Phospholipid peroxidation fuels ExoU phospholipase-dependent cell necrosis and supports *Pseudomonas aeruginosa*-driven pathology

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Summary

Regulated cell necrosis supports immune and anti-infectious strategies of the body; however, dysregulation of these processes drives pathological organ damage. *Pseudomonas aeruginosa* expresses a phospholipase, ExoU that triggers pathological host cell necrosis through a poorly characterized pathway. Here, we investigated the molecular and cellular mechanisms of ExoU-mediated necrosis. We show that cellular peroxidised phospholipids enhance ExoU phospholipase activity, which drives necrosis of immune and non-immune cells. Conversely, both the endogenous lipid peroxidation regulator GPX4 and the pharmacological inhibition of lipid peroxidation delay ExoU-dependent cell necrosis and improve bacterial elimination *in vitro* and *in vivo*. Our findings also pertain to the ExoU-related phospholipase from the bacterial pathogen *Burkholderia thailandensis*, suggesting that exploitation of peroxidised phospholipids might be a conserved virulence mechanism among various microbial phospholipases. Overall, our results identify an original lipid peroxidation-based virulence mechanism as a strong contributor of microbial phospholipase-driven pathology.

Key words: lipid peroxidation, microbial phospholipases, cell necrosis, *Pseudomonas aeruginosa*

Introduction

Regulated cell necrosis (RCNs) drives physiological and immune processes, yet dysregulation of this process promotes pathological responses such as organ-failure and sepsis (Bedoui et al., 2020; Galluzzi et al., 2018; Place et al., 2021; Tang et al., 2020). Mechanistically, oxygen-dependent cell death is an evolutionary conserved process that involves the production of reactive oxygen species (ROS), transition metals (e.g. iron) and peroxidised lipid accumulation (Bogacz and Krauth-Siegel, 2018; Conrad et al., 2018; Dixon et al., 2012; Jenkins et al., 2020). In addition to cell necrosis, lipid peroxidation broadly involves cellular processes essential to mediate optimal efferocytosis of dead cells, cellular communication resulting from the formation of lipids derived from peroxided phospholipids (e.g. isoprostanes, platelet activating factor) or the production of bioactive lipids (eicosanoids) from arachidonic acid (Bochkov et al., 2010; Tyurina et al., 2019). In addition, the peroxidation of the mitochondrial phospholipid cardiolipin initiates apoptosis while the accumulation of peroxidised phosphatidyl ethanolamines (PE) promote the cellular necrosis, ferroptosis (Bersuker et al., 2019; Conrad and Pratt, 2019; Doll et al., 2017, 2019; Kagan et al., 2017; Wiernicki et al., 2020; Yang et al., 2016). Specifically, the dysregulation of lipid peroxidation processes is associated with various human pathologies such as cancer chemoresistance, brain and ischemia injuries, neurological alterations, metabolic diseases as well as tuberculosis susceptibility (Amaral et al., 2019; Dar et al., 2018; Li et al., 2020; Meunier and Neyrolles, 2019; Stockwell et al., 2020; Zhu et al., 2019). In this context, the enzymes glutathione peroxidase 4 (GPX4) and ferroptosis-suppressor protein-1 (FSP1) that belongs to the CoQ antioxidant system, detoxify phospholipid hydroperoxide accumulation, hence allowing lipid peroxide amounts to be balancedin cells (Bersuker et al., 2019; Dixon et al., 2012; Doll et al., 2019; Friedmann Angeli et al., 2014; Yang et al., 2016). On the contrary, iron excess, lipoxygenase activity or cytochrome P450 oxidoreductase (CYPOR) all promote phospholipid peroxidation, which can end with ferroptosis induction in the absence of proper regulation (Dixon et al., 2012; Doll et al., 2017; Kagan et al., 2017; Yan1 et al., 2020; Yang et al., 2016; Zou et al., 2020).

In this regard, the bacterial pathogen *Pseudomonas aeruginosa (P. aeruginosa)* expresses,ExoU, an A2 phospholipase from the patatin family, that triggers a necrosis-dependent pathology through a poorly understood pathway (Anderson et

al., 2015; Dessen, 2000; Diaz and Hauser, 2010; Gendrin et al., 2012; Howell et al., 2013; Phillips et al., 2003; Rabin and Hauser, 2003; Sato and Frank, 2004; Sitkiewicz et al., 2006; Wilson and Knoll, 2018). In presence of cellular co-factors such as ubiquitin (Anderson et al., 2015) or the trafficking chaperone DNAJC5 (Deruelle et al., 2020), ExoU activity rapidly cleaves at the sn-2 position of host membrane phospholipids, liberating large amounts of arachidonic acid that are then metabolized into eicosanoids by cellular enzymes cyclooxygenases, cytochrome P450 or lipoxygenases (Machado et al., 2011; Pazos et al., 2017; Saliba et al., 2005; Wilson and Knoll, 2018). Importantly, *in vivo*, ExoU expression by *P. aeruginosa* is associated with a robust production of oxidized lipids such the platelet activating factor (PAF) or isoprostanes (da Cunha et al., 2015; Machado et al., 2011). In this context, we explored the possibility that *P. aeruginosa* ExoU mediates a necrosis-dependent host pathology involving lipid peroxidation.

P. aeruginosa infection triggers ExoU-dependent alarmin and peroxidised lipid production in mice

P. aeruginosa ExoU is injected into cells by the Type-3 Secretion System (T3SS) (Gendrin et al., 2012; Rabin and Hauser, 2003), which triggers a fast and violent cellular necrosis. Therefore, we first monitored the profile of ExoU-dependent pathology in mice infected with the clinical isolate pp34 *exoU*$^{+}$ or its isogenic mutant (*exoU*$^{-}$). Similar to previous studies (Howell et al., 2013; Al Moussawi and Kazmierczak, 2014; Phillips et al., 2003; Shaver and Hauser, 2004), intranasal instillation with either *exoU*$^{+}$ or *exoU*$^{-}$ strains highlighted a *P. aeruginosa*-induced acute pathology mainly due to ExoU, as mice infected with *exoU*$^{-}$ bacteria showed improved survival to infection (**Fig. 1A**). This observation was paralleled with lower bacterial loads of *P. aeruginosa exoU*$^{-}$ than *exoU*$^{+}$ in the bronchoalveaolar lavage fluids (BALFs), the lungs, the blood and the spleen, suggesting that ExoU also promotes bacterial dissemination (**Fig. 1B**). As *P. aeruginosa* triggers NLRC4-, NLRP3- and Caspase-11-dependent inflammasome response (Balakrishnan et al., 2018; Bitto et al., 2018; Cohen and Prince, 2013; Eren et al., 2019; Faure et al., 2014; Franchi et al., 2007; Iannitti et al., 2016; Miao et al., 2008; Al Moussawi and Kazmierczak, 2014; Santos et al., 2018; Sutterwala et al., 2007), we infected inflammasome-deficient mice (*Casp1/Casp11*$^{-/-}$, *Nlrc4*$^{-/-}$ and *GasderminD*$^{-/-}$) and observed that those mice were not protected against *P. aeruginosa exoU*$^{+}$, hence suggesting that ExoU-promoted mouse pathology occurs independently from the inflammasome machineries **(Figs. S1A, B)**. A hallmark of host cell necrosis is the release of intracellular mediators such as alarmins that contribute to the initiation and the development of an inflammatory reaction, which occurs upon *P. aeruginosa* infection (Aoyagi et al., 2017; Al Moussawi and Kazmierczak, 2014). Therefore, we primarily focused our analysis on alarmin release. We observed a strong ExoU-dependent alarmin production in BALFs 6 h after infection, such as IL-1 family alarmins IL1α, IL-33 or IL-36γ (Yang et al., 2017) (**Fig.1C**). In addition, we also detected that *exoU*-expressing *P. aeruginosa* triggered a strong production of phospholipid- and arachidonic acid (aa)-derived mediators such as prostaglandin E2 and leukotriene B4, which correlates with the robust phospholipase activity of ExoU (**Fig.1D**) (Machado et al., 2011; Pazos et al., 2017; Saliba et al., 2005). Importantly, BALFs of mice infected with *exoU*-expressing *P. aeruginosa* also exhibited a marked

presence of oxidized lipid (by)-products such as isoprostanes (8-iso PGF2α) or Malondialdehyde (MDA), which suggests that *exoU*-expressing *P. aeruginosa* also drives an exacerbated lipid oxidation response in mice (**Fig.1E**) (da Cunha et al., 2015; Saliba et al., 2006).

Lipid peroxidation contributes to ExoU-induced cell necrosis and *P. aeruginosa* escape from phagocyte-mediated killing

The observation that *exoU*-expressing *P. aeruginosa* infection associates to a lipid peroxidation signature *in vivo*, encouraged us to determine the importance of lipid peroxidation on ExoU-induced cellular necrosis. As *P. aeruginosa* strains that do not express E xoU can promote an NLRC4 inflammasome response in macrophages (Sutterwala et al., 2007), we used mouse Bone-Marrow-Derived Macrophages (BMDMs) that lack *Nlrc4* expression to specifically address the importance of lipid peroxidation on ExoU-dependent cell necrosis. We infected *Nlrc4*$^{-/-}$ primary murine BMDMs with *P. aeruginosa* strains expressing or not expressing ExoU.. The pharmacological inhibition of various regulated necrosis pathways (e.g. pyroptosis, necroptosis, apoptosis, parthanatos) showed that only ferrostatin-1, a potent and well characterized inhibitor of phospholipid peroxidation (Skouta et al., 2014), repressed ExoU-dependent cell necrosis (**Figs. 2A, S2A; Movies S1-S6**). In addition, ExoU-induced IL-1α and HMGB1 alarmin release in macrophages was reduced in presence of ferrostatin-1 whereas TNFα levels remained similar (**Fig. 2B**), suggesting that lipid peroxidation contributes to alarmin release in response to ExoU. We noticed that ExoU-triggered ferrostatin-1-sensitive necrosis was not restricted to murine BMDMs as primary human macrophages, the human U937 monocytic cell line, human and murine neutrophils and eosinophils, the human bronchial epithelial (HBEs), A549 or HELA epithelial cells were all sensitive to lipid peroxidation inhibition upon infection with *exoU*-expressing *P. aeruginosa* (**Fig. S2B**). ExoU exhibits a calcium-independent phospholipase A2-like activity (Dessen, 2000). Hence, we transfected recombinant ExoU protein (rExoU) or its enzymatically inactive mutant ExoU^{S142A} (Tamura et al., 2004) in WT BMDMs and monitored for cell necrosis. Only macrophages transfected with active ExoU underwent to cell death, a process that was inhibited by the use of ferrostatin-1 or the phospholipase inhibitor MAFP (**Fig. 2C**). In line, we found that ferrostatin-1 itself did not alter bacterial growth or ExoU

secretion (**Figs. S2C, D**), suggesting that ferrostatin-1 does not directly alter bacterial physiology nor expression/secretion of ExoU. Upon phospholipase activation arachidonic acid release can be metabolized and oxidized by various cellular enzymes, including cyclooxygenases 1 and 2 (COX1, COX2), lipoxygenases (ALOX5 and ALOX12/15 in mice) or cytochrome p450 (CYPs) enzymes. Therefore, we transfected recombinant ExoU in WT, *Alox5*$^{-/-}$ or *Alox12/15*$^{-/-}$ BMDMs in presence or absence of various COX, CYP or different lipid peroxidation inhibitors (a-tocopherol, liproxstatin-1, Resveratrol, ferrostatin-1). Although we observed that all lipid peroxidation inhibitors have a strong inhibitory impact on cell death, cyclooxygenase, cytochrome P450 or lipoxygenase targeting did not interfere with ExoU-dependent cell necrosis, hence suggesting that those enzymes do not regulate lipid-peroxidation-dependent cell necrosis upon ExoU exposure (**Fig. 2D**). Importantly, we also observed that ferrostatin-1 delayed ExoU-induced cell necrosis, suggesting that either the phospholipase activity of ExoU promotes lipid peroxidation-independent cell death or that the inhibitory effect of ferrostatin-1 is unstable over time (**Fig. 2E**). Finally, we evaluated if the inhibition of lipid peroxidation would modulate macrophage and neutrophil microbicidal response upon *exoU*-expressing *P. aeruginosa* infection. We observed that ferrostatin-1 strongly improved both macrophage and neutrophil microbicidal activities to a level close to those observed in response to *exoU*-deficient *P. aeruginosa* (**Fig. 2F**), hence suggesting that *P. aeruginosa* ExoU relies on lipid peroxidation-dependent cell necrosis to escape from phagocyte attack. Together, our results suggest that host cell lipid peroxidation is important for ExoU-induced host cell necrosis and release of alarmins.

Lipid peroxidation fuels ExoU phospholipase activity

Lipid-peroxidation requires reactive oxygen species (ROS), such as H_2O_2, that can oxidize various phospholipids (Dixon et al., 2012). Therefore, we evaluated the ability of ExoU to induce ROS-dependent lipid peroxidation in macrophages. Although we observed that, 30 minutes after transfection, ExoU but not its catalytically inactivated mutant ExoU^{S142A}, triggered an acute ROS production in BMDMs, we surprisingly failed to detect a robust lipid peroxidation accumulation as measured by the C11 Bodipy probe (**Figs. 3A, S3A**). As control, the well-known lipid peroxidation inducer Cumene hydroperoxide (CuOOH) promoted cellular lipid peroxidation (**Fig. 3A**)

(Lovatt et al., 2020). In contrast, we observed that basal lipid peroxidation in cells was reduced upon ExoU transfection, a process that was further strengthened in presence of ferrostatin-1 (**Fig. 3A**).

These results suggest that, instead of promoting pathological lipid peroxidation, ExoU might actually use cellular lipid peroxidation to promote cell necrosis. To this regard, various host phospholipase A2 enzymes have been described to specifically cleave and remove peroxidised phospholipids from membranes (Beatty et al.; Beharier et al., 2020; Lu et al., 2019). To address this hypothesis, we performed a redox phospholipidomic approach to determine if ExoU could interfere with the endogenous levels of peroxidised phospholipids. We used a 45 min time-point to perform our experiments, as a point where plasma membrane permeabilization (propidium uptake monitoring) is not observed. This design excludes the possibility that a decrease in peroxidised phospholipids is due to cell necrosis induced by ExoU (**Fig. S3C**). We observed that *exoU*-treated macrophages had a decrease in peroxidised phospholipids as measured by the reduction in hydroperoxil (-OOH)- and hydroxyl (-OH)-phosphoinositols (PIs)/- phosphoserines (PSs) and - phosphocholines (PCs) with arachidonic acid (C20:4/C22:4) acid side chains (**Figs. 3B, S3B).**

In cells, peroxidised phospholipids are detoxified by various factors, one of the most important being the ferroptosis regulator glutathione peroxidase 4 (GPX4) (Dixon et al., 2012). Consequently, the use of pro oxidant molecules or *Gpx4* genetic inactivation both induce a strong accumulation of various peroxidised phospholipids in cell membranes (Dixon et al., 2012). Therefore, we hypothesized that prestimulation of macrophages with non-cytotoxic doses of the lipid peroxidation inducer Cumene hydroperoxide (20µM, 1h) might sensitize cells to ExoU-induced cell necrosis. We transfected recombinant *exoU* in WT BMDMs in presence or absence of non-toxic doses of the pro-oxidant Cumen hydroperoxide (CuOOH, 20µM, 1h) (Lovatt et al., 2020). Although CuOOH promoted lipid peroxidation but not BMDM cell death, *exoU* transfection specifically induced an increased cell necrosis in CuOOH-primed BMDMs, a process that was inhibited by the use of ferrostatin-1 **(Figs. 3C, D)**. In agreement with this result, we measured a strong decrease in lipid peroxidation in CuOOH-primed cells transfected with *exoU*, confirming that ExoU efficiently targeted lipid peroxides induced by CuOOH **(Figs. 3C, D)**. Finally, using Crispr-Cas9, we generated *Gpx4*$^{-/-}$ immortalized BMDMs (**Fig. S3D**). As previously observed by others

in other cell lines (Friedmann Angeli et al., 2014; Kagan et al., 2017), *Gpx4*$^{-/-}$ immortalized BMDMs exhibited increased basal levels of peroxidised lipids **(Fig. S3E)**. Therefore, *exoU* transfection triggered faster cell death of *Gpx4*$^{-/-}$ macrophages than their WT counterpart, suggesting that lipid peroxidation of cells enhances ExoU-dependent toxicity **(Figs. 3E, S3D, E)**.

Upon phospholipid peroxidation, arachidonic acid-containing phospholipids from isoprostanes, potent intra- and extra-cellular mediators (Bochkov et al., 2010; Tyurina et al., 2019). Once formed, these isoprostanes are released from phospholipids by the action of phospholipases (Bochkov et al., 2010; Tyurina et al., 2019). Therefore, we reasoned that if ExoU targets peroxidised phospholipids, this would promote ExoU phospholipase-dependent release of endogenous pre-formed isoprostanes. Accordingly, the release of the 8-PGF2α isoprostane was specifically induced by ExoU in WT macrophages, a process that was further amplified by the co treatment of cells with non-toxic concentrations of Cumen hydroperoxide (CuOOH 20µM, 1 h) and ExoU (**Fig. 3F**). Of importance, ferrostatin-1 strongly inhibited ExoU- and ExoU/CuOOH-induced 8-PGF2α release (**Fig. 3F**). In addition, we also detected that in CuOOH-primed macrophages, the amount of arachidonic acid-derived eicosanoids leukotriene B4 and prostaglandin E2, which are an indirect indication of the phospholipase activity of ExoU, were also strongly increased after the exposure to ExoU, hence suggesting that ExoU-targeted peroxidised phospholipids might increase its phospholipase activity toward all phospholipids (peroxidized or not) (**Fig. S3F**). Consequently, we measured the phospholipase activity of ExoU in cell lysates where we chemically induced non-lethal lipid peroxidation with Cumene hydroperoxide (CuOOH, 20µM) for 1 h or not. We observed that in CuOOH-primed cell lysates, ExoU exhibited a stronger activity than in unprimed samples after 4 h of incubation (**Fig. 3G**). Importantly, after 18 h incubation, we observed the same accumulation of hydrolysed substrate in CuOOH-primed and unprimed samples, which suggests that lipid peroxidation exacerbates the early activation of ExoU (**Fig. 3G**). As control, ExoU^{S142A}- treated cell lysates did not show a significant phospholipase activity induction, suggesting that we mostly measured the PLA2 activity from ExoU, but not from cellular phospholipases (**Fig. 3G**). Finally, we aimed at challenging our findings by determining if other toxic phospholipases also had a similar activation pattern to ExoU. Hence, we transfected macrophages with the

closely related patatin-like phospholipase A2 from *Burkholderia thailandensis* (ExoU^{BtU}) (Anderson et al., 2015). We observed that recombinant ExoU^{BtU} transfection induced BMDMs necrosis, a process that was exacerbated by CuOOH priming and inhibited by the use of ferrostatin-1, suggesting that ExoU^{BtU} also follows a pattern involving host cell lipid peroxidation (**Fig. 3H**). Altogether, our results suggest a surprising mechanism by which ExoU exploits cellular lipid peroxidation to trigger necrosis, a process that can be extended to the action of *B. thailandensis* ExoU^{BtU}-related phospholipase.

Ferrostatin-1 improves mouse resistance to infection by *exoU*-expressing *P. aeruginosa*

ExoU-induced necrosis promotes host lung pathology, which leads to a sepsis like response as well as respiratory failure syndrome. Therefore, we hypothesized that ferrostatin-1 use could protect mice against *exoU*-expressing *P. aeruginosa*. Intranasal infection of mice using *P. aeruginosa exoU*$^{+}$ showed that mice intraperitoneally pre-treated with ferrostatin-1 (6 h before infection, 6mg.k^{-1}) had diminished bacterial loads in BALFs, lungs and spleen. Ferrostatin-1 pre-treatment did not significantly modify bacterial loads of *exoU*-deficient bacteria, suggesting that ferrostatin-1 mainly modulates ExoU-dependent processes in mice (**Fig. 4A**). Similarly, ferrostatin-1 also attenuated ExoU-dependent alarmin release (e.g. IL-36γ, IL33, IL1α) and the level of oxidized lipids (isoprostanes, MDA) in the BALs (**Fig. 4B, C**). Additionally, evaluation of the cellular contents in BALFs showed that ferrostatin-1 significantly protected a pool of alveolar macrophage upon *P. aeruginosa* challenge simultaneously decreasing the number of recruited neutrophils, eosinophils and monocytes (**Figs. 4D, S4A**). Although a pathological function of recruited immune cells such as neutrophils is probable, we hypothesize that ferrostatin-1-inhibited resident alveolar macrophage death in response to *exoU*-expressing *P. aeruginosa* might confer an improved immune protection characterized by lower immune cell recruitment and lower tissue damages. Regarding this, lung histological observations showed that the inflammatory status of mice infected with non-lethal doses of ExoU-expressing *P. aeruginosa* (1.10^5 CFUs) was improved in presence of ferrostatin-1 (**Fig. 4E**). Next, we addressed survival upon ExoU-expressing *P. aeruginosa* challenge. We observed that ferrostatin-1-treated mice (4-6 h before infection, 6mg.k^{-}

[1]) had an improved survival rate than those treated with PBS after 40 h after infection (**Fig. 4F**). We validated that ferrostatin-1 specifically protected mice against ExoU-induced pathology as ferrostatin-1-treated mice did not show enhanced protection (survival) against ExoU-deficient *P. aeruginosa* (**Fig. 4F**). Finally, we aimed toevaluate if *P. aeruginosa* ExoU would rigger pathological lipid peroxidation-dependent cell necrosis. in human bronchial organoids. Organoids were derived from normal lung tissue adjacent to tumors obtained from patients undergoing lung resection due to non-small cell lung carcinoma (NSCLC). Live cell imaging of organoids microinjected with *P. aeruginosa* showed that ExoU triggered complete organoid collapse (**Fig. 4G; Movies S7-S12**). Importantly, ferrostatin-1 strongly attenuated *P. aeruginosa*-dependent organoid damages (**Fig. 4G; Movies S7-S12**). Altogether, our results identified lipid peroxidation as a pathological mechanism induced by the *P. aeruginosa* ExoU phospholipase both in mice and in human bronchial organoids.

Discussion

As a preferential extracellular pathogen, *P. aeruginosa* uses its Type 3-Secretion System (T3SS) to inject virulence factors (Exo S, T, Y and U), allowing bacterial escape from phagocytic uptake and killing. Although *exoS*-expressing *P. aeruginosa* strains associate to the development of chronic infections, *exoU*-expressing *P. aeruginosa* triggers acute deadly infections that associate with a strong oxidative imbalance. In this study, we describe that endogenous basal lipid peroxidation contributes to ExoU-dependent cellular toxicity and mouse pathology. Though we do not exclude that *in vivo*, lipid peroxidation might play various pathological roles that go beyond the sole regulation of cell necrosis, such processes appear to be linked to ExoU expression. In this context, previous studies showed that ExoU promotes production of the platelet-activating factor or the 8-PGF2α isoprostane, two oxidized lipids (da Cunha et al., 2015). In addition, ExoU directly promotes a strong release of arachidonic acid from phospholipids. Enzymes such as cytochrome P450/COXs/LOXs can enzymatically produce oxygenated arachidonic products such as prostaglandin E2/leukotriene B4 involved in pathological signalling pathways upon *P. aeruginosa* infection (Machado et al., 2011; Sadikot et al., 2007; Saliba et al., 2005). However, results from others and ours mostly suggest that, taken individually, those enzymes only play a negligible role in ExoU-induced cell necrosis (Machado et al., 2011; Sadikot et al., 2007; Saliba et al., 2005).

Although controlled phospholipid peroxidation is of importance for the cells to perform various processes such as efferocytosis through the engagement of peroxidised-PS, mitochondria-dependent apoptosis through cardiolipin peroxidation, signal transduction through peroxidised PC-derived lipids, unrestricted accumulation of peroxidised PEs drives ferroptosis (Bochkov et al., 2010; Tyurin et al., 2014; Tyurina et al., 2019). Specifically, ferroptosis is thought to be a constitutively activated form of cell death that is kept under control through the activity of endogenous regulators of lipid peroxidation such as GPX4, FSP1-mediated coQ10 production, α-tocopherol (vitamin E). In addition, the host cellular calcium (Ca^{2+})-independent PLA2γ, the peroriredoxin Prdx6 PLA2 or the PLA2G6 (Ca^{2+}-independent PLA2β) can cleave and

remove preferentially peroxidised phospholipids, hence contributing to phospholipid peroxide detoxification (Beharier et al., 2020; Kinsey et al., 2008; van Kuijk et al., 1987; Lu et al., 2019; Miyamoto et al., 2003; Sevanian et al., 1988; Yedgar et al., 2006). The activity of those phospholipases is tightly regulated by various cellular systems (e.g. ROS levels, calcium fluxes, phospholipid composition) that ensure an optimal but not dysregulated phospholipid cleavage (Yedgar et al., 2006). To this regard, our findings that cellular phospholipid peroxidation is a strong enhancer of ExoU-induced pathological necrosis appears in first view counter intuitive. In this context, we envision that, as a virulence factor, ExoU activity does not follow host regulation and uses host peroxided phospholipids to boost its patatin-like A2 phospholipase activity allowing to aberrantly target and cleave host (peroxidised) phospholipids. Consequently, the use of lipid peroxidation inhibitors such as resveratrol, liproxstatin-1 or ferrostatin-1 attenuates the potency and the speed of ExoU-induced cell necrosis. This offers a key time window for macrophage and neutrophil-mediated bacterial uptake and killing. To the contrary, lipid peroxidation accumulation upon *Gpx4* removal or oxidant stress enhances ExoU-induced cellular necrosis. It is intriguing that endogenous peroxidised phospholipids favour ExoU-induced cell necrosis, suggesting that ExoU-expressing strains of *P. aeruginosa* take advantage of the host ferroptosis pathways to maximally damage host tissues. Hence, studying the importance of additional regulators of ferroptosis such as the cytochrome P450 oxidoreductase or the ACSL and LPCAT acyl transferases on ExoU-dependent toxicity warrants further investigations (Doll et al., 2017).

Phospholipases are also present in venoms or various microbial pathogens (e.g. *M. tuberculosis*, *L. monocytogenes*, *S. pyogenes*) and can also promote fast cell necrosis (Flores-Díaz et al., 2016; Hiu and Yap, 2020; Sitkiewicz et al., 2007). Conversely, we extended our findings to the ExoU closely related $ExoU^{BtU}$ phospholipase from *B. thailandensis*. Remarkably, snake, scorpion or spider venoms are a complex mixture of various components, including the L-amino acid oxidase, able to generate H_2O_2-driven lipid peroxidation, and secreted phospholipases able to cleave phospholipids (Hiu and Yap, 2020). In this context, it is tempting to speculate that venoms have all components necessary to mediate cell damage in a complex single-injection mixture. L-amino acid oxidase-induced lipid peroxidation might work with venom PLA2 to optimize phospholipid cleavage and subsequent cell necrosis.

Related to this, Sevanian and colleagues made pioneer observations that the PLA2 activity from the snake *Crotalus adamanteus* is exacerbated in contact of liposomes constituted of peroxidised phospholipids, a process that is thought to be due to the better accessibility of the sn2-peroxidized fatty acid to phospholipase (Sevanian et al., 1988). Whether ExoU and its relatives follow a similar pathway of activation will be studied in future studies.

In a broader point of view, it is interesting to note that phospholipases can promote allergic shock associated with a strong release of the allergic alarmin interleukin-33 (Palm et al., 2013), a signature we also observed in mice infected with ExoU-expressing *P. aeruginosa*. Should lipid peroxidation be involved in IL33-driven allergy or asthma in response to phospholipases or other allergens (e.g. proteases) (Cayrol et al., 2018) will require additional study.

Understanding the mechanisms of regulated cell necrosis and their physio-pathological consequences is currently driving intensive research and debates. While the importance of lipid peroxidation in antigen presentation, anti-cancer treatments or in exacerbating neurodegenerative diseases becomes more and more clear, its function in infectious diseases remains less studied. Regarding this, Dar et al., recently described that, upon chronic infection, secreted *P. aeruginosa* lipoxygenase (loxA) could sensitize cells to lipid peroxidation-induced ferroptosis (Dar et al., 2018). In addition, Kain and colleagues recently linked regulation of host lipid peroxidation and ferroptosis to restriction of liver-stage malaria, which suggests that host peroxidised phospholipids might play yet unsuspected functions in immunity or susceptibility to various pathogens (Kain et al., 2020). Thus, our findings that the bacterial patatin-like phospholipase A2 ExoU contributes to pathology by exploiting target cells lipid peroxidation adds an additional piece of significance for the role of lipid peroxidation in infectious diseases but also offers novel insights to target host lipid peroxidation pathways in the frame of infectious diseases.

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AUTHOR CONTRIBUTIONS

SB, RP and EM designed the experiments. SB, RP and EM wrote the manuscript. SB and RP performed the experiments with the help of SLI, MP, AH, KS, EE, PJB, EN, AB, CB, NI, AM, YR and CC. RP and EM supervised the entire study. LL, AC, IA, DWF, HC and PJP provided essential tools, reagents and expertise to conduct the project.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

FIGURE LEGENDS

Figure 1: ExoU-dependent lung pathology in mice associates to an alarmin and peroxidized lipid signature

(A) Survival of WT mice intranasally infected (n=7 animals per condition) with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. Graphs represent one experiment (8 mice/group) out of three independent *in vivo* experiments. Log-rank Cox-Mantel test was used for survival comparisons. ***p ≤ 0.001

(B) Bronchoalveolar (BAL), lung, blood and spleen bacterial loads from WT mice (n=8) 18 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. Graphs represent one experiment (8 mice/group) out of three independent *in vivo* experiments. **p ≤ 0.01, Mann-Whitney analysis test.

(C) Alarmin levels in bronchoalveolar fluids (BALFs) from WT mice (n=8) 6 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. Graphs represent one experiment (8 mice/group) out of three independent *in vivo* experiments; *p ≤ 0.05; **p ≤ 0.01, Mann-Whitney analysis test.

(D) Prostaglandin E2 (PGE2) and Leukotriene B4 (LTB4) levels in bronchoalveolar fluids (BALFs) from WT mice (n=8) 6 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. Graphs represent one experiment (8 mice/group) out of three independent *in vivo* experiments; **p ≤ 0.01, Mann-Whitney analysis test.

(E) Peroxidized lipid product (isoprostanes and MDA) levels in bronchoalveolar fluids (BALFs) from WT mice (n=8) 6 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. Graphs represent one experiment (8 mice/group) out of three independent *in vivo* experiments; *p ≤ 0.05; **p ≤ 0.01, Mann-Whitney analysis test.

Data information: Data shown as means (**Graphs B-E**) and are representative of one experiment performed three times; *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001, Mann-Whitney analysis test (**B-E)** and log-rank Cox-Mantel test for survival comparisons (**A**).

Figure 2: Lipid peroxidation inhibition delays ExoU-induced cell necrosis

Otherwise specified, cells were infected with an MOI of 0.5 of *P. aeruginosa* PP34, PP34^{exoU-} or PP34^{exoUS142A} for various times. ***p ≤ 0.001, T-test with Bonferroni correction.

(A, B) Measure of LDH and alarmin/cytokine release in *Nlrc4*$^{-/-}$ BMDMs infected with PP34 or PP34^{exoU-} in presence of Z-VAD (40µM), olaparaib (10µM), Necrostatin-1s (Ne1s, 40µM) or Ferrostatin-1 (Fe1, 10µM) for 2 hours. ***p ≤ 0.001, T-test with Bonferroni correction.

(C) Measure of LDH release in WT BMDMs transfected with recombinant ExoU (100ng), in presence of MAFP (50µM) or Ferrostatin-1 (Fe1, 10µM) for 3 hours. ***p ≤ 0.001, T-test with Bonferroni correction.

(D) Heat map representing measure of LDH release in WT, *ALOX5*$^{-/-}$ and *ALOX12/15* BMDMs transfected with recombinant ExoU in presence/absence of Cox1 inhibitor (Ketorolac Tromethamine, 10µM), Cox2 inhibitor (NS 398, 25µM), Cyp450 epoxygenase activity inhibitor (PPOH, 10µM), phospholipase inhibitor MAFP (50µM) or lipid peroxidation inhibitors Ferrostatin-1 (Fe1, 20µM), Resveratrol (5µM), Liproxstatin-1 (30µM), a-Tocopherol (20µM) for 2 hours. The heat map shows the mean of three combined independent experiments, each performed in triplicate.

(E) Time course measure of plasma membrane permeabilization using propidium iodide incorporation in *Nlrc4*$^{-/-}$ BMDMs infected with PP34, PP34^{exoU-} or PP34^{exoUS142A} in presence of Ferrostatin-1 (Fe1, 20µM). ***p ≤ 0.001, T-test with Bonferroni correction.

(F) Microbicidal activity of macrophages (5h) and neutrophils (3h) after infection with *P. aeruginosa exoU*$^{+}$ and *exoU*$^{-}$ (MOI 0.5) in presence/absence of ferrostatin-1 (10µM). ***p ≤ 0.001, T-test with Bonferroni correction.

Data information: Data are represented as means+/- SEM (graphs A- F) from n = 3 independent pooled experiments; ***P ≤ 0.001 for the indicated comparisons using t-test with Bonferroni correction.

Figure 3: ExoU-induced cell death involves ROS-induced lipid peroxidation but proceeds in a ferroptosis independent manner

(A) Cytometry detection and quantification of (phosphor)lipid peroxidation using the probe C11-bodipy in WT BMDMs treated with CuOOH (20µM) or transfected with rExoU (500ng) or its catalytically dead mutant rExoU^{S142A} (500ng) for 1 hour in presence or absence of Ferrostatin-1 (20µM). Sample were acquired using FACSCalibur™ (BD). The graphs shows the mean+/-SEM of one experiment performed in triplicate out of three independent experiments. **P≤0.001, ***P ≤ 0.001 for the indicated comparisons using t-test with Bonferroni correction.

(B) (Redox) lipidomic analysis of phospholipid peroxidation in BMDMs transfected with recombinant ExoU or its catalytically dead mutant ExoU^{S142A} for 45 minutes. Each value is standardized to the corresponding phospholipid content shown in (**Fig S3B**). The heat map shows the mean of one experiment performed in triplicate.

(C) Cytometry detection and quantification of (phosphor)lipid peroxidation using the probe C11-bodipy in WT BMDMs pre-treated or not for 1 hour with CuOOH (20µM) in presence or absence of Ferrostatin-1 (20µM) and then transfected with rExoU (500ng) for 1 hour. Sample were acquired using FACSCalibur™ (BD). The graphs shows the mean+/-SEM of one experiment performed in triplicate out of three independent experiments. *P ≤ 0.05, **P≤0.001, for the indicated comparisons using t-test with Bonferroni correction.

(D) Measure of LDH release in WT BMDMs pre-treated or not for 1 hour with CuOOH (20µM) in presence or absence of Ferrostatin-1 (20µM) and then transfected with rExoU (500ng) for 3 hours. ***p ≤ 0.001, T-test with Bonferroni correction.

(E) Time course measure of plasma membrane permeabilization using propidium iodide incorporation in immortalised WT and *Gpx4*$^{-/-}$ BMDMs transfected with rExoU (500ng) for 7 hours. ***p ≤ 0.001, T-test with Bonferroni correction.

(F) Measure of 8-iso PGF2α isprostane in cell supernatant in WT BMDMs pre-treated or not for 1 hour with CuOOH (20µM) in presence or absence of Ferrostatin-1 (20µM) and then transfected with rExoU (500ng) for 3 hours. ***p ≤ 0.001, T-test with Bonferroni correction.

(G) ExoU phospholipase activity determination in WT BMDM lystaes pre-treated or not with CuOOH (20µM, 1hour). 100 pmols of ExoU were used and phospholipase hydrolysis rate (nmoles of substrate hydrolysed/nmole of ExoU) was measured after 4 h and 16 hours. ***p ≤ 0.001, T-test with Bonferroni correction.

(H) Measure of LDH release in WT BMDMs primed or not with CuOOH (20µM, 1hour) in presence or absence of ferrostatin-1 (20µM) and transfected for 3 hours with 5µg of rExoU^{BtU} or 500ng rExoU. ***p ≤ 0.001, T-test with Bonferroni correction.

Data information: Data are plotted as means+/- SEM (**E-H**) from n = 3 independent pooled experiments; ***P ≤ 0.001 for the indicated comparisons using t-test with Bonferroni correction.

Figure 4: Ferrostatin-1 protects mice against ExoU-induced lung pathology

(A) Bronchoalveolar (BAL), lung and spleen bacterial loads from WT mice (n=7/group) 18 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. When specified, mice were intraperitoneally pretreated with ferrostatin-1 (6mg.k^{-1} or PBS) 4 hours before intranasal infection. Graphs represent one experiment (7 mice/group) out of three independent *in vivo* experiments. **p ≤ 0.01, Mann-Whitney analysis test. NS: Not significant

(B, C) Alarmin and lipid peroxide products levels in bronchoalveolar fluids (BALFs) from WT mice (n=7 mice/group) 6 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. When specified, mice were intraperitoneally pretreated with ferrostatin-1 (6mg.k^{-1} or PBS) 4 hours before intranasal infection. Graphs represent one experiment (7 mice/group) out of three independent *in vivo* experiments; *p ≤ 0.05; **p ≤ 0.01, Mann-Whitney analysis test.

(D) Immune cell (CD45+) populations in bronchoalveolar fluids (BALFs) from WT mice (n=7 mice/group) 6 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. When specified, mice were intraperitoneally treated with ferrostatin-1 (6mg.k^{-1} or PBS) 4-6 hours before intranasal infection. Graphs represent one experiment (7 mice/group) out of three independent *in vivo* experiments; *p ≤ 0.05; **p ≤ 0.01, Mann-Whitney analysis test.

(E) Histological observation and scoring of bronchial and lung cellular infiltrations upon *exoU*-expressing *P. aeruginosa* intranasal infection. When specified, mice were intraperitoneally pretreated with ferrostatin-1 (6mg.k^{-1} or PBS) 4-6 hours before intranasal infection. Stars show the cellular infiltrates. *p ≤ 0.05; Mann-Whitney analysis test.

(F) Mice survival (n=7 mice/group) 40 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. Mice were intraperitoneally pretreated with ferrostatin-1 (6mg.k^{-1} or PBS) 4 hours before intranasal infection. Graphs represent one experiment (7 mice/group) out of three independent *in vivo* experiments; **p ≤ 0.01, Log-rank Cox-Mantel test was used for survival comparisons.

(G, H) Time-lapse microscopy and the associated quantifications of the measure of plasma membrane permeabilization using propidium iodide incorporation in human primary bronchial organoids infected (microinjection) with *P. aeruginosa* expressing *exoU*$^+$ or its isogenic mutant (*exoU*$^-$) in presence or absence of ferrostatin-1 (40µM) for 15 hours. Data are plotted as means+/- SEM. ***p ≤ 0.001, T-test with Bonferroni correction.

Data information: Data shown as means (**Graphs A-E**) and are representative of one experiment performed three times; *p ≤ 0.05; **p ≤ 0.01, Mann-Whitney analysis test (**A-E)** and log-rank Cox-Mantel test for survival comparisons (**F**).

STAR Method

Table 1: Resource of reagents used in this study

Information and reagents are available upon request to Etienne.meunier@ipbs.fr

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GPX4, 1/1000	abcam	ab125066
ExoU, 1/1000	Ina Attree/CNRS, France.	(Deruelle et al., 2020)
Gapdh 1/10000	Gentex	GTX100118
Goat anti-Rabbit HRP (1/10000)	Advansta	R-05072-500
Bacterial and Virus Strains		
PAO1	J. Buyck/Univ of Poitiers/France	N.A.
PP34	Ina Attree/CNRS, France.	(Deruelle et al., 2020)
PP34*exoU*$^{-}$	Ina Attree/CNRS, France.	(Deruelle et al., 2020)
PP34*exoU*^{S142A}	Ina Attree/CNRS, France.	(Deruelle et al., 2020)
CHA	Ina Attree/CNRS, France.	(Deruelle et al., 2020)
CHAdST	Ina Attree/CNRS, France.	(Deruelle et al., 2020)
CHAdST*exoU*$^{+}$	Ina Attree/CNRS, France.	(Deruelle et al., 2020)
PA103	J. Buyck/Univ of Poitiers/France	N.A.
PA103*exoU*$^{-}$	J. Buyck/Univ of Poitiers/France	N.A.
PA14	J. Buyck/Univ of Poitiers/France	N.A.
PA14 *exoU*$^{-}$	J. Buyck/Univ of Poitiers/France	N.A.
Biological Samples		
Human lung biopsy	Hospital of Toulouse	CHU 19 244 C CNRS 205782

Human blood	EFS	21PLER2017-0035AV02
Chemicals, Peptides, and Recombinant Proteins		
Recombinant ExoU	This study	(Anderson et al., 2015)
Recombinant ExoUS142A	This study	(Anderson et al., 2015)
FCS	Fisher Scientific	16010-159
mMCSF	L929 cell supernatant	NA
HEPES	Fisher Scientific	SH30237.01
Non-essential amino acids	Invitrogen	
ECL Clarity Max Substrate	BioRad	1705060
ECL Clarity Max Substrate	BioRad	1705062
Western Blot Strip Buffer	Diagomics	R-03722-D50
Tris base	euromedex	200923-A
SDS ultra-pure (4x)	Euromedex	1012
Acrylamide / Bisacrylamide 37.5/1 30%	Euromedex	EU0088-B
Temed	Sigma	T9281-25ML
Ammonium persulfate	Sigma	248614-100g248614-100g
Page Ruler 10-180 kDa	Fisher Scientific	15744052
Triton X-100	Euromedex	2000
DMEM	Fisher Scientific	41965-039
LB	Fisher Scientific	BP1426-2
LB Agar	INVITROGEN	22700025
Roche protease inhibitor cocktail	Sigma	0000000116974980 01
BSA	SIGMA	A9647-100G
Propidium iodide	Invitrogen	P3566
Beads Neutrophils human	Miltenyi biotec	130-104-434
Beads Neutrophils murine	Miltenyi biotec	130-120-337
Kit de coloration bleue et fixable des cellules mortes LIVE/DEAD™ pour excitation UV	ThermoFisher Scientifique	L34961
APC/Cyanine7 anti-mouse CD45 Antibody	BioLegend	103116
PE/Dazzle™ 594 anti-human CD64 Antibody	BioLegend	305032
FITC anti-mouse MERTK (Mer) Antibody	BioLegend	151504
CD170 (Siglec F) Monoclonal Antibody (1RNM44N), Super Bright 780,	eBioscience	78-1702-82
Ly-6G Monoclonal Antibody (1A8-Ly6g), APC	eBioscience	17-9668-82
Brilliant Violet 650™ anti-mouse/human CD11b Antibody	BioLegend	101259
Brilliant Violet 421™ anti-mouse Ly-6C Antibody	BioLegend	128032
PE/Cyanine7 anti-mouse CD11c Antibody	BioLegend	117318
Eosinophil differentiation cocktail (IL-5)	R&D Systems	405-ML-005
Eosinophil differentiation cocktail (SCF)	Biolegend	579706
Eosinophil differentiation cocktail (Flt-3)	Biolegend	550706
Puromycin	ThermoFisher Scientifique	A1113803
G418 (Geneticin)	invivoGen	ant-gn-1

Blasticidin	nvivoGen	ant-bl-1
Cumene hydroperoxide	Sigma-Aldrich	247502-5G
RSL3	Sigma-Aldrich	SML2234
Ferrostatin-1	Sigma-Aldrich	SML0583
Liproxstatin-1	Sigma-Aldrich	SML1414
DFO	Sigma-Aldrich	D9533
a-tocopherol	Sigma-Aldrich	258024
MAFP	Sigma-Aldrich	M2689
PPOH	CaymanChem	75770
Cox1 inhibitor	Ab142904 (Abcam)	Ab142904
Cox2 inhibitor	NS 398 (Abcam)	Ab120295
cPLA2 assay kit	Cayman Chemical	765021
CD14+ beads	Miltenyi biotec	130-050-201
RPMI	Fisher Scientific	72400-021
OPTIMEM	Fisher Scientific	31985-04
Z-VAD	Invivogen	tlrl-vad
Olaparib	CaymanChem	10621
Necrostatin-1s	Sigma-Aldrich	N9037 10MG
hMCSF	Miltenyi biotec	170-076-171
Fisher BioReagents™ Lymphocyte Separation Medium-LSM	Fisher Scientific	BP2663500
ExoU	This study	N.A.
ExoUS142A	This study	N.A.
Human bronchial organoid culture reagents		
Advanced DMEM/F12	Invitrogen	12634028
Gibco™ L-Glutamine (200 mM)	Fisher	11500626
Hepes 1 M	Fisher	11560496
Penicillin/Streptomycin	Fisher	11548876
Primocin	Invivogen	ant-pm-1
Basic Media	In house	NA
Rspol	In house	NA
Noggin	In house	NA
B27	Gibco/Invitrogen	17504044
N-Acetylcysteine	Sigma	A9165-5g
Nicotinamide	Sigma	N0636
Y-27632	Cayman	10005583
A83-01	Tocris	2939
SB 202190	Sigma	S7067
FGF-7	Peprotech	100-19
FGF-10	Peprotech	100-26
Critical Commercial Assays		
mIL-1alpha ELISA kit	Fisher Scientific	88-5019-88
mIL-36g ELISA kit	Ray Biotech	ELM-IL36G
LDH Cytotoxicity Detection Kit	Takara	MK401
mTNFalpha ELISA kit	Fisher Scientific	88-7324-22

mIL-33 ELISA kit	Fisher Scientific	88-7333-88
mHMGB1 ELISA kit	Clinisciences	LS-F4040-1
TBAR MDA colorimetric kit	Cayman	10009055
PGE2 EIA Kit	Cayman	514010
LTB4 EIA kit	Cayman	520111
8-PGF2 EIA kit	Cayman	516351
H2DCFDA ROS detecting probe	Invitrogen	D399
C11 bodipy phospholipid peroxide detection probe	Invitrogen	D3861
Experimental Models: Cell Lines		
WT Mouse Bone marrow derived macrophages	This study	
Alox5-/- Mouse Bone marrow derived macrophages	This study	
Alox12/15-/- Mouse Bone marrow derived macrophages	This study	
Nlrc4-/- Mouse Bone marrow derived macrophages	This study	
Casp1-/-/Casp11-/- Mouse Bone marrow derived macrophages	This study	
GsdmD-/- Mouse Bone marrow derived macrophages	This study	
WT Mouse bone marrow derived eosinophils	This study	
WT Mouse bone marrow derived neutrophils	This study	
Human blood monocyte derived macrophages	This study	
Human blood neutrophils	This study	
Immortalized WT murine bone marrow derived macrophages	This study	
Immortalized Gpx4-/- murine bone marrow derived macrophages	This study	
Human Bronchial epithelial cells	This study	
Human Alveolar epithelial A549 cell line	This study	
Human intestinal epithelial HELA cell line	This study	
Experimental Models: Organisms/Strains		
WT C57Bl6J mice	C. Rivers	
WT C57Bl6N mice	C. Rivers	
Alox5-/- C57Bl6 mice	A.Coste	(Lefèvre et al., 2015)
Alox12/15-/- C57Bl6 mice	A.Coste	(Lefèvre et al., 2015)
Nlrc4-/- C57Bl6 mice	C.Bryant	(Man et al., 2014)
Casp1-/-/Casp11-/- C57Bl6 mice	B.Py/ Junying Yuan	(Li et al., 1995)
GsdmD-/- C57Bl6 mice	P.Broz	(Demarco et al., 2020)
Human Bronchial organoids	This sudy	(Bartfeld and Clevers, 2015; Sachs et al., 2019)
Oligonucleotides		
Guide Crispr mGpx4- Exon1 Forward	Sigma-Aldrich	GGACGCTGCAGA CAGCGCGG
Recombinant DNA		
Plasmid: ExoU	(Anderson et al., 2015)	(Anderson et al., 2015)
Plasmid: ExoUS142A	(Anderson et al., 2015)	(Anderson et al., 2015)
Plasmid: $ExoU^{BtU}$	(Anderson et al., 2015)	(Anderson et al., 2015)
LentiGuide-Puro	Feng Zhang lab	Addgene #52963
Lenti-multi-Guide	From Qin Yan	Addgene #85401

pMD.2G	Didier Trono lab	Addgene #12259
p8.91	Didier Trono lab	N.A.
LentiCas9-Blast	Feng Zhang lab	Addgene #52962
Software and Algorithms		
Graph Pad Prism 8.0		
Image J		
Snapgene	GSL Biotech LLC, Chicago, U.S.A	
FlowJO Benchling Software	FlowJo LLC	
Other		

Mice

Nlrc4$^{-/-}$, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *GsdmD*$^{-/-}$, *ALOX12/15*$^{-/-}$ and *ALOX5*$^{-/-}$ mice were generated and described in previous studies (Demarco et al., 2020; Lefèvre et al., 2015; Li et al., 1995; Man et al., 2014). Mice were bred at the IPBS (Toulouse, France) animal facilities in agreement to the EU and French directives on animal welfare (Directive 2010/63/EU). Charles Rivers provided WT C57BL/6 mice.

Animal infection models

6-10 mice/group were intranasally infected with 5.10^5 Colony Forming Units (CFUs) of *P. aeruginosa* PP34 strain (*ExoU*$^+$) or its isogenic mutant (*ExoU*$^-$) and animal survival was followed over 40-50 hours after infection. When specified, mice were intraperitoneally treated with 100µL of PBS or ferrostatin-1 ($6mg.k^{-1}$) 4-6 hours before intranasal infections with bacterial strains.

Regarding bacterial loads assays, 6-10 mice/group were intranasally infected with 2.10^5 bacteria for 24 hours, and Bronchoalveaolar (BALs), lung spleen and blood bacterial numbers were evaluated using CFU plating. BAL fluids (BALFs) were also used to address cytokine, alarmin and lipid levels using ELISA, EIA and colorimetric kits. There were no randomization or blinding performed.

Animal experiments were approved (License APAFIS#8521-2017041008135771, Minister of Research, France) and performed according to local guidelines (French ethical laws) and the European Union animal protection directive (Directive 2010/63/EU).

Histological experiments and scoring

Mice were intraperitoneally treated with 100µL of PBS or ferrostatin-1 (6mg.k^{-1}) 4-6 hours before intranasal infections with sub-lethal doses (2.10^5 CFUs) of *exoU*-expressing *P. aeruginosa*. 6 hours later, lung tissues were fixed for 48 h in 10% buffered formalin, washed 3 times in ethanol 70% and embedded in paraffin. 5 µm sections were stained with hematoxylin and eosin (HE). Histopathological scoring from 0 to 3 were attributed based on the severity of peribronchial, perivascular, and interstitial cell infiltration, resulting in a maximum score of 9.

Bacterial cultures

P. aeruginosa (PP34, PA103, CHA, PAO1, PA14) bacteria and their isogenic mutants were grown overnight in Luria Broth (LB) medium at 37°C with aeration and constant agitation in the presence or absence of EGTA (10mM) to ensure T3SS expression. Bacteria were sub-cultured the next day by dilution overnight culture 1/50 and grew until reaching an optical density (OD) O.D600 of 0.6 – 1. Bacterial strains and their mutants are listed in Table S1.

Bone Marrow-derived Macrophage (BMDMs), Eosinophil (BMDEs) or Neutrophil (BMDNs) isolation and culture.

Murine Bone Marrow-Derived Macrophages (BMDMs) from bone marrow progenitors were differentiated in DMEM (Invitrogen) supplemented with 10% v/v FCS (Thermo Fisher Scientific), 10% v/v MCSF (L929 cell supernatant), 10 mM HEPES (Invitrogen), and nonessential amino acids (Invitrogen) for 7 days as previously described (Eren et al., 2020).

Murine Bone Marrow-Derived Eosinophils were differentiated *in-vitro* from bone marrow as previously described (Dyer et al., 2008). cells were resuspendent and cultured at 10^6/mL in RPMI glutamax medium with HEPES containing 20% FBS, 100 IU/ml penicillin and 10 µg/ml streptomycin, 1 mM sodium pyruvate (Life Technologies), and 50 µM 2-ME (Sigma-Aldrich) supplemented with 100 ng/ml stem cell factor (SCF; PeproTech) and 100 ng/ml FLT3 ligand (FLT3-L; PeproTech) from days 0 to 4. On day 4, the medium containing SCF and FLT3-L was replaced with

685 medium containing 10 ng/ml recombinant mouse (rm) IL-5 (R&D Systems) only.
686 Medium was replaced every 4 days and the concentration of the cells was adjusted
687 each time to106/ml. After 10 to 14 days of culture, cells were recovered by gentle
688 pipetting and used as Eosinophils in our experiments. Over 95% of cells had the
689 standard phenotype of Eosinophils : CD11b+ Siglec F + after FACS analysis.

690 Murine Bone Marrow-derived Neutrophils were isolated and purified from fresh bone
691 marrows using Anti-Ly-6G micro bead kit (Miltenyi Biotec). Analysis of cell purity by
692 FACS show that over 95% of cells had the standard phenotype of Neutrophils
693 Ly6G+/Ly6C+.

694 $2.5x10^5$ BMDMs or 1.10^6 BMDEs/BMDNs were seeded in 24 well-plates and infected
695 or exposed to various treatments. Regarding ferroptosis experiments, BMDMs were
696 infected with various bacterial strains of *P. aeruginosa* expressing or not *exoU* at an
697 MOI 0.1-1 for various times. When specified, recombinant microbial phospholipases
698 (10ng-1µg) were transfected in BMDMs using Fugene (3µl per 1µg of transfected
699 protein) for 2-4 hours. Compound-induced ferroptosis was achieved using RSL-3
700 (10µM, 8H) or Cumene hydroperoxide (CuOOH, 500µM, 3H).

701 When required, BMDMs were pretreated for 2 hours with pharmacological inhibitors
702 necrostatin-1s (40µM), Z-VAD (40µM), olaparib (10µM), ferrostatin-1 (1-40µM),
703 MAFP (50µM), liproxstatin (30µM), a-tocopherol (20µM).

704 For all stimulations, cell culture medium was replaced by serum-free and antibiotic-
705 free Opti-MEM medium and triggers were added to the cells for various times.

706

707 **Cell line culture**

708 Immortalized murine bone-marrow derived macrophages have been described
709 previously (Eren et al., 2020). U937 cells were cultured in RPMI glutamax medium
710 containing 10% FBS, 100 IU/ml penicillin and 10 µg/ml streptomycin, 1 mM sodium
711 pyruvate (Life Technologies), and 50 µM 2-ME (Sigma-Aldrich). Medium was
712 renewed every 3 days and the concentration of the cells was adjusted each time to
713 $5x10^5$/ml. A549, HeLa and HBE cells were cultured in DMEM glutamax medium with
714 HEPES containing 10% FBS, 100 IU/ml penicillin and 10 µg/ml streptomycin, 1 mM
715 sodium pyruvate (Life Technologies). When the cells reach approximately 90%

confluency, cells are detached with Trypsin 0.05% (Gibco), cell suspension is diluted 1/10 in fresh medium, and placed back in the incubator for culture.

Purification and generation of human blood neutrophils and monocyte-derived Macrophages

Monocytes were isolated from Peripheral Blood Mononuclear Cells (PBMCs) from the buffy coat of healthy donors obtained from the EFS Toulouse Purpan (France) as described previously (Troegeler et al., 2014). Briefly, PBMCs were isolated by centrifugation using standard Ficoll-Paque density (GE Healthcare) (Eren et al., 2020). The blood was diluted 1:1 in phosphate-buffered saline (PBS) pre-warmed to 37°C and carefully layered over the Ficoll-Paque gradient. The tubes were centrifuged for 25 min at 2000 rpm, at 20°C. The cell interface layer was harvested carefully, and the cells were washed twice in PBS (for 10 min at 1200 rpm followed by 10 min at 800 rpm) and re-suspended in RPMI-1640 supplemented with 10% of foetal calf serum (FCS), 1% penicillin (100 IU/mL) and streptomycin (100 µg/ml). Monocytes were separated from lymphocytes by positive selection using CD14+ isolation kit (Myltenyi biotec). To allow differentiation into monocyte-derived macrophages, cells were cultured in RPMI medium (GIBCO) supplemented with 10% FCS (Invitrogen), 100 IU/ml penicillin, 100µg/ml streptomycin, 10 ng/ml M-CSF for 7 days.

Human blood neutrophils were isolated from whole blood of healthy donors obtained from the EFS Toulouse Purpan (France). Neutrophils were enriched using MACSxpress Whole Blood Neutrophil Isolation Kit whole blood neutrophil isolation kit (Myltenyi biotec) according to manufacturer instructions. Red blood cells (RBC) were removed by 10 min incubation in RBC Lysis Buffer (BioLegend).

Genetic invalidation of *Gpx4* genes in immortalized BMDMs

Targeted genes were knocked-out using the crispr/cas9 system in immortalized BMDMs. Single guide RNAs (sgRNA) specifically targeting *Gpx4* exon1 (for 5' GGACGCTGCAGACAGCGCGG 3' were designed using Benchling tool (Benchling.com), and oligonucleotides were synthesized by Sigma-Aldrich. Crispr guide RNA oligonucleotides were hybridized and subsequently cloned into the vector Lenti-gRNA-Puromycin using BsmBI restriction enzyme (Addgene 52963, Feng Zhang lab). Generated constructs were then transfected in lipofectamine 2000 into HEK293T for 48 hours together with the lentiviral packaging vector p8.91 (Didier Trono lab, EPFL, Switzerland) and the envelop coding VSVg plasmid (pMD.2G, Addgene 12259, Didier Trono lab). Viral supernatants were harvested, filtered on 0.45 µm filter and used to infect cells expressing Cas9 (1,000,000 cells/well in 6-well plates. Efficient infection viral particles was ensured by centrifugating cells for 2 h at 2900 rpm at 32°C in presence of 8µg/ml polybrene. 48 h later, medium was replaced and Puromycin selection (10µg/mL) was applied to select positive clones for two weeks. Puromycin-resistant cells were sorted at the single cell level by FACS (Aria cell sorter). Individual clones were subjected to western blotting to confirm the absence of targeted proteins.

Human bronchial organoid production and maintenance

Airway organoids were derived from lung biopsies as described (Bartfeld and Clevers, 2015; Sachs et al., 2019). Briefly, Human lung tissue was provided by the CHU of Toulouse under the CNRS approved protocols CHU 19 244 C and CNRS 205782. All patients participating in this study consented to scientific use of their material. Biopsies (1 mm3) of normal lung tissue adjacent to the tumor obtained from patients who underwent lung resection due to Non-small cell lung carcinoma (NSCLC) were minced and digested with 2 mg ml−1 collagenase (Sigma) on an orbital shaker at 37°C for 1h. The digested tissue suspension was sheared using flamed glass Pasteur pipettes and strained over a 100-µm cell strainer (Falcon). The resultant single cell suspensions were embedded in 10 mg ml−1 of Cultrex growth factor reduced BME type 2 (R & D Systems) and 40µl drops were seeded on Nunclon Delta surface 24-well plates (Thermo Scientific). Following polymerization, 500 µl of Advanced DMEM/F12 (Invitrogen) supplemented with 1x L-Glutamine

(Fisher Scientific), 10mM Hepes (Fisher Scientific), 100 U ml-1 / 100 µg ml-1 Penicillin / Streptomycin (Fisher Scientific), 50 µg ml-1 Primocin (InvivoGen), 10% Noggin (homemade), 10% RspoI (homemade), 1x B27 (Gibco), 1.25mM N-Acetylcysteine (Sigma-Aldrich), 10mM Nicotinamide (Sigma-Aldrich), 5µM Y-27632 (Cayman Chemical), 500nM A83-01 (Tocris Bioscience), 1µM SB 202190 (Sigma-Aldrich), 25 ng ml−1 FGF-7 (PeproTech), 100 ng ml−1 FGF-10 (PeproTech) was added to each well and plates transferred to humidified incubator at 37°C with 5% CO2. The organoids were passaged every 4 weeks.

Organoid infections

Before infection, 35µl drops of Matrigel (Fisher Scientific) containing organoids were seeded on Nunclon Delta surface 35x10mm Dish (Thermo Scientific) and 2ml of Advanced DMEM/F12 supplemented with 1x L-Glutamine and 10mM Hepes was added to each plate. Depending on the indicated conditions, organoids were pretreated or no with 40µM Ferrostatin-1 for 1hr before infection. Ferrostatin-1 was maintained throughout the experiment. PP34 *exoU* or *exoU*^{S142A} were grown as previously described until reach OD600 = 1. Bacterial density was adjusted to OD600 = 0.0005, and phenol red added at 0.005% to visualize successful microinjection (2). Injected organoids were individually collected and re-seeded into fresh matrix for subsequent analysis. For time-lapse imaging, injected and stimulated organoids were stained with 50 µg ml-1 Propidium Iodide (Thermo Scientific). Images were acquired every 15 minutes for the duration of experiments under an EVOS M7000 (Thermo Scientific) Imaging System (10x, at 37°C with 5% CO2). Data was analyzed using Fiji/ImageJ.

Cell necrosis, alarmin/cytokine and lipid release assays

LDH Cytotoxicity Detection Kit (Takara) was used to determine the percentage of cell lysis. Normalization of spontaneous lysis was calculated as follows: (LDH infected – LDH uninfected)/(LDH total lysis – LDH uninfected)*100.

Murine Il-1α, IL-33, IL-36α, IL-36γ, HMGB1, TNFα, cytokine levels in cell supernatants or in BALFs were measured by ELISA listed in resource Table 1.

Oxidized lipids isoprostanes, eicosanoids PGE2 and LTB4 were detected in cellular supernatants or BALFs using EIA kits listed in resource Table 1.

Plasma membrane permeabilization assays

Cells are plated at density of 1 x 10^5 per well in 96-well Plates or at 2x10^5/well in 24-well plates (Corning 356640) in complete culture medium. The following day, medium is replaced by Opti-MEM supplemented with Propidium iodide (100 ng/ml) or SYTOX green (100ng/mL). Pharmacological inhibitors are added 1h before infection. Red (Propidium Iodide) or green (SYTOX) fluorescence are measured in real-time using Clariostar plate reader or an EVOS7000 microscope, both equipped with a 37°C cell incubator.

Malondialdehyde (MDA) assays

Malondialdehyde production was addressed using the MDA lipid peroxidation kit according to the manufacturer's instructions (Abcam, ab118970). Cells were lysed using 500 µl of lysis buffer supplemented with butylated hydroxytoulene. Cell lysates were centrifuged for 10 min at 13,000 g (RCF) and the supernatants were used for MDA assay. TBA solution was added to each replicate, and samples were then incubated at 95°C for 1 hour. 100µL of each sample was then processed for fluorometric assay at Ex/Em = 532/553 nm. BAL levels of MDA were normalized to the total protein concentration.

Recombinant protein production

Plasmids coding for *exoU*^{BtU}, *exoU* or *exoU*^{S142A} were a kind gift from Dara W. Frank's lab. All recombinant proteins were expressed in BL21(DE3) pLysS strain in LB medium, according to Anderson DM et al. (Anderson et al., 2015). Proteins fused with an N-terminus hexahistidine-tag were purified as previously described with slight modifications. Briefly, after cell harvest, bacteria were lysed by sonication under ice

and recombinant proteins were purified by nickel metal affinity chromatography (Takara). After sample concentration, Superose 6 was exchanged for a Superdex 200 size exclusion column (GE Healthcare) as a final purification step. Samples were either used fresh or keep at -80°C for long-term storage.

Cytometry quantification of immune cells in mice BAL fluids (BALFs)

C57BL/6 mice received an injection of Ferrostatine (6mg/kg) or PBS as control intraperitoneally. 4-6h after, mice were infected by intranasal instillation of 50 µL of PBS containing or not $5x10^6$ bacteria (PP34) in presence or absence of Ferrostatine (6mg /kg). 18h after infection, BALFs were collected and quality/quantity of immune cells content was assayed by flow cytometry. Briefly, cells were pelleted (1000 rpm, 5 minutes), Red blood cells (RBC) were removed by 10 min incubation in RBC Lysis Buffer (BioLegend), monocytes, macrophages, neutrophils, and eosinophils were subsequently stained with a cocktail of fluorochrome-conjugated antibodies detailed in the material section. Cells were then fixed in 4% PFA before fluorescence associated cell sorting (FACS) analysis using a LSRII instrument. AccuCheck Counting Beads (ThermoFisher) were used to determine absolute cell number. Data analysis and processing were performed using FlowJO software.

Cytometry-based Lipid peroxidation or ROS production

To measure lipid peroxidation or ROS production, cells were first washed with PBS 1X, and then incubated with either C11-BODIPY(581/591) (ThermoFisher) at 2 µM, or H2DCFDA (ThermoFisher) at 10 µM in Opti-MEM medium for 30 min at 37°C. After three washes with PBS 1X cells are resuspended in Opti-MEM medium and infected in presence or absence of pharmacological inhibitors. After 1-3h of infection, cells are washed with PBS, detached in MACS buffer (PBS-BSA 0,5%-EDTA 2mM) and samples were acquired within one hour using a flow cytometer (BD FORTESSA LSR II). Data were analysed with FlowJO software (version 10).

Immunoblotting

867 Cell lysate generation has been described previously (Eren et al., 2020). Briefly,
868 proteins were loaded in 12% SDS-PAGE gels and then transferred on PVDF
869 membranes. After saturation for 1 hour in Tris-buffered saline (TBS) supplemented
870 with 0.05% Tween 20 containing 5% non-fat milk (pH8), membranes were exposed
871 with antibodies at 4°C overnight (Table 1). Next day, membranes were washed 3
872 times in TBS 0.1% Tween 20 and incubated with the corresponding secondary
873 antibodies conjugated to horseradish peroxidase (HRP) (Table 1) for 1h at room
874 temperature. Immunoblottings were revealed using a chemiluminescent substrate
875 ECL substrate (Biorad) and images were acquired on a ChemiDoc Imaging System
876 (Biorad). All antibodies and their working concentrations are listed in Table 1.

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878

879 **(Redox) lipidomic**

880 1 million bone-marrow-derived macrophages were seeded into 6-well plates. Next
881 day, BMDMs were transfected with recombinant ExoU or ExoU^{S142A} proteins
882 (500ng/well) for one hour. Then, supernatant was removed, cells were washed two
883 times in PBS. Finally, 500µL of a cold solution of 50% PBS/50% Methanol was added
884 to cells and samples were transferred to -80°C for storage and subsequent analyses.

885 After thawing, lipids were extracted using a methyl-tert-butyl ether (MTBE)-based
886 liquid-liquid extraction method. Cell suspensions (500 µL in PBS/methanol 1:1, v/v)
887 were thawed on ice before adding 100 µL methanol MeOH containing 50 ng each of
888 the internal standards PC(15:0/18:1-d7), PE(15:0/18:1- d7), PG(15:0/18:1-d7),
889 PI(15:0/18:1-d7) and PS(15:0/18:1-d7) (EquiSPLASH, Avanti Polar Lipids). Samples
890 were then transferred into 8-mL screw-cap tubes, and then 1.125 methanol and 5 mL
891 MTBE were added. After vigorous mixing, samples were incubated at room
892 temperature on a tabletop shaker for 45 min. For phase separation, 1.25 mL water
893 was added, and samples were vortexed and centrifuged for 15 min at 2000 x g. The
894 upper organic phase of each sample was carefully removed using a Pasteur pipette,
895 transferred into an empty glass round-bottom tube, and dried under vacuum in a
896 SpeedVac concentrator. The dried lipid extracts were resuspended in 200 µL HPLC
897 mobile phase A/mobile phase B 3:1 (v/v) for targeted lipidomic analysis of oxidized
898 phospholipids. For LC-MS/MS, using a Sciex ExionLC Integrated System, 20 µL of

each lipid extract was injected using Column Kinetex 2.6 µm HILIC 100 Å 100x2.1 mm, Phenomenex and a Flow Rate of 200 µL/min. Then, the analyte-specific m/z transition profile was determined and the area under the peak (ion intensity vs. elution time) was calculated using MultiQuant, Sciex software.

Data calculation was performed by doing ratio between the values of "area ratio analyte/internal standard" of each oxidized phospholipid and its non-oxidized phospholipid. The fold induction in oxidized phospholipid was then calculated by doing a ratio between each oxidized ratio and the non-stimulated condition. Accordingly, the unstimulated condition oxidized ratios were 1 or 0 when no peroxidation was detected in any condition.

Phospholipase activity measurement

Evaluation of ExoU phospholipase activity was performed using the Cayman Chemical cPLA2 kit and performed as previously described with minor modifications (Deruelle et al., 2020). Briefly, 10 µL of a 1mg/mL (160pmols) solution of recombinant ExoU or ExoU^{S142} proteins were mixed in 96-well plates with 10µL of lysed cell samples and 10µL of Assay Buffer. Then, samples were incubated for 1 hour at room temperature with 250µL of substrate solution (1.5 mM arachidonyl thiophosphatidylcholonie) and then for additional 4 or 16 hours in dark. Reaction was stopped using 25mM solution of DTNB according to maniufacturer instructions and absorbance was detected at 405nm using a Clariostar plate reader. Phospholipase activity of ExoU or ExoU^{S142} was calculated as the hydrolysis rate accordingly to the manufacturer instructions.

Ethics statements

The use of human cells was performed under the agreement of the Research Ethical Committee, Haute-Garonne, France. Buffy coats came anonymously by the EFS (établissement français du sang, Toulouse, France). For each donor, a written informed consent was obtained according to the EFS contract agreement n°

21PLER2017-0035AV02, according, to "Decret N° 2007-1220 (articles L1243-4, R1243-61)".

Statistical analysis

Statistical data analysis was performed using Prism 8.0a (GraphPad Software, Inc.). We used T-test with Bonferroni correction for comparison of two groups. Data are reported as mean with SEM. Regarding animal experiments, we used Mann-Whitney tests and mouse survival analysis were done using log-rank Cox-Mantel test. P values in figures have the following meaning; NS non-significant and Significance is specified as *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001.

Supplemental information

Figure S1: ExoU-dependent lung pathology in mice occurs in an inflammasome-independent manner

(A) Survival of WT, *Casp1*$^{-/-}$/*Casp11*$^{-/-}$, *Nlrc4*$^{-/-}$ and *GsdmD*$^{-/-}$ mice intranasally infected (n=6 animals per condition) with 5.10^5 CFUs of *P. aeruginosa* PP34. Graphs represent one experiment (6 mice/group) out of three independent *in vivo* experiments. NS: Not significant using Log-rank Cox-Mantel test for survival comparisons.

(B) Bronchoalveolar (BAL) and lung bacterial loads from WT, *Casp1*$^{-/-}$/*Casp11*$^{-/-}$, *Nlrc4*$^{-/-}$ and *GsdmD*$^{-/-}$ mice (n=6) 18 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34. Graphs represent one experiment (6 mice/group) out of three independent *in vivo* experiments. NS: Not significant using Mann-Whitney analysis test.

Figure S2: Lipid peroxidation contributes to ExoU-induced necrosis in various cell types

(A) LDH release in *Nlrc4*$^{-/-}$ BMDMs infected with various *P. aeruginosa* strains expressing or not *exoU* in presence of Ferrostatin-1 (Fe1, 10µM) for 2 hours.

(B) Measure of LDH release in various human and murine cell types infected with various *P. aeruginosa* strains expressing or not *exoU* in presence of Ferrostatin-1 (Fe1, 10µM) for 2 hours.

(C) Immunoblotting of ExoU secretion by *P. aeruginosa* in presence of ferrostatin-1 (20µM).

(D) Measure of bacterial growth (O.D 600) in presence or absence of ferrostatin-1 (10, 20µM) for 14 hours)

Figure S3: L**ipid peroxidation fuels ExoU-dependent necrosis**

(A) ROS production in WT BMDMs transfected with ExoU or its catalytically dead mutant ExoU^{S142A} for 45 minutes using H2DCFDA (1µM) probe.

(B) Lipidomic analysis of the relative amount of each phospholipid upon rExoU transfection analysed in **Figure 3B**.

(C) Representative microscopy images of propidium iodide uptake in WT BMDMs transfected with rExoU or its catalytically inactive mutant ExoU^{S142A} (500ng). Images show two independent experiments, each performed three times at 45 minutes or 3 hours post transfection.

(D) Immunoblotting of Crispr Cas9-mediated *Gpx4* gene deletion in immortalized BMDMs. The Gpx4#1 (red) was selected for further analysis. CD8 and GFP means that cells were transduced with sgRNA targeting *Gfp* or *Cd8* genes and used as controls.

(E) Cytometry detection and quantification of phospholipid peroxidation using the probe C11-bodipy in immortalized WT or *Gpx4*$^{-/-}$ BMDMs using a fortessa cytometer.

(F) PGE2 and LTB4 eicosanoid release in WT BMDMs transfected with 100ng of ExoU or its catalytically dead mutant ExoU^{S142A} for 3 hours in presence or absence of ferrostatin-1 (20µM).

Figure S4: Ferrostatin-1 protects mice against ExoU-induced lung pathology

(A) Gating strategy to analyse Immune cell populations in bronchoalveolar fluids (BALFs). Immune cells were identified as CD45+ cells. Among CD45+ cells, different subset of immune cells including Interstitial/Alveolar Macrophages, Eosinophils and Neutrophils are identified based on specific cell surface marker expression.

Graphical abstract: Host lipid peroxidation fuels ExoU-induced cell necrosis-dependent pathology. Upon injection into host target cells ExoU becomes hyper-activated by host cell peroxidised phospholipids, which drives an exacerbated cell necrosis and contributes to the subsequent pathology. Consequently, targeting lipid peroxidation (ferrostatin-1) inhibits ExoU-dependent cell necrosis and attenuates the host deleterious consequences.

Movie S1: Live cell imaging of uninfected immortalized murine *Nlrc4*$^{-/-}$ BMDMs cell death using SYTOX green. 1 "time point" corresponds to 150s.

Movie S2: Live cell imaging of uninfected immortalized murine *Nlrc4*$^{-/-}$ BMDMs cell death in presence of 20µM of ferrostatin-1 using SYTOX green. 1 "time point" corresponds to 150s.

Movie S3: Live cell imaging of immortalized murine *Nlrc4*$^{-/-}$ BMDMs cell death infected with *exoU*-expressing *P. aeruginosa* (MOI1) using SYTOX green. 1 "time point" corresponds to 150s.

Movie S4: Live cell imaging of immortalized murine *Nlrc4*$^{-/-}$ BMDMs cell death infected with *exoU*-expressing *P. aeruginosa* (MOI1) in presence of ferrostatin-1 (20µM) using SYTOX green. 1 "time point" corresponds to 150s.

Movie S5: Live cell imaging of immortalized murine *Nlrc4*$^{-/-}$ BMDMs cell death infected with *exoU*-deficient *P. aeruginosa* (MOI1) using SYTOX green. 1 "time point" corresponds to 150s.

Movie S6: Live cell imaging of immortalized murine *Nlrc4*$^{-/-}$ BMDMs cell death infected with *exoU*-deficient *P. aeruginosa* (MOI1) in presence of ferrostatin-1 (20µM) using SYTOX green. 1 "time point" corresponds to 150s.

Movie S7: Live cell imaging of uninfected human bronchial organoids using Propidium Iodide up to 12 hours.

Movie S8: Live cell imaging of uninfected human bronchial organoids in presence of ferrostatin-1 (40µM) using Propidium Iodide up to 12 hours.

Movie S9: Live cell imaging of human bronchial organoids microinjected with *exoU*-expressing *P. aeruginosa* using Propidium Iodide up to 12 hours.

Movie S10: Live cell imaging of human bronchial organoids microinjected with *exoU*-expressing *P. aeruginosa* in presence of ferrostatin-1 (40µM) using Propidium Iodide up to 12 hours.

Movie S11: Live cell imaging of human bronchial organoids microinjected with *exoU*-deficient *P. aeruginosa* using Propidium Iodide up to 12 hours.

Movie S12: Live cell imaging of human bronchial organoids microinjected with *exoU*-deficient *P. aeruginosa* in presence of ferrostatin-1 (40µM) using Propidium Iodide up to 12 hours.

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