Helicobacter pylori extract induces purified neutrophils to produce reactive oxygen species only in the presence of plasma

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Abstract. *H. pylori* is a bacterial pathogen infecting over half of the world's population and induces several gastric and extra-gastric diseases through its various virulence factors, especially *cag*A. These factors may be released from the bacteria during interactions with host immune cells. Neutrophils play key roles in innate immunity, and their activity is regulated by plasma factors, which can alter how these cells may interact with pathogens. The aim of the present study was to determine whether purified neutrophils could produce reactive oxygen species (ROS), one of the key functions of their anti-microbial functions, in response to extracts of *cagA*+ and *cagA*- *H. pylori*. Extracts from either *cagA*+ or *cagA*- *H. pylori* were co-cultured with human neutrophils in the presence or absence of plasma, and the neutrophil ROS production was measured. In the absence of plasma, extracts from *cag*A+ and *cag*A- *H. pylori* did not induce neutrophil ROS production, whereas in the presence of plasma, extracts from both *cagA*+ and *cagA*- *H. pylori*-induced ROS production. Furthermore, when peripheral blood mononuclear cells (PBMCs) were added to the purified neutrophils in the absence of plasma, there was no neutrophil ROS production after challenging with extracts from either *cagA*+ or *cagA*- *H. pylori*. Thus, it is suggested that plasma contains immunological components that change the responsiveness of neutrophils, such that when neutrophils encounter the bacterial antigens in *H. pylori* extracts, they become activated and produce ROS. This study also revealed a potential novel immunopathogenic pathway by which *cag*A activation of neutrophils contributed to inflammatory damage.

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Introduction

H. pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium that can survive in the highly acid environment of the human stomach. Most infected individuals are asymptomatic; however, for a significant number of individuals, infection with *H. pylori* causes the development of gastritis, gastric-duodenal ulcers, and even cancer. For this reason, *H. pylori* is listed as a Class I carcinogen (1). The first virulence factor of *H. pylori* to be identified *cag*A, is an oncoprotein encoded by the *cag*A gene localized on the Cag pathogenicity island (2). *cag*A protein is delivered into host cells by a type-4 secretory system (T4SS) and then induces cellular alterations that can lead to pathological changes, via activation of signaling pathways leading to gene expression (3). The presence of *cagA*+ *H. pylori* is associated with the infiltration of neutrophils and peripheral mononuclear cells (PBMCs) with the secretion of pro-inflammatory cytokines such as IL-1b, IL-8, IL-6, and TNF-a in the gastric mucosa (4). *cagA*+ *H. pylori* induces gastric epithelial cells to secrete IL-8, which attracts neutrophils and causes mucosal tissue damage (5). Therefore, *cagA*+ *H. pylori* strains are associated with strong inflammatory responses and severe clinical outcomes (6).

Neutrophils are the most abundant circulating immune cells. They play a crucial role in innate immune responses through the secretion of toxic molecules, such as reactive oxygen species (ROS) to kill invading bacteria. To generate ROS, the nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase becomes activated and provides electrons to oxygen (O_2) to generate superoxide (O_2-). Then superoxide dismutase catalyzes O_2- to hydrogen peroxide (H_2O_2) which is a substrate for myeloperoxidase to generate hypohalous acids (7). Although ROS is generated as part of the mechanisms used to kill invading pathogens, if unregulated, bystander effects of ROS can cause tissue injury including cellular DNA damage to host tissues (8,9). Hence, neutrophil functions are usually highly regulated by serum/plasma factors such as complement proteins and immunoglobulins (10).

The majority of studies on neutrophil *H. pylori* interactions involve experiments using live bacteria and purified neutrophils (11,12). While these experiments can shed light on live pathogen: immune cell interactions, they may fail to give insights into the full range of effects of pathogenicity factors as the immune cells may not be exposed to intra-bacterial molecules. In the present study, novel activation of neutrophils by *H. pylori* extracts that was only observed when neutrophils were co-incubated with plasma was identified. When PBMCs were co-incubated with neutrophils and extracts, this activity was not seen. It was also shown that extracts containing *cag*A generated significantly higher levels of ROS compared to extracts devoid of this protein. These novel data identify a new and pathologically important process whereby *cag*A can stimulate adverse immune processes that contribute to tissue damage.

Material and methods

Study workflow. Neutrophils, PBMCs, and plasma were separated from whole blood using the Ficoll density gradient separation method (Fig. 1A). Stocks of *cagA*+ and *cagA*- *H. pylori* strains were grown on *Brucella* agar plates for 3 days before being expanded into broth media for 7 days. Bacterial cells were disrupted using an ultra-sonicator and centrifuged to collect total protein extract (Fig. 1B). As a positive control for ROS production, purified neutrophils were activated by PMA. Experiment 1 was designed to investigate whether *H. pylori* extract could stimulate ROS of neutrophils in the absence of plasma. Experiment 2 was designed to investigate whether cell-to-cell contact between purified neutrophils and PBMCs could induce ROS production by extracts in the absence of plasma. In this experiment, PBMCs were added to purified neutrophils in ratios of 1:1 and 5:1. These mixtures were then co-cultured with extracts from *cagA*+ and *cagA*- *H. pylori*. Experiment 3 was designed to determine whether *H. pylori* extract could trigger neutrophils to produce ROS in the presence of plasma. In this experiment, neutrophils were mixed with autologous plasma (1:1 v/v) and co-incubated with *H. pylori* extract at 37˚C for 60 min. All experiments were performed in technical duplicates, n=3 donors.

Participants. Blood was provided by healthy blood donors from the Blood Bank of Srinagarind University Hospital. The present study was approved by the Ethics Committee of Khon Kaen University, Faculty of Medicine, Khon Kaen, Thailand (approval no. HE651442). The 6 donors were 30-65 years old, with a male: female ratio of 1:1. Patients did not disclose any underlying infections or inflammatory conditions.

H. pylori strains and extract preparation. *cag*A + and *cag*A- *H. pylori* strains were provided by the Tropical Disease Research Center, Khon Kaen University. *Cag*A+ and *Cag*A- *H. pylori* isogenic strains of P12 (13) were generously provided by Professor R. Haas (Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Ludwig-Maximilians-Universität, München, Germany) and grown on Brucella solid agar plates containing *H. pylori* selective supplement (Dent) (cat. no. SR0147, Oxoid Limited), at 37˚C in microaerophilic conditions for 7 days. Colonies were then inoculated into Dent-supplemented Brucella broth-culture flasks and incubated at 37˚C, 10% CO_2.

From broth media, bacterial cells were pelleted and washed twice by centrifugation at 800 x g, 18˚C, for 5 min, then re-suspended in 1 ml PBS. The bacterial cells were fragmented by an ultrasonic processor at 22 kHz for 3 min on ice. The sonicated suspension was centrifuged at 7,000 x g, 4˚C, for 10 min. The supernatant containing total protein extract was removed and the protein concentration was measured using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.; OD A_{280}-A_{310}). The extracts were stored at -20˚C until required.

Neutrophil isolation. Neutrophils were isolated from heparinized whole blood using the density gradient separation method. HetaSep™ (cat. no. 7906; Stem Cell Technologies, Inc.) was added to whole blood at a ratio of 1:5. The mixture was incubated at 37˚C for 30 min until the RBC interface was 50% of the total volume. The leucocyte-rich plasma was removed and overlaid gently onto Ficoll-Hypaque (cat. no. 17144002; Cytiva), at a ratio of 1:1, and centrifuged at 500 x g for 30 min at 25˚C. The upper layer (containing platelets and plasma) and the second interface (containing PBMCs) were collected and retained, while the Ficoll suspension above the cell pellet was discarded. To the pellet, 1 ml RPMI 1640 (cat. no. SH303555.02; Cytiva) was added, and gently re-suspended before adding 9 ml ammonium chloride lysis buffer (13.4 mM $KHCO_3$, 155 mM NH_4Cl, 96.7 μM EDTA) and then incubated for 3 min at 25˚C to disrupt the red blood cells. This mixture was centrifuged at 500 x g, 25˚C for 3 min. The supernatant was discarded, and neutrophils were resuspended in 2 ml RPMI 1640. An aliquot of the purified neutrophils was stained with Trypan Blue (0.4%, w/v) for 1 min at 25˚C (cat. no. 15250061, Thermo Fisher Scientific, Inc.) and counted on a hemocytometer slide before adjusting the neutrophil concentration to $2x10^6$ cells/ml with RPMI 1640 medium. Purity was determined using Wright's staining and morphological staining (14) and was routinely >95% neutrophils.

ROS measurement. A total of 250 μl plasma and/or 250 μl RPMI 1640 were added sequentially to 250 μl purified neutrophils containing $5x10^5$ cells, then co-incubated with 200 μg/ml *cagA*+ and *cagA*- *H. pylori* extracts for 1 h at 37˚C. A total of 2 μg/ml dihydrodichlorofluorescein (cat. no. 309825, MilliporeSigma), used to detect H_2O_2, was added and incubated for a further 15 min at 37˚C. Phorbol myristate acetate (PMA, final concentration 0.1 μg/ml) (cat. no. P1585-1MG; MilliporeSigma) was used as a positive control. ROS production was detected using flow cytometry on a BD FACSCanto™ II flow cytometer (BD Biosciences). The flow cytometry results were analyzed using FlowJo™ v10.8 Software (BD Biosciences).

Gating strategy. The gating strategy for ROS detection is shown in Fig. 2. Neutrophils were gated by forward and side scatter (Fig. 2A) and then single neutrophils were analyzed by forward scatter area and forward scatter high (Fig. 2B). The cut-off value for a positive signal was identified based on comparisons of negative control values (Fig. 2C) vs. positive control values with PMA stimulation (Fig. 2D).

Statistical analysis. All data are presented as the mean ± SEM. Statistical comparisons were performed using

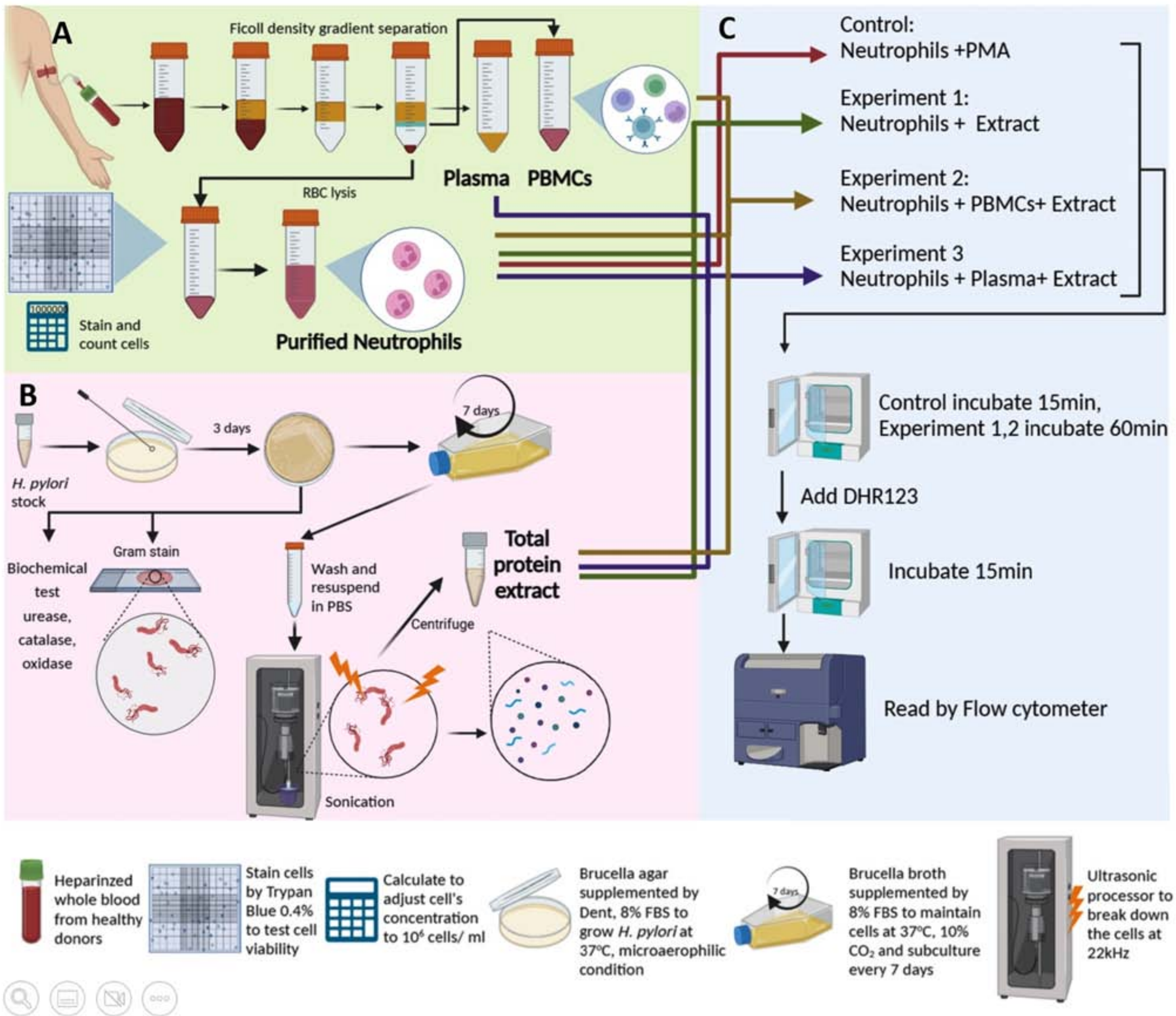

Figure 1. Schematic overview of the experimental design. (A) Isolation of purified neutrophils. (B) Preparation of *H. pylori* extracts. (C) ROS measurement by flow cytometry ROS, reactive oxygen species; PBMC, peripheral blood monocytes; PMA, Phorbol myristate acetate; DHR123, dihydrodichlorofluorescein.

a Mann-Whitney U test between groups. P<0.05 was considered to indicate a statistically significant difference. All data were analyzed using GraphPad Prism version 8.0 (GraphPad Software, Inc.).

Results

cagA+ and cagA- H. pylori extracts do not directly trigger ROS production in purified neutrophils. To identify whether *H. pylori* extracts can trigger ROS production directly, purified neutrophils were co-cultured with *cagA*+ and *cagA*- *H. pylori* extracts. Very few neutrophils produced ROS in the untreated control samples (<5%, Fig. 3A), whereas PMA effectively stimulated the majority of the neutrophils to release ROS. Therefore, the control system was reliable.

Neither *cagA*+ (P=0.200) nor *cagA*- (P=0.3429) *H. pylori* extract increased the number of ROS-producing cells (Fig. 3B and C, respectively) when compared to the untreated control (Fig. 3D). Thus, it was concluded that these extracts could not induce ROS production directly, or otherwise, neutrophils require other factors to prime them, such as cytokines from PBMCs before encountering the antigens.

cagA+ and cagA- H. pylori extracts do not trigger the production of ROS by neutrophils in the presence of PBMCs. To determine whether cell-to-cell contact with PBMCs induced neutrophil ROS production, purified PBMCs were added to the neutrophils before incubation with *cagA*+ and *cagA*- *H. pylori* extracts, and the ROS levels were measured. There were no notable levels of ROS detected in the control group (only neutrophils and PBMCs) (Fig. 4A) nor in the *cagA*+ (Fig. 4B) or in the *cagA*- *H. pylori*-treated neutrophils (Fig. 4C). we increased the ratio of PBMCs to neutrophil was increased to 5:1, there was still no measurable ROS production detected (Fig. 4E-G).

cagA+ and cagA- H. pylori extracts induce ROS production by neutrophils in the presence of human plasma. To investigate whether human plasma affects ROS production by neutrophils in response to *cagA*+ and *cagA*- *H. pylori* extracts, autologous plasma was used to pre-treat neutrophils, with or

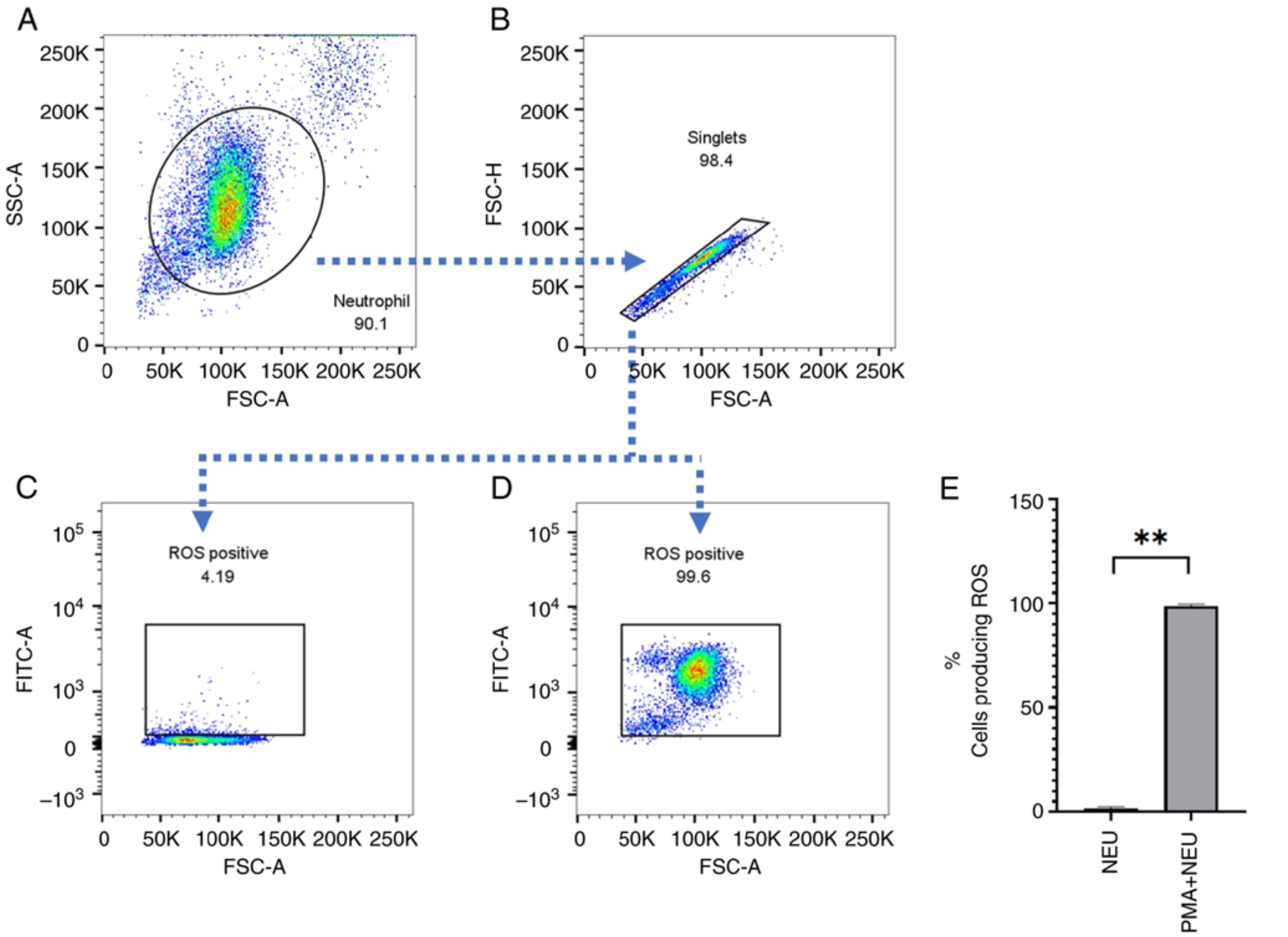

Figure 2. Gating strategy. (A) Neutrophil population. (B) Single neutrophils. (C) Negative control (neutrophils only, without stimulus). (D) Positive control (PMA-treated neutrophils). (E) ROS production in the negative control (neutrophil), and PMA-treated neutrophils (neutrophils + PMA). n=3. **P<0.01. ROS, reactive oxygen species; PMA, Phorbol myristate acetate; NEU, neutrophils; FSC, forward scatter; SSC, side scatter.

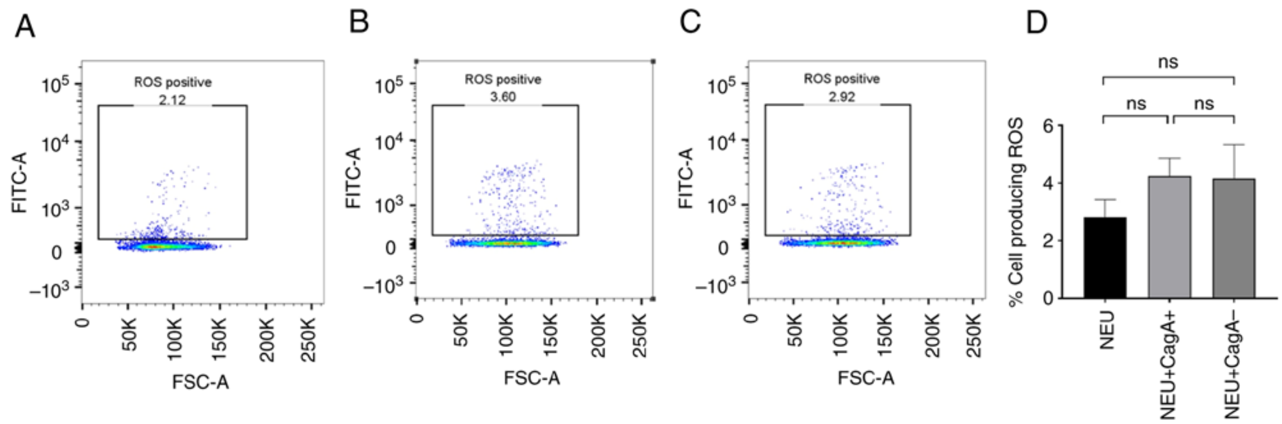

Figure 3. *H. pylori* extract does not induce ROS production by human neutrophils. (A) Neutrophils only (no extract)-untreated controls. (B) ROS production by neutrophils treated with *cagA+ H. pylori* extract. (C) ROS production by neutrophils treated with *cagA- H. pylori* extract. (D) Summary of ROS production induced by *cagA+* and *cagA- H. pylori* extracts compared to untreated neutrophils. All experiments were performed in technical duplicates. n=3 donors. ns, not significant; ROS, reactive oxygen species; NEU, neutrophils; FSC, forward scatter.

without co-culture with *cagA+* and *cagA- H. pylori* extracts, and measured ROS production after 1 h. There was no ROS production in the negative controls (plasma-treated neutrophils without *H. pylori* extracts; Fig. 5A). However, in the presence of plasma, both *cagA+* (Fig. 5B; P=0.0286) and *cagA- H. pylori* extracts stimulated neutrophils to produce significantly more ROS than the negative controls (Fig. 5C; P=0.0286). Additionally, *cagA+ H. pylori* extract induced the production of significantly more ROS by neutrophils than *cagA- H. pylori* extract in the presence of plasma (Fig. 5D).

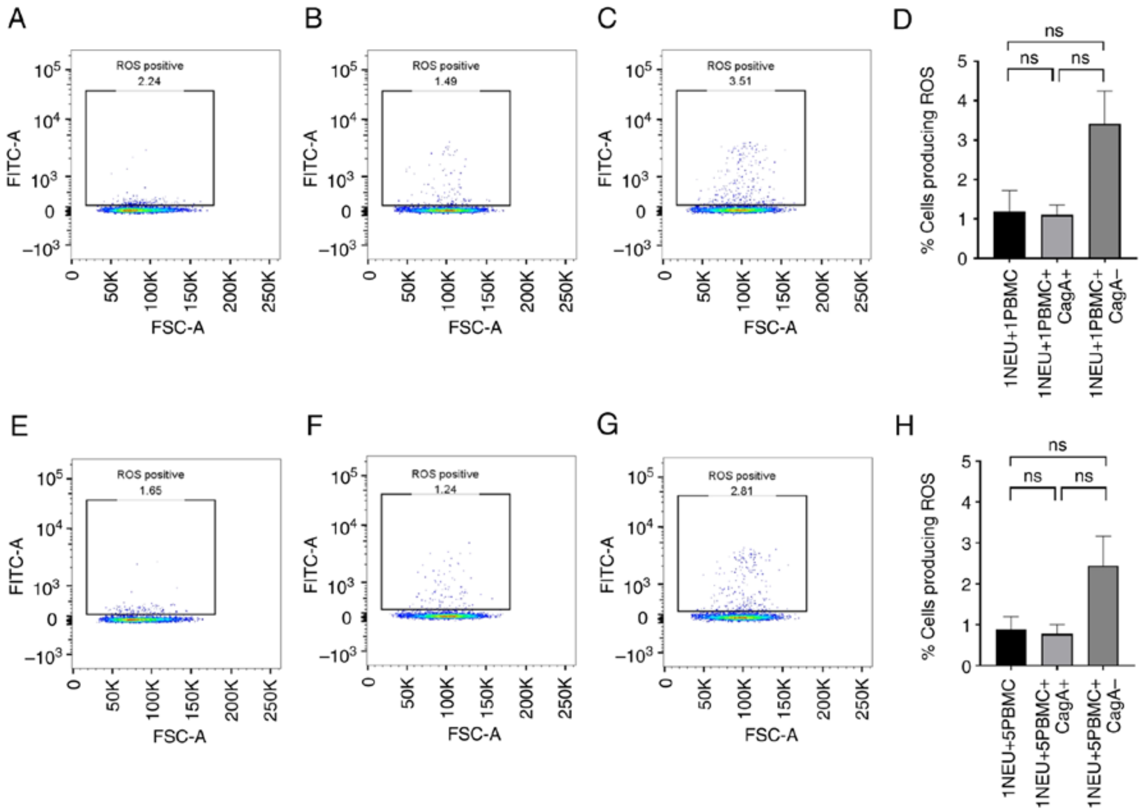

Figure 4. Neutrophil-PBMC cell-to-cell contact did not induce ROS production by neutrophils stimulated with *H. pylori* extracts. (A) Neutrophils mixed with PBMCs at a ratio=1:1. (B) Neutrophils and PBMCs (1:1) co-cultured with *cagA+ H. pylori* extract. (C) Neutrophils and PBMCs (1:1) co-cultured with *cagA- H. pylori* extract. (D) Summary of ROS production of neutrophil co-incubation with PBMCs (ratio 1:1) and *cagA+* and *cagA- H. pylori* extracts. (E) Neutrophils mixed with PBMCs at a ratio=1:5. (F) Neutrophils mixed with PBMCs (1:5) co-cultured with *cagA+ H. pylori* extract. (G) Neutrophils mixed with PBMCs (1:5) co-cultured with *cagA- H. pylori* extract. (H) Summary of ROS production by neutrophils co-incubated with PBMCs (1:5) and *cagA+* and *cagA- H. pylori* extracts. All experiments were performed in technical duplicates. n=3 donors. ROS, reactive oxygen species; PBMC, peripheral blood monocytes; ns, not significant; NEU, neutrophils; FSC, forward scatter.

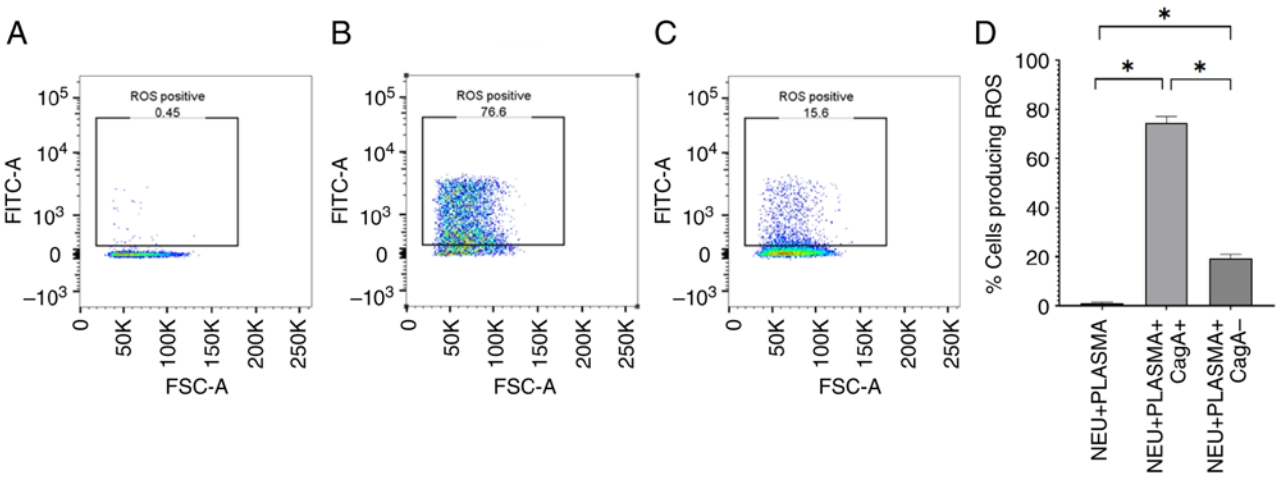

Figure 5. Plasma primes neutrophils for generation of ROS induced by *H. pylori* extracts. (A) Neutrophils were cultured in the presence of plasma (no *H. pylori* extract). (B) ROS production by neutrophils in the presence of plasma after addition of *cagA+ H. pylori* extract. (C) ROS production by neutrophils in the presence of plasma after addition of *cagA- H. pylori* extract. (D) Summary data of ROS production in the absence or presence of *cagA+* and *cagA- H. pylori* extract. All experiments were performed in technical duplicates. n=3 donors. *P<0.05. ROS, reactive oxygen species; NEU, neutrophils; FSC, forward scatter; SSC, side scatter.

Discussion

This study shows, for the first time, that complex host-pathogen inflammatory processes regulate the activation of human neutrophils using *H. pylori* extracts. It was found that these extracts could only activate ROS production by human neutrophils in the presence of plasma, and that this activation could not be replicated by the addition of PBMCs. It was also shown that *H. pylori* extracts containing the *cag*A protein generated significantly higher levels of ROS than extracts devoid of this protein. This is an important observation in view of the fact that infection with cagA+ *H. pylori* strains usually results in more adverse pathological outcomes, such as an increased risk of gastric cancer, than those that do not express this pathogenicity factor (15). The results of the present study indicated that serum factors are necessary to prime neutrophils to generate ROS after incubation with these extracts, but also showed that extracellular CagA activated neutrophils in the presence of these plasma factors.

Several components in plasma may be involved in the process of ROS activation of neutrophils. Firstly, following interaction with immune cells, activated platelets can release chemokines (to attract neutrophils) and proinflammatory cytokines such as CD40L and IL-1β (16), which may prime neutrophil functions. Second, immunoglobulins in plasma may elicit neutrophil ROS production, either alone or via the formation of immune complexes after interactions with their cognate antigen. It has been shown that immunoglobulins for intravenous use, named Gamimune N, Sandoglobuin, and Intraglobin F can enhance neutrophil respiratory-burst activity *in-vitro* (17). Moreover, these immunoglobulins also promote killing activity towards multi-drug-resistant bacteria and autophagy of neutrophils in immunocompromised patients (18). However, whether the plasma of the volunteers contained anti-Helicobacter antibodies was not determined, although this is now being explored in the follow-up studies. Third, complement proteins such as C3a, C5a, and the surface-bound opsonins, C3b and C4b, can enhance the ability of neutrophils to phagocytose opsonized particles, release proinflammatory cytokines, generate ROS, and form neutrophil extracellular traps (NETs) (19). It is postulated that these components in plasma can prime neutrophils, and once these primed neutrophils encounter specific antigens in *H. pylori* extracts, they become activated. It is also possible that components in plasma interact with *H. pylori* proteins, subsequently activating neutrophils. Nevertheless, these results are the first to demonstrate that neutrophil ROS production can be stimulated by *H. pylori* extracts even without phagocytosis of intact live bacteria; however, factors present within plasma are required for this ROS production to occur.

It is hypothesized that cell-to-cell contact with PBMCs may facilitate neutrophil ROS production in the presence of *H. pylori* extracts. However, this was shown not to be the case. The results of the present study suggest that cell-to-cell contact with, or cytokines from, PBMCs do not contribute to neutrophil ROS production at least after incubation with *cagA*+ and *cagA*- *H. pylori* extract under the experimental conditions employed in the present study.

H. pylori has been studied largely given its role in gastric cancer development and several *in vitro* experiments support this property. For example, co-culture of *H. pylori* extract with gastric epithelial cell lines leads to elevated cell proliferation as well as inhibition of cell apoptosis and autophagy (20). In addition, *H. pylori* extract induces mRNA expression of gastric cancer biomarkers such as chloride channel-3 and slingshot protein phosphatase 1, which suggests that *H. pylori* extract may contribute to gastric cancer progression (20). *H. pylori* extract also causes extra-gastric disorders. For example, an animal-model study indicated that this extract promoted the expression of chemokines and elevated the levels of TGF-β1/NF-κB-mediated inflammation in a rat hepatic stellate cell line (21). For these reasons, *H. pylori* extract has been used for vaccine development. Flagella are important for *H. pylori* motility and colonization, and flagella-sheath proteins or total protein lysate have been used to vaccinate mice (22). These immunized mice were then infected with *H. pylori* orally and both forms of vaccination led to an equally significant decrease in *H. pylori* burden relative to non-vaccinated controls (22).

A major finding of the present study was that *cagA*+ *H. pylori* extract induced significantly higher ROS levels than *cagA*-extracts, which suggests a novel immunopathogenic pathway induced by *cagA*+ *H. pylori*. As the two strains that were used in the present study were isogenic (that is, the *cagA*-strain was identical to the wild-type strain except that it was specifically depleted of *cagA*), the only difference in protein composition of the two extracts used was the absence or presence of CagA. Thus, this pathogenicity factor induced high levels of ROS production in plasma-treated neutrophils, and it is hypothesized that this mechanism plays a role in tissue damage associated with this organism in human diseases.

*cag*A is normally inserted into host cells via the type-4 secretory system and once within the cytoplasm, it becomes phosphorylated and then interacts with and activates intracellular signaling pathways leading to altered gene expression and oncogenesis (3). Intact *H. pylori* are phagocytosed by human neutrophils and can survive intracellularly and delay neutrophil apoptosis (23). It is noteworthy that in the present study, extracellular *cag*A activated human neutrophils (in the presence of serum) to generate ROS. Previously, it has been reported that in addition to their role in gastric diseases, *Helicobacter spp* are responsible for a number of hepatobiliary pathologies including several types of liver cancer (24-27). In addition, cagA+ *H. pylori* is detected at considerably higher levels in individuals infected with the liver fluke, *Opisthorchis viverrini* (compared to uninfected controls) and at even higher levels in those with advanced periductal fibrosis, which is an outcome of liver fluke infection (28). *O. viverrini* is a reservoir for *H. pylori* and hence carries this bacterial pathogen to the bile ducts during liver fluke infection (29). At a 2-year follow-up, >40% of those initially diagnosed with liver fibrosis were now parasite free but had persistent or worsening fibrosis (30) and these individuals had significantly higher levels of *cag*A + *H. pylori* (31).

The present study highlights the importance of *cag*A as an important pathogenicity factor that can activate neutrophils to generate molecules that may damage host tissues, as ROS may induce oxidative stress, resulting in DNA damage. Identification of the molecular mechanisms responsible for the receptor/intracellular signaling processes mediated by *cag*A is important in order, not only to define this mechanism, but to identify ways by which this pathway can be experimentally blocked. Further work will also include identifying the factor(s) within plasma that can regulate this process and establish the full range of neutrophil functions (including secreted proteases and cytokines/chemokines) that are activated by this novel mechanism.

In conclusion, total extracts from *cagA*+ and *cagA*- *H. pylori* had little effect on neutrophil ROS production in the absence of plasma. However, the addition of plasma significantly primed human neutrophils to generate ROS in response to the extracts. *cagA*+ *H. pylori* extract-treated neutrophils produced significantly more ROS than did neutrophils treated with *cagA*- *H. pylori* extracts. These data show that *H. pylori* proteins, perhaps actively secreted by the bacteria or after release from dead bacteria, can, in the presence of plasma factors, activate ROS by neutrophils. *CagA*+ *H. pylori* extract significantly induced higher ROS than *cagA*-extract, which suggests a novel immunopathogenic pathway of *cagA*+ *H. pylori*.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

TDT, SWE and KS conceived and designed the study. TDT, CC, DW, and KS performed the experiments. TDT, KF, BS, SWE and KS analysed and interpreted the data. TDT and KS wrote the first draft of the manuscript. TDT, KF, BS, SE and KS edited and finalized the manuscript. All authors have read and approved the final manuscript. TDT, SWE and KS confirm the authenticity of all the raw data.

Ethics approval and consent to participant

The present study was approved by the Ethics Committee of Khon Kaen University, Faculty of Medicine (Khon Kaen, Thailand; approval no. HE651442) and written informed consent was obtained from each participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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