THE FUNCTION OF NATURAL KILLER CELLS IN *HELICOBACTER PYLORI* INFECTION AND GASTRIC CANCER

ÅSA LINDGREN

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

INSTITUTE OF BIOMEDICINE

THE SAHLGRENSKA ACADEMY, UNIVERSITY OF GOTHENBURG

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ABSTRACT

Helicobacter pylori infection is one of the most wide-spread infections in the world and causes a chronic inflammation in the gastrointestinal mucosa characterised by increased production of IFN-γ and is associated with an increased risk of developing gastric cancer. The mechanisms behind the development of gastric cancer in *H. pylori* infected individuals are unclear but probably constitute a combination of bacterial factors and host susceptibility. Since the persistent *H. pylori*-induced inflammation may promote tumour development and tumour cells must acquire the ability to evade the immune system, it is important to study the immune response to *H. pylori* to understand how gastric cancer develops.

The presence of Natural Killer (NK) cells in the gastric mucosa and the ability of NK cells to produce IFN-γ suggest an important role of NK cells in the immune response towards *H. pylori*. NK cells in the gastrointestinal mucosa are likely to encounter *H. pylori* as well as other bacteria and may play an important role in the mucosal innate immune defence.

The focus of this project has been the ability of human NK cells to respond to bacterial components with IFN-γ production. We have investigated the mechanisms for recognition of *H. pylori* as well as the NK-cell subsets involved in the recognition. Furthermore, we have examined the ability of NK cells derived from gastric cancer patients to respond to bacterial stimuli.

We have demonstrated that in contrast to peripheral blood, most NK cells in the human gastrointestinal mucosa lack CD8 expression. Importantly, we show that $CD8^-$ and $CD8^+$ NK cells have different functional properties; only $CD8^-$ NK cells were capable of responding with IFN-γ production to stimulation with lysate from *H. pylori* and other bacteria.

Our studies also indicate an involvement of Toll-like receptors (TLRs), and in particular TLR2 in the recognition of *H. pylori*. Furthermore, we have shown that the *H. pylori* specific membrane bound lipoprotein HpaA induce IFN-γ production from NK cells through TLR2.

In addition, we have examined the IFN-γ producing ability of NK cells from gastric cancer patients. Our results show that NK cells from gastric cancer patients have a severely suppressed ability to produce IFN-γ after stimulation with *H. pylori* lysate and the synthetic bacterial lipoprotein FSL-1. We propose that the suppression is due to tumour-derived TGF-β, since TGF-β treatment of NK cells from healthy individuals leads to a similar suppression of NK-cell activity.

In conclusion we have shown that (i) $CD8^-$ NK cells are the predominant NK-cell subset in the gastric mucosa, (ii) $CD8^-$ NK cells are especially adapted to respond to bacterial stimuli, (iii) NK cells recognise *H. pylori* via TLR2, (iv) NK cells from gastric cancer patients have an impaired ability to produce IFN-γ and (v) that the impaired IFN-γ production may be due to tumour-derived TGF-β. These findings may have important implications for the understanding of NK-cell subsets and the innate defence against gastrointestinal bacterial infections and the development and progression of gastric cancer caused by chronic *H. pylori* infection.

Keywords: Natural killer cells, *Helicobacter pylori*, gastric cancer, TLR, IFN-γ

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ORIGINAL PAPERS:

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-III):

I. Åsa Lindgren[*], Cheol-Heui Yun[*], Anna Lundgren, Åsa Sjöling, Lena Öhman Ann-Mari Svennerholm, Jan Holmgren & Samuel B. Lundin. * These authors contributed equally

$CD8^-$ natural killer cells are greatly enriched in the human gastrointestinal tract and have the capacity to respond to bacteria. *J Innate Immun.* 2010 In press

II. Åsa Lindgren, Voja Pavlovic, Carl-Fredrik Flach, Åsa Sjöling & Samuel Lundin

Interferon-gamma secretion is induced in IL-12 stimulated human NK cells by recognition of Helicobacter pylori or TLR2 ligands. *Innate Immun.* 2010 In press

III. Åsa Lindgren[*], Cheol-Heui Yun[*], Åsa Sjöling, Camilla Berggren, Jia-Bin Sun, Erik Jonsson, Jan Holmgren, Ann-Mari Svennerholm & Samuel B. Lundin. * These authors contributed equally

Impaired IFN-γ production after stimulation with bacterial components by natural killer cells from gastric cancer patients. *Submitted*

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ABBREVATION LIST

ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GATA-3	GATA binding protein 3
HpaA	*Helicobacter pylori* adhesin A
IFN-γ	Interferon-gamma
IL	Interleukin
LPL	Lamina Propria Lymphocytes
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activate protein kinase
MHC	Major histocompatibility complex
mRNA	Messenger Ribonucleic acid
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cells
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase Polymerase chain reaction
T-bet	T-cell-specific T-box transcription factor
TGF-β	Transforming growth factor beta
Th	T helper cell
TLR	Toll-like receptor
T_{reg}-cell	Regulatory T cell

INTRODUCTION

1. THE IMMUNE SYSTEM

The human body is constantly exposed to potentially harmful agents such as microbes and chemicals that may cause infections, tissue damage and/or tumour development. Therefore there is a need for effective protection and resistance towards pathogens and tumours and means to distinguish healthy normal cells from infected or dysfunctional cells. This is provided by the immune system.

The human immune system is a complex structure that is generally subdivided into innate and adaptive immunity. While the adaptive immune system is the specific part of the immune system with highly antigen-specific cells such as T- and B-lymphocytes, the innate immune system is the first line of defence.

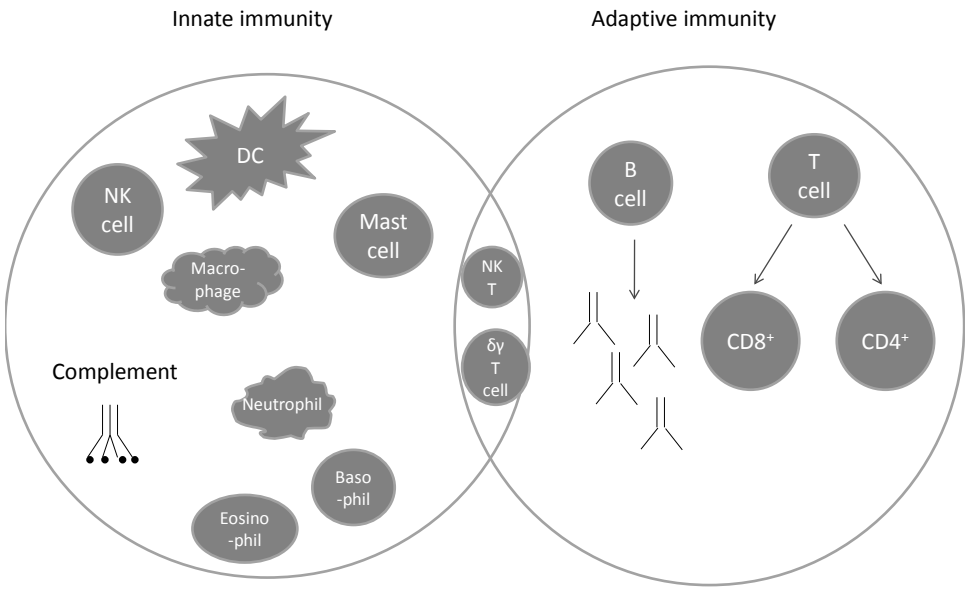

Figure 1: *The cells of innate and adaptive immunity*

1.1. INNATE IMMUNITY

The innate immune system includes mechanical barriers such as the skin and mucosal surfaces but also soluble proteins in the blood and extracellular fluids, for example the complement system. Furthermore, there are a number of different innate immune cells (Figure 1). These immune cells include phagocytic cells; neutrophils, monocytes and macrophages, antigen presenting cells (APC); dendritic cells (DC) and lymphocytes; natural killer cells (NK

cells). In addition there are some lymphocytes in the interface between innate and adaptive immunity; NKT cells and γδ T lymphocytes (Abbas et al. 2007).

Innate immune cells recognise a wide variety of microbial structures via pattern recognition receptors. Several classes if these receptors are known, e.g. Toll-like receptors (TLRs) and Nod-like receptors (NLRs). TLRs are expressed on the surface of cells or on intracellular vesicles, while NLRs are cytoplasmic (Kumar et al. 2009).

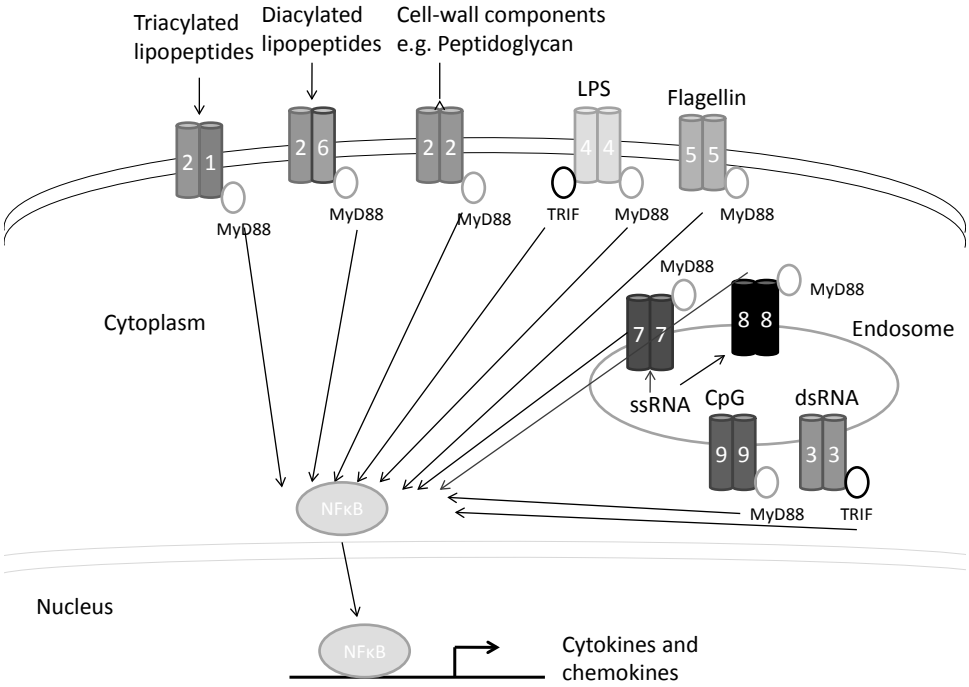

Figure 2. *Toll-like receptors*

TLRs (Figure 2) are evolutionary conserved structures and were first characterised as homologues of the signalling molecule Toll in the fruit fly *Drosophila melanogaster*, hence the name Toll-like receptors. In humans there are currently 10 known TLRs (Janssens and Beyaert 2003, Kumar et al. 2009) and they are mainly present on innate immune cells, but also on for example epithelial cells and to some extent on adaptive immune cells. TLRs can be both extra- (TLR1, 2, 4, 5 & 6) and intracellular (TLR3, 7, 8 & 9) and most often signal via the adaptor protein MyD88, although there are a growing number of examples of MyD88 independent signalling (for example via TRIF) (Janssens and Beyaert 2003). TLR signalling requires that the receptors are located together as dimers and most often result in activation of

the transcription factors NFκB and AP-1, with the end result of pro-inflammatory cytokine production (Netea et al. 2004).

The innate immune system is not only the first line of defence but also initiates and regulates the adaptive immune system by presentation of antigens and production of cytokines that recruit and activate cells.

2. NATURAL KILLER CELLS

The main lymphocyte population in the innate immune system are the NK cells, which were originally described in 1975 (Kiessling et al. 1975a, Kiessling et al. 1975b) as large granular lymphocytes with natural cytotoxicity towards tumour cells. The effector functions of NK cells were later extended to recognition of stressed and infected cells and subsequent cytotoxicity as well as cytokine production to recruit and activate other immune cells. NK cells regulate both the innate and the adaptive immune response by boosting the maturation and activation of DCs, macrophages and T cells. NK cells are also able to prevent a faulty immune response through killing of immature DCs, activated $CD4^+$ T cells and hyper activated macrophages (Ferlazzo et al. 2003).

While lymphocytes in the adaptive part of the immune system rely on receptor rearrangement for antigen specificity, NK cells have a fixed set of receptors recognising microbial, stress-related and tumour associated patterns (e.g. down regulation of MHC class I molecules).

The majority of human NK cells are present in peripheral blood, lymph nodes, spleen and bone marrow, but NK cells express chemokine receptors such as CXCR1 and CXCR3 that induce migration to sites of inflammation in response to inflammatory chemokines (Gregoire et al. 2007, Robertson 2002). NK cells are also present in peripheral tissue such as the liver, the peritoneal cavity and the gastrointestinal mucosa (Cerwenka and Lanier 2001). NK cells have generally been considered to develop in the bone marrow but recent findings show that NK cells may develop in secondary lymphoid organs as well (Freud and Caligiuri 2006).

Human NK cells comprise 5-15% of the lymphocytes in the peripheral blood (Cerwenka and Lanier 2001) and are characterised based upon presence of CD56 ($CD56^+$), which is an isoform of the neural cell adhesion molecule, with unknown function in NK cells, and absence of the T-cells receptor associated molecule CD3 ($CD3^-$) (Caligiuri 2008).

2.1. NK-CELL SUBSETS

The NK cells are far from a homogenous population and can be organised into a large number of subsets. The most common categorisation is based on the level of surface expression of CD56 into the $CD56^{bright}$ (high expression) and $CD56^{dim}$ (low expression) NK-cell subsets. Approximately 5% of the NK cells in peripheral blood are $CD56^{bright}$ and the rest are $CD56^{dim}$ (Jonges et al. 2001). The $CD56^{bright}$ NK cells are generally regarded as more adapted towards cytokine production and the $CD56^{dim}$ NK cells as the more cytotoxic subset, although there is substantial overlap between the two populations (Cooper et al. 2001b).

Human NK cells can be further categorised based on expression of the CD16 molecule, the low affinity FcγRIII, which bind opsonised targets and signal to direct antibody dependent cell-mediated cytotoxicity (ADCC) (Mandelboim et al. 1999). The majority of $CD56^{bright}$ NK cells are $CD16^{-}$ and the majority of $CD56^{dim}$ NK cells are $CD16^{+}$.

The $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ subsets also have different homing abilities, while $CD56^{bright}CD16^{-}$ NK cells express the chemokine receptor CCR7 (Berahovich et al. 2006) $CD56^{dim}CD16^{+}$ NK cells express CXCR1 and CXCR3 (Moretta et al. 2008, Robertson 2002). CCR7 bind the ligands MIP-3β and SLC (expressed in for example secondary lymph nodes) (Kim et al. 1999) and expression of CCR7 is crucial for leukocyte entry into lymph nodes while the CXCR1 and CXCR3 receptors bind to inflammation induced chemokines such as IL-8 and IP-10. This could explain why most NK cells found in secondary lymphoid organs are $CD56^{bright}CD16^{-}$ (Fehniger et al. 2003) and it would make the $CD56^{dim}CD16^{+}$ NK-cell subset more prone to be recruited into sites of pathogen induced inflammation where they exert their cytotoxic effect on damaged cells.

Furthermore, it has been suggested that the $CD56^{dim}CD16^{+}$ NK cells are more mature than the $CD56^{bright}CD16^{-}$ subset (Ferlazzo et al. 2003). The NK-cell precursors leave the bone marrow and enter the lymph nodes via peripheral blood and there they differentiate under the influence of cytokines into $CD56^{bright}CD16^{-}$ cells. The NK cells then further mature to $CD56^{dim}$ $CD16^{+}$ NK cells and return to the circulation (Caligiuri 2008).

NK cells can be further divided into subsets based on phenotypic expression of a large number of surface molecules such as a recently discovered NK-cell subset that has been designated as NK22 cells. This NK-cell subset is found in the mucosa associated lymphoid tissue (MALT) and differs from the subsets found in peripheral blood. It produces the Th17 cytokine IL-22, which induce the anti-inflammatory cytokine IL-10 from epithelial tissue (Cooper et al. 2009).

In addition it is well established that NK cells can be divided into subsets based on the absence or presence of the CD8 molecule (Jonges et al. 2001, Lanier et al. 1983, Lucia et al. 1995). The only functional difference between the subset lacking CD8 ($CD8^-$ NK cells) and the subset with CD8 ($CD8^+$ NK cells) reported up to now has been that the ligation of the CD8 molecule would enhance the cytolytic activity of the NK cells towards target cells (Addison et al. 2005), making $CD8^+$ NK cells more cytotoxic than $CD8^-$ NK cells. However, in this thesis we have demonstrated that the $CD8^-$ NK cells are better adapted to respond to bacterial components with IFN-γ production than the $CD8^+$ NK cells (paper I).

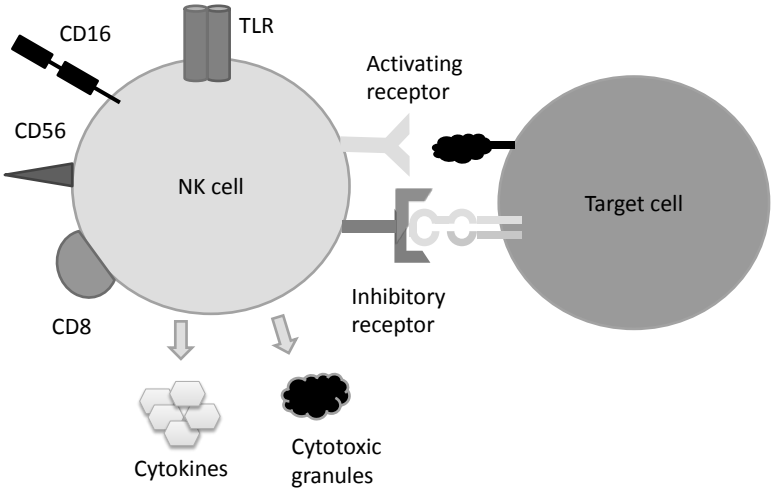

Figure 3: *Overview of NK-cell function and surface molecules*

2.2. CYTOTOXICITY

The missing self theory states that NK cells recognise and kill target cells lacking the MHC class I molecule (Ljunggren and Kärre 1985). MHC class I molecules are expressed on all healthy cells, but may be down-regulated or lost on cells that are malignantly transformed or infected. To control the NK-cell mediated killing, NK cells have two sets of receptors with opposing functions; inhibitory and activating receptors (Biassoni 2009).

The inhibitory receptors are specific for MHC class I and are the brake on the system preventing attack on normal tissue. The inhibitory receptors belong to two major families; The Killer Ig-like receptors (KIR) and the C-lectin superfamily (e.g. CD94/NKG2A). In

addition to controlling NK-cell activity the inhibitory receptors are also important for the maturation of NK cells to a fully functional state (Biassoni 2009).

The activating receptors induce cytotoxicity upon ligand engagement in the absence of sufficient inhibitory signals. The major NK-cell receptors that are able to induce NK-cell mediated killing are the Natural cytotoxicity receptors (NCR, e.g. NKp30, NKp44 and NKp46) and the lectin like receptor NKG2D. The cellular ligands for the NCRs are currently not known, while for NKG2D seven different ligands have been identified, whose expression is altered in stressed cells (e.g. MICA and MICB) (Raulet and Guerra 2009).

NK cells mediate cytotoxicity through release of granules, which are specialised secretory lysosomes that contain perforin; which is crucial for the delivery of the granule content into the target cell (Voskoboinik et al. 2006) and a family of serine proteases called granzymes. Granzymes activate caspases that result in disruption of mitochondrial membranes, DNA damage and hence induce apoptotic pathways in the target cell (Lieberman 2003). Granules in human NK cells also contain another membrane-damaging protein; granulysin, that kill bacteria, fungi and mycobacteria by increasing the membrane permeability (Clayberger and Krensky 2003).

2.3. CYTOKINE PRODUCTION

NK cells are among the most important sources of IFN-γ production, but depending on the nature of the stimuli NK cells can also produce TNF-α, IL-10, IL-13, IL-5 and GM-CSF. A subdivision of human NK cells parallel to the Th1/Th2 subsets in T-lymphocytes has been proposed, dividing NK cells into NK1 and NK2 cells, where the NK1 cells produce IFN-γ, TNF-α and IL-10 and the NK2 cells produce IL-5 and IL-13 (Deniz et al. 2002).

The main function of IFN-γ is as an activator of effector cells of the immune system and it is produced by the Th1 subset of helper T cells, NK cells, NKT cells and activated cytotoxic $CD8^+$ T cells.

The production of IFN-γ by NK cells regulate the Th1 response, activate DCs and macrophages and have anti-proliferative effects on virally infected and malignant transformed cells (Caligiuri 2008). The subset of NK cells that is the most potent inducer of IFN-γ ($CD56^{bright}CD16^-$ NK cells) is primarily located in the T-cell and DC rich regions of secondary lymphoid tissue. This enables the regulatory role of the NK cells since the close proximity of the cells facilitate the NK-cell mediated killing of immature DCs, IFN-γ induced activation of DCs and regulation of T-cell priming.

NK cells usually require two signals to produce IFN-γ and one of them is often IL-12. The second can be IL-1β (Cooper et al. 2001a), IL-2 (Hodge et al. 2002), IL-15 or IL-18 (Mavropoulos et al. 2005) or engagement of a NK-cell activating receptor such as NKG2D or CD16 (Caligiuri 2008). In addition, other combinations of cytokines such as IFNα/IL-18 (Malmgaard and Paludan 2003) can also induce IFN-γ production. Furthermore, the two-signal requirement can be overridden by high levels of IL-12 (Hodge et al. 2002, Yun et al. 2005).

NK-cell function is regulated directly by TGF-β (Bellone et al. 1995, Meadows et al. 2006) and indirectly by IL-10 (Couper et al. 2008) which are produced by for example regulatory T cells (T_{reg}-cells) (Baecher-Allan et al. 2004). IL-10 mainly down-regulates the IFN-γ production while TGF-β is involved in the down regulation of both cytotoxicity and IFN-γ production.

Human NK cells express all currently known TLRs (TLR1-10) (Hornung et al. 2002) indicating an intrinsic ability of NK cells to respond directly to bacterial and viral antigens. Indeed several studies indicate that NK cells are able to respond with cytotoxicity and IFN-γ production after TLR engagement. Both viral (Hart et al. 2005, Schmidt et al. 2004, Sivori et al. 2004) and bacterial patterns (Becker et al. 2003, Marcenaro et al. 2008) induce IFN-γ production; however a second signal such as IL-2 or IL-12 is required (paper II).

2.4. DC-NK CROSSTALK

DCs are critical for the initiation of the immune response. Immature DCs function as sentinels in the peripheral tissue where they sample the environment and sense the presence of pathogens via TLRs and other pattern recognition receptors. The DCs secrete cytokines and chemokines to recruit and activate other inflammatory cells (Sabatte et al. 2007).

NK cells are recruited to the site of inflammation where they interact with immature DCs by close physical contact. This contact promotes a series of events including DC-induced NK-cell proliferation, NK-cell dependent DC maturation and NK-cell mediated killing of immature DCs (Della Chiesa et al. 2005). NK cells and DCs can also simultaneously recognise pathogens via TLRs. Recognition of pathogens via TLRs activate the NK cells and induce IL-12 production from the DCs which in turn induce IFN-γ production from the NK cells that induce DC maturation. Presence of IL-12 also make the activated NK cells up-regulate their cytolytic ability and kill the immature DCs (Della Chiesa et al. 2005). This can take place in the lymph node as well as the site of inflammation since peripheral TLR

stimulated NK cells migrate to the lymph nodes where they interact with DCs (Lucas et al. 2007). It has been suggested that the NK-cell mediated killing of immature DCs serve to control the maturation process of the DCs and the quality and quantity of the DCs undergoing maturation. DC that fail to express sufficient amounts of the MHC class I molecule would be removed by the NK cells and thereby prevent the survival of faulty DCs that could induce inappropriate low-affinity T-cell priming resulting either in Th2 response or tolerisation (Moretta et al. 2008).

3. *HELICOBACTER PYLORI*

The stomach was long considered to be sterile, but in the early 1980´s Barry Marshall and Robin Warren were able to culture the gram-negative bacterium *Helicobacter pylori* from the stomach mucosa (Marshall and Warren 1984). They were awarded the Nobel Prize in medicine for their discovery in 2005.

H. pylori is a rod shaped curved bacterium that lives under microaerobic conditions in the human stomach. The bacterium colonises the mucus layer of the gastric epithelium and has evolved mechanisms to survive in the hostile acidic environment in the stomach. *H. pylori* have several virulence factors such as urease, BabA, VacA, CagA and HpaA. Some are important for colonisation (e.g. HpaA (Carlsohn et al. 2006) and urease (Tsuda et al. 1994)) and presence of other virulence factors are associated with more severe disease (VacA and CagA (Huang et al. 2003)). *H. pylori* are generally considered to be an extracellular bacterium but recent findings indicate that the bacteria can also grow intracellularly (Dubois and Boren 2007). *H. pylori* has been demonstrated to be able to induce endocytosis and hence uptake of the bacteria into epithelial cells (Evans et al. 1992).

H. pylori is one of the most common infections world-wide, infecting approx. 50% of the world population (Rothenbacher and Brenner 2003). The prevalence of *H. pylori* infection is decreasing in the industrialised part of the world, while in developing countries the infection affects up to 90% of the population (Brown 2000). The bacterium colonises the antrum and/or corpus of the human stomach, and is generally acquired during early childhood and cause a lifelong infection (Rowland et al. 2006). The routes of transmission of *H. pylori* are unclear, however epidemiological studies indicate a primarily oral-oral or fecal-oral route of transmission, where vomiting has been proposed to be a risk factor for spread of the bacteria (Janzon et al. 2009). Furthermore, recognised risk factors for infection are poor hygiene, socioeconomic factors and infected family members (Rowland et al. 2006).

Although most infected individuals are asymptomatic, *H. pylori* infection is associated with both acute and chronic inflammation the gastric mucosa and about 10-15% develops peptic (duodenal and gastric) ulcers and 2% gastric cancer as a result of the infection (Ernst and Gold 2000, Uemura et al. 2001). *H. pylori* was classified as a class I carcinogen in 1994 by the World Health Organisation (WHO).

The current treatment against *H. pylori* infection includes proton pump inhibitor treatment in combination with two different antibiotics (Wong et al. 1999). The treatment is effective in terms of eradication of the bacteria and healing of ulcers; however it does not protect against re-infection and there are escalating problems with antibiotic resistance of *H. pylori* (Boyanova et al. 2002, Koletzko et al. 2006). Hence the development of a vaccine towards *H. pylori* would reduce the risk of re-infection and a further development of antibiotic resistant *H. pylori* strains.

3.1. IMMUNE RESPONSES TO *H. PYLORI*

As mentioned above, *H. pylori* infection results in a chronic inflammation in the gastric mucosa and is generally skewed towards a Th1-mediated immune response. The infection is characterised by increased infiltration of a variety of immune cells linked both to the innate - neutrophils and macrophages (Ernst and Gold 2000) - and the adaptive branch of the immune system - T- and B-lymphocytes specific for *H. pylori* antigens (Lundgren et al. 2005a, Mattsson et al. 1998).

In contact with the gastric epithelium *H. pylori* induce IL-8 production (Ernst and Gold 2000), which is an important chemotactic and activating factor for neutrophils. Infiltration of neutrophils leads to increased accumulation of reactive oxygen species (ROS) that may damage the epithelial layer and induce production of several pro-inflammatory cytokines, such as IFN-γ from NK cells and $CD8^+$ T cells (Smythies et al. 2000, Sommer et al. 1998) and IL-12 (Akhiani et al. 2002, Pellicano et al. 2007, Trinchieri 2003). The failure of the immune system to eradicate the bacterium despite a massive inflammatory response renders the inflammation to become chronic and may progress to ulceration and gastric cancer development.

In contact with the gastric epithelium *H. pylori* is recognised by epithelial cells and immune cells such as DCs by TLRs (Rad et al. 2007) and in particular TLR2 and TLR5 (Smith et al. 2003, Torok et al. 2005) via a MyD88-dependent signalling pathway (Hirata et al. 2006). Several ligands for *H. pylori* recognition has been proposed, including *H. pylori* LPS and

flagellin, however there is inconsistent data concerning the ability of these antigens to be recognised by the immune system. The ligand for TLR4 is typically LPS, however *H. pylori* LPS does not seem to signal via TLR4, but is rather a TLR4 antagonist (Lepper et al. 2005) and signal via TLR2 (Mandell et al. 2004, Smith et al. 2003, Torok et al. 2005).

In the case of *H. pylori* flagellin, there are conflicting data concerning whether it can be recognised by the immune system at all. While flagellins from other species, such as *Salmonella thyphimurium,* are TLR5 agonists, there is data indicating that the flagellae of *H. pylori* as well as several other bacteria (e.g. *Campylobacter jejuni, Bartonella bacilliformis*) are mutated to avoid recognition by TLR5 of the host, possibly as a mechanism to evade the immune system (Andersen-Nissen et al. 2005).

Furthermore, the TLR2 dependent recognition of *H. pylori* may be due to recognition of either *H. pylori* LPS or lipoproteins, which are considered the typical TLR2-ligands, such as the *H. pylori* specific lipoprotein HpaA (paper II).

An increased infiltration of $CD4^+$ T-helper cells as well as regulatory $CD4^+CD25^{high}$ T cells (T_{reg}-cells) is observed in the infected antral and duodenal mucosa in comparison to uninfected mucosa (Kindlund et al. 2009, Lundgren et al. 2005b). T_{reg}-cells have the ability to down-regulate the T-cell response to *H. pylori* (Enarsson et al. 2006, Lundgren et al. 2003) and hence may suppress the immune response in order to minimise the tissue damage. However, this may at the same time contribute to persistence of the infection as well as increasing the risk of gastric cancer development by reducing the anti-tumour responses via TGF-β and IL-10 production.

The interaction between NK cells and *H. pylori* is a poorly explored field. However, it has been shown that NK cells are important producers of IFN-γ and are able to respond to *H. pylori in vitro* with IFN-γ production (Tarkkanen et al. 1993, Yun et al. 2005). This indicates that NK cells may be an important factor in the inflammatory response to *H. pylori* and in the development of *H. pylori* induced gastric cancer.

4. GASTRIC CANCER

Gastric cancer is the second most common cause of cancer deaths in the world. The process leading to gastric cancer development is slow but accelerates with age and hence the disease is most common in elderly people. More than 70% of the people that develop gastric cancer have a history of *H. pylori* infection (Ekström et al. 2001).

There are two major categories of gastric cancer; adenocarcinoma – which accounts for approx. 90% of all gastric cancers and MALT lymphomas – which are diffuse large B-cell lymphomas (Mbulaiteye et al. 2009). *H. pylori* infection is associated with development of both MALT lymphomas and adenocarcinomas in the distal part of the stomach i.e. non-cardia gastric cancer. Adenocarcinomas can be of two different types; intestinal type and diffuse adenocarcinomas. Intestinal type adenocarcinomas are tumour cells that form functional glands that resemble the intestinal mucosa and are the most common type of non-cardia gastric cancer. The *H. pylori* associated non-cardia intestinal type of adenocarcinomas develops through distinct sequential steps (Figure 4) of nonatrophic gastritis, atrophy, intestinal metaplasia, dysplasia and gastric cancer (Correa and Houghton 2007). The diffuse type of adenocarcinomas are non-functional tumour cells lacking organisation, are more common in the cardia and are more prone to metastasis and hence have a poorer prognosis (Mbulaiteye et al. 2009).

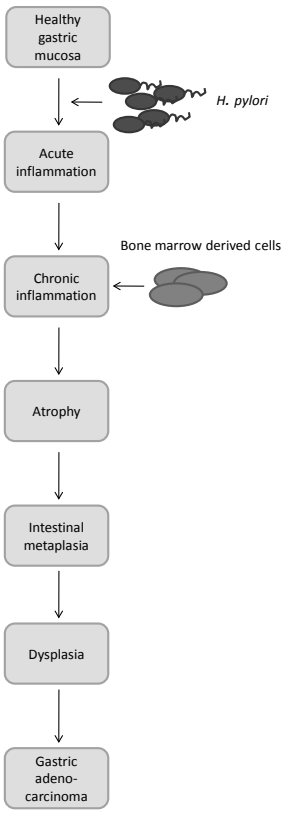

***Figure 4**: The steps of gastric adenocarcinoma development*

As mentioned above, *H. pylori* infection is an important risk factor for development of gastric cancer, however only a minor portion of the *H. pylori* infected individuals develop gastric cancer. The reason behind this is not completely known, however several factors that may increase the risk of gastric cancer development have been identified. These include dietary factors, socioeconomic factors (Hamajima et al. 2006) and bacterial factors, as well as genetic predisposition. The bacterial risk factors include the presence of several virulence factors such as CagA, which has been linked to an increased risk for development of intestinal type adenocarcinomas (Huang et al. 2003, Shibata et al. 2002).

Furthermore, the genotype of the infected individual may also play a substantial role for the development of gastric cancer. Polymorphisms of a number of pro-inflammatory cytokines (e.g. IL-1β, TNF-α and IL-10) have been demonstrated to increase the risk of non-cardia gastric cancer development (El-Omar et al. 2003, El-Omar et al. 2000). Presence of these polymorphisms may skew the immune response during *H. pylori* infection to a more severe chronic inflammatory phenotype, with reduced gastric acid secretion and increased oxidative stress to the gastric mucosa.

Chronic infection with *H. pylori* has been suggested to lead to recruitment of bone-marrow derived cells that home to the gastric mucosa in order to prevent extensive tissue damage (Houghton et al. 2004). It has been suggested that these cells may transform into tumour cells, through unknown mechanisms. It is possible that these bone-marrow derived cells are cancer stem cells of mesenchymal origin (Cao et al. 2009). The hallmark of a stem cell is continuous self-renewal and division, which make it prone to accumulate mutations. It has been demonstrated in a number of different cancers (e.g. leukaemia, lung and ovarian cancer) that only a minor portion of the tumour cells have tumourgenic properties such as extensive proliferation and metastatic ability. These cells were then proposed to be cancer stem cells (Reya et al. 2001).

4.1. THE IMMUNE SYSTEM VS. CANCER

The six hallmarks of cancer - evasion of apoptosis, insensitivity to anti-growth signals, self sufficiency in growth signals, sustained angiogenesis, tissue invasion/metastasis and limitless replicative potential – have been established as the acquired abilities required for tumour development (Hanahan and Weinberg 2000). In addition to this a seventh hallmark of cancer has been proposed; the acquired capacity of developing tumours to escape control by the immune system (Colotta et al. 2009). In agreement with this, the hypothesis of cancer

immunoediting states that pressure from the immune system can block the tumour growth, development and survival. But the immune system may also facilitate tumour development by shaping the tumour immunogenicity or by inhibition of the protective anti-tumour responses (Dunn et al. 2006).

The process of cancer immunoediting can be divided in three stages; elimination (protection), equilibrium (persistence) and escape (progression). The stage of elimination involves the recognition of transformed cells by the immune system, resulting in the killing of these cells by cytotoxic T cells and NK cells. If the tumour is not eliminated, the process of tumour development can progress to the stage of equilibrium where the tumour persists but is prevented from expansion by the immune system. When the balance between the immune system and the tumour is disrupted in favour of the tumour, the tumour can evade the immune pressure and enter the stage of escape resulting in tumour growth.

4.2. ANTI-TUMOUR RESPONSES

The recognition of tumour cells and the anti-tumour responses is thought to mainly be mediated by NK cells, T cells and macrophages. Identification of tumours can be mediated via activating NK-cells receptors such as NKG2D, which is also expressed on cytotoxic $CD8^+$ T cells and that recognise absence of MHC class I molecules. $CD8^+$ T cells are also able to recognise tumour antigens via MHC class I presentation (Shrikant and Mescher 1999). Furthermore, $CD4^+$ T helper cells have the ability to recognise tumour antigens via MHC class II presentation. However, the most important effector function of $CD4^+$ T helper cells is production of cytokines which activates and recruits immune cells to the tumour (Gerloni and Zanetti 2005).

IFN-γ is mainly produced by NK cells and T cells and is not only important for the promotion of host responses to microorganisms but also for the anti-tumour responses. Part of the anti-tumour ability of IFN-γ is mediated by the capacity to up-regulate the MHC class I pathway of antigen presentation and processing in tumour cells, making the tumours targets for NK- and T-cell mediated cytotoxicity (Shankaran et al. 2001). The anti-tumour capacities of IFN-γ also include the ability to inhibit cellular proliferation and to inhibit angiogenesis (Dunn et al. 2006). Furthermore, IFN-γ has been demonstrated to be able to block the generation and activation of T_{reg}-cells (Nishikawa et al. 2005) as well as the immunosuppressive actions of these cells.

4.3. TUMOUR ESCAPE MECHANISMS

Tumour cells acquire the ability to escape recognition by the immune system by a variety of mechanisms, including loss or alteration of MHC class I molecules which results in impaired T-cell mediated recognition of the tumour. Alteration rather than loss of MHC class I molecules further prevent NK-cell mediated lysis of the tumour cells. The tumour can also release soluble ligands for the NKG2D receptor that block the NKG2D activation of cytolytic effector cells (Poggi and Zocchi 2006). In addition, tumours can produce cytokines such as TGF-β and IL-10 that can have an immunosuppressive effect (Cerwenka and Lanier 2001).

The tumour can also induce the activation of T_{reg}-cells. This was observed in gastric adenocarcinoma patients, in which the increased numbers of T_{reg}-cells suppressed the non regulatory T-cell proliferation and IFN-γ production (Enarsson et al. 2006). T_{reg}-cells also control NK-cell proliferation and cytotoxicity in a TGF-β dependent manner (Ghiringhelli et al. 2005). This has implications on the survival of the patients, cancer patients with reduced NK-cell mediated responses and infiltration into tumours have been shown to have a poor prognosis with high risk of metastasis and cancer related death (Ishigami et al. 2000, Takeuchi et al. 2001).

Furthermore, the immune response to tumours also results in production of ROS from monocytes and macrophages. ROS may inactivate NK-cell and T-cell function and induce apoptosis of these cells, by rendering NK and T cells unresponsive to IL-2 (Hansson et al. 1999).

4.4. NK-CELL IMMUNOTHERAPY IN CANCER

Due to the anti-tumour properties of NK cells, there is growing interest towards using NK cells in treatment strategies for a variety of cancers. A number of different approaches have been used in order to increase the anti-tumour effect of NK cells, such as modulation of the NK-cell activity, adoptive transfer of NK cells or genetic modification of NK cells (Sutlu and Alici 2009).

Modulation of NK-cell activity can be performed through the administration of cytokines, most commonly IL-2; alone or in combination with other cytokines (Margolin 2008) such as IL-12 or IL-15 or in combination with histamine (Brune et al. 2006). Histamine has the ability to prevent the formation of ROS from monocytes and macrophages and hence prevent the ROS-mediated unresponsiveness to IL-2 in NK and T cells (Hansson et al. 1999). Histamine and IL-2 treatment have been used in phase III clinical trials for treatment of Acute Myeloid

Leukemia and has been demonstrated to have a positive effect on the long-term survival of the patients (Brune et al. 2006).

The major drawback with the use of IL-2 is the potential risk of activating T_{reg}-cells as well, which can suppress the NK-cell activity (Baecher-Allan et al. 2004). Furthermore, only the $CD56^{bright}$ NK-cell subset have the ability to respond to IL-2, since $CD56^{dim}$ NK cells miss the α-chain of the IL-2R (CD25) (Caligiuri et al. 1990).

Another approach to use NK cells in cancer treatment is the use of adoptive transfer of NK cells; either autologous NK cells or from a donor. The NK cells are purified and expanded and activated *ex vivo* before being transferred to the recipient. The major drawback with this method is the current lack of a large scale clinical grade NK-cell expansion method.

Genetic modification of NK cells and subsequent transfer to the recipient has also been performed. The NK cells may be modified by inducing the proliferation/survival of the NK cells via cytokine gene therapy (e.g. IL-2 (Konstantinidis et al. 2005)) which diminish the systemic effects of the cytokines. The NK cells can also be modified by over expression of activating receptors or silencing inhibitory receptors, enhancing the cytotoxicity to the tumour cells (Sutlu and Alici 2009).

AIMS OF THE THESIS

The general aim of this thesis is to characterise the activity and phenotype of NK cells in *H. pylori* infection as well as in gastric adenocarcinoma.

The specific aims of the thesis are to;

- Characterise the $CD8^+$ and $CD8^-$ NK-cell subsets

- Investigate the mechanisms behind the *H. pylori* induced IFN-γ production from NK cells

- Indentify potential *H. pylori* components responsible for IFN-γ production

- Examine the activity of NK cells from individuals suffering from gastric adenocarcinoma

MATERIAL & METHODS

HUMAN VOLUNTEERS

The experiments in this thesis were approved by the Ethical Review Board at the University of Gothenburg and informed consent was obtained from each volunteer.

Peripheral blood was drawn from asymptomatic *H. pylori* carriers (paper I &III), uninfected volunteers (paper I), *H. pylori* infected gastric adenocarcinoma patients (paper III), colon cancer patients (paper III) and pancreatic cancer patients (paper III). The asymptomatic *H. pylori* did not have any previous history of gastrointestinal illness or symptoms and were not under medication during the preceding 3 weeks before recruitment to the studies. Blood samples were drawn at the time of endoscopy from asymptomatic carriers and at the time of surgery from the cancer patients. Blood samples were also drawn from a number of gastric cancer patients not undergoing surgery (paper III).

Gastric tissue samples were also collected from the antrum of gastric adenocarcinoma patients (paper III) and asymptomatic carriers (paper I &III). The tissue samples from the gastric adenocarcinoma patients were collected at gastrectomy from "tumour-free" mucosa. Tissue was considered tumour-free if collected at least 5cm from the tumour edge. The tissue samples were transported in PBS on ice and immediately used for isolation of lymphocytes.

Peripheral blood was also collected from healthy blood donors (paper I, II &III) at the hospital in Kungälv and the Sahlgrenska University hospital in Gothenburg. The infection status of these individuals is not known.

Table I: Cancer patients recruited to the study (paper III)

	Number	Age (median, max, min)	Gender	Location of tumour	Cancer type
Gastric cancer	13	72.5; 84; 49	9 men, 5 women	Antrum 57 % Corpus 29 % Fundus 7 % Cardia 7%	Intestinal:71% Diffuse: 29%
Pancreatic cancer	6	61; 72; 57	3 men, 3 women		
Colon cancer	2	59; 60; 58	1 man, 1 woman		

ANALYSIS OF *H. PYLORI* INFECTION STATUS

Infection status of *H. pylori* of volunteers (paper I & III) was determined by serology test as described previously (Lundgren et al. 2005b) and confirmed by culturing of *H. pylori* from antral biopsy specimens or using Pyloriset EIA-G III ELISA (Orion Diagnostica, Espoo, Finland). Asymptomatic individuals and gastric cancer patients were considered to be *H. pylori* positive if at least two of the three tests were positive.

PREPARATION OF CELLS

Lamina propria lymphocytes (LPL, paper I & III) were isolated by sequential EDTA-collagenase treatment of biopsies, as previously described (Lundgren et al. 2005b). Briefly, the tissue was first stripped of the muscle and fat layers, cut into small pieces and incubated in Hank's balanced salt solution without calcium or magnesium containing 1 mM EDTA and 1 mM dithiothreitol to remove the epithelium and intraepithelial lymphocytes. The remaining tissue where then incubated in a collagenase-DNase solution and the resulting cell suspension was filtered and the number of LPL was counted.

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using Ficoll-Paque gradient centrifugation.

NK cells were further isolated and purified from PBMCs and LPL using a magnetic bead isolation kit (Human NK isolation kit, Miltenyi Biotech, Germany) according to the recommendations of the manufacturer. $CD8^+$ and $CD8^-$ NK-cell populations were also purified using magnetic beads (Miltenyi Biotec, paper I). The NK-cell fractions were >90% pure when purified from PBMCs and >85% pure when purified from LPL, estimated by FACS analysis. The bead-purified NK cells were then either used directly or sorted using a flow cytometry sorter (FACS Vantage SE or FACS Aria, BD Biosciences, San José, CA).

FLOW CYTOMETRY SORTING

Bead-purified NK cells (paper I, II &III), LPL (paper I &III) and PBMCs (paper III, from gastric adenocarcinoma and pancreatic cancer patients) were sorted using a flow cytometry sorter (FACSVantage or FACSAria) to obtain 99-100% pure NK-cell populations.

Different combinations of fluorescently labelled antibodies were used to sort the NK cells:

-α-CD56-PE (BD Bioscience), α-CD3-FITC (BD Bioscience) and α-CD8-APC (Diatech or Miltenyi Biotec, Germany) (paper I)

-α-CD56-PE and α-CD3-FITC (paper II & III)
-α-CD56-PECy5 (BD Bioscience), α-CD3-FITC, α-CD8-APC and α-CD16-PE (BD Bioscience) (Thesis)

FLOW CYTOMETRY ANALYSIS

PBMCs and NK cells were stained intra- and extra-cellularly with fluorescently labelled antibodies;

Paper I:

Intracellular staining:

- α-CD3-FITC, α-CD56-PECy5, α-CD8-APC and α-Granzyme A-PE (BD Bioscience) or α-perforin-PE (BD Bioscience)

Extracellular staining:

- α-CD3-FITC, α-CD56-PE and α-CD8-APC
- α-CD3-PerCP (BD Bioscience), α-CD56-AF488 (BD Bioscience), α-CD8-APC and α-CD16-PE

Paper II:

Intracellular staining:

- α-CD3-FITC, α-CD56-PECy5 and α-IFN-γ-PE (BD Bioscience)

Extracellular staining:

- α-CD3-APC (BD Bioscience), α-CD56-PE, primary antibody: polyclonal rat IgG anti-TLR1/2/4/5/6 (InvivoGen, San Diego, CA) and secondary antibody goat α-rat IgG FITC (Caltag-Medsystems UK)

The cells were stained for cell surface expression using optimal concentrations of antibodies. Intracellular staining (paper II) were performed using FSL-1 and/or IL-12 stimulated bead-purified NK cells. Five hours prior to the end of the incubation, GolgiPlug (BD Bioscience) was added to the culture. Thereafter the cells were stained for cell surface expression and resuspended in Cytofix/cytoperm (BD Bioscience) followed by perm/wash buffer (BD

Bioscience) according to the protocol of the manufacturer, and stained for intracellular IFN-γ expression using optimal concentrations of antibodies.
Fluorescently labelled cells were analysed by flow cytometry using a FACSCalibur (BD Bioscience).

PREPARATION OF BACTERIA

Lysates of *H. pylori* strain Hel305 ($cagA^+$ and $vacA^+$, isolated from a patient with duodenal ulcer, paper I, II &III), *H. pylori* strain SS1 (both wild-type and a ΔHpaA strain, paper II), enterotoxigenic *Escherichia coli* (ETEC strain E11881/9, ST^+ and LT^+, paper I) and *Streptococcus mitis* (paper I) was prepared as previously described (Raghavan et al. 2002). The protein contents were determined by spectrophotometry. The lysates were snap frozen in liquid nitrogen and were stored in aliquots at -70^{o}C until use.

REAL-TIME RT PCR

To determine gene expression levels bead-purified (paper II) or flow cytometry sorted (paper III) NK cells were used directly after isolation/sorting or incubated for four (paper II) or two hours (paper III), stimulated as described in Table II. After sorting/incubation the cells were resuspended in RLT lysis buffer (Qiagen, Hilden, Germany) supplemented with 1% β-mercaptoethanol and stored at -70^{o}C until further processing. The mRNA was then extracted using the RNeasy Micro (paper II) or Mini kit (Qiagen, paper III) according to the manufacturer´s instructions and was then either stored at -70^{o}C or directly used for cDNA preparation. cDNA was prepared using the Sensiscript Reverse Transcription kit (Qiagen, paper II) or the QuantiTect Rev. Transcription kit (Qiagen, paper III) according to the manufacturer's instructions.
The cDNA was then used for Real-time RT-PCR using Taqman Gene Expression assays (Applied Biosystems, Foster City, CA) for TLR1, -2, -4, -5 & -6 (paper II), GATA-3, STAT-4, T-bet (paper III), IFN-γ and HPRT (paper II& III), followed by analysis using the 7500 Real Time PCR system (Applied Biosystems). Standard conditions for relative gene expression analysis as recommended by the manufacturer were used. The results were normalised to the expression level of the housekeeping gene HPRT.

STIMULATION OF NK CELLS AND PBMCS

NK cells (paper I, II & III) and PBMCs (paper II) were cultured in the presence or absence of antigenic stimulants (see Table II for details) for 48 or 72 hours in X-vivo 15 medium supplemented with L-glutamine (Lonza, Belgium). Supernatants from the cultures were collected at selected time-points and kept at -70^{o}C until analysis of IFN-γ with an in-house ELISA (Lundin et al. 2002). The minimum detectable concentration of IFN-γ was 6 pg/ml.
Analysis of cytokine production was also made using CBA Th1/Th2 (IL-2, IL-4, IL-5, IL-10, TNF & IFN-γ) and Inflammation (IL-8, IL-1β, IL-6, IL-10, TNF & IL-12p70) kits (both BD Biosciences, San Jose, CA) (paper II).

Table II: Summary of stimulations used

Stimulation	**Application**	**Paper**
***H. pylori* lysate**	ELISA/mRNA/Cytotoxicity/ Inhibition/CBA	I, II &III /II&III/III/II/II
***E. coli* lysate**	ELISA	I
***S. mitis* lysate**	ELISA	I
IL-2	ELISA/ Cytotoxicity	II&III/III
IL-10	ELISA	III
IL-12	ELISA/mRNA/Cytotoxicity /Inhibition/CBA	I, II &III /II&III/III/II/II
IL-18	ELISA	I
TGF-β	ELISA/mRNA	III/III
IFN-α	ELISA	I
HpaA	ELISA/Inhibition	II/II
HpaA$_{trunc}$	ELISA	II
***H. pylori* flagellin**	ELISA	II
TLR agonist	ELISA/Inhibition/CBA	II&III/II/II

CYTOTOXICITY ASSAYS

Cytotoxicity was assessed by analysis of NK-cell mediated killing of the NK-cell sensitive target cell line K562. NK cells were incubated with unlabelled (paper I) or $^{51}CrO_4^-$ labelled (paper III) K562 cells. The unlabelled K562 cells were harvested and stained with anti-CD71-APC (BD Biosciences) and propidium iodide and analysed by flow cytometry. Target cells

were identified by scatter profile combined with analysis of CD71; K562 cells but not NK cells express CD71.

The supernatants of the $^{51}CrO_4^-$ labelled K562 cells were collected by a tissue collecting system and assayed for radioactivity using a γ-counter. NK-cell cytotoxicity was calculated as percent specific lysis at various effector/target ratios based on the following formula: 100 x ((experimental counts-spontaneous release) / (maximal release- spontaneous release)) = % specific killing.

INHIBITION ASSAYS

Inhibition of MyD88 activity (paper II) was analysed by incubation of NK cells with MyD88 homodimerisation peptide (Imgenex, San Diego, CA) prior to stimulation of NK cells with antigenic stimulants (details see Table II). After culture the NK cells were collected and frozen in RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol and stored at -70^{o}C until Real-time RT-PCR analysis of IFN-γ expression.

Inhibition with the PI3K inhibitor Wortmannin (Sigma, St. Louis, MO), the MAPK p38 inhibitor SB203580 (Sigma), the calcium uptake channel inhibitor La3+ (Sigma) and anti-TLR2 (eBioscience, San Diego, CA paper II) was analysed by incubation of the inhibitors with NK cells in the absence or presence of antigenic stimulants (details see Table II). Supernatants were collected and IFN-γ content analysed with ELISA (Lundin et al. 2002).

STATISTICS

Comparative data were analysed with GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA) using Wilcoxon signed rank test (paper II&III), Mann-Whitney test (paper II&III) and Student's paired (paper I& II) and unpaired t-tests (paper I). A p-value less than 0.05 were considered statistically significant.

RESULTS & DISCUSSION

The role of NK cells in bacterial infection in general and *H. pylori* infection in particular is not clear; however previously it has been demonstrated that NK cells have the ability to respond to bacterial components with IFN-γ production (Iho et al. 1999, Yun et al. 2005).

1. NK-CELL SUBSETS

1.1. $CD8^+$ AND $CD8^-$ NK CELLS

NK cells can as previously described, be divided into a number of different subsets including the $CD8^+$ and $CD8^-$ NK cells. To further characterise these subsets we investigated the distribution of these cells in blood and mucosal tissue. In peripheral blood both subsets are present, with a slight majority of $CD8^-$ NK cells (mean frequencies 31% $CD8^+$ and 69% $CD8^-$ NK cells, Figure 5). However, in the gastrointestinal mucosa the $CD8^-$ NK cells are almost exclusively present (paper I). The same distribution of $CD8^{+/-}$ NK cells could be observed in the antrum of the stomach (Figure 5), in the duodenal mucosa as well as in the colonic mucosa. The exclusive presence of $CD8^-$ NK cells in the gastrointestinal mucosa could be due to preferential migration of this subset into the tissue, because of differences in chemokine receptor distribution on the subsets. However, this remains to be investigated. In addition there could be higher survival of the $CD8^-$ NK cells in the tissue, although this seems unlikely considering the fact that presence of CD8 seems to prevent apoptosis of the NK cells rather than to induce it (Addison et al. 2005).

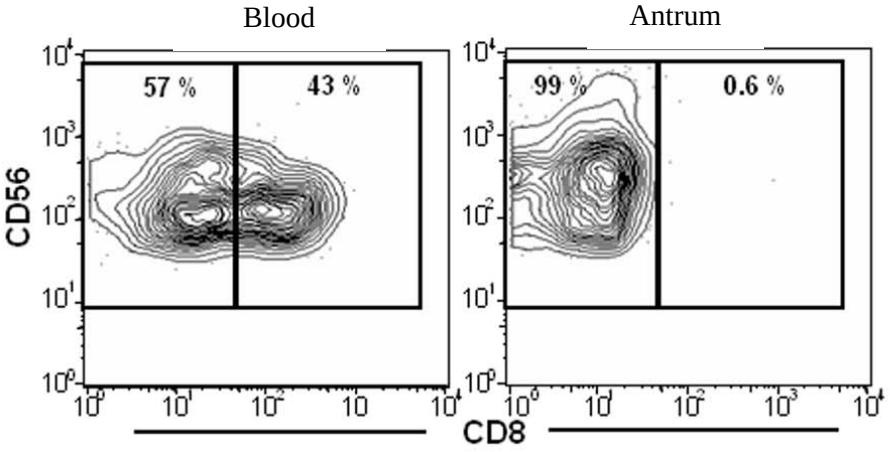

Figure 5: *Distribution of $CD8^+$ and $CD8^-$ NK cells in peripheral blood and antrum*

Further characterisation of the $CD8^{+/-}$ NK-cell subsets in peripheral blood revealed that there are a higher proportion of $CD56^{bright}$ cells among the $CD8^-$ NK cells than in the $CD8^+$ population (paper I). In addition there is a higher proportion of $CD56^{bright}$ NK cells in the gastric mucosa compared to peripheral blood (paper I), which is in contrast with previous observations (Fehniger et al. 2003) in which the $CD56^{bright}$ cells are considered to be the lymph node homing population.

Moreover, in peripheral blood the majority of the $CD8^+$ NK cells are $CD16^+$ while the $CD8^-$ cells can be both $CD16^+$ and $CD16^-$, however the majority of the $CD8^-$ NK cells are $CD16^+$ (paper I). This is in contrast with mucosal tissue where the NK cells are shown to be predominantly $CD16^-$ (Möller et al. 1998, Pang et al. 1993). Taken together, this indicates that it might be the $CD8^-CD56^{bright}CD16^-$ NK-cell subset that predominantly home to mucosal tissue.

1.2. $CD8^+$ AND $CD8^-$ NK CELLS RESPOND DIFFERENTLY TO BACTERIAL COMPONENTS

As previously shown *H. pylori* lysate in combination with IL-12 induce IFN-γ production from NK cells (Yun et al. 2005). Stimulation of $CD8^+$ and $CD8^-$ NK cells from peripheral blood with *H. pylori* lysate and IL-12 revealed the intriguing information that the $CD8^-$ NK cells were much better producers of IFN-γ than the $CD8^+$ NK cells. The $CD8^-$ NK cells produced more than 10-fold more IFN-γ than the $CD8^+$ NK cells in response to *H. pylori* (Figure 6). Even increasing the amount of $CD8^+$ NK cells up to three-fold did not induce IFN-γ production anywhere near the magnitude produced by the $CD8^-$ NK cells. Stimulation with lysate of both gram-negative (enterotoxigenic *E. coli;* ETEC) and gram-positive (*S. mitis*) bacteria revealed a similar result as observed after *H. pylori* stimulation, although the effect was not as pronounced as for *H. pylori* (paper I). This indicates that this is not a *H. pylori* specific phenomenon.

In order to rule out that the $CD8^+$ NK cells have an intrinsic defect that make them less prone to produce IFN-γ, the NK cells were then stimulated with well-established IFN-γ inducing agents; cytokines (IFN-α/IL-18) and PMA/Ionomycin. Treatment with these agents revealed an equally good ability of the $CD8^+$ NK cells to produce IFN-γ as the $CD8^-$ NK cells (paper I). This indicates that there is a preferential activation of $CD8^-$ NK cells after stimulation with bacterial components.

Furthermore, the higher ability of the $CD8^-$ NK cells to produce IFN-γ is not due to increased apoptosis of the $CD8^+$ NK cells after stimulation with *H. pylori*, since around 70% of both the

unstimulated and the *H. pylori* stimulated $CD8^+$ NK cells as well as $CD8^-$ NK cells were alive at the end of *in vitro* culture (paper I).

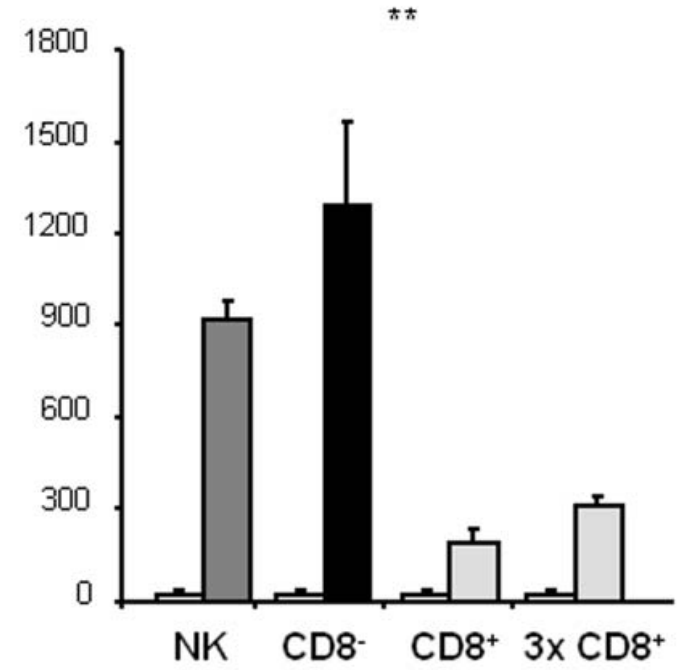

***Figure 6**: H. pylori stimulated $CD8^+$ and $CD8^-$ NK cells*

Since the $CD16^-$ NK cells generally is considered to be the more cytokine producing NK-cell subset (Cooper et al. 2001b) one might argue that the effect observed in the $CD8^-$ NK cells is due to preferential IFN-γ production by the $CD8^-CD16^-$ subset. It is also possible that the $CD8^+CD16^+$ NK cells are poorer IFN-γ producers due to the presence of the less cytokine producing $CD16^+$ subset. However, stimulations of the $CD8^+CD16^+$, $CD8^-CD16^-$ and $CD8^-CD16^+$ NK-cell subsets with both *H. pylori* lysate/IL-12 and IFN-α/IL-18 reveal that both the $CD8^-CD16^-$ and the $CD8^-CD16^+$ NK-cell subsets are equally good at IFN-γ production. Furthermore, the $CD8^+CD16^+$ population were as good at IFN-γ production as both the $CD8^-$ subsets, after stimulation with IFN-α/IL-18 (Figure 7). These results indicate that it is not the presence or absence of CD16 that is the main determinant for the ability to produce IFN-γ after bacterial stimulation.

In addition, a two-fold higher proportion of $CD56^{bright}$ cells could be detected among the $CD8^-$ NK cells in blood (paper I), which is considered to be more prone to produce IFN-γ compared to the $CD56^{dim}$ NK cells (Cooper et al. 2001b). However, this quite small increase in the number of $CD56^{bright}$ cells would not be likely to account for the more than ten-fold difference in IFN-γ production between the $CD8^+$ and $CD8^-$ NK cells observed after *H. pylori*

stimulation. Furthermore, the ability of the $CD8^+$ NK cells to produce equal amounts of IFN-γ after cytokine stimulation further strengthen the hypothesis that it is not the difference in either CD56 or CD16 expression between the $CD8^+$ and $CD8^-$ NK-cell populations that determine their ability to produce IFN-γ.

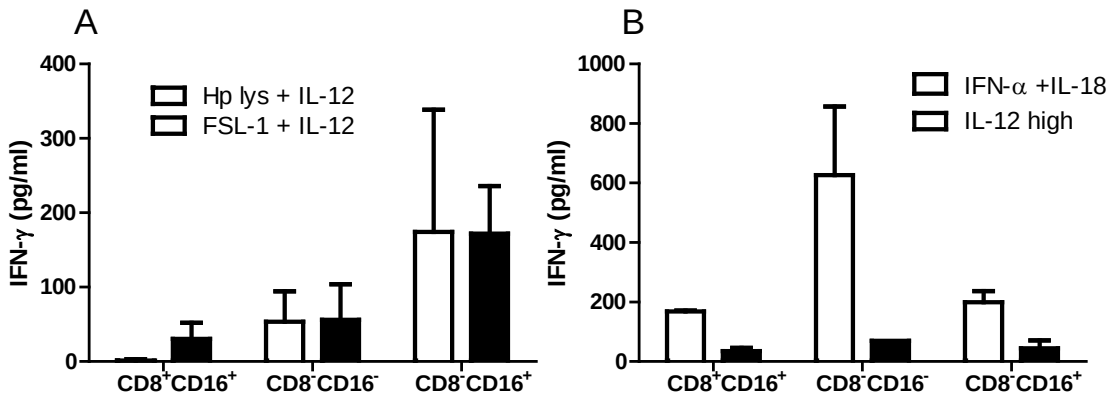

Figure 7: *Flow cytometry sorted NK-cell subsets stimulated with (A) bacterial components and (B) cytokines for 48 h. IFN-γ production was measured using ELISA. n=2.*

1.3. CYTOTOXICITY

Previously it has been reported that $CD8^+$ and $CD8^-$ NK cells differ in their cytolytic capacity (Addison) due to ligation of the CD8 molecule, which would prevent activation-mediated apoptosis of the NK cells. However, in our settings, we could not detect any difference in the cytotoxic capacity of the $CD8^+$ and $CD8^-$ NK cells. Both subsets were equally efficient at cytolytic killing of the NK-cell sensitive cell-line K562 (paper I), even though the $CD8^+$ NK cells were predominantly $CD56^{dim}$ and $CD16^+$, which would indicate a higher cytotoxic capacity. In addition both $CD8^+$ and $CD8^-$ NK cells were analysed by flow cytometry for expression of the cytotoxic substances Granzyme A and perforin, which reveal no major difference between the two subsets (paper I).

The differences in the results between our setting and the experiments by Addison *et al.* (Addison et al. 2005) could be due to the differences in incubation time. We used a shorter incubation time, which did not induce any differences in the cytotoxic capacity and it is possible that pro-longed incubation would reveal the differences observed by Addison *et al.* However, we can conclude that both the $CD8^+$ and $CD8^-$ NK-cell populations have an initial capacity to be cytotoxic.

2. *HELICOBACTER PYLORI*

2.1. MECHANISMS OF *H. PYLORI* INDUCED IFN-γ PRODUCTION

The mechanisms behind the *H. pylori* induced IFN-γ production from NK cells are not clear. In order to elucidate this further, our approach was to first examine the potential role of some major signalling cascades. Since the p38 MAPK previously have been shown to be involved in IFN-γ production (Pisegna et al. 2004, Zhang and Kaplan 2000), we used an inhibitor of p38 MAPK which revealed that the p38 MAPK is important in *H. pylori* induced IFN-γ production as well. Inhibition of the p38 MAPK reduced the *H. pylori* induced IFN-γ production from NK cells with up to 75% and the mRNA expression of IFN-γ with up to 52% (paper II).

Furthermore, since the adaptor protein MyD88 have previously been shown to be a critical signal transducer in *H. pylori* infected epithelial cells (Hirata et al. 2006) as well as necessary for IFN-γ production by NK cells in *Legionella pneumophilia* infection (Spörri et al. 2006), we next investigated the effects of inhibition of MyD88 signalling. In fact inhibition of MyD88 signal transduction resulted in 74% reduction of the *H. pylori* induced IFN-γ mRNA expression (paper II).

Taken together, the findings that the p38 MAPK and MyD88 are crucial for the production of IFN-γ from NK cells in the presence of *H. pylori* indicate that TLRs are involved in the recognition of the bacterium.

NK cells have previously been shown to express all currently known TLRs (TLR1-10) (Hornung et al. 2002), and to confirm this we quantified the mRNA expression of TLR1, 2, 4, 5 & 6, all recognising bacterial components. The highest expression levels were observed for TLR1, 2 & 6; however TLR4 & 5 could be detected as well (paper II). The gene expression of the TLRs was confirmed by flow cytometry staining (paper II).

To get a hint of which, if any, of the TLRs that may be involved in the recognition of *H. pylori* by NK cells we treated NK cells with well-established TLR agonists in combination with IL-12. The agonists that best mimicked the *H. pylori* induced IFN-γ production were the bacterial lipoproteins PAM_3CSK_4 (TLR1/2 agonist) and FSL-1 (TLR2/6 agonist) and also flagellin from *S. thyphimurium* (TLR5 agonist). None of the viral agonists did induce any IFN-γ in combination with IL-12; however in combination with IL-2 IFN-γ production was induced (Figure 8). This indicates a difference in the co-stimulatory requirements for bacterial and viral immune responses by NK cells. This might be a regulatory mechanism for the immune system to provide a specific immune response depending on the nature of the

infection. This is in line with that both IL-12 and IL-2 have been used as adjuvants (Tovey and Lallemand 2010) to induce immune responses in combination with vaccine antigens and may have the ability to steer the immune response.

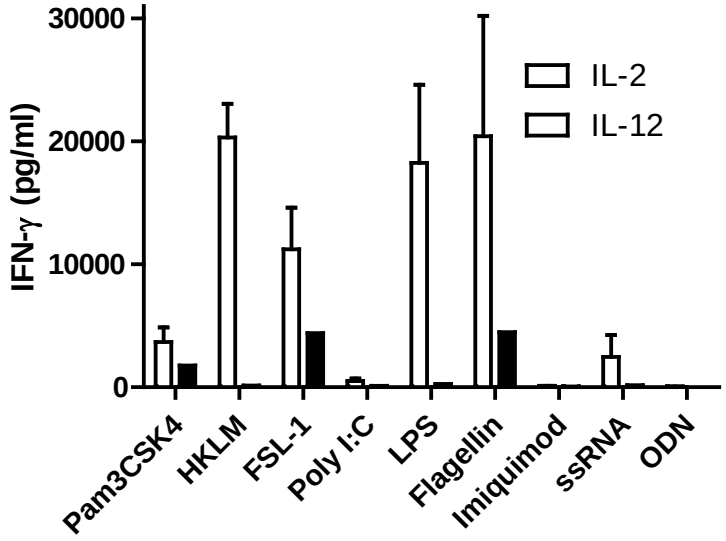

Figure 8: *NK cells stimulated with TLR agonists in the presence of IL-2 or IL-12*

Since the bacterial TLR agonists only induce IFN-γ production in combination with IL-12, we then investigated the relative contribution of the bacterial components and the IL-12 in the process resulting in IFN-γ release from the NK cell. FSL-1 and/or IL-12 stimulated NK cells were examined for IFN-γ mRNA expression (transcription, paper II), intracellular presence of IFN-γ with flow cytometry (production, paper II) and release of IFN-γ from the NK cells using ELISA (secretion, paper II). This revealed that both the bacterial components as well as the cytokine are required for all steps of bacterially induced IFN-γ production by NK cells.

2.2. HPAA BUT NOT *H. PYLORI* FLAGELLIN INDUCES IFN-γ PRODUCTION

Since TLR1, 2, 6 and 5 agonists were shown to induce IFN-γ production from NK cells, the ability of putative *H. pylori* TLR1, 2, 6 and 5 agonists to induce IFN-γ were tested.

The flagellin from *S. thyphimurium* (TLR5 agonist) was efficient in induction of IFN-γ production; hence *H. pylori* flagellin was also tested to determine if it too could be important in the recognition by NK cells. *H. pylori* flagellin did only induce IFN-γ in the presence of IL-12 as expected, but at about 20-fold lower IFN-γ levels compared to *S. thyphimurium* (paper II). This indicates that *H. pylori* flagellin may not be the most important component of *H. pylori* for recognition by NK cells. In addition, there actually is some debate regarding the

ability of *H. pylori* flagellin to be recognised by TLR5. Analysis of the *H. pylori* flagellin indicates that it may be mutated to avoid recognition by TLR5 of the host as a mechanism for evasion of the immune system (Andersen-Nissen et al. 2005). The flagellin of *S. thyphimurium* could be experimentally altered in a similar way with a subsequent loss of TLR5 recognition. However, the flagellae of the mutated strain would be fully functional and required for the survival and motility of the bacteria. The IFN-γ produced as a response to *H. pylori* flagellin could then either be due contaminations or that *H. pylori* flagellin might be recognised by another TLR than is traditionally associated with recognition of flagellin by the immune system. This has been shown to be the case with *H. pylori* LPS, which is not detected by TLR4 but by TLR2 (Lepper et al. 2005).

As the TLR2 agonists Pam_3CSK_4 and FSL-1 also induced IFN-γ production, the putative TLR2 ligand HpaA was analysed with respect to IFN-γ production. HpaA is a *H. pylori* specific lipoprotein found in the membrane of *H. pylori* that previously have been demonstrated to be required for the colonisation of *H. pylori* in mice (Carlsohn et al. 2006). HpaA was able to induce IFN-γ production when combined with IL-12, at levels similar to or exceeding those of *H. pylori* lysate (paper II). In addition, a truncated version of HpaA, lacking the lipid portion of the protein failed to induce IFN-γ production. The IFN-γ production was reduced with more than 97% compared to wild-type HpaA (paper II). This indicates that it is the putative TLR2 binding lipid portion of the protein which is recognised by the immune system. However, stimulation of NK cells with a *H. pylori* strain lacking the HpaA protein did not result in a complete reduction of IFN-γ production (paper II). This indicates that although HpaA may be important for recognition, it is not the only *H. pylori* component recognised by NK cells.

Database searches of the *H. pylori* genome have revealed that in *H. pylori* about 20 membrane lipoproteins or putative membrane lipoproteins are encoded for in the genome, including rlpA, Lpp20 and cag12. These lipoproteins could also be candidate proteins involved in the recognition of *H. pylori* by TLR2 in parallel to HpaA.

To further investigate the potential role of TLR2 in the *H. pylori* induced IFN-γ production, the effects on IFN-γ production were analysed in NK cells treated with an inhibitor of TLR2 activity. The inhibitor reduced the IFN-γ production induced by FSL-1 as expected, but also the IFN-γ production induced by *H. pylori* lysate (paper II). This further strengthens the hypothesis that TLR2 is important for the recognition of *H. pylori* by NK cells. In addition,

the TLR2 inhibitor also reduced the HpaA induced IFN-γ production (paper II), providing further evidence that HpaA is in fact a TLR2 ligand.

3. GASTRIC CANCER

3.1. NK CELLS FROM GASTRIC CANCER PATIENTS HAVE IMPAIRED ACTIVITY

Presence of NK cells in tumours is often a positive finding for the patient, since high NK-cell infiltration and activity is associated with a better prognosis and higher survival rate of gastric cancer patients (Ishigami et al. 2000, Takeuchi et al. 2001). Furthermore, it has been shown that the cytotoxic activity of NK cells is often impaired in patients suffering from a variety of cancers, including gastric cancer (Aparicio-Pages et al. 1991, Kono et al. 2002, Yoon et al. 1998). These findings indicate that NK cells are important in the defence against gastric tumours and that reduced activity of the NK cells may lead to an impaired response to the tumour. To that end, we investigated the activity of NK cells from patients suffering from gastric cancer. The results reveal that NK cells isolated from these patients have a severely impaired ability to produce IFN-γ in response to *H. pylori* lysate in combination with IL-12 and/or IL-2 as compared to asymptomatic *H. pylori* carriers and healthy blood donors (paper III, Figure 9). To test if this suppression is specific to gastric cancer patients, NK cells from patients with pancreatic and colon cancer were analysed as well. NK cells from pancreatic cancer patients had an intermediate IFN-γ production while NK cells from colon cancer patients produced similar levels of IFN-γ as NK cells from gastric cancer patients (paper III). The same results were obtained both from NK cells isolated from the peripheral blood and the gastric mucosa, indicating a systemic rather than a local effect (paper III).

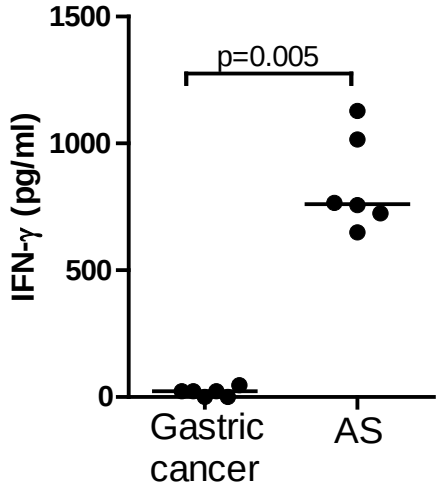

Figure 9: *IFN-γ production from NK cells from gastric cancer patients and asymptomatic H. pylori carriers (AS) after H. pylori + IL-12 stimulation*

Since the samples from the patients were collected at the time of surgery, it could be argued that the observed suppression of IFN-γ production is due to the stress of surgery. However, NK cells isolated from patients not undergoing surgery were as suppressed in their IFN-γ producing ability (paper III).

NK cells from cancer patients were not only suppressed in their IFN-γ producing capacity, but had also a reduced ability to kill the NK-cell sensitive cell-line K562 compared to asymptomatic *H. pylori* carriers (paper III).

3.2. MECHANISMS OF SUPPRESSION

The general suppression of NK-cell activity observed in the cancer patients could be due to presence of suppressive cytokines, such as TGF-β and/or IL-10. Both these cytokines are present in the *H. pylori* infected mucosa as well as in gastric cancer patients (Ellmark et al. 2006). TGF-β and IL-10 are produced by T_{reg}-cells and have the ability to suppress both cytokine production and cytotoxicity (Cerwenka and Lanier 2001, Ghiringhelli et al. 2005).

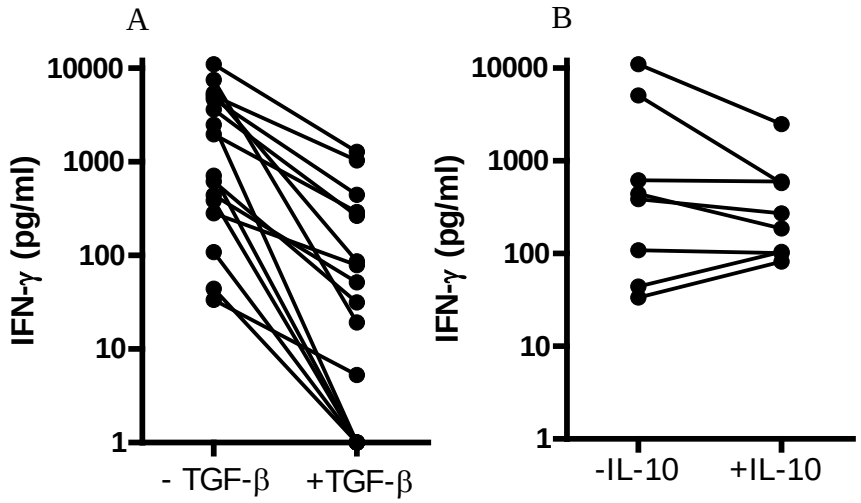

Figure 10: *NK cells stimulated with H. pylori and IL-12, in the absence or presence of (A) TGF-β and (B) IL-10*

To investigate the potential role of the suppressive cytokines on IFN-γ production, NK cells from healthy blood donors were treated with TGF-β or IL-10 and stimulated with bacterial components. Addition of TGF-β efficiently reduced the IFN-γ production, while IL-10 treatment did not have much effect (paper III, Figure 10). The decrease in IFN-γ production after TGF-β treatment was not a transient effect since pre-stimulation with TGF-β prior to stimulation with bacterial components induced as efficient reduction in IFN-γ production as

co-culture of TGF-β with IFN-γ inducing agents (paper III). This demonstrates that TGF-β does not have to be present when the NK cell encounters the bacterial stimuli for suppression to occur.

The impaired IFN-γ production in NK cells from gastric cancer patients may thus be due to presence of TGF-β. To look further into the mechanisms of suppression the mRNA expression of the transcription factors T-bet and GATA-3 were analysed. These transcription factors are considered to have opposing effect on IFN-γ production from T-cells. T-bet is regarded as a Th1-inducing transcription factor promoting IFN-γ production, while GATA-3 is regarded a Th2-inducing transcription factor that inhibits IFN-γ production (Agnello et al. 2003). In fact it has been suggested that the main role of T-bet is to negatively regulate GATA-3 rather than positively regulate IFN-γ gene expression (Usui et al. 2006). Furthermore, GATA-3 expression has previously been shown to be up-regulated in both gastric and pancreatic cancer (Gulbinas et al. 2006, Yang et al. 2010) while T-bet has been demonstrated to be down-regulated in PBMCs from gastric cancer patients (Yang et al. 2010). The expression of these transcription factors was examined in NK cells from gastric cancer patients and revealed a substantial increase in GATA-3 expression in gastric cancer patients as compared to healthy blood donors (paper III). This is in agreement with the impaired IFN-γ production observed in NK cells from gastric cancer patients. The mRNA expression of T-bet would then be expected to be decreased in the cancer patients as compared to the healthy individuals, since GATA-3 and T-bet are considered to have opposing effects. However, this is not the case since T-bet expression was also up-regulated in gastric cancer patients, similar to the situation with GATA-3 expression (paper III). It is not clear whether or not the increased T-bet expression has any consequence for the IFN-γ production, since IFN-γ production in NK cells may be T-bet independent (Way and Wilson 2004).

Since TGF-β was shown to reduce the IFN-γ production, the effects of TGF-β was also investigated on mRNA expression of GATA-3 and T-bet in NK cells from healthy individuals. Treatment with TGF-β induced a similar up regulation of GATA-3 as observed in the gastric cancer patients (paper III), indicating that presence of TGF-β could be a mechanism that contributes to the impaired IFN-γ production. However, T-bet expression was not altered after TGF-β treatment (paper III), which indicates that the increase in T-bet expression observed in the cancer patients may be of no consequence for the IFN-γ production.

Furthermore, T-bet did only moderately increase in NK cells from healthy individuals treated with *H. pylori* lysate and IL-12 (paper III), a treatment that does induce IFN-γ production (Yun et al. 2005) further strengthening the hypothesis that T-bet is not crucial for IFN-γ production in NK cells.

GENERAL DISCUSSION

H. pylori colonise the gastric mucosa and hence this is the most likely place of encounter between NK cells and *H. pylori*. In agreement with this it has been shown that NK cells are present in the gastrointestinal mucosa and comprise up to 15% of the mucosal lymphocytes (Yun et al. 2005) and thus there is a possibility for interaction between the NK cells and the bacterium. *H. pylori* infection is characterised by a Th1-skewed immune response and hence IFN-γ production (Pellicano et al. 2007, Sommer et al. 1998). In fact IFN-γ production has been shown to be associated with protection against *H. pylori* infection in mice (Akhiani et al. 2002). The presence of NK cells in the gastric mucosa and their ability to produce IFN-γ when stimulated by *H. pylori* components indicates that this cell type is important in the immune response to *H. pylori* infection. Furthermore, IFN-γ has been shown to be involved in tumour suppression by prevention of tumour growth and metastasis formation (Ikeda et al. 2002, Kaplan et al. 1998, Shankaran et al. 2001) and high infiltration of NK cells in cancer tissue appears to increase the survival rate of gastric cancer patients (Ishigami et al. 2000, Takeuchi et al. 2001). Therefore, we believe that the activation of NK cells by bacterial components might play a significant role in the suppression of tumour development, and that a diminished NK-cell activity may consequently increase the risk of acquiring gastric cancer.

The role of NK cells in bacterial infections in general and *H. pylori* infection in particular is a poorly explored field. However, the work in this thesis has identified a subset of NK cells; $CD8^-$ NK cells that may be most important in the defence against this bacterium. Our data shows that $CD8^-$ NK cells dominate in the gastric, duodenal and colonic mucosa, but it remains to be investigated whether this subset is specific for the gastrointestinal mucosa or whether it is homing to/accumulating in peripheral tissues in general.

The $CD8^+$ and $CD8^-$ NK-cell subsets has previously been described (Jonges et al. 2001) and has been attributed with different cytotoxic abilities (Addison et al. 2005) but this is to our knowledge the first attempt to characterise the cytokine producing ability of these subsets. The difference in IFN-γ production induced by bacterial components but not by cytokine stimulation indicate that the $CD8^-$ NK cells are a unique NK-cell subset that are especially adapted to respond to bacterial stimuli. Further examination will be required to determine if this is true for all bacteria or only some. The findings that the $CD8^+$ NK cells and the $CD8^-$ NK cells produce IFN-γ at similar levels in response to cytokines regardless of CD16

expression and to some extent also regardless of CD56 expression somewhat challenge the common idea that $CD56^{bright}$ and $CD16^-$ NK cells are more prone to produce cytokines. Further studies on these subsets are required, however it is plausible that the NK-cell function may not be determined in full by the phenotypic subset but it may be the surrounding environment that determine if the NK cell is able to produce cytokines or be cytotoxic.

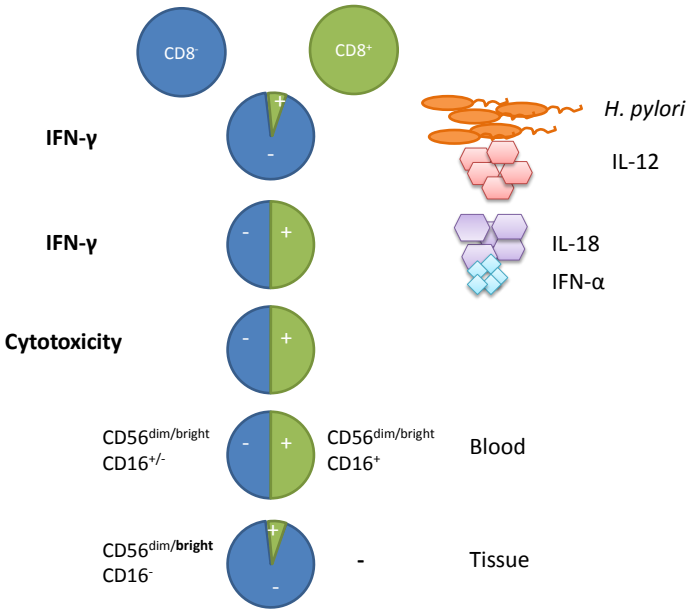

Figure 11: *Overview of $CD8^+$ and $CD8^-$ NK cells*

The mechanisms responsible for the differential response remains to be examined, however the most appealing theory is that the $CD8^+$ and $CD8^-$ NK-cell subsets differ in TLR expression either on the mRNA or protein level. This would explain why the $CD8^+$ NK cells are able to induce IFN-γ production in response to cytokines but not to bacteria. It is of course also possible that the subsets differ in the signalling down-stream to the TLRs, such MyD88 signal transduction and/or p38 MAPK activation. In addition there could be differences in the expression and activation of important transcription factors such as NFκB and/or GATA-3 which needs to be investigated in future studies.

This thesis has also provided knowledge concerning the mechanisms of recognition of *H. pylori* by NK cells. We have shown that NK cells can recognise *H. pylori* directly without the presence of antigen presenting cells (APC) such as DCs; however the presence of APC-derived IL-12 is required for IFN-γ production to occur. *H. pylori* is recognised by NK cells

via TLR2 in combination with either TLR1 or TLR6 and the signalling is dependent on both the MyD88 adaptor protein and the p38 MAPK. This is in agreement with previous results, identifying TLR2 as a recognition receptor for *H. pylori* by epithelial cells (Mandell et al. 2004, Smith et al. 2003). This information is of course of interest not only for understanding the immune response to *H. pylori* but also provides additional information of the function of human NK cells. The consequences of the ability of NK cells to recognise *H. pylori* remains to be investigated *in vivo*, however it may be possible that the ability of NK cells to produce IFN-γ could determine the outcome of the infection. The initial IFN-γ production in response to the bacteria may be positive in order to control the infection, but the immune system most often fails to clear the infection resulting in chronic inflammation. However, one might speculate that the sustained IFN-γ production could be one reason why only a minority of the *H. pylori* infected individuals develop gastric cancer since IFN-γ is involved in suppression of tumour development.

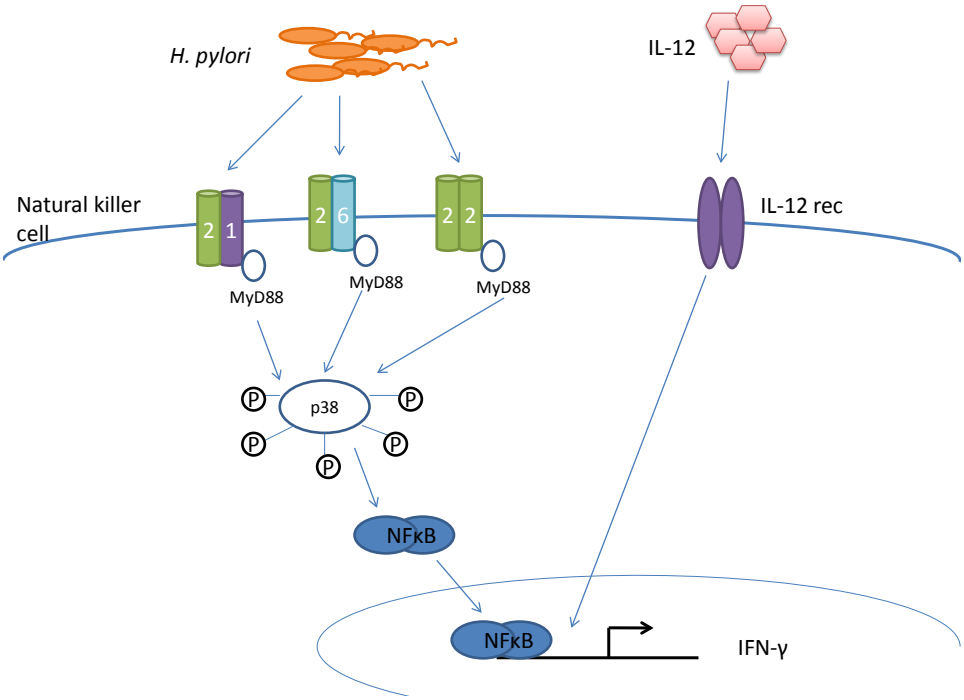

***Figure 12**: Proposed mechanism for H. pylori induced IFN-γ production in NK cells*

We have demonstrated an impaired ability of NK cells from gastric cancer patients to respond to bacterial components and propose that this diminished activity is due to the presence of the

suppressive cytokine TGF-β. TGF-β is produced by for example T_{reg}- cells, which have been demonstrated to be recruited to the site of tumour in gastric cancer (Enarsson et al. 2006) and to reduce IFN-γ production in a TGF-β-dependent manner (Ghiringhelli et al. 2005).

We also suggest that the reduced IFN-γ production is mediated by TGF-β via increasing the expression of the Th2-associated transcription factor GATA-3, rather than reducing the expression of the Th1-associated transcription factor T-bet as could be expected based on data obtained from T cells (Agnello et al. 2003). Since we only have studied the mRNA expression of the transcription factors, it still remains to be confirmed whether or not GATA-3 is being activated and translocated into the nucleus in NK cells from gastric cancer patients.

We believe that this thesis provides knowledge that are important for understanding the immune response in the gastric mucosa to bacterial infections and the long-term consequences of chronic infections, such as development of gastric cancer. We also believe that our findings might aid the development of strategies for treatment of gastric cancer such as modulation of NK-cell activity and cytokine production by for example IL-2 and IL-12 treatment. However, the most likely application would be to use the impaired NK-cell activity in gastric cancer for the diagnosis of gastric cancer rather than as a treatment. Thus it may be possible to use the observations of elevated GATA-3 mRNA expression in combination with the decreased IFN-γ mRNA expression in NK cells as a biomarker for the diagnosis of gastric cancer. This could result in earlier diagnosis and hence a better prognosis for the gastric cancer patients.

SWEDISH SUMMARY – POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Bakterien *Helicobacter pylori* (*H. pylori)* infekterar slemhinnan i magsäcken och orsakar en av de vanligaste infektionerna i världen. *H. pylori* ger i princip alltid upphov till en kronisk inflammation i slemhinnan, men majoriteten av de som drabbas har inga symptom. Däremot får ca en sjättedel av de som infekteras magsår/tolvfingertarmsår och några få procent magsäckscancer. Mekanismerna bakom utvecklingen av magsäckscancer hos vissa personer med *H. pylori* infektion är ännu oklara, men beror troligtvis på en rad egenskaper hos bakterien i kombination med miljöfaktorer samt genetiska faktorer hos den drabbade. Symptomen är diffusa och därför upptäcks magsäckscancer ofta i ett sent skede av sjukdomen och har därför en mycket dålig prognos.

För att förstå hur magsäckscancer utvecklas från en kronisk inflammation orsakad av *H. pylori* är det av intresse att studera de immunceller som är inblandade i immunförsvaret mot *H. pylori*. I denna avhandling har vi undersökt hur en viss typ av immunceller som kallas Natural killer (NK) celler (”Naturliga mördarceller”) reagerar på *H. pylori*. NK-celler är en viktig del av immunförvaret och är inblandade både i immunförsvaret mot bakterie- och virusinfektioner samt mot tumörer. NK-celler har dels förmågan att vara cytotoxiska, dvs. döda infekterade celler eller tumörceller och dels kan de producera s.k. cytokiner, som är immunförsvarets signalämnen. En viktig cytokin som bildas av NK-celler är interferon gamma (IFN-γ), som aktiverar och rekryterar andra immunceller till inflammerad eller skadad vävnad. IFN-γ har även en roll i immunförsvaret mot tumörer genom att förhindra tumörtillväxt och spridning.

NK-celler finns bl.a. i magslemhinnan där även *H. pylori* finns och NK-cellernas förmåga att producera IFN-γ som svar på *H. pylori* gör att det finns anledning att tro att NK-cellerna kan ha en viktig roll i immunförsvaret mot *H. pylori.*

Rollen för NK-celler i bakterieinfektioner i allmänhet och *H. pylori* infektion i synnerhet är ett dåligt utforskat område. Men NK-celler finns bl.a. i magslemhinnan där även *H. pylori* finns och NK-cellernas förmåga att producera IFN-γ som svar på *H. pylori* gör att det finns anledning att tro att NK-cellerna kan ha en viktig roll i immunförsvaret mot *H. pylori.*

Vi har identifierat en grupp NK-celler som kan vara viktiga för immunförsvaret mot *H. pylori* och visat att de NK-celler som definieras av avsaknad av CD8 molekylen ($CD8^-$ NK-celler) dominerar i magtarmslemhinnan. Dessutom har vi visat att $CD8^-$ NK-celler är mångfalt bättre

på att producera IFN-γ efter att ha exponerats för bakterier jämfört med de NK-celler som har CD8 molekylen ($CD8^+$ NK-celler).

Vi har även undersökt mekanismerna bakom hur NK-cellerna känner igen *H. pylori*. Vi har visat att det sker via s.k. Toll-lika receptorer (TLR), framförallt TLR2 i kombination med antingen TLR1 eller TLR6. Denna information är intressant inte bara för förståelsen av immunförsvaret mot *H. pylori* infektion utan ger också information om NK-cellers funktion vid bakterieinfektioner.

Ett högt antal NK-celler i tumörvävnad har tidigare visats vara sammankopplat med en bättre prognos och ökad överlevnad för magsäckscancerpatienter. Därför tror vi att aktivering av NK-celler av bakterier kan spela en viktig roll i förhindrandet av tumörutveckling och att en försvagad NK-cellsaktivitet kan öka risken för att utveckla cancer.

Vi har visat att NK-celler från magsäckscancerpatienter har en kraftigt försämrad förmåga att svara på bakterier och vi föreslår att denna försämrade förmåga beror på förekomsten av cytokinen TGF-β. TGF-β produceras bl.a. av regulatoriska T-celler (T_{reg}-celler), som ansamlas i magsäckscancertumörer och har förmågan att förhindra IFN-γ produktion med hjälp av TGF-β. Vi föreslår även att den reducerade IFN-γ produktionen hos NK-cellerna från magsäckcancerpatienter beror på att TGF-β ökar genuttrycket av transkriptionsfaktorn GATA-3 som i sin tur hämmar genuttrycket av IFN-γ och därmed IFN-γ produktionen.

Vi tror att resultaten i denna avhandling är viktiga för förståelsen av immunförsvaret mot bakteriella infektioner i magslemhinnan och långtidskonskvenser av kroniska infektioner så som utveckling av magsäckscancer. Vår förhoppning är att informationen kan användas för att ta fram nya behandlings- eller diagnosmetoder för magsäckscancer. En möjlighet skulle vara att använda det ökade genuttrycket av GATA-3 samt det låga genuttrycket av IFN-γ som en biomarkör för diagnos av magsäckscancer. Detta skulle kunna betyda en möjlighet att diagnosticera magsäckscancer på ett tidigt stadium vilket därmed skulle betyda en bättre prognos för magsäckscancerpatienter.

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