ORIGINAL RESEARCH

Induction of ferroptosis-like cell death of eosinophils exerts synergistic effects with glucocorticoids in allergic airway inflammation

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ABSTRACT

Introduction Eosinophils are critical in allergic disorders, and promoting eosinophil death effectively attenuates allergic airway inflammation. Ferroptosis is a recently described novel form of cell death; however, little is known about ferroptosis in eosinophils and related diseases. This study aimed to investigate the effects of ferroptosis-inducing agents (FINs) on eosinophil death and allergic airway inflammation, and to explore their potential synergistic effect with glucocorticoids (GCs).

Methods Eosinophils isolated from the peripheral blood of humans or mice were incubated with FINs, and eosinophil ferroptosis was assessed. The in vivo effects of FINs alone or in combination with dexamethasone (DXMS) were examined in a mouse model of allergic airway inflammation. Bronchoalveolar lavage fluid and lung tissue were collected to examine airway inflammation.

Results Treatment with FINs time and dose dependency induced cell death in human and mouse eosinophils. Interestingly, FINs induced non-canonical ferroptosis in eosinophils, which generated morphological characteristics unique to ferroptosis and was iron dependent but was independent of lipid peroxidation. The antioxidants glutathione and N-acetylcysteine significantly attenuated FIN-induced cell death. Treatment with FINs triggered eosinophil death in vivo and eventually relieved eosinophilic airway inflammation in mice. Furthermore, FINs exerted a synergistic effect with DXMS to induce eosinophil death in vitro and to alleviate allergic airway inflammation in vivo.

Conclusions FINs induced ferroptosis-like cell death of eosinophils, suggesting their use as a promising therapeutic strategy for eosinophilic airway inflammation, especially due to the advantage of their synergy with GCs in the treatment of allergic disorders.

Key messages

What is the key question?

► Here, we questioned whether the induction of eosinophil ferroptosis could be a new effective strategy against allergic airway inflammation.

What is the bottom line?

► Treatment with ferroptosis-inducing agents (FINs) triggered eosinophil death and eventually relieved eosinophilic airway inflammation; moreover, FINs exerted a synergistic effect with dexamethasone to induce eosinophil death in vitro and to alleviate allergic airway inflammation in vivo.

Why read on?

► This is the first study to show that FINs induce ferroptosis-like cell death in eosinophils, suggesting their use as a promising therapeutic strategy for eosinophilic airway inflammation, especially due to the advantage of their synergy with glucocorticoids in the treatment of allergic disorders.

INTRODUCTION

Eosinophils, terminally differentiated granulocytic cells, have been implicated in the pathogenesis of diverse inflammatory responses.[1] They mature from pluripotent progenitors in the bone marrow and are released into the circulation in a phenotypically mature state.[2] Eosinophils normally account for less than 5% of leucocytes in the blood.[3] In the absence of external stimuli, circulating eosinophils usually end physiologically by spontaneous apoptosis within 2–5 days.[4] In response to diverse stimuli, eosinophil production is increased. Activated eosinophils migrate into the bloodstream and are subsequently recruited to inflammatory foci, where their lifespan is believed to be prolonged due to the presence of prosurvival factors in the local microenvironment.[5] Eosinophils are clearly recruited for defence against invading pathogens at the inflamed site. On the other hand, eosinophils have also been shown to serve as major effector cells that induce tissue injury and dysfunction by secreting toxic granule proteins and lipid mediators.[6]

Overwhelming evidence shows that eosinophil infiltration in the airways is a key feature of allergic asthma and is believed to be associated with the pathogenesis of this disease. The results of both clinical studies and studies in allergic mouse models have also demonstrated that eosinophils contribute to asthma pathogenesis, ongoing inflammation, airway hyper-responsiveness and tissue remodelling.[5] An increasing number of studies

have proposed that the course of allergic airway inflammation not only depends on eosinophil recruitment but also partly depends on the increased lifespan of eosinophils within inflamed tissue.[2 7] The delay of eosinophil apoptosis is a critical mechanism of eosinophil accumulation at an inflammatory site.[8] Glucocorticoids (GCs) are the most effective therapy for eosinophilic disorders by both their direct induction of eosinophil apoptosis and their suppression of prosurvival signals, but the pleiotropic effects of corticosteroids limit their therapeutic use, especially at high doses.[9 10] Recent studies have focused on the development of new agents to block eosinophil recruitment and/or to decrease eosinophil survival and activation.[11 12] We recently uncovered that Bcl-2 inhibitors that promote eosinophil apoptosis eventually reduced allergic inflammation.[13] Thus, selective induction of eosinophil death is likely to resolve allergic inflammation and restore tissue homeostasis.

Ferroptosis, a novel form of non-apoptotic cell death, is characterised by the accumulation of reactive oxygen species (ROS) derived from iron metabolism and lipid peroxidation.[14] Multiple small molecules called ferroptosis-inducing agents (FINs), including experimental compounds, for example, erastin and Ras-selective lethal small molecule 3 (RSL3), and clinical drugs, for example, artesunate (ART), sulfasalazine and sorafenib, have been discovered. Cell death triggered by erastin was found to result in glutathione (GSH) depletion and lipid peroxidation accumulation through its ability to inhibit the import of cystine by directly inhibiting cystine/glutamate antiporter system x_c^- activity.[15] RSL3 was demonstrated to induce ROS production from lipid peroxidation by inactivating glutathione peroxidase 4 (GPX4).[15] In addition, some FINs trigger ferroptosis through a Fenton-like reaction.[16] Generally, ferroptosis should be pharmacologically inhibited by both an iron chelator and a lipid peroxidation inhibitor and should involve accumulation of lipid hydroperoxides. In cases of cell death postulated to be ferroptosis, other known forms of cell death, such as apoptosis and necrosis, should be ruled out. Ferroptotic cells lack the morphological and biochemical characteristics of cells undergoing other forms of cell death, which exhibit smaller mitochondria with condensed mitochondrial membrane densities, outer mitochondrial membrane rupture and a reduction in mitochondrial crista.[17]

Recent reports have revealed the connection between ferroptosis and the pathological processes of several diseases and conditions, including neurodegenerative diseases,[18] heart transplantation[19] and neoplastic diseases.[20] Ferroptosis has been suggested as a potential contributor for the treatment of cell death-related diseases. Recently, high levels of lipid peroxides were found in asthmatic airway epithelial cells, contributing to epithelial dysfunction, cell death and asthma exacerbation.[21] However, the effects of ferroptosis on eosinophils and eosinophilic inflammation have not been studied. A previous study showed that eosinophils have the highest level of catalytic ferrous iron (Fe(II)) in normal status, which would further increase in allergic status.[22] Fe(II) is considered as an initiator of the Fenton reaction and ROS generated through Fenton reaction is known to contribute to the initiation of ferroptosis.[23] Thus, eosinophils appear to be more likely to occur ferroptosis due to their abundance of iron.

In this study, we show that FIN treatment is associated with eosinophil death in vitro and exerts therapeutic efficacy in an allergic airway inflammation model in vivo. Furthermore, FINs have a synergistic effect with GCs to induce eosinophil death and alleviate allergic airway inflammation. Accordingly, the objective of the current study is to identify FINs as a promising therapeutic strategy for allergic airway inflammation.

METHODS

Additional methods are presented in the online supplementary file.

Human subjects

Nine hypereosinophilic patients were recruited from the clinical population at the Department of Respiratory and Critical Care Medicine of the Second Affiliated Hospital of Zhejiang University School of Medicine. All patients provided written informed consent and understood that their samples would be used for research.

Experimental animals and treatments

Male C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co. (Shanghai, China). Mice from each litter were randomised to different groups. Protocols were approved by the Ethics Committee for Animal Studies at Zhejiang University, China.

Erastin (25 mg/kg) and RSL3 (10 mg/kg) were dissolved in dimethyl sulfoxide (DMSO) and administered intraperitoneally 2 hours after each ovalbumin (OVA) challenge. The controls were received equal dosage of DMSO for erastin or RSL3 group. ART (10 or 20 mg/kg) was dissolved in normal saline (NS) and delivered intraperitoneally once a day for 3 days before the first challenge, and 2 hours after each challenge. Dexamethasone (DXMS) (0.25 or 0.5 mg/kg) was diluted in NS and administered intraperitoneally 2 hours after each challenge. In ART/DXMS groups, mice were injected with ART (10 mg/kg) alone once a day for 3 days before the first challenge, and then cotreated with ART (10 mg/kg) and DXMS (0.25 mg/kg) 2 hours after each challenge. Control mice were received the same volume of NS for ART, DXMS or ART/DXMS group. Twenty-four hours after the last administration, all mice were sacrificed for analysis.

Statistics

All related data are presented as the mean ± SEM. Comparisons between two groups were calculated by two-tailed Student's t-test, and significant differences between multiple groups were evaluated by one-way analysis of variance with Tukey's honestly significant difference (HSD) post hoc testing using GraphPad Prism 8 software (GraphPad Software, La Jolla, California, USA). The test statistics have been transformed into adjusted p values following Tukey multiple comparison testing. Differences were considered statistically significant when the p value was less than 0.05.

RESULTS

FINs triggered the cell death of eosinophils from human subjects and mice

To explore whether FIN stimulation could lead to eosinophil death, we initially harvested peripheral leucocytes from patients with asthma and non-asthma with increased eosinophils and incubated the leucocytes with FINs. Eosinophils among leucocytes were distinguished by flow cytometry for Siglec-8 and CCR3 staining (Siglec-8^+/$CCR3^+$)[24] (online supplementary figure 1A). Treatment of peripheral eosinophils from both patients with asthma and non-asthma with a series of FINs (erastin, RSL3, and ART) resulted in concentration-dependent cell death, as assayed by flow cytometry (figure 1A, online supplementary figure 1B,C). However, in this experimental setting, we could not exclude the potential influence of other types of leucocytes on eosinophil death. We subsequently isolated eosinophils from the whole blood of patients with asthma to examine the effects

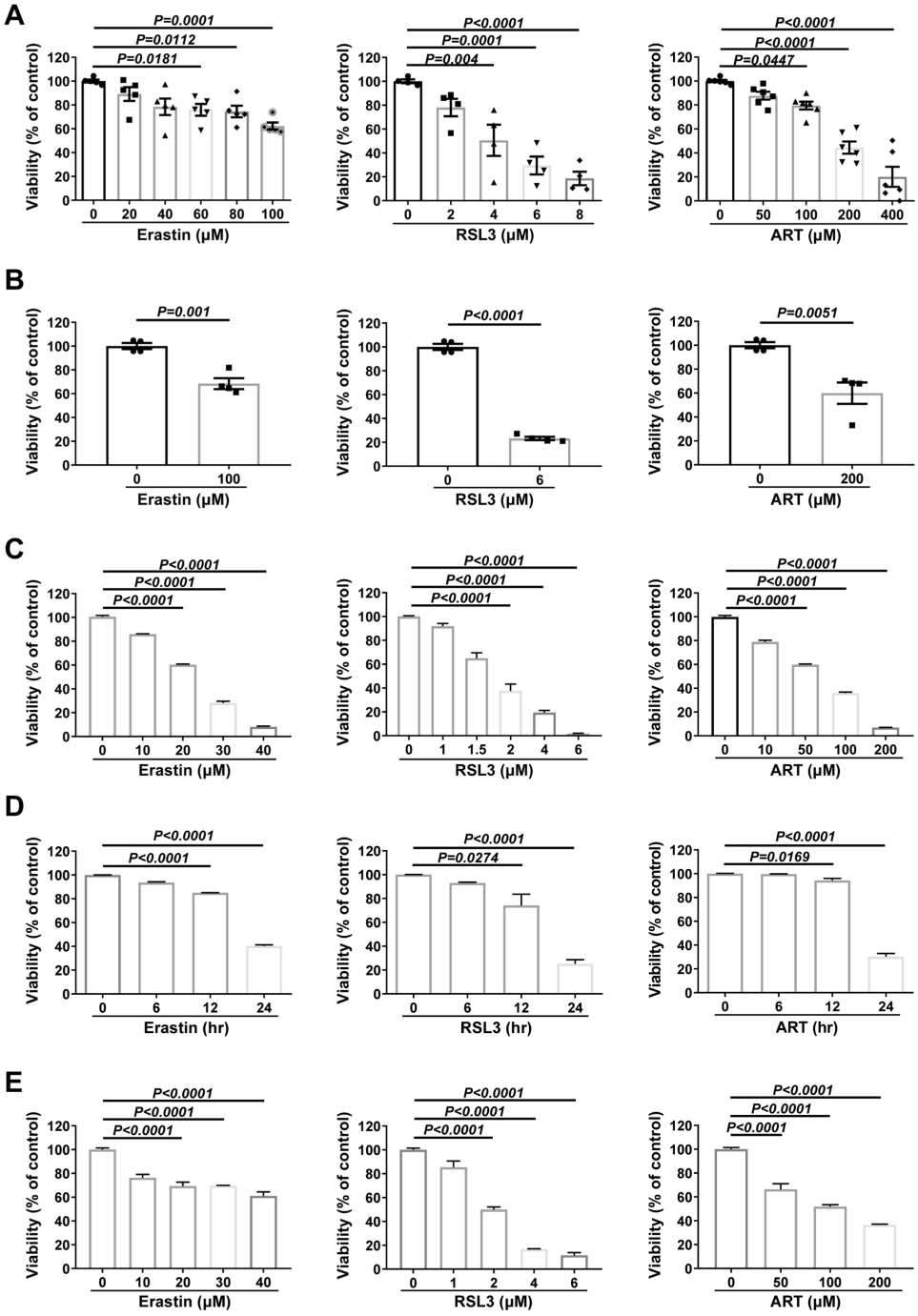

Figure 1 Ferroptosis-inducing agents (FINs) triggered the cell death of eosinophils from patients with asthma and allergic mice. A total of six patients with asthma were recruited. (A) Peripheral leucocytes (n=4–6) from patients with asthma were cultured with various concentrations of FINs for 24 hours prior to viability determination by flow cytometry. Siglec-8$^+$/CCR3$^+$/annexin V$^-$/4',6-diamidino-2-phenylindole (DAPI$^-$) cells were distinguished as viable peripheral eosinophils. (B) Eosinophils were purified from four of the six patients with asthma and cultured with indicated concentrations of FINs for 24 hours. Annexin V$^-$/propidium iodide (PI$^-$) cells were defined as viable cells. (C and D) Eosinophils isolated from the peripheral blood of *interleukin 5* transgenic mice were cultured with various concentrations of FINs for 24 hours (C) or FINs (erastin 30 μM, Ras-selective lethal small molecule 3 (RSL3) 2 μM, artesunate (ART) 100 μM) for the indicated duration before harvest (D). Annexin V$^-$/PI$^-$ cells were defined as viable cells. (E) Bronchoalveolar lavage (BAL) cells from allergic mice were cultured with various concentrations of FINs for 24 hours. Siglec-F$^+$/CD11c$^-$/annexin V$^-$/DAPI$^-$ cells were distinguished as viable BAL eosinophils by flow cytometry. All data are shown as mean±SEM, analysed by one-way analysis of variance (A, C, D, E) or Student's t-test (B).

of FINs on purified eosinophils. A similar effect in response to FINs was observed in purified eosinophils and eosinophils among leucocytes, indicating that FIN-treated eosinophil death was not affected by the presence of other cell types (figure 1B).

To further test whether FINs could exert similar effects on mouse eosinophils, we isolated eosinophils from the peripheral blood of *Cd3δ* promoter interleukin *5* transgenic (Tg) mice. Similarly, FINs dose and time dependency induced cell

death in mouse eosinophils (figure 1C,D, online supplementary figure 2A). To confirm that FINs can induce cell death in local inflammatory eosinophils, we next collected bronchoalveolar lavage (BAL) cells from allergic mice and then stimulated the BAL cells with FINs. Mouse BAL eosinophils were distinguished by Siglec-F and CD11c staining (Siglec-F$^+$/CD11c$^-$)[25] (online supplementary figure 2B). Again, FIN treatment efficiently triggered concentration-dependent cell death in BAL eosinophils, though eosinophils in the bronchoalveolar lavage fluid (BALF) were slightly more resistant to erastin and ART stimulation than those in blood (figure 1E, online supplementary figure 2C). Taken together, these results indicate that FINs could remarkably trigger the cell death of eosinophils from both human subjects and mice, regardless of their allergic status.

FINs induced non-canonical ferroptosis in eosinophils

We further examined the mode of cell death caused by FINs in eosinophils. We first investigated whether the observed cytotoxicity was related to apoptotic or necrotic death or autophagy. As expected, Z-VAD-FMK (a pan-caspase inhibitor), necrostatin-1 (a potent necroptosis inhibitor targeting the death domain kinase RIP), or spautin-1 (a specific autophagy inhibitor targeting the activity of ubiquitin-specific peptidases) failed to reverse erastin-induced or RSL3-induced cell death (figure 2A,B). In ART-treated

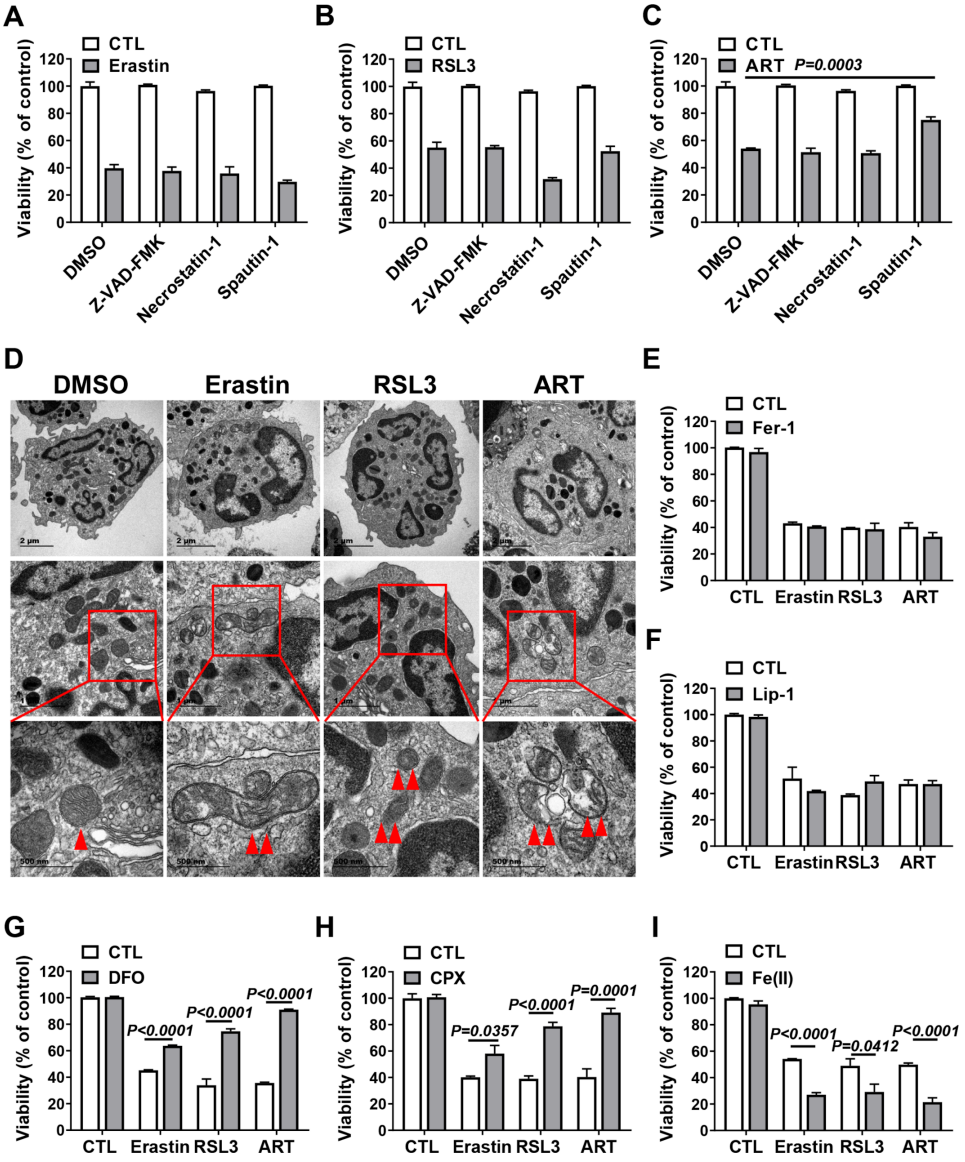

Figure 2 Ferroptosis-inducing agents (FINs) induced non-canonical ferroptosis in eosinophils. Eosinophils were isolated from the peripheral blood of interleukin *5* transgenic mice. Annexin V$^-$/PI$^-$ cells were defined as viable eosinophils. (A–C) Eosinophils were cultured with FINs (erastin 30 µM, Ras-selective lethal small molecule 3 (RSL3) 2 µM, artesunate (ART) 100 µM) with or without Z-VAD-FMK (100 µM), necrostatin-1 (100 µM) and spautin-1 (5 µM) for 24 hours prior to viability determination by flow cytometry. (D) Transmission electron microscopy of eosinophils treated with dimethylsulfoxide (DMSO) or FINs for 12 hours. Single red arrowheads point to normal mitochondria; paired red arrowheads point to damaged mitochondria. (E and F) Effects of ferrostatin-1 (fer-1; 2 µM) and liproxstatin-1 (lip-1; 2 µM) on the cell viability of eosinophils treated with FINs for 24 hours. (G and H) Effects of deferoxamine (DFO; 100 µM) and ciclopirox olamine (CPX; 500 nM) on the cell viability of eosinophils treated with FINs for 24 hours. (I) Effects of ferrous iron (Fe(II); 200 µM) and FIN cotreatment on the cell viability of eosinophils for 24 hours. All data are shown as mean±SEM, analysed by one-way analysis of variance. CTL, control; PI, propidium iodide.

eosinophils, cell death was partly suppressed by spautin-1, but not by Z-VAD-FMK or necrostatin-1 (figure 2C). The bioactivity of these two compounds was confirmed in other cases (online supplementary figure 3A,B). These data suggest that FINs induce predominantly non-apoptotic cell death in eosinophils and that autophagy is likely involved in ART-induced cell death.

We next sought to test whether ferroptosis was genuinely activated in the eosinophils treated with FINs. As ferroptosis has its unique morphological characteristics,[14] we used transmission electron microscopy to monitor morphological changes caused by FINs. FIN-treated eosinophils exhibited shrunken and damaged mitochondria, features unique to ferroptosis, with no other obvious changes prior to the occurrence of cell death (figure 2D). For direct comparison, eosinophils were treated with staurosporine or hydrogen peroxide solution to trigger apoptosis or necroptosis, respectively, and the representative morphologies induced by these types of cell death were clearly different from that induced by FINs (online supplementary figure 3C).

Lipid peroxidation is a defining event in ferroptosis. We stained FIN-treated eosinophils with C11-BODIPY, a membrane-targeted lipid ROS sensor, to detect changes in lipid ROS by flow cytometry. Not surprisingly, the levels of lipid ROS were significantly increased on FIN treatment (online supplementary figure 3D). We then pretreated eosinophils with ferrostatin-1 (fer-1) or liproxstatin-1 (lip-1) to eliminate the accumulation of lipid peroxides. Surprisingly, these lipid ROS inhibitors failed to exert any protective effect against FIN-induced cell death in eosinophils (figure 2E,F), although lipid ROS were effectively abolished (online supplementary figure 3E). These results indicate that lipid ROS accumulation by FINs may not be necessary for FIN-induced cytotoxicity in eosinophils.

We confirmed the effects of these ferroptosis inhibitors in classical ferroptosis-sensitive cells. Mouse embryonic fibroblasts (MEFs), in which ferroptosis had been verified, were stimulated with FINs at lethal concentrations. Cell death induced by FINs in MEFs was almost completely suppressed by fer-1 or lip-1 (online supplementary figure 3F), and lipid ROS were eliminated from the cells (online supplementary figure 3G).

Ferroptotic cell death has been characterised by its dependency on iron; thus, we investigated the effects of the iron chelators deferoxamine (DFO) and ciclopirox olamine (CPX) on FIN-induced cell death in eosinophils. Interestingly, both iron chelators markedly reversed FIN-induced cell death in eosinophils (figure 2G,H). On the other hand, the addition of Fe(II) to the eosinophil culture medium further augmented cell death induced by FINs (figure 2I).

Altogether, these data indicate that FINs induce non-classical cell death in eosinophils, which display the cardinal morphological features of ferroptosis, and that this cell death is iron dependent but independent of lipid ROS. We therefore suggest that FIN-induced cell death of eosinophils is non-canonical ferroptosis or ferroptosis-like cell death.

FINs induced ferroptosis-like cell death through cytosolic ROS

Since lipid ROS were not critical for FIN-induced cell death in eosinophils, we next attempted to identify the other types of ROS that might be involved. All eosinophils treated with FINs exhibited increased CM-H_2DCFDA fluorescence, indicating the production of cytosolic ROS (figure 3A,B). Moreover, we examined the functional requirement of cytosolic ROS during ferroptosis by treating eosinophils with FINs in the presence of the ROS scavengers N-acetylcysteine (NAC) or GSH. Notably, the addition of NAC or GSH completely reversed RSL3-induced cell death and partly blocked erastin-induced or ART-induced cell death (figure 3C,D). Indeed, NAC and GSH could suppress cytosolic ROS accumulation induced by all FINs, but fer-1 failed to decrease the generation of cytosolic

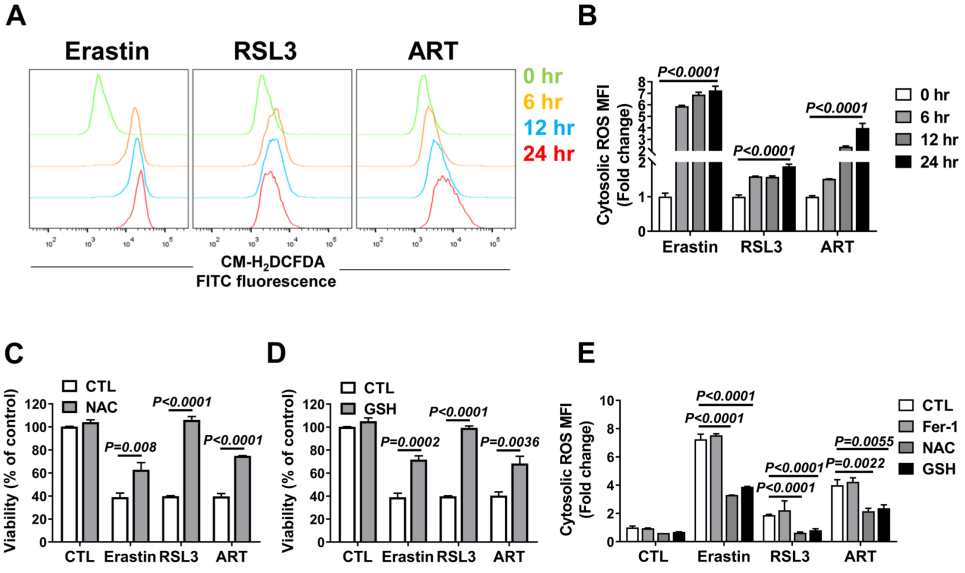

Figure 3 Ferroptosis-inducing agents (FINs) induced ferroptosis-like cell death through cytosolic reactive oxygen species (ROS). Eosinophils were isolated from the peripheral blood of *interleukin 5* transgenic mice. Annexin V^-/PI^- cells were defined as viable eosinophils. (A and B) Eosinophils were cultured with FINs (erastin 30 µM, Ras-selective lethal small molecule 3 (RSL3) 2 µM, artesunate (ART) 100 µM). Cytosolic ROS production at indicated times (6, 12 and 24 hours) was assessed by flow cytometry using CM-H_2DCFDA. Representative histograms are shown in (A), and cumulative data expressed relative to the control are represented in (B). (C and D) Effects of N-acetylcysteine (NAC; 5 mM) and glutathione (GSH; 5 mM) on the lethality of FINs in eosinophils. All drug treatments were administered for 24 hours. (E) Ability of ferrostatin-1 (fer-1; 2 µM), NAC (5 mM) and GSH (5 mM) to prevent accumulation of cytosolic ROS when used to cotreat FINs for 24 hours. All data are shown as mean±SEM, analysed by one-way analysis of variance. CTL, control; PI, propidium iodide.

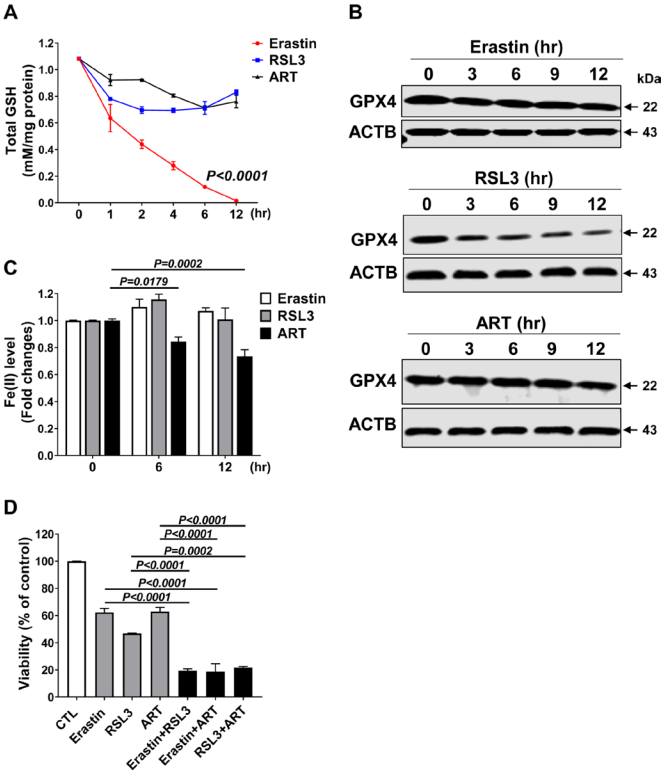

Figure 4 Ferroptosis-inducing agents (FINs) induced ferroptosis-like cell death in eosinophils via differential mechanisms and exerted synergistic effects. Eosinophils were isolated from the peripheral blood of *interleukin 5* transgenic mice and cultured with FINs (erastin 30 µM, Ras-selective lethal small molecule 3 (RSL3) 2 µM, artesunate (ART) 100 µM). (A) Total glutathione (GSH) levels were assessed over the indicated duration. (B) Glutathione peroxidase 4 (GPX4) protein at indicated times (3, 6, 9 and 12 hours) was assessed by western blot analysis. Actin beta (ACTB) was used as a loading control. (C) Cellular Fe(II) levels at indicated times (6 and 12 hours) were assessed. (D) Synergistic effect of FINs (erastin 20 µM, RSL3 1.5 µM, ART 50 µM) on the cell viability of eosinophils administered in pairs for 24 hours. Annexin V^-/PI^- cells were defined as viable eosinophils. All data are shown as mean±SEM, analysed by one-way analysis of variance. CTL, control; Fe(II), ferrous iron; PI, propidium iodide.

ROS (figure 3E). In ART-treated eosinophils, we observed a time-dependent increase in mitochondrial ROS production, whereas no change was observed in erastin-treated or RSL3-treated cells (online supplementary figure 4A,B). However, MitoTEMPO, a mitochondria-targeted antioxidant, exerted no appreciable effects on the ART-induced cell death of eosinophils (online supplementary figure 4C).

Altogether, these data suggest that FINs induce ferroptosis-like cell death in eosinophils most likely through cytosolic ROS, but not lipid or mitochondrial ROS, and that ferroptosis pathways in eosinophils differ from the canonical pathways previously identified in tumour cells.[14 17]

FINs induced ferroptosis-like cell death in eosinophils via differential mechanisms and exerted a synergistic effect

Ferroptosis induced by erastin or RSL3 is due to GSH depletion or reduced GPX4 activity, respectively[15]; however, little is known about the mechanisms of ART in regulating of ferroptosis. Consistent with previous reports, erastin time dependency decreased the cellular levels of GSH in eosinophils, while the other two FINs tested only slightly decreased GSH levels (figure 4A). Interestingly, RSL3 time dependency attenuated the expression of GPX4 in eosinophils, whereas neither erastin nor ART affected GPX4 expression (figure 4B). As ART-triggered cell death was totally dependent on iron, we hypothesised that ART generates a Fenton-type reaction to initiate oxidative damage in eosinophils. We then measured the oxidation state of iron and observed that cellular Fe(II) levels were modestly reduced with ART treatment, while erastin-treated and RSL3-treated cells showed no change in the abundance of Fe(II) (figure 4C).

As erastin, RSL3 and ART likely induce eosinophil death through different intracellular signals, we stimulated eosinophils with these three compounds in pairs. Every pair of compounds exerted a synergistic effect (figure 4D), laterally suggesting that these three compounds induce ferroptosis-like cell death in eosinophils via distinct mechanisms.

FINs attenuated allergic airway inflammation and induced eosinophil death in vivo

We next questioned whether facilitating the ferroptosis-like cell death of eosinophils would attenuate allergic airway inflammation in vivo. In the classical OVA-induced model of allergy, FINs were administered 2 hours after each OVA challenge, except ART was also treated for 3 days before the first challenge. BALF and lung tissues were collected 24 hours after the last administration of FINs (online supplementary figure 5A). Significantly decreased infiltration of total BAL cells and eosinophils was observed in the FIN-treated group (figure 5A–C). H&E staining further revealed that the accumulation of airway inflammatory cells in the peribronchiolar and perivascular regions was clearly reduced in FIN-treated mice (figure 5D,E). The mRNA levels of *Il13* or *Il25* were also dramatically decreased by FIN treatment (figure 5F). Moreover, mucus hyperproduction induced by OVA was dramatically attenuated in response to FIN treatment, as evidenced by periodic acid-schiff staining (online supplementary figure5B,C). Each compound alone exhibited no appreciable toxicity in mouse lungs (figure 5A–F), and mouse weight and BAL protein levels were not affected by FINs (online supplementary figure 5D,E), indicating the inappreciable pulmonary toxicity of these FINs in vivo.

To further examine possible eosinophil cell death in vivo, we acquired BAL cells from allergic mice treated with FINs or vehicle control. BAL cells were collected and subsequently stained with anti-CD11c and anti-Siglec-F (online supplementary figure 5F). As expected, FIN treatment led to reduced cell viability (Annexin V^-/4',6-diamidino-2-phenylindole$^-$) of BAL eosinophils (figure 5G,H). In addition, BAL eosinophils from allergic mice treated with FINs exhibited morphological changes indicative of ferroptosis (shrunken and damaged mitochondria) (figure 5I).

Synergistic effect of FINs with DXMS in vitro and in vivo

GCs such as betamethasone or DXMS are conventionally used as an effective anti-inflammatory therapy for asthma, and the partial anti-inflammatory effects of GCs have been ascribed to their ability to facilitate eosinophil apoptosis.[26] As expected, DXMS induced eosinophil death in a concentration-dependent manner, which could be effectively prevented by Z-VAD-FMK (figure 6A,B). However, DXMS-induced eosinophil death was not responsive to iron chelation or supplementation. In addition, fer-1 also failed to protect eosinophils from DXMS-induced cell death (figure 6C). These results suggest that DXMS-induced death of eosinophils is likely apoptosis. A recent study found that the combination of ferroptosis and apoptosis serves as a promising modality to improve anticancer treatment efficacy.[27] As long-term use of GCs in high

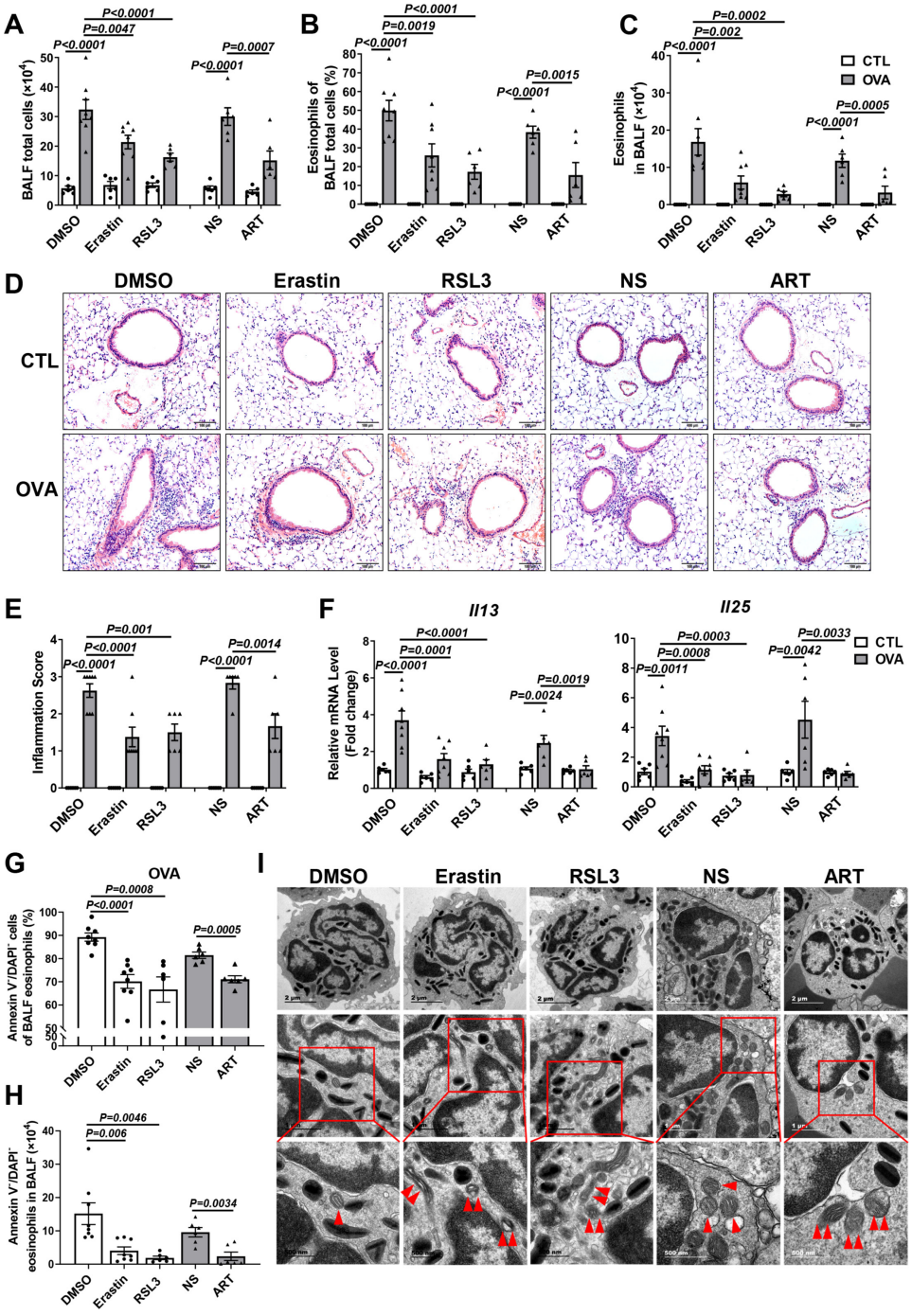

Figure 5 Ferroptosis-inducing agents (FINs) attenuated allergic airway inflammation and induced eosinophil death in vivo. (A) Total bronchoalveolar lavage (BAL) cell counts. (B) BAL eosinophil percentage. (C) BAL eosinophil counts. (D) Representative lung tissue sections stained with H&E. (E) Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. (F) Cytokine levels in lung tissues were determined by qPCR. (G) The percentage of viable cells of total BAL eosinophils was determined by flow cytometry. Siglec-F$^+$/CD11c$^-$/annexin V$^-$/4',6-diamidino-2-phenylindole (DAPI$^-$) cells were distinguished as viable BAL eosinophils. (H) Viable BAL eosinophil counts. (I) Transmission electron microscopy of BAL eosinophils from allergic mice treated with control or FINs. Single red arrowheads point to normal mitochondria; paired red arrowheads point to damaged mitochondria. All data are shown as the mean±SEM of six to eight mice per group, analysed by one-way analysis of variance (A, B, C, E, F) or Student's t-test (G, H). ART, artesunate; BALF, bronchoalveolar lavage fluid; CTL, control; DMSO, dimethyl sulfoxide; Il, interleukin; NS, normal saline; OVA, ovalbumin; RSL3, Ras-selective lethal small molecule 3.

doses is associated with considerable side effects, we were therefore interested in whether FINs could synergize with GCs to induce cell death in eosinophils. To investigate the efficacy of FINs and DXMS combined, we administrated both FINs and low dose of DXMS (0.4 mM) to eosinophils in vitro. Intriguingly, eosinophil death was markedly enhanced when the eosinophils were cotreated with FINs and DXMS, indicating the apparent synergistic effect of FINs and DXMS (figure 6D–F).

As ART is now widely used for clinical treatment and is well tolerated, we studied the possible synergistic effect of ART with

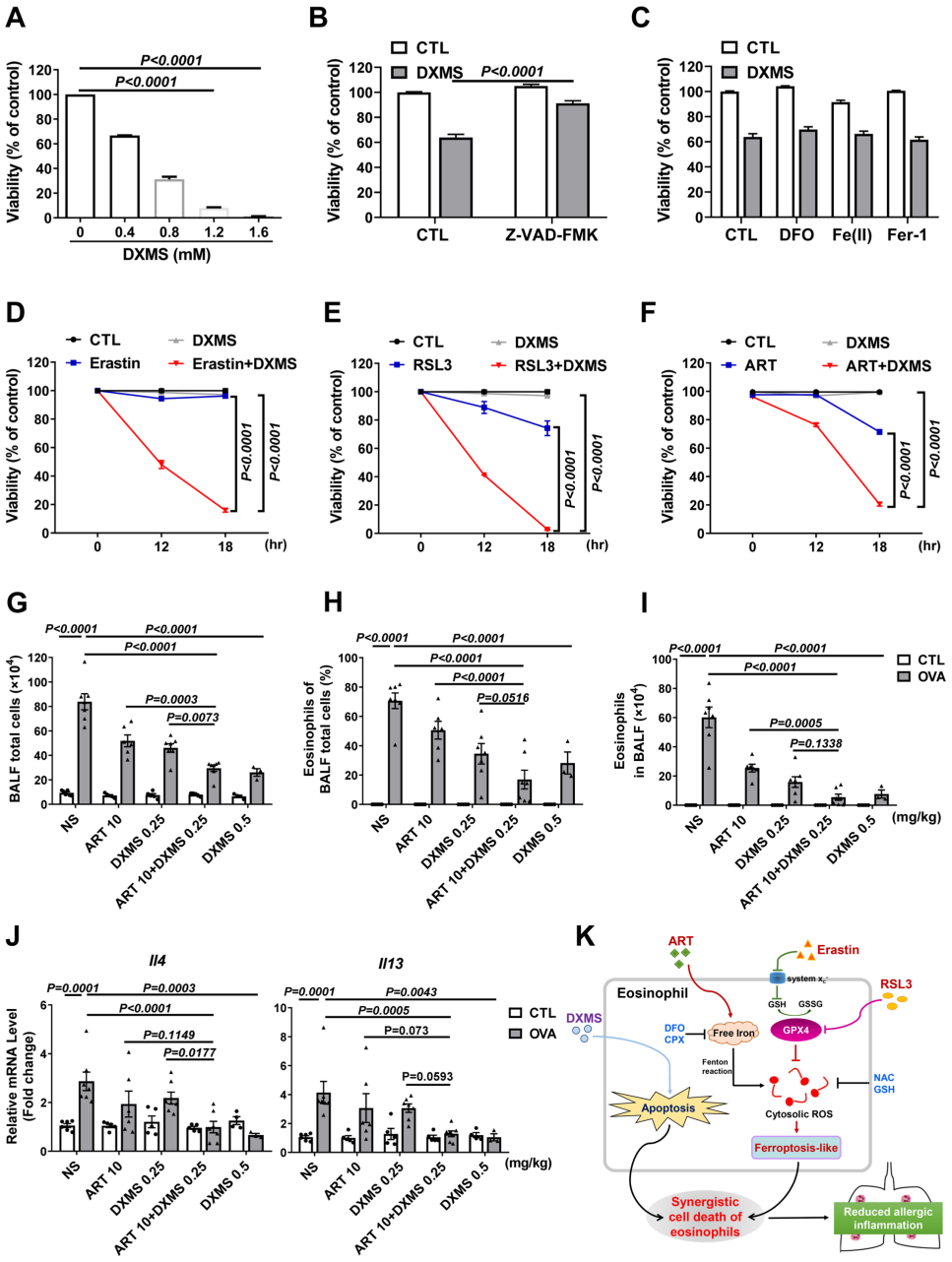

Figure 6 Ferroptosis-inducing agents (FINs) showed a synergistic effect with dexamethasone (DXMS) in vitro and in vivo. Eosinophils were isolated from the peripheral blood of interleukin (*Il*) *5* transgenic mice. Annexin V⁻/PI⁻ cells were defined as viable eosinophils. (A) Eosinophils were cultured with various concentrations of DXMS for 24 hours. (B and C) Effects of Z-VAD-FMK (100 µM), deferoxamine (DFO; 100 µM), ferrous iron (Fe(II); 200 µM) and ferrostatin-1 (fer-1; 2 µM) on the cell viability of eosinophils treated with DXMS (0.4 mM) for 24 hours. (D–F) Viability of eosinophils coadministrated FINs (erastin 30 µM, Ras-selective lethal small molecule 3 (RSL3) 2 µM, artesunate (ART) 100 µM) and DXMS (0.4 mM) at indicated times (12 and 18 hours) was assessed by flow cytometry. (G–J) ART (10 mg/kg) and DXMS (0.25 mg/kg or 0.5 mg/kg) were administered alone or in combination (ART 10 mg/kg and DXMS 0.25 mg/kg), with acquisition and analysis 24 hours after the last administration. Total bronchoalveolar lavage (BAL) cell counts (G), BAL eosinophil percentage (H), BAL eosinophil counts (I) and cytokine levels in lung tissues determined by qPCR (J) are shown. (K) Schematic summarising the synergistic effects of FINs with glucocorticoids in allergic airway disease. All data are shown as mean±SEM, analysed by one-way analysis of variance. CTL, control; PI, propidium iodide.

DXMS in vivo. ART and DXMS at doses of 10 and 0.25 mg/kg/day, respectively, were injected intraperitoneally alone or in combination. Interestingly, the infiltration of total BAL cells and eosinophils in the combination group was lower than that in the groups treated with either ART or DXMS (figure 6G–I). Consistently, levels of the T_H2-related cytokines *Il4* and *Il13*, as measured by real-time quantitative PCR, were more significantly decreased in the combined treatment group (figure 6J). Figure 6K summarises the potential synergistic effects of FINs with GCs in attenuating allergic airway inflammation.

DISCUSSION

In this study, we demonstrated that FINs induced the ferroptosis-like cell death of eosinophils in a time-dependent and concentration-dependent manner. However, unlike the

ferroptosis of engineered tumour cells, the ferroptosis of eosinophils occurred via a non-canonical pathway that was iron dependent and likely required cytosolic ROS, but not lipid or mitochondrial ROS, to trigger cell death. We further demonstrated that promoting the ferroptosis-like cell death of eosinophils relieved allergic airway inflammation in mice. Moreover, FINs exerted a synergistic effect when combined with DXMS in vitro, and the combined administration of ART and DXMS improved the therapeutic effect and reduced the required dosage of DXMS in vivo.

Eosinophils are thought to play important roles in both the maintenance of tissue homeostasis and exacerbation of disease in allergic diseases.[28] Timely resolution of inflammation is essential for the host to prevent severe tissue damage and regain homeostasis. Otherwise, non-resolving inflammation can lead to host tissue injury and organ failure.[2] Of note, it is increasingly recognised that eosinophils at an inflammatory site have a prolonged life span, which may impede the timely clearance of inflammatory cells and delay the resolution of airway inflammation.[8] Recently, pharmacological agents targeting eosinophil apoptosis have been studied, and consequent benefits were observed in preclinical models of inflammation.[29 30] Thus, treatments that specifically promote eosinophil resolution are likely to be effective in controlling allergic inflammatory diseases.

Ferroptosis is a non-apoptotic form of cell death that was recently identified during exposure to FINs, including erastin and RSL3, and other clinical drugs, including ART.[31] Emerging studies suggest that FINs exhibit strong antitumour activity in different cancer cells.[32] We first showed here that FINs could effectively induce ferroptosis-like cell death in eosinophils, which eventually protected against allergic airway inflammation in vivo. This study may provide a novel strategy for airway inflammation therapy similar to other antieosinophil therapies, such as GCs or anti-IL5 treatment. More importantly, we found that FINs showed a synergistic effect with DXMS in triggering eosinophil death in vitro. The drug combination of ART and DXMS also obviously improved the therapeutic effect of steroids in vivo. Therefore, treatments that specifically target eosinophil ferroptosis seem to be effective in controlling allergic airway inflammation and may reduce the dose and adverse effects of GCs. ART, a drug for falciparum malaria, shows remarkable safety and is widely used in the clinic. Thus, our results suggest that combined ferroptosis-apoptosis therapy might serve as a safe and effective treatment for allergic airway inflammation.

Although erastin, RSL3 and ART serve as canonical ferroptosis inducers, accumulating evidence indicates that the mode of cell death may vary in different cell types.[33] Ferroptosis is distinct from known forms of cell death and characterised by the iron-dependent accumulation of ROS, especially lipid ROS, to lethal levels.[14 17] We found that erastin-induced or RSL3-induced eosinophil death could not be prevented by small-molecule inhibitors of apoptosis, necroptosis or autophagy. However, we found that eosinophil death triggered by FINs was iron dependent, as it was inhibited by the presence of the iron chelators DFO and CPX. Mitochondrial changes typical of cell ferroptosis were observed in FIN-treated eosinophils, but few morphological characteristics of other types of cell death were found. Therefore, we assumed that the form of cell death induced by FINs in eosinophils was non-canonical ferroptosis or ferroptosis-like cell death. Nonetheless, we verified that these FINs could trigger typical ferroptosis in MEFs, which was dependent on lipid ROS. Thus, our results suggest that the mechanisms of FIN-triggered ferroptosis are cell type specific and provide a paradigm showing that ferroptosis is not always dependent on lipid ROS.

Ferroptosis is related to metabolic dysfunction that leads to the accumulation of both cytosolic and lipid ROS, independent of mitochondria.[14] It is possible that different types of ROS are involved in mediating cell death in response to lethal stimulation with different FINs. We found that cytosolic ROS were induced by FINs in eosinophils and that FIN-stimulated cytosolic ROS production and cell death could be reversed by NAC or GSH. However, although mitochondrial ROS accumulated in only ART-treated eosinophils, they were not essential for ART lethality, as MitoTEMPO failed to protect against ART-induced cell death. Thus, the initiation of ferroptosis in eosinophils seems to be solely dependent on cytosolic ROS.

Previous studies have reported that the antimalarial mechanism of ART is Fe(II) dependent, as Fe(II) can cause the cleavage of an endoperoxide bridge in ART and lead to the generation of ROS.[34] In addition, ART derivatives have been shown to induce iron-dependent programmed ferroptosis in tumour cells.[35 36] Consistently, in our study, we observed that ART-induced ferroptosis-like cell death of eosinophils was iron dependent. Moreover, it is worth noting that autophagy is associated with ferroptosis in eosinophils following ART treatment but does not contribute to erastin-induced or RSL3-induced ferroptosis. Autophagy has been shown to contribute to ferroptosis by degrading ferritin in fibroblasts and cancer cells.[37] Increased ferritin expression is thought to limit ferroptosis.[38] Thus, increased autophagy might increase iron levels leading to oxidative injury by the Fenton reaction.

In conclusion, our study suggests that induction of the ferroptosis-like cell death of eosinophils might be a promising therapeutic strategy for allergic airway inflammation, especially due to the advantage of its synergy with GCs in the treatment of eosinophil-related disorders.

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Contributors ZC, HS and WL designed and supervised the study. YW, HC, NX, LZ, YW, CZ, ML, QW, JS, ZL, YZ and MW performed experiments. XX, HZ, BZ, FL and LX assisted in the collection of human samples. YW, HC and ZC prepared figures and drafted manuscript. SY, WL, HS and ZC analysed data and revised manuscript. All authors approved the final manuscript.

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