

# Interferon-gamma secretion is induced in IL-12 stimulated human NK cells by recognition of *Helicobacter pylori* or TLR2 ligands

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*Helicobacter pylori* induce a chronic inflammation in the human gastric mucosa characterized by increased production of interferon-gamma (IFN- $\gamma$ ). The presence of natural killer (NK) cells in the human gastric mucosa and the ability of NK cells to produce IFN- $\gamma$  suggest an important role of NK cells in the immune response directed towards *H. pylori* infection. Since NK cells previously have been shown to respond to bacterial components with IFN- $\gamma$  production, we investigated the mechanisms for the recognition of *H. pylori*. We found that inhibition of MyD88 homodimerization resulted in decreased production of IFN- $\gamma$  and that inhibition of the p38 MAPK decreased the production as well as the secretion of IFN- $\gamma$ . Further studies indicated an involvement of Toll-like receptors (TLRs), in particular TLR2. Finally, we showed that the *H. pylori* specific membrane bound lipoprotein HpaA induced IFN- $\gamma$  production from NK cells through recognition by TLR2. In conclusion, we suggest an involvement of TLR2 in the recognition of *H. pylori* by human NK cells and that HpaA is a TLR2 ligand important for recognition.

**Keywords:** Human, NK cells, bacterial infection, cell surface molecules, cytokines, *H. pylori*, IFN- $\gamma$

## INTRODUCTION

It is well established that natural killer (NK) cells play a critical role in the innate immune response against viral infections and tumour cells; however, during recent years, it has become increasingly clear that NK cells also can be involved in the innate response to bacterial infection.<sup>1,2</sup> Natural killer cells are important for host defence both through their cytolytic capacity and their ability to produce cytokines (*e.g.* IFN $\gamma$ ), which provides an important bridge between innate and adaptive immunity.<sup>3</sup> In humans, NK cells represent up to 15% of the lymphocytes in peripheral blood,<sup>4</sup> and are phenotypically characterized by their expression of the adhesion molecule CD56 and lack of expression of CD3.<sup>3</sup> Natural killer cells are also found in peripheral tissues such as the

gastrointestinal mucosa, the liver and the peritoneal cavity.<sup>5</sup>

The Gram-negative bacterium *Helicobacter pylori* is the cause of one of the most wide-spread infections in the world. *Helicobacter pylori* causes a chronic inflammation in the gastric and duodenal mucosa and is associated with an increased risk of developing ulcers and gastric adenocarcinomas.<sup>6</sup> The mechanisms behind the development of gastric cancer in infected individuals are unclear, but it is possible that genetic susceptibility in combination with the prolonged immune response and inflammation driven by the chronic presence of bacteria is promoting tumour development.

Chronic gastric inflammation induced by *H. pylori* is characterized by increased production of IFN- $\gamma$  and interleukin (IL)-12 and is, therefore, considered to be a

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T-helper cell (Th) 1-mediated process.<sup>7,8</sup> Generally, IL-12 is produced in response to bacterial infections by phagocytes and dendritic cells.<sup>9</sup> Neutralization of IL-12 has been shown to reduce the levels of IFN- $\gamma$ , indicating a requirement for IL-12 in the induction of IFN- $\gamma$  production.<sup>7</sup> Furthermore, in mouse studies, it has been shown that protective immunity against *H. pylori* infection is associated with the presence of IL-12 and up-regulation of IFN- $\gamma$  production by Th1 cells.<sup>10</sup>

As both T-cells and NK cells produce IFN- $\gamma$ , interest has been directed towards investigating the role of NK cells in *H. pylori* infection. Thus, it was previously shown by us and others that NK cells respond to Gram-negative bacterial components with IFN- $\gamma$  production, and we demonstrated that stimulating NK cells with *H. pylori* and IL-12 has a synergistic effect on the IFN- $\gamma$  production.<sup>11</sup> Furthermore, the *H. pylori* induced IFN- $\gamma$  secretion was accompanied with activation of surface markers such as CD69 and CD25.<sup>11</sup> Due to their ability to produce IFN- $\gamma$ , the presence of NK cells in the gastric mucosa may indicate that this cell type is important in the immune response to *H. pylori* infection. Although the characteristic of mucosal and peripheral blood derived NK cells are slightly different (*e.g.* higher percentage of CD56<sup>bright</sup> cells among mucosal NK cells) previous results (Lindgren *et al.*, submitted) demonstrate that mucosal NK cells respond in a similar manner as peripheral blood derived NK cells to *H. pylori* stimulation. For practical reasons, peripheral blood derived NK cells have been used throughout this study and we think that the results obtained on peripheral blood derived NK cells are relevant also for mucosal NK cells.

Interferon- $\gamma$  has been shown to be involved in tumour suppression<sup>12</sup> and high infiltration of NK cells in gastric cancer tissue appears to increase the survival rate of cancer patients.<sup>13,14</sup> This is of high relevance, since the majority of gastric cancers are considered to be caused by *H. pylori* infection.<sup>15</sup> Thus, the activation of NK cells by bacterial components might play a significant role in the suppression of tumour development, and a diminished NK-cell activity may consequently increase the risk of acquiring gastric cancer.

The Toll-like receptors (TLRs), which recognize a wide range of microbial molecular patterns, are a part of the innate immune system and are expressed on a variety of cell-types. At present, there are 10 known human TLRs and mRNAs for all are expressed, but at low levels, on human NK cells.<sup>16</sup> The TLRs, in particular TLR2 and TLR5, have previously been described to be involved in the recognition of *H. pylori* by monocytes, macrophages<sup>17</sup> and gastric epithelial cells.<sup>18</sup>

In this study, we investigated the molecular mechanisms behind the recognition of bacterial products, and in particular *H. pylori*, by human NK cells.

## MATERIALS AND METHODS

### Preparation of bacteria

*Helicobacter pylori* (strain Hel305, cagA<sup>+</sup> and vacA<sup>+</sup>, isolated from a patient with duodenal ulcer) from  $-70^{\circ}\text{C}$  stock culture was grown on Columbia iso-agar plates followed by liquid culture in Brucella broth. Lysate of *H. pylori* (Hel 305) was prepared as previously described.<sup>19</sup> The protein contents were determined by spectrophotometry. The lysate was snap frozen in liquid nitrogen and was stored in aliquots at  $-70^{\circ}\text{C}$  until use.

### Purification of NK cells

Peripheral blood NK cells were purified from Buffy coats enriched in leukocytes, obtained from healthy blood donors. Mononuclear cells (PBMCs) were separated from the Buffy coats using Ficoll-Paque gradient centrifugation and NK cells (CD56<sup>+</sup> CD3<sup>-</sup>) were isolated with negative selection magnetic beads (human NK cell isolation kit, Miltenyi Biotec, Germany) according to the manufacturer's instructions. Bead-purified NK cells had a purity of >90% (median purity, 96.1%). The bead-purified NK cells were then either used directly or stained with anti-CD56-PE and anti-CD3-FITC antibodies (both BD Biosciences) and sorted using a flow cytometry cell sorter (FACSVantage SE; BD Biosciences). The flow cytometry sorted NK cells were >99% pure.

### Stimulation of NK cells and PBMCs

The NK cells were cultured in X-vivo 15 medium (Lonza, Belgium) supplemented with 1% L-glutamine in round-bottom, 96-well plates, each well contained  $1 \times 10^5$  cells. The cells were cultured in the presence or absence of IL-12 (50 pg/ml) and Hel 305 lysate (2  $\mu\text{g}/\text{ml}$ ).

HpaA, recombinantly produced and purified, as previously described<sup>20</sup> (1  $\mu\text{g}/\text{ml}$  or 2  $\mu\text{g}/\text{ml}$ ; kindly provided by AstraZeneca, Boston, MA, USA) and recombinant *H. pylori* flagellin (5  $\mu\text{g}/\text{ml}$ ; US Biological, Swampscott, MA, USA) were also used in combination with IL-12. In addition, a recombinantly-produced, truncated form of HpaA (HpaA<sub>trunc</sub>) containing the protein part of the mature HpaA but devoid of the lipid portion was also used in the experiments (2  $\mu\text{g}/\text{ml}$ ). *H. pylori* lysate from the SS1 strain was used for stimulation of NK cells at 20  $\mu\text{g}/\text{ml}$ , 5  $\mu\text{g}/\text{ml}$  and 2  $\mu\text{g}/\text{ml}$ , and a mutant strain lacking HpaA (HpaA<sup>-</sup>) was also used.

The NK cells and PBMCs were stimulated with known TLR agonists (Table 1; TLR agonist kit, InvivoGen, San Diego, CA, USA) with or without IL-12 (50 pg/ml) or IL-2 (50 U/ml) for 48 h at  $37^{\circ}\text{C}$ . The TLR agonists were endotoxin-free according to the

**Table 1.** TLR agonists used throughout the study, and IFN- $\gamma$  levels after stimulation of PBMC

Agonist	TLR activated	Concentration used	IFN- $\gamma$ (pg/ml) in PBMC-cultures		
			No cytokine (n = 6)	IL-12 (n = 4)	IL-2 (n = 3)
Pam <sub>3</sub> CSK <sub>4</sub>	1/2	1 $\mu$ g/ml	0	83	3590
HKLM (heat-killed <i>L. monocytogenes</i> )	2	10 <sup>8</sup> cells/ml	237	1990	3590
FSL-1	2/6	1 $\mu$ g/ml	11.85	584.5	6668
Poly I : C	3	25 $\mu$ g/ml	0	7	3890
LPS ( <i>E. coli</i> K-12)	4	5 $\mu$ g/ml	16	1300	11590
Flagellin ( <i>S. enterica</i> sv. Typhimurium)	5	5 $\mu$ g/ml	8	1740	3980
Imiquimod	7	5 $\mu$ g/ml	0	0	0
ssRNA40	8	5 $\mu$ g/ml	400	1690	11,120
ODN 2006	9	5 $\mu$ M	0	10	81
No TLR agonist	–	–	0	0	15

manufacturer with the exception of *Salmonella enterica* sv. Typhimurium flagellin and *Escherichia coli* LPS. Supernatants from the NK cell cultures were collected at selected time-points and kept at  $-70^{\circ}\text{C}$  until analysis of IFN- $\gamma$  content with an in-house ELISA as previously described.<sup>21</sup> The minimum detectable concentration of IFN- $\gamma$  was 6 pg/ml. Both sorted and bead-purified NK cells were used with similar results.

Analysis of cytokine production was made using CBA Th1/Th2 and Inflammation kits (both BD Biosciences, San Jose, CA, USA). Rabbit anti-human IL-15 (0.2  $\mu$ g/ml; PeproTech EC Ltd, UK) and mouse IgG<sub>1</sub> anti-human IL-18 (1  $\mu$ g/ml; MBL, Woburn, MA, USA) were used to analyze involvement of IL-15 and IL-18 in IFN- $\gamma$  production. Normal rabbit sera and mouse anti-human IgG1 antibody (eBioscience, San Diego, CA, USA) was used as control.

#### Real-time RT-PCR

To determine gene expression levels bead-purified NK cells ( $3 \times 10^5$  cells/sample) were either used directly after cell isolation or incubated in triplicate ( $10^5$  cells/well) with Hel 305 lysate (2  $\mu$ g/ml), IL-12 (50 pg/ml) and the p38 MAPK inhibitor SB203580 (1  $\mu$ M; Sigma, St Louis, MO, USA) at  $37^{\circ}\text{C}$  for 4 h. After sorting/incubation, the cells were resuspended in RLT lysis buffer (Qiagen, Hilden, Germany) supplemented with 1%  $\beta$ -mercaptoethanol and stored at  $-70^{\circ}\text{C}$  until further processing. The mRNA was then extracted using RNeasy Micro kit (Qiagen) according to the manufacturer's instructions and was then either stored at  $-70^{\circ}\text{C}$  or directly used for cDNA preparation. The cDNA was prepared using the Sensiscript Reverse Transcription kit (Qiagen) according to the manufacturer's instructions.

The cDNA was then used for real-time (RT)-PCR using Taqman Universal PCR Master Mix (Applied

Biosystems, Foster City, CA, USA) and Taqman Gene Expression assays (Applied Biosystems) for TLR1, TLR2, TLR4, TLR5 and TLR6, IFN- $\gamma$  and HPRT followed by analysis using the 7500 Real Time PCR system (Applied Biosystems) using standard conditions for relative gene expression analysis as recommended by the manufacturer. The results were normalized to the expression level of the house-keeping gene HPRT.

#### Flow cytometry

Intracellular IFN- $\gamma$  was analyzed using bead-purified NK cells stimulated with FSL-1 (1  $\mu$ g/ml; Invivogen) and IL-12 at  $37^{\circ}\text{C}$  for 24 h. At 5 h prior to the end of the incubation, GolgiPlug (BD Biosciences) was added to the cell culture. Thereafter, the cells were stained with anti-CD3 FITC and anti-CD56 PE-Cy5 (both BD Biosciences). The cells were then resuspended in Cytofix/cytoperm (BD Biosciences) followed by Perm/wash buffer (BD Biosciences) and stained for IFN- $\gamma$  with anti-IFN- $\gamma$  PE antibodies (BD Biosciences) and then analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

The presence of TLRs was determined with flow cytometry on bead-purified NK cells. As primary antibody, polyclonal rat IgG anti-TLR1, anti-TLR 2, anti-TLR 4, anti-TLR 5 and anti-TLR 6 (Invivogen) and as secondary antibody goat anti-rat IgG FITC (Caltag-MedSystems, UK) were used. Normal rat sera were used as control.

#### Inhibition experiments

Inhibition of MyD88 activity were performed using bead-purified NK cells ( $1 \times 10^5$  cells/well) pre-incubated in triplicate in 96-well plates with MyD88 homodimerization inhibitory peptide (100  $\mu$ M, Imgenex, San Diego,

CA, USA) for 24 h at 37°C. The cells were then stimulated with FSL-1 (1 µg/ml; Invivogen)/Hel305 lysate (2 µg/ml) with or without the addition of IL-12 (50 pg/ml) for another 4 h. The NK cells were stimulated with a high concentration of IL-12 (1 ng/ml) as MyD88-independent control. After culture, the cells were collected and frozen in RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol and stored at -70°C until real-time RT-PCR analysis of IFN-γ expression as described above.

Sorted NK cells were stimulated with the PI3K inhibitor Wortmannin (10 µM; Sigma), the MAPK p38 inhibitor SB203580 (1 µM, Sigma) and the calcium uptake channel inhibitor La<sup>3+</sup> (as lanthanum chloride heptahydrate, 5 µM; Sigma) and then incubated with or without IL-12 and Hel305 lysate for 48 h at 37°C. Supernatants were collected and IFN-γ content analyzed by ELISA.<sup>21</sup>

Inhibition of TLR2 activity was performed using anti-human TLR2 (10 µg/ml; eBioscience) on sorted NK cells (1 × 10<sup>5</sup> cells/well) stimulated with Hel 305 lysate (2 µg/ml), wild-type HpaA (2 µg/ml) or FSL-1 (1 µg/ml; Invivogen) in combination with IL-12 (50 pg/ml) for 48 h at 37°C.

#### Statistical analysis

Comparative data were analyzed with GraphPad Prism v.5.0 (GraphPad Software Inc., La Jolla, CA, USA) using Wilcoxon signed ranks test, Mann-Whitney test or Student's paired *t*-test, with a *P*-value of less than 0.05 considered being statistically significant.

## RESULTS

#### Inhibition of MyD88 and p38 reduce IFN-γ production

Human NK cells produce IFN-γ in response to *H. pylori* lysate in combination with IL-12.<sup>11</sup> To elucidate the signalling pathways involved in *H. pylori* induced IFN-γ production, NK cells were stimulated with *H. pylori* lysate and IL-12 in combination with inhibitors of some key components of major signalling cascades.

Treatment with the calcium uptake channel inhibitor La<sup>3+</sup> (data not shown) and the PI3K inhibitor Wortmannin (Fig. 1A) had no statistically significant effect on IFN-γ production. However, treatment with Wortmannin gave an increase in IFN-γ production in some of the tested individuals (3 out of 8), while in others it induced a decrease in IFN-γ production. These results demonstrate that the calcium uptake channels are not involved in the signalling pathways resulting in *H. pylori* induced IFN-γ production, but that PI3K cannot be discarded as a component in the activated pathways.

Inhibition of the p38 MAPK with SB203580 in NK cells stimulated with *H. pylori* lysate and IL-12, led to a 75% reduction of the IFN-γ levels in the cell supernatants (Fig. 1B; *P* = 0.023). No effect on IFN-γ production were observed in cells stimulated with SB203580 alone or in combination with *H. pylori* lysate (data not shown), but only in the presence of both IL-12 and *H. pylori* lysate.

To clarify on which level the p38 MAPK is involved in the production of IFN-γ, mRNA of NK cells stimulated with SB203580 in combination with *H. pylori* lysate and/or IL-12 for 4 h, was analyzed for IFNG mRNA expression (Fig. 1C). Treatment with the p38 MAPK inhibitor reduced the levels of IFNG expressed upon stimulation with both IL-12 and lysate (52% mean reduction; *P* = 0.020) but no difference in expression was observed when IL-12 or lysate alone were combined with the inhibitor (data not shown).

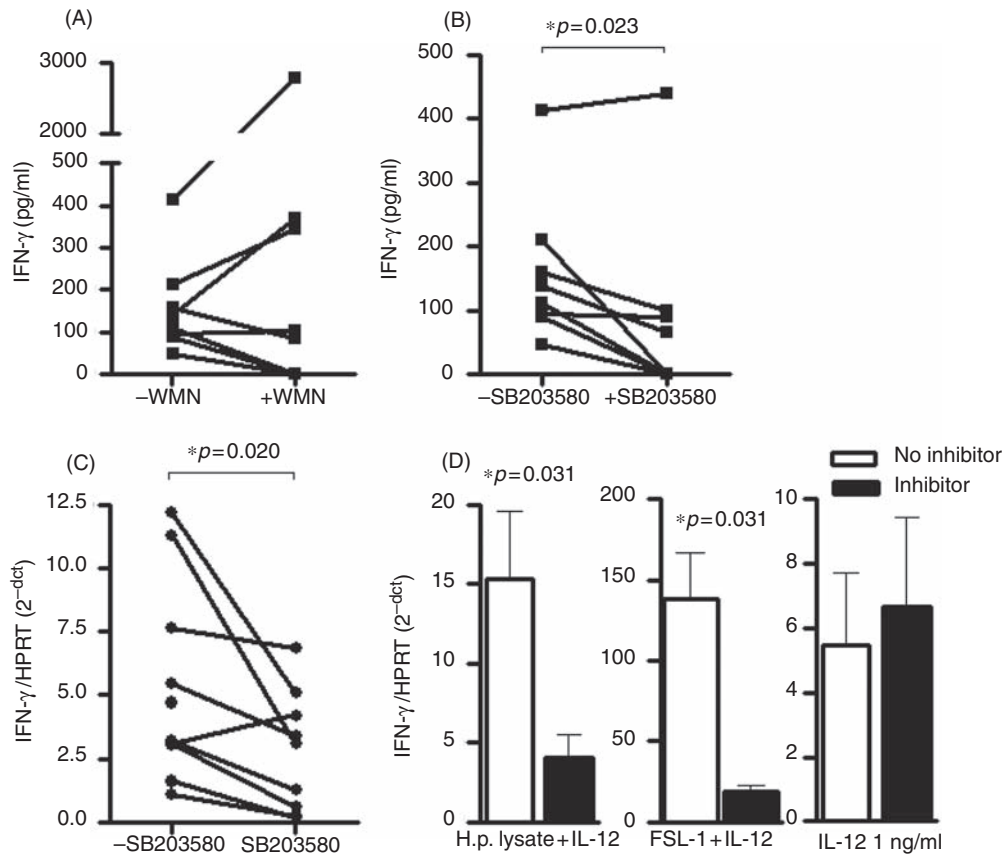
To investigate further the *H. pylori* induced signalling resulting in IFN-γ production by NK cells, homodimerization of the MyD88 adaptor protein was inhibited. Addition of the inhibitory peptide to NK cells prior to *H. pylori* lysate and IL-12 stimulation lead to an inhibition of the IFNG mRNA (Fig. 1D; mean inhibition 74%). As a MyD88-independent control, a high concentration of IL-12 (1 ng/ml) was used to stimulate the NK cells after pre-treatment with the inhibitory peptide, and this did not have any inhibitory effect on the IFN-γ production (Fig. 1D). FSL-1 was used as a MyD88-dependent positive control (Fig. 1D).

Taken together, these results indicate that both p38 MAPK and MyD88 are important components in the *H. pylori* induced signalling cascade resulting in IFN-γ production and that p38 MAPK is involved in the transcriptional regulation of IFN-γ production.

#### No effect of contaminating non-NK cells

The NK cells used in this study were purified from healthy blood donors using magnetic beads and had a purity of >90% (median purity, 96.1%) while the flow cytometry sorted NK cells were 99–100% pure. To rule out contribution to IFN-γ production by contaminating cells, the non-NK cells in the bead-purified population were analyzed by flow cytometry. The majority of the contaminating cells among the bead-purified NK cells were NKT cells and T cells. Less than 0.02% (median value, 0.0125%) of the bead-purified population were DCs (Fig. S1; supporting information).

Also, analysis of cytokine production was made using CBA Th1/Th2 and inflammation kits to assess whether any pro-inflammatory cytokines (IL-2, IL-12, tumor-necrosis factor (TNF)-α, IL-1β, IL-6) produced by contaminating cells after stimulation with lysate or TLR



**Fig. 1.** *Helicobacter pylori* induced IFN- $\gamma$  production is MyD88 and p38 MAPK dependent in NK cells. Sorted NK cells were stimulated with *H. pylori* lysate and IL-12 and treated with (A) Wortmannin (WMN, inhibits PI3K activity) or (B) SB203580 (inhibits p38 activity) for 48 h. Interferon- $\gamma$  secretion in the supernatant was measured by ELISA ( $n=7$  individuals). \* $P<0.05$  compared to samples not treated with inhibitor using Wilcoxon matched pairs test. (C) Bead-purified NK cells were stimulated with *H. pylori* lysate and IL-12 in the presence and absence of SB203580 for 4 h and IFN- $\gamma$  mRNA levels were investigated with real-time RT-PCR. Results are expressed as  $2^{-\Delta C_t}$  and show the relative expression of IFN- $\gamma$  compared to the housekeeping gene HPRT ( $n=9$  individuals). Unstimulated NK cells treated with the inhibitors did not produce any IFN- $\gamma$ . (D) Bead-purified NK cells were pre-incubated with MyD88 homodimerization inhibitory peptide for 24 h and then stimulated with *H. pylori* lysate/FSL-1 and IL-12 for another 4 h and IFN- $\gamma$  expression were analyzed with real-time RT-PCR. Interleukin-12 (1 ng/ml) was used as MyD88-independent control. Results are expressed as  $2^{-\Delta C_t}$  and show the relative expression of IFN- $\gamma$  compared to the housekeeping gene HPRT. ( $n=2-6$  individuals). \* $P<0.05$  compared to samples untreated with inhibitor using Wilcoxon matched pairs test.

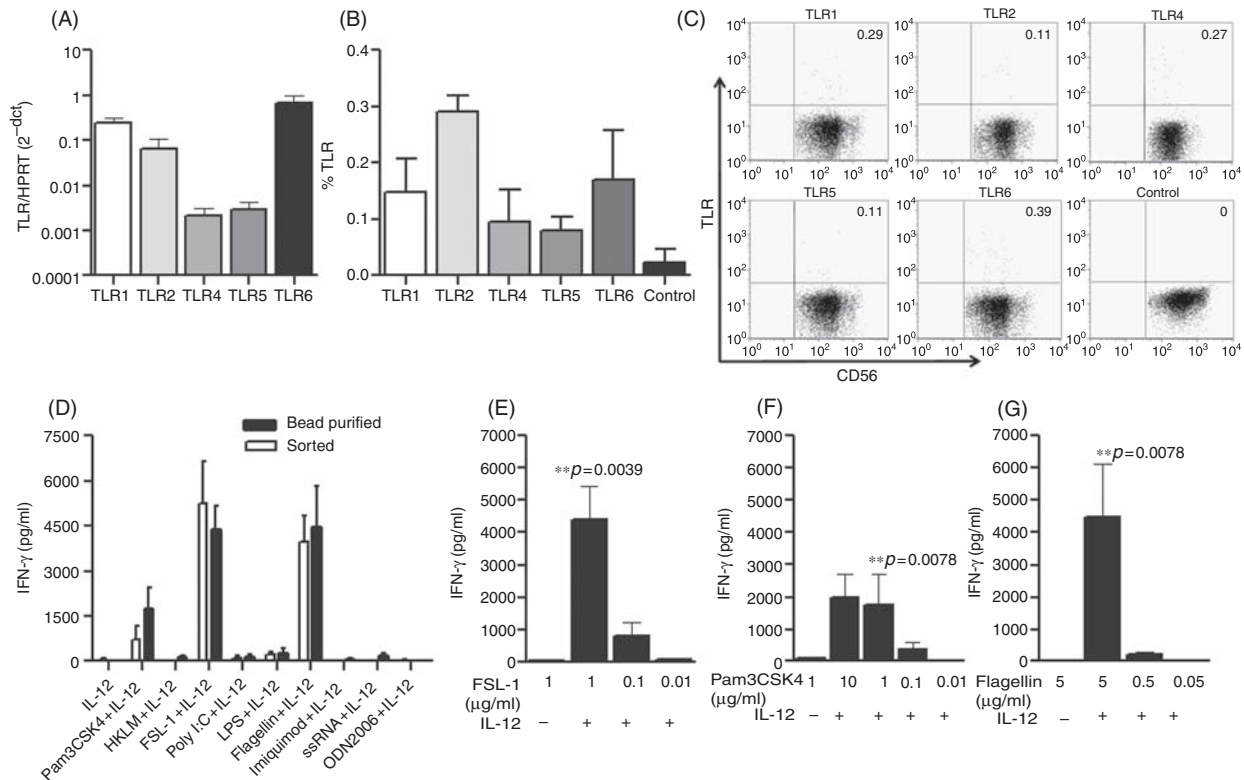
ligands of bead-purified NK cells could contribute to IFN- $\gamma$  production by NK cells (Fig. S2A,B; supporting information). The results showed that TLR ligands did not induce any consistently detectable levels of IL-1 $\beta$ , IL-2, IL-6 or IL-12. tumor-necrosis factor- $\alpha$  was produced in low levels, but these levels did not correlate with the IFN- $\gamma$  levels induced by the stimulations.

To rule out involvement of IL-15 and IL-18 in IFN- $\gamma$  production induced in NK cells,<sup>22</sup> neutralizing antibodies against IL-15 and IL-18 were used. Both bead-purified NK cells and PBMCs were analyzed (Fig. S3; supporting information) and neutralization of IL-15 or IL-18 in bead-purified NK cells did not cause any change in IFN- $\gamma$  production.

Taken together, these results indicate that, even though the bead-purified NK cells are not 100% pure, the contaminating cells were not influencing the production of IFN- $\gamma$  by NK cells in our system.

#### *TLR2 and TLR5 ligands activate IFN- $\gamma$ production in combination with IL-12*

The importance of MyD88 and p38 in the *H. pylori* induced signalling leading to increased IFN- $\gamma$  production by NK cells suggests a possible mechanism involving binding of bacterial membrane associated structures to TLRs on the NK cells. To confirm that NK cells express TLR mRNA, gene expression levels of TLR1, TLR2, TLR4, TLR5, and TLR6, all recognizing bacterial components, were quantified in unstimulated purified NK cells using real-time RT-PCR (Fig. 2A). All the examined TLRs were expressed at the mRNA level (Fig. 2A), confirming previous results.<sup>16</sup> The highest expression levels were found for TLR1, TLR2 and TLR6 while TLR4 and TLR5 were expressed at lower levels. Also flow cytometry staining for TLR1, TLR2, TLR4,



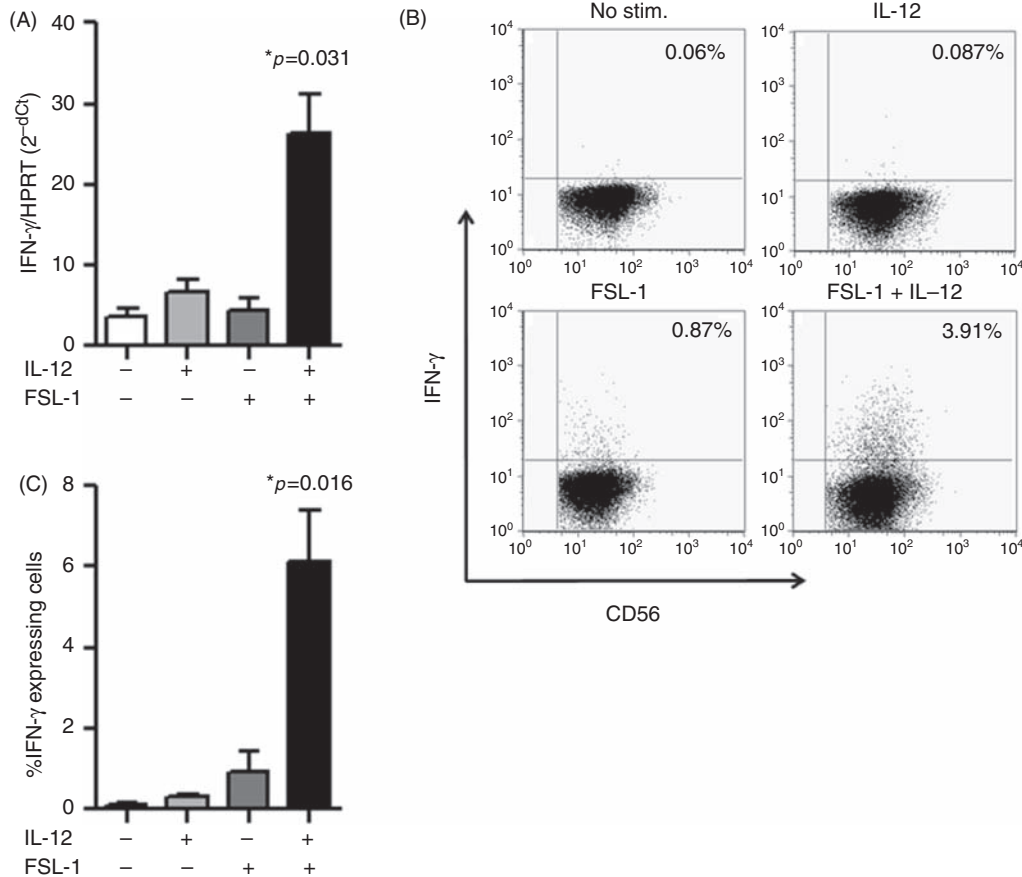
**Fig. 2.** Flagellin, Pam<sub>3</sub>CSK<sub>4</sub> and FSL-1 in combination with IL-12 induce IFN- $\gamma$  in NK cells. (A) The presence of selected TLR was analyzed using real-time RT-PCR in unstimulated bead-purified NK cells ( $n=4$  or 5 individuals). Results are expressed as 2<sup>-dct</sup> and show the relative expression of TLR in relation to the relative expression of the housekeeping-gene HPRT. (B,C) Flow cytometry analysis of TLR protein expression on NK cells ( $n=4$  individuals). Results are expressed as (B) percentage TLR expressing NK cells and as (C) flow cytometric analysis of one representative experiment. (D) NK cells were stimulated with commercially available TLR agonists (see Table 1 for details) for 48 h, in the presence or absence of IL-12. Interferon- $\gamma$  production was measured in the supernatants with ELISA. Both bead-purified and sorted cells are included in the figure, no difference between them could be observed. In an initial experiment, NK cells from 3–5 individuals were treated with TLR agonists. These experiments indicated that three of the agonists induce a response in combination with IL-12; therefore, these three agonists were used in further experiments ( $n=3$ –11 individuals). \*\**P* < 0.01 compared to samples stimulated with IL-12 alone using Wilcoxon matched pairs test. (E–G) Bead-purified NK cells were stimulated with different concentrations of FSL-1, Pam<sub>3</sub>CSK<sub>4</sub> and *S. enterica* sv. Typhimurium flagellin, respectively, with and without IL-12. Interferon- $\gamma$  production was measured by ELISA ( $n=3$  or 4 individuals).

TLR5, and TLR6 (Fig. 2B,C) confirmed the observations on mRNA expression.

To investigate if TLRs are potential upstream recognition receptors for the signalling cascade resulting in IFN- $\gamma$  production, NK cells were treated with established TLR agonists (Fig. 2D; see Table 1 for details regarding stimulation) with or without the addition of IL-12, to try to mimic the situation when NK cells are stimulated with *H. pylori* lysate and IL-12. Initially, it could be observed that none of the viral TLR agonists (poly I:C, ssRNA and Imiquimod) induced any significant IFN- $\gamma$  production in combination with IL-12 (Fig. 2D). However, when IL-12 was used instead of IL-12, the viral agonists did induce some IFN- $\gamma$  (data not shown). The same phenomenon was observed when PBMCs were used (Table 1), indicating differences in requirement for co-stimulation for viral and bacterial immune responses.

Pam<sub>3</sub>CSK<sub>4</sub>, FSL-1 and recombinant flagellin (*S. enterica* sv. Typhimurium) in combination with IL-12 induced the most prominent effects on IFN- $\gamma$  production (Fig. 2D); therefore, these agonists were further investigated in additional individuals. For these three agonists, IFN- $\gamma$  production was induced only in the presence of IL-12 (Fig. 2D,E–G), in accordance with *H. pylori* lysate stimulation of NK cells. This effect was dose-dependent, lower levels of the agonists reduced the levels of IFN- $\gamma$  (Fig. 2E–G).

Both FSL-1 and Pam<sub>3</sub>CSK<sub>4</sub> are synthetic lipoproteins and agonists to TLR2. However, the specificity of the two lipoproteins differ, FSL-1 binds to the TLR2/TLR6 heterodimer<sup>23</sup> and Pam<sub>3</sub>CSK<sub>4</sub> binds to the TLR2/TLR1 heterodimer.<sup>24</sup> An important difference between the two TLR2 agonists could be observed in our experiments, FSL-1-induced IFN- $\gamma$  production by the NK cells from all tested individuals, while Pam<sub>3</sub>CSK<sub>4</sub>-induced IFN- $\gamma$



**Fig. 3.** Both bacterial components and IL-12 are required for production of IFN- $\gamma$  protein but not for transcription of IFN- $\gamma$  mRNA. (A) Bead-purified NK cells were stimulated with FSL-1 and/or IL-12 for 4 h and then analyzed for IFN- $\gamma$  mRNA expression with real-time RT-PCR. Results are expressed as  $2^{-dCt}$  and show the relative expression of IFN- $\gamma$  compared to the housekeeping gene HPRT ( $n=6$  individuals). \* $P<0.05$  compared to unstimulated samples using Wilcoxon matched pairs test. (B,C) Bead-purified NK cells were stimulated with FSL-1 and IL-12 for 24 h and then stained for intracellular IFN- $\gamma$  content. Results are shown as (B) flow cytometric analysis of one representative experiment and (C) percentage IFN- $\gamma$  positive cells within the NK-cell population ( $n=7$  individuals) \* $P<0.05$  compared to unstimulated samples using Wilcoxon matched pairs test.

production only from some (7 of 11 individuals). This indicates that there is an individual variation in the ability to respond with IFN- $\gamma$  production after Pam<sub>3</sub>CSK<sub>4</sub> stimulation.

Taken together, these results identify TLR2 in combination with TLR1 or TLR6 and also TLR5, as candidates for recognition of *H. pylori* and other bacteria by NK cells.

#### *Both IL-12 and bacterial components are required for the transcription, production and secretion of IFN- $\gamma$*

Both IL-12 and bacterial components are required for the secretion of IFN- $\gamma$ , as previously shown.<sup>11</sup> Therefore, we wanted to investigate the individual contribution of the components used in the induction of IFN- $\gamma$  production and secretion.

First, the mRNA expression of IFN- $\gamma$  was investigated with real-time RT-PCR. NK cells were stimulated with

IL-12 and FSL-1 alone or in combination for 4 h and then mRNA was extracted. Analysis revealed that FSL-1 and IL-12 in combination induced the highest expression of IFN- $\gamma$  and that IL-12 or FSL-1 alone were not able to induce an IFN- $\gamma$  mRNA expression significantly higher than that of unstimulated cells (Fig. 3A).

Next, to examine the intracellular levels of IFN- $\gamma$  and hence the production of the IFN- $\gamma$  protein, NK cells were stimulated with IL-12 and FSL-1. Several time points (from 6–72 h) were tested and 24 h was selected as the optimal time point for this assay (data not shown). Intracellular staining of IFN- $\gamma$  in NK cells stimulated with *H. pylori* lysate was also performed; however, the numbers of IFN- $\gamma$  producing NK cells were then too low to be determined with accuracy using the current method. FSL-1 was used since it mimics the response seen with *H. pylori* lysate regarding IFN- $\gamma$  production but with a higher frequency of IFN- $\gamma$  producing cells. The intracellular staining of IFN- $\gamma$  revealed that both

FSL-1 and IL-12 are required for the production, as well as the secretion, of the IFN- $\gamma$  protein (Fig. 3B,C). Furthermore, neither FSL-1 nor IL-12 alone induced any significant IFN- $\gamma$  production (Fig. 3B,C).

Taken together, these results indicate that both bacterial components and IL-12 are required for inducing both translation and secretion of the IFN- $\gamma$  protein.

#### *Helicobacter pylori* flagellin does not induce substantial IFN- $\gamma$ production in combination with IL-12

The TLR5 agonist *S. enterica* sv. Typhimurium flagellin used in the TLR agonist experiments induced high levels of IFN- $\gamma$ . Since there are conflicting data regarding if flagellin from *H. pylori* is a TLR5 agonist or not,<sup>18,25</sup> we investigated whether *H. pylori* flagellin had the same ability as *S. enterica* sv. Typhimurium flagellin to induce IFN- $\gamma$  production from NK cells. Therefore, recombinant *H. pylori* flagellin was used to stimulate NK cells with and without IL-12. *H. pylori* flagellin alone did not induce IFN- $\gamma$  production, though in combination with IL-12 four individuals out of six responded with detectable IFN- $\gamma$  production to *H. pylori* flagellin (Fig. 4A). However, the median level of IFN- $\gamma$  induced was more than 20-fold lower than the median level of IFN- $\gamma$  induced by the same concentration of *S. enterica* sv. Typhimurium flagellin (Fig. 4A;  $P=0.016$ ). To test whether *H. pylori* flagellin could induce higher levels of IFN- $\gamma$  using other conditions, several concentrations of *H. pylori* flagellin were tested in combination with IL-12. However, increasing the concentration of

*H. pylori* flagellin did not have any significant effect on the production of IFN- $\gamma$  (Fig. 4B).

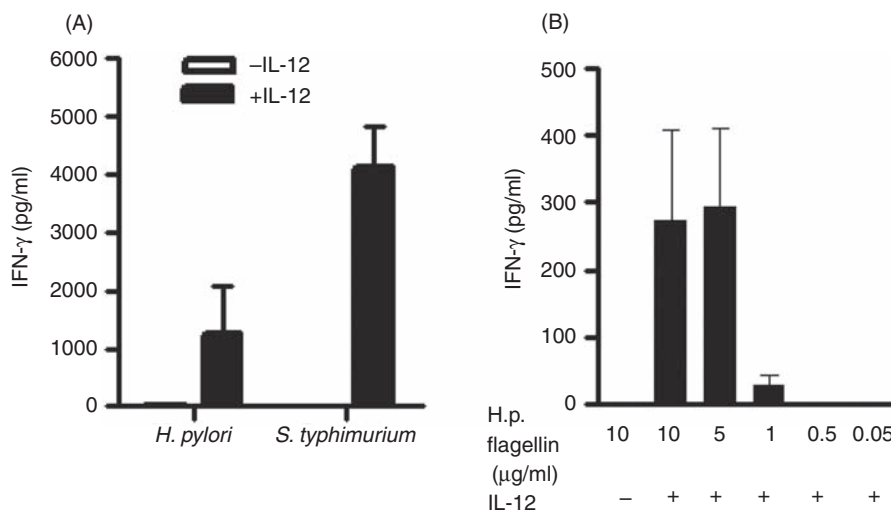
These results indicate that *H. pylori* flagellin is not a major component involved in recognition of *H. pylori* by NK cells.

#### *Helicobacter pylori* lipoprotein HpaA induced IFN- $\gamma$ production in combination with IL-12

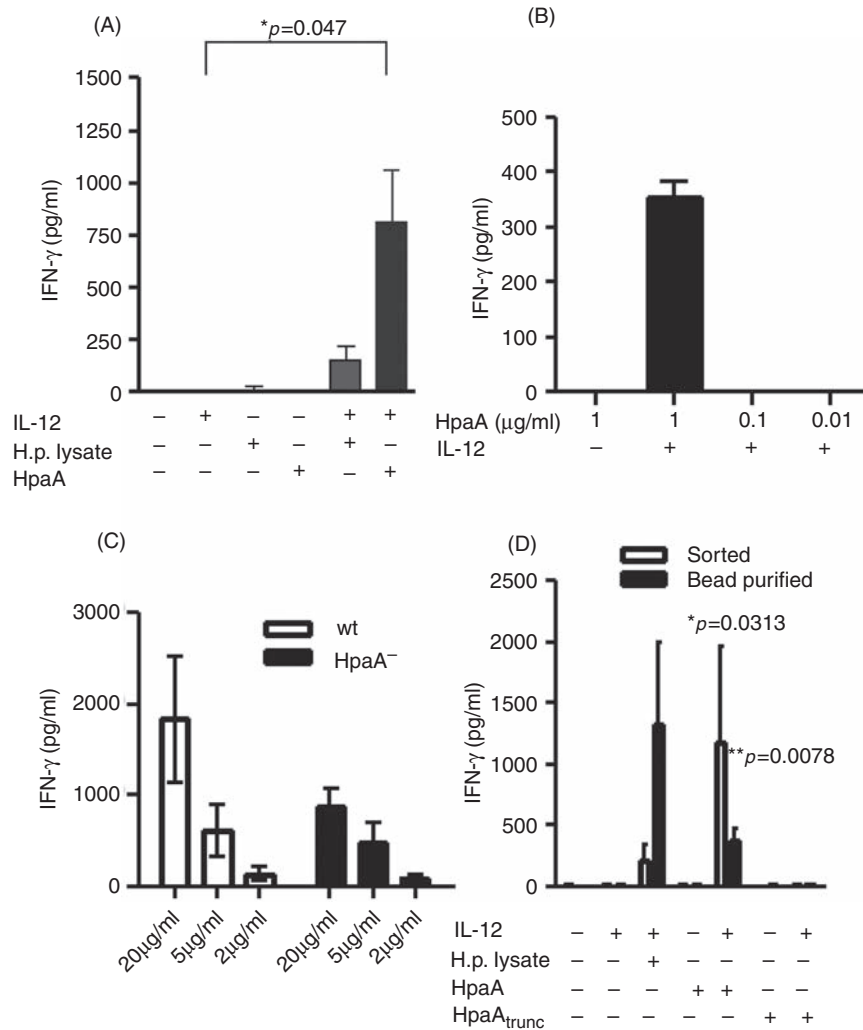
Since *H. pylori* flagellin might not be an effective TLR5 agonist, we suggest that lipopeptides recognized by TLR2/6 or TLR1/2 are more likely to be involved in the recognition of *H. pylori* by NK cells. To test this hypothesis, the *H. pylori* specific putative lipoprotein HpaA, shown to be required for colonization in mice,<sup>26</sup> was used for stimulation of NK cells with and without IL-12. HpaA alone did not elicit any IFN- $\gamma$  production; however, in combination with IL-12, IFN- $\gamma$  was produced in high amounts (Fig. 5A;  $P=0.047$ ), with levels similar to, or exceeding, those seen when whole *H. pylori* lysate was used. The effect of HpaA was dose-dependent, since the effect of HpaA diminished when the concentration was lowered (Fig 5B).

Stimulation of NK cells with *H. pylori* lysate from the SS1 strain and with the HpaA<sup>-</sup> SS1 strain reveal that lack of HpaA does indeed decrease IFN- $\gamma$  production to some extent, although not completely (Fig. 5C).

When HpaA<sub>trunc</sub>, a form of HpaA lacking the TLR2-recognition (presumably important) lipid portion, was used with IL-12 to stimulate NK cells, IFN- $\gamma$  production was markedly lower compared to when NK cells were stimulated with HpaA and IL-12 (Fig. 5D);



**Fig. 4.** *Helicobacter pylori* flagellin does not induce IFN- $\gamma$  in NK cells. (A) Bead-purified NK cells were stimulated with 5  $\mu$ g/ml *H. pylori* recombinant flagellin and 5  $\mu$ g/ml *S. enterica* sv. Typhimurium flagellin in the presence or absence of IL-12 for 48 h and IFN- $\gamma$  secretion were measured with ELISA ( $n=5-10$  individuals). \* $P<0.05$  compared to *H. pylori* flagellin and IL-12, using Mann-Whitney test. (B) Bead-purified NK cells were stimulated with *H. pylori* flagellin in different concentrations with and without IL-12. Interferon- $\gamma$  production was measured with ELISA ( $n=3$  individuals).



**Fig. 5.** The *H. pylori* specific putative lipoprotein HpaA (*H. pylori* adhesin A) induces IFN- $\gamma$  in NK cells. (A) Flow cytometry sorted NK cells were stimulated with 2  $\mu$ g/ml *H. pylori* lysate or 1  $\mu$ g/ml recombinant HpaA in the absence or presence of IL-12 for 48 h and IFN- $\gamma$  secretion was measured by ELISA ( $n=3$  or 4 individuals). \* $P<0.05$  compared to samples stimulated with IL-12 alone using paired  $t$ -test. (B) Sorted NK cells were stimulated with HpaA in different concentrations in the absence or presence of IL-12 for 48 h and IFN- $\gamma$  secretion was measured by ELISA ( $n=2$  individuals). (C) Sorted NK cells were stimulated with wild-type (wt) and mutant (HpaA<sup>-</sup>) SS1 lysate (20, 5 and 2  $\mu$ g/ml) with IL-12 for 48 h and IFN- $\gamma$  secretion was measured by ELISA ( $n=5$  or 6 individuals). (D) Sorted and bead-purified NK cells were stimulated with 2  $\mu$ g/ml HpaA or HpaA<sub>trunc</sub> (truncated HpaA) with or without IL-12 for 48 h and IFN- $\gamma$  production was measured by ELISA ( $n=6-8$  individuals) \* $P<0.05$  compared to samples stimulated with HpaA<sub>trunc</sub> and IL-12 using Wilcoxon matched pairs test.

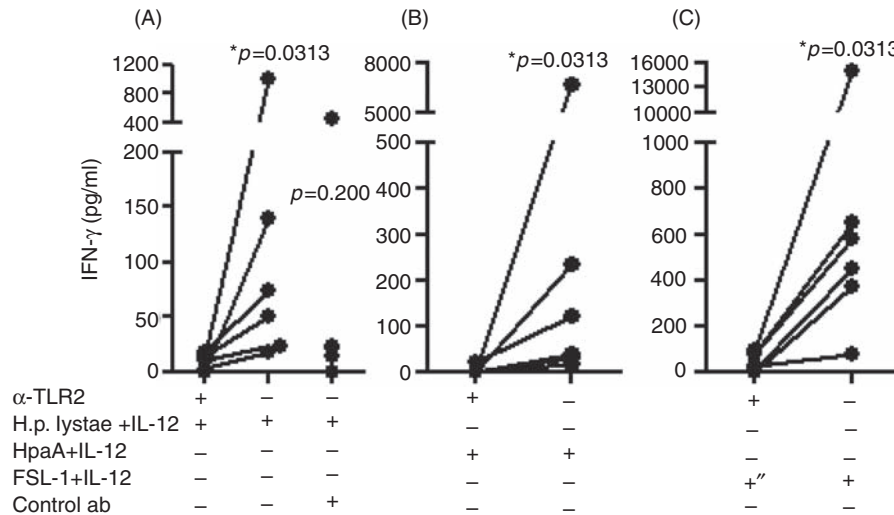
99.5% and 96.7% reduction for sorted and bead-purified NK cells, respectively). This suggests the lipid portion of HpaA is an important part of the protein for the induction of IFN- $\gamma$  production.

Taken together, these results support the hypothesis that lipopeptides or lipoproteins, and in particular HpaA, are important recognition factors involved in NK cell activation by *H. pylori*.

#### Inhibition of TLR2

Since we suggest that lipoproteins and lipopeptides are important for recognition of *H. pylori* by NK cells, we

investigated the effects of blockade of TLR2 activity. natural killer cells were stimulated with *H. pylori* lysate, wild-type HpaA or FSL-1 in combination with IL-12 and then treated with a neutralizing anti-TLR2 antibody. Interferon- $\gamma$  production was clearly reduced in both NK cells stimulated with *H. pylori* lysate (Fig. 6A;  $P=0.0313$ ) and HpaA (Fig. 6B;  $P=0.0313$ ). As a control, NK cells were stimulated with the TLR2/6 agonist FSL-1; as expected, IFN- $\gamma$  production in response to FSL-1 was reduced by anti-TLR2 antibody (Fig. 6C). These results strongly indicate that TLR2 is an important recognition receptor for induction of IFN- $\gamma$  production by *H. pylori* in NK cells, and that HpaA is a TLR2 ligand.



**Fig. 6.** Interferon- $\gamma$  production induced by *H. pylori* or HpaA is TLR2 dependent. Sorted NK cells were stimulated with 2  $\mu$ g/ml *H. pylori* lysate (A), 2  $\mu$ g/ml HpaA (B) or 1  $\mu$ g/ml FSL-1 (C) in combination with IL-12 in the absence or presence of neutralizing anti-TLR2 antibody or control antibody for 48 h. Interferon- $\gamma$  secretion was measured by ELISA ( $n=6$  individuals). *P*-values were calculated compared to samples stimulated without neutralizing anti-TLR2 antibody, using Wilcoxon matched pairs test.

## DISCUSSION

The hallmark of *H. pylori* infection is a Th1 response dependent on production IL-12 and IFN- $\gamma$ . The association between NK cells and *H. pylori* infection is a poorly explored field; however, recent studies have shown that NK cells in the presence of IL-12 are able to respond to the bacterium with IFN- $\gamma$  production.<sup>11</sup> We have shown, in this study, that the molecular mechanisms underlying this response is mainly due to recognition of *H. pylori* membrane lipoproteins by TLR2 in combination with TLR1 or TLR6 on NK cells.

To study the molecular mechanisms in the NK cell-*H. pylori* interaction, we examined if inhibition of major signalling pathways had any effect on *H. pylori* induced IFN- $\gamma$  production. The reduction in IFN- $\gamma$  production observed both when p38 MAPK and the adaptor protein MyD88 were inhibited suggest an involvement of TLRs in the recognition of *H. pylori* by NK cells. MyD88 activation has previously been shown to be necessary for IFN- $\gamma$  production by NK cells stimulated with *Legionella pneumophila*.<sup>2</sup> Also, in agreement with our results, it has been shown in human epithelial cells that MyD88 is a critical signal transducer in *H. pylori* infection.<sup>27</sup> Furthermore, p38 MAPK is often implicated in the signalling subsequent to TLR activation,<sup>28</sup> as well as in the production of IFN- $\gamma$  induced by IL-12 signalling.

We investigated if activation of TLRs with well-established TLR agonists could evoke a similar response to that seen with whole *H. pylori* lysate. Since all currently known TLRs are expressed at the mRNA level in NK cells,<sup>16</sup> this suggests an inherent function of

the cells to respond to a wide variety of microbial components. However, our results indicated that only TLR5, TLR1/2 and TLR2/6 are involved in IFN- $\gamma$  production when NK cells are stimulated with TLR agonists in the presence of suboptimal doses of IL-12. Contrary to this, recent studies show IFN- $\gamma$  production from NK cells using viral TLR agonists and IL-12.<sup>29,30</sup> However, the concentration of IL-12 used in those studies was 20-fold higher (1 ng/ml) than the concentration used in our experiments. Others have claimed that TLR3 ligands directly stimulate NK cells;<sup>31,32</sup> however, in one of the studies,<sup>31</sup> maintenance levels of IL-2 were used in the cell culture media that corresponded to the concentrations used for stimulation in our study (data not shown). Furthermore, TLR7/8 ligands have also been implicated as inducing IFN- $\gamma$  from NK cells;<sup>33</sup> however, that experimental system involved the presence of monocytes making it difficult to consider the contribution of cytokines. Other cytokines than IL-12 have also been suggested to induce IFN- $\gamma$  production, such as IFN- $\alpha/\beta$  and IL-18.<sup>34</sup> Activation of NK cells via TLRs might induce cytotoxicity from the NK cells;<sup>32,33</sup> however, in our hands, *H. pylori* recognition did not seem to affect the cytotoxic ability of NK cells (data not shown).

Taken together, our results demonstrate the unexpected finding that NK cells are more capable of responding with IFN- $\gamma$  production by stimulation with bacterial compared to viral components.

The TLR5 agonist *S. enterica* sv. Typhimurium flagellin that was used in the initial TLR agonist experiments (Fig. 2D) did evoke a strong response from the NK cells. The *H. pylori* flagellin did not have

the same prominent effect, although the response differed markedly between individuals (Fig. 4A). Indeed, there are conflicting data concerning whether *H. pylori* flagellin is a TLR5 agonist at all. Although several studies claim *H. pylori* flagellin to be a TLR5 ligand,<sup>18,35</sup> it was recently shown that the flagellae of *H. pylori* and other chronically infecting bacteria are mutated to avoid recognition by host TLR5 as a mechanism for evasion of the immune response.<sup>25</sup> It might be that the response induced by *H. pylori* flagellin in previous studies<sup>18,35</sup> is not TLR5-dependent but rather due to contamination or that *H. pylori* flagellin may be able to signal via TLRs other than TLR5. Our results indicate that *H. pylori* flagellin may, in some individuals, evoke some IFN- $\gamma$  production from NK cells, but it is most likely not the main component involved in the induction of IFN- $\gamma$ .

The *H. pylori* specific antigen HpaA is a putative lipoprotein found in the membrane of *H. pylori* that has been shown to be essential for colonization in mice and present in all *H. pylori* strains.<sup>26</sup> The production of IFN- $\gamma$  that was induced in NK cells by HpaA in combination with IL-12 indicates a possible involvement of this antigen in the recognition of *H. pylori*. However, the HpaA<sup>-</sup> *H. pylori* SS1 strain (Fig. 5C) only partially decreased *H. pylori* induced IFN- $\gamma$  production, which indicates that HpaA is only one of several antigens recognized by NK cells. Database searches of the *H. pylori* genome revealed that about 20 membrane lipoproteins or putative membrane lipoproteins are encoded in the genome; for example, *rlpA*, *Lpp20* and *cag12*. These lipoproteins are also candidate proteins that may be involved in the recognition of *H. pylori* by TLR2 similar to HpaA. This will be investigated in future studies.

Furthermore, the reduction in IFN- $\gamma$  production when NK cells were stimulated with HpaA<sub>trunc</sub> instead of HpaA (Fig. 5D) indicates that HpaA is recognized via TLR2 in combination with TLR1 or TLR6, since the lipid portion of the protein seems to be essential for IFN- $\gamma$  production. Toll-like receptor 2 has previously been shown to be the key receptor for direct NK cell recognition of *Mycobacterium bovis*<sup>36</sup> and of lipophosphoglycan (LPG) from *Leishmania*,<sup>37</sup> indicating that recognition of bacterial components by NK cells is not a *H. pylori* specific phenomenon.

In the infected stomach, NK cells interact with accessory cells such as dendritic cells (DCs). It has previously been demonstrated that bacterial<sup>1,38</sup> and viral<sup>30,33</sup> components activate DCs and NK cells via TLRs. This primes the NK cells for IFN- $\gamma$  production and induces IL-12 production in DCs which activates the NK cells.<sup>39</sup> However, although the presence of DCs might enhance the response, NK cells are able to produce IFN- $\gamma$  even in the absence of DC-derived

IL-12.<sup>11,32,37,40</sup> This is in agreement with our previous observations that *H. pylori* up-regulates expression of CD69 and CD25 in NK cells and that high concentrations of *H. pylori* lysate without the presence of IL-12 are able to induce IFN- $\gamma$  production even in highly purified NK cells.<sup>11</sup> This suggests that the NK cells themselves are capable of responding to bacterial components via TLRs but that IL-12 enhances this response.

We suggest that NK cells recognize *H. pylori* via TLR2, which induces up-regulation of the IL-12 receptor,<sup>11</sup> making the NK cells more sensitive to IL-12. At the same time, *H. pylori* induces IL-12 production from, for example, DCs and monocytes,<sup>40</sup> which in turn induce IFN- $\gamma$  production from the NK cells. Direct cell-cell contact between the NK cells and the IL-12 producing cells is not necessary, since the presence of IL-12 alone is sufficient for IFN- $\gamma$  production.

From the hypothesis that NK cells recognize *H. pylori* through TLRs, the question of how the NK cells encounter the bacterium *in vivo* inevitably follows. First, NK cells have been shown to be present in the gastrointestinal mucosa.<sup>11</sup> *Helicobacter pylori* infection induces a life-long chronic inflammation in the epithelium, which leads to a greater permeability of the tight junctions of the cells, resulting in a leakage of antigens into the tissue<sup>41</sup> and, hence, the NK cells in the tissue are constantly exposed to *H. pylori* components. *Helicobacter pylori* has been shown to bud off membrane vesicles which, in turn, have the ability to modulate the immune response.<sup>42</sup> Therefore, it is plausible that NK cells encounter the antigens or secreted vesicles and, via TLRs, recognize *H. pylori* specific membrane proteins. Recently, reports have also shown the presence of intact invading *H. pylori* in gastric tissue.<sup>43</sup> Thus, there is also the possibility that even intact bacteria may be encountered by NK cells *in vivo*.

Whether activation of NK cells has any relevance for the progress of *H. pylori* infection *in vivo* in a positive or negative manner has not yet been investigated. However, the involvement of NK cells in several other bacterial infections such as *Listeria monocytogenes*,<sup>44</sup> *Shigella flexneri*<sup>45</sup> and *S. enterica* sv. Typhimurium<sup>46</sup> indicates that the main role of NK cells in bacterial infections is to control the infection rather than to be involved in clearance. Furthermore, in a model of another *Helicobacter* species, *Helicobacter felis*, it was shown that IFN- $\gamma$  was essential for controlling bacterial numbers.<sup>47</sup> This indicates that IFN- $\gamma$  from NK cells might limit the infection also in *H. pylori* infection. In addition, in mouse models for some other Gram-negative bacteria, such as *L. pneumophila*<sup>2</sup> and *Bordetella pertussis*,<sup>1</sup> NK cells have been shown to be crucial for clearance of the infection via IFN- $\gamma$  production.

It is well recognized that the chronic inflammation induced by *H. pylori* leads to an increased risk of developing gastric cancer. When the cancer has been established, prolonged survival rates of the patients are correlated with high numbers of NK cells in the tumour.<sup>13,14</sup> In addition, we speculate that the presence of NK cells may be associated with a decreased risk of developing gastric cancer, since, in the presence of *H. pylori*, NK cells do not produce any pro-inflammatory cytokines other than IFN- $\gamma$  (Fig. S2B, supporting information). Thus, while NK cell-derived IFN- $\gamma$  production may contribute to tumour immunosurveillance, NK cells do not contribute to production of the cancer-inducing pro-inflammatory cytokine IL-6 (Fig. S2B, supporting information). We, therefore, believe that the production of IFN- $\gamma$  by NK cells responding to *H. pylori* antigens may lead to protective effects on the severe long-term consequence of chronic *H. pylori* infection – gastric cancer.

### CONCLUSIONS

We have shown that: (i) NK cells are activated by bacterial TLR agonists; (ii) it is likely that NK cells recognize *H. pylori* via TLRs, in particular TLR2; and (iii) this process is MyD88 and p38-dependent. We have also shown that the *H. pylori* specific lipoprotein HpaA is important for the recognition of the bacteria and that the recognition is TLR2-dependent.

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