Inhibiting The xCT Antiporter Sensitizes High Risk DLBCL to Doxorubicin-Containing Therapy

Xiaolei Wei

Nanfang hospital, Southern medical university <https://orcid.org/0000-0002-4271-9588>

Yongqiang Wei

Nanfang hospital, Souther medical university

Jialin Song

Southern Medical University Nanfang Hospital

Yun Mai

Albert Einstein College of Medicine

Fei Yuan

Albert Einstein College of Medicine

Jinghang Zhang

Albert Einstein College of Medicine

Ru Feng

Southern Medical University Nanfang Hospital

Bihui Hilda Ye (✉ hilda.ye@einsteinmed.org)

Albert Einstein College of Medicine

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Abstract

Background. Although outcomes in diffuse large B-cell lymphoma (DLBCL) have dramatically improved following the introduction of R-CHOP two decades ago, one-third of the patients fail to attain durable remission and require salvage and additional therapies. Many attempts to improve upon R-CHOP efficacy by incorporating targeted agents have failed, suggesting a need to better understand the mechanisms underlying R-CHOP resistance/responsiveness. We have previously reported that Doxorubicin (Dox), the main cytotoxic ingredient in CHOP, has subtype-specific mechanisms of cytotoxicity in DLBCLs due to differences in its subcellular distribution pattern. Specifically, Dox-induced cytotoxicity in the therapy refractory ABC-DLBCLs is largely dependent on oxidative stress rather than the DNA damage response. These findings suggest that R-CHOP outcome could be modulated by manipulating oxidative stress response in DLBCL cells. This study focuses on xCT, the light chain of the cystine/glutamate antiporter x_c^- that controls the rate-limiting step for glutathione (GSH) biosynthesis. We demonstrate novel roles of the xCT-GSH-ROS axis in maintenance and therapeutic response of high risk DLBCLs.

Methods. Prognostic significance of xCT protein expression in a cohort of 87 DLBCL patients treated with R-CHOP were evaluated. Dynamic changes of the cellular GSH pool to Dox treatment were examined. xCT inhibition was achieved using a small molecular inhibitor sulfasalazine (SASP) and through shRNA-mediated knock-down. The ability of SASP to sensitize ABC-DLBCL/double-hit lymphomas to Dox-containing therapy was investigated in cell line and NSG-based xenograft models.

Results. Dox elicits a rapid antioxidant response characterized by a GSH spike that can be eliminated by suppressing the xCT antiporter function. Mechanistically, xCT inhibition depleted the total GSH pool and markedly enhanced the p38-dependent apoptosis response following Dox treatment. Furthermore, supporting a role of the xCT-GSH axis in clinical response to Dox-containing therapies, xCT expression predicts poor prognosis among ABC-DLBCL but not GCB-DLBCL patients treated with R-CHOP. Finally, we demonstrate that combinatorial treatment with Dox and SASP produced marked synergy in xenograft models for ABC-DLBCL/double-hit lymphomas.

Conclusions. In ABC-DLBCL, the xCT-GSH-p38 axis plays a critical role in survival response to Dox-containing therapies. xCT inhibition may hold significant promise to improve the first-line treatment outcome of patients with high risk DLBCL.

Background

Diffuse large B cell lymphoma (DLBCL) is the most common and aggressive lymphoid malignancy in adults. When managed with R-CHOP, or one of its variations including R-ACVBP and DA-EPOCH-R, one-third of the patients fail to attain durable remission and instead develop relapsed/refractory disease^{17,20}. Till now, all FDA approved targeted therapies and immunotherapies for DLBCL are for second line or later use. Over the past 13 years, significant efforts have been made to improve the efficacy of R-CHOP; yet none of the many clinical trials could demonstrate superiority over R-CHOP^{4,20}. We argue that these

clinical trial experiences underscore the need for a greater understanding of the mechanisms that lead to the failure of R-CHOP among the primary resistant patients.

Transformed from normal B cells positioned at different stages of the germinal center (GC)-to-plasma cell maturation process, DLBCL has a high degree of biological and clinical heterogeneity³². By far, the most informative sub-classification approach for DLBCL pathogenesis understanding and treatment improvement is the cell-of-origin (COO) algorithm^{1,33}. When treated with either the combination chemotherapy CHOP or the immuno-chemotherapy R-CHOP, the response rate and the ultimate survival outcome of the ABC-DLBCL group is always much worse than that of the GCB-DLBCL variant^{13,23}. In addition, the high-grade B-cell lymphoma group, which contains the so-called double-hit and triple-hit cases (MYC, BCL2, and/or BCL6 translocations) that straddle the GCB and ABC subgroups, also has particular poor outcomes with R-CHOP^{30,31}. Yet, the molecular mechanisms underlying CHOP/R-CHOP resistance remain poorly defined. Based on observations that mutated p53 and activated STAT3 predict poor prognosis exclusively in GCB-DLBCL and ABC-DLBCL, respectively^{19,36}, we postulate that a) there exist subtype-specific resistance mechanisms directed against the chemotherapy drugs in the original CHOP formulation, and b) the addition of rituximab, while overall improving the response rate, does not adequately address the ABC-specific resistance mechanism. In support of the first hypothesis, we have previously reported that Doxorubicin (Dox), the main cytotoxic ingredient in CHOP, has subtype-specific mechanisms of cytotoxicity in DLBCLs due to differences in its subcellular distribution pattern²⁹. In particular, Dox-induced cytotoxicity in ABC-DLBCLs is largely dependent on oxidative stress rather than the DNA damage response. We also showed that the basal oxidative stress status is predictive of treatment outcome for the ABC-DLBCL subgroup but not the GCB-DLBCL variant²⁹. These findings predict that agents capable of disrupting the redox balance in ABC-DLBCL cells could augment therapeutic activity of Dox-containing first line therapies.

xCT is the light chain of the cystine/glutamate antiporter x_c^- that controls the rate-limiting step for glutathione (GSH) biosynthesis^{24,27}. Elevated expression of xCT has been reported in several types of cancers and shown to critically regulate tumor cell proliferation, invasion, and response to several cancer treatment modalities^{27,28}. However, the role of xCT in DLBCL has not been directly examined. In vivo, xCT has a fairly restricted expression pattern, with its protein stability positively regulated by a splicing variant of CD44, CD44V6²¹. We have previously reported that expression of CD44 and CD44V6 are poor prognosticators for DLBCL³⁴. While SLC7A11, which encodes xCT, is the main driver in the 16-gene oxidative stress signature that demonstrated a prognostic impact among ABC-DLBCL cases in our previous work²⁹. In that study, we discovered that the xCT-GSH-ROS axis plays a very active role in deciding the cell death-survival outcome of Dox-treated ABC-DLBCL cells in a p38-dependent manner. Accordingly, high xCT protein expression in diagnostic samples correlates with poor prognosis in ABC-DLBCL but not GCB-DLBCL. Most importantly, combining Dox with an xCT inhibitor, sulfasalazine (SASP)¹¹, produced marked synergy in cell line and xenograft models representing ABC-DLBCL and

double-hit lymphomas. These findings support the concept that GSH restriction can potentially improve first-line treatment outcomes for high risk DLBCLs.

Materials And Methods

Additional methods and reagent details are described in Supplementary Methods, available online at the journal's Web site.

Cell culture and transfection

Human DLBCL cell lines, Ly1, Ly3, SuDHL2, SuDHL5, SuDHL6, TMD8 and Val were cultured in IMDM medium supplemented with 10% FBS (Gemini Bio-Products). Ly10 was cultured in IMDM plus 20% human serum. HBL-1, Riva, U2932, Pfeiffer, and Karpas-422 was cultured in RPMI medium supplemented with 10% FBS. For xCT silencing, short hairpin RNAs targeting xCT (Ambion siRNA Select s24290) and control scrambled oligonucleotides were transfected into Riva and TMD8 cells as previously described⁹. Cell lines stably transfected with shRNA vectors were sorted by flow cytometry before selection with puromycin.

Cell viability assays

Cells were pretreated with SASP for 16 h using IC₅₀ doses and then seeded in 96-well plates at the density of 3×10^4 cells per well before exposure to varying concentrations of Dox for 48 hrs. MTT solution (0.5 mg/ml) was then added and incubated at 37°C for 4 hours. The crystals were dissolved by the addition of 150 μ l of DMSO per well and the absorbance at OD₄₉₀ was read using a Microplate spectrophotometer. IC₁₀, IC₂₅, and IC₅₀ concentrations for SASP were defined as the concentration of SASP required to inhibit cell viability by 10%, 25%, and 50%, respectively. When exogenous GEE was used, cells were pre-treated with 1 mM GEE for 2 hrs before exposure to Dox/SASP.

Flow cytometry based ROS and cell viability measurements

Endogenous ROS was detected by DCFDA (Invitrogen) staining according to the manufacturer's instruction followed by flow cytometry. The results were shown as mean fluorescent intensity (MFI). To quantify apoptotic and total viable cell fractions, cells were stained with Annexin V-APC/SYTOX Green (Invitrogen) followed by flow cytometry.

Determination of cellular GSH levels

A glutathione assay kit (Cayman Chemical, Cat 703002) was used to measure intracellular GSH levels according to the manufacturer's instruction. This assay reflects total glutathione including GSH and GSSG.

Western blotting and Quantitative RT-PCR

For Western blotting, cells were lysed using the RIPA buffer. Standard procedure was followed for the subsequent steps⁹. Total RNA was isolated using Trizol (Invitrogen), and the results from qRT-PCR were normalized using the geometric mean methods with 2 housekeeping genes, HPRT, and 18S rRNA. Primary antibodies and RT-PCR primer sequences are summarized in Supplementary sTable 1 and sTable 4, respectively.

Statistical analysis

Unpaired 2-tailed Student t-test was performed to compare numerical variables in two sample groups. The Kaplan-Meier method was used to estimate the survival distributions with the log-rank test performed to compare the survival curves. The statistical analyses were performed with GraphPad Prism 7 or Stata v12 software. Statistical significance was set at a level of $p < 0.05$.

Results

xCT maintains cellular GSH store and prevents oxidative cell death in ABC-DLBCL cells.

We first examined the expression pattern of xCT in DLBCL cell lines. In a panel of 9 DLBCL cell lines examined, moderate-to-high levels of the xCT protein were detected in about half of those in the ABC category (4/9) but only 1 of 4 in the GCB subtype (Fig. 1A). Interestingly, 2 cell lines (Ly3-S and Ly10) express readily detectable xCT mRNA but have no xCT protein while the opposite is true for TMD8 (Supplementary Fig. 1A), suggesting roles played by post-transcriptional regulation. For additional experiments in this study, 3 ABC-DLBCL cell lines with readily detectable levels of xCT protein were studied, i.e. Riva, SuDHL2, and U2932. Detailed mechanistic analyses were performed in Riva and SuDHL2, both of which described as double-hit lymphomas^{8, 25}.

To pharmacologically suppress xCT activity, we used Sulfasalazine (SASP), a specific inhibitor of xCT-mediated cystine uptake¹¹. In each of the 3 cell lines tested, Riva, SuDHL2, and U2932, endogenous ROS was significantly elevated following 20 hrs of SASP treatment at individually determined IC50 concentrations (Fig. 1B, Supplementary Fig 1B). As expected, SASP markedly reduced the endogenous GSH pool although the kinetic response was different among the 3 cell lines. In Riva cells, SASP treatment triggered an initial rise in GSH level followed by a rapid return to the baseline at 8 hrs. In SuDHL2 cells, the GSH pool was completely depleted within 8 hrs. In U2932, a gradual decline was observed, resulting in only ~ 50% loss at 24 hrs (Fig. 1C) possibly due to a marked upregulation of the xCT protein following SASP treatment (Supplementary Fig. 1C). SASP-triggered ROS increase could be significantly attenuated by exogenous glutathione monoethyl ester (GEE) supplement (Fig. 1D), confirming that a diminished GSH pool was directly responsible for the ROS spike in SASP treated cells. We also examined cellular consequences of SASP treatment in Riva and SuDHL2. p-p38 signals were markedly increased at 12 and 24 hrs, indicating activation of the antioxidant response (Fig. 1E). Consistent with apoptotic cell death (PARP cleavage, Fig. 1E and data not shown), we detected reduction in pro-survival BCL2 family proteins (MCL1 and BCL2) and increase in pro-death BCL2 family members

(Bim and Bax) following SASP treatment (Fig. 1E). Collectively, these results suggest that at steady state, xCT protects ABC-DLBCL cells from oxidative cell death by enabling GSH synthesis and maintaining the endogenous GSH store.

Dox triggers an early surge in cellular GSH as the result of an xCT-dependent, anti-oxidant defense response.

Dox is the cornerstone ingredient of chemotherapy regimens for DLBCL. In our previous study, we showed that in 4 out of 6 ABC-DLBCL cell lines tested, Dox-induced cytotoxicity was dependent on oxidative stress. We found that the STAT3-regulated SOD2 can antagonize this process.²⁹ Other cellular redox regulators were not examined. As shown in Fig. 2A, Dox triggered a rapid decrease in the GSH store within 4 hrs followed by a quick rebound at 8 hrs. By 24 hrs, the GSH pool was depleted in U2932 while remaining well-above the basal levels in Riva and SuDHL2 cells, in which there appeared to be a more robust antioxidant response. This Dox-triggered GSH resurgence in Riva and SuDHL2 could be eliminated if cells were pre-treated with SASP (Fig. 2B). SASP pre-treatment also augmented Dox-triggered ROS build-up significantly (Fig. 2C).

To validate the role of xCT using a genetic approach, we established 2 xCT stable knock-down (KD) Riva cell lines using lentivirus-mediated gene knock-down (shxCT#1 and shxCT#3, Fig. 2D). Interestingly, in control cells (Ctrl), 24 hr Dox treatment led to nearly 3-fold increase in xCT protein level possibly because xCT is a redox-sensitive gene^{14,35}. In comparison, both the basal and Dox-induced xCT levels were much lower in the two KD cell lines with the reduction being more pronounced in shxCT#1 (Fig. 2D), which correlated with an impaired ability of these cells to replenish their GSH store after Dox treatment (Fig. 2E). Specifically, Dox triggered a 4.8-fold GSH increase in control cells compared to only 1.9-fold in shxCT#3. In shxCT#1, the pre-treatment level was only 47% of the control cells, which crashed to a mere 10% after 24-hr Dox exposure (Fig. 2E). Taken together, these results demonstrate that xCT is an integral part of the protective, antioxidant response that ABC-DLBCL cells mount following Dox exposure.

xCT inhibition sensitizes ABC-DLBCL cell lines to Dox-induced cytotoxicity.

Next, we investigated whether inhibiting xCT could potentiate Dox-induced oxidative stress and therapeutic response in ABC-DLBCL cells. As shown in Fig. 3A & 3B, in 48-hr cell viability assays, both Riva and SuDHL2 were highly resistant to Dox when it was used as a single agent. At the highest dose of Dox tested, 2 ug, ~ 50% Riva cells and ~60% SuDHL2 cells remained viable. Yet, when the cells were pretreated with SASP at IC10 or IC25 dosages, the Dox-resistant fraction was markedly reduced to 15-17% for Riva and 22-26% for SuDHL2. We also note that in Riva cells, the maximum kill was reached at 500 nM Dox under all conditions while SuDHL2 cells showed a dose response from 100 nM to 2 uM with and without SASP combination. Corroborating the SASP effect, xCT KD by shRNA also improved Dox sensitivity (supplementary Fig. S2). To characterize the nature of cell death, we measured apoptosis based on SYTOX Green and Annexin V double staining. In all three cell lines tested, the combination of Dox and SASP increased the fraction of Annexin V+ cells relative to either Dox or SASP treatment only

(Fig. 3C), confirming additive effects between these two drugs. Mechanistically, SASP synergized with Dox treatment to reduce MCL1 and increase BIM and BAX in Riva cells while BCL2 expression was largely unaffected. A similar trend was observed in SuDHL2 cells which express very little BCL2 and BIM (Fig. 3D). These changes in apoptosis regulators are consistent with more PARP cleavage being detected in cells treated with Dox/SASP combination. For c-Myc downregulation, a clear synergy between SASP and Dox was seen in Riva cells. In SuDHL2, while c-Myc was resistant to SASP, it was completely abolished by Dox and Dox/SASP combination. The pattern of p27/Kip1 alteration is unique. As single agents, both SASP and Dox reduced p27/Kip1, yet cells treated with Dox/SASP combination contained more of this protein than those in Dox treatment alone. The significance of this apparent anti-synergistic effect on p27/Kip1 is unclear (Fig. 3D). Taken together, these data suggest that xCT inhibition sensitized ABC-DLBCL cells to Dox-induced apoptosis through additive/synergistic effects on key cell death and cell cycle regulators.

Cell death triggered by Dox/SASP combination requires p38 activation.

To characterize the mechanism that connects oxidative stress and apoptosis in Dox-treated ABC-DLBCL cells, we examined the stress-activated protein kinases p38 and JNK in Riva and SuDHL2 cells 24 hours after single agent or combination treatment. As shown in Fig. 4A, untreated Riva and SuDHL2 cells did not contain activated p38, which was weakly activated by SASP alone, moderately activated by Dox alone and maximally activated by Dox/SASP combination. JNK has two isoforms, the p46 JNK1 and the p54 JNK2. In Riva cells, neither was activated by SASP but Dox activated them strongly and the Dox/SASP combination treatment activated them either further. A similar trend was seen in SuDHL2 cells except that p54 JNK2 already existed in an activated form in untreated and SASP-treated cells. Supporting the concept that a diminished GSH store is the mechanistic basis of p38 activation, exogenously supplied GSH efficiently antagonized p-p38 upregulation following Dox/SASP treatment (sFig. 4B). Next, we investigated the contribution of p38 activation to cell death with the use of SB203580, a specific p38 inhibitor. One-hour pretreatment with SB203580 was sufficient to inhibit p38 activity as evidenced by marked reduction of p-MAPKAPK-2, a downstream target protein of p38 in both Riva and SuDHL2 cells (Fig. 4B). In Riva cells, SB203580 substantially negated the SASP-associated sensitization effect on Dox cytotoxicity (Fig. 4C). In SuDHL2 cells, the SASP effect was nearly completely abolished by SB203580 pretreatment (Fig. 4D). Similar results were confirmed by direct measurement of apoptosis. Specifically, SB203580 reduced Dox/SASP-triggered apoptosis by 82% and 55%, in Riva and SuDHL2 cells, respectively (Fig. 4E). These observations indicate that p38 signaling is a major mediator of cytotoxicity triggered by Dox/SASP combination.

High xCT expression correlates with poor outcome in patients with non-GCB DLBCL but not GCB-DLBCL.

To examine the expression pattern and prognostic significance of xCT in DLBCL, we first detected xCT protein by IHC staining of diagnostic specimens from 87 DLBCL patients, all of which were treated with R-CHOP (Table 1). Representative examples of staining results are provided in Fig. 5A. Receiver operating characteristic curve analysis dichotomized samples into xCT high (n = 34) and low (n = 53) groups with a

cutoff value of 75% positive staining ($p = 0.046$, supplementary Fig. S4). These two groups have comparable clinic-pathological features including age distribution, Ann Arbor stage, LDH level, IPI score, and COO status (Table 1). Cases with high xCT expression showed a tendency towards shorter overall survival (OS) and progression-free survival (PFS) although the difference did not reach statistical significance (OS, $p = 0.273$; PFS, $p = 0.191$, Fig. 5 B, bottom row). We then performed the same analysis after separating the cases into the two COO subtypes defined using the Han's algorithm¹⁵. Among the 87 cases, 31 (35.6%) were classified as GCB-DLBCL while 56 (64.1%) fell into the non-GCB category. Survival analysis showed that within the non-GCB subtype, high xCT expression was significantly associated with inferior OS (5-years OS of 59.8% versus 87.0%, $p = 0.038$) and PFS (5-years PFS of 48.7% versus 78.4%, $p = 0.043$, Fig. 5B, top row). However, this prognostic association was absent among the GCB cases (5-years OS of 88.9% versus 78.0%, $p = 0.299$; 5-years PFS of 66.7% versus 68.4%, $p = 0.543$, Fig. 5B, middle row). These results suggest that the xCT-GSH axis is engaged in the therapeutic response of non-GCB DLBCLs, but not in GCB-DLBCLs.

xCT inhibition sensitizes ABC-DLBCL cell lines to Dox-induced cytotoxicity in ABC-DLBCL xenograft models.

Given our cell line-based mechanistic findings and the prognostic association of xCT among ABC-DLBCLs, we speculated that ABC (or IHC-defined non-GCB) cases with high xCT expression may have added capacity to replenish intracellular GSH stores when treated with R-CHOP, which is key to their poor treatment outcome. To test this hypothesis, we turned to mouse models. First, we evaluated the impact of xCT KD on in vivo tumor growth in NSG mice. Compared to the control Riva cell line (sh-Ctrl), the two xCT KD cell lines (sh-xCT#1 and #3) grew much more slowly (Fig 6A). In addition, at all 6 graft sites, sh-Ctrl tumors grew to > 400 mg, but only 3/6 and 1/8 tumors reached this size for the sh-xCT#1 and #3 groups (sFig 6A). Weight of tumors harvested at necropsy on Day 43 produced a similar result (sFig 6B), indicating a requirement for xCT for tumor initiation in vivo.

To generate proof-of-concept for therapeutic targeting of xCT, we next tested the CHOP/SASP combination treatment using the Riva-based NSG xenograft model. Tumor-bearing mice were randomly assigned to one of the 5 treatment arms when tumor size reached 250-300 mg, and the treatment began immediately. When used at 150 mg/kg, SASP alone did not show any activity against Riva tumors (Fig. 6B). When SASP was administered at the same time as the CHOP regimen, it did not provide any additional therapeutic benefit (Fig. 6B). Nevertheless, when SASP was used as a priming treatment and administered 3 days prior to the initiation of CHOP, a marked synergistic effect was observed after day 14 (Fig. 6B). On day 20, when compared to the CHOP response, the pre-SASP+CHOP treatment generated a tumor growth inhibition ratio (TGI) of 69.4%. These data indicate that xCT promotes in vivo growth of ABC-DLBCL cells and that targeting xCT can substantially sensitize CHOP-resistant ABC-DLBCLs to this first line regimen.

Discussion

We report in this study that the endogenous redox response pathway, specifically the xCT-GSH-ROS-p38 axis, is actively engaged during cellular response to Dox (Fig 6C). The role played by xCT is most likely specific to ABC-DLBCL as the prognostic value of xCT expression was detected only among non-GCB cases but not in GCB cases (Fig 5B). We made the novel discovery that in 3 ABC-DLBCL cell lines, including 2 that fall into the “double-hit” category, Dox-triggered ROS insurgence rapidly depleted the endogenous GSH pool (Fig 2), so that the death/survival outcome is critically dependent upon the cell's capability to replenish the GSH store. Accordingly, manipulations that either shored up the endogenous GSH level or inhibited p38 signaling significantly attenuated Dox-triggered apoptosis (Fig 1D, Fig 4B-E), while genetic and pharmacological suppression of xCT impaired GSH regeneration and sensitized ABC-DLBCL cells to Dox cytotoxicity (Fig 3, Fig 6).

To the best of our knowledge, we are the first to study the role of the xCT-GSH system in ABC-DLBCL. In addition to its function in lymphoma cells exposed to Dox-containing therapies, the xCT antiporter is likely important for spontaneous, in vivo development of DLBCL as well. This is because inhibiting xCT negatively impacted in vitro and in vivo proliferation of ABC-DLBCL cells (supplementary sFig 3, sFig 5). shxCT#1 reached a much deeper loss of xCT protein compared with shxCT#3. Accordingly, shxCT#1 showed a greater defect in GSH regeneration (Fig 2C-D). Yet, it was shxCT#3 that suffered a greater in vivo growth impairment, suggesting that the role played by xCT in the complex tumor microenvironment could be different from the in vitro setting. xCT is likely to have a physiological role in GCB-DLBCL, too, because as a group, the GCB lines had comparable SASP IC50 values compared to the ABC lines (Supplementary sFig 1B).

This study adds further support to the concept that oxidative stress plays a selective role in first line response of the ABC-DLBCL subgroup. We previously reported that Dox kills GCB-DLBCL cells through the classic pathway, i.e. by damaging DNA and triggering the DNA damage response; in contrast, in 4 of 6 ABC-DLBCL cell lines examined, Dox is largely excluded from the nucleus and trapped in cytoplasmic organelles including mitochondria where it generates ROS and causes oxidative cell death²⁹. Research into anthracycline resistance in solid tumors suggests that cytoplasmic trapping of Dox may be linked to a certain cellular metabolic state which, in turn, translates to alterations in mitochondria mass and pH gradient across the mitochondria membrane^{2, 16, 22}. The high level c-Myc expression found in many cancer cells, including the double-hit and double-expressor DLBCLs, has a profound influence on cell metabolism^{6, 7, 31}. It will be interesting to explore the potential link between persistent, high level c-Myc expression and the ROS-based Dox cytotoxicity in the future.

Our work also provides two relevant insights regarding the optimum approach to target xCT. First, the expression level of xCT only predicts poor prognosis in the non-GCB group of DLBCL patients treated with R-CHOP (Fig 5B). This suggests a possibility that a new prognosticator may be developed that combines the COO sub-classification with a xCT expression score. The majority of our results were obtained from Riva and SuDHL2, two ABC-DLBCL cell lines that fall into the double-hit lymphoma category. When larger patient cohorts can be accessed, it will be helpful to explore the relationship between xCT protein expression and prognostically relevant oncogenes, such as activated STAT3^{26, 29} and the c-Myc/BCL2

translocation status³¹. Second, the sequence of Dox/SASP combination is very important. For our in vitro experiments, SASP was always used as a pre-treatment 16 hrs prior to Dox. When two strategies were used to combine SASP and CHOP in the xenograft setting, however, very different outcomes were obtained. When SASP and CHOP were administered concurrently, SASP had no impact at all on tumor growth. In stark contrast, when animals were pretreated with the same dose of SASP 3 days prior to CHOP, potent synergy was generated (Fig 6B). Considering that in Dox-treated cells, the depletion and restoration of intracellular GSH is a very rapid process (Fig 2A), this result suggests that xCT suppression must be achieved in a priming step before the CHOP therapy.

Although our working model (Fig 6C) is supported by multiple lines of in vivo and in vitro evidences, its main premises await validation by independent studies. Furthermore, much remains to be learned about the mechanistic details underlying functional interactions between the xCT-GSH-ROS-p38 axis and Dox, especially in the R-CHOP treatment setting in patients. In the solid tumor arena, targeting cellular redox homeostasis in general and xCT in particular has attracted significant attention as a potential strategy to improve therapeutic outcomes. There was also an exciting discovery that xCT inhibition can suppress tumor growth while preserving antitumor immunity³. Recently, some spot light has also been given to the role of redox homeostasis in B-cell lymphoma development and therapeutics^{10, 12}. For a long time, xCT-directed cancer treatment has been hindered by the lack of potent and bioavailable xCT inhibitors. This is starting to change. A new xCT inhibitor was recently identified that is 100-fold more active than SASP and showed potent activity in KRAS-mutant lung cancer models¹⁸. A nontoxic approach to target the metastatic support from the cancer stroma has also demonstrated efficacy in nonhuman primates⁵. We look forward to more advances in this area and the eventual development of a redox-based strategy to improve the efficacy of R-CHOP in high risk DLBCLs.

Conclusion

xCT plays a critical role in sustaining in vivo GSH production and detoxification of Dox-induced ROS in ABC-DLBCL cells. High xCT expression predicted poor survival outcome in DLBCL patients treated with R-CHOP. Inhibiting xCT function not only prevented Dox-induced endogenous GSH increase, but also potentiated Dox-induced ROS accumulation and cytotoxicity in a p38-dependent manner. Most importantly, our findings in xenograft mouse model support the concept that xCT inhibition may hold significant promise to improve the first-line treatment outcome of patients with high risk DLBCL.

Abbreviations

ABC: activated B-cell-like

CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone

R-CHOP: rituximab plus CHOP

COO: cell-of-origin

DLBCL: Diffuse large B cell lymphoma

Dox: Doxorubicin

PFS: progression free survival

GCB: germinal center B-cell– like

GEE: glutathione monoethyl ester

GSH: glutathione; MFI: mean fluorescent intensity

OS: overall survival; ROS: reactive oxygen species

SASP: sulfasalazine.

Declarations

Ethics approval and consent to participate

Eighty-seven diagnostic samples were collected, with the informed consent of DLBCL patients, from Nanfang Hospital. This study was approved by the Ethics Committee of Southern Medical University affiliated Nanfang Hospital before study initiation.

Consent for publication

All authors reviewed and approved to the publication of our work entitled “Inhibiting the xCT antiporter sensitizes high risk DLBCL to doxorubicin-containing therapy” to *Journal of Experimental & Clinical Cancer Research*.

Availability of data and materials

Materials and data are available from the corresponding author according to NIH policies governing data and material sharing.

Competing interests

The authors declare no relevant conflict of interest.

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Authors' contributions

XW, RF, and BHY designed the research, XW, YW, JS, YM, FY, JZ performed experiments, XW, YW, and RF obtained IRB approval and collected clinical data, XW, YW, and BHY analyzed the data. BHY and RF supervised the study; XW and BHY wrote the manuscript.

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Tables

Table 1. Clinical characteristics of patients according to xCT expression.

Characteristics	Total	xCT		P-value
		low	High	
Age				0.995
≤60y	64(73.6%)	39(73.6%)	25(73.5%)	
>60y	23(26.4%)	14(26.4%)	9(26.5%)	
Gender				0.280
Female	29(33.3%)	20(37.7%)	9(26.5%)	
Male	58(66.7%)	33(62.3%)	25(73.5%)	
Systemic symptoms				0.498
A	55(63.2%)	35(66.0%)	20(58.8%)	
B	32(36.8%)	18(34.0%)	14(41.2%)	
Performance status				0.480
0-1	71(81.6%)	42(79.2%)	29(85.3%)	
2-4	16(18.4%)	11(20.8%)	5(14.7%)	
Extranodal sites				0.856
0-1	45(51.7%)	27(50.9%)	18(52.9%)	
≥2	42(48.3%)	26(49.1%)	16(47.1%)	
Ann Arbor stage				0.976
I/II	36(41.4%)	22(41.5%)	14(41.2%)	
III/IV	51(58.6%)	31(58.5%)	20(58.8%)	
LDH				0.163
Normal	44(50.6%)	30(56.6%)	14(58.8%)	
Elevated	43(49.4%)	23(43.4%)	20(41.2%)	
IPI				0.957
0-1	28(32.2%)	16(30.2%)	12(35.3%)	
2	27(31.0%)	17(32.1%)	10(29.4%)	
3	19(21.8%)	15(28.3%)	4(11.8%)	
4-5	13(14.9%)	5(9.4%)	8(23.5%)	
Ki-67				0.839

<80%	50(57.5%)	30(56.6%)	20(58.8%)
>80%	37(42.5%)	23(43.4%)	14(41.2%)
COO	0.958		
GCB	31(35.6%)	19(35.8%)	12(35.3%)
Non-GCB	56(64.4%)	34(64.2%)	22(64.7%)

Mann-Whitney test was applied to assess mean differences between groups.

Figures

Figure 1

xCT inhibition reduced cellular GSH stores leading to ROS accumulation in ABC-DLBCL cells. A, Protein expression of xCT examined by Western blotting in a panel of DLBCL cell lines. B, The effect of xCT inhibition on endogenous ROS levels in three ABC-DLBCL cell lines. Riva, SuDHL2, and U2932 were exposed to IC50 concentrations of SASP for 20 h. ROS measurement was based on mean fluorescence intensity (MFI) of DCFDA staining. C, Alterations in cellular GSH levels following SASP treatment at

different time points (0, 8, 16, 24 and 48 h). D, Exogenous GSH (GEE) markedly attenuated SASP-triggered endogenous ROS increase. E, Western blotting analysis of apoptosis related proteins in Riva and SuDHL2 treated with IC50 concentrations of SASP for 12 and 24 h. Bar graphs depict the mean $\pm$ SEM and represent two or three experiments. **, $p < 0.01$, ***, $p < 0.001$.

Figure 2

xCT inhibition suppressed Dox-induced initial surge in cellular GSH levels leading to augmented p38 and JNK activation. A, Changes in endogenous GSH levels following Dox treatment at indicated time points. B, Inhibiting xCT by SASP suppressed Dox-induced initial surge in cellular GSH levels. Riva and SuDHL2

cell lines were pre-treated with SASP for 16 h before exposure to IC50 concentrations of Dox for 20 h. The impact of xCT shRNA on endogenous xCT protein (C) and GSH levels (D) in untreated and Dox-treated Riva cells after 24 h. E, The effect of SASP on Dox-induced ROS in three ABC-DLBCL cell lines. Samples were pre-treated with SASP for 16 h before exposing to IC50 concentrations of Dox for 20 h. ROS measurement was based on mean fluorescence intensity (MFI) of DCFDA staining followed by flow cytometry. Bar graphs depict the mean $\pm$ SEM and represent two or three experiments. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

Figure 3

xCT inhibition by either SASP or shRNA-mediated gene silencing sensitizes ABC-DLBCL cell lines to Dox-induced cytotoxicity. Riva (A) and SuDHL2 (B) were pre-treated with the IC10 / IC25 concentrations of SASP overnight before exposure to different concentrations of Dox for 48 h. Cell viability was measured by the MTT assay. C, MTT assay-based cell viability assay in xCT KD Riva cell treated with different concentrations of Dox for 48 h. D, Western blotting analysis of apoptosis and cell cycle related markers in 2 double-hit cell lines, Riva and SuDHL2. Samples were pre-treated with SASP for 16 h before exposure to IC50 concentrations of Dox for 24 h. A-C each represents two or three experiments. Bar graphs depict the mean $\pm$ SEM and **, $p < 0.01$, ***, $p < 0.001$.

Figure 4

p38 activation is critical to cytotoxicity induced by Dox/SASP combination. A, Western blotting analysis of p-p38 and p-JNK in 2 double-hit lymphoma cell lines Riva and SuDHL2. Samples were pre-treated with SASP for 16 h before exposure to IC50 concentrations of Dox for 24 h. B, Western blot analysis of PARP, p-p38 and p-MAPKAPK-2 in Riva and SuDHL2 cells. Samples were pre-treated either with DMSO or with the p38 inhibitor, SB203580, for 1 h. Dox/SASP combination was administered as described for Fig. 3. Riva (C) and SuDHL2 (D) were pre-treated with SASP for 16 h and the p38 inhibitor, SB203580, for 1 h before exposure to different concentrations of Dox for 48 h. Viability was measured by the MTT assay. E, p38 inhibition suppressed apoptosis triggered by Dox/SASP combination. Riva and SuDHL2 were pre-treated with SASP for 16 h and the p38 inhibitor, SB203580, for 1 h before exposure to IC50 concentrations of Dox for 24 h. Annexin V and Sytox green staining was performed followed by flow cytometry. Apoptosis was defined as all Annexin V positive cells. C-E, each represents two or three experiments. Bar graph depicts the mean $\pm$ SEM and *, $p < 0.05$, **, $p < 0.01$.

A **B****Non-GCB** **GCB** **All cases** **Figure 5**

xCT expression correlates with poor outcome in patients with ABC-DLBCL but not GCB-DLBCL. A, Expression of xCT protein in DLBCL specimens was examined by IHC. Shown are images representative of results scored as (1): <25%, (2): 26-50%, (3): 51-75%, (4): >75%. B, Kaplan-Meier analysis of overall survival and progression-free survival according to xCT expression and cell-of-origin status.

Figure 6

SASP markedly enhanced the activity of CHOP in an ABC-DLBCL xenograft model. A, In vivo growth curves of 2 xCT shRNA KD and 1 control Riva cell line. B, NSG mice bearing established Riva tumors were treated with vehicle, SASP (150 mg / kg), CHOP, SASP+CHOP and pre-SASP (150 mg / kg 3 days before CHOP) + CHOP. Results presented are group means with error bars indicating standard error. Tumor weight in SASP+CHOP and pre-SASP+CHOP groups was compared to that in the CHOP group. 2-tailed t-test was performed, *, $p < 0.05$, **, $p < 0.01$. C, Overall working model depicting the role played by the xCT-GSH-ROS-p38 axis during Dox treatment.

Supplementary Files

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