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IRF1-mediated thioredoxin (TXN) protects multiple myeloma cells from ferroptosis by regulating polyunsaturated fatty acids (PUFAs) and PUFA-containing phospholipid metabolism

Aili He

heaili@xjtu.edu.cn

the Second Affiliated Hospital of Xi'an Jiaotong University https://orcid.org/0000-0003-2521-3806

Jiaxi Liu

the Second Affiliated Hospital of Xi'an Jiaotong University

Jiyu Miao

The Second Affiliated Hospital of Xi'an Jiaotong University

Yang Lv

The Second Affiliated Hospital of Xi'an Jiaotong University

Bingjie Fu

The Second Affiliated Hospital of Xi'an Jiaotong University

Xiaomin Ren

The Second Affiliated Hospital of Xi'an Jiaotong University

Xuezhu Xu

the Second Affiliated Hospital of Xi'an Jiaotong University

Gongzhizi Gao

The Second Affiliated Hospital of Xi'an Jiaotong University

Zujie Lin

The Second Affiliated Hospital of Xi'an Jiaotong University

Wenxiang Zhang

The Second Affiliated Hospital of Xi'an Jiaotong University

Bide Zhao

The Second Affiliated Hospital of Xi'an Jiaotong University

Ying Shen

the Second Affiliated Hospital of Xi'an Jiaotong University

Yachun Jia

Second Affiliated Hospital, Xi'an Jiaotong University Health Care Center

Wanhong Zhao

The Second Affiliated Hospital of Xi'an Jiaotong University

Rui Liu
the Second Affiliated Hospital of Xi'an Jiaotong University https://orcid.org/0000-0001-5229-829X

Article

Keywords: multiple myeloma, ferroptosis, PUFA, PUFA-PL, bortezomib, drug-resistance

Posted Date: March 10th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8988709/v1

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Additional Declarations: (Not answered)

Abstract

Multiple myeloma (MM) is a hematologic malignancy characterized by uncontrolled plasma cell proliferation. Patients with relapsed or refractory MM face poor survival, highlighting the need for novel therapeutic targets. Ferroptosis, an iron-dependent form of regulated cell death, is governed by a complex network of enzymes, proteins, pathways, and organelles, and has been implicated in various diseases. In this study, we identified thioredoxin (TXN), as a key suppressor of ferroptosis, was among the top ferroptosis-related genes linked to poor prognosis in MM patients. Functionally, TXN loss impaired MM progression by enhancing ferroptosis largely through regulating polyunsaturated fatty acids (PUFAs) and PUFA-containing phospholipids (PUFA-PLs), the major substrates for lipid peroxidation. TXN deficiency promoted ferroptosis by increasing PUFA biosynthesis and their incorporation into lipid peroxidation pathways. We further discovered that interferon regulatory factor 1 (IRF1), downregulated in MM, acts as a transcriptional repressor of TXN. Additionally, we demonstrated that bortezomib (BTZ)-resistant MM displayed elevated TXN expression, which enabled them to evade ferroptosis and diminished their sensitivity to BTZ.

In conclusion, our findings revealed that IRF1-mediated regulation of TXN modulates PUFA/PUFA-PL metabolism to protect MM cells from ferroptosis, establishing TXN as a promising therapeutic target for overcoming ferroptosis resistance and improving treatment outcomes in MM.

INTRODUCTION

Multiple myeloma (MM) is the second most common hematologic malignancy, driven by the uncontrolled clonal proliferation of malignant plasma cells in the bone marrow[1]. The age-adjusted global incidence of MM is approximately 2.1% in men and 1.5% in women, and MM caused more than 120,000 deaths in 2022[2, 3]. The introduction of proteasome inhibitors (PIs), monoclonal antibodies, and chimeric antigen receptor-engineered T cells (CAR-T)—has markedly improved patient outcomes[4]. Nevertheless, MM remains incurable; most patients relapse and eventually acquire drug resistance, underscoring the need to better understand disease pathogenesis and develop new therapeutic strategies.

Ferroptosis, first proposed by Dixon in 2012[5], is an iron-dependent form of regulated cell death (RCD) characterized by mitochondrial shrinkage, reduced cristae, and structural damage[6]. Unlike apoptosis or necrosis initiating by protein cascades, ferroptosis is governed by three interconnected processes: initiation, defense, and regulation network[7]. Initiation involves the Fenton reaction, in which ferrous iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) to generate reactive oxygen species (ROS). These ROS subsequently drive the oxidation of PUFA-PLs in cell membranes, causing lethal lipid peroxidation [5, 8]. The primary defense system relies on the glutathione peroxidase 4 (GPX4)–glutathione (GSH) axis[9], with radical-trapping antioxidants (RTAs) providing additional protection against lipid peroxidation[10]. Beyond these, a broad regulatory network of enzymes, proteins, pathways, and organelles modulates ferroptosis susceptibility[11, 12, 13, 14]. Over the past decade, ferroptosis has

been implicated in tumorigenesis [15] and immune escape[16], but its contribution to MM progression remains poorly defined.

TXN, an antioxidant protein that forms the thioredoxin system alongside thioredoxin reductase (TXNRD1) and NADPH, regulates multiple cellular processes including proliferation, antioxidant defense, apoptosis, and ferroptosis[17, 18, 19, 20]. Cancer cells frequently exploit TXN to buffer redox stressand support survival under hostile microenvironmental conditions[21]. For example, TXN maintains STAT3 in a reduced, active state, thereby promoting proliferation, metabolic reprogramming, and immune evasion [22]. Inhibition of TXN enhances p53 activity and triggers cell death in castration-resistant prostate cancer, underscoring its role in mutant p53-driven tumors[23]. Moreover, the tumor-promoting effect of TXN is partly attributable to its ability to suppress ferroptosis: the TXN inhibitor ferroptocide (FCD) efficiently induces ferroptosis in breast cancer cells[20], while TXN overexpression rescues GPX4 and GSH depletion in Parkinson's disease models, preventing ferroptosis[24]. Despite these findings, the role of TXN in MM remains unclear.

In this study, we demonstrate that TXN is highly expressed in MM and correlates with poor prognosis. Mechanistically, TXN promotes MM cell proliferation and ferroptosis resistance by regulating lipid peroxidation through the IRF1/TXN/PUFA/PUFA-PL axis. We further show that bortezomib (BTZ) resistance is linked to TXN upregulation, which enables MM cells to evade ferroptosis and reduces their sensitivity to BTZ. Together, these results identify TXN as a potential therapeutic target in MM.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Human multiple myeloma (MM) cell lines MM.1S, RPMI-8226, and NCI-H929 were obtained from ATCC (USA). ARD and KMS-11 cells were purchased from Procell (Wuhan, China), and OPM2 and U266 cells were kindly provided by Professor Jinsong Hu (Xi'an Jiaotong University Health Science Center, China). All cell lines were cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Biological Industries, Israel), 10,000 U/L penicillin, and 100 mg/L streptomycin (BioSharp, China). Cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Patient Samples

Bone marrow specimens were collected from MM patients at the Department of Hematology, Second Affiliated Hospital of Xi'an Jiaotong University (2019–2023). Sample collection and analysis were approved by the institutional ethics committee (Approval No. 2015186). Written informed consent was obtained from all patients for the use of their blood and bone marrow samples in this study.

Reagents

RSL3 (HY-100218A, purity ≥ 99.9%), Ferrostatin-1 (HY-100579 , purity ≥ 99.71%), Z-VAD (HY-164388, purity ≥ 99.20%), Necrostatin-1 (HY-15760, purity ≥ 99.89%), and Bortezomib (HY-10227, purity ≥ 98.0%)

was purchased from MCE. Erastin(HY-15763, purity ≥99.62%) was purchased from Solarbio. Ferroptocide (2505218-38-0, purity ≥ 90%) was purchased from Aladdin. All compounds were dissolved in DMSO, aliquoted, and stored at −80 °C.

Public Dataset Analysis and GSEA

Gene expression data and clinical information were retrieved from GEO (https://www.ncbi.nlm.nih.gov/geo/), including datasets GSE6477, GSE6691, GSE13591, GSE47552, GSE136400, GSE24080, and GSE31161. Ferroptosis-related genes were obtained from FerrDb (http://www.zhounan.org/ferrdb/current/). Differentially expressed genes were identified across GSE6477, GSE6691, GSE13591, and GSE47552. In GSE24080, patients were stratified into poor- (<2 years) and good-prognosis (>4 years) groups for target screening. TXN expression across MM progression (MGUS, SMM, NDMM, and RRMM) was analyzed using GSE6477. Associations with ISS and R-ISS stages were assessed in GSE24080 and GSE136400. Survival analysis was performed using Kaplan-Meier curves and log-rank tests ($p < 0.05$ considered significant).

Cell Viability Assays

Cell viability was measured using the CCK-8 kit (Beyotime, China). MM cells (7×10^3/well) were seeded in 96-well plates and treated with siRNA, single agents, or drug combinations for up to five days. At designated timepoints, 10 μL of CCK-8 solution was added to each well for 1-4 h before measuring absorbance at 450 nm. Synergy was assessed using CompuSyn software, with combination index (CI) <1 indicating synergy.

Gene Knockdown

TXN knockdown was achieved using siRNAs (GenePharma, China) or lentiviral shRNA constructs (GeneChem, China). IRF1 knockdown siRNAs were purchased from Stingke (Beijing, China). Transfection efficiency was confirmed by qRT-PCR and Western blot.

Lipid Peroxidation Assay

Lipid peroxidation was assessed using BODIPY™ 581/591 C11 (Thermo Fisher Scientific). Cells were incubated with the probe for 30 min at 37 °C, washed, and analyzed by flow cytometry. A fluorescence shift from red to green indicated lipid peroxidation.

Intracellular ROS Measurement

Cells were incubated with 10 μM DCFH-DA (Sigma-Aldrich) for 30 min at 37 °C. After washing, ROS levels were quantified by flow cytometry (excitation 488 nm, emission 525 nm).

RNA Sequencing

RNA-seq was performed on siNC- and siTXN-transfected RPMI-8226 cells, and on BTZ-sensitive and BTZ-resistant RPMI-8226 cells (LC-Bio Technologies, Hangzhou, China). Differential expression was analyzed using DESeq2. GO and KEGG analyses were performed to identify dysregulated pathways.

Lipidomic Analysis

Lipidomics was conducted on siNC- and siTXN-transfected RPMI-8226 cells, and on Ferroptocide-treated cells (8 μM, 6 h) (LC-Bio). Lipids were extracted using organic solvent precipitation and analyzed by LC-MS. Data processing was performed with XCMS. Metabolites were annotated using MS/MS spectra matched to an in-house library and the LIPID MAPS database. Pathway enrichment was analyzed using KEGG.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using a commercial kit (Beyotime, China). Crosslinked chromatin was sonicated and incubated with anti-IRF1 antibody or IgG control. Immunoprecipitated DNA was analyzed by qPCR. IRF1-binding motifs in the TXN promoter were predicted using JASPAR(https://jaspar.genereg.net/)[48].

Promoter Reporter and Dual-Luciferase Assay

The TXN promoter was cloned into pGL3 reporter vectors (Tsingke, China). Cells were co-transfected with firefly luciferase constructs, Renilla luciferase control plasmid, and where indicated, IRF1 siRNA. Luciferase activity was measured after 48 h using the Dual-Luciferase Reporter Assay System (Yeasen, China).

Apoptosis Assay

Apoptosis was assessed using Annexin V-PE/RedNucleus II (Bioscience, China). Cells were transfected with siRNA, stained, and analyzed by flow cytometry (FlowJo software).

Transmission Electron Microscopy (TEM)

Cells were fixed with 2.5% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated, embedded in epoxy resin, sectioned (~90 nm), stained with uranyl acetate and lead citrate, and imaged with a Hitachi HT7800 TEM a 120 kV.

Quantitative PCR (qPCR)

Total RNA from human samples, mouse tumors and MM cell lines was extracted using TRIzol reagent (GenStar, China) and reverse transcribed into cDNA using HiFiScript cDNA Synthesis Kit (Cwbio, China). qPCR was performed using SuperStar Universal SYBR Master Mix (Cwbio, China) according to the manufacturer's protocol. The primers used are listed in Table S1.

Immunoblotting

Total protein from human samples, mouse tumors and MM cell lines was extracted with RIPA lysis buffer (Cwbio, China), and the protein concentration was measured using the BCA Protein Quantification Kit (Yeasen, China). The primary antibodies used were as follows: anti-TXN (1:5000, 12 kDa, 66475-1-Ig, Proteintech, China), anti-GAPDH (1:5000, 36 kDa, 10494-1-AP, Proteintech, China), anti-IRF1 (1:2000, 50 kDa, Proteintech, China).

Animal Studies

All animal experiments were approved by the Ethics Committee of Xi'an Jiaotong University Health Science Center (Approval No. 2025-2662) and were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. Male BALB/c nude mice (3 weeks old) were purchased from GemPharmatech (China). Mice were allowed to acclimate for at least one week prior to any experimental procedures. Mice were housed under specific pathogen-free (SPF) conditions in individually ventilated cages (IVCs).

Animal health and welfare were monitored daily by trained personnel. To establish subcutaneous xenograft models, RPMI-8226 cells stably transduced with LV-NC and LV-TXNkd were injected into the right/left flank of each mouse. Tumor dimensions (length and width) were measured every two days using a caliper, and tumor volume was calculated using the formula: Volume (mm^3) = ($width^2 \times length$) / 2. Once tumors reached 100 mm^3, mice received intraperitoneal BTZ (0.5 mg/kg, every other day) or PBS.

Mice were humanely euthanized when any individual tumor diameter reached 15 mm to prevent undue suffering. Euthanasia was performed by carbon dioxide (CO_2) asphyxiation in a gradual-fill chamber, followed by cervical dislocation as a secondary confirmation of death. Immediately after euthanasia, tumors were resected, weighed, and divided for subsequent analyses: one portion was fixed in 4% paraformaldehyde for immunohistochemistry (IHC), and the remainder was snap-frozen in liquid nitrogen for ROS and lipid ROS assays.

Tumor size/burden limitations and compliance: The animal study protocol, as approved by the Ethics Committee of Xi'an Jiaotong University Health Science Center, stipulated a maximum permitted tumor burden of 1.5 cm in the longest diameter to prevent undue animal suffering. Throughout the study, tumor growth was closely monitored. We confirm that this maximum tumor size/burden was not exceeded in any animal. Humane endpoints were strictly enforced to ensure compliance with this ethical standard. In accordance with journal policy, no images of live animals bearing tumors are presented; all excised tumors were photographed adjacent to a scale ruler for size documentation.

Statistical Analysis

All experiments were performed with 3-6 biological replicates. Differences between two groups were analyzed using Student's t-test or Mann-Whitney U test. Multiple group comparisons were performed by one-way ANOVA with Bonferroni post hoc test. $p < 0.05$ was considered statistically significant.

RESULTS

TXN is highly expressed in MM and associated with poor prognosis

To identify ferroptosis-related genes linked to aggressive MM, we first analyzed four MM-related RNA sequencing datasets from GEO (GSE6477, GSE6691, GSE13591, and GSE47552) and identified 14 differentially expressed genes (DEGs) were identified between MM patients and healthy donors (Fig. 1a), including CKS2 [25], GADD45A [12], and TXN, which are associated with cancer progression and ferroptosis. Next, we examined prognosis-related ferroptosis regulators using the GSE24080 dataset and ferroptosis-related gene sets from FerrDb. Comparing patients with good prognosis (overall survival >4 years) versus poor prognosis (overall survival <2 years), gene set enrichment analysis (GSEA) revealed 41 ferroptosis suppressor genes associated with poor outcomes (Fig. 1b). TXN was among these prognosis-related suppressors.

Comprehensive analysis across GEO datasets showed that TXN expression was significantly higher in MM compared with normal plasma cells (NPCs), monoclonal gammopathy of undetermined significance (MGUS), and smoldering MM (SMM) (Fig. 1c). High TXN expression correlated with advanced International Staging System (ISS) and Revised ISS (R-ISS) stages (Fig. 1d-e) and was significantly elevated in relapsed versus newly diagnosed patients, indicating a role in disease recurrence (Fig. 1f). Kaplan–Meier analysis confirmed that high TXN expression predicted shorter overall survival (OS) and event-free survival (EFS) (Fig. 1g-h).

Furthermore, we examined TXN expression in both MM patients and MM cell lines. Compared with healthy donors, MM patients showed higher TXN expression than healthy donors, with relapsed/refractory MM (RRMM) patients exhibiting higher levels than newly diagnosed MM (NDMM) patients (Fig. 1i). TXN expression also increased with more advanced ISS and R-ISS stages (Fig. 1j). MM cell lines (MM.1S, U266, ARD, RPMI-8226, H929, OPM2, and KMS-11) expressed higher TXN at both mRNA and protein levels compared with bone marrow stromal cells (HS-5) (Fig. 1k).

Taken together, these findings suggested that TXN, as a key regulator of ferroptosis, is highly expressed in MM and orrelates with advanced disease stage and poor prognosis.

Silencing TXN inhibited MM cell proliferation and tumor growth

To determine whether TXN contributes to MM progression through ferroptosis, we silenced TXN expression in two human MM cell lines with high endogenous TXN levels (RPMI-8226 and MM.1S) using specific small interfering RNAs (siRNAs) (Fig. 2a-b). CCK-8 assays demonstrated that TXN knockdown (siTXN) significantly reduced cell viability compared with negative control siRNA-treated cells (siNC) (Fig. 2c). Similarly, treatment with the TXN inhibitor Ferroptocide (FCD) decreased MM cell viability (Fig. 2d, Supplementary Fig. 1d-e). Notably, TXN knockdown did not significantly affect apoptosis in MM cells (Supplementary Fig. 1a-b), suggesting that reduced proliferation rather than apoptosis contributed to the observed growth inhibition.

To evaluate the effects of TXN inhibition *in vivo*, we generated stable TXN knockdown MM cell lines using lentiviral transduction (LV-TXNkd) (Supplementary Fig. 1c). BALB/c nude mice injected with LV-TXNkd MM cells exhibited significantly reduced tumor burden than controls (Fig. 2e), concomitant with successful knockdown of TXN protein (Fig. 2f). Slower tumor growth, and higher body weight compared with mice bearing control LV-NC MM cells (Fig. 2g-h). Immunohistochemistry (IHC) analysis confirmed markedly decreased TXN and Ki-67 expression in tumors derived from LV-TXNkd cells relative to controls (Fig. 2i, Supplementary Fig. 1f).

Collectively, these findings indicate that TXN is critical for MM cell proliferation and tumor progression, highlighting its potential as a therapeutic target in MM.

TXN protects MM cells from lipid peroxidation-induced ferroptosis

To further investigate whether TXN promotes MM progression by suppressing lipid peroxidation-induced ferroptosis, we measured lipid ROS levels in TXN-knockdown MM cells. Compared with control siRNA-treated cells (siNC), TXN knockdown (siTXN) significantly increased lipid ROS (Fig. 3a) and total intracellular ROS levels (Fig. 3b). Similarly, treatment with the TXN inhibitor Ferroptocide (FCD) markedly elevated both lipid ROS and total ROS in MM cells (Fig. 3c-d).

To confirm that these effects were mediated by ferroptosis, we performed rescue experiments using ferroptosis inhibitor Ferrostatin-1 (Fer-1). Fer-1 significantly reversed FCD-induced lipid peroxidation, ROS accumulation, and cell death, whereas the apoptosis inhibitor Z-VAD and the necroptosis inhibitor Necrostatin-1 (NEC-1) had minimal effect (Fig. 3e-f, Supplementary Fig. 2a). Consistent with these *in vitro* findings, tumor cells derived from mice bearing LV-TXNkd MM cells exhibited elevated lipid ROS and total ROS compared with LV-NC controls (Fig. 3g-h). IHC results further confirmed increased levels of 4-Hydroxynonenal (4-HNE), a lipid peroxidation byproduct and well-recognized ferroptosis marker, in tumors from LV-TXN knockdown mice (Fig. 3i, Supplementary Fig. 2b).

To further validate the role of TXN in ferroptosis, we treated TXN-knockdow MM cells with the ferroptosis inducer RSL3. siTXN cells displayed higher lipid peroxidation and ROS levels than siNC cells (Supplementary Fig. 2c-d). Additionally, we examined mitochondrial morphology in MM cells treated with siNC, siTXN, FCD (8 μM, 6 h), or RSL3 (5 μM, 6 h) using transmission electron microscopy (TEM). Mitochondria in TXN-deficient, FCD-treated, or RSL3-treated cells exhibited classic ferroptotic changes, including swelling, reduced electron density, and cristae loss (Fig. 3j).

Taken together, these results demonstrate that TXN protects MM cells from lipid peroxidation-induced ferroptosis, highlighting its role as a key ferroptosis suppressor in MM.

Negligible impact of TXN knockdown on transcriptomic profiles

To explore how TXN regulates lipid peroxidation-induced ferroptosis in MM cells, we performed transcriptomic sequencing in MM cells transfected with either siNC or siTXN. Surprisingly, TXN knockdown induced only modest changes in gene expression, with 115 genes upregulated and 139 genes downregulated, and did not significantly alter classical ferroptosis-related genes such as SLC7A11, GPX4, or ACSL4 (Fig. 4a). DEGs were subsequently subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. No significant enrichment of ferroptosis pathways was observed in TXN-deficient cells (Fig. 4b-d, Supplementary Fig. 3a-b). However, DEGs were enriched in PUFA-related enzymatic activities and PUFA metabolism pathways, including linolenic acid and arachidonic acid (AA) epoxygenase activity (Fig. 4b-d).

These results suggest that TXN, as an antioxidant protein, was more likely functions downstream in the redox cascade—modulating the terminal stages of lipid peroxidation—without broadly impacting upstream gene expression networks. Based on this, we focused subsequent investigations on the regulation of PUFA metabolism.

TXN knockdown promoted ferroptosis by facilitating PUFA and PUFA-PL biosynthesis

Given the potential role of TXN in lipid metabolism, we performed a comprehensive lipidomic profiling of TXN-knockdown (TXN-KD) MM cells using liquid chromatography-mass spectrometry (LC-MS) (Fig. 4e). Cells treated with the TXN inhibitor Ferroptocide (FCD, 8 μM, 6 h) were also profiled. Over 2,000 lipid metabolites were identified (Fig. 4f) and classified into six major categories: fatty acids (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), and prenol lipids (PR). In total, 1,969 lipid species were quantified, of which 183 were significantly altered in TXN-KD cells compared with controls (Fig. 4g-h). KEGG enrichment analysis revealed significant enrichment in PUFA metabolism pathways, consistent with transcriptomic results (Fig. 4i). A regulatory network based on KEGG annotations highlighted alterations in linoleic acid, α-linolenic acid, and arachidonic acid metabolism (Fig. 4j).

Global lipid composition analysis revealed that glycerophospholipids (GP) constituted the largest proportion of lipids, followed by GL and SP (Fig. 5a). FCD treatment increased GL and decreased GP, prompting a detailed analysis of GP subclasses, which included phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI)—collectively referred to as phospholipids (PLs). Cardiolipin (CL), lysophospholipids (Lyso-PL), and phosphatidylinositol phosphates (PLP) were also included in the GP category, with PLs being the most abundant (Supplementary Fig. 4a). The relative composition of PLs was broadly similar across groups, dominated by PC and PE (Fig. 5b). PLs were further subclassified into diacyl-PLs, ether-linked PLs (PL-O), and vinyl ether-linked PLs (PL-P), with diacyl-PC and diacyl-PE prevailing in all groups (Fig. 5c, Supplementary Fig. 4b).

Analysis of FA composition revealed that TXN deficiency, whether by knockdown or pharmacological inhibition, induced broad alterations in FA profiles (Fig. 5d-e). Notably, PUFA abundance was significantly increased in both TXN-KD and FCD-treated cells (Fig. 5f-g), including key substrates for PL biosynthesis

such as FA 22:4 and FA 20:5, as well as FA 18:2 (linoleic acid), FA 18:3 (α-linolenic acid), and FA 22:5 (docosapentaenoic acid) (Supplementary Fig. 4c). Treatment with AA further confirmed the role of PUFA in ferroptosis, significantly reducing MM cell viability in a dose-dependent manner (Supplementary Fig. 4d).

PLs were categorized by fatty acid content into saturated (SFA-PLs), monounsaturated (MUFA-PLs), and polyunsaturated (PUFA-PLs), the latter serving as terminal substrates for lipid peroxidation during ferroptosis. TXN knockdown altered both MUFA-PLs and PUFA-PLs (Fig. 5h, Supplementary Fig. 4e), while FCD treatment caused more pronounced increases in PUFA-PLs (Fig. 5i, Supplementary Fig. 4f). Then, we calculated an estimated cellular phospholipid peroxidation index (CPI) based on the known relative hydrogen atom transfer propagation rate constants of fatty acids[26, 27]. The results showed that FCD-treated cells had the highest CPI, with TXN-KD cells showing a trend toward increased CPI (Fig. 5j). Analysis of PL double bond content showed no significant change in TXN-KD cells, whereas FCD treatment enriched PLs with more than two double bonds (Supplementary Fig. 4g-h), further supporting enhanced ferroptosis susceptibility. Finally, examination of specific PUFA-PL species showed that TXN knockdown increased PUFA-PC 22:4 levels, a key pro-ferroptotic lipid (Fig. 5m), whereas FCD treatment broadly elevated total PUFA-PLs, reduced MUFA-PLs, and specifically increased PUFA-PC and PUFA-PE species containing 22:4 and 20:4 (Fig. 5k-o).

In summary, TXN deficiency enhances PUFA and PUFA-PL biosynthesis, indicating that TXN protects MM cells from lipid peroxidation-induced ferroptosis by suppressing the formation of pro-ferroptotic lipids.

IRF1 promoted ferroptosis in MM cells by repressing TXN expression

To identify upstream regulators of TXN in ferroptosis, we analyzed the promoter of *TXN* gene using the NCBI, JASPAR, and UCSC Genome Browser databases. These analyses revealed several candidate transcription factors (TFs), including KLF5, EGR1, and IRF1. To determine which TF regulates TXN in MM, we examined their expression in bone marrow samples from healthy donors and MM patients and performed Kaplan-Meier survival analysis. Among the candidates, IRF1 was significantly downregulated in MM patients compared with healthy donors (Fig. 6a), and lower IRF1 expression correlated with poorer overall survival (Fig. 6b). An analysis of MM patient samples demonstrated a significant inverse correlation between the expression of IRF1 and TXN (Fig. 6c).

Next, we knocked down IRF1 in MM cells using siRNA. This led to a marked increase in TXN expression at both the mRNA and protein levels (Fig. 6d-e), suggesting that IRF1 may functions as a transcriptional repressor of TXN. To confirm this regulatory relationship, we identified a potential IRF1-binding motif on the TXN promoter using JASPAR (Fig. 6f) and performed chromatin immunoprecipitation (ChIP) assays. These assays demonstrated strong IRF1 binding to the TXN promoter in MM.1S cells (Fig. 6g-h). We then constructed PGL3-BASIC luciferase reporter plasmids containing either the wild-type (WT) or mutated (Mut) IRF1-binding motif. Dual-luciferase assays showed that IRF1 knockdown partially restored TXN promoter activity (Fig. 6i), further supporting its role as a negative regulator of TXN transcription.

Finally, we assessed whether IRF1 modulates ferroptosis in MM cells through TXN. While IRF1 knockdown alone did not significantly affect lipid ROS levels, it markedly reduced RSL3-induced lipid ROS and total ROS accumulation (Fig. 6j), indicating that downregulating IRF1 could relieve RSL3-induced ferroptosis in MM cells by upregulating TXN.

In summary, IRF1 is downregulated in MM and acts as a transcriptional repressor of TXN. Reduced IRF1 expression elevates TXN levels, which protects MM cells from ferroptosis and may contribute to disease progression.

TXN deficiency-induced ferroptosis synergized with bortezomib to effectively target MM cells

Bortezomib (BTZ), the first proteasome inhibitor approved by the U.S. Food and Drug Administration (FDA) for multiple myeloma (MM) [28], exerts its therapeutic effects through multiple mechanisms, including the induction of apoptosis [29] , endoplasmic reticulum and Golgi stress [30], and disruption of diverse cellular pathways [31]. We hypothesized that ferroptosis may act synergistically with BTZ to enhance its anti-myeloma efficacy. To test this, we treated MM cells with the ferroptosis inducers RSL3 or erastin in combination with BTZ. Compared to monotherapy, the combination treatment significantly enhanced the inhibition of MM cell viability (Fig. 7a-b). Consistently, co-treatment with the TXN inhibitor FCD and BTZ further suppressed cell viability compared with either agent alone (Fig. 7c), suggesting that TXN inhibition can potentiate the anti-myeloma effect of BTZ. The calculated combination index (CI) values, which quantify the drug synergy, are summarized in Table 1.

Table.1 Synergistic effect of bortezomib and ferroptosis inducers

BTZ (nM)	RSL3 （μM）	RPMI-8226		MM.1S	
		Effect	CI	Effect	CI
12.5	0.1	0.68299	0.50239	0.68797	0.74150
12.5	0.2	0.51537	0.51607	0.41920	0.67980
12.5	0.4	0.44158	0.78583	0.32532	0.99838
25.0	0.1	0.56054	0.34467	0.58795	0.73805
25.0	0.2	0.39133	0.34737	0.35666	0.67694
25.0	0.4	0.33386	0.54319	0.22850	0.82191
50.0	0.1	0.41539	0.22930	0.26200	0.47361
50.0	0.2	0.32644	0.28802	0.21705	0.57825
50.0	0.4	0.25912	0.41062	0.19542	0.84727
	Erastion （μM）	Effect	CI	Effect	CI
12.5	5.0	0.88105	1.33501	0.97224	3.56292
12.5	10.0	0.74353	1.18148	0.91211	1.73011
12.5	20.0	0.56578	1.27673	0.69590	0.76883
25.0	5.0	0.83072	1.24596	0.44208	0.20032
25.0	10.0	0.64335	0.95094	0.14310	0.06821
25.0	20.0	0.44096	0.95541	0.12395	0.08961
50.0	5.0	0.70118	0.96899	0.35307	0.24255
50.0	10.0	0.25657	0.33671	0.11693	0.08278
50.0	20.0	0.23386	0.53647	0.06712	0.06603
	Ferrotpcide（μM)	Effect	CI	Effect	CI
25.0	1.0	0.71841	0.65960	0.80794	0.55951
50.0	1.0	0.65624	0.79739	0.76495	0.46769
100.0	1.0	0.59104	0.99473	0.72070	0.41067
25.0	2.0	0.50542	0.51309	0.71820	0.46462
50.0	2.0	0.44228	0.55140	0.74633	0.61884
10.0	2.0	0.38902	0.62127	0.64657	0.38916
25.0	4.0	0.30667	0.76359	0.71285	0.86104

BTZ (nM)	RSL3 (μM)	RPMI-8226		MM.1S	
		Effect	CI	Effect	CI
50.0	4.0	0.27898	0.77853	0.60198	0.58272
100.0	4.0	0.23881	0.79394	0.53656	0.47799

Two multiple myeloma cell lines (RPMI-8226 and MM.1S) were treated with bortezomib (BTZ) in combination with either RSL3, erastin, or ferroptocide at the indicated concentrations for 24 h. “Effect” corresponds to cell viability. The combination index (CI) was calculated using CompuSyn software to evaluate drug synergy, where CI < 1 indicates a synergistic interaction.

We next evaluated this synergistic effect *in vivo*. Nude mice were subcutaneously injected with RPMI-8226 cells transfected with either LV-NC or LV-TXNkd. Once tumors reached ~100 mm^3, mice received intraperitoneal BTZ (0.5 mg/kg, every other day) or PBS (Fig. 2g). As expected, BTZ alone suppressed tumor growth, but the LV-TXNkd + BTZ group showed a significantly greater reduction in tumor volume and growth rate compared with the LV-NC + BTZ group (Fig. 7d-e).

Collectively, these findings demonstrated that TXN deficiency-induced ferroptosis could synergize with BTZ both to suppress MM progression *in vitro* and i*n vivo*.

TXN overexpression promotes bortezomib resistance in MM cells via ferroptosis evasion

To investigate whether TXN contributes to BTZ resistance, we established BTZ-resistant (BTZ-R) RPMI-8226 and OPM2 cell lines by stepwise exposure to increasing BTZ concentrations. RNA sequencing showed TXN was significantly upregulated in BTZ-R RPMI-8226 cells (Fig. 8a), which was validated by RT-qPCR and Western blot (Fig. 8b).

We next examined ferroptosis sensitivity. When treated with the ferroptosis inducer RSL3, BTZ-R RPMI-8226 cells exhibited significantly higher viability than parental cells (IC50: 0.981 μM vs. 1.784 μM), and similar results were observed in BTZ-R OPM2 cells (IC50: 2.059 μM vs. 6.462 μM) (Fig. 8c-d). Consistently, BTZ-R MM cells accumulated less lipid ROS and total ROS than parental cells following RSL3 treatment (Fig. 8e-f), indicating that ferroptosis evasion contributes to BTZ resistance, likely through elevated TXN expression.

To directly assess the role of TXN, we established stable TXN knockdown in BTZ-R RPMI-8226 cells via lentiviral transduction (Fig. 8g). TXN knockdown increased lipid ROS and total ROS levels in BTZ-R cells (Fig. 8h), suggesting that loss of TXN restores susceptibility to lipid peroxidation–driven ferroptosis. Moreover, RSL3 treatment further reduced cell viability in TXN-deficient BTZ-R cells compared with controls (IC50: 2.044 μM vs. 1.868 μM) (Fig. 8h).

In summary, BTZ-resistant MM cells evade ferroptosis by upregulating TXN, thereby reducing their sensitivity to BTZ. Targeting TXN may therefore help overcome BTZ resistance in MM.

DISCUSSION

Ferroptosis, a recently recognized form of RCD, has emerged as a critical process in cancer biology and a promising therapeutic target[5]. MM is a highly heterogeneous hematological malignancy. Dysregulated iron and lipid metabolism in MM contributes to uncontrolled cell proliferation, abnormal differentiation, and drug resistance[32, 33], and may also influence the process of ferroptosis. Yet, the role of ferroptosis in MM progression has remained unclear. In this context, we identify thioredoxin (TXN) as a ferroptosis suppressor in MM. Mechanistically, TXN limits lipid peroxidation and protects cells from ferroptotic death through the IRF1–TXN–PUFA/PUFA-PL axis. We also show that BTZ-resistant MM cells upregulate TXN, enabling ferroptosis evasion and diminishing drug sensitivity. Together, these findings establish TXN as a central regulator of ferroptosis and a potential therapeutic target in MM, especially in the setting of drug resistance.

TXN is a key antioxidant protein that maintains redox homeostasis by reducing protein disulfides and modulating redox-sensitive signaling pathways. Prior studies have implicated TXN in ferroptosis regulation. For example, Bai et al.[24] showed that TXN protects against MPP+/MPTP-induced ferroptosis through GPX4 upregulation. While in lung adenocarcinoma, pharmacological activation of retinoic acid receptor α enhanced TXN expression and suppressed ferroptosis[34]. These observations underscore the relevance of the System XC^-/Cys/TXN/TXNRD axis in ferroptosis control. However, the ferroptosis-related role of TXN in MM has been largely unexplored. Our data demonstrate that TXN is significantly upregulated in MM, correlating with disease progression, advanced stage, relapse, and poor prognosis. Both genetic silencing and pharmacological inhibition of TXN reduced MM cell viability and triggered ferroptosis, consistent with prior work showing that thioredoxin inhibitors such as ferroptocide induce ferroptosis in solid tumors[35].

A defining feature of ferroptosis is the peroxidation of PUFAs incorporated into phospholipids, particularly those with chains longer than 20 carbons[36, 37, 38]. Our lipidomic analysis showed that TXN knockdown or pharmacological inhibition enriched PUFA species, such as FA 18:2, FA 18:3, FA 20:5, FA 22:4, and FA 22:5. Treatment with arachidonic acid (AA, FA 20:4) alone induced MM cell death in a dose- and time-dependent manner, supporting the functional role of PUFA enrichment. Consistently, Lee et al.[36] conducted a lipid profiling analysis in the mesenchymal-type gastric cancer cells following upregulation of ELOVL5 and FADS1. Their results showed enrichment of AA (FA 20:4) and adrenic acid (AdA, FA 22:4), which are required for ferroptosis, thereby enhancing ferroptotic sensitivity. Dierge et al[34]. reported that excess uptake of n-3 and n-6 PUFA could trigger ferroptosis in cancer cells under ambient acidosis. Collectively, these findings suggest that PUFAs are primary targets of lipid peroxidation in ferroptosis, and modulation of PUFA biosynthesis can influence ferroptotic sensitivity across different cancer cells. PUFA-PLs are equally critical, as their incorporation into membranes makes them primary substrates for lethal lipid peroxidation. We observed that TXN knockdown preferentially elevated PUFA-PC species containing FA 22:4, while pharmacological inhibition produced a broader increase in PUFA-PLs, especially PUFA-PC and PUFA-PE species containing FA 20:4 and FA 22:4. This suggests that pharmacological inhibition exerts a stronger metabolic effect than knockdown alone.

Kagan et al.[37] performed global redox phospholipidomics LC-MS/MS analysis of RSL3-treated Pfa1 cells, demonstrating that AA- and AdA-containing phospholipid species were most responsive, highlighting these species as key lipid death signals. Additionally, Morgan et al.[39] demonstrated that variations in PUFA-PLs content underpin the differential susceptibility of immune cells to ferroptosis, specifically, with low PUFA-PL content conferring resistance in activated neutrophils. Collectively, our findings align with reports that AA- and AdA-containing phospholipids act as key death signals and that PUFA-PL content determines cell-type-specific ferroptotic susceptibility.

We further identified IRF1 as a direct transcriptional repressor of TXN. IRF1 downregulation in MM was associated with worse prognosis, and IRF1 knockdown increased TXN levels and reduced ferroptotic sensitivity. IRF1 has been widely studied for its tumor-suppressive functions[40, 41]. These findings extend the tumor-suppressive role of IRF1 and establish a new regulatory axis in which IRF1 represses TXN to promote ferroptosis.

To explore therapeutic relevance, we combined BTZ with the TXN inhibitor FCD or ferroptosis inducers. Co-treatment produced strong synergy in suppressing MM cell viability *in vitro* and tumor growth *in vivo*. This suggests that the efficacy of BTZ can be enhanced by promoting ferroptosis. Prior studies support this concept: RSL3 and BTZ have been shown to synergize through glutathione depletion[42]; AP-1 inhibition sensitizes MM cells to BTZ by inducing ferroptosis[43]; and BTZ promotes ferritinophagy-mediated iron release, reinforcing synergy with ferroptosis inducers[44]. Importantly, we demonstrated that BTZ-resistant MM cells display reduced sensitivity to ferroptosis and upregulate TXN, suggesting that TXN-mediated ferroptosis evasion contributes to drug resistance. Similar mechanisms have been reported in other malignancies, where ferroptosis modulation impacts BTZ sensitivity[45, 46]. TXN has also been implicated in BTZ resistance through mitophagy suppression and mTOR/ERK activation, underscoring its multifaceted role in therapy resistance[47]. Together, these findings indicated that ferroptosis dysregulation underlies BTZ resistance in MM, and that targeting TXN or related pathways may provide a strategy to overcome relapse.

However, our study has several limitations. Firstly, most of our functional experiments were performed using MM cell lines rather than primary MM cells from patients. Secondly, it remains unclear whether the combination treatment could impact extramedullary infiltration of MM.

In summary, we show that TXN protects MM cells from ferroptosis by suppressing PUFA and PUFA-PL accumulation, and that IRF1 represses TXN transcription to enhance ferroptotic sensitivity. Elevated TXN expression contributes to BTZ resistance by enabling ferroptosis escape. These findings identify TXN as a potential therapeutic target for MM and highlight ferroptosis modulation as a promising strategy to improve outcomes, particularly in relapsed or refractory disease.

Declarations

ACKNOWLEGEMENT

We acknowledge the colleagues at the Department of Hematology, The Second Affiliated Hospital of Xi'an Jiaotong University, for their assistance in sample collection.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The raw sequencing data generated in this study have not been deposited in a public repository at the time of submission. These data will be made available in the NCBI Gene Expression Omnibus (GEO) prior to publication. During the review process, the data are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study involving human participants was approved by the Institutional Ethics Committee of The Second Affiliated Hospital of Xi'an Jiaotong University (Approval No.: 2015186). The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. All participants provided written informed consent prior to participation. The animal experiments were approved by the Ethics Committee of Xi'an Jiaotong University Health Science Center (Approval No.: 2025-2662). All procedures were conducted in accordance with institutional and national guidelines for the care and use of laboratory animals. The approved protocol specified a maximum tumor size limit, which was not exceeded during the study.

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