregulation in hematologic malignancy.

TECHNICAL ADVANCES IN SINGLE-CELL METABOLOMICS

Metabolomics has gradually exceeded the powers of genomics, transcriptomics, and proteomics to facilitate an understanding and assessment of the clinical phenotype. Numerous technologies and strategies are available for metabolism research. To determine what the individual cell is actually doing in nature, however, requires single-cell metabolomics. A broad array of new techniques allow researchers to catalog the chemical contents at single-cell resolution.

Mass Spectrometry-Based Single-Cell Metabolomics Approaches

Mass spectrometry has emerged as the most widely used technique for single-cell metabolomics owing to its high sensitivity, broad molecular coverage, and wide dynamic ranges. MS is coupled with capillary electrophoresis (CE) and nano liquid chromatography (nanoLC), allowing efficient separation, sensitive detection, and identification of complex cellular contents (91–93). These hyphenated MS techniques enabled to identify hundreds to thousands of molecules with attomole to zeptomole sensitivity (94, 95). However, the application of these hyphenated MS techniques is limited by the relatively low throughput of cell analysis. Cell pretreatment inevitably causes strong cellular perturbation.

Mass spectrometry imaging (MSI) is an attractive approach to simultaneously image different compounds in a high-throughput manner, overcoming the limited number of molecules detected in traditional optical imaging (96, 97). Specially, MSI allows for visualization of the spatial distribution of biomolecules without extraction, purification, separation or labeling, which is in stark contrast to most label-based imaging methods.

The rapid development of single-cell metabolomics is attributed to single-cell separation and injection techniques, such as cell micro-array, single-cell droplet printing, and flow cytometry (93), that can then spawn new single-cell metabolomics technologies, such as high-density micro-arrays for mass spectrometry (MAMS), droplet-based electrospray ionization (ESI)-MS, and label-free mass cytometry (CyESI-MS). MSI has been coupled with many typical ionization techniques, such as matrix-assisted laser desorption ionization (MALDI), ion beam ionization, electrospray ionization, nanoelectrospray ionization (nESI), and matrix-free laser desorption ionization (LDI). MSI-based single-cell metabolomics technologies have evolved as the best suited platforms.

MALDI-MSI is one of the most popular techniques for single-cell metabolic analysis. With its minimal sample preparation and high throughput, MALDI–MS is well suited to analyzing large populations of cells, and has been used successfully to reveal cellular heterogeneity and to discover rare cell subtypes (98). High spatial resolution MALDI-MSI can achieve high-precision metabolite positioning at the cellular and subcellular levels *in situ*, which advances our understanding of complex biological processes by revealing unprecedented details of metabolic biology (99).

The direct injection of single cells separated by microfluidic devices or micropipettes into MS provides novel ways for highly sensitive metabolite analysis in single cells (100). Zhang et al. proposed a novel strategy integrating spiral inertial microfluidics and ion mobility mass spectrometry (IM-MS) for single-cell metabolite detection and identification, which offered a simple and efficient method for single-cell lipid profiling, with additional ion mobility separation of lipids significantly improving the confidence toward identification of metabolites (100).

Single-Cell Metabolic Profiling by Flow Cytometry-Based Methods

With high sensitivity, broad molecular coverage, wide dynamic range, and structural identification capabilities, flow cytometry has become a widely used analytical tool for single-cell metabolomics. Met-Flow, a flow cytometry-based method, is capable of interrogating the network of metabolic pathways at the single-cell level within a heterogeneous population. Using Met-Flow, Patricia et al. captured the metabolic state of immune cells by targeting key proteins and rate-limiting enzymes across multiple pathways and discovered that glucose restriction and metabolic rewiring drive the expansion of an inflammatory central memory T cell subset (101).

Single-cell energetic metabolism by profiling translation inhibition (SCENITH) is a simple method for complex metabolic profiling samples *ex vivo*, that allows for the study of metabolic responses in multiple cell types in parallel by flow cytometry, particularly for rare cells. The ability of SCENITH to reveal global metabolic functions and determine complex and linked immune-phenotypes in rare cell subpopulations is helpful for evaluating therapeutic responses or patient stratification (102).

Single-Cell Spatial Metabolomics

Dissection of spatiotemporal differences in metabolic activities of singular immune cells in the tumor microenvironment (TME) is the key to understanding their complex communication networks and the immune landscape that exists within compromised tissues. With its rapidly evolving methods, single-cell metabolomics technology is expanding to high spatio-temporal resolution, providing new platforms for spatial cell atlases and *in situ* visualization of metabolic processes.

High-spatial resolution MALDI-MSI has been applied to map and visualize the three-dimensional spatial distribution of phospholipid classes (103). Alexandrov et al. developed SpaceM, a method that integrates MALDI imaging with light microscopy and digital image processing to precisely match up the mass spectrometry data with the cells, which preserved the spatial relationships between the cells without requiring special preparation (104). The spatial single nuclear metabolomics (SEAM) method is a flexible platform combining high-spatial-resolution imaging mass spectrometry and a set of computational algorithms that can display multi-scale and multi-color tissue tomography together with the identification and clustering of single nuclei by their *in situ* metabolic fingerprints. SEAM is able to explore the spatial metabolic profile and tissue histology at the single-cell level, leading to a deeper understanding of tissue metabolic organization (105).

Metabolic Modeling at the Single-Cell Level

Metabolic measurements at the single-cell level bring new insights into cellular function, which can often not be captured on other omics layers. However, single-cell metabolomics is limited by insufficient scalability and sensitivity, and is not yet widely available due to resource intensiveness. Metabolic modeling represents an interesting alternative strategy to infer latent cellular metabolism states from widely available information about reaction networks and other single-cell omics. Three main classes of modeling approaches used for prediction of metabolism on the single-cell level have been recently well reviewed, including pathway-level analysis, constraint-based modeling, and kinetic models (106). scMetNet constructs a metabolic network based on pathway repositories and then identifies metabolic rewiring across different cell populations (107). Single-cell flux estimation analysis (scFEA) infers the cell-wise fluxome from single-cell RNA-sequencing data by modeling the metabolic map with a graph neural network (108).

While an array of approaches for modeling cellular metabolic state have been proposed, a set of limitations restrict larger applicability. As experimental, technical, and biological reasons, intrinsic heterogeneity between different single-cell omics places a barrier to single-cell metabolic modeling from single-cell RNA-seq data. Single-cell proteomics measurements provide a promising alternative as they are more informative for prediction of enzyme activity. Modeling single-cell metabolism depends on well-established metabolic network models, which are basic to feasible metabolic conversions. Unpredictable metabolite exchanges between different cells and between

individual cells and the environment pose additional challenges to modeling metabolism at the single-cell level. New metabolic modeling paradigms based on advanced computational approaches, such as deep learning, may enable efficient modeling at the single-cell and multi-omics levels.

Single-Cell Multi-Omics

Single-cell multi-omics provides multiple biomolecular profilings of phenotypically heterogeneous cells on different biological layers. Multi-omics profiling of single-interacting cells in the native TME is essential for deeper understanding of the complex communication networks and the immune landscape that exist within compromised tissues. Tian et al. developed a new methodology called high-energy gas cluster ion beam-secondary ion mass spectrometry (GCIB–SIMS), which combined the chemical specificity of mass spectrometry with imaging resolution approaching 1 micron, small enough to image a single cell (109). GCIB–SIMS can comprehensively identify lipidomic and metabolomic profiling in different cell types, leading to new insights into the role of lipid reprogramming and metabolic response in normal regulation or pathogenic discoordination of cell-cell interactions in a variety of tissue microenvironments. Xiong et al. created a single-lysosome mass spectrometry (SLMS) platform that combined lysosomal patch-clamp recording with induced nanoelectrospray ionization mass spectrometry, which allowed the simultaneous detection of the electrophysiological properties and metabolome of the lysosome. These multimodal approaches open the door to much richer investigations into the interactions between cancer cells and immune cells, as well as cell–cell interactions in other systems (110).

APPLICATION AND POTENTIAL OF SINGLE-CELL METABOLOMICS IN HEMATOPOIESIS AND HEMATOLOGICAL MALIGNANCIES

Tumor Biology

Metabolic reprogramming is one of the hallmarks of malignant tumors, which provides energy and material basis for tumor proliferation, invasion, metastasis as well as immune escape. Therefore, identifying the key metabolic factors that regulate cell cancerous changes and immune responses has become a major challenge. In recent years, single-cell metabolomics has emerged as a breakthrough technique that enable to directly measure metabolic states and defines unique cell types at unparalleled high resolution. Patricia et al. used Met-Flow to simultaneously measure divergent metabolic profiles and dynamic remodeling in human peripheral blood mononuclear cells and discovered that glucose restriction and metabolic remodeling drive the expansion of an inflammatory central memory T cell subset (101). Met-Flow is able to capture the complex metabolic state of individual hematopoietic cells, which will lead to a greater understanding of the role of metabolic reprogramming in hematopoiesis and hematological malignancies.

Cellular Heterogeneity

Compositional heterogeneity is an inherent property of cell populations, presenting a major challenge in understanding the function of specific cellular subpopulations. Blocking the energy and material supply of tumor cells is one of the strategies for tumor treatment, however, metabolic heterogeneity of tumor cells hinders metabolic-based anti-tumor treatment. Metabolic differences can provide additional information to accurately identify cell state heterogeneity. To characterize cellular heterogeneity and discern specific subpopulations, molecular analysis at the single-cell level is necessary. Single-cell metabolomics analysis has revealed tremendous heterogeneity, conflicting with the classical view of hematopoiesis.

High-resolution mass spectrometry (HRMS) technology is an attractive approach to ultrasensitively detect proteins, peptides, and metabolites in limited amounts of samples, even single cells. Using single-cell capillary electrophoresis HRMS, Nemes et al. documented the differences in metabolite composition between left and right dorsal-animal blastomeres from the eight-cell frog embryo, indicating that metabolites trigger the differentiation of the stem cells into organ-specific lineages, and metabolite changes can alter cell fate (111). To extract trace-level signals from metabolic datasets with low abundances, the Trace framework was adopted, which incorporated machine learning (ML) to automate feature selection and optimization (112).

Precise discrimination of leukocyte subsets is very helpful for the clinical diagnosis of many diseases, especially for hematological malignancies. In order to rapidly discriminate various leukocyte subsets with specific functions, CyESI-MS was proposed to reveal leukocyte heterogeneity at the single-cell level. The single-cell metabolic fingerprints acquired by CyESI-MS as well as metabolite biomarkers can be used to distinguish different subtypes of leukemia cells from normal leukocytes, reflecting the application potential in clinical research (113).

Immunometabolism

Metabolic reprogramming is vital for immune cell differentiation, function and fate (114, 115). The capacity of immune cells to respond to changing environments by metabolic reprogramming is crucial to their effector function. Single-cell metabolomics analysis offers robust solutions for profiling metabolites in a high-throughput manner and has substantially deepened our understanding of metabolic networks in immune cells. Cytometry by time of flight (CyTOF) platform uses metal-tagged antibodies to estimate the metabolic configurations within single cells and has largely expanded its capability in single-cell omics by combining additional markers, such as acetylation marks, metabolic signaling, and lineage markers. Levine et al. reported a CyTOF-based approach to define the metabolic features of $CD8^+$ T cells in response to pathogen challenge at the single-cell level (116). This approach identified a transition state during an earlier stage of T-cell activation, characterized by high glycolytic and oxidative activity. Interestingly, analogous metabolic dynamics were observed in chimeric antigen receptor (CAR) T cells interrogated longitudinally in advanced lymphoma patients (116).

Therapeutic Resistance

During tumor therapy, some patients develop therapeutic resistance, resulting in treatment failure and tumor recurrence. Metabolic rewiring is an effective way to evade immune cell antitumor activity, which is favored by cancer cells. Metabolic competition between tumor and immune cells limits nutrient availability and leads to microenvironmental acidosis in the tumor ecosystem, which hinders immune cell antitumor activity. By analyzing the genetic and metabolite information of individual cells, we can distinguish genes and regulatory pathways driving drug resistance development.

A wide multitude of research activities have been focused on immune evasion and drug resistance to overcome therapeutic resistance. Chen et al. utilized the single-probe mass spectrometry technique to analyze live irinotecan-resistant (IRI) cells under different treatment conditions, demonstrating a metformin-IRI synergistic effect overcoming drug resistance (117). Inhibition of fatty acid synthase (FASN) is a potential mechanism related to metformin treatment of drug-resistant cancer cells, which results in the downregulation of lipids and fatty acids. Liu et al. reported an analytical approach that combines single-cell mass spectrometry (SCMS)-based metabolomics with machine learning (ML) models to monitor the degree of drug resistance in early chemotherapeutic stage from single cells in their native microenvironment (118). This method can be potentially employed to evaluate chemotherapeutic efficacy in the clinic.

Drug Development and Discovery

In recent decades, single-cell metabolomics has demonstrated enormous potential in many fields, including drug research and development. Current data suggest distinctive metabolic aberrations in hematological malignancies. Thus, molecular hallmarks of cancer cell metabolism provide opportunities for novel therapeutic interventions that will be complementary to existing diagnostic and treatment options. Preclinical or clinical trial studies using metabolic agents alone or in combination with other remedies have demonstrated promising outcomes. Numerous medications targeting cell metabolism are used in the care of patients with hematological malignancies (**Figure 2**), representing a promising endeavour in the search for effective treatment of hematological diseases.

Single-cell metabolomics promises to characterize metabolic reprogramming of cells in cancer, and shed light on metabolic effects of drugs (119). The application of single-cell metabolomics in drug discovery requires high throughput. Alexandrov et al. explored the potential of the recently developed method SpaceM for integration with high-content imaging and high-throughput applications in drug discovery, and successfully scaled up SpaceM to tens of samples (120).

Even though most of the findings on tumor metabolism derive from metabolomics analysis at the bulk level, single-cell metabolomics holds promise to further advance research on hematopoiesis and hematological malignancies. Given the ease of accessibility of liquid tumor biopsies in hematology, embedding single-cell techniques in routine laboratory diagnostics is feasible. Leveraging single-cell metabolomics to evaluate serial patient samples through diagnosis and the course of therapy provides a powerful means to stratify disease, evaluate tumor evolution, inform prognostication, and assist with treatment decisions. From this perspective, we foresee that single-cell metabolomics will boost the molecular diagnosis of hematological diseases, and open a new door to personalized medicine and the development of more effective therapies.

CHALLENGES AND PERSPECTIVES OF SINGLE-CELL METABOLOMICS

Single-cell metabolomics is hitting its stride and is beginning to be widely employed to profile cellular metabolism. As we have shown in this review, single-cell metabolomics has many applications and enormous potentials, however, several limitations and challenges need to be further addressed. Firstly, sample preparation is a foundation for taking an accurate and complete snapshot of the metabolome. Due to rapid metabolic changes, sample preparation can alter the metabolome from its native state. Freeze-drying and chemical fixation are common methods for quenching metabolism, which allow some metabolites to diffuse away from the cell and eventually cause the loss of biological information. Different strategies, such as frozen hydration and capillary extraction, can be used to prepare samples for single-cell metabolomics analysis. Frozen hydration preserved the integrity and compartmentalization of the pristine molecular constituents of cells, reflecting a near-natural state of the metabolome (109). Capillary extraction efficiently separates the sample molecules with less damage to biological specimens than traditional dissection methods (111). Its resampling ability enables the combination of metabolomics with other omics techniques within the same cell, laying a foundation for obtaining single-cell multimodal profiles.

Secondly, the magnitudes of metabolite abundances vary wildly, emphasizing the need for single-cell metabolomics (SCM) techniques with ultrahigh sensitivity. Thus, heightening sensitivity is a common objective to maximize the chemical information obtained in single-cell metabolomics. For MS-based single-cell metabolomics, many efforts have been made to improve metabolite extraction, ionization techniques, and proprietary algorithms. A suitable extraction procedure is a prerequisite for detecting and identifying metabolites, especially for a tiny quantity of metabolites. Onjiko et al. employed capillary electrophoresis coupled with single-cell high-resolution mass spectrometry (CE-MS) to uncover small molecules (111). Then, Trace was used to extract trace-level signals to enhance sensitivity for metabolomics analysis (111). To achieve adequate sensitivity for metabolomics analysis, Takayuki et al. developed a "nanoCESI" emitter that allowed up to sub-nM detectability by establishing a reproducible fabrication process (95). Compared with a conventional sheathless emitter, the nanoCESI emitter improved the sensitivity by 3.5-fold, and by coupling with large-volume dual preconcentration by isotachophoresis and stacking (LDIS), further achieved up to 800-fold enhanced sensitivity. As new technologies and algorithms for single-cell metabolomics continue to develop and

improve, researchers will be able to catalog the chemical contents of individual cells with high sensitivity.

The extreme complexity of the metabolome poses another challenge for single-cell metabolomics (121). The mass, charge, shape, and modification of metabolites must be considered in metabolite identification. Unpredictable new products generated by side reactions exacerbated this complexity. Another thorny issue is how to distinguish various isomers of large biomolecules. Definitively identifying and measuring the full and complete chemical contents from the cell is tricky. Spatial single-cell metabolomics allows hundreds of metabolites to be detected *in situ*. It is important to accurately match up metabolome data with the physical characteristics and neighborhood of the cell in the native context. The complexity of this analysis is sure to create new computational challenges. The integration of multidimensional data from single-cell omics is computationally challenging because of the intrinsic heterogeneity of these data (122). Fortunately, the recent explosion in molecular biology techniques and computational approaches makes it possible to overcome the above problems. Artificial intelligence (AI)-based methods have been successfully applied to several tasks of single-cell omics, representing powerful and promising tools for biological discovery (123). The power of single-cell metabolomics can be improved by combining artificial intelligence-based algorithms. New algorithms for metabolite identification and open-platforms will allow neophytes and seasoned investigators alike to make sense of the jumble peaks in the spectra, such as Trace (112) and METASPACE (124).

Finally, with respect to clinical applications, the clinical translation of single-cell metabolomics techniques is another key challenge in this field. Considering the complexity and sensitivity of SCM, the results derived from SCM must be interpreted with caution in clinical decisions. Additionally, the high cost of single-cell metabolomics creates a barrier to its widespread implementation in routine testing. With the advent of increasingly faster and cheaper high-throughput technologies, the integration of single-cell omics across modalities will expand our horizons and deepen our understanding of the interactions among the different biological layers. It is easy to imagine that single-cell methodologies will be applied routinely in clinical diagnostics, prognosis prediction as well as disease monitoring, which will revolutionize the diagnosis and therapy of patients.

AUTHOR CONTRIBUTIONS

FZ and XH contributed to investigation, data curation, illustration, writing, and editing. XH and JY contributed to writing-review and editing. All authors have read and agreed to the published version of the manuscript.

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REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144(5):646–74. doi: 10.1016/j.cell.2011.02.013
2. Judge A, Dodd MS. Metabolism. *Essays Biochem* (2020) 64(4):607–47. doi: 10.1042/ebc20190041
3. Cantor JR, Sabatini DM. Cancer Cell Metabolism: One Hallmark, Many Faces. *Cancer Discovery* (2012) 2(10):881–98. doi: 10.1158/2159-8290.Cd-12-0345
4. Cairns RA, Harris IS, Mak TW. Regulation of Cancer Cell Metabolism. *Nat Rev Cancer*. (2011) 11(2):85–95. doi: 10.1038/nrc2981
5. Murphy MP, O'Neill LA. Krebs Cycle Reimagined: The Emerging Roles of Succinate and Itaconate as Signal Transducers. *Cell* (2018) 174(4):780–4. doi: 10.1016/j.cell.2018.07.030
6. Pollizzi KN, Powell JD. Integrating Canonical and Metabolic Signalling Programmes in the Regulation of T Cell Responses. *Nat Rev Immunol* (2014) 14(7):435–46. doi: 10.1038/nri3701
7. Zasłona Z, O'Neill LA. Cytokine-Like Roles for Metabolites in Immunity. *Mol Cell* (2020) 78(5):814–23. doi: 10.1016/j.molcel.2020.04.002
8. Voss K, Hong HS, Bader JE, Sugiura A, Lyssiotis CA, Rathmell JC. A Guide to Interrogating Immunometabolism. *Nat Rev Immunol* (2021) 21(10):637–52. doi: 10.1038/s41577-021-00529-8
9. Cameron AM, Lawless SJ, Pearce EJ. Metabolism and Acetylation in Innate Immune Cell Function and Fate. *Semin Immunol* (2016) 28:408–16. doi: 10.1016/j.smim.2016.10.003
10. Fan J, Krautkramer KA, Feldman JL, Denu JM. Metabolic Regulation of Histone Post-Translational Modifications. *ACS Chem Biol* (2015) 10(1):95–108. doi: 10.1021/cb500846u
11. Lv Y, Tariq M, Guo X, Kanwal S, Esteban MA. Intricacies in the Cross Talk Between Metabolic Enzymes, Rna, and Protein Translation. *J Mol Cell Biol* (2019) 11(10):813–5. doi: 10.1093/jmcb/mjz089
12. Martinez-Outschoorn UE, Lin Z, Trimmer C, Flomenberg N, Wang C, Pavlides S, et al. Cancer Cells Metabolically "Fertilize" the Tumor Microenvironment With Hydrogen Peroxide, Driving the Warburg Effect: Implications for PET Imaging of Human Tumors. *Cell Cycle* (2011) 10 (15):2504–20. doi: 10.4161/cc.10.15.16585
13. Yuneva MO, Fan TW, Allen TD, Higashi RM, Ferraris DV, Tsukamoto T, et al. The Metabolic Profile of Tumors Depends on Both the Responsible Genetic Lesion and Tissue Type. *Cell Metab* (2012) 15(2):157–70. doi: 10.1016/j.cmet.2011.12.015
14. DeBerardinis RJ. Is Cancer a Disease of Abnormal Cellular Metabolism? New Angles on an Old Idea. *Genet Med* (2008) 10(11):767–77. doi: 10.1097/GIM.0b013e31818b0d9b
15. Wike-Hooley JL, Haveman J, Reinhold HS. The Relevance of Tumour pH to the Treatment of Malignant Disease. *Radiother Oncol* (1984) 2(4):343–66. doi: 10.1016/s0167-8140(84)80077-8
16. Shime H, Yabu M, Akazawa T, Kodama K, Matsumoto M, Seya T, et al. Tumor-Secreted Lactic Acid Promotes IL-23/IL-17 Proinflammatory Pathway. *J Immunol* (2008) 180(11):7175–83. doi: 10.4049/jimmunol.180.11.7175
17. Hirschhaeuser F, Sattler UG, Mueller-Klieser W. Lactate: A Metabolic Key Player in Cancer. *Cancer Res* (2011) 71(22):6921–5. doi: 10.1158/0008-5472.Can-11-1457
18. Liu T, Kishton RJ, Macintyre AN, Gerriets VA, Xiang H, Liu X, et al. Glucose Transporter 1-Mediated Glucose Uptake Is Limiting for B-Cell Acute Lymphoblastic Leukemia Anabolic Metabolism and Resistance to Apoptosis. *Cell Death Dis* (2014) 5(10):e1470. doi: 10.1038/cddis.2014.431

19. Matsumoto T, Jimi S, Migita K, Takamatsu Y, Hara S. Inhibition of Glucose Transporter 1 Induces Apoptosis and Sensitizes Multiple Myeloma Cells to Conventional Chemotherapeutic Agents. *Leuk Res* (2016) 41:103–10. doi: 10.1016/j.leukres.2015.12.008
20. Song K, Li M, Xu XJ, Xuan L, Huang GN, Song XL, et al. HIF-1α and GLUT1 Gene Expression is Associated With Chemoresistance of Acute Myeloid Leukemia. *Asian Pac J Cancer Prev* (2014) 15(4):1823–9. doi: 10.7314/apjcp.2014.15.4.1823
21. Bentley J, Walker I, McIntosh E, Whetton AD, Owen-Lynch PJ, Baldwin SA. Glucose Transport Regulation by P210 Bcr-Abl in a Chronic Myeloid Leukaemia Model. *Br J Haematol* (2001) 112(1):212–5. doi: 10.1046/j.1365-2141.2001.02428.x
22. Boag JM, Beesley AH, Firth MJ, Freitas JR, Ford J, Hoffmann K, et al. Altered Glucose Metabolism in Childhood Pre-B Acute Lymphoblastic Leukaemia. *Leukemia* (2006) 20(10):1731–7. doi: 10.1038/sj.leu.2404365
23. McBrayer SK, Cheng JC, Singhal S, Krett NL, Rosen ST, Shanmugam M. Multiple Myeloma Exhibits Novel Dependence on GLUT4, GLUT8, and GLUT11: Implications for Glucose Transporter-Directed Therapy. *Blood* (2012) 119(20):4686–97. doi: 10.1182/blood-2011-09-377846
24. Adekola KU, Dalva Aydemir S, Ma S, Zhou Z, Rosen ST, Shanmugam M. Investigating and Targeting Chronic Lymphocytic Leukemia Metabolism With the Human Immunodeficiency Virus Protease Inhibitor Ritonavir and Metformin. *Leuk Lymphoma*. (2015) 56(2):450–9. doi: 10.3109/10428194.2014.922180
25. Jitschin R, Hofmann AD, Bruns H, Giessl A, Bricks J, Berger J, et al. Mitochondrial Metabolism Contributes to Oxidative Stress and Reveals Therapeutic Targets in Chronic Lymphocytic Leukemia. *Blood* (2014) 123(17):2663–72. doi: 10.1182/blood-2013-10-532200
26. Galicia-Vázquez G, Aloyz R. Metabolic Rewiring Beyond Warburg in Chronic Lymphocytic Leukemia: How Much do We Actually Know? *Crit Rev Oncol Hematol* (2019) 134:65–70. doi: 10.1016/j.critrevonc.2018.12.003.
27. Chen WL, Wang JH, Zhao AH, Xu X, Wang YH, Chen TL, et al. A Distinct Glucose Metabolism Signature of Acute Myeloid Leukemia With Prognostic Value. *Blood* (2014) 124(10):1645–54. doi: 10.1182/blood-2014-02-554204
28. Ju HQ, Zhan G, Huang A, Sun Y, Wen S, Yang J, et al. ITD Mutation in FLT3 Tyrosine Kinase Promotes Warburg Effect and Renders Therapeutic Sensitivity to Glycolytic Inhibition. *Leukemia* (2017) 31(10):2143–50. doi: 10.1038/leu.2017.45
29. Wang YH, Israelsen WJ, Lee D, Yu VWC, Jeanson NT, Clish CB, et al. Cell-State-Specific Metabolic Dependency in Hematopoiesis and Leukemogenesis. *Cell* (2014) 158(6):1309–23. doi: 10.1016/j.cell.2014.07.048
30. Fujiwara S, Wada N, Kawano Y, Okuno Y, Kikukawa Y, Endo S, et al. Lactate, a Putative Survival Factor for Myeloma Cells, is Incorporated by Myeloma Cells Through Monocarboxylate Transporters 1. *Exp Hematol Oncol* (2015) 4:12. doi: 10.1186/s40164-015-0008-z
31. Hanson DJ, Nakamura S, Amachi R, Hiasa M, Oda A, Tsuji D, et al. Effective Impairment of Myeloma Cells and Their Progenitors by Blockade of Monocarboxylate Transportation. *Oncotarget* (2015) 6(32):33568–86. doi: 10.18632/oncotarget.5598
32. Mikkilineni L, Whitaker-Menezes D, Domingo-Vidal M, Sprandio J, Avena P, Cotzia P, et al. Hodgkin Lymphoma: A Complex Metabolic Ecosystem With Glycolytic Reprogramming of the Tumor Microenvironment. *Semin Oncol* (2017) 44(3):218–25. doi: 10.1053/j.seminoncol.2017.10.003
33. Chen Y, Xu Q, Ji D, Wei Y, Chen H, Li T, et al. Inhibition of Pentose Phosphate Pathway Suppresses Acute Myelogenous Leukemia. *Tumour Biol* (2016) 37(5):6027–34. doi: 10.1007/s13277-015-4428-5
34. Poulain L, Sujobert P, Zylbersztejn F, Barreau S, Stuani L, Lambert M, et al. High Mtorc1 Activity Drives Glycolysis Addiction and Sensitivity to G6PD Inhibition in Acute Myeloid Leukemia Cells. *Leukemia* (2017) 31(11):2326–35. doi: 10.1038/leu.2017.81
35. Kreitz J, Schönfeld C, Seibert M, Stolp V, Alshamleh I, Oellerich T, et al. Metabolic Plasticity of Acute Myeloid Leukemia. *Cells* (2019) 8(8):805. doi: 10.3390/cells8080805
36. Chen WL, Wang YY, Zhao A, Xia L, Xie G, Su M, et al. Enhanced Fructose Utilization Mediated by SLC2A5 Is a Unique Metabolic Feature of Acute Myeloid Leukemia With Therapeutic Potential. *Cancer Cell* (2016) 30(5):779–91. doi: 10.1016/j.ccell.2016.09.006
37. Yan TY, Naren DL, Gong YP. [The Roles of Glut5 in Imatinib Resistance in the Ph(+) Acute Lymphoblastic Leukemia Cell]. *J Sichuan Univ Med Sci edition* (2017) 48(3):389–93.
38. Zhao P, Huang J, Zhang D, Zhang D, Wang F, Qu Y, et al. SLC2A5 Overexpression in Childhood Philadelphia Chromosome-Positive Acute Lymphoblastic Leukaemia. *Br J haematology*. (2018) 183(2):242–50. doi: 10.1111/bjh.15580
39. Vangapandu HV, Alston B, Morse J, Ayres ML, Wierda WG, Keating MJ, et al. Biological and Metabolic Effects of IACS-010759, an OxPhos Inhibitor, on Chronic Lymphocytic Leukemia Cells. *Oncotarget* (2018) 9(38):24980–91. doi: 10.18632/oncotarget.25166
40. Mayer RL, Schwarzmeier JD, Gerner MC, Bileck A, Mader JC, Meier-Menches SM, et al. Proteomics and Metabolomics Identify Molecular Mechanisms of Aging Potentially Predisposing for Chronic Lymphocytic Leukemia. *Mol Cell Proteomics*. (2018) 17(2):290–303. doi: 10.1074/mcp.RA117.000425
41. Carew JS, Nawrocki ST, Xu RH, Dunner K, McConkey DJ, Wierda WG, et al. Increased Mitochondrial Biogenesis in Primary Leukemia Cells: The Role of Endogenous Nitric Oxide and Impact on Sensitivity to Fludarabine. *Leukemia* (2004) 18(12):1934–40. doi: 10.1038/sj.leu.2403545
42. Martinez Marignac VL, Smith S, Toban N, Bazile M, Aloyz R. Resistance to Dasatinib in Primary Chronic Lymphocytic Leukemia Lymphocytes Involves AMPK-Mediated Energetic Re-Programming. *Oncotarget* (2013) 4(12):2550–66. doi: 10.18632/oncotarget.1508
43. Bruno S, Ledda B, Tenca C, Ravera S, Orengo AM, Mazzarello AN, et al. Metformin Inhibits Cell Cycle Progression of B-Cell Chronic Lymphocytic Leukemia Cells. *Oncotarget* (2015) 6(26):22624–40. doi: 10.18632/oncotarget.4168
44. Kishton RJ, Barnes CE, Nichols AG, Cohen S, Gerriets VA, Siska PJ, et al. AMPK Is Essential to Balance Glycolysis and Mitochondrial Metabolism to Control T-ALL Cell Stress and Survival. *Cell Metab* (2016) 23(4):649–62. doi: 10.1016/j.cmet.2016.03.008
45. Sriskanthadevan S, Jeyaraju DV, Chung TE, Prabha S, Xu W, Skrtic M, et al. AML Cells Have Low Spare Reserve Capacity in Their Respiratory Chain That Renders Them Susceptible to Oxidative Metabolic Stress. *Blood* (2015) 125(13):2120–30. doi: 10.1182/blood-2014-08-594408
46. Song K, Li M, Xu X, Xuan LI, Huang G, Liu Q. Resistance to Chemotherapy is Associated With Altered Glucose Metabolism in Acute Myeloid Leukemia. *Oncol letters*. (2016) 12(1):334–42. doi: 10.3892/ol.2016.4600
47. Suganuma K, Miwa H, Imai N, Shikami M, Gotou M, Goto M, et al. Energy Metabolism of Leukemia Cells: Glycolysis Versus Oxidative Phosphorylation. *Leuk Lymphoma*. (2010) 51(11):2112–9. doi: 10.3109/10428194.2010.512966
48. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, et al. Cancer-Associated IDH1 Mutations Produce 2-Hydroxyglutarate. *Nature* (2009) 462(7274):739–44. doi: 10.1038/nature08617
49. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 Mutations Result in a Hypermethylation Phenotype, Disrupt TET2 Function, and Impair Hematopoietic Differentiation. *Cancer Cell* (2010) 18(6):553–67. doi: 10.1016/j.ccr.2010.11.015
50. Amary MF, Bacsi K, Maggiani F, Damato S, Halai D, Berisha F, et al. IDH1 and IDH2 Mutations are Frequent Events in Central Chondrosarcoma and Central and Periosteal Chondromas But Not in Other Mesenchymal Tumours. *J Pathol* (2011) 224(3):334–43. doi: 10.1002/path.2913
51. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, et al. Recurring Mutations Found by Sequencing an Acute Myeloid Leukemia Genome. *N Engl J Med* (2009) 361(11):1058–66. doi: 10.1056/NEJMoa0903840
52. Yen KE, KonteatisZenon D, Zhihua S, Artin E, Dang L, Straley KS, et al. Abstract B126: AG-881, A Brain Penetrant, Potent, Pan-Mutant IDH (mIDH) Inhibitor for Use in mIDH Solid and Hematologic Malignancies. *Mol Cancer Ther*, Vol. 17. (2018) B126–B126. doi: 10.1158/1535-7163.TARG-17-B126
53. Salama V, Brooks NA, Skwarska A, Kays LM, Milligan PL, Newell KJ, et al. Abstract 6417: LY3410738, A Novel Inhibitor of Mutant IDH1 Is More Effective Than Ivosidenib and Potentiates Antileukemic Activity of Standard

Chemotherapy in Preclinical Models of Acute Myeloid Leukemia (AML). *Cancer Res* (2020) 80:6417–7. doi: 10.1158/1538-7445.AM2020-6417
54. Chan WK, Horvath TD, Tan L, Link T, Harutyunyan KG, Pontikos MA, et al. Glutaminase Activity of L-Asparaginase Contributes to Durable Preclinical Activity Against Acute Lymphoblastic Leukemia. *Mol Cancer Ther* (2019) 18(9):1587–92. doi: 10.1158/1535-7163.Mct-18-1329
55. Zelenetz AD. Chronic Lymphocytic Leukemia: Individualizing Treatment Approach. *J Natl Compr Canc Netw* (2017) 15(5s):713–5. doi: 10.6004/jnccn.2017.0081
56. Petronini PG, Urbani S, Alfieri R, Borghetti AF, Guidotti GG. Cell Susceptibility to Apoptosis by Glutamine Deprivation and Rescue: Survival and Apoptotic Death in Cultured Lymphoma-Leukemia Cell Lines. *J Cell Physiol* (1996) 169(1):175–85. doi: 10.1002/(SICI)1097-4652(199610)169:1<175::AID-JCP18>3.0.CO;2-C
57. Gregory MA, Nemkov T, Park HJ, Zaberezhnyy V, Gehrke S, Adane B, et al. Targeting Glutamine Metabolism and Redox State for Leukemia Therapy. *Clin Cancer Res* (2019) 25(13):4079–90. doi: 10.1158/1078-0432.Ccr-18-3223
58. Willems L, Jacque N, Jacquel A, Neveux N, Maciel TT, Lambert M, et al. Inhibiting Glutamine Uptake Represents an Attractive New Strategy for Treating Acute Myeloid Leukemia. *Blood* (2013) 122(20):3521–32. doi: 10.1182/blood-2013-03-493163
59. Bolzoni M, Chiu M, Accardi F, Vescovini R, Airoldi I, Storti P, et al. Dependence on Glutamine Uptake and Glutamine Addiction Characterize Myeloma Cells: A New Attractive Target. *Blood* (2016) 128(5):667–79. doi: 10.1182/blood-2016-01-690743
60. Herranz D, Ambesi-Impiombato A, Sudderth J, Sánchez-Martín M, Belver L, Tosello V, et al. Metabolic Reprogramming Induces Resistance to Anti-NOTCH1 Therapies in T Cell Acute Lymphoblastic Leukemia. *Nat Med* (2015) 21(10):1182–9. doi: 10.1038/nm.3955
61. Miraki-Moud F, Ghazaly E, Ariza-McNaughton L, Hodby KA, Clear A, Anjos-Afonso F, et al. Arginine Deprivation Using Pegylated Arginine Deiminase has Activity Against Primary Acute Myeloid Leukemia Cells *In Vivo*. *Blood* (2015) 125(26):4060–8. doi: 10.1182/blood-2014-10-608133
62. Mussai F, Egan S, Higginbotham-Jones J, Perry T, Beggs A, Odintsova E, et al. Arginine Dependence of Acute Myeloid Leukemia Blast Proliferation: A Novel Therapeutic Target. *Blood* (2015) 125(15):2386–96. doi: 10.1182/blood-2014-09-600643
63. De Santo C, Booth S, Vardon A, Cousins A, Tubb V, Perry T, et al. The Arginine Metabolome in Acute Lymphoblastic Leukemia can be Targeted by the Pegylated-Recombinant Arginase I BCT-100. *Int J Cancer.* (2018) 142(7):1490–502. doi: 10.1002/ijc.31170
64. Du H, Wang L, Liu B, Wang J, Su H, Zhang T, et al. Analysis of the Metabolic Characteristics of Serum Samples in Patients With Multiple Myeloma. *Front Pharmacol* (2018) 9:884. doi: 10.3389/fphar.2018.00884
65. Kourelis TV, Nasr SH, Dispenzieri A, Kumar SK, Gertz MA, Fervenza FC, et al. Outcomes of Patients With Renal Monoclonal Immunoglobulin Deposition Disease. *Am J Hematol* (2016) 91(11):1123–8. doi: 10.1002/ajh.24528
66. Fallarino F, Grohmann U, Puccetti P. Indoleamine 2,3-Dioxygenase: From Catalyst to Signaling Function. *Eur J Immunol* (2012) 42(8):1932–7. doi: 10.1002/eji.201242572
67. Platten M, Wick W, Van den Eynde BJ. Tryptophan Catabolism in Cancer: Beyond IDO and Tryptophan Depletion. *Cancer Res* (2012) 72(21):5435–40. doi: 10.1158/0008-5472.Can-12-0569
68. Chamuleau ME, van de Loosdrecht AA, Hess CJ, Janssen JJ, Zevenbergen A, Delwel R, et al. (Indoleamine 2,3-Dioxygenase) mRNA Level in Blasts of Acute Myeloid Leukemic Patients Predicts Poor Clinical Outcome. *Haematologica* (2008) 93(12):1894–8. doi: 10.3324/haematol.13113
69. Mabuchi R, Hara T, Matsumoto T, Shibata Y, Nakamura N, Nakamura H, et al. High Serum Concentration of L-Kynurenine Predicts Unfavorable Outcomes in Patients With Acute Myeloid Leukemia. *Leuk Lymphoma.* (2016) 57(1):92–8. doi: 10.3109/10428194.2015.1041388
70. Vonka V, Humlova Z, Klamova H, Kujovska-Krcmova L, Petrackova M, Hamsikova E, et al. Kynurenine and Uric Acid Levels in Chronic Myeloid Leukemia Patients. *Oncoimmunology* (2015) 4(3):e992646. doi: 10.4161/2162402x.2014.992646
71. Atene CG, Fiorcari S, Mesini N, Alboni S, Martinelli S, Maccaferri M, et al. Indoleamine 2, 3-Dioxygenase 1 Mediates Survival Signals in Chronic Lymphocytic Leukemia *via* Kynurenine/Aryl Hydrocarbon Receptor-Mediated MCL1 Modulation. *Front Immunol* (2022) 13:832263. doi: 10.3389/fimmu.2022.832263
72. Masaki A, Ishida T, Maeda Y, Ito A, Suzuki S, Narita T, et al. Clinical Significance of Tryptophan Catabolism in Hodgkin Lymphoma. *Cancer Sci* (2018) 109(1):74–83. doi: 10.1111/cas.13432
73. Akagi T, Yin D, Kawamata N, Bartram CR, Hofmann WK, Wolf I, et al. Methylation Analysis of Asparagine Synthetase Gene in Acute Lymphoblastic Leukemia Cells. *Leukemia* (2006) 20(7):1303–6. doi: 10.1038/sj.leu.2404216
74. Broome JD. Evidence That the L-Asparaginase of Guinea Pig Serum is Responsible for its Antilymphoma Effects. I. Properties of the L-Asparaginase of Guinea Pig Serum in Relation to Those of the Antilymphoma Substance. *J Exp Med* (1963) 118(1):99–120. doi: 10.1084/jem.118.1.99
75. Egler RA, Ahuja SP, Matloub Y. L-Asparaginase in the Treatment of Patients With Acute Lymphoblastic Leukemia. *J Pharmacol pharmacotherapeutics.* (2016) 7(2):62–71. doi: 10.4103/0976-500x.184769
76. Clavell LA, Gelber RD, Cohen HJ, Hitchcock-Bryan S, Cassady JR, Tarbell NJ, et al. Four-Agent Induction and Intensive Asparaginase Therapy for Treatment of Childhood Acute Lymphoblastic Leukemia. *N Engl J Med* (1986) 315(11):657–63. doi: 10.1056/nejm198609113151101
77. Sallan SE, Hitchcock-Bryan S, Gelber R, Cassady JR, FreiE3rd, Nathan DG. Influence of Intensive Asparaginase in the Treatment of Childhood non-T-Cell Acute Lymphoblastic Leukemia. *Cancer Res* (1983) 43(11):5601–7.
78. Raffel S, Falcone M, Kneisel N, Hansson J, Wang W, Lutz C, et al. BCAT1 Restricts αkg Levels in AML Stem Cells Leading to IDHmut-Like DNA Hypermethylation. *Nature* (2017) 551(7680):384–8. doi: 10.1038/nature24294
79. Hattori A, Tsunoda M, Konuma T, Kobayashi M, Nagy T, Glushka J, et al. Cancer Progression by Reprogrammed BCAA Metabolism in Myeloid Leukaemia. *Nature* (2017) 545(7655):500–4. doi: 10.1038/nature22314
80. Rozovski U, Grgurevic S, Bueso-Ramos C, Harris DM, Li P, Liu Z, et al. Aberrant LPL Expression, Driven by STAT3, Mediates Free Fatty Acid Metabolism in CLL Cells. *Mol Cancer Res* (2015) 13(5):944–53. doi: 10.1158/1541-7786.Mcr-14-0412
81. Rozovski U, Harris DM, Li P, Liu Z, Jain P, Ferrajoli A, et al. STAT3-Activated CD36 Facilitates Fatty Acid Uptake in Chronic Lymphocytic Leukemia Cells. *Oncotarget* (2018) 9(30):21268–80. doi: 10.18632/oncotarget.25066
82. Goldberg IJ. Lipoprotein Lipase and Lipolysis: Central Roles in Lipoprotein Metabolism and Atherogenesis. *J Lipid Res* (1996) 37(4):693–707. doi: 10.1016/S0022-2275(20)37569-6
83. Rozovski U, Hazan-Halevy I, Barzilai M, Keating MJ, Estrov Z. Metabolism Pathways in Chronic Lymphocytic Leukemia. *Leuk Lymphoma.* (2016) 57(4):758–65. doi: 10.3109/10428194.2015.1106533
84. Kersten S. Integrated Physiology and Systems Biology of Pparα. *Mol Metab* (2014) 3(4):354–71. doi: 10.1016/j.molmet.2014.02.002
85. Rozovski U, Harris DM, Li P, Liu Z, Jain P, Ferrajoli A, et al. Ibrutinib Inhibits Free Fatty Acid Metabolism in Chronic Lymphocytic Leukemia. *Leuk Lymphoma.* (2018) 59(11):2686–91. doi: 10.1080/10428194.2018.1439167
86. Liu PP, Liu J, Jiang WQ, Carew JS, Ogasawara MA, Pelicano H, et al. Elimination of Chronic Lymphocytic Leukemia Cells in Stromal Microenvironment by Targeting CPT With an Antiangina Drug Perhexiline. *Oncogene* (2016) 35(43):5663–73. doi: 10.1038/onc.2016.103
87. Tucci J, Chen T, Margulis K, Orgel E, Paszkiewicz RL, Cohen MD, et al. Adipocytes Provide Fatty Acids to Acute Lymphoblastic Leukemia Cells. *Front Oncol* (2021) 11:665763. doi: 10.3389/fonc.2021.665763
88. Butturini AM, Dorey FJ, Lange BJ, Henry DW, Gaynon PS, Fu C, et al. Obesity and Outcome in Pediatric Acute Lymphoblastic Leukemia. *J Clin Oncol* (2007) 25(15):2063–9. doi: 10.1200/jco.2006.07.7792
89. Panaroni C, Fulzele K, Mori T, Siu KT, Onyewadume C, Maebius A, et al. Multiple Myeloma Cells Induce Lipolysis in Adipocytes and Uptake Fatty Acids Through Fatty Acid Transporter Proteins. *Blood* (2022) 139(6):876–88. doi: 10.1182/blood.2021013832

90. Tabe Y, Konopleva M, Andreeff M. Fatty Acid Metabolism, Bone Marrow Adipocytes, and AML. *Front Oncol* (2020) 10:155. doi: 10.3389/fonc.2020.00155
91. Zhou W, Zhang B, Liu Y, Wang C, Sun W, Li W, et al. Advances in Capillary Electrophoresis-Mass Spectrometry for Cell Analysis. *TrAC Trends Analytical Chem* (2019) 117:316–30. doi: 10.1016/j.trac.2019.05.011
92. Ali A, Abouleila Y, Shimizu Y, Hiyama E, Emara S, Mashaghi A, et al. Single-Cell Metabolomics by Mass Spectrometry: Advances, Challenges, and Future Applications. *TrAC Trends Analytical Chem* (2019) 120:115436. doi: 10.1016/j.trac.2019.02.033
93. Xu S, Yang C, Yan X, Liu H. Towards High Throughput and High Information Coverage: Advanced Single-Cell Mass Spectrometric Techniques. *Anal Bioanal Chem* (2022) 414(1):219–33. doi: 10.1007/s00216-021-03624-w
94. Cong Y, Liang Y, Motamedchaboki K, Huguet R, Truong T, Zhao R, et al. Improved Single-Cell Proteome Coverage Using Narrow-Bore Packed NanoLC Columns and Ultrasensitive Mass Spectrometry. *Anal Chem* (2020) 92(3):2665–71. doi: 10.1021/acs.analchem.9b04631
95. Kawai T, Ota N, Okada K, Imasato A, Owa Y, Morita M, et al. Ultrasensitive Single Cell Metabolomics by Capillary Electrophoresis-Mass Spectrometry With a Thin-Walled Tapered Emitter and Large-Volume Dual Sample Preconcentration. *Anal Chem* (2019) 91(16):10564–72. doi: 10.1021/acs.analchem.9b01578
96. Stoeckli M, Chaurand P, Hallahan DE, Caprioli RM. Imaging Mass Spectrometry: A New Technology for the Analysis of Protein Expression in Mammalian Tissues. *Nat Med* (2001) 7(4):493–6. doi: 10.1038/86573
97. Passarelli MK, Ewing AG. Single-Cell Imaging Mass Spectrometry. *Curr Opin Chem Biol* (2013) 17(5):854–9. doi: 10.1016/j.cbpa.2013.07.017
98. Hansen RL, Lee YJ. High-Spatial Resolution Mass Spectrometry Imaging: Toward Single Cell Metabolomics in Plant Tissues. *Chem Rec.* (2018) 18 (1):65–77. doi: 10.1002/tcr.201700027
99. Feng D, Xu T, Li H, Shi X, Xu G. Single-Cell Metabolomics Analysis by Microfluidics and Mass Spectrometry: Recent New Advances. *J Anal Testing* (2020) 4(3):198–209. doi: 10.1007/s41664-020-00138-9
100. Zhang L, Xu T, Zhang J, Wong SCC, Ritchie M, Hou HW, et al. Single Cell Metabolite Detection Using Inertial Microfluidics-Assisted Ion Mobility Mass Spectrometry. *Anal Chem* (2021) 93(30):10462–8. doi: 10.1021/acs.analchem.1c00106
101. Ahl PJ, Hopkins RA, Xiang WW, Au B, Kaliaperumal N, Fairhurst AM, et al. Met-Flow, a Strategy for Single-Cell Metabolic Analysis Highlights Dynamic Changes in Immune Subpopulations. *Commun Biol* (2020) 3(1):305. doi: 10.1038/s42003-020-1027-9
102. Argüello RJ, Combes AJ, Char R, Gigan JP, Baaziz AI, Bousiquot E, et al. SCENITH: A Flow Cytometry-Based Method to Functionally Profile Energy Metabolism With Single-Cell Resolution. *Cell Metab* (2020) 32(6):1063–75.e7. doi: 10.1016/j.cmet.2020.11.007
103. Dueñas ME, Essner JJ, Lee YJ. 3d MALDI Mass Spectrometry Imaging of a Single Cell: Spatial Mapping of Lipids in the Embryonic Development of Zebrafish. *Sci Rep* (2017) 7(1):14946. doi: 10.1038/s41598-017-14949-x
104. Rappez L, Stadler M, Triana S, Gathungu RM, Ovchinnikova K, Phapale P, et al. SpaceM Reveals Metabolic States of Single Cells. *Nat Methods* (2021) 18 (7):799–805. doi: 10.1038/s41592-021-01198-0
105. Yuan Z, Zhou Q, Cai L, Pan L, Sun W, Qumu S, et al. SEAM is a Spatial Single Nuclear Metabolomics Method for Dissecting Tissue Microenvironment. *Nat Methods* (2021) 18(10):1223–32. doi: 10.1038/s41592-021-01276-3
106. Hrovatin K, Fischer DS, Theis FJ. Toward Modeling Metabolic State From Single-Cell Transcriptomics. *Mol Metab* (2022) 57:101396. doi: 10.1016/j.molmet.2021.101396
107. Jatav S, Malhotra S, Miller FD, Jha A, Goyal S. Inferring Metabolic Rewiring in Embryonic Neural Development Using Single Cell Data. *bioRxiv* [preprint] (2020). doi: 10.1101/2020.09.03.282442
108. Alghamdi N, Chang W, Dang P, Lu X, Wan C, Gampala S, et al. A Graph Neural Network Model to Estimate Cell-Wise Metabolic Flux Using Single-Cell RNA-Seq Data. *Genome Res* (2021) 31(10):1867–84. doi: 10.1101/gr.271205.120
109. Tian H, Sparvero LJ, Anthonymuthu TS, Sun WY, Amoscato AA, He RR, et al. Successive High-Resolution (H(2)O)(n)-GCIB and C(60)-SIMS Imaging Integrates Multi-Omics in Different Cell Types in Breast Cancer Tissue. *Anal Chem* (2021) 93(23):8143–51. doi: 10.1021/acs.analchem.0c05311
110. Zhu H, Li Q, Liao T, Yin X, Chen Q, Wang Z, et al. Metabolomic Profiling of Single Enlarged Lysosomes. *Nat Methods* (2021) 18(7):788–98. doi: 10.1038/s41592-021-01182-8
111. Onjiko RM, Nemes P, Moody SA. Altering Metabolite Distribution at Xenopus Cleavage Stages Affects Left-Right Gene Expression Asymmetries. *Genesis* (2021) 59(5-6):e23418. doi: 10.1002/dvg.23418
112. Liu Z, Portero EP, Jian Y, Zhao Y, Onjiko RM, Zeng C, et al. Trace, Machine Learning of Signal Images for Trace-Sensitive Mass Spectrometry: A Case Study From Single-Cell Metabolomics. *Anal Chem* (2019) 91(9):5768–76. doi: 10.1021/acs.analchem.8b05985
113. Yao H, Zhao H, Pan X, Zhao X, Feng J, Yang C, et al. Discriminating Leukemia Cellular Heterogeneity and Screening Metabolite Biomarker Candidates Using Label-Free Mass Cytometry. *Anal Chem* (2021) 93 (29):10282–91. doi: 10.1021/acs.analchem.1c01746
114. Johnson MO, Siska PJ, Contreras DC, Rathmell JC. Nutrients and the Microenvironment to Feed a T Cell Army. *Semin Immunol* (2016) 28:505–13. doi: 10.1016/j.smim.2016.09.003
115. Boothby M, Rickert RC. Metabolic Regulation of the Immune Humoral Response. *Immunity* (2017) 46(5):743–55. doi: 10.1016/j.immuni.2017.04.009
116. Levine LS, Hiam-Galvez KJ, Marquez DM, Tenvooren I, Madden MZ, Contreras DC, et al. Single-Cell Analysis by Mass Cytometry Reveals Metabolic States of Early-Activated CD8(+) T Cells During the Primary Immune Response. *Immunity* (2021) 54(4):829–44.e5. doi: 10.1016/j.immuni.2021.02.018
117. Chen X, Sun M, Yang Z. Single Cell Mass Spectrometry Analysis of Drug-Resistant Cancer Cells: Metabolomics Studies of Synergetic Effect of Combinational Treatment. *Analytica Chimica Acta* (2022) 1201:339621. doi: 10.1016/j.aca.2022.339621
118. Liu R, Sun M, Zhang G, Lan Y, Yang Z. Towards Early Monitoring of Chemotherapy-Induced Drug Resistance Based on Single Cell Metabolomics: Combining Single-Probe Mass Spectrometry With Machine Learning. *Analytica chimica Acta* (2019) 1092:42–8. doi: 10.1016/j.aca.2019.09.065
119. Nassar SF, Raddassi K, Wu T. Single-Cell Multiomics Analysis for Drug Discovery. *Metabolites* (2021) 11(11):729. doi: 10.3390/metabo11110729
120. Alexandrov T. Alexandrov T. Integrating High-Content Imaging With Mass Spectrometr (HCS+ M) for High-Throughput Single-Cell Metabolomics and Drug Discovery. in*ATTRACT Final Conference*.
121. Seydel C. Single-Cell Metabolomics Hits its Stride. *Nat Methods* (2021) 18 (12):1452–6. doi: 10.1038/s41592-021-01333-x
122. Forcato M, Romano O, Bicciato S. Computational Methods for the Integrative Analysis of Single-Cell Data. *Briefings Bioinf* (2021) 22(1):20–9. doi: 10.1093/bib/bbaa042
123. Campillo-Marcos I, Alvarez-Errico D, Alandes RA, Mereu E, Esteller M. Single-Cell Technologies and Analyses in Hematopoiesis and Hematological Malignancies. *Exp hematology.* (2021) 98:1–13. doi: 10.1016/j.exphem.2021.05.001
124. Alexandrov T, Ovchinnikova K, Palmer A, Kovalev V, Tarasov A, Stuart L, et al. METASPACE: A Community-Populated Knowledge Base of Spatial Metabolomes in Health and Disease. *bioRxiv* [preprint] (2019) 539478. doi: 10.1101/539478

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REVIEW
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Novel Agents For Relapsed and Refractory Classical Hodgkin Lymphoma: A Review

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Classical Hodgkin lymphoma (cHL) is the most common type of HL that occurs mainly in people aged between 15–30 and over 55 years. Although its general prognosis is favorable, 10%–30% of patients with cHL will ultimately develop relapsed or refractory disease (r/r cHL). Improving the cure rate of r/r cHL has proven to be challenging. Some novel agents, such as brentuximab vedotin and immune checkpoint inhibitors, which have been used in conventional regimens for patients with r/r cHL in the past decade, have been shown to have good curative effects. This paper reviews the conventional regimens for patients with r/r cHL and focuses on the newest clinical trials and treatment measures to prolong prognosis and reduce adverse events. The evaluation of prognosis plays a vital role in analyzing the risk of relapse or disease progression; thus, finding new predictive strategies may help treat patients with r/r cHL more efficaciously.

Keywords: novel agents, brentuximab vedotin, nivolumab, pembrolizumab, relapsed and refractory classical hodgkin lymphoma

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1 INTRODUCTION

Hodgkin lymphoma (HL) is a frequent hematological malignancy, with 8,830 new cases reported in the United States in 2021 (1). Classical HL (cHL) is one of the most common types of HL and is defined as a B lymphatic cell malignancy characterized by the presence of malignant Hodgkin and Reed–Sternberg (HRS) cells in the tumor microenvironment (2). It occurs mainly in people aged between 15–30 and over 55 years, with an incidence of approximately three newly diagnosed cases per 100,000 individuals per year (3, 4). cHL is mainly divided into stages I–II and stages III–IV, the former of which is further classified into three subgroups, namely, stages IA–IIA (favorable), stages I–II (unfavorable with non-bulky disease), and stages I–II (unfavorable with bulky disease) (5). Patients with different stages may have different therapeutic choices. However, the prognosis of patients with HL is generally favorable, with approximately 80% of young adults cured of the disease after receiving initial standard chemotherapeutic treatment (3). However, 10%–30% of patients ultimately develop relapsed or refractory cHL (r/r cHL) disease (3). Patients with r/r cHL, especially those aged 60 years or older, have a much poorer prognosis, with a reported 3-year progression-free survival (PFS) rate of 50% and an overall survival (OS) rate of 68% (6). In addition, the toxic effects of the therapeutic approach on the number of years lost from productive life are remarkable. The

common adverse events of conventional chemotherapeutic agents, such as peripheral neuropathy (7), also negatively affect the prognosis. Therefore, finding novel agents and therapeutic modalities is still of great significance in improving the overall cure rates and prolonging PFS and OS in patients with r/r cHL. Herein, we introduce the current chemotherapies used in clinical practice and novel treatments that show improved prognosis in the latest clinical trials to provide a more comprehensive understanding of r/r cHL treatment and promising directions for future research in this field.

2 CURRENT STANDARD TREATMENT OF R/R CHL

2.1 Treatment for adult patients aged 18–60 years

Combined modality therapy (chemotherapy and radiotherapy) remains a top priority for patients with newly diagnosed cHL. For stage I–II patients aged 18–60, two cycles of ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) followed by positron emission tomography/computed tomography (PET/CT) evaluation are routinely used as the primary treatment. Further decisions regarding combined therapy are based on lymphoma remission status assessed by PET/CT. The escalated BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone in escalated doses) regimen is also widely used in early-stage young adults as an additional therapy. For stage III–IV patients, apart from the ABVD regimen, escalated BEACOPP or brentuximab vedotin (BV) combined with AVD (doxorubicin, vinblastine, and dacarbazine) is recommended as the primary treatment in certain cases (8). However, the prognosis is significantly poorer for a small proportion of patients who may have a limited response to first-line therapy or relapse. Therefore, salvage treatment, high-dose therapy, and autologous stem cell transplantation (HDT-ASCT) play a key role in prolonging PFS and OS.

Patients with r/r cHL are recommended to be treated with salvage regimens, which mainly include dexamethasone, cisplatin, high-dose cytarabine (DHAP); etoposide, methylprednisolone, high-dose cytarabine, cisplatin (ESHAP); ifosfamide, carboplatin, etoposide (ICE); gemcitabine, vinorelbine, liposomal doxorubicin (GVD); BV; and a combination of bendamustine and nivolumab (5). A brief summary of the newly reported clinical trials on salvage systemic therapies is listed in **Table 1**. If PET/CT re-evaluation indicates a complete response (CR) or partial response (PR), additional therapy with HDT-ASCT would be feasible and effective if not contraindicated. Radiation should also be considered for selected sites that have not previously been irradiated. Response to salvage treatment is an important predictor of prognosis in patients with r/r cHL since individuals achieving metabolic CR after salvage treatment have a higher chance of being cured (9). In contrast, patients who experience disease progression after salvage treatment may benefit from further systemic therapy with or without radiation, and if they respond, autologous or allogeneic hematopoietic stem cell transplantation (HSCT) is also favorable. However, half of the patients who undergo HSCT cannot be cured, and their prognosis is poor (10). Therefore, maintenance therapy, such as BV and other novel modalities, is vital for patients at a high risk of relapse. For those who underwent multiple-line therapy and still suffered from disease relapse, regimens such as chimeric antigen receptor (CAR) T-cell therapy (11) may offer sustained relief.

2.2 Treatment for older patients

cHL in patients over 60 years of age is highly linked to poorer prognosis since these patients generally suffer from inferior efficacy but great toxicity, owing to comorbidity and geriatric fitness (12). The management of older patients should be individualized and usually requires clinical judgment. Indeed, choosing agents with mild toxicities and at suitable doses remains challenging when aiming to improve the OS and PFS of this group of patients (5). ACCRU, as a multicenter phase II trial, reported the combination of BV and nivolumab as first-line therapy in older patients, and 22 out of 46 (48%) individuals achieved complete response, although grade 3–5 adverse events occurred in 80% of the patients (13). However, for older patients with r/r cHL, no consensus has been reached regarding the suggested treatment regimens.

2.3 Treatment for pediatric patients

Regimen options for pediatric cHL patients are distinct from those for adult patients, and the treatment of pediatric patients is highly based on risk stratification (14, 15). OEPA (vincristine, etoposide, prednisone, and doxorubicin), AVPC (doxorubicin, vincristine, prednisone, and cyclophosphamide), and ABVE-PC (doxorubicin, bleomycin, vincristine, etoposide, cyclophosphamide, and

TABLE 1 | Newly reported clinical trials of salvage treatment of r/r cHL.

Agents	Patients	No. of patients	CR (%)	ORR (%)	Severe toxicity rate (%)	NCT number
V	Relapsed or ineligible post-ASCT	153	24	54.2	11	NCT02684292
BV + bendamustine	Refractory or relapsed after multiple-line chemotherapy	37	43	78	14 (lung infections) 25 (neutropenia)	NCT01657331
BV + nivolumab	Refractory or relapsed after first-line chemotherapy	93	67	85	18 (immune-related)	NCT02572167
BV + ESHAP	Refractory or relapsed after first-line chemotherapy	66	70	91	33	–
BV + ICE	Refractory or relapsed after first-line chemotherapy	45	74	91	29	NCT02227199
Pembrolizumab	Relapsed or ineligible post-ASCT	210	27.6	71.9	12.0	NCT02453594
Pembrolizumab	Relapsed or ineligible post-ASCT	151	25	65.6	16	NCT02684292
Pembrolizumab + GVD	Refractory or relapsed after first-line chemotherapy	39	95	100	31	NCT03618550

prednisone) regimens are conventionally used in underage patients as first-line systemic therapy (16) but are rarely used in adult patients. In contrast, for adolescents with suspected r/r cHL, available clinical trials are favored if the biopsy site is positive. Otherwise, re-induction therapy is required to avoid relapse or prolong PFS. HDT-ASCT, radiotherapy, and maintenance therapy are optional based on the metabolic condition of patients. Subsequent therapy was considered when relapse occurred after maintenance treatment. Re-induction and subsequent therapy are similar to those in adults, such as DHAP, IGEV (ifosfamide, gemcitabine, vinorelbine), ICE, nivolumab (17), BV (18), and the combination of BV with bendamustine, gemcitabine, or nivolumab.

3 LATEST UPDATES OF TARGETED AGENTS FOR R/R CHL

3.1 BV

BV is a CD30-directed antibody–drug conjugate (ADC) that links an antineoplastic agent, monomethyl auristatin E (MMAE), to a monoclonal antibody that can direct MMAE to CD30-positive lymphoma cells (19). BV was initially approved for patients who failed ASCT or at least two prior lines of chemotherapy by the US Food and Drug Administration (FDA) in 2011, based on its great drug efficacy in a phase II clinical trial, with an overall response rate (ORR) of 75% (20). BV is now commonly used as salvage or post-ASCT maintenance therapy for patients with r/r cHL, but it has also been proven to be effective and recommended in patients with stage III/IV cHL as first-line therapy when combined with the AVD modality (8). Notably, severe adverse events of BV are negligible due to a high incidence rate of 32%, among which the most common and severe one is neutropenia (21). The latest update of the ECHELON-1 study illustrated that the BV plus AVD regimen could significantly improve the 5-year PFS compared to the standard ABVD regimen for the first-line treatment of patients with stage III/IV cHL (82.2% vs. 75.3%, $p = 0.0017$) (22). Another phase II trial further investigated the outcomes of BV-AVD treatment in patients with non-bulky stage I/II cHL, concluding a favorable PFS (94%) and OS (97%) during a median follow-up of 38 months (23).

For salvage treatment, BV monotherapy administered prior to ASCT in the treatment of r/r cHL yielded a CR rate (CRR) of 24%–35% (24, 25), which is not a favorable outcome, probably due to BV chemoresistance (26). In contrast, BV combined therapy has been intensively studied and demonstrated to markedly improve CR (25, 27, 28). Clinical trials of BV combined with conventional salvage chemotherapy have been widely performed over the last decade, including the combination of BV with ICE, DHAP, and ESHAP. The concurrent treatment with BV and ICE produced a high CRR of 74% and improved post-HSCT outcomes (29). Bendamustine is a purine analog with antitumor activity by damaging DNA and inducing cell apoptosis and has been approved for the treatment of chronic lymphocytic leukemia and non-HL. The combination of BV and bendamustine (BVB) as salvage therapy has shown a high CRR of 73.6% (30). BVB therapy has been widely researched in developed countries and has displayed impressive outcomes as a second-line treatment, with a CR of over 70% (30). In comparison, the CR of BVB combined therapy in a middle-income setting in India was lower at 62% (31). BVB is also highly active in patients with prior BV exposure since the PFS duration was similar to that of patients that had not received BV before (32). Another retrospective analysis indicated that BVB salvage therapy is highly effective in children and young adults under 30, with a high CR of 79% (33). A phase II transplant BRaVE trial recruited 55 patients with r/r cHL and administered BV-DHAP combined therapy as the first salvage treatment. CR was achieved in 81% of the patients before HDT-ASCT (34), which is a high rate compared to BV in combination with bendamustine (73.6%) (30) and ICE (74%) (29).

For maintenance therapy, the phase 3 AETHERA trial demonstrated the efficacy of BV in patients with a high risk of relapse after auto-HSCT treatment. The 5-year PFS was 59% in BV-treated patients compared with 41% in the placebo group [hazard ratio (HR), 0.521; 95% CI, 0.379–0.717] (35). Maintenance therapy with BV is comparatively safe, and 90% of the patients are diagnosed with peripheral neuropathy, which is the most common adverse event of BV (35, 36). The AETHERA trial showed that BV is an effective post-ASCT maintenance agent. However, since the AETHERA trial excluded individuals who had previously received BV, the efficacy of BV in this group remains unclear. A recent multicenter retrospective study of 105 cases, including both BV-naive and BV-exposed patients, reported 3-year PFS and OS rates of 54% and 71%, respectively, for BV-naive patients and 77% and 96%, respectively, for BV-exposed patients (37).

3.2 Immune checkpoint inhibitors

Immune checkpoint inhibitors (ICIs) have been widely used to treat hematologic malignancies, including cHL. Anti-PD-1 agents have been widely used to treat cHL. Tumor cells expressing PD-L1 and PD-L2 can escape the immune surveillance of mature cytotoxic T cells *via* the PD-1 pathway, which is one of the most critical mechanisms that leads to cHL. 9p24 Genomic amplification of this.1, which may cause overexpression of PD-1, PD-L1, and PD-L2 in malignant HRS cells, is prevalent in this disease (38). PD-L1 blockers can restore immunoactivity in the tumor microenvironment (TME) and suppress the viability of HRS cells that express PD-1, PD-L1, and PD-L2. Nivolumab and pembrolizumab, which are both fully human IgG4 monoclonal PD-1 antibodies, were approved by the FDA in 2016 and 2017, respectively, as treatment options for patients with r/r cHL. The safety and efficacy of these two agents are not yet weighted, but healthcare insurance data suggest that the pembrolizumab cohort had a lower hospitalization rate than the nivolumab cohort (39). To date, finding novel and highly effective anti-PD-1 agents remains a major challenge worldwide. In China, several anti-PD-1 agents have been approved by the National Medical Products Administration for use in the treatment of r/r cHL from 2018 to 2022, including sintilimab (40, 41), tislelizumab (42, 43),

camrelizumab (44–50), penpulimab (51), and zimberelimab (52). A summary of these agents is provided in **Table 2**.

The side effects of ICIs are non-specific, including infection, inflammation, pyrexia, digestive symptoms, etc. Notably, the side effect of nivolumab is statistically higher than that of pembrolizumab, and neutropenia is the most common side effect of nivolumab (53).

3.2.1 Nivolumab

Nivolumab was the first ICI approved by the FDA to treat patients with r/r cHL. CheckMate 205 showed a durable response to nivolumab monotherapy in r/r cHL in 2018 (54), and the latest real-life experience in Turkey is consistent with this result in heavily pretreated r/r cHL (55). Analysis of cohort D of CheckMate 205 suggested that nivolumab combined with the AVD regimen (N-AVD) significantly increased ORR and CRR. The ORR and CRR at the end of nivolumab monotherapy were 69% and 18%, respectively, whereas, after two combination cycles of N-AVD, the figures increased to 90% and 51%, respectively (53). The randomized phase II NIVAHL trial explored the efficacy of N-AVD in an earlier stage of cHL as first-line therapy, which also resulted in an attractively high CR (56).

Nivolumab combined with BV has shown great outcomes as a second-line regimen, reporting an ORR of 82% and CR of 61% in r/r cHL (57). Another phase I study explored BV combined with nivolumab, nivolumab, and ipilimumab and found a similar ORR of 76% and CRR of 57% in the BV-nivolumab group and a high ORR of 82% and CRR of 73% in the triplet group (58). The next phase II study will compare these two modalities as a bridge to HCST (58). A comparison of the efficacy of nivolumab monotherapy and nivolumab in combination with ICE (NICE) multiagent therapy was conducted in a phase II trial (NCT03016871). Of the 42 evaluable patients, 34 received monotherapy, and nine received NICE therapy. The ORR and CRR in the NICE group were noticeably higher than those in the single-agent group (ORR 81% versus 93%; CRR 71% versus 91%) (59), although the number of samples was limited.

The ORR of anti-PD-1 agents was initially high. However, long-term treatment with nivolumab may decrease the drug efficacy and lead to chemoresistance. Nivolumab as salvage treatment without ASCT consolidation displayed a markedly higher relapse rate than those with ASCT consolidation (62.2% versus 0%), based on a retrospective analysis (60).

3.2.2 Pembrolizumab

Pembrolizumab is another commonly used anti-PD-1 antibody approved by the FDA that has shown great PFS improvement (61–63). The phase III clinical trial of the KEYNOTE-204 study reported pembrolizumab as a preferred agent compared to BV in treating r/r cHL individuals who were ineligible for ASCT or had relapsed after ASCT. The median PFS reached 13.2 months for pembrolizumab versus 8.3 months for BV ($p = 0.0027$), although the ORR and CR did not show statistical significance ([65.6% (57.4–73.1) versus 54.2% (46.0–62.3) and 25% versus 24%, respectively) (64). Furthermore, pembrolizumab can improve health-related quality of life compared to BV in patients with r/r cHL (65). The duration of pembrolizumab response was observed when BV monotherapy was ineffective in the KEYNOTE-013 study (NCT01953692), and it was found that some patients may benefit from long-term pembrolizumab treatment since the duration of response was not reached in a 4-year follow-up (66).

Despite the remarkable efficacy of pembrolizumab as monotherapy, combinations of pembrolizumab and other agents are more widely suggested. A phase II study reported that pembrolizumab followed by AVD as first-line therapy was effective and well-tolerated in untreated patients with advanced stage cHL. Similarly, for r/r cHL patients, pembrolizumab also showed some favorable outcomes (67). A retrospective analysis reported a pembrolizumab-BV regimen in 10 patients with multirefractory cHL, and the final CR reached 80%. Seven out of 10 proceeded to ASCT directly, although the number of patients was limited, and further studies on two-drug therapy are of great significance (68). GVD, another commonly used salvage regimen of r/r cHL with a CRR of less than 50% (69), combined with pembrolizumab (pembro-GVD), was studied in a phase II clinical trial (NCT03618550) to evaluate its efficacy and safety. After two to four cycles of the pembro-GVD regimen, 95% of the patients achieved CR. Moreover, 36 out of 38 patients underwent HDT-ASCT, and no relapses were observed during a median follow-up of 13.5 months (70). AFM13, a CD30/CD16A-bispecific antibody that can stimulate innate immune cells, combined with pembrolizumab, has been reported to display

TABLE 2 | Anti-PD-1 antibodies approved by the National Medical Products Administration in China for r/r cHL.

Anti-PD-1 agent	Approved year	Patients	No. of patients	CRR (%)	ORR (%)	Severe toxicity (%)	NCT number
Sintilimab	2018	Relapsed or refractory after two or more lines of therapy	96	34	80·4	18	NCT03114683
Tislelizumab	2019	Failed or were ineligible for ASCT	70	67.1	87.1	31.4	NCT03209973
Camrelizumab	2019	Had prior ASCT or at least two lines of prior chemotherapy	75	28.0	76.0	26.7	NCT03155425
Enpulimab	2021	Had prior ASCT or at least two lines of prior chemotherapy	94	47.1	89.4	4.3	NCT03722147
Zimberelimab	2022	Relapsed or refractory after two or more lines of therapy	85	32.9	90.6	22.4	NCT03655483

good tolerance and an ORR of 88% in patients with r/r cHL (71); however, further investigation is needed.

3.2.3 Sintilimab

Sintilimab is a highly selective PD-1 blocker that revealed an ORR of 80.4% in heavily pretreated cHL patients from 18 hospitals in China in a single-arm phase II trial (NCT03114683) (41). A new case was reported in which one cHL-HIV patient, who failed ABVD and GDP chemotherapeutic regimens, showed CR after receiving sintilimab for nine cycles, with acceptable adverse events (40).

3.2.4 Tislelizumab

Tislelizumab is a humanized IgG4 monoclonal antibody that binds PD-1 with high affinity. A phase II trial in China evaluated the efficacy and safety of r/r cHL in patients who failed or were ineligible for ASCT, reporting an ORR of 87.1% and CR of 62.9% after a median of 9.8 months of follow-up (43). The latest update revealed that the PFS and OS rates were 40.8% and 84.8%, respectively, after a 3-year follow-up.

3.2.5 Camrelizumab

Camrelizumab is an anti-PD-1 agent that has shown good efficacy against various advanced malignancies (44). It achieved a high rate of objective response (76.0%; 95% CI, 64.7–85.1) in cHL patients failing or ineligible for ASCT (45), and the median PFS was 22.5 months and the 36-month OS was 82.7%, according to the latest updates (46). However, some patients still have progressive disease or relapse after receiving anti-PD-1 agents, probably due to the overexpression of PD-1 in T cells in the microenvironment or upregulation of PD-L1 in HRS cells (47). Decitabine is a DNA methyltransferase inhibitor with the potential to reduce camrelizumab resistance (50). Therapy with camrelizumab plus decitabine markedly improved the CRR (71%) in patients with r/r cHL compared with camrelizumab monotherapy (32%) (48), and after a median follow-up of 34.5 months, the complete remission rate reached 79% with dual therapy (49). Further study of this combined therapy was conducted in two phase II trials (NCT02961101 and NCT03250962) in r/r cHL patients with PD-1 resistance, and the ORRs in two separate cohorts were 52% and 68%, respectively, with a longer PFS compared to PD-1 monotherapy (50). This suggests a high synergistic antitumor activity and long-term benefit of the camrelizumab plus decitabine combination. However, grade 3–4 adverse events of leukocytopenia occur more frequently with this combined therapy than with camrelizumab monotherapy.

4 NOVEL THERAPEUTIC MEASURES TO TREAT R/R CHL

Clinical Trials of potential effective novel agents in the treatment of r/r cHL under recruiting are summarized in **Table 3**. Although conventional therapeutic regimens, BV, and ICIs have greatly improved the prognosis, heavily chemoresistant patients still have a limited choice of effective drugs. Moreover, the high rate of adverse events among the aforementioned regimens remains a major obstacle, especially in adolescents and older patients. Therefore, novel agents that prolong PFS and OS and reduce adverse events are of great significance.

4.1 Radioimmunotherapy

Since BV and ICIs are increasingly used in either the first-line or second-line treatment of cHL, the efficacy of these drugs may not be as effective as post-HCST maintenance therapy.

TABLE 3 | Clinical trials of novel agents in the treatment of r/r cHL under recruiting.

Agents	Function	Intervention	Phase	NCT number
AZD7789	Anti-PD-1 and anti-TIM-3 antibody	Drug: AZD7789	Phase 1/2	NCT05216835
THOR-707	Non-α-selective IL-2	Drug: THOR-707 Drug: pembrolizumab	Phase 2	NCT05179603
Azacitidine	Cytosine nucleoside analogs	Biological: nivolumab Drug: oral azacitidine	Phase 1	NCT05162976
Ipilimumab	Anti-CTLA-4 monoclonal antibody	Drug: brentuximab vedotin Biological: ipilimumab Biological: nivolumab	Phase 1/2	NCT01896999
AZD4573	Cyclin-dependent kinase 9 inhibitor	Drug: AZD4573	Phase 2	NCT05140382
Ruxolitinib	JAK1/2 inhibitor	Drug: ruxolitinib Drug: nivolumab	Phase 1/2	NCT03681561
Decitabine	2-Deoxycytidine analogs	Drug: camrelizumab and decitabine	Phase 2/3	NCT04510610
Chidamide	Histone deacetylase inhibitor	Drug: chidamide Drug: camrelizumab Drug: decitabine	Phase 2	NCT04233294
TQB2450	Anti-PD-1 antibody	Drug: TQB2450	Phase 2	NCT03800706
CD30.CAR-EBVST cells	CD30.CAR in EBV-specific T cells	Biological: CD30.CAR-EBVST cells	Phase 1	NCT04288726
HSP-CAR30	Anti-CD30 CAR-T cells	Biological: HSP-CAR30	Phase 1/2	NCT04288726
AFM13	Anti-CD30/CD16A monoclonal antibody	Biological: AFM13 Drug: cyclophosphamide Drug: fludarabine Drug: fludarabine phosphate Biological: genetically engineered lymphocyte therapy	Phase 1	NCT04074746

Radioimmunotherapy is a novel targeted approach that may reduce the risk of relapse after HSCT in patients with radiosensitivity. 90Y-basiliximab/DOTA is a specially designed agent that conjugates basiliximab (anti-CD25 antibody) and 1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA) and radiolabels 90Y for therapeutic dosing. 90Y-basiliximab/DOTA was administered in combination with the BEAM regimen (carmustine, etoposide, cytarabine, and melphalan) as maintenance therapy, and the estimated 5-year PFS and OS rates were 68% and 95%, respectively (72). Further evaluation of this approach is ongoing in phase II trials.

4.2 Novel ADCs

ADC is a group of agents that connect small molecule bioactive drugs to monoclonal antibodies; hence, the antibodies can transport bioactive drugs to target cells directly. Because of the huge success of BV as an ADC in the treatment of cHL, novel ADCs against other key receptors are being investigated in both preclinical and clinical studies (73).

CD25 is a receptor of interleukin-2 (IL-2) that is abundantly distributed on the surface of both hematological tumor cells and Treg cells. Camidanlumab tesirine is a novel agent that conjugates anti-CD25 antibody to a pyrrolobenzodiazepine dimer and causes cell death by damaging the DNA structure. A phase I study assessed the efficacy of camidanlumab tesirine in patients with r/r cHL who had no available therapies. The median duration of response was 7.2 months, and the ORR was 71% (74, 75). A later phase II study demonstrated that 18 (38.3%) and 20 (42.6%) of 47 heavily treated patients attained CR and PR, respectively (76), showing that camidanlumab tesirine is a promising ADC agent next to BV.

CD123 is an alpha subunit of the interleukin 3 receptor (IL3RA) that is enriched in acute myeloid leukemia and cHL cells. IL3RA-directed ADC, such as BAY-943, seems to be effective in suppressing cHL development. BAY-943 has demonstrated noticeable antiproliferative efficacy in HL cell lines and xenograft models (77), and further clinical trials of BAY-943 are warranted.

4.3 Anti-CD30 CAR-T-cell therapy

CAR-T-cell immunotherapy has been increasingly studied in a wide range of cancers. Engineered T cells express CARs on the cell surface so that they can recognize and eliminate cells expressing specific target antigens. CAR-T therapy targeting the CD30 antigen (CD30.CAR-T) can be effective in hematological malignancies, including cHL (78). Adoptive transfer of CD30.CAR-T preceded by fludarabine-based lymphodepletion chemotherapy was performed in 41 heavily pretreated patients with r/r cHL who developed chemoresistance to the aforementioned agents, including BV and IPIs, with an ORR of 72%, CR of 59%, 1-year PFS of 36%, and OS of 94% (11). Although individuals that achieve CR after CD30.CAR-T could experience relapse again, possibly due to insufficient persistence of CAR-Ts, the combination of CD30.CAR-Ts and ICIs may improve their PFS, and further clinical trials are needed (11).

4.4 Potential effective chemotherapeutic agents and targeted agents

New combination regimens of standard chemotherapeutic agents have also been tested in an attempt to expand the range of options and achieve a higher prognosis. For example, gemcitabine and bendamustine are used in different conventional regimens, while their concurrent combination was first reported in a phase I/II study as late-line therapy for heavily pretreated patients, showing a well-tolerated and effective result (ORR 69%; CRR 46%) but a potentially high pulmonary toxicity risk (79).

Novel chemotherapeutic agents are also being researched in both preclinical and clinical studies. Lenalidomide is an immunomodulatory drug used to treat myelodysplastic syndrome, and a low dose displays some activity in patients with r/r cHL who fail or are ineligible for ASCT, with 64% of the patients remaining stable and 11% achieving a PR. However, it is not suggested for post-ASCT treatment (80, 81). A phase I/II clinical trial combined lenalidomide with the mTOR inhibitor temsirolimus in patients with r/r cHL and reported an ORR of 80% and a CR of 35% (82), which is encouraging. Trabectedin is a tetrahydroisoquinoline alkaloid with antitumor activity targeting both TME and HRS cells in cHL. Preclinical studies have demonstrated that trabectedin induces DNA skeleton cleavage and cancer cell necrosis by binding to the DNA structure and blocking the transcription level of stress-induced proteins. It also reduced HRS cell secretion of important factors and reduced immunosuppressive immunocytes in the TME, indicating that trabectedin can be a rational candidate in patients with r/r cHL (83). Furthermore, the combination of trabectedin and the CCR5 antagonist maraviroc enhanced DNA damage and antitumor activity (84).

5 CONCLUSION

cHL is a generally curable disease that occurs mainly in those aged 15–30 and over 55 years (3). However, a small group of patients are refractory to treatment or suffer a relapse after receiving primary systemic therapy and HDT-ASCT. The prognosis for these patients is notably poor. In this review, we briefly summarized the current treatment principles and conventional regimens and provided an update on the latest clinical trials of leading cHL agents (BV and ICIs) and novel potential drugs that may be used in the near future. In addition, prognosis evaluation plays a vital role in directing the next treatment strategy and analyzing the risk of relapse or disease progression. The International Prognostic Score (IPS) is composed of seven potential risk factors [albumin < 4 g/dl, hemoglobin < 10.5 g/dl, male, age ≥ 45 years, stage IV disease, leukocytosis, and lymphocytopenia (5)] and is intensively applied for risk classification. PET/CT is currently the most effective method to assess treatment efficacy and predict individual prognosis. The Deauville criteria is the most commonly used scale to evaluate the degree of lymphoma remission. However, when comparing different clinical trials, ORR and CRR may be based on different criteria, among which the Lugano 2014 classification and 2007 revised response criteria are frequently used. Although the guidelines for adult patients with cHL are mature, the treatment

of certain groups of patients (including pediatric, older, and HIV-related individuals) (85) remains controversial. Further studies should focus on these population groups.

AUTHOR CONTRIBUTIONS

YZ, TW, and WW designed and analyzed the manuscript and were responsible for the funding acquisition and manuscript writing—review and editing. ZX, LM, ZL, and JZ supervised the study and drafted the manuscript. All authors reviewed and approved the manuscript.

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REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2021. *CA Cancer J Clin* (2021) 71(1):7–33. doi: 10.3322/caac.21654
2. Dores GM, Curtis RE, Dalal NH, Linet MS, Morton LM. Cause-Specific Mortality Following Initial Chemotherapy in a Population-Based Cohort of Patients With Classical Hodgkin Lymphoma, 2000-2016. *J Clin Oncol* (2020) 38(35):4149–62. doi: 10.1200/jco.20.00264
3. Ansell SM. Hodgkin Lymphoma: 2018 Update on Diagnosis, Risk-Stratification, and Management. *Am J Hematol* (2018) 93(5):704–15. doi: 10.1002/ajh.25071
4. Brice P, de Kerviler E, Friedberg JW. Classical Hodgkin Lymphoma. *Lancet* (2021) 398(10310):1518–27. doi: 10.1016/s0140-6736(20)32207-8
5. *NCCN Clinical Practice Guidelines in Oncology, Hodgkin Lymphoma (Version 2.2022 — February 23, 2022)*. Available at: https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1439.
6. Evens AM, Advani RH, Helenowski IB, Fanale M, Smith SM, Jovanovic BD, et al. Multicenter Phase II Study of Sequential Brentuximab Vedotin and Doxorubicin, Vinblastine, and Dacarbazine Chemotherapy for Older Patients With Untreated Classical Hodgkin Lymphoma. *J Clin Oncol* (2018) 36 (30):3015–22. doi: 10.1200/jco.2018.79.0139
7. Straus DJ, Długosz-Danecka M, Alekseev S, Illés Á, Picardi M, Lech-Maranda E, et al. Brentuximab Vedotin With Chemotherapy for Stage III/IV Classical Hodgkin Lymphoma: 3-Year Update of the ECHELON-1 Study. *Blood* (2020) 135(10):735–42. doi: 10.1182/blood.2019003127
8. Ramchandren R, Advani RH, Ansell SM, Bartlett NL, Chen R, Connors JM, et al. Brentuximab Vedotin Plus Chemotherapy in North American Subjects With Newly Diagnosed Stage III or IV Hodgkin Lymphoma. *Clin Cancer Res* (2019) 25(6):1718–26. doi: 10.1158/1078-0432.Ccr-18-2435
9. Moskowitz CH, Matasar MJ, Zelenetz AD, Nimer SD, Gerecitano J, Hamlin P, et al. Normalization of Pre-ASCT, FDG-PET Imaging With Second-Line, non-Cross-Resistant, Chemotherapy Programs Improves Event-Free Survival in Patients With Hodgkin Lymphoma. *Blood* (2012) 119(7):1665–70. doi: 10.1182/blood-2011-10-388058
10. Smith SD, Moskowitz CH, Dean R, Pohlman B, Sobecks R, Copelan E, et al. Autologous Stem Cell Transplant for Early Relapsed/Refractory Hodgkin Lymphoma: Results From Two Transplant Centres. *Br J Haematol* (2011) 153 (3):358–63. doi: 10.1111/j.1365-2141.2011.08616.x
11. Ramos CA, Grover NS, Beaven AW, Lulla PD, Wu MF, Ivanova A, et al. Anti-CD30 CAR-T Cell Therapy in Relapsed and Refractory Hodgkin Lymphoma. *J Clin Oncol* (2020) 38(32):3794–804. doi: 10.1200/jco.20.01342
12. Orellana-Noia VM, Isaac K, Malecek MK, Bartlett NL, Voorhees TJ, Grover NS, et al. Multicenter Analysis of Geriatric Fitness and Real-World Outcomes in Older Patients With Classical Hodgkin Lymphoma. *Blood Adv* (2021) 5 (18):3623–32. doi: 10.1182/bloodadvances.2021004645
13. Cheson BD, Bartlett NL, LaPlant B, Lee HJ, Advani RJ, Christian B, et al. Brentuximab Vedotin Plus Nivolumab as First-Line Therapy in Older or Chemotherapy-Ineligible Patients With Hodgkin Lymphoma (ACCRU): A Multicentre, Single-Arm, Phase 2 Trial. *Lancet Haematol* (2020) 7(11):e808–15. doi: 10.1016/s2352-3026(20)30275-1
14. Johnston RL, Mottok A, Chan FC, Jiang A, Diepstra A, Visser L, et al. A Gene Expression-Based Model Predicts Outcome in Children With Intermediate-Risk Classical Hodgkin Lymphoma. *Blood* (2022) 139(6):889–93. doi: 10.1182/blood.2021011941
15. Parikh RR, Kelly KM, Hodgson DC, Hoppe BS, McCarten KM, Karolczuk K, et al. Patterns of Initial Relapse From a Phase 3 Study of Response-Based Therapy for High-Risk Hodgkin Lymphoma (AHOD0831): A Report From the Children's Oncology Group. *Int J Radiat Oncol Biol Phys* (2022) 112 (4):890–900. doi: 10.1016/j.ijrobp.2021.10.152
16. *NCCN Clinical Practice Guidelines in Oncology, Pediatric Hodgkin Lymphoma (Version 1.2022 — April 8, 2022)*. Available at: https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1439.
17. Davis KL, Fox E, Merchant MS, Reid JM, Kudgus RA, Liu X, et al. Nivolumab in Children and Young Adults With Relapsed or Refractory Solid Tumours or Lymphoma (ADVL1412): A Multicentre, Open-Label, Single-Arm, Phase 1-2 Trial. *Lancet Oncol* (2020) 21(4):541–50. doi: 10.1016/s1470-2045(20)30023-1
18. Metzger ML, Link MP, Billett AL, Flerlage J, Lucas JTJr., Mandrell BN, et al. Excellent Outcome for Pediatric Patients With High-Risk Hodgkin Lymphoma Treated With Brentuximab Vedotin and Risk-Adapted Residual Node Radiation. *J Clin Oncol* (2021) 39(20):2276–83. doi: 10.1200/jco.20.03286
19. Francisco JA, Cerveny CG, Meyer DL, Mixan BJ, Klussman K, Chace DF, et al. Cac10-vcMMAE, an Anti-CD30-Monomethyl Auristatin E Conjugate With Potent and Selective Antitumor Activity. *Blood* (2003) 102(4):1458–65. doi: 10.1182/blood-2003-01-0039
20. Younes A, Gopal AK, Smith SE, Ansell SM, Rosenblatt JD, Savage KJ, et al. Results of a Pivotal Phase II Study of Brentuximab Vedotin for Patients With Relapsed or Refractory Hodgkin's Lymphoma. *J Clin Oncol* (2012) 30 (18):2183–9. doi: 10.1200/JCO.2011.38.0410
21. Moskowitz CH, Nademanee A, Masszi T, Agura E, Holowiecki J, Abidi MH, et al. Brentuximab Vedotin as Consolidation Therapy After Autologous Stem-Cell Transplantation in Patients With Hodgkin's Lymphoma at Risk of Relapse or Progression (AETHERA): A Randomised, Double-Blind, Placebo-Controlled, Phase 3 Trial. *Lancet* (2015) 385(9980):1853–62. doi: 10.1016/s0140-6736(15)60165-9
22. Straus DJ, Długosz-Danecka M, Connors JM, Alekseev S, Illés Á, Picardi M, et al. Brentuximab Vedotin With Chemotherapy for Stage III or IV Classical Hodgkin Lymphoma (ECHELON-1): 5-Year Update of an International, Open-Label, Randomised, Phase 3 Trial. *Lancet Haematol* (2021) 8(6): e410–21. doi: 10.1016/s2352-3026(21)00102-2
23. Abramson JS, Arnason JE, LaCasce AS, Redd R, Barnes JA, Sokol L, et al. Brentuximab Vedotin, Doxorubicin, Vinblastine, and Dacarbazine for Nonbulky Limited-Stage Classical Hodgkin Lymphoma. *Blood* (2019) 134 (7):606–13. doi: 10.1182/blood.2019001272
24. Chen R, Palmer JM, Martin P, Tsai N, Kim Y, Chen BT, et al. Results of a Multicenter Phase II Trial of Brentuximab Vedotin as Second-Line Therapy Before Autologous Transplantation in Relapsed/Refractory Hodgkin Lymphoma. *Biol Blood Marrow Transplant* (2015) 21(12):2136–40. doi: 10.1016/j.bbmt.2015.07.018
25. O'Connor OA, Lue JK, Sawas A, Amengual JE, Deng C, Kalac M, et al. Brentuximab Vedotin Plus Bendamustine in Relapsed or Refractory Hodgkin's Lymphoma: An International, Multicentre, Single-Arm, Phase 1-2 Trial. *Lancet Oncol* (2018) 19(2):257–66. doi: 10.1016/s1470-2045(17) 30912-9

26. Chen R, Herrera AF, Hou J, Chen L, Wu J, Guo Y, et al. Inhibition of MDR1 Overcomes Resistance to Brentuximab Vedotin in Hodgkin Lymphoma. *Clin Cancer Res* (2020) 26(5):1034–44. doi: 10.1158/1078-0432.Ccr-19-1768
27. Advani RH, Moskowitz AJ, Bartlett NL, Vose JM, Ramchandren R, Feldman TA, et al. Brentuximab Vedotin in Combination With Nivolumab in Relapsed or Refractory Hodgkin Lymphoma: 3-Year Study Results. *Blood* (2021) 138 (6):427–38. doi: 10.1182/blood.2020009178
28. Garcia-Sanz R, Sureda A, de la Cruz F, Canales M, Gonzalez AP, Pinana JL, et al. Brentuximab Vedotin and ESHAP is Highly Effective as Second-Line Therapy for Hodgkin Lymphoma Patients (Long-Term Results of a Trial by the Spanish GELTAMO Group). *Ann Oncol* (2019) 30(4):612–20. doi: 10.1093/annonc/mdz009
29. Lynch RC, Cassaday RD, Smith SD, Fromm JR, Cowan AJ, Warren EH, et al. Dose-Dense Brentuximab Vedotin Plus Ifosfamide, Carboplatin, and Etoposide for Second-Line Treatment of Relapsed or Refractory Classical Hodgkin Lymphoma: A Single Centre, Phase 1/2 Study. *Lancet Haematol* (2021) 8(8):e562–71. doi: 10.1016/s2352-3026(21)00170-8
30. LaCasce AS, Bociek RG, Sawas A, Caimi P, Agura E, Matous J, et al. Brentuximab Vedotin Plus Bendamustine: A Highly Active First Salvage Regimen for Relapsed or Refractory Hodgkin Lymphoma. *Blood* (2018) 132 (1):40–8. doi: 10.1182/blood-2017-11-815183
31. Radhakrishnan VS, Bajaj R, Raina V, Kumar J, Bhave SJ, Sukumaran Nair RK, et al. Relapsed Refractory Hodgkin Lymphoma and Brentuximab Vedotin-Bendamustine Combination Therapy as a Bridge to Transplantation: Real-World Evidence From a Middle-Income Setting and Literature Review. *Front Oncol* (2021) 11:796270. doi: 10.3389/fonc.2021.796270
32. Moretti M, Liberati AM, Rigacci L, Puccini B, Pulsoni A, Gini G, et al. Brentuximab Vedotin and Bendamustine Produce Long-Term Clinical Benefit in Patients With Relapsed or Refractory Classical Hodgkin Lymphoma: A Multicenter Real-Life Experience. *Clin Lymphoma Myeloma Leuk* (2022) 22 (3):198–204. doi: 10.1016/j.clml.2021.09.018
33. McMillan A, O'Neill AT, Townsend W, Lambert J, Virchis A, Shah R, et al. The Addition of Bendamustine to Brentuximab Vedotin Leads to Improved Rates of Complete Metabolic Remission in Children, Adolescents and Young Adults With Relapsed and Refractory Classical Hodgkin Lymphoma: A Retrospective Single-Centre Series. *Br J Haematol* (2021) 192(3):e84–7. doi: 10.1111/bjh.17274
34. Kersten MJ, Driessen J, Zijlstra JM, Plattel WJ, Morschhauser F, Lugtenburg PJ, et al. Combining Brentuximab Vedotin With Dexamethasone, High-Dose Cytarabine and Cisplatin as Salvage Treatment in Relapsed or Refractory Hodgkin Lymphoma: The Phase II HOVON/LLPC Transplant BRaVE Study. *Haematologica* (2021) 106(4):1129–37. doi: 10.3324/haematol.2019.243238
35. Moskowitz CH, Walewski J, Nademanee A, Masszi T, Agura E, Holowiecki J, et al. Five-Year PFS From the AETHERA Trial of Brentuximab Vedotin for Hodgkin Lymphoma at High Risk of Progression or Relapse. *Blood* (2018) 132 (25):2639–42. doi: 10.1182/blood-2018-07-861641
36. Nademanee A, Sureda A, Stiff P, Holowiecki J, Abidi M, Hunder N, et al. Safety Analysis of Brentuximab Vedotin From the Phase III AETHERA Trial in Hodgkin Lymphoma in the Post-Transplant Consolidation Setting. *Biol Blood Marrow Transplant* (2018) 24(11):2354–9. doi: 10.1016/j.bbmt.2018.05.026
37. Massaro F, Pavone V, Stefani PM, Botto B, Pulsoni A, Patti C, et al. Brentuximab Vedotin Consolidation After Autologous Stem Cell Transplantation for Hodgkin Lymphoma: A Fondazione Italiana Linfomi Real-Life Experience. *Hematol Oncol* (2022) 40(1):31–9. doi: 10.1002/hon.2939
38. Green MR, Monti S, Rodig SJ, Juszczynski P, Currie T, O'Donnell E, et al. Integrative Analysis Reveals Selective 9p24.1 Amplification, Increased PD-1 Ligand Expression, and Further Induction *via* JAK2 in Nodular Sclerosing Hodgkin Lymphoma and Primary Mediastinal Large B-Cell Lymphoma. *Blood* (2010) 116(17):3268–77. doi: 10.1182/blood-2010-05-282780
39. Laliberté F, Raut M, Yang X, Germain G, Nahar A, Desai KD, et al. Real-World Healthcare Resource Utilization in Patients With Classical Hodgkin Lymphoma Treated With Pembrolizumab and Nivolumab in the USA. *Target Oncol* (2021) 16(1):85–94. doi: 10.1007/s11523-020-00778-y
40. Shi Y, Li Q, Zhang W, Nan Y, Yang T, Liang X, et al. Sintilimab as Salvage Treatment in an HIV Patient With Relapsed/Refractory Hodgkin: A Case Report. *Ann Palliat Med* (2020) 9(4):2414–9. doi: 10.21037/apm-20-1333
41. Shi Y, Su H, Song Y, Jiang W, Sun X, Qian W, et al. Safety and Activity of Sintilimab in Patients With Relapsed or Refractory Classical Hodgkin Lymphoma (ORIENT-1): A Multicentre, Single-Arm, Phase 2 Trial. *Lancet Haematol* (2019) 6(1):e12–9. doi: 10.1016/s2352-3026(18)30192-3
42. Song Y, Gao Q, Zhang H, Fan L, Zhou J, Zou D, et al. Tislelizumab for Relapsed/Refractory Classical Hodgkin Lymphoma: 3-Year Follow-Up and Correlative Biomarker Analysis. *Clin Cancer Res* (2022) 28(6):1147–56. doi: 10.1158/1078-0432.Ccr-21-2023
43. Song Y, Gao Q, Zhang H, Fan L, Zhou J, Zou D, et al. Treatment of Relapsed or Refractory Classical Hodgkin Lymphoma With the Anti-PD-1, Tislelizumab: Results of a Phase 2, Single-Arm, Multicenter Study. *Leukemia* (2020) 34(2):533–42. doi: 10.1038/s41375-019-0545-2
44. Wang CY, Sheng CC, Ma GL, Xu D, Liu XQ, Wang YY, et al. Population Pharmacokinetics of the Anti-PD-1 Antibody Camrelizumab in Patients With Multiple Tumor Types and Model-Informed Dosing Strategy. *Acta Pharmacol Sin* (2021) 42(8):1368–75. doi: 10.1038/s41401-020-00550-y
45. Song Y, Wu J, Chen X, Lin T, Cao J, Liu Y, et al. A Single-Arm, Multicenter, Phase II Study of Camrelizumab in Relapsed or Refractory Classical Hodgkin Lymphoma. *Clin Cancer Res* (2019) 25(24):7363–9. doi: 10.1158/1078-0432.Ccr-19-1680
46. Wu J, Song Y, Chen X, Lin T, Cao J, Liu Y, et al. Camrelizumab for Relapsed or Refractory Classical Hodgkin Lymphoma: Extended Follow-Up of the Multicenter, Single-Arm, Phase 2 Study. *Int J Cancer* (2022) 150(6):984–92. doi: 10.1002/ijc.33852
47. Sasse S, Reddemann K, Diepstra A, Oschlies I, Schnitter A, Borchmann S, et al. Programmed Cell Death Protein-1 (PD-1)-Expression in the Microenvironment of Classical Hodgkin Lymphoma at Relapse During Anti-PD-1-Treatment. *Haematologica* (2019) 104(1):e21–4. doi: 10.3324/haematol.2018.196279
48. Nie J, Wang C, Liu Y, Yang Q, Mei Q, Dong L, et al. Addition of Low-Dose Decitabine to Anti-PD-1 Antibody Camrelizumab in Relapsed/Refractory Classical Hodgkin Lymphoma. *J Clin Oncol* (2019) 37(17):1479–89. doi: 10.1200/jco.18.02151
49. Liu Y, Wang C, Li X, Dong L, Yang Q, Chen M, et al. Improved Clinical Outcome in a Randomized Phase II Study of Anti-PD-1 Camrelizumab Plus Decitabine in Relapsed/Refractory Hodgkin Lymphoma. *J Immunother Cancer* (2021) 9(4):e002347-17. doi: 10.1136/jitc-2021-002347
50. Wang C, Liu Y, Dong L, Li X, Yang Q, Brock MV, et al. Efficacy of Decitabine Plus Anti-PD-1 Camrelizumab in Patients With Hodgkin Lymphoma Who Progressed or Relapsed After PD-1 Blockade Monotherapy. *Clin Cancer Res* (2021) 27(10):2782–91. doi: 10.1158/1078-0432.Ccr-21-0133
51. Song Y, Zhou K, Jin C, Qian Z, Hou M, Fan L, et al. A Phase II Study of Penpulimab, an Anti-PD-1 Antibody, in Patients With Relapsed or Refractoryclassic Hodgkin Lymphoma (cHL). *J Clin Oncol* (2021) 39 (15_suppl):7529. doi: 10.1200/JCO.2021.39.15_suppl.7529
52. Lin N, Zhang M, Bai H, Liu H, Cui J, Ke X, et al. Efficacy and Safety of GLS-010 (Zimberelimab) in Patients With Relapsed or Refractory Classical Hodgkin Lymphoma: A Multicenter, Single-Arm, Phase II Study. *Eur J Cancer (Oxford Engl 1990)* (2022) 164:117–26. doi: 10.1016/j.ejca.2021.07.021
53. Ramchandren R, Domingo-Domènech E, Rueda A, Trněný M, Feldman TA, Lee HJ, et al. Nivolumab for Newly Diagnosed Advanced-Stage Classic Hodgkin Lymphoma: Safety and Efficacy in the Phase II CheckMate 205 Study. *J Clin Oncol* (2019) 37(23):1997–2007. doi: 10.1200/jco.19.00315
54. Armand P, Engert A, Younes A, Fanale M, Santoro A, Zinzani PL, et al. Nivolumab for Relapsed/Refractory Classic Hodgkin Lymphoma After Failure of Autologous Hematopoietic Cell Transplantation: Extended Follow-Up of the Multicohort Single-Arm Phase II CheckMate 205 Trial. *J Clin Oncol* (2018) 36(14):1428–39. doi: 10.1200/jco.2017.76.0793
55. Bekoz H, Ozbalak M, Karadurmus N, Paydas S, Turker A, Toptas T, et al. Nivolumab for Relapsed or Refractory Hodgkin Lymphoma: Real-Life Experience. *Ann Hematol* (2020) 99(11):2565–76. doi: 10.1007/s00277-020-04077-4
56. Bröckelmann PJ, Goergen H, Keller U, Meissner J, Ordemann R, Halbsguth TV, et al. Efficacy of Nivolumab and AVD in Early-Stage Unfavorable Classic Hodgkin Lymphoma: The Randomized Phase 2 German Hodgkin Study Group NIVAHL Trial. *JAMA Oncol* (2020) 6(6):872–80. doi: 10.1001/jamaoncol.2020.0750
57. Herrera AF, Moskowitz AJ, Bartlett NL, Vose JM, Ramchandren R, Feldman TA, et al. Interim Results of Brentuximab Vedotin in Combination With Nivolumab in Patients With Relapsed or Refractory Hodgkin Lymphoma. *Blood* (2018) 131(11):1183–94. doi: 10.1182/blood-2017-10-811224

58. Diefenbach CS, Hong F, Ambinder RF, Cohen JB, Robertson MJ, David KA, et al. Ipilimumab, Nivolumab, and Brentuximab Vedotin Combination Therapies in Patients With Relapsed or Refractory Hodgkin Lymphoma: Phase 1 Results of an Open-Label, Multicentre, Phase 1/2 Trial. *Lancet Haematol* (2020) 7(9):e660–70. doi: 10.1016/s2352-3026(20)30221-0
59. Mei MG, Lee HJ, Palmer J, Chen RW, Tsai NC, Chen L, et al. Response-Adapted Anti-PD1 Based Salvage Therapy for Hodgkin Lymphoma With Nivolumab +/- ICE (NICE). *Blood* (2022) 139(25):3605–16. doi: 10.1182/blood.2022015423
60. Manson G, Mear JB, Herbaux C, Schiano JM, Casasnovas O, Stamatoullas A, et al. Long-Term Efficacy of Anti-PD1 Therapy in Hodgkin Lymphoma With and Without Allogenic Stem Cell Transplantation. *Eur J Cancer* (2019) 115:47–56. doi: 10.1016/j.ejca.2019.04.006
61. Armand P, Chen YB, Redd RA, Joyce RM, Bsat J, Jeter E, et al. PD-1 Blockade With Pembrolizumab for Classical Hodgkin Lymphoma After Autologous Stem Cell Transplantation. *Blood* (2019) 134(1):22–9. doi: 10.1182/blood.2019000215
62. Chen R, Zinzani PL, Fanale MA, Armand P, Johnson NA, Brice P, et al. Phase II Study of the Efficacy and Safety of Pembrolizumab for Relapsed/Refractory Classic Hodgkin Lymphoma. *J Clin Oncol* (2017) 35(19):2125–32. doi: 10.1200/jco.2016.72.1316
63. Chen R, Zinzani PL, Lee HJ, Armand P, Johnson NA, Brice P, et al. Pembrolizumab in Relapsed or Refractory Hodgkin Lymphoma: 2-Year Follow-Up of KEYNOTE-087. *Blood* (2019) 134(14):1144–53. doi: 10.1182/blood.2019000324
64. Kuruvilla J, Ramchandren R, Santoro A, Paszkiewicz-Kozik E, Gasiorowski R, Johnson NA, et al. Pembrolizumab Versus Brentuximab Vedotin in Relapsed or Refractory Classical Hodgkin Lymphoma (KEYNOTE-204): An Interim Analysis of a Multicentre, Randomised, Open-Label, Phase 3 Study. *Lancet Oncol* (2021) 22(4):512–24. doi: 10.1016/s1470-2045(21)00005-x
65. Zinzani PL, Ramchandren R, Santoro A, Paszkiewicz-Kozik E, Gasiorowski R, Johnson NA, et al. Quality-Of-Life Analysis of Pembrolizumab vs Brentuximab Vedotin for Relapsed/Refractory Classical Hodgkin Lymphoma. *Blood Adv* (2022) 6(2):590–9. doi: 10.1182/bloodadvances.2021004970
66. Armand P, Kuruvilla J, Michot JM, Ribrag V, Zinzani PL, Zhu Y, et al. KEYNOTE-013 4-Year Follow-Up of Pembrolizumab in Classical Hodgkin Lymphoma After Brentuximab Vedotin Failure. *Blood Adv* (2020) 4 (12):2617–22. doi: 10.1182/bloodadvances.2019001367
67. Allen PB, Savas H, Evens AM, Advani RH, Palmer B, Pro B, et al. Pembrolizumab Followed by AVD in Untreated Early Unfavorable and Advanced-Stage Classical Hodgkin Lymphoma. *Blood* (2021) 137(10):1318–26. doi: 10.1182/blood.2020007400
68. Massaro F, Meuleman N, Bron D, Vercruyssen M, Maerevoet M. Brentuximab Vedotin and Pembrolizumab Combination in Patients With Relapsed/Refractory Hodgkin Lymphoma: A Single-Centre Retrospective Analysis. *Cancers (Basel)* (2022) 14(4):982–9. doi: 10.3390/cancers14040982
69. Bartlett NL, Niedzwiecki D, Johnson JL, Friedberg JW, Johnson KB, van Besien K, et al. Gemcitabine, Vinorelbine, and Pegylated Liposomal Doxorubicin (GVD), a Salvage Regimen in Relapsed Hodgkin's Lymphoma: CALGB 59804. *Ann Oncol* (2007) 18(6):1071–9. doi: 10.1093/annonc/mdm090
70. Moskowitz AJ, Shah G, Schöder H, Ganesan N, Drill E, Hancock H, et al. Phase II Trial of Pembrolizumab Plus Gemcitabine, Vinorelbine, and Liposomal Doxorubicin as Second-Line Therapy for Relapsed or Refractory Classical Hodgkin Lymphoma. *J Clin Oncol* (2021) 39(28):3109–17. doi: 10.1200/jco.21.01056
71. Bartlett NL, Herrera AF, Domingo-Domenech E, Mehta A, Forero-Torres A, Garcia-Sanz R, et al. A Phase 1b Study of AFM13 in Combination With Pembrolizumab in Patients With Relapsed or Refractory Hodgkin Lymphoma. *Blood* (2020) 136(21):2401–9. doi: 10.1182/blood.2019004701
72. Herrera AF, Palmer J, Adhikarla V, Yamauchi D, Poku EK, Bading J, et al. Anti-CD25 Radioimmunotherapy With BEAM Autologous Hematopoietic Cell Transplantation Conditioning in Hodgkin Lymphoma. *Blood Adv* (2021) 5(23):5300–11. doi: 10.1182/bloodadvances.2021004981
73. Dela Cruz Chuh J, Go M, Chen Y, Guo J, Rafidi H, Mandikian D, et al. Preclinical Optimization of Ly6E-Targeted ADCs for Increased Durability and Efficacy of Anti-Tumor Response. *MAbs* (2021) 13(1):1862452. doi: 10.1080/19420862.2020.1862452
74. Hamadani M, Collins GP, Caimi PF, Samaniego F, Spira A, Davies A, et al. Camidanlumab Tesirine in Patients With Relapsed or Refractory Lymphoma: A Phase 1, Open-Label, Multicentre, Dose-Escalation, Dose-Expansion Study. *Lancet Haematol* (2021) 8(6):e433–45. doi: 10.1016/s2352-3026(21)00103-4
75. Epperla N, Hamadani M. A New Target for Hodgkin Lymphoma - Camidanlumab Tesirine. *Curr Hematol Malig Rep* (2021) 16(1):19–24. doi: 10.1007/s11899-021-00604-w
76. Herrera AF, Carlo-Stella C, Collins GP, Maddocks KJ, Bartlett NL, Savage KJ, et al. Preliminary Results of a Phase 2 Study of Camidanlumab Tesirine (Cami), a Novel Pyrrolobenzodiazepine-Based Antibody-Drug Conjugate, in Patients With Relapsed or Refractory Hodgkin Lymphoma. *Blood* (2020) 136 (Supplement 1):21–3. doi: 10.1182/blood-2020-137451
77. Kirchhoff D, Stelte-Ludwig B, Lerchen HG, Wengner AM, Ahsen OV, Buchmann P, et al. IL3RA-Targeting Antibody-Drug Conjugate BAY-943 With a Kinesin Spindle Protein Inhibitor Payload Shows Efficacy in Preclinical Models of Hematologic Malignancies. *Cancers (Basel)* (2020) 12 (11):3464–80. doi: 10.3390/cancers12113464
78. Voorhees TJ, Zhao B, Oldan J, Hucks G, Khandani A, Dittus C, et al. Pretherapy Metabolic Tumor Volume is Associated With Response to CD30 CAR T Cells in Hodgkin Lymphoma. *Blood Adv* (2022) 6(4):1255–63. doi: 10.1182/bloodadvances.2021005385
79. Cohen JB, Wei L, Maddocks KJ, Christian B, Heffner LT, Langston AA, et al. Gemcitabine and Bendamustine is a Safe and Effective Salvage Regimen for Patients With Recurrent/Refractory Hodgkin Lymphoma: Results of a Phase 1/2 Study. *Cancer* (2020) 126(6):1235–42. doi: 10.1002/cncr.32640
80. Ma H, Cheng B, Montanari F, Lue JK, Deng C, Marchi E, et al. Low Dose Continuous Lenalidomide in Heavily Pretreated Patients With Relapsed or Refractory Classical Hodgkin Lymphoma: A Retrospective Case Series. *Ther Adv Hematol* (2020) 11:2040620720947340. doi: 10.1177/2040620720947340
81. Shea L, Watkins MP, Wan F, Cashen AF, Wagner-Johnston ND, Jacoby MA, et al. A Pilot Study of Lenalidomide Maintenance Therapy After Autologous Transplantation in Relapsed or Refractory Classical Hodgkin Lymphoma. *Biol Blood Marrow Transplant* (2020) 26(12):2223–8. doi: 10.1016/j.bbmt.2020.08.017
82. Major A, Kline J, Karrison TG, Fishkin PAS, Kimball AS, Petrich AM, et al. Phase I/II Clinical Trial of Temsirolimus and Lenalidomide in Patients With Relapsed and Refractory Lymphomas. *Haematologica* (2021) 107:1609–18. doi: 10.3324/haematol.2021.278853
83. Casagrande N, Borghese C, Favero A, Vicenzetto C, Aldinucci D. Trabectedin Overcomes Doxorubicin-Resistance, Counteracts Tumor-Immunosuppressive Reprogramming of Monocytes and Decreases Xenograft Growth in Hodgkin Lymphoma. *Cancer Lett* (2021) 500:182–93. doi: 10.1016/j.canlet.2020.12.015
84. Casagrande N, Borghese C, Aldinucci D. In Classical Hodgkin Lymphoma the Combination of the CCR5 Antagonist Maraviroc With Trabectedin Synergizes, Enhances DNA Damage and Decreases Three-Dimensional Tumor-Stroma Heterospheroid Viability. *Haematologica* (2022) 107(1):287–91. doi: 10.3324/haematol.2021.279389
85. Lievin R, Hendel-Chavez H, Baldé A, Lancar R, Algarte-Génin M, Krzysiek R, et al. Increased Production of B-Cell Activating Cytokines and Altered Peripheral B-Cell Subset Distribution During HIV-Related Classical Hodgkin Lymphoma. *Cancers (Basel)* (2021) 14(1):128–41. doi: 10.3390/cancers14010128

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A novel strategy to fuel cancer immunotherapy: targeting glucose metabolism to remodel the tumor microenvironment

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The promising results of immunotherapy in tumors have changed the current treatment modality for cancer. However, the remarkable responses are limited to a minority of patients, which is due to immune suppression in the tumor microenvironment (TME). These include the pre-exists of suppressive immune cells, physical barriers to immune infiltration, antigen and antigen presentation deficiency, and expression of inhibitory immune checkpoint molecules. Recently, increasing evidence reveal that tumor metabolism, especially abnormal glucose metabolism of tumors, plays an essential role in tumor immune escape and is a potential target to combine with immunotherapy. By glucose uptake, tumor cells alter their metabolism to facilitate unregulated cellular proliferation and survival and regulate the expression of inhibitory immune checkpoint molecules. Meanwhile, glucose metabolism also regulates the activation, differentiation, and functions of immunocytes. In addition, tumor mainly utilizes glycolysis for energy generation and cellular proliferation, which cause the TME to deplete nutrients for infiltrating immune cells such as T cells and produce immunosuppressive metabolites. Thus, therapeutics that target glucose metabolism, such as inhibiting glycolytic activity, alleviating hypoxia, and targeting lactate, have shown promise as combination therapies for different types of cancer. In this review, we summarized the functions of glucose metabolism in the tumor cells, immune cells, and tumor microenvironment, as well as strategies to target glucose metabolism in combination with immune checkpoint blockade for tumor therapy.

KEYWORDS

glucose metabolism, tumor microenvironment, aerobic glycolysis, immune checkpoint inhibitors, immunotherapy

Introduction

During the past decade, multiple immunotherapies, such as immune checkpoint blockade, adoptive cell therapy, and cancer vaccines, have revolutionized the treatment of cancer (1–3). Notably, immune checkpoint inhibitors (ICIs) that restore the antitumor immune response of T-cells by blocking the ligation of coinhibitory signaling molecules (PD-1/PD-L1, CTLA4/B7) have become one of the most promising immunotherapies (4). Indeed, remarkable efficacy of ICIs has been shown in patients with several types of cancer (5, 6). However, most patients with cancer remain unresponsive to immunotherapy due to the tumor adopting multiple mechanisms to weaken the antitumor immune response. On one hand, tumor cells dampen immune responses through intrinsic mechanisms, such as loss of antigen presentation or expression of immunosuppressive molecules. On the other hand, the tumor microenvironment (TME), such as stromal barriers, hypoxia, insufficient vascularization, nutrient deficiencies, and metabolic disorders, also facilitates immunosuppression and limits anticancer immune responses.

Recently, the role of glucose metabolism in immune microenvironment has gained increased attention. Growing evidence reveals that glucose metabolism is able to aid tumor escape from immune surveillance and impede immunotherapy. Elevated glucose metabolism in tumor cells facilitates unregulated cellular proliferation and regulates the expression of inhibitory immune checkpoint molecules, such as PD-L1 (7). Meanwhile, glucose metabolism also regulates the activation and differentiation of immunocytes. It was reported that glycolysis could promote the function of T-cells and enhance the secretion of IFN-γ (8). Tumor-associated macrophage enhances glucose uptake and promotes differentiation of M1 macrophage, which increases the expression of pro-inflammatory cytokines and promotes tumor progression (9). Additionally, abnormal glucose metabolism also promotes the formation of the immunosuppressive microenvironment. Tumors mainly utilize glycolysis for energy generation, which causes depletion of nutrients for infiltrating immune cells and production of immunosuppressive metabolites (8, 10). Moreover, high glucose metabolism transforms TME into acidic and hypoxic phenotypes, which dampen the cytotoxicity activity of T-cells (11–13). Indeed, targeting tumor cell glycolysis not only inhibits tumor cell growth, but also preserves the antitumor T-cell function (14).

In conclusion, aberrant glucose metabolism in tumors may lead to immune cell dysfunction, which may account for the failure of immunotherapy. An in-depth understanding of tumor immunometabolism may contribute to discovering novel promising approaches to boost T-cells activity. Here, we reviewed the characteristics of glucose metabolism in tumor microenvironment, discussed the regulatory role of glucose metabolism in antitumor immune response, and summarized the potential therapeutic strategies targeting glucose metabolism to enhance the efficacy of cancer immunotherapy, especially ICIs.

Characteristics of glucose metabolism in the tumor microenvironment

Highly proliferative cancer cells uptake large amounts of glucose for energy generation in the TME. Likewise, the differentiation and activation of immunocytes in the TME also rely on glucose metabolism, thus creating a competition for glucose between cancer and immune cells (15). The intensive glycolysis of tumor cells impedes T-cell access to glucose, thereby impairing their appropriate proliferation and function (16). Dysregulation of glucose metabolism in the TME can affect the biological activity of tumor cells and also regulate the effector functions of immune cells. Therefore, a better understanding the changes of glucose metabolism in the TME and their impacts on antitumor immune response may help uncover potential metabolic targets to inhibit tumor growth and enhance the efficacy of immunotherapy.

Glucose metabolism of tumor cells

Notably, the level of glycolysis is increased in tumors despite sufficient oxygen levels. This phenomenon of metabolic reprogramming, also known as the Warburg effect, is considered a crucial feature of cancer metabolism (17, 18). Although oxidative phosphorylation (OXPHOS) is a more efficient pathway for ATP generation, tumor cells have a lower rate of OXPHOS (18). Apparently, glycolysis is an important metabolic pathway for highly proliferative cancer cells, which can supply essential metabolic intermediates to synthesize biomolecules, such as nucleotides, lipids, and amino acids (19). The programs of glucose metabolism of tumor cells consume lots of glucose, then convert glucose into pyruvate and generate ATP. Further metabolic reactions of pyruvate release lactate into the TME. Activation of PI3K/AKT signaling in tumor cells increases glucose transporters (GLUTs) at the plasma membrane and promotes glucose uptake. AKT has also been found to activate the glycolytic enzymes (20). Upregulation of glycolytic metabolism in tumors results in glucose depletion, hypoxia, and lactate accumulation in the TME. In return, hypoxia further promotes glycolysis in tumor cells by stabilizing hypoxia-inducible factor-1α (HIF-1α), which induces the transcription of glycolytic enzymes (21). The high levels of lactate result in acidic conditions that promote tumor progression and metastasis (22, 23). However, glucose depletion and hypoxia impose metabolic stress on T-cells and impair the cytolytic activity (16). In addition, lactate has diverse effects on immune cells, including polarizing macrophages towards the anti-inflammatory M2 type,

supporting regulatory T-cells or suppressive myeloid populations, and interfering with dendritic cell (DC) maturation (24, 25). Collectively, these findings indicate that glycolytic activity not only provides advantages to cancer cells to grow under hypoxia and glucose deprivation conditions but also creates an immunosuppressive microenvironment to regulate surrounding cells that contributes to tumor immune evasion. Therefore, targeting glucose metabolism to improve the TME is a rational and promising strategy for anticancer therapy.

Glucose metabolism of immune cells in tumor

Importantly, metabolic reprogramming also occurs within immune cells (26). T-cells are critical executors in tumor cell killing. The reprogramming of glucose metabolism is involved in the differentiation and activation of T-cells. Briefly, naïve $CD4^+$ and $CD8^+$ T-cells have minimal metabolic activity and engage in OXPHOS to generate energy. Upon activation, the activated T-cells are accompanied by metabolic changes that increase glucose uptake and upregulate glycolysis to support their growth and function (27). The glucose metabolism of activated T-cells is regulated by several signaling pathways, including the PI3K/AKT/mTOR and AMPK pathways (28, 29). The PI3K/AKT/mTOR pathway senses nutrient availability and promotes glucose uptake and glycolysis (30), whereas AMPK induces a metabolic switch toward OXPHOS by inhibiting mTOR signaling (31, 32). Hence, glucose depletion within the TME enhances AMPK activation, which may inhibit effector function of T-cells (33). Nutrient competition between cancer and immune cells contributes to functional exhaustion of $CD8^+$ T-cells (34, 35). Exhausted T-cells also exhibit dysregulated metabolism with repressed glycolytic and mitochondrial function (36, 37). In addition, $CD8^+$ memory T-cells are an important member of the adaptive immune system and play a crucial role in long-term tumor control. During the differentiation of activated effector T-cells into memory T-cells, there is also a metabolic shift to increase mitochondrial metabolism for energy production (38). Inhibition of mTOR signaling enhances $CD8^+$ memory T cell formation but compromises effector T-cell activity (39). Antitumor $CD4^+$ T-cells share metabolic profiles with activated $CD8^+$ T-cells (40). However, not all $CD4^+$ T-cells undergo metabolic transition after activation. Regulatory T-cells (Treg), a subset of $CD4^+$ T-cells, play a critical role in dampening antitumor immune response. Low-glucose availability induces FOXP3 expression, increasing T cell differentiation to Tregs (39). This immunosuppressive subset has low levels of glycolysis and predominantly depends on mitochondrial respiration and fatty acid oxidation for their metabolism (41, 42). In addition, lactate is preferentially utilized by Tregs since they prefer oxidative metabolism (42). The distinct metabolic programs enable Treg cells to maintain optimal function in the lactate-rich TME (42).

The activation of natural killer (NK) cells depends on glycolysis and OXPHOS to supply energy (43). Therefore, the lack of glucose in the TME affects the function of NK cells. It has been reported that the glycolytic rate of NK cells was suppressed in lung cancer, which further weakened the cytotoxicity and cytokine production (44). Glycolysis is not the only way of glucose metabolism, gluconeogenesis also plays a significant role in TME (45). Gluconeogenesis is the process of generating glucose from non-carbohydrate substrates. Fructose-1,6-bisphosphatase (FBP1), a key enzyme in gluconeogenesis, is upregulated in murine NK cells (44). FBP1 represses the expression of glycolytic genes and reverses the direction of glycolysis to promote glucose synthesis (46). Therefore, inhibition of FBP1 strongly restores glycolytic metabolism and improve the function of NK cells (44).

Similarly, other innate immune cells employ specific metabolic programs upon activation. DCs are professional antigen presenting cells that phagocytize and present antigen to T-cells after maturation. This process is accompanied by an increase in glycolysis through PI3K/AKT pathway in response to Toll-like receptor (TLR) signals (47). Activated DCs require high glycolytic metabolism, which is critical for DC continued survival and secretion of pro-inflammatory cytokines (48). Thus, glucose competition in the TME may affect DC function and limit the activity of T-cells (49). Macrophages reprogram their metabolism in the TME through elevated glycolysis while maintaining a tumor-promoting function (50). Tumor-associate macrophages (TAMs) are categorized into inflammatory (M1) and immunosuppressive (M2) phenotypes, which have distinct metabolic programs. The pro-inflammatory M1 macrophages enhance glycolysis and fatty acid synthesis (FAS) to support their function (51). Conversely, like Treg cells, anti-inflammatory M2 macrophages rely more on fatty acid oxidation (FAO) and OXPHOS to maintain their tumor-promoting function (50, 52). The myeloid-derived suppressor cells (MDSCs) also exhibit characteristic metabolic phenotypes. It is generally believed that both aerobic glycolysis and OXPHOS were upregulated in MDSCs (53). Recent study shows that MDSCs consume the most glucose per cell in the TME and account for one-third of glucose uptake in the whole tumor, limiting the availability of glucose to other immune cells (54). And blocking glycolysis has been shown to inhibit the expansion and function of MDSCs (55). Hypoxia and lactate in the TME can polarize macrophages toward M2 anti-inflammatory phenotype, promote MDSC suppressive function, and reduce dendritic cell activation and stimulatory effects (56–58).

Targeting glucose metabolism to enhance cancer immunotherapy

Multiple researches have shown that glycolytic activity of glucose metabolism not only provides an intrinsic growth

advantage for cancer cells but also exerts inhibitory effects on antitumor immune cells by creating an immunosuppressive microenvironment. Thus, improving the immune state of TME *via* metabolic intervention is expected to provide promising strategies for enhancing the therapeutic effect of tumors. To date, several drugs have been proposed to target tumor glucose metabolism for cancer treatment (**Table 1**). Here, we summarize the therapeutic strategies targeting glucose metabolism and advances in glucose metabolism intervention combined with ICI immunotherapy.

Strategies of targeting glucose metabolism within the TME

Inhibiting glycolytic activity of tumor cells

The enhanced glycolytic activity of cancer cells results in deprivation of glucose and accumulation of lactate, which inhibit the effector function of antitumor immune cells and promote the differentiation and recruitment of immunosuppressive cell populations. Therefore, weakening tumor aerobic glycolysis by either inhibiting glycolysis-related enzymes or using the competitive glucose analog may help to inhibit tumor growth. 2-Deoxyglucose (2-DG) is a glucose analog and inhibitor of hexokinase used to reduce glycolysis. Studies revealed it could inhibit cancer cell proliferation and promote the formation of memory $CD8^+$ T-cells, but it also impairs the effector function of T-cells (59, 60). Dichloroacetate (DCA) is an inhibitor of phosphofructokinase-1 and can induce a shift from glycolysis to OXPHOS and inhibits tumor cells' growth (61, 62). However, these two drugs also inhibit T-cell function and promote immunosuppression because they are not specific to tumor cell metabolism. Therefore, the targeting specificity of tumor cells is critical for applying glycolysis inhibitors.

Targeting lactate in the TME

The enhanced glycolytic activity of tumor cells can release large amounts of lactate, leading to the formation of acidic TME (63). Lactic acid increases the expression of IL-8 and VEGF, which promotes tumor metastasis and progression (22). Yet, lactate inhibits the proliferation and cytotoxicity of T and NK cells, but promotes the survival of Treg and MDSC cell populations (42, 64). Hence, inhibiting the production of lactate or neutralizing lactate in the TME may improve the efficacy of immunotherapy. Lactate dehydrogenase (LDH), which is comprised of two major subunits, LDH-A and LDH-B, catalyzes the reversible reaction between pyruvate and lactate. LDH-A has a higher affinity for pyruvate and favors the conversion of pyruvate to lactate, making LDH-A a target of interest (65). LDH-A inhibitors, such as FX11 and galloflavin, have been reported to reduce tumor growth (66). Another approach to targeting lactate is to inhibit lactate transporters monocarboxylate transporter (MCT) 1-4. MCT inhibitor lenalidomide has been reported to suppress tumor cell proliferation and enhance IL-2 and IFN-γ secretion of T-cells (67). In a mouse model of breast cancer, blockade of MCT1/MCT4 combined with ICI enhances antitumor immune responses compared to ICI alone (14). Further, neutralizing lactic acid with bicarbonate or proton pump inhibitors can improve the low pH of TME. Importantly, oral bicarbonate combined with anti-PD-1 immunotherapy inhibits tumor growth in a melanoma mouse model (68, 69).

Alleviating hypoxia in the TME

The rapid proliferation of tumor cells results in massive oxygen consumption and hypoxia in the TME. Under hypoxic conditions, HIFs are stabilized to promote the expression of glycolytic genes and cytokines that promote tumor metabolism and angiogenesis (70). Furthermore, intra-tumoral hypoxia inhibits the function of T-cells and supports the generation of immunosuppressive cells, such as

TABLE 1 Potential targets for modulating glucose metabolism.

Intervention	Target	Representative drugs
Inhibiting glycolytic activity	HK	2-DG
	PFK-1	DCA
	GLUT1	WZB117
Targeting lactate in the TME	LDH-A	FX11, Galloflavin
	MCTs	Lenalidomide, AZD3965
	Acidic pH	Bicarbonate
	Proton pump	Omeprazole
Alleviating hypoxia in the TME	Hypoxia	Supplemental oxygen, Evofosfamide
	Mitochondrial respiratory complex I	Metformin
Targeting PI3K/AKT/mTOR signaling pathway	mTOR	Rapamycin
	PI3K isoforms	AZD8835, BKM120
	AKT	AKTi, Ipatasertib

HK, Hexokinase; 2-DG, 2-Deoxyglucose; PFK-1, Phosphofructokinase-1; DCA, Dichloroacetate; GLUT1, glucose transporter 1; LDH-A, Lactate dehydrogenase-A; MCTs, Monocarboxylate transporters; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol-3 kinase.

Treg and M2 macrophages (71, 72). As oxygen is a determinant of energy production needed for the differential and activation of effector T-cells, alleviating hypoxia can boost antitumor immune responses. Studies have confirmed that supplemental oxygen increased T-cell infiltration and inflammatory cytokine production, and decreased Treg cell, which can enhance the antitumor immunity of T-cells in mice with lung tumors (73). Similar results can be obtained with the use of metformin, which reduces tumor hypoxia and improves T-cell response in a mouse model of melanoma (89).

Targeting PI3K/AKT/mTOR signaling pathway

The PI3K/AKT/mTOR is an important pathway involved in cell proliferation. This pathway also senses nutrients and plays a key role in promoting glycolysis in tumor cells and effector T-cells (39). Analogs of rapamycin can reduce cancer cell aerobic glycolysis and proliferation. However, these drugs can also suppress the function of effector T-cells and endow T-cell with a memory phenotype (74). For example, the mTORC1 inhibitor rapamycin enhances the formation of $CD8^+$ memory T-cells and also inhibits immune response of $CD8^+$ T-cells (75). Likewise, PI3K inhibitor skews T-cell differentiation toward memory phenotypes and improves *in vivo* persistence and antitumor activity in mice with acute myeloid leukemia (76). Among the PI3K isoforms, PI3Kα and PI3Kβ are ubiquitously expressed, whereas PI3Kγ and PI3Kδ are found primarily in leukocytes. PI3Kα/δ inhibition promotes antitumor immunity through enhancement of $CD8^+$ T-cell activity and suppression of Tregs (77). Interestingly, recent evidence suggests that rapamycin combined with immunotherapy promote cytotoxic and memory function of T-cells in glioblastoma (78). Therefore, blockade of PI3K/AKT/mTOR signaling may be an attractive strategy to be used in combination with immunotherapy.

Advances in targeting glucose metabolism combined with immune checkpoint inhibitors

Immune checkpoint inhibitors restore the antitumor immunity of T-cells by blocking the binding of inhibitory immune checkpoint ligands and receptors. Undoubtedly, ICIs have achieved good results in some malignant tumors. Recent evidence has indicated that immune checkpoint signaling can regulate metabolism of cancer cells and T-cells. Indeed, several studies suggest that the interaction of PD-1 and PD-L1 impairs the metabolic feature of T-cells, including inhibiting aerobic glycolysis *via* suppression of PI3K/AKT/mTOR signaling (79, 80). Similar to PD-1signaling, CTLA-4 ligation to CD80/CD86 inhibits the glucose metabolism of T-cells by reducing AKT phosphorylation and activation (81). Hence, checkpoints signaling would impact the activation and antitumor function of T-cells (80). In addition, immune checkpoints also affect metabolism of tumor cells. PD-L1 in tumor cells has been shown to upregulate aerobic glycolysis by increasing the activity of PI3K/AKT/mTOR pathway (82). Consequently, PD-1/PD-L1 axis supports the survival and progression of cancer cells. Thus, inhibition of PD-1/PD-L1 signaling might restore the metabolic requirements of T-cells while simultaneously inhibiting glycolysis levels in tumor cells. Indeed, the ICIs differentially influence the metabolic programs in the TME by increasing the glucose availability to T-cells and in contrast inhibiting the rate of glycolysis in cancer cells. In tumors, the TME imposes many metabolic stresses on immune cells, especially the antitumor effector T-cells. Therefore, the metabolic treatments that modulate glucose metabolism to improve the TME could be attractive adjuvants to be used in combination with ICIs.

Recently, researchers constructed a nanosystem embedded with lactate oxidase (LOX) and a glycolysis inhibitor for lactate consumption and modulation of metabolism. This nanosystem can inhibit tumor growth by blocking glycolysis and removing lactic acid in the TME. Importantly, this metabolic regulation strategy can effectively improve the therapeutic effect of anti-PD-L1 therapy and avoid systemic toxicity (83). In addition, other studies also found that neutralizing tumor acidity with oral bicarbonate impaired the tumor growth in melanoma mice, further combining bicarbonate with anti-PD1 improved antitumor response and prolonged mouse survival (68). Metformin can inhibit oxygen consumption in tumor cells to reduce intra-tumoral hypoxia. A combination of metformin with PD-1 antibody improves T-cell function and tumor clearance in mice with melanoma (84). Favorable treatment outcomes were also observed in melanoma patients receiving metformin in combination with ICIs (85).Similarly, patients with non-small cell lung cancer receiving concurrent metformin and ICIs showed higher response rate and overall survival (86). A Phase I clinical trial reported that evofosfamide in combination with ipilimumab improved immune activity in advanced solid tumors (87). Future studies focusing on metabolic modulation therapy in combination with ICIs are clearly warranted.

Conclusion and perspectives

Increasing evidence indicates that cellular metabolism could remodel TME and regulate antitumor immunity. These provide opportunities to target metabolism as a means to enhance immunotherapy. Varieties of strategies targeting glucose metabolism have been developed for tumor therapy, but their efficacy needs to be further explored. Moreover, the similarity of metabolism between tumor cells and T-cells raises the concern that targeting tumor metabolism might undermine the immune response of activated T-cells. In fact, several studies have suggested that drugs targeting tumor aerobic glycolysis also significantly inhibit T-cell function and promote immunosuppression (59). Therefore, special attention should be devoted to targeting tumor cells and avoiding

systemic toxicity. Using more specific inhibitors or nano-delivery technology may be the way to address the toxic effects.

In addition to glucose metabolism, tumor cells also obtain energy and substances through other metabolic pathways, such as amino acid and fatty acid metabolism. Hence, it is unlikely that a single enzyme or transporter targeting specific pathway will provide a perfect solution. Instead, approaches intervening metabolic targets in combination with other therapies, including ICIs, may offer the most significant potential to improve clinical efficacy.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Darvin P, Toor SM, Sasidharan Nair V, Elkord E. Immune checkpoint inhibitors: Recent progress and potential biomarkers. *Exp Mol Med* (2018) 50 (12):1–11. doi: 10.1038/s12276-018-0191-1

2. Papaioannou NE, Beniata OV, Vitsos P, Tsitsilonis O, Samara P. Harnessing the immune system to improve cancer therapy. *Ann Transl Med* (2016) 4(14):261. doi: 10.21037/atm.2016.04.01

3. Zhang YY, Zhang ZM. The history and advances in cancer immunotherapy: Understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications. *Cell Mol Immunol* (2020) 17(8):807–21. doi: 10.1038/s41423-020-0488-6

4. Zavala VA, Kalergis AM. New clinical advances in immunotherapy for the treatment of solid tumours. *Immunology* (2015) 145(2):182–201. doi: 10.1111/imm.12459

5. Mahoney KM, Freeman GJ, McDermott DF. The next immune-checkpoint inhibitors: Pd-1/Pd-L1 blockade in melanoma. *Clin Ther* (2015) 37(4):764–82. doi: 10.1016/j.clinthera.2015.02.018

6. Ivashko IN, Kolesar JM. Pembrolizumab and nivolumab: Pd-1 inhibitors for advanced melanoma. *Am J Health Syst Pharm* (2016) 73(4):193–201. doi: 10.2146/ajhp140768

7. Yu YB, Liang Y, Li D, Wang LP, Liang ZJ, Chen YB, et al. Glucose metabolism involved in pd-L1-Mediated immune escape in the malignant kidney tumour microenvironment. *Cell Death Discovery* (2021) 7(1):15. doi: 10.1038/s41420-021-00401-7

8. Chang CH, Curtis JD, Maggi LB, Faubert B, Villarino AV, O'Sullivan D, et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* (2013) 153(6):1239–51. doi: 10.1016/j.cell.2013.05.016

9. Shi R, Tang YQ, Miao H. Metabolism in tumor microenvironment: Implications for cancer immunotherapy. *MedComm* (2020) 2020) 1(1):47–68. doi: 10.1002/mco2.6

10. Ho PC, Bihuniak JD, Macintyre AN, Staron M, Liu XJ, Amezquita R, et al. Phosphoenolpyruvate is a metabolic checkpoint of anti-tumor T cell responses. *Cell* (2015) 162(6):1217–28. doi: 10.1016/j.cell.2015.08.012

11. Diaz FE, Dantas E, Geffner J. Unravelling the interplay between extracellular acidosis and immune cells. *Mediat Inflammation* (2018) 2018:1218297. doi: 10.1155/2018/1218297

12. DePeaux K, Delgoffe GM. Metabolic barriers to cancer immunotherapy. *Nat Rev Immunol* (2021) 21(12):785–97. doi: 10.1038/s41577-021-00541-y

13. Yin ZP, Bai L, Li W, Zeng TL, Tian HM, Cui J. Targeting T cell metabolism in the tumor microenvironment: An anti-cancer therapeutic strategy. *J Exp Clin Canc Res* (2019) 38(1):403. doi: 10.1186/s13046-019-1409-3

14. Renner K, Bruss C, Schnell A, Koehl G, Becker HM, Fante M, et al. Restricting glycolysis preserves T cell effector functions and augments checkpoint therapy. *Cell Rep* (2019) 29(1):135–50.e9. doi: 10.1016/j.celrep.2019.08.068

15. Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell* (2015) 162(6):1229–41. doi: 10.1016/j.cell.2015.08.016

16. Sanli T, Steinberg GR, Singh G, Tsakiridis T. Amp-activated protein kinase (Ampk) beyond metabolism: A novel genomic stress sensor participating in the DNA damage response pathway. *Cancer Biol Ther* (2014) 15(2):156–69. doi: 10.4161/cbt.26726

17. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *J Gen Physiol* (1927) 8(6):519–30. doi: 10.1085/jgp.8.6.519

18. DeBerardinis RJ, Chandel NS. We need to talk about the warburg effect. *Nat Metab* (2020) 2(2):127–9. doi: 10.1038/s42255-020-0172-2

19. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. *Sci Adv* (2016) 2(5):e1600200. doi: 10.1126/sciadv.1600200

20. Hoxhaj G, Manning BD. The Pi3k-akt network at the interface of oncogenic signalling and cancer metabolism. *Nat Rev Cancer* (2020) 20(2):74–88. doi: 10.1038/s41568-019-0216-7

21. Zhao H, Jiang H, Li Z, Zhuang Y, Liu Y, Zhou S, et al. 2-methoxyestradiol enhances radiosensitivity in radioresistant melanoma mda-Mb-435r cells by regulating glycolysis *Via* hif-1alpha/Pdk1 axis. *Int J Oncol* (2017) 50(5):1531–40. doi: 10.3892/ijo.2017.3924

22. Hirschhaeuser F, Sattler UG, Mueller-Klieser W. Lactate: A metabolic key player in cancer. *Cancer Res* (2011) 71(22):6921–5. doi: 10.1158/0008-5472.CAN-11-1457

23. Wang J, Wang H, Liu AF, Fang CG, Hao JG, Wang ZH. Lactate dehydrogenase a negatively regulated by mirnas promotes aerobic glycolysis and is increased in colorectal cancer. *Oncotarget* (2015) 6(23):19456–68. doi: 10.18632/oncotarget.3318

24. Dietl K, Renner K, Dettmer K, Timischl B, Eberhart K, Dorn C, et al. Lactic acid and acidification inhibit tnf secretion and glycolysis of human monocytes. *J Immunol* (2010) 184(3):1200–9. doi: 10.4049/jimmunol.0902584

25. Colegio OR, Chu NQ, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* (2014) 513(7519):559–63. doi: 10.1038/nature13490

26. Andrejeva G, Rathmell JC. Similarities and distinctions of cancer and immune metabolism in inflammation and tumors. *Cell Metab* (2017) 26(1):49–70. doi: 10.1016/j.cmet.2017.06.004

27. Patel CH, Leone RD, Horton MR, Powell JD. Targeting metabolism to regulate immune responses in autoimmunity and cancer. *Nat Rev Drug Discov* (2019) 18(9):669–88. doi: 10.1038/s41573-019-0032-5

28. Zeng H, Cohen S, Guy C, Shrestha S, Neale G, Brown SA, et al. Mtorc1 and Mtorc2 kinase signaling and glucose metabolism drive follicular helper T cell differentiation. *Immunity* (2016) 45(3):540–54. doi: 10.1016/j.immuni.2016.08.017

29. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. Ampk phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* (2008) 30(2):214–26. doi: 10.1016/j.molcel.2008.03.003

30. Waickman AT, Powell JD. Mtor, metabolism, and the regulation of T-cell differentiation and function. *Immunol Rev* (2012) 249:43–58. doi: 10.1111/j.1600-065X.2012.01152.x

31. Eichner LJ, Brun SN, Herzig S, Young NP, Curtis SD, Shackelford DB, et al. Genetic analysis reveals ampk is required to support tumor growth in murine kras-dependent lung cancer models. *Cell Metab* (2019) 29(2):285. doi: 10.1016/j.cmet.2018.10.005

32. Ma EH, Poffenberger MC, Wong AHT, Jones RG. The role of ampk in T cell metabolism and function. *Curr Opin Immunol* (2017) 46:45–52. doi: 10.1016/j.coi.2017.04.004

33. Blagih J, Coulombe F, Vincent EE, Dupuy F, Galicia-Vazquez G, Yurchenko E, et al. The energy sensor ampk regulates T cell metabolic adaptation and effector responses in vivo. *Immunity* (2015) 42(1):41–54. doi: 10.1016/j.immuni.2014.12.030

34. Kouidhi S, Ben Ayed F, Elgaaied AB. Targeting tumor metabolism: A new challenge to improve immunotherapy. *Front Immunol* (2018) 9:353. doi: 10.3389/fimmu.2018.00353

35. Beckermann KE, Dudzinski SO, Rathmell JC. Dysfunctional T cell metabolism in the tumor microenvironment. *Cytokine Growth Factor Rev* (2017) 35:7–14. doi: 10.1016/j.cytogfr.2017.04.003

36. Bengsch B, Johnson AL, Kurachi M, Odorizzi PM, Pauken KE, Attanasio J, et al. Bioenergetic insufficiencies due to metabolic alterations regulated by the inhibitory receptor pd-1 are an early driver of Cd8(+) T cell exhaustion. *Immunity* (2016) 45(2):358–73. doi: 10.1016/j.immuni.2016.07.008

37. Scharping NE, Menk AV, Moreci RS, Whetstone RD, Dadey RE, Watkins SC, et al. The tumor microenvironment represses T cell mitochondrial biogenesis to drive intratumoral T cell metabolic insufficiency and dysfunction. *Immunity* (2016) 45(2):374–88. doi: 10.1016/j.immuni.2016.07.009

38. van der Windt GJW, Everts B, Chang CH, Curtis JD, Freitas TC, Amiel E, et al. Mitochondrial respiratory capacity is a critical regulator of Cd8(+) T cell memory development. *Immunity* (2012) 36(1):68–78. doi: 10.1016/j.immuni.2011.12.007

39. Bader JE, Voss K, Rathmell JC. Targeting metabolism to improve the tumor microenvironment for cancer immunotherapy. *Mol Cell* (2020) 78(6):1019–33. doi: 10.1016/j.molcel.2020.05.034

40. Leone RD, Powell JD. Metabolism of immune cells in cancer. *Nat Rev Cancer* (2020) 20(9):516–31. doi: 10.1038/s41568-020-0273-y

41. Field CS, Baixauli F, Kyle RL, Puleston DJ, Cameron AM, Sanin DE, et al. Mitochondrial integrity regulated by lipid metabolism is a cell-intrinsic checkpoint for treg suppressive function. *Cell Metab* (2020) 31(2):422. doi: 10.1016/j.cmet.2019.11.021

42. Angelin A, Gil-de-Gomez L, Dahiya S, Jiao J, Guo L, Levine MH, et al. Foxp3 reprograms T cell metabolism to function in low-glucose, high-lactate environments. *Cell Metab* (2017) 25(6):1282–93.e7. doi: 10.1016/j.cmet.2016.12.018

43. Keppel MP, Saucier N, Mah AY, Vogel TP, Cooper MA. Activation-specific metabolic requirements for nk cell ifn-gamma production. *J Immunol* (2015) 194(4):1954–62. doi: 10.4049/jimmunol.1402099

44. Cong J, Wang X, Zheng X, Wang D, Fu B, Sun R, et al. Dysfunction of natural killer cells by Fbp1-induced inhibition of glycolysis during lung cancer progression. *Cell Metab* (2018) 28(2):243–55.e5. doi: 10.1016/j.cmet.2018.06.021

45. Bose S, Zhang C, Le A. Glucose metabolism in cancer: The warburg effect and beyond. *Adv Exp Med Biol* (2021) 1311:3–15. doi: 10.1007/978-3-030-65768-0_1

46. Huangyang P, Simon MC. Hidden features: Exploring the non-canonical functions of metabolic enzymes. *Dis Model Mech* (2018) 11(8):dmm033365. doi: 10.1242/dmm.033365

47. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, et al. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* (2010) 115(23):4742–9. doi: 10.1182/blood-2009-10-249540

48. Guak H, Al Habyan S, Ma EH, Aldossary H, Al-Masri M, Won SY, et al. Glycolytic metabolism is essential for Ccr7 oligomerization and dendritic cell migration. *Nat Commun* (2018) 9(1):2463. doi: 10.1038/s41467-018-04804-6

49. Giovanelli P, Sandoval TA, Cubillos-Ruiz JR. Dendritic cell metabolism and function in tumors. *Trends Immunol* (2019) 40(8):699–718. doi: 10.1016/j.it.2019.06.004

50. Netea-Maier RT, Smit JWA, Netea MG. Metabolic changes in tumor cells and tumor-associated macrophages: A mutual relationship. *Cancer Lett* (2018) 413:102–9. doi: 10.1016/j.canlet.2017.10.037

51. Rabold K, Netea MG, Adema GJ, Netea-Maier RT. Cellular metabolism of tumor-associated macrophages - functional impact and consequences. *FEBS Lett* (2017) 591(19):3022–41. doi: 10.1002/1873-3468.12771

52. Vitale I, Manic G, Coussens LM, Kroemer G, Galluzzi L. Macrophages and metabolism in the tumor microenvironment. *Cell Metab* (2019) 30(1):36–50. doi: 10.1016/j.cmet.2019.06.001

53. Hossain F, Al-Khami AA, Wyczechowska D, Hernandez C, Zheng L, Reiss K, et al. Inhibition of fatty acid oxidation modulates immunosuppressive functions of myeloid-derived suppressor cells and enhances cancer therapies. *Cancer Immunol Res* (2015) 3(11):1236–47. doi: 10.1158/2326-6066.CIR-15-0036

54. Reinfeld BI, Madden MZ, Wolf MM, Chytil A, Bader JE, Patterson AR, et al. Cell-programmed nutrient partitioning in the tumour microenvironment. *Nature* (2021) 593(7858):282–8. doi: 10.1038/s41586-021-03442-1

55. Jian SL, Chen WW, Su YC, Su YW, Chuang TH, Hsu SC, et al. Glycolysis regulates the expansion of myeloid-derived suppressor cells in tumor-bearing hosts through prevention of ros-mediated apoptosis. *Cell Death Dis* (2017) 8(5):e2779. doi: 10.1038/cddis.2017.192

56. Corzo CA, Condamine T, Lu L, Cotter MJ, Youn JI, Cheng P, et al. Hif-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J Exp Med* (2010) 207(11):2439–53. doi: 10.1084/jem.20100587

57. Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, et al. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood* (2006) 107(5):2013–21. doi: 10.1182/blood-2005-05-1795

58. Nasi A, Fekete T, Krishnamurthy A, Snowden S, Rajnavolgyi E, Catrina AI, et al. Dendritic cell reprogramming by endogenously produced lactic acid. *J Immunol* (2013) 191(6):3090–9. doi: 10.4049/jimmunol.1300772

59. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting glycolytic metabolism enhances Cd8+ T cell memory and antitumor function. *J Clin Invest* (2013) 123(10):4479–88. doi: 10.1172/JCI69589

60. Zhang D, Li J, Wang F, Hu J, Wang S, Sun Y. 2-Deoxy-D-Glucose targeting of glucose metabolism in cancer cells as a potential therapy. *Cancer Lett* (2014) 355(2):176–83. doi: 10.1016/j.canlet.2014.09.003

61. Vella S, Conti M, Tasso R, Cancedda R, Pagano A. Dichloroacetate inhibits neuroblastoma growth by specifically acting against malignant undifferentiated cells. *Int J Cancer* (2012) 130(7):1484–93. doi: 10.1002/ijc.26173

62. Tong J, Xie G, He J, Li J, Pan F, Liang H. Synergistic antitumor effect of dichloroacetate in combination with 5-fluorouracil in colorectal cancer. *J BioMed Biotechnol* (2011) 2011:740564. doi: 10.1155/2011/740564

63. Choi SY, Collins CC, Gout PW, Wang Y. Cancer-generated lactic acid: A regulatory, immunosuppressive metabolite? *J Pathol* (2013) 230(4):350–5. doi: 10.1002/path.4218

64. Husain Z, Huang Y, Seth P, Sukhatme VP. Tumor-derived lactate modifies antitumor immune response: Effect on myeloid-derived suppressor cells and nk cells. *J Immunol* (2013) 191(3):1486–95. doi: 10.4049/jimmunol.1202702

65. Khan AA, Allemailem KS, Alhumaydhi FA, Gowder SJT, Rahmani AH. The biochemical and clinical perspectives of lactate dehydrogenase: An enzyme of active metabolism. *Endocr Metab Immune Disord Drug Targets* (2020) 20(6):855–68. doi: 10.2174/1871530320666191230141110

66. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, et al. Inhibition of lactate dehydrogenase a induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci U S A* (2010) 107(5):2037–42. doi: 10.1073/pnas.0914433107

67. Gorgun G, Calabrese E, Soydan E, Hideshima T, Perrone G, Bandi M, et al. Immunomodulatory effects of lenalidomide and pomalidomide on interaction of tumor and bone marrow accessory cells in multiple myeloma. *Blood* (2010) 116(17):3227–37. doi: 10.1182/blood-2010-04-279893

68. Pilon-Thomas S, Kodumudi KN, El-Kenawi AE, Russell S, Weber AM, Luddy K, et al. Neutralization of tumor acidity improves antitumor responses to immunotherapy. *Cancer Res* (2016) 76(6):1381–90. doi: 10.1158/0008-5472.CAN-15-1743

69. Fais S. Proton pump inhibitor-induced tumour cell death by inhibition of a detoxification mechanism. *J Intern Med* (2010) 267(5):515–25. doi: 10.1111/j.1365-2796.2010.02225.x

70. Palazon A, Tyrakis PA, Macias D, Velica P, Rundqvist H, Fitzpatrick S, et al. An hif-1alpha/Vegf-a axis in cytotoxic T cells regulates tumor progression. *Cancer Cell* (2017) 32(5):669–83.e5. doi: 10.1016/j.ccell.2017.10.003

71. Westendorf AM, Skibbe K, Adamczyk A, Buer J, Geffers R, Hansen W, et al. Hypoxia enhances immunosuppression by inhibiting Cd4+ effector T cell function and promoting treg activity. *Cell Physiol Biochem* (2017) 41(4):1271–84. doi: 10.1159/000464429

72. Multhoff G, Vaupel P. Hypoxia compromises anti-cancer immune responses. *Adv Exp Med Biol* (2020) 1232:131–43. doi: 10.1007/978-3-030-34461-0_18

73. Hatfield SM, Kjaergaard J, Lukashev D, Schreiber TH, Belikoff B, Abbott R, et al. Immunological mechanisms of the antitumor effects of supplemental oxygenation. *Sci Transl Med* (2015) 7(277):277ra30. doi: 10.1126/scitranslmed.aaa1260

74. Zhang L, Tschumi BO, Lopez-Mejia IC, Oberle SG, Meyer M, Samson G, et al. Mammalian target of rapamycin complex 2 controls Cd8 T cell memory differentiation in a Foxo1-dependent manner. *Cell Rep* (2016) 14(5):1206–17. doi: 10.1016/j.celrep.2015.12.095

75. Chaoul N, Fayolle C, Desrues B, Oberkampf M, Tang A, Ladant D, et al. Rapamycin impairs antitumor Cd8+ T-cell responses and vaccine-induced tumor eradication. *Cancer Res* (2015) 75(16):3279–91. doi: 10.1158/0008-5472.CAN-15-0454

76. Zheng W, O'Hear CE, Alli R, Basham JH, Abdelsamed HA, Palmer LE, et al. Pi3k orchestration of the in vivo persistence of chimeric antigen receptor-modified T cells. *Leukemia* (2018) 32(5):1157–67. doi: 10.1038/s41375-017-0008-6

77. Carnevalli LS, Sinclair C, Taylor MA, Gutierrez PM, Langdon S, Coenen-Stass AML, et al. Pi3kalpha/Delta inhibition promotes anti-tumor immunity through direct enhancement of effector Cd8(+) T-cell activity. *J Immunother Cancer* (2018) 6(1):158. doi: 10.1186/s40425-018-0457-0

78. Mineharu Y, Kamran N, Lowenstein PR, Castro MG. Blockade of mtor signaling *Via* rapamycin combined with immunotherapy augments antiglioma cytotoxic and memory T-cell functions. *Mol Cancer Ther* (2014) 13(12):3024–36. doi: 10.1158/1535-7163.MCT-14-0400

79. Freemerman AJ, Johnson AR, Sacks GN, Milner JJ, Kirk EL, Troester MA, et al. Metabolic reprogramming of macrophages glucose transporter 1 (Glut1)-mediated glucose metabolism drives a proinflammatory phenotype. *J Biol Chem* (2014) 289(11):7884–96. doi: 10.1074/jbc.M113.522037

80. Patsoukis N, Bardhan K, Chatterjee P, Sari D, Liu B, Bell LN, et al. Pd-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. *Nat Commun* (2015) 6:6692. doi: 10.1038/ncomms7692

81. Li X, Wenes M, Romero P, Huang SC, Fendt SM, Ho PC. Navigating metabolic pathways to enhance antitumour immunity and immunotherapy. *Nat Rev Clin Oncol* (2019) 16(7):425–41. doi: 10.1038/s41571-019-0203-7

82. Lim S, Liu H, Madeira da Silva L, Arora R, Liu Z, Phillips JB, et al. Immunoregulatory protein B7-H3 reprograms glucose metabolism in cancer cells by ros-mediated stabilization of Hif1alpha. *Cancer Res* (2016) 76(8):2231–42. doi: 10.1158/0008-5472.CAN-15-1538

83. Gao F, Tang Y, Liu WL, Zou MZ, Huang C, Liu CJ, et al. Intra/Extracellular lactic acid exhaustion for synergistic metabolic therapy and immunotherapy of tumors. *Adv Mater* (2019) 31(51):e1904639. doi: 10.1002/adma.201904639

84. Scharping NE, Menk AV, Whetstone RD, Zeng X, Delgoffe GM. Efficacy of pd-1 blockade is potentiated by metformin-induced reduction of tumor hypoxia. *Cancer Immunol Res* (2017) 5(1):9–16. doi: 10.1158/2326-6066.Cir-16-0103

85. Afzal MZ, Mercado RR, Shirai K. Efficacy of metformin in combination with immune checkpoint inhibitors (Anti-Pd-1/Anti-Ctla-4) in metastatic malignant melanoma. *J Immunother Cancer* (2018) 6(1):64. doi: 10.1186/s40425-018-0375-1

86. Afzal MZ, Dragnev K, Sarwar T, Shirai K. Clinical outcomes in non-Small-Cell lung cancer patients receiving concurrent metformin and immune checkpoint inhibitors. *Lung Cancer Manag* (2019) 8(2):LMT11. doi: 10.2217/lmt-2018-0016

87. Hegde A, Jayaprakash P, Couillault CA, Piha-Paul S, Karp D, Rodon J, et al. A phase I dose-escalation study to evaluate the safety and tolerability of evofosfamide in combination with ipilimumab in advanced solid malignancies. *Clin Cancer Res* (2021) 27(11):3050–60. doi: 10.1158/1078-0432.CCR-20-4118

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Enhanced lipid metabolism confers the immunosuppressive tumor microenvironment in CD5-positive non-MYC/BCL2 double expressor lymphoma

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Lymphoma cells expressing CD5 (CD5+) confer inferior outcome of diffuse large B-cell lymphoma (DLBCL), especially in non–MYC/BCL2 double expressor (non-DE) patients. In tumor microenvironment, CD5+ non-DE tumor revealed increased proportion of immunosuppressive M2 macrophages and enhanced pathways related to macrophage activation and migration. In accordance to M2 activation, lipid metabolism was upregulated, including fatty acid uptake and fatty acid oxidation, which supplied energy for M2 macrophage polarization and activation. Meanwhile, CD36 expression was upregulated and strongly correlated to the proportion of M2 macrophages in CD5+ non-DE DLBCL. *In vitro*, a DLBCL cell line (LY10) overexpressing CD5 significantly increased M2 proportion in comparison with control when cocultured with peripheral blood mononuclear cells (PBMCs). The addition of metformin significantly decreased the M2 proportion and the CD36 expression level in the coculture systems, indicating that metformin could target altered lipid metabolism and decrease M2 macrophages in DLBCL, especially in CD5+ non-DE lymphoma. In conclusion, enhanced lipid metabolism and M2 macrophage activation contributed to the immunosuppressive tumor microenvironment and could be potential therapeutic targets in CD5+ non-DE DLBCL.

KEYWORDS

diffuse large B cell lymphoma (DLBCL), CD5 positive, non-MYC/BCL2 double expressor, tumor microenvironment, lipid metabolism

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most prevalent type of non-Hodgkin lymphoma and is heterogeneous in clinical and molecular features (1). Although standard immunochemotherapy (R-CHOP regimen: rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) has cured about 60% of DLBCL patients, 40% DLBCL patients experience relapse and remain refractory to conventional treatment (2).

Accumulating data have revealed that CD5-positive (CD5+) DLBCL patients fail to benefit from rituximab-based immunochemotherapy and intensive regimens, with the 5-year survival rate as only 40% (3–5). CD5+ DLBCL, a rare disease, accounts for 5%–10% of DLBCL and is defined as an immunohistochemical subtype according to the World Health Organization classification (6). As reported, CD5+ DLBCL is related to an increased serum lactate dehydrogenase level, an advanced Ann Arbor stage, high risk scores of an international prognostic index (IPI), and more frequencies of central nervous system (CNS) relapse (7, 8). Although CD5 is regarded as a poor prognosis biomarker of DLBCL, several studies indicate the heterogeneities of CD5+ DLBCL. Salles et al. reported that CD5 expression over 75% by immunohistochemistry indicated poor outcome (9). Recently, Shen et al. reported that CD5+ double expressor (DE; co-expression of MYC/BCL2) had inferior survival as compared with CD5+ non–double expressor (non-DE) patients (10). Therefore, it is important to explore the genetic alterations in CD5+ DLBCL.

Tumor microenvironment (TME) has been documented to have an essential role in the progression and chemoresistance of DLBCL (11, 12). Immunosuppressive TME accelerates tumor progression through impeding effector T cell and natural killer (NK) cell activation and recruiting immunosuppressive cells such as regulatory T cells, myeloid-derived suppressor cells, and macrophages (13, 14). Tumor-associated macrophages (TAMs) are notorious immune suppressors, supporting tumorigenesis, vascularization, and immunoevasion (15).

Extensive studies have illustrated the profound influence of metabolic reprogramming on TME (16). Immune cells are characterized by different metabolic phenotypes in their proliferation and activation. Activated T and NK cells preferentially use aerobic glycolysis for energy supplement. By contrast, other immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells rely on fatty acid oxidation and oxidative phosphorylation for cell function (17). Metabolic changes can interfere the polarization of TAMs to either pro-inflammatory M1 or anti-inflammatory M2. Transcriptional analyses distinguish M1 and M2 in several metabolic pathways. M1 is enriched by glycolysis, glycerophospholipid metabolism, and fructose and mannose metabolism, whereas M2 is enriched by glutamate, purine, arginase, and fatty acid metabolisms (18).

Enhanced fatty acid metabolism is one of the critical metabolic adaptations of M2. Fatty acid transporter (CD36), fatty acid–binding proteins (FABPs), and fatty acid synthase (FASN) are the main essential proteins involved in the M2 lipid metabolism (19). CD36 is strongly expressed on M2 macrophages, contributing to prolonged survival and activation of M2 macrophages (20). Uptake of triacylglycerol substrates *via* CD36 enhances downstream peroxisome proliferator-activated receptor-γ (PPAR-γ) and arginase expression, also following mitochondrial biogenesis and fatty acid oxidation in M2 (21). Stimulation of ox-LDL induces M2 polarization, with a subsequent increase in the expression of arginase-1, interleukin-10 (IL-10), and transforming growth factor-beta (TGF-β), which represent M2 activation (22). Thus, targeting CD36 on M2 provided a possible approach in targeting lipid metabolism to interfere immunosuppressive TME.

Here, we focus on the clinical, genetic, and microenvironment characteristics in CD5+ non-DE DLBCL and further explore the influence of lipid metabolism on the TME.

Methods

Patients

As shown in the flowchart (Figure 1), a total of 658 newly diagnosed DLBCL patients treated in Ruijin Hospital from 2006 to 2019 were included in this study, namely 64 patients with CD5+ and 594 patients negative for CD5 (CD5–). Patients with primary CNS lymphoma, with primary mediastinal lymphoma, or who received supportive care were excluded. All patients were treated with the R-CHOP–based therapy (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) and were included in the survival analysis. There were 529 non-DE DLBCL patients, namely 48 CD5+ non-DE patients and 481 CD5– non-DE patients. Among the 48 CD5+ non-DE patients, eight were recruited in a prospective, single-arm phase II clinical trial (ChiCTR-OIN-17012130) to evaluate the therapeutic effect of metformin as maintenance therapy, in which patients received metformin orally at a dose of 1.0 g, twice a day for 2-year maintenance after complete remission (23). The remaining 40 patients did not receive metformin maintenance therapy. Among 658 patients, 355 had available DNA sequencing, and 208 had available RNA sequencing data.

Clinical features and pathological data

Clinical data including age, Eastern Cooperative Oncology Group performance status, Ann Arbor stage, serum lactate dehydrogenase, IPI, and sites of extranodal involvement were collected. Immunohistochemistry was performed for CD10, BCL6, MUM1, BCL2, MYC, and CD5 using an indirect immunoperoxidase method on the paraffin sections of tumor

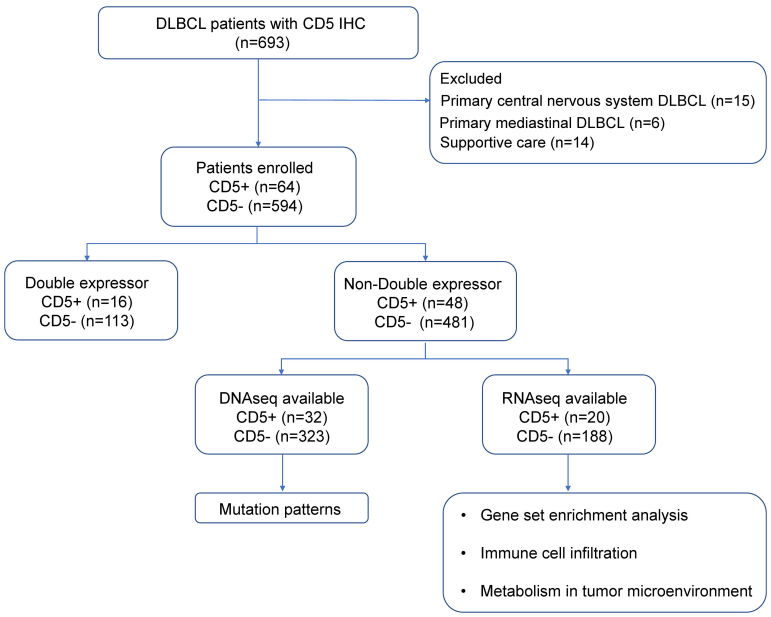

FIGURE 1
Flowchart describing the procedure of patient selection. DLBCL, diffuse large B-cell lymphoma; IHC, immunohistochemistry.

tissues according to standard protocols. The histological diagnosis and percentage of positive tumor cells were confirmed by two experienced pathologists. Germinal center B-cell or non–germinal center B-cell origin was classified by Hans algorithm. MYC/BCL2 DE lymphoma was defined as MYC expressed on ≥40% of tumor cells and BCL2 expressed on ≥50% of tumor cells (24).

DNA sequencing

Genomic DNA from frozen tumor tissues was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany); DNA from formalin-fixed paraffin-embedded tumor tissues was extracted using a GeneRead DNA Formalin-Fixed Paraffin-Embedded Tissue Kit (Qiagen). For whole genome sequencing (WGS), sequencing was performed with 150-bp paired-end strategy on an Illumina HiSeq platform in WuXi NextCODE, Shanghai. For whole exome sequencing (WES), exome regions were captured by a SeqCap EZ Human Exome Kit (version 3.0), and sequencing was performed with 150-bp paired-end strategy on a HiSeq 4000 platform in Righton, Shanghai. Burrows–Wheeler Aligner version 0.7.13-r1126 was applied to align read pairs to Human Reference Genome version hg19 (downloaded from UCSC Genome Browser, URLs). Samtools version 1.3 was used to remove PCR duplications and generate chromosomal coordinate-sorted bam files. Genome Analysis Toolkit version 3.4 Haplotype Caller and Genome Analysis Toolkit Unified Genotyper were applied to call single-nucleotide variations (SNVs) and indels. Annotation of SNVs and indels was applied using the UCSC Genome Browser (http://genome.ucsc.edu). The filter of SNVs and indels was based on the following pipelines: (1) germline mutations detected in paired blood samples were excluded, (2) mutations with low frequency (<0.05) were excluded, and (3) mutations included in the single-nucleotide polymorphism database and not reported in COSMIC (the Catalogue of Somatic Mutations in Cancer) version v77 were excluded.

For targeted sequencing, PCR primers were designed by Primer 5.0 software. Multiplexed libraries of tagged amplicons from tumor tissue samples were generated by Shanghai Righton Bio-Pharmaceutical Multiplex–PCR Amplification System.

All data of DNA sequencing including targeted sequencing (n = 178), WES (n = 119), and WGS (n = 58) were collected from our previous report (14).

RNA sequencing

RNA was extracted from frozen tumor tissue samples with a Trizol and RNeasy Mini Kit (Qiagen) and quantified with NanoDrop. Details of RNA sequencing procedures and RNA sequencing data of 208 patients were all cited from our previous report (14). The raw reads were aligned to human reference genome hg19 with a Burrows-Wheeler Aligner (v0.7.13-r1126). Transcript counts table files were generated with HTSeq.

Normalization of raw reads and analysis of differentially expressed genes were carried out using R package "limma" (v3.48.1) (25). Gene set enrichment analysis (GSEA) was performed with R package "clusterProfiler" (v4.0.0) based on MSigDB-curated gene sets (c2.cp.keggADD.v7.0.symbols.gmt and c5.bp.v7.0.symbols.gmt) (26). Metabolic signature scores were calculated using single-sample GSEA through R package "GSVA" (1.40.1); gene sets of lipid metabolism were acquired from the KEGG database. A CIBERSORT deconvolution algorithm (v 1.03) was applied in the calculation of immune cell populations based on its reference list. A total of 22 immune cell phenotypes were included in the analysis according to the signatures of bulk RNA-sequencing profile.

Cells and reagents

The B-lymphoma cell line OCI-LY-10 was provided by Huang CX. DB and THP-1 cells were purchased from the American Type Culture Collection. PBMCs from a healthy donor were obtained *via* Ficoll–Paque density gradient centrifugation. Cells were cultivated in IMDM or RPMI 1640, with the addition of 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (15140122, Gibco, Carlsbad, CA, USA) under a humidified atmosphere containing 95% air–5% CO_2 at 37 °C. Metformin was obtained from Selleck (Houston, TX, USA). Sulfosuccinimidyl oleate (SSO) was obtained from MedChemExpress (Shanghai, China).

Cell transfection

CD5 overexpression cells or negative control cells were constructed using lentiviral transfection. A purified plasmid MSCV-IRES-EGFP-CD5 (GV358) retroviral vector or control vector was transfected into package HEK-293T cells. Lentiviral particles were collected from the supernatant of HEK-293T cells and condensed to a viral concentration of ~2×10^8 transducing units/ml. Then, LY-10 cells or DB cells were cultivated, with the addition of lentiviral particles for 72 h in IMDM or RPMI 1640 containing polybrene (8 μg/ml). Flow cytometry was applied to select stably transduced clones expressing EGFP fluorescence protein.

Flow cytometry

PBMCs were cocultured with CD5 overexpressed OCI-LY-10 or vector OCI-LY-10 in IMDM and CD5 overexpressed DB in RPMI 1640 for 72 h. The following anti-human antibodies were used: APC anti-CD14 (555397, BD Biosciences), PE-CY7 anti-CD68 (565595, BD Biosciences), PE anti-CD163 (556018, BD Biosciences), and Percp-Cy5.5 anti-CD36 (336224, Biolegend). Flow cytometry was applied on a FACS Calibur cytometer (BD Biosciences) and analyzed by FlowJo software (v10.4).

Generation of M2 macrophages from THP-1 cell line

To induce M2 macrophages, 320 nM phorbol myristate acetate was added to THP-1 cells for 6 h, followed by phorbol myristate acetate plus 20 ng/ml of IL-4 and IL-13 for 18 h (27).

Oil red O staining

Oil red O staining was applied using a modified Lipid Staining Kit (C0158S, Beyotime) following its staining protocol. Cells were fixed with 10% formalin for 10 min and washed with phosphate-buffered saline twice. Staining wash buffer was added for 20 s and then removed. Then, cells were stained with oil red O solution for 20–30 min, and then the stain solution was removed. Oil red O staining was observed under a microscope.

Quantitative real-time PCR

Total RNA was extracted from M2 cells using a Trizol reagent and reverse-transcribed into cDNA using a HiScript III RT SuperMix for qPCR with a gDNA wiper (R323-01, Vazyme). Quantitative real-time PCR was performed with a ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme) using ABI ViiA7 (Applied Biosystems, Bedford). Forward primer of CD36: 5′-GGCTGTGACCGGAACTGTG-3′; reverse primer: 5′-AGGTCTCCAACTGGCATTAGAA-3′; forward primer of FASN: 5′-CCGAGACACTCGTGGGCTA-3′; reverse primer: 5′-CTTCAGCAGGACATTGATGCC′; forward primer of FABP5: 5′-TGAAGGAGCTAGGA GTGGGAA-3′; reverse primer: 5′-TGCACCATCTGTAAAG TTGCAG′. Relative expression was calculated and described using $^{2-\Delta\Delta}$CT.

Statistical analysis

The clinical characteristics of patients were assessed using Pearson's χ^2 test or Fisher's exact test. Differences of immune cell populations and normalized gene expression in two groups were carried out using unpaired *t*-test or Mann–Whitney *U* test. Progression-free survival (PFS) was determined as the time from the date of diagnosis to the date when the disease progression of relapse was recognized or the date of last follow-up. Overall survival (OS) was determined as the time from the date of

diagnosis to the date of death or the date of last follow-up. Survival functions were analyzed using the Kaplan–Meier method and compared by the log-rank test. Statistical significance was defined as $p < 0.05$. All statistical analyses were performed using R software (version 4.1.0; http://www.R-project.org) and Statistical Package for the Social Sciences (SPSS, 25.0) software (SPSS, Inc., Chicago, IL, USA).

Results

Clinical characteristics and survival

The median follow-up of 658 patients was 41.3 months, and the 5-year PFS and the 5-year OS were 70.6% and 80.0%, respectively. However, CD5+ DLBCL patients had poor 5-year PFS (54.2% vs. 72.4%, p = 0.003; Figure 2A), but without statistical difference in the 5-year OS (71.0% vs. 80.9%, p = 0.077; Figure 2B) when compared with CD5− DLBCL patients. Among the DE patients, no significant difference was observed between CD5+ and CD5− patients in terms of PFS (5-year PFS 40.0% vs. 59.3%, p = 0.110; Figure 2C) and OS (5-year OS 65.6% vs. 65.9%, p = 0.879; Figure 2D). Nevertheless, CD5+ non-DE patients had poor outcome, as compared with CD5− non-DE patients: 5-year PFS 58.8% vs. 76.3%, p = 0.016 (Figure 2E) and 5-year OS 72.9% vs. 84.7%, p = 0.037 (Figure 2F).

Based on these findings that CD5+ had prognostic indications in non-DE DLBCL, further clinical, genetic, and microenvironment profiles were analyzed in non-DE patients. Among non-DE, CD5+ patients had advanced Ann Arbor stage (III–IV) (p = 0.021), high-risk IPI (p = 0.010), more frequent bone marrow involvement (p = 0.018), and high CNS relapse (p = 0.040; Table 1).

Mutation patterns

Oncogenic mutations related to DLBCL progression were detected in WES, WGS, and targeted sequencing. Mutation patterns in non-DE patients were displayed in Figure 3A. The mutation frequencies of MYD88 (28.1% vs. 13.0%, p = 0.020), FOXO1 (18.8% vs. 6.5%, p = 0.013), and TMSB4X (15.6% vs. 4.6%, p = 0.010) were significantly higher in CD5+ patients than in CD5− patients.

Gene expression analysis and tumor microenvironment analysis

Gene ontology analysis revealed the activation of multiple pathways, including the MYD88-dependent Toll-like receptor pathway, monocyte- and macrophage-related pathways (monocyte chemotaxis, macrophage migration, macrophage activation, positive regulation of response to cytokine stimulus, regulation of myeloid cell differentiation, tumor necrosis factor superfamily cytokine production, and CCR chemokine receptor binding), and pathways related to inhibit immune responses (negative regulation of immune system process, TGF-β receptor signaling pathway), in CD5+ non-DE patients (Figures 3B,C). Following analysis of an immune cell proportion in TME was carried out through CIBERSORT. As displayed in Figure 3D, an elevated proportion of monocytes (p = 0.019), M2 macrophage ($p < 0.001$), and a decreased proportion of NK cells were observed ($p < 0.001$) in CD5+ non-DE when compared with CD5− non-DE patients. Meanwhile, the normalized expression level of HAVCR2 (also called TIM3) was significantly increased in CD5+ than CD5− non-DE patients (p = 0.018) (Figure 3E). Moreover, the normalized expression levels of HAVCR2 (p = 0.033) and TGFBI ($p < 0.001$) were positively correlated with the M2 proportion in TME, and the normalized expression of HAVCR2 was positively correlated with TGFBI ($p < 0.001$) (Figure 3F).

Variation in lipid metabolism in RNA sequencing

Regarding the variation in metabolic pathways between CD5+ and CD5− non-DE DLBCL, as revealed by GSEA, upregulation of lipid metabolic pathways, including the PPAR signaling pathway, the ATP-binding cassette transporter pathway, ether lipid metabolism, the fatty acid metabolism pathway, and arachidonic acid metabolism, was observed in CD5+ patients. However, the metabolic pathway enriched in CD5− patients included pyrimidine metabolism, porphyrin and chlorophyll metabolism, pentose and glucuronate interconversions, and glyoxylate and dicarboxylate metabolism (Figure 4A). Further comparing the correlation between metabolic pathways and immune cell proportion, all these upregulated metabolism pathways in CD5+ non-DE patients were positively correlated with the M2 proportion (Figure 4B). Among differentially expressed genes, the normalized expression level of CD36 was significantly higher in CD5+ than CD5− non-DE patients (p = 0.035; Figure 4C) and positively correlated with the M2 proportion in TME ($p < 0.001$; Figure 4D). Additionally, the expression level of CD36 was also positively correlated with the expressions of multiple chemokines and chemokine receptors including CCL16, CCL18, CCL2, CCL8, CCR2, CXCR1, and CXCR2 (Figure 4E).

Metformin attenuated CD36 expression and lipid metabolism

After PBMC cocultured with CD5-overexpressed (LY-10/CD5) or vector-transfected (LY-10/vector) cells at the ratio of 5:1

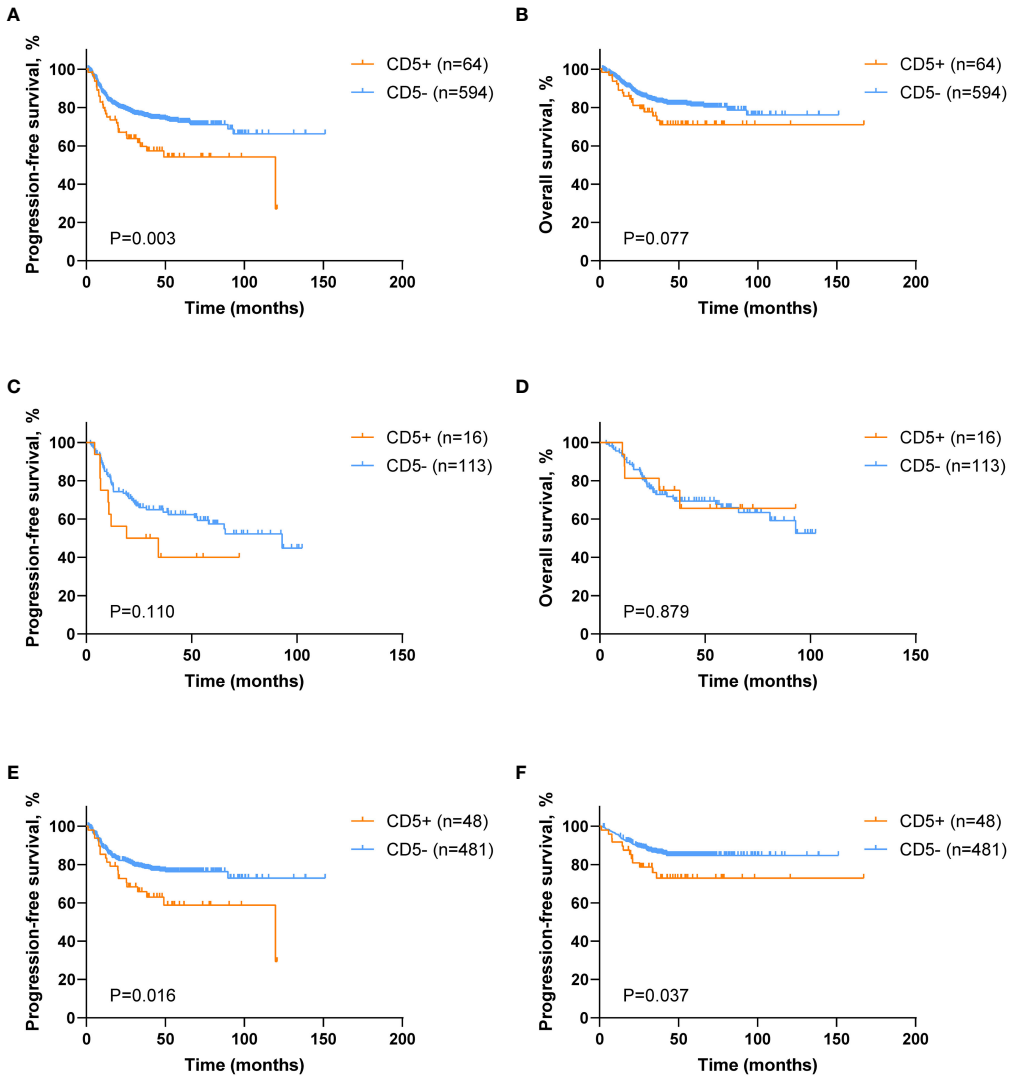

FIGURE 2
Survival analysis of DLBCL patients according to CD5 positivity. **(A, B)** Kaplan–Meier survival curves of progression-free survival (PFS) and overall survival (OS) comparing CD5+ (n = 64) and CD5– (n = 594) DLBCL patients. **(C, D)** Kaplan–Meier survival curves of PFS and OS in CD5+ (n = 16) and CD5– (n = 113) double expressor (DE) DLBCL patients. **(E, F)** Kaplan–Meier survival curves of PFS and OS in CD5+ (n = 48) and CD5– (n = 481) non–double expressor (non-DE) DLBCL patients.

for 72 h, flow cytometry was applied to calculate the proportion of M2 and CD36 expression on M2. As shown in Figure 5A, the coculture with LY-10/CD5 or LY-10/vector increased the M2 proportion (13.0% ± 3.3%, p = 0.004 or 6.9% ± 1.7%, p = 0.007) in PBMC as compared with PBMC alone (1.4% ± 0.9%). Of note, the M2 proportion was significantly high in the LY-10/CD5 coculture system than in the LY-10/vector coculture system (p = 0.047; Figure 5A). Metformin treatment (200 μM) significantly decreased the proportion of M2 in both coculture systems, from 13.0% ± 3.3% to 4.8% ± 1.4% (p = 0.017) in the LY-10/CD5 coculture system and from 6.9% ± 1.7% to 3.6% ± 0.9% (p = 0.039) in the LY-10/vector coculture system (Figure 5B). Further exploring the efficacy of metformin on the expression of CD36, flow cytometry analysis revealed that, after treatment with metformin, the CD36 expression level on M2 was significantly downregulated in the LY-10/CD5 coculture system (p = 0.025) than the LY-10/vector coculture system (p = 0.052; Figure 5C). PBMCs cocultured with DB/CD5 cells were also treated with 200 μM metformin for 72 h, M2 cells decreased from 10.8% ± 2.1% to 6.3% ± 1.5% (p = 0.043), and CD36 expression on M2 cells also decreased significantly (p = 0.018) (Supplementary Figure S1). M2 cells induced from THP-1 cells were treated with 200 μM metformin for 72 h. The mRNA expression levels of CD36, FABP5, and FASN were significantly decreased after metformin

TABLE 1 Clinical characteristics of CD5+ and CD5− non-DE DLBCL patients. .

Characteristic	CD5+ (n = 48)	2.1.1.1 CD5− (n = 481)	p
Median age, years (range)	57 (25–90)	58 (16–90)	
Gender			0.079
Men	32 (66.7%)	257 (53.4%)	
Women	16 (33.3%)	224 (46.6%)	
Ann Arbor, n (%)			0.021
I–II	21 (43.7%)	293 (60.9%)	
III–IV	27 (56.3%)	188 (39.1%)	
LDH, n (%)			0.241
> Normal	26 (54.2%)	218 (45.3%)	
≤ Normal	22 (45.8%)	263 (54.7%)	
ECOG, n (%)			0.330
< 2	43 (89.6%)	449 (93.3%)	
≧ 2	5 (10.4%)	32 (6.7%)	
IPI, n (%)			0.010
0–2	27 (56.2%)	355 (73.8%)	
3–5	21 (43.8%)	126 (26.2%)	
Age > 60 years, n (%)	19 (39.6%)	202 (42%)	0.747
Extranodal sites, n (%)			0.231
≧ 2	15 (31.3%)	113 (23.5%)	
< 2	33 (68.7%)	368 (76.5%)	
Cell of origin			0.443
Non-GCB	30 (62.5%)	373 (56.8%)	
GCB	18 (37.5%)	208 (43.2%)	
Response			0.413
CR	38 (79.2%)	403 (83.8%)	
Non-CR	10 (20.8%)	78 (16.2%)	
Bone marrow involvement, n (%)	8 (16.7%)	31 (6.4%)	0.018
CNS relapse, n (%)	3 (6.3%)	6 (1.3%)	0.040

DLBCL, diffuse large B-cell lymphoma; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; IPI, international prognostic index; GCB, germinal center B-cell; CR, complete remission; CNS, central nervous system.

treatment (p = 0.019, p = 0.021, and p = 0.037, respectively) (Figure 5D). Also, oil red O staining showed reduced lipid droplets accumulation after metformin treatment (Figure 5E).

Metformin decreased immune checkpoint expression

In the LY10/CD5 and PBMC coculture system, after the addition of metformin (200 μM) for 72 h, the expressions of PD-1 on both CD4 T cells (from 7.0% ± 2.6% to 2.8% ± 0.4%, p = 0.048) and CD8 T cells (from 8.8% ± 2.6% to 4.4% ± 0.4%, p = 0.044) decreased significantly. Meanwhile, TIM3 expression decreased from 5.4% ± 2.6% to 0.8% ± 0.5% on CD8 T cells (p = 0.038) and decreased from 5.0% ± 2.3% to 1.1% ± 0.5% (p = 0.046) on CD4 T cells (Figure 5F).

Consistent with the *in vitro* results that metformin targeted immunosuppressive TME, CD5+ non-DE patients with metformin maintenance tended to have longer PFS and OS than those without metformin maintenance (5-year PFS, 87.5% vs. 50.3%, p = 0.096, and 5-year OS, 87.5% vs. 69.3%, p = 0.329; Figure 5G).

CD36 inhibitor interfered lipid metabolism and immune checkpoint expression

To further explore the impact of CD36 inhibition on lipid metabolism in the coculture system, irreversible CD36 inhibitor SSO (100 μM) was applied in PBMC cocultured with LY-10/CD5 or LY-10/vector for 72 h; flow cytometry was used for the quantification of the M2 proportion and surface CD36 proportion. After SSO treatment, M2 proportion was significantly decreased from 13.0% ± 3.3% to 4.4% ± 0.5% (p = 0.012) in the LY-10/CD5 coculture system and decreased

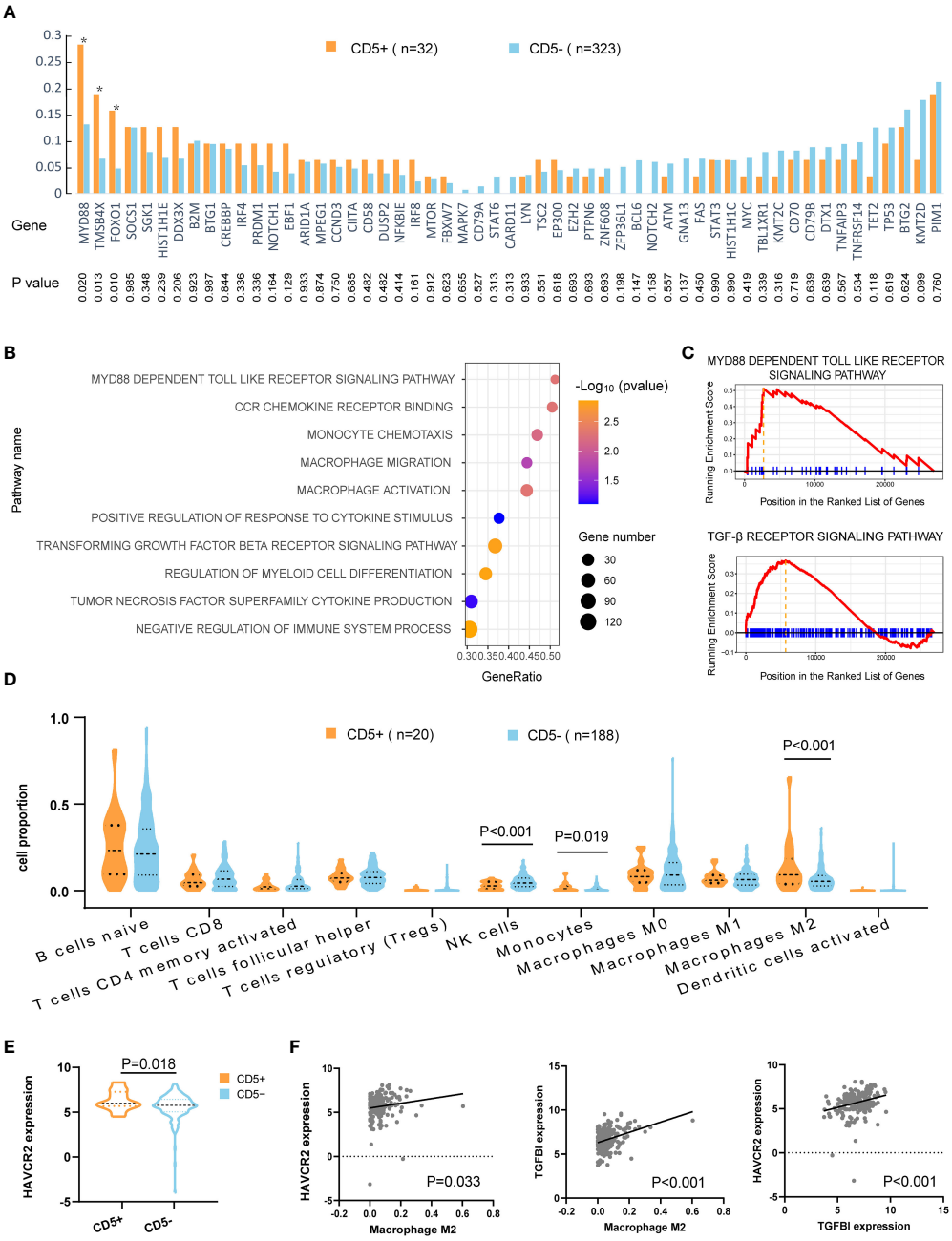

FIGURE 3
Mutation patterns and tumor microenvironment of CD5+ non-DE patients. **(A)** Genetic mutations of 55 genes in CD5+ (n = 32) and CD5− (n = 323) non-DE patients. P values are listed in the lower part of the graph. **(B)** Upregulated gene ontology (GO) pathways in CD5+ non-DE (n = 20) compared with CD5− non-DE (n = 188) patients. The color gradient represents $-\log_{10}$ (p value) of GO pathways, the point size indicates the gene numbers involved in each gene set, and the position of the *x*-axis indicates the gene ratio of genes enriched in each gene set. **(C)** Enrichment plot of MYD88-dependent Toll-like receptor signaling pathway and TGF-β receptor signaling pathway. **(D)** Proportion of immune cells in the tumor microenvironment of CD5+ non-DE (n = 20) and CD5− non-DE (n = 188) patients. **(E)** Normalized expression of HAVCR2 (TIM3) comparing CD5+ non-DE (n = 20) and CD5− non-DE (n = 188) patients. **(F)** Correlation between M2 macrophage proportion and normalized expression of HAVCR2 or TGFBI, and correlation between normalized expression of HAVCR2 and TGFBI. P values were listed in each plot.

from 6.9% ± 1.7% to 3.8% ± 0.4% (p = 0.035) in the LY-10/vector coculture system (Figure 6A), as well as the reduction of surface CD36 proportion on M2 (p < 0.001) (Figure 6B). However, SSO treatment did not affect the mRNA expression level of CD36 but significantly decreased the mRNA level of FABP5 (p = 0.014) and FASN (p = 0.002) in M2 (Figure 6C). Consequently, the accumulation of lipid droplets was significantly inhibited in M2 upon SSO treatment (Figure 6D).

Comparable with the results of metformin treatment, blockage of CD36 with SSO in the coculture system (LY10/CD5 and PBMC) significantly decreased the immune checkpoint expression on CD4 T cells (PD-1: from 7.0% ± 2.6% to 0.7% ± 0.4%, p = 0.013; TIM3: from 5.0% ± 2.3% to 0.4% ± 0.2%, p = 0.026) and CD8 T cells (PD-1: from 8.8% ± 2.6% to 1.3% ± 0.7%, p = 0.009; TIM3: from 5.4% ± 2.6% to 0.8% ± 0.6%, p = 0.037) (Figure 6E).

Discussion

CD5+ DLBCL accounts for approximately 10% of DLBCL, which is commonly reported with inferior prognosis and higher relapse probability of CNS. Our study revealed that CD5 positivity conferred inferior outcome in non-DE patients, but not in DE patients, indicating that CD5+ should be paid more attention among non-DE patients.

In our cohort, MYD88 was the most frequently mutated in CD5+ non-DE DLBCL, consistent with the previous results that MYD88 mutation is supposed to be a genetic feature of CD5+ DLBCL (28, 29). MYD88 is an adaptor protein, receives stimulation from Toll-like and interleukin receptors, and drives the activation of the nuclear factor-κB pathway (30). Interestingly, it has recently been demonstrated that DLBCL with mutant MYD88 exhibited with a macrophage-activating

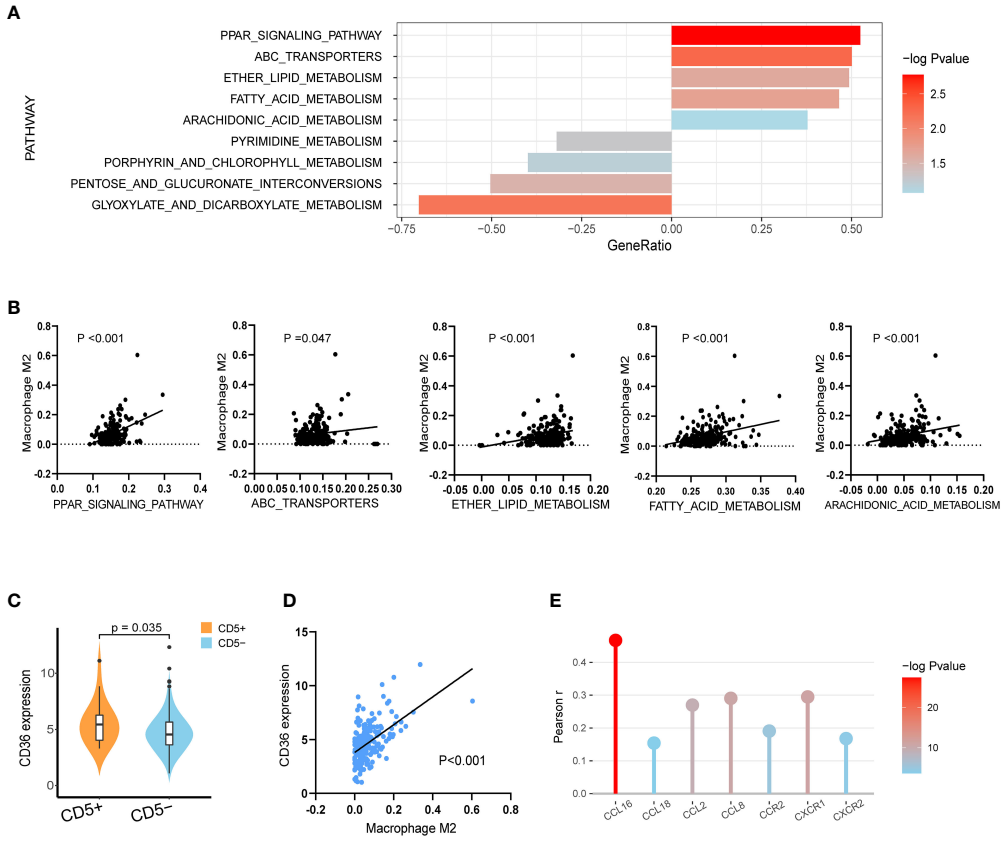

FIGURE 4
Transcriptional analysis of lipid metabolism alterations in CD5+ non-DE patients. **(A)** The most variable metabolic process upregulated in CD5+ non-DE (n = 20) patients (gene ratio above 0.00) or CD5− non-DE (n = 188) patients (gene ratio below 0.00) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. **(B)** Correlation between M2 proportions and signature scores of PPAR signaling pathway, ATP-binding cassette transporters pathway, ether lipid metabolism, fatty acid metabolism pathway, and arachidonic acid metabolism pathway in non-DE patients (n = 208). **(C)** Comparison of normalized CD36 expression between CD5+ non-DE (n = 20) and CD5− non-DE (n = 188) patients. **(D)** Correlation between M2 proportion and normalized CD36 expression. P value was labeled in the graph. **(E)** Correlation between normalized expression of CD36 and expression of chemokines or chemokine receptors including CCL16, CCL18, CCL2, CCL8, CCR2, CXCR1, and CXCR2. The color gradient represents $-\log_{10}$ (p value), and the *y*-axis represents Pearson *r*.

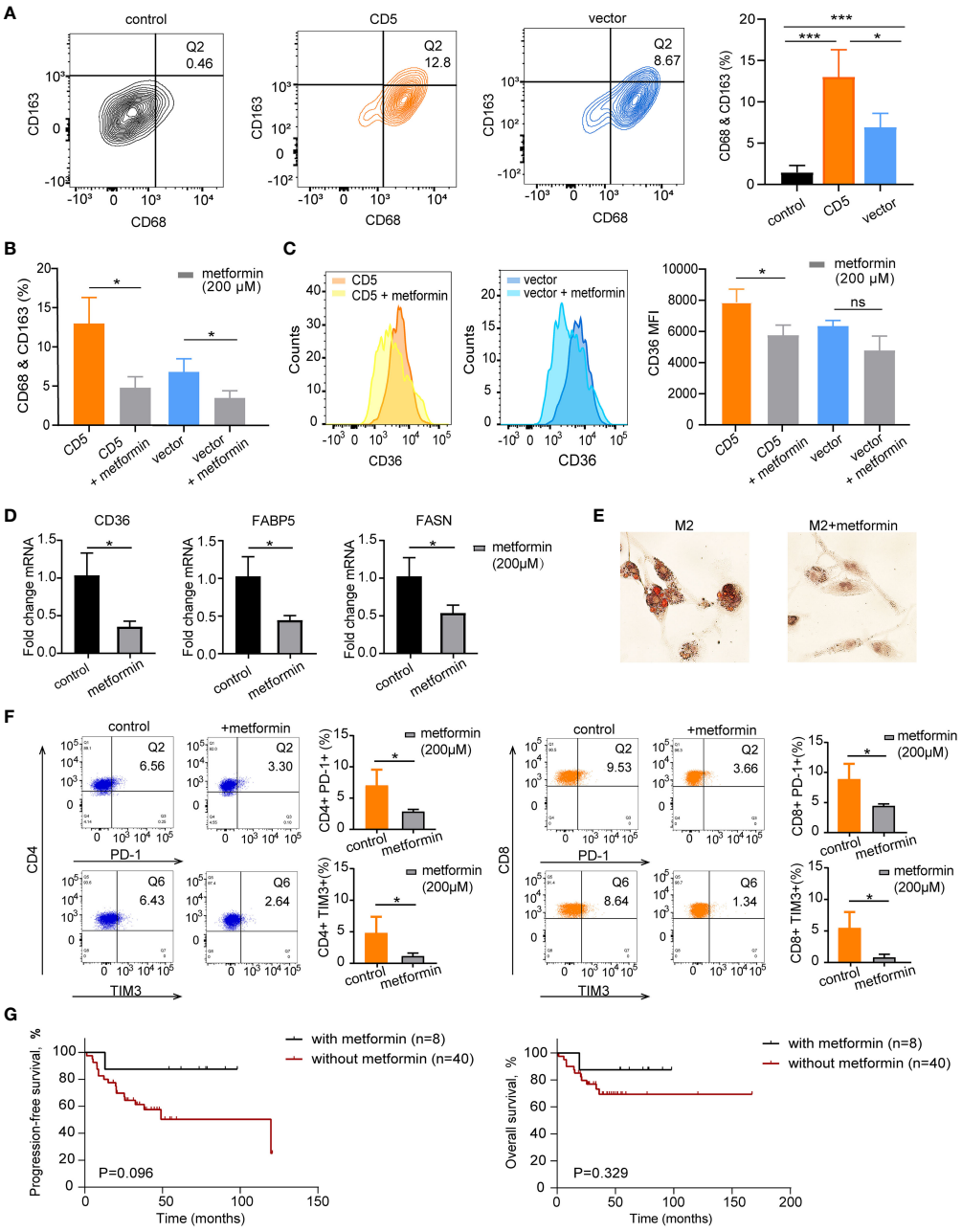

FIGURE 5
The therapeutic impacts of metformin on M2 lipid metabolism and CD36 expression. **(A)** Flow cytometry analysis of M2 macrophages (Q2) proportion based on co-expression of CD68 and CD163 in PBMC control, coculture of PBMC and LY-10/CD5, and coculture of PBMC and LY-10/vector for 72 h **(B)** Bar plot of M2 proportion in PBMC cocultured with LY-10/CD5 or LY-10/vector with or without 200 μM metformin treatment for 72 h, summarized as mean ± SD (n = 3). **(C)** Flow cytometry analysis of CD36 expression on M2 macrophages comparing the counts and median fluorescence intensity (MFI) of CD36 on M2 in PBMC cocultured with LY-10/CD5 or LY-10/vector with or without 200 μM metformin treatment. **(D)** The normalized mRNA expression of CD36, FABP5, and FASN in THP-1–induced M2 cells with or without 200 μM metformin treatment. **(E)** Oil red O staining of lipid droplets in THP-1–induced M2 cells with or without 200 μM metformin treatment. **(F)** Expression of immune checkpoint PD-1 and TIM3 on CD4 and CD8 T cells after LY-10/CD5 cocultured with PBMC with or without 200 μM metformin treatment for 72 h **(G)** Kaplan–Meier survival curves of PFS and OS in CD5+ non-DE patients with (n = 8) or without (n = 40) metformin maintenance. * represented significant difference of P<0.05.

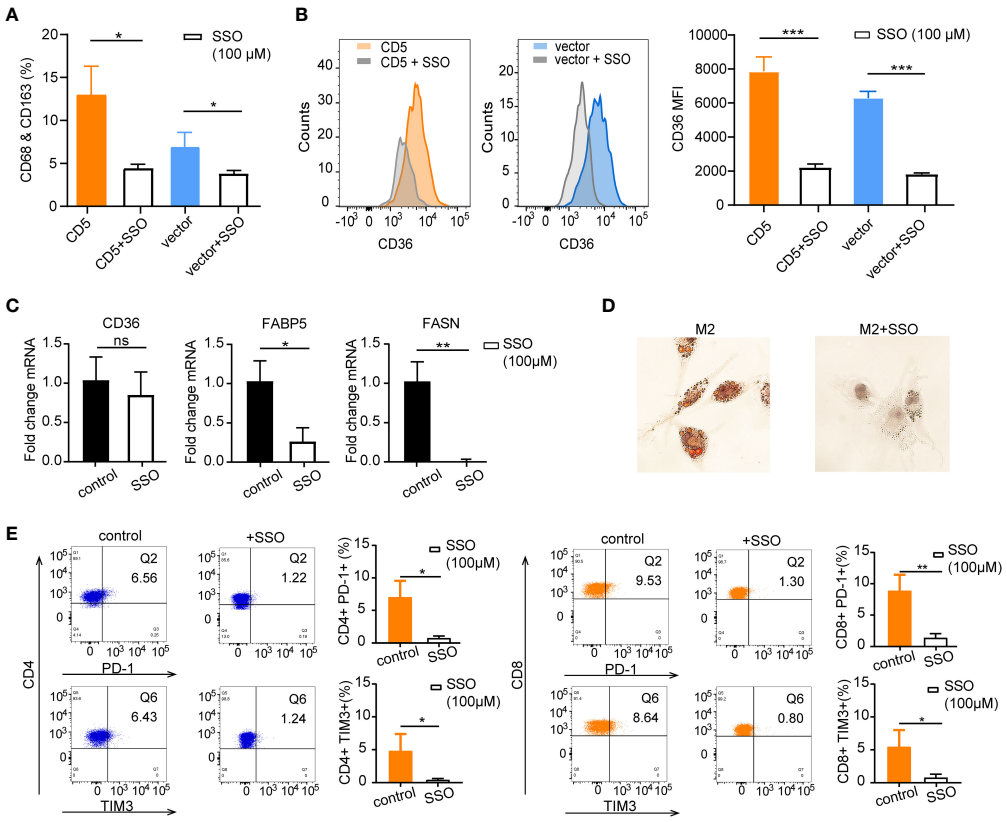

FIGURE 6
The therapeutic impacts of sulfosuccinimidyl oleate (SSO) on M2 lipid metabolism and CD36 expression. **(A)** Bar plot of M2 proportion in PBMC cocultured with LY-10/CD5 or LY-10/vector with or without 100 μM SSO treatment for 72 h, summarized as mean ± SD (n = 3). **(B)** Flow cytometry analysis of CD36 expression on M2 macrophages comparing the counts and median fluorescence intensity (MFI) of CD36 on M2 in PBMC cocultured with LY-10/CD5 or LY-10/vector with or without 100 μM SSO treatment. **(C)** The normalized mRNA expression of CD36, FABP5, and FASN in THP-1–induced M2 cells with or without 100 μM SSO treatment. **(D)** Oil red O staining of lipid droplets in THP-1–induced M2 cells with or without 100 μM SSO treatment. **(E)** Expression of immune checkpoint PD-1 and TIM3 on CD4 and CD8 T cells after LY-10/CD5 cocultured with PBMC with or without 100 μM metformin treatment for 72 h. * represented significant difference of P<0.05; ** represented significant difference of P<0.01; *** represented significant difference of P<0.001. ns, no significance

secretion profile through exhibiting elevated CCL2 expression, followed by an increase in macrophage-derived TGF-β1 secretion (31). Meanwhile, higher frequencies of FOXO1 mutation and TMSB4X mutation were also detected in CD5+ non-DE DBLCL. FOXO1 mutation is an unfavorable prognostic factor in DLBCL (32). TMSB4X is regulated by TGF-β and facilitated tumor metastasis in melanoma (33).

Comparable to MYD88 mutation, according to RNA sequencing data, significant upregulation of macrophage activation pathways and infiltration of M2 macrophages were presented in CD5+ non-DE DLBCL, indicating the crucial role of immunosuppressive cells in this subgroup. TGF-β–induced protein (TGFBI) is predominantly secreted by CD163-positive macrophages, which contributes to the suppressive TME (34). In concordance with the increased proportion of M2 in non-DE CD5+ DLBCL, the expression of immune checkpoint TIM3 (HAVCR2) was positively correlated with the proportion of M2 in our study. TIM3 expression on TAMs is conditionally fostered by TGF-β and enhances macrophage activation to promote tumor progression (35). On the other hand, the fraction of NK cells was lower in the TME of CD5+ non-DE DLBCL, which also supports the immunosuppressive TME condition.

Moreover, comparable with the activation and infiltration of macrophages in TME, the activation of macrophage-related metabolism pathways such as the fatty acid metabolism pathway (36) and the PPAR signaling pathway (37) and the regulation of the lipid metabolic pathway were significantly increased in CD5+ non-DE DLBCL. Fatty acid uptake is initialized through CD36 internalization and contributes to M2 polarization (19). Fatty acid metabolism not only supports energy for M2 macrophages (38) but also impairs the antitumor function of NK cells (39) and CD8+ T cells (40). In our cohort of patients, the expression of CD36 was significantly elevated in

CD5+ non-DE lymphoma than its CD5− counterpart, consistent with the previous findings that CD36 is one of the top variant genes that distinguished CD5+ from CD5− DLBCL (41, 42).

Metformin, a traditional medicine for the regulation of glucose metabolism, has additional functions on lipid metabolisms. Lipid biogenesis can be downregulated by metformin treatment, which targets sterol regulatory element-binding protein-1c in fatty acid synthesis (43). The expression of scavenger receptor CD36 can also be attenuated by metformin and thus reduces triglyceride contents in cells (44, 45). As displayed in our results, metformin significantly decreased the M2 proportion and inhibited the expression of CD36, FABP5, and FASN, leading to the reduction of lipid droplets in M2, which indicated that lipid metabolism in M2 cells was interfered by metformin. Interestingly, immune checkpoints (PD-1 and TIM3) expression on T cells was downregulated, suggesting that metformin treatment might overcome the immunosuppressive TME in CD5+ non-DE DLBCL. Metformin maintenance therapy did not achieve significantly higher PFS in CD5+ non-DE DLBCL patients; this might be due to the limited number of patients and the short duration of follow-up.

Taken together, in non-DE DLBCL, CD5+ lymphoma represented an increased proportion of M2 and immunosuppressive TME through reprogrammed lipid metabolism and conferred poor prognosis of patients upon R-CHOP treatment. Metformin could target dysregulated fatty acid metabolism through inhibiting CD36 expression on M2 and M2 polarization, rendering the clinical rationale of metformin treatment in CD5+ non-DE DLBCL.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.biosino.org/node/project/detail/OEP001143.

Ethics statement

The studies involving human participants were reviewed and approved by Shanghai Ruijin Hospital Ethics Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

W-LZ, LW, P-PX, and SC designed and supervised the study. M-KL, H-MY, YH, and YQ collected clinical data and carried out statistical analysis. M-KL, L-LC, and DF performed biological experiments and made the figures. Y-TD, HF, YF, and QL provided technical support for bioinformatic analysis, and W-LZ, LW, and M-KL drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.885011/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The therapeutic impacts of metformin and SSO on M2 proportion and CD36 expression in DB cell lines. **(A)** Flow cytometry analysis of M2

macrophages (Q2) proportion based on co-expression of CD68 and CD163 in PBMCs cocultured with DB/CD5, with or without metformin/SSO treatment for 72 h. Bar plot of M2 proportion in PBMC cocultured with DB/CD5 with or without metformin/SSO treatment for 72 h, summarized as mean ± SD (n = 3). **(B)** Flow cytometry analysis of CD36 expression on M2 macrophages comparing the counts and median fluorescence intensity (MFI) of CD36 on M2 in PBMC cocultured with DB/CD5 with or without metformin/SSO treatment.

References

1. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the world health organization classification of lymphoid neoplasms. *Blood* (2016) 127(20):2375–90. doi: 10.1182/blood-2016-01-643569

2. Coiffier B, Thieblemont C, Van Den Neste E, Lepeu G, Plantier I, Castaigne S, et al. Long-term outcome of patients in the lnh-98.5 trial, the first randomized study comparing rituximab-chop to standard chop chemotherapy in dlbcl patients: A study by the groupe d'etudes des lymphomes de l'adulte. *Blood* (2010) 116 (12):2040–5. doi: 10.1182/blood-2010-03-276246

3. Ennishi D, Takeuchi K, Yokoyama M, Asai H, Mishima Y, Terui Y, et al. Cd5 expression is potentially predictive of poor outcome among biomarkers in patients with diffuse Large b-cell lymphoma receiving rituximab plus chop therapy. *Ann Oncol* (2008) 19(11):1921–6. doi: 10.1093/annonc/mdn392

4. Yamaguchi M, Nakamura N, Suzuki R, Kagami Y, Okamoto M, Ichinohasama R, et al. *De novo* Cd5+ diffuse Large b-cell lymphoma: Results of a detailed clinicopathological review in 120 patients. *Haematologica* (2008) 93 (8):1195–202. doi: 10.3324/haematol.12810

5. Chuang WY, Chang H, Shih LY, Wang PN, Chang YS, Lin TL, et al. Cd5 positivity is an independent adverse prognostic factor in elderly patients with diffuse Large b cell lymphoma. *Virchows Arch* (2015) 467(5):571–82. doi: 10.1007/s00428-015-1845-1

6. Miyazaki K, Yamaguchi M, Suzuki R, Kobayashi Y, Maeshima AM, Niitsu N, et al. Cd5-positive diffuse Large b-cell lymphoma: A retrospective study in 337 patients treated by chemotherapy with or without rituximab. *Ann Oncol* (2011) 22 (7):1601–7. doi: 10.1093/annonc/mdq627

7. Alinari L, Gru A, Quinion C, Huang Y, Lozanski A, Lozanski G, et al. *De novo* Cd5+ diffuse Large b-cell lymphoma: Adverse outcomes with and without stem cell transplantation in a Large, multicenter, rituximab treated cohort. *Am J Hematol* (2016) 91(4):395–9. doi: 10.1002/ajh.24299

8. Huang H, Li Z, Huang C, Rao J, Xie Q, Cui W, et al. Cd5 and Cd43 expression are associate with poor prognosis in dlbcl patients. *Open Med (Wars)* (2018) 13:605–9. doi: 10.1515/med-2018-0089

9. Salles G, de Jong D, Xie W, Rosenwald A, Chhanabhai M, Gaulard P, et al. Prognostic significance of immunohistochemical biomarkers in diffuse Large b-cell lymphoma: A study from the lunenburg lymphoma biomarker consortium. *Blood* (2011) 117(26):7070–8. doi: 10.1182/blood-2011-04-345256

10. Shen Z, Wang L, Zhang B, Li T, Li D, He C, et al. Development and validation of a novel prognostic nomogram for Cd5-positive diffuse Large b-cell lymphoma: A retrospective multicenter study in China. *Front Oncol* (2021) 11:754180. doi: 10.3389/fonc.2021.754180

11. Kotlov N, Bagaev A, Revuelta MV, Phillip JM, Cacciapuoti MT, Antysheva Z, et al. Clinical and biological subtypes of b-cell lymphoma revealed by microenvironmental signatures. *Cancer Discovery* (2021) 11(6):1468–89. doi: 10.1158/2159-8290.CD-20-0839

12. Autio M, Leivonen SK, Bruck O, Karjalainen-Lindsberg ML, Pellinen T, Leppa S. Clinical impact of immune cells and their spatial interactions in diffuse Large b-cell lymphoma microenvironment. *Clin Cancer Res* (2021) 28(4):781–92. doi: 10.1158/1078-0432.CCR-21-3140

13. Huang YH, Cai K, Xu PP, Wang L, Huang CX, Fang Y, et al. Crebbp/Ep300 mutations promoted tumor progression in diffuse Large b-cell lymphoma through altering tumor-associated macrophage polarization *Via* Fbxw7-Notch-Ccl2/Csf1 axis. *Signal Transduct Target Ther* (2021) 6(1):10. doi: 10.1038/s41392-020-00437-8

14. Shen R, Xu PP, Wang N, Yi HM, Dong L, Fu D, et al. Influence of oncogenic mutations and tumor microenvironment alterations on extranodal invasion in diffuse Large b-cell lymphoma. *Clin Transl Med* (2020) 10(7):e221. doi: 10.1002/ctm2.221

15. Vitale I, Manic G, Coussens LM, Kroemer G, Galluzzi L. Macrophages and metabolism in the tumor microenvironment. *Cell Metab* (2019) 30(1):36–50. doi: 10.1016/j.cmet.2019.06.001

16. Bader JE, Voss K, Rathmell JC. Targeting metabolism to improve the tumor microenvironment for cancer immunotherapy. *Mol Cell* (2020) 78(6):1019–33. doi: 10.1016/j.molcel.2020.05.034

17. Leone RD, Powell JD. Metabolism of immune cells in cancer. *Nat Rev Cancer* (2020) 20(9):516–31. doi: 10.1038/s41568-020-0273-y

18. Jha AK, Huang SC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity* (2015) 42(3):419–30. doi: 10.1016/j.immuni.2015.02.005

19. Munir R, Lisec J, Swinnen JV, Zaidi N. Too complex to fail? targeting fatty acid metabolism for cancer therapy. *Prog Lipid Res* (2022) 85:101143. doi: 10.1016/j.plipres.2021.101143

20. Huang SC, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, et al. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol* (2014) 15(9):846–55. doi: 10.1038/ni.2956

21. Griffiths HR, Gao D, Pararasa C. Redox regulation in metabolic programming and inflammation. *Redox Biol* (2017) 12:50–7. doi: 10.1016/j.redox.2017.01.023

22. Rios FJ, Koga MM, Pecenin M, Ferracini M, Gidlund M, Jancar S. Oxidized ldl induces alternative macrophage phenotype through activation of Cd36 and pafr. *Mediators Inflamm* (2013) 2013:198193. doi: 10.1155/2013/198193

23. Fan X, Zhong H-J, Zhao B-B, Ou Yang B-S, Zhao Y, Ye J, et al. Metformin prolonged the survival of diffuse Large b-cell lymphoma and grade 3b follicular lymphoma patients responding to first-line treatment with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone: A prospective phase ii clinical trial. *Trans Cancer Res* (2018) 7(4):1044–53. doi: 10.21037/tcr.2018.07.20

24. Herrera AF, Mei M, Low L, Kim HT, Griffin GK, Song JY, et al. Relapsed or refractory double-expressor and double-hit lymphomas have inferior progression-free survival after autologous stem-cell transplantation. *J Clin Oncol* (2017) 35 (1):24–31. doi: 10.1200/JCO.2016.68.2740

25. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for rna-sequencing and microarray studies. *Nucleic Acids Res* (2015) 43(7):e47. doi: 10.1093/nar/gkv007

26. Yu G, Wang LG, Han Y, He QY. Clusterprofiler: An r package for comparing biological themes among gene clusters. *OMICS* (2012) 16(5):284–7. doi: 10.1089/omi.2011.0118

27. Tjiu JW, Chen JS, Shun CT, Lin SJ, Liao YH, Chu CY, et al. Tumor-associated macrophage-induced invasion and angiogenesis of human basal cell carcinoma cells by cyclooxygenase-2 induction. *J Invest Dermatol* (2009) 129 (4):1016–25. doi: 10.1038/jid.2008.310

28. Takeuchi T, Yamaguchi M, Kobayashi K, Miyazaki K, Tawara I, Imai H, et al. Myd88, Cd79b, and Card11 gene mutations in Cd5-positive diffuse Large b-cell lymphoma. *Cancer* (2017) 123(7):1166–73. doi: 10.1002/cncr.30404

29. Nishimura N, Takeuchi K, Asaka R, Tuyama N, Inoue N, Kusano Y, et al. Myd88 L265p mutation detected by digital pcr as a prognostic factor in patients with diffuse Large b-cell lymphoma in rituximab era. *Leuk Res* (2020) 97:106426. doi: 10.1016/j.leukres.2020.106426

30. Yu X, Li W, Deng Q, Liu H, Wang X, Hu H, et al. Myd88 L265p elicits mutation-specific ubiquitination to drive nf-kappab activation and lymphomagenesis. *Blood* (2021) 137(12):1615–27. doi: 10.1182/blood.2020004918

31. Reimann M, Schrezenmeier J, Richter-Pechanska P, Dolnik A, Hick TP, Schleich K, et al. Adaptive T-cell immunity controls senescence-prone Myd88- or Card11-mutant b-cell lymphomas. *Blood* (2021) 137(20):2785–99. doi: 10.1182/blood.2020005244

32. de Miranda NF, Georgiou K, Chen L, Wu C, Gao Z, Zaravinos A, et al. Exome sequencing reveals novel mutation targets in diffuse Large b-cell lymphomas derived from Chinese patients. *Blood* (2014) 124(16):2544–53. doi: 10.1182/blood-2013-12-546309

33. Morita T, Hayashi K. Tumor progression is mediated by thymosin-Beta4 through a Tgfbeta/Mrtf signaling axis. *Mol Cancer Res* (2018) 16(5):880–93. doi: 10.1158/1541-7786.MCR-17-0715

34. Lecker LSM, Berlato C, Maniati E, Delaine-Smith R, Pearce OMT, Heath O, et al. Tgfbi production by macrophages contributes to an immunosuppressive microenvironment in ovarian cancer. *Cancer Res* (2021) 81(22):5706–19. doi: 10.1158/0008-5472.CAN-21-0536

35. Yan W, Liu X, Ma H, Zhang H, Song X, Gao L, et al. Tim-3 fosters hcc development by enhancing tgf-Beta-Mediated alternative activation of macrophages. *Gut* (2015) 64(10):1593–604. doi: 10.1136/gutjnl-2014-307671

36. Nomura M, Liu J, Rovira II, Gonzalez-Hurtado E, Lee J, MJ W, et al. Fatty acid oxidation in macrophage polarization. *Nat Immunol* (2016) 17(3):216–7. doi: 10.1038/ni.3366

37. Chawla A. Control of macrophage activation and function by ppars. *Circ Res* (2010) 106(10):1559–69. doi: 10.1161/CIRCRESAHA.110.216523

38. Yan J, Horng T. Lipid metabolism in regulation of macrophage functions. *Trends Cell Biol* (2020) 30(12):979–89. doi: 10.1016/j.tcb.2020.09.006

39. Kobayashi T, Lam PY, Jiang H, Bednarska K, Gloury R, Murigneux V, et al. Increased lipid metabolism impairs nk cell function and mediates adaptation to the lymphoma environment. *Blood* (2020) 136(26):3004–17. doi: 10.1182/blood.2020005602

40. Xu S, Chaudhary O, Rodriguez-Morales P, Sun X, Chen D, Zappasodi R, et al. Uptake of oxidized lipids by the scavenger receptor Cd36 promotes lipid peroxidation and dysfunction in Cd8(+) T cells in tumors. *Immunity* (2021) 54(7):1561–77 e7. doi: 10.1016/j.immuni.2021.05.003

41. Ueno S, Yamaguchi M, Kimura M, Odagiri H, Shiraki K, Uemoto S, et al. Expression of Cd29 on lymphoma cells and/or Cd36 on microvascular endothels correlates with high serum ldh level in diffuse Large b-cell lymphomas (Dlbcls) and is frequent in *De novo* Cd5-positive dlbcls. *Int J Oncol* (2005) 27(5):1241–6.

42. Kobayashi T, Yamaguchi M, Kim S, Morikawa J, Ogawa S, Ueno S, et al. Microarray reveals differences in both tumors and vascular specific gene expression in *De novo* Cd5+ and Cd5- diffuse Large b-cell lymphomas. *Cancer Res* (2003) 63(1):60–6.

43. van Stee MF, de Graaf AA, Groen AK. Actions of metformin and statins on lipid and glucose metabolism and possible benefit of combination therapy. *Cardiovasc Diabetol* (2018) 17(1):94. doi: 10.1186/s12933-018-0738-4

44. Zhou B, Jia L, Zhang Z, Xiang L, Yuan Y, Zheng P, et al. The nuclear orphan receptor Nr2f6 promotes hepatic steatosis through upregulation of fatty acid transporter Cd36. *Adv Sci (Weinh)* (2020) 7(21):2002273. doi: 10.1002/advs.202002273

45. Hyun B, Shin S, Lee A, Lee S, Song Y, Ha NJ, et al. Metformin down-regulates tnf-alpha secretion *Via* suppression of scavenger receptors in macrophages. *Immune Netw* (2013) 13(4):123–32. doi: 10.4110/in.2013.13.4.123

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