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The Ferroptosis Inducer Erastin Promotes Proliferation and Differentiation in Human Peripheral Blood Mononuclear Cells

Ding Wang^{a,*}, Nan Xie^b, Wanli Gao^a, Rui Kang^c, and Daolin Tang^{a,c,*}

^aThe Third Affiliated Hospital, Center for DAMP Biology, Key Laboratory for Major Obstetric Diseases of Guangdong Province, Key Laboratory of Protein Modification and Degradation of Guangdong Higher Education Institutes, School of Basic Medical Sciences, Guangzhou Medical University, Guangzhou, Guangdong, 510510, China

^bDepartment of Oral Pathology, Guanghua School of Stomatology, Research Institute of Stomatology, Guangdong Province Key Laboratory of Stomatology, Sun Yat-sen University, Guangzhou, Guangdong 510055, P.R. China.

^cDepartment of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania 15219, USA

Abstract

Peripheral blood mononuclear cells (PBMCs) contain multipotent progenitor cell populations and possess the potential to differentiate into various types of immune cells under both physiological and pathological conditions. Ferroptosis is a type of oxidative stress-associated cell death that is mainly mediated by lipid peroxidation. However, the function of ferroptosis in cell differentiation remains unknown. Here, we showed that the ferroptosis inducer erastin did not cause cell death in human PBMCs. In contrast, erastin-induced lipid peroxidation promoted human PBMC proliferation and differentiation into B cells and natural killer cells through inhibition of bone morphogenetic protein family expression. These findings uncover a new immune modulation function of erastin in promoting PBMC proliferation and differentiation.

Keywords

ferroptosis; erastin; PBMCs; immunity; lipid peroxidation

1. Introduction

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the activity of antioxidant defense systems [1]. It is clear that oxidative stress is implicated in the pathogenesis of aging-associated diseases such as atherosclerosis,

*Correspondence to: Ding Wang (largestone_1984@163.com) or Daolin Tang (tangd2@upmc.edu).

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Conflict of interest

The authors declare no conflicts of interest or financial interests.

hypertension, diabetes mellitus, neurodegenerative diseases, and malignancies [2]. Targeting oxidative stress through antioxidants such as vitamins C and E, carotene, lycopene, and lutein may play a role in helping to delay aging and oxidative stress-related human diseases [3]. At the cellular level, oxidative stress plays a dual role in the regulation of cell survival or death. It not only causes hazardous events such as membrane disruption, protein degradation, lipid peroxidation, DNA damage, and regulated cell death, but also is an adaptation phenomenon to help the survival or differentiation of cells in response to environmental and intracellular stimuli [4]. Thus, the type of oxidative stress varies depending on the type of cell and the level of insult [5].

Ferroptosis is a type of oxidative stress-associated cell death that is mainly mediated by lipid peroxidation [6]. Although the precise mechanism remains unclear, ferroptosis can be induced through inhibition of several antioxidant defense systems such as the antiporter system Xc^- [7], the NRF2 (nuclear factor erythroid 2-related factor 2) pathway [1, 8], and selenocysteine-containing enzyme GPX4 (glutathione peroxidase 4) [9]. The numbers of ferroptosis inducers have increasingly grown over the last five years [6]. Nevertheless, erastin, a cell-permeable piperazinyl-quinazolinone compound, is the first identified reagent to induce ferroptosis in cancer cells [7]. Molecular pharmacology studies have shown that erastin has at least two direct drug binding targets, including system Xc^- and VDAC (voltage-dependent anion channel), responsible for ferroptosis induction [7, 10].

In the current study, we aimed to investigate the impact of erastin on human peripheral blood mononuclear cells (PBMCs), a critical component of the immune system associated with human disease. Surprisingly, erastin did not cause ferroptosis in PBMCs. In contrast, erastin-induced lipid peroxidation promoted proliferation and differentiation of PBMCs into B cells and natural killer (NK) cells through modulation of BMP (bone morphogenetic protein) signaling. These studies provide new insights about the pro-survival role of ferroptosis inducer.

2. Methods

2.1 Regents

Erastin (Cat#S7242) and ferrostatin-1 (Cat#S7243) were purchased from Selleck Chemicals (Houston, TX, USA). Phytohemagglutinin (Cat#11249738001) was purchased from Sigma (Minneapolis, MN, USA).

2.2 PBMC isolation and cell culture

Five healthy volunteers were enrolled; the subjects were male, 25–42 years old, without infection or immunity disease, and without drug usage for three months. This study was approved by the Ethics Committee of The Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). Venous blood was obtained and injected into a vacuum blood collection tube (Cat# 180408, Improvacuter, Guangzhou, China); 20 IU/ml heparin sodium was pre-added as anticoagulant agent. Anticoagulant blood was diluted with two volumes of 0.9% sodium chloride. Two volumes of diluted blood were gently added above lymphocyte separation medium (Cat#6300837, Sinopharm Chemical Reagent Co., Ltd,

Shanghai, China) and centrifuged at 1500rpm for 30 min. The PBMC layer was drawn after washing with culture medium twice; then PBMCs were immediately used for drug treatment. PBMCs were maintained in 1640 medium (Cat#8116123, GIBCO, Invitrogen China Limited, Shanghai, China) with 10% fetal bovine serum (FBS) (Cat#104-001-1A, BI, Biological Industries Israel Beit Haemek LTD., Kibbutz Beit-Haemek, Israel). PBMCs were counted and suspended in certain drug concentration mediums for drug treatment. PBMCs treated with 5 ug/ml phytohemagglutinin (PHA) were regarded as classic activation. PANC1 cells were cultured in Dulbecco's Modified Eagle's Medium (Cat#12491-023, Gibco) supplemented with 10% FBS (Biological Industries Israel Beit Haemek LTD.), 2 mM L-glutamine, and 100 U/ml of penicillin and streptomycin (Cat#10378016, Invitrogen, Grand Island, NY, USA) in 37C and 5% CO₂, passaged by 0.25% trypsin (Cat#1930117, GIBCO).

2.3 Cell viability assay

Cell viability assay was performed using a commercialized Cell Counting Kit-8 (CCK8) (Cat#C0039, Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

2.4 Quantitative real time polymerase chain reaction (Q-PCR) analysis

Total RNA was extracted and purified from cultured cells using the TRIzol™ Plus RNA Purification Kit (Cat#12183555, Invitrogen, Carlsbad, CA, USA). RNA concentration was determined by absorbance at 260 nm. 1.5ug RNA was used to synthesize 20ul cDNA by Reverse Transcriptase M-MLV (RNase H-) Kit (Cat#639524, TaKaRa, Dalian, China). Quantitative real-time polymerase chain reaction was performed using AceQ™qPCR SYBR® Green Master Mix (Cat#p411, Vazyme, Nanjing, China) on Applied Biosystems ViiA™ 7 Real-Time PCR System (Thermofisher Scientific, Waltham, MA, USA) analysis by ViiA7™ System software (Thermofisher Scientific) according to the manufacturer's protocol. The expression of target genes was calculated using the ddCt method relative to the expression of a housekeeping gene, 18s RNA [11]. The 'specific primers were listed as follows: TNFα: 5'-GGATCATTGCCCTGTGAGGAGGA-3' and 5'-GCCAGAAGAGGTTGAGGGTGTCT-3'; CD40: 5'-AAGTTTGGTGGTGGTGGTGTGG-3' and 5'-GGCATCTGTGTATATGGCTTCCTGG-3'; CD154: 5'-GAGCAACAACCTGGTAACCCTGGAA-3' and 5'-CCGATTGGAACAGAAGGTGACTTGG-3'; BMP2: 5'-CTGACGAGGTCCTGAGCGAGTT-3' and 5'-TGACCTGAGTGCCTGCGATACA-3'; BMP4: 5'-ATCGTTACCTCAAGGGAGTGGGA-3' and 5'-CCACATCGCTGAAGTCCACATAGAG-3'; BMP6: 5'-GCGACACCACAAAGAGTTCAAGTT-3' and 5'-AGTCTCTGTGCTGATGCTCCTGTA-3'; and BMP7: 5'-AGAACAGCAGCAGCGACCAGa-3' and 5'-TCACAGTAGTAGGCGGCGTAGC-3'.

2.5 Malondialdehyde (MDA) assay

Lipid peroxidation assay was performed by measuring the concentration of MDA (Cat#S0131, Beyotime Biotechnology, Jiangsu, China) and normalized by protein concentration, according to the manufacturer's instructions. Protein concentration was

assayed using a Pierce™ BCA Protein Assay Kit (Cat#23227, Thermofisher Scientific) according to the manufacturer's instructions.

2.6 GSH assay

After cell harvest, cell numbers were counted using a cell-count board before cell lysis. The total quantities of glutathione and oxidized glutathione (GSSG) were measured using a GSH and GSSG Assay Kit (Cat#S0053, Beyotime Biotechnology, Jiangsu, China) and normalized by cell number according to the manufacturer's instructions. GSH quantity was calculated using the formula of total glutathione minus twofolds GSSG.

2.7 Flow cytometric analysis

Lymphocyte subset percentages were determined using a Multitest 6-color TBNK kit (Cat#644611, BD Biosciences, San Jose, CA, USA) and analyzed on the BD FACSCanto II flow cytometer using BD FACS Diva software v6.1.3. BD Multitest 6-color TBNK reagent was provided in 1 mL of buffered saline with 0.1% sodium azide. It contained FITC-labeled CD3, clone SK7; PE-labeled CD16, clone B73.1, and CD56, clone NCAM16.2; PerCP-Cy™5.5-labeled CD45, clone 2D1 (HLe-1); PE-Cy™7-labeled CD4, clone SK3; APC-labeled CD19, clone SJ25C1; and APC-Cy7-labeled CD8, clone SK1.

2.8 Statistical analysis

Quantitative results were expressed as means $\pm$ SD. Proportional results were expressed in a scatter diagram. Unpaired Student's t tests were used to compare the means of two groups. Tukey's test was used for comparison among the different groups. A p-value of <0.05 was considered significant.

3. Results

3.1 Erastin promotes cell proliferation in PBMCs

We previously demonstrated that human pancreatic ductal adenocarcinoma (PDAC) cells are generally sensitive to erastin-induced cell death [12–14]. Cell viability assay confirmed that erastin dose-dependently inhibited cell growth in PANC1 cells, a human PDAC cell line (Fig. 1A). In contrast, erastin at the same dose promoted, but did not inhibit, growth of human PBMCs (Fig. 1A). Consistent with cell viability assay, microscopic observation further showed that erastin was time-dependently toxic to PANC1 cells and resulted in morphological patterns of necrosis such as rupture of the plasma membrane and loss of intracellular content (Fig. 1B). In contrast, the number of PBMCs was not affected after treatment with erastin for 24–96 hours as observed using microscopy (Fig. 1C). PHA is a mitogenic agent with potent cell agglutinating activities [15]. Unlike PHA, erastin did not induce colony formation in PBMCs (Fig. 1C). Both PHA and erastin increased cell viability in PBMCs following treatment for 24–96 hours (Fig. 1D). These findings indicate that erastin exhibited different pro-survival activities in PBMCs compared to cancer cells.

3.2 Erastin increases the differentiation of B cells and NK cells from PBMCs

Lymphocytes, including T cells, B cells, and NK cells, are the major PBMC subtype, comprising 70–90% of PBMCs [16]. The frequencies of these subpopulations vary by cytokine, chemokine, and growth factor. To determine whether erastin can change the number of immune cell subpopulations, we treated PBMCs with erastin and PHA. Flow cytometric analysis showed that the percentage of CD4⁺ T cells, CD8⁺ T cells, NK T cells (CD16⁺CD56⁺CD3⁺), and suppressor T (Ts) cells (CD3⁺CD8⁺) were not affected by PHA and erastin (Fig. 2A and 2B). In contrast, the percentage of helper T (Th) cells (CD4⁺CD8⁺) was downregulated by both PHA and erastin (Fig. 2A and 2B). Notably, PHA and erastin exhibited a distinct role in the regulation of subpopulations of B cells and NK cells in PBMCs (Fig. 2A and 2B). Flow cytometric analysis showed that PHA significantly reduced, whereas erastin increased the subpopulation of B cells (CD19⁺) and NK cells (CD16⁺CD56⁺) in PBMCs (Fig. 2A and 2B). These findings suggest that erastin is a potential immune reagent to regulate PBMC differentiation.

3.3 Lipid peroxidation contributes to erastin-induced cell proliferation

Given that erastin can trigger lipid peroxidation in ferroptosis [7], we next tested whether lipid peroxidation is required for erastin-induced PBMC proliferation. Ferrostatin-1 is a potent inhibitor of ferroptosis through reduction of lipid soluble ROS. As expected, ferrostatin-1 reversed erastin-induced growth inhibition in PANC1 cells (Fig. 3A). In contrast, ferrostatin-1 reduced erastin-induced PBMC proliferation (Fig. 3A). Malondialdehyde (MDA) is one of the final products of lipid peroxidation in cells [17]. Glutathione (GSH) is an important antioxidant capable of preventing oxidative damage from free radicals, peroxides, lipid peroxides, and heavy metals [18]. Ferrostatin-1 inhibited MDA production (Fig. 3B) and restored GSH levels (Fig. 3C) in both PANC1 cells and PBMCs in response to erastin. These findings suggest that lipid peroxidation plays a dual role in the regulation of survival and death in different cells.

3.4 Erastin inhibits BMP family expression

We next investigated how erastin affects the growth and differentiation of PBMCs. Studies over the past decade have revealed that the expressions of CD40 and CD154 (also known as CD40 ligand) are important regulators of immune cell activation, especially T cells [19]. The mRNA expression of CD40 (Fig. 4A) and CD154 (Fig. 4B) was upregulated by PHA, but not by erastin. Thus, erastin may have no impact on the CD154-CD40 pathway. BMPs belong to the transforming growth factor- β superfamily and play central roles in controlling cellular proliferation, differentiation, and activity in B cells, NK cells, and myeloid cells [20–23]. The effects of erastin and PHA on the expression of BMPs were further assayed. Both erastin and PHA suppressed BMP2 expression in PBMCs (Fig. 4C). In contrast, erastin and PHA exhibited different impacts on mRNA expression of other BMP family members. Notably, PHA increased whereas erastin inhibited mRNA expression of BMP4 (Fig. 4D), BMP6 (Fig. 4E), and BMP7 (Fig. 4F). These findings indicate that erastin may regulate PBMC differentiation through selective control of BMP expression.

4. Discussion

PBMCs contain multipotent progenitor cell populations and possess the potential to differentiate into various types of immune cells to regulate innate and adaptive immunity under both physiological and pathological conditions [24]. In this study, we demonstrated that the differentiation of PBMCs into immune cells, especially B cells and NK cells, can be modulated with the small molecule erastin. These findings uncover a new function of erastin in cell proliferation and differentiation.

Erastin is a small molecule capable of initiating ferroptosis in cancer and non-cancer cells. Mechanistically, erastin binds and inhibits VDAC2, VDAC3, or cystine-glutamate antiporter system X_c^- , which leads to mitochondrial dysfunction, excessive lipid peroxidation, and finally ferroptosis [7, 10]. Impairment of antioxidant defenses such as GPX4 accelerates ferroptotic cell death. However, GPX4 depletion-mediated immune cell death is both ferroptosis-dependent and -independent. For example, GPX4-deficient T cells are more sensitive to ferroptosis, but not necroptosis [25]. In contrast, GPX4-deficient myeloid cells are more sensitive to pyroptosis due to increased proinflammatory caspase-1 and caspase-11 activity [26]. In addition, system X_c^- dysfunction is also involved in types of non-ferroptotic-regulated cell death such as apoptosis [27]. Thus, lipid peroxidation could be an initiating signal, but not the effector mechanism of cell death.

Our study indicates that erastin promotes the growth and differentiation of PBMCs through lipid peroxidation. Lipid peroxidation can produce a wide variety of oxidation products including MDA and 4-hydroxy-2-nonenal (4-HNE). MDA and 4-HNE can induce the inflammation response in tissue injury and repair [28]. Moreover, oxidation product-mediated signaling can largely influence a cell's fate through modulating autophagy, a lysosome-dependent degradation pathway [29]. Autophagy can function as a survival mechanism in response to starvation or stress [30]. However, excessive or uncontrolled autophagy can lead to autophagic cell death [31]. We recently demonstrated that ferroptosis is a type of autophagic cell death due to degradation of ferritin or inhibition of system X_c^- activity [14, 32]. These findings support the notion that oxidative stress and autophagy promote survival or death in a cell context-specific manner.

To gain more specific information about erastin in the regulation of PBMC differentiation, we assayed clone formation and immune cell subpopulations in response to erastin and PHA. Unlike PHA, erastin did not induce large clone formation in PBMCs, indicating that erastin may not promote cell-cell adhesion. Remarkably, erastin promotes the production of B cells and NK cells, which are components of the immune system, by secreting antibodies or perforin/granzymes, respectively. In contrast to NK cells, erastin did not affect NK cells, which are regulatory T cells that bridge the innate and adaptive immune systems. To better illustrate the mechanism of action of erastin in PBMC differentiation, we assayed the expression of core components of the CD40 and BMP pathways. Our studies indicated that erastin significantly regulates the expression of BMP, but not CD40. BMPs have been reported to both positively and negatively regulate innate and adaptive immune responses, which are dependent on the target cells and the type of BMP [20–23, 33–35].

In summary, we demonstrated that the ferroptosis inducer erastin promotes human PBMC proliferation and differentiation through activation of lipid peroxidation and downregulation of BMPs. Additional studies are needed to determine if other ferroptosis inducers have similar functions in the regulation of PBMC activity and to further define their capacity in autoimmune diseases or antitumor immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

PBMCs	peripheral blood mononuclear cells
GSH	reduced glutathione
GSSG	oxidized glutathione
BMP	bone morphogenetic protein
CD	cluster of differentiation
Ts cells	suppressor T cells
Th cells	helper T cells
NK cells	natural killer cells
PHA	phytohemagglutinin
PDAC	pancreatic ductal adenocarcinoma
ROS	reactive oxygen species
NRF2	nuclear factor erythroid 2-related factor 2
GPX4	glutathione peroxidase 4
Q-PCR	quantitative real time polymerase chain reaction
VDAC	voltage-dependent anion channel

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Fig. 1. Erastin promotes PBMC proliferation.

(A) PANC1 cells and PBMCs were treated with erastin (1.25–40 μM) for 72 h and then cell viability was assayed ($n=3$, $*P<0.05$ versus control group). (B–D) PANC1 cells and PBMCs were treated by erastin (10 μM) or PHA (5 $\mu\text{g/ml}$) for 24–72 h and then cell morphology was assayed. (D) PBMCs were treated by erastin (10 μM) or PHA (5 $\mu\text{g/ml}$) for 24–72 h and then cell viability was assayed ($n=3$, $*P<0.05$ versus control group).

Fig. 2. Erastin increases the differentiation of B cells and NK cells from PBMCs.

(A) PBMCs were treated with PHA (5 µg/ml) and erastin (10 µM) for 72 hours and then cell population was detected via flow cytometry. (B) Statistical analysis of population change in PBMCs following PHA (5 µg/ml) and erastin (10 µM) treatment for 72 hours (n=5, *P<0.05 versus control group).

Fig. 3. Lipid peroxidation contributes to erastin-induced cell proliferation.

PANC1 cells and PBMCs were treated with erastin (10 μ M) in the absence or presence of ferrostatin-1 (1 μ M) for 72 h. Cell viability (A), MDA (B), and GSH (C) were assayed (n=3, *P<0.05).

Fig. 4. Erastin inhibits BMP family expression.

PBMCs were treated with erastin (10 μ M) and PHA (5 μ g/ml) for 72 h. mRNA expression of CD40 (A), CD154 (B), BMP2 (C), BMP4 (D), BMP6 (E), and BMP7 (F) were assayed (n=3, *P < 0.05 versus control group).