THE ROLE OF INTERLEUKIN-10 IN

HELICOBACTER PYLORI INFECTION.

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ABSTRACT

Background & Aims: *Helicobacter pylori* (*H. pylori*) chronic infection is the major cause of peptic ulceration and gastric cancer. Strains with the *cag* pathogenicity island (*cag*PaI) are more strongly associated with disease. In earlier studies, higher levels of interleukin-10 (IL-10) were found in human gastric biopsies with the highest *H. pylori* colonisation density grading. In the current study, experiments were performed to determine if this effect could be replicated *in vitro* using human gastric epithelial cell lines. Although IL-10 is abundant in the gastric mucosa, the nature of its interaction with the epithelium is unknown. IL-10 receptor (IL-10R) expression on gastric epithelial cells was therefore investigated.

Materials & Methods: *H. pylori* was co-cultured with the human gastric epithelial cell lines AGS, MKN28 and MKN45 in the presence or absence of recombinant human IL-10 (rIL-10). Bacterial densities were assessed by viable count assays. Immunofluorescence microscopy, flow cytometry, RT-PCR and western blotting were used to investigate the presence of IL-10R (IL-10Rα and IL-10Rβ subunits) on gastric epithelial cell lines. An IL-10 Fluorokine Kit (R&D Systems) was also used to quantify IL-10 binding onto gastric epithelial cells.

Results: Addition of rIL-10 significantly increased the density of *H. pylori cag*PaI positive strains indirectly through effects on gastric epithelial cells. No such differences were observed with *cag*PaI negative strains. AGS cells expressed both IL-10Rα and IL-10Rβ constitutively but IL-10Rβ expression was increased with infection. Conversely, the binding of IL-10 was decreased when AGS cells were infected with *H. pylori.*

Conclusion: This study highlighted a novel finding of constitutive IL-10R expression in AGS cells. Interestingly, *H. pylori* infection of AGS cells downregulated the binding of IL-10 to its receptor. The higher level expression of IL-10Rβ following infection was in opposition to this. Possibly IL-10Rβ could be sequestered into other cytokine receptor complexes and therefore is not participate in IL-10 binding. These studies have shown that IL-10 has a more complex role in the interaction between *H. pylori* and its host. The IL-10 response now also appears to influence the density of *H. pylori* in the stomach, via *cag*PaI-dependent effects on epithelial cells. More work must be carried out to investigate the mechanisms behind this effect.

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API-1 – Activator protein-1
CARD4 – caspase recruitment domain 4
CTLA-4 – Cytotoxic T lymphocyte antigen 4
DCs – dendritic cells
DU – duodenal ulcer
ECL – enhanced chemiluminescene
hBD-2 – human β-defensin-2
HIF-1α – hypoxia inducible factor-1α
H_2O_2 – hydrogen peroxide
HSV – herpes simplex virus
IAP – inhibitor of apoptosis protein
IFNγ – interferon gamma
iNOS – inducible nitric oxidase
LPS – lipopolisaccaride
mAb – monoclonal antibody
MALT – mucosa-associated lymphoid tissue
Mcl-1 – myeloid cell leukemia-1
MMP-7 – matrix metalloproteinase-7
MMP-9 – matrix metalloproteinase-9
MOI – multiplicity of infection
NFAT – nuclear factor of activated T-cells
NF-κB – nuclear factor kappa B
NK – natural killer cells
NO – nitric oxide
NOD1 – nucleotide-binding oligomerization domain-containing protein 1
O_2^- - superoxide
OD – optical density
PAMPs – pathogen-associated molecular patterns
PPI – proton pump inhibitor
PRRs – pattern recognition receptors
rIL-10 – recombinant human IL-10
ROS – reactive oxygen species
SNP – single nucleotide polymorphism
T4SS – type IV secretion system
Th – T helper
TLR – toll-like receptor
TNFα – tumour necrosis factor alpha
TPM – tyrosine phosphorylation motif
UBT – urea breath test
VEGF – vascular endothelial growth factor
WHO – World Health Organization

CHAPTER 1:

INTRODUCTION

1.1. *Helicobacter pylori* background.

1.1.1. History.

H. pylori was first discovered by Robin Warren following his three-year observation of small curved and s-shaped bacilli in sections from 135 gastric biopsy specimens. During that time, this Gram-negative, flagellate (Figure 1) and microaerophilic bacterium was regarded as a new species related to the genus *Campylobacter* due to its similarity in morphology and physiology (Marshall & Warren, 1984).

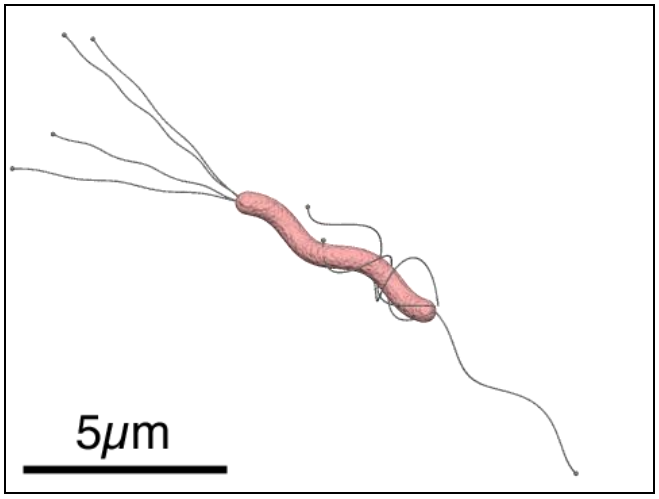

Figure 1. The *Helicobacter pylori* (Image published in http://commons.wikimedia.org/wiki).

Helicobacters in general are the indigenous biota of the gastro-intestinal tract of virtually all mammals from small rodents to dolphins (Blaser, 1998). *H. pylori* is considered to be the human-specific inhabitant of the stomach (Blaser & Atherton, 2004). This bacterium

has only been seen in the gastric mucus layer and rarely invades the lamina propria and gastric cells (Ko *et al.*, 1999). *H. pylori* infection is predominantly acquired in childhood (Banatvala *et al.*, 1993) and usually persists for the life of the host unless treatment is given (Blaser, 1999).

This bacterium is very well adapted to survive in the very harsh acidic and nutrient-poor environment of the stomach. It releases the enzyme urease to convert urea from the host to ammonia which will raise the gastric pH surrounding its niche (Marshall *et al.*, 1990). It also avoids contact with the acidic gastric juice by crossing the thick layer of the mucus lining of the stomach using its flagella (Eaton *et al.*, 1992). Once it reaches the host cells, it adheres to the gastric epithelial cells using adhesion proteins such as BabA, SabA and HpaA (Ilver *et al.*, 1998) and finally secretes factors to initiate inflammation as well as damaging the host cells.

The actual route of transmission for *H. pylori* is unknown but it is more likely to be through gastro-oral contact as a higher rate of *H. pylori* infection was found to be associated with history of vomiting in siblings (Luzza *et al.*, 2000). Although live *H. pylori* has been isolated from human faeces (Thomas *et al.*, 1992), this is rare and the faecal-oral route is unlikely to be a common mode of transmission (Malaty *et al.*, 2003). It may be significant, however in certain areas which lack good sanitary practices (Moreira *et al.*, 2005) and there is also some evidence for this from animal studies (Lee & Kim, 2006). Some researchers have also proposed a person-to-person transmission such

as from mother to child (Escobar & Kawakami, 2004), through unhygienic food preparation or *H. pylori*-contaminated water (Ahmed *et al.*, 2006).

Invasive and non-invasive tests have been used to detect the presence of *H. pylori* in an individual. Invasive tests such as bacterial cultures, *Campylobacter*-like organism (CLO) test and histology are based on gastric biopsy samples (Lee *et al.*, 1990). The currently used non-invasive tests include rapid ^{13}C-urea breath test (UBT), fecal antigen test and serology. The UBT, which is the most commonly used, is thought to be the most reliable method of detection with overall accuracy of almost 95% (Kato *et al.*, 2004). This test requires the patient to ingest urea with ^{13}C or ^{14}C instead of normal 12C and if present, urease enzyme will hydolyse the urea to give out labelled carbon dioxide which will be deteced in breath (Bell *et al.*, 1991). The fecal antigen test is normally used to detect *H. pylori* in children and the serology test is usually applied for large case studies. Both of these tests are based on enzyme-linked immunosorbent assay (Brown, 2000; Kabir, 2001). A single non-invasive test may give a false-positive result and therefore should be confirmed with another test (Nurgalieva *et al.*, 2006).

The first choice of treatment for *H. pylori*-infected individuals would be the triple therapies composed of a proton pump inhibitor (PPI) and two antibiotics (clarithromycin and amoxicillin) (Malfertheiner *et al.*, 2007). Sometimes, a quadruple therapy is administered if infection still persists after triple therapy (Veldhuyzen van Zanten *et al.*, 2000). This is using a combination of a PPI, bismuth substrate and two different antibiotics.

1.1.2. Epidemiology.

H. pylori has been known to infect half of the world's population (as reviewed by Wilson & Crabtree, 2007). In Western countries, such as the USA, the prevalence of *H. pylori* infection was not more than 30% for individuals below the age of thirty years and more than 75% of those above the age of 60 years (Graham *et al.*, 1991). There may also be differences in prevalence in different parts of the same country (Alpizar-Alpizar *et al.*, 2005; Palli *et al.*, 2005) and these may be due to differences in socio-economic status, since poor and crowded living conditions especially during childhood are associated with increased infection rates (Malaty & Graham, 1994). *H. pylori* prevalence in Western countries is now decreasing (Roosendaal *et al.*, 1997) and this is thought to be due to improved living standards, good hygiene practice (Rehnberg-Laiho *et al.*, 2001), less crowded living condition and the common use of antibiotics in children (Banatvala *et al.*, 1993).

In Asia, the rate of *H. pylori* infection is very high but the occurrence of gastric cancer, which is associated with the infection, is different between regions. Less developed countries such as Thailand and Indonesia are known to have a very high incidence of *H. pylori* infection, but the risk of gastric cancer development is very low in these countries. Whereas, countries such as Japan and China have high *H. pylori* infection rates as well as high gastric cancer occurrence (Matsukura *et al.*, 2003). This puzzling scenario, termed the Asian enigma (Miwa *et al.*, 2002) was initially thought to be due to the presence of less-pathogenic strains in areas with low gastric cancer rates. However, pathogenic *H. pylori* strains were also found in those countries with low gastric cancer rates (Kumar *et*

al., 1998; Singh *et al.*, 2003). Other possible reasons may be variations in the host response, polymorphisms in the host genetic reservoir, dietary and other environmental factors (as reviewed by Singh & Ghoshal, 2006).

A similar phenomenon, called the African enigma, was also found in Africa where *H. pylori* infection is ubiquitous, but gastric adenocarcinoma was rare in some countries (Holcombe, 1992). However, data collected from prospective endoscopic studies in African populations has shown that the actual association of *H. pylori* infection rates and disease outcomes is actually similar to those seen in industrialised countries (Agha & Graham, 2005). The authors suggested that this could be due to the selected African populations being biased towards those who have very limited access to health care and a shorter life expectancy. This may be another possible reason for the Asian enigma.

1.1.3. *H. pylori*-mediated diseases.

1.1.3.1. Gastroduodenal diseases.

a) Gastritis.

During gastric mucosal colonisation, *H. pylori* adheres to gastric epithelial cells by means of adhesion molecules. Bacterial factors such as the CagA protein (Konturek *et al.*, 2000) are inserted into host cells which will eventually initiate a pro-inflammatory response. Immune cells and lymphocytes are drawn towards the inflamed epithelial cells. This chronic gastritis (persistent inflammation of the gastric mucosa) is the initial stage which

preceeds the development of peptic ulcers, gastric cancer and MALT lymphoma (Graham *et al.*, 2004).

Acute gastritis is rare but has been reported from individuals who deliberately ingested *H. pylori* (Marshall *et al.*, 1985; Sobala *et al.*, 1991). These individuals showed symptoms such as fullness, nausea and vomiting. Asymptomatic chronic *H. pylori*-gastritis is more common, but only in a minority of cases will this develop into disease such as peptic ulceration. This is depends on the distribution of gastritis and acid level (Figure 2).

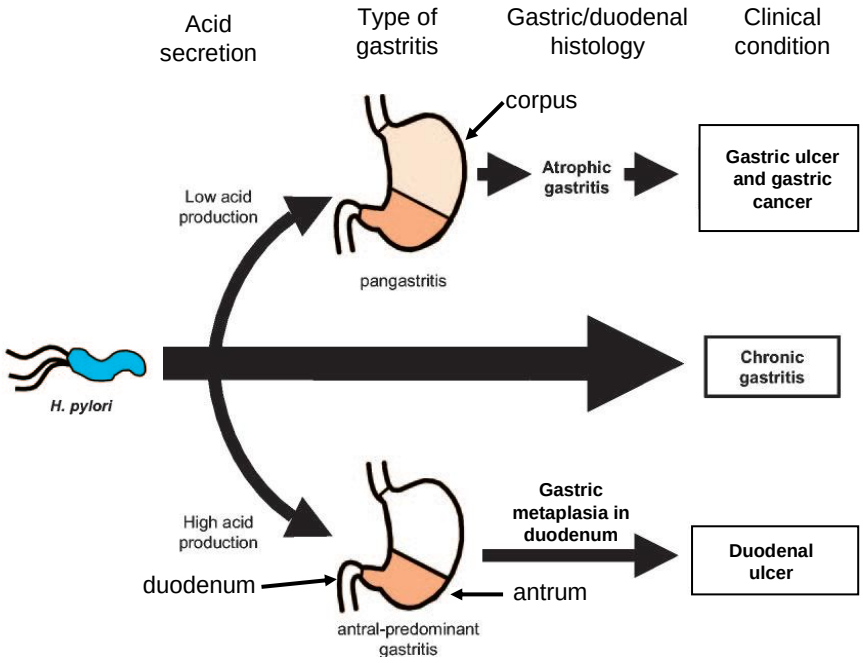

Figure 2. Gastritis pattern and disease outcome.
H. pylori-chronic infections in humans generally cause asymptomatic chronic gastritis. However, bacterial colonisation in the antrum contributes to antral-predominant gastritis which results in high acid production. This leads to gastric metaplasia in the duodenum thus duodenal ulcer. Colonisation in the corpus predisposes to pangastritis (or corpus-predominant gastritis) which results in low acid production and eventually the occurance of gastric ulcer or gastric cancer (Adapted from Kusters *et al.*, 2006).

b) Peptic ulcers (duodenal and gastric ulcers).

Approximately 90% of duodenal ulcers and 70% of gastric ulcers are attributable to infection by *H. pylori* (as reviewed by Ernst & Gold, 2000). A study performed in a large series of patients in Italy has confirmed that *H. pylori* infection is associated with duodenal ulcer (Zambon *et al.*, 2005).

The pattern of *H. pylori*-gastritis and the level of acid load are very important to determine the site of ulcer. *H. pylori* normally colonises the gastric antrum because the corpus has a higher number of acid-secreting parietal cells and thus a high production of acid (as reviewed by Kusters *et al.,* 2006). Antral-predominant gastritis suppresses D cells from producing somatostatin and induces G cells to secrete gastrin (Zavros *et al.*, 2003). Gastrin is a gastric hormone which induces gastric acid secretion. Somatostatin suppresses gastrin production. Hypergastrinaemia stimulates high production of acid from parietal cells in the corpus. Over time, high acid levels will be delivered into the duodenum and damage its epithelial cells. Gastric metaplasia (replacement of the duodenal epithelial cells with those of a gastric type) occurs and the duodenum can then be colonised by *H. pylori* causing inflammation and eventually duodenal ulcer (DU) (Robinson & Atherton, 2009). Thus, DU is associated with an antral-dominant gastritis (Tham *et al.*, 2001).

For some reason, acid secretion may be impaired in some individuals and *H. pylori* may colonise the corpus leading to corpus-predominant gastritis or pan-gastritis. This type of gastritis leads to hypochlorhydria, and hypergastrinaemia occurs, which in turn leads to

increased prevalence of atrophy and intestinal metaplasia (El-Omar *et al.*, 1997). Hypochlorhydria may be caused by the presence of pro-inflammatory cytokines such as IL-1β which are acid suppressant (Beales & Calam, 1998). Atrophy causes the loss of acid-secreting parietal cells and further reduces the acid level. Hypergastrinaemia was found to correlate with production of COX-2, (Konturek *et al.*, 2000), expression of anti-apoptotic proteins (Hartwich *et al.*, 2001) and also induction of epithelial cells proliferation (Wang *et al.*, 2000). Thus, the combination of the increased rate of cell proliferation with less apoptosis in the presence of DNA-damaging compounds, increases mutation levels and eventually the risk for carcinogenesis (as reviewed by Fox & Wang, 2007).

c) Gastric adenocarcinoma.

In 2007, 13% of all deaths in that year were attributable to cancer with stomach cancer is the second most frequent cause of cancer-associated death as reported by the World Health Organization (WHO) (http://www.who.int/). Gastric cancer has an extremely poor prognosis, the 5-year survival rate is less than 20% (Cenitagoya *et al.*, 1998). *H. pylori* was classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC) (1994). Since *H. pylori* infection is known to be the first stage in gastric carcinogenesis, studies related to *H. pylori* pathogenesis are very important.

Strong evidence supports the hypothesis that *H. pylori* infection is closely-linked to the occurrence of gastric cancer. A study showed that only those infected with *H. pylori* had gastric cancer cancer but not uninfected individuals (Uemura *et al.*, 2001). The long-term

effect of *H. pylori* gastritis on the gastric mucosa is also a significant risk factor for development of atrophic gastritis and intestinal metaplasia (Kuipers *et al.*, 1995) which are the precursors of gastric cancer formation. This was further supported by the fact that antibiotic eradication of *H. pylori* before the appearance of pre-malignant pathology, could prevent carcinogenesis (Wong *et al.*, 2004).

Correa's model of intestinal type gastric carcinogenesis suggested that the initial stage towards gastric cancer is changing of the gastric mucosal morphology from normal to superficial gastritis and this is where *H. pylori* infection intervenes. This then lead to gastric atrophy (loss of glands), which resulted in intestinal metaplasia, followed by dysplasia and eventually gastric cancer (Correa, 1992). However, *H. pylori* infection alone is not sufficient to cause gastric carcinogenesis. Other factors such as excessive salt intake and smoking may play a role.

d) Gastric mucosa-associated lymphoid tissue (MALT) lymphoma.

Lymphoma, as the name indicates, is a form of cancer that affects immune cells, the lymphocytes, and can be categorised as Hodgkin lymphoma or non-Hodgkin lymphomas (http://www.lymphomation.org/aboutNHL.htm). Non-Hodgkin lymphomas can be further divided into B-cells, T-cells and Natural Killer (NK) cells neoplasms. The WHO has renamed low grade gastric B cell lymphoma as "Extranodal marginal zone B cell lymphomas of mucosa-associated lymphoid tissue but the short term MALT lymphoma was retained. Low grade gastric MALT lymphomas could be transformed to high grade which have a lower prognosis than low grade (Chan *et al.*, 1990).

The normal gastric mucosa has no lymphoid tissue but the vigorous immune and inflammatory response to chronic *H. pylori* infection could induces lymphoid follicle formation (Stolte & Eidt, 1989). This may eventually lead to the development of gastric MALT lymphoma (Eidt *et al.*, 1994). Furthermore, *H. pylori* infection also promotes expansion of the transformed cells, as shown in an *in vitro* study where heat killed *H. pylori* increased the growth of lymphoma cells (D'Elios *et al.*, 1999). It has been found that complete reduction of the lymphoma can be achieved by *H. pylori* eradication alone in many cases, provided that the lymphoma has not spread beyond the stomach (Montalban *et al.*, 2001).

e) Others.

Vitamin B-12 (cobalamin) deficiency has been suggested to be connected with *H. pylori* infection. Cobalamin malabsorption is affected by low acid level in the stomach (Andres *et al.*, 2004) and *H. pylori*-associated gastritis can initiate the change of acid level. A study on a group of Finnish elderly males showed that in vitamin B-12 deficiency cases, 75% had caused by *H. pylori*-induced atrophic gastritis (Sipponen *et al.*, 2003). Kaptan *et al.* have detected *H. pylori* in 56% of patients with vitamin B-12 deficiency and eradication of the bacterium has corrected vitamin B-12 level in 40% of those infected (Kaptan *et al.*, 2000).

1.1.3.2. Extragastric diseases.

H. pylori has also been linked to extragastric diseases including idiopathic thrombocytopenic purpura, ischemic heart disease, hepatobiliary diseases and iron-deficiency anaemia.

H. pylori may initiate autoimmune diseases such as idiopathic thrombocytopenic purpura (ITP) through the production of autoreactive antibodies via B-1 cell activation (Yamanishi *et al.*, 2006). ITP is a haematological condition where there is destruction of platelets through auto-immune mediation (as reviewed by Franceschi & Gasbarrini, 2007). It has been reported that the levels of these autoantibodies decreased after *H. pylori* eradication (Kohda *et al.*, 2002) and elimination of *H. pylori* from ITP patients is effective (Satake *et al.*, 2007). In some cases, ITP may not be directly induced by *H. pylori* infection but by an antibody produced by MALT lymphoma B cells (Noda *et al.*, 2004).

Iron-deficiency anaemia (IDA) is another haematological condition that has a connection with *H. pylori* infection. This is thought to be due to bleeding ulcers (Gisbert *et al.*, 2007) but *H. pylori*-associated IDA was not reported to have gastrointestinal bleeding (Sarker *et al.*, 2008). Thus, the most likely reason for this connection would be that iron absorption is disturbed by reduced levels of gastric acid and ascorbic acid (Annibale *et al.*, 2003) which is needed in iron uptake. *H. pylori* eradication has helped to cure this condition without the need to continue oral iron treatment in 10 out of 14 *H. pylori*-positive patients (Hershko *et al.*, 2007). The elimination of this bacterium has been proved to enhance the

efficacy of ferrous succinate therapy in IDA patients with chronic gastritis (Chen & Luo, 2007).

Mendall *et al.* were first to suggest that there was a connection between *H. pylori* and atherosclerotic diseases (Mendall *et al.*, 1994) and this was supported by a study showing that patients with ischaemic heart disease had a higher prevalence of CagA positive strain infections (Pasceri *et al.*, 1998). Little evidence of this association was found in other studies (Stone *et al.*, 2001) but a few serological studies have reported that there is an association between *H. pylori* infections, especially *cagA*-positive strains, with the risk of coronary heart disease in middle aged white men (Gunn *et al.*, 2000; Singh *et al.*, 2002). The link with atherosclerosis remains controversial although it has been reported that *H. pylori* can cause damage to the walls of blood vessels and cause inflammation (Kowalski *et al*, 2001). *H. pylori* infection is also reported to be one of the risk factors for the development of cardiovascular diseases (CVD) among Korean adults (Sung *et al.*, 2005). This was supported by association of antibodies against *H. pylori*-heat shock protein 60 (*H. pylori*-HSP60) and CVD risk factors (Okada *et al.*, 2007).

A link between *Helicobacter* infection and hepatitis C-associated cirrhosis has been reported (Rocha *et al.*, 2005). *H. pylori* DNA has been found in liver samples from patients with hepatitis C virus-positive cirrhosis with and without hepatocellular carcinoma. *Helicobacter* DNA was found in 8 out of 20 liver samples from patients with primary liver carcinoma (Huang *et al.*, 2004).

1.1.4. Determinants of *H. pylori* disease outcome.

H. pylori infections do not always result in the development of disease and the vast majority (85-90%) of infected people remain asymptomatic. Who develops disease depends on several factors including the virulence of the infecting *H. pylori* strain, the genetic susceptibility of the host, environmental co-factors such as smoking, diet and concurrent infections (Atherton, 1997).

1.1.4.1. Major *H. pylori* virulence factors.

a) Cytotoxin-associated gene (*cag*) pathogenicity island (*cag*PaI).

The *cag*PaI is a region of DNA made up of about 30 genes which encode a type IV secretion system (T4SS). Infection with *H. pylori* strains possessing the *cag*PaI are approximately twice as likely to lead to peptic ulceration and gastric adenocarcinoma. CagA, a protein encoded by this island, is injected into the epithelial cytosol through the T4SS (Fischer *et al.*, 2001) and is used as a marker for the presence of the PaI (Akopyants *et al.*, 1998). Delivery of CagA is also triggered by interactions between CagL, a specialized adhesin encoded on *cag*PaI, with integrin $\alpha_5\beta_1$ receptor on gastric epithelial cells (Kwok *et al.*, 2007).

The T4SS itself has been found to induce expression of pro-inflammatory cytokines when interacting with host cells (Segal *et al.*, 1999) by a mechanism which does not involve CagA but *H. pylori* cell wall components e.g. peptidoglycan instead. Peptidoglycan is recognised by the host defence molecule, NOD1, and this leads to NF-κB activation

(Viala *et al.*, 2004) and increased expression of pro-inflammatory genes such as interleukin-8 (Holck *et al.*, 2003). Other than CagA and peptidoglycan, this T4SS may also translocate other important bacterial factors (Christie & Vogel, 2000) with a role in pathogenesis. The importance of the *cag*PaI was demonstrated when its deletion or disruption of several *cag* genes, blocked *H. pylori*-mediated induction of the proinflammatory chemokine interleukin-8 (IL-8) by gastric epithelial cells *in vitro* (Akopyants *et al.*, 1998). This factor is an important chemoattractant for neutrophils and lymphocytes. Neutrophil infiltration in the gastric mucosa was significantly more severe in patients who were infected with *cagE*-positive strain compared to *cagE*-negative strain (Maeda *et al.*, 2002). These conditions show that the presence of the *cag*PaI makes a big contribution towards induction of inflammation.

Upon its injection into epithelial cells via the T4SS, the CagA protein is recognized by Src family kinases (Stein *et al.*, 2002) and phosphorylated at the tyrosine residues in EPIYA motifs (Asahi *et al.*, 2000). It is this phosphorylated-CagA which interacts with host signalling molecules, e.g. tyrosine phosphatase SHP-2 (Higashi *et al.*, 2002), which leads to changes in epithelial cell morphology (Figure 3). CagA proteins from different *H. pylori* isolates vary in their tyrosine phosphorylation motifs (TPMs) (Stein *et al.*, 2002). An *In vitro* study reported that *H. pylori* strains which deliver CagA with more TPMs, induced more profound cytoskeletal changes to gastric epithelial cell lines (Argent *et al.*, 2004). Strains producing CagA with more TPMs, increase its binding to SHP-2 and thus increase the cellular effects. This was reported for Asian strains which were associated with increase risk of gastric cancer (Azuma *et al.*, 2002).

In addition, many reports show the linked between clinical disease outcome and presence of *cag*. The risk for the development of gastric cancer is higher in infection with a *cagA*-positive *H. pylori* strain in comparison with a *cagA*-negative strain (Blaser *et al.*, 1995). Furthermore, CagA enhances production of reactive oxidative species (ROS) and might induce oxidative stress to the gastric mucosa (Farinati *et al.*, 2003). Infection with a *cagA*-positive *H. pylori* strain is also associated with an increased prevalence and intensity of antral atrophy and intestinal metaplasia (Sozzi *et al.*, 1998).

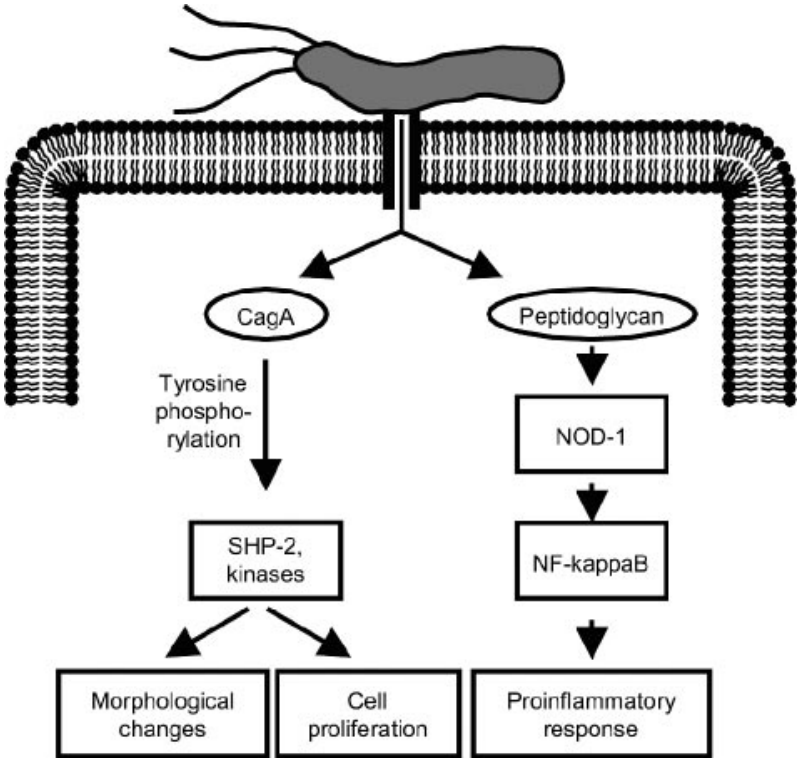

Figure 3. CagA interaction with host molecules and cellular consequences.
Once transported into host epithelial cells via the T4SS, CagA is phosphorylated and interacts with SHP-2, the host signalling molecule. This interaction leads to morphological changes and proliferation of host cells. Other than CagA, peptidoglycan is also translocated into host cells and recognised by NOD1. This promotes NF-κB activation and induces inflammatory responses (Taken from Kusters *et al.*, 2006).

b) Vacuolating cytotoxin A (VacA).

All *H. pylori* strains possess a *vacA* gene (Phadnis *et al.*, 1994) and approximately half of them secrete an active VacA protein (Leunk *et al.*, 1988). This protein is a toxin which induces massive vacuole formation in epithelial cells *in vitro* and reduces the proliferation of T-cells (Smoot *et al.*, 1996). It has been suggested that this inhibition of T cell clonal expansion may allow *H. pylori* to evade the adaptive immune response and establish chronic infection (Sundrud *et al.*, 2004). The toxin is capable of forming pores in gastric epithelial cells which transport interstitial fluids together with urea towards the bacterium (Tombola *et al.*, 2001). In this way, the bacterium gains nutrients as well as balancing the pH in its surrounding niche by converting urea to ammonia which eventually helps *H. pylori* to grow. VacA also acts by loosening the tight junctions between cells and causes damage to the epithelium (Pelicic *et al.*, 1999).

The *vacA* gene is polymorphic and may contain s1 or s2, i1 or i2 and m1 or m2 alleles (Figure 4). The i1 type has been closely linked with peptic ulcer disease (Basso *et al.*, 2008). Type s1/m1 strains produce a higher level of cytotoxin activity *in vitro* than type s1/m2 (Atherton *et al.*, 1995). Several studies have shown that infection by more virulent and highly pathogenic s1/m1 strains of *H. pylori* increase the risk of gastric cancer (Enroth *et al.*, 2000). Although s1/m1 strains were associated with the disease, VacA is not thought to act via inflammation but due to inducing tissue damage.

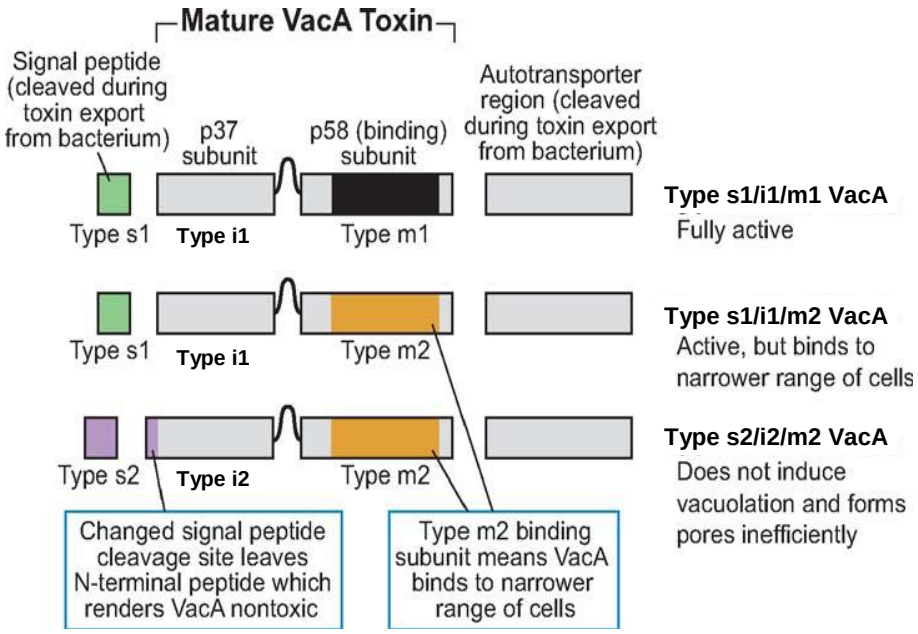

Figure 4. Polymorphism in the *vacA* gene.
Between strains, the *vacA* gene in *H. pylori* varies in the s (signal), i (intermediate) and m (mid) regions. The s region encodes the signal peptide (determines cytotoxic activity and level of secretion) and may exist as type s1 or s2. The i region is located within the p37 subunit (also determines cytotoxic activity) and may exist as type i1 or i2. The m region encodes part of the p58 binding subunit (determines binding to host cells) and may exist as type m1 or m2. Type s1/i2/m2 is also exist and has similar activity with type s2/i2/m2 (Adapted from Atherton, 2006).

c) Duodenal ulcer promoting gene A (*dupA*).

This gene is present in the genome sequence of *H. pylori* strain J99 as two overlapping genes but is present as one open reading frame in most other strains (as reviewed by Robinson *et al*., 2007). The *dupA* gene was found mostly to be connected with the presence of peptic ulcers but not gastric cancer. In one study, this gene was significantly associated with increased risk of duodenal ulcer but appeared to protect against *H. pylori*-induced gastric cancer in patients from South Korea, Japan and Colombia (Lu *et al.*,

2005a). Others have shown a significant association of *dupA* with peptic ulcer diseases in an Iraqi population (Hussein *et al.*, 2008) and that *dupA* positive strains are inversely associated with gastric cancer in Iranian patients (Douraghi *et al.*, 2008).

In addition, a study in China recently showed that duodenal ulcer patients had the highest prevalence of *dupA* positive strains compared to gastric cancer and gastric ulcer patients (Zhang *et al.*, 2008). However, in Brazil, *dupA* positive strains are highly prevalent but infection with them was not significantly associated with gastric cancer or duodenal ulcer, both in adults and children (Gomes *et al.*, 2008). Importantly, *Lu et al.* showed that *dupA*+ infections are associated with increased levels of IL-8 in the gastric mucosa and a more severe neutrophil infiltration (Lu *et al.*, 2005a). This indicates that the high risk of disease is driven by induction of inflammation.

d) Outer inflammatory protein (OipA).

The *oipA* gene can also induce IL-8 expression from gastric epithelial cells (Yamaoka *et al.*, 2000). The presence of OipA is correlated with duodenal ulceration (Yamaoka *et al.*, 2002) and gastric cancer (Yamaoka *et al.*, 2006). Additional evidence of the importance of this gene comes from the fact that an *oipA* knockout mutant strain of *H. pylori* failed to colonise Mongolian gerbils (Akanuma *et al.*, 2002) and it induced lower levels of gastric inflammation in mice than its wild-type parent (Yamaoka *et al.*, 2002).

e) Other outer membrane proteins.

Multiple *H. pylori* outer membrane proteins can mediate *H. pylori* adherence to gastric epithelial cells. Examples of these proteins are BabA, SabA, HpaA, Omp18, AlpA, AlpB and HopZ. BabA (blood group antigen binding adhesion A), one of the most-studied adherence factors, binds to Lewis B blood group antigen, which is found on epithelial cells (Ilver *et al.*, 1998). It facilitates *H. pylori* colonization and increased IL-8 responses, which in turn increase mucosal inflammation (Rad *et al.*, 2002). SabA (sialic acid-binding adhesion) expression was increased in strains obtained from patients with gastric cancer, intestinal metaplasia gastric atrophy (Yamaoka *et al.*, 2006). Interactions between bacterial adhesion and a host receptor can alter disease outcome in an animal without affecting colonization levels (Guruge *et al.*, 1998). If certain *H. pylori* strains adhere more strongly to the host cells, then toxins or other effector molecules can be efficiently delivered to the cells which may increase the level of damage and inflammation.

f) HP-NAP.

HP-NAP is another factor that can activate neutrophils. HP-NAP activates mast cells to release the contents of granules and the pro-inflammatory cytokine IL-6. These factors can then recruit monocytes and neutrophils to the site of infection (Montemurro *et al.*, 2002). The gene for this *H. pylori* neutrophilic activating factor is called *napA* (Evans *et al.*, 1995) and is able to induce neutrophils to adhere to endothelial cells (Evans *et al.*, 1995). It can also mediate the binding of *H. pylori* to cell surface via interaction with carbohydrates (Teneberg *et al.*, 1997). HP-NAP has also been found to induce a strong

Th1 response, induces neutrophils to produce reactive oxygen species and thus increase inflammation level as well as damaging host cells (Montecucco & de Bernard, 2003).

1.1.4.2. Host genetics.

Inflammation caused by *H. pylori* infection is mediated by an array of pro- and anti-inflammatory cytokines (as reviewed by Lochhead & El-Omar, 2007). Individuals with a more pro-inflammatory genotype are at more risk of developing disease as a consequence of this infection. Single-nucleotide polymorphisms in several genes encoding pro-inflammatory cytokines can influence the clinical outcome of the *H. pylori* infection (as reviewed by Algood & Cover, 2006).

A group of researchers have reported that *IL-1* gene cluster polymorphisms suspected of enhancing production of IL-1β are associated with an increased risk of both *H. pylori*-induced hypochlorhydria and gastric cancer (El-Omar *et al.*, 2000). These polymorphisms were also associated with more severe inflammation and an increased prevalence of intestinal metaplasia and atrophic gastritis (Rad *et al.*, 2004). In addition, polymorphisms that enhance the expression of TNF-α are associated with an increased risk of gastric cancer development (Machado *et al.*, 2003), and duodenal and gastric ulcers (Zambon *et al.*, 2005). *IL-8* gene polymorphism has also been reported to play a role in the severity of gastric inflammation. The *IL-8*-251T allele is associated with increased production of IL-8 in the *H. pylori*-infected gastric mucosa (Lee *et al.*, 2005).

Polymorphisms in a range of other genes (e.g. IL-17F, IL-7R IFNGR2 and TLR4) are also reported to be associated with increased risk of gastric cancer and its precursors (as reviewed by Lochhead & El-Omar, 2007).

Polymorphisms in the *IL-10* gene are also important in modulating disease outcome. This will be discussed in detail in section 1.4.5.1.

1.1.4.3. Environmental factors.

Other than host genetics and bacterial virulence factors, environmental factors such as smoking and diet can also contribute to the development of gastritis, peptic ulceration and gastric cancer.

Cigarette smoking has been linked to many diseases including gastric cancer and peptic ulcers (as reviewed by Sherman, 1991). A study conducted in Venezuela has reported that people who were current smokers or had smoked previously, had significantly increased risks of intestinal metaplasia, dysplasia and premalignant pathology (Kato *et al.*, 2004). Tobacco smoking has also been associated with a higher risk of gastric cancer (You *et al.*, 2000) and risk of duodenal ulceration (Martin *et al.*, 1989) for those infected with *H. pylori*.

Although diet does not play a major role in duodenal disease, greater consumption of fresh fruits and vegetables has been shown to protect against risk of gastric cancer. In the study of Mayne *et al.* antioxidants such as vitamin C have been shown to have an inverse

association with the risk of noncardia gastric carcinoma (Mayne *et al.*, 2001). A vitamin C dietary supplement may also protect from progression of gastric mucosal atrophy (Sasazuki *et al.*, 2003). However, a high salt intake and especially high dietary nitrite from meat is directly associated with noncardia gastric cancer risk (Mayne *et al.*, 2001). Research has also found that excessive intake of sodium chloride augments colonization of *H. pylori* in mice and humans, which would in turn aggravate gastritis (Fox *et al.*, 1999). Others have observed a dose-dependent increase in gastric cancer risk with the consumption of highly salted food such as salted fish roe and salted fish preserves among middle-aged Japanese men and women (Tsugane *et al.*, 2004). Furthermore, a high salt diet increases the expression of interleukin-1β in the gastric epithelial cells *in vitro* (Zhang *et al.*, 2006).

1.2. Host response.

H. pylori infection induces vigorous innate and adaptive immune responses but the host is still unable to clear the organism from the mucosa. Gastric epithelial cells, the first-line of defense during *H. pylori* infection, express a number of innate pattern recognition receptors (PRRs) such as TLR2, 4, 5 and 9 (as reviewed by Lee & Josenhans, 2005) which recognise bacterial components such as flagellin and lipopolysaccharide (LPS). NOD1, an intracellular pattern recognition molecule that specifically responds to peptidoglycan products, is activated by cell wall component of *H. pylori* which enters gastric epithelial cells when the *cag* T4SS penetrates the cells (Viala *et al.*, 2004). TLR2 could also respond to peptidoglycan but not from *H. pylori* (Travassos *et al.*, 2004).

Activation of PRRs on gastric epithelial cells leads to expression of pro-inflammatory factors such as interleukin-8 (IL-8) (Holck *et al.,* 2003). Many of these molecules act as a chemotactic factor for neutrophils and lymphocytes, which also respond to *H. pylori* by producing further pro-inflammatory factors such as IFN-γ and antibacterial effectors such as defensin.

1.2.1. The gastric mucosal barrier.

Gastric mucosa is protected by a thick layer of mucus comprised mostly of epithelial mucins MUC5AC and MUC6 (Taylor *et al.*, 1998). *H. pylori* swims through this and can interact with other mucins e.g. MUC1, which is expressed on the surface of the gastric epithelium cells (Linden *et al.,* 2004). *H. pylori* infection can alter the expression levels of some mucin genes. In a study by Wang and Fang, gastric cancer patients with *H. pylori* infection had lower expression of MUC1, MUC5AC and MUC6 but higher MUC2 expression (Wang & Fang, 2006). MUC2 is normally expressed in intestinal (Carrato *et al.*, 1994) but its present in the gastric mucosa is a marker for intestinal metaplasia (Babu *et al.*, 2006).

MUC1 is a large, heavily glycosylated mucin expressed on the apical surface of a variety of epithelia including the mammary gland, gastrointestinal, respiratory, urinary and reproductive tract (as reviewed by Gendler, 2001). Recently, the epithelial mucin MUC1 has been demonstrated to limit the level of *H. pylori* colonization in the mouse gastric mucosa (McGuckin *et al.,* 2007) by acting as a releasable decoy (Linden *et al.,* 2004). The binding of *H. pylori* to MUC1 depends on the size of the MUC1 variable number of

tandem repeats (VNTR) domain as well as bacterial pathogenicity. It was found that larger VNTR regions and infection with a more pathogenic strain increased adhesion (Costa *et al.*, 2008). The most likely reason for this is that larger VNTR regions may provide more sites for *H. pylori* adhesion factors to bind.

The MUC1 extracellular domain is highly polymorphic (Gendler *et al.*, 1990) and the presence of different alleles of this membrane glycoprotein affects susceptibility to gastritis. It was found that the shorter the alleles of MUC1, the more risk of acquiring gastritis (Vinall *et al.*, 2002) and gastric cancer (Carvalho *et al.*, 1997) for that specific individual. What is more, mice deficient in MUC1 had 5-fold higher *H. pylori* colonisation densities and developed atrophic gastritis (McGuckin *et al.*, 2007).

Another important molecule that is expressed on gastric epithelial cells during *H. pylori* infection is B7-H1 (programmed death-1 ligand) (Das *et al.*, 2006). The B7 family provides signal that is very important for stimulating and inhibiting T cell activation (as reviewed by Coyle & Gutierrez-Ramos, 2001). When the gastric epithelial cells are infected with *H. pylori*, naive T cells can develop into regulatory T-cells with a CD4+CD25+FoxP3+ phenotype and this depends upon expression of B7-H1 (Beswick *et al.*, 2007). Up-regulating the expression of this molecule may help *H. pylori* to inhibit T cell responses in an indirect way.

1.2.1.1. Gastric cytokines and inflammatory cell infiltrates.

Cells of the gastric epithelial layer, the first line of defense during *H. pylori* infection, secrete cytokines and chemokines which stimulate the migration of granulocytes, monocytes and lymphocytes into the inflamed mucosa (as reviewed by Robinson *et al.*, 2007). Increased expression of the chemokine receptor CCR4 on antral CD4+ T cells from infected compared to uninfected stomach mucosa tissue has been found (Lundgren *et al.*, 2005b) which guides homing of these cells to the stomach. In all *H. pylori*-infected subjects, levels of IL-8, IL-1β, IL-6, IFN-γ and TNF-α are increased in the gastric mucosa compared to uninfected healthy individuals (Lindholm *et al.*, 1998). Some of these factors may be secreted by epithelial cells, but the source of many factors is the infiltrating immune and inflammatory cells. These cells release cytokines in response to encountering bacteria or bacterial components in the lamina propria.

IL-8 is one of the important factors that drive the influx of neutrophil towards the infected cells and the amount of IL-8 expressed by gastric epithelial cells in response to *H. pylori* is sufficient to induce neutrophil chemotaxis (Yamada *et al.*, 2001). Both *in vivo* and *in vitro* studies have shown increased levels of IL-8 in association with *H. pylori* infection (Kim *et al.*, 2001; Ren *et al.*, 2001; Seo *et al.*, 2004; Robinson *et al.*, 2008). Higher serum IL-8 has also been seen in gastric cancer patients than in controls without gastric cancer or *H. pylori*-infected controls (Konturek *et al.*, 2003). The induction of IL-8 expression is thought to be mediated through NF-κB and activator protein-1 (AP-1) (Chu *et al.*, 2003).

Neutrophils are generally the most abundant cells that populate the infected gastric epithelial cells. However, *H. pylori* evades the killing and phagocytosis activity by neutrophils. *H. pylori* strains, especially *cag*PaI-positive strains, could induce neutrophils to produce reactive oxygen species (ROS) and therefore cause tissue damage (as reviewed by Allen, 2001). Other than being phagocytes, neutrophils also produce inflammatory cytokines in response to *H. pylori* infection, such as IL-8, IL-1β and TNF-α (Alvarez-Arellano *et al.*, 2007). A study demonstrated that, wild type and neutrophil-depleted mice have similar intensities of gastric inflammation indicating that neutrophils may play a minor role in the response to *H. pylori* infection (Ismail *et al.*, 2003).

Macrophages are another set of immune cells that mediate phagocytosis but *H. pylori* also avoids killing by these cells. However, it was found that the type II strains, which are *cag*PaI-negative, could be rapidly engulfed and killed by macrophages compared to *cag*PaI-positive type I strains. The type I strains were found residing in megasomes, a combination of phagosomes of cultured macrophages (Allen *et al.*, 2000). *H. pylori* has been reported to invade the lamina propria and be captured by macrophages (Ito *et al.*, 2008). Another possible strategy of *H. pylori* to persistently colonise its host is by inducing arginase from macrophages (Gobert *et al.*, 2002) to counter the activity of inducible nitric oxidase (iNOS) also by macrophages (Fu *et al.*, 1999).

Dendritic cells (DCs) are potent connectors between the innate and adaptive immune responses because they can capture, process and present the antigen especially to T-cells (as reviewed by Banchereau *et al.*, 2000). It was found that immature DC from either *H.*

pylori-positive or *H. pylori*-negative human donors could be activated by *H. pylori* (Kranzer *et al.*, 2004) for example by inducing IL-12 production by these cells (Guiney *et al.*, 2003). It is still unclear how *H. pylori* actually interacts with DCs, but both *H. pylori* and DCs were found to manipulate epithelial tight junctions. It has been suggested that DCs could open up tight junctions in the gastric epithelium and project dendrites between cells to sample bacteria from the lumenal surface (Rescigno *et al.*, 2001).

1.2.1.2. Reactive oxygen and nitrogen species.

In chronic *H. pylori* infection, there is usually a persistent gastric neutrophilia and this is thought to be particularly important in pathogenesis through release of damaging inflammatory mediators such as reactive oxygen species (ROS) (as reviewed by Robinson *et al.*, 2007). *H. pylori*-induced chronic gastritis is the initial step towards carcinogenesis, since prolonged exposure of the tissue to ROS causes DNA damage and leads to mutation (Farinati *et al.*, 1998). TNFα and IL-8 are considered to be the main oxidative stress markers that trigger increased levels of ROS (Augusto *et al.*, 2007). Xu *et al.* identified one source of oxygen radicals in human gastric epithelial cells to be hydrogen peroxide (H_2O_2). This is specifically produced via *H. pylori*-induced spermine oxidase activity and resulted in apoptosis and DNA damage (Xu *et al.*, 2004). Superoxide (O_2^-) is another major source of intracellular ROS (as reviewed by Imlay, 2003). When various *H. pylori* strains were used to infect primary cultures of human gastric mucosal cells, the CagA protein was found to play an important role in the induction of oxidative stress and DNA damage (Bagchi *et al.*, 2002). This was confirmed *in vivo* where gastric

biopsies from patients infected with *cagA*-positive *H. pylori* had higher scores for gastritis and greater oxidative DNA damage overall (Farinati *et al.*, 2003).

In addition, nitric oxide (NO) generated by the enzyme inducible nitric oxide synthase (iNOS) during inflammation may also injure gastric epithelial cells. NO has antimicrobial activity and can inhibit growth of viruses, parasites and Gram-positive organisms by causing DNA damage (as reviewed by Jaiswal *et al.*, 2001). However, too much exposure to NO is lethal for cells and continued release of NO by the tumour cells is reported to promote tumour growth (Jenkins *et al.*, 1995). NO plays several roles in cancer development: an endogenous mutagen; an angiogenesis factor; an enhancer of protooncogene expression and an inhibitor of apoptosis (as reviewed by Jaiswal *et al.*, 2001). iNOS expression in *H. pylori*-stimulated gastric epithelial cells is thought to be regulated by NF-κB activation (Lim *et al.*, 2001).

1.2.1.3. Cyclooxygenase-2 (COX-2).

Cyclooxygenase (COX) is a rate limiting enzyme in the synthesis of prostaglandins and exists initially in two isoforms, COX-1 and COX-2 (as reviewed by Konturek *et al.*, 2005). A third isoform of this enzyme, COX-3 has also been characterized. COX enzymes may be inhibited selectively or non-selectively by non-steroidal anti-inflammatory drugs (NSAIDs) (Chandrasekharan *et al.*, 2002). Prostaglandins have diverse functions including maintaining mucosal integrity and blood flow (Takeuchi *et al.*, 1999). They also induce inflammation, fever and pain. Prostaglandins are also reported to be tumourogenic (Plummer *et al.*, 1995) and mutagenic (Boolbol *et al.*, 1996).

COX-2 is well known for its role in gastrointestinal carcinogenesis, and the growth of tumours is markedly attenuated in COX-2 deficient ($COX\text{-}2^{-/-}$) mice but not in $COX\text{-}1^{-/-}$ or wild type mice (Williams *et al.*, 2000). Chulada *et al.* found that both COX-1 and COX-2 are important in intestinal tumourogenesis (Chulada *et al.*, 2000). COX-2 inhibition using its specific inhibitors rofecoxib (Konturek *et al.*, 2003) and celecoxib (Steinbach *et al.*, 2000), in clinical studies significantly reduced the development of colonic cancer.

In connection with *H. pylori* infection, COX-2 mRNA expression is significantly increased in patients with gastritis compared to uninfected patients with and without gastritis (Fu *et al.*, 1999; Tatsuguchi *et al.*, 2000). *H. pylori* infection induces expression of mRNA and protein for COX-2 via activation of NF-κB in a time-dependent manner (Kim *et al.*, 2001). Meyer *et al.* reported that when peripheral blood mononuclear cells (PBMCs) were infected with *H. pylori*, COX-2 is induced and prostaglandin E_2 is produced (Meyer *et al.*, 2003). Prostaglandin E_2 is reportedly involved in the proliferation of human gastric cancer cells (Lim *et al.*, 2002).

1.2.2. Innate immunity and inflammation.

1.2.2.1. Pattern recognition receptors.

Generally, the initiation of innate immune response to bacterial pathogens begins when the host recognizes the pathogens through its pathogen-associated molecular patterns

(PAMPs) such as LPS, flagellins and DNA. The host uses pattern recognition receptors (PRRs) to recognize PAMPs. In the case of *H. pylori* infection, NOD1, the host PRR, is activated by *H. pylori* peptidoglycan which enters gastric epithelial cells when the *cag* T4SS penetrates the cells (Viala *et al.*, 2004). NOD1 is encoded by the caspase-recruitment domain 4 gene (*CARD4*) (Strober *et al.*, 2006). Interaction of peptidoglycan with NOD1 leads to activation of NF-κB (Figure 5) which is the key transcription factor controlling expression of pro-inflammatory cytokines genes, including IL-8 (Chu *et al.*, 2003; Fox & Wang, 2007). The T4SS transports CagA into host cells and induces host cell signalling which leads to NF-κB activation. NF-κB was also found to be activated by *cagE*-positive strains (Maeda *et al.*, 2002), further supporting the presence of *cag* which is essential to initiate inflammation.

The other well-known PRRs are the Toll-like receptors (TLRs), a family of 11 proteins expressed on cell surfaces. TLRs are involved in the development of many pathological conditions including responses to infectious diseases, tissue damage, autoimmune & neurodegenerative diseases and cancer (as reviewed by Chen *et al.*, 2007). TLRs recognize a wide range of molecules and some of them recognize Gram-negative bacteria including TLR2 (recognizes lipoprotein and peptidoglycan), TLR4 (recognizes Gram-negative LPS), TLR5 (recognizes flagellin) and TLR9 (recognizes bacterial CpG DNA motifs) (as reviewed by Algood & Cover, 2006). It has been suggested that TLR2 maybe the dominant innate immune receptor for recognition of gastrointestinal *Helicobacter* species (Mandell *et al.*, 2004).

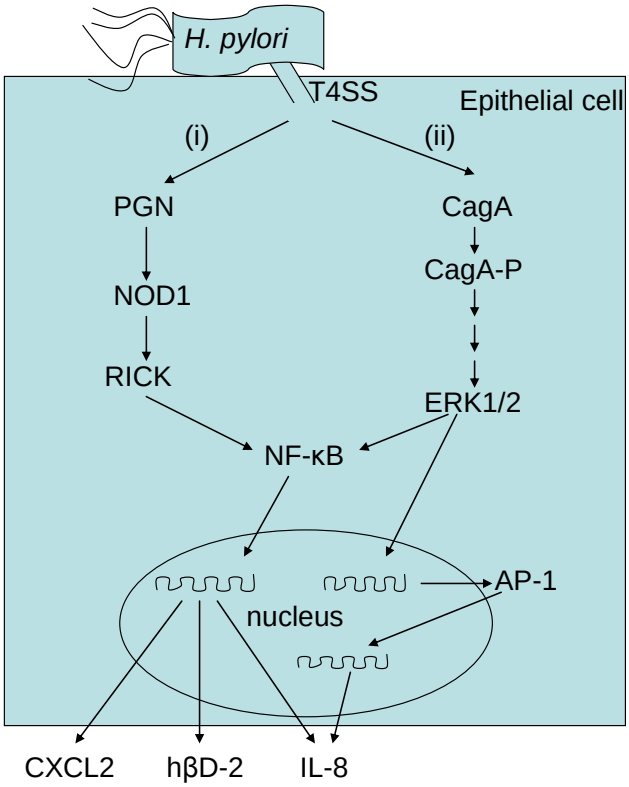

Figure 5. The induction of pro-inflammatory responses by *H. pylori*.
When (i) peptidoglycan (PGN) or (ii) the CagA protein are delivered into infected gastric epithelial cells via a functional T4SS, they will be recognized by host molecules. (i) PGN recognition by NOD1 leads to NF-κB activation. This activation is via the interactions of NOD1 with receptor-interacting serine-threonine kinase (RICK). The NF-κB complexes are translocated into the nucleus and eventually expression of IL-8, CXC-chemokine ligand 2 (CXCL2) and the antimicrobial peptide human β-defensin-2 (hBD-2) will be up-regulated. (ii) Phosphorylated CagA protein triggers a signalling cascade that also leads to activation of NF-κB as well as activator protein-1 (AP-1) and eventually IL-8 expression by host cells (Adapted from Fox & Wang, 2007).

There is evidence that *H. pylori*-derived LPS is an important virulence factor for induction of gastritis. *H. pylori* infection increases levels of TLR4 located in both the cytoplasm and at the apical surface of the epithelial cells (as reviewed by Chen *et al.*, 2007). However, TLR4 stimulation by *H. pylori* is weak and this helps *H. pylori* to evade the immune response (as reviewed by Lee & Josenhans, 2005). *H. pylori* infection of epithelial cells also induces NF-κB cellular responses via TLR2 and TLR5 *in vitro* (Smith *et al.*, 2003) and NF-κB activation is important for the induction of the inflammatory response.

1.2.2.2. Antibacterial peptides.

Antibacterial peptides are compounds used by the host immune system to attack invading pathogens. Their major killing mechanism is by disrupting microbial membranes. These compounds can also exhibit extracellular effects including inhibition of DNA, RNA or protein synthesis as well as activating antimicrobial enzymes that degrade components of the microbe (Kindt *et al.,* 2007). Defensins and cathelicidins are among the peptides isolated from diverse sources in the body.

It has been shown that human β-defensin 2 (hBD-2) can be induced by *H. pylori* infection of the MKN45 cell line, and this was restricted to strains containing the *cag*PaI (Wada *et al.*, 1999). Activation of NF-κB is essential for the induction of the hBD-2 gene (Wada *et al.*, 2001), which has been detected in gastric cancers from patients with *H. pylori* infection (Uehara *et al.*, 2003). On the other hand, human β-defensin 1 (hBD-1) is constitutively expressed by gastric epithelial cells *in vitro* and *in vivo*, and not up-

regulated by *H. pylori* infection (O'Neil *et al.*, 2000). It has been reported that hBD-2 but not hBD-3 expression is NOD1-dependent (Boughan *et al.*, 2006).

Besides defensins, cathelicidins have also been found in relation to *H. pylori* infection. This is another group of antimicrobial peptide found in mammals (as reviewed by Gennaro & Zanetti, 2000). It has been reported that the production of LL-37/human cationic antimicrobial peptide 18 (hCAP18), the single known cathelicidin in man, is up-regulated in the epithelium and gastric secretion of *H. pylori*-infected patients (Hase *et al.*, 2003). This human cathelicidin has also been found elsewhere in the human body such as the lung (Bals *et al.*, 1998), in sweat (Murakami *et al.*, 2002b) and in the oral cavity (Murakami *et al.*, 2002a).

1.2.3. Adaptive immune responses.

1.2.3.1. Humoral responses.

H. pylori infection induces strong antibody responses in the human gastric mucosa, both in asymptomatic carriers and duodenal ulcer patients. In a study, higher frequencies of both IgA- and IgM-secreting cells are found in infected subjects compared to noninfected subjects but there was no difference in numbers of IgG-secreting cells (Mattsson *et al.*, 1998). This could be due to the difference in endothelial expression of adhesion molecules directing trafficking of B cells to the inflamed gastric mucosa. In addition, the presence of serum antibodies specific for *H. pylori* antigen was also reported and has been utilised in a diagnostic test (Kindermann *et al.*, 2001).

However, production of antibodies may damage the gastric inflammatory response and hinder elimination of the bacteria (Akhiani *et al.*, 2004). It was reported that IgA together with IL-10 helps *H. pylori* to evade host resistance against infection (Akhiani *et al.*, 2005). Furthermore, antibodies are unnecessary for protection, since vaccination is possible in B-cell deficient mice (Sutton *et al.*, 2000).

1.2.3.2. T-cell responses.

The prominent type of T-cells during the immune response to *H. pylori* is the CD4+ T-cell but CD8+ are also present in the gastric mucosa. *H. pylori* virulence factors are able to alter the T-cell response. For example, VacA may reduce T-cell proliferation (Smoot *et al.*, 1996) and impair the priming of immune responses by disrupting antigen presentation (Molinari *et al.*, 1998). Nevertheless, VacA can indirectly induce IL-12 secretion which is needed for T- cell expansion (Gebert *et al.*, 2003). *H. pylori* has mechanisms for evading cellular immunity. Some *H. pylori* strains that are capable of binding to Lewis antigens can interact with DCs and eventually block the development of T helper (Th) 1 cells (Bergman *et al.*, 2004). *H. pylori* infection has caused the up-regulation of B7-H1 expression by gastric epithelial cells which can suppress T-cell activity when engaged (Das *et al.*, 2006). The role of different T-cell subsets (Table 1) which are important in *H. pylori*-mediated disease is discussed below.

	Th1	Th2	Th17	Treg
Inflammation and pathology	More severe (D'Elios *et al.*, 1997)	Less severe (Smythies *et al.*, 2000)	Increased neutrophil infiltration (Shiomi *et al.*, 2008)	Suppressed (Lundgren *et al.*, 2005a)
High levels and disease outcome	Peptic ulcers (Robinson *et al.*, 2008)	Lack of peptic ulcers (Fox *et al.*, 2000)	Peptic ulcers and gastric cancer (Mizuno *et al.*, 2005)	Cancer progression (Enarsson *et al.*, 2006)
Successful vaccination	Required (Taylor *et al.*, 2008)	Not required (Akhiani *et al.*, 2002)	Required (DeLyria *et al.*, 2009)	Inhibits efficacy in mice (Watanabe *et al.*, 2002)
Colonization	Reduced in mice and humans (Holck *et al.*, 2003)	Increased in mice (Fox *et al.*, 2000)	Reduced in mice (Shiomi *et al.*, 2008)	Increased in mice (Raghavan *et al.*, 2003) and humans (Robinson *et al.*, 2008)

Table 1. T-helper subsets in connection with *H. pylori* infection (Adapted from Robinson & Atherton, 2009).

a) CD4+ T-helper cell response.

Many studies have reported the importance of CD4+ T-cells in the response to *H. pylori* infection. They are present in the gastric antral infiltrates of *H. pylori*-infected patients with duodenal ulcers (D'Elios *et al.*, 1997). An increased number of CD4+ T cells have been found in the gastric antrum but not in the duodenum of *H. pylori*-positive

individuals (Lundgren *et al.*, 2005b). CD4+ T-cells can be further divided into subsets of T helper (Th) 1, Th2, Th17 and regulatory T-cells.

Th1

The importance of Th1 cells in modulating *H. pylori*-mediated disease was shown when there was no induction of gastritis in mice deficient in this subset of T-cells after *H. pylori* infection (Eaton *et al.*, 2001). This was supported by the finding that *H. pylori*-infected IFNγ deficient mice induce less inflammation and do not develop gastritis (Akhiani *et al.*, 2002). In addition, CD4+IFNγ+ are the most prevalent subset of CD4+ T cells during *H. pylori* infection (Bamford *et al.*, 1998). A study in rhesus macaques showed an increased prevalence of IFNγ+CD4+ T cells during acute *H. pylori* infection (Mattapallil *et al.*, 2000).

The presence of some *H. pylori*-virulence factors may have a role in skewing the response towards Th1. Strains that posses the *cag*PaI and *vacA* were correlated with the induction of IL-12 in samples from patients with *H. pylori*-gastritis (Takeshima *et al.*, 2009). *oipA* gene was also found to promote the expression of Th1-associated cytokines (Yamauchi *et al.*, 2008). Furthermore, administration of HP-NAP was found to promote Th1 response as well as suppress Th2 response in *in vivo* allergy model (D'Elios *et al.*, 2009).

Human response with Th1 polarization showed a more severe disease outcome. Th1 cytokines (IL-12, IFNγ and TNFα), but not Th2 cytokine (IL-4) were found in samples

from antral biopsies of patients with peptic ulcers (D'Elios *et al.*, 1997). Furthermore, peptic ulcers as well as a more severe gastritis level were associated with an increase in the number of IFNγ-secreting Th1 cells in the infected human gastric mucosa (Robinson *et al.*, 2008).

In addition, the presence of a Th1 response is important for successful *H. pylori*-immunization as shown with decrease in number of bacteria colonising the gastric mucosa of wild type but not the IL-12 and IFNγ deficient mice, after challenge with *H. pylori* (Akhiani *et al.*, 2002; Taylor *et al.*, 2008).

Th2

In patients with gastritis, a Th1 response was found to predominate but for those with gastric cancer and its related pathology, a Th2 response was strongly induced (Ren *et al.*, 2001). The ability of Th2 or IL-4 deficient mice to be equally protected as wild type mice after immunization and challenge implies that a Th2 response is not required for successful *H. pylori* vaccination (Akhiani *et al.*, 2002). However, a Th2 response is needed to suppress inflammation as IL-4 deficient mice elicit a more severe gastritis compared to wild type animals (Smythies *et al.*, 2000).

Th17

Th17 is another important subset and was recently added in the studies of *H. pylori*-mediated immune responses. When IL-17 deficient mice were infected with *H. pylori*, the levels of neutrophils as well as bacterial numbers in the gastric mucosa were found to

be lower than in wild type mice (Shiomi *et al.*, 2008). Together with a study showing high IL-17 concentrations at gastric ulcer sites and correlations with IL-8 expression (Mizuno *et al.*, 2005), these suggested that IL-17 may promote neutrophil influx and inflammation. This was also supported by a study which found increased IL-17 expression as well as neutrophil-attracting chemokines in *H. pylori*-infected mice (DeLyria *et al.*, 2009). Recent findings showed that IL-17 neutralising antibody administered to immunised and *Helicobacter felis*-challenged mice prevented the clearance of bacteria and reduction of gastric inflammation (Velin *et al.*, 2009).

Regulatory T-cells (Tregs).

Tregs have suppressive effects on other cells and their association with *H. pylori* infection will be discussed with further detail in section 1.3.4.

b) CD8+ cytotoxic T-cells.

Together with CD4+ T-cells, CD8+ T-cell responses have also been found in relation to *H. pylori* infection but these have been studied much less intensively. An absence of CD4+ T cells in *H. pylori*-infected mice resulted in increased gastritis and influx of CD8+ T cells, indicating that CD4+ T-cells usually suppress the inflammatory response to *H. pylori* (Tan *et al.*, 2008). A study on samples from duodenal ulcer patients and asymptomatic *H. pylori* carriers showed a substantial production of IFN-γ, mainly by CD8+ cells (Quiding-Jarbrink *et al.*, 2001). This type of T-cells may also express inflammatory cytokines such as IL-17 (Caruso *et al.*, 2008). They are mostly found as memory cells in the gastric mucosa of *H. pylori*-infected individuals (Azem *et al.*, 2006).

In peripheral blood of *H. pylori*-infected duodenal ulcer patient, elevated levels of CD8+ T-cells were found (Figueiredo Soares *et al.*, 2007). However, one study contradicted this finding where the presence of CD8+ T-cells were correlated with non-ulcer *H. pylori* infection (Goll *et al.*, 2005).

1.3. Regulatory T-cells.

1.3.1. Background and function.

T cells are an important component of the human adaptive immune response to *H. pylori* and among these are the regulatory T cells (Tregs). This is a subset of mostly CD4+ T cells which have suppressive effects on other cell types. There appear to be two main types of Tregs depending on their origin. The natural Tregs mature in the thymus (Ng *et al.*, 2001) while the inducible Tregs (Tr1 and Th3) arise from naive T-cells in the periphery when stimulated (Chen *et al.*, 1994). CD8+ Tregs also exist and fall into the latter group (Steinbrink *et al.*, 1999).

Tregs are often characterised as CD4+CD25^{hi} cells, due to their ability to constitutively express high levels of the IL-2Rα-chain (CD25) (Sakaguchi *et al.*, 1995). They also constitutively express other markers such as cytotoxic T lymphocyte antigen 4 (CTLA-4) (Read *et al.*, 2000) and glucocorticoid-induced tumour necrosis factor (TNF) receptor-related protein (GITR) (McHugh *et al.*, 2002). Several types of Tregs express the

transcription factor forkhead box P3 (FoxP3) which is a vital regulator of suppressive gene expression and is required for their development (Fontenot *et al.*, 2003). However, the Tr1 Treg subset was reported not to express FoxP3 (Roncarolo *et al.*, 2006). It has been demonstrated that, together with promoting the generation of Tregs, FoxP3 also suppresses the activity of pro-inflammatory cytokine expression and transcription factors involved in T-cell activation such as nuclear factor of activated T-cells (NFAT) and NF-κB (Bettelli *et al.*, 2005). The inducible Tregs, Tr1 and Th3, are known to secrete suppressive cytokines, for example IL-10 (Groux *et al.*, 1997) and transforming growth factor beta (TGF-β) (Nakamura *et al.*, 2001).

1.3.2. Mechanisms of action.

The subject of how Tregs work is a complicated matter as new factors and mechanisms emerged recently. Firstly, Tregs may act through a contact-dependent mechanism as shown by an *in vitro* study where Tregs were unable to suppress T effector cells when separated by a permeable membrane (Takahashi *et al.*, 1998). Recent research has investigated how Tregs initiate this type of mechanism. One possibility is that Tregs induce apoptosis by depriving T effector cells of an important cytokine, IL-2 (Pandiyan *et al.*, 2007). However, some have found that failure to provide IL-2 is not solely sufficient to induce apoptosis (Duthoit *et al.*, 2005). Other have suggested that Tregs have high expression of granzyme B which is used to kill target cells directly (Cao *et al.*, 2007). Tregs may also release adenosine nucleosides which can cause metabolic disruption of their target cells (Bopp *et al.*, 2007).

Secondly, Tregs have been found to act on T effector cells via interaction with dendritic cells through CTLA-4 or lymphocyte activation gene 3 (LAG3). CTLA-4 was found to be constitutively expressed on Tregs (Read *et al.*, 2000) and the suppression activity of Tregs was reduced in the absence of CTLA-4 (Serra *et al.*, 2003). Alternatively, suppression of T effector could be induced via dendritic cells through a CLTA4:CD80/CD86-dependent mechanism (Mellor & Munn, 2004). Another suitable candidate, LAG3 has been found to be important for maximal Treg function (Huang *et al.*, 2004).

Thirdly, Tregs may act on target cells by expressing regulatory effector molecules which are IL-10, TGF-β and IL-35. IL-10 produced by Tregs was found to be important in suppressing colitis, an Inflammatory Bowel Disease (IBD) (Asseman *et al.*, 1999). However, an adoptive transfer of allergen-specific Tregs induced production of IL-10 after allergen challenge, by CD4+ T effector instead of Tregs themselves (Kearley *et al.*, 2005).

Studies on the role of TGF-β produced by Tregs are rather confusing. *In vitro*, TGF-β was able to suppress IFNγ production by Th1 cells (Lin *et al.*, 2005). A study showed that TGF-β is needed for the expansion of Tregs as well as for their suppressive activity *in vivo* (Huber *et al.*, 2004). However, CD4+CD25+ T-cells could be induced in TGF-β-deficient mice and these cells still exhibit their suppressive activity in absence of TGF-β (Mamura *et al.*, 2004).

Another inhibitory cytokine produced by Tregs is the recently discovered factor IL-35. This has been found to be important for maximal suppressive activity and could suppress T-cell proliferation *in vitro* (Collison *et al.*, 2007). Another *in vitro* study confirmed this and showed that IL-35 could also induce Treg proliferation (Niedbala *et al.*, 2007). The latter study also showed that IL-35 could suppress Th17 differentiation and, under certain conditions, inhibit or expand IFNγ production by CD4+CD25- T-cells.

1.3.3. Tregs and disease.

Tregs play a key role in preventing allergy (Jutel *et al.*, 2003), autoimmunity (Cao *et al.*, 2003), cancer (Sasada *et al.*, 2003) and harmful inflammatory diseases such as IBD (Asseman *et al.*, 1999). Some studies report that Tregs downregulate immunity to pathogens. Belkaid *et al.* showed that an endogenous Treg response is responsible for controlling the persistence of *Leishmania major* in the skin after healing in resistant C57BL/6 mice (Belkaid *et al.*, 2002). Others have shown that herpes simplex virus (HSV) infection induced the immunosuppressive function of Tregs (Suvas *et al.*, 2003). Furthermore, filamentous hemagglutinin of *Bordetella pertussis* could induce formation of Tr1 cells at mucosal surface to suppress *B. pertussis*-induced Th1 response *in vivo* (McGuirk *et al.*, 2002).

Patients with gastrointestinal malignancies tend to have a higher proportion of CD4+CD25+ T cells in their peripheral blood (Sasada *et al.*, 2003). This has also been

reported by Perrone *et al*, who found significantly higher levels of Tregs in gastric carcinoma tissue than in normal surrounding tissue (Perrone *et al.,* 2008). Furthermore, FOXP3+ Tregs were present in significantly higher numbers amongst tumour-infiltrating lymphocytes in gastric cancer compared to lymphocytes from normal gastric tissues (Mizukami *et al.,* 2008). These indicate that Tregs may impair anti-tumour immunity.

1.3.4. The relationship between Tregs and *H. pylori* infection.

Unless treated, *H. pylori* infection usually persists lifelong despite a vigorous immune and inflammatory response. This persistence may be established by inducing the suppressive activity of Tregs. *H. pylori* infected patients have been reported to have a high frequencies of Tregs in the gastric mucosa which dampen immune and inflammatory responses (Lundgren *et al.,* 2005a). A study from my research group found that *H. pylori*-infected gastric tissue contained greatly increased number of Treg cells. A low-level Treg response was significantly associated with the presence of peptic ulcer disease which indicates that peptic ulcer disease occurs when there is an insufficient Treg response to suppress inflammation (Robinson *et al.,* 2008). It has been shown that Tregs can suppress *H. pylori*-induced T cell responses and anti-tumour immunity, which probably contributes to persistence of the infection as well as unchecked tumour growth (Enarsson *et al.,* 2006).

Goll *et al.* measured regulatory cytokine gene expression in the *H. pylori* infected gastric mucosa and found high levels of IL-10 expression which also indicates that IL-10-secreting Tregs play a role in *H. pylori* infection (Goll *et al.,* 2007). Higher numbers of

CTLA-4 positive mononuclear cells and a higher level of TGF-β expression have been found in the lamina propria of *H. pylori*-infected duodenal ulcer patients as compared to asymptomatic carriers (Stromberg *et al.*, 2005). These Tregs were demonstrated to have suppressive activity on IL-8 production by gastric epithelial cells.

1.4. Interleukin-10.

1.4.1. Background and function.

IL-10 is a potent immunoregulatory cytokine and circulates as a homodimer consisting of two tightly packed 160-amino-acid proteins (as reviewed by Opal & DePalo, 2000). Its gene is located on the human chromosome 1q31-32 and consists of approximately 5.2 kbp organized in 5 exons (Kim *et al.*, 1992). IL-10 was first described as cytokine synthesis inhibiting factor (CSIF) due to its ability to suppress cytokine synthesis in certain T cells (Fiorentino *et al.*, 1989). IL-10 shares nucleic acid and amino acid sequence homology with an uncharacterized gene in the Epstein-Barr virus genome, BCRF1 (viralIL-10) (Moore *et al.*, 1990). IL-10 is a member of a wider IL-10 family of cytokines which includes IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 (as reviewed by Commins *et al*., 2008).

IL-10 is produced by many cell types including B cells (Fillatreau *et al.*, 2002), T cells, especially Tregs (Lundgren *et al.*, 2005), monocytes and macrophages (Edwards *et al.*,

2006) and dendritic cells (McGuirk *et al.*, 2002), but not epithelial cells. A study using IL-10-deficient mice showed that Tregs are an important source of IL-10 (Sydora *et al.*, 2008). The production of IL-10 from immature dendritic cells can promote the differentiation of CD4+ T cells into Tr1 Tregs which express IL-10 (Levings *et al.*, 2005). Initially, only T helper (Th) 2 cells were known to produce IL-10 (Gazzinelli *et al.*, 1992) but recently Th1 cells were reported to produce IL-10 (O'Garra & Vieira, 2007).

IL-10 and IL-10-secreting Tregs are known to be important for preventing disease in the gastrointestinal tract. A study reported that IL-10 was strongly expressed by intraepithelial lymphocytes in the small intestine and colonic lamina propria lymphocytes after multiple T cell receptor stimulations (Kamanaka *et al.*, 2006). Kullberg *et al.* have shown that IL-10 produced by Tr1 cells can suppress Th1-derived IFN-γ expression in a mouse model of IBD using *H. hepaticus* infection (Kullberg *et al.*, 2002). Their work also reported that those Tregs subset inhibited *H. hepaticus*-induced colitis.

Furthermore, IL-10 could also control inflammatory responses towards intestinal antigens (Asseman *et al.*, 1999) and epithelial damage in ulcerative colitis (van der Sluis *et al.*, 2008). In addition, the constitutively-expressed IL-10 by human normal colonic mucosa is important in preventing intestinal barrier disruption by strong IFN-γ responses (Jarry *et al.*, 2008). In mice with colon cancer, the presence of IL-10-secreting Tregs reduced cancer progression (Erdman *et al.*, 2003). However, high serum level of IL-10 has been associated with poor prognosis of gastric cancer patients (Ikeguchi *et al.*, 2009).

1.4.2. Regulatory effects of IL-10.

The major role of IL-10 is to suppress inflammation and regulate T-cell subsets responses. IL-10 can specifically suppress IFN-γ and IL-2 production by Th1 cells (Groux *et al.,* 1998), which was the initial reason for it being named CSIF. Th1 cells are the major source of IFN-γ, which is a potent activator of monocytes and inhibitor of Th2 proliferation (Mamata *et al.,* 2007). By suppressing IFN-γ, IL-10 helps to maintain polarized T helper cells in a Th2 phenotype (Ito *et al.*, 1999).

IL-10 can also inhibit IFN-γ production by natural killer (NK) cells (Gazzinelli *et al.*, 1992) and block NF-κB activation. This transcription factor controls expression of many pro-inflammatory and antiapoptotic genes (Table 2) and is very important in the development of acute and chronic inflammatory diseases (Schottelius *et al.*, 1999). Through the blockade of NF-κB activation, IL-10 inhibited IL-1-mediated induction of COX-2 protein synthesis (Al-Ashy *et al.*, 2006) and COX-2 protein expression in neutrophils (Niiro *et al.*, 1997). Furthermore IL-10 can inhibit monocyte adherence to endothelial cells (Mostafa Mtairag *et al.,* 2001). The expression of IL-17, IL-22, and the Th17-cell associated transcription factor retinoic acid-related orphan receptor (ROR)γt by macrophages and T cells were negatively regulated by IL-10 (Gu *et al.*, 2008).

On the other hand, IL-10 exhibits immunostimulatory properties on B cells and endothelial cells (MacDonald *et al.*, 1999). IL-10 can influence mast cell function and significantly enhances the growth activity of stem cell factor on these cells (as reviewed by Lin & Befus, 1997). When IL-10 is combined with IL-3 and IL-4, the number of mast

cell colonies is increased and the production of pure mast cells is augmented (Thompson-Snipes *et al.*, 1991).

***H. pylori*-induced host factors regulated by NF-κB activation**	**Role**	**References**
IL-8	Chemotaxis for neutrophils and lymphocytes	(Chu *et al.*, 2003)
iNOS	Enzyme that generates cell-damaging NO	(Lim *et al.*, 2001)
COX-2	The rate limiting enzyme in the synthesis of prostaglandins	(Kim *et al.*, 2001)
hBD-2	Anti-bacterial peptide	(Wada *et al.*, 2001)
MMP-9 and -7	Matrix metalloproteinases, tumour invasiveness	(Mori *et al.*, 2003; Wroblewski *et al.*, 2003)
IAP and Mcl-1	Anti-apoptotic genes	(Chang *et al.*, 2004; Maeda *et al.*, 2002)
IL-12p40, TNF-α, IFN-γ, IL-2, IL-6	Pro-inflammatory cytokines	(Lu et al., 2005; Takeshima et al., 2009; Toyoda et al., 2009)
VEGF, HIF-1α	Angiogenic growth factors	(Yeo *et al.*, 2006)
Bax	Apoptotic gene	(Cha *et al.*, 2009)
PAI-2	Inhibit fibrinolysis (degradation of blood clots)	(Varro *et al.*, 2004)

Table 2. Some of the host factors regulated by NF-κB activation in response to *H. pylori* infection.

1.4.5. IL-10 and therapy.

1.4.5.1. Administering IL-10.

IL-10 is an important anti-inflammatory cytokine which is well-known for its suppressive effects. These properties of IL-10 made it a suitable candidate therapeutic for some diseases. Numerous studies using animal models reported that administering IL-10, either by delivering the *IL10* gene, injecting recombinant human IL-10 (rhIL-10), or administering agents to promote the induction of IL-10, may prevent diseases such as liver fibrosis, airway inflammation and renal impairment (Chou *et al.*, 2006; Nakagome *et al.*, 2005; Tipping *et al.*, 1997).

Cua *et al.* used a replication-defective adenovirus as a vector to directly deliver IL-10 to the central nervous system in an animal model of multiple sclerosis and found that IL-10 effectively blocked disease progression (Cua *et al.*, 2001). Multiple injections of plasmid DNA vectors encoding IL-10 into the spinal canal of rats relieved neuropathic pain for more than forty days (Milligan *et al.*, 2006). In the latter study, the administration of the IL-10 protein only resolved the pain for few hours. These studies showed the importance of IL-10 being delivered close to targeted sites and the need for it to be in an active state to be useful.

In addition, Lee *et al.* used non-obese-diabetic mice to see the effect of *IL10* gene delivery to combat type 1 diabetes, with IL-10 plasmid (pSI-IL-10-NFκB) (Lee *et al.*, 2006). They found that a single injection of the *IL10* gene completely prevented disease. *IL10* gene therapy could also prevent sepsis in mice (Oberholzer *et al.*, 2002) and

experimental colitis in rats (Barbara *et al.*, 2000). In the latter study, timing of gene delivery was crucial as a beneficial effect was seen only when the *IL10* gene was delivered prior to induction of colitis. This is also true in another experimental colitis study, where adenoviral vector encoding IL-10 was injected prior to trinitrobenzene sulphonic acid induced-colitis to successfully cure the disease (Lindsay *et al.*, 2002).

The role of IL-10 as anti-inflammatory substance has also attracted many therapeutic studies for skin diseases. In a mouse model for allergic contact dermatitis (an inflammatory skin disorder) animals had less inflammatory cell infiltration and lower oedema at the dermis when injected with IL-10 (Kondo *et al.*, 1994). IL-10 was found to work by suppressing IFN-γ production, thus further supporting its role as an inflammatory inhibitor. Furthermore, IL-10 helped to decrease disease severity in mice with Pemphigus vulgaris, an autoimmune bullous skin disease (Toto *et al.*, 2000).

IL-10 has also been used as adjuvant in a mouse model study of an anti-cancer vaccine (Kaufman *et al.*, 1999). Mice with induced lung cancer were vaccinated with recombinant vaccinia virus followed by injections of IL-10. Although the results were promising; with decreasing rates of tumour metastases and low viral titres, its application for use in humans would need more validation. In another cancer study, IL-10 was also found to significantly inhibit tumour growth and eliminate metastasis in mice with melanoma (Huang *et al.*, 1999).

In addition, the importance of IL-10 in suppressing inflammation has been demonstrated in IL-10$^{-/-}$ mice, which responded with high expression of TNFα and excessive inflammation upon infection with *Mycobacterium tuberculosis* (Higgins *et al.*, 2009). A similar effect was also shown when neutralising IL-10 antibodies were administered to *Streptococcus*-infected mice (Puliti *et al.*, 2002). In the latter study, a reduction in pathology as well as in the expression of inflammatory cytokines was also seen when IL-10 was administered.

In contrast to the animal studies, IL-10 therapy has shown a variety of outcomes in humans. rIL-10 given subcutaneously to patients with Crohn's disease had little efficacy in treating the disease (Tilg *et al.*, 2002). This could be due to inappropriate timing or localization of the rIL-10 (as reviewed by O'Garra *et al.*, 2008). Others have demonstrated a significant effect of subcutaneous rIL-10 application to individuals with psoriasis (Asadullah *et al.*, 2001), where IL-10 acted to decrease IFN-γ and TNF-α (Reich *et al.*, 2001). Friedrich *et al.* reported that after remission of psoriasis, the use of long-term subcutaneous IL-10 at a low dose is safe and could prevent the condition from recurring (Friedrich *et al.*, 2002). The safe-application of rIL-10 was shown in other studies, which also found no side effects from injecting a single large dose of IL-10 to healthy volunteers (Fuchs *et al.*, 1996). In the treatment of asthma, agents have been administered (e.g. glucocorticoids) which increase IL-10 production. Such treatments have been correlated with significant increases in *FOXP3* mRNA expression and therefore an increased Treg response in asthmatic patients (Karagiannidis *et al.*, 2004).

However, IL-10 can also promote pro-inflammatory activity making it a risky choice as a therapeutic agent. Administration of rIL-10 to healthy volunteers, either prior to or after LPS infection, enhanced IFNγ production and NK cell activity (Lauw *et al.*, 2000). These data showed that IL-10 administration to individuals with a deficiency in their inflammatory response could initiate unwanted pro-inflammatory outcomes. Some mouse model studies also demonstrated IL-10 as an agent that worsens disease. IL-10-transgenic mice, which over expressed IL-10, had increased tissue inflammation, mucus metaplasia and airway fibrosis (Lee *et al.*, 2002). Furthermore, inoculation with *Streptococcus pneumoniae* plus rIL-10 shortened the life expectancy of mice (van der Poll *et al.*, 1996).

1.4.5.2. Neutralising IL-10.

Several animal studies have shown that blocking IL-10 activity also has a therapeutic benefit. Neutralising IL-10 activity either by administering anti-IL-10R monoclonal antibody (mAb) or using *IL10* gene deletion has effectively been used in combating bacterial and viral infection. For example, Brooks *et al.* demonstrated that the viral load after infection with lymphocytic choriomeningitis virus (LCMV) persisted in the presence of IL-10 (Brooks *et al.*, 2006). When anti-IL-10R mAb was administered, the viral density decreased and the infection was eliminated. This work was supported by another study which cleared LCMV infection without causing pathology (Ejrnaes *et al.*, 2006). In addition, blocking IL-10 activity enhanced the effectiveness of Hepatitis C antiviral treatment (Rigopoulou *et al.*, 2005).

Blockade of IL-10R has also been used in treating mice infected with *Mycobacterium tuberculosis* (Beamer *et al.,* 2008). In this study, administering anti-IL-10R1 mAb to the infected mice, controlled bacterial growth and increased mouse survival rates. Another study on *Mycobacterium* infection also demonstrated the effectiveness of administering anti-IL10R mAb to eliminate infection (Silva *et al.*, 2001). This study not only showed that blocking IL-10R improves the effectiveness of chemotherapy when the infection is latent or chronic, but that the anti-IL-10R mAb is also useful as an adjuvant in vaccination against *M. avium.*

The use of anti-IL-10R mAb as an adjuvant has also proved valuable in some animal studies of *Leishmania major* infection. Vaccination with killed *L. major* (Okwor *et al.*, 2009), its DNA (Roberts *et al.*, 2005) or plasmid containing some genes of this parasite (Stober *et al.*, 2005) together with anti-IL-10R mAb prior to challenge reduced parasite proliferation effectively. In addition, anti-IL-10 treatment itself has proved to be a beneficial therapy in Leishmaniasis, at least in animal studies. In the absence of IL-10 or with the blockade of IL-10R, rapidly decreased parasite loads of *L. major* (Kane & Mosser, 2001) and *Leishmania donovani* (Murray *et al.*, 2002) were seen in infected mice.

Neutralising IL-10 therapeutic studies have also been tested in oncology. When CpG immunostimulatory oligonucleotides and anti-IL-10R mAb were administered simultaneously in mouse tumour models, the anti-tumour immune response was enhanced compared to administering CpG alone (Vicari *et al.*, 2002). Furthermore,

priming for Th1 responses with soluble ovalbumin preparation containing LPS, was found successful with the use of anti-IL-10R mAb as adjuvant (Castro *et al.*, 2000). This study may be helpful in designing vaccines for immunotherapy.

The therapeutic strategy of silencing the *IL10* gene has been applied to skew the immune response against tumours or pathogens. Dendritic cells (DCs) transfected with small interfering RNA (siRNA) that is specific for the *IL10* gene will produce high levels of IL-12 (Liu *et al.*, 2004) and help CD4+ T cells to increase IFNγ and decrease IL-4 production (Singh *et al.*, 2008). These also promote antiviral activity in cytotoxic T cells. However, the increased production of pro-inflammatory cytokines due to the inactivation of *IL10* gene in T-cells, resulted in severe pathology after *Toxoplasma gondii* infection (Roers *et al.*, 2004). In a human melanoma *in vitro* study, silencing the *IL10* gene in DCs increased IL-12 production and improved the cytotoxic T cells response (Chhabra *et al.*, 2008).

1.4.3. IL-10 receptor and signaling.

Interleukin-10 bears close topological resemblance to IFN-γ and their receptor complexes also belong to the same cytokine receptor family (Ding *et al.*, 2001). The IL-10 receptor (IL-10R) is made of at least two subunits, the α-chain (IL-10R1) and the β chain (IL-10R2) (as reviewed by Moore *et al.*, 2001). The α-chain, which is a 110-kDa polypeptide, has the dominant role in mediating high affinity ligand binding and signal transduction (Riley *et al.*, 1999). Its gene is located on human chromosome 11 (Liu *et al.*, 1994). It has been shown that IL-10 binds to IL-10R1 with high affinity (Figure 6) and induces

changes to the conformation of the IL-10-IL-10R1 complex for the IL-10R2 to come into contact with (Yoon *et al.,* 2006). The gene for IL-10R2 is in the same cluster as the IFN-γR2 and both genes are located on chromosomes 21 (Reboul *et al.*, 1999).

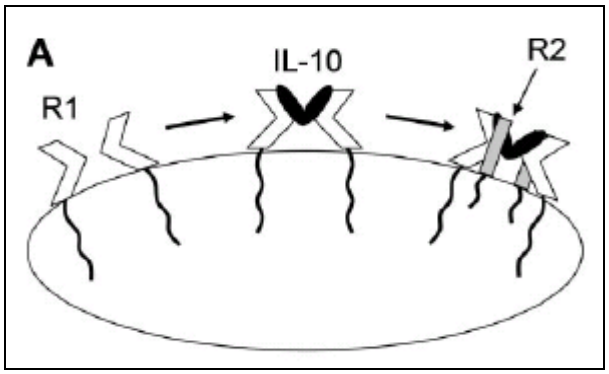

Figure 6. A schematic model of IL-10 binding to its receptor.
IL-10 binds to IL-10R1 with high affinity, followed by low affinity interactions between IL-10R2 and the IL-10-IL-10R1 complex (Taken from Yoon *et al.*, 2006).

The expression of IL-10R on different types of human cells is a particularly understudied area. Most is known about IL-10R on leukocytes but there is very little published on other cell types. This is important as we need to understand which cells are capable of responding to IL-10 thus modulating their inflammatory responses.

Immune cells are important during inflammation and well known for their high IL-10R expression so that they can switch this response off when needed to prevent tissue damage. NK cells constitutively express cell surface IL-10R at low density (Carson *et al.*, 1995). Neutrophils and macrophages were found to express the receptor at cell surface but internalised the receptor when stimulated (Du *et al.,* 2006; Elbim *et al.*, 2001).

Monocytes express IL-10R at low levels but expression was increased in association with diseases such as arthritis (Takasugi *et al.*, 2006). Dendritic cells (MacDonald *et al.*, 1999), B-cells and T-cells (CD4+ and CD8+) were also reported to express IL-10R (Lai *et al.*, 1996). In a study on leukocyte subpopulations of asthmatic patients, monocytes and CD4+CD25^{hi} T-cells were found to express the highest level of IL-10R while expression of the receptor on CD8+ T-cells, NK cells and neutrophils was low (Moniuszko *et al.*, 2009). These indicate that different type of leukocytes may express IL-10R differently compared to each other in disease states.

Some non-leukocytes also express the receptor for IL-10 especially cells throughout the gastrointestinal tract which are frequently in contact with harmful pathogens. A study on *Prevotella intermedia*, a pathogen in periodontal disease, has confirmed that the expression of IL-10R in cultured human dental pulp fibroblasts is induced by the presence of bacterial LPS (Tokuda *et al.*, 2003). Evidence from experiments with the murine small and large intestine has shown that IL-10 binds to a specific receptor which is constitutively expressed on intestinal epithelial cells (Denning *et al.*, 2000). This was also shown *in vitro*, whereby the receptors were found to be constitutively expressed on Mode-K cells (a murine intestinal cell line) (Al-Ashy *et al.*, 2006).

The best characterized IL-10 signalling pathway is the Jak/Stat system (as reviewed by Moore *et al.*, 2001). The Jak/Stat family consist of four Janus Tyrosine Kinases (Jak1, Jak2, Jak3 and Tyk2) and seven latent cytosolic transcription factors (Stat1-Stat6) (Weber-Nordt *et al.*, 1996). When the receptors for IL-10 interact with their ligand,

members of the Jak family will be phosphorylated and eventually the Jak/Stat pathway will be activated (Ding *et al.*, 2001). Specifically, IL-10 affects the activation of Jak1 (associated with IL-10 receptor α chain) and Tyk2 (associated with the IL-10 receptor β) (Riley *et al.*, 1999). In monocytes and T cells, IL-10 stimulates tyrosine phosphorylation of Tyk2 and Jak1 together with the activation of STAT1 and STAT3 (as reviewed by Finbloom & Winestock, 1995). The binding of IL-10 to its receptor also activates STAT5 in a murine B-cell line (Wehinger *et al.*, 1996). To date, there is no published data on the expression of IL-10R by gastric epithelial cells.

1.4.4. IL-10 super family and sharing of IL-10 receptor subunits.

IL-19, IL-22, IL-24, IL-26, IL-28 and IL-29 are also members of the IL-10 family (as reviewed by Commins *et al.*, 2008). These cytokines have approximately 20% amino acid sequence identity to IL-10 (as reviewed by Pestka *et al.*, 2004) and have been further subdivided into two groups according to the sharing of receptor subunits and gene localization. IL-19, IL-20 and IL-24 share the IL-20R2 in their receptor complexes (Dumoutier *et al.*, 2001a) and their genes are located close to *IL10* gene on chromosome 1 (Gallagher *et al.*, 2000; Blumberg *et al.*, 2001; Huang *et al.*, 2001). IL-22, IL-26, IL-28 and IL-29 on the other hand, share the IL-10R2 subunit (Donnelly *et al.*, 2004) and except for the later, their genes are located in the same cluster as the gene encoding IFNγ, on chromosome 12 (as reviewed by Pestka *et al.*, 2004). IL-28 and IL-29 are the most

recent addition to this family and their genes are located on chromosome 19 (as reviewed by Uze & Monneron, 2007).

Unlike IL-10, IL-19 is a monomer (Chang *et al.,* 2003) produced by monocytes and has a direct effect on immune cells (Gallagher *et al.,* 2000). Another member of this family, IL-20, is also expressed by monocytes (Wolk *et al.,* 2002) and has been associated with skin disease. Overexpression of this cytokine leads to psoriasis (Blumberg *et al.,* 2001). The melanoma differentiation-associated gene-7 (mda-7) or IL-24, discovered by Jiang *et al.* (1995) has been shown to induce apoptosis in some cancer cells (Saeki *et al.,* 2000). This cytokine has also been found to have suppressive effects on the inflamed intestinal mucosa in IBD (Andoh *et al.*, 2009). Although IL-19, IL-20 and IL-24 share components of their receptor complexes among them, it has been noted that IL-19 and IL-24 are growth inhibitory but not IL-20 (Parrish-Novak *et al.*, 2002).

IL-10-related T cell-derived inducible factor (IL-TIF) or IL-22 was discovered in 2000 and has been intensively studied (Dumoutier *et al.*, 2000a). It is produced by activated T cells and NK cells (Wolk *et al.,* 2002). IL-22 has the opposite function from IL-10, including pro-inflammatory activity on hepatocytes (Dumoutier *et al.,* 2000b).

AK155 or IL-26 is expressed mainly by T cells and NK cells (Wolk *et al.*, 2002) and targets epithelial cells, colon carcinoma cells and keratinocytes (Hor *et al.,* 2004). IL-26 was initially isolated from herpesvirus saimiri-transformed T cells and formed a homodimer, similar to IL-10 (Knappe *et al.*, 2000). High expression of IL-26 has been

correlated with increased IL-8 expression which is opposite to the activity of IL-10 (Dambacher *et al.*, 2008).

IL-28/29 or IFN-λs are type III IFNs which were discovered in 2003 (Kotenko *et al.*, 2003) and showed anti-tumour activity against B16 melanoma (Lasfar *et al.*, 2006). They also have antiviral activity against encephalomyocarditis virus (EMCV) and herpes simplex virus type-2 (HSV-2) (Ank *et al.*, 2006).

All IL-10 family members utilise heterodimeric receptor complexes which are composed of a type 1 receptor chain (R1) and a type 2 receptor chain (R2) to exert their biological effects (as reviewed by Wolk & Sabat, 2006). IL-10 and its receptor have been described previously (section 1.4.3). Several IL-10 family cytokines utilise an IL-10R subunit in their receptor complexes.

The IL-10R2 subunit is shared with the receptor complexes of IL-10, IL-22, IL-26 and IL-28/29 (Figure 7) (Donnelly *et al.*, 2004). Some researchers found that IL-22 binds directly to IL-10R2 (Xie *et al.*, 2000) but others have found that it binds to both IL-22R1 and IL-10R2 subunits (Kotenko *et al.*, 2001). It has a natural antagonist, called IL-22 binding protein (IL-22BP) which, when bound, inhibits its activity in hepatocytes and intestinal epithelial cells (Dumoutier *et al.*, 2001b). The binding of IL-22 to its receptor complex also activates STAT1/3, similar to IL-10 and most other IL-10 family cytokines (Brand *et al.*, 2006).

The IL-26 receptor complex consists of IL-20R1 and IL-10R2 (Hor *et al.*, 2004). These receptor subunits have been found to be expressed by intestinal epithelial cell lines (Dambacher *et al.*, 2008). The IL-28/29 receptor complex consists of IL-10R2 and IL-28RA (Sheppard *et al.*, 2003).

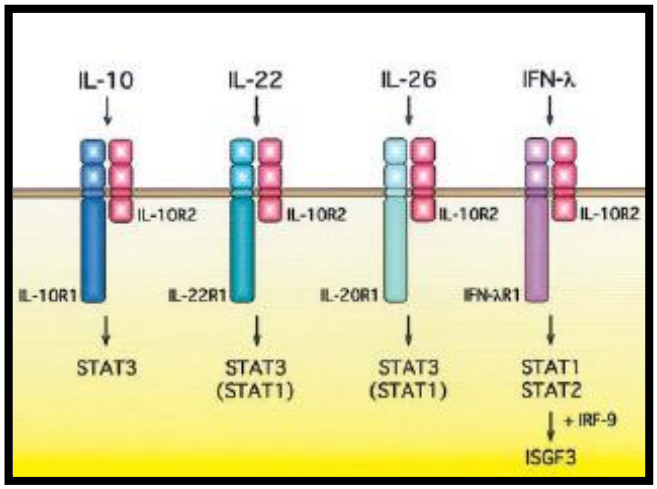

Figure 7. The IL-10R2 chain is shared amongst several IL-10 superfamily cytokine receptor complexes.
The binding of ligands (IL-10, IL-22, IL-26 and IFN-λ (IL-28/IL-29)) to their specific ligand binding chain, the R1, formed intermediate complexes with the required conformation for the binding of IL-10R2. These will then activate JAK and Tyk2 followed by activation of specific STATs. STAT3 is the major transcription factor activated by IL-10, IL-22 and IL-26. IFN-λ, however, activates both STAT1 and STAT2. These two STATs can combine with IRF-9, another cytosolic protein to form the ISGF3 transcription factor complex (Taken from Donnelly *et al.*, 2004).

1.4.5. IL-10 responses in *H. pylori* infection.

1.4.5.1. IL-10 gene polymorphisms.

Studies on IL-10 gene polymorphisms and disease have been conflicting due to this cytokine having both immunosuppressive (potentially cancer promoting) and anti-angiogenic (potentially cancer inhibiting) properties (as reviewed by Howell & Rose-Zerilli, 2006). Most of the research has been done on SNPs of the *IL-10* promoter region which includes position -1082(G/A), -819(C/T) and -592(C/A). Carriers of the *IL-10*-1082G/-819C/-592C alleles (GCC haplotype) had higher mucosal IL-10 mRNA levels than ATA haplotype carriers (Rad *et al.*, 2004). Furthermore, the ATA/ATA genotype has been confirmed to have an association with low IL-10 production (Crawley *et al.*, 1999).

Polymorphisms that resulted in reduced expression levels of IL-10 have been reported to be associated with a higher risk of gastric cancer (El-Omar *et al.*, 2003). Subjects with the *IL-10*-1082AA genotype were at a two-fold higher risk of gastric cancer compared to those with an *IL-10*-1082GG genotype. Furthermore, Kato *et al.* found a 60% increase in the risk of intestinal metaplasia and dysplasia subsequent to *H. pylori* infection, among the carriers of the *IL-10-1082* low activity allele (Kato *et al.*, 2006). The subjects for that study were known to have extremely high *H. pylori* infection rates. Another study done in an Italian population also showed a higher risk for non-cardia gastric cancer and intestinal metaplasia with individuals having *IL-10*-819TT genotype (Zambon *et al.*, 2005). This was also true in another case-control study where subjects with T allele at

position -819 of the IL-10 promoter region were more at risk of gastritis (46.5%) compared to controls (35.7%) (Achyut *et al.*, 2008).

In contrast, others have reported that polymorphisms resulting in increased IL-10 expression are associated with the increased risk of *H. pylori*-mediated diseases. For example, two studies in a Chinese population have shown that a high IL-10 producer genotype was significantly associated with gastric cancer (Lu *et al.*, 2005b; Wu *et al.*, 2003). Sicinschi *et al.* (2006) also reported that subjects having the *IL-10*-592CC genotype were associated with more than double of the risk of the intestinal-type gastric cancer, and the risk was higher in subjects infected with CagA positive *H. pylori*. Furthermore, in a Japanese population, the presence of an ATA/GCC haplotype of *IL-10*-1082/-819/-592 polymorphisms significantly increased the risk of gastric cancer development compared to ATA/ATA haplotype (Sugimoto *et al.*, 2007).

1.4.5.2. IL-10 and *H. pylori*-mediated diseases.

H. pylori infections were found to be associated with elevated IL-10 expression in the human gastric mucosa. Goll *et al*. reported that the samples from *H. pylori*-positive patients showed an increased of 6.7 fold in IL-10 production compared to the samples from *H. pylori*-negative patients (Goll *et al.*, 2007). A study in a German population has confirmed that colonization by more virulent *cagA*$^+$, *vacAs1*$^+$ and *babA2*$^+$ *H. pylori* strains were associated with high level mucosal IL-10 production (Rad *et al.*, 2004).

Similar findings also supported that infection with a CagA+ strain is associated with an increase in IL-10 mRNA expression (Hida *et al.*, 1999; Robinson *et al.*, 2008).

Studies of *H. pylori* infections using mouse models have shown a relationship between the IL-10 response, intensity of gastric mucosal inflammation and bacterial load (as reviewed by Algood & Cover, 2006). Research with IL-$10^{-/-}$ mice has shown that, after 6 weeks of inoculation, colonization of the gastric mucosa by *H. pylori* was significantly decreased and this reduction was accompanied with enhanced gastric inflammation and elevated levels of specific IgA and IgG (Chen *et al.*, 2001). Another similar but longer study showed complete eradication of *H. pylori* and more severe inflammation in IL-$10^{-/-}$ mice (Matsumoto *et al.*, 2005).

In addition, a high level of IL-10 was also associated with increased numbers of Tregs in the gastric mucosa of *H. pylori*-infected patients (Lundgren *et al.*, 2003). This was also supported by the study of Lundin *et al.* which reported high amounts of IL-10 productions from both peripheral blood and gastric mucosa of *H. pylori*-infected gastric cancer patients (Lundin *et al.*, 2007). In the later study, the IL-10 production from blood T cells of *H. pylori*-infected asymptomatic subject was low.

Furthermore, the presence of high levels of IL-10 produced by Tregs was also linked to reduced pathology as found in a study (Lee *et al.*, 2007), using recombination-activating gene 2-deficient (Rag$2^{-/-}$) mice, which are unable to produce T-cells (Vieira *et al.*, 2004). Previous studies in our laboratory have shown that gastric biopsy samples with the

highest *H. pylori* densities (graded 3 on a scale of 0-3) contained 6-fold higher levels of *IL10* mRNA than samples with a grade of 1 (Robinson *et al.,* 2008). When Harris *et al*. evaluated gastric histopathology and Treg cell responses in *H. pylori*-infected children and adults; they found that the level of IL-10 in the antral mucosa of *H. pylori*-infected children was significantly 2 fold higher than the level in infected and uninfected adults and 3 fold higher than in uninfected children (Harris *et al.*, 2008). This was correlated with infected children having reduced gastritis and higher number of Tregs in the gastric mucosa compared to infected adults.

In addition, previous *in vitro* work in our laboratory also showed a significantly increased bacterial density with the addition of 20ng/ml rIL-10 in AGS-*H. pylori* co-cultures (Robinson *et al.,* 2008). This effect was shown via AGS cells because addition of rIL-10 to *H. pylori* alone has no direct effect. This indirect effect suggested that AGS cells may express the receptor for IL-10 but further experiments were needed to verify this. Furthermore, in the same study, *H. pylori*-induced IL-8 expression was suppressed in dose-dependent manner with the addition of rIL-10 in co-cultures of *H. pylori* with AGS cells *in vitro*. What is more, *H. pylori*-induced NF-κB activation (important for IL-8 expression) was also inhibited in the presence of rIL-10 in the same co-cultures. Another pro-inflammatory marker, ICAM1, which expression was elevated in AGS-*H. pylori* co-cultures, was significantly reduced by the addition of rIL-10 (Zakaria *et al.,* 2008). These data indicate that IL-10 may suppress *H. pylori*-induced inflammatory responses which allow the bacterium to grow to higher densities and the effects were mediated indirectly through gastric epithelial cells.

1.5. Aims.

Reports have shown that IL-10 is abundant in the gastric mucosa during *H. pylori* infection (Lundin *et al.,* 2007; Goll *et al.,* 2007). IL -10 responses have also been associated with increased bacterial density and reduced pathology. Previous experiments showed that addition of rIL-10 to co-cultures of *H. pylori* and AGS cells resulted in reduced IL-8 response (Robinson *et al.,* 2008). Therefore, it was hypothesised that IL-10 helps to maintain bacterial colonisation and exerts its effects directly on gastric epithelial cells. The aims of this study were:

1. To investigate the expression of IL-10R subunits by gastric epithelial cells,
2. To characterise the effects of IL-10 on *H. pylori*-infected gastric epithelial cell lines,
3. To determine how IL-10 influences *H. pylori*-induced responses.

CHAPTER 2:

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

2.1. *H. pylori* strains and culture conditions.

H. pylori strains were grown on blood agar base no.2 plates supplemented with 5% (v/v) horse blood (Oxoid Ltd.) at 37^{o}C in a 5% CO_2 humidified incubator. The strains used were Tx30a –*cag*PAI-negative strain (Rieder *et al.*, 1997), *vacA* s2/m2 (Letley *et al.*, 2003), 60190 – *cag*PAI-positive strain (Rieder *et al.*, 1997), *vacA* s1/m1 (Letley *et al.*, 2003), 60190 *cagA* mutant (Bebb *et al.*, 2006), 60190 *cagE* mutant (Bebb *et al.*, 2003b) and 60190 *vacA* mutant (Bebb *et al.*, 2003a).

2.2. Cell culture.

2.2.1. AGS cells.

AGS cells (ECACC) are human gastric epithelial cells originally derived from an adenocarcinoma of the stomach of a 54 year-old Caucasian female with no prior anti-cancer treatment (http://www.ecacc.org.uk). These cells were grown in F12 Ham's nutrient mixture medium, supplemented with 10% (vol/vol) heat inactivated foetal calf serum (FCS) and 2mM L-glutamine, at 37^{o}C in a 5% CO_2 humidified incubator as described by Rieder *et al.*, (Rieder *et al.*, 1997). Once fully confluent, cells were washed twice with sterile phosphate buffered saline (PBS) (Oxoid Ltd.) and Trypsin-EDTA (Gibco) solution was used to remove cells from the flask. F12 Ham's nutrient mixture

medium was added after trypsinization and cells were washed twice before resuspension in an appropriate volume of F12 Ham's culture medium. The cell suspension was then diluted (split ratio 1:12) into new T75 flasks (Nunclon) with fresh medium and incubated as before for maintenance, or cells were seeded into multi-well plates and used for an experiment.

2.2.2. MKN28.

This gastric carcinoma cell line was derived from the stomach of a 70 year-old female (http://cellbank.nibio.go.jp/celldata/jcrb0253.htm). The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) with 2 mM L-glutamine and 10% (vol/vol) FCS at 37^{o}C in a 5% CO_2 humidified incubator. Once fully confluent, the cells were split using the same methods as the AGS cells for maintenance or were used in an experiment.

2.2.3. MKN45.

This gastric carcinoma cell line, derived from the stomach of a 62 year-old female (http://cellbank.nibio.go.jp/celldata/jcrb0254.htm), was kindly donated by the Cancer Studies Unit, University of Nottingham. The cells were grown in RPMI 1640 medium with 2mM L-glutamine and 10% (vol/vol) FCS at 37^{o}C in a 5% CO_2 humidified incubator. The same routine as AGS cells was used when passaging the cells into a new flask. After treatment with trypsin, a cell scraper was used to detach cells from the surface of the flask. The cells were washed and diluted into new culture flasks.

2.2.4. THP-1.

THP-1 is a non-adherent human monocytic leukaemia cell line which was derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia (http://www.ecacc.org.uk). The medium used for this cell line was RPMI 1640 medium with 2mM L-glutamine and 10% (vol/vol) FCS. For counting, a sample from the cells suspension was diluted with trypan blue and applied to a haemocytometer slide. The cells were observed and counted under light microscopy. The cells were kept in exponential growth by maintaining cultures between 3-5 x 10^5 cells/ml. Cultures were incubated at 37^{o}C in a 5% CO_2 humidified atmosphere with the flask in standing position.

2.3. Experimental analysis of IL-10R expression by gastric epithelial cells.

2.3.1. Immunofluorescence microscopy.

Chamber slides (4-well; BD Biosciences) were seeded with AGS cells from a confluent T75 flask and were incubated at 37^{o}C in a 5% CO_2 humidified incubator until the next day. The total volume of medium used was 1.5ml for each chamber. A *H. pylori* 60190 strain suspension was prepared at an optical density of 0.05 (wavelength 600nm) (OD_{600}) using F12 medium. Medium from each chamber was replaced with either 1ml of bacterial suspension or fresh F12 Ham's nutrient mixture medium appropriately. The cells were incubated at 37^{o}C in a 5% CO_2 humidified incubator.

After 24 hours, staining procedures were carried out. Cells were fixed by replacing medium with 1ml of methanol per chamber. Cells were washed twice with 1.5ml PBS/0.5% bovine serum albumin (BSA). The solution was removed and a primary antibody (mouse anti-IL-10Rα (R&D systems), rabbit anti-*H. pylori* (Dako) or an isotype control (AbD Serotec)) was added. The cells were incubated at room temperature for 1 hour and then were washed 3 times with PBS/0.5% BSA solution. The secondary antibody (anti-mouse-PE or anti-rabbit-FITC) was added and the cells were incubated in the dark at room temperature for 1 hour. The cells were washed as before and the manifold of the chamber slide was removed. Coverslips were mounted using anti-fade mounting medium (Slow fade Gold; Invitrogen) and the edges of the slides were sealed. Cells were visualised by fluorescence microscopy.

2.3.2. Flow cytometry.

2.3.2.1. FACS analysis using antibodies.

AGS cells from one fully confluent T75 flask were seeded into 6-well plates and incubated at 37^{o}C in a 5% CO_2 humidified incubator for 24 hours. Once fully confluent, the medium from each well was removed and replaced with 3ml of medium alone or medium with a suspension of *H. pylori* 60190 strain at OD_{600} 0.05.

The THP-1 cell line, known to express IL-10R constitutively and at high-level, was used as a positive control (Tan *et al.*, 1993). On the day of infection the number of cells per ml was counted using a haemocytometer. Cells were then centrifuged at 200*x*g and

resuspended in an appropriate volume of medium. Cells were suspended in FACS tubes at 1x 10^6 cells per ml. The tubes received either medium alone or bacterial suspension at a final OD_{600} of 0.05.

For AGS cultures, after 24 hours of incubation at 37°C in a 5% CO_2 humidified incubator, supernatants were removed and PBS was added to wash each well. The PBS was removed and 3ml of non-enzymatic cell dissociation agent was added to each well to detach the cells from the plate. The cells were incubated for 10 to 15 minutes. Using a pipette, cells were scraped from the surface and together with the agent, were transferred into FACS tubes. The cells from each well were transferred into separate tubes.

Both AGS and THP-1 cells were washed twice with 1ml PBA/2% FCS and placed on ice. Primary antibodies (anti-IL-10Rα (R&D systems) or an isotype control (AbD Serotec)) were added to designated cell pellets and incubated for 1 hour on ice. The cells were washed again 3 times with 1ml PBA/2% FCS.

Secondary antibody anti-mouse-PE was added to the cell pellets and cells were incubated for 1 hour on ice. The cells were washed again 3 times and 0.5ml of cell fix solution (0.5% formaldehyde in PBS) was added to the cell pellets. Fluorescent labelling was detected using a coulter EPICS XL-MCL flow cytometer and WinMDI was used for data analysis.

2.3.2.2. Measurement of IL-10 binding to cultured cell lines.

This was done using an IL-10 Fluorokine kit (R&D Systems), following the manufacturer's instructions. For AGS cultures, after 24 hours of incubation, supernatants were removed and cells were washed twice with 5ml/well PBS. Then, 3ml/well of non-enzymatic cell dissociation agent was added and cells were incubated for 10 to 15 minutes before being counted. 1x 10^6 AGS or THP-1 cells were centrifuged at 200*x*g for 5 minutes and washed twice with 2ml wash buffer provided with the kit. Cells were then resuspended in 250µl sterile PBS. 25µl cells were added to each tube. 10µl of biotinylated rhIL-10 was added to test tubes. 10µl of biotinylated soybean trypsin inhibitor was added instead for negative control staining.

To ensure specific staining, controls were included where the interaction of biotinylated IL-10 with cells was blocked with anti-IL-10 antibody. 1µl of human IgG (1mg/ml) was added into a tube containing 25µl of cells incubated for 15 minutes at room temperature (tube A). In a second tube (B), 20µl of anti-human IL-10 antibody was mixed with 10µl of biotinylated rhIL-10 and incubated for 15 minutes at room temperature. The contents of tube B were added to tube A and then treated as the others from this point.

All tubes were incubated for 60 minutes at 4^{o}C. 10µl of avidin-FITC reagent was added to each tube and the cells were further incubated for 30 minutes at 4^{o}C in the dark. Cells were washed twice with 2ml wash buffer and centrifuged at 200*x*g for 5 minutes. 200µl wash buffer was used to resuspend cells for final flow cytometric analysis. Nonviable cells were excluded by the addition of 10µl/2x10^5 cells propidium iodide (PI)

(Invitrogen). Fluorescent labelling was detected using a Coulter EPICS Altra flow cytometer, gating out cells that stained with PI. WinMDI 2.8 software was used for data analysis.

2.3.3. Quantitative real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR).

Total RNA was purified from cultured AGS with and without *H. pylori* infection using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's recommendations. The mRNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) with an oligo(dT) primer as per the manufacturer's instructions (see Appendix for further information). Controls, adding RNase-free water instead of reverse transcriptase (-RT), were prepared in parallel for each sample.

The real-time PCR reactions were performed on a Rotor-Gene 3000 (Corbett Research, Cambridge, UK) using a Dynamo HS SYBR green qPCR kit (GRI, Braintree, UK). Amplification was carried out over 40 cycles of 15 seconds at 95^{o}C, 30 seconds at 61^{o}C, 30 seconds at 72^{o}C and 15 seconds at 80^{o}C. The IL-10Rα and IL-10Rβ data were normalised to GAPDH. The results were analysed using the Pfaffl method (Pfaffl, 2001).

Gene	Forward primer	Reverse primer
GAPDH	CCACATCGCTCAGACACCAT	GGCAACAATATCCACTTTACCAGAGT
IL-10RA	CTCACCAGGCAGTATTTCACGG	CTCTGGGGAGGGACGCTG
IL-10RB	TTCCACAGCACCTGAAAGAGTT	AGGATTCTGCTTGCCGCTC

Table 3. Primer sequences for real-time PCR. Sequences are shown in the 5'-3' orientation.

2.3.4. SDS-PAGE and Western blotting.

AGS cells were grown to 80-90% confluence in 6-well plate and were infected the next day with *H. pylori* strain 60190 at an MOI of 10. After 24 hours of infection, cells were scraped from the triplicate wells and solubilized in 2x sodium dodecyl sulphate (SDS) sample buffer (pH 6.8). Proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membrane using the Mini Protean 3 apparatus (Bio-Rad, Hertfordshire, UK) as per the manufacturer's instructions. Blots were blocked for 1 hour at room temperature with PBS/0.05% Tween 20/2% dried skimmed milk powder and probed with primary antibodies overnight at 4^{o}C. The primary antibodies used were mouse anti-human IL-10R alpha monoclonal antibody (final concentration, 2µg/ml; R&D Systems) and rabbit anti-human IL-10R beta polyclonal antibody (final concentration, 1µg/ml; Santa Cruz Biotechnology, Heidenberg, Germany). Blots were washed and incubated with anti-rabbit and anti-mouse peroxidise conjugate antibodies (dilutions used were as per manufacturer's instructions). Both primary and secondary antibodies were diluted in SignalBoost Immunoreaction Enhancer

Solution (Calbiochem®, Nottingham, UK). Ingredients for buffers and solutions for these protocols are in the Appendix.

Chemiluminescent ECL Western Blotting Detection Reagents (GE Healthcare Ltd, Buckinghamshire, UK) and autoradiography films (ECL hyperfilm; GE Healthcare Ltd) were used to develop the blots with an exposure time of 10 minutes. Films were developed using developer and fixer chemicals (Ilford PQ Universal, Cheshire, UK) before being washed well with water and allowed to dry.

Staining for β-actin was subsequently used to control for protein loading. Briefly, blots were washed 2 times in PBS-Tween and re-probed with rabbit- anti-human β-actin antibody using the same protocol as mentioned earlier.

2.4. Bacterial-epithelial cell co-culture experiments and quantification of bacterial densities.

2.4.1. Preparation of AGS cells.

AGS cells from a fully confluent flask were trypsinised, washed and resuspended in 10ml F12 Ham's nutrient mixture medium. The cells were seeded in 24-well plates (Costar, Dorset, UK) and incubated as before. For use the next day, cells were seeded at full confluence. For use after 2 days, cells were seeded at 50% confluence. For the

experiment the cells were at 80-90% confluence and 20ng/ml of recombinant human IL-10 (rIL-10) (AbD Serotec, Kidlington, UK) was added to appropriate wells. The cells were incubated at 37^{o}C in a 5% CO_2 humidified atmosphere.

2.4.2. Preparation of *H. pylori.*

The growth from two blood agar plates which had been inoculated with *H. pylori* 24 hours previously was removed using sterile cotton swabs. The bacteria were suspended in 5ml serum-free F12 Ham's nutrient mixture medium and the optical density was measured on a spectrophotometer at a wavelength of 600nm (OD_{600}). A cuvette containing 1ml of medium was used as a blank. It was estimated that an OD_{600} of 0.01 contained approximately $5x10^6$ *H. pylori* colony-forming units (CFU) per ml. The dilution required for adding bacteria to the culture wells was calculated so that there would be a final OD_{600} of 0.01 in the well with a total volume of 0.5ml.

2.4.3. Viable count assay.

After 24 hours, 10-fold dilution series were set up from each co-culture well. Sterile PBS with 10% saponin was prepared and 50µl of this solution was added to each well. The mixtures were mixed well and 20µl aliquots were removed from each well into the top row of a 96-multiwell plate (Nunc). 180µl of fresh sterile PBS was added into this making a 1/10 (10^{-1}) dilution. 20µl of this dilution was transferred into the next well down and 180µl of PBS was added into this second dilution making a 1/100 (10^{-2}) dilution. These steps were continued until the final well which made-up a 1/100,000,000

(10^{-8}). Tips were changed between wells. One dilution series was prepared for each replicate.

Blood agar plates were marked with four quadrants and were labelled with 10^{-1} to 10^{-8} respectively. 4 times 10µl drops of each dilution were placed onto the blood agar with designated quadrant. As a control, 4 times 10µl of PBS were pipetted onto one blood agar plate to check for contamination. The spots were allowed to sink before the plates were turned over and incubated as before. The colonies were counted after 3 to 5 days of incubation. Colony forming units per ml were calculated:

CFU/ml= mean count from replicate 10µl spots x 100 x dilution factor

2.5. Pro-inflammatory chemokine IL-8 assay.

AGS cells were cultured with and without *H. pylori* 60190 (MOI=10) in a 6-well plate and supernatants were collected after 24 hours of infection. IL-8 was assayed using a Human IL-8 CytoSet ELISA kit (Biosourse, Belgium) as per the manufacturer's instructions. The limit of sensitivity of the assay (typically less than 1.26 pg/ml) was calculated as shown below:

Limit of sensitivity = (mean + 3 x standard deviation) of 6 replicate 0pg/ml IL-8 wells.

2.6. Statistical analysis.

All analyses for statistically significant differences were performed with a paired or unpaired Student *t* test. $P<0.05$ was considered statistically significant.

CHAPTER 3: RESULTS

3.1. Measurement of IL-10 receptor expression by gastric epithelial cells.

It has been reported that IL-10 is abundant in the gastric mucosa during *H. pylori* infection (Goll *et al.*, 2007) but to date, there have been no reports on whether the IL-10 receptor (IL-10R) is expressed on gastric epithelial cells. Therefore, experiments were done to determine whether IL-10 can act on cells of the epithelium through the IL-10R in the presence of *H. pylori*.

3.1.1. Immunofluorescence microscopy.

These were qualitative experiments carried out to determine the expression of IL-10Rα on AGS cells with or without the presence of *H. pylori*. Cells were grown in chamber slides and were infected with *H. pylori* 60190 strain (MOI = 143). After 24 hours of incubation, AGS cells were fixed with methanol and the staining procedure was done using primary and secondary antibodies. The primary antibodies used were mouse-anti-IL-10Rα, rabbit-anti-*H. pylori* (as a positive control) and an isotype control (mouse IgG1, negative control). The secondary antibodies used were anti-mouse-PE (phycoerythrin conjugate) and anti-rabbit-FITC (fluorescein isothiocynate isomer 1 (FITC) conjugate). Stained cells were then examined under fluorescence microscopy.

Red fluorescence was detected when AGS cells, with or without *H. pylori* 60190 strain, were stained with anti-IL-10Rα antibody. The staining was mostly punctate but there

were also areas of very high-level staining which maybe non-specific (Figure 8a and b). This shows it is likely that AGS cells do express the IL-10R. Green fluorescence was detected when AGS cells infected with *H. pylori* were stained with rabbit-anti-*H. pylori* antibody and anti-rabbit-FITC (Figure 8c). No fluorescence was detected when AGS cells were stained with only anti-mouse-PE (figure not shown) illustrating that the binding of this secondary antibody is specific. For controls, stained AGS cells alone with anti-*H. pylori* and anti-rabbit-FITC antibodies or with an isotype control antibody (figure not shown) also showed no fluorescence.

The microscopy data indicated that the gastric epithelial cell line can express the IL-10R either constitutively or induced by *H. pylori* infection. However, this experiment has a limitation since it is a qualitative method. It is not known whether the expression was up-regulated during infection. In further experiments, flow cytometry was therefore used.

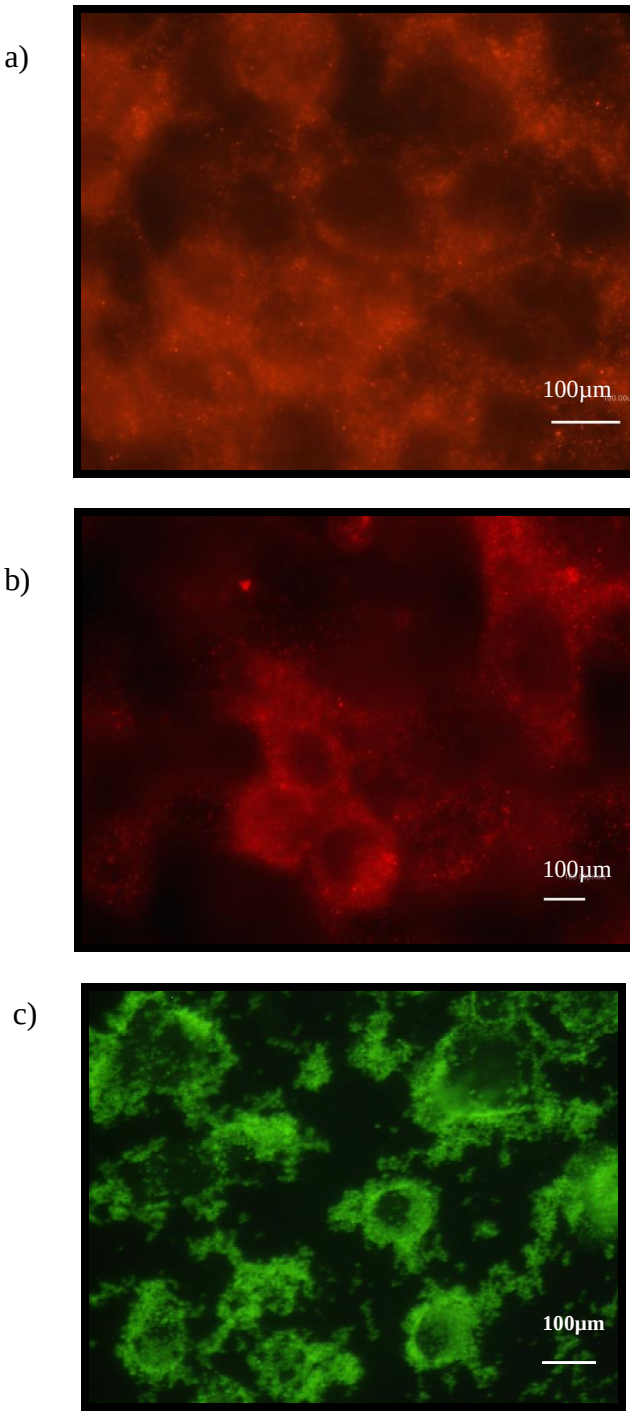

Figure 8. Stained AGS cells under fluorescence microscopy:
AGS cells were left uninfected (a) or infected with *H. pylori* 60190 strain (b and c) and incubated for 24 hours. Cells were then stained with mouse-anti-IL-10Rα antibody (a and b), rabbit-anti-*H. pylori* antibody (c). Anti-mouse-PE (a and b) and anti-rabbit-FITC (c) were used as secondary antibodies. The presence of IL-10Rα is indicated with the red fluorescence**.**

3.1.2. FACS analysis.

3.1.2.1. Measurement of IL-10R expression using antibodies.

The microscopy data showed that gastric epithelial cells did express IL-10R, but it was difficult to see whether the expression was increased when the cells were infected with *H. pylori*. The FACS analysis was carried out to determine if IL-10R expression is influenced by *H. pylori* infection. This quantitative analysis was performed to give two sets of information: the intensity of fluorescence from the staining of cells with anti-IL-10Rα antibody (shown by the position of the peak on the histogram plots) which is related to the amount of IL-10R expressed, and the proportion of cells expressing IL-10R as shown by the percentage of positively stained cells. For these flow cytometry experiments, cells were stained with different primary antibodies and anti-mouse-PE as the secondary antibody.

The AGS cells, with and without *H. pylori* infection, were stained with mouse-anti-IL-10Rα (primary) antibody and with anti-mouse-PE (secondary) antibody. There was no increase in the geometric mean fluorescence (position of the peak) but there was an increase in the proportion of positively stained cells when *H. pylori* was added to the AGS cells (Figure 9a and b). These have shown that more AGS cells expressed IL-10R but the expression level was not up- regulated within those cells already expressing the receptor.

It could be seen that the percentage of IL-10R+ cells was increased by 1.5 fold when the AGS cells were infected with the bacterium (Figure 9a and b). Unstained AGS cells, with and without the presence of *H. pylori*, resulted in only very small proportions of cells within the selected range of fluorescence (M1), 0.21% and 0.49% respectively (Figure 9c and d).

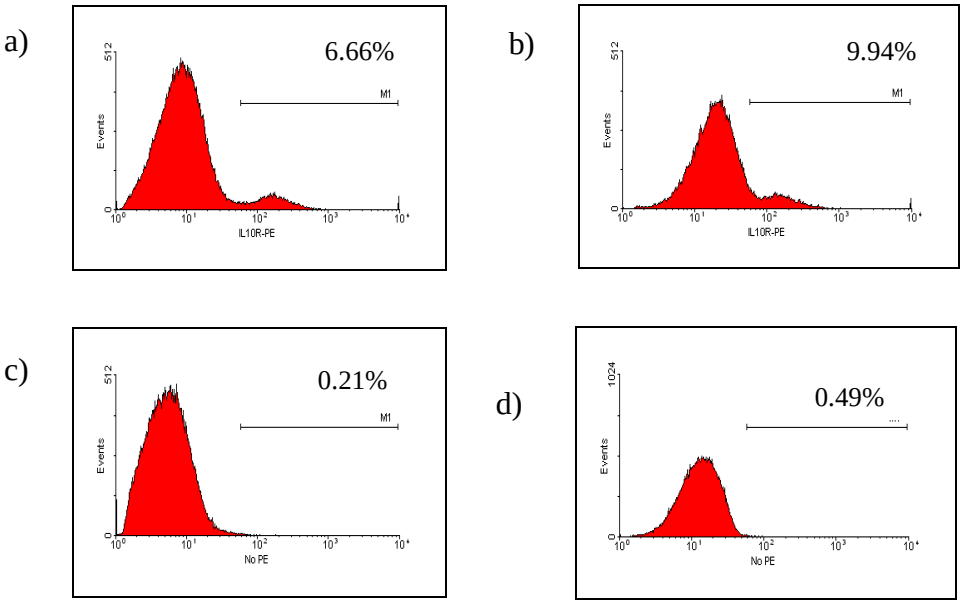

Figure 9. IL-10R FACS analysis of AGS cells:
AGS cells were either left uninfected (a and c) or infected with *H. pylori* 60190 strain (b and d). Cells were then stained with mouse-anti-IL-10Rα antibody (a and b) or left unstained (c and d). Anti-mouse-PE was used as the secondary antibody. These histogram plots show intensity of fluorescence on the x-axis and number of cells (events) on the y-axis. M1 is the marker region which indicates the fluorescence from positive staining. Percentage figures indicate the proportion of events within region M1.

The experiment was repeated a further two times and the isotype control was included to act as the negative control. Again, there was an increase in IL-10Rα expression on infected AGS cells compared to uninfected cells (Figure 10). The increment was 1.7 fold, similar to the previous experiment.

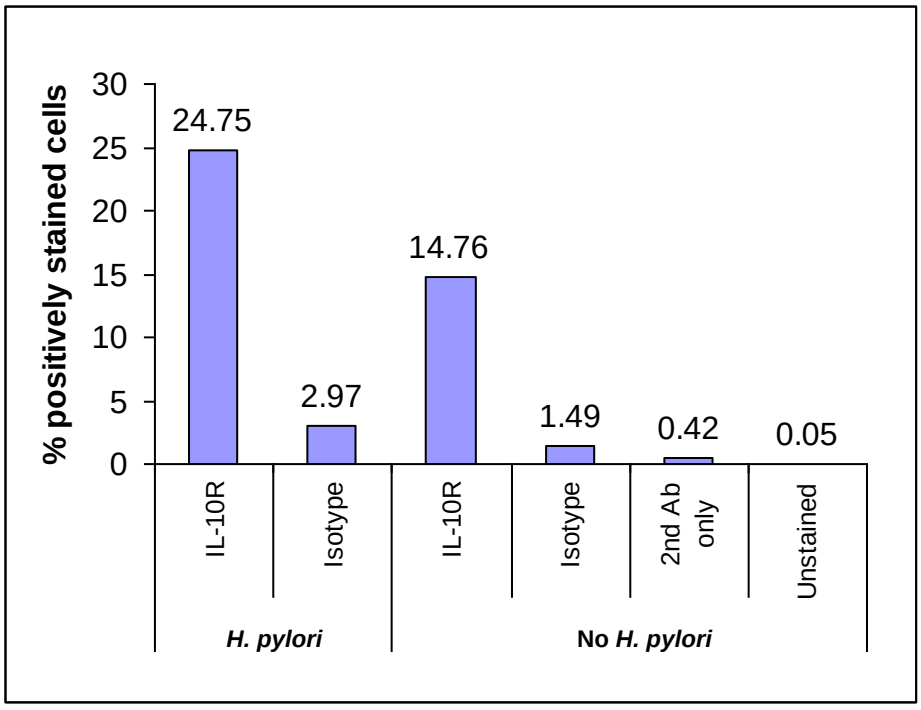

Figure 10. Fluorescence-sorted IL-10R-positive AGS cells:
Bars represent the proportion of positively stained AGS cells within the selected range of fluorescence (M1). The x-axis is the percentage of positively stained cells and the y-axis is the condition of cells during staining.

In parallel, the THP-1 cell line was included as a positive control because it has been previously reported to express IL-10R constitutively and at high-level (Tan *et al.*, 1993). The primary antibodies used were again mouse-anti-IL-10Rα antibody and the isotype control. The same secondary antibody was used. 78.08% of the THP-1 cells expressed IL-10R constitutively and this was increased to 94.19% with *H. pylori* infection (Figure 11a and b).

In contrast to AGS cells, a shift in the position of the peak was observed when THP-1 cells were cultured with *H. pylori* and together with the increase on the percentage of IL-10R positive cells (Figure 11a and b) indicates that there were more THP-1 cells expressing IL-10R and each cell expressed IL-10R at an increased level when infected with *H. pylori*. When the cells were stained with secondary antibody only or with no primary antibody, 5.97% and 0.04% of the THP-1 cells emitted the fluorescent signal respectively (Figure 11e and f). The experiment was repeated once with consistent results.

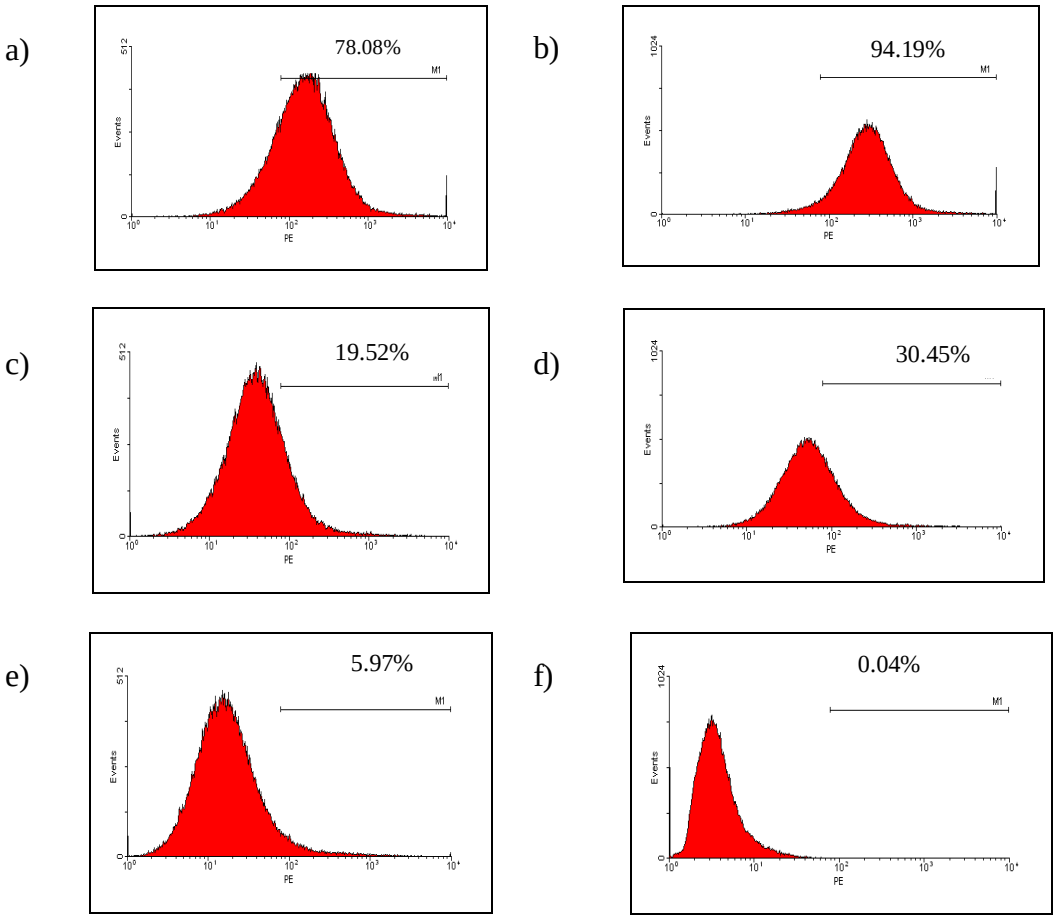

Figure 11. IL-10R FACS analysis of THP-1 cells:
Cells were either left uninfected (a, c, e and f) or infected with *H. pylori* 60190 strain (b and d). Cells were then stained with mouse-anti-IL-10Rα antibody (a and b), the isotype control (c and d) or left unstained (e and f). Anti-mouse-PE was used as the secondary antibody (except in f). These histogram plots show intensity of fluorescence on the x-axis and number of cells (events) on the y-axis. M1 is the marker region which indicates the fluorescence from positive staining. Percentage figures indicate the proportion of events within region M1.

Although the amount of isotype control used was the same as with AGS cells, the percentage of positively-stained THP-1 cells with isotype control was very high (Figure 12). High level staining with isotype control antibody indicates that the antibody should be used at lower concentrations and that perhaps there was non-specific staining. Due to this, another assay needs to be carried out to validate these experiments.

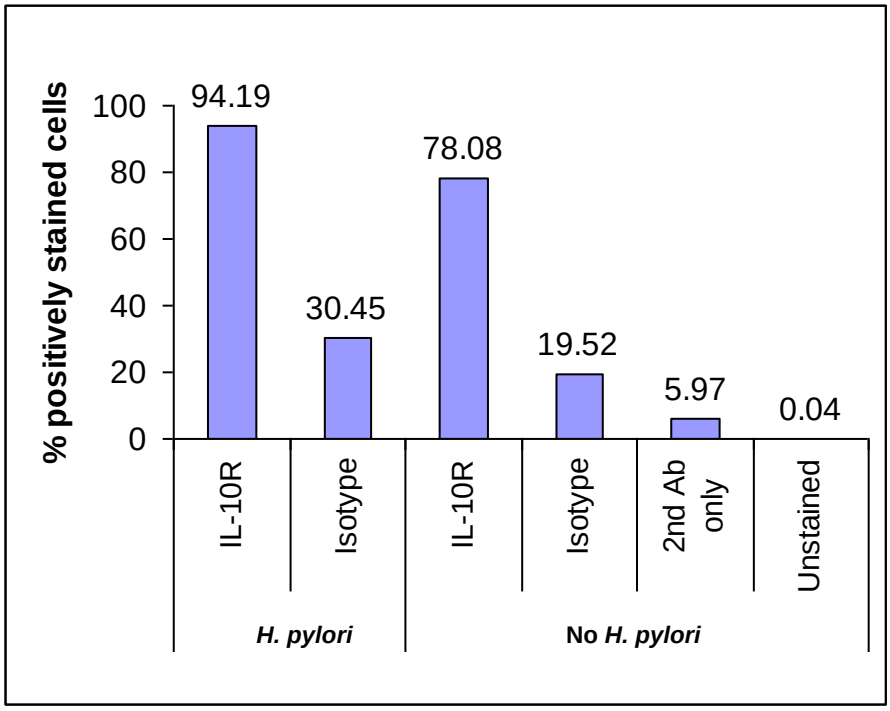

Figure 12. Fluorescence-sorted IL-10R-positive THP-1 cells:
Bars represent the proportion of positively stained THP-1 cells within the selected range of fluorescence (M1). The x-axis is the percentage of positively stained cells and the y-axis is the condition of cells during staining.

3.1.2.2. Measurement of IL-10 binding to gastric epithelial cells.

The results from FACS assay using antibodies were not adequate. In search of other reagents and methods, it was apparent that an IL-10 Fluorokine assay (R&D Systems Ltd, Abingdon, UK) has been used by many others to investigate IL-10 interactions (Fernandez *et al.*, 2004; Ji *et al.*, 2003; Du *et al.*, 2006). This flow cytometry based assay detects the binding of fluorescently labelled IL-10 to cells. It was used to determine IL-10 binding to epithelial cells cultured with and without *H. pylori*. Briefly, AGS and THP-1 cells were infected with *H. pylori* 60190 strain or left uninfected. They were then incubated with biotinylated IL-10, followed by avidin-FITC. The kit included a control biotinylated protein to ensure that IL-10 binding was specific. An additional control involved pre-incubating the IL-10 with blocking antibodies prior to adding it to the cells.

The mean percentage of IL-10-binding cells was very high for the uninfected cells which was about 73% (Figure 13). Surprisingly, the binding was decreased when AGS cells were infected with *H. pylori* 60190 (*cag*+ strain) and Tx30a (*cag*- strain) to 40% and 22% respectively (Figure 13). This decrement was statistically significant ($p<0.05$). However, this was the reversed for THP-1 cell line where the percentage of binding was increased with infected cells compared to uninfected cells (Figure 14). Also, the percentage of IL-10-binding cells was not as high as AGS cells.

The fluorokine assay was repeated four times with consistent results. The results from this assay did not agree with FACS assay using antibodies where a higher proportion of

cells expressed IL-10Rα. IL-10R expression was therefore investigated using the third technique, RT-PCR.

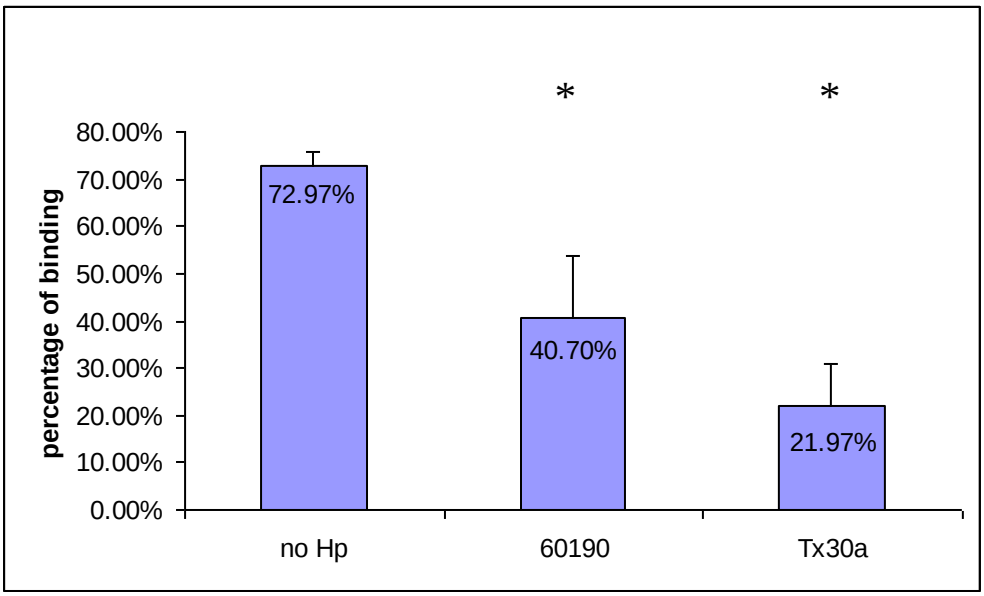

Figure 13. IL-10 binding by AGS cells.
Cells were left uninfected or infected with *H. pylori* 60190 at MOI of 10 and were used in Fluorokine assay. Bars represent the mean percentage of binding from 5 separate experiments and error bars display the standard deviations. * indicates significant decrease in IL-10 binding compared with cultures in the absence of *H. pylori* ($p<0.05$). Hp= *H. pylori*.

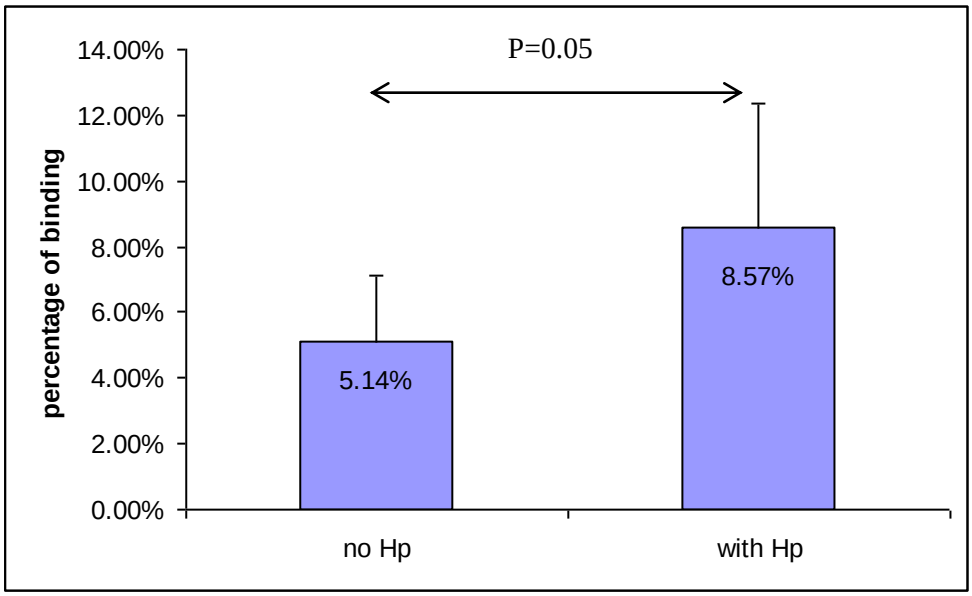

Figure 14. IL-10 binding by THP-1 cells.
Cells were left uninfected or infected with *H. pylori* 60190 at MOI of 10 and were used in Fluorokine assay. Bars represent the mean percentage of IL-10 binding from 5 separate experiments and error bars display the standard deviations. Hp= *H. pylori.*

3.1.3. *IL10R* mRNA expression by real-time RT-PCR.

Primers were designed for the human *IL10RA* and *IL10RB* genes. The real-time PCR was carried out to measure the relative mRNA expression of *IL10RA* and *IL10RB* between uninfected and infected AGS cells. Total RNA was extracted from uninfected and infected AGS cells. mRNA was reverse-transcribed and used in real-time RT-PCR. The ratios of gene expression levels were calculated using the Pfaffl equation (Pfaffl, 2001). The data were normalised against *GAPDH*. Results were reproduced in three individual experiments, with each parameter tested in four replicates.

From these experiments, it could be seen that the level of mRNA expression for both *IL10RA* and *IL10RB* were increased when AGS cells infected with *H. pylori*. But the increment for *IL10RB* mRNA level (25-fold) was far higher than that of *IL10RA* (2.5-fold) (Figure 15). To validate this, IL-10R expression at the protein level was quantified by western blotting.

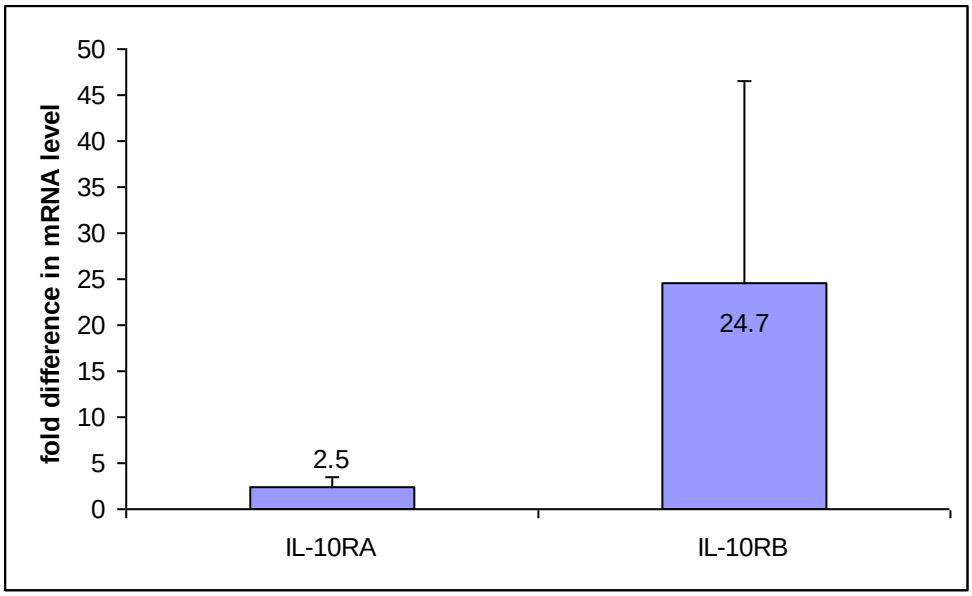

Figure 15. Upregulation of IL-10R mRNA expression in infected AGS cells:
Total RNA was extracted and reverse-transcribed from uninfected and infected AGS cells. Each experiment consists of 4-analysed samples. Bars represent the mean fold difference in *IL-10RA* and *IL-10RB* mRNA expression levels between uninfected and infected cells. Error bars represent the standard deviation of the mean. The results were reproduced in three individual experiments, with each parameter replicated four times. Data were normalised against *GAPDH* expression.

3.1.4. Quantifying IL-10R expression by SDS-PAGE and western blotting.

To confirm results from real-time RT-PCR, IL-10R expression by AGS cells was investigated using western blotting. AGS cells in 6-well plates were infected with *H. pylori* 60190 strain or left uninfected. After 24 hours of incubation, cells were solubilised in 2x SDS buffer and electrophoresed on 10% SDS-PAGE gels. Proteins were then transferred onto nitrocellulose membrane and probed with anti-IL-10Rα or anti-IL-10Rβ antibodies. Membranes were re-probed with anti-β actin antibody to act as a loading control.

Figure 16 showed the Western blot results where 63 kDa, 37 kDa and 42 kDa are the apparent molecular weight of IL-10Rα, IL-10Rβ and β-actin respectively. From this, it can be seen that the expression of IL-10Rα was similar between uninfected and infected AGS cells (Figure 16 (ai)). Since the IL-10Rα bands were weak, it was not possible to see if there was any difference in intensity.

In contrast, the IL-10Rβ expression was increased in infected cells compared to uninfected cells (Figure 16 (bi)). This result was similar to the real-time PCR data. There were no bands detected in the control lanes which received no primary antibodies. The experiment was repeated and gave consistent results.

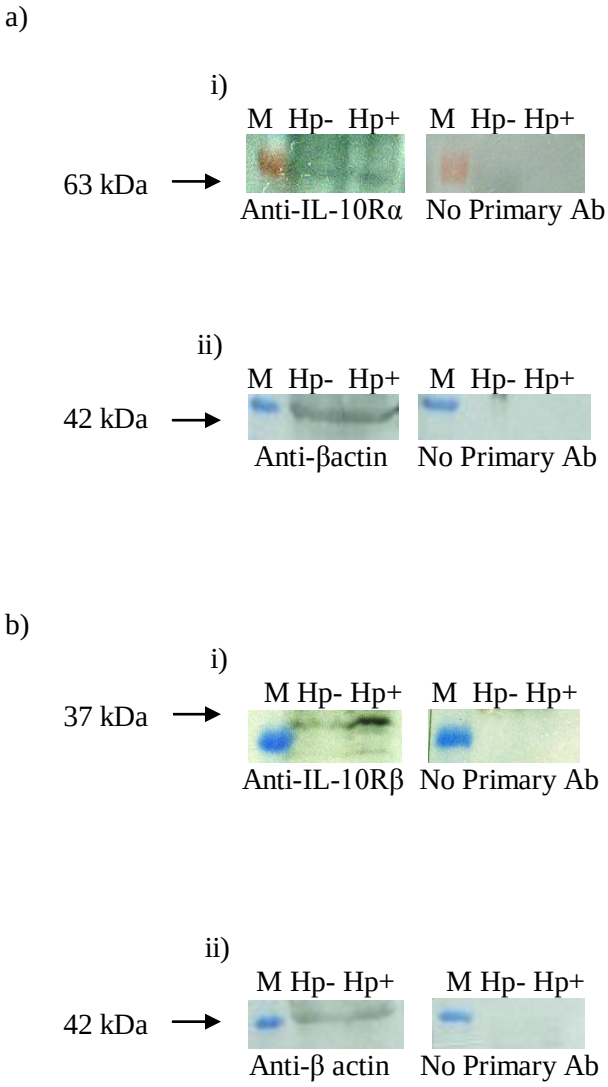

Figure 16. Western blot of IL-10Rα and IL-10Rβ expression in AGS cells
AGS cells were grown in 6-well plates and infected with *H. pylori* 60190 strain (MOI = 10) or left uninfected. Proteins from solubilised cells were electrophoresed in 10% SDS-PAGE gels and transfer to nitrocellulose membrane. Blots were probed with anti-IL-10Rα antibody (ai) and anti-IL-10Rβ antibody (bi). Blots were re-probed with anti-β actin antibody (aii and bii) to serve as a loading control. M=marker, Hp-= uninfected AGS cells and Hp+ = infected AGS cells with *H. pylori* 60190 strain.

3.2. Bacterial-epithelial cell co-culture experiments and quantification of bacterial densities.

Previous studies in the lab have shown that 20ng/ml of rIL-10 induced a significantly increased bacterial density ($p<0.002$) and gastric biopsies with the highest *H. pylori* density grading (3 on a scale of 0-3) contained 6-fold higher levels of *IL-10* mRNA than the samples with a grade of 1 (Robinson *et al.*, 2008). Further co-culture experiments were therefore carried out to confirm or refute the previous work and to determine whether it is reproducible.

3.2.1. Dose-dependent effect of rIL-10.

From previous work, an effect was observed with 20ng/ml but as it is difficult to determine whether this concentration is physiologically relevant, effects from lower concentrations were tested. As a starting point, the effects of rIL-10 at 5ng/ml, 10ng/ml and 20ng/ml of rIL-10 were tested in co-cultures of AGS cells and the 60190 *H. pylori* strain. Each culture condition was carried out in 6 replicates. There was a significant increase in bacterial densities with 20ng/ml ($p=0.0015$), 10ng/ml ($p=0.0001$) and 5ng/ml ($p=0.001$) of rIL-10 (Figure 17). The experiment was repeated once with consistent results. Lower concentration of rIL-10 at 2.5ng/ml was also tested in separate experiment but there was no significant effect.

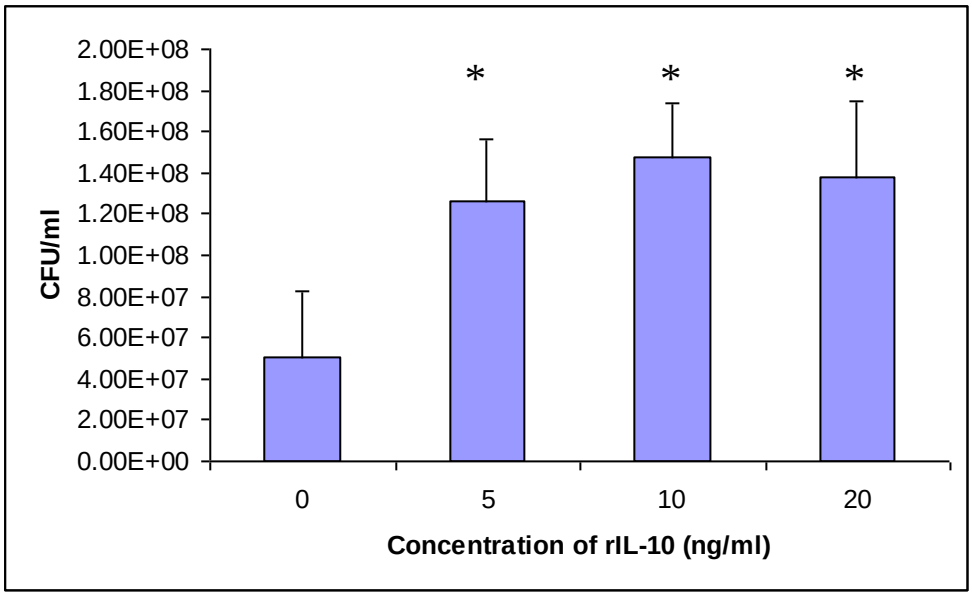

Figure 17. Effect of rIL-10 concentration on the density of *H. pylori* 60190 in dose-dependent manner:
Cells were infected with 60190 *H. pylori* strain at OD_{600} 0.01 and rIL-10 was added at different concentration. Bars show the mean of colony forming units per ml from 6 replicates. Error bars represent the standard deviation of the mean. * indicates significant increase compared with cultures in the absence of IL-10 ($p<0.05$).

3.2.2. Determination of whether growth effects of IL-10 were direct or indirect.

This experiment was to determine whether growth effects of IL-10 mediated directly on the bacteria or indirectly via the AGS cells. Infected and uninfected AGS cells were added with and without 20ng/ml of rIL-10. There was a significant increase in bacterial numbers when rIL-10 was added to the infected AGS cells. No significant increase was observed on the addition of rIL-10 to the bacterium alone (Figure 18). The experiment was repeated once with consistent results.

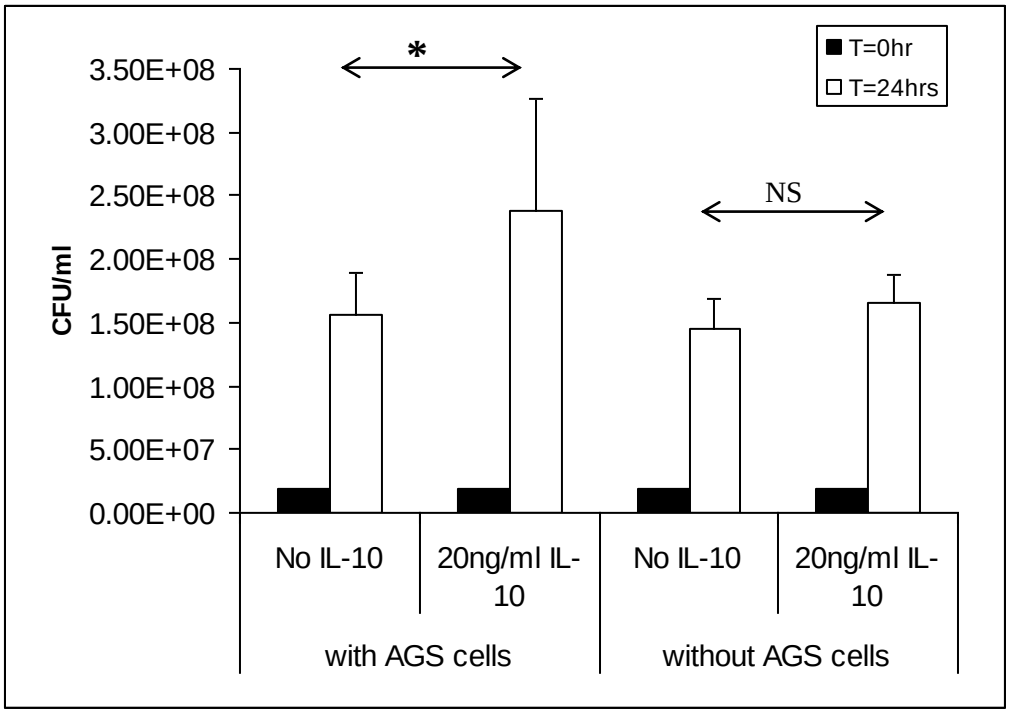

Figure 18. Effect of adding 20ng/ml rIL-10 on *H. pylori* 60190, with and without AGS cells:
The bacteria were grown alone or with AGS cells and treated with or without 20ng/ml rIL-10. After 24 hours (T=24hrs), the number of colony-forming units/ml (CFU/ml) in 6 replicates with and without 20ng/ml was determined (open bars). Closed bars show the number of colony forming units/ml at 0 hours (T=0hr). Bars and error bars represent the mean and standard deviation. * indicates a significant increase compared with cultures in the absence of IL-10 ($p<0.05$) and NS denotes a not significant increase compared with cultures in the absence of IL-10.

3.2.3. Effect of rIL-10 on different strains.

Since the last two experiments only examined the effects of IL-10 on the 60190 *H. pylori* which is a *cag*PaI-positive strain, the next experiment was conducted to see if rIL-10 had effects on other strain. Therefore, an experiment was carried out to examine the effect of rIL-10 on the *cag*PaI-negative strain Tx30a. The result showed that rIL-10 significantly increased the density of *H. pylori* 60190 strain bacteria ($p=0.004$) but no such effects

were observed with the *cag*PaI-negative *H. pylori* Tx30a strain (p=0.347) (Figure 19). The experiment was repeated once with consistent results.

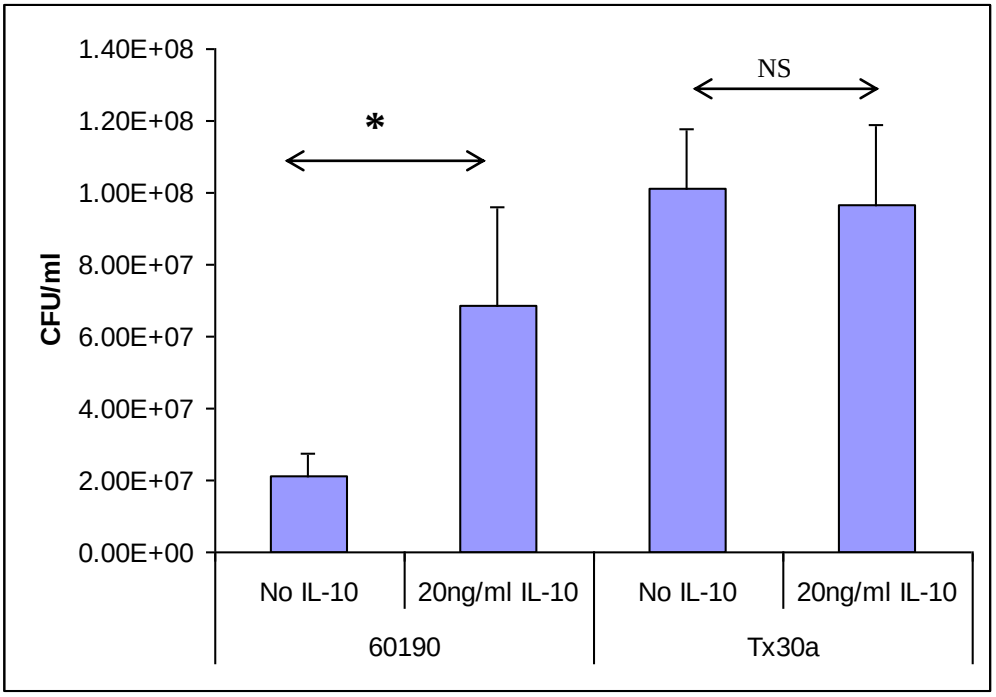

Figure 19. Effect of adding 20ng/ml rIL-10 on the bacterial density of *H. pylori* 60190 and Tx30a strains:
AGS cells were infected with 60190 or Tx30a *H. pylori* strains and were treated with 20ng/ml rIL-10 or left untreated. After 24 hours, the number of colony-forming units/ml (CFU/ml) in 6 replicates with and without 20ng/ml was determined. Bars show the mean and error bars show the standard deviation. * indicates a significant increase compared with cultures in the absence of IL-10 ($p<0.05$) and NS denotes a not significant increase compared with cultures in the absence of IL-10.

3.2.4. Determination of whether the effect of rIL-10 depends on the *cag* pathogenicity island.

The strains Tx30a and 60190 vary in more than just being positive or negative for the *cag*PaI. Therefore an experiment was carried out to compare the 60190 wild type (WT)

with its isogenic *cagE* mutant strain which cannot express a functional *cag* type IV secretion system.

After 24 hours incubation, there was no significant increase in the density of *H. pylori* 60190 WT bacteria when 10ng/ml rIL-10 was added compared to the number of WT bacteria with no addition of rIL-10 (Figure 20). There was no obvious difference in bacterial density *H. pylori* 60190 *cagE* mutant with or without the addition of rIL-10.

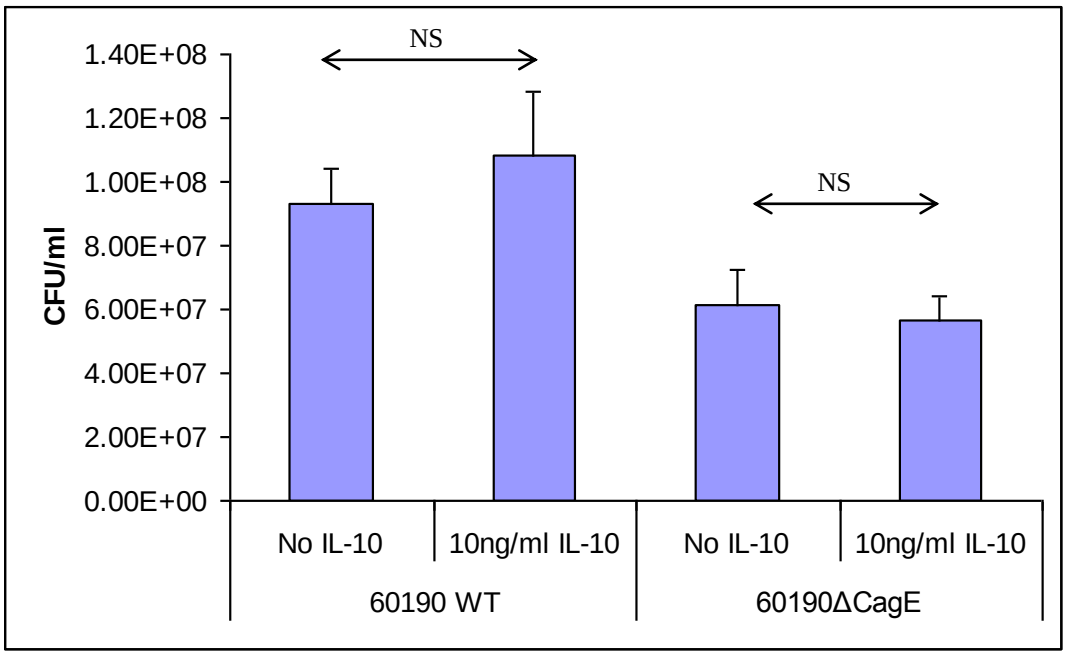

Figure 20. The effect of 10ng/ml rIL-10 on the density of *H. pylori* 60190 WT and *cagE* mutant: AGS cells were infected with 60190 WT or its isogenic *cagE* mutant strains at OD_{600} 0.01 and were treated with 20ng/ml rIL-10 or left untreated. After 24 hours, the number of colony-forming units/ml (CFU/ml) in 6 replicates with and without 20ng/ml was determined. Bars show the mean and error bars show the standard deviation. NS indicates a not significant increase compared with cultures in the absence of IL-10.

3.3. Quantification of IL-8 release.

Expression of the pro-inflammatory chemokine IL-8 was measured to confirm that interaction between AGS cells and *H. pylori* exists. Supernatants from co-culture of AGS cells and *H. pylori* 60190 strain were collected and IL-8 was assayed after 24 hours. Figure 21 showed that the production of IL-8 was much higher in infected AGS cells compared to uninfected cells and the difference is statistically significant ($p<0.0005$). The experiment was repeated once with consistent results.

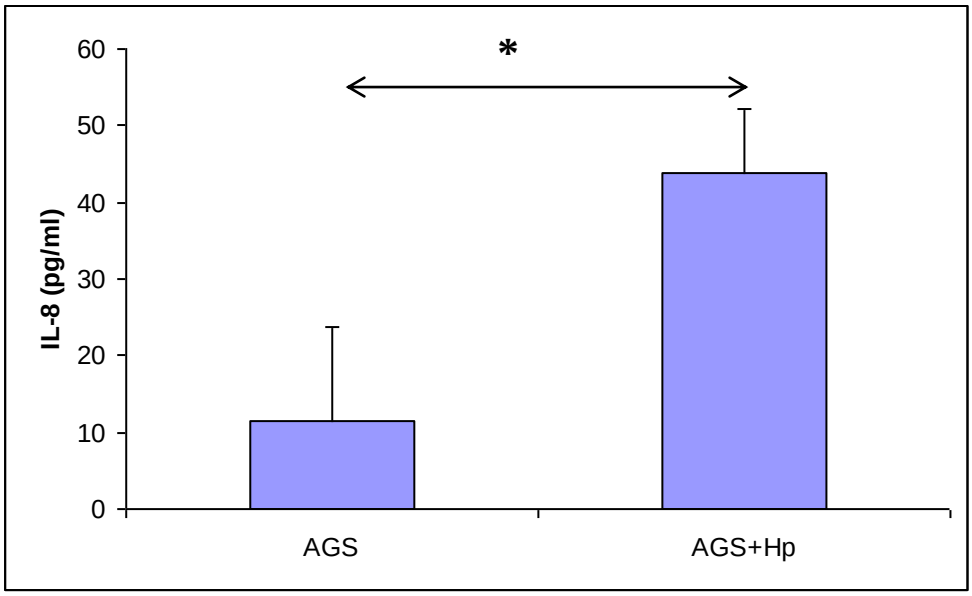

Figure 21. Expression of IL-8 by AGS cells:
Cells were left uninfected or infected with 60190 *H. pylori* strain at MOI=10. Bars show the mean of IL-8 concentration from six culture wells. Error bars represent the standard deviation of the mean. * indicates significant increase of IL-8 release from infected cells compared with cultures of AGS alone ($p<0.0005$). Hp= *H. pylori*.

CHAPTER 4:

DISCUSSION

Chronic *Helicobacter pylori* infection is associated with gastritis (Kuipers *et al.*, 1995), and may cause diseases such as peptic ulceration, gastric cancer (Graham *et al.*, 2004) and gastric MALT lymphoma (Eidt *et al.*, 1994). Only a minority of infected individuals suffer symptomatic disease. This is thought to be due to bacterial, host and environmental factors which are known to influence the outcome of an infection (Atherton, 1997). One of the important host factors involved in modifying the disease outcome is IL-10, an anti-inflammatory cytokine that exerts regulatory effects on many cells.

Reports have shown that IL-10 is abundant in the human gastric mucosa during *H. pylori* infection (Lundin *et al.*, 2007; Goll *et al.*, 2007). Furthermore, the IL-10 response has been correlated with increased bacterial density and reduced pathology (Robinson *et al.*, 2008) in *H. pylori* infection. Therefore, in the light of these findings, more detailed information on the role of IL-10 in modulating *H. pylori*-mediated diseases is now required.

The purpose of the current study was to firstly investigate IL-10 receptor expression by gastric epithelial cells (which orchestrate the inflammatory response to *H. pylori* infection) and secondly to study the effects of IL-10 on these cells when infected *in vitro*.

4.1. IL-10 receptor expression by gastric epithelial cell lines.

To date, nothing has been reported in the literature concerning the expression of IL-10R by gastric epithelial cells. It is very important to understand this as gastric epithelial cells are probably the main effector cells in defence against *H. pylori* and IL-10 is a major factor in the host response. The important question to address here is whether gastric epithelial cells can bind to IL-10 and respond to it.

Although there is no published data on IL-10R expression by gastric epithelial cells, studies have shown that epithelial cells from the murine small and large intestine constitutively express the receptor for IL-10 (Denning *et al.*, 2000). This was confirmed by another study which performed RT-PCR analysis to detect *IL10RA* (the α-chain of the IL-10 receptor) mRNA, in a mouse intestinal epithelial cell line (Al-Ashy *et al.*, 2006). They also utilised FACS analysis to show that IL-10 was able to bind to these cells. One interesting study showed induction of IL-10R expression by human dental pulp cells, in the presence of bacterial LPS (Tokuda *et al.*, 2003). For that reason, it was hypothesised that the levels of IL-10R expression would also be increased upon *H. pylori*-infection of human gastric epithelial cells.

In this study, experiments using immunofluorescence microscopy, flow cytometry, RT-PCR and western blotting were carried out to characterise IL-10R expression by gastric epithelial cell lines.

4.1.1. Immunofluorescence microscopy.

AGS cells, a human gastric epithelial cell line, were cultured with or without the cagPaI$^+$ *H. pylori* strain 60190 in chamber slides and assayed for IL-10R expression after 24 hours. Using an anti-IL-10Rα monoclonal antibody, immunofluorescence microscopy indicated that AGS cells did express IL-10R. Several experiments were performed to optimise staining conditions; however, the final results were not completely satisfactory. Although the staining was mostly punctuate, the background was high, indicating that staining may not be specific. Both uninfected and infected AGS cells in this microscopy experiments stained positive for the receptor (Figures 7a and b), but it was not possible to discern whether the level of expression was changed in response to *H. pylori* infection using this qualitative method.

As an alternative approach, the human IL-10 Fluorokine kit (R&D Systems), which permits detection of labelled IL-10 binding, was used with the microscopy method described by Michel *et al.* for staining tissue sections (Michel *et al.*, 1997). No strong signals were obtained, even when using a good anti-fade mounting medium. One major difference between the methods was the area of staining. The chamber slides tended to cause the reagents to accumulate around the edges and the wells were of larger surface area. Scaling up the staining volumes did not make any difference. Given the availability of smaller multi-well slides, we would like to repeat these experiments.

Microscopy could provide basic information about IL-10R expression by gastric epithelial cells, but it is unlikely to be the best technique for analysing IL-10R.

Microscopy is most useful when the molecules of interest are expressed at fairly high level. Since IL-10R has been found to be expressed at low level in some cells such as NK cells (Carson *et al.*, 1995), the level of IL-10R expression on gastric epithelial cells may be similar. In this work, the anti-IL-10R antibody may bind to its target molecules but the fluorescent signals may be too low to be visible by eye. A more sensitive and quantitative method is needed to determine if IL-10R expression is influenced by *H. pylori* infection. Therefore, flow cytometry was used as quantitative method can detect levels of fluorescence not apparent by eye.

4.1.2. FACS analysis.

The flow cytometry experiments were done in two parts, firstly detecting the IL-10Rα expression using anti-IL-10Rα antibody, then measuring the extent of IL-10 binding to gastric epithelial cell lines. As an additional positive control, the human monocytic THP-1 cell line was included as these cells are known to express IL-10R (Tan *et al.*, 1993).

4.1.2.1. Measurement of IL-10R expression using antibodies.

Infected and uninfected AGS cells were stained with anti-IL-10Rα antibody and analysed using a flow cytometer for the level of IL-10R expression. Controls included cells stained with an irrelevant antibody (isotype control), cells stained only with the secondary antibody, or totally unstained cells. All controls showed very low fluorescent signals indicating the staining was specific and this assay might be a reliable technique for detecting IL-10R expression by gastric epithelial cells.

THP-1 cells were found to express much higher levels of IL-10R compared to AGS cells. Monocytic cells respond rapidly to infection or components of infectious organisms. They express high levels of toll-like receptors and also play an important role in inflammation (Bas *et al.,* 2008). Thus, it is a must for them to exhibit high IL-10R expression so that they can turn this response off when needed to prevent tissue damage. Unfortunately, with this cell line, the controls used were not satisfactory. The percentage of IL-10R-positive cells in the isotype control, for both infected and uninfected cells, were very high (Figure 11) which meant that the results from AGS cells were unreliable.

Since the antibody specificity was questionable and the level of staining was low, FACS experiments using the human IL-10 Fluorokine assay were carried out. This is widely used in investigating responsiveness to IL-10, and far better controls were employed.

4.1.2.2. Measurement of IL-10 binding by gastric epithelial cell lines.

Due to unsatisfactory results from previous IL-10R antibody and flow cytometry experiments, this work was extended using a human IL-10 Fluorokine assay. Instead of directly measuring the level of IL-10R expression, this assay provided data on the proportion of gastric epithelial cells which bound IL-10.

As expected, IL-10 binding of *H. pylori*-infected THP-1 cells was higher than the uninfected cells (Figure 13). THP-1 cells are monocytic cells which are known to play an important role in inflammation and should respond rapidly to infection (Bas *et al.,* 2008). These cells have been found to express IL-10R upon stimulation (Takasugi *et al.,* 2006).

Unexpectedly, a very high percentage of uninfected AGS cells bound IL-10 (73%). The proportion was decreased when AGS cells were infected with *H. pylori* strains 60190 and Tx30a (Figure 12). What is more, the percentage of cells that bound IL-10 was the lowest when cells were infected with the *H. pylori cag*PaI-negative strain Tx30a compared to the uninfected cells and those infected with the *cag*PaI-positive strain 60190 cells. Although the differences were not statistically significant, the trend indicates that *cag*PaI activity may have caused the cells to bind more IL-10 and would lead to increased IL-10-mediated effects on infected gastric epithelial cells.

The result should be reliable as two different specificity controls as provided with the kit were assayed simultaneously with the test sample. One control consisted of an irrelevant protein which was biotinylated to the same degree as IL-10. The other used anti-IL-10 antibody to specifically block IL-10 binding to its receptor.

Two other human gastric epithelial cell lines MKN 28 and MKN 45 were tested for IL-10 binding, but only AGS cells provided a consistent result. The trends may be peculiar to the AGS cell line only and this is a weakness of the study. Since there is no literature on IL-10R or IL-10 binding to normal human gastric epithelial cells, this research needs to be done.

Although, these cell lines are designated as gastric epithelial cell lines, they exhibit different characteristics. AGS cells have an intestinal epithelial cell morphology whereas, MKN45 cells exhibit a gastric cell phenotype (Matsuda *et al.*, 2008). Both

originate from tumours, therefore it is not possible for them to exhibit many properties, such as expression of genes involved in carcinogenesis, differently between them (Zheng *et al.*, 2009). They also have different rates of apoptosis (Yang *et al.*, 2004), levels of NF-κB activation (Li *et al.*, 2005) and defensin expression (Bajaj-Elliott *et al.*, 2002). Since these were also originally associated with *H. pylori* infections, therefore they may respond differently during infection. Furthermore, it was reported that, STAT3 activity which is activated following IL-10 stimulation, was high in AGS and MKN 45 but low in MKN 28 (Yu *et al.*, 2004). Thus, these cells are likely to respond differently to IL-10.

Since IL-10 binding to AGS cells decreased upon *H. pylori* infection, it was speculated that IL-10R may be internalized as previously reported for human neutrophils (Elbim *et al.*, 2001) and macrophages (Du *et al.*, 2006). In the latter study, the human IL-10 Fluorokine assay was used which showed a rapid decrease of IL-10 binding to macrophages upon stimulation. Although these reports are on phagocytic cells, other cytokine receptors e.g. IL-13Rα2, have also been found to be predominantly intracellular molecules in human primary bronchial epithelial cells and fibroblasts (Konstantinidis *et al.*, 2008). There have been some reports that *H. pylori* can be found within gastric epithelial cell (Lozniewski *et al.*, 2003; Papadogiannakis *et al.*, 2000). This could mean that *H. pylori* as well as other molecules on gastric epithelial cell surface e.g. IL-10R could be internalized simultaneously. The fluorokine assay was adapted to determine if the IL-10R could be intracellularly located in the AGS cells. For intracellular staining, the cells were fixed with 0.5% formaldehyde prior to permeabilisation and staining. Unfortunately this resulted in a dramatic reduction in IL-10 binding to uninfected cells.

This is probably due to formalin-induced protein cross-links causing structural changes in the IL-10R. Other methods to check for localization of IL-10R should be investigated.

To support this work and confirm its findings, IL-10R expression was further investigated using RT-PCR.

4.1.3. Measurement of *IL10R* mRNA expression by real time RT-PCR.

Real-time PCR was carried out to measure the expression of *IL10RA* and *IL10RB* in *H. pylori*-infected AGS cells in comparison to uninfected cells. Cells were cultured with and without *H. pylori* 60190 strain in 6-well plates. Cells were harvested and total RNA was extracted from these cells. mRNA was reverse-transcribed and used in real-time RT-PCR. Primers for the human *IL10RA* and *IL10RB* genes were designed so that they cross at intron-exon boundaries. Before using them on test samples, primers were validated using commercial human cDNA and products were run on 1.5% agarose gel to confirm that they were at the required size. Both *IL10RA* and *IL10RB* data were normalised against *GAPDH* expression. Results were reproduced in three individual experiments, with each parameter tested in four replicates.

It was shown here that the transcripts for *IL10RA* and *IL10RB* were expressed in infected AGS cells after 24 hours with *H. pylori* infection (Figure 14) thus confirming the previous data. In contrast to the IL-10 Fluorokine data however, expression of both genes was up-regulated upon infection. The increment for the *IL10RB* was far greater than that

of *IL10RA*. It is possible that the high level up-regulation of *IL10RB* may be due to it being utilised in other cytokine receptor complexes such as IL-22R (Donnelly *et al.*, 2004). Although IL-22RA and IL-10RA are reportedly not expressed on the same cell type (Wolk *et al.*, 2004), however, these authors did not study gastric epithelial cells. A few human organs have been found to express IL-22R, including the digestive system (Wolk *et al.*, 2004). Epithelial cells are one of the cell types found to express IL-22RA (as reviewed by Wolk & Sabat, 2006). The gastric epithelial cells may be expressing both types of receptor complexes but further investigations need to be done to confirm this.

A limitation of the data is that a comparative method was used. It was not possible to determine the actual expression levels of *IL10RA* and *IL10RB*. In order to set up a fully quantitative PCR, the *IL10RA* and *IL10RB* coding sequences could have been cloned into a plasmid to be used as a standard curve. Unfortunately, there was no time for this. In addition the real time RT-PCR was only measuring IL-10R expression at the transcript level, the amounts of translated protein of both subunits were not known. Since there are several steps between transcription and translation which control protein expression, RT-PCR may give an inaccurate representation of IL-10R protein. In order to address this, western blotting was then used to measure IL-10R protein expression.

4.1.4. IL-10R protein expression by SDS-page and western blotting.

AGS cells were cultured with and without *H. pylori* 60190 strain in 6-well plates. Cells were solubilised in SDS buffer and protein were electrophoresed on 10% SDS-PAGE gels before being transferred onto nitrocellulose membranes. Anti-IL-10Rα or anti-IL-10Rβ antibodies were used to probe the membranes.

Using this analysis, the IL-10Rα band density appeared to be approximately the same for uninfected and infected cells (Figure 15 (ai)). However, it was difficult to measure the density of the bands as the signals were not strong enough. Even SignalBoost, which has been reported to enhance the signal, was used in this experiment but still did not give any difference. This result may indicate that the IL-10R was constitutively expressed by AGS cells. This may be so since some cell lines such as SF-MA (a human skin fibroblast cell line) and U937 (a human monocyte cell line) also have been reported to constitutively express IL-10R, and there was no up-regulation when stimulated with LPS (Tokuda *et al.*, 2003).

Interestingly, the IL-10Rβ expression was clearly being expressed more in the infected cells compared to the uninfected cells (Figure 15 (bi)). This result agrees with data from the RT-PCR experiments. As mentioned earlier, the very high increment in IL-10Rβ expression could be due to it being incorporated into other cytokine receptor complexes such as IL-22R (Donnelly *et al.*, 2004). This has yet to be confirmed as there is no published report on IL-22R expression by AGS cells or other gastric epithelial cell lines.

A study reported that IL-10 and IL-22 has been found simultaneously in both systemic and local inflammation but, apart from competition for IL-10Rβ, they did not influence each other (Wolk *et al.*, 2005).

4.1.5. Summary of the findings for IL-10R expression.

In summary, immunofluorescence microscopy was not a suitable method to detect IL-10R expression as there was high level background staining indicating that the staining may not be specific (Table 4). The FACS analysis using anti-IL-10Rα antibody also failed as the isotype control for the THP-1 cells showed very high fluorescent signals and therefore the results obtained with AGS may be unreliable. The human IL-10 Fluorokine assay, however, may be the most reliable method to use as the controls utilised in this method worked well and the results were reproducible.

The IL-10 Fluorokine assay showed that a very high proportion of uninfected AGS cells bound IL-10. This could mean that the AGS gastric epithelial cell line expressed IL-10R constitutively. The data from this work surprisingly showed a rapid decrease in cell surface binding of IL-10 upon *H. pylori* infection. It is hypothesised that this may be due to the receptor being internalised when the cells were infected, as described in another study (Du *et al.*, 2006) but experiments to test this were unsuccessful. In addition to using the Fluorokine assay, Du *et al.* confirmed receptor internalization by showing a decrease in cell surface IL-10RB subunit using immunoblotting. Similar experiments should be carried out to validate the Fluorokine assay data in this study.

With RT-PCR and western blotting experiments, this study showed an increase in protein and transcript for the IL-10RB subunit when cells were infected with *H. pylori*. This is may be due to the subunit being shared by other receptor complexes such as IL-22R. Although there is no published data on IL-10RA and IL-22RA expression in gastric epithelial cells so far, these cells may be the target cells for IL-22 as this cytokine has been reported to target epithelial cells.

Techniques	**Detection of:-**		
	IL-10Rα	**IL-10Rβ**	**IL-10 binding (Fluorokine)**
Monoclonal antibodies and immunofluorescence microscopy	Questionable specificity	Not done	—
Fluorescent IL-10 and immunofluorescense microscopy	—	—	Very weak signals
Monoclonal antibodies and flow cytometry	Questionable specificity	Not done	—
Fluorescent IL-10 and flow cytometry	—	—	Strong specific binding to AGS cells. Binding decreased upon infection with *H. pylori*
RT-PCR	Expression increased 2.5-fold upon infection with *H. pylori*	Expression increased 25-fold upon infection with *H. pylori*	—
Western blotting	Expression similar for infected and uninfected cells	Expression increased in infected cells	—

Table 4. Techniques used for detection of IL-10R expression and IL-10 binding to AGS cells.

4.2. Bacterial-epithelial cell co-culture experiments and effects of IL-10 on bacterial densities.

Previous experiments in this study have confirmed that the gastric epithelial cell line, AGS does express IL-10R. Although the proportion of cells binding IL-10 was decreased when infected with *H. pylori*, there is still a clear possibility that IL-10 may exert its effects during *H. pylori* infection. Furthermore, previous studies in the lab have shown that human gastric biopsies with the highest *H. pylori* density grading (3 on a scale of 0-3) contained 6-fold higher levels of *IL10* mRNA than the samples with grade of 1 and 20 ng/ml rIL-10 induced a significantly increased bacterial density ($p<0.002$) in *H. pylori*-AGS co-cultures *in vitro* (Robinson *et al.,* 2008). Therefore, *H. pylori*-AGS co-culture experiments need to be done to investigate if IL-10 can exert its properties *in vitro* and also to confirm or refute previous findings.

Using viable counting on *H. pylori*-AGS co-cultures, this study has shown and confirmed that adding rIL-10 promoted the growth of *H. pylori* in dose-dependent manner. Additionally, the effect of IL-10 was found to be mediated through gastric epithelial cells since there was no significant effect on bacterial growth when *H. pylori* was cultured alone. It was hypothesised that, in the presence of IL-10, gastric epithelial cells may produce less antibacterial effector molecules allowing bacteria to survive, or IL-10 may

induce the cells to provide a more favourable environment for *H. pylori* to grow (for example, expressing growth factors or increasing intracellular uptake).

The positive effect of IL-10 on the densities of a *cag*PaI-positive strain but not a *cag*PaI-negative strain, shown in this co-culture study, proved that the presence of this set of genes is important for IL-10 to modulate the outcome of infection. For additional evidence on the importance of the *cag*PaI for IL-10-mediated effects, an experiment comparing effects of IL-10 in AGS co-cultures with the *H. pylori* 60190 wild type strain and its isogenic *cagE* mutant was carried out. The results showed that there was an increase in growth of the wild type strain when rIL-10 was added but not the mutant. Although this did not reach statistical significance, the trend was similar to the experiments using 60190 and Tx30a *H. pylori* strains. IL-10 may therefore be suppressing *cag*PaI-dependent effects on cells.

It has been reported that the *cag*PaI-encoded T4SS delivers CagA protein (Fischer *et al.*, 2001) and peptidoglycan (Viala *et al.*, 2004) into the host epithelial cells and activate NOD1. This activation will lead to activation of NF-κB which induces the expression of genes encoding pro-inflammatory cytokines, including IL-8 (Chu *et al.*, 2003) and defensins such as hBD-2 (Wada *et al.*, 2001). A recent study reported that *H. pylori* induces a *cag*PaI-dependent increase in mucosal inflammation which is accompanied by expression of an array of antimicrobial peptides such as hBD-2 (Hornsby *et al.*, 2008). There are many other antibacterial effectors during *H. pylori* infection but hBD-2 could be used as a starting point. Compared to hBD-1, only the gene encoding hBD-2 has the

binding sites for NF-κB in the promoter sequence of hBD-2 (Liu *et al.,* 1997). Since rhIL-10 has been found to inhibit NF-κB activation and IL-8 expression in *H. pylori*-AGS co-cultures (Robinson *et al.,* 2008), it is tempting to speculate that IL-10 also inhibits hBD-2 expression by gastric epithelial cells through suppression of NF-κB activation.

IL-10 is also known to act on immune and inflammatory cells such as T cells and neutrophils (Groux *et al.,* 1998; Niiro *et al.,* 1997) by decreasing their activity. Furthermore, *H. pylori*-infected IL-$10^{-/-}$ mice suffered from severe inflammation then cleared the infection (Matsumoto *et al.,* 2005). IL-10 has also been found to suppress IL-8 secretion and expression of intracellular adhesion molecule-1 (ICAM-1) in *H. pylori*-AGS co-culture assays (Zakaria *et al.,* 2008). All of these showed that IL-10 has the potential to suppress of the inflammation and support prolonged colonisation of *H. pylori* in the gastric mucosa.

However, increases in bacterial density may lead to higher production of bacterial toxins and oxygen radicals in the gastric mucosa long term. These may then promote oxidative damage in the host cells and eventually, the host cells may be at higher risk of cancer development. This type of damage is not easily repaired and *H. pylori* eradication must be given before the appearance of pre-malignant pathology to completely avoid gastric cancer (Farinati *et al.,* 2004).

4.3. IL-8 release by AGS cells.

Many studies have confirmed that increased levels of IL-8 in the gastric mucosa occur in association with *H. pylori* infection (Kim *et al.*, 2001; Ren *et al.*, 2001). Work from our research group reported that there were 17-fold higher *IL8* mRNA levels gastric tissue from infected patients compared to uninfected patients ($p<0.0001$) (Robinson *et al.*, 2008). IL-8 expression is a predominant inflammatory marker which is commonly used to show that gastric epithelial cells in culture are capable of initiating changes related to acute inflammation (Crowe *et al.*, 1995). IL-8 expression was shown previously to be down-regulated upon the addition of rhIL-10 in *H. pylori*-AGS co-cultures in a dose-dependant manner (Robinson *et al.*, 2008). The significant ($p<0.0005$) production of IL-8 by infected AGS cells in the current study (Figure 20) showed that the *in vitro* model in this study was working as expected, giving similar results to previous published work (Robinson *et al.*, 2008) and thus demonstrating the important role of epithelial cells in the inflammatory process.

4.4. Critical analysis of the methods.

4.4.1. Cell lines.

In this study, human gastric epithelial cell lines originating from adenocarcinoma tissue were used since gastric epithelial cells do not survive for longer than 24 hours after extraction from normal tissue and are difficult to obtain. It would also be extremely difficult to eliminate immune cells and other cells that are present in the gastric

epithelium. This would complicate the results as such cell types have been found to express IL-10R (Elbim *et al.*, 2001) and IL-10 (Edwards *et al.*, 2006; Lundgren *et al.*, 2005). Since cancer cells were used, these may exhibit different properties from normal cells. In particular, they may not show the same level of IL-10R expression as the non-malignant gastric epithelium.

Alternative methods for analysis of normal gastric epithelium may be utilized such as immunohistochemistry. Sections from biopsies of *H. pylori*$^{+}$ and *H. pylori*$^{-}$ patients could be stained with anti-IL-10R antibody or human IL-10 Fluorokine reagents. However, endogenous IL-10 may be present and could interfere with the result.

In addition, epithelial cells could be extracted from tissue sections using laser capture microdissection (LCM). LCM is a method of isolating cells of interest from heterogenous tissue that has been sectioned, with the aid of a microscope, specialist equipment and software (Emmert-Buck *et al.*, 1996). These samples could be assayed for IL-10R expression using RT-PCR, as described by others (Makino *et al.*, 2008).

4.4.2. Standardising the number of cells added to the wells or tubes.

The precise number of cells in each well may differ in each experiment and this could cause variation in the overall outcome of each experiment. Although care has been taken to equally distribute the cells into each well during seeding, pipetting errors and cell

clumping may have caused some wells to have a slightly different number of cells. Moreover, incubation with *H. pylori* causes some cells to apoptose and therefore there would be a reduction in cells remaining after 24 hours.

These differences will contribute to variation within experiments where replicates give a broader range. A bigger effect is then needed to be able to observe a statistically significant outcome. Furthermore, unequal distribution of cells will also lead to variation between experiments as seen when some assays were not very reproducible for example human IL-10 Fluorokine assay using MKN28 and MKN45 cell lines.

4.4.3. IL-10R antibodies.

In this study, only one monoclonal antibody was used for the detection of IL-10Rα and β subunit. Availability of such reagents for immunofluorescence is limited and the anti-IL-10Rα mAb used in this study was not actually recommended for this technique. Many of these mAbs are recommended to be used with western blotting or immunoprecipitation methods. For western blotting, two more antibodies, anti-IL-10Rα (C-20) polyclonal antibody (Santa Cruz) and anti-IL-10R (CD210) mAb (BD Bioscience) were also used, but the results were not any better. Between these two, only anti-CD210 is also recommended for immunofluorescence but the manufacturer of this antibody could not specify to which subunit of the receptor it will bind. Nothing is known about IL-10R expression by gastric epithelial cells. It is therefore important to use mAb specific for the

IL-10Rα subunit since this truly represents the IL-10R. The IL-10Rβ subunit has been found to be part of other cytokine receptor complexes.

4.4.4. Standardising the number of bacteria added to the wells.

Although numbers of bacteria were carefully distributed equally, it is impossible to eliminate errors in putting the same number of bacteria per well. The optical density (OD) was used to estimate the concentration of bacteria for infecting AGS or THP-1 cells, but this is not an actual method for counting viable bacteria as non-viable bacteria also absorb light. Furthermore, multiplicity of infection (MOI) was calculated from OD value but the actual MOI in the wells maybe different. In order to standardise the inocula as much as possible, growth was scraped from plates inoculated 24 hours previously. This should ensure that the proportions of viable and non-viable bacteria were of similar levels on each occasion. This was important to minimise variation within and between experiments.

4.4.5. Quantitative RT-PCR method.

This technique was used since the IL-10 receptor is thought to be expressed at only a few hundred molecules per cell (Tan *et al.*, 1993), and the mRNA expression may also be at low levels. It is a sensitive and accurate method but also has some disadvantages such as detecting nonspecific PCR products and primer dimers. Fortunately, our hot start PCR technique could minimise primer dimer formation (Kellogg *et al.*, 1994). The presence of nonspecific products was distinguished by testing non-reverse transcribed RNA in

parallel with the test samples. In addition, the melting points for the specific products were also used as guidelines. The PCR products were run on 1.5% agarose gels to confirm that the predicted size for the required products was correct. It would be more specific if Taqman probes were used instead of the SYBR Green method.

Since this is a comparative method of looking at the mRNA level of IL-10R expression, the initial amount was not known. A quantitative RT-PCR method could be utilized but time was short and this would have required more work as this involves cloning cDNA into a plasmid for use in a standard curve. Furthermore, *GAPDH* gene expression may not be the most appropriate housekeeping gene. It would have been useful if an experiment was done to confirm that *GAPDH* expression levels are unchanged upon infection of gastric epithelial cells.

4.4.6. Loading control for western blotting.

A gel loading control is important when using western blotting or any protein measurement techniques. β-actin, used in this study, may not be a reliable loading control for western blot analysis as depicted by another study (Dittmer & Dittmer, 2006), which showed that the antibody specific to β-actin was not able to differentiate different levels of actin protein. It would be useful, if an experiment is done to check the suitability of loading controls used as mentioned by a study (Bauer *et al.*, 2009). Furthermore, some have found that using two total protein stains (such as SYPRO Ruby and Amido Black) may be an acceptable alternatives to a single-protein control such as β-actin or GAPDH (Aldridge *et al.*, 2008).

4.5. Conclusions.

Taken together, the data in this study highlight a novel and important finding regarding IL-10R expression by gastric epithelial cells. This was convincingly shown by RT-PCR and western blotting. The finding was expected as the research group had previously shown that addition of rIL-10 reduced the IL-8 response of *H. pylori* infected AGS cells (Robinson *et al.,* 2008). However, work on the effects of *H. pylori* on IL-10 binding capacity of AGS cells and IL-10R expression were conflicting. Data indicated that IL-10 binding was reduced upon infection, but IL-10R expression was increased. These finding have not yet been explained but possibly IL-10R molecules may not all be present at the surface, or possibly the IL-10Rβ subunit is incorporated into the IL-22 receptor complex instead.

Results obtained in the viable counting experiments suggest that rIL-10 promotes the growth of *H. pylori,* especially *cag*PaI+ strains, and the effect is mediated through gastric epithelial cells. The human IL-10 Fluorokine assay also showed that a higher proportion of *H. pylori-cag*PaI+ infected AGS cells bound IL-10 compared to cells infected with a *cag*PaI- strain. These data suggest that the presence of the *cag*PaI is important for the ability of *H. pylori* to modulate the capacity of AGS cells to bind IL-10 and therefore IL-10 could carry out its effects on cells such as suppressing NF-κB activation. This is thought to be the mechanism of suppressing IL-8 and hBD-2 which help to promote bacterial growth.

4.6. Future work.

In the future, the immunofluorescence microscopy experiments should be repeated using the IL-10 Fluorokine kit as mentioned in a previous study (Michel *et al.*, 1997), and perhaps more sensitive methods such as ^{125}Iodine-labelled IL-10 would be useful (Denning *et al.,* 2000). More gastric epithelial cell lines would be tested for their IL-10R expression to determine if the findings of this study are not just a phenomenon restricted to AGS cells only. The IL-10R expression in the human gastric tissue also needs to be examined. Monoclonal antibodies of different sources or from different manufacturers which are specific for IL-10Rα and IL-10Rβ as well as those recommended for the techniques used, should be considered.

Furthermore, rIL-10 addition to co-culture experiments on *cag* mutants should be repeated to confirm that IL-10 mediates its effect through a *cag*PaI-dependent pathway. Other *cag*PaI-positive and *cag*PaI-negative wild type strains should also be considered. This has already been completed by another member of the research group (Zakaria *et al.*, 2008) which showed a significant increased of bacterial density when administering rIL-10 AGS infected with *H. pylori* wild-type J99 and 11673 (*cag*PaI+) strains but not J63 and J68 (*cag*PaI-) strains.

Work on the effects of IL-10 upon growth or survival of *H. pylori* in epithelial cell co-cultures should be closely investigated. Western blot analysis could be carried out to examine IL-10-mediated inhibition of antibacterial effector expression example for defensins, reactive oxygen and nitrogen derivatives. Experiments could also be carried

out to test whether IL-10 induces the expression of beneficial factors for growth of *H. pylori*. This could be done by adding culture supernatants from rIL-10-treated and untreated AGS cells to *H. pylori* cultures. The effect of the supernatants on bacterial growth could be determined by optical density measurements and viable counting.

The best characterized IL-10 signalling pathway, the Jak/STAT system, will be elucidated in details. Microarrays could be used for the expression profiling of gastric epithelial cells to determine which genes are differentially expressed in response to *H. pylori* and with or without the exposure to IL-10. The genes of particular interest might include cytokines, chemokines, adhesion molecules, antibacterial genes (such as defensins and cathelicidins) and other genes thought to be associated with pro-inflammatory responses.

Finally, experiments would investigate whether the pro-inflammatory genes influenced by IL-10 *in vitro* are also differentially expressed *in vivo*. Human gastric biopsy specimens from infected patients would be tested by real-time qPCR for transcript levels of IL-10 and other genes that are differentially express in response to IL-10 for example IL-8. In addition, the influence of IL-10 *in vivo* may be tested directly by administering either rIL-10, neutralising anti-IL-10 monoclonal antibodies or a placebo to mice.

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APPENDIX

Reagents.

Flow cytometry buffers

PBS/0.5% BSA (PBA)
0.5g Albumin from bovine serum (BSA)
100ml PBS

PBA/2% FCS
100ml PBS
0.1g BSA
0.5ml 20% azide
2ml FCS

SDS-PAGE and western blotting buffers

Reducing sample buffer
5ml 0.5M Tris-HCl pH6.8
0.8g SDS
0.04g bromophenol blue
20ml glycerol
0.5ml 2-mercaptoethanol
10ml distilled water

0.5M Tris-HCl pH6.8
90.8g Tris base
300ml distilled water
HCl for adjusting pH to 6.8

Blocking solution
1g skimmed milk powder (Marvel)
50ml PBS-Tween

PBS-Tween
1L distilled water
10 PBS tablets
0.5ml Tween20 (Polyoxythylenesorbitan monolaurate)

Blotting buffer
5.8g glycine
11.6g tris base
7.4ml 10% SDS
400ml methanol
Distilled water to total volume 2L

Resolving gel
2.5ml 1.5M Tris-HCl pH 8.8
4.0 distilled water
0.1ml 10% SDS
3.4ml acrylamide:bis acrylamide (30%:0.8%)
50µl 10% ammonium persulphate
10µl TEMED

Stacking gel
1.25ml 0.5M Tris-HCl pH6.8
3.0ml distilled water
50µl 10%SDS
650µl acrylamide:bis acrylamide (30%:0.8%)
50µl 10% ammonium persulphate
5µl TEMED

Real-time RT-PCR

cDNA synthesis reagents

Reagents	Volume per tube(µl)
Oligo(dT) primer	0.5
RNA template (25µg/µl)	4
dNTP mix (10mM each)	1
RNAse free water	6.5
5x first strand buffer	4
RNAseOut	1
0.1M DTT	2

Real-time PCR reaction mixture

Reagents	Volume per tube (µl)
Dynamo HS SYBR Green	10
Forward primer	2
Reverse primer	2
RNAse free water	2
cDNA template	4

Glossary

Intestinal metaplasia – replacement of gastric epithelial cells with those of intestinal type
Dysplasia – abnormality of maturation of cells within a tissue
Gastric antrum – the lower part of the stomach
Lymphoma – cancer that affects lymphocytes
Hypochlorhydria – low level of gastric acid in the stomach
Atrophic gastritis – loss of gastric glands
Oedema – abnormal accumulation of fluid beneath the skin or cavities of the body