A new thiol-independent mechanism of epithelial host defense against *Pseudomonas aeruginosa*: iNOS/NO• sabotage of theft-ferroptosis

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** Article Info**

**Abstract**

Ferroptosis is a redox-driven type of regulated cell death program arising from maladaptation of three metabolic pathways: glutathione homeostasis, iron handling and lipid peroxidation. Though GSH/Gpx4 is the predominant system detoxifying phospholipid hydroperoxides (PLOOH) in mammalian cells, recently Gpx4-independent regulators of ferroptosis like ferroptosis suppressor protein 1 (FSP1) in resistant cancer lines and iNOS/NO• in M1 macrophages have been discovered. We previously reported that *Pseudomonas aeruginosa* (PA) utilizes its 15-lipoxygenase (pLoxA) to trigger ferroptotic death in epithelial cells by oxidizing the host arachidonoyl-phosphatidylethanolamine (ETE-PE) into pro-ferroptotic 15-hydroperoxy- arachidonyl-PE (15-HpETE-PE). Here we demonstrate that PA degrades the host Gpx4 defense by activating the lysosomal chaperone-mediated autophagy (CMA). In response, the host stimulates the iNOS/NO•-driven anti-ferroptotic mechanism to stymie lipid peroxidation and protect Gpx4/GSH-deficient cells. By using a co-culture model system, we showed that macrophase-produced NO• can distantly prevent PA stimulated ferroptosis in epithelial cells as an inter-cellular mechanism. We further established that suppression of ferroptosis in epithelial cells by NO• is enabled through the suppression of phospholipid peroxidation, particularly the production of pro-ferroptotic 15-HpETE-PE signals. Pharmacological targeting of iNOS (NO• generation) attenuated its anti-ferroptotic function. In conclusion, our findings define a new inter-cellular ferroptosis suppression mechanism which may represent a new strategy of the host against *P. aeruginosa* induced theft-ferroptosis.

1. Introduction

Upon infection, host cells and bacteria enter a tug-of-war [1] in which the invader attempts to make the host environment more favorable to survival and dissemination, whereas the host cells try to mount an effective defense [1,2]. Multiple mechanisms aimed at dampening the host response, including hijacking of mammalian programs of regulated cell death, are enacted by bacteria [3]. Commonly, a number of cell death programs - apoptosis, pyroptosis, necroptosis, autophagic cell death - are employed by the host to counteract the infection [4–6]. However, these same programs may be manipulated and used by pathogens to reverse the role of the cell death process and its outcome in

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their favor [7–9]. One of the latest examples of this is the use of a newly discovered regulated cell death (RCD) program, ferroptosis, by an opportunistic Gram-negative bacterium *Pseudomonas aeruginosa* [10].

In mammalian cells, ferroptosis is triggered by the imbalanced synchronization of iron, thiols and lipids resulting in the selective peroxidation of arachidonoyl-PE (ETE-PE) into pro-ferroptotic 15-hydroperoxy-arachidonyl-PE (15-HpETE-PE) [11–14]. One of the likely catalysts of this reaction is 15-lipoxygenase (15LOX) complexed with a scaffold protein, PE-binding protein-1 (PEBP1) [15–17]. Ferroptosis has also been reported to play a pathogenic role in bacterial infection induced host cell damage [10,18]. We described that *P. aeruginosa* (PA) exploits “theft-ferroptosis” as a virulence mechanism utilizing a specialized 15LOX, pLOXA [10]. This is achieved by targeting and hijacking the host redox lipid remodeling pathway leading to the accumulation of proferroptotic phospholipid hydroperoxides in human bronchial epithelial cells [10]. To counter, host cells usually employ a unique selenoenzyme from the glutathione peroxidase family, GPx4, that specifically reduces phospholipid hydroperoxides to the respective non-ferroptotic alcohols [19,20]. In addition to this main “watchdog” of ferroptotic cell death, recent studies have identified several new GPx4 independent regulators of ferroptosis, explaining the resistance mechanisms exhibited by some cell types [21–24]. In this context, we demonstrated that among the host immune cells, macrophages in M1 activation state expressing INOS/NO*+* are resistant to ferroptosis [23].

NO*+* is a reactive molecule produced by nitric oxide synthase (NOS) family of proteins. NO*+* directly binds and inactivates Fe-containing enzymes [25,26] or reacts with superoxide anion-radical O2*•−* to form a highly reactive peroxynitrite (OONO−) attacking pathogen’s...
membrane lipids and proteins, particularly protein thiols [27,28]. In this way, NO⁺ exerts bactericidal and bacteriostatic propensities within macrophages acting as an essential part of the host defense against pathogens [29].

Similar to other types of regulated cell death, ferroptosis uses intrinsic cellular machinery for the program execution. However, quite distinctively, ferroptosis is a non-cell autonomous program, that can spread and impact the neighboring cells [30,31]. Given the diffusible signaling characteristics of NO⁺ and its ability to protect M1 macrophages from ferroptosis, we envisioned a unique intercellular anti-ferroptotic protection by NO⁺, particularly in the context of host-pathogen interactions. Here, using a two-cell epithelial and macrophage coculture system, we demonstrate that i) PA targets host GPx4/GSH system prompting degradation of GPx4 and promoting ferroptosis; ii) PA-stimulated macrophages generate NO⁺ which prevents phospholipid peroxidation, particularly the production of pro-ferroptotic 15-HpETE-PE signals, and hence protecting against ferroptosis in macrophages as well as in neighboring epithelial cells; iii) using si-RNA mediated knock-down (KD) approach in epithelial cells, we validated that even under GPx4 insufficient conditions, INOS/NO⁺ protects cells against ferroptosis.

2. Results

2.1. PA targets host GPx4/GSH system

PA produces, secretes and utilizes outer membrane vesicles (OMVs) as virulence factors which interact and alter host cell biology [32–34]. Among them are inducers of ferroptosis, including pLoxA [10]. We previously observed that pLoxA activity of the pathogen OMV (supernatants) and the GSH levels of host cells collectively are promising predictors of ferroptotic cell death [10]. However, to assess their individual contributions and to understand whether the changes induced by the pathogen and host responses are dependent on one another, we performed partial correlation analysis between pLoxA activity of P. aeruginosa clinical isolates and ferroptosis while controlling for host GSH levels (Fig. 1A, left panel). Conversely, controlling for pLoxA activity, we performed partial correlation analysis of host GSH levels after treatment with P. aeruginosa clinical isolates and cell death (Fig. 1A, right panel). At constant GSH levels in host cells, the pLoxA activity elicits a robust positive correlation with cell death ($r$ value of 0.76, $p = 2.4 \times 10^{-6}$). Similarly, maintaining pLoxA activity of the supernatants constant, the host GSH levels displayed a strong negative correlation with cell death ($r$ value of -0.68 and $p = 7.5 \times 10^{-5}$), (Fig. 1A, right panel). This suggests that the two factors, pLoxA activity of the pathogen and the GSH levels of host, are independent of each other; however, collectively they produce a synergistic effect. It also implies that for proficient theft-ferroptosis to occur, PA supernatant in addition to containing the pLoxA also targets the GPx4/GSH defense system of the host.

GSH-dependent selenoenzyme GPx4 is a central anti-ferroptotic defense system of the host that controls ferroptosis by reducing the pro-ferroptotic signal 15-HpETE-PE to non-ferroptotic 15-hydroxy-arachidonoyl-phosphatidylethanolamine (15-HETE-PE) [12,20,35]. Sufficiency of this system maintains cellular homeostasis and resistance to ferroptotic death program [28]. We found that treatment with pLoxA-containing supernatant targeted GPx4 and degraded it in a pLoxA dependent manner (Fig. 1B). Treatment with PA supernatant was also accompanied by a significant decrease in the activity of GPx4 (Supplementary Fig. 1A). Expectedly, the treatment caused enhanced “lipid ROS” as indicated by BODIPY oxidation (Fig. 1B). In contrast, the supernatant from pLoxA deficient mutant (loxA::Tn) was not able to affect either the GPx4 protein levels or induce lipid peroxidation (Fig. 1B).

2.2. PA activates host CMA pathway for the degradation of GPx4

Host protein regulatory pathways are one of the major targets of pathogens. It has been reported that P. aeruginosa exploits the host ubiquitination signaling pathways for its successful progression [36]. Therefore, we next explored the host protein degradation pathway targeted by PA supernatant to manipulate the GPx4 protein levels. For this we incubated HBE cells with PA supernatant and commonly used inhibitors of proteasome (MG132 and PS341) or lysosomal (NH₄Cl and Chloroquine) degradation pathways (Fig. 1C). Only NH₄Cl and Chloroquine (CQ) displayed significant inhibition of ferroptosis suggesting the involvement of lysosomal pathway in GPx4 degradation (Fig. 1C). Both NH₄Cl and CQ also inhibited lipid ROS, while the proteasomal inhibitors (MG132 and PS341) were inept in protecting epithelial cells from BODIPY oxidation (Fig. 1D). Further, we confirmed the involvement of lysosomal degradation pathway by testing activation of Lamp2a after treatment with PA supernatant and found that the Lamp2a levels were considerably increased (Fig. 1E, left panel). Lamp2a is a hallmark for CMA-a specific lysosomal protein degradation pathway [37,38]. Notably, the decrease in levels of GPx4 protein by PA treatment were rescued by both NH₄Cl and CQ (Fig. 1E, right panel).

There are several types of ferroptosis inducers [39,40]. Two of them affect either the pathways controlling GSH levels (e.g., erastin inhibiting cystine/glutamate antiporter) [39,41] or GPx4 activity (e.g., RSL3, ML162). Our results indicate that CMA-inhibitors, NH₄Cl and CQ, rescued cells from erastin induced ferroptotic death but not from RSL3 induced ferroptosis (Fig. 1F). None of the inhibitors affected erastin dependent decrease in GSH/GSSG (Supplementary Fig. 1B). Based upon these findings, we conclude that co-incubation of epithelial cells with PA supernatant leads to inactivation of the “host” GPx4/GSH defense system yielding ferroptosis.

2.3. iNOS/NO⁺ rescues epithelial cells from PA induced ferroptotic cell death

Pulmonary epithelial cells are surrounded by host immune cells such as macrophages whose job is to remove microbial threats. In the context of ferroptosis, macrophages are of particular importance since INOS makes them resistant to ferroptosis [23]. Interestingly, incubation of macrophages with PA supernatant led to their transition to M1 phenotype with high level of iNOS expression (Fig. 2A) and resulting in resistance to ferroptosis (Fig. 2B). Counting the amounts of macrophages after PA treatment revealed no difference in the cell numbers after activation (Supplementary Fig. 2A, left panel). Based on these findings, we explored whether expression of iNOS in macrophages may be sufficient to prevent the epithelium from PA induced ferroptosis. When epithelial cells were co-incubated with PA supernatant in the presence of macrophages, the ferroptotic death of epithelial cells was significantly suppressed (Fig. 2C). Since lipid peroxidation is a requirement for ferroptosis, we used BODIPY oxidation as an indicator of lipid peroxidation in our co-culture experiment and found that macrophages significantly blunted the generation of lipid peroxidation in epithelial cells (Fig. 2D).

2.4. Epithelial cells and iNOS

iNOS can also be expressed by epithelial cells in response to cytokines or bacterial products [25,42]. Therefore, we examined the levels of iNOS in HBE cells incubated in the presence of PA supernatant. Treatment with PA supernatant did not stimulate the expression of iNOS in these cells (Fig. 2E). Next, we wanted to know whether INOS/NO⁺ can function as anti-ferroptotic regulator in epithelial cells. For this, we expressed iNOS in epithelial cells and then treated them with PA supernatant or RSL3 or erastin to trigger ferroptosis. Indeed, we found that iNOS expressing epithelial cells were resistant not only to PA supernatant initiated ferroptotic insult but also to other commonly used ferroptosis inducers like RSL3 and erastin (Fig. 2F and G). Expression of
iNOS did not increase epithelial cell proliferation (Supplementary Fig. 2, right panel). To confirm that the protection was due to iNOS/NO, we tested the stability of GPx4 in the presence of sets of experiments; i) we tested the stability of GPx4 in the presence of (right panel) and by flow cytometry (right panel) (n = 3). (B) Macrophages were activated with PA supernatant for 24 h and then treated with erastin (left panel) or RSL3 (right panel) along with ferrostatin 1 (Fer-1, 0.4 μM). Cell death was measured by LDH release assay after 14 h. ***p < 0.0001 relative to samples without (erastin/RSL3), **p < 0.001 vs RSL3 or erastin alone (n = 3). (C & D) Epithelial cells were co-cultured with macrophages (1:1) in 6 well plates and then treated with PA supernatant. Cell death and Lipid ROS was measured by Zombie staining and BODIPY using the flow cytometry. ***p < 0.0001; **p < 0.001 relative to HBE + PA Sup.; *p < 0.0001 vs UT (untreated HBE only), (n = 3). (E) Expression of iNOS in epithelial cells after PA treatment. (F & G) Epithelial cells were transfected with vector only (pCMV6) or with iNOS (pCMV6-iNOS) and then treated with PA supernatant (F) or with RSL3 (500 nM) or erastin (10 μM) (G) in the absence or presence Fer-1 or iNOS inhibitors. *p < 0.0001 vs UT (untreated); ***p < 0.0001 relative to PA Sup./RSL3/erastin; **p < 0.001 vs pCMV6-iNOS treated PA Sup./RSL3/erastin. (n = 3). (H) Epithelial cells with or without iNOS were treated with PA Sup. Lipid ROS was determined by BODIPY staining and analyzed by flow cytometry. *p < 0.0001 vs UT; ***p < 0.0001 relative to PA Sup. without iNOS. (n = 3).

### 2.6. iNOS/NO* induced protection is independent of GPx4

Next, we asked whether iNOS/NO* can protect epithelial cells from PA stimulated ferroptotic cell death under GPx4 limiting conditions. For this, we first knocked-down (KD) GPx4 using si-RNA and then expressed iNOS. KD was confirmed by western blotting (Fig. 3D, left panel). We observed that the decrease in the amount of GPx4 protein sensitized epithelial cells to PA induced ferroptosis and lipid ROS (Fig. 3D and E). Remarkably, expression of iNOS/NO* rescued GPx4-deficient cells from both lipid ROS and ferroptotic death (Fig. 3D and E).

### 2.7. Macrophage mediated protection is achieved via intercellular transfer of NO* from macrophages to epithelial cells

Under conditions of PA induced deficiency of GPx4/GSH anti-ferroptotic defense in host epithelial cells, the potential of iNOS/NO* to act remotely and independently of GPx4 bestows the host with a unique intercellular mechanism to protect the epithelial cells from PA. To examine this role of NO*, we performed a co-culture experiment in the presence of specific inhibitor of iNOS, which significantly eliminated the protection of epithelial cells and made them sensitive to PA induced ferroptosis (Fig. 4A). As a second line of evidence, we employed si-RNA to knock-down iNOS in macrophages and then used them for co-culture experiment. Again, we found that iNOS deficient macrophages were incompetent to rescue epithelial cells from PA supernatant induced ferroptotic cell death (Fig. 4B).
2.8. Redox phospholipidomics reveals reduction of proferroptotic signal (15-HpETE-PE) in epithelial cells cocultured with macrophages

Lipid peroxidation is the driver of ferroptotic cell death [12,43]. Since macrophage generated NO\(^\bullet\) does not regulate ferroptotic cell death at GPx4 level, we wondered whether NO\(^\bullet\) generated by macrophages could hinder the ferroptotic death signals. For this we labeled epithelial cells with Cell Tracker Green and co-cultured them with macrophages (1:1). After treatment with PA supernatant, we sorted epithelial cells by flow-cytometry and performed LC-MS.
lipidomics analysis (Fig. 4C). Indeed, we found a significant decrease in the previously established proferroptotic signals 15-HpETE-PEs in HBE cells co-cultured with macrophages (Fig. 4D and E) [10,12].

3. Discussion

The association of ferroptosis with various disease conditions ranging from neurodegenerative disorders, ischemia-reperfusion injury, organ failure to resistant cancers is now well established [31]. This also rescued ferroptosis through NAD(P)H/FSP1/CoQ10 axis, independently inducible nitric oxide synthase (iNOS) [23]. Overexpression of FSP1 rescues ferroptosis through NAD(P)/FSP1/CoQ10 axis, independently of GPx4 [21,22]. Similarly, activation of iNOS in M1 macrophages or microglia confers resistance to ferroptosis by preventing lipid peroxidation [23].

In this study, we attempted to investigate the role of host NO© as an intercellular anti-ferroptotic mechanism against PA induced ferroptosis. We demonstrate that after activation, macrophage generated NO© disseminates to surrounding epithelial cells, where it disrupts pathogen triggered ferroptotic process, and protects epithelial cells from ferroptotic death. This unique intercellular anti-ferroptotic function of NO is due to its small size, lipophilic and highly diffusible nature and its ability to prevent lipid peroxidation, including enzymatic peroxidation catalyzed by 15LOXecs: pLOxA in PA supernatants and 15LOX/PBP1 complexes in mammalian cells [23,44].

For the host, GPx4/GSH anti-oxidant system is the central shield against lipid hydroperoxide driven ferroptotic cell death and is emerging as a prime target for pathogens to chase and exploit for their benefit [10,18,45]. Even though the breakdown of this shield is sufficient to trigger ferroptosis, our previous work and the results of this study suggest that P. aeruginosa targets multiple host pathways. On the one hand, P. aeruginosa secreted lipoxigenase hijacks host lipid modelling pathway to generate pro-ferroptotic signals and, on the other hand, the pathogen manipulates the host defense system responsible for eliminating such signals (Fig. 1) [10]. Similarly, in tuberculosis, Mycobacterium tuberculo- sis induced ferroptosis is considered to be one of the primary mechanisms of infection and is postulated as a promising target for therapeutic intervention [18]. Hence, assuming ferroptosis a part of the virulence repertoire of PA, we have uncovered an important protective (anti-)ferroptotic role for host NO©. This is important for two main reasons i) host epithelial cells are defenseless and overtaken by the pathogen; ii) the pathogen utilizes outer membrane vesicles (OMV) as a primary source of virulence executioners which interact and alter the host biology without any need for an actual direct contact [32–34].

As GPx4 is a central armor for the cell against formation of lipid hydroperoxides [12,20,46], its degradation is a mandatory signaling event in the execution of ferroptotic cell death [12,20]. Similar to classical ferroptosis inducers like erastin or RSL3, PA supernatant induces degradation of GPx4 leading to intensified lipid hydroperoxide generation and ferroptosis (Fig. 1) [10]. Recently, the mechanisms leading to depletion of GPx4 has attracted a lot of attention and a hope that it will give us additional tools to control, study and understand ferroptotic cell death pathway [46]. The mechanisms are emerging but still are not completely clear. Two independent studies have demonstrated the involvement of lysosomal protein degradation pathway in erastin induced GPx4 degradation-one through activation of Lamp2a [47] and the other one via acid sphingomyelinase (ASM) mediated redox amplification [48]. In this study, we demonstrate that treatment of PA supernatant leads to Lamp2a mediated GPx4 depletion through CMA activation. The presence of lysosomal degradation inhibitors (NH4Cl and CQ), resulted in diminished PA induced lipid peroxidation and restoration of GPx4 protein levels.

NO© is a potent signaling molecule with the ability to act in a paracrine manner during smooth muscle relaxation or vascular dilation or act as intracellular secondary messenger regulating anti-oxidant response [25,44]. Inducible nitric oxide synthase (iNOS) generated NO©- through interactions with superoxide radicals to produce peroxynitrite, is considered as the first line of host immune defense response against invading pathogens particularly the intracellular ones [25,26,49]. In addition, NO©’s ability to interact with different radicals, including lipid radical intermediates during ferroptosis, can facilitate post-translational nitrosylation of proteins modifying their activity, stability or localization [27]. We found that expression of iNOS did not stabilize or prevent degradation of GPx4, but prevented generation of lipid hydroperoxides and ferroptosis even under GPx4 insufficient conditions. This highlights the role of iNOS/NO© as GPx4 independent regulator of ferroptosis. The fact that NO© is protective against different types of ferroptosis inducers (RSL3, erasin or PA) and is competent locally as well as remotely far away from the source of its generation makes it an important anti-ferroptotic regulatory factor. These features of NO© may have crucial implications in tumors or in chronic P. aeruginosa or M. tuberculosis lung infections, where the neighboring microenvironment influences the outcome of the target cells. It has been reported that in epithelial cancer cells, E-cadherin mediated intercellular signaling via E-cadherin-NF2-Hippo-YAP axis enhances the resistance to ferroptosis of all cells involved [50]. It is well known that normally low abundance of P. aeruginosa, may be substantially elevated in conditions leading to the immune-compromised states such as massive irradiation of cancer patients [51,52]. It is possible that employment of NO© donors may represent a promising therapeutic strategy in these patients as well in cases of exposure to total body irradiation after accidental or intentional catastrophic circumstances [53].

Lipid peroxidation is one of hallmarks of ferroptosis [12,43,54]. In biological systems, lipid peroxidation can proceed via enzymatic or non-enzymatic mechanisms [55]. While free ionic iron or complexes of iron with low molecular weight ligands represent major non-enzymatic catalysts of free radical lipid peroxidation, lipoxigenases, cyclo-oxygenases, and cytochromes P450 are the major enzymes involved in lipid peroxidation [14,55]. Under normal conditions, these enzymes predominantly utilize free polyunsaturated fatty acids as their substrates of oxygenation reactions yielding different lipid mediators, mostly regulating inflammatory responses [56,57]. 15LOXs can also catalyze peroxidation of polyunsaturated fatty acid residues of phospholipids [16]. Ferroptosis is marked by accumulation of unstable
hydroperoxy-phosphatidylethanolamines and subsequent failure of GSH/GPX4 system to reduce them to the respective alcohols. While both non-enzymatic and enzymatic pathways may be involved in ferroptosis, the selectivity and specificity of characteristic products, hydroperoxy-phosphatidylethanolamines, suggests the involvement of enzymatic pathways in the initiation of the process [58]. Our data demonstrate that irrespective of the source of NO*, either from over-expression in HBE cells or from neighboring macrophages, it decreases lipid peroxidation and prevents epithelial ferroptosis induced not only by PA but also by other inducers. In addition, in the co-culture system we observed that the epithelial cells generate a significantly lower amount of pro-ferroptotic 15-HpETE-PE signals. This protection from different types of ferroptotic cell death inducers suggests a general anti-ferroptotic regulatory role of NO*. Inhibition of lipid peroxidation by nitric oxide may be realized via several mechanisms [59, 60]. Among them are the reduction of the catalytic 15LOX iron, scavenging of lipid radical intermediates during peroxidation and competitive inhibition by NO* of molecular oxygen delivery through specialized channels in the complex of 15LOX with a scaffold-protein, PEBP1, catalyzing peroxidation of polyunsaturated phosphatidylethanolamines (Unpublished results) [60, 61]. While all three modes of action can participate in the inhibition of ferroptosis, further detailed studies are needed to quantitatively assess their specific contribution.

Overall, this study highlights a new intercellular anti-ferroptotic role of NO* with immense implications in the context of host-pathogen interaction. The anti-ferroptotic function of NO* is fueled by its small size, diffusible nature and ability to react with enzymatic machinery generating hydroperoxy-PE as well as with radical intermediates of pro-ferroptotic signals. Importantly, iNOS/NO* functions independently of GPx4, protecting GPx4 deficient cells from ferroptosis. Furthermore iNOS/NO* system is equally competent against different ferroptosis inducers (PA supernatant, RSL3, erasin), thus providing another universal layer of protection against ferroptotic death of the host epithelium.

4. Materials and methods

4.1. Reagents

MEM (Thermo Fisher Scientific, 11095-080), FCS (Gibco, 10437-028), Lysogeny broth (LB) (Sigma-Aldrich, L3152), RSL3 (Selleck Chemicals, S8155), erasin (Selleck Chemicals, S7242), ferrostatin-1 (Sigma-Aldrich, SML0583), Chloroquine (Sigma, C6628), NH4Cl (Sigma, A9434), MG132 (Selleck Chemicals, S2619), P3421 (Selleck Chemicals, S1013), 1400W (Cayman, 81520), L-NIL (Cayman, 80310), BSA (Sigma-Aldrich), DETA-NONOate and DPTA-NONOate (Cayman Chemicals, S1013), 1400W (Cayman, 81520), L-NIL (Cayman, 80310), BSA (Sigma-Aldrich), DETA-NONOate and DPTA-NONOate (Cayman Chemical), NADPH (Sigma), Glutathione peroxidase (Sigma G6137), Cumene hydroperoxide (Sigma C0524), Thiol fluorescent probe IV (Millipore 595504), Glutathione reductase (Sigma), Glutathione reduced (Sigma G4251), 15-HpETE-PE (Caymen Chemical), Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 232225), anti- GPx4 (rabbit monoclonal, Abcam, ab125066), anti-Lamp2a (Abcam, ab18528), anti-iNOS (Abcam, ab3522), anti-actin (mouse monoclonal, Sigma-Aldrich, A3854, clone AC-15), secondary antibody, goat anti-rabbit (Sigma-Aldrich, A0545).

4.2. Cell culture

Macrophages (RAW 264.7) from ATCC were cultured at 37 °C and 5% CO2 in DMEM (ATCC) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich) and 100 U ml−1 penicillin–streptomycin (ThermoFisher Scientific). HBE (bronchial epithelial cell line originally established by D. Gruenert) was maintained in minimum essential medium (MEM; ThermoFisher) supplemented with 10% FCS (Gibco), 50 μl ml−1 penicillin–streptomycin (ThermoFisher), plasmocin (InvivoGen) and uglutamine (ThermoFisher) as described previously [10, 23, 62]. HT-1080 cells from ATCC were cultured in EMEM media (ATCC) containing 10% fetal calf serum (FCS; Sigma-Aldrich) and 100 U ml−1 penicillin–streptomycin (ThermoFisher Scientific) and incubated at 37 °C and 5% CO2.

4.3. Bacterial strains

The P. aeruginosa hyper-biofilm mutant (Δ wspF) and the pLoxA deficient (loxA::Tn) strains used in this study was obtained from the P. aeruginosa transposon mutant library, University of Washington (Seattle), as described previously [10]. P. aeruginosa respiratory isolates (Tobramycin-resistant) from ICUs were identified at the clinical microbiology laboratory of University of Pittsburgh Medical Center (IRB no. PRO12066302) and designated TRPA002–TRPA122 as described previously [10].

4.4. PA supernatant collection

P. aeruginosa was grown in LB medium at 37 °C, 220 r.p.m for 14 h and then re-inoculated in MEM medium (without phenol red) at an OD600 of 0.05 in 96-well microtitre plates (100 μl per well; Costar). Plates were then incubated at 37 °C without agitation for 24 h. Supernatant was collected by centrifugation (2X) at 3,000 g for 8 min and then frozen before further use in cell culture experiments [10].

4.5. siRNA knockdown experiments

Two Dicer-substrate si-RNAs against iNOS (mm.Ri. Nos2.13.1 and mm.Ri. Nos2.13.2) or GPx4 (hs.Ri.GPx4.13.1 and hs.Ri.GPx4.13.2) or control scrambled DsiRNA (51-01-14-04) (Integrated DNA Technology) were transfected into macrophages or epithelial cells (HBE) using RNAiMax (Life Technology) according to the manufacturer’s instructions. After transfection (12 h or 8 h), cells were counted and seeded into a 6-well plate for co-culture experiments or 6 cm dishes for western blotting, and treated with PA supernatant for 48 h.

4.6. Overexpression of iNOS

Epithelial or HT-1080 cells were transfected with 1 μg of vector only (pCMV6) or pCMV6-iNOS DNA using Lipofectamine 3000 (Life Technology) for 12 h. Cells were then counted and seeded for cell death or Lipid ROS experiments in the absence or presence of Fer-1 or iNOS inhibitors (L-NIL and 1400W) as indicated. Epithelial cells were transfected with 1 μg of vector only (pCMV6) or pCMV6-GPx4 DNA using Lipofectamine 3000 (Life Technology) for 12 h. Cells were then counted and seeded for Lipid ROS experiments.

4.7. GPx4 activity

HT-1080 untreated and PA supernatant treated cell pellet was resuspended in 0.1 M Tris (pH 8.0) and 150 mM NaCl containing protease/phosphatase inhibitor (1X) and sonicated (3X, 10s on 15s off, at 20%). After sonication, cell lysate was centrifuged at 12000 rpm for 14 min and the supernatant was collected and estimated for protein concentration using Bio-Rad protein assay (Biorad) according to the manufacturer’s instructions. Supernatant (2 μg protein in buffer containing 0.1 M Tris (pH 8.0), 1.25% triton X-100 and 50 μM of H2O2) was incubated with horseradish peroxidase (HRP) conjugated with anti-GPx4 antibody and incubated at room temperature for 15 min. Reaction was performed using 120 μg of protein in buffer containing 0.1 M Tris (pH 8.0), 1.25% triton X-100 and 50 μM of H2O2, horseradish peroxidase (3 mM), 120 μM of H2O2 and 0.1% H2S. Consumption of NADPH was measured by fluorescence using Shimadzu spectrofluorimeter RF-5301 PC. Fluorescence measurements were performed in 50 μl quartz cuvette using an excitation wavelength of 340 nm, emission wavelength 460 nm (excitation and emission slits of 5 nm and 3 nm, respectively).

4.8. Ferroptosis assay

Epithelial cells were seeded in 24-well plates (20,000 cells per well) for 48 h and then treated with PA Sup or RSL3 or erasin along with
ferrostatin 1 or protein degradation inhibitors MG132, PS341, NH4Cl and Chloroquine (CQ) or NO - donors for 20 h. Cell death was monitored by PI staining using flow cytometry as described previously [10]. For macrophages, cells were plated in 24 well plates and then activated with PA supernatant for 24 h. Then cells were incubated in fresh media along with RSL3 or erastin and cell death estimated after 12 h by LDH release assay as previously reported [12,23]. HT-1080 untreated or pCMV6 or iNOS expressing cells (20,000 per well) after seeding in 24 well plate for 24 h were treated with PA supernatant with Fer.1 or NO – donors for 20 h. Cell death was monitored by PI staining using flow cytometry as described previously [10].

4.9. Lipid ROS analysis by BODIPY-C11

To analyze lipid ROS, epithelial cells were seeded in 12 well plates for 48 h before treating with PA supernatant. After 14 h of treatment, cells were incubated with 5 μM BODIPY 581/592-C11 (Invitrogen, D3861) for 25–30 min at 37 °C. Cells were then trypsinized, washed with HBSS (2X) and resuspended in HBSS followed by flow cytometric analysis using FL1 channel in BD Canto-II flow cytometer (BD Biosciences). For data analysis FlowJo software was used.

4.10. Co-culture experiments

Epithelial cells 0.08 × 10^6 per well were seeded in 6well plates for 48 h and then labeled with CellTracker Green (Invitrogen, C7025) (5 μM) for 45 min as previously described. After washing (3X) with PBS, macrophages (0.08 × 10^6) were added and incubated for 6 h before treating the co-cultured cells with PA supernatant along with Fer.1 or iNOS inhibitor L-NIL. Cells were trypsinized and labeled with fixable Zombie NIR viability kit (BioLegend, 234105) as per the instructions of the manufacturer. Briefly, after washing with PBS, cells were incubated in 100 μl Zombie NIR in PBS (1:1000 diluted) for 30 min in the dark and then washed with PBS containing 0.1% BSA. Finally, cells were fixed with 4% paraformaldehyde and analyzed by flow cytometry. Acquisition was performed by multicolor flow cytometry using LSRII cytometer with FACS DIVA software (BD Bioscience). Double positive (Cell tracker Green+/Zombie−) epithelial cells were considered for analysis using FlowJo software.

4.11. Western blot analysis

Cells lysates were prepared after washing cells with PBS and lysing in buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1% SDS) or by resuspending the pellet in RIPA buffer (ThermoFisher) containing freshly added protease-phosphatase cocktail inhibitor mix (Thermo-Fisher). Samples were sonicated and total protein amount was estimated by bicinchoninic acid (BCA) protein assay (ThermoFisher) and diluted in 2x Laemml buffer before loading in 8–16% Tris-glycine gradient gels (Life Technologies). Subsequently, proteins were transferred to PVDF membrane and blocked with PBST (5% milk) for 1 h and the proteins were detected using anti-GPX4 (1:2000), anti-iNOS (1:1000), anti-Lamp2a (1:1000) and anti-f-actin (1:1000 for h), antibodies following overnight incubation at room temperature. Membranes were washed with PBST (3X) and then incubated with HRP-conjugating goat anti-rabbit (1:2000 1 h) and then developed with SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher) using Amersham Imager 600 (GE Health Care, Life Sciences).

To determine expression of iNOS protein in macrophages by flow cytometry, cells were stained with anti-CD11b antibody conjugated with BUV605 (Biolegend) followed by the intracellular staining with fixation and permeabilization using anti-iNOS antibody conjugated with AlexaFl700 (BD Bioscience).

4.12. GSH measurements

GSH measurements were performed as described previously [10]. Briefly, cells were resuspended in 50 μl of PBS and then lysed by freeze-thaw and bath sonication. Samples 10 μl in triplicate were incubated with 10 μM Thiol fluorescent probe IV (Millepro) for 5 min and then analyzed with an excitation and emission wavelength of 400 nm and 465 nm using the Cytation 5 imaging reader (BioTek). GSH amount was normalized by protein concentration measured by BCA method. To estimate the contribution of other small molecular weight thiols, we utilized untreated (control) and erastin treated HBE cells and pre-incubated the samples with Glutathione peroxidase from bovine erythrocytes and cumene hydroperoxide to selectively oxidize GSH. After this, we added the excess of a thiol fluorescent probe (10 μM). We found that the contribution of other small thiols was minor and did not exceed 10% of the response in the presence of endogenous GSH (Supplementary Fig. 3).

For GSH/GSSG measurement, the sample was treated with 2 U ml⁻¹ of glutathione reductase (Sigma) and 30 μM of NADPH (Sigma Aldrich) at room temperature for 30 min to reduce all present GSSG to GSH. After reaction, untreated and reduced sample was taken in duplicate at 10 μl amounts and incubated with 10 μM Thiol Fluorescent Probe IV (Millipore) at room temperature for 5 min and analyzed with an excitation and emission wavelength of 400 nm and 465 nm using the Cytation 5 Imaging Reader (BioTek). Thiol Fluorescent Probe IV contains reactivity toward 2-ME, GSH, and cysteine but not non-thiol-containing GSSG or cystine. Therefore, the GSSG amount was calculated by taking the GSH difference of reduced and untreated samples and divided by two (1 GSSG yields 2 GSH). GSH amounts were normalized by protein concentration measured by BCA method.

4.13. Determination of pLoxA activity in P. aeruginosa clinical isolates

Supernatant from clinical isolates (50 μl) was mixed with 50 μl of 20 mM HEPES buffer, pH 7.4, containing 100 μM DTPA, 10 μM AA (arachidonic acid), and 0.5 μM 15-HOO-AA-PE and incubated for 10 min at 37 °C. Then samples were extracted by Folch procedure [12,16] and analyzed by LC-MS. Data was normalized to the amount of protein and presented as AAoxidation (pmol/min/μg of protein).

4.14. LC–MS analysis of phospholipids

MS analysis of phospholipids was performed on an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher). Phospholipids were separated on a normal phase column (Luna 3 μm Silica (2) 100 Å, 150 × 2.0 mm, Phenomenex) at a flow rate of 0.2 ml min⁻¹ on a Dionex Ultimate 3000 HPLC system. The column was maintained at 35 °C. Analysis was performed using gradient solvents (A and B) containing 10 mM ammonium acetate. Solvent A contained propanol:hexane:water (285:215:5, vol/vol/vol) and solvent B contained propanol:hexane:water (285:215:40, vol/vol/vol). All solvents were LC–MS grade. The column was eluted for 0–23 min with a linear gradient of 10–32% B; 23–32 min using a linear gradient of 32–65% B; 32–35 min with a linear gradient of 65–100% B; 35–62 min held at 100% B; 62–64 min with a linear gradient from 100% to 10% B followed by and equilibration from 64 to 80 min at 10% B. The instrument was operated with the electro-spray ionization probe in negative polarity mode. Analysis of LC-MS data was performed using software package Compound Discoverer (ThermoFisher) with an in-house generated analysis workflow and oxidized phospholipid database. Lipids were further filtered by retention time and confirmed by fragmentation mass spectrum.

4.15. Partial correlation analysis

Partial correlation analysis was performed using the function pcor.test from ppcor package in R languages. Partial correlation analysis was
performed between ferroptotic cell death and GSH or pLoxA while controlling for pLoxA or GSH levels respectively. Residual scatter plot was plotted using ggplot2 package of R language.

4.16. Statistical analysis

The data in figure legends are presented as mean ± s.d. values from at least three experiments unless otherwise specified. In general, the exact value of sample size (n) is presented in the figure legends and reflects either the number of experimental replications with cells or biochemical model systems. Statistical analyses were performed by One-way ANOVA, Tukeys multiple comparisons test, unless otherwise specified.

Author contributions

H. H. D., H. B and V. E. K conceived the study. H. H. D., L. A. P. and A. B. performed all cell experiments. T. S. A. performed lipidomics experiments and analyzed the data. H. H. D., A. B. and B. S. performed flow analysis and harvested data. O. K. performed GSH measurements and analyzed the data. V. A. T performed pLoxA activity experiments and LC-MS analysis. J. S. L. participated in selection and testing of clinical isolates. R. K. M. and E. W. participated in formulating the idea and interpreted the results. J. S. L. and H. B. participated in writing the manuscript. H. H. D. and V. E. K wrote the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflicting interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102045.
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