Studying of host-pathogen interactions to develop new Helicobacter pylori therapies and prevent adverse consequences of its eradication.

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Abstract

Helicobacter pylori (*Hp*) colonizes the human gastric mucosa with a high worldwide prevalence. Currently, *Hp* is eradicated by the use of antibiotics. However, elevated antibiotic resistance suggests new therapeutic strategies need to be envisioned. One promising approach is the prophylactic vaccination. Pre-clinical and clinical data show that a urease-based vaccine is efficient in decreasing *Hp* infection through the mobilization of T helper (Th) and their associated immune effectors cells. Among them Th17 cells and eosinophils have been identified.

In our studies, we demonstrate that GM-CSF$^+$ IL-17$^+$ Th17 cells accumulate in the stomach mucosa of *Hp* infected mice during the vaccine-induced reduction of *Hp* infection. Then, we provide evidence that vaccinated GM-CSF deficient mice only modestly reduce *Hp* infection. Conversely, we observe that an increase in GM-CSF availability reduces *Hp* burden in chronically infected mice. Finally, we show that GM-CSF, by acting on gastric epithelial cells, promotes the production of βdefensin3, which exhibits *Hp* bactericidal activities. In parallel, we confirm that activated eosinophils infiltrate the gastric mucosa during vaccine-induced reduction of *Hp* infection. However, the absence of eosinophils does not decrease the efficacy of our *Hp* vaccine in vivo. Indeed, vaccinated eosinophils deficient mice, display a lower *Hp* colonization compared to their eosinophil sufficient counterparts. Although the vaccine induces similar urease-specific humoral and Th responses in both eosinophil sufficient and deficient mice, a decreased production of anti-inflammatory cytokines, such as IL-10, TGFβ, and calgranulin B, was specifically observed in eosinophil depleted mice.

Taken together, we demonstrate a key role of GM-CSF, most probably originating from Th17 cells, in the vaccine-induced reduction of *Hp* infection. In addition, our results suggest that gastric eosinophils maintain an anti-inflammatory environment, thus sustaining chronic *Hp* infection. Because eosinophils are one of the main immune effectors mobilized by Th2 responses and that GM-CSF originates from Th17 cells, our studies strongly suggest that the formulation of an *Hp* vaccine needs to include an adjuvant that preferentially primes *Hp*-specific Th17 responses.

Résumé

Helicobacter pylori (*Hp*) est une bactérie colonisant la muqueuse gastrique avec une haute prévalence mondiale. Le traitement de l'infection par *Hp* requiert l'usage de plusieurs antibiotiques. Malheureusement, l'augmentation croissante de la résistance à ces antibiotiques suggère que de nouveaux traitements doivent être développés. Parmi les solutions envisagées, la vaccination prophylactique retient particulièrement l'attention et semble prometteuse. En effet, des études précliniques et cliniques ont démontré qu'un vaccin à base d'uréase (exprimée à la surface de *Hp*) diminue efficacement la charge bactérienne. En effet, cette vaccination induit la mobilisation des cellules Th ainsi que de leurs cellules effectrices associées. Parmi elles, les cellules Th17 et les éosinophiles ont été identifiés.

Dans nos études, nous avons démontré que les cellules Th17 GM-CSF$^+$ IL-17$^+$ s'accumulent dans la muqueuse gastrique des souris infectées avec *Hp* grâce à la vaccination. En parallèle, la vaccination de souris déficientes en GM-CSF n'induit qu'une modeste réduction de l'infection. De plus, lors d'une supplémentation en GM-CSF, l'infection à *Hp* est diminuée chez les souris infectées chroniquement. Enfin, nous avons démontré que le GM-CSF stimule les cellules épithéliales et induit la production du peptide antimicrobien βdefensine3 possédant des propriétés antibactériennes contre *Hp*.

En parallèle, nous avons confirmé que des éosinophiles activés infiltrent la muqueuse gastrique lors de l'élimination de *Hp* grâce à la vaccination. Cependant, l'ablation des éosinophiles ne diminue pas l'efficacité de la vaccination. Au contraire, les souris vaccinées éliminent mieux l'infection en l'absence d'éosinophiles. Nous avons également observé que la réponse humorale spécifique à l'uréase et les réponses Th ne varient pas avec la présence ou non d'éosinophiles. Cependant, nous avons observé une augmentation des cytokines anti-inflammatoires comme l'IL-10, le TGFβ et la calgranuline B lorsque les souris ne possèdent pas d'éosinophiles.

En conclusion, nous avons démontré que le GM-CSF, probablement issu des cellules Th17, a un rôle important dans l'élimination médiée par la vaccination de l'infection avec *Hp*. De plus, nos résultats suggèrent que les éosinophiles présents dans la muqueuse gastrique maintiennent un environnement anti-inflammatoire propice à l'établissement d'une infection chronique. Sachant que les éosinophiles sont les principales cellules effectrices mobilisées lors d'une réponse immunitaire de type Th2, et que le GM-CSF provient des cellules Th17, nos études suggèrent que la formulation d'un vaccin contre *Hp* devrait privilégier un adjuvant qui stimule une réponse spécifique contre *Hp* de type Th17.

Abbreviations

Ag	Antigen
AlpA/B	Adherence-associated lipoprotein A and B
AMP	Anti-microbial peptides
APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
BabA	Blood group antigen-binding adhesion
BAX	Bcl-2-associated X-protein
Cag	cytotoxin-associated gene
CagPAI	cytotoxin-associated gene pathogenicity island
cAMP	Cyclic adenosine 3,5-monophosphate
CD	Cluster of differentiation
cDC	Classical dendritic cell
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CLR	C-type lectin receptors
CNS	Central nervous system
COX-2	Cyclooxygenase 2
CT	Cholera toxin
CTB	Cholera toxin subunit B
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DC-SIGN	Dendritic cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
dmLT	Double mutant heat labile toxin
DNA	Deoxyribonucleic acid
EAE	Experimental allergic encephalomyelitis
ECP	Eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EGFR	Epidermal growth factor receptor
EoE	Eosinophilic esophagitis
EPO	Eosinophil peroxidase
ERK	Extracellular signal-regulated kinases
FasL	Fas ligand
FDA	Food and Drug Administration
FoxP3	Forkhead box P3
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM-CSFR	GM-CSF receptor
gp130	Glycoprotein 130
hBD	Human beta-defensin
Hop	*Helicobacter* outer membrane protein
Hp	*Helicobacter pylori*
***HP*-NAP**	*Helicobacter pylori* neutrophil-activating protein
IARC	International Agency for Research on Cancer
IBD	Inflammatory bowel disease
IDO	Indoleamine 2-3 deoxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
JAK	Janus kinase

JAM	Junctional adhesion molecule
LCN2	Lipocalin2
LN	Lymph nodes
LPS	Lipopolysaccharide
LT	Heat labile toxin
LTB	Heat labile toxin subunit B
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MHCII	Major histocompatibility complex class II
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NAP	Neutrophil-activating protein
NET	Neutrophil extracellular trap
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
OipA	Outer inflammatory protein A
OMP	Outer membrane protein
p53	Tumor protein P53
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death 1
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed death-ligand 1
PI3K	Phosphoinositide 3-kinase
PPI	Proton pump inhibitor
Prg2	Proteoglycan2
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RNase2	Ribonuclease A family member 2
RORγt	Retinoic acid-related orphan receptor gamma t
ROS	Reactive Oxygen Species
RPTPβ	Receptor protein tyrosine phosphatase beta
SAA	Serum amyloid A
SabA	Sialic acid-binding adhesion
S*HP*2	Src homology-2 domain-containing protein tyrosine phosphatase-2
SiglecF	Sialic acid-binding immunoglobulin-like lectin F
STAT3	Signal transducer and activator of transcription 3
T4SS	Type IV secretion system
T-bet	T-box expressed in T cells
TCR	T cells receptor
TGF	Tumor growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T regulatory
VacA	Vacuolating cytotoxin A
γGT	Gamma-glutamyltranspeptidase

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CHAPTER 1: INTRODUCTION

1. *Helicobacter pylori*

Helicobacter pylori (*Hp*) is a gram-negative bacterium that colonizes the gastric mucosa. This infection is acquired commonly during childhood and persists lifelong if not treated. The transmission of the infection is not fully understood but lots of evidence prone to gastro-oral, oral-oral or fecal-oral contamination routes, especially in context of intra-familial clusters or mother to child transmission [1].

In 1982, B. Marshall and R. Warren identified for the first time this small-curved bacilli on gastric epithelium of half of their patients with chronic gastritis, coming for gastroscopy [2]. After further investigation, *Hp* infection have been associated with gastric inflammation, asymptomatic in most of cases, which could progress to more severe pathologies or cancers [3]. In 1994, the International Agency for Research on Cancer (IARC) has recognized *Hp* as type 1 carcinogen [4, 5] due to its infection-induced cancers in humans. Finally, in 1996, the Food and Drug Administration (FDA) approved for the first time 3 different regimens to eradicate *Hp* in patients with active duodenal ulcers [6]. Thanks to these treatments leading to *Hp* clearance, the risk of recurrences of duodenal ulcers as well as the incidence of gastric cancer have significantly decreased [7, 8]. Unfortunately, due to the emergence of antibiotics resistance and poor sanitary conditions in some developing countries, *Hp* infection remains a public concern. Indeed, a strong epidemiology study published in 2015 reveals that more than half of the world population is infected with *Hp* [9]. In addition, *Hp* remains responsible of approximatively 90% of gastric cancers, which are the third more common cancer worldwide.

1.1. Gastric environment

As part of the gastrointestinal (GI) tract, the stomach is located between the esophagus and the duodenum. It is composed of the cardia, the fundus, the body and the pyloric part. Its functions are mechanical and chemical digestion of ingested food, nutrients absorption and hormones secretion such as gastrin, cholecystokinin, secretin, and gastric inhibitory peptide.

The stomach contain a gastric juice made of various enzyme and hydrochloric acid, which maintain an acidic environment at pH 1-2. Due to its low pH, majority of microorganisms are not able to colonize the stomach. However, one of them is *Hp,* which chronically colonizes the antrum of the stomach.

To infect efficiently the stomach, *Hp* has developed a large panel of strategies to overcome the low pH, escape innate and adaptive immune responses and adhere to the gastric epithelium. Despite several virulence factors, the pathogenicity of *Hp* first relies on its ability to survive in a low pH environment. In this attempt, *Hp* has developed mechanisms to neutralize the acidic pH mainly thanks to urease [10]. Urease is a nickel-containing enzyme that is produced in large quantities by *Hp* intracellularly but is also present at its surface. Low gastric pH enhance intracellular urease production. The substrate of urease is urea. At low environmental pH, urea can enter into the intracellular space through porins and passive diffusion mediated by permease protein (UreI). This urea is hydrolyzed into ammonia (NH_3) and carbamate by urease in presence of nickel as cofactor. Then, carbamate is decomposed into NH_3 and broken down into CO_2 and H_2O. Finally, NH_3 is protonated to form ammonium (NH_4^+). This NH_4^+ neutralizes the stomach acidity, creates a neutral microenvironment around the bacterium, and allows its survival [11].

In addition to the low pH, a viscoelastic mucus gel that protects the epithelium against acidity and pathogen invasion coats the stomach. To colonize efficiently the gastric environment, *Hp* needs to cross this gel, to get close to the epithelium. In this attempt, *Hp* motility provided by its flagella is crucial but not sufficient. Indeed, viscoelasticity of gastric mucins, which are glycoproteins constitutive of the mucus, is dependent of the pH. At low pH, mucins form a viscous gel where *Hp* is not able to swim. Conversely, at raised pH, viscoelasticity is decreased significantly to reach a solution form. In this context, *Hp* can easily swim due to its helical shape and motility, far from the acidic lumen, and reach the gastric epithelium [12].

Once *Hp* reaches the epithelium, it must binds to the epithelial cells surface otherwise it would be quickly eliminated. In this attempt, adhesins play a central role for *Hp* binding to gastric epithelial cells. The outer membrane proteins (OMPs), characteristic of gram-negative bacteria, are essential adhesins in this context. Among them, the blood group antigen-binding adhesion (BabA) and the sialic acid-binding adhesion (SabA) adhesins are known to recognize lewis antigens presents at the gastric epithelial cells surface [13]. Other adhesins, such as adherence-associated lipoprotein A and B (AlpA/B), the outer inflammatory protein A (OipA), and *Helicobacter* outer membrane protein (Hop) Z are also implicated into the adhesion processes but their respective roles need to be further clarified. Once bounded to the cell surface, the cytotoxin-associated gene pathogenicity island (CagPAI) virulence factor, encoding Type IV secretion system (T4SS)-dependent host cell signaling plays a key role. Its activation induces the transcription of genes that enhance inflammation, development of intestinal metaplasia, and is associated precancerous transformations [14].

In parallel, *Hp* can influence the epithelial barrier by modulating the tight and adherent junctions. Tight and adherent junctions are multiprotein junctional complexes

composed by occluding, claudins, junctional adhesion molecules (JAMs), catenin and cadherin. These junctions play a major role in the barrier functions of the gastric mucosa. Theses junctional complexes regulate the diffusion of small and large molecules across the epithelium. Several studies showed that *Hp* in disrupting these junctions, in part through dissolution of the E-cadherin/ β-catenin complex, leads to the modification of epithelial cell proliferation and polarity [15, 16].

Finally, a continuous turnover of gastric epithelial cell constitutes an efficient defense mechanism against bacterial colonization. In order to constrain this cell renewal, *Hp* is able to prevent epithelial cells apoptosis and decrease the rapid turnover of these cells favoring its persistent [17].

Once *Hp* is well established in close vicinity and/or adhere to epithelial cells, *Hp* interacts with gastric epithelial cells through the production of several virulence factors to dysregulate various signaling pathways and host immune responses.

1.2. Modulation of innate immune responses by *Hp*

To ensure its persistence, *Hp* highjacks the host innate immune responses. These immune responses are provided by gastric epithelial cells, resident and recruited immune cells (e.g. dendritic cells (DCs), macrophages and neutrophils) upon *Hp* detection through pattern recognition receptors (PRRs) [18].

1.2.1. Pattern Recognition Receptors

PRRs are receptors able to recognize molecular patterns from pathogens (pathogen-associated molecular patterns (PAMPs)) or damaged cells (damage-associated molecular patterns (DAMPs)). Once PRRs are activated, they trigger innate immune responses through secretion of pro-inflammatory cytokines such as type I interferon (IFN). PRRs also regulate several cell death programs [19]. There are four types of

PPRs. However, in context of *Hp* infection, two of them are crucial: the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs).

1.2.1.1. TLRs

Gastric epithelial cells and innate immune cells such as DCs, macrophages and neutrophils, express TLRs. TLRs can be either expressed on cell surface (TLR2, TLR4, TLR5) or intracellularly, located in the endosome (TLR8, TLR9) [20].

TLR2 and TLR4 are specifics of PAMPs and specifically recognizes lipoteichoic acid or lipopolysaccharides (LPS) which are constitutive of the outer membrane of gram-negative bacteria [21].

TLR5 is specific of flagellin, which is the central protein of bacterial flagella.

TLR8 and TLR9 recognize bacterial nucleic acids. For instance, TLR9 detects bacterial DNA, especially in DCs [22].

After PAMPs detection, all TLRs recruit myeloid differentiation primary response gene 88 (MyD88) which activates nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) for the induction of type I IFN and inflammatory cytokine genes [23].

Remarkably, *Hp* possesses a strong ability to avoid TLRs recognition due to the expression of modified LPS and flagellin. LPS are composed of three parts: the lipid A, which is the most toxic part, the oligosaccharide core related to the O-antigen, which is in direct contact with extracellular milieu. First, the lipid A of *Hp* is highly modified compared to other gram-negative bacteria allowing it to attenuate its recognition by TLRs [24]. Second, *Hp* O-antigen exhibits structures that mimic human Lewis antigens expressed at the surface of gastric epithelial cells of the host allowing its evasion from TLRs detection [25]. Third, *Hp* displays flagellin mutations leading to its dampened recognition by TLR5. However, TLR5 is detected in gastric lesion of *Hp* infected

humans, suggesting its implication upon infection. Indeed, instead of flagellin detection, TLR5 system is highjacked by the T4SS of *Hp* via CagL (T4SS pilus-exposed protein) allowing a modulation of immune responses to its advantage [26]. Taken together, *Hp* uses efficient tools to escape immune recognition or modulate innate immune responses to its advantage.

1.2.1.2. CLRs

Apart from TLRs, *Hp* interacts with another type of PRRs: the CLRs. Among CLRs, dendritic cell-specific Intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) are expressed by myeloid DCs and interact with pathogens such as *Hp* through mannose or Lewis antigen sugars. Once recognized, pathogens are usually internalized and degraded for further antigen presentation and T helper (Th) cells induction [27]. Interestingly, recognition of *Hp* Lewis antigens by DC-SIGN blocks the differentiation of naïve T cells into Th1 cells by DCs. Moreover, the binding of *Hp* lewis antigens to DC-SIGN induced increased production of the anti-inflammatory cytokine interleukin (IL)-10. This increased of IL-10 is in part protective, since it limits damages caused by inflammation. However, it dampens immune responses against *Hp,* leading to its persistence. Finally, the variable expression switch on/off of Lewis antigens at *Hp* cell surface allows efficient modulation of the immune system contributing to *Hp* persistence [28, 29].

1.2.2. Inhibition of innate immune recognition

1.2.2.1. Resistance to phagocytosis

Once detected through different PAMPs, the presence of *Hp* enhances recruitment of immune cells such as macrophages, DCs and many neutrophils through chemoattractant IL-8 and others bacterial factors secretion [30]. These cells are antigen-presenting cells (APCs) capable of killing pathogens by phagocytosis.

Normally, once bound through adhesins, bacteria are rapidly ingested by APCs. Then, through endosomal pathway, bacteria are degraded and eliminated in a mature phagolysosome. However, *Hp* has developed several mechanisms to escape phagocytosis. Instead of most of bacteria, *Hp* is recognized via its T4SS that acts as an adhesin and slows ingestion by APCs. In parallel, as *Hp* secretes ammonia, the pH of the *Hp*-containing phagosome is increased. This elevated pH inhibits formation of mature phagolysosome and stimulate megasome formation. Megasomes consist in clustering of several phagosomes and contain multiple bacteria. Finally, these megasomes confer resistance to intracellular killing [31, 32]. Additionally, *Hp* is auxotrophic for cholesterol and extracts it from epithelial membranes. Thanks to *Hp* cholesterol-α-glucosyltransferase, intrinsic α-glucosylation of cholesterol allows *Hp* to escape phagocytosis [33].

However, it is important to notice that not all *Hp* strains are able to avoid phagocytosis. Indeed, *Hp* bacteria are divided in two groups. First, the type I group expressing both vacuolating cytotoxin (VacA) and CagPAI, which is able to resist to phagocytosis and is responsible of severe symptoms. Second, the type II group lacking these virulence factors, which is often asymptomatic and sensitive to macrophages killing [32, 34].

1.2.2.2. Resistance and production of Reactive Oxygen Species and Nitric Oxide

In addition to phagocytosis inhibition, *Hp* can resist to killing by reactive oxygen species (ROS). They are responsible of irreversible bacterial DNA damages but also oxidative stress that can impair cell integrity. First, *Hp* produces the pro-inflammatory molecule neutrophil-activating protein (NAP), which activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase for ROS production [35]. Second, upon APCs activation due to *Hp* engulfment, ROS are produced. However, to overcome damages, *Hp*

produces catalase and superoxide dismutase, which detoxify ROS and allow *Hp* survival [32]. In parallel, *Hp* disrupts NADPH oxidase system to avoid accumulation of ROS in the phagosome during phagocytosis. Consequently, ROS are released directly in the intracellular space, apart from *Hp*-containing phagosomes, which avoid *Hp* killing but is detrimental for host cell integrity [36].

In parallel, another line of defense against pathogens is the production of inducible nitric oxide synthase (iNOS) by macrophages. This iNOS enzyme catalyzes the conversion of L-arginine in nitric oxide (NO) and is another mechanism to kill *Hp*. Urease expressed by *Hp* triggers iNOS production, which is detrimental for *Hp* survival. Despite this production, *Hp* infection persists thanks to its production of arginase. Indeed, L-arginine is an essential nutrient for *Hp* and serves as substrate for urea production catalyzed by arginase. Consequently, the presence of *Hp* decreases L-arginine availability for macrophage production of NO [37]. In parallel, macrophages primed by *Hp* produce Arginase II. Some data highlighted Arginase II also dampen L-arginine availability and dampen NO production [38]. Altogether, these mechanisms limit ROS and NO productions by macrophages and protect *Hp* against killing.

1.2.3. Anti-microbial peptides

Upon *Hp* stimulation, gastric epithelial cells have the ability to secrete anti-microbial peptides (AMPs). These small peptides, mostly cationic, are an important part of the innate immune response against pathogens. Since bacterial membrane are electronegatively charged, strong electrostatic interactions occur between AMPs and bacteria [39]. In addition, AMPs trigger several immune responses such as cytokine production, wound healing or chemotaxis. Among AMPs, defensins play a critical role upon *Hp* infection. In human, *Hp* infection triggers human beta-defensin2 (hBD2), hBD3, hBD4 and the amphipathic α-helical cathelicidin LL37. hBD3 and LL37 efficiently

kill *Hp* [18], as well as hBD2 in a lesser extent [40]. Unfortunately, some studies demonstrate *Hp* has the ability to subvert recognition by AMPs. Indeed, expression of hBD3 relies on epidermal growth factor receptor (EGFR) activation. However, the virulence factor CagA induces EGFR dephosphorylation, which abrogates hBD3 expression and leads to *Hp* survival [41]. In addition, high levels of hBD2 have been detected but correlated with chronic gastritis of *Hp* infected patients compared to non-infected one [42], suggesting that high local production of defensins might not necessarily beneficial. Consequently, additional studies are important to better characterize the defensin responses, to understand the mechanisms regarding resistance or *Hp* sensitivity to these peptides and to determine whether defensin analogs could be an alternative to antibiotics.

1.3. Adaptive immune response : Dendritic cells

DCs are important APCs, mediating innate and adaptive immune responses through T cells activation. The DC family contain two major division: the classical DCs (cDC1 and 2), and plasmacytoid DCs (pDCs). They both share the same precursor but differ in their life cycle as well as in their functions. In their immature state, all the DCs are found into the periphery and some tissues and act as sentinels before migrating into lymphoid and nonlymphoid organs for Ag presentation. The cDCs possess a strong ability to sense tissue damages as well as to capture and present Ag to T cells. As a result, cDCs reinforce immunity through an efficient naïve T cells priming. Instead of cDCs, pDCs express low level of MHCII but display TLR7/9 at their surface for pathogen nucleic acid recognition. Upon activation, pDCs secrete large amount of type I IFN and present antigen to T cells in LNs [43].

Upon *Hp* infection, DCs are able to cross the epithelium by extending their dendrites through the tight junctions and to take up *Hp* in order to initiate immune responses.

However, *Hp* induces DCs maturation toward a tolerogenic phenotype expressing less MHCII and co-stimulatory molecules cluster of differentiation (CD) 80/CD86 [32]. Upon CagA stimulation, these tolerogenic DCs produce high amounts of IL-10 that activates the signal transducer and activator of transcription 3 (STAT3) pathway and dampen IL-1β production [44]. In parallel, CagA activates the Src homology-2 domain-containing protein tyrosine phosphatase-2 (S*HP*2) leading to inhibition of the interferon regulatory factor (IRF) 3 translocation and IFNγ production. These two pathways dampen pro-inflammatory IL-12p40 and tumor necrosis factor (TNF) α production by DCs [32, 45]. In parallel, *Hp* enzymatic activity of the γGT virulence factor converts glutamine into glutamate. This glutamate is recognized by glutamate receptors presents at DCs surface and inhibits cyclic adenosine 3,5-monophosphate (cAMP) signaling for the pro-inflammatory cytokine IL-6 production [46]. In addition, some studies prone for an increased expression of the T cells co-inhibiting molecule programmed death-ligand 1 (PD-L1) on DCs upon chronic infection [47]. Altogether, the *Hp*-dependent modulation of DC functioning dampens pro-inflammatory cytokines production and favor anti-inflammatory molecules production. Consequently, naïve T cells priming will generate high number of T regulatory (Treg) and low number of Th1 and Th17 cells.

1.4. Adaptive immune response : effector T cells

Apart from APCs, $CD4^+$ T cells and $CD8^+$ T cells are also recruited into the gastric mucosa of infected individuals. In addition, IFNγ, TNFα, IL-1, IL-6, IL-7, IL-8 and IL-18 cytokines production are also increased, suggesting a Th1 polarized response in infected hosts. Later, additional studies demonstrate that Th17, Th22 and Treg cells are also recruited into the gastric mucosa during *Hp* infection [48].

1.4.1. Effector T cells

Th1 cells produce mainly IFNγ and are in general more implicated in the control of intracellular bacterial infection. This Th1 subtype is under the control of the transcription factor T-box expressed in T cells (T-bet). Moreover, these cells are the major Th cells subtype mobilized in context of *Hp* infection. Indeed, antigen presentation by APCs coupled with local cytokines production, trigger Th1 proliferation in the LN and Th1 reactivation in the gastric mucosa. Altogether, these Th1 stimulations lead to IFNγ, IL12 and IL-18 production, which are significantly present in the gastric biopsies of infected individuals.

Although, Th1 response in *Hp*-mediated immune responses is well recognized, Th2 cytokines have been detected, in a lesser extent, in the gastric mucosa of infected patients. Th2 cells secrete IL-4, IL-5 and IL-6 via the transcription factor GATA-3. Several studies report their protective role from extracellular pathogen-mediated infections as increased *Hp* infection load was detected in IL-4 deficient mice [49]. However, some efforts left to better characterize the role of Th2 response during *Hp* infection.

Few years ago, apart from Th1 and Th2 cells, Th17 cells that produce IL-17 were identified in *Hp* infected host. Th17 cells differentiation is mediated by tumor growth factor (TGF) β, IL-6 and IL-23 through the upregulation of the transcription factor retinoic acid-related orphan receptor gamma t (RORγt). In addition, it is now well established that Th17 derived-cytokines such as IL-6, IL-8, Granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-17 and IL-21 (for autocrine Th17 stimulation) are important to fight against pathogens. In human infection, IL-17 has a regulatory role on IL-8 production, a strong neutrophils chemoattractant. Indeed, IL-17 is capable of stimulating IL-8 release by gastric epithelial cells, promoting neutrophils recruitment

in the gastric mucosa [50]. In addition, several studies probe for a synergistic role of IL-17 and IL-22 to promote secretion of AMPs such as lipocalin2 (LCN2) and βdefensins. Consequently, some studies described IL-17 as a promising target to promote protective immune responses against *Hp* [51].

As producer of IL-22, Th22 are important for mucosal immunity, in response to IL-6 and TNFα, to trigger secretion of anti-microbial peptides by epithelial cells. Remarkably, IL-22 receptor are only present in tissues, such as gastric epithelial cells, allowing a direct communication with the immune system [52].

Instead of Th cells, Treg cells are capable of suppressing Th cells proliferation to maintain a balance between immunity and inflammation. However, Treg cells also limit beneficial immune responses (e.g. in context of vaccine-induced responses) meaning they can have either beneficial or deleterious effects. For instance, Treg cells are important to prevent or limit autoimmune and chronic inflammatory diseases. In another hand, they can block beneficial responses against some pathogen such as *Hp* by preventing sterilizing immunity. Treg cells display several suppressive mechanisms: secretion of inhibitory cytokines (e.g. IL-10, IL-35 and TGFβ), cytolysis (through granzymes and perforins secretion), metabolic disruption (due to IL-2 consumption) and DCs modulation. The DCs modulation occurs via interaction of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) present at Treg cells surface and CD80/86 of DCs. This interaction triggers secretion of the immunosuppressive molecule indoleamine 2-3 deoxygenase (IDO) by DCs [53]. Apart from Th1 and Th17, DCs are also able to drive Treg responses through secretion of IL-10 and IL-18, which trigger forkhead box P3 (FoxP3) production and Treg differentiation [54]. In context of *Hp* infection, these cells are found in the circulation of infected individuals suggesting these cells are recruited into the gastric mucosa to limit inflammation, but contributing

to *Hp* persistence. For instance, children infected with *Hp* have a greater amount of Treg than infected adults have and are less prone to develop gastritis [55].

Altogether, a balance between Th and Treg cells is important to maintain gastric integrity. However, *Hp* have developed multiple tools to dysregulate this balance on its advantage.

1.4.2. Modulation of T cells populations by *Hp*

1.4.2.1. T helper cells inhibition

In order to insure its persistence in the gastric mucosa of its host, *Hp* virulence factors are important to dampen T cells responses.

1.4.2.1.1. Role of VacA

The virulence factor VacA is important for T cells highjacking by *Hp*. By arresting G1/S cell cycle, VacA inhibits T cells signaling and proliferation. Indeed, it interferes with the T cells receptor (TCR)/IL-2 signaling leading to the suppression of the translocation of nuclear factor of activated T cells (NFAT). As NFAT is one of the main regulator of T cells, its modulation dampens *Hp*-activated T cells expansion [56, 57]. In addition, VacA decreases the mitochondrial membrane potential of T cells thanks to its N-terminal hydrophobic region, leading to inhibition of T cells proliferation [58]. Finally, VacA mediates apoptosis in T cells via an intrinsic pathway. Indeed, through T cells mitochondrial destabilization, *Hp* leads to increased expression of the pro-apoptotic protein Bcl-2-associated X-protein (BAX) and dampens expression of the anti-apoptotic Bcl-2 family proteins. BAX interacts with the transcription factor tumor protein P53 (p53), which is increased upon cellular stress or deoxyribonucleic acid (DNA) damages and leads to cell death [59]. Altogether, VacA contributes to *Hp* persistence in the gastric environment.

1.4.2.1.2. Role of γGT

The γGT virulence factor also influences T cell responses. Indeed, γGT arrests cell cycle at G1 leading to compromised proliferation, activation and effector cytokine expression of T-cells. As it mediates extracellular cleavage of glutathione and ROS production, γGT limits T cells cycle by depriving them from glutamine [60, 61]. This modification of the extracellular milieu compromises c-Myc and IRF4 (both important for T cells proliferation and activation) expression and leads to a decreased effector T cells response, favorable to *Hp* persistence [61].

However, Wüstner et al. recently demonstrated that the enzymatic activity of γGT is also important to initiate gastric colonization through glutamine deprivation. In addition, it triggers infiltration of $CD8^+$ T cells as well as elevated IFNγ and consequently can modify the outcome of the infection [62].

1.4.2.1.3. Role of Arginase

Arginase is also implicated in *Hp*-induced T cells response dampening. Like macrophages, L-arginine is important for T cell functions. As *Hp* also need L-arginine for urea production, T cells are deprived. Consequently, T cell functions are impaired as well as the expression of the CD3ξ-chain of the TCR is decreased [63].

1.4.2.1.4. Role of CagPAI and CagA

CagPAI and its effector protein CagA are also important virulence factors. Indeed, this factor is responsible of T cell apoptosis through the induction of the Fas ligand (FasL) expression [64] but its principal function is to modulate signal transduction in epithelial cells [65].

Taken together, *Hp* virulence factor are crucial for inhibition of T cell responses and constitute a major evasion mechanism for persistence of the infection (**Figure 1**).

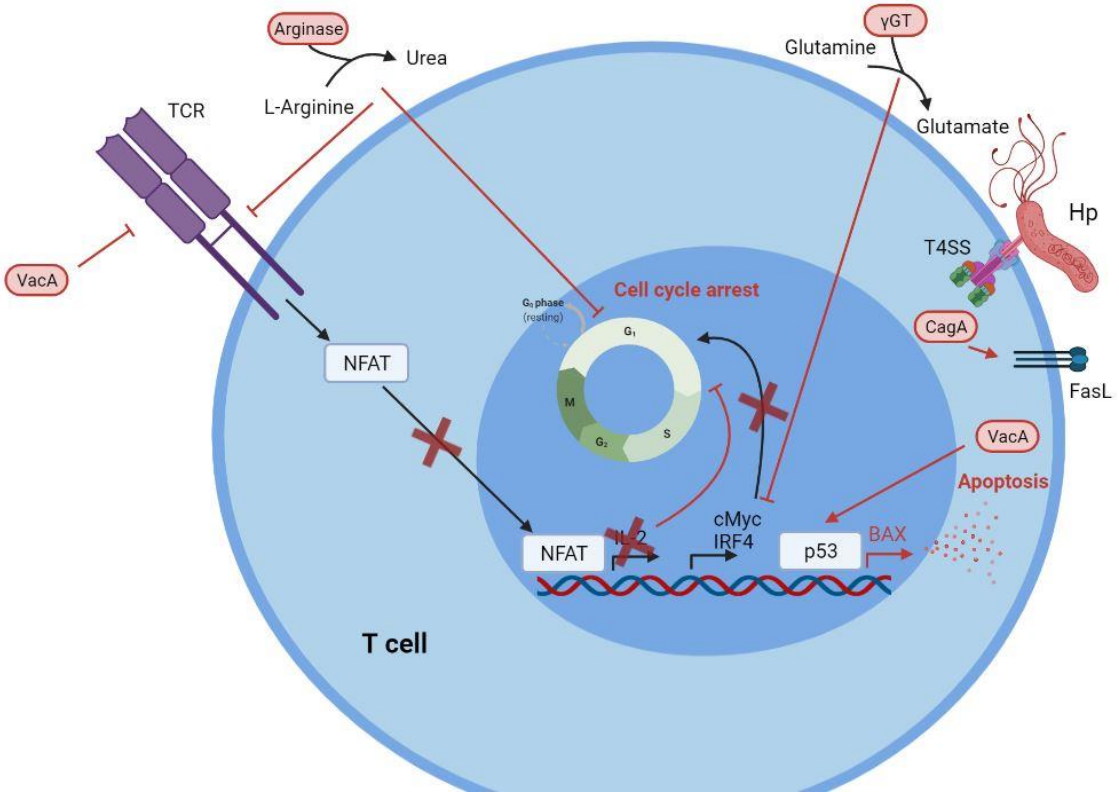

Figure 1. Modulation of effector T cells by Hp virulence factors. *Hp* infection triggers massive Th response in the gastric mucosa. However, *Hp* displays a panel of virulence factors (in red circles) to highjack T cells response and maintain its persistence. VacA interacts with TCR. Through the NFAT pathway, VacA stop IL-2 production leading to cell cycle arrest. In parallel, VacA stimulates p53 transcription factor leading to pro-apoptosis protein BAX production. In addition, γGT deprives cell environment of glutamine, which compromise c-Myc/IRF4 signaling and impairs T cell survival. *Hp* secretes arginase important to catalyze L-Arginine to urea reaction. Depravation of L-Arginine triggers cell cycle arrest and decrease TCR expression. Finally, *Hp* T4SS allows intracellular secretion of CagA, which enhances FasL activation leading to apoptosis.

1.4.2.2. Modulation of Treg cells generation

Apart from Th dampening, *Hp* trigger accumulation of Treg cells in the gastric mucosa. As immunosuppressive cells, Treg maintain effector T cells function at a suboptimal level to facilitate *Hp* colonization. Indeed, Treg population increases in the gastric mucosa of infected patients [32]. However, the *Hp*-induced Treg cells production is dependent of the age of the patient when the infection occurred. As mentioned before, *Hp* infection occurring during neonatal period triggers many Treg cells, leading to immune tolerance and protection against T cells-mediated immunopathology such as gastritis and gastric cancers. However, infection at adult stage leads to elevated Th1/Th17 responses and *Hp*-induced pathologies [66].

1.5. Bacterial Plasticity and Virulence factors

1.5.1. Bacterial plasticity

Hp is one of the most genetically diverse bacterial species since it exhibits extensive diversity among strains [67]. Due to its strong mutagenesis abilities, *Hp* have developed powerful tools to escape the immune system. In addition, this diversity leads to lots of different immune responses in infected patients and complicates treatments development. Adhesins and virulence factors variation is particularly efficient to persist in the gastric mucosa. Indeed variability of adhesins relies on allele differences as well as on on/off mechanisms [68]. In addition, some studies of virulence factors such as CagPAI demonstrate high mutation rates and high recombination frequencies in human [68]. Taken together this bacterial plasticity helps *Hp* to colonize the gastric mucosa on the long run and leads to resistance to antibiotic treatments [69].

1.5.2. Virulence factors

As mentioned before for T cells modulation, *Hp* has a strong virulence potential thanks to several bacterial factors [70]. These factors are divided into several categories depending on their properties: cell binding molecules (e.g. BabA, SabA, OipA, HopQ, HopZ, CagL, CagY), immunoresponsive elements, (e.g. NapA, γGT, peptidoglycan) and survival proteins (e.g. urease, flagellin, arginase). Among them, two major virulence determinants are key for *Hp* pathogenesis: VacA and CagPAI.

1.5.2.1. VacA

VacA is a toxin that is secreted by *Hp* and causes many effects on epithelial cells, APCs, phagocytic cells, mast cells and T cells [71]. Indeed, it alters late endocytic compartments, mitochondrial membrane permeability and cellular signaling pathways. It has been firstly characterized as it triggers formation of intracellular vacuoles in

mammalian cells [72]. Later, several studies suggests *Hp* expressing VacA has a selective advantage for stomach colonization as it contributes to initial colonization and also have immunosuppressive properties enabling its persistence [73].

First, VacA modulates epithelial cells function. Indeed, VacA binds to the plasma membrane of cells through various receptors such as receptor protein tyrosine phosphatase beta (RPTPβ), sphingomyelin, EGFR and heparin/heparan sulfate [74] and induces cell vacuolation [75]. In addition, VacA induces mitochondria alterations resulting of an impairment of cell-cycle progression [76]. Cellular signal-transduction pathways is also altered through activation of MAPK since VacA can directly bind to RPTPβ without any internalization. Finally, VacA increases epithelial permeability [77] and increases transepithelial flux of molecules through formation of channels in the plasma membrane [78].

Secondly, VacA modulates the functions of immune cells. VacA induces the formation of megasomes to protect *Hp* from macrophages phagocytosis. Moreover, VacA dampen T cell response, as described earlier. In addition, VacA promote maturation of DCs through a tolerogenic phenotype that promote IL-10 secretion and Treg expansion [79]. However, VacA is also reported to bind and directly activate mast cells for migration and production of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 [80]. Finally, VacA induces the pro-inflammatory enzyme cyclooxygenase 2 (COX-2) expression in neutrophils and macrophages [81]. Taken together, modulation of immune cells by VacA is complex since it is a balance between immunostimulatory and immunosuppressive actions.

1.5.2.2. CagPAI and T4SS

As many gram-negative bacteria, *Hp* expresses T4SS. This pilus structure allows translocation of *Hp* virulence factors into gastric epithelial cells and modify their cellular

machinery for its benefit. The CagPAI, with its substrate protein CagA, [82] encodes this T4SS. CagA have been detected in highly virulent *Hp* infection and is associated with development of aggressive forms of gastric diseases [83]. In *Hp*, T4SS is important to trigger two major mechanisms in infected epithelial cells: induction of IL-8 through transcription factor NF-κB and delivery of CagA.

Delivery of CagA into epithelial cells is mediated by integrin receptors at host cell surface. These integrins are located at the basolateral surface of cells meaning *Hp* has to disrupt cell junctions made through serine protease secretion [84]. Mainly, *Hp* uses integrin β1 thanks to several Cag components (CagA, CagI, CagL, and CagY) for CagA translocation [65].

Using several subset of the cited above signaling factors, injected CagA hijacks multiple cellular signal transduction cascades and thereby induces different cell responses such as anti-autophagy, cell proliferation, anti-apoptosis and cell differentiation [85]. For instance, CagA interacts with glycoprotein 130 (gp130) receptor. This complex can interact with janus kinase (JAK)/STAT3 (for inflammation, anti-apoptotic and pro-metastatic gene activation such as Bcl-2 proteins family) or tyrosine phosphatase S*HP*2/extracellular signal-regulated kinases (ERK) pathways [86]. The phosphorylation status of CagA balances the activation of these pathways to modulate anti-apoptotic effects and overcome self-renewal of the gastric epithelium [17].

1.5.2.3. γGT

Hp γGT is a key bacterial virulence factor that support initial colonization as well as pro-inflammatory immune responses. First studies probe for an essential role of γGT for colonization [87]. However, despite γGT facilitates *Hp* colonization, it appears to be not essential in recent studies [62, 88]. However, its implication into glutamine and

glutathione consumption are beneficial for *Hp* colonization since it leads to production of ammonia and generation of ROS. It triggers gastric epithelial cells apoptosis (through caspase activation), necrosis (via depletion of adenosine triphosphate) and cell cycle arrest [89]. Apoptosis is mediated by the up-regulation of proapoptotic proteins (e.g. BAX) and the downregulation of its anti-apoptotic counterpart. However, it can also enhance cell proliferation through NF-κB activation, leading to COX-2, IL-8, iNOS and others growth factors induction [90]. Apart from its modulation on T cells responses (as previously described), and similarly to VacA, γGT reprograms DCs toward a tolerogenic phenotype promoting Treg expansion [79]. This tolerogenic phenotype is mediated by glutamate generated via γGT enzymatic activity, which inhibit cAMP signaling and IL-6 secretion [46]. Finally, high activities of γGT have been associated with peptide ulcer disease [91].

1.5.2.4. Other adhesion molecules

The blood group antigen-binding adhesion BabA facilitates the adherence of *Hp* to Lewis^{b} antigens and enhance pathogenicity of *Hp* via T4SS. Sialic acid binding adhesion protein (SabA) facilitates the adherence of *Hp* to sialyl-Lewis^{x} antigens. In early stage of infection, BabA/ Lewis^{b} is essential to colonization. However, upon gastric inflammation, sialyl-Lewis^{x} antigens expression increase and promote SabA adhesion to enflamed tissues [16]. In addition, SabA has an on/off state allowing modulation of its activities.

HopZ is also important for adhesion but its relationship with other virulence factors and clinical outcomes remain unclear [92].

Recently carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family was shown to serve as receptors for the *Hp* outer membrane protein HopQ. The HopQ-CEACAM1 interaction allows translocation of CagA and trigger IL-8 expression [93].

As HopQ, the outer inflammatory protein A (OipA) seems to interact with CagA, leading to the induction of IL-8 expression and neutrophils recruitment. It also inhibits apoptosis of gastric cells and has an on/off state [92].

The *Hp* neutrophil-activating protein (*HP*-NAP) is a potent immunomodulator that promotes Th1 and Th17 responses, leading to progression of inflammation and tissue damages [94]. It also stimulates neutrophils adherence to gastric epithelial cells and promotes ROS production. Furthermore, it promotes release of chemokines and cytokines such as IL-8, TNFα, IL-6 and activates neutrophils to release IL-12 and IL-23 for Th1 and Th17 promotion. *HP*-NAP also increases anti-apoptotic proteins from the Bcl-2 family, protecting cells from apoptosis [70].

Altogether, these virulence factors maintain a complex equilibrium between pro and anti-inflammatory responses to maintain *Hp* persistence via T cells modulation (**Figure 1**) and epithelial cells highjacking (**Figure 2**).

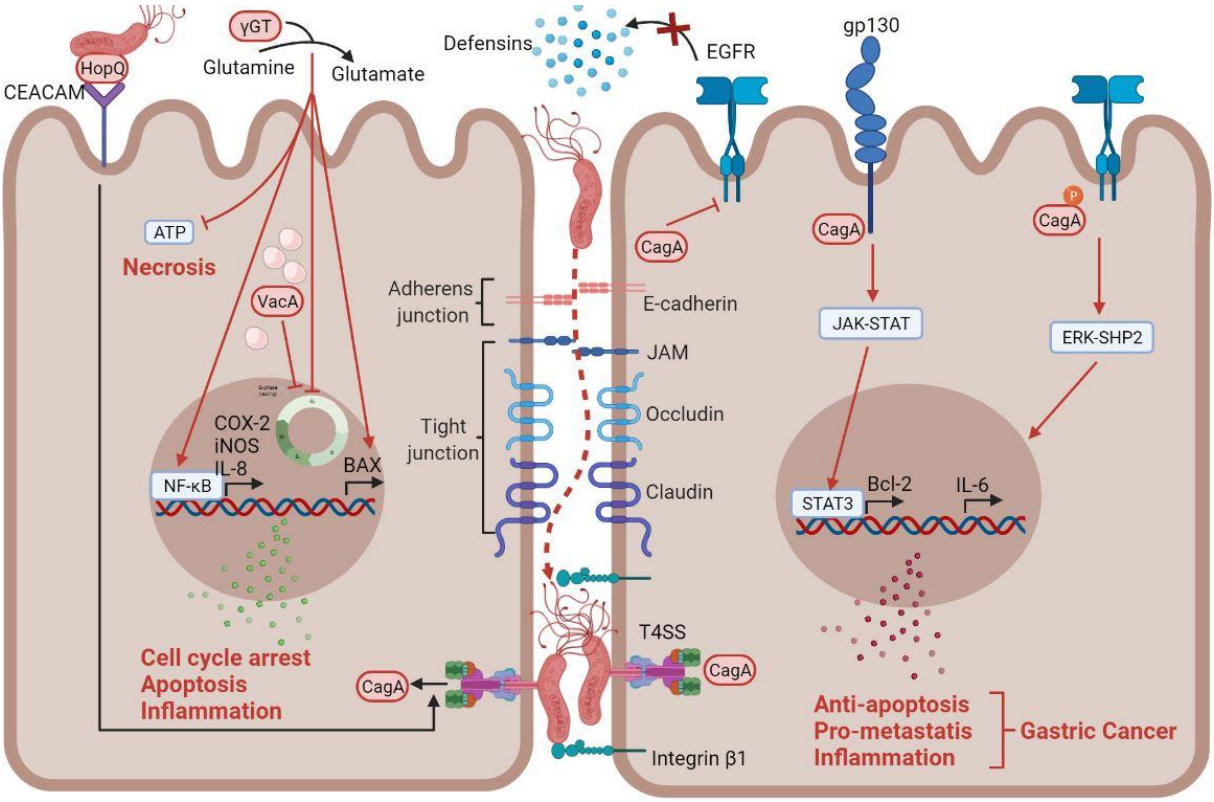

Figure 2. Hp infection highjack epithelial cells machinery. Upon *Hp* infection, epithelial cells are highly impacted. Thanks to vacuolation, VacA reaches the intracellular space and triggers cell cycle arrest. In addition, γGT enhances glutamine consumption, ATP depletion leads to necrosis. In parallel it also stop cell cycle and promote pro inflammatory molecules secretion via NF-kB activation as well as pro-apoptosis BAX protein production. Altogether, these mechanisms impair the epithelial barrier integrity and are favorable for *Hp* persistence. In parallel, *Hp* disrupts adherens (E-cadherin) and tight (JAM, occluding, claudin) junctions and gets access to the basolateral part of epithelial cells. With help of integrin β1 and HopQ/CEACAM interaction, T4SS releases CagA in the intracellular space. CagA interact with gp130 via the JAK/STAT pathway to enhance STAT3-mediated Bcl-2 anti-apoptotic protein secretion. Upon phosphorylation, CagA promotes IL-6 production via ERK-S*HP*2 signaling. Finally, CagA blocks the EGFR responsible of the release of defensins. Using several mechanisms, CagA creates an inflammatory anti-apoptotic environment and enhance cell metastasis leading to severe gastric cancer.

Due to the multiple interactions between *Hp* and its host, a constant improvement of our understanding of these mechanisms is crucial to develop new therapeutics and prophylactic strategies [95] (**Figure 3**).

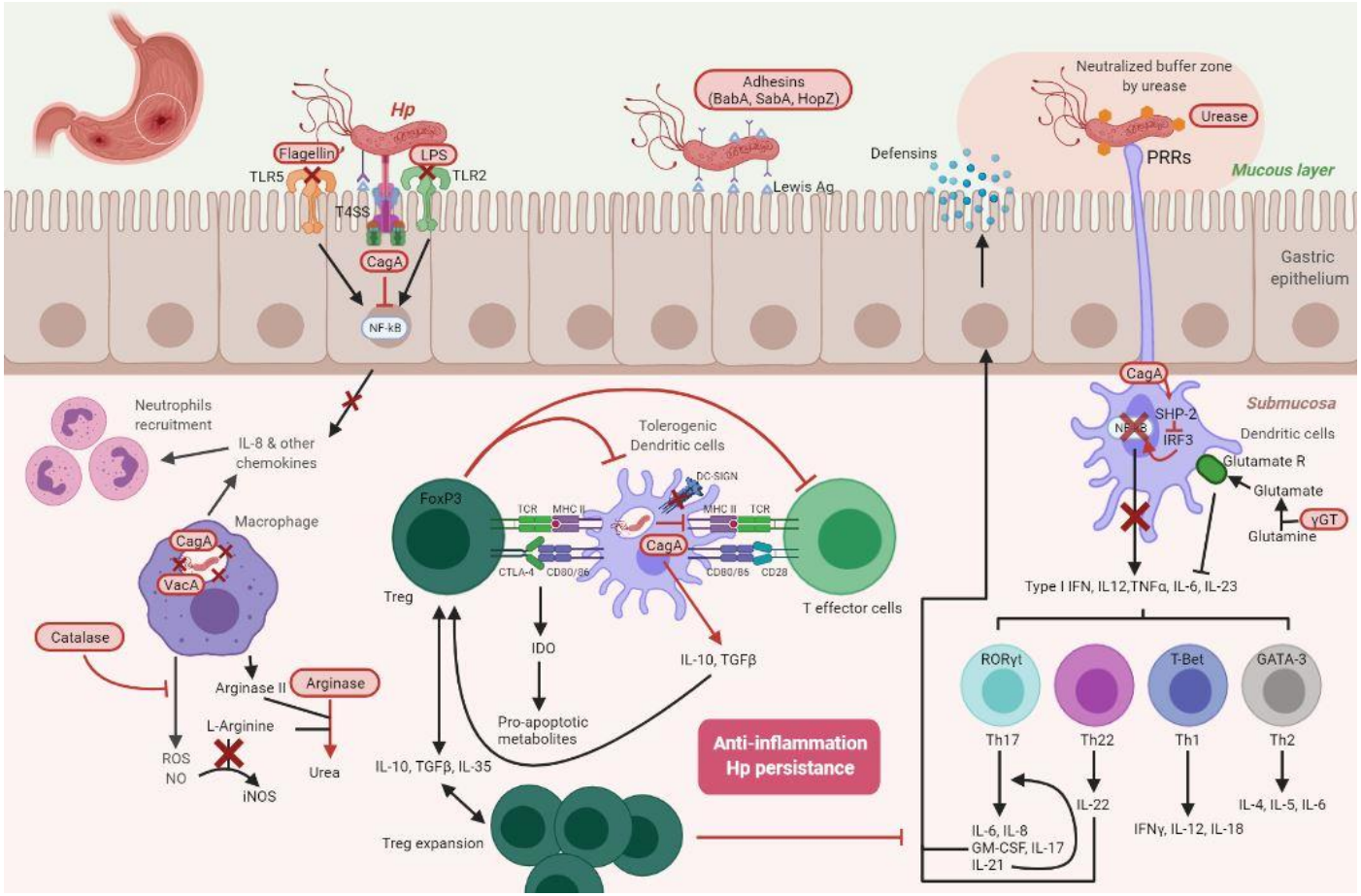

Figure 3. Immune responses to Hp infection. Hp neutralizes the acidic pH thanks to urease and creates a buffered zone to allow *Hp* persistence. Using its adhesins, *Hp* binds to lewis antigens at the cell surface. Thanks to its special fragellin and LPS, *Hp* avoid recognition respectively by TLR5 and TLR2-4 to block neutrophils attracting chemokine IL-8 secretion. In parallel DCs sense *Hp* through PRR and DC-SIGN. However, CagA enhances S*HP*-2 pathway leading to IRF3-mediated NF-κB dampening. Associated with increased glutamate receptor activation mediated by γGT, this leads to inhibition of pro-inflammatory cytokines secretion responsible of effector T cells maturation. In parallel, *Hp* resists to phagocytosis. In macrophages and DCs, once phagocytosed, *Hp* avoid formation of phagolysosome thanks to VacA and CagA. In addition *Hp* catalase blocks secretion of ROS and NO by macrophages. *Hp* triggers arginase II secretion by macrophages, which associated with its own arginase, deprave cells from L-arginine to create urea and avoid formation of iNOS. Moreover, CagA block MHCII and DC-SIGN in DCs to dampen interactions with effector T cells. DCs are deflected toward a tolerogenic phenotype and produce IL-10 and TGFβ. These cytokines promote Treg generation and CTLA-4 interaction with CD80/86 present at the cell surface of DCs trigger IDO and secretion of pro-apoptotic metabolites. Treg expansion maintains an anti-inflammatory environment by inhibiting effector T cells and their pro-inflammatory cytokines. The absence of these cytokines also dampen defensins secretion by gastric epithelial cells.

1.6. Therapies

Due to its ability to highjack the host immune system, *Hp* persists on the long run. Responsible of around 80% of gastric cancers, it is the second most common causes of cancer death in 2020 worldwide [96]. In addition, eradicating the infection cures gastritis and prevents gastric cancers [97]. Consequently, developing efficient therapies such as antibiotics treatment or vaccination is a public health concern.

1.6.1. Antibiotics

When *Hp* was discovered, 7 days triple therapy with twice daily administration of clarithromycin, metronidazole (or amoxicillin), and proton pump inhibitors (PPIs) produced eradication rates of up to 90% in clinical trials and rapidly became the standard of care [98]. Due to clarithromycin resistance, a popular alternative is bismuth-based quadruple therapy consisting of a PPI combined with bismuth, tetracycline, and metronidazole treatment. Unfortunately, this treatment is not convenient for patients since it involves taking lots of pills at multiple time a day, reduced compliance [99]. Therefore, according to Savoldi et al, “resistance of *H pylori* to antibiotics has reached alarming levels worldwide” [100].

To address this challenge, three separate expert groups in the USA [101], Canada [102, 103] and Europe [104] have recently discussed treatment guidelines. Altogether, they recommend using the antibiotic sensitivity data of each patients to avoid inappropriate antibiotic use. However, the lack of antibiotic sensitivity in some part of the world is a strong limitation of this recommendation [105]. Altogether, with the constant declining of these therapies efficiency, it is important to consider and develop new therapeutic approaches such as vaccination.

1.6.2. The development of Vaccines

Vaccination against *Hp* appears as a promising approach to overcome antibiotic resistance. To develop an efficient *Hp* vaccine, *Hp* infection mouse models have been developed, first using *Helicobacter felis*, and finally with clinical isolate of *Hp*. In these mouse models, oral immunizations with *Hp* antigens coupled with mucosal adjuvant such as cholera toxin (CT) or heat labile toxin from *Escherichia coli* (LT) only partially reduce the infectious load [106]. Therefore, it is important to characterize the vaccine-induced immune responses to improve vaccine efficacy.

It was previously shown that the vaccine-induced reduction of *Hp* infection rely on $CD4^+$ T cells but not on antibodies. Th1, Th17 and Th22 are particularly important during the vaccine-induced reduction of *Hp* infection [48, 107, 108]. In mouse model, multiple *Hp* proteins (e.g. urease, CagA, VacA) were demonstrated to confer protection. In addition, several mucosal routes of vaccine administration were shown to efficiently provide protection (e.g. oral, intranasal and rectal) [109]. Finally, *Hp* urease was the first recombinant bacterium protein used for oral vaccination, in combination with LT in human [110]. Unfortunately, as observed in mice, this immunization gave only a partial reduction of *Hp* and was associated with side effects such as diarrhea. From there, several clinical trials were performed without reaching sterilizing immunity [109, 111]. One might hypothesize is that since *Hp* triggers immune tolerance by stimulating Treg generation, it also limit the vaccine-induced immune responses. Consequently, therapeutic vaccines are not able to generate fully protective Th responses. The *Hp*-induced Treg will not be an obstacle for prophylactic vaccination. However, it is important to consider that *Hp* infection almost occurs during childhood. Consequently, prophylactic vaccine should be given very early in life to be effective, and this would be challenging since many vaccines are already done during this period [112]. Zeng et

al. perform for the first time in 2015 a phase 3 clinical trial of a prophylactic *Hp* vaccine in children. Three oral administrations of the vaccine composed of urease B subunit and heat-labile enterotoxin B subunit were performed in children. Within the first year, vaccination displayed an efficacy of 71.8%. However, the efficacy drop to 55% in the second and the third year [113]. In conclusion, although those results are encouraging, some efforts left to obtain an efficient vaccine [114].

1.7. Beneficial effects of *Hp* on different diseases

Although the eradication of *Hp* infection prevents gastric cancer, it is also important to consider that *Hp* infection could display beneficial effects on several diseases [112]. These are almost allergic Th2-related diseases such as asthma and eosinophilic esophagitis (EoE).

1.7.1. Protection against Asthma

Asthma is a chronic inflammatory disease characterized by airways hyper-responsiveness and obstruction consecutive to allergen exposure. Asthma is caused by a breakdown of immune tolerance toward specific allergens, causing an aberrant immune response. Mast cells, eosinophils, Th17 and Th2 cells participate to the allergic response. Importantly, several studies have reported an inverse correlation between asthma incidence and chronic infection with *Hp*, especially when *Hp* carries the CagA virulence factor [115, 116].

One hypothesis is that *Hp* interferes in Th2 mediated disease through the generation of T regulatory (Treg) cells. Indeed, induction of Tregs in *Hp* infected mice was shown to be a key factor to prevent allergic asthma in mice [66]. Some data suggest that *Hp* possesses the ability to profoundly influence DCs maturation processes through IL-18 production and leading to Treg differentiation [54, 117]. The key role of Treg in asthma

protection have also been demonstrated recently in human, suggesting that the IL-10 secreting Treg are mandatory to regulate allergen-specific IgE production [118].

1.7.2. Role of *Hp* in EoE

Eosinophilic esophagitis (EoE) is a chronic immune-mediated disease characterized by esophageal dysfunction and eosinophilic inflammation localized to the esophagus. In addition to the high number of eosinophils, the esophagus of EoE patients displays a thickened mucosa and basal layer hyperplasia associated with a collagen deposition [119]. Some evidences suggest that these symptoms are triggered by food and aeroallergen hypersensitivity and by an impaired epithelial barrier function [120]. Moreover, the environment plays a key role into the development of the disease and especially alteration of the microbiome during early life (e.g. antibiotics use, C-section delivery or breastfeeding) [121].

In industrialized countries such as Switzerland, the incidence and prevalence of EoE has rapidly increased in the past 20 years [122]. Now, this disease has become the second most frequent esophageal disease after the reflux, resulting in an important public and economic concern.

Currently, treatment strategies are very restrictive for the patients. Indeed, there is no cure for EoE and the treatment cannot be ended without recrudescence of the disease.

Like other atopic diseases, EoE involves Th2 cytokines signaling which drives the recruitment and proliferation of eosinophils and mast cells to the esophagus. This accumulation of cells leads to stricture formation, dysphagia and food impaction [123].

Recently, an inverse correlation between EoE or asthma severity and *Hp* infection has been described [124]. In this study, they founded evidence for a significant association between *Hp* exposure and reduced odds of EoE.

However, further studies are needed to better characterize the mechanisms of this association.

2. Eosinophils

Paul Ehrlich has identified eosinophils in 1879 by using a staining blood smear technique. First, these cells were described as containing intracytoplasmic cationic granules, with an affinity for acidic dyes [125].

2.1. Structure and development

Eosinophils are generated in the bone marrow, circulate in the bloodstream and recruit to non-inflamed and enflamed tissues. They are multifunctional leukocytes involved in many defense and inflammatory processes such as parasitic helminths infection and allergic diseases [126].

2.1.1. Cell structure

2.1.1.1. Granules

As a member of the granulocyte family, eosinophils are characterized by the presence in their cytoplasm of large granules. These granules are made of a dense crystalline core and matrix. They contain multiple active molecules such as bioactive lipids, granule-derived cationic proteins and a large number of chemokines and cytokines that are key to mediate their inflammatory functions [127]. Among these granule-derived cationic proteins, major basic protein (MBP; also known as proteoglycan2 (Prg2)) is located in the core, while the matrix contains eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN, also known as ribonuclease A family member 2 (RNase2)). When MBP, EPO and ECP are released in the extracellular space, they can be toxic for several tissues. ECP and EDN display

antiviral activities. ECP forms toxin pores in the membrane of cells to facilitate the entry of cytokines and plays several noncytotoxic roles for T and B cells responses such as suppression of T cells responses, IgG synthesis by B cells, as well as airway mucus secretion. MBP alters smooth muscle contraction activities and modulates peripheral nerve plasticity. Finally, EPO is involved in ROS formation leading to oxidative stress and cell death [128] (**Figure 4**).

2.1.1.2. Surface molecules and receptors

As contributors of innate and adaptive immune responses, eosinophils display a wide repertoire of surface molecules and receptors.

One of the most important is the IL-5 receptor (IL-5R). Indeed, IL-5 is crucial for eosinophils development. This receptor is made of two different chains: the IL-5Rα and IL-5Rβ. IL-5Rα is specific of IL-5, while IL-5Rβ is share with the GM-CSF receptor. Moreover, eosinophils display receptors for other cytokines and growth factors such as IL-4, IL-13, IL-33, TLSP and TGFβ. In parallel, eosinophils express CCR3, which is an important homing receptor recognizing eotaxin [129]. Apart from their roles during allergic diseases, eosinophils control pathogen infections through PAMPs and DAMPs recognition. They constitutively expressed a panel of TLRs, especially TLR7 and 8 [130]. Ig Fc receptors are also expressed by eosinophils facilitating their interactions with the adaptive immune responses. Finally, eosinophils express single-pass transmembrane cell surface proteins siglecs (sialic acid-binding, immunoglobulin-like lectins). In particular, human eosinophils display siglec-8 (functional paralog siglec-F in mouse) at their surface, which promotes apoptosis upon its engagement through the generation of ROS [131].

Taken together, eosinophils display a large panel of receptors and surface molecules allowing their pleiotropic functions in innate and adaptive immune responses.

2.1.2. Development and maturation

Eosinophils develop from pluripotent granulocyte progenitors in the bone marrow. Under the control of transcription factors such as GATA-1, PU.1, and C/EBP members, they differentiate into eosinophils. The cytokines IL-3, IL-5 and GM-CSF contribute to the development of mature eosinophils. Then, IL-5 triggers their migration into the circulation. Once in the blood stream, a subsequent panel of chemokines (eotaxin-1), eosinophil integrins (α4β1, α4β7, αmβ2, αLβ2) and adhesion molecules (MAdCAM-1, VCAM-1, and ICAM-1) allow eosinophils to cross the endothelium and reach different organs during homeostatic conditions [128].

2.2. Tissue resident eosinophils and homeostasis

Infiltration of eosinophils in tissues is mediated by the constitutive expression of eotaxin-1 (CCL-11), eotaxin-2 (CCL-24) and eotaxin-3 (CCL-26) which bind to CCR3 present at eosinophil cell surface. Whereas local eotaxins drive eosinophils infiltration in tissues, IL-5 is important in mediating eosinophilia [132]. Usually, Th2 immune response leads to IL-5 production. However, it has been demonstrated that ILC2 are major IL-5-expressing cells within tissues, especially for homing into the GI tract [133]. Indeed, alarmins (IL-25, IL-33 and TSLP) secreted by epithelial cells promote eosinophilia by stimulating ILC2 production of IL-5 [134] (**Figure 4**). In addition to IL-5, IL-3 and GM-CSF are also important for generation and maintenance of eosinophils in tissues.

Under baseline conditions, eosinophils traffic into the thymus, mammary gland, uterus, and particularly into the gastrointestinal tract. Some data suggest that this accumulation in the gastrointestinal tract is independent of lymphocyte and intestinal flora [135]. Eosinophils are present in the female reproductive tract, during the estrus.

In addition, some studies reveal their implication for the development of mammary gland at puberty, during pregnancy and premature labor [136-138]. This migration in the uterus and mammary gland is mediated by eotaxin-1 local secretion, under hormonal control. Eosinophils also migrate into the thymus during the neonatal period. Thymic eosinophils express high levels of MHC-II molecules, are CD11b/CD11c positive and express messenger ribonucleic acid (mRNA) for several proinflammatory cytokines [128]. Finally, eosinophils are also recruited to all regions of the GI tract (excepted in the esophagus) via eotaxin-1 [135]. Some studies demonstrates that eosinophils are crucial to maintain GI homeostasis. Indeed, they are implicated into secretory IgA production and integrity of intestinal microbiome and mucosal barrier. Moreover, they are important for peyer's patches as well as for T cells and DCs development [139].

Taken together, eosinophils residing within the tissues are important to maintain tissue integrity and immune homeostasis at steady state.

2.3. Effector functions: anti-pathogen activities

In addition to their implication during allergic diseases and tissues homeostasis, eosinophils display effector functions against several pathogens. Indeed, eosinophil degranulation can be toxic for pathogens and several studies argue that eosinophils can "trap" bacteria.

2.3.1. Degranulation

As described earlier, eosinophils detect PAMPs (e.g. LPS or β-glucans) from bacteria and fungi thanks to the presence of PRRs at their surface. This detection allows specific release of eosinophil granules, containing cytotoxic molecules for pathogens. For instance, MBP is toxic for pathogens due to its extreme basic nature [140, 141].

ECP binds to LPS and peptidoglycans of bacteria and displays antiviral properties through the formation of pore in target membranes [142]. EPO triggers generation of ROS responsible of cellular stress [141]. EDN induces DCs maturation and activation [143] (**Figure 4**).

In addition to their cytotoxic molecules, eosinophils granules store preformed cytokines, chemokines, and growth factors [127]. Among others, IFNγ, IL-4, IL-6, TNFα, IL-10, IL-12 and IL-13 were found in human eosinophils [144]. The same observation have been done in mouse eosinophils.

Degranulation occurs thanks to three secretory processes. First the classical exocytosis happening when eosinophils adhere to the cell surface of a parasite and release granule content directly into the pathogen cytoplasm through a pore. Secondly, the cytolysis with granule release. In this case, eosinophils undergo a cytolytic cell death leading to their nuclear and plasma membrane dissolution. Some cell-free membrane-bound granules maintain an intact membrane with functional receptors and can release their content upon different stimuli within tissues. Lastly, the piecemeal degranulation through formation of vesicle fusing with eosinophil plasma membranes to release granule in the extracellular milieu [127].

2.3.2. Extracellular traps

Apart from their cytotoxic granules, eosinophils can release extracellular traps. In 2008, Yousefi et al. demonstrated for the first time that eosinophils can release mitochondrial DNA. This process occurs after LPS stimulation of IL-5 or IFNγ-primed eosinophils and is dependent of ROS generation. Moreover, this release is independent of cell death since eosinophils remain intact and do not release nuclear DNA. Finally, this mitochondrial DNA forms extracellular structures able to bind and kill bacteria under

inflammatory conditions [145]. Recently, these observations have been confirmed in mice, in context of *Citrobacter rodentium* (*C. rodentium*) infection [146]. This study provides evidence that *C. rodentium* infection triggers formation of eosinophil extracellular DNA traps, leading to bacterial killing.

2.3.3. Tissue damage, wound healing and remodeling

In response to DAMPs secreted by injured cells, eosinophils are activated through their PRRs and migrate to the tissue-injured site. Then, eosinophils promote wound healing to restore tissue integrity. However, depending on the stage of the inflammatory disease (e.g. EoE), excessive eosinophils activation promote fibrogenic processes especially during chronic phases. In this context, eosinophils secrete a large panel of mediators such as TGFβ, Th2 cytokines and granules. These mediators promote epithelial and smooth muscle cells hyperplasia as well as tissue fibrosis, leading to tissue remodeling [129, 147] (**Figure 4**).

2.3.4. Role of eosinophils during *Hp* infection

Since many years, gastric eosinophils infiltration has been detected in *Hp* infected patients, this infiltration being associated with gastritis. Moreover, severity of the gastritis is correlated with eosinophil infiltration [148]. From now, some studies demonstrate that the crosstalk between *Hp* and gastric epithelial cells is implicated in eosinophil recruitment. This phenomenon is mediated by MAPK signaling through p38 and ERK-dependent pathways in gastric epithelial cells as well as by CagPAI [149]. The recruited eosinophils interact with gastric epithelial cells, which induce their degranulation. In parallel, eosinophils also play a direct role in immunomodulation.

2.1. Immunomodulatory roles

2.1.1. Maintenance of immune homeostasis

As described earlier, eosinophils are present at steady state in some tissues. These eosinophils play a key role in maintaining homeostasis. In the bone marrow, eosinophils are important for plasma cells maintenance through secretion of A proliferation-inducing ligand (APRIL) and IL-6. These molecules sustain the survival of plasma cells [150] and consequently sustain Ig production.

The major proportion of tissue resident eosinophils are located in the GI tract. There, eosinophils are important for the development of peyer's patches and immunoglobulin (Ig) A-producing plasma cells. Indeed, the constant production of secretary IgA production is key to maintain microbial homeostasis within the gut [139]. In addition, lamina propria eosinophils secrete TGFβ to induce Treg diffenciation and protect from inflammation [151].

2.1.2. Th2/Th1 modulation

2.1.2.1. Th2 stimulation through antigen presentation and cytokine secretion

In addition to the release of granules and mitochondrial DNA, eosinophils display antigen presentation abilities [152]. Indeed, several studies highlight the ability of eosinophils to internalize, process and present antigenic peptides from microbes, virus and parasites [153]. In parallel of surface MCHII expression, eosinophils also display co-stimulatory molecules such as CD80, CD86 and CD40 [154]. Consequently, eosinophils pulsed with parasitic antigens were shown to prime naïve T cell toward a Th2 polarization associated with cytokine secretion as well as to robustly trigger $CD4^+$ T cells proliferation within the draining lymph node [155]. Moreover, eosinophils display

superior abilities to phagocyte antigens than DCs suggesting they could play an important role in antigen presentation. Finally, the presence of Fc receptors at their surface could also facilitate uptake of antigens in immune complex. In addition, eosinophils produce IL-4, which specifically enhance Th2 polarization [156]. Recently, new evidence indicate that eosinophils also secrete IL-25 [157]. This cytokine, a member of the IL-17 family, promote Th2 response and enhance memory Th2 cells. Altogether, the antigen-presenting ability of eosinophils associated with pro-Th2 cytokine secretion seems important to trigger Th2 responses against pathogens and/or asthma (**Figure 4**).

2.1.2.2. Th1 inhibition

Additionally to Th2 promotion, eosinophils dampen Th1 responses.

IDO is an IFNγ inducible enzyme that catalyzes the oxidative catabolism of tryptophan to kynurenines. Eosinophils constitutively express IDO and increase kynurenines generation. Kynurenines inhibit proliferation and promote apoptosis of Th1 cells, valorizing Th2 response [158].

In parallel, Arnold et al. demonstrate that in context of infection with *Hp* or *C. rodentium*, eosinophils express PD-L1. In this context, direct contact between PD-L1+ eosinophils and PD-1+ T cells in the enflamed gastric mucosa, allows Th1 immunosuppression. In addition, the authors demonstrate that this interaction is dependent of IFNγ expression in the gastric environment [146] (**Figure 4**).

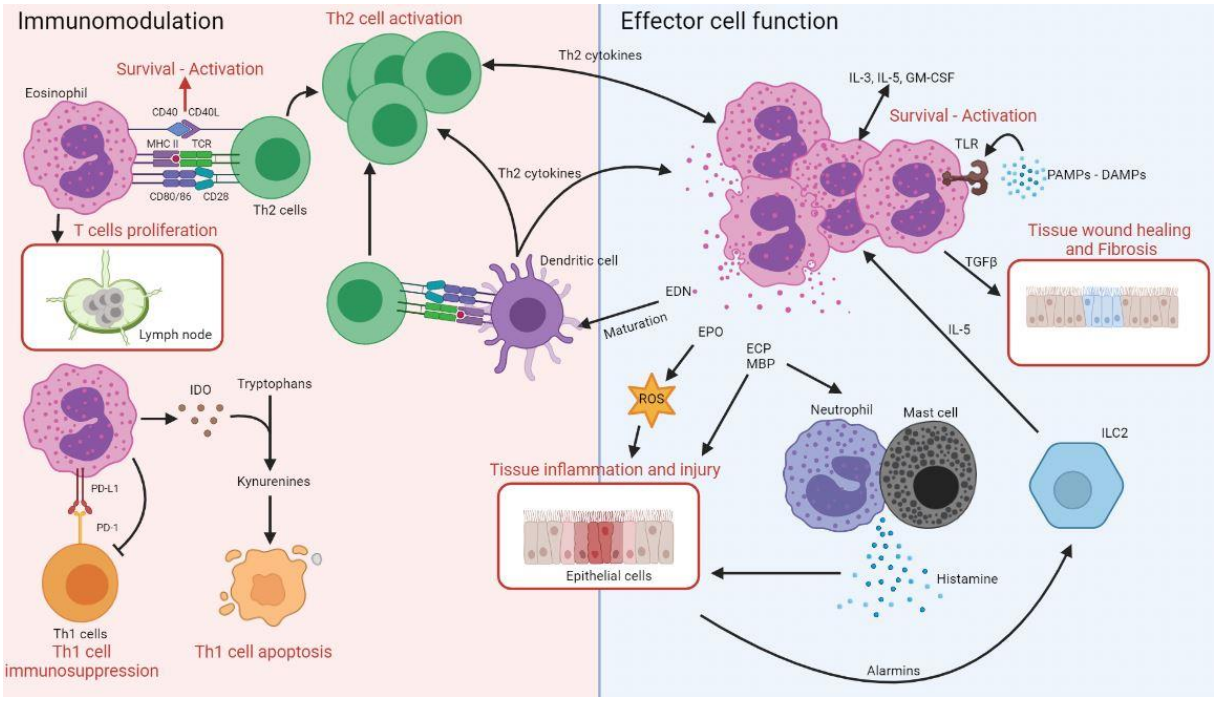

Figure 4. Eosinophils functions. Eosinophils display effector cell functions. Their maintenance is mediated by IL-3, IL-5 and GM-CSF. Upon activation (e.g. via TLR detection of PAMPs or DAMPs), eosinophils are able to secrete a large panel of cytotoxic granules as well as pro-inflammatory cytokines. Cytotoxic granules trigger maturation of DCs, activation of granulocytes, ROS production and tissue pro-inflammatory damages. Upon maturation, DCs are able to present antigens to T cells leading to their activation. Granulocytes (e.g. neutrophils and mast cells) release histamine and mediate allergic diseases. Inflamed tissues secrete several alarmins. These alamins trigger IL-5 secretion by ILC2 that stimulates eosinophils. Eosinophils also secrete TGFβ implicated in tissue wound healing but could lead to fibrosis. In parallel, eosinophils play an immunomodulatory role on T cells. First, eosinophils can act as an APC and induce T cells proliferation within the lymph node as well as Th2 cell local activation. Secondly, eosinophils dampen Th1 cells by activating PD-1/PD-L1 immune blockade and by secreting IDO. IDO catalyzes transformation of tryptophan in kynurenines that leads to Th1 cells apoptosis.

3. GM-CSF

In the 70's, Burgess et al. identified the GM-CSF physiological activities in a lung-conditioned medium. GM-CSF was shown to specifically stimulate the proliferation and differentiation of mouse bone marrow cells into granulocytes and macrophages [159]. Then, evidence shows its implication to stimulate multipotent progenitor and leukemic cells [160]. In the 80's, GM-CSF has been cloned for the first time. With this

achievement, recombinant GM-CSF allows extensive *in vivo* and *in vitro* studies to better characterize its functionalities [161].

3.1. Sources, receptors and signaling

A large panel of cells such as macrophages, mast cells, T cells, fibroblasts, epithelial and endothelial cells produces GM-CSF [162]. Its receptor (GM-CSFR) is a heterodimeric complex composed of a GM-CSF-specific α chain and a β chain shared with IL-3 and IL-5 receptors. This receptor is present at the surface of myeloid progenitors and on mature monocytes, neutrophils, eosinophils, basophils and DCs [163]. GM-CSF level is barely detectable at steady state in the circulation but is rapidly elevated during infection or inflammation. Its secretion is triggered by pro-inflammatory cytokines such as IL-1β, TNFα and IL-12, and inhibited by IL-4, IFNγ and IL-10 [164].

Once secreted, GM-CSF displays several properties. First, it modulates the development of myeloid cells (e.g. granulocytes and monocytes) from their immature to mature state. Secondly, upon its activation, GM-CSFR triggers the activation of JAK2, STAT5, phosphoinositide 3-kinase (PI3K) pathway, MAPK and NF-κB promoting cell differentiation, inflammation, and cell survival [165, 166]. Finally, its production by many different cell types (as mentioned before), upon DAMPs of PAMPs stimulation, strongly modulates immune responses. Although the majority of GM-CSF studies probe for its pro-inflammatory role, it is also important to consider its immune-suppressive role, especially in tumor setting [167].

3.2. Action on immune cells

GM-CSF is secreted in context of inflammation and modulates the activities of immune cells such as macrophages, DCs, neutrophils, B and T cells (**Figure 5**).

3.2.1. Macrophages and dendritic cells

Macrophages and DCs derived from the same myeloid precursors. Interestingly, GM-CSF modulates differentiation fates of both cell types. Consequently, understanding in which conditions GM-CSF impacts differentiation toward one cell type, rather than another, retain attention. Indeed, in the context of GM-CSF grown bone marrow cells followed by LPS stimulation, macrophages and DCs cells do not display the same functions: macrophages are more prone to secrete pro-inflammatory cytokines, whereas DCs display high abilities to present antigens [168]. It is suggested that intermediate dose of GM-CSF favors DCs while high doses promote macrophages differentiation in inflamed tissues [169, 170].

3.2.1.1. Macrophages

In contrast to other cytokines, GM-CSF not only promote survival of macrophages but also enhances their differentiation toward a pro-inflammatory phenotype called M1. GM-CSF induces pro-inflammatory cytokines and chemokines secretion by M1 such as TNFα, IL-6, IL-12, IL-23, IL-1β and several CCL (e.g. CCL24, CCL22, CCL5 and CCL1) [164] which, in turn, promote Th1, Th2 and Th17 responses. Moreover, GM-CSF enhances phagocytosis by increasing expression of PAMPs receptors at macrophages surface (e.g. TLR2 and TLR4).

In parallel, some studies report the implication of activin A for M1 polarization via GM-CSF. Activins are pluripotent growth and differentiation factors that belong to the TGFβ family. Their expression is elevated in inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). Indeed, M1 macrophages polarized by GM-CSF secrete lots of activin A that, in an autocrine manner, promote M1 differentiation. In addition, activin A impairs the acquisition of M2 markers and dampens IL-10 production, promoting a pro-inflammatory environment [171]. However,

GM-CSF has also been associated with the development of M2 macrophages in context of tumor environment but the mechanism remains unclear [167].

3.2.1.2. DCs

The pivotal role of GM-CSF on DCs have been first described *in vitro*, since it promotes DCs differentiation from human peripheral blood monocytes as well as from human and mouse hematopoietic progenitor cells. Then, its implication for DCs maturation have been extensively studied but some misunderstanding remain. For instance, splenic and thymic DC numbers increase when GM-CSF is overexpressed. However, GM-CSF depletion only leads to a slight reduction of DCs in lymphoid organs but to a significant decrease of migratory DCs present in non-lymphoid tissues [166]. Since GM-CSF circulates at low level at steady state, its action on DCs is more pronounced during inflammation. Some studies show that GM-CSF and IL-4 induce the development of inflammatory DCs. These inflammatory DCs secrete Th1 (IFNγ) and Th2 (IL-10) cytokines and CCL2 chemokine [172] that recruits monocytes, memory T cells, and DCs to the sites of inflammation. However, other studies suggest that the development of inflammatory DCs is GM-CSF-independent [173]. Consequently, the role of GM-CSF on DCs differentiation is depend on the inflammatory context. At steady state, it promotes the differentiation of non-lymphoid tissue migratory DCs that maintain self-tolerance and the induction of specific immune responses. However, in context of inflammation, its role in DC differentiation need to be deeply investigated [167] before drawing conclusions.

3.2.2. Neutrophils

As for macrophages, GM-CSF is involved in differentiation, survival and activation of neutrophils in context of inflammation. In addition, some studies report that GM-CSF has a very potent chemoattractant activity for neutrophils [174]. Finally, as observed

for eosinophils, neutrophils are able to release neutrophil extracellular traps (NETs), which bind and kill microorganisms. Yousefi et al. demonstrated that, upon GM-CSF and TLR4 stimulation, viable neutrophils release mitochondrial DNA to form NETs in a ROS dependent manner [175] and participate to pathogens killing.

3.2.3. B cells

The role of GM-CSF on the development and functions of B is not clear. Indeed, the functions of B cells are not limited to the production of Igs, since B cells are known to also participate to the development of diseases in an Ig-independent manner. Some studies demonstrate that B cells are able to produce GM-CSF that contribute to their survival in an autocrine feedback loop [176]. Moreover, in context of Multiple sclerosis (MS), GM-CSF-producing B cells activate myeloid cells, leading to the development of inflammatory environment in the central nervous system. It has been suggested that the depletion of these GM-CSF-producing B cells would be beneficial for MS patients [177].

3.2.4. T cells

GM-CSF has a direct and an indirect impact on T cells. First, GM-CSF plays a crucial role in various inflammatory diseases. For instance, several studies highlight that the administration of anti-GM-CSF blocking antibodies decrease the severity of RA [178, 179]. Since this disease is T cells mediated, it has been concluded that GM-CSF was able to primed autoreactive T cells responses. Indeed, GM-CSF is produced by T cells only upon TCR activation and resting T cells are not able to produce it. However, both $CD4^+$ (Th1 and Th2) and $CD8^+$ T cells produce GM-CSF upon activation.

Second, a majority of studies report the impact of GM-CSF on T cells via the modulation of APCs maturation. Indeed, as mentioned before, GM-CSF is important

for DCs development and maturation. This ability to modulate antigen presentation has been largely studied in context of anti-tumor responses. Indeed, associated with anti-CTLA-4 therapy, GM-CSF positively influences DC population to increase antigen-specific T cells as well as IFNγ secretion [177].

Altogether, GM-CSF immunobiology in T cells is important and need to be more clarified [180].

GM-CSF is classified as a Th17 cytokine. However, although Th1 and Th2 can produce it, a new GM-CSF-producing Th cells has recently been identified and named pathogenic Th17 cells (or Th1/17 cells).

3.2.4.1. Th17 and pathogenic Th17 cells (Th1/17)

It is now well established that Th17 cells represent an independent Th subtype. First, the Th17 subset was described based on its ability to secrete IL-17. Then several cytokines were added to their secretion activities such as IL-17F, IL-21 and GM-CSF. Many studies demonstrated their key roles the immune responses during pathogen invasion or during the development of autoimmune diseases [181]. Moreover, at the difference of Th1 and Th2 cells, Th17 show an important magnitude of plasticity and are able to modulate their functions. Several cytokines such as TGFβ, IL-6, IL-21, IL-1β and IL-23 are particularly important for Th17 plasticity. At the difference of TGFβ and IL-21, which promote Th17 differentiation from naïve $CD4^+$ T cells, IL-1β and IL-23 are important for the generation of Th17 from memory cells. The IL-1β and IL-23-dependent Th17 cells displays a high inflammatory potential and have been particularly involved in the development of autoimmune diseases and consequently named pathogenic Th17 cells [182, 183]. These pathogenic Th17 cells are characterized by their ability to produce IL-17, IFNγ and GM-CSF. In this context, GM-CSF is crucial for

the pathogenicity of Th17 cells [184]. These pathogenic Th17 cells have been detected in several inflammatory pathologies such as IBD and MS [185].

3.2.4.2. Th1

Th1 cells have the ability to produce GM-CSF but in a lesser extent than pathogenic Th17 cells. Indeed, upon IL-12 stimulation, naïve $CD4^+$ T cells differentiate into Th1 cells. These IL-12-dependent Th1 are able to produce GM-CSF and trigger a strong pro-inflammatory response, leading to autoimmune diseases such as experimental allergic encephalomyelitis (EAE) [186].

3.2.4.3. Th2

In vitro, some studies demonstrate that Th2 cells produces GM-CSF [187]. In addition, in the context of allergic diseases, Th2 cells correlate with $GM\text{-}CSF^+$ cells. However, further investigations are needed to elucidate the role of GM-CSF in Th2 cells [185].

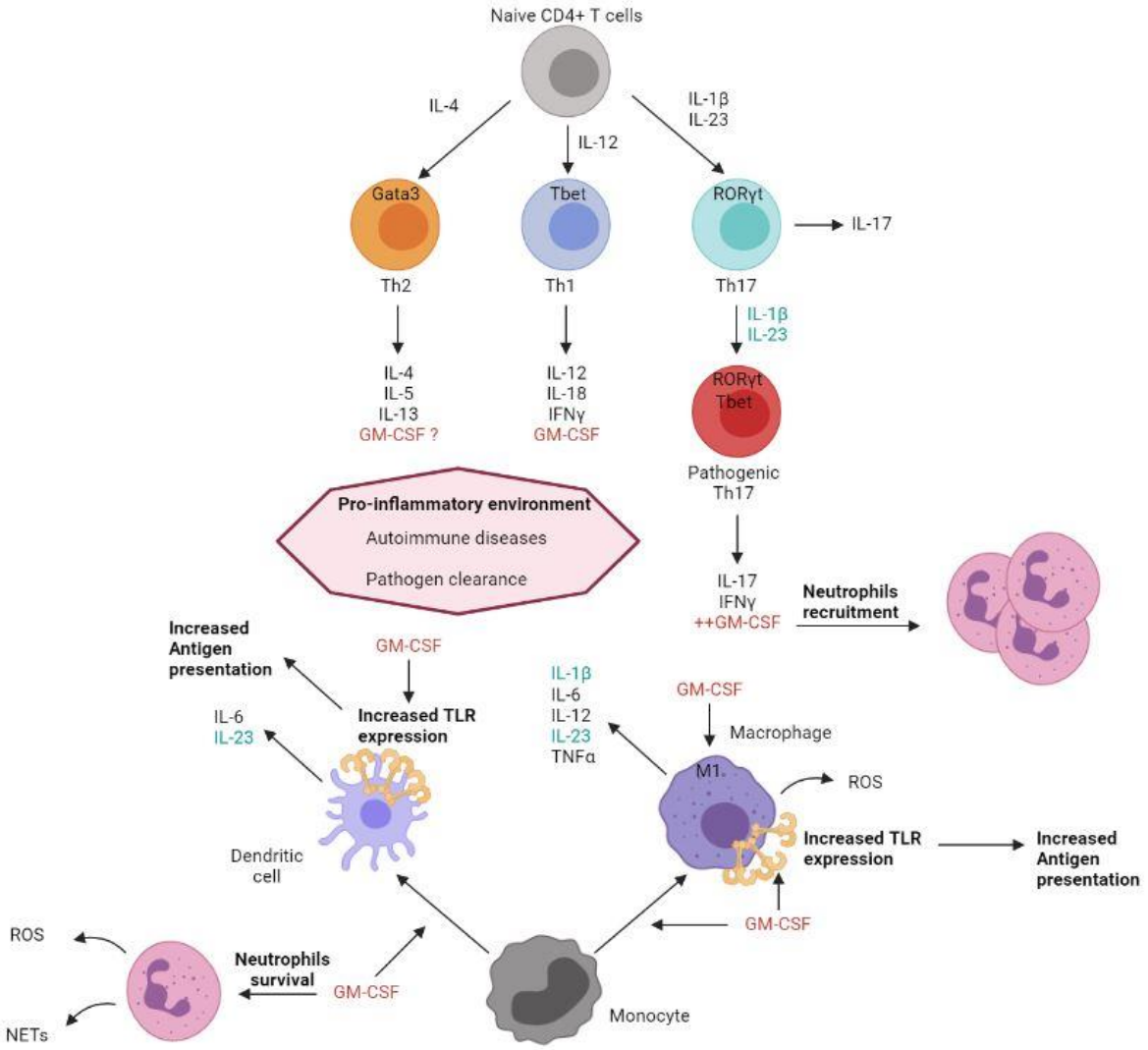

Figure 5. The role of GM-CSF in immune responses. Naïve CD4+ T cells differentiate in Th2, Th1 or Th17 depending on the stimulus. Th17 cells have the ability to become pathogenic and secrete lots of GM-CSF. This GM-CSF promote neutrophils recruitment and survival as well as the ability of monocyte to differentiate into dendritic cells (in presence of low GM-CSF) or in macrophages (in presence of high GM-CSF). On both cell types, GM-CSF promote expression of TLR that increase antigen presentation to T cells, leading to their activation. In addition, macrophages switched into M1 phenotype and produce pro-inflammatory cytokines. Maturation of DCs is also increased. Neutrophils released NETs and ROS upon stimulation. Altogether, GM-CSF enhance a pro-inflammatory environment favorable for autoimmune diseases but also pathogen clearance.

3.3. Role of GM-CSF in antimicrobial responses

Apart from its implication in autoimmune and inflammatory responses, GM-CSF is a component of the antimicrobial responses. Indeed, during *Mycobacterium tuberculosis* infection, macrophages are central players of the immune response, leading to bacterial control. Recently Bryson et al. demonstrated that GM-CSF plays a key role in the control *Mycobacterium tuberculosis* infection by macrophages. This study

highlights the positive effect of GM-CSF on phagolysosomal fusion leading to decrease bacterial growth [188]. The same observation has been done in context of *Candida albicans* infection where GM-CSF is also associated with the reduction of yeast growth [189]. Remarkably, several studies demonstrate that *Hp* enhances GM-CSF production by gastric epithelial cells, suggesting that GM-CSF might participate to the immune responses in the stomach of patients infected with *Hp* [190, 191].

Finally, recent evidences suggest that GM-CSF could be involved in pathogenicity of the COVID-19 since serum levels of GM-CSF is upregulated in infected patients [192].

CHAPTER 2: RESULTS

Hp is a major public concern due to its high global prevalence and its implication in gastric cancer. Current therapies use a mix of several antibiotics. However, the increase of antibiotics resistance suggests that other strategies need to be envisioned. Prophylactic vaccination is a promising alternative. However, all clinical trials failed due to a lack of vaccine efficacy over the time. To develop an efficient vaccine in human, we perform pre-clinical studies to identify the vaccine-induced immune mechanisms that efficiently clear *Hp* from the gastric mucosa.

Publication 1: Gastric eosinophils are detrimental for *Helicobacter pylori* vaccine efficacy

In this study, we examined whether eosinophils could be implicated in vaccine-induced *Hp* clearance. First, we observed that activated eosinophils, expressing CD63, CD40, MHCII and PD-L1 at their cell surface, infiltrate the gastric mucosa during the vaccine-induced reduction of *Hp* infection.

As described in the introduction, eosinophils display anti-bacterial functions that rely on the secretion of cationic granules [127]. To assess the bactericidal activity of eosinophils, we co-cultivated *Hp* and bone marrow-differentiated eosinophils. Strikingly, we provide evidence that bone marrow-differentiated eosinophils efficiently kill *Hp in vitro*, suggesting that eosinophils may participate to the vaccine-induced reduction of *Hp* infection.

However, conversely to our expectations, the absence of eosinophils does not decrease the efficacy of this *Hp* vaccine *in vivo*. Indeed, vaccinated mice that have been genetically ablated of the eosinophil lineage (ΔdblGATA mice) or that are receiving anti- sialic acid-binding immunoglobulin-like lectin F (SiglecF) depleting antibodies, display a lower *Hp* colonization when compared to their eosinophil sufficient counterparts. Although the vaccine induces similar urease-specific humoral and Th responses in both eosinophil sufficient and deficient mice, a decreased production of anti-inflammatory cytokines, such as IL-10, TGFβ, and calgranulin B, was specifically observed in eosinophil depleted mice.

Taken together, as already suggested by Arnold et al [146], our results suggest that gastric eosinophils maintain an anti-inflammatory environment, which sustains chronic *Hp* infection (**Figure 6**). Because eosinophils are one of the main immune effectors mobilized by Th2 responses, our study strongly suggests that the formulation of an *Hp* vaccine needs to include an adjuvant that preferentially primes *Hp*-specific Th1/Th17 responses.

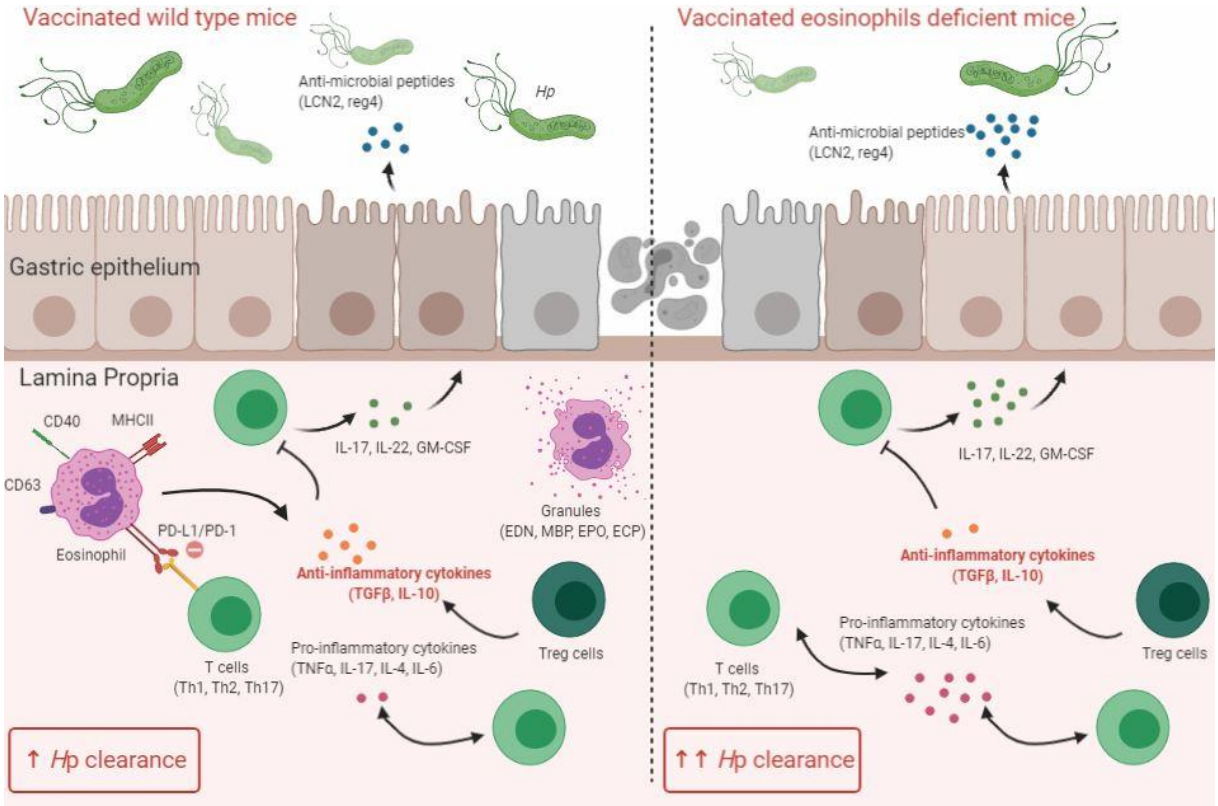

Figure 6. Gastric eosinophils are detrimental for Hp vaccine efficacy. In context of Hp infection of vaccinated mice, eosinophils are recruited in the lamina propria of the stomach. In parallel, vaccination triggers an important influx of Th cells, secreting pro-inflammatory cytokines. However, eosinophils dampen Th response through PD-L1/PD-1 interaction and favor anti-inflammatory cytokines as well as Treg expansion. Altogether, eosinophils probe for an anti-inflammatory environment leading to altered AMPs secretion and limiting Hp clearance. On the opposite, the absence of eosinophils allow the recruitment of many Th cells and the production of pro-inflammatory cytokines leading to an efficient Hp clearance.

Publication 2: GM-CSF is key in the efficacy of vaccine-induced reduction of *Helicobacter pylori* infection

In this second study, we examined whether GM-CSF could be implicated in vaccine-induced *Hp* clearance. Indeed, vaccination with urease with CT adjuvant triggers Th17 responses [108]. As mentioned in the introduction section, Th17 cells display important plasticity properties and can be differentiate into a pathogenic Th17 cells upon IL-23 stimulation [183, 193]. As these pathogenic Th17 cells produce GM-CSF, which is involved in many pro-inflammatory mechanisms, we wanted to better characterize the vaccine-induced Th17 response mediating *Hp* clearance.

Firstly, we characterized the cytokine production in the gastric mucosa of vaccinated infected mice. Mice were vaccinated with urease and CT before getting *Hp* infected. We showed that the gastric mRNA expression of IL-17 and GM-CSF is elevated in vaccinated and infected mice compared to non-vaccinated and infected counterparts. In addition, vaccine-induced Th17 cells, which accumulate in the mouse gastric mucosa during the reduction of *Hp* infection, secrete GM-CSF. Suggesting that these Th17 cells display a pathogenic phenotype.

Next, by using two different approaches (αGM-CSF mAb injection and GM-CSFRko mice), we showed that inhibition of the biological activity of GM-CSF jeopardizes vaccination efficacy. Indeed, in absence of GM-CSF, the vaccine-induced reduction of *Hp* burden is attenuated. In parallel, gastric mRNA expression level of βdefensin3 (an AMP involved in *Hp* clearance [41] is not up-regulated upon vaccination in absence of GM-CSF compared to GM-CSF sufficient mice. This result suggests that GM-CSF is most probably key to promote βdefensin3 production.

In parallel, we performed hydrodynamic gene delivery injection of GM-CSF in chronically *Hp* infected mice. As expected, therapeutic injection of GM-CSF decreases *Hp* infection burden. In addition, we observed that GM-CSF administration induces increased gastric βdefensin3 mRNA expression, confirming a GM-CSF-driven mechanism allowing βdefensin3 production.

Finally, by using an *in vitro* model of gastric epithelial cells, we observed that GM-CSF stimulates gastric epithelial cells to produce AMPs and to kill *Hp*.

Taken together, we demonstrate that in the context of vaccination, the generation of pathogenic Th17 response is beneficial for the vaccine-induced *Hp* clearance. The

GM-CSF, produced by pathogenic Th17, most probably directly stimulates the epithelial cells inducing βdefensin3 *Hp* clearance (**Figure 7**).

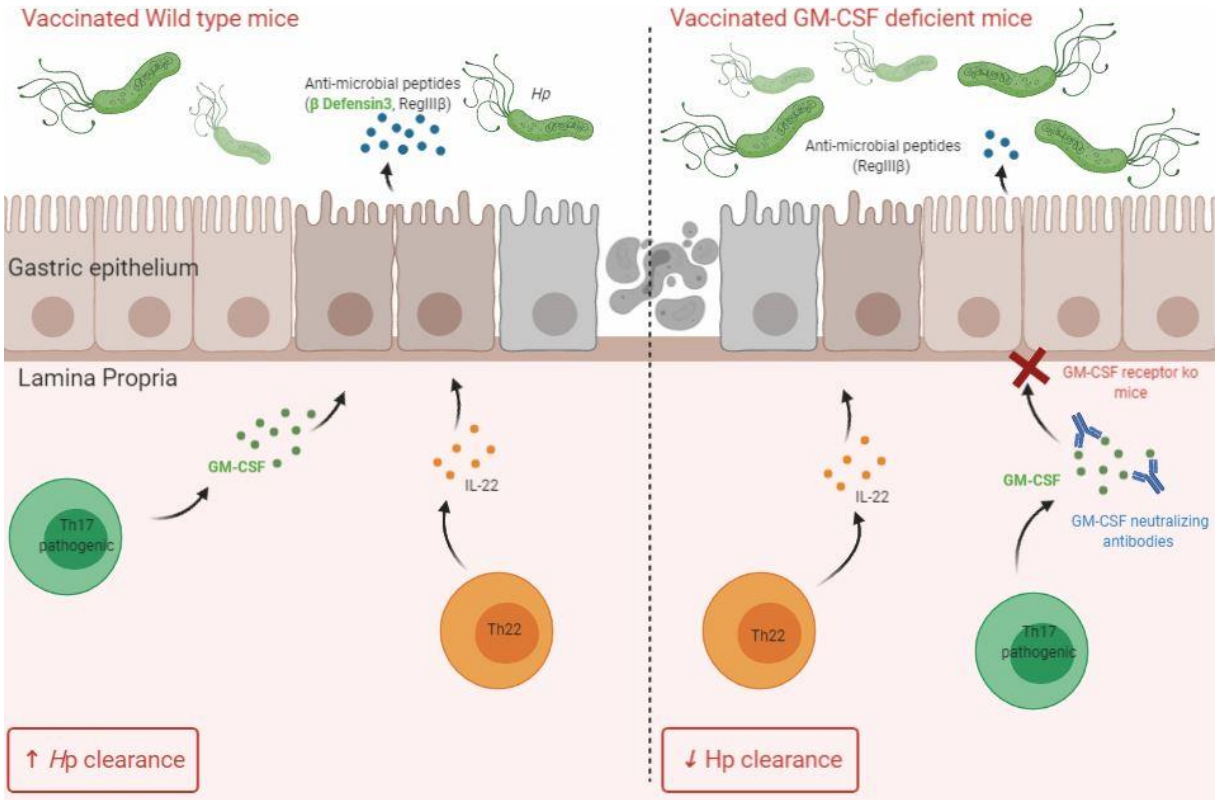

Figure 7. GM-CSF is key in the efficacy of vaccine-induced reduction of Hp infection. Upon vaccination, Hp infection triggers Th22 and pathogenic Th17 cells recruitment in the gastric lamina propria. These cells respectively produce IL-22 and GM-CSF, which stimulate AMPs production by gastric epithelial cells. These AMPs, respectively RegIIIβ and βdefensin3, are efficient Hp killers. In absence of GM-CSF, less AMPs are produced leading to a decreased Hp clearance.

CHAPTER 3: DISCUSSION AND PERSPECTIVES

The development of an efficient vaccine against *Hp* relies on the identification of positive and negative immune effectors, which act remotely and/or locally to clear *Hp*. Previous pre-clinical and clinical data have clearly identified the urease-based vaccine as a promising prophylactic approach to protect the host against *Hp* infection.

♦ Prophylactic vs therapeutic vaccine

A vaccine could be either prophylactic or therapeutic. Both have been shown to be efficient in pre-clinic [114, 194]. To be efficient, an *Hp* vaccine should induces strong local adaptive and innate immune responses [195, 196]. *Hp* infection is mainly

acquired during childhood, meaning that a prophylactic vaccine would be useful for young children [197]. However, therapeutic vaccine would be more relevant for chronically infected adults with gastric ulcers or cancer. Although not 100% effective, a therapeutic vaccine would be useful to reduce bacterial load or to protect against reinfection. However, therapeutic vaccination can be associated with post immunization gastritis [198].

Another challenge for vaccine development and identification of protective immune mechanisms is that only few patients develop major symptoms such as peptic ulcer or gastric cancer. In addition, the development of symptoms is not predictable and depend on the *Hp* virulence factors, the genetic and physiological factors of the host. Moreover, the immune responses of a host that develop symptoms differs from ones that remains asymptomatic. This suggests that the ideal *Hp* vaccine should trigger an immune response in context of asymptomatic carriage, but dampen the inflammation in patients with *Hp*-associated diseases [199]. However, this ideal vaccine is, from now, unachievable.

In addition to the variable presence of *Hp* symptoms, it is important to consider that the prevalence of *Hp* infection is not homogeneous across the world. Although the *Hp* prevalence in developed countries (e.g. Europe, US) is decreasing, it remains extremely high in developing countries. This suggests that a prophylactic vaccine is more adapted for developing countries, whereas the therapeutic vaccination would be useful worldwide. In addition, previous infection do not provide protection against re-infection. In high endemic places, the risk of re-infection is particularly high. Recently, a study suggests that a protection is achieved when an adjuvant is administrated concomitantly to the first infection [198] but only prophylactic vaccine could dampens the *Hp* prevalence in these countries.

Treg cells are involved in the immune escape of *Hp* [200]. Upon infection, *Hp* has the ability to promote Treg generation as well as a number of immunosuppressive responses leading to persistence of the infection for decade. During the neonatal period, the *Hp*-induced Treg response is particularly important and rarely leads to symptoms during childhood [201]. Usually symptoms appear from 50 years old. Consequently, a therapeutic vaccination, which induce a T cell response, would be difficult to achieve in presence of Treg cells. In this context, a prophylactic vaccine would be a more promising alternative, especially for developing countries. However, this vaccination should be given before the age of six since a Chinese clinical study demonstrates that 20% of children older than this age are already infected [113].

Due to the complexity of *Hp*-induced immune responses, a better characterization of the protective immune responses are crucial to improve vaccine efficacy. Indeed, from now, vaccine candidates only achieved a modest reduction of *Hp* burden or acquisition. However, for all the reasons detailed above, our studies focused on prophylactic vaccination.

- Publication 1: our results and contribution

In our first study, we assessed the role of the eosinophils for the vaccine-induced *Hp* clearance. We demonstrated that eosinophils recruitment was not beneficial for vaccine efficacy since eosinophils promote an anti-inflammatory environment. Our results are reminiscent of the work of Arnold et al. that recently made similar observation [146]. Indeed, they observed that activated PD-L1^{+} eosinophils interact with PD-1 at the surface of CD4^{+} T cells leading to T cell inactivation. This PD-L1/PD-1 interaction might lead to the reduction of the efficacy of the vaccine-induced *Hp* clearance.

Consequently, the recruitment and the activation of eosinophils, a Th2–dependent effector cell, in the gastric mucosa of *Hp* infected and vaccinated mice is detrimental for the efficacy of the vaccine. Our study has important clinical implications, as it reveals that *Hp* vaccines developed in the future should avoid promoting Th2 responses which are known to stimulate the production of eosinophils by the bone marrow and their systemic and local activation. Indeed, during the *Hp* infection of immunized host, the activation of resident memory $CD4^+$ T cells, which occurs within 2 to 3 days post antigen challenge [202, 203], will produce Th1, Th2, or Th17 cytokines depending on the adjuvant used in the vaccine formulation. For instance, adjuvants such as aluminum hydroxide that stimulates Th2 responses are not appropriate. While recombinant cholera toxin subunit B (CTB) [204] or the non-toxic form of Escherichia coli heat-labile toxin (LT) [205], that trigger Th1/17 responses represent better choices to avoid the generation of a eosinophil-mediated suppressive environment that will jeopardize the vaccine-induced reduction of *Hp* infection. In conclusion, it is imperative that the formulation of an *Hp* vaccine includes an adjuvant and administration protocols that preferentially primes Th1/Th17 anti-*Hp* responses.

- Pro Th1/Th17 adjuvants: CTB and LT

CTB (e.g. Dukoral® vaccine), the *Escherichia coli* LT subunit B (LTB), or double mutant LT (dmLT) are promising adjuvants to trigger specific Th1/17 immune responses. CTB as adjuvant has been almost evaluated fused to the vaccine antigen [206]. Indeed, CTB receptor (called GM1) is widely present at the epithelial cells surface of the gut as well on APCs. Consequently, administration of the vaccine antigen-CTB fusion protein triggers an important mucosal and systemic T cell responses and might confers protection. In their *in vivo* study, Wiedinger K et al. demonstrate that intranasal co-administration of CTB and pneumococcal surface protein A followed by lethal bacterial

challenge, provides efficient protection against *Staphylococcus pneumoniae* through a Th1 cells increase [207]. This suggests CTB could be an interesting adjuvant candidate to improve *Hp* vaccine efficacy. In addition to CTB, LTB could also be envisioned as adjuvant. Indeed, in the Lee J et al. study a protein from *Naegleria fowleri* combined with LTB leads to 60% of survival rate [208]. However, as with CTB, the main drawback of this molecule is its high potent enterotoxicity in human. To overcome these side effects, a LT mutant called dmLT has been designed and efficiently acts as a mucosal adjuvant [205] and specifically induces expression of IL-1β and IL-17 in a very recent clinical trial evaluating ETVAX (a leading oral enterotoxigenic *Escherichia coli* vaccine candidate) [209].

- Pro Th1/Th17 adjuvants: Recent alternatives to CTB and LT

In addition to CTB, LTB and dmLT, recent adjuvant alternatives have been also evaluated. In their study, Van Dis et al. demonstrate that mucosal administration of cyclic dinucleotides adjuvant in a protein subunit vaccine against *Mycobacterium tuberculosis*, confers enhanced protection through the generation of specific $CD4^+$ T cells and a Th17 immune response [210]. Cyclic dinucleotides are ubiquitous second messengers in bacteria and recognized as PAMPs by the host. Consequently, this adjuvant could be envisioned in context of *Hp* vaccine development. In parallel, Miller et al. made the bet of TLR agonist adjuvant to improve *influenza* vaccine. Interestingly, they provide evidences that lipidated imidazoquinolines TLR7/8 agonists elicit a Th1-biased influenza specific immune response in mice and when combined with a TLR4 agonist, elicit a Th17 response as well [211]. Protective immune responses against *Hp* involving TLRs activation could be another option to improve *Hp* vaccine efficacy.

♦ mRNA vaccination against *Hp* infection

From now, all the *Hp* vaccine trials were based on the use of *Hp* derived proteins (e.g. urease). Currently, mRNA vaccines retain attention thanks to the commercialization of several vaccines against the COVID-19. Based on the fact that mRNA delivery that is a potent stimulator of the innate response, an *Hp*-mRNA vaccine could be efficient for *Hp* clearance.

The successful use of mRNA vaccine has been firstly described in the 90's [212]. However, some concern such as mRNA instability, its high innate immunogenicity and challenging delivery have limited mRNA vaccine development. In the last decades, this technology have been efficiently improved and currently the use of mRNA vaccine displays several advantages. First, this approach is safe since mRNA is non infectious and degraded by normal processes. Second, as mRNA is stable and highly translatable, this technology is efficient. Indeed, mRNA can be delivered using carrier molecules, allowing their rapid expression in cell cytoplasm. Third, the production of mRNA vaccine is rapid, low cost and transferable to high scale manufacturing [213]. In addition, they induce strong and potent T and B cells responses, and can be developed as therapeutic or prophylactic [214]. Consequently, mRNA vaccines would be a promising approach for *Hp* vaccination. Depending on the mRNA synthesis, purity, carrier and administration route, the vaccine-induced immune responses are heterogenous [213, 214].

♦ Publication 2: our results and contribution

To better understand the vaccine-induced immune response upon *Hp* infection, we interrogated whether GM-CSF plays a role in the vaccine-induced reduction of *Hp* infection. Firstly, we clearly establish that GM-CSF^{+} IL-17^{+} pathogenic Th17 cells accumulate in the stomach mucosa during the vaccine-induced reduction of *Hp*

infection. Secondly, we provided evidence that vaccinated GM-CSF deficient mice only modestly reduce *Hp* infection. Conversely, we observed that increased availability of GM-CSF reduces *Hp* burden in chronically infected mice. Thirdly, we show that GM-CSF, by acting on gastric epithelial cells, promotes the production of βdefensin3, which exhibits *Hp* bactericidal activities.

Our observations are reminiscent of the results of Annemann M et al showing that pathogenic Th17 cells are protective against C*itrobacter rodentium* infection [215]. In the context of vaccine development, it is important to consider this information to select an adjuvant that promotes Th17 responses [216]. Bacterial components, including muramyl dipeptide (MDP), lipopolysaccharide (LPS), and CpG, are known to augment Th17 responses [217-220] and are very good candidates to be considered in vaccine formulation. The differentiation of Th17 cells into pathogenic Th17 cells has been recently shown to occur in inflamed tissue where Th17 cells are recruited and the differentiation into pathogenic Th17 cells can be mediated by the local production of serum amyloid A (SAA) proteins [221]. Interestingly, SSA proteins are known to be upregulated in the stomach mucosa of *Helicobacter* infected mice [222]. Therefore, it can be hypothesized that the vaccine–induced *Hp* specific Th17 cells, during their homing into the gastric mucosa of *Hp* infected hosts, will differentiate into pathogenic Th17 cells and will efficiently reduce *Hp* infection burden. In addition, we demonstrate for the first time the role of GM-CSF, most probably originating from pathogenic Th17 cells, in the vaccine-induced reduction of *Hp* infection. Mechanistically, we show that GM-CSF directly acts on gastric epithelial cells to induce the production of βdefensin3 and to kill *Hp*. Altogether, these findings highlight several potential alternatives and/or combination therapies to eradicate *Hp* infection in humans.

♦ GM-CSF as adjuvant

Interestingly, several studies probe for the use of GM-CSF as vaccine adjuvant. However, even some studies report that GM-CSF helps in generating an immune response, others claimed it induced a suppressive effect [223]. From now, several trials of human tumor cells transduced with the GM-CSF gene highlight the generation of an anti-tumor immune response in many types of cancer [223]. For instance, GVAX (a gene-transduced tumor vaccine), has been shown to induce reliable antitumor immunity in mice without any toxicity. Moreover, several human clinical trials confirm these preclinical observations [224]. In the context of increased immune responses, GM-CSF impact lies in the local recruitment and maturation of DCs leading to an increased antigen presentation in the LN [225]. Indeed, GM-CSF induces a DC subset more prone to phagocytosis with higher level of costimulatory molecules [224]. It is well known that DCs plays an important function during *Hp* infection, meaning that the use of GM-CSF as adjuvant in vaccination against *Hp* could be relevant. In addition to cancer, GM-CSF pretreatment before HBV antigen administration lead to a robust antigen-specific immune responses in mice through induction of DCs. This protocol has also been successfully adapted in humans [226], confirming that GM-CSF as vaccine pretreatment or adjuvant could be a possible option to improve *Hp* clearance.

♦ AMPs analogs for co-treatments

Apart from taking part to the challenge of increasing *Hp* vaccine efficacy, our contribution suggests a role for βdefensin3 in *Hp* clearance. Indeed, AMPs are already known to be efficient in *Hp* killing and several studies highlight the promising potential of cathelicidin AMPs analogs for *Hp* clearance in combination or not with antibiotics [227-229]. However, due to the physiological conditions in the stomach, designing and administrating by oral route an AMP analog remain very challenging. Concerning the

synthesis of defensin analogs, challenges remain in their size and complexity of disulfide pairing [41]. Nevertheless, even some efforts left to generate stable and efficient analogs of defensins, Pero et al. recently demonstrate that this challenge is achievable [42].

♦ Protective role of *Hp* infection

Developing an efficient therapeutic and/or prophylactic vaccine against *Hp* is a global public concern. However, before its eradication, it is important to determine whether this treatment could negatively affect concomitant diseases. As mentioned before, several observations prone for a positive role of *Hp* infection on the severity of several diseases. For instance, patients with EoE concomitantly to *Hp* infection have a decreased odds of the EoE disease, compared to EoE patients non infected with *Hp* [124]. The same observation has been done in context of asthma, esophageal reflux, as well as in IBD [115, 230, 231]. This means that *Hp* eradication should not be an automatism. In parallel, Oster P, Vaillant L et al. recently demonstrate that *Hp* seropositivity in non-small-lung cancer patients undergoing treatment with immune checkpoint inhibitors, do not respond to these powerful anticancer drugs. In addition, eradicating *Hp* infection do not restore the response, meaning alternative for cancer treatment need to be envisioned for these patients [232]. Altogether, these observations suggest that before *Hp* eradication, concomitant pathologies of the patient need to be considered. to be considered.

♦ Current attempts for vaccine development

Finally, another challenge for developing an efficient *Hp* vaccine is the investment by pharmaceutical companies. In the past decades, small companies or academic institutions have unsuccessfully initiated several preclinical and clinical studies [233]. Unfortunately, no major pharmaceutical companies engaged research on this field. To

be continued, vaccine development needs significant investment from major companies, especially for the late stage development. Consequently, it is important to reconsider the global disease burden in order to incentivize a strong investment by pharmaceutical companies and worldwide organizations.

♦ Concluding remark

This thesis attempts to better characterize immune responses involved in the vaccine-induce *Hp* clearance. In the two manuscripts, one published and the other submitted to publication, we offer a new understanding of the Th responses involved in the vaccine-induced *Hp* clearance, as well as potent treatment alternatives using βdefensin3 analogs or GM-CSF. Taken together, these studies contribute to pave the way for the development of an efficient therapy against the strongest known risk factor for gastric cancer: *Hp* infection.

Publications

Publication 1: Gastric eosinophils are detrimental for *Helicobacter pylori* vaccine efficacy

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Gastric eosinophils are detrimental for *Helicobacter pylori* vaccine efficacy

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ABSTRACT

Helicobacter pylori (*Hp*) colonizes the human gastric mucosa with a high worldwide prevalence. Currently, *Hp* can be eradicated by the use of antibiotics. Due to the increase of antibiotic resistance, new therapeutic strategies need to be devised: one such approach being prophylactic vaccination. Pre-clinical and clinical data showed that a urease-based vaccine is efficient in decreasing *Hp* infection through the mobilization of T helper (Th)-dependent immune effectors, including eosinophils. Preliminary data have shown that upon vaccination and subsequent *Hp* infection, eosinophils accumulate in the gastric mucosa, suggesting a possible implication of this granulocyte subset in the vaccine-induced reduction of *Hp* infection.

In our study, we confirm that activated eosinophils, expressing CD63, CD40, MHCII and PD-L1 at their cell surface, infiltrate the gastric mucosa during vaccine-induced reduction of *Hp* infection. Strikingly, we provide evidence that bone marrow derived eosinophils efficiently kill *Hp* in vitro, suggesting that eosinophils may participate to the vaccine-induced reduction of *Hp* infection. However, conversely to our expectations, the absence of eosinophils does not decrease the efficacy of this *Hp* vaccine in vivo. Indeed, vaccinated mice that have been genetically ablated of the eosinophil lineage or that have received anti-Sialic acid-binding immunoglobulin-like lectin F eosinophil-depleting antibodies, display a lower *Hp* colonization when compared to their eosinophil sufficient counterparts. Although the vaccine induces similar urease-specific humoral and Th responses in both eosinophil sufficient and deficient mice, a decreased production of anti-inflammatory cytokines, such as IL-10, TGFβ, and calgranulin B, was specifically observed in eosinophil depleted mice.

Taken together, our results suggest that gastric eosinophils maintain an anti-inflammatory environment, thus sustaining chronic *Hp* infection. Because eosinophils are one of the main immune effectors mobilized by Th2 responses, our study strongly suggests that the formulation of an *Hp* vaccine needs to include an adjuvant that preferentially primes *Hp*-specific Th1/Th17 responses.

1. Introduction

Helicobacter pylori (*Hp*) is a gram negative bacteria that colonizes the mucus layer of the stomach mucosa and is among the most common chronic bacterial infections in humans [1]. Although the majority of cases remain asymptomatic for decades, *Hp* infection can trigger chronic inflammation of the gastric mucosa. Moreover, this infection is associated with increased occurrences of peptic ulcers and more seriously with the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [2,3]. Due to its high global prevalence of approximately 50%, the eradication of this bacterial infection is an important public health concern. Currently, *Hp* eradication is possible using a combination of antimicrobials and antisecretory drugs [4,5]. However, the rapid emergence of antibiotic resistance and the lack of specificity of the currently available therapies, suggests that protocols of eradication need to be improved and/or alternative therapies developed.

Therapeutic alternatives currently envisioned involves the development of a prophylactic and/or therapeutic vaccine, a strategy that spares the microbiota and does not induce resistance. An efficient vaccine requires the selection of immunogenic and protective antigens mixed with appropriate adjuvants. In their seminal work, Czinn et al. demonstrated that the oral administration of a bacterial lysate plus cholera toxin (CT) in mice conferred protection against *Helicobacter felis* (a close relative of *Hp*) [6]. Several protective antigens have been characterized, including urease, a

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protein expressed at the cell surface of all *Hp* strains [7]. This antigen is considered as one of the most promising candidates for vaccine development against *Hp* [8,9]. In humans, a phase III clinical trial evaluated a urease-based vaccine, which was shown to protect 70% of children from the acquisition of *Hp* infection [10]. Unfortunately, this protection was not stable, and its efficacy decreased to 56% after one year. Although this study clearly indicates that the urease-based vaccine is protective in humans, major efforts are still needed in order to increase its protective effect and induce long term protective immunity.

In order to improve vaccine-induced protection, an in depth understanding of the immune-protective mechanisms involved in conferring *Hp* immunity is a pre-requisite to select the best vaccine candidate. Key studies have demonstrated that $CD4^+$ T helper cells, which are involved with mobilizing gastric immune effectors, confer protection against *Hp* [11,12]. Different T helper cell subsets produce interleukins such as IL-5, IL-17 and IL-22, among others. IL-22, produced by Th22 cells, triggers the production of antimicrobial peptides such as RegIIIβ by the gastric epithelial cells, which is one of the key molecules involved in vaccine-induced reduction of *Hp* colonization [13]. IL-17, produced by Th17 cells, plays a major role in stimulating granulopoiesis, in mobilizing granulocytes into sites of inflammation and in stimulating fibroblasts, endothelial cells, macrophages, and epithelial cells to produce multiple proinflammatory mediators, leading to the vaccine-induced reduction of *Hp* infection [14,15]. IL-5, produced by Th2 cells, acts directly on B cells and eosinophils to promote their mobilization and survival at the site of ongoing immune responses [16]. Although it has been demonstrated that an efficient *Hp* vaccine requires the induction of Th1, Th17 and Th22 responses [11,17] concerns pertaining to the benefit of Th2 cells remain. It is not yet understood whether the immune effectors mobilized by the Th2 immune response will increase or decrease vaccine-induced reduction of *Hp* colonization [18].

In both humans and mice, eosinophils are known to infiltrate and engulf *Hp* in the gastric mucosa of infected hosts [19,20]. Preliminary data published by Akhiani et al. showed that eosinophils are recruited into the gastric mucosa, close to epithelial cells [21] during vaccine-induced reduction of *Hp* colonization. The ability of eosinophils to harbor antimicrobial activities [22] suggests a possible role of these immune cells in vaccine-induced immune mechanisms, conferring protection against *Hp*. Contrastingly, recent reports highlight key roles of eosinophils in the downregulation of Th1 and Th17 mucosal responses, leading to the possibility that eosinophils might be detrimental for *Hp* vaccine efficacy [20,23].

The objective of this study was to probe for possible roles of eosinophils and Th2-dependent granulocytes in the control of *Hp* infection in non-immune or vaccinated hosts.

2. Materials and methods

2.1. Mice

Female BALB/c OlaHsd (Balb/c) mice (6–8 weeks old) were purchased from Envigo (Ad Horst, Netherlands). $Gata1^{tm6Sho}$/J (ΔdblGATA) mice and their BALB/cJ controls were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility. This study was approved by the State of Vaud Veterinary Office (authorization no. 836.11/2). Mice were bred under specific-pathogen-free conditions.

2.2. Hp infection

Helicobacter pylori P49 (*Hp*49), a human clinical isolate adapted to mice, was grown in brain heart infusion (BHI, Becton Dickinson, Heidelberg, Germany) supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaillé, France) in microaerophilic conditions. Adult mice were infected twice with 5×10^8 *Hp*49 bacteria. Bacteria were administered by oral gavage in 200 μl of BHI at a 2-day interval. The control group received 200 μl of BHI.

2.3. Assessment of Hp colonization

Quantification of *Hp* colony forming units (CFU) was used to assess infection status [24]. CFU were determined immediately after stomach collection. One-third of the stomach was immersed in 200 μl of CFU medium (10 μg/ml Vancomycin (Sigma, St. Louis, MO), 20 μg/ml Bacitracin (Sigma), 5 μg/ml Amphotericin B (Sigma), 0.3 μg/ml Polymyxin B (Sigma), 1.07 μg/ml Nalidixic acid (Sigma) in BHI) and homogenized with a fitted plastic pestle in a sterile Eppendorf tube (Vaudaux-Eppendorf, Basel, Switzerland). Serial 10-fold dilutions of the homogenate were then plated on *Helicobacter* plates (Becton Dickinson). Plates were incubated for 3–4 days in microaerophilic conditions after which the CFU were counted. Identification of *Hp* was based on the appearance of colonies on plates and gram staining. Results were expressed as number of CFU per one-third of stomach.

2.4. Culture of bone marrow-derived eosinophils

Tibiae and femur marrow cells were obtained via flushing bones with cold RPMI 1640 (Gibco, Invitrogen Corporation, Carlsbad, CA) and washed through a 40 μm cell strainer with RPMI 1640. The bone marrow cells were cultured at a concentration of 10×10^6 cells/ml in RPMI 1640 supplemented with 20% FBS, 100 IU/ml penicillin (BioConcept, Allschwil, Switzerland), 100 μg/ml streptomycin (BioConcept), 1 × nonessential amino acids solution (Gibco), 60 μM 2-Mercaptoethanol (Gibco) supplemented with 10 μg/ml stem cell factor (SCF; PeproTech, London, UK) and 10 μg/ml FLT3 ligand (FLT3-L; PeproTech) from days 0 to 4. On day 4, the cells were harvested and re-suspended in medium containing SCF, FLT3-L and 1 μg/ml recombinant mouse IL-5 (rmIL-5, PeproTech). On days 7 and 9, medium was replaced with fresh medium containing SCF, FLT3-L and rmIL-5. On day 10, bone marrow-derived eosinophils were enumerated and used to perform in-vitro experiments.

2.5. In vitro killing assay

Mesenteric lymph nodes (mLN) were recovered and incubated in 1 ml RPMI 1640 containing 1% collagenase IV (Sigma) and 0.1% DNAse I recombinant (Roche, Basel Switzerland) for 20 min at 37 °C. Cells isolated from the mLN were then filtered through a 40 μm cell strainer and washed in RPMI 1640 supplemented with 10% FBS. 4×10^5 eosinophils or mLN cells and 4×10^5 *Hp*49 bacteria were plated into a 96 well U-Bottom plate (Falcon, Becton Dickinson) in 110 μl of RPMI 1640. Plates were incubated for 4 h under microaerophilic conditions. Efficacy of the killing was assessed by CFU quantification.

2.6. Eosinophil depletion

BALB/c mice were injected intraperitoneally on days −1, 1, 2, 3 and 5 post *Hp* infection with 15 μg of anti-mouse Siglec-F (R&D Systems, Minneapolis, MN). Monoclonal Rat IgG_{2a} (BioXcell, West Lebanon, NH) was used as an isotype-matched control antibody.

2.7. Vaccination

Mice were immunized intranasally 4 times at 1-week intervals with 30 μg of recombinant *Hp* urease (kindly provided by Sanofi-

Pasteur, Lyon, France) combined with 5 μg of cholera toxin (Calbiochem, Lucerne, Switzerland). Control mice were administered only cholera toxin [14,24].

2.8. Determination of the anti-urease antibody response

Blood samples were recovered from submandibular vein using a sterile glodenrod™ animal lancet (Medipoint Inc, Mineola, USA) in an Eppendorf tube at least 2 weeks after the last immunization. Sera samples were recovered after 2 h of blood coagulation at room temperature. The anti-urease antibody response was determined as described elsewhere [24].

2.9. Flow cytometry

The spleen, blood and stomach were processed immediately after sacrifice. Spleens were recovered in RPMI 1640 and injected with 1 ml of RPMI containing 1% collagenase IV (Sigma) and 0.1% DNAse I recombinant (Roche) for 20 min at 37 °C. Digested spleens were then filtered through a 40 μm cell strainer and washed in RPMI 1640 supplemented with 10% FBS and were centrifuged for 10 min at 1500 rpm, 4 °C. Cells were then resuspended in fluorescence-activated cell sorter (FACS) buffer (1% bovine serum albumin (Sigma) 2% FBS and 2 mM EDTA (Sigma) in phosphate-buffered saline (PBS, Gibco)). Blood was recovered just before sacrificed in a 15 ml falcon® polystyrene conical tube (Corning, Tamaulipas, Mexico) containing 20 μl heparin (Braun Medical AG, Sempach, Switzerland) in 2 ml PBS. Red blood cells were removed by adding 5 ml of red blood cell (RBC) lysis buffer (1.5×10^{-5} M NH_4Cl (Sigma), 1×10^{-2} M $KHCO_3$, (Sigma)). The mixture was incubated for 5 min on ice, completed with PBS and centrifuged for 10 min at 1500 rpm at 4 °C. Lysis cycles were repeated until supernatant had become clear. Lastly, blood cells were resuspended in FACS buffer. The isolation of the gastric immune cells was performed as described elsewhere [25]. Isolated gastric immune cells were resuspended in FACS buffer. All cells were counted using cellometer® Auto T4 cell counter (Nexcelom Bioscience, Lawrence, MA) before staining.

For extracellular stainings, cells were incubated with anti-mouse CD16/CD32 (Clone 2.4G2; Becton Dickinson) for 20 min on ice. Then, cells were stained for viability assessment with LIVE/DEAD™ fixable Aqua Dead cell stain kit (Invitrogen, Life technology corporation, OR) for 20 min on ice. Cells were then stained with anti-mouse antibodies to CD63- Bright™ fluorescein isothiocyanate (Bright™ FITC; Clone REA563, Miltenyi Biotec, Auburn, CA), CD11b-PerCP/Cyanine5.5 (PerCP/Cy5.5, Clone M1/70, BioLegend, San Diego, CA), PD-L1- Allophycocyanine (APC, Clone 10F.9G2, BioLegend), Ly6G-AlexaFluor 700 (AF700, Clone 1A8, BioLegend), CD4-APC/Cy7 (Clone GK1.5, BioLegend), CD45-VioBlue® (Clone REA 737, Miltenyi biotec), Siglec-F- phycoerythrin (PE, Clone REA 798, Miltenyi biotec), CD11b-PacificBlue™ (Clone M1/70, BioLegend), MHC-II-AF700 (Clone M5/114.15.2, BioLegend), CD40-PE/Vio®615 (Clone REA965, Miltenyi biotec), CD3-PerCP/Cy5.5 (Clone 17A2, BioLegend), CD45-AF700 (Clone 30-F11, BioLegend) for 20 min on ice. Cells were then fixed using BD Cytofix/Cytoperm™ solution (BD Biosciences, Basel Switzerland) for 20 min on ice. Lastly, the cells were resuspended in FACS buffer.

For intracellular staining, gastric immune cells were first activated for 5 h at 37 °C in RPMI 1640 supplemented with 10% FBS, 1% Penicillin/Streptomycin (BioConcept), 1 mM sodium pyruvate (Sigma), 0.05 mM 2-Mercaptoethanol (Gibco), 10 mM HEPES (BioConcept) in the presence of Brefeldin A (BioLegend Switzerland), Phorbol 12-myristate 13-acetate (Sigma), and Ionomycin calcium salt (Sigma). The cells were then stained extracellularly as described above. Finally, cells were stained with anti-mouse antibodies to IL-17A-FITC (Clone TC11-18H10.1, BioLegend), GM-CSF-APC (Clone MP1-22E9, Biolegend) and resuspended in FACS buffer.

Cells were acquired using Attune NxT Flow Cytometer (ThermoFisher, Waltham, MA). Samples were analysed with FlowJo V.10 software (FLOWJO LLC, Ashland, OR).

2.10. Urease-induced specific proliferation

A total of 1×10^5 splenocytes labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Enzo life Science, Lausen, Switzerland) [26] were stimulated with 10 μg/ml purified urease in RPMI 1640 supplemented with 5% FBS. After 4 days of incubation at 37 °C, cells were stained with CD3-AF700 (Clone 17A2, BioLegend), CD4-APC/Cy7 (Clone GK1.5, BioLegend), and 7-Amino-Actinomycin D (7-AAD) for discrimination of viable from non-viable cells (Beckman Coulter, Brea, CA). CFSE dilution was measured by FACS using Attune NxT Flow Cytometer (ThermoFisher) and samples were analysed with FlowJo V.10 software. The proliferation index was determined using proliferation with Dynabeads Mouse T-Activator CD3/CD28 (Gibco) to normalize urease-specific proliferation.

2.11. Quantitative PCR (qPCR)

RNA extraction was performed on stomach tissue using Trizol (Invitrogen Corporation, Carlsbad, CA) and an RNeasy minikit (Qiagen, Valencia, CA). RNA (300 ng) was reverse transcribed into cDNA using a PrimeScript reverse transcriptase (RT) reagent kit (TaKaRa Bio Inc., Otsu, Japan). qPCR amplification was performed on a QuantStudio 6 Flex Real-Time PCR Systems apparatus (ThermoFisher), using 96 or 384-well plates (ThermoFisher). The qPCR was performed in duplicate with FASTSTART SYBR GREEN MASTER (Roche). The primers used were as follows: GAPDH (5′-GCTAAG CAGTTGGTGGTGCA-3′ and 5′-TCACCACCATGGAGAAGGC-3′, Microsynth AG, Balgach, Switzerland), LCN2 (QT00113407, Qiagen), Reg4 (QT00121513, Qiagen), CD4 (QT00096166, Qiagen), TNFα (5′-TGG GAGTAGACAAGGTACAACCC-3′ and 5′-CATCTTCTCAAAATTCGAGT GACAA-3′, Microsynth AG), IL-17 (5′-GCTCCAGAAGGCCCTCAGA-3′ and 5′-AGCTTTCCCTCCGCATTGA-3′, Microsynth AG), IL-4 (5′-GAA GCCCTACAGACGAGCTCA-3′ and 5′-ACAGGAGAAGGGACGCCAT-3′, Microsynth AG), IL-6 (5′-CACGATTTCCCAGAGAACATGTG-3′ and 5′-ACAACCACGGCCTTCCCTACTT-3′, Microsynth AG), β-Defensin3 (QT00265517, Qiagen), IL-1β (5′-CAACCAACAAGTGATATTCTC CATG-3′ and 5′-GATCCACACTCTCCAGCTGCA-3′, Microsynth AG), IL-12p40 (5′-GGAAGCACGGCAGAATA-3′ and 5′-AACTTGAGGGA GAAGTAGGAATGG-3′, Microsynth AG), TGFβ (5′-GGTTCATGTCATG GATGGTGC-3′ and 5′TGACGTCACTGGAGTTGTACGG-3′, Microsynth AG), CalgranulinB (QT00105252, Qiagen), IP-10 (5′-GCCGTCATTTT CTGCCTCAT-3′ and 5′-GCTTCCCTATGGCCCTCATT-3′, Microsynth AG), CCR3 (QT00262822, Qiagen), IL-10 (5′-ACCTGCTCCACTGCCTT GCT-3′ and 5′-GGTTGCCAAGCCTTATCGGA-3′, Microsynth AG), RNase2b (QT00325878, Qiagen), RegIIIβ (QT00239302, Qiagen), RegIIIγ (QT00147455, Qiagen).

Quantification of input cDNA from the unknown samples was performed by including a standard curve as described elsewhere [24].

2.12. Statistical analysis

The distribution of the data was compared using Mann-Whitney tests and two-way analysis of variance (ANOVA) using GraphPad software (GraphPad Software, San Diego, CA), with a P value of 0.05 being considered as the limit of significance.

3. Results

3.1. Eosinophils are recruited within the gastric mucosa shortly after Hp infection

First, we studied the time course of gastric infiltration of eosinophils upon *Hp* infection of non-immune mice (Fig. 1 A). In as little as two days post *Hp* infection, the number of gastric eosinophils substantially increased as compared to non-infected mice (Fig. 1B.1, p = 0.12). Remarkably, on days 2 and 4 post infection, gastric eosinophils displayed an activated phenotype, as determined by a significant increase in the expression of the degranulation marker, CD63 [27], and the activation marker, PD-L1 [28], at the cell surface (Fig. 1B.2 & B.3). We also observed an increased absolute number of gastric $CD4^+$ T cells (Fig. 1B.4) on day 2 post infection. From day 4 post infection onward, the absolute number of $CD4^+$ T cells and the activation status of eosinophils returned to values similar to what was observed in non-infected mice. It is known that activation of eosinophils in the blood is a prerequisite for their recruitment into inflamed tissues [29]. As expected, we observed that the degranulation and activation status and absolute number of blood eosinophils increased on day 2 and 6 post infection (Fig. 1C.1, C.2, C.3). We also observed a slight progressive increase of the proportion of eosinophils, especially those expressing CD63 and PD-L1, in the spleen of *Hp* infected mice (Fig. 1D.1, D.2, D.3). Here, the spleen could be acting as a reservoir of bone marrow-derived eosinophils and/or as a site of elimination of unused activated and/or aged eosinophils [30,31].

Taken together, our results demonstrate that after *Hp* infection, activated eosinophils, together with $CD4^+$ T cells, accumulate in the gastric mucosa. Gastric hypereosinophilia was detected two days post infection and vanishes very rapidly.

3.2. Activated eosinophils are recruited into the gastric mucosa of Hp infected vaccinated mice

We showed in Fig. 1 that eosinophils are recruited transiently into the gastric mucosa of naïve mice infected with *Hp*. We next studied the recruitment of eosinophils in the gastric mucosa of vaccinated mice challenged with *Hp*. To this end, we immunized Balb/c mice with urease adjuvanted with CT via intra-nasal vaccination and infected them with *Hp*. Infection status, assessed by CFU, confirms vaccine-induced *Hp* clearance but was only detectable from day 7 post infection (Fig. 2 A). As compared to naïve mice, the gastric hypereosinophilia induced by *Hp* infection was two-fold higher on day 2 post infection and tend to stabilize on day 7 (Fig. 2 B). In addition, these gastric eosinophils displayed a very strong activation status compared to eosinophils of non-vaccinated mice. Indeed, there was an upregulation of the degranulation marker CD63, the activation markers MHCII [32] PD-L1, and the survival marker CD40 at their cell surface [33] (Fig. 2 C). Taken together, these results show that vaccine-induced immune responses strongly increase the recruitment and activation of eosinophils into the stomach mucosa shortly after *Hp* infection. Our results are in agreement with the work of Akhiani et al., showing a major accumulation of eosinophils in the gastric mucosa during the vaccine-induced reduction of *Hp* colonization [21].

3.3. Eosinophils display antimicrobial activity against Hp in vitro

Eosinophils display antimicrobial activities [22] which are dependent on proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPX) and eosinophil-derived neurotoxin (EDN or RNase2b) which are stored in their granules [34]. To evaluate whether eosinophils display antimicrobial activity against *Hp*, we derived eosinophils from bone marrow cells and incubated them with *Hp* for 4 h. At the end of the incubation, we determined *Hp* viability by plating the bacteria and by CFU quantification. As compared to *Hp* incubated alone or with lymph node cells, only a small proportion of *Hp* remained viable after co-incubation with eosinophils (Fig. 3 A). These results demonstrate that eosinophils exhibit antimicrobial activity against *Hp*. Noteworthy, we observed an increased expression, in the stomach of vaccinated mice, of mRNA encoding RNase2b on day 2 post *Hp* infection (Fig. 3 B), suggesting that eosinophils are armed to kill *Hp* in vivo.

3.4. Lower level of Hp colonization in eosinophil-deficient mice

As activated eosinophils are recruited into the gastric mucosa of infected mice and display *Hp* killing activities in vitro, we reasoned that eosinophils might participate in the immune-induced reduction of *Hp* infection. In order to probe for a role of eosinophils in the immune control of *Hp* infection, we first chronically infected eosinophil-deficient ΔdblGATA mice. These mice displayed a deletion of a palindromic GATA-binding site in the GATA1 promoter. This promoter mediates positive autoregulation of GATA-1 expression and the knockout results in the complete ablation of the eosinophil lineage [35].

Unexpectedly, eosinophil-deficient mice display lower levels of *Hp* colonization as compared to wild type (WT) counterparts two months following infection (Fig. 4 A). Interestingly, as compared to WT mice, the gastric mucosa of noninfected eosinophil-deficient mice already display increased mRNA encoding antimicrobial peptides (AMPs) such as Lipocalin2 (LCN2) and Regenerating Family Member 4 (Reg4). These AMPs are major actors in the control of bacterial infection of mucosal surfaces [36,37] (Fig. 4 B). Moreover, we also observed higher expression of mRNA encoding inflammatory cytokines such as TNFα, IL-17, IL-6, IL-4 together with the mRNA encoding CD4, a protein that is preferentially expressed by Th cells in the gastric mucosa of noninfected ΔdblGATA mice as compared to wild type counterparts (Fig. 4 C). Altogether, these results suggest that in the absence of eosinophils, the gastric mucosa of ΔdblGATA noninfected mice is already inflamed, as it expresses high levels of AMPs and cytokines usually expressed by activated Th1, Th2 and Th22 cells.

Two months post infection, both eosinophil-deficient and sufficient mice expressed similarly high levels of TNFα, IL-17, IL-6, IL-4, IL-1β, IL-12p40 and βdefensin 3 as compared to their noninfected counterparts (Fig. 4 C). Strikingly, the mRNA encoding two pro-repair and anti-inflammatory molecules, transforming growth factor beta (TGFβ) and calgranulin B, although upregulated in the gastric mucosa of WT mice, remain unchanged in ΔdblGATA mice [38–40] (Fig. 4 D).

In summary, we observed that two months post infection, eosinophil deficient mice display lower *Hp* infection burden as compared to their WT counterparts. The lower *Hp* infection burden is associated with an inhospitable niche of the gastric mucosa of noninfected ΔdblGATA mice. Indeed, as compared to WT mice, the gastric mucosa of noninfected eosinophil-deficient mice is already inflamed and there is a high expression of AMPs. Lower *Hp* burden is also associated with low expression levels of mRNA encoding anti-inflammatory molecules such as TGFβ and calgranulin B in the gastric mucosa of eosinophils deficient mice as compared to their WT counterparts. Taken together, these results show that eosinophils favor *Hp* colonization in naive mice.

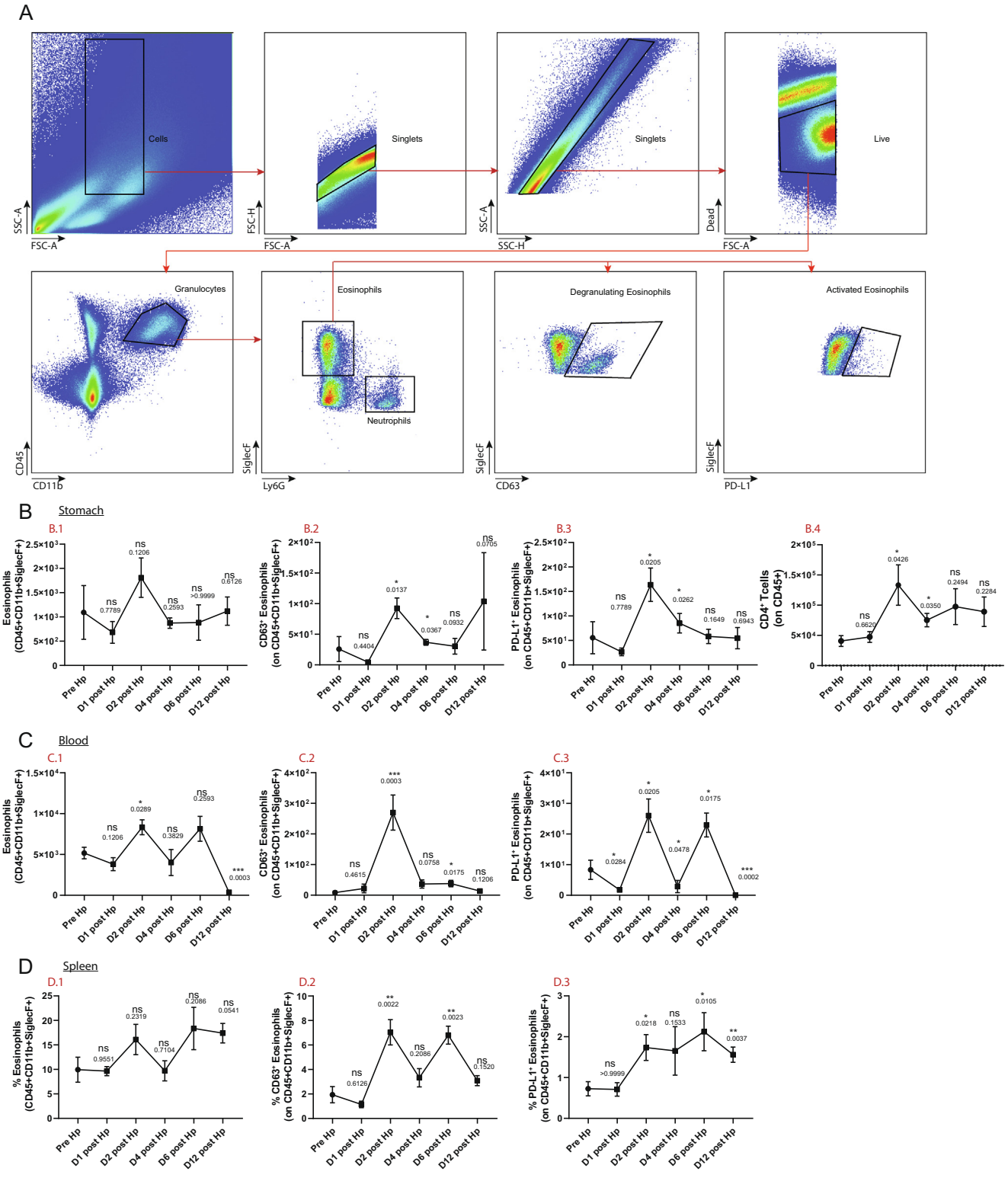

Fig. 1. Comparative timeline analysis of eosinophils infiltration upon *Hp* infection of naïve mice. Eight week old Balb/c mice were infected with *Hp*. Eosinophils and/or Th cells were analyzed in the stomach, blood and spleen. **(A)** Gating strategies used in flow cytometry to characterize the absolute cell number and degranulation/activation status of eosinophils isolated from the stomach mucosa. **(B)** Analysis of stomach eosinophils and $CD4^+$ Th cells. Total number of eosinophils (B.1), number of eosinophils expressing the degranulation marker CD63 (B.2), number of eosinophils expressing the activation marker PD-L1 and (B.3) total number of $CD45^+CD4^+$ Th cells (B.4). **(C)** Analysis of blood eosinophils. Total number of eosinophils (C.1), number of eosinophils expressing the degranulation marker CD63 (C.2), number of eosinophils expressing the activation marker PD-L1 (C.3). **(D)** Analysis of spleen eosinophils. Percentages of eosinophils (D.1), percentages of eosinophils expressing the degranulation marker CD63 (D.2), percentages of eosinophils expressing the activation marker PD-L1+ (D.3). ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney test). Bar graphs show the mean ± SEM. For each time point, 7–8 mice were analyzed.

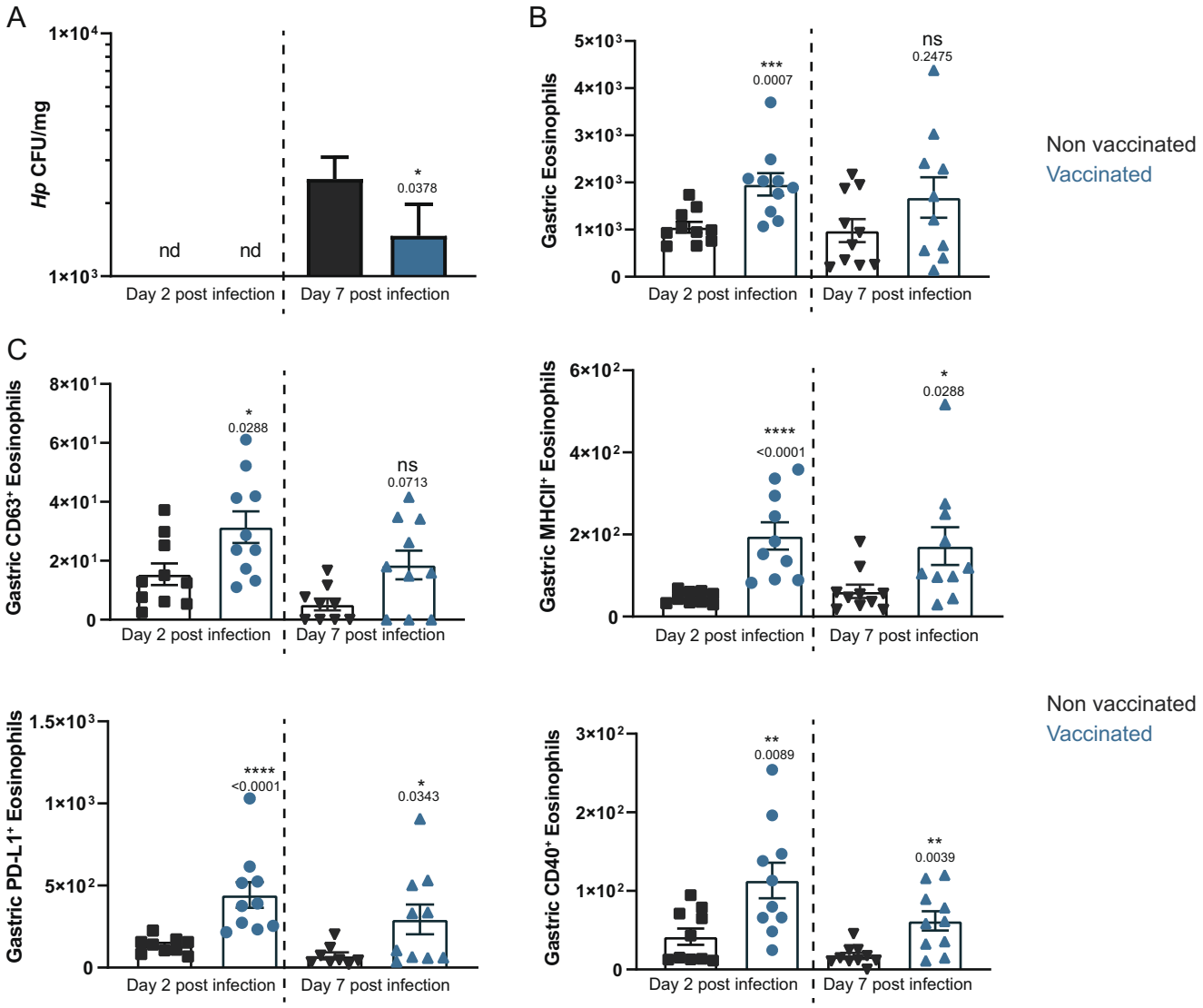

Fig. 2. Comparative timeline analysis of stomach eosinophil infiltration upon *Hp* infection of vaccinated mice. Eight week old Balb/c mice were immunized and two weeks later, mice were infected with *Hp*. Eosinophils infiltrating the stomach were analyzed by flow cytometry on day 2 and 7 post infection. **(A)** At sacrifice, the stomachs were recovered and *Hp* colonization was assessed by CFU per mg of stomach, each group contain 8 to 10 mice, nd not detectable **(B)** Total number of eosinophils ($CD45^+CD11b^+SiglecF^+$). **(C)** Total number of $CD63^+$, PD-$L1^+$, $MHCII^+$ or $CD40^+$ eosinophils. Each dot represents one mouse. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Mann-Whitney test). Bar graphs show the mean ± SEM.

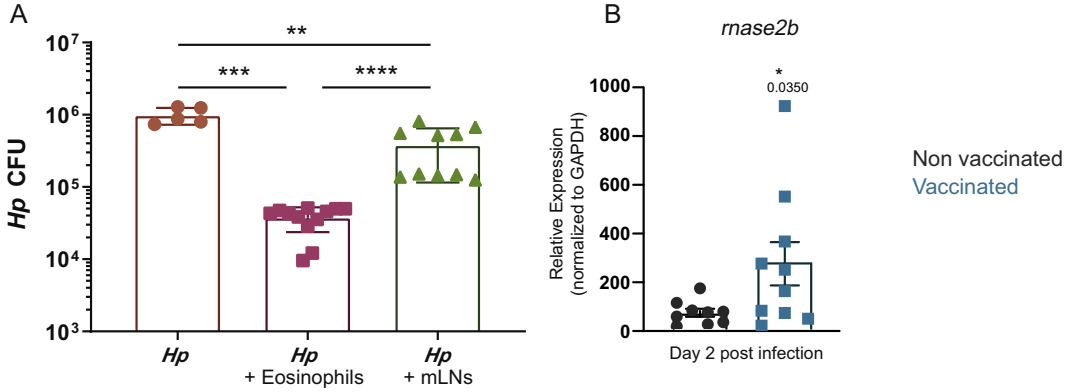

Fig. 3. Antimicrobial activities of eosinophils against *Hp*. **(A)** Bone marrow-derived eosinophils were co-cultured with *Hp* for 4 h before plating and CFU counting. As controls, *Hp* alone and *Hp* co-cultured with cells isolated from mesenteric lymph nodes (mLNs) were used. Each dot represents the CFU number from one co-culture well. **(B)** Gastric mucosal expression of the mRNA encoding the eosinophil marker *rnase2b*. Each symbol represents one mouse. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Mann-Whitney test). Bar graphs show the mean ± SEM.

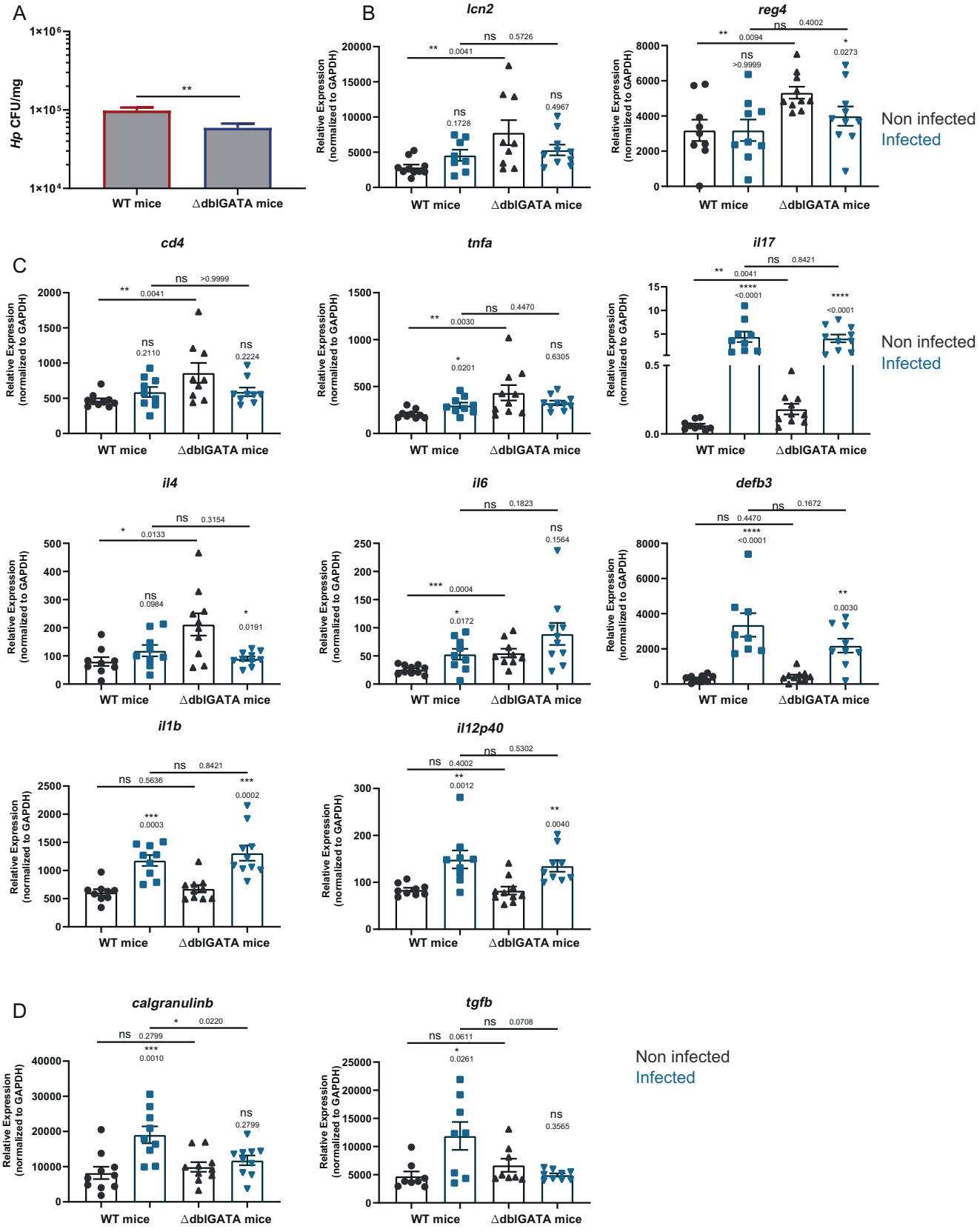

Fig. 4. Characterization of the gastric inflammatory status of *Hp* infected or uninfected eosinophil deficient ΔdblGATA mice. Eight week old ΔdblGATA and wild type (WT) mice control Balb/c mice were infected with *Hp* by oral gavage and sacrificed two months later. **(A)** At sacrifice, the stomachs were recovered and *Hp* colonization was assessed by CFU per mg of stomach, each group contain 8 to 10 mice. **(B-D)** Gastric mucosal expression of mRNA encoding *lcn2*, *reg4*, *cd4*, *tnfa*, *il17*, *il4*, *il6*, *defb3*, *il1b*, *il12p40*, *calgranulinb*, and *tgfb*. Each symbol represents one mouse. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Mann-Whitney test). Bar graphs show the mean ± SEM.

3.5. Eosinophil deficiency boosts vaccine-induced reduction of Hp infection

We next determined the role of eosinophils in the vaccine-induced decrease of *Hp* colonization.

We vaccinated and infected ΔdblGATA mice as previously described. As expected in this model, circulating eosinophils were completely absent regardless of the infection or vaccination status (Fig. 5 A). Moreover, vaccination induces similar humoral and cellular responses in ΔdblGATA and WT mice (Fig. 5B.1 & B.2). Remarkably, we observed that vaccinated eosinophil-deficient mice displayed lower levels of *Hp* colonization as compared to vaccinated WT counterparts (Fig. 5 C). The decreased *Hp* burden in the gastric mucosa of vaccinated ΔdblGATA mice was not associated with an increase of effector Th responses and AMPs production. Indeed, we did not observe an increase of $CD4^+$ T cells, $CD4^+$ T cells secreting GM-CSF or IL-17, or major increases of mRNA levels encoding AMPs or pro/anti-inflammatory cytokines in the stomach mucosa of vaccinated ΔdblGATA mice as compared to their WT counterparts (Fig. 5 D & E). On day 7 post infection, the lower *Hp* burden of vaccinated ΔdblGATA mice may originate from the absence of eosinophils. However, the apparent increase of the vaccine efficacy in ΔdblGATA mice may also come from a lower capacity of *Hp* to infect ΔdblGATA mice (Fig. 5 C, $p < 0.001$).

3.6. Eosinophils reduce the efficacy of vaccine-induced reduction of Hp colonization

In order to circumvent the decreased infectivity of *Hp* in ΔdblGATA mice, we used another experimental approach to deplete eosinophils on demand. Sialic acid-binding immunoglobulin-like lectins – F (Siglec-F) is a receptor that is highly expressed at the cell surface of murine eosinophils [41,42] and the injection of anti-Siglec-F monoclonal antibodies (αSiglec-F) into mice has been reported to deplete eosinophils [43]. We injected vaccinated and non-vaccinated mice with αSiglec-F antibodies one day before *Hp* infection and 1, 2, 3 and 5 days post infection. Firstly, the αSiglec-F-induced eosinophil depletion was confirmed by flow cytometry analysis and qPCR. As expected, the number of blood and splenic eosinophils were markedly reduced by αSiglec-F injection (Fig. 6 A). Moreover, we observed a substantial decrease in the expression level of mRNA encoding CCR3 in the gastric mucosa of mice injected with αSiglec-F. CCR3 is highly expressed by eosinophils and constitutes as an evaluative marker of eosinophil infiltration into inflamed tissues [44] (Fig. 6 B). Moreover, the substantially decreased expression levels of mRNA encoding CCR3 in the gastric mucosa of αSiglec-F injected mice highly suggests that the depletion of eosinophils is efficient at the site of *Hp* infection. Remarkably, the αSiglec-F –induced depletion of eosinophils does not decrease the *Hp* infectivity in non-vaccinated mice but clearly promotes the efficacy of the vaccine-induced decrease *Hp* colonization (Fig. 6 C). As observed in vaccinated and *Hp* infected ΔdblGATA mice, the vaccine-induced Th responses and AMP production were similar between eosinophil depleted and non-depleted mice. Indeed, no major modifications of the mRNA levels encoding AMPs or pro-inflammatory cytokines were observed in the stomach mucosa of control or αSiglec-F injected vaccinated mice (Fig. 6 D). Strikingly, we observed a reduction of mRNA encoding IL-10 and TGFβ; two major anti-inflammatory molecules in the stomach mucosa of αSiglec-F injected mice (Fig. 6 E).

Taken together, these results show that eosinophils are detrimental for the efficacy of vaccine-induced reduction of *Hp* colonization.

4. Discussion

The development of an efficient vaccine against *Hp* relies on the identification of positive and negative immune effectors which act remotely and/or locally to remove *Hp* from the gastric mucosa of the infected host. Previous pre-clinical and clinical data have clearly identified the urease-based vaccine as a promising prophylactic approach to protect the host against *Hp* infection. However, there exist a myriad of opportunities to improve the efficacy of vaccine-induced protection.

In this paper, we interrogated whether eosinophils are critical players in vaccine-induced reduction of *Hp* infection. Activated/degranulated eosinophils colonized the gastric mucosa of vaccinated or non-vaccinated mice on two days post *Hp* infection (Figs. 1 and 2). This early recruitment of eosinophils to the gastric mucosa is likely the consequence of the *Hp* infectious challenge. Indeed, as previously demonstrated by Stenfeldt and colleagues, intestinal epithelial cell damage or necrosis are potent signals for eosinophil chemotaxis [45]. Very similarly, it can be postulated that *Hp* infection generates gastric epithelial cell stress, which may lead to the secretion of DAMPS (Damage Associated Molecular Patterns) and PAMPS (Pathogen Associated Molecular Patterns) that trigger local secretion of chemoattractants such as CCL2, CCL5 or GM-CSF as well as the recruitment and activation of eosinophils within the gastric epithelium [46].

Eosinophils recruited to the gastric mucosa have been shown to engulf *Hp* [47] and displayed surface markers of degranulation and activation (Figs. 1 and 2). In vitro, we observed that bone marrow-derived eosinophils kill *Hp* (Fig. 3). Conversely, Arnold et al. did not observe in vitro *Hp* killing by eosinophils [20]. Our experimental conditions are markedly different, in particular in the determination of bacterial viability. Consequently, it can be postulated that the early recruitment of eosinophils to the gastric mucosa might participate in the reduction of *Hp* colonization.

To investigate this hypothesis, we infected eosinophil deficient ΔdblGATA mice. Mice were sacrificed on day 7 or 2 months post infection and we observed that in the absence of eosinophils, *Hp* burden is reduced (Figs. 4 and 5). These results do not support a role of eosinophils in the immune response leading to the reduction of *Hp* infection, but on the contrary, they suggest that eosinophils may favor *Hp* colonization of non-vaccinated mice.

Next, we vaccinated ΔdblGATA and WT mice to probe for a role of eosinophils in the vaccine-induced reduction of *Hp* colonization. On day 2 post *Hp* infection, vaccinated WT mice have a significant increase in number and activation status of recruited gastric eosinophils as compared to their non-vaccinated counterparts (Fig. 2). Moreover, we detected a clearly defined increase of mRNA expression levels that encode eosinophilic antimicrobial proteins in vaccinated WT mice (Fig. 3). This vaccine-induced potentiation of eosinophil recruitment/activation in the gastric mucosa on day 2 post *Hp* infection (Fig. 2) is likely dependent on immune complexes that are known to be very potent eosinophil activation stimuli [48].

Although the vaccine promotes eosinophil recruitment and activation in WT mice, the absence of eosinophils in vaccinated ΔdblGATA mice or αSiglec-F injected WT mice does not prevent the reduction of *Hp* colonization. To our surprise, *Hp* colonization was promoted. Hence, it can be concluded that eosinophils favor *Hp* colonization in non-vaccinated and vaccinated mice.

Prior to infection, we detected the expression of mRNA encoding inflammatory cytokines and AMPs in the gastric mucosa of ΔdblGATA mice. Consequently, it may be possible that eosinophils that are known to colonize the gastric mucosa of WT mice during the weaning period [20] are key cells to dampen gastric inflammation in the long run. The low grade inflammation of the gastric mucosa likely offers poorer growth conditions and explains, in

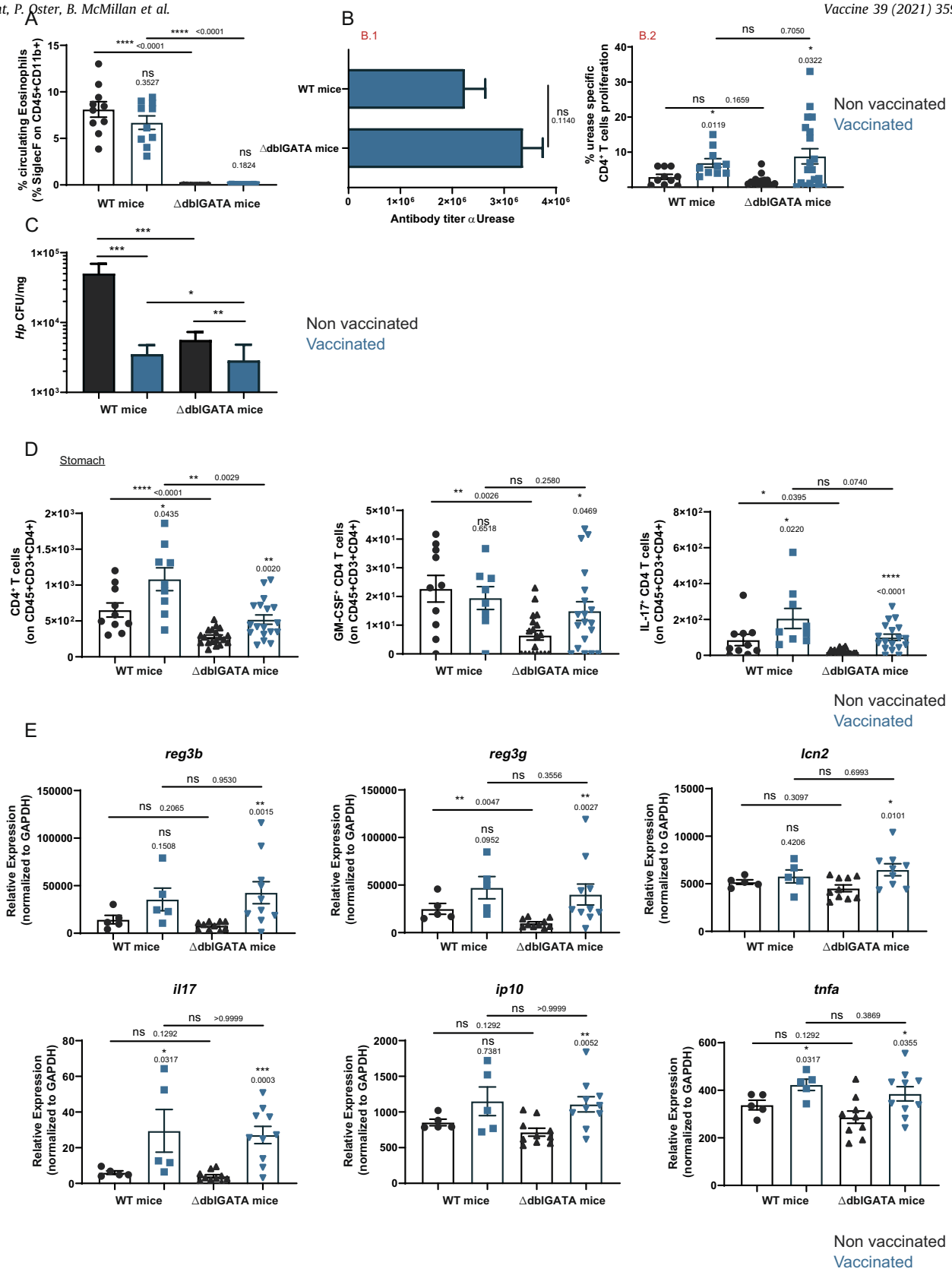

Fig. 5. Increased vaccine-induced reduction of *Hp* infection in eosinophil deficient ΔdblGATA mice. Eight week old ΔdblGATA and WT mice were intranasally immunized and two weeks later, infected with *Hp*. They were sacrificed seven days 7 post infection. **(A)** At sacrifice, blood was collected, and the percentages of circulating eosinophils were determined by flow cytometry. **(B)** Vaccine-induced urease immune response characterization in WT and ΔdblGATA mice. (B.1) Serum urease specific IgG titers were determined by ELISA. (B.2) At sacrifice, splenocytes were recovered and the urease-induced $CD3^+CD4^+$ T cell proliferation was assessed by CSFE staining and flow cytometry. **(C)** At sacrifice, the stomachs were recovered and *Hp* colonization was assessed by CFU quantification per mg of stomach, each group contain 8 to 10 mice. **(D)** Total number of $CD4^+$ T cells (gated on $CD45^+CD3^+$), GM-CSF^+CD4^+ T cells (gated on $CD45^+CD3^+CD4^+$), and IL-17^+CD4^+ T cells (gated on $CD45^+CD3^+CD4^+$) isolated from the stomach of naïve or vaccinated ΔdblGATA and WT mice, 7 days post *Hp* infection. **(E)** Gastric mucosal expression of mRNA encoding *reg3b, reg3g, lcn2, il17, ip10* and *tnfα*. Each symbol represents one mouse. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Mann-Whitney test). Bar graphs show the mean ± SEM.

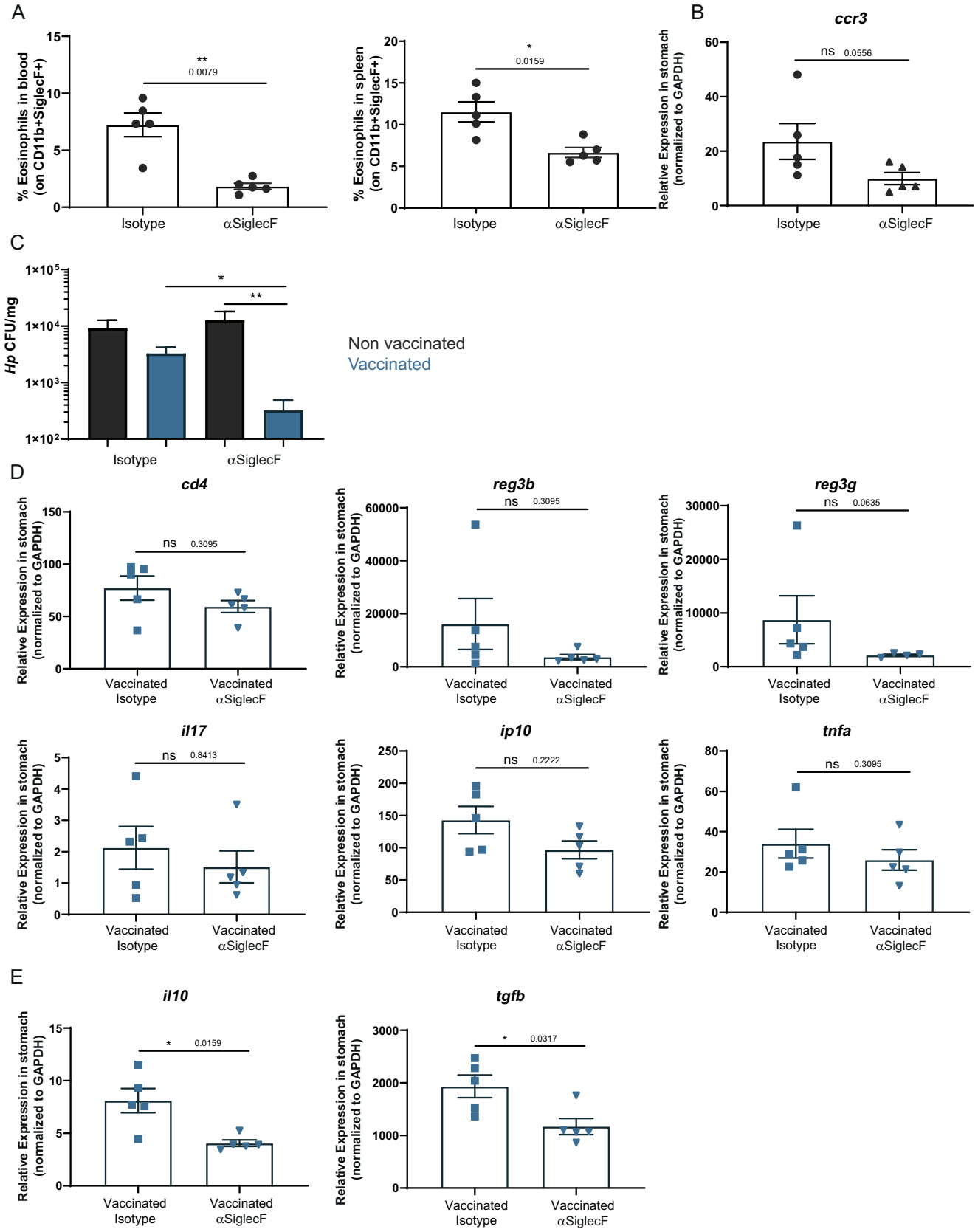

Fig. 6. Increased vaccine-induced reduction of *Hp* infection in eosinophil-depleted mice. Eight week old Balb/c mice were intranasally immunized, two weeks later, mice were infected with *Hp*. Mice were intraperitoneally administered on days −1, 1, 2, 3, 5 post infection with 15 μg of anti-mouse Siglec-F (αSiglecF) or isotype- control antibodies. Mice were sacrificed 7 days post infection. **(A)** Characterization of the anti-mouse Siglec-F-induced eosinophil depletion. Percentage of eosinophils in the blood and spleen of mice at sacrifice. **(B)** Characterization of the anti-mouse Siglec-F-induced eosinophil depletion in the stomach. Gastric mucosal expression of mRNA encoding *ccr3*. **(C)** At sacrifice, the stomachs were recovered and *Hp* colonization was assessed by CFU quantification per mg of stomach, each group contain 5 mice. **(D, E)** Gastric mucosal expression of mRNA encoding *cd4, reg3b, reg3g, il17,ip10, tnfα, il10*, and *tgfβ*. Each symbol represents one mouse. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Mann-Whitney test). Bar graphs show the mean ± SEM.

part, the lower *Hp* burden observed in non-vaccinated ΔdblGATA mice as compared to WT counterparts on day 7 and 2 months post infection (Figs. 4 and 5). In addition, although chronically infected ΔdblGATA mice had very similar levels of mRNA encoding inflammatory markers and AMPs, mRNA encoding TGFβ was clearly decreased in infected eosinophil deficient mice. Indeed, eosinophils are a chief source of TGFβ, which is a master regulator of tissue repair and anti-inflammatory processes [49–51]. Moreover, TGFβ is known to promote the differentiation of regulatory T cells, which are key in the evasion of immune mechanisms that are used by *Hp* to chronically infect its host [52]. Additionally, it is well established that the interaction between PD-L1 and PD-1 expressing T cells leads to their inhibition [53]. As activated eosinophils express PD-L1 at their surface (Figs. 1 and 2), the pro-inflammatory environment in mice lacking eosinophils might also be linked to the absence of PD-L1^{+} eosinophils to dampen Th1 responses [20]. Finally, Sugawara et al. demonstrated the ability of small intestinal eosinophils to produce high levels of IL-1 receptor antagonist (IL-1Ra), a natural inhibitor of IL-1β, which decreases Th17 survival [23]. Taken together, it can be hypothesized that the absence of these eosinophilic anti-inflammatory pathways may contribute to the constitutive pro-inflammatory environment present in the stomach of ΔdblGATA mice.

Although αSiglec-F injected vaccinated mice were clearly more prone to decreased *Hp* colonization than their eosinophil sufficient counterparts, no major modifications of the levels of mRNA encoding AMPs or pro-inflammatory cytokines were observed in the stomach mucosa (Fig. 6 D), perhaps because these mice do not constitutively lack eosinophils. However, we observed a reduction of mRNA encoding two major anti-inflammatory molecules, TGFβ and IL-10, both of which are known to mediate immune evasion mechanisms used by *Hp* to chronically infect the hosts. As discussed above, TGFβ is a key molecule in promoting the generation of regulatory T cells, which secrete an important effector molecule, IL-10, which mediates the suppressive function of regulatory T cells. Unfortunately, we could not find an increase gastric expression of foxp3, a master regulator of regulatory T cell subset, in WT mice as compared to eosinophils-deficient mice. This is most probably the consequence of the experimental conditions used in our study. Indeed, in one hand, the adult *Hp* infection has been shown to induction the differentiation of very limited number of regulatory T cells [54]. In the second hand, in αSiglec-F injected WT mice, we sacrificed mice on day 7 post *Hp* infection, a very early time that most probably prevent the characterization of regulatory T cells population.

One limitation of our study could be that we performed our experiments with BALB/c mice. It is well documented that BALB/c mice are prone to develop Th2 responses, leading to the possibility that we may over-estimate the role of eosinophils during the chronic *Hp* infection and/or the vaccine-induced reduction of *Hp* infection. However, Arnold et al. also observed eosinophils recruitment in the gastric mucosa of *Hp* infected C57BL/6 mice [20]. In the same study, the authors showed that those eosinophils down-regulated intestinal Th1 responses [20], demonstrating that, in C57/BL/6 background, eosinophils also display inflammatory roles. Taken together, the conclusions of our study is in alignment with study of Arnold et al showing that eosinophils at mucosal surfaces display anti-inflammatory properties irrespectively of the genetic background.

Collectively, it can be concluded that the recruitment and the activation of eosinophils, a Th2 –dependent effector cell, in the gastric mucosa of *Hp* infected vaccinated mice is detrimental for the efficacy of the vaccine. Our study has important clinical implications, as it reveals for the first time, that *Hp* vaccines developed in the future should avoid promoting Th2 responses which are known to stimulate the production of eosinophils by the bone marrow and their systemic and local activation. Indeed, during the *Hp* infection of immunized host, the activation of resident memory CD4+ T which occurs within 2 to 3 days post antigen challenge [55,56] will produce Th1, Th2, or Th17 cytokines depending on the adjuvant used in the vaccine formulation. For instance, adjuvants such as aluminium hydroxide that stimulates Th2 responses [57] are not appropriate. While recombinant cholera toxin subunit B (i.e. Dukoral® vaccine) [58] or the non-toxic form of *Escherichia coli* Heat-Labile Toxin, LT(R192G/L211A) [59 60], that trigger Th1/17 responses represent better choices to avoid the generation of a very potent eosinophil-mediated suppressive environment that will jeopardize the vaccine-induced reduction of Hp infection.

In conclusion, it is imperative that the formulation of an *Hp* vaccine includes an adjuvant and administration protocols that preferentially primes Th1/Th17 anti-*Hp* responses.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Publication 2: GM-CSF is key in the efficacy of vaccine-induced reduction of *Helicobacter pylori* infection

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GM-CSF is key in the efficacy of vaccine-induced reduction of *Helicobacter pylori* infection

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Abstract: *Helicobacter pylori* (*Hp*) colonizes the human gastric mucosa with a high worldwide prevalence. Currently, *Hp* is eradicated by the use of antibiotics. However, elevated antibiotic resistance suggests new therapeutic strategies need to be envisioned: one approach being prophylactic vaccination. Pre-clinical and clinical data show that a urease-based vaccine is efficient in decreasing *Hp* infection through the mobilization of T helper (Th) cells, especially Th17 cells. Th17 cells produce interleukins such as IL-22 and IL-17, among others, and are key players in vaccine efficacy. Recently, granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing Th17 cells have been identified. This study explores the possibility that GM-CSF plays a role in the reduction of *Hp* infection following vaccination. We demonstrate that GM-CSF$^+$ IL-17$^+$ Th17 cells accumulate in the stomach mucosa of *Hp* infected mice during the vaccine-induced reduction of *Hp* infection. Secondly, we provide evidence that vaccinated GM-CSF deficient mice only modestly reduce *Hp* infection. Conversely, we observe that an increase in GM-CSF availability reduces *Hp* burden in chronically infected mice. Thirdly, we show that GM-CSF, by acting on gastric epithelial cells, promotes the production of βdefensin3, which exhibits *Hp* bactericidal activities. Taken together, we demonstrate a key role of GM-CSF, most probably originating from Th17 cells, in the vaccine-induced reduction of *Hp* infection.

Keywords: Helicobacter pylori, vaccine, Th17 response, GM-CSF, antimicrobial peptide, defensin

1. Introduction

Helicobacter pylori (*Hp*) is one of the most common chronic bacterial infections of the human stomach mucosa [1]. This infection is acquired commonly during childhood and persists lifelong if not treated. The transmission of the infection is not fully understood but lots of evidence prone gastro-oral, oral-oral or fecal-oral contamination routes, especially in context of intra-familial clusters or mother to child transmission [2]. Although the majority of cases remain asymptomatic for decades, *Hp* infection is associated with increased occurrences of peptic ulcers, and more seriously with the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [3, 4]. Currently, a combination of antimicrobials and antisecretory drugs [5, 6] is considered the best way to eradicate *Hp* infection. However, its high global prevalence of approximately 50% [7] is associated with the rapid emergence of antibiotic resistance and the lack of specificity of the available therapies suggest the eradication of this bacterial infection is an important public health concern. Because *Hp* displays many immune evasion strategies to persist in the mucus layer of the stomach mucosa [8], it is of clinical interest to better characterize immune responses elicited upon infection and further improve therapeutic protocols.

Currently, alternative prophylactic and therapeutic vaccines have been conceptualized. Indeed, as vaccination is specific and does not trigger resistance, this therapeutic strategy has clinical merit and as such, should be improved. To be efficient, a vaccine requires the selection of immunogenic and protective antigens mixed with appropriate adjuvants. Antigen selection requires extensive knowledge of *Hp* bacterium as well as an understanding of the immune responses involved in its clearance. First, oral administration of a bacterial lysate plus cholera toxin (CT) in mice, conferred protection against *Helicobacter felis* (a close relative of *Hp*) [9]. Then, several

protective antigens have been characterized, including urease, a protein expressed at the cell surface of all *Hp* strains [10] and is now considered a promising protective antigen candidate [11, 12].

In humans, a phase III clinical trial evaluated that a urease-based vaccine protects 71.8% of children from the acquisition of *Hp* infection [13]. Unfortunately, this protection was not stable, and its efficacy decreased to 55.8% after one year. Although this study clearly indicates that the urease-based vaccine is protective in humans, major efforts are still needed to increase its protective effect and induce long-term protective immunity.

In order to improve vaccine-induced protection, understanding the immune-protective mechanisms involved in conferring *Hp* immunity is essential to select the best vaccine candidate. Key studies have demonstrated that $CD4^+$ Th cells (Th1, Th2 and Th17 cells) confer protection against *Hp* [14-17]. Th17 cells produce a number of cytokines, such as IL-17 and IL-22, which are key players in the mediation of adaptive immune responses against *Hp* infection, as well as in the vaccine-induced clearance of *Hp* [17-19].

IL-22, in response to *Hp* infection, triggers the production of anti-microbial peptides (AMPs) such as regenerating islet-derived protein 3-beta (RegIIIβ) by gastric epithelial cells. RegIIIβ is one of the key molecules involved in vaccine-induced reduction of *Hp* colonization in mice [20]. Indeed, AMPs are an important part of the innate immune response against *Hp* by protecting the gastrointestinal mucosa from pathogen invasion. Apart from RegIIIβ, βdefensins and Lipocalin2 also play a pivotal role upon *Hp* infection and alteration of the gut microbiota [21-23]. These small proteins are efficient gram-positive and negative bacteria killers and are inducible after exposure to

lipopolysaccharides (LPS) and/or pro-inflammatory cytokines [24], suggesting their possible role in vaccine-induced *Hp* clearance.

In parallel, IL-17 plays a major role in stimulating granulopoiesis, mobilization of granulocytes into sites of inflammation, and stimulating fibroblasts, endothelial cells, macrophages, and epithelial cells to produce multiple proinflammatory mediators, leading to the vaccine-induced reduction of *Hp* infection [16, 17]. Recently, a new Th cell subset was identified. This subset, Th1/Th17 cells, produce IL-17, interferon gamma (IFNγ) and GM-CSF [25]. Th cell differentiation into the Th1/Th17 subset is mediated, in part, by IL-23 [26, 27], a cytokine secreted by dendritic cells (DCs) and macrophages during *Hp* infection [28]. Since their identification, several studies demonstrated that these Th1/Th17 cells, also named pathogenic Th17 cells, are involved in the pathogenesis of inflammatory diseases including autoimmune encephalomyelitis, multiple sclerosis, and colitis [27, 29]. One of pathogenic Th17 cells product, GM-CSF, is a key factor known to play an important role in gut homeostasis [30-32]. Indeed, GM-CSF is secreted by many other cell types such as epithelial cells [33] and is a key factor in sustaining and promoting innate and adaptive mucosal immune responses [34, 35].

The objective of this study was to probe for a role of GM-CSF in the vaccine-induced reduction of *Hp* infection. We observed that the inhibition of the biological activities of GM-CSF jeopardizes the vaccine-induced reduction of *Hp* infection. We detected GM-CSF-producing pathogenic Th17 cells in mice stomach and show that GM-CSF stimulated gastric epithelial cells produce βdefensin3, which has the capacity to kill *Hp*.

2. Materials and Methods

2.1. Mice

Female BALB/c OlaHsd (Balb/c) mice (6 to 8 weeks old) were purchased from Envigo (Ad Horst, Netherlands). BALB/c GM-CSFR-deficient (GM-CSFRko) mice were generously provided by Prof. Angel Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia). This study was approved by the State of Vaud Veterinary Office (authorization no. 836.11/2). Mice were bred under specific-pathogen-free conditions in our animal facility.

2.2. H. pylori infection

Helicobacter pylori P49 (*Hp*), a human clinical isolate adapted to mice, was grown in brain heart infusion (BHI, Becton Dickinson, Heidelberg, Germany) supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaillé, France) under microaerophilic conditions for 36 hours. Adult mice were infected twice with 5 x 10^8 *Hp* bacteria. Bacteria were administered by oral gavage in 200 µl of BHI at a 2-days interval. The control group received 200 µl of BHI.

2.3. Assessment of H. pylori colonization

Quantification of *Hp* CFU was used to assess infection status [36]. CFU were determined immediately after stomach ressection. One-third of the stomach was immersed in 200 µL of selective culture medium (10µg/ml Vancomycin (Sigma, St. Louis, MO), 20 µg/ml Bacitracin (Sigma), 5 µg/ml Amphotericin B (Sigma), 0.3 µg/ml Polymyxin B (Sigma), 1.07 µg/ml Nalidixic acid (Sigma) in BHI) and homogenized with a fitted plastic pestle in a sterile Eppendorf tube (Vaudaux-Eppendorf, Basel, Switzerland). Serial 10-fold dilutions of the homogenate were then plated on Helicobacter plates (Becton Dickinson). Plates were incubated for 3–4 days in microaerophilic conditions, after which the CFU were counted. Identification of *Hp* was based on the appearance of colonies on plates and gram staining. Results were expressed as number of CFU per one-third of stomach.

A rapid biochemical test based on the urea reaction (named Rapid Urease Test, RUT) (Cleartest Histo *HP*; servoprax GmbH, Wesel, Germany) was also used to assess infection status. Briefly, stomachs were resected, and one-third of the stomach was immersed in 500 µL of the supplier's suspension and incubated at 37 °C for two hours. Specimens were centrifuged, and the supernatant used for spectrophotometric quantification at an optical density of 550 nm.

2.4. GM-CSF neutralization

BALB/c mice were injected intraperitoneally on days -1 and 2 post *Hp* infection with 125 µg of anti-mouse GM-CSF monoclonal antibody (MP1-22E9, Biolegend). Monoclonal Rat IgG2a (BioXcell, West Lebanon, NH) was used as an isotype-matched control antibody.

2.5. Vaccination

Mice were immunized intranasally four times at one-week intervals with 30 µg of recombinant *Hp* urease (kindly provided by Sanofi-Pasteur, Lyon, France) combined with 5 µg of cholera toxin (CT) (Calbiochem, Lucerne, Switzerland). Control mice were administered only with cholera toxin [17].

2.6. Hydrodynamic gene delivery (HGD) injection

Anesthetized mice received one injection of either 20 or 50 µg of IL-22, GM-CSF and Control expression plasmid (respectively MR225471, MC208342 and PS100001 OriGene Technologies, Rockville, US). Injections were performed intravenously in 2 ml of Ringer “Bichsel” solution (Ringer-Lösung “Bichsel”, Laboratorium Dr. G. Bichsel AG, Unterseen, Switzerland) as described by Liu F et al. [37].

2.7. Flow cytometry

Isolation of gastric immune cells was performed as described elsewhere [38]. Isolated gastric immune cells were resuspended in FACS buffer. All cells were counted using cellometer® Auto T4 cell counter (Nexcelom Bioscience, Lawrence, MA) prior to activation.

Gastric immune cells were first activated for five hours at 37 °C in RPMI 1640 supplemented with 10% FBS, 1% Penicillin/Streptomycin (BioConcept), 1 mM sodium pyruvate (Sigma), 0.05 mM 2-Mercaptoethanol (Gibco) and 10 mM HEPES (BioConcept) in the presence of Brefeldin A (BioLegend Switzerland), Phorbol 12-myristate 13-acetate (Sigma), and Ionomycin calcium salt (Sigma). The cells were then stained extracellularly as described below.

For extracellular staining, cells were incubated with anti-mouse CD16/CD32 (Clone 2.4G2; Becton Dickinson) for 20 minutes on ice. Then, cells were stained for viability assessment with LIVE/DEAD™ fixable Aqua Dead cell stain kit (Invitrogen, Life technology corporation, OR) for 20 minutes on ice. Cells were then stained with anti-mouse antibodies CD4-PE/Cy7 (Clone GK1.5, BioLegend), CD3-PerCP/Cy5.5 (Clone 17A2, BioLegend), CD45-AF700 (Clone 30-F11, BioLegend) for 20 minutes on ice. Cells were then fixed using BD Cytofix/Cytoperm™ solution (BD Biosciences, Basel Switzerland) for 20 minutes on ice.

For intracellular staining, cells were stained with anti-mouse antibodies IL-17A-FITC (Clone TC11-18H10.1, BioLegend), GM-CSF-APC (Clone MP1-22E9, Biolegend) and resuspended in FACS buffer.

Cells were acquired using Attune NxT Flow Cytometer (ThermoFisher, Waltham, MA). Samples were analyzed with FlowJo V.10 software (FLOWJO LLC, Ashland, OR).

2.8. Quantitative PCR (qPCR)

RNA extraction was performed on stomach tissue and AKP cells using Trizol (Invitrogen Corporation, Carlsbad, CA) and an RNeasy mini kit (Qiagen, Valencia, CA). RNA (300 ng) was reverse transcribed into cDNA using a PrimeScript reverse transcriptase (RT) reagent kit (TaKaRa Bio Inc., Otsu, Japan). qPCR amplification was performed on a QuantStudio 6 Flex Real-Time PCR Systems apparatus (ThermoFisher), using 96 or 384-well plates (ThermoFisher). The qPCR was performed in duplicate with FASTSTART SYBR GREEN MASTER (Roche). The primers used were as follows: GAPDH (5'-GCTAAGCAGTTGGTGGTGCA-3′ and 5′-TCACCACCATGGAGAAGGC-3′, Microsynth AG, Balgach, Switzerland), Lipocalin2 (QT00113407, Qiagen), IL-17 (5′-GCTCCAGAAGGCCCTCAGA-3′ and 5′-AGCTTTCCCTCCGCATTGA-3′, Microsynth AG), βdefensin3 (QT00265517, Qiagen), RegIIIβ (QT00239302, Qiagen), RegIIIγ (QT00147455, Qiagen), Csf2 (QT00251286, Qiagen), GM-CSFRb (5′-TGTTCCAGGATGGAGGTAAA-3′ and 5′-CCCACACTGCACATCCATAG-3′, Microsynth AG), IL-22R1 (5′-AAGCGTAGGGGTTGAAAGGT-3′ and 5′-CTACGTGTGCCGAGTGAAGA-3′, Microsynth AG), GM-CSFRa (5′-TGCGGGGCCAGTGCGGTTCCT-3′ and 5′-CAGTGCTTCATCCTCGTGTCG-3′, Microsynth AG).

Quantification of input cDNA from the unknown samples was performed by including a standard curve as described elsewhere [36]. Briefly, To construct the standard DNA curve, amplicons generated by RT-PCR using the primers described above were purified on silica columns (QiAquick PCR purification, Qiagen) and cloned into pGEM-Teasy (Promega Corp, Madison, WI). Ligated fragments were transformed into DH5-competent cells and plasmid DNA was prepared using silica cartridges (Qiagen). The sequence of the cloned amplicons was determined by cycle sequencing. DNA plasmid concentrations were measured by optical density spectrophotometry and the

corresponding copy numbers calculated using the following equation: 1 µg 1000-bp DNA = 9.1 x 10^{11} molecules. Serial 10-fold dilutions of plasmids ranging from 10^7 to 10^2 DNA copies were used as standard curve in each PCR run. The calculated number of mRNA copies for the gene of interest was then normalized per million of mRNA copies obtained for GAPDH, which was used as a house keeping gene.

2.9. Generation of AKP cells

The antral glands of the stomach of Apcfl/fl ;KrasLsl-G12D;Tp53fl/fl ;villin-CreERT2 mice [39] were isolated [40] and infected with an adenovirus encoding Cre recombinase [41]. Limited dilutions were performed and an AKP cell clone was isolated. AKP cells expressing markers characteristic of gastric epithelial cells were determined by PCR, see 'PCR for AKP characterization' section. In addition, AKP cells expressing mRNA encoding IL-22 and GM-CSF receptors were confirmed by qPCR, see 'Quantitative PCR (qPCR)' section.

2.10. PCR for AKP characterization

DNA of AKP cells was isolated using DNeasy Blood and Tissue kit (Qiagen). The primers used were as follows: Villin (5′-CAGTGGGGATGAGAGGGAGA-3′ and 5′-CCTGCTTCACCACGATGATA-3′, Microsynth AG) Gastrin (5′-TGTGGACAAGATGCCTCGAC-3′ and 5′-TGGTCCCTGGTCCAGATGAT-3′, Microsynth AG) Somastotastin (5′-CTGCGACTAGACTGACCCAC-3′ and 5′-GAAACTGACGGAGTCTGGGG-3′, Microsynth AG) H+/K+/ATPase (5′-GTTCCAGTGGTGGCTGGT-3′ and 5′-GCTGATAGTGGAGAGATG-3′, Microsynth AG) Lrg5 (5′-TGCCATCTGCTTACCAGTGTTGT-3′ and 5′-ATTCCGTCTTCCCACCACGC-3′, Microsynth AG) Olfm4 (5′-GCCACTTTCCAATTTCAC-3′ and 5′-GAGCCTCTTCTCATACAC-3′, Microsynth AG) Gif (5′-TGAATCCTCGGCCTTCTATG-3′ and 5′-CAGTTAAAGTTGGTGGCACTT-3′,

Microsynth AG) Apc (5′-TGAGGAATTTGTCTTGGCGAG-3′ and 5′-GCACTTCCCATGGCAATCATT-3′, Microsynth AG). DNA samples were amplified by PCR using a mix containing the primers cited above, PCR Rxn buffer (inVitrogen, CA), dNTP mix (Promega, WI USA), MgCl2 (inVitrogen), Taq DNA polymerase recombinant (inVitrogen) (annealing temperature 55 °C, 35 cycles). PCRs were loaded on a 2% agarose gel.

2.11. AKP cells co-culture with H. pylori

AKP cells were cultured in collagen type I solution from rat tail (Sigma) pre-coated T flasks (Falcon) in Minigut medium composed of DMEM/F12 (Gibco), 1% Penicillin/streptomycin, 1% N-2 supplement (Gibco), 1% B-27 supplement (Gibco), 10% FBS in a 37°C - 5% CO2 incubator. Then, 4 x 10^5 cells were added on a collagen pre-coated filter of a transwell plate (costar, 0.4µm) in the presence of Minigut medium supplemented with FBS. Twenty-four hours later, cells were basolaterally stimulated with 100 ng/ml of IL-22 or GM-CSF (Peprotech). Twenty-four hours later, 4 x 10^5 cells of 36h-old *Hp*49 were added apically to each transwell and incubated 12 or 24h in a 37°C in a 5% CO2 incubator. Bacterial killing was assessed by CFU counting according to the 'Assessment of *Hp* colonization' section.

2.12. Immunohistochemistry

One-third sections of mouse stomachs were recovered and fixed in 10% (v/v) buffered formalin for subsequent paraffin embedding and histological analysis. Paraffin-embedded stomach tissue sections (4 mm) were decorated with rabbit anti-β-defensing 3 antibodies (Alpha Diagnostic International, San antonio, USA) followed by Dako EnVision®+, Peroxidase (Agilent Technologies, California, USA) and Dako, DAB+, substrate buffer and chromogen (Agilent Technologies). Finally, Harris hematoxylin was used as a nuclear counterstain.

2.13. Antimicrobial assay

To evaluate the antimicrobial activity of βdefensin3 on *Hp*, *Hp* was cultured as described in '*Hp* infection' section. Bacterial suspension at 8 x 10^6 CFU/ml was co-cultured in the presence of 50 µg/ml of recombinant mouse βdefensin3 protein (NBP2-35146, Novusbio, Colorado, USA) and incubated for one hour under microaerophilic conditions. Bacteria treated with PBS alone served as negative controls. At the end of the incubation period, CFU counting was performed to assess bacterial viability.

2.14. Statistical analysis

The distribution of the data was compared using Mann-Whitney tests using GraphPad software (GraphPad Software, San Diego, CA), with a P value of 0.05 being considered as the limit of significance.

3. Results

3.1. Pathogenic Th17 cells accumulate in the gastric mucosa during the vaccine-induced reduction of H. pylori infection

As it has been shown that the infection of vaccinated mice triggers massive urease-specific Th17 responses [17, 19], we determined whether these Th17 cells secrete GM-CSF. To this end, we immunized Balb/c mice with urease adjuvanted with cholera toxin (CT). After immunization and challenge with *Hp* (**Figure S 1 A**), gastric colonization was assessed by Rapid Urease Test (RUT), 7 days post infection. As expected, vaccinated mice were infected to a lesser extent than non-vaccinated mice, confirming the vaccine efficacy (**Figure 1 A**). This decreased *Hp* burden is concomitant with massive immune cell infiltration into the gastric mucosa (**Figure 1 B.1**). Among these infiltrating cells, $CD4^+$ T cells were particularly numerous (**Figure 1 B.2**). Interestingly, the expression of mRNA encoding IL-17 and GM-CSF are respectively two fold and forth fold increased in vaccinated infected mice compared to non-

vaccinated infected mice (**Figure 1 C**). Remarkably, flow cytometric analysis revealed that the stomach mucosa of vaccinated and *Hp* infected mice is infiltrated by pathogenic Th17 cells secreting IL-17 and GM-CSF (**Figure 1 D & Figure S 2**). Taken together, our results demonstrated that the vaccine-induced *Hp* reduction is associated with the gastric accumulation of pathogenic Th17 cells.

3.2. Inhibition of the biological activity of GM-CSF jeopardizes vaccination efficacy

Next, as GM-CSF is secreted by vaccine-primed Th17 cells, we evaluated whether the absence of GM-CSF is detrimental for vaccine efficacy. To assess the role of GM-CSF, we used two different approaches. As a first approach, vaccinated and non-vaccinated GM-CSF receptor knockout (GM-CSFRko) mice were challenged with *Hp*. In parallel, vaccinated and non-vaccinated wild type (WT) mice, challenged with *Hp*, were injected with neutralizing anti-GM-CSF monoclonal antibodies (αGM-CSF mAb) (**Figure S 1 A & B**). In non-immunized mice, the absence of GM-CSF biological activity had no impact on *Hp* infection (**Figure 2 A**). As expected, in GM-CSF sufficient mice, only 33% of vaccinated WT mice remained infected compared to non-vaccinated mice. However, the vaccine-induced reduction of *Hp* infection was jeopardized in GM-CSFRko mice as well as in WT mice injected with αGM-CSF mAb. Indeed, 67% and 60% of GM-CSFRko mice and αGM-CSF mAb-injected WT mice, respectively, remained colonized by *Hp* after vaccination (**Figure 2 A**). Consequently, these results showed that the vaccine efficacy is dependent on the biological activities of GM-CSF.

Knowing that AMPs produced by gastric epithelial cells are key molecules involved in the vaccine-induced reduction of *Hp* colonization, we evaluated whether the absence of GM-CSF lead to a reduction of AMP production. Gastric mRNA expression levels of Lipocalin2 and βdefensin3 were significantly increased in vaccinated WT mice as compared to non-vaccinated WT mice (**Figure 2 B.1 & B.2**). The mRNA expression

level of Lipocalin2 was also found to be significantly upregulated in vaccinated GM-CSFRko mice, but not in the αGM-CSF mAb treated mice, when compared against their non-vaccinated counterparts (**Figure 2 B.1**). Remarkably, we did not detect any significant difference in the mRNA expression level of βdefensin3 amongst the vaccinated and non-vaccinated mice with deficient GM-CSF biological activity. This result suggests that GM-CSF directly and/or indirectly promotes an increased expression of gastric βdefensin3 during the vaccine-induced reduction of *Hp* infection (**Figure 2 B.2**).

3.3. Therapeutic injection of GM-CSF decreases H. pylori infection burden

As deficiency in GM-CSF dampens *Hp* vaccine efficacy, we determined whether therapeutic administration of GM-CSF could decrease *Hp* infection burden. To this end, we performed hydrodynamic gene delivery (HGD) injection of plasmid DNA coding for GM-CSF or IL-22 to chronically *Hp* infected mice (**Figure S 1 C**). HGD is recognized as a simple method to induce, in the short term, a massive production of cytokines into the circulatory system [42, 43]. Twelve days post HGD, *Hp* colonization was assessed by RUT or colony forming unit (CFU) numeration. Moyat et al. already demonstrated that IL-22 is a key cytokine for vaccine-induced *Hp* clearance, as it stimulates AMP production by gastric epithelial cells [20]. Consequently, plasmid DNA coding for IL-22 was used as a positive control in this experimental setting. As expected, IL-22 HGD induced a decrease in *Hp* infection burden in chronically infected mice as compared to control mice (**Figure 3**). Remarkably, GM-CSF HGD decreased *Hp* infection as observed with IL-22 HGD (**Figure 3**). Consequently, like IL-22, therapeutic GM-CSF administration promotes the reduction of *Hp* burden.

3.4. Therapeutic GM-CSF administration induces gastric βdefensin3 expression

The absence of the biological activities of GM-CSF decreases the mRNA expression levels of gastric AMPs (**Figure 2 B.1 & B.2**). Therefore, we checked whether GM-CSF and IL-22 HGD increases the gastric expression levels of mRNA encoding AMPs. It is known that IL-22 up-regulates RegIIIβ expression by gastric epithelial cells [20]. As expected, we found that IL-22 HGD increases the gastric expression levels of mRNA encoding not only RegIIIβ, but also of RegIIIγ in chronically infected mice (**Figure 4 A**). Contrastingly, GM-CSF HGD did not increase the gastric expression levels of RegIIIβ nor RegIIIγ. However, GM-CSF HGD stimulated the production of mRNA encoding βdefensin3 in the gastric mucosa of *Hp* infected mice (**Figure 4 B**). Lastly, by performing immuno-chemistry, we detected an increased expression of βdefensin3 in the glands of the stomach of chronically infected mice injected with GM-CSF HGD as compared to IL-22 HGD or mice injected with an empty plasmid (**Figure 4 C**).

Taken together, our results show that the GM-CSF-induced reduction of *Hp* infection burden is associated with an increased βdefensin3 gastric expression.

3.5. GM-CSF stimulates gastric epithelial cells to produce AMPs, and to kill H. pylori

To determine whether GM-CSF has a direct effect on gastric epithelial cells in promoting βdefensin3 expression and *Hp* killing, we performed a series of *in vitro* experiments. We have developed a new *in vitro* co-culture assay using AKP cells, a mouse gastric epithelial cell line, and *Hp* bacteria. Polarized AKP cell monolayers were cultivated on filters allowing for basolateral activation with GM-CSF or IL-22 and apical infection with *Hp*. Epithelial cell induced *Hp* killing was then evaluated by CFU counting.

The AKP cells expressed different molecular markers characteristic of antral gastric epithelial cells, as they expressed mRNA encoding gastrin, somatostatin, Olfactomedin

4 (Olfm4), leucine rich repeat containing g protein-coupled receptor 5 (lrg5), but not the mRNA encoding villin, H+/K+ATPase and gastric intrinsic factor (Gif) (data not shown). AKP cells also express mRNA encoding IL-22R1α chain and the GM-CSFRα and βc chains (**data not shown**).

Remarkably, in line with our *in vivo* observations, we confirmed that AKP cells stimulated by IL-22 induce *Hp* killing. Similarly, AKP cells stimulated by GM-CSF also promote *Hp* killing (**Figure 5 A**). Moreover, we documented the increased expression of mRNA encoding βdefensin3 in AKP cells upon GM-CSF stimulation for 12 hours and 24 hours (**Figure 5 B**). These results are reminiscent of our *in vivo* results showing that GM-CSF induces the production of βdefensin3 by gastric epithelial cells (**Figure 2 B & Figure 4**). To firmly establish a link between βdefensin3 expression and *Hp* killing, *Hp* was co-incubated with 50 µg/µl of recombinant mouse βdefensin3 for one hour and plated to enumerate CFU. As expected, we observed that βdefensin3 displays direct anti-microbial activities against *Hp*, leading to the conclusion that GM-CSF-induced βdefensin3 may play a key role in the decrease of *Hp* infection burden (**Figure 5 C**). Taken together, our results highlight that GM-CSF can directly stimulate gastric epithelial cells to trigger βdefensin3 production. In addition, we demonstrated that βdefensin3 displays AMP properties against *Hp* and is therefore is involved in the vaccine-induced reduction of *Hp* infection.

4. Discussion

Improving *Hp* vaccine and/or treatment efficacy relies on the elucidation of immune mechanisms involved in the vaccine-induced reduction of *Hp* infection. The identification of key molecules modulating both innate and adaptive immune responses toward the promotion of bactericidal *Hp* activities will undoubtedly improve the development of new strategies to treat *Hp* infection.

In this study, we interrogated whether GM-CSF plays a role in the vaccine-induced reduction of *Hp* infection. Firstly, we clearly established that GM-CSF+ IL-17+ pathogenic Th17 cells accumulate in the stomach mucosa during the vaccine-induced reduction of *Hp* infection. Secondly, we provided evidence that vaccinated GM-CSF deficient mice only modestly reduce *Hp* infection. Conversely, we observed that increased availability of GM-CSF reduces *Hp* burden in chronically infected mice. Thirdly, we showed that GM-CSF, by acting on gastric epithelial cells, promotes the production of βdefensin3, which exhibits *Hp* bactericidal activities.

In order to identify new therapies to reduce *Hp* burden, we focused our research on molecules produced by CD4+ T cells that accumulate in the stomach mucosa during the vaccine-induced reduction of *Hp* infection. Indeed, it is well described that vaccine-primed *Hp* specific CD4+ T cells, especially those belonging to the Th17 subsets, are major contributors to vaccine efficacy [16, 17, 44]. In addition to IL-17, Th17 cells secrete GM-CSF (**Figure 1**). These GM-CSF producing Th17 cells have already been identified in the literature as pathogenic Th17 cells. Interestingly enough, although these cells have been mainly characterized in the context of autoimmunity and inflammatory diseases [25, 45], our study is the first to show that pathogenic Th17 are recruited into the stomach mucosa of vaccinated and infected mice during the vaccine-induced reduction of *Hp* infection (**Figure 1 D**). This accumulation of pathogenic Th17 was accompanied by an increased expression of mRNA encoding IL-17 and GM-CSF in the gastric mucosa (**Figure 1 C**). Remarkably, by decreasing the biological activity of GM-CSF in vaccinated mice, the vaccine efficacy largely declines and no longer reduces *Hp* infection burden (**Figure 2 A**). The lower vaccine efficacy was found to be associated with the decreased expression of mRNA encoding AMPs, such as βdefensin3 and Lipocalin2, in the gastric mucosa of vaccinated and infected GM-CSF

deficient mice (**Figure 2 B**). Inversely, we demonstrated that without vaccination, the increased bioavailability of GM-CSF in chronically infected mice leads to increased expression of mRNA encoding βdefensin3 (**Figure 4**) and thus the reduction of *Hp* burden (**Figure 3**). These results clearly demonstrated that GM-CSF, most probably originating from the infiltration of pathogenic Th17 cells, is a key molecule capable of reducing *Hp* burden. Until now, the anti-bacterial effect of GM-CSF has been mainly described in the lung and was mostly attributed to a direct effect of GM-CSF on macrophages, dendritic cells and/or neutrophils [46]. In addition, several studies have probed for roles of a lung-protective effect of GM-CSF by a direct impact on alveolar epithelial cells leading to improvement of epithelial repair processes [47]. Similarly, we show that the anti-bacterial effect of GM-CSF is also dependent on the direct activation of epithelial cells (**Figure 5**). Indeed, our *in vitro* experiments provide evidence that GM-CSF-stimulated gastric epithelial cells upregulate the expression of mRNA encoding βdefensin3, which demonstrate *Hp* bactericidal activity (**Figure 5**). Altogether, our study reveals for the first time that GM-CSF mobilizes the bactericidal activities of gastric epithelial cells by increasing the production of βdefensin3. In humans, the murine βdefensin3 ortholog is β-defensin2. Interestingly, several studies have revealed that human β-defensin2 also displays antimicrobial *Hp* properties [48, 49], supporting the notion that our pre-clinical data may be translatable to humans.

Strongly rooted in our observations from this study, GM-CSF could be a potential alternative to treat *Hp* infection alone or in combination with other drugs to avoid antibiotic resistance and/or increase therapeutic vaccine efficacy. Indeed, recombinant human GM-CSF (sargramostim) is FDA-approved for multiple cancer immunotherapies [50] and has been envisioned as a therapeutic strategy against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [51]. In addition,

as GM-CSF triggers βdefensin3 secretion, this AMP alone or in combination with RegIIIβ [20] could also be an alternative treatment for *Hp* clearance. Indeed, several synthetic AMP analogs have been synthetized and have been shown to efficiently kill different type of bacteria [21]. This suggests that AMP analogs could be envisioned to alleviate or ameliorate antibiotic therapies for *Hp* eradication.

Our results show that pathogenic Th17 cells are involved in the vaccine-induced reduction of *Hp* infection and are reminiscent of the results of Annemann M et al showing that pathogenic Th17 cells are protective against *citrobacter rodentium* infection [52]. In the context of vaccine development, it is important to consider this information to select an adjuvant that promotes Th17 responses [53]. Bacterial components, including muramyl dipeptide (MDP), lipopolysaccharide (LPS), and CpG, are known to augment Th17 responses [54-57] and are very good candidates to be considered in vaccine formulation. The differentiation of Th17 cells into pathogenic Th17 cells has been recently shown to occur in inflamed tissue where Th17 cells are recruited and the differentiation into pathogenic Th17 cells can be mediated by the local production of serum amyloid A (SAA) proteins [58]. Interestingly, SSA proteins are known to be upregulated in the stomach mucosa of Helicobacter infected mice [59]. Therefore, it can be hypothesized that the vaccine–induced *Hp* specific Th17 cells, during their homing into the gastric mucosa of *Hp* infected hosts, will differentiate into pathogenic Th17 cells and will efficiently reduce *Hp* infection burden.

In this study, we demonstrate for the first time the role of GM-CSF, most probably originating from pathogenic Th17 cells, in the vaccine-induced reduction of *Hp* infection. Mechanistically, we show that GM-CSF directly acts on gastric epithelial cells to induce the production of βdefensin3 and to kill *Hp*. Altogether, these findings

highlight several potential alternatives and/or combination therapies to eradicate *Hp* infection in humans.

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Figure legend

Figure 1. Upregulation of GM-CSF gastric expression during the vaccine-induced reduction of H. pylori infection. Eight-week-old Balb/c mice were either vaccinated with urease and cholera toxin or treated with cholera toxin alone as a control. Two weeks after vaccination, mice were challenged with *Hp*. Immune cells infiltrating the stomach were analyzed by flow cytometry and qPCR respectively on days 7 and 6 post infection. **(A)** At sacrifice, the stomachs were recovered and *Hp* colonization was assessed using RUT (optical density (OD) at 550 nm). **(B)** Absolute number of cells (B.1) and frequency of $CD4^+$ T cells ($CD45^+CD3^+CD4^+$) (B.2) in the stomach of mice 7 days post infection. **(C)** Gastric mucosal expression of mRNA encoding csf2 and il17. **(D)** Relative and absolute number of gastric pathogenic Th17 cells ($CD45^+CD3^+CD4^+IL\text{-}17^+GM\text{-}CSF^+$) on day 7 post *Hp* infection. Each dot represents one mouse. * $P<0.05$, *** $P<0.001$ (Mann-Whitney test). Bar graphs show the mean ± SEM.

Figure 2. GM-CSF deficiency jeopardizes the efficacy of the vaccine-induced reduction of H. pylori infection. Eight-week-old WT or GM-CSFRko Balb/c mice were either vaccinated with urease and cholera toxin or treated with cholera toxin alone as a control, and two weeks later infected with *Hp*. WT Balb/c mice received either αGM-CSF mAb or its isotype control on days -1 and 2 post *Hp* infection. Mice were sacrificed 7 days post *Hp* infection. **(A)** At sacrifice, the stomachs were recovered. *Hp* colonization was assessed by RUT and results are expressed in percentage of *Hp* infected mice. Each group contains 5 to 6 mice. **(B)** Gastric mucosal expression of the mRNA encoding lipocalin2 (B.1) and DEFB3 (βdefensin3) (B.2). Each dot represents one mouse. ns, not significant; * $P<0.05$ (Mann-Whitney test). Bar graphs show the mean ± SEM.

Figure 3. Therapeutic administration of GM-CSF reduces H. pylori colonization. Eight-week-old Balb/c mice were infected with *Hp*. Twenty-eight days later; mice received hydrodynamic gene delivery (HGD) injection of 20µg plasmid encoding IL-22 (pIL-22), GM-CSF (pGM-CSF) or an empty plasmid (pControl). Mice were sacrificed 12 days post injection. *Hp* colonization was assessed by RUT and CFU. Graphs are from two different experiments. Each symbol represents one mouse; statistics are related to the control condition. ns, not significant; ** P<0.01; **** P<0.0001 (Mann-Whitney test). Bar graphs show the mean ± SEM.

Figure 4. Therapeutic administration of GM-CSF upregulates gastric βdefensin3 expression. Eight-week-old Balb/c mice were infected with *Hp*. Twenty-eight days later; mice received hydrodynamic gene delivery (HGD) injection of 20µg plasmid encoding IL-22 (pIL-22), GM-CSF (pGM-CSF) or an empty plasmid (pControl). **(A & B)** Gastric mucosal expression of mRNA encoding reg3b (RegIIIβ), reg3g (RegIIIγ) and DEFB3 (βdefensin3), 12 days post HGD injection. **(C)** Detection of βdefensin3 by immunohistochemical staining on day 7 post *Hp* infection. Stomach tissue sections of mice were decorated with anti-βdefensing3 Rabbit antibodies followed by biotinylated goat anti-rabbit and system-HRP for detection. Pictures are representative of data obtained from three individual mice per group. Bars: 50 µm (left panel) or 100 µm (right panel). Each dot represents one mouse. ns, not significant; * P<0.05 (Mann-Whitney test). Bar graphs show the mean ± SEM.

Figure 5. βdefensin3 displays antimicrobial properties against H. pylori *in vitro*. AKP cells were activated for 24 hours with IL-22 or GM-CSF before co-culture with *Hp*. **(A)** *Hp* killing was assessed by CFU 24 hours after beginning the co-culture. Results from 3 different experiments **(B)** GM-CSF stimulated AKP cell expression of mRNA encoding lipocalin2 and DEFB3 (βdefensin3) 12 or 24 hours post co-culture with *Hp*.

(C) 1 x 10^5 *Hp* bacteria were cultured in presence of 50 µg/µl of mouse βdefensin3 for one hour. *Hp* killing by βdefensin3 was assessed by CFU. Each dot represents one well. * P<0.05, ** P<0.01, **** P<0.0001 (Mann-Whitney test). Bar graphs show the mean ± SEM.

Figure S 1. Description of *in vivo* models. **(A)** Vaccination against *Hp* followed by two round of oral gavage with *Hp*. **(B)** Depletion of GM-CSF using αGM-CSF monoclonal antibody and *Hp* infection performed either in vaccinated or non-vaccinated mice. **(C)** HGD by intravenous hydrodynamic injection of pIL-22, pGM-CSF or pControl in chronically *Hp* infected mice.

Figure S 2. Gastric expression of GM-CSF by Th17 cells. Gating strategy used for flow cytometry to characterize the absolute cell number of Th17 producing GM-CSF cells in the stomach mucosa.

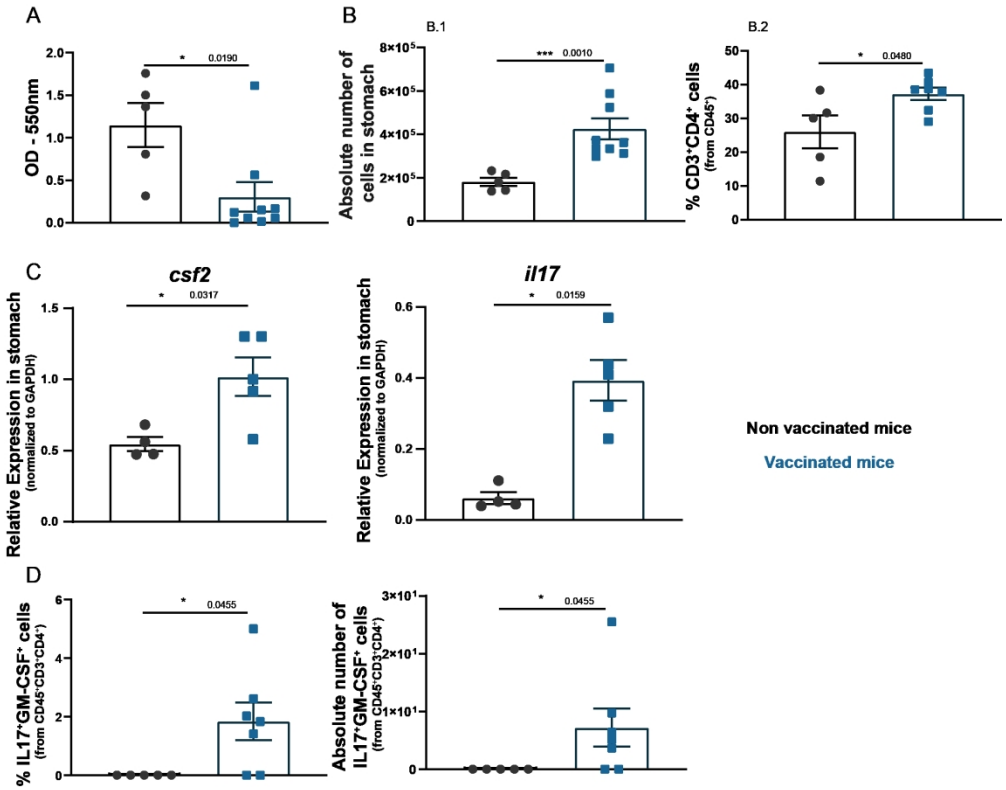

Figure 1. Upregulation of GM-CSF gastric expression during the vaccine-induced reduction of H. pylori infection. Eight-week-old Balb/c mice were either vaccinated with urease and cholera toxin or treated with cholera toxin alone as a control. Two weeks after vaccination, mice were challenged with Hp. Immune cells infiltrating the stomach were analyzed by flow cytometry and qPCR respectively on days 7 and 6 post infection. (A) At sacrifice, the stomachs were recovered and Hp colonization was assessed using RUT (optical density (OD) at 550 nm). (B) Absolute number of cells (B.1) and frequency of CD4+ T cells (CD45+CD3+CD4+) (B.2) in the stomach of mice 7 days post infection. (C) Gastric mucosal expression of mRNA encoding csf2 and il17. (D) Relative and absolute number of gastric pathogenic Th17 cells (CD45+CD3+CD4+IL-17+GM-CSF+) on day 7 post Hp infection. Each dot represents one mouse. * P<0.05, *** P<0.001 (Mann-Whitney test). Bar graphs show the mean ± SEM.

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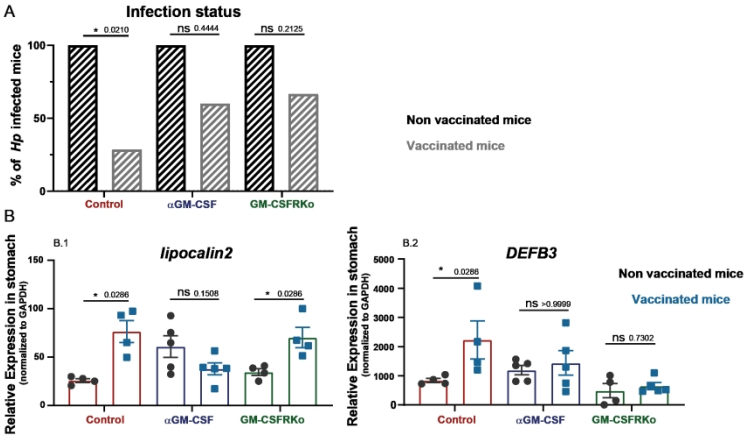

Figure 2. GM-CSF deficiency jeopardizes the efficacy of the vaccine-induced reduction of H. pylori infection. Eight-week-old WT or GM-CSFRko Balb/c mice were either vaccinated with urease and cholera toxin or treated with cholera toxin alone as a control, and two weeks later infected with Hp. WT Balb/c mice received either αGM-CSF mAb or its isotype control on days -1 and 2 post Hp infection. Mice were sacrificed 7 days post Hp infection. (A) At sacrifice, the stomachs were recovered. Hp colonization was assessed by RUT and results are expressed in percentage of Hp infected mice. Each group contains 5 to 6 mice. (B) Gastric mucosal expression of the mRNA encoding lipocalin2 (B.1) and DEFB3 (βdefensin3) (B.2). Each dot represents one mouse. ns, not significant; * P<0.05 (Mann-Whitney test). Bar graphs show the mean ± SEM.

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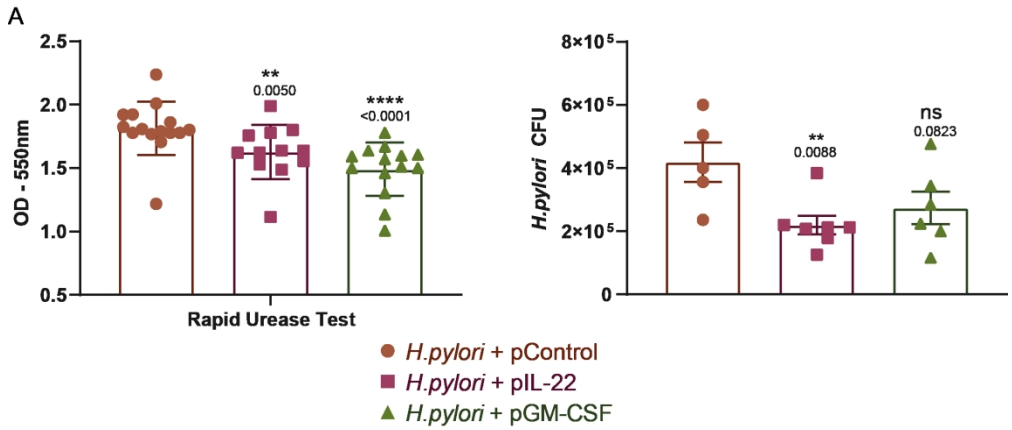

Figure 3. Therapeutic administration of GM-CSF reduces H. pylori colonization. Eight-week-old Balb/c mice were infected with Hp. Twenty-eight days later; mice received hydrodynamic gene delivery (HGD) injection of 20µg plasmid encoding IL-22 (pIL-22), GM-CSF (pGM-CSF) or an empty plasmid (pControl). Mice were sacrificed 12 days post injection. Hp colonization was assessed by RUT and CFU. Graphs are from two different experiments. Each symbol represents one mouse; statistics are related to the control condition. ns, not significant; ** P<0.01; **** P<0.0001 (Mann-Whitney test). Bar graphs show the mean ± SEM.

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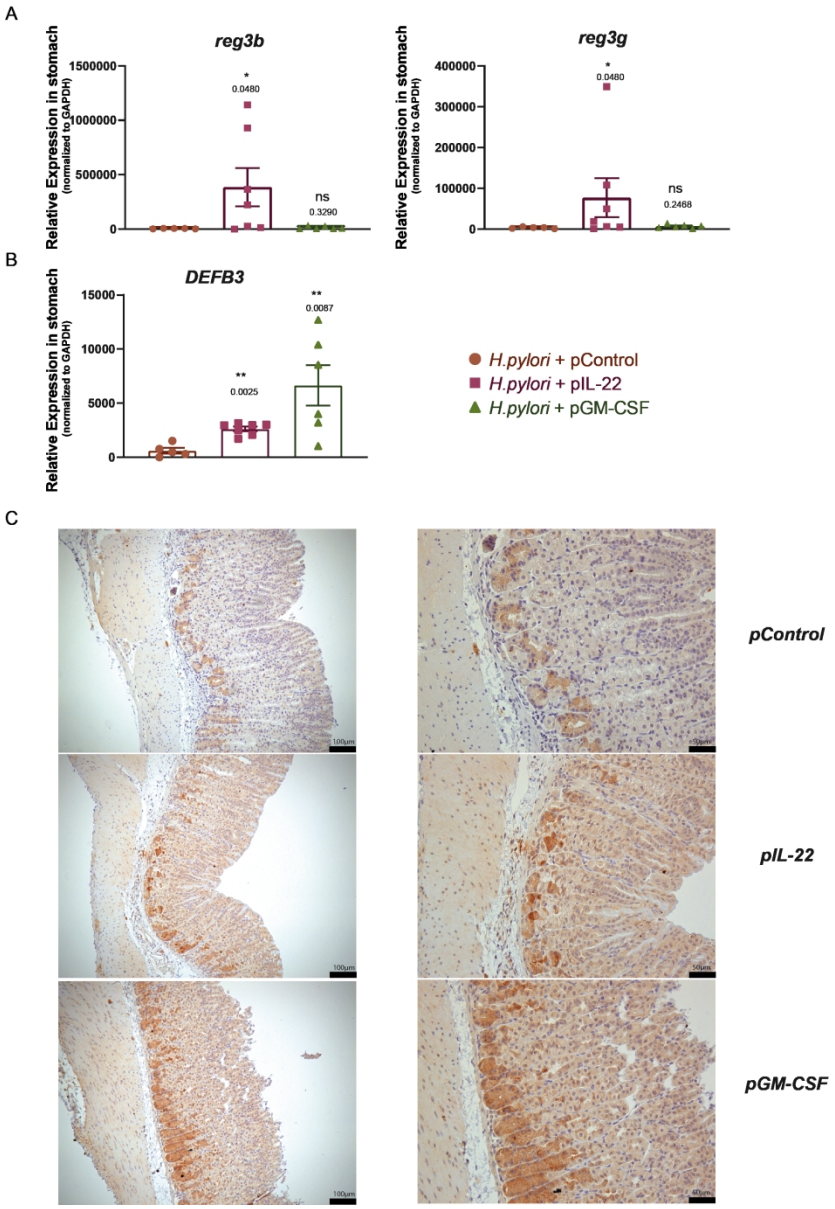

Figure 4. Therapeutic administration of GM-CSF upregulates gastric βdefensin3 expression. Eight-week-old Balb/c mice were infected with Hp. Twenty-eight days later; mice received hydrodynamic gene delivery (HGD) injection of 20μg plasmid encoding IL-22 (pIL-22), GM-CSF (pGM-CSF) or an empty plasmid (pControl). (A & B) Gastric mucosal expression of mRNA encoding reg3b (RegIIIβ), reg3g (RegIIIγ) and DEFB3 (βdefensin3), 12 days post HGD injection. (C) Detection of βdefensin3 by immunohistochemical staining on day 7 post Hp infection. Stomach tissue sections of mice were decorated with anti-βdefensing3 Rabbit antibodies followed by biotinylated goat anti-rabbit and system-HRP for detection. Pictures are representative of data obtained from three individual mice per group. Bars: 50 μm (left panel) or 100 μm (right panel). Each dot represents one mouse. ns, not significant; * P<0.05 (Mann-Whitney test). Bar graphs show the mean ± SEM.

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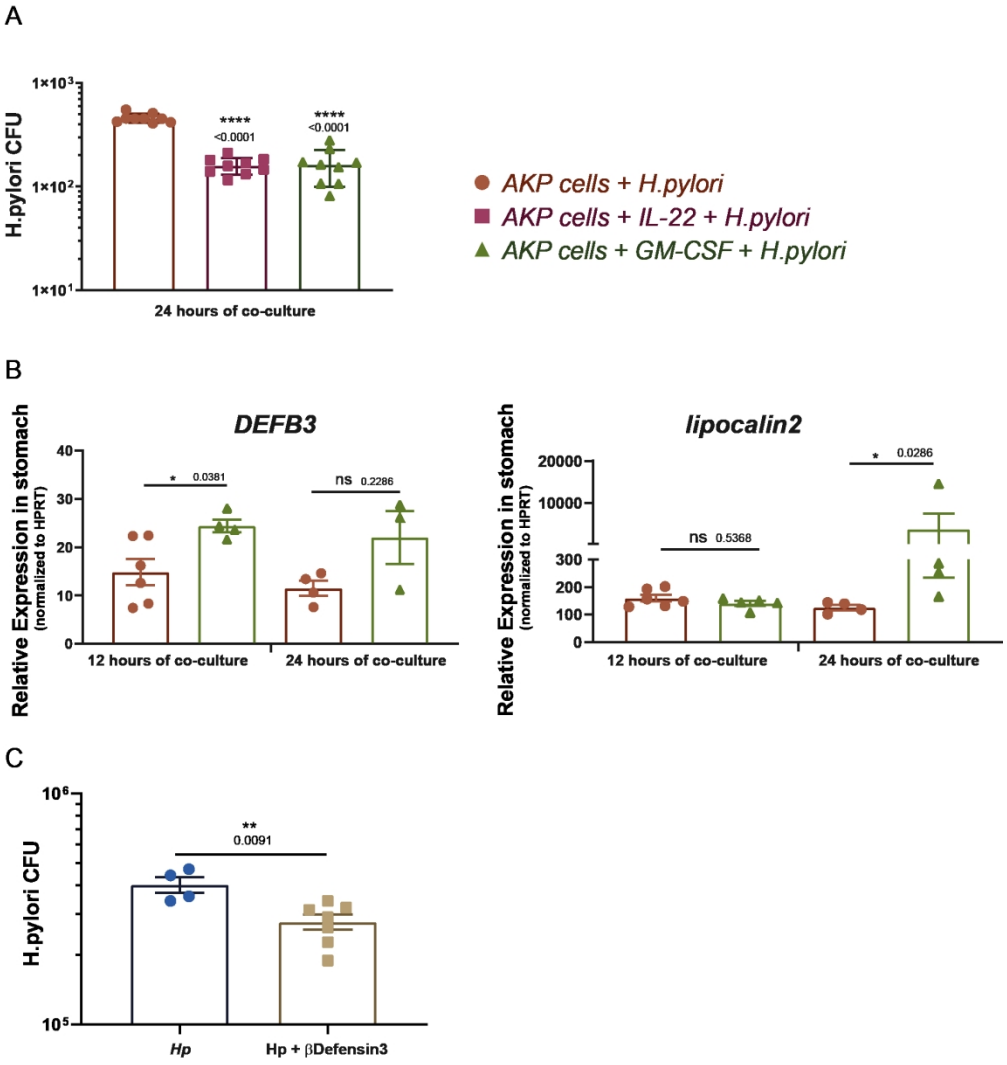

Figure 5. βdefensin3 displays antimicrobial properties against H. pylori in vitro. AKP cells were activated for 24 hours with IL-22 or GM-CSF before co-culture with Hp. (A) Hp killing was assessed by CFU 24 hours after beginning the co-culture. Results from 3 different experiments (B) GM-CSF stimulated AKP cell expression of mRNA encoding lipocalin2 and DEFB3 (βdefensin3) 12 or 24 hours post co-culture with Hp. (C) 1 x 105 Hp bacteria were cultured in presence of 50 µg/µl of mouse βdefensin3 for one hour. Hp killing by βdefensin3 was assessed by CFU. Each dot represents one well. * P<0.05, ** P<0.01, **** P<0.0001 (Mann-Whitney test). Bar graphs show the mean ± SEM.

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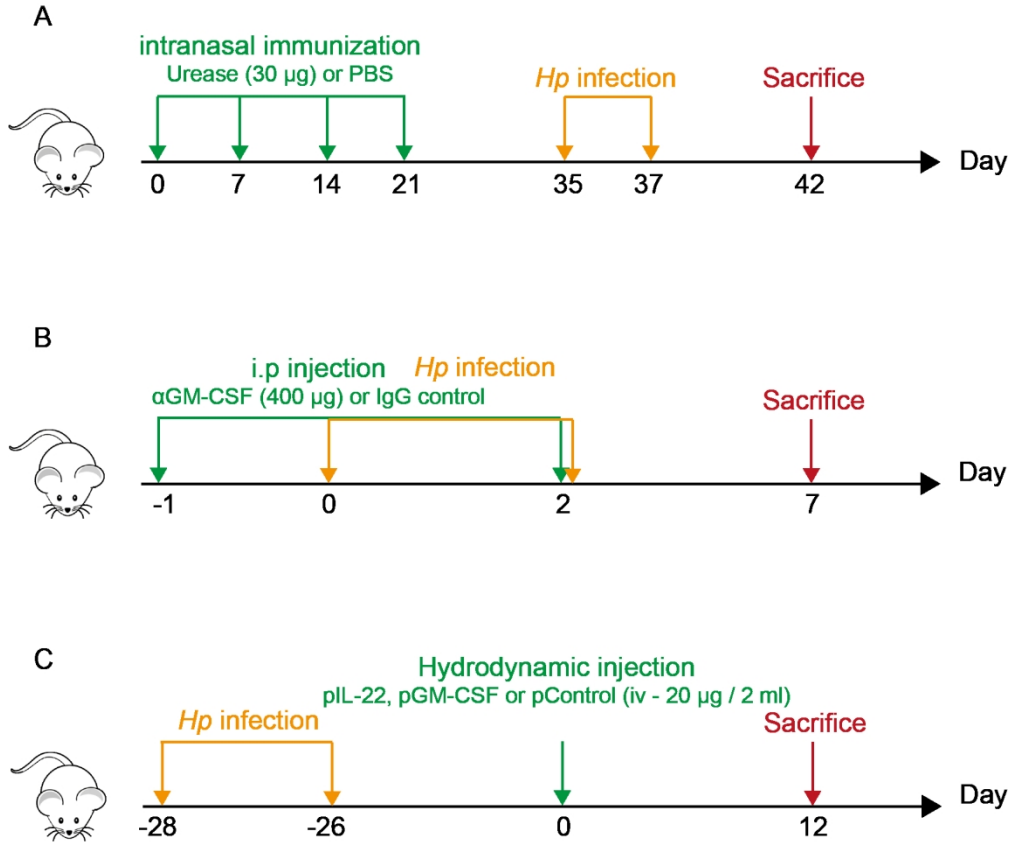

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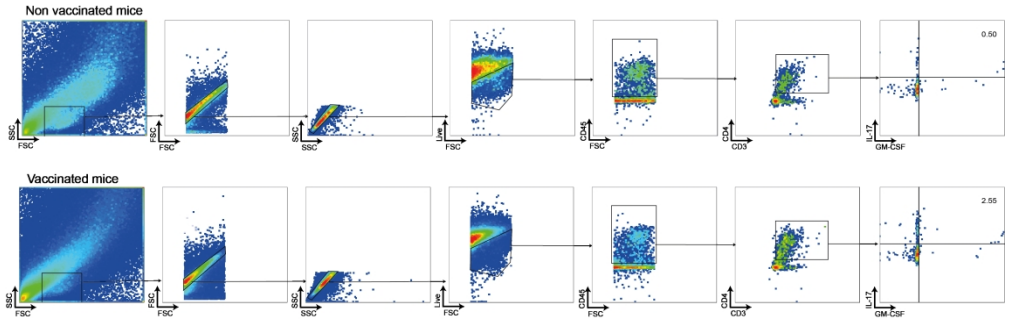

711x223mm (118 x 118 DPI)

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