NOD1 is required for *Helicobacter pylori* induction of IL-33 responses in gastric epithelial cells

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**Abstract**

*Helicobacter pylori* (*H. pylori*) causes chronic inflammation which is a key precursor to gastric carcinogenesis. It has been suggested that *H. pylori* may limit this immunopathology by inducing the production of interleukin 33 (IL-33) in gastric epithelial cells, thus promoting T helper 2 immune responses. The molecular mechanism underlying IL-33 production in response to *H. pylori* infection, however, remains unknown. In this study, we demonstrate that *H. pylori* activates signalling via the pathogen recognition molecule Nucleotide-Binding Oligomerisation Domain-Containing Protein 1 (NOD1) and its adaptor protein receptor-interacting serine-threonine Kinase 2, to promote production of both full-length and processed IL-33 in gastric epithelial cells. Furthermore, IL-33 responses were dependent on the actions of the *H. pylori* Type IV secretion system, required for activation of the NOD1 pathway, as well as on the Type IV secretion system effector protein, CagA. Importantly, *Nod1*+/− mice with chronic *H. pylori* infection exhibited significantly increased gastric IL-33 and splenic IL-13 responses, but decreased IFN-γ responses, when compared with *Nod1*−/− animals. Collectively, our data identify NOD1 as an important regulator of mucosal IL-33 responses in *H. pylori* infection. We suggest that NOD1 may play a role in protection against excessive inflammation.
Chronic inflammation caused by infection with the Gram-negative bacterium <i>Helicobacter pylori</i> is an essential precursor to gastric carcinogenesis (Peek & Crabtree, 2006). Upon infection, <i>H. pylori</i> interacts with host cells within the gastric mucosa, resulting in activation of multiple innate immune signalling pathways that shape host adaptive immune responses (Tran, Chonwerawong, & Ferrero, 2017). Innate immune recognition of <i>H. pylori</i> results in T helper (Th) 1 and 17 responses which promote the development of gastric immunopathologies (Hitzler, Kohler, Engler, Yazgan, & Muller, 2012; Sayi et al., 2009). It has been shown, however, that <i>H. pylori</i> can limit excessive gastric inflammation and promote bacterial persistence through the induction of Th2 immune responses (Berg, Lynch, Lynch, & Lauricella, 1998; Chen, Shu, & Chadwick, 2001; Smythies et al., 2000).

The IL-1 cytokine family comprises 11 members and has been described as important drivers of the host adaptive cytokine profile (Lopetuso, Chowdhry, & Pizarro, 2013). A recently identified member of this family, IL-33, has been shown to act as an alarmin and be associated with Th2 signature cytokine production (e.g., IL-4, IL-5, and IL-13) by Group 2 innate lymphoid cells and Th2 lymphocytes during allergic inflammation or parasite infection (Liew, Girard, & Turnquist, 2016). In addition, IL-33 was shown to promote the proliferation of regulatory T cells and to function as an anti-inflammatory cytokine mediating tissue repair (Martin & Martin, 2016; Molofsky, Savage, & Locksley, 2015).

A recent study reported upregulated IL33 gene expression in human gastric mucosa in response to <i>H. pylori</i> infection (Shah et al., 2015). Buzzelli et al. (Buzzelli et al., 2015) demonstrated increased IL-33 responses in the gastric mucosa of <i>H. pylori</i>-infected mice in the acute phase of infection, whereas its expression was reduced during chronic infection. Despite the observed association between IL33 expression and <i>H. pylori</i> infection, the upstream signalling mechanism underlying <i>H. pylori</i> regulated IL-33 production remains elusive.

We have previously reported that the cytosolic receptor Nucleotide-Binding Oligomerisation Domain-Containing Protein 1 (NOD1) is an important pathogen recognition receptor regulating host epithelial cell responses to <i>H. pylori</i> (Viala et al., 2004). NOD1 was shown to sense Gram-negative peptidoglycan (PG), delivered into the cytoplasm of host cells via the <i>H. pylori</i> Type IV secretion system (T4SS), encoded by the cag pathogenicity island (CagPAI; Allison, Kufer, Kremmer, Kaparakis, & Ferrero, 2009; Boonyanugomol et al., 2013; Viala et al., 2004). In addition to the T4SS, <i>H. pylori</i> can activate the NOD1 pathway via the actions of outer membrane vesicles (OMVs) which function as a transport mechanism for bacterial PG into the host cell cytosol (Irving et al., 2014; Kaparakis et al., 2010; Olofsson et al., 2010). Upon recognition of PG, NOD1 undergoes self-oligomerisation, leading to the recruitment of the scaffolding kinase protein, receptor-interacting serine–threonine kinase 2 (RIPK2; also known as RIP2, RICK, or CARDIAK; Allison et al., 2009; Baker et al., 2017; Hasegawa et al., 2008; Olofsson et al., 2010). This leads to activation of nuclear factor-kappa B (NF-kB) and mitogen-activated protein kinases signalling pathways and the subsequent transcriptional activation of multiple pro-inflammatory cytokine genes (Allison et al., 2009).

Given that IL-33 is downstream of the NF-kB signalling pathway (Farias & Rousseau, 2015; Kobori et al., 2010), we hypothesised that NOD1 sensing of <i>H. pylori</i> infection may regulate IL-33 responses in the stomach. Here, we show that NOD1 is required for <i>H. pylori</i> upregulation of IL-33 gene expression, synthesis, and processing in gastric epithelial cells (GECs) in vitro and in vivo. Furthermore, NOD1 regulation of IL-33 production was shown to be associated with the development of Th2-type immune responses in mice with chronic <i>H. pylori</i> infection, suggesting a protective role for NOD1 against excessive inflammation.

2 | RESULTS

2.1 | NOD1 sensing of <i>H. pylori</i> mediates IL-33 responses in GECs

It has been shown that IL-33 production is mediated by NF-kB signaling and that sensing of <i>H. pylori</i> infection by NOD1 leads to the activation of NF-kB signalling in GECs (Allison et al., 2009; Farias & Rousseau, 2015; Kobori et al., 2010; Viala et al., 2004). In order to initially investigate the link between NOD1 signalling and IL-33 regulation in response to <i>H. pylori</i>, we used the mouse GSM06 gastric epithelial cell line. To block NOD1 signalling in GSM06 cells, we pretreated cells with the NOD1-specific inhibitor ML130 (Correa et al., 2011). As expected, inactivation of NOD1 signalling by treatment with ML-130 significantly attenuated the production of keratinocyte chemoattractant (KC), a cytokine downstream of <i>H. pylori</i> infection (Figure 1a; p < .05). Likewise, the stimulation of cells with <i>H. pylori</i> bacteria induced upregulated IL33 gene expression and this was significantly reduced in cells pretreated with ML130 (Figure 1b; p < .05). Consistent with the gene expression data, full-length IL-33 (33 kDa) production was increased in the lysates of GSM06 cells in response to <i>H. pylori</i> stimulation, whereas the levels were reduced when NOD1 signalling was inhibited by ML130 treatment (Figure 1c). Furthermore, we detected higher levels of processed IL-33 (22 kDa) in the lysates and cultured supernatants of GSM06 cells following <i>H. pylori</i> stimulation as compared with ML130-treated cells (Figure 1c). Lower levels of total IL-33 were also detected in the culture supernatants of ML-130-treated GSM06 cells, as compared with control cells (Figure S1a). These data showed that NOD1 promotes both gene transcription and protein production of IL-33 in mouse GECs. To further investigate the contribution of NOD1 in IL-33 regulation, we isolated primary mouse GECs from Nod1<sup>−/−</sup> or Nod2<sup>−/−</sup> mice. The purity of the GEC preparations was confirmed by immunofluorescence staining with an antibody to the epithelial cell-specific marker, EpCAM (Figure S1b,c). The reduction in KC and macrophage inflammatory protein 2 (MIP2) production in Nod1<sup>−/−</sup> GECs further confirmed the important role of NOD1 in activation of NF-kB in response to <i>H. pylori</i> stimulation (Figure S1d,e). The GECs were then co-cultured with the <i>H. pylori</i> clinical Strain 10700 or its mouse-adapted variant, Sydney Strain 1 (SS1). Consistent with findings in GSM06 cells, the levels of full-length and processed IL-33 were markedly lower in Nod1<sup>−/−</sup> GECs as compared with Nod1<sup>+/−</sup> cells in response to either 10700 or SS1 stimulation (Figure 1d). To extend our findings, we used
human AGS GEC lines, stably expressing an shRNA specific for NOD1 or an irrelevant gene EGFP (hereafter referred to as sh.NOD1 AGS or sh. EGFP, respectively [Grubman et al., 2010]). Increased levels of IL33 gene expression, as well as full-length IL-33 synthesis and processing, were observed in sh.EGFP AGS cells in response to stimulation with H. pylori 26695 bacteria (Figure 1e,f). In contrast, these responses were completely ablated in sh.NOD1 AGS cells (Figure 1e,f). To investigate the role of NOD1 sensing of bacterial PG in IL-33 responses, we stimulated AGS cells with wild type or isogenic mutant bacteria that lack the lytic transglycosylase, Slt (HP0645). These slt− mutant bacteria release less NOD1 ligand and therefore also have a reduced capacity to activate the NOD1 signalling pathway (Viala et al., 2004). AGS cells that were co-
cultured with *H. pylori* silt bacteria exhibited reduced IL-33 responses when compared with the parental strain (Figure 1e,f). Furthermore, *H. pylori* OMVs harbouring PG induced higher levels of full-length and processed IL-33 in sh.EGFP AGS cells when compared with sh.NOD1 cells (Figure S2). These findings confirm the importance of NOD1 sensing of *H. pylori* PG for IL-33 responses in GECs.

To confirm our findings, we generated NOD1 knockout (KO) AGS cells using CRISPR/Cas9 technology to target the CARD domain of NOD1, which is required for its interaction with downstream adaptor molecules and activation of NF-κB signalling (Inohara et al., 1999). The isolated NOD1 KO clone#1 and clone#2 were shown to carry 71-bp and 31-bp deletions, respectively, introducing premature stop codons that resulted in truncated proteins (Figure S3a,b). As expected, the two KO clones displayed undetectable levels of NOD1 gene expression (Figure S3c) and attenuated IL-8 production in response to NOD1 agonist C12-EiDAP or *H. pylori* stimulation (Figure S3d). Importantly, both NOD1 KO AGS clones express significantly lower levels of IL33 gene expression (Figure 1g; p < .05) and produce less full-length and mature IL-33 protein than control AGS cells (Figure 1h). Taken together, these findings show that activation of NOD1 signalling by *H. pylori* enhances IL-33 production in both mouse and human GECs.

In addition to NOD1, recent studies have reported that the host tumour necrosis factor receptor-associated factor (TRAF)-interacting protein with forkhead-associated domain (TIFA) drives NF-κB activation upon detection of heptose-1,7-bisphosphate, an intermediate product of lipopolysaccharide biosynthesis (Gall, Gaudet, Gray-Owen, & Salama, 2017; Gaudet et al., 2017; Stein et al., 2017). To address the contribution of TIFA in *H. pylori*-induced IL-33 production, we “knocked down” its expression by transfecting AGS cells with TIFA-specific siRNA prior to bacteria stimulation. Transfection with TIFA siRNA resulted in significant reductions in TIFA gene expression and IL-8 responses (Figure S4a,b), yet the levels of both full-length and mature forms of IL-33 in the lysates of *H. pylori*-stimulated cells remained unchanged (Figure S4c). Interestingly, AGS cells in which TIFA expression was reduced by siRNA treatment secreted lower levels of mature IL-33 into the culture supernatants, as compared with control AGS cells (Figure S4c). These data suggest that unlike NOD1, TIFA is required for IL-8 production and proteolytic processing of full-length IL-33 but is dispensable for IL-33 synthesis.

### 2.2 RIPK2 is required for NOD1-mediated production and processing of IL-33

RIPK2 has been described to be a central down-stream mediator of NOD1 signalling (Hasegawa et al., 2008). To test the role of RIPK2 in *H. pylori*-mediated IL-33 responses, AGS cells were either transfected with RIPK2-specific siRNA or pretreated with the RIPK2 inhibitor, WEHI-345, prior to *H. pylori* stimulation (Nachbur et al., 2015). siRNA-mediated knock down of RIPK2 expression was confirmed by qPCR (Figure 2a; p < .05). Additionally, inhibition of RIPK2 function was confirmed by measuring IL-8 responses as the production of this chemokine in response to *H. pylori* stimulation is known to be
NOD1-dependent (Figure 2b,c). Importantly, RIPK2 inhibition resulted in reduced levels of IL-33 production and processing (Figure 2d,e). These data show that classical NOD1/RIPK2 signalling is important in mediating the IL-33 responses induced by H. pylori.

2.3 Loss of nuclear localisation of IL-33 in response to H. pylori stimulation is NOD1-dependent

It has been reported that under basal conditions, IL-33 predominantly resides in the nucleus of epithelial cells, whereas loss of its nuclear location is associated with enhanced release of IL-33 and induction of inflammatory responses (Kuchler et al., 2008; Mousson, Ortega, & Girard, 2008). Consistent with the published literature, IL-33 expression was predominantly localised within the nucleus of unstimulated control and NOD1 KO AGS cells (Figure 3a,b). H. pylori stimulation promoted the relocalisation of IL-33 from the nucleus to the cytoplasm in control cells, but not in NOD1 KO AGS cells, which instead displayed a predominantly nuclear localisation for IL-33 similar to that in non-stimulated cells (Figure 3c,d). This observation was further confirmed by a significant reduction in the ratio of nuclear to cytoplasmic IL-33 in control cells but not in NOD1 KO AGS cells after stimulation with H. pylori (Figure 3e; p < .001). These data indicate that NOD1 is involved in promoting the nuclear release of IL-33. Thus, NOD1 not only promotes the synthesis of IL-33 protein but also release of this cytokine, which may allow host epithelial cells to activate other immune cell subsets and shape the downstream immune responses to H. pylori infection.

2.4 The H. pylori T4SS and CagA are required for induction of IL-33 in human GECs

The H. pylori T4SS is one mechanism by which bacteria can deliver their PG into the cytoplasm of host cells, leading to the activation of NOD1 signalling (Viala et al., 2004). Therefore, we addressed whether bacterial T4SS plays a role in NOD1-mediated IL-33 responses by co-culturing human AGS cells with wild type H. pylori 251 or isogenic mutant strains having either defective T4SSs (i.e., ΔcagPAI and ΔcagM) or lacking the T4SS effector protein, CagA (ΔcagA). These mutant H. pylori bacteria induced significantly reduced levels of IL-8 production when compared with wild type bacteria (Figure 4a; p < .01 and p < .05, respectively). H. pylori mutant bacteria with defective T4SSs also induced significantly lower levels of IL33 expression (Figure 4b; p < .05), as well as reduced production of both full-length and processed IL-33 (Figure 4c; p < .05). Interestingly, H. pylori ΔcagA bacteria also induced significantly reduced IL-33 responses in AGS cells (Figure 4c; p < .05). Taken together, our data show that T4SS, which is required for the activation of NOD1 signalling, as well as for translocation of the CagA oncoprotein, contributes to H. pylori induced IL-33 production in human GECs.

2.5 NOD1 drives IL-33 production and Th2 cytokine responses during chronic H. pylori infection in vivo

To study the role of NOD1 in IL-33 production in vivo, Nod1−/− and Nod1−/− mice were inoculated with H. pylori and left for 1 or 8 weeks, respectively. At 1 week post-infection, we observed no significant changes in Il33 gene expression (Figure 5a) or IL-33 production (Figure 5b) in the stomachs of either H. pylori-infected Nod1−/− or Nod1−/− mice, when compared with control uninfected animals. In contrast, at 8 weeks post-infection, Nod1−/− animals displayed significantly down-regulated levels of Il33 gene expression (Figure 5a; p < .05) and protein production (Figure 5b; p < .001), when compared with H. pylori-infected Nod1−/− animals. The former also exhibited lower levels of both full-length and processed IL-33 forms (Figure 5c). Thus,

![Figure 3: NOD1 mediates loss of nuclear location of IL-33 in human GECs in response to H. pylori stimulation.](image-url)
consistent with our in vitro data, NOD1 mediates IL-33 production during chronic infection with H. pylori.

Given that IL-33 is a key driver of Th2 immunity in a wide range of inflammatory settings, we next investigated the cytokine profiles of...
splenocytes isolated from Nod1+/− or Nod1−/− mice at 8 weeks post-infection. Although splenocytes from Nod1+/+ and Nod1−/− mice produced similar levels of the Th2 cytokines, IL-4 and IL-10, significant differences were observed for another Th2 cytokine, IL-13 (Figure 5d–f; p < .05). Conversely, the splenocytes from Nod1−/− mice produced significantly higher levels of the Th1 cytokine IFN-γ, as compared with Nod1+/+ splenocytes (Figure 5g; p < .05). No differences were observed for the Th-17 cytokine, IL-17 (Figure 5h). Thus, during chronic infection with H. pylori, NOD1 is involved in IL-33 production thereby shaping host adaptive immune responses towards a Th2-dominant phenotype.

3 | DISCUSSION

IL-33 is well established as an important mediator of both innate and adaptive immune responses (Martin & Martin, 2016). Although recent studies have revealed an association between mucosal IL-33 production and H. pylori infection, the underlying mechanism by which the bacterium drives IL-33 production remains unknown (Buzzelli et al., 2015; Shahi et al., 2015). In this study, we show that H. pylori activation of the innate immune molecule NOD1 promotes IL33 gene expression and processing in GECs.

It has recently been reported that IL-33 induction is driven by NF-kB signalling in epithelial cells in response to bacterial infection and inflammation (Farias & Rousseau, 2015; Kobori et al., 2010). This led us to hypothesise that in response to H. pylori infection, NOD1 may regulate IL-33 production in epithelial cells. Consistent with this hypothesis, we showed that disruption of NOD1 signalling by either pharmacological inhibition, siRNA knockdown or CRISPR/Cas9 gene targeting resulted in attenuation of both classical NF-kB-dependent cytokine production and IL-33 responses. Furthermore, we showed that blockade of RIPK2, a critical downstream mediator of NOD1 signalling responsible for NF-kB activation (Inohara et al., 2000), resulted in decreased IL-33 production. Thus, these findings suggest that NOD1 sensing of H. pylori promotes IL-33 production via activation of the NF-kB signalling pathway.

It is thought that IL-33 possesses a nuclear localisation sequence within its N terminus and is thus normally stored in the nucleus in order to be protected from cleavage and inappropriate release (Carriere et al., 2007; Gautier et al., 2016). In this study, we observed that NOD1 activation by H. pylori caused loss of nuclear IL-33 and enhanced secretion of processed IL-33 in the culture supernatants of control AGS cells but not those from NOD1 KO cells (Figure 3). Thus, it is possible that NOD1 mediates the release of IL-33 via cleavage of an N-terminal nuclear localisation signal required for tethering IL-33 in the nucleus. However, the mechanism(s) whereby these processed forms are secreted into the extracellular compartment remain(s) largely unknown. The processed form of IL-33 may be biologically active or inactive, depending on the proteases mediating the cleavage (Martin & Martin, 2016). In common with other members of the IL-1 cytokine family, IL-33 was initially reported to become active after processing by caspase-1 (Schmitz et al., 2005). However, subsequent studies showed that caspase-1 and other apoptotic caspases, including caspases-3 and -7, could cleave IL-33 into an inactive form (Cayrol & Girard, 2009). IL-33 can also be processed by neutrophil proteases, resulting in highly active forms (Lefrancais et al., 2012). Thus, future studies are needed to assess the protease(s) responsible for the processing of IL-33 in GECs, the secretion mechanism involved in release of the processed form, as well as the role of NOD1 in IL-33 processing and its impact on H. pylori-induced pathology.

The turnover and release of PG, mediated by the lytic transglycosylase Slt, has been shown to be important for H. pylori to activate NOD1 signalling (Viala et al., 2004). In the present study, we show that AGS cells stimulated with H. pylori slt deficient bacteria induced significantly lower levels of IL-33 mRNA and protein than those stimulated with wild type bacteria (Figure 1e,f). These data suggest the importance NOD1 sensing of PG in IL-33 responses. Consistent with this suggestion, H. pylori OMVs induced IL-33 responses via a NOD1-dependent mechanism (Figure S2). In addition, H. pylori mutant bacteria lacking a functional T4SS system, which is required to activate NOD1 signalling via the delivery of PG into host cells (Viala et al., 2004), were affected in their ability to induce IL-33 responses in human GECs (Figure 4). It is also noteworthy that we observed higher levels of processed IL-33 in the culture supernatants of mouse GECs after stimulation with the clinical isolate 10700, which has a functional T4SS, when compared with those from cells stimulated its mouse-adapted variant (Strain S51), which lacks a functional T4SS (Philpott et al., 2002; Figure 1d). Although the T4SS is not essential for H. pylori induction of NF-kB-dependent responses in mouse GECs (Ferrero et al., 2008), it is possible that this secretion system may be required for maximal IL-33 responses in GECs of murine origin. Altogether, these data suggest that the metabolism and delivery of PG into host cells are crucial for H. pylori activation of NOD1-driven IL-33 responses in GECs.

The induction of both IL-8 and IL-33 production was not completely abrogated in GECs in which NOD1 signalling had been dampened by either pharmacological inhibition, shRNA knockdown or gene deletion (Figure 1), suggesting the potential involvement of a NOD1-independent mechanism in these responses. Although the contribution of CagA in H. pylori induced NF-kB responses remains controversial, we found that H. pylori ΔcagA bacteria induced less IL-8 production compared with wild type bacteria and, moreover, these responses were not completely abolished in shNOD1 AGS cells (Figure 5a). Additionally, we showed that H. pylori ΔcagA bacteria induced lower levels of IL-33 production and processing (Figure 4c, d), suggesting that CagA may act independently of NOD1 signalling to drive IL-33 production. Although beyond the scope of the current study, the mechanism whereby H. pylori CagA regulates IL-33 responses warrants further investigation.

Unlike NOD1, we found that TIFA is dispensable for the production of full-length IL-33 in cell lysates. IL-33 is known as an “alarmin” which is released rapidly during the early phase of infection to alert the host immune system to the presence of bacteria (Molofsky et al., 2015). We speculate that NOD1 is the first sensor recruited to the cell membrane upon initial contact with H. pylori, hence predominantly mediating a rapid response. This speculation is supported by a recent study by Gaudet et al. (Gaudet et al., 2017) showing that NOD1 and TIFA independently contribute to NF-kB activation by the invasive Gram-negative bacterium, Shigella flexneri, and that the activation of NOD1 pathway precedes TIFA. In contrast, however, Gall et al. (Gall et al., 2017) claimed that in H. pylori infection, TIFA is activated prior to the
NOD1 response. Nevertheless, there is currently no experimental evidence to support this sequential activation of NOD1 and TIFA during *H. pylori* infection. Future studies are therefore required to delineate the links between NOD1 and TIFA activation during *H. pylori* stimulation. Interestingly, a reduction in the levels of mature IL-33 was observed in the culture supernatants of cells in which TIFA had been knocked down using siRNA (Figure S4c). Thus, the involvement of both NOD1 and TIFA in IL-33 processing indicates that these proteins might be components of a protein complex that mediates proteolytic cleavage of IL-33 at later phases of infection.

To establish the in vivo significance of NOD1-mediated IL-33 production, we assessed IL-33 production in both the acute and chronic phases of infection (1 and 8 weeks post-infection). In line with a recent clinical study that reported a strong correlation between chronic *H. pylori* infection and IL-33 gene expression in human gastric biopsies (Shahi et al., 2015), we found enhanced IL-33 production in the chronic phase of infection (Figure 5a–c). A recent study by Buzzelli et al. (Buzzelli et al., 2015), however, reported that IL-33 gene expression was increased in the early phase of infection (1 week) but reduced after long term infection. This discrepancy could be attributed to differences in the *H. pylori* strains used in the two studies. Indeed, Buzzelli et al. used the *H. pylori* SS1 strain that was previously shown to have lost its T4SS functions during in vivo colonisation (Philpott et al., 2002). In contrast, we used the *H. pylori* 245 m3 strain (Philpott et al., 2002) in our study which has an intact cagPAI and still possesses a functional T4SS (L. S. Tran, K. D’Costa, unpublished data). It is possible that this allows the bacterium to sustain IL-33 production during long term infection.

Importantly, the levels of gastric IL-33 mRNA and protein were significantly higher in *Nod1−/−* than *Nod1+/−* mice (Figure 5a,b). These IL-33 responses were associated with up-regulation of IL-13 and down-regulation of IFN-γ in splenic lymphocytes (Figure 5d,g). Based on previous findings (Sawai et al., 1999; Sayi et al., 2009), these responses would be expected to attenuate the development of gastric inflammation and affect bacterial clearance. One possible mechanism underlying the beneficial effects of IL-33 is that this cytokine can drive the generation of regulatory T cells which was reported to limit inflammatory responses and reduce bacterial loads in mice infected with *H. pylori* (Rad et al., 2006). In contrast, IL-33 was shown to elicit Th1 immune responses in tumour tissues by inducing IFN-γ production in CD8+ T cells and NK cells (Gao et al., 2015). Thus, future studies should explore the biological consequences of IL-33 production in the context of *H. pylori* infection and disease.

Collectively, in this work, we report for the first time that *H. pylori* infection promotes IL-33 secretion in GECs via a NOD1-dependent mechanism. Additionally, NOD1-driven IL-33 production during chronic *H. pylori* infection is associated with a Th2-type immune response, which may protect against excessive inflammatory responses and favour the persistence of *H. pylori* infection.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cell lines, bacterial, and mouse strains

The mouse GSM06 GEC line (RCB1779) was obtained from Riken Cell Bank and grown as described previously (Ferrero et al., 2008). Briefly, the cells were maintained in Dulbecco’s modified Eagle medium–nutrient mixture/F-12 medium, supplemented with 10% foetal calf serum, 1% (w/v) insulin/transferring/selenium (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.

Human AGS gastric cancer cells stably expressing shRNA to either *EGFP* (sh.EGFP) or NOD1 (sh.NOD1) were generated as described previously (Grubman et al., 2010). These cells were maintained in complete RPMI medium supplemented with 10% (v/v) foetal calf serum, 1% (w/v) L-glutamine and 1% (w/v) penicillin/streptomycin (Gibco; ThermoFisher Scientific, VIC, Australia).

AGS cells harbouring CRISPR/Cas9 mediated NOD1 gene KO were generated by using a “Cas9 nickase” nuclease with pairs of guide RNAs (gRNAs) to introduce double-strand breaks (Ran et al., 2013). The following pair of gRNAs were designed to target the CARD domain of NOD1: 1) gRNA1: GCTGAAGAATGACTTCTTAC; and gRNA2: GCTTTTCGAACTTGAAAGT. These gRNAs were cloned into pSpCas9n(BB)-2A-GFP (PX461; Addgene plasmid # 48140) and pSpCas9n(BB)-2A-Puro (PX462; Addgene plasmid # 48141). The ligated vectors were transfected into AGS cells using LipoFectamine LTx (ThermoFisher Scientific), and then cells were selected with 1 μg/mL puromycin. As a control, cells were transfected with PX461 and PX462 plasmids not carrying gRNAs. Limiting dilution was performed to obtain mutant clones derived from a single cell. Frame-shift KO clones were screened by polymerase chain reaction and pyrosequencing with primers flanking the target region: Fwd-GGCCACAGTGAGATGGAAAT and Rev-GGCCAGCACAACACATCTC. The KO status of NOD1 in AGS cells was further verified by measuring the levels of NOD1 gene expression and NOD1 induced IL-8 production in response to NOD1 agonist C12-IEDAP (10 μg/mL, Invivogen).

*H. pylori* Strains 26695 (wild type, *slt+*; Viala et al., 2004), 251 (wild type, *cagM*, *cagPAI* and *cagA*; Allison et al., 2009), SS1 and 10700 (Lee et al., 1997) were used for in vitro experiments. A mouse-adapted, *cagPAI−* *H. pylori* strain (245 m3; Philpott et al., 2002) was employed for in vivo experiments. All bacterial strains were grown on Blood Agar Base No. 2 (Oxoid; Thermo Fisher Scientific), supplemented with 5% (v/v) whole horse blood (Australian Ethical Biologicals, VIC, Australia), Skirrow’s selective supplement (155 μg/mL Polymyxin B, 6.25 μg/mL vancomycin, 3.125 mg/mL trimethoprim, and 1.25 mg/mL Amphotericin B; Sigma, MO, USA), at 37 °C under microaerobic conditions.

*Nod1+/+ and Nod1−/−* C57BL/6 mice were maintained under specific pathogen-free conditions at the Animal Research Facility (Monash Medical Centre). All animal procedures complied with guidelines approved by Monash Medical Centre Animal Ethics Committee (MMCA/2015/43).

4.2 | Primary GEC isolation

Stomachs were removed from 4 to 5-week-old *Nod1+/+* or *Nod1−/−* mice and the gastric tissues excised and finely minced in 5 ml of Hank’s Balanced Salt Solution without Ca2+ and Mg2+ (Gibco), supplemented with 0.125% (w/v) bovine serum albumin (Sigma), 0.072% (w/v) dispase (Roche Life Science, NSW, Australia), and 0.1% (w/v) Collagenase A (Roche). Tissues were digested by incubation for 2 hr at 37 °C, in 5% CO2 and shaking at 150 rpm. Digested tissues were pelleted and
washed in DMEM/F12 medium by centrifugation at 30 × g for 5 min. Cells were plated at 10⁶ cells/ml per well in 24-well plates coated with 2 mg/ml rat Tail Collagen I (ThermoFisher Scientific). The culture medium was changed every 2 days to remove fibroblasts and epithelial cells were grown for 5 days before performing co-culture assays. Epithelial cell purity was confirmed by immunofluorescence using the epithelial-cell-specific marker, EpCAM (Abcam, Cambridge, UK, 1:200) and isotype control rabbit IgG (Dako).

### OMV isolation

OMVs were purified from mid-exponential phase cultures that were pelleted at 4,000 g for 40 min at 4 °C (Kaparakis et al., 2010). Supernatant fractions were collected and filtered through 0.22-μm filters (Merck Millipore, VIC, Australia). Filtered supernatants were subjected to ultracentrifugation as described previously (Kaparakis et al., 2010). Supernatants were discarded and the pellets resuspended in Brain Heart Infusion medium (Thermo Fisher Scientific). OMV protein concentrations were quantified using the Bradford Protein Assay (BioRad, CA, USA).

### Inhibitor treatment

The small molecule inhibitor, ML130 (CID-1088438), was purchased from Tocris (Bristol, UK) and has been confirmed to selectively inhibit NOD1 signalling (IC₅₀ = 0.56 μM) with no cytotoxicity in previous studies (Correa et al., 2011). The RIPK2 selective inhibitor WEHI-345 was used as described previously (Nachbur et al., 2015). Briefly, cells were pretreated with ML130 (5 μM) or WEHI-345 (0, 5, or 10 μM) for 1 hr prior stimulation with H. pylori, and then maintained in inhibitor-containing medium throughout the experiment.

### siRNA transfection

TIFA or RIPK2-specific silencer and negative control siRNA (40 μmol of each; ThermoFisher Scientific) were diluted in Opti-MEM medium (Gibco) supplemented with 2 μl of Lipofectamine® 2000 reagent (Thermo Fisher Scientific). The mixtures were incubated at room temperature for 20 min then added in a drop-wise manner to each well of 12-well plates containing 10⁵ AGS cells. Transfected cells were incubated at 37 °C for 24 hr prior to co-culture with bacteria.

### Cell co-culture assay

*H. pylori* liquid cultures were obtained by growing bacteria in BHI containing 10% (v/v) heat-inactivated foetal calf serum (Thermo Fisher Scientific) and Skirrow’s selective supplement, in a shaking incubator for 16–18 hr. Bacteria were pelleted and washed twice with phosphate-buffered saline (PBS) by centrifugation at 4,000 × g for 10 min at 4 °C prior to resuspension in culture medium for co-culture assays. Viable counts were performed by serial dilution of bacterial suspensions on horse blood agar plates.

Cells were seeded in 12-well plates at 1 × 10⁵ cells/ml and allowed to grow overnight. The culture medium was removed and replaced with serum- and antibiotic-free medium prior to stimulation with bacteria. Cells were incubated with *H. pylori* wild type or isogenic mutant strains at a multiplicity of infection of 10:1. The bacteria were removed after 1 hr. RNA extraction was performed at 4 hrs post-stimulation, whereas culture supernatants and lysates were collected at 24 hrs post-stimulation for ELISA and Western blot analyses.

### qRT-PCR analyses

RNA was extracted using the PureLink® RNA mini kit (Thermo Fisher Scientific). cDNA was generated from 500 μg of RNA using the Tetro cDNA synthesis kit (Bioline, NSW, Australia), as per the manufacturer’s instructions. qPCR reactions consisted of 4 μl of diluted synthesised cDNA (1:10), 5 μl of SYBR® Green qPCR MasterMix (Thermo Fisher Scientific), and 1 μl of oligonucleotide primers (1 μM) for the tested genes. Oligonucleotide sequences were as follows: Rn18s, Fwd-GTAAACCGTTGAACCCATT and Rev-CCATCCATCAGGTGATG AGCG; Rn18s1, Fwd-CGGCTACCATGCAAGGAGA and Rev-GCGAATTCCGCGCT; Il33, Fwd-CTCAACTCCAGATTCC and Rev-CAAACGGAGTCTCATGCAG; Il33, Fwd-AGTCTCAAC ACCCTCATAAG and Rev-CTTTTGTAGGACTCAGGTACC; RIPK2, Fwd-GCCACCTGGAACACTGAAACC and Rev-CTGCAAGATGTGGTGACATC. TIFA, Fwd-TCTATCCCTGCCGTCG and Rev-CCGGTA TCTGGAGACAGTTAAC. qPCR assays were performed in an Applied Biosystems™ 7900 Fast Real-Time PCR machine (Thermo Fisher Scientific; MHTP Medical Genomics Facility), using the following program: 50 °C, 2 min, followed by 95 °C, 10 min, then 40 successive cycles of amplification (95 °C, 15 s; 60 °C, 1 min). Gene expression levels were determined by the Delta–Delta Ct method to measure relative gene expression levels to the 18S rRNA gene.

### 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 (Thermo Fisher Scientific), supplemented with complete protease and phosphatase inhibitors (Roche). Fifty micrograms of total protein lysate, as determined using the Qubit™ fluorometer (Thermo Fisher Scientific), were resuspended in 30 μl of Laemmli buffer (Thermo Fisher 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 (Thermo Fisher Scientific). The separated proteins were transferred onto membranes using the iBlot® transfer system (Thermo Fisher Scientific), as per the manufacturer’s instructions. The membranes were blocked using Odyssey® blocking buffer (Oddysey; LI-COR, NE, USA). Membranes were incubated with 0.5 ng/ml of goat-antihuman IL-33 antibody (clone AF3625; R&D Systems, MN, USA) overnight, at 4 °C. Membranes were then washed in PBS-Tween 0.05% (v/v) and incubated for 2 hr with 0.67 ng/ml of rabbit-antigot secondary antibody-Alexa Fluor® 680 conjugate (Thermo Fisher Scientific). Membranes were washed and developed on the Odyssey Infrared Imaging System (LI-COR). As loading controls, lysate samples were probed with 0.9 ng/ml of rat-antihuman TUBULIN (Rockland, PA, USA), followed by 0.33 ng/ml of goat-antirat secondary antibody-Alexa Fluor® 800 conjugate (Thermo Fisher Scientific). The relative intensity figures for all blots are now presented in Table S1.
4.9 | Immunofluorescence

GECs were fixed with 4% (w/v) paraformaldehyde for 15 min, at room temperature. Cells were washed in PBS and incubated in blocking buffer (3% BSA [w/v] and 0.1% [w/v] saponin in PBS). After 1 hr, cells were incubated overnight, at 4 °C with 5 μg/ml of goat antihuman IL-33 primary (clone AF3625; R&D Systems). Samples were washed thrice with blocking buffer and incubated with antigas Alexa Fluor® 680-conjugated secondary antibody (1:500) for 1 hr. After washing, cell nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific; 1:1,000) for 5 min before mounting. Imaging was performed on a confocal microscope (Nikon Instruments Inc., NY, USA; Monash Micro Imaging). Cytoplasmic to nuclear ratio measurements were carried out in the FIJI image analysis software on a single image plane extracted from the z-stacks captured by confocal microscopy. All images were captured using the same settings. Images of the cytoplasmic fractions were created by subtracting Hoechst-stained images from IL-33 stained images using the image calculator in FIJI. A threshold was applied to the stained area in the resulting cytoplasmic fraction image. The mean intensity was measured for the area under the threshold. After measuring the intensity, the threshold of the cytoplasmic fraction image was converted to a binary, which was then subtracted from the original IL-33 stained image, using the FIJI image calculator, to generate a nuclear fraction image. A threshold was then applied to the nuclear fraction image and mean intensity measured.

4.10 | ELISA

Human CXCL8 (BD Biosciences, NSW, Australia) and mouse KC, MIP-2 and IL-33 (DuoSet ELISA kit, R&D Systems) were quantified by ELISA, as per the manufacturer's instructions. Absorbance values were measured at 450 nm with a FLUOStar® Optima microplate reader (BMG Labtech, VIC, Australia). Cytokine levels were determined by linear or 4-parameter fit analysis.

4.11 | Mouse H. pylori infection

Nod1+/+ and Nod1−/− mice (6–8-week old) were inoculated via oral gavage with 10⁸ colony-forming units of H. pylori Strain 245 m3 (Philpott et al., 2002), using previously described techniques (Ferrero et al., 2012). Bacterial viability and numbers were determined by agar plate dilution (Ferrero et al., 2012). Stomachs and spleens were harvested from these animals at 1 and 8 weeks post-infection. Gastric tissues were subjected to homogenisation using a gentleMACS™ Dissociator (Miltenyi Biotec, NSW, Australia). Bacterial infection status was confirmed by bacteriological culture (Ferrero et al., 2012). Gastric homogenates were then centrifuged at 13,000 × g at 4 °C and the supernatants collected for analysis by the Qubit™ protein assay (Thermo Fisher Scientific), ELISA, and/or Western blotting.

4.12 | Cytokine production in splenocytes

Spleens were collected from euthanised mice. Single cell suspensions were obtained by grinding through 70 μm cell strainers. Splenocytes were recovered in RPMI complete medium and seeded at 2 × 10⁶ cells/ml in 24-well plates. Cells were left untreated or stimulated with 5 μg/ml Concanavalin A (ConA; Sigma) then incubated for 3 days at 37 °C in 5% CO₂.

ProcartaPlex Multiplex Immunoassays (eBioscience; Thermo Fisher Scientific) were performed according to the manufacturer’s instructions to simultaneously quantify the levels of IL-4, IL-10, IL-13, IL-17, and IFN-γ in splenocyte culture supernatants. Briefly, culture supernatants (50 μl) or standards were mixed with 50 μl magnetic beads and incubated overnight at 4 °C. After washing thrice with washing buffer, 50 μl of streptavidin-PE was added and incubated with shaking for 30 min at RT. The mixtures were resuspended in 120 μl of reading buffer after washing thrice with wash buffer. Data were acquired on the Luminex® 100/200™ instrument (Bio-Rad, NSW, Australia). The concentrations of each cytokine were calculated using the ProcartaPlex® Analyst 1.0 Software (eBioscience).

4.13 | Statistical analyses

GraphPad Prism (GraphPad Software, CA, USA) was used for graph preparation and statistical analyses. For qPCR assays and ELISA, data were analysed by the Mann–Whitney test. Differences were considered statistically significant for p < .05.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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**SUPPORTING INFORMATION**

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