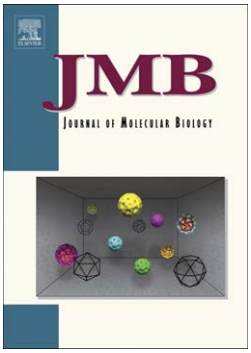

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Consequences of Epithelial Inflammasome Activation by Bacterial Pathogens

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Abstract

Inflammasome signalling impinges on activation of inflammatory caspases, i.e. caspase-1 and caspase-4/5/11, and endows host cells with a sentinel system to sense microbial intrusion and thereby initiate appropriate immune responses. Lately, it has become evident that mammalian inflammasome-dependent responses to infection are not confined solely to cells of hematopoietic origin. Epithelial cells that line the body´s mucosal surfaces use inflammasome signalling to sense and counteract pathogenic microorganisms that compromise barrier integrity. Many of the molecular mechanisms of epithelial inflammasome signalling remain unexplored. However, it now seems clear that epithelial inflammasome activation has a profound impact both on the infected cell itself and on its ability to communicate with other cell types of the mucosa. Here, we summarize current knowledge regarding the output of epithelial inflammasome activation during bacterial infection. Well established downstream effects include epithelial cell death, release of soluble mediators, and subsequent recruitment of effector cell types, including NK cells, mast cells and neutrophils, to sites of mucosal infection. We discuss the implications of recent findings for anti-bacterial defense in the mucosa and sketch out areas for future exploration.

Key words: Epithelium, Inflammasomes, NLRC4, NLRP3, Interleukin-18

Epithelial inflammasomes and bacterial infection

The body´s mucosal linings constitute the predominant entry portals and toxin target sites for pathogenic microorganisms. This places the epithelial cell layer(s) at the front line of the ensuing battle between microbes and the host. How epithelial cells respond to interactions with microbes, and how they differentiate between non-hostile and pathogenic interactions, is consequently of key importance to allow switching of the mucosa between homeostasis and defense. Recent work has highlighted a central role of epithelial inflammasome signalling in the making of these decisions.

Inflammasomes consist of cytosolic pattern-recognition receptors (PRRs), commonly of the Nod-like receptor family (denoted NLRs; nucleotide-binding domain leucine-rich repeat-containing receptors), and their binding partners. A single cell may express several inflammasome PRRs, each capable of sensing distinct pathogen- or damage-associated ligand(s) [1,2]. NLRC4 detects flagellin or type-III-secretion systems of gram-negative bacteria through Neural apoptosis inhibitor proteins (NAIPs) [3]. NLRP1 detects anthrax lethal toxin, whereas NLRP3 indirectly recognizes a multitude of stimuli, including pore-forming toxins, crystals, and extracellular ATP. Absent in melanoma 2 (AIM2) detects double stranded cytosolic DNA of both microbial and host origin [1]. Finally, the Pyrin inflammasome reacts to modification of cellular Rho GTPases as a proxy for pathogen perturbation [2,4]. Some proposed inflammasomes may also recognize additional as of yet poorly defined or unknown ligands.

Despite the differences in ligand specificity, inflammasome PRRs converge with minor exceptions on a common intracellular signalling cascade. The activated receptor multimerizes, binds the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), associates with and activates caspase-1, thereby initiating proteolytic cleavage of a large panel of substrates, including interleukin-1 (IL-1) family cytokines [1]. Moreover, a group of caspase-1-related proteases, comprising caspase-4/-5 in humans (caspase-11 in mouse) can directly bind to cytosolic bacterial lipopolysaccharide (LPS), and initiates a “non-canonical” inflammasome signalling cascade [2,5]. Inflammasome activation as a rule results in the demise of the affected cell and the release of soluble pro-inflammatory mediators.

Recently, it has become evident, that in addition to the influence of inflammasomes on myeloid immune cell responses, inflammasome-dependent programs also impact on the physiology and anti-pathogen defense of epithelial tissues, including those of the oral cavity, intestine, lung and urogenital tract. A sentinel system for pathogens compromising the epithelium makes sense intuitively, since pathogenic microorganisms have to expose key virulence factors, i.e. flagella and type-III-secretion systems, to reach and engage with this site. Thus, at this site the respective ligands cannot be "concealed" by down-regulation of their expression, a phenomenon commonly encountered during systemic infection [6]. The strategic position, combined with the fact that epithelial cells (similarly to many blood-derived cell types) as a rule express more than one type of inflammasome [7–9], may leave room for tailored responses against multiple mucosal insults. The literature suggests a functional relevance for both NAIP/NLRC4 [10–12], NLRP3 [13,14], NLRP6 [15], AIM2 [16,17] and caspase-4/-11 [18–20] signalling in intestinal epithelial cells during bacterial infection. Dual NLRP3 and NLRC4 responses have also been reported in the infected oral epithelium [21–23]. Moreover, detectable albeit variable expression of NLRP1, NLRP3, NLRC4 and AIM2 has been confirmed in normal and cancerous lung epithelial cells [24], with implications for protection

against at least influenza virus infection [25]. At other sites, such as the urogenital epithelium, the set of expressed inflammasomes is not fully defined, but inflammasome-dependent responses to *Chlamydia trachomatis*, and uropathogenic *Escherichia coli* (UPEC), have been described [26,27]. Future work will have to establish the comprehensive panel of inflammasomes expressed at each epithelial surface and how this impacts on pathogen-specific and/or stereotypic responses towards infectious agents.

Much work remains to uncover the detailed molecular mechanisms of epithelial inflammasome signalling. This includes validation of the eliciting ligands and intracellular chains-of-events (Figure 1), which are now often inferred from studies of cultured macrophages. Moreover, the epithelial responses *in vivo* have only been probed using a few selected bacterial pathogens [7,9], or a purified PRR ligand [12]. What appears clear at this stage, however, is that the output of epithelial inflammasome activation results in both cell-autonomous, local and global effects in the mucosa, all of which may contribute to anti-pathogen defense. In this piece, we delineate the main downstream consequences of epithelial inflammasome activation during bacterial infection, namely i) epithelial cell death, ii) release of soluble pro-inflammatory mediators, and iii) recruitment/activation of effector cell types through intercellular communication (Figure 1). We discuss the findings in context of mucosal protection against bacteria-inflicted disease.

Figure 1. Schematic depiction of epithelial inflammasome signalling pathways and downstream consequences for mucosal anti-bacterial defense. Note that the wiring of the intracellular signalling cascades (dashed arrows) is partly based on inference from non-epithelial cell types. For epithelial NLRP6, both the eliciting ligand and the extent of signalling through a classical inflammasome pathway (question mark) remain unclear.

Inflammasome-dependent death of infected epithelial cells

Epithelial tissues experience a constant shower of chemical, physical, and biological insults. This necessitates a high removal rate for worn epithelial cells, and their replacement through stem cell driven proliferation. As a consequence, the typical life-span of a differentiated epithelial cell often amounts to only a few days. Conditions of mucosal infection and inflammation may further augment epithelial turnover rates [28].

Intimate interactions between neighbouring cells, as well as high-affinity integrin-mediated adhesion to the basal membrane, ensures the integrity of epithelial tissues. This interconnected assembly has implications for the execution of cell death responses. The processes of cell death, removal of the dying cell, and sealing of any structural gaps, have to be tightly linked to guarantee tissue integrity. For stratified epithelia, cell death involves sloughing of superficial cells upon loosening of connections to underlying cell layers. Within single-layered epithelia, e.g. in the

intestine, dying cells are instead removed through a process coordinated by actin cytoskeleton contraction in neighbouring cells, commonly referred to as “extrusion” [29]. Inflammasome-triggered cell death has been described in both types of epithelial tissues *in vivo* [10,12,18,19,27,30].

Data from the current literature cannot be reconciled with a single molecular pathway driving inflammasome-dependent cell death in all epithelial cell types. Based on the morphological characteristics of dying cells, both lytic [12,19,27,30] and non-lytic [31–33] modes of epithelial cell death have been linked to inflammasome PRR activation (Figure 2A). Furthermore, the underlying mucosal wiring of the response could give rise to an additional classification into three broad categories (Figure 2B). Several lines of evidence favour the presence of epithelial cell-autonomous death pathway(s) [18,19] (Figure 2B, model I). Infected epithelial cells can also locally recruit other cell types, which exhibit lytic/apoptotic activity towards the epithelial cell itself [27] (Figure 2B, model II). Finally, pro-inflammatory cytokine release sparks a state of global inflammation and leukocyte influx throughout the surrounding mucosa [34], thereby boosting general epithelial turnover and indiscriminate removal of infected and uninfected epithelial cells alike (Figure 2B, model III). Local, limited infections, will most likely elicit limited areas of inflammation while the infection of larger mucosal areas may elicit an inflammation-associated cytokine storm with elevated levels of several apoptosis-inducing soluble factors, e.g. tumour necrosis factor (TNF). Here, we first examine the evidence for and properties of cell-autonomous death of bacteria-infected epithelial cells. We revisit local and tissue-scale circuits in subsequent sections.

Figure 2. A. Two fundamentally different modes of epithelial cell death that have been linked to epithelial inflammasome activation. **B.** Three conceptual models for the circuits executing epithelial cell death downstream of inflammasome activation.

Infection of macrophages with *Salmonella* Typhimurium, *Shigella flexneri*, and related bacteria sparks a prompt lytic form of cell death denoted pyroptosis [35,36]. This was first seen as a process driven by the pathogen to escape confinement, but is now rather viewed as a means for the host to expose the pathogen to secondary attack by recruited effector cells, most notably neutrophils [6]. Pyroptosis can be initiated both upon caspase-1 activation by inflammasome PRRs and downstream of caspase-4/-5/-11 [37]. Recent work has now converged on a molecular explanation for macrophage lysis. Inflammatory caspases cleave a pyroptosis executioner protein, gasdermin D [38–40], and this processing generates an N-terminal gasdermin D fragment that forms nm-sized pores by insertion into the plasma membrane [41–44]. Several related gasdermins share this lysis-inducing capacity [38,41]. Hence, a complete cell-autonomous program has been delineated, whereby sensing of pathogen components, e.g. flagellin and type-III-secretion proteins through NAIP/NLRC4 and

cytosolic and LPS through caspase-4/-5/-11, results in activation of canonical caspase-1 or non-canonical caspase-4/-5/-11, cleavage of gasdermin D, and subsequent plasma membrane lysis.

A similar process to macrophage pyroptosis seems to exist also in infected or bacterial-ligand-exposed epithelial cells [5,12,18,19,30]. *Salmonella* Typhimurium infection of a polarized human adenocarcinoma cell line caused the shedding of bacteria-filled dead epithelial cells from the monolayer [30]. This process shared morphological features with the epithelial cell extrusion events noted during steady state turnover [29,30]. Notably however, in this model, shed infected cells exhibited a permeabilized membrane and allowed re-entry of bacteria into the extracellular luminal environment juxtaposed to the infected epithelium [30]. Initial work suggested involvement of caspase-1 in the epithelial death response [30], but later follow-up studies rather pointed to the importance of non-canonical caspase-4 signalling [19]. An independent study reached similar overall conclusions [18]. Specifically, the authors found that *Shigella*, *Salmonella* and pathogenic *E. coli* infection of a panel of human epithelial cell lines caused lytic cell death, that in the case of *Shigella* was suppressed by the bacterial effector OspC3. Furthermore, OspC3 was found to bind and inactivate caspase-4, hence blocking lytic cell death and allowing prolonged epithelial infection [18]. A similar caspase-4-suppressing activity was subsequently reported also for pathogenic *E. coli* [20].

The mechanism driving human epithelial cell lysis downstream of caspase-4 activation has not been fully elucidated. In this context, it is interesting to note that members of the gasdermin family appear highly expressed in epithelial cells of the digestive tract [45]. Moreover, as a recent study implicates gasdermin D at least in caspase-1-dependent epithelial cell death [12] (discussed further below), it appears plausible that the general mechanism for lysis is conserved between macrophages and epithelial cells. In either case, several studies support that in human adenocarcinoma cell lines, caspase-4-dependent lytic cell death is a prominent consequence of gram-negative bacterial infection.

Caspase-4 appears constitutively and abundantly expressed in human intestinal and other epithelial cell lines [5,18,19]. It is however noteworthy that these cells as a rule feature dramatically lower, or even undetectable levels of caspase-1 mRNA and protein, as compared to primary untransformed epithelial cells from the digestive tract (e.g. [19]). Consequently, the impact of caspase-1-dependent processes and upstream inflammasome PRRs may be underestimated in this type of experimental model.

In the mouse mucosa, we found that an epithelial NAIP/NLRC4 response promotes death and removal of *Salmonella*-infected epithelial cells. This response reduced mucosal pathogen loads by ≥50-fold during acute infection [10]. A similar dependence on epithelial NLRC4 was also noted for defense against *Citrobacter rodentium* infection [11]. Importantly, lack of the inflammasome-dependent soluble mediators IL-1β and IL-18 (which delays the mounting of overt tissue

inflammation; see below) did not phenocopy the effect of NAIP/NLRC4-deficiency with respect to pathogen loads [10,34]. This hinted towards the presence of an epithelium-autonomous NAIP/NLRC4-induced cell death mechanism. A subsequent study provided definitive proof that epithelial NLRC4 can drive intestinal epithelial cell death in the absence of other cell types [12]. The authors used an elegant genetic engineering approach to selectively re-express NLRC4 in epithelial or blood-derived cell types in *Nlrc4*$^{-/-}$ mice, and challenged these animals with flagellin delivered through a toxin fusion ("FlaTox"). Epithelial cells expressing NLRC4 underwent rapid cell death and expulsion into the lumen upon FlaTox treatment. Strikingly, the same response was also observed in pure epithelial organoids derived from the animals. These data show that NLRC4-induced epithelial cell death can occur independently of cross-talk with non-epithelial cell types present in the intestinal mucosa [12] (Figure 2B, model I).

By examining the retention of (transgenic) cytosolic red-fluorescent protein in *Salmonella*-infected gut epithelial cells, we previously concluded that shedding *Salmonella*-infected epithelial cells *in vivo* retained an intact plasma membrane until they arrived in the lumen, where disintegration ensued [10]. This observation favours a mode of cell death that is not strictly analogous to the inflammasome-dependent pyroptosis response of macrophages. By contrast, FlaTox treatment of NLRC4-expressing intestinal epithelial cells both *in vivo* and in culture caused prompt cell lysis, characterized by rapid uptake of propidium iodide and a genetic dependence on gasdermin D [12]. Whether this discrepancy can be attributed to differences in experimental readouts (i.e. different gasdermin D pore permeability for a large fluorescent protein vs. a small fluorescent dye), or rather reflect differential responses that depend on ligand dosage remains to be elucidated.

Finally, there is also evidence that inflammasome PRRs may in some settings drive a typical apoptotic death of epithelial cells. Ectopic expression of constitutively active NLRC4 variants in a lung epithelial cell line induced a caspase-8-dependent, but caspase-1-independent cell death [32,46]. This response shared the classical morphological features of apoptosis [32], and required downstream activation of caspase-3 [46]. In addition, extrinsic apoptosis elicited by TNF+cycloheximide stimulation of primary mouse tubular epithelial cells was surprisingly found to depend on an NLRP3/ASC platform for recruitment of caspase-8 [33]. These observations are in line with work on macrophages, showing that NLRP3/ASC and AIM2/ASC can direct either pyroptotic or apoptotic cell death depending on the concentration of the eliciting stimulus [47]. Genetic deletion of caspase-1 or gasdermin D may further skew the mode of macrophage cell death towards apoptosis [48,40]. Such duality was indeed also recently noted for intestinal epithelial cells exposed to the NLRC4-ligand FlaTox [12]. Genetic deletion of either caspase-1 or gasdermin D blocked epithelial cell lysis, but still allowed death and luminal expulsion of epithelial cells in a manner dependent on ASC/caspase-8 and occurring without compromising plasma membrane integrity [12].

Hence, inflammasome PRR activation may under some conditions favour induction of apoptosis in macrophages and epithelial cells alike.

Inflammasome-dependent release of soluble mediators from the infected epithelium

It is by now well established that inflammasome activation can trigger the maturation and release of two prototypical cytokines, IL-1β and IL-18, from innate immune cells [49]. These cytokines recruit effector cell types and orchestrate immune responses to infection both at systemic sites and in mucosal tissues.

IL-1β is a multifunctional cytokine. Due to highly pro-inflammatory properties, expression of IL-1β is tightly regulated in a two-step process, involving first Toll-like receptor (TLR)-induced expression of the inactive ~31-34kDA precursor pro-IL-1β, and second, caspase-1-dependent cleavage and secretion of the active form [50]. Upon bacterial infection of the gut mucosa, IL-1β contributes to reduction of bacterial loads through an array of mechanisms, including stimulation of effector cell responses and fortification of the epithelial barrier [51–53]. However, intestinal epithelial cells often do not produce significant levels of IL-1β themselves [14,19]. Instead lamina propria phagocytes may constitute the main source for this cytokine in the infected intestine [53], although the literature is not entirely consistent on the topic [8]. By contrast, some stratified epithelial cell types, including gingival and bladder epithelial cells, have been shown to express and secrete considerable amounts of bioactive IL-1β upon bacterial challenge [23,27]. In both cases, IL-1β production was attributed to NLRP3 inflammasome activation [23,27]. The potential significance of these findings for differential responses between epithelial cell types remains unexplored.

For IL-18, on the other hand, ample evidence for expression and secretion by diverse epithelial cell types comes from both *in vivo* [12,19,54–57] and cell line culture [14,18,19,31] experiments. Unlike IL-1β, the inactive 24kDa precursor protein, pro-IL-18, is constitutively expressed in the mucosa and maturation and secretion follows on inflammasome activation [34]. The best-characterized property of IL-18 is the ability to induce IFNγ-production by diverse cell types [58]. Recent work has also highlighted additional roles in recruitment of mucosal effector cells, a topic discussed in detail below. Taken together, pathogen-induced epithelial inflammasome activation as a rule results in secretion of the cytokine IL-18, and under some conditions also IL-1β.

In addition to IL-1 family cytokines, inflammasome activation affects the secretion of several other bioactive factors from immune cells. The alarmin HMGB1 is a DNA-binding protein. When released, it triggers broad pro-inflammatory effects and promotes endotoxic shock symptoms during sepsis [59,60]. Both LPS-transfected intestinal epithelial cells, *Legionella pneumophila*-infected lung epithelial cells and *Fusobacterium nucleatum*-infected gingival epithelial cells have been shown to release HMGB1 [5,23,61], which in the latter case coincided with ASC and IL-1β secretion upon

NLRP3 inflammasome activation [23]. The effect of epithelial HMGB1 release on the mucosal response to infection remains to be clarified, however. Similarly, basic Fibroblast Growth Factor (bFGF), another protein linked to inflammasome-dependent unconventional secretion, is found in the supernatant, at least upon Rhinovirus-infection of airway epithelial cells [62]. However, the repercussions remain unclear. A systematic examination of the inflammasome-dependent secretome of infected epithelial cells, and its functional impact, represents an important area for future studies. Such work would also have to address the relative contribution of non-proteinaceous inflammatory mediators, including e.g. pro-inflammatory eicosanoids [63,64]. The eicosanoid prostaglandin PGE_2 was in fact recently found to be secreted by murine intestinal epithelial cells upon NLRC4 activation and might contribute to gut diarrheal symptoms [12].

In conclusion, we have only begun to characterize the panoply of bioactive substances released upon bacteria-elicited epithelial inflammasome activation. In the following sections, we explore three prominent examples of how such released factors, specifically the IL-1 family cytokines, may shape the ensuing innate mucosal tissue response through intercellular communication.

Recruitment of NK cells to mount mucosal inflammation and prevent bacterial spread

During infection of mucosal tissues, natural killer (NK) cells play an important role in early innate immune defenses [65]. In addition to local tissue-resident NK cells that are quickly activated in response to the intruding pathogen, peripheral NK cells are recruited in response to an acute infection to augment anti-pathogen defense. This NK cell response is in turn regulated by various cytokines, including IL-15, IL-12 and notably IL-18 [34,65].

Given the early inflammasome-dependent secretion of IL-18 from infected epithelial cells (augmented by other sources), the cytokine is ideally positioned to stimulate early NK cell recruitment and function during mucosal infection. Indeed, in the cecal mucosa, IL-18 has been shown to drive NK cell accumulation in the infected tissue at least via two pathways, first by upregulating NK cell recruiting chemokines (e.g. Cxcl 9, -10 and -11), and second by stimulating the migratory capacity of NK cells [34] (Figure 3).

Besides stimulating NK cell accumulation, IL-18 plays a pivotal role in activating NK cell effector functions. In accordance with its known IFNγ-inducing capacity, IL-18 is essential to stimulate early IFNγ production from mucosal NK cells [34,66,67]. NK cell-derived IFNγ can help to restrict mucosal pathogen loads and subsequent systemic pathogen spread. These observations suggest that the inflammasome/IL-18/IFNγ axis is a central element of the innate immune defense [68–72], and IFNγ may moreover also affect remission kinetics [73]. However, compared to intestinal epithelial cell death, which is able to reduce epithelial pathogen loads already within the first hours of infection [10], NK cell IFNγ-dependent pathogen restriction sets in later, on a scale of one to several days after

the initial insult [34] (Figure 3). Furthermore, NK cells have been shown to drive local monocyte responses in the infected tissue, first by IL-18-dependent secretion of monocyte recruiting chemokines (e.g. CCL3) and second via local IFNγ-dependent monocyte differentiation in the infected mucosa. This program regulates inflammatory cell dynamics during mucosal infection and enhances the local inflammatory response [74,75] (Figure 3).

Surprisingly, while NK cell-derived IFNγ is important for pathogen control and shaping of mucosal inflammatory cell dynamics, it appears to be redundant in early pathogen-induced inflammation [34]. Besides the production of pro-inflammatory cytokines, another effector function of NK cells is the ability to exert a cytolytic response towards compromised host cells. This NK cell cytotoxicity has classically been attributed to anti-viral or anti-tumor defense [76,77]. However, recent studies have implicated NK cell cytotoxicity also in the defense against bacterial as well as parasitic infections [34,72,78–80]. In a model of acute intestinal *C. rodentium* infection, the lack of PD-1 receptor signaling was found to impair the downstream production of NK cell cytolytic effector molecules (i.e. perforin and granzyme B) and rendered the host susceptible to acute enteric bacterial infection [80]. Moreover, recent findings from systemic *C. violaceum* infection have pointed to a coordination of IL-18-induced NK cell cytolysis and neutrophil bactericidal activity in the control of bacterial tissue loads [79].

In the infected gut mucosa, we have found that perforin-mediated NK cell cytotoxicity, induced by IL-18, forms an early response axis against *Salmonella* Typhimurium already during the first hours after challenge [34]. However, by contrast to observations during systemic infection, NK cell cytotoxicity did not significantly alter early mucosal pathogen loads. Instead, the response promoted acute tissue inflammation, e.g. phagocyte influx, tissue erosion and development of submucosal edema [34] (Figure 3). More work is needed to uncover the exact mechanism(s) driving this perforin-dependent inflammatory response. Several questions remain unanswered, including: What specific cell type(s) are targeted by perforin activity in the mucosa? What soluble pro-inflammatory mediators are released from targeted cells? And, how do these contribute to the onset of acute inflammation?

In summary, present findings suggest that IL-18 secreted from epithelial cells (and possibly supplemented by additional sources) stimulate mucosal NK cell responses during bacterial infection. These NK cell responses encompass secretion of IFNγ (Figure 3, I), recruitment and regulation of other inflammatory cells (Figure 3, II), and perforin-dependent cytotoxicity resulting in inflammation (Figure 3, III). Hence, the mucosal inflammasome-IL-18-NK cell axis can be regarded as a potent amplifier, translating a local input (infection of specific mucosal cells) into a global tissue defensive state.

Figure 3. IL-18-dependent NK cell recruitment and subsequent effects within the infected mucosa.

Recruitment of mast cells that interact with the infected epithelium

Following UPEC infection of the urinary bladder, bacteria attach in a type I-pili-dependent manner to the epithelial surface [81]. Within hours, the host response elicits extensive death and shedding of superficial epithelial cells from the mucosal surface, concomitant with a drastic drop in bacterial titers. Pan-caspase inhibitor administration blocks epithelial cell death and causes elevated bacterial colonization, hence pointing to a host-protective effect of this epithelial cell shedding response [81].

A human bladder epithelial cell line was recently shown to undergo lytic cell death upon *Salmonella* Typhimurium infection, but notably not upon UPEC infection [27]. The *Salmonella*-triggered cell death appears reminiscent of the caspase-4-dependent cell-autonomous response that has been observed in human intestinal epithelial cells [18,19]. However, it seemed harder to reconcile the lack of UPEC-induced cell death with the pronounced death and shedding of epithelial cells occurring in the bladder *in vivo* [27]. Notably, the bladder epithelial cell line was found to secrete high levels of IL-1β in response to UPEC infection, an observation that sparked a tour-de-force to assess the impact of IL-1β-recruited cell types in the infected bladder. This work uncovered a mechanism whereby NLRP3, ASC and caspase-1/11-dependent IL-1β recruits mast cells to the infected bladder epithelium, followed by mast cell-induced lytic epithelial cell death [27] (Figure 4).

Figure 4. An epithelial inflammasome – mast cell circuit fuelling death and shedding of infected bladder epithelial cells.

Mast cells have traditionally been linked to parasitic worm infections and allergic conditions, but an increasing number of studies also highlight important roles in anti-bacterial defense [82]. Mature mast cells are morphologically characterized by a cytoplasm packed with electron-dense granules. These granules contain a myriad of bioactive components, including vasoactive substances such as histamine, cytokines such as TNF, and a panel of proteases. Upon cross-linking of the IgE-receptor or other cell surface receptor(s), mast cells degranulate, releasing this bioactive arsenal into their immediate environment [83]. Strikingly, during UPEC infection of the bladder, infected epithelial cells were found to internalize mast cell granules prior to their demise [27]. Furthermore, administration of purified mast cell granules, but not protease-inhibitor-treated granules, alone into the bladder sparked epithelial cell shedding. Finally, mice deficient in granule-associated mast cell chymase (mMCPT4) failed to elicit bladder epithelial cell death and exhibited elevated levels of UPEC colonization [27]. These data fit with a model in which mast cell recruitment downstream of epithelial inflammasome activation results in uptake of lytic granules and protease-dependent lysis

of infected epithelial cells. It remains to be formally established if NLRP3/ASC-dependent IL-1β comes exclusively from an epithelial source also in the intact bladder. Nevertheless, this study provides a compelling example of a local inflammasome-driven cell circuit sparking epithelial cell death (i.e. Figure 2, model II above). Future work will have to determine if the epithelial inflammasome and IL-1β-dependent mast cell recruitment circuit can be generalized also to other mucosal tissues and microbial threats.

Recruitment of neutrophils and coordination of bacterial clearance

Neutrophils are key effector cells in the innate immune defense towards bacterial infection. Upon microbial breach of a mucosal barrier, neutrophils accumulate in the infected tissue within hours to limit further pathogen influx and subsequent spread to sterile sites. A complex interplay of various chemotactic mediators, cell adhesion molecules and cytokines orchestrates the processes of neutrophil recruitment, tissue invasion and transepithelial migration [84–86].

Recent work has pointed to a functional contribution of the mucosal inflammasome-IL-1/IL-18 axis to the coordination of neutrophil recruitment and activity during bacterial infection. IL-1β, possibly of both epithelial and hematopoietic origin, was found to enhance neutrophil recruitment to infected lung and intestinal tissues, by inducing expression of endothelial cell adhesion molecules, or by stimulating the production of other neutrophil-recruiting chemokines by the intestinal epithelium [53,87,88]. In addition, IL-18 from epithelial and other sources boosted neutrophil recruitment to the intestinal mucosa early during *Salmonella* infection, presumably via the induction of neutrophil-recruiting chemokines [34]. This suggests a concerted action of the two cytokines in the mucosa (Figure 5), similar to what has been recently shown in a model of systemic *Salmonella* infection. Here, the interplay of IL-1β, IL-18 and eicosanoids fueled neutrophil recruitment to infected pyroptotic macrophages [64], thus ensuring efficient bacterial clearance from the spleen.

Due to their broad arsenal of antimicrobial peptides, toxic products, and prodigious epithelial transmigration, neutrophils are thought to inflict significant collateral damage on the surrounding tissue during pathogen defense [89,90]. Interestingly, even though neutrophil accumulation in the bacteria-infected mucosa occurs nearly simultaneous with development of pathology, recent studies favor that the onset of tissue inflammation does not require neutrophils [34,91]. Importantly, this does not exclude the involvement of neutrophils in processes driving tissue pathology in general.

The predominant role of neutrophils in the bacteria-infected mucosa, however, is to eliminate or immobilize the invading microbes. Similarly to at systemic sites, neutrophils can reduce bacterial loads in the mucosal lamina propria, i) by direct engulfment and pathogen killing, ii) by release of IFNγ to activate the microbiocidal activity of mononuclear phagocytes, and/or iii) by production and secretion of IL-22 and subsequent reinforcement of epithelial barrier functions

[6,53,87,88,92–95]. Moreover, transmigrated neutrophils have been found to kill or physically contain also the gut-luminal pathogen population [96,97]. Finally, transmigrating neutrophils can form a transient “intraluminal cast” (also referred to as “pseudo-membrane") to prevent translocation of commensals or pathobionts following damage to the epithelial barrier [98]. Hence, neutrophils suppress pathogen transit through the mucosa by multiple mechanisms.

Besides direct killing of extracellular bacteria, neutrophils were recently found to eliminate bacteria lodged within macrophages through a striking inflammasome-dependent mechanism. As described earlier, inflammasome PRR activation in macrophages results in gasdermin D insertion into the cell membrane and prompt permeabilization [99]. This partial rupture facilitates the release of IL-1 family cytokines, nucleotides and other cytoplasmic material. By contrast, cellular organelles, cytoskeletal proteins and notably intracellular bacteria remain confined within the cellular remnants. These structures have been dubbed "Pore induced intracellular cellular traps" (PITs [100]). The trapped bacteria suffer limited outer membrane damage, but remain viable. Hence, the pathogen is not directly killed during macrophage pyroptosis, but instead, elimination hinges on a dedicated subsequent step, i.e. recruitment of neutrophils, phagocytosis of trapped bacteria ("efferocytosis"), and NADPH-oxidase-dependent killing. For *Salmonella* Typhimurium, the macrophage-PIT-neutrophil mechanism was demonstrated both in tissue culture infections and in mice intraperitoneally inoculated with infected macrophages [100]. It remains to be examined if the same mechanism also helps to defend infected mucosae. In the intestine, this appears plausible as orogastric *Salmonella* Typhimurium infection yields significant numbers of bacteria lodged within lamina propria macrophages [101–103], and copious numbers of neutrophils are recruited into the infected tissue [34,96].

More central for the scope of this review, the “Macrophage-PIT-neutrophil” conjecture raises questions about the fate of bacteria originally lodged within dying epithelial cells. The epithelial NAIP/NLRC4 and/or non-canonical caspase-4 inflammasome drives death and disintegration of enterobacteria-infected epithelial cells [7,10,12,18,19]. But what happens thereafter to the epithelial cell-lodged bacteria? In the gut lumen, the pathogen may break free from the dying cell and engage in additional rounds of epithelium re-infection [30,104] (Figure 5). Alternatively, the pathogen may remain trapped (in a PIT-like fashion) within the epithelial cell remnants. We have indeed observed numerous bacteria within or associated with epithelial cell debris in the gut lumen during early *Salmonella* Typhimurium infection [10]. It remains to be explored if these bacteria are really trapped and if such a fate poises them for e.g. NADPH oxidase-dependent attack by transmigrated neutrophils (Figure 5). In either case, inflammasome-promoted death (and shedding) of infected epithelial cells and recruitment plus transmigration of neutrophils co-occurs during the initial phases

of mucosal infection [10,34]. Thus, it is tempting to speculate that this represents a coordinated anti-bacterial defense.

Figure 5. Inflammasome-dependent coordination of epithelial cell death and recruitment of neutrophils during mucosal infection. Note that the possible implications of bacterial trapping within epithelial cell remnants remain speculative.

Perspectives

Murine bone-marrow-derived macrophages have been used as a golden standard experimental model for molecular inflammasome research. This stable point-of-reference has contributed tremendously to the current consensus on the salient features of inflammasome signalling [1], despite the large number of contributing laboratories. By contrast, research on epithelial responses suffers from the shear diversity of experimental models that may display both common and disparate features. Herein, we have highlighted the impact of epithelial inflammasome responses on mucosal anti-bacterial defense. However, it should be noted that the underlying mechanisms are often described in broad strokes and in some cases remain completely unknown. Furthermore, it often remains to be assessed whether a proposed response can be generalized also to other epithelial cell types and infectious agents. Do different microbes trigger distinct inflammasomes in the epithelium? Do different epithelial tissues use distinct inflammasomes to optimize defense against one and the same pathogen? And, does this lead to specifically tailored downstream responses? Consequently, a systematic cataloguing of i) the repertoire of inflammasomes expressed within distinct epithelial tissues, ii) the eliciting ligands for each epithelial inflammasome, iii) the intracellular signalling cascades that transmit the signal onwards, and iv) how such cascades may differ between epithelial cell types represents an important future undertaking.

In the initial step, epithelial inflammasome activation results in two prototypic effects - cell death and release of soluble mediators. The nature of the cell death response constitutes another key topic for future studies. There is still conflicting data on both the fundamental properties of the epithelial cell death response (e.g. lytic vs. non-lytic) and its underlying molecular wiring (e.g. caspase-4/11- vs. caspase-1-dependent). This might be explained by actual physiological differences in such a response between distinct epithelial cell types, between species, or between specific eliciting ligands and their dosage or timing. In either case, these recalcitrant issues warrant further comparative research.

We have only begun to map the effects of released soluble mediators in the infected mucosa. Interleukin-1 family cytokines, in particular IL-18, are secreted by diverse infected epithelia and, as delineated above, contribute to recruitment and activation of innate immune cells. But, besides such

cytokine(s), what other bioactive substances transmit physiologically relevant signals from the activated epithelial inflammasome? And, through what downstream mechanism(s)? Finally, multiple findings suggest that inflammasome-dependent secreted factors do not solely impact on innate immune cell recruitment and activation within the mucosa. They can additionally e.g. foster adaptive immune responses and immune homeostasis [55,56,105], promote epithelial barrier fortification, and stimulate secretion of both mucus and antimicrobial peptides [16,57]. Such effects may even impact on long-term gut lumen pathogen colonization in unexpected ways [106]. Hence, future work should also include systems level analysis of the multicellular mucosal programs sparked upon epithelial inflammasome activation.

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Figure 1

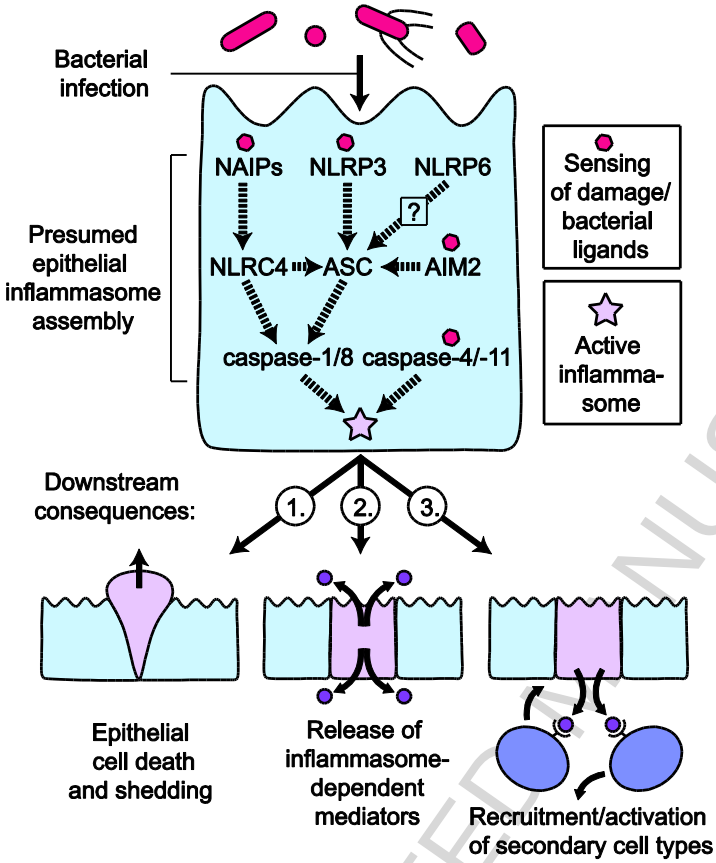


Figure 2

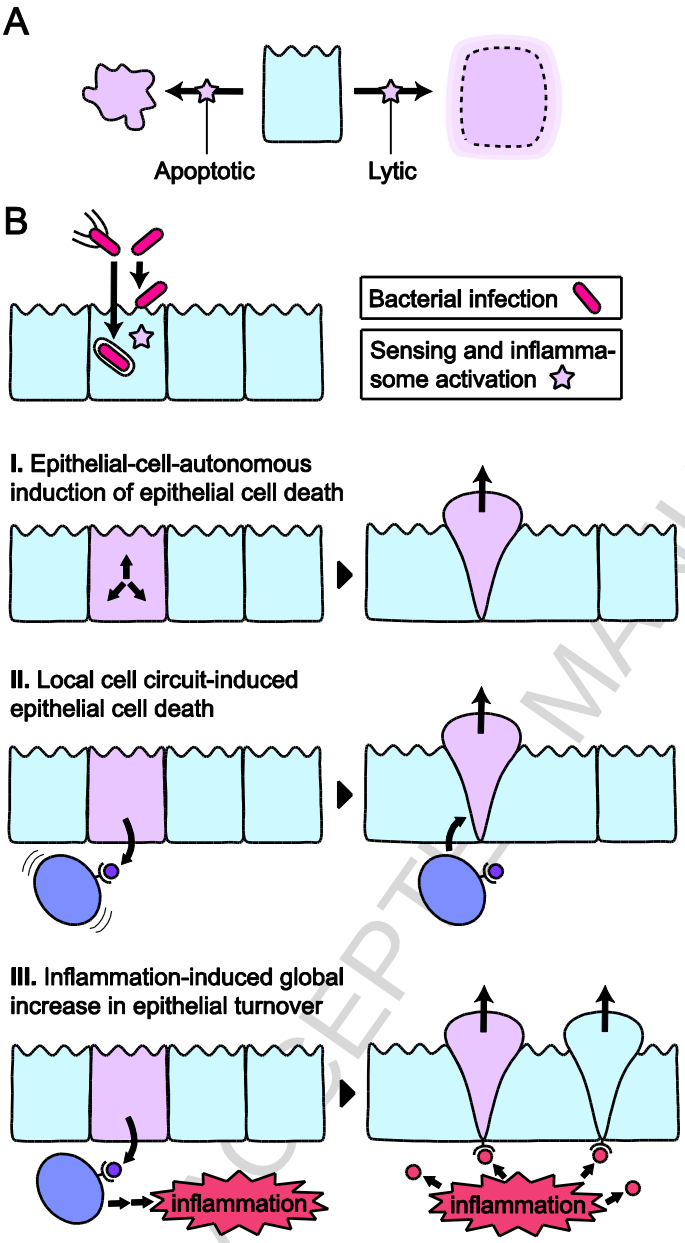
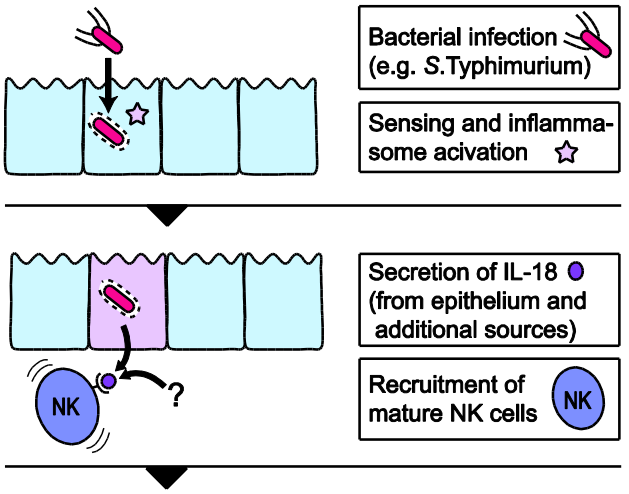
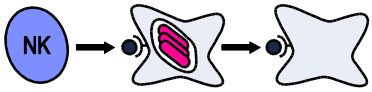


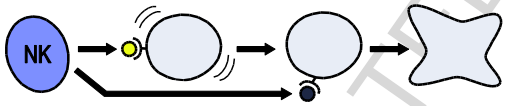
Figure 3



I. NK cell secretion of IFNγ, which promotes phagocyte clearance of bacteria and restricts systemic spread



II. NK cell-dependent control of phagocyte precursor recruitment and local differentiation



III. NK cell perforin attack on (unknown) target cells, resulting in mucosal inflammation

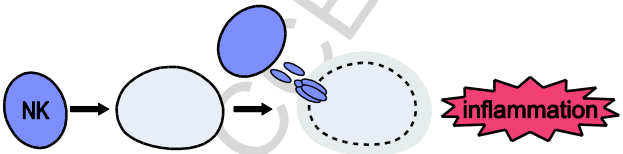


Figure 4

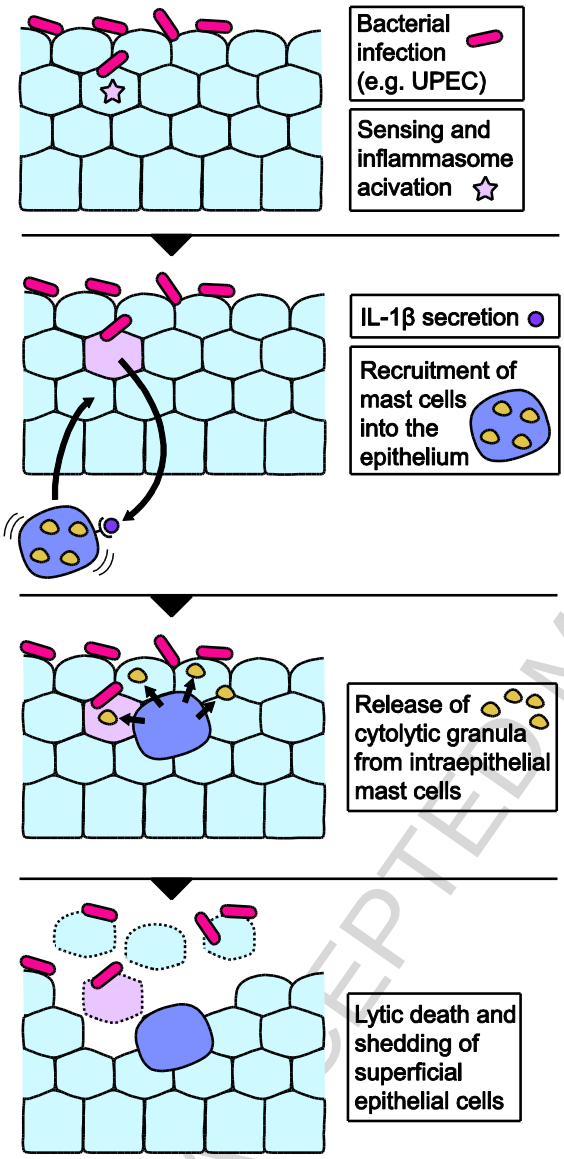
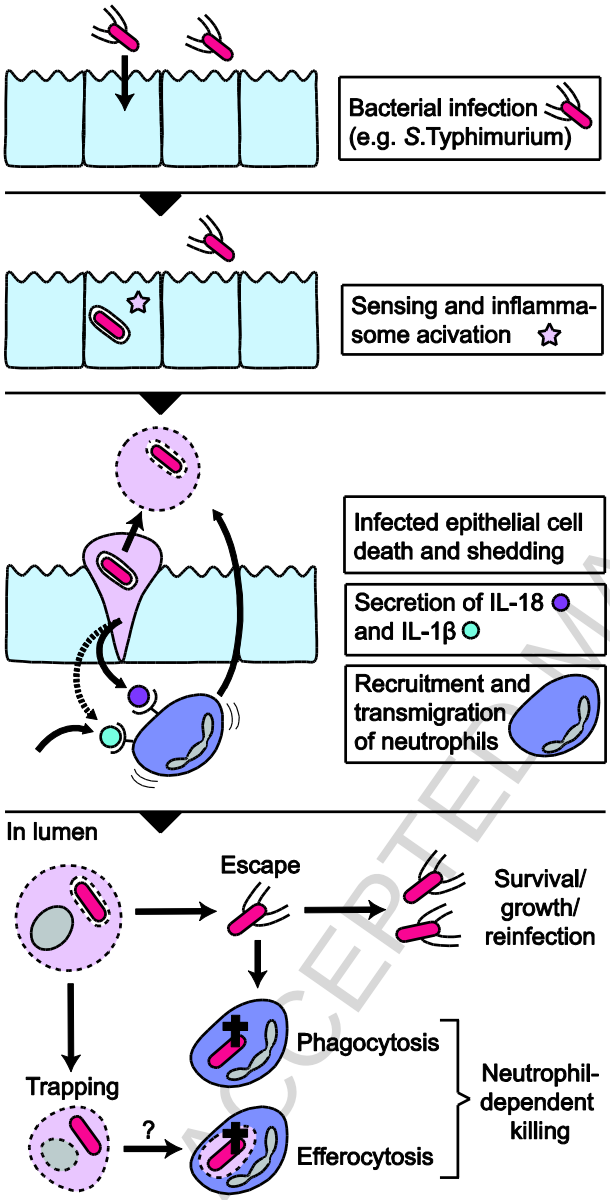
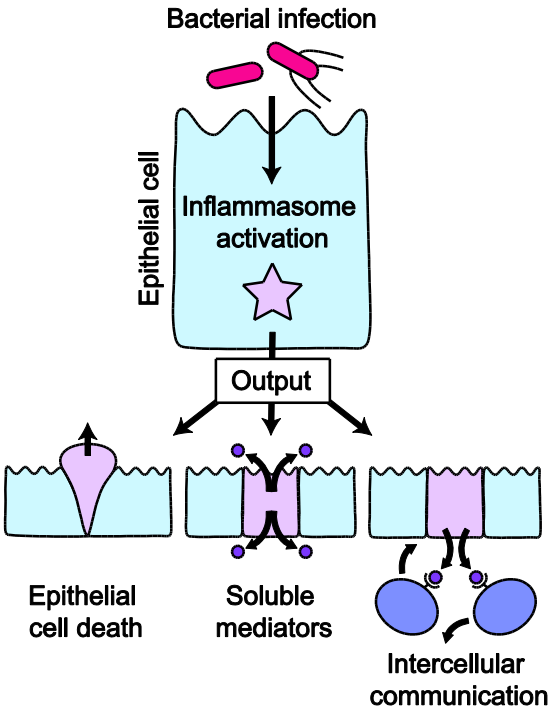


Figure 5
A





Graphical abstract

Highlights

- Epithelial tissues use inflammasome signalling to counter bacterial infection
- Epithelial inflammasome activation drives cell death and soluble mediator release
- Released IL-1β and IL-18 recruit NK cells, mast cells and PMNs into mucosae