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Caspase-4: A Therapeutic Target for Peptic Ulcer Disease

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INTRODUCTION

Several cell death mechanisms are capable of fueling inflammatory responses, causing tissue damage and contributing to the pathologic condition of inflammatory disease (1, 2). Pyroptosis is a highly inflammatory type of cell death that is triggered by the pore-forming N-terminal cleavage fragment of gasdermin D (GSDMD) (3). Inflammatory caspases (caspase-1, -4, and -5 in humans; caspase-1 and -11 in mice) are responsible for GSDMD cleavage in mice and humans (3–6). Caspase-11, and its human orthologs caspase-4 and -5, can directly bind cytosolic LPS from Gram-negative bacteria, leading to GSDMD processing, the pore-forming N-terminal cleavage fragment of gasdermin D (3). In mice and humans (3–6). Caspase-11, and its human orthologs caspase-4 and -5, can directly bind cytosolic LPS from Gram-negative bacteria, leading to GSDMD processing, pyroptosis, and NLRP3-dependent activation of caspase-1, known as the noncanonical inflammasome (6–8). Murine caspase-11 is an essential mediator of sepsis (8). Although originally limited to bacterial infection, pyroptosis has recently been implicated in the pathogenesis of disorders, including asthma and psoriasis (9–12), in which caspase-11 plays an essential role (12, 13). As caspase-4 expression is induced in asthmatic patients and correlates with disease severity, this suggests that its inhibition may have therapeutic potential (13, 14).

In this study, we report that caspase-4 is induced in peptic ulcer tissue, suggesting that noncanonical inflammasome activation and pyroptosis are processes participating in ulcer formation. Caspase-4 has previously been linked to decreased adenosine deaminase activity and implicated as a factor inducing gastric ulcers (15). Immune cells, such as macrophages, contribute to peptic ulcer formation through bacterial-induced inflammation (16). Helicobacter pylori infection alters the gastric microbiome (17) and induces inflammatory responses by recruiting immune cells, including macrophages, dendritic cells, neutrophils, and lymphocytes (18, 19). The main cytokine involved in modulating the inflammatory response during ulcer disease is IL-1β (20).

Perforated peptic ulcers can lead to peritonitis and, subsequently, sepsis—a common emergency condition worldwide with associated
mortality rates of up to 30% (21). This study demonstrates that PGE2 inhibits IL-1β production and delays mortality in a murine model of LPS-induced peritonitis. We show that PGE2 inhibits caspase-11–driven pyroptosis in macrophages, which can also subsequently limit canonical and noncanonical inflammasome activation to effectively block the inflammatory cascade. Our in vivo data provide a possible explanation for the beneficial effects of misoprostol (PGE1 analogue) observed in the management of peptic ulcers. Because misoprostol has side effects and is poorly tolerated (22), we suggest that caspase-4 inhibition represents a novel therapeutic strategy for the management of peptic ulcers and the prevention of ulcer perforation and its associated complications.

MATERIALS AND METHODS

Mice

Casp11−/− and wild-type (WT) mice were bred under specific pathogen-free conditions and under license and the approval of the local animal research ethics committee and the Irish Health Protection Regulatory Agency.

Cell culture

WT and Casp11−/− bone marrow–derived macrophages (BMDM) were isolated and cultured in 20% L929 media until day 6, after which they were plated for experimentation. THP-1 monocytes were differentiated into macrophages with 10 ng/ml PMA for 72 h prior to H. pylori infection.

Patient samples

The study was conducted after ethical approval was granted from the St. James’ Hospital/Adelaide and Meath hospital, incorporating the National Children’s Hospital Research Ethics Committee in accordance with European Communities (Clinical Trials on Medicinal Products for Human Use) regulations 2004 and International Conference on Harmonisation – Good Clinical Practice guidelines. Patients participating in the study were between 18 and 80 y of age, referred by their treating clinician for a gastroscopy based on standard clinical assessment. Peptic biopsies were taken at the side of the antral ulcer, and antral biopsies from unaffected patients served as controls. Patients with a known history of peptic malignancy (carcinoma or lymphoma), stromal tumor, or previous upper gastrointestinal surgery, as well as those with the endoscopic description of a mass, polyp, nodule, or lump anywhere in the esophagus, stomach, or small intestine, were excluded from the study. Biopsies were anonymized and stored at −80°C until analysis. Procedures had no effect on patient care, and primary consultant interactions were carried out as normal.

List of Abs for Western blot

β-Actin (1:15,000, AC-74; Sigma-Aldrich), IL-1β (pro–IL-1β and cleaved; 1:1000, AF-401; R&D Systems), caspase-11 (1:1000, 17D9; Sigma-Aldrich), caspase-1 p20 (Asp297; 1:1000, D57A2; Cell Signaling Technology), pro–caspase-1 p45 (A-19, 1:2000, sc-622; Santa Cruz Biotechnology), caspase-4 (1:1000, no. 4450; Cell Signaling Technology).

FIGURE 1. Caspase-4 expression is increased in patient ulcers and following H. pylori infection in macrophages.

A) Caspase-4 and β-actin levels in biopsy tissue from control and antrum ulcer patients were assessed by Western blot, and (B) subsequent densitometric analysis of caspase-4 is presented relative to actin as a housekeeping gene, n = 5. *p < 0.05, two-tailed Student t test. (C) Relative mRNA expression levels of caspase-4 in gastric epithelium (GE) biopsies taken from the antrum region of H. pylori (HP)-infected patients with normal, mild, and severe gastritis were analyzed using the published dataset GSE60427. Mann–Whitney U test for nonparametric data were used to evaluate significant differences between the groups. *p < 0.05.
Technology), and HRP-conjugated secondary Abs were from Jackson ImmunoResearch Laboratories.

**ELISA** IL-1β concentrations in supernatants were measured by ELISA (R&D Systems) or by MesoScale Diagnostics according to the manufacturer’s instructions.

**Lactate dehydrogenase assay** Lactate dehydrogenase (LDH) release into the cell culture supernatants was determined using CytoTox 96 Non-Radioactive Cytotoxicity Assay Technical Bulletin (Promega) according to the manufacturer’s protocol.

**Statistical analysis** Data are presented as mean ± SEM. Statistical significance was analyzed using the GraphPad Prism 5.0 statistical program (GraphPad Software, La Jolla, CA). Datasets were analyzed using the parametric Student t test or ANOVA. Data were considered significant when *p < 0.05, **p < 0.01, and ***p < 0.001.

**RESULTS**

**Increased caspase-4 expression in patient ulcer biopsies** The majority of gastric ulcers are situated in the gastric antrum, as gastritis is usually more severe in the antrum, with little or no inflammation observed in the gastric corpus (23). We observed increased caspase-4 expression in gastric antrum biopsies from ulcer patients, compared with healthy patients (Fig. 1A, 1B). Moreover, dataset analysis reveals that CASP4 mRNA expression in antrum biopsies correlates with disease severity in *H. pylori*-infected patients (Fig. 1C). These findings confirm previously reported findings, suggesting that caspase-4 contributes to the inflammation and mucosal damage that occur during ulcer formation (15).

**Caspase-11 regulates caspase-1 activity and IL-1β production in *H. pylori*-infected macrophages** *H. pylori* is one of the most common risk factors for peptic ulcer disease, with colonization occurring predominantly in the gastric antrum (24). To determine whether *H. pylori* can induce caspase-4 expression in macrophages, mRNA levels of caspase-4 and pro–IL-1β were determined in THP-1 cells infected over a 24-h time course. Results revealed that *H. pylori* infection significantly increased caspase-4 and pro–IL-1β expression (Fig. 2A, 2B). Caspase-11 is the murine ortholog of caspase-4 (25); therefore, to assess the importance of caspase-4 during *H. pylori* infection, we compared the effects of infection in primary macrophages from WT and caspase-11–deficient (*Casp-11−/−*) mice. *H. pylori*-infected BMDM from *Casp-11−/−* mice had an impaired ability to upregulate pro–IL-1β and expressed lower levels of cleaved caspase-1 p10 when compared with WT BMDM (Fig. 2C). Importantly, mature IL-1β, which contributes to gastric ulcer pathogenesis (26), was also

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**FIGURE 2. Caspase-4 expression is increased following *H. pylori* infection in macrophages.** (A and B) PMA-differentiated THP-1 macrophages were *H. pylori* NCTC11638 (multiplicity of infection [MOI] 100) infected for 0–24 h, as indicated, prior to RNA extraction and RT qPCR determination of (A) caspase-4 and (B) IL-1β mRNA levels. Changes in expression were evaluated using the comparative cycle threshold method. Results were normalized to GAPDH levels and expressed relative to uninfected cells. Paired Student t test was used to compare gene expression in cell culture samples. *p < 0.05, **p < 0.01. (C and D) *H. pylori* strain NCTC11638 infection (MOI 100) of WT and Caspase-11−/− (*C11−/−*) BMDM for 0–48 h, as indicated. (C) Inflammatory caspase-1 and -11, pro–IL-1β, and β-actin levels were determined by Western blot. (D) Secreted levels of IL-1β were determined by ELISA (MesoScale Discovery). Values represent the mean ± SEM; two-way ANOVA was used to compare mean values WT and C11−/− BMDM. Blots shown are representative of three independent experiments. **p < 0.01. HP, *H. pylori* strain NCTC11638.

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significantly decreased in Casp-11−/− BMDM after *H. pylori* infection (Fig. 2D). We have previously shown that the PGE₁ analogue, misoprostol, decreases caspase-11 expression in vivo (13).

**PGE₂ inhibits IL-1β production and pyroptosis in a murine model of peritonitis**

We have initially verified that *H. pylori* is capable of inducing caspase-11 expression, and more importantly, this induction is completely inhibited by PGE₂ treatment (27) (Fig. 3A). Peritonitis is a complication that occurs following ulcer perforation. Non-canonical inflammasome activation is driven by caspase-11, rather than canonical caspase-1, and is responsible for LPS-induced mortality in a murine model of peritonitis. PGE₂ is an inhibitor of caspase-11–driven pyroptosis (13). Given the clinically proven protective role of the stable PGE₁ analogue, misoprostol, against ulcers, we tested its effects in a peritonitis model. We observed that mice treated with misoprostol before LPS challenge had significantly decreased IL-1β serum levels and delayed LPS-induced lethality (Fig. 3B, 3C). Consistent with previous findings (10), data revealed that PGE₂ inhibited caspase-11–induced pyroptosis (Fig. 3D).

**Caspase-11 regulates caspase-1 activity and IL-1β production**

Caspase-11–mediated pyroptosis causes the release of damage-associated molecular patterns (DAMPs) and ATP, which can subsequently activate NLRP3 and caspase-1, which will serve to further boost pyroptosis levels. PGE₂ would therefore have potential to limit this process based on its well-established tissue protective characteristics (28). We designed an experiment to determine whether PGE₂ could limit DAMP production and thereby indirectly inhibit NLRP3 and caspase-1 activation (Fig. 4A). WT BMDM were pretreated with PGE₂ or vehicle before a 4-h LPS prime and subsequent LPS transfection to activate the noncanonical, caspase-11–dependent pathway. To support the hypothesis that protective effects of PGE₂-driven caspase-11 inhibition can indirectly attenuate canonical inflammasome (caspase-1) activation, Casp-11−/− BMDM were similarly treated to compare their responses against PGE₂-treated WT BMDM. In vitro, canonical inflammasome activation requires LPS priming, followed by addition of ATP or nigericin to imitate DAMPs. Western blotting revealed that supernatants from PGE₂-pretreated and LPS-primed and -transfected WT BMDM (donors), incubated with LPS-primed WT BMDM (recipients), caused significantly lower secretion levels of mature IL-1β and processed caspase-1 (p10) when compared with donor supernatants from

**FIGURE 3. PGs inhibit caspase-11 upregulation, IL-1β production, and pyroptosis in mice.**

(A) BMDM were pretreated with 1 μM PGE₂ for 30 min and then treated with 10 ng/ml LPS from *H. pylori* NCTC11637 for 4 h. Caspase-11 expression was assessed in protein lysates by Western blot. (B) Mice (n = 6 per group) were i.p. injected with 100 μl saline containing 1% DMSO or 50 μg misoprostol in 1% DMSO (misoprostol). Following 2 h, half of the mice from each group were administered LPS (2.5 mg/ml, i.p. injection) or PBS. All mice were sacrificed 90 min later, and blood serum was analyzed for IL-1β by ELISA. Data are representative from three independent experiments. *p < 0.05. (C) Mice (n = 15 per group) were i.p. injected with 100 μl saline containing 1% DMSO or 50 μg misoprostol in 1% DMSO. Following 2 h, mice were administered LPS (i.p., 25 mg/ml), and survival was monitored over 72 h. (D) Murine BMDM were primed with LPS (100 ng/ml, 4 h) prior to 30 min pretreatment with PGE₂ (1 μM) or DMSO. Cells were transfected with LPS (2 μg) using FuGENE 0.25% v/v liposomes o/n. Supernatants were collected and analyzed for cell death using LDH assay. Data shown as percentage of total cell death from three independent experiments, each using three mice per group. Data are represented as means ± SEM. Statistical analysis was performed using unpaired two-tail Student t test to compare mean values. ***p < 0.001.

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similarly stimulated WT BMDM that received no PGE2 pre-treatment (Fig. 4B; compare LPS prime [pLPS] supernatant [Sup. A1] with pLPS + Sup.B1). Importantly, PGE2 alone did not affect IL-1β secretion (Fig. 4C). Fig. 4C revealed that recipient LPS-primed WT BMDM exhibited significantly less cell death when incubated with donor supernatants from PGE2-treated WT (compare A2 with B2) or Casp-11−/− BMDM (compare A2 with C2). The inhibitory effect of PGE2 on IL-1β secretion was also confirmed by ELISA (Fig. 4D). These data confirm that PGE2 can indirectly limit NLRP3 and caspase-1–dependent IL-1β production. We therefore propose that in vivo inhibition of caspase-11 will effectively attenuate the inflammatory and pyroptotic cascades that occur during ulcer development or perforation.

**DISCUSSION**

Caspase-11/4 expression and functions are studied in the context of bacterial infection and LPS recognition (29). Infection with *H. pylori* and associated Gram-negative microbiota activates pyroptosis in ulcers of damaged and infected gastric mucosa. A hypothesis that combination of *H. pylori* and other bacteria, possibly *Escherichia coli* infection, activates caspase-4 and pyroptosis was suggested by our in vitro study that demonstrated that LPS from *H. pylori* alone was not able to induce caspase-4 expression or activation; however, in vivo biopsies from patients with gastric ulcers exhibited a clear induction of caspase-4 expression. Therefore, a model is likely in which *H. pylori* may sensitize macrophages for caspase-4 activation by other infectious bacteria or even commensals capable of effectively activating caspase-4–driven noncanonical pyroptosis. Inhibiting inflammatory cascades that lead to abdominal and systemic inflammation in this context is critically important, as peptic ulcer complications include perforations that lead to peritonitis and often sepsis. Although an anti-inflammatory role for PGE2 in tissue homeostasis has been known for years (32), the mechanism underlying its tissue protective activity has not been fully elucidated. We have shown that *H. pylori* induces caspase-11, mediating caspase-1 activation and IL-1β production. As LPS extracted from *H. pylori* is a weak inducer of inflammatory responses (33), additional virulence factors are probably needed to induce inflammasome activation (18, 19).
PGE$_2$ concentrations increase during inflammatory disease (34) and are suppressed following treatment with anti-inflammatory drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs) (35). Although we have not been able to demonstrate it with our animal model, it is likely that caspase-4 expression and activity may be altered in scenarios beyond bacterial infection, namely during NSAID treatment, increasing susceptibility to conditions such as peptic ulcers. Aspirin is a common cause of peptic ulcers in humans (36). Since its discovery at the end of the nineteenth century, the inflammatory effect of aspirin on the lining of the stomach and small intestine has been known. Aspirin inhibits PGE$_2$ production, and misoprostol has been used clinically to treat peptic bleeding in these patients (37). PGE$_2$ is well known as a facilitator of tissue regeneration and a return to homeostasis (32, 38, 39) actively involved in the process of peptic ulcer healing (28). PGE$_2$ defense against ulcers is currently believed to be linked to mucosal protection (40), possibly by inhibition of peptic acid secretion (41) as well as increase in mucus secretion (42). Data provided in this manuscript strongly suggest that the protective nature of PGE$_2$ acts beyond its positive effect on mucus production (40). Based on a recent publication, our observations may extend beyond the stomach and could also offer insights into small bowel ulcers (43).

Our observations may additionally explain the specific effects of misoprostol in ulcer treatment and, to our knowledge, shed new light on the mechanism of ulcer formation (40). Caspase-4 expression data from trials examining the effects of NSAIDs, like low-dose aspirin trials (44, 45), would increase the value of our findings. Studies of this nature would establish the relative contribution of drugs such as NSAIDs in the presence/absence of H. pylori infection on caspase-4 expression and activity. Not all individuals that are $H.$ pylori infected or take NSAIDs develop peptic ulcer disease, suggesting that individual susceptibility to bacterial virulence and drug toxicity is essential to the initiation of mucosal damage (22). Screening a population of ulcer patients for bacterial virulence and drug toxicity is essential to the initiation of pyroptosis. Overall, we demonstrate that PGE$_2$ attenuates caspase-11/4-driven pyroptosis by forming membrane pores.

DISCLOSURES

The authors have no financial conflicts of interest.

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