NOD1 mediates interleukin-18 processing in epithelial cells responding to *Helicobacter pylori* infection

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Abstract

The interleukin-1 family members, IL-1 β and IL-18, are processed into their biologically active forms by multi-protein complexes, known as inflammasomes. Although the inflammasome pathways that mediate IL-1 β processing in myeloid cells have been well defined, those involved in IL-18 processing, particularly in non-myeloid cells, are still not well understood. Here, we report that the host defence molecule NOD1 regulates IL-18 processing in epithelial cells to the mucosal pathogens, *Helicobacter pylori* and *Pseudomonas aeruginosa*. We show that IL-18 is important in protecting against pre-neoplastic changes induced by gastric *H. pylori* infection *in vivo*. NOD1 mediates IL-18 processing via homotypic CARD-CARD interactions with caspase-1, and independently of canonical inflammasome proteins (NLRP3, ASC). These findings reveal an unanticipated role for NOD1 in the formation of bioactive IL-18, thereby underlining the differences in inflammasome functions between haematopoietic and non-haematopoietic cells.

Introduction

Interleukin-1 (IL-1) family cytokines (*e.g.* IL-1 α , IL-1 β , IL-18) play important roles in inflammatory disorders of the gastrointestinal tract ¹. These cytokines undergo proteolytic cleavage to become biologically active ¹. The processing of IL-1 family members IL-1 β and IL-18 is mediated by multi-protein complexes, known as “inflammasomes”, which can play both beneficial and deleterious roles in the host during infection ^{2,3}. Five proteins were reported to form “canonical inflammasomes”, with several others awaiting confirmation ². The confirmed proteins are: nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-containing (NLR) protein containing a pyrin domain 1 (NLRP1); NLRP3; NLR caspase activation and recruitment domain-containing protein 4 (NLRC4); absent in melanoma 2 (AIM2); and pyrin. These proteins act as sensors, forming molecular scaffolds for the recruitment of pro-caspase-1, leading to cytokine processing and induction of inflammatory cell death ².

According to the current model of canonical inflammasome formation, sensor proteins with a pyrin domain (PYD) respond to cellular signals by forming oligomeric structures, then bind via PYD–PYD interactions with the adaptor protein, apoptosis-associated speck-like protein (ASC) ². In turn, ASC interacts with pro-caspase-1 through homotypic caspase-activation recruitment domain (CARD) binding, resulting in cleavage of pro-caspase-1 and subsequent processing of IL-1 β /IL-18 into their biologically active forms ². Alternatively, inflammasome sensor proteins may bind directly with caspase-1 via CARD–CARD interactions to initiate cytokine processing (*e.g.* NLRC4) ². These models of canonical inflammasome activation have been developed from studies on IL-1 β responses in haematopoietic cells, particularly those of the myeloid cell lineage. In contrast, knowledge regarding inflammasome functions in non-haematopoietic cell lineages, such as epithelial cells, is much less well understood ³. It was suggested that inflammasomes in epithelial cells are more likely to be directed at IL-18 and not IL-1 β processing ³.

IL18 is expressed constitutively in the gastrointestinal tract and is upregulated in response to infection by the gastric mucosal pathogen, *Helicobacter pylori*⁴⁻⁶. The role of IL-18 in gastric *H. pylori* infection is currently unclear, with both pro-⁴⁻⁶ and anti-⁷ inflammatory activities having been assigned to this cytokine. *H. pylori* generally causes chronic gastritis, but in approximately 1% of cases, infection leads to gastric cancer⁸. According to the “Correa model” of gastric carcinogenesis, there is a histological progression from chronically inflamed stomach mucosa to increasingly severe changes in the glandular epithelium, characterised by atrophic gastritis, intestinal metaplasia and dysplasia, resulting finally in invasive carcinoma⁹. Several of these pre-neoplastic changes are observed in mouse gastric cancer models, however, these changes are more reflective of repair and regeneration rather than carcinogenesis¹⁰.

H. pylori strains that harbour a type IV secretion system (T4SS) are more frequently associated with gastric carcinogenesis than strains lacking this factor¹¹. The *H. pylori* T4SS, which is encoded by the *cag* pathogenicity island (*cagPAI*), is essential for *H. pylori* induction of nuclear factor κ B (NF- κ B)-dependent pro-inflammatory responses in human epithelial cells¹². It is now clear that *H. pylori* strains harbouring a functional T4SS can activate NF- κ B via at least three pathways¹². One of these pathways involves sensing by the NLR family member nucleotide-binding oligomerisation domain 1 (NOD1), which is expressed in gastric epithelial cells and responds to cell wall peptidoglycan fragments (muropeptides) delivered by the T4SS¹³. Furthermore, *H. pylori* and other Gram-negative bacteria can activate the NOD1 signalling pathway independently of the T4SS via the release of membrane vesicles (MVs) which carry muropeptides into the cytosol of host cells^{14,15}. Upon activation, NOD1 undergoes self-oligomerisation, leading to the exposure of its CARD and recruitment of the scaffolding kinase protein, receptor-interacting serine-threonine kinase 2 (RIPK2)¹⁶. This leads to activation of NF- κ B and mitogen-activated protein kinase (MAPK) cascades, resulting in cytokine and chemokine production^{13,14,17}.

Here, we identify a new role for NOD1 in the production of bioactive IL-18 by epithelial cells in response to *H. pylori* and another NOD1-activating mucosal bacterium, *Pseudomonas aeruginosa*¹⁸. Furthermore, we show that IL-18 plays a protective role in mouse *H. pylori* infection models and that epithelial cells are the major source of this cytokine in the stomach. Importantly, NOD1-mediated IL-18 processing is independent of the key canonical inflammasome components, NLRP3 and ASC. Instead, NOD1 interacts with caspase-1 via CARD–CARD interactions to promote the production of bioactive IL-18. Finally, we show that NOD1 maintains mucosal tissue homeostasis by regulating epithelial cell proliferation and apoptosis. We propose that NOD1-mediated formation of bioactive IL-18 prevents excessive pathology due to chronic *H. pylori* infection and may play a similar role in other mucosal infections. These findings highlight the importance of considering inflammasome functions in a cell specific-context that is relevant to the particular infection or disease.

Results

Epithelial cells are a major source of IL-18 in the gastric mucosa

IL-18 is expressed by many different tissues throughout the body, including the gastrointestinal tract, where it is produced by epithelial cells, macrophages and dendritic cells^{1,19}. In the stomach, IL-18 expression is constitutive, but is upregulated in response to infection by the pathogenic bacterium, *H. pylori*⁴⁻⁷. Given this observation, together with the fact that epithelial cells are a major target cell population for *H. pylori*-host interactions¹², we sought to investigate inflammasome-mediated processing of IL-18 in epithelial cell responses to *H. pylori* infection. It was first necessary, however, to demonstrate epithelial cells as a source of IL-18 in the stomach.

For this, we infected mice with the mouse-adapted *H. pylori* SS1 strain and then assessed the *Il18* expression levels of the gastric cell populations isolated from these animals by fluorescence-activated cell sorting (FACS). As a comparison, we also measured *Il1b* expression, because it has a very different expression profile in tissues to that of IL-18¹⁹. Consistent with studies in mice and humans⁴⁻⁷, we detected constitutive IL-18 production in mice, whereas its production was significantly increased in animals at both 1 and 8 weeks post-infection (p.i.), when compared with control animals given Brain Heart Infusion (BHI) broth alone (Fig. 1a; $p = 0.03$ and $p = 0.004$, respectively). In contrast, gastric IL-1 β production was only increased at 8 weeks p.i. (Fig. 1b; $p = 0.05$). FACS analyses of isolated gastric mucosal cells from *H. pylori*-infected mice demonstrated that *Il18* gene expression levels in gastric epithelial cells (EpCAM⁺ CD45⁻) were 22-fold higher than those of the immune cell (EpCAM⁻ CD45⁺) population, whereas *Il1b* was highly expressed in immune cells but undetectable in epithelial cells (Fig. 1c). Immunofluorescence analysis further confirmed that EpCAM⁺ cells represent an important source of IL-18 in the gastric mucosa (Fig. 1d). Hence, although both IL-18 and IL-1 β production is induced in the gastric mucosa during *H. pylori* infection, these cytokines display distinct cellular sources of origin, with IL-18 predominantly originating from epithelial cells.

Non-haematopoietic cells produce IL-18 which protects against pre-neoplastic lesions during chronic *H. pylori* infection

To further confirm the epithelial cell origin of IL-18 and also assess its role in *H. pylori* infection, we employed a bone marrow (BM) reconstitution mouse model. Consistent with the idea that epithelial cells are a major source of gastric IL-18, γ -irradiated *Il18*^{-/-} recipient mice that were reconstituted with BM cells either from *Il18*^{+/+} or *Il18*^{-/-} donor mice and infected for 5 weeks with *H. pylori* SS1 produced significantly lower levels of IL-18 than *Il18*^{+/+} recipient mice, (Fig. 2a; $p = 0.006$ and 0.004 , respectively). Similar findings were observed at 18 weeks p.i. (data not shown). Comparable levels of IL-1 β and bacterial loads were observed in both *Il18*^{+/+} and *Il18*^{-/-} recipient mice, suggesting that epithelial cell-derived IL-18 does not affect IL-1 β production nor bacterial colonisation (Fig. 2b, 2c). Other studies also reported that IL-18 was not required for protection against *H. pylori* infection in mouse challenge²⁰ and prophylactic immunisation models^{7,20}. Importantly, we observed that mice specifically lacking IL-18 in the non-haematopoietic compartment, and reconstituted with IL-18-producing BM, had increased stomach weights (Fig. 2d; $p = 0.002$), mucosal thickness (gastric hyperplasia; Fig. 2d, e) and acid mucin production

(Fig. 2f) when compared with wild-type (WT) animals. The *Il18*^{-/-} mice reconstituted with *Il18*^{+/+} BM also displayed increased stomach weights (Fig. 2d; *p* = 0.004) and mucosal thickness (Fig. 2e; *p* = 0.0001) when compared with *Il18*^{-/-} BM/*Il18*^{-/-} recipient mice. This finding suggests that IL-18-secreting haematopoietic cells may be pathogenic in mice lacking IL-18 production in the non-haematopoietic compartment.

Similar to the *Il18*^{-/-} recipient mice reconstituted with *Il18*^{-/-} BM (Fig. 2d), *Il18*^{-/-} total knockout (KO) mice exhibited significantly increased stomach weights at 2 months p.i. with *H. pylori*, as compared with *Il18*^{+/+} animals (Supplementary Fig. 1a, 1b). Despite this difference, however, similar expression levels of the inflammatory cytokine genes, *Il1b* and *Cxcl2*, were observed in the *Il18*^{+/+} and *Il18*^{-/-} mice (Supplementary Fig. 1c). Consistent with these data, *Il18*^{-/-} mice that were infected for 13 months with *Helicobacter felis*, which is more pathogenic than *H. pylori* in mice ²¹, also displayed larger stomachs than WT animals (Supplementary Fig. 1d, 1e). Taken together, our data show that non-haematopoietic cells in the stomach produce IL-18 which plays a protective role against the development of the pre-neoplastic lesions typically observed in ¹⁰ during chronic *Helicobacter* infection.

IL-18 responses to *H. pylori* infection are independent of the canonical inflammasome

We next sought to determine the inflammasome signalling molecules required for epithelial cell production of IL-18 in response to *H. pylori* infection. For this, primary gastric epithelial cells were isolated from mice deficient in the canonical inflammasome molecules, Nlrp3, Asc (encoded by the *Pycard* gene) and caspase-1. Similar to primary human gastric epithelial cells ⁶, some IL-18 was produced constitutively by primary mouse gastric epithelial cells (Fig. 3a). This may be attributed to the presence of NOD1 ligand (peptidoglycan) in the serum used for tissue culture which is able to promote low level NOD1 activation ²². Primary epithelial cells from *Casp1*^{-/-} mice, but not those from *Nlrp3*^{-/-} nor *Pycard*^{-/-} animals, produced significantly less IL-18 in response to stimulation with *H. pylori* SS1 bacteria, when compared with WT cells (Fig. 3a; *p* = 0.0004). *H. pylori* bacteria did not induce any IL-18 production in BM-derived macrophages (BMDMs) (Supplementary Fig. 2). As reported previously ²³, the bacterium induced IL-1 β responses in BMDMs (Supplementary Fig. 2). These findings underscore the differences in epithelial and myeloid cell responses to *H. pylori* infection.

Next, we performed *H. pylori* infection studies in WT, *Nlrp3*^{-/-}, *Pycard*^{-/-} and *Casp1*^{-/-} mice. Although all animal groups showed similar levels of bacterial loads at 6 months post-infection (Fig. 3b), significantly increased inflammatory scores and stomach weights were only observed in *Casp1*^{-/-} mice when compared with WT animals (Fig. 3c, 3d; *p* = 0.027 and *p* = 0.018, respectively). Moreover, we infected mice deficient in other known canonical inflammasome molecules, Nlrp1 and Nlrc4, the latter responding to bacterial flagellin and rod proteins ², but observed no significant differences in colonisation levels nor stomach pathology when compared with WT animals (Supplementary Fig. 3). Taken together, the findings suggest that the regulation and functions of IL-18 in gastric epithelial cells in response to *H. pylori* infection is dependent on caspase-1, but independent of canonical inflammasome pathways,

including the key inflammasome molecules NLRP3 and ASC, which have been implicated in responses to bacterial pathogens.

NOD1 in epithelial cells mediates IL-18 processing in response to *H. pylori* infection

To further characterise the inflammasome pathway involved in *H. pylori*-induced IL-18 processing, we used human AGS gastric epithelial cells, a standard cell line used in the *H. pylori* field and in which *IL18* mRNA⁶ and protein²⁴ are upregulated in response to *H. pylori* stimulation. Consistent with the *in vivo* data (Fig. 3), we were unable to detect the presence in AGS cells of either NLRP3 or ASC, nor other important inflammasome proteins, NLRC4 or AIM2 (Supplementary Fig. 4). These findings suggested that a non-classical inflammasome protein may be involved in *H. pylori*-induced IL-18 processing in human gastric epithelial cells.

We hypothesised that this protein may be NOD1 which plays important roles in the sensing of *H. pylori* bacteria^{13,14,17} and is strongly expressed in AGS cells (Supplementary Fig. 4d). Indeed, interfering with NOD1 signalling in AGS cells, by either short hairpin RNA (shRNA) knockdown (sh*NOD1*;^{14,25}) or CRISPR/Cas9 knockout (*NOD1* KO;¹⁷), resulted in the loss of IL-18 processing in response to live *H. pylori* bacteria (Fig. 4a, b). A similar NOD1-dependent effect was observed for IL-18 processing in response to *H. pylori* MVs, which contain peptidoglycan^{14,15}. The BHI broth, from which MVs were isolated, also induced IL-18 processing, most likely due to serum in the medium²². As reported²⁶, we also did not detect any IL-1 β production in the AGS cell line (data not shown).

We next studied the contribution of the *H. pylori* T4SS on IL-18 processing using strains lacking a functional T4SS *i.e.* Δ *cagPAI*, Δ *cagM* isogenic mutants and SS1¹³. The levels of mature IL-18 production in cell culture supernatants of control (sh*EGFP*) AGS cells were reduced in response to strains lacking a functional T4SS when compared with the respective WT (251) and parental human clinical (10700) isolates (Fig. 4b). The reduced levels of pro-IL-18 production induced in sh*NOD1* AGS cells by the T4SS-deficient strains, when compared with the WT isolates (Fig. 4a), may be attributed to the ability of the latter to activate T4SS-dependent but NOD1-independent pathways that converge on NF- κ B¹², resulting in the upregulation of pro-IL-18 production. These data show that *H. pylori* T4SS activation of the NOD1 signalling pathway is required for maximal IL-18 production and processing in human gastric epithelial cells. This result is consistent with previous findings for NOD1-dependent IL-8 production^{13,14} and interleukin-33 (IL-33) processing¹⁵ in human gastric epithelial cells. Importantly, no differences in cell death were observed between the *NOD1* KD and WT AGS cells in response to *H. pylori* stimulation (Fig. 4d), suggesting that the presence of mature IL-18 in culture supernatants was not due to cell lysis.

To further investigate the role of NOD1 in IL-18 processing to *H. pylori* infection, we next used immortalised and primary mouse gastric epithelial cells. Total levels of IL-18 production were increased in the culture supernatants of the mouse GSM06 cell line²⁷ in response to *H. pylori* SS1 stimulation, whereas it was significantly reduced when NOD1 self-oligomerisation was inhibited by pre-treatment with the NOD1-specific inhibitor, ML130²⁸ (Fig. 4a; $p = 0.0002$). ML130 had an effect on the formation of both

precursor and mature forms of IL-18 in response to *H. pylori* stimulation (Fig. 4b). Similarly, primary gastric epithelial cells from *Nod1*^{-/-} mice stimulated with *H. pylori* bacteria produced significantly lower levels of total IL-18 (Fig. 4c; $p = 0.0005$), as well as pro- and mature IL-18 (Fig. 4c, 4d), when compared with *Nod1*^{+/+} cells. Although a functional T4SS was required for *H. pylori* bacteria to induce optimal IL-18 processing in human gastric epithelial cells (Fig. 4a, b), this did not appear to be the case in epithelial cells of murine origin. We also found that Nod1 was not required for IL-18 (nor IL-1 β) production by BMDMs in response to either *H. pylori* or the canonical inflammasome activators, LPS and nigericin (Supplementary Fig. 5), again indicating that NOD1 mediates IL-18 processing in a cell-type-specific manner.

NOD1 in epithelial cells mediates IL-18 processing in response to *P. aeruginosa* infection

To investigate whether NOD1 may mediate IL-18 processing in response to other mucosal pathogens, we stimulated human A549 pulmonary epithelial cells with *P. aeruginosa*¹⁸. Total IL-18 levels were significantly decreased in *P. aeruginosa*-stimulated cells that had been transfected with either of two validated *NOD1* siRNAs, siRNA-1 and -2 (Fig. 5a; $p = 0.0004$ versus control), when compared with cells transfected with a scramble siRNA (Fig. 5b; $p = 0.002$). IL-18 processing was also affected in cells that were transfected with *NOD1* siRNA-2, but not in those transfected with siRNA-1 (Fig. 5c). This may be explained by the fact that *NOD1* siRNA-2 targets exon 6, encoding the NOD, which is required for NOD1 oligomerisation, whereas *NOD1* siRNA-1 targets exon 7, encoding a region of the LRR that is dispensable for NOD1 detection of its ligand²⁹. Taken together, these data suggest that NOD1 may play a broad role in regulating IL-18 processing in epithelial cells responding to various bacterial infections.

NOD1 mediates IL-18 processing independently of RIPK2–NF- κ B signalling

Classically, NOD1 activation results in the recruitment of the adaptor molecule RIPK2, induction of the NF- κ B signalling pathway and upregulation of pro-inflammatory cytokine production^{13,14,16,17}. We investigated the role of RIPK2 in *H. pylori*-induced IL-18 processing in epithelial cells by small interfering RNA (siRNA) transfection or blocking its activity with the kinase inhibitor, WEHI-345³⁰. siRNA-mediated KD of *RIPK2* was confirmed by qPCR detection of *RIPK2* expression and by measuring IL-8 production as a read-out for classical NOD1 signalling¹⁷. Blocking RIPK2 signalling had no effect on IL-18 processing in response to *H. pylori* stimulation in AGS cells (Fig. 6a, 6b). In agreement with this finding, IL-18 processing was unaffected in both primary gastric epithelial cells and BMDMs from *Ripk2*^{-/-} mice, whereas epithelial cell production of the NOD1-regulated chemokines, Cxcl1/keratinocyte chemoattractant (KC) and Cxcl2/macrophage inflammatory protein 2 (MIP2)^{13,15,31,32}, was reduced in response to *H. pylori* stimulation (Supplementary Fig. 6; $p = 0.0005$ and $p = 0.0002$, respectively). To demonstrate that NF- κ B activation by NOD1 is dispensable for IL-18 processing in epithelial cells, we restored NOD1 expression in sh*NOD1* AGS cells with plasmid constructs expressing either WT or a mutant form of NOD1 (K208R NOD1) that is unable to activate NF- κ B signalling³³. As expected, NOD1 sh*NOD1* AGS cells transfected with plasmid encoding WT NOD1 exhibited significantly higher IL-8

responses to *H. pylori* stimulation than cells transfected with either the K208R NOD1 mutant NOD1 or empty control plasmids (Fig. 6c; $p = 0.0004$ and $p = 0.0002$, respectively). Conversely, WT and K208R mutant NOD1 plasmids were equally able to rescue IL-18 processing in shNOD1 AGS cells (Fig. 6d), suggesting that NOD1 mediates gastric epithelial IL-18 processing in an NF- κ B independent manner. We propose that *H. pylori* induces IL-18 processing via a non-classical type of NOD1 signalling pathway.

It was recently reported that NOD1 plays a non-redundant role with the host adapter protein, tumour necrosis factor (TNF) receptor-associated factor-interacting protein with a forkhead-associated domain (TIFA), in regulating inflammatory responses by gastric epithelial cells to *H. pylori*³⁴. Consistent with this finding, we showed that transfection with TIFA siRNA significantly reduced IL-8 responses to *H. pylori* in AGS cells, when compared with scramble siRNA (Supplementary Fig. 7a, b; $p < 0.0001$). Moreover, TIFA siRNA transfection abrogated IL-18 processing in AGS cells stimulated with *H. pylori* bacteria (Supplementary Fig. 7c), thus suggesting that cross-talk between NOD1 and TIFA pathways may be important for *H. pylori*-induced IL-18 processing in epithelial cells.

NOD1 activates caspase-1 to promote IL-18 processing in epithelial cells

It is well established that caspase-1 is a key protease responsible for the cleavage of pro-IL-18 to its mature form in innate immune cells^{1,19}. Although early studies reported potential CARD–CARD interactions between NOD1 and pro-caspase-1³³, these findings have never been re-visited. Therefore, to examine whether NOD1 mediates IL-18 processing in epithelial cells via caspase-1 activation, shNOD1 AGS cells that were transiently expressing yellow fluorescent protein (YFP)-labelled NOD1 were incubated with anti-caspase-1 antibody. As can be observed (Fig. 7a), NOD1 is normally proximal to the cell membrane under basal conditions, but moved towards the cytosol and formed yellow punctate stained aggregates with endogenous caspase-1, in response to *H. pylori* stimulation (Fig. 7a). This suggested that NOD1 may associate with caspase-1 upon activation by *H. pylori*. To demonstrate NOD1-mediated activation of caspase-1, we used the fluorescently labelled inhibitor of caspase (FLICA) reagent that binds irreversibly to active caspase-1. WT (shEGFP) AGS cells exhibited enhanced staining with FLICA after stimulation with *H. pylori*, whereas staining was barely detected in shNOD1 AGS cells (Fig. 7b). Furthermore, we could only detect the mature, active form of caspase-1 (p20) in the culture supernatants of WT but not shNOD1 AGS cells in response to *H. pylori* stimulation (Fig. 7c). We also observed reduced levels of active caspase-1 in AGS cells stimulated with *H. pylori* mutant strains lacking either a functional T4SS (Δ cagPAI or Δ cagM) or defective in activating the NOD1 pathway (slt^- ; ¹³) (Fig. 7c). The data suggest that *H. pylori* activation of the NOD1 pathway contributes to caspase-1 activation in gastric epithelial cells, but that other pathway(s) may also be involved. Collectively, these data show that NOD1 sensing of *H. pylori* promotes IL-18 processing via activation of caspase-1.

NOD1 interacts with caspase-1 via CARD–CARD interactions independently of ASC

We next sought to elucidate potential NOD1-caspase-1 molecular interactions and their involvement in IL-18 processing. It is well established that NLRP3 and AIM2 need the adaptor protein, ASC, to recruit and

activate caspase-1. However, as reported above (Supplementary Fig. 4), the AGS cell line does not produce detectable levels of ASC protein, suggesting that NOD1 may directly interact with caspase-1 via homotypic CARD–CARD interactions. This hypothesis was addressed using the fluorescence lifetime imaging microscopy–Förster resonance energy transfer (FLIM-FRET) technique in live cells. For this, we transfected sh*NOD1* AGS cells with NOD1-mOrange plasmid (donor alone control) or both NOD1-mOrange and the acceptor plasmid, encoding caspase-1-GFP (Fig. 8a). We found that *H. pylori* stimulation led to a significant reduction in the lifetime of the donor fluorochrome, indicative of quenching of the donor by the acceptor caspase-1-GFP due to close proximity (< 10 nm) of NOD1 and caspase-1 proteins (Fig. 8a; $p < 0.0001$). In contrast, the lifetime of the donor remained unchanged in unstimulated cells. Reconstitution of NOD1 expression with full-length NOD1 in *NOD1* KO AGS cells resulted in maximal IL-18 processing in response to *H. pylori* (Fig. 8b). In contrast, cells transfected with CARD-deficient NOD1 exhibited background levels of IL-18 processing (Fig. 8c), which was most likely due to the effect of *H. pylori* on pro-IL-18 synthesis. Collectively, these results suggest that NOD1 sensing of *H. pylori* in epithelial cells activates caspase-1 via direct CARD–CARD interactions, resulting in the processing of IL-18.

NOD1 maintains epithelial homeostasis in response to *H. pylori* infection

As IL-18 appeared to be important for protecting tissues against excessive pathology during chronic *H. pylori* infection *in vivo* (Fig. 2, Supplementary Fig. 1), we next asked whether NOD1-mediated IL-18 secretion may play a role in regulating gastric epithelial cell survival responses. For this, we performed *in vitro* assays including MTT assay and Annexin V/propidium iodide (PI) staining to assess cell proliferation and apoptosis, respectively. sh*NOD1* KD AGS cells exhibited significantly higher levels of both cell growth and apoptosis in response to *H. pylori* stimulation, when compared with sh*EGFP* AGS cells (Fig. 9a, 9b; $p = 0.005$ and $p = 0.01$, respectively). Differences in apoptosis were not observed in cells stimulated with the apoptotic-inducing agent, etoposide (Fig. 8b). These data suggest that NOD1 is involved in regulating both epithelial cell growth and death in response to *H. pylori* stimulation. To confirm these findings, we established a 3-D gastric organoid model from *Nod1*^{+/+} and *Nod1*^{-/-} mice. *H. pylori* bacteria were added to the lumen of the organoids by microinjection (Fig. 9c). Consistent with data for AGS cells (Fig. 2a, b), *Nod1*^{-/-} organoids exhibit higher levels of cell proliferation and apoptosis, as compared with *Nod1*^{+/+} organoids (Fig. 9d, 9e; $p = 0.02$ and $p = 0.003$, respectively). Collectively, these results show that NOD1 is important for maintaining homeostasis in gastric epithelial cell turnover in response to *H. pylori* infection.

Discussion

Current understanding of canonical inflammasome functions has largely been shaped by studies in haematopoietic cells and, in particular, the processing of the pro-inflammatory cytokine, IL-1 β . In contrast, the functions of inflammasomes in non-haematopoietic cell lineages, including epithelial cells, are much

less well understood³. The inflammasome molecules AIM2, NLRP3, NLRP6 and NLR Family Apoptosis Inhibitory Protein (NAIP)/NLRC4 were reported to play important roles in epithelial cells by protecting against infection and maintaining tissue homeostasis^{3, 35-39}. An important distinction between epithelial inflammasomes and those in mononuclear phagocytes is that the former are primarily involved in IL-18 production, whereas the latter are a major source of IL-1 β ³. IL-18 was initially identified as promoting intestinal inflammation, but there is now overwhelming evidence to support the contention that this cytokine can also play protective roles against colitis^{3, 37, 40-42}. In the current study, we show that IL-18 plays a protective role against the development of pre-neoplastic changes in the stomach due to chronic *H. pylori* infection. Moreover, we identify a new type of inflammasome, involving NOD1, as being important for epithelial cell production of mature, biologically active IL-18 in response to *H. pylori* and *P. aeruginosa* infection. Interestingly, it was reported that *Chlamydomophila trachomatis* also promotes IL-1 family cytokine processing in a NOD1-dependent and NALP3/ASC-independent manner⁴³, however, that work studied IL-1 β and not IL-18 secretion. Furthermore, IL-1 β production was assessed in a specialised cell type (trophoblasts) and not epithelial cells.

Intestinal epithelial cells constitutively produce IL-18, suggesting that epithelial inflammasomes have a “ready-to-go” phenotype that allows rapid responses to pathogens and other damage to the gut^{3, 37}. In agreement with this suggestion, we (Fig. 4, 5) and others⁴⁻⁶ have established that IL-18 is expressed constitutively in the gastric mucosa, with gene expression and protein levels upregulated in response to *H. pylori* infection. Nevertheless, the role of IL-18 responses in *H. pylori* infection and associated diseases is unclear. A gene polymorphism study identified certain haplotypes as possibly being associated with susceptibility to *H. pylori* infection in the Korean population⁴⁴, but functional data for IL-18 responses were not provided. Other studies could not find any correlations between IL-18 haplotype, phenotype and clinical outcome, including gastric cancer^{4, 45}. Conversely, a correlation was found between certain IL-18 haplotypes, higher gastric IL-18 levels and more severe mononuclear cell infiltration in *H. pylori*-infected subjects⁴. Nevertheless, this does not prove causality and may simply reflect IL-18 production by the mononuclear cells recruited to the site of infection. Yamauchi *et al.* isolated mononuclear and epithelial cells from the gastric mucosa and showed that both cell types produce IL-18 in response to *H. pylori* stimulation *ex vivo*⁶, but was unable to determine the relative contributions of these cells to IL-18 production *in vivo*. Our data provide unequivocal evidence that epithelial cells are an important source of IL-18 production in the stomach during *H. pylori* infection (Fig. 1c, d; 2, a, b). Using primary and immortalised epithelial cells, we show that these cells can also process IL-18 to its mature form in response to *H. pylori* bacteria or MVs, which contain peptidoglycan (Fig. 4a-c, f, h). We suggest that in order to better understand the role of IL-18 in *H. pylori* disease, it is necessary to not only investigate the cellular source of this cytokine, but also the forms (pre-formed and mature, rather than just the total amount) that are present in the gastric tissues from *H. pylori*-infected subjects presenting with diseases of varying severity.

It has been reported by several groups that *H. pylori* induces NLRP3 inflammasome activation and IL-1 β responses in dendritic cells, neutrophils and macrophages/monocytes^{23, 46-50}. *H. pylori* was shown to

upregulate IL-18 production in these immune cells ^{7, 47–49} via an NLRP3-dependent mechanism ^{48, 49}. Similarly, we observed NLRP3-dependent regulation of IL-1 β and IL-18 responses in BMDMs (Supplementary Fig. 2), however, no role could be found for this inflammasome, nor for its adaptor ASC, in IL-18 production by primary mouse gastric epithelial cells (Fig. 3a). *H. pylori* upregulated IL-18 production and processing in the human AGS gastric epithelial line (Fig. 4e, f, 5d) which, contrary to previous reports ^{51, 52}, does not appear to express either NLRP3 or ASC (*PYCARD*) (Supplementary Fig. 4). These findings are confirmed by another study, which also reported that AGS cells do not produce ASC ⁶. RNA sequencing data also revealed very low expression levels of *NLRP3* and *PYCARD* in the AGS cell line, equivalent to 0.01 and 0.54 reads/kilobase of transcript/million mapped reads (RPKM), respectively (GEO database Accession number GSM1056529). Consistent with these data, no differences in *H. pylori* bacterial loads or resulting inflammation were observed in *Nlrp3*^{-/-} or *Pycard*^{-/-} mice, when compared with WT animals (Fig. 3b-d). One group reported increased bacterial loads and decreased gastric inflammation in *Nlrp3*^{-/-} mice ²³, whereas the complete opposite was reported in another study ⁴⁷. The reasons for the divergent results between these three studies are unclear but may stem from differences in the composition of the mouse microbiota and not to the bacterial strains used, as the latter two studies used the same strain *i. e.* *H. pylori* 10700 (PMSS1). Notwithstanding the different findings, the data presented here clearly establish that the canonical NLRP3/ASC inflammasome is not required for gastric epithelial cell production of IL-18 responses to *H. pylori* infection (Fig. 3; 4e, f).

Additionally, our data suggest that the inflammasome protein NLRC4 does not play a significant role in the infection (Fig. Supplementary Fig. 3g, h). This finding is compatible with the known inability of *H. pylori* ²³ or its flagellin ⁵³ to promote caspase-1 processing and IL-1 β secretion in macrophages. Our findings are, however, different to those of another group who recently reported that NLRC4 is important for *H. pylori* T4SS-dependent induction of IL-18 responses in gastric epithelial cells, using primary gastric epithelial cells from WT and *Nlrc4*^{-/-} mice ²⁶. The authors used the *H. pylori* PMSS1/10700 strain which, like the B128 7.13 strain used here (Supplementary Fig. 3g, h), has a functional T4SS ⁵⁴. It is important to also note that although the authors demonstrated that the MKN7 human gastric epithelial cell line produced IL-18 in response to *H. pylori*, they did not confirm a role for NLRC4 signalling in these cells by siRNA or CRISPR/Cas9 gene editing. Our work (Supplementary Fig. 4) and RNA sequencing data suggest that AGS cells express very low levels (0.58 RPKM), if any, of *NLRC4* (GEO database Accession number GSM1056529). Although it cannot be discounted that NLRC4 plays a role in the regulation of inflammation *in vivo* via epithelial production of IL-18 ²⁶, further mechanistic insights are required to understand how *H. pylori* might activate NLRC4 signalling in gastric epithelial cells.

A key and novel discovery of the current work is the observation that NOD1 interacts with caspase-1 to mediate *H. pylori* induction of IL-18 processing in epithelial cells. Interestingly, this IL-18 processing occurs independently of CARD-CARD interactions between NOD1 and its adapter molecule, RIPK2 (Fig. 5). Early studies in the field reported NOD1 interactions with caspase-1 ^{33, 55}, resulting in IL-1 β secretion in cells ⁵⁵. A caveat to those studies, however, is that they were undertaken using over-expression approaches which can be susceptible to experimental artefacts. We now provide several lines of

supportive evidence to show that NOD1 interacts with caspase-1 to mediate tissue protective IL-18 responses in epithelial cells. Firstly, we have confirmed the findings of previous investigations by performing similar over-expression experiments (Fig. 8b) and used fluorescence and FLIM-FRET imaging to show close interactions between NOD1 and caspase-1 in response to *H. pylori* stimulation (Fig. 7a, b; Fig. 8a). Secondly, we demonstrate that caspase-1 processing is itself dependent on NOD1 (Fig. 7c). Thirdly, we show that IL-18 production is abrogated in primary gastric epithelial cells from *Casp1*^{-/-} mice (Fig. 3a) and in cells pre-treated with the caspase-1 inhibitor, Z-YVAD-fmk (data not shown). Finally, *Casp1*^{-/-} mice infected with *H. pylori* reproduce aspects of the severe pathology observed in *Il18*^{-/-} animals (Fig. 3b-d) and is consistent with the enhanced inflammatory changes also observed in *Casp1*^{-/-} mice infected with *H. felis*⁷.

NLRs were originally identified for their roles in host defence and inflammation. However, more recent work in colitis models has shown that these proteins also play important roles in tissue repair and the maintenance of homeostasis. NLRs were shown to mediate these protective effects via various mechanisms, including the production of antimicrobial peptides, control of the gut microbiota^{36,38}, prevention of tumour development^{35,56} and regulation of NF-κB and MAPK signalling⁵⁷. A key downstream mediator of these tissue protective responses is IL-18^{36,38,58}. Indeed, administration of IL-18 to *Casp1*^{-/-} mice protected animals from tissue damage and death caused by dextran sulphate sodium treatment⁵⁸. We propose that IL-18 may play a similar protective role against tissue damage caused by chronic *H. pylori* infection. Our data show that NOD1 is an important regulator of bioactive IL-18 production by epithelial cells in response to the infection and, moreover, that this NLR plays a protective role in tissue homeostasis through its control of cell proliferation and apoptosis (Fig. 9). Supportive evidence for this suggestion comes from the Mongolian gerbil model in which treatment with a NOD1 agonist prior to *H. pylori* challenge was shown to suppress the development of inflammation and carcinogenesis in animals³².

In conclusion, we have identified a new NOD1 inflammasome pathway that mediates different cellular responses to those regulated by classical NOD1/RIPK2/NF-κB signalling. We also identified the existence of potential cross-talk between this NOD1 inflammasome pathway and the cytosolic adaptor protein, TIFA (Supplementary Fig. 7), which was previously reported to play a non-redundant role with NOD1 in mediating inflammatory responses by epithelial cells to *H. pylori* and *Shigella flexneri*³⁴. Similar to other NLR inflammasomes, activation of NOD1 signalling via a single stimulus (*i. e.* *H. pylori*) promotes both inflammatory and tissue repair responses in cells. These responses occur simultaneously in cells *in vitro*, but may be temporally regulated *in vivo*, particularly over long periods, such as chronic *Helicobacter* infection. This regulation may occur via the actions of CARD-containing proteins which have been shown to differentially regulate inflammatory and cell death pathways⁵⁹. Indeed, it was shown that the CARD-containing protein, CARD6, could selectively modulate NOD1/NF-κB signalling, independently of caspase-1/IL-1β secretion⁶⁰. Interrogation of the STRING database identified two protein interaction nodes for NOD1: one node comprising proteins associated with inflammatory responses (*e.g.* RIPK2) and

autophagy (e.g. ATG16L1); and the other comprising proteins, including caspase-1, involved in cell death pathways (Supplementary Fig. 8). Future studies are required to determine how NOD1 is able to regulate these different downstream signalling pathways, as well as its interactions with these different proteins. Finally, we showed that *P. aeruginosa* induces NOD1-dependent IL-18 processing in epithelial cells, suggesting that other Gram-negative bacteria that colonise mucosal surfaces may activate NOD1 signalling to promote tissue repair.

Methods

Mouse strains, cell lines and bacterial strains

Casp1^{-/-} (*Casp1*^{-/-} *Casp11*^{-/-})⁶¹, *Il18*^{-/-}⁶¹, *Nlrp1*^{-/-}⁶², *Nlrp3*^{-/-}⁶³, *Nlrc4*^{-/-}⁶⁴, *Pycard*^{-/-}⁶¹, *Ripk2*^{-/-}³⁰ and WT C57BL/6J mice were bred as litter mates at the Walter Eliza Hall Institute of Medical Research (WEHI). *Nod1*^{-/-}¹⁷, *Nod1*^{fl/fl} and *Nod1*^{fl/fl} x LysM-Cre mice (Supplementary Fig. 9) and WT mice were bred at the Animal Research Facility, Monash Medical Centre (MMC). KO mice had been backcrossed at least ten generations on the C57BL/6J background and maintained under specific pathogen-free conditions in micro-isolator cages with access to food and water *ad libitum*. Experiments were performed using age- and sex-matched animals. Mouse primary gastric epithelial cells¹⁷ and BMDMs⁶¹ were isolated, as described previously. All animal procedures complied with guidelines approved by the Animal Ethics Committees at the WEHI (2014.004, 2011.014, 2008.022) and MMC (MMCA/2015/43), respectively.

The human AGS gastric cancer cells stably expressing shRNA to either *EGFP* (sh*EGFP*) or *NOD1* (sh*NOD1*) were generated as described previously²⁵. Human *NOD1* KO and control AGS cells were generated by CRISPR Cas/9 technology and their KO status validated previously¹⁷. AGS and human A549 pulmonary epithelial cells were maintained in RPMI¹² and F-12K⁶⁵ media, respectively, supplemented with 10% (v/v) foetal calf serum (FCS), 1% (w/v) L-glutamine and 1% (w/v) penicillin/streptomycin (Gibco; ThermoFisher Scientific, VIC, Australia).

The mouse GSM06 GEC line (Riken Cell Bank RCB1779) was grown as described previously²⁷. Briefly, the cells were maintained in Dulbecco's modified Eagle medium–nutrient mixture/F-12 medium, supplemented with 10% FCS, 1% (w/v) insulin/transferrin/selenite (Gibco) and 10 ng/ml epidermal growth factor. Cells were grown in 5% CO₂ at the permissive temperature of 33°C, then moved to 37°C prior to experiments.

H. pylori strains 251 (WT, Δ *cagPAI*, Δ *cagM*,^{13,14}), 26695 (WT, *slt*⁻,¹³), 245 m3⁶⁶, B128 7.13⁵⁴ the mouse-adapted SS1 strain and its clinical progenitor isolate 10700 (also known as PMSS1)⁶⁷ were routinely grown on blood agar medium containing selective antibiotics at 37°C under microaerobic conditions²⁷. MVs were isolated from *H. pylori* bacteria grown in overnight liquid cultures grown in Brain Heart Infusion (BHI) broth, as described previously¹⁵. *P. aeruginosa* PA103 *pilA* was grown on Luria-Bertani (LB) agar, as described previously¹⁵.

Mouse *H. pylori* infection

Mice (6–8 week-old) were inoculated via oral gavage with 10^8 colony-forming units of *H. pylori* strain SS1²⁷, B128 7.13⁵⁴ or 245 m3²⁷. Bacterial viability and numbers were determined by agar plate dilution. Stomachs and spleens were harvested at 1 and 8 weeks p.i. Gastric tissues were subjected to homogenisation using a gentleMACS™ Dissociator (Miltenyi Biotec, NSW, Australia). Bacterial infection status was confirmed by bacteriological culture²⁷. Gastric homogenates were centrifuged at 13,000 x g at 4°C and the supernatants collected for analysis by the Qubit™ protein assay (ThermoFisher Scientific) and ELISA.

BM reconstitution experiments were performed by subjecting recipient mice to two 5.5-Gy doses of irradiation given 3 hr apart and then injecting 1×10^6 donor bone marrow cells via intravenous injection⁶¹. At 6 weeks post-transfer, recipient mice were infected with *H. pylori* SS1 and sacrificed at 5 and 18 weeks p.i.

Inhibitor treatment

Cells were pre-treated with ML130 (5 µM; CID-1088438; Tocris, UK)²⁸ or WEHI-345 (0, 5 or 10 µM)³⁰ for 1 hr prior to stimulation with *H. pylori*, then maintained in inhibitor-containing medium throughout the experiments.

Histological analysis

Gastric tissues were formalin-fixed and embedded in paraffin. Tissues were stained with Mayer's Haematoxylin & Eosin or Periodic acid-Schiff (PAS)-Alcian Blue (AB) stains and imaged at 20 x magnification using a bright-field microscope (Nikon). Inflammatory scores for neutrophils and lymphocytes were graded in a blinded fashion (JSP) according to a previously described grading scheme²⁷. The mucosal thickness of stomach tissues was determined in a blinded fashion (JE) on sections that had been digitised using an Aperio slide scanner (Leica Biosystems, VIC, Australia) and ImageScope software (Leica Biosystems). Three measurements were taken for each tissue section, with 3–4 sections available per stomach sample.

Immunofluorescence

Antigen retrieval was performed by immersion in 10 mM sodium citrate buffer (pH 6) and boiling for 8 min in a microwave. Sections were washed twice with PBS and immersed in blocking buffer (5% horse serum, 1% Triton X100). After 1 hr, sections were incubated overnight at 4°C with an antibody mix containing rat anti-mouse IL-18 (5 µg/ml; D047-3; R&D Systems, MN, USA) and rabbit anti-EpCAM (Clone ab71916, Abcam). An isotype control for IL-18 staining was performed using mouse IgG (Dako). Samples were washed thrice with blocking buffer and incubated for 2 hr with anti-rat Alexa Fluor® 488- and anti-rabbit Alexa Fluor® 594-conjugated secondary antibodies (ThermoFisher Scientific, 1:400). After washing, cell nuclei were stained with Hoechst 33342 (ThermoFisher Scientific; 1:1000) for 5 min before

mounting. Imaging was performed on a confocal microscope (Nikon Instruments Inc., NY, USA). All images were captured using the same settings.

Cytokine ELISA

Mouse IL-1 β (BD Biosciences, NSW, Australia), IL-18 (R&D Systems), Cxcl1/KC (R&D Systems), Cxcl2/MIP2 (R&D Systems), human IL-18 (R&D Systems) and CXCL8 (BD Biosciences) were quantified by ELISA, as per the manufacturers' instructions.

Cell sorting

Single-cell suspensions were prepared from stomach tissues of *H. pylori* infected mice. Briefly, whole stomachs were finely minced in disassociation buffer (2% FCS in Hank's Balanced Salt Solution without calcium and magnesium (Life Technologies) and 5 mM EDTA) and incubated at 37°C with shaking at 180 rpm for 30 min. Tissues were passed through 70 μ m cell strainers (Falcon, ThermoFisher Scientific) and the filtrates were collected. The remaining pieces of tissues were further digested at 37°C with shaking at 180 rpm for 45 min in 2% FCS in RPMI (Life Technologies), containing 1 mg/ml collagenase Type 1 (Life Technologies), 0.4 units Dispase (Life Technologies), and 0.01 mg/ml DNase (Roche). Samples were passed through cell strainers (70 μ m) and the filtrates combined and centrifuged at 630 x g for 10 min at RT. Cell pellets were resuspended in FACS buffer (5% FCS in PBS, Life Technologies). After adding Fc γ receptor block (1:100 dilution; BD Biosciences) and incubating on ice for 10 min, dead cells were stained with PI (0.5 μ g/ml; Life Technologies) and incubated for 30 min at 4°C with antibodies specific for the following markers: CD45.2-PE-Cy7 (1:200 dilution; BD Biosciences); EpCAM-APC (1:100 dilution; eBioscience, CA, USA). Viable CD45.2⁺ EpCAM⁻ and CD45.2⁻ EpCAM⁺ cells were sorted (FACS Aria Fusion, Beckman Coulter), verified for cell purity and the RNA isolated using Trizol (Life Technologies).

Cell co-culture assay

H. pylori bacteria were grown in BHI broth (Oxoid, ThermoFisher Scientific) supplemented with 10% (v/v) heat-inactivated foetal calf serum (ThermoFisher Scientific) and antibiotics, in a shaking incubator for 16–18 hr at 37°C under microaerobic conditions²⁷. *P. aeruginosa pilA* was grown in LB broth (Oxoid, ThermoFisher Scientific), with shaking for 16 hr, at 37°C. Bacteria were pelleted and washed twice with phosphate-buffered saline (PBS) by centrifugation at 4,000 x g for 10 min at 4°C prior to resuspension in RPMI medium for co-culture assays. Viable counts were performed by serial dilution of bacterial suspensions on blood agar or LB plates, respectively.

Cells were seeded in 12-well plates at 1 $\times$ 10⁵ cells/ml and allowed to grow overnight. The culture medium was removed and replaced with serum- and antibiotic-free media prior to stimulation with bacteria. Cells were incubated with *H. pylori* or *P. aeruginosa* at multiplicities of infection (MOI) of 10:1¹⁴ and 100:1^{18, 65}, respectively. The bacteria were removed after 1 hr. Culture supernatants and lysates were collected at 24 hr post-stimulation for ELISA and Western blot analyses. FLICA staining was performed as per the manufacturer's instructions (ImmunoChemistry Technologies, MN, USA). Cell death in AGS cells was assessed using the lactate dehydrogenase (LDH) assay after treatment with *H. pylori* or the pro-apoptotic

drug, etoposide (50 μ m). Cell proliferation was determined using the MTT assay (Promega) on adherent AGS cells, while apoptosis was determined in AGS cells detached from culture plates by the addition of 0.25% Trypsin and 1 mM EDTA for 2 min. The isolated cells were subjected to Annexin V/PI (Life Technologies) staining and analysed by Flow cytometry.

Cell transfection

Specific silencer siRNA for *TIFA* (40 nM; s40985) ¹⁵ (20 nM; s20322, siRNA1; s20324, siRNA2) ⁶⁸ were diluted in Opti-MEM medium containing Lipofectamine® 2000 or RNAiMAX (all reagents from ThermoFisher Scientific), respectively, then incubated at room temperature for 20 min. The mixtures were added drop-wise to each well of 12-well plates containing AGS (10^5) or A549 (6×10^4) cells. As a control, cells were transfected with scramble siRNA (Silencer® Select Negative control No.1 siRNA; ThermoFisher Scientific). Transfected cells were incubated at 37°C for 24 hr (*TIFA*) or 48 h (*NOD1*), prior to co-culture with bacteria. Cells were transfected with plasmids pSCFP3a-C1-NOD1 ³³, pSCFP3a-C1-NOD1 K208R ³³, Caspase-1-GFP ⁶⁹, NOD1-mOrange ⁶⁹ or NOD1-YFP (TK71; T. Kufer, unpublished), diluted in Opti-MEM medium and supplemented with 2 μ l Lipofectamine® 2000 reagent (ThermoFisher Scientific). Cells were then incubated at 37°C for 24 hr, prior to co-culture with bacteria.

FLIM-FRET analysis

AGS cells were co-transfected with caspase-1-GFP (donor) ⁷⁰ and NOD1-mOrange ⁶⁹ (acceptor) before either *H. pylori* stimulation or control. FLIM data was recorded on an Olympus FV1000 confocal microscope equipped with a PicoHarp300 FLIM extension and 485 nm pulsed diode (PicoQuant, Germany). The pixel integration time for FLIM imaging was maintained at 40 μ s/pixel and fluorescence lifetime histograms were accumulated to at least 20,000 counts to ensure sufficient data collection for FLIM-FRET analysis. Data was collected with photon count rates at 5% of the laser repetition rate to prevent pileup. FLIM-FRET analysis was performed using Symphotime 64 software (PicoQuant, Germany), with individual cells selected as regions of interest (ROIs) for analysis. In each experiment, ten random fields of view were recorded for each treatment and a minimum of five cells from each field were analysed. The fluorescence lifetime decay of each cell was deconvolved using the measured instrument response function (IRF), then fitted with a biexponential decay. The amplitude-weighted average lifetime was obtained from each fit and averaged over all cells for each condition.

Western blot analyses

Cell culture supernatants were harvested and concentrated with StrataClean resin beads (Agilent Technologies, CA, USA), as per the manufacturer's instructions. Cell lysates were prepared using NP-40 lysis buffer (ThermoFisher Scientific), supplemented with complete protease and phosphatase inhibitors (Roche). Total protein lysates (50 μ g) were resuspended in Laemmli buffer (30 μ l; ThermoFisher Scientific). All samples were heated at 98°C for 10 min, loaded onto NuPAGE® 4–12% gels and run at 120 V in 1 X MES buffer (ThermoFisher Scientific). The separated proteins were transferred onto membranes using the iBlot® transfer system (ThermoFisher Scientific), as per the manufacturer's instructions. The membranes were blocked using Odyssey® blocking buffer (Odyssey; LI-COR, NE, USA).

Membranes were incubated overnight at 4°C, with rat anti-mouse IL-18 (1 µg/ml; D047-3; R&D Systems), rabbit anti-human IL-18 (0.2 µg/ml; sc-7954; Santa Cruz Biotechnology), mouse anti-human caspase-1 (0.1 µg/ml; sc56036; Santa Cruz Biotechnology), rabbit anti-ASC (1 µg/ml; clone AL177; AdipoGen, CA, USA), rabbit anti-human NLRP3 (1 µg/ml; Clone D2P5E; Cell Signaling, CA, USA) or mouse anti-FLAG (1 µg/ml; F1804; Sigma). Membranes were washed in PBS-0.05% (v/v) Tween 20 and incubated for 2 hr with goat-anti-mouse secondary antibody-Alexa Fluor® 680 conjugate or goat anti-rabbit secondary antibody-Alexa Fluor® 800 (0.67 ng/ml; ThermoFisher Scientific). Membranes were washed and developed on the Odyssey Infrared Imaging System (LI-COR). As loading controls, lysate samples were probed with rat-anti-human Tubulin (0.9 ng/ml; Rockland, PA, USA), followed by goat-anti-rat secondary antibody-Alexa Fluor® 800 conjugate (0.33 ng/ml; ThermoFisher Scientific).

Quantitative PCR (qPCR) analyses

RNA was extracted from cells using the PureLink® RNA mini kit (ThermoFisher Scientific). cDNA was generated from 500 µg of RNA using the Tetro cDNA synthesis kit (Bioline, NSW, Australia), as per the manufacturer's instructions. cDNA (1 µl) was used per reaction, consisting of 5 X Green GoTaq Flexi Buffer (Promega), dNTP mix (0.2 mM per dNTP) and primer (1 µM). Oligonucleotide sequences were as follows: *RNA18S1*, Fwd-CGGCTACCACATCCAAGGAA and Rev-GCTGGAATTACCGCGGCT; *AIM2*, Fwd-TGAAACCCCGAAGATCAACAC and Rev-CCCAGTACTTCCATTTTCCCAG; *PYCARD*, Fwd-TCACCGCTAACGTGCTG and Rev-TGGTCTATAAAGTGCAGGCC; *NOD1*, Fwd-ACGATGAAGTGGCAGAGAGTT and Rev-GGCAGTCCCCTTAGCTGTGA; *NLRC4*, Fwd-CAGTCCCCTCACCATAGAAG and Rev-TCAAGTTACCCAAGCTGTCAG; *NLRP1*, Fwd-GGACTGACGATGACTTCTGG and Rev-ATCACAAAGCAGAGACCCG; *NLRP3*, Fwd-GTGTTTCGAATCCCACTGTG and Rev-TCTGCTTCTCAGTACTTTCTG; *RIPK2*, Fwd-GCCACCTGAAAACCTATGAACC and Rev-CTGCAAAGGATTGGTGACATC¹⁷; *TIFA*, Fwd-TCGATTCCCCTCGCTCTG and Rev-CCGTCATCTGGAGACAAGTTAC¹⁷; *Rn18s*, Fwd-GTAACCCGTTGAACCCCAT and Rev-CCATCCAATCGGTAGTAGCG; *Cxcl2*, Fwd-AACATCCAGAGCTTGAGTGTGA and Rev-TTCAGGGTCAAGGCCAACTT; *Il1b*, Fwd-ACGGACCCCAAAAGATGAAG and Rev-TTCTCCACAGCCACAATGAG; *Il18*, Fwd-GCCTCAAACCTTCAAATCAC and Rev-GTTGTCTGATTCCAGGTCTCC; *Nod1*, Fwd-GGAGGCCAACAGACGCCT and Rev-ACTGACCTAGAGGGTATCG. Denaturation was performed at 95 °C for 15 seconds, then 20 successive cycles of amplification (95°C, 15 sec; 60°C, 1 min) and extension at 72 °C for 30 sec. PCR products were run at 100 V for 20 min on 2% (w/v) agarose gels and stained with SYBR Safe DNA Gel Stain (Invitrogen; Carlsbad, California, USA). Gels were visualised in a UV trans-illuminator and imaged using Quantum-Capture software.

For qPCR, reactions consisted of synthesised cDNA (4 µl, 1:10 dilution), SYBR® Green qPCR MasterMix (5 µl; ThermoFisher Scientific) and primer (1 µl, 1 µM). Assays were performed in an Applied Biosystems™ 7900 Fast Real-Time PCR machine (ThermoFisher Scientific), using the following program: 50°C, 2 min, followed by 95°C, 10 min, then 40 successive cycles of amplification (95°C, 15 sec; 60°C, 1 min). Gene expression levels were determined by the Delta-Delta Ct method using the *18S rRNA* gene as a reference.

Organoid cultures and microinjection

Gastric organoids were generated using an adaptation of previously described methods⁷¹. Briefly, mouse stomachs were dissected, cut open along the greater curvature, and washed thoroughly with PBS. The outer muscle layer was removed using fine-tipped forceps and the forestomach discarded. The remaining tissue was cut into 2 mm pieces and washed in cold PBS before digestion with 10 mM EDTA for 4 hr at 4 °C. To isolate glands, tissue fragments were vigorously suspended in cold PBS using 10 ml pipettes and the supernatants were collected. This procedure was repeated a total of three times. Crypts were pelleted by centrifugation at 240 x g for 5 min, at 4 °C. Pellets were resuspended in PBS, passed through cell strainers (70 µm; Falcon) and centrifuged. Supernatants were discarded and the pellets containing the glands were resuspended in Matrigel (Corning). Matrigel was seeded into each well of a 24-well plate (Nunc) and incubated for 10 min at 37 °C until solidified. Crypt culture media (DMEM/F12 (Gibco), B27 (Gibco), Glutamax (Gibco), N2 (Gibco), 10 mM HEPES (Gibco), N-acetyl cysteine (1.25 µM; Sigma Aldrich), EGF (50 ng/ml; Peprotech), Noggin (100 ng/ml; Peprotech), Gastrin (10 nM; Sigma Aldrich), FGF10 (100 ng/ml; Peprotech), 10% (v/v) R-spondin 1-conditioned media, and 50% (v/v) Wnt3a-conditioned media) was added to each well. Established organoids were microinjected with 20 nl of overnight culture *H. pylori* 251 suspension (10⁷ bacteria/ml) using the IM-300 pneumatic microinjector (Narishige). The presence of bacteria in the organoids was confirmed by immunofluorescence staining with rabbit anti-*H. pylori* antibody (in-house) and anti-cadherin (5 µg/ml; clone DECMA-1, Abcam), followed by the appropriate secondary antibodies. Organoid viability was assessed using PrestoBlue reagent (Life Technologies). Cell proliferation and apoptosis in the organoids were assessed using the MTT assay and Annexin V/PI staining, respectively.

Statistical analyses

GraphPad Prism (GraphPad Software, CA, USA) was used for statistical analyses and graphical preparation. Data were analysed by the Student's T-test (unpaired, two-tailed), one-way or two-way ANOVA (Tukey's multiple comparisons test), as indicated. Differences were considered statistically significant for *p* values < 0.05.

Declarations

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Author contributions

L. S. T. designed and performed experiments, analysed data and drafted sections of the manuscript. L. Y. performed experimental work, designed experiments and analysed data. K. D'C., G. W.-M., L. L., H. C., J. E., A. De P., M. K.-L. and J. C. performed experiments. G. K. designed and performed the organoid experiments. J. D., A. M., P. A. J., D. J. P., H. E. A. and U. N. provided expertise and essential research tools. S. C. and K. E. provided expertise and analysed imaging data. C. C. A., J. F. and T. A. K. generated important research tools. J. S. P. performed histological analyses. B. A. C. and S. L. M. designed and performed experiments. R. L. F. designed and performed experiments, analysed data, conceptualised the project and drafted the manuscript.

Competing Interests

There are no competing interests.

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Figures

Figure 1

Figure 1

Epithelial cells are a major source of gastric IL-18 in response to *H. pylori* infection. a IL-18 and b IL- β levels in stomach homogenates from mice administered either BHI broth or *H. pylori* SS1 at 1 and 8 weeks p.i. c Epithelial (EpCAM⁺ CD45⁻) and immune (CD45.2⁺ EpCAM⁻) cells were isolated by FACS from the gastric tissues of *H. pylori*-infected mice and tested for Il18 and Il1b expression by qPCR. d Gastric tissue sections from *H. pylori*-infected Il18^{+/+} and Il18^{-/-} mice were reacted with rat anti-mouse IL-18 and rabbit anti-EpCAM antibodies, followed by anti-rat Alexa Fluor® 488- and anti-rabbit Alexa Fluor® 594 conjugated secondary antibodies. Mouse IgG was used as an isotype control for IL-18 staining. Cell nuclei were stained with Hoechst 33342 and the sections analysed by confocal microscopy. Scale bar = 50 μ m. a, b Data pooled from two independent experiments. Significance was determined by the Student's t-test. Data correspond to the mean $\pm$ SEM.

Figure 2

Figure 2

Non-haematopoietic cells produce IL-18 which protects against pre-neoplastic lesions in *H. pylori* infection. BM reconstitution experiments were performed by transferring BM from either II18^{+/+} or II18^{-/-} donor mice to γ-irradiated II18^{+/+} or II18^{-/-} recipient mice which were challenged with *H. pylori* SS1 then culled at 5 weeks p.i. a IL-18 and b IL-β levels in stomach homogenates; c stomach *H. pylori* bacterial loads and d weights; e-f mucosal thickness measurements (e) and images of PAS-AB-stained gastric

tissue sections (f). Arrows show acid mucins (blue). a-e Data pooled from two independent experiments. Individual points refer to either individual mice (a-d) or individual data points analysed from 2-4 sections per stomach sample (e). Significance was determined by one-way ANOVA. NS = not significant. Data correspond to the mean $\pm$ SEM.

Figure 3

H. pylori induces IL-18 production independently of the canonical inflammasome. a IL-18 production was assessed in primary gastric epithelial cells that had been isolated from WT, *Nlrp3*^{-/-}, *Pycard*^{-/-} and *Casp1*^{-/-} mice and stimulated with H. pylori (HP) SS1 bacteria or left untreated (UT). b-d WT and KO mouse strains were infected with H. pylori, sacrificed at 6 months p.i. and then their stomachs analysed

for (b) bacterial loads, inflammatory scores (c) and weights (d). Representative data for three independent experiments (a). Significance was determined by one- or two-way ANOVA (b-d and a, respectively). Data correspond to the mean $\pm$ SEM.

Figure 4

Figure 4

NOD1 is required for *H. pylori*-induced IL-18 processing in epithelial cells. a-c Pro- and mature IL-18 production in human AGS gastric epithelial cells. a AGS cells stably expressing shRNA to either EGFP

(shEGFP) or NOD1 (shNOD1) were left untreated (1), or stimulated with *H. pylori* 251WT (2), Δ cagPAI (3), cagM (4), 10700 WT (5) or SS1 WT (6) bacteria. b AGS NOD1 CRISPR/Cas9 KO (two clones) or Cas9 control (CON) cells were stimulated with *H. pylori* (HP) or left untreated (UT). c shEGFP or shNOD1 AGS cells were left untreated (1), or stimulated with BHI (2), *H. pylori* 10700 (3) or SS1 (4) MVs. Tubulin (a, b) or total protein (c) were used as loading controls. d Cell death was assessed in shEGFP or shNOD1 AGS cells, treated with *H. pylori* 251 (HP) or etoposide (ETP), and assessed relative to that in the respective untreated cells. e Mouse GSM06 gastric epithelial cells that had been pre-treated with NOD1 inhibitor (ML130; 5 μ M) or vehicle (control) were stimulated with *H. pylori* (HP) SS1 or left untreated (UT). IL-18 levels were measured in culture supernatants (e), while pro-IL-18 and mature IL-18 were detected in cell lysates (LYS) and supernatants (SUP), respectively (f). g, h Primary gastric epithelial cells from Nod1^{+/+} and Nod1^{-/-} mice were analysed for IL-18 production (g), pro-IL-18 and mature IL-18 (h). Representative data for three independent experiments. Significance was determined by one-way ANOVA (a, c). NS = not significant. Data correspond to the mean $\pm$ SEM.

Figure 5

Figure 5

NOD1 regulates *P. aeruginosa*-induced IL-18 processing in epithelial cells. a NOD1 expression was assessed in A549 human pulmonary epithelial cells which had been pre-treated with either scramble siRNA, or two species targeting NOD1 (siRNA-1, -2). b Total IL-18 levels in culture supernatants of A549 cells that had been pre-treated with siRNA then either left untreated (UT) or stimulated with *P. aeruginosa* (Pa). c Pro-IL-18 and mature IL-18 were detected in cell lysates (LYS) and supernatants (SUP). Actin was

used as a loading control. Representative data for three independent experiments. Significance was determined by one- or two-way ANOVA (a and b, respectively). NS = not significant. Data correspond to the mean $\pm$ SEM.

Figure 6

Figure 6

The NOD1 adapter protein *RIPK2* is dispensable for NOD1-dependent IL-18 processing in epithelial cells. a Human AGS gastric epithelial cells were either pre-treated with scramble or *RIPK2* siRNA then stimulated with *H. pylori* (HP) 251 or left untreated (UT). IL-18 processing was assessed in culture supernatants (SUP). b AGS cells were pre-treated with varying concentrations of the *RIPK2* inhibitor WEHI-345 then stimulated or not with *H. pylori*. c, d shNOD1 AGS cells were transfected with either a mutant form of NOD1 (K208R NOD1), WT NOD1 or the vector (control plasmid). IL-8 production (c) and IL-18 processing (d) was assessed in culture supernatants. Representative data for three independent experiments. Significance was determined by one-way ANOVA. NS = not significant. Data correspond to the mean $\pm$ SEM.

Figure 7

Figure 7

NOD1 activates caspase-1 to promote IL-18 processing in epithelial cells. a Human AGS gastric epithelial cells stably expressing shRNA to NOD1 (shNOD1) were transfected with YFP-labelled NOD1, stimulated with *H. pylori* (HP) 251 or left untreated (UT), then incubated with an anti-caspase-1 antibody. In response to *H. pylori* stimulation, NOD1 and caspase-1 molecules can be observed co-localising (arrows). Cell nuclei were stained with Hoechst 33342, the images merged and the sections analysed by confocal

microscopy. Scale = 10 μ m. b AGS cells stably expressing shRNA to either EGFP (shEGFP) or NOD1 (shNOD1) were stimulated with *H. pylori* or left untreated and then incubated with FLICA, a fluorescently labelled caspase inhibitor that binds irreversibly to caspase-1. Increased levels of fluorescence can be seen in the shEGFP cells responding to *H. pylori* stimulation (arrows). Cell nuclei were stained with Hoechst 33342 and the images merged. Scale bar = 50 μ m. c shEGFP and shNOD1 AGS cells were left untreated (UT), or stimulated with the indicated *H. pylori* WT and mutant strains. Pro- (p45) and mature (p20) forms of caspase-1 were detected by Western blotting of cell lysates (LYS) and supernatants (SUP). IL-18 processing was determined by Western blotting. Representative data for two independent experiments.

Figure 8

Figure 8

NOD1 interacts with caspase-1 via CARD–CARD interactions. **a** Human AGS gastric epithelial cells stably expressing shRNA to NOD1 (shNOD1) were transfected with NOD1-mOrange plasmid (Donor alone) or both NOD1-mOrange and caspase-1-GFP (Donor + Acceptor). Cells were then stimulated with *H. pylori* (HP) 251 or left untreated (UT) and NOD1–caspase-1 interactions determined by the FLIM-FRET technique. A decrease in the lifetime of the donor fluorochrome, indicative of quenching of the donor by

the acceptor, indicates close proximity ($< 10\text{ nm}$) between NOD1 and caspase-1 proteins. b NOD1 KO AGS cells, generated by CRISPR/Cas9 gene editing, were transfected with either full-length NOD1 (NOD1) or CARD-deficient NOD1 (Δ CARD NOD1), then stimulated with *H. pylori* or left untreated. Proteins were detected in the cell lysate (LYS) or supernatant (SUP) using anti-NOD1 or anti-IL-18 antibodies, respectively. Representative data for two independent experiments. Data were pooled from two independent experiments and significance was determined by one-way ANOVA (a). Data correspond to the mean $\pm$ SEM.

Figure 9

Figure 9

NOD1 maintains epithelial homeostasis in response to *H. pylori* infection. a, b Human AGS gastric epithelial cells stably expressing shRNA to either EGFP (shEGFP) or NOD1 (shNOD1) were stimulated with *H. pylori* (HP) 251 or left untreated (UT). Cell proliferation (a) and apoptosis (b) were determined using the MTT assay and Annexin V/PI staining, respectively. As a control, cells were treated with the apoptotic-inducing agent, etoposide (5 μ M). c-e Gastric organoids were isolated from Nod1^{+/+} and Nod1^{-/-} mice, then either left untreated (UT) or treated with *H. pylori* (HP) or etoposide. *H. pylori* bacteria (green) were microinjected into the lumen of organoids consisting of E-cadherin⁺ cells (red) (c). Cell nuclei were stained with Hoechst 33342, the images merged and the sections analysed by confocal microscopy. d, e Gastric organoid cultures from Nod1^{+/+} and Nod1^{-/-} mice were assessed for changes in cell proliferation (d) and apoptosis (e), using Presto Blue assay and Annexin V/propidium iodide (PI) staining, respectively. Representative data for two independent experiments (a-e). Significance was determined by two-way ANOVA. Data correspond to the mean $\pm$ SEM.

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