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Étude du rôle des morts cellulaires inflammatoires dans la régulation des infections bactériennes pulmonaires : Identification de nouveaux facteurs impliqués dans la pathologie pulmonaire médiée par l'Exotoxine U de *P. aeruginosa*

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Abbreviations

NCCD: Nomenclature Committee on Cell Death

RCD: Regulated cell death

PAMP: Pathogen-associated molecular patterns,

DAMPs: Danger-associated molecular patterns,

PRRs: Pattern Recognition Receptor,

TNF: Tumor Necrosis Factor

TRIF: TIR-domain-containing adapter-inducing interferon-β

TLRs: Toll-like receptors,

TRAIL: TNF Related Apoptosis Inducing Ligand,

AIF: Apoptosis inducing factor,

Apaf1: Apoptotic protease activating factor1,

CARD: Caspase Activation Recruitment Domain,

TNFR1: Tumour necrosis factor receptor 1,

ZBP1: Z-DNA binding protein 1,

ATP: Adenosine triphosphate,

ROS: Reactive oxygen species,

MLKL: Lineage kinase domain like pseudo-kinase,

RIPK1: Receptor-interacting protein kinase 1,

RIPK3: Receptor-interacting protein kinase 3,

RHIM: RIP homotypic interaction motif,

Pam3CSK4: Pam3CysSerLys4 (synthetic triacylated lipopeptide)

HMGB1: High mobility group box 1,

LDH: Lactate deshydrogenase,

NINJ1: Nerve injury–induced protein 1,

IRF: Interferon Regulated Factors,

ISGs: Interferons Stimulated Genes

MCP-1; Monocyte Chemoattractant Protein-1,

MIP-2 (CXCL2): Macrophage inflammatory protein (: Chemokine (C-X-C motif) ligand 2),

CXCL1: Chemokine (C-X-C motif) ligand 1,

CXCL10: Chemokine (C-X-C motif) ligand 10,

GPX4: Glutathione (GSH) peroxidase 4,

GSH: Glutathione,

RSL-3/-5: Ras-selective lethal small molecules 3/-5

PARP-1: Poly (ADP-ribose) polymerase-1,

Fer-1: Ferrostatin-1

RLRs: Rig-Like Receptors,

NLRs: Nod-Like Receptors,

ALRs: AIM-2-Like Receptors,

SLRs: Sequestrosome-Like Receptors,

LPS: Lipopolysaccharide,

T3SS : Type 3 Secretion system,

ExoU: Exotoxin U,

PLP: Patatin like phospholipases,

PLA2: Phospholipases A2,

PIP2: Phosphatidylinositol 4, 5-bisphosphate,

SOD1: Superoxide dismutase,

DNAJC5 (CSPα): DnaJ homolog subfamily C member 5 (cysteine string protein),

LTB4: Leukotriene B4,

PG: Prostaglandins,

COX: Cyclooxygenase,

LOX: Lipoxygenase,

GTP: Guanosine-5'-triphosphate

GBPs: Guanylate Binding Proteins

Irgs: Immunity-related GTPases.

Abstract

In the context of pulmonary infections, the host and the pathogens have developed diverse mechanisms to regulate the host cell death process. Such mechanisms are initiated after recognition of dangerous signals associated with the pathogens by the innate immune system, which leads to an inflammatory response at the site of infection and promote pathogen clearance. Therefore, various cell deaths have been described as proinflammatory, in particular the most inflammatory pyroptosis and ferroptosis. These cell deaths are regulated (controlled at the genetic level) and allow the release of molecules (cytokines and chemokines) that activate the immune system. They are real arms for the organism to fight against infectious agents. However, deregulation of these deaths induces tissue damage, which can lead to fatal sepsis and the escape of infectious agents.

Many bacteria modulate the host cell death for proliferate, such as *Pseudomonas aeruginosa* (*P. aeruginosa*). Today, this bacterium is a major global health concern due to its involvement in nosocomial infections and fatal infections in patients with cystic fibrosis. *P. aeruginosa* presents several virulence factors but in specific, the strains expressing exotoxin U (ExoU), are associated with antibiotic resistance but also with higher mortality and morbidity in immunocompromised patients. In fact, ExoU causes cell death and tissue damage via its phospholipase activity upon infection. However, the mechanisms involved, and the role of ExoU-dependent death remain unknown. Therefore, we have shown that *P. aeruginosa* ExoU destroys cells by necrosis using cellular oxidized lipids as in ferroptosis. The production of oxidized lipids can be inhibited by lipophilic antioxidants (vitamin E derivatives) or ferroptosis inhibitors (ferrostatin-1). Indeed, the treatment of immune (macrophages, neutrophils) and non-immune (epithelial cells) with these molecules before infection protects them from ExoU toxicity. Moreover, pretreatment of mice with these molecules significantly attenuates the sepsis (death) induced by ExoU in mice. Finally, we observed the same phenotype with human lung organoids suggesting that humans could also be protected against this infection upon treatment. This work was published in the journal Plos Pathogens.

Interferon cytokines are important during bacterial infections; indeed, they stimulate the expression of more than 2000 genes that modulate the inflammation. In this second part, we were focused on the role of Irgm2, one of the interferon-induced genes, during

bacterial infection. Indeed, lipopolysaccharide (LPS, one component of Gram-negative bacteria) activates the cytosolic receptor caspase11 which leads to the infected cells pyroptosis. In this study, we showed that Irgm2 and the ATG8 family member Gate-16 cooperatively regulate caspase11-dependent pyroptosis and the release of proinflammatory cytokines such as interleukin -1 and -18. Therefore, mice mutated for Irgm2 have a rapid death following infection compared to WT mice. These proteins act as a balance to avoid exacerbation of inflammation and thus sepsis. This work was published in the Embo Reports journal.

Altogether, the data generated during my thesis highlight the importance of regulated cell death to modulate lung infections and tissue alteration leading to sepsis.

Key words: Cell death, innate immunity, Exotoxin U, oxidized lipids, Irgm2, pulmonary pathology.

Résumé

Dans le contexte des infections pulmonaires, l'hôte et les pathogènes ont développé divers mécanismes pour réguler le processus de mort cellulaire de l'hôte. Ces mécanismes sont initiés après la reconnaissance des signaux de danger associés aux pathogènes par le système immunitaire inné ; qui entraîne une réponse inflammatoire au site de l'infection et favorise l'élimination des pathogènes. Par conséquent, diverses morts cellulaires ont été décrites comme pro-inflammatoires, en particulier la pyroptose et la ferroptose. Ces morts cellulaires sont régulées (contrôlées au niveau génétique) et permettent la libération de molécules (cytokines et chimiokines) qui activent le système immunitaire. Ce sont de véritables armes pour l'organisme afin de lutter contre les agents infectieux. Cependant, une dérégulation de ces morts induit une lésion tissulaire, qui peut conduire à un sepsis fatal, donc un échappement de ces agents infectieux.

De nombreuses bactéries modulent les morts cellulaires pour proliférer, comme *Pseudomonas aeruginosa* (*P. aeruginosa).* A ce jour, cette bactérie constitue une préoccupation majeure de santé mondiale en raison de son implication dans les infections nosocomiales et les infections fatales chez les patients atteints de mucoviscidose. *P. aeruginosa* a plusieurs facteurs de virulences mais les souches exprimant l'exotoxine U (ExoU), sont associées à la résistance aux antibiotiques mais aussi à une mortalité et une morbidité plus élevée chez les patients immunodéprimés. En effet, ExoU entraîne la mort des cellules et un dommage tissulaire via son activité phospholipase. Cependant les mécanismes impliqués et le rôle de la mort dependant d'ExoU restent inconnus. Ainsi, nous avons demontré que la toxine U de la bactérie *P. aeruginosa* détruit les cellules par nécrose grâce à l'utilisation des lipides oxydés cellulaires comme dans la ferroptose. La production de lipides oxydés peut être inhiber par des antioxidants de types lipophilique (dérivés de la vitamine E) ou inhibiteurs de la ferroptose (ferrostatin-1). En effet, le traitement de cellules immunitaires (macrophages, neutrophiles) et ou non immunitaires (épithéliales) avec ces molecules avant infections les protègent contre la toxicité de toxine U. De plus, le prétraitement des souris avec ces molécules atténue de manière très significative le sepsis (la mort) induit par la toxine U chez les souris. Pour finir, nous avons observé le même phénotype avec des organoïdes pulmonaires humains ce qui suggère que l'homme pourrait être aussi protégé contre cette infection à la suite du traitement. Ces travaux ont été publiés dans le journal Plos Pathogens.

Les cytokines type interféron sont importantes lors des infections bactériennes, en effet elles stimulent l'expression de plus de 2000 gènes qui modulent l'inflammation. Dans cette deuxième partie, nous nous sommes intéressés au rôle d'Irgm2, un des gènes induits par les interférons, lors d'infections bactériennes. En effet, le lipopolysaccharide (LPS, un composant des bactéries Gram négatives) active le récepteur cytosolique caspase11 qui conduit à la pyroptose des cellules infectées. Dans cette étude, nous avons montré que Irgm2 et le membre de la famille ATG8 Gate-16 régulent de manière coopérative la pyroptose dependante de la caspase11 et la libération de cytokines proinflammatoires tels que l'interleukine -1β et -18. Ainsi, les souris mutées pour ce gène ont une mort rapide suite à une infection par des bactéries Gram négatives par rapport aux souris sauvages. Cette protéine sert de balance afin d'éviter l'exacerbation de l'inflammation et ainsi donc le sepsis. Ces travaux ont été publiés dans le journal Embo *Reports*.

Finalement, l'ensemble des données de cette thèse témoignent de l'importance de la mort cellulaire dans la modulation des infections pulmonaires et à altération des tissus pouvant conduire au sepsis.

Mots clés : Mort cellulaire, immunité innée, exotoxine U, lipides oxydés, Irgm2, pathologie pulmonaire.

Part 1:

Introduction

Part 1: Summary of knowledge

Chapter 1: Cell death and inflammation

The name necrosis comes from ancient Greek denoting ‘to kill’. Necrosis is a term used to designate cellular injuries that trigger the premature death of cells in living tissue plants or animals (Proskuryakov et al., 2003). Cell necrosis is caused by various internal and external hazards such as ischemia or trauma. During necrosis several alterations occur, including membrane disruption, mitochondrial swelling, and cytoplasmic swelling; the nucleus may swell or retract, followed by nuclear disruption into the cytoplasm (karyolisis) (Proskuryakov et al., 2003).

Regarding this, targeting and inhibiting necrosis has always been a goal of the pharmaceutical and medical sciences. For instance, in the 1000s, flies and maggots were used to treat chronic wounds (gangrene) or ulcers to prevent or arrest necrotic spread (E. Shi & Shofler, 2014). These methods were stopped after the introduction of antibiotics and enzymes to the range of treatments for wounds. In spite of these cures, some wounds progress to gangrene (putrefaction of tissues) (Figure1), which was the cause of many people died throughout the world. However, the amputation of the members (arms or legs) affected could help in some cases.



Figure 1: Dry gangrene of thumb and first finger (right hands).(Kenney, 1946)

I-Cell death in general

Cell deaths are essential processes that cover all areas of life. In this context, those extremely regulated processes can be induced upon embryo development, various attacks (biological, physical and chemicals) or to restrict the appearance of

autoimmune or cancerous cells. Since the first observation of cell death by Karl Vogt in toad in 1842, one century later cell deaths were classified in two forms with the formal characterization of apoptosis in 1972. First, apoptosis has largely been considered as a regulated and non-immunogenic death whereas necrosis was considered as unregulated and inflammatory cell death. Nowadays, both scientific interests and the progress of research allowed a better characterization of the molecular mechanisms of cell deaths, which led to the discovery of various regulated forms of cell necrosis, involving cellular regulators. (Figure 2). To avoid confusion between these newly described regulated cell deaths, one committee on cell death (NCCD, Nomenclature Committee on Cell Death) was created. This committee provides a widely accepted and updated nomenclature on cell death, which permits investigating the molecular mechanisms that regulate all those cells deaths (Galluzzi et al., 2011, 2014, 2018; Kroemer et al., 2005, 2008).

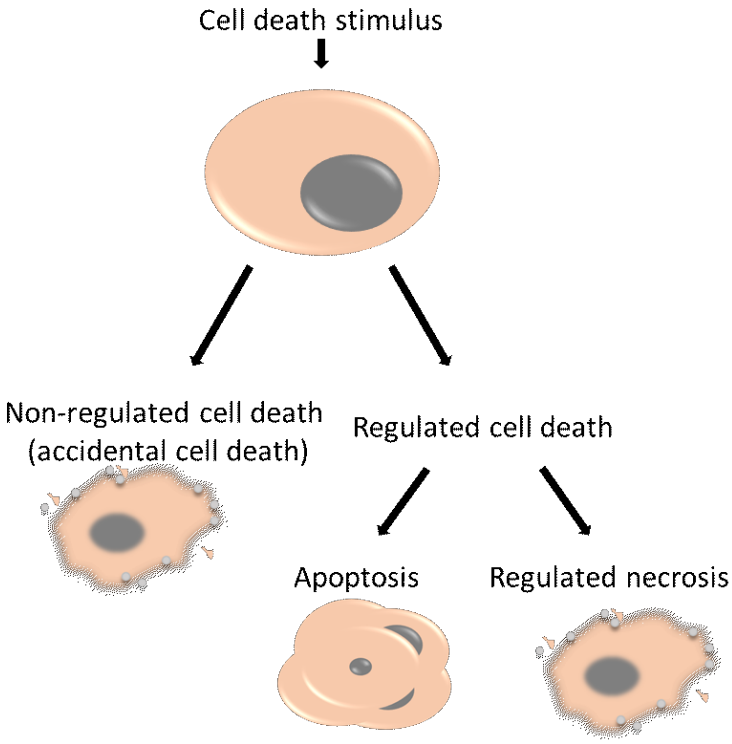

Figure 2: The different types of cell death: ways of dying after receiving stimuli.

Morphology of cell death:

The cell in the process of necrosis presents the following morphology: cellular swelling, loss of membrane integrity following early permeabilization, thinning of the cytoplasm, swelling of cellular organelles, mitochondrial dysfunction, and permeabilization of the lysosomal membrane, slight condensation of chromatin, intact nucleus and release of cytoplasmic contents into the extracellular space (Galluzzi et al., 2014, 2018). However, for apoptosis cell death, there are some differences that are well described in section III-2-1 of chapter 1.

II-The different types of cells deaths: Why so many ways to die?

1-Non-regulated necrosis (until they get characterized)

Necrosis caused by physicochemical or mechanical events (elevated temperature or pressures, potent detergent or shearing) outside the cell is called “accidental” cell necrosis. Those forms of cell necrosis are independent of any known molecular machinery and occur almost immediately after cell contact with the trigger. This has led various studies to suggest that such cell necrosis cannot be influenced either by genetic intervention or by a pharmacological molecule due to the lack of intracellular regulation (Galluzzi et al., 2014, 2018). Although no studies could link already described regulators of cell deaths with those of cell necrosis, does not mean that those forms of cell necrosis are not regulated. For instance, the discovery of the mechanosensitive ion channels, namely PIEZO channels, has led to the development of numerous axes of research on the ability of our cells to detect pressure, temperature modifications (Coste et al., 2010). Therefore, having mecano- or or temperature-sensitive receptors able to modulate non-regulated cell necrosis is also a hypothesis that warrants investigations.

2-Regulated cell death

Cell death dependent on cellular machinery is assumed to be programmed or regulated (Figure 3). They can be modulated by molecular intervention or pharmacological treatment. It is vital for the body to control cell death in order to avoid an uncontrolled inflammatory response that is harmful to the proper functioning of a tissue, organ (or more) or the immune system.

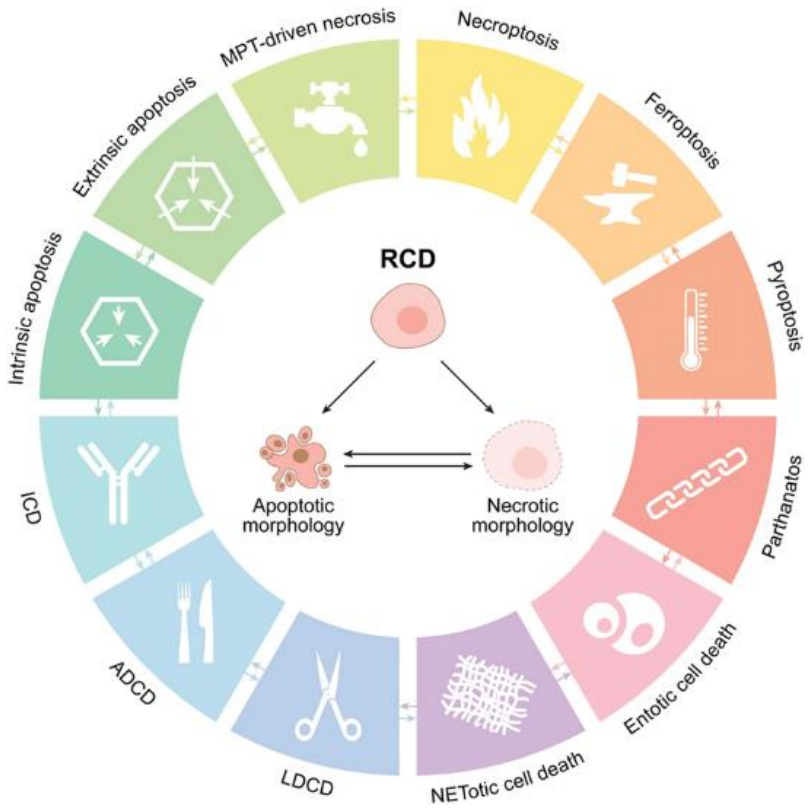

Figure 3: Classification of regulated cell death. from (Galluzzi et al., 2018)

2-1-Apoptosis

Apoptosis is distinct by different morphological features described in 1972 by John Kerr, Andrew Wyllie and Alastair Currie: budding of the plasma membrane, cytoplasmic and chromatin condensation as well as nuclear fragmentation (Kerr et al., 1972). The fragmentation of the cell into "apoptotic bodies" defined by a membrane containing micronuclei (nuclear fragments) is a hallmark of apoptosis. According to the observations of Kerr et al., apoptosis is frequent during embryonic development and renewal of healthy tissues as well as in certain pathological situations such as tissue ischemia and tumour regression (Kerr et al., 1972). This death occurs in response to many stimuli either external by the activation of death receptors (DRs) or pathogen-associated molecular pattern (PAMPs) receptors (Pattern Recognition Receptors (PRRs)), or internal via stress factors, a loss of integrity of the DNA following damage or a major change in cell homeostasis (Ziegler & Groscurth, 2004). These events will lead to the activation of intracellular enzymes, called the caspases which are a cysteine dependent aspartate specific protease. These will generate the cleavage of proteins essential to the structural integrity of the cell, known as " essential " and thus

indispensable to the maintain, the organization and the attachment of the cytoskeleton as well as the proteins necessary to the structural integrity of the nucleus by maintaining the mitotic apparatus (Ziegler & Groscurth, 2004). This death was for a long time considered as the only programmed death pathway as opposed to necrosis, which is considered accidental. There are two distinct pathways of apoptosis regulation: extrinsic and intrinsic (Figure 4).

Extrinsic activation of apoptosis:

The extrinsic pathway is mainly activated by the binding of ligands to the TNF (*Tumor Necrosis Factor*) superfamily receptor: TNF-α, FasL and TRAIL (TNF Related Apoptosis Inducing Ligand), allowing the formation of complex IIa in the case of TNF-α or complex called "Death Inducing Signalling Complex" (DISC) in the case of FasL or TRAIL. These complexes are necessary for the activation of the initiating caspase 8, which plays a central role in the activation of effector caspases -3, -6 and -7. Extrinsic apoptosis is commonly activated by immune cells such as NK (Natural Killer) cells and cytotoxic T cells following activation of DRs and PRRs to eliminate infected, damaged or precancerous cells as well as by host cells to prevent viral replication in host cells and the dissemination of viruses in the body. The apoptotic cells are then cleared by phagocytic cells such as macrophages via a mechanism call “efferocytosis” (Green et al., 2018).

Intrinsic activation of apoptosis:

The intrinsic pathway, on the other hand, is triggered in response to a major intracellular damage or a stress signal and is regulated by pro-apoptotic and anti-apoptotic proteins of the Bcl2 family. This pathway is characterized by the permeabilization of the mitochondrial outer membrane (MOMP) and the release of cytochrome-c into the cytosol as well as other pro-apoptotic molecules (Smac, Endonuclease G, AIF (apoptosis inducing factor)). Seven molecules of apoptotic protease activating factor 1 (Apaf1) then interact with cytochrome-c and recruit the procaspase 9 through their CARD domain for "Caspase Activation Recruitment Domain" to form the apoptosome, responsible for the activation of caspase 9 and then of caspase 3 (Bratton et al., 2001).

These two pathways can be engaged successively via the cleavage of Bid into tBid (truncated Bid) by caspase 8 allowing an amplification of the pro-apoptotic signal,

responsible for the caspase 3 activation, which leads to irreversible cell dismantling resulting in apoptosis. Apoptosis ensures cell death related to the organisms development and tissue homeostasis of adults. This death has long been described as non-inflammatory in opposition to necrosis since it prevents the escape of any intracellular material by the formation of apoptotic bodies. Moreover, apoptosis generates chemoattractant and recognition signals to ensure rapid elimination of the cell by phagocytosis, termed "find me" and "eat me" (S. P. Cullen et al., 2013) such as externalization of phosphatidylserine that will allow their recognition and elimination. However, when the destruction of apoptotic bodies is incomplete, there may be degradation of their plasma membrane and release of their contents into the tissue, leading to secondary necrosis (Honda et al., 2000) which results in autoimmunity or an uncontrolled inflammatory response (Kawane et al., 2010; Mukae et al., 2002). Thus, apoptosis can be a source of inflammation and contribute to the development of pathologies as described in the liver where hepatocyte death can be followed by inflammation due to phagocytic of apoptotic bodies by Kupffer cells or by liver star cells, producing TNF-α, FasL, and TRAIL (Malhi et al., 2010).

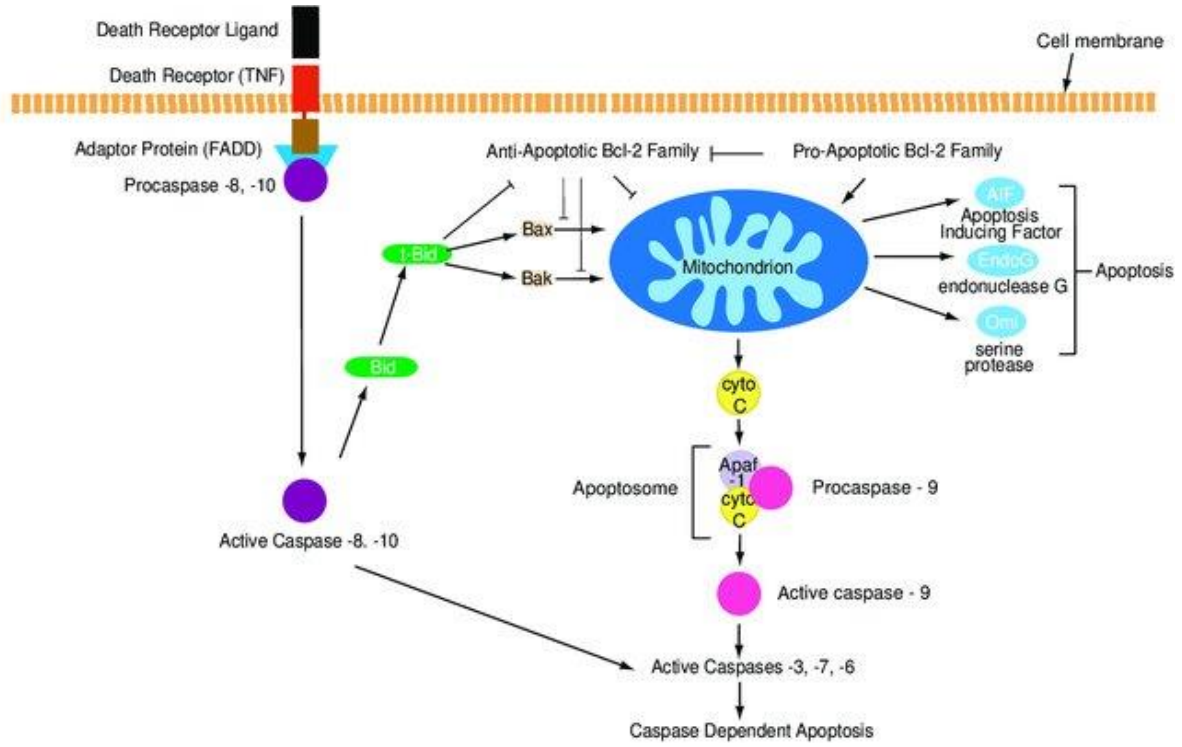

Figure 4: The two apoptosis pathways. The extrinsic pathway (left) and intrinsic pathway (centre). The extrinsic and intrinsic pathways share a common end point at the level of caspase-3/7/6 activation (Guerin et al., 2006).

2-2-Regulated necrosis:

For a long time, necrosis was considered as the incapacity of cells to perform regulated apoptosis, hence leading to a consensus that necrosis is only a side effect of dysregulated responses. However, the last decades have seen the discovery and characterization of numerous genetic and pharmacologic regulators of cell necrosis, which induced a total opinion change in the view and understanding of the functions of regulated cell necrosis (Tang et al., 2019). These inflammatory and immunogenic deaths are beneficial in the fight against pathogenic bacteria, as well as viral and parasitic infections in a context of danger for the organism (Matzinger, 1998); moreover, they promote healing. They can become deleterious when deregulated in the case of autoimmune, inflammatory, ischemic or degenerative diseases. The term regulated necrosis is not a synonym for necroptosis, as was widely used in the literature between 2005 and 2008 (Figure 3). Regulated necrosis groups together different types of death which all present a necrotic morphology. In fact, regulated necrosis contains: pyroptosis (B. Cookson in 2001) (Cookson & Brennan, 2001), NETosis (A. Zychlinsky in 2004) (Brinkmann et al., 2004), necroptosis (J. Yuan in 2005) (Degterev et al., 2005), entosis (J. Brugge in 2007) (Overholtzer et al., 2007), parthanatos (V.L. Dawson in 2009) (K. K. David et al., 2009), ferroptosis (B. Stockwell in 2012) (Dixon et al., 2012), immunogenic cell death (G. Kroemer in 2005) (Casares et al., 2005), lysosomal cell death (J. Franko in 2000) (Franko et al., 2000), autosis (B. Levine in 2013) (Y. Liu et al., 2013), Oxeiptosis (A. Pichlmair in 2018) (Holze et al., 2017), alkaliptosis (D. Tang in 2018) (Song et al., 2018) (Tang et al., 2019) (Figure 3 and 5). They are controlled by different signalling pathways but share the same characteristics such as loss of membrane integrity, excessive production of reactive oxygen species (ROS) or ATP depletion (Galluzzi et al., 2011; Vandenabeele et al., 2010).

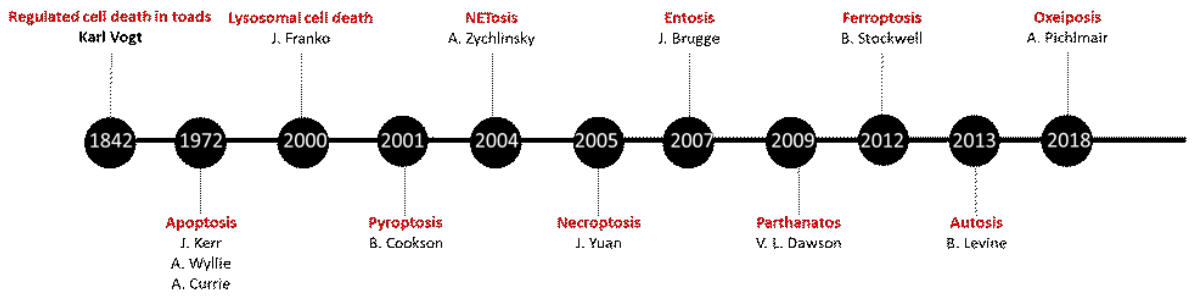

Figure 5: Discovery of the different cell deaths through time.

In this section, I will briefly describe some regulated necrosis, focusing in particular on Pyroptosis and Ferroptosis that are two deaths studied during this thesis.

A-Necroptosis:

Necroptosis is introduced by perturbations of the extracellular or intracellular microenvironment detected by specific death receptors, including (but not limited to) FAS and Tumour necrosis factor receptor 1 (TNFR1), or PRRs, including TLR3, TLR4, and Z-DNA binding protein 1 (ZBP1). Moreover, various studies have shown that necroptosis participates in developmental programs (Galluzzi et al., 2018).

Mechanistically, necroptosis depends on the activation of RIPK3 and mixed lineage kinase domain like pseudo-kinase (MLKL) (Linkermann & Green, 2014; J. M. Murphy et al., 2013). During the initiation necroptosis by TNFR1, RIPK3 is activated by RIPK1 (if Caspase 8 is inactive) through a mechanism involving the physical interaction between their respective RIP homotypic interaction motif (RHIM) domains and RIPK1 catalytic activity (Y. S. Cho et al., 2009; Li et al., 2012; Vandenabeele et al., 2010). Therefore, chemical inhibitors of RIPK1 including necrostatin-1 (Nec-1) and derivatives robustly inhibit TNFR1-driven necroptosis, *in vitro* and *in vivo* (Degterev et al., 2005, 2008). Additionally, RIPK3 can be activated as a result of RHIM-dependent interaction with (1) TRIF upon activation of TLR3 by double-stranded RNA (dsRNA) or of TLR4 by lipopolysaccharide (LPS) (Kaiser et al., 2013); or (2) ZBP1, which is a cytosolic DNA sensor promoting type I interferon (IFN) synthesis and NF-κB activation (Lin et al., 2016; Maelfait et al., 2017; Newton et al., 2016). The active RIPK3 catalyzes the phosphorylation of MLKL, resulting in the formation of MLKL oligomers (probably trimers or tetramers) that translocate to the plasma membrane, where they bind to specific phosphatidylinositol phosphate species by a rolling mechanism and thus trigger plasma membrane permeabilization (Galluzzi et al., 2018; Linkermann & Green, 2014).

B-Pyroptosis:

Cookson and Brennan define a specific type of RCD partially resembling apoptosis but dependent on inflammatory caspase 1, call “Pyroptosis” (Cookson & Brennan, 2001). Initially, only monocytes or macrophages was thought to die upon caspase1 activation (Bergsbaken et al., 2009; Zychlinsky et al., 1992). However, recent findings indicate that pyroptosis can occur in other cell types from monocytes lineage (J. Shi et al., 2014a). It can be driven by several other caspases such as caspase 3 (J. Shi et al.,

2017) and present a major role in innate immunity against intracellular pathogens (Jorgensen & Miao, 2015; Lamkanfi & Dixit, 2010). In some pathological conditions such as lethal septic shock pyroptosis is importantly involved (Aziz et al., 2014; vanden Berghe et al., 2014).

This form of regulated necrosis would follow an infection or cellular stress (Lamkanfi & Dixit, 2010). At the molecular level, pyroptosis generally relies on the activation of cellular sensors. In fact, all cells of the organism express membrane and cytosolic receptors, called PRRs, allowing them to detect both extracellular and intracellular pathogens but also danger signals released by the stressed cells. Indeed, one type of cytosolic PRRs, form a multi-protein complex upon activation, call inflammasomes. The inflammasome is composed of a PPR and an adaptor protein call ASC (for apoptosis associated speck-like protein containing A card) and an activator protein caspase1 (Man & Kanneganti, 2015). The receptors capable to form an inflammasome are canonical: AIM2, IFI16 from ALR family, NLRP1/NLRP3/NLRC4 of NLR family, the PYRIN receptor and non-canonical: Caspase11 (mouse), Caspase-4 and 5 (human). Upon activation of receptor, ASC interacts with both the receptor and caspase1 via his CARD domain (Figure 6). These lead to an auto-activation of caspase1, which will do the maturation of inflammatory cytokines of interleukin-1 (IL-1) family (IL1-β and IL-18) and the pore forming protein gasdermin (gsdm). On one hand, gsdm cleaved, his N-terminal fragment make a pore to the cell membrane leading to the pyroptosis and secretion of cytokines and DAMPs (Figure 6). It leads to ROS production, loss of mitochondrial function, cell swelling, plasma membrane permeabilization and nuclear condensation and DNA fragmentation (Lamkanfi & Dixit, 2010).

Very recently, a new protein call nerve injury-induced protein 1 (NINJ1) was described to play a crucial role in the last step of pyroptosis unlike gsdm-D (Kayagaki et al., 2021; Newton et al., 2021). Indeed, *Ninj1* lacking cell die after cleavage of gsdm-D but persist ballooned and fail to release large protein such as LDH (a commonly used marker of cell rupture) and HMGB1 (DAMP). NINJ1 is expressed on the cell surface and has two transmembrane domains plus an evolutionarily conserved extracellular amphipathic α-helix. The latter appears to contribute to NINJ1 oligomerization and membrane disruption (Newton et al., 2021). Kayagaki N et al demonstrated that NINJ1 is essential for also the plasma membrane rupture following necrotic and apoptotic cell death signals.

Pyroptosis is not only associated to sepsis and infectious diseases, but also described in chronic inflammatory conditions such as the inherited disease CAPS (for Cryopyrin-associated periodic syndrome) (Lachmann et al., 2009; vande Walle & Lamkanfi, 2016) as well as other multifactorial diseases such as diabetes, atherosclerosis, asthma (Conforti-Andreoni et al., 2011) and Alzheimer's disease (Venegas et al., 2017).

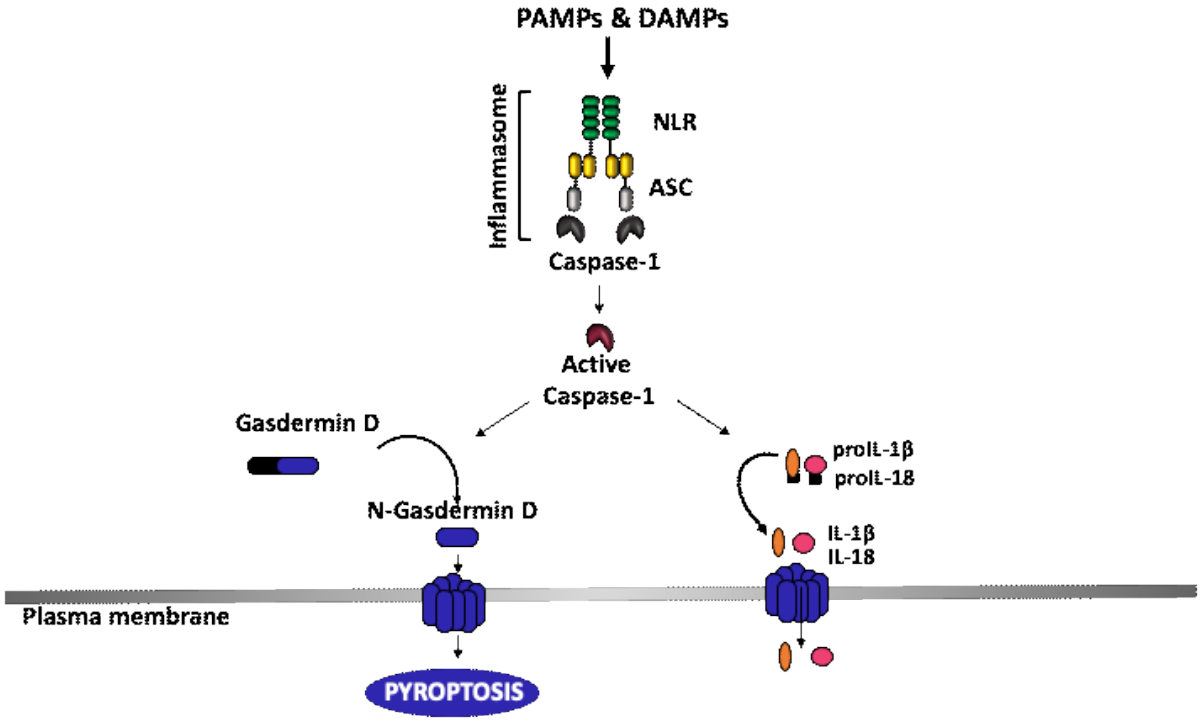

Figure 6: Pyroptosis induction upon activation of NLR receptors.

C-Ferroptosis:

Studies on ferroptosis and its mechanisms emerged several years before the establishment of the term “ferroptosis”. Murphy's group discovered that glutamate caused neuronal cell death by inhibiting system xc- (T. H. Murphy et al., 1989), which was later named “oxytosis” by Maher's group in 2001 (Shirlee Tan et al., 2001). Recent studies found that ferroptosis and oxytosis had several common characteristics, such as the role of lipoxygenase, ROS production, and gene expression (Lewerenz et al., 2018; Neitemeier et al., 2017). However, there are discrepancies in a few protein-signalling pathways between ferroptosis and oxytosis (Lewerenz et al., 2018). In 2008, two compounds, ras-selective lethal small molecules 3 (RSL3) and 5 (RSL5), were screened using a high-throughput method that could selectively induce cell death in cells carrying RAS mutant subtype genes (W. S. Yang & Stockwell, 2008). The results

facilitated the identification of the lethal mechanism of the compound erastin (Dolma et al., 2003). Erastin engages tumorigenic cells in a regulated necrosis dependent on the RAS-RAF-MEK signalling pathway (Yagoda et al., 2007). This inducer binds to mitochondrial voltage-dependent anion channels (VDAC) and alters the outer membrane of mitochondria (Yagoda et al., 2007). In addition, it inhibits the Xc- transporter, a cysteine/glutamate (Cys/Glu) antiporter, responsible for the accumulation of oxidized cysteines (Dixon et al., 2012). Inhibition of this transporter decreases glutathione (GSH) biosynthesis responsible for loss of function of GPX4 (Glutathione (GSH) peroxidase 4) and ROS dependent lipid peroxidation (Dixon et al., 2012; W. S. Yang et al., 2014). Different groups agree that increased concentration of lipid hydroperoxides is responsible for ferroptosis.

This caspase-independent death does not induce cytochrome-c release, PARP-1 (Poly(ADP-ribose)polymerase-1) cleavage, or ATP depletion (Dixon et al., 2012; Dolma et al., 2003; Yagoda et al., 2007). It is characterized by the accumulation of lethal concentrations of peroxidized lipids which can be inhibited by antioxidants, due to the loss of activity of GPX4, which is the main protein in animals that can reduce lipid hydroperoxides in a membrane phospholipid (Yagoda et al., 2007; W. S. Yang et al., 2014). This death was named ferroptosis by Dixon et al. since it depends solely on intracellular iron metabolism (Dixon et al., 2012; W. S. Yang & Stockwell, 2008). There are different inducers of this death: erastin and RSL3 and RSL5 (Ras Selective Lethal 3 or 5) which have similar mechanisms of action and BSO (buthionine sulfoxamine), an irreversible inhibitor of GSH biosynthesis (Dixon et al., 2012; Yagoda et al., 2007). In 2018, the NCCD defined ferroptosis as “a form of regulatory cell death initiated by oxidative perturbations of the intracellular microenvironment that is under constitutive control by GPX4 and can be inhibited by iron chelators and lipophilic antioxidants” (Figure7) (Galluzzi et al., 2018).

The first chemical inhibitor described was named ferrostatin-1 (Fer-1) (Dixon et al., 2012). It is a chelator of peroxidized lipids *in vitro*; this one is unstable *in vivo*. The Fer-1-derived molecule 16- 86 has since been developed as it offers better plasma and metabolic stability and better *in vivo* efficacy in a renal ischemia-reperfusion injury model (Linkermann & Green, 2014). Peter Vandenabeele's group has recently developed a new series of Fer-1 analogues that offer improved stability and anti-ferroptotic efficacy (in the nM range) that can be used in mice (Hofmans et al., 2016).

Other molecules also prevent ferroptosis such as Liproxstatin-1, zileuton, lipophilic antioxidants, iron chelators or kinase inhibitors (U0126: MEK inhibitor; SU6656: Src inhibitor) (Li et al., 2020). From a pathophysiological perspective, this regulated necrosis appears to be involved in neurodegeneration in Parkinson's disease (do Van et al., 2016; Seiler et al., 2008), Huntington's disease (Skouta et al., 2014), cancer (LACHAIER et al., 2014), renal or hepatic ischemia-reperfusion (Friedmann Angeli et al., 2014; Linkermann & Green, 2014), and infectious diseases (Bagayoko & Meunier, 2021; Jiang et al., 2021).

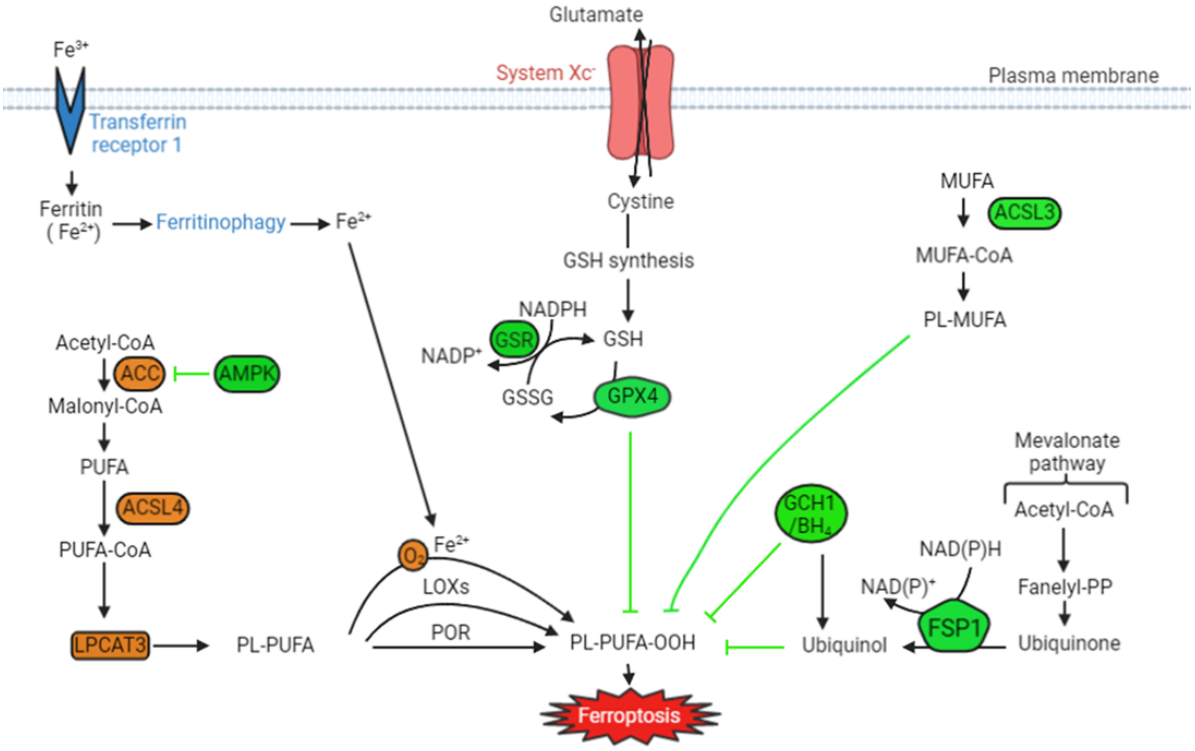

Figure 7: Ferroptosis induction and inhibition (green) pathways (Bagayoko & Meunier, 2021). During ferroptosis, phospholipids with polyunsaturated acyl tails (PL-PUFAs) are peroxidized (bottom left) due to their intrinsic susceptibility to peroxidation. PL-PUFAs are generated by different enzymes such as ACSL4 and LPCATs (orange, bottom left) which incorporate free PUFAs into phospholipids. Acetyl CoA can be used for synthesizing PUFA via acetyl CoA carboxylase (ACC) (orange, bottom left). The 5' adenosine monophosphate-activated protein kinase (AMPK) can suppress ferroptosis by inhibiting ACC (green, bottom left). Once PL-PUFAs are produced, iron-dependent enzymes and labile iron use molecular oxygen (O2) to make a peroxidation reaction, generating PL-PUFA-OOH (lipoxygenases and cytochrome P450 oxidoreductase (POR) enzymes). Labile iron is imported through the transferrin receptor 1 (TfR1) and stowed in ferritin (blue, top left). Ferritin can be degraded via an autophagy-like

process, ferritinophagy, which releases labile iron and facilitates the lipid peroxidation driving ferroptosis (top left). Three main pathways for removing peroxidized PL-PUFAs (green, middle and bottom right): the GPX4-glutathione axis , the FSP1-CoQ10 axis, and the GCH1-BH4 or MUFA axis : The canonical ferroptosis controlling axis involves uptake of cystine through the cystine-glutamate antiporter (system xc−), glutathione (GSH)-, GSH biosynthesis, and glutathione peroxidase 4 (GPX4)-mediated reduction of phospholipid hydroperoxides (PL-PUFA-OOH). Glutathione-disulfide reductase (GSR) allows recycling of oxidized glutathione (GSSG) using electrons provided by NADPH. Reduced coenzyme Q10, also known as ubiquinol, prevents lipid peroxidation and so ferroptosis. FSP1 (previously known as AIFM2) (green, bottom right), regenerates ubiquinol from ubiquinone, which is produced through the mevalonate pathway (bottom right). The last ferroptosis-suppressive mechanisms consist of GCH1 / BH4 pathway, which permits the production of reduced ubiquinol and the remodeling lipids to disfavor lipid peroxidation. Moreover, monounsaturated fatty acids (MUFAs), when incorporated into phospholipids via ACSL3, act through an unknown mechanism to suppress ferroptosis (green, top right).

In only a decade, these various molecular mechanisms of regulated necrosis have been highlighted. Contrary to apoptosis, necrosis groups together accidental death and regulated deaths that share the same morphological characteristics, which makes their classification more complex. The signalling pathways involved in the different types of death are identified using specific chemical inhibitors or biological models genetically modified for the expression of their key players (Galluzzi et al., 2011, 2014, 2018). The current classification of regulated necrosis is thus based on the molecular signalling pathways involved (Galluzzi et al., 2014, 2018).

III- Pattern Recognition Receptors (PRRs) and inflammation:

1- PRRs/cell death/inflammation

Our cells display a large number of mechanisms to fight against endogenous or exogenous dangers, the last one being death. Cell autonomous immunity is the intrinsic capacity of the cell to defend itself against aggression. Actually, immune cells and some non-immune cells, especially endothelial or epithelial cells, express receptors of danger signals. These sensors, called PRRs (Mostowy & Shenoy, 2015), are classified according to their biochemical and structural characteristics. Thus, we

distinguish between membrane receptors such as TLRs (Toll-Like Receptors), CLRs (C-type Lectin Receptors), scavenger receptors or even opsonin receptors. The intracellular compartment of cells is scrutinized by receptors such as RLRs (Rig-Like Receptors), NLRs (Nod-Like Receptors), ALRs (AIM-2-Like Receptors) or SLRs (Sequestrosome-Like Receptors) (Mostowy & Shenoy, 2015). For instance, macrophages, one of the first immune cells in the site of infection engulf (e.g. phagocytosis and endocytosis) the pathogens via some membrane PRRs during an infection. Then, the pathogen is enclosed in a locked compartment (phagosome or endosome) and followed by its degradation via the maturation of this compartment. Unlike PRRs involved in phagocytosis, many PRRs induce transcriptional remodelling of the cell after activation, resulting in the expression of numerous genes encoding communication effectors such as cytokines, chemokines, lipid mediators and also microbicide molecules helping the efficiency of cell autonomous immunity. The activation of PRRs, as described above (part chapter 1- III) triggers also cell death such as apoptosis or pyroptosis (Table 1), this contributes greatly to the activation of the immune system and the induction of inflammation.

Table 1: Some PRRs described to trigger cell death upon recognition of the ligands adapted from (Amarante-Mendes et al., 2018).

PAMPs/DAMPs	Receptors	Signal effectors	Cell death induced
Pam3CSK4	TLR1	**FADD/MyD88/**	Apoptosis
Pam3CSK4 **Lipoproteins** **Poly(I:C)** **LPS/HMGB1**	TLRs (2,3,4)	**FADD/MyD88/** **RIPK1**(TLR3)/**RIPK3/MLKL**	Apoptosis, Necroptosis
Flagellin	TLR5	**RIPK3/MLKL**	Pyroptosis, Necroptosis
CpG DNA	TLR9	**RIPK3/MLKL**	Necroptosis
LPS	Caspase11	**Active caspase 11**	Pyroptosis
dsDNA	NLRs, ALRs (AIM2)	**Caspase 1**	Pyroptosis
ssRNA **shRNA**	RLRs (RIG-I)	**FADD/MyD88/** **RIPK1/RIPK3/MLK**	Necroptosis, Apoptosis

2-Inflammation

Independently of the regulation or not of such forms of cell necrosis, a conserved consequence is their inflammatory potential due to the release of elevated cellular content within the tissue. The release of components such as actin or vimentin that are not supposed to be present in the extracellular environment triggers a strong

inflammatory reaction. Hence, in opposition to the pathogen exogenous activating signals, namely PAMPs, those cellular pro-inflammatory components have been named DAMPs. The detection of DAMPs and PAMPs is carried out by the NLRs or the TLRs. These activated receptors mobilize transcription factors such as Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) or Interferon Regulated Factors (IRFs), involved in the production of inflammatory cytokines and chemokines (Figure 8). Essentially, NF-κB pathway a common signalling event of the PRRs upon activation, is responsible for transcriptional induction of pro-inflammatory cytokines such as interleukin -1, -2, -6, -8, -12, -18 and TNFα, chemokines like MCP-1, MIP-2, CXCL1, and CXCL10 (T. Liu et al., 2017). Whereas IRFs are a family of master transcription factors that regulate interferon production. Nowadays, three major subfamilies of interferons are described: type I (α), type II (IFNγ) and type III (IFNλ). Interferons are microbicide cytokines of the host and play a critical role during infection. Further, these cytokines have a large direct or indirect effect on various cell types during bacterial or viral infections, due to their capacity to trigger more than 2,000 novel ISGs (Interferons Stimulated Genes) which display anti-microbicide properties (Green et al., 2018; Schneider et al., 2014).

Establishment of inflammation

These pro-inflammatory molecules establish the inflammation. Indeed, they trigger amongst others, the recruitment of immune cells such as neutrophils, monocytes to the site of inflammation through the induction of molecules that allow for diapedesis at the endothelial level as well as the increase of body temperature via the production of prostaglandins (PGs) (Dinarello, 2018). The combination of IL-18 with IL-12 leads to the production of Interferon gamma (IFNγ), a highly microbicidal and inflammatory cytokine, by T cells and Natural Killers (NKs) cells (Dinarello, 2018).

Different types of inflammation

It is widely accepted that inflammatory reactions can be profoundly different depending on the initial stimulus inducing the response. Microbial (bacteria) infections elicit a “type 1” inflammatory response characterized by neutrophil and macrophage recruitment and release of pro-inflammatory cytokines. During these inflammatory responses, the subsequent T-cell responses, dominated by T helper 1 (TH1) cells trigger macrophages activation and polarization via the production of interferon (Helming,

2011). Hence, they become more efficient at killing infectious agents. In contrast, infections with large extracellular parasites, such as helminths, provoke a “type 2” inflammatory response with a recruitment of eosinophils and basophils which release the cytokine interleukin-4 (IL-4), subsequently stimulating a T helper 2 (TH2) response (Voehringer et al., 2004). Under these conditions, there is also macrophages activation and polarization. Instead of an increased killing capacity, these macrophages actually display decreased phagocytic and microbial killing capacity, express a different set of cellular markers and are associated with healing processes and protective responses to helminth infection (Martinez et al., 2009; Voehringer et al., 2004).

3-Resolution of inflammation:

Neutrophils, monocytes, and macrophages are closely related phagocytic cells that cooperate during the initiation, progression and resolution of inflammation (Kourtzelis et al., 2020; Soehnlein & Lindbom, 2010). Once these cells have entered the site of infection, they collaborate to remove pathogens (e.g. bacteria). After the elimination of infectious agents, the ongoing inflammatory response must be resolved to prevent excessive tissue damage and to initiate the healing process. During the resolution of inflammation, a series of processes prevent further leukocyte infiltration and promote the removal of debris from the inflamed site, thereby restoring tissue homeostasis. The resolution process is an active process requiring signals that turn off neutrophil infiltration and, at the same time, promote the uptake and clearance of apoptotic cells. Lipid mediators appear to play a key role in this process (Serhan et al., 2008), and the resolution of inflammation is accompanied by an active switch in the types of lipid mediators present at the inflamed site. During the initial inflammatory response, prostaglandins and leukotrienes that amplify inflammation are generated by different cell types, including endothelial and epithelial cells, neutrophils, monocytes, and macrophages (Funk, 2001). Then, prostaglandin e2 (PGe2) and PGD2 progressively promote the synthesis of mediators with anti-inflammatory and pro-resolving activity, such as lipoxins. This process is known as lipid mediator class-switch ((Serhan et al., 2008). Moreover, some previous studies have shown that uptake of apoptotic neutrophils can stimulate macrophages to release mediators that suppress the inflammatory cytokines transforming growth factor-β (TGFβ) and IL-10 (Bellingan et al., 1996; Fadok et al., 1998; Voll et al., 1997). It also triggers the release of vascular endothelial growth factor (veGF) and other growth factors that are crucial for repair.

However, the release of TGFβ, IL-10 and PGe2 contribute to the restoration of homeostasis, but also dampen all the anti-microbicidal mechanisms.

In conclusion, the process of inflammation (initiation progression and the resolution) needs to be tightly controlled to prevent major tissue damages and pathogens persistence.

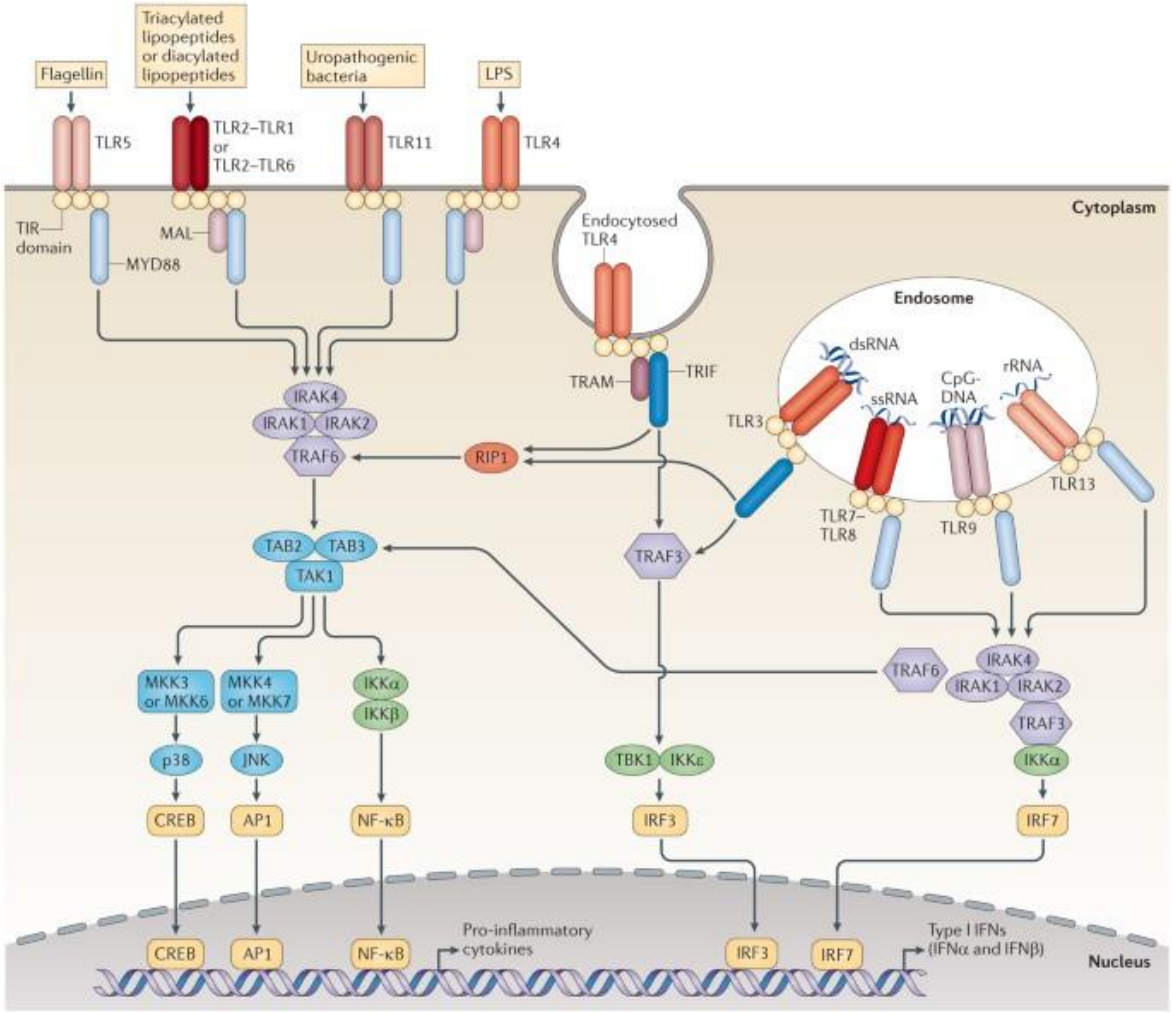

Figure 8: PRRs (TLRs) signalling leading to proinflammatory cytokines (O'Neill et al., 2013).

Chapter 2: Lipid peroxidation/Ferroptosis and its activation of immunity during infectious diseases:

Lipid peroxidation

The Review in FEBS journal: “Emerging roles of ferroptosis in infectious diseases”

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STATE-OF-THE-ART REVIEW

Emerging roles of ferroptosis in infectious diseases

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Keywords
ferroptosis; immunity; infections; lipid peroxidation

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In living organisms, lipid peroxidation is a continuously occurring cellular process and therefore involved in various physiological and pathological contexts. Among the broad variety of lipids, polyunsaturated fatty acids (PUFA) constitute a major target of oxygenation either when released as mediators by phospholipases or when present in membranous phospholipids. The last decade has seen the characterization of an iron- and lipid peroxidation-dependent cell necrosis, namely, ferroptosis, that involves the accumulation of peroxidized PUFA-containing phospholipids. Further studies could link ferroptosis in a very large body of (physio)-pathological processes, including cancer, neurodegenerative, and metabolic diseases. In this review, we mostly focus on the emerging involvement of lipid peroxidation-driven ferroptosis in infectious diseases, and the immune consequences. We also discuss the putative ability of microbial virulence factors to exploit or to dampen ferroptosis regulatory pathways to their own benefit.

Introduction

Addressing the role of cell death in various contexts often sends back to our own societal conception of life and death and our trials to postpone or escape it, a process that mostly cancer cells succeed to achieve. At the biological and homeostatic level, cells do not seem to integrate such notions, which leads to very stringent decisions to self-kill to the benefit of a whole population. To this regard, cell death constitutes an essential

Abbreviations
4-HNE, 4-Hydroxynonenal; ACSL4, acyl-CoA synthetase long-chain family member 4; AGER receptor, advanced glycosylation end products; AMPK, adenosine monophosphate-activated protein kinase; BRCA1, breast cancer type 1; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; COX, cyclooxygenase; CYPOR, cytochrome p450 oxidoreductase; DAMP, damage associated molecular patterns; FSP1(AIFM2), ferroptosis suppressor protein 1; GPX, glutathione peroxidase; GSH, glutathione; GSH/GSSG, reduced glutathione/glutathione di- sulfide; HETE, hydroxyeicosatetraenoate; HMGB1, is nuclear factor of the High Mobility Group; HO-1, heme oxygenase-1; HODE, hydroxyoctadecadienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; ICAM-1, intercellular adhesion molecule 1; LO·, lipid oxyl radical; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; LOX, lipoxygenase; LPCAT3, lysophosphatidylcholine acyltransferase 3; LPO, lipid peroxidation or oxidation; LTB4, leukotrienes B4; MDA, malondialdehyde; MHC CD1, Major histocompatibility complex (MHC) Cluster of Differentiation 1 (CD1); MLKL, Mixed Lineage Kinase Domain Like Pseudokinase; MPO, neutrophil myeloperoxidase; Mt-ETC, mitochondrial electron transport chain; MUFA, monounsaturated fatty acid; NADPH/NAD(P)H, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappa B; NINJ1, nerve injury-induced protein 1; NLR, Nod-like receptors; NRF2, nuclear factor erythroid 2-related factor 2; PAMP, pathogen-associated molecular pattern; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphotidylinosotol; PLA 2, phospholipase-A 2; PLOOH, phospholipid hydroperoxide; POV-PC, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidylcholine; PRDX6, Peroxiredoxin-6; PRR, pattern recognition receptor; PS, phosphotidylserine; PUFA, polyunsaturated fatty acid; PUFA-ePL, polyunsaturated ether phospholipids; ROS, reactive oxygen species; RSL3, RAS-selective lethal molecule; SLC7A11, Solute Carrier Family 7 Member 11; SOD1, superoxide dismutase 1; STING, stimulator of interferon genes; TFR1, transferrin receptor 1; VCAM-1, vascular cell adhesion protein 1; XCT, cystine/glutamate antitransporter.

genetically encoded process to remove damaged, aged, or nonessential cells, which allows maintaining an optimal and still-adapted tissue environment [1]. Consequently, the lack of proper regulation of cell death can either lead to the establishment of various pathologies, including cancer, auto-inflammation, autoimmunity, metabolic, and neurological disorders, but also organ dysfunction and failure in the frame of infectious diseases. Since the 1970s and the characterization of the first regulated cell death, apoptosis, the following decades have seen the discovery and characterization of numerous regulated cell deaths such as the regulated cell necrosis pyroptosis, necroptosis, NETosis, or ferroptosis with strong involvements in physiology and pathology including immunity, metabolism, or tumoral processes [1]. Hence, all this knowledge already allows designing novel therapeutic strategies that integrate cell death modalities in the frame of autoinflammatory, autoimmune, infectious, tumoral, or neurodegenerative diseases.

While the molecular and cellular mechanisms that drive apoptosis, pyroptosis, necroptosis, or NETosis are under extensive investigations and rely on a large body of fundamental and applied research, those that control ferroptosis have just 10 years of research and offer novel and large avenues of research [2]. Paradoxically, before being formally characterized in 2012 [2], ferroptosis was already studied since the 1960s, long before the discovery of pyroptosis, necroptosis, and NETosis [3]. However, the lack of genetic regulators and pharmacological modulators for a long time have impaired the deep study of this cell necrosis in various contexts. Since 2012, a very dense literature has studied the mechanisms regulating ferroptosis but also the importance of ferroptosis in neurological, cancer, and metabolic contexts [4]. However, the importance of ferroptosis upon infectious diseases has remained under-investigated [5]. Therefore, in this review, we specifically discuss the current knowledge that link lipid peroxidation, ferroptosis, and infectious diseases but also try to integrate it into the immune and host–microbial interaction contexts.

Ferroptosis induction and inhibition

Over the last 10 years, an important batch of studies has characterized various inducers of ferroptosis but also addressed various aspects of the molecular mechanisms that regulate ferroptosis [4]. Common to all contexts is the role of accumulated peroxidized phospholipids (PLOOHs) and more specifically peroxidized phosphatidylethanolamines (PE-OOHs) as being critical for ferroptosis execution [6–8]. Phospholipid peroxidation can occur in the presence of exogenous activators such as environmental or chemical pollutants, chemotherapy, or antibiotic exposure but also the frame of genetic or metabolic diseases [4,9–15]. This has allowed isolating iron-driven nonenzymatic lipid peroxidation through the Fenton reaction but also enzymes able to directly promote lipid peroxidation such as lipoxygenases (LOXs) and the cytochrome p450 oxidoreductase (CYPOR) [2,6,7,16–18]. In addition, deciphering the mechanisms of PL-OOH generation also led to the characterization and the discovery of PUFA synthesis pathways that start in peroxisomes as well as specific regulators of ferroptosis, such as the acyl-CoA synthetase long-chain family member 4 (ACSL4) [8], lysophosphatidylcholine acyltransferase 3 (LPCAT3) [19], and recently ACSL1 [12]. Similarly, various signalling pathways that directly or indirectly regulate lipid peroxidation were identified as critical modulators of ferroptosis, including the cysteine–glutathione (GSH)–GSH peroxidase 4 (GPX4) axis in the host cell cytosol–lipid interface, the dihydroorotate dehydrogenase pathway [20], the GPX4-independent regulator of lipid peroxides ferroptosis suppressor protein 1 (FSP1) [21,22], the cellular phospholipases A2 (PLA2) [23,24], the antioxidant transcriptional factor nuclear factor erythroid 2-related factor 2 (NRF2), or the autophagy machinery [25,26].

Lipid peroxidation process

Lipid peroxidation or oxidation (LPO) is the reaction that involves lipids containing carbon-carbon double bond(s), and more specifically PUFAs, with prooxidants such as free radicals present in a cell [27]. LPO involves hydrogen abstraction from a carbon of PUFA, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides as described previously [28]. Glycolipids, phospholipids (PLs), and cholesterol (Ch) are well-known targets of damaging and potentially lethal peroxidative modification. LPO in biologic systems could occur under enzymatic control, for example, via the activation of cyclooxygenases (COXs), LOXs, cytochrome p450s (CYPs), and additional oxidoreductase enzymes [cytochrome oxidoreductase CYPOR; nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, squalene oxidase] or nonenzymatically via the iron-catalyzed Fenton/Haber–Weiss reaction (Fig 1) [28].

Over the last four decades, many studies regarding LPO have shown its major role in cell biology and human pathology [29,30]. The peroxide lipids signalling mediators like prostaglandins, hydroperoxyeicosatetraenoic acid (H(p)ETE), lipoxins, leukotrienes

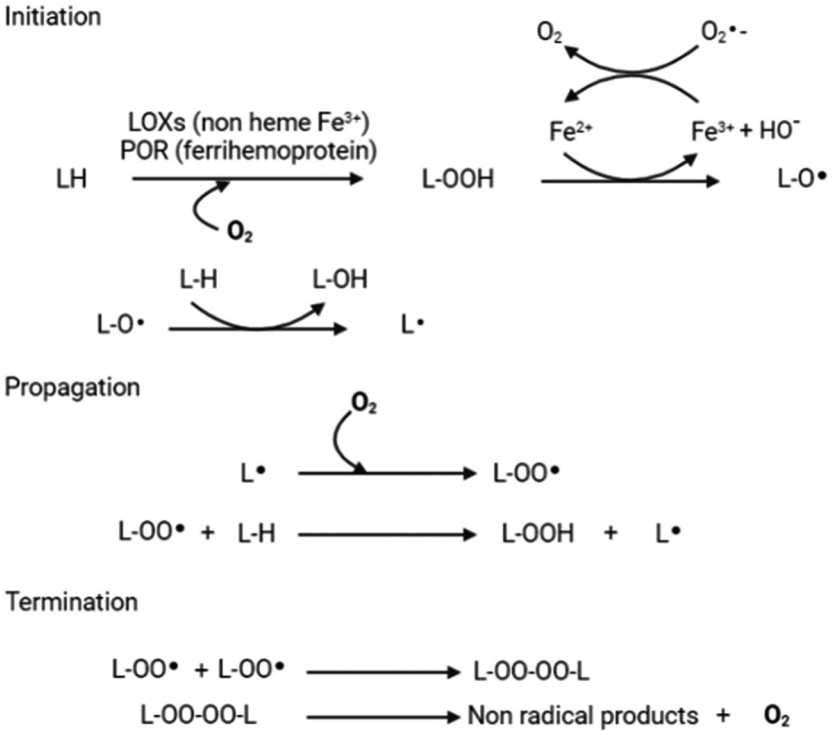

Fig. 1. Enzymatic and nonenzymatic processes that promote different steps of lipid peroxidation. Lipid peroxidation can be distributed in three phases, initiation, propagation, and termination. LOXs and/or CYPOR have been involved in initiating the process of lipid peroxidation by dioxygenation of lipids (LH). Then, thanks to Fenton reaction, lipid radicals (L•) are generated, which is the end of initiation process. In the propagation step, lipid radicals react with dioxygen (O_2), forming new lipid radicals (peroxyl radical, L-OO•). This step repeats until the termination step, where radicals reacted with another radical or antioxidants.

epoxyeicosatrienoic acids, leukotoxins and thromboxanes are really crucial in pathology [28,30–36]. Specifically, both prostaglandins and leukotriene play major roles in modulating inflammatory [33,37] and immune [38] processes such as but not restricted to, the recruitment of neutrophils [39].

Lipid peroxidation involves three steps: initiation, propagation, and termination (Fig. 1) [28,40]. The initiation step consists of the abstraction of the allylic hydrogen by prooxidants like hydroxyl radical result in carbon-centered lipid radical (L•). During the propagation part, lipid radical (L•) rapidly reacts with oxygen to form a lipid peroxy radical (LOO•), which abstracts hydrogen from another lipid molecule generating a new L• (that continues the chain reaction) and lipid hydroperoxide (LOOH). In the termination reaction, antioxidants like vitamin E donate a hydrogen atom to the LOO• species and form a corresponding vitamin E radical that reacts with another LOO• forming nonradical products (Fig. 1). Once lipid peroxidation is initiated, a propagation of chain reactions will take place until termination products are produced. There are two different products from lipid peroxidation. The primary products are lipid hydroperoxides, produced during the propagation step of lipid peroxidation [28,40]. The hydroperoxide group may be attached to various lipid structures, for example, free fatty acids, triacylglycerols, phospholipids, and sterols. Aldehydes are the secondary products of lipid peroxidation such as malondialdehyde (MDA), 4-Hydroxynonenal (4-HNE), propanal, hexanal. Among them, MDA and 4-HNE are the most studied and are well known to generate toxicity in cells [41].

In response to membrane lipid peroxidation, and according to specific cellular metabolic conditions and repair capacities [42], the cells may promote survival or induce cell death [43,44]. However, under medium or high lipid peroxidation rates the extent of oxidative damage exceed the cell repair capacity [4,11]. Hence, various signalling networks will set up cell death programs, including apoptosis or ferroptosis [44].

Phospholipid peroxidation and ferroptosis

Morphologically, ferroptosis presents smaller mitochondria, diminished mitochondria crista, and condensed

mitochondrial membrane densities [2]. The nuclear structure of ferroptotic tumor cells is intact without karyorrhexis and margination of chromatin. These features are essential for distinguishing ferroptosis from apoptosis, pyroptosis, autophagy, and necrosis [1].

Although inhibition of the activity/expression of the peroxidase GPX4 emerges as a central regulator of ferroptosis, a second pathway, independent of GPX4 also emerges (Fig. 2).

First, the direct or indirect GPX4 inactivation leads to an iron-dependent uncontrolled accumulation of peroxidized phospholipids (PL-OOHs) in membranes and ferroptosis induction. This pathway arises from a pharmacological screening of the Stockwell lab initiated in 2003 that identified novels compounds able to trigger cancer cell death in a nonapoptotic manner [45]. GPX4 is a selenoprotein that catalyses the reduction of PL-OOHs discovered in 1982 [46]. It uses reduced GSH as an electron donor, which allows the reduction of hydroperoxide-containing phospholipids and cholesterol into alcohols. Specifically, GSH provides from the amino acid transporter XC^--mediated cysteine entry into cells or by the transsulfuration pathway [47,48] involving cysteinyl–transfer RNA

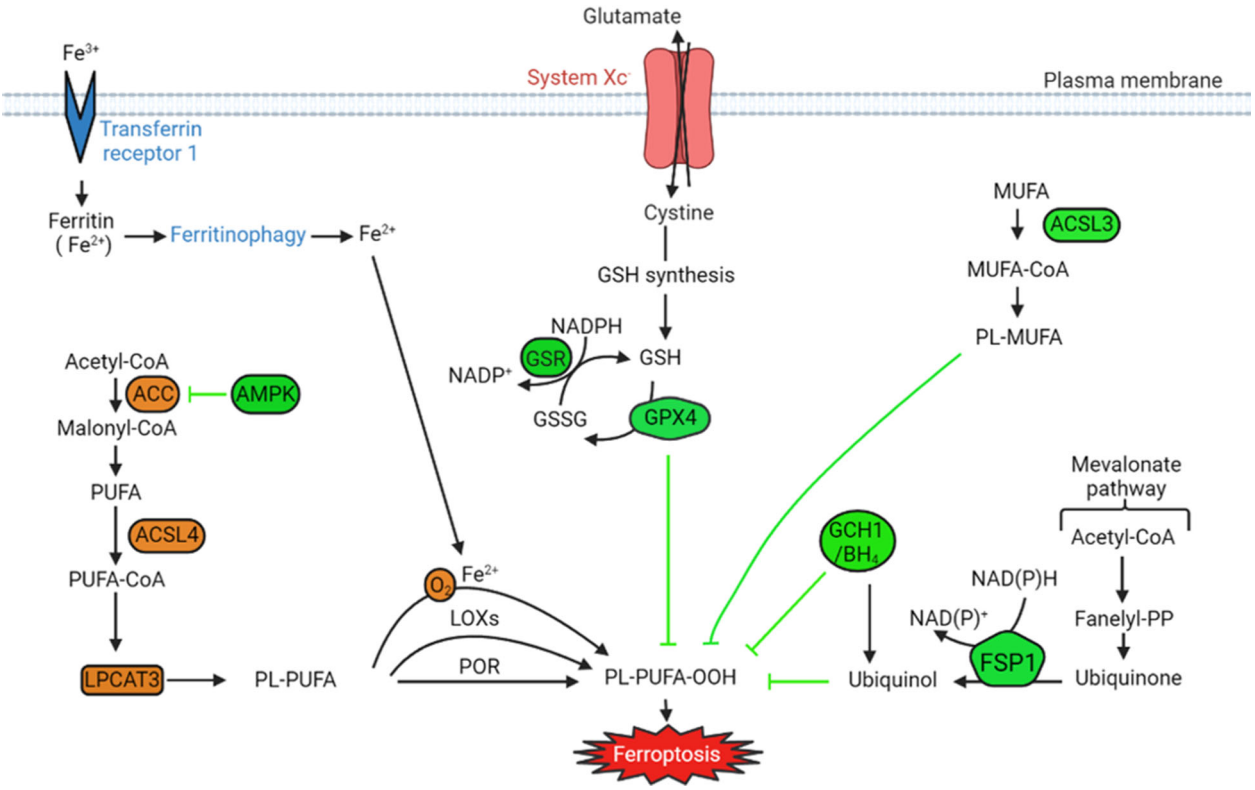

Fig. 2. Molecular mechanisms of ferroptosis. During ferroptosis, phospholipids with polyunsaturated acyl tails (PL-PUFAs) are peroxidized (bottom left) due to their intrinsic susceptibility to peroxidation. PL-PUFAs are generated by different enzymes such as ACSL4 and LPCATs (orange, bottom left) which incorporate free PUFAs into phospholipids. Acetyl CoA can be used for synthesize PUFA via acetyl CoA carboxylase (ACC; orange, bottom left). The 5′ AMPK can suppress ferroptosis by inhibiting ACC (green, bottom left). Once PL-PUFAs are produced, iron-dependent enzymes and labile iron use molecular oxygen (O_2) to make a peroxidation reaction, generating PL-PUFA-OOH (LOXs and CYPOR enzymes). Labile iron is imported through the TfR1 and stowed in ferritin (blue, top left). Ferritin can be degraded via an autophagy-like process, ferritinophagy, which releases labile iron and facilitates the lipid peroxidation driving ferroptosis (top left). Three main pathways for removing peroxidized PL-PUFAs (green, middle, and bottom right): the GPX4-GSH axis, the FSP1-CoQ10 axis, and the GCH1-BH4 or MUFA axis: The canonical ferroptosis controlling axis involves uptake of cystine through the cystine-glutamate antiporter (system xc−), GSH-, GSH biosynthesis, and GPX4-mediated reduction of phospholipid hydroperoxides (PL-PUFA-OOH). GSH-disulfide reductase (GSR) allows recycling of oxidized GSH (GSSG) using electrons provided by NADPH. Reduced coenzyme Q10, also known as ubiquinol, prevents lipid peroxidation and so ferroptosis. FSP1 (previously known as AIFM2; green, bottom right), regenerates ubiquinol from ubiquinone, which is produced through the mevalonate pathway (bottom right). The last ferroptosis-suppressive mechanisms consist of GCH1/BH4 pathway, which permits the production of reduced ubiquinol and the remodeling lipids to disfavor lipid peroxidation. Moreover, MUFAs, when incorporated into phospholipids via ACSL3, act through an unknown mechanism to suppress ferroptosis (green, top right).

synthetase 1 [49–52]. As consequences, direct genetic and pharmacological [RAS-selective lethal molecule (RSL3), ML1210, FIN56) inhibition/inactivation of GPX4 activity [53,54] or indirect targeting of GSH levels (erastin, sorafenib, sulfasalazine inhibited XC^- transporter) [2] strongly sensitives cells to ferroptosis and impairs mouse development [9,10,13,16,19,45,55]. Conditional *Gpx4* KO mice could show its importance at regulating lipid peroxidation-dependent cell necrosis [53,54]. The recent generation of mice expressing a mutated form of GPX4 where the amino acid selenocysteine (Sec) was replaced by a cysteine (Cys) unveiled that those mice were surprisingly viable until the weaning stage and then die of epileptic damages [54]. However, of importance, cells from those mice showed an amplified sensitivity to peroxide-induced cell ferroptosis, a process driven by the increased oxidation of the cysteines to oxidation-mediated GPX4 inactivation. This suggests that the selenocysteine amino acid might confer some protection to GPX4 against irreversible oxidation-driven inactivation of this enzyme.

Second, hyper-lipid peroxidation induced by various triggers (e.g., Tert-Butyl hydroperoxide TBOOH, Cumene hydroperoxide CuOOH) forces fast ferroptosis induction, a process that overwhelms the repair capacities of the cells, including those under the control of GPX4 [56]. The ability of various chemicals such as Tert-Butyl and cumene hydroperoxides to promote lipid peroxidation has been described for a long time. However, in 2008 Linden and colleagues started characterizing the molecular and cellular mechanisms by which those products trigger oxidative cell death [57]. Hence, studies found that TBOOH triggers ferroptosis in lipid peroxidation- and iron-dependent manner [56–58]. The authors of those studies also assessed the role of described regulators of oxidative cell deaths such as p53, poly(ADP)ribose polymerase-1 (PARP-1), the stress kinases p38 and c-Jun N-terminal kinases 1/2 (JNK1/2), or receptor-interacting serine/threonine protein kinase 1 (RIPK1) and 3 (RIPK3) and found none of them involved in TBOOH-induced cell death [56–58]. Although GPX4 inhibition can potentiate TBOOH-induced ferroptosis, such a process could also occur in presence of GPX4, hence suggesting that inhibiting GPX4 might not be the only way to trigger ferroptosis [58]. Curiously, another study also unveiled that the chemical lipid peroxidation inducer CuOOH triggers ferroptosis in corneal cells in a GPX4-independent manner [59], which suggests that CuOOH and TBOOH-induced ferroptosis might involve additional regulatory pathways independently of GPX4 inhibition. To this regard, recent studies found that the oxidoreductase CYPOR critically controls the generation of phospholipid peroxides, a process that we found to be central in CuOOH-induced phospholipid peroxidation and ferroptosis [17,18,60]. How those chemical compounds trigger CYPOR-dependent, yet partially GPX4 inhibition-independent ferroptosis will warrant further investigations.

Common to both pathways is the requirement of iron. Multiple sources of iron have been described so far [61]. For instance, the exogenous sources of iron can be provided by the dietary. Specifically, heme is obtained from meat sources and the consumption of grains and vegetables provide nonheme iron [61]. These irons fund the cellular iron stocking. Another source of cellular iron is the recycling of senescent red blood cells (RBCs), a process where senescent RBCs are phagocytosed and degraded. The heme is broken down into biliverdin and Fe^{2+} by heme oxygenase 1 (HO-1) [62]. Fe^{2+} can be stored in endosome or exported from the macrophage by ferroportin1 (Fpn1). Once exported, Fe^{2+} is oxidized by ceruloplasmin (Cp) and binds to transferrin receptor 1 (TFR1), where it may be delivered to target cells [61]. Hence, TFR1 imports Fe^{3+} then it is stored in the endosome where Fe^{3+} is converted to Fe^{2+} [63–65]. Free Fe^{2+} can also circulate as non-transferrin-bound iron (NTBI) when iron levels exceed the binding capacity of the available TFR1 [61]. The NTBI appears to contribute to cellular iron loading. Regarding this, a study suggests that the SLC39A zinc transporter, Zip14, may be involved in the uptake of NTBI [66]. Later, Fe^{2+} could be released from the endosome into a labile iron pool (LIP) in the cytoplasm through divalent metal transporter 1 (DMT1). This free Fe^{2+} can react with H_2O_2 as described above (Fig. 1) and generate reactive oxygen species [ROS; hydroxyl radicals (•OH) and superoxide ($•O^{2-}$)]. These ROS could peroxide lipids to produce oxidized lipids. Hence, silencing of iron-responsive element binding protein 2, a master transcription factor of iron metabolism, significantly limits erastin-induced ferroptosis [2,65]. This illustrates the important function of iron in ferroptosis process. However, the precise role of iron in ferroptosis remains largely unknown.

Common to all those activators is the terminal involvement of peroxidized phospholipids, and more specifically phosphatidylethanolamine (PE-OOHs). As described earlier, phospholipids are hydrocarbons that constitute the essential elements of membranes and are extremely prone to lipid peroxidation events. Such process mostly depends on their degree of unsaturation and more specifically on their PUFA chains. Upon ferroptosis, PE-containing PUFAs are peroxidized, a process that is thought to promote plasma membrane

destabilization and rupture through the generation of PE-containing hydroperoxides (15-HpETE-PE) [7,8,67]. As arachidonic acid (aa) incorporation into phospholipids requires several enzymes, both the acyl-coenzyme A [CoA] synthetase long-chain family member 4 (ASCL4) [8] and the LPCAT3 [19] enzymes have been found to play a major role in aa integration into phospholipids. Consequently, cells deficient for ACSL4 expression show a strong resistance to ferroptosis induction. To the contrary, monounsaturated fatty acids (MUFAs), which are less prone than PUFA to oxidation, displace PUFAs from phospholipids, including Pes, hence conferring some protection against ferroptosis induction. Recently, peroxisomes were characterized as a major synthesis factory of polyunsaturated ether phospholipids [68,69], hence providing substrates for lipid peroxidation and subsequent ferroptosis induction.

Although it appears more and more clearly that phospholipid peroxidation is a key driver of ferroptosis, a strongly debated question lies on how lipid peroxidation is initiated and persists during ferroptosis. To this regard, iron-driven nonenzymatic peroxidation of PUFA in phospholipids appears as a strong component of lipid peroxidation initiation and amplification [2,4,9,11]. However, enzymatic systems can also promote phospholipid peroxidation, including LOXs [6,7] and the cytochrome oxidoreductase p450 (CYPOR) [17,70]. Both enzymes require iron to perform lipid peroxidation and previous studies associated either LOXs or CYPOR to PUFA-containing PE peroxidation and the subsequent ferroptosis induction. Indeed, some LOXs, including LOX15-1 in Humans can directly oxidize phospholipids upon certain circumstances, such as when complexed with the PE-binding protein-1 [6]. However, genetic removal of 12/15 lox in GPX4-deficient mice failed to prevent ferroptosis [53]. In addition, the use of various LOX inhibitors showed that those compounds exhibited strong Radical Trapping activities independently of their LOX inhibitory action [71]. To the contrary, pan lox genes silencing in human cells inhibited erastin-induced ferroptosis but not RSL3 (GPX4 inhibitor)-induced ferroptosis, suggesting that LOXs involvement in ferroptosis might be context and cell type specific [67]. Recently, CYPOR enzyme has also been described as triggering phospholipid peroxidation, through a yet to describe mechanism in various cell types [17,70].

Finally, a recent study unveiled that the neutrophil myeloperoxidase (MPO) was also able to promote ferroptosis in neighboring cells when secreted, a process involving lipid peroxidation-induced ferroptosis. Specifically, neutrophils recruited to the tumor site released MPO-containing granules in the extracellular environment. Such process allowed MPO catalyzing iron-dependent phospholipid peroxidation and subsequent ferroptosis induction in neighboring hypoxic tumor cells, a process that exhibited a strong antitumor function [72].

Yet, at this step, the respective importance of each described enzyme in ferroptosis remains strongly debated and further studies will help to clarify their function in lipid peroxidation-induced ferroptosis. Depending on the context and the cell type targeted, it is probable that various enzymatic and nonenzymatic processes requiring iron might be involved at promoting phospholipid peroxidation-dependent ferroptosis. Another yet unanswered question also lies on determining the identity and location of specific cellular membranes/organelles that drive ferroptosis. So far, an integrated network of multiple membranes and organelles have been characterized for their functions in ferroptosis including the plasma membrane, the mitochondria, the peroxisomes, and the endoplasmic reticulum. How those compartments cooperate in ferroptosis regulation has not yet been defined but its characterization will help to understand the molecular and cellular hierarchical events that govern ferroptosis induction in cells.

Additional processes of peroxidized lipid removal/detoxification

Beyond the central and crucial function of GPX4 at removing peroxidized phospholipids, various enzymes and signaling pathways have been described for their role at helping detoxifying peroxidized lipids from cells.

FSP1 was initially named AIFM2 for 'Apoptosis-Inducing Factor Mitochondria Associated 2' and has been characterized for its role in lipid peroxidation regulation in the frame of a CRISPR screen targeting antiferroptotic effectors [21,22]. The authors initially observed that several cell lines lacking GPX4 still showed marked resistance to ferroptosis induction, despite the loss of GPX4. The identification of FSP1 oxidoreductase as a GPX4-independent regulator of phospholipid peroxidation allowed to show its role in promoting the generation of ubiquinone (coenzyme Q10) in presence of the NAD(P)Hs system in nonmitochondrial membranes. As consequence, reduced Ubiquinone (coQ10) forms ubiquinol a strong lipid peroxidation trapping agent, hence limiting pathological phospholipid peroxidation. However, in cells expressing competent GPX4, FSP1 inhibition alone does not induce or sensitive cells to ferroptosis induction as

GPX4 inhibition or removal does, which suggests that the FSP1 pathway appears secondary to GPX4. Yet, several pathologies, including cancer or neurodegenerative diseases, might actually benefit from FSP1 activation or inhibition (Fig. 2).

PLA2 enzymes cleave aa in phospholipids at the sn2 position to generate free aa that will then be transformed into various lipid mediators through its direct oxidation (isoprostanes) or via the direct action of COXs, LOXs and Cyp450 enzymes (eicosanoids) [73]. Intriguingly, deficiency or pharmacological inhibition of various PLA2s [74,75], including the Ca2+-independent iPLA2β (iPLA2β) or the Peroxiredoxin-6 (PRDX6), leads to both accumulation peroxidized phospholipids and to the increased susceptibility of cells to oxidant agent-driven ferroptosis [23,24,76]. Hence, recent studies showed that iPLA2β directly cleaves hydroperoxides in membranes [23], hence lowering the sensitivity of cells to oxidant (ferroptosis)-driven damages. To the contrary, loss-of-function mutations in various patients link iPLA2β to exacerbated oxidative stress and neurological pathologies [75,77,78]. Similarly, deficiency or inhibition of the phospholipase activity of PRDX6 leads to the deleterious accumulation of PL-OOHs upon GPX4 inhibition (RSL3, Erastin), which exacerbates ferroptosis induction [76]. Alternatively, a broad set of signalling pathways have also been involved in regulating lipid peroxidation, including but not restricted to, cell density-induced E-Cadherin pathway, hypoxia or glucose-sensitive adenosine monophosphate-activated protein kinase (AMPK) and amino acid-sensitive mTOR pathways [79–85].

Indirectly, expression and activation of the GPX4 and XC^- systems is regulated at various levels, including BECLIN-1/Autophagy pathway [26,85,86], ubiquitin aldehyde binding 1-regulated protein degradation through the proteasome [87], Nrf2-, tumor protein p53- or breast cancer type 1-associated protein 1-mediated transcriptional regulation of XC^-/antioxidant components [e.g., Solute Carrier Family 7 Member 11 (SLC7A11), superoxide dismutase 1, HO-1] [84,85,88].

Ferroptosis repair mechanism and execution

Despite a large body of information on novels ferroptosis inducers and on the mechanisms that govern phospholipid peroxidation, little is known about specific effectors, which can act downstream of phospholipid peroxidation to execute lytic cell death. Linking ferroptosis to other lytic forms of cell death it has been found that the endosomal sorting complexes required for transport machinery could repair plasma membrane damages induced upon ferroptosis, hence protecting cells against cell lysis [42]. Regarding the active execution of ferroptosis, MDA, and 4-HNE peroxidized by-products were suggested to exert strong toxicity on cells [41], but so far, no direct evidence could link them to cell lysis execution upon ferroptosis. Similarly, CYPOR-mediated lipid peroxidation-dependent membrane damages in liposomes have been suggested to be executioner of ferroptosis, yet further research will be required to see if this applies in various ferroptotic contexts and how peroxidized lipids directly destabilize membranes [70]. Finally, the recent discovery of the cell shrinkage executioner nerve injury-induced protein 1 (NINJ1) in the context of pyroptosis rises the role of NINJ1 at promoting cell lysis downstream of ferroptosis induction in various cell types [89].

Finally, an intriguing feature of ferroptosis is its ability under certain circumstances to spread a ferroptotic signal to neighboring cells following a ‘wave’ model [90]. This process involves in ferroptotic cells exposed to erastin the transfer of ferroptosis activating signals (e.g., peroxidized lipids) through a so-called ‘swelling effect’ to other cells before complete cell lysis, which suggests that an active mechanism might regulate it [90]. In such model, the authors used osmoprotectant (i.e., PEG1540) to inhibit cell rupture and observed that the spread of ferroptosis from one cell to another did not require cell rupture. Relying on the fact that calcium waves have already been described [91], the authors also used the calcium fluorescent reporter GCaMP6 and found that Calcium waves between ferroptotic cells and nonferroptotic cells also occurred before cell lysis. Whether and how in tissues such process might occurs remains yet to be investigated.

Ferroptosis and innate immune activation

Many regulated cell necrosis, including pyroptosis, necroptosis, or NETosis, drive the processing and the release of numerous intracellular components, the alarmins, and the damage-associated molecular patterns (DAMPs) [92,93]. Those DAMPs will then be recognized either directly or indirectly by various pattern recognition receptors (PRRs), which will contribute to and sustain the inflammatory reaction [94]. Regarding the types of DAMPs generated and released upon ferroptosis but also their importance at driving inflammatory processes, some studies could already identify several various factors and their associated molecular pathways.

Protein DAMPs detected upon ferroptosis induction

HMGB1 is a nuclear factor of the High Mobility Group (HMG) family proteins that contributes to chromatin architecture and gene expression in addition to Histones. Upon various regulated cell necrosis, HMGB1 is released and perform various inflammatory processes through its direct or indirect interactions with different membrane bound PRRs, including TLRs or the advanced glycosylation end products (AGER) receptor [95]. In addition, due to its strong affinity for LPS, HMGB1 has been recently found to be one of the most important contributors of endotoxemia-induced sepsis by promoting LPS access and detection in host cell cytosol by the pro-inflammatory caspase-11 [96]. In the context of ferroptosis, it has been demonstrated that AGER, one PRR that detects HMGB1 plays a robust role at driving ferroptosis-dependent inflammation, notably by promoting TNFα production [95]. Similarly, another study could link ferroptosis-released exosome-containing mutated KRAS12D as a signal for AGER-mediated uptake and immune polarization [95].

Various other alarmins and DAMPs, including IL1α, S100, IL16, IL36, and Galectins 1 and 3 are susceptible to be released upon ferroptosis, yet their respective function in such context has not yet been studied [92,93].

Peroxidized phospholipids/oxidized lipids as ferroptotic DAMPs

As an oxidative cell death, ferroptosis associates to the production/generation of large amount of peroxidized lipids and theirs products such as 4-HNE or MDA [32,41,43,44,86]. Upon oxidant injury, various cellular phospholipases will cleave oxidized and nonoxidized aa from phospholipids [73,75]. This generates one hand large amount of substrate for the COX, LOX, and Cyp450 enzymes that will then metabolize aa into various eicosanoid species, including the proinflammatory leukotrienes, prostaglandins, or the anti-inflammatory lipoxins [32,36,39,43]. In addition, direct arachidonic peroxidation into phospholipids also drives the generation of isoprostanes lipids, extremely potent inflammatory lipids that are released from membranes under the action of phospholipases [97]. Specifically, peroxidized phosphatidylcholine generated upon oxidative attack holds the capacity to promote platelet activation and aggregation, neutrophil, monocyte, and endothelial cell responses in a similar pathway than the platelet-activating factor [98–100]. The oxidized (ox)PAPC lipid carries numerous biological and inflammatory functions that go from TLR4 antagonism to inflammasome activation, hence sustaining or inhibiting the inflammatory process according to the context oxidized phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine is produced [101]. Regarding this, a recent comparison of phospholipid peroxidation upon various cell deaths, including ferroptosis, Caspase-1-, and Caspase-11-driven pyroptosis or apoptosis showed that Caspase-11 can also promote through a yet to determine mechanism phospholipid peroxidation, although ferroptosis does not contribute to pyroptosis [102]. This suggests that phospholipid peroxidation might generate novel lipid DAMPs able to trigger additional signalling pathways upon lipid peroxidation-independent cell deaths. For instance, upon apoptosis, phosphatidylserine peroxidation does not generate DAMPs but promotes phosphotidylserine externalization, essential for efficient efferocytosis of dead cells [103].

Lipid peroxidation- and Ferroptosis-modulated STING pathway

Upon oxidative stress, DNA is also a strong target of modifications. In this context, a recent study has recently identified the ability of ferroptotic cells to inhibit the stimulator of interferon genes (STING)-driven type I Interferon pathway through the action of 4-HNE-mediated STING carbonylation [104]. Of note, pyroptosis and apoptosis also involve caspases that through their proteolytic activities also inactivate the production of Interferon [105–108]. For instance, apoptotic Caspase-3 and pyroptotic Caspase-1 directly cleave the DsDNA sensor cyclic GMP–AMP synthase, hence limiting the generation of the sting ligand 2′3′ cyclic GMP–AMP [107,108]. In addition, gasdermin D activation has also be linked to the limitation of type I IFN upon pyroptosis [105]. Therefore, it is quite intriguing that most of the regulated cell death pathways all converge to a limitation of type I IFN production pathways, albeit through different mechanisms.

Finally, another study from Dai *et al.* [109] described that upon ferroptosis of cancer cells, the production and release of the oxidized nucleic acid 8-hydroxyguanosine drives a STING-dependent DNA sensory pathway in surrounding cells, hence promoting type I IFN production.

This suggests that ferroptosis is not a silent form of cell death at the level of DAMP release. If ferroptosis also behaves as an immunogenic cell death [110], by shaping the adaptive immunity will warrant some investigations. In addition, the generation of various (oxidized) lipids also raises the question of

self-lipid presentation by the nonclassical MHC CD1 and their putative importance in the frame of infectious, pathological, or autoimmune contexts [111,112]. Finally, next studies combining redox lipidomic, cell biology, biochemistry, and immunology will probably enrich ferroptosis DAMP catalogue with novel and specific oxidized lipids or peroxidized phospholipids, which will allow specifically determining the inflammatory and immunological signature of ferroptosis [113].

Ferroptosis in disease

Lipid peroxidation, which drives cell death, has been demonstrated in multiple normal and cancerous mammalian cells and different tissues, where it has been implicated in the pathogenesis of chronic and acute diseases such as ischemia-reperfusion of the liver [114], acute kidney injury [16,25], diabetes [25,115–117], iron overload [118], and neurodegenerative diseases (e.g., Huntington's, Alzheimer's, and Parkinson's diseases; Fig. 3) [119]. Ferroptosis has been also documented in plants [120]. Here, we voluntary focus on infectious and immune-related diseases and ferroptosis as being less addressed and partly discuss the dense literature of ferroptosis in cancer and neurodegenerative diseases but include very complete reviews on these themes.

Inflammatory diseases

Atherosclerosis is a chronic inflammatory disease, which is characterized by specific invasion of monocytes and T cells into the vascular wall, while neutrophils are not present in atherosclerotic lesions. Some oxidized phospholipids trigger monocytes adhesion to endothelial cells such as oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylethanolamine and oxidized 1-palmitoyl-2-arachidonoylsn-glycero-3-phosphoserine [115]. Monocyte adhesion to endothelial cells after stimulation with these oxidized lipids involves the activation of an$\alpha 5\beta 1$ integrin and increased deposition of connecting segment-1 (CS-1)-containing fibronectin on the cell surface [115].

Cystic fibrosis (CF) is a genetic disorder in Caucasian people. It is due to the mutation of CF transmembrane conductance regulator (*CFTR*) gene located on the long arm of the chromosome 7, which encodes for CFTR protein [121]. The latter, an adenosine triphosphate binding cassette, is a transmembrane chloride channel that is also involved in GSH transport. As GSH/GSH disulfide constitutes the most important pool of cellular redox systems, CFTR defects could thus disrupt the intracellular redox balance. During the last decade, many studies have shown a higher lipid peroxidation and lower antioxidant status in CF patient compare to the heath control

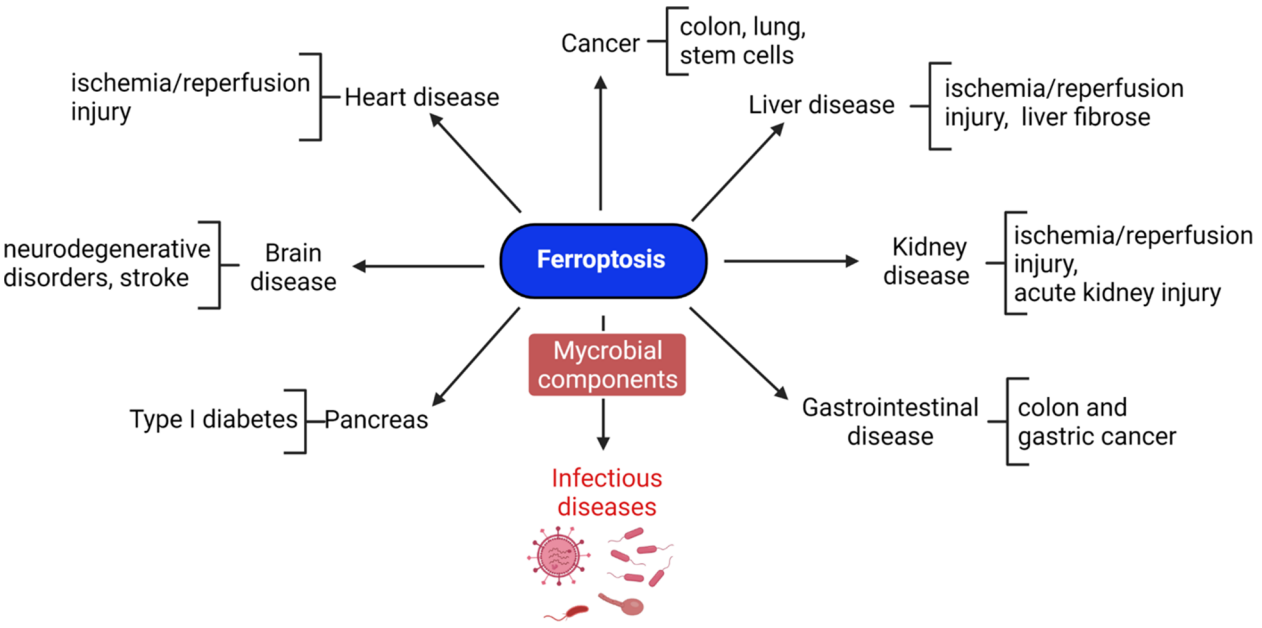

Fig. 3. Ferroptosis in disease. Ferroptosis involvement in multiple diseases can be highlighted in cancer (lung, stem cells, colon), brain diseases (stroke, neurodegenerative disorders), heart diseases (ischemia/reperfusion injury), liver diseases (fibrose, ischemia/reperfusion injury), gastrointestinal diseases (colon and gastric cancer), kidney diseases (ischemia/reperfusion injury, acute kidney injury), pancreatic diseases (type I diabetes), and in infectious diseases (bacteria, viruses, parasites, fungi).

[34,122,123]. CF patients present a significantly increase level of the MDA, autoantibodies against oxidized low-density lipoproteins [34,122,123]. The level of antioxidant such as alpha-tocopherol and beta-carotene is lower in CF patients than in controls [34,122,123]. These studies indicate that both lipid peroxidation biomarkers and antioxidant status were disturbed in CF patients, despite medical assistance. This perturbation or imbalance of oxidative stress in CF patients may contribute to their susceptibility to lung infections.

Cancer

The recent research on cancer has shown a clear involvement of oxidative stress level in cancer development or therapies. It is well known that lipid metabolisms play an important role in tumorigenesis, invasion, and metastasis [2,9,10,20,45,55,72,83,85,124,125]. For example, high saturation levels of membrane lipids protect cancer cells from damage induced by reactive oxygen, since saturated membrane lipids are less sensitive to peroxidation. More important, peroxide lipids could trigger signals for cell proliferation invasion and migration, which are important for cancer establishment. Knowing that the modulation redox imbalance can triggers cell death. Some studies shown that the induction of lipid peroxidation led to cell necrosis and cancer size reduce significantly. Ferroptosis, which is a lipid peroxidation-induced cell death, has been suggested to have potential as an anticancer therapeutic strategy. Unlike apoptosis, which many cancer cells can evade, ferroptosis is lethal to many tumor cells that have become dependent on the suppression of ferroptosis for their survival, including some of the most drug-resistant and aggressive cancer cells, such as persisted cells, and cells that have undergone epithelial-mesenchymal transition [55,125]. Thus, triggering ferroptosis may open new therapeutic avenues for treating drug-resistant cancers.

Ischemia reperfusion injury

Ischemia reperfusion injury (IRI) is an inflammatory context induced by the consequences of a lack of blood supply to organs followed by a re-funding of blood and oxygen. Various organs can be affected by such process, including liver, heart, gut, kidney, or brain. Recent reports have shown that both necroptosis and ferroptosis could play a major deleterious and inflammatory role in such contexts. In this context, the use of ACSL4 or lipid peroxidation inhibitors significantly improves protection against IRI in rodent models [65,113,114,126]. Further studies using genetically invalidated targets will then help determining the respective contribution of necroptosis and ferroptosis in IRI but also be helpful to translate it into clinical studies.

Ferroptosis in infectious diseases

Reactive oxygen species production is rapidly increased during infection, which facilitates pathogen clearance as well as contributes to signaling cascades related to cell proliferation, inflammation, and immune responses [127]. The best-characterized sources of ROS during infection are the membrane associated NADPH oxidase complex and the mitochondrial electron transport chain (Mt-ETC) [128]. In this context, few studies or reports addressed the importance of ferroptosis upon microbial infections, yet there is various indirect evidence that link lipid peroxidation, iron and we speculate, ferroptosis, to host–pathogen interactions.

Parasite infections

The first study aiming at determining the importance of ferroptosis in the frame of infections has been performed by the lab of Manfred Kopf. They showed that proper regulation of lipid peroxidation in T lymphocytes is essential to set up an efficient adaptive immune response against the Choriomeningitis virus and the parasite *Leishmania major* infections [129]. Specifically, GPX4 removal in T-cell compartment triggered ferroptosis of TCD8 cells, which prevented the immune system activation.

Another study studied the links between ferroptosis and the parasite *Plasmodium falciparum*, the causative agent of malaria and the first transmitted to mammalian hosts by infected *Anopheles* mosquitos. After transmission, *P. falciparum* first infects hepatocyte to form a liver stage parasite. Once the liver stage is completed, the parasite enters in the bloodstream and infect erythrocytes then initiate symptomatic malaria. Heather S. K and collaborators found that inhibition of GPX4 or SLC7a11 dramatically reduces *Plasmodium* liver stage infection [130,131]. Conversely, knockdown of the ferroptosis inducers NOX1 or TFR1 or the use of lipophilic antioxidants lead to an increase in livers stage infection. Thus, induction of lipid peroxidation via SLC7a11-GPX4 pathway serves to eliminate the liver stage parasites during *Plasmodium* infection. Furthermore, in cattle *Theileria annulata* triggers an oxidative stress followed by lipid peroxidation in erythrocytes, and it seems that this might be the cause of increased erythrocyte fragility due to membrane lysis [132].

Viral infections

During viral infections, there is also a modulation of infected-cell lipid peroxidation. For example, it has been reported that serum lipid peroxidation products are increased in patients with liver disease such as chronic hepatitis C patient [133]. Related to this, several studies found that HCV triggers ROS production then production of lipid peroxidation products such as MDA [134–136]. In this context, lipid peroxidation has been suggested in addition to iron to promote pathological hepatitis infection with detrimental liver damages [134–136].

The human immunodeficiency virus (HIV) also associates with oxidative stress and the reduction of GSH contents in plasma and lung epithelial lining fluid [137]. The decrease in GSH is also associated with an increase in the concentration of lipid peroxidation products (MDA) in serum of HIV seropositive children [138–140]. Oxidative stress can stimulate viral replication via the activation of inflammatory pathways, including the nuclear factor kappa B (NF-κB) in T lymphocytes, thus favoring the progression of the disease [141]. In this context, *N*-acetylcysteine (NAC), which provides cysteine, essential to generate GSH, inhibits HIV replication in infected T cells, chronically infected monocytic cells and in normal PMBCs infected *in vitro* [142]. GSH and GSH esters also block cytokine-stimulated HIV transcription [142]. The mechanism of GSH (thiol) regulation of HIV activation could mainly be explained by the influence of thiols on NF-κB transcription factor. NF-κB is a nuclear factor that binds to the enhancer of the κ light chain17 and greatly increases HIV transcription and replication [143].In addition, low GSH levels were also associated with poor survival of HIV-infected patients [144]. Regarding this, it has also been demonstrated that some antioxidant substances might inhibit the induction of lipid peroxidation-driven ferroptosis *in vitro* [145]. These substances have been proposed as adjuvant therapy in HIV infection [146]. As previously described, *Gpx4*$^{-/-}$ mice exhibit T cell populations unable to mount a proper immune response during acute lymphocytic choriomeningitis virus infection [129]. Indeed, CD8(+) and CD4(+) T cells lacking *Gpx4* failed to protect against choriomeningitis virus infection; however, the diet supplementation with high dosage of vitamin E rescued this phenotype. Hence, the lack of *Gpx4*$^{-/-}$ T lymphocytes efficient response seem to parallel to a certain extend the observations regarding HIV-infected T cells that exhibit low GSH levels.

Another layer of consequences of low GSH levels in HIV-infected T lymphocytes is their inability to respond properly to subsequent infection with the bacterial pathogen *Mycobacterium tuberculosis* (*M. tuberculosis*). In this context, T cells with low GSH show lower capacity to produce Interferon-gamma and IL-12 cytokines, hence favoring *M. tuberculosis* proliferation in infected myeloid cells. Inline, preclinical studies using liposome-encapsulated NAC in HIV-associated patients showed improved cytokine production [147,148]. Related to this, further studies that NAC-replenished GSH levels helped T-cell-driven cytokine production as well as macrophage-driven clearance of *M. tuberculosis* in HIV/*Mtb* co-infected cells [148–150]. Finally, recent randomized phase two clinical trials unveiled that NAC treatment also improved the redox status of HIV-associated tuberculosis patients, characterized by a decrease in production of in lipid peroxide products such as MDA [151,152]. Altogether, those compelling studies suggest that lipid peroxidation and theoretically, ferroptosis, contribute to HIV-derived pathogenesis, including tuberculosis development.

In general, viral infection lead to ROS production and depletions of antioxidants [153]. It is also well known the generation of ROS in RNA viruses' infection [153,154] in which belong coronavirus, including the coronavirus 2 (Cov2), responsible of the Severe acute respiratory syndrome (Sars) [155–157]. One source of this ROS could be a 'cytokine storm' with release of IL-2, IL-6, IL-7, and TNF α, which has been described in coronavirus disease 2019 (COVID-19) [158]. The cytokine storm has been described to be accompanied by hyperferritinemia, which is known to generate via the Fenton reaction, the production of ROS [153]. In addition, a study found that Sars-Cov2 infection decreases the gene expression of various anti-ferroptotic enzymes, including those coding for the expression of the GPX4 protein [156].

Therefore, we can speculate that Sars-Cov2 infection may be followed by an increase in iron availability, ROS production and so lipid peroxidation. We can also postulate that this lipid peroxidation triggers ferroptosis, which can participate in various pathological states of the COVID-19, including those driving cytokine storm and he multiorgan failures. Beyond speculations, current and future studies will help at deciphering the putative role of ferroptosis in the development of COVID-19.

Fungal infections

Studies in plant–pathogen interaction recently observed that the fungus M. Oryzae drives ferroptosis in rice, a process druggable by iron chelators and lipid

peroxidation inhibitors [120,159]. Accordingly, inhibition of lipid peroxidation enhances rice protection against M. Oryzae, suggesting that ferroptosis might play a deleterious function in this context.

One of the most common fungal infections in the central nervous system is Cryptococcal neoformans meningitis (CM), also a leading cause of mortality among HIV-infected patients [160]. Some studies have observed rapid iron accumulation and lipid peroxidation within the brain, all of which are hallmarks of ferroptosis [161,162]. In this context, one can speculate that ferroptosis could be involved in the pathogenesis induced by Cryptococcal neoformans meningitis. Regarding this, additional studies found that the infection of alveolar macrophages by Cryptococcal neoformans meningitis, *Candida albicans* or *Aspergillus fumigatus* leads to the induction of lipid peroxidation and detachment of these cells [163]. Lipid peroxidation could be counteracted by vitamin E. Those studies bring new interesting leads regarding the putative importance of lipid peroxidation-driven ferroptosis in fungal infections.

Bacterial infections

In 2000, Azenabor and Mahony demonstrated that monocytes, Sup-T1 cells, and Hep-2 cells infected with Chlamydia trachomatis results in the formation of membrane hydroperoxide lipids [164]. Additionally, this lipid peroxidation stimulated the induction of cell death.

Recently, *M. tuberculosis* (*Mtb*), the agent of tuberculosis, has been characterized as a trigger of pathological ferroptosis in macrophages [165]. Specifically, Amaral *et al.* demonstrated that the lipophilic antioxidant ferrostatin-1 and iron chelators significantly inhibited *Mtb*-induced macrophage necrosis. Consequently, inhibiting lipid peroxidation protected lung mice against *Mtb*-induced damages and favored its elimination. In a follow-up study, the authors also described that the transcription factor BACH-1 inhibited GPX4 expression in macrophages, hence favoring *Mtb*-induced detrimental ferroptosis in macrophages [166,167]. If a specific virulence factor drives GPX4 degradation still remains to be explored. However, the mechanisms involving *Mtb*-induced macrophage necrosis remain deeply discussed as additional cell deaths, including necroptosis [168–170], NLRP3 inflammasome-driven pyroptosis [171] but also an Interferon-inducible process [172], yet ferroptosis-independent mechanism, have also been described as a way by which *Mtb* triggers macrophage necrosis.

The most intriguing reports on ferroptosis and infectious diseases come from *Pseudomonas aeruginosa* (*P. aeruginosa*) studies. *Pseudomonas aeruginosa* is an opportunistic pathogen that thrives in the lungs of immune-compromised patients (e.g., CF, nosocomial infections). The group of V. E. Kagan relied on two key observations: First, airways of CF patients carrying *P. aeruginosa* infection are extremely enriched with peroxidized phospholipid-containing aa, a hallmark of ferroptosis, and second, *P. aeruginosa* secretes a virulence factor carrying a 15 lipoxygenase-like activity, lipoxygenase A (LoxA) [173]. Therefore, the authors showed that secretion of LoxA drives phospholipid peroxidation, which sensitives bronchial epithelial cells to ferroptosis upon *P. aeruginosa* infection [174]. Such process was blocked upon lipid peroxidation inhibitor use such as ferrostatin-1, suggesting that ferroptosis might be exploited as a virulence mechanism by *P. aeruginosa*. *Pseudomonas aeruginosa* also expresses and secretes an elastase-like protease, namely *P. aeruginosa* elastase. Among various targets, this protease can cleave transferrin-bound to iron in order to promote bacterial iron uptake [175]. In this process, released iron drives the generation of peroxidized phospholipids on neighboring cells through the Haber–Weiss reaction. Should this promotes also ferroptosis in various infectious contexts will warrant further investigations.

Related to this, we recently found that another strain of *P. aeruginosa*, expressing the lytic phospholipase toxin ExoU [176] exploits host endogenous phospholipid peroxidation to trigger cell necrosis and pathology [60]. Nevertheless, such mechanism does not rely on the induction of ferroptosis as we observe a decrease of phospholipid peroxidation upon ExoU exposure. However, it appears that ExoU phospholipase activity is exacerbated by the presence of peroxidized phospholipids, which drives an exacerbated necrosis. In this context, targeting ferroptosis pathways that drive lipid peroxidation (e.g., CYPOR enzyme) or directly lipid peroxidation (ferrostatin-1, α-tocopherol) diminishes the sensitivity of cells to ExoU-induced cell necrosis and pathology [60]. In the same study, we also showed that other microbial phospholipases could use a similar process to kill host target cells, including *Burkholderia thailandensis* or *Pseudomonas fluorescence* exoU-like toxins. Beyond bacterial phospholipases, venoms from snakes, scorpions, spiders, or animals are enriched with various toxic phospholipases but also with an L-amino acid oxidase, able to generate H_2O_2-driven lipid peroxidation in a similar manner than CYPOR enzyme. Therefore, it is tempting to speculate that venoms toxicity might somewhere intersect with ferroptosis pathways [177].

Therefore, it is likely that numerous pathogens and organisms might have developed various strategies to exploit or modulate ferroptosis pathways to their own benefit.

Ferroptosis in Infectious sepsis

Sepsis refers to an overwhelming inflammatory reaction that provokes extremely deleterious organ damages are associates with high mortality rates. In a study on polymicrobial sepsis, the conditional deletion of GPX4 in the myeloid compartment exacerbated pathological inflammation induced by the LPS sensor Caspase-11 [178]. In such process, the authors found that caspase-11-induced pyroptosis relied on GPX4 expression. Specifically, the authors found that lipid peroxidation helped the active pro-pyroptotic fragment of gasdermin D (GSDMD) to trigger pyroptosis. Conversely, the use of vitamin E, a lipophilic lipid peroxidation inhibitor inhibited GSDMD-induced pyroptosis in macrophages. Hence, mice lacking Gpx4 expression in the myeloid compartment exhibited extreme sensitivity to polymicrobial sepsis, a process that was described to involve exacerbated caspase-11 and gasdermin-D-dependent responses. However, a recent study comparing various types of cell necrosis, including ferroptosis and pyroptosis, did not find a role for lipid peroxidation at regulating caspase-11-dependent pyroptosis. However, the authors used the inhibitor of lipid peroxidation ferrostatin-1 and not Vitamin E, suggesting that both molecules might regulate different peroxidized phospholipids or processes involving gasdermin-D [102]. Finally, the laboratory of M. Kopf also used $Gpx4^{-/-}$ mice in the myeloid compartment [179]. Testing the inflammasome response of those macrophages, the authors failed to detect a defect in canonical (Caspase-1) inflammasome response in those cells. Therefore, the interactions between the canonical/noncanonical inflammasome and GPX4 will warrant further studies to decipher the contribution of GPX4/Lipid peroxidation to pyroptosis induction. The interactions between pyroptosis and ferroptosis remain unknown but future studies should help at determining their exact links. It should be noticed that GPX8, a member of the GPX family, has recently been shown as a critical regulator of caspase-11 activation through a direct interaction [180], suggesting that GPX family members might contribute in a ferroptosis independent manner to various caspases activation.

Beyond the dedicated functions of GPXs enzymes in various cell deaths, various sepsis status, either in patient cohorts or in study animals found that upon bacterial infection-driven organ failure or sepsis, the levels of lipid peroxides and their byproducts (e.g., MDA, 4-HNE) were strongly increased [181–185]. In this context, preclinical studies could unveil the benefit for the organ protection of giving a-tocopherol, selenium, or vitamin E to rats or mice undergoing microbial sepsis, including lipopolysaccharide (LPS)-dependent sepsis [185–187]. However, LPS, a major component of the Gram-negative bacterial membrane, triggers endotoxemia through the hyper activation of the protease Caspase-11 [188]. To this regard, the study from Wiernicki and colleagues suggests that Caspase-11 activation drives the generation of peroxidized phospholipids [102]. Hence, determining whether Caspase-11-dependent pyroptosis promotes ferroptosis in other cell types through the release of peroxidized phospholipids and if this process plays a role in sepsis, will help determining the respective contribution and interactions of the whole spectrum of regulated cell necrosis in infectious sepsis.

Ferroptosis as a microbicidal mechanism

Lipid peroxidation-mediated pathogen restriction and killing has a long history. For instance, mechanisms involving aa, the NADPH oxidase or various lipid peroxidation-based systems (nanoparticles, oxidases, iron, etc.) were shown to be efficient against various pathogens [189–197]. To this regard, if some of those pathogens are targeted by a ferroptosis-like process and if ferroptosis induced in cells can actually target pathogens has not been yet studied to our knowledge. A recent study could determine that the parasite *Trypanosoma cruzi* could actually die in a process that resembles ferroptosis when targeted by ferroptosis inducer RSL3 [198]. Authors found that this GPX4 inhibitor targeted the distant relatives of GPX4 Tryparedoxin peroxidase in *T. cruzy*, hence driving parasite ferroptosis. In another study, the authors found that arachidonic overload in *Staphylococcus aureus* bacterial pathogen drives lipid peroxidation-dependent bacterial death [191], hence suggesting that ferroptosis induction might be a way for cells to eliminate various pathogens.

Whether ferroptosis inducers might also be of interest in infectious diseases will be of importance in the frame of the finding of novel antimicrobial strategies, but so far some currently anti infectious therapies were recently found to trigger ferroptosis [199,200].

Conclusion and perspectives

Ferroptosis studies are in full expansion. With the strong impact of ferroptotic inducers on various cancers, translation of fundamental research to therapeutic

applications is going extremely fast. However, many aspects of ferroptosis remain fuzzy. First, is there a strong biological function of ferroptosis? So far, most of the studies showed that ferroptosis plays a pathological role in various contexts, but the conservation of this cell death among species and over evolution suggests a robust benefit for the organisms. Regarding the signature of ferroptosis, there is also a lot to determine. As a nonapoptotic cell death, many DAMPs that are also found released in pyroptosis, NETosis or necroptosis contexts will also be detected in ferroptosis. However, a major hallmark of ferroptosis is the induction of a robust lipid peroxidation; Therefore, the use of modern redox lipidomic approaches should help at determining if there are specific oxidized lipids that are specific DAMP features of ferroptosis, in a similar model of IL1beta signature for inflammasomes. In addition, the development of genetic models (e.g., ACSL4, GPX4 or Cypor KO cells/mice/organoids) will also help at discriminating ferroptosis from other cell deaths.

Is ferroptosis an important cell death in the development of immunity and inflammation? If so, what type of DAMPs/signals are required for such process? What type of immune polarization does it promote? At the cell-autonomous level, is ferroptosis conferring any protection against infections? What are the microbial and host derived signals that can trigger ferroptosis? How do cells execute the final step of ferroptosis? Are there shared process/effectors between pyroptosis, necroptosis, apoptosis and ferroptosis?

Answering all these questions will constitute exciting future challenges that will probably help translating ferroptosis to various clinical contexts, including infectious diseases.

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Conflict of interest

The authors declares no conflict of interest.

Author contributions

SB and EM wrote and edited the manuscript.

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Chapter 3: *Pseudomonas aeruginosa* infection

I-Introduction: Genus's *Pseudomonas*

The name *Pseudomonas* was proposed in 1894 by the botanist Walter Emil Friedrich August Migula for a type of cell described as "cell with motility organs, which make spore but in specific species". Nowadays, there is more than 200 bacteria species in *Pseudomonas* group. This bacterium presents a rod-shaped and measuring between 0.5 to 1.0µm in diameter by 1.5 to 5.0 µm in length (Jun et al., 2015). These bacteria are Gram-negative, and only ~25 species are pathogens for humans, animals and plants. For instance, the species *P. syringae*, a major plant pathogen, responsible for the emergence of diseases in fruit trees, or *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. stutzeri* and *P. chlororaphis*, which are associated with infections in humans and animals (Peix et al., 2009). These pathogenic species are opportunists, they infect immune deficient or compromised hosts. *Pseudomonas* bacteria are found in diverse places such as water or ground (Figure7; (Scales et al., 2014)). *Pseudomonas* has a strong adaptability due to a genetic plasticity in effect 10% of their genome (6,3 millions of base pairs with almost 6000 genes) code for elements involved in gene regulation (Moore et al., n.d.). Belong *Pseudomonas* gender aeruginosa species is the most known and studied due to its association with human diseases.

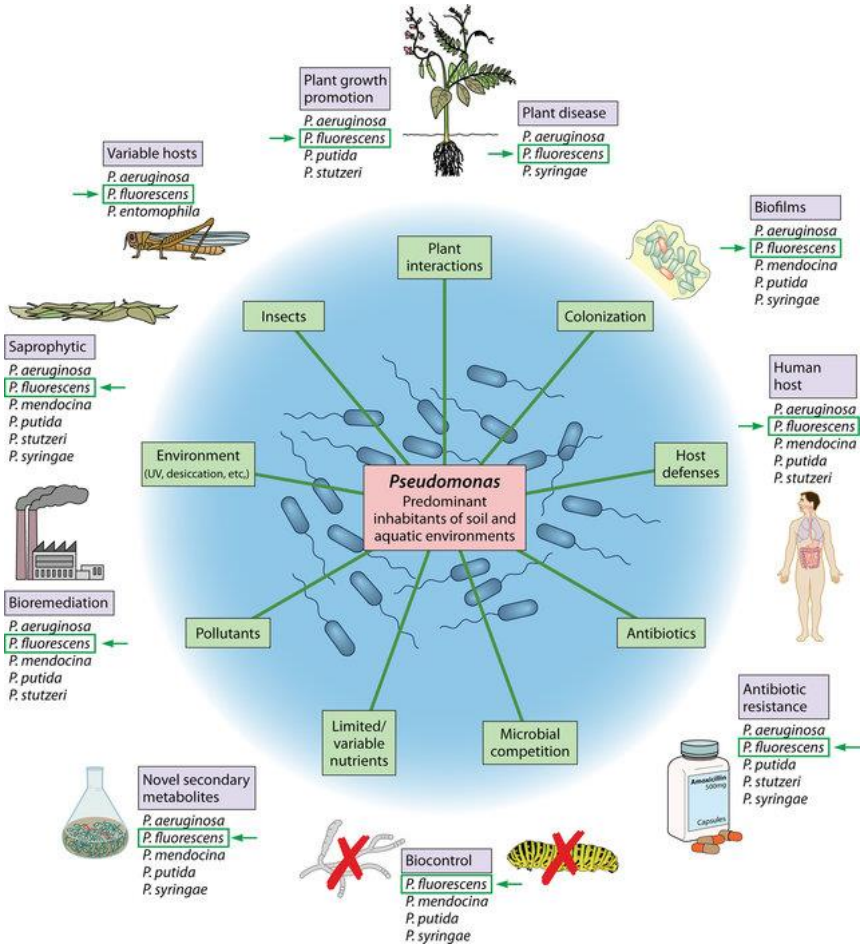


Figure 9 : Environmental niches of Pseudomonas highlighting the wide distribution of these species (Scales et al., 2014).

1-*Pseudomonas* aeruginosa (*P. aeruginosa*)

Pseudomonas aeruginosa has been isolated for the first time in 1882 by Carle Gessard from two patients with the infected wound (Lyczak et al., 2000). For a long time, multiple names have been attributed to this opportunistic pathogen such as *Bacillus pyocyaneus*, *Bakterium aeruginosa*, *Pseudomonas polycolor*, and *Pseudomonas pyocyaneus*, before the final designation. These names are related to the bacterium shape and the blue/green colour, which characterize the bacterial culture or infected injuries. In fact, *P. aeruginosa* products a mix of two pigments, a pyocyanin (blue) and pyoverdin fluorescein (yellow) (Botzenhart & Döring, 1993).

It is a nosocomial opportunistic bacterium. In fact, *P. aeruginosa* resides mainly in humid and community environments like hospitals, ground, sinks and showers as well as artificial water systems (Pellett et al., 1983; Rutherford et al., 2018). *P. aeruginosa* is able to form a biofilm, which is useful for human tissues colonisation during chronic

infection also for the persistence of bacteria. In some conditions, the bacteria from biofilm can be detached, become free bacteria, and so disseminate in other tissues (Dunne & Jr., 2002). Moreover, it is one of the four nosocomial species in France public health (with *Escherichia coli*, *S. aureus*, *Enterococcus faecalis*) (*Enquête Nationale de Prévalence Des Infections Nosocomiales et Des Traitements Anti-Infectieux En Établissements de Santé, Mai-Juin 2017*, n.d.). These microorganisms are the most frequent and isolated from nosocomial infections. *P. aeruginosa* presents also a crucial capacity of antibiotics resistance, in a normal way (by constitutive expression of β-lactamases and/or efflux pumps, or low permeability of external membrane), upon exposition to antibiotics or environmental stresses or the acquisition of resistant genes, mutations, overexpression of efflux pump or down expression of porins (Barbier & Wolff, 2010; Bonomo & Szabo, 2006; Hwang & Yoon, 2019; Mesaros et al., 2007).

2-Virulence factors of *P. aeruginosa*

P. aeruginosa has a similar external cell wall structure to other Gram-negative bacteria, lipopolysaccharide (LPS, or endotoxin), Type IV pili and flagella, adhesins, and lectins (Lec) (Kazmierczak et al., 2015). All these membrane components are virulence factors, for instance, LPS is known as the most potent microbial mediator implicated in the pathogenesis of sepsis and septic shock (Opal, 2010). Nowadays, it is known that LPS triggers sepsis through the generation of cell death (pyroptosis) and the release of a huge amount of inflammatory cytokines (Kayagaki et al., 2011a, 2015). The feature of motility for *P. aeruginosa* is an advantage, as it can move from one niche to another with no difficulty (Jain et al., 2012). Three types of motilities, including swarming, swimming, and twitching motility, enable *P. aeruginosa* to be present in a wide range of different habitats with a diversity of environmental factors (Kazmierczak et al., 2015). Lectins are proteins on the outer membrane of *P. aeruginosa*, which recognize glycosylated carbohydrates on host tissues, helping the adherence of bacterial cells. For example, LecA (which binds to galactose) and LecB (which binds to fucose) mediate the adherence of this pathogen to epithelial cells in the lung (Chemani et al., 2009; Thuenauer et al., 2020). These cell-mediated virulence elements present essential functions in the initial phase of colonization, persistence, and in the establishment of infections *in vivo* (Behzadi et al., 2021; Moradali et al., 2017). However, most virulence factors associated with *P. aeruginosa* are secreted elements (Figure10). These may be synthesized and secreted to the environment or

neighbourhood cells of these bacteria and can damage surrounding tissues, and immune cells. In addition, they may be introduced directly into host cells via a type III secretion system (T3SS) (Vanderwoude et al., 2020; Veesenmeyer et al., 2009). Secreted virulence factors are crucial in the later stages of the infection and invasion, during which bacterial cells proliferate and subsequent damage occurs in tissue cells at the site of infection, and the host immune response is deficient.

These secreted virulence factors in *P. aeruginosa* include: pigments, siderophores (e.g., achromobactin), and inorganic compounds (e.g., hydrogen cyanide), which have roles in iron scavenging, protection against damage caused by reactive oxygen species (ROS; originating from immune cells), and competition against other bacterial genera. Exotoxins, which are cytotoxic: exotoxin A (ETA), exotoxin S, exotoxin U, exotoxin T, exotoxin Y and exolysin A. Exotoxin A is a lethal toxin that inhibits protein synthesis in mammalian cells (B Wretlind, n.d.). ExoT and ExoS are similar proteins, possessing an N-terminal GAP (Rho-GTPase activating) domain and a C-terminal ADP-ribosyltransferase domain (Goehring et al., 1999; J. Sun & Barbieri, 2003). Exotoxin U (ExoU) presents a phospholipase activity, which rapidly leads to cell lysis and has roles in inducing septic shock upon infection (Phillips et al., 2003; Sato et al., 2003). Exotoxin Y (ExoY) has adenylyl cyclase activity (Yahr et al., 1998) and induces pro-apoptotic processes. The last one exolysin A (ExlA), which is secreted by a two-partner secretion system (TPS) triggers cell necrosis (Elsen et al., 2014).

Proteases and other enzymes: lipases, alkaline protease, elastase A (LasA), and B (LasB), heat-stable hemolysin/phospholipase H (PLH), phospholipase C (PLC), and DNase. *P. aeruginosa* express also some secretion systems: among which, Types I (T1SS), II (T2SS), and III (T3SS) are involved in the virulence of this pathogen. T1SS and T2SS are relevant in the secretion of various proteases and lipases, ETA, LasA, LasB, and PLH. On the other hand, the role of T3SS is to expel flagellar proteins and the previously mentioned effector toxins (such as ExoU and ExoS) into the cytoplasm of mammalian cells (A. R. Hauser, 2009; Vanderwoude et al., 2020; Veesenmeyer et al., 2009). In contrast to cell-mediated virulence factors (which are considered to be constitutive), the production of secreted virulence factors is largely dependent on the environmental factors and the niche surrounding the pathogen. Regarding the type 4 secretion system, it is involved in the transfer of large molecules (such as genes, proteins) allowing resistance or adaptation to the environment (Behzadi et al., 2021).

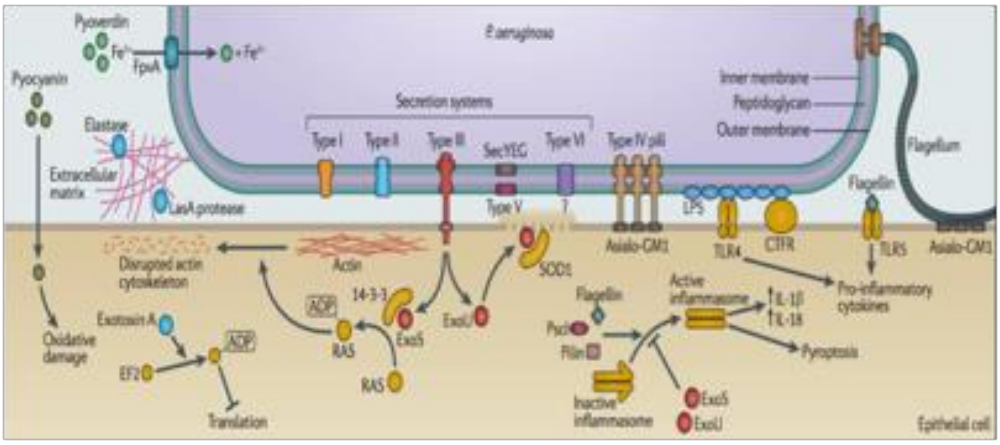


Figure 10 : *Pseudomonas aeruginosa* virulence factors (A. R. and O. E. A. Hauser, 2011).

II-Acute and chronic infection of *Pseudomonas aeruginosa*:

1-Site of infections

P. aeruginosa is an opportunistic pathogen, able to infect vertebrates and non-vertebrates. In humans, *P. aeruginosa* is able to establish an infection when the immune system is impaired, or the physical barrier of the body is broken due to an injury. The most susceptible person to infection is cystic fibrosis patients, resulting in thickening of the airway mucus and patients in hospitals. These patients may have severe damage or deficiency of the immune system upon chemotherapy or HIV (Human Immunodeficiency Virus), which favour the infection (Franzetti et al., 1992; Lyczak et al., 2000). Catheter insertion, intubation or placement of other medical devices are also risk factors in hospitalized patients (A. David et al., 2005). *P. aeruginosa* is associated to pulmonary infection (13,94%) and to other infections such as skin or smooth tissues (9.29%) (*Enquête Nationale de Prévalence Des Infections Nosocomiales et Des Traitements Anti-Infectieux En Établissements de Santé, Mai-Juin 2017*, n.d.). *P. aeruginosa* is also responsible for the infection in the eyes (due to the injury in cornea and contamination of the contact lenses or washing solution) and the infection in the ear (otitis infections) (Gellatly & Hancock, 2013; Schaefer & Baugh, 2012; Y. Sun et al., 2012).

Table 2: Associated risk factors and prevalence of *P. aeruginosa* for various types of nosocomial infections (*Enquête Nationale de Prévalence Des Infections Nosocomiales et Des Traitements Anti-Infectieux En Établissements de Santé, Mai-Juin 2017*, n.d.).

Infection site	Risk factors	Pathology	*P. aeruginosa* rank
Lung	Intubation, assisted breathing, HIV cystic fibrosis, chronic obstructive pulmonary disease	Pneumonia	2nd
Skin and smooth tissues (muscles, tendons, ligaments, adipose tissue)	Profound injury: wound, burn, surgical procedure	Cutaneous infection	2nd
Urinary system	Placement of urinary catheter or obstructed urinary tract	Urinary infection	5th
Blood	Intravascular catheter, contaminated needle, low white blood cell count (chemotherapy), infected organ with access to the bloodstream	bacteremia	5th

*Belong isolated bacteria from the site of infection

2-Acute infections

Acute infection of *Pseudomonas* could be pulmonary, cutaneous, urinary, gastro-intestinal or in the bloodstream (Silby et al., 2011). These infections often spread rapidly and can cause tissue damage and sepsis with a high mortality rate. Acute infections at specific sites, such as the lungs of cystic fibrosis patients, can evolve into chronic infections. This is due to adaptive changes in the infecting clonal type, including downregulation of acute virulence genes with upregulation of chronic infection phenotypes and antibiotic resistance (Balasubramanian et al., 2013). During acute infection some virulence factors are involved include type II and III secretion systems as well as the virulence factors secreted by them, flagella, type IV pili and virulence factors regulated by Quorum sensing (QS) (proteases, elastase, pyocyanin) (Figure11). These virulence factors cause damage to the epithelial barriers, which promote the spread of the bacteria (Gellatly & Hancock, 2013). This dissemination will be even more important if the host's immune system is deteriorated and therefore

unable to handle the pathogen. In addition, *P. aeruginosa* is also responsible for chronic infections. These infections occur after acute infections if they are not handled in time or properly, but some other factors can contribute to this chronicity.

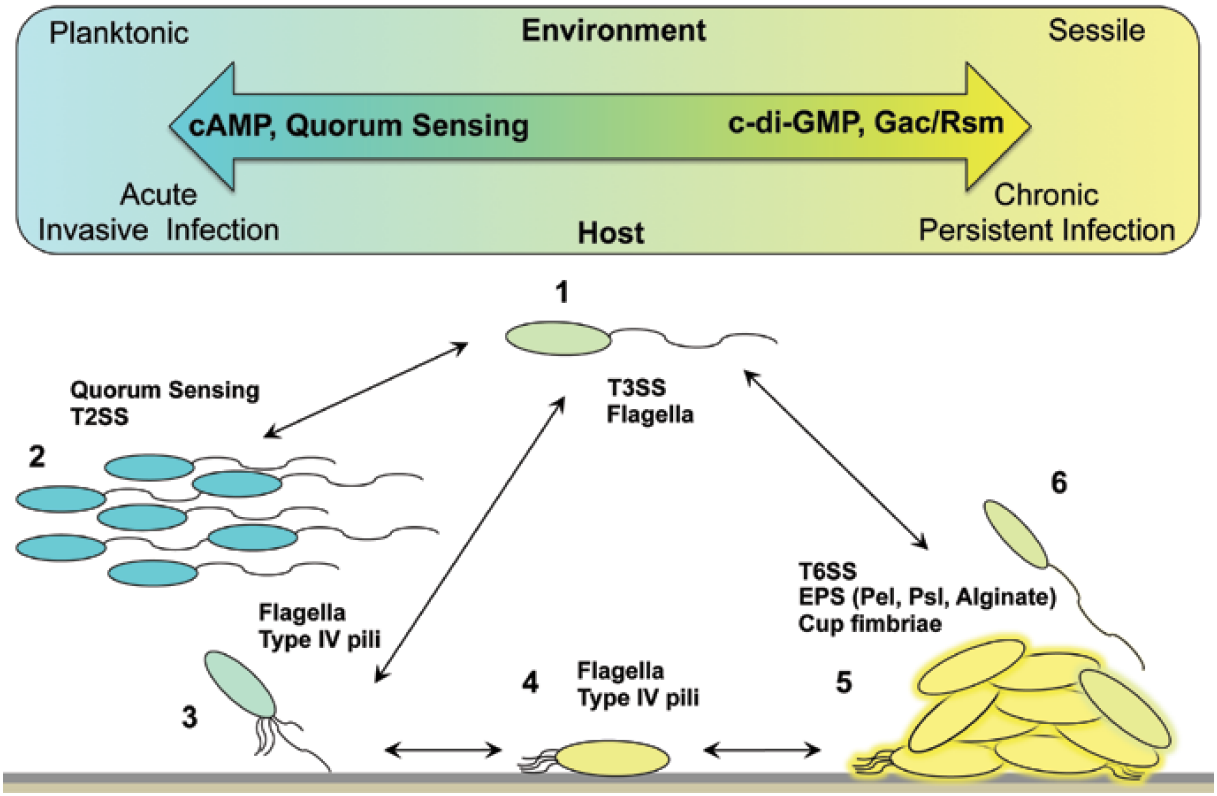


Figure 11: *P. aeruginosa* has two different modes of life, either planktonic (1-3) or attached forming a biofilm (5-6). Four regulatory pathways (cAMP, c-di-GMP, Quorum sensing, and Gac/Rsm) play similar roles in infection by monitoring acute and chronic infection phenotypes. From (Coggan & Wolfgang, n.d.).

3-Chronic infections

Chronic infections, on the other hand, persist for weeks, months, or years despite intensive clinical intervention (Gellatly & Hancock, 2013; Turner et al., 2014). One or more bacteria could induce this type of infection. The number and type of bacteria can change over time. These bacteria have a lifestyle in the form of biofilm. Among chronic infections, *P. aeruginosa* is mostly identified in people with the implantation of medical equipment (urinary catheters or prostheses), or the production of abnormally thick bronchial mucus, such as the case of cystic fibrosis or chronic obstructive pulmonary disease (*SFM 2019*, n.d.). Moreover, during the establishment of a chronic infection,

there is an overproduction of extracellular exopolysaccharides (EPS), up-regulation of the type VI secretion system and formation of biofilm and small colony variants (Figure 11).

A- P. aeruginosa infection in Cystic Fibrosis (CF) patients

Cystic fibrosis is an inherited disease incurable. It is due to the mutation of the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene, which encodes for an ATP-binding cassette (ABC) transporter superfamily or also CFTR protein (Lyczak et al., 2002). This causes sticky mucus to build up in the lungs and digestive system and triggers lung infections and problems with digesting food. CFTR protein functions as a chloride channel, and controls ion and water secretion and absorption in epithelial tissues and allow hydrating and fluidifying mucus, a substance involved in the evacuation of dust and bacteria through ciliary movement. Cystic fibrosis is the most common lethal genetic disorder in populations of Northern European descent. In France, this disease affects one child out of 4,500 newborns and the life expectancy does not exceed 46 years. Most of them die of respiratory failure. Currently, different mutations (~1,500) have been reported to be responsible for the appearance of the disease. These mutations lead to inactivation, malformation or even the absence of the CFTR protein. The most common (70% of cases) is the deltaF508 mutation, which leads to impaired folding of the protein after synthesis, preventing its localization on the apical surface of epithelial cells. CFTR mutation causes a blockage of chloride ions in the cells and results in increased mucus thickness. The accumulation of mucus in the bronchial tubes block the bronchioles and therefore become a favourable environment for bacterial growth, especially *P. aeruginosa*. CF patients (adults) are infected by *P. aeruginosa* at 80%. Chronic respiratory infections are the leading cause of death in these patients (Bhagirath et al., 2016; Lyczak et al., 2002; Rossi et al., 2021).

It is known that *Pseudomonas* infection in cystic fibrosis patients evolves from childhood to adulthood with a high percentage in adulthood. It is not the only bacterium colonizing the lung of these patients for example *S. aureus* is mainly found in young patients. The inversion from *S. aureus* to *P. aeruginosa* is due to the ability of *P. aeruginosa* to secrete or to induce the production by the host, of antimicrobials, capable of eliminating *S. aureus*. In fact, during iron deficiency, *P. aeruginosa* secretes molecules called 2-alkyl-4 (1H)-quinolones, which target and kill *S. aureus*. In addition,

P. aeruginosa stimulates the production of an enzyme by epithelial cells, type-IIA phospholipase A2, which can kill Gram-positive bacteria (A. T. Nguyen et al., 2015; Pernet et al., 2014). Other factors are also responsible for the persistence of *P. aeruginosa* in CF patients, such as oxygen and nutrient limitation, antibiotic use and high immune response, leading *P. aeruginosa* to evolve towards clones more adapted to this new environment. In particular, the mucoid clones present an overexpression of genes coding for exopolysaccharides (Bhagirath et al., 2016; L. Cullen & McClean, 2015; Rossi et al., 2021; Smith et al., 2006). These latter play an important role in biofilm formation that provide protection of *P. aeruginosa* against the host immune system and antibiotics, in order to ensure the survival of the bacteria in this hostile environment (Bjarnsholt et al., 2009; Breidenstein et al., 2011).

B-Other chronic infection of Pseudomonas

P. aeruginosa is chronically associated with other pathologies besides cystic fibrosis. Other diseases affect the lung or urinary tract, skin and ears. Indeed, *P. aeruginosa* is also responsible for chronic infections in patients with chronic obstructive pulmonary disease (Martínez-Solano et al., 2008; Rosell et al., 2005); patients with bronchiectasis, chronic asthma, or lung cancer (Davies et al., 2006; Gao et al., 2018; Q. Zhang et al., 2012).

In general, prompt and appropriate treatment of the patient is crucial to increase the probabilities of survival from a potentially fatal *P. aeruginosa* infection. However, many strains have developed resistance to commonly used antibiotics, which poses a major public health problem worldwide.

III-Treatment against *P. aeruginosa*

1-Treatments and resistance of *P. aeruginosa*

The outcome of *P. aeruginosa* infections depends on the state of the host's defence system, previous unsuccessful treatments, and the virulence of the bacteria. Furthermore, the intense and recurrent use of antibiotics in the treatment of patients infected with *P. aeruginosa* has led to the emergence of multi-resistant strains to the different classes of antibiotics, which are the β-lactams (penicillins, cephalosporins and monobactams), carbapenems, fluoroquinolones and aminoglycosides (Barbier & Wolff, 2010; Williams et al., 2010). Due to its remarkable ability to resist antibiotics, *P. aeruginosa* has moreover been classified by the World Health Organization (*WHO*

Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed, n.d.), among the critical group of "priority pathogens" resistant to antibiotics. Therefore, the development of new antibacterial strategies is necessary. Antibiotic resistant strains are isolated through different mechanisms detailed in the section "*Pseudomonas aeruginosa*" (Figure 12). These multi-resistant strains can accumulate several of the resistance mechanisms, considerably limiting the therapeutic options to eradicate them and thus treat patients.

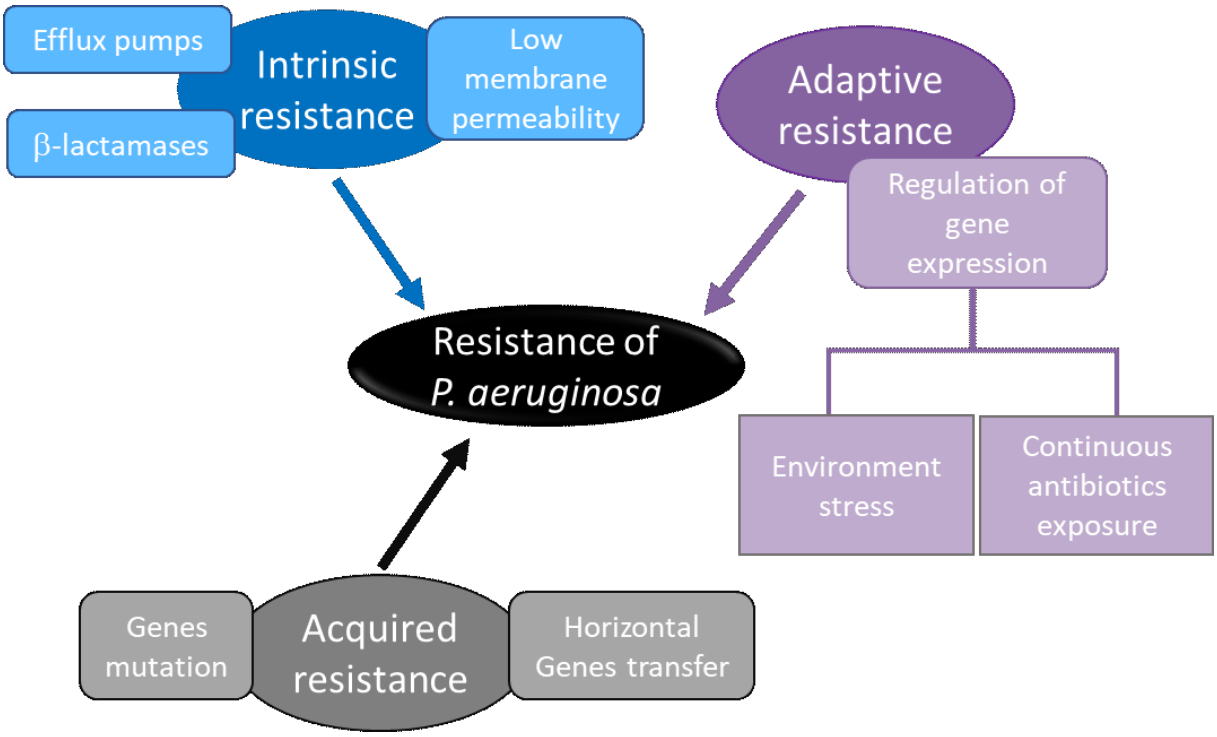


Figure 12: Different mechanisms of *P. aeruginosa* resistance.

2-New therapeutically strategy

Currently, a large number of new approaches are emerging to limit infections by multi-resistant strains of *P. aeruginosa*. These approaches involve the use of different combinations of antibiotics, as well as the development of new antibiotics (Doripenem, plazonmicin, POL7001). New therapeutic strategies are also emerging, either alone or in combination with conventional therapies. These strategies span from elimination (novel antibiotics) to disarming (anti-virulence) of the pathogen (Bassetti et al., 2018). Indeed, some of the therapies aim to prevent biofilm formation (quorum-sensing and bacterial lectin inhibitors, iron ion chelators), improve antibiotic penetration into the

bacteria (nanoparticles), prevent infection (vaccines), or lyse these bacteria (antimicrobial peptides and phage therapy) (Pang et al., 2019).

In general, the severity of *P. aeruginosa* infections is related to the bacteria capacity to adapt and so survive in hostile environments, but also to its incredible resistance to multiple antibiotics.

IV-Immune response upon *P. a*eruginosa infection

During *Pseudomonas* infection, the bacteria crosse the host natural barriers (skin and mucous membranes), leading to the activation of the immune system. First, the bacteria induce innate immunity, which plays an essential role in controlling infections caused by *P. aeruginosa*. Innate immunity is triggered rapidly, following the recognition of PAMPs associated with certain structures of the pathogen, such as the flagellum, pili and LPS, by PRR receptors. The activation of these receptors leads to different cellular mechanisms, including the expression of new genes. The main cells involved in this defence are epithelial cells, monocytes/macrophages, neutrophils. These cells induce among other procedures the production of antimicrobial agents. Regarding adaptive immunity, it is later and allows the production of specific antibodies to *Pseudomonas* (Lavoie et al., 2011; Moser et al., 2021).

1-Innate immunity: monocyte/macro and neutrophils

A- Role of pattern recognition receptors during infection:

As previously described, PAMPs are detected by PRRs, which include cell surface and endosomal Toll-like receptors (TLRs), and cytosolic Nod-like receptors (NLRs). MyD88, an adaptor molecule for almost all Toll-like receptors (TLRs), is required for the control of *P. aeruginosa* in the lung (Lavoie et al., 2011). For example, TLR4 and TLR5, which recognize LPS and flagellin, respectively, can initiate protective responses to *P. aeruginosa* infection. This is illustrated by the survival of wild-type animals after infection with *P. aeruginosa* versus reduced survival of TLR4/5 double knockout mice (Feuillet et al., 2006; Ramphal et al., 2008). Other TLRs that are expressed by lung epithelium and alveolar macrophages, such as TLR2 and TLR9, do not appear to mediate *P. aeruginosa* recognition in the lung.

P. aeruginosa as previously described can inject virulence factors into host cells. Among these virulence factors such as flagellin which is known to induce the assembly of the inflammasome (Pyroptosis section). *P. aeruginosa* induced caspase-1 activation requires NLRC4 (also known as IPAF, one NLRs) in different immune cells such as alveolar or bone marrow-derived macrophages (Sutterwala et al., 2007; Zhao et al., 2011). In fact, NAIP5 which is one of the BIR-domain NLR protein family, engagement by flagellin promoted a NAIP5–NLRC4 association, then the inflammasome assembly ((Zhao et al., 2011). This explains a huge inflammatory cytokine interleukin-1β (IL-1β) release during the early pulmonary infection. Of note, the T3SS effectors ExoU (Sutterwala et al., 2007) and ExoS (Galle et al., 2008) can inhibit caspase-1 activation, by mechanisms that require their respective phospholipase A2 and ADP-ribosyltransferase activities. *In vivo*, caspase-1 activation and subsequent IL-1R dependent signalling are required for rapid neutrophil recruitment to the site of infection due to chemokines productions (Mijares et al., 2011; Wangdi et al., 2010). Of note, IL-1R signalling in non-bone marrow-derived cells, such as airway epithelial cells, is necessary and sufficient for these early host responses (Lavoie et al., 2011).

B-Monocyte/macrophages

The first immune cells to encounter *P. aeruginosa* in the lung are resident alveolar macrophages. Macrophages can phagocyte and kill bacteria; however, their role in pathogen sensing is critical during *P. aeruginosa* infections. *In vitro*, murine alveolar macrophages secrete chemokines (KC) and cytokines (TNF-α and IL-6) after activation of TLR-4 and TLR-5 by *P. aeruginosa* LPS and flagellin, respectively (Lavoie et al., 2011; Moser et al., 2021); thus, macrophages can produce chemokines that recruit neutrophils. As noted above, alveolar macrophages also respond to a signal associated with *P. aeruginosa* T3SS by activating caspase-1. IL-1β produced by macrophages can be detected by airway epithelial cells, which in turn secrete neutrophil chemokines (Mijares et al., 2011).

B-1Phagocytosis and phagosome formation

Macrophages eliminate invading microorganisms and other foreign particles by first ingesting them into an intracellular vacuole derived from the plasma membrane called a phagosome. The formed phagosomes undergo a series of fission and fusion events that affect their membrane and content composition, in a process that mimic the progression of the endocytosis pathway organized into a continuum of organelles from

early endosomes to lysosomes. This process of phagosome maturation allows the vacuole to acquire a multitude of degradation products, which are central to its microbicidal function (Vieira et al., 2002). During the maturation of the phagosome, the internal pH becomes more acidic around 5.5 and the vacuole is enriched in hydrolytic enzymes, which is referred to as the late phagosome. The phagolysosome is finally formed when the late phagosomes fuse with the lysosomes filled with hydrolases (nucleases, lipases, glycosidases, proteases and cathepsins). The internal pH then becomes very acidic (pH <5.5), which ensures the optimal activity of degradation enzymes and compromises the survival of many microorganisms. The lysosomes also bring membrane proteins as well as the ATPase of type V. The latter is a proton pump that contributes to the acidification of the phagolysosomal contents (Vieira et al., 2002).

B-2Microbicide effect of monocyte/macrophage:

There are two types of antimicrobial systems in the macrophage: oxygen-dependent systems that involve the production of reactive oxygen and nitrogen species (ROO) (i), and oxygen-independent systems that may involve, nutrient deprivation (ii), acidification of the phagolysosomal vacuole (iii), production of defensins and other antimicrobial peptides (iv), and action of lysosomal hydrolytic enzymes (v).

The most important antimicrobial systems in phagocytic cells are the NADPH phagocyte oxidase (PHOX) and Inducible nitric oxide synthase (iNOS) pathways, which are responsible for generating superoxide radicals (O2-) and nitric oxide (NO) respectively. These ROS are rapidly essential for the microbicidal function of the phagocyte (Nunes et al., 2013). Depriving the pathogen of nutrients is another important antimicrobial function of the phagocyte, which includes spatial isolation and active molecular mechanisms of nutrient deprivation. For example, iron and manganese are actively excluded from the phagosome by the action of a metal transporter Nramp1 (Natural Resistance-Associated Membrane Protein) (Cellier et al., 2007). In addition, studies have shown a deficiency in other nutrients essential for bacterial multiplication (amino acids, pyrimidines, purines and vitamins) (Appelberg, 2006). Macrophages produce antimicrobial peptides, which are small peptides that exert microbicidal activity by disrupting the membrane or creating pores at the pathogen's membrane. They are present in the granulations of phagocytic cells and are able to eliminate a broad spectrum of microorganisms (Wassing et al., 2015). The

final mechanism used to eliminate bacteria involves more than 50 different lysosomal acid hydrolases. Thus, the concerted action of proteases, lipases, nucleases, glycosidases, and phosphatases allows for the complete disintegration of many complex structures such as dying microorganisms and allows for direct antimicrobial action (Flannagan et al., 2012).

C-Neutrophils cells

The recruited cells, mainly composed of neutrophils, which eliminate the bacteria. Complement factors also participate in this elimination by forming pores in the bacterial membrane, thanks to the membrane attack complex (MAC) (Lavoie et al., 2011). Thus, infection of the lungs by *P. aeruginosa* triggers an intense inflammatory response in the host. As evidence, cystic fibrosis patients infected with *P. aeruginosa* typically exhibit excessive inflammation, including high expression of pro-inflammatory cytokines such as interleukins 1, -6 and -8 (IL-1β, IL-6 and IL-8) (Bonfield et al., 2012; DiMango et al., 1995; Kube et al., 2001).

Except for neutropenia, a neutrophil response will be involved in each *P. aeruginosa* lung infection, and any possibility of complete resolution will require this response. In both acute and chronic *Pseudomonas* lung infections, the neutrophil response is usually robust. Murine models of acute *Pseudomonas* pneumonia in which neutrophils were depleted showed increased mortality of individuals, highlighting the importance of neutrophils in host defence against *P. aeruginosa* (Williams et al., 2010). Similar to macrophages, neutrophils generate a number of important antimicrobial molecules, including reactive oxygen species (ROS), produced by NADPH oxidase, reactive nitrogen species, via iNOS, elastase and antimicrobial peptides, including α-defensin. Lactoferrin and lysozyme are two important products of the neutrophil. Lactoferrin is both bactericidal and bacteriostatic to *P. aeruginosa* and inhibits the development of bacteria in the form of a biofilm. Lysozyme is a cationic polypeptide important in defence against *P. aeruginosa* by disrupting the bacterial membrane (Williams et al., 2010).

D-Macrophage and Neutrophil interaction during the infection

Neutrophils are recruited, following secretion of chemokines and cytokines (TNF-α and IL-6) by murine alveolar macrophages or airway epithelial cells (Lavoie et al., 2011). Thus, neutrophils and monocytes/macrophages coordinate their activities, leading to alternating waves of recruitment of these two cell types to the site of infection (Figure

13) (Amulic et al., 2012). Alveolar macrophages during pulmonary infection engulf dying neutrophils and initiation of resolution and repair.

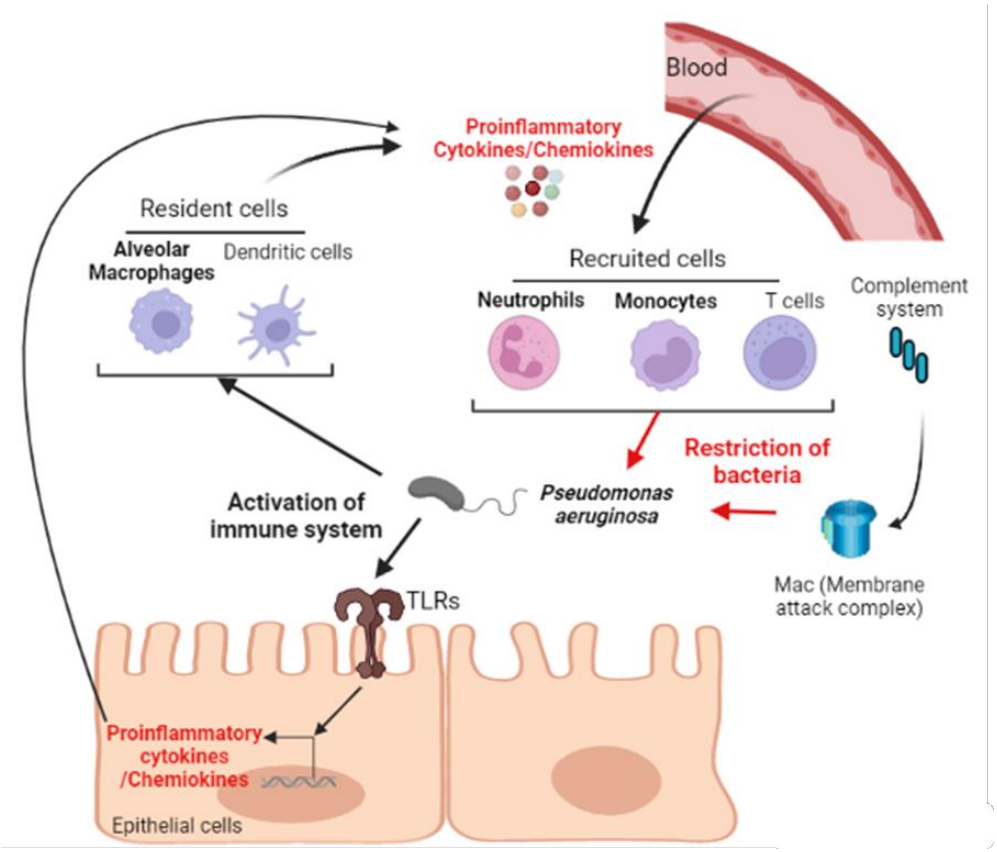


Figure 13: Activation of immune cells during *Pseudomonas aeruginosa* infection.

2-Adaptive immunity

Acquired immunity is triggered later, sometimes several days after the first contact with the pathogen. It is activated after digestion of the pathogen by phagocytes, in particular dendritic cells. These cells attach bacterial peptides to MHC (Major Histocompatibility Complex) receptors and present them to T and B lymphocytes in the lymph nodes, allowing the production of specific antibodies and memory T and B cells. To persist and disseminate in the body, *P. aeruginosa* will attenuate the host immune system using several virulence factors (Diaz et al., 2008; Lavoie et al., 2011; Moser et al., 2021). Activation of innate immunity is necessary for pathogen elimination but is

double-edged: the incessant stimulation of the inflammatory response related to the persistence of the bacteria is deleterious to the organism and causes severe injury.

In the next part, we will focus on the modulation of *Pseudomonas* infection by the exotoxin U that is the main subject of this thesis.

V-Exotoxin U: modulation of *P. aeruginosa* infection

In 1997, Frank DW's laboratory identified the toxin U (ExoU) in the cytotoxic strains of *P. aeruginosa* that cause severe acute lung injury and sepsis in animal models of pneumonia (Finck-Barbançon et al., 1997). Exotoxin U was then characterized as a T3SS toxin, therefore injected directly into the target cell. Since its discovery, other clinical studies have revealed that *P. aeruginosa* isolates possessing ExoU cause bacteraemia, sepsis and high mortality (Roy-Burman et al., 2001). In addition, ExoU and Exos are mutually expressed in clinical strains of *Pseudomonas* (A. R. Hauser, 2009). ExoU positive bacteria (ExoU+) is accounts for 28-40 % of *Pseudomonas aeruginosa* isolated from patients. However, one exception is PA99, a clinical isolate that naturally secretes ExoS, ExoU and ExoT (Shaver & Hauser, 2004). Moreover, several studies have been focused on the characterization of ExoU, its mechanism of action and its effects on cells and organisms affected by *P. aeruginosa*. Indeed, ExoU is the most toxic effector secreted by SST3 and induces rapid cell death. This cell death is necrotic (loss of plasma membrane integrity) (A. R. Hauser, 2009).

1-Exotoxin U and PLP A2 activity

A-ExoU protein structure

Genetic analyses also showed that the ExoU gene (of 2064 bp) located in the pathogenicity island 2 (PAPI-2; 11-kb insertion sequence), is in the form of an insertion gene cluster in the chromosomal genome of ExoU expressing strains. The spcU gene that encodes the ExoU chaperone protein is situated downstream of ExoU gene (Finck-Barbançon et al., 1997; He et al., 2004). Furthermore, there is a 1:1 stoichiometry of the ExoU/SpecU complex in bacteria (Gendrin et al., 2012; Halavaty et al., 2012). Indeed, the structure of ExoU was solved in complex with SpcU due to ExoU instabiliy to crystallization alone (Benson et al., 2011).

ExoU and SpcU are respectively of 74 kDa (687 amino acids) and 15 kDa (137 amino acids). ExoU is composed of four main domains playing an important function in its

toxicity (Figure 14) (Gendrin et al., 2012; Halavaty et al., 2012). The first fifteen N-terminal amino acids of the toxin might be involved in the recognition of ExoU by T3SS in order to be secreted (Finck-Barbançon et al., 1997; A. R. Hauser, 2009). The next domain called Chaperone Binding Site (CBS) located from amino acids 52 to 100 is a specific toxin interaction sequence with SpcU (Finck-Barbançon et al., 1997; Gendrin et al., 2012; Halavaty et al., 2012). From amino acids 106 to 471 is the so-called Patatin-Like Phospholipase (PLP) domain, responsible for the phospholipase activity of the toxin (Finck-Barbançon & Frank, 2001). This sequence contains a catalytic dyad consisting of serine (Ser-142) and aspartic acid (Asp-344), which are identical to the cytosolic and calcium-independent phospholipases A2 present in mammals (cPLA2 and iPLA2, respectively) (Sato et al., 2003). Finally, the C-terminus is composed of two domains. One domain is required for the membrane localization of the toxin called MLD (Membrane Localization Domain) and composed of residues 588 to 687, and a bridging domain, comprising residues 480 to 580 (Rabin et al., 2006; Rabin & Hauser, 2005). Substitution or deletion mutations of residues within the MLD domain result in a loss of membrane localization of the toxin (Gendrin et al., 2012; Rabin et al., 2006; Rabin & Hauser, 2005; Schmalzer et al., 2010; Stirling et al., 2006; Tessmer et al., 2017; Tyson et al., 2015). For the bridging domain, it could serve as a platform to place the adjacent domains, the catalytic and membrane-binding domains in proximity (Gendrin et al., 2012). These two domains located at the C-terminus of ExoU, interact with eukaryotic factors to activate the lipase activity of the toxin and induce cell death. The interaction of these eukaryotic cofactors with the different domains of ExoU plays a specific role in the activity, conformation and localization of the toxin in host cells (Finck-Barbançon & Frank, 2001; Rabin & Hauser, 2005).

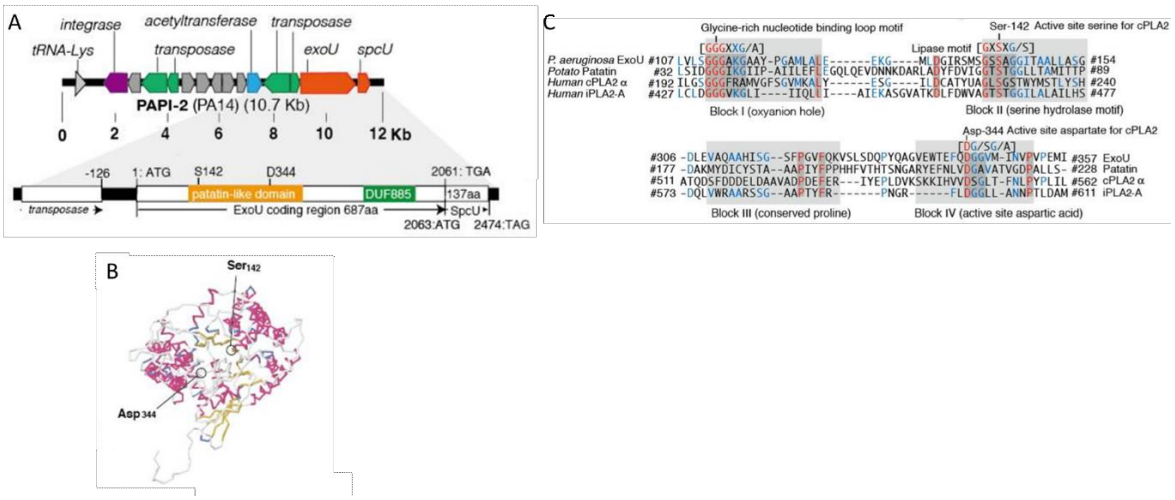


Figure 14: Exotoxin U: A) Exotoxin U gene in PIP2, B) structure of ExoU protein and C) Different domain of ExoU and its analogues (Sawa et al., 2016).

B- Patatin like phospholipases (PLP), PLA2 and Exotoxin U activity

a) Phospholipase activity of ExoU

Phospholipases A2 (PLA2s) are a family of enzymes that catalyze the hydrolysis of the ester bond of the acyl group at the sn-2 position of phospholipids of membrane phospholipids, releasing a free fatty acid, such as arachidonic acid (AA) and a lysophospholipid (Figure 15). PLA2s are involved in several cellular processes such as lipid metabolism, signal transduction, host defence and modulation of the inflammatory response via lipid mediators produced (Burke & Dennis, 2009; Ricciotti & Fitzgerald, 2011). In fact, the lipase activity of ExoU has a broad range of substrates, including phospholipids, lysophospholipids, and neutral lipids (Phillips et al., 2003; Sato et al., 2003; Tamura et al., 2004).

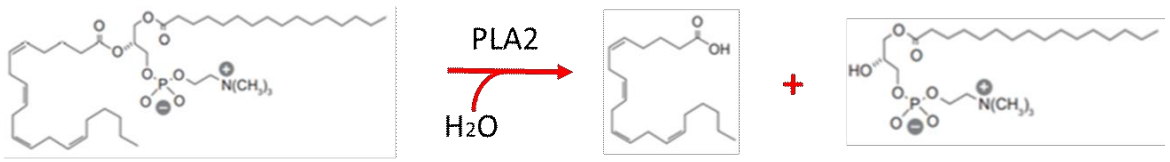


Figure 15: Phospholipase activity on phospholipid.

b) Patatin like phospholipases

Patatin is a family of glycoproteins, abundantly found in potato (Solanum tuberosum) tubers. They permit protein storage but also present a lipase activity (Andrews et al., 1988). This last function is performed on a large number of lipid substrates, such as phospholipids (Jiménez-Atiénzar et al., 2003). In 2003, Sato et al. first identify a patatin-like phospholipase (PLP) domain in the ExoU sequence (Sato et al., 2003). Alignments of ExoU, plant patatins and mammalian PLA2 such as iPLA2 (calcium-independent PLA2) and cPLA2 (cytosolic PLA2) show three highly conserved regions, typical of the PLP domains (Figure 14):

-A glycine-rich nucleotide-binding motif, G-X-G-X-X-G, forms an oxyanion hole, allowing the positioning of the ester function of the substrate and stabilising the enzyme-substrate complex (Dessen et al., 1999).

-A serine hydrolase motif, G-X-S-X-G.

-An active site aspartate residue is conserved in the motif D-X-G/A. These last two motifs form a catalytic dyad involved in the nucleophilic attack of the substrate ester in the sn-2 position (Dessen et al., 1999). Therefore, this Ser-Asp dyad is responsible for ExoU cytotoxicity during infection. In fact, the PLA2 inhibitor pseudolipasin A reduce the cytotoxicity effect of ExoU in yeast and mammalian cells pre-treated (Phillips et al., 2003; Sato et al., 2003; Tamura et al., 2004). Moreover, the nonsense mutations in the dyad region, impair the lipase activity of ExoU and so its cytotoxicity (Gendrin et al., 2012; Phillips et al., 2003; Sato et al., 2003). These studies confirm the previous observation of cell lysis and death by necrosis upon *Pseudomonas* expressing ExoU (Apodaca et al., 1995; A. R. Hauser & Engel, 1999). The PLP-like domains have also been identified in proteins from other bacteria, both pathogenic and non-pathogenic, both positive and negative Gram bacteria such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Yersinia pestis*, *Vibrio cholerae* and *Pseudomonas putida* (Banerji & Flieger, 2004).

C-Expression and secretion of ExoU

Previous studies suggest that both T3SS expression and injection, then ExoU expression and secretion appear at the first 24h during infection (Diaz & Hauser, 2010; Howell et al., 2013). Howel H A et al. showed, by using inducible ExoU-expression

bacteria, that the early expression of ExoU is critical for mice survival to pneumonia. Therefore, we can conclude that bacteria need a rapid expression of toxin U to avoid clearance.

Regarding ExoU secretion, the toxin is associated with its chaperone SpcU, which helps to sequester the toxin in the bacterial cytosol in an inactive form. During the approach of bacteria to target cells, the ExoU-SpcU complex migrates to the SST3, where ATP hydrolysis by the ATPase PscN, dissociates ExoU from its chaperone. The toxin is then unfolded and secreted through the needle of this system to be injected into the cytoplasm of target cells(Akeda & Galán, 2005; Halder et al., 2019). Importantly, SpcU is required for maximal ExoU secretion, but this chaperone is not secreted into the host cell with the toxin (Finck-Barbançon et al., 1997). The internal diameter of the SST3 needle is small 2.0-2.5 nm, implying that effectors are in an unfolded state to move through this structure (Matteï et al., 2011). Thus, ExoU is injected in an unfolded form into the cytoplasm of target cells and is therefore inactive. Although its mechanism of activation remains poorly defined, some eukaryotic proteins have been identified as cofactors, interacting directly with the toxin to modify its conformation and activate its phospholipase activity. These factors are intracellular since ExoU is not cytotoxic when the toxin is added to the cell surface, which is a consistent with-it injection into cells and not secreted into the external environment (Phillips et al., 2003). Distinct residues are involved in the interaction between ExoU and these cofactors and will be detailed in the following paragraphs. ExoU requires eukaryotic factors in order to be active, which explains its specificity regarding host cell lipids and its inability to lyse the bacterial membrane.

2-Exotoxin U and its cellular cofactors

Once in the host cell, ExoU interacts with different cellular factors. It has been reported that it requires ubiquitin and ubiquitylated proteins, then Phosphatidylinositol 4,5-bisphosphate (PIP2) and very recently DNAJC5 or cysteine string protein α (CSPα).

A-ExoU and ubiquitin-protein

Indeed, in 2006, ExoU was reported to be activated by superoxide dismutase (SOD1) (Sato et al., 2006). Interestingly SOD1 enzymatic activity was not necessary for this effect, suggesting that SOD1 activated ExoU by other mechanisms such as inducing a conformational change or linking ExoU to other factors. Subsequent studies revealed that SOD1 acts as the ubiquitin donor and the ubiquitination of the carboxyl terminal

domain of ExoU are the mechanism of PLP activation (Anderson et al., 2011; Schmalzer et al., 2010). The carboxyl-terminal of ExoU contains a conserved domain called DUF885, and mutant proteins lacking this region cannot be activated (Anderson et al., 2011; Sawa et al., 2016; Schmalzer et al., 2010). Moreover, once inside the host cell, two ubiquitin molecules are added to lysine 178 of this effector protein (Stirling et al., 2006).

B-Interaction ExoU and PIP2 and oligomerization

Upon injection, exoU is found in the plasma membrane via its interaction with PIP2 (Tessmer et al., 2017; Tyson et al., 2015). PIP2 is a biologically important phospholipid in a eukaryotic cell. It is mainly localized in the inner plasma membrane at low concentrations (1%) (Wenk et al., 2003). PIP2 is involved in cell signalling pathways, which govern cell adhesion, motility, cytoskeletal organization and dynamics and membrane trafficking (di Paolo & de Camilli, 2006; Raucher et al., 2000). In addition, it directly binds to focal adhesion molecules such as talin and vinculin and other adaptor proteins that have a crucial role in cell-matrix and cell-cell adhesion (di Paolo & de Camilli, 2006).

The C-terminal MLD of ExoU, containing the four-helical bundle, presents a high binding affinity for PIP2 (Gendrin et al., 2012; Sato & Frank, 2014). The model suggested by several studies is, that the binding to PIP2 triggers conformation changes in the structure of ExoU, including the conformational rearrangement of the four-helical bundle of ExoU, allowing it to insert into the lipid membrane (Tessmer et al., 2017; A. Zhang et al., 2017). PIP2 binding has also been demonstrated to promote ExoU multimerization (A. Zhang et al., 2017). SEC-MALS (Multiangle light scattering) analysis and phospholipase assays indicate that, *in vitro*, in the presence of PIP2, ExoU can form multimers that have greatly enhanced catalytic activity, in the presence of ubiquitin, when compared to ExoU and ubiquitin alone (Sawa et al., 2016; A. Zhang et al., 2017). ExoU binding to PIP2 is followed by ExoU hydrolyses of substrates and its cytotoxicity (Gendrin et al., 2012; Sato & Frank, 2014; Tyson et al., 2015).

C-DNAJC5 (CSPα) key cellular factor of ExoU cytotoxicity

Recently, Duruelle et al shown that ExoU needs DNAJC5 for trigger host cell lysis (Deruelle et al., 2021). In fact, the genetic screen using CRISPR-Cas9 method identify DNAJC5 as a key cellular factor of the toxin cytotoxicity induction. DNAJC5 is a cytoplasmic protein present on the surface of late endosomes (LEs), and it functions

as a co-chaperone in association with Hsc70 or Hsp70, which play a central role in protein homeostasis (Gundersen, 2020). In this study, it is its new function in the secretion misfolding proteins pathways that is important. In fact, misfolding proteins are translocated into DNAJC5+ LEs, and this latter is transported to the membrane where they fuse with it, hence releasing of misfolding proteins outside the cell. They demonstrated that DNAJC5 + LEs colocalizes with ExoU, however ExoU can still be transported to the plasma membrane in DNAJC5-/- (mutated) cells (Figure 16)(Deruelle et al., 2021). Further studies are needed to identify ExoU receptors at the vesicle's membrane. It would also be interesting to know if the ubiquitination of ExoU allowing its stabilization and increasing its catalytic activity, is upstream or downstream of its association to DNAJC5-containing vesicles.

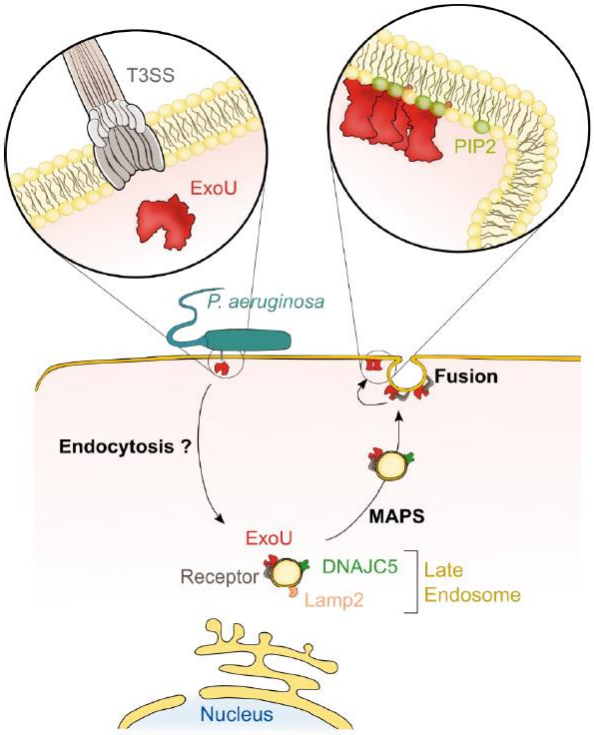


Figure 16 : ExoU cofactors ubiquitin, PIP2 and DNAJC5 (Deruelle et al., 2021).

3-Role of exotoxin U in *Pseudomonas* infection

Exotoxin U of *Pseudomonas* needs ubiquitin, PIP2 and DNAJC5 to be active. These factors are conserved and found in the eukaryotic cell include yeast, which explains the cytotoxic effect of the ExoU in these living organisms. Phillips et al, demonstrated that 300 to 600 ExoU molecules are necessary to trigger cell death which can happen during the first 10min upon injection (Phillips et al., 2003). However, it has been

suggested that ExoU modulates particular cellular signalling pathways involving host innate immunity activation and so inflammation (Figure 17).

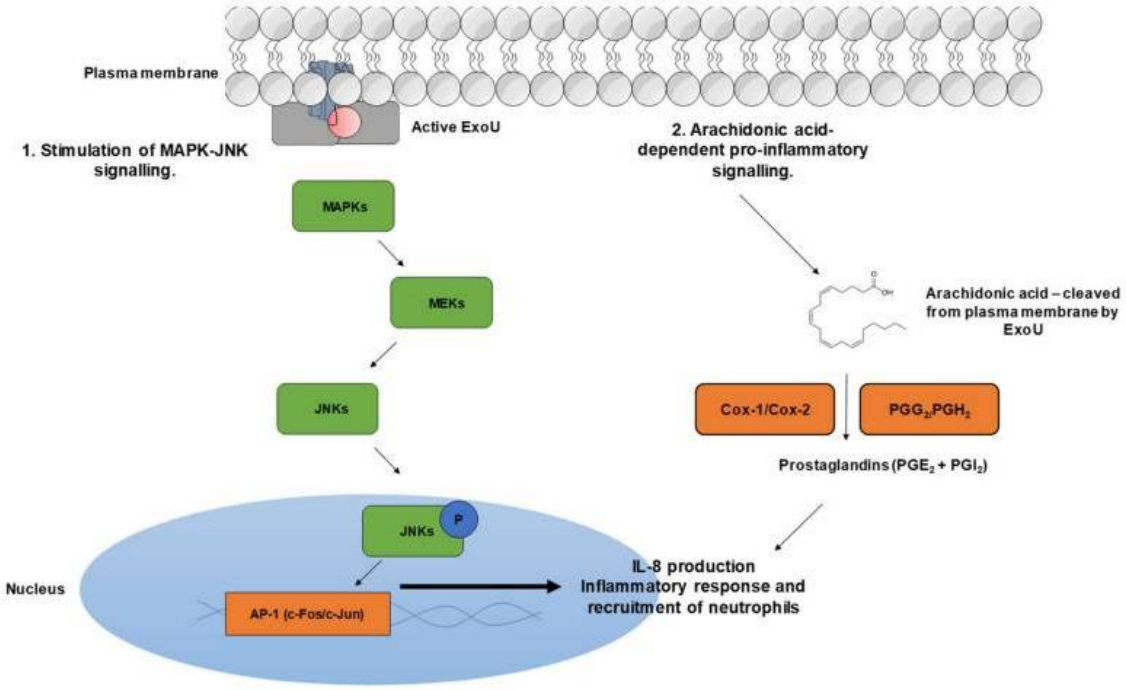


Figure 17: ExoU modulates innate immunity activation and so inflammation. ExoU mediated activation of host cell signalling pathways. 1. The injected active ExoU stimulates the mitogen-activated protein kinase (MAPK) signalling cascade through an unknown mechanism. MEKs phosphorylate c-Jun NH2 terminal kinase (JNKs), which translocate to the nucleus and activate c-Fos and c-Jun transcription factors to stimulate the inflammatory response. 2. Active ExoU cleaves membrane phospholipids at the sn2 position to yield arachidonic acid (AA). Arachidonic acid is converted to prostaglandins PGE_2 and PGI_2 by cyclooxygenases Cox-1 and Cox-2 and endoperoxides PGG_2 and PGH_2. (Foulkes et al., 2019).

a) Inflammatory responses modulation by ExoU

The activation of host immune response via recruitment of immune cells (monocytes/macrophages, neutrophils/granulocytes) aims to eliminate the pathogen.

However, *P. aeruginosa* possesses different effectors which modulate the immune system in order to increase its virulence and escape to the host control.

In 2003, MacMorrane et al., demonstrated that *P. aeruginosa* ExoU+ induced the early and transient up-regulation of gene expression in the AP1 transcription factor complex (McMorran et al., 2003). Later, Cuzick et al., shown in a human bronchial cell line that *P. aeruginosa* ExoU+ could activate the c-Jun NH2 terminal kinase (JNK) mitogen-activated protein kinase (MAPK) pathway, resulting in increased production of active AP-1 transcription factor and so increased IL-8 production (Cuzick et al., 2006; Foulkes et al., 2019). They also demonstrated the JNK and MAPK modulation is dependent on ExoU phospholipase activity by using the active and inactive ones (catalytic dead) (S142A) (Cuzick et al., 2006). The clear molecular mechanism which governs JNK and MAPK regulation by ExoU is still unknown.

ExoU exacerbated arachidonic acid-dependent inflammatory cascade with the increased eicosanoids production such as prostaglandins (PGE2 and PGI2) (Saliba et al., 2005). Indeed, the phospholipase activity of ExoU triggers the release of arachidonic acids, which are subtracts for Cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. These enzymes permit the production of inflammatory mediators such as prostaglandins (PGE2 or PGI2). These molecules are huge inducers of IL-6 and -8 production (J. S. Cho et al., 2014; Kawahara et al., 2015). Another study unveils that infection with *P. aeruginosa* ExoU+ presents an increased concentration of lipid hydroperoxides, 8-isoprostane, reactive oxygen intermediates, peroxynitrite and nitric oxide (NO), when compared to cells infected with ExoU-deficient mutants (da Cunha et al., 2015). This is another potential mechanism of tissue damage during infection.

The interleukins (IL-6; -8) and mediators produce during infection are crucial in establishment of inflammation via the recruitment of neutrophils. Neutrophils recruitment is important in bacterial clearance; however, ExoU triggers a huge recruitment through IL-8 and leukotriene B4 (LTB4) which tissue damage and subvert the innate immune response by increasing epithelial cell permeability and, in turn, potentiate invasion of the bacteria (Cuzick et al., 2006; Pazos et al., 2017). In fact, ExoU+ bacteria preferentially infect and inject ExoU into neutrophils and are capable of rapidly lysing these cells as well as alveolar macrophages and epithelial cells (Diaz et al., 2008; Diaz & Hauser, 2010). In addition, ExoU inhibits the production of other pro-inflammatory cytokines such as IL-1β (Cuzick et al., 2006; Diaz & Hauser, 2010;

Saliba et al., 2005). Therefore, the toxin causes local immunosuppression that allows *P. aeruginosa* to avoid bacterial clearance and promotes its persistence (Diaz et al., 2008).

b) ExoU toxicity

In models of acute pulmonary infection or in infected patients, ExoU+ strains are associated with severe pathologies, such as pneumonia or bacteremia (Allewelt et al., 2000; Finck-Barbançon et al., 1997; A. R. Hauser et al., 1998, 2002; Kurahashi et al., 1999; Pankhaniya et al., 2004; Roy-Burman et al., 2001; Schulert et al., 2003; Shaver & Hauser, 2004). Expression of the toxin U induces epithelial damage and rapid cell death (Finck-Barbançon et al., 1997; Phillips et al., 2003; Shaver & Hauser, 2004). These ExoU-induced lesions also compromise the epithelial barrier, allowing dissemination of the bacteria to other organs and infiltration of cytokines into the systemic circulation, leading to sepsis (Kurahashi et al., 1999).

The toxicity of ExoU in eukaryotic cells is directly linked to its phospholipase A2 activity (Phillips et al., 2003). ExoU intoxication causes disruption of focal adhesions and linkages between integrins, the actin cytoskeleton and the cell membrane, which leads to cell detachment, cytoskeletal collapse, and cell rounding. Subsequently, 3.5 h after infection, membrane blebbing becomes apparent, followed by the loss of plasma membrane integrity, function, and rupture (Sato & Frank, 2014).

Mechanistically, according to Sato and Frank, ExoU would start with hydrolysis of PIP2 which leads to the collapse of the cytoskeleton and the loss of membrane integrity (Sato & Frank, 2014). Indeed, PIP2 is an essential phospholipid, to the organization and dynamics of the cytoskeleton. Thus, the cleavage of PIP2 dislocates the connections between the cytoskeleton and the extracellular matrix, resulting in the rounding and then shrinking of cells, which then detach from their support (Sato & Frank, 2014). Furthermore, the loss of membrane integrity, observed following infection of cells with ExoU+ strains, would result in PIP2 and other neighbouring membrane lipids hydrolysis.

4-Pharmacological target of ExoU

Although the mechanisms of ExoU regulation are not fully understood, however the importance of ubiquitin-binding or ubiquitination, the PIP2 and DNAJC5 have been clearly demonstrated in ExoU phospholipase A2 activity and therefore its associate pathology upon infection. All this result in multiple dynamic conformational changes

that could be targeted by small molecules to attenuate ExoU activity in clinical infections (Foulkes et al., 2019).

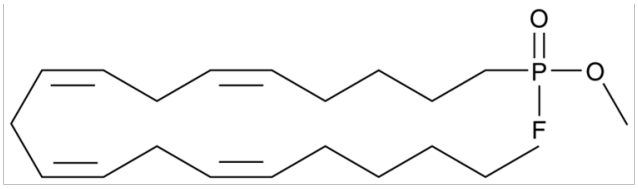
First in 2003, methyl arachidonyl fluorophosphonate (MAFP), an irreversible active phospholipase inhibitor, protects Chinese Hamster Ovary (CHO) cells from ExoU mediated cell lysis after infection with the PA103 clinical isolate strain of *P. aeruginosa* (Phillips et al., 2003). MAFP is an arachidonic acid analogue and a pan PLA2 inhibitor (Figure 18). It possesses a phosphate group that covalently binds to the phospholipase catalytic serine residue. The measurable inhibition of CHO cell lysis by MAFP requires a high concentration (67.5 µM). MAFP molecule was the first proof that ExoU catalytic activity could be targeted by a small molecule inhibitor, and suggests that clinical phospholipase inhibitors, developed to target endogenous human phospholipases for some specific diseases (Nikolaou et al., 2019) could inhibit ExoU activity.

Later, the 9H-fluorene-4-carboxamide, the designated pseudolipasin A (Figure 18), was identified as a promising molecule to inhibit ExoU activity in CHO cells (V. T. Lee et al., 2007). For CHO cells inhibition only 5-7 µM are required, and the IC50 (7 µM) confirm this value *in vitro* with recombinant ExoU.

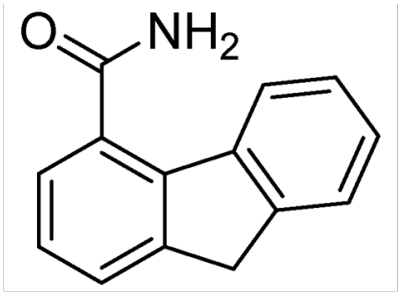
Another independent screen of molecule, identify one derived from sulfonamide, arylsulphonamide, as effective to mitigate the cytotoxic effect of recombinant ExoU expression in yeast (D. Kim et al., 2014). In fact, during the study Saccharomyces cerevisiae were transformed with pDH105, which encoded ExoU cDNA. Upon induction with copper, ExoU expression led to S. cerevisiae lysis except in the presence of 5 µM of arylsulfonamide compound. This phenotype has been confirmed in HEK293 cells (Foulkes et al., 2019; D. Kim et al., 2014). However, this component is less efficient than pseudolipasin A for the same concentration. Very recently, Foulkes et al demonstrated that arylsulphonamide does not inhibit ExoU activity *in vitro* and in HeLa cells (Foulkes et al., 2021).

Two other compounds A and B have been identified as ExoU inhibitors. Foulkes and collaborators showed that 10µM of compound A or B is enough to inhibit cell lysis and Lactate deshydrogenase (LDH) release (cell cytotoxicity assay) from HeLa cells upon infection (Foulkes et al., 2021). Mechanistically these small molecules seem to destabilize ExoU by binding in a non-covalent manner on one region close to the catalytic domain (Foulkes et al., 2021).

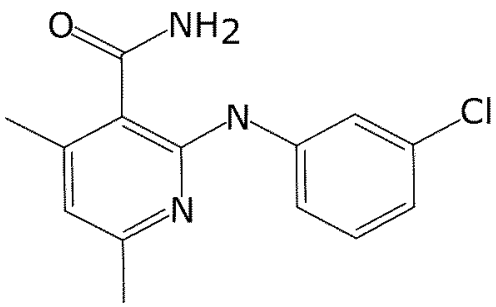
The structure of previously mentioned molecules is completely different so by modulating each on other we can elaborate other molecules more effective in ExoU inhibition and so the improvement of the associated pathology. On the other hand, the molecular mechanism of ExoU after secretion in a cell can be used to target the inhibition of its activity. Indeed, ExoU binding to PIP2, the DNAJC5 recently described as crucial for its activity can be targeted to develop new inhibitors. Furthermore, a strategy for the treatment of *Pseudomonas* ExoU+ related infections may be a combination of antibiotics with ExoU inhibitors, targeting bacteria as well as its U effector.



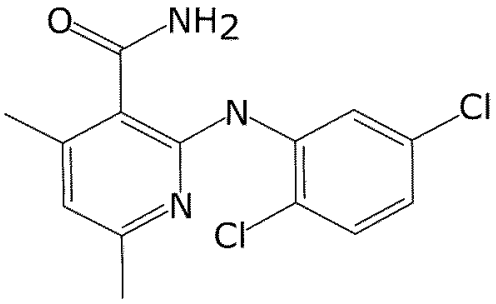
MAFP



Pseudolipasin A



Composé A



Composé B

Figure 18: Inhibitors of ExoU: MAFP (Methoxy arachidonyl fluorophosphonate), Pseudolipasin A, compounds A and B.

Work context and objectives of the thesis

As stated in the introduction, *P. aeruginosa* infection is, to date, one of the major global health issues. About 30% of *P. aeruginosa* isolates express ExoU, and expression of this toxin is associated with antibiotics resistance but also higher mortality and morbidity. ExoU injected into host cells by T3SS, results in necrosis of target cells (e.g., alveolar epithelial cells, macrophages and neutrophils) and tissue necrosis (Diaz & Hauser, 2010). This promotes bacterial proliferation and a fatal risk for cystic fibrosis and immunocompromised patients. ExoU toxicity is dependent on its phospholipase activity, however, the mechanisms involved, and the role of this necrosis remain unknown. Moreover, a better understanding of this necrosis is an important key for the development of therapeutic strategies against *P. aeruginosa* infections. Considering that ferroptosis, one of the regulated cell deaths described in the introduction, is related to membrane lipids and more precisely to the peroxidation of these lipids, we hypothesized that ExoU dependent necrosis could be regulated (ferroptosis). Moreover, upon infection with ExoU+ bacteria there is an increase in the production of lipid peroxidation products and also a decrease in the enzyme GPX4 and its cofactor glutathione (very important in ferroptosis) (da Cunha et al., 2015).

Therefore, the main objectives of my thesis work were to characterize the molecular mechanisms leading to cell death following *P. aeruginosa* ExoU+ infection; and subsequently to analyze the molecules involved during acute pneumonia in mice. I focused on the death of the main immune cells involved in the response to *P. aeruginosa*, macrophages and neutrophils. These cells are the main targets of ExoU and succumb to its toxicity very quickly.

As detailed in part 1, different types of cell death have been characterized as well as the main genes and proteins involved. Thus, the first step was to characterize the type of cell death induced by ExoU. Regarding that, I first used pharmacological inhibitors specific to the types of cell death to make the first screen. Then, cells mutated for specific genes involved in certain deaths were used to confirm the involvement or not of a type of cell death in *P. aeruginosa* ExoU+ infection. Briefly, we have shown that only the ferroptosis inhibitors are able to block ExoU death in macrophages and neutrophils. Moreover, we confirmed that this toxicity is observed in neutrophils,

macrophages but also in the cell lines A549, HeLa HEK, HBE, HAP1. We then showed that ExoU uses the oxidized phospholipids of the cell to exert its activity (see more on Part 3rd Results - chapter 4). Chronologically this study took the last two years of the thesis.

Regarding the first year of the thesis, it was focused on the study of the IRGM2 protein involvement in the regulation of intracellular bacterial infections. Indeed, IRGM2 is a protein induced by IFN cytokines produced during inflammation. We have shown that IRGM2 controls infectious sepsis by regulating pyroptotic death. Thus, mice mutated for this gene have a rapid death following infection compared to WT mice (see Part 3rd Results - chapter 5). Thus, the two studies conducted during this thesis were the subject of two scientific publications (see Part 2 Results).

Part 2:

Results

Part 2: Results

Chapter 4: Host phospholipid peroxidation fuels ExoU-dependent cell necrosis and supports *P. aeruginosa*-driven pathology

I-Study context and objectives

As a consequence of its resistance to antibiotic treatments, the opportunistic bacterium *P. aeruginosa* has been extensively studied over the past 30 years to find new therapeutic strategies. The molecular mechanisms of the different virulence factors have been demonstrated, in particular T3SS and its secreted effectors. Among these effectors, ExoU is the most toxic when injected into all immune and non-immune cell types. It has been shown that during acute pneumonia ExoU is preferentially injected into recruited immune cells including neutrophils and monocytes/macrophages within a few hours of infection (Diaz & Hauser, 2010). Macrophages and neutrophils are privileged targets of bacteria because of their great ability to destroy them via different mechanisms (described in the 1st part of chapter 3). This demonstrates that ExoU is very rapidly employed to modulate the host immune system in order to better persistence. Indeed ExoU, through its phospholipase activity, degrades plasma membrane lipids, which leads to the release of cytosolic contents. These mechanisms result in the death of the recruited immune cells, thus no restriction but a strong bacterial proliferation. It is therefore important to understand which cellular factors allow this cell lysis by ExoU in order to help the immune system to fight the infection. Thus, we were interested in this ExoU-dependent necrosis during infection, knowing that cell death can be regulated, we hypothesized that ExoU death can be regulated, and more particulate can be ferroptosis. Indeed, ferroptosis is a form of cell necrosis that involves an accumulation of oxidized lipids and/or a defect in the cell's ability to repair lipids (e.g., deficiency in Gpx4 enzyme activity) or detoxification of reactive oxygen species (ROS) (see Chapter 2). Therefore, the accumulation of peroxidized lipids promotes plasma membrane rupture and necrosis. Thus, the objectives of this part of my thesis work were to characterize: (1) the immune modulation triggers by ExoU, (2) the type of cell death induced by ExoU, (3) the consequences of ExoU-dependent cell death the modulation in the pulmonary pathology and bacteria persistence.

II-Results

A-Scientific contribution to the paper

The results of this study were published in the following article:

"**Host phospholipid peroxidation fuels ExoU-dependent cell necrosis and supports *Pseudomonas aeruginosa*-driven pathology**"

Salimata Bagayoko, Stephen Adonai Leon-Icaza, Miriam Pinilla, Audrey Hessel ,Karin Santoni, David Péricat, Pierre-Jean Bordignon, Flavie Moreau, Elif Eren, Aurélien Boyancé, Emmanuelle Naser, Lise Lefèvre, Céline Berrone, Nino Iakobachvili, Arnaud Metais, Yoann Rombouts, Geanncarlo Lugo-Villarino, Agnès Coste, Ina Attrée, Dara W. Frank, Hans Clevers, Peter J. Peters, Céline Cougoule, Rémi Planès, Etienne Meunier

PLoS Pathogens Journal 2021 Sep. doi: 10.1371/journal.ppat.1009927

In this study, I performed or participated in the achievement of all the experiments except the production of the recombinant protein ExoU, the phospholipid redox lipidomic experiments, the generation of human bronchial organoids and mice infection that was performed in Biosecurity level 3 (BSL3). Actually, the human organoids have been generated by Celine Cougoule and Stephen Leon-Icaza and I provided assistance in terms of bacterial culture and analysis of the results. Regarding, the recombinant ExoU protein, it was produced by Audrey Hessel, and the phospholipid redox lipidomic experiments were performed by Cayman Chemical Company (Ann Arbor, USA) Remi Planes, David Pericat and Etienne Meunier performed the mice infection and I helped with the bacteria and the inhibitor molecules preparation.

B-Paper

doi.org/10.1371/journal.ppat.1009927 (to add)

PLOS PATHOGENS

RESEARCH ARTICLE

Host phospholipid peroxidation fuels ExoU-dependent cell necrosis and supports *Pseudomonas aeruginosa*-driven pathology

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Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files S1 and S2 Datas.

Abstract

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.

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Competing interests: The authors have declared that no competing interests exist.

Author summary

Although a proper activation of various regulated cell necrosis confer a significant advantage against various infectious agents, their dysregulation drives host tissue damages that can end up with fatal sepsis. Specifically, 30% of the bacterial strains of *Pseudomonas aeruginosa* (*P. aeruginosa*) express the phospholipase A2-like toxin ExoU that is injected into host target cells through the Type-3 Secretion System. This toxin induces, through a yet unknown mechanism, a strong and fast necrotic cell death that supports fatal respiratory infections. Therefore, in this study, we sought to determine the cellular mechanisms by which ExoU triggers host cell necrosis. In this context, we found that ExoU exploits basal cellular phospholipid peroxidation to promote cell necrosis. Mechanistically, host cell lipid peroxidation stimulates ExoU phospholipase activity, which then triggers a pathological cell necrosis both *in vitro* and *in vivo*. Altogether, our results unveil that targeting host cell lipid peroxidation constitutes a virulence mechanism developed by microbial phospholipases, a process that contributes to *P. aeruginosa*-mediated pathology.

Introduction

Regulated cell necrosis (RCNs) drives physiological and immune processes, yet dysregulation of this process promotes pathological responses such as organ-failure and sepsis [1–4]. 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 [5–8]. 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 [9,10]. In addition, the peroxidation of the mitochondrial phospholipid cardiolipin initiates apoptosis while the accumulation of peroxidised phosphatidyl ethanolamines (PE) promote the cellular necrosis, ferroptosis [11–17]. 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 [18–23]. 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 balanced in cells [5,11,12,14,24]. 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 [5,14–16,25,26].

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 [27–36]. In presence of cellular co-factors such as ubiquitin [31] or the trafficking chaperone DNAJC5 [37], 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 [32,38–40]. 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 [38,41]. In this context, we explored the possibility that *P. aeruginosa* ExoU mediates a necrosis-dependent host pathology involving lipid peroxidation.

Results

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) [28,36], 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 [27,29,42,43], 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 [42,44,45–52,53], 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 (**S1A and S1B Fig**). 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 [42,54]. Therefore, we primarily focused our analysis on alarmin release. We observed a strong ExoU-dependent alarmin production in BALFs 6

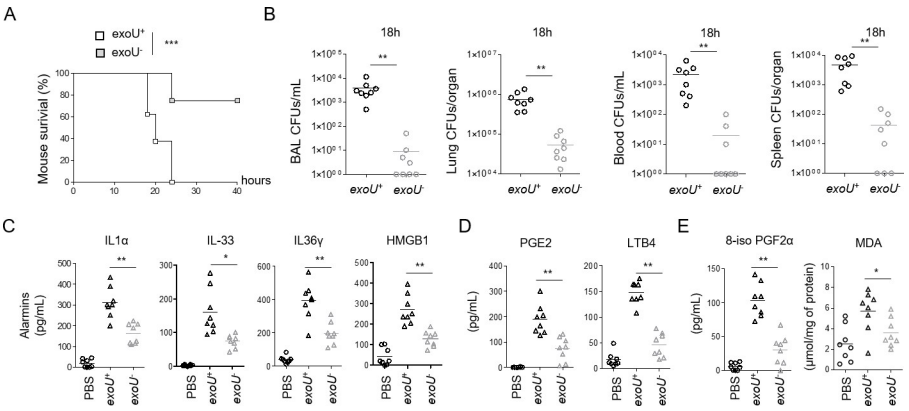

Fig 1. ExoU-dependent lung pathology in mice associates to an alarmin and peroxidized lipid signature. (A) Survival of WT mice intranasally infected (n = 7 animals per condition) with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. Graphs represent one experiment (8 mice/group) out of three independent *in vivo* experiments. Log-rank Cox-Mantel test was used for survival comparisons. $^{***}p \leq 0.001$. **(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. $^{**}p \leq 0.01$, 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; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, 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; $^{**}p \leq 0.01$, 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; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, Mann-Whitney analysis test. Data information: Data shown as means (**Graphs B-E**) and are representative of one experiment performed three times; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, Mann-Whitney analysis test (**B-E**) and log-rank Cox-Mantel test for survival comparisons (**A**).

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h after infection, such as IL-1 family alarmins IL1α, IL-33 or IL-36γ [55] (**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**) [38–40]. 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**) [41,56].

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 ExoU can promote an NLRC4 inflammasome response in macrophages [50], 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 [57], repressed ExoU-dependent cell necrosis (**Figs 2A and 2B and S2A and S1–S6 Movies**). Ferrostatin-1 action was specific to lipid peroxidation-dependent cell necrosis as it also inhibited Cumene hydroperoxide-induced ferroptosis (CuOOH, 400μM) but not Flagellin-/LPS-induced pyroptosis or TCPA-1/Z-VAD/TNFα-dependent necroptosis (**Fig 2B**). 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 2C**), 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* (**S2A and S2B Fig**). ExoU exhibits a calcium-independent phospholipase A2-like activity [33]. Hence, we transfected recombinant ExoU protein (rExoU) or its enzymatically inactive mutant ExoU^{S142A} [58] 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 (**S2C Fig**). In line, we found that ferrostatin-1 itself did not alter bacterial growth or ExoU secretion (**S2D and S2E Fig**), 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,

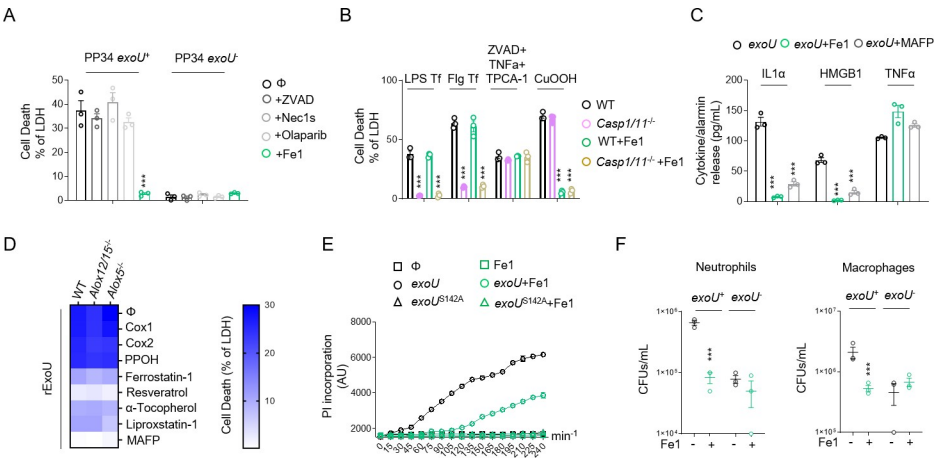

Fig 2. Lipid peroxidation inhibition delays ExoU-induced cell necrosis. Otherwise specified, cells were infected with an MOI of 0.5 of *P. aeruginosa* PP34, PP34^{exoU-} or PP34^{exoUS142A} for various times. $^{***}p \leq 0.001$, t-test with Bonferroni correction. **(A)** Measure of LDH release in *Nlrc4*$^{-/-}$ 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. $^{***}p \leq 0.001$, t-test with Bonferroni correction. **(B)** Measure of LDH release in WT or *Casp1*$^{-/-}$/*Casp11*$^{-/-}$ BMDMs transfected (Lipofectamin 2000) with 1μg of LPS or Flagellin (Flg) to induce pyroptosis, treated with Z-VAD (40μM)/TNFα (500UI/mL)/TPCA-1 (5 μM) to induce necroptosis or with Cumene hydroperoxide (CuOOH, 400μM) to induce ferroptosis in presence or absence of Ferrostatin-1 (Fe1, 10μM) for 6 hours. $^{***}p \leq 0.001$, t-test with Bonferroni correction. **(C)** Measure of 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. $^{***}p \leq 0.001$, 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 or PP34^{exoUS142A} in presence/absence of Ferrostatin-1 (Fe1, 20μM). $^{***}p \leq 0.001$, 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). $^{***}p \leq 0.001$, t-test with Bonferroni correction. Data information: Data are represented as means +/- SEM (graphs A- F) from n = 3 independent pooled experiments; $^{***}P \leq 0.001$ for the indicated comparisons using t-test with Bonferroni correction.

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suggesting either that 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). Regarding this, the replenishment of *P. aeruginosa*-infected cells with fresh ferrostatin-1 each hour strongly improved cell viability, suggesting that the instability of Fe1 might also account in the delayed ExoU-induced cell necrosis we observed (S2F Fig). 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 [5]. 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 and S3A and S3B**). As control, the well-known lipid peroxidation inducer Cumene hydroperoxide (CuOOH) promoted cellular lipid peroxidation (**Fig 3A**) [59]. In contrast, we observed that basal lipid peroxidation in cells was reduced upon ExoU transfection or PP34 infection, a process that was further strengthened in presence of ferrostatin-1 (**Figs 3A and S3B**).

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 [60–62]. To address this hypothesis, we performed a redox phospholipidomic approach to determine if ExoU could interfere with the endogenous levels of peroxidised phospholipids (**Figs 3B and S3C).** 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 (**S3D Fig**). 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 and S3C).**

In cells, peroxidised phospholipids are detoxified by various factors, one of the most important being the ferroptosis regulator glutathione peroxidase 4 (GPX4) [5]. Consequently, the use of pro oxidant molecules or *Gpx4* genetic inactivation both induce a strong accumulation of various peroxidised phospholipids in cell membranes [5]. Therefore, we hypothesized that prestimulation of macrophages with non-cytotoxic doses of the lipid peroxidation and ferroptosis inducer Cumene hydroperoxide (20μM, 1h) might sensitize cells to ExoU-induced cell necrosis. We transfected recombinant (r)ExoU in WT BMDMs in presence or absence of non-toxic doses of the pro-oxidant Cumen hydroperoxide (CuOOH, 20μM, 1h) [59]. Although CuOOH promoted lipid peroxidation but not BMDM cell death, rExoU transfection specifically induced an increased cell necrosis in CuOOH-primed BMDMs, a process that was inhibited by the use of ferrostatin-1 (**Fig 3C and 3D**). In agreement with this result, we measured a strong decrease in lipid peroxidation in CuOOH-primed cells transfected with rExoU (**Fig 3C and 3D**), confirming that ExoU efficiently targeted lipid peroxides induced by CuOOH. In addition, microscopy observations of CuOOH-primed cells highlighted a decrease of peroxidized lipids at the plasma membrane upon infection by ExoU-expressing strain of *P. aeruginosa* (PP34), suggesting that ExoU mostly target plasma membrane peroxidized phospholipids to promote cell necrosis (**Fig 3E**). The enzyme cytochrome p450 oxidoreductase (CYPOR) has recently been found to be an important provider of peroxidized phospholipids upon ferroptosis induction; we hypothesized that ExoU function might be regulated by CYPOR-regulated phospholipid peroxidation. We acquired *Cypor*-deficient HeLa cells but also generated *Cypor*$^{-/-}$ immortalized (i)BMDMs using CRISPR (**S3E Fig**) and evaluated the importance of CYPOR on ExoU-driven cell necrosis. PP34 infection of WT and *Cypor*$^{-/-}$ immortalized BMDMs triggered similar cell deaths, suggesting that in resting cells, CYPOR does not promote the basal lipid peroxidation involved in ExoU-dependent cell necrosis (**Fig 3G and 3H**). However, in CuOOH-primed macrophages, where phospholipid peroxidation is induced, we observed that CYPOR was a major contributor of phospholipid peroxidation (**S3F Fig**). This was associated to enhanced ability of PP34 to trigger cell necrosis in CuOOH-primed WT but not in *Cypor*$^{-/-}$ iBMDMs and HeLa cells (**Fig 3G and 3H**), which suggests that CYPOR-induced lipid peroxidation heightens ExoU-dependent toxicity. However, in resting cells,

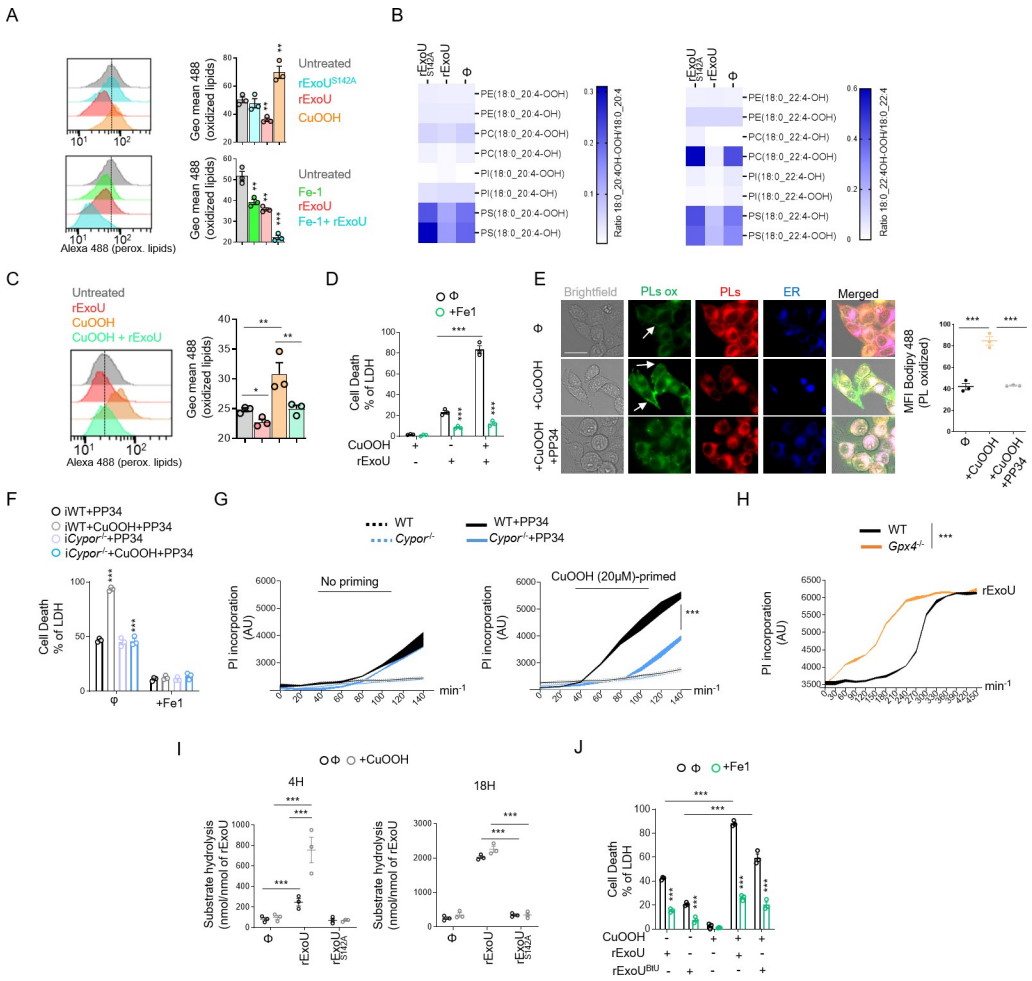

Fig 3. ExoU-induced cell death involves ROS-induced lipid peroxidation but proceeds in a ferroptosis independent manner. (A) Cytometry detection and quantification of (phospho)lipid peroxidation using the probe C11-bodipy in WT BMDMs treated with CuOOH (20μM) or transfected with rExoU (500ng) or its 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. $^{**}P \leq 0.001$, $^{***}P \leq 0.001$ 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 (S3B Fig). 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. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$, 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. $^{***}p \leq 0.001$, T-test with Bonferroni correction. **(E)** Representative microscopy images (phospho)lipid peroxidation and quantifications using the probe C11-bodipy in CuOOH-primed (20μM) HELA cells infected with PP34 (MOI5) for 2 hours. Images show two independent experiments, each performed three times at 2 hours post infection. Scale bar 20μm; Green, oxidized bodipy (oxidized phospholipids, PLs ox); Red, bodipy (phospholipids, PLs); Blue (Endoplasmic Reticulum, ER tracker probe, 1μM). Arrows show enriched peroxidised phospholipids in the plasma membrane area. Quantifications show the Mean Fluorescence Intensity (MFI) quantification of Peroxidized lipids from one experiment performed three times (50–60 cells counted). $^{***}P<0.001$ by T-test. **(F)** Measure of LDH release in immortalized (i) WT or *Cypor*$^{-/-}$ BMDMs primed or not with CuOOH (20μM, 1hour) in presence or absence of ferrostatin-1 (20μM) and infected for 2 hours with PP34. $^{***}p \leq 0.001$, T-test with Bonferroni correction. **(G)** Time course measure of plasma membrane permeabilization using propidium iodide incorporation in WT and *Cypor*$^{-/-}$ HELA cells primed or not with CuOOH (20μM, 1hour) and infected with PP34 (MOI5) for 2 hours. $^{***}p \leq 0.001$, T-test with Bonferroni correction. **(H)** Time course measure of plasma membrane permeabilization using propidium iodide incorporation in immortalised WT and *Gpx4*$^{-/-}$ BMDMs transfected with rExoU (500ng) for 7 hours. $^{***}p \leq 0.001$, T-test with Bonferroni correction. **(I)** ExoU phospholipase

activity determination in WT BMDM lysates 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. $^{***}p \leq 0.001$, T-test with Bonferroni correction. **(J)** 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. $^{***}p \leq 0.001$, T-test with Bonferroni correction. Data information: Data are plotted as means+/- SEM (**D, F-J**) from n = 3 independent pooled experiments; $^{***}P \leq 0.001$ for the indicated comparisons using t-test with Bonferroni correction.

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basal lipid peroxidation appears to be regulated by other processes/enzymes. Finally, using Crispr-Cas9, we generated *Gpx4*$^{-/-}$ immortalized BMDMs (S3G Fig). As previously observed by others in other cell lines [16,24], *Gpx4*$^{-/-}$ immortalized BMDMs exhibited increased basal levels of peroxidised lipids (S3H Fig). Therefore, rExoU transfection triggered faster cell death of *Gpx4*$^{-/-}$ macrophages than their WT counterpart, suggesting that lipid peroxidation of cells enhances ExoU-dependent toxicity (**Figs 3H and S3G and S3H**).

Upon phospholipid peroxidation, arachidonic acid-containing phospholipids form isoprostanes that are potent intra- and extra-cellular inflammatory mediators [9,10]. Once formed, these isoprostanes are released from phospholipids by the action of phospholipases [9,10]. 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 (S3I Fig). Of importance, ferrostatin-1 strongly inhibited ExoU- and ExoU/CuOOH-induced 8-PGF2α release (S3I Fig). 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) (S3J Fig). 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 3I). 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 3I). 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 3I). 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* ($ExoU^{BtU}$) [31]. 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 3J). 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 and 4C). 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 **and** 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 to evaluate if *P. aeruginosa* ExoU would trigger 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 **and** S7–S12 **Movies**). Importantly, ferrostatin-1 strongly attenuated *P. aeruginosa*-dependent organoid damages (Fig 4G **and** S7–S12 **Movies**). Altogether, our results identified that *P. aeruginosa* ExoU phospholipase benefits from lipid peroxidation to trigger pathology 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 [41]. 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 [38,40,63]. However, results from others and ours mostly suggest that, taken individually, those enzymes only play a negligible role in ExoU-induced cell necrosis [38,40,63]. Regarding the central cell types involved in ExoU-induced pathology, previous studies identified macrophages and

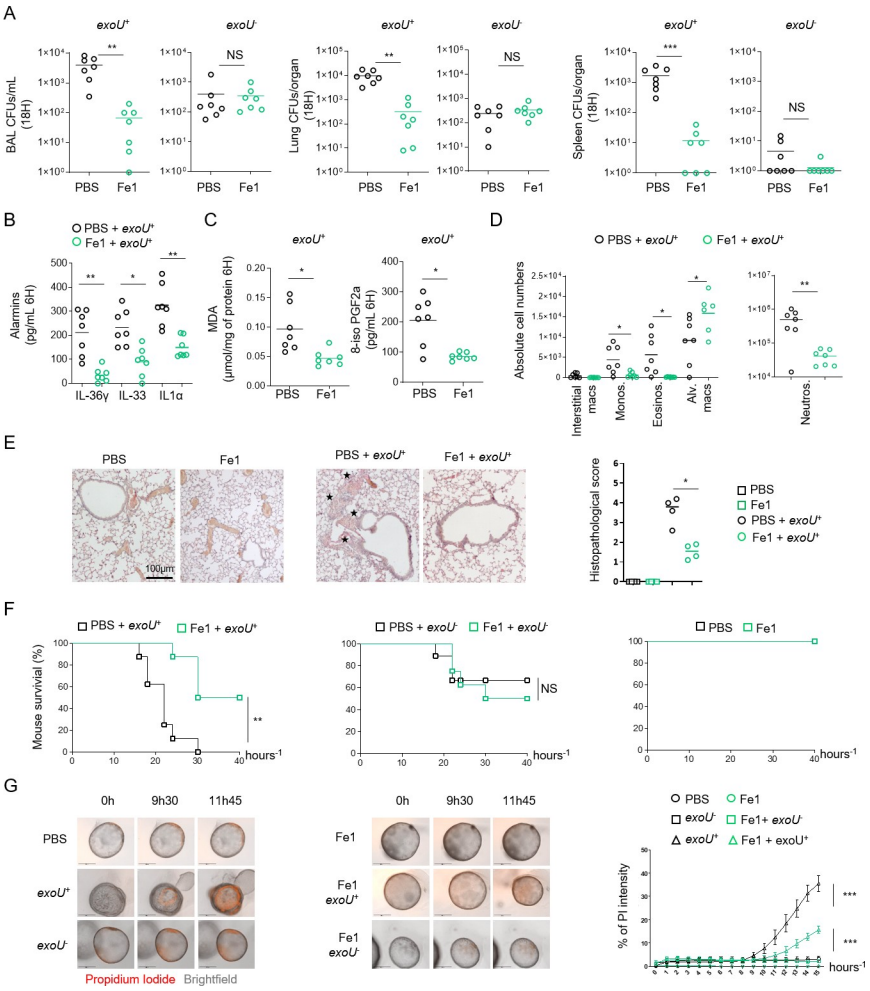

Fig 4. Ferrostatin-1 protects mice against ExoU-induced lung pathology. (A) Bronchoalveolar (BAL), lung and spleen bacterial loads from WT mice (n = 7/group) 18 hours after intranasal infection with 5.10^5 CFUs of *P. aeruginosa* PP34 or its isogenic mutant PP34^{exoU-}. When specified, mice were intraperitoneally 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. $^{**}p \leq 0.01$, 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; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, 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; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, 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. $^{*}p \leq 0.05$; 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; $^{**}p \leq 0.01$, 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. $^{***}p \leq 0.001$, T-test with Bonferroni correction. Data information: Data shown as means (**Graphs A-E**) and are representative of one experiment performed three times; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, Mann-Whitney analysis test (**A-E**) and log-rank Cox-Mantel test for survival comparisons (**F**).

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neutrophils as central targets of ExoU injection by *P. aeruginosa*. Therefore, future studies will be of importance to determine if the respective contribution of each cell type in pathology induced by ExoU-exploited lipid peroxidation. Regarding this, our *in vivo* observations that targeting lipid peroxidation confers some protection of mice against ExoU-dependent pathology is to put in the light of a decrease in some eicosanoid production such as LTB4 and PGE2, two important modulators of ExoU-driven pathology [38,40,63]. Therefore, the use of *Alox5*$^{-/-}$ or *Cox*$^{-/-}$ mice, unable to generate LTB4 or various prostaglandins respectively, will also help to determine the respective importance of eicosanoid burst or cell necrosis upon lipid peroxidation-driven ExoU activity.

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 [9,10,64]. A question in both basal lipid peroxidation and ferroptosis-induced lipid peroxidation lies on the compartment phospholipid peroxidation occurs. Peroxisiomes are key at providing ether-phospholipids that will be peroxidised upon ferroptosis induction, the Endoplasmic reticulum is also a central regulator of phospholipid turn over and plasma membrane constitutes the probable location of lipid peroxidation-driven cell lysis upon ferroptosis induction [16,65,66]. Our observations also suggest that although lipid peroxidation can occur in various cellular compartments, ExoU-induced cell necrosis mostly arises from plasma membrane cleaved peroxidized phospholipids. Yet, this does not exclude at all that phospholipid peroxidation could occur in other intracellular organelles, including the endoplasmic reticulum.

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 [61,62,67–71]. It is important to notice that both the iPLA2beta and iPLA2g belong to the patatin-like phospholipase family, as ExoU, which suggests that this family of phospholipases might have some conserved affinities to peroxidzed phospholipids [72]. 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 [71]. 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. Although, the identification of cellular enzymatic systems that promote basal lipid peroxidation remains to be explored and characterized, 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, oxidant-activated cytochrome P450 oxidoreductase CYPOR, a crucial regulator of ferroptosis, strongly enhanced ExoU-dependent cell necrosis, which suggests a important link between ferroptosis-regulated pathways and ExoU activity. Should other regulators of ferroptosis such as ACSL and LPCAT acyl transferases on ExoU-dependent toxicity warrants further investigations [15].

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 [73–75]. 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 [73]. 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 [70]. 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 [76], 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) [77] 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 [22]. 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 [78]. Thus, our findings that the bacterial patatin-like phospholipase A2 ExoU contributes to pathology by exploiting target cell 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 (**S1 Graphical Abstract**).

Material and method

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)".

Animal experiments were approved by local (CE01 committee) and national ethic committees (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).

Mice

Nlrc4$^{-/-}$, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *GsdmD*$^{-/-}$, *ALOX12/15*$^{-/-}$and *ALOX5*$^{-/-}$mice were generated and described in previous studies [79–82]. 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.

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 1.

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 [85].

Murine Bone Marrow-Derived Eosinophils were differentiated *in-vitro* from bone marrow as previously described [86]. 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 medium containing 10 ng/ml recombinant mouse (rm) IL-5 (R&D Systems) only. Medium was replaced every 4 days and the concentration of the cells was adjusted each time to106/ml. After 10 to 14 days of culture, cells were recovered by gentle pipetting and used as Eosinophils in our experiments. Over 95% of cells had the standard phenotype of Eosinophils: CD11b+ Siglec F + after FACS analysis.

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.	[37]
CYPOR 1/1000	abcam	ab180597
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.	[37]
PP34*exoU*$^{-}$	Ina Attree/CNRS, France.	[37]
PP34*exoU*^{S142A}	Ina Attree/CNRS, France.	[37]
CHA	Ina Attree/CNRS, France.	[37]
CHAdST	Ina Attree/CNRS, France.	[37]
CHAdST*exoU*$^{+}$	Ina Attree/CNRS, France.	[37]
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	[31]
Recombinant ExoUS142A	This study	[31]
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	248614-100g
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	000000011697498001
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

(*Continued*)

Table 1. (Continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
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
TPCA-1	Tocris	2559
mTNFa	abcam	ab259411
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
RspoI	In house	NA
Noggin	In house	NA
B27	Gibco/Invitrogen	17504044

(*Continued*)

Table 1. (Continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
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
ER-Tracker Blue-White DPX, for live-cell imaging	Invitrogen	E12353
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	[79]
Alox12/15-/- C57Bl6 mice	A.Coste	[79]
Nlrc4-/- C57Bl6 mice	C.Bryant	[80]
Casp1-/-/Casp11-/- C57Bl6 mice	B.Py/ Junying Yuan	[81]
GsdmD-/- C57Bl6 mice	P.Broz	[82]
Human Bronchial organoids	This sudy	[83,84]
Oligonucleotides		

(*Continued*)

Table 1. (Continued)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Guide Crispr mGpx4- Exon1 Forward	Sigma-Aldrich	GGACGCTGCAGACAGCGCGG
Guide Crispr mCypor- Exon1 Forward	Sigma-Aldrich	
Recombinant DNA		
Plasmid: ExoU	[31]	[31]
Plasmid: ExoUS142A	[31]	[31]
Plasmid: ExoU^{BtU}	[31]	[31]
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	FlowJo LLC	
Benchling Software		
Other		

https://doi.org/10.1371/journal.ppat.1009927.t001

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

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

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

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

Cell line culture

Immortalized murine bone-marrow derived macrophages have been described previously [85]. U937 cells were cultured in RPMI glutamax medium containing 10% FBS, 100 IU/ml penicillin and 10 μg/ml streptomycin, 1 mM sodium pyruvate (Life Technologies), and 50 μM 2-ME (Sigma-Aldrich). Medium was renewed every 3 days and the concentration of the cells was adjusted each time to 5x10^5/ml. A549, HeLa and HBE cells were cultured in DMEM glutamax medium with HEPES containing 10% FBS, 100 IU/ml penicillin and 10 μg/ml streptomycin, 1 mM 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 [87]. Briefly, PBMCs were isolated by centrifugation using standard Ficoll-Paque density (GE Healthcare) [85]. 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 and Cypor* 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' GGACGCTGCAGA-CAGCGCGG 3' *Cypor* exon2 (for 5' AGTGTCTCTATTCAGCACAA 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 [83,84]. 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. [31]. 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. ExoU and ExoUS142A activities were validated on cellular lysates (**Fig 3I**) based on the advices and experience of our collaborator [37].

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 Ferrostatin-1 (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 and Method" 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.

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/treated 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 or a FACS Calibur). Data were analysed with FlowJO software (version 10). When specified, adherent cells loaded with Bodipy probes where infected at indicated MOIs of *P. aeruginosa* and lipid peroxidation is observed using an EVOS7000 microscope. For live imaging, the GFP brightness threshold was kept equal for all the independent experiments. Mean fluorescence intensity (MFI) was analyzed using Fiji/ImageJ.

Immunoblotting

Cell lysate generation has been described previously [85]. Briefly, proteins were loaded in 12% SDS-PAGE gels and then transferred on PVDF membranes. After saturation for 1 hour in Tris-buffered saline (TBS) supplemented with 0.05% Tween 20 containing 5% non-fat milk (pH8), membranes were exposed with antibodies at 4˚C overnight (Table 1). Next day,

membranes were washed 3 times in TBS 0.1% Tween 20 and incubated with the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) (Table 1) for 1h at room temperature. Immunoblottings were revealed using a chemiluminescent substrate ECL substrate (Biorad) and images were acquired on a ChemiDoc Imaging System (Biorad). All antibodies and their working concentrations are listed in **Table 1**.

(Redox) lipidomic

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

After thawing, lipids were extracted using a methyl-tert-butyl ether (MTBE)-based liquid-liquid extraction method. Cell suspensions (500 μL in PBS/methanol 1:1, v/v) were thawed on ice before adding 100 μL methanol MeOH containing 50 ng each of the internal standards PC (15:0/18:1-d7), PE(15:0/18:1- d7), PG(15:0/18:1-d7), PI(15:0/18:1-d7) and PS(15:0/18:1-d7) (EquiSPLASH, Avanti Polar Lipids). Samples were then transferred into 8-mL screw-cap tubes, and then 1.125 methanol and 5 mL MTBE were added. After vigorous mixing, samples were incubated at room temperature on a tabletop shaker for 45 min. For phase separation, 1.25 mL water was added, and samples were vortexed and centrifuged for 15 min at 2000 x g. The upper organic phase of each sample was carefully removed using a Pasteur pipette, transferred into an empty glass round-bottom tube, and dried under vacuum in a SpeedVac concentrator. The dried lipid extracts were resuspended in 200 μL HPLC mobile phase A/mobile phase B 3:1 (v/v) for targeted lipidomic analysis of oxidized 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 [37]. 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 manufacturer 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.

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 $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Supporting information

S1 Data. Original immunoblotting membranes.
(TIF)

S2 Data. Numerical values obtained in the current study.
(XLSX)

S1 Fig. 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.
(TIF)

S2 Fig. Lipid peroxidation contributes to ExoU-induced necrosis in various cell types. (A, 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)** LDH release in BMDMs transfected with recombinant ExoU (100ng) or its catalytically inactive mutant ExoU^{S142A}, in presence of MAFP (50μM) or Ferrostatin-1 (Fe1, 10μM) for 3 hours. $^{***}p \leq 0.001$, T-test with Bonferroni correction. **(D)** Immunoblotting of ExoU secretion by *P. aeruginosa* in presence of ferrostatin-1 (20μM). Star (*) show non-specific bands. **(E)** Measure of bacterial growth (O.D 600) in presence or absence of ferrostatin-1 (10, 20μM) for 14 hours). **(F)** Measure of LDH release in *Nlrc4*$^{-/-}$ BMDMs infected with PP34 (MOI5) in presence of Ferrostatin-1 (Fe1, 10μM) for 3 hours. Each hour, fresh Ferrostatin-1 (10μM) was added to cells (+) or not (φ). "pi" refers to post-infection.
(TIF)

S3 Fig. Lipid 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)** Cytometry detection and quantification of (phospho)lipid peroxidation using the probe C11-bodipy in WT BMDMs infected with PP34^{ExoU+} or PP34^{ExoU-} (MOI 5) for 1 hour. Sample were acquired using FACSCalibur (BD). The graph shows the mean+/-SEM of one experiment performed in triplicate out of three independent experiments. $^{*}P \leq 0.05$, for the indicated comparisons using t-test with Bonferroni correction. **(C)** Lipidomic analysis of the relative amount of each phospholipid upon rExoU transfection analysed in Fig 3B. **(D)** Representative microscopy images and time course experiment of propidium iodide uptake in WT BMDMs transfected with rExoU or its catalytically inactive mutant ExoU^{S142A} (500ng) in presence or not of ferrostatin-1 (Fe1, 10μM). Images show two independent experiments, each performed three times at 45 minutes or 3 hours post transfection. **(E)** Immunoblotting of Crispr Cas9-mediated *Cypor* gene deletion in immortalized (i)BMDMs or of *Cypor*-deficient HELA cells. The *Cypor*#2 (red) was selected for further analysis. GFP means that cells were transduced with sgRNA targeting *Gfp* and used as control. **(F)** Cytometry detection and

quantification of (phospho)lipid peroxidation using the probe C11-bodipy in WT or *Cypor*$^{-/-}$ immortalized (i)BMDMs pre-treated or not for 1 hour with CuOOH (20μM) in presence or absence of Ferrostatin-1 (20μM) and then infected with PP34^{ExoU+} or PP34^{ExoU-} (MOI 5) 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. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$, for the indicated comparisons using t-test with Bonferroni correction. **(G)** 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. **(H)** Cytometry detection and quantification of phospholipid peroxidation using the probe C11-bodipy in immortalized WT or *Gpx4*$^{-/-}$ BMDMs using a fortessa cytometer. **(I)** 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. $^{***}p \leq 0.001$, T-test with Bonferroni correction. **(J)** PGE2 and LTB4 eicosanoid release in WT BMDMs pre-treated or not for 1 hour with CuOOH (20μM) and then transfected with 100ng of ExoU or its catalytically dead mutant ExoU^{S142A} for 3 hours.
(TIF)

S4 Fig. 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.
(TIF)

S1 Graphical Abstract. Host lipid peroxidation fuels ExoU-induced cell necrosis-dependent pathology. In resting cells or in cells with induced lipid peroxidation (e.g. ferroptosis pathway), ExoU (purple) becomes hyper-activated by host cell peroxidised phospholipids, which drives an exacerbated cell necrosis, alarmin and lipid release and contributes to the subsequent pathology. Consequently, targeting lipid peroxidation (ferrostatin-1) inhibits ExoU-dependent cell necrosis and attenuates the host deleterious consequences. EM and SB used Biorender.com to create this figure.
(TIF)

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

S2 Movie. 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.
(AVI)

S3 Movie. 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.
(AVI)

S4 Movie. 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.
(AVI)

S5 Movie. 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.
(AVI)

S6 Movie. 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.
(AVI)

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

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

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

S10 Movie. 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.
(AVI)

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

S12 Movie. 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.
(AVI)

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III-Discussion and perspectives

P. aeruginosa is a ubiquitous Gram-negative pathogen that typically causes disease in immune-compromised patients. Its infection in the airway is a significant source of morbidity and mortality in patients with cystic fibrosis (CF). *P. aeruginosa*, a preferential extracellular pathogen, 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 are mainly associated with the development of chronic infections, exoU-expressing *P. aeruginosa* essentially triggers acute deadly infections. Indeed, ExoU-associated toxicity is mediated by its lysophospholipase activity (PLA2). In addition to inducing cytolysis/toxicity, ExoU PLA2 function has been associated with a strong oxidative imbalance in particular modulation of arachidonic acid (AA) and subsequent eicosanoid generation, such as PGE2 (Pazos et al., 2017; Plotkowski et al., 2008; Saliba et al., 2005).

Therefore, in this study, we first confirmed with our strains of bacteria the previous observation of *P. aeruginosa* ExoU+ infected mice in comparison with the ExoU-. As well described in our paper and previous ones, upon infection with ExoU expressing bacteria there is a huge release of alarmins (IL-1a, -33, -38g and HMGB1) and peroxided lipids (PGE2, LTB4, 8isoPGF2a, MDA), and high bacterial load in blood, Broncho Alveolar Lavage (BAL), lung and spleen. All together, these confirmed that ExoU is responsible for the vast pathology in mice that lead to animal death. Regarding ExoU dependent cell necrosis, we demonstrated that the host oxidized phospholipid is one the key regulators of ExoU dependent cell necrosis and alarmins release and that targeting of these peroxidation mechanisms could alleviate the pulmonary pathology in mice models and human bronchial organoids. In this context, previous studies showed that ExoU promotes the production of the platelet-activating factor or the 8-PGF2α isoprostane, two oxidized lipids (da Cunha et al., 2015). In addition, enzymes such as cytochrome P450, Cyclooxygenases (COXs) a Lipoxygenases (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; Pazos et al., 2017; Sadikot et al., 2007; Saliba et al., 2005). However, the use of bone marrow-derived macrophages (BMDMs) WT and ALOX5$^{-/-}$ and ALOX12/15$^{-/-}$ in the presence or absence of COXs and cytochrome P450 inhibitors and recombinant ExoU protein (Figure19) shows that these enzymes taken

individually, display a negligible role in ExoU-induced cell necrosis. This observation is consistent with some previous studies (Machado et al., 2011; Pazos et al., 2017; Sadikot et al., 2007; Saliba et al., 2005). Therefore, the use of *Alox*$^{-/-}$, *Cox*$^{-/-}$, *cytochrome P450*$^{-/-}$ or double knock out mice, unable to generate LTB4 or various prostaglandins, will allow determining the importance of eicosanoid burst in ExoU dependent pathology. Moreover, this knowledge would allow a better understanding of the *in vivo* mechanisms and so the development of adapted treatment strategies.

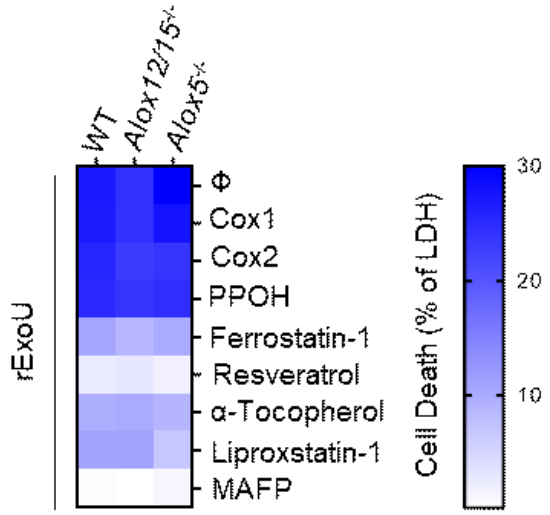

Figure 19: Heat map of cell death (by measure of LDH release) in WT, ALOX5-/- and ALOX12/15 BMDMs transfected with recombinant ExoU in presence/absence of Cox1 inhibitor (Ketorolac Tromethamine, 10µM), Cox2 inhibitor (NS 398, 25µM), Cyp450 epoxygenase activity inhibitor (PPOH, 10µM), phospholipase inhibitor 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.

About ExoU dependent cell necrosis characterization, due to the release of huge amounts of lipid peroxidation subproducts upon infection, we make a hypothesis that ExoU could induce ferroptosis or at least one described pathway relied on ferroptosis. In fact, ferroptosis is thought to be a constitutively activated form of cell death that is retained under control through the activity of endogenous regulators of lipid peroxidation such as GPX4/GSH, FSP1-mediated coenzyme Q10 production, α-tocopherol (vitamin E). In addition, previous studies described that host cellular calcium

(Ca2+)-independent PLA2γ, the peroxiredoxin Prdx6 PLA2 or the PLA2G6 (Ca2+-independent PLA2β) can cleave and remove preferentially peroxidised phospholipids, hence contributing to peroxidized phospholipid detoxification and then preventing ferroptosis (Beharier et al., 2020; Kinsey et al., 2008; Lu et al., 2019; Miyamoto et al., 2003; van Kuijk et al., 1987; Yedgar et al., 2006). It is important to notice that both the iPLA2beta and iPLA2g belong to the patatin-like phospholipase family, as ExoU, which suggests that this family of phospholipases might have some conserved affinities to peroxidized phospholipids (Kienesberger et al., 2009). In order to avoid dysregulated phospholipid cleavage, these phospholipases activity is tightly regulated by various cellular systems (e.g. ROS levels, calcium fluxes, phospholipid composition) (Yedgar et al., 2006). In this regard, we investigate the molecular mechanisms used by ExoU to trigger cell death. And surprisingly, we found that cellular phospholipid peroxidation is a strong enhancer of ExoU-induced pathological necrosis. In this context, we envision that, as a virulence factor, ExoU activity does not follow host regulation and uses host peroxidized phospholipids to boost its patatin-like A2 phospholipase activity allowing it to aberrantly target and cleave host (peroxidized) 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.

Furthermore, in this study, we were essentially interested in macrophages and neutrophils, given that they are the key cells in the fight against *Pseudomonas* and the first targets of the bacteria. We can wonder if these cells have a high level of lipid peroxidation which explains in the case of ExoU that they are the most targeted and injected by the bacterium (Diaz & Hauser, 2010). Indeed, in the paper of Diaz & hauser, they demonstrated that a few hours after infection with using translational fusions of ExoU with a β-lactamase reporter (ExoU-Bla) expressing bacteria, the neutrophils, macrophages, and monocytes are the main cells injected by *Pseudomonas* with ExoU. Hence, it would be interesting to know if the oxidative profile of these cells made them susceptible to the toxin compared to other cell types. In line with this, a new project has been developed that relied on neutrophils importance in ExoU expressing *Pseudomonas* infection. In fact, we observed that neutrophils

displayed more lipid peroxidation than all cells contained in the blood, and they die in Myeloperoxidase (MPO) dependent manner (*paper in preparation in the team*).

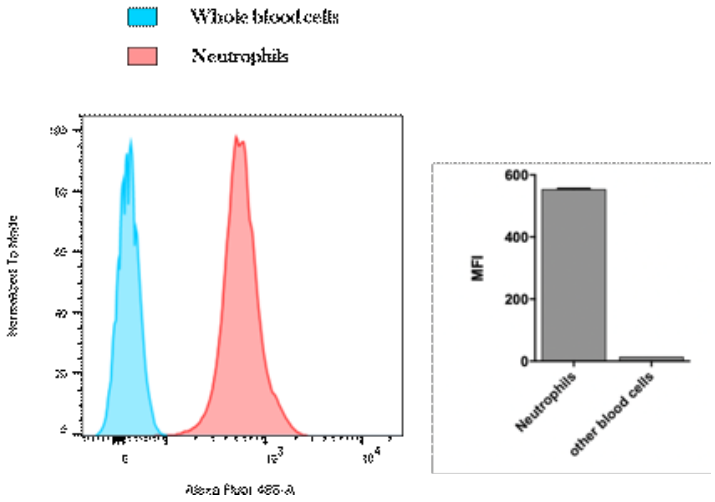

Figure 20: The Human whole blood cells were stained by C11-BODIPY581/591 to determine the lipid peroxidation level. Neutrophils present high level of peroxidised lipids.

Although the identification of cellular enzymatic systems that promote basal lipid peroxidation remains to be explored and characterized, lipid peroxidation accumulation upon Gpx4 removal or oxidant stress enhances ExoU-induced cellular necrosis. We can hypothesize that at the early stage of infection there is a quick induction of ROS and so lipid peroxides in the cell via PRRs activation by *P. aeruginosa* which could favour ExoU activity. All this will be the subject of future studies knowing that lipid peroxidation could be a non-active mechanism (non-enzymatic). Moreover, it is interesting that endogenous peroxidized phospholipids promote ExoU-induced cell necrosis, suggesting that ExoU-expressing strains of *P. aeruginosa* take advantage of the host ferroptosis pathways to extremely damage host tissues. Hence, oxidant-activated (such as Cumene Hydroperoxide or Tertbutyl Hydroperoxide) cytochrome P450 oxidoreductase CYPOR, a crucial regulator of ferroptosis, strongly enhanced ExoU-dependent cell necrosis, which suggests an important link between ferroptosis-regulated pathways and ExoU activity. Regarding other regulators of ferroptosis such as Acyl-CoA synthetase long-chain family member 1 or 3 or 4 (ACSL-1 or -3 or -4) and Lysophospholipid acyltransferase 3 (LPCAT) (Beatty et al., 2021; Kagan et al., 2017; Magtanong et al., 2019), further investigations are required to evaluate their involvement in ExoU-related pathology.

The enzymes with phospholipases activity are present in various microbial pathogens (e.g. M. tuberculosis, L. monocytogenes) or in venom, and can promote fast cell necrosis (Bagayoko & Meunier, 2021; Flores-Díaz et al., 2016; Hiu & Yap, 2020; Sitkiewicz et al., 2007). Therefore, we extended our studies to the ExoU closely related ExoUBtU or ExoUPfU phospholipases from respectively *Bulkoderia thailandensis and Pseudomonas fluorescens* (Anderson et al., 2015). Then, we find that rExoUBtU or rExoUPfU are less cytotoxic (release of LDH) than rExoU at least in our condition, but trigger cell death modulable by lipophilic antioxidants ((Bagayoko et al., 2021) Figure 2j; data unpublished Figure 20). Remarkably, snake, scorpion or spider venoms are a complex mixture of various components, including the L-amino acid oxidase and secreted phospholipases, respectively able to generate H2O2-driven lipid peroxidation, and cleave phospholipids (Hiu & Yap, 2020). In this context, we can speculate that venoms have all components necessary to mediate cell and tissue damage. Related to this, Sevanian and colleagues observe that the PLA2 activity from the snake Crotalus adamanteus is exacerbated in contact with liposomes constituted of peroxidized 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). Future studies will determine whether ExoU and the analogous toxins follow a similar activation pathway.

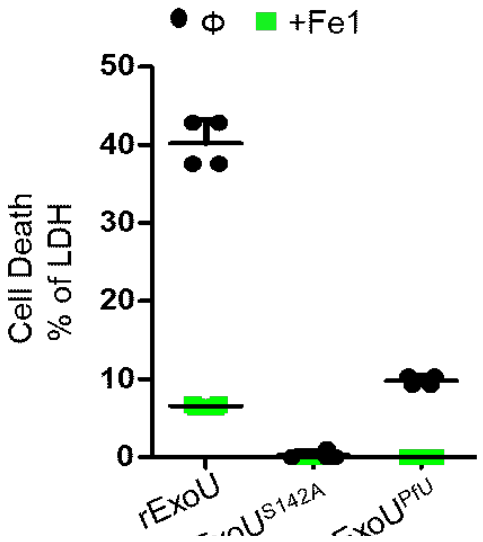

Figure 21: LDH release in WT BMDMs transfected for 3 hours with 5µg of rExoUPfU or 500ng rExoU or the catalytic death mutant rExoU^{S142A} in presence or absence of ferrostatin-1 (20µM).

Finally, we demonstrated that ExoU-dependent necrosis is linked to the ferroptosis process, we can speculate that ExoU and ExoU-like toxins could trigger huge damage in the patients with mutations in ferroptosis regulators genes. Indeed, GPX4/GSH or FSP1/Coenzyme Q10 are the keys ferroptosis negative regulators, mutations that alter their function have been shown to conduct to several diseases such as Sedaghatian-type Spondylometaphyseal Dysplasia (SSMD) or Leigh syndrome with CoQ10 deficiency (Cheff et al., 2021; López et al., 2006; Yan et al., 2021). We already demonstrated that GPX4 deficiency in mice cells increases ExoU activity, it will be interesting to confirm GPX4/GSH and FSP1/Coenzyme Q10 in mice and human (by using cells and organoids).

Among inherited diseases resulting from a defect in the control of the lipid peroxidation process, we are interested in cystic fibrosis (CF). At first sight, it does not seem to be linked to the process of ROS and oxidized lipids production. However, different previous studies have shown a defect in the control of ROS and oxidized lipid production in these patients. In addition, *P. aeruginosa* is one the main bacteria found in CF patients and responsible for the high morbidity and mortality of these patients (Bagayoko & Meunier, 2021; Benabdeslam et al., 1999; Kleme & Levy, 2015a; Portal et al., 1995). Furthermore, recent studies validated that the links between cystic fibrosis, *Pseudomonas* and ferroptosis (Dar et al., 2018; Ousingsawat et al., 2021). In one they showed that in chronic infection of CF patients *Pseudomonas aeruginosa* lipoxygenase LoxA peroxidizes lipids which induces cell sensibility to ferroptosis (Dar et al., 2018), the second paper stated that *Pseudomonas aeruginosa* triggers ferroptosis in CF patients' context (Ousingsawat et al., 2021). However, in the last one, they did not identify how, and which *Pseudomonas aeruginosa* gene is responsible for ferroptosis in CF (Ousingsawat et al., 2021). In line with that, we are currently studying the susceptibility of CF cells and organoids to ExoU infection and to ferroptosis described inducers.

On the other hand, this high lipid peroxidation environment presented in CF patients could be an active process (Bagayoko & Meunier, 2021; Kleme & Levy, 2015b; Mailhot et al., 2010; Portal et al., 1995). In fact, fatty acid desaturase 1 or 2 (FADS1 or 2) are enzymes involved in the biosynthesis of polyunsaturated fatty acids (PUFAs) which is important in ferroptosis induction. Very recently, some studies demonstrated their involvement in cell sensitivity to ferroptosis (J. Y. Lee et al., 2020; Yamane et al., 2021).

Thus, we postulated that these enzymes might be associated with the susceptibility of CFTR $^{-/-}$ cells to ferroptosis, and prove the crucial role of PUFAs biosynthesis in the predisposition to ferroptosis of cystic fibrosis patients. We are currently investigating this hypothesis in the laboratory. Therefore, we hope to show that cystic fibrosis patients are susceptible to infections with ExoU-like toxins but also to offer a new avenue of therapy for CF patients. All together, our findings during this thesis highlight, for the first time, one interesting role of lipid peroxidation in infectious diseases.

Chapter 5: Irgm2 and Gate-16 cooperatively dampen Gram-negative bacteria-induced caspase-11 response

I- Study context

As described above (cell death section chapter 1, Proptosis), inflammasomes are cytosolic innate immune complexes that initiate inflammatory responses upon sensing of microbe and danger signals MAMPs and DAMPs, respectively (Galluzzi et al., 2014; Hayward et al., 2018). Specifically, the rodent protease caspase-11 (and its human orthologs caspase-4 and caspase-5) detects, via its caspase activation and recruitment domain (CARD), the presence of the Gram-negative bacterial cell wall component LPS within the host cell cytosol (Aachoui et al., 2013; Broz et al., 2012; Hagar et al., 2013; Kayagaki et al., 2011b, 2013; J. Shi et al., 2014b; D. Yang et al., 2015). LPS interaction with CARD promotes caspase-11 oligomerization and activation, which triggers the initiation of the non-canonical inflammasome (D. Yang et al., 2015). Indeed, unlike canonical inflammasomes, the non-canonical inflammasome proceeds in two steps (Figure 21). (1) Upon activation (B. L. Lee et al., 2018), caspase-11 cleaves and activates the pyroptosis executioner gasdermin-D (gsdmD) into the p30 active fragment (Kayagaki et al., 2015; J. Shi et al., 2014b). Cleaved gsdmD forms a pore into phosphatidylinositol-4,5-bisphosphate (PIP2)-enriched domains at the plasma membrane, which lead to pyroptosis (Aglietti et al., 2016; X. Liu et al., 2016; Sborgi et al., 2016; J. Shi et al., 2014b). (2) In parallel, gsdmD pore-induced ionic perturbations such as potassium efflux, which are activation signals for the canonical NLRP3 inflammasome. The induction of this last one results in the caspase-1-dependent maturation of the pro-inflammatory cytokines interleukins (IL)-1b and IL-18 (Kayagaki et al., 2011b; Schmid-Burgk et al., 2015).

Therefore, caspase-11 confers host protection against intracellular Gram-negative bacteria (Aachoui et al., 2013; Cerqueira et al., 2018; Chen et al., 2018). However, some cytosolic Gram-negative bacteria display mechanisms avoiding caspase-11 recognition such as *Shigella flexneri* and *Francisela tularensis* which express hypo-acetylated LPS (tetra acetylated) during infection, whereas caspase-11 recognizes hexa-acetylated LPS (Hagar et al., 2013; Okan & Kasper, 2013; Paciello et al., 2013). Similarly, vacuolar Gram-negative bacteria such as *Salmonella typhimurium* reside in protective vacuoles, making detection of their cytosolic LPS difficult by caspase-11 (Broz et al., 2012).

On the other hand, like all inflammasomes, caspase-11 uncontrolled activation provokes irreversible organ failure, blood clotthing and sepsis (Cheng et al., 2017; Deng et al., 2018; Kayagaki et al., 2011b, 2013, 2015; Rathinam et al., 2019; X. Yang et al., 2019). This suggests that host regulators might fine-tune the non-canonical inflammasome to optimize caspase-11-dependent response. In this context, interferons, microbicide cytokines play a primordial role. Indeed, one the interferon-induced gene call GTPases family are crucial regulators of the non-canonical inflammasome activation pathway (Cerqueira et al., 2018; Finethy et al., 2015; Lagrange et al., 2018; B. C. Liu et al., 2018; Man et al., 2015; Meunier et al., 2014, 2015; Wallet et al., 2017; Zwack et al., 2017). GTPases consist of Mx GTPases, Very Large GTPases (GVIN) Guanylate Binding Proteins (GBPs) and finally immunity-related GTPases (Irgs), (Meunier & Broz, 2016). Precisely, GBPs (1, 2, 3, 4 and 5) are recruited on LPS-enriched structures such as cytosolic Gram-negative bacteria and their derived products outer membrane vesicles (OMVs) (Fisch et al., 2019; Lagrange et al., 2018; Man et al., 2015; Meunier et al., 2014; Santos et al., 2018). Thus, these GBPs then engage caspase-11 which binds to LPS, thereby promoting the non-canonical inflammasome pathway (Fisch et al., 2019). As described in the inflammation section in chapter 1, IFNs induce more than 2,000 ISGs antimicrobial genes, among them, many genes counterbalance overactivation of the cells such as SOCS1 and USP18 that balance the level of the host cell response (Basters et al., 2018; Liau et al., 2018).

In this context, we hypothesized that IFNs might also induce negative regulators of the non-canonical inflammasome. In this regard, some articles suggest that the Irgm proteins belonging to the Irgs subfamily may play a regulatory role for GBPs (Feeley et al., 2017; Haldar et al., 2013; B. H. Kim et al., 2012; Pilla-Moffett et al., 2016). Irgms lack the ability to hydrolyse the GTP due to a mutation in their catalytic domain (GMS), whereas other Irgs are GTPase active (GKS) (Coers, 2013). Human being possesses one IRGM protein, with various spliced variants, that is not IFN-inducible but that requires IFN signalling to be functional (S. Kim et al., 2019). By contrast, mice display three different Irgms, namely Irgm1, Irgm2 and Irgm3 (B. H. Kim et al., 2012). Previous studies demonstrated an inhibitory role of Irgm1 and Irgm3 on the recruitment and/or activation of the GBPs and Irg-GKS on microbial membranes although independent processes can also occur (Feeley et al., 2017; Haldar et al., 2013). Further, some

studies identified Irgm1 and its human homologous IRGM, as being critical for the NLRP3 canonical inflammasome regulation by modulating the autophagy pathway, suggesting a close link between Irgm proteins and inflammasomes (Mehto et al., 2019; pei et al., 2017). In light of all this knowledge, we hypothesized that Irgm proteins might be IFN-inducible regulators of the non-canonical inflammasome activation.

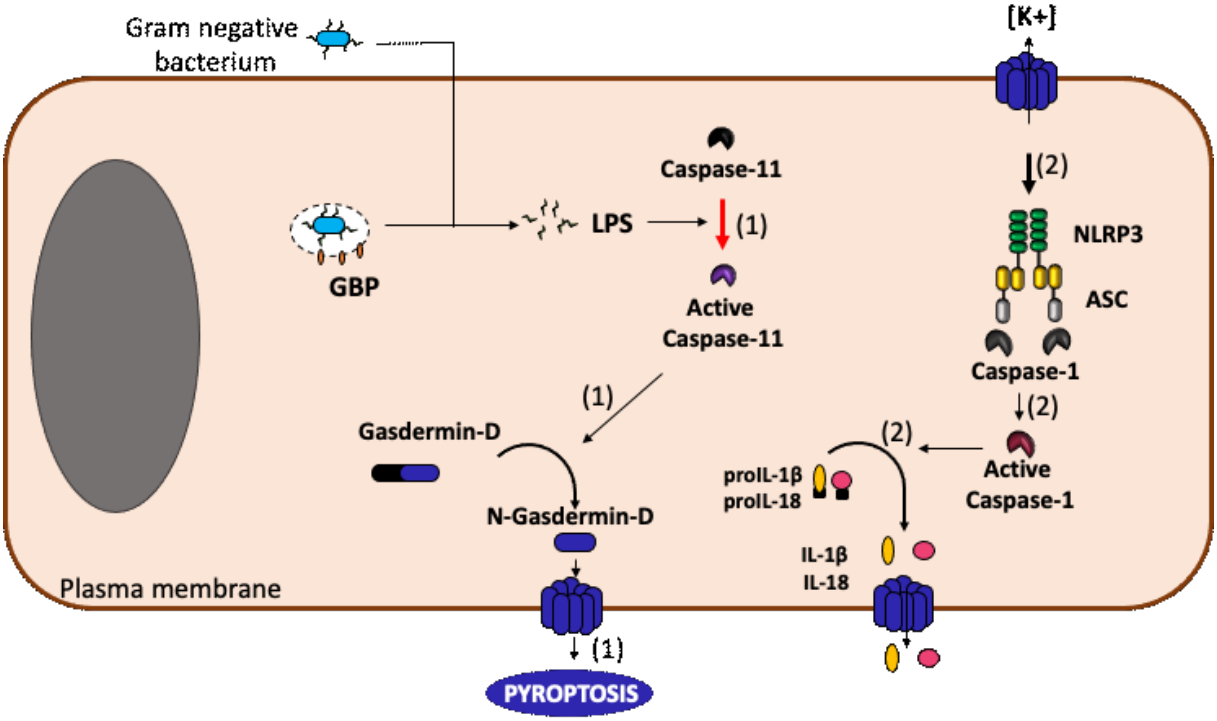

Figure 22: Non canonical inflammasome activation: (1) activation of pro caspase-11 by LPS from Gram bacterium in cytosol, the active caspase-11 triggers the cleavage of Gasdermin-D and thus the formation of pore on plasma membrane which lead to the cell pyroptosis. (2) there is a release of potassium K+ from cell which active NLRP3 inflammasome, this is followed by caspase1 activation and so cleavage of proinflammatories cytokines (IL1b and IL18).

II-Results

A-Scientific contribution to the paper

The results of this study were published in Embo Journal in 2020:

"**Irgm2 and Gate-16 cooperatively dampen Gram-negative bacteria-induced caspase-11 response**"

Elif Eren, Rémi Planès, Salimata Bagayoko, Pierre-Jean Bordignon, Karima Chaoui, Audrey Hessel, Karin Santoni, Miriam Pinilla, Brice Lagrange, Odile Burlet-Schiltz, Jonathan C Howard, Thomas Henry, Masahiro Yamamoto, Etienne Meunier.

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In this study, I participated in the realization of the *in vitro* (cells) experiments under the supervision of Elif Eren and Etienne Meunier (during my Master internship and at the first part of my PhD), specifically in infection/stimulation/electroporation of macrophages, cytotoxicity test (LDH release), western blot, and Elisa test.

B-Paper

doi.org/10.15252/embr.202050829 (to add)

Article

SOURCE DATA | TRANSPARENT PROCESS

EMBO reports

Irgm2 and Gate-16 cooperatively dampen Gram-negative bacteria-induced caspase-11 response

Elif Eren[1,†], Rémi Planès[1,†], Salimata Bagayoko[1], Pierre-Jean Bordignon[1], Karima Chaoui[1,2], Audrey Hessel[1], Karin Santoni[1], Miriam Pinilla[1], Brice Lagrange[3], Odile Burlet-Schiltz[1,2], Jonathan C Howard[4], Thomas Henry[3], Masahiro Yamamoto[5,6] & Etienne Meunier[1,‡,*]

Abstract

Inflammatory caspase-11 (rodent) and caspases-4/5 (humans) detect the Gram-negative bacterial component LPS within the host cell cytosol, promoting activation of the non-canonical inflammasome. Although non-canonical inflammasome-induced pyroptosis and IL-1-related cytokine release are crucial to mount an efficient immune response against various bacteria, their unrestrained activation drives sepsis. This suggests that cellular components tightly control the threshold level of the non-canonical inflammasome in order to ensure efficient but non-deleterious inflammatory responses. Here, we show that the IFN-inducible protein Irgm2 and the ATG8 family member Gate-16 cooperatively counteract Gram-negative bacteria-induced non-canonical inflammasome activation, both in cultured macrophages and *in vivo*. Specifically, the Irgm2/Gate-16 axis dampens caspase-11 targeting to intracellular bacteria, which lowers caspase-11-mediated pyroptosis and cytokine release. Deficiency in *Irgm2* or *Gate16* induces both guanylate binding protein (GBP)-dependent and GBP-independent routes for caspase-11 targeting to intracellular bacteria. Our findings identify molecular effectors that fine-tune bacteria-activated non-canonical inflammasome responses and shed light on the understanding of the immune pathways they control.

Keywords Caspase-11; Gate-16; infections/Interferons; Irgm2; non-canonical inflammasome

Subject Categories Autophagy & Cell Death; Immunology; Microbiology, Virology & Host Pathogen Interaction

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See also: **R Finethy *et al*** and **A Linder & V Hornung** (November 2020)

Introduction

Inflammasomes are cytosolic innate immune complexes that initiate inflammatory responses upon sensing of microbe- and damage-associated molecular patterns (MAMPs and DAMPs, respectively) (Hayward *et al*, 2018). Specifically, the rodent caspase-11 (and its human orthologs caspase-4 and caspase-5) detects the presence of the Gram-negative bacterial cell wall component lipopolysaccharide (LPS) within the host cell cytosol (Kayagaki *et al*, 2011, 2013; Broz *et al*, 2012; Aachoui *et al*, 2013; Hagar *et al*, 2013; Yang *et al*, 2015). LPS interaction with the caspase activation and recruitment domain (CARD) of caspase-11 promotes its oligomerization and activation, which triggers the activation of the non-canonical inflammasome (Yang *et al*, 2015). Upon activation (Lee *et al*, 2018), caspase-11 cleaves and activates the pyroptosis executioner gasdermin-D (gsdmD) into the p30 active fragment (Kayagaki *et al*, 2015; Shi *et al*, 2015). Cleaved gsdmD then forms a pore into phosphatidylinositol-4,5-bisphosphate (PIP2)-enriched domains at the plasma membrane, which triggers pyroptosis, a pro-inflammatory form of cell death (Shi *et al*, 2015; Aglietti *et al*, 2016; Liu *et al*, 2016; Sborgi *et al*, 2016). In parallel, gsdmD pore-induced ionic perturbations also trigger activation of the canonical NLRP3 inflammasome, which results in the caspase-1-dependent maturation of the pro-inflammatory cytokines interleukins (IL)-1β and IL-18 (Kayagaki *et al*, 2011; Rühl & Broz,

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2015; Schmid-Burgk *et al*, 2015). Although caspase-11 confers host protection against intracellular Gram-negative bacteria (Aachoui *et al*, 2013; Cerqueira *et al*, 2018; Chen *et al*, 2018), its unrestrained activation provokes irreversible organ failure, blood clothing and sepsis (Kayagaki *et al*, 2011, 2013, 2015; Napier *et al*, 2016; Cheng *et al*, 2017; Deng *et al*, 2018; Rathinam *et al*, 2019; Yang *et al*, 2019). This suggests that host regulators might fine-tune the non-canonical inflammasome in order to optimize caspase-11-dependent response. To date, only few of them were described including SERPINB1-inhibited caspase-11/-4/-1 activation in resting cells or ESCRT-mediated plasma membrane repair (Rühl *et al*, 2018; Choi *et al*, 2019).

Crucial at regulating the activation the non-canonical inflammasome pathway are the IFN-inducible GTPases, the so-called guanylate binding proteins (GBPs) and the immunity-related GTPase (Irg) Irgb10 (Meunier *et al*, 2014, 2015; Pilla *et al*, 2014; Finethy *et al*, 2015; Man *et al*, 2015, 2016; Wallet *et al*, 2017; Zwack *et al*, 2017; Cerqueira *et al*, 2018; Costa Franco *et al*, 2018; Lagrange *et al*, 2018; Liu *et al*, 2018). Specifically, GBPs (1, 2, 3, 4 and 5) are recruited on LPS-enriched structures such as cytosolic Gram-negative bacteria and their derived products outer membrane vesicles (OMVs) (Meunier *et al*, 2014; Man *et al*, 2015; Finethy *et al*, 2017; Lagrange *et al*, 2018; Santos *et al*, 2018; Fisch *et al*, 2019). As such, these GBPs then engage caspase-11 that will bind the LPS moiety lipid A, hence promoting the non-canonical inflammasome pathway (Fisch *et al*, 2019). Beyond their role at triggering GBP expression, IFNs induce more than 2,000 antimicrobial genes (Green *et al*, 2018). Among them, many IFN-inducible regulatory genes also counter-balance overactivation of the cells (Green *et al*, 2018). For instance, SOCS1 and USP18 are ISGs that balance the level of the host cell response (Basters *et al*, 2018; Liau *et al*, 2018). In this context, we hypothesized that IFNs, in addition to their ability to promote GBP expression, might also induce negative regulators of the non-canonical inflammasome. In this regard, Irgm proteins belong to the IFN-inducible immunity-related GTPase (Irg) family proteins (Kim *et al*, 2012, 2019; Pilla-Moffett *et al*, 2016). Human being possess one IRGM protein, with various spliced variants, that is not IFN-inducible but that requires IFN signalling to be functional (Kim *et al*, 2019). By contrast, mice display three different Irgms, namely Irgm1, Irgm2 and Irgm3 (Kim *et al*, 2012). All Irgms lack the ability to hydrolyse the GTP due to a mutation in their catalytic domain (GMS), whereas other Irgs are GTPase active (GKS) (Coers, 2013). Previous studies underscored an inhibitory role of Irgm1 and Irgm3 on the recruitment and/or activation of the GBPs and Irg-GKS on microbial membranes although independent processes can also occur (Haldar *et al*, 2013, 2015; Feeley *et al*, 2017). In addition, recent studies identified Irgm1 and its human homologous IRGM, as being critical for the NLRP3 canonical inflammasome regulation by modulating the autophagy pathway, suggesting a close link between Irgm proteins and inflammasomes (Pei *et al*, 2017; Mehto *et al*, 2019a, b). In this context, we hypothesized that Irgm proteins might be IFN-inducible regulators of the non-canonical inflammasome activation threshold.

Here, we report that IFN-inducible Irgm2 and the non-canonical autophagy effector Gate-16 indirectly fine-tune non-canonical inflammasome activation by intracellular bacteria, which protects against endotoxemia.

Results and Discussion

IFN-inducible protein Irgm2 restrains Caspase-11-dependent responses to Gram-negative bacteria

IFN-inducible Irgms control Irg and GBP microbicidal activity against intracellular pathogens (Pilla-Moffett *et al*, 2016). In this context, we sought to determine whether Irgms might also modulate the non-canonical inflammasome response. Using an RNA interference approach (siRNA), we silenced the three murine Irgms in primary murine bone marrow-derived macrophages (BMDMs) and measured their ability to undergo caspase-11-dependent cell death and IL-1β maturation upon *Salmonella* Typhimurium challenge. To ensure that the inflammasome response in macrophages is caspase-11-dependent, we used an isogenic mutant of *Salmonella* (*orgA*$^{-}$) lacking expression of SP1-encoded T3SS secretion system (Broz *et al*, 2012). As previously published, *Casp11* and *Gbp2* silencing reduced macrophage death (LDH release) and IL-1β release after 16 h of infection (Figs 1A and EV1A; Meunier *et al*, 2014). Importantly, *Irgm2*-silenced BMDMs had higher levels of cell death and IL-1β release than the wild-type (WT) macrophages (Figs 1A and EV1A). Such process was specific to Irgm2 given that *Irgm1*- and *Irgm3*-targeted siRNAs did not induce significant variation in macrophage death and IL-1β release upon *Salmonella* (*orgA*$^{-}$) infection, despite the fact that their mRNA levels were efficiently reduced (Figs 1A and EV1A). To further validate that Irgm2 is a regulator of the non-canonical inflammasome response, we challenged WT, *Irgm2*$^{-/-}$, *Casp11*$^{-/-}$ and *GBP*$^{Chr3-/-}$ BMDMs with a panel of Gram-negative bacteria all known to activate the non-canonical inflammasome. Immunoblotting experiments in WT and *Irgm2*$^{-/-}$ BMDMs showed that Irgm2 is IFN-inducible and that *Irgm2* deficiency does not lead to a defect in caspase-1, caspase-11, GBP2 or GBP5 expression, all involved in the non-canonical inflammasome pathway (Fig EV1B and C). Yet, when challenged with various Gram-negative bacteria, *Irgm2*$^{-/-}$ macrophages showed an exacerbated cell death, IL-1β release and gasdermin-D p30 (active) and processed caspase-1 p20 (inactive) fragments compared with their WT counterparts (Fig 1B and C). In addition, Irgm2-regulated cell pyroptosis upon Gram-negative bacterial challenge was independent of NLRP3 as the use of the NLRP3 inhibitor MCC950 or *Nlrp3*$^{-/-}$ BMDMs did not drive any defect in cell death but significantly reduced NLRP3-dependent IL-1β release (Fig EV1D). As expected, both *Casp11*$^{-/-}$ and *GBP*$^{Chr3-/-}$ BMDMs were protected against Gram-negative bacteria-induced non-canonical inflammasome response (Fig 1B). Importantly, CRISPR-deleted *Irgm2* gene expression in immortalized (i) *Casp11*$^{-/-}$ BMDMs (referred as *Casp11*$^{-/-}$ sg*Irgm2*) did not reinduce pyroptosis and IL-1β release upon Gram-negative bacterial infections (*S.* Typhimurium *orgA*$^{-}$ and *Escherichia coli*) or *E. coli*-derived OMVs exposure, thus confirming that Irgm2 negatively regulated caspase-11-dependent response (Figs 1D and EV1E). Next, we assessed whether Irgm2 directly or indirectly regulates the non-canonical inflammasome response. To this end, we electroporated LPS into the host cell cytosol of IFNγ-primed WT, *Irgm2*$^{-/-}$ and *Casp11*$^{-/-}$ BMDMs and evaluated their ability to undergo pyroptosis. Surprisingly, we observed that WT and *Irgm2*$^{-/-}$ macrophages engaged cell death to the same extent 4 h after LPS electroporation whereas *Casp11*$^{-/-}$ BMDMs were protected against LPS-induced cell death (Fig 1E). This suggests that

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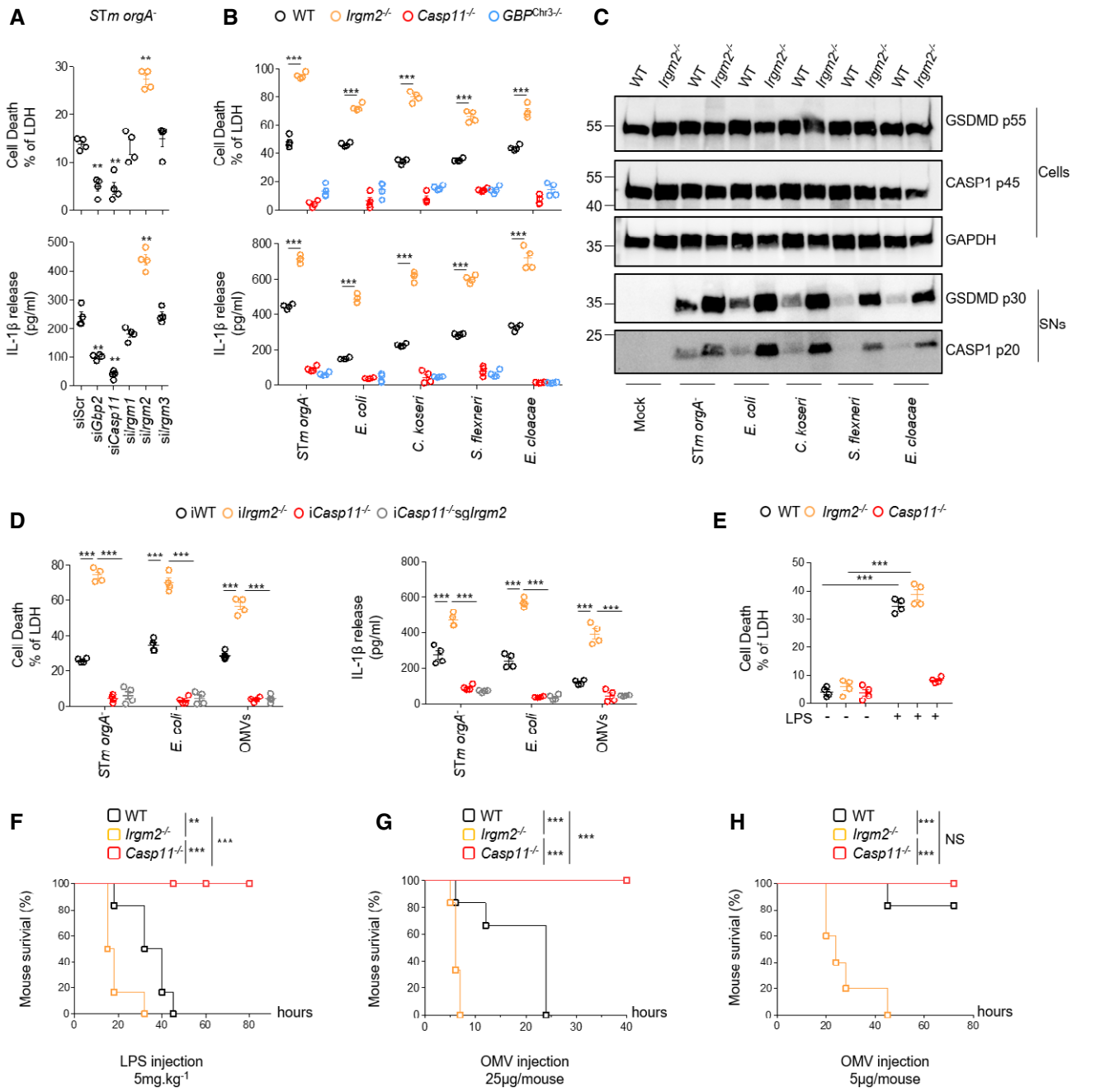

Figure 1. IFN-inducible protein Irgm2 restrains caspase-11-dependent response to Gram-negative bacteria.

Unless otherwise specified, BMDMs were either infected with various Gram-negative bacterial strains (MOI25) or stimulated with outer membrane vesicles (OMVs) for 16 h.

A siRNA-treated BMDMs were infected for 16 h with *S.* Typhimurium (*orgA*$^{-}$), and LDH and IL-1β release were measured.

B Cell death (LDH) and IL-1β release evaluation in WT, *Irgm2*$^{-/-}$, *GBP*$^{Chr3-/-}$ and *Casp11*$^{-/-}$ BMDMs infected for 16 h with different Gram-negative bacteria (MOI 25).

C Western blot examination of processed caspase-1 (p20) and gasdermin-D (p30) in supernatants and pro-caspase-1 (p45), pro-gasdermin-D (p55) and GAPDH in cell lysates of WT and *Irgm2*$^{-/-}$ BMDMs infected for 16 h with different Gram-negative bacterial strains.

D IL-1β and cell death (% LDH) evaluation in immortalized WT, *Irgm2*$^{-/-}$, *Casp11*$^{-/-}$ and *Casp11*$^{-/-}$*Irgm2*$^{-/-}$ (referred as sg*Irgm2*) BMDMs after 16 h of *Escherichia coli*, *S.* Typhimurium *orgA*$^{-}$ and OMV treatment.

E Cell death (% LDH) evaluation in IFNγ-primed WT, *Irgm2*$^{-/-}$ and *Casp11*$^{-/-}$ BMDMs 4 h after electroporation or not with 1 µg of *E. coli* LPS.

F–H Survival of WT, *Casp11*$^{-/-}$ and *Irgm2*$^{-/-}$ mice primed with 100 µg poly(I:C) for 6 h and injected (i.p.) with 5 mg/kg LPS or 5 and 25 µg of OMVs (n = 6 animals per condition).

Data information: Data shown as means ± SEM (graphs A, B, D and E) from n = 4 independent pooled experiments; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ for the indicated comparisons using t-test with Bonferroni correction. Image (C) is representative of one experiment performed three times. (F–H) are representative of three independent experiments; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, log-rank Cox–Mantel test for survival comparisons (F–H).
Source data are available online for this figure.

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Irgm2-inhibited caspase-11 response occurs upstream from LPS sensing by caspase-11.

Based on these results, we next determined whether Irgm2 also inhibited canonical inflammasomes. We treated WT, *Irgm2*$^{-/-}$, *Casp11*$^{-/-}$ and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs with various inflammasome activators, including flagellin (NLRC4), poly-dAdT (AIM2), Nigericin (NLRP3) and TcdB (PYRIN), and measured their ability to commit pyroptosis and to release IL-1β. Although all canonical inflammasome activators induced significant caspase-1-dependent response, cell death and IL-1β release levels remained similar in both WT and *Irgm2*$^{-/-}$ BMDMs (Fig EV1F). In addition, activation of the NLRC4 inflammasome by T3SS-expressing *Pseudomonas aeruginosa* and *S*. Typhimurium remained similar between WT and *Irgm2*$^{-/-}$ BMDMs (Fig EV1G), suggesting that Irgm2 specifically regulates the non-canonical inflammasome response to Gram-negative bacteria.

As caspase-11 also drives mouse susceptibility to LPS-induced inflammatory-related damages, we also evaluated whether *Irgm2* deficiency might sensitize mice to sepsis. We used two LPS-dependent sepsis models, where WT, *Irgm2*$^{-/-}$ and *Casp11*$^{-/-}$ mice were intraperitoneally injected with poly(IC) to induce ISG expression (Kayagaki *et al*, 2013; Santos *et al*, 2018). Then, mice were injected either with pure LPS (5 mg/kg) or with OMVs (25 μg/ml; Vanaja *et al*, 2016; Santos *et al*, 2018). Mouse survival showed that while *Casp11*$^{-/-}$ mice had resistance to LPS- and OMV-induced sepsis, WT mice succumbed faster, hence validating our sepsis model (Fig 1F and G). Noticeably, *Irgm2*$^{-/-}$ mice were even more susceptible than WT mice to both LPS- and OMV-induced sepsis (Fig 1F and G). Therefore, we used a sub-lethal model of OMV-induced sepsis by injecting 5 μg of OMVs in mice. In such model, both WT and *Casp11*$^{-/-}$ mice recovered from OMV injection whereas all *Irgm2*$^{-/-}$ mice did succumb (Fig 1H). Moreover, cytokine assays showed that *Irgm2*$^{-/-}$ mice had an exacerbated release of all pro-inflammatory and inflammasome-related cytokines tested upon OMV challenge, a phenotype that was reduced in *Casp11*$^{-/-}$ mice, hence confirming that Irgm2 expression is crucial to temperate the activation level of the non-canonical inflammasome (Fig EV1H). Altogether, our data suggest that Irgm2 indirectly inhibits caspase-11-dependent endotoxemia, which protects against sepsis.

Irgm2 regulates GBP-independent caspase-11 targeting to Gram-negative bacteria

IFN-inducible GBPs are important regulators of the non-canonical inflammasome response. Specifically, GBP-1 and GBP-2 regulate human caspase-4/-5 activation while GBP-2 and GBP-5 control mouse caspase 11. Therefore, we hypothesized that Irgm2 might control caspase-11 response through the modulation of the GBPs. To this end, we silenced *Irgm2* in WT and *GBP*$^{Chr3-/-}$ BMDMs (lacking 5 GBPs, 1-3, 5 and 7) and evaluated the caspase-11 response upon OMV stimulation (Fig EV2A). While OMV-induced both cell death and IL-1β release was strongly reduced in *GBP*$^{Chr3-/-}$, *Irgm2*-silenced *GBP*$^{Chr3-/-}$ BMDMs partially recovered a caspase-11-dependent response, suggesting that Irgm2-inhibited caspase-11 response could occur independently of GBPs (Fig 2A). Other and we previously showed that GBPs also controlled canonical AIM2 inflammasome activation upon *Francisella tularensis* spp *novicida* infection. In this context, we evaluated the importance of Irgm2 at controlling AIM2 inflammasome response upon *F. novicida* infection. Surprisingly, IL-1β and cell death levels were not different between WT and *Irgm2*$^{-/-}$, although they were strongly reduced in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ and *GBP*$^{Chr3-/-}$ BMDMs (Figs 2B and EV2B). In addition, we observed that *Irgm2*-silenced *GBP*$^{Chr3-/-}$ BMDMs did not recover an inflammasome response upon *F. novicida* infection. Then, we generated i*GBP*$^{Chr3-/-}$ *Irgm2*$^{-/-}$ (referred hereafter as i*GBP*$^{Chr3-/-}$sg*Irgm2*) by crispr Cas9 and evaluated their response upon *S*. Tm (*orgA*$^{-}$) challenge. i*Irgm2*$^{-/-}$ BMDMs showed time-dependent increased cell death compared with iWT cells (Fig EV2C and D). While cell death in i*GBP*$^{Chr3-/-}$ BMDMs was strongly impaired, it was partially reversed in i*GBP*$^{Chr3-/-}$sg*Irgm2*, alluding that *Irgm2* deficiency was sufficient to specifically promote caspase-11-dependent response in the absence of GBPs (Fig EV2C and D). GBP enrichment on microbial ligand is of importance for efficient caspase-11 and human caspase-4 recruitment (Thurston *et al*, 2016; Fisch *et al*, 2019). However, monitoring for GBP loading on mCherry-expressing *S*. Typhimurim did not show a significant change in the percentage of bacteria targeted by GBP2 (10–15%) in WT and *Irgm2*$^{-/-}$ BMDMs (Fig 2C), which suggests that Irgm2-inhibited non-canonical inflammasome response does not involve GBP2 recruitment modulation.

As caspase-11 activation needs binding to LPS, we hypothesized that Irgm2 expression regulates caspase-11 recruitment to bacterial LPS. In order to monitor this, we generated WT, *Irgm2*$^{-/-}$, *GBP*$^{Chr3-/-}$ and *GBP*$^{Chr3-/-}$*Irgm2*$^{-/-}$ iBMDMs that expressed a catalytically inactive mutant of caspase-11 coupled to GFP (Thurston *et al*, 2016) and primed them with IFNγ to induce ISG expression. The recruitment of CASP11-C254G-GFP on *S*. Typhimurium (*orgA*$^{-}$) occurred after 4 h of infection in *Irgm2*$^{-/-}$ iBMDMs, whereas the percentage of caspase-11-targeted bacteria in iWT *GBP*$^{Chr3-/-}$ and *GBP*$^{Chr3-/-}$*Irgm2*$^{-/-}$ iBMDMs remained low or null (Fig EV2E). After 8 h of infection, however, the levels of CASP11-C254G-GFP-associated bacteria increased in iWT (10%), albeit the percentage of CASP11-C254G-GFP^{+} bacteria remained below those observed in *Irgm2*$^{-/-}$ cells (15–16%) (Fig 2D). Strikingly, we noticed that CASP11-C254G-GFP targeting to *Salmonella* was partially restored in *GBP*$^{Chr3-/-}$*Irgm2*$^{-/-}$ iBMDM after 8 h of infection, although it was strongly impaired in *GBP*$^{Chr3-/-}$ cells (Fig 2D). Altogether, our results point out that *Irgm2* deficiency accelerates caspase-11 recruitment on bacterial membranes. Although we cannot entirely exclude that Irgm2 might also regulate GBP-dependent caspase-11 recruitment to bacterial membranes, our result show that an *Irgm2* deficiency opens a GBP^{Chr3} alternative road for caspase-11 response.

Irgm2 cooperates with GATE16 to dampen Gram-negative bacteria-induced non-canonical inflammasome activation

As *Irgm2* deficiency can promote caspase-11 activation independently of *GBP*^{Chr3}, we next searched for additional regulators. We used a GFP-Trap coupled to mass spectrometry (MS) strategy using IFNγ-primed i*Irgm2*$^{-/-}$ BMDMs complemented with a doxycycline-inducible Irgm2-GFP construct (Fig 3A). Although we detected some important described immune regulators (e.g., galectin-8), the three independent MS datasets (Fig EV3A) showed that one protein, namely gamma-aminobutyric acid (GABA)-A-receptor-associated protein (GabarapL2, referred as Gate-16), was reproducibly enriched in the top 10 hits of the Irgm2-GFP fraction (Figs 3A and EV3A). Therefore, we decided to investigate whether Gate-16 played a role in Irgm2-inhibited caspase-11 response. Co-immunoprecipitation

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experiments confirmed that Gate-16 was present in the Irgm2-GFP fraction, hence validating our MS results (Fig 3B). Gabarap proteins (Gabarap, GabarapL1 and Gate-16) belong to the ATG8 superfamily proteins, all involved in autophagy/membrane remodelling regulation. While *Gabarap* deficiency leads to increased canonical NLRP3 inflammasome response in mice, there is no information regarding the putative function of Gate-16 at regulating the non-canonical inflammasome. In this context, we found that silencing of *Gate-16*, but no other *gabaraps,* increased OMV-induced caspase-11-dependent cell death and IL-1β release (Figs 3C, and EV3B and C). As a control, *Gate-16* silencing did not alter BMDM response to canonical NLRP3 inflammasome activators (Fig EV3D). Then, we sought to determine whether Gate-16-inhibited caspase-11 response was part of the Irgm2 path. Consequently, we silenced *Gate-16* gene expression in WT, $GBP^{Chr3-/-}$, $Casp11^{-/-}$ and $Irgm2^{-/-}$ BMDMs and evaluated the ability of OMVs to induce a caspase-11-dependent response. *Gate-16*

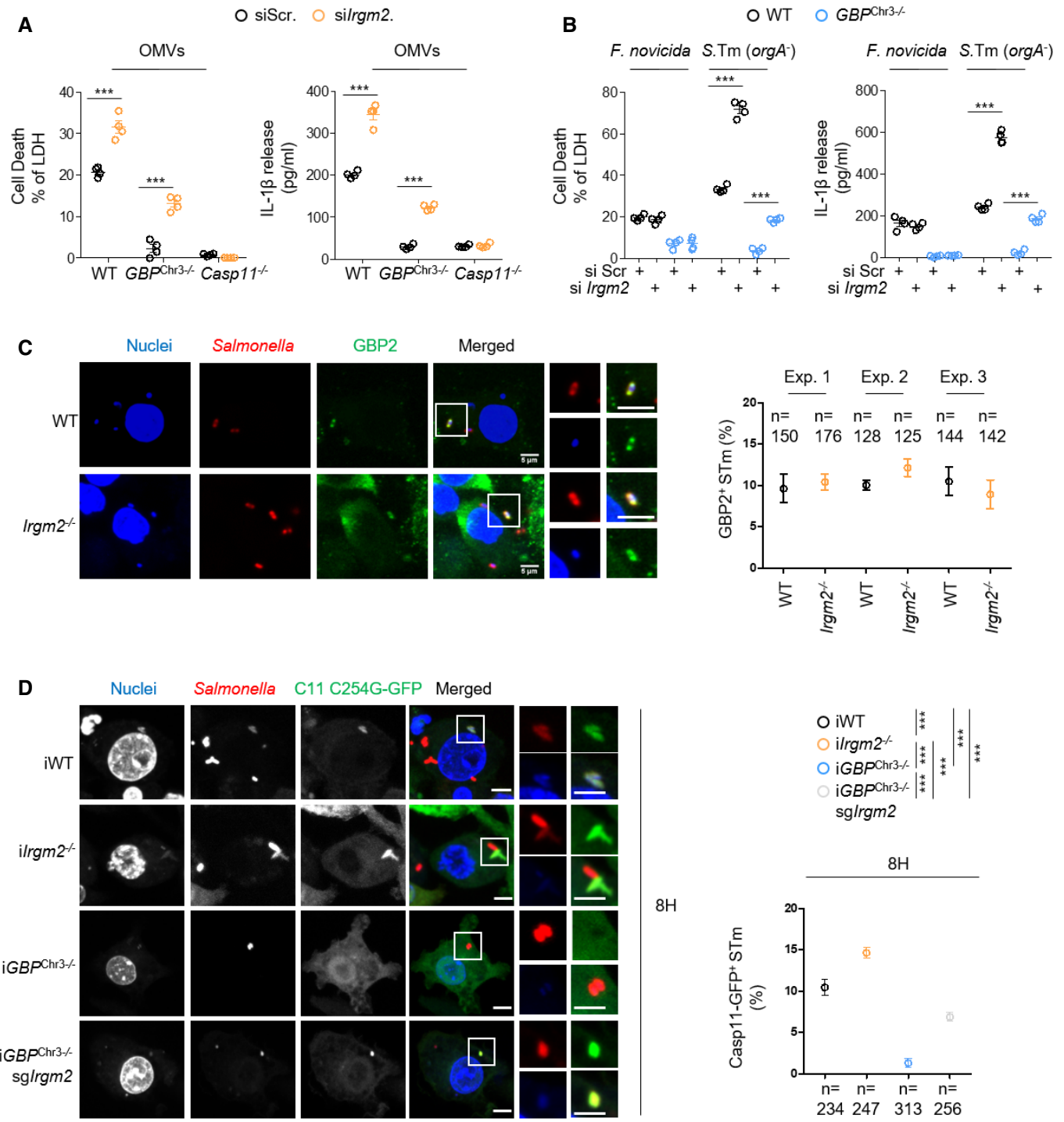

Figure 2.

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Figure 2. Irgm2 regulates GBP-independent caspase-11 targeting to Gram-negative bacteria.

Unless otherwise specified, BMDMs were treated with 2.5 μg/2 × 10^5 cells of OMVs or infected with either *S.* Typhimurium *orgA*$^-$ (*S.* Tm *orgA*$^-$) or *F. tularensis* spp *novicida* (*F. novicida*) with an MOI of 25 for various times.

A Measure of LDH and IL-1β release in WT, *GBP*$^{Chr3-/-}$ and *Casp11*$^{-/-}$ BMDMs were Irgm2 was knocked down 16 h after exposure to 2.5 μg/2 × 10^5 cells of OMVs. Si Scramble (siScr.) refers to RNAi pools with non-targeting sequences.

B Cell death (LDH) and IL-1β release evaluation in *Irgm2*-silenced WT and *GBP*$^{Chr3-/-}$ BMDMs infected for 16 h with either *S.* Tm *orgA*$^-$ or *F. novicida* (MOI 25). Si Scramble (siScr.) refers to RNAi pools with non-targeting sequences.

C Florescence microscopy and associated quantifications of GBP-2 (green) recruitments to intracellular *S.* Tm *orgA*$^-$-mCherry (MOI 10, red) in IFNγ-primed WT and *Irgm2*$^{-/-}$ BMDMs. Nucleus was stained with Hoechst (blue). Confocal images shown are from one experiment and are representative of $n = 3$ independent experiments; scale bars 5 μm. For quantifications, the percentage of GBP-associated bacteria was quantified and "*n*=" refers to the number of intracellular bacteria counted in each experiment; quantifications from $n = 3$ independent experiments were then plotted and expressed as mean ± SEM.

D Confocal fluorescence microscopy images and associated quantifications of caspase-11-C254G-GFP (green) recruitment to *S.* Tm-mCherry (*orgA*$^-$, red) in IFNγ-primed iWT, i*Irgm2*$^{-/-}$, i*GBP*$^{Chr3-/-}$ and i*GBP*$^{Chr3-/-}$/sg*Irgm2* BMDMs after 8 h of infection. Nucleus (blue) was stained with Hoescht, scale bar 5 μm. For quantifications, the percentage of bacteria positive for caspase-11-C254G-GFP was determined by combining the bacterial counts from $n = 3$ independent experiments and expressed as mean ± SEM. ***$P \leq 0.001$ for the indicated comparisons using *t*-test with Bonferroni correction.

Data information: Data shown as means ± SEM (graphs A, B) from $n = 4$ independent pooled experiments; ***$P \leq 0.001$ for the indicated comparisons using *t*-test with Bonferroni correction. Images (C, D) are representative of one experiment performed three times.

silencing in WT BMDMs increased the non-canonical inflammasome response while *Casp11*$^{-/-}$ macrophages remained unresponsive to OMV-induced cell death, IL-1β release (Fig 3D). Interestingly, we observed that *GBP*$^{Chr3-/-}$ macrophages silenced for Gate-16 partially recovered their ability to respond to caspase-11 activators (Fig 3E). Finally, *Gate-16* knock down in *Irgm2*$^{-/-}$ BMDMs did not exacerbate cell death and IL-1β release nor gasdermin-D or caspase-1 cleavages (Fig 3D and E), suggesting that Irgm2 and Gate-16 work together to restrain non-canonical inflammasome response. Since *Irgm2* deficiency leads to caspase-11 enrichment to bacterial membranes, we wondered about the role of Gate-16 in this process. We silenced *Gate-16* in iWT-expressing CASP11-C254G-GFP and checked for its recruitment on *S.* Tm membranes. Consequently, iWT BMDMs knocked down for *Gate-16* had a more pronounced accumulation of caspase-11 on *S.* Tm membranes than the controls after 4 and 8 h of infection (Fig 3F), which mirrored what we previously observed in *Irgm2*$^{-/-}$ macrophages. By contrast, *Gate-16* silencing in *Irgm2*$^{-/-}$ did not increase the percentage of bacteria targeted by CASP11-C254G-GFP after 4 h of infection (Fig EV3E). Recent work suggested that caspase-11 activation could restrict *Salmonella* proliferation in macrophages and epithelial cells (Meunier *et al*, 2014; Thurston *et al*, 2016). Therefore, we monitored bacterial load in WT and *Casp11*$^{-/-}$ BMDMs in which we silenced *Irgm2* and *Gate-16*. Whereas *Gate-16* silencing led to slight increased colony forming unit (CFUs) 24 h after infection, *Irgm2* silencing did not modify intracellular bacterial loads, suggesting that Gate-16 also covers a caspase-11-independent cell autonomous immune process (Fig EV3F). Altogether, these results suggest that Irgm2 and Gate-16 cooperate to restrict the non-canonical inflammasome response by restraining caspase-11 targeting to bacterial membranes.

Irgm2- and Gate16-inhibited *Salmonella*-induced non-canonical inflammasome responses do not involve canonical autophagy

As Gate-16 is involved in the canonical and non-canonical autophagy regulation, we wondered if the exacerbated non-canonical inflammasome response observed in the absence of Gate-16 and Irgm2 relied on autophagy modulation. Therefore, we pharmacologically inhibited canonical autophagy (3-MA or Wortmannin) in WT and *Casp11*$^{-/-}$ BMDMs targeted with siRNA against *Irgm2* or *Gate-16*. Autophagy inhibition in BMDMs triggered exacerbated *Salmonella* (*orgA*$^-$)-induced cell death and IL-1β release, a process that required caspase-11 (Fig EV4A). However, although Irgm2 and Gate16 knock down drove increased caspase-11-dependent response, autophagy inhibition also exacerbated the macrophage response to *Salmonella* infection (Fig EV4A). LC3b targeting to bacteria is also a hallmark of anti-bacterial autophagy (Masud *et al*, 2019; Wu & Li, 2019). Consequently, we infected WT BMDMs silenced for *Irgm2* or *Gate16* with *Salmonella* and analysed by fluorescence microscopy the recruitment of LC3b on bacterial compartments. We found that the percentage of LC3b$^+$ bacteria in control siRNA-treated BMDMs was not significantly altered in BMDMs silenced for *Irgm2* or *Gate16*, although we noticed a trend for LC3b accumulation in si*Gate16*-treated cells (Fig EV4B). This suggests that *Gate16* and *Irgm2* deficiencies modulate the non-canonical inflammasome response independently of canonical autophagy. This is in agreement with the findings of Finethy *et al* (2020) that underlined a lack of canonical autophagy markers (e.g., LC3 lipidation) alteration in *Irgm2*$^{-/-}$ BMDMs. Yet, BMDMs deficient for canonical autophagy regulators had an exacerbated non-canonical inflammasome response. Altogether, these results suggest that Gate-16/Irgm2-inhibited non-canonical inflammasome response does not involve canonical autophagy modulation.

GATE16 inhibits non-canonical inflammasome activation in human myeloid cells

Gate-16 is expressed in both humans and rodents, yet humans only express one IRGM, although mice have three (Irgm1-3). Therefore, we performed siRNA-based experiments in primary human monocyte-derived macrophages (hMDMs) to determine whether IRGM and GATE16 might also regulate the caspase-4/5 non-canonical inflammasome. Although the use of the caspase-4/5 inhibitor LEVD and of the NLRP3 inhibitor MCC950 showed that hMDMs responded to *Salmonella orgA*$^-$ infection by activating the non-canonical inflammasome, we failed to observe any regulatory role for IRGM at regulating such a process (Figs 4A and EV5A). However, *GATE16* silencing increased their ability to respond to *Salmonella* through the non-canonical inflammasome (Figs 4A and EV5A). When we used Nigericin to trigger the canonical NLRP3 inflammasome, hMDMs knocked down for *GATE16* did not show cell death and IL-1B alterations (Fig 4B). To the contrary, *IRGM*-silenced hMDMs had

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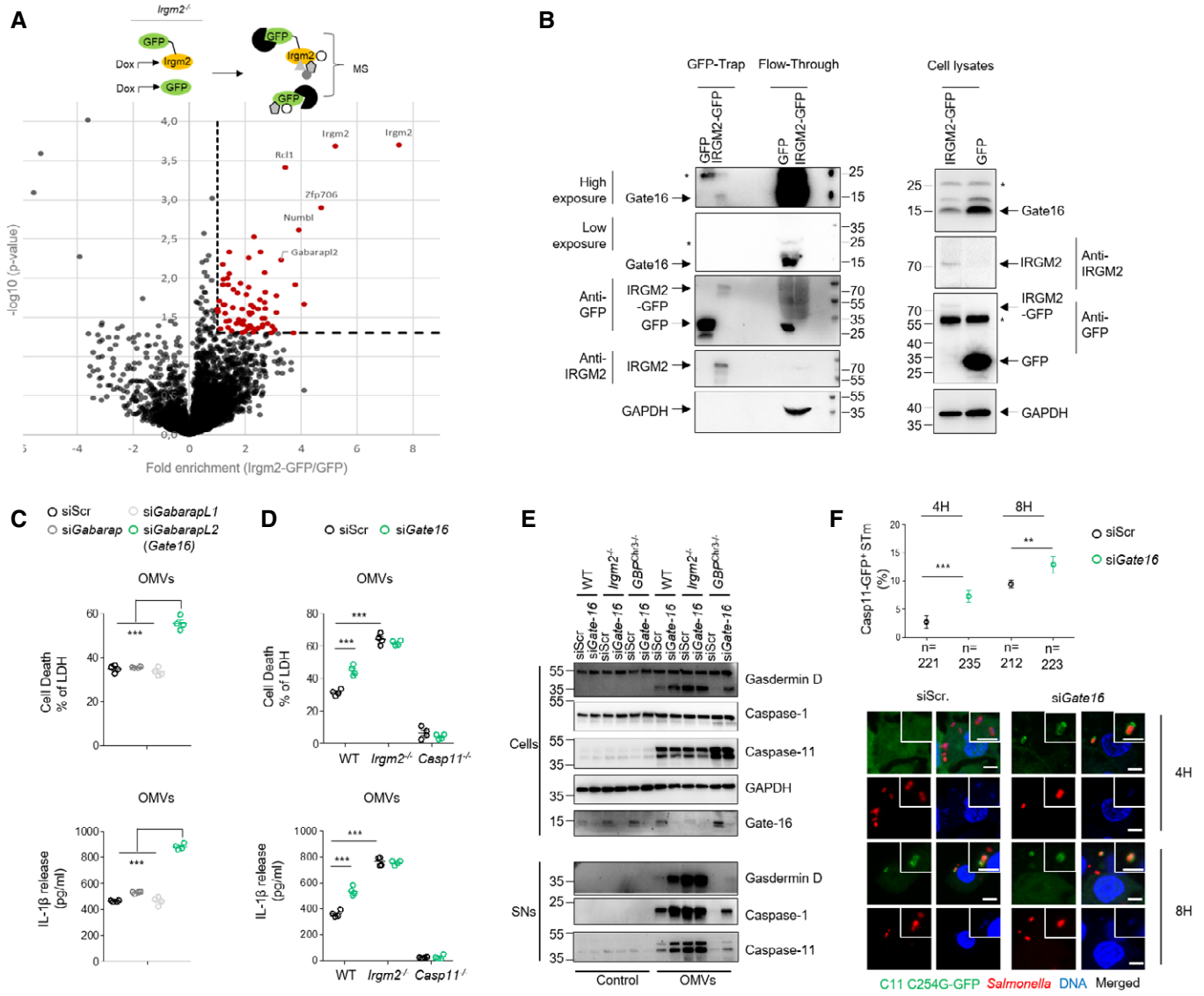

Figure 3. Irgm2 cooperates with GATE16 to dampen Gram-negative bacteria-induced non-canonical inflammasome activation.

A GFP-Trap coupled to mass spectrometry strategy used. The volcano plot represents three independent combined experiments. Threshold selection of enriched proteins (red dots) in Irgm2-GFP fraction was set at 2-fold enrichment (*x*-axis) and *P*-value < 0.05 (*y*-axis). Blacks and grey dots indicate proteins that did not reach a *P*-value < 0.05 using *t*-test with Bonferroni correction. Labelled proteins represent the top 6 enriched proteins in the Irgm2 fraction.

B Green florescent protein (GFP)-Trap assay of the presence of Gate16 in Irgm2-GFP-enriched fraction from the lysates of IFNγ-primed i$Irgm2^{-/-}$ BMDMs complemented with lentiviral constructs coding for a fusion of Irgm2 with GFP (Irgm2-GFP) or GFP alone. Arrows show the presence or not of Gate16, Irgm2-GFP and GAPDH in the Irgm2-GFP-enriched fractions, flow-through and total cell lysates. * means non-specific band.

C LDH and IL-1β release from siRNA-treated WT BMDMs, and then exposed to 2.5 μg/2 × 10^5 cells of OMVs for 16 h.

D LDH and IL-1β release from WT, $Irgm2^{-/-}$ and $Casp11^{-/-}$ BMDMs silenced for *Gate16* and treated for 16 h with 2.5 μg/2 × 10^5 cells of OMVs. Si Scramble (siScr.) refers to RNAi pools with non-targeting sequences.

E Western blot examination of caspase-11 and processed caspase-1 (p20) and gasdermin-D (p30) in supernatants and pro-caspase-1 (p45), pro-gasdermin-D (p55), pro-caspase-11, Gate16 and GAPDH in cell lysates of *Gate16*-silenced WT, $Irgm2^{-/-}$ and $GBP^{Chr3-/-}$ BMDMs exposed to 2.5 μg/2 × 10^5 cells of OMVs for 16 h. Si Scramble (siScr.) refers to RNAi pools with non-targeting sequences.

F Representative confocal fluorescence microscopy images and associated quantifications of caspase-11-C254G-GFP (green) recruitment to *S.* Tm-mCherry ($orgA^-$, red, MOI 10) in IFNγ-primed iWT BMDMs silenced for *Gate16* after 4 and 8 h of infection. Nucleus (blue) was stained with Hoechst, scale bar 5 μm. For quantifications, the percentage of bacteria positive for caspase-11-C254G-GFP was determined by combining the bacterial counts from $n = 3$ independent experiments and expressed as mean ± SEM. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ for the indicated comparisons using *t*-test with Bonferroni correction. Si Scramble (siScr.) refers to RNAi pools with non-targeting sequences.

Data information: Data shown as means ± SEM (graphs C, D) from $n = 4$ independent pooled experiments; $^{***}P \leq 0.001$ for the indicated comparisons using *t*-test with Bonferroni correction. Image (B) is representative of one experiment performed two times, and (E) represents one experiment out of three. (A) represents one experiment out of three independent experiments.
Source data are available online for this figure.

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higher IL-1B and cell death levels than their respective controls, which is reminiscent of previous studies that showed a regulatory role for IRGM on the canonical NLRP3 inflammasome (Fig 4B). Although the research of a protein with a similar function of rodent Irgm2 in humans warrants further investigations, our results suggest that GATE16 function is conserved between both species. Next, we wondered if GATE16-inhibited non-canonical inflammasome response to *Salmonella* depended on human GBPs. GBP1, GBP2 and GBP5 have recently been described as being important for efficient CASP4 recruitment and activation on bacterial membranes. Hence, we used human monocytic cell line U937 genetically invalidated for GBP1, GBP2 and GBP5 (*GBP1/2/5*$^{-/-}$) (Fig EV5B). Infection of IFNγ-primed WT or *GBP1/2/5*$^{-/-}$ U937 cells with *Salmonella* triggered both GBP-dependent cell death and IL-1B release (Fig 4C). Importantly, *GATE16* silencing in *GBP1/2/5*$^{-/-}$ cells reinsured significant cell death and IL-1B release, suggesting that the human non-canonical inflammasome response relies on GBP-dependent and GBP-independent mechanisms (Fig 4C). Finally, we aimed at determining if GATE16 is a direct regulator of the non-canonical inflammasome response in human cells. Therefore, we electroporated LPS in WT or *GBP1/2/5*$^{-/-}$ U937 cells in the presence or absence of *GATE16* siRNA (Fig 4D). Our results showed that electroporated LPS-induced cell death and IL1B release did not involve GATE16 (Fig 4D), suggesting that GATE16 acts upstream of the non-canonical inflammasome. Altogether, our results identified Irgm2 and GATE16 that cooperatively restrict Gram-negative bacteria-induced non-canonical inflammasome activation in both mice and humans.

Tight regulation of the non-canonical inflammasome pathway is of major importance as its uncontrolled non-canonical inflammasome response drives endotoxic shock. Conversely, two recent studies have uncovered that the IRF2 transcription factor (and to a lower extent IRF1) transcriptionally controls murine gasdermin-D and human caspase-4 expression (Benaoudia *et al*, 2019; Kayagaki *et al*, 2019). In addition, SERPINB1 has also been found to directly interact and inhibit activation of the inflammatory caspase-1, caspase-4 and caspase-11 (Choi *et al*, 2019). Here, both Finethy *et al* (2020) and us report a critical role of Irgm2 and Gate-16 at balancing the non-canonical inflammasome activity. The Irgm2/Gate-16 axis was required to inhibit caspase-11 recruitment to Gram-negative bacteria in the host cell cytosol, which provided controlled caspase-11 response and protection against sepsis. These findings open many yet unanswered questions such as at which step the Irgm2/Gate16 axis is regulating caspase-11 recruitment to bacterial products. Gate-16 has been found to control proper cytosolic localization of various GBPs (Park *et al*, 2016; Sasai *et al*, 2017), including GBP2, crucial at regulating caspase-11 recruitment on intracellular pathogen PAMPs. However, results from Finethy *et al* (2020) and ours indicate that *Gate16/Irgm2* removal in GBP-deficient macrophages (lacking GBPs 1, 2, 3, 5 and 7) partially restores a caspase-11-dependent response, suggesting that the Gate-16/Irgm2 path might regulate caspase-11, at least to certain extent, independently of these GBPs. Recently, two publications described that hGBP4 participated with GBP1 and GBP3 to the recruitment of CASP4 to cytosolic Gram-negative bacterial membranes in human

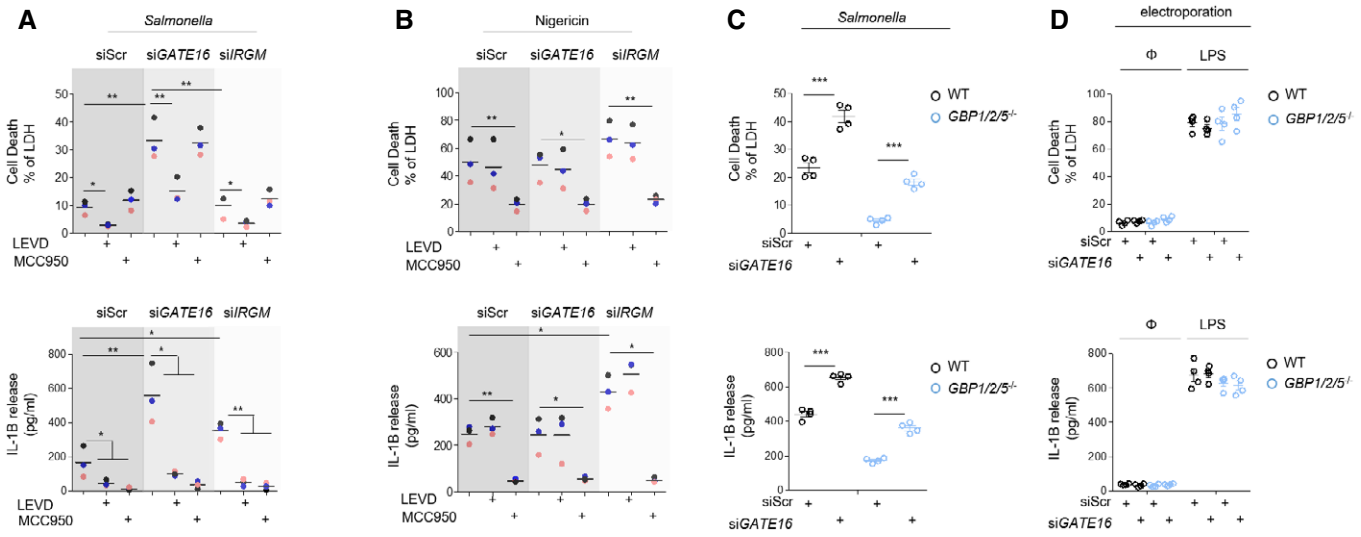

Figure 4. GATE16 inhibits non-canonical inflammasome activation in human myeloid cells.

A LDH and IL-1B release from siRNA-treated primary human monocyte-derived macrophages (hMDMs) infected with *S.* Typhimurium *orgA*$^{-}$ (MOI25) for 16 h. When specified, the caspase-4/5 inhibitor Z-LEVD (25 μM) or the NLRP3 inhibitor MCC950 (10 μM) was added to the experiments.

B LDH and IL-1B release from siRNA-treated primary human monocyte-derived macrophages (hMDMs), primed with IFNγ (10 UI/ml) and PAM3CSK4 (100 ng/ml) and then stimulated with Nigericin (20 μM) for 4 h. When specified, the caspase-4/5 inhibitor Z-LEVD (25 μM) or the NLRP3 inhibitor MCC950 (10 μM) was added to the experiments.

C, D LDH and IL-1B release from siRNA-treated WT or *GBP1/2/5*$^{-/-}$ U937 monocytic cell line, primed with IFNγ (10 UI/ml) and PAM3CSK4 (100 ng/ml) and then infected with (C) with *S.* Typhimurium *orgA*$^{-}$ (MOI25) for 10 h or (D) electroporated with 1 μg of *Escherichia coli* LPS for 4 h. Φ indicates that no LPS electroporation was performed. Data shown as means ± SEM from $n = 4$ independent pooled experiments; ***$P \leq 0.001$ for the indicated comparisons using *t*-test with Bonferroni correction.

Data information: Data shown as means (graphs A, B) from $n = 3$ different donors; each donor is represented with a coloured circle; *$P \leq 0.05$, **$P \leq 0.01$ for the indicated comparisons using one-way ANOVA with multiple Bonferroni correction.

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epithelial cells (Santos *et al*, 2020; Wandel *et al*, 2020). Whether Irgm2/Gate-16-inhibited caspase-11 (or hCASP4) enrichment on bacterial membranes involves other GBPs (4 and 6) remains to be addressed. So far, due to the lack of information, a possible function of Irgm2 remains elusive, but Irgm1 and its human homologous IRGM have been described to participate in the autophagy/xenophagy processes (Maric-Biresev *et al*, 2016; Azzam *et al*, 2017). In addition, Gate-16 belongs to the ATG8-like proteins, including LC3 (abc), Gabarap and GabarapL1, all involved in various autophagy/membrane remodelling step regulation such as lysosome biogenesis, autophagosome formation and closure (Lee & Lee, 2016; Nguyen *et al*, 2016; Gu *et al*, 2019). To this regard, both Irgm1/IRGM (Finethy *et al*, 2020) and Gabarap proteins inhibit the activation of the Nlrp3 inflammasome (Pei *et al*, 2017; Mehto *et al*, 2019a,b). In addition, Irgm-1 and Irgm-3 deficiencies also rescue exacerbated inflammasome response from Irgm2-deficient macrophages (Finethy *et al*, 2020). Therefore, one can hypothesize that *Gate-16* and *Irgm2* deficiencies could lead to defective autophagy, which would promote cytosolic LPS accumulation and an exacerbated caspase-11 activation. However, results from Finethy *et al* (2020) and our own suggest that Gate-16 and Irgm2 regulate Gram-negative bacteria-induced non-canonical inflammasome response in an autophagy-independent manner. Should those effectors modulate the non-canonical autophagy pathway is an attractive hypothesis that deserves further investigations. Another possible explanation relies on the Golgi enrichment of both Irgm2 and Gate-16 (Sagiv, 2000; Zhao *et al*, 2010). Indeed, Gate-16 also regulates Snare-dependent vesicular trafficking, independently of its autophagy function (Sagiv, 2000). Various groups previously found that endocytosed and intracellular monomeric LPS could be targeted to the Golgi apparatus (Thieblemont & Wright, 1999; Latz *et al*, 2002). Caspase-4, caspase-5 and caspase-11 need accessible lipid A to oligomerize and auto activate, which can be extremely difficult in the presence of multimeric and hydrophobic LPS particles to the contrary of monomeric LPS that might present a more accessible lipid A. An attractive hypothesis is that GBP-mediated bacterial membrane damages allow LPS retrieval from aggregates in order to ensure proper lipid A exposure to caspase-11 (Santos *et al*, 2018, 2020; Kutsch *et al*, 2020; Wandel *et al*, 2020). Therefore, one could speculate that Golgi-regulated monomeric LPS trafficking might be impaired in the absence of either Irgm2 or Gate-16, which would allow direct caspase-11/lipid A interactions without the need for GBPs.

Our results showed that both murine and human Gate-16 regulate the non-canonical inflammasome response to LPS-containing particles. Yet, we failed to isolate IRGM as a human functional homologous of Irgm2. Given the strong role of Irgm2 at regulating the non-canonical inflammasome in mice, there is a possibility that another, yet unidentified, human protein holds a similar function of the one carried out by Irgm2. Therefore, this warrants future investigations to identify such Irgm2-like human protein. Humans and mice have different sensitivities to LPS. Indeed, LPS-induced sepsis in mice requires 1–25 mg/kg of LPS whereas humans have a 100–1,000,000 time lower sepsis threshold (2–4 ng/kg of LPS) (Fink, 2014). Another explanation could be that the evolutionary loss of Irgm2 in humans would leave human cells with only Gate-16, which would greatly lower the sensitivity of human cells to cytosolic LPS-activated non-canonical inflammasome.

In summary, our work identified two negative regulators of caspase-11 recruitment to bacterial membranes, namely Irgm2 and Gate-16. Additional investigations will be necessary to understand how both effectors balance caspase-4, caspase-5 and caspase-11 sensitivity to Gram-negative bacteria, and what specific physiological and cellular processes Irgm2 and Gate-16 cover together.

Materials and Methods

Reagents, biological samples and their concentration of use are referenced in the Appendix Table S1.

Mice

Casp11$^{-/-}$, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Nlrp3*$^{-/-}$ and *GBP*$^{Chr3-/-}$ mice have been described in previous studies (Li *et al*, 1995; Wang *et al*, 1998; Martinon *et al*, 2006; Yamamoto *et al*, 2012). *Irgm2*$^{-/-}$ mice were provided by the Jackson laboratory (USA). All mice were bred at the IPBS institute (Toulouse, France) animal facilities according to the EU and French directives on animal welfare (Directive 2010/63/EU). Charles Rivers provided WT C57BL/6J and C57BL/6N mice.

Animal sepsis models

8- to 12-week-old mice (sex-matched, 6–10 per group) were injected intraperitoneally with a solution of 100 µl of poly(IC) LMW (invivoGen, 100 µg/animal) for 6 h. Then, mice were intraperitoneally injected with 5 mg/kg of LPS (InvivoGen, O111:B4) or 5 or 25 µg/animal of outer membrane vesicles (*E. coli*, InvivoGen). Mouse survival was monitored over 80 h. For cytokine assays, poly(IC)-primed mice were injected with 25 µg/animal of OMVs for 8 h and plasma cytokine amounts were addressed using ELISA kits (listed in the Appendix Table S1). There was no randomization or blinding performed.

Animal experiments were approved (Licence 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).

BMDM isolation and culture

Murine bone marrow-derived macrophage (BMDM) generation has previously been described. Briefly, 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. For experiments, 1.25×10^6, 2.5×10^5 or 5×10^4 BMDMs were seeded in 6-, 24- or 96-well plates, respectively, when described BMDMs were prestimulated overnight with either PAM3CSK4 (InvivoGen, 100 ng/ml) or IFNγ (PeProtech, 100 UI/ml). For non-canonical inflammasome stimulation, we used pure LPS (O111B4, InvivoGen, 1 µg/ml), outer membrane vesicles (*E. coli*, InvivoGen, 0.5, 1 and 2.5 µg/2×10^5 cells) or various Gram-negative bacterial strains were used including, *Shigella flexneri* (M90T, MOI25), *Salmonella* Typhimurium *orgA*$^-$ (SL1344, MOI25), *E. coli* (K12, MOI25), *Citrobacter koseri* (MOI25) and *Enterobacter cloacae* (MOI25). When required, Wortmannin (Wort, 10 µM) and 3-metyladenine (3-MA, 1 mM) were added 2 h after infection in order to inhibit autophagy. When

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specified, 1 μg of *E. coli* ultrapure LPS (O111:B4) was electroporated with Neon™ Transfection System (Thermo Fisher) according to manufacturer's protocol. Briefly, 5×10^5 cells were resuspended in Buffer R and 1 μg/ml of LPS was electroporated in 10 μl tips using two pulses of 1,720 V and 10 width. Cells were then plated in 24-well plates.

For canonical inflammasome stimulations, overnight (ON) IFNγ-primed BMDMs were then prestimulated with PAM3CSK4 (InvivoGen, 100 ng/ml) for 4 h to induce pro-IL1β expression. Then, Nigericin (NLRP3 activator, 40 μM, InvivoGen), dA:dT (AIM2 activator, 1 μg/ml, InvivoGen), TcdB toxin (PYRIN inducer, 0.05 μg/ml, List Biological Laboratories) or flagellin (NLRC4 trigger, 1 μg/ml, InvivoGen) was used to stimulate various canonical inflammasomes. Both flagellin and poly(dA:dT) were transfected into cells using FuGeneHD (Promega) transfection reagent in Opti-MEM culture medium. When specified, *P. aeruginosa* (PAO1) and *S*. Tm strains (SL1344) (MOI 5) were used to trigger NLRC4 inflammasome response.

For all stimulations, macrophage medium was replaced by serum-free and antibiotic-free Opti-MEM medium and inflammasome triggers were added to the macrophages for various times.

Specific to infections, plates were centrifuged for 1 min, 800 rpm to ensure homogenous infections. Then, extracellular bacteria were eliminated with gentamicin (100 μg/ml, Invitrogen).

Bacterial cultures

Bacteria were grown overnight in Luria Broth (LB) medium at 37°C with aeration and constant agitation in the presence or absence of antibiotics (specified in the Appendix Table S1), stationary phase (OD of 2–2.5) bacteria when then used for infections. Stimulation of the NLRC4 inflammasome by *S*. Typhimurium SL1344 and *P. aeruginosa* PAO1 bacteria required proper T3SS and flagellin expression; therefore, bacteria were sub-cultured the next day by dilution overnight culture 1/50 and grew until reaching an O.D600 of 0.6–1.

CFU evaluation

2.5×10^5 BMDMs were infected with stationary phase *Salmonella orgA*$^-$ (MOI10) for 1 h. Cells were treated with gentamicin (100 μg/ml) for 30 min to kill extracellular bacteria and then washed three times in PBS. Medium was replaced with BMDM medium supplemented with 20 μg/ml of gentamicin to avoid extracellular bacterial replication. At the end of the experiment, cells were lysed in Triton 0.1% and intracellular bacterial loads were evaluated using CFU plating.

Gene knock down

Gene silencing was achieved using siRNA pools (Dharmacon, 25 nM/well listed in Appendix Table S1) as previously described (Meunier *et al*, 2015; Santos *et al*, 2018) or accell siRNA technology. SiRNA smart pools from Dharmacon were transfected into cells using the DharmaFECT 4 transfection reagent (Dharmacon) for 48 h. Primary human macrophages were treated with 1 μM siRNA Accell (Dharmacon, smart pool) in the absence of transfection reagent for 72 h. Then, murine BMDMs and human macrophages were stimulated with 1 μg/2×10^5 cells of OMVs or infected with *Salmonella* Typhimurium (*orgA*$^-$) to trigger non-canonical inflammasome response. For siRNA experiments, gene knock down efficiency was monitored by qRT–PCR or immunoblotting (WB) assays.

Quantitative real-time PCR

Cellular RNAs were extracted from 2.5×10^5 cells using RNeasy Mini Kit (Qiagen). mRNAs were reverse transcribed with the Verso cDNA Synthesis Kit (Thermo Scientific). Regarding qPCR experiments, 1 μM of primers (Appendix Table S1), SYBR™ Select Master Mix (Thermo Scientific) and 15 ng of cDNA were mixed in a 10 μl reaction in a QuantStudio 5 device (Applied Biosystems). Primers were generated using primer3 software.

Cytokine and pyroptosis measurement

Murine Il-1α, IL-1β, TNF-α, IL12, IL18, IFNγ, IL-6, and human IL-1B cytokine levels were measured by ELISA (listed in Appendix Table S1). LDH cytotoxicity detection kit (Takara) allowed to monitor for cell lysis. Normalization of spontaneous lysis was calculated as follows: (LDH infected − LDH uninfected)/(LDH total lysis − LDH uninfected) × 100.

Immunoblotting

Preparation of cell lysates and supernatants has been described previously. Proteins were loaded and separated in 12% SDS–PAGE gels and then transferred on PVDF membranes. After 1 h of saturation in Tris-buffered saline (TBS) with 0.05% Tween 20 containing 5% non-fat milk (pH 8), membranes were incubated overnight with various antibodies (referenced in Appendix Table S1). The next day, membranes were washed three times in TBS 0.1% Tween 20 and incubated with appropriate secondary horseradish peroxidase (HRP)-conjugated antibody (dilution 1/5,000–10,000, listed in Appendix Table S1) for 1 h at room temperature. Then, after three washes, immunoblottings were revealed with a chemiluminescent substrate ECL substrate (Bio-Rad) and images were acquired using ChemiDoc Imaging System (Bio-Rad). All antibody references and working dilutions are presented in Appendix Table S1.

Microscopy

2.5×10^5 BMDMs on glass coverslips were infected with *S*. Typhimurium (MOI10) expressing an mCherry fluorescent protein. At the indicated times, cells were washed three times with PBS and fixed with 4% PFA for 10 min at 37°C. 0.1 M glycine was used to quench excess of PFA for 10 min at room temperature. Then, cells were permeabilized and incubated with primary antibodies O/N at 4°C in saponin 0.1%/BSA 3% solution. Cellular stainings were achieved using Hoescht (DNA labelling), GBP2 antibody (gift from J Howard). Coverslips were then washed with Saponin/BSA solution and further incubated with the appropriate secondary antibodies coupled to fluorochromes (1/1,000; Appendix Table S1). After three washes with PBS, cells were mounted on glass slides using VECTASHIELD (Vectalabs). Coverslips were imaged using confocal Zeiss LSM 710 (Image core Facility, IPBS, Toulouse or an Olympus/Andor CSU-X1 Spinning dick microscope using a 63× oil objective. Otherwise specified, 5–10 fields/experiment were manually counted using ImageJ software.

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Transduction of iBMDMs

HEK 293-based retroviral packaging cell line (GP2-293) was plated in 10-cm Petri dish in DMEM + 10% FCS + 1% PS. When cell's confluency reached 60–80%, cells were placed in serum and antibiotic-free Opti-mem medium and transfected with VSV-G encoding vector (pMD.2G) along with CASP11-C254G-GFP or pRetro (-GFP or –Irgm2-GFP) vectors using PEI transfection reagent. 10 h after transfection, cell medium was replaced by DMEM + 10% FCS + 1% PS. At 48 h post-transfection, cell's supernatant containing retroviral particles were collected, filtered 0.45 μm and used to transduce target cells. After 48 h, puromycin (5 μg/ml) was used to select cells positively transduced with the transgene. When vectors contained GFP fusions, cells were sorted using fluorescence-activated cell sorting.

Immunoprecipitation and GFP-Trap

Irgm2$^{-/-}$ immortalized macrophages were transduced with retroviral vectors carrying a doxycycline-inducible Irgm2-GFP, or GFP alone constructs, cloned into Retro-X™ Tet-On® 3G vector (Clontech Laboratories, Inc.). To ensure proper Irgm2-GFP expression, cells were incubated 16 h with doxycycline 1 μg/ml in the presence of IFNγ. Irgm2-GFP and associated protein complexes were pull-down using GFP-Trap magnetic beads according to manufacturer's instructions (chromotek). Briefly, cells were lysed in CoIP lysis buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40, 0.09% Na-Azide) supplemented with a protease inhibitor cocktail (Roche). Cell lysates were then incubated with GFP-Trap-MA beads for 1 h at 4°C. After two washes with wash-buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA, 0.018% Na-Azide), GFP-Trap complexes were boiled for 10 min at 95°C in RIPA buffer + Laemmli before separation on SDS–PAGE and mass spectrometry or immunoblotting.

Mass spectrometry analysis

Immuno-purified protein samples were reduced with β-mercaptoethanol by heating at 95°C for 5 min, and cysteines were alkylated by addition of 90 mM iodoacetamide. Samples were loaded on a 1D SDS–PAGE gel, and proteins were isolated in a single gel band, which was excised and washed with several cycles of 50 mM ammonium bicarbonate–acetonitrile (1:1). Proteins were in-gel digested using 0.6 μg of modified sequencing grade trypsin (Promega) in 50 mM ammonium bicarbonate overnight at 37°C. Resulting peptides were extracted from the gel by successive incubations in 50 mM ammonium bicarbonate and 10% formic acid–acetonitrile (1:1), then dried in a speed-vac and resuspended with 22 μl of 5% acetonitrile, 0.05% trifluoroacetic acid (TFA) for MS analysis. Peptides were analysed by nanoLC-MS/MS using an UltiMate Nano/Cap System NCS-3500RS coupled to a Q-Exactive HFX mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Separation was performed on a C-18 column (75 μm ID × 50 cm, Reprosil C18) equilibrated in 95% solvent A (5% acetonitrile, 0.2% formic acid) and 5% solvent B (80% acetonitrile, 0.2% formic acid), using a gradient from 10 to 45% gradient of solvent B over 60 min at a flow rate of 350 nl/min. The mass spectrometer was operated in data-dependent acquisition mode with the Xcalibur software. Survey MS scans were acquired in the Orbitrap on the 350–1,400 m/z range, with the resolution set to 60,000, and the 12 most intense ions were selected for fragmentation by higher-energy collisional dissociation (HCD) using a normalized collision energy of 28. MS/MS scans were collected at 15,000 resolution with an AGC target value of 1e5 and a maximum injection time of 22 ms. Dynamic exclusion was used within 30 s to prevent repetitive selection of the same peptide. Three replicate MS analyses were performed for each sample.

Bioinformatic processing of mass spectrometry data

Raw mass spectrometry files were searched using Mascot (Matrix Science) against the Mouse entries of the SwissProt–TrEmbl protein database. The enzyme specificity was "trypsin", with a maximum of two misscleavages. Cysteine carbamidomethylation was set as a fixed modification, and N-terminal protein acetylation and methionine oxidation were specified as variable modifications. For the search, mass tolerance parameters were set at 5 ppm on the parent ion and 20 mmu on the fragment ions. Protein identification results were then validated with the Proline software by the target-decoy approach using a reverse database at a both a peptide and a protein FDR of 1%. To perform label-free relative quantification of proteins, the "abundance" metric retrieved by Proline was used, after global normalization of the MS signal across all MS runs. For each protein, a mean abundance value was computed from technical LC-MS replicate runs, and $\log_2$-transformed. Missing protein abundance values were then replaced by a noise value estimated for each analysis as the 1% lowest percentile of the protein abundance values distribution. Bona fide Irgm2 interactors were identified by comparing Irmg2-GFP immuno-purified samples and GFP control samples. For each protein, an enrichment ratio relative to the control and a Student *t*-test *P*-value was calculated from the protein abundance values derived from three independent biological replicate experiments. Relevant interactors were selected based on an enrichment ratio higher than 2 and a Student *t*-test *P*-value lower than 0.05.

Genetic invalidation of *Caspase-11* and *Irgm2* genes in immortalized BMDMs

Casp11 and *Irgm2* genes were knocked out using the crispr/cas9 system in onco J2-immortalized (i) bone marrow-derived macrophages (BMDMs) iWTs or i*Irgm2*$^{-/-}$macrophages. Single guide RNAs (sgRNA) specifically targeting caspase-11 exon 2 forward (5′CACCGCTTAAGGTGTTGGAACAGCT3′) reverse (5′AAACAGCTG TTCCAACACCTTAAGC3′), Irgm2 exon 2 forward (5′CACCGTTCCA TGTTGTCGAGCAACG3′) reverse (5′AAACCGTTGCTCGACAACATG GAAC3′) were designed using Benchling tool (Benchling.com), and oligonucleotides were synthesized by Sigma-Aldrich. Crispr guide RNA oligonucleotides were then hybridized and cloned in LentigRNA-Puromycin vector using BsmBI restriction enzyme (lentiGuide-Puro, Addgene 52963, Feng Zhang Lab). HEK293T cells were transfected for 48 h with all constructs (Lipofectamine 2000) together with the lentiviral packaging vector p8.91 (Didier Trono Lab, EPFL, Switzerland) and the envelop coding VSV-G plasmid (pMD.2G, Addgene 12259, Didier Trono Lab). 48 h later, viral supernatants were harvested and subsequently filtered on 0.45-μm filter. Recipient cells expressing Cas9 (1,000,000 cells/well in 6-well plates) were generated using lentiviral transduction with a Cas9-expressing lentiviral vector (lentiCas9-Blast, Addgene 52962, Feng Zhang Lab). Then, Cas9^{+} cells were infected with packaged viral

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particles. To ensure efficient infection, viral particles were centrifugated for 2 h at 1,081 *g* at 32°C in presence of 8 μg/ml polybrene. 48 h later, medium was replaced and puromycin selection (10 μg/ml) was applied to select positive clones for 2 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.

Genetic invalidation of human GBPs in U937 cell line

To generate GBP1/2/5 knock-out cell line, deletion of genes was performed in a Cas9-expressing U937 clone obtained by transduction with the plasmid LentiCas9-Blast (from Feng Zhang; Addgene plasmid # 52962) followed by blasticidin selection and clonal isolation using the limit dilution method. A clone strongly expressing Cas9 was selected based on Western blot analysis using anti-Cas9 antibody (Millipore; # MAC133; 1:1,000 dilution). gRNAs targeting GBP1, GBP2 and GBP5 (Appendix Table S1) were cloned into the pKLV-U6gRNA(BbsI)-PGKpuro2ABFP vector (from Kosuke Yusa; Addgene plasmid # 50946). For each gene, two pairs of gRNAs were used. Lentiviral particles were produced in 293T cells using pMD2.G and psPAX2 (from Didier Trono, Addgene plasmids #12259 and #12260), and pKLV-U6gRNA(BbsI)-PGKpuro2ABFP. Cas9-expressing U937 cells were transduced by spinoculation. Gene deletion invalidation was verified by Western blotting analysis (Appendix Table S1).

Generation of human monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat of healthy donors obtained from the EFS Toulouse Purpan (France). Briefly, PBMCs were isolated by centrifugation using standard Ficoll-Paque density (GE Healthcare). 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 514 *g*, at 20°C. The cell interface layer was harvested carefully, and the cells were washed twice in PBS (for 10 min at 185 *g* followed by 10 min at 800 rpm) and resuspended 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 (Miltenyi Biotec). To allow differentiation into monocyte-derived macrophages, cells were cultured in RPMI medium (GIBCO) supplemented with 10% FCS (Invitrogen), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml MCSF for 7 days.

Ethics statements

The use of human cells was approved by the Research Ethical Committee, Haute-Garonne, France. Buffy coats were provided anonymously by the EFS (établissement français du sang, Toulouse, France). Written informed consent was obtained from each donor under EFS contract no 21PLER2017-0035AV02, according, to "Decret No 2007-1220 (articles L1243-4, R1243-61)".

Statistical analysis

Statistical data analysis was performed using Prism 5.0a (GraphPad Software, Inc.). *t*-Test with Bonferroni correction was used for comparison of two groups. For multiple comparisons, one-way ANOVA with multiple Bonferroni correction test was used. Data are reported as mean with SEM. For animal experiments, Mann–Whitney tests were performed, and for mouse survival analysis, log-rank Cox–Mantel test was selected. *P*-values are given in figures, NS means non-significant. Significance is specified as $*P \leq 0.05$; $**P \leq 0.01$, $***P \leq 0.001$.

Data availability

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al*, 2019) partner repository with the data set identifier PXD020457 (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD020457).

Expanded View for this article is available online.

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Author contributions
EE and EM designed the experiments with the help of RP. EE, RP and EM wrote the manuscript. EE and RP performed the experiments with the help of SB, P-JB, AH, KS and MP. KC and OB-S performed essential mass spectrometry run acquisitions and analysis. TH, BL, MY and JCH provided essential reagents to conduct the project.

Conflict of interest
The authors declare that they have no conflict of interest.

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III-Summary of the paper and discussion

Inflammasomes are very effective in fighting pathogens, however, their overactivation leads to tissue damage and specifically to endotoxic shock in the case of the non-canonical inflammasome. Hence, an understanding of the molecular mechanisms of caspase-4, -5 and -11. So many recent studies have been conducted on non-canonical inflammasome, such as the SERPINB1 effect on activation of the inflammatory caspase-1, caspase-4 and caspase-11 (Choi et al., 2019). In this paper, we report with Finethy et al (Finethy et al., 2020) that IFN-inducible protein Irgm2 and ATG8 family member Gate-16 cooperatively regulate Gram-negative (e.g., *S. typhimurium; E. Coli*) bacteria-induced non-canonical inflammasome activation, both in cultured macrophages and *in vivo*. Precisely, these two proteins target caspase-11 activation by LPS which lead to lower caspase-11-mediated pyroptosis and cytokine release, and so protects against endotoxemia.

These results open many questions, such as at what stage Irgm2 and Gate16 regulate caspase-11 recruitment to bacterial products and thus its activation. Indeed, Gate-16 was found to control the correct cytosolic localization of various GBPs (Park et al., 2016; Sasai et al., 2017), including GBP2, crucial at regulating caspase-11 recruitment on intracellular pathogen PAMPs. However, results from Finethy et al (Finethy et al., 2020) and ours indicate that Gate16/Irgm2 removal in GBP- deficient macrophages (lacking GBPs 1, 2, 3, 5 and 7) partially restores a caspase-11-dependent response, suggesting that the Gate- 16/Irgm2 path might regulate caspase-11, at least to a certain extent, independently of these GBPs.

Further, Irgm1 (same family of Irgm2) and its human homologous IRGM have been described to participate in the autophagy/xenophagy processes (Azzam et al., 2017; Maric-Biresev et al., 2016). In addition, Gate-16 is involved in various autophagy membrane remodelling step regulations such as lysosome biogenesis, autophagosome formation and closure (Gu et al., 2019; Y. K. Lee & Lee, 2016; T. N. Nguyen et al., 2016). Therefore, we can hypothesize that Gate-16 and Irgm2 could use the autophagy pathways to control cytosolic LPS accumulation and an exacerbated caspase-11 activation. However, results from Finethy et al (Finethy et al., 2020) and our own suggest that Gate-16 and Irgm2 regulate Gram-negative bacteria-induced non-canonical inflammasome response in an autophagy-independent manner.

Contrary to Irgm2, Gate16 modulate in both murine and human the non-canonical inflammasome response to LPS-containing particles. In fact, the conversion of Irgm2 discovery in mice to humans is more challenging. Indeed, IRGM is the only gene coding for a protein with a similar structure to murine Irgm1, 2 and 3 (Bekpen et al., 2009, 2010). However, IRGM has 5 isoforms, one of them or another different protein could be the functional ortholog of Irgm2 in human. Thus, identification of this Irgm2-like protein in human will be interesting.

Therefore, these results demonstrate that the Irgm2 and GATE-16 proteins negatively regulate the activation of the non-canonical inflammasome in response to Gram-negative bacteria. However, further research will be warranted to understand how both effectors balance the sensitivity of caspase-4, caspase-5, and caspase-11 to Gram-negative bacteria. This project opens perspectives for fundamental research on the mechanisms of action of this GTPase but also on the consequences for the host and the bacterium.

Part 3:

Conclusion

Part 3: Conclusion

The concept of cell death in animals and plants, since the discovery and description of apoptosis, has fully evolved with the characterization of different forms of regulated cell death. These various types of cell death have shown to be necrotic with a release of cell content which can promote the initiation of inflammation. Indeed, this particularity in cell death is hijacked by numerous pathogens for disseminate *Pseudomonas aeruginosa* ExoS+ triggering pyroptosis (Sutterwala et al., 2007) and or *Shigella* and *Francisella* which inhibit its own recognition by caspase 11 to avoid the activation of pyroptosis (Okan & Kasper, 2013; Paciello et al., 2013). The same regulation has been shown for other human diseases such as cancer diseases where some cancerous cells inhibit all the other cell death pathways, but they are sensitive to ferroptosis (LACHAIER et al., 2014; Li et al., 2020). Considering that, understanding of mechanisms that cover their induction or inhibition will allow modulating in disease dependant manner. In this context, this thesis work has thus allowed the identification of a new cellular pathway: lipid peroxidation, involved in cell death induced by *Pseudomonas aeruginosa* exotoxin U.

On the other hand, this thesis enabled the discovery of modulating molecules (lipophilic antioxidants, IRGM2 and Gate16) of regulated necrotic cell death and bacterial sepsis. Our data allow to enrich our knowledge concerning the possibilities of modulation (inhibition) of inflammation during bacterial infections, and to consider these targets for a therapeutic strategy in the context of acute infectious diseases. Lipid peroxidation is a process generally present in all our cells and is known to be modulated during different bacterial, viral, or parasitic infections (Bagayoko & Meunier, 2021). It could be interesting to investigate the involvement of this process in the establishment and development of chronic infections such as in cystic fibrosis patients.

Considering that resistance to antibiotics is one of the major public health problems in the world. In recent years, researchers have focused on the study of molecular and cellular mechanisms during infections (*Pseudomonas aeruginosa*). And since then, various new therapeutic approaches have been described, including the results of this thesis. Thus, the major challenge of our team at the end of this work is now to uncover the role of basal lipid peroxidation in *Pseudomonas* infections and its development

from acute to chronic infection. Finally, we hope to propose molecules to complement classical antibiotics in order to improve the living condition of these patients.

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