Crosstalk between intracellular pathogens and cell death

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ABSTRACT

Infections with bacterial pathogens often results in the initiation of programmed cell death as part of the host innate immune defense, or as a bacterial virulence strategy. Induction of host cell death is controlled by an elaborate network of innate immune and cell death signaling pathways and manifests in different morphologically and functionally distinct forms of death, such as apoptosis, necroptosis, NETosis and pyroptosis. The mechanism by which host cell death restricts bacterial replication is highly cell-type and context depended, but its physiological importance is highlighted the diversity of strategies bacterial pathogens use to avoid cell death induction or blocks cell death signaling. In this review, we discuss the latest insight into how bacterial pathogens elicit and manipulate cell death signaling, how different forms of cell death kill or restrict bacteria and how cell death and innate immune pathway crosstalk to guard against pathogen-induced inhibition of host cell death.

KEYWORDS:

Programmed cell death, Innate Immunity, Infection, Pyroptosis, Apoptosis, Necroptosis

MAIN TEXT

1) INTRODUCTION

Cell death is a common feature of many bacterial infections, which is often detected as accumulations of pus at sites of infection, necrotizing granulomas or necrotic skin infections. Depending on the underlying signaling pathways, cellular morphology and its outcome, cell death can be classified as programmed or accidental, lytic or non-lytic, immunogenic or immunologically silent[1]. Apoptosis, for example, is regarded as a classically non-lytic and immunologically silent form of cell death. By contrast, lytic forms of cell death, such as pyroptosis, necroptosis, ferroptosis and others, are thought to be highly pro-inflammatory and immunogenic.

Which type of cell death is induced during a bacterial infection depends not only on the type of pathogen and the virulence factors it expresses, but also on which tissues and cell types are infected, and the state of the host inflammatory response. Consequently, the outcome for both the host and the pathogen varies significantly. While induction of cell death was initially thought to be a strategy by pathogens to eliminate host immune cells, programmed cell death (PCD) is now overwhelmingly seen as a host innate immune defense mechanism[2]. PCD can for example eliminate the replicative niche of intracellular pathogens, initiate inflammation and the recruitment of specialized effector immune cells, trap pathogens within dead cells, or release neutrophil extracellular traps (NETs) and other antimicrobial factors. By consequence, this has led to an evolutionary arms race between pathogens and the host, in which pathogens develop strategies to evade or block cell death induction, while the host counters with establishing new surveillance and back-up pathways. The result is an intricate and complex network of crosstalk between cell death and innate immune signaling pathways that the research by countless groups uncovered in recent years[3].

In this review, we will discuss the molecular pathways that regulate PCD during bacterial infections, with the main focus on the best-studied pathways - pyroptosis, apoptosis, ferroptosis and necroptosis - the crosstalk that guards these pathways against the manipulation by pathogens and the question how cell death restricts or eliminates bacterial pathogens during infections.

2) PROGRAMMED CELL DEATH DURING BACTERIAL INFECTION

2.1. Inflammasomes and the induction of pyroptosis

The term pyroptosis, derived from the Greek '*pyro*' (fire, fever) and '*ptosis*' (to fall off), was first coined in the early 2000s[4] to describe a form of cell death that was first observed in mouse macrophages that were either treated with lethal toxin from *Bacillus anthracis*[5] or infected with the bacteria *Shigella flexneri*[6] or *Salmonella enterica* serovar Typhimurium (referred to as *S.* Typhimurium)[7]. While this form of cell death was initially mistaken for apoptosis, later studies showed that pyroptosis occurred independently of apoptotic executor caspases-3 and -7, but instead required the inflammatory caspase-1 [7,8]. Consistently, pyroptosis was found to be associated with the release of mature interleukin-(IL)-1β and IL-18, cytokines known to be processed by active caspase-1[4]. Following the identification of the inflammasome complex as the caspase-1 activating platform in 2002[9], pyroptosis was redefined as an inflammasome-induced cell death and as one of the major effector mechanisms of this signaling pathway[10]. Currently, two distinct inflammasome pathways, known as the canonical and non-canonical inflammasome, are described[10]: Canonical inflammasomes activate caspase-1 and are assembled by pyrin, the Pyrin- and HIN domain-containing (PYHIN) family member absent in melanoma 2 (AIM2) or members of the nucleotide-binding oligomerization domain, leucine-rich repeat-containing (NLR) protein family; proteins that serve as stress sensors by detecting pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) or the disruption of cellular homeostasis. The non-canonical pathway, on the other hand, appears to be simpler and involves the activation of mouse caspase-11 or its human orthologs caspase-4 and -5 by cytosolic Gram-negative bacterial lipopolysaccharide (LPS)[10]. Despite the differences in the signals and proteins that control their activation, all of these caspases induce pyroptosis by the very same mechanism – the cleavage of their common substrate gasdermin D (GSDMD)[11,12]. GSDMD is a member of the gasdermin protein family, which can be found in mammals and higher vertebrates and which features 6 members in humans and 11 in mice[13]. Gasdermins are characterized by a common structure that consists of an N-terminal cytotoxic domain ($GSDM^{NT}$), a flexible linker and a C-terminal repressor domain. Caspase cleavage in the linker domain of GSDMD releases its $GSDMD^{NT}$, which then targets the plasma membrane to form large pores[14–17]. Formation of these pores mediates unconventional secretion of the cytokines IL-1β and IL-18 as long as the levels of pore formation are kept in check[18–20], but eventually GSDMD pores disrupt ion homeostasis and the electrochemical gradient, resulting in the typical membrane ballooning and the loss of membrane integrity that are the hallmarks of pyroptotic cells[13].

While pyroptosis is commonly observed in infected cultured cells upon inflammasome activation, it is much less studied to what extent pyroptosis occurs *in vivo*, and how it contributes to inflammasome-mediated restriction or clearance of pathogens. *Casp-1*-, *Casp-11*- and *Gsdmd*-deficient mice have been shown to be highly susceptible to numerous bacteria as well as viruses, fungi and parasites[13]. However, since pyroptosis and IL-1β/-18 release both require caspase activity and GSDMD pores, the individual contribution of these two effector mechanisms can only be deduced by comparing the resistance of the above-mentioned mice to *IL-1b*$^{-/-}$*IL-18*$^{-/-}$ mice. However, such a comparison of different genotypes is complicated by the high level of redundancy and cross-talk between cell death pathways (see respective chapter), which results in the activation of apoptotic caspases and lytic cell death in both *Casp1*$^{-/-}$ and *Gsdmd*$^{-/-}$ animals. Furthermore, tools to unmistakably identify pyroptotic cells *in vivo* are still missing, although the recent development of cleavage-specific GSDMD antibodies might lead the way[21]. Finally, it needs to be considered that efficient clearance by pyroptosis might be impossible without the concomitant cytokine-dependent recruitment and activation of effector immune cells. Nevertheless, a number of different mechanisms by which pyroptosis restricts bacteria *in vitro* and *in vivo* have emerged recently and are discussed in more detail below.

Activation of pyroptosis by bacteria

Bacteria activate all currently known inflammasome sensors, and studying host-bacteria interaction proved to be essential in the identification of many of the sensor proteins and signaling events that control the activation of inflammatory caspases and pyroptosis induction (**Fig. 1**)[10]. One of the first inflammasome sensors shown to exclusively detect bacterial pathogens was NLRC4[22], which responds to bacterial flagellin as well as the rod and needle subunits of bacterial type 3 secretion systems (T3SSs)[23–26]. To detect these distinct ligands, mouse NLRC4 uses NLR family, apoptosis inhibitory proteins (NAIPs) as direct upstream receptors[27,28]. Binding of their cognate ligand — the T3SS rod protein by NAIP2, the T3SS needle protein by NAIP1, and flagellin by NAIP5 and NAIP6 — allows the NAIPs to interact with NLRC4 and initiate inflammasome assembly. By contrast, human NAIP combines the ability to detect all 3 ligands, but the underlying mechanism remains unknown[29]. Direct recognition of a pathogen-derived ligand is also employed the PYHIN family member AIM2, which features a DNA binding domain (HIN200) and acts as a cytosolic sensor for host or pathogen-derived double-stranded DNA[30(p2),31]. Although DNA binding viruses might be the main targets of AIM2, it also detects DNA from

Francisella novicida, Listeria monocytogenes and other cytosolic bacteria[32–35], and significantly contributes to *in vivo* host defense against these intracellular bacteria. Bacteria and their virulence factors also played an essential role in characterizing the activation of the inflammasomes sensors pyrin and NLRP1. The pyrin inflammasome for example, is engaged by bacterial toxins or effector proteins such as TcdA/B (*Clostridium difficile*), C3 toxin (*Clostridium botulinum*), VopS (*Vibrio parahaemolyticus*), IbpA (*Histophilus somni*) and TecA (*Burkholderia cenocepacia*) that inactivate the small GTPase RhoA by modifying its switch 1 region[36], or by *Yersinia* spp. YopE a GTPase activating protein (GAP) and YopT, a cysteine protease that cleaves the plasma membrane associated RhoA[37,38]. The loss of RhoA activity results in reduced activity of the kinases PKN1/2, which is necessary to keep pyrin in a phosphorylated inactive state, bound by 14-3-3 proteins[39,40]. Another bacterial toxin – *B. anthracis* protease lethal factor (LF) – was the first activator of pyroptosis to be identified[5]. Later studies showed that LF engaged NLRP1B, one of the 3 NLRP1 isoforms in mice[41]. Mechanistically, NLRP1B activation involves N-terminal cleavage by the LF, which generates a new N-terminus that is ubiquitinated (N-end rule) and directed to the proteasome for degradation[42,43]. Since NLRP1 is auto-processed in its function-to-find domain (FIIND), proteasomal degradation releases its C-terminal CARD (Caspase recruitment domain) that engages caspase-1. Interestingly, the effector IpaH7.8 from *S. flexneri* can also induce NLRP1 activation by directly ubiquitinating NLRP1 and directing it to the proteasome[42].

NLRP3, which responds to permeabilization of the plasma membrane, potassium efflux and the disruption of the trans Golgi network (TGN)[44(p3)], is also activated by many bacterial pathogens. Bacterial pore forming toxins, such as α-hemolysin, listeriolysin and pneumolysin[45], are well-known activators of NLRP3, but in most cases bacteria-induced NLRP3 activation is linked to the sensing of LPS by the non-canonical inflammasome. Since its discovery in 2011[46] this pathway emerged as the common sensing mechanism for cytosolic Gram-negative bacteria[10]. The LPS motif that is detected is the LipidA moiety of LPS[47,48], which is highly conserved compared to other parts of the molecule, such as the O-antigen. LPS and LipidA were both shown to directly bind to caspase-11 (-4/-5) and induce caspase oligomerization and activation[49]. This has led to a model in which no additional factors besides the caspase are necessary for non-canonical inflammasome activation. However, it is well known that both the human and mouse non-canonical inflammasomes also strongly depend on interferon signaling and the upregulation of guanylate-binding proteins (GBPs) and immune-related GTPases (IRGs)[50–53],

which belong to the larger family of IFN-induced GTPases. Cells and animal deficient in these GTPases are attenuated in non-canonical inflammasome signaling in response to Gram-negative bacterial infections and even to cytosolic LPS[52,54]. How these GTPases promote caspase activation is still a matter of debate, but it is currently assumed that it involves the destabilization and lysis of the bacterial pathogen. While it helps to contain bacterial infections, the non-canonical inflammasome is however a double-sided sword. A number of studies have demonstrated that *Casp11*-, *Gsdmd*- and to some degree *Gbp*^{chr3}-deficient are protected in different models of LPS-induced lethality[11,47,48,52,54], suggesting that at least in mice overactivation of the pathway is main driver of bacteria-induced septic shock syndrome.

Inhibition of pyroptosis by bacteria

The importance of inflammasomes-induced pyroptosis in anti-bacterial host defense is highlighted by the different strategies that pathogenic bacteria use to prevent pyroptosis induction. The simplest strategy is to avoid inflammasome activation from the beginning by evading recognition by inflammasome sensors. As many studies have documented, this strategy is for example employed by the enteric pathogen *S.* Typhimurium. *S.* Typhimurium virulence involves the forced invasion of gut enterocytes early during the infection, process which requires flagellin-based mobility and the *Salmonella* pathogenicity island 1 (SPI-1) T3SS[55]. During this phase *S.* Typhimurium strongly activates NLRC4 in infected enterocytes and macrophages[23,24,26], either via the SPI-1 T3SS structural components PrgJ and PrgI, or T3SS-injected flagellin. Later, once the infection spreads systemically and the bacteria reside within infected cells, *S.* Typhimurium however escapes NLRC4 detection altogether by downregulating flagellin and SPI-1 expression and expressing the *Salmonella* pathogenicity island 2 (SPI-2) T3SS[24,56], whose structural components are not recognized by NLRC4[26]. The importance of avoiding NLRC4 activation is highlighted by the experiments that showed that strains which express flagellin constitutively are highly attenuated and rapidly cleared *in vivo*[57]. Interestingly, mutating flagellin to escape host immune surveillance does not appear to be a viable strategy for bacterial pathogens, since NAIP5 recognizes multiple conserved region of flagellin that are required for flagellar motility[58]. By contrast, modification of LPS appears to come at lower costs, since several bacteria use this strategy to evade or mitigate non-canonical inflammasome activation, and at the same time LPS recognition by myeloid differentiation 2 (MD2)/Toll-like receptor 4 (TLR4). These pathogens,

such as *F. novicida*[48], usually feature an under-acylated LPS (penta- or tetra-acylated) are known to be poor activators of caspase-11, even though human caspase-4 appears to recognize these types of LPS better[59].

If inflammasome activation cannot be avoided, bacteria directly interfere with receptor activation or signal propagation. Several forms of LPS, such as from *Rhizobium galegae, Rhodobacter sphaeroides* or *Helicobacter pylori,* are known to not only inhibit TLR4 activation, but to also act as antagonists of caspase-11/-4[47–49]. Escaping non-canonical inflammasome activation is especially important for professional cytosolic Gram-negative bacteria like *F. novicida* and *S. flexneri*, that cannot hide within a vacuolar compartment. While *F. novicida* expresses a modified, under-acylated LPS[48], *S. flexneri* not only modifies its LPS[60], but also inhibits the non-canonical inflammasome signaling pathway by two effectors proteins: IpaH9.8, a E3 ubiquitin ligase that targets GBPs and promotes their degradation, and OspC3 which inhibits caspase-4 directly[61,62,63(p3)]. Consequently mutants lacking these two effectors are highly attenuated in human cells and cannot avoid GBP-mediated restriction of intracellular motility and GBP-dependent caspase-4 activation[61,62,63(p3)].

More difficult to block are pathways that sense the disturbance of cellular homeostasis, for example by bacterial effector proteins, since often such alterations of homeostasis are often associated with processes that are essential for bacterial virulence, such as the entry into host cells and manipulation of the host cytoskeleton. For example, *Yersinia* spp. virulence requires the activity of YopT and YopE, which inhibit Rho GTPase activity[37,38]. This manipulation however is detected by the sensor pyrin that will trigger inflammasome assembly. To counteract pyrin activation *Yersinia* employs another effector, YopM, which activates serine/threonine protein kinase C-related kinases that phosphorylate pyrin again and thus ensure that the sensor remains inactive[37,38]. On the other hand, YopM has also been shown to directly bind caspase-1 and inhibit its activity[64]. While a number of other bacterial effectors have been reported to inhibit inflammasome activation, these reports need to be carefully evaluated. The observation that the lack of virulence factors or effectors results in higher inflammasome activation does not necessarily indicate that these proteins function as inhibitor; deletion of effectors maintaining vacuolar stability for example are known to result in a mislocalisation of bacteria to the cytosol and activation of pathways that would normally be avoided by the wild-type bacterium[65]. Nevertheless, the examples listed above highlight part of the fascinating arsenal of strategies that bacteria developed to

escape surveillance or even to inhibit the steps leading up to pyroptosis (Table 1) and underline the importance of this type of cell death as a host defense mechanism.

2.2. Apoptosis/ Necroptosis

Apoptosis is characterised by activation of apoptotic caspases, nuclear fragmentation, cellular disintegration into small apoptotic bodies and inactivation cleavage of innate immune sensing pathways[66]. While these hallmarks of apoptosis are generally considered anti-inflammatory and are designed to ensure that apoptosis remains immunologically silent during development and homeostasis, an emerging body of evidence indicate that apoptosis may also promote inflammation and host defence during bacterial infection. The best examples of apoptotic cell death in driving anti-bacterial defence were arguably demonstrated in the context of enteric bacterial infection, as mice that were deficient in apoptotic signalling machineries were more susceptible to *Citrobacter rodentium* and *Yersinia pseudotuberculosis* infection compared to wild type animals[67–70]. Detection of pathogens by pattern recognition receptors (PRRs) such as TLRs drive the expression of pro-inflammatory cytokine production such as tumor necrosis factor (TNF) and IL-6, which are critical to mediate anti-bacterial defence. Successful enteric pathogens in turn inject effector proteins using their T3SS or T4SS into host cells to inhibit innate immune signalling pathways. For example, pathogenic *Yersinia* species including *Y. pseudotuberculosis* and *Y. enterocolitica* inject the effector protein, YopJ, an acetyltransferase to block the kinase activity of transforming growth factor beta-activated kinase 1 (TAK1) and IκB kinase (IKK) to suppress nuclear factor-κB (NF-κB) activation and pro-inflammatory cytokine production[71]. Other enteric bacterial pathogens, such as enteropathogenic *Escherichia coli* (EPEC), *S.* Typhimurium and *S. flexneri* were also documented to block NF-κB activation via injection of effector proteins NleE, SseL and IpaH9.8 respectively[72–75]. Although these virulence factors have different cellular substrates, they ultimately block NF-κB activation and production of pro-inflammatory cytokines. In the case of *Yersinia* infection, coincidence detection of TNF signalling and blockade of TAK1 or IKK kinase activity in turn triggers the assembly of a cytoplasmic multiprotein complex comprised of FAS-associated death domain (FADD), receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and apoptotic caspase-8, termed TNF Complex IIb. Assembly of this complex enables caspase-8 dimerization and autoactivation, which cleaves downstream effectors caspase-3 and -7 to drive apoptotic cell death[69]. This form of pathogen-induced apoptotic cell death is believed to prime

cytokine production from bystander innate immune cells to mediate anti-bacterial defence[69]. In addition, recent studies have also uncovered additional mechanisms by which apoptotic cell death drives host defence. First, two studies demonstrated that apoptotic caspase-8 directly cleaves GSDMD to drive pyroptosis during *Yersinia* infection[76,77]; second, caspase-8 activation has also been observed to direct inflammasome assembly[76,78–80] although the mechanisms by which this occurs is still debated and is discussed in detail in the later paragraphs.

Given that pathogen-induced caspase-8 activation drives such potent antimicrobial defence, it is not surprising that several pathogens have developed strategies to inhibit caspase-8 activity, although this is more commonly observed during viral infection. While inhibition of caspase-8 activity suppresses apoptotic death, this unleashes host cells to undergo an alternative, backup form of lytic cell death known as necroptosis[81]. This occurs because the RIP homotypic interaction motif (RHIM)-containing protein receptor-interacting serine/threonine-protein kinase 3 (RIPK3), a key molecule required for necroptosis, is no longer cleaved and inactivated by caspase-8, thus, enabling RIPK3 recruitment through homotypic RHIM-RHIM interaction with RIPK1 on Complex IIb[82,83]. In this new complex (the necrosome), RIPK3 is believed to undergo autoactivation and further phosphorylates the pseudokinase mixed lineage kinase domain-like protein (MLKL) to execute necroptotic death. Interestingly, in TAK1-deficient cells, TLR stimulation appears to trigger the assembly of Complex II, which is able to trigger a mixture of apoptosis, pyroptosis and necroptosis, dubbed as 'PANoptosis' (PMID: 31869420). A recent study revealed that several enteric pathogens including enterohemorrhagic *Escherichia coli* (EHEC), EPEC and *C. rodentium* encode a novel bacterial cysteine protease, EspL which cleaves RHIM-containing adaptor proteins such as RIPK1, RIPK3, TIR-domain-containing adapter-inducing interferon-β (TRIF) and Z-DNA-binding protein 1 (ZBP1) to that are associated with both apoptotic and necroptotic signalling[67]. Although it is unclear at this point whether enteric bacteria encode any caspase-8 inhibitors and are able to directly induce necroptosis, the *C. rodentium ⊿espL* mutant that is unable to cleave RHIM-containing proteins is clearly less virulent than its isogenic wild-type counterpart *in vivo*, suggesting that apoptotic and/or necroptotic cell death is host defence mechanism against enteric bacterial infection[67]. In addition, EPEC and *C. rodentium* were also documented to express several other inhibitory effector molecules, such as NleB, a N-acetylglucosamine (GlcNAc) transferase that inactivates several death receptor signalling proteins including tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), FADD, RIPK1 and tumor necrosis factor receptor 1

(TNFR1) to block death receptor signalling[68,84]. These evidences, together with the observations that *ΔnleB* mutants are less able to establish an infection compared to their isogenic wild-type counterparts, highlight the importance of apoptotic cell death in controlling enteric bacterial infection.

2.3 Ferroptosis

One of the most recent forms of PCD is ferroptosis. It was first described by Dixon et *al.* as an iron-dependent form of necrotic cell death that was induced by RAS-selective lethal small molecule compounds, like erastin or RSL3, that are toxic to RAS mutant tumor cells[85]. Ferroptosis was shown to be dependent on intracellular iron, but not on other metals, and morphologically, biochemically and genetically distinct from other cell death pathways, e.g. apoptosis, necroptosis, pyroptosis and autophagy. Ferroptosis is characterized by a destabilization of the plasma membrane and cell lysis, which is initiated by Fenton reaction–induced hydrogen peroxides that upon interaction with membrane lipids produce toxic lipid peroxides[1,86]. The glutathione peroxidase 4 (GPX4) enzyme reduces lipid peroxides under steady-state condition and thus provides the primary mode of protection against ferroptosis induction. Iron overload for example can inhibit GPX4 and lead to ferroptosis induction. Another factor that appears to play a major role is apoptosis-inducing factor mitochondria-associated-2 (AIFM2, renamed as ferroptosis suppressor protein 1, FSP1), since deletion of this protein sensitizes cells to ferroptosis induction[87(p1),88(p1)]. Since the genetics of ferroptosis are still largely unstudied, it is usually distinguished from other cell death pathways by its susceptibility to lipid peroxidation inhibitors, such as ferrostatin, its association with increased levels of iron and lipid peroxides, and the reduced levels of glutathione (GSH) and GPX4 activity[1].

Ferroptosis is now recognized as a major cell death pathway and it has in the last few years been associated with a wide spectrum of diseases, including diabetes, cancer, neurodegenerative diseases, and renal failure[86]. Yet links to infectious diseases, e.g. bacterial infections, have only now been emerging. Dar and colleagues for example recently reported that during lung infection with *Pseudomonas aeruginosa,* the bacteria can express and release lipoxygenase (pLoxA) which oxidizes host arachidonic acid-phosphatidylethanolamines (AA-PE) to 15-hydroperoxy-AA-PE (15-HOO-AA-PE), and thereby induce ferroptotic death of human bronchial epithelial cells[89]. While the importance of pLoxA for bacterial virulence was not studied *in vivo*, it could be implied from other experiments that detected oxidized AA-PE in airway tissues from *P. aeruginosa*-infected cystic fibrosis patients[89].

Another recent study revealed that the necrosis of macrophages that was commonly observed during *Mycobacterium tuberculosis* infections is caused by ferroptosis [90]. The involvement of ferroptosis was implied since the authors found that macrophage death was associated with reduced levels GSH and GPX4, and an increase in free iron, mitochondrial superoxide and lipid peroxidation. Consistent with ferroptosis inducing death of *M. tuberculosis*-infected macrophages, death was suppressed by the synthetic antioxidant ferrostatin-1 and iron chelation. Ferroptosis induction in this case appeared to favor the pathogen, since ferrostatin-1 treatment reduced bacterial loads in infected animals[90]. Both of these studies suggest that, unlike other types of necrotic cell death, ferroptosis induction favors pathogen dissemination or virulence and not host defense. That ferroptosis might worsen the outcome of bacterial infections is also supported by a new study that showed that GPX4-deficiency in myeloid cell increases the severity of polymicrobial sepsis in mice, since GPX4 was necessary to block phospholipase C gamma 1 (PLCG1)-mediated GSDMD activity and pyroptosis [91]. However due the recent emergence of the ferroptosis pathway and the lack of genetic tools, we currently know too little about this pathway to definitely conclude that ferroptosis cannot be protective as well. Actually, since ferroptosis is known to cause inflammation, it would be rather surprising if ferroptosis induction would not to some degree favor host immunity against pathogens. Additional studies will thus be necessary to further determine the immune function of this new fascinating cell death mode, and to determine how bacterial pathogens induce or inhibit the pathway.

2.4 NETosis

Neutrophils are the most abundant innate immune cell and are critical drivers of host defence, especially during bacterial and fungal infection. Although neutrophils are notorious for their short lifespan, these cells are also surprisingly resistant to several PCD pathways such as caspase-1-dependent pyroptosis [92(p4)] and TLR4-driven necroptosis[93], in contrast to their myeloid counterparts. Neutrophils presumably evolved these mechanisms to extend their lifespan in order to neutralise pathogens via their classical antimicrobial mechanisms (e.g. phagocytosis, degranulation, extracellular reactive oxygen species (ROS) production) at a site of infection. However, successful pathogens have developed an array of evasion strategies to subvert these antimicrobial programmes, for example by inhibiting phagocytosis, suppressing fusion of phagolysosome with neutrophilic granules, and escaping the phagolysosome into the host cytosol. In response to these perturbations, neutrophils undergo a suicide programme, where

they launch the release of a meshwork of DNA and antimicrobial peptides, called neutrophil extracellular traps (NETs) in a last-ditch attempt to ensnare and neutralise these pathogens.

Although the phenomenon of NET extrusion (called NETosis) was observed more than 15 years ago[94], the exact molecular mechanism of NETosis is still not completely understood and widely debated. It is likely that neutrophils have developed multiple signalling strategies to extrude NETs in order to provide a rapid response against a wide variety of microbial threats. Here, we will summarise the three major pathways known to trigger suicidal (lytic) NETosis. We would like to highlight that an alternative, non-lytic NETosis pathway has also been described[95], which is not discussed in this review.

ROS-NE-MPO pathway

The ROS-NE-MPO pathway is activated by a number of bacteria, as well as fungal and parasitic pathogens (Table 1). Engagement of this pathway occurs after recognition of microbial products by cell surface PRRs including TLRs, dectin receptors and complement receptors. Ligation of these receptors culminate in the production of NADPH oxidase-dependent ROS, which liberates neutrophil elastase (NE) from azurophilic granules into the cytosol. Surprisingly, release of NE from the granule to cytosol appears to occur in the absence of granule lysis. Instead, a fraction of NE is believed to form part of a membrane-spanning multiprotein complex called the 'azurosome' which permits NE release upon ROS sensing. This event also requires myeloperoxidase (MPO), another major component of the azurosome. Interestingly, the enzymatic activity of MPO is dispensable for NE release[96,97]. Once in the cytosol, NE cleaves the pore-forming protein, GSDMD, and liberates the $GSDMD^{NT}$ and disrupts the nuclear membrane[98]. This enables NE-dependent histone H4 cleavage which drives nuclear decondensation and expansion[99]. $GSDMD^{NT}$ also disrupts azurophilic granules, which amplifies the release of NE and drive nuclear decondensation in a feed forward mechanism. Finally, GSDMD-mediated plasma membrane damage triggers lytic cell death and drive the release of decondensed DNA and antimicrobial peptides to the extracellular space[98].

Although ROS production is commonly observed after pathogen recognition, single bacteria that are efficiently phagocytosed and digested appear to be weak inducers of NETosis, while large pathogens or those that resist phagocytosis elicit potent NET extrusion[100]. This highlights that phagocytosis is the primary mechanism of pathogen clearance and NET extrusion occurs after 'frustrated phagocytosis'. Indeed, following phagocytosis, fusion of the phagolysosome and azurophilic granules sequesters NE and prevent its translocation to the cytosol, ensuring neutrophils get the necessary lifespan to digest

ingested cargos via the classical endocytic pathway[100]. By contrast during 'frustrated phagocytosis', NE accumulates and is allowed to access the cytosol to initiate NET release and neutralise extracellular pathogens. Collectively, these studies highlight that NETosis occurs via a tightly regulated process.

PAD4 drives histone citrullination and NET extrusion

Protein arginine deiminase 4 (PAD4) is an enzyme that catalyse the deimination of arginine to citrulline and is implicated as a key regulator of NETosis[101,102(p4),103(p4)]. PAD4 is a calcium-dependent enzyme and is activated upon a spike in intracellular calcium, for example, upon exposure to microbial ionophores such as ionomycin and nigericin[104] or other microbial pathogens (Table 2). While ROS scavengers have minimal impact on PAD4-dependent NETosis, neutrophil elastase deficient murine neutrophils display a slight defect in extruding NETs compared to WT cells upon ionomycin treatment[104,105]. PAD4- and NE-driven NETosis display similar characteristics including chromatin relaxation, nuclear delobulation and disintegration of nuclear and plasma membrane rupture [106,107]. In agreement with a minor role of NE in driving PAD4-dependent NETosis, the GSDMD inhibitor, LDC7559, provided minimal inhibition to ionophore-driven NETosis[98]. The effector molecule(s) that drives PAD4-dependent NETosis has not been identified.

Activation of death effector proteins GSDMD and MLKL drives NETosis via distinct mechanisms

Although NETosis is most commonly associated as a host defence mechanism against extracellular pathogens, we recently demonstrated that NETs also drive host defence against Gram-negative bacteria that have escaped into the host cytosol[108]. Activation of this pathway requires the non-canonical inflammasome, in which cytosolic LPS from Gram-negative bacteria activates caspase-4/11 and GSDMD, and is termed non-canonical NETosis[109]. Analogous to the ROS-NE-MPO pathway, active GSDMD promotes nuclear membrane damage and licenses caspase-11-dependent clipping of histone H3 and nuclear decondensation. GSDMD-dependent pore formation also drives calcium influx, PAD4 activation, and histone citrullination, however, neither PAD4, NE nor MPO are required for non-canonical NETosis. In addition to driving plasma membrane pores and NET release, $GSDMD^{NT}$ additionally promotes IL-1β maturation via the NLRP3 inflammasome to drive neutrophil recruitment and phagocytic clearance of the trapped pathogens.

In agreement with the observation that death effector proteins drive NET expulsion, a recent study likewise reported that MLKL, the death effector in the necroptosis pathway, promotes NETosis in necroptotic neutrophils. Mechanistically, MLKL-driven plasma membrane pores promote calcium influx, PAD4 activation and NET release [110(p4)]. Importantly, *Pad4*-deficient neutrophils fail to undergo NETosis in necroptotic neutrophils, while PAD4 is dispensable for non-canonical NETosis. Whether NE and MPO is required for this pathway was not investigated in that study.

3. EFFECTOR MECHANISMS OF CELL DEATH

Since bacterial pathogen developed different immune strategies to either avoid or inhibit cell death induction, it can be assumed that PCD is highly relevant for host defense. However, unlike viruses, most bacteria that replicate within cells are only facultative intracellular pathogens and thus removing their intracellular replicative niche would not restrict their replication. However, dying cells release a plethora of find-me and eat-me signals that promote efferocytosis, the phagocytosis of dead cells, which might be used to remove bacteria through new phagocytes. Furthermore, emerging evidence suggest that dying cells might even be intrinsically antimicrobial and thus able to kill of damage intracellular bacteria. Below we discuss in detail the diverse mechanisms by which PCD promotes antibacterial immunity (**Fig. 2**).

3.1 PITs

One effector mechanism by which cell death restricts intracellular bacteria is the formation of so-called *pore-induced intracellular traps* (PITs). The PIT concept was proposed in 2016 by Jorgensen et al. to describe the entrapment of *S*. Typhimurium within pyroptotic macrophages, in analogy to the entrapment of extracellular bacteria by NETs[111]. Pyroptosis was not the only type of cell death capable of forming PITs, as similar entrapment of bacteria was also observed *in vitro* upon induction of necroptosis with TNF/BV6/ZVAD or upon saponin treatment (necrosis). Interestingly, disruption of microtubules or actin filaments did not abrogate bacteria trapping, which suggested that cytoskeleton is not required for PIT formation. Electron microscopy analysis showed that PIT-contained *S*. Typhimurium remained alive, but also revealed membrane ruffling which indicated potential damage to the bacterial outer membrane. Indeed, further analysis showed that bacteria isolated from PITs are more susceptible to stressors such as H_2O_2, polymyxin B and ciprofloxacin, and display reduced fitness

as they were less able to reinfect cells. Interestingly, PIT-contained *L. monocytogenes* did not present a disrupted outer membrane, which could be potentially linked to the thicker peptidoglycan layer of Gram-positive bacteria. What causes the damage to the Gram-negative bacterial membrane is so far unknown, but one possibility could be the formation of GSDMD pores (further discussed below). However, since it also is observed in necroptotic/necrotic cells, it might be the consequence of other processes that happen during cell necrosis, such as the destruction of lysosomes.

The function of PITs in restricting bacterial replication appears to be mainly linked to their ability to attract neutrophils. Previous work by Miao *et al.* had already shown that flagellin-expressing *S*. Typhimurium induce exaggerated pyroptosis[57], and that the bacteria are cleared by neutrophils in a manner requiring the production of ROS by the NADPH oxidase enzyme complex. Following up on these findings, Jorgensen et *al*. showed that pyroptotic *S*. Typhimurium-infected macrophages are phagocytosed by neutrophils upon injection into mice and that neutrophils killed the entrapped *S*. Typhimurium in a ROS-dependent manner. So, what attracts neutrophils to PITs *in vivo*? It is well known that apoptotic bodies release eat-me and find-me signals to promote efferocytosis[66]. While ATP is an essential find-me signal during apoptosis, PIT-induced efferocytosis is independent of ATP sensing, but requires scavenger receptors and the complement system to promote neutrophil recruitment to PITs[111]. An additional role of the cytokines IL-1β and IL-18 together with eicosanoids was observed in a further study by infecting mice with a lower dose[112], but this mechanism might apply to pyroptotic corpses only. Overall, these findings demonstrated that PIT formation restricts *S. Typhimurium* and potentially other Gram-negative bacteria *in vitro*. Additional studies with other bacteria, both Gram-negative and Gram-positive will be required to confirm the concept, and additional *in vivo* work will be necessary to investigate its physiological relevance. Besides, a particular question that will need to be answered is whether PITs are beneficial for the host in all cases, or whether some pathogens might highjack necrotic cell corpses to spread within the host and re-infect new phagocytes.

3.2 Efferocytosis

The term efferocytosis has been coined to describe the engulfment of apoptotic cells by phagocytes but it also applies also to the uptake of necrotic cells by phagocytes. In the case of apoptotic cells, this efferocytosis is generally thought to occur prior to cellular membrane disruption, thereby avoiding the start of secondary necrosis and an inflammatory response[113]. Consequently, defect in efferocytosis

have been shown to result in auto-inflammatory diseases. Since the previous chapter covered the uptake of necrotic, necroptotic and pyroptotic cells by phagocytes, we will mainly focus on efferocytosis of infected apoptotic cells in this chapter.

In the last years, a host of studies revealed that efferocytosis is an important host defense mechanism but can also favour bacterial dissemination. For instance, during *M. tuberculosis* infection, efferocytosis of infected macrophages confers host resistance by delivering apoptotic cell debris containing *M. tuberculosis* to the recipient cell for lysosomal degradation[114]. Although *M. tuberculosis* is able to block phagolysosome maturation as a survival mechanism[115], it is unclear why the bacteria is unable to do so when it is efferocytosed. In that regard it has been speculated that *M. tuberculosis* is unable to secrete effectors through the extra layer of cellular membrane when efferocytosed, but experimental evidence is still lacking. Interestingly, once efferocytosis is blocked, cells undergo secondary necrosis, which promotes *M. tuberculosis* dissemination. Neutrophilic efferocytosis of infected macrophages is also beneficial for the host upon *Mycobacterium marinum* infection in a zebrafish model[116], but efferocytosis does not always need to involve professional phagocytes. Epithelial cells have been shown to phagocytose apoptotic cells together with attached *P. aeruginosa* and thereby promote bacterial clearance by phagolysosome maturation[117]. Conversely, other studies report that efferocytosis can also be detrimental for the host. For example, Dallenga et al. showed that engulfment of *M. tuberculosis*-infected necrotic neutrophils by macrophages promotes bacterial dissemination[118], which is contradictory to the data obtained in macrophages[114]. Furthermore, deficiency of the scavenger receptor TIM-4 promotes *L. monocytogenes* clearance in mice[119]. While this could indicate that efferocytosis favours the pathogen in this case, it could also be linked to other functions of TIM-4, which for example was shown to be associated with suppression of pro-inflammatory cytokines production[120]. Indeed, Czuczman et al. also reported elevated basal levels of pro-inflammatory cytokines in *Timd4*$^{-/-}$ mice prior infection[119]. Thus, further studies are required to discriminate whether the enhanced *L. monocytogenes* dissemination in *Tim4*$^{-/-}$ mice is due to abrogated efferocytosis or the enhanced pro-inflammatory cytokines production.

The argument that efferocytosis serves as an antimicrobial mechanism is supported by observations that various pathogens such as *Staphylococcus aureus* and *Klebsiella pneumoniae* have developed evasion strategies against efferocytosis. In infected neutrophils, *S. aureus* upregulates CD47, a membrane receptor known to function as a ‘don`t eat me’ signal, thereby reducing neutrophil ingestion

by macrophages[121]. Furthermore, by *S. aureus* secretes alpha-toxin that reduces CCN1 expression in the lung and alters surface localization of DD1α in aveolar macrophages, both of which reduce efferocytosis of infected neutrophils and thus promote bacterial dissemination[122]. *K. pneumoniae* on the other hand evades killing by inducing IL-10 production, which reduces pyroptosis of infected cells and subsequent efferocytosis[123]. In summary, these findings reveal highly-context depended outcomes for the efferocytosis of infected cells. The protective or detrimental roles of efferocytosis for the host appear to vary significantly based on the type of pathogens that is involved, the cell type that is efferocytosed and the type of cell death it underwent, as well the phagocytes themselves. Thus, pro- or anti-microbial roles of efferocytosis will need to be analysed on an individual basis. These studies will however be important, since the strategies that pathogens use to evade or exploit efferocytosis might significantly contribute to our understanding of the process as such, and potentially even harness the development of potential anti-bacterial therapies in the future.

3.3 NETs

NETs are large, sticky extracellular structures that comprise of DNA, histones and antimicrobial proteins such as NE, MPO and lactoferrin. So far two major mechanisms were proposed by which NETs could drive host defence[94]. First, since NETs bind pathogens, they were proposed to serve as a physical barrier that entraps pathogens and thus limits their dissemination in the host. This model was put forward after observations that *S.* Typhimurium, *S. flexneri* and *S. aureus* associate with NETs *in vitro*[94]*.* In support of this model, a large number of studies subsequently reported that the application of exogenous DNase I, in order to dismantle NETs, promoted bacterial replication compared to control groups *in vitro* and *in vivo*[103(p4),104,108,124]. The model is further strengthened by the observation a variety of bacterial mutants that are deficient in nuclease production are reported to be less virulent compared to isogenic controls (**Table 2**).

The second mechanism by which NETs or NETosis are thought to promote host defence is via the release of antimicrobial factors. For example, NE has been documented to cleave and inactivate virulence factors from enteric bacteria[125], and MPO promotes bacterial killing by catalysing the conversion of chloride and hydrogen peroxide to hypochlorite, which has superior antimicrobial activity. Histones, which are a major component of NETs, were also documented to exert potent antimicrobial

activities[126]. Therefore, NETs are likely to promote pathogen clearance by preventing its dissemination while at the same time also increasing the local concentration of antimicrobial peptides.

3.4 Expulsion of dead cells

The intestinal epithelium is formed by a single layer of cells that serves as a mechanical barrier to separate the body from the content of the gut lumen. Seminal studies have provided evidence that intestinal epithelial cells express inflammasomes components, and actively promote host defence during infections (Extensively reviewed in [127]). In 2010, Knodler et al. reported that during S. Typhimurium infections infected epithelial cells get extruded from the epithelial layer and that these extruded cells presented structural features typical of cell death[128]. Experiments in human colonic epithelial cells showed that extruded infected cells harbour active caspase-1 and present disrupted plasma membrane. Later studies also showed that cell extrusion also occurs *in vivo* and that it functions as a major host defense mechanism against *S.* Typhimurium infection[129,130]. Using the streptomycin pre-treated mouse model of *Salmonella* colitis, Sellin et al. (2014) showed that intraepithelial *S*. Typhimurium loads increased until 12 h post-infection, but that bacteria numbers reached a plateau after 18 h post-infection and even declined at later time points[129]. The authors observed that the bacterial restriction correlated with the expulsion of infected cells into the lumen. Extracellular bacteria were also observed in the lumen, suggesting that *S.* Typhimurium can escape from the expelled cells[129]. Consistent with previous studies that showed that the NLRC4 inflammasome is critical for *S*. Typhimurium clearance in the intestine[131], mice lacking NLRC4 carried higher bacterial loads in epithelial cells compared to littermate control[129]. Importantly, bacterial restriction required activity of the NLRC4 inflammasome in epithelial cells and not immune cells, and was found to be independent on the NLRP3 inflammasome or the pro-inflammatory cytokines IL-1a, IL-1β and IL-18, thus indicating that pyroptosis was the main driver of bacterial restriction. At first sight, these findings are in contradiction with studies using systemic models of *S*. Typhimurium infection, which reported that host defense requires both the NLRC4 and the non-canonical inflammasome[132,133], but the exact infection model employed and the timepoint that is analysed might be key in understanding this discrepancy. Indeed, while Sellin et al. did not observe a role for caspase-11 in restricting *S.* Typhimurium colonization in the early phase of the infection[129], Knodler et al. demonstrated that mice lacking *Casp11* showed delayed shedding of *S.* Typhimurium-infected epithelial cells in the gall bladder at later timepoints[130]. The same

authors also found that *Casp11*$^{-/-}$ mice presented higher bacterial loads in cecal tissues and lumen at 7 days post-infection[130]. Recently, Crowley et al. further unravelled this discrepancy by comparing *S.* Typhimurium burdens of streptomycin pre-treated *Casp1*$^{-/-}$, *Casp11*$^{-/-}$, and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice [134]. The authors concluded that both caspases effectively contribute to *S*. Typhimurium infection control, but that they are part of a layered defense. Consistent with their previous work[130], the caspase-11-dependent restriction occurs in the later course of infection[134]. To mimic the *in vivo* conditions, Crowley et al. pre-treated enteroid-derived monolayers with IFN-γ, reported to upregulate caspase-11[46,133], and observed enhanced bacterial restriction by caspase-11. Overall, these findings suggest that bacterial restriction is mainly NLRC4/caspase-1-dependent during the initial infection phase. Meanwhile, the infected epithelial cells are expelled into the lumen and the epithelium releases pro-inflammatory cytokines such as IFN-γ to prime uninfected cells. Primed-cells upregulates caspase-11 that confers further host protection against a followed infection.

Recently, Rauch et al. investigated the mechanism of inflammasome-driven cell extrusion in more detail by challenging mice that exclusively express NLRC4 in epithelial or myeloid cells with FlaTox, a fusion of LT-flagellin fusion protein or *S*. Typhimurium infections[135]. In agreement with Sellin et al. (2014)[129], NLRC4-expressing intestinal epithelial cells were found to be required for cell expulsion and *S*. Typhimurium restriction. But in contrast to previous observations by Sellin et al., extruded infected cells showed membrane permeabilization (presumably induced by GSDMD pores) already prior to cell expulsion. This discrepancy might be explained by the fact that Sellin et al. monitored membrane permeabilization by analysing the release of a tandem fluorescence protein (60 kDa), which should be too large to be secreted through GSDMD pores[19]. Interestingly, infected intestinal cells lacking CASP1 or GSDMD were found to be still expelled, but lacked signs of membrane permeabilization[135]. Consistent with *in vitro* work that showed that ASC specks can activate caspase-8 in absence of caspase-1, *Casp1*$^{-/-}$*Casp8*$^{-/-}$*Ripk3*$^{-/-}$, but not *Casp1*$^{-/-}$*Ripk3*$^{-/-}$ or *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ mice were protected from cell expulsion and phenocopied the resistance of *Nlrc4*$^{-/-}$ mice to FlaTox treatment. Consistently, the authors further showed that caspase-8 formed specks in the enterocytes of FlaTox-treated mice, and that caspase-8 co-immunoprecipitated with NLRC4 and NAIP5 only in the presence of ASC *in vitro*. These findings demonstrate that not only pro-inflammatory caspases but also the apoptotic caspase-8 promote intestinal immune defence during *S.* Typhimurium infection.

Overall, these emerging studies agree that cell expulsion is a host defense mechanism to clear infected cells from the intestinal epithelium and to restrict *S. Typhimurium* replication. An open question is whether inflammasomes in epithelial cells follow the same rules as in myeoloid cells. Crowley and colleagues elucidated the mechanisms by how infected epithelial cells switch from the NLRC4-mediated to caspase-11-mediated restriction and prime bystander cells to promote host defense[134]. Interestingly, GBPs are also upregulated upon IFN-γ priming and are known to be required for full activity of the non-canonical inflammasome in macrophages by facilitating caspase-11 binding to LPS[50,52,53(p10)]. This raises the question whether GBPs also contribute to the caspase-11-driven *S. Typhimurium* restriction in epithelial cells.

All studies have so far mainly focused on *S.* Typhimurium, and it is thus still unclear whether expulsion of infected epithelial cells is beneficial for the host upon infection with other bacteria. It is not excluded that some pathogens might exploit cell expulsion by escaping from infected cells in the lumen followed by reinfection to other cells. Furthermore, studies that focus on the mechanisms by which infected epithelial cells trigger and synchronize their expulsion and cell death would be essential to further elucidate this fascinating host response mechanism.

3.5 Direct antimicrobial effectors

Although cell death can promote cell-extrinsic mechanisms to resist pathogenic infection, such as the recruitment and priming of bystander cells, an emerging body of evidence now indicate that necrotic cell death, in particular pyroptosis, can have direct antimicrobial effects. Targeting of the pore-forming $GSDMD^{NT}$ to the plasma membrane requires the binding to acidic phospholipids such as phosphoinositides, phosphatidic acid and phosphatidylserine[14,15]. Interestingly, the same studies also reported that $GSDMD^{NT}$ bound to cardiolipin, which is present in the inner membrane of eukaryotic mitochondria and in bacterial membranes, leading to the hypothesis that $GSDMD^{NT}$ might also target these membranes. In line with this hypothesis, *in vitro* experiments showed that $GSDMD^{NT}$ over-expression is toxic to bacteria, and that $GSDMD^{NT}$ lyses cardiolipin-containing single membrane protoplast of the Gram-positive *Bacillus megaterium*[15]. Furthermore, Liu and colleagues reported, that pyroptotic cell supernatant reduced *Escherichia coli* and *L. monocytogenes* colony-forming units (CFU) in a GSDMD-dependent manner[14]. Analysis of propidium iodide uptake confirmed bacterial membrane disruption, presumably induced by GSDMD pores. Important to note, it is unclear whether the bacterial

restriction that was observed was due to bacteria killing by GSDMD or another effect of the host cell death. However additional experiments with recombinant $GSDMD^{NT}$ showed a similar effect on *E. coli* and *S. aureus*, suggesting that GSDMD was responsible for reduced viability[14]. In agreement with this notion, Wang et al. recently demonstrated that GSDMD has antimicrobial activity on *Burkholderia thailandensis in vitro*[136]. Higher *B. thailandensis* numbers were recovered from infected *Gsdmd*-deficient macrophage compared to wild-type controls, and these bacteria were more resistant to stressors such as H_2O_2 and β-defensin-3, although if these was due to GSDMD pore formation in the bacterial membranes could not be determined. This appears to be reminiscent of the reduced fitness of PIT-contained bacteria, but since necrosis and necroptosis cause a similar fitness reduction it is unclear if GSDMD is the only anti-microbial effector in necrotic cells. Conversely, Thurston et al. reported that caspase-1 and caspase-11 restrict the replication of the intracellular bacterium *S.* Typhimurium but found it to be independent of GSDMD[137], which suggest that other unidentified caspase substrates might exert antimicrobial functions. Thus, further studies are required to elucidate whether bacteria killing by GSDMD occurs in parallel to caspase-1/-11-dependent bacteria restriction.

The necroptosis executor protein MLKL is activated by RIPK3-dependent phosphorylation and oligomerizes at the plasma membrane to form pores that induce cell lysis. Similar to GSDMD, MLKL has also been reported to bind to negatively charged lipids, among them cardiolipin. This raises the question whether MLKL might also promote bacterial restriction by directly targeting bacteria. A recent study provided the first evidence for this concept by demonstrating that the Gram-positive bacterium *L. monocytogenes* activates the RIPK3-MLKL pathway in infected cells and is restricted by RIPK3 *in vivo*[138]. Unexpectedly, activation of RIPK3-MLKL did not induce host cell death, but only suppressed *Listeria* replication. Interestingly, the authors found that MLKL is recruited to intracellular *Listeria*, but not to the Gram-negative bacteria *E. coli* or *S*. Typhimurium. The MLKL specificity to *L. monocytogenes* might be explained by the fact that cardiolipin levels are higher in the outer membrane in Gram-positive bacteria. However, it remains to be elucidated whether MLKL also targets other Gram-positive bacteria, by which mechanisms are MLKL recruited to the bacteria, and how MLKL-targeting restricts growth. It is also unclear how *Listeria*-infected cells prevent necroptosis while activating MLKL, but it is possible that low levels of MLKL pore formation are tolerated due to the activity of ESCRT (Endosomal sorting complexes requires for transports) membrane repair[139].

Overall, these emerging studies demonstrated that cells have developed direct antimicrobial effectors to prevent the potential dissemination of viable bacteria prior undergoing cell death, which might prevent unnecessary tissue damage. It is still to be discovered which other pathogens are susceptible to these antimicrobial effectors and the physiological relevance remains untested.

4. CROSSTALK

An emerging theme in the field of cell death research is the surprisingly high level of crosstalk between the different PCD pathways that has been uncovered in the last years. This crosstalk is mainly driven by the ability of signaling adaptors like RIPK1 and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) to interact with different downstream signaling components in a context or time-dependent manner, and the observation that inflammatory and apoptotic caspases share certain common substrates, which they cleave with different efficiency. Another level of complexity is added by the fact that apoptosis, necroptosis and pyroptosis are also closely linked to pro-inflammatory signaling, in that these pathways are either initiated by the engagement of pattern recognition receptors (inflammasome sensors, TLR-TRIF, ZBP1 and others) or upon inhibition of inflammatory signaling. Overall this has resulted in an intricate network of signaling pathways, which researchers have only now begun to uncover. A defining feature of this network is that many pathways are only detectable or engaged if another signaling pathway is inhibited. The best-described examples here are the activation of caspase-8 upon pathogen-driven inactivation of NF-κB signaling, or the activation of necroptosis when caspase-8 activity is blocked, as discussed above. The fact that these pathways lie hidden does not make them less relevant: Indeed, they appear to serve specifically as important back-up pathway for situations, such as during viral or bacterial infections, where pathogens inhibit host pro-inflammatory or cell death signaling. Below we highlight some of the known crosstalk between different pathways and discuss its implication for bacterial infections.

4.1. Apoptosis engagement in absence of caspase-1 or GSDMD

Early studies on *F. novicida*-induced AIM2 inflammasome activation had noted that the phenotype of *Asc*- and *Casp1*-deficient macrophages and mice varied, in that *Asc*-deficiency caused a stronger reduction in cell death and cytokine release, as well as higher susceptibility *in vivo*[140]. This observation

could be explained when it was shown that the ASC speck recruits and activates caspase-8 in *Casp1*-deficient cells and thus induces death and cytokine release[141]. These results were corroborated by other studies that found that ASC also induced caspase-8-dependent death after NLRP3, NLRC4 and NLRP1 inflammasome activation in *Casp1*-deficient BMDMs[142–144]. Interestingly, the interaction between the ASC speck and caspase-8 was found to be mediated by the PYD (pyrin domain) of ASC and the DED (death effector domain) of caspase-8, unlike for caspase-1 which is recruited by the ASC CARD[145,146]. Conversely, the interaction between ASC and caspase-8 can go both ways, in that caspase-8 initiates receptor-independent ASC speck formation, and subsequent caspase-1-dependent pyroptosis as recently proposed by several studies[147,148]. Collectively, the unusual link between ASC and caspase-8 might serve as a back-up pathway in the case of pathogen-induced inactivation of the protease activity of caspase-1 or caspase-8, respectively.

Interestingly, deletion of *Gsdmd* results in a complete abrogation of caspase-11-induced death, while it only delays cell lysis induced upon caspase-1 activation[11,12,149]. This observation suggests that caspase-1 can cleavage additional substrates that drive a lytic form of cell death. We and others have now shown that this death requires the catalytic activity of caspase-1 and the activation of apoptotic initiators caspase-8 and -9, and the executor caspase-3[150,151]. A critical driver of this death is a direct caspase-8-independent cleavage of Bid by caspase-1, which results in the permeabilization of mitochondria and in the release of both cytochrome c and SMAC. Both of these factors are then important, since cytochrome c is necessary to activate caspase-9, and second mitochondria-derived activator of caspases (SMAC) relives inhibitor of apoptosis proteins (IAP) inhibition and thereby allows auto-processing of caspase-3 to the fully active p17/p10 form, which is necessary to drive death. Morphologically, *Gsdmd*$^{-/-}$ cells start to undergo apoptotic blebbing, but quickly switch to a necrotic phenotype with extensive membrane ballooning, thus suggesting that caspase-1 activation in absence of *Gsdmd* results in rapid secondary necrosis[150]. While not yet proven, it can be assumed that the ability of caspase-1 to induce rapid secondary necrosis by activating apoptotic caspases might have evolved as a safeguard against pathogens that inhibit GSDMD. While no bacterial inhibitor of GSDMD has yet been identified, the pathogenic enterovirus 71, that is known to trigger the NLRP3-inflammasome, was shown to interfere with GSDMD activation[152]. In particular the viral protease 3C was shown to cleave GSDMD at Q193/194, interfering with N-terminal fragment formation, oligomerization and GSDMD pore formation. GSDMD-independent secondary necrosis does contribute to the clearance of bacterial

infection, as it was shown that *Gsdmd*$^{-/-}$ mice are less susceptible to infection with *F. novicida* compared to *Casp1*- or *Aim2*-deficient animals[143,153]. Along the same lines, *Gsdmd*-deficient mice infected with *B. thailandensis* show lower CFUs and lower IL-1β levels than *Casp1/Casp11*-deficient animals[136]. Similarly, it was reported that peritoneal IL-1β levels are higher in *S.* Typhimurium-infected *Gsdmd*$^{-/-}$ mice than *Casp1*$^{-/-}$ controls [154]. These studies thus allow the conclusion that GSDMD-independent cell death is also engaged *in vivo* and that it allows partial protection against intracellular bacterial pathogens. Unexpectedly however, GSDMD-independent secondary necrosis does not appear to be engaged in models of autoinflammatory diseases, since *Gsdmd*-deficiency rescues mice expressing mutant NLRP3 or Pyrin, linked to Neonatal Onset Multisystem Inflammatory Disease (NOMID) and Familial Mediterranean Fever (FMF)[155,156]. How this discrepancy arises is unclear, but it might be linked to differences in the cell types that are activating caspase-1 or to the more complex inflammatory setting elicited by bacterial infections.

4.2. GSDMD as a common substrate of inflammatory and apoptotic caspases

The cell death executor GSDMD was initially identified as the executor of inflammasome-induced pyroptotic cell death and shown to be processed by the inflammatory caspases-1 and -11 in mice and caspases-1, -4 and -5 in humans. Surprisingly, several groups have now reported that GSDMD is also a substrate of apoptotic initiator and executor caspases, however the outcome of GSDMD cleavage by apoptotic caspases varies dramatically[76–78,157]. Cleavage by caspase-3/-7 at D88 in mouse GSDMD or D87 in human GSDMD results in the inactivation of the pore-forming GSDMD^{NT}, and thus has a negative regulatory function[78,157]. On the other hand, cleavage by caspase-8 at D276 in mice and D275 in humans, e.g. at the same site that is also cleaved by inflammatory caspases, activates the protein[76–78]. Notably, cleavage by caspase-8 is around 30-fold less efficient than cleavage by caspase-1[78], nevertheless we and other have found that treatment of BMDMs with extrinsic apoptosis triggers or infection with *Yersinia* spp., which activates caspase-8, results in GSDMD-dependent LDH release[76–78]. At the same time, it can be observed that a large part of active GSDMD p30 is processed by caspase-3/-7 into the inactive p20 subunit. The physiological function of GSDMD cleavage by apoptotic caspases is so far unclear and can only be speculated on. It is possible that inactivation of GSDMD by executor caspases-3, -7 evolved to reduce unwanted or overarching activation of inflammasome-dependent pyroptosis in apoptotic cells which has been reported before (see below), thus ensuring an

immunologically silent outcome of cell death. It is more difficult to explain the purpose of caspase-8-dependent activation of GSDMD, but it is conceivable that it is part of a crosstalk between pyroptosis and apoptosis that evolved as a mechanism against pathogen-induced inactivation of inflammasomes (see above). Importantly, in the context of pathogen-induced inactivation of inflammatory caspases it might not only be of advantage to activate apoptotic caspases, but also to endow these with the ability to induce a lytic type of cell death by cleaving GSDMD. Incidentally, caspase-8 not only processes GSDMD but also IL-1β[143], thus assuming another function normally executed by caspase-1. Although appealing, this explanation does not address how cells safeguard against accidental engagement of caspase-8-driven GSDMD activation during apoptosis that happens as part of development and homeostasis. In that regard it would be possible that the caspase-8-GSDMD pathway requires additional pathogen-induced triggers, linked for example to the inactivation of NF-κB signaling and/or to the activity of the executor caspases. Thus, cell with low caspase-3/-7 activity might be especially prone to engage pyroptosis. Alternatively, it is possible that not all types of apoptotic signaling complexes (complex IIa/b, DISC etc.) are able to activate this pathway. Thus, further research is necessary to understand this unexpected link between apoptosis and the gasdermins.

4.3. Apoptosis-driven NLRP3 activation

Early observations that death receptor ligation promoted maturation and secretion of the proinflammatory cytokine, IL-1β, provided strong indication that the apoptotic machinery triggers inflammasome assembly[158]. Indeed, an increasing list of 'classical apoptosis agonists' such as TNF/cycloheximide, SMAC mimetics, BH3 mimetics and staurosporine have all been demonstrated to activate the NLRP3 inflammasome in myeloid cells[78,159–162]. Activation of the NLRP3 inflammasome during intrinsic apoptosis appears to be dependent on both caspase-3 and -7, as *Casp3* or *-7* single deficiency has no major impact on caspase-1 autoprocessing, a hallmark of inflammasome activation[162]. This suggest that cleavage of a common caspase-3 and -7 substrate licenses NLRP3 assembly in apoptotic cells. In agreement with this, we recently demonstrated that the caspase-3 and -7 substrate, pannexin-1, a membrane glycoprotein, is required for NLRP3 assembly in apoptotic cells[78]. Cleavage of pannexin-1 by caspase-3 and-7 relieves its auto-inhibitory domain, allowing pannexin-1 channel activity, membrane permeability, potassium efflux and NLRP3 inflammasome assembly. In support of this, blockade of pannexin-1 channel activity but not pannexin-1 cleavage using small

molecule inhibitors abrogated NLPR3 activation in apoptotic cells[79]. Interestingly, under conditions of intrinsic apoptosis, caspase-8 appears to be equally important as the NLRP3 inflammasome in promoting IL-1β maturation. Mechanistically, SMAC released from permeabilized mitochondria promotes the assembly of caspase-8 activating ripoptosome complex by depleting cellular levels of IAPs[162,163]. The mechanisms by which extrinsic apoptosis activates the NLRP3 inflammasome is less straightforward and still debatable. A study proposed that direct cleavage of GSDMD by caspase-8 promotes plasma membrane permeability and potassium efflux to activate the NLRP3 inflammasome[76]. However, we found that loss or gain of GSDMD pores had no impact on caspase-1 processing during extrinsic apoptosis. Instead, we observed that similar to the mechanism described for intrinsic apoptosis, pannexin-1 is likewise required for NLRP3 inflammasome assembly when macrophages were treated with TNF/SMAC mimetic or TNF/TAK1i to activate extrinsic apoptosis[78]. The reasons for this discrepancy are still unclear, and further studies will be required to clarify this.

5. CONCLUSIONS/OUTLOOK

Since the first reports that described pathogen-induced activation of host cell death over 20 years ago, the field has come a long way in characterizing the signaling pathways that control programmed cell death induction during infection and demonstrating its physiological significance. Work on host-pathogen interaction often proved to be essential in identifying novel forms of cell death and dissecting the network of innate immune and cell death signaling pathways that control its activation. Nevertheless much work remains to be done, in particular when it comes to understanding host cell death as an innate immune mechanism *in vivo*. Tools that allow to visualize and clearly identify different forms of cell death *in vivo* remain limited, and genetic approaches are confounded by the high-level of redundancy that can induce back-up cell death pathways. Nevertheless, the understanding of cell death has progressed considerably in recent years, as highlighted by the identification of the cell death executors like MLKL and the gasdermins. The latter in particular have proven to be a highly exciting field of research, and it will be interesting to see how the activation of orphan gasdermins is controlled and if they function in host defense as well. Last but not least it remains to be shown if and how all of this newly obtained knowledge will be translated into treatments for new anti-bacterial therapies. Inhibitors of all major death signaling pathways have been identified and thus allow the complete block of certain forms of cell death, or even the rerouting from one form of death into another outcome. This

might not only prove to be important in cases where bacterial pathogens profit from cell death induction, but also helpful to convert a highly pro-inflammatory outcome into another less inflammatory forms of cell death, such as during sepsis. Thus further research into the molecular basis of cell death signaling, the cross-talk between pathways and the strategies by which bacterial pathogens manipulate cell death pathways will undoubtedly provide insight into new therapeutic approaches to control bacterial infection and inflammatory disease progression.

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Table 1. Inhibition of inflammasomes and pyroptosis by bacterial pathogens.

Pathogen	Effector	Target	Reference
Rhizobium galegae *Rhodobacter sphaeroides* *Helicobacter pylori*	Antagonist LPS	Caspase-11/-4	47,49
Yersinia spp.	YopM YopK	Pyrin inflammasome/direct caspase-1 binding Modulation of inflammasome activity	37,38,64,164
Shigella flexneri	IpaH7.8 IpaH9.8 OspC3 LPS modifications	NLRP1B Guanylate-binding proteins Caspase-4	42,60–62,63(p3)
Coxiella burnetii	IcaA	Caspase-11	165

Table 2. List of pathogens known to induce NETosis

Pathogen	Known regulators	Subversion	Reference
Gram-negative bacteria			
Shigella flexneri	PAD4		94

Burkholderia pseudomallei	ROS	Expression of Bsa T3SS and capsule suppresses NET formation	166
Yersinia species	ROS	Unidentified nuclease	167,168
Neisseria gonorrhoeae	ROS	NET degradation by Nuc	169,170
Pseudomonas aeruginosa	NE, PAD4	Downregulation of flagellar motility	171,172
Vibrio cholerae	-	NET degradation by Dns and Xds	173
Salmonella Typhimurium	Caspase-11, GSDMD		108
Citrobacter rodentium	Caspase-4/5, GSDMD, PAD4		108,174
Haemophilus influenzae	TLR4, MyD88		175
Gram-positive bacteria			
Staphylococcus aureus	ROS, NE (lytic) TLR2, C3 (non-lytic)	Conversion of NETs to deoxyadenosine, which induces apoptosis in immune cells. Net degradation by Nuc	94,95,107,176–178
Group A Streptococcus	PAD4	DNase Sda1, Sda2	103(p4),179–181
Group B Streptococcus	ROS, NE	Molecular mimicry of host sialylated glycans	104,182
Streptococcus pneumonia	-	NET degradation by EndA	183
Listeria monocytogenes	ROS, CLEC5A		184
Fungi			
Candida albicans	ROS, NE	Cell wall remodelling, production of biofilms	104,185,186
Aspergillus fumigatus	ROS, PAD4	Expression of RodA	187–189
Parasites			
Leishmania amazonensis	-		190
Leishmania mexicana	-		191
Leishmania donovani	ROS-independent	Expression of lipophosphoglycan	192
Leishmania infantum	Likely ROS-dependent	NET degradation by 3′-Nucleotidase/Nuclease	190

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FIGURES

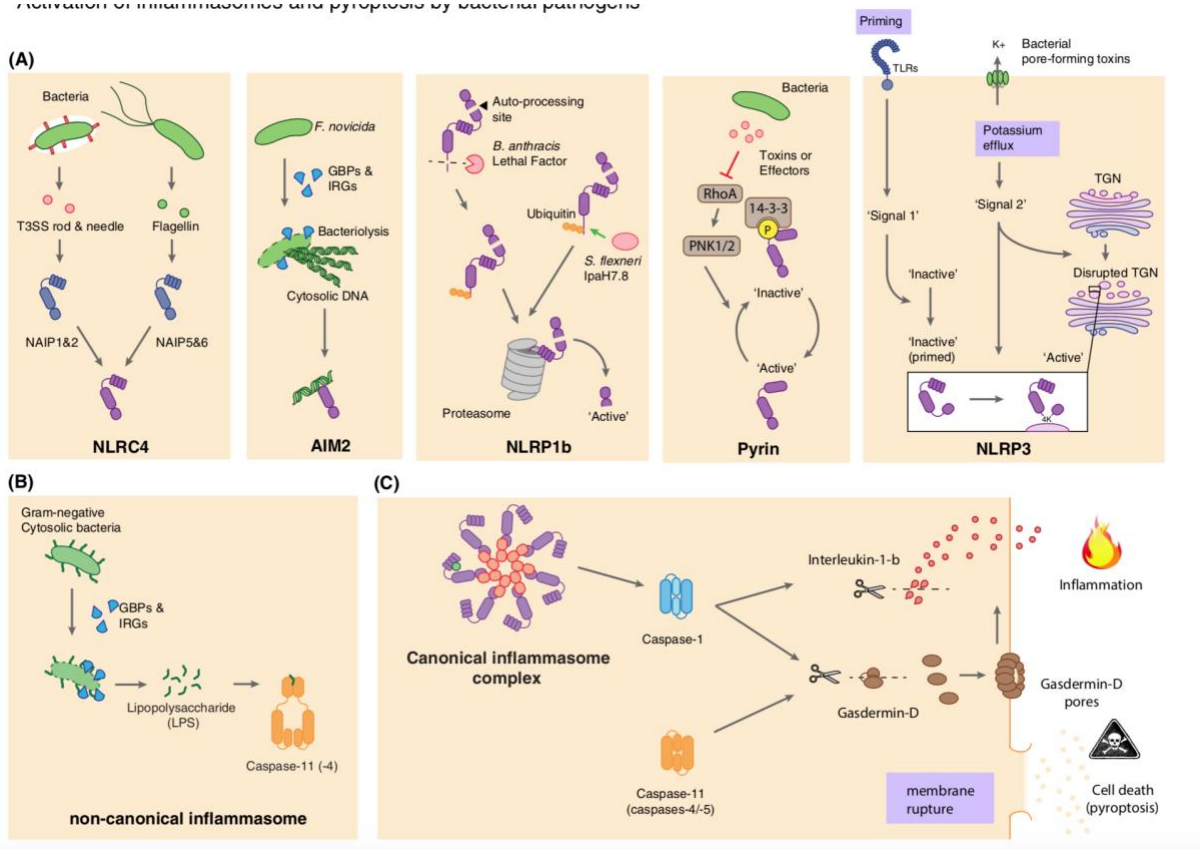

Figure 1: Schematic overview of inflammasome activation and pyroptosis during bacterial infections. (A) Canonical inflammasomes: NLRC4 responds to bacterial flagellin, rod and needle subunits of the bacterial type 3 secretion system (T3SSs). Detection of these ligands occurred via NAIPs as direct upstream receptors. The absent in melanoma 2 (AIM2) inflammasome detects DNA from cytosolic bacteria such as *F. novicida*. Guanylate-binding proteins (GBPs) and immune-related GTPase (IRGs) facilitates bacterial detection by AIM2. Protease lethal factor from *B. anthracis* cleaves the NLRP1b in its N-terminus. Cleaved NLRP1b is ubiquitinated and targeted to the proteasome for degradation, which releases its C-terminal CARD that engages with caspase-1. The effector IpaH7.8 from *S. flexneri* can directly ubiquitinate NLRP1 and promote its targeting to the proteasome. The pyrin inflammasome is activated by bacterial toxins or effector proteins that inactivate the small GTPAse RhoA, leading to reduced activity of the kinases PKN1/2. PKN1/2 activity is necessary to keep pyrin in a phosphorylated inactive state, bound by 14-3-3 proteins. Toll-like receptor (TLR) priming induces NLRP3 transcriptional induction (Signal 1). The NLRP3 inflammasome is activated by diverse stimuli (signal 2) such as potassium efflux induced by permeabilization of the plasma membrane, the disruption

of the trans Golgi network (TGN) or many bacterial pathogens (not depicted here). **(B)** Noncanonical inflammasome: LPS from cytosolic bacteria binds to caspase-11 (-4) thus inducing the oligomerization and activation of the caspase. GBPs /IRGs assist in the process or ensure accessibility of LPS. **(C)** The cleavage of gasdermin D (GSDMD) in its linker domain by caspase-11 (caspase-4 in humans) and caspase-1 liberates its N-terminal cytotoxic domain, which targets the plasma membrane to form pores and induce the lytic cell death pyroptosis. Caspase-1 also cleaves the pro-inflammatory cytokines IL-1β and IL-18 (not depicted here) to its biologically active forms which is secreted in an GSDMD-dependent manner.

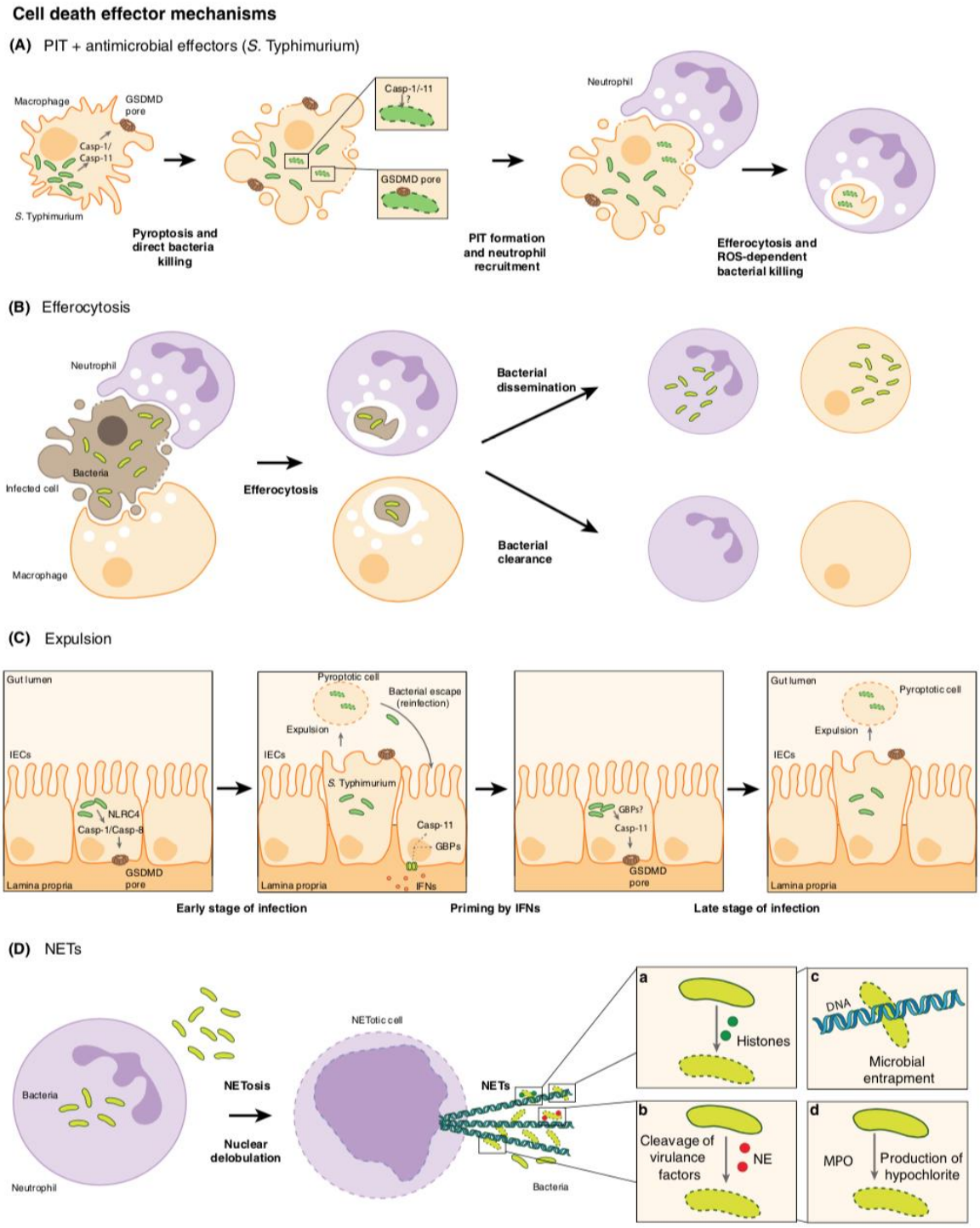

Figure 2: Schematic overview of effector mechanisms of cell death. (A) The detection of intracellular *S*. Typhimurium by macrophages triggers caspase-1/-11 activation and gasdermin D (GSDMD) processing. The N-terminal cytotoxic domain ($GSDMD^{NT}$) targets the plasma membrane to form pores that triggers the lytic cell death pyroptosis. Intracellular *S*. Typhimurium is damaged by antimicrobial effectors such as GSDMD or by caspase-1/-11-dependent mechanisms. After pyroptosis, *S*. Typhimurium is entrapped in *pore-induced intracellular traps* (PITs). Entrapped bacteria present

damaged outer membrane, which is caused either by the mentioned effector mechanisms or by other unknown mechanisms. Recruited neutrophils efferocytose the infected macrophages containing entrapped *S*. Typhimurium and promote bacterial clearance in an ROS-dependent manner. **(B)** Phagocyte cells such as macrophages and neutrophils efferocytose infected cells. The protective or detrimental roles of efferocytosis for the host depends on the type of pathogen and the phagocytic cell. **(C)** In an early stage of infection, intestinal epithelial cells (IEC) detect intracellular *S*. Typhimurium by the NLRC4 inflammasome that triggers caspase-1/-8 activation and GSDMD processing. $GSDMD^{NT}$ induces cell lysis and expulsion of infected cell. How cells synchronize these two events it is not completely clear. Cell death from expulsed cell triggers the release of pro-inflammatory cytokines such as IFN-γ that primes neighboring cells. Upregulated caspase-11 confers a further protection at late stage of infection against *S*. Typhimurium that has escaped from expulsed cells. Whether guanylate-binding proteins (GBPs), reported to be upregulated by IFN-γ and to facilitate caspase-11 sensing to bacterial LPS, promotes bacterial clearance in IECs has not been investigated. **(D)** Infected neutrophils undergo NETosis promoting bacterial clearance by diverse mechanisms: (a) Histones are reported to have antimicrobial activities. (b) Neutrophil elastase (NE) cleave and inactivate virulence factors. (c) Expelled DNA entraps bacteria limiting their dissemination in the host. (d) Myeloperoxidase (MPO) promotes bacterial killing by catalyzing the conversion of chloride and hydrogen peroxide to hypochlorite, which has antimicrobial activity.