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Helicobacter pylori-induced NF- κ B: Trailblazer for gastric pathophysiology

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To the Editor of
Trends in Molecular Medicine
Dr. Claudia G. Willmes
tmm@cell.com

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Magdeburg, 13 December 2021

Dear Dr. Willmes,

Please find our revised Review article entitled "*Helicobacter pylori-induced NF- κ B: Trailblazer for gastric pathophysiology*" for publication in Trends of Molecular Medicine.

We would like to thank the reviewers for their appreciative comments and you for the helpful suggestions to improve our manuscript.

Kind regards

Michael Naumann

1 **Highlights**

2
3 *H. pylori*-induced classical and alternative NF- κ B pathways in gastric epithelial cells
4 converges at the ADP-heptose - ALPK1 - TIFA signaling module.

5
6 NF- κ B activation in *H. pylori* infection not only affects the gastric epithelial
7 homeodynamics, but the cellular response to *H. pylori* affects also NF- κ B.

8
9 The *H. pylori*-induced NF- κ B controls cellular processes to establish persistent
10 infection. This together with genomic predisposition plays an important role in gastric
11 pathophysiology.

12
13 Gastric 3D organoids and especially its derivative the 2D polarized monolayers in air-
14 liquid-interphase culture are versatile tools for the analysis of *H. pylori*- and NF- κ B-
15 dependent gastric pathophysiology under *in vivo*-like conditions.

16
17 Recent understanding of the impact of *H. pylori*-induced NF- κ B on gastric
18 pathophysiology including malignancies imply the contemplation of new therapeutic
19 avenues like epigenetic therapy.

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***Helicobacter pylori*-induced NF- κ B: Trailblazer for gastric pathophysiology**

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Keywords

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Abstract

NF- κ B signaling pathways, induced by a variety of triggers, play a key role in regulating the expression of genes involved in the immune response and cellular responses to stress. The human pathogen *Helicobacter pylori* induces classical and alternative NF- κ B signaling pathways via its effector ADP-glycero- β -D-manno-heptose. We review *H. pylori*- and NF- κ B-dependent alterations in cellular processes and associated maladaptation leading to deleterious gastric pathophysiology that have implications for the diagnosis and treatment of gastric diseases. Therapeutic options for gastric cancer include clinically relevant small molecule inhibitors of NF- κ B and epigenetic therapy approaches. In this context, gastric organoid biobanks originated from patient material represent a valuable platform for translational applications to predict patient responses to chemotherapy, with a view to personalized medicine.

***H. pylori*-induced NF- κ B signaling in colonized gastric epithelial cells**

H. pylori is present in about half of the world's population and poses a risk factor for the occurrence of malignant gastric diseases in a subgroup of individuals [1,2]. Here, the bacteria colonize the gastric epithelium and trigger a variety of signaling processes, prominently the NF- κ B pathways. The initiated processes leading among others to the alteration of the polarized epithelium and persistent inflammation [3,4]. The recent identification of a long missing mechanism of *H. pylori*-induced NF- κ B is beginning to refine our understanding of its involvement in gastric diseases. Although the incidence of gastric cancer (GC) is on the decline worldwide [5], the question how the *H. pylori*-triggered NF- κ B and its regulation by a diverse set of molecules contributes to metabolic, genetic and epigenetic changes in the gastric epithelium leading to the development of gastric malignancies is of utmost importance. Given that *H. pylori* is a human pathogen, the most relevant *in vivo* model to study this bacterium is the human stomach. Therefore, efforts to establish a reliable *ex vivo* technique by the generation of gastric organoids, representing the *in vivo* situation, have tremendously increased recently. This technique will further improve the identification of therapeutic options in the fight against gastric diseases.

Recently, it has been discovered that ADP-glycero- β -D-manno-heptose (ADP-heptose), a soluble metabolite of the LPS synthesis pathway of different Gram-negative bacteria [6-8], is the missing bacterial effector that enters the cytosol and elicits NF- κ B activation in *H. pylori* infection [9,10]. Although the mechanism through which *H. pylori*-synthesized ADP-heptose enters the host cytosol is still unclear, the *H. pylori*-induced NF- κ B activation is strictly type IV secretion system (T4SS)-dependent [11].

In the cytosol, ADP-heptose binds to alpha-protein kinase 1 (ALPK1) [8]. ALPK1 is a serine/threonine kinase that belongs to the family of atypical protein kinases [12]. ALPK1 is required for the phosphorylation of the threonine residue T9 of tumor necrosis factor receptor-associated factor (TRAF)-interacting protein with forkhead-associated domain (TIFA) dimers, leading to the self-oligomerization of TIFA [13,14]. Upon *H. pylori* infection, TIFA binds to different TRAF molecules to activate the classical as well as the alternative NF- κ B pathways [9]. Binding of TIFA to TRAF6 followed by recruitment of transforming growth factor (TGF)- β -activated kinase 1 (TAK1) in complex with TAK1-binding proteins (TABs) activates the inhibitor of nuclear factor κ B kinase (IKK) complex and therefore the classical NF- κ B pathway [15,16] (Figure 1). In contrast, the interaction

of TIFA with TRAF2 in the NF- κ B-inducing kinase (NIK) regulatory complex destabilizes cellular inhibitor of apoptosis 1 (cIAP1) resulting in its degradation. The ensuing NIK accumulation leads to activation of the alternative NF- κ B pathway [9] (Figure 1). The interaction of *H. pylori*'s HopQ protein with different carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) is essential for bacterial adherence and the T4SS-dependent translocation of the cytotoxin-associated gene A (CagA) protein [17-20]. CEACAMs are a diverse group of cell surface glycoproteins that are part of the immunoglobulin superfamily involved in cell-cell recognition and modulate cellular processes [21]. The binding of recombinant HopQ to human CEACAM1, 3, 5, and 6 has been demonstrated [17]. This interaction also leads to an enhanced T4SS functionality regarding the classical and alternative NF- κ B activation by *H. pylori* [22,23]. The necessity of the enhanced binding of *H. pylori* to the surface of gastric epithelial cells might hint at a translocation of ADP-heptose in part via secretion and endocytosis. This is supported by the finding that the dynamin GTPase inhibitor Dynasore hampers the induction of the classical and the alternative NF- κ B pathways [9]. The cellular response to *H. pylori*-triggered NF- κ B could be detrimental for the gastric epithelium, ultimately leading to gastric diseases. The number of molecules affecting or affected by NF- κ B is ever-increasing and this knowledge serves to improve our understanding of disease mechanisms.

Effects of *H. pylori* on molecules affecting or affected by NF- κ B

H. pylori infection initiates different responses of the infected gastric epithelium including NF- κ B, which in turn alter the **homeodynamics** (see glossary) of gastric epithelial cells [1, 24]. Molecules like receptor of activated protein C kinase 1 (RACK1), mucin 17 (MUC17) and microRNA-204 that are suppressed upon *H. pylori* infection have been shown to inhibit NF- κ B via indirect mechanisms [25-27]. RACK1 is a cytosolic scaffold protein belonging to the Trp-Asp (WD) repeat protein family. Downregulated upon *H. pylori* infection *in vivo* and *in vitro*, RACK1 overexpression experiments in GES-1 cells showed reduced NF- κ B activation via the regulation of integrin- β 1, whose overexpression restored NF- κ B activation [26]. Downregulation of MUC17 as well as microRNA-204 by *H. pylori* infection is mediated by promoter hypermethylation, which in the case of MUC17 could be attributed to DNA methyltransferase 1. Both molecules were reduced in clinical samples of GC patients [25,27]. MUC17 overexpression suppressed the expression of CEACAM 1-3 through the inactivation of NF- κ B in

different cell lines, leading to reduced CagA translocation [27]. On the other hand, the downregulation of microRNA-204 by *H. pylori* augmented NF- κ B activation that could be attributed to the increase of *baculoviral IAP repeat containing 2 (BIRC2)* expression in MGC-803 cells, which is a target of microRNA-204 [25].

Upon *H. pylori* infection, the NF- κ B-dependent upregulation of hepatocyte nuclear factor 4 alpha (HNF4 α), peroxiredoxin 2 (PRDX2) and caudal type homeobox 2 (CDX2) is evident [28-30]. The upregulation of HNF4 α leads to a self-sustaining feedback loop by upregulating IL-1R1, therefore amplifying the epithelial response to IL-1 β *in vivo* and *in vitro* [29], while the extent of CDX2 activation *in vitro* and *in vivo* seems to be dependent on the presence of nucleotide binding oligomerization domain containing 1 [28]. The antioxidant enzyme PRDX2 inhibits *H. pylori*-induced ROS and protects against oxidative DNA damage and DNA double strand breaks in GC cell lines AGS and SNU-1. In contrast, the prognosis of overall and progression-free survival of GC patients with high levels of PRDX2 is significantly worse than for those with low levels [30]. The overexpression of HNF4 α as well as IL-1R1, CDX2 and PRDX2 led to reduced survival of GC patients, making these molecules potential therapeutic targets.

The disturbance of cellular processes by NF- κ B can benefit the outcome of the infection by *H. pylori* by ensuring its long-term colonization, making *H. pylori* one of the best adapted microorganisms. Moreover, NF- κ B-regulated factors could contribute to maladaptive cellular changes and pathophysiological processes.

Versatile control of cellular processes by NF- κ B

Besides its prominent role in innate immunity and inflammation, NF- κ B also regulates physiological processes involving apoptotic cell death [31,32]. Dysregulated apoptotic cell death could lead to mutations and malignant transformation of gastric epithelial cells due to accumulating DNA damage. The malignant transformation involves an increase in proliferation, invasion and the migratory phenotype of epithelial cells.

NF- κ B-dependent regulation of apoptotic cell death

Apoptotic cell death is a tightly regulated process that plays a pivotal role in normal development and tissue homeodynamics [33]. On the other hand, hallmarks of cancer development are sustaining proliferative signaling, evading growth suppressors and resisting cell death [34]. *H. pylori*-induced NF- κ B regulates apoptosis by different means

[35-38], thereby possibly contributing to the genesis of gastric tumors [1]. For instance, the **deubiquitinylase** A20 and the p62 protein are upregulated by NF- κ B in *H. pylori* infection. In AGS cells, p62 facilitates the interaction of A20 with procaspase-8, which supports the depolymerization of K63-linked ubiquitinated procaspase-8 to compromise its activation [36]. This undermines apoptotic cell death that could be advantageous for *H. pylori* to establish persistent infection [39]. In addition, *H. pylori* induces dopamine and cAMP-regulated neuronal phosphoprotein 32 (DARPP32) expression *in vitro* and *in vivo*, depending on the NF- κ B binding to the DARPP32 promoter. Immunohistochemistry (IHC) staining showed a strong increase of DARPP32 in gastric tissue samples of **intestinal metaplasia**, high-grade **dysplasia** and adenocarcinoma. DARPP32 counteracts *H. pylori*-associated apoptotic cell death via the activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase / AKT serine/threonine kinase pathway [38]. Infection by *H. pylori* also led to the NF- κ B-dependent downregulation of Fas-associated factor 1 (FAF1) expression. Overexpression of FAF1 leads to an increase of apoptosis in the GC cell line HGC-27 [37]. Accordingly, low expression of FAF1 in GC patients was observed and this was predictive for poor overall survival if stratified for FAF1 expression.

NF- κ B-dependent promotion of apoptotic cell death via the induction of p53 upregulated modulator of apoptosis (PUMA) has been demonstrated during *H. pylori* infection. PUMA is upregulated in gastritis tissue on the mRNA and protein levels, as well as on the mRNA level in gastric tumor cell line AGS upon *H. pylori* infection. *H. pylori*-associated apoptotic cell death was reduced in PUMA-knockout AGS cells and PUMA-deficient mice, as confirmed by flow cytometry and caspases-3 and -8 cleavage, as well as by IHC, respectively [35]. These data present the delicate balance between molecules regulating apoptotic cell death and cell survival established by *H. pylori*-induced NF- κ B activation.

H. pylori-induced NF- κ B-dependent changes in proliferation, migration and invasion

H. pylori is a class I carcinogen for gastric malignancies with sufficient evidence in humans for **carcinogenicity** [40]. Tumor development is always linked to aberrant proliferation, migration and invasion of transformed cells [34]. Therefore, changes in genes influencing the proliferation and migration of gastric epithelial cells by *H. pylori* support the mechanism of *H. pylori*-associated carcinogenesis. For example, although the upregulation of long non-coding RNA H19 in GC tissue *in vivo* was not strictly linked

to *H. pylori* infection, the increase of H19 in GES-1 and SCG-7901 upon *H. pylori* infection promotes cell viability, migration, invasion and inflammatory response. These effects depended on NF- κ B activation as shown by knockdown experiments of H19 [41]. In another study, the expression of a proliferation-inducing ligand (APRIL) was correlated to the infection with *H. pylori* *in vivo* and *in vitro*. *H. pylori* infection in SGC-7901 and BGC-823 cell lines decreased the expression of microRNA-145 which was inversely correlated with the APRIL expression. A knockdown of APRIL significantly inhibited proliferation, migration and invasiveness of SGC-7901 cells. A xenograft mouse model showed an impaired tumor growth of the cell line with knockdown of APRIL. The overexpression of APRIL in GC cell lines led to an increase in nuclear NF- κ B/RelA and the expression of pro-survival target genes via activation of the AKT pathway [42]. In summary, these results emphasize the contribution of *H. pylori* infection and its accompanying NF- κ B activation in the development of a proliferative, invasive and migratory phenotype of gastric epithelial cells. The impact of an aberrantly activated NF- κ B is also emphasized by polymorphisms present in its genes contributing to peptic ulcer and GC.

NF- κ B polymorphisms and *H. pylori*-induced gastric pathophysiology

H. pylori-induced NF- κ B activity and its contribution to pathological processes including malignant transformation is closely connected to chronic inflammatory conditions like ulcerative colitis [43] (Box 1). One major cause affecting NF- κ B activity is represented by polymorphisms in NF- κ B genes. For example, various **single nucleotide polymorphisms (SNPs)** located in the promoter of *NFKB1* (p105/p50) appear to be associated with GC progression. Here, gastric mucosal inflammation was more severe in *H. pylori*-infected del/del ATTG homozygotes, suggesting that *NFKB1* -94 del/del homozygote may accelerate severe gastric inflammation [44], whereas *NFKB1* polymorphism -94 ins/del ATTG (rs28362491) is closely associated with the development of the diffuse type of GC [45]. The expression of the *NFKB2* gene (p100/p52) in peptic ulcer cases was increased relative to control tissues, whereas in GC cases, it was decreased compared to normal stomach tissue [46].

In addition to genetic alterations of NF- κ B genes themselves, aberrantly activated NF- κ B signaling molecules have also been associated with gastric carcinogenesis. Polymorphism rs696 of *NFKBIA* (I κ B α gene) in the 3'-UTR region was linked to the susceptibility of cardia cancer while *NFKBIA* rs2233406 mutation in the promoter region

was associated with the susceptibility of non-cardia cancer in the Chinese population [47]. Further, GC patients with SNP in *IKBKB* rs2272736 A allele, which encodes IKK β , had significantly prolonged overall survival time compared to those with the G allele [48]. In addition to polymorphisms, there exist manifold alterations in NF- κ B and NF- κ B signaling molecules related to *H. pylori*-associated gastric diseases [3,31], the complex interplay of which needs further investigations. These are hampered by limitations of conventional experimental techniques, spawning attempts to develop new cell culture modalities, which represent a first step towards *in vivo* cell culture.

Ex vivo analysis of gastric homeodynamics and pathophysiology in organoids

Previous attempts to study epithelial biology relied on mouse models that are unnatural hosts for *H. pylori* or on cell lines that are far from representing the complex structure of an epithelium. Organoids is an innovative tool to study host-pathogen interactions (Box 2). We reflect on the possible applications of organoids to understand the role of NF- κ B in regulating tissue homeodynamics.

Gastric epithelial stem cells and differentiation

As organoids are driven by stem cells with **multilineage differentiation capacity**, they enable research into the regulation of epithelial homeodynamics. The generation of the first gastric organoids from adult tissue has shown the importance of the activation of the Wnt/ β -catenin signaling pathway for the maintenance of adult gastric stem cells [49, 50]. Lack of Wnt activation drives the cells specifically toward differentiation into mucus-producing cells at the pit of the gastric glands [49-51]. Gastric organoids cultivated into 2D monolayers in **air-liquid-interphase** (also called “mucosoids” [51]) revealed that combinations of epidermal growth factor (EGF), bone morphogenetic protein (BMP) and noggin regulate foveolar, chief and parietal cells differentiation [52]. How inflammation interferes with stem cell maintenance and differentiation is still not known. NF- κ B is crucial for initiating and maintaining inflammation, and the transcription of cytokines and chemokines is central for the recruitment of immune cells in the mucosa. These cells might produce ligands that interfere with the pathways regulating stem cells and differentiation. In addition, there are multiple evidences in other tissues of direct crosstalk between NF- κ B, EGF and Wnt signaling [53,54], and BMP4 is known to be an NF- κ B target gene [55] in epithelial cells. Establishing models of co-culture of organoids

or mucosoids with immune cells will give insight into a possible direct or indirect role of the activation of NF- κ B in influencing epithelial homeodynamics (Figure 2A).

*Infection of gastric organoids with *H. pylori**

The exceptional colonization ability of *H. pylori* highly depends on its capacity to localize and move toward the epithelium by swimming through the protective mucus layer [56]. Mucosoid cultures were used to demonstrate that the inner mucus layer is a physical barrier and the outer mucus layer is toxic to bacteria only after infection. This suggests the epithelial secretion of bactericidal compounds in the mucus [51], which is likely controlled by NF- κ B (Figure 2B) [57].

Co-culture of organoids with cells of the immune system has shown a chemokine-dependent recruitment of dendritic cells [58] and cytotoxic T cells [59], pointing to a central role of the epithelium in coordinating immune cell response to infection. It is known that NF- κ B regulate the expression of several chemokines including C-C motif chemokine ligand 20 (CCL20), C-X-C motif chemokine ligand 1 (CXCL1), 2, 3, 5 and 8, interleukin 23 (IL23), and IL32, which are involved in the recruitment of neutrophil, macrophages and leukocytes [51]. However, transcription of NF- κ B target genes in infected organoids and mucosoids depends on the differentiation status of the cells [49,51], suggesting the hypothesis that humoral response and cytokine secretion might be different at the pit or inside the gland.

Gastric organoids in cancer science and translational applications

Although there are strong epidemiological and biological clues suggesting a causal link between *H. pylori* infection and GC, it is still unclear how healthy cells became transformed after infection. GC is characterized by somatic mutations in the epithelium and the human healthy cells derived from organoids offer the unprecedented opportunity to study the genomic consequences of infection. While infection with *H. pylori* induces DNA double-stranded breaks in organoid-derived cells [60,61], mutations have not been found yet. This suggests that the timeframe and the low complexity of *in vitro* infections might not be sufficient to observe mutagenesis in empirical settings. We can hypothesize that during infection, the inflammatory microenvironment of the stomach might give a selective advantage to cells with specific mutations acquired after an incorrect resolution of the DNA damage. Understanding this microenvironment might shed new light on the role of inflammation in carcinogenesis (Figure 2C).

Mutations in GC occur frequently on specific genes suggesting their possible role in driving carcinogenesis. To test this hypothesis, healthy organoids can be genetically engineered to carry specific mutation. It has been found that combined mutation in the genes of E-cadherin and TP53 makes the organoids independent from R-spondin [62]. Altered regeneration is a key aspect in carcinogenesis and independency from R-spondin suggests that mutated cells can regenerate outside their natural stem cell niche. However, the question remains about the origin of these mutations. All known hallmarks of cancer involve NF- κ B activation, which has been shown to not only enhance cell proliferation and survival, but also to promote genetic alteration and acquisition of stem cell properties [63]. Experiments using healthy organoids harboring genetic alteration of NF- κ B-related genes will clarify the role of this potent transcription factor in driving carcinogenesis (Figure 2C).

GC exist in genetically different subtypes [64] (**Epstein Barr virus-associated, microsatellite instable, genomically stable and chromosomally instable**) and these genetic differences can be perpetuated in organoid cultures [65,66]. Patient-derived GC organoids showed a divergent response to 5-fluorouracil, oxaliplatin, irinotecan, epirubicin and docetaxel treatment. In particular, trastuzumab is efficient in eliminating organoids harboring Erb-B2 receptor tyrosine kinase 2 (ERBB2) alterations while palbociclib is specific for cyclin-dependent kinase inhibitor 2A (CDKN2A) loss [67]. A larger drug screen revealed the sensitivity of organoids to unexpected compounds including napabucasin, abemaciclib and the Ataxia telangiectasia- and Rad3-related inhibitor VE-822 [65]. Overall, the gastric organoids biobanks offer a valuable platform to predict patient response to chemotherapy with an outlook into personalized medicine. These inhibitors act on specific pathways involved in cellular life including DNA damage response and cell cycle. NF- κ B activation might interfere with the response of these drugs and promote cell survival [3]. Organoids in culture are usually not inflamed and future experiments to predict drug response using stem cell-derived models should consider the role of inflammation in altering cellular response to drugs. **This new *in vivo*-adapted experimental tool enables research to pursue new avenues in the choice of therapeutic options.**

NF- κ B-related and epigenetic therapy options in GC

In contemplating therapeutic options for GC, one should consider the implications of *H. pylori* infection on the transformation of gastric epithelial cells. As reviewed in the

sections above, these frequently comprise of the NF- κ B-dependent regulation of gene expression or regulation of NF- κ B activity by different entities like enzymes, non-coding RNAs, **epigenetic modifications** and DNA-interacting proteins. Based on this current knowledge, therapeutic options can be envisioned (see [Clinician's Corner](#)). The activation of NF- κ B could be addressed with inhibitors as one broad therapeutic option. Clinically relevant small molecule inhibitors of NF- κ B are comprehensively reviewed in Ramadass *et al.* and are therefore not discussed here [68]. The notion that *H. pylori* infection can induce epigenetic modifications implies the consideration of an epigenetic therapeutic approach, which will be briefly discussed below.

Epigenetics describe heritable molecular determinants of a specific phenotype which are independent of DNA sequence. These include features like DNA methylation, histone modifications, and chromatin structure. The epigenetic status provides information about the current state of a cell. Transformation is characterized by a defined epigenome [69]. There exists ample evidence that *H. pylori* infection leads to aberrant epigenetic modifications, inducing a **CpG island methylator phenotype (CIMP)** [70] and that these modifications contribute to gene regulation [25,27,71,72] and to the development of GC [73-76]. The impact of a single methylation in a position adjacent to the NF- κ B binding site on the function of NF- κ B has been shown recently [77]. Insight into *H. pylori*-mediated epigenetic changes in gastric epithelial cells leading to excessive inflammation, partially mediated by NF- κ B, justifies the contemplation of an epigenetic therapy. Recently, newly developed drugs show clinical success in hematological tumors and therapeutic potential in solid tumors [78]. The feasibility of this therapeutic approach was recently documented in a study of a bromodomain extra-terminal motif (BET) inhibitor (JQ1) in *H. pylori* infection. The publication clearly shows the involvement of bromodomain containing 4 (Brd4), a BET protein, in the NF- κ B-dependent regulation of inflammatory genes. JQ1 inhibited the recruitment of Brd4 to RelA and suppressed inflammation and cell proliferation in *H. pylori*-infected mice [79], but this approach still lacks specificity for defined genes. Further research will be necessary for induced targeted epigenetic modifications [80,81]. A glimpse of what might be possible in the future is shown in two recent publications [82,83]. Kang *et al.* demonstrate the usefulness of a **CRISPR/Cas9-mediated epigenetic editing system** to modulate the methylation at specific CpG sites of the Octamer-Binding Protein 4 promoter to elicit gene expression [82]. Liao *et al.* show the *in vivo* delivery of a Cas9-based epigenetic gene activation system, which ameliorates disease phenotypes in mouse models [83].

We are at the start to the development of new outstanding tools for therapeutic interventions. In order to implement them and yield effective results, we need to better understand the molecular mechanisms in gastric disorders.

Concluding remarks

Although the activity driven by NF- κ B is increasingly recognized as an important facilitator of disease processes including gastric pathophysiology [3], pivotal challenges to comprehensively understand the essential molecular mechanisms and their role in the disease remain to be resolved (see [Outstanding questions](#)). At this point, fundamental knowledge of *H. pylori*-associated molecular processes represent a key issue [84,85]. A first step is the use of organoids and the recently developed 2D polarized monolayers [51] of the stomach as innovative tools to study the role of *H. pylori*-triggered NF- κ B in regulating tissue homeodynamics. The stomach-on-chip will be the next step to study the *H. pylori*-induced pathophysiology upon long term infection and the coordinating role of NF- κ B in this process. Herein, the implementation of stromal and immune cells will enhance our comprehension of the role of the microenvironment of the gastric mucosa [86]. Furthermore, the use of organoids derived from cancer patients are already contributing to the refinement of treatments specific for the genotype of the patient, paving the way to personalized medicine. The realization of the impact of epigenetic changes on the development of *H. pylori*-associated diseases will lead to new therapeutic approaches. The application of newly evolved drugs targeting the inducer or modifier of epigenetic marker will also find its way into the treatment of gastric diseases. Last but not least, the advent of the CRISPR/Cas9-mediated epigenetic editing system will give us the right instruments to precisely correct changes which contribute to gastric malignancies.

Glossary

Air-liquid-interphase

Cell culture method formed upon removal of medium from the apical side of cells seeded on transwell inserts still exposed to medium at the basal side of the cells.

Carcinogenicity

The measure for the carcinogenic potential of a substance.

Chromosomally instable (CIN) gastric cancer

Refers to a higher than normal degree of missegregation of chromosomes or parts of chromosomes during mitosis. Form of gastric cancer.

CpG island methylator phenotype (CIMP)

Classification of a cancer type with a high degree of methylation in CpG-rich promoters.

Clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9)-mediated epigenetic editing system

A new technique of genome-editing using a guide RNA (gRNA) to specifically target a genome region and change the epigenetic modification, like methylation, in this region.

Deubiquitinylase

Deubiquitinylases are enzymes that cleave ubiquitin attached to proteins.

Dysplasia

The visible, abnormal development of cells within an organ or tissue.

Epigenetic modification

Heritable, temporally regulated and reversible modifications of chromosomal components leading to changes in gene expression patterns without altering the underlying DNA sequence.

Epstein Barr virus associated gastric cancer

442 Most common malignancy associated with Epstein-Barr virus (human Herpes virus)
443 infection. Form of gastric cancer.

444

445 **Genomically stable (GS) gastric cancer**

446 These cancers have a low mutation burden and low somatic copy number aberrations.
447 Frequently mutated in E-cadherin and RhoA gene. Form of gastric cancer.

448

449 **Homeodynamics**

450 Describes the dynamic regulation and interaction among different levels of
451 organization within a complex biological system.

452

453 **Intestinal metaplasia**

454 Intestinal metaplasia is a condition in which the gastric epithelial cells are changed or
455 replaced and resemble the intestinal lining.

456

457 **Microsatellite instable (MSI) gastric cancer**

458 Associated with a hypermutated genome, DNA hypermethylation, MLH1 silencing and
459 frequent *PIK3CA* mutations. Form of gastric cancer.

460

461 **Multilineage differentiation capacity**

462 Describes the ability of a cell to differentiate in multiple cell types within a cell lineage.

463

464 **Peptic ulcer**

465 Peptic ulcers are lesions in the lining of the stomach, lower esophagus, or small
466 intestine, usually formed because of inflammation, as well as from erosion from
467 stomach acids.

468

469 **Single nucleotide polymorphism (SNP)**

470 Describes the substitution of a single nucleotide at a specific position in the genome.

Box 1. *H. pylori* type-B-gastritis and development of gastric neoplasia

H. pylori infection of the stomach elicits NF- κ B signaling via ADP-heptose [9] and this contributes to chronic inflammation (lymphocytes and plasma cells) in combination with a varying active component (neutrophilic granulocytes). Chronic and active inflammatory infiltrates are graded histologically according to the updated Sydney System [87].

Besides *H. pylori* type-B-gastritis, there are also other forms of chronic gastritis often with no active inflammatory component such as chemical reactive gastritis (type-C-gastritis) (Figure I). Such non-active forms of gastritis almost never undergo malignant transformation. Historically, this leads to an etiological typing of the form of gastritis into type-A for autoimmune gastritis, type-B for bacterial gastritis and type-C for chemical reactive gastritis (due to NSAID/ASA or bile reflux) [88]. It is mainly type-A and -B gastritis with the varying degree of active inflammatory infiltrates that predispose to the development of neoplasia [85].

Chronic and active inflammation can proceed to the development of atrophy of the glands, intestinal metaplasia (development of goblet cells) and subsequent development of dysplasia (blue caskets) and carcinoma (green gaskets) [89] (Figure I). Such precancerous conditions can be assessed endoscopically as well as by the Kyoto classification risk scoring system [90]. *H. pylori*-positive first-degree relatives of gastric carcinoma patients show an odds ratio (OR) of 15 to develop carcinoma. Those individuals showing a markedly active inflammation in the middle and upper stomach compared to the distal stomach (corpus dominant gastritis) have an OR of 32 for developing GC [91]. A pilot study indicates an improvement of atrophy and intestinal metaplasia upon treatment and thus potentially reducing the risk of malignant transformation [92].

Besides a strong indication that NF- κ B is dysregulated during gastric carcinogenesis, the detailed processes are still somewhat unclear [31]. The gastric neoplasm itself can be subgraded into those arising from the gastric surface epithelium (foveolar differentiation), those from deep gastric mucous glands (pyloric gland differentiation) and from specialized gastric glands (chief cell or oxyntic gland differentiation) [84] (Figure I). Therapeutically, there is little difference in these differentiations of gastric neoplasia and almost all of them occur in an inflamed gastric mucosa, with two exceptions. These are some cases of foveolar neoplasia, chief cell/oxyntic gland

neoplasia and a genetically-caused signet ring cell carcinoma [93]. They cannot be prevented with a *H. pylori* eradication therapy and a regular follow-up is the only option.

Figure I, Box1. Schematic representation *H. pylori* type-B-gastritis and development of gastric neoplasia. Created with [BioRender.com](https://www.biorender.com).

Box 2. Organoids and mucosoids represent important tools for studies close to the *in vivo* setting

Chopping tissue into small pieces (<1 mm), incubation with solutions containing chelating agents (agents) followed by a compression enables the release of gland and single cells from stomach tissue. Cells can be obtained from healthy or cancer [94] biopsies or surgical resections. The commercial extracellular matrix known as MatrigelTM and a cocktail of growth factors [49, 50, 94] enable the formation of cyst-like structures known as organoids (Figure I A, step I). Gastric organoids are hollow cyst-like structures that can be up to ~300 µm in diameter. They are composed of a single layer of columnar epithelial cells with their apical side facing the lumen (Figure I B). The apical side of the epithelium is in the body the part that faces the luminal content of the stomach. Organoids can be propagated every 7 to 10 days by mechanical and enzymatic dissociation for several passages (Figure I A, steps I and II). Alternatively, single dissociated cells from tissue or from organoid cultures can be seeded at confluency in collagen-coated polycarbonate filters in transwells (Figure I A, step III). Cells are left for 3 days in culture before starting air-liquid interphase. Removal of liquid from the apical side enables the cells to grow in height and start secreting mucus after approximately 10-13 days (Figure I C). In the mucosoids, the apical side is more accessible compared to organoids and any factor included in the cultivation medium will access the basal side. This is a great advantage in infection studies as *H. pylori* can be added directly into the mucus while for organoids, it must be injected. Mucosoids can be propagated by deriving single cells from step IV and reiterating steps III and IV (Figure I A, steps III and IV). Alternatively, single cells from mucosoids can be used to seed organoids (from step IV to step I) indicating that these two methods are totally interconvertible.

Figure I, Box2. Generation of organoids and mucosoids from healthy and diseased gastric tissue. Created with [BioRender.com](https://www.biorender.com).

Box 3. Clinician's corner

The *H. pylori*-induced NF- κ B pathways influence the behavior of gastric epithelial cells by regulating the expression of a great variety of molecules, which in turn affect other cellular pathways. These changes to the gastric epithelial cells can be paramount for a transformation of the gastric mucosa as demonstrated for DARPP32, which is increased in gastric tissue samples of intestinal metaplasia, high-grade dysplasia and adenocarcinoma. The search and analysis of such predisposing factors have the potential to identify diagnostic markers.

In order to develop new therapeutic options, the study of GC is transferring to a new level of experimental systems closer to the *in vivo* situation. Gastric organoids derived from different patients are useful for studying drug susceptibility and a valuable platform for translational applications with an outlook towards personalized medicine. A license-based biobank of cancer organoids called HUB Organoids has been established to foster further clinical research.

The implication of *H. pylori*-induced NF- κ B-regulated molecules in the onset of gastric malignancies makes NF- κ B a candidate for therapeutic approaches. Clinically relevant small molecule inhibitors of NF- κ B are in clinical trials in phases 1 to 3. They target membrane receptors, adaptor molecules, the IKK complex, the ubiquitin-proteasome system, nuclear translocation, DNA binding and transcriptional activation of the NF- κ B pathways.

Newly developed epigenetic drugs show clinical success in hematological tumors and therapeutic potential in solid tumors. A number of them are already approved (Vidaza, Dacogen, Zolinza, Idhifa and Tazverik) and many more are in clinical trials. Furthermore, epigenetic modifications in GC tissue can be used as diagnostic and prognostic tools to assess patients in the clinic.

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Figure legends

Figure 1. *H. pylori*-induced NF- κ B pathways.

Cytosolic bacterial ADP-heptose binds and activates ALPK1, which leads to the phosphorylation of existing TIFA dimers at threonine nine. These phosphorylated TIFA dimers interact with different targets in so called TIFAsomes to facilitate either TRAF6 K63-linked ubiquitinylation or cIAP1 K48-linked ubiquitinylation and degradation to activate the classical or alternative NF- κ B pathway, respectively. Interaction with TRAF6 triggers the binding of TAK1 via TABs and the activation of the IKK complex, leading to I κ B α degradation, liberation and nuclear entry of RelA/p50. On the other hand, interaction with the NIK regulatory complex (TRAF2, TRAF3, cIAP1) and the ensuing degradation of cIAP1 stabilizes NIK, initiates the phosphorylation of IKK α followed by the phosphorylation and proteasomal processing of p100 to p52. The resultant RelB/p52 dimer translocates to the nucleus. The NF- κ B dimers bind the respective target promoter to regulate gene expression. Created with [BioRender.com](https://www.biorender.com).

Figure 2. Hypotheses on the role of NF- κ B that can be tested using stem cell driven culture models

(A) WNT EGF and BMP regulate healthy epithelial homodynamic, but infection and inflammation might alter the signalling microenvironment thereby inducing changes in the gland cell composition as observed in chronic, atrophic gastritis and intestinal metaplasia. (B) The apical and the basal side of an epithelial are actively secreting proteins and we can hypothesize that NF- κ B is central in controlling antimicrobial secretion in the mucus to attack bacteria and chemokine on the basal side to coordinate the response of the immune system. (C) Cancer starts from mutated cell that can outcompete the normal one under specific circumstances. We hypothesize that inflammation is the microenvironment that can promote mutation but also induce the expansion of mutated cells at the cost of the normal epithelium. Created with [BioRender.com](https://www.biorender.com).

Outstanding questions

How to analyze the trigger-dependent spatio-temporal dynamics of NF- κ B regulation in cell physiology/pathophysiology?

Molecular and biomedical studies addressing NF- κ B-dependent cellular processes, mostly focus on one single mechanism, but what is the overall effect of dysregulated NF- κ B function on gastric cell physiology?

How to develop a diagnostic evaluation of NF- κ B-associated alterations taking into account the presence of gene polymorphisms, overexpression of genes and epigenetic alterations?

How to identify the single molecule/or protein modification in the regulation of NF- κ B that leads to a specific cellular response in order to target it therapeutically without affecting the other processes?

How to design therapeutics, which specifically target cells containing dysregulated NF- κ B without interrupting its function in all other cell types?

What kind of co-culture systems implementing gastric organoids with cells of the micromilieu are necessary to investigate close to the *in vivo* situation NF- κ B-dependent tissue homeodynamics and the *H. pylori*-associated gastric pathophysiology?

Will it be possible to engineer personalized biomimetic tissue models for the development of safer therapeutic targeting strategies to a variety of human diseases?

Response to the comments of the editor and reviewers

EDITORIAL COMMENTS

Editor

1. In the introduction ("H. pylori-induced NF- κ B signaling 80 in colonized gastric epithelial cells"), please ensure that the significance, motivation and timeliness of the topic of discussion for a TMM broad readership are clearly stated. Why is this topic of discussion important in this day and age? The timeliness should also be connected to the description of the sub-topics covered. (In other words the connection between why you are writing this piece with the subtopics covered should be made obvious). A final statement in the intro drawing in the reader and conveying the excitement in the field should be included.

We have now stated the importance, motivation and timeliness of the topic for a TMM broad readership and have linked the text of the introduction to the description of the sub-topics (see **lines 85-96**). In addition, a statement has also been included to draw the reader into the topic and express enthusiasm for it (see **lines 128-132**).

2. Please note that the Clinician's Corner should not be a second summary of the text. The aim of the Clinician's corner is to capture the attention of medical research scientists. It differs from the Outstanding Questions and Highlights in that it comprises very simple and succinct information and take-home messages that would be needed in the medical field and guide practicing clinicians on the topic. Relevant points are disease prevalence, diagnostics, clinical trials, as well as current and novel therapeutics.

We improved the text in the revised manuscript and have addressed diagnostic markers, personalized medicine, clinical trials and novel therapeutics (see **lines 546-567**).

3. Overall, I felt the "organoid" section is not well connected to the other section of the manuscript, and especially the jump from the organoid section to the therapy section seems abrupt. The manuscript needs to be more cohesive and flow a little better, and so it would be important to include more transition words and checking how each of the paragraphs connect to each other to deliver the key points and take-home messages across. Ask yourself what are the key arguments that you are trying to make, what are the implications and interpretations of the findings for the next section? The manuscript should follow a logical thread to make a more unifying story. I also highly recommend a summarizing statement and transition to "concluding remarks"

To improve coherence, we added more transitional words to better connect the paragraphs and convey the key points and take-home messages (see **lines 164-167; 226-230; 254-256; 343-345; 384-386**).

4. The Concluding Remarks section is a rather short roundup of the manuscript and omits the discussion of the section on (epigenetic) therapy options. Please ensure that the Concluding Remarks section is not just a summary of the main body of the text, but rather, that it opens up new ground and future perspectives (and I am sure this is what you intend). It needs to direct readers to a fundamental question of "where do we go from here... and what is the big picture?"

As requested we now included a statement on (epigenetic) therapy options in the concluding remarks and address some fundamental questions of the topic (see **lines 392-400; 402-408**).

5. Other minor points:

The manuscript contains some sentences that were complicated or incomplete and there are some small areas for corrections that may not be noticed or misinterpreted at the proof stage. Therefore, please carefully proof-read your revised manuscript prior to resubmitting.

We have carefully proof-read the revised manuscript.

I recommend referring to both references 81 and 82 in the format of "XY et al " as I was confused why one was cited as "XY et al" and the other as "XY and colleagues" (unless there is a reason to do so that I did not see.

We have changed to *et al.* in both cases-

You can remove the following terms from the Glossary, as they can be assumed common knowledge of TMM readers: Inflammation, mutagenesis, Stem cells, organoids, xenograft mouse model

We have removed the proposed terms in the revised version of the manuscript.

For Figure 1, I suggest to shorten the title and remove "Schematic representation "

We have shortened the title as recommended (see **line 806**)

Please note that Box figures should not be accompanied by a legend. Instead, all relevant figure legend text should be incorporated into box text. Please also note that Box figures need to be submitted as editable files, labelled as "Figure (roman numeral), Box (arabic numeral)".

We have removed the figure legends from the figures in the boxes. We now included the text in Fig. I in Box 1 (see **lines 501-505**).

If biorender was used for creation of figures, please credit biorender in the acknowledgement as "figures were created with biorender".

We have now acknowledged biorender.

6. Another important point to keep in mind: please double-check that your review of the literature is updated at this point. The number of references should be limited to 150 (including requests to add additional references), so please remove redundant and dated ones and avoid dwelling on your own publications. Please ensure that reliance on reviews is only 10% of total references and that scientifically sound, primary research papers (especially from the last 0-5 years) represent the core support of your article.

We have kept the number of references to a minimum and most of the original references are from the last 5 five years. We have cited a sufficient number of reviews in our article and due to the editorial limitation about this number we refrain from including more reviews as requested by Reviewer #3.

Reviewer #1

The abbreviations should be uniformed after its first occurrence in the manuscript, such as gastric cancer (GC). In summary, the manuscript meets the requirements for publication.

We have standardized the abbreviations according to their first occurrence.

Reviewer #2

Minor points for improvement:

1.) The authors should acknowledge previous NF-kB / H. pylori review articles such as (doi: 10.1016/j.tim.2016.12.004; doi: 10.1016/j.tim.2010.08.003).

We had already included one of the suggested reviews in our submitted manuscript, the other review was published 2010. As recommended by the editor (item 6), we have cited a sufficient number of reviews in our article and due to the editorial limitation about this number we refrain from including further reviews.

2.) The authors should at least mention in brief that the effector protein CagA plays some role in NF-kB signaling, especially at late stages of infection (doi: 10.1073/pnas.0409873102).

The suggested reference is a single observation published in 2005. As recommended by the editor (item 6), we have mainly cited original references from the last five five years and therefore refrain from citing this reference.

3.) *The authors should also discuss the role of NF-kB activation in the inflammasome (doi: 10.1016/j.micinf.2017.06.005; doi: 10.1007/978-3-319-41171-2_6).*

The role of NF-kB in the inflammasome and the proposed reviews are not relevant to our review article. Therefore, we refrain from discussing this topic.

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