Mitochondria supply sub-lethal signals for cytokine secretion and DNA-damage in *H. pylori* infection

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The bacterium *Helicobacter pylori* induces gastric inflammation and predisposes to cancer. *H. pylori*-infected epithelial cells secrete cytokines and chemokines and undergo DNA-damage. We show that the host cell’s mitochondrial apoptosis system contributes to cytokine secretion and DNA-damage in the absence of cell death. *H. pylori* induced secretion of cytokines/chemokines from epithelial cells, dependent on the mitochondrial apoptosis machinery. A signalling step was identified in the release of mitochondrial Smac/DIABLO, which was required for alternative NF-κB-activation and contributed to chemokine secretion. The bacterial *cag*-pathogenicity island and bacterial muropeptide triggered mitochondrial host cell signals through the pattern recognition receptor NOD1. *H. pylori*-induced DNA-damage depended on mitochondrial apoptosis signals and the caspase-activated DNAse. In biopsies from *H. pylori*-positive patients, we observed a correlation of Smac-levels and inflammation. Non-apoptotic cells in these samples showed evidence of caspase-3-activation, correlating with phosphorylation of the DNA-damage response kinase ATM. Thus, *H. pylori* activates the mitochondrial apoptosis pathway to a sub-lethal level. During infection, Smac has a cytosolic, pro-inflammatory role in the absence of apoptosis. Further, DNA-damage through sub-lethal mitochondrial signals is likely to contribute to mutagenesis and cancer development.

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INTRODUCTION

*Helicobacter pylori* (*Hp*) colonizes the gastric mucus layer of approximately half of the world’s human population. In most cases *H. pylori* is transmitted in families in childhood and remains associated with its host for decades. An innate and adaptive immune response to the infection ensues, causing chronic gastritis. Infection is mostly asymptomatic but is a major risk factor for gastric and duodenal ulcers and gastric malignancies [1, 2]. *H. pylori* uses a type IV-secretion system, encoded on the 37 kb *cag*-pathogenicity island (*cag*-PAI), to interact closely with gastric epithelial cells and to deliver the cytotoxin associated antigen A (*CagA*) into the cells [3, 4]. Epithelial cells recognize *Hp* and activate a number of signalling pathways. Various pattern recognition receptors (PRR) have been implicated, and the cells secrete cytokines/chemokines [5]. This epithelial response likely contributes to the initiation of inflammation. *Hp* can induce DNA-damage, manifesting as an increased mutation rate [6, 7] but also double-strand breaks (DSBs) [8, 9]. DSBs depended on the type IV-secretion system and received a contribution from host cell factors [8, 10, 11]. DNA-damage induced by direct contact of *Hp* with gastric epithelial cells may introduce genomic mutations, contributing to cancer development.

Apoptotic cell death can contribute to protecting the replicative niche and to pathogen dissemination. Further, some pathogens can drive and inhibit more than one form of regulated cells death [12]; *Hp* has also been found to be able to induce pyroptosis in myeloid cells [13]. Many cases of apoptosis are orchestrated through the mitochondrial pathway. In this pathway, an apoptotic stimulus drives the release of the mitochondrial intermembrane space proteins cytochrome c and Smac into the cytosol, where they activate caspases. The intriguing feature of low-level activation of the apoptotic apparatus in the absence of cell death is a recent discovery [14, 15]. Mitochondrial release of cytochrome c does not have to be the point of no return. Rather, during mitochondrial pro-apoptotic signalling, small amounts of cytochrome c may be released, inducing little caspase activity. Small-scale mitochondrial outer membrane permeabilization (‘minority MOMP’ [16]) may however activate the caspase-activated DNAse (CAD), which can introduce persistent DNA-mutations [16, 17].

The apoptosis system often plays a role in infection, and many pathogens have pro- or anti-apoptotic effects, sometimes both [18]. We have recently reported that sub-lethal mitochondrial apoptosis signalling occurs during infection of epithelial cells with intracellular pathogens, from viruses to bacteria and a parasite.
Intriguingly, the mitochondrial apoptosis apparatus contributed to cytokine/chemokine secretion. The apoptosis apparatus can be very easily activated. Its low-level activation may therefore be a sensitive way to sense stimuli including infectious agents.

High numbers of Hp in cell culture induce apoptosis [20, 21] but apoptosis is not a regular feature during Hp-infection of the stomach. We therefore hypothesized that ‘physiological’, lower numbers of Hp-infection activate the apoptosis pathway in...
Cytokine/chemokine secretion is reduced in cells lacking mitochondrial apoptosis. a AGS cell lines were infected with *H. pylori* G27 strain with a multiplicity of infection (MOI) of 100 for different time periods. IL-8 in cell culture supernatant was measured by ELISA in three independent experiments. b, c AGS cell lines were infected with *H. pylori* G27 strain at a MOI of 100 for 18 h, CXCL-1 (b) and VEGF-α (c) were measured by a bead-based immunoassay and by ELISA for VEGF-α in three (b) or six (c) independent experiments. d AGS cell lines were infected with *H. pylori* clinical isolates 902, 903 at an MOI of 100 for 18 h. IL-8 and CXCL-1 were measured by ELISA in at least three independent experiments. The values of detection limit were used for statistical analysis if the measured chemokines were under detection limit. One data point was removed after Grubbs outlier testing. e, f HeLa cell lines were infected with *H. pylori* G27 (e) or T26695 (f) strains at an MOI of 100 for 24 h. IL-8 (e, f) and CXCL-1 (e) were measured by ELISA in eight (e), seven (e) and five (f) independent experiments. One data point was removed (e) after Grubbs outlier testing. All infected samples were normalized to CTRL infected. Not normalized data are shown in SBA-B. g AGS cell lines were infected with *H. pylori* G27 strain at an MOI of 100 for 18 h. The cell culture supernatant was passed through a 0.2 µm filter and co-incubated with human neutrophils for 24 h. Neutrophil survival was determined by AnnexinV/Live-Dead staining. (Data are from three individual donors, with supernatants from three independent infection experiments tested for each donor). h AGS cell lines were infected with *H. pylori* G27 strain at an MOI of 100 for 18 h. The cell culture supernatant was passed through a 0.2 µm filter and co-incubated with human neutrophils derived from a healthy donor. Neutrophil supernatants were harvested after 24 h and assayed for IL-8 by ELISA. Neutrophil IL-8 secretion was calculated as the difference between total IL-8 amounts in supernatants after co-incubation and IL-8 amounts of AGS supernatants prior co-incubation; all secretion data are shown in Fig. S8C (data are from supernatants derived from three independent infection experiments). Lines connect data point from the same individual experiments. i HeLa cell lines were infected with *H. pylori* G27 strain at an MOI of 100 for 20 h. The cell culture supernatant was passed through a 0.2 µm filter and co-incubated with human neutrophils for 24 h. Neutrophil survival was determined by AnnexinV/PI staining. (Data are from supernatants derived from three independent infection experiments). j HeLa cell lines were infected with *H. pylori* G27 strain at an MOI of 100 for 20 h. The cell culture supernatant was passed through a 0.2 µm filter. Human neutrophil chemotaxis was measured in a transwell migration assay (data are from six independent experiments). Lines demonstrate connected individual experiments. Data information: Bars represent the mean and dots the value of independent experiments. Hollow dots represent detection limit. Error bars show standard error of mean. Ns: p > 0.05, *, p < 0.05, **, ***, p < 0.001, ****, p < 0.0001. The significance were tested by parametric two-Way ANOVA with Dunnett’s post hoc testing (a, d: IL-8), parametric one-Way ANOVA with Sidak’s post hoc testing (b, c: CXCL-1), one sample T-Test (e, f), unpaired T-Test (g, h, i, j), CTRL, non-targeting control gRNA; Bax−/−Bak−/−, double knockout of Bax and Bak by CRISPR/Cas9; Bcl-XL tg, overexpressing Bcl-XL; CAD−/−, depletion of CAD by CRISPR/Cas9.

**RESULTS**

Inactivation of mitochondrial apoptosis reduces the cytokine response during *Hp*-infection of epithelial cells

Mitochondrial apoptosis occurs through the activity of Bcl-2-family members. Anti-apoptotic Bcl-2 proteins such as Bcl-Xl block apoptosis by binding to the pro-apoptotic members, including the trigger molecules (BH3-only proteins) and the two effectors, Bax and Bak [22]. To test for a potential contribution of the mitochondrial apoptosis system to *Hp*-induced inflammation, we first infected AGS gastric carcinoma cells with *Hp*. Infected cells secreted increasing amounts of IL-8 over time, and this was consistently reduced in Bax/Bak-deficient cells (Fig. 1a). A screen for soluble products secreted by AGS cells upon *Hp*-infection we also detected CXCL1 and VEGFα (not shown; notably, the inflammasome products IL1β and IL-18 were not detected), and secretion of both was reduced from AGS cells lacking Bax and Bak (Fig. 1b, c; the confirmation of gene-modified cells made in this study is shown in Fig. S1). Chemokine-induction by a clinical *Hp*-isolate was also reduced (Fig. 1d), as was secretion from HeLa cells lacking Bax and Bak or overexpressing Bcl-Xl (Fig. 1e; for some experiments, the results were normalized. The original data from these experiments are all shown in Fig. S8). The same was seen for IL-8 with a second laboratory strain (Fig. 1f). IL-8-secretion from PMA-treated mutant cells was unaltered (Fig. S1o–q).

Supernatants from *Hp*-infected AGS cells increased neutrophil survival but to a smaller extent when supernatants were from Bax/Bak-deficient cells (Fig. 1g). Neutrophils secreted high amounts of IL-8, and this was reduced in supernatants from Bax/Bak-deficient AGS cells (Fig. 1h). Neutrophil survival in supernatants from *Hp*-infected HeLa cells showed the same pattern (Fig. 1i), and supernatants from Bax/Bak-deficient HeLa cells induced less neutrophil migration than supernatants from control cells (Fig. 1j). Thus, the mitochondrial apoptosis machinery contributes to chemokine secretion and inflammatory activity in *Hp*-infected epithelial cells.

**Hp-infection induces sub-lethal caspase-activation**

High MOI of *Hp* have been reported to activate caspase-3 [21]. Effector caspase activity during sub-lethal signalling may be too low to be detectable by standard assays [16, 19]. No caspase-3 activation was observed by flow cytometry in *Hp*-infected AGS or KATOIII cells (Fig. 2a, S2A). No cytotoxicity (LDH-release and Trypan blue uptake) was seen up to 24 h (Fig. 2b, Fig. S2B). Infection up to MOI = 30 did not reduce long-term colony formation in AGS cells. At MOI = 100, there was some reduction, possibly because of the effect of *Hp* on the cytoskeleton [4]. This was however Bax/Bak independent (Fig. 2c) and therefore independent of mitochondrial apoptosis.

Biotinylated caspase-inhibitory peptide (bio-VAD) was however able to precipitate active caspase-3 from lysates of infected AGS cells (Fig. 2d; staurosporine was used as a positive control). Fluorogenic enzyme assay further showed a moderate but significant activation of effector caspases in lysates from *Hp*-infected AGS cells (Fig. 2e), which was blocked by the caspase-inhibitor Q-VD-OPH (Fig. S2B). A second gastric cell line, KATOIII, was more sensitive to *Hp*-induced apoptosis: caspase-3-positive cells were detectable upon infection at an MOI of 30 (Fig. 2f), with a trend to small amounts of DEVD-cleaving activity at MOI = 10 (Fig. 2g), where no caspase-3-positive cells were detected (Fig. 2f) and other measurements of cell death were negative (Fig. 2h, i). In *Hp*-infected HeLa cells, no active effector caspase was detectable using a reporter line [19] (Fig. S2A, C), and no signal was obtained by enzyme assay (Fig. S2D). No active effector caspases were precipitated from these cells (Fig. S2E), and no cytotoxicity was observed (Fig. S2F). A trend to a higher number of HeLa cells with reduced mitochondrial membrane potential was seen (Fig. S2G). A time course over 48 h of infection of AGS control and Bax/Bak-deficient cells with *Hp* and the read-outs of Trypan blue uptake, LDH-release and DEVD-cleaving activity is shown in Fig. S3.

Low-level, sub-lethal caspase-activation can thus be measured in *Hp*-infected AGS and KATOIII but not HeLa cells in conditions where the mitochondrial apoptosis apparatus contributed to cytokine secretion. Thus, *Hp*-infection can generate a sub-lethal signal in the apoptosis pathway.
Hp-infection induces release of Smac
Mitochondrial cytochrome c-release is required for caspase-activation, so small amounts of cytochrome c were likely to be released in the conditions here. Smac is released during (full) apoptosis concomitant with cytochrome c-release [23]; it therefore seemed possible that it is also released upon the Hp-dependent, sub-lethal signals. Smac is a mitochondrial protein but all known molecular functions of Smac are in the cytosol. Cytosolic Smac binds the X-linked inhibitor of apoptosis protein (XIAP), releasing its caspase-inhibitory function. Cytosolic Smac further inactivates cIAP1/2 [24–26]. Small-molecule Smac-
mimetics have pro-inflammatory activity, primarily through the activation of alternative NF-κB: inactivation of cIAP1/2 by Smac and Smac-mimetics increases the levels of NIK and triggers alternative NF-κB, detectable as its active form, p52 [26, 27].

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deficient AGS cells (Fig. 3a–c). By immunostaining, mitochondria of Hp-infected wt but not Bax/Bak-deficient cells showed cytochrome c but little Smac-fluorescence, indicating preferential loss of mitochondrial Smac (Fig. 3d, S4A). We further measured the loss of Smac and cytochrome c in AGS cells single-Bax or -Bak-deficient by microscopy. As shown in Fig. 4B, there was a trend towards a loss of both Smac and cytochrome c in wt cells and in cells lacking Bax but not AGS cells lacking Bak, suggesting it is Bak-activation that drives this loss. Subcellular fractionation showed detectable release of both Smac and cytochrome c into the cytosol of Hp-infected cells (Fig. 3e). A similar effect was observed by measuring total immunofluorescence in KATOIII cells; a trend to Bax/Bak-independent reduction in cytochrome c-fluorescence was also noted in these cells (Fig. 3f, S4C). In HeLa cells, we observed mitochondrial loss of Smac-GFP but retention of cytochrome c during Hp-infection (Fig. 4G) and found Bax/Bak-dependent loss of Smac and Bak/Bak-independent decrease in cytochrome c-levels (Fig. 4H). There was no detectable loss of mitochondrial membrane potential or mitochondrial mass during Hp-infection of AGS or KATOIII cells (Fig. S4D, E).

The results strongly suggest that Smac is released due to the sub-lethal action of Bax/Bak upon Hp-infection and is degraded in the cytosol. Cytosolic Smac is degraded by the proteasome [28]. During apoptosis, caspases counter-regulate degradation [23], probably through proteasome-inhibition [29]. We reproduced this effect: Smac was released from mitochondria by staurosporine treatment and Smac-GFP fluorescence was not detectable in the presence of a caspase inhibitor (Fig. S4I). The low activity of caspases induced by Hp is likely insufficient for proteasomal degradation, and therefore cytosolic Smac is degraded. Proteasome inhibition protected Smac from degradation (Fig. S4J). These results indicate that Hp-infection induces Bax/Bak-dependent, non-apoptotic mitochondrial Smac-release.

Smac triggers the activation of NF-kB p100

When experimentally expressed in the cytosol, Smac stimulates alternative NF-kB, as do small molecule Smac-mimetics [26, 30]. We hypothesized that Smac, released from mitochondria during sub-lethal apoptosis signalling, has the same activity. The activation of alternative NF-kB during Hp-infection (processing of p100 to p52), has been described in vitro, as has its role in gene-deficient mice in vivo [31, 32]. We confirmed NF-kB p100-processing (Fig. 4a). Processing was not seen in Bax/Bak-deficient AGS (Fig. 4a) or HeLa (Fig. S5A) cells. Deletion of Smac reduced p100-processing in AGS (Fig. 4b, S5B), KATOIII (Fig. 4c) and HeLa cells (Fig. S5A; normal p100-processing in response to Smac-mimetic in Bax/Bak and Smac-mutant AGS cells was confirmed, Fig. S1M, N). Phosphorylation of NF-kB p65, i.e. classical NF-kB-signalling, was unaltered (Fig. S5C). We further performed reporter assays in HeLa cells. An NFkB-reporter construct was introduced into control, Bax/Bak-deficient and Smac-deficient cells. These cells showed induction of the reporter upon infection with Hp, and this induction was smaller in Bax/Bak and in Smac-deficient cells (Fig. S5D).

Smac-mimetic-signalling involves the inhibition of cIAP1/2, activating NIK and IKKα [26]. As predicted, KATOIII cells lacking Ikkα showed no p100-processing (Fig. 4c). Hp-infection reduced cIAP1 but not XIAP-levels, depending on Smac in KATOIII cells (Fig. 4d, S5E). There was no clear difference in cIAP2-levels in KATOIII cells, and no reproducible loss of cIAP1/2 in AGS or HeLa cells (not shown). In AGS cells infected with the G27-strain, a contribution of Smac to IL-8-secretion was seen at an early time point (6 h, Fig. 4e) but not later on (18 h; not shown). Restoring Smac-expression in the Smac-deficient AGS cells also restored IL-8-secretion (Fig. 4f). Upon infection with the clinical isolate, chemokine secretion at 18 h also required a Smac-contribution (Fig. 4g). In HeLa cells, Smac-deficiency reduced secretion of IL-8 and CXCL1 (Fig. 4h) as well as migration-inducing capacity towards neutrophils (Fig. 4i). Thus, Hp causes the Bax/Bak-dependent release of Smac, which provides a major part of the p100-processing signal, and which contributes to chemokine secretion and neutrophil attraction.

Hp-induced DNA-damage is due to sub-lethal apoptosis signalling

Hp-infection can cause DNA-strand breaks, which may contribute to malignant transformation [8]. Sub-lethal apoptosis signalling can activate the caspase-activated DNase (CAD), causing DNA-damage and inducing permanent mutations [16]. CAD is activated by the caspase-mediated cleavage of its inhibitor ICAD [33]. Activation of CAD has been described in the published situations of sub-lethal apoptosis signalling, and the CAD-dependent DNA-damage response, detectable as the phosphorylation of the histone H2AX (γH2AX), is a very sensitive way to detect sub-lethal apoptosis signalling [16, 19].

We hypothesized that sub-lethal apoptosis signalling may contribute to the DNA-damage during Hp-infection. As reported, there was a clear γH2AX response in Hp-infected AGS cells. This response was almost abrogated in CAD-deficient cells (Fig. 5a, S6A,B; two bacterial strains were used). The γH2AX-response was also reduced in Bax/Bak-deficient AGS cells (Fig. 5a, S6A). To test for actual DNA-damage, we scored infected AGS cells for CHK1-activation, which can form when a cell with damaged genomic DNA goes through mitosis [34, 35]. Hp-infection of AGS cells caused the CAD-dependent formation of micronuclei (Fig. 5b). The γH2AX-signal was abolished by the deletion of CAD in KATOIII
(Fig. 5c) and HeLa cells (Fig. 5d, e) and not detectable in HeLa cells when caspase activity was inhibited or mitochondrial apoptosis had been disabled (Bax/Bak-deficiency or Bcl-X₇-overexpression, Fig. 5e, S6C,D). The γH2AX-signal was not seen in cells deficient in caspase-9, while individual caspase-3- or caspase-7-deficiency did not block the signal (Fig. 5f). Thus, Hp-infection induces DNA-damage and a DNA-damage-response through the sub-lethal activation of the mitochondrial apoptosis apparatus and CAD.
Both caspase-3 and -7 appear to be able to activate CAD downstream of caspase-9.

**Upstream signals in the engagement of mitochondria**

Our results show that Hp-recognition by epithelial cells generates a signal that triggers the release of mitochondrial Smac. To approach the question of the upstream signals, we analysed bacterial factors and host cell receptor candidates. We infected AGS or HeLa cells with Hp-mutants lacking CagA, the Cag-pathogenicity island (PAI) or the adhesin protein BbA. As has been reported, the appearance of yH2AX and the activation of alternative NF-κB depended on the PAI and BbA but not CagA (Fig. S7A–D) [8, 31, 36]. Two receptor systems can mediate the PAI-dependent recognition of Hp in human cells. The TIFA-signalling axis acts as a recognition machinery of the LPS biosynthesis precursor, peptidoglycan fragments (muropeptides) [39].

Loss of Smac upon Hp-infection (Fig. 6a, S7E, G, H) and yH2AX-induction (Fig. S7G) were unaltered in TIFA-deficient cells (as a control in HeLa cells, Smac-loss is also shown, Fig. 6a, S7E). We used a chemical inhibitor of NOD1, ML-130 [40]. This inhibitor blocked release (Fig. 6a, S7E) and loss of Smac (Fig. 6b, c), as well as the activation of NF-κB p100 (Fig. 6b, c) and the yH2AX-signal in AGS cells (Fig. 6b, c). The NOD1-inhibitor reduced p100-upregulation, the appearance of p52 and Smac-loss also in HeLa cells (Fig. S7F). Genomic deletion of the NOD1-gene further reduced IL-8 secretion (Fig. S4F) and the yH2AX-DNA-damage response in AGS cells (Fig. 6d).

The signaling pathway upstream of mitochondria therefore appears to originate from NOD1 while TIFA, which is required for the activation of classical NF-κB upon Hp-infection, does not partake in mitochondrial signaling. The ligands of NOD1 are Hp peptidoglycan-fragments (muropeptides). While it has not been possible to generate a peptidoglycan-deficient strain of Hp, a strain deficient in lytic transglycosylase activity, which has a defect in the release of the NOD1-binding muropeptides, has been found to be less active in NOD1-dependent induction of IL-8 in AGS cells [39]. This strain had lost the ability to generate a yH2AX-signal upon infection of AGS cells (Fig. 6e), consistent with the interpretation that NOD1 is required to drive this signalling.

**Evidence of sub-lethal apoptosis signaling in Hp-patients**

We analyzed biopsies from a cohort of Hp-positive gastritis patients. Most patients showed histological evidence of gastritis (Sydney score for acute inflammation of 1–2, Fig. 7a, d, for chronic inflammation 1–3, Fig. 7e). In a substantial number of samples we detected epithelial cells in the gastric glands that gave a clear signal when stained with an antibody recognizing active caspase-3. The frequency of positive cells varied (mostly in the range of 5–50%); typically, large fractions of the cells in the neck region of the gastric glands, where Hp is commonly seen, were positive (Fig. 7a). Hardly any apoptoses were observed (under 1% of cells). No correlation between caspase-3-activation and inflammatory score was observed (Fig. 7b).

Parietal cells gave a generally stronger Smac-signal, and the corpus (more parietal cells) was more strongly positive than the antrum (Fig. 7c). Intriguingly, the overall proportion of Smac-positive cells inversely correlated with acute and chronic inflammatory scores, with a similar trend for acute inflammation in the antrum and chronic inflammation in the body separately (Fig. 7d, e). This is reminiscent of the Smac content of cell lines, where Hp –infection reduced Smac. Biopsies from patients without (known) acute gastric inflammation, who had undergone gastric resection during bariatric surgery, were also stained and showed low levels of caspase-3-positive cells (Fig. 7b). In vitro, the most sensitive parameter of sub-lethal signals in the apoptosis pathway has been the DNA-damage response. We tested for DNA-damage response in these patient samples, using phosphorylation of ATM-kinase as a read-out. The signal for pATM showed a clear correlation with the level of cells expressing active caspase-3 (Fig. 7f), consistent with the model where Hp activates caspase-3 to a sub-lethal level of activity, causing DNA-damage and a DNA-damage response.

**DISCUSSION**

It is clear now that the mitochondrial apoptosis pathway can be triggered to sub-lethal activity but potential physiological roles and their pathways need to be worked out. This study shows that the pathway is triggered by a PRR during infection with a common bacterium, which causes chronic and oncogenic inflammation. It identifies a signalling role for Smac: Smac can be released in non-apoptotic cells and assume the pro-inflammatory role previously identified for Smac-mimetics. The results further suggest that sub-lethal mitochondrial apoptosis signals contribute to the development of infection-associated cancer.

We detected release of small amounts of both cytochrome c and Smac upon Hp-infection, and the data suggest that Smac was preferentially released. How such preferential release of Smac is...
achieved is unclear: during apoptosis, Smac-release occurs alongside the release of cytochrome c [23]. However, loss of the mitochondrial fission protein Drp1 reduced the release of cytochrome c but not Smac [41], and the apoptotic release of cytochrome c itself is far from clear [42]. Release of Smac has been reported in human cells infected with Shigella bacteria, where it has been suggested to block anti-bacterial immunity through interference with XIAP [43].
Most information of a pro-inflammatory role of Smac comes from investigations of Smac-mimetics, with some studies of experimental overexpression of Smac. In these studies, a dramatic cIAP-downregulation has regularly been observed (see for example [26]). We observed a relatively small loss of cIAP1 only in one of the cell lines we used, although deletion and reconstitution experiments clearly identified a role of Smac in IL-8-secretion upon H. pylori infection. It seems conceivable that the generation of a signal activating alternative NF-kB through Smac may occur in the absence of obvious loss of cIAPs, especially if classical NF-kB is activated at the same time. Indeed, it may be the case that the massive loss of these proteins that are seen during Smac-mimetic stimulation is not necessarily what occurs during perhaps physiological, small stimuli. Further, cIAP-levels may be concurrently up-regulated by other signalling pathways, such as canonical NF-kB [44] or, as recently shown for cIAP2 and Hp-infection, through Brd4 [45].

Our data suggest a model where peptidoglycan, whose delivery required PRR but not CagA, as well as bacterial adhesion, stimulate NOD1 as the most upstream sensor. It has been reported earlier that ABC transporters, for example TIFA (CSB-606866), or other PRR, can be involved in a role of Smac in the IL-1β-induced NF-kB activation [46]. How Hp regulates its activities in the human stomach during long-term infection is an intriguing question, and how this is linked to the induction of sub-lethal signals in the mitochondrial apoptosis pathway will need careful future investigation.

It may be surprising that a PRR, a receptor from a class much better known to activate NF-kB and interferon responses, triggers the mitochondrial apoptosis apparatus. However, there are many examples where in experimental situations PRR can cause apoptotic cell death. This has been found for NOD1 itself [47], examples where in experimental situations PRR can cause mitochondrial apoptosis. However, there are many examples where in experimental situations PRR can cause mitochondrial apoptosis. However, there are many examples where in experimental situations PRR can cause mitochondrial apoptosis. However, there are many examples where in experimental situations PRR can cause mitochondrial apoptosis. However, there are many examples where in experimental situations PRR can cause mitochondrial apoptosis. However, there are many examples where in experimental situations PRR can cause mitochondrial apoptosis. However, there are many examples where in experimental situations PRR can cause mitochondrial apoptosis.
Signaling #14334). Secondary antibodies: anti mouse IgG-Cy5 (715-175-151, Dianova), donkey anti rabbit IgG-Alexa Fluor647 (711-605-152, Dianova), anti rabbit IgG-Alexa488 (711-545-152, Dianova), anti mouse Ig-HRP (115-035-166, Dianova), anti rabbit-HRP (A6667, Sigma).

Reagents
DAPI, etoposide, Hoechst, PMA, staurosporine (Sigma); Fugene (Promega), LCL161 (Active Biochem), Mito Tracker Deep Red (Life Technologies), ML-130 (Tocris), Z-VAD-fmk (Gentaur) were used as indicated.
**Fig. 6  NOD1 activated by H. pylori induces low-level activation of the apoptosis apparatus.** a HeLa cell lines were infected with *H. pylori* G27 strain at an MOI of 100 for 18 h. The NOD1-inhibitor ML-130 was added to CTRL cells at the same time as the *H. pylori* infection. Endogenous levels of Smac (green) and cytochrome c (red) were detected by immunofluorescence. Shown are representative pictures and quantification of five independent experiments with at least 640 cells per condition. Two outliers were removed after Grubbs outlier testing. Scale bars represent 50 µm. A larger magnification and individual fluorescence channels are shown in Fig. S7E. b, c AGS cell lines were infected with *H. pylori* G27 at an MOI of 100 for 18 h. Various concentrations of the NOD1-inhibitor ML-130 were added at the same time as the *H. pylori* infection. Processing of NF-κB p100 to p52, endogenous level of Smac in whole cell lysates and the DNA-damage response by appearance of a γH2AX-signal were measured by Western blotting. Shown is a representative Western blot (b) and quantification (c) of five individual experiments. Not normalized data are shown in S8I. d AGS CTRL or NOD1−/− cells were infected with *H. pylori* G27 at an MOI of 100 for 24 h. The DNA-damage response by appearance of a γH2AX-signal was measured by Western blotting. Shown is one Western blot representative of two independent experiments. e AGS cell lines were infected at an MOI of 100 using a *H. pylori* T26695 deletion strains in lytic transglycosylase activity or wild type strain for 18 h. The DNA-damage response was measured as γH2AX-signal by Western blotting. Shown is a representative Western blot and quantification of three independent experiments. Data information: Bars represent the mean and dots the value of independent experiments. Error bars show standard error of mean. Ns: p > 0.05; ** p < 0.01; *** p < 0.001. Significance was tested by two-way ANOVA (a), one-way ANOVA (c, e; Dunnett’s post hoc test; c: Sidak’s post hoc test) or one sample T-Test (c). CTRL, non-targeting control; NOD1−/−, deletion of NOD1 by CRISPR/Cas9; slt, T26695 deletion strains in lytic transglycosylase activity; WT, *H. pylori* T26695 wild typ; ML-130, NOD1 inhibitor.

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**a** Active caspase3

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**b**

![Sydney Score for acute inflammation](Image)

- **antrum + body**
  - *H.p. patients*
  - *controls*
  - p=0.4548

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**c**

- Smac
  - body
  - antrum

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**d**

![](Image)

- **antrum + body**
  - H.p. patients
  - controls
  - p=0.0413

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**e**

- **body**
  - Sydney Score for chronic inflammation
  - p=0.0087

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**f**

- pATM
  - 100x
  - 400x
RIPA buffer. Laemmli buffer was added and beads were boiled, followed by beads (Thermo Fischer) at 4 °C overnight. The beads were washed with 95 °C in Laemmli buffer. Supernatants were incubated with neutravidin harvesting. Cells were lysed with RIPA buffer. Aliquots were boiled at caspases, biotinylated VAD-fmk (Santa Cruz) was added 3 h before DEVD-AMC (Bachem), 100 µg/ml BSA, 0.1% CHAPS) in triplicates. Analyses lysate were incubated with reaction buffer (90 µl, MDB buffer, 11 µM Ac-FDG-PVFl-Ala-Arg-Val-AMC). Neutralized with ECL substrate. Signal intensity was calculated with ImageJ.

ELISA
We identified soluble AGS-products in a screen by bead array (Eve Technologies, Calgary). Cytokines in supernatants were measured by ELISA: IL-8 (Biolegend), CXCL-1 (RnD Systems), VEGF-a (Boster Bio).

Primary peripheral blood neutrophils
Neutrophils were obtained from healthy adult volunteers by negative selection with a magnetic cell separation system (EasySep kit, Stem Cell Technologies). Purity of cell preparations was confirmed by Giemsa staining.

Transwell migration assay
A 24-well transwell system (3 µm pore, Corning Costar) was used. HeLa cell supernatants (400 µl) were added into the lower chamber, and 3.5 × 10^6 freshly isolated neutrophils in 200 µl complete medium were placed into the upper chamber. Negative controls (medium) and positive controls (human IL-8 (5 ng/ml)) were included. After 75 min incubation migration was stopped. Cells in the lower chamber were harvested and counted (CASY cell counter, Omni Life Science).

Neutrophil function
Neutrophils (3 × 10^5) were co-incubated with supernatants from AGS cells infected with Hp G27 (diluted 1:2) for 24 h. Cells were harvested, stained with Annexin V-FITC (Thermo Fisher) and Live/Dead Fixable Far Red Dye (Thermo Fisher), fixed in 4% PFA (Morphisto) and analyzed by flow cytometry (FACS Calibur, BD). Supernatants from neutrophils were collected for ELISA.

Caspase activity assays
Cells (1.5 × 10^5) were seeded in 6-well plates. Following infection, cells were counted and plated (500 cells/well) in triplicates (medium contained 100U/ml Ampicillin). Seven days later, colonies were stained with crystal violet and counted.

Immunofluorescence
Cells were fixed on IBIDI-slides and permeabilized with 0.2% Triton-X100 in PBS (Smac and cytochrome c) or with methanol at −20 °C (γH2AX). Staining was done by consecutive incubation with primary and secondary antibody in the same buffer. Nuclei were stained with Hoechst. Pictures were taken blinded with a Zeiss LSM 880 (Smac, cytochrome c) or with a Keyence BZ-9000 (γH2AX). H2AX dots per cell were counted with ImageJ. In KATOIII cells, corrected total cell fluorescence (CTCF) was determined with ImageJ. Photos of at least 50 Smac and cytochrome c-co-stained cells per condition and per experiment were acquired (Zeiss LSM 880). CTCF was calculated using the formula CTCF = integrated density − (area of selected cell × mean fluorescence of background readings).

Western blotting
Cells (1.5 × 10^5) were seeded in 6-well plates. Cells were lysed with Laemmli buffer in the wells. Samples were sonicated and heated to 95 °C before loading to SDS PAGE. PVDF membranes were blocked with 5% milk. Proteins were detected with ECL substrate. Signal intensity was calculated with ImageJ. Full length original western blots are provided in Supplementary File 1.

Subcellular fractionation to analyze cytochrome c and Smac subcellular localization
AGS cells containing an empty vector were either mock- or Helicobacter-infected (G27, MOI 100) for 18 h to assess the release of Smac and cytochrome c. Cells were harvested, washed and resuspended in MB-EDTA buffer. Mitochondria were obtained by flashing cells through a 27 G needle using 1 mL syringe as described [56]. Mitochondrial fractions were isolated and supernatants were centrifuged for 60 min at 4 °C and 120,000xg. The resulting supernatants (cytoplasmic fractions) together with mitochondrial fractions were analyzed by immunoblotting using VDAC and α-tubulin as marker proteins for mitochondrial and cytoplasmic fractions.

Micronuclei assay
AGS cells in IBIDI 8 µm microscopy well were fixed with 4% PFA and stained with anti-tubulin antibody and DAPI in 1% BSA/0.1% Tween-20/ PBS. Approximately 200 cells per experiment in five pictures (Zeiss LSM 880 confocal microscope) were analyzed in a blinded fashion.

Human stomach samples
Sampling of biopsies from H. pylori-infected patients was approved by the local ethics board. Thirty-six samples from 18 patients (20x antrum, 16x body) were analyzed. Tissues were blocked and immunostained for Smac, pATM and cleaved caspase-3 using a Dako detection system and counterstained.
with hematoxylin. Epithelial cells (at least 400 cells per biopsy) were counted in high power fields of representative areas. Inflammation was scored in H&E stains using the Sydney classification [57].

Statistics

Statistics were calculated with Prism (V7, GraphPad). Unpaired T-test was used when comparing two samples. One-way and two-way ANOVA were used for multiple testing. Normalized data were analyzed by one-sample T-test. All statistical tests were performed two-sided. Linear regression was used to compare biopsies from Hp-infected patients.

DATA AVAILABILITY

All data supporting the findings of this study are available from the corresponding author upon reasonable request. Information on the human stomach biopsies can be accessed upon request from the CCCF tumour bank Freiburg.

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AUTHOR CONTRIBUTIONS
Conceptualization: GH, BD; Formal analysis: BD; Funding acquisition: GH; Investigation: BD, MTB, AH, LF, JV, BE, AM, PB, KA, JH, CWK, SK, IP, AW; Methodology: GH, BD, AH, JV, BE, SK; Project administration: GH, BD; Resources: GH, SK, PB, JH; Supervision: GH; Validation: GH, SK, KA; Visualization: GH, BD, KA; Writing—original draft: GH, BD; Writing—review and editing: MTB, AH, LF, JV, BE, AM, PB, KA, JH, CWK, SK, AW.

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COMPETING INTERESTS
The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Informed patient consent has been obtained. Sampling of biopsies from Hp-infected patients was approved by the local ethics board.

ADDITIONAL INFORMATION
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