ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATION OF BAFF EXPRESSION AND SIGNALING PATHWAY IN *HELICOBACTER*-INFECTED GASTRIC EPITHELIAL CELLS ON MOLECULAR LEVEL

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

HELİKOBAKTER – İNFEKTE MİDE EPİTEL HÜCRELERİNDEN BAFF ÜRETİMİNİN VE SİNYAL YOLAĞININ MOLEKÜLER DÜZEYDE ARAŞTIRILMASI

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AĞUSTOS 2014

Miray KARAYILAN, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID 521111122, successfully defended the **thesis** entitled "**INVESTIGATION OF BAFF EXPRESSION AND SIGNALING PATHWAY IN *HELICOBACTER*-INFECTED GASTRIC EPITHELIAL CELL ON MOLECULAR LEVEL"**, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family and friends,

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Miray KARAYILAN

(Molecular Biologist and Genetics)

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ABBREVIATIONS

Mg : Microgram
µM : Micromolar
µm : Micrometer
∆BAFF : delta BAFF
∆APRIL : delta APRIL
AP-1 : Activator factor-1
APC : Antigen presenting cell
APRIL : A proliferation inducing ligand
bp : Base pair
BabA : Blood group antigen binding adhesion
BAFF : B cell activating factor
BAFF-R : BAFF Receptor
BCA : Bicinchoninic Acid
BCMA : B cell maturation antigen
BIR : Baculoviral inhibitor of apoptosis repeat
BSA : Bovine Serum Albumin
Cag A : Cytotoxin- asssociated gene –A
Cag E : Cytotoxin -asssociated gene –E
cagPAI : cag Pathogenicity island
Cag Y : Cytotoxin- asssociated gene –Y
Cag I : Cytotoxin -asssociated gene –I
Cag L : Cytotoxin -asssociated gene-L
CARD : Caspase activation and recruitment domain
cDNA : Complementary DNA
CIITA : Class II, major histocompatibility complex, transactivator
CLR : C-type lectin like receptor
cm^2 : Centimeter square
CRD : Cystein rich domain
Csk : C-terminal Src kinase
Ct : Cycle threshold
DAMP : Danger-associated molecular pattern
DMEM : Dulbecco's modified Eagle medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide
dsRNA : Double- stranded RNA
EAE : Experimental autoimmune encephalomyelitis
EDTA : Ethylenediaminetetraacetic acid
ELISA : Enzyme-Linked Immunosorbent Assay
FBS : Fetal Bovine Serum
g : Gram
h : Hour

H.pylori **:** *Helicobacter pylori*
HSP **:** Heparin sulphated proteoglycan
IFN **:** Interferon
IFN-α : Interferon- alpha
IFN-β **:** Interferon –beta
IFN-γ **:** Interferon –gamma
IFNAR **:** Interferon alpha / beta receptor alpha chain
IFNGR **:** Interferon gamma receptor
IL-8 **:** Interleukin-8
ISGF3 **:** Interferon stimulated gene factor-3
IRF7 **:** Interferon regulatory transcription factor 7
iE-DAP **:** γ-d-glutamyl-meso-diaminopimelic acid
JAK **:** Janus Kinase
JAK I **:** Janus Kinase Inhibitor
JNK **:** c-Jun N-terminal Kinase
kbp **:** Kilo base pair
kDa **:** Kilo dalton
L **:** Liter
LPS **:** Lipopolysaccharide
LRR **:** Leucine rich repeat
M **:** Molar
Mal **:** MyD88-adapter –like
MALT **:** Mucosa-associated lymphoid tissue lymphoma
MAPK **:** Mitogen Activating Protein Kinase
MDP **:** muramyl di-peptide
min **:** minute
mBAFF **:** membrane –bound BAFF
mL **:** Mililiter
mM **:** Milimolar
mm **:** Milimeter
MM **:** Multiple myeloma
mRNA **:** Messenger Ribonucleic Acid
MyD88 **:** Myeloid Differentiation primary response gene -88
MZ **:** Marginal zone
NFAT **:** Nuclear factor activated T cell
NF-κB **:** Nuclear factor-kappa B
ng **:** Nanogram
NHL **:** Non Hodgkins lymphoma
NLR **:** Nod-like receptor
nm **:** Nanometer
nM **:** Nanomolar
NOD1 **:** Nucleotide oligomerization domain-1
NOD2 **:** Nucleotide oligomerization domain-2
OipA **:** Outer inflammatory protein A
OMP **:** Outer membrane protein
OMV **:** Outer membrane vesicle
PCR **:** Polymerase Chain Reaction
pH **:** Power of Hydrogen
RICK **:** Receptor-interacting serine/threonine-protein kinase 2
PAMP **:** Pathogen-associated molecular patter

PGN : Peptidoglycan
PRR : Pattern –recognation receptor
PYD : Pyrin domain
qRT-PCR : Quantitative Real Time Polymerase Chain Reaction
RA : Rheumatoid arthiritis
RLR : RIG-I like receptor
RNA : Ribonucleic Acid
rpm : Recolutions per minute
SabA : Sialic acid-binding adhesin
SARM : Sterile alpha and HEAT/Armadillo motif
sBAFF : Soluble BAFF
sec : Second
SGECs : Salivary gland epithelial cells
SHP-2 : Src homology 2 domain
SLE : Systemic lupus ertyhematosus
STAT : Signal Transducer and Activator of Transcription
S/N : Supernatant
SS : Sjögren's Syndrome
ssRNA : Single-standed RNA
T1 : Transitional type 1
T2 : Transitional type 2
T4SS : Type IV secretion system
TACI : Transmembrane activator and calcium modulator cyclophilin interacting ligand
TAK1 : TGF-beta activated kinase-1
TBK1 : TANK-binding kinase 1
Th1 : T helper 1
Th 17 : T hepler 17
TIR : Toll – IL-1 receptor
TLR : Toll-like receptor
TRAF : TNF receptor – associated factor
TRAM : Toll-like receptor 4 adaptor protein
TRIF : TIR domain- containing adapter-inducing interferon-β
Tm : Melting Temperature
TNF : Tumor necrosis factor
TRAF : Tumor necrosis factor receptor-associated factors
TWEAK : TNF- related weak inducer of apoptosis
UV : Ultraviolet
V : Volt
Vac A : Vacuolating cytotoxin A
WCE : Whole cell extract

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INVESTIGATION OF BAFF EXPRESSION AND SIGNALING PATHWAY FROM *HELICOBACTER*-INFECTED GASTRIC EPITHELIAL CELLS ON MOLECULAR LEVEL

SUMMARY

Helicobacter pylori (H.pylori) is a gram negative, spiral –shaped, microaerophilic bacterium which colonize in the human stomach and is significantly associated with chronic gastritis, gastric adenocarcinoma, gastric ulcers and gastric lymphoma. In developing countries, 80% of the population is infected with *Helicobacter pylori* but most of them are asymptomatic. The bacterial virulence factors, host genetic traits, and environmental parameters are responsible for the *Helicobacter*-associated diseases. *H.pylori*-driven inflammation in stomach leads to follicular gastritis in childhood and MALT (mucosa-associated lymphoid tissue) lymphoma in adults, both of which are characterized by the presence of B cell follicles.

To understand the contribution of *H.pylori* on gastric immunopathology formation indirectly through affecting gastric epithelial cells in molecular level, we focus on B cell activating factor (BAFF), which was shown to be produced in *H. pylori*-infected gastric epithelial cells. BAFF is a member of TNF (tumour necrosis factor) family and first identified in 1999. BAFF is also termed as BLys, THANK, TALL-1, zTNF4, TNFSF13B. BAFF is located on chromosome 13 in humans and has 6 exons. BAFF is responsible for regulation innate and adaptive immunity. It has a role on B cell maturation, proliferation, development and survival. BAFF is produced by many cell types including monocytes, neutrophils, dendritic cells, activated T cells, epithelial cells and macrophages. BAFF production is also reported from cancer cells. Elevated levels of BAFF were found in sera of several autoimmune diseases patients such as rheumatoid arthritis (RA), Sjögren's syndrome (SS), experimental autoimmune encephalomyelitis (EAE), systemic lupus erythematosus (SLE).

APRIL (a proliferation inducing ligand) is a homologue of BAFF and a member of TNF family cytokines. We also focus on *H.pylori* G27 WT sonicate- stimulated APRIL production from gastric epithelial cells. APRIL is also termed as TRDL-1, TALL-2 and TNFSF-13a. APRIL is located on chromosome 17 in humans and has 6 exons. APRIL is expressed by monocytic, dendritic, epithelial cells, macrophages and cancer cells. APRIL has a role on plasma cell survival and stimulation of tumour cells. High levels of APRIL were also found in sera of autoimmune disease patients. In this study, the effect of *H.pylori* virulence factor Cag A (cytotoxin- associated gene-A) on BAFF & APRIL production from gastric epithelial cells and their signalling pathway were investigated. It was reported that *H.pylori* virulence factor Cag A is associated with development of gastric malignancies. For that reason, a gastric epithelial cell line KATO-III was treated with wild- type (WT) *H. pylori* G27 strain sonicate and its ΔCag A mutant sonicate. The differences of BAFF expression in mRNA level were determined by quantitative real-time PCR (qRT-PCR). Our

research indicates that BAFF is produced from *H.pylori* G27 WT sonicate- treated KATO-III cells. It was observed that BAFF expression is significantly decreased from *H.pylori* G27 ΔCag A sonicate- treated KATO-III cells compared to *H.pylori* G27 sonicate- treated cells. This result suggests that *H.pylori* virulence factor Cag A has an effect on BAFF production in KATO-III cells. BAFF protein levels were detected by using Western Blotting and ELISA. BAFF protein levels were also significantly increased in *H.pylori* G27 WT sonicate- treated KATO-III cells compared to untreated controls and *H.pylori* ΔCagA mutant sonicates- treated KATO-III cells had less BAFF production compared to *H.pylori* wild-type counterpart. Findings revealed a correlation between RNA and protein levels of BAFF in response to *H.pylori* G27 WT sonicate.

APRIL was found to be expressed less than BAFF. Findings suggest that APRIL expression is significantly increased in *H.pylori* G27 WT sonicate- treated KATO-III cells. qRT-PCR and Western Blotting results indicates that *H.pylori* virulence factor Cag A has a role in APRIL expression and production.

NOD1 (nucleotide –binding oligomerization domain) is an intracellular pattern recognition receptor and is found in the cytoplasm. NOD1 is a member of NLRs and contains CARD (caspase recruitment domain domain). NOD1 is present in all gram – negative bacteria and some gram - positive bacteria. It recognizes peptide derived peptidoglycans (PGNs) derived from bacterial cell walls. It is reported that *H.pylori* PGNs induce NOD1 expression in intestinal epithelial cells. In our work, NOD1 is also found to be expressed by *H.pylori* sonicates- treated KATO-III cells. It was observed that NOD1 expression is increased in *H.pylori* G27 WT sonicate-treated cells compared to untreated controls. It is known that activation of NOD1 leads to IFN-β (interferon-beta) expression via IRF7 translocation to nucleus. Secreted type I cytokine IFN-β, binds to its own receptor on the cell surface and activates JAK/STAT signalling pathway. In the light of this knowledge, we investigated IRF7 expression and IFN-β expression levels in *H.pylori* sonicates-treated KATO-III cells by using qRT-PCR. qRT-PCR results suggest that IRF7 expression level is increased in *H.pylori* G27 WT sonicate- treated KATO-III cells. Following IRF7 expression levels, IFN-β expression levels were also investigated by qRT- PCR. It was observed that IFN-β expression level was increased in *H.pylori* G27 WT sonicate- treated cells compared to untreated control.

In literature, BAFF production is reported from different epithelial cells such as airway epithelial cells and salivary gland cells via JAK/STAT signalling pathway. We aimed to elucidate *H.pylori* G27 WT sonicate- stimulated BAFF signalling pathway in gastric epithelial cells. In order to investigate *H.pylori* G27 WT sonicate-induced JAK/STAT signalling pathway, KATO-III cells were treated with different doses of JAKI and then stimulated with *H.pylori* G27 WT sonicate for 24 h. According to Western Blotting results, STAT1 phosphorylation levels were decreased in the presence of JAKI in a dose –dependent manner of JAKI. Our results suggest that JAK/STAT signalling pathway plays a role in BAFF&APRIL expression and secretion in *H.pylori* G27 WT sonicate- treated KATO-III cells.

HELİKOBAKTER – İNFEKTE MİDE EPİTEL HÜCRELERİNDEN BAFF ÜRETİMİNİN VE SİNYAL YOLAĞININ MOLEKÜLER DÜZEYDE ARAŞTIRILMASI

ÖZET

Helikobakter pilori (*H.pilori*) gram –negatif, spiral şekilli, mikroaerofilik bir bakteri olup insan midesinde kolonize olmakla beraber kronik gastrit, gastrit adenokarsinoma , ülcer ve gastik lenfomayla yakından ilişkili olan bir bakteridir. Gelişmekte olan ülkelerin %80'i *Helikobakter pilori* ile infektedir fakat bireylerin çoğu asemptomatiktir. Bakteri virülans faktörleri, konağın genetik özellikleri ve çevresel etkenler *Helikobakter* ile ilişkili hastalıklardın gelişmesinden sorumludur. *H. pilori* kaynaklı mide enflamasyonu çocuklarda foliküler gastrite, yetişkinlerde ise MALT(mukoza ilişkili lenfoid doku) lenfomaya sebep olmaktadır. Bu iki hastalık da B hücre folikülleri varlığı ile karakterizedir.

H. pilori'nin mide epitel hücrelerini etkileyerek gastrik immunopatoloji oluşumunda katkısını anlayabilmek amacıyla, araştırmamızda *H. pilori* ile enfekte mide epitel hücreleri tarafından üretildiği gösterilen B hücre aktive edici sitokin (BAFF) üzerinde yoğunlaşmaktayız. TNF (tümör nekrozis faktör) ailesi üyelerinden olan BAFF ilk olarak 1999'da keşfedilmiş olup, BLys, THANK, TALL-1, zTNF4, TNFSF13B adları ile de anılmakadır. BAFF, insanlarda 13. kromozomun uzun kolunda kodlanmakta ve 6 adet ekzonu bulunmaktadır. BAFF, doğustan ve sonradan kazılan bağışıklıkta regüle edici özelliği bulunan bir sitokindir. Aynı zamanda B hücre matürasyonu, proliferasyonunda, yaşamı, geşilmesi, farklılaşmasında görev alan bir sitokindir. BAFF çeşitli hücrelerce üretilmektedir. Örneğin monositler, nötrofiller, dendritik hücreler, aktive olan T hücreleri, epitel hücreler, makrofajlar tarafından üretildikleri gösterilmiştir. BAFF'ın aynı zamanda kanser hücrelerinden de üretildiğini gösteren çalışmalar vardır. Romatoid artrit (RA), Sjögren sendromu (SS), multiple skleroz (MS), deneysel otoimmün ensefalomiyelitis (EAE) ve sistemik lupus eritamatozus (SLE) gibi otoimmün hastaların serumlarında yüksek düzeyde BAFF saptanmıştır.

APRIL (proliferasyon indükleyici ligand), BAFF'ın homoloğu olan ve yine TNF ailesine ait bir sitokindir. Çalışmalarımız *H.pilori* G27 WT sonikatı ile stimule edilen gastrik epitel hücrelerinden APRIL ekspresyonu ve üretimi ile devam etmiştir. APRIL, TRDL-1, TALL-2 and TNFSF-13a gibi isimlerle de terimlendirilir. İnsanlarda 17. kromozomun küçük kolunda yer alıp , 6 ekzona sahiptir. APRIL da monositik, dendritik, epitel , makrofaj ve çeşitli kanser hücrelerince üretilmektedir. APRIL'ın plasma B hücrelerinin proliferasyonunu ve daha çok tümor hücrelerini stimüle edici özelliği olduğu literatürde gösterilmiştir. APRIL'ın da yüksek düzeyde otoimmün hastaların serumlarında bulunduğu gösterilmiştir.

Bu çalışmamızda *H.pilori* virulans faktörlerinden Cag A (sitotoksin- ilişkili gen A) virulans faktörünün gastik epitel hücrelerinden BAFF ve APRIL üretimine etkisi ve sinyal yolakları araştırılmıştır. Cag A, *H.pilori'*nin en önemli virulans faktörlerinden biri olup gastrik hastalıklarının gelişmesinde önemli rol oynadığı gösterilmiştir.

Bu amaçla mide epitel hücresi olan KATO-III, yabanıl (WT) *H.pilori* G27 suşu ve onun ΔCag A (Cag A virulans faktörü bulunmayan) mutantı ile muamele edilmiştir. mRNA seviyelerindeki BAFF ekspresyon farkları kantitatif- gerçek zamanlı PZR ile tayin edildi. Elde edilen bulgular *H.pilori* G27 WT sonikatının incelenen KATO-III hücre hattında BAFF üretimine sebep olduğunu göstermektedir. *H.pilori* G27 ΔCag A mutantı sonikatı ile muamele edilmiş KATO-III hücrelerindeki BAFF ekspresyonu, *H.pilori* G27 WT sonikatı ile muamele edilmiş hücrelere göre daha düşük seviyelerde bulunmuştur. Bu sonuç bize Cag A virulans faktörünün BAFF üretimi üzerindeki etkisini göstermektedir. Protein düzeyinde yapılan Western Blotlama ve BAFF ELIZA çalışmalarının sonuçları gösterdi ki, BAFF üretimi *H. pilori* G27 WT ile muamele edilmiş hücrelerde daha fazla saptanmıştır. RNA ve protein düzeyindeki çalışmalar birbirini tamamlamaktadır.

APRIL eksresyonu da BAFF kadar yüksek düzeylerde olmasa da *H.pilori* G27 WT ile muamele edilen KATO-III hücrelerinde APRIL ekspresyonu daha fazla miktarda gözlenmiştir. Protein düzeyindeki Western Blotlama çalışmalarına göre de APRIL üretiminde Cag A virulans faktörünün önemli rol oynadığı gösterilmiştir.

NOD1 (nükleotid bağlayıcı oligomerizasyon domain proteini 1), sitoplazmada bulunan bir hücre içi patojen tanıma reseptörü olup NLR (NOD-benzeri reseptör) ailesinin CARD bölegesi taşıyan bir üyesidir. Genellikle tüm gram-negatif ve bazı gram-pozitif bakterilerde bulunup, hücre duvarından kökenlenen peptidoglikanları tanımakla görevlidir. *Helikobakter pilori'*den kökenlenen peptidoglikanların NOD1 ekspresyonunu arttırtığı daha önce bağırsak epitel hücrelerinden gösterilmiştir. Bizim çalışmamızda da *H.pilori* G27 WT sonikatı ile muamele edilmiş mide epithel hücrelerinden üretilen NOD1 ekspresyonu ekspresyonu gerçek zamanlı PZR tekniği kullanılarak analiz edilmiştir. NOD1 ekspresyon sonuçlarına göre *H.pilori* G27 WT sonikatı ile muamele edilen hücreler, *H.pylori* sonikatı ile muamele edilmeyen hücrelere göre daha fazla NOD1 ekspres etmektedirler. Literatürde NOD1'ın IRF7 (interferon regülator faktör 7) 'nin nukleusa translokasyonunu sağlayarak IFN-β (İnterferon-beta) eksprese edilemesine yol açtığı bilinmektedir. Üretilen tip-I sitokin olan IFN-β 'nın da hücre yüzeyinde bulunan İnterferon-beta reseptörüne bağlanıp JAK/STAT yolağını aktive edici özelliği vardır. Bu bilgiler ışığında, bizim çalışmalarımızda *H.pilori* sonikatı ile muamele edilmiş KATO-III hücrelerinden IRF7'nin ve IFN-β'nın ekpresyonlarını kantitatif- gerçek zamanlı PZR tekniği kullanılarak araştırılmıştır. Kantitatif- gerçek zamanlı PZR sonuçlarına göre, *H.pilori* G27 WT sonikatı muamele edilen hücrelerdeki IRF7'nin ekspresyonudaki artışın *H.pilori* sonikatı ile muamele edilmeyen hücrelere göre fazla olduğu gözlenmiştir. IRF7 ekspresyon sonuçlarını takiben *H.pilori* G27 WT sonikatı ile muamele edilmiş hücrelerden IFN-β sitokinin ekpresyon düzeyindeki analizleride kantitatif- gerçek zamanlı PZR ile araştırılmıştır. Elde edilen bulgulara göre, *H.pilori* G27 WT sonikatı varlığında IFN-β'nın üretiminin *H.pilori* sonikatı ile muamele edilmeyen KATO-III hücrelerine göre daha fazla ekprese edildiği gösterilmiştir.
Literatürde hava yolu aracılı epitel ve tükürük bezi epitel hücrelerinden BAFF üretiminin JAK/STAT (Janus tirozin kinaz /sinyal ileticisi ve transkripsiyon aktivitörü) aktive edici yolağı aracılı ile olduğu gösterilmiştir. Çalışmamızda *H.pilori* G27 WT sonikatı ile infekte mide epitel hücrelerinde ki BAFF üretim yolağının

aydınlatılması amaçlanmıştır. Bu amaçla *H.pilori* G27 WT sonikatı ile indüklenen hücrelerden JAK/STAT sinyal yolağını aydınlatmak için KATO-III hücreleri çeşitli dozlarda JAKI (JAK inhibitörü) ile muamele edilip ardıdan *H.pilori* G27 WT sonikatı ile stimule edilmiştir. Elde edilen Western Blotlama sonuçlarına göre JAKI kullanıldığında STAT1' in fosforilasyonundaki azalma gösterilmiştir. BAFF ve APRIL ekspresyonu ve üretimide JAKI kullanıldığında azalmaktadır. BAFF ve APRIL üretiminde JAK/STAT sinyal yolağının mide epitel hücrelerinde de rol oynadığını göstermiştir.

1. INTRODUCTION

1.1 *Helicobacter pylori*

Helicobacter pylori (*H.pylori*) is a gram-negative, spiral-shaped, microaerophilic, flagellate bacterium that can successfully colonizes the human stomach. More than 50% of the world population is infected with *H.pylori* but most of the infected individuals are asymptomatic [1]. In early childhood, infection can be passed through gastric - oral route within families and can be established as a long- term infection if not treated. Also host's genetical backgrounds, environmental factors, virulence factors of *Helicobacter* strains are important in the adaptation of this bacterium to the host stomach. *Helicobacter pylori* was first identified by two Australian scientists Dr. Barry Marshall and Dr. Robin Wallen in 1982. In 1994, *H.pylori* has been classified as class I carcinogen bacterium by the International Agency for Research on Cancer and reported as the main factor for developing gastric cancer [2]. The discovery of *H.pylori* and its relation to the gastric malignancies won the Nobel Prize in Medicine in 2005.

All *Helicobacter pylori* strains possess urease, catalase and oxidase enzymes. Urease facilitates neutralization of the gastric acidic environment so that *H.pylori* can survive in human stomach. Urease also plays a role in degradation of urea into ammonia and this ammonia is used for amino acid biosynthesis. Oxidase protects bacteria against free- radicals. Catalase enzyme is required for the growth and survival of *H.pylori* and also protects bacteria against hydrogen peroxide.

Infection with *Helicobacter pylori* is associated with the development of chronic gastritis, peptic ulcers, duodenal ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (MALT) [3]. Gastric adenocarcinoma was listed as a second cause of cancer-associated death in world - wide [4]. It was shown that *Helicobacter* – specific virulence genes are important in the progression of gastric malignancies [5].

1.2 *Helicobacter pylori* Virulence Factors

Most of the *Helicobacter pylori*-infected patients do not develop any gastric complications. This situation led to notion that some *H.pylori* strains may be more virulent than others. Adhesion molecules are responsible for the adherence of bacteria to host cells. Also, these molecules are required for *H.pylori* colonization in human stomach (Figure 1.1). *H.pylori* adherence molecules are members of outer membrane protein (OMP) family and named as Hop family. Hop family contains BabA (blood group antigen binding adhesion), OipA (the outer inflammatory protein A), SabA (sialic acid-binding adhesion), HopZ and AlpA/B [7]. Apart from *H.pylori* virulence factors and adhesion molecules, flagella, LPS (lipopolysaccharides), peptidoglycan (PGN), superoxide dismutase, catalase, and protease enzymes of *H.pylori* may play a role in severity of diseases.

Several *H.pylori* virulence factors have been identified and found that some of these factors play an important role in developing gastric malignancies. Most of these virulence factors are located in the cag Pathogenicity Island (cagPAI). Another important *H.pylori* virulence factor is VacA (vacuolating cytotoxin A). VacA augments the risk factor for developing gastric cancer [6] (Figure 1.1).

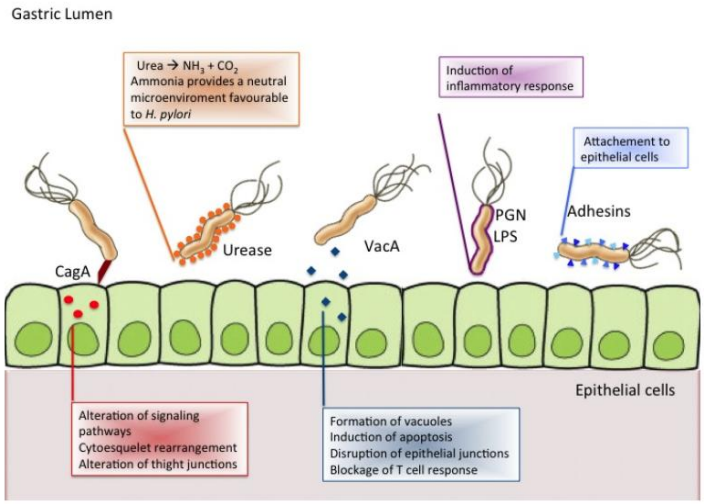


Figure 1.1 : *Helicobacter pylori* virulence factors (adapted from ref. 6)

1.2.1 The *H.pylori* cag Pathogenicity island

The cag Pathogenicity Island (cagPAI) was first identified in 1996. cagPAI is a 40 kb DNA insertion element and includes approximately 31 genes. cagPAI is present in 60-70% of Western *H.pylori* strains and approximately 100% of East –Asian *H.pylori* strains [8]. This island encodes a type IV secretion system (T4SS). T4SS also exists in other bacteria such as Agrobacterium, Legionella, Bartorella and Bordetella. T4SS is required for bacterial conjugation and translocation of bacterial effector proteins into the host gastric epithelial cells [9,10]. *Helicobacter pylori* uses T4SS injection apparatus for translocation of Cag A protein and peptidoglycans (PGNs) into the host cell [11].

CagPAI contains several virulence factors such as Cag A, Cag E, Cag L, Cag Y, and Cag I [12] (Figure 1.2).

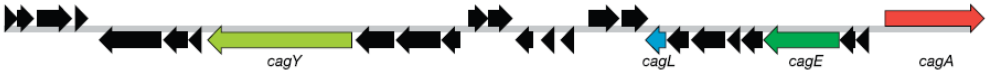


Figure 1.2 : *Helicobacter pylori* virulence genes located on cagPAI (adapted from ref.12)

1.2.1.1 Cag A virulence factor

The most important *H.pylori* virulence factor Cag A is encoded by the cytotoxin-associated gene A and localized in the pathogenicity island. Cag A is a highly immunogenic, 120-140 kDa protein. Cag A protein is closely associated with the severe gastric diseases such as peptic ulcers and gastric cancer. *H.pylori* strains have been divided in two types as type I and type II [13]. Type I strains possess Cag A and Vac A, and they are important for the development of gastric malignancies. Type II strains lack these virulence factors. Cag A oncoprotein has repeated sequences located in 3' region. These repeated regions consist of Glutamine- Proline-Isoleucine- Tyrosine- Alanine (EPIYA) motifs and these motifs include a tyrosine phosphorylation site. There are four different EPIYA segments: EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D. Cag A from Western *H.pylori* isolates have EPIYA-A, EPIYA-B and EPIYA-C (EPIYA-ABC type) whereas CagA from East Asian *H.pylori* isolates possess EPIYA-A, EPIYA-B and EPIYA-D (EPIYA-ABD type) [14]. EPIYA-D motif is unique for East Asian Cag A and is associated with the gastrointestinal diseases.

1.2.1.2 Other virulence factors in cagPAI

One of the other *H.pylori* effector protein is Cag E (cytotoxin- associated gene-E) which is conserved among strains. Cag E is located in the right half of the cagPAI and helps the translocation and phosphorylation of Cag A protein. Cag E also has a role in induction of proinflammatory cytokines such as IL-8 (Interleukin-8) (neutrophil-activating chemokine). IL-8 secretion is mediated by NF-κB (Nuclear Factor -kappa B) pathway [15].

Cag L is located at the surface of the pilus and this virulence factor has a specific Arginine-Glycine-Aspartate (RGD) motif. This motif is recognized by the $\alpha_5\beta_1$ integrin receptor on the target epithelial cell. CagL helps translocation of Cag A protein into the cytoplasm of host cell [16]. Also, CagL activates host Src and host focal adhesion kinase (FAK) for phosphorylation of Cag A protein.

Cag Y virulence factor is found at the pilus and both Cag Y and Cag I are required for Cag A translocation.

1.2.2 Vac A (vacuolating cytotoxin A) virulence factor

Vac A is an important key toxin of *H.pylori*. Beside Cag A, *H.pylori* virulence factor Vac A protein also leads to gastric malignancies. *H.pylori* strains which possess cag A and Vac A are associated related with development of gastric cancer [5]. Among all *H.pylori* strains possess vac A gene and there is no close homologous of this virulent factor in other *Helicobacter* species or other bacteria. Vac A has several functions including vacuole formation and membrane channel formation in epithelial cell. Vac A initiates apoptotic cascade by inducing cytochrome c release from mitochondria via increasing expression of a pro-apoptotic protein Bax, and decreasing expression of an anti-apoptotic protein Bcl2 which in turn disrupts mitochondrial functions. Inhibition of T cell proliferation is maintained by Vac A through inactivation of transcription factor NFAT (nuclear factor of activated T cells), and blockage of IL-2 secretion. IL-2 is an important cytokine for T cell activation and survival [17, 18].

Vac A has allelic variations in signal (s), intermediate (i) and middle (m) regions. s regions has s1 and s2 alleles, and s1 allele is more related with an increased risk for peptic ulceration and gastric cancer than s2 [19]. Almost all Cag A positive strains contain s1 allele and Cag A negative strains possess s2/m2. The i and the m regions

also have i1 and i2, m1 and m2 alleles respectively. Vac A s1/m1 strains are more virulent than s2/m2 strains [20]. vacA gene encodes 140 kDa pro-toxin protein and has three components: a signal sequence, a passenger domain and an auto-transporter domain. Signal sequence helps the translocation of pro-toxin. The passenger domain contains two subunits p33 and p53 respectively. p33 subunit has a role in the pore formation, cell binding and initiation of apoptosis via going to mitochondria. p53 subunit has a role in the cell binding, anionic membrane channels and vacuole formation [21,22,23,24].

1.2 Cag A Translocation via Type IV Secretion System

Cell membrane- attached *H.pylori* injects Cag A protein into host cell. Once Cag A is translocated into host cell it localizes at the inner surface of the plasma membrane and then undergoes tyrosine phosphorylation at EPIYA motifs by the host Src and Abl kinases. Src family kinases are c-Src, Lyn, Fyn, and Yes. These kinases are expressed by gastric epithelial cells. These kinases are required for Cag A phosphorylation and important for CagA-SHP2 complex formation. After phosphorylation of Cag A, SHP-2 (Src homology 2 domain - containing phosphatase) binds to tyrosine phosphorylated EPIYA motifs: EPIYA-C and EPIYA-D. Cag A positive strains which possess EPIYA-D binds stronger to SHP-2 than EPIYA-C segment containing ones. SHP-2 kinase has a role in the transmission of signals and cell motility. Activation of SHP-2, dephosphorylates host cell proteins and leads to cellular morphological changes such as humming bird phenotype (more motile phenotype leading to cell scattering), cell elongation, apoptosis, spreading, migration of cells, disruption the cytoskeleton, epithelial cell functions, cell polarity and tight junctions [11,25,26,27] (Figure 1.3).

Phosphorylated form of Cag A may also bind to C-terminal Src kinase (Csk) by SH2 domain. Formation of CagA- Csk complex phosphorylates Src family kinases, down-regulates CagA-SHP2 domain complex formation, inhibits c-Src activity and reduce phosphorylation levels of Cag A. This negative feedback signalling pathway leads to morphological changes in epithelial cells, cytoskeletal change, cell elongation and cellular rearrangements via tyrosine dephosphorylation of some proteins such as actin-binding proteins cortactin, ezrin and vinculin. Csk and SHP-2 compete with each other to bind tyrosine phosphorylated [28,29,30].

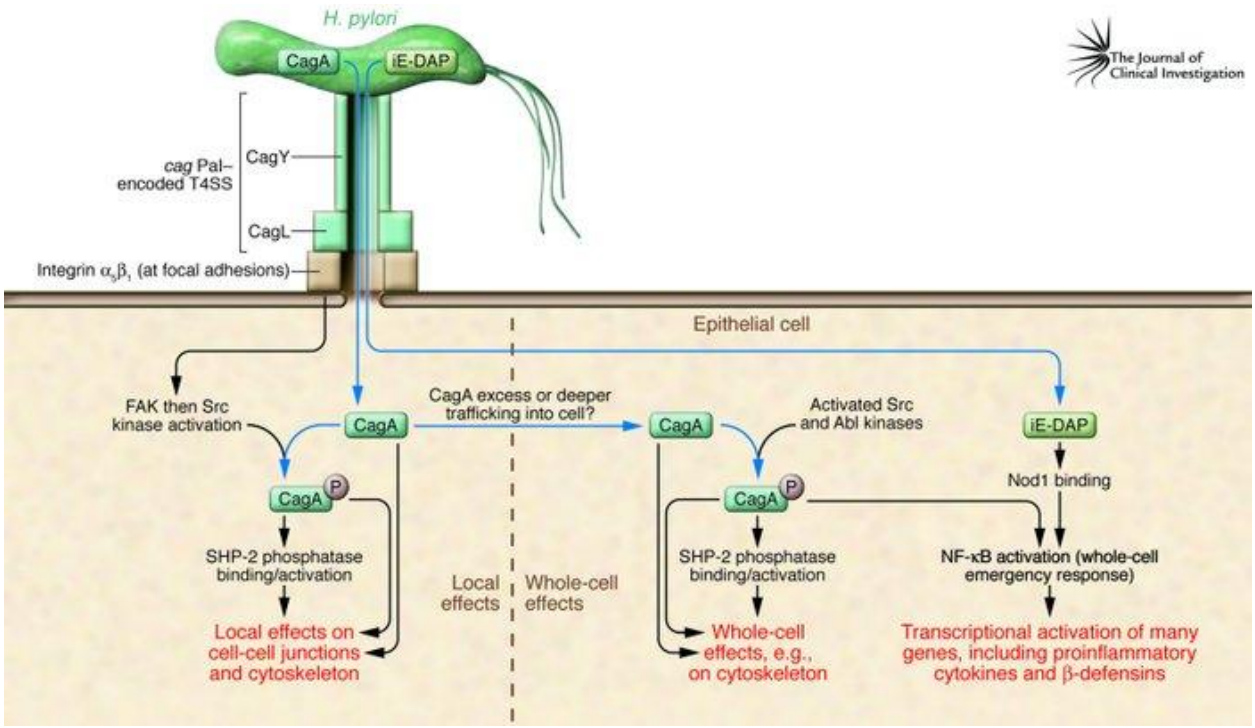


Figure 1.3 : Translocation of Cag A into host cell (adapted from ref. 27)

1.3 B Cell Activating Factor (BAFF)

BAFF, which is a short name of B cell activating factor, belongs to Tumor Necrosis Factor (TNF) super family. BAFF is identified in 1999 and also known as BLys, THANK, TALL-1, zTNF4, TNFSF13B. BAFF is located on chromosome 13q34 in humans and has six exons [31,32]. BAFF is an important cytokine for regulating innate and adaptive immunity. Like other TNF family ligands BAFF regulates lymphocyte functions, development of lymphocyte organs and immune tolerance. BAFF is a vital cytokine for B cells. This cytokine plays an important role in homeostasis of B cells, B cell maturation, B cell survival, B cell differentiation and development, plasma cell survival, antibody response promotion, Ig class switch recombination. It was demonstrated that BAFF deficient mice have failed to convert transitional type 1 (T1) B cells to transitional type 2 (T2) B cells [33,34,35,36].

BAFF is expressed by various cell types such as monocytes, neutrophils, dendritic cells, activated T cells, epithelial cells, salivary gland cells, myeloid cells, macrophages and also by cancer cells such as B-CLL, Non Hodgkin's lymphoma (NHL) and multiple myeloma (MM). In humans, high production of BAFF promotes

the survival of autoreactive B cells that leads to autoimmune diseases. Autoreactive B cells also produce BAFF and this cytokine acts as an autocrine survival factor for malignant B cells. Elevated levels of BAFF have been found in sera of autoimmune diseases patients such as rheumatoid arthritis (RA), Sjögren's syndrome (SS), experimental autoimmune encephalomyelitis (EAE), systemic lupus erythematosus (SLE) and autoimmune thyroid disease [37,38,39,40].

Like other TNF family ligands, BAFF (285 amino acid protein) is a member of transmembrane II proteins and it shares 75 % homology with mice. It is initially synthesized as membrane-bound monomers and then can be oligomerized as trimers. Trimer oligomerization of BAFF leads to formation of virus like assembly. This homotrimeric structure of BAFF is required for receptor binding. BAFF can be also secreted as soluble form after cleavage from the cell surface by furin convertase. Souble BAFF (sBAFF) can be self –oligomerized and form a 20-mer and also can be found like trimer and 60- mer form [36,41].

An alternatively spliced form exon 3 of BAFF have been discovered in humans and named as ΔBAFF (delta BAFF). ΔBAFF (lacks 57 bp) can only exist as a membrane-bound form and it can form a heterodimer with full length BAFF. A variant form of BAFF inhibits BAFF activity, and reduces BAFF release [41,42].

1.5 A Proliferation Inducing Ligand (APRIL)

APRIL is a sister molecule of BAFF and classified in TNF family. It shares 50% homology with BAFF. APRIL is also termed as TRDL-1, TALL-2 and TNFSF-13a. It is found in chromosome 17p13.1 in humans and has six exons. APRIL is produced by dendritic cells, monocytes, macrophages, T cells and epithelial cells. Different tumour cells like gastrointestinal, duodenum, rectum, colon and stomach also express APRIL. High levels of APRIL were also reported in the sera of autoimmune patients [36,43,44].

Unlike BAFF, APRIL is cleaved in the Golgi apparatus by a furin- convertase and is able to perform its function as soluble form. Secreted APRIL cannot form a multimer virus-like cluster. Alternatively spliced form of APRIL, which is called ΔAPRIL, can also exist as membrane-bound. ΔAPRIL lacks a furin cleavage site. APRIL can make a fusion protein with another TNF family ligand TWEAK (TNF- related weak

inducer of apoptosis, TNFSF12) called TWE-PRIL also be found on the cell surface (Figure 1.4). Heterodimer of BAFF and APRIL have been reported just once in autoimmune diseases [41,45,46].

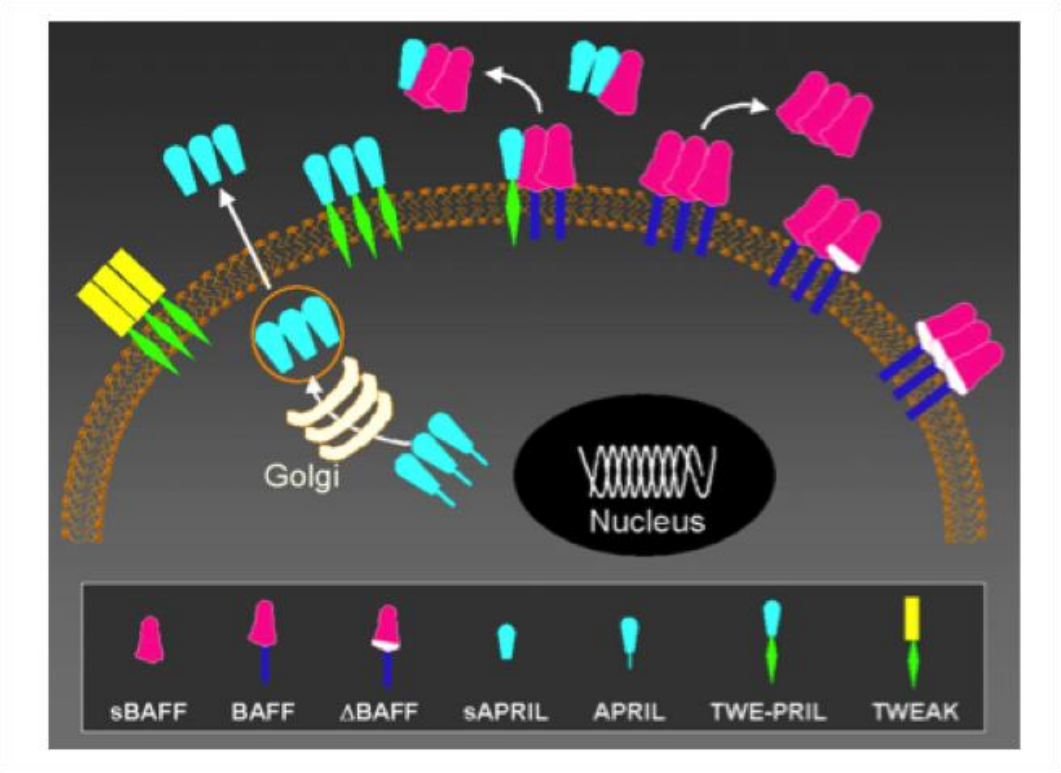


Figure 1.4 : Various forms of BAFF and APRIL (adapted from ref. 41)

APRIL plays a role in the development and expansion of B cells. It also stimulates tumour cells growth. APRIL binds CD95 and induces apoptosis of tumour cells. [47]. It has been reported that this TNF family ligand has a role in the long-term survival of plasma cells in the bone marrow [36,48].

1.6 Receptors of BAFF & APRIL

BAFF and APRIL are ligands for TNF family receptors. These receptors can be associated with TRAFs (TNF receptor associated factors) that have an inducible effect on survival, differentiation and function of B cells. BAFF and APRIL can both bind B cell maturation antigen (BCMA, TNFRSF17) and the transmembrane activator and calcium modulator cyclophilin interacting ligand (TACI, TNFRSF13b). APRIL have a high affinity to bind to BCMA than BAFF. BAFF can also specifically bind to BAFF receptor (BR3) which APRIL can not bind [49]. BR3 is

located on the human chromosome 22q13.2 and expressed by memory and naive B cells. BCMA is discovered on chromosome 16p13.1 in humans and is an important receptor for long-lived plasma cells. Third receptor TACI is found mainly on short-lived plasma cells and located on 17p11.2 human chromosome. BCMA receptor contains a single cysteine- rich domain (CRD) whereas TACI receptor has two. BR3 receptor has no CRD, but it is comprised of a typical module [40,49].

After cleavage from the cell membrane, soluble homotrimer form of BAFF can bind to its specific BR3 (TNFRSF13c) receptor. Soluble 60-mer form of BAFF can bind to TACI receptor whereas homotrimers of BAFF cannot activate TACI receptor. Only multimerized - membrane BAFF and multimerized APRIL can bind to TACI receptor. Beside BCMA and TACI receptors, APRIL can bind heparan sulphated proteoglycans (major component of extracellular matrix) on the cell surface of B cells. Heparan sulphated proteoglycans (HSPG) are known as receptors for APRIL. Heterodimer of BAFF and APRIL share receptors with APRIL such as TACI and BCMA [50] (Figure 1.5).

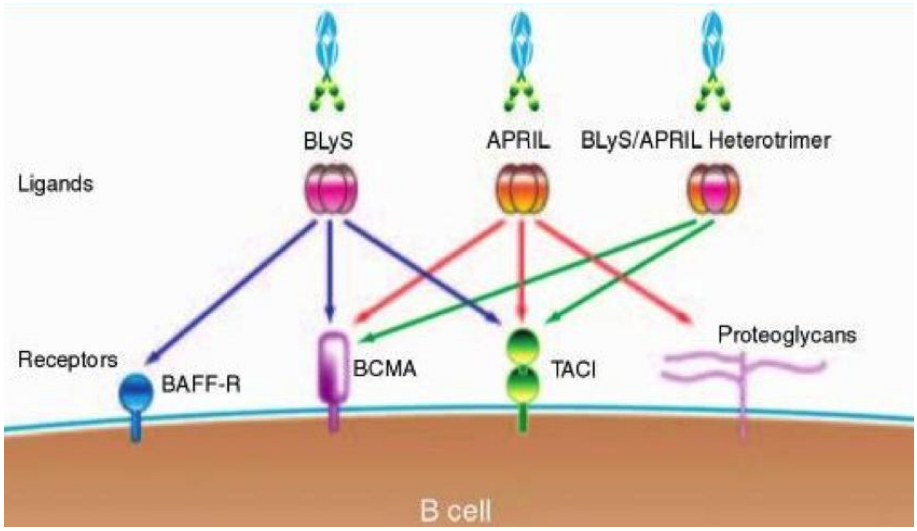


Figure 1.5 : Receptors of BAFF and APRIL (adapted from ref. 50)

These BAFF/APRIL receptors are expressed during different time points of B cell differentiation. BR3 is essential for survival and maturation of immature B cells. BR3 is expressed by Marginal zone (MZ) B cells and T cells. It has been shown that BR3 - deficient mice lacks mature B cells [40,51]. TACI receptor is also detected in MZ-B cells, B1-B cells and memory B cells. This receptor is required for the T – cell independent responses and known to suppress B cell proliferation and survival.

BCMA receptor expression was found in plasma cells.

BAFF or APRIL activates NF-κB and MAPK (Mitogen-activated protein kinase) pathways through binding to TACI and BCMA receptors. These receptors interact with death domain- containing proteins (TRAFs) which activates these pathways. This interaction leads to apoptotic or survival signals. BCMA receptor induction can also leads to activation of p38 MAPK and JNK (c-Jun N terminal kinase). TACI receptor induction can induce transcription factors AP-1 (activator protein-1) and NF-AT (nuclear factor of activated T cells), which are important proteins for lymphocyte survival [52].

1.7 Innate Immunity

Innate immunity is the first line of defence against microorganisms in a non-specific manner. Pathogens can be recognized by host cell and destroyed in few minutes or hours. It serves as an immediate response to pathogens. Innate immunity is an evolutionary conserved defence system that is found in insects, fungi, plants and vertebrates.

Monocytes, neutrophils, eosinophils, basophils, natural killer cells, and epithelial barriers are components of innate immunity. According to Charles A. Janeway theory, pathogens or pathogen derived components termed pathogen-associated molecular patterns (PAMPs) and molecules released by injured and dying cells termed as danger –associated molecular patterns (DAMPs) are recognized specifically by the immune system via pattern –recognition receptors (PRRs) or the other name is germ line- encoded receptors and activate innate immune cells [53]. These recognitions and activation of immune cells results in secretion of cytokines or chemokines and starts inflammatory responses. PRRs are essential receptors for the innate immunity. They are expressed by immune cells such as macrophages, dendritic cells, antigen - presenting cells (APCs), and by non- immune cells including epithelial cells. PRRs are divided in four families: Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and RIG-I like receptors (RLRs). TLRs can be found either on the cell surface or endosomal compartments. NLRs are cytoplasmic receptors and they recognize intracellular pathogens or pathogen–derived components [54]. CLRs recognize bacterial and fungal components whereas RLRs recognize viral double-stranded RNA (dsRNA).

1.7.1 Toll-like Receptors

Toll receptor was first identified in *Drosophila melanogaster* by Christiane Nüsslein-Volhard in 1985 [55]. Toll was found as an essential receptor for fungal infection in *Drosophila*. After the discovery of Toll, its homologue Toll like-receptor (TLR4) was identified in mammals. Toll-like receptors (TLRs) are important sensors of innate immune system and these receptors can be activated via PAMPs and DAMPs. They are common in various species and expressed by immune and non-immune cells. They recognize pathogens or particles of pathogens and activate immune cell response. 13 TLRs have been identified in mammals. TLR 1 to 9 was conserved in human and mouse, but only mice express TLRs 11,12 and 13 [56]. Cytoplasmic domain of Toll resembles IL-1 receptor so it's named as TIR (Toll-IL-1 receptor) domain. TIR domain mediates intracellular signalling. TLRs have leucine- rich repeats, which are required for ligand binding. There are 5 TIR adaptor molecules that play a role in signalling pathway: MyD88 (myeloid differentiation primary response gene -88), Mal (MyD88-adapter -like), TRIF (TIR domain- containing adapter-inducing interferon-β), TRAM (Toll-like receptor 4 adaptor protein), and SARM (sterile alpha and HEAT/Armadillo motif). These adaptor proteins activate kinases and induce the secretion of cytokines by NF-κB and MAPK pathways. TLR stimulation also leads to activation of JAK/STAT signalling pathway, which is important in cytokine secretion [57].

TLRs recognize different microbial components. TLR1 recognizes peptidoglycan and lipoproteins in the interaction with TLR2. TLR2 can recognize lipoproteins/lipopeptides from different pathogens, peptidoglycan and lipoteichoic acid from gram-positive bacteria. TLR3 recognize double-stranded RNA (Figure 1.6).

TLR4 is required for LPS recognition. Bacterial flagella are recognized by TLR5 receptor. TLR7 and TLR8 recognize single-stranded RNA (ssRNA) and nucleic acid structure of viruses. TLR9 is a critical receptor for CpG oligodeoxynucleotide (a synthetic DNA oligonucleotide in which a guanine is preceded by a cytosine nucleotide) recognition [58].

Some of these TLRs such as TLR1, TLR2 and TLR4 are expressed on the cell membrane and some of them like TLR3, TLR7, TLR8 and TLR9 are localized in intracellular compartments such as endosomes [59] (Figure 1.6).

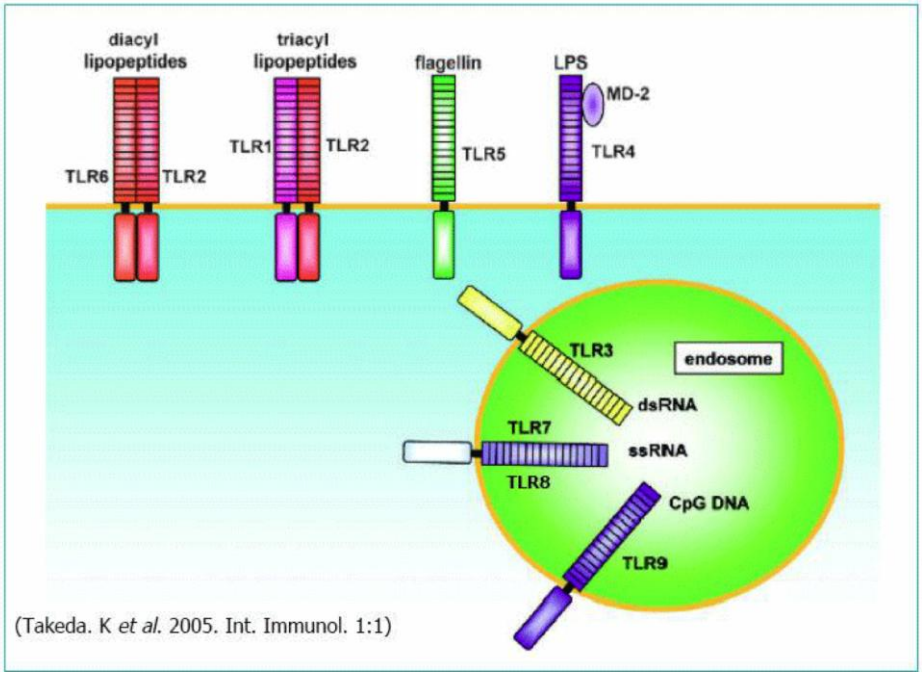


Figure 1.6 : Toll-like receptors and ligands (adapted from ref. 59)

1.7.2 NOD-like Receptors

NOD-like receptors (NLRs) are intracellular sensors of PAMPs and DAMPs. They also play a key role in regulation of innate immunity. NLRs are expressed by immune cells such as lymphocytes, macrophages, dendritic cells and non-immune cells like epithelial cells. They are strongly conserved during evolution. Homologues of mammalian NLRs are discovered in plants and zebra fishes [60]. 22 members of NLRs exist in humans. NLRs consist of three domains: N-terminal domain (CARD, PYD, BIRs or AD) which is required for homophilic protein-protein interaction, a central NOD (NACHT or NBD) domain which is common to all NLRs and it facilitates self oligomerization, and C-terminal leucine-rich repeat (LRR) which is essential for ligand recognition.

NLRs are divided in four groups according to their N-terminal domains. First family, NLRA have an acidic transactivation domain: CIITA (class II, major histocompatibility complex, transactivator). NLRB family contains BIR (baculoviral inhibitor of apoptosis repeat) domain: NAIP. NLRC families contain CARD (a

caspase recruitment domain): NOD1, NOD2, NLRC3, NLRC4, NLRC5. NLRP subgroup has a pyrin domain (PYD): NLRP1-14. CARD and PYD containing proteins are involved in apoptosis and inflammatory processes [61]. The last group is NLRX, which is newly discovered dsRNA receptor and have no homology to other N-terminal domain of NLRs (Figure 1.7).

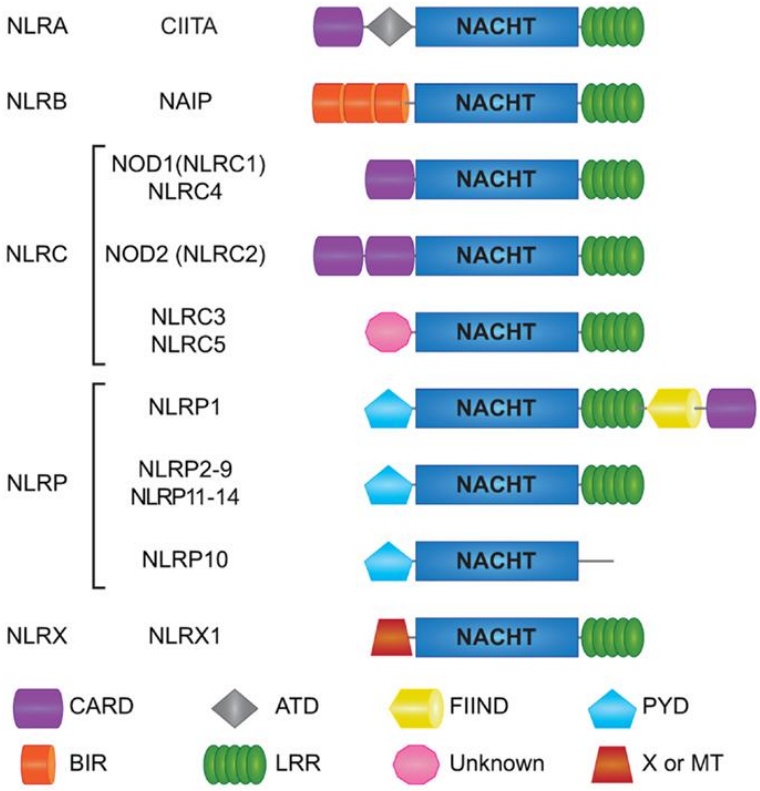


Figure 1.7 : Classification of NOD- like receptors according to their N-terminal domains (adapted from ref.61)

1.7.2.1 NOD1

Helicobacter infection induces adaptive Th1 (T helper 1) and Th17 (T helper 17) immune responses, which are important for the protective immune response against *H.pylori*. Apart from the adaptive immunity, innate immunity also becomes activated by *H.pylori* infection in gastric epithelial cells. Nucleotide-binding oligomerization domain - containing protein 1 (NOD1) is an important component of innate immunity. NOD1 is a well - known member of NLR family. NOD1 is a mammalian protein that is expressed in the cytosol and recognizes the peptides derived from peptidoglycan (PGN) of bacterial cell wall and stimulate immune response. NOD1 is

expressed by gastrointestinal epithelial cells, primary epithelial cells, and antigen - presenting cells. Another well described NLR family member NOD2 is expressed in specialized epithelial cells such as Paneth cells. γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP) containing bacterial molecules are ligands for NOD1 whereas muramyl di-peptide (MDP) can recognized by NOD2. All bacteria possess MDP but only some gram-positive and almost all gram-negative bacteria have iE-DAP [62]. It was demonstrated that *H.pylori* infected gastritis patients have higher NOD1 expression compared to non-infected patients or *H.pylori* non –associated gastric patients [63]. Activation of NOD1 and NOD2 both promote secretion of pro-inflammatory cytokines.

NOD1 consist of three domains; N-terminal CARD, a central NOD and C-terminal leucine rich domain (LRR) [63,64] (Figure 1.8).

Figure 1. 8 : Structure of NOD1 (adapted from ref. 63)

Helicobacter pylori- derived peptidoglycan can enter to the host cell via T4SS and induce activation of NF-κB pathway and IL-8 secretion. In the absence of T4SS, the infection with *H.pylori* can still deliver its peptidoglycan and cause inflammation. It has been reported that *H. pylori* OMVs containing peptidoglycan can enter the cell through specialized domains of cell membranes called lipid rafts. *H.pylori* peptidoglycans bind to LRR domain of NOD1 and molecule (NOD1) undergoes conformational change [65]. CARD domain of NOD1 and CARD domain of downstream molecule serine/threonine kinase RICK have an interaction with each other. This interaction leads to ubiquitination of RICK, which promotes binding and activation of TAK1 (TGF-beta activated kinase-1). TAK1 inhibits the NF-κB pathway by degradation of IkBa complex. Activated RICK binds to TNF receptor-associated factor 3 (TRAF3). Interaction between RICK and TRAF3 leads to activation of TANK-binding kinase 1 (TBK1) and induction of Interferon regulatory factor 7 (IRF7). Translocation of IRF7 into nucleus initiates IFN-β expression from host cell (Figure 1.9).

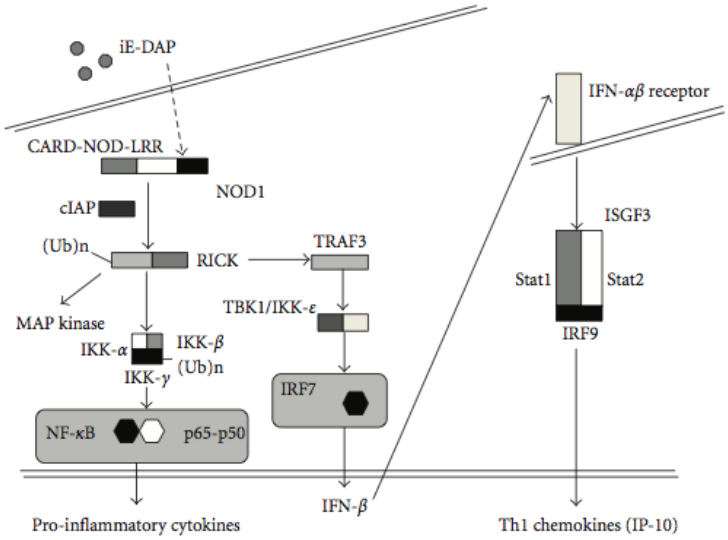


Figure 1.9 : NOD1 activation leads to IFN-β secretion (adapted from ref. 62)

Secreted IFN-β induces expression of Th1 cytokines via JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription) signalling pathway and also induces expression of BAFF from airway epithelial cells [66] and salivary gland cells [75] through unknown mechanism.

The Janus kinase/Signal Transducer and Activator of Transcription pathway transmits the extracellular chemical signals through the cell membrane and then to the nucleus and activates the targeted gene transcriptions. This signalling pathway induces cell proliferation, migration, differentiation and apoptosis. Activation occurs when ligand binds and induces multimerization of receptor subunits. Cytoplasmic domain subunits of induced receptors interact with the JAK tyrosine kinase [68,69,70]. JAK has a SH2 domain, which has tyrosine kinase activity and JAK can bind to cell surface receptors via this domain and autophosphorylates itself. JAK1, JAK2, JAK3 and Tyk2 are the members of JAK family in mammals. Activated JAKs phosphorylates receptors from their tyrosine residues. STATs are the transcription factors and are found in the cytoplasm until getting activated. They have an important role in signal transduction [70]. STATs can bind to induced receptors and that are phosphorylated by JAKs. Phosphorylated STATs form dimer with another phosphorylated STAT via SH2 domain and activated STAT dimer translocates to the nucleus and binds to the specific regions on DNA to activate or repress the target genes [71,72,73,74] (Figure 1.10).

1.7.3 Interferon Regulatory Factor 7 (IRF7)

IRF7 is a member of the interferon regulatory transcription factor (IRF) family. It has been shown that NOD1 is induced by PAMPs or DAMPs from its LRR domain and gets self- oligomerized. NOD1 interacts with its downstream molecule RICK/RIP2 and activates TAK1, which is an activator of the NFκB and MAPK pathways. It has been reported that *Helicobacter pylori* - infected gastric epithelial cells induce type I IFN signalling via NOD1 [62,63,65]. NOD1 becomes activated by *H.pylori* derived PGN and results in interaction between RICK and TRAF3. Following this interaction, TBK1, which is known as T2K and NAK, becomes activated. TBK1 is an essential serine-threonine kinase for IRF7 phosphorylation. Activated IRF7 translocates to nucleus and initiates transcription of type I cytokines such as IFN-α (interferon-alpha) and IFN-β (interferon-beta). This interferon regulatory transcription factor family member IRF7 is a critical transcription factor for IFN-β expression.

1.7.4 Interferon-beta (IFN-β)

Interferons (IFNs) are divided into three subgroups: type I, type II and type III. Type I family members are IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. All type I IFNs binds to cell surface receptor called IFNAR (Interferon alpha / beta receptor alpha chain). This receptor consists of two subunits; IFAR1 and IFAR2. Type II IFN cytokine family member is IFN-γ and it binds to IFNGR on the cell surface. IFNGR have IFNGR1 and IFNGR2 chains. The type III IFN group members are IFN-λ molecules called IFN-λ1, IFN-λ2 and IFN-λ3. Their receptors are IL10R2 and IFNLR1.

IFN-β is one of the member of type I cytokine family. These family members of cytokines are present in mammals, birds, amphibians and fish species. IFN-β cytokine is expressed by immune cells such as B cells, T cells, natural killer cells (NK), macrophages and some epithelial cells. IFN-β induction plays a role in innate immunity against bacterial infections and some viruses (e.g., influenza virus H5N1). PAMPs and DAMPs can be recognized by TLRs and NLRs and induce IFN-β secretion. Several studies showed that IRF7 is transcription factor for IFN-β [62,63,65]. Secreted IFN- β binds and activates its receptor IFNAR, which is localized on the cell. A member of the JAK family, JAK1 interacts with the activated IFNAR receptor, and then STATs can bind to receptor and induce JAK/STAT (Janus

kinase/Signal Transducer and Activator of Transcription) signalling pathway. Published studies showed that IFN-β gene expression is accomplished via JAK/STAT signalling pathway. IFN-β induces secretion of its own along with Th1 cytokines in an autocrine manner. It was reported that activation of STAT1 by JAK1 induces IFN-β expression in airway epithelial cells [66]. IFN-β also leads to complex formation of ISGF3 (interferon-stimulated gene factor 3), which is composed of STAT1, STAT2 and IRF9. This transcription factor is also important for Th1 cytokine and IFN-β secretion against *H.pylori* and some other pathogens [67] (Figure 1.9).

1.8 The role of JAK/STAT signalling pathway on BAFF expression

It has been reported that JAK/STAT signalling pathway is important for BAFF expression in airway epithelial cells. dsRNA – stimulation induces IFN-β secretion from airway epithelial cells [74]. Secreted IFN-β binds to its receptor on the cell surface and JAK1 interacts with IFNAR. STAT1 becomes activated and forms a dimer with other STAT molecules. Activated STAT dimers (either homo or heterodimers) can translocate to nucleus in order to induce IFN-stimulated response elements and initiate gene transcriptions of type I IFN cytokines (IFN-α and IFN-β). It was shown that IFN-β expression induces BAFF production in an autocrine manner. It has been also demonstrated that inhibition of IFN pathway reduces partially BAFF production in epithelial cells [75]. Intracellular signalling pathway that is responsible from induction of BAFF production and secretion from *Helicobacter pylori*- induced gastric epithelial cells still remains unclear.

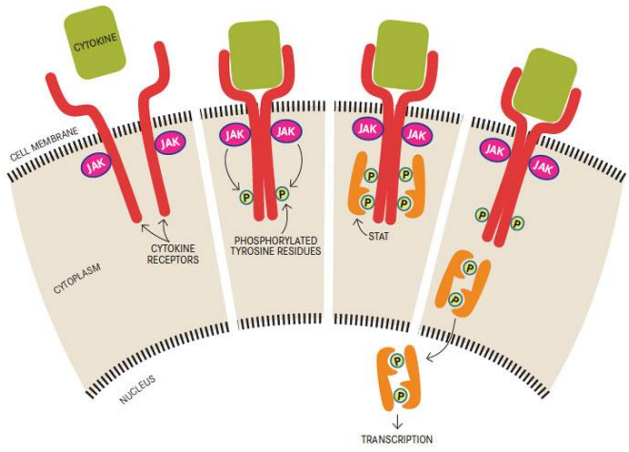


Figure 1.10 : JAK/STAT signalling pathway (adapted from ref. 74)

1.9 Aim of the Study

BAFF is expressed by dendritic cells, macrophages, monocytes, neutrophils, cancer cells and epithelial cells and is known to be a vital cytokine for B cell maturation and survival. It was recently reported that *H.pylori* induces BAFF expression from gastric epithelial cell lines. However, BAFF production from *H.pylori*- stimulated gastric epithelial cell line has not been investigated yet. Also, the role of *Helicobacter pylori* Cag A virulence factor on BAFF expression, production, and BAFF signalling pathway in gastric epithelial cells still remain unclear.

It was shown that JAK/STAT pathway plays a crucial role in BAFF expression from dsRNA- treated airway epithelial cells. The aim of this study is to determine the BAFF/APRIL expression and production level of *H.pylori* G27 WT- treated KATO-III cells (gastric adenocarcinoma cell line). Then it is aimed to investigate the role of *H.pylori* virulence factor Cag A on BAFF/ APRIL expression and production. The aim also includes the investigation of BAFF signalling pathway in *H.pylori* - treated KATO-III cells. Based on previous studies on signalling pathways responsible for BAFF production from epithelial cells, JAK/STAT signalling pathway was selected for investigation. For this purpose, KATO-III cells were treated with *Helicobacter pylori* G27 WT in a time-dependent manner to investigate the exact time for noticeable BAFF expression. Then KATO-III cells were stimulated with *H.pylori* G27 WT and its mutant ΔCag A sonicate and the role of this *H.pylori* virulence factor on BAFF expression and secretion were investigated. In order to determine the role of JAK/STAT signalling pathway in our model, specific (JAK inhibitor) against JAK1 was used in a dose-dependent manner and its effect on STAT1 phosphorylation was examined in *H.pylori* G27 WT- treated cells. Since BAFF expression was shown to be induced by various cytokines such as IFN-β, involvement of IFN-β in BAFF expression from *H.pylori* sonicate- stimulated gastric epithelial cells was also investigated in this study.

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 *Helicobacter* strains

Helicobacter pylori strains: G27 (wild- type) and its ΔCagA mutant were kindly provided by Prof. Anne Müller at University of Zurich, Zu□rich, Switzerland. Components of *Helicobacter* growth agar were given in Table 2.1. Antibiotics and components of antibiotic cocktails were shown in Table 2.2, 2.3. and 2.4 respectively. For liquid culture of *H.pylori,* solutions are given in Table2.5. and the freezing solution ingredients are shown in 2.6.

Table 2.1 : Components of *Helicobacter* growth agar.

Component	**Amount**
Colombia agar	42,5 g
Horse blood	50 ml
β-cyclodexin	10 ml
1000 X antibiotic cocktail 200 X antibiotic cocktail	1 ml 5 ml

Table 2.2 : Antibiotics used in *Helicobacter* agar.

Antibiotic	**Supplier Company**
Trimethoprim	HiMedia
Amphotericin B	HiMedia
Vancomysin	HiMedia
Cefsulodin	HiMedia
Polymixin B	HiMedia
β-cyclodextrin	HiMedia

Table 2.3 : 1000X Antibiotic Cocktail.

Content	Amount
Trimethoprim	100 mg
Amphotericin B	160 mg
DMSO	20 ml

Table 2.4 : 200X Antibiotic Cocktail.

Content	Amount
Vancomysin	100 mg
Cefsulodin	50 mg
Polymixin B	3.3. mg
ddH_2O	50 ml

Table 2.5 : Solutions used for *Helicobacter* growth.

Brucella Broth	50 ml
FBS (10% (v/v))	5 ul
Vancomycin	5 ul

Table 2.6 : Freezing medium of *Helicobacter pylori.*

Brucella Broth	25 ml
Glyserol	25 ml

2.1.2 KATO-III Cell Line

The KATO-III human gastric adenocarcinoma cell line was kindly provided by Doç.Dr. Rengül Çetin Atalay at Bilkent University, Ankara, Turkey.

2.1.3 Buffers and solutions

2.1.3.1 Cell culture

Table 2.7 : Solutions and media used in cell culture.

0.5%Tyripsin-EDTA 10 X	Lonza, Switzerland

DMSO	Fisher Scientific
Dulbecco s Modified Eagle Medium (DMEM)	Lonza
Fetal Bovine Serum (FBS)	Lonza
Penicilin / Streptomycin 100X	GibcoBRL, USA

Buffer and solutions used in cell culture experiments are given in table 2.8.

Table 2.8 : Buffers and solutions used in cell culture.

Freezing Medium	% 50 FBS 1X Pen/Strep DMEM %5 DMSO
PBS 1X	9,55g in 1 L ddH2O

2.1.4 Agarose Gel Electrophoresis

Table 2.9 : Buffers and solutions used for agarose gel electrophoresis.

50 X Tris Acetate acid EDTA(TAE)	242 g Tris base 57.1 ml Glacial Acetic Acid 100 ml 0.5 M EDTA up to 1L ddH_2O
DNA Ladder	100 bp DNA Ladder Mix GeneON, Germany
Sybr Safe	Invitrogen
Loading Dye	6X Loading dye Fermantas, Canada

2.1.5 Chemicals

Chemicals used in this study are shown in Table 2.10.

Table 2.10 : Chemical list in this study.

Chemical	**Supplier Company**
Acetic Acid (glacial)	Merck
Agarose	Lonza

dNTP	GeneON
EDTA	Applichem
Ethanol (absolute)	Merck
Sybr Safe DNA Gel Stain	Invitrogen
$MgCl_2$	GeneON
NaCl	Merck
Glycerol	Merck
Culture Media Dulbecco's Modified Eagle Medium (DMEM)	Lonza
Fetal Bovine Serum (FBS)	Lonza
Penicillin/Streptomycin 10X	Gibco
Trypan Blue	Lonza
Phosphate-Buffered Saline (PBS)	Lonza
Ammonium persulfate (APS)	Fisher-Scientific
Tween-20	Fisher-Scientific
Bovine Serum Albumin (BSA)	Santa Cruz
DMSO	Fisher Scientific
Sodium Deodecyl Sulfate (SDS)	Fisher Scientific
TEMED	Sigma-Aldrich
Acrylamide- Bis-acrylamide (40%)	Fisher Scientific
Bromophenol Blue	Fisher Scientific
Nonfat dry milk powder	Organic valley
Protein Ladder	New England Biolabs
PMSF	Applichem

Table 2.10 (cont'd.) : Chemical list in this study.

Nonidet P-40 (NP-40)	Applichem
HALT Protease Inhibitor (100X)	Thermo Scientific
PhosSTOP Phosphatase Inhibitor (10X)	Roche
DTT	Applichem
β-Mercaptoethanol	Sigma-Aldrich
Ponceau S	Fisher Scientific
Methanol	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
HCl	Sigma-Aldrich
NaOH	Sigma-Aldrich
Glycine	Fisher Scientific
Tris base (Trizma base)	Sigma-Aldrich
NaF	Alfa Aesar
Columbia Agar	BD
Brucella Broth	BD
CampyGen 2.5L	Oxoid

2.1.6 Protein isolation

Whole Cell Extract (WCE) Buffer was used for protein isolations and the components & amounts used in this study are shown in Table 2.11. After protein isolation, protein concentrations were measured by BCA. Diluted scheme for BCA is given in Table 2.12.

Table 2.11 : WCE components and amounts.

Content	**Amount**
1 M HEPES	2 ml of 1M
0.4 mM NaCl	8 ml of 5mM
25% glycerol	25 ml of 50% glycerol
1 mM EDTA	200 µl of 0.5 M
15 mM NaF	100 µl of 0.5 M
0.1 % NP-40	1 ml of 10% NP-40

Table 2.11 (cont'd) : WCE components and amounts.

ddH_2O	up to 100 ml
10X Phosphatase Inhibitor	Final concentration 1X
100 X Protease inhibitor Cocktail	Final concentration 1X

Table 2.12 : Dilution scheme for BCA Assay standards

Vial	**Volume of diluents ddH_2O (µl)**	**Volume & source of BSA (µL)**	**Final BSA Concentration (µg/ml)**
A	0	300 of stock	2,000
B	125	375 of stock	1,500
C	325	325 of stock	1,000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

2.1.7 Western Blotting

Table 2.13 : Buffers, solutions and supplements used for Western Blotting

Blocking Solution	1g non-fat dry milk powder in 20 ml 1X TBS-T
Ponceau Staining Solution	0.1 g Ponceau S (0.1% (w/v)) 5 ml Glacial Acetic Acid (5% (v/v)) up to 100 ml ddH_2O
Protein Ladder	New England Biolabs
10 X Running Buffer (pH 8.3)	15 g Tris base (25 mM) 71.2 g Glycine (192 mM) 50 ml 10% SDS (0.1%) up to 1L ddH_2O

Table 2.13 (cont'd) : Buffers, solutions and supplements used for Western Blotting

10X Transfer Buffer	58.2 g Tris Base 29.3 g Glycine 37.5 ml 10% SDS up to 1L ddH_2O
1X Transfer Buffer	100 ml 10 X Transfer Buffer 200 ml Methanol 700 ml ddH_2O
10 X TBS (Tris Buffered Saline) (pH:7.4)	24.23 g Tris Base 80.06 g NaCl up to 1L ddH_2O
1X TBS/T	100 ml 10 X TBS 900 ml ddH_2O 1ml Tween-20
Laemmli Buffer 5X	1.5 g SDS 3.75 ml 1 M Tris-HCl (pH 6.8) 0.015 g Bromophenol Blue 1.16 g DTT 7.5 ml Glycerol up to 15 ml total volume ddH_2O
%10 SDS-polyacrylamide Separation Gel	4 ml ddH_2O 3.3 ml 30% Acrylamide mix 2.5 ml 1.5 M Tris (pH 8.8) 0.1 ml 10% SDS 0.1 ml 10% APS 0.005 ml TEMED
10% SDS-polyacrylamide Stacking Gel	3.4 ml ddH_2O 0.83 ml 30% Acrylamide mix 0.63 ml 1 M Tris (pH 8.8) 0.05 ml 10% SDS 0.05 ml 10% APS 0.006 ml TEMED

2.1.8 Primers

Primers and sequences used in this study are given in below Table 2.14.

Table 2.14 : Primers and sequences used for qRT-PCR.

Primer name	Sequence (5'-3')	Species	GC content	Tm	Product size
BAFF fw	ACCGCGGGACTGAAAATCT	h	52.63%	59°C	129 bp
BAFF rv	TGCAATCAGTTGCAAGCAGTC	h	47.62%		
APRIL fw	ATAGCGCAGGTGTCTTCCAT	h	50%	59°C	117 bp
APRIL rv	ACAGTTTCACAAACCCCAGGA	h	47.62%		
NOD1 fw	GTGGACAACTTGCTGAAGAATGAC	h	45.83%	60 ^{0}C	108 bp
NOD1 rv	CTGTACCAGGTCCAGAATTTTGC	h	47.83%		
IFN- β fw	AATTGCTCTCCTGTTGTGCTTC	h	45.45%	60 ^{0}C	133 bp
IFN-β rv	GCAGTATTCAAGCCTCCCAT	h	50%		
IRF7 fw	CACCCCCATCTTCGACTTCA	h	55%	65 ^{0}C	101 bp
IRF7 rv	CGAAGCCCAGGTAGATGGTATAG	h	52.17%		
18SrRNA fw	GGCCCTGTAATTGGAATGAGTC	m/h	50%	59 ^{0}C	146 bp
18s rRNA rv	CCAAGATCCAACTACGAGCTT	m/h	47.62%		

2.1.9 Antibodies and inhibitor

Table 2.15 : Antibodies and inhibitor used in this study.

Antibody	Catalog number	Supplier Company
Rat anti-BAFF mAb	ab16082	Cell Signaling
Goat anti-BAFF mAb	af124	R&D Systems
Rabbit anti- APRIL mAb	ab64967	Abcam
Rabbit anti-GAPDH	5174	Cell Signaling
Mouse total anti-STAT1	9176	Cell Signaling
Rabbit phospho anti- STAT1	9177	Cell Signaling
Anti-rat IgG, HRP-linked Antibody	7077	Cell Signaling
Anti-rabbit IgG, HRP-linked	7074	Cell Signaling

Table 2.15 (cont'd) : Antibodies and inhibitor used in this study

Anti-goat IgG, HRP-linked Antibody	sc-2020	Santa Cruz
JAK Inhibitor	cas457081-03-7	Santa Cruz

2.1.10 Equipment

The equipment used in this study is listed in Table 2.16.

Table 2.16 : The equipment used in this study.

Equipment	**Supplier Company**
Laminar Air Flow Cabinets	FASTER BH_EN 2003
Pipettes	10 µl, 20 µl, 100 µl, 200 µl, 1000 µl Socorex and 10 µl, 100 µl, 1000 µl Biohit
Electronic Pipette	CappAid
Centrifuges	Beckman Coulter Allegra ™ 25 R Centrifuge Scanspeed 1730 R Labogene Scanspeed mini
Microplate Spectrophotometer	BIO-RAD Benchmark Plus
Microwave	White-Westinghouse
Eppendorfs	Interlab
Nitrocellulose Transfer Packs	BIO-RAD
Scale	Precisa
pH Meter	Mettler Toledo MP220
Examination Gloves	Beybi
Incubator with CO_2	BINDER
Nitrogen tank	Air liquide
Tissue culture flasks (25 cm^2, 75 cm^2)	Sarstedt
Culture plate (6-well, 12-well, 96-well U bottom, 96-well F bottom)	TPP
Petri dishes	Isolab
Serological pipette (5 ml, 10 ml, 25 ml)	Sarstedt

Table 2.16 (cont'd) : The equipment used in this study

Centrifuge tubes	Sarstedt, Isolab
Anaerobic Jar	Anaerocult
Nitrocellulose Paper (0.2 µm pore size)	Santa Cruz
Thermo Cycler	Techne 512
Vortex	Mixer Uzusio VTX-3000L,LMS
Quick spin	LMS
Incubator	BINDER
Whatman Filter Paper (3 mm)	Whatman
Magnetic stirrer	WiseStir MSH-20D, Wisd
pH Meter	InoLab
Light Microscope	Olympus CH30
Hemacytometer	Isolab
Ice Machine	Scotsman AF10 Siemens (-20 ^{0}C) Haier (- 80 ^{0}C)
Electrophoresis Gel System	BIO-RAD
SDS-PAGE Gel Electrophoresis System	BIO-RAD
UVIPhoto MW Version 99.05 for Windows 95&98	Microsoft
Shakers	Heidolp Duomax 1030
Lightcycler 480	Roche
Lightcycler 480 Multiwell plate	Roche
Lightcycler 480 Sealing foil 50 foils	Roche
Applied Biosystems Multiwell plate	Applied Biosystems
Applied Biosystems Sealing foil	Applied Biosystems
Kodak Medical X-Ray Processors	Kodak
Densitometer	G8 8000
Thermo shaker	Biometra TS1 ThermoShaker
Spectrophotometers	Thermo Scientific Nanodrop ^{TM} 2000 c, BIO-RAD Benchmark Plus
Sonicator	Bandelin Sonopuls
Nitrogen Tank	Air Liquid
Tissue flask	Sarstedt
Power Supply	BIO-RAD

2.1.11 Commercial kits

Commercial kits are given in below Table 2.17.

Table 2.17 : Commercial kits used in this study.

Kit	Supplier Company
Innu-Prep RNA Mini isolation Kit	Analytic jena, Biometra
High capacity cDNA syntesis Kit, 200 rxns	Applied Biosystems
Human BAFF ELISA	Abcam
Lightcycler 480 Sybr-Green I Master	Roche
Power SYBR® Green PCR Master Mix	Applied Biosystems
Maximo Taq	mSuperHot GeneON
20 X LumiGlo®Reagent and 20 X	Cell Signaling
Pierce ® BCA ^{TM} Protein Assay	Thermo Scientific

2.2 METHODS

2.2.1 Maintenance of *Helicobacter* species

Helicobacter pylori Wild type (G27) and its ΔCagA mutant were kindly provided by Prof. Anne Müller at University of Zurich, Zurich, Switzerland. Bacteria were seeded on Columbia Agar plates for 2 days at 37 ^{0}C in the presence of Oxoid Campygen sheet. After 2 days, bacteria viability was checked under microscope and transferred to liquid culture containing Brucella broth, 10%FBS and vancomycin.

2.2.2 Growth of *Helicobacter* strains

2.2.2.1 Colombia agar preparation

In order to growth *Helicobacter pylori* strains, 42,5 g Colombia agar was dissolved in 1000 ml water and autoclaved. Then the bottle was put in 50^{0}C water for 1 hour. 50 ml horse blood was added to agar. 5 ml β-cyclodexin was added on them. 0,5 ml of 1000 X antibiotic cocktail containing 100mg Trimethoprim and 160mg Amphotericin B and dissolve in 20ml DMSO and 2,5 ml from 200 X antibiotic cocktail containing 100mg Vancomycin, 50mg Cefsulodin, 3.3mg Polymixin B and dissolve in 50ml H_2O was additionally added to *H.pylori* plates.

2.2.2.2 Liquid culture

Helicobacter pylori strains were cultured in Brucella broth containing 10%FBS and 10.000X vancomycin. Oxoid Campygen sheet was added on the aerobic jar and bacteria were left growing at 37 ^{0} C.

2.2.3 Sonication of *Helicobacter pylori* Strains

Helicobacter pylori's mobility was checked under light microscope. 120- 200 ml liquid culture of *Helicobacter pylori* was aliqouted to 15 ml falcons. Falcons were centrifuged at 3000 rpm for 10 minutes. Supernatants were discarded. 10 ml PBS was used to washing step and was centrifuged at 3000 rpm for 7 minutes. Supernatants were discarded. 3.5 ml PBS was added on pellet and mixed. Tube was taken to sonication on ice. Sonication was performed as 30 sec pulse on, 50 sec pulse off for 6.30 minutes at 50 watt (MS 72 probe was used). They were aliquoted to 1.5 ml eppendorfs at 500 μl for each and then were centrifuged at 15000rpm for 20 minutes. Supernatant is taken to new eppendorf tubes and their concentrations were measured with BCA.

2.2.4 Protein Bicinchoninic Acid (BCA) Assay

The bicinchoninic acid assay (BCA) is similar to Bradford assay. This technic is used for the determining the total concentration of protein in a solution. After sonication of *Helicobacter pylori* strain, their concentrations were measured with Thermo Scientific's Pierce Protein BCA Assay Kit. Bovine Serum Albumin (BSA) standards (prepared from 2000 μg/ml top standard solution were prepared as 0 μg/ml, 25μg /ml, 125 μg /ml, 250 μg/ml, 500 μg/ml, 750 μg/ml, 1000 μg/ml, 1500 μg/ml, 2000 μg/ml with dilution instructions provided by the manufacturer**,** working reagent (200 μl per sample) and samples were prepared. Working reagent was a mixture of Solution B and Solution A with a ratio of 1:50, respectively. Standards and samples were used as duplicates**.** Prepared working reagent was added to each 96-well F bottom plate as 200 μl. Then 10 μl of each standard and diluted samples were added into the corresponding wells. Plate was covered and incubated at 37°C for 30 minutes. After 30 minutes incubation, absorbances of proteins were measured at 562 nm at Microplate Reader.

2.2.5 Cell Culture

2.2.5.1 Maintenance of KATO-III Cell Lines

KATO-III cells were grown in complete DMEM (supplemented with %10 FBS, 2mM L-Glutamine, 1 X Penicillin and Streptomycin). Cells were grown at 37 ^{O}C and 5% CO_2 incubator. Trypsin was used to subculture. KATO-III cells were frozen in freezing medium containing complete DMEM with %50 FBS and **%**10 DMSO.

2.2.5.2 Cell thawing

9 ml required DMEM medium was added to a 15 ml falcon tube. Frozen cells were thaw with the heat of your hand briefly. The cells were dissolved with complete DMEM medium. The falcon tube was centrifuged at 900 rpm for 5 min to remove DMSO (which is toxic for cells) from cells. The supernatant was discarded carefully following centrifugation. ~5 ml fresh DMEM medium for T25 flask or ~12 ml fresh DMEM medium for T75 flask was added to the pellet and the pellet was dissolved in the medium. The viability of cells was checked under the microscope before placing the flask to the incubator (37°C + 5% CO_2).

2.2.5.3 Cell passage

Cell density was determined on light microscope. Suspension KATO-III cells were put on a 15 ml falcon tube and centrifuged at 900 rpm for 5 minutes. The supernatant was discarded and the pellet was dissolved in 1 ml culture medium. The flask was washed once with 12 ml 1X PBS and 1500 µl of trypsin 100X was added on flask. The flask was incubated at 37 ^{0}C for 4 minutes. Then the flask was taken and mechanic force was applied to flask. 2,5 ml of culture medium and 1 ml dissolved cells were added to inhibit the effect of trypsin. 1ml cells ($2x10^6$ cells) were taken in a new flask with a fresh culture medium

2.2.5.4 Cell freezing

The medium was discarded from the flask. PBS was added flask to wash cells, then discard PBS. 1,5 ml trypsin was added to T75 flask and incubate the flask for 4 min. at 37°C. Attachment of cells were controlled under the microscope.

Approximately 9 ml DMEM medium to T75 flask to inactivate trypsin and transfer the mix into a 15 ml falcon tube. Cells were centrifuged at 900 rpm for 5 min.

The supernatant was discarded and 500 µL-1000 µL freezing medium was added (supplemented with growth media and 10% DMSO) to the pellet and dissolve the pellet in the freezing medium. Frozen cells were stored at -80°C.

2.2.5.5 Cell counting

Growth medium was removed from the flask and washed once with 1X PBS. 1.5 ml of Trypsin treatment from 10X stock was added to flask to breaks down the disulphate bounds and waited 4 min. at 37^0C incubator. Then 4 ml growth medium was added to the flask for inhibition of Trypsin solution.10 µl suspension was taken into 1.5 µl eppendorf tube and 1µl of trypan blue was added to check the viability of cells. Then 10 µl of cell suspension was put onto the hemacytometer and the cells were counted.

2.2.5.6 Treatment of KATO-III cells with *Helicobacter pylori* Sonicates

KATO-III cells were seeded on 6-well plates as $5x10^5$ cells/well one day before the treatment. The gastric adenocarcinoma cells were treated *H.pylori* G27 (wild- type) sonicate and its ΔCag A sonicate for 24 h.

2.2.5.7 Cell Harvesting

After 24 h waited treatment, 6-well plates were checked under microscope. Suspension cells were collected in a 1.5 ml eppendorf tubes and centrifuged at 3000 rpm for 8 minutes. Supernatants were discarded and cell pellet was dissolved in 500 µl of culture medium. 6-wells were washed with 1ml 1X PBS. 400 µl of trypsin was added to wells and incubated at 37 ^{0}C for 4 minutes. After the incubation, 1ml culture medium was added into wells. Cells from wells and also dissolved cells were transferred into 2 ml eppendorf tubes. They were centrifuged at 3000 rpm for 8 minutes. The supernatants were discarded. Cell pellets were put on -80 ^{0}C for further experiments.

2.2.6 RNA isolation from eukaryotic KATO-III cells

RNA isolations were performed by using Innu-prep RNA Mini kit (Analyticjena, Biometra Germany). 400µl of Lysis RL Solution was added onto the cell pellet and incubated for 2 minutes at room temperature. Cell pellet was re-suspended completely up and down by pipetting. The sample was incubated 3minutes at room temperature. A Spin D Filter was placed into a 2 ml Receiver tube. The lysed sample

was transferred onto the Spin Filter D and the tube was centrifuged at 10.000x g (12.000 rpm) for 2 minutes. Spin D Filter was discarded. The filtrate was not discarded because the filtrate contains the RNA. A Spin Filter R was placed in a new 2 ml receiver tube. An equal volume (app.400 µl) of 70% ethanol was added to the filtrate. The sample was mixed by pipetting sometimes up and down. The sample was transferred onto the Spin Filter R and centrifuged at 10.000 x g for 2 minutes. 2ml receiver tube was discarded and Spin Filter R was placed in new receiver tube. The Spin Filter R was opened and 500 µl Washing solution HS was added. The cap was closed and centrifuged at 10.000 x g for 1 minute. The receiver tube was discarded with filtrate and new one was placed. The Spin Filter R was opened and 700 µl Washing solution LS was added. The cap was closed and centrifuged at 10.000 x g for 1 minute. The receiver tube was discarded with filtrate and new one was placed. The tube was centrifuged at 10.000 x g for 3 minutes to remove all traces of ethanol. The receiver tube was discarded. The Spin Filter R was placed into a 1.5 ml Elution tube. The cap of Spin Filter R was opened carefully and 30-80 µl of RNase- free water was added. The tube was incubated at room temperature for 1 minute and centrifuged at 6.000 x g for 1 minute.

2.2.7 cDNA Synthesis

After RNA isolation and nanodrop measurements, samples were calculated as total RNA would be 1 µg and then ddH_2O to obtain 10 µl final volume for each tube. Reagents for cDNA synthesis is given in Table 2.18 and cDNA PCR conditions are given Table 2.19.

Table 2.18 : Reagents for cDNA synthesis.

Components	Reaction Volume
Buffer	2 µl
dNTP	0,8 µl
OligodT	1 µl
Reverse Transcriptase	1 µl
RNase Inhibitor	0,125 µl
ddH_2O	5,075 µl

Table 2.19 : cDNA PCR conditions.

25 ^{0}C	37 ^{0}C	80 ^{0}C	4 ^{0}C
10 min	120 min	5 min	∞

2.2.8 Primer design

Forward and reverse primers specific for expression of BAFF, APRIL, NOD1, IFN-β, IRF7 and 18S rRNA were design by using Primer blast program. Melting temperatures and GC content of primers, self-dimer formation were checked. All primers are given in Table 2.14.

2.2.9 18S rRNA PCR

Polymerase Chain reaction (PCR) is developed in 1983 by Kary Mullis. Specific DNA regions can be amplified by the *in vitro* enzymatic replications. PCR is composed of three steps including denaturation, annealing and extension. In denaturation step, two DNA strands were separated at 94 ^{0}C. Specifically designed primers were bind to complementary region of the DNA in the annealing step.

Annealing temperatures change according to primers melting temperature. Extension and final extension steps occur at 72^{0}C.

In order to check the cDNA, 18S rRNA primers were used for the conventional PCR. 18S rRNA primers are given in Table 2.14. Required PCR reagents are shown in below Table 2.20. and Conventional PCR conditions are indicated Table 2.21.

Table 2.20 : Reagents for PCR.

Components	**Reaction Volume**
10 X Taq Buffer	2,5 µl
$MgCl_2$ (25 mM)	1,5 µl
dNTP (10 mM)	0,5 µl
Forward Primer (10 µM)	0,75 µl
Reverse Primer (10 µM)	0,75 µl
Maximo Taq	0,1 µl
ddH_2O	17,9 µl
Template	1 µg

Table 2.21 : Conventional PCR conditions.

Initial Denaturation	95 ^{o}C	5 min.	1 cycle
Denaturation	95 ^{o}C	30 sec.	35 cycle
Annealing	59 ^{o}C	30 sec.	
Extension	72 ^{o}C	45 sec.	
Final extension	72 ^{o}C	7 min	1 cycle
Hold	4 ^{o}C	∞	-

2.2.10 Agarose Gel Electrophoresis

2% agarose gel is sufficient for the observation of our expected PCR products. The agarose gel contains 2 g agarose in 100 ml 1X TAE. The gel solution was boiled in the microwave until the agarose was dissolved. After the cooling, 10 µl sybr safe solution was added in to the gel and poured in to the gel cassette**.** Marker was loaded as 2.5 µl always in the first well of the gel to evaluate the length of the PCR products. 7.5 µl PCR products were mixed with 1.5 µl 6X loading dye. Loading dye was used to facilitate the observation. Gel was run at 110 V for 17 minutes. The bands, which are Sybr- safe stained was observed with UVtrans- illuminator by the help of UV PhotoMW software.

Table 2.22 : Agarose gel preparation.

Components	**Amounts**
1X TAE	100 ml
Agarose	2 g
Sybr safe	10 µl
6X loading dye (Fermantas)	1.5 ul
Template	7.5 ul

2.2.11 Real-time PCR

qRT-PCR which is a short name of quantitative Real-time PCR, a technique that based on the PCR. qRT-PCR is used to amplify and simultaneously quantify the DNA target molecule. In this study, expression levels of target genes were normalized to their house- keeping gene. Sybr Green I is used as a nucleic acid stain in these RT-PCR experiments. Sybr Green I is a dye which binds to DNA and gives

the emission that can be detected. 18S rRNA was used as a reference gene for qRT-PCR. qRT-PCR's were performed in Roche Light Cycler 480-II. qRT-PCR reagents are indicated in Table 2.23. The Light Cycler program is given in the Table 2.24.

Table 2.23 : qRT-PCR reagents.

Components	**Amounts**
cDNA	2.5 µl
Forward Primer (10 µM) Reverse Primer (10 µM)	0.5 µl 0.5 µl
Sybr Green I Master Mix	5 µl
ddH_2O	1.5 µl
Total mix	10 µl

Reaction mix was added to each well as 7.5 µl and then 2.5 µl of cDNAs were started to put as duplicates. Plate was covered with the seal and centrifuged at 1000 rpm for 1 min.

Table 2.24 : Real-time PCR conditions.

Step	**Temperature**	**Time**	**Cycle**
Initial denaturation	95 ^{0}C	1 min	1
Denaturation	95 ^{0}C	15 sec.	45
Amplification	59 ^{0}C	1 min	
Extension	72 ^{0}C	1 min	

For analysing the results, concentration of target genes normalized to reference gene. Efficiency values and the error rates were in expected ranges.

2.2.12 Protein isolation

For protein isolation, pellets were resuspended in Whole Cell Extract Buffer (WCE) containing freshly added 0.1 M PMSF (final conc.: 0.5 mM), 100X HALT Protease Inhibitor Cocktail (final conc.: 1X) and 10X Roche Phosphatase Inhibitor Cocktail (final conc.: 1X). Samples were incubated on ice for 30 min. Then they were centrifuged at 14000 rpm for 10 min. Supernatants were aliquoted as total protein samples and stored at -80°C. Protein concentrations were determined by Protein BCA Assay.

2.2.13 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis is a technique widely used to separate proteins according to electrophoretic mobility. The proteins are separated in gel according to their size. In this study, 5% stacking gel and 10 % separating gel were used. Prepared gels were poured between SDS-glasses and left for polymerization. After the polymerization, samples were mixed 5X Laemmli Buffer and boiled at 100°C for 5 minutes. The gels were placed into the tank and were run in running buffer at 90V-150V for approximately 2 h.

In order to detect the specific proteins Western Blot technique is used in this study. After SDS-PAGE, proteins were transferred from gel to the nitrocellulose membrane using Wet Transfer blotting (at 160V for 1h at 4°C). Transferred proteins were stained with Ponceau S. After visualizing the proteins, membrane was blocked at 4°C overnight in blocking solution (TBS/T containing 5% non-fat dry milk) to prevent non-specific binding. Then its incubated with 1:1000-1:2000 diluted primary antibodies (in TBS/T containing 5% BSA) at 4°C overnight with gentle shaking. The day after, membrane was washed with TBS-T buffer 3 times for 10 minutes in the shaker. 1:3000 diluted secondary antibody which was conjugated with HRP enzyme prepared again in blocking solution and then membrane was incubated with secondary antibody solution for 1-2 hour at 4°C in the shaker. After washing steps of membrane 3 times with TBS/T, the HRP substrate 20X LumiGLO ® Reagent (Cell Signaling) was diluted in to 1X with distilled sterile water (ddH_2O). This substrate is required for the visualization of the proteins. The membrane was exposed to X-ray film and then developed in Kodak Medical X-ray Processor according to

manufacturer's instruction. For the detection of other proteins in the same membrane, membrane can wash with TBS/T and can be incubated in other primary antibody.

2.2.14 BAFF ELISA

In order to determine the amount of secreted BAFF levels from gastric epithelial cell line, KATO-III, supernatants of treated and untreated cells were used in Human BAFF ELISA. ELISA (Enzyme-Linked Immunosorbent Assay) is a quantitative method that can be detect secreted proteins.

All reagents, working standards and samples were prepared to room temperature. Standards were serially diluted before use. 10 ng/ml Stock standard was prepared by reconstituting one vial of the BAFF standard with the addition of 1 mL Sample Diluent Buffer and hold at room temperature for 10 min. Standard tubes were labelled with 1 to 8. 400 µL of the 10 ng/mL Stock Standard was put into standard tube #1 and mixed with 600 µL Sample Diluent Buffer. 300 µL of sample diluent buffer was added into #2 to #8 standard tubes. Standard #2 was prepared by transferring 300 µL from standard #1 to standard #2. Standard #3 was prepared by transferring 300 µL from standard #2 to standard #3. Serial dilutions were repeated for tubes #4 to #7. Standard #8 is the blank control.

Table 2.25 : Standard preparation of Human BAFF ELISA.

Standard#	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc.(pg/ml)	Final Conc.(pg/ml)
1	Stock	400	600	10,000	4,000
2	Standard #1	300	300	4,000	2,000
3	Standard #2	300	300	2,000	1,000
4	Standard #3	300	300	1,000	500
5	Standard #4	300	300	500	250
6	Standard #5	300	300	250	125
7	Standard #6	300	300	125	62,5
8	None	-	300	-	-

After standard preparation, 100 µL of standard and undiluted samples were added to wells, which were pre-coated with specific mouse monoclonal anti BAFF antibody.

Plate was covered and incubated at 37 ^{0}C for 90 min. Cover was removed and contents were discarded from each well, plate was blotted onto paper towels. Wells do not let completely dry. 100 µL of 1X Biotinylated anti-Human BAFF antibody was added into each well and the plate was incubated at 37°C for 60 minutes. Plate was washed 3X with 1X PBS. Washing buffer was stayed in wells for one minute. Washing buffer was discarded and the plate was blotted onto paper towels. 100 µL of 1X Avidin-Biotin-Peroxidase Complex working solution was added into each well and incubated at 37°C for 30 minutes. Plate was washed 5X with 1X PBS and washing buffer was discarded as previously described. 90 µL of prepared TMB colour developing agent was added into each well and the plate was incubated at 37°C in dark for 20 - 25 minutes. 100 µL of prepared TMB Stop Solution was added into each well and the colour changes into yellow were seen immediately. The absorbance at 450 nm was read in a microplate reader within 30 min. after adding stop solution.

2.2.15 Densitometric analyses

Densitometric analyses were performed to detect the densities of protein bands of the target proteins and normalize their density to that of housekeeping protein (GAPDH). X-ray films were scanned in Bio-Rad GS-800 densitometer and densitometric analyses were performed using Adobe PhotoShop CS5 Software. Densitometric values of protein bands were determined by multiplying index and mean levels of each protein band and these values were normalized to their corresponding housekeeping controls (GAPDH levels). Phosphorylation percentages of target proteins were determined by the ratio of densitometric values of phospho-proteins over total-proteins normalized to their internal control. Phosphorylation percentages were graphed using GraphPad Prism 5.0 software. Standard deviations of the mean are indicated by vertical bars in column bar graphs.

2.2.16 Statistical analyses

All p values were calculated using GraphPad Prism 5.0 software. Significancies were determined by Student t test. In all analyses, a two-tailed p-value of less than 0.05 was considered statistically significant. In column bar graphs, vertical bars indicate standard deviations of the mean, n.s. denotes not significant.

3. RESULTS

3.1 Effect of *H.pylori* on BAFF expression in KATO-III cells

3.1.1 Investigation of BAFF expression from *H.pylori* G27 WT sonicate- treated KATO-III gastric epithelial cells in a time-dependent manner

It was reported that *H.pylori* induces the BAFF expression from other gastric epithelial cell lines [76]. In order to assess BAFF expression from *H.pylori* G27 WT sonicate- treated gastric epithelial cells, human KATO-III cells were investigated for BAFF expression on RNA level in a time-dependent manner upon *Helicobacter pylori*- infection (Figure 3.1).

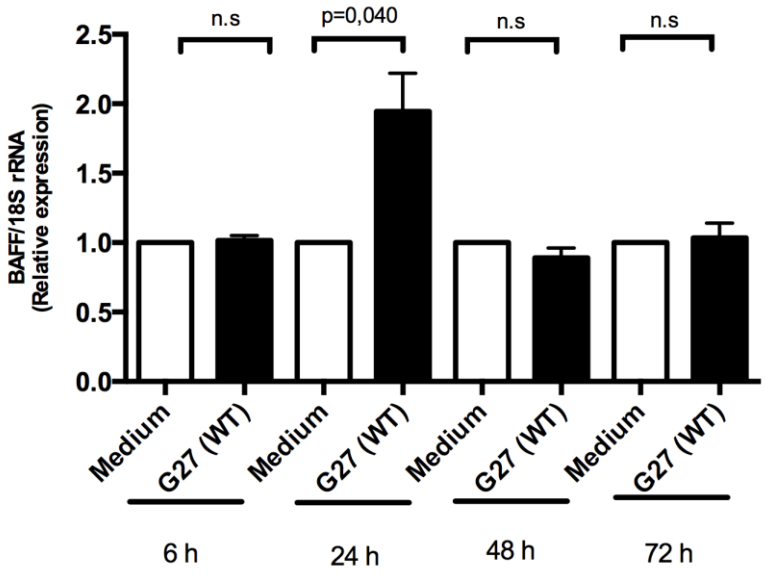


Figure 3.1 : *Treatment with H.pylori G27 WT sonicate leads to BAFF expression in* in *KATO-III cells.* KATO-III cells were seeded on 6 - well plates as $5x10^5$ cells and they were treated with *H.pylori* G27 WT sonicate (10 μg/ml) or left unstimulated (medium) in a time- dependent manner. Then, cells were harvested at indicated time-points (6 h, 24 h, 48 h and 72 h) and RNA isolations were performed. Following RNA isolations, they were converted to cDNA and analysed for BAFF and 18S rRNA

expressions using realtime - PCR. 18S rRNA was used as a reference gene for qRT-PCR. For every each sample BAFF concentrations were normalised to 18S rRNA concentrations. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Figure represents three independent experiments (n=3), n.s.: not significant.

BAFF mRNA expression level was significantly 2- fold increased in *H.pylori* G27 WT sonicate- treated cells compared to untreated control at 24 h in KATO-III cells. It was observed that BAFF mRNA expression levels were not significantly induced in other indicated time points. This result suggests that *Helicobacter pylori* G27 WT sonicate can induce BAFF expression from KATO-III cells. Further experiments were performed at 24 hours.

3.1.2 Effect of *H.pylori* virulence factor Cag A on BAFF expression in KATO-III cells

Cag A is a well – known virulence factor of *H.pylori*. The effect of Cag A on BAFF expression is not- defined in gastric epithelial cells. In order to assess the effect of *H.pylori* virulence factor Cag A on BAFF expression, KATO-III cells were investigated for expression of BAFF upon stimulation with *Helicobacter pylori* G27 WT or its Cag A mutant strains sonicates at 24 h time point. Figure 3.2 indicates the BAFF expression levels of *H.pylori* G27 WT sonicate, *H.pylori* G27 ΔCag A sonicate- treated or untreated KATO-III cells.

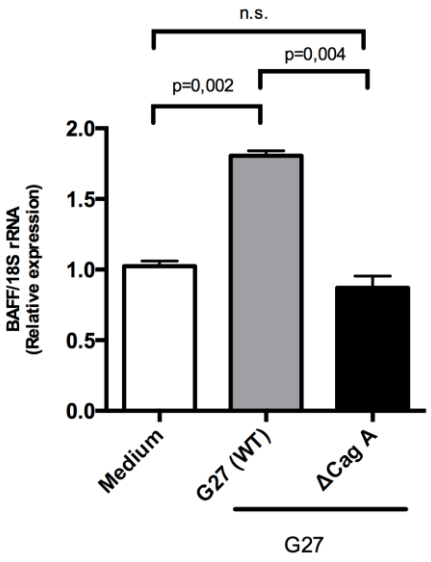


Figure 3.2 : *BAFF expression level is significantly decreased in H.pylori G27ΔCag A sonicate treated KATO-III cells at 24h.* KATO-III cells were seeded on 6 - well plates as $5x10^5$ cells and were treated with *H.pylori* G27 WT

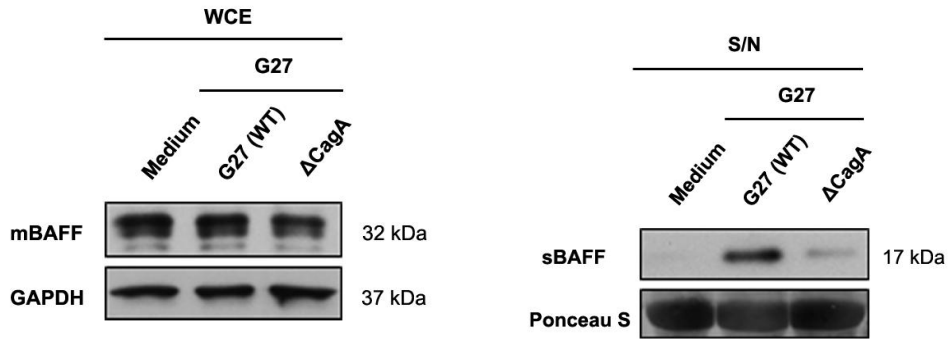
sonicate and *H.pylori* G27 isogenic mutant Cag A (lacks of cagA gene- ΔCag A) at 10 μg/ml final concentration or left untreated (medium) for 24 h. Cells were harvested at 24 h and RNA isolations were performed. Isolated RNAs were converted to cDNA and BAFF, 18S rRNA expression levels were determined by qRT -PCR. 18S rRNA was used as an endogenous control. BAFF mRNA expression levels were normalized to endogenous control. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of three independent experiments (n=3), n.s.: not significant.

BAFF expression level was significantly 1.7- fold increased in *H.pylori* G27 sonicate- treated cells compared to untreated cells at 24 h time-point. There was no significant induction of BAFF expression in *H.pylori* G27 ΔCag A sonicate- treated cells compared to untreated cells. This study represents the importance of *H.pylori* virulence factor Cag A on BAFF expression in KATO-III cells.

3.2 Effect of *H.pylori* sonicate- treated KATO-III cells on BAFF production

3.2.1 *H.pylori* sonicate induced membrane-bound and soluble BAFF production

H.pylori induction causes BAFF expression in RNA level from gastric epithelial cell line (MKN28) [76]. However, production of BAFF in protein level has not been reported. In order to assess effect of *H.pylori* sonicates on BAFF production, KATO-III cells were treated with *H.pylori* G27 WT sonicate and *H.pylori* G27 ΔCag A sonicate at 24h. Then BAFF protein levels were detected upon stimulation with *H.pylori* G27 WT sonicate and *H.pylori* G27 ΔCag A sonicate by Western Blotting (Figure 3.3). To determine different forms of BAFF protein, different BAFF antibodies targeted against membrane-bound BAFF and soluble BAFF proteins were used.



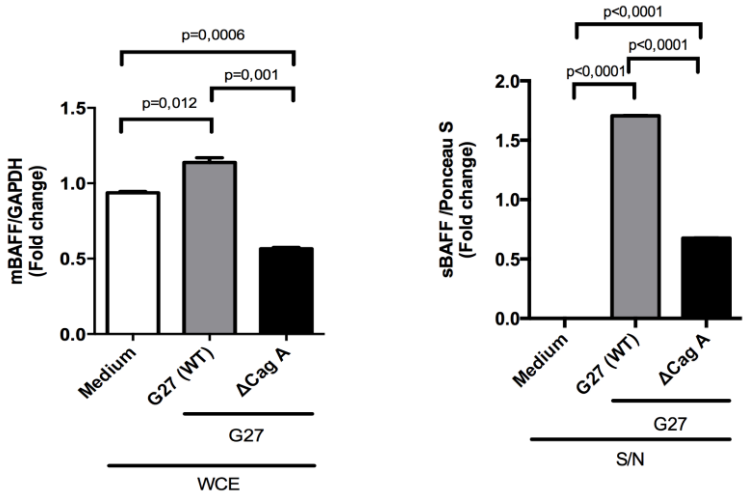


Figure 3.3 : *BAFF protein level is significantly decreased in H.pylori G27 ΔCag A* sonicate *treated KATO-III cells at 24h*. Cells were stimulated with *H.pylori* G27 WT sonicate and *H.pylori* G27 ΔCag A at 10 μg/ml final concentration or left untreated (medium) for 24 h. KATO-III cells were harvested for protein isolations and culture supernatants were aliquoted for detection of sBAFF on SDS-PAGE. Cells were lysed and immunoblotted for membrane-bound BAFF (32 kDa). Supernatants were immunoblotted for soluble BAFF (17 kDa). GAPDH (37 kDa) and Ponceau S were used as loading controls respectively. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of two independent experiments (n=2), n.s.: not significant.

According to our results membrane- bound BAFF was 1.2- fold increased in *H.pylori* G27 WT sonicate- treated KATO-III cells compared to untreated cells. sBAFF Western Blotting results shows 1.7-fold significant increase of BAFF protein from *H.pylori* G27 WT sonicate- treated cells compared to untreated controls. It was observed that mBAFF production was not increased in *H.pylori* G27 ΔCagA sonicate- treated cells compared to untreated cells. Soluble BAFF was not detected in untreated control. Both *H.pylori* G27 WT and *H.pylori* G27 ΔCagA sonicate induce soluble BAFF production. However, secreted sBAFF was 2.4-fold increased in *H.pylori* G27 WT sonicate- treated KATO-III cells when compared to *H.pylori* G27 ΔCagA sonicate- treated cells. Overall, our data indicate the role of Cag A on BAFF production and secretion from *H.pylori* sonicates- treated KATO-III cells.

3.2.2 Effect of *H.pylori* sonicate on BAFF secretion in KATO-III cells

In order to confirm the BAFF secretion from *H.pylori* G27 WT sonicate- treatment in KATO-III cells and effect of *H.pylori* virulence factor CagA on BAFF secretion, BAFF ELISA (Abcam) was performed from culture supernatants of *H.pylori* sonicates- treated or untreated cells (Figure 3.4).

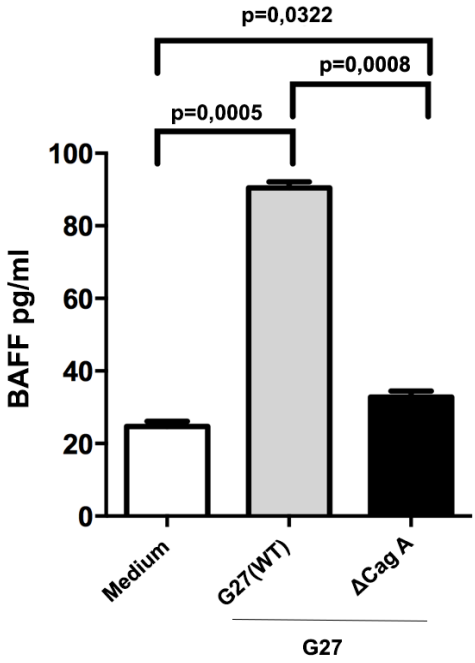


Figure 3.4 : *BAFF secretion is significantly induced in H.pylori* G27 WT *sonicate-treated KATO-III cells.* KATO-III cells ($5x10^5$ cells/well) were incubated for 24 h with *H.pylori* G27 WT sonicate (10 μg/ml), and *H.pylori* G27 ΔCagA sonicate 10 μg/ml) or left untreated (medium). Secreted BAFF levels were detected by ELISA in culture supernatants of KATO-III cells treated with *H.pylori* sonicates. Results are graphed using *Graphpad Prism* 5.0. p values were determined by Student's *t*-test.

As expected, BAFF secretion level was significantly 4-fold increased in *H.pylori* G27 WT sonicate- treated cells compared to untreated cells. BAFF secretion level was not induced in *H.pylori* G27 ΔCag A sonicate- treated cells as much as *H.pylori* G27 WT sonicate treatment. This result indicates that stimulation of gastric epithelial cells with *H.pylori* G27 WT sonicate induces BAFF secretion at 24 h.

3.3 Effect of *H.pylori* on APRIL expression in KATO-III cells

3.3.1 Investigation of APRIL expression from *H.pylori* G27 (WT) sonicate treated KATO-III gastric epithelial cells in a time-dependent manner

It was shown in this study that *H.pylori*- stimulated gastric epithelial cells significantly express BAFF at 24 h (Figure 3.1). APRIL is a homologue cytokine of BAFF and also expressed by epithelial cells [36]. For that reason, APRIL expression levels were investigated in KATO-III cells, which were treated with *H.pylori* G27 WT sonicate. In order to determine APRIL expression levels from KATO-III cells stimulated by *H.pylori* G27 sonicate in time-dependent manner via qRT-PCR (Figure 3.5).

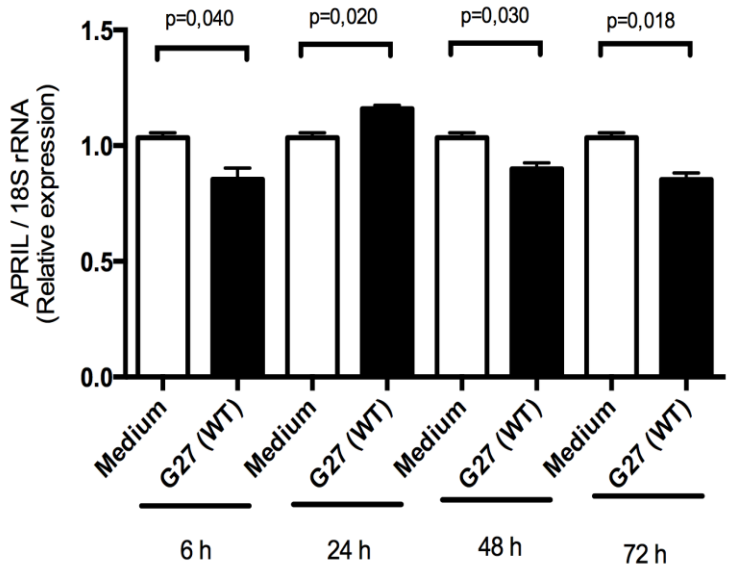


Figure 3.5 : *Treatment with H.pylori G27 WT sonicate leads to APRIL expression in KATO-III cells.* KATO-III cells ($5x10^5$ cells/well) were stimulated with *H.pylori* G27 sonicate (10 μg/ml) final concentration or left unstimulated (medium). Then, cells were harvested at indicated time-points (6 h, 24 h, 48 h and 72 h) and RNA isolations were performed. Following RNA isolations, they were converted to cDNA and analysed for BAFF and 18S rRNA expressions using qRT-PCR. 18S rRNA was used as endogenous control. Results are graphed using *Graphpad Prism* 5.0. p values were determined by Student's *t*-test. Figure 3.5 represents three independent experiments (n=3), n.s: not significant.

It was observed that APRIL mRNA expression levels were lower than BAFF expression levels (Figure 3.1). *APRIL mRNA expression level was significantly 1.2*

fold increased in H.pylori G27 WT sonicate- treated cells only at 24 h time-point. At other time points APRIL expression levels were not induced in *H.pylori* G27 WT sonicate- treated samples compared to untreated controls. These results indicate that APRIL is induced by *H.pylori* G27 WT sonicate treatment in KATO-III cells at 24 hours. Therefore, we treated KATO-III cells with *H.pylori* sonicates at 24 hour time-point in our further experiments.

3.3.2 Effect of *H.pylori* virulence factor Cag A on APRIL expression in KATO-III cells

H.pylori virulence factor Cag A plays a role in development of gastric malignancies such as gastric cancer [6]. Figure 3.2 shows the importance of Cag A on BAFF expression. Also, the presence of Cag A has an effect on BAFF production and secretion in KATO-III cells at 24 h (Figure 3.2). In order to assess the effect of Cag A on APRIL expression, KATO-III cells were stimulated with *H.pylori* G27 WT sonicate and *H.pylori* G27 ΔCag A sonicate at 24 hours. APRIL expression levels were detected by qRT-PCR (Figure 3.6).

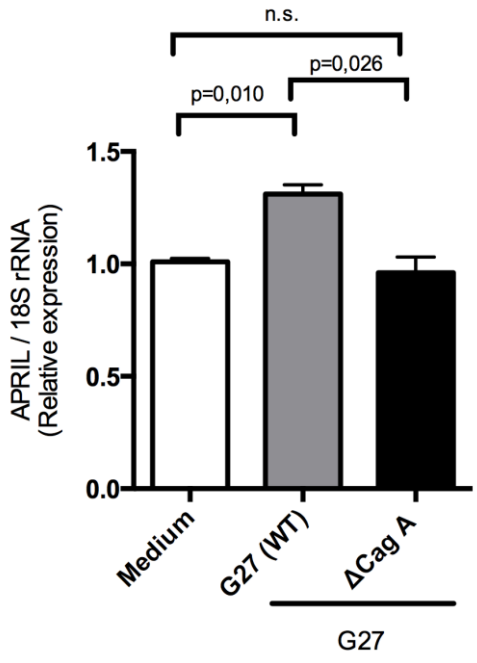


Figure 3.6 : *APRIL expression level is decreased in H.pylori G27 ΔCag A sonicate treated KATO-III cells at 24h.* KATO-III cells ($5x10^5$ cells/ml) were stimulated for 24 h *H.pylori* G27 WT sonicate (10 μg/ml), and *H.pylori* G27 ΔCag A sonicate(10 μg/ml) or left untreated (medium). Cells were harvested at 24h and RNA isolations were performed. Isolated RNAs were converted to cDNA and APRIL, 18S rRNA expression levels were determined by qRT-PCR. 18S rRNA was used as endogenous control.

APRIL mRNA levels were normalised to 18S rRNA concentrations. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of three independent experiments (n=3), n.s: not significant.

Expression levels of APRIL were found to be less than BAFF expression levels. APRIL expression level was significantly 1.2-fold increased in *H.pylori* G27 WT sonicate- treated KATO-III cells compared to untreated cells. There was no significant induction of APRIL expression level in *H.pylori* G27 ΔCag A sonicate-treated cells compared to untreated cells. Our results show the effect of *H.pylori* virulence factor Cag A on APRIL expression in KATO-III cells.

3.4 Effect of *H.pylori* sonicate- treated KATO-III cells on APRIL production

3.4.1 Effect of *H.pylori* virulence factor CagA on APRIL production

Our previous results showed that *H.pylori* G27 WT sonicate- treated KATO-III cells express APRIL cytokine and *H.pylori* virulence factor Cag A has an effect on APRIL expression (Figure 3.5 and Figure 3.6). In order to confirm the Cag A effect on APRIL production, KATO-III cells were treated with *H.pylori* G27 WT sonicate and *H.pylori* G27 ΔCag A sonicate for 24 hours. APRIL production levels were investigated by Western Blotting (Figure 3.7).

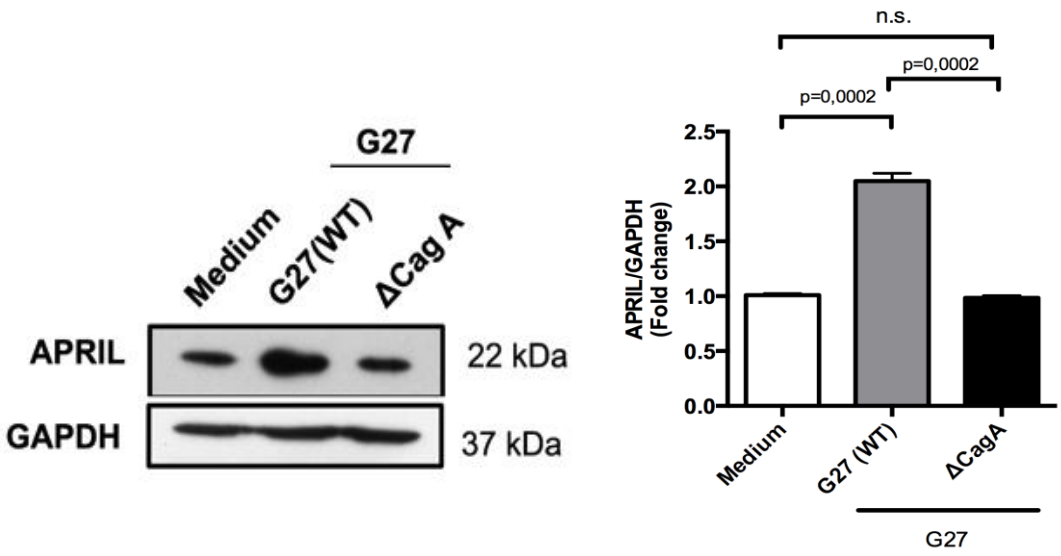


Figure 3.7 : *APRIL protein level doesn't change in H.pylori G27 ΔCag A sonicate treated KATO-III cells at 24h.* Cells ($5x10^5$ cells/well) were seeded on 6-well plates and stimulated for 24 h with *H.pylori* G27 WT sonicate (10 μg/ml), *H.pylori* G27 ΔCagA sonicate (10 μg/ml) or left untreated

(medium).KATO-III cells were harvested for protein isolations and culture supernatants were aliquoted for detection of sBAFF on SDS-PAGE. Cells were lysed and immunoblotted for APRIL (22 kDa). GAPDH (37 kDa) was used as loading control. Samples were loaded as 20 μg. Densitometric analysis was performed by using GAPDH as endogenous controls for APRIL. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of two independent experiments (n=2), n.s: not significant.

According to our results, APRIL production was significantly 2-fold increased in *H.pylori* G27 WT sonicate- treated KATO-III cells compared to untreated cells whereas *H.pylori* G27 ΔCag A sonicate- treated cells had no effect on APRIL production in KATO-III cells compared to untreated control. It was observed that *H.pylori* G27 WT sonicate- treated KATO-III cells produce APRIL protein at 24 h time-point. Overall, our results indicate the role of Cag A on APRIL production in *H.pylori* sonicate- treated KATO-III cells.

3.*5 H.pylori* G27 WT sonicate induces NOD1 expression in KATO-III cells

3.5.1 Effect of *H.pylori* G27 WT sonicate on NOD1 expression in a time-dependent manner

Up to this point, BAFF and APRIL expression were shown to be Cag A dependent both in RNA and protein level. It has been reported that *H.pylori* has a role in induction of NOD1 expression from gastric epithelial cells [62]. NOD1 signalling can both activate the production of pro-inflammatory cytokines via canonical NF-κb pathway or type I cytokines such as IFN-β via IRF signalling. In order to determine the role of *H.pylori* G27 WT sonicate on NOD1 expression and NOD1 signalling pathway, KATO-III cells were treated with *H.pylori* G27 WT sonicate in a time-dependent manner. NOD1 mRNA expression levels were detected by qRT-PCR. (Figure 3.8)

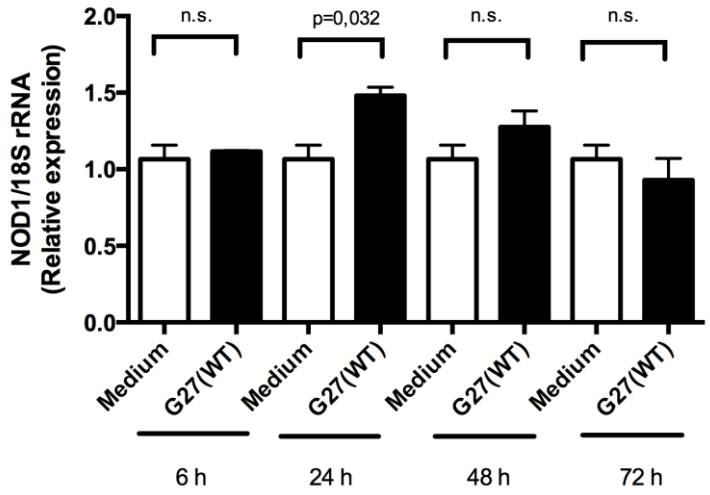


Figure 3.8 : *NOD1 expression is induced by H.pylori G27* WT *sonicate- treated KATO-III cells at 24h.* KATO-III cells ($5x10^5$ cells/well) were stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) or left unstimulated (medium). Then, cells were harvested at indicated time-points (6h, 24h, 48h and 72h) and RNA isolations were performed. Following to RNA isolations, they were converted to cDNA and analysed for BAFF and 18S rRNA expressions using qRT-PCR. 18S rRNA was used as endogenous control. Results are graphed using *Graphpad Prism* 5.0. p values were determined by Student's *t*-test. Figure 3.8 represents three independent experiments (n=3), n.s.: not significant.

NOD1 mRNA expression level was significantly 1.5- fold increased by stimulation with *H.pylori* G27 WT sonicate in KATO-III cells compared to untreated controls at 24 h. It was observed that NOD1 mRNA expression levels were not significantly induced in other indicated time points. This result indicates that NOD1 expression levels were induced in *H.pylori* G27 WT sonicate in KATO-III cells at 24 hours. Further experiments were performed at 24 h time-point.

3.5.2 Effect of *H.pylori* virulence factor CagA on NOD1 expression

One of the most important virulence factors of *H.pylori* Cag A was shown to play a role in BAFF and APRIL expression and production. Figure 3.8 indicates that *H.pylori* G27 WT sonicate- treated KATO-III cells express NOD1 at 24 hours. In order to assess the role of *H.pylori* virulence factor Cag A on NOD1 expression, KATO-III cells were investigated for NOD1 expression by qRT-PCR (Figure 3.9).

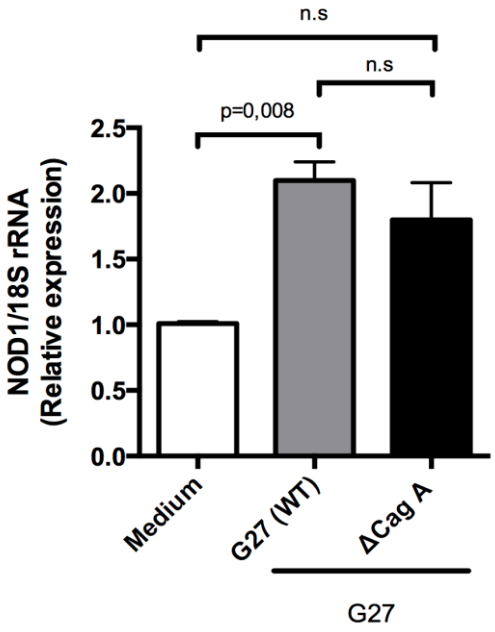


Figure 3.9 : *NOD1 expression is significantly increased in H.pylori G27 WT sonicate treated KATO-III cells.* KATO-III cells ($5x10^5$ cells/well) were stimulated for 24 h with *H.pylori* G27 WT sonicate (10 μg/ml), *H.pylori* G27 ΔCagA sonicate (10 μg/ml) or left untreated (medium). Cells were harvested at 24 h and RNA isolations were performed. Isolated RNAs were converted to cDNA and NOD1, 18S rRNA expression levels were determined by qRT-PCR. 18S rRNA was used endogenous control. *Graphpad Prism* 5.0 was used to graph expression levels of NOD1. p values were determined by Student's *t*-test. Data are representative of three independent experiments (n=3), n.s: not significant.

NOD1 expression level of *H.pylori* G27 sonicates- treated KATO-III cells was significantly 2-fold increased compared to untreated cells. It was observed NOD1 expression was not significantly altered in *H.pylori* G27 ΔCag A sonicate- treated KATO-III cells compared to *H.pylori* G27 WT sonicate- treated cells. Figure 3.9 indicates that there is no significant effect of Cag A on NOD1 expression. Then, research was carried on by investigation of downstream molecules of NOD1 in *H.pylori* sonicates- treated KATO-III cells.

3.6 Effect of *H.pylori* G27 WT sonicate on IRF7 expression in KATO-III cells

3.6.1 Investigation of IRF7 expression from *H.pylori* G27 WT sonicate treated KATO-III gastric epithelial cells in a time-dependent manner

NOD1 signalling pathway may lead to expression of type I IFNs from epithelial cells [63]. Figure 3.8 showed that NOD1 expression was significantly induced by

stimulation of *H.pylori* G27 WT sonicate in KATO-III cells at 24 h time-point. IRF7 is a downstream effector of NOD1 and an important transcription factor for expression of type I IFNs. In order to understand the *H.pylori*- induced NOD1 signalling pathway, KATO-III cells were examined for IRF7 expression by qRT-PCR (Figure 3.10).

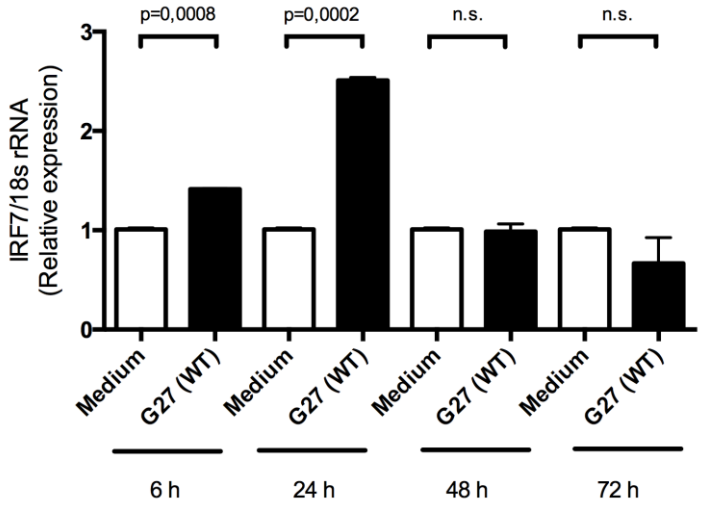


Figure 3.10 : *Treatment with H.pylori G27 WT sonicate induces IRF7 expression in KATO-III cells.* KATO-III cells ($5x10^5$ cells/well) were induced with *H.pylori* G27 sonicate (10 μg/ml) or left unstimulated (medium). KATO-III cells were harvested as indicated time-points (6 h, 24 h, 48 h and 72 h) and RNA isolations were performed. Following RNA isolations, they were converted to cDNA and analysed for IRF7 and 18S rRNA expressions using qRT-PCR. 18 S rRNA was used as endogenous control. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Figure 3.10 represents three independent experiments (n=3), n.s: not significant.

IRF7 is a downstream molecule of NOD1 and its translocation into nucleus induces type I IFNs. According to Figure 3.9, NOD1 expression level was significantly 2-fold increased after stimulation with *H.pylori* G27 WT sonicate at 24 h. IRF7 mRNA expression level was significantly 2.5-fold induced by stimulation with *H.pylori* G27 WT sonicate in KATO-III cells compared to untreated cells at 24 h. It was observed that IRF7 mRNA expression levels were not significantly induced in other indicated time points except for 6 h time-points (1.2-fold). NOD1 expression leads to induction of IRF7 expression at 24 h in *H.pylori* sonicate- stimulated KATO-III gastric

epithelial cell line. Further experiments were performed at 24 h time-point to investigate the role of *H.pylori* virulence factor Cag A on IRF7 expression.

3.6.2 Effect of *H.pylori* virulence factor Cag A on IRF7 expression

In order to understand the role of *H.pylori* Cag A on the expression of IRF7, KATO-III cells were investigated for IRF7 expression via qRT-PCR.

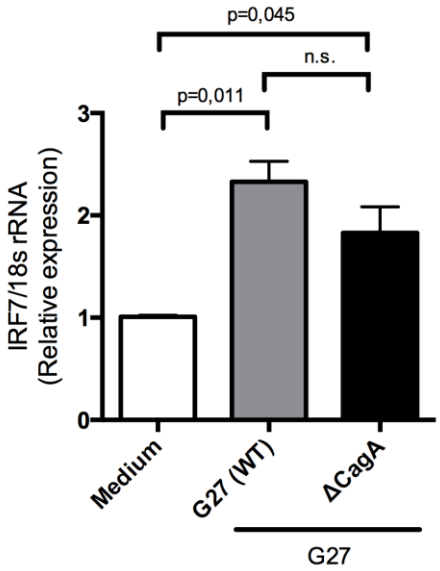


Figure 3.11 : *IRF7 expression is significantly increased in H.pylori G27 sonicate treated KATO-III cells.* KATO-III cells ($5x10^5$ cells/well) were stimulated for 24 h with *H.pylori* G27 WT sonicate (10 μg/ml) and *H.pylori* G27 ΔCag A sonicate (10 μg/ml) or left untreated (medium). Cells were harvested at 24h and RNA isolations were performed. Isolated RNAs were converted to cDNA and IRF7, 18S rRNA expression levels were determined by qRT-PCR. IRF7 mRNA expression levels were normalised to 18S rRNA expression levels. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of three independent experiments (n=3), n.s.: not significant.

According to our results, IRF7 mRNA expression level was significantly 2.5-fold increased in *H.pylori* G27 WT sonicate- treated cells compared to untreated cells. It was observed that IRF7 expression level was not significantly altered in *H.pylori* CagA sonicate- treated cells compared to *H.pylori* G27 WT sonicate- treated cells. Figure 3.11 indicates that there is no significant effect of *H.pylori* virulence factor Cag A on IRF7 expression in KATO-III cells. In further experiments, we focus on IFN-β expression level in *H.pylori* sonicates- treated cells.

3.7 Role of *H.pylori* sonicate on IFN-β expressions in KATO-III cells

3.7.1 Investigation of IFN-β expression from *H.pylori* G27 WT sonicate treated KATO-III gastric epithelial cells in a time-dependent manner

IRF7 induces the expression of IFN-β, which is a member of type I IFNs. Previous studies have shown that IFN-β induces BAFF expression in monocytes, macrophages, dendritic cells, salivary gland epithelial cells and airway epithelial cells [66,75]. In order to determine IFN-β expression in a time-dependent manner, IFN-β expression from *H.pylori*- treated gastric epithelial KATO-III cells were examined by using qRT-PCR (Figure 3.12).

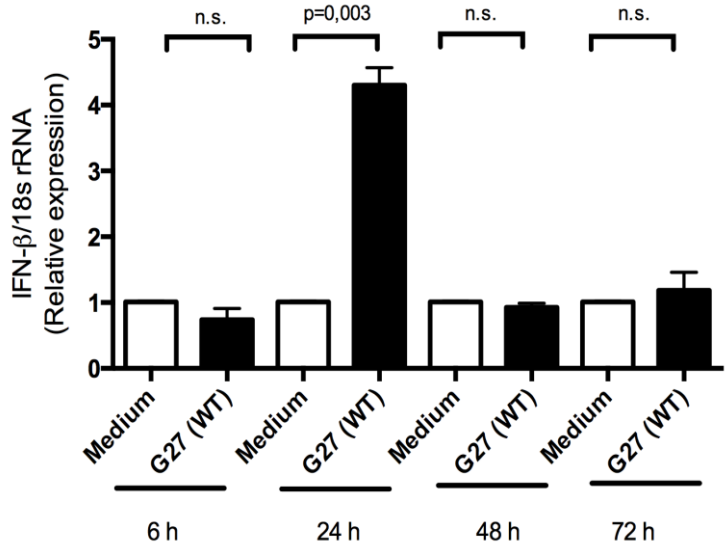


Figure 3.12 : *H.pylori G27 WT sonicate induces IFN-β expression in KATO-III cells at 24 h.* KATO-III cells (5x 10^5 cells/well) were induced with *H.pylori* G27 WT sonicate (10 μg/ml) or left unstimulated (medium). Then, KATO-III cells were harvested as indicated time-points (6 h, 24 h, 48 h and 72 h) and RNA isolations were performed. Following to RNA isolations, they were converted to cDNA and analysed for IFN-β and 18S rRNA expressions using qRT-PCR. 18S rRNA was used as endogenous control. *Graphpad Prism* 5.0 was used to graph expression levels of IFN-β. p values were determined by Student's *t*-test. Figure 3.12 represents three independent experiments (n=3), n.s: not significant.

According to IFN-β qRT-PCR results, IFN-β beta expression was significantly 4-fold increased in KATO-III cells when they were treated with *H.pylori* G27 WT sonicate compared to untreated controls at 24 h time-point. Correlating with significant increase of NOD1 (Figure 3.8) and IRF7 expression levels (Figure 3.10)

by stimulation with *H.pylori* G27 WT sonicate for 24 h, IFN-β mRNA expression was also found to be significantly elevated at 24 h upon treatment with *H.pylori* G27 sonicate. Overall, these results indicate that *H.pylori* G27 WT sonicate may induce IFN-β expression through NOD1-IRF7 signalling.

3.7.2 Effect of *H.pylori* virulence factor Cag A on IFN- β expression

Figure 3.12 indicates the increase of IFN- β expression when KATO-III cells were treated with *H.pylori* G27 WT sonicate at 24 h. Then we aimed to understand the role of *H.pylori* virulence factor of Cag A on IFN-β expression in KATO-III cells. For this purpose, KATO-III cells were investigated for IFN-β expression by qRT-PCR (Figure 3.13)

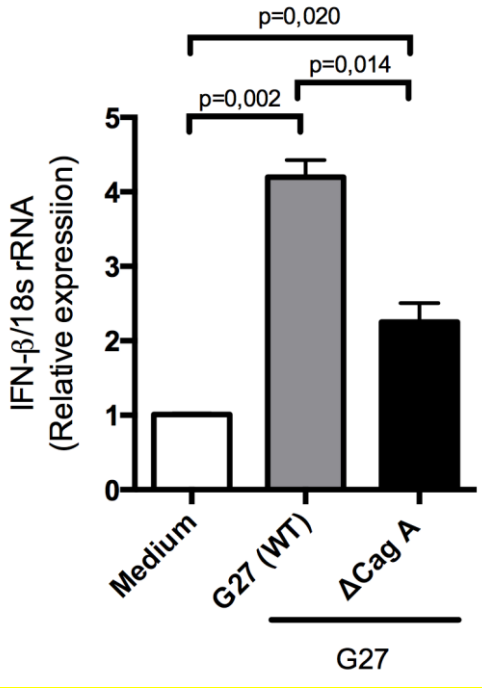


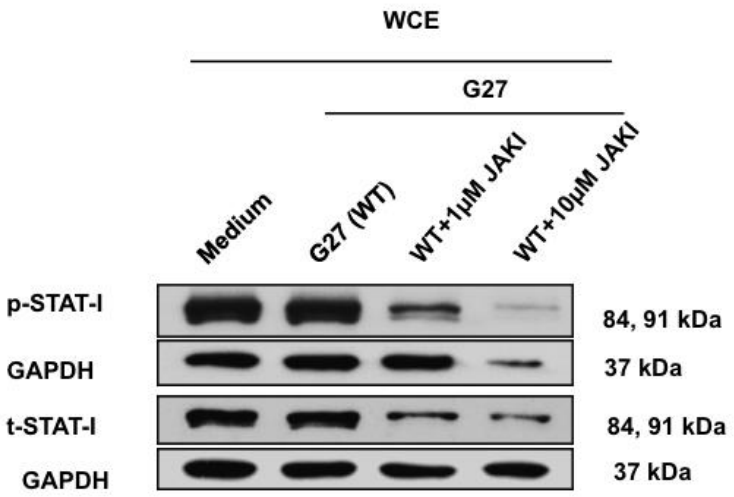
Figure 3.13: *IFN- β expression level is significantly decreased in H.pylori G27 ΔCag A sonicate treated KATO-III cells at 24h.* KATO-III cells (5x10 ^{5} cells/well) were induced for 24 h with *H.pylori* G27 WT (10 μg/ml) sonicate and *H.pylori* G27 ΔCag A sonicate (10 μg/ml) or left untreated (medium). Cells were harvested at 24 h and RNA isolations were performed. Isolated RNAs were converted to cDNA and IFN-β, 18S rRNA expression levels were determined by qRT-PCR. IFN- β were normalized to 18 S rRNA expression levels. *Graphpad Prism* 5.0 was used to graph expression levels of IFN- β. p values were determined by Student's *t*-test. Data are representative of three independent experiments (n=3), n.s: not significant.

According to IFN- β qRT-PCR results, IFN-β mRNA expression level was significantly 4-fold increased in *H.pylori* G27 WT sonicate- treated cells compared to untreated cells. It was also observed that IFN- β expression level was also significantly induced (2-fold) in *H.pylori* G27 ΔCag A sonicate- treated cells but not as much as *H.pylori* G27 WT sonicate- treated cells. This results show that Cag A has a role on IFN-β expression.

3.8 Investigation of JAK/STAT signalling pathway in *H.pylori* G27 WT sonicate treated KATO-III cells

3.8.1 Effect of *H.pylori* G27 WT sonicate on STAT1 Phosphorylation

Previous studies have shown that IFN- β expression induces high expression of BAFF in airway epithelial cells and salivary gland cells in an autocrine/paracrine pathway. It was also reported that BAFF expression and secretion was found to be dependent on JAK/STAT signalling pathway in these epithelial cells [66,75]. In our study, it was aimed to investigate JAK/STAT signalling pathway on BAFF expression and production from *H.pylori* G27 WT sonicate- stimulated gastric epithelial cell line, KATO-III. In order to assess the STAT1 phosphorylation, we used specific JAK inhibitor I (JAKI) which inhibits STAT1 activation (Figure 3.14).



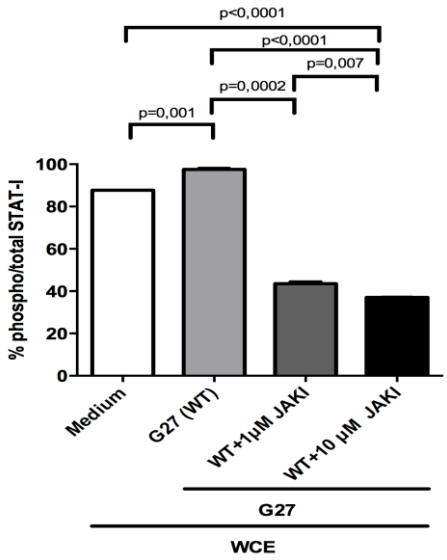


Figure 3.14 : *Treatment with H.pylori G27 WT sonicate induces STAT1 phosphorylation in KATO-III cells.* KATO-III cells were either pre-incubated with 1 or 10 μM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) without pre-treatment with JAKI or left untreated (medium). Protein isolations were performed and lysate samples were loaded on gel as 20 μg and immunoblotted for anti-phophoSTAT-1antibody. Phosphorylation levels of STAT1 were determined by using Western Blotting. Phospho- STAT1 bands were normalized to total STAT1 protein levels. *Graphpad Prism* 5.0 was used to graph expression levels of IFN- β. p values were determined by Student's *t*-test. Data are representative of two independent experiments (n=2), n.s: not significant.

Figure 3.14 shows that STAT1 phosphorylation was increased from 80% to 100% in *H.pylori* G27 WT sonicate- treated KATO-III cells compared to untreated control. Phosphorylation of STAT1 was reduced in 1 μM JAKI and 10 μM JAKI pre- treated and *H.pylori* G27 WT sonicate- stimulated KATO-III cells compared to *H.pylori* G27 WT sonicate- treated cells (42%, 38% respectively). These results demonstrate the efficiency of JAKI on blocking phosphorylation of STAT1.

3.8.2. Effect of JAKI on BAFF expression

It is known that JAK/STAT signalling pathway plays a role in IFN- β secretion via IFNARs. BAFF is produced by JAK/STAT signalling pathway in airway epithelial cells [66]. In order to investigate the role of JAK/STAT signalling pathway on BAFF expression, BAFF expression levels of *H.pylori* G27 WT sonicate- stimulated

KATO-III cells (either pre-treated with JAKI or not) were determined by qRT-PCR (Figure 3.15).

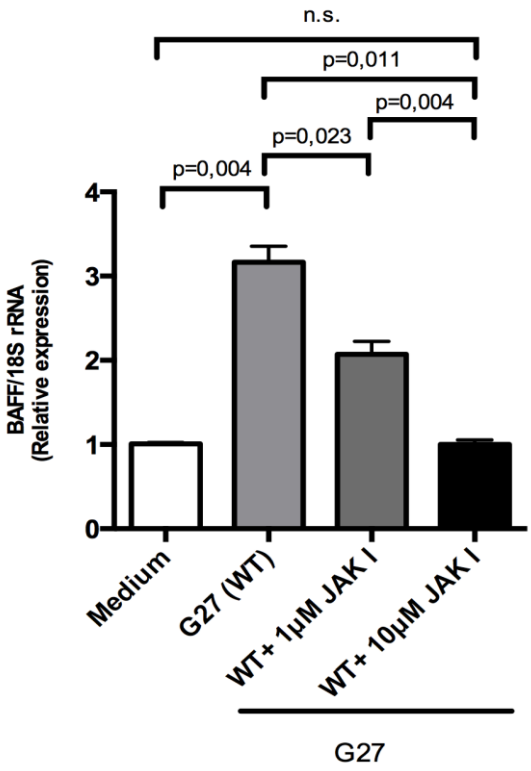


Figure 3.15 : *Treatment with JAKI inhibits BAFF expression in KATO-III cells.* KATO-III cells (5x 105 cells/well) were either pre-incubated with 1 or 10 µM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 µg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 µg/ml) without pre-treatment with JAKI or left untreated (medium). Isolated RNAs were converted to cDNA and BAFF, 18S rRNA expression levels were determined by qRT-PCR. 18S rRNA was used as endogenous control. BAFF mRNA expression levels were normalised to 18S rRNA. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of three independent experiments (n=3), n.s.: not significant.

Figure 3.15 shows that BAFF expression was dependent on JAK/STAT signalling pathway in *H.pylori* G27 WT sonicate- stimulated gastric epithelial cell line KATO-III. JAK1 inhibits STAT1 phosphorylation and BAFF expression levels were decreased in the presence of JAKI. BAFF expression level of *H.pylori* G27 WT sonicate- treated cells was significantly increased compared to untreated control (3-

fold). BAFF expression from *H.pylori* G27 WT sonicate- treated KATO-III cells with 1μM pre-treated JAKI was significantly decreased compared to *H.pylori* G27 WT sonicate- treated cells. When KATO-III cells were pre-incubated with 10 μM JAKI, BAFF expression level was significantly decreased compared to 1μM pre-treated JAKI with *H.pylori* G27 WT sonicate- treated cells and *H.pylori* G27 WT sonicate- treated cells. BAFF mRNA expression level of 10 μM JAKI pre-treated and *H.pylori* G27 WT sonicate- stimulated KATO-III cells was similar to untreated control cells.

3.8.3. Effect of JAKI on BAFF production

In order to investigate the importance of JAK/STAT signalling pathway on *H.pylori* G27 WT sonicate- stimulated KATO-III cells, BAFF production from these cells were determined using Western Blotting (Figure 3.16).

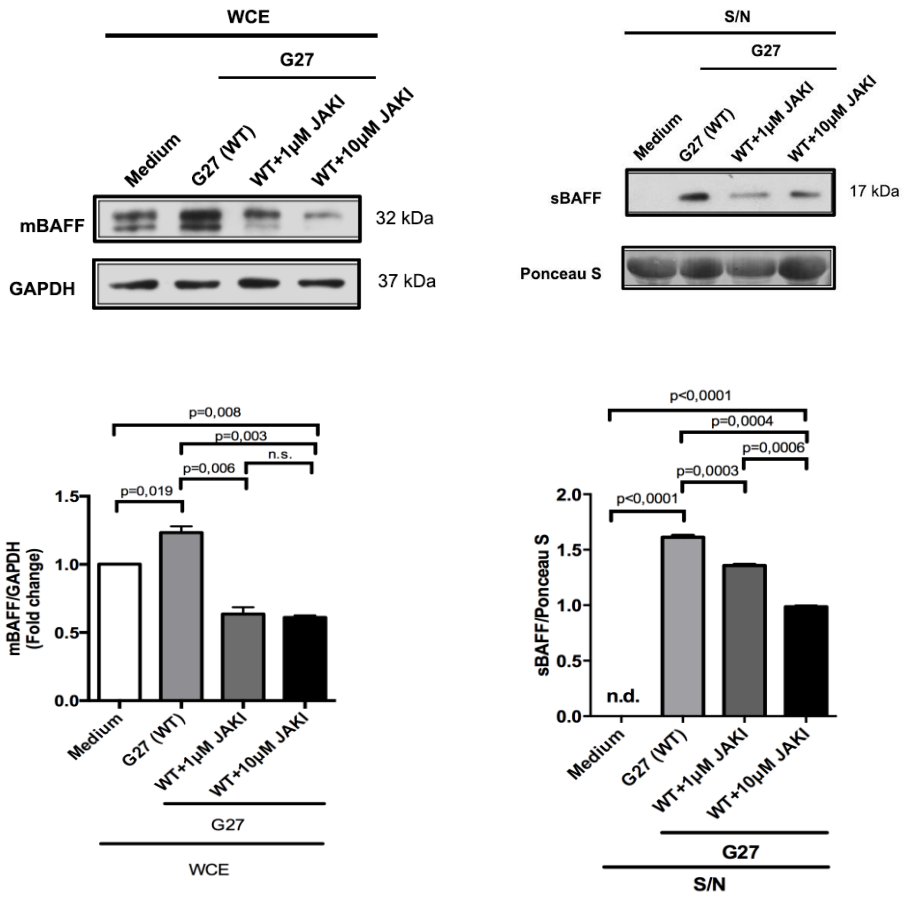


Figure 3.16 : *mBAFF and sBAFF protein levels were decreased in the presence of JAKI.*

Figure 3.16 (cont'd): KATO-III cells (5x 105 cells/well) were either pre-incubated with 1 or 10 μM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) without pre-treatment with JAKI or left untreated (medium). KATO-III cells were harvested for protein isolations. Culture supernatants and lysate samples concentrations were measured by BCA assay kit. Samples were loaded on gel as 20 μg. mBAFF and sBAFF levels were analysed by using Western Blotting. Densitometric analysis was performed by using GAPDH as endogenous controls for mBAFF. Ponceau S was used for normalizing sBAFF protein levels. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of two independent experiments (n=2), n.s: not significant.

According to membrane-bound BAFF (mBAFF) Western Blotting results, BAFF production was significantly 1.3-fold increased in *H.pylori* G27 WT sonicate- treated cells compared to untreated KATO-III cells. Both pre-treatment with 1μM JAKI or 10 μM JAKI significantly inhibited mBAFF production level compared to *H.pylori* G27 WT sonicate- treated cells. Soluble BAFF (sBAFF) Western Blotting results also showed that sBAFF was significantly 1.6-fold increased in *H.pylori* G27 sonicate- treated cells compared to untreated control. In a dose-dependent manner of pre-treatment of JAKI, sBAFF protein levels were significantly inhibited compared to *H.pylori* G27 WT sonicate- treated cells. These results suggest that JAK/STAT signalling pathway is important in BAFF expression and production from *H.pylori* G27 WT sonicate- treated KATO-III cells.

3.8.4 Investigation of JAKI on BAFF secretion

Figure 3.15 and Figure 3.16 showed that JAKI has an inhibitory effect on BAFF expression and production. In order to verify the inhibition of JAKI on BAFF secretion, BAFF ELISA was performed in JAKI pre-treated and then stimulated with *H.pylori* G27 WT sonicate- induced KATO-III cells (Figure 3.17).

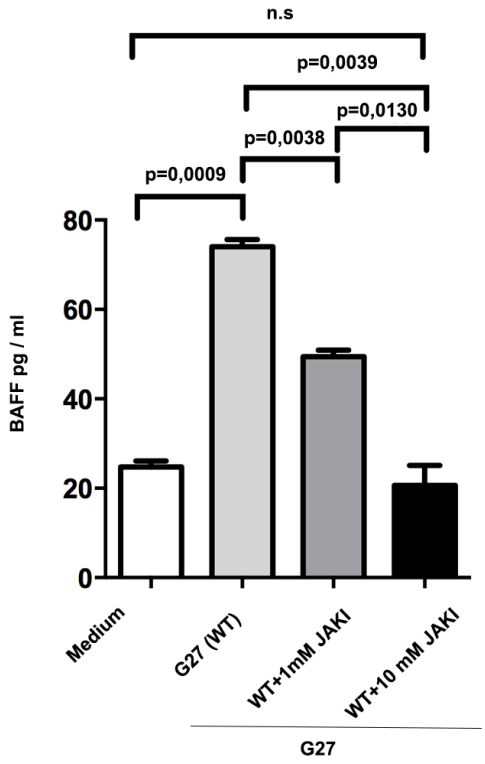


Figure 3.17 : *BAFF secretion levels were decreased in JAKI treated KATO-III cells.* KATO-III cells (5x 105 cells/well) were either pre-incubated with 1 or 10 μM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) without pre-treatment with JAKI or left untreated (medium). Secreted BAFF levels were detected by ELISA in culture supernatants of KATO-III cells. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test.

BAFF ELISA results showed the similar pattern with BAFF expression and BAFF production results in the presence of JAKI. According to BAFF ELISA results, BAFF secretion was significantly 3.5-fold increased in *H.pylori* G27 WT sonicate-treated KATO-III cells when compared to untreated control. Dose –dependent JAKI treatment were found to result in significant inhibition of secreted BAFF levels when compared to *H.pylori* G27 WT sonicate- treated cells. Overall, our results show that JAKI inhibits the BAFF secretion from gastric epithelial cell line KATO-III at 24 h time-point. In addition to that, BAFF secretion is dependent on JAK/STAT signalling pathway in gastric epithelial cells like airway epithelial cells.

3.8.5. Effect of JAKI on APRIL expression

Inhibition effect of JAKI on BAFF expression was shown in figure 3.15. JAK1 is an essential factor for BAFF expression, it was continued by investigating APRIL signalling pathway in *H.pylori* G27 WT sonicate- stimulated KATO-III cells. For

this purpose, APRIL mRNA expression levels were determined by qRT-PCR in JAKI pre-treated and *H.pylori* G27 WT sonicate- induced KATO-III cells (Figure 3.18).

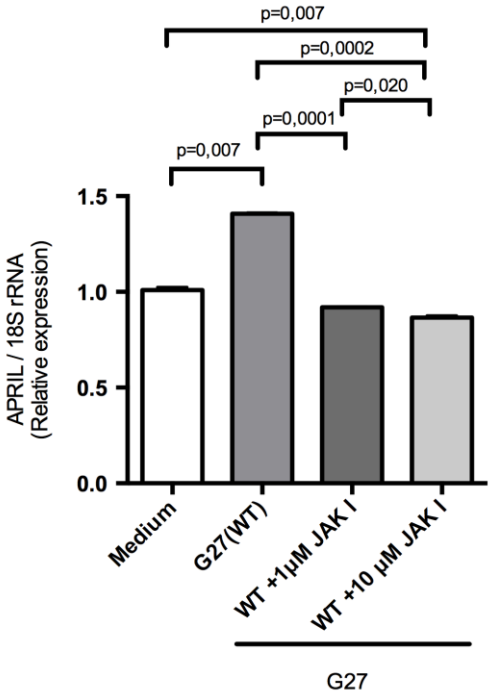


Figure 3.18 ***:*** *Treatment with JAKI inhibits APRIL expression in KATO-III cells.* KATO-III cells (5x 10^5 cells/well) were either pre-incubated with 1 or 10 μM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) without pre-treatment with JAKI or left untreated (medium). Cells were harvested after 24 and RNA isolations were performed. Isolated RNAs were converted to cDNA. APRIL and 18S rRNA expression levels were determined by qRT-PCR. APRIL mRNA expression levels were normalized to 18 S rRNA expression levels. *Graphpad Prism* 5.0 was used to prepare the graphs representing the expression levels of APRIL. p values were determined by Student's *t*-test. Data are representative of three independent experiments (n=3).

Our data suggests that JAK/STAT signalling pathway induces APRIL expression from *H.pylori* G27 WT sonicate- treated KATO-III cells at 24 h time-point. APRIL expression level was significantly 1.3-fold increased in *H.pylori* G27 WT sonicate-treated KATO-III cells compared to untreated cells. It was observed that JAKI inhibits APRIL expression from KATO-III cells. This result suggests that APRIL is

also expressed through by JAK/STAT signalling pathway in gastric epithelial cell line KATO-III when treated with *H.pylori* G27 WT sonicate.

3.8.6. Effect of JAKI on APRIL production

Following APRIL expression analysis in JAKI studies, in order to understand the effect of JAKI on APRIL production, APRIL protein levels were investigated in JAKI pre- incubated and *H.pylori* G27 WT sonicate-stimulated KATO-III cells at 24 h time-point (Figure 3.19)

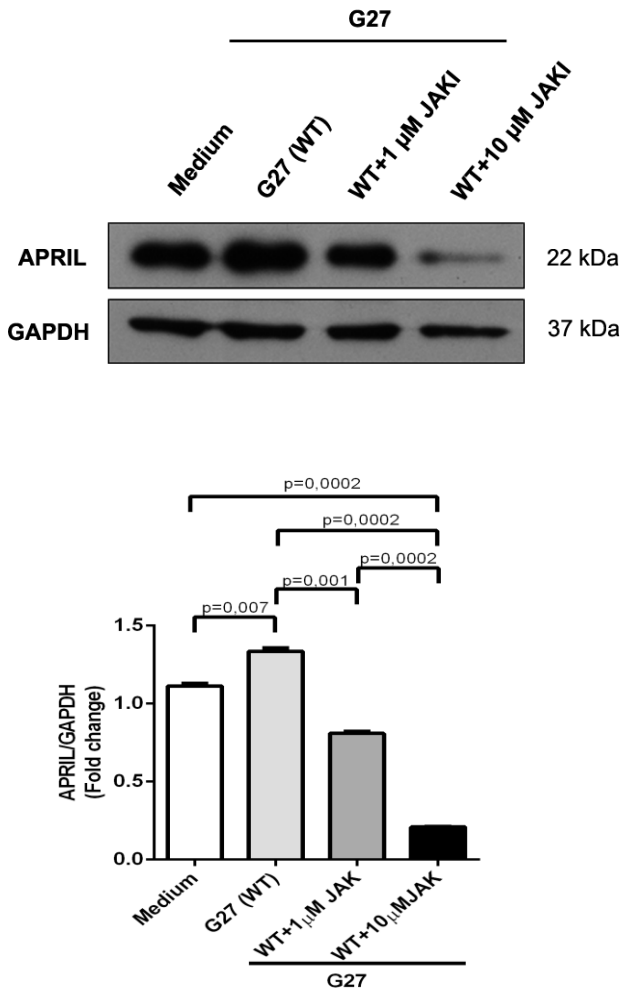


Figure 3.19 : *APRIL protein levels were decreased in JAKI treated KATO-III cells.* KATO-III cells (5x 10^5 cells/well) were either pre-incubated with 1 or 10 μM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) without pre-treatment with JAKI or left untreated (medium). KATO-III cells were harvested for protein isolations. Densitometric analysis was performed by using GAPDH as endogenous

controls for APRIL protein. *Graphpad Prism* 5.0 was used to prepare the graphs representing the production levels of APRIL. Data are representative of two independent experiments (n=2), n.s.: not significant.

According to APRIL Western Blotting results, it was observed that JAKI have an also inhibitory effect on APRIL production in 24 h time-point. APRIL production was significantly 1.3-fold increased in *H.pylori* G27 WT sonicate- treated cells compared to untreated cells. JAKI treatment in dose-dependent manner (1-10 μM JAKI) showed a significant decrease in APRIL production from KATO-III cells. APRIL production level in KATO-III cells pre-treated with 10 μM JAKI and stimulated with *H.pylori* G27 WT sonicate was decreased compared to untreated cells. These results suggest that *H.pylori* G27 WT sonicate- treated KATO-III cells produce APRIL via JAK/STAT signalling pathway.

3.8.7. Effect of JAKI on NOD1 expression

Inhibitory effect of JAKI on NOD1 has not been investigated yet. Figure 3.8 shows that NOD1 expression is significantly 2-fold increased in *H.pylori* G27 WT sonicate-treated KATO-III cells compared to untreated cells. In order to assess the effect of JAKI on NOD1 expression, KATO-III cells were investigated for NOD1 expression by qRT -PCR (Figure 3.20).

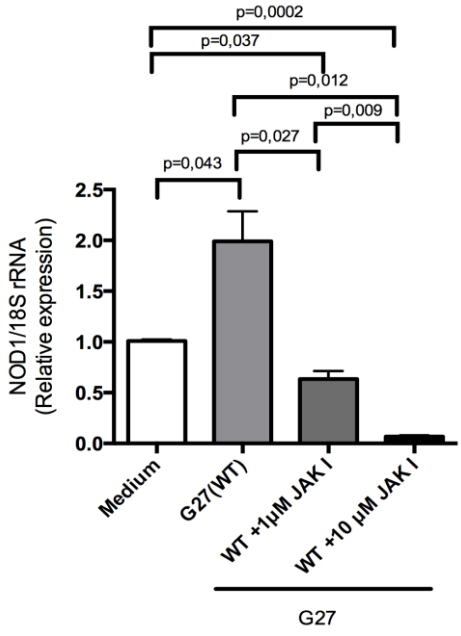


Figure 3.20 : *JAKI inhibits NOD1 expression in KATO-III cells.*

Figure 3.20 (cont'd): KATO-III cells ($5x10^5$ cells/well) were either pre-incubated with 1 or 10 μM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) without pre-treatment with JAKI or left untreated (medium). Isolated RNAs were converted to cDNA. NOD1 and 18S rRNA expression levels were determined by qRT-PCR. 18S rRNA was used as endogenous control. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of two independent experiments (n=2).

According to qRT-PCR results of NOD1 expression, *H.pylori* G27 WT sonicate-treated cells express significantly 2-fold increased levels of NOD1 compared to untreated cells. It was observed that the pre-treatment with JAKI in dose-dependent manner inhibits NOD1 expression in *H.pylori* G27 WT sonicate-stimulated KATO-III cells. This study needs further experiments to understand the JAKI effect on NOD1 expression.

3.8.8. Effect of JAKI on the expression of IRF7

IRF7 translocation into nucleus is known to be important for IFN-β expression in epithelial cells. IFN-β can bind to its receptor on the cell surface and induce Th1 cytokines and its own production through JAK/STAT signalling pathway and also induces BAFF expression in epithelial cells [66,75] (Figure 1.9). In order to understand the effect of JAKI on IRF7 expression, KATO-III cells were investigated for expression of IRF7 in RNA level (Figure 3.21)

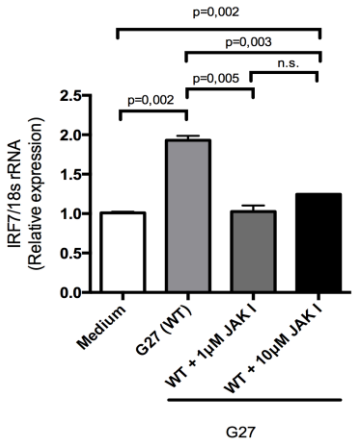


Figure 3.21 : *IRF7 expression is decreased in JAKI treated KATO-III cells.*

Figure 3.21 (cont'd): KATO-III cells (5x 10^5 cells/well) were either pre-incubated with 1 or 10 μM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) without pre-treatment with JAKI or left untreated (medium). Cells were harvested and RNA isolations were performed. Isolated RNAs were converted to cDNA. IRF7 and 18S rRNA expression levels were determined by qRT-PCR. RF7 mRNA expression levels were normalised to 18S rRNA expression levels. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of two independent experiments (n=2).

Figure 3.21 indicates that IRF7 expression level was significantly 2-fold increased in *H.pylori* G27 WT sonicate- treated KATO-III cells compared to untreated cells. IRF7 expressions from JAKI pre-treated KATO-III cells were significantly inhibited compared to *H.pylori* G27 WT sonicate- treated KATO-III cells at 24 h time-point. This result suggests that JAKI significantly inhibits IRF7 expression in KATO-III cells.

3.8.9. Role of JAKI on IFN-β expression

Published studies showed that IFN-β beta secretion is involved in JAK/STAT signalling pathway [66,75]. It was also reported that IFN-β induces high levels of BAFF expression in epithelial cells. In order to assess the effect of JAKI on IFN-β expression, KATO-III cells were investigated for IFN-β expression by qRT-PCR (Figure 3.22).

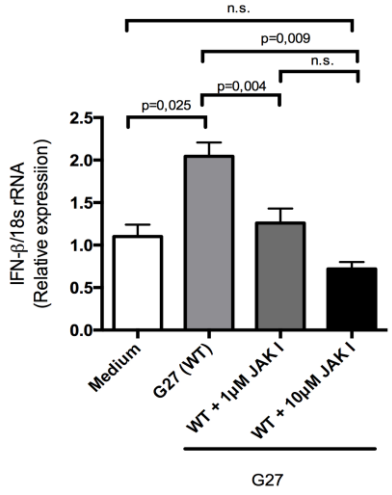


Figure 3.22 : *IFN-β expression is decreased in JAKI treated KATO-III cells.*

Figure 3.22 (cont'd): KATO-III cells (5x 10^5 cells/well) were either pre-incubated with 1 or 10 μM JAKI for 1 h and then stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) for 24 h or stimulated with *H.pylori* G27 WT sonicate (10 μg/ml) without pre-treatment with JAKI or left untreated (medium). Cells were harvested and RNA isolations were performed. Isolated RNAs were converted to cDNA. IFN-β and 18S rRNA expression levels were determined by qRT-PCR. IFN-β expression levels were normalized to 18S rRNA expression levels. Results are graphed using *Graphpad Prism* 5.0. Statistical analysis was conducted using Student's *t*-test. Data are representative of two independent experiments (n=2).

According to qRT-PCR results of IFN-β expressions, it was observed that IFN-β expression was significantly 2-fold increased in *H.pylori* G27 WT sonicate- treated KATO-III cells compared to untreated control. The increase in IFN-β mRNA expression levels in *H.pylori* G27 WT sonicate- treated cells were significantly inhibited by both 1 μM JAKI or 10 μM JAKI treatment. It is known from the published studies that IFN-beta is expressed by JAK/STAT signalling pathway in epithelial cells. This study also showed that JAK/STAT signalling pathway has a role in IFN-β expression. Figure 3.22 shows the effect of JAKI on IFN-β expression in RNA level.

4.DISCUSSION AND CONCLUSION

TNF family members, BAFF and APRIL are the regulator and activator cytokines for B cells. BAFF is expressed by monocytes, macrophages, activated-T cells, dendritic cells, epithelial cells and cancer cells. APRIL is also produced by immune cells such as macrophages, monocytes, dendritic cells, T cells and also non-immune cells such as epithelial cells [36,37,43]. APRIL has also a role in stimulation of tumour growth. Elevated levels of BAFF and APRIL have been found in several autoimmune diseases such as rheumatoid arthritis (RA), Sjögren's syndrome (SS), experimental autoimmune encephalomyelitis (EAE), systemic lupus erythematosus (SLE) and autoimmune thyroid disease [37,38,39,40].

Different forms of BAFF have been characterized. First of all, BAFF is synthesized as membrane–bound form and then cleaved from the cell surface by furin convertase. Therefore, it can both exist as a membrane-bound (32 kDa) and soluble form (17kDa). This TNF family cytokine can form a homodimer, heterodimer, 20 –mer and 60-mer forms to bind its receptors on the cell surface [36,41]. BAFF expression and secretion from *Helicobacter pylori*–infected gastric epithelial cell line was shown in this study. In order to understand the importance of Cag A virulence factor on BAFF expression, KATO-III cells were stimulated with *H.pylori* G27 (WT) and its isogenic Cag A mutant (ΔCag A) at 24 h time-point. Quantitative Realtime- PCR results indicated that BAFF was significantly increased in *Helicobacter pylori* G27 (WT) when compared to untreated control cells, whereas BAFF expression was found to be significantly inhibited in *H.pylori* G27 ΔCagA-treated KATO-III cells compared to *H.pylori* G27 WT- treated cells (Figure 3.2). In order to investigate BAFF production from *H.pylori*- treated KATO-III cells, Western Blotting and ELISA experiments were performed. Both membrane-bound and soluble forms of BAFF were shown in Western Blotting experiments (Figure 3.3). These results correlated with the results of BAFF mRNA expression levels. Both membrane-bound and soluble forms of BAFF were significantly increased in *H.pylori* G27- treated KATO-III cells compared to *H.pylori* G27 ΔCagA- treated KATO-III cells and

untreated controls in protein levels. The role of Cag A, which is one of the most important virulence factors of *H.pylori*, on BAFF expression and secretion from gastric epithelial cell line was shown in this study. APRIL, a homologue of BAFF, was significantly increased in both RNA and protein level in *H.pylori* G27 WT sonicate- treated KATO-III cells compared to untreated control cells. Like in the case of BAFF, lack of Cag A leads to decrease of APRIL both in RNA and protein levels (Figure 3.7). These results suggest that expression and secretion of BAFF and APRIL from gastric epithelial cells depend on the presence of *H.pylori* virulence factor CagA.

NOD1 is a NLR family member expressed by antigen-presenting cells and gastric epithelial cells. It has been reported that NOD1 plays an important role for innate immune response in gastric epithelial cells against *Helicobacter pylori* [62]. NOD1 is an intracellular key receptor for recognition of PAMPs and DAMPs. NOD1 can be activated by the peptides derived from peptidoglycan (PGN), which is present in most of the gram-negative bacteria. *H.pylori* can deliver its PGN into host cell by using Type-IV secretion system or outer membrane vesicles and induce NOD1 activation [65]. Upon ligand binding, NOD1 becomes activated and induce activation of down-stream molecule RICK. Activated RICK can both interact with TAK1 or TRAF3. Activation of TAK1 induces NF-κB pathway. Interaction between RICK and TRAF3 activates TBK1 and induces IRF7, a transcription factor for type I interferons, expression that leads to production of IFN-β. Secreted IFN-β binds to its IFNARs on the cell membrane and activates JAK/STAT signalling pathway and induce secretion of Th1 cytokines and IFN-β (Figure 1.9).

Recently it was reported that IFN-β induce BAFF production from airway epithelial cells and salivary gland cells [66,75]. In order to investigate the role of NOD1 signalling pathway in BAFF expression and BAFF production from *H.pylori*- treated KATO-III cells, NOD1, downstream IRF7 and IFN-β expression levels were examined by qRT-PCR in this study. qRT-PCR results demonstrated that NOD1 expression was increased in *H.pylori* G27 WT sonicate- treated KATO-III cells (Figure 3.9). Moreover, *H.pylori* G27 WT sonicate- induced IRF7 expression in RNA level (Figure 3.11). In parallel with IRF7 expression levels, IFN-β mRNA expression levels were also increased in *H.pylori* G27 WT sonicate- treated cells

compared to untreated control cells (Figure 3.13). Therefore, involvement of IFN-β in BAFF and APRIL production from gastric epithelial cells may be speculated.

The effect of JAK inhibitor (JAKI) on BAFF production in airway epithelial cells has been recently demonstrated [66]. In this study, we have shown that STAT1 phosphorylation levels were significantly inhibited in the presence of increasing doses of JAKI (Figure 3.14). BAFF expression and protein levels were also significantly inhibited by JAKI in a dose –dependent manner in KATO-III cells. BAFF expression was highly increased in *H.pylori* G27 WT sonicate- treated KATO-III cells whereas pre-treatment with JAKI significant inhibited this increase in BAFF expression and production. Western Blotting and qRT-PCR results demonstrated that APRIL was significantly inhibited in both RNA and protein level in the presence of JAKI (Figures 3.18 and 3.19). These results suggest that production of BAFF and APRIL is mediated by JAK/STAT signalling pathway in *H.pylori* G27 WT sonicate- treated KATO-III cells.

In the light of this study, induction of BAFF expression and production in *H.pylori* G27 WT sonicate- stimulated may be mediated by IFN-β. IFN-β is induced by possibly PGNs of *H.pylori* which in turn leads to its own production of IFN-β along with BAFF and APRIL through interaction with IFNARs (Figure 4.1). Silencing of IFNARs with relevant siRNAs may provide more information regarding IFN-β dependent BAFF signalling. Intracellular signalling mechanism(s) of IFN-β mediated BAFF expression still remain unclear. In order to elucidate the induction of IFN-β in *H.pylori* sonicate stimulated KATO-III cells, *H.pylori* derived PGNs can be investigated further.

In this study, the effect of JAKI on NOD1 expression was also demonstrated for the first time. In order to elucidate the intracellular mechanisms of NOD1 signalling in BAFF expression further studies including siRNA silencing of NOD1 and determining phosphorylation levels of RICK (a downstream molecule of NOD1) may be performed.

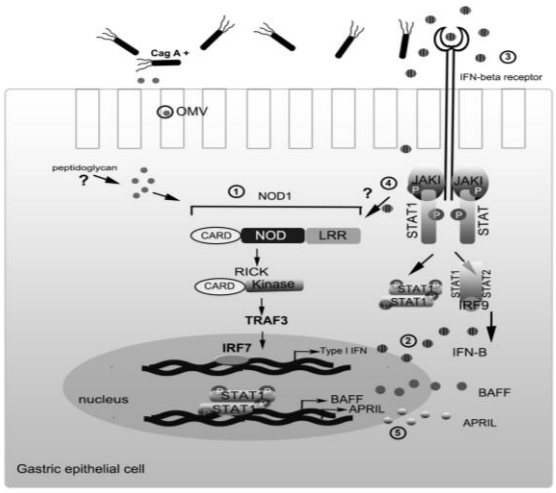


Figure 4.1 : *Proposed model of BAFF and APRIL expression in H.pylori G27 WT sonicate- treated KATO-III cells.* PGNs of *H.pylori* can induce NOD1 expression and IRF7 translocation to nucleus. IRF7 initiates IFN-β expression from KATO-III cells. Secreted IFN-β binds to its own receptor on the cell surface and initiates JAK/STAT signalling pathway. BAFF expression is possibly dependent on IFN-β mediated JAK/STAT signalling.

These studies may be also repeated in primary gastric epithelial cells and the effect of BAFF and APRIL, which were secreted from gastric epithelial cells, on B cell survival and proliferation can be investigated in further studies.

In conclusion, this study has significant contribution to literature since it shows for the first time the involvement of Cag A virulence factor on BAFF and APRIL expression and importance of JAK/STAT signalling pathway on production of these cytokines from *H.pylori* G27 WT sonicate treated gastric epithelial cell line, KATO-III.

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