Accepted Manuscript

*Helicobacter pylori* Depletes Cholesterol in Gastric Glands to Prevent Interferon gamma Signaling and Escape the Inflammatory Response


PII: S0016-5085(17)36712-4
DOI: 10.1053/j.gastro.2017.12.008
Reference: YGAST 61583

To appear in *Gastroenterology*
Accepted Date: 14 December 2017


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Defence — Protected Niche — Defence

Cholesterol Extraction

IFN-γ / IL-22

Activated $T_H$ cells

IL-8
*Helicobacter pylori* Depletes Cholesterol in Gastric Glands to Prevent Interferon gamma Signaling and Escape the Inflammatory Response

**Short title:** *H pylori* blocks IFNG signaling

**Authors:** Pau Morey\(^1\*\), Lennart Pfannkuch\(^3\*\), Ervinna Pang\(^1\*\), Francesco Boccellato\(^1\), Michael Sigal\(^1\,2\), Aki Imai-Matsushima\(^1\), Victoria Dyer\(^1\), Manuel Koch\(^1\), Hans-Joachim Mollenkopf\(^1\), Philipp Schlaermann\(^1\) and Thomas F. Meyer\(^1\*\)

\(^1\)Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany

\(^2\)Department of Hepatology and Gastroenterology, Charité University Medicine

\(^*\)These authors contributed equally to this work

**Grant Support:** P.M. was supported by the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°316682, E.P. and L.P. received support from the Deutsche Forschungsgemeinschaft through grant SFB633 to T.F.M., M.S. was funded as a clinician scientist by the Berlin Institute of Health (BIH). The funders played no role in the design of the study or the collection, analysis and interpretation of the data.

**Abbreviations**

aCAG cholesteryl-6’-O-acyl-a-glucoside

aCPG cholesteryl-6’-O-phosphatidyl-a-glucoside

ALI air-liquid interface

AMP antimicrobial peptides
CagA  cytotoxin-associated gene A

cagPAI  cag pathogenicity island

CFU  colony-forming units

Cgt  cholesterol-α-glucosyltransferase

CTxB  cholerae toxin B subunit

CXCL  C–X–C motif chemokine

DRM  detergent-resistant membranes

EGFR  epidermal growth factor receptor

GGT  γ-glutamyl transpeptidase

GM1  ganglioside M1

hBD3  human beta defensin 3

HK  heat killed

IFNAR1  interferon α receptor 1

IFNGR1  interferon γ receptor 1

IFN  interferon

IL22  interleukin 22

IL6  interleukin 6

IP-10  IFNG-inducible protein 10

IRF  interferon regulatory factors

JAK  Janus Kinase

MAPK  mitogen-activated protein kinase

MIG  monokine induced by IFNG

MOI  multiplicity of infection

MZ  myriocin and zaragozic acid

mβCD  methyl-β-cyclodextrin

NF-κB  nuclear factor κ B

p.i.  post-infection

PEG-cholesterol  Poly(ethylene glycol)-derived cholesterol
PVDF  polyvinylidene fluoride
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
SHP-2  Src homology 2 domain–containing protein tyrosine phosphatase
STAT1  signal transducer and activator of transcription-1
TGFBR  transforming growth factor-β receptor
vacA  vacuolating cytotoxin A

Correspondence: Prof. Dr. Thomas F. Meyer, Department of Molecular Biology, Max Planck Institute for Infection Biology, Charitéplatz 1, 10117 Berlin, Germany
E-mail: tfm@mpiib-berlin.mpg.de
Tel: +49 30 28 460 400
Fax: +49 30 28 460 401

Disclosures: The authors declare no conflict of interest

Transcript profiling
Microarray data have been deposited in the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) of the National Center for Biotechnology Information and can be accessed with the GEO accession number GSE76589.

Author Contributions: P.M., E.P. and L.P. designed and performed the experiments and analyzed the data; V.D. and M.K. performed the in vivo experiments; H-J.M. analyzed the microarray data; P.S., F.B., M.S., A.I.M., M.K, and T.F.M. provided experimental guidance during the study; P.M., L.P. and T.F.M. wrote the manuscript; T.F.M. conceived the study and provided conceptual guidance.
Abstract:

Background & Aims: Despite inducing an inflammatory response, Helicobacter pylori can persist in the gastric mucosa for decades. H pylori expression of cholesterol-α-glucosyltransferase (encoded by cgt) is required for gastric colonization and T-cell activation. We investigated how cgt affects gastric epithelial cells and the host immune response.

Methods: MKN45 gastric epithelial cells, AGS cells, and human primary gastric epithelial cells (obtained from patients undergoing gastrectomy or sleeve resection or gastric antral organoids) were incubated with interferon gamma (IFNG) or IFNB and exposed to H pylori, including cagPAI and cgt mutant strains. Some cells were incubated with methyl-β-cyclodextrin (to deplete cholesterol from membranes) or myriocin and zaragozic acid to prevent biosynthesis of sphingolipids and cholesterol and analyzed by immunoblot, immunofluorescence, and quantitative reverse transcription PCR analyses. We compared gene expression patterns among primary human gastric cells, uninfected or infected with H pylori P12 wt or P12Δcgt, using microarray analysis. Mice with disruption of the IFNG receptor 1 (Ifngr1–/– mice) and C57BL6 (control) mice were infected with PMSS1 (wild-type) or PMSS1Δcgt H pylori; gastric tissues were collected and analyzed by reverse transcriptase PCR or confocal microscopy.

Results: In primary gastric cells and cell lines, infection with H pylori, but not cgt mutants, blocked IFNG-induced signaling via JAK and STAT. Cells infected with H pylori were depleted of cholesterol, which reduced IFNG signaling by disrupting lipid rafts, leading to reduced phosphorylation (activation) of JAK and STAT1. H pylori infection of cells also blocked signaling by IFNB, interleukin 6 (IL6), and IL22 and reduced activation of genes regulated by these signaling pathways, including cytokines that regulate T-cell function (MIG and IP10) and anti-microbial peptides such as human β-defensin 3 (hBD3). We found that this mechanism allows H pylori to persist in proximity to infected cells while inducing inflammation only in the neighboring, non-infected epithelium. Stomach tissues from mice infected with PMSS1 had increased levels of IFNG, but did not express higher levels of interferon-response genes. Expression of the IFNG-response gene IRF1 was substantially higher in PMSS1Δcgt-infected mice than PMSS1-infected mice. Ifngr1–/– mice were colonized by PMSS1 to a greater extent than control mice.

Conclusions: H pylori expression of cgt reduces cholesterol levels in infected gastric epithelial cells and thereby blocks IFNG signaling, allowing the bacteria to escape the host inflammatory response. These findings provide insight into the mechanisms by which H pylori might promote gastric carcinogenesis (persisting despite constant inflammation) and ineffectiveness of T cell-based vaccines against H pylori.

KEY WORDS: hBD3, JAK/STAT, IL-22, hBD3
About half the world’s population is chronically infected with the gram-negative bacterium *Helicobacter pylori*, which is implicated in severe gastric disease, including peptic ulcer and adenocarcinoma\(^1\). Colonization takes place in the gastric mucus and eventually involves bacterial adherence to the glandular epithelium\(^2\). The infection is characterized by a rapid and strong NF-κB-driven response\(^3\), which leads to the recruitment and activation of immune cells: CD4+ T cells, dominated by the Th1 lineage\(^4, 5\) play a decisive role in controlling the *Helicobacter* load via secretion of IFNG\(^6\). This Th1 response is also fueled by type I IFNs (α/β) released from gastric epithelial cells themselves\(^7\). Th17 and Th22 T cells, characterized by IL17 and IL22 production, also promote the inflammatory milieu, helping to control infection\(^8, 9\). Yet, *H pylori* is able to escape full elimination by host immunity through an unknown mechanism, resulting in a severe chronic inflammatory condition that represents a crucial aspect of its pathogenesis.

Gastric epithelial cells display receptors for type I (α/β) and type II (γ) interferons, the subunits of which (IFNAR1/IFNAR2 and IFNGR1/IFNGR2, respectively) are assembled in specialized cholesterol-rich membrane microdomains, known as lipid rafts\(^10\). Microdomain-dependent receptor activation\(^11\) triggers JAK/STAT signaling via STAT1/2 phosphorylation and nuclear translocation to promote downstream expression of genes involved in inflammation and defense, including interferon regulatory factors (IRF), which further amplify the IFN response through positive feedback via STATs\(^12\). Gastric epithelial cells also express receptors for IL22\(^9\), which can stimulate the production of antimicrobial factors that defend against mucosal pathogens\(^13\).

The effector mechanisms controlling colonization by *H pylori* remain sparsely understood. Epithelial cells can produce antimicrobial peptides (AMP), such as hBD3, which effectively kills *H pylori*\(^14, 15\) and is induced through MAPK and JAK/STAT signaling and stimulation with IFNG or IL22\(^14, 16, 17, 18\). Strikingly, despite continued inflammation, hBD3 is not detected in infected human gastric biopsies\(^15, 19\). We have previously shown that *H pylori* prevents hBD3 expression through a CagA-dependent mechanism\(^14\). However, how hBD3 remains blocked despite IFNG and IL22 stimulation is unclear.
H pylori, which is auxotroph for cholesterol, extracts the lipid from host membranes to incorporate it into its outer membrane as an α-glucosylated derivative, using the enzyme Cgt encoded by the gene HP0421 (cgt)\textsuperscript{20-22}. Cgt is the first in a series of H pylori enzymes that cause additional modifications to generate cholesteryl-6'-O-aycl-α-glucoside (αCAG) or cholesteryl-6'-O-phosphatidyl-α-glucoside (αCPG)\textsuperscript{18}. Cholesterol glucosylation and extraction from host cells result in lipid raft destruction and/or alteration of the membrane architecture\textsuperscript{21, 22}, which has been linked to immune evasion and bacterial persistence\textsuperscript{22-24}. We previously reported that a cholesterol-rich diet leads to a reduction of the H pylori load concomitant with an increased Th1 response\textsuperscript{22}.

Here, we provide insight into the underlying mechanism by showing that the IFN response is subverted by H pylori in gastric epithelial cells. This is caused by Cgt-dependent cholesterol depletion, resulting in the destruction of lipid rafts, failure of IFN receptor subunit assembly and, ultimately, lack of downstream signaling. Similarly, Cgt blocks IL6 and IL22 signaling. Our data provide evidence for the highly effective destruction of the responsiveness of H pylori infected epithelium, even in the presence of strong cytokine signals from the adjacent micro-environment, thus impairing mucosal defense.
Materials and Methods

Ethical permissions

Human gastric tissue specimens were obtained from individuals undergoing gastrectomy or sleeve resection, under the ethics approval by the Charité Ethics Committee (EA1/058/11 and EA1/129/12). Animal experiments were performed in mice maintained under pathogen-free conditions based on approval by the Ethics Committee for Animal Experimentation of the State of Berlin (G0205/12).

Cell culture infection and treatment

Bacteria were collected from plates and resuspended in RPMI 1640 (serum-free). All cells were grown to 60% confluency, washed twice with PBS and serum starved with RPMI 1640 for 16 h prior to experiments. Infection was carried out under serum-starved conditions at 37 °C, 5% CO₂ for the indicated times at MOI 20 or 50. Treatment with mβCD (Sigma) was carried out for 5 h at indicated concentrations. Biosynthesis of sphingolipids and cholesterol was inhibited by treating cells with myriocin (50 µM, Sigma) for 72 h and zaragozic acid (50 µM, Cayman Chemical) for the last 18 h. In selected experiments, bacteria were treated with water-soluble cholesterol (Sigma) at 1 mg/ml for 1 h prior to infection. Alternatively, PEG-cholesterol (10 mg/ml, Sigma) was added to cultures during the last hour of infection, while mock-infected cells were treated with an equal volume containing cholesterol. After infection, recombinant human IFN G (R&D Systems, 10 ng/ml), IFNB (PBL Assay Science, 2300 U/ml), IL6 (Peprotech, 25 ng/ml) or IL22 (Peprotech, 50 ng/ml) were added to selected wells and maintained for indicated times until the end of the experiment.

Primary gastric epithelial air-liquid interface (ALI) cultures

Cells from organoids or freshly isolated glands were seeded in trans-well inserts (Millipore PIHP01250) in 24-well plates and wells filled with 400 µl primary cell culture medium. Once cells had formed a confluent monolayer, medium on top of the cells was removed to start ALI culture. Cultures
were kept at 37°C, 5% CO$_2$ in a humidified incubator. Ten days later cells were infected by placing 50 µl of bacteria in PBS (MOI 100) on top of the filters for 3 days.

For further information see Supplementary Materials and Methods

**Data deposition**

Microarray data have been deposited in the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) of the National Center for Biotechnology Information under GEO accession number GSE76589.
Results

*H pylori* blocks JAK/STAT signaling upon IFNG treatment.

To assess the influence of *H pylori* on the response to IFNG, we infected MKN45 gastric epithelial cells with strains P12 and P1 for 6 or 24 h, followed by treatment with IFNG for 30 min. Immunoblotting revealed STAT1 phosphorylation in response to IFNG treatment in non-infected cells, cells infected with heat-killed bacteria, and cells infected for 6 h (Figure 1A). Surprisingly, after prolonged infection (24 h) IFNG stimulation failed to activate STAT1, irrespective of the *H pylori* strain (Figure 1A). To analyze the dynamics of this phenotype, we performed a time course experiment, showing that STAT1 phosphorylation was diminished after 16 h and completely blocked from 24 until 96 h of infection with P12 (Supplementary Figure 1A). To investigate the underlying mechanisms, we thus chose the 24 h time point of infection as a reference. In AGS cells, too, 24 h of infection inhibited the response to IFNG (Supplementary Figure 1B). MOI 10 was sufficient to partially block IFNG signaling in MKN45 cells within 24 h and a complete block was observed with MOI 50 (Supplementary Figure 1C), which was therefore chosen for further experiments.

Upon IFNG stimulation, an activated IFNGR complex phosphorylates JAK1 and JAK2 kinases, which in turn phosphorylate STAT1. After 24 h infection with P12 or P1, neither JAK1 nor JAK2 was phosphorylated any more in MKN45 cells (Figure 1B and 1C middle panel; Supplementary Figure 1D). Notably, JAK1 (Figure 1B) and JAK2 (data not shown) were activated in non-infected MKN45 cells even without IFNG stimulation; however this was also diminished after infection. Infection conditions did not compromise cell viability (Supplementary Figure 1E).

*Cgt* is required for inactivation of IFNG-JAK/STAT1 pathway

To identify the bacterial factor involved in the block of IFNG signaling, we infected MKN45 cells with *H pylori* wild type and mutant strains. As a recent report linked CagA translocation to STAT1 dephosphorylation via SHP-2 activation\(^25\), we tested deletion mutants for *cagPAI*, which encodes the...
entire type IV secretion system, \textit{cagA} and \textit{cgt}. While the \textit{cagA} and \textit{cagPAI} mutant strains still inhibited JAK/STAT1 signaling upon IFNG stimulation, the \textit{cgt} mutant did not (Figure 1D and Supplementary Figure 1B). Immunofluorescence (IF) analysis showed that P12\textDelta cgt (Figure 1C and Supplementary Figure 1D) did not block JAK2 activation upon IFNG treatment, despite adhering to epithelial cells at levels comparable to wild type. Similarly, STAT1 signaling upon IFNG treatment was still inhibited in a Cgt-dependent manner at 48 h.p.i. (Supplementary Figure 1F). Although genetic complementation of the \textit{cgt}-mutant strain only partially reconstituted Cgt expression (Supplementary Figure 1G), it was enough to restore the ability to impair JAK/STAT1 activation (Figure 1E) in MKN45 cells. Finally, we evaluated the consequences of disrupted JAK/STAT1 signaling by analyzing expression of downstream genes after IFN treatment, by infecting MKN45 cells for 24 h with P12 wild type or P12\textDelta cgt prior to treatment with IFNG for 2.5 or 5 h, followed by RT-qPCR. Expression of IRF1, as well as the T cell attractant chemokines MIG and IP-10 (encoded by CXCL9 and CXCL10 genes, respectively), was strongly upregulated in non-infected cells upon IFNG treatment (Supplementary Figure 1H). Cells infected with P12\textDelta cgt also responded to IFNG, but after infection with wt P12 the response was significantly reduced. Accordingly, Cgt activity blocks the JAK/STAT1 pathway, as well as transcription of downstream genes involved in amplification of the IFNG response.

\textbf{Inhibition of IFNG response is linked to host cholesterol depletion, lipid raft disruption and IFNGR assembly}

Cholesterol acquisition by \textit{H pylori} has been linked to the destruction of lipid rafts in epithelial cells\textsuperscript{22}. Lipid rafts serve as platforms for cell signaling cascades, including IFN signaling\textsuperscript{26}. As previously described\textsuperscript{22} we observed cholesterol depletion in cells infected with wild type \textit{H pylori} (Supplementary Figure 2A). To connect this to the block of the IFNG response, we treated cells with methyl-\textbeta-cyclodextrin (m\textbeta CD), which depletes cholesterol from eukaryotic membranes. Treatment did not affect cellular viability (Supplementary Figure 2B) but impaired both JAK1 and STAT1
phosphorylation (Figure 2A). We also inhibited the biosynthesis of sphingolipids and cholesterol by combined treatment with myriocin and zaragozic acid (MZ) to disrupt lipid raft function\textsuperscript{11}. MZ treatment indeed significantly blocked the induction of IRF1 expression in response to IFNG (Supplementary Figure 2C). In addition, when we coated \textit{H pylori} with water-soluble cholesterol before infection to abolish cholesterol transfer from host cells, they did not block the cellular response to IFNG treatment (Figure 2B). Cholesterol-coating also restored the capacity of wt \textit{H pylori}-infected cells to upregulate IRF1 in response to IFNG, at levels comparable to non-infected cells (Supplementary Figure 2D). Cholesterol-coating did not affect bacterial viability (Supplementary Figure 2E). To control for any effects on the initial host-pathogen interplay (e.g. by masking bacterial adhesins), we repeated the experiment by adding PEG cholesterol to the medium during the last hour of infection. This partially rescued the capacity of infected cells to respond to IFNG treatment, further highlighting the importance of cholesterol as a mediator of inflammation in response to \textit{H pylori} (Figure 2C).

The IFNGR subunits 1 and 2 need to merge at cholesterol-rich micro-domains\textsuperscript{11} in order to respond to IFNG stimulation and initiate downstream signaling via JAK1 and JAK2\textsuperscript{10, 27}. We investigated the impact of \textit{H pylori} on the integrity of lipid rafts by assessing potential alterations in the assembly of functional IFNGR. According to IF analysis, surface accumulation of glycosphingolipid ganglioside GM1, a constituent marker of lipid rafts, is lost after infection with P12 wt and P12ΔcagA (Figure 2D, quantification of the relative membrane GM1 signal in Supplementary Figure 2F). In contrast, infection with P12Δcgt did not notably alter GM1 distribution (Figure 2D and Supplementary Figure 2F). In addition, we performed a membrane fractionation to separate lipid rafts as detergent-resistant membranés (DRM). In control cells, fractions containing cholesterol-rich micro-domains, identified by the presence of raft markers GM1 or caveolin, partitioned in top fractions (Supplementary Figure 2G). Combining these fractions for Western blot analysis showed that GM1 as well as IFNGR1 were lost in DRMs upon infection with wild type P12 but not P12Δcgt, regardless of IFNG treatment (Figure 2E). In total lysates, IFNGR1 and IFNGR2 were detected in all conditions.
Finally, we performed immunoprecipitation of IFNGR subunit 2 to test co-precipitation of IFNGR1. IP-specificity was controlled for by absence of the transferrin receptor CD71, a protein not associated with lipid rafts or the IFNGR complex (Supplementary Figure 2I). In line with recent findings\textsuperscript{11} MKN45 cells showed constitutive oligomerization of IFNGR subunits even in the absence of IFNG (Figure 2F). Despite this, prolonged wt \textit{H pylori} infection but not P12\textDelta{}cgt abolished assembly of receptor subunits. Coating \textit{H pylori} with exogenous cholesterol prior to infection restored assembly of IFNGR subunits (Figure 2G). Together, these data suggest that subversion of JAK/STAT1 signaling by \textit{H pylori} takes place at the very top of the pathway, by preventing assembly of IFNG receptor subunits through lipid raft destruction.

\textbf{\textit{H pylori} blocks the IFNG response \textit{in vivo} in a Cgt-dependent manner}

Reportedly, \textit{H pylori} cgt mutants are unable to colonize mice\textsuperscript{21, 22}. Since Cgt activity is required for the block of the IFNG response, which in turn protects against \textit{H pylori} \textit{in vivo} \textsuperscript{6}, we investigated whether the IFNG response is linked to bacterial clearance. After 3 days of infection PMSS1\textDelta{}cgt was already undetectable in wild type mice (Figure 3A). Mice infected with PMSS1 or PMSS1\textDelta{}cgt presented similar IFNG levels in the stomach, however the induction of the IFNG downstream response gene IRF1 was substantially higher in PMSS1\textDelta{}cgt-infected mice (Figure 3B). Next, we infected mice for two weeks with PMSS1 to allow a stronger Th1 response to develop. We observed a corpus-predominant gland occupation by \textit{H pylori} in all analyzed mice (Figure 3C). Notably, the IFNG expression (Figure 3D, left) was higher compared to the less colonized antrum. However, IRF1 expression remained at similar levels (Figure 3D, right). Infection with wild type \textit{H pylori} thus leads to increased levels of IFNG, but fails to increase expression of interferon response genes consistent with the notion of a Cgt-dependent block. Finally, although we reasoned that \textit{H pylori} PMSS1\textDelta{}cgt might be able to infect Ifngr1-knock out mice, (Figure 3A, right) this was not the case. Knockout mice did show increased colonization by wild type PMSS1, indicating that additional defense-related pathways apart from IFNG might contribute to the clearance of PMSS1\textDelta{}cgt.
Cholesterol depletion by *H pylori* inhibits type I IFN, IL6 and IL22 signaling and downstream hBD3 response

Lipid raft integrity is also known to be required for type I IFNs and IL6 signaling\textsuperscript{10, 28}. In congruence, we observed that *H pylori* P12 also inhibited IFNB-induced STAT1 signaling in MKN45 cells, and this effect was dependent on the presence of *cgt* but not *cagA* or *cagPAI* (Figure 4A). Also, mβCD treatment inhibited IFNB signaling in a dose-dependent manner (Supplementary Figure 3A). Similarly, wild type infection and mβCD treatment, but not infection with the *cgt* mutant, prevented STAT3 phosphorylation upon IL6 treatment (Figure 4B). IL22 is a crucial cytokine for mediating the epithelial defense against mucosal pathogens. Binding to receptors in epithelial cells triggers STAT3 activation, inducing expression of antimicrobial factors\textsuperscript{13}. Again, 24 h infection with wt P12 but not a P12Δ*cgt* mutant strain inhibited IL22 signaling transduction in epithelial cells (Figure 4C); the block was also observed upon mβCD treatment (Figure 4C). Genetic rescue of the *cgt* mutant partially restored the bacterial capacity to block IL22 signaling (Supplementary Figure 3B). Since IL22 signaling has not previously been reported to depend on cholesterol, we demonstrated restoration of IL22 signaling by adding PEG-cholesterol to MKN45 cells prior to IL22 stimulation (Supplementary Figure 3C).

As IFNG and IL22 are reported to induce epithelial expression of hBD3\textsuperscript{29}, a potent defensin against *H pylori*\textsuperscript{15, 19}, we tested the impact of infection on the cytokine-induced hBD3 response. Infection of MKN45 cells for 24 h with P12Δ*cgt* but not P12 wt, followed by treatment with IFNG for 5 h, induced a significant increase of hBD3 transcription (Figure 4D, left), which was even more dramatic after 24 h IFNG treatment (Figure 4D, right). In contrast, the increase over time in non-infected and P12 wt infected cells was minimal. Similarly, IL22 treatment also induced hBD3 expression in P12Δ*cgt*-infected cells, albeit to a lower extent, following a similar time-course as IFNG (Figure 4E). In summary, *H pylori* blocks the production of hBD3 in epithelial cells stimulated with IFNG or IL22. Interestingly, co-stimulation with a *cgt*-deficient strain induced substantially higher hBD3 expression.
compared to cytokine treatment alone. Overall, these data confirm that cholesterol depletion by \textit{H pylori} not only inhibits the response to IFNG, but also type I IFNs, IL6 and IL22.

Inhibition of JAK/STAT signaling in human primary gastric epithelial cells

We next used human gastric primary cultures\textsuperscript{30,31} to validate our observations during authentic host-pathogen interaction. Cells derived from primary gastric antral organoids were seeded on plastic and infected with \textit{H pylori} under serum starvation prior to IFNG or IFNB treatment for 30 min. Similar to results obtained with MKN45, \textit{H pylori} P12, but not P12\textsuperscript{Δcgt}, prevented JAK1/STAT1 phosphorylation (Figure 5A). These differences were also observed at higher MOI and in cells isolated from the corpus region (Supplementary Figure 4A), independent of donors (Supplementary Figure 4B). In contrast to cancer cell lines, primary gastric epithelial cells did not exhibit constitutive JAK1 activation (Figure 5A). Block of IFNG signaling was observed at 24 h post infection, but not at earlier time points (Supplementary Figure 4C) or with a low MOI (Supplementary Figure 4D). Cholesterol-coated bacteria, however, failed to block JAK/STAT1 signaling (Figure 5B). Further, block of IFN signaling, as determined by the upregulation of the type II IFN-activated gene CXCL9 in response to IFNG stimulation, was observed in \textit{H pylori} P12 wt, P12\textsuperscript{ΔcagA}, P12\textsuperscript{ΔcagPAI} or P12\textsuperscript{ΔvacA} but not in P12\textsuperscript{Δcgt}-infected cells (Supplementary Figure 4E). Moreover, \textit{H pylori} P12wt, but not P12\textsuperscript{Δcgt} inhibited STAT3 phosphorylation upon IL22 treatment at 24 h post infection (Figure 5C). Genetic rescue of P12\textsuperscript{Δcgt} restored its capacity to block the response to IFNG and IL22 in primary epithelial cells (Supplementary Figure 4F and G).

To assess the global cellular response to infection with \textit{H pylori} P12 wt or P12\textsuperscript{Δcgt}, we performed microarray analysis with human primary gastric cells infected with P12 wt or P12\textsuperscript{Δcgt}. GO term enrichment analysis revealed responsiveness for genes involved in ‘response to external stimuli’, ‘signal transduction’ and ‘immune response’, which was similar for both wt and P12\textsuperscript{Δcgt}-infected cells (Figure 5D, and data not shown). Many of the pro-inflammatory genes upregulated upon
infection with either strain are related to NF-κB signaling (Figure 5E). Therefore, while *H pylori* effectively blocks the response to IFNG, IFNB and IL22 in normal human gastric epithelial cells, initial sensing of the pathogen by NF-κB \(^3\) remains largely unaffected.

*H pylori* generates micro-niches of diminished inflammatory response

To spatially resolve the effects *H pylori* exerts, we examined the resulting cellular phenotypes over longer periods of time using a novel infection model of human gastric primary cells in air-liquid interphase culture\(^32\). This model enables longer infection times and resembles the *in vivo* situation more closely, since it features greater cell type diversity, a protective layer of mucin and cell polarization (Supplementary Figure 5A). After 3 days of infection with P12 wt at MOI 100, cells were treated with IFNG for 30 min and analyzed by immunofluorescence. Interestingly, IFNG treatment led to nuclear translocation of phospho-STAT1 (Figure 6A, middle panel). Micro-colonies of spiral-shaped bacteria (red) formed infection foci (Figure 6B and Supplementary Figures 5A and B) correlating with areas of reduced phospho-STAT1 exhibition (Figure 6A, bottom panel). At lower magnification, areas of reduced STAT1 activation appeared to clearly correlate with infected areas, while non-infected areas of the same filter responded normally to IFNG (Figure 6B). Next, we quantified the number of phospho-STAT1-positive cells in infected compared to non- or less well-infected microscopic fields of the same filter. The vast majority of infected areas displayed phospho-STAT1 levels below the activation threshold while non-infected fields exhibited a robust IFNG response (Supplementary Figure 5C). Infection with the P12Δcgt mutant at MOI 200 led to similar colonization densities, yet the infected monolayer exhibited full STAT1 phosphorylation upon IFNG similar to non-infected cells (Supplementary Figure 5D). Similarly, *H pylori* infection also hampered STAT3 phosphorylation upon IL22 treatment in infected areas (Figure 6C). Thus, using an advanced epithelial cell culture model, we demonstrate that *H pylori* prevents infected cells from responding to IFNG or IL22. This favors the formation of micro-colonies at micro-niches devoid of STAT1 or STAT3 activation. Although non-
infected sites of the same culture retain full responsiveness, this does not halt micro-colony formation at protected sites.
Discussion

A hallmark of gastric infections with *H pylori* is the strong NF-κB-driven response of the epithelium. This initiates a chronic inflammatory condition fueled by the recruitment of immune cells, which produce IFNs\(^4\)\(^6\) and other cytokines, including IL22\(^1\)\(^3\). These cytokines trigger a second wave of epithelial responses, characterized by the release of defensins and other bactericidal factors, to restrict pathogen growth\(^2\)\(^9\). As we report here, *H pylori* evolved a powerful means to prevent infected epithelial cells from responding to this cytokine burst – shutting down bactericidal activity right at the site of infection. This intriguing phenomenon, exerted by the bacterial enzyme Cgt, is in line with previous observations that point to an unexplained inhibition of IFNG-induced nuclear translocation of STAT1 during *H pylori* infection\(^3\)\(^3\). Our findings now provide the mechanistic clue to our earlier characterization of *cgt* as a critical determinant of *H pylori* immune evasion\(^2\)\(^2\).

The gastric mucosa of patients exhibits a marked prevalence of pro-inflammatory cytokines and mediators that provoke innate and Th1-, Th17- and Th22-driven immune responses\(^8\),\(^9\),\(^3\)\(^4\),\(^3\)\(^5\). However, IFNG and IL17 merely control, rather than clear, established infections. Similarly, IL22 induces the production of relevant antimicrobial factors in gastric epithelial cells, but surprisingly IL22 knockout and wt mice show similar *H pylori* colonization rates\(^1\)\(^8\). Previous work identified the *H pylori* factors VacA and GGT as immune modulators that impair the activation and proliferation of T cells\(^5\),\(^3\)\(^6\). They skew normal T cell maturation from Th1 and Th17 towards a Treg phenotype and thereby elicit a considerable degree of local and systemic tolerance\(^3\)\(^7\). Although this deficit of T cell maturation can be rescued by vaccination, vaccine-driven T cell activation normally achieves only a reduction of the bacterial load concomitant with an increased inflammation\(^3\)\(^8\). This paradox points to a block of the T cell-mediated immunity at the effector side of host epithelial defense, which can be explained by the action of Cgt. Since Cgt acts only on infected cells, this gives rise to an intriguing scenario: While inflammation prevails in the infected tissue, Cgt generates protected islands of diminished defense, promoting pathogen survival. Indeed, this notion is corroborated by observations with chronically infected mice challenged with a secondary *H pylori* infection\(^3\)\(^9\). Newly incoming bacteria were unable
to colonize infected mice except for a few glands already occupied by the primary infection, pointing towards an overall anti-microbial environment in the infected stomach. Together these observations suggest that *H pylori* forms protected micro-niches surrounded by an otherwise inflamed and microbicidal milieu (Figure 7).

Amongst the T cell chemotactic factors affected by Cgt are MIG and IP-10, which contribute to protection against *H pylori*\(^7, 40\). Homeostasis of the mucosal colonization by *H pylori*, avoiding an excessive bacterial load, is thought to be achieved through secretion of a variety of innate factors, including defensins, mucins and oxidative metabolites\(^15, 41, 42\). Many of these are produced by the epithelium in response to IFNG and IL22\(^13, 16, 17\). hBD3, one of the most potent factors against *H pylori*, is induced in an IFNG-dependent manner in the context of chronic gastrointestinal inflammation\(^15, 16\).

We have previously observed that EGFR-dependent induction of hBD3 is downregulated upon translocation of *H pylori* CagA via the activation of SHP-2\(^14\) and CagA-dependent activation of SHP-2 also interferes with IFN signaling\(^25\). Thus, CagA acts synergistically with Cgt in preventing IFN signaling and hBD3 synthesis, with Cgt expressed also in strains that lack the *cag*PAI T4SS. Our results, however, show a minimal contribution of CagA in blocking JAK/STAT signaling, possibly due to variations in the active motifs of CagA proteins in our European strain as compared to Asian strains\(^43\), such as the one used by Wang and colleagues\(^25\). Consistently, biopsies from European patients exhibited poor bactericidal activity against *H pylori* and lack of hBD3, regardless of whether they are *cag*PAI/cagA positive or negative\(^15, 19\).

Lipid rafts are also targeted by other pathogens known to block IFN signaling like West Nile virus, which modifies the distribution of lipid rafts in the membrane\(^44\) and *Leishmania donovani*, which disrupts lipid rafts in macrophages to inhibit assembly of IFNGR subunits\(^27\). Here, using *H pylori*, we achieve striking mechanistic insights into microbial cholesterol depletion from host cells, an apparently common, yet little appreciated virulence strategy. Accordingly, cholesterol depletion prevents the partitioning of IFNGR1 to lipid rafts and association with IFNGR2 subunits\(^27\). In MKN45 cells, we observed an association of IFNGR2 and IFNGR1 subunits even in the absence of infection,
consistent with the abnormal, constitutive JAK1/2 activation. However, this association collapsed upon *H pylori* infection, rendering these cells unresponsive, independently of the presence of interferons.

Our *in vivo* experiments confirmed a Cgt-dependent block of the IFNG response, with the results obtained in Ifngr1 knockout mice indicating that additional pathways contribute to preventing colonization with the PMSS1Δcgt mutant. This finding is consistent with the inhibitory action of Cgt on multiple lipid raft-dependent pathways, including IL6 and IFNB\textsuperscript{10, 28, 44} and IL22, in addition to IFNG. Vice versa, Cgt may also cause receptor activation such as for EGFR and TGFBR, where cholesterol depletion increases the number of molecules available for ligand binding\textsuperscript{26, 45, 46}. Apart from this, many signaling routes, particularly those involving the pro-inflammatory pathway NF-κB, appear to function normally *in H pylori* infected cells, even at a time when cholesterol depletion has progressed.

We have substantiated our findings in primary epithelial cells from different donors. Importantly, the use of cells from human gastric organoids allowed us to address biological questions in a mutation-free background\textsuperscript{30}. Although primary cell models are increasingly used to address questions of infection biology\textsuperscript{47} little data related to *H pylori* are available yet. Here, we also utilized an advanced ALI model, which offers several improvements: (i) cultures grow under the influence of Wnt to maintain stemness; (ii) cells differentiate towards a diversity of cells, including pit, neck, chief, parietal and neuroendocrine cells; (iii) cell polarization and secretion of a protective mucus layer supports an authentic equilibrium between cells and bacteria, enabling long-term infection\textsuperscript{32}.

As we have previously shown, dietary cholesterol supplementation significantly reduces the *H pylori* burden in mice\textsuperscript{22}. The decreased colonization came at the cost of substantially increased IFNG-driven inflammation. Our current results, together with the established link between lipid rafts and IFN signaling\textsuperscript{10, 27}, are in complete agreement with previous *in vivo* findings\textsuperscript{22}. Accordingly, cholesterol supply in the context of *H pylori* infection enhances JAK/STAT signaling together with the release of antibacterial effectors. Such increased Th1 responses, however, are also thought to promote
preneoplastic lesions\textsuperscript{6, 48}. This notion, in turn, is consistent with the situation in patients suffering from increased blood cholesterol levels, especially in the form of low-density lipoprotein-cholesterol, who often exhibit severe \textit{H pylori}-induced gastritis\textsuperscript{49}. Accordingly, a low-cholesterol diet may reduce the pathology of \textit{H pylori} gastric infections. This places Cgt function, cholesterol metabolism, and inflammation at the crossroads of gastric pathogenesis and cancer.
Acknowledgements:

We thank Jörg Angermann, Kirstin Hoffmann, Stefanie Mülllerke and Ina Wagner for technical assistance, Robert Hurwitz for generating the Cgt antibody, Toni Aebischer, Bianca Bauer, Michael Fehlings, June Ghosh-Guha and Eric Perret for fruitful discussions and Rike Zietlow for expert editing of the manuscript.
References


**Figure Legends**

Figure 1. Prolonged infection prevents IFNG-induced activation of JAK1, JAK2 and STAT1 through Cgt activity.

MKN45 cells were infected with the indicated strains (HK=heat killed) at MOI 50 and treated (+) or mock-treated (-) with IFNG (10 ng/ml, 30 min) at the indicated times post-infection (p.i.). A) and B) Immunoblot analysis of total protein level and phosphorylation of JAK1 or STAT1, respectively. C) Analysis of JAK2 phosphorylation (red) by confocal IF, maximum projection. Cells were non-infected (NI), or infected with indicated *H pylori* strains (green) and treated with IFNG (10 ng/ml, 30 min). Scale bar = 5µm. D) and E) Immunoblot analysis JAK1 and STAT1 phosphorylation and total protein levels of MKN45 cells infected or mock-infected (NI) for 24 h at MOI 50 with the indicated *H pylori* strains and treatment with IFNG (10 ng/ml, 30 min).

Figure 2. Cholesterol depletion by *H pylori* blocks IFNG signaling by disrupting lipid rafts, impairing IFNGR complex assembly

A) Immunoblot analysis of JAK1/STAT1 phosphorylation after 5 h mβCD treatment at indicated concentrations or in non-treated cells (-), with or without IFNG treatment (10 ng/ml, 30 min). B) Immunoblot analysis of JAK1 and STAT1 phosphorylation upon IFNG treatment (+). MKN45 cells were infected for 24 h with the indicated strains or non-infected, then treated with IFNG (+) (10 ng/ml, 30 min). Cholesterol-coating of bacteria was started 1 h before infection and applied to NI cells accordingly. C) Immunoblot analysis of STAT1 phosphorylation upon IFNG and/or PEG-cholesterol treatment. MKN45 cells were infected for 24 h with *H pylori* wt or non-infected. In selected wells (+), PEG-cholesterol (5 mg/ml) was added during the final hour of infection. Then, cells were treated with IFNG (10 ng/ml, 30 min) (+) or mock-treated (-). Quantification: band intensity ratio STAT1pY:actin. D) Confocal microscopy analysis (x-y sections with lateral views along dotted lines: x-z, bottom; y-z right) of the lipid raft marker GM1 (green) in membranes of cells non-infected or infected for 24 h with
indicated strains. Nuclei stained with DAPI (blue). Scale bar: 5 µm. E) Immunoblot analysis of detergent-resistant membranes for IFNGR1, IFNGR2, caveolin or dot blot analysis with CTxB-HRP. Cells were non-infected or infected for 24 h with indicated strains, followed by treatment (+) or mock-treatment (-) with IFNG (10 ng/ml, 30 min). F) and G) Immunoprecipitation (IP) and immunoblot analysis of IFNGR subunits 1 and 2. IFNGR2 was pulled down using IFNGR2 antibody. MKN45 cells were infected with *H. pylori* P12 wt or coated with water-soluble cholesterol before infection. Results representative of two independent experiments.

Figure 3. IFN response triggered by *H. pylori* in vivo is ineffective.

A) Bacterial clearance in C57BL6 wt and Ifngr1 -/- mice orally infected with PMSS1 wt or PMSS1Δcgt. Each point represents CFU counts for individual mice. Mean ± SD is also represented. ** p < 0.01. B) RT-qPCR analysis of IFNG and IRF1 expression in stomachs of infected wt and Ifngr1 -/- mice, shown as fold-change to respective expression of mGAPDH. Data represented as mean ± SD. ns, non-significant, * p < 0.05. C) Confocal microscopy image of longitudinal stomach sections of mice orally infected with PMSS1 for 2 weeks, labelled for actin (phalloidin, red), *H. pylori* (green) and nuclei (DAPI, blue). Scale bars: 1000 µm (upper) and 100 µm (lower). D) RT-qPCR analysis of IFNG and IRF1 expression in samples taken from corpus or antrum of the mice used in (C), results represented as in (B).

Figure 4. Cgt prevents IFNB, IL6 and IL22-induced signaling in epithelial cells upon prolonged infection and impairs hBD3 expression.

A) Immunoblot analysis of STAT1 pathway activation in MKN45 cells. Cells were infected for 24 h with indicated strains or non-infected, then treated (+) or mock-treated (-) with IFNB (2300 U/ml) for 30 min. B) and C) Immunoblot analysis of STAT3 activation in MKN45 cells infected for 24 h with indicated strains or treated with mβCD (5 mg/ml) for 5 h, followed by 30 min with B) IL6 (100 ng/ml)
or C) IL22 (50 ng/ml). D) and E) RT-qPCR analysis of hBD3. MKN45 cells were non-infected or infected with indicated strains for 24 h. IFNG (10 ng/ml) (D) or IL22 (50 ng/ml) (E) were then added to selected wells for the specified times. To represent the net response to cytokines, results are normalized against relative gene expression of corresponding non-infected, P12 wt and P12Δcgt infected cells. Data represented as mean ± SD. ns, non-significant, ** p < 0.01.

Figure 5. Cgt blocks IFN signaling in primary gastric epithelial cells

Human primary gastric cells were infected with indicated strains at MOI 20 for 24 h or non-infected. A) Immunoblot analysis of JAK1/STAT1 pathway activation. After infection cells from antrum were mock-treated or treated for 30 min with IFNG (5 ng/ml) or IFNB (2300 U/ml). Quantification: band intensity ratio STAT1pY: actin. B) Cholesterol-coating of bacteria was started 1 h before infection and applied to NI cells accordingly. Afterwards cells were mock-treated or treated for 30 min with IFNG (5 ng/ml). Result representative of two independent experiments. C) Immunoblot analysis of STAT3 activation. Cells from antrum were infected for 24 h with indicated strains and after infection treated for 30 min with IL22 (50 ng/ml). D) and E) Primary human cells were infected with H pylori P12 wt or P12Δcgt for 26.5 h and microarray results compared to those obtained in non-infected (n.i.) cells. Results shown are from two independent experiments with cells obtained from two independent donors. D) GO processes enrichment (Panther) from upregulated genes in n.i. vs. P12 wt data set. In all cases P < 0.05. E) NF-κB-response genes differentially modulated in n.i. vs. P12 wt and n.i. vs P12Δcgt datasets. In all cases, fold change > 1.5 and P value < 0.05.

Figure 6. Block of cytokine responses in infected ALI cultures.

Primary human cells grown on filters were infected with P12 wt at MOI 100 for 3 days or mock-infected (NI), then mock-treated or treated for 30 min with IFNG (10 ng/ml) (A and B) or IL22 (50 ng/ml) (C) and fixed with PFA. Filters were labelled with DAPI (DNA, blue) and antibodies against H
*pylori* (red) and analyzed by whole-mount IF. A) Analysis of STAT1 phosphorylation. Filters were labelled with phalloidin (green) and STAT1-pY (white). Scale bar: 20 µm. B) Analysis of STAT1 phosphorylation, lower magnification. Filters were labelled with antibodies against STAT1-pY (white). Scale bar: 50 µm. C) Analysis of STAT3 phosphorylation. Filters were labelled with antibodies against STAT3-pY (white). Scale bar: 20 µm.

Figure 7. Summary of cholesterol-dependent subversion of immune response

Extraction of host cell cholesterol by H pylori’s Cgt disrupts lipid rafts and thereby prevents assembly of IFNG receptors. In infected cells this leads to a shutdown of JAK/STAT1 signaling, inhibiting the production of T cell chemo-attractants and antimicrobial peptides like hBD3. With this mechanism, *H pylori* creates micro-niches of locally diminished inflammation despite a marked global immune response.
Figure 6

A

<table>
<thead>
<tr>
<th>DAPI</th>
<th>H. pylori</th>
<th>STAT1 pY</th>
<th>Phalloidin</th>
<th>H. pylori + STAT1 pY</th>
</tr>
</thead>
<tbody>
<tr>
<td>NI NT</td>
<td>NI + IFN-γ</td>
<td>P12 wt</td>
<td>+ IFN-γ</td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>DAPI</th>
<th>H. pylori</th>
<th>STAT1 pY</th>
<th>H. pylori + STAT1 pY</th>
</tr>
</thead>
<tbody>
<tr>
<td>P12 wt</td>
<td>+ IFN-γ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>DAPI</th>
<th>H. pylori</th>
<th>STAT3 pY</th>
<th>H. pylori + STAT3 pY</th>
</tr>
</thead>
<tbody>
<tr>
<td>P12 wt</td>
<td>+ IL-22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7
Supplementary Materials and Methods

Cell lines and bacterial strains

The human gastric adenocarcinoma cell lines MKN45 (DSMZ- ACC 409) and AGS (ATCC) were grown in RPMI 1640 medium containing 4 mM glutamine (Invitrogen) and 10% FCS (Gibco) in a humidified atmosphere with 5% CO₂. Cell lines were periodically confirmed to be mycoplasma-free and morphologically authenticated. Type I \textit{H pylori} wild type strains P1 (strain collection no. P381) and P12 (no. 243), as well as the mouse-adapted strain PMSS1 (no. 504) were grown on GC agar plates with vancomycin as described elsewhere \textsuperscript{1, 2}. For \textit{H pylori} P12 isogenic mutants P12Δcgt (no. P451), P12ΔcagPAI (no. P387), P12ΔcagA (no. P378), P12ΔvacA (no. 14), and PMSS1Δcgt (no. P520) GC agar plates with vancomycin were further supplemented with selective chloramphenicol (4 μg/ml) and/or kanamycin (8 μg/ml), depending on the strain used. Bacteria were incubated 1 day before infection at 37 °C in a humidified microaerobic atmosphere containing 5% CO₂, 5% O₂ and N₂/H₂ (9:1).

Complementation of the P12Δcgt mutant

Genetic complementation of the isogenic \textit{H pylori} mutant P12Δcgt was performed using a derivative \textit{E. coli}-\textit{H pylori} shuttle plasmid pHel3 (kanR), designated as pIB6 (unpublished). Cgt was amplified from chromosome of the \textit{H pylori} strain P12 using the HP0421-forward primer containing a \textit{Sal}I restriction site and the HP0421-reverse primer containing a \textit{Bgl}II restriction site. The purified PCR product was digested with \textit{Sal}I and \textit{Bgl}II, and ligated into the pIB6 vector on the corresponding restriction sites that resulted in pMW713 plasmid. Conjugation of these shuttle vectors carrying the \textit{cgt} gene to \textit{H pylori} was performed according to Heuermann and Haas\textsuperscript{4}. Single colonies isolated from double selective vancomycin agar plates containing kanamycin and chloramphenicol antibiotics were grown on double selective BHI medium, and subsequently subjected to bacterial genomic DNA isolation using genomic DNA purification
kit (Fermentas). Presence of cgt was determined via Western blot using a specific antibody generated in-house.

**Western blot and dot blot**

For Western blot we used antibodies against STAT1 pY, STAT1, JAK1 pY, JAK1 (Cell Signaling, Cat. No. 9171, 9172, 3331, 3332, respectively), α-actin (Sigma, Cat. No. A5441), IFNγR1 (clone GIR94, Santa Cruz, Cat. No. sc-12755) IFNγR2 (clone MMHGR-2, Santa Cruz, Cat. No. sc-57215 or PBL, Cat. No. 21585), caveolin (BD, Cat. No. 610060) and horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham). For dot blots, 3 μl of sample were pipetted onto nitrocellulose membranes and incubated with cholera-toxin B conjugated to HRP (Life Technologies, Cat. No. C34780). In all cases, signal was detected with the WESTERN LIGHTNING™ western blot kit system for ECL immunostaining. In all figures, blots from the same panel come from the same membrane. Quantification was performed using ImageJ. Unless otherwise indicated, all blots shown are representative of three independent experiments.

**Immunoprecipitation**

Cells were lysed on ice in RIPA buffer containing 1% Triton X-100, 50 mM Tris HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% sodium azide, 1mM Na3VO4, and complete protease inhibitor. The lysates were passaged 15 times through a 20-gauge syringe. Cell debris was removed by centrifugation at 12,000 x g for 5 min. Lysates were incubated with 2 μg of the indicated bait antibody (in 1x lysis buffer) overnight at 4 °C. Subsequently, protein agarose beads were added α/n at 4 °C to precipitate antigen-antibody complexes. After incubation, the antigen-antibody complexes were washed three times with 1X lysis
buffer, before eluting precipitate by heating to 95 °C in 1X Laemmli buffer and separating proteins by SDS-PAGE, followed by immunoblotting.

**Immunofluorescence microscopy**

MKN45 cells were grown on poly-L-lysine coated coverslips, starved and infected at MOI 50 for 24 h, followed by 30 min treatment with IFNG in selected wells. For GM1 labelling cells were then placed on ice, washed with ice cold PBS and incubated with Alexa Fluor 488-linked CTxB (1:100, Thermo Fisher, Cat. No. C-34775) in PBS for 30 min, before fixing with 1% PFA for 10 min. For JAK2 pY labelling cells were washed, fixed with 4% PFA for 15 min, permeabilized using 0.1% TritX100 for 10 min and blocked with 5% horse serum, before incubating with JAK2 pY antibody (1:50, Santa Cruz, Cat. No. Sc21870) and polyclonal antibody against whole *Helicobacter* lysate (1: 50, US Biological, Cat. No. H1840-10) for 1 h at RT, followed by anti goat-Cy3 (Cat. No. 705-165-003) and anti-rabbit-Alexa Fluor 647 (Cat. No. 711-605-152, both Dianova) and DAPI (1:750, Roche, Cat. No. H1840-10). Coverslips were mounted with Mowiol and analyzed by laser scanning microscopy (Leica TCS SP-8), using 63x and 100x oil immersion objectives. Images were processed using LAS X (Leica) and Fiji.

For whole mount staining of ALI cultures cells on the filter were permeabilized with methanol for 10 min at -20°C, washed with PBS, blocked with 5% donkey serum in PBS/0.3% Triton-X 100 for 1 h and incubated overnight with the primary antibodies STAT3 pY (CST, Cat. No 9134S), STAT1 pY (CST, Cat. No 7649P) and anti-HP (SC, Cat. No 57778), all at 1:100 in PBS/0.1% BSA/0.3% Triton-X 100. Secondary staining with fluorescently labeled antibodies was performed using donkey anti-rabbit-Cy3 (1:100, Cat. No. 711-166-020), donkey anti-mouse-Cy2 (1:100 Cat. No 715-225-150), DAPI (1:300, Roche, Cat. No. H1840-10) and phalloidin-Alexa647 (1:100, Invitrogen, Cat. No A22287) for 2 h at RT in same buffer as primary staining. Samples were mounted in Mowiol and analyzed by laser scanning microscopy (Leica TCS SP-8).
TCS SP-8). Unless otherwise indicated, images shown are representative of three independent experiments.

To visualize gland-associated *H pylori*, stomach sections of infected mice were generated as described previously. In brief, stomach tissue was fixed in 4% paraformaldehyde for 1 h followed by three washes with PBS. Samples were embedded in 4% agarose and cut into 300 μm thick longitudinal sections with a vibratome (Leica). Sections were stained overnight at 4°C with primary antibody (rabbit anti-*H pylori*, 1:300). Secondary staining was performed for 2 h at room temperature using DAPI (1:300, Roche, Cat. No. H1840-10), chicken anti-rabbit-Alexa488 (1:100 Life Technologies, Cat. No A21441) and phalloidin-Alexa647 (1:100, Invitrogen, Cat. No A22287). Samples were mounted in Vectashield and visualized by laser scanning microscopy (Leica TCS SP-8).

Quantification of microscopic images

To quantify STAT1 pY-positive cells in infected ALIs, images of P12 wt-infected and IFN-treated ALIs were analyzed using FIJI. 1-3 regions of filters from three independent experiments were manually classified as infected or uninfected, depending on the presence of bacteria. Number and area of single nuclei were detected automatically. Mean intensity of the STAT1 pY staining within the nuclear areas was measured independently for each cell.

To distinguish between positive and negative cells, a threshold of 75% of the average STAT1 pY intensity from non-infected regions was set up for each filter. Cells that showed a stronger signal were counted as positive and the ratio of positive to total cells was calculated.

For GM1 quantification, confocal images from three different experiments were analyzed using FIJI. Within a microscopic field, single cells were selected and the plasma membrane was transformed into a line using a plugin developed by E. Donnelly and F. Mothe (http://rsbweb.nih.gov/ij/plugins/polar-
transformer.html). Based on the transformed image, the outer border of the cell was manually selected and the plasma membrane was set as the area starting from this line 1 µM into the cytosol. The average intensity of GM1 staining within both plasma membrane and cytoplasm was measured and the ratio of average intensities from membrane to cytoplasm was calculated for each cell and normalized to the average intensity ratio of non-infected cells of each corresponding experiment.

**ATP-luciferase cellular viability assay**

Cells were seeded in 96-well plates and infection performed as described above. ATP-luciferase (Promega) assay was performed according to manufacturer’s instructions.

**Cholesterol measurement**

Cells were seeded in 12-well plates and infection performed as described above. Cells were collected with a scraper and cholesterol measured using the Amplex Red Cholesterol Assay Kit according to the manufacturer’s instructions.

**Isolation of DRMs**

2-3 × 10⁷ cells were infected in T-150 flasks at MOI 50 for 24 h followed by 30 min of IFN treatment (10 ng/ml IFNG or 2300 U/ml IFNB) in selected flasks. Cells were then washed and lysed with ice-cold TNE buffer (25 mM Tris-Cl, pH 7.4; 150 mM NaCl; 5mM EDTA) containing 1% Triton X-100 and protease inhibitors (Complete, Roche). Cell lysates were mixed with Optiprep (Sigma) to a final concentration of 35% and transferred to an ultracentrifuge tube. A density gradient was overlaid by adding fractions of
decreasing Optiprep concentrations (30%, 25%, 20% and 0%) in TNE buffer. Tubes were ultracentrifuged (4 °C, 135,000 x g, 16 h) and content divided into 10 fractions.

**RT-qPCR**

RNA extraction was performed using the GeneJet RNA purification kit (Fermentas) or RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. Conversion of RNA into cDNA and subsequent cDNA amplification was achieved together by using Power Sybr® Green RNA-to-CT™ 1-step kit (Applied Biosystems) and 7500 fast realtime PCR system (Applied Biosystems) following manufacturer’s instructions. Primer sequences are listed in Supplementary Table 1. For hBD3 and mouse IFNG analysis, TaqMan® probes were used. In this case, RNA was analyzed using TaqMan® Fast Virus 1-Step Master Mix and commercial labeled probes against DEFB103A/DEFB103B and GAPDH were used, as well as probes against mouse IFNG and mouse GAPDH (TaqMan® Expression Assays, Applied Biosystems). Unless otherwise indicated, data shown correspond to a minimum of three independent biological experiments with technical duplicates.

**Microarray analysis**

Microarray experiments were performed as independent dual-color dye-reversal color-swap hybridizations using two biological replicates. Quality control and quantification of total RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and a NanoDrop 1000 UV-Vis spectrophotometer (Kisker). Total RNA was isolated using the GeneJet RNA purification kit (Fermentas) according to manufacturer’s instructions. RNA labeling was performed with the dual-color Quick-Amp Labeling Kit (Agilent Technologies). In brief, mRNA was reverse transcribed and amplified using an oligo-dT-T7 promoter primer, and resulting cRNA was labeled with Cyanine 3-CTP or Cyanine 5-CTP. After
precipitation, purification, and quantification, 1.25 μg of each labeled cRNA was fragmented and hybridized to whole genome human 4 × 44k multipack microarrays (Agilent-026652 Human Gene Expression 4x44K v2 Microarray Kit) according to the manufacturer’s protocol (Agilent Technologies). Scanning of microarrays was performed with 5 μm resolution using a G2565CA high-resolution laser microarray scanner (Agilent Technologies) with extended dynamic range (XDR). Microarray image data were analyzed with the Image Analysis/Feature Extraction software G2567AA v. A.11.5.1.1 (Agilent Technologies) using default settings and the GE2_1105_Oct12 extraction protocol. The extracted MAGE-ML files were analyzed further with the Rosetta Resolver Biosoftware, Build 7.2.2 SP1.31 (Rosetta Biosoftware). Ratio profiles comprising single hybridizations were combined in an error-weighted fashion to create ratio experiments. A 1.5-fold change expression cut-off for ratio experiments was applied together with anti-correlation of dye-swapped ratio profiles, rendering the microarray analysis highly significant (P < 0.01), robust, and reproducible. Upregulated genes were further analyzed with Panther (www.pantherdb.org) to assess enrichment in GO processes.

Mouse infection model.

Animals were kept in autoclaved microisolator cages, provided with sterile drinking water and chow was given ad libitum. Female C57BL/6 or C57BL/6 Ifngr1 –/– mice (6 weeks old) were infected with an oral dose of 10^8 PMSS1wt or PMSS1Δcgt suspended in PBS/0.1 M NaHCO₃. After two days, mice were reinfected (oral dose, 10^8 bacteria). Three mice from each group were euthanized 4 days after the first infection. The stomach was removed, weighed, homogenized, serially diluted and plated out on GC agar plates containing vancomycin to determine the number of colony-forming units of H pylori. Similarly, for the 2 week-infection experiments, 6 week-old male mice in a C57Bl6 background were inoculated with a single oral dose of 10^8 H pylori PMSS1. Following euthanization, the forestomach was cut off and the glandular stomach cut open following the lesser curvature and spread on Whatman paper. The stomach
was cleaned and the tissue cut into two halves at the greater curvature. For imaging, longitudinal
sections at the midline along the greater curvature were used comprising tissue from duodenum to the
antral region. A corresponding piece of tissue was divided between corpus and antrum and used for RNA
analysis.

Statistical analysis

RT-qPCR data are shown as mean ± SD. To ensure statistical significance, main results were obtained
after three independent biological experiments with at least two technical replicates each. In case higher
statistical size was required, the number of independent biological experiments is indicated in the figure
legends. In all cases, data sets were normally distributed, equal variance was assumed and paired two-
tailed t-tests were performed. For Western blot quantification, we combined data from independent
experiments, equal variance was assumed and unpaired two-tailed t-tests were performed. For STAT-
positive cell quantification and in vitro experiments, equal variance was assumed and unpaired two-
tailed t-tests were performed.
Supplementary References

Supplementary Figure 3
Supplementary Figure 5

A

B

H. pylori (Fig. 6B)  H. pylori (Fig. 6B, Zoom)

C

Uninfected field + IFN-γ

Infected field + IFN-γ

D

P12.5γ/Δf   + IFN-γ

H. pylori

STAT1 γY

H. pylori + STAT1 γY

% STAT1 γY-positive cells

Uninfected fields

Infected fields

**
Supplementary Figures

Supplementary Figure 1. A) Time course analysis of IFNG induced STAT1 phosphorylation by immunoblot. MKN45 cells were infected with H pylori P12 wild type at MOI 50 or non-infected (NI). After indicated infection times, cells were treated (+) with IFNG (10 ng/ml, 30 min). B) Immunoblot analysis of IFNG induced STAT1 phosphorylation in AGS gastric epithelial cell line upon infection with indicated H pylori strain at MOI 50 and in non-infected cells. After 24 h of infection, cells were treated (+) with IFNG (10 ng/ml, 30 min). C) MOI titration analysis of IFNG induced STAT1 phosphorylation by immunoblot. MKN45 cells were infected with H pylori P12 wild type at indicated MOIs or non-infected (NI). After 24 h of infection, cells were treated (+) with IFNG (10 ng/ml, 30 min). D) Analysis of JAK2 phosphorylation (red) by confocal IF at low magnification, confocal section. Cells were non-infected (NI), or infected with indicated H pylori strains (bacteria labeled in green). In all cases, cells were treated with IFNG (10 ng/ml, 30 min). Scale bar: 10 μm. The result is representative of three independent experiments. E) Cellular viability of MKN45 cells infected with indicated H pylori strains, or heat-killed P12 (P12 HK) at indicated MOIs for 24 h. Viability was measured via a fluorescence-based ATP assay (Promega). All readings were normalized against non-infected (NI) cells. Cells treated for 5 min with Triton X-100 serve as control. Data shows mean ± SD of two independent biological experiments. SD is derived from three technical replicates. F) Immunoblot analysis of IFNG induced JAK1 and STAT1 phosphorylation in MKN45 gastric epithelial cells after 48 h of infection with indicated H pylori strains at MOI 50 and in non-infected cells. At the end of infection, cells were treated (+) or mock-treated (-) with IFNG (10 ng/ml) for 30 min. Quantification shows band intensity ratio of STAT1pY:actin. G) Immunoblot analysis of CGT expression after genetic complementation of CGT mutant strain. 4x10^6 bacteria of H pylori P12 wt, P12Δcgt, ΔcagA, ΔcagPAI, and 2 clones of the recomplemented strains Δcgt/cgt were lysed in Laemmli buffer and checked for CGT expression by using an in-house created monoclonal anti-CGT antibody. H) Response to IFNG for genes IRF1, CXCL9, and CXCL10 quantified by RT-qPCR analysis. MKN45 cells were non-infected or...
infected with indicated *H pylori* strains for 24 h. IFNG (10 ng/ml) was then added to selected wells for the specified times. To represent the net response to IFN, results were normalized against relative gene expression of corresponding non-infected, P12 and P12Δcgt infected cells. Data are represented as mean ± SD; ns, non-significant, * p < 0.05, ** p < 0.01.

**Supplementary Figure 2.** A) Cholesterol quantification of MKN45 upon *H pylori* infection. Cells were non-infected (NI) or infected with indicated strains of *H pylori* for 24 h at MOI 50. After this period, cholesterol was quantified using Amplex kit (Invitrogen). All readings were normalized against protein content and further normalized against cholesterol levels in non-infected cells. Data show mean ± SD of two independent biological experiments with two technical replicates each. B) Cellular viability of MKN45 cells treated with methyl-β-cyclodextrin (5 mg/ml) for 6 h. Viability was measured via a fluorescence based ATP assay (Promega). All readings were normalized against the NI and mock-treated cells. Cells treated for 5 min with TritonX-100 served as control. Data show mean ± SD of two independent biological experiments with two technical replicates each. C) Response to 2.5 h treatment with IFNG (10 ng/ml) after mock treatment or treatment with myriocin (72 h, 50 μM) plus zaragozic acid (18 h, 50 μM) (MZ). IRF1 mRNA levels obtained by qRT-PCR were normalized against respective GAPDH mRNA expression, and further normalized against non-infected (NI) cells. Data show mean ± SD of two independent biological experiments with two technical replicates each. For statistical analysis, MZ treated samples were compared to mock, IFNG treated controls: ns non-significant, ** p < 0.01. D) Response to 2.5 h treatment with IFNG for IRF1 gene in cells infected with bacteria with or without cholesterol coating. MKN45 cells were non-infected or infected with indicated *H pylori* strains for 24 h. IFNG (10 ng/ml) was then added to selected wells for the 2.5 h. To represent the net response to IFN, results were normalized against relative gene expression of respective corresponding non-infected, P12 and P12Δcgt infected cells. Data are represented as mean ± SD; ns, non-significant, ** p < 0.01. E) Viability of cholesterol-coated bacteria. Bacteria (10^8/ml) in serum-free medium were coated or mock-
coated with exogenous cholesterol (1 mg/ml) for 1 h. Colony forming units (CFU)/ml were quantified 3 days later. Data are expressed as percentage bacterial survival relative to the untreated P12 (without cholesterol coating). Data show mean ± SD of two independent biological experiments with two technical replicates each. F) Confocal microscopic analysis of the lipid raft marker GM1 (stained with CTxB-Alexa488, green) in cellular membranes of cells non-infected (NI) or infected for 24 h with indicated *H pylori* strains. Left panel corresponds to the quantification of relative superficial GM1 signal. Results are shown as a box-plot (2nd and 3rd quartiles, median represented as horizontal line inside the box) of the ratio between cellular membrane and correspondent cytoplasm average intensities and further normalized to the average intensity ratio of non-infected cells of each corresponding experiment. Data correspond to three independent experiments; n = number of analyzed cells; ** p < 0.01. Right panel, representative images used for quantification. Scale bar: 10 μm. G) Immunoblot analysis of detergent resistant membrane fractions (1-10, from top to bottom) of uninfected MKN45 cells after density gradient centrifugation for distribution of caveolin or GM1 distribution detected by dot blot with cholera toxin B conjugated to HRP. Fractions inside the box were pooled for further analysis. H) Immunoblot analysis of whole cell lysate of MKN45 cells for IFNGR1, IFNGR2 or caveolin. Cells were non-infected (NI) or infected for 24 h with indicated *H pylori* strains. After infection time, cells were treated (+) or mock-treated (-) with IFNG (10 ng/ml, 30 min). I) Immunoprecipitation (IP) and immunoblot analysis of IFNGR subunits 1, 2 and CD71. IFNGR2 was pulled down using anti-IFNGR2 antibody.

**Supplementary Figure 3.** A) Immunoblot analysis of STAT1 phosphorylation after 5 h mβCD treatment at the concentrations indicated or in non-treated cells (-). IFNB treatment (2300 U/ml, 30 min) was applied in selected wells. The result is representative of three independent experiments. Quantification shows band intensity ratio of STAT1pY:actin B) Immunoblot analysis of STAT3 phosphorylation of MKN45 cells infected with indicated *H pylori* strains or mock-infected (NI) for 24 h at MOI 50 with the indicated *H pylori* strains, followed by treatment with IL22 (50 ng/ml) for 30 min. C) Immunoblot analysis of STAT3
activation in MKN45 cells. Cells were infected for 24 h with *H pylori* P12 wild type or mock infected. In selected wells PEG-cholesterol was added during the final hour of infection. Cells were then treated with IL22 (50 ng/ml, 30 min) (+) or mock-treated (-). Quantification shows band intensity ratio of STAT1pY:actin.

**Supplementary Figure 4.** Experiment performed in 2D primary gastric human cells from different donors infected with indicated *H pylori* strains at MOI 20 or 50 for 24 h or non-infected (NI). A) Immunoblot analysis of STAT1 pathway activation. Cells from the corpus region were infected with indicated *H pylori* strains at different MOIs. After infection time cells were mock-treated or treated with IFNG (5 ng/ml) B) Immunoblot analysis of the activation of JAK1/STAT1 pathway. After infection, cells were mock-treated or treated 30 min with IFNG (γ) (5 ng/ml) or IFNB (β) (2300 U/ml). C) Time course analysis of IFNG-induced STAT1 phosphorylation by immunoblot. Cells were infected with *H pylori* P12 wt at MOI 20 or non-infected (NI). After indicated infection times, cells were treated (+) with IFNG (5 ng/ml, 30 min). D) Immunoblot analysis of STAT1 phosphorylation. Cells were infected with *H pylori* wild type at different MOIs or left uninfected. After infection time cells were mock-treated or treated with IFNG (5 ng/ml). E) Response to 2.5 h treatment with IFNG (5 ng/ml) after infection with indicated *H pylori* strains or non-infected (NI). CXCL9 mRNA levels obtained by qRT-PCR were normalized against respective GAPDH mRNA levels, and further normalized against non-infected (NI) cells. Data show mean ± SD of four independent biological experiments (performed with cells from two different donors) with two technical replicates each. For statistical analysis, infected samples were compared to uninfected, IFNG treated controls: ns non-significant, ** p < 0.01. F) and G) Immunoblot analysis of STAT1 (F) or STAT3 (G) phosphorylation of primary cells mock-infected (NI) or infected with *H pylori* P12 wt, P12Δcgt or 2 clones of the recomplemented strains Δcgt/cgt for 24 h at MOI 50, followed by treatment with (F) IFNG (5 ng/ml, 30 min) or (G) IL22 (50 ng/ml, 30 min). Quantification of (F) shows band intensity ratio of STAT1pY:actin.
Supplementary Figure 5. Blockage of cytokine response in infected ALI cultures. Human gastric primary cells were grown on polycarbonate filters, infected with *H. pylori* and analyzed by confocal IF. A) Schematic representation of an infected ALI monolayer. Microscopic image corresponds to a paraffin section of cells infected with P12 at MOI 100 for 3 days. Monolayers were labelled with DAPI and antibodies against cagA (red) and E-cadherin (green) followed by IF analysis. B) Detail at high magnification showing infecting *H. pylori* bacteria (labeled in red) from Fig. 6B. Right image corresponds to the square-delimited area on the left image. Scale bars: 50 μm (left), 5 μm (right). C) Representative microscopic fields from infected filters used for quantification of STAT1 pY-positive cells and quantification of nuclear STAT1 pY-positive cells in infected fields. After 3 days of infection with *H. pylori* wild type (MOI 100), cells were treated for 30 min with IFNG (10 ng/ml). Filters were labelled with DAPI (DNA, blue), and antibodies against STAT1 pY (white) and *H. pylori* (red), followed by whole mount IF. Scale bar: 100 μm. For quantification, microscopic fields homogeneously infected (number of cells n = 9,548) were compared to non- or minimally infected ones (number of cells n = 15,682). Results were obtained from four independent infected filters and are expressed as percentage of nuclear STAT1 pY-positive cells determined by ImageJ software. ** p < 0.01. D) Analysis of STAT1 phosphorylation. After 3 days of infection with *H. pylori* P12Δcgt (MOI 200), cells were treated for 30 min with IFNG (10 ng/ml), and labelled with DAPI (DNA, blue), and antibodies against STAT1 pY (white) and *H. pylori* (red), followed by whole mount IF. Scale bar: 50 μm.
Supplementary Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH Fwd</td>
<td>GGTATCGTGGAAGGACTCATGAC</td>
</tr>
<tr>
<td>GAPDH Rev</td>
<td>ATGCCAGTGAGCTCCCGTTCAG</td>
</tr>
<tr>
<td>IRF1 Fwd</td>
<td>ATGCCCATCACTCGGATGC</td>
</tr>
<tr>
<td>IRF1 Rev</td>
<td>CCCTGCCTTTGTATCGGCCCTG</td>
</tr>
<tr>
<td>CXCL9 Fwd</td>
<td>ATTTGAGTGCAAGGAAACCC</td>
</tr>
<tr>
<td>CXCL9 Rev</td>
<td>GGGCTTGGGGAATTGT</td>
</tr>
<tr>
<td>CXCL10 Fwd</td>
<td>GTGGCATTAAGGAGTACCT</td>
</tr>
<tr>
<td>CXCL10 Rev</td>
<td>GCATCGATTGCTCCCCTC</td>
</tr>
<tr>
<td>IRF7 Fwd</td>
<td>GAGCTGTGCTGGCGAGAAG</td>
</tr>
<tr>
<td>IRF7 Rev</td>
<td>TGGTGGGACATGGATGCTG</td>
</tr>
<tr>
<td>HP0421 Fwd</td>
<td>GATCGTCACTGATGTTGTTGAGT</td>
</tr>
<tr>
<td>HP0421 Rev</td>
<td>GATCAGATCTTTATGATAAGGTTAAGA</td>
</tr>
<tr>
<td>Mouse IRF1 Fwd</td>
<td>ATCTCGGGCATTTTCGCTT</td>
</tr>
<tr>
<td>Mouse IRF1 Rev</td>
<td>TCTGACATCTAGCCAGG</td>
</tr>
<tr>
<td>Mouse GAPDH Fwd</td>
<td>TACCATCTCCAGGAGCG</td>
</tr>
<tr>
<td>Mouse GAPDH Rev</td>
<td>AAGCAGTTGGGTGGTCAGG</td>
</tr>
</tbody>
</table>