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A clinically attainable dose of L-asparaginase targets glutamine addiction in lymphoid cell lines

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L-asparaginase (L-ASNase) is an important branch of chemotherapy for acute lymphoblastic leukemia (ALL) and some types of non-Hodgkin's lymphoma, including natural killer (NK)-cell lymphoma. Although it mediates hydrolysis of asparagine (Asn) and glutamine (Gln), which are variably required for cancer cell survival, the relative contribution of Asn and Gln depletion to the anti-tumor activity in therapeutic doses is unclear in ALL and malignant lymphoma. Here we demonstrate that L-ASNase exerts cytotoxicity through targeting the Gln addiction phenotype in lymphoid cell lines. A clinically attainable intermediate dose of L-ASNase induced massive apoptosis in ALL Jurkat and mantle cell lymphoma Jeko cell lines, while a low dose of L-ASNase effectively killed NK-cell lymphoma cells. In the lymphoid cell lines Jurkat and Jeco, deprivation of Gln but not Asn specifically suppressed cell growth and survival, and phenocopied the action of L-ASNase. L-ASNase treatment and Gln deprivation dramatically disrupted the refilling of the tricarboxylic acid (TCA) cycle by intracellular glutamate (Glu) and disturbed the mitochondrial integrity, which were alleviated by various anaplerotic TCA cycle intermediates, suggesting a direct contribution of glutaminase activity of L-ASNase. The action of L-ASNase differs between Jurkat cells and NK-cell lymphoma cells, according to their dependence on Gln and Asn. Furthermore, we observed that high expression of glutaminase GLS1 is associated with increased sensivity to L-ASNase in pediatric B lineage ALL. Our results redefine L-ASNase as a therapeutic agent targeting Gln addiction in certain lymphoid cells and offer an additional basis for predicting L-ASNase sensitivity and engineering selective L-ASNase derivatives for leukemia and lymphoma.

L-asparaginase (L-ASNase) is an important anti-tumor agent used in the treatment of acute lymphoblastic leukemia (ALL) and some types of non-Hodgkin's lymphoma, including natural killer (NK)-cell lymphoma.[1,2] We previously reported its high anti-tumor activity at low doses (around 0.01 U/mL) against NK-cell lymphoma cell lines and samples.[3] However, the mechanism of cytotoxic activity of L-ASNase and the determinants of its sensitivity in various cancer types have not been fully understood.[4] Although we previously found a good inverse correlation between *in vitro* sensitivity to L-ASNase and the expression level of glutamine-dependent asparagine synthetase (ASNS), which opposes the action of L-ASNase, at least in nasal-type NK-cell lymphoma,[3] the relationship between the expression level of ASNS and the resistance to L-ASNase remains controversial in other cancer types, especially in pediatric ALL.[4–7] Besides being bona fide asparaginase against asparagine (Asn), L-ASNase possesses some glutaminase (GLS) activity that hydrolyzes extracellular glutamine (Gln) to glutamate (Glu) and ammonia, thereby blocking Gln uptake into the cell.[8,9] Km values for the hydrolysis of Asn and Gln by L-ASNase are 15 μM and 3.5 mM, respectively.[10] Although some reports have suggested a potential contribution of GLS activity to the anti-tumor effect of L-ASNase,[8,9,11] others have insisted that accompanying GLS activity should cause only various side effects like hepatotoxicity and immunosuppressive actions.[12,13] The relative contribution of Asn and Gln depletion to anti-tumor activity of L-ASNase in therapeutic doses and the relationship with Gln dependence in cancer cells is unclear in ALL and malignant lymphoma.

Glutamine, the most abundant amino acid in the human body, plays a role as a major nitrogen donor in nucleotide and amino acid biosynthesis.[14] In addition, Gln has recently been found to function as a carbon source to supply tricarboxylic acid (TCA) cycle intermediates in Gln-addicted cells.[15,16] In this process, intracellular Gln is first deaminated to Glu and ammonium by intrinsic GLS. Glu is then converted to α-ketoglutarate by transaminases or glutamate dehydrogenase (GDH), and enters the TCA cycle in the mitochondrion, resulting in the production of NADPH and acetyl coenzyme A (acetyl-CoA), essential for redox control and lipid synthesis, respectively.

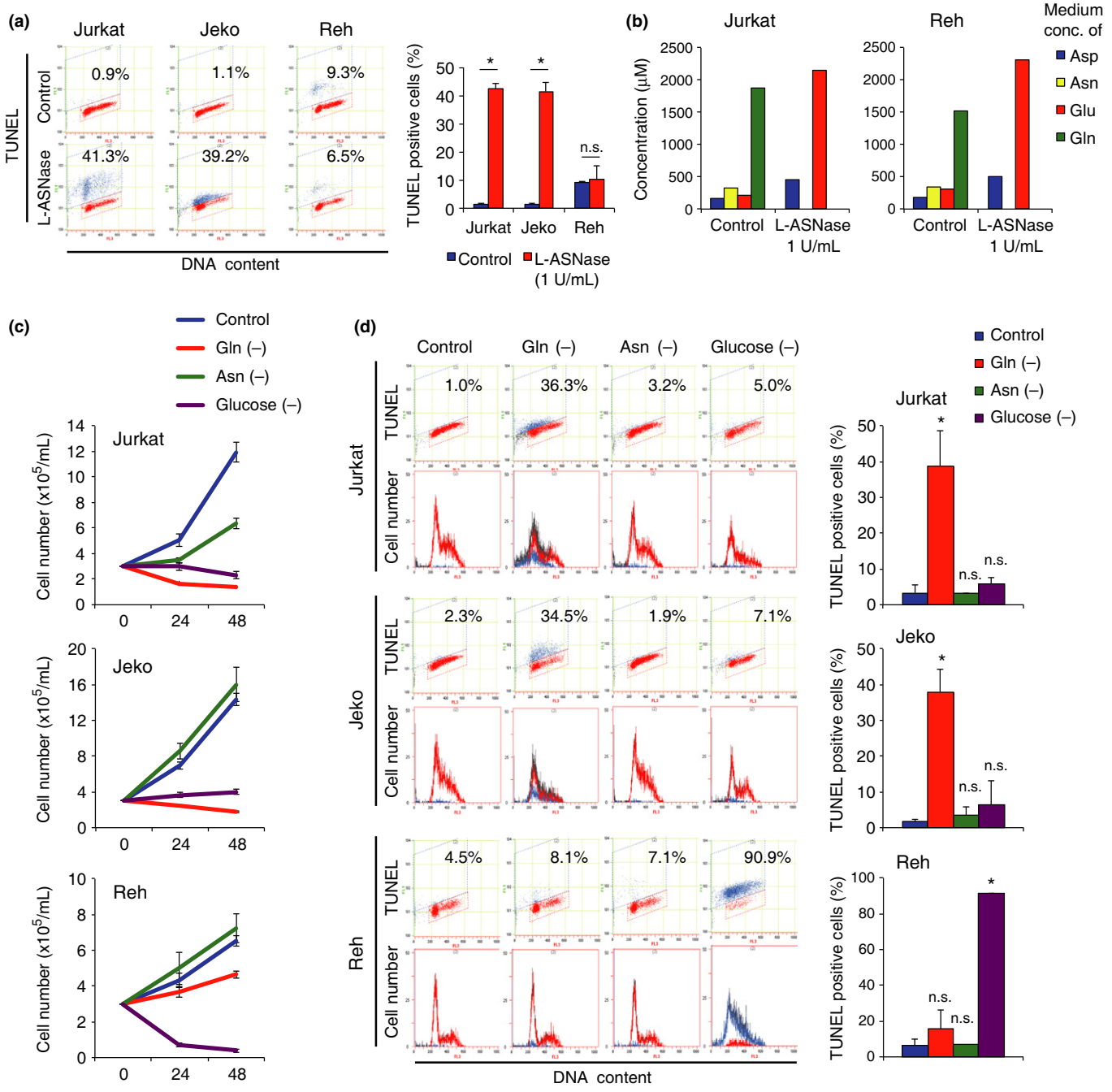

Fig. 1. L-asparaginase (L-ASNase)-sensitive Jurkat and Jeko cells are glutamine (Gln)-dependent. (a) Flow cytometric TUNEL assay in Jurkat, Jeko and Reh cells treated without (control) or with 1 U/mL of L-ASNase for 24 h. Percentages of apoptotic cells (blue) are indicated in each panel. (b) Concentrations of aspartate (Asp), asparagine (Asn), glutamate (Glu) and Gln in Jurkat and Reh cell culture supernatant after treatment without/with 1 U/mL of L-ASNase (24 h). (c) Cell growth curves of Jurkat, Jeko and Reh cells in culture media selectively lacking Gln, Asn or glucose for 48 h. Results are expressed as the means ± SD (n = 3). (d) Flow cytometric TUNEL assay showing apoptotic cell percentage and cell cycle distribution of Jurkat, Jeko and Reh cells in culture media selectively lacking Gln, Asn or glucose for 24 h. *P < 0.05; n.s., not significant.

Many types of cancer cells uptake glucose and simultaneously produce lactic acid at a higher rate than normal tissues, as initially observed by Otto Warburg.[17] Although seemingly wasteful from the standpoint of glucose usage, the altered metabolism of glucose in cancer cells should be beneficial for rapid incorporation of nutrients into the biomass necessary for high-speed proliferation.[18,19] Such cancer cells often exhibit Gln addiction; that is, they fully depend on Gln as a source of macromolecular synthesis and total bioenergetic production necessary for cell growth and survival. Some reports show that Gln addiction can be a direct consequence of high expression of MYC.[20–22] Thus, recent advances have highlighted Gln as one of the key molecules of cancer metabolism and its versatile metabolic functions beyond its role as a major nitrogen donor.

In the present study, we investigated the relationship between L-ASNase anti-tumor activity and the Gln addiction phenotype.

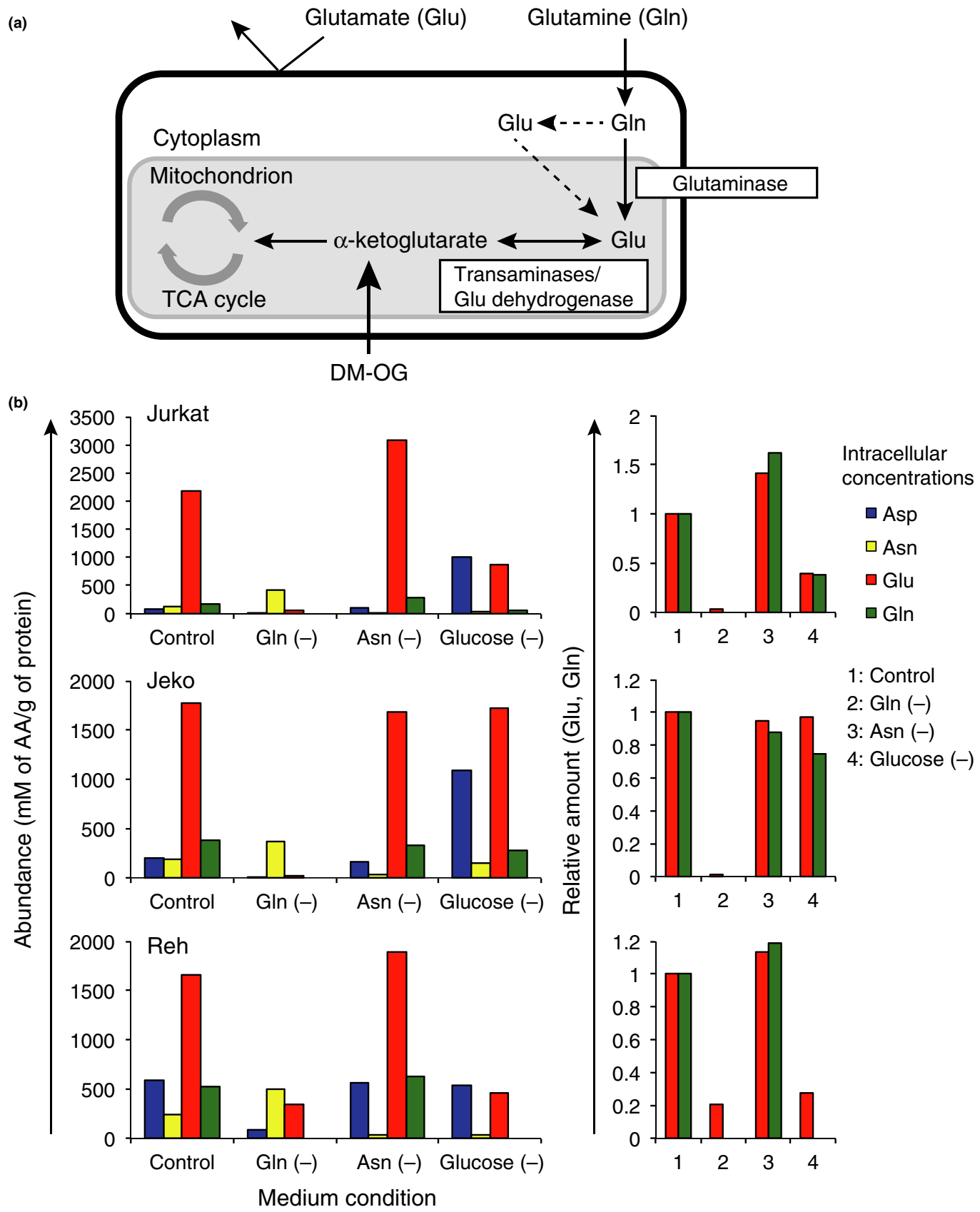

Fig. 2. Glutamine (Gln) deprivation results in depletion of intracellular glutamate (Glu) in Jurkat and Jeko cells. (a) Scheme of Gln metabolism showing the metabolic flow from extracellular Gln to α-ketoglutarate in the mitochondrion. (b) Intracellular levels of aspartate (Asp), asparagine (Asn), Glu and Gln in Jurkat, Jeko and Reh cells under depletion of Gln, Asn or glucose for 24 h. Left panels show intracellular amounts of these amino acids corrected for protein level. Right panels show relative amounts of Glu and Gln.

Materials and Methods

Cell culture and drug treatment. Jurkat, Jeko and Reh cell lines were maintained in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 10% FCS, 100 units/mL penicillin and 100 μg/mL streptomycin. NK-YS cell line was grown according to previous reports.[3,23] Cells were split to keep cell density from 3×10^5 to 1×10^6 cells/mL. To deplete Gln, Asn or glucose, cells were cultured in RPMI1640 completely lacking one of these nutrients (Sigma) supplemented with 10% dialyzed FCS. Logarithmically growing cells were treated with indicated doses of L-ASNase (Kyowa Hakko Kirin), whose 1 U is equivalent to 1 E of Asparaginase medac (medac GmbH, Wedel, Germany). Dimethyl-2-oxoglutarate (DM-OG), methylpyruvate, oxaloacetate, aminooxyacetate (AOA) and epigallocatechin gallate (EGCG) were purchased from Sigma.

Flow cytometer analysis. Flow cytometric terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assays were performed using a Cell Lab Quanta SC flow cytometer (Beckman Coulter, Fullerton, CA, USA), according to our previous report.[24] Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were evaluated by staining with 1 nM of 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6[3]$) (Molecular Probes, Eugene, OR, USA). Representative flow cytometry plots are shown in the figures.

Analysis of free amino acids in cell and medium. PBS-washed cells and cell culture supernatants were deproteinized with 5% sulphosalicylic acid. After centrifugation at 15000 *g* and 4°C for 15 min, free amino acids in supernatants were analyzed using an L-8900FF amino acid analyzer (Hitachi High-Technologies, Tokyo, Japan).

Gas chromatography-mass spectrometry. Harvested cells were washed once with PBS. Metabolites were extracted with cold ethanol including 1,6-$^{13}C_2$-adipic acid as an internal standard and derivatized by 1% methoxylamine-hydrochloride in pyridine and N,O-bis (trimethylsilyl) acetamide. Each sample underwent GC-MS analysis using a Triplus autosampler (Thermo Fisher Scientific, Waltham, MA, USA). Gas chromatography was performed on a Trace GC Ultra gas chromatographer (Thermo Fisher Scientific) equipped with a DB1301 fused silica capillary column (20 m length, 0.25 mm inner diameter, 0.25 μm firm thickness; Agilent Technologies, Santa Clara, CA, USA) connected to a TSQ Quantum GC mass spectrometer (Thermo Fisher Scientific). Analytical conditions were as follows: splitless injector at 240°C; column at 100°C for 1 min raised by 15°C/min up to 250°C for 5 min; transfer line at 250°C; helium was used as carrier gas at 1 mL/min. Molecules were detected by chemical ionization/selected-reacting monitoring mass spectrometry. The flow of methane as an ionization gas was 1.5 mL/min; the flow of argon gas for collision was 1.0 mTorr. Collision energy was set to 5 to 25 eV.

Gene expression profiling analysis. Gene expression profiling of pediatric B lineage ALL and classification of drug sensitivity were described previously.[25] mRNA expression data (GSE2351) were downloaded from Gene Expression Omnibus and analyzed after quantile normalization.

Statistical analysis. Statistical analysis was performed using the two-tailed Student's *t*-test.

Results

L-asparaginase-sensitive Jurkat and Jeko cells are glutamine-dependent but not asparagine-dependent. In our previous study, Jurkat T-cell ALL cell line was resistant to a low dose of L-ASNase (0.01–0.1 U/mL) relative to NK-cell lymphoma cell lines.[3] To extend the understanding of L-ASNase action in lymphoid cell lines, we compared the effects of a clinically readily attainable intermediate dose of L-ASNase (1 U/mL)[26,27] for three lymphoid malignant cell lines: Jurkat (T-ALL), Jeko (mantle cell lymphoma) and Reh (pre-B ALL). Treatment with 1 U/mL of L-ASNase for 24 h induced massive apoptosis in Jurkat and Jeko cells but not in Reh cells (Fig. 1a). Capillary electrophoresis coupled to mass spectroscopy (CE-MS) confirmed complete depletion of both Asn and Gln in the culture supernatant of Jurkat and Reh cells after 24 h of L-ASNase treatment, although Gln was the most abundant among these amino acids in the control supernatant (Fig. 1b). Increased aspartate (Asp) and Glu concentrations by L-ASNase were consistent with enzymatic conversion of Asn and Gln, respectively. To dissect the mechanisms of L-ASNase action, we incubated these cells in culture media completely lacking Gln, Asn or glucose (Fig. 1c). The proliferation of L-ASNase-sensitive Jurkat and Jeko cells was dramatically inhibited by deprivation of Gln but not of Asn (Fig. 1c). Glucose deprivation also retarded the growth of these cells, although more weakly than Gln deprivation. In contrast, L-ASNase-insensitive Reh cells grew even in the absence of Gln. Reh cell growth was largely dependent on the presence of glucose in the culture medium. TUNEL assay demonstrated that deprivation of Gln but not Asn for 24 h induced massive apoptosis in Jurkat and Jeko cells but little if any in Reh cells (Fig. 1d). Interestingly, glucose deprivation failed to cause considerable apoptosis in Jurkat and Jeko cells during the observed time period (24–48 h, Fig. 1d), although both Gln and glucose withdrawal resulted in remarkable retardation of growth (Fig. 1c). Most Reh cells, however, became apoptotic after 24 h of glucose deprivation. Overlap between L-ASNase response and

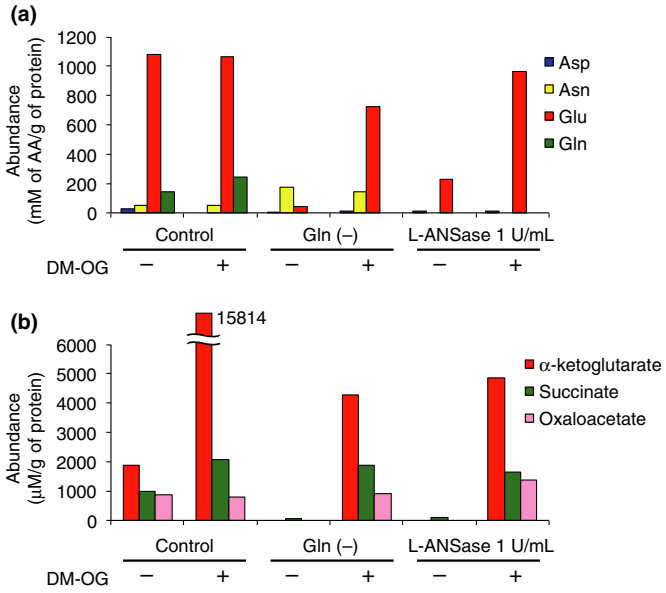

Fig. 3. Dimethyl-2-oxoglutarate (DM-OG) restores intracellular glutamate (Glu) and tricarboxylic acid (TCA) cycle intermediates in glutamine (Gln)-depleted or L-asparaginase (L-ASNase)-treated Jurkat cells. (a,b) Intracellular levels of aspartate (Asp), asparagine (Asn), Glu, Gln (a), α-ketoglutarate, succinate and oxaloacetate (b) in Jurkat cells mock-treated, Gln-deprived, or treated with 1 U/mL of L-ASNase for 24 h in the absence or presence of DM-OG (2.1 mM).

Gln dependence in Jurkat and Jeko cells suggested the possibility that L-ASNase targets the Gln addiction phenotype in these cell lines.

Intracellular glutamate level depends on exogenous glutamine in Jurkat and Jeko cells. As illustrated in Figure 2a, extracellular Gln but not Glu can directly enter into the cell. Gln is deaminated to Glu in the mitochondrion or in the cytoplasm. Then, Glu is reversibly converted to α-ketoglutarate and enters into the TCA cycle. We next determined the intracellular levels of Asp, Asn, Glu and Gln upon withdrawal of extracellular Gln, Asn or glucose. Glu was the most abundant intracellular amino acid in rapidly growing Jurkat, Jeko and Reh cells (Fig. 2b). Gln deprivation from the culture medium for 24 h almost completely depleted the intracellular Glu and Gln in Jurkat and Jeko cells (Fig. 2b). In contrast, lack of Asn or glucose failed to induce such a sharp fall of the intracellular Glu and Gln levels in these cell lines. In Reh cells, reduction of intracellular Glu by Gln deprivation was not as drastic as in Jurkat and Jeko cells. Glucose deprivation, however, induced reduction of intracellular Glu and Gln levels comparable to Gln depletion in Reh cells, suggesting that Glu could be synthesized from glucose in this cell line. These results indicate that maintenance of intracellular Glu level heavily relies on uptake of exogenous Gln in Jurkat and Jeko cells.

Anaplerotic flux of glutamine into the tricarboxylic acid cycle in Jurkat cells. Considering the Gln addiction phenotype in Jurkat and Jeko cells, we investigated whether or not Gln is used as a carbon source to supply TCA cycle intermediates and is involved in the maintenance of mitochondrial membrane potential integrity. CE-MS analysis confirmed that both Gln deprivation and L-ASNase treatment resulted in considerable depletion of intracellular Glu (Fig. 3a) and of three representative TCA cycle intermediates (i.e. α-ketoglutarate, succinate and oxaloacetate; Fig. 3b) in Jurkat cells. Addition of DM-OG, a cell-permeable pre-form of α-ketoglutarate, reversed these suppressive effects (Fig. 3a,b). These results confirmed that Gln provides anaplerosis for refilling the mitochondrial carbon pool in Jurkat cells.

Dimethyl-2-oxoglutarate attenuates apoptosis caused by glutamine depletion or L-asparaginase in Jurkat and Jeko cells. We next examined whether supplement of DM-OG could ameliorate cell death caused by Gln withdrawal and L-ASNase treatment. Addition of DM-OG reduced the apoptosis of Jurkat and Jeko cells deprived of Gln or treated with 1 U/mL of L-ASNase for 24 h (Fig. 4a). DM-OG attenuated L-ASNase-induced apoptosis in a dose-dependent manner in these cells (Fig. 4b). These results suggested that L-ASNase may exert its cytotoxic effects through depletion of α-ketoglutarate or its derivative(s).

L-asparaginase disrupts the mitochondrial membrane potential maintained by glutamine in Jurkat cells. Analysis of mitochondrial membrane potential ($\Delta\Psi_m$) demonstrated that $\Delta\Psi_m$ was clearly lowered in the total population and even in the viable population of Gln-deprived or L-ASNase-treated Jurkat cells (Fig. 5a), indicating irreversible loss of mitochondrial function. Addition of DM-OG restored $\Delta\Psi_m$ levels in Jurkat cells under these conditions. Because TCA cycle reactions take place in the mitochondrion, apparent reduction of TCA cycle components should lead to impaired mitochondrial membrane potential. Thus, both Gln depletion and L-ASNase may exert cytotoxic activity through depletion of TCA cycle intermediates and subsequent disruption of mitochondrial membrane potential integrity.

Inhibition of intracellular glutamine-to-α-ketoglutarate conversion induces apoptosis in Jurkat cells. To confirm the importance of extracellular Gln to supply the TCA cycle, we treated Jurkat cells with amino oxyacetate (AOA) or epigallocatechin gallate (EGCG), inhibitors of transaminases and GDH, respectively (Fig. 5b).[20,28] Both agents induced massive apoptosis, and DM-OG suppressed apoptosis induced by them (Fig. 5c). Our results suggest that transaminases and GDH play a similar important role in glutaminolysis in Jurkat cells, while Choo

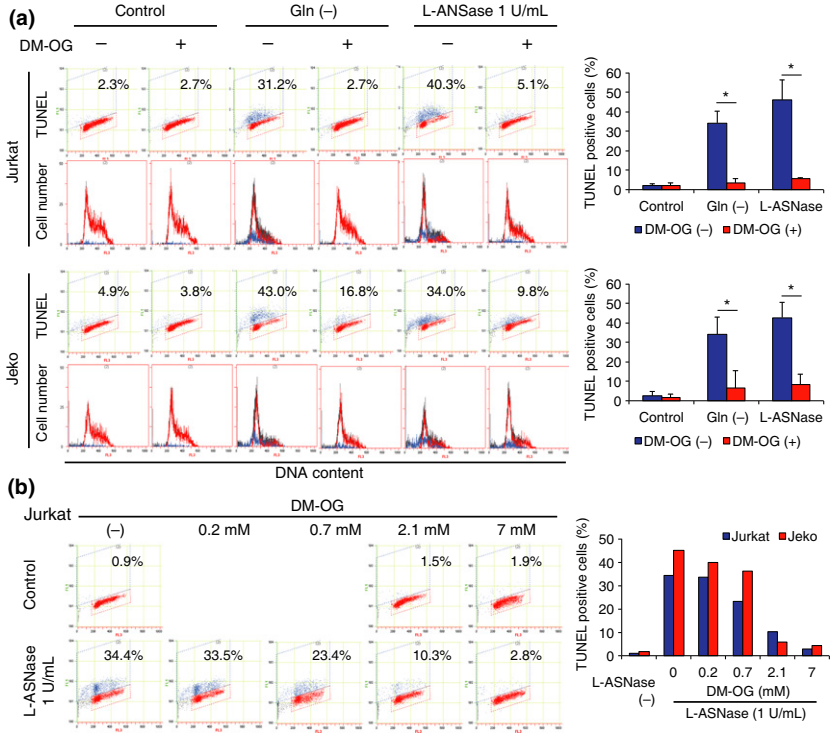

Fig. 4. Dimethyl-2-oxoglutarate (DM-OG) attenuates apoptosis induced by glutamine (Gln) depletion or L-asparaginase (L-ASNase) in Jurkat and Jeko cells. (a) TUNEL assay showing the percentage of apoptotic cells (top) and cell cycle distribution (bottom) of Jurkat and Jeko cells mock-treated (control), deprived of Gln, or treated with 1 U/mL of L-ASNase for 24 h in the absence or presence of DM-OG (2.1 mM). *$P < 0.05$. (b) Dose-dependent inhibitory effects of DM-OG on L-ASNase-induced apoptosis in Jurkat and Jeko cells.

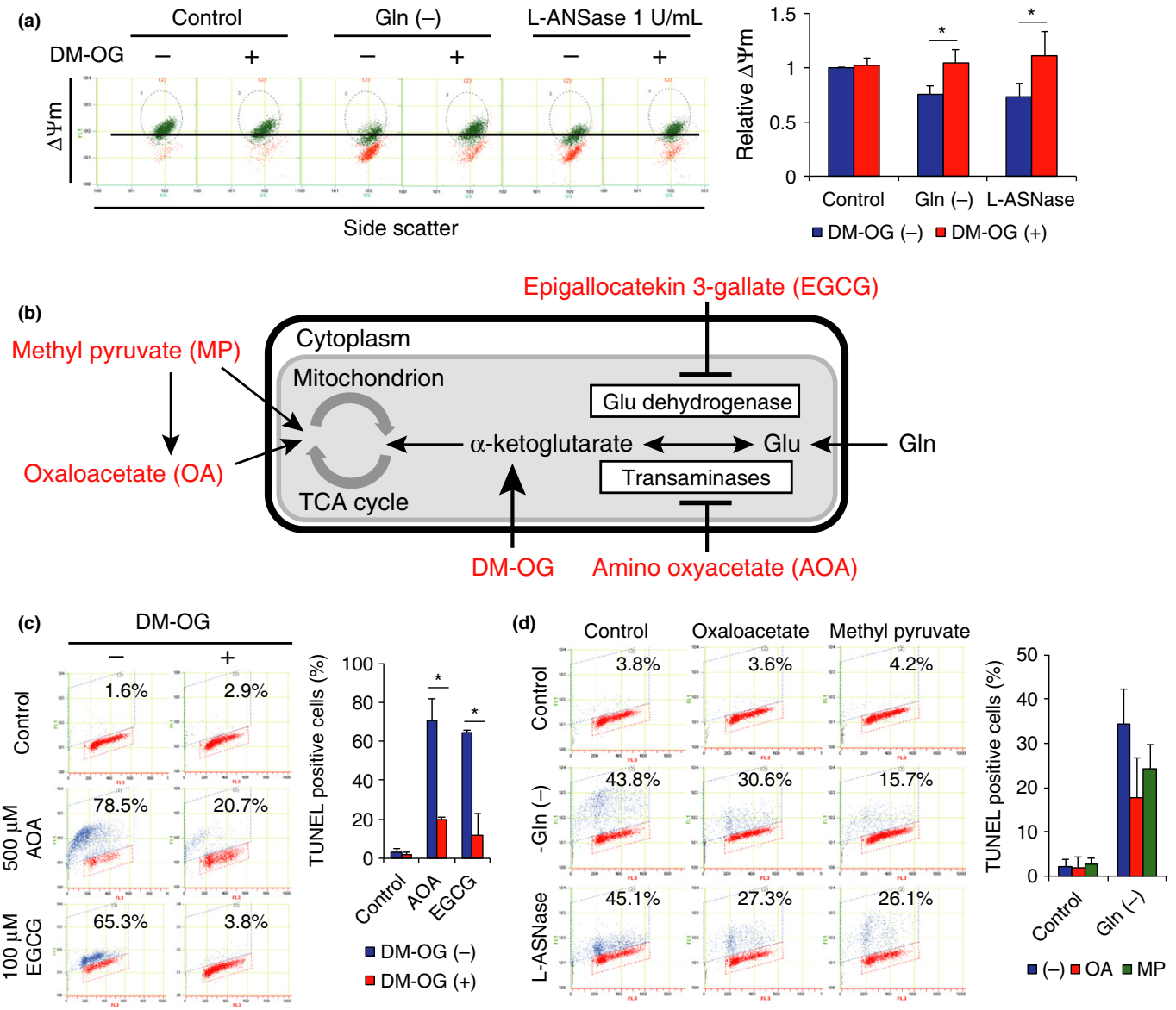

Fig. 5. Blockage of extracellular glutamine (Gln) usage leads to decreased $\Delta\Psi_m$ and apoptosis in Jurkat cells through inhibition of anaplerotic function of Gln for tricarboxylic acid (TCA) cycle. (a) Mean values of mitochondrial membrane potential ($\Delta\Psi_m$) in the viable population (top) and two-dimensional flow cytometric analyses with side scatter and $\Delta\Psi_m$ (bottom) of control, Gln-deprived or L-asparaginase (L-ASNase)-treated Jurkat cells in the absence or presence of DM-OG (2.1 mM). In the flow cytometric panels, viable and non-viable groups are colored green and orange, respectively. *$P < 0.05$. (b) Modification of the metabolic flow from extracellular Gln to α-ketoglutarate by extracellular agents. (c) Flow cytometric TUNEL assay on Jurkat cells treated with mock (control), 500 μM of amino oxyacetate (AOA), or 100 μM of epigallocatechin gallate (EGCG) for 24 h in the absence or presence of DM-OG (2.1 mM). (d) TUNEL assay on Jurkat cells mock-treated (control), deprived of Gln, or treated with 1 U/mL of L-asparaginase (L-ASNase) for 24 h without or with 3 mM of oxaloacetate or methyl pyruvate.

et al. reported that GDH, but not transaminases, was the critical enzyme responsible for Gln metabolism under glucose-limited conditions in mouse embryonic fibroblasts.[28] In addition, we confirmed that other cell-permeable anaplerotic TCA cycle intermediates, oxaloacetate and methyl pyruvate, could attenuate the cytotoxicity mediated by L-ASNase and Gln withdrawal in Jurkat cells (Fig. 5d).

NK-YS cells are asparagine-dependent and therefore sensitive to a low dose L-asparaginase. We previously reported a rather specific anti-tumor activity of L-ASNase against NK-YS NK-cell lymphoma cells and clinical samples even at a low dose (0.01 U/mL).[3]

In nasal-type NK-cell lymphoma, ASNS expression levels inversely correlates with *in vitro* sensitivity to L-ASNase, and the expression of ASNS is very low in NK-YS cells.[3] Although deprivation of Gln and glucose increased the apoptotic cell percentage from approximately 10 to 20–30%, only Asn deprivation resulted in massive apoptosis of NK-YS cells (Fig. 6a). Addition of DM-OG failed to alleviate the apoptosis induced by Asn deprivation, although DM-OG decreased apoptosis induced by Gln depletion. A very low dose (0.01 U/mL) was enough to deplete Asn from the medium in 24 h (Fig. 6b), while 1 U/mL of L-ASNase was necessary to deplete Gln; 0.01 or 0.03 U/mL of L-ASNase induced the same extent of apoptosis as 1 U/mL in NK-YS cells (Fig. 6c). Therefore, the cytotoxic activity through Asn depletion seemed to be independent of mitochondrial TCA cycle maintenance for cell survival of NK-YS cells.

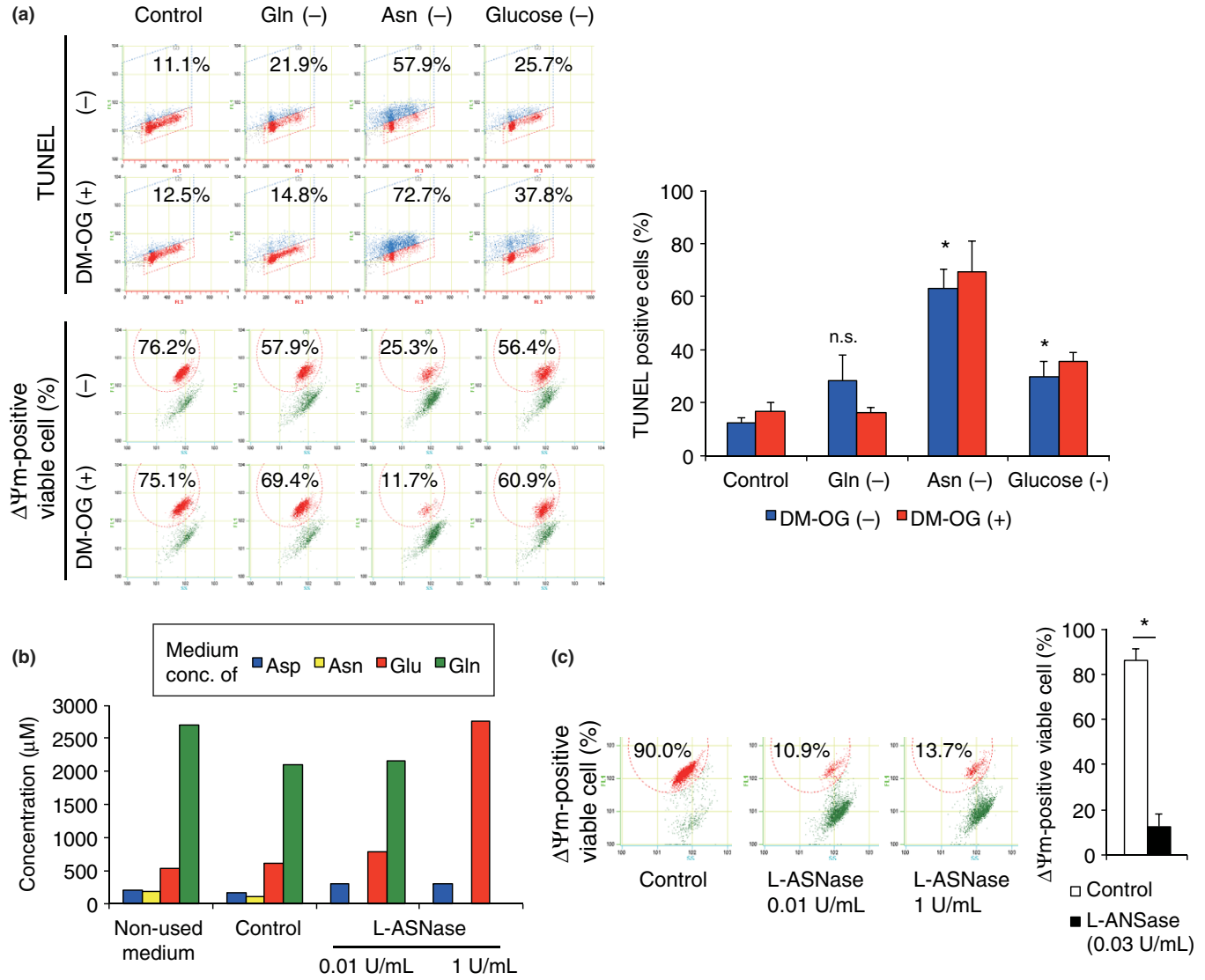

Fig. 6. NK-YS cells are asparagine (Asn)-dependent and sensitive to a low dose of L-asparaginase (L-ASNase). (a) TUNEL assay and $\Delta\Psi_m$-positive cell measurement of NK-YS cells mock-treated, deprived of glutamine (Gln), Asn or glucose for 24 h with or without DM-OG (2.1 mM). (b) Concentrations of aspartate (Asp), Asn, glutamate (Glu) and Gln in the NK-YS cell culture supernatant after 24 h of L-ASNase treatment measured by CE-MS analysis. (c) $\Delta\Psi_m$-positive cell measurement of NK-YS cells after 24 h of mock or L-ASNase treatment. *$P < 0.05$; n.s., not significant.

Expression of glutaminase and asparagine synthetase in pediatric B lineage ALL. Finally, to assess the relationship between L-ASNase sensitivity and the expression levels of molecules involved in Gln and Asn metabolism, we analyzed a transcriptome database of pediatric B lineage ALL, which includes LC50 values for L-ASNase, prednisolone (PRED), vincristine (VCR) and daunorubicin (DNR) in 129 patients.[25] The present study found that drug resistance patterns consist of two patterns: cross-resistance for four drugs and discordant resistance to L-ANSase and vincristine.[25] As shown in Figure 7, we observed that the expression levels of GLS1 carboxy terminal splice variant (glutaminase C, GAC) and ASNS significantly differed between L-ANSase-sensitive plus VCR-resistant patients and L-ANSase-resistant plus VCR-sensitive patients, while we failed to observe clear differences for other molecules (Fig. 7a). L-ANSase-sensitive plus VCR-resistant patients were associated with high expression of GAC and low expression of ASNS (Fig. 7b,c). This is consistent with several reports proposing that GLS is a biomarker of Gln dependence.[29–31] Our results suggest that L-ASNase sensitivity may be explained by a combination of ASNS and GLS.

Discussion

Our study showed that Jurkat cells heavily depend on extracellular Gln to supply the TCA cycle and to maintain mitochondrial integrity and their survival, and that Jurkat and Jeko cell lines exhibited this Gln addiction phenotype. In these cells, L-ASNase essentially phenocopied deprivation of Gln but not Asn and the effects were reversed by DM-OG. Recently, a study using L-ASNase derivatives revealed the importance of secondary GLS activity for cancer cell cytotoxicity, especially in ASNS-positive cancer cells, which can synthesize Asn by themselves.[32,33] Although a contribution of GLS activity in anti-tumor activity of L-ASNase has been also suggested,[16] the present study has definitely shown that L-ASNase should

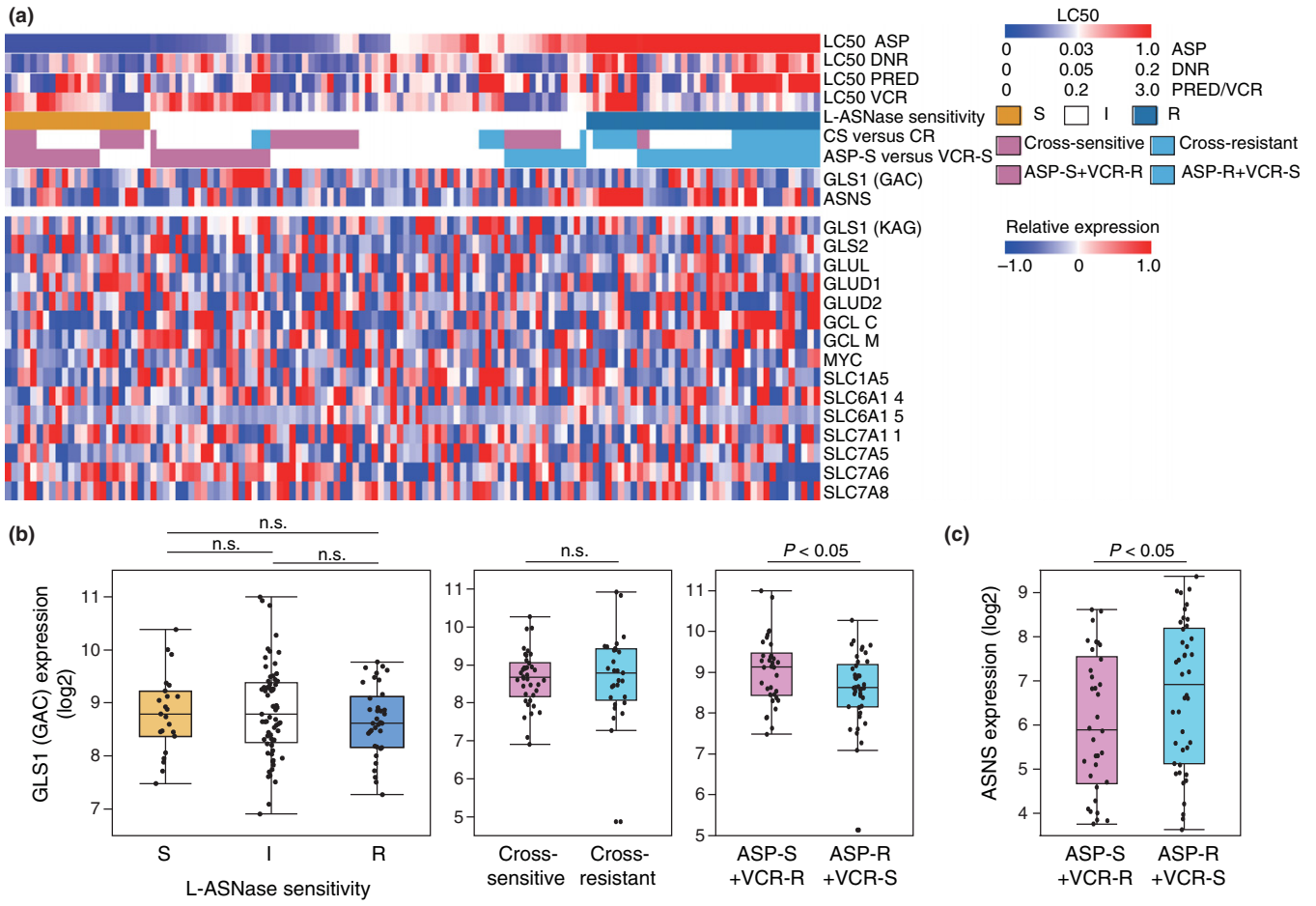

Fig. 7. Expression of glutaminase (GLS) and asparagine synthetase (ASNS) in pediatric B lineage acute lymphoblastic leukemia (ALL). (a) Heatmap showing LC50 values for four anti-leukemic agents, drug sensitivity patterns, and the expression of various molecules associated with Gln metabolism in primary ALL cells from 129 patients with pediatric B lineage ALL. ASP, L-ASNase; CR, cross-resistant; CS, cross-sensitive; DNR, daunorubicin; GAC, glutaminase C; I, intermediate; KGA, kidney glutaminase; PRED, prednisolone; R, resistant (LC50 > 0.912 IU/mL); S, sensitive (LC50 < 0.033 IU/mL); VCR, vincristine. (b, c) Expression levels of GLS1 (GAC) (b) and ASNS (c) in pediatric B lineage ALL.

exert its cytotoxic activity through blockage of Gln addiction in lymphoid cell lines.

The TCA cycle, essential for cell growth and survival, was recently suggested as being not only as a catabolic pathway of glucose but also a hub of many macromolecular biosynthetic pathways of lipids, proteins and nucleic acids.[18,34] Various types of cancer cells use extracellular Gln to replenish the TCA cycle molecules instead of glucose, which is often secreted as lactate even in the presence of sufficient oxygen.[35] Gln is also utilized for conversion to glutathione (GSH) to decrease reactive oxygen species (ROS) and to protect cells from oxidative stress. Accordingly, it was reported that Gln depletion induces a decrease in GSH levels and an elevation in ROS in Gln-dependent acute myeloid leukemia (AML) cell lines.[36] Although the roles of redox control by Gln should be further investigated in Gln-dependent ALL cell survival, these findings suggest that Gln has multiple roles in cancer cell survival and that these roles of Gln could be targeted by L-ASNase.

The present study has delineated two independent cytotoxic activities of L-ASNase; that is, Asn depletion at low doses and Gln depletion at clinically attainable intermediate doses. The refractoriness of Asn-depletion-mediated apoptosis to DM-OG (Fig. 6a) suggests that the cytotoxic activity through Asn depletion is different from that by Gln depletion and is independent of mitochondrial TCA cycle maintenance for cell survival. Consistently, a recent report by Zhang *et al.* showed that Asn functions outside of mitochondrial TCA cycle and is a critical suppressor of apoptosis but not growth retardation under Gln withdrawal in *MYC*-amplified glioma cells.[37] In addition, in glucose-dependent Reh cells, glucose depletion induced a decrease in intracellular Asn in contrast to Gln depletion (Fig. 2b). On the other hand, while intracellular Asn and Asp decreased upon Gln withdrawal in the report by Zhang *et al.*,[37] the levels of Asn did not show a decline under Gln depletion in our analysis (Fig. 2b). This suggests that roles of Asn in adaptive stress responses may differ among cancer cell types and types of cell stress.

Primary samples and cell lines of NK-cell malignancies were sensitive to as low as 0.01 U/mL of L-ASNase,[3] and we confirmed that main target of L-ASNase in NK-YS cells is genuinely Asn, but not Gln (Fig. 6). However, several studies have reported that serum L-ASNase concentration is sustained above 1 U/mL even 24 h after the administration of clinically applicable doses of L-ASNase.[26,27] This intermediate dose of L-ASNase mainly used in the present study can deplete both

Asn and Gln, indicating that our findings are basically relevant in clinical settings.

The determinants of L-ASNase sensitivity in various cancer types have not been fully understood.[4] Several studies have shown the difficulty to clearly correlate the sensitivity to L-ASNase with any one parameter like ASNS in ALL,[4–7] while ASNS mRNA levels correlate well with L-ASNase sensitivity among Asn-dependent NK cell tumor samples.[3] Gln addiction, this new conceptual framework of L-ASNase activity, may explain the rather enigmatic observation that TEL-AML1-positive pediatric ALL cases with higher expression levels of ASNS are more sensitive to L-ASNase than TEL-AML1-negative cases;[5,6] that is, TEL-AML1-positive ALL cases may heavily depend on extracellular Gln.

In this study, we observed that high expression of GAC GLS variant is associated with L-ANSase-sensitive plus VCR-resistant phenotype in pediatric B lineage ALL (Fig. 7). In cancer biology, MYC is reported to facilitate Gln metabolism through upregulation of Gln transporters and GLS.[20–22] Expression of GLS1 or GLS1 GAC is reported to be associated with Gln dependence in breast cancer, lung cancer and AML.[29–31] Furthermore, a recent report demonstrated that inhibition of GLS1 induces suppression of glutaminolysis and cell death in AML and that this phenomenon is suppressed by α-ketoglutarate.[31] It was also reported that silencing of GLS induces pyruvate carboxylase-mediated glucose-dependent anaplerosis.[38] Taken together, these reports and our findings suggest that GLS could be a biomarker of Gln dependence and a therapeutic target. In contrast, another report stated that changes in the expression of apoptosis-regulatory genes (especially NF-κB-related genes) are associated with L-ASNase susceptibility in B-ALL cell lines.[39] These findings therefore suggest that multiple factors other than Asn-dependency and ASNS, including GLS1, can influence L-ASNase action and susceptibility depending on the cellular context.[39] Future studies using clinical samples may provide insights for the precise mechanisms of L-ASNase in ALL and malignant lymphoma.

In conclusion, we demonstrated that clinically attainable intermediate doses of L-ASNase target the Gln addiction phenotype in lymphoid cell lines. This study provides a better perspective for the cytotoxic mechanism of L-ASNase and development of L-ASNase derivatives against leukemia and lymphoma cells and for the prediction of their sensitivity to L-ASNase.

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