Elucidating the role of TLR2 in STAT3-mediated gastric tumourigenesis

A thesis submitted for the degree of
Doctor of Philosophy

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September 2012

Contents

ABSTRACT

Gastric cancer (GC) is the second most lethal form of cancer world-wide, which represents a subset of inflammation-associated carcinogenesis. While it is accepted that deregulated interactions between gastric microbes (i.e. *Helicobacter pylori)* and the host innate immune system are likely to be involved in the pathogenesis of gastric inflammation (gastritis) and the development GC, the identity of oncogenic inflammatory regulators in the host gastric mucosa remains obscure. On this note, uncontrolled activation of cytokine signalling pathways, especially the pro-inflammatory and oncogenic transcription factor signal transducer and activator of transcription (STAT) 3, is implicated in various inflammation-associated cancers, including up to 50% of human GC cases. However, the downstream molecular consequences of aberrant STAT3 activation in promoting gastritis and subsequently gastric tumorigenesis are ill-defined.

This thesis addresses the role of toll-like receptors (TLRs) which are key components of the innate immune system primarily known to trigger an inflammatory response upon pathogen detection during gastric tumourigenesis. Our mouse model for GC, was generated by a specific "knock-in" phenylalanine substitution at tyrosine 757 in the interleukin (IL)-6 cytokine family co-receptor gp130 (*gp130*$^{F/F}$), which abolishes the negative feedback mechanism, thus leading to gp130-STAT3 hyper-activation. These mice spontaneously developed gastric hyperplasia and adenomatous lesions that resembled histopathological features similar to human intestinal-type GC. Notably, aberrant STAT3 activation in the gastric compartment of these mice directly caused a significant increase in the expression of TLR2, a receptor that is responsible for recognising a broad range of

microbial products. In addition, genetic deletion of TLR2 in *gp130*$^{F/F}$ mice severely reduced the gastric tumour mass by approximately 50%. However, unexpectedly, the level of gastritis was comparable to *gp130*$^{F/F}$ mice. Interestingly, immunohistochemical analyses revealed that *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice had increased TUNEL-positive apoptotic cells and reduced PCNA-positive cells in the gastric mucosal epithelium, therefore implicating a role for TLR2 in gastric epithelial cell proliferation and survival. Consistent with our mouse data, we identified that activating TLR2, using synthetic lipopeptides, in human gastric epithelial cell lines promoted gastric epithelial cell proliferation. Collectively, our data depicts an unexpected role for TLR2 in gastric tumourigenesis, whereby increased STAT3 activation resulted in over-expression of TLR2 to promote gastric tumour cell growth.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge and belief, contains no material published or written by another person, except where due reference is made in the text. Where the work in this thesis is part of joint research, the relative contributions of the respective persons are listed in the text.

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ACKNOWLEDGEMENTS

I am going to keep this short and sweet because to everyone that has helped throughout my PhD I should have already thanked you in person, if not I give you permission to slap me on the head and remind me to do so. However, I would like to express my appreciation and gratitude to my supervisor/mentor, A/Prof. Brendan Jenkins, for his continuous support (especially during the submission of our Cancer Cell paper) as I felt like I was banging my head against a brickwall, and the tremendous guidance in improving my writing skills.

Special thanks to both Meri Najdovska and Dr. Saleela Ruwanpura-Pratheep for all the help they have provided me in the lab and maintaining my sanity during the last 4 years, I don't think I could have made it without you guys. Additionally, I would also like to thank the other members of the Jenkins group (Catherine Kennedy, Louise McLeod, Alistair Miller, Gavin Brooks and Alison Browning) for all the ample supply of help and fun, especially in the lab!

Next, I would also like to thank all our collaborators: A/Prof Patrick Tan, Prof Massanobu Oshima and Prithi Bhatal for their input and knowledge as they have greatly filled in the gaps to this story.

Finally, a special thanks to my family and friends (you know who you are) who have helped and supported me during the last 4 years of this long and emotional roller-coaster ride that has contributed to my Permanent Head Damage (i.e. PhD). Thanks all ☺

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ABBREVIATIONS

AD	Activation domain
AOM	Azoxymethane
AP-1	Activator protein-1
ASC	Apoptosis-associated speck-like protein containing a CARD
Bax	Bcl-associated X
Bcl-2	B-Cell lymphoma 2
BIR	Baculovirus IAP repeats
Bcl-x_L	B cell lymphoma extra large
CAC	Colitis-associated colon cancer
***cag*PAI**	cag pathogenicity island
CagA	Cytotoxin-associated gene A
CARD	Caspase-activation and recruitment domain
C/EBP	CCAAT/enhancer binding protein
CIITA	Class II transactivator
CNTF	Ciliary neurotrophic factor
COX-2	Cyclooxygenase 2
CREB	CAMP response element-binding protein
CSK	C-terminal Src kinase
CT	Cardiotrophin
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DD	Death domain
DMEM	Dulbecco's modified eagle medium
ERK	Extracellular-signal regulated kinase
FCS	Fetal calf serum
FIIND	Function to be identified domain
GC	Gastric cancer
GF	Germ-free
GFP	Green fluorescent protein
gp130	Glycoprotein 130
hBD	Human beta-defensin

HBSS	Hank’s buffered salt solution
H&E	Hemotoxylin and eosin
HMGB1	High mobility group box 1
HMVEC	Human microvascular endothelial cell
HUVEC	Human endothelial cell
i.p.	Intraperitoneal
i.v.	Intravenous
IBD	Inflammatory bowel disease
ID	Inter-domain
IκB	Inhibitor of kappa B
IL	Interleukin
IRF	Interferon-regulated factor
ISG	Interferon stimulated gene
JAK	Janus kinase
LB	Luria broth
LDL	Low density lipoprotein
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAL	MyD88 adaptor like
MALT	Mucosal-associated lymphoid tissue lymphoma
MAPK	Mitogen activated protein kinase
mDAP	Meso-diaminopimelic acid
MDP	Muramyl dipeptide
MDSC	Myeloid-derived suppressor cell
MIP-2	Macrophage inflammatory protein 2
MMP	Matrix metalloproteinase
mPGES	Microsomal prostaglandin E synthase
MyD88	Myeloid differentiation 88
NAD	NACTH-associated domain
NCGC	Non-cardia GC
NF-κB	Nuclear factor kappa B
NLR	Nod-like receptor

NOD	Nucleotide binding and oligomerisation domain
OD	Optical density
OSM	Oncostatin M
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PG	Peptidoglycan
pGEC	primary gastric epithelial cell
PGE2	Prostaglandin E2
PIAS	Protein inhibitor of activated STAT3
PIP2	Phosphatidylinositol (4,5) biphosphate
PRR	Pathogen recognition receptor
PUD	Peptic ulcer disease
PYD	Pyrin domain
RE	Response element
RHIM	RIP homotypic interaction motif
RICK	Receptor interacting protein serine/threonine kinase 2
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
SAA	Serum amyloid A
SFK	Src family of tyrosine kinase
SH2	Src homology 2
SHP2	Tyrosine phosphatase 2
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signalling 3
STAT3	Signal transducer and activation of transcription 3
T4SS	Type 4 secretion system
T6BM	TRAF6 binding motif
TAD	Transactivation domain
TAM	Tumour-associated macrophage
TGF	Transforming growth factor
TIR	Toll-IL-1 receptor
TIRAP	Toll-IL-1 receptor domain containing adaptor protein

TLR	Toll like receptor
TMB	3, 3', 5, 5' Tetramethylbenzidine
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAM	Trif-related adaptor molecule
TRIF	Toll-IL-1 receptor domain containing adaptor inducing IFN-β
TRAF6	Tumour necrosis factor receptor-associated factor
VacA	Vacuolating toxin A
VEGF	Vascular endothelial growth factor

LIST OF CONFERENCE PRESENTATIONS

Part of the work in this thesis was presented as oral presentations at the following conferences:

2012: JAK/STAT meeting, Clayton, Australia

Elucidating the role of STAT3 in gastric tumourigenesis. *H. Tye (presenting author)*

2012: Conference meeting, Cancer Immunotherapeutics and Tumour Immunology, City of Hope, California, USA

Defining the role of TLR2 in gp130/STAT3-mediated gastric tumourigenesis. *H. Tye (presenting author)*

2011: MIMR postgraduate symposium Clayton, Australia

Elucidating the role of TLR2 in gp130/STAT3-mediated gastric tumourigenesis. *H. Tye (presenting author)*

2011: Three minute thesis competition, Clayton, Australia

The promiscuous role of TLR2 in gastric tumourigenesis. *H. Tye (presenting author)*

2011: Walter and Eliza Hall Institute (WEHI) student symposium, Melbourne, Australia

The unexpected role of TLR2 in gastric tumourigenesis. *H. Tye (presenting author)*

2011: Victorian Infection and Immunity Network (VIIN) student symposium, Melbourne, Australia

Elucidating the role of TLR2 in gp130/STAT3-mediated gastric tumourigenesis. *H. Tye (presenting author)*

2010: Victorian Infection and Immunity Network (VIIN) student symposium, Melbourne, Australia

Elucidating the role of TLR2 in gp130/STAT3-mediated gastric tumourigenesis. *H. Tye (presenting author)*

2010: Australian society for medical research (ASMR) student symposium, Melbourne, Australia.

Elucidating the role of pathogen recognition receptors in gastric tumourigenesis. *H. Tye (presenting author)*

Part of the work in this thesis was presented as posters at the following conferences:

2012: Southern health research week, Clayton, Australia.

Elucidating the role of TLR2 in the progression of gastric tumourigenesis. *H. Tye (presenting author)*

2012: Innate Immunity: Sensing the Microbes and Damage Signals Keystone Symposia, Colorado, USA.

Elucidating the role of TLR2 in the progression of gastric tumourigenesis. *H. Tye (presenting author)*

2011: ASMR student symposium, Melbourne, Australia.

Elucidating the role of pathogen recognition receptors in gastric tumourigenesis. *H. Tye (presenting author)*

2011: Southern health research week, Clayton, Australia.

Elucidating the role of pathogen recognition molecules in the progression of gastric tumourigenesis. *H. Tye (presenting author)*

2011: Lorne 1st infection and immunity conference, Lorne, Australia

Elucidating the role of TLR2 in gp130/STAT3-mediated gastric tumourigenesis. *H. Tye (presenting author)*

2010: Lorne 22nd cancer conference, Lorne, Australia

Elucidating the role of pathogen recognition molecules in the progression of gastric tumourigenesis. *H. Tye (presenting author)*

2010: Southern health research week, Clayton, Australia.

Elucidating the role of pathogen recognition molecules in the progression of gastric tumourigenesis. *H. Tye (presenting author)*

2009: VIIN student symposium, Melbourne, Australia.

Gastric inflammatory responses in the regulation of STAT signaling. *H. Tye (presenting author)*

2009: Lorne 21st Cancer conference, Lorne, Australia.

The role of pathogen recognition receptors in the development of gastric tumours in gp130F/F mice. *H. Tye (presenting author)*

CHAPTER 1

LITERATURE REVIEW

1.1 Background

Gastric cancer (GC) is the second leading cause of mortality worldwide with adenocarcinoma (90% of cases) being the most common type of GC (Crew and Neugut, 2006). Gastric adenocarcinoma consists of 2 histopathological types; differentiated intestinal metaplasia which represents 95% of cases, and the remaining 5% of cases are undifferentiated diffused type adenocarcinoma (Crew and Neugut, 2006). Intestinal type is commonly found in males of older age group, and in high risk areas which include East Asia, Eastern Europe, Central and South America (Crew and Neugut, 2006). However, diffuse-type has equal male-to-female ratio, is commonly seen in the younger population and has no geographical distribution bias (Crew and Neugut, 2006). Diagnosis of GC is often at an advance stage with limited treatment options, such as chemotherapy and surgical resection of the stomach, which are often associated with poor prognosis of this disease resulting in a 5-year survival rate of less than 20% of patients (Crew and Neugut, 2006, Meyer and Wilke, 2011). Thus, there is an urgent need for a better understanding of factors that contribute to the initiation and progression of GC to facilitate new advances in the early detection and treatment of disease.

The development of GC is a multi-factorial process with both environmental (e.g. diet and gastric pathogen infection) and genetic (e.g. polymorphisms in pro-inflammatory mediators) factors being implicated (Milne *et al*., 2009). In humans, elevated mucosal

immune responses following colonisation by the gastric pathogen, *Helicobacter pylori*, results in chronic gastric inflammation (gastritis), and highly correlate with the risk of developing gastric adenocarcinoma (Aggarwal and Gehlot, 2009). *H. pylori* infection is known to trigger a series of events, according to Correa's model of gastric tumourigenesis, comprising of chronic gastritis, gastric atrophy, intestinal metaplasia, dysplasia, and finally, gastric adenocarcinoma (Crew and Neugut, 2006). However, it must be noted that approximately 25% of human GC cases develop in the absence of *H. pylori*, and the overall survival of *H. pylori*-negative GC patients is several-fold less than their *H. pylori* counterparts (Meimarakis *et al*., 2006). Therefore collectively, it suggests the presence of other ill-defined genetic factors which predispose certain individuals to GC independent of *H. pylori*. With regard to this, the transcription factor signal transducer and activation of transcription 3 (STAT3), has been shown to be up-regulated in several cancers including up to 50% of human GC (Yu *et al*., 2009). STAT3 is a potent regulator of genes involved in cell cycle progression, survival, angiogenesis and pro-inflammatory mediators, however, its role in tumourigenesis remains unclear (Yu *et al*., 2009).

1.2 Inflammation-associated carcinogenesis

Pathogen-induced abnormal immune responses have been associated as mechanisms to some inflammation-associated carcinogenesis. Colitis associated colon cancer (CAC) is a classic example of inflammation-associated carcinogenesis as studies have indicated that inflammatory bowel disease (IBD) is strongly linked to CAC (Uronis *et al*., 2009). Uronis *et al*., (2009*)* proposed that the intestinal microbiota triggers the host immune response, whereby uncontrolled immune signalling can occur as a consequence of a defect in immunosuppressive mechanisms. A mouse colitis model that lacks interleukin (IL)-10 (*Il-10*$^{-/-}$), a potent anti-inflammatory cytokine, was shown to only develop CAC in the

presence of intestinal microflora but not in germ free conditions when treated with azoxymethane (AOM) a carcinogen known to induce CAC in mice (Uronis *et al*., 2009). Furthermore, the importance of intestinal microflora in CAC was demonstrated using a STAT3 conditional knockout mouse model, whereby mice receiving antibiotic treatment displayed a significant reduction in inflammation and the formation of colonic tumours compared to sham animals (Deng *et al*., 2010b). More importantly, Uronis *et al.*, (2009) demonstrated that Toll-like receptor (TLR) signalling plays an important role in AOM-induced CAC, as genetic deletion of myeloid differentiation 88 (MyD88), which is a common adaptor molecule for most TLR signalling pathways, in *Il-10*$^{-/-}$ mice fail to develop neoplastic lesions and/or CAC. Given that abnormal immune responses have been linked to inflammation-associated carcinogenesis; which also include chronic viral hepatitis to liver cancer and, more importantly, *H. pylori* infection to gastric cancer, investigators have been studying the role of pathogen recognition receptors (PRRs) in GC (Fukata and Abreu, 2008, Lin and Karin, 2007).

1.3 Helicobacter pylori-mediated gastric cancer

H. pylori is a gram-negative pathogen which is considered a type 1 carcinogen as it is known to cause gastritis, gastric adenocarcinoma, peptic ulcer disease (PUD) and gastric mucosal-associated lymphoid tissue lymphoma (MALT) (Dorer *et al*., 2009). Studies have shown that *H. pylori* influences the progression of intestinal metaplasia to intestinal type gastric adenocarcinoma, however this process can also occur in the absence of *H. pylori* (Pimentel-Nunes *et al*., 2011).

Chronic inflammation induced by *H. pylori* infection in the gastric mucosa often results in the recruitment of macrophages and neutrophils, which produce a milieu of cytokines,

chemokines and subsequently the production of reactive oxygen species (ROS) by the gastric mucosa at the site of infection (Kabir, 2009). Moreover, ROS insult can cause oxidative stress leading to DNA damage and transformation within the gastric mucosa of the host to promote gastric carcinogenesis (Kabir, 2009).

H. pylori strains harbouring the *cag* pathogenicity island (*cag*PAI), which encodes the type four secretion system (T4SS), aids in the translocation of effector molecules, such as cytotoxin-associated gene A (CagA) and peptidoglycan (PG), into the cytoplasm of the host cells to cause disease (Kusters *et al*., 2006). The virulence factors, vacuolating toxin (VacA), urease, adhesins (*babA2)* and outer-membrane proteins also contribute to the pathogenesis of *H. pylori,* which are encoded outside the *cag*PAI (Kusters *et al*., 2006, Yamaoka *et al*., 2002). The acidic environment found in the stomach prevents the colonisation and multiplication of bacteria, however *H. pylori* is able to overcome this through the production of urease, which is an enzyme that breaks down urea into ammonia and carbon dioxide (Algood and Cover, 2006, Kusters *et al*., 2006). Ammonia is capable of neutralising the microenvironment by increasing the pH level, thus allowing *H. pylori* to colonise. Another well characterised virulence factor is VacA which forms small pores in the membrane of epithelial cells to induce apoptosis (Kusters *et al*., 2006). Studies show a strong correlation between *H. pylori* strains harbouring specific VacA genotypes and the development of peptic ulcer disease and GC (Atherton *et al*., 1995).

1.3.1 CagA-mediated gastric disease

The translocation of CagA into host cells, mediated by the T4SS, enables CagA to interact with a number of host proteins containing a Src homology 2 (SH2) domain (Higashi *et al.,*

2005). These proteins can be tyrosine kinases or phosphatases, such as C-terminal Src kinase (CSK) or tyrosine phosphatase 2 (SHP-2), respectively, that are involved in cytoskeletal rearrangement, cell proliferation, survival and motility (Wen and Moss, 2009). Studies have shown that individuals colonised with CagA-positive compared to CagA-negative strains have increased risk of developing GC (Kabir, 2009). In this regard, CagA-positive *H. pylori* strains have been associated with increased levels of cyclooxygenase-2 (COX-2) expression in human GC (Thiel *et al*., 2011). COX-2 is an enzyme that facilitates the production of prostaglandin E2 (PGE2), which is known to regulate inflammation by promoting vasodilation and the activation of neutrophils, macrophages, mast cells and dendritic cells (Kalinski, 2012). Interestingly, a transgenic mouse model that over-expressed COX-2 and microsomal prostaglandin E synthase (mPGES)-1 spontaneously developed hyperplastic gastric tumours with high levels of macrophage infiltration, therefore suggesting that PGE2 is involved in macrophage recruitment in gastric disease (Oshima *et al*., 2004). More importantly, a study revealed that *H. pylori*-infected wild type mice that were treated with a COX-2 inhibitor significantly suppressed gastric hyperplasia compared to *H. pylori*-infected wild-type mice that did not receive the inhibitor (Xiao *et al*., 2001). Furthermore, eradication of *H. pylori* in patients with *H. pylori*-associated gastritis and adenocarcinoma resulted in a significant decrease in COX-2 expression, thus indicating a strong correlation between *H. pylori* infection and the production of COX-2 (Sung *et al*., 2000).

In addition, it has also been reported that CagA-positive *H. pylori* strains containing a functional T4SS can activate STAT3 (Bronte-Tinkew *et al*., 2009). More recently, Lee *et al.*, (2010) demonstrated that unphosphorylated CagA is responsible for *H. pylori*-induced STAT3 activation resulting in nuclear translocation and transcription of STAT3 target

genes, such as *c-myc* which is known to play an important role in cell cycle progression. Although CagA is not known for its potent ability to induce a pro-inflammatory response in the host cell, a study has shown that CagA can interact with NF-κB to induce the production of a chemokine, IL-8, which is responsible for the influx of neutrophils thereby contributing to gastritis in the host (Brandt *et al*., 2005). These authors analysed the sequence of CagA and showed that the number of Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, which are tyrosine phosphorylated by the Src family of tyrosine kinases (SFK), and single amino acid mutations or deletions upstream of these motifs, distinguished the high IL-8 and low IL-8 inducers. There has been contradicting evidence on the role that CagA has on pro-inflammatory cytokine production. Nevertheless, Nozawa *et al*. (2002) demonstrated that isogenic *H. pylori* CagA mutants were still able to produce similar levels of IL-8 to that of the wild-type *H. pylori* strain, thus strongly suggesting that CagA has little effect on the induction of IL-8 and that another mechanism is warranted.

1.4 Pathogen recognition receptors (PRRs)

PRRs are our first line of defence during an infection and injury (Li *et al*., 2010). Two major classes of PRRs, are TLRs and nucleotide binding and oligomerisation domain (Nod)-like receptors (NLRs), which represent innate immune sensors that are either expressed on the cell surface, within endosomes or in the cytoplasm of host cells.

1.4.1 Toll-like receptors

TLRs were first discovered in the drosophila fly, where they were shown to play an important role in development and host defense (O'Neill, 2004). The term Toll came from the German word for "weird" because Nusslein-Volhard noticed that flies lacking TLRs developed in an unusual way (O'Neill, 2004). Interestingly, it was observed that flies

lacking Toll would succumb to fungal infections, thus suggesting their role in the host immune response. In 1997 the first human Toll was discovered, and in the following year it was demonstrated that TLR4 plays a role in pathogen sensing as mice that were resistant to lipopolysaccharide (LPS) had mutations within the *TLR4* gene (Medzhitov *et al*., 1997, Poltorak *et al*., 1998).

To date, 13 TLRs have been identified in mammals (Brikos and O'Neill, 2008). TLRs contain leucine rich repeats (LRRs) that are responsible for the recognition of pathogen-associated molecular patterns (PAMPs), a transmembrane domain and a cytosolic Toll-IL-1 receptor (TIR) domain, which mediates the activation of downstream signalling events (Kawai and Akira, 2011), as shown in Figure 1.1. TLRs recognise specific PAMPs from bacteria, viruses, fungi and parasites and these include lipopeptides (TLR1, TLR2, TLR6), double stranded RNA (TLR3), LPS (TLR4), flagellin (TLR5), single stranded RNA (TLR7 and TLR8), unmethylated CpG-DNA (TLR9), profilin-like molecule (TLR11, whereby the human homologue is inactive), which are listed in Table 1.1. Unlike human TLR8, the mouse TLR8 is unable to initiate a signalling response. In addition, although the ligands for TLR10, TLR12 and TLR13 have not yet been identified, only humans express TLR10, whilst the latter two are expressed in mice (Brikos and O'Neill, 2008).

In the stomach, TLR2, TLR4, TLR5 and TLR9 have been shown to be expressed on gastric epithelial cells (Fukata and Abreu, 2008). Of all the members of the TLR family, TLR2 can recognise a broad range of microbial products as it has the ability to form TLR1/2 or TLR2/6 heterodimers to recognise triacyl or diacyl lipopeptides, respectively (Medzhitov, 2001) (Table 1.1).

The recognition of PAMPs by TLRs results in the activation of several signalling pathways which are necessary for the induction of an inflammatory response. There are two arms to TLR signalling, the MyD88 dependent (Figure 1.1) and independent (Figure 1.2) pathways (Takeda and Akira, 2004). In the MyD88-dependent TLR signalling pathway, activation of TLRs recruit TIR-domain-containing adaptor proteins such as MyD88, Toll-IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF) (utilised by TLR3 and TLR4 signalling), Trif-related adaptor molecule (TRAM) (utilised by TLR4 signalling) and Toll-IL-1 receptor domain-containing adaptor protein (TIRAP)/ MyD88 adaptor-like (Mal), whereby MyD88 is a common adaptor protein for all TLR signalling except for TLR3 (Kawai and Akira, 2011) (Figure 1.3). MyD88 recruits IL-1 receptor associated kinase 1 (IRAK1) and IRAK4 through the interaction of their death domains, which subsequently recruits tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) to initiate the activation of nuclear factor (NF)-κB and mitogen activated protein kinase (MAPK) signalling pathways to induce an inflammatory response through the transcriptional inducer of cytokine and chemokine genes (Kawai and Akira, 2011). However, in the MyD88-independent signalling pathway, which is common to TLR3 and TLR4, the recruitment of TRIF results in the activation NF-κB and interferon-regulated factor 3 (IRF3), which is required for the transcription of inflammatory mediators and interferon-regulated genes (ISGs) to initiate an anti-viral response, respectively (Takeda and Akira 2004) (Figure 1.2). With regard to TLR2 signalling, it is worthy to note that although both TLR2/6 and TLR2/1 heterodimers require MyD88, Mal is not required for TLR2/1 signalling, since mouse macrophages isolated from *Mal*$^{-/-}$ mice displayed equivalent protein levels of TNF-α in response to Pam_3CSK4 (TLR2/1 agonist) but were significantly decreased in mouse macrophages isolated from *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$ mice (Santos-Sierra *et al.*, 2009).

1.4.2 Other non-proinflammatory roles for Toll-like receptors

TLRs have been implicated in wound healing by promoting angiogenesis, whereby a study revealed that *Myd88*$^{-/-}$ mice displayed a delayed response in wound healing, which was determined by the formation of new blood vessels identified by immunohistochemical staining with CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1) (Macedo *et al*., 2007). Furthermore, *Myd88*$^{-/-}$ mice expressed significantly lower levels of VEGF, a pro-angiogenic factor produced by macrophages, in their wounds compared to *Myd88*$^{+/+}$ mice. In addition, a study demonstrated that LPS was able to specifically induce angiogenesis in a TRAF6 dependent manner, as endothelial cells that expressed the dominant-negative TRAF6 construct were unable to induce endothelial sprouting in the presence of LPS (Pollet *et al*., 2003). Interestingly, in a recent study, it showed that the activation of TLR2 not only promoted angiogenesis, but also induced endothelial cell invasion and migration when human microvascular endothelial cells (HMVEC) were cultured in the presence of Pam3CSK4 (Saber *et al*., 2011).

Furthermore, there is mounting evidence to suggest that TLR2 plays an important role in cell proliferation and survival as a study revealed that *Listeria monocytogenes* accelerated the growth of tumour cell proliferation via TLR2 and not TLR4 signalling in hepatocarcinoma H22 cell lines (Huang *et al*., 2007). Similarly, another group reported that *Staphylococcus aureus* and various TLR2 agonists were able to promote epithelial wound repair, cell proliferation and survival (Shaykhiev *et al*., 2008). With respect to this, Rakoff-Nahoum and Medzhitov (2007) demonstrated that MyD88 is required for promoting tumourigenesis in a mouse model that spontaneously developed intestinal adenomas due to a mutation in the *APC* gene (*Apc*$^{Min-/+}$) (Rakoff-Nahoum and Medzhitov, 2007). *Apc*$^{Min-/+}$:*Myd88*$^{-/-}$ mice were shown to have a significant decrease in intestinal adenomas and polyps, which presented smaller in size. Interestingly, these authors also demonstrated that

the loss of MyD88 in *Apc*$^{Min-/+}$ mice had a significant increase in apoptotic cells compared to *Apc*$^{Min-/+}$ mice even though the level of proliferative cells remained unchanged (Rakoff-Nahoum and Medzhitov, 2007). Therefore, their results suggest that MyD88 may play an essential role in promoting tumour growth by regulating cell cycle progression. Although, the literature on the secondary role of TLRs in cell proliferation and survival is limited, the evidence warrants further investigation because this dual mechanism of TLR2 may apply to the pathogenesis of inflammation-associated cancer, especially GC.

1.4.3 Nod-like receptors

The NLR family of proteins (Figure 1.4) are found in the cytoplasm of host cells and have a diverse range of functions (Elinav *et al*., 2011), see also (Table 1.2). Family members such as nucleotide binding and oligomerisation domain (NOD1) 1 and NOD2 play a role in microbial recognition, whereas other NLR family members such as NLRP1, NLRP3 and NLRC4 play an important role in forming multi-protein complexes known as inflammasomes (Elinav *et al*., 2011). NLR proteins are characterised by a conserved NOD or NACHT domain which has ATPase activity and mediates self-oligomerisation for the activation of downstream effector molecules, and a C-terminal LRR for ligand binding (Fritz *et al*., 2006). In addition, NLRs can either contain a caspase-activation and recruitment domain (CARD) or a pyrin domain (PYDs) as shown in Figure 1.4.

1.4.4 NOD1 and NOD2

NOD1 and NOD2 recognise specific PG motifs that are found in the cell wall of bacteria, which are listed in Table 1.2 (Elinav *et al*., 2011). More specifically, NOD1 recognises muro-peptides (iE-DAP) that are predominantly found in gram-negative bacteria and in some gram-positive bacteria, while NOD2 recognises a common motif found in all PG,

muramyl dipeptide (MDP) (Elinav *et al*., 2011). This family of NLR proteins play a very important role in host antimicrobial responses through the activation of NF-κB and MAPK signalling pathways (Elinav *et al*., 2011). In addition, *in vitro* studies have shown the importance of NOD1 in the recognition of *H. pylori* PG (Viala *et al*., 2004). Viala *et al.*, (2004) identified by mass-spectrometry that the *H. pylori* PG motif recognised by NOD1 is *meso*-diaminopimelate (mDAP) containing N-acetylglucosamine N-acetylmuramic acid (GM-tripeptide) to induce a NF-κB response in HEK293 cells that express NOD1 and not TLR2 or TLR4. Upon activation of NOD1 and NOD2, the N-terminus CARD domain is essential for the recruitment of other downstream adaptor molecules that contain a CARD domain, such as the serine/threonine kinase, receptor-interacting serine/threonine-protein kinase (RICK) 2 (Inohara and Nunez, 2003) (Figure 1.5). The activation of RICK results in a series of phosphorylation events that eventually lead to the activation and translocation of NF-κB into the nucleus (Strober *et al*., 2006). In addition, both NOD1 and NOD2 can signal via the mitogen-activated protein kinase (MAPK) signalling pathway, which results in the activation of activator protein (AP)-1, another transcription factor that is also responsible for the transcription of pro-inflammatory mediators (Strober *et al*., 2006).

1.4.5 Inflammasome components

Some NLR family members (e.g. NLRP1, NLRP3, NLRC4) and AIM2 can form the inflammasome complex (Elinav *et al*., 2011) (Figure 1.6). Inflammasomes are essential for the activation of inflammatory caspases, such as caspase 1, caspase 4, caspase 5, caspase 11 and caspase 12, with caspase 1 being the most prominent member of the family. The activation of inflammatory caspases are necessary for the processing of immature pro-IL-18 and pro-IL-1β into their mature forms for secretion (Elinav *et al*., 2011). It is also known that inflammasome activity requires two signals; the first signal requires ligand-

induced activation of PRRs to induce gene transcription of pro-IL-18 and pro-IL-1β mRNA, and the second signal involves the activation of NLRPs/AIM2 via specific ligands to enable inflammasome assembly, that is required for the processing of pro-IL-18 and pro-IL-1β (Figure 1.7). Inflammasome assembly occurs through CARD-CARD and PYD-PYD interactions, whereby a member of the NLR family interacts with apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase 1 (Elinav *et al*., 2011) (See Figure 1.6).

1.5 Sterile Inflammation

While PRRs are classically considered to recognise and respond to molecular patterns associated with invading microbes (i.e. PAMPs), there is emerging evidence that indicates a role for PRRs in recognising non-microbial injury, termed sterile inflammation. In sterile inflammation, activation of PRRs is mainly mediated through danger-associated molecular patterns (DAMPs), such as heat shock proteins, β-defensins, uric acid crystals, high mobility group box 1 (HMGB1) and others that may be released during cell death (Barton, 2008).

For instance, HMGB1, a non-histone, chromatin-associated nuclear protein, which can be released by the host cell during an acute inflammatory response, can activate TLR2 and TLR4 present on neutrophils and macrophages (Ashizawa *et al*., 2005). In addition, Stewart *et al.,* (2010) demonstrated that molecules such as oxidised low-density lipoprotein (LDL) and amyloid-β contributed to the pathogenesis of atherosclerosis and Alzheimer's disease, respectively and required the dimerisation of TLR4 and TLR6 together with the co-receptor CD36 to initiate IL-1β gene transcription. Their data indicated that non-

microbial compounds can activate TLRs to initiate a sterile inflammatory response which results in IL-1β expression.

1.6 PRR recognition of *H. pylori* in gastric disease

A PRR that is known to be predominantly involved in the recognition of *H. pylori* is NOD1 (Girardin *et al*., 2003). As mentioned earlier the delivery of *H. pylori* PG into the host cell is mediated by the T4SS, where it is recognised by NOD1 (Viala *et al*., 2004). Studies have shown that NOD1 is essential for the activation of transcription factors, such as NF-κB and AP-1, to induce a strong pro-inflammatory response, which leads to the secretion of IL-8 from human gastric epithelial cells (Allison *et al*., 2009). More importantly, NOD1-deficient mice had increased *H. pylori cag*PAI-positive isolates in the stomach, thus suggesting the importance of NOD1 in host defence (Viala *et al*., 2004, Boughan *et al*., 2006). Viala *et al.* (2004) discovered that this phenomenon was due to the reduced ability of these mice to respond to *H. pylori,* as primary gastric epithelial cells isolated from NOD1-deficient mice co-cultured with *H. pylori* did not display increased p65 nuclear translocation or induce MIP-2 (mouse analogue to human IL-8) secretion. In addition, epithelial-derived antimicrobial peptides known as human β-defensins (hBDs) specifically hBD2, was shown to be regulated by NOD1 (Boughan *et al*., 2006). Interestingly, Grubman *et al.,* (2010) recently identified that hBD2 plays a crucial role in killing *H. pylori*, which further explains the increased bacterial load in NOD1-deficient mice.

The stomach from patients with gastric dysplasia have been shown to highly express TLR2, TLR4 and TLR5 compared to patients with normal gastric mucosa, thus suggesting that these receptors may also play a role in the pathogenesis of GC (Pimentel-Nunes *et al*.,

2011). Although TLR4 commonly recognises bacterial LPS and TLR2 recognises lipoproteins, several studies have showed that *H. pylori* LPS is recognised by TLR2 instead of TLR4 in an NF-κB-dependent manner (Mandell *et al*., 2004, Lepper *et al*., 2005, Smith *et al*., 2003). These authors showed that HEK293 cells that over-expressed the TLR2 receptor alone were able to respond to *H. pylori* LPS. Furthermore, TLR2 knock-out macrophages were unable to induce a pro-inflammatory response when exposed to *H. pylori*, however this response was intact in TLR4 knock-out macrophages (Mandell *et al*., 2004). Furthermore, a recent study discovered that *H. pylori* LPS can up-regulate TLR4 expression and gastric epithelial cell proliferation via TLR2 in an extracellular-signal-regulated kinase (ERK)1/2 MAPK-dependent manner, suggesting that up-regulation of TLR4 by TLR2 may augment pro-inflammatory responses mediated by other immunogenic bacterial LPS, for example *Escherichia coli* (Yokota *et al*., 2010). Smith *et al.,* (2003) also demonstrated that TLR5 detects *H. pylori* flagella and induced NF-κB-dependent IL-8 gene expression in a human gastric epithelial cell line, MKN45, and HEK293 cells expressing TLR5. However unlike the other PRRs, TLR5 has not been extensively studied. Given that 25% of GC patients are *H. pylori*-negative, it is tempting to speculate that other mechanisms may be contributing to the pathogenesis of GC, such as the genetic make-up of individuals.

1.7 Genetic polymorphisms in PRRs predisposes individuals to GC

Considering host genetic variation, such as single nucleotide polymorphisms (SNPs), insertion, deletion and microsatellite repeat polymorphisms, are associated with many cancers, together with the role of PRRs in *H. pylori* induced pro-inflammatory and oncogenic responses, it is not surprising that numerous studies have investigated genetic

polymorphisms of PRRs and their effect on the risk of GC (El-Omar *et al*., 2008). Since TLR2 and TLR4 have been widely studied in *H. pylori*-associated gastric carcinogenesis, the genetic variants of these PRRs will be discussed. It must be noted that even though NOD1 is important for the recognition of *H. pylori*, studies on the genetic polymorphisms of NOD1 are limited and therefore will not be discussed.

Several genetic polymorphisms in TLR2 have been identified in gastric disease, whereby the most commonly studied genetic variant is -196 to -174del (Delta22) (Kutikhin, 2011). A study demonstrated that the TLR2 -196 to -174ins/ins genetic variant, in the Japanese population, is associated with increased severity to intestinal-type GC (Tahara *et al*., 2008). Interestingly, a luciferase reporter activity assay indicated that the deletion of -196 to -174 in the TLR2 5′ promoter region impaired the transcriptional activity of the TLR2 gene compared to TLR2 -196 to -174ins/ins construct (Noguchi *et al*., 2004). Therefore, Tahara *et al* (2008) proposed that the increased transcriptional activity seen with the TLR2 -196 to -174ins/ins genetic polymorphisms may contribute to gastric disease due to the increased TLR2 response in the stomach.

With regard to TLR4, two polymorphisms (Asp299Gly and Thr399Ile) in TLR4 are commonly found in the Caucasian population and are very rare in the Japanese population (Tahara *et al*., 2007, Arbour *et al*., 2000). Studies have demonstrated that this genetic polymorphism in individuals is a determining factor for only *H. pylori*-associated non-cardia GC (NCGC), which may be due to the inability of TLR4 to detect *H. pylori*, thus allowing this pathogen to cause chronic gastritis in the individual through the activation of other PRRs (Hold *et al*., 2007). Interestingly, primary airway epithelial cells isolated from surgical polypectomies from non-cystic fibrosis patients and organ donation indicated that

individuals that were heterozygous for both TLR4 polymorphisms did not respond to LPS, however LPS responsiveness was restored when the airway epithelial cells over-expressed the wild-type allele of both polymorphisms (Arbour *et al*., 2000). Furthermore, organ donors with both Asp299Gly and Thr399Ile polymorphisms expressed lower levels of the TLR4 receptor on the surface airway epithelium (Arbour *et al*., 2000), therefore suggesting that these SNPs play an important role in the transcriptional activity of TLR4. However, as mentioned earlier, it has been questionable as to whether TLR2 or TLR4 detects *H. pylori* LPS and whether *H. pylori* LPS is immunogenic (Yokota *et al*., 2007, Lepper *et al*., 2005).

1.8 Alternative microbial causes for GC

Since not a lot is known about the microbes that resides in the gastric mucosa of 25% of individuals with *H. pylori*-negative GC (Meimarakis *et al*., 2006), it is debatable as to whether other gastric microbes play a role in gastric tumourigenesis. There is evidence indicating that an infection with other bacteria, such as Gram-negative *Acinetobacter lwoffii*, in mice can cause chronic gastritis similar to what is observed with *H. pylori*, thus supporting the role of non-*H. pylori* bacteria in gastric disease (Zavros *et al*., 2002a). It has long been thought that the overgrowth of microbes in the stomach is capable of promoting gastric carcinogenesis, potentially through the activation of the host immune system (Burkitt *et al*., 2009). A likely explanation for gastric microbial overgrowth is due to the increase in gastric pH levels, as this was demonstrated in mice lacking the gastrin gene, which developed severe gastric inflammation compared to wild-type mice (Zavros *et al*., 2002b). Nevertheless, inflammation was alleviated during the elimination of gastric microbes by antibiotic treatment, again suggesting that the detection of microbes by PRRs play an important role in gastric disease (Zavros *et al*., 2002b). In addition, IL-1β is known to be a potent inhibitor of gastric acid secretion, thereby allowing the colonisation

of other bacteria in the stomach due to the high gastric pH levels (El-Omar, 2001). Interestingly, as mentioned earlier, *H. pylori* LPS is able to augment TLR4 expression via TLR2 and since it is known that *H. pylori* can induce IL-1β secretion it is plausible that the increased gastric pH levels allow for other microbes to colonise and activate pro-inflammatory responses via TLR4 signalling (Yokota *et al*., 2010). However, the underlying mechanisms, in particular the role of indigenous (i.e. non-*H. pylori*) gastric microbes and the identity of the host mucosal immune regulators in gastric disease pathogenesis is poorly understood.

1.9 Inflammatory cytokines that play an important role in gastric disease

Cancer is often linked with persistent inflammation due to the over-production of ROS and reactive nitrogen species (RNS) by the infiltrating inflammatory cells which can result in DNA damage (Kim and Karin, 2011). Furthermore, it has also been hypothesised that cytokines produced by the infiltrating inflammatory cells can act as growth factors in the tumour microenvironment (Lin and Karin, 2007). The inflammation-associated tumour microenvironment can be characterised by the recruitment of inflammatory cells of haemopoietic origin, which are namely tumour-associated macrophages (TAMs), mast cells, dendritic cells (DCs) and T-cells (Beales, 2002). The recruitment of inflammatory cells to the site of injury and infection results in the production of pro-inflammatory mediators, such as IL-1β, TNF-α, IL-8 and IL-6, which all have the potential to induce and regulate other cytokines, chemokines, matrix-metalloproteinases (MMPs) and pro-angiogenic factors.

1.9.1 Interleukin 1-β

IL-1β is a pro-inflammatory cytokine that is a potent regulator of inflammation as it induces the transcription of other pro-inflammatory mediators, such as TNF-α, IL-8 and IL-6 (El-Omar, 2001). The importance of IL-1β in gastric tumourigenesis was further illustrated by over-expression of human IL-1β. in transgenic mice which spontaneously developed gastric inflammation and tumours, of which the phenotype was further exacerbated upon *Helicobacter* infection (Tu *et al*., 2008). Moreover, genetic polymorphisms that enhance the production of this pro-inflammatory cytokine are strongly associated with an increased risk of developing GC (El-Omar *et al*., 2001). As mentioned earlier, one of the biological effects of IL-1β in the stomach is the inhibition of gastric acid secretion leading to the pathogenesis of PUD, possibly by allowing the overgrowth of gastric microbes in the stomach to promote chronic gastritis and gastric atrophy, which is the destruction of parietal cells that are involved with gastric acid secretion (Beales and Calam, 1998, El-Omar, 2001). In addition, Beales *et al*.,(2002) demonstrated that IL-1β was able to induce gastric epithelial cell proliferation, which may contribute to gastric hyperplasia. Furthermore, IL-1β has been shown to have pro-tumour properties as mice bearing tumour cells secreting IL-1β had increased levels of $CD11b^{+}$/ $Gr1^{+}$ (myeloid-derived suppressor cells (MDSCs)) in the spleen as compared to normal mice or mice receiving mock-transfected tumour cells (Song *et al*., 2005). MDSC support tumourigenesis by promoting angiogenesis and tumour growth by suppressing tumour immunosurveillance (Schmid *et al*., 2010). More importantly, transgenic mice that over-express human IL-1β in the stomach had an accumulation of MDSCs in the stomach, which was significantly reduced when IL-1RA (neutralising antibody) was administered (Tu *et al*., 2008).

1.9.2 Tumour necrosis factor α

Both tumour epithelial cells and/or inflammatory cells produce TNF-α, which is highly linked to inflammation-associated cancers (Lin and Karin, 2007). Similar to IL-1β, TNF-α secretion is also able to inhibit gastric acid secretion but to a lesser extent, thus causing gastric atrophy (Lin and Karin, 2007, Beales and Calam, 1998). More importantly, a study demonstrated that *Tnf*-$\alpha^{-/-}$ mice did not develop *Helicobacter*-induce gastritis or hyperplasia in the gastric mucosa, suggesting that TNF-α plays an important role in the pathogenesis of *H. pylori*-mediated GC (Hasegawa *et al*., 2004). It was shown that TNF-α produced by activated macrophages induced the Wnt/β-catenin signalling pathway, which is known to play an important role in GC (Oguma *et al*., 2008, Oshima *et al*., 2006). Furthermore, TNF-α contributed to tumour initiation by promoting the production of ROS, thus resulting in DNA damage (Lin and Karin, 2007). More importantly, genetic polymorphisms predicted to enhance TNF-α production have increased risk of developing GC (Lin and Karin, 2007, Wilson *et al*., 1997, Gorouhi *et al*., 2008).

1.9.3 Interleukin 8

IL-8, also known as CXCL8, is a pro-inflammatory chemokine produced by epithelial cells, monocytes and macrophages (Waugh and Wilson, 2008). In a tumour microenvironment, IL-8 is known to promote angiogenesis, proliferation, survival and migration of vascular endothelial cells (Waugh and Wilson, 2008). A study demonstrated that human endothelial cells (HUVEC) cultured in the presence of IL-8 displayed increased cell proliferation and cell migration *in vitro* (Li *et al*., 2003). Furthermore, HUVEC cells that were exposed to IL-8 had augmented levels of *Bcl-*x_L (anti-apoptotic) mRNA, an up-regulation of Bcl-2 (anti-apoptotic) protein and reduced levels of Bax (pro-apoptotic) protein, thus supporting the role of IL-8 in inducing endothelial cell survival. More importantly, similar results were observed in human gastric epithelial cells, whereby over

expression of IL-8 in MKN45 cells resulted in an increase in gastric epithelial cell migration and invasion (Kuai *et al*., 2012). Since *H. pylori* is known to significantly induce IL-8 secretion in human gastric epithelial cell lines, IL-8 production in the tumour microenvironment may support tumourigenesis (Torok *et al*., 2005). With regard to this, genetic polymorphisms of IL-8 have been extensively studied and are strongly associated with gastric carcinogenesis in *H. pylori*-infected individuals (Song *et al*., 2010, Xue *et al*., 2012).

1.9.4 Interleukin 6 family of cytokines

The IL-6 family of cytokines are characterised by 4 α-helix bundle structures, which consists of IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin (CT)-1 and several others (Heinrich *et al*., 1998). IL-6 is a pro-inflammatory cytokine that is produced by a diverse range of cell types and is known to promote inflammation, angiogenesis, tumour cell growth and survival by activating the Janus kinase (JAK)/STAT pathway via the glycoprotein (gp)130 receptor (Heinrich *et al*., 1998, Lin and Karin, 2007). Studies have shown that patients with advanced GC have significantly higher IL-6 levels in their serum (Ashizawa *et al*., 2005, Ikeguchi *et al*., 2009). Furthermore, genetic polymorphisms of IL-6 that increases the levels of IL-6 in individuals strongly correlates with poor prognosis in gastric cancer (Yu *et al*., 2011).

1.10 Mouse models for gastric cancer

Mouse models have proven to be a very useful tool in understanding the molecular mechanisms that contribute to the pathogenesis of GC as summarised in Table 1.3. As mentioned earlier, a transgenic mouse model that over-expressed human IL-1β in the

stomach spontaneously developed gastritis and hyperplasia within 12 months in the absence of *Helicobacter* (Tu *et al*., 2008). Interestingly, Tu and colleagues (2008) showed that 30% of mice that highly expressed IL-1β developed adenocarcinoma, whereas mice that lowly expressed IL-1β did not, thus demonstrating a relationship between IL-1β expression and the severity of gastric carcinogenesis. Moreover *H. felis* infection in IL-1β transgenic mice exacerbated gastric disease with approximately 10% of mice developing invasive carcinoma regardless of the level IL-1β expression. Although this model demonstrated the importance of IL-1β in the development of gastric carcinogenesis, the question still remains as to whether there is a link between increased production of IL-1β in *H. pylori*-independent GC. Based on our knowledge of the biological properties of IL-1β, further investigation into the molecular mechanisms that drives IL-1β production (i.e. recognition of PAMPs/DAMPs by PRRs) in the gastric compartment is warranted. Therefore, in inflammation-associated carcinogenesis it would be interesting to identify if there were any significant changes in the gastric microflora, as the change in gastric pH levels may result in the colonisation of pathogenic bacteria to exacerbate gastric disease. In contrast, given that IL-1β is implicated in sterile inflammation it is important to determine if mice still develop gastritis and gastric hyperplasia in the absence of microbes (i.e. germ free).

A mouse model that nicely demonstrates the interplay between multiple signalling pathways is the *K19-Wnt1/C2mE* transgenic mice (Gan) (Oshima *et al*., 2006). Since GC patients that were colonised by *H. pylori* displayed and increased levels of COX-2, the role of COX-2 and mPGE in gastric disease was first investigated upon over-activation of these signalling pathways in the gastric mucosa of *K19-C2mE* mice, which resulted in the spontaneous development of gastric tumours at 48 weeks of age (Oshima *et al*., 2004).

However, Oshima and colleagues illustrated that increased activation of both Wnt and COX-2/PGE2 signalling pathways in the gastric mucosa of Gan mice spontaneously lead to the development of gastric tumours at 30 weeks, thus confirming the synergistic interaction of multiple signalling pathways in GC (Oshima *et al*., 2006). In addition, there was an increase in the number of proliferative cells in the gastric mucosa of SPF Gan compared to germ-free (GF) Gan mice, which suggests that gastric microbes may contribute to gastric tumour growth (Oshima *et al*., 2011). Since intestinal-type metaplasia is the most common type of GC, it is worthy to note that the gene signature of gastric tumours isolated from Gan mice resembled that of human intestinal-type GC (Itadani *et al*., 2009), thus suggesting that this mouse model serves as a useful tool for understanding the molecular mechanisms involved in human intestinal-type GC. Similarly, another useful mouse model that displays STAT3 hyperactivation (discussed below) can also provide molecular mechanistic insights into human intestinal-type GC, since these mice spontaneously develop gastric hyperplasia and adenomatous lesions with histopathological features that resemble human intestinal-type GC (Judd *et al*., 2006).

1.11 Signal transducer and activator of transcription 3 (STAT3) in gastric cancer

STAT3 is up-regulated in a vast array of human cancers, including gastric, colon, liver and lung (Corvinus *et al*., 2005, Yu *et al*., 2009). This molecule is one of seven members of the STAT family of transcription factors (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6), which are ubiquitously expressed except for STAT4 which is predominantly found in myeloid cells (Heinrich *et al*., 2003). STAT3 is a potent pro-inflammatory and oncogenic regulator that contains a coiled-coil (also known as oligomerisation), DNA binding, linker, Src-homology (SH) 2 and transactivation domain as depicted in Figure 1.8 (Heinrich *et al*., 2003). Activation of STAT3 is mediated via the IL-6 cytokine family via the gp130 receptor (Yu *et al*., 2009). Members of the STAT

family have been shown to form homo- and hetero-dimers with other STAT molecules, which is a prerequisite for binding to STAT3 target genes (Heinrich *et al*., 2003). For example, STAT3 has been shown to form hetero-dimers with STAT1 and the p65 subunit of NF-κB (Yang *et al*., 2007, Heinrich *et al*., 2003). There are two splice variants of STAT3, which are denoted as STAT3α and STAT3β, whereby the transactivation domain is deleted in the STAT3β isoform (Bowman *et al*., 2000) (Figure 1.8). Although it is known that tyrosine phosphorylation is essential for the activation of STAT3, it has been shown that serine phosphorylation, within the transactivation domain, contributes to maximal transcriptional activity of STAT3 (Wen *et al*., 1995), therefore STAT3β may inhibit the actions of STAT3α in a dominant negative manner (Bowman *et al*., 2000)

STAT3 immunohistochemical staining of adenocarcinoma gastric tissue samples revealed that patients with high STAT3 expression correlated with a lower survival rate than the STAT3-negative group regardless of their *H. pylori* status (Kim *et al*., 2009a). The very first report that illustrated the oncogenic property of STAT3 was demonstrated by Bromberg *et al.,* (1999), whereby the constitutively active STAT3 (STAT3-C) construct was created by substituting cysteine residues for A661 and N663 to enforce the formation of di-sulphide bonds between two STAT3 monomers. Bromberg *et al.*, (1999) revealed that STAT3-C was able to translocate into the nucleus and initiate the transcription of STAT3 target genes. In addition, it was discovered that cells that stably over-expressed the STAT3-C construct had enhanced cellular transformation and expressed augmented levels of genes involved in cell cycle progression (CyclinD1 and c-Myc) and survival (Bcl-$_{xL}$) (Bromberg *et al*., 1999).

More importantly, the complexity of genes regulated by STAT3 is clearly cell type-dependent, since in one study STAT3 had an anti-apoptotic role in mammary gland epithelial cells from STAT3 conditional knock-out mice as it failed to up-regulate Bcl-$_{xL}$ (Chapman *et al*., 1999). However, in another study the authors noted that STAT3 activation could directly regulate Bcl-$_{xL}$ expression in myeloma tumour cells, thus effecting the survival and pathogenesis of this disease (Catlett-Falcone *et al*., 1999).

In the gastric compartment, Kanda *et al.*, (2004) identified that STAT3 inhibition resulted in the reduction of CyclinD1 protein expression and survivin (inhibitor of apoptosis) expression levels. Furthermore, our collaborators have revealed that STAT3 regulated Bcl-$_{xL}$, c-Myc and vascular endothelial growth factor (Vegf)α (pro-angiogenic) in mice that displayed increased levels of STAT3 activation (Jenkins *et al*., 2005). Interestingly, these authors showed that transforming growth factor (TGF)-β, a negative regulator of epithelial cell proliferation, is indirectly regulated by STAT3 activation through the transcription of Smad7, which is known to inhibit TGF-β signalling, thereby promoting gastric epithelial cell proliferation in these mice. In humans, a study revealed that approximately 50% of human gastric tumour tissues expressed phospho-STAT3 (pSTAT3), which is an indicator of active STAT3, whereas only 11% of pSTAT3 was detected in the normal gastric epithelium of individuals (Deng *et al*., 2010a). In addition, a significantly positive correlation between STAT3 activation and the expression of survivin and Bcl-2 (inhibitor of apoptosis) was observed, whereby high STAT3 expression was also strongly associated with lymph node metastases and a lower survival rate (Deng *et al*., 2010a, Kim *et al*., 2009a).

STAT3 has also been shown to be a potent regulator of pro-inflammatory cytokines, as the gene expression levels of *Il-6*, *Il-1β* and *Tnf-α* were significantly augmented in the stomachs of mice with STAT3 hyperactivation but were substantially reduced in mice that had reduced levels of STAT3 activation (Judd *et al*., 2006). It was also observed by Judd *et al.*, (2006) that the recruitment of leukocytes to the gastric mucosa, as well as the gene expression level of chemokines that regulate the migration of macrophages and neutrophils (i.e. *Ccl-1*, *Ccl-12* and *Cxcl-2*), were significantly down-regulated in mice that displayed reduced levels of STAT3 activation compared to mice with STAT3 hyperactivation, thus suggesting a crucial role for STAT3 in leukocyte recruitment. Collectively, the above studies demonstrate a role for STAT3 in mediating cell proliferation, survival, angiogenesis and inflammation which contributes to the development of gastric tumourigenesis.

1.11.1 The IL-6 family of cytokines-STAT3 signalling pathway

The IL-6 family of cytokines bind to their alpha-receptor subunits, which interact and activates the common gp130 receptor, resulting in the activation of the MAPK and JAK/STAT pathway (Figure 1.9) (Amieva *et al*., 2003). As shown in Figure 1.9, subsequent activation of the gp130 receptor involves the recruitment of JAKs to tyrosine phosphorylated (pY) residues on the cytoplasmic domain of the gp130, which also facilitates the tyrosine phosyphorylation of STAT3 by JAKs (Amieva *et al*., 2003). STAT3 phosphorylation (pY-STAT3) results in the formation of homodimers or heterodimers with other STAT molecules, predominantly STAT1, upon which they enter the nucleus to initiate transcription of target genes. In addition, tyrosine phosphorylation of residue 757/759 in the gp130 cytoplasmic tail of mouse/human leads to the binding of tyrosine phosphatase SHP-2, which results in the activation of the MAPK signalling pathways

(Heinrich *et al*., 2003). Under normal homeostatic conditions, these signalling pathways are tightly regulated by suppressor of cytokine signalling (SOCS) 3, whereby SOCS3 competes with SHP2 by interacting with the gp130 receptor, specifically at residue 757, to target the receptor complex for proteosomal degradation (Heinrich *et al*., 2003). Moreover, a protein inhibitor of activated STAT3 (PIAS) 3 has been shown to specifically interact with pY-STAT3, and not STAT1, to inhibit the transcription of STAT3 target genes (Heinrich *et al*., 2003)

1.11.2 STAT3 and NF-κB interaction

Very little is known about the precise mechanisms by which STAT3 promotes pro-inflammatory processes. However, recent reports of interactions between NF-κB, which has well characterised roles in inflammation, and STAT3 may provide some explanation. It has been shown that STAT3 and NF-κB can interact regardless of their phosphorylation status (Bronte-Tinkew *et al*., 2009). A study conducted by Yang *et al.,* (2007) revealed that the accumulation of unphosphorylated STAT3 (U-STAT3) in the cytoplasm facilitated the interaction of U-STAT3 with unphosphorylated NF-κB, leading to the translocation of this complex into the nucleus to initiate transcription of NF-κB dependent and independent genes. More interestingly, another study found that pY-STAT3 can form a complex with the p65 NF-κB subunit following IL-1β and IL-6 stimulation to actively transcribe serum amyloid A (SAA) despite there not being a typical STAT3 response element (RE) in the serum amyloid A (SAA) promoter (Hagihara *et al*., 2005). This suggests that dimerisation of STAT3 and NF-κB confers unique target specificity to STAT3. In addition, it has also been documented that the hyper-active STAT3-C mutant resulted in hyper-acetylation of p65, prolonging the activity of NF-κB in the nucleus, through the interaction with acetyltransferase p300 to maintain p65 acetylation (Fukata and Abreu, 2008). De-

acetylation of p65 is crucial for inactivating NF-κB activation by increasing its affinity to its inhibitor IκBα. Although, these studies support the role of STAT3 in inflammation, the exact mechanisms linking STAT3 with chronic inflammatory responses during tumourigenesis are not well understood.

1.11.3 The gp130$^{F/F}$ mouse model of STAT3-mediated gastric tumourigenesis

A mouse model (*gp130*$^{F/F}$) with a homozygous phenylalanine "knock-in" substitution at tyrosine 757 of the gp130 receptor abrogated SOCS3 binding, resulting in STAT3/1 hyper-activation. Furthermore, due to the Y757F mutation in the gp130 receptor, these mice were unable to signal via SHP2-mediated MAPK signalling pathways. Notably, these mice spontaneously developed gastritis, gastric hyperplasia and adenomatous lesions, with histopathological resemblance to human intestinal-type GC, in the absence of *H. pylori* (Figure 1.10) (Tebbutt *et al*., 2002, Judd *et al*., 2006). In addition, genetic reduction of the STAT3 activation level in *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice suppressed the onset of gastritis and tumourigenesis emphasising the importance of STAT3 in gastric adenocarcinoma (Jenkins *et al*., 2005). It has also been demonstrated that STAT3-driven IL-11, but not IL-6, is the primary cytokine that drives gastric disease in this model as gastric inflammation and tumourigenesis were absent in compound mutant *gp130*$^{F/F}$:*IL-11R*$^{-/-}$ knock-out mice, but not *gp130*^{FF}:*IL-6*$^{-/-}$ mice (Ernst *et al*., 2008). The expression levels of genes that were involved in cell cycle progression and survival were markedly reduced in the *gp130*$^{F/F}$:*IL-11R*$^{-/-}$ mice compared to *gp130*$^{F/F}$ mice. In addition, the lack of inflammatory cell infiltrates in the gastric mucosa of *gp130*$^{F/F}$:*IL-11R*$^{-/-}$ mice suggested that IL-11-mediated STAT3 activation plays a crucial role in initiating the gastritis stage of gastric carcinogenesis, whereby IL-11 has been touted as a potential gastric disease biomarker (Necula *et al*., 2012, Ernst *et al*., 2008). The involvement of PRRs in the development of gastric disease

in *gp130*$^{F/F}$ mice was first suggested in a study that reported that *gp130*$^{F/F}$ mice placed on a broad-spectrum antibiotic regimen for 8 weeks to eliminate gastric flora resulted in a 70% reduction in tumour formation compared to untreated *gp130*$^{F/F}$ mice (Judd *et al*., 2006). Importantly, my preliminary unpublished data show that among all TLRs investigated, in *gp130*$^{F/F}$ mice gastric tumours correlate with i) augmented TLR2 expression levels in the stomach antrum and ii) heightened gene expression levels of TLR2-regulated pro-inflammatory mediators.

1.12 Transcriptional regulation of TLR2

Despite the evolutionary conservation of mouse and human TLR2 proteins, the 5' promoter regions of the mouse and human TLR2 gene have a very low sequence homology (6-10% homology) (Haehnel *et al*., 2002). Furthermore, unlike the mouse TLR2 promoter, the promoter region of human TLR2 does not respond to microbial product-activated NF-κB (Haehnel *et al*., 2002). According to Musikacharoen and colleagues (2001) the 5' mouse TLR2 promoter region contained 2 putative NF-κB (at positions -1115 to -1106 and -160 to -150), 2 CCAAT/enhancer binding protein (C/EBP) (at positions -1591 to -1578 and -777 to -765), a CAMP response element-binding protein (CREB) (at position -1267 to -1260) and a STAT (at position -279 to -271) binding sites (Figure 1.11). In addition, to identify which binding sites were essential for *Tlr2* gene transcription Musikacharoen and colleagues (2001) truncated the 5′ promoter of *Tlr2* at several regions and measured the luciferase activity. They proposed that the 5′ *Tlr2* promoter region between -2332 to -1486 may be required for negative regulation of *Tlr2* gene transcription as the luciferase activity was diminished in the truncated TLR2 promoter in response to LPS or TNFα, which are known inducers of TLR2 gene transcription. Interestingly, 2 NF-κB binding sites were

required for full *Tlr2* gene transcription, however mutation of either one NF-κB binding site did not completely abolish luciferase activity thus suggesting that another transcription factor may be involved (Musikacharoen *et al*., 2001). More importantly, (2001) demonstrated that STAT5 is recruited to the STAT binding site within the 5′ TLR2 promoter and transfections performed in conjunction with a dominant negative form of STAT5 abrogated TLR2 luciferase activity. Surprisingly, a 5′ TLR2 promoter construct containing a STAT binding site and the deletion of NF-κB binding site at position -160 to -150 was unable to respond to IL-15 thus suggesting that the cooperation of both NF-κB and STAT5 may be required. It is worthy to note that these studies were performed more than 10 years ago, therefore more recent analyses should be carried out with more up to date databases.

1.13 Hypothesis

Approximately 50% of GC patients over-express STAT3, which is known to play an essential role in GC, as STAT3 is a potent regulator of genes involved in cell cycle progression, survival, angiogenesis and inflammation. Interestingly, more than 10 years ago, a bioinformatic study revealed the presence of a STAT binding site within the 5′ promoter region of the TLR2 gene, thus suggesting that STAT3 may also transcriptionally regulate TLR2. With regard to TLRs, the role of TLRs have been implicated in STAT3-mediated gastric tumourigenesis, since antibiotic-treated *gp130*$^{F/F}$ mice displayed a 70% reduction in tumour mass compared to un-treated *gp130*$^{F/F}$ mice. Given that TLRs have been extensively studied for it role in initiating an inflammatory response and, to a lesser extent, its role in mediating wound healing, angiogenesis, cell proliferation and survival, the importance of investigating the link between STAT3-mediated GC and TLRs is needed. Therefore, I hypothesised that STAT3 hyper-activation augments TLR2 signalling

in the stomach to promote gastric disease through TLR2-mediated gastritis and/or cell proliferation and survival.

1.14 Aims

1.14.1 Aim 1

The first aim of this PhD project was to investigate whether TLR2 is a STAT3 target gene. A global gene expression analyses of 84 genes involved in TLR signalling was initially examined in the stomachs of *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ mice and since it is known that the IL-11/STAT3 signalling axis is crucial for the development of gastritis and adenomatous lesions, quantitative gene expression analyses were further validated in *gp130*$^{F/F}$ compound mutant mice.

Gp130 mutant mouse models:

1. *gp130*$^{+/+}$: displays normal STAT3/1 activity
2. *gp130*$^{F/F}$: displays increased STAT3/1 activity and mice develop hyper-proliferative lesions and gastritis at 6 weeks of age.
3. *gp130*$^{F/F}$*:Stat3*$^{+/-}$: displays increased STAT1 but relatively normal STAT3 activity and mice develop hyper-proliferative lesions and gastritis with 30% penetrance.
4. *gp130*$^{F/F}$*:Il-11r*$^{-/-}$: displays normal STAT3/1 activity and mice do not develop hyper-proliferative lesions and gastritis.

To demonstrate if STAT3 directly augments TLR2 gene expression levels in the stomach, several *in vitro* experiments involving over-expression of the hyper-active STAT3 (STAT3-C/GFP) construct in mouse (IMGE-5) and human (MKN-28, AGS) gastric epithelial cell lines will be conducted. Furthermore, bioinformatic analyses, using the

TRANSFAC and MATCH databases, will be employed to determine the putative STAT binding sites in the 5′ promoter region of the TLR2 gene.

1.14.2 Aim 2

Since preliminary data suggest that *Tlr2* gene expression levels are augmented in the stomachs of *gp130*$^{F/F}$ mice, my second aim of this project was to identify the role for TLR2 signalling in STAT3-mediated gastric tumourigenesis. To do this, *gp130*$^{F/F}$ mice were crossed with *Tlr2*$^{-/-}$ mice to generate *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice. Initially, since TLR2 is an important regulator of inflammation, the level gastritis and the role of hematopoietic cell lineages were examined in *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice compared to *gp130*$^{F/F}$ mice. In addition, given that studies have shown that TLR2 also plays a crucial role in mediating other cellular processes, such as wound healing, angiogenesis, cell proliferation and survival, I also examined these cellular processes in our *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice.

CHAPTER 2

Methods and Materials

2.1 Patient sampling and histological grading

Gastric biopsies were collected from 25 patients undergoing gastroscopy or gastric resection and the samples were either stored in 10% formalin for embedding in paraffin blocks or snap-frozen in liquid nitrogen for RNA extraction. *H. pylori* colonisation and pathological changes in the gastric biopsies, such as moderate gastritis, severe gastritis and gastric adenocarcinoma were determined by hemotoxylin and eosin (H&E) and cresyl fast violet-stained tissue sections, following the revised version of the Sydney system (Dixon *et al*., 1996) by an independent pathologist, Prithi Bhatal (Melbourne pathology). The *H. pylori* status was further confirmed using *16S* rDNA PCR, as described in Boon jakuakul *et al.,* (2005). Informed consent was obtained from all patients and the collection of gastric biopsies was approved by the Southern Health Human Research Ethics Committee.

2.2 Animal work

2.2.1 Mouse generation and housing

The generation of *gp130*$^{F/F}$ mice and compound mutant mice that are heterozygous for *Stat3* (*gp130*$^{F/F}$:*Stat3*$^{+/-}$) has been previously described (Jenkins *et al*., 2005). Mice homozygous null for the *Tlr2* gene (Takeuchi *et al*., 1999) and *Tlr4* gene (Hoshino *et al*., 1999) have been previously generated. These mice were then crossed with *gp130*$^{F/F}$ mice to generate compound mutant *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ and *gp130*$^{F/F}$:*Tlr4*$^{-/-}$. All experiments were performed following Animal Ethics approval from the Monash Medical Center "A" committee, and included *gp130*$^{+/+}$ (wild-type) littermate controls that were genetically

matched on a mixed 129Sv x C57BL/6 background. All mice were kept in a specific pathogen-free area and all mice were age-matched for each experiment.

2.2.2 Genotyping

Genomic tail DNA was extracted from mouse tails for genotyping by polymerase chain reaction (PCR). Mouse tails were digested in tail buffer (Appendix II) for 3 hours at 55˚C. The samples were mixed with 5M NaCl, incubated at room temperature for 10 minutes, centrifuged and 800µl of the supernatant was collected in a fresh tube containing 500µl of 100% isopropanol. The DNA was collected at the bottom of the tube by centrifugation and washed with 70% ethanol. Dry the DNA pellet and resuspend in 125µl of MilliQ (Millipore) water for 3 hours at 37˚C. The DNA was purified using a UNIFILTER ® 800 (Whatman) plate under vacuum using PBB binding buffer (Qiagen) and PE wash buffer (Qiagen) and eluted in 0.25% (v/v) TE buffer (Appendix II).

PCR reactions were setup with the following reagents (Promega): 100ng of genomic DNA, 6µl 5X Buffer, 3µl of $MgCl_2$, 0.6µl of dNTPs, 200ng forward and reverse oligo primers (Appendix III).

DNA fragments were visualised on a 2% (w/v) agarose gel made with 1X Tris-acetate ethylenediamine tetraacetic acid (EDTA) (TAE) buffer (Appendix II) and SYBR safe. The samples were loaded into the wells and were separated at 100 volts for 1 hour and the bands were viewed using the gel doc (Fisher Biotech).

2.2.3 Irradiation and bone marrow reconstitution of mice

Recipient mice were irradiated with a split dose of 550 rads x 2 (1100 rads in total) in a mouse irradiation facility. The femurs from donor mice were harvested on the same day and their bone marrows were flushed using a 30 gauge needle with sterile PBS. Cell counts were performed and the cell pellet was resuspended in sterile phosphate buffered saline (PBS) to give 25 x 10^6 cells/ml. Recipient mice received 200µl of cell supernatant via intravenous (i.v.) injection.

2.2.4 Administration of OPN301 to mice

The TLR2 response in mice was blocked using a TLR2-blocking antibody (OPN301). Mice were administered with either OPN301 or IgG1 isotype control by intraperitoneal (i.p.) injection at 10mg/kg.

In the *in vivo* acute study, mice were pre-treated with this antibody for 30 minutes followed by 10mg/kg of Pam_3CSK4 (InvivoGen). At 1, 3 and 6 hour, mice were culled and the stomachs were harvested.

In the *in vivo* chronic study, mice at 12 weeks of age were subjected to 10mg/kg of OPN301 antibody twice a week by intraperitoneal (i.p.) injection for 12 weeks. Upon completion of the study, the mice were culled and the stomachs were harvested and weighed.

2.2.5 Organ collection

Mouse stomachs were harvested and were separated into tumour, underlying antrum and fundus for snap freezing in liquid nitrogen. Regardless of whether the tissue was snap

frozen or used to culture primary gastric epithelial cells, the fore stomach was removed and washed PBS (Appendix II).

Mouse stomachs that were collected for immunohistochemistry were cut longitudinally on the inner curvature of the stomach and washed in PBS. The stomachs were then pinned against a cork board and soaked in 20% (v/v) formalin in PBS for 24 hours and then replaced with 70% (v/v) ethanol. The stomachs were processed and embedded in paraffin-blocks for further analyses.

2.3 Cell culture

2.3.1 Passage and Maintenance of human and mouse gastric epithelial cells

Mouse gastric epithelial cell line, IMGE-5, and human gastric epithelial cell lines, AGS and MKN-28, were grown in Roswell Park Memorial Institute (RPMI) (Gibco) complete media containing 10% (v/v) foetal bovine serum (FCS), 1% (v/v) penicillin/streptomycin (Gibco) in a 75 cm^2 or 175 cm^2 tissue culture flask. Cell lines that stably over express either a wild type or hyperactive form of STAT3 (STAT3-C/GFP) were selectively grown and maintained in RPMI complete media with the addition of 0.4% (v/v) geneticin (Gibco).

Human embryonic kidney (HEK) 293 cells were cultured in Advanced Dulbecco's modified Eagle's Medium (DMEM) (Gibco) complete media containing 5% (v/v) FCS, 1% (v/v) L-Glutamine (Gibco) and 1% (v/v) penicillin/streptomycin.

Cell lines were passaged by replacing the media in the 75 cm^2 or 175 cm^2 flasks with either 1.5ml or 2.5 ml of Trypsin-EDTA (Gibco) respectively and were incubated at 37°C for 5 minutes. The cells were collected in their respective media and centrifuged for 5 minutes at

200g. The supernatant was discarded and the pellet was resuspended in 5ml or 10 ml of the appropriate media. Cell maintenance was performed by appropriately diluting the cells with RPMI or DMEM complete media, respectively, in tissue culture flasks and incubated at 37°C with 5% carbon dioxide (CO_2).

Prior to stimulation with various TLR2 ligands (LTA, FSL-1 and Pam_3CSK4) (InvivoGen), cells were serum starved in media that did not contain FCS for 16 hours. Experiments that involved the use of specific pathway inhibitors, cells were pre-treated for 30 minutes with either 0.1% (v/v) DMSO vehicle, U0126 (Sigma), Wortmannin (Sigma), MG132 (Calbiochem), SB203580 (Calbiochem), SP600125 (Calbiochem) at the indicated concentrations prior to stimulation with Pam_3CSK4.

2.3.2 Isolation of primary gastric epithelial cells from mice

Mice were culled at 4 weeks of age using a CO_2 chamber and cervical dislocation, whereby the isolation of the antrum and fundus of the gastric compartment was cleaned in PBS and collected in 5ml of media 1 (Hanks balance salt solution (HBSS) (Gibco) with 0.2% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich)). The stomachs were transferred into $10cm^2$ plates with 5ml of fresh media 1 and were minced with a scalpel blade. Subsequently, the minced stomachs were centrifuged for 5 minutes at 200g and resuspended in media 2 (HBSS, 0.2% (w/v) BSA, 0.4mg/ml collagenase A (Roche) and 1% (v/v) penicillin/streptomycin). To activate collagenase activity the suspension was transferred into a $75cm^2$ tissue culture flask and incubated for 1 hour at 37°C with 5% CO_2. The cells were dissociated by aspirating up and down for 5 minutes and then transferred into a 50ml tube containing media 3 (DMEM, 20% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 0.1% (v/v) fungizone amphotericin B(Gibco)). To obtain a single cell suspension cells

were further dissociated by aspirating up and down for another 5 minutes and cell debris was allowed to settle to the bottom of the tube. The supernatant was carefully collected in a fresh 50ml tube and cells were centrifuged for 5 minutes at 200g. The cell pellet was resuspended with 3 ml of media 3 and cell counts were performed using the Sysmex KX-21N (Roche). Cells were seeded at 5 x 10^5 cells per well in a 12 well plate format.

Prior to stimulation with various ligands or agonists, cells were serum starved in media that did not contain FCS for 16 hours.

2.4 Cell transfections

2.4.1 Cell transfection - FuGene 6

Cell transfections with Fugene 6 (Roche) were conducted on HEK293 cells for a TLR2 reporter luciferase assay. HEK293 cells were seeded in a 96 well plate format at a cell density of 2 x 10^4 cells per well in plain DMEM. The following day, HEK293 cells were transfected with 20ng/well of various TLR2 reporter constructs, 50ng/well and 100ng/well with STAT3-C/GFP, 50ng/well with dTK Renilla vector (Promega) and the total amount of DNA was kept constant at 230ng/well by the addition of an EGFP empty vector. FuGene 6 was added drop-wise, based on a ratio of 4µl of FuGene to 1µg of total DNA, to the plain tissue culture media. The FuGene/media suspension was incubated at room temperature for 5 minutes and the diluted DNA was added drop-wise into this suspension. This mixture was further incubated at room temperature for 15 minutes and 100µl of this mixture was added to each well drop-wise. Transfected cells were incubated for 24 hours at 37°C in 5% CO_2 before luciferase reporter activity was measured.

HEK293 cells that were grown in 24-well and 96-well plates were lysed with 100µl/well and 50µl/well of Reporter Lysis buffer (Promega), respectively. The plates were incubated on ice for 10 minutes, until the cells appear to be lysed, and were stored at -20°C prior to the luciferase assay.

2.4.2 Cell transfection - Amaxa

Cell transfections using the Amaxa cell line Nucleofector kit V (Lonza) were performed on IMGE-5 cells. These experiments were done to over-express STAT3-C/GFP transiently or stably in IMGE-5 cells. Prior to transfection, cells were approximately 70% confluent in a tissue culture flask. The protocol was followed according to the manufacturer specifications. IMGE-5 cells were transfected with 500ng of STAT3-C/GFP per 1 x 10^6 cells and to maintain stable expression of STAT3-C/GFP the cells were grown in RPMI complete media supplemented with G418 (Sigma-Aldrich). After transfection, the cells were plated in a 6 well plate format and fresh media was replaced 24 hours later. IMGE-5 cells that transiently over-expressed STAT3-C/GFP were used for experiments 24 hours after the transfection, while IMGE-5 cells that stably over-expressed STAT3-C/GFP were grown in selective media and sorted for IMGE-5 cells that only contained STAT3-C/GFP.

2.5 Reporter luciferase assay

Prior to conducting the luciferase assay HEK293 cells were lysed with 50µl/well of Reporter Lysis buffer (Promega) on ice for 10 minutes, until the cells appear to be lysed. Sample aliquots of 20µl were transferred in to two 96-well white polystyrene plates. On one plate 30µl of the Luciferin substrate (Promega) was added to each well, whereas, on another plate 50µl of coelenterazine or Renilla substrate was added per well. All samples were done in triplicate and luminescence was measured using an Infinite M200 (Tecan)

immediately after the addition of the substrate. Results were analysed using the Mars software.

2.6 Enzyme linked immunosorbent assay (ELISA)

The concentration of various mouse and human chemokines and cytokines were determined in supernatants collected 12 and 24 hours after stimulation. All ELISA kits were obtained from BD Biosciences and the protocol was followed according to the manufacturer specifications. All antibodies were diluted at 1:250 in assay diluent except for the capture antibody, which was diluted in coating buffer. The 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution and hydrogen peroxide (Thermoscientific) was prepared at a ratio of 1:1 for the development of the ELISA and kept away from light. Prior to reading the absorbance at 450nm, 50µl of stop solution (2N H_2SO_4) was added to each well. The absorbance was read using the Infinite M200 and results were analysed by the Mars software, using a four-parameter scale to determine the concentration present in each sample.

2.7 RNA extraction

2.7.1 Tissues

RNA extraction of gastric tissues were homogenised in Trizol (Invitrogen) reagent. The tissue suspensions were transferred into 1.5ml tube containing 200µl of chloroform and were vigorously mixed for 15 seconds. After the 3 minutes incubation at room temperature the samples were centrifuged at 12000xg for 30 minutes at 4°C. The aqueous layer containing RNA was carefully collected into a fresh 1.5ml tube and the RNA was precipitated by adding 500µl of 100% isopropanol. Centrifugation at 12000xg for 30

minutes at 4°C was performed to pellet the RNA. The RNA pellet was washed in 1ml of 75% (v/v) ethanol followed by centrifugation at 7500xg for 15 minutes at 4°C and the supernatant was discarded. Diethylpyrocarbonate (DEPC) (Sigma-Aldrich) -treated water (Appendix II) was used to resuspend the pellet and the concentrations of RNA were determined using the Nano-drop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc.), by measuring the optical density (OD) at 230, 260 and 280nm. Once the concentrations were determined, 100µg of RNA from each sample was diluted in DEPC-treated water to give a final volume of 100µl and the Qiagen RNeasy Kit (Qiagen) was used to DNase-treat each sample. To the diluted RNA sample, 350µl of RLT buffer with 1% (v/v) β-mercaptoethanol was added to each tube followed with the addition of 250µl of 100% ethanol and samples were transferred into an RNeasy column (see 2.7.3).

2.7.2 Cells

Cells were directly lysed in the well with 350µl of RLT lysis buffer (Qiagen) and 1% (v/v) β-mercaptoethanol (Amresco). The suspension was passed through a syringe with a 30 gauge needle to ensure complete lysis of the cells and collected in 1.5ml tubes. Samples can either be stored at -20°C or 350µl of 70% (v/v) ethanol was added. Proceed to on column DNase treatment step.

2.7.3 On column DNase treatment

The samples were immediately transferred into an RNeasy column and centrifuged for 30 seconds at 10000xg. The flow through was discarded and the column was washed with RW1 buffer. For on column DNase-treatment, the DNase mixture (70µl RDD buffer and 10µl DNase per column) was directly aliquoted onto the column and allowed to sit at room

temperature for 15 minutes. The column was washed with RW1 buffer followed by 2 washes of RPE buffer, and the column was dried by centrifugation for 2 minutes before the RNA was eluted in DEPC-treated water. The RNA concentration was determined using the Nano-drop® ND-1000 Spectrophotometer and the quality of the RNA was confirmed by running 5µl of RNA sample on a 1% agarose gel. The gel apparatus and tank were cleaned with 5M NaOH and 1X TAE buffer was made up with DEPC-treated water.

2.8 Reverse transcription (RT)-polymerase chain reaction (PCR)

For the synthesis of cDNA, the same total concentration of each RNA sample was used in each experiment. The SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) was used. Each reaction contained 1µl of random hexamers and 1µl dNTP mix and the final volume of 10ul was achieved with DEPC-treated water. The samples were incubated at 65°C for 5 minutes and then chilled on ice for at least 1 minute. Meanwhile, a cDNA synthesis mix consisting of 4µl of 5x First-strand buffer, 4µl of $MgCl_2$, 2µl DTT, 1µl of RNaseOut and 1µl of superscript III enzyme was prepared. Superscript III enzyme was substituted for DEPC treated water in the negative RT-PCR control. To each RNA sample, 10µl of cDNA synthesis mix was added and the samples were incubated at 25°C for 10 minutes, 50°C for 1 hour and the enzyme was heat inactivated at 85°C for 5 minutes. To ensure that the samples did not contain single stranded RNA, 1µl of RNase H was added to each tube and incubated at 37°C for 20 minutes. The cDNA was either stored at -20°C prior to quantitative-PCR (qPCR) analysis or used immediately.

2.9 Quantitative real-time PCR (qRT-PCR)

A relative comparative threshold method was employed in the analysis of qRT-PCR. β-Actin or 18S, which is a house-keeping gene, were commonly used as a reference gene.

A mastermix, with a final volume of 8µl, consisted of 5µl of SYBR magic mix (Applied Biosystems), 0.2µl of forward and reverse primers (100µM), 2.6µl of DEPC-treated water, per well of 384 well detection plate (Applied biosystems). The sequences of the forward and reverse primers used in qPCR are shown in Appendix III. Once the mastermix was aliquoted into the appropriate wells, 2µl of cDNA was added to each well. The plates were sealed and subjected to qPCR conditions which were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A melting curve was included at the end of the 40 cycle reaction to detect the formation of primer-dimers. The reactions took place in a 7900HT Fast Real-time PCR system and the results were analysed using the SDS2.3 software.

2.10 Protein analysis

2.10.1 Preparing protein lysates from tissues

A small piece of tissue was isolated and placed into a tube containing 800µl of lysis buffer, which consisted of 1ml of KalB buffer (Appendix II). Once the tissue was completely homogenised, the supernatant was transferred into a 1.5ml tube and placed on the wheel in the cold room for 30 minutes. The suspension was centrifuged at 10000xg for 3 minutes to remove the debris and the supernatant was collected in a fresh tube. To this, 20µl of sepharose G beads (Phamacia Biotech) was added and the samples were placed on the wheel in the cold room for another 30 minutes. The sepharose G beads were removed by

centrifugation at 3000xg for 3 minutes and the concentration of protein present was determined by performing a Lowry assay.

2.10.2 Preparing protein lysates from cells

To obtain protein lysates from cells, the same lysis buffer, as mentioned above, was used. The lysis buffer was directly added into each well and the cells were scrapped off using a cell scrapper. The lysates were collected in 1.5ml tubes and placed on the wheel in the cold room for 30 minutes. The samples were then centrifuged at 10000xg for 3 minutes to remove any cell debris and the supernatants were collected in a fresh tube. Protein concentration can be determined using the Lowry protein assay.

2.10.3 Lowry protein assay

BSA protein standards (Sigma-Aldrich) were prepared, in duplicate, with a starting concentration of 20µg and serial dilutions were performed to generate a standard curve. Once 5µl of protein standard and samples were added to each well, 25µl of working reagent (20µl of Reagent S to 1ml of Reagent A, Biorad) was added, followed by the addition of 200µl of Reagent B (Biorad). The plate was gently agitated to mix the reagents and after 15 minutes the absorbance was read at 490nm using the Infinite M200 and results were analysed by the Mars software. From the standard curve, the total protein concentration was determined for each sample.

2.10.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein lysates were diluted with 2x sample buffer (Appendix II) and proteins were reduced by heating at 99°C for 5 minutes. The proteins were separated on a 10% lower

resolving SDS-PAGE gel (Appendix II) with a 4.5% upper stacking gel (Appendix II) at 100 volts for approximately 90 minutes in SDS Running Buffer (Appendix II).

The proteins were transferred onto a nitrocellulose membrane (pore size 45µm) (Amersham) using either the conventional wet transfer method or with IBlot (Invitrogen) according to manufacturer's specifications.

2.10.5 Wet transfer

Prior to the transferring procedure; the nitrocellulose membrane, blotting paper and the polyacrylamide gels were soaked in pre-chilled transfer running buffer (Appendix II). The transfer sandwich was assembled in the following order on the black frame: foam, blotting paper, SDS-PAGE gel, nitrocellulose membrane, blotting paper and foam. The gel was transferred at 100 volts for 90 minutes at 4°C.

2.10.6 Western blot analysis

Western blot analyses were conducted on the nitrocellulose membranes and were blocked with 5% (w/v) BSA in Odyssey blocking buffer (Licor) and 0.1% (v/v) Tween 20 for 1 hour at room temperature or overnight with gentle agitation at 4°C. The primary antibody was added to the membrane with gentle agitation overnight at 4°C, whereby the antibody concentrations are listed in Appendix IV. The membranes were washed using PBS with 0.05% (v/v) tween-20, three times for 5 minutes on a shaker and then incubated with the a fluorescent secondary antibody for 1 hour in the dark (Appendix IV). The Western blots were scanned using the Odyssey scanner (Licor) at the appropriate wavelengths.

Antibodies were removed from the hybridised membranes by incubating the membranes with stripping buffer (Appendix II) for 10 minutes at 55°C with regular shaking. The membranes are washed 2 times for 10 minutes each in PBS and 0.05% (v/v) Tween-20. Membranes are then re-blocked with 5% BSA in Odessey blocking buffer and 0.1% (v/v) Tween 20 for 1 hours with gentle shaking and the western blot analysis was carried out as mentioned above with the appropriate antibodies.

2.11 Proliferation assay

Cell proliferation assays were performed using the EdU Click-iT kit (Invitrogen). Cells were plated at 1 x 10^4 cells per well in a 96-well plate format in the morning and given 6 hours to adhere to the well. Prior to the addition of EdU reagent (10µM per well) the media was replaced with serum-free media and cells were either pre-treated with various signaling pathway inhibitors for 30 minutes followed by P3C stimulation at 10µg/ml. The cells were allowed to proliferate overnight and the assay was completed according to the manufacturer specifications. The fluorescent signal was detected by the Infinite M200 plate reader whereby, the excitation and emission filters were set to 544λ and 590λ, respectively.

2.12 Immunohistochemistry for paraffin-embedded tissue sections

Slides containing 4µm thick tissue sections were dewaxed in the following order for 5 minutes in each step: twice in xylene, twice in 100% ethanol and once in 70% (v/v) ethanol. The slides were thoroughly washed in distilled water twice. Refer to Appendix IV for the optimised protocol for each antibody. To perform the antigen retrieval step, slides were boiled in the appropriate antigen retrieval buffer in the microwave for 2 minutes on

high, followed by another 4 minutes on med-low heat and allowed to cool in the fumehood. The slides were thoroughly washed in distilled water twice and the sections were incubated in 3% hydrogen peroxide in PBS for 30 minutes to quench peroxidase activity. All antibody incubations were conducted in a humidified box. Prior to blocking the sections with CAS block (Invitrogen) for 30 minutes at room temperature, the sections were washed thoroughly in PBS. All primary antibodies were incubated overnight at 4°C and were diluted appropriately in CAS blocking buffer. The sections were washed thoroughly in PBS for 5 minutes and incubated in a biotinylated secondary antibody for 30 minutes at room temperature (diluted appropriately in PBS) whereby, the sections were once again washed thoroughly in PBS for 5 minutes. Whilst the secondary antibody incubation is taking place, the Vectastain ABC reagent (Vector Laboratories) was prepared and kept away from light. The sections were incubated in Vectastain ABC reagent for 30 minutes at room temperature and washed thoroughly in PBS for 5 minutes prior to signal development in peroxidase solution DAB (Dako). This reaction was stopped by rinsing the sections in distilled water. The sections were counterstained in haemotoxylin for 15 seconds and washed thoroughly in distilled water, followed by incubating the sections in Scott's tap water (Appendix II) for 30 seconds and rinsed in distilled water for 2 minutes. The sections were dehydrated in the following order for 10 seconds in each step: 70% (v/v) ethanol, twice in 100% ethanol and twice in xylene.

2.13 Site directed mutagenesis

Site directed mutagenesis was performed on the pTLR2-*luc* construct using the QuikChange II XL Site-Directed Mutagenesis Kit (Strategene). The protocol was carried out according to the manufacturer's specifications whereby, a total of 10ng of pTLR2-*luc* construct was used in each sample reaction.

XL10-Gold ultracompetent cells (Strategene) were used for the transformation of the site directed mutant pTLR2-*luc* construct whereby, a total of 10ng was used for the transformation. The transformation protocol was performed according to the manufacturer's protocol and the competent cells were plated on Luria Broth (LB) agar (Appendix II) with 100µg/ml of ampicillin overnight at 37°C to obtain single colonies. Plasmid preparations were then carried out.

2.14 Plasmid preparations

A single colony was used to inoculate 100ml of LB with 100µg/ml of ampicillin for approximately 16 hours on a shaker (125rpm) at 37˚C. The LB culture was centrifuged at 4000 x g for 10 minutes and the supernatant was discarded. Plasmids were purified using the Maxi PureLink HiPure Plasmid Filter Purification Kit (Invitrogen) and performed according to the manufacturer's protocol. Elution of plasmid DNA was performed using endotoxin-free water and were stored at -20˚C.

CHAPTER 3

The transcriptional regulation of TLR2 by STAT3

3.1 Introduction

PRRs act as our first line of defence against pathogens to elicit an inflammatory response, and as such have attracted much interest as potential candidate contributors to inflammation-associated carcinogenesis (Li *et al*., 2010). For example, up-regulation of TLR4 protein expression in 72% of patients with colorectal adenomas correlated with an increased incidence of tumour metastasis and a poorer prognosis compared to patients that displayed low TLR4 expression (Wang *et al*., 2010). In human GC, genetic variants and/or increased expression of numerous TLRs, especially TLR2, TLR4, TLR5 and TLR9, have been reported (Harris *et al*., 2006), albeit without any definitive evidence for a crucial role in disease pathogenesis. Nonetheless, consistent with these observations, the human gastric epithelium expresses TLR2, TLR4, TLR5 and TLR9, and collectively these receptors have been associated with the recognition of *H. pylori* to promote gastritis-mediated carcinogenesis in the host (Fukata and Abreu, 2008). However, it is important to note that the role of TLR4 in *H. pylori*-mediated GC still remains controversial, as it is questionable as to whether TLR4 recognises whole *H. pylori* bacterium or just *H. pylori* LPS, which has low immunogenicity (Yokota *et al*., 2007).

Of the TLRs that are expressed in the human gastric epithelium, TLR2 has been shown to induce COX-2 expression, an enzyme that mediates gastric inflammation through the production of prostaglandin, in an NF-κB-dependent manner (Chang *et al*., 2004). In addition, TLR2 has been shown to facilitate *H. pylori* induced IL-8 secretion in human

gastric epithelial cell lines via NF-κB and P38 MAPK signalling pathways (Torok *et al*., 2005, Smith *et al*., 2003). With regard to the involvement of TLR2 in *H. pylori*-dependent GC, the TLR2 -196 to -174ins/ins genetic polymorphism is strongly associated with human intestinal-type GC (Tahara et al., 2008). Given that Noguchi *et al.,* (2004) demonstrated that the TLR2 -196 to -174del/del genetic polymorphism displays reduced transcriptional activity compared to the TLR2 -196 to -174ins/ins genetic polymorphism, Tahara *et al.,* (2008) proposed that increased TLR2 activity may play a role in promoting gastric carcinogenesis.

As discussed earlier in Chapter 1, there is strong clinical evidence for the importance of deregulated STAT3 activation in GC (Yu *et al*., 2009). These observations have also been supported by the *gp130*$^{F/F}$ GC mouse model displaying IL-11 driven hyper-activation of STAT3, which spontaneously develop gastritis and gastric adenomatous lesions (in a STAT3-dependent manner) from 6 weeks of age onwards (Tebbutt *et al*., 2002). To investigate whether there was a correlation between STAT3 hyper-activation and the up-regulation of specific TLRs in gastric tumourigenesis, this well characterised *gp130*$^{F/F}$ mouse model for gastritis associated tumourigenesis was initially utilised.

3.2 STAT3 hyper-activation in the gastric tumours of *gp130*$^{F/F}$ mice correlates with augmented expression levels of *Tlr2*.

The initial step of this project was to identify if there were any significant changes in the expression of PRRs in the gastric tumours of *gp130*$^{F/F}$ mice. Gene expression profiling of 84 TLR-associated genes was performed on the gastric antral tumours of 24 week old *gp130*$^{F/F}$ mice compared to *gp130*$^{+/+}$ antrum samples. As listed in Table 3.1, 9 out of the 13 genes that were at least 2-fold up-regulated in the gastric tumours of *gp130*$^{F/F}$ mice are NF-

κB regulated genes. Interestingly, among the TLR family members, it was observed that *Tlr2* and *Tlr9* were up-regulated in the gastric tumours of *gp130*$^{F/F}$ mice. Given the association between TLR2 and GC, *Tlr2* gene expression was further validated using quantitative real-time PCR (qRT-PCR), while *Tlr4* was used as a negative control as it was not up-regulated in *gp130*$^{F/F}$ gastric tumours. A significant increase in the expression level of *Tlr2* was detected in the gastric tumours of *gp130*$^{F/F}$ mice compared to *gp130*$^{+/+}$ non-tumour gastric tissues (Figure 3.1). To illustrate that the significant increase in *Tlr2* gene expression was a direct consequence of increased STAT3 activation and target gene expression and not due to the transformation status of the *gp130*$^{F/F}$ gastric epithelium versus non-transformed *gp130*$^{+/+}$ gastric epithelium, gene expression data was compared between gastric tumour tissue from *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice, the latter displaying lesser STAT3 activation (Judd *et al*., 2006). My results demonstrated that the expression profile of *Tlr2* mRNA correlated with that of mRNA levels for the STAT3 target genes, *Stat3* and *Il-11*, since *Tlr2* mRNA levels were significantly reduced in the gastric tumour tissue of *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice (Figure 3.1). Furthermore, augmented levels of *Tlr2* gene expression were strongly associated with the severity of gastric disease, since *Tlr2* mRNA levels in *gp130*$^{F/F}$:*Il11r*$^{-/-}$ mice, that do not develop gastric disease, were reduced to *gp130*$^{+/+}$ levels (Ernst *et al*., 2008).

Further qRT-PCR analyses were performed on *Nod1* and *Nod2* since these NLRs have been implicated in *H. pylori*-mediated GC (Girardin *et al*., 2003). Since approximately 20% of *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice develop gastric adenomatous lesions, qRT-PCR analyses were conducted on only two gastric tumour samples from *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice, and as expected there were no gene expression differences in the gastric tumour and non-tumour tissue of *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice (Figure 3.1). Surprisingly, a significant reduction in *Nod1*

mRNA was observed in the gastric tumours of *gp130*$^{F/F}$ mice when compared to *gp130*$^{+/+}$ antral tissue, while no differences in *Nod2* gene expression was observed in the compound *gp130*$^{F/F}$ mutants (Figure 3.1). By contrast, a significant increase in the expression of TLR2-regulated inflammatory genes, such as *IL-1β* and *Cxcl2*, was also observed in the *gp130*$^{F/F}$ gastric tumour tissue that displayed elevated *Tlr2* gene expression levels (Figure 3.2). Collectively, these results suggest that in the gastric tumours of *gp130*$^{F/F}$ mice there is a strong correlation between the up-regulation of *Tlr2* gene expression and augmented mRNA levels of STAT3-target genes, such as *Stat3* and *Il-11*.

3.3 Over-expression of a hyper-active STAT3 mutant in gastric epithelial cell lines results in a significant increase in *TLR2* gene expression levels.

To determine whether STAT3 could directly up-regulate *Tlr2* gene expression, preliminary transient transfection experiments were conducted in a mouse gastric epithelial cell line (IMGE-5) to identify the optimal transfection concentration of a hyper-active STAT3 green-fluorescent protein (GFP)-fusion expression construct (STAT3-C/GFP). Transient transfection of increasing concentrations of STAT3-C/GFP in IMGE-5 cells resulted in an increase in endogenous *Tlr2*, but not *Tlr4*, mRNA as determined by qRT-PCR (Figure 3.3), with 500ng appearing as an optimal dose with no obvious toxicity to cells (based on cell morphology under light microscopy). As illustrated in Figure 3.3B, IMGE-5 cells that stably over-express 500ng of STAT3-C/GFP displayed a significant increase in endogenous *Tlr2* gene expression and not *Tlr1, Tlr4* and *Tlr6* when compared to IMGE-5 cells that over-express a GFP-empty vector (control). GFP immunoblot assays were performed on both IMGE-5 cells that were stably transfected with a GFP-empty vector or STAT3-C/GFP expression construct and these confirmed that the transfections were efficient (Figure 3.3C).

Due to the low sequence homology (6-10%) between the 5′ promoter region of human and mouse TLR2, these findings were also validated in 2 human cancer cell lines, MKN-28 and AGS cells (Haehnel *et al*., 2002). As expected, MKN-28 cells that stably over-express 500ng of STAT3-C/GFP only displayed a significant increase in *TLR2* but not *TLR1, TLR4* and *TLR6* mRNA (Figure 3.4A). GFP immunoblot assays performed on MKN-28 cells confirmed that good transfection efficiencies were achieved with the STAT3-C/GFP construct (Figure 3.4B). In addition, gene expression data were observed in AGS cells that stably over-express 500ng of STAT3-C/GFP, with again only endogenous *TLR2* mRNA levels being significantly up-regulated in STAT3-C/GFP transfected cells (Figure 3.4C). Therefore, these results strongly suggest that STAT3 can directly up-regulate *TLR2* gene expression levels in gastric epithelial cells.

3.4 STAT3 binds to the 5′ promoter region of TLR2 at position -1241 to initiate transcription of TLR2.

Over 10 years ago, promoter analyses demonstrated that within the 5′ promoter region of the mouse *Tlr2* gene there are two putative NF-κB, one C/EBP, one CREB and one STAT binding site (Musikacharoen *et al*., 2001). The consensus sequence for a STAT binding site is $TTCN_{2\text{-}4}GAA$, however it was demonstrated by Ehret *et al* (2001) that many variations of this consensus sequence exist due to the formation of STAT homodimers and heterodimers (e.g. STAT1/STAT3). Given the advancements in recent years with bioinformatic tools for predicting transcription factor binding sites within promoters, often based on experimentally-validated data, we performed our own bioinformatics analysis of the 5′ promoter region of mouse TLR2 to thoroughly investigate the presence of additional putative STAT3 binding sites using TRANSFAC and MATCH databases (Pairo *et al*.,

2012). These analyses revealed that five putative STAT3 binding sites, as shown in Figure 3.5A. Importantly, bioinformatic analyses also revealed four putative STAT3 binding sites in human *TLR2* gene promoter, and for simplicity, the putative STAT3 binding sites in the 5′ promoter region of mouse and human TLR2 are represented schematically in Figure 3.5B. To identify which STAT3 binding sites are important for the recruitment of STAT3 to the mouse TLR2 promoter, chromatin immunoprecipitation (ChIP) assays were carried out in our laboratory (by Catherine Kennedy) on IL-11 stimulated IMGE-5 cells that stably over-express the wild-type STAT3 (STAT3-WT/GFP) construct. In this experiment, 3 primer sets were designed to amplify the five putative STAT3 binding sites and it was discovered that tyrosine-phosphorylated STAT3 was recruited to the 5′ Tlr2 promoter region at position -1241 (Figure 3.6A). SOCS3 was used as a positive control as it has been shown that the bona fide STAT3 binding site is located at position -70 in the 5′ promoter region of the mouse *Socs3* gene (Zhang *et al*., 2006). In addition, qRT-PCR amplification of the TLR2 promoter region containing the -1241 to -1248 STAT3 binding site that was immunoprecipitated by pY-STAT3 antibody was up-regulated in response to IL-11 stimulation (Figure 3.6B).

Transcriptional regulation of TLR2 by STAT3 was further demonstrated through TLR2-promoter luciferase reporter assays in human embryonic kidney (HEK)-293 cells. To determine the necessity of the STAT3 binding site at position -1241 in the 5′ promoter region of the mouse *Tlr2* gene, a truncated (TLR2-1248) and site directed mutant (TLR2 -1241 TT>AA) TLR2-promoter luciferase reporter constructs were used, as described in Figure 3.7A. In support of the ChIP assays, transient over-expression of STAT3-C/GFP could only positively trans-activate the full length wild-type TLR2-promoter reporter

construct (Figure 3.7B). Collectively, these results suggest that the -1241 STAT3 binding site is essential for the recruitment and transcription of TLR2 (in the mouse) by STAT3.

3.5 Discussion

In this chapter, my results strongly support the notion that TLR2 is a novel STAT3 target gene, and the up-regulation of TLR2 correlated with hyper-activation of STAT3 and the severity of gastric disease. My results showed that gastric tumours of *gp130*$^{F/F}$ mice expressed augmented levels of *Tlr2* mRNA, which are reduced to wild-type levels in the gastric tumours of *gp130*$^{F/F}$:Stat3$^{-/+}$ mice, thus indicating that STAT3-driven up-regulation of TLR2 plays a role in gastric disease. These findings are of significance considering that STAT3 is up-regulated in approximately 50% of human GC patients, thus suggesting (at this point of my thesis) that increased activation of STAT3 in human GC may result in augmented levels of TLR2 to enhance the responsiveness of the gastric epithelium to TLR2 ligands (currently unknown) and subsequently cause chronic gastritis. Importantly, my results illustrated that augmented mRNA levels of *Il-11*, *Stat3* and *Tlr2* were detected in gastric tumour tissue from *gp130*$^{F/F}$ mice and not in gastric tumour tissue of *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice, indicating that the altered *Tlr2* gene expression pattern correlated with the extent of IL-11/STAT3 signalling and is not due to differences in the transformation status of the gastric tissue. This correlation between *Il-11*, *Stat3* and *Tlr2* gene expression in the intestinal-type tumours of *gp130*$^{F/F}$ mice is also of note considering that Nakayama *et al* (2007) demonstrated a statistically significant increase in IL-11 protein expression in intestinal-type adenocarcinoma compared to diffuse-type adenocarcinoma, although the STAT3 activation status was not documented. Furthermore, TLR2-regulated inflammatory genes which have also been implicated in GC, such as *Il-1β* and *Cxcl2* (IL-8) (Tu *et al*., 2008, Torok *et al*., 2005) were significantly increased in

gp130$^{F/F}$ gastric tumours where TLR2 expression was highest, which further invokes the possibility that that TLR2 regulated genes may be playing a role in gastric tumourigenesis

A key finding in this chapter was that STAT3 binds to the 5′ promoter region of TLR2 at position -1241. However, at present it is unknown whether the regulation of *Tlr2* by STAT3 is mediated via either STAT3 homodimers or heterodimers with other STAT proteins, for instance STAT1. My results from Figure 3.3 and Figure 3.4 suggest that the transcription of *Tlr2* is most likely regulated by STAT3 homodimers, since upon transfection of STAT3-C/GFP in mouse and human gastric epithelial cells a direct up-regulation of *TLR2* mRNA was observed. The STAT3-C construct was initially generated by Bromberg *et al* (1999) by substituting cysteine residues at position A661 and N663 to enable the formation of disulphide bonds between two STAT3 monomers. In addition, Bromberg *et al* (1999) demonstrated that STAT3-C was able to translocate into the nucleus to initiate the transcription of CyclinD1 (STAT3 target gene) regardless of the phosphorylation status. To address the involvement of STAT3 and STAT1 heterodimers in the transcription of the *Tlr2* gene, ChIP analyses can be performed on the gastric tumour tissues from *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice, since these mice lack STAT1. Furthermore, as discussed in Chapter 1, phosphorylated and unphosphorylated STAT3 can act as a transcription factor, whereby the latter can interact with NF-κB to initiate the transcription of unique STAT3 target genes, such as *Il-6* (Yang *et al*., 2007). Therefore it is worthy to note that the interaction of unphosphorylated STAT3 with NF-κB may facilitate the transcription of the *Tlr2* gene. Nevertheless, ChIP analyses in Figure 3.6 revealed that pY-STAT3 is recruited to the 5′ promoter region of TLR2 at position -1241, following which it would be presumed to initiate *Tlr2* gene transcription, thus suggesting that the phosphorylation of STAT3 is necessary for the transcription of the *Tlr2* gene.

Surprisingly, a significant down-regulation of *Nod1* mRNA levels was observed in the gastric tumours of *gp130*$^{F/F}$ mice**.** NOD1 has been shown to play an essential role in the recognition of *H. pylori* PG to initiate an inflammatory response in gastric epithelial cells (Watanabe *et al*., 2010). One possible explanation for this observation could be that the loss of SHP2-mediated ERK MAPK or PI3K/AKT signalling pathways in *gp130*$^{F/F}$ mice (Tebbutt *et al*., 2002) may be important in the regulation of NOD1. In addition, it is conceivable that STAT3 activation may suppress the transcription of *Nod1*, especially since *gp130*$^{F/F}$ *Nod1* gene expression levels were restored to wild-type levels in *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice.

While another TLR that was up-regulated in the gastric tumours of *gp130*$^{F/F}$ mice is TLR9 (Table 3.1), I chose to further explore the role of TLR2 in gastric tumourigenesis for a number of reasons. Firstly, our recent bioinformatic analyses suggest that the 5′ TLR2 promoter region contains five putative STAT3 binding sites. Secondly, among TLRs, TLR2 can recognise the broadest range of PAMPs by forming hetero-dimers with TLR1 and TLR6. Thirdly, TLR2 has been more extensively studied in human GC than TLR9, as it recognises *H. pylori* to induce the transcription of pro-inflammatory mediators (Mandell *et al*., 2004, Smith *et al*., 2003). Finally, the TLR2 -196 to -174ins genetic polymorphism is strongly associated with human intestinal-type GC (Tahara *et al*., 2008), as this polymorphism is known to regulate TLR2 promoter activity (Noguchi *et al*., 2004). However, I do acknowledge the importance of further investigating TLR9 in gastric tumourigenesis as a study suggested that TLR9 can recognise *H. pylori* to induce COX2-mediated gastric epithelial cell invasion and angiogenesis (Chang *et al*., 2005). Furthermore, a human SNP of TLR9 (-1237T/C) that increases the transcription of TLR9 is

associated with increased risk to *H. pylori*-induced GC in the Caucasian population (Ng *et al*., 2010). Interestingly, it was demonstrated that the -1237T/C TLR9 polymorphism introduces an IL-6 response element and as a consequence of this it was proposed that IL-6-mediated STAT3 activation results in augmented levels and responsiveness of TLR9 to unmethylated CpG DNA (Carvalho *et al*., 2011).

CHAPTER 4

TLR2 promotes tumourigenesis independent of inflammation in *gp130*$^{F/F}$ mice

4.1 Introduction

The role of PRRs in microbial-driven inflammation-associated carcinogenesis has been widely investigated because PRRs are known to initiate an inflammatory response to PAMPs (O'Neill, 2008). For instance, the importance of TLRs was demonstrated in the AOM-induced mouse model for CAC (*Il-10*$^{-/-}$), since the absence of MyD88 in AOM-treated *Il-10*$^{-/-}$ mice prevented the development of neoplastic lesions compared to *Il10*$^{-/-}$ mice, thus suggesting a role for MyD88-dependent TLR signaling in promoting inflammation-associated carcinogenesis (Uronis *et al*., 2009). In GC, *H. pylori* has been identified as a causative agent, whereby it is known to strongly induce gastritis in the host, through the recognition of PRRs such as NOD1 and TLR2 (Viala *et al*., 2004, Mandell *et al*., 2004). Despite this, a clinical study revealed that 25% of individuals with GC are *H. pylori*-negative, which is associated with a lower survival rate compared to *H. pylori*-positive patients, thus suggesting that other factors (e.g. gastric microflora composition, genetic polymorphisms in PRRs and inflammatory genes) in these patients may contribute to disease pathogenesis (Meimarakis *et al*., 2006). In this respect, Chapter 1 discussed the involvement of TLR2 -196 to -174ins/ins genetic polymorphism, which displayed higher transcriptional activity compared to the TLR2 -196 to -174ins/del mutant (Noguchi *et al*., 2004) and is associated with the severity of intestinal-type GC (Tahara *et al*., 2008). Furthermore, antibiotic treatment experiments in mouse models for inflammation-

associated gastro-intestinal carcinogenesis demonstrated that the reduction of bacteria suppressed the onset of inflammation and tumour formation (Judd *et al*., 2006, Uronis *et al*., 2009). With respect to the latter, the treatment of *gp130*$^{F/F}$ mice with broad-spectrum antibiotics for 8 weeks significantly reduced the gastric tumour burden by approximately 70% (Judd *et al*., 2006). Intriguingly, the amount of pY-STAT3 in the gastric tumours of *gp130*$^{F/F}$ and antibiotic-treated *gp130*$^{F/F}$ mice were unchanged, thus suggesting that a mediator downstream of IL-11/STAT3 signalling is playing a crucial role in gastric tumourigenesis (Judd *et al*., 2006). Given that I have demonstrated that TLR2 is a novel STAT3 target gene, these data highlight the significance of investigating the role of TLR2 inflammatory responses in STAT3-driven tumourigenesis in *gp130*$^{F/F}$ mice.

4.2 Genetic deletion of TLR2 in *gp130*$^{F/F}$ mice suppresses gastric tumourigenesis

The *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mouse was generated to investigate the role of TLR2 in STAT3-driven gastric tumourigenesis. Since *gp130*$^{F/F}$ mice start to develop hyper-proliferative lesions and gastritis from 6 weeks of age, and fully develop severe gastric hyperplasia and adenomatous lesions by 24 weeks, *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice were aged to 24 weeks (Jenkins *et al*., 2005). Interestingly, macroscopic examination of the gastric compartment of *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice revealed that the gastric tumour burden was significantly reduced by approximately 50% compared to *gp130*$^{F/F}$ male mice (Figure 4.1A). However, there appeared to be a gender bias, since the reductions in stomach and tumour mass were more pronounced in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ (versus *gp130*$^{F/F}$) male mice compared to *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ (versus *gp130*$^{F/F}$) female mice (Figure 4.1B-C). In addition, the number of tumours, which are classified according to size, were significantly reduced in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice compared to *gp130*$^{F/F}$ mice but these reductions were not observed in the female counterparts (Figure 4.1D-E).

My data presented in Chapter 3 demonstrated that TLR4 was not a STAT3 target gene nor was it up-regulated in the gastric tumours of *gp130*$^{F/F}$ mice (Figure 3.1). Therefore, *gp130*$^{F/F}$:*Tlr4*$^{-/-}$ mice were generated to confirm the specificity of the role of TLR2 in STAT3-mediated gastric disease. Data collected in our laboratory (by Meri Najdovska), revealed that the appearance of the gastric compartment of *gp130*$^{F/F}$:*Tlr4*$^{-/-}$ mice was identical to *gp130*$^{F/F}$ mice at 24 weeks of age (Figure 4.2A), whereby the stomach and tumour mass, and the number of tumours in *gp130*$^{F/F}$:*Tlr4*$^{-/-}$ mice resembled *gp130*$^{F/F}$ mice (Figure 4.2B-D). In addition, H&E-stained gastric cross-sections of 24 week old *gp130*$^{F/F}$:*Tlr4*$^{-/-}$ mice revealed the presence of marked gastritis, as determined by inflammatory aggregates in the sub-mucosa layer and the infiltration of neutrophils, mast cells, plasma cells and eosinophils. Therefore, these results suggest that TLR2, but not TLR4, is playing an important role in promoting gastric tumourigenesis in *gp130*$^{F/F}$ mice.

4.3 TLR2 is expressed in the gastric surface epithelium and in immune cells

Since TLR2 promotes gastric tumourigenesis in *gp130*$^{F/F}$ mice, I wanted to determine where TLR2 is expressed in the stomach. The expression of TLR2 was determined by immunohistochemical staining of gastric cross-sections from 24 week old *gp130*$^{F/F}$ male mice. Representative photomicrographs of TLR2-stained gastric cross-sections indicated that TLR2 was specifically expressed within the surface epithelial layer of the gastric mucosa, and also in immune cells that are present in the sub-mucosa (Figure 4.3A-B). In contrast, immunohistochemical staining with the matching IgG control to the TLR2 antibody revealed that there was no non-specific binding of the TLR2 antibody in the gastric cross-sections from 24 week old *gp130*$^{F/F}$ male mice (Figure 4.3C).

4.4 TLR2-mediated inflammatory response is impaired in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice

To ensure that the significant reduction in tumour size observed in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice was not due to changes in the IL-11/STAT3 signalling axis but was rather specifically TLR2-mediated, the levels of pY-STAT3 and IL-11 proteins were determined. As shown in Figure 4.4, immunoblot analyses of representative protein lysates from the gastric tumours of 24 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice confirmed that the levels of pY-STAT3 and IL-11 were unchanged in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice. The amounts of total STAT3 and actin proteins were also determined and served as loading controls in this experiment.

Given that TLR2 is an important regulator of inflammation, qRT-PCR analyses were performed on gastric tumours from 24 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice to determine if genetic ablation of TLR2 affected the transcription of TLR2-regulated pro-inflammatory mediators in the gastric compartment. TLR2-regulated inflammatory genes, such as *Cxcl2*, *Il-1β* and *Tnf-α,* but not *Il-6*, were significantly reduced in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice compared to *gp130*$^{F/F}$ mice (Figure 4.5). In addition, since the gastric epithelium is the first line of defense against pathogens, primary gastric epithelial cells (pGECs) were isolated from 4 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice in order to investigate whether TLR2-regulated inflammatory genes were down-regulated specifically in the gastric epithelium, where TLR2 is also expressed as shown in Figure 4.3. Upon stimulation of pGECs with 24 week old *gp130*$^{F/F}$ gastric microbial-containing homogenate for 12 hours, the secretion of IL-6 and TNF-α proteins by pGECs was determined in the culture supernatants by ELISA. As shown in Figure 4.6, there was a significant reduction in the secretion of TNF-α in pGECs isolated from *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice compared to pGECs from *gp130*$^{F/F}$ mice, and there was also a slight reduction in IL-6 secretion, albeit not significant. Collectively, these data indicate that genetic deletion of TLR2 in *gp130*$^{F/F}$ mice

significantly dampens the TLR2-regulated inflammatory responses in the gastric epithelium.

4.5 Gastric inflammation is not suppressed in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice

In light of the findings in the previous sections of this chapter, I next investigated whether the reduced tumourigenesis and gastric epithelial inflammatory responses in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice correlated with reduced gastritis. For this purpose, H&E stained cross-sections of gastric tissue from 24 week old *gp130*$^{+/+}$, *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice were initially generated (Figure 4.7A). As indicated by the arrows in the photomicrographs in Figure 4.7A, both *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice displayed large inflammatory foci in the gastric sub-mucosa. Furthermore, these inflammatory foci were predominantly composed of B-lineage cells as shown by the B220 immunohistochemical staining of the sub-mucosa from 24 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice (Figure 4.7B). High powered magnification of H&E stained cross-sections of the gastric sub-mucosa of 24 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice also indicated that there were no considerable differences in the recruitment of inflammatory cell types, such as plasma cells, eosinophils, neutrophils, lymphocytes and mast cells (Figure 4.7C). To confirm this result, FACS analyses performed in our laboratory (by Catherine Kennedy) were conducted on gastric single cell suspension from 24 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice using inflammatory cell markers to identify the percentages of inflammatory cells present in the stomach (Figure 4.8A). These results indicated that the percentage of cells that were B220^{+} (B cells), CD3^{+} (T cells), CD11b^{+}Gr-1^{+} (MDSCs) and CD11b^{+}F4/80^{+} (monocytes/macrophages) remained unchanged in both genotypes. In addition, to ensure that TLR2 was not playing a role in the differentiation and activation of B and T cells, FACS analyses showed that the proportions of B220^{+}CD86^{+}, CD4^{+}CD69^{+} and

$CD8^+CD69^+$ cells were similar in gastric single cell suspensions from 24 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice (Figure 4.8B). As previously mentioned in Chapter 4.4, since the TLR2-mediated inflammatory response is significantly suppressed in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice, these data suggest the existence of other mechanisms for gastritis, which will be further discussed in Chapter 4.7.

4.6 TLR2-expressing hematopoietic cells do not contribute to gastric tumourigenesis

Since TLR2 is also expressed on immune cells, bone marrow chimera experiments were carried out to exclude the role of TLR2-expressing hematopoietic cells in STAT3-mediated gastric tumourigenesis. Reconstitution of recipient *gp130*$^{F/F}$ mice, that were lethally-irradiated, with autologous (*gp130*$^{F/F}$) and heterologous (*gp130*$^{F/F}$:*Tlr2*$^{-/-}$) bone marrow was performed at 8 weeks of age and mice were culled at 22 weeks. My results confirm that TLR2-expressing hematopoietic cells play a limited role in gastric tumourigenesis, since gastric disease was not alleviated in *gp130*$^{F/F}$ recipient mice receiving heterologous *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ donor bone marrow (Figure 4.9A). Furthermore, reciprocal reconstitution of recipient *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice with *gp130*$^{F/F}$ donor bone marrow did not exacerbate gastric disease (Figure 4.9B), thus demonstrating that TLR2-expressing hematopoietic cells do not play a primary role in promoting gastric tumourigenesis.

4.7 Discussion

My results in this chapter demonstrated that genetic ablation of TLR2 suppressed gastric tumour formation in *gp130*$^{F/F}$ mice, which suggests, for the first time, that TLR2 has a pro-tumour role in GC. This observation is consistent with a previous report showing that MyD88, a downstream effector molecule which is required for most TLR signalling, including that of TLR2, played an important role in intestinal tumourigenesis (Rakoff-

Nahoum and Medzhitov, 2007). Specifically, genetic ablation of *MyD88* in *Apc*$^{min/+}$ mice, which spontaneously developed intestinal adenomas in the small intestine, significantly reduced the number and mass of intestinal adenomas, therefore suggesting that TLR signalling plays a crucial role in promoting tumourigenesis (Rakoff-Nahoum and Medzhitov, 2007). Interestingly, my data showed that the reduction in gastric tumour growth was more pronounced in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ versus *gp130*$^{F/F}$ male mice compared to the female counterparts, thus suggesting that a component of gastric tumourigenesis may be hormonally regulated. In this respect, a study demonstrated that estrogen has an inhibitory effect on IL-6, whereby 100% of male mice with higher levels of circulating serum IL-6 compared to 10-30% of female mice develop diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC), which suggested that there was an inverse correlation between estrogen levels and IL-6 production (Naugler *et al*., 2007). In addition, Naugler *et al* (2007) showed a significant reduction in HCC tumour incidence in *Il6*$^{-/-}$ male mice compared to wild-type male mice but no differences were observed in the female counterparts, therefore indicating that increased IL-6 production in male mice, due to low levels of estrogen, facilitated the formation of DEN-induced HCC. Although the mechanism(s) underlying the marked decrease in the gastric tumour burden in male versus female *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice remain unknown, it is plausible that a sex hormone, such as testosterone or estrogen, may be regulating gastric tumourigenesis, which itself is worth further investigation.

Surprisingly, the reduction in gastric tumour burden in *gp130*$^{F/F}$ mice was not due to the suppression of TLR2-mediated gastritis. Since the prominent decrease in gastric tumour burden was only observed in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$, and not *gp130*$^{F/F}$:*Tlr4*$^{-/-}$ mice, compared to *gp130*$^{F/F}$ mice these results suggest that this phenomenon is specific to TLR2 signalling in

gastric tumourigenesis. Quantitative gene expression analyses revealed that TLR2-regulated inflammatory genes, such as *Cxcl2, Tnf-α* and *Il-1β* were significantly reduced in the gastric tumours of *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice compared to *gp130*$^{F/F}$ mice. Furthermore, *Il-6* gene expression levels in the gastric tumour of *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice, as well as IL-6 secretion from *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ pGECs stimulated with microbial-containing *gp130*$^{F/F}$ gastric homogenate were reduced but not statistically significant is consistent with a previous report that *Il-6* gene expression is largely regulated by STAT3 (Greenhill *et al*., 2011), which remains hyperactivated in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice. In addition, these results confirm that the down-regulation of TLR2-regulated inflammatory genes in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice, are due to the reduced responsiveness of the *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ gastric epithelium instead of the inflammatory cells that are present in the gastric mucosa.

Studies show that pro-inflammatory mediators such as CXCL2 and IL-1β are involved in the recruitment of immune cells (Tu *et al*., 2008, Waugh and Wilson, 2008). Interestingly, transgenic mice that over-express *hIL-1β* and spontaneously developed gastritis and gastric hyperplasia, display increased MDSCs, T cells, macrophages and neutrophils in the gastric mucosa compared to wild-type mice, thus suggesting that IL-1β may be involved in the recruitment of these inflammatory cell subsets (Tu *et al*., 2008). Since my data demonstrated that similar inflammatory cell subsets, such as plasma cells, neutrophils, eosinophils, mast cells and lymphocytes were observed in the gastric mucosa of both *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ male mice, these results suggest that CXCL2 and IL-1β may not be essential for the recruitment of leukocytes during gastritis in this mouse model. Moreover, FACS analyses also confirmed that there were no significant changes in the activation status and recruitment of B cells (B220^{+}), T cells (CD3^{+}), MDSCs (CD11b^{+}GR-1^{+}) and monocytes/macrophages (CD11b^{+}F4/80^{+}) in the stomach. Consistent with my data,

Rakoff-Nahoum and Medzhitov (2007) also noted that there were no differences in leukocyte infiltrates in the intestine of *Apc*$^{Min/+}$*:Myd88*$^{-/-}$ mice and *Apc*$^{Min/+}$ mice (the former protected against spontaneous intestinal adenoma formation), which suggested MyD88-dependent TLR signalling does not contribute to intestinal tumourigenesis through the recruitment of inflammatory cells.

Previously, our laboratory has performed bone marrow reconstitution studies to demonstrate that the role of hematopoietic cells in gastric disease is negligible (Jenkins *et al*., 2005). Consistent with this, I showed in this chapter that TLR2-expressing hematopoietic cells do not promote gastric tumourigenesis in *gp130*$^{F/F}$ mice. The bone marrow chimera studies in this chapter also revealed that recipient *gp130*$^{F/F}$ mice receiving donor *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ bone marrow do not display reduced gastric tumour burden, and conversely recipient *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice receiving donor *gp130*$^{F/F}$ bone marrow did not show an increase in gastric disease. Collectively, these data suggest that TLR2 promotes gastric disease that is independent of inflammation, thus suggesting an alternative mechanism by which TLR2 contributes to gastric disease is warranted.

In support for the initiation of gastric tumourigenesis in *gp130*$^{F/F}$ mice being independent of inflammatory cells, the role of T and B cells have previously been shown to not play an essential role in STAT3-mediated gastric tumourigenesis, since *gp130*$^{F/F}$*:Rag*$^{-/-}$ mice, that lack T and B cells, developed gastric adenomatous lesions that were similar to *gp130*$^{F/F}$ mice (Howlett *et al*., 2005). Despite these observations, the recruitment of adaptive (and for that matter innate) immune inflammatory cells is most likely attributed to increased STAT3 activity in these mice, since a previous study demonstrated that *gp130*$^{F/F}$:Stat3$^{-/+}$ (which have normalised STAT3 activity and suppressed gastric disease) display

dramatically reduced levels of inflammatory cells in the gastric mucosa compared to *gp130*$^{F/F}$ mice (Jenkins *et al*., 2005). Although the exact molecular basis by which STAT3 promotes gastritis in *gp130*$^{F/F}$ mice remains unknown, it may involve STAT regulating pro-inflammatory chemokine production to recruit inflammatory cells, as has previously been reported by our laboratory (McLoughlin *et al*., 2005, Fielding *et al*., 2008).

As demonstrated in Table 3.1, it is also worth noting that TLR9 was up-regulated in the gastric tumours of *gp130*$^{F/F}$ mice. Although TLR9 was not further investigated in STAT3-mediated gastric tumourigenesis, it is possible that the level of gastritis observed in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice is attained through TLR9 signalling, as TLR9 recognises unmethylated CpG DNA that is commonly found in bacteria (Kawai and Akira, 2011). With regard to this, little is known about the role of TLR9 in GC, however one group has demonstrated that the recognition of *H. pylori* by TLR9 resulted in the activation of COX-2 (Chang *et al*., 2004), which is known to facilitate gastritis through the production of PGE2 (Kalinski, 2012). As previously discussed in Chapter 1, a transgenic mouse model that over-expressed COX-2 and mPGES-1 spontaneously developed gastric hyperplasia and displayed increased levels of macrophage infiltration (Oshima *et al*., 2004), which may suggest that TLR9 could play a role also in mediating gastritis. In addition, a *TLR9* -1237T/C SNP, which introduces an IL-6 response element (RE) (Carvalho *et al*., 2011) in the 5′ TLR9 promoter region is associated with *H. pylori*-induced GC (Ng *et al*., 2010). While beyond the scope of my thesis, such observations warrant future work to investigate, at the very least, the responsiveness of *gp130*$^{F/F}$ pGECs to TLR9 ligand stimulation, and whether in fact increase TLR9 expression correlates with the extent of STAT3 hyperactivation and gastric disease using various *gp130*$^{F/F}$ compound mutant mice (*gp130*$^{F/F}$*:Stat3*$^{-/-}$, *gp130*$^{F/F}$*:Il-11r*$^{-/-}$).

CHAPTER 5

TLR2 promotes gastric epithelial cell proliferation and survival

5.1 Introduction

While TLR2 is well documented for its inflammatory role, more recently TLR2 has been shown to play an important role in wound healing, angiogenesis, cell proliferation and survival (Macedo *et al*., 2007, Rakoff-Nahoum and Medzhitov, 2007). Angiogenesis is defined by the formation of new blood vessels by endothelial cells to aid in the distribution of nutrients and oxygen, a process which is crucial for tumour growth (Raica *et al*., 2009). As discussed in Chapter 1, mice lacking the TLR-downstream adaptor molecule MyD88 (*Myd88*$^{-/-}$) exhibited a delayed angiogenic response, as there was a decrease in CD31-positive blood vessel formation in the excisional skin wounds of *Myd88*$^{-/-}$ compared to *Myd88*$^{+/+}$ mice (Macedo *et al*., 2007). These observations are also supported by the report that activation of the TLR2/1 heterodimer with a synthetic ligand (Pam3CSK4) in an endothelial cell line, HMVEC, induced the formation of angiogenic tube-like structures and cellular processes that support angiogenesis, such as endothelial cell migration and invasion (Saber *et al*., 2011). With regard to epithelial cell migration during wound healing, it has been shown that the rate of wound closure in human airway epithelial cells (NCI-H292) was accelerated upon stimulation with Pam_3CSK4 (Shaykhiev *et al*., 2008).

While the above studies allude to a role for TLR2 signalling in angiogenesis and wound healing, more compelling evidence has emerged for a role in promoting the growth and survival of epithelial cells. As briefly discussed in Chapter 1, intestinal tumourigenesis was ameliorated in *Apc*$^{Min/+}$:*Myd88*$^{-/-}$ mice compared to *Apc*$^{Min/+}$ mice and the intestinal epithelium of *Apc*$^{Min/+}$:*Myd88*$^{-/-}$ mice displayed increased numbers of TUNEL-positive apoptotic cells, but not BrdU-positive proliferative cells compared to *Apc*$^{Min/+}$ mice, therefore suggesting that TLR signalling may contribute to tumourigenesis by regulating genes involved in anti-apoptosis/cell survival (Rakoff-Nahoum and Medzhitov, 2007). With respect to TLR2 itself, a number of studies have emerged over recent years implicating a role for TLR2 in promoting cell proliferation and survival (Huang *et al*., 2007, Shaykhiev *et al*., 2008). For instance, NCI-H292 cells displayed increased levels of BrdU incorporation when stimulated with Pam_3CSK4, thus demonstrating that TLR2 activation promotes cell proliferation in NCI-H292 cells (Shaykhiev *et al*., 2008). Given the emerging evidence for the non-canonical role of TLR2 in promoting non-immune (e.g. epithelial) cell proliferation, survival and migration, and to a lesser extent angiogenesis, in inflammation-associated carcinogenesis, Chapter 5 will address these cellular processes in gastric tumourigenesis.

5.2 Gastric epithelial cell migration is unaffected upon TLR2 activation

As discussed earlier, although TLR2 activation has been shown to mediate human airway epithelial cell migration, the role of TLR2-mediated cell migration in gastric epithelial cells has not yet been examined. Therefore to address this, my initial experiment was to determine if Pam_3CSK4 could induce gastric epithelial cell migration by performing scratch assays. Given that IL-11 augments *Tlr2* gene expression levels via STAT3 in gastric epithelial cells, IMGE-5 cells were either un-treated or pre-treated with IL-11 for 30

minutes and subsequently stimulated with Pam_3CSK4 for 16 hours. Cell migration of IMGE-5 cells was monitored over a period of 16 hours and the migration rate was calculated in each treatment group. No significant differences in the migration rates were observed in IMGE-5 cells cultured in either the absence or presence of Pam_3CSK4, regardless of whether IMGE-5 cells had been pre-treated with IL-11 (Figure 5.1A). In addition, to mimic the STAT3-hyperactivation seen in *gp130*$^{F/F}$ mice, IMGE-5 cells that stably over-expressed the hyper-active STAT3-C/GFP mutant were subjected to the same *in vitro* experiment. Similarly, no significant differences in the migration rates were observed in Pam_3CSK4-treated IMGE-5 cells stably over-expressing STAT3-C/GFP compared to the un-treated controls (Figure 5.1B). Collectively, these data suggest that Pam_3CSK4 does not mediate mouse gastric epithelial cell migration.

5.3 Genetic ablation of TLR2 in *gp130*$^{F/F}$ mice affects cell survival and proliferation in the gastric epithelium, but not angiogenesis

As shown in Chapter 4, genetic ablation of TLR2 in *gp130*$^{F/F}$ mice dramatically suppressed gastric tumourigenesis and this was more marked in male versus female mice. Accordingly, the immunohistochemical-based studies in this section of my thesis were performed on male mice. To determine if TLR2 promoted angiogenesis in the gastric tumours of *gp130*$^{F/F}$ mice, I performed CD31 immunohistochemical staining on gastric cross-sections of 24 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ male mice. As shown in Figure 5.2A-B, no differences in CD31-positive stained cells were observed in the gastric mucosa and sub-mucosa of *gp130*$^{F/F}$ and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ male mice, while the matching IgG negative control revealed no non-specific binding of this antibody (Figure 5.2C). Collectively, this data indicates that TLR2 does not play a role in promoting angiogenesis in *gp130*$^{F/F}$ mice.

In light of the aforementioned reports demonstrating a role for TLR2 in promoting the proliferation of non-immune cells (e.g. epithelial) (Shaykhiev *et al*., 2008, Huang *et al*., 2007), I examined whether TLR2 may be playing a role in gastric epithelial cell proliferation. To demonstrate this, I performed immunohistochemical staining on gastric cross-sections of 24 week old *gp130*$^{F/F}$ and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ male mice. As shown in Figure 5.3A, in *gp130*$^{F/F}$ mice, PCNA staining was observed throughout the glandular epithelium of the gastric mucosa. By contrast, the staining pattern of PCNA in *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ male mice resembled *gp130*$^{+/+}$ mice, whereby PCNA-positive proliferative cells were only detected within the zone of proliferation and absent in the surface epithelium. I next investigated whether TLR2 was also important for the survival of gastric epithelial cells. As shown in Figure 5.3B, TUNEL-positive apoptotic cells were distinctly present in the surface epithelial layer of *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice, whereas such cells were largely absent in *gp130*$^{F/F}$ or *gp130*$^{+/+}$ mice. Notably, these observations were consistent with my prior data showing that TLR2 expression was restricted to the surface epithelium of the gastric mucosa (Figure 4.3). Therefore, these data imply that TLR2 provides an anti-apoptotic and pro-proliferative role within the gastric tumour epithelium of *gp130*$^{F/F}$ mice.

5.4 TLR2 promotes human gastric epithelial cell proliferation via multiple signalling pathways

To confirm that TLR2 was also playing a role in promoting the proliferation of human gastric epithelial cells, *in vitro* EdU proliferation assays were conducted using MKN-28 cells. The incorporation of EdU in MKN-28 cells was measured 24 hours following stimulation with increasing concentrations of Pam_3CSK4 and these experiments demonstrated a dose-dependent increase in gastric epithelial cell proliferation (Figure

5.4A). In addition, in collaboration with Professor Massanobu Oshima (University of Kanazawa, Japan), the dose-dependent increase in TLR2-induced gastric epithelial cell proliferation in response to Pam_3CSK4 was confirmed in another human gastric epithelial cell line (NUGC4), which is known to express high levels of TLR2 (unpublished data).

Various signalling pathways, such as ERK1/2, JNK and p38 MAPK, PI3K/AKT and NF-κB, have been shown to play a role in cell proliferation (Ding *et al*., 2008, Huang *et al*., 2007, Harashima *et al*., 2012), and it has also been shown that these signalling pathways are also activated by TLR2. Accordingly, I investigated the involvement of these signalling pathways in TLR2-mediated gastric epithelial cell proliferation by performing EdU proliferation assays on MKN-28 cells that were pre-treated with either specific inhibitors that block MAPK, AKT and NF-κB signalling pathways, or DMSO vehicle, prior to Pam_3CSK4 stimulations. Inhibition of ERK1/2 (U0126) and JNK (SP600125) MAPK, and AKT signalling pathways significantly suppressed TLR2-induced MKN-28 cell proliferation in a dose-dependent manner, thus demonstrating that several signalling pathways may be contributing to TLR2-mediated gastric epithelial cell proliferation (Figure 5.5). By contrast, blockade of p38 MAPK with the SB203580 inhibitor had no effect on cell proliferation (Figure 5.5). Interestingly, also shown in Figure 5.5, the blockade of NF-κB (MG132) signalling in MKN-28 cells severely reduced the percentage of proliferative cells in a dose-dependent manner compared to the un-treated controls, therefore suggesting that the inhibition of NF-κB signalling in MKN-28 cells may also induce cell death. Such an observation was previously described by Fan *et al.*, (2001), whereby the treatment with 10μM of MG132 induced DNA fragmentation and apoptosis in AGS and MKN-28 cells. Furthermore, to confirm that each specific inhibitor reduced the signalling activity of their respective signalling pathway, I performed immunoblot assays

on MKN-28 cells that were treated with DMSO or each inhibitor prior to Pam_3CSK4 stimulations for 30 minutes. As shown in Figure 5.5, the immunoblot assays confirmed that each specific inhibitor successfully reduced the signal of their respective signalling pathway.

5.5 The identification of genes involved in TLR2-mediated gastric epithelial cell survival and proliferation

To gain molecular insights into the gene networks that are involved in TLR2-mediated gastric epithelial cell proliferation, in collaboration with Associate Professor Patrick Tan (Genome Institute of Singapore, Singapore) gene microarray analyses were performed on TLR2 agonist-treated human GC cell lines known to either express high or low levels of *STAT3* and *TLR2* (unpublished). As shown in Figure 5.6, both NUGC4 and MKN1 human GC cell lines expressing high levels of *STAT3* and *TLR2* were the most responsive to gene expression changes upon TLR2 activation. By contrast, AZ521 cells which display low levels of *STAT3* and *TLR2* expression displayed the lowest extent of differentially-expressed genes in response to TLR2 stimulation. Interestingly, there was no overlap in the gene expression profile between the cells that express high levels (NUGC4 and MKN1) versus low levels (AZ521) of *STAT3* and *TLR2* in response to TLR2 agonists. However, 19 mutual genes were up-regulated in both NUGC4 and MKN1 cells, of which 3 of these genes are anti-apoptotic molecules (*BCL2A1*, *BIRC3* and *BCL3*) (Figure 5.6). In addition, another gene involved in cell cycle progression (*IER3*) which was highly expressed in NUGC4 cells (also up-regulated in MKN1 cells, but not statistically significant) stimulated with TLR2 agonists was also identified as a potential TLR2-regulated gene. Together with *BCL2L1* (anti-apoptotic), *C-MYC* and *CCND1* genes (pro-proliferative) which have been previously documented to be up-regulated in the tumours of $gp130^{F/F}$ mice (Ernst *et al*.,

2008, Jenkins *et al*., 2005), *BIRC3*, *BCL2A1*, *BCL3* and *IER3* were used as a representative panel of potential TLR2-regulated genes for further validation studies, along with a known TLR2-regulated gene, *IL-8.* Accordingly, MKN-28 cells were stimulated with TLR2 agonists, and then qRT-PCR gene expression assays were performed. My results confirmed that TLR2 signalling induced a significant increase in the expression levels of all 8 genes in MKN-28 cells (Figure 5.7). In addition, since I showed in Figure 4.1 that *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice displayed a reduction in gastric tumour mass, I investigated if these TLR2-regulated genes were also reduced due to the loss of TLR2 signalling in *gp130*$^{F/F}$ mice. As demonstrated in Figure 5.8, all genes examined were significantly down-regulated in *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ tumours compared to *gp130*$^{F/F}$ tumours, except for *Ier3*, thus indicating that the absence of TLR2 in *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice resulted in the down-regulation of genes involved in cell cycle progression and anti-apoptosis. It is therefore tempting to speculate that these genes may contribute to TLR2-induced gastric tumourigenesis.

Given that the inhibition of ERK1/2 and JNK MAPK, PI3K/AKT and NF-κB signalling pathways suppressed gastric epithelial cell proliferation (Figure 5.5), the involvement of these signalling pathways in regulating the expression of these genes downstream of TLR2 was investigated. To demonstrate this, MKN-28 cells were either pre-treated with DMSO or specific inhibitors followed by Pam_3CSK4 stimulation and the gene expression levels were assessed by qRT-PCR. Most genes, such as *BCL2A1*, *BIRC3*, *CCND1* and *C-MYC* were significantly down-regulated by several signalling pathways, however a significant down-regulation of *BCL2L1* and *BCL3* was only observed in MKN-28 cells that had been pre-treated with MG132 (Figure 5.9). Furthermore, no changes in the regulation of *IER3* were observed between DMSO pre-treated-MKN-28 cells compared to cells that were pre-

treated with specific inhibitors, suggesting that an as yet unknown pathway(s) downstream of TLR2 (or a combination of known pathways) plays a role in its regulation.

5.6 Discussion

My results in this Chapter demonstrated, for the first time, that the activation of TLR2 signalling plays an important role in gastric tumourigenesis by promoting gastric epithelial cell proliferation. My data revealed that the activation of TLR2 with Pam_3CSK4 induced gastric epithelial cell proliferation in MKN-28 cells, which was also further validated independently in another human GC cell line, NUGC4. In concordance with my results, Shaykhiev *et al.,* (2008) illustrated that Pam_3CSK4-treated human airway epithelial cells proliferated faster compared to untreated control cells (Shaykhiev *et al*., 2008), thus further confirming the role of TLR2 in epithelial cell proliferation.

Despite these prior studies, my results show for the first time that increased STAT3 activation (up-regulated in 50% of human GC) (Yu *et al*., 2009) results in the direct transcriptional induction of the *TLR2* gene (Chapter 3), which in turn augments gastric epithelial cell proliferation upon ligand engagement of TLR2. Interestingly, Huang *et al* (2007) also demonstrated that *H. pylori* isolated from patients with *H. pylori*-positive GC accelerated the growth of human gastric tumour cells from *H. pylori*-negative GC patients, therefore suggesting that *H. pylori* infection can exacerbate gastric disease by promoting tumour growth. Although these authors did not address which TLR is responsible for *H. pylori*-induced gastric tumour cell proliferation, one could speculate a role for TLR2 given that several studies have shown that TLR2 can recognise *H. pylori* (Smith *et al*., 2003, Torok *et al*., 2005).

Of the 7 anti-apoptotic and pro-proliferative genes investigated in this chapter, the most striking gene was *BCL2A1,* as it was up-regulated by 8-fold upon Pam_3CSK4 stimulation versus un-treated MKN-28 cells. Furthermore, the significant TLR2-driven up-regulation of *BCL2A1* (~100-fold, $P \leq 0.01$, data not shown) was independently verified in human NUGC4 cells by our collaborators in Japan. *BCL2A1* is a BCL2 family member that sequesters pro-apoptotic proteins, such as BAX and BAK, to inhibit apoptosis (Vogler, 2012). Over 10 years ago, bioinformatic analyses performed on the 5′ promoter region of *BCL2A1* revealed a putative NF-κB binding site at position -833, whereby BCL2A1 luciferase reporter constructs confirmed the importance of this NF-κB binding site in the transcriptional regulation of *BCL2A1* (Zong *et al*., 1999). In this regard, my data consistently showed that the blockade of NF-κB signalling by MG132 down-regulated the gene expression levels of *BCL2A1,* however, thus far, there is no evidence on the transcriptional regulation of *BCL2A1* by the ERK1/2 MAPK signalling pathway. Given that we are not aware of other studies investigating the signalling events that regulate TLR2-driven gene networks investigated in this chapter, it is possible that TLR2 may regulate the transcription of *BCL2A1* in an ERK1/2-dependent manner in human gastric epithelial cells. Interestingly, a study demonstrated that i.p. injection of recombinant human *BCL2A1* protein (rhBCL2A1) protected mice from ischemia reperfusion injury by reducing the number of apoptotic cardiomyocytes in a TLR2-dependent manner, as *Tlr2*$^{-/-}$ mice were not protected from ischemia reperfusion injury by rhBCL2A1 protein (Iwata *et al*., 2010). In addition, the expression of BCL2A1 is increased in a number of cancers, such as colon, breast and most importantly gastric (Vogler, 2012). Based on my results and what is known in the literature, I hypothesise that increased TLR2 activation results in augmented levels of BCL2A1, which can feed back and activate TLR2 in an autocrine or a paracrine manner to augment gastric epithelial cell proliferation and survival.

Given that the inhibition of NF-κB signalling by MG132 significantly down-regulated genes that inhibit apoptosis, such as *BCL2A1*, *BCL2L1* and *BIRC3*, it is possible that the loss of NF-κB signalling induces apoptosis in MKN-28 cells. As demonstrated in Figure 5.5, the percentage of proliferative MKN-28 cells that were pre-treated with MG132 was reduced compared to control cells, thus suggesting that the blockade of NF-κB signalling induced apoptosis. This data is consistent with a previous study showing that MG132 induced apoptosis in AGS and MKN-28 cells, which was characterised by DNA fragmentation, caspase 3 activation and augmented levels of a pro-apoptotic protein (BAX), commonly known to be negatively regulated by the BCL-2 family members (BCL2A1 and BCL2L1) (Fan *et al*., 2001). Therefore, one can speculate that the significant down-regulation of *BCL2A1* and *BCL2L1* in MG132-treated MKN-28 cells (Figure 5.5) may result in the inability of these BCL-2 family members to inhibit the actions of BAX in promoting apoptosis.

Despite studies illustrating the role for TLR2 signalling in promoting endothelial cell migration and angiogenesis (Saber *et al*., 2011, Chang *et al*., 2005), my results indicated that TLR2 signalling does not induce gastric epithelial cell migration and angiogenesis. As shown by the CD31 immunohistochemical staining of gastric tumour cross-sections from *gp130*$^{F/F}$ and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice, the level of staining in the gastric tumours of both genotypes was similar, therefore suggesting that the ablation of TLR2 signalling in *gp130*$^{F/F}$ mice did not reduce the angiogenic response. It has been previously shown that the angiogenic response in *gp130*$^{F/F}$ mice is mediated via STAT3, as the number of blood vessels formed in the gastric tumours of *gp130*$^{F/F}$*:Stat3*$^{-/+}$ mice was significantly reduced compared to *gp130*$^{F/F}$ mice (Judd *et al*., 2006). However, the exact molecular and cellular

basis by which STAT3 promoted angiogenesis in this GC mouse model remains unresolved. Similarly, Chen *et al* (2012) showed that the inhibition of STAT3 activation by the natural compound nitidine chloride significantly reduced tumour angiogenesis, since human GC xenografts in mice that were treated with nitidine chloride expressed a significantly lower number of CD31-positive stained cells. Taken together, these results suggest that TLR2 in endothelial cells may not be contributing to gastric tumour angiogenesis, but rather an unknown STAT3-dependent molecular process does.

With regard to cell migration, my results demonstrated that TLR2 activation does not mediate gastric epithelial cell migration. In contrast, a study showed that a by-product of gastrin, glycine-extended gastrin, promotes IMGE-5 cell migration (Hollande *et al*., 2001), thus suggesting that a TLR2-independent mechanism may be involved in gastric epithelial cell migration.

CHAPTER 6

Therapeutic blockade of TLR2 suppresses gastric tumourigenesis

6.1 Introduction

GC remains the second highest cause of cancer-related mortalities world-wide, which is largely due to a combination of late detection of advanced disease leading to a poor prognosis, inefficient treatment options (see below) and the aggressive nature of this disease (Crew and Neugut, 2006). The highest incidence of GC is found in Japan, Korea, China, South America and Eastern Europe (Bickenbach and Strong, 2012) and it has been noted that the survival rates in, for instance, Asian populations are higher than Western populations due to the implementation of regular screening programs, such as the barium meal examination (Kunisaki *et al*., 2006, Bickenbach and Strong, 2012). The 5 year overall survival rate for patients with GC in Japan is approximately 77% (Yamashita *et al*., 2011), however in Western countries the 5 year overall survival rate drops to a staggering 25% (Meyer and Wilke, 2011). Thus far, the treatment options for GC are fairly limited, whereby complete resection of the tumour is the standard treatment option, which may include subtotal distal gastrectomy or total gastrectomy (Meyer and Wilke, 2011). Furthermore, surgical resection with adjuvant chemotherapy marginally improved the survival rate by 4%-6% and is only most beneficial to patients with advance GC (Meyer and Wilke, 2011). To date, the only therapeutic agent for GC that is in Phase III clinical trials is trastuzumab (Meyer and Wilke, 2011), which is a monoclonal neutralising antibody against the human epidermal growth factor receptor 2 (HER2) (Chua and Merrett, 2012). Although clinical trials revealed that trastuzumab improved the prognosis of GC patients (Bang *et al*., 2010), this drug is only efficacious in approximately 20% of GC patients that over-express HER2 in the stomach (Chua and Merrett, 2012). Therefore, the

identification of novel drug targets is vital for improving the prognosis of GC. Given that my results thus far suggest that genetic ablation of TLR2 can suppress gastric tumour growth in *gp130*$^{F/F}$ mice, this Chapter will explore the therapeutic potential of blocking TLR2 signalling in gastric tumourigenesis.

6.2 Augmented levels of the *TLR2* gene in human gastric tumour samples correlate with increased expression of TLR2-regulated anti-apoptotic and cell cycle proliferative genes.

My results presented in Chapter 3 revealed that there is a strong correlation between increased expression of STAT3-target genes (i.e. *Stat3* and *Il-11*) with augmented expression levels of the *Tlr2* gene in the gastric tumour of *gp130*$^{F/F}$ mice. Accordingly, I wanted to confirm these observations in human gastric tumour samples to validate the relevance of my work to human GC. Gastric biopsies were collected (by Louise McLeod) by either gastroscopy or gastrectomy from fully-informed and consenting patients following which they were 1) stored in 10% formalin for histopathology, and 2) snap-frozen in liquid nitrogen for RNA isolation and gene expression profiling (Chapter 2.1). *H. pylori* colonisation and pathological changes in the gastric biopsies, such as moderate or severe gastritis, and gastric adenocarcinoma were determined from H&E and cresyl fast violet-stained tissue sections, using the revised version of the Sydney system (Dixon *et al*., 1996) by an experienced pathologist, Prithi Bhatal (Melbourne Pathology, Australia). Quantitative real-time PCR assays performed on tumour and matching non-tumour tissue from GC patients, and disease-free tissue from healthy individuals as controls revealed that similar to what was observed in our *gp130*$^{F/F}$ mice (Figure 3.1), the increased expression of *STAT3* and *IL-11* correlated with augmented *TLR2* gene expression levels in human gastric tumour compared to tumour-free tissues (i.e. normal and non-tumour) (Figure 6.1). Based

on these results, I next investigated if the expression of TLR2-regulated anti-apoptotic and cell cycle genes, identified in Chapter 4, was augmented in the gastric tumour of GC patients that over-express the *TLR2* gene. In concordance with my results in Chapter 4, I observed that the expression levels of *BCL2A1, BCL3* and *BIRC3* genes were significantly augmented in human gastric tumour tissues compared to tumour-free samples (Figure 6.2), thus suggesting that these genes may contribute to the transformation status of the gastric epithelium. In addition, my results revealed that the gene expression levels of *BCL2L1* were increased in the gastric tumour samples, albeit not significantly ($P \leq 0.1$), whereas *C-MYC* gene expression levels were comparable among the normal, non-tumour and tumour tissues (Figure 6.2). In contrast to these data, the gene expression levels of *CCND1* were not detected in any of the gastric tissues examined (data not shown). Importantly, it is worthy to note that there were no significant differences in gene expression analyses between *H. pylori*-positive versus *H. pylori*-negative tumour samples, and thus *H. pylori*-positive samples were included in the analyses. Collectively, these data confirm that the up-regulation of *TLR2* in the gastric tumour from patients with intestinal-type GC display augmented levels of TLR2-regulated anti-apoptotic genes, such as *BCL2A1, BCL3* and *BIRC3.*

6.3 Therapeutic targeting of TLR2 alleviates gastric disease.

Given that there are limited treatment options that provide long-term health benefits for GC, the discovery of novel "druggable" targets for the design of highly efficacious therapies in human GC is highly desired. In this regard, I propose that TLR2 serves as a potential therapeutic target for GC, as my data not only show that the gene expression levels of *TLR2* are augmented in human GC patient tumour biopsies (Figure 6.1) but also that genetic ablation of *Tlr2* in *gp130*$^{F/F}$ mice significantly reduced the gastric tumour

burden by approximately 50% (Figure 4.1).To formally test this notion, a TLR2 blocking antibody (OPN-301) (Ultaigh *et al*., 2011) was used to initially confirm that the inhibition of TLR2 signalling suppressed TLR2-regulated inflammatory genes in *gp130*$^{F/F}$ mice, as OPN-301 has been shown to inhibit Pam_3CSK4-induced cytokine production in rheumatoid arthritis *ex vivo* tissue explant cultures (Ultaigh *et al*., 2011). OPN-301 or the isotype control was administered by i.p. injection to *gp130*$^{F/F}$ mice for 30 minutes prior to i.p. injection with Pam_3CSK4 for 3 or 6 hours. Quantitative real-time PCR gene expression analyses confirmed that the gene expression levels of *Il-6*, *Cxcl2*, and *Tnf-α* were down-regulated in the gastric antrum tissues from *gp130*$^{F/F}$ mice treated with OPN-301 and Pam_3CSK versus the isotype-treated *gp130*$^{F/F}$ mice (Figure 6.3).

Since it has been documented that the onset of gastritis and gastric hyperplasia occurs at 6 weeks of age in *gp130*$^{F/F}$ mice and progresses in severity up to 24 weeks of age (Jenkins *et al*., 2005), 12 week old *gp130*$^{F/F}$ mice displaying established disease were selected for long-term treatment with OPN-301 to ensure the clinical relevance of this study. The initial macroscopic analyses indicated that the administration of OPN-301 to *gp130*$^{F/F}$ mice via i.p. injection twice a week for 10 weeks resulted in the appearance of smaller stomachs and a reduced gastric tumour burden compared to isotype-treated *gp130*$^{F/F}$ mice (Figure 6.4A). Moreover, significant reductions in gastric tumour mass and tumour incidence were observed in *gp130*$^{F/F}$ mice receiving OPN-301 compared to the isotype control (Figure 6.4B-C), thus suggesting that the blockade of TLR2 signalling in *gp130*$^{F/F}$ male mice reduced both the formation of new tumours and growth of existing tumour.

To confirm that the blockade to TLR2 signalling abrogated gastric tumour cell proliferation, I performed *in vitro* EdU proliferation assays on MKN-28 cells which

transiently over-expressed the TLR2 expression construct and which were either pre-treated with OPN-301 or the isotype control prior to Pam_3CSK4 stimulation. As shown in Figure 6.5, Pam_3CSK4-induced cell proliferation was suppressed in MKN-28 cells that were pre-treated with OPN301. Therefore, my results suggest that TLR2 may serve as a potential therapeutic target for GC, as the OPN-301 TLR2 blocking antibody reduced the gastric tumour burden of *gp130*$^{F/F}$ mice probably by impeding gastric epithelial tumour cell proliferation.

6.4 Discussion

My results in Chapter 6 highlight the importance of TLR2 signalling in human GC, since my data showed that the *TLR2* gene expression levels are augmented in the gastric tumours of GC patients with intestinal-type metaplasia. In this regard, Tahara *et al.*, (2008) demonstrated that the *TLR2* -196 to -174in/ins genetic polymorphism, whereby deletion of this region has been shown to diminish the transcriptional regulation of *TLR2* (Noguchi *et al*., 2004), is associated with the severity of intestinal-type metaplasia, thus suggesting that *TLR2* signalling in the stomach is linked to poorer prognosis of intestinal-type GC. Since my results confirm that the expression levels of TLR2-regulated anti-apoptotic genes, such as *BCL2A1*, *BCL3* and *BIRC3* are also significantly augmented in the gastric tumours of patients with intestinal-type metaplasia, one could speculate that the increased TLR2 activity, in the gastric epithelium of GC patients favour gastric epithelial cell survival and proliferation. As previously discussed in Chapter 5, the cytoprotective effects of rhBCL2A1 in a mouse model for ischemia-reperfusion injury is dependent on the TLR2/MyD88 signalling pathway (Iwata *et al*., 2010), and since my data suggests that *BCL2A1* is a TLR2-regulated gene it is tempting to assume that TLR2-driven up-regulation of BCL2A1 in gastric tumours can feedback and activate TLR2 to further promote gastric

epithelial growth. Therefore, despite the small sample size of my current study, future genome-wide expression profiling studies on larger cohorts of patients exploring the correlation between augmented TLR2 gene expression levels with *BCL2A1* (Figure 6.2) are now strongly warranted. Furthermore, such studies have the power to identify correlations between the gene expression patterns of TLR2 and additional "potential TLR2-regulated genes" (e.g. cell cycle), which may form the basis of a gene expression signature to identify patients suitable (i.e. potentially responsive) to anti-TLR2 based therapies. It is also important to note that such studies would be greatly complemented in the future by performing immunohistochemical analyses to identify correlations between the increased expression of these genes (and their expressed proteins) and activation of the signalling pathways believed (from my data in Chapter 5) to regulate their expression.

Another key finding in this Chapter revealed that TLR2 is a potential candidate for therapeutic targeting, since Figure 6.4 showed that *gp130*$^{F/F}$ mice receiving OPN301 displayed reduced gastric tumour burden compared to isotype-treated mice. Furthermore, the blockade of TLR2 signalling in MKN-28 cells prevented Pam_3CSK4-induced cell proliferation, thus confirming that the mechanism by which TLR2 mediates gastric tumourigenesis is through gastric epithelial cell proliferation and survival. With respect to TLR2 activation, studies showed that TLR2 not only recognises microbial products (Kawai and Akira, 2011), but has also been reported to recognise non-microbial products, such as BCL2A1, versican and serum amyloid A (SAA) (Iwata *et al*., 2010, Cheng *et al*., 2008, Kim *et al*., 2009b). Regarding these non-microbial products, Zhang *et al.,* (2012) discovered that rhIL-11-treated human gastric cancer cell lines (AGS and MKN-45) over-expressed versican, which is a component of the extracellular matrix, while the levels of SAA are augmented in the serum of GC patients (Chan *et al*., 2007). This is noteworthy,

since 50% of GC patients over-express activated STAT3, and based on my results in this thesis, it is tempting to assume that the activation of STAT3-driven TLR2 in the gastric tumour epithelium results in a positive feed-back loop to further promote gastric disease through the recognition of these non-microbial products, thus making TLR2 an excellent therapeutic target. Due to time constraints, I acknowledge that further experiments are required to address the efficacy of the therapeutic benefits of OPN301 in human GC, which will be discussed in more detail in the next Chapter.

CHAPTER 7

Summary, Discussion and Conclusion

7.1 Summary and discussion

The aims of this thesis were to, firstly demonstrate that TLR2 is a novel STAT3 target gene, and secondly to elucidate the role of TLR2 in STAT3-mediated gastric tumourigenesis. In Chapter 3, I successfully demonstrated that TLR2 is a novel STAT3 target gene, whereby over-expression of the hyper-active STAT3-C/GFP mutant in mouse (IMGE-5) and human (MKN-28 and AGS) gastric epithelial cell lines resulted in a significant increase in endogenous TLR2 gene expression levels. More importantly, ChIP assays revealed that the STAT3 binding site located -1241 upstream of the transcriptional start site of the *Tlr2* gene is necessary for the interaction of pY-STAT3. Additionally, *Tlr2* promoter luciferase reporter assays demonstrated that the STAT3 binding site at position -1241 is required for full transcriptional activity of the *Tlr2* gene, since STAT3-C/GFP did not transcriptionally induce luciferase activity with the truncated (TLR2-1248) and site-directed (TLR2- TT>AA) mutant TLR2-reporter constructs which both disrupted the -1241 binding site. Although the *Tlr2* gene promoter-reporter was of mouse origin, bioinformatic analyses indicated that there are 4 putative STAT3 binding sites in the 5' promoter region of the human *TLR2* gene. In concordance with this work, our collaborator (Patrick Tan) identified that there was a strong correlation ($P \leq 0.05$) between *STAT3* and *TLR2* gene expression levels in patients with GC, whereby patients that expressed high levels of *STAT3* and *TLR2* displayed a significantly lower survival rate compared to those who expressed low *STAT3* and *TLR2* levels (Tye *et al.*, accepted). In addition, 18 human GC

cell lines that were previously characterised by gene expression data to represent the activity status of various oncogenic signalling pathways (Ooi *et al*., 2009), including STAT3, demonstrated a strong correlation between STAT3 activity and *TLR2* gene expression ($P \leq 0.05$) (Tye *et al.*, accepted).

Given that the *Tlr2* gene is regulated by pY-STAT3, which is up-regulated in 50% of human GC patients (Deng *et al*., 2010a), Chapter 4 investigated the role of TLR2 in STAT3-mediated GC. To formally elucidate the role for TLR2 in gastric tumourigenesis our $gp130^{F/F}$ mouse model for GC, that spontaneously develop gastritis and adenomatous lesions, resembling that of human intestinal-type metaplasia (Tebbutt *et al*., 2002), was used to generate the $gp130^{F/F}$*:*$Tlr2^{-/-}$ mouse. Since the activation of TLR2 is implicated in inducing an inflammatory response, I initially investigated whether the level of gastritis was reduced in $gp130^{F/F}$*:*$Tlr2^{-/-}$ mice. As discussed in Chapter 4, despite the significant reduction in gastric tumour burden observed in $gp130^{F/F}$*:*$Tlr2^{-/-}$ mice, genetic ablation of TLR2 in $gp130^{F/F}$ mice did not reduce the level of gastritis determined by recruitment and activation of inflammatory cells to the gastric mucosa. With regard to this, STAT3 is most likely playing an important role in mediating gastritis and the recruitment of inflammatory cells, since our laboratory has previously shown that the levels of inflammatory cells in the gastric mucosa are reduced in $gp130^{F/F}$:$Stat3^{-/+}$ mice compared to $gp130^{F/F}$ mice (Jenkins *et al*., 2005, Judd *et al*., 2006). To further support this notion, studies have shown that the IL-6/STAT3 signalling axis plays an important role in regulating chemokines for T cell and neutrophil recruitment, such as chemokine (C-C motif) ligand 5 (CCL5) (McLoughlin *et al*., 2005) and chemokine (C-X-C motif) ligand 1 (CXCL1) (Fielding *et al*., 2008), respectively. However, the molecular basis by which STAT3 promotes gastric submucosal infiltration of inflammatory cells requires further investigation. Additionally, although it

was not further investigated and beyond the scope of my PhD, perhaps the up-regulation of *Tlr9* in the gastric compartment of *gp130*$^{F/F}$ mice may also be contributing to the level of gastritis observed in *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice. To support this hypothesis, Chang *et al.*, (2005) have demonstrated that TLR9 is involved in *H. pylori*-mediated gastritis and therefore to formally address the role of STAT3-driven up-regulation of TLR9 in gastric disease, initial studies demonstrating the responsiveness of *gp130*$^{F/F}$ pGECs to TLR9 agonists and determining the gene expression levels of *Tlr9* in our *gp130*$^{F/F}$ compound mutant mice should be considered. Interestingly, the *TLR9* -1237T/C SNP introduces an IL-6 RE in the 5′ promoter region of the *TLR9* gene (Carvalho *et al*., 2011), which is associated with *H. pylori*-induced GC (Ng *et al*., 2010), therefore the role of TLR9 in gastric tumourigenesis warrants further investigation.

The above observations of my PhD studies dissociating gastric inflammation from tumourigenesis suggested that immune/inflammatory cells may not be playing a key role in disease pathogenesis in the *gp130*$^{F/F}$ mouse model. This notion was strongly supported by the bone marrow chimera experiments performed during my PhD, which indicated that TLR2-expressing hematopoietic cells do not play a role in our model for gastric tumourigenesis. In further support of this, a previous study demonstrated that recipient wild-type mice receiving donor *gp130*$^{F/F}$ bone marrow did not develop gastric hyper-proliferative lesions, thus confirming that hematopoietic cells play a minimal (if any) role in gastric tumourigenesis (Ernst *et al*., 2008). Stimulation of pGECs isolated from *gp130*$^{F/F}$ and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice with microbial-containing gastric homogenates from 6 month old *gp130*$^{F/F}$ mice confirmed that the TLR2 inflammatory response (as determined by the production of inflammatory cytokines IL-6 and TNF-α) was diminished in *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ pGECs. Given that inflammatory cells are still present in the gastric mucosa of

gp130$^{F/F}$*:Tlr2*$^{-/-}$ mice, the reduced gastric tumour burden observed in these mice is presumably due to the diminished TLR2 response in the gastric epithelium that is independent of gastric inflammation.

In light of the above observations, I therefore investigated other TLR2-mediated cellular processes that may account for this phenomenon. Interestingly, as shown in Chapter 5, both my *in vivo* and *in vitro* data suggest that TLR2-mediated gastric epithelial cell growth. With regard to TLR2-mediated epithelial cell proliferation, studies have also shown that activation of TLR2 by TLR2 agonists promote cell proliferation in other epithelial cell lines, such as hepatocarcinoma H22 (Huang *et al*., 2007) and lung airway NCI-H292 cells (Shaykhiev *et al*., 2008). More importantly, Huang *et al.*, (2007) also demonstrated that *H. pylori* isolates from *H. pylori*-positive GC patients augmented cell proliferation of human primary gastric epithelial cells isolated from *H. pylori*-negative patients, thus demonstrating that *H. pylori* recognition can promote gastric epithelial cell proliferation, albeit by an unknown mechanism. Given that several studies have implicated TLR2 in the recognition of *H. pylori* (Mandell *et al*., 2004, Yokota *et al*., 2007, Smith *et al*., 2003), it is therefore tempting to propose that in human disease the detection of *H. pylori* by TLR2 may drive gastric tumour growth by promoting gastric epithelial cell proliferation. This hypothesis is further supported by a study performed by Yokota *et al.*, (2010), which demonstrated that, similar to synthetic TLR2 agonists, *H. pylori* LPS also induced TLR2-dependent gastric epithelial cell proliferation via the ERK MAPK signalling pathway compared to untreated-cells, however my data suggest that multiple signalling pathways are involved in promoting the expression of various anti-apoptotic and cell proliferative genes in human GC.

Despite studies such as those highlighted in the previous paragraph, which demonstrated a role for TLR2 in gastric epithelial cell proliferation, the TLR2 receptor complex involved in promoting gastric epithelial cell proliferation remains unclear. Since there is compelling evidence to suggest that there is variation in signalling pathways associated with TLR2/6 and TLR2/1 heterodimers, such as Mal being absolutely essential for PI3K/AKT-dependent TLR2/6 but not TLR2/1 signalling (Santos-Sierra *et al*., 2009), it is therefore important to identify whether TLR2/1 and/or TLR2/6 heterodimer(s) are involved in gastric tumourigenesis. In this regard, my results showed that Pam_3CSK4, a TLR2/1 ligand, augmented the expression levels of anti-apoptotic and cell proliferative genes, and also induced cell proliferation in MKN-28 cells. These preliminary findings now pave the way for future experiments to investigate in detail whether cell proliferation as well as the expression of anti-apoptotic and cell proliferative genes (identified in chapter 5) are differentially regulated in human GC epithelial cells treated with a TLR2/6 ligand, such as FSL-1 or MALP-2.

The importance of determining whether gastric epithelial cell proliferation is mediated through a TLR2/1 and/or TLR2/6 heterodimer(s) is underscored by studies showing that in GC several endogenous TLR2 ligands are up-regulated, such as versican (TLR2/6) (Kim *et al*., 2009b, Zhang *et al*., 2012), SAA (TLR2/1) (Cheng *et al*., 2008, Chan *et al*., 2007) and BCL2A1 (TLR2/1) (Iwata *et al*., 2010, Vogler, 2012). Regarding the latter, my data suggest that *BCL2A1* may be a TLR2-regulated gene, as the gene expression levels of the *BCL2A1* gene were significantly augmented in Pam_3CSK4-treated human GC epithelial cell lines compared to un-treated cells. Therefore, it is tempting to speculate that TLR2-driven up-regulation of BCL2A1 can feedback and activate TLR2 in an autocrine or paracrine manner to further promote gastric epithelial cell proliferation. To demonstrate

this, initial experiments would involve performing quantitative real-time PCR and immunoblot assays on Pam_3CSK4-treated NUGC4 (high *TLR2* gene expression levels) and AZ521 (low *TLR2* gene expression levels) human GC cell lines to determine if the expression of BCL2A1 is further augmented in NUGC4 compared to AZ521 upon TLR2 activation. In addition, proliferation assays could be conducted on human GC cell lines to confirm that BCL2A1 can augment cell proliferation. Furthermore, given the link between versican and SAA in GC, it is therefore necessary to confirm whether the anti-apoptotic and cell cycle genes (identified in Chapter 5) are up-regulated in human gastric biopsy tumour samples and whether these TLR2 ligands can also promote gastric epithelial cell proliferation. With regard to the former endogenous ligand, a recent study demonstrated that IL-11 augmented the production of versican V0 and V1 protein isoforms in AGS and MKN-45 (Zhang *et al*., 2012), whereas versican V1 has been shown to accelerate TLR2-mediated cell proliferation in a lung cancer epithelial cell line (Kim *et al*., 2009b). Additionally, although a STAT3 binding site has not been identified in the 5′ promoter region of the human SAA gene (Hagihara *et al*., 2005), a study revealed that phosphorylated STAT3 can form a heteromeric complex with NF-κB p65 and p300 to initiate the transcription of the SAA gene (Hagihara *et al*., 2005). Nonetheless, since the common link among these endogenous ligands is TLR2, it suggests that TLR2 may represent a new therapeutic candidate for GC.

The poor prognosis of GC is largely due to the paucity of both robust early detection methods and highly efficacious treatment options for GC patients (Crew and Neugut, 2006), which places a large emphasis on discovering novel biomarkers and therapeutic drug targets. With regard to GC treatment, chemotherapy (epirubicin, cisplatin and flurouracil: ECF) combined with surgical resection of the stomach is still the gold-standard

treatment option for advanced GC patients with perioperative chemotherapy significantly improving the patient's overall survival compared to surgery alone (5-year survival rate, 36% vs 23%) (Cunningham *et al*., 2006). However 36% is still an unacceptably low survival rate, and thus there is a need for additional adjuvant (targeted) therapies to try and further improve patient survival. In this respect, another key finding of my thesis was that the therapeutic blockade of TLR2 suppressed gastric tumourigenesis. As shown in Chapter 6, 12 week old *gp130*$^{F/F}$ mice receiving OPN-301 TLR2 blocking antibody for 10 weeks appeared to be healthy and displayed reduced gastric tumour growth compared to *gp130*$^{F/F}$ mice receiving the isotype control. In addition, my *in vitro* data revealed that the blockade of TLR2 signalling in human GC epithelial cells diminished the proliferative response to Pam_3CSK4, thus confirming that TLR2 promotes gastric tumour cell proliferation. These data convincingly suggest that the OPN-301 TLR2 blocking antibody has great potential for therapeutic targeting possibly as an adjuvant to ECF chemotherapy in human GC, and now set the stage for additional experiments to be carried out. For instance, immunohistochemical staining of gastric cross-sections from OPN-301-treated *gp130*$^{F/F}$ mice could investigate if OPN-301 affected the level of gastritis (H&E) and/or gastric epithelial cell proliferation and survival (PCNA and TUNEL). While in the *gp130*$^{F/F}$ GC model it would be expected that blocking TLR2 with this antibody would not affect gastritis, nonetheless it should be noted that OPN-301 has been previously shown to significantly reduce inflammation in other inflammation-associated diseases, such as rheumatoid arthritis (Ultaigh *et al*., 2011) and ischemia reperfusion injury (Farrar *et al*., 2012). In addition, since my results suggest that anti-apoptotic and pro-proliferative genes are up-regulated in response to a TLR2 agonist, qRT-PCR gene analyses can also be used to confirm that these genes are down-regulated in the stomach of OPN-301-treated *gp130*$^{F/F}$ mice.

Additional experiments, such as administering OPN-301 (and isotype as a control) with or without ECF chemotherapy in nude mice with established xenografts from either human GC cell lines (e.g. NUGC4) or primary GC patient tumours will enable us to determine the potential efficacy of OPN-301 in suppressing the growth of human gastric tumours. If successful, OPN-301 would provide an attractive candidate adjuvant therapy in concert with ECF chemotherapy in advance GC patients to suppress tumour growth, and thus improve the patient's overall survival rate. Assuming that OPN-301 is more effective in patients that express high levels of the *TLR2* gene, in such a scenario it will be essential to perform qRT-PCR gene expression analyses (e.g. *TLR2* and *BCL2A1)* on tissue biopsies to stratify potentially responsive GC patients to OPN-301.

7.2 Conclusion

In summary, my data have established a novel mechanism by which STAT3 mediates gastric tumourigenesis through the up-regulation of TLR2 to promote gastric tumour growth, whereby we have shown that the blockade of TLR2 signalling in our mouse model for GC proves to be effective in reducing gastric tumour burden, which is illustrated in Figure 7.1. Futhermore, my data described in this thesis demonstrated, for the first time, that STAT3-driven up-regulation of TLR2 promoted gastric tumourigenesis independent of gastritis. The over-expression of STAT3 in the gastric mucosa, which is seen in approximately 50% of human GC patients, is strongly associated with lymph-node metastases and a lower survival rate (Kim *et al*., 2009a). Given that my results demonstrated that TLR2 is a STAT3 target, the over-expression of STAT3 in the gastric mucosa is associated with a lower survival rate possibly due to increased *TLR2* gene expression levels, which augments gastric epithelial cell proliferation. This notion was

further confirmed in collaboration with Patrick Tan (Genome Institute of Singapore, Singapore), whereby it was revealed that GC patients had a lower survival rate when they expressed high *STAT3* and *TLR2* gene expression levels compared to patients with low *STAT3* and *TLR2* gene expression levels (Tye *et al.*, accepted). More importantly, the mechanism by which TLR2 is mediating gastric tumourigenesis has been discussed in my thesis, whereby I have shown that TLR2 plays an important role in gastric epithelial cell proliferation. Another important aspect of my thesis is that the inhibition of TLR2 signalling with the OPN-301 TLR2 blocking antibody significantly reduced cell proliferation in MKN-28 cells, and suppressed gastric disease in $gp130^{F/F}$ mice with established gastric disease, which is the highlight of this work.

APPENDIX I

ACCEPTED MANUSCRIPTS GENERATED FROM THIS THESIS

***Tye, H*.,** Kennedy, C.L., Najdovska, M., McLeod, L., McCormack, W., Hughes, N., Dev, A., Sievert, W., Ooi, C.H., Ishikawa, T., Oshima, H., Bhathal, P.S., Parker, A.E., Oshima, M., Tan, P. And Jenkins, B.J. (2012). STAT3-driven upregulation of TLR2 promotes gastric tumourigenesis independent of tumour inflammation. *Cancer cell*. **Accepted.**

APPENDIX II

BUFFERS AND SOLUTIONS

1X TAE:

0.04M Tris-HCl, 0.001M EDTA, 5.7% (v/v) glacial acetic acid, adjusted to pH 8.0

Tail Buffer:

0.05M Tris-HCl, 0.025M EDTA, 0.05M NaCl, 0.005% SDS

T.E Buffer

10mM Tris-HCl, 1mM EDTA, adjusted to pH 8.0

DEPC-treated water:

0.1% (v/v) DEPC in 1L MilliQ water. Incubated overnight at 37˚C then autoclaved

DNA loading dye:

0.25% Bromophenol blue, 0.25% Xylene cyanol, 10mM EDTA, 20% (v/v) glycerol, adjusted to pH 8.0

1X PBS:

1.4mM NaCl, 0.3mM KCl, 1mM $Na_2HPO_4.12H_2O$, 0.18mM KH_2HPO_4, adjusted to pH 7.4

KalB Lysis Buffer:

50mM Tris, 150mM NaCl, 1% (v/v) Trition X-100, 1mM EDTA, adjusted to pH 7.4 and stored at 4˚C. Prior to use 1% (v/v) NaF, 1% (v/v) $NaVO_4$, 1% (v/v) and 1 tablet of protease inhibitor (EDTA-free).

SDS-PAGE resolving gel buffer (RGB):

2M Tris-HCl and 10% SDS, adjusted to pH 8.8

SDS-PAGE stacking gel buffer (SGB):

1M Tris-HCl and 10% SDS, adjusted to pH 6.8

10% SDS-PAGE resolving gel (1x gel):

10% (v/v) polyacrylamide, 0.26% (v/v) RGB, 0.1% (v/v) ammonium persulfate, 0.004% TEMED

4.5% SDS-PAGE stacking gel (1x gel):

4.5% (v/v) polyacrylamide, 13.5% (v/v) SGB, 0.01% (v/v) ammonium persulfate, 0.004% TEMED

2X SDS-PAGE sample buffer:

2.5% (v/v) SDS, 25% (v/v) glycerol, 0.04% (w/v) bromophenol blue, 0.125M Tris-HCl, adjusted to pH 6.8. Prior to use add 5% (v/v) β-mercaptoethanol.

1X SDS-PAGE running buffer:

25mM Tris-HCl, 192mM glycine, 0.1% (v/v) SDS.

Membrane stripping buffer:

0.2M Glycine, 0.05% (v/v) Tween-20, 0.7% (v/v) β-mercaptoethanol.

APPENDIX III

PRIMER SEQUENCES

GP130 genotyping PCR

GP130 common forward primer:

5’ – TGAAGCCACTCGTCTTTAGC – 3’

GP130 wild-type reverse primer:

5’ – CAAGTGTTCTCAAGGTCCGAGTCCAC – 3’

GP130 mutant reverse primer:

5’ – CTGAATGAACTGCAGGACGA – 3’

STAT3 genotyping PCR

STAT3 common forward primer:

5’ – AGCAGCTGACAACGCTGGCTGAGAAGCT – 3’

STAT3 wild-type reverse primer:

5’ – TTGCTGCTCTCGCTGAAGCGCAGTAGG – 3’

STAT3 knock-out reverse primer:

5’ – ATCGCCTTCTATCGCCTTCTTGACGAG – 3’

TLR2 genotyping PCR

TLR2 common forward primer:

5’ – TTGGATAAGTCTGATAGCCTTGCCTCC – 3’

TLR2 wild-type reverse primer:

5’ – GTTTAGTGCCTGTATCCAGTCAGTGCA- 3’

TLR2 knock-out reverse primer:

5’ – ATCGCCTTCTATCGCCTTCTTGACGAG – 3’

Mouse qRT-PCR forward and reverse primer sequences (5' to 3')

Gene	Forward primer	Reverse primer
18S	GTAACCCGTTGAACCCCATT	CATCCAATCGGTAGTAGCG
Actin	AGCCATGTACGTAGCCATCCA	TCTCCGGAGTCCATCACAATG
Bcl2l1	CACTGTGCGTGGAAAGCATA	AAAGTGTCCCAGCCGCC
Bcl2a1	CTTCAGTATGTGCTACAGGTACC	TGGAAACTTGTTTGTAAGCACGT
Bcl3	CGCAGCCGCAGGGTCATTGAT	TTGGCGAGGACTGGAGGCCA
Birc3	TGTCAGCCAAGTTCAAGCTG	ATCTTCCGAACTTTCTCCAGGG
C-myc	TGAGCCCCTAGTGCTGCAT	AGCCCGACTCCGACCTCTT
Ccnd1	CACAACGCACTTTCTTTCCA	GACCAGCCTCTTCCTCCAC
Cxcl2	AACATCCAGAGCTTGAGTGTGA	TTCAGGGTCAAGGCAAACTT
Ier3	TCCACCGCGCGTTTGAACACT	GTAGCTGGCGCCGGACCACTC
Il-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Il-6	ATGGATGCTACCAAACTGGAT	TGAAGGACTCTGGCTTTGTCT
Il-11	CTGCACAGATGAGAGACAAAT	GAAGCTGCAAAGATCCCAATG
Nod1	GGAGGCCAACAGACGCCT	ACTGACCTAGAGGGTATCG
Nod2	CTTATCACAGCCACAAGCATT	CACCCATTCACACTGACCTCT
Stat3	GTGCTGCCCCGTACCTGAAGA	GCAGGTGTCTCAAGTCAC
Tlr1	GGACCTACCCTTGCAAACAA	GGTGGCACAAGATCACCTTT
Tlr2	CCCTGTGCCACCATTTCC	CCACGCCCACATCATTCTC
Tlr4	TCTGAGCTTCAACCCCTTG	TGCCATGCCTTGTCTTCA
Tlr6	CCAAGAACAAAAGCCCTGAG	TGTTTTGCAACCGATTGTGT
Tnf-α	CAAATTCGAGTGACAAGCCTG	GAGATCCATGCCGTTGGC

Human qRT-PCR forward and reverse primer sequences (5’ to 3’)

Gene	Forward primer	Reverse primer
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
ACTIN	GATGAGATTGGCATGGCTTT	CACCTTCACCGTTCCAGTTT
BCL2L1	GGTCGCATTGTGGCCTTT	TCCGACTCACCAATACCTGCA
BCL2A1	CGACAGCAAATTGCCCCGGAT	TGTGCCATTTCCCCCAGCCT
BCL3	GCCATGGGCTCCCCGTTTCC	TGGAGAGGCGTGTCTCCGTCC
BIRC3	TGTTGGGAATCTGGAGATGA	CGGATGAACTCCTGTCCTTT
C-MYC	TCAAGAGGCGAACACACAAC	GGCCTTTTCATTGTTTTCCA
CCND1	CGCCCCACCCCTCCAG	CCGCCCAGACCCTCAGACT
IL-8	GTTTGATACTCCCAGTCTTGTCATTG	CTGTGGAGTTTTGGCTGTTTTAATCG
IER3	TACCCTCGAGTGGTCCGGCG	GCCAGGGATGCGGCGTTAGG
IL-1β	TCCCCAGCCCTTTTGTTGA	TTAGAACCAAATGTGGCCGTG
IL-11	TCTCTCCTGGCGGACACG	AATCCAGGTTGTGGTCCCC
STAT3	GGAGGAGGCATTCGGAAAG	TCGTTGGTGTCACACAGAT
Tlr1	CAGTGTCTGGTACACGCATGGT	TTTCAAAAACCGTGTCTGTTAAGAGA
Tlr2	GCCTCTCCAAGGAAGAATCC	TCCTGTTGTTGGACAGGTCA
Tlr4	AAGCCGAAAGGTGATTGTTG	CTGAGCAGGGTCTTCTCCAC
Tlr6	GAAGAAGAACAACCCTTTAGGATAGC	AGGCAAACAAAATGGAAGCTT

APPENDIX IV

DILUTIONS OF ANTIBODY FOR WESTERN BLOTTING AND IMMUNOHISTOCHEMISTRY

Table 2.1 Antibodies used in western blot analyses

Primary antibody	**Secondary antibody**
Anti-β-Actin antibody (Epitomics) **Molecular weight:** 42kDa **Dilution:** 1:1000	Goat anti-rabbit (Chemicon) **Dilution:** 1:2000
SAPK/JNK antibody (Cell Signalling Technology) **Molecular weight:** 46, 54kDa **Dilution:**1:1000	Goat anti-rabbit **Dilution:** 1:2000
Phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cell Signalling Technology) **Molecular weight:** 46, 54kDa **Dilution:**1:1000	Goat anti-rabbit **Dilution:** 1:2000
p44/42 MAPK antibody (Cell Signalling Technology) **Molecular weight:** 42, 44kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000
Phospho-p44/42 MAPK (Thr202/Tyr204) antibody (Cell Signalling Technology) **Molecular weight:** 42, 44kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000

p38 MAPK antibody (Cell Signalling Technology) **Molecular weight:** 43kDa **Dilution: 1:1000**	Goat anti-rabbit **Dilution:** 1:2000
Phospho-p38 MAPK (Thr180/Tyr182) antibody (Cell Signalling Technology) **Molecular weight:** 43kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000
Akt antibody (Cell Signalling Technology) **Molecular weight:** 60kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000
Phospho-Akt (Ser473) antibody (Cell Signalling Technology) **Molecular weight:** 60kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000
p65 antibody (Santa Cruz) **Molecular weight:** 65kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000
Phospho–p65 (ser276) antibody (Cell Signalling Technology) **Molecular weight:** 65-80kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000
TLR2 antibody (Epitomics) **Molecular weight:** 90kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000

STAT3 antibody (Santa Cruz) **Molecular weight:** 86kDa **Dilution:** 1:1000	Rabbit anti-mouse (Chemicon) **Dilution:** 1:2000
Phospho-STAT3 (Tyr705) antibody (Santa Cruz) **Molecular weight:** 86kDa **Dilution:** 1:1000	Goat anti-rabbit **Dilution:** 1:2000
IL-11 (Santa Cruz) **Molecular weight:** 21kDa **Dilution:** 1:100	Goat anti-rabbit **Dilution:** 1:2000

Table 2.2 Antibodies used in immunohistochemical analyses

Primary Antibody	**Antigen retrieval**	**Secondary antibody**	**Comments**
B220 (BD Pharmingen) **Dilution:** 1:50	n/a	Goat anti-rat (Vector Labs) **Dilution:** 1:200	
PCNA (Cell signalling Technologies) **Dilution:** (1:4000)	Citrate buffer (Appendix II)	Rabbit anti-mouse (Vector labs) **Dilution:** 1:200	Mouse on mouse (M.O.M) kit (Vector Labs). Followed manufacturer's specifications
TUNEL (Milipore)	n/a	n/a	Followed manufacturer's specifications
CD-31(Abcam) **Dilution:** 1:400	Citrate Buffer	Goat anti-rat (Vector Labs) **Dilution:** 1:200	
OPN-301 (Opsona therapeutics) **Concentration:** 12µg	Citrate Buffer	Rabbit anti-mouse **Dilution:** 1:200	Mouse on mouse (M.O.M) kit

APPENDIX X

Suppliers

Applied Biosystems	Foster City, CA, USA
Ameresco	Solon, OH, USA
BD Biosciences	Bedford, MA, USA
BioRad	Hercules, CA, USA
Cell Signalling Technology	Danvers, MA, USA
Dako	Glostrup, Denmark
EMD Milipore	Darmstadt, Germany
Eppendorf	Hamburg, Germany
GE Healthcare	Rydalmere, NSW, Australia
Gibco	Paisley, UK
Invitrogen	San-Diego, California, USA
Invivogen	San-Diego, California, USA
LI-COR	Lincoln, NE
Lonza	Basel, Switzerland
Millipore	Bedford, MA, USA
Nanadrop Technologies	Wilmington, DE, USA
Promega	Madison, WI, USA
Qiagen	Germany
R&D Systems	Minneapolis, MN, USA
Roche Molecular Biochemicals	Mannhein, Germany
Santa Cruz Biotechnology	Santa Cruz, CA, USA

Sigma-Aldrich	Saint Louise, MO, USA
Strategene	Santa Clara, CA, USA
Tecan	Männedorf, Switzerland
Thermo Fisher Scientific	Waltham, WA, USA
Vector Laboratories	Burlingame, CA, USA

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Figure 1.1. MyD88-dependent TLR signalling pathways (Adapted from Buchholz and Bauer, 2010 and Sierra *et al.*, 2009). Activation of TLRs result in the initiation of multiple signalling pathways, such as JNK, ERK and p38 MAPK, NF-κB and PI3K/AKT.

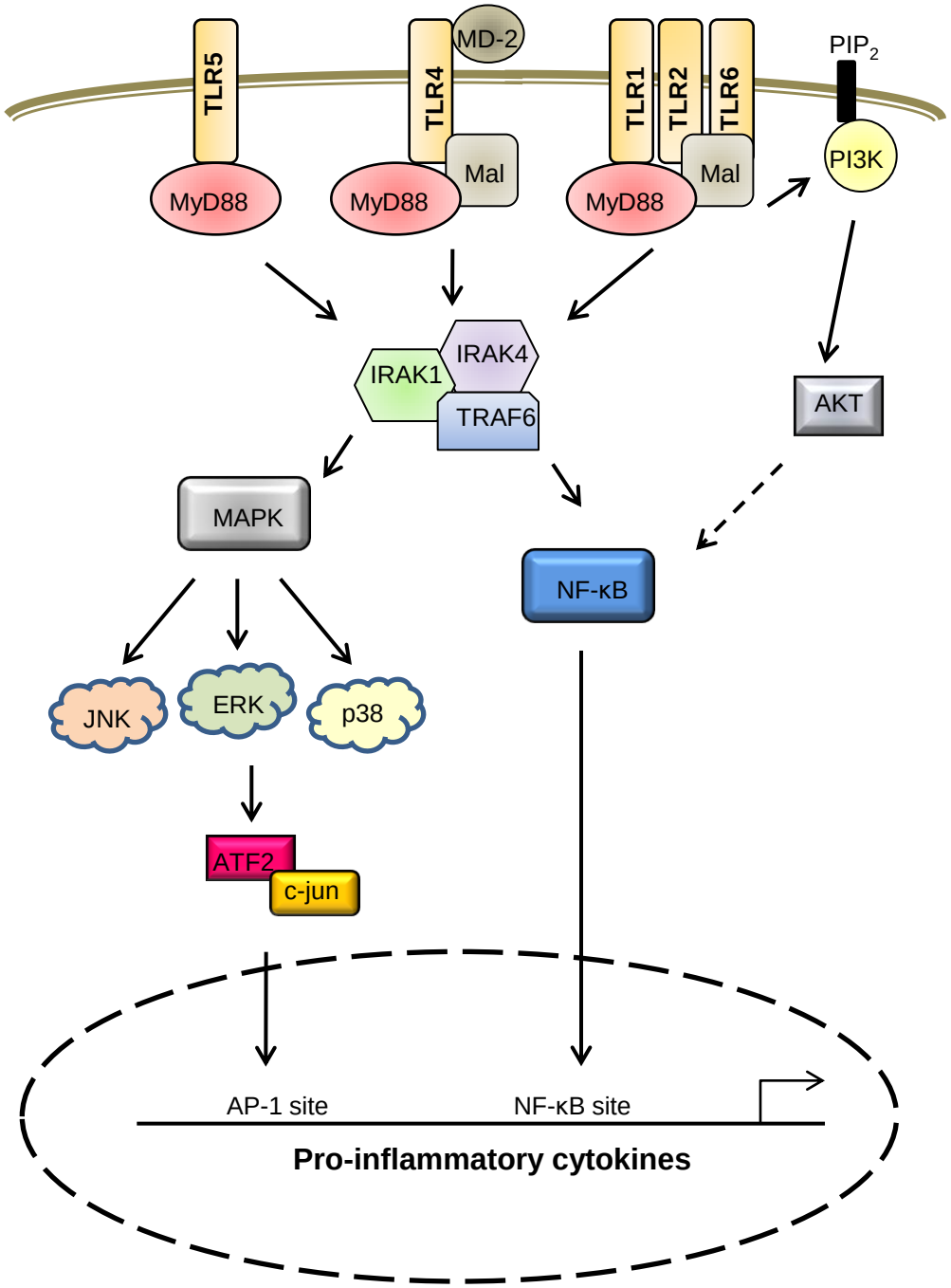

TLR5
MD-2
TLR4
TLR1
TLR2
TLR6
PIP2
MyD88
MyD88
Mal
MyD88
Mal
PI3K
IRAK4
IRAK1
TRAF6
AKT
MAPK
NF-κB
JNK
ERK
p38
ATF2
c-jun
AP-1 site
NF-κB site
Pro-inflammatory cytokines

Figure 1.2. MyD88-independent TLR signalling pathways (Adapted from Buchholz and Bauer, 2010 and Sierra *et al*., 2009). Activation of TLR3 and TLR4 result in the recruitment of TRIF, whereby TRAM is required to facilitate the interaction between TLR4 and TRIF to initiate IRF3 and NF-κB signalling pathways.

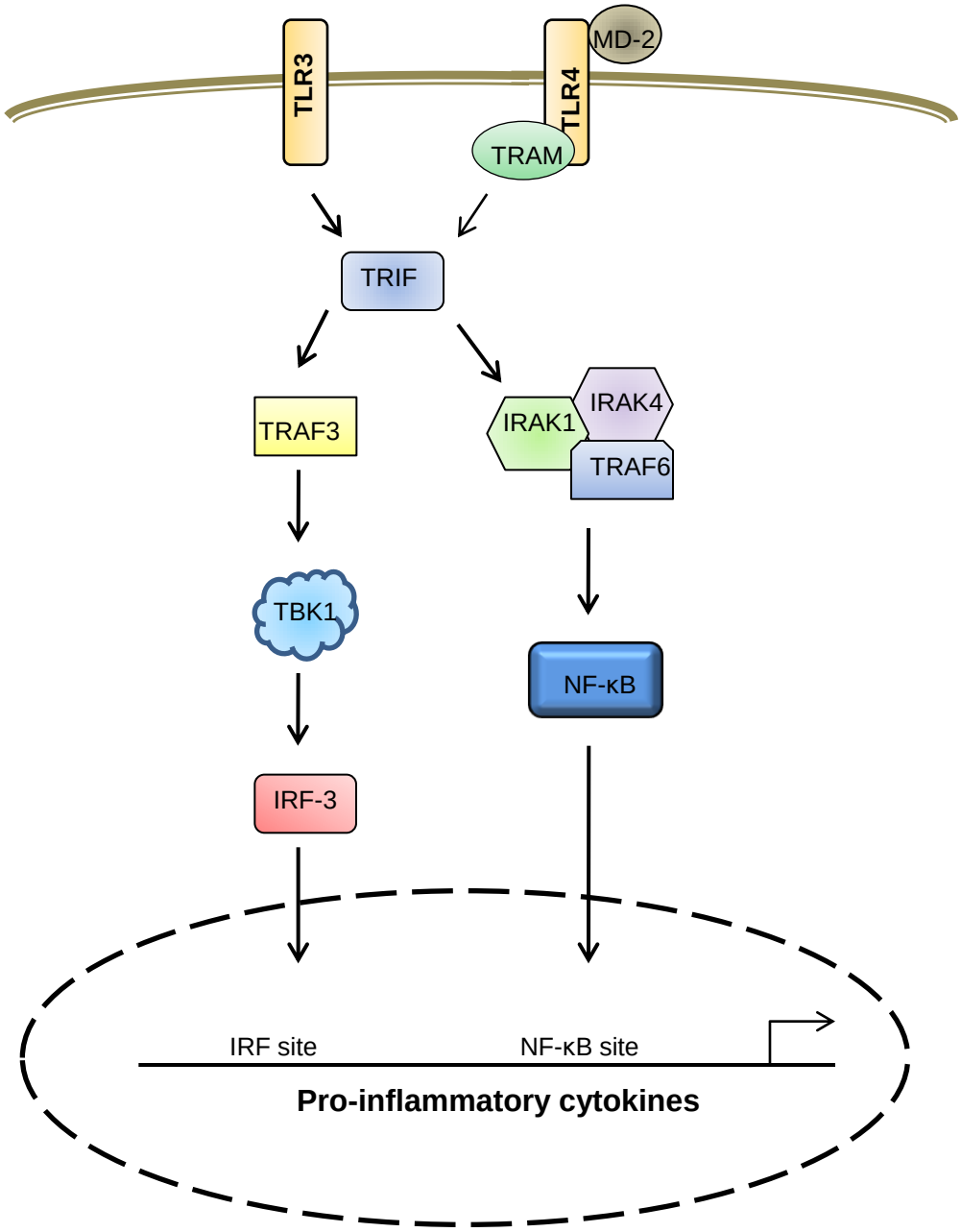

TLR3
TLR4
MD-2
TRAM
TRIF
TRAF3
IRAK1
IRAK4
TRAF6
TBK1
NF-κB
IRF-3
IRF site
NF-κB site
Pro-inflammatory cytokines

Figure 1.3. Structural representation of the molecules involved in TLR signalling (Adapted from Jenkins and Mansell, 2010). TLRs contain a LRR domain that is necessary for the recognition of PAMPs/DAMPs. Upon activation of TLRs, TIR containing molecules interact with TLRs through their TIR domains to initiate a signalling cascade. Toll-IL-1 receptor (TIR) (orange) domain. Leucine-rich repeat (LRR) (yellow). Other abbreviations include: Death domain (DD) (green), Interdomain (ID) (blue), Phosphatidylinositol 4,5 biphosphate (PIP2) (bright yellow), TRAF6 binding-motif (T6BM) (red), Receptor-interacting protein (RIP) homotypic interaction motif (RHIM) (dark green).

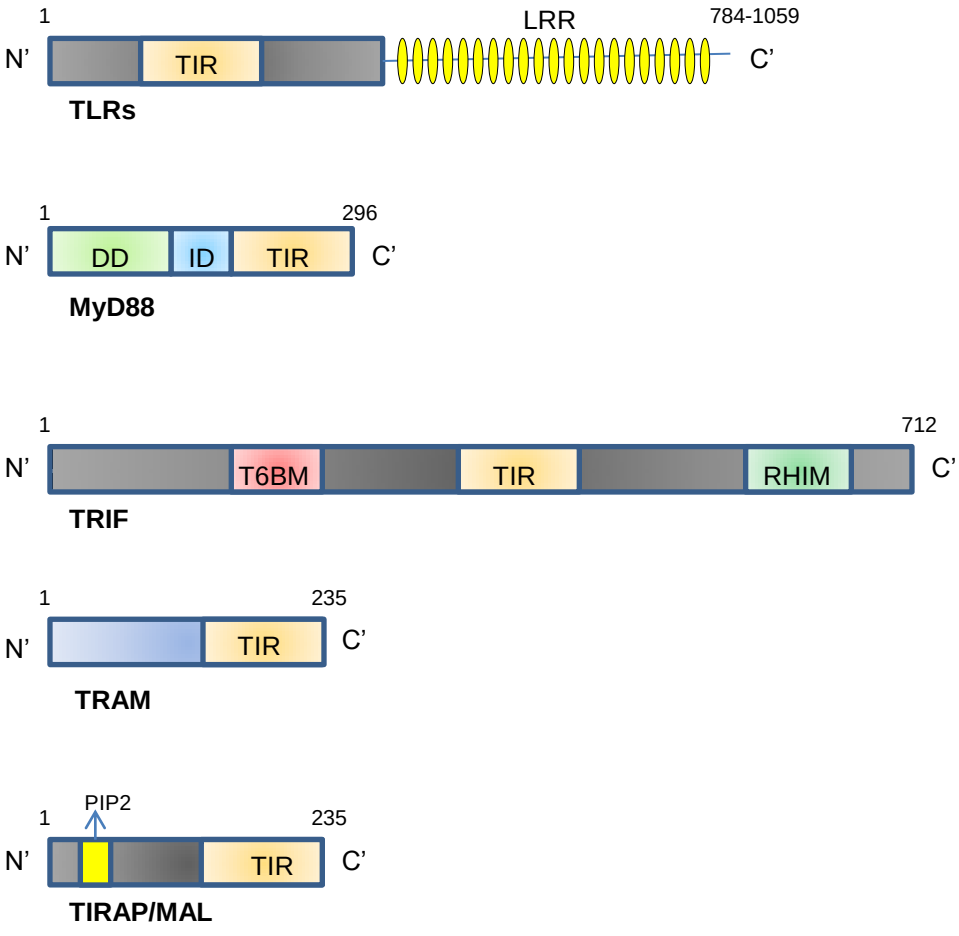
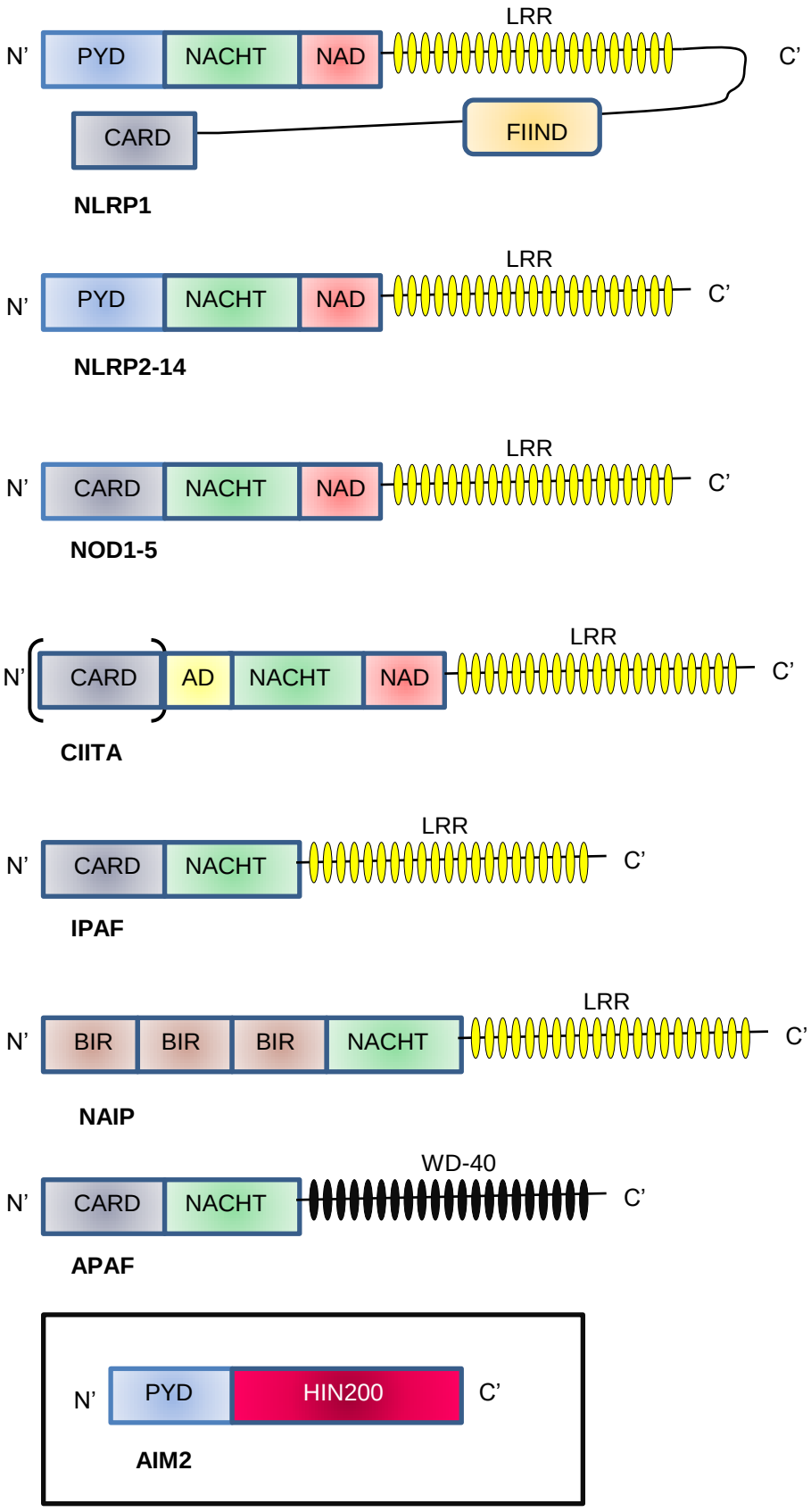

1
LRR
784-1059
N’
TIR
C’
TLRs
1
296
N’
DD
ID
TIR
C’
MyD88
1
712
N’
T6BM
TIR
RHIM
C’
TRIF
1
235
N’
TIR
C’
TRAM
PIP2
1
235
N’
TIR
C’
TIRAP/MAL

Figure 1.4. Schematic representation of the members of the NLR family and AIM2 (Adapted from Martinon and Tschopp, 2005). Members of the NLR family are characterised by three domains: an N terminus that contains either a pyrin (PYD) (light blue) or a caspase recruitment domain (CARD) (dark blue), a NACHT (green) domain and a LRR domain at the C terminus. In addition, NALPs, NODs and CIITA contain a NACHT-associated domain (NAD) (red), while the parenthesis surrounding the CARD for CIITA indicates that this domain is only present in dendritic cells (DCs). Other abbreviations include: BIR (brown) (baculoviral inhibitor-of-apoptosis repeat), FIIND (orange) function to be identified domain, AD (yellow) activation domain and HIN200 (pink) , which is only present in AIM2 (a non-NLR family member) and is required for ligand binding.


LRR
N'
PYD
NACHT
NAD
C'
CARD
FIIND
NLRP1
LRR
N'
PYD
NACHT
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C'
NLRP2-14
LRR
N'
CARD
NACHT
NAD
C'
NOD1-5
LRR
N'
CARD
AD
NACHT
NAD
C'
CIITA
LRR
N'
CARD
NACHT
C'
IPAF
LRR
N'
BIR
BIR
BIR
NACHT
C'
NAIP
WD-40
N'
CARD
NACHT
C'
APAF
N'
PYD
HIN200
C'
AIM2

Figure 1.5. Signalling of NOD1 and NOD2 (Adapted from Strober *et al.*, 2006). Activation of NOD1 and NOD2 by their respective bacterial peptidoglycan (PGN) induces the NF-κB and MAPK signalling pathways to initiate the transcription of pro-inflammatory genes.

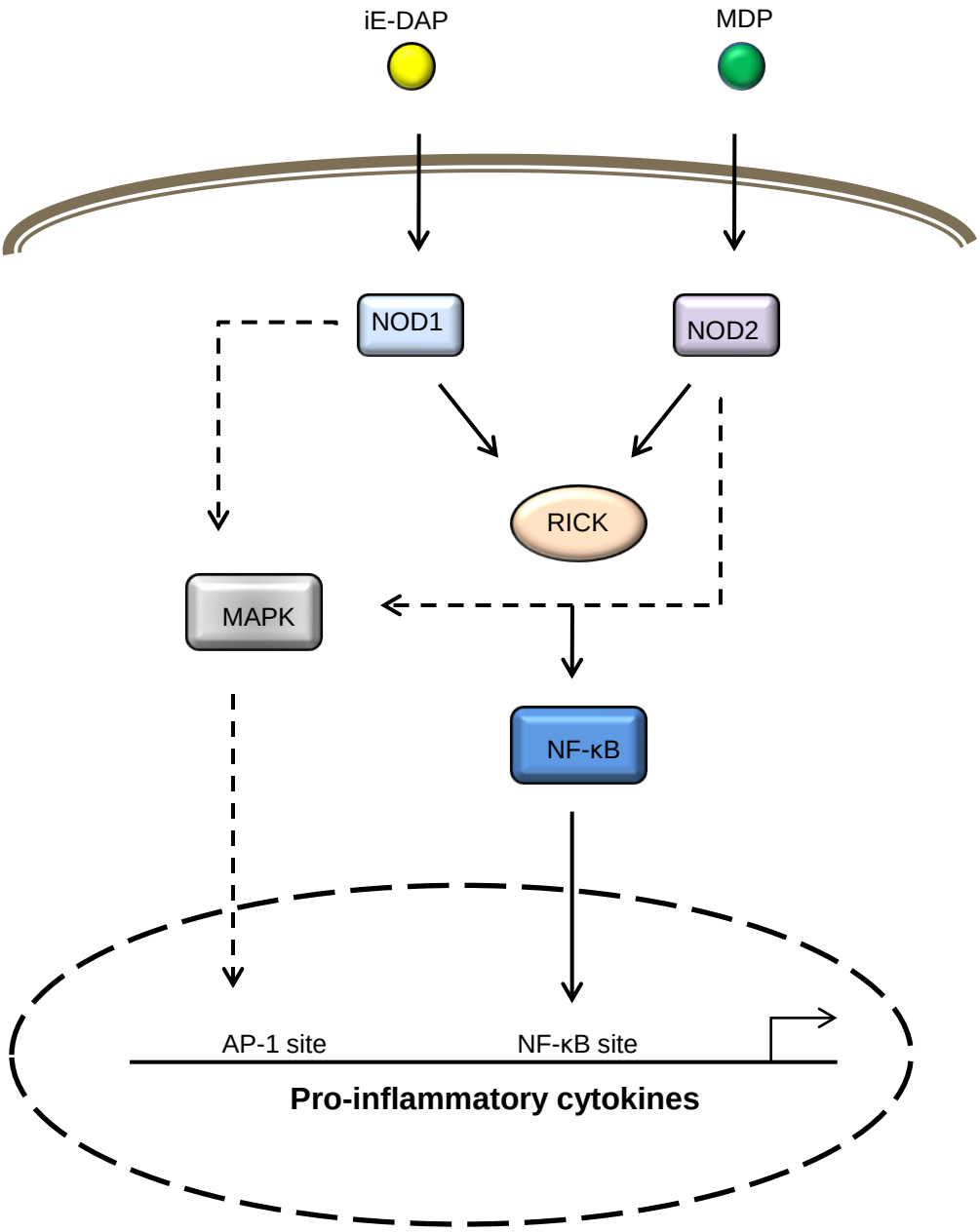

iE-DAP
MDP
NOD1
NOD2
RICK
MAPK
NF-κB
AP-1 site
NF-κB site
Pro-inflammatory cytokines

Figure 1.6. Schematic representation of the inflammasome complexes (Adapted from Jones *et al.*, 2011). The inflammasome complex consists of the interaction of apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase 1 with a member of the NLR family or AIM2. The formation of this complex is necessary for the processing of pro-IL-1β and pro-IL-18 into their mature forms.

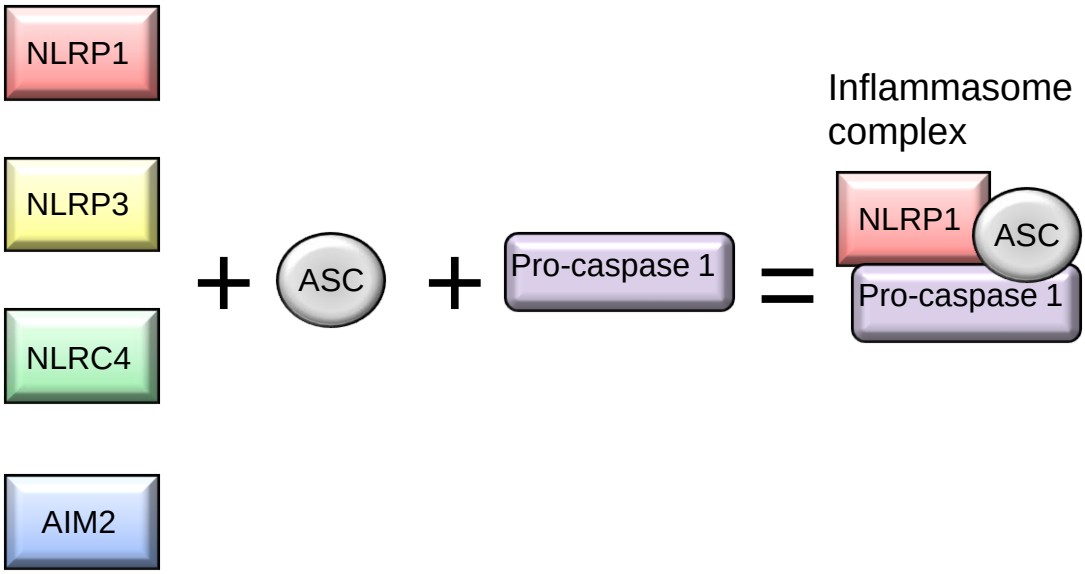

NLRP1
NLRP3
NLRC4
AIM2
+
ASC
+
Pro-caspase 1
=
Inflammasome complex
NLRP1
ASC
Pro-caspase 1

Figure 1.7. Schematic diagram of the inflammasome signalling pathway (Adapted from Elinav *et al.*, 2011). Activation of the inflammasome requires two signals. Signal 1 consists of the recognition of PAMPs by TLRs to induce the transcription of pro-IL-1β and pro-IL-18 mRNA. Signal 2 is the recognition of DAMPs by NLRs/AIM2 for inflammasome assembly, which is required for the processing of pro-IL-1β and pro-IL-18 mRNA into their mature forms.

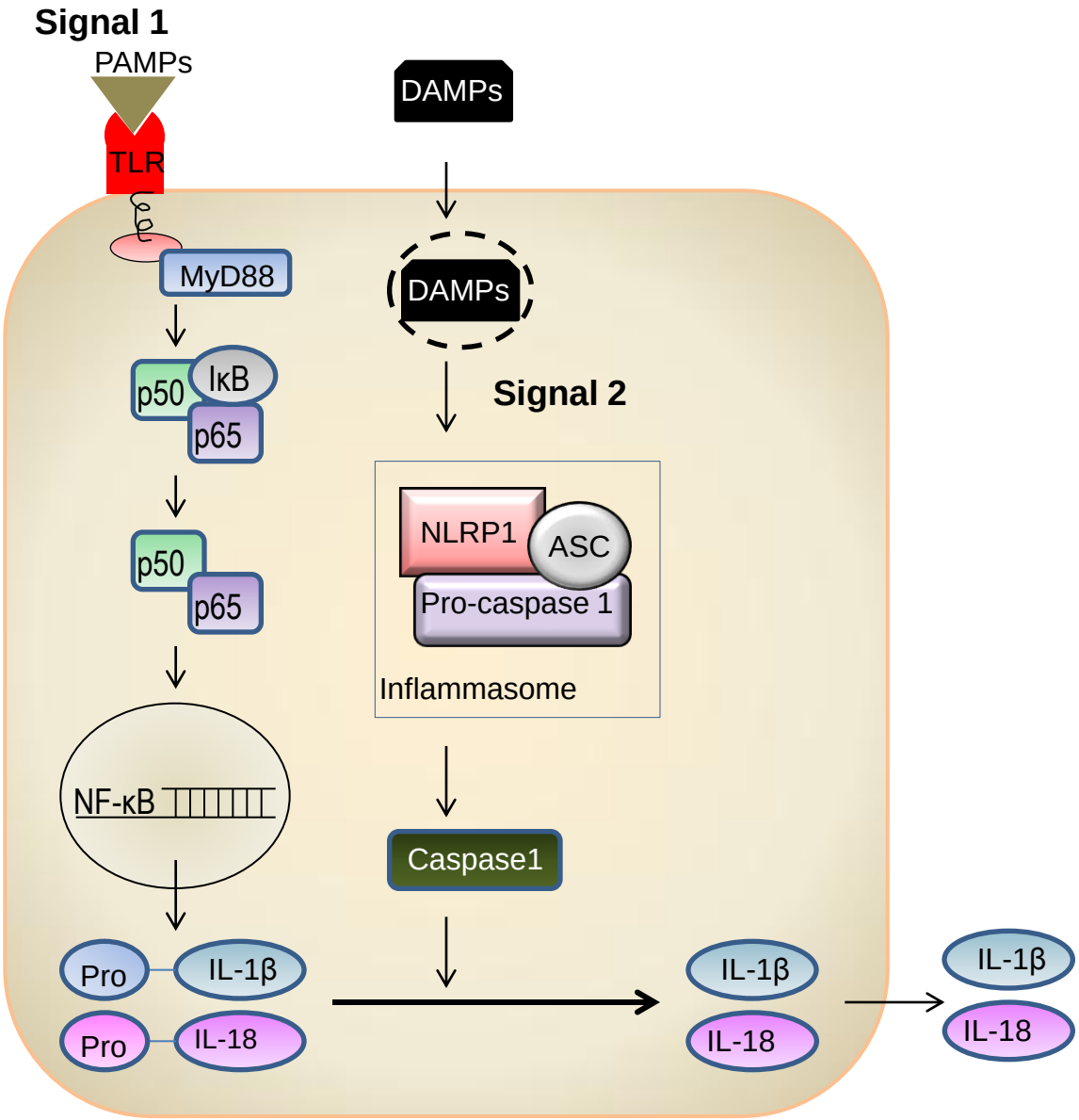
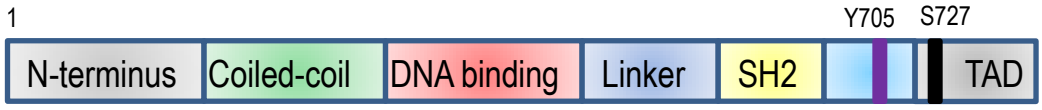
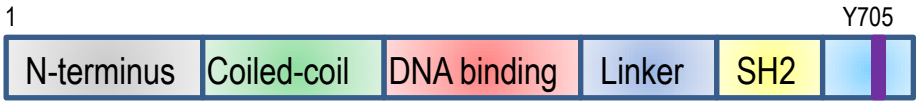

Signal 1
PAMPs
TLR
MyD88
p50
IκB
p65
p50
p65
NF-κB
Pro
IL-1β
Pro
IL-18
DAMPs
DAMPs
Signal 2
NLRP1
ASC
Pro-caspase 1
Inflammasome
Caspase1
IL-1β
IL-18
IL-1β
IL-18

Figure 1.8. Schematic representation of the structure of STAT3α and STAT3β isoforms (Adapted from Bowman *et al.*, 2000). STAT3 typically contains coiled-coil domain, also known as the oligomerisation domain, a DNA binding domain, a linker domain, a Src-homology 2 (SH2) domain and a trans-activation domain (TAD), which is absent in the STAT3β isoform. The Y705 and S727 shown in this diagram represent important phosphorylation sites for the activation of STAT3.



STAT3α



STAT3β

Figure 1.9. A schematic representation of the IL-6 signalling pathway. Activation of gp130 homodimers with members of the IL-6 family of cytokines result in the recruitment of Janus kinases (JAKs) to phosphorylate (P) tyrosine (Y) residues along the cytoplasmic tail of gp130. Phosphorylation of Y757 mediates the interaction of SH2 domain-containing tyrosine phosphatase (SHP) 2 to initiate MAPK and AKT signalling pathways, and suppressor of cytoking signalling (SOCS) 3, which is known to negatively regulate STAT3 activity by targeting the receptor complex to the proteosome for degradation. The recruitment and activation of STAT3 results in the formation of homodimers and heterodimers with STAT1, whereby it translocates into the nucleus to initiate the transcription of STAT3 target genes.

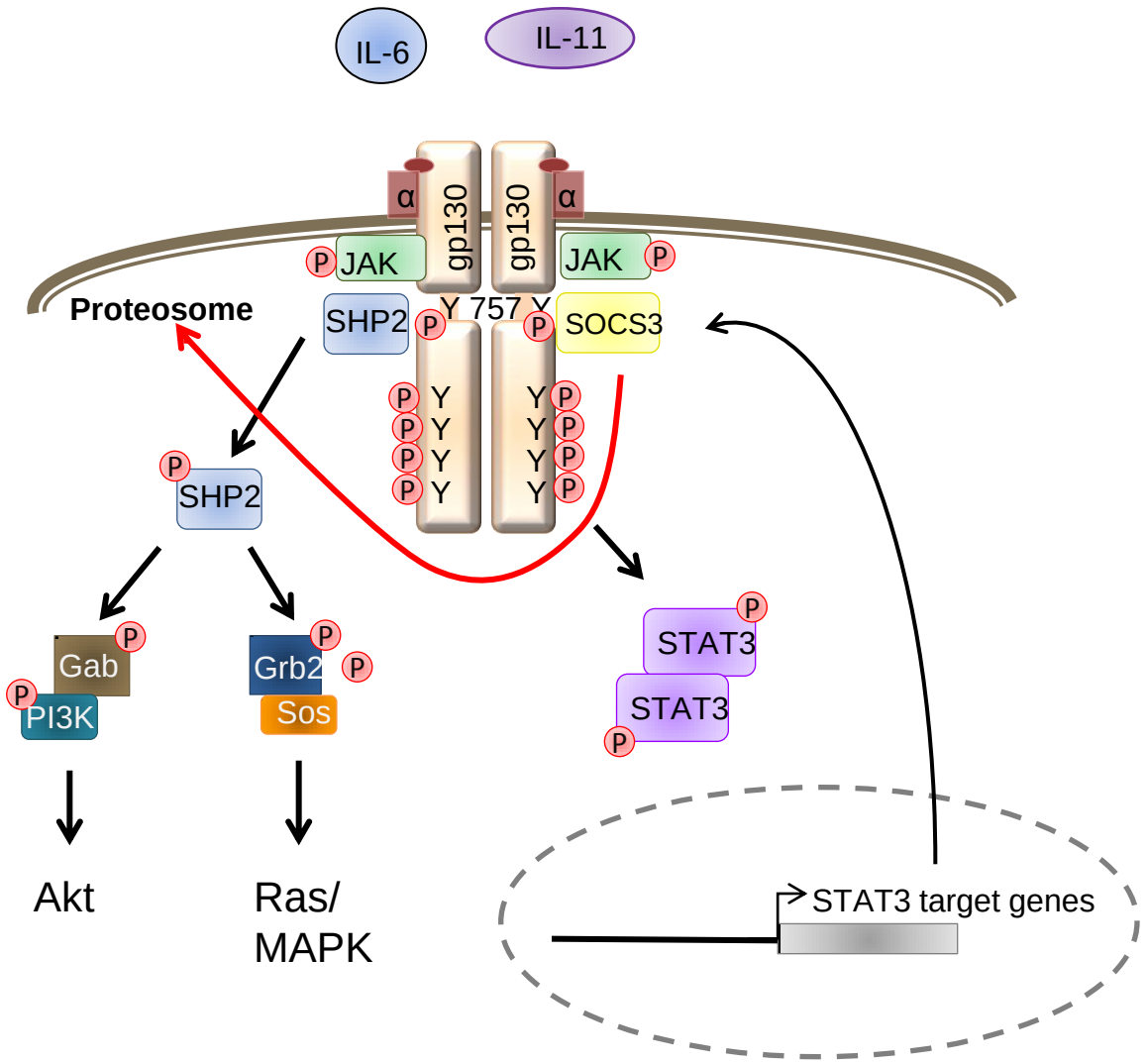

IL-6
IL-11
α
gp130
gp130
α
JAK
JAK
Proteosome
SHP2
Y 757 Y
SOCS3
Y
SHP2
Gab
PI3K
Grb2
Sos
STAT3
STAT3
Akt
Ras/
MAPK
STAT3 target genes

Figure 1.10. A schematic representation of the IL-6 signalling pathway in *gp130*$^{F/F}$ mice. A phenylalanine substitution of tyrosine 757 hinders the interaction of SOCS3 and thus abrogates the negative regulation of STAT3 activity. As shown in this figure *gp130*$^{F/F}$ mice are unable to signalling via SHP2 in an IL-11-dependent manner and display augmented levels of STAT3 activation.

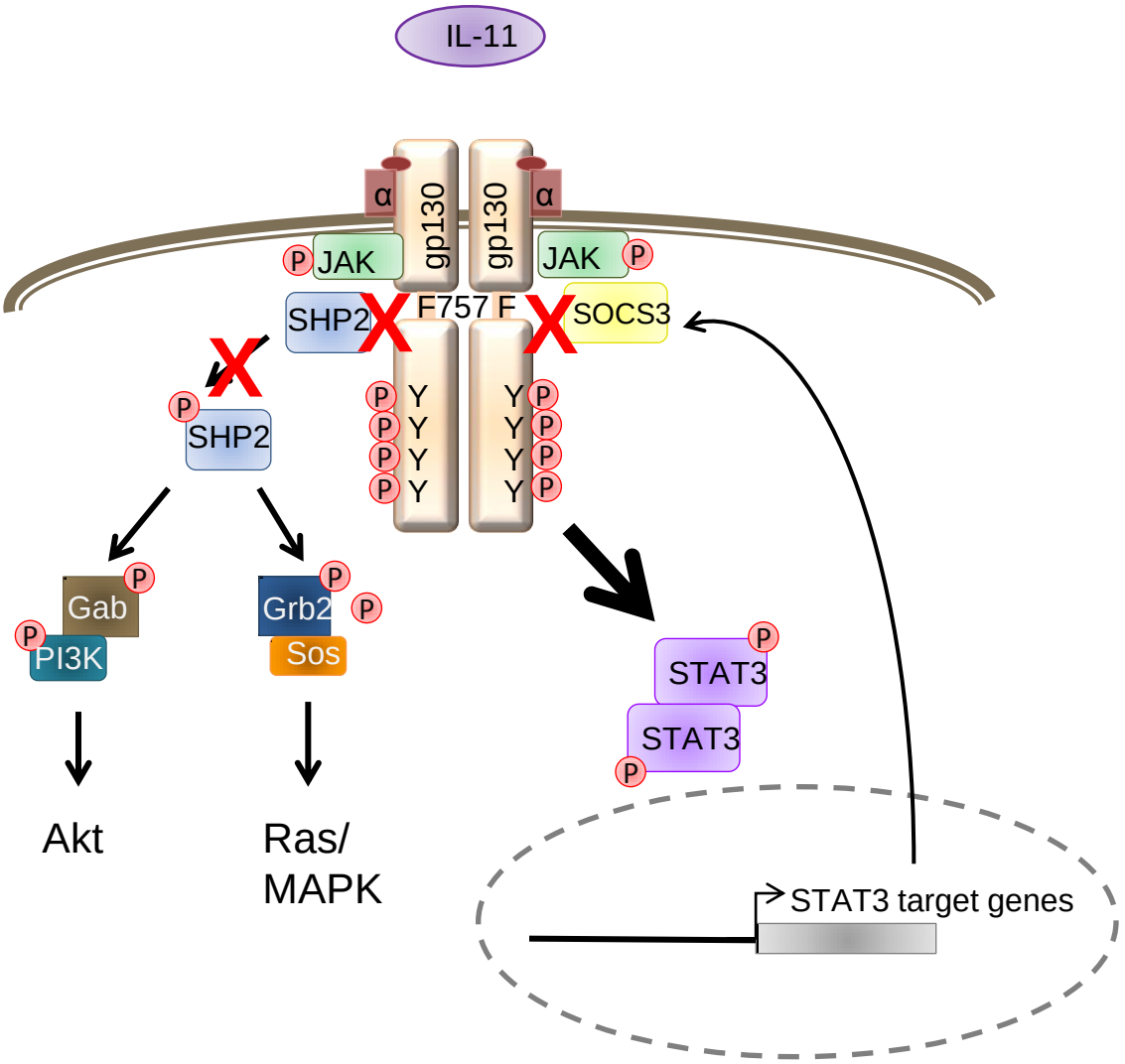
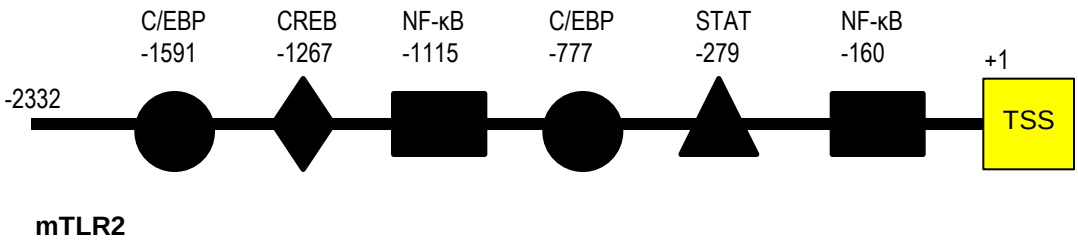

IL-11
α
gp130
gp130
α
P
JAK
JAK
P
SHP2
F757 F
SOCS3
P
SHP2
P Y
P Y
P Y
P Y
Y P
Y P
Y P
Y P
Gab
P
P
PI3K
Grb2
P
P
Sos
STAT3
P
STAT3
P
Akt
Ras/
MAPK
STAT3 target genes

Figure 1.11. Schematic representation of the 5' promoter region of the mouse *Tlr2* gene (Adapted from Musikacharoen *et al.*, 2001). The putative transcription binding sites upstream of the transcription start site (TSS) were determined using TRANSFAC.


C/EBP
-1591
CREB
-1267
NF-κB
-1115
C/EBP
-777
STAT
-279
NF-κB
-160
+1
-2332
TSS

mTLR2

Table 1.1. The TLR family members

TLR	Species expression	Ligand
TLR1	Mouse, Human	Bacterial and mycobacterial diacyl lipopeptides Synthetic triacylated lipoproteins (Pam3Cys)[1]
TLR2	Mouse, Human	Lipopeptides from Gram positive bacteria Lipomannans from mycobacteria Phospholipomannan from fungi Glycosylphosphatidylinositolmucin from parasites Proteins from Gram negative bacteria (i.e. porins) and viruses
TLR3	Mouse, Human	Double stranded RNA Synthetic polyriboinosinic: polyribocytidylic acid (poly(I:C))
TLR4	Mouse, Human	Bacterial lipopolysaccharide (LPS) from Gram negative bacteria Mannan from Fungi Glycoinositolphospholipids from parasites Viral proteins
TLR5	Mouse, Human	Bacterial flagellin
TLR6	Mouse, Human	Diacylated lipoproteins from mycoplasma Bacterial glycolipid lipotechoic acid (LTA)[2]
TLR7	Mouse, Human	Single stranded RNA
TLR8	Mouse	Unable to signal
TLR8	Human	Single stranded RNA Imidazoquinolines (imiquimod)
TLR9	Mouse, Human	Unmethylated CpD-DNA motifs from bacteria and mycobacteria
TLR10	Human	Ligand not identified
TLR11	Mouse, Human	Profilin-like molecule
TLR12	Mouse	Ligand not identified
TLR13	Mouse	Ligand not identified

[1, 2] As a heterodimer with TLR2

Table 1.2. The NLR family members

NLR Subfamily	Name	N-terminus	Ligand/function
NLRA	CIITA	CARD	Regulate MHC class II expression
NLRB	NAIP	Bir	Involved in inflammasome activation
NLRC	NOD1	CARD	Muro-peptides (iE-DAP)
	NOD2	CARD	Muramyl dipeptide (MDP)
	NLRC3	CARD[2]	Unknown
	NLRC4[1]	CARD	Mainly Gram negative bacteria containing a type 3 secretion system (T3SS) or T4SS with flagella (e.g. *Salmonella, shigella* and *legionella*)
	NLRC5	CARD[2]	Anti-viral properties Regulates MHC class 1 expression
NLRP	NLRP1[1]	PYD	Infectious signals (e.g. *Bacillus anthracis* lethal toxin and MDP)
	NLRP2	PYD	Activate caspase-1 through inflammasome activation
	NLRP3[1]	PYD	Non-infectious signals (e.g. monosodium urate (MSU), silica and alum) Infectious signals (e.g. pore forming toxins pneumolysin by *Streptococcus pneumonia* and viral single/double stranded RNA)
	NLRP4	PYD	Unknown
	NLRP5	PYD	Unknown
	NLRP6	PYD	Unknown
	NLRP7	PYD	Unknown
	NLRP8	PYD	Unknown
	NLRP9	PYD	Unknown
	NLRP10	PYD	Unknown
	NLRP11	PYD	Unknown
	NLRP12	PYD	Possesses anti-inflammatory regulatory properties
	NLRP13	PYD	Unknown
	NLRP14	PYD	Unknown
NLRX1	NLRX1	CARD[2]	Anti-viral response

[1] Forms the inflammasome complex
[2] Unconfirmed CARD domain

Table 1.3. Mouse models of GC

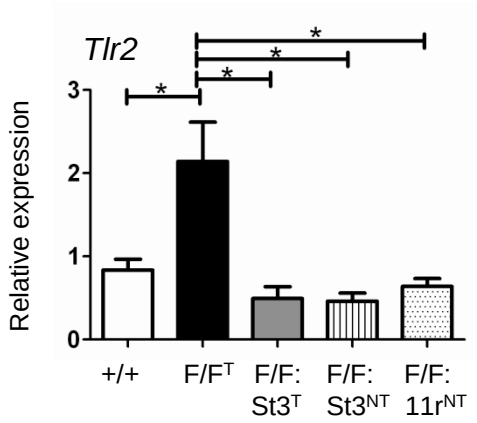
Mouse model	Molecular outcome	Phenotype	Reference
Cdx2 Tg	Mice display increase levels of Cdx2 (transcription factor) in the gastric mucosa	Hypergastrinemia and intestinal metaplasia	Mutoh *et al.,* (2002)
***Runx3*$^{-/-}$**	*Runx3* (transcription factor) KO	Born with gastric hyperplasia due to increased gastric epithelial cell proliferation as cells are resistant to TGF-β (cytostatic tumour activity)	Li *et al.,* (2002)
***Tgfb1*$^{C33S/C33S}$**	Cys33Ser mutation prevents the formation of TGF-β1 complex	Multi-organ inflammation by 10 weeks and adenocarcinoma in the glandular stomach by 8 weeks. Inflammation is not as severe as *Tgfb1*$^{-/-}$ mice.	Yoshinaga *et al.,* (2008)
INS-GAS Tg	Mice display increase gastrin production	Gastric adenocarcinoma with *H. pylori* infection 7 months post-infection	Fox *et al.,* (2003)
h*IL-1β* Tg	Over-express human IL-1β in the stomach	Spontaneous gastritis and hyperplasia at 12 months. 30% of mice that highly over-express IL-1β will develop adenocarcinoma. Infection with *H. felis* exacerbated the phenotype with approximately 10% of mice developing invasive carcinoma.	Tu *et al.,* (2008)
K19-Wnt1/C2mE (Gan)	Mice display increase activation of Wnt, COX-2 and prostaglandin E_2 signalling pathways in the gastric mucosa	Metaplasia by 5 weeks and dsyplastic tumours by 20 weeks of age with 100% penetrance.	Oshima *et al.,* (2006)

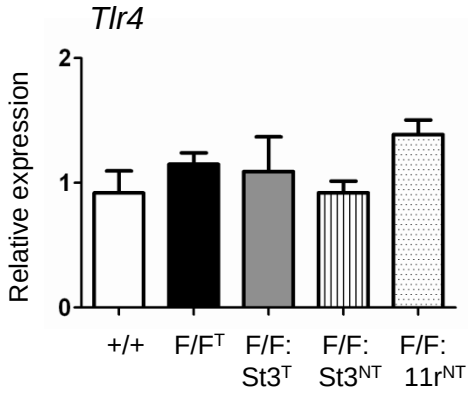
Tg = transgenic, KO = knock-out

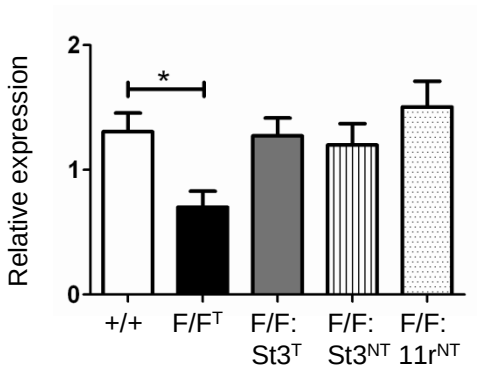
Table 3.1. Differential expression of genes (>2 fold expression) involved in the toll-like receptor signalling pathway that are up-regulated in the gastric antral tumours of 24 week old *gp130*$^{F/F}$ mice compared to the gastric antrum from age-matched *gp130*$^{+/+}$ mice. Data are from 4 mice per genotype and are presented as the mean fold difference relative to *gp130*$^{+/+}$ antrum and data are normalised to *Actin*.

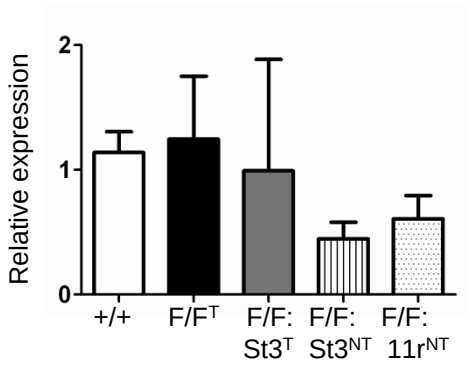
Gene Symbol	Fold Difference	Protein Function
Il6	24.9292	Downstream Pathways and Target Genes: NF-kB Pathway
Il1a	12.0407	Downstream Pathways and Target Genes: NF-kB Pathway
Csf3	9.6946	Downstream Pathways and Target Genes: NF-kB Pathway
Il1b	9.5919	Downstream Pathways and Target Genes: NF-kB Pathway
Tnfa	8.2091	Downstream Pathways and Target Genes: NF-kB Pathway
Tnfaip3	6.1455	Downstream Pathways and Target Genes: NF-kB Pathway
Lta	5.5133	Downstream Pathways and Target Genes: NF-kB Pathway
Il1r1	3.0707	Downstream Pathways and Target Genes: NF-kB Pathway
Rel	2.0665	Downstream Pathways and Target Genes: NF-kB Pathway
Cd14	5.8313	Adaptors & Toll-Like Receptor Interacting Proteins
Tlr9	2.4266	Toll-Like Receptors
Tlr2	2.1586	Toll-Like Receptors
Cd80	2.9662	Regulation of Adaptive Immunity

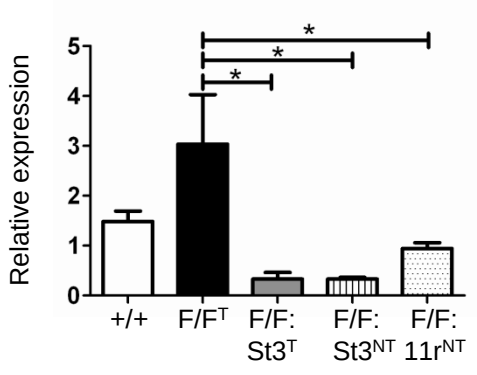
Figure 3.1. Up-regulation of *Tlr2* gene expression in the tumours of *gp130*$^{F/F}$ mice strongly correlates with augmented expression levels of STAT3 target genes, *Stat3* and *Il-11*. Quantitative real-time PCR analyses were performed on gastric tissues from 24 week old *gp130*$^{+/+}$ antrum (+/+), *gp130*$^{F/F}$ tumour (F/F^{T}), *gp130*$^{F/F}$*:Stat3*$^{+/-}$ tumour (F/F:St3^{T}) and tumour-free antrum (F/F:St3^{NT}), and *gp130*$^{F/F}$*:IL-11r*$^{-/-}$ tumour-free antrum (F/F:11r^{NT}). Data from at least 4 mice per genotype are shown following normalisation for *18S* and are expressed relative to *gp130*$^{+/+}$ antrum as the mean SEM. n = 2 for *Nod1* and *Nod2* qRT-PCR analyses on F/F:St3^{T} . * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.


Tlr2
Relative expression
0
1
2
3
*
*
*
*
+/+
F/F^{T}
F/F: St3^{T}
F/F: St3^{NT}
F/F: 11r^{NT}


Tlr4
Relative expression
0
1
2
+/+
F/F^{T}
F/F: St3^{T}
F/F: St3^{NT}
F/F: 11r^{NT}


Nod1
Relative expression
0
1
2
*
+/+
F/F^{T}
F/F: St3^{T}
F/F: St3^{NT}
F/F: 11r^{NT}


Nod2
Relative expression
0
1
2
+/+
F/F^{T}
F/F: St3^{T}
F/F: St3^{NT}
F/F: 11r^{NT}


Stat3
Relative expression
0
1
2
3
4
5
*
*
*
+/+
F/F^{T}
F/F: St3^{T}
F/F: St3^{NT}
F/F: 11r^{NT}

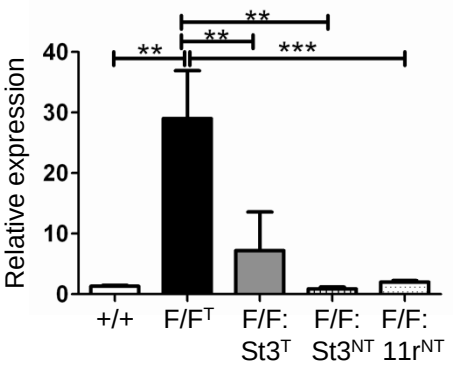
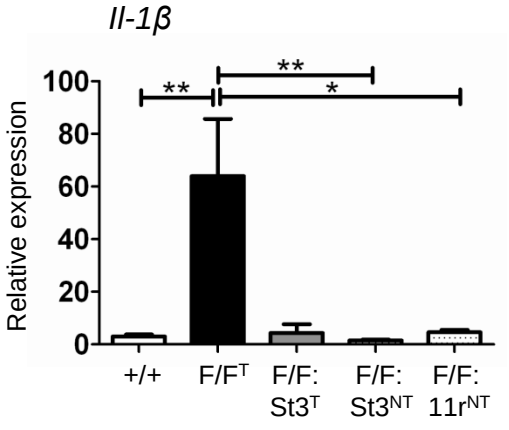

Il-11
Relative expression
0
10
20
30
40
**
**
**
***
+/+
F/F^{T}
F/F: St3^{T}
F/F: St3^{NT}
F/F: 11r^{NT}

Figure 3.2. Pro-inflammatory mediator gene expression levels are significantly augmented in the gastric tumours of *gp130*$^{F/F}$ mice. The gene expression levels of *Il-1β* and *Cxcl2* were measured by quantitative real-time PCR in 24 week old tissue samples from *gp130*$^{+/+}$ antrum (+/+), *gp130*$^{F/F}$ tumour (F/F^{T}), *gp130*$^{F/F}$*:Stat3*$^{+/-}$ tumour (F/F:St3^{T}) (n=2) and antrum (F/F:St3^{NT}) and *gp130*$^{F/F}$*:IL-11r*$^{-/-}$ antrum (F/F:11r^{NT}). Data from at least 4 mice per genotype are shown following normalisation for *18S* and are expressed relative to *gp130*$^{+/+}$ antrum as the mean SEM. * $P \leq 0.05$ and ** $P \leq 0.01$.


Il-1β
Relative expression
100
80
60
40
20
0
**
**
*
+/+
F/F^T
F/F: St3^T
F/F: St3^NT
F/F: 11r^NT

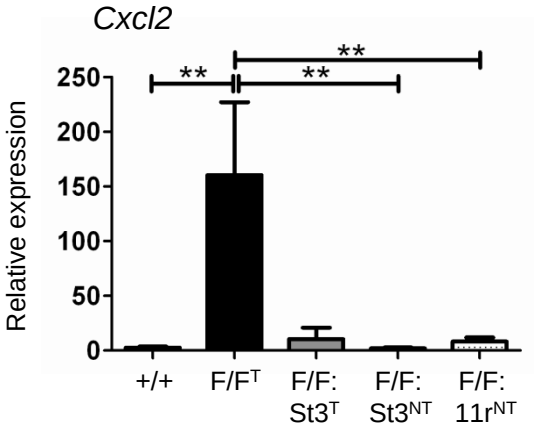
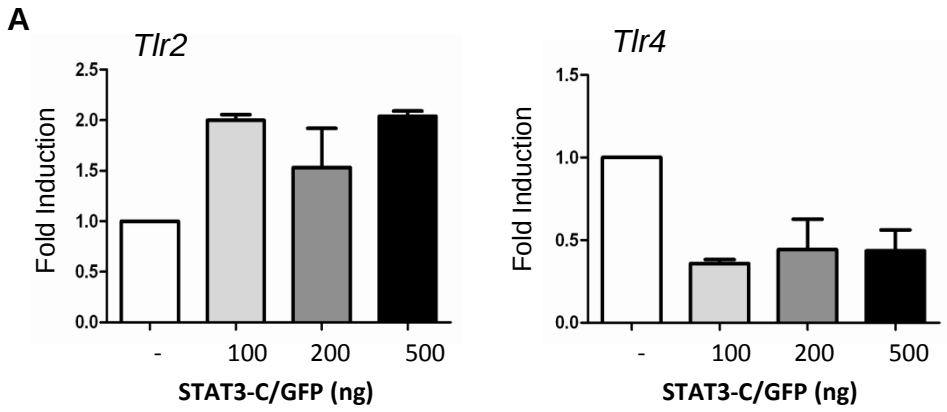
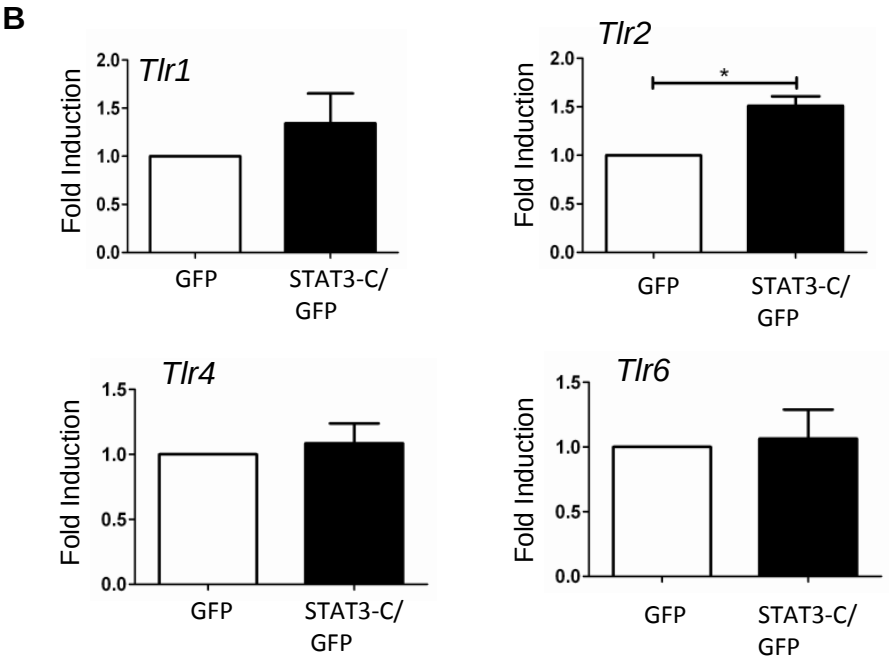

Cxcl2
Relative expression
250
200
150
100
50
0
**
**
**
+/+
F/F^T
F/F: St3^T
F/F: St3^NT
F/F: 11r^NT

Figure 3.3. Over-expression of a hyper-active STAT3 construct (STAT3-C/GFP) in a mouse gastric epithelial cell line (IMGE-5) augments *Tlr2* gene expression. Quantitative real-time PCR gene expression analyses were performed on **(A)** IMGE-5 cells that transiently over-express an empty GFP vector (control), or 100ng, 200ng and 500ng of STAT3-C/GFP (n=2) and **(B)** IMGE-5 cells that stably over-express 500ng of STAT3-C/GFP (n=3). **(C)** GFP immunoblotting of IMGE-5 cells that stably over-express STAT3-C/GFP. Data from 3 independent experiments are shown following normalisation to *18S* and are expressed relative to control cells as the mean SEM. * $P \leq 0.05$


A
Tlr2
Fold Induction
2.5
2.0
1.5
1.0
0.5
0.0
-
100
200
500
STAT3-C/GFP (ng)
Tlr4
Fold Induction
1.5
1.0
0.5
0.0
-
100
200
500
STAT3-C/GFP (ng)


B
Tlr1
Fold Induction
2.0
1.5
1.0
0.5
0.0
GFP
STAT3-C/ GFP
Tlr2
Fold Induction
*
GFP
STAT3-C/ GFP
Tlr4
Fold Induction
1.5
1.0
0.5
0.0
GFP
STAT3-C/ GFP
Tlr6
Fold Induction
1.5
1.0
0.5
0.0
GFP
STAT3-C/ GFP

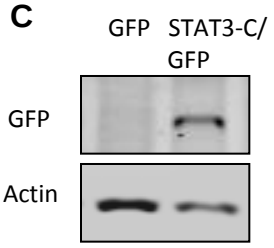

C
GFP
STAT3-C/ GFP
GFP
Actin

Figure 3.4. Human gastric epithelial cell lines that stably over-express STAT3-C/GFP display augmented levels of *TLR2* gene expression. Quantitative real-time PCR analyses were performed on **(A)** MKN-28 cells and **(B)** AGS cells that stably over-express 500ng of empty GFP vector (control) or STAT3-C/GFP. **(C)** GFP immunoblotting of MKN28 cells that over-express STAT3-C/GFP. Data from 3 independent experiments are shown following normalisation to *18S* and are expressed relative to control cells as mean SEM. * $P \leq 0.05$, ** $P \leq 0.01$

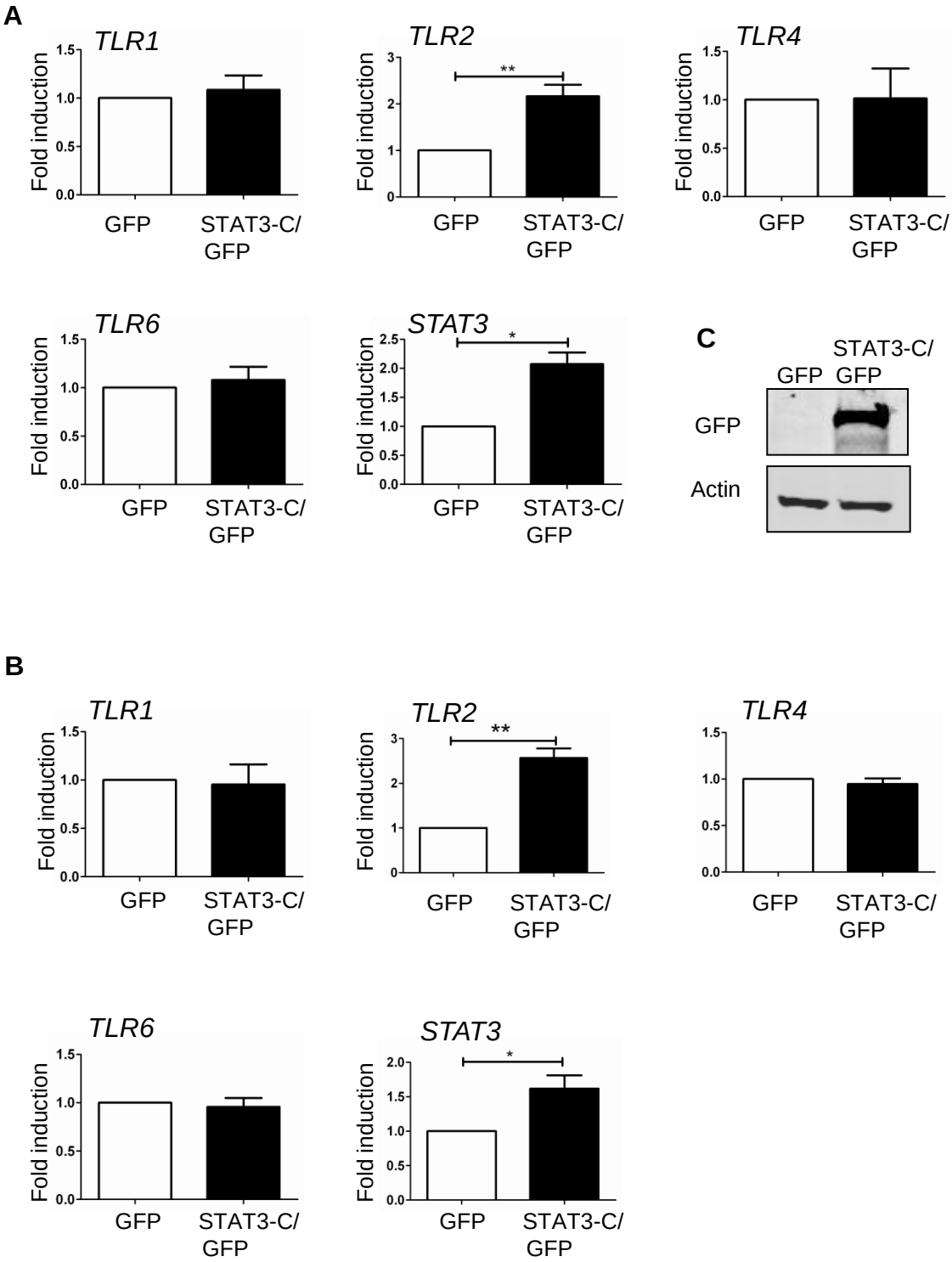

A
TLR1
TLR2
TLR4
TLR6
STAT3
Fold induction
GFP
STAT3-C/ GFP
**
*
C
STAT3-C/ GFP
GFP
Actin
B
TLR1
TLR2
TLR4
TLR6
STAT3
Fold induction
GFP
STAT3-C/ GFP
**
*

Figure 3.5. Promoter sequence analyses of mouse TLR2 reveal that there are five putative STAT3 binding sites. (A) The sequence of the 5′ promoter region of mouse TLR2. Sequences that are underlined depict the putative STAT3 binding sites, while the blue, green and red highlighted sequences show the forward and reverse primers designed for chromatin immunoprecipitation (ChIP) assays. **(B)** Schematic representation of the mouse and human TLR2 5 ′ promoter region and the location of putative STAT3 binding sites.

A

TGACAGCATCTCATGTAGCCCGGGCTGGCCTAATTACTCCATGAAATCCTTCTTCCTTGGCCTCCCCAATCGTGGG

ATTCCATGTGAGTCCCCACAGCAGGCAGAAATCAACGGCTTTTGCTCAGCTTTGCTTTTAGGATCACAGAGCAATA

AACTCAGTTAACCCTGGACGGCCAATTCGAGAAGTCAGCGTAGAGAAGAGCAGCTTCCAGTGTTAGGGAAAAGAAG

CTCAAAGGAGTTTTTGCTCAGTGTTTGAGAAATCCGGAGGAAGGAAGGCAGGCATCTGCTGTAGAGCCCGAGCCGC

TGAAGCTGCACAGTCACATGGGAACCTGTAAAACCAGACTTTGCAGAGCACAGAAGTTCACTCTCAAGCTTGAATT

GGTGCTTATTGATCTAGTTTCACTGATATTTGAAGGGCTAAGGGGTCAGAAAATGATAGTCCACAGGAAGTCACTT

TGGAATCCAAACTCACAGTGTTTGTGACAGCAAAGATAGCAGGGGCTTTGTCTGAGTCTTCTTATCTATACAACAC

TGGATCAGCCAAAGAGGAAGAGCATTGCCTCTGGCTCT**TTCAGAGA**TGAAGCTCACAGGGAAATGCCAACCCATGC

TAGAGCTCAGAGGCCTATGGCTGCATTTTC**TTCCCTGGAA**GCAAACCTCCTGGAACTGTAAACTCTTGCTCGTGAA

GAGGTTTTTGACGCACCAACACACTGGCCCTTCTTTGAGTCTCCTTGCCTTGCATGGCTGCCAGGGACATGTGTAT

GGGAGGAAATTTGCTTCTCTCTTGTTTTCGGTTGGTTTCAGCAGGTTTAAGGAATGTAGCTTTCAGAGAGAGAGAG

AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAACTTCTCTATAGGGCAGTGGTTCTCAACCCGTGGGTT

GAGACCCCATGGTGGTTGCATATATATTTTCATTACTATTCTTAACAGTAGGAAAATTACAATTATAAAGTACCAA

CAAAAATAATTTTATGGTTGGGAGTCATCACAACATGAGGAACCGTACTGAAGGGTTGCAGGGTTAGGAAGGTTGA

CAACCTATAAGGACAAGGGATGTGGAGCTGAGGGGAAGAGACCCCCTGGGTTTAAGGGAAAAATTCTGACTTCAAA

TCTCAGCTGCTATTTCTGA**TTCACAGGAC**ATTTTCTTGTGAAAATGGGGGTAAGGTTGCTTGCTCTGCATCCAATC

TCGCCTTGCCGAGGTCAAATGGGATCACAGGCACCCGAGGCAGGTGATTAACTTGTTTTAAAGTAGGGGGTTTCCC

CTTACAACTGGAATATGGAGCCACATCTCCACCATGAAGCAAACCAATCTACGCTGTTTTCAGAATCACTCTGCGA

TGCAGCTATGGGATAGAGAAAGGGAAGTACTTAGCTTCAAAGCCATAGATAGAAGTGGGGGCCCCGAATCTTCCCC

ATGCTTAGAAGGGGGGCTGTTCTGGGGGCCAAGGAAAGAGAAACATATCTGTGCCATCCTTATCCATTCATATCCC

AAGCACAATGCTGCCATTCAGGTCTGCATGGAGGCTGGTAGACTCTGTTATCAGATGCTGAGCAGAAATGGATCCC

AGAGACATAAGGGCAGGGGGACAAAGTGTTGAAAAGGAGGAGAAAGAGAAAATATTCCTGAGCCCAAGTTGCAACC

TCTGAGGATGGAGAGATAGCAAAAGAGTTCAAAAGATGTGTTTGAGGGAGAAGTGAGATTCTGAGATTAGAGTAGT

TAGAGTTAGGACCTGGGTCTTGGCAGCCCTGGCTGTCTAGCCTATCCTTTTGGGCCTCGATAGGGTATTTAAATGA

TTATCAGTAGACAGCAGAAAAGTAGCTATTCAGTGTAGCCACATCAGGAAGACAACTAAATAATGAGGGTGTACGA

CGCCCCCATCCTAAGTTCAGCATCCTGTCACCACAGGTTTAGATTTAGATAATACAGAAAGAATCCATAAGTAGGC

AGTTTTGGTCAAGGTGTGCTATCCCCCCGAGTCATTCAGCCATCATTGTCCAGGCCTCCAGTGTTTTCCTCATGGT

GAACTGATAAAAACTCCCTGTGACTGGCACAGGGCTGTAATTTTTTAACGT**TTCCTAGC**TGGAGCA**TTCCAATAA**C

CAAAGAGAGTGCGTCTCTTCAGAATATCCTCTTCCTTCCAGAATCCTTGCAGCAGCTCAACTCAGCCAAAAGGCGA

GCTGGGAGGCAGCTTTCTCTTTAGGACACCTGGGGAATTCCCACACGGAGCCTCTGGACTTTCAGTTCTGTTTTGC

CTGCCCTGTGGCTCCTGCCAGCTCTGATGCCAGGCTCCGTTCCCCTTGCAGACCCCTGTGGGCGGCGCTTGCCGCA

CCGGGGGCGGTGCTGGCGACCGGGAAGTTCGGGGCCCTGACCTGGGGACATC

B

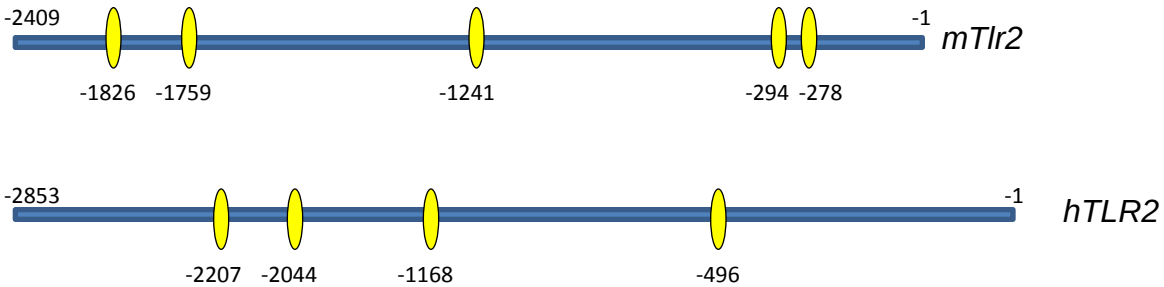
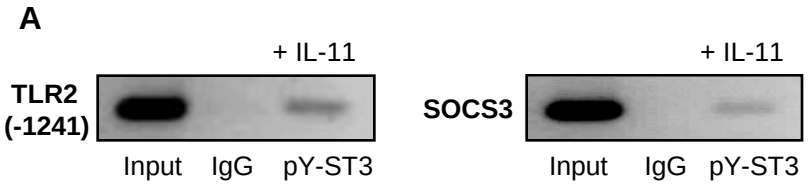


Figure 3.6. Tyrosine phosphorylated STAT3 is recruited to the STAT3 binding site located at position -1241 on the 5′ promoter region of TLR2. (A) IMGE-5 cells that stably over-express STAT3-WT were stimulated with IL-11 for 1 hour and ChIP analyses were performed using tyrosine phosphorylated STAT3 antibody and IgG control antibody. Gels represent semi-quantitative RT-PCR with primers spanning the -1241 STAT3 site in the TLR2 promoter, as well as control primers spanning the STAT3 site at position -70 in the *Socs3* gene promoter region. **(B)** Quantitative real-time PCR analyses were performed on the immunopreciptated pY-STAT3 TLR2 promoter region containing the -1241 to -1248 STAT3 binding site from cells either un-treated or treated with 200ng of IL-11.


A
+ IL-11
TLR2
(-1241)
Input
IgG
pY-ST3
+ IL-11
SOCS3
Input
IgG
pY-ST3

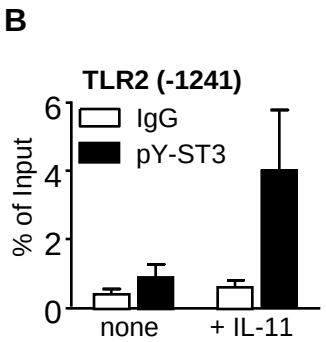

B
TLR2 (-1241)
IgG
pY-ST3
% of Input
6
4
2
0
none
+ IL-11

Figure 3.7. TLR2 is transcriptionally regulated by STAT3. (A) Schematic representation of the full length, truncated mutant and site-directed mutant 5′ TLR2 promoter luciferase constructs. **(B)** Human embryonic kidney (HEK) 293T cells were co-transfected with either an empty GFP vector or STAT3-C/GFP together with various 5′ TLR2 promoter luciferase constructs and the luciferase activity was measured, following which samples were made relative to cells transfected with the 5′ TLR2 promoter luciferase construct alone. Data are from 3 independent experiments and are presented as the mean luciferase activity SEM. *** $P \leq 0.001$

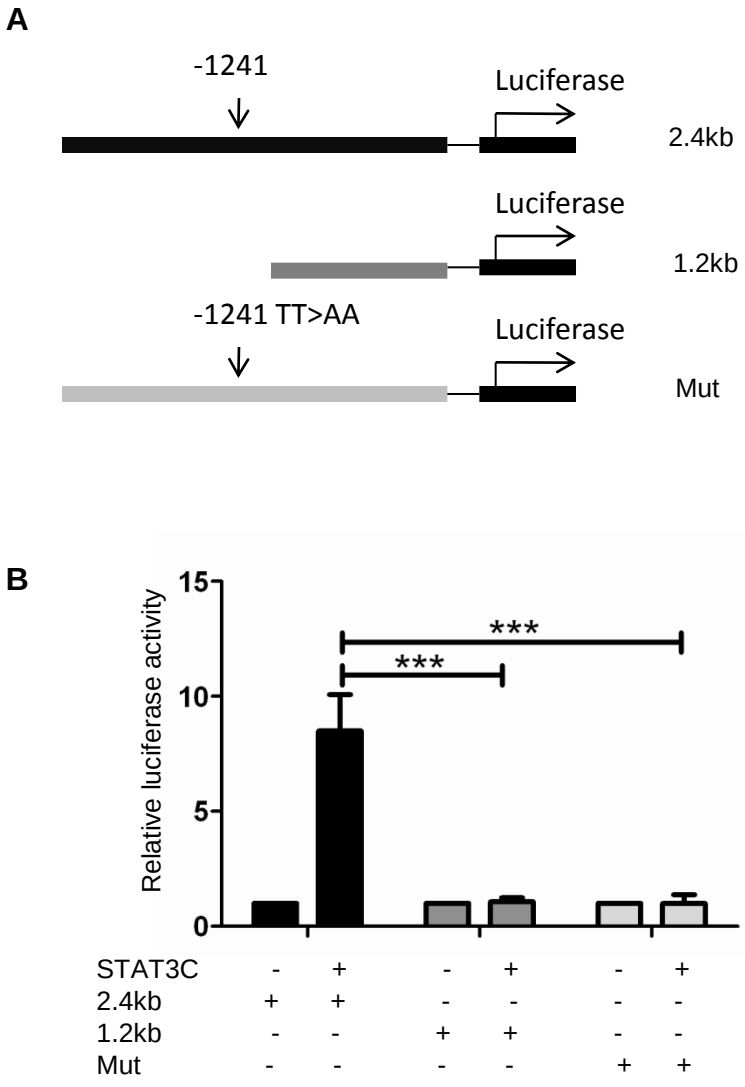

A
-1241
Luciferase
2.4kb
Luciferase
1.2kb
-1241 TT>AA
Luciferase
Mut
B
Relative luciferase activity
15
10
5
0
***
***
STAT3C - + - + - +
2.4kb + + - - - -
1.2kb - - + + - -
Mut - - - - + +

Figure 5.1. Activation of TLR2 does not promote mouse gastric epithelial cell migration *in vitro*. A scratch assay was performed with **(A)** IMGE5 cells and **(B)** IMGE5 cells that stably over-express STAT3-C/GFP. Cells were either untreated or treated with Pam_3CSK4 (P_3C) (100ng/ml) and rhIL-11 (200ng/ml) for a period of 16 hours, and the migration rate was quantified by measuring the area that is not occupied by cells at t=0 versus t=16 hours. Data are from 4 independent experiments and are presented as migration rate SEM.

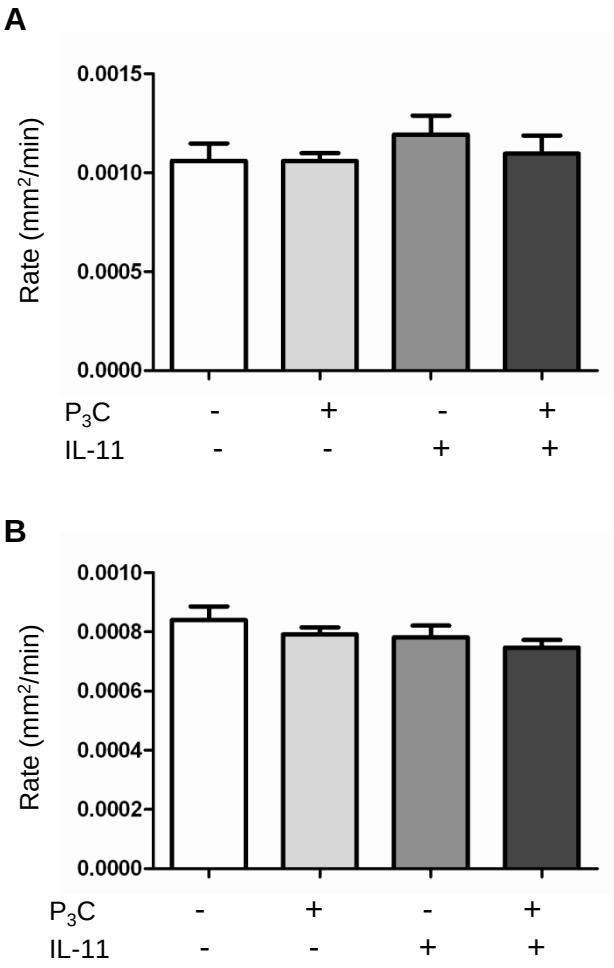

A
0.0015
0.0010
0.0005
0.0000
Rate (mm²/min)
P3C - + - +
IL-11 - - + +
B
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
Rate (mm²/min)
P3C - + - +
IL-11 - - + +

Figure 5.2. Angiogenesis is unaffected in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice. Representative photomicrographs (from n = 3 mice per genotype) stained with CD31 of the **(A)** gastric tumours and **(B)** gastric sub-mucosa of 24 week old *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ (F/F:T2$^{-/-}$) male mice. **(C)** Representative photomicrograph stained with Rat IgG1 isotype control. Scale bars = 200μm.

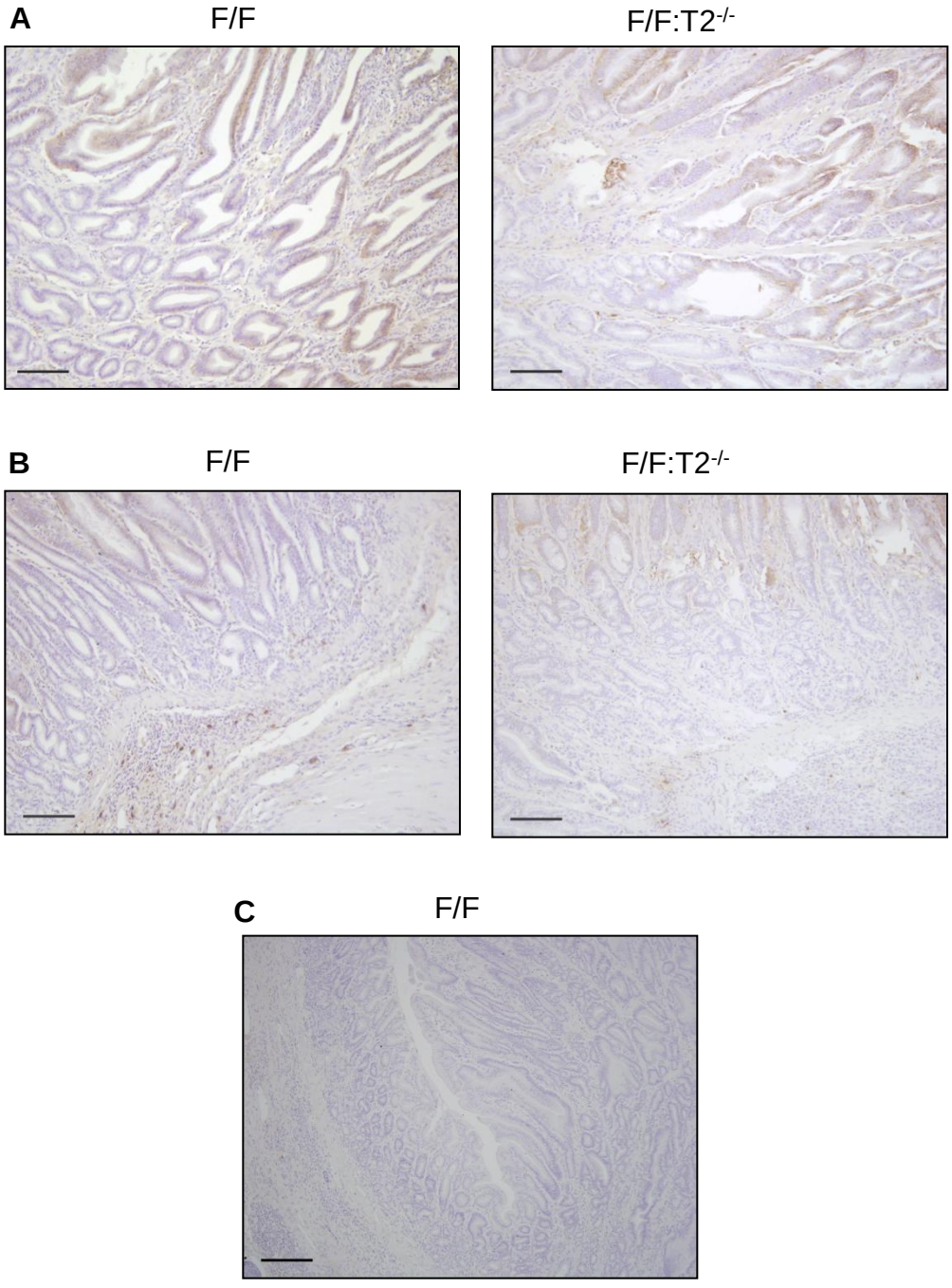

A
F/F
F/F:T2-/-
B
F/F
F/F:T2-/-
C
F/F

Figure 5.3. Genetic ablation of TLR2 in *gp130*$^{F/F}$ mice results in increased numbers of apoptotic cells in the gastric surface epithelium. Representative photomicrographs (from n = 3 mice per genotype) showing **(A)** PCNA-stained and **(B)** TUNEL-stained gastric cross-section of 24 week old *gp130*$^{+/+}$ (+/+), *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ (F/F:T2$^{-/-}$) male mice. Scale bars = 100μm.

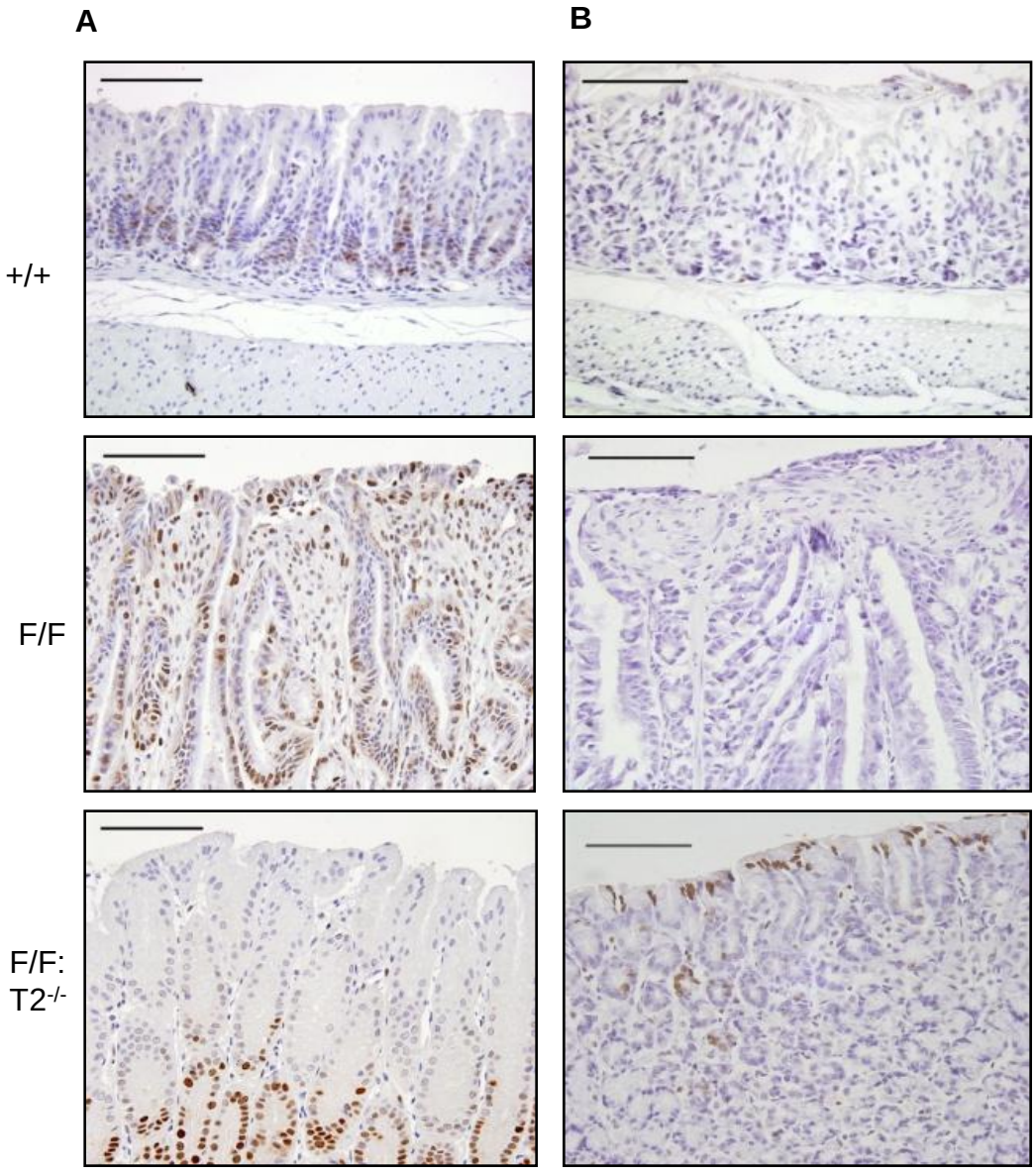

A
B
+/+
F/F
F/F:
T2-/-

Figure 5.4. Activation of TLR2 signalling in human gastric cancer cell lines induces cell proliferation. *In vitro* proliferation assays were performed on **(A)** MKN-28 cells and **(B)** NUGC4 cells that were either un-treated or treated with 1μg/ml or 10μg/ml of Pam_3CSK4 for 24 hours. Data are from 3 independent experiments and are expressed relative to un-treated cells as the mean percentage increase in proliferation SEM. *$P \leq 0.05$, **$P \leq 0.01$.

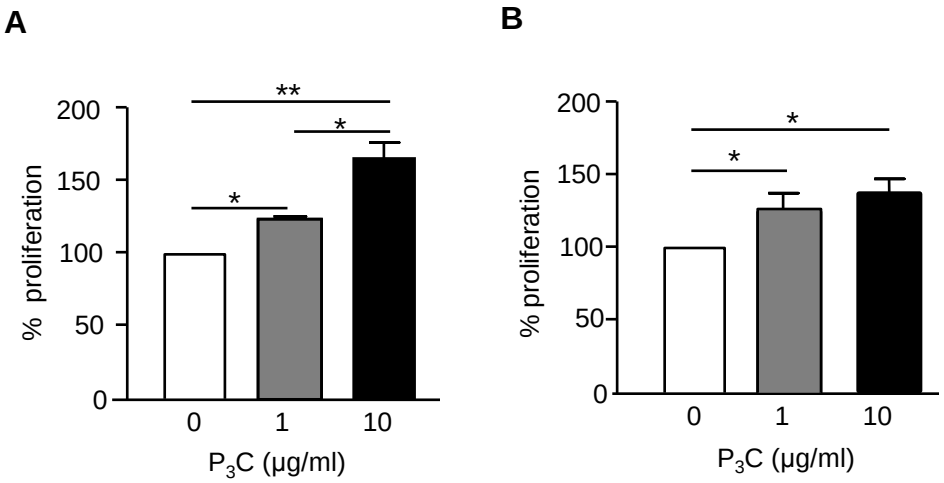
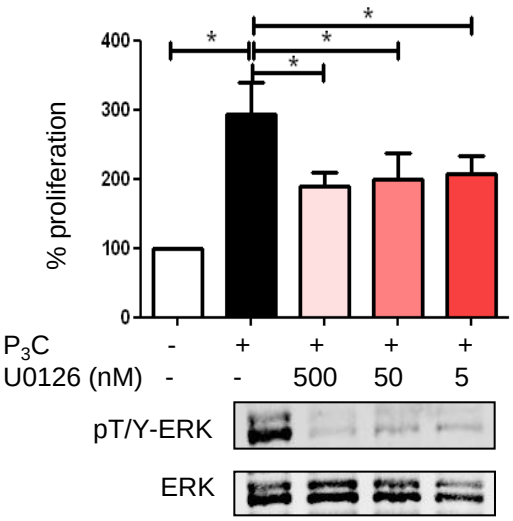
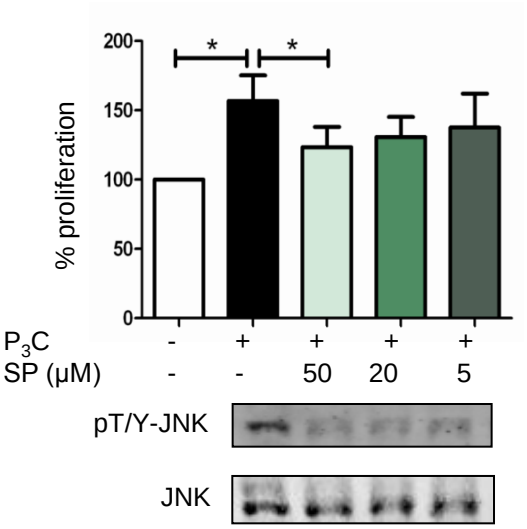
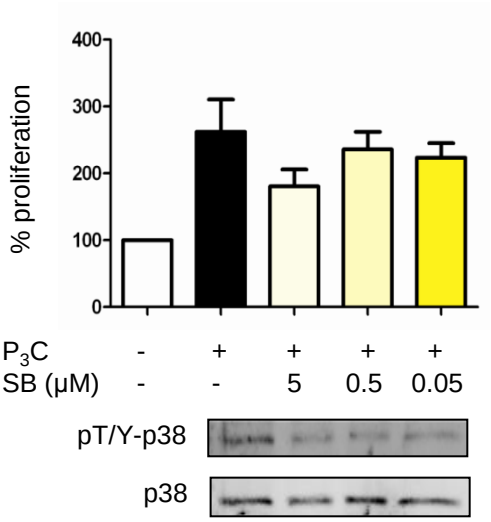
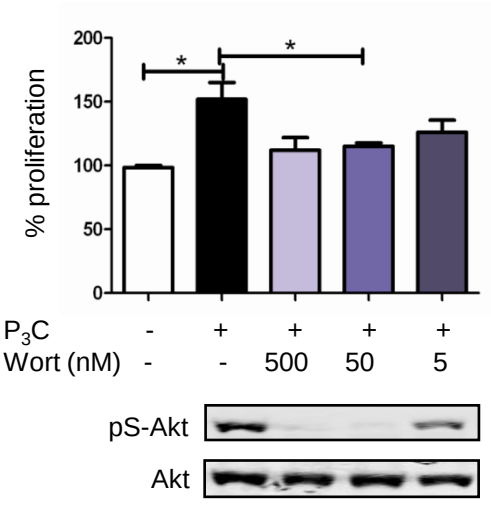

A
B
200
150
100
50
0
% proliferation
**
*
*
0
1
10
P3C (µg/ml)

Figure 5.5. The involvement of multiple signalling pathways in TLR2-mediated gastric epithelial cell proliferation. MKN-28 cells were pre-treated with specific inhibitors at 3 different concentrations that suppress ERK1/2, p38 and JNK MAPKs, PI3K/AkT and NF-κB pathways for 30 minutes, followed by Pam_3CSK4 (10μg/ml) stimulation for 24 hours. The fluorescent intensity of EdU was then measured. Below each individual bar graph is a representative immunoblot assay illustrating the reduced activity of each signalling pathway by their respective specific inhibitor. U0126 = ERK1/2, SP600125 (SP) = JNK, SB203580 (SB) = p38, Wortmanin (Wort) = PI3K/AkT, MG132 = NF-κB. Proliferation assay data are from at least 4 independent experiments and are presented as the mean SEM. $*P \leq 0.05$, $**P \leq 0.01$


% proliferation
400
300
200
100
0
*
*
*
*
P3C - + + + +
U0126 (nM) - - 500 50 5
pT/Y-ERK
ERK


% proliferation
200
150
100
50
0
*
*
P3C - + + + +
SP (μM) - - 50 20 5
pT/Y-JNK
JNK


% proliferation
400
300
200
100
0
P3C - + + + +
SB (μM) - - 5 0.5 0.05
pT/Y-p38
p38


% proliferation
200
150
100
50
0
*
*
P3C - + + + +
Wort (nM) - - 500 50 5
pS-Akt
Akt

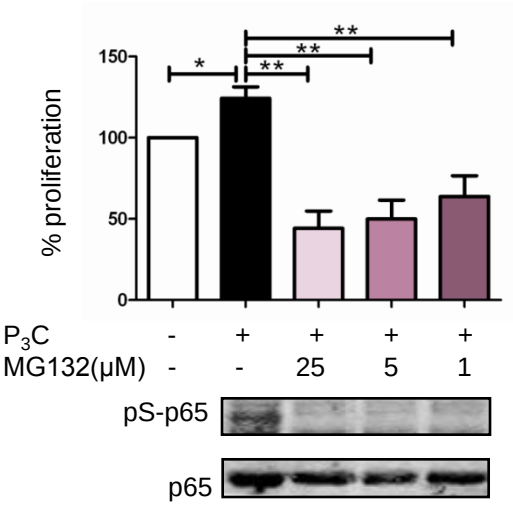
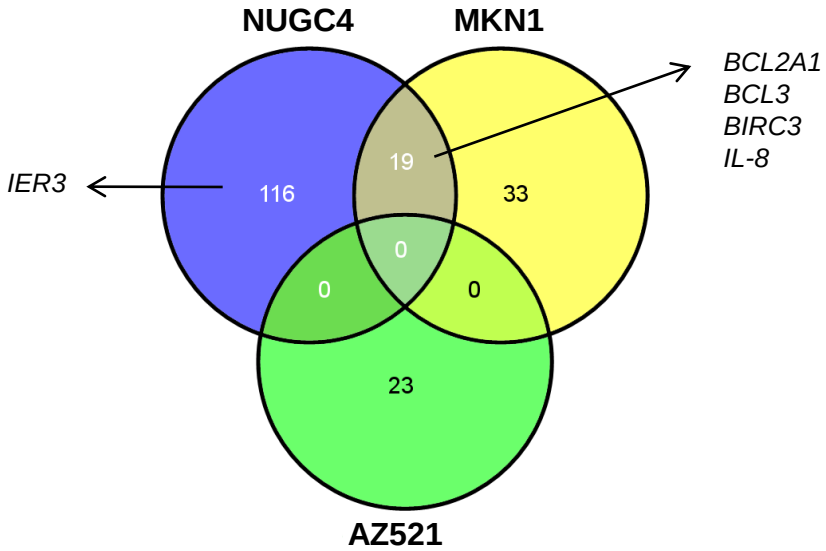

% proliferation
150
100
50
0
*
**
**
**
P3C - + + + +
MG132(μM) - - 25 5 1
pS-p65
p65

Figure 5.6. Microarray analyses on human gastric cancer cell lines stimulated with TLR2 agonists. Gene microarray analyses were performed on total RNA from human gastric cancer cell lines, expressing either high levels of STAT3 and TLR2 (NUGC4 and MKN1) or low levels of STAT3 and TLR2 (AZ521), that were stimulated with TLR2 agonists (combination of 100ng/ml each of FSL-1, LTA and Pam_3CSK4) for 6 hours. The venn diagram indicates the number of unique and overlapping genes that were up-regulated by ≥ 2-fold in stimulated cells relative to unstimulated cells and statistical significance ($P \leq 0.05$) was determined using Student t-tests.

(high STAT3/TLR2 expression)



(low STAT3/TLR2 expression)

Figure 5.7. TLR2 activation in human gastric cancer cells augments the expression of anti-apoptotic and cell cycle progression. Quantitative real-time PCR analyses were performed on MKN-28 cells that were either un-treated or treated with TLR2 agonists (combination of 100ng/ml each of FSL-1, LTA and Pam_3CSK4) for 6 hours. Data from 3 independent experiments are shown following normalisation for *ACTIN* and are expressed relative to un-treated MKN-28 cells as the mean SEM. * $P \leq 0.05$, ** $P \leq 0.01$

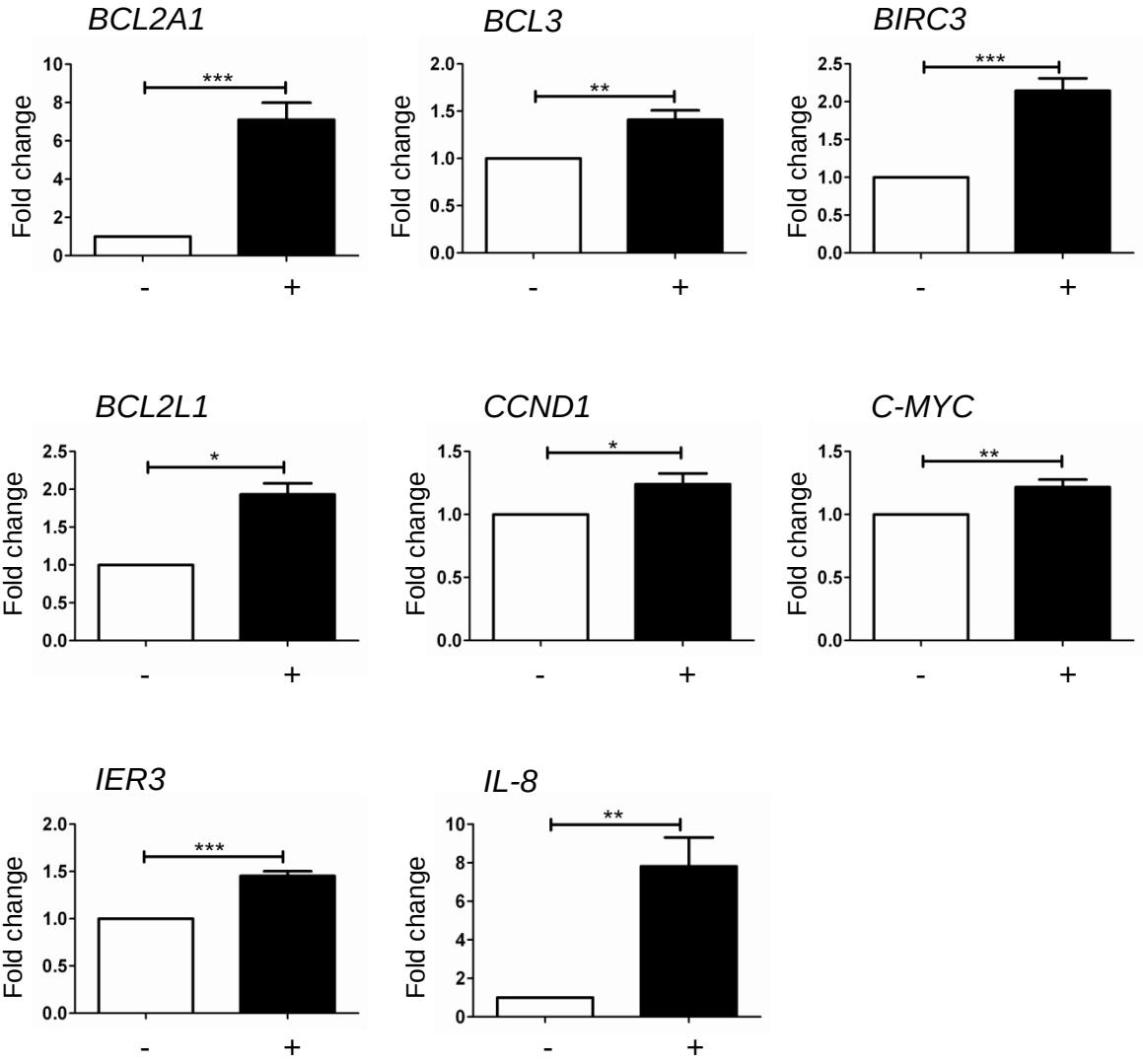

BCL2A1
Fold change
10
8
6
4
2
0
***
-
+
BCL3
Fold change
2.0
1.5
1.0
0.5
0.0
**
-
+
BIRC3
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
***
-
+
BCL2L1
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
*
-
+
CCND1
Fold change
1.5
1.0
0.5
0.0
*
-
+
C-MYC
Fold change
1.5
1.0
0.5
0.0
**
-
+
IER3
Fold change
2.0
1.5
1.0
0.5
0.0
***
-
+
IL-8
Fold change
10
8
6
4
2
0
**
-
+

Figure 5.8. Genetic ablation of TLR2 in *gp130*$^{F/F}$ mice results in the down-regulation of anti-apoptotic and cell cycle progression genes. Quantitative real-time PCR analyses were performed on gastric tumour tissues from 24 week old *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ (F/F:T2-/-) mice. Data from at least 5 mice per genotype are shown following normalisation to *18S* and are expressed relative to F/F samples as the mean SEM. *$P \leq 0.05$, **$P \leq 0.01$

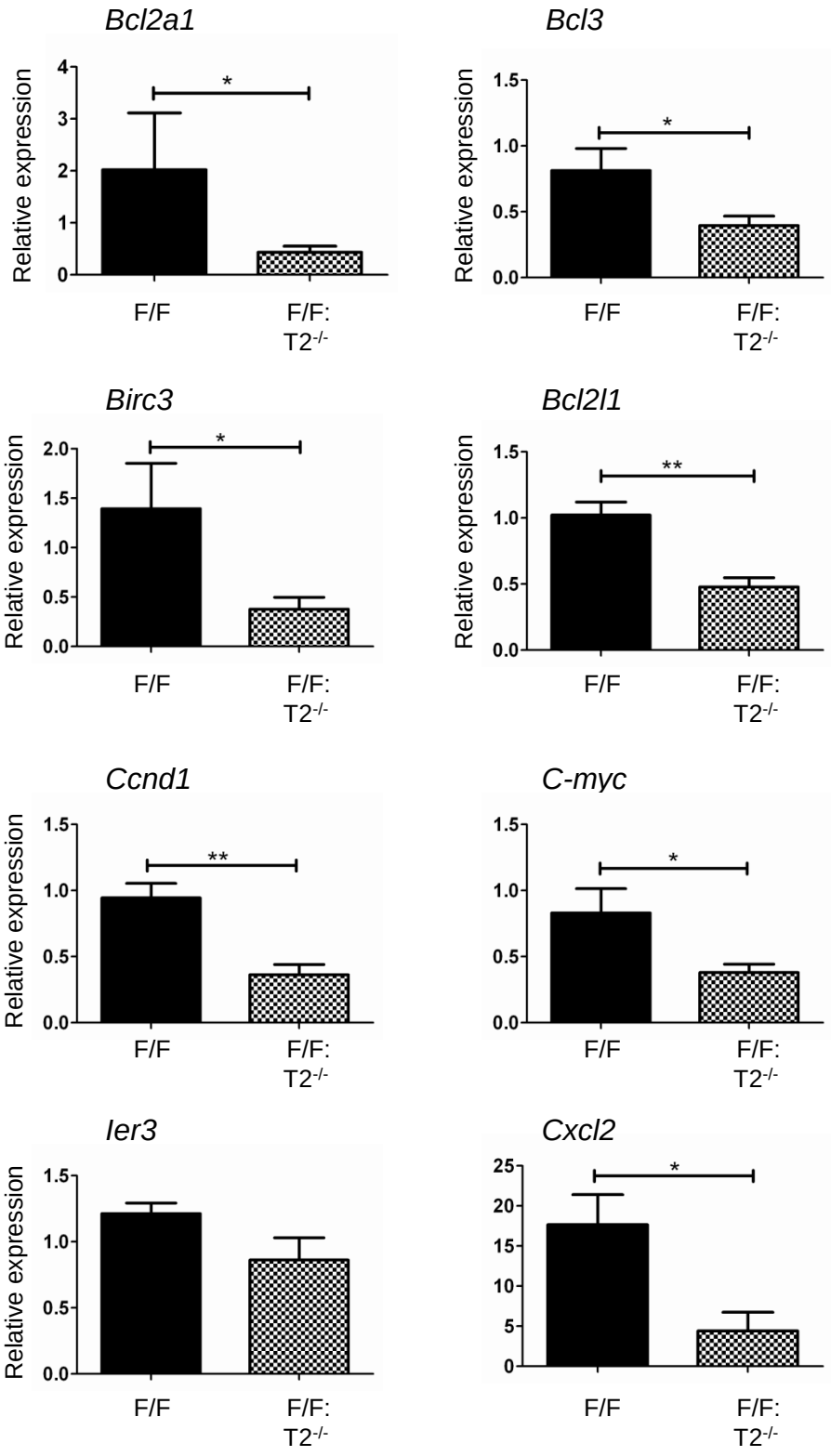
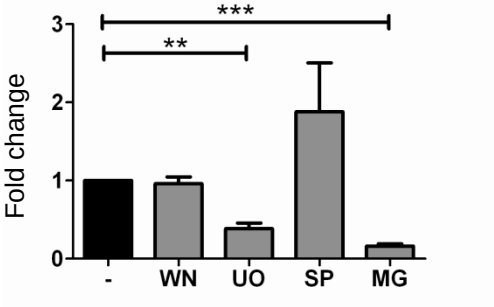
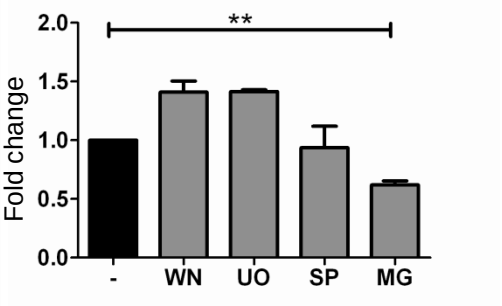

Bcl2a1
Relative expression
4
3
2
1
0
*
F/F
F/F:
T2-/-
Bcl3
Relative expression
1.5
1.0
0.5
0.0
*
F/F
F/F:
T2-/-
Birc3
Relative expression
2.0
1.5
1.0
0.5
0.0
*
F/F
F/F:
T2-/-
Bcl2l1
Relative expression
1.5
1.0
0.5
0.0
**
F/F
F/F:
T2-/-
Ccnd1
Relative expression
1.5
1.0
0.5
0.0
**
F/F
F/F:
T2-/-
C-myc
1.5
1.0
0.5
0.0
*
F/F
F/F:
T2-/-
Ier3
Relative expression
1.5
1.0
0.5
0.0
F/F
F/F:
T2-/-
Cxcl2
25
20
15
10
5
0
*
F/F
F/F:
T2-/-

Figure 5.9. Multiple signalling pathways are involved in the regulation of TLR2-induced anti-apoptotic and cell cycle genes. Quantitative real-time PCR gene expression analyses were conducted on MKN-28 cells that were un-treated or treated with wortmanin (Wn), U0126 (U0), SP600125 (SP) and MG132 (MG) prior to Pam_3CSK4 stimulation (100ng/ml) for 6 hours. Data from 3 independent experiments are shown following normalisation for *ACTIN* and are expressed relative to un-treated cells as mean SEM. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$*

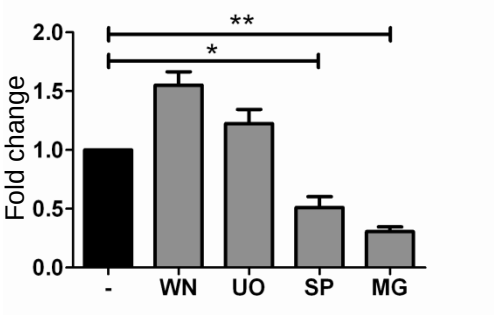
BCL2A1



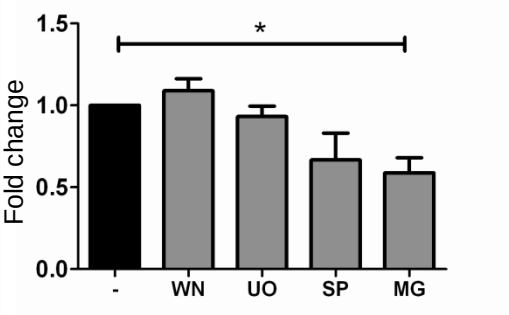
BCL3



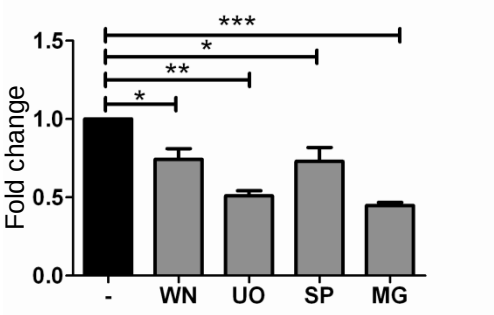
BIRC3



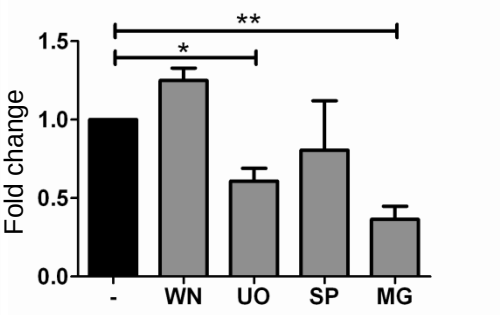
BCL2L1



CCND1



C-MYC



IER3

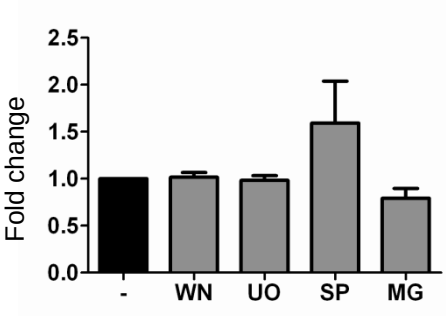


Figure 4.1. Genetic ablation of TLR2 in *gp130*$^{F/F}$ mice results in a significant decrease in STAT3-driven gastric tumourigenesis. (A) Macroscopic examination of the gastric compartment of 24 week old male *gp130*$^{+/+}$ (+/+), *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ (F/F:T2-/-) mice. Arrows indicate gastric adenomatous lesions (>4mm). **(B-E)** Dot plots of **(B)** stomach mass (grams, g), **(C)** tumour mass (g) and **(D, E)** number of gastric tumours present in male and female mice. Data are from 10 mice per genotype and are expressed as mean SEM. a = antrum. f = fundus.* *P≤0.05*, ** *P≤0.01* and *** *P≤0.001*

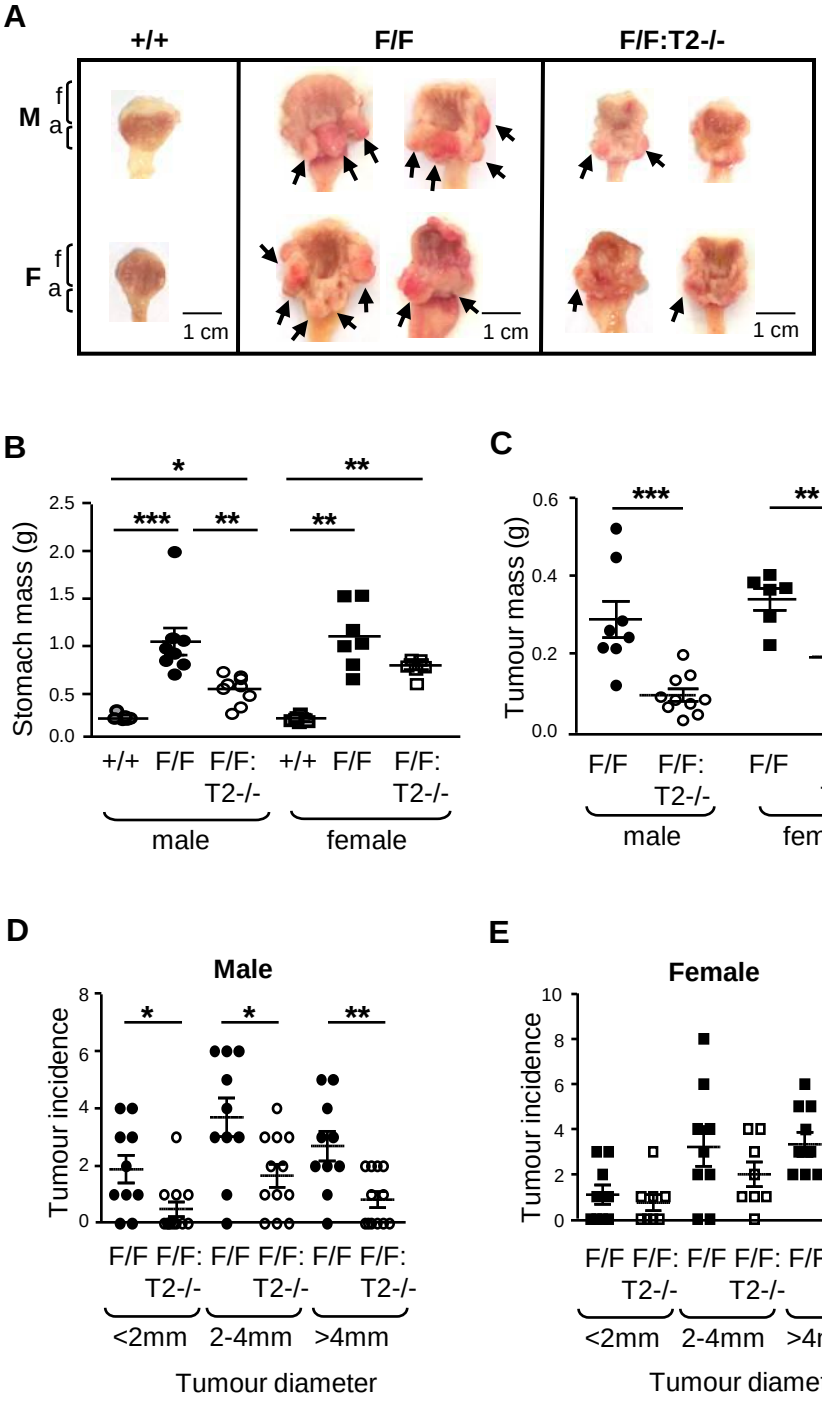

A
+/+
F/F
F/F:T2-/-
M
f
a
F
f
a
1 cm
1 cm
1 cm
B
Stomach mass (g)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
***
**
**
+/+
F/F
F/F: T2-/-
+/+
F/F
F/F: T2-/-
male
female
C
Tumour mass (g)
0.6
0.4
0.2
0.0
***
**
F/F
F/F: T2-/-
F/F
F/F: T2-/-
male
female
D
Male
Tumour incidence
8
6
4
2
0
*
*
**
F/F
F/F: T2-/-
F/F
F/F: T2-/-
F/F
F/F: T2-/-
<2mm
2-4mm
>4mm
Tumour diameter
E
Female
Tumour incidence
10
8
6
4
2
0
F/F
F/F: T2-/-
F/F
F/F: T2-/-
F/F
F/F: T2-/-
<2mm
2-4mm
>4mm
Tumour diameter

Figure 4.2. Genetic ablation of TLR4 in *gp130*$^{F/F}$ mice does not affect gastric tumour burden. (A) Macroscopic examination of 24 week old *gp130*$^{+/+}$ (+/+), *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$*:Tlr4*$^{-/-}$ (F/F:T4-/-) mice. Arrows indicate gastric adenomatous lesions (>4mm). **(B-D)** Dot plots of F/F and F/F:T4-/- depicting the **(B)** stomach mass (g), **(C)** tumour mass (g) **(D)** tumour incidence. **(E)** Representative H&E-stained cross-sections of 6 month old *gp130*$^{F/F}$*:Tlr4*$^{-/-}$ gastric sub-mucosa (Scale bar = 200μm) including a high-powered magnification showing the inflammatory cell types present (Scale bar = 50μm). P = plasma cell, E = eosinophil, M = mast cell and N = neutrophil. Data are from 5 mice per genotype and are expressed as mean SEM. a = antrum. f = fundus.

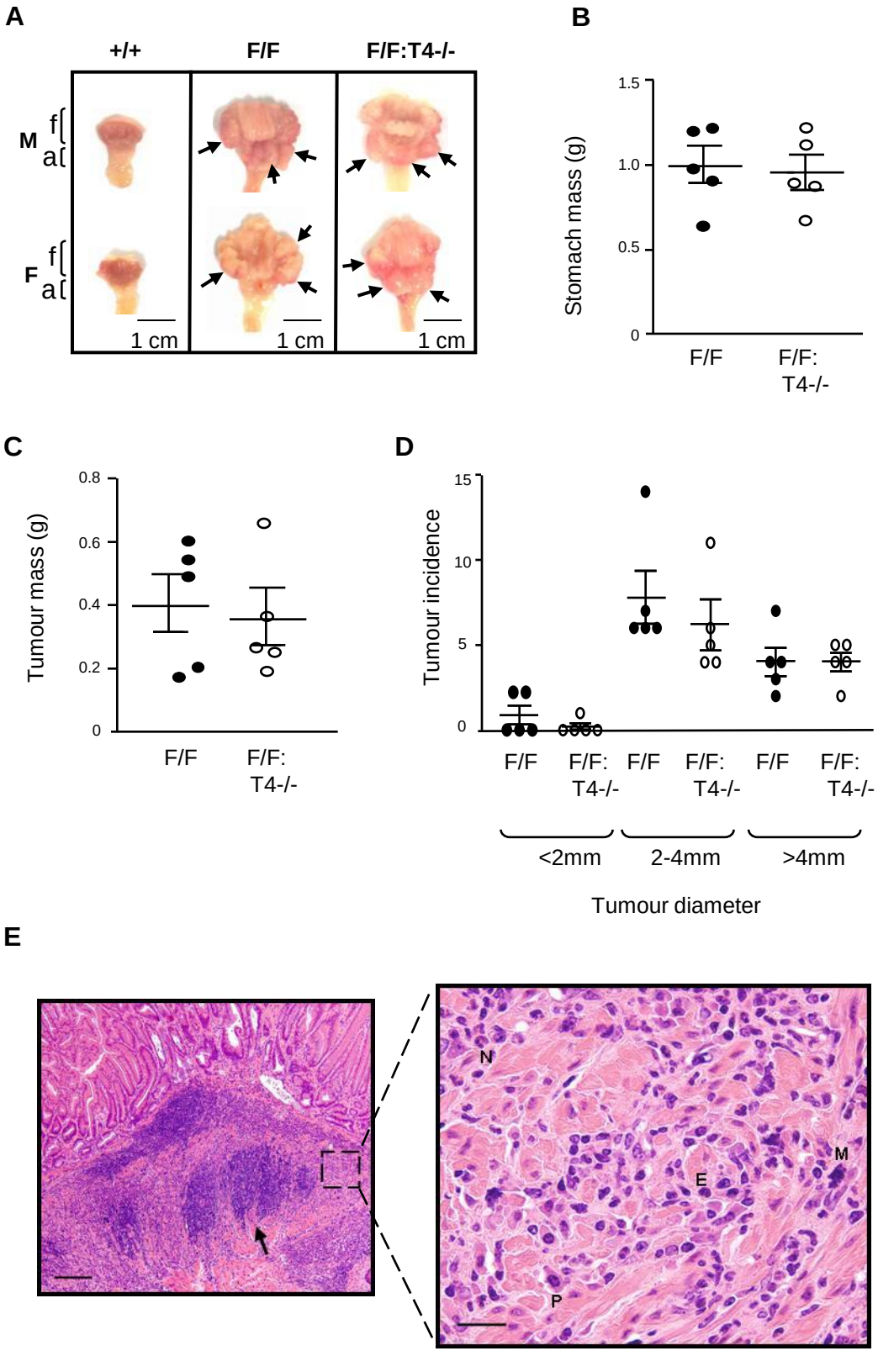
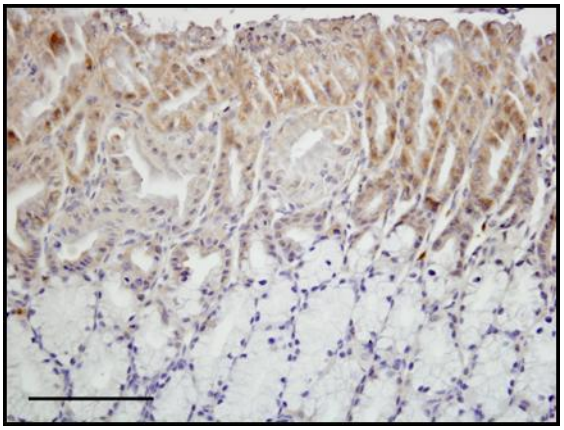

A
+/+
F/F
F/F:T4-/-
M
f
a
F
f
a
1 cm
1 cm
1 cm
B
Stomach mass (g)
1.5
1.0
0.5
0
F/F
F/F:
T4-/-
C
Tumour mass (g)
0.8
0.6
0.4
0.2
0
F/F
F/F:
T4-/-
D
Tumour incidence
15
10
5
0
F/F
F/F:
T4-/-
F/F
F/F:
T4-/-
F/F
F/F:
T4-/-
<2mm
2-4mm
>4mm
Tumour diameter
E
N
M
E
P

Figure 4.3. TLR2 protein expression is mainly observed in the gastric surface epithelial layer. Representative photomicrographs of TLR2-stained gastric cross-sections of the **(A)** epithelial layer and **(B)** sub-mucosa of 24 week old *gp130*$^{F/F}$ (F/F) mice. **(C)** Representative photomicrograph of the grastric sub-mucosa stained with mouse IgG1 isotype control. Data are representative of 3 mice per genotype. Scale bars = 200µm.

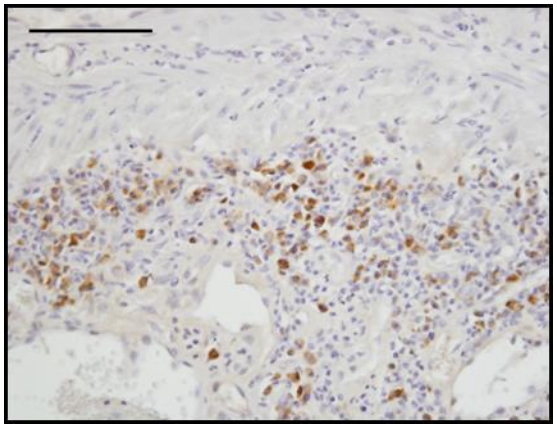
A

F/F



B

F/F



C

F/F

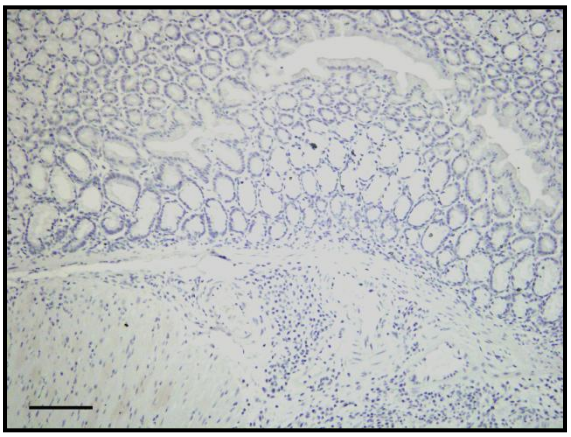


Figure 4.4. The protein expression of IL-11 and the activation status of STAT3 is not altered in *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice. The expression level of IL-11, tyrosine phosphorylated STAT3, total STAT3 and actin was determined in the gastric tumour tissues of 24 week old *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ (F/F:T2$^{-/-}$) mice by immunoblotting, actin and total STAT3 were used as a loading control. Data are from 2 mice per genotype.

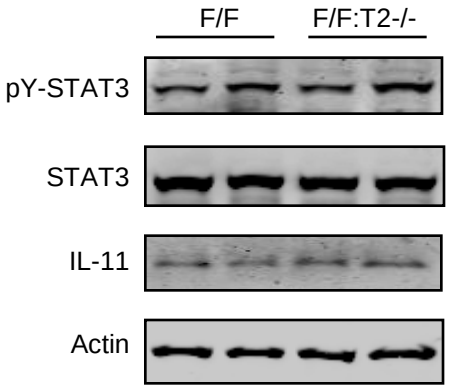
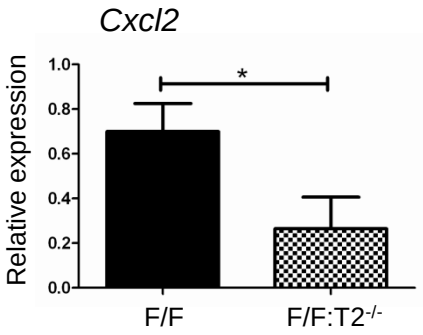
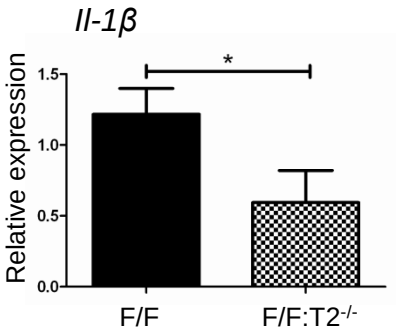
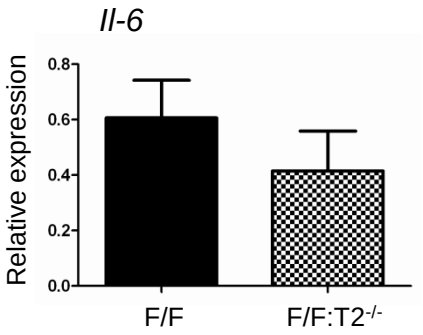

F/F
F/F:T2-/-
pY-STAT3
STAT3
IL-11
Actin

Figure 4.5. TLR2-regulated inflammatory gene expression levels are down-regulated in gastric tumours of *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ mice. Quantitative real-time PCR gene expression analyses on pro-inflammatory mediators were performed on gastric tumour tissues of 24 week old *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$:*Tlr2*$^{-/-}$ (F/F:T2$^{-/-}$) mice. Data from at least 4 mice per genotype are shown following normalisation for *18S* and are expressed relative to F/F tumour samples as the mean SEM relative to *gp130*$^{F/F}$ gastric tumour tissue. * $P \leq 0.05$, ** $P \leq 0.01$


Cxcl2
Relative expression
1.0
0.8
0.6
0.4
0.2
0.0
*
F/F
F/F:T2-/-


Il-1β
Relative expression
1.5
1.0
0.5
0.0
*
F/F
F/F:T2-/-


Il-6
Relative expression
0.8
0.6
0.4
0.2
0.0
F/F
F/F:T2-/-

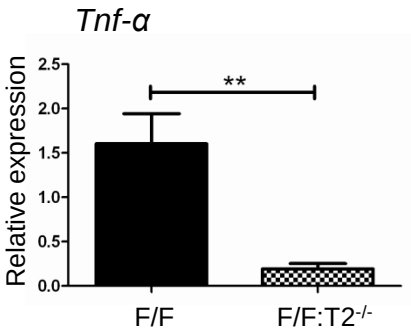

Tnf-α
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
**
F/F
F/F:T2-/-

Figure 4.6. TLR2-regulated inflammatory cytokine production is impaired in primary gastric epithelial cells (pGECs) isolated from *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice. The isolation of pGECs from *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ (F/F:T2-/-) mice were co-cultured with 6 month old microbial-containing gastric homogenate for 12 hours and the protein levels of IL-6 and TNF-α present in the supernatant were measured by ELISA. Data are from 3 independent experiments and are presented as mean concentration SEM. *$P \leq 0.05$*

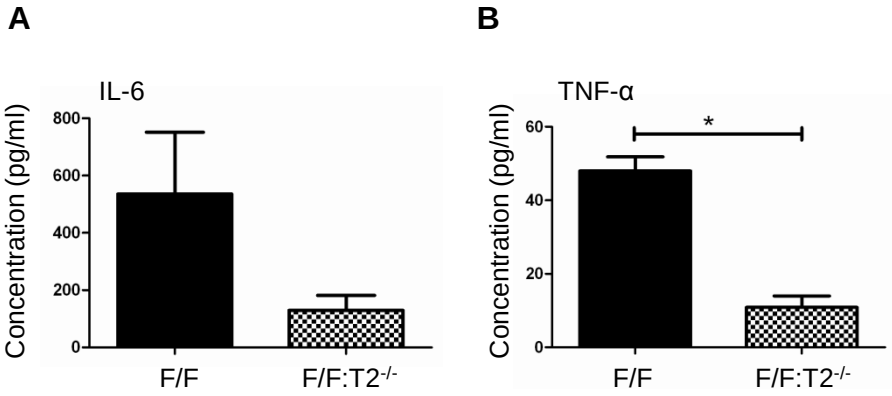
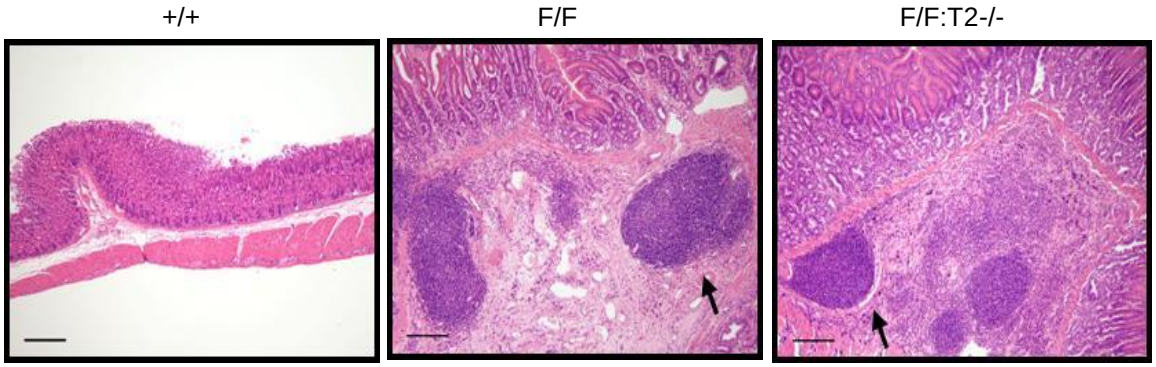
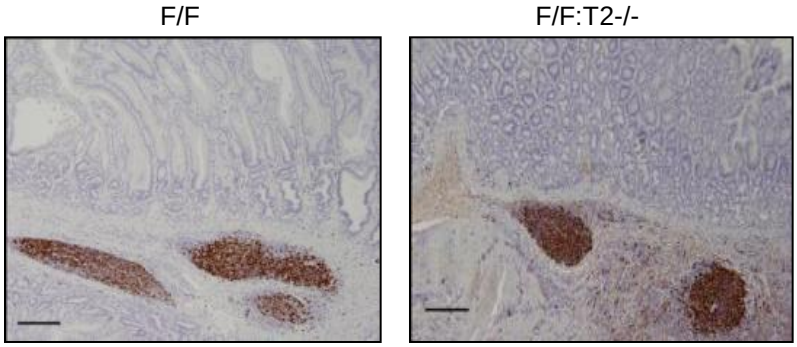

A
IL-6
Concentration (pg/ml)
800
600
400
200
0
F/F
F/F:T2-/-
B
TNF-α
Concentration (pg/ml)
60
40
20
0
*
F/F
F/F:T2-/-

Figure 4.7. Genetic ablation of TLR2 in g*p130*$^{F/F}$ mice does not affect the recruitment of inflammatory cells to the gastric mucosa. (A) Representative photomicrographs of H&E stained gastric cross-sections of 24 week old *gp130*$^{+/+}$ (+/+), *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ (F/F:T2-/-) mice. Arrows indicate inflammatory cell aggregates present in the gastric sub-mucosa (Scale bars = 200µm). **(B)** Representative photomicrographs of B220 stained cross-sections of the gastric sub-mucosa from 24 week old F/F and F/F:T2-/- mice (Scale bars = 200µm). **(C)** High powered representative photomicrographs of H&E stained gastric cross-sections of 24 week old F/F and F/F:T2-/- mice showing the inflammatory cell types present in the gastric mucosa. P = plasma cell, E = eosinophil, M = mast cell, N = neutrophil and L = leukocyte. Scale bars = 50µm. Data are representative of 3 mice per genotype.

A



B



C

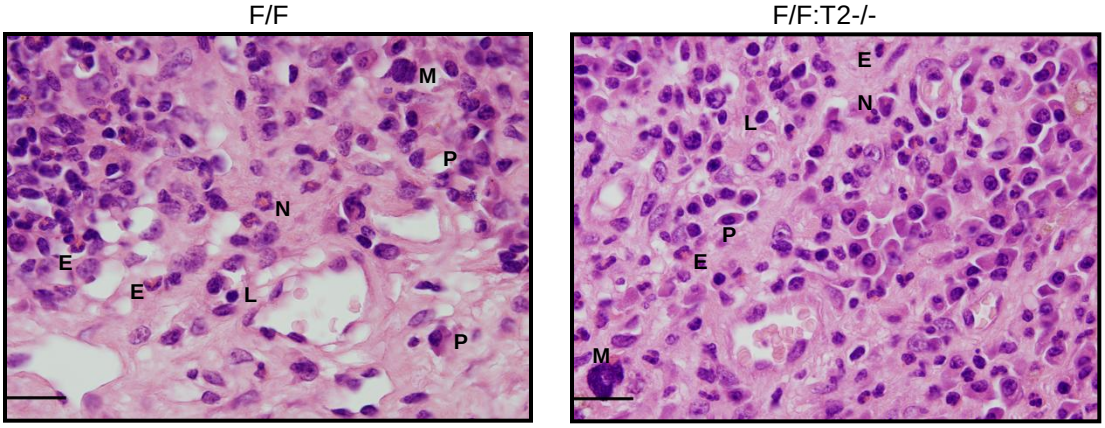


Figure 4.8. No changes in the inflammatory cell population was observed in the stomach of *gp130*$^{F/F}$ and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ mice. (A) Representative flow cytometry scatter plots showing the B cells (B220^{+}), T cells (CD3^{+}), myeloid-derived suppressor cells (MDSC) (CD11b^{+}Gr-1^{+}) and monocytes/macrophages (CD11b^{+}F4/80^{+}) in the stomach of 24 week old *gp130*$^{F/F}$ (F/F) and *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ (F/F:T2-/-) mice, with the bar graphs depicting the mean SEM percentages of the inflammatory cell population. **(B)** Bar graphs showing the percentage of activated T (CD4^{+}CD69^{+} and CD8^{+}CD69^{+}) and B (B220^{+}CD86^{+}) cell populations in the stomach of 24 week old F/F and F/F:T2-/- mice by flow cytometry. Data are from 3 mice per genotype and are expressed mean SEM.

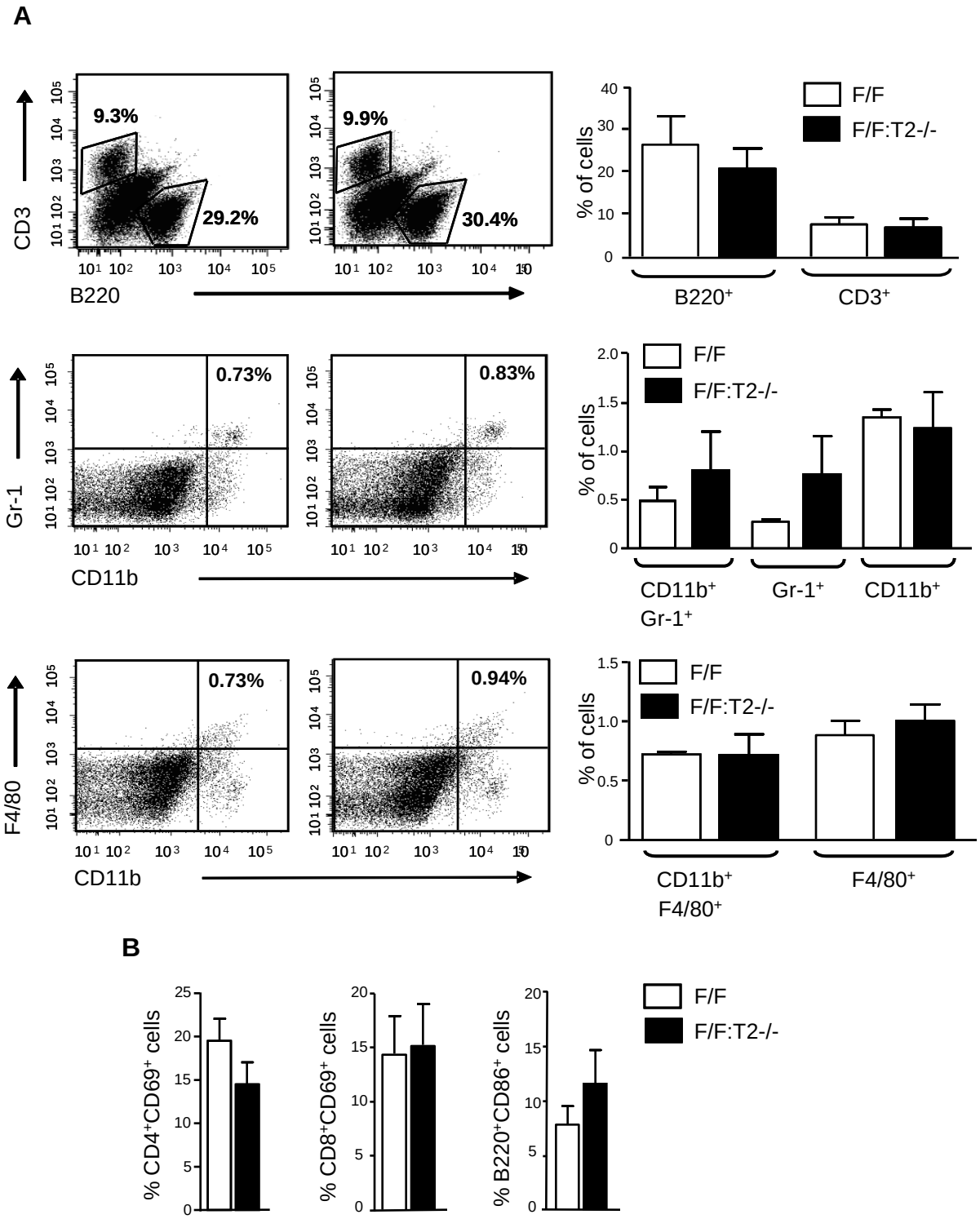

A
CD3
B220
9.3%
29.2%
9.9%
30.4%
% of cells
F/F
F/F:T2-/-
B220+
CD3+
Gr-1
CD11b
0.73%
0.83%
CD11b+
Gr-1+
F4/80
0.73%
0.94%
F4/80+
B
% CD4+CD69+ cells
% CD8+CD69+ cells
% B220+CD86+ cells

Figure 4.9. TLR2-expressing hematopoietic cells do not play a critical role in gastric tumourigenesis. (A) Representative macroscopic observation of the stomachs from 22 week old *gp130*$^{F/F}$ (F/F) naïve mice (n = 3) or F/F mice receiving donor bone marrow from autologous (F/F$^{F/F}$) (n = 3) or heterologous (F/F$^{F/F:T2-/-}$) (n = 10) mice. **(B)** Representative macroscopic observation of the stomachs from 22 week old *gp130*$^{F/F}$*:Tlr2*$^{-/-}$ (F/F:T2-/-) (n = 3) mice, or F/F:T2-/- mice reconstituted with autologous (F/F:T2$^{F/F:T2}$) (n = 5) or heterologous (F/F:T2$^{F/F}$) (n = 7) mice. a = antrum. f = fundus.

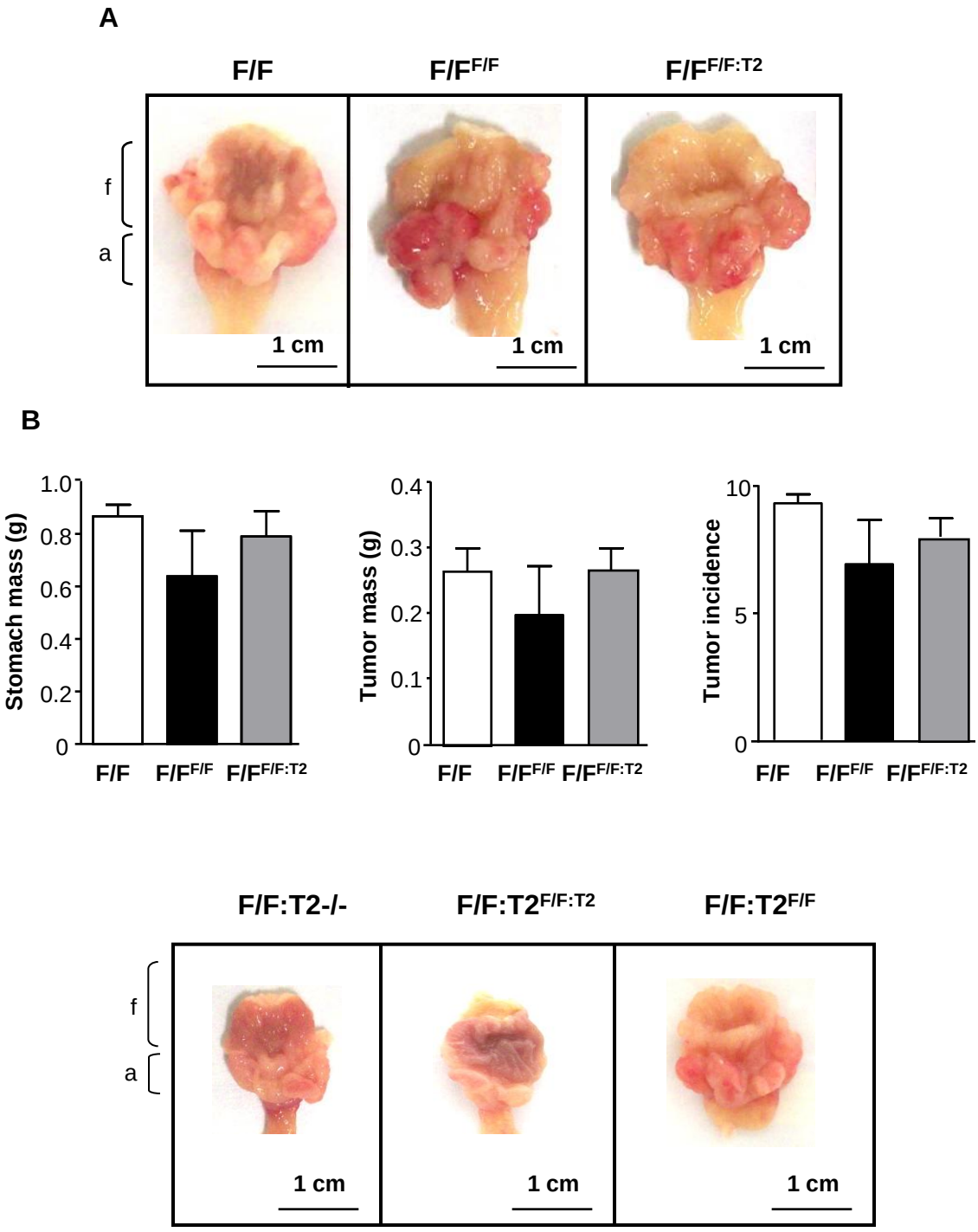
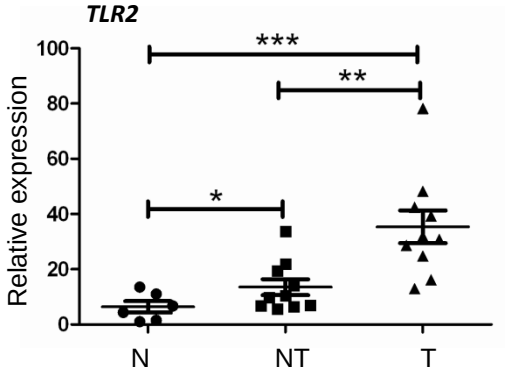
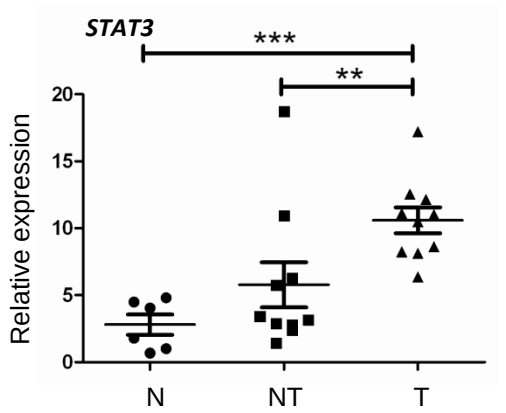

A
F/F
F/F^F/F
F/F^F/F:T2
f
a
1 cm
1 cm
1 cm
B
Stomach mass (g)
1.0
0.8
0.6
0.4
0.2
0
F/F
F/F^F/F
F/F^F/F:T2
Tumor mass (g)
0.4
0.3
0.2
0.1
0
F/F
F/F^F/F
F/F^F/F:T2
Tumor incidence
10
5
0
F/F
F/F^F/F
F/F^F/F:T2
F/F:T2-/-
F/F:T2^F/F:T2
F/F:T2^F/F
f
a
1 cm
1 cm
1 cm

Figure 6.1. Increased expression of *TLR2* in human gastric tumours correlate with augmented levels of *STAT3* and *IL-11* expression. Quantitative real-time PCR analyses were performed on human gastric biopsies from patients that were classified as normal (N) (n = 6), and from tumour tissue (T) (n = 10) and adjacent non-tumour tissue (NT) (n = 10) of patients with gastric cancer. Data are shown following normalisation to *18S* and are expressed relative to normal tissues as the mean SEM. *$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.001$


TLR2
Relative expression
100
80
60
40
20
0
***
**
*
N
NT
T


STAT3
Relative expression
20
15
10
5
0
***
**
N
NT
T

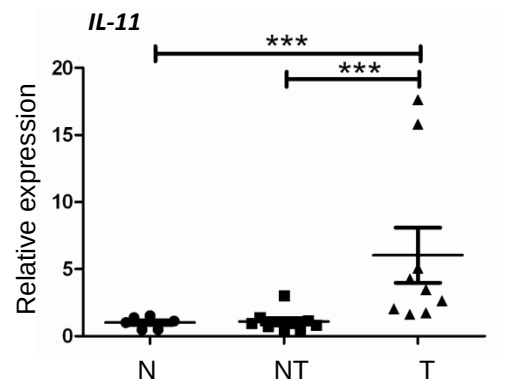
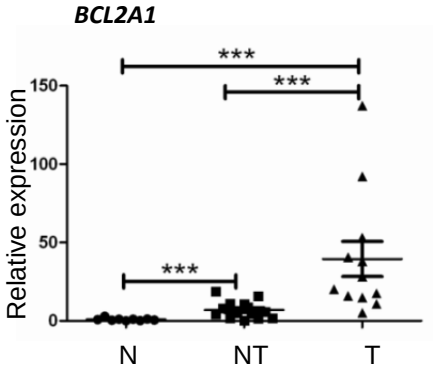
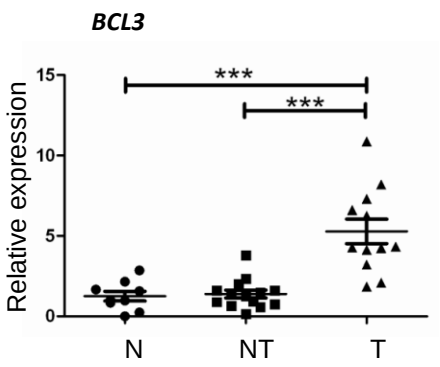
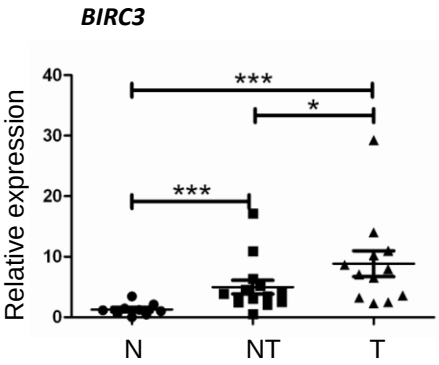
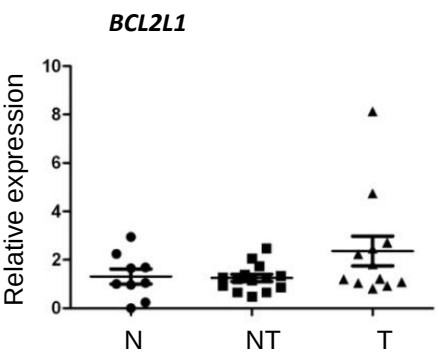

IL-11
Relative expression
20
15
10
5
0
***
***
N
NT
T

Figure 6.2. Elevated expression of TLR2-regulated anti-apoptotic genes in human gastric tumour tissues. Quantitative real-time PCR analyses were performed on human gastric biopsies from patients that were classified as normal (N) (n = 9), and from tumour tissue (T) (n = 12) and adjacent non-tumour tissue (NT) (n = 14) of patients with gastric cancer. Data are shown following normalisation to *18S* and are expressed relative to normal tissues as the mean SEM. *$P \leq 0.05$, ***$P \leq 0.001$


BCL2A1
Relative expression
150
100
50
0
***
***
***
N
NT
T


BCL3
Relative expression
15
10
5
0
***
***
N
NT
T


BIRC3
Relative expression
40
30
20
10
0
***
*
***
N
NT
T


BCL2L1
Relative expression
10
8
6
4
2
0
N
NT
T

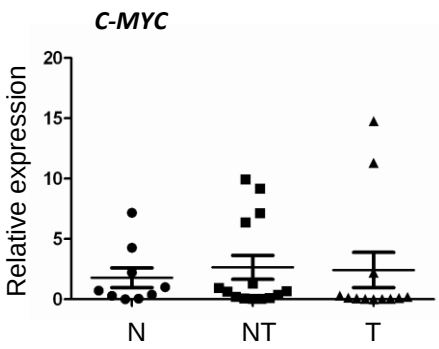
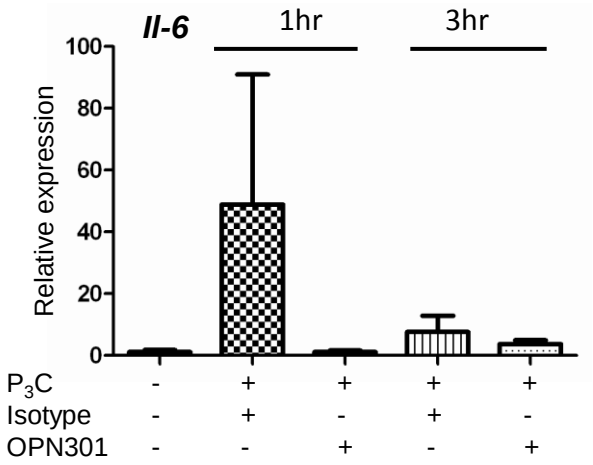
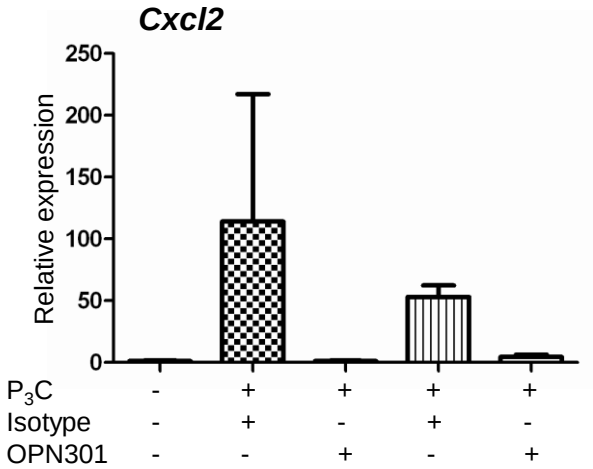

C-MYC
Relative expression
20
15
10
5
0
N
NT
T

Figure 6.3. TLR2 blocking antibody reduces the expression levels of TLR2-regulated inflammatory genes in *gp130*$^{F/F}$ mice. Quantitative real-time PCR analyses were performed on 12 week old gastric tumour tissues from *gp130*$^{F/F}$ mice that were i.p. injected with either the TLR2 blocking antibody (OPN301) (10mg/kg) or the matching isotype control (10mg/kg) for 30 minutes prior to administration with Pam_3CSK4 (P3C) (2mg/kg) for 1 hour and 3 hours. Data from 2 mice per treatment group are shown following normalisation to *18S*, and are expressed relative to untreated *gp130*$^{F/F}$ mice as mean SEM.


Il-6
1hr
3hr
Relative expression
100
80
60
40
20
0
P3C - + + + +
Isotype - + - + -
OPN301 - - + - +


Cxcl2
Relative expression
250
200
150
100
50
0
P3C - + + + +
Isotype - + - + -
OPN301 - - + - +

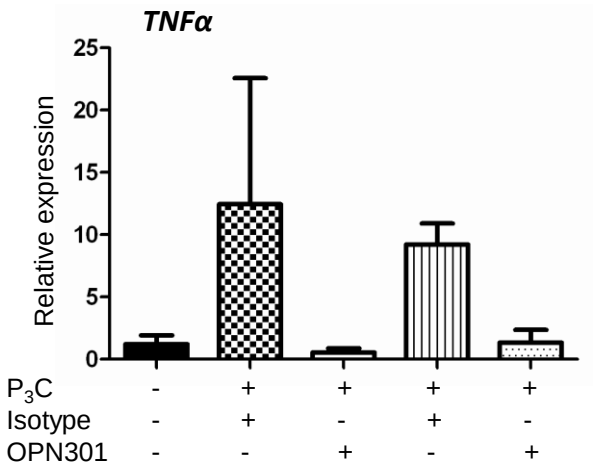

TNFα
Relative expression
25
20
15
10
5
0
P3C - + + + +
Isotype - + - + -
OPN301 - - + - +

Figure 6.4. Therapeutic blocking of TLR2 responses in *gp130*$^{F/F}$ mice significantly suppresses gastric tumour growth. **(A)** Representative image of stomachs from 22 week old *gp130*$^{F/F}$ mice that were administered with 10mg/kg of either isotype (ISO) control or OPN301 antibody by i.p. injection twice a week for 10 weeks. Arrows indicate adenomatous lesions (>4mm). **(B-C)** Dot plots of **(B)** tumour mass (g) and **(C)** tumour incidence of gastric tumours present in *gp130*$^{F/F}$ mice treated with either isotype (n = 3) or OPN-301 (n = 5) antibody. a = antrum, f = fundus. Data are represented as mean SEM. * $P \leq 0.05$

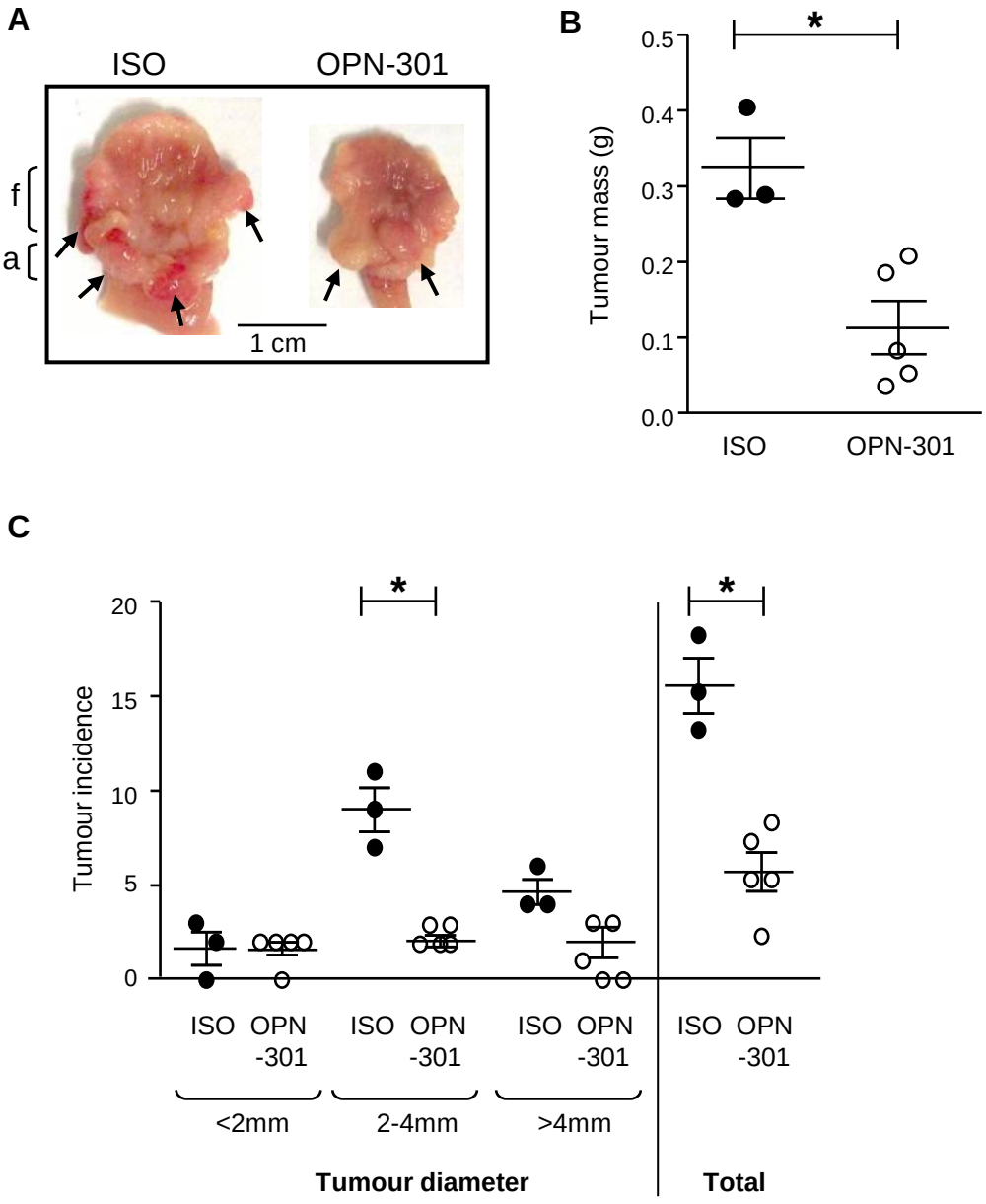

A
ISO
OPN-301
f
a
1 cm
B
*
Tumour mass (g)
0.5
0.4
0.3
0.2
0.1
0.0
ISO
OPN-301
C
*
*
Tumour incidence
20
15
10
5
0
ISO
OPN -301
ISO
OPN -301
ISO
OPN -301
ISO
OPN -301
<2mm
2-4mm
>4mm
Tumour diameter
Total

Figure 6.5. OPN-301 significantly reduces Pam_3CSK4-induced human gastric epithelial cell proliferation. *In vitro* EdU proliferation assays were performed on MKN-28 cells that were pre-treated with 1μg/ml of either isotype control or OPN-301 for 30 minutes followed by Pam_3CSK4 (10μg/ml) stimulation for 24 hours. Data from 3 independent experiments are normalised to isotype-treated cells, and are expressed as the mean percentage increase in proliferation SEM. *$P \leq 0.05$

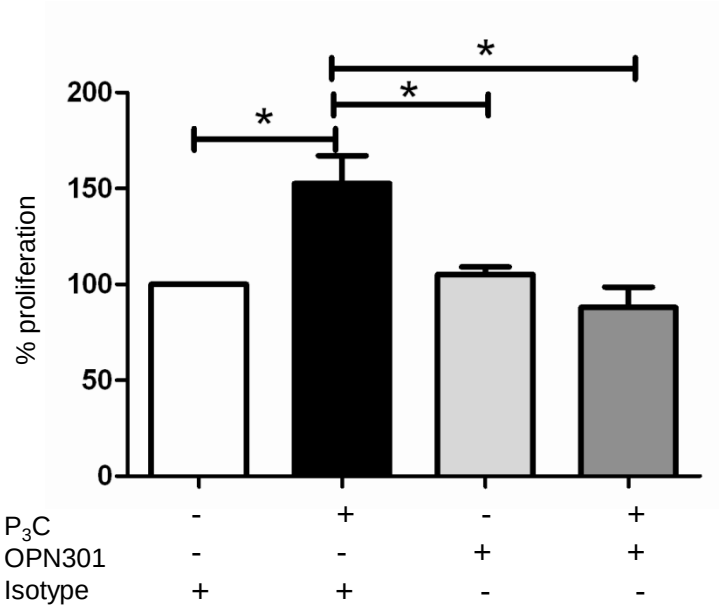
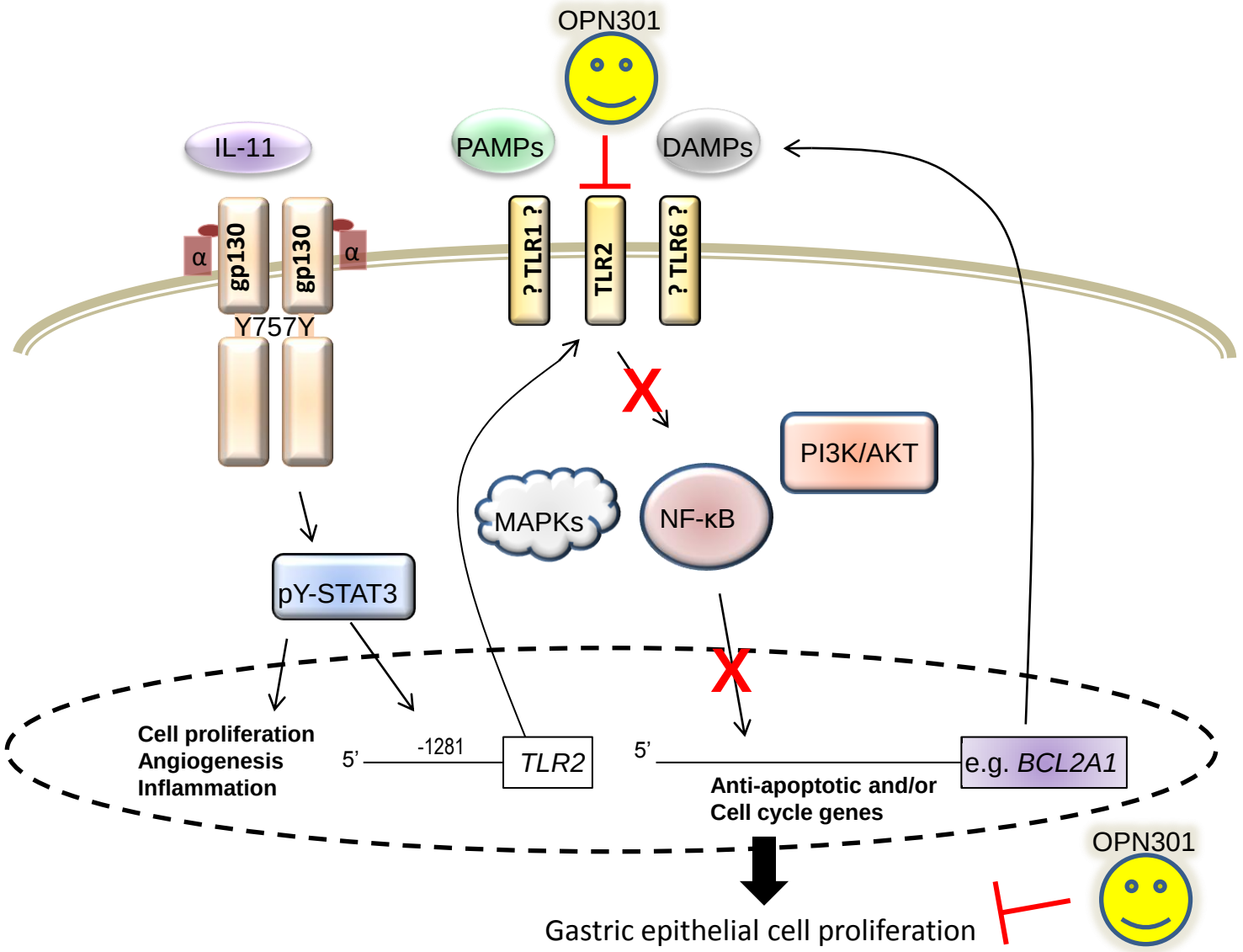

*
*
*
200
150
100
50
0
% proliferation
P3C - + - +
OPN301 - - + +
Isotype + + - -

Figure 7.1. Proposed model for STAT3-driven TLR2-mediated gastric tumourigenesis. Increased activation of STAT3 directly augments *TLR2* gene expression levels, which increases the responsiveness of TLR2 to PAMPs/DAMPs to promote gastric epithelial cell proliferation by regulating anti-apoptotic and/or cell proliferative genes through multiple signalling pathways (i.e. MAPKs, NF-κB and PI3K/AKT). Additionally, my preliminary studies have shown that OPN-301 TLR2 blocking antibody prevented gastric epithelial cell proliferation, and thus serves as a potential therapeutic candidate for human GC.


OPN301
IL-11
PAMPs
DAMPs
α
gp130
gp130
α
Y757Y
? TLR1 ?
TLR2
? TLR6 ?
X
PI3K/AKT
MAPKs
NF-κB
pY-STAT3
X
Cell proliferation
Angiogenesis
Inflammation
5'
-1281
TLR2
5'
e.g. BCL2A1
Anti-apoptotic and/or
Cell cycle genes
OPN301
Gastric epithelial cell proliferation