

The role of IL-17A and IFN γ in vaccine-induced protection against *Helicobacter pylori*

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Cover Illustration: Adapted image of IL-17A staining in mouse stomach tissue.

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Kompendiet

♥ Till min älskade familj och käraste ♥

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Abstract

It is estimated that half the world's population is infected with *Helicobacter pylori* in the stomach. Chronic *H. pylori* infection can lead to peptic ulcer disease or gastric cancer, but only in a sub-population of infected individuals. Eradication of the bacteria with antibiotic treatment can be successful, but the emergence of antibiotic resistant strains of *H. pylori* is a problem in areas endemic with *H. pylori* infection. A mucosal vaccine would have the potential for boosting the immune response to *H. pylori*, preventing and thus reducing the prevalence of the infection. In spite of decades of intense research, no vaccine has yet been found to be effective against *H. pylori* infection in humans. The work in this thesis aimed to evaluate the impact of varying the adjuvant and route of mucosal vaccinations on the gastric immune response and protection against *H. pylori* infection in a mouse model. In particular, the role of cytokines induced by *H. pylori* infection was evaluated, with an objective to separate the protective and pathogenic immune response in the stomach. In the first part of the thesis, the adjuvant effect of a detoxified mucosal adjuvant based on the *E. coli* heat labile toxin LT, double mutant heat-labile toxin R192G/L211A (dmLT) addressed the differences if any, in immune responses and protection against *H. pylori* infection after sublingual (SL; under the tongue) and intragastric (IG) route of vaccination with *H. pylori* antigens and the prototype mucosal adjuvant cholera toxin (CT). And finally, using gene knockout mice and neutralizing antibodies, the impact of cytokines IFN γ and IL-17A on bacterial load and immune responses was addressed.

Sublingual vaccination with *H. pylori* antigens and dmLT as an adjuvant was efficient in reducing the bacterial load in the stomach of mice, similar to when using the potent adjuvant CT, which is highly toxic in humans. Compared to infected unvaccinated mice, sublingual vaccination with dmLT enhanced stomach IFN γ and IL-17A secretion and proliferative responses to *H. pylori* antigens in mesenteric lymph nodes and spleen. Furthermore, we could show that there was a tendency for the sublingual route to be more efficient than the intragastric route of vaccination in reducing the bacterial load in the stomach. And that the sublingual route of vaccination enhanced both IFN γ and IL-17A responses in the draining lymph nodes compared to unvaccinated mice. Studies on the role of individual cytokines in vaccine-induced responses revealed that after sublingual vaccination, IFN γ knockout (IFN $\gamma^{-/-}$) mice were protected against *H. pylori* infection and had elevated IL-17A production and lower inflammation scores in the stomach compared to vaccinated wild-type mice. Furthermore, neutralization of IL-17A in sublingually vaccinated IFN $\gamma^{-/-}$ mice abrogated protection against *H. pylori* infection. As IL-17A was found to be important for vaccine-induced protection, we next examined the mechanisms for induction and maintenance of IL-17A after sublingual vaccination by studying the role of cytokines IL-1 β and IL-23. Our results show that after sublingual vaccination, IL-23, but not IL-1 β , deficient mice were protected against *H. pylori* infection. Gastric IL-17A responses could not be induced after challenge in the absence of IL-1 β , but could be maintained in the absence of IL-23.

In summary, we report that dmLT can be considered as a strong candidate mucosal adjuvant for use in a *H. pylori* vaccine in humans particularly when administered via the sublingual route. Furthermore, we show that IL-17A might contribute to protective immune responses, while IFN γ may promote inflammation. The results presented in this thesis will facilitate the design and administration of a vaccine against *H. pylori* infection in humans.

Keywords: *Helicobacter pylori*, vaccination, CT, dmLT, Sublingual, IFN γ , IL-17A, IL-1 β and IL-23.

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- II. Defining the roles of IFN γ and IL-17A in inflammation and protection against *Helicobacter pylori* infection
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ABBREVIATIONS

ADP	Adenosine diphosphate
APC	Antigen presenting cells
CagA	Cytotoxin associated gene A
CagPAI	Cag Pathogenicity Island
cAMP	cyclic adenosine monophosphate
CFU	Colony forming units
CLN	Cervical lymph nodes
CT	Cholera Toxin
CTA	Cholera Toxin A subunit
CTB	Cholera Toxin B subunit
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dmLT	double mutant heat-labile toxin
HpaA	<i>H. pylori</i> adhesion A
IFN γ	Interferon gamma
IG	Intragastric
ILC	Innate lymphoid cells
IL	Interleukin
IN	Intranasal
i.p.	intraperitoneally
LT	Heat-labile toxin
LTA	Heat-labile toxin A subunit
LTB	Heat-labile toxin B subunit
LMIC	Low and middle income countries
MHC	Major Histocompatibility Complex
MLN	Mesenteric lymph nodes
NAP	Neutrophil Activating Protein
NLR	Nod-like receptor
Nod	Nucleotide-binding Oligomerization Domain
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
SL	Sublingual
TGF β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNF α	Tumor necrosis factor alpha
Treg	Regulatory T cell
VacA	Vacuolating cytotoxin A

Introduction

History and Epidemiology of *Helicobacter pylori* infection

Helicobacter pylori was first discovered by Warren and Marshall in 1983 when they regularly found spiral shaped bacteria in biopsies from patients with gastritis and peptic ulcer disease. Later, Marshall cultured the bacteria *ex-vivo* and, by ingesting the spiral shaped bacilli, proved that it is the major cause of chronic inflammation in the stomach [1]. This discovery gave new insights into the mechanisms of gastric disorder in subsets of infected individuals with gastritis, acid reflux disease and peptic ulcers, which could now be attributed to *H. pylori* infection. Since the discovery of *H. pylori*, the pathogenesis of associated inflammatory and malignant diseases have been intensively investigated.

Approximately half of the world's population is infected with *H. pylori* in the stomach. *H. pylori* infection is most prevalent in the low and middle income countries (LMIC) in which it is estimated that 80-90% of the population are infected while the prevalence of the infection has decreased in developed countries in which less than 40% are infected [172]. *H. pylori* infection in LMIC is typically acquired during early childhood [63, 136]. The transmission of *H. pylori* occurs most often via the fecal-oral route or the oral-oral route in crowded and unsanitary living conditions [75, 169]. Whether contaminated environmental and drinking water can be a reservoir for the bacteria and the source for new infections has been debated. This is due to fact that it has been extremely difficult to detect *H. pylori* DNA in environmental and drinking water samples [91]. Transcriptionally active bacteria were instead found in, for example, vomitus and fecal samples of *H. pylori* -infected individuals with ETEC diarrhea, supporting the proposed oral-oral or fecal-oral routes of transmission [29, 76, 91, 171].

Helicobacter pylori colonization and clinical aspects

H. pylori colonize the human stomach and duodenum and reside in the gastric mucus layer and also in close proximity to the epithelium [34, 63, 226-227]. The bacteria colonize mainly the corpus and antrum regions of the stomach and in areas of gastric metaplasia in the duodenum [208, 227]. *H. pylori* have evolved mechanisms of protection against the gastric acid in the human stomach. For example, the colonization factor flagellae allow for rapid movement through the mucus, while secreted urease neutralizes the low pH, and adherence factors, for example, BabA and SabA¹ help the bacteria bind to gastric epithelial cells [108, 138, 166, 187]. Other putative virulence factors are, for example, cytotoxin-associated gene A (CagA), vacuolating cytotoxin protein A (VacA), neutrophil activating protein (NAP) and the putative *H.*

¹ BabA: blood group antigen-binding adhesion; SabA: sialic acid-binding adhesin

pylori adhesion A (HpaA) [152, 187, 189]. Some of these components facilitate the evasion and dampening of the host immune response [189]. CagA is encoded by the *cag* pathogenicity island (CagPAI) gene cluster, which is believed to encode many more virulence factors. CagA is injected into the cytoplasm of gastric epithelial cells via the type IV secretion system, which is also encoded in the CagPAI [187]. Translocation of CagA into the cytoplasm induces gastric epithelial cell changes and pro-inflammatory immune responses via activation of the nuclear factor kappa B (NF- κ B) pathway and binding to intracellular nucleotide-binding oligomerization domain-containing protein 1 (Nod1) receptors [156]. Translocation of CagA also results in CXCL8 production by epithelial cells [97]. VacA can initiate the formation of trans-membrane pores and induce vacuolization and apoptosis of epithelial cells [41-42]. In addition, it has been proposed that VacA also disturbs antigen processing and presentation by antigen presenting cells (APC) and inhibits T cell proliferation [41]. NAP secreted by the bacteria attracts neutrophils and induces the production of IL-6, CXCL8, IL-12 and IL-23 from monocytes, mast cells and neutrophils *in vitro* [16, 64, 153, 191]. HpaA is a surface lipoprotein and a putative adhesion that has been shown to be highly conserved amongst strains of *H. pylori* [93, 165]. HpaA has also been shown to be a colonization factor in mice [164]. Additionally, studies in mice have shown that immunization with recombinant or purified HpaA can confer protection against *H. pylori* infection and might be a promising antigen to include in a *H. pylori* vaccine together with urease [33, 164].

H. pylori infection causes gastritis in all infected individuals but only a subset of those infected develop clinical symptoms [108]. About 10-15% of chronically infected individuals develop symptoms e.g. dyspepsia and peptic ulcers and 1-2 % have an increased risk of developing gastric cancer [63]. The so-called “triple therapy” is a treatment regimen against *H. pylori* infection in symptomatic individuals. It consists of combination of two antibiotics taken with a proton pump inhibitor two times a day for two weeks [73]. In spite of good eradication rates and cure of symptoms related to the infection, epidemiological studies have reported that a previous infection does not protect against reinfection after antibiotic treatment [82, 114, 137]. Furthermore, the emerging antibiotic resistance among *H. pylori* strains and poor patient compliance make the antibiotic eradication treatment unsuitable particularly in LMIC where *H. pylori* are highly prevalent and reinfections common. For these reasons, vaccination has been suggested as an approach for the control of *H. pylori* infection and disease [158].

Developing vaccines against pathogens that cause chronic infections for e.g. HIV, malaria and tuberculosis has proven to be challenging and *H. pylori* is no exception in this regard [186]. The main hurdle in the development of a vaccine against *H. pylori* infection has been the selection of appropriate antigens, mucosal adjuvant and route of immunization to induce immune responses strong enough to eradicate the infection. Much basic knowledge about *H. pylori* and its interaction with the human host is now known, which can help in the design of a vaccine, some of which are discussed below [29, 76, 91, 171].

Immune responses to *Helicobacter pylori*

Innate immune responses

Most of the bacteria reside in the mucus layer and occasionally some bacteria will relocate close to the epithelial layer and bind to host cells [17]. The innate response is initiated by the binding of bacteria to gastric epithelial cells. The pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and the Nod-like receptor (NLR) recognize pathogen-associated molecular patterns (PAMPs) on the bacteria [189]. TLRs and NLRs are present on many cell types such as epithelial cells, macrophages, dendritic cells (DCs) and lymphocytes. It has been suggested that *H. pylori* flagellin and lipopolysaccharide (LPS) may escape from being recognized by TLR5 and TLR4 respectively, but that instead intact *H. pylori* bacteria may be recognized via TLR2 and thereby initiate the NF- κ B pathway and consequent pro-inflammatory response [140, 178]. In mice it has been shown that *H. pylori* DNA is recognized by intracellular TLR9 in DCs and that the adaptor signaling molecule MyD88 is essential for cytokine responses [178]. Peptidoglycan can be translocated together with CagA into the host cell and recognized by the intracellular nuclear oligomerization domain 1 (Nod1) which will also lead to NF- κ B activation [222]. Triggering of, for example, Nod1 initiates the assembly of the inflammasome and eventually the secretion of pro-inflammatory molecules such as IL-1 β (discussed below) [140].

The *H. pylori* bacterial components, for example, virulence factors and colonization factors binding to gastric human epithelial cells, cause activation via TLRs and NLRs leading to a pro-inflammatory cascade in the stomach that involves the secretion of IL-1 β , IL-2, IL-6, CXCL8 and tumor necrosis factor alpha (TNF α) [43, 107]. A major cause of influx of innate cells is the triggering by *H. pylori* of gastric epithelial cells to produce high concentrations of CXCL8 which acts as a chemoattractant for neutrophils [44]. In infected individuals, the above-mentioned cytokines and chemokine CXCL8, together with transforming growth factor β (TGF β) and IFN γ , can cause additional immune cell infiltration of neutrophils, macrophages, mast cells and NK cells to the gastric mucosa [24, 36, 122, 124, 228]. The immune cells recruited to the stomach in response to bacterial antigens further amplify the response, leading to continuous recruitment of immune cells to the stomach during chronic infection.

APCs such as macrophages or DCs can phagocytose the bacteria and their components and become activated and start to express chemokines and cytokines. The number of DCs is increased in the stomach of *H. pylori* infected individuals [27, 96]. *In vitro* studies have suggested that culturing DCs together with *H. pylori* and human natural killer (NK) cells can stimulate IFN γ secretion from the NK cells, suggesting a role for DCs in presenting the bacteria to NK cells [78]. Immature DCs are attracted to the stomach by the chemokine, CCL20, which binds CCR6 on DCs [40]. In the stomach of *H. pylori*-infected individuals, an increased CCL20 gene expression and subsequent protein production, together with an influx of DCs has been reported [27, 231]. Similar increases in CCL20 and CCR6 can also be found in the stomach of

vaccinated mice post-challenge [67]. In addition, DCs may also bind directly to *H. pylori* via the DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) receptor [19]. Upon stimulation with *H. pylori*, immature DCs express CCR7 (binds CCL19/21 in the lymph nodes) and subsequently migrate to the draining lymph node to present antigen to T cells [22, 79]. Interestingly, in mice, it has also been shown that DCs have the ability to sample bacterial components directly in the gut lumen by extending dendrites through the epithelial layer [185]. This has also been suggested to occur in human *H. pylori*-infected stomach, in which intraepithelial DCs were observed and their dendrites extended through the epithelial layer and sampled the bacteria [157].

Macrophages can be activated by IFN γ which, early in infection, is mainly secreted by NK cells. Subsequently the activated macrophages in turn secrete more IFN γ together with microbicidal products, such as nitric oxide and reactive oxygen species and can also directly phagocytose the bacteria or infected neutrophils in the stomach [154]. *H. pylori*-infected individuals have increased levels of molecules associated with macrophage activation such as inducible nitric oxide synthase (iNOS) as well as the macrophage chemoattractant CCL3 [109, 177, 229]. Furthermore, mice infected with *H. pylori* have increased numbers of macrophages in the stomach and in the paragastric lymph nodes draining the stomach [14].

Adaptive immune responses

The adaptive immune response generally includes a humoral response with activation of B cells followed by production of antibodies together with activation of effector T cells and their recruitment to the local site of infection. Lymphocyte-attracting chemokines, such as CCL28, CXCL10, CXCL13 and CCL5 are produced in the infected gastric mucosa. In *H. pylori*-infected individuals, circulating peripheral blood lymphocytes express the homing receptor $\alpha 4\beta 7$ and respond *in vitro* to *H. pylori* stimulation [176]. Activation of naïve lymphocytes occurs in the lymph nodes when DCs present antigen on MHC class I (presentation to CD8⁺ T cells) or MHC class II (presentation to CD4⁺ T cells) together with the appropriate co-stimulatory molecules [28]. Presentation on MHC-I activates CD8⁺ T cells that differentiate into cytotoxic T lymphocytes (CTL) whereas presentation on MHC-II activates CD4⁺ T cells that differentiate into different T helper cell populations. CD4⁺ T helper (Th) cell responses are polarized, depending on the cytokines produced by the DCs presenting the antigens on MHC-II. During *H. pylori* infection, DCs can produce IL-1 β , IL-6, IL-10, IL-4, IL-12 and IL-23 which will promote T helper 1 (Th1) or T helper 17 (Th17) cells and to a lesser extent T helper 2 (Th2) cells [27, 66, 96].

T cell responses

H. pylori infection can activate both CD4⁺ and CD8⁺ T cells in humans, and subsequent IFN γ production which can be detected locally in the gastric mucosa [209]. The majority of T cells infiltrating the human stomach during chronic *H. pylori* infection are CD4⁺ and furthermore, of

Th1 phenotype, secreting IFN γ [203]. Th2 cells are mainly activated during extracellular parasitic infections such as helminth infection or to allergen exposure which would explain why only a minority of T cells are of the Th2 phenotype in *H. pylori* infection [203]. Interestingly, during recent years, another CD4⁺ effector T cell population has been investigated in *H. pylori* infection, namely Th17 cells, which are characterized by the production of the cytokine IL-17A (discussed below).

In addition to the increase in number of T effector cells in the stomach during *H. pylori* infection, there is also an accumulation of CD4⁺CD25⁺ regulatory T cells (Tregs), which can dampen the T cell effector functions [129-130]. It has been shown that *H. pylori*-infected individuals have increased numbers of Tregs in the stomach that secrete IL-10 which subsequently dampens the secretion of CXCL8 by gastric epithelial cells *in vitro* [188]. Further IL-10 secreting Tregs may also suppress Th1 responses *in vivo* although this has not been shown in humans [50, 81, 188]. It has also been reported that DCs stimulated with *H. pylori* antigens *in vitro* induce proliferation of Tregs in humans [150]. This may explain the increase in frequency of Tregs found in the circulation and stomach of *H. pylori*-infected individuals [129, 188]. Studies in the *H. pylori* mouse model have shown that transient depletion or absence of Tregs results in decreased bacterial colonization in the stomach upon challenge with live bacteria but at the cost of exacerbated inflammation [179-180, 192]. Thus, by preventing excessive inflammation, Tregs were considered to be beneficial to the host during *H. pylori* infection. It has been proposed in chronic *H. pylori* infection in humans however that, the persistence of bacterial infection in the stomach followed by development of peptic ulcer disease may be due to the increase of Tregs in the tissue [188].

Th1 responses

As mentioned previously, *H. pylori* infection can induce DCs to secrete cytokines that will induce Th1 and Th17 responses. A CD4⁺Th1 response to *H. pylori* infection is initiated by DCs presenting the antigen on MHC-II molecule, followed by engagement of co-stimulatory molecules. Further, this will initiate the secretion of IL-12 which promotes Th1 responses and which has been found to be increased in the stomach of infected individuals [81]. The Th1 response is defined by CD4⁺ T cell producing IFN γ and IL-2 which activate macrophages and induces T cell proliferation, respectively. In the context of *H. pylori* infection, IFN γ -producing cells have been shown to correlate with the severity of gastritis in infected human gastric mucosa [15, 85, 117, 124]. Similar results have been reported in mouse models in which a predominantly elevated Th1 response is correlated with more severe gastritis in the stomach [202].

The recruitment of Th1 cells to the gastric mucosa during *H. pylori* infection occurs due to the expression of a number of specific chemokines. *H. pylori* bacteria can induce the expression of CCL5 and CXCL10 by human gastric epithelial cell lines and these chemokines have also been found to be elevated in the stomach tissue of *H. pylori*-infected individuals [15, 60, 105, 229]. In

human *H. pylori* infection, the CCL5 is secreted by epithelial cells, fibroblasts, CD8⁺ T cells and platelets and functions as a chemotactic molecule, mainly for T cells among other cells through binding to its receptors CCR1, CCR3, CCR4 and CCR5. Notably, CCR4 is found to be elevated in *H. pylori*-infected stomach tissue [20, 131]. In mice, CCL5 and CXCL10 are upregulated in the stomach after vaccination and challenge with live *H. pylori* [67].

IFN γ

IFN γ is produced by Th1, NK cells, CD8⁺ T cells, NK T cells, B cells and macrophages. IFN γ is a cytokine of diverse functions: it can induce isotype switching in B cells; it can also activate macrophages to enhance the ability to kill pathogens; it can induce apoptosis in epithelial cells; it plays a role in regulating antigen presentation, enhancing MHC-I- and MHC-II molecule expression on APCs; it can also upregulate the expression of CXCL10 and adhesion molecules in the endothelium and thereby increase leukocyte migration into the affected tissue [11, 15]. Furthermore, as mentioned previously, IFN γ is elevated in *H. pylori*-infected individuals and in infected mice [68, 203].

Th17 responses

In addition to IL-12, DCs may also produce IL-1 β , IL-6, TGF β and IL-23 in response to *H. pylori* [96]. The combinations of these cytokines are responsible for the induction and maintenance of the CD4⁺ Th17 response in mice and humans [39, 103, 219, 224]. However, although it has been shown that TGF β has an important role in inducing Th17 responses in mice, its role in the induction of Th17 cells in humans is unclear. Further, it has been shown in humans that DCs stimulated with *H. pylori* antigens can induce IL-17A production from CD4⁺ T cells *in vitro* in culture [96]. Th17 cells are defined by secretion of the cytokines IL-17A, IL-17F, IL-21, and IL-22 in both humans and mice. Additionally IL-26 secretion and expression of the chemokine receptor CCR6 have also been observed in humans [4, 103]. Th17 cells are attracted to the stomach by the expression of tissue CCL20 binding to CCR6 on human Th17 cells. The Th17 cell secreting cytokines attract neutrophils, activate epithelial cells, generally amplify local inflammation, but also induce antibody secretion [151]. Importantly, Th17 cells are multi-functional cells that produce cytokines involved in both pathogenicity and host defense in humans and mice [103]. In the case of *H. pylori* infection, Th17 cells and their associated cytokine IL-17A, are increased in *H. pylori*-infected individuals and in mouse models (discussed below) [88, 98, 133, 173, 194, 199].

IL-17A

IL-17A belongs to the IL-17 family of cytokines in which IL-17F has similar functions [59]. IL-17A binds to the IL-17 receptor IL-17RA and IL-17RC in humans, but only to IL-17RA in mice

[106]. CD4⁺ Th17 cells are not the only cell type secreting IL-17A in humans or in mice. IL-17A can also be secreted by $\gamma\delta$ T cells, CD3⁺ invariant natural killer T (iNKT) cells, lymphoid-tissue inducer (LTi) cells, Group 3 innate lymphoid cells (ILCs), NK cells, neutrophils and macrophages [46, 207]. IL-17A is a multi-functional cytokine that targets many different cells such as epithelial cells, fibroblasts, endothelial cell and neutrophils and can induce the production of e.g. IL-1 β , IL-6, CXCL8, granulocyte colony stimulating-factor (G-CSF), granulocyte macrophage colony stimulating-factor (GM-CSF), TNF α and anti-microbial peptide production [103, 120, 170]. An important function of IL-17A is to attract neutrophils to the site of infection. Thus, deficiencies of IL-17 in mice have been shown to be associated with neutrophil defects leading to disease susceptibility [102, 121]. For example, mice deficient in IL-17RA are highly susceptible to extracellular pathogens, including the bacteria *Klebsiella pneumoniae*, the yeast *Candida albicans*, and the parasite *Toxoplasma gondii*, [89, 94, 230]. In the field of *H. pylori*, IL-17A is elevated in the gastric tissue of *H. pylori* infected compared to in uninfected individuals and can induce CXCL8 secretion by epithelial cells in a dose-dependent manner [35, 98, 133, 194]. In addition, the increase of IL-17A in *H. pylori*-infected individuals compared to uninfected individuals is also associated with gastritis [35]. Notably, it has been shown that reduced IL-17A responses are associated with lower gastritis score in children infected with *H. pylori* and interestingly the IL-17A response and gastritis were higher in adults [195]. However, the association of IL-17A with gastritis may be related to its function in recruiting neutrophils to the site of infection, as gastritis is often correlated with neutrophil numbers in the tissue. Further, the increase in IL-17A is also associated with increased IL-23 in *H. pylori*-infected individuals [35].

IL-1 β

IL-1 β is a pro-inflammatory cytokine that is elevated in the stomach of *H. pylori* infected individuals [24]. IL-1 β is secreted in large amounts by macrophages and DCs but can also be secreted by neutrophils, monocytes, mast cells, T cells, B cells, endothelial cells and epithelial cells [55, 200]. As mentioned previously, it has been shown to enhance IL-17A secretion by T cells and ILCs in the presence of IL-6 and IL-23 [38, 200, 211-212]. The receptor for IL-1 β is the IL-1 receptor I (IL-1RI) which is expressed on a wide range of cells including epithelial cells, endothelial cells, and innate and adaptive leukocytes [200]. IL-1 signaling is regulated by the IL-1R antagonist (IL-1Ra) which is constitutively expressed and competes with binding to the IL-1RI and thereby inhibiting the IL-1 signaling [54]. Importantly, a lack of IL-1 signaling in mice, results in a defect in the generation of IL-17A-producing T cells [211]. A subset of NLRs which is activated during microbial infection is involved in the formation of the inflammasome [30]. Furthermore, activation via TLRs will lead to the synthesis of the inactive form of IL-1 β , pro-IL-1 β [143]. Notably, in *H. pylori* infection, activation via Nod1 can in turn lead to activation of the transcription factor NF- κ B, activation of the inflammasome and caspase-1 that can cleave pro-IL-1 β to biologically active IL-1 β which then can be secreted [143, 189].

IL-23

IL-23 consists of the p40 subunit, paired with a distinct second chain p19 subunit [167]. IL-23 is secreted mainly by macrophages and DCs [9]. IL-23 binds to the IL-23 receptor (IL-23R) which is expressed by macrophages, DCs, T cells, NK cells, $\gamma\delta$ T cells, iNKT cells and ILCs [46, 111]. As discussed earlier, secretion of both IL-1 and IL-23 has been suggested to promote IL-17A responses in chronic inflammatory disorders [7, 38-39, 45, 211]. However, IL-23 was later shown to be necessary for the maintenance of the Th17 cells and not in the induction phase. In *H. pylori* infection, IL-23 has been proposed to be an important cytokine in promoting chronic gastritis in humans as it can be found in higher levels in *H. pylori*-infected gastric mucosa [35, 96]. In addition, in mice it has been shown that *H. pylori*-infected IL-23p19^{-/-} mice have reduced inflammation compared to infected wild-type mice which may be related to the effect of IL-23 on IL-17A levels and consequently reduced neutrophil recruitment into the tissue [88].

B cell responses

H. pylori also induces local and systemic humoral immune responses with antibody production. B cells expressing CXCR5 can be recruited to the site of infection by chemokines such as CCL28 and CXCL13 and subsequently form germinal centers [116, 155]. Symptomatic and asymptomatic *H. pylori*-infected individuals have elevated *H. pylori*-specific secretory IgA antibodies [144]. In addition, *H. pylori*-infected individuals have increased levels of serum IgG and IgA, which can be used as a marker for diagnosis of the infection [132, 145]. In a birth cohort study in Bangladesh it was shown that passive transfer of *H. pylori*-specific IgA via breast milk to infants, from mothers with high antibody titers, resulted in later acquisition of infection than those in infants receiving breast milk with low antibody titers [26]. In addition, it has been shown that *H. pylori* antigen-specific IgA locally in the stomach and IgG in serum are elevated in infected compared to naïve mice [62, 215].

Gastritis

Inflammation in the stomach tissue (gastritis) is characterized by a massive infiltration of cells which often occurs in response to the cytokines and chemokines induced during *H. pylori* infection. Interestingly, *H. pylori* infection causes gastritis in all infected individuals, but not all individuals develop clinical symptoms. In symptomatic individuals, evaluating the severity of gastritis is an important aspect that can be related to the symptoms, making scoring and classifying highly important. The gastritis can be classified and graded based on The Sydney System which is a standardized system in which cell infiltration, tissue changes and overall damage can be assessed [58]. The pattern of gastritis in the stomach has been associated with increased risk of developing gastric cancer or peptic ulcer disease depending on the location in the stomach at which the gastritis is most prominent - corpus or antrum, respectively [108].

Furthermore, chronic infection may also induce gastric atrophy (loss of normal mucosa and destruction of parietal and chief cells in the corpus), intestinal metaplasia (the formation of intestine-like epithelium) and dysplasia (changes in epithelial cells) which are all risk factors for developing gastric cancer [108]. The association of inflammation with multiple pro-inflammatory cytokines is evident from studies in both humans and animal models [111, 200].

Despite a strong pro-inflammatory response induced in the stomach of *H. pylori*-infected individuals, the infection is chronic and is rarely spontaneously eradicated. One explanation could be the presence of Tregs that dampen excessive inflammation at the cost of persistent colonization of the bacteria [128, 188]. Clearly, this is a problem, as some *H. pylori*-infected individuals can remain asymptomatic but still develop gastric malignancies due to the chronic inflammation (if they are colonized with bacteria with certain virulence factors and have a genetic predisposition). Treatment of the infection using antibiotics may be effective in individual cases, but as many individuals are asymptomatic they would not seek treatment. In symptomatic *H. pylori*-infected individuals and those individuals carrying risks for gastric malignancies, a vaccine as mentioned previously would protect against infection and minimize the likelihood of re-infection, thus also minimizing medical and clinical health problems associated with symptomatic infection.

***Helicobacter pylori* mouse model**

To be able to study the immune responses after vaccination, mouse models of *H. pylori* have been utilized and the most well established and widely used model is the *H. pylori* Sydney strain 1 (SS1) infection in C57BL/6 mice. *H. pylori* SS1 was originally isolated from a patient with peptic ulcer disease and was mouse adapted [115]. The mouse model allows studies of host interactions, pathology and more specifically the evaluation of vaccine candidates and vaccination strategies. *H. pylori* SS1 bacteria colonize at high levels in the mouse stomach starting two weeks post-infection and stable for up to eight weeks after infection [181].

Mucosal vaccination against *Helicobacter pylori* infection

In mouse models it has been shown that vaccination can boost the infection-induced immune responses leading to reduction in bacterial load in the stomach and importantly can protect against reinfection [74, 183]. In addition to specific *H. pylori* antigens, an effective adjuvant is required to induce protection against *H. pylori* infection after mucosal immunization [68, 216]. Key features of a successful vaccination are the choice of antigens, adjuvant and route of vaccination. These combinations have been evaluated in animal models such as mice, with immunizations both prophylactically (before infection) and therapeutically (after infection).

Antigens

H. pylori bacteria have been shown to induce a strong and robust immune response in both humans and mouse models. Several strategies can be identified regarding the choice of the antigen used for the immunizations e.g. (i) whole cell formalin-inactivated bacteria (ii) vectors expressing *H. pylori* antigens e.g. Salmonella-expressing urease (iii) immunodominant antigens, e.g., CagA and urease (iv) boost responses to weakly immunogenic antigens for example, HpaA, BabA and SabA. In mouse models, several antigen preparations have been used and shown to induce protection against *H. pylori* infection. Whole cell vaccines have been widely used and induce protection after vaccination in mice and enhanced immune responses in infected human volunteers [104, 181]. Recombinant antigens such as urease B (large subunit) (UreB), CagA, VacA, HpaA and NAP have been evaluated, either alone or in different combinations and they have been found to be protective in mice [69, 74, 99, 142, 191, 214]. In addition, combinations of purified recombinant antigens of the urease (UreA/UreB) have been used in human volunteers and induce *H. pylori*-specific responses [23]. Clinical trials performed with a candidate *H. pylori* vaccine containing a *Salmonella enteric serovar* vector expressing *H. pylori* urease A and B and subsequently challenged with *H. pylori* [6] showed lack of protection against *H. pylori* infection. However, it was evident that enhanced CD4⁺ T cell responses correlated with lower bacterial load irrespective of immunization status and that the vaccination did not exacerbate the *H. pylori* infection-induced gastritis. In mice, intranasal (IN) or sublingual (SL) immunization with recombinant HpaA or UreB alone induced immune responses, although weak. However, when both antigens were combined, together with cholera toxin (CT), strong immune responses were generated and induced protection against *H. pylori* infection [69, 164].

Adjuvants

In vaccines, adjuvants are used to enhance the immune response towards a specific antigen. Mucosal adjuvants have the ability to enhance antigen presentation by APCs and thus T cell activation occurs more efficiently [2]. In mice, the standard mucosal adjuvant used most often is CT from *Vibrio cholerae*. It induces strong humoral and cellular immune responses to co-administered antigen [182-183, 214]. Studies in the *H. pylori* mouse model have shown that an adjuvant such as CT is essential for protection against *H. pylori* infection after mucosal immunization [18]. CT can promote strong T cell responses and more specifically T helper (Th1) and T helper (Th17) responses to the co-administered antigen through activation of APCs [18, 52, 68, 217]. Only a few adjuvants are licensed for use in humans thus far, and a strong and safe mucosal adjuvant is still lacking. Clinical trials have used native or attenuated enterotoxins from enterotoxigenic *Escherichia coli* (ETEC) in humans, but thus far the majority have still shown safety and toxicity problems. A major focus in mucosal adjuvant research has been the generation of non-toxic derivatives of CT or the heat labile toxin (LT) from ETEC while still retaining significant adjuvanticity [174].

CT and LT toxins belong to the AB toxin group which is typically characterized by an enzymatically active A subunit and a binding B subunit. The A subunit consists of the components A1 (CTA1/LTA1), which is the enzymatically active ADP-ribosyltransferase, and A2 (CTA2/LTA2) which links the A subunit to the B subunit (CTB/LTB). CT binds via CTB to the membrane bound ganglioside, GM1, present on most nucleated cells, while LT binds through LTB to not only GM1 but also other receptors present in the intestine. When CTB or LTB has bound to its receptor, the molecule including the A subunits is internalized by endocytosis into the host cells and transported to the endoplasmic reticulum (ER). The A and B subunit subsequently dissociate and the A1 subunit is translocated to the cytoplasm by an unknown pathway. Once in the cytosol, the A1 subunit ADP-ribosylates Gs proteins, resulting in activation of adenylate cyclase, and increased intracellular levels of cAMP (toxicity). The increased cAMP will cause electrolyte imbalance in the host cell and ultimately cause water secretion and diarrhea [190]. Mutations introduced in the enzymatically active A1 subunit can detoxify these molecules while still retaining their adjuvant function.

CTA1-DD is an attenuated form of CT consisting of an A1 subunit genetically fused to the dimer of the Ig-binding D region (DD) of *Staphylococcus aureus* protein A (SpA). The DD moiety in CTA1-DD targets all B cells [8, 162]. In mice, IN immunization with *H. pylori* lysate antigens and CTA1-DD does induce protection against *H. pylori* infection although not as efficiently as when CT is used as an adjuvant [13]. Both native LT and attenuated versions of LT e.g. LTK63 have been used in mice and humans and confers protection against *H. pylori* infection although native LT predictably, caused side effects in humans [23, 74, 142]. Another version of attenuated LT is the LT (R192G) which has a single amino acid substitution, resulting in reduced enterotoxicity compared to native LT. In humans, LT (R192G) alone induced antibody responses in the stomach of *H. pylori*-negative individuals [125], and was found to be safe and well tolerated [104]. However, when included in an *H. pylori* vaccine, a subset of the volunteers experienced mild diarrhea [104]. To overcome this, a second mutation was introduced in the LT molecule at site 211 which resulted in the double mutant LT (dmLT) (R192G/L211A). The second mutation dramatically decreased cAMP production compared to native LT *in vitro* in epithelial cell lines and *in vivo* as measured by fluid secretion in the intestine in a patent mouse assay [160]. In mice, dmLT has been shown to induce strong antibody and Th17 responses [113, 161].

The question still remains: How can dmLT, with its minimal ADP-ribosylating activity [160-161], be as potent as CT in inducing mucosal immune responses? In an attempt to elucidate the adjuvant mechanism of dmLT, Norton et al [160] reported that the mutations in the dmLT molecule that prevent the proteolytic cleavage of LT-A subunit into A1 and A2 subunits, also led to rapid degradation of the A-subunit in the cytosol of intestinal epithelial cells [160]. They also showed that a higher dose of dmLT was needed to induce cAMP in Caco-2 cells *in vitro*, compared to native LT [160]. Thus, dmLT not only induced much reduced cAMP formation in

cells, but its A-subunit rapidly degraded, thus generating limited enterotoxicity. It is possible that the very short half-life of the A subunit of dmLT within the cells allows stimulation of low but sufficient amounts of cAMP for adjuvant activity without leading to enterotoxicity as also proposed by Norton et al [160]. Further studies to elucidate the effect of dmLT on different cell types and the adjuvant function of dmLT and CT in the presence or absence of cAMP inhibitors may help to clarify the level of cAMP important for the adjuvant function of dmLT.

Currently, the vaccines against *H. pylori* infection that have been evaluated in clinical trials have either not studied the effect on bacterial load or have reported meager T responses to co-administered antigen [139]. In addition, an effective non-toxic mucosal adjuvant is of the essence as those that have been used in clinical trials together with *H. pylori* antigens components have shown adverse effects in human volunteers [23, 31, 104, 149].

Routes of immunization

Mucosal vaccination has been extensively investigated due to the large number of pathogens invading and causing disease at our mucosal surfaces. In mice, the most commonly investigated routes of immunizations are intragastric (IG), IN and SL while rectal and intravaginal routes have been evaluated to a less extent. Rectal immunization against *H. pylori* infection has been evaluated in humans and in mice and although this route of immunization confers immune responses, other routes of immunization are preferable [99, 206]. In human volunteers, intravaginal immunization has been shown to induce local immune responses, but does not induce immune responses in the gut [148]. In humans, the IG route has been by far the most often used for vaccination against *H. pylori* infection [23, 104, 125]. In mice, protection against *H. pylori* infection has been demonstrated after prophylactic and therapeutic IG vaccination [142, 183, 214]. However, as the IG route delivers the antigen and adjuvant directly into the stomach, there are concerns that the antigens and adjuvants might be degraded because of the harsh environment and thus a large dose may be required to induce protection. The IN route of vaccination has been evaluated in mice and confers protection against *H. pylori* infection [13, 99]. This route eliminated the problem of components of the vaccine degrading but instead introduced new safety issues such as translocation of antigen and adjuvant to the olfactory bulb of the brain in addition to lack of efficacy in humans relative to mice [92]. The SL route of immunization has been evaluated in mice as an alternative route to the nasal route for inducing mucosal immune responses [49]. The SL route has previously been used as a route of sensitizing and treating allergic asthma in humans and for vaccination against influenza virus in mice with promising results [197]. In mice, immunization via the SL route using the adjuvant CT and ovalbumin as antigen increases the number of CD11c⁺ DCs in the SL mucosa which migrate to the cervical lymph node (CLN) to present the antigens and generate antigen-specific T cell responses [47, 204]. Immunizing via the SL route also increase antibody responses both systemically and mucosally and protects against a lethal dose of influenza virus [47, 205]. The

SL route of immunization also induces a strong *H. pylori* antigen specific responses and protection against *H. pylori* infection in mice [69, 182]. The SL route of immunization can also induce immune responses in a wide range of mucosal tissues (e.g. genital tract, respiratory system, small intestine and stomach) in mice, and is a preferable route for immunization in humans [49, 110, 159]. In addition, the antigen or adjuvant when delivered via the SL route does not translocate to the olfactory bulb [198], minimizing safety concerns.

Immune responses after mucosal vaccination in mice

Vaccine-induced protection is associated with increased levels of antibodies. However, studies using gene knockout mice have shown that mice deficient in mature B cells and antibody responses are still protected after IG vaccination and thus the humoral response does not seem to be essential for vaccine-induced protection [62]. Studies have shown that CD4⁺ T cells and not CD8⁺ T cells are essential for vaccine-induced protection against *H. pylori* infection [62, 168]. More specifically the Th1 and Th17 responses, with subsequent IFN γ and IL-17A production strongly correlate with vaccine-induced protection [52, 68, 182, 221]. The robust immune response induced after vaccination and challenge with *H. pylori*, manifests itself as inflammation in the stomach referred to as “post-immunization gastritis” [72, 77, 183]. Post-immunization gastritis has been shown to be transient and resolves when the bacteria are eradicated from the stomach of vaccinated mice [72]. The gastritis is accompanied by a major influx of hematopoietic cells and secretion of cytokines and chemokines. It has been shown that there is a strong influx of neutrophils and increase in the chemokines and receptors for neutrophil influx, CXCL2, CXCL5 and CXCR2 within 7 days after vaccination and infection with *H. pylori* [14, 67]. Subsequently, vaccine-induced protection against *H. pylori* infection has been shown to be neutrophil dependent [52]. In addition, mast cells have been shown to be important for vaccine-induced protection in a *H. felis* model in which mice had increased levels of bacteria in their stomach when mast cells were depleted [220]. Furthermore, mast cell-deficient mice have been shown to be partially protected against *H. pylori* infection after vaccination, but, with lower numbers of neutrophils in the stomach [57]. Vaccination and infection with *H. pylori* also induced the expression in the mouse stomach of CXCL10 (attracts Th1 cells), CCL20 (attracts Th17/DCs) and CCL8 (attracts eosinophils) and their receptors [67]. Furthermore, it has also been shown that there is an increased frequency of Th1 cells, eosinophils and DCs in the stomach which correlated with protection against *H. pylori* infection [67].

Aims

In a *Helicobacter pylori* mouse model;

- ❖ To evaluate immune responses after sublingual vaccination with a candidate *H. pylori* vaccine containing *H. pylori* antigens and a de-toxified double mutant heat labile toxin (dmLT) from enterotoxigenic *Escherichia coli* as a mucosal adjuvant.
- ❖ To assess the role of IL-17A and IFN γ in protection against *H. pylori* infection and inflammation after sublingual and intragastric vaccination.
- ❖ To investigate the role of IL-1 signaling and IL-23 in inducing IL-17A responses after sublingual vaccination

Key Methodologies

Mice

For all experiments, wild-type (WT) specific-pathogen-free C57BL/6 mice were purchased from Taconic, Denmark or Harlan Laboratories, Horst, Netherlands. Interferon- γ knockout (IFN γ ^{-/-}) mice from Genentech were bred at the Laboratory for Experimental Biomedicine and used in paper II. Interleukin-1 receptor I knock-out (IL-1RI^{-/-}) mice were obtained from the Jackson Laboratory, ME and bred at the Laboratory for Experimental Biomedicine and Interleukin-23 p19 subunit knock-out (IL-23p19^{-/-}) mice were provided by Dr. Rene de Waal Malefyt, Merck Research Laboratories, CA, were used in paper III. All gene-knockout mice were obtained on a C57BL/6 background. In Appendix I, ovalbumin T cell transgenic mice (OT-II) were used which are homozygous for a transgene that encodes a T-cell receptor specific for chicken ovalbumin (amino acids 323-339), presented on the MHC class II molecule I-A^b (from in house breeding). All mice were housed in microisolators at the Laboratory for Experimental Biomedicine, Göteborg University for the duration of the study with food and water provided ad libitum. All experiments were approved by the Ethical Committee for Laboratory Animals in Gothenburg, Sweden.

Human volunteers

Appendix II

Antrum and corpus stomach tissue biopsies were taken from Swedish volunteers as previously described [5]. Written consent was obtained from each volunteer before participation and the study approved by the Human Research Ethics Committee in Gothenburg as described in [5]. Briefly, blood was collected from all volunteers and screened using a whole blood quick test to detect *H. pylori* positivity (Quick Vue *H. pylori* gII test; Quidel, San Diego, CA, USA) and the infection was also confirmed by culture from biopsies on *H. pylori* selective plates and ELISA to detect *H. pylori* specific serum antibody responses. Biopsies were also taken and used for histopathological scoring and immunohistochemistry. Three different groups of volunteers were analysed (i) *H. pylori*-negative (Hp-) individuals; (ii) Asymptomatic *H. pylori*-positive (Hp+) individuals without corpus atrophy or intestinal metaplasia and; (iii) *H. pylori*-positive with corpus atrophy (Hp+ CA) and none or mild intestinal metaplasia. Biopsies were frozen and kept at -70 °C until use for immunohistochemistry.

Cultivation of *H. pylori* SS1 for infection

The *H. pylori* SS1 was used for infecting the mice [115]. The bacteria were cultured on agar plates and subsequently transferred to a broth and cultured additionally overnight [182]. The bacteria were visualized under a microscope to check for motility before infection of mice. Before IG infection of mice, the optical density (OD) was adjusted to 1.5, and a single dose of approximately 3×10^8 viable bacteria was given intragastrically to each mouse [183].

***H. pylori* lysate antigens and recombinant antigen preparation for immunizations and ELISA**

For immunizations, *H. pylori* lysate antigens from the strain Hel 305 (CagA⁺, VacA⁺) isolated from a duodenal ulcer patient was prepared as previously described [123, 181-182]. The bacteria were grown to confluence on agar plates and the bacterial harvest was suspended phosphate-buffered saline (PBS) and sonicated on ice. After centrifugation to remove the bacterial cell membranes the supernatant was sterile filtered to remove any possible contamination with whole bacteria. For SL immunizations, thawed aliquots of the *H. pylori* lysate antigens were immediately freeze-dried and reconstituted to a protein concentration of 20 mg/ml (Paper II-III) or 40 mg/ml (paper I) to reduce the volume used for the immunization to a maximum of 10 µl (including the adjuvant). For IG immunizations the same concentration of the antigens was used as for SL immunizations. The coating antigen for enzyme-linked immunosorbent assay (ELISA) to detect *H. pylori* specific antibodies, *H. pylori* membrane protein (MP) antigen, from strain Hel 305 (MP Hel 305) was prepared as described in detail in paper I. The antigen preparations were aliquoted and stored at -70°C until further use and not subjected to multiple freeze-thaw cycles. Purified recombinant *H. pylori* antigens HpaA (rHpaA) and UreB (rUreB) used for immunizations were prepared as described previously [69] and (Paper I).

Reconstitution of adjuvants for immunization

Lyophilized CT from *Vibrio cholerae* (Sigma Aldrich) and dmLT (R192G/L211A) from *E. coli* were prepared as described [160] and reconstituted to a concentration of 1 mg/ml and stored at -70°C and 4°C respectively until further use.

***In vivo* neutralizing antibodies**

For *in vivo* neutralization of IL-17A in paper II, a rat anti-IL-17A IgG monoclonal antibody clone JL7.1D10 (Merck Research Labs, Palo Alto, CA) was used [37]. Purified rat IgG antibody (Sigma Aldrich) was used as an isotype control. A concentration of 300 µg /mouse and occasion of neutralizing antibodies or isotype control antibodies was administered intraperitoneally (i.p.) in a volume of 300 µl.

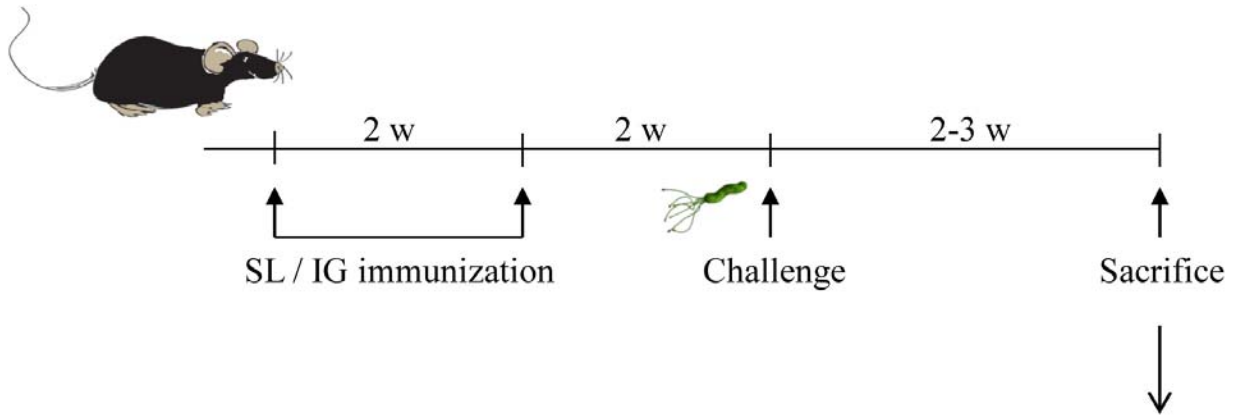
Experimental setup

Prophylactic SL immunization was carried out by carefully placing a total volume of 10 µl of *H. pylori* lysate antigens reconstituted in CT, dmLT or PBS without bicarbonate buffer through a micropipette under the tongue of the mice. Prophylactic IG immunization was performed using a feeding needle, placing a total volume of 300 µl of *H. pylori* lysate antigens and CT or dmLT in 3% sodium bicarbonate buffer directly into the stomach.

Both immunizations were administered under deep anesthesia (Isoflurane; Abbott Scandinavia AB, Solna, Sweden).

Mice strains

- C57Bl/6 (WT)
- IFN γ ^{-/-}
- IL-1RI^{-/-}
- IL-23p19^{-/-}



Antigen and adjuvant combinations

- *H.pylori* lysate antigens + CT
- *H.pylori* lysate antigens + dmLT
- *H.pylori* lysate antigens
- CT
- dmLT
- rHpaA + UreB + CT
- rHpaA + UreB + dmLT

Taken
Blood
Spleen
Stomach
CLN
MLN

Figure 1. Experimental setup of the *in vivo* experiments included in the thesis.

Paper I

Each mouse was prophylactically immunized according to one of the following protocols.

- 1) **SL** immunization at biweekly intervals with 400 μ g *H. pylori* lysate antigens and 10 μ g CT or 10 μ g dmLT.
- 2) **IG** immunization at biweekly intervals with 400 μ g *H. pylori* lysate antigens and 10 μ g CT or 10 μ g dmLT.

- 3) SL immunization at biweekly intervals with 400 µg of *H. pylori* lysate antigens **alone** or 20 µg dmLT **alone** (antigen and adjuvant controls).
- 4) **SL** immunization at biweekly intervals with 50 µg **rHpaA**+50 µg **rUreB** and 10 µg of CT or 20 µg dmLT

Two weeks after the last immunization the mice were challenged with live *H. pylori* SS1 bacteria. Animals were sacrificed at two-three weeks after challenge, and the number of *H. pylori* bacteria in the stomach of individual mice was determined (Fig. 1).

Paper II and III

Before starting these next set of experiments described below, the amount of *H. pylori* lysate antigens needed for SL and IG immunization was titrated. The results showed that 200 µg *H. pylori* lysate antigens and CT induced the similar immune responses and protection against *H. pylori* infection as 400 µg and thus we continued our experiments using 200 µg *H. pylori* lysate antigens for the immunizations (*data not shown*).

- 1) **SL** immunization at biweekly intervals with 200 µg *H. pylori* lysate antigens and 10 µg CT (Paper II and III).
- 2) **IG** immunization at biweekly intervals with 200 µg *H. pylori* lysate antigens and 10 µg CT (Paper II).

Two weeks after the last immunization, mice were challenged with live *H. pylori* SS1 bacteria and sacrificed 2, 3 or 5 weeks after challenge and the number of bacteria in the stomach was determined by quantitative culture (Paper II and III). In Paper II sublingually immunized and unimmunized IFN γ ^{-/-} mice were injected i.p. with 300 µg/mouse neutralizing anti-IL-17A or isotype IgG antibody on day 5, 8 and 11 after challenge. At day 14 after challenge the mice were sacrificed and bacterial counts in the stomach assessed along with cellular immune responses, antibody production and cytokine responses.

Appendix I

Mice were immunized via the SL or IG route and challenged as described in (1) and (2) for Paper I. Stomach tissue was assessed for IL-17A positive cells by immunohistochemistry.

To block the egress of lymphocytes from the lymph nodes, the drug FTY720 (Cayman Chemicals, Ann Arbor, MI), a structural analog of sphingosine and potent agonist of the sphingosine-1-phosphate receptors was used. FTY720 was diluted to 0.5 mg/ml in physiological saline and each mouse was administered 0.025 mg i.p. every 48 h for maximum 6 days. Mice were immunized via the SL or IG route on day 1 (after FTY720 injection) and sacrificed on day 8. To ensure that the FTY720 treatment was efficient and that no T cells exited the lymph nodes, blood was collected on day 6 and day 8 stained for lymphocytes CD3, CD4 and CD8 and analyzed by Flow cytometry.

Expansion of CD11c⁺ dendritic cells *in vivo*

Appendix I

Flt3L secreting B16-F10 melanoma was obtained from Nicolas Mach [135] and was stored in liquid nitrogen, thawed and cultured for 2-3 days in a 25 mm² flask in Iscove's basal medium (Biochrome, Berlin, Germany) supplemented with fetal calf serum, 2-mercaptoethanol, 1mM L-glutamine and gentamycin (Iscove's complete medium) at 37°C and in 5% CO₂ atmosphere. On the day of injection, the cells were collected, washed and resuspended in fresh Iscove's complete medium and counted. Each mouse was administered a total of 1 x 10⁶ cells injected subcutaneously. The tumor cells upon injection into mice forms a solid tumor which secrete Flt3L that has the ability to expand CD11c⁺ dendritic cells in lymph nodes and spleen. The mice were palpated daily to follow tumor growth and sacrificed when the tumor size had reached no more than 10 mm in diameter.

Quantitative culture of *H. pylori* SS1 from the stomach

Papers I-III

To evaluate bacterial colonization in the stomachs of the mice, one half of each stomach was homogenized in brucella broth using a tissue homogenizer (Ultra Turrax, IKA Laboratory Technologies, Staufen, Germany). Serial dilutions of the homogenates were plated on blood skirrow agar plates (BD Biosciences). After 7 days of incubation at 37°C under microaerophilic conditions, visible colonies with typical *H. pylori* morphology were counted, and a urease test was performed for any uncertain colonies. Calculations of the number of bacteria per stomach were done as previously reported [163].

Determination of cytokines IL-17A and IFN γ in stomach tissue extracts

Papers II-III

IL-17A and IFN γ in the stomach was determined in tissue extracts prepared using a 2% saponin-PBS solution as previously described [163]. IL-17A in the extracted stomach tissue supernatant were analyzed using the mouse cytometric bead array (CBA) flex set kit (BD Biosciences) according to manufacturer's instructions. To ensure that cytokines could be detected correctly, an experiment was performed where saponin tissue extracts were spiked with different concentrations of the recombinant cytokine and analyzed. This experiment showed that the CBA flex set kit was able to specifically detect and bind to cytokines in saponin tissue extracts. Furthermore, two different kits; CBA Th1/Th2/Th17 kit and CBA flex set kit were used during the study to detect cytokines in saponin tissue extracts and supernatants from proliferation assays and comparison of the two kits showed that they gave similar results.

Determination of gene expression in stomach tissue

Papers I, III

RNA isolation and cDNA preparation

The stomach was excised and dissected along the greater curvature. Any loose stomach contents were removed by washing in PBS. One longitudinal strip including the corpus and antrum were cut and placed directly into RNAlater and stored at -70°C . For RNA isolation, the tissue was thawed and transferred to RLT lysis buffer and homogenized using a Tissue Lyser II. Total RNA was extracted using RNeasy mini kit (Qiagen) and then stored at -70°C . RNA purity and concentration was measured using the fluorospectrometer Nanodrop ND-1000. RNA ($2\ \mu\text{g}$) was reverse transcribed into cDNA using the Omniscript kit.

Real Time PCR

All real-time PCRs (RT-PCRs) were run in 96-well plates using the standard amplification conditions described for the 7500 RT-PCR system and 40 ng cDNA, 10 μl 2 \times Power SYBR green master mix (Applied Biosystems, Foster City, CA), and 1 μl of gene-specific oligonucleotide primers (Eurofins MWG Operon, Ebersberg, Germany). The reactions were run in duplicate, and β -actin was used as the reference gene in all experiments. The difference between β -actin and the target gene (ΔCT) was determined, and the relative expression was calculated using the formula $2^{\Delta\text{CT}}$. The values were adjusted so that the mean in the infection control group was set to 1. The negative control (lacking reverse transcriptase) giving the lowest CT value was used to determine the detection limit. The sequences used in paper I, II and III are shown in Table 1 and have been previously published [201].

Table 1. Oligonucleotide primers used for quantitative PCR

Gene	Primer sequence	
	Forward	Reverse
β -actin	5'-CTGACAGGATGCAGAAGGAGATTACT-3'	5'-GCCACCGATCCACACAGAGT-3'
CD4	5'-ACTGGTTCGGCATGACACTCT-3'	5'-TCCGCTGACTCTCCCTCACT-3'
IL-17	5'-CCCTTGGCGCAAAAGTG-3'	5'-TCTTCATTGCGGTGGAGAGT-3'
IFN γ	5'-GCATAGATGTGGAAGAAAAGAGTCTCT-3'	5'-GGCTCTGCAGGATTTTCATGT-3'

Grading inflammation in stomach tissue

Papers I, II, III

A longitudinal strip from the entire longer curvature of the stomach was taken and fixed in 4% phosphate-buffered formalin and then embedded in paraffin. Sections, 8 μm thick were cut and stained with hematoxylin and eosin. The slides were then examined by light microscopy (x100), and the extent of gastritis was graded based on the Sydney system of scoring gastritis as described previously [180]. Briefly, sections were graded and given a score for parietal cell destruction (1-6), chief cell destruction (1-6) and cell infiltration (1-4) giving a maximum score of 16. Grading was done as follows: Grade 0–1, normal gastric mucosa that contained few lymphocytes scattered throughout the submucosa; Grade 2, small aggregates containing three to

four layers of cells in the mucosa or sparse infiltrates of cells in the submucosa covering ~5% of the section; Grade 3, frequent and larger infiltrates extending into the mucosa; Grade 4, infiltrates spanning half to the entire width of the mucosa; Grade 5, partial or complete (grade 6) obliteration of parietal and chief cells with hyperplasia of mucous and epithelial cells.

Immunohistochemistry staining for IL-17A in mouse stomach tissue

Appendix I

A vertical strip from the entire stomach was taken and frozen in embedding medium OCT (Histolab, Göteborg, Sweden) in ice-cold isopentane cooled by liquid nitrogen and the frozen blocks were stored at -70°C until further use. Sections (7 µm) were cut and allowed to dry followed by fixation with 100% ice-cold acetone for 10 minutes at 4 °C. Sections were permeabilized with 0.5 % Triton X-100 (Sigma Aldrich) for 10 min and then blocked with 10 % goat serum (Sigma Aldrich) and Fc-γ receptor block for 45 minutes. The blocking solution was blotted out and primary antibody rat anti mouse IL-17A-FITC (eBioscience) or rat isotype-FITC control (eBioscience) was added in 0.1 % Triton X-100 and incubated for 60 minutes. Sections were washed in PBS and mounted using mounting medium prolong® gold antifade (Life Technologies, Carlsbad, CA) containing DAPI (nuclear stain). The IL-17A staining was then visualized using a fluorescence microscope (Zeiss Axioplex 2) and pictures of the positive staining in the entire stomach tissue were taken. The pictures were analyzed using the Biopix software (Biopix, Göteborg, Sweden) for quantification of IL-17A⁺ cells.

Immunohistochemistry staining for IL-17A in human stomach tissue

Appendix II

Biopsies from human volunteers were taken from the antrum and corpus of *H. pylori* negative patients (n=6), *H. pylori* positive patients (n=6) and *H. pylori* positive patients with corpus atrophy (n=5). The biopsy of the stomach was taken and frozen in embedding medium OCT (Histolab) in ice-cold isopentane cooled by liquid nitrogen and the frozen blocks were stored at -70°C until further use. Sections (7 µm) were cut and allowed to dry followed by fixation with 100% ice-cold acetone for 10 minutes at 4 °C. Sections were permeabilized with 0.5 % Triton X-100 (Sigma Aldrich) for 10 min and then blocked with 10 % human AB serum 2 % naïve mouse serum and 10 % bovine serum albumin (Sigma Aldrich) for 60 minutes. The blocking solution was blotted out and primary antibody mouse anti human IL-17A-FITC (eBioscience) or mouse isotype-FITC control (eBioscience) was added in 0.1 % Triton X-100 and incubated for 60 minutes. Sections were washed in PBS and mounted using mounting medium prolong® gold antifade (Life Technologies) containing DAPI. The IL-17A staining was then visualized using a fluorescence microscope (Zeiss Axioplex 2) and pictures of the positive staining in the entire stomach tissue were taken. The pictures were analyzed using the Biopix software (Biopix) for histological quantification of IL-17A⁺ cells.

Antibody responses

Papers I-III

H. pylori-specific IgA antibodies in the stomach against MP Hel 305 were determined in the stomach tissue extracts by ELISA as described in detail in Paper I.

Serum antibody titers were also determined by ELISA against MP Hel 305. For IgA the end point values that was used was the optical density measured at 490 nm. And for serum IgG measurements, the antibody titers are defined as the reciprocal serum dilution giving an absorbance of 0.4 above the background.

Determination of *H. pylori* specific cellular immune responses and subsequent cytokine responses in culture supernatant

Papers I-III

For the proliferation assays, single cell lymphocyte suspensions were prepared from the spleen, mesenteric lymph nodes (MLN) and cervical lymph nodes (CLN). Cells were seeded (2×10^5 cells per well) in the presence or absence of boiled *H. pylori* Hel 305 lysate antigens (4 $\mu\text{g/ml}$) and cultured for 72 hours in Iscove's complete medium. Supernatants were collected and stored at -70°C for subsequent cytokine analysis. To determine proliferation (radioactive thymidine incorporation assay), the cells were pulsed with 1 μCi of $[^3\text{H}]$ thymidine (Amersham Bioscience, Buckinghamshire, United Kingdom) for the last 6 to 8 hours of culture. The cellular DNA was collected with a cell harvester (Skatron) on glass fiber filters (Wallac) and assayed for ^3H incorporation using a liquid scintillation counter (Beckman, LKB, Bromma, Sweden). Cytokines in culture supernatants was measured using the mouse cytometric bead array kit (BD Biosciences) and analyzed according to the manufacturer's instructions. Supernatant from the proliferation assay was analyzed for cytokines IL-17A, $\text{IFN}\gamma$ and $\text{TNF}\alpha$ using Mouse Th1/Th2/Th17 Cytometric Bead Array kit or Cytometric Bead Assay Flex Set beads according to manufacturer's protocol (BD Biosciences).

Determination of ovalbumin specific cellular responses

Appendix I

Preparation and stimulation of DCs

Single cell suspensions were prepared from the MLN and CLN of Flt3L treated wild-type mice. CD11c^+ cells were enriched from total MLN and CLN cells by CD11c^+ positive selection using magnetically labeled MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of the CD11c^+ DCs in repeated isolations was typically $>90\%$. Enriched CD11c^+ dendritic cells from MLN or CLN were incubated alone or in the presence of 2 mg/ml Ovalbumin grade VII (Sigma Aldrich) together with or without 10^{-3} $\mu\text{g/ml}$ of CT or dmLT and cultured for 4 hours in Iscove's complete medium. After incubation the cells were washed thoroughly 3-4 times to completely remove any residual antigen or adjuvant.

Preparation of OT-II T CD4⁺ T cells and co-culture with stimulated DCs

Spleen CD4⁺ T cells ovalbumin transgenic (OT-II) mice were prepared using CD4 positive selection kit (Miltenyi Biotech). CD4⁺ cells in repeated isolations was typically >90%. DCs (stimulated or unstimulated) were seeded (1×10^4 DCs per well) together with CD4⁺ OT-II spleen T cells (1×10^5 per well) in a sterile 96-well plate and incubated for 5 days in 37°C in a 5% CO₂ atmosphere. Proliferation of the cells was determined by the radioactive thymidine incorporation assay.

Determination of *H. pylori* specific CD4⁺ T cell responses

Appendix I

Preparation and stimulation of DCs

Enriched CD11c⁺ dendritic cells from MLN or CLN were incubated alone or in the presence of *H. pylori* Hel 305 lysate antigens (20 µg/ml), together with or without 10^{-3} µg/ml of CT or dmLT and cultured for 4 hours in Iscove's complete medium. The purity of the CD11c⁺ DCs was typically >90%. After incubation, cells were washed thoroughly in order to completely remove any residual antigens or adjuvant.

Preparation of CD4⁺ T cells and co-culture with stimulated DCs

The MLN was isolated from mice sublingually immunized with *H. pylori* lysate antigens and CT and challenged with *H. pylori* bacteria. The CD4⁺ T cells from the MLN were enriched by CD4⁺ positive selection using magnetically labeled MACS beads (Miltenyi Biotech). DCs (stimulated or unstimulated) were seeded (1×10^4 DCs per well) together with CD4⁺ T cells isolated from the MLN (1×10^5 + thymidine incorporation assay).

Gene expression in isolated CD11c⁺ dendritic cells exposed to adjuvant CT or dmLT

Dendritic cell culture

CD11c⁺ cells were enriched from total spleen cells of Flt3L treated C57BL/6 wild-type mice by CD11c⁺ positive selection using magnetically labeled MACS beads (Miltenyi Biotech). CD11c⁺ DCs cultured for RNA extraction were seeded (4×10^6 DCs per well) in the presence or absence of 1 µg/ml CT or dmLT and cultured for 4 h in Iscove's complete medium at 37°C in a 5% CO₂ atmosphere. Cells were collected after incubation, thoroughly washed to remove all culture medium and additives and resuspended in 350 µl RLT lysis Buffer (Qiagen) for downstream RT-PCR application. The samples were stored at -70°C.

RNA extraction and cDNA preparation

For RNA isolation, the cells were thawed and run through a shredder column (Qiagen for homogenization). RNA was extracted by using RNeasy mini kit (Qiagen) and purity and concentration was measured using the fluorospectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific). RNA (2 µg) was reverse transcribed into cDNA using the Quantitect kit (Qiagen).

RT-PCR array

cDNA (2 µg) were run in 96-well array plates (Mouse dendritic and antigen presenting cell array, SA Biosciences/Qiagen) according to manufacturer's instruction using the 7500 RT-PCR Applied Biosystems system and 2x Power SYBR green master mix (Applied Biosystems). An average of the housekeeping genes β -actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), β -2 microglobulin (β 2m), β -glucuronidase (Gusb) and heat shock protein 90 α b1 (Hsp90 α b1) was used as a controls. The difference between the sample gene and the average from the housekeeping genes (Δ CT value) was obtained and the relative fold change and expression was calculated by $2^{\Delta\text{CT}}$. The values for all samples were compared to non-stimulated cells and expressed as fold-change from non-stimulated samples. Validation was carried out with RNA (2 µg) that was first reverse transcribed into cDNA using the Quantitect kit (Qiagen). The samples were run in 96-well plates using the standard amplification settings for 7500 RT-PCR system and 20 ng cDNA (same amount as used for the array), 10 µl 2x Power SYBR green master mix (Applied Biosystems) and 1 µl of gene-specific oligonucleotide primers (Eurofins MWG Operon) (table 1). Each sample was run in duplicates and β -actin was used as a housekeeping gene control for the validation experiment. The difference between the sample gene and β -actin (Δ CT value) was obtained and the relative expression was calculated by $2^{\Delta\text{CT}}$. The values for all samples were calculated as fold change from non-stimulated cells.

Flow cytometric analysis

Paper II and Appendix I

For cytokine staining, MLN and CLN cells were stimulated with 20 ng/ml Phorbol 12-Myristate 13-Acetate (PMA, Sigma Aldrich) and 1 µg/ml Ionomycin (Sigma Aldrich) in Iscove's complete medium (Iscove's medium supplemented with 10% heat-inactivated fetal calf serum, 50 µM 2-mercaptoethanol, 1 mM L-glutamine, and 50 µg/ml gentamicin) for 2 h at 37°C in a 5% CO₂ atmosphere. After 2 h, 10 µg/ml Brefeldin A (BFA, Sigma Aldrich) in Iscove's complete medium was added to the cells and left for additional 2 h at 37°C in a 5% CO₂ atmosphere. Surface and intracellular staining was carried out using anti-mouse CD3-PerCP, CD4-Alexa Fluor 700, IFN γ -APC (all BD Biosciences) and IL-17A-FITC (eBioscience) and Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies). Stained cells were acquired using the LSR-II Flow Cytometer and the Diva software (BD Biosciences) and analysis were carried out using FlowJo software (FlowJo, Ashland, OR, USA). Stimulation with PMA and Ionomycin reduced the frequency of CD3⁺CD4⁺ T cells by 5% (*data not shown*).

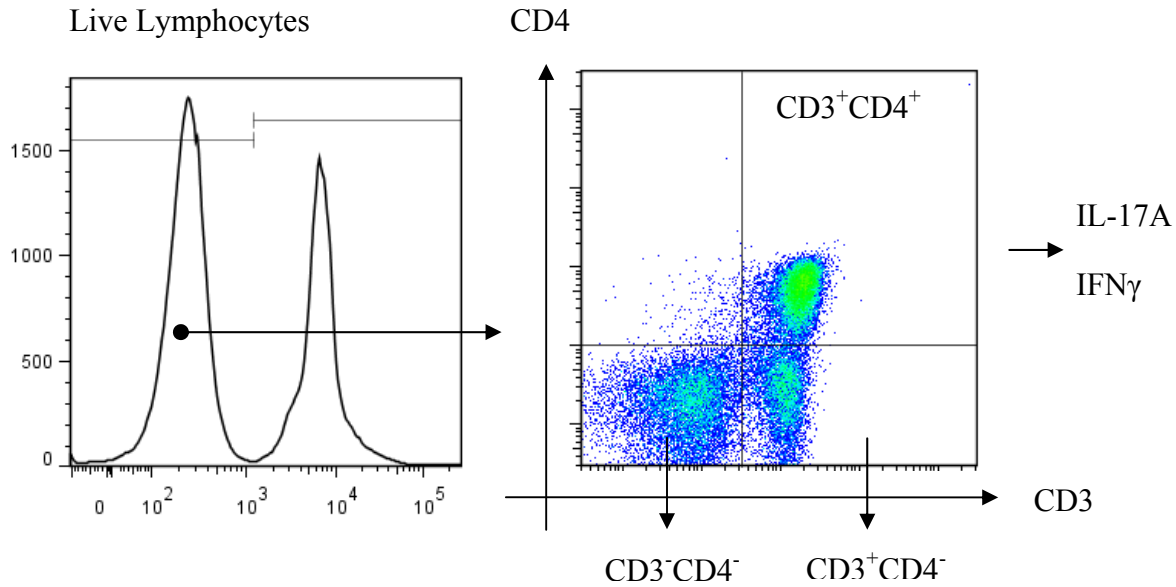


Figure 2. Gating procedure for Flow Cytometry analysis of IL-17A⁺ and IFN γ ⁺ in MLN and CLN cells.

Statistical analysis.

Papers I-III and Appendix I-II

Analysis of variance (ANOVA) with Bonferroni's (Paper I) or Dunette's (Paper I) posttest or student's t-test with Welch correction (Paper II, III, Appendix I) was used to compare multiple groups of mice using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). For all tests, a *p* value of <0.05 was considered to be statistically significant.

Results and comments

PAPER I

Evaluation of dmLT as an adjuvant in a vaccine against *H. pylori* infection

The use of CT as an adjuvant in a vaccine against *H. pylori* infection has provided important insights into the protective immune mechanisms against *H. pylori* infection. However, the inherent enterotoxicity of CT precludes its use in a vaccine for humans. This is due to the fact that as discussed previously, CT and the related LT when translocated into the host cell can ADP ribosylate the stimulatory Gs proteins of adenylate cyclase resulting in an increase in cyclic AMP. These events lead to electrolyte secretion in the intestinal epithelial cells with massive watery diarrhea as a consequence. In the last decade several targeted mutations in the CT or LT molecule have been constructed with the aim to remove or attenuate the toxic effects while still retaining the adjuvant function. One promising molecule is dmLT. The aim of this study was to evaluate the efficacy of the newly developed double mutant LT (dmLT) as a mucosal adjuvant and compare it with the well-established but toxic adjuvant CT in inducing immune responses and protection against *H. pylori* infection.

Protection against H. pylori infection after sublingual immunization with H. pylori lysate antigens and dmLT as an adjuvant

SL immunization using *H. pylori* lysate antigens and dmLT led to a significant reduction in bacterial load post-challenge which was similar to the reduction seen using CT as adjuvant (Fig 3, paper I). To confirm the adjuvant effect of dmLT after SL immunization, mice were immunized with either dmLT or *H. pylori* lysate antigens alone which did not decrease the bacterial load in the stomach after challenge (paper I). Thus in agreement with our earlier reports [182] that, vaccination with a combination of antigen and adjuvant is required for protection against *H. pylori* infection in mice. In addition, IG immunization with either dmLT or CT as adjuvant together with *H. pylori* lysate antigens also reduced the bacterial load in the stomach of mice after challenge with *H. pylori* compared to unimmunized infected mice, but not to the same extent as after SL immunization (paper I).

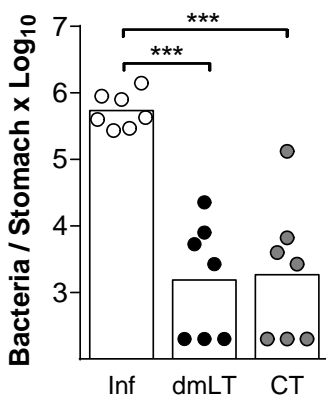


Figure 3. Sublingual immunization using dmLT or CT as adjuvant and *H. pylori* lysate antigens protects against *H. pylori* infection.

Mice were immunized via SL route with *H. pylori* lysate antigens and dmLT or CT or left unimmunized (Inf) and subsequently challenged with live *H. pylori* bacteria. Three weeks post-challenge, reduction in bacterial load as a measure of protection was evaluated by quantitative culture and expressed as mean log₁₀ values of bacteria per stomach. n=7 mice/group, representative of two independent experiments. Statistically significant difference in bacterial load between groups was assessed by unpaired two-tailed t-test with Welch correction and denoted by *** (p<0.001).

Our studies utilized *H. pylori* lysate antigens for vaccination. Although, the lysate preparation contains protective antigens, it also contains several toll-like receptor agonists and thus may not be suitable in a future vaccine for humans. Whole cell bacteria and recombinant antigens are more likely to be components of a *H. pylori* vaccine. Indeed, we have previously shown that SL immunization with recombinant *H. pylori* HpaA and *H. pylori* urease B subunit (UreB) and CT was protective against *H. pylori* infection [69]. Therefore we wanted to evaluate whether vaccination with HpaA and UreB with the non-toxic dmLT adjuvant could reduce the bacterial load in the stomach of mice. Our results show that, SL immunization with a combination of HpaA and UreB and dmLT significantly reduced the bacterial load in the stomach after challenge compared to unimmunized infected mice (paper I).

Stomach cytokine responses and inflammation after sublingual immunization with *H. pylori* lysate antigens and dmLT

Previous immunization studies using CT as adjuvant have shown that enhanced Th1 and Th17 response in the stomach was correlated with reduction in bacterial load in the stomach of mice [67-68]. Whether similar immune responses to co-administered antigens are induced in the stomach by dmLT is not known. Thus, we evaluated the CD4, IL-17A and IFN γ responses after SL vaccination. SL immunization using dmLT or CT as adjuvant resulted in a similar increase in gene transcripts for CD4, IL-17A and IFN γ compared to unimmunized infected mice (Fig 4A-C, paper I).

If dmLT is to be used as an adjuvant in a *H. pylori* vaccine in humans, it is important to evaluate whether protection is associated with enhanced inflammation in the stomach (post-immunization gastritis). Stomach tissue from vaccinated and unvaccinated mice was evaluated and given a score for cell infiltration (0-4), parietal cell destruction (0-6) and chief cell destruction (0-6). The score for each mouse from the different criteria was added to give a cumulative maximum score of 16. In sublingually immunized mice, a statistically significant increase in inflammation score was seen when using a vaccine containing *H. pylori* lysate antigens and the dmLT as adjuvant compared to that in unimmunized infected mice (Table 2, paper I).

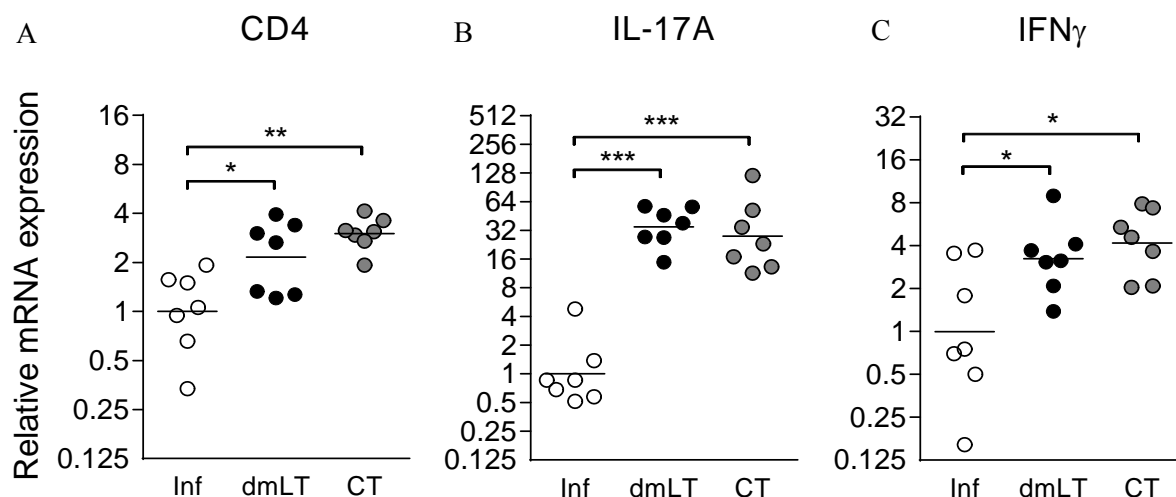


Figure 4. Stomach cytokine and CD4 gene expression is upregulated after sublingual immunization against *H. pylori* infection

Mice were unimmunized or immunized via SL route with *H. pylori* lysate antigens and dmLT or CT or left unimmunized (Inf) and challenged with live *H. pylori* bacteria. Gene expression of **A.** CD4, **B.** IL-17A and **C.** IFN γ was evaluated by RT-PCR in stomach tissue two weeks post-challenge. The difference between β -actin and the target gene (ΔCT) was determined, and the relative expression was calculated using the formula $2^{\Delta CT}$. The values were adjusted so that the mean in the infection control group was set to 1. The bars represent mean, data representative of two independent experiments. Statistically significant difference in gene expression between groups was assessed by unpaired two-tailed t-test with Welch correction and denoted by * ($p < 0.05$) ** ($p < 0.01$) and *** ($p < 0.001$).

Table 2. Inflammation score after sublingual immunization and challenge

	Inflammation Score
<i>H. pylori</i> lysate antigens + dmLT	3.4 ± 0.6 ***
<i>H. pylori</i> lysate antigens + CT	3.2 ± 0.7 **
Infection control	0.4 ± 0.1

§ Inflammation score in the stomachs of mice sublingually immunized or unimmunized mice post-challenge with live *H. pylori* bacteria. Data represents mean values \pm SEM with $n = 10$ mice /group and pool of two experiments. Statistically significant difference between group scoring values for immunized mice compared to unimmunized infected mice was assessed by unpaired two-tailed t-test with Welch correction and denoted ** ($p < 0.01$) and *** ($p < 0.001$).

Our results from paper I show that after SL immunization, the dmLT adjuvant induce strong mucosal and systemic immune responses with reduction in bacterial load in the stomach of mice. Remarkably, the removal of the toxicity of the molecule did not have a large impact on the adjuvant effect of dmLT since the immune responses were similar to those induced by CT.

PAPER II**Comparison of immune responses after sublingual or intragastric vaccination against *H. pylori* infection**

Previous studies have shown that both IG and SL immunization routes can enhance immune responses and reduce bacterial load in the stomach of mice [182]. We have previously observed reduced colonization of the bacteria in the stomach of sublingually compared to intragastrically immunized mice [182] (Fig. 5A, Paper I and II). However, to date, protection against *H. pylori* infection and immune responses have not been evaluated and compared directly between the two immunization routes (SL and IG) in the same study. Therefore, the aim of the study was to understand the underlying immune mechanisms behind the differences in bacterial colonization in sublingually and intragastrically immunized mice.

Post-immunization gastritis in the stomach of sublingually or intragastrically immunized mice

As mentioned previously, post-immunization gastritis was associated with reduction in bacterial load and we were therefore interested in comparing the gastritis scores after SL and IG immunization in these mice and its relation to the bacterial load. We observed that the inflammation score was significantly increased in the stomach of sublingually immunized mice compared to unimmunized infected mice (Fig. 5B). The inflammation score in the intragastrically immunized mice was also increased compared to unimmunized mice, but the difference was not statistically significant (Fig. 5B, paper II). However, there was a statistically significant decrease ($p < 0.01$) in the inflammation observed in intragastrically immunized mice compared to sublingually immunized mice (Fig. 5B). Thus the lower bacterial load seen in the stomach of sublingually immunized compared to intragastrically immunized mice might be related to the differences in the induction of local inflammatory response. Indeed, a statistically significant inverse correlation was found between the bacterial colonization and inflammation score in individual mice (*data not shown*).

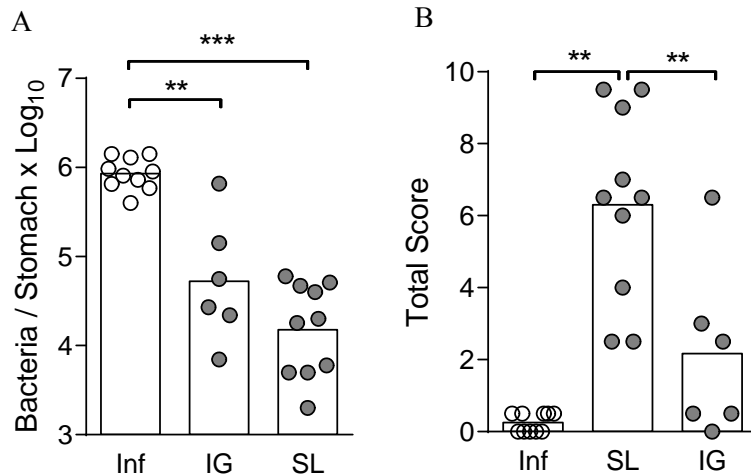


Figure 5. *H. pylori* bacterial load and inflammation score in the stomach of sublingually compared to intragastrically immunized mice. Mice were immunized via the SL or IG route with *H. pylori* lysate antigens and CT (SL or IG) or left unimmunized (Inf) and then challenged with live *H. pylori* bacteria. Two weeks post-challenge mice were evaluated for **A.** Protection by quantitative culture and expressed as mean log₁₀ values of bacteria per stomach and **B.** Inflammation score in formalin fixed stomach tissue stained with hematoxylin and eosin. Data are shown as mean values with n=6-10 mice/group and pool of two experiments. Statistically significant difference in bacterial load and inflammation score between groups was assessed by unpaired two-tailed t-test with Welch correction and denoted by ** (p<0.01) and *** (p<0.001).

Cytokine responses in the stomach in sublingually and intragastrically immunized mice

Next we studied in further detail the immune response in the stomach of sublingually and intragastrically immunized mice. As strong Th1 and Th17 responses have been associated with reduction in bacterial load in the stomach of immunized mice, we compared the IL-17A and IFN γ response in saponin extracts of the stomach tissue after SL and IG immunization. SL and IG immunization both induced IL-17A secretion in the stomach to a similar extent compared to unimmunized infected mice (Fig. 6A, paper II). There was a tendency for IFN γ secretion to be high in some intragastrically immunized mice compared to both sublingually immunized mice and unimmunized infected mice (Fig. 6B, paper II). The inflammation score was not related to the levels of IL-17A in the stomach as both sublingually and intragastrically immunized mice had comparable levels of IL-17A.

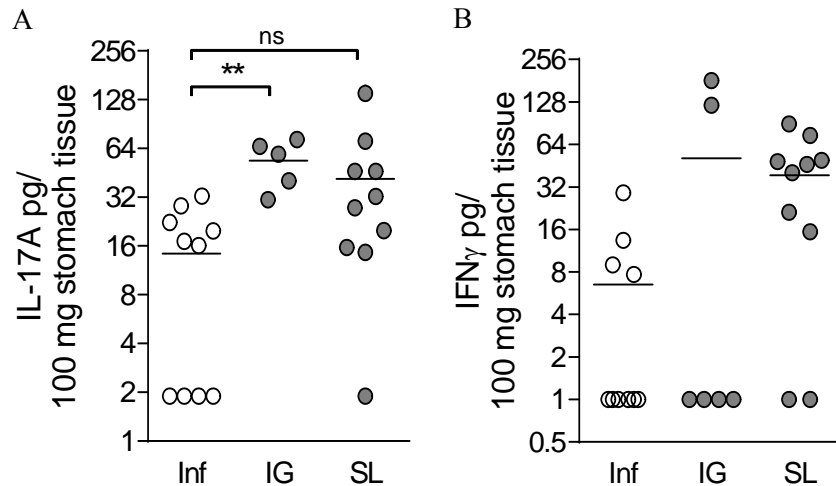


Figure 6. IL-17A and IFN γ secretion in the stomach after sublingual or intragastric immunization, post-challenge. Mice were immunized via SL or IG route with *H. pylori* lysate antigens and CT (SL or IG) or left unimmunized (Inf) and then challenged with live *H. pylori* bacteria. Two weeks post-challenge stomach tissue was evaluated for **A.** IL-17A and **B.** IFN γ levels in saponin extracts showing pg/ 100 mg stomach tissue; n=6-10 mice/group and mean values depicted on a Log₂ scale and pool of two experiments. Statistically significant difference in cytokine levels between groups was assessed by unpaired two-tailed t-test with Welch correction and denoted by ** (p<0.01) and ns (non-significant).

The main source of the IL-17A in MLN after vaccination is CD4⁺CD3⁺ T cells

As we observed increased levels of IL-17A in the stomach of vaccinated mice post-challenge, we next analyzed possible cellular source of the IL-17A. MLN cells were isolated from sublingually and intragastrically immunized and unimmunized infected mice after challenge. Single cell suspensions were stimulated with PMA and ionomycin for four hours and IL-17A and IFN γ secretion were evaluated in gated (i) CD3⁺CD4⁻, (ii) CD3⁺CD4⁺ and (iii) CD3⁻CD4⁻ cell populations. Flow cytometric analysis of MLN cells isolated from sublingually and intragastrically immunized mice revealed that the IL-17A was mainly produced by CD3⁺CD4⁺ cells and to some extent by CD3⁺CD4⁻ cells (paper II). In addition, CD3⁻CD4⁻ cells also produced IL-17A upon stimulation although not to the same extent as CD3⁺CD4⁺ cells (paper II). However, IL-17A production from other cell subsets than CD3⁺CD4⁺ cells were not increased compared to unimmunized infected mice (paper II). When analyzing the IFN γ secretion in the same cells, CD3⁺CD4⁻ cells and not CD3⁺CD4⁺ cells were the main source of the cytokine (paper II).

In summary, our data suggests that post-immunization gastritis is, inversely correlated with bacterial numbers and not IL-17A responses in the stomach of sublingually and intragastrically immunized mice

PAPERS II and III

Addressing the role of the cytokines IFN γ , IL-1 β and IL-23 in vaccine-induced protection and post-immunization gastritis

SL immunization was more efficient in reducing bacterial load in the stomach than IG immunization, but it was also associated with increased post-immunization gastritis. As cytokine levels of IL-17A and IFN γ was increased after SL immunization, the aim of the study was to determine whether protective inflammation can be separated from pathogenic inflammation by studying the role of IFN γ and IL-17A in these two processes. We found that IL-17A was important to the antimicrobial response to *H. pylori* infection in the stomach of sublingually immunized mice. Thus, to further validate the role of IL-17A in *H. pylori* infection we also studied the role of IL-17A-associated cytokines IL-1 β and IL-23 in vaccine-induced protection after SL immunization.

In order to study the function of each cytokine in the *H. pylori* infection model, we used gene-knockout mice for IFN γ , IL-1RI and IL-23p19 and evaluated the protection and immune responses in these mice after SL immunization and subsequent challenge with live *H. pylori* bacteria.

Vaccine-induced protection against H. pylori infection after sublingual immunization was induced in the absence of IFN γ and IL-23 but was partly dependent on IL-1 signaling

All three knockout strains of mice (IFN γ , IL-1RI and IL-23p19) were susceptible to *H. pylori* infection and were colonized to the same extent as wild-type mice. Sublingually immunized IFN γ ^{-/-} and wild-type mice were protected against *H. pylori* infection and were able to reduce their bacterial load significantly when compared with unimmunized infected mice (Fig. 7A, paper II). Additionally, the bacterial load continued to remain low in sublingually immunized IFN γ ^{-/-} mice 5 weeks post-challenge. (Fig. 7D, paper II). Remarkably, sublingually immunized IFN γ ^{-/-} mice administered IL-17A neutralizing antibody *in vivo* after challenge with *H. pylori* bacteria were not protected with increased bacterial load in the stomach compared to sublingually immunized mice receiving isotype control antibody (paper II).

In sublingually immunized IL-1RI^{-/-} mice, protection against *H. pylori* infection was affected by the absence of IL-1 signaling (Fig. 7B, paper III). Even though the bacterial load in the stomach was decreased in sublingually immunized IL-1RI^{-/-} mice compared to unimmunized mice, it was significantly higher than sublingually immunized wild-type mice (Fig. 7B, paper III). Sublingually immunized IL-23p19^{-/-} mice had a statistically significant decrease in bacterial load in the stomach compared to unimmunized infected mice, and the reduction was similar to that seen in sublingually immunized wild-type mice compared to unimmunized infected mice (Fig. 7C, paper III).

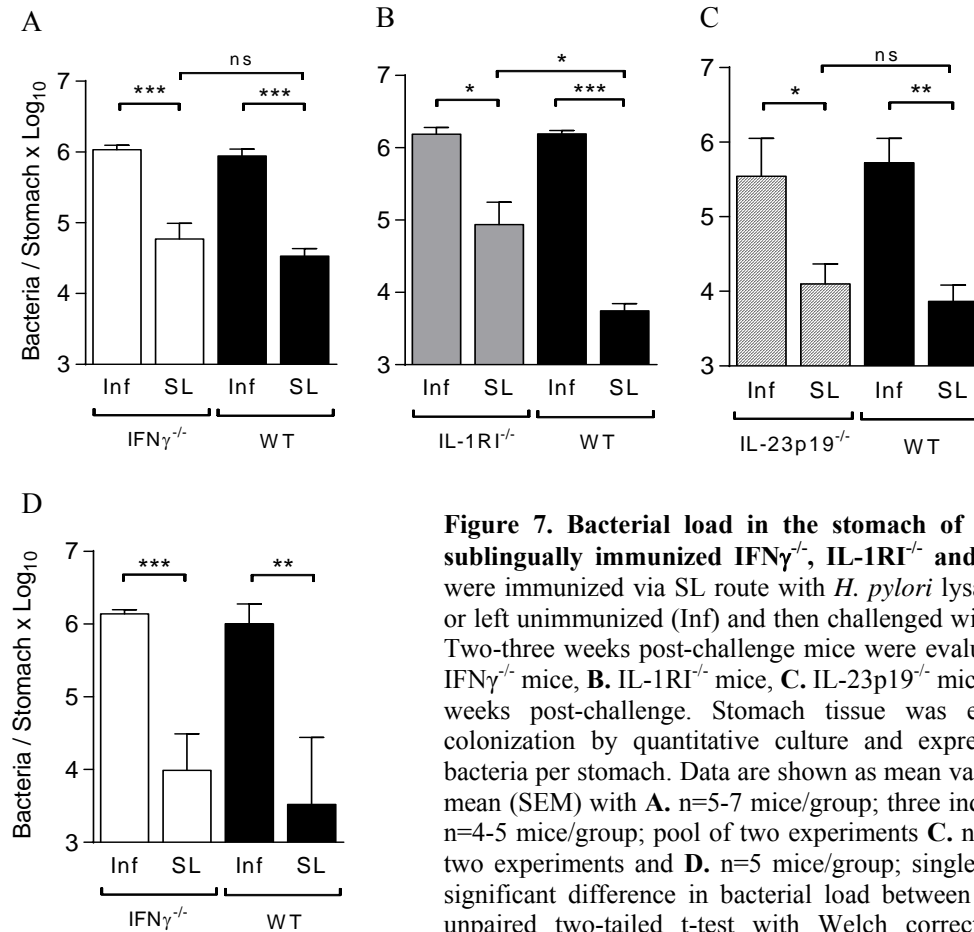


Figure 7. Bacterial load in the stomach of mice, post-challenge in sublingually immunized IFN $\gamma^{-/-}$, IL-1RI $^{-/-}$ and IL-23p19 $^{-/-}$ mice. Mice were immunized via SL route with *H. pylori* lysate antigens and CT (SL) or left unimmunized (Inf) and then challenged with live *H. pylori* bacteria. Two-three weeks post-challenge mice were evaluated for protection in **A**. IFN $\gamma^{-/-}$ mice, **B**. IL-1RI $^{-/-}$ mice, **C**. IL-23p19 $^{-/-}$ mice and **D**. IFN $\gamma^{-/-}$ mice at 5 weeks post-challenge. Stomach tissue was evaluated for *H. pylori* colonization by quantitative culture and expressed as log₁₀ values of bacteria per stomach. Data are shown as mean values and standard error of mean (SEM) with **A**. n=5-7 mice/group; three independent experiments **B**. n=4-5 mice/group; pool of two experiments **C**. n=5-7 mice/group; pool of two experiments and **D**. n=5 mice/group; single experiment. Statistically significant difference in bacterial load between groups was assessed by unpaired two-tailed t-test with Welch correction and denoted by * (p<0.05), ** (p<0.01), *** (p<0.001) and ns (non-significant).

Role of cytokines IFN γ and IL-17A in promoting inflammation in the stomach of sublingually immunized mice

Since IFN γ , IL-1 and IL-23 have been associated with inflammation and/or IL-17A responses, we evaluated the influence of these cytokines on the immune response in the stomach and post-immunization gastritis. Histopathology, saponin extracts for cytokine analysis and gene expression in the stomach was analyzed. The results showed that sublingually immunized IFN $\gamma^{-/-}$ mice had decreased inflammation in the stomach, but increased IL-17A secretion compared to sublingually immunized wild-type mice (Table 3, paper II). The already low inflammation score could be further dampened even more when neutralizing IL-17A antibody was administered *in vivo* to sublingually immunized IFN $\gamma^{-/-}$ mice (paper II). Sublingually immunized IL-1RI $^{-/-}$ mice also showed a decrease in inflammation score and the IL-17A and IFN γ gene expression was also decreased compared to sublingually immunized wild-type mice (Table 3, paper III). Furthermore, sublingually immunized IL-23p19 $^{-/-}$ mice had a decrease in inflammation score, compared to that of sublingually immunized wild-type mice (Table 3, paper III). Considering the role of IL-23 in maintaining IL-17A responses, the IL-17A secretion in the stomach of

sublingually immunized IL-23p19^{-/-} was unexpectedly not impaired and was instead similar compared to sublingually immunized wild-type mice (paper III). The sublingually immunized IL-23p19^{-/-} mice instead had an increased secretion of IFN γ compared to sublingually immunized wild-type mice (paper III). Taken together, after SL immunization, these results indicate that IFN γ and IL-1 signaling is important for inducing inflammation in the stomach while IL-23 appears to play a minor.

Table 3. Inflammation score after sublingual immunization in cytokine gene deficient mice

	<i>H. pylori</i> lysate antigens + CT	Infection control
IFN γ ^{-/-}	2.86 ± 1.04*	0.08 ± 0.08
WT	6.70 ± 1.25**	0.10 ± 0.10
IL-1RI ^{-/-}	3.46 ± 2.03	0.38 ± 0.13
WT	7.69 ± 1.76**	0.19 ± 0.09
IL-23p19 ^{-/-}	5.50 ± 2.84	0.70 ± 0.34
WT	10.33 ± 2.21**	0.55 ± 0.05

§ Inflammation score in stomachs of sublingually immunized or unimmunized mice post-challenge with live *H. pylori* bacteria. Data represents mean values ± SEM with n=4-9 mice /group (figure 7). Statistically significant difference between group scoring values for immunized mice compared to unimmunized infected mice was assessed by unpaired two-tailed t-test with Welch correction denoted *(p<0.05) and ** (p<0.01).

Neutralization of IL-17A in the sublingually immunized IFN γ ^{-/-} mice post-challenge also showed that IL-17A is important for reduction of bacterial load in the stomach following SL immunization. However this IL-17A production *in vivo* is independent of IL-23 while partially dependent on IL-1 signaling.

Cellular and antibody responses are intact in the absence of IFN γ , IL-23 and IL-1 signaling

Cellular proliferative responses from MLN and spleen cells and subsequent levels of cytokines in *in vitro* culture supernatants were evaluated in sublingually immunized wild-type and knockout mice. MLN and spleen cells were isolated from the respective groups of mice and stimulated *in vitro* with *H. pylori* lysate antigens and proliferative response as well as the cytokine production in culture supernatants were measured. Sublingually immunized mice deficient for IFN γ , IL-23 or IL-1 signaling showed no impairment in proliferative responses of MLN or spleen cells *in vitro* which were comparable to those of sublingually immunized wild-type mice (paper II and III). However, despite a strong proliferative response, IL-17A secretion was decreased in culture supernatants from MLN and spleen cells from sublingually immunized IL-1RI^{-/-} and IL-23p19^{-/-} mice compared to sublingually immunized wild-type mice (paper III). These results suggest that in spite of a vigorous proliferative response against *H. pylori* lysate antigens, IL-17A secretion is affected in the MLN cells from mice in the absence of IL-1 signaling, or IL-23, but not IFN γ .

In conclusion, the results from papers II and III showed that SL immunization is more efficient in reducing bacterial colonization of *H. pylori* in than IG immunization using *H. pylori* lysate antigens and CT as a vaccine. In addition, SL immunization induces increased IL-17A secretion in the stomach. Furthermore, we could also show that the IL-17A response was important for bacterial reduction while IFN γ promoted post-immunization gastritis. Finally, vaccine-induced IL-17A responses and reduction in bacterial load, post-challenge could be seen in the absence of IFN γ or IL-23. However, an intact IL-1 signaling was important for induction of IFN γ and IL-17A responses in the stomach and reduction in bacterial load post-challenge.

APPENDIX I

Evaluation of dmLT and CT adjuvants administered via the SL or IG route of immunization

Previous results presented in this thesis have provided knowledge about the vaccine-induced immune response after SL and IG immunization but they were evaluated post-challenge long after the immunization event. We were thus interested in the early priming of the immune responses in the draining lymph nodes after immunization. As DCs are among the first cell type to come into contact with the antigen and adjuvant, we evaluated the effect of contact with of the two adjuvants CT and dmLT on DC activation and the ability of DCs in the CLN (for SL immunization) and MLN (for IG immunization) to prime T cell proliferation and cytokine secretion after immunization using *H. pylori* lysate antigens and CT or dmLT. Finally, to localize the IL-17A response in the stomach we also quantitated the IL-17A staining in the stomach of sublingually and intragastrically immunized mice when CT or dmLT was used as an adjuvant.

Adjuvants dmLT and CT induce expression of genes related to activation and co-stimulation in DCs

To address the contribution of the adjuvants in the priming of the T cell response in the draining lymph nodes, we first compared the effect of CT and dmLT on DC activation. CD11c⁺ DCs were isolated using MACS beads from the spleen of mice injected with Flt3L-secreting melanoma and stimulated *in vitro* with 1 µg/ml each of CT or dmLT. DCs were collected after 5 hours of stimulation with the adjuvants and gene expression in the cells was evaluated by PCR array (Mouse Dendritic & Antigen Presenting Cell; SA Biosciences/Qiagen). The DCs upregulated activation markers such as CD80 and CD86 in the presence of 1 µg/ml of CT or dmLT (1.5-2-fold), compared to un-stimulated DCs (Table. 4). In addition, CXCL2 (10.8-fold) and thrombospondin-1 (TSP-1) (67-fold) were also highly upregulated after stimulation with CT and to a lesser extent after stimulation with dmLT (2-fold and 29-fold respectively) (Table 4). CXCL2 attracts neutrophils, while thrombospondin-1 is known to (i) activate TGFβ and (ii) promote angiogenesis by inhibiting vessel dilation [100, 112]. CT also induced upregulation of IL-6 greater than dmLT while dmLT induced higher levels of IFNγ transcription than CT in DCs (Table 4). In summary, compared to dmLT, CT induced elevated levels of specifically TSP-1, CXCL2 and IL-6 in DCs, whereas dmLT preferentially induced transcription of IFNγ. Validation of the PCR array results on selected genes (IL-6, IFNγ and CXCL2) was performed using in-house primers and confirmed the results of the RT-PCR array (*Data not shown*).

Table 4: Gene expression in dendritic cells stimulated with adjuvant CT or dmLT

Up-regulated genes			
Gene	1 μ g CT	1 μ g dmLT	Responding cell
CCR1	2.05	1.43	T cells
CD80	2.03	1.54	T cells
CD86	2.05	1.57	T cells
CXCL2 (Mip-2 α)	10.80	2.28	PMNs*, stem cells
Fc γ R1 (CD64)	2.99	1.41	M \emptyset , Monocytes
IFN γ	3.47	4.81	M \emptyset , T and B cells, NK cells
IL-6	5.92	2.71	B cells, Neutrophils
TSP-1 (Thrombospondin-1)	67.23	29.07	M \emptyset , Endothelial cells, PMNs*
Tnfsf11 (RANKL)	2.68	2.98	Osteoclast cells, Stromal cells

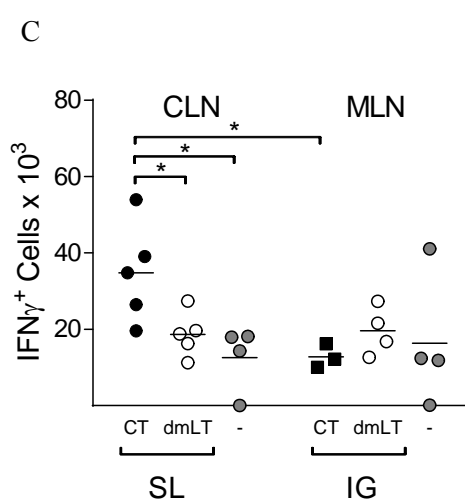
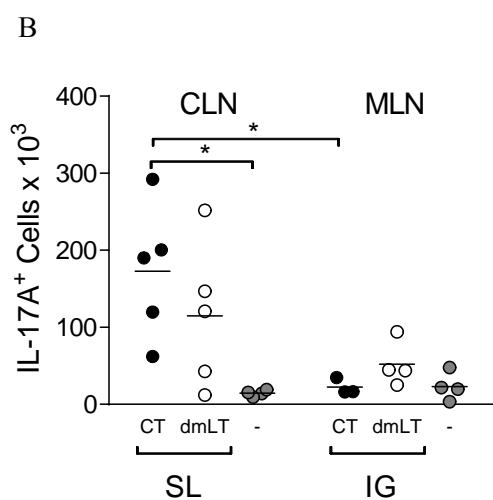
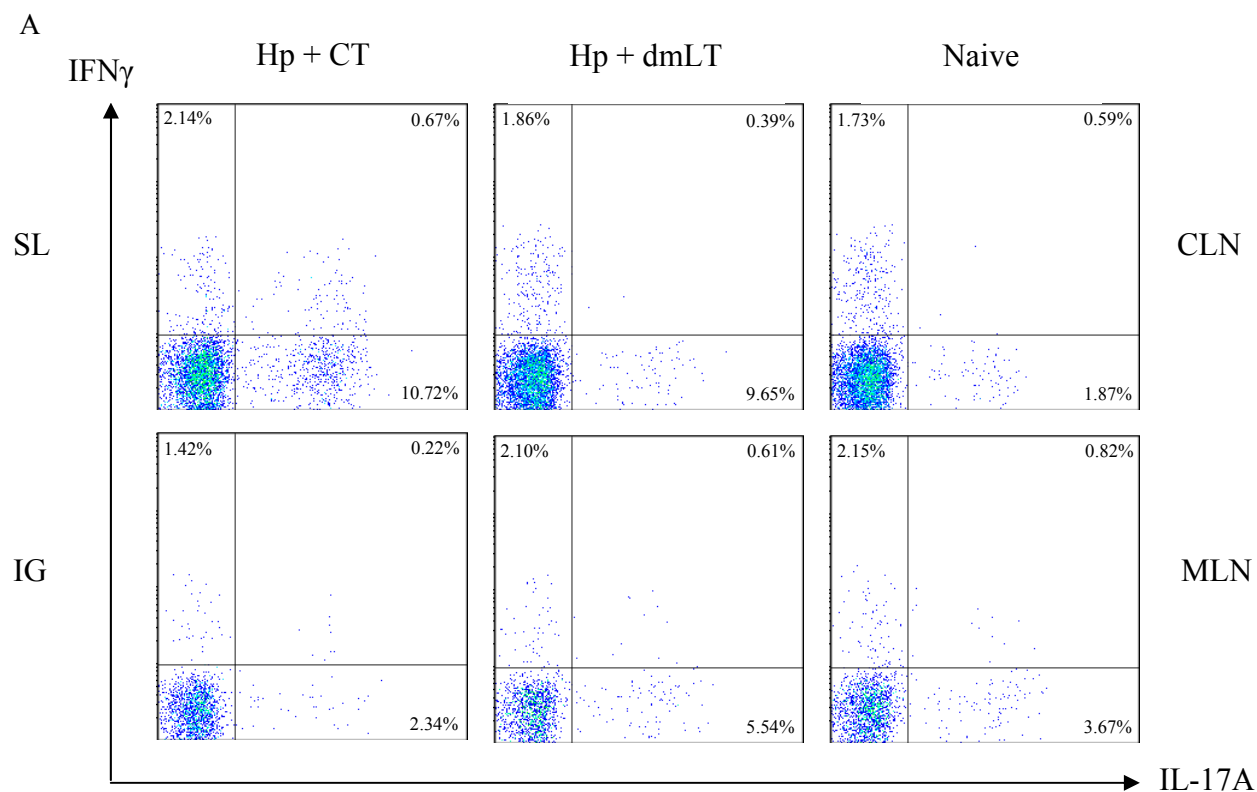
*PMN = polymorphonuclear cells, M \emptyset = Macrophages

Fold-Change	<0.5	0	2	4	8	16	32	64<
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§ RT-PCR array on cultured DCs. The difference between housekeeping genes and the target gene (ΔCT) was determined, and the relative expression was calculated using the formula $2^{\Delta CT}$. Values are then expressed as fold change compared to unstimulated DCs.

Sublingual immunization induces enhanced secretion of IL-17A and IFN γ in CLN T cells

We next evaluated the induction of IL-17A and IFN γ responses in the draining lymph nodes after SL or IG immunization. To block the exit of lymphocytes during the experiment, FTY720 was injected i.p. on day 1 and then every 48 h for 8 days. On day 2 mice were sublingually and intragastrically immunized using adjuvants CT and dmLT together with *H. pylori* lysate antigens. On day 7 after the immunizations, CLN and MLN cells were isolated and stained for Flow cytometry analysis. Our preliminary results show that, when comparing the routes of immunization, both adjuvants induced a stronger IL-17A and IFN γ responses in CLN cells than in MLN cells (Fig. 8A-C). However, when the two adjuvants were compared, CT induced strong IL-17A and IFN γ immune responses from CLN cells after SL immunization while CT and dmLT were comparable in the induction of IL-17A and IFN γ immune responses from MLN cells after IG immunizations (Fig. 8A-C).



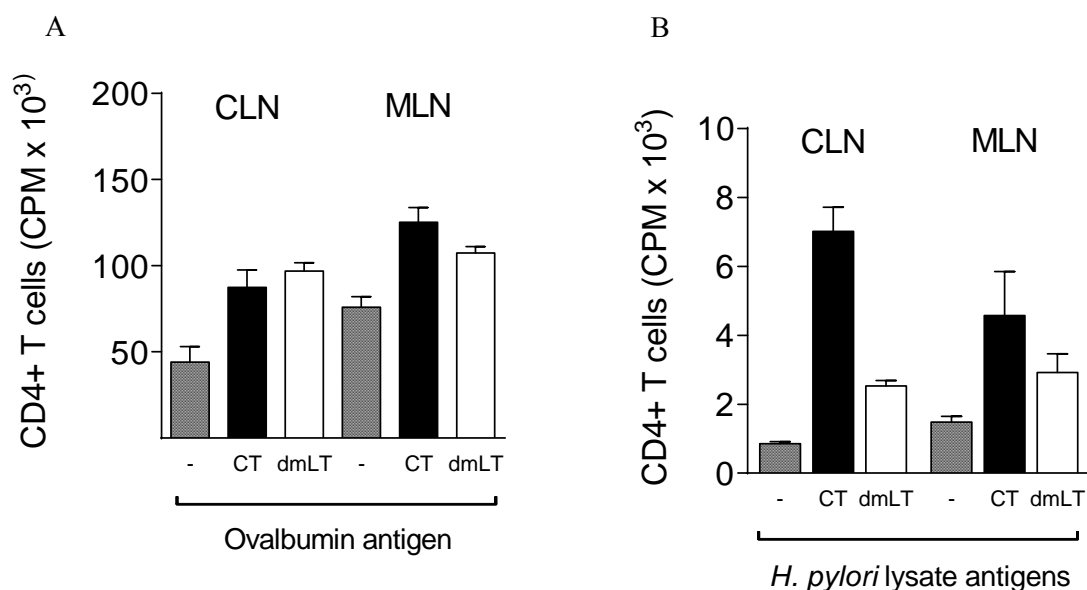
Appendix I. Figure 8. IL-17A and IFN γ responses in the lymph nodes after immunization

Mice were administered FTY720 i.p. on day 0 and every 2 days and mice were immunized via the SL or IG route with *H. pylori* lysate antigen and CT or dmLT or unimmunized (day 1). Seven days post-immunization, mice were sacrificed and CLN was taken from sublingually immunized mice and MLN from intragastrically immunized mice and single cell suspension was prepared for Flow cytometric analysis. Cells were stimulated with PMA + ionomycin and then stained for live cells, CD3, CD4, IL-17A and IFN γ . **A.** Flow cytometric analysis of IL-17A and IFN γ on cells gated on live, CD3⁺CD4⁺ cells. Percentage of Live CD3⁺CD4⁺IL-17A⁺ and Live CD3⁺CD4⁺IFN γ ⁺ cells **B.** number of total Live IL-17A⁺ IFN γ ⁺ cells in the lymph nodes after immunization. Bars represent mean with n=3-5 mice/group; pool of two experiments. Statistically significant difference was assessed by unpaired two-tailed t-test with Welch correction and denoted * ($p < 0.05$).

Dendritic cells isolated from MLN or CLN and pulsed with antigens and adjuvant CT induce enhanced CD4⁺ T cell proliferation

To further evaluate the T cell priming at the two draining lymph nodes after SL or IG immunization, we next addressed the induction of T cell responses by DCs from CLN and MLN cells and the effect of the adjuvants CT and dmLT on proliferation of T cells and cytokine secretion. We setup the *in vitro* culture assay system by using a model antigen ovalbumin and responder T cells from ovalbumin T cell transgenic (OT-II) mice (Fig. 9A). DCs were isolated from CLN and MLN of Flt3L-producing melanoma-injected mice and stimulated *in vitro* with 10^{-3} $\mu\text{g/ml}$ of CT or dmLT together with ovalbumin or *H. pylori* lysate antigens (Fig. 9A, B). DCs were collected after four hours of stimulation and thoroughly washed. CD4⁺ T cells were either isolated from spleens of OT-II mice, or from MLN of sublingually immunized mice challenged with *H. pylori* and were co-cultured with DCs pulsed with either 2 mg/ml ovalbumin or 20 $\mu\text{g/ml}$ *H. pylori* lysate antigens, respectively, with or without 10^{-3} $\mu\text{g/ml}$ CT or dmLT adjuvants. Pulsed DCs and antigen-specific CD4⁺ T cells were co-cultured for 5 days and proliferative and cytokine responses were measured. The results showed that pulsing DCs isolated from either MLN or CLN with ovalbumin and adjuvants induced higher proliferative responses by the CD4⁺ T cells than with ovalbumin alone (Fig. 9A). There was a tendency for DCs from MLN pulsed with CT and ovalbumin to induce increased proliferation in CD4⁺ T cells, compared to DCs from CLN pulsed with CT and ovalbumin (Fig. 9A). No such difference was observed in the ability of DCs from either MLN or CLN pulsed with dmLT and ovalbumin to induce CD4⁺ T cell proliferation. The results from these initial experiments provided information on DC activation, the optimal concentration of adjuvants, and the time point for measurement of the cytokine responses.

Using the conditions established for the proliferation of OT-II mice, priming of *H. pylori*-specific T cell responses by DCs in the presence or absence of adjuvant was then addressed. Our preliminary results showed only minor differences in the CD4⁺ T cell proliferative response induced by DCs isolated from MLN or CLN when *H. pylori* lysate antigens with or without adjuvant are used for pulsing (Fig. 9B). However the data also showed that DCs pulsed *in vitro* with *H. pylori* antigens and CT induced a more effective proliferation of CD4⁺ T cells than DCs pulsed with *H. pylori* antigens and dmLT (Fig. 9B).



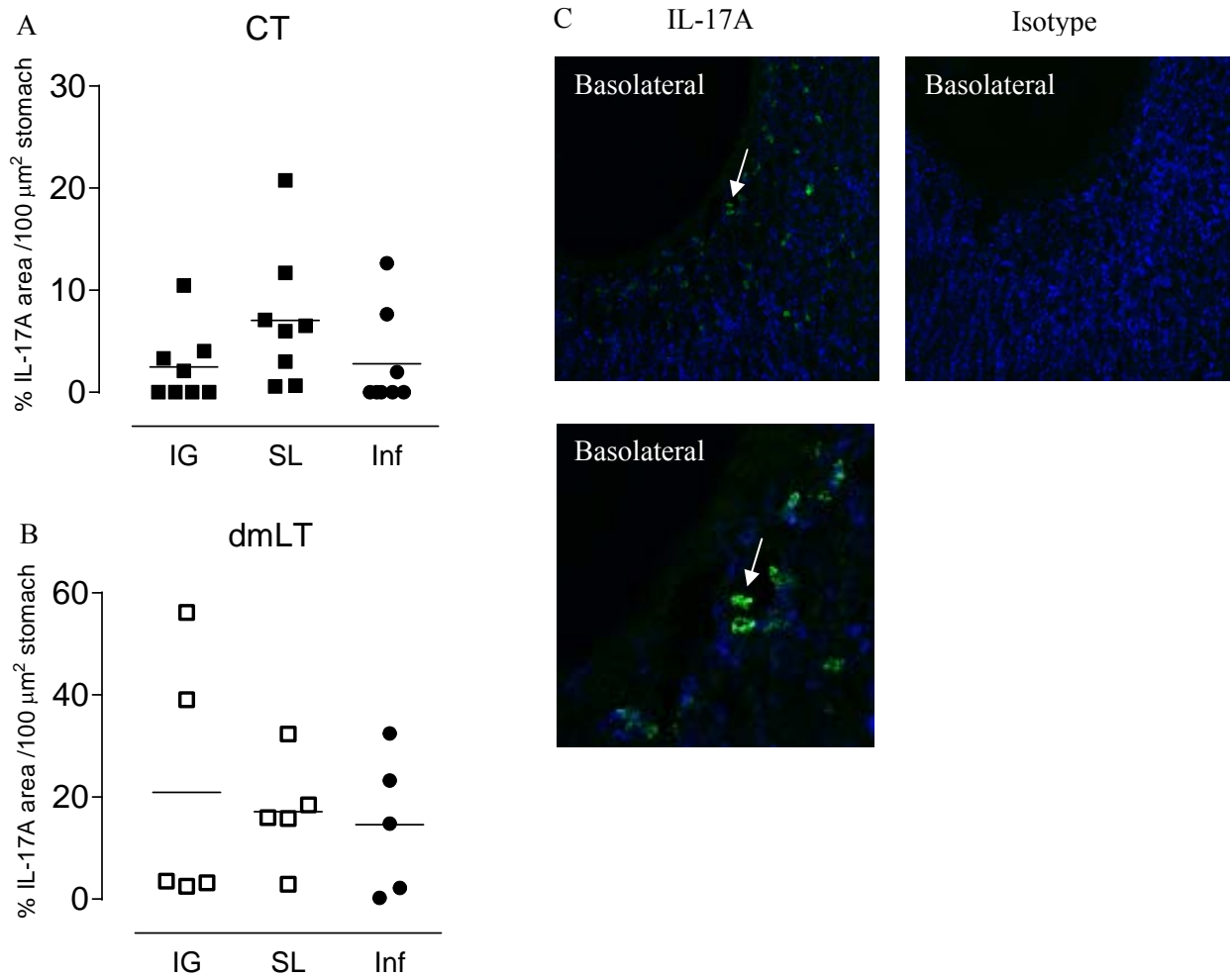
Appendix I. Figure 9. *In vitro* culture of DCs from MLN and CLN and proliferation of CD4⁺ T cells after exposure to *H. pylori* antigens

Mice were injected s.c. with Flt3L secreting melanoma cells to increase the number of DCs. DCs were isolated from MLN and CLN and pulsed with **A.** 2 mg/ml Ovalbumin antigen or **B.** 20 μ g *H. pylori* antigen with or without 10⁻³ μ g CT or dmLT for 4 h. **A.** CD4⁺ T cells from OT-II transgenic mice or **B.** CD4⁺ T cells from *H. pylori* infected mice were isolated and cultured together with pulsed DCs for 5 days. Thymidine incorporation was then measured and proliferation of CD4⁺ T cells determined. Data are shown as mean values of counts per minute in triplicate wells with standard deviation in one experiment. No statistical analysis was carried out on the data.

Sublingual and intragastric immunization with *H. pylori* lysate antigens induces an increase in IL-17A⁺ cells in the stomach

As we observed an increased frequency of IL-17A secreting cells in CLN of sublingually immunized mice and in the stomach post-challenge, we hypothesized that cells induced in the CLN possibly migrate to the stomach after challenge with live bacteria to perform their effector functions. Thus, we quantitated the numbers of IL-17A⁺ cells in the stomach of sublingually and intragastrically immunized mice and unimmunized infection controls by immunohistochemistry. The IL-17A⁺ staining reside mainly in the corpus region with some additional scattered cells in the antrum region of the stomach (Fig. 10C). The IL-17A⁺ cells were located in the lamina propria and submucosa (Fig. 10C). There was a tendency for the mean percent stained area of IL-17A to be higher in sublingually immunized compared to intragastrically immunized mice when using CT as adjuvant, although the difference was not statistically significant (Fig. 10A). When dmLT was used as adjuvant in the vaccine, the IL-17A⁺ staining in the stomach after *H. pylori* challenge was increased in intragastrically immunized groups compared to infection controls, although again the difference was not statistically significant (Fig. 10B). In summary, both SL and IG immunization induce IL-17A responses in the stomach after challenge with *H. pylori*.

In summary, our results show that immunizing mice via the SL route with either CT or dmLT as adjuvant together with *H. pylori* lysate antigens tend to induce a higher CD4⁺ IL-17A response in the CLN compared to IG immunization in the MLN although the IL-17A staining pattern in the stomach tissue was similar in sublingually or intragastrically immunized mice. One reason for the lack of difference in the IL-17A staining pattern in the stomach of immunized mice compared to the infection controls could be that the source of IL-17A might be different. Experiments were carried to co-stain IL-17A⁺ cells with CD4. However, the two staining protocols (IL-17A and CD4) were incompatible and thus made it difficult to assess the source of the IL-17A in the tissue. The data also show that CT and dmLT have similar effects on activation and co-stimulatory molecule expression in DCs, which might explain the high proliferative response by CD4⁺ T cells *in vitro* and possibly *in vivo*.



Appendix I. Figure 10. IL-17A⁺ staining in the stomach of mice after challenge with *H. pylori*.

Mice were immunized via the SL or IG route with *H. pylori* lysate antigens and CT (SL or IG) or dmLT (SL or IG) or left unimmunized (Inf) and then challenged with live *H. pylori* bacteria. Two weeks post-challenge the mice were sacrificed and the stomach tissue was taken for immunohistochemistry staining for IL-17A. IL-17A staining in mice immunized with **A. CT** or **B. dmLT**. **C.** Representative picture of IL-17A staining in the stomach (in green) and nuclear DAPI staining (in blue) and IL-17A isotype control. Data are shown as % stained area of IL-17A per 100 μm^2 area of stomach in individual mice with 5-8 mice/group, in pool of two independent experiments. Filled squares represent mice immunized with adjuvant CT and open squares represent mice immunized with adjuvant dmLT and filled circles unimmunized infection controls (Inf). No statistical significance was detected.

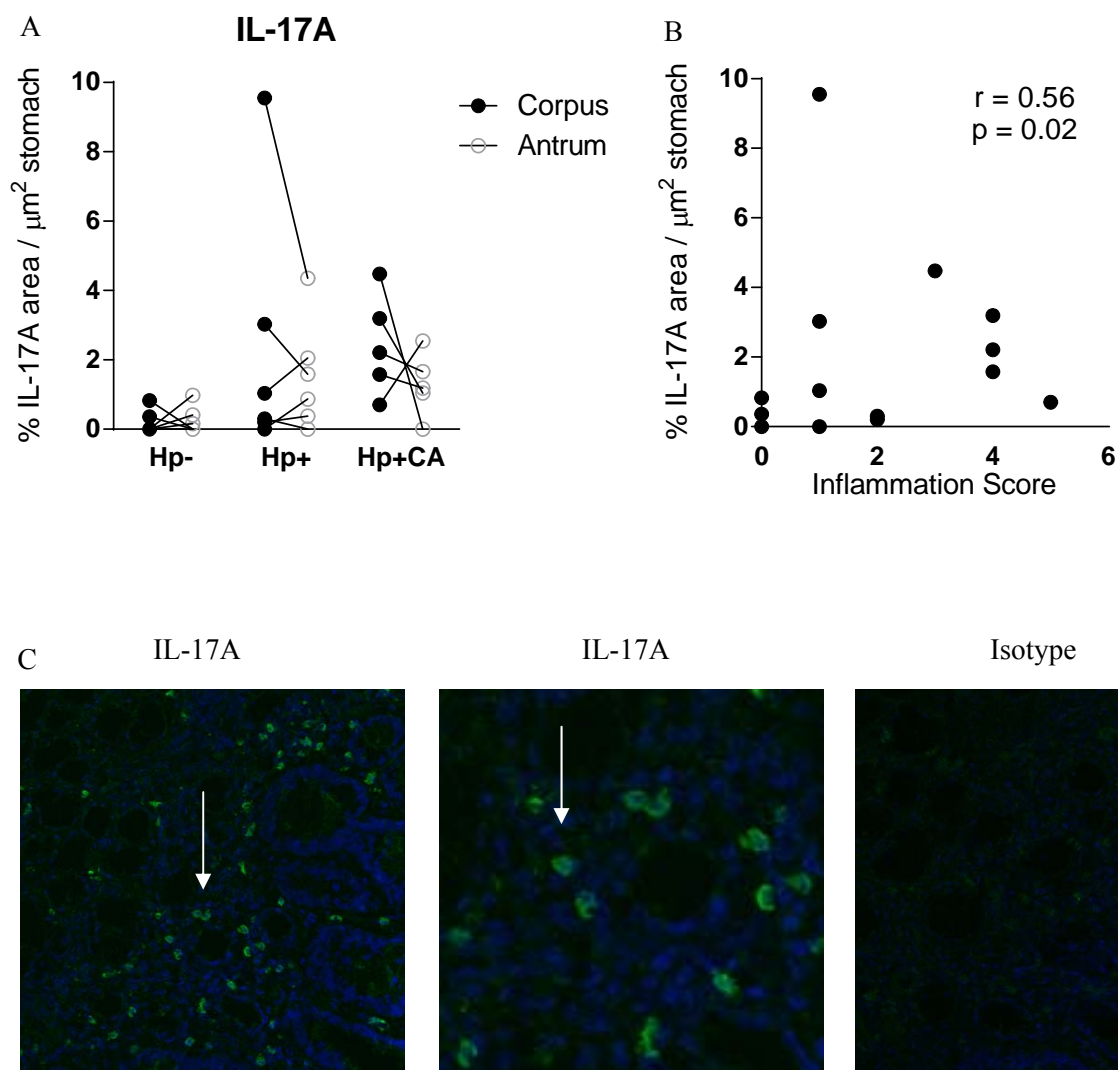
APPENDIX II

IL-17A staining pattern in the stomach of *H. pylori*-infected individuals and its relation to inflammation score

We could show in the *H. pylori* mouse model that IL-17A responses are increased upon vaccination and are associated with the decreased bacterial load seen in the stomach. However, the relevance of our results to the human *H. pylori* infection and disease is not known. Human gastric cells and human gastric cell lines have been shown to secrete IL-17A in response to *H. pylori* antigens [96, 193-194]. In addition, increased IL-17A responses in the stomach of *H. pylori*-infected individuals have been reported [35, 98, 133, 194]. However, this has not been correlated to bacterial load or inflammation. Thus, our aim was to evaluate the IL-17A response in biopsies from *H. pylori*-infected individuals and to correlate this with inflammation. We analyzed in a small cohort of individuals the IL-17A response in the stomach and its relation to bacterial load and inflammation.

Increased staining for IL-17A in the stomach of H. pylori-infected individuals

Antrum and corpus gastric biopsies obtained from adult volunteers were stained for IL-17A by immunohistochemistry; a separate biopsy from the same individual was evaluated by a pathologist for inflammation score according to the Sydney system [175]. Biopsies were obtained from *H. pylori*-positive (Hp^+) individuals with ongoing inflammation but no corpus atrophy, from *H. pylori*-positive individuals with corpus atrophy (Hp^+ CA) and for control purposes from *H. pylori*-negative (Hp^-) individuals [5]. IL-17A staining in the gastric mucosa was quantified in individual biopsies from the antrum and corpus, respectively and related to the inflammation score from the same individual. Corpus biopsies had increased IL-17A⁺ staining compared to antral biopsies from Hp^+ individuals although the difference was not statistically significant. A tendency for increased IL-17A⁺ staining of corpus biopsies from Hp^+ CA compared to Hp^+ individuals was also observed (Fig. 11A,B). Histopathological evaluation of these biopsies revealed that an increase in IL-17A⁺ staining correlated with inflammation score in corpus biopsies (Fig. 11C). This relationship could not be observed for the antral biopsies. Thus, our analyses show that IL-17A is increased in gastric antral and corpus biopsies collected from *H. pylori* infected individuals, in particular from those with an ongoing inflammation in the corpus mucosa.



Appendix II. Figure 11. Increased IL-17A⁺ staining of stomach corpus biopsies from *H. pylori* infected individuals.

Antral and corpus biopsies were obtained from *H. pylori*-negative (Hp-) individuals with non-inflamed mucosa, *H. pylori*-positive (Hp+) individuals and *H. pylori*-positive individuals with corpus atrophy (Hp+CA) which were stained for IL-17A. **A.** Data are shown as % stained area of IL-17A per μm^2 of individual antral and corpus biopsies from Hp- (n=6), Hp+ (n=6) and Hp+CA (n=5) individuals in each group; antral and corpus biopsies have been taken from the same individual. Filled circles represent corpus biopsies and open circles represent antral biopsies **B.** Non-parametric two-tailed Spearman correlation analysis between the % IL-17A stained area in the corpus and inflammation score in the same individual. A statistically significant correlation between increased IL-17A staining and inflammatory score was detected, meaning that those individuals with increased IL-17A staining had increased inflammation score in the corpus. **C.** Representative picture of IL-17A staining (in green) and DAPI nuclear staining (in blue) and IL-17A isotype control staining in the stomach biopsies.

Discussion

Several mucosal routes of vaccination have been evaluated with different combinations of antigens and adjuvants reporting varying levels of protection against *H. pylori* infection. However, the most effective route for immunization against *H. pylori* infection and its impact on the immune responses is not known. This thesis has evaluated IG and SL route of mucosal immunization and the use of the adjuvants dmLT and CT. The work in the thesis has also addressed the role of IL-17A and IFN γ in the protective immune responses against *H. pylori* infection and post-immunization gastritis. Knowing the appropriate immune response necessary for eradication of *H. pylori* infection with minimal post-immunization gastritis is essential for the rational design of vaccine and for planning vaccination strategies in countries where *H. pylori* infection is highly prevalent in the children and adults.

SL immunization with *H. pylori* lysate antigens and dmLT as a mucosal adjuvant was efficient in inducing immune responses and protection against *H. pylori* infection in mice. Notably the effect on immune responses was comparable to that of CT. CT and LT belong to the family of AB toxins and are strong mucosal adjuvants, but extremely toxic in humans. Non-toxic derivatives have been developed by introducing mutations in the A subunit which leads to attenuation of ADP-ribosylating activity and induction of intracellular cAMP. Protection against *H. pylori* infection after IG immunization has been reported in mice using two such derivatives, LTK63 and mLT (R192G) [53, 74, 142, 210]. However, in humans, intranasal administration of the adjuvant LTK63 with human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* antigens led to a serious side-effect called Bell's palsy (facial paralysis). The use of the adjuvant in vaccines was discontinued indefinitely which was very discouraging for the field of mucosal vaccines [119]. The adjuvant mLT (R192G) have also been evaluated in humans together with inactivated *H. pylori* whole cell vaccine via the IG route. However the study reported diarrheal side-effects in volunteers that received the adjuvant with or without the inactivated bacteria [104] which again resulted in the adjuvant being discontinued for use in vaccines. Despite these drawbacks and with the ambition to find an alternative adjuvant, a new mutant of LT was constructed carrying a two mutations in the A1 subunit (dmLT), again with the aim of attenuating the ADP-ribosylating activity and minimizing the cAMP production [160]. The potential for dmLT to function as a mucosal adjuvant has been evaluated in mice using a lyophilized *H. pylori* whole-cell (HWC) vaccine proving to be efficient in reducing the live *H. pylori* bacterial load in the stomach of mice [210]. SL immunization with dmLT and a pneumococcal whole cell vaccine was shown to confer protection against pneumococci infection in mice [126]. Given the history of failures using detoxified enterotoxins as mucosal adjuvants, further studies of dmLT in larger clinical trials are warranted before it can be considered suitable for use in a mucosal vaccine particularly in children.

In our study we show that SL immunization with *H. pylori* antigens and dmLT induces strong *H. pylori* specific proliferative responses in MLN and spleen cells. This proliferative response was accompanied by an increase in IL-17A and IFN γ cytokine secretion by these cells which was comparable to when CT was used as an adjuvant in the vaccine. Further, SL immunization using either CT or dmLT together with *H. pylori* lysate antigens induced enhanced *H. pylori*-specific stomach IgA and serum IgG antibodies. In support of our findings, studies using dmLT together with *H. pylori* antigens, Enterotoxigenic *E.coli* (ETEC)-expressing colonization factors, *Shigella spp.* antigens or tetanus toxoid for IG or IN immunization, have confirmed that antigen-specific antibody responses are enhanced by dmLT as well as an IL-17A response in mice [80, 86, 160-161, 183, 210]. In addition, the *in vitro* IL-17A response to the purified protein derivative (PPD) by peripheral blood mononuclear cells, from individuals vaccinated with *Bacillus Calmette-Guérin* (BCG) can be enhanced by dmLT [113]. Importantly, in phase I clinical trials dmLT administered intragastrically to volunteers alone or in combination with an ETEC vaccine was well tolerated ([61] and Lundgren et al Personal communication). Thus, the results presented in this thesis together with preclinical and phase I trials performed in volunteers suggests that dmLT is a safe non-toxic adjuvant with potential for use in human vaccines administered via the mucosal route. Remarkably, our preliminary data suggests that SL immunization with recombinant antigens from *H. pylori* together with dmLT confers protection against *H. pylori* infection (Paper I), making dmLT also a promising candidate mucosal adjuvant for inclusion in a *H. pylori* vaccine in humans. The safety issues regarding the translocation of components of adjuvants and bacteria through the olfactory bulb when immunizing via the IN route are not likely to occur if the SL or IG routes of vaccination were used.

Several mechanisms have been proposed whereby dmLT is able to induce such strong mucosal immune responses which are comparable to using a highly toxic adjuvant, CT. The binding of CT to cells is mediated through cholera toxin B subunit (CTB) binding to the GM1 ganglioside which is present on most nucleated cells [87]. The B subunit of LT also has the ability to bind to GM1 and some additional receptors [174]. Since dmLT is a derivative of the LT molecule, it is possible that it may bind to additional receptors compared to CTB. Also dmLT is known to induce low amounts of cAMP in host cells which may be sufficient for the adjuvant function but not for the toxicity [160]. It has been proposed that the ADP-ribosylation activity and adjuvant function of CT, LT or their derivatives are closely related [134, 184, 190]. However, the precise mechanism whereby dmLT performs its functions inside the host cells is largely unknown. Our preliminary data showed that dmLT can induce cytokine and chemokine gene expression such as IFN γ , CXCL2 and IL-6 in DCs, although to a somewhat lesser extent than CT, suggesting that dmLT is less pro-inflammatory than CT. However, it is interesting that the adjuvant functions of dmLT and CT are almost comparable despite their molecular differences in toxicity.

Our studies have also focused on the evaluation of the IG and SL routes of immunization in protection against *H. pylori* infection. We report that both IG and SL routes of immunization

using *H. pylori* lysate antigens and CT induce significant reduction in the bacterial load in the stomach of mice. But the sublingually immunized mice had lower bacterial load in the stomach than intragastrically immunized mice. IG immunization is the route which has been extensively evaluated in the field of vaccine-induced protection against *H. pylori* infection which is not surprising as the bacteria colonizes in the stomach. Prophylactic or therapeutic IG route of vaccination using CT as adjuvant together with inactivated *H. pylori* whole-cell bacteria or lysate antigens, has been shown to provide protection against *H. pylori* infection and against reinfection in mice [163-164, 168, 181-183]. IG immunization in humans would be the obvious route of immunization as the stomach is the environment the bacteria first encounter. In addition a drinkable vaccine would be highly desirable due to the ease in administration to both children and adults. Furthermore needle-free vaccines reduce the risk associated with spreading disease through infected needles and the need of trained personal for administration of the vaccine, although a cold chain handling of the vaccine maybe required as it is for the Cholera vaccine, Dukoral®. Indeed, encouraging for the *H. pylori* vaccine field, based on preclinical studies in mice (203) a recent phase I clinical trial has shown that IG immunization using formalin-killed *E.coli* bacteria over-expressing colonization factors for ETEC and the hybrid binding subunit protein (LCTBA) generates strong mucosal and systemic antibody responses in human volunteers against ETEC [86, 127]. In humans, the IG immunization route has already been in use for a wide range of clinical trials with *H. pylori* antigens and mucosal adjuvants. As only antibody and not T cell responses were evaluated, the antibody titers in the volunteers cannot be correlated to the protective effects of the vaccine [216]. Despite the obvious advantages of the IG route of immunization for a *H. pylori* vaccine, there are some concerns regarding the stability of the antigen and adjuvants in the harsh acidic environment and the difficulty in determining the dose required for an effective immune response and protection against *H. pylori* infection. Furthermore, the studies have evaluated a single route of immunization and it is unknown whether the IG route is the most efficient in inducing immune responses against *H. pylori*.

SL immunization has previously been reported to induce protection against *H. pylori* infection when CT and *H. pylori* lysate antigens have been used [182]. In addition, as mentioned previously, we report now that when dmLT and *H. pylori* lysate antigens were administered via the SL immunization route, bacterial loads were highly reduced, to the same extent as when CT was used. When comparing SL and IG route of immunization against *H. pylori* infection using CT and *H. pylori* lysate antigens, there was a tendency in our study and in earlier reports [182] for the SL route of immunization to be more efficient in reducing bacterial load in the stomach than the IG route. This was particularly evident when dmLT was used with *H. pylori* lysate antigens [201] or when recombinant antigens HpaA and UreB and CT were used in the vaccine [69]. The difference in protection against *H. pylori* infection after SL and IG immunization reported in the two studies was statistically significant [69, 201]. The SL route of immunization is not confined to the *H. pylori* field of research, but has been used in administering vaccines against other mucosal infections in mice. For example, recently, SL immunization using

recombinant influenza virus hemagglutinin protein 1 (sHA1) and CT was shown to confer protection in mice against the pandemic influenza virus H1N1 and also induced antibody responses in mucosal and systemic tissues [196]. The SL mucosa is a site where small molecular weight drugs and molecules can be applied and transported rapidly via the blood stream, and more importantly the route is already used in humans. However, the mechanisms of lymphocyte recirculation after SL immunization are not completely defined and the ability to induce immune responses at other mucosal sites in humans is not known.

In addition to evaluating the bacterial load in mice after immunization via SL and IG route, we also evaluated the inflammation and cytokine responses generated against *H. pylori* infection. It has previously been reported that both IG and intranasal immunization using *H. pylori* lysate antigens and CT induce inflammation in the stomach post-challenge referred to as post-immunization gastritis in mice [72, 77, 213]. As inflammation is generally associated with protection, it introduces a problem when proceeding to clinical trials [72, 77]. However, at least in mice it has been shown that when the infection is cleared the post-immunization gastritis will subside. [72]. When comparing post-immunization gastritis after challenge, intragastrically immunized mice had a lower inflammation score in the stomach compared to SL immunized mice. However, in the stomach, only IL-17A was elevated in intragastrically immunized mice while both IFN γ and IL-17A were elevated in sublingually immunized after challenge with live bacteria.

In our preliminary experiments we could show that SL immunization increased the number of CD4⁺ T cells secreting IL-17A⁺ and IFN γ ⁺ in the CLN compared to the number of CD4⁺ T cells secreting IL-17A⁺ and IFN γ ⁺ cells in MLN after IG immunization. Surprisingly, the IL-17A secretion and staining in the stomach was comparable in intragastrically and sublingually immunized mice, suggesting that post-challenge CD3⁺CD4⁺IL-17A⁺-producing cells are expanded in the MLN (Paper II) that migrate to the stomach. Taken together, our preliminary data suggests that there is a bias for priming IL-17A and IFN γ in CLN than in MLN. However, it is possible and not tested in the current study, if repeated immunizations would induce IL-17A responses in the MLN.

Previous studies evaluating the role for IFN γ in vaccine-induced protection against *H. pylori* infection in mice have unfortunately generated indecisive results [12, 71]. Our data showed that sublingually immunized IFN γ ^{-/-} mice had a similar reduction in bacterial load compared to immunized wild-type mice. Interestingly, similar to our observations in sublingually immunized IFN γ ^{-/-} mice, the intranasal route of immunization using *H. pylori* lysate antigens and CT in IFN γ ^{-/-} mice also results in reductions in bacterial load. Our study also confirms previous observations that *in vivo* neutralization of IFN γ after SL immunization had no effect on the bacterial reduction against *H. pylori* [68]. In addition, the SL immunized IFN γ ^{-/-} mice also had

lower level of inflammation in the stomach compared to immunized wild-type mice, indicating instead a pivotal role for IFN γ in inflammation. Although IFN γ has been shown to have a protective role in intracellular bacterial infections, for example, in *Mycobacterium* and *Salmonella* infections [70] a dominant role in protection against extracellular pathogens might be questioned. Instead, considering the known functions of IL-17A at mucosal sites (see next section), it is possible that IL-17A alone can fight extracellular infections. Indeed, susceptibility to chronic mucocutaneous candidiasis in humans is associated with a deficiency in IL-17A responses which further confirms the protective role of IL-17A in extracellular mucosal infections [48]. The sublingually immunized IFN γ ^{-/-} mice had elevated levels of IL-17A in the stomach compared to immunized wild-type mice and similar bacterial load suggesting that IL-17A can lower the bacterial numbers in the stomach. In agreement with our results, a recent study in mice deficient in Th1 responses (IL-12p35^{-/-}) showed that immunization via the intranasal route, resulted in increased IL-17A responses in the stomach and protection against *H. pylori* infection [56].

When IL-17A was neutralized in SL immunized IFN γ ^{-/-} mice post-challenge, bacterial load was increased to the level of unimmunized infected mice. These results suggest a role for IL-17A in bacterial reduction after SL immunization. The role of IL-17A in protection after IG immunization in IFN γ ^{-/-} mice however, has not yet been addressed. That IL-17A may play a pivotal role in the anti-bacterial response may not be surprising considering the many effects of IL-17A on the immune system including, recruitment of neutrophils and inducing anti-microbial peptide production by epithelial cells [101]. In addition IL-17A has also been shown to be important for induction of IgA responses [32, 83]. Since IL-17A was only neutralized at the time of challenge the effects can be quite different from lacking IL-17A at the time of immunization and through the entire experiment. Thus we wanted to further study importance of IL-17A during the priming phase of the immune response in inducing immune responses and protection against *H. pylori* infection. This was addressed by using knockout mice lacking cytokines IL-1 and IL-23 that are important in the induction or maintenance of IL-17A responses [25, 219]. We could have utilized IL-17A^{-/-} mice; but, when we initiated the study it was already reported that intranasally immunized IL-17A^{-/-} mice are protected against *H. pylori* infection [51]. Although not discussed in the article, we found that in IL-17A^{-/-} mice intranasally immunized with *H. pylori* lysate antigens and CT elevated levels of IL-17F which may compensate for the lack of IL-17A as they bind to the same receptor and have overlapping functions ([118] *and unpublished observations*). Thus we chose to use IL-23p19^{-/-} mice that lack the cytokine important for the maintenance of IL-17A⁺ cells. IL-23p19^{-/-} mice were first described to be resistant to experimental autoimmune encephalomyelitis (EAE) which is known to be promoted by IL-17A-secreting CD4⁺ T cells. In humans, IL-1 β is important together with IL-6 and IL-23 in the induction of IL-17A responses [3, 141, 223, 225]. During *H. pylori* infection, IL-1 β , IL-23, IL-17A and IFN γ have been shown to be elevated in the stomach of *H. pylori* infected individuals compared to uninfected individuals indicating that the conditions necessary for the polarization

of the T cell response to IL-17A production are present in *H. pylori* infected individuals [21, 35, 65, 124, 194, 203].

IL-23 has been shown to play an important role in promoting intestinal inflammation in a T cell transfer model of colitis as IL-23R^{-/-} mice and IL-23p19^{-/-} mice were resistant to colitis development [10, 218]. Previous studies on colonization of *H. pylori* SS1 in IL-23p19^{-/-} mice have reported a slightly higher bacterial load and reduced inflammation 3-4 month post-infection [88]. However, when we initiated our study, the role of IL-23 in maintaining vaccine-induced IL-17A responses was unknown. Sublingually immunized IL-23p19^{-/-} mice were able to reduce the bacterial load to a similar extent as sublingually immunized wild-type mice. These mice also had IL-17A and IFN γ production in the stomach, to a similar extent as sublingually immunized wild-type mice. Although this was a surprising observation, studies in the T cell transfer model of colitis in IL-23p19Rag1 double knockout mice have shown that IL-17A can be secreted by T cells in the intestine during inflammation despite the lack of IL-23 [10, 90]. Thus in the stomach and colon, IL-23 may be dispensable for the maintenance of IL-17A responses. Indeed, a recent study also presented findings similar to our own showing that intranasally immunized IL-23p19^{-/-} were protected against *H. pylori* infection albeit with a low level of IL-17A gene expression the stomach [56]. In the SL immunized IL-23p19^{-/-} mice, protection against *H. pylori* infection was associated with a lower inflammation score in the stomach compared to SL immunized wild-type mice. There have been studies suggesting that IL-23 can drive a pathogenic Th17 population [90], and that in the absence of IL-23, a preferential IL-10 producing Th17 population develops [146]. In addition, IL-23 has been shown to block regulatory T cell expansion which might further suggest a role for the IL-23 to generate a pathogenic immune responses [9]. We have not evaluated in our studies the IL-10 production in the stomach of sublingually immunized IL-23p19^{-/-} mice, but this would be of interest as the non-pathogenic Th17 cells may be the protective against *H. pylori* infection.

IL-1 β has been shown to be essential for the induction of IL-17A responses to co-administered antigen using CT as an adjuvant [84]. Our studies showed that SL immunization was less efficient in reducing the bacterial load in IL-1RI^{-/-} mice, suggesting an important role for IL-1 β signaling in vaccine-induced protection against *H. pylori* infection. The requirement for IL-1 β signaling has also been observed in mice after IG immunization in vaccine-induced protection against *H. pylori* infection [84]. The same study also showed that IFN γ and IL-17A gene expression was disturbed in the stomach of vaccinated IL-1RI^{-/-} mice. As both IFN γ and IL-17A responses were reduced in the stomach of IL-1RI^{-/-} mice the higher colonization in the sublingually immunized mice may be due to either reduced IFN γ or reduced IL-17A. However, as we have seen that protection after SL immunization was not dependent on IFN γ we believe that the reduced protection that we observe the stomach of IL-1RI^{-/-} mice may be due to a defect in IL-17A secretion. IL-1 β signaling has also been shown to be critical for the development of

Th17 cells in an EAE mouse model in which IL-1RI^{-/-} mice were shown to be resistant to the development of EAE with a greatly reduced number of CD4⁺ T cells secreting IL-17A in the brain lesions [211]. One caveat to using IL-1RI^{-/-} mice, to study the role of this cytokine in protection against *H. pylori* infection is that the adjuvant functions of CT might be impaired in these mice. Thus it cannot be ruled out, that the reduced protection we see in SL immunized IL-1RI^{-/-} mice might be due to an impaired adjuvant activity of CT in the absence of IL-1 β . We have addressed this possibility by studying the immune responses in the wild type and IL-1RI^{-/-} mice after SL immunization with *H. pylori* antigens alone or *H. pylori* antigens and CT before challenge with live bacteria. We observed no difference in *H. pylori*-specific serum IgG response but a reduction in *H. pylori*-specific IgA responses in SL immunized IL-1RI^{-/-} compared to wild-type mice. Our results suggest that, as discussed previously, the adjuvant function of CT is necessary for protection against *H. pylori* infection and that it may be mediated through IL-1 β during immunization.

Our studies in mice have suggested a role for IL-17A in protection and to a lesser extent in inflammation against *H. pylori* infection. The role of IL-17A in human *H. pylori* infection is only now beginning to be elucidated. Two elegant studies comparing the IL-17A responses in the stomach of children (>10 years) and adults have shown that lesser gastritis in *H. pylori* infected children is associated with less IL-17A responses compared to *H. pylori* infected adults that had a higher IL-17A secretion in the stomach and increased gastritis score. These association studies in *H. pylori* infected individuals suggest that IL-17A might be pro-inflammatory [95, 195]. Co-staining of IL-17A and bacteria or neutralization of IL-17A in *H. pylori*-infected individuals and its impact on bacterial load and inflammation might give a clue as to whether IL-17A is indeed pro-inflammatory. Thus, when designing a vaccine for human use, it is important to keep in mind that children and adults may respond differently to the vaccine with respect to the IL-17A response and the efficacy may therefore be different, if the IL-17A response is protective.

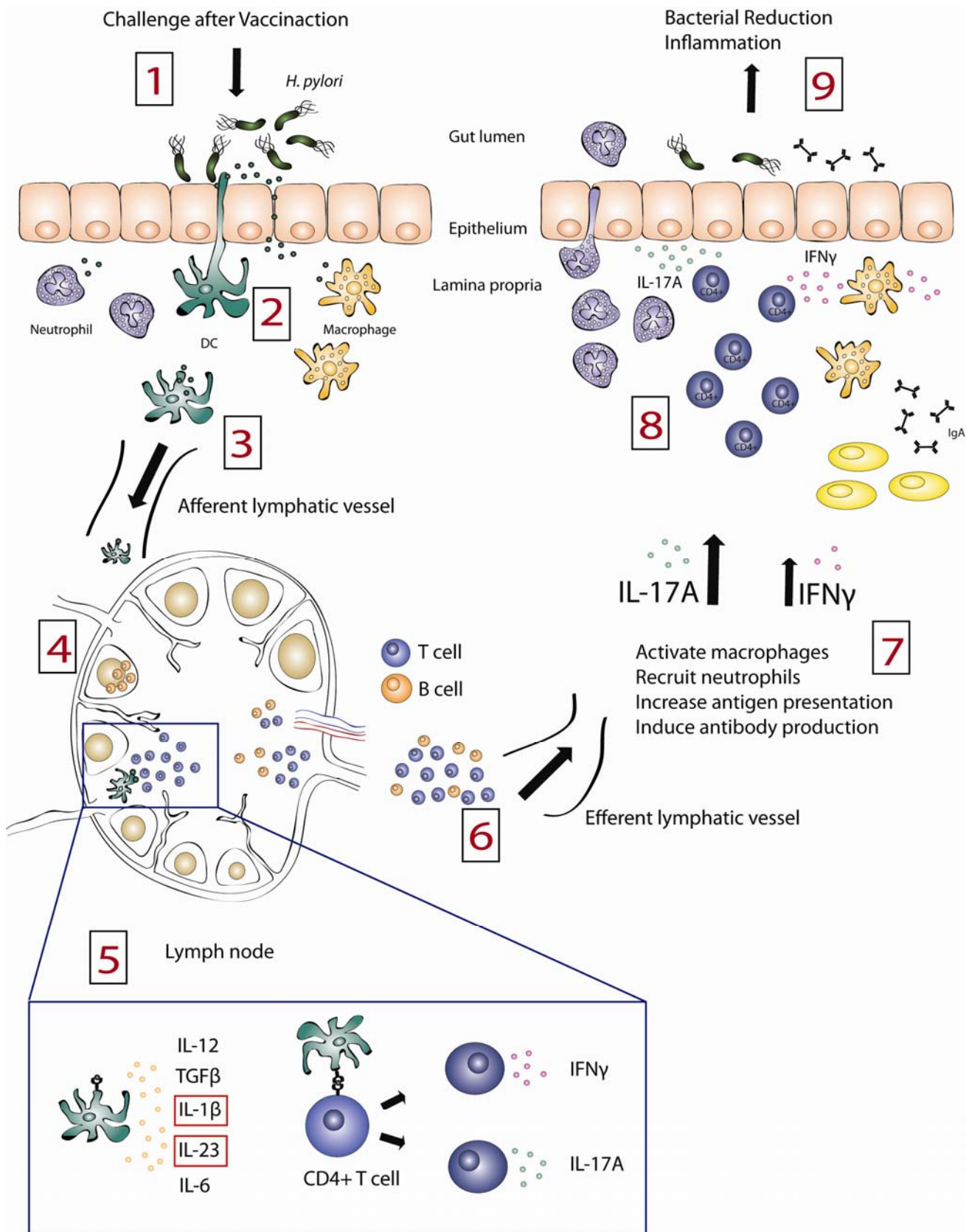
Concluding remarks

The focus in this thesis has been to elucidate immune mechanisms of vaccine-induced protection against *H. pylori* infection with emphasis on the role of IL-17A and IFN γ using the mouse model of *H. pylori*. An essential point to be considered in our studies is that mice are not men and it remains to see if the results in this study hold true when evaluated in humans. Possible differences in the anatomy and cellular composition of the mucosal compartment can lead to different outcomes for the immune response generated [147]. In particular, the function of IL-17A responses that we report in mice remains to be confirmed in humans. Nevertheless, we could show in our preliminary study that the frequency of IL-17A⁺ cells was elevated in the stomach of *H. pylori*-infected individuals and particularly in those with corpus atrophy and higher inflammation score. The IL-17A may also come from other sources than CD4⁺ T cells and it would therefore be of interest to evaluate the stomach for innate sources of IL-17A to determine to what extent they may be important for the protection or inflammation. Another

important function of Th17 cells is the production of IL-22 which induces anti-microbial peptides and acts, together with IL-17A, on the epithelial cells and it would be of interest to evaluate IL-22 in relation to protection and inflammation [120].

In summary, our results show that dmLT is a strong mucosal adjuvant that induces similar immune responses and most importantly, protection against *H. pylori* infection in mice comparable to the gold standard adjuvant, CT. Its pre-clinical and clinical use so far has established that dmLT would be a good candidate for inclusion in a human vaccine. Furthermore, the SL route of immunization has proven in mouse studies to generate strong mucosal and systemic immune responses together with protection against *H. pylori* infection. The SL route is already in use in humans, although not for vaccination against bacterial pathogens and thus evaluation of the SL route for induction of mucosal immune responses in humans is needed. Our studies suggest that a key feature of the SL route of immunization seems to be the generation of IL-17A and IFN γ responses against *H. pylori* infection (Fig 12). The IL-17A and IFN γ responses may be induced in the absence of IL-23 but requires IL-1 signaling. The mechanisms of protection and post-immunization gastritis may be separated in that IL-17A is important for protection while IFN γ promotes inflammation (Fig 12).

The results from the work presented in this thesis provide a basis for the design of an appropriate clinical trial with a *H. pylori* vaccine in humans. The recommendation based on our work would be to use a combination of recombinant antigens such as HpaA, UreA or UreB together with the mucosal adjuvant, dmLT. The SL route of immunization could be considered since it has also been proven safe in humans and to induce immune responses in the stomach at least in mice. As a comparison, we also recommend the IG route of immunization as reference.



Induction of Th1 and Th17 responses in the lymph node by cytokine secretion from DCs

Figure 12. Summary of series of events in mice after sublingual vaccination. **1.** SL immunization with *H. pylori* lysate antigens and CT followed by challenge with live *H. pylori* bacteria. **2.** The bacteria will relocate close the epithelium in the stomach where bacterial antigens can either be sampled by DCs or secreted by the bacteria which will activate the PPRs in the epithelium, tissue resident macrophages and DCs. This will also induce influx of a few neutrophils and macrophages through epithelial secretion of CXCL8. **3.** DCs will migrate to the draining lymph node with the sampled antigens. **4.** In the lymph node, expansion of B cells with T cell help will occur resulting in formation of germinal centers, antibody production and isotype switching. **5.** DCs will present the sampled antigen to primed CD4⁺ T cells that will recognize *H. pylori* antigens presented on MHC-II and expansion of T cells occur. DCs will secrete cytokines such as IL-6, IL-12, IL-23, IL-1 β , TNF α and IFN γ that will further differentiate the T cells into IL-17A and IFN γ producing cells. In our studies, we have also addressed the role of cytokine IL-1 β and IL-23 and evaluated their impact on IL-17A responses. **6.** Due to the ongoing infection, T and B cells will be recruited back to the stomach via the efferent lymph guided by homing receptors. **7-8.** In the stomach, production of IL-17A and IFN γ will occur which will further lead to activation of macrophages, influx of neutrophils and antibody production. **9.** These series of events will result in bacterial reduction and to some extent inflammation. Our results in the IFN γ ^{-/-} mice showed that post-immunization gastritis is attenuated and neutralization of IL-17A abrogates the protection. We believe that, in *H. pylori* infection induced post-immunization gastritis; we have been able to separate the protective effects of inflammation from the pro-inflammatory.

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