ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATION OF INTERLEUKIN-10 (IL-10) PRODUCTION FROM *HELICOBACTER*-ACTIVATED IL-10^{+} B CELLS ON MOLECULAR LEVEL

M.Sc. THESIS

Emre SOFYALI

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

HELİKOBAKTER-AKTİVE İNTERLÖKİN-10^{+} (IL-10^{+}) B HÜCRELERİNDEN IL-10 ÜRETİMİNİN MOLEKÜLER DÜZEYDE İNCELENMESİ

YÜKSEK LİSANS TEZİ

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Ağustos 2014

Emre SOFYALI, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID 521111104, successfully defended the **thesis** entitled **"INVESTIGATION OF INTERLEUKIN-10 (IL-10) PRODUCTION FROM *HELICOBACTER*-ACTIVATED IL-10^{+} B CELLS ON MOLECULAR LEVEL"**, which he 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,

FOREWORD

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August 2014

Emre SOFYALI
(Molecular Biologist & Geneticist)

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ABBREVIATIONS

µg : Microgram
µm : Micrometer
µM : Micromolar
AMP : Adenosine monophosphate
AP-1 : Activator protein-1
APC : Antigen presenting cell
APS : Ammonium persulfate
ATF : Activating transcription factor
BAFF : B-cell activating factor
BCA : Bicinchoninic Acid
Breg : Regulatory B cell
BSA : Bovine Serum Albumin
CagA : Cytotoxin-associated gene A
CD : Cluster of differentiation
CD40L : CD40 ligand
C/EBPα : CCAAT/enhancer binding protein α
c-FOS : Cellular Finkel-Biskis-Jinkins osteosarcoma
CIA : Collagen-induced arthritis
cm^2 : Centimeter square
CpG ODN : CpG oligodeoxynucleotides
CREB : cAMP response element-binding protein
CRF2 : Class II cytokine receptor family
CSIF : Cytokine synthesis inhibiting factor
DC : Dendritic cell
DC-SIGN : DC-specific ICAM3-grabbing non-integrin
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
DTT : Dithiothreitol
DUSP1 : Dual specificity phosphatase 1
EAE : Experimental autoimmune encaphalomyelitis
ECL : Enhanced chemiluminescence
EDTA : Ethylenediaminetetraacetic acid
ELISA : Enzyme-linked immunosorbent assay
ELK : ETS domain-containing protein
ERK : Extracellular Signal Regulated Kinase
ETS : E-twenty six
FBS : Fetal Bovine Serum
FoxO : Forkhead transcription factor
g : Gram
GSK-3β : Glycogen synthase kinase 3 beta
GTP : Guanosine-5'-triphosphate
h : Hour

IκB : Inhibitor of kappa B
ICAM3 : Intercellular adhesion molecule 3
IFN-β : Interferon-beta
IKK : Inhibitor of nuclear factor-κB kinase
IL-1β : Interleukin-1 beta
IL-4 : Interleukin-4
IL-5 : Interleukin-5
IL-6 : Interleukin-6
IL-8 : Interleukin-8
IL-10 : Interleukin-10
IL-13 : Interleukin-13
IL-10R : IL-10 receptor
IRAK : IL-1R-associated protein
IRF : Interferon-regulatory factors
JAK : Janus kinase
JNK : JUN N-terminal kinase
kDa : Kilodalton
L : Liter
LPS : Lipopolysaccharide
LTβ : Lymphotoxin-β
MAF : Musculoaponeurotic fibrosarcoma
MALT : Mucosa-associated lymphoid tissue
MAPK : Mitogen Activating Protein Kinase
MAPKAPK : MAPK-activated protein kinases
MDM : Mouse double minute
MEF : Myocyte enhancer factor
MEK : Mitogen/extracellular signal-regulated kinase
MHC-II : Major histocompatibility complex class II
min : Minute
MIP-2α : Macrophage inflammatory protein 2-alpha
ml : Mililiter
mM : Milimolar
MNK : MAPK-interacting kinase
MSK : Mitogen- and stress-activated kinase
mTOR : Mammalian target of rapamycin
mTORC2 : mTOR-rictor complex
MyD88 : Myeloid differentiation primary response gene 88
NEMO : NF-κB essential modulator
NF-κB : Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK : NF-κB inducing kinase
NOD2 : Nucleotide-binding oligomerization domain 2
NP-40 : Nonidet-P40
PAMP : Pathogen associated molecular pattern
PBS : Phosphate buffered saline
PDK-1 : 3-phosphoinositide dependent protein kinase-1
PDTC : Pyrrolidinedithiocarbamate ammonium
pg : Picogram
pH : Power of Hydrogen
PI3K : Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP_2 : Phosphatidylinositol 4,5-bisphosphate

PIP_3 : Phosphatidylinositol (3,4,5)-trisphosphate
PKB : Protein kinase B
PMA : Phorbol 12-myristate 13-acetate
PMSF : Phenylmethylsulfonyl fluoride
PRR : Pattern recognition receptor
PTEN : Phosphatase and tensin homolog
RAF : Rapidly accelerated fibrosarcoma
RAS : Rat sarcoma
RIP1 : Receptor-interacting protein 1
RIPA : Radioimmunoprecipitationassay
rpm : Revolutions per minute
RPMI : Roswell Park Memorial Institute
RSK : Ribosomal S6 kinase
RTK : Receptor tyrosine kinase
SAPK : Stress-activated protein kinases
SDS : Sodium deodecyl sulfate
SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOCS3 : Suppressor of cytokine signaling 3
STAT : Signal transducer and activator of transcription
SYK : Spleen tyrosine kinase
TAB2 : TAK1-binding protein 2
TAB3 : TAK1-binding protein 3
TAK1 : TGF-β activated kinase 1
TANK : TRAF associated NF-κB activator
TBK1 : TANK-binding kinase 1
TBS : Tris buffered saline
TBS/T : Tris buffered saline/Tween-20
TEMED : N-N-N-'N'-Tetramethylethylenediamine
TF : Transcription Factor
Th1 : T helper 1
Th2 : T helper 2
Th17 : T helper 17
TIR : Toll-IL-1R
TLR : Toll-like receptor
TNF-α : Tumor necrosis factor-alpha
TPL2 : Tumor progression locus 2
Tr-1 : T regulatory-1
TRAF : TNF receptor-activated factors
TRAM : TRIF-related adaptor molecule
Treg : Regulatory T cell
TRIF : TIR-domain-containing adaptor protein inducing interferon-β
Ub : Ubiquitin
V : Volt
VacA : Vacuolating cytotoxin A
WHO : World Health Organization

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INVESTIGATION OF INTERLEUKIN-10 (IL-10) PRODUCTION FROM *HELICOBACTER*-ACTIVATED IL-10^{+} B CELLS ON MOLECULAR LEVEL

SUMMARY

Helicobacter pylori (*H.pylori*) is a gram-negative, spiral-shaped, microaerophilic bacterium that is defined to be a common risk factor for development of gastric malignancies that may lead to gastric cancer. Approximately 50% of the world's population is infected with *H.pylori*. The prevalence of *H.pylori* infection goes up to 80% in developing countries. Although majority of world population is infected, only 20% of infected individuals may develop peptic ulcer, MALT (mucosa-associated lymphoid tissue) lymphoma and gastric adenocarcinoma. Majority of infected individuals are asymptomatic. Anti-inflammatory cytokine interleukin-10 (IL-10) represses immune response against pathogens and attenuates damage to host which may occur due to excessive inflammation. B and T cells are known to have regulatory subtypes. Recent researches indicate that IL-10 producing B cells have suppressive and immune regulatory role in mouse models of *Helicobacter felis* infection. *Helicobacter felis (H.felis)* is closely related to *Helicobacter pylori. Helicobacter felis* is widely used in animal models since it has higher immunogenicity for mice.

The signals received through ligands binding to their cognate receptors on cell surface are relayed by various intracellular signaling routes. The signal relayed through intracellular signaling pathways elicits a cellular response proper for the stimuli. It has been recently reported that IL-10 is produced and secreted through Toll-like receptor 2 (TLR2) signaling in *Helicobacter*-activated IL-10 producing B cells. It is known that TLR-mediated IL-10 secretion is maintained through MAPK (mitogen-activated protein kinase), PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase) and NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathways in macrophages and dendritic cells, antigen presenting cells of the innate immune system. Fundamental intracellular signaling pathways that are investigated in this study are therefore MAPK, PI3K and NF-κB signal transduction pathways.

There are many proteins that that part in intracellular signal transduction. Proteins that enable phosphorylation of themselves and other proteins by enzymatic reactions are termed as kinases. Aforementioned intracellular signaling pathways require activity of many kinases. These pathways induce sequential phosphorylation of protein kinases which leads to activation of various transcription factors that will translocate into nucleus and initiate transcription of genes related to proper cellular response to the stimuli. Phosphorylation of two protein kinases of MAPK signaling pathway, p38 MAPK and p44/42 MAPK (ERK-1/2) were shown to be required for induction of IL-10 production. PI3K signaling pathway requires phosphorylation of Akt. These separate pathways induce the phosphorylation, thereby activation of

downstream signaling molecules such as CREB (cAMP response element-binding protein) which leads to IL-10 expression in macrophages. Phosphorylation of each element in intracellular signaling pathway is generally closely related with their activation status. Therefore, activation of MAPK and PI3K signaling pathways can be determined by investigating phosphorylation status of p38 MAPK, ERK-1/2, Akt and CREB. For activation check on canonical pathway of NF-κB, phosphorylation of p65 subunit can be examined.

Although intracellular signaling pathways that play a role in IL-10 production from immune cells such as macrophages, dendritic cells or T cells have been characterized, intrinsic signal transduction pathways responsible for expression and secretion of IL-10 in *Helicobacter*-activated B cells have been ill-defined. It was recently reported that *Helicobacter*-activated IL-10^+ B cells have important contribution to prevention of *Helicobacter*-associated gastric pathology. Identification of signaling pathways that take part in induction of IL-10 production from *Helicobacter*-activated IL-10^+ B cells will pave the way for immune therapeutic researches that target these cells. For this reason, the focus of this study was identification of intracellular signaling pathways responsible for induction of IL-10 production from TLR2-stimulated B cells. For that purpose, splenic naïve B cells of C57BL/6 mice were stimulated with *H.felis* sonicate in order to induce IL-10 production and secretion. Then, *H.felis* sonicate-stimulated B cells were separated magnetically according to their IL-10 production capacities. Magnetic separation resulted in two fractions: *Helicobacter*-activated IL-10^- B cells and IL-10^+ B cells.

Protein samples of murine naïve B cells, *Helicobacter felis* sonicate-stimulated B cells, *Helicobacter*-activated IL-10^- B cells and IL-10^+ B cells were investigated for phosphorylation status of specific kinases which constitute significant junctions in MAPK, PI3K and NF-κB signaling pathways. Phosphorylation levels were determined via Western Blotting by making use of specific antibodies that recognize total- and phosphorylated forms of related signaling molecules. For investigation of NF-κB pathway, phosphorylation of p65 subunit was determined via signaling node sandwich ELISA method.

Following determination of phosphorylation status, the changes in IL-10 secretion from *Helicobacter*-activated B cells upon blocking of examined signaling pathways with specific inhibitors that inhibit activation of related kinases was investigated in order to identify the role of blocked signaling pathway on IL-10 production and secretion. For this purpose, p38 MAPK and ERK-1/2 modules of MAPK signaling pathway, PI3K signaling pathway and canonical pathway of NF-κB were blocked in murine naïve B cells and *H.felis* sonicate-stimulated B cells by chemical inhibitors. Then, the amount of secreted IL-10 culture supernatants was determined quantitatively by IL-10 ELISA.

The results of this study regarding identification of intracellular pathways taking part in induction of IL-10 production and secretion from *Helicobacter*-activated IL-10^+ B cells revealed that ERK-1/2 got significantly more phosphorylated in *Helicobacter*-activated IL-10^+ B cells following a comparison between *Helicobacter*-activated IL-10^- B and IL-10^+ B cells. On the other hand, phosphorylation levels of both p38 MAPK and Akt were found to be significantly elevated in *Helicobacter*-activated IL-10^- B cells. No significant change in phosphorylation status of p65 subunit of NF-κB canonical signaling pathway was determined. These findings suggest a more prominent role for ERK-1/2 activation in induction of IL-10 production from

Helicobacter-activated B cells. Moreover, investigation regarding the effect of blocked signaling pathways on IL-10 secretion showed that only inhibition of signal transduction through ERK-1/2 module of MAPK signaling pathway resulted in significant reduction of secreted IL-10 from *Helicobacter*-activated B cells. This finding supports the hypothesis on the prominent role of ERK-1/2 signaling in IL-10 production from *Helicobacter*-activated B cells. Significance of this research underlies in its contribution to the understanding of unexplored intrinsic signal transduction pathways of TLR2-mediated IL-10 production from B cells and the involvement of ERK-1/2 module of MAPK pathway in induction of IL-10 production from *Helicobacter*-activated B cells.

HELİKOBAKTER-AKTİVE İNTERLÖKİN-10^{+} (IL-10^{+}) B HÜCRELERİNDEN IL-10 ÜRETİMİNİN MOLEKÜLER DÜZEYDE İNCELENMESİ

ÖZET

Helikobakter pilori (H.pilori) gram-negatif, spiral yapıda, mikroaerofilik bir bakteri olmakla beraber gastritten mide kanserine kadar uazanan gastrik patolojilerin temel risk faktörü olarak tanımlanmıştır. Dünya nüfusunun yaklaşık %50'si *H.pilori* ile enefektedir. Bu oran gelişmekte olan ülkelerde %80'e çıkabilmektedir. Ancak dünya genelinde enfekte olan kişi sayısı oldukça fazla olmasına karşın, enfekte bireylerin yalnızca % 20'sinde ise peptik ülser, MALT lenfoma ve mide kanseri gibi patolojiler görülmektedir. Enfekte bireylerin büyük bir kısmı herhangi bir semptom göstermemektedir. İnterlökin-10 (IL-10) patojenlere karşı oluşan immün cevabı baskılaması ve dolayısıyla patojene karşı oluşacak immün cevap esnasında üretilebilecek pro-inflamatuvar sitokinlerin aşırı inflamasyon nedeniyle konağa zarar vermesini engellemesi ile anti-inflamatuvar özellik gösteren bir sitokindir. B ve T hücrelerinin IL-10 üreten regülatör alt tipleri olduğu bilinmektedir. Güncel araştırmalar, *Helikobakter felis* (*H.felis*) enfeksiyonu fare modellerinde IL-10 üreten B hücrelerinin immün cevabı baskılayıcı ve düzenleyici rolü olduğunu ortaya koymuştur. *H.felis*, *H.pilori* ile aynı aileden gelmektedir ancak farelerde immün cevap oluşturma olasılığı daha yüsek olduğundan fare modellerinde kullanılmaktadır.

Reseptörlere bağlanan ligandlar aracılığı ile alınan sinyaller hücre içinde çeşitli yollardan iletilmektedir. Hücre içi sinyal yolakları tarafından iletilen uyarı, sinyale uygun bir cevap verilmesini sağlamaktadır. IL-10 üreten *Helikobakter*-aktive B hücrelerinden IL-10 salınımının Toll-benzeri reseptör 2 (TLR2) aracılığıyla sağlandığı yakın zaman önce gösterilmiştir. Doğal bağışıklık sisteminin antijen sunan hücreleri olan makrofajlar ve dendritik hücrelerden TLR-uyarımı ile IL-10 üretilmektedir. Bu hücrelerden IL-10 üretim ve salınımının mitojen aktive protein kinaz (MAPK), fosfatidilinositol-3 kinaz (PI3K) ve nükleer faktör-kappa B (NF-κB) sinyal yolakları aracılığıyla olduğu bilinmektedir. Dolayısıyla, bu çalışmada incelenen önemli hücre içi sinyal iletim yolları MAPK, PI3K ve NF-κB sinyal yolaklarıdır.

Hücre içi sinyal iletiminde bir çok protein görev almaktadır. Enzimatik reaksiyonla kendinin veya başka proteinlerin fosforilasyonunu sağlayan proteinler kinaz adını almaktadır. Bahsedilen sinyal iletim mekanizmaları bir çok kinazın aktivitesini gerektirmektedir. İlgili yolaklar bir dizi kinazın fosforilasyonunun ardından sinyal yolağını tetikleyen sinyale uygun immün cevap oluşturmak amacıyla nükleusa gidecek ve ilgili gen bölgesine bağlanarak immün cevapla ilintili gen veya genlerin transkripsiyonunu sağlayacak çeşitli transkripsiyon faktörlerinin aktivasyonuna neden olurlar. MAPK sinyal kaskadında IL-10 üretiminde rol oynadığı gösterilen iki kinaz p38 MAPK ve p44/42 MAPK (ERK-1/2)'dır. PI3K sinyal yolağında sinyal iletimi Akt kinazın fosforilasyonu aracılığıyla gerçekleşmektedir. CREB (siklik AMP tepki

elemanı bağlayıcı protein) ise bahsedilen sinyal yolakları tarafından aktive olduğu bilinen ve makrofajların IL-10 transkripsiyonunda aktif rol alan bir transkripsiyon faktörüdür. Hücre içi sinyal yolaklarında bir sinyal molekülünün fosforilasyonu genel olarak aktivasyonu anlamına gelmektedir. Diğer bir deyişle MAPK ve PI3K sinyal yolaklarının aktivasyonu p38 MAPK, ERK-1/2, Akt ve CREB fosforilasyonuna bakılarak tayin edilebilir. NF-κB klasik sinyal yolağında ise p65 sinyal molekülünün fosforilasyonu aktivasyon ile ilintilidir.

Makrofajlar, dendritik hücreler ve T hücreleri gibi bağışıklık sistemi hücrelerinde IL-10 üretiminde rol oynayan hücre içi sinyal yolakları karakterize edilmiş olmasına karşın, TLR2-aracılığı ile aktive olan B hücrelerinde IL-10 üretimini sağlayan hücre içi sinyal yolakları bilinmemektedir. *Helikobakter*-aktive IL-10^{+} B hücrelerinin *Helikobakter*-aracılıklı mide patolojisini önlemede önemli bir katkısı olduğu bilinmektedir. *Helikobakter*-aktive IL-10^{+} B hücrelerinden IL-10 üretiminde rol oynayan sinyal iletim yolaklarının tespiti, ilgili hücrelerin kullanılacağı immün tedavi araştırmalarına da zemin hazırlaması bakımından önem teşkil etmektedir. Bu nedenle, bu çalışmanın odak noktası B hücrelerinin TLR2-uyarımı ile IL-10 üretmesini sağlayan hücre içi sinyal yolaklarının tespit edilmesidir. Bu amaçla, C57BL/6 dalağından elde edilen naif B hücreleri *H.felis* sonikatı ile uyarılmış ve ilgili hücrelerden TLR2-aracılıklı IL-10 üretimi ve salınımı gerçekleşmesi sağlanmıştır. Ardından *H.felis* sonikatı ile uyarılmış B hücreleri IL-10 üretme kapasitelerine göre manyetik yöntemle ayrılmış ve IL-10 negatif (IL-10^{-} B hücreleri) ve IL-10 pozitif (IL-10^{+} B hücreleri) fraksiyonlar elde edilmiştir.

Fare-kaynaklı naif B hücreleri, *H.felis* sonikatı ile uyarılmış B hücreleri, *Helikobakter*-aktive IL-10^{-} B ve IL-10^{+} B hücrelerinden elde edilen protein örnekleri MAPK, PI3K ve NF-κB yolaklarında kilit rol oynayan sinyal moleküllerinin fosforilasyonu açısından incelenmiştir. İlgili sinyal moleküllerinin fosforilasyonları total ve fosforile formlarını tanımak için özelleşmiş antikorlar kullanılarak Western blotlama yöntemi ile tayin edilmiştir. NF-κB klasik sinyal yolağında önemli rol oynayan p65 sinyal molekülünün fosforilasyonu ise sinyal yolağı protein ELISA yöntemi ile tayin edilmiştir.

Fosforilasyon tayininin ardından, hücre içi yolaklarda görev alan kinazların aktivitesini baskılayan spesifik inhibitörler yardımıyla ilgili yolakların bloklanması sonucu *Helikobakter*-aktive B hücrelerinden IL-10 salınımındaki değişim incelenmiştir. Bu sayede, baskılanan sinyal yolağının IL-10 üretimindeki etkisinin anlaşılması amaçlanmıştır. Bu amaçla, fare-kaynaklı naif B hücreleri ve *H.felis* sonikatı ile uyarılmış B hücrelerinin MAPK sinyal yolağının p38 MAPK modülü ve ERK-1/2 modülü, PI3K sinyal yolağı ve NF-κB klasik sinyal yolakları kimyasal inhibitörler kullanılarak bloklanmıştır. Ardından hücre üst-fazları toplanmış ve hücre üst-fazlarındaki IL-10 miktarı IL-10 ELISA yöntemi uygulanarak kantitatif olarak belirlenmiştir.

Helikobakter-aktive IL-10^{+} B hücrelerinden IL-10 üretimini ve salınmasını sağlayan hücre içi sinyal yolaklarının incelenmesi ve belirlenmesini amaçlayan çalışmanın sonucunda elde edilen verilere gore *Helikobakter*-aktive IL-10^{-} B ve IL-10^{+} B hücre örnekleri arasında yapılan karşılaştırma sonrasında ERK-1/2 proteinlerinin *Helikobakter*-aktive IL-10^{+} B hücrelerinde anlamlı düzeyde daha fazla fosforile olduğu görülmüştür. İncelenen diğer kinazların (p38 MAPK ve Akt) ise *Helikobakter*-aktive IL-10^{-} B hücre örneklerinde anlamlı düzeyde daha fazla fosforile olduğu gözlemlenmiştir. NF-κB sinyal proteini olan p65'in fosforilasyonunun ise

örnekler arasında belirgin bir artış veya azalış göstermediği tespit edilmiştir. Bu durum ERK-1/2 aktivasyonunun *Helikobakter*-aktive B hücrelerinden IL-10 üretiminde daha etkin rol oynuyor olabildiğini düşündürmüştür. Bunun yanı sıra, spesifik inhibitörler kullanılarak bloklanmış sinyal iletim yolaklarının IL-10 salınımı üzerindeki etkisinin araştırılması sonucunda yalnızca ERK-1/2 inhibisyonunun B hücrelerinden IL-10 salınımında anlamlı bir azalışa yol açtığı görülmüştür. Bu bulgu da *Helikobakter*-aktive B hücrelerinden IL-10 üretiminde MAPK sinyal yolağı modülü olan ERK'in etkin rolü olduğu hipotezini güçlendirmektedir. Bu çalışma B hücrelerinden TLR2-aracılıklı IL-10 üretiminde etkili hücre içi sinyal yolaklarını aydınlatması ve MAPK sinyal iletim yolağının ERK-1/2 modülünün *Helikobakter*-aktive B hücrelerinden IL-10 üretimindeki rolünü göstermesi açısından önemlidir.

1. INTRODUCTION

1.1 *Helicobacter felis (H. felis)*

Helicobacter felis is a small, gram negative, helical shaped, microaerophilic bacterium that colonizes in the stomach of cats and mice. Lee and his colleagues first isolated *H. felis* from stomach of cats in 1988 [1]. *H. felis* is closely related to *Helicobacter pylori*. *Helicobacter pylori* is a gastric pathogen that has higher immunogenicity for humans. *Helicobacter pylori* infection can result in chronic gastritis. The step-by-step clinical manifestations of chronic *Helicobacter* infection leading to gastric adenocarcinoma are depicted in Figure 1.1. Perpetual exposure to pathogen leads to gastric atrophy. Gastric mucus layer is distorted and gastric pH goes up due to loss of gastric chief and parietal cells, which are key players in maintaining gastric low pH [2]. Distortions in the protective gastric mucus layer enable microbial components and carcinogens to work their way into gastric epithelial cells in order to exert their effects on DNA and induce a number of changes in the cell that may lead to gastric adenocarcinoma [3]. Chronic gastric inflammation leads to transformation of epithelial layer of gastric tissue into intestinal-like epithelium, a process which is termed intestinal metaplasia. Perpetual exposure to the bacteria and its microbial components results in abnormal epithelial growth, which is termed dysplasia and subsequent gastric adenocarcinoma [4]. Microbial components of *Helicobacter pylori* are various virulence factors. The most significant ones that are closely associated with disease-causing capacity of the bacterium and severity of clinical outcome are cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) [5].

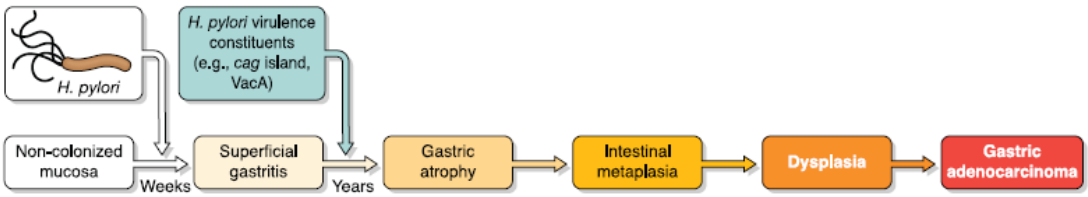

Figure 1.1: *Helicobacter pylori* infection leading to gastric adenocarcinoma (adapted from ref. 5).

Helicobacter felis does not have CagA or VacA virulence factors. Nevertheless, the *Helicobacter felis* infection and prognosis of disease in mouse models have a striking resemblance to *Helicobacter pylori* infection in humans [6, 7].

1.2 Mouse Models of *Helicobacter* Infection

Apart from the virulence factors of the pathogen, host genetic background is another determinant for the clinical outcome of the disease. Immune response to *Helicobacter* infection is divergent in two different strains of mice, which are commonly and widely used in *Helicobacter* infection studies [8].

1.2.1 C57BL/6 mice

T helper 1 (Th1) type immune response is induced in *Helicobacter*-infected C57BL/6 mice (Fig. 1.2). T helper 1 type immune response is associated with secretion of pro-inflammatory cytokines that decreases the bacterial burden while damaging the tissue due to perpetual gastric inflammation induced by *Helicobacter felis* infection [9,10]. Persistent *H.felis* infection can lead to histological alterations in gastric tissue of C57BL/6 mice eventually leading into gastric adenocarcinoma in the presence of salt diet or carcinogens [11].

Figure 1.2: C57BL/6 mice (adapted from ref. 12).

1.2.2 BALB/c mice

T helper 2 (Th2)-type immune response is predominant in BALB/c strain mice (Fig 1.3) in response to *Helicobacter*-infection. Regarding *Helicobacter*-infection, in contrast to Th1-type response, Th2-type response is more of a tolerogenic immune response in which the host bacterial burden levels remain high with minimal damage to tissue [6]. Long-term exposure of BALB/c to *Helicobacter* leads to aggregation of lymphocytes. Therefore, BALB/c is widely used as a model for mucosa-associated lymphoid tissue (MALT) lymphoma [8].

Figure 1.3: BALB/c mice (adapted from ref. 12).

Approximately half of the world's population is infected with *Helicobacter pylori*. Due to well- defined causative role of *H. pylori* in gastric adenocarcinoma formation, the bacterium was classified as a class I carcinogen by World Health Organization (WHO) in 1994 [13]. Increasing prevalence of *Helicobacter* infection (80%) is observed in developing countries. All individuals that are infected with the pathogen develop chronic gastritis, however majority of infected individuals are asymptomatic [14, 15]. 20% of infected individuals suffer from severe *Helicobacter*-associated gastric diseases such as peptic ulcer, MALT lymphoma or gastric cancer [16]. Persistent infection over time, virulence factors of pathogen, genetic and environmental background of the host are important determinants for development of severe gastric complications [17]. The complex mechanisms underlying the asymptomacity in majority of *Helicobacter*-infected individuals have been ill-defined [18].

1.3 Interleukin-10 (IL-10)

Interleukin-10 is an anti-inflammatory immune mediator. IL-10 was first described by Mosmann and colleagues as a cytokine which T helper 2 (Th2) cells produce and which inhibits production of Th1 cell-derived interferon-γ (IFN-γ) and interleukin-2 (IL-2) [19]. Initially named as "cytokine synthesis inhibiting factor (CSIF)" due to its ability to suppress cytokine synthesis, this immune mediator was later known as "IL-10" in cytokine nomenclature. Today, it is known that various immune cells such as $CD4^+$ T cells, $CD8^+$ T cells, B cells, monocytes, macrophages and dendritic cells are capable of producing IL-10 [20]. IL-10, owing to its pleiotropic effects, has significant implications on immune regulation. IL-10 represses immune response against pathogens and attenuates damage to host, thereby prevents autoimmunity [21].

1.3.1 IL-10 gene and protein

The gene responsible for IL-10 expression is localized to chromosome 1 in both human and mouse [22]. hIL-10 is comprised of 4 exons whereas mIL-10 is comprised of 5 exons over a span of 5.1 kb pairs. IL-10 protein is a homodimer that is composed of two monomers encoded by *il-10* gene. Each monomer is a protein comprised of 178 amino acids [23]. Cleavage of 18 amino acid-long signal peptide reveals the mature 160 amino acid-long mature segment. Human and murine IL-10 proteins are found to be 75% alike regarding their amino acid sequences. Various viral homologs of IL-10 exist: the Epstein Barr virus (BCRF1) [24], the Herpes type 2 virus [25], the Orf virus [26], the Cytomegalovirus [27]. Regarding amino acid sequence, Epstein Barr virus (BCRF1) and hIL-10 is 83% alike [28]. Due to this striking resemblance, acquisition of this gene was associated with the ability of virus to repress host antiviral immune response. [29].

1.4 IL-10 Producing Regulatory B Cells

B cells are significant for the humoral immune response due to their capacity to produce and secrete antibodies targeted against pathogens upon activation. Like macrophages and dendritic cells (DCs), B cells are antigen presenting cells which present soluble antigens to $CD4^+$ T cells [30]. B cells are also able to produce and secrete various cytokines which mediate the immune response. Effector B cells can

be categorized into subsets depending on the type of cytokine that they produce. This categorization originates from the classification of cytokines produced and secreted by T helper (Th) cells. Th1-type cytokines such as IFN-γ are pro-inflammatory, whereas Th2-type cytokines such as IL-4, IL-5 and IL-13 are anti-inflammatory and associated with a tolerant response [31, 32] (Fig. 1.4).

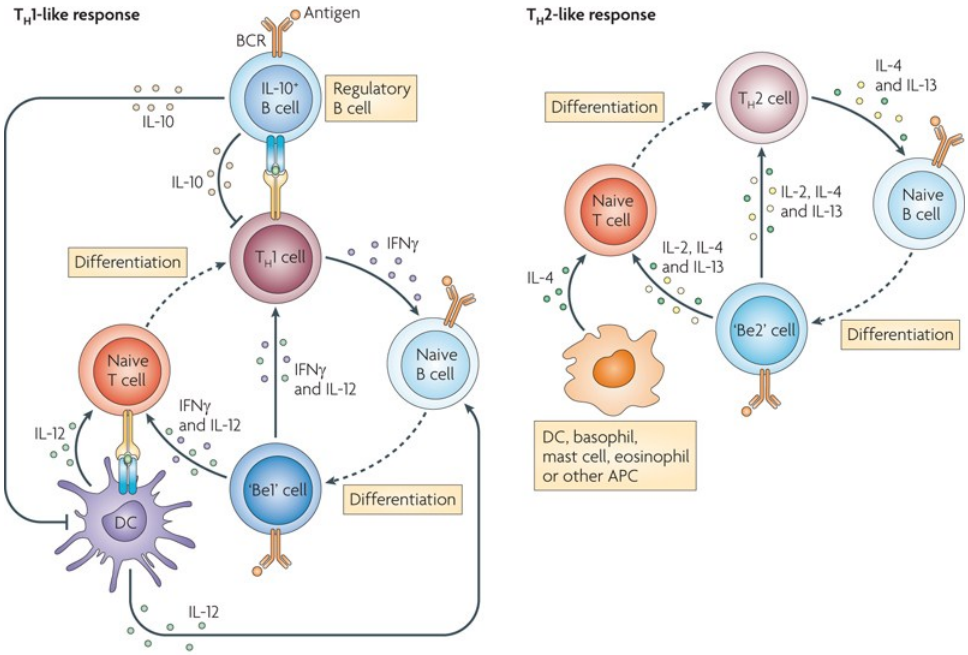

Figure 1.4: Th1 and Th2 type immune responses (adapted from ref. 33).

Therefore effector B cells can be categorized as "Be1" and "Be2", due to their secretion of Th1-type cytokines and Th2-type cytokines, respectively. Be1 cells present their antigen to Th1 cells whereas Be2 cells present their antigen to Th2 cells. Being antigen presenting cells (APCs), effector B cells can also present the antigen recognized by their B cell receptor (BCR) to naive $CD4^+$ T cells and induce them to take part in the immune response. The immune mediators secreted by effector B cells ensure sustenance of the immune response either Th1- or Th2-type, depending on the cytokine profile [31, 33] (Fig. 1.4).

Reminiscent of regulatory T (Treg) cells, recently a new subset of B cells with regulatory function has been defined. These B cells are characterized by their production and secretion of anti-inflammatory cytokine interleukin-10 (IL-10). IL-10 hampers antigen-presenting capacity of APCs, represses immune response of effector T cells and induces Treg formation and proliferation [33].

Experimental autoimmune encephalomyelitis (EAE) mouse model revealed the function of B cells producing IL-10 *in vivo* for the first time. Exacerbation of the disease in B-cell deficient mice was shown to be due to lack of IL-10 production by B cells. Excessive Th1-derived pro-inflammatory immune response persisted when there was no IL-10 production and secretion by B cells. Mice could not recover from EAE due to excessive inflammation [34, 35]. IL-10 producing B cells have been shown to play an important role in restraining of excessive Th1-type immune response in a number of other autoimmune diseases including lupus, collagen-induced arthritis (CIA), and colitis. [36-38]. These IL-10 producing B cells were called regulatory B (Breg) cells by Bhan and colleagues in 2002 due to their immune regulatory function [36].

Recent findings revealed that murine splenic B cells produce and secrete IL-10 upon *Helicobacter*-infection *in vitro* as well as *in vivo*. *Helicobacter felis* was found to be recognized by Toll-like receptor 2 (TLR2) of B cells and subsequent activation of myeloid differentiation primary response gene 88 (MyD88) is required for induction of IL-10 since TLR2 or MyD88 knock-out mice ($TLR2^{-/-}$ or $MyD88^{-/-}$) had significantly less IL-10 secretion when compared to wild type (WT) mice. Gastric pathology developed upon *Helicobacter* infection was similar to WT in $TLR4^{-/-}$ and $TLR9^{-/-}$ mice. Moreover, both $TLR2^{-/-}$ and $MyD88^{-/-}$ mice developed accelerated gastric histopathology compared to WT mice. IL-10 producing regulatory B cells restrain excessive Th1-type pro-inflammatory immune response and gastric immunopathology of C57BL/6 mice via suppression of $CD4^+$ effector T cells [18]. The interaction between regulatory B cells and T cells were also denoted as required for the function of regulatory B cells. Bregs were shown to be able to convert $CD4^+$ T cells into IL-10-producing T regulatory 1 (Tr-1) cell through direct interaction. Tr-1 cells and Bregs work in harmony in order to restore the immune balance in *Helicobacter*-infection by ameliorating excessive gastric immunopathology while preventing bacterial clearance in the gastric mucosa [18]. This study was significant for demonstrating a B cell subset with regulatory function in a bacteria-associated disease model for the first time.

1.5 Sensors of Immune System: Toll-like Receptors (TLRs) and TLR Ligands

Several immune cells have been known to produce and secrete IL-10 through Toll-like receptor (TLR) stimulation [75]. Toll-like receptors are sensors of the immune system. They recognize molecular patterns termed pathogen-associated molecular patterns (PAMPs) of pathogens. That is the reason why they are called pattern recognition receptors (PRRs) [39]. Toll protein was found to bear developmental significance for *Drosophila* when it was first identified. The importance of Toll in immunity as an anti-fungal protein was later delineated [40]. TLRs in mammals are homologous family of Toll receptors as the name suggests [41]. TLRs are found to be structurally reminiscent of IL-1 receptors (IL-1R) especially in their cytoplasmic domains which are termed as Toll-IL-1R (TIR) domains [39].

Humans have been shown to possess TLR1-10 whereas 12 murine TLR members have been identified, TLR1-9, TLR11, TLR12 and TLR13. Mice do not have a functional TLR10 [42]. TLRs may recognize intracellular as well as extracellular pathogen-related microbial patterns. Some of TLRs span the plasma membrane whereas some of them are localized to endosomal membrane (Fig. 1.5). Toll-like receptors mostly function as dimers (homodimers or heterodimers) and different TLRs respond to different stimuli. Known ligands of TLRs are listed in Table 1.1.

Table 1.1: Physiological and synthetic agonists of TLRs (adapted from 43).

TLR	Physiological ligands	Synthetic ligands
TLR1-2	Triacylated lipopeptides (bacteria and mycobacteria)	Pam_3CSK_4
TLR2	Peptidoglycan (gram positive bacteria), multiple lipopeptides, multiple lipoproteins (bacteria)	
TLR2-6	Diacylated lipopeptides (*Mycoplasma*), zymosan (*Saccharomyces cerevisiae*)	Pam_2CSK_4
TLR3	dsRNA (viruses)	Poly I:C

Table 1.1 (cont'd.): Physiological and synthetic agonists of TLRs (adapted from 43).

TLR4	LPS (gram-negative bacteria), several heat shock proteins (HSP60, HSP70), heparan sulfate fragments, proteoglycans, fibrinogen	
TLR5	Flagellin (flaggellated bacteria)	
TLR7-8	ssRNA (RNA viruses)	Resquimod (R848)
TLR9	CpG DNA (bacteria)	CpG ODNs
TLR11	Profilin-like proteins	
TLR13	23S rRNA (bacteria) [44]	

Due to their ability to recognize pathogens, TLRs are key players of innate immunity. However, recognition of PAMPs through TLRs can also lead to cytokine production and secretion from antigen presenting cells and activation of naive T and B cells which brings about induction of acquired immune system (either cell-mediated or humoral immune response). Therefore, it would not be wrong to claim that TLRs form an essential bridge between innate and adaptive immunity [45].

The signal received through recognition of microbial patterns is relayed through either MyD88-dependent or MyD88-independent pathways. All of TLRs relay their signal through MyD88-dependent pathway except for TLR3. TLR4 is unique in such a way that it can utilize both MyD88-dependent and MyD88-independent pathways for signal transduction. Translocation of TLR4 from plasma membrane to endosomal membrane brings about switching of the signal transduction from MyD88-dependent to MyD88-independent. [46] (Fig. 1.5).

As an adaptor protein containing TIR domain, MyD88 enables interaction between IL-1R and IL-1R-associated protein (IRAK). MyD88-adaptor-like protein (MAL) can also act as an adaptor protein alongside MyD88 for relaying received signal. Ligand binding to TLR stimulates activation of IRAK through phosphorylation and interaction of IRAK with tumor necrosis factor (TNF) receptor-activated factors (TRAFs) [46, 47] (Fig. 1.5).

In MyD88-independent signal transduction, ligand binding to endosomal TLR3 (and TLR4) leads to activation of TIR-domain-containing adaptor protein inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). TRIF and TRAM activation brings about the activation of TRAFs. This step is where the two signaling pathways are converged. Downstream signaling steps are common for two distinct pathways [46, 48] (Fig. 1.5).

Activation of TRAFs results in activation of either p38 or JUN N-terminal kinase (JNK) of mitogen-activated protein kinase (MAPK) pathway or inhibitor of nuclear factor-κB kinase (IKK) of NF-κB pathway. Downstream transcription factors of these distinct pathways are cyclic AMP-responsive element-binding protein (CREB), activator protein-1 (AP-1), NF-κB or interferon-regulatory factors (IRFs). CREB, AP-1 and NF-κB are mainly responsible for transcription of genes coding for pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α whereas endosomal TLR-induced IRFs are responsible for transcription of type I interferons interferon-α (IFN-α) and interferon-β (IFN-β) as an initial immune response to pathogens [46-48] (Fig. 1.5).

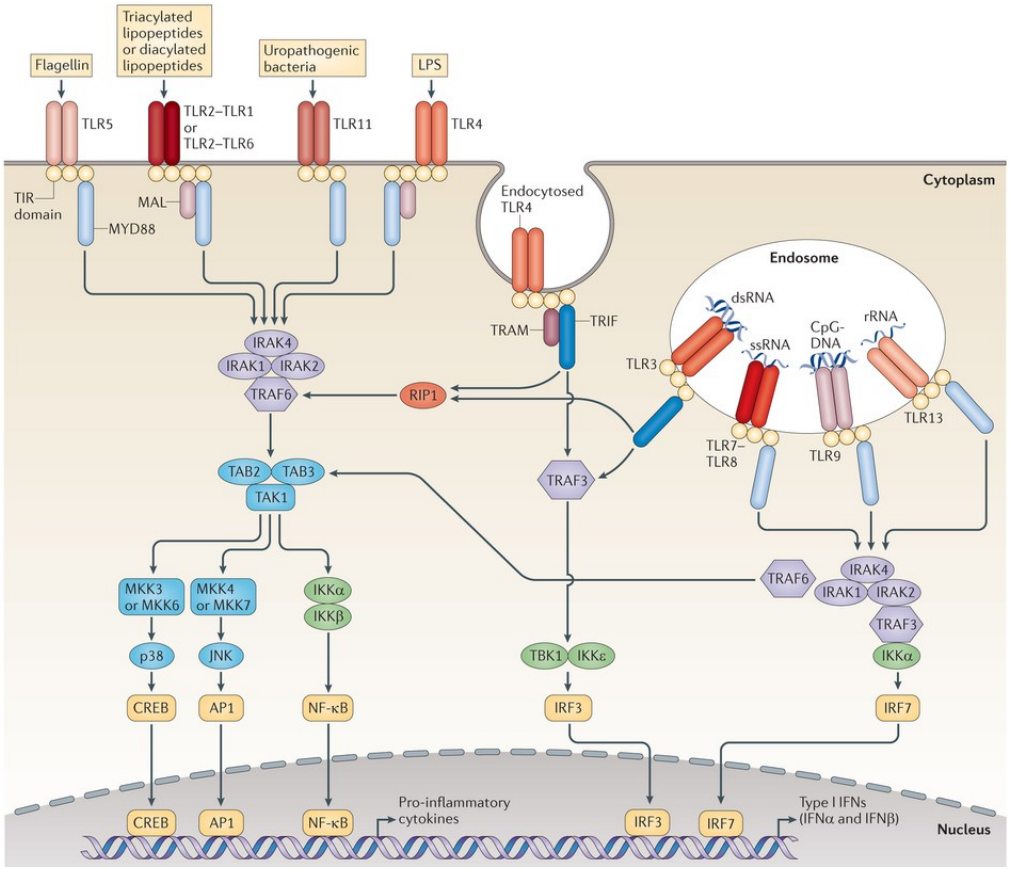

Figure 1.5: TLRs and TLR signaling pathways (adapted from ref. 46).

1.6 Intracellular Signaling Pathways That Are Important for Induction of IL-10 Production From Immune Cells

1.6.1 MAPK/ERK signaling pathway

The role of MAPK/ERK signaling pathway on induction of TLR-mediated IL-10 production from various immune cells such as macrophages, dendritic cells (DCs) and T helper cell subsets has been recently identified [75]. The mitogen-activated protein kinase/extracellular signal-regulated kinase **(**MAPK/ERK) signaling pathway that relays a signal received by cell surface receptors through a cascade of cellular proteins in order to induce proper cellular response to stimuli in the form of transcription of related genes coding for related response proteins. The activation of each element in the cascade is achieved through phosphorylation by its predecessor [49]. Activation of cell surface receptors such as guanosine nucleotide-binding protein (G-protein)-coupled receptor, receptor tyrosine kinases (RTKs), B cell receptors (BCRs) or T cell receptors (TCRs) is the first step in an intracellular signaling cascade which involves phosphorylation, therefore activation of several kinases including MAP kinase kinase kinases (MAP3K), MAP kinase kinases (MAP2K) and MAP kinases (MAPK) [50-54]. Phosphorylation and activation of relevant MAPK molecule induces a transcription factor for transcription of a response gene. Ras-Raf-MEK-ERK pathway is an example for MAPK signaling pathway. Ligands of the receptors induce activation of G-protein which acts as a molecular switch. When they are bound to GTP, they are “on” whereas being bound to GDP renders them “off”. Ras is an important G-protein that is upstream of many intracellular signaling routes. Upon activation Ras activates its effector Raf kinase, which activates mitogen/extracellular signal-regulated kinase (MEK) that in turn activates ERK. That is why this pathway was originally called as Ras-Raf-MEK-ERK pathway. Cancer is a likely outcome of a malfunction in signal transduction which renders a kinase in perpetual “on” or “off” state. Six different MAPK signaling modules have been identified: ERK-1/2, JNK-1/2/3, p38 MAPKs (α/β/γ/δ), ERK-5, ERK-3/4 and ERK-7/8. Each MAPK gets activated by a different signaling cascade [50]. Most researched MAPKs are ERK-1/2, JNK-1/2/3, p38 MAPKs. Signals elicited through p38 MAPK and ERK are relayed to relevant transcription factors directly or indirectly through activation of MAPK-activated protein kinases (MAPKAPKs) that activate specific transcription factors [51]. MAPKAPKs activated

through ERK-1/2 modules are ribosomal S6 kinase 1 (RSK1), RSK-2, RSK-3, RSK-4 and MAPK-interacting kinase 2 (MNK2). p38 MAPK module activates MAPKAPK2, MAPKAPK3. Mitogen- and stress-activated kinase 1 (MSK-1), MSK-2 and MNK-1 are downstream effectors of both p38 MAPK and ERK-1/2. MAPK modules are inactivated by dual-specificity phosphatases (DUSPs) by dephosphorylation via negative feedback loops [51, 52] (Fig. 1.7).

Chemical inhibitor of p38 MAPK widely used for research purposes is SB203580. SB203580 blocks p38 MAPK signaling by inhibiting activation of downstream MAPKAPK2. It does not affect the activation of p38 MAPK module. Specific inhibitor of MEK-1/2 widely used for research purposes is U0126. U0126 inhibits phosphorylation, therefore activation of both MEK-1 and MEK-2 and downstream effectors [51] (Fig. 1.6).

Cellular responses elicited through activation of MAPK/ERK pathway depend upon stimuli. ERKs mainly respond to growth factors and mitogens and their response mainly involve cell proliferation and differentiation. JNKs are also known as stress-activated protein kinases (SAPKs) and as the name suggests JNKs along with p38 MAPKs are responders of DNA damage, oxidative stress, heat shock and cytokines. The typical responses elicited through these signaling nodes are cell differentiation, apoptosis and cytokine production [50-54]. Figure 1.7 demonstrates the stimulants, modules, kinases and effectors of MAPK signaling pathway.

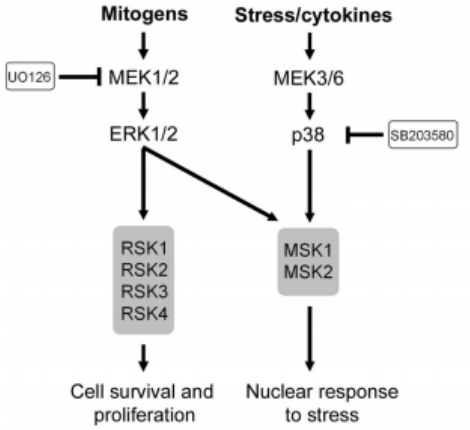

Figure 1.6: MAPK signaling pathways leading to downstream effector MAPKAPKs (modified from ref. 51).

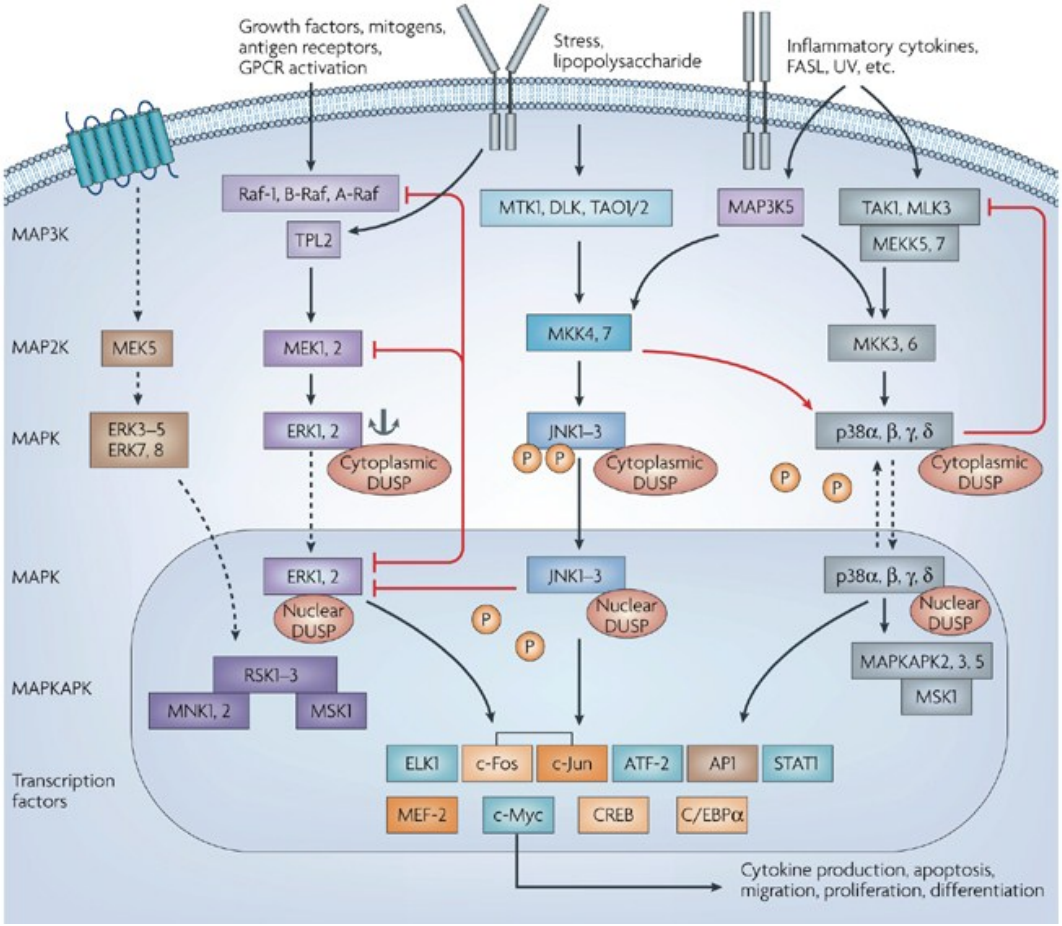

Figure 1.7: Overview of MAPK signaling pathway (adapted from ref. 52).

1.6.2 PI3K pathway

The contribution of PI3K signaling pathway on IL-10 production upon TLR-stimulation has been revealed with studies performed in macrophages [75]. Phosphoinositide 3‑kinase (PI3K) pathway is pivotal for cell proliferation, survival and growth in response to cytokines, growth factors and hormones. PI3K is activated when ligands bind to their receptors such as a receptor tyrosine kinase (RTK). Activated PI3K turns membrane-bound Phosphatidylinositol 4,5-bisphosphate (PIP_2) into Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). Akt (a.k.a. Protein kinase B (PKB)) gets translocated to plasma membrane in order to interact with PIP3. The phosphorylation, therefore activation of Akt is achieved in two-steps. 3-phosphoinositide dependent protein kinase-1 (PDK1) phosphorylates Akt at Thr308 whereas mammalian target of rapamycin (mTOR)-rictor complex (mTORC2) phosphorylates Akt at Ser473 (Fig. 1.8). Phosphatase and tensin homolog (PTEN) is the designated negative-regulator of PI3K/Akt signaling pathway as it reverses

conversion of PIP_2 to PIP_3. Overactivity of Akt signaling can lead to malignancies such as cancers due to uncontrolled cell proliferation [54-56].

Dual phosphorylation of Akt results in phosphorylation of many downstream effector molecules. mTOR is one of the most important effectors of Akt as it leads to cell cycle progression and cell growth. Glycogen synthase kinase-3 (GSK-3) is also another effector. GSK-3 differs from other kinases as its activity is inhibited through phosphorylation. Phosphorylation at Ser21 and Ser9 renders GSK-3α and GSK-3β inactive, respectively. Phosphorylated GSK-3 has pleiotropic effects on many cellular processes including cell cycle progression (Cyclin D1), glycogen metabolism. Akt inhibits apoptosis by inhibitory phosphorylation of many pro-apoptotic effectors such as Bad, Bim and Forkhead transcription factors (FoxO1, FoxO3a) [54-56]. Figure 1.8 depicts the molecules, kinases and effectors of PI3K signaling pathway.

Selective inhibitors of PI3K or Akt block PI3K signaling pathway. One of the widely used inhibitors of PI3K is LY294002. LY294002, the first synthetic PI3K inhibitor abrogates activity of PI3K and its downstream effector molecules [55].

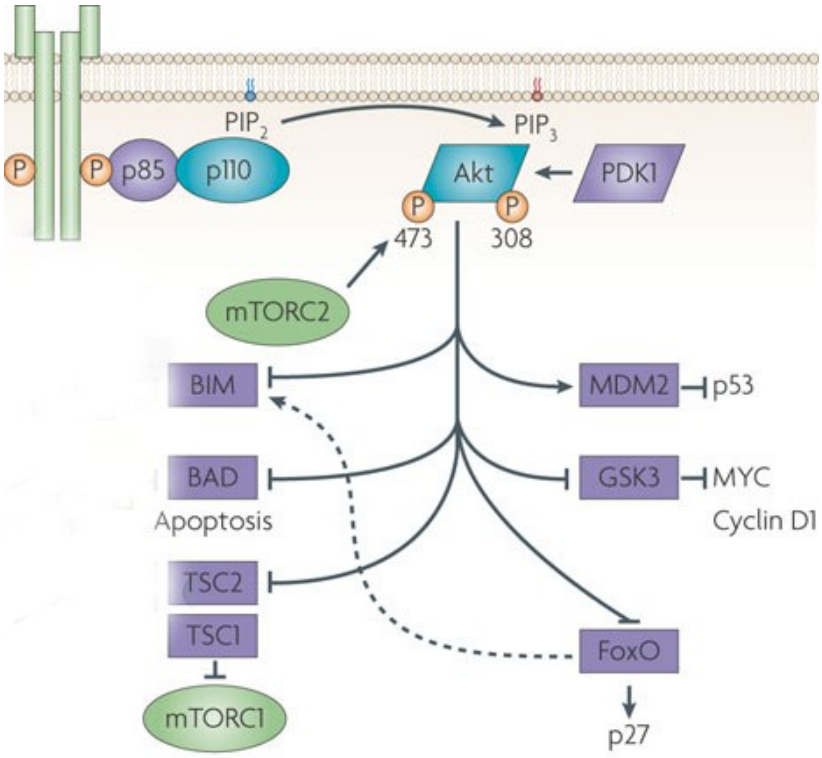

Figure 1.8: Overview of PI3K signaling pathway (adapted from ref. 56).

1.6.3 NF-κB pathway

The involvement of NF-κB signal transduction pathway in TLR-stimulated IL-10 production from immune cells such as macrophages has been designated [75]. Nuclear factor-κB (NF-κB) pathway elements play a central role in innate and adaptive immune responses and inflammation. NF-κB/Rel proteins are p50/105 (NF-κB1), p52/100 (NF-κB2), p65 (RelA), c-Rel and RelB. Functional dimers of these proteins act as transcription factors. There are two distinct pathways for NF-κB signaling: canonical and non-canonical pathways [57].

1.6.3.1 Canonical (classical) pathway

Classical pathway is induced with ligands such as LPS, growth factors, pro-inflammatory cytokines and antigens recognized by their cognate receptors. Received signals activate IKK complex which is composed of IKKβ, IKKα, and NF-κB essential modulator (NEMO) by phosphorylation. Activated IKK complex brings about phosphorylation of nuclear factor of kappa light polypeptide gene enhancer in B-cells (IκB) proteins which are bound to NF-κB1/RelA proteins and when unphosphorylated inhibit their activation. IκB proteins are ubiquitinated and targeted for degradation upon phosphorylation. Degradation of IκB renders NF-κB1/RelA proteins functionally active. Phosphorylated and active p50/p65 heterodimer translocates into the nucleus and acts as transcription factor or co-transcription factor of AP-1, signal transducer and activator of transcription (STAT) for expression of several response genes [57,58] (Fig. 1.9).

1.6.3.2 Non-canonical (alternative) pathway

NF- κB2 p100/RelB proteins reside in cytoplasm in inactive form when there are no stimuli. Ligands such as lymphotoxin-β (LTβ), CD40L or B-cell activating factor (BAFF) exert their effect by inducing a signaling cascade involving sequential phosphorylation of NF-κB inducing kinase (NIK), IKKα complex and NF-κB2 p100 subunit. Processing of precursor p100 into mature p52 occurs upon phosphorylation and ubiquitination of p100. Functionally active p52/RelB heterodimer goes into the nucleus and induce expression of response genes [57-59] (Fig.1.9).

There are many chemical inhibitors of NF-κB signaling. Being one of them, PDTC (Pyrrolidinedithiocarbamate ammonium) exerts its effect by inhibiting degradation of

IκBα. IκBα is normally ubiquitinated and targeted for degradation by proteosomal proteolytic pathway in order to enable formation and translocation of p50/p65 heterodimer into nucleus [60].

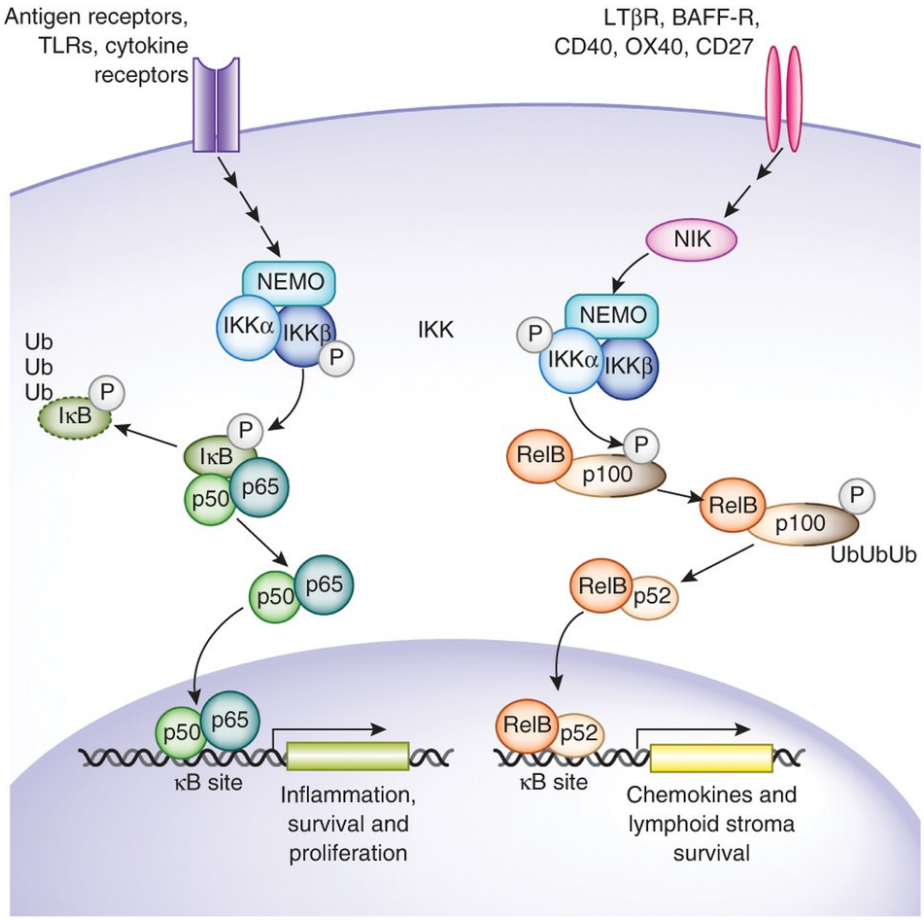

Figure 1.9: NF-κB canonical and non-canonical signal transduction pathways (adapted from ref. 58).

1.7 IL-10 Receptor and IL-10/IL-10R Signaling

Biological activity of IL-10 is mediated by its specific transmembrane receptor complex (IL-10R). IL-10R is comprised of IL-10R1 (the ligand-binding chain) and IL-10R2 (the accessory chain), both of which are members of the class II cytokine receptor family (CRF2) [23]. Binding between IL-10 and IL-10R complex is a two-step process. IL-10 first binds to IL-10R1 and this interaction between IL-10 and IL-10R1 induces a conformational change in cytokine structure, revealing an epitope to enable interaction between IL-10 and IL-10R2. Therefore, the interaction of IL-10/IL-10R1 complex enables binding of IL-1R2 since IL-10R2 is unable to interact with IL-10 alone [61] (Fig. 1.10).

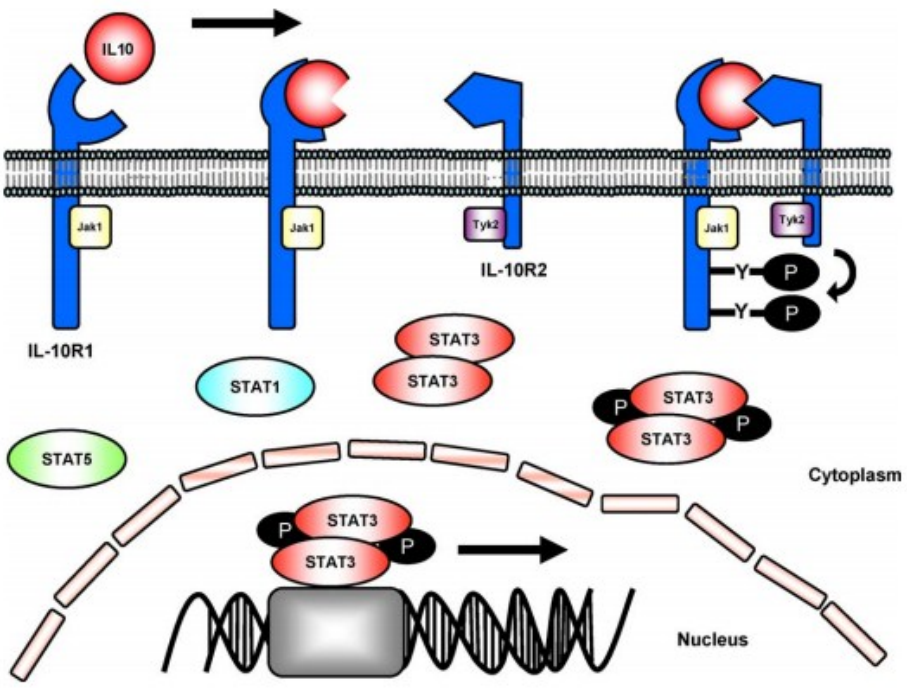

Figure 1.10: Interaction scheme between IL-10 and IL-10 receptor complex (adapted from ref. 23).

IL-10R1 is predominantly found on immune cells. Unlike IL-10R1, various cells express IL-10R2 [62]. IL-10 exerts its effects through Janus kinase (JAK) / Signal transducer and activator of transcription (STAT) pathway. Jak1 and Tyk2 are activated following binding of IL-10 to its receptor complex. These two kinases belong to Janus Kinase family and interaction partners of Jak1 and Tyk2 are IL-10R1 and IL-10R2, respectively. This activation leads to phosphorylation of IL-10R1 on tyrosine residues Tyr 446 and Tyr 496. This is followed by binding of STAT3, a transcription factor, to phosphorylated tyrosine domains and phosphorylation, therefore activation of STAT3 by receptor-associated Janus kinases [63]. STAT1 and STAT5 were also found to be activated in response to IL-10 in murine pre-B cell line transfected with mIL-10 receptor coding gene [64]. Activated STAT transcription factors are translocated into nucleus in the form of phosphorylated homo- or hetero-dimers and start transcription of IL-10-induced genes by binding to the response elements of STAT molecules [63]. The suppressor of cytokine signaling 3 (SOCS3) is one of the IL-10-induced genes. As the name suggests, SOCS3 inhibits expression of pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), IL-1, IL-6. Expression of SOCS3 in response to IL-10 in monocytes and macrophages is

found to be closely related to inhibition of expression of endotoxin-inducible (LPS- or IFN-induced) genes [29, 65].

1.8 Effects of IL-10 on Immune Cells

As previously implicated, IL-10 has various pleiotropic effects on immune cells. Antigen presenting cells, especially monocytes and macrophages are characterized by their hallmark features like phagocytosis, antigen presentation and cytokine release to elicit an immune response. One of the impacts of IL-10 is limiting the secretion of macrophage-derived pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8, thereby hampering innate immune response-related cytokine release [66]. Another aspect of IL-10 influence on monocytes/macrophages is repression of antigen presentation. This inhibition is accomplished by decreased expression of major histocompatibility complex class II (MHC-II) molecules, co-stimulation molecules such as CD86 and adhesion molecules such as CD54, each of which do take an active role in binding and antigen presentation between antigen presenting cell and its interaction partner (e.g. $CD4^+$ T cell) [23]. IL-10 is also capable of limiting Th1- and Th17-mediated immune responses by reducing the secretion of IL-12 and IL-23 from APCs, respectively. IL-12 is the cytokine responsible for Th1-type T cell conversion from naive $CD4^+$ T cells whereas IL-23 is known to be pivotal for Th17 immune response [67]. Phagocytosis efficiency of APCs is enhanced by IL-10 via increasing expression of cell surface receptors that enable internalization of pathogens. It has been shown that treatment of monocytes with IL-10 induces expression of CD16, CD32, CD64 Fc receptors that are crucial for uptake of opsonized (being marked for phagocytosis) material and CD14 which acts as a co-receptor for the internalization of microorganisms that are not opsonized [68]. IL-10 also prevents killing of internalized pathogens while circumventing the complement system attack on phagocytes [69].

The effects of IL-10 on T cells, apart from its indirect effects elicited through APCs, are mainly impeded cell proliferation and repression of Th1- (e.g. IL-2, IFN-γ) or Th2-type cytokine (e.g. IL-4, IL-5) secretion from $CD4^+$ T helper cells [70]. IL-10 produced by TLR2-activated B cells has also been implicated with their potency to convert $CD4^+$ T cells into IL-10 producing regulatory T cells (Treg), which are also termed as T regulatory-1 (Tr-1) cells, *in vitro* in a contact-dependent manner [18].

IL-10 acts on B cells as a growth factor which enables and enhances expansion, differentiation and Ig class switching of B cells. IL-10 is also capable of increasing the expression of MHC-II on cell surface [23].

Since NF-κB signaling is central for expression of many pro-inflammatory cytokines, NF-κB has been a potential target for IL-10 to exert its limiting effects on cytokine or chemokine secretion [71]. Indeed, in monocytes and macrophages, IL-10 was shown to inhibit canonical pathway of NF-κB signaling, that is translocation of functionally active p50/p65 heterodimer into nucleus [72]. Inhibition of NF-κB signaling by IL-10 may be via bilateral molecular mechanisms as depicted in Figure 1.11. IL-10 may hamper the translocation of functionally active p50/p65 heterodimer via IKK inhibition or inhibit binding of translocated heterodimer to relevant DNA region [73]. An IL-10 favored p50/p50 homodimer translocation instead of p50/p65 heterodimer was also suggested. These p50/p50 homodimers are known for their inhibitory effect on transcription unlike their p50/p65 heterodimer counterparts [74].

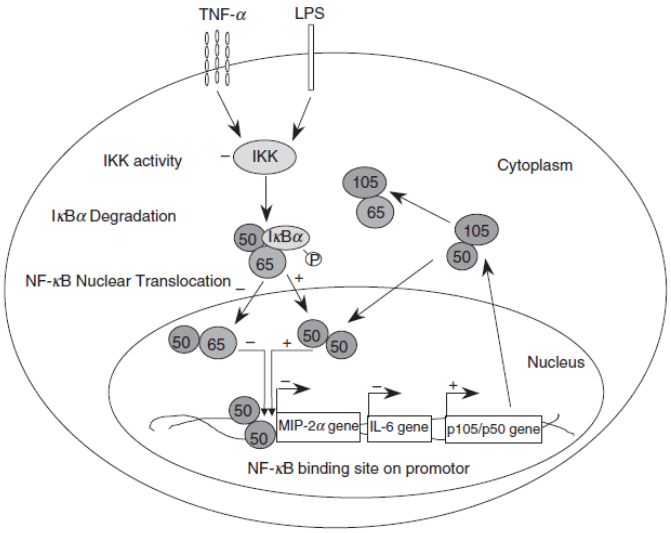

Figure 1.11: Molecular mechanisms used by IL-10 to inhibit NF-κB activity (adapted from ref. 74).

1.9 Regulation of IL-10 Production From Immune Cells

Macrophages, dendritic cells (DCs) and neutrophils have been shown to secrete IL-10 both *in vitro* and *in vivo* upon stimulation of relevant PRRs [75]. IL-10 production of antigen presenting cells is found to be stimulated by ligands for TLR2 as seen in the case of *Mycobacterium tuberculosis*-activated DCs [76, 77]. IL-10 production

pathways independent from TLR2 signaling have also been defined like the designated role of nucleotide-binding oligomerization domain 2 (NOD2) in macrophages activated by pneumoccocal cell wall [78]. LPS and CpG, ligands for TLR4 and TLR9, respectively, are also found to be alternative signals that elicit IL-10 production in macrophages, myeloid DCs and B cells [79, 80] In myeloid DCs, induction of IL-10 production can also be maintained through cell surface proteins DC-specific ICAM3-grabbing non-integrin (DC-SIGN) inducing RAF1 or PRR dectin-1 inducing spleen tyrosine kinase (SYK). Subsequent activation of ERK by SYK is required for IL-10 production [81, 82].

Upon stimulation through TLR, activation of ERK1/2 was found to be central for IL-10 production since lack of ERK of chemical suppression of phosphorylation capacity of ERK resulted in less IL-10 production [76, 83-84]. A correlation between expression and activation levels of ERK and IL-10 production levels has been revealed. Indeed, macrophages, that produce high amounts of IL-10, had high levels of ERK activation when compared to myeloid DCs which do produce moderate amounts IL-10 due to moderately activated ERK. Plasmacytoid DCs on the other hand, had no IL-10 production owing to low levels of activated ERK [84] (Fig. 1.12).

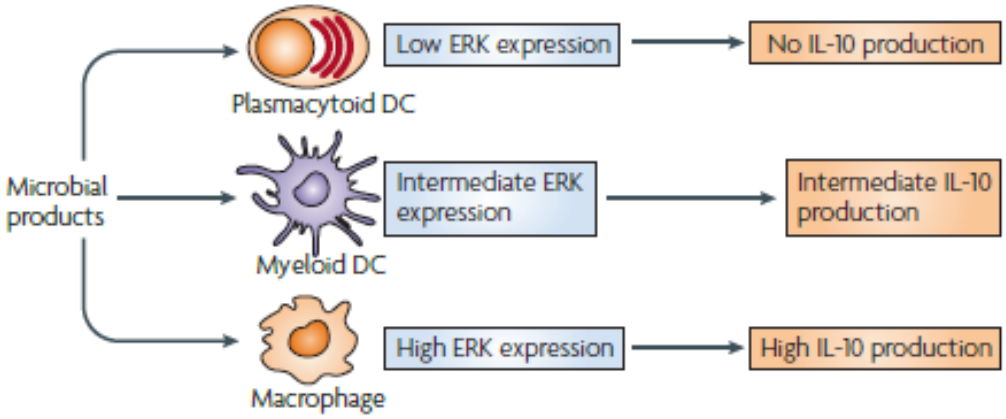

Figure 1.12: ERK expression-dependent IL-10 production from different immune cell types (adapted from ref. 75).

In macrophages and myeloid DCs TLR-mediated signals are relayed either in MyD88-dependent of MyD88-independent manner. MyD88-dependent pathway utilizes activation of MAPKs (p38 or ERK), PI3K or NF-κB whereas in MyD88-independent way TRIF-TRAF3 interactions are pivotal for IL-10 production. ERK

gets activated by tumor progression locus 2 (TPL2), a MAP3K. The interaction between p105 subunit of NF-κB and TPL2 is important for ERK activation. TLR-mediated signal enables TPL2 to detach from p105/TPL2 complex, which then can induce activation of ERK. p105/TPL2 complex protects of TPL2 from degradation, thereby ensures activation of ERK. Macrophages lacking either p105 or TPL2 were shown to produce less IL-10 upon TLR-stimulation when compared to wild-type controls [84-86]. CREB, as the common downstream effector of TLR-mediated signal relayed through different cascades is activated by phosphorylation at Ser133. PI3K activation leads to phosphorylation and activation of Akt. Akt, then phosphorylates GSK-3β. As previously mentioned, GSK-3β is active when unphosphorylated. Active GSK-3β is known to inhibit binding of CREB to DNA by phosphorylating CREB at Ser129 residue rendering it inactive [87]. Functional GSK-3β also inhibits DNA binding activity of AP1. Phosphorylation by Akt inactivates GSK-3β, enabling binding of CREB and AP1 to DNA for expression of IL-10 [88] (Fig. 1.13).

IL-10 production from APCs (macrophages and DCs) is under both negative and positive regulation. IFN-γ acts as negative regulator of both MAPK- and PI3K-mediated IL-10 production [88]. IFN-γ exerts its effect on GSK-3β by inhibiting its phosphorylation therefore blocking its inactivation. Active GSK-3β inhibits IL-10 expression. IL-10 induces a negative feedback loop on its own production through activation of dual-specificity phosphatase 1 (DUSP1). DUSP1 dephosphorylates, therefore inactivates p38 MAPK signaling [89]. Production of IL-10 is positively regulated through increased TPL2 expression [90]. Also, IL-10 leads to an increase of its own production via STAT3 activation [91]. Figure 1.13 demonstrates regulation of IL-10 production in antigen presenting cells of the innate immune system.

p65 subunit of NF-κB was found to interact with IL-10 coding DNA region in murine macrophages stimulated by LPS for IL-10 production [92]. On the other hand, p50 subunit of NF-κB was found to interact with promoter region of IL-10 in HuT78 cells, a human T cell lymphoma cell line [75]. p50/p50 homodimers, which were thought to have an inhibitory effect on NF-κB-induced gene expression, were found to induce IL-10 production in LPS-stimulated primary murine macrophages [93].

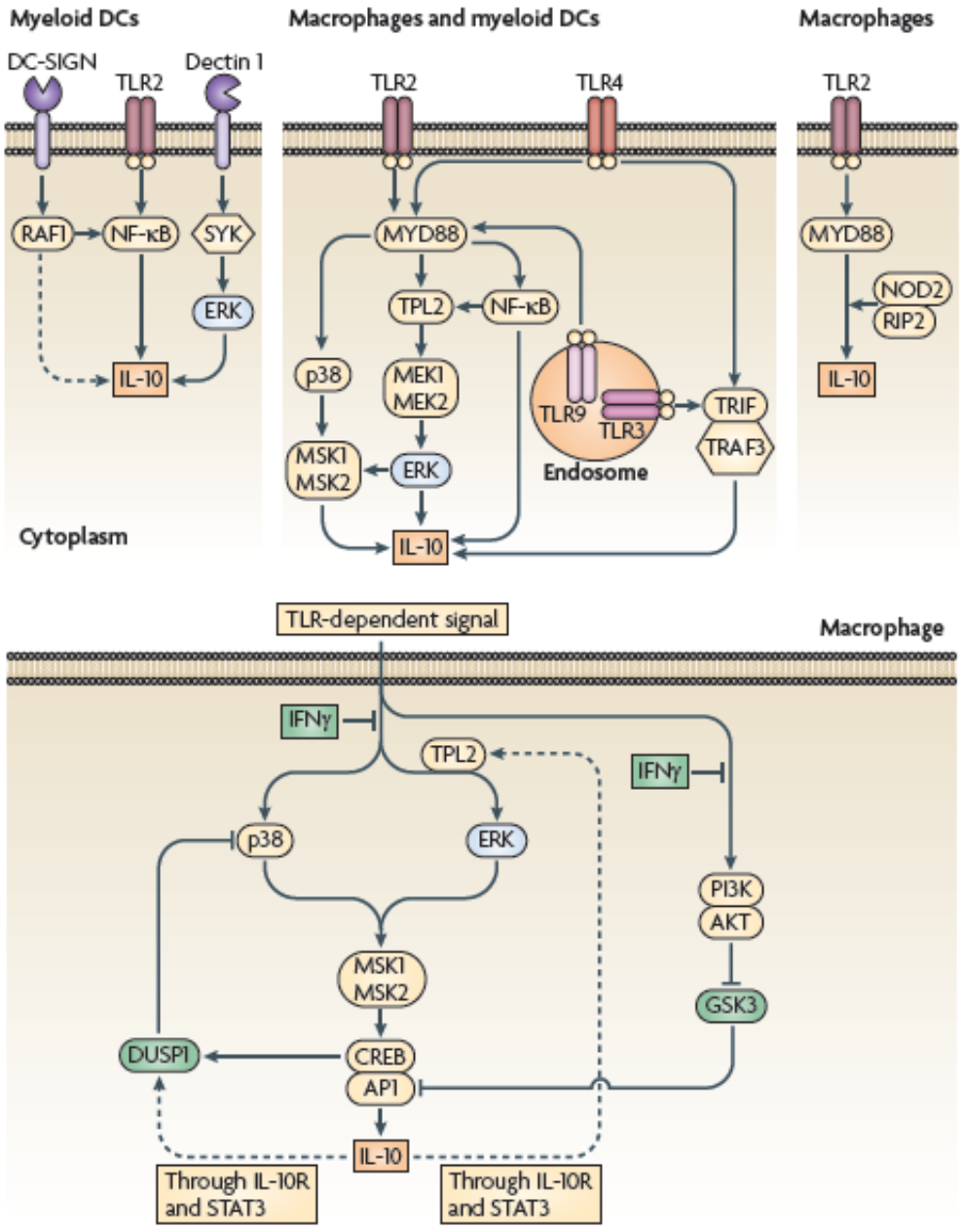

Figure 1.13: Regulation of IL-10 production in APCs of innate immune system (adapted from ref. 75).

$CD4^+$ Th1 cells are known for their signature pro-inflammatory immune response (IFN-γ secretion), therefore they do not normally secrete IL-10. However, it has been shown that high antigen exposure and IL-12 together can elicit IL-10 secretion from Th1 cells [94]. Th1 cells utilize ERK and STAT4 pathways for IL-10 expression [95]. Th2 cells produce IL-10 through IL-4 mediated ERK and STAT6 signaling. T cell receptor-triggered IL-10 production from Th17 subset depends on ERK and STAT3 signaling [75, 95-96]. ERK signaling for IL-10 production seems to be

shared by many immune cells. On the other hand, unlike macrophages and DCs, T helper subsets do not utilize p38 MAPK or GSK-3β signaling for IL-10 expression since abrogation of p38 or GSK-3β signaling with appropriate inhibitor did not result in alteration of IL-10 production detected by intracellular flow staining [95]. Figure 1.14 depicts IL-10 production pathways utilized by T helper cell subsets.

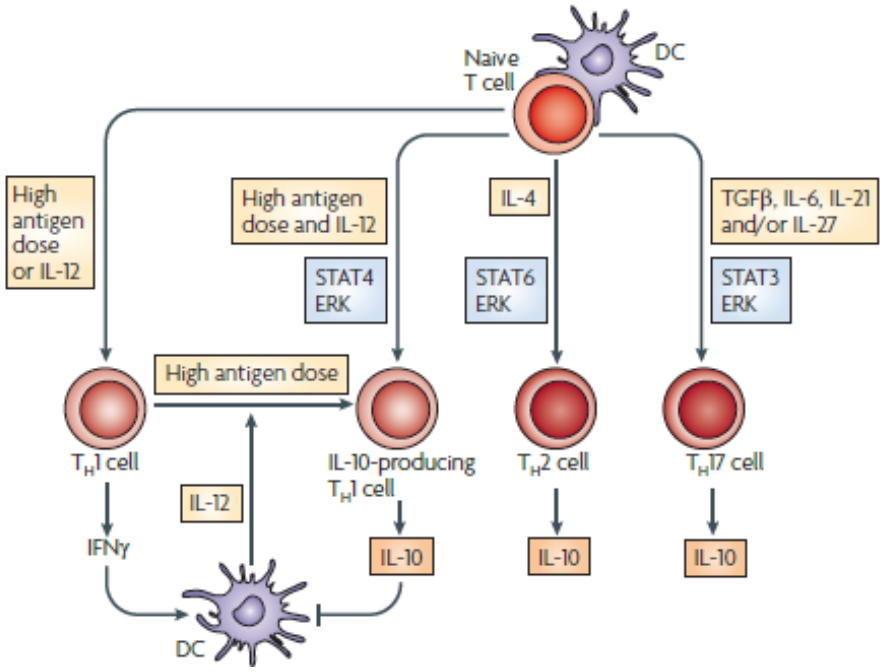

Figure 1.14: IL-10 production pathways in T helper cell subsets (adapted from ref. 75).

IL-10 has been shown to be produced by other immune cell subsets such as regulatory T cells (Treg), $CD8^+$ T cells, B cells. B cells are known to produce IL-10 via TLR4 or TLR9 stimulation [37, 96-99]. Recently B cell derived IL-10 production via TLR2 stimulation by *Helicobacter felis* was identified in mouse models [18]. Human B cells are shown to produce IL-10 upon stimulation with TLR7/8 and TLR9 ligands [100]. However, the intrinsic signaling pathways of IL-10 production in B cells have not been researched extensively. Recently, the role of p38 MAPK signaling pathway has been identified for murine B cell-derived IL-10 expression and secretion upon TLR4 and TLR9-stimulation [80]. In the case of human B cells, ERK and STAT3 pathways have been designated as central pathways for TLR7/8 or TL9-mediated IL-10 production [100]. Nevertheless, TLR2-mediated signal transduction pathways leading to IL-10 production and secretion still remain ill-defined.

1.10 Aim of the Study

B cells are known to produce IL-10 in TLR-dependent manner [75]. TLR2-, TLR4-, TLR7/8 and TLR9-stimulation elicit IL-10 production from B cells [75, 80, 100]. TLR4- and TLR9-stimulated murine B cells were found to utilize p38 MAPK module of MAPK pathway rather than ERK-1/2 module [80] whereas ERK-1/2 and STAT3 activation were required for induction of IL-10 production from TLR7/8-stimulated human primary B cells [100].

Helicobacter felis sonicate is recognized by TLR2 on B cells and elicits IL-10 production and secretion [18]. Even though TLR2-dependent IL-10 production from other APCs (macrophages and DCs) has been extensively researched [75], the intrinsic pathway(s) regulating IL-10 production from B cells upon TLR2-stimulation has/have not been clearly identified. The aim of this study is to identify and elucidate the signaling nodes that take part in TLR2-mediated IL-10 production and secretion *in vitro*. For this purpose, splenic murine B cells are stimulated with *Helicobacter felis* sonicate to determine activation of certain signaling kinases that take part in induction of IL-10 production. Based upon previous studies on IL-10 production from immune cells such as macrophages, dendritic cells, T helper cell subsets [75], MAPK/ERK, PI3K and NF-κB signaling pathways were chosen as the main focus of investigation. In order to further elucidate the role of putative signaling node(s) on IL-10 production from *Helicobacter*-activated B cells, these signaling pathways were blocked with specific chemical inhibitors and any change in IL-10 secretions were investigated.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

Helicobacter felis (H. felis) strain was kindly provided by Prof. Dr. Anne Müller from University of Zurich. They were grown in Columbia Agar plates supplemented with 1000X antibiotic cocktail. Ingredients of Columbia Agar (BD, U.S.A.) plate and 1000X antibiotic cocktail were given in Table 1 and Table 2, respectively. Solutions and chemicals that are used in maintenance of *Helicobacter felis* are given in Tables 1.1 and 2.1.

Table 2.1: Components of Columbia Agar Plates.

Component	Amount
Columbia Agar	42,5 g
Horse Blood	50 ml
β-cyclodexin	10 ml
1000X Antibiotic Cocktail	1 ml

Table 2.2: Components of 1000X Antibiotic Cocktail.

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

2.1.1.1 Antibiotics

Antibiotics that are used in *Helicobacter felis* culture are listed in Table 2.3.

Table 2.3: Antibiotics used in *Helicobacter felis* culture.

Content	Supplier Company
Trimethoprim	HiMedia
Amphotericin B	HiMedia

2.1.1.2 Liquid culture

Ingredients of liquid culture of *Helicobacter felis* are shown in Table 2.4 with a representative volume of 50 ml. The volume of ingredients may change depending on the required volume of components for proper growth of bacteria.

Table 2.4: Components of *Helicobacter felis* liquid culture.

Content	Amount
Brucella Broth	50 ml
FBS [10% (v/v)]	5 ml
Vancomysin (1000X)	5 µl

2.1.1.3 Freezing of *Helicobacter felis*

The medium suitable for freezing *Helicobacter felis* for stock purposes is depicted in Table 2.5 with its ingredients. Upon preparation the medium can be stored at 4°C.

Table 2.5: Freezing medium for *Helicobacter felis*.

Component	Amount
Brucella Broth	25 ml
Glycerol	25 ml

2.1.2 Primary cell line

$CD19^+$ splenic naive B cells were obtained from C57BL/6 mice via magnetic separation (Macs Miltenyi, Germany). Primary B cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, 1% Penicillin/Streptomycin. Purified $CD19^+$ B cells were stimulated with either *Helicobacter felis* sonicate, lipopolysaccharide (LPS) or CpG. CpG was kindly provided by Prof. Dr. İhsan Gürsel from Bilkent University, Ankara.

2.1.3 Buffers and solutions

Buffers and solutions that were used in different steps of this study are listed in Tables 2.6-2.9.

2.1.3.1 Cell culture

Culture media and solutions that were used in cell culture studies can be seen in Table 2.6 and buffers that are used in cell culture studies are listed in Table 2.7.

Table 2.6: Solutions and media used in cell culture studies.

Solution	Supplier Company
Roswell Park Memorial Institute (RPMI) Medium	Lonza
Fetal Bovine Serum (FBS) (10%)	Lonza
Penicillin/Streptomycin (1%)	Gibco
Trypan Blue	Lonza
DMSO	Fisher-Scientific

Table 2.7: Buffers used in cell culture studies.

Buffer	Content and Amount
PBS 1X	9,55 g in 1L ddH_2O
MACS Buffer	0.5% BSA 2 mM EDTA in PBS 1X
FACS Buffer	2% FBS in PBS 1X

2.1.3.2 Protein extraction

Components and amounts of Radioimmunoprecipitationassay (RIPA) Buffer that was used in extraction of total proteins from cell pellets are listed in Table 2.8.

Table 2.8: RIPA Buffer used in protein extraction.

Content	Amount
50 mM Tris-HCl (pH:7.4)	5 ml of 1M
1% NP-40	1 ml
0.5% Na-deoxycholate	2.5 g
0.1 % SDS	0.5 g
150 mM NaCl	3 ml of 5M
2 mM EDTA	0.4 ml of 0.5 M
50 mM NaF	1.05 g
ddH_2O	up to 100 ml

2.1.3.3 Western Blotting

Buffers and solutions that were used in Western Blotting studies are listed in Table 2.9.

Table 2.9: Buffers and solutions used in Western Blotting.

Buffer/Solution	Content and Amount
Protein Ladder (7-175 kDa)	New England Biolabs
Blocking solution	5% nonfat dry milk or 5% BSA in TBS/T

Table 2.9 (cont'd.): Buffers and solutions used in Western Blotting.

10% SDS-polyacrylamide separating 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 5 µl TEMED
5% SDS-polyacrylamide stacking gel	3.4 ml ddH_2O 0.83 ml 30% Acrylamide Mix 0.63 ml 1.0 M Tris (pH 6.8) 0.05 ml 10% SDS 0.05 ml 10% APS 6 µl TEMED
Ponceau S staining solution	0.1 g Ponceau S 5 ml Glacial Acetic Acid up to 100 ml ddH_2O
Running Buffer 10X (pH: 8.3)	15 g Tris base (25 mM) 71.2 g Glycine (192 mM) 50 ml 10% SDS (0.1%) up to 1 L ddH_2O
Transfer Buffer 10X	58.2 g Tris base 29.3 g Glycine 37.5 ml 10% SDS up to 1 L ddH_2O
Transfer Buffer 1X	100 ml Transfer Buffer 10X 200 ml Methanol 700 ml ddH_2O
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 ddH_2O
Tris Buffered Saline (TBS) 10X (pH: 7.4)	24.23 g Tris base 80.06 g NaCl up to 1 L ddH_2O
TBS/T 1X	100 ml TBS 10X in 1L ddH_2O 1 ml Tween-20

2.1.3.4 IL-10 ELISA

Solutions that were used in IL-10 ELISA studies are given in Table 2.10.

Table 2.10: Solutions used in IL-10 ELISA experiments.

Solution	Content
PBS/T 1X	0.05% Tween-20 in PBS 1X
Stop Solution	2N H_2SO_4 in ddH_2O

2.1.4 Equipment

Laboratory equipments which were used in this study are shown in Table 2.11 with their suppliers.

Table 2.11: Laboratory 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
Incubator with CO_2	BINDER
Vortex	Mixer Uzusio VTX-3000L,LMS
Quick spin	LMS
Magnetic stirrer	WiseStir MSH-20D, Wisd Laboratory Equipment
pH Meter	Mettler Toledo MP220
Light Microscope	Olympus CH30
Hemacytometer	Isolab
Ice Machine	Scotsman AF10
Freezers	Altus (+ 4 ^{0}C) Siemens (-20 ^{0}C) Haier (- 80 ^{0}C)
Power Supply	BIO-RAD
SDS-PAGE Gel Electrophoresis System	BIO-RAD
Shakers	Heidolp Duomax 1030
Kodak Medical X-Ray Processors	Kodak
Flow Cytometer	BD Accuri C6
Densitometer	GS-800
Thermo shaker	Biometra TS1 ThermoShaker
Sonicator	Bandelin Sonopuls
Nitrogen Tank	Air Liquid
Microplate Spectrophotometer	BIO-RAD Benchmark Plus
Tissue flask	Sarstedt
Serologic pipettes	Dispenser
Centrifuge tubes	Interlab
Eppendorf tubes (0.6 ml, 1.5 ml, 2 ml)	Interlab
Nitrocellulose membrane (0.2 µm pore size)	Santa Cruz
Scale	Precisa
Examination Gloves	Beybi
Cell strainer (70 µm)	BD

Table 2.11 (cont'd.): Laboratory equipment used in this study.

Tissue culture flasks (25 cm^2, 75 cm^2)	Sarstedt
Anaerobic Jar	Anaerocult
Whatman Filter Paper (3 mm)	Whatman
Erlens	Isolab
Falcons (15 ml, 50 ml)	Isolab
Slides	Interlab
Coverslips	Interlab
Cotton Swap	Interlab
96-well F plate (for ELISA studies)	Nunc

2.1.5 Commercial kits

Commercial kits that were used in this study are listed with their supplier companies in the table below.

Table 2.12: Commercial kits used in this study.

Kit	Supplier Company
Mouse B Cell Isolation Kit	MACS, Miltenyi Biotec
Mouse Regulatory B cell Isolation Kit	MACS, Miltenyi Biotec
BCA™ Protein Assay Reagent Assay	Thermo Scientific
20 X LumiGLO®Reagent and 20 X Peroxide	Cell Signaling
Mouse IL-10 Deluxe Max ELISA	Biolegend
PathScan® Signaling Nodes Multi-Target Sandwich ELISA Kit	Cell Signaling

2.1.6 General chemicals

General chemicals used in this study are listed with their supplier companies in Table 2.13.

Table 2.13: General chemicals used in this study.

Chemical	Supplier Company
Acetic Acid (glacial)	Merck
EDTA	Applichem
Ethanol (absolute)	Merck
NaCl	Merck
Glycerol	Merck
Phosphate-Buffered Saline (PBS) 10X	Lonza
Ammonium persulfate (APS)	Fisher-Scientific
Tween-20	Fisher-Scientific
Bovine Serum Albumin (BSA)	Santa Cruz
DMSO	Fisher Scientific

Table 2.13 (cont'd): General chemicals used in this study.

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
PMSF	Applichem
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
Lipopolysaccharide (LPS)	Sigma-Aldrich
Fixation Buffer 1X	Biolegend
Permeabilization Buffer 10X	Biolegend

2.1.7 Antibodies

Antibodies that were used in this study are given in Table 2.14.

Table 2.14: Antibodies used in this study.

Antibody/Inhibitor	Clone	Supplier Company	Application
Rabbit anti-CREB mAb	48H2	Cell Signaling	WB
Rabbit anti-Phospho-CREB (Ser133) mAb	87G3	Cell Signaling	WB
Mouse anti-GSK-3β mAb	3D10	Cell Signaling	WB
Rabbit anti-Phospho-GSK-3β (Ser9) mAb	D85E12	Cell Signaling	WB
Rabbit anti-p44/42 MAPK (Erk1/2) mAb	137F5	Cell Signaling	WB
Rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mAb	D13.14.4E	Cell Signaling	WB
Rabbit anti-Akt (Pan) mAb	C67E7	Cell Signaling	WB

Table 2.14 (cont'd.) : Antibodies used in this study.

Rabbit anti-Phospho-Akt (Thr308) mAb	C31E5E	Cell Signaling	WB
Rabbit anti-p38 MAPK mAb	D13E1	Cell Signaling	WB
Rabbit anti-Phospho-p38 MAPK (Thr180/Tyr182) mAb	D3F9	Cell Signaling	WB
Mouse anti-Vinculin mAb	hVIN-1	Sigma	WB
Anti-rabbit IgG, HRP-linked Antibody	-	Cell Signaling	WB
Anti-mouse IgG, HRP-linked Antibody	-	Cell Signaling	WB
Rat anti-mouse CD19-FITC	6D5	Biolegend	FACS
Rat anti-mouse IL-10-PE	Mouse Regulatory B Cell Isolation kit component	MACS Miltenyi	FACS
7-AAD Staining Solution	-	BD	FACS

2.1.8 Inhibitors

Inhibitors which were used in this study are listed with their suppliers and working concentrations in Table 2.15.

Table 2.15 : Inhibitors used in this study.

Inhibitor	Working concentration	Supplier Company
U0126 (MEK-1/2 inhibitor)	15 µM	Cell Signaling
SB203580 (p38 MAPK inhibitor)	10 µM	Cell Signaling
LY294002 (PI3K inhibitor)	15 µM	Cell Signaling
PDTC (NF-κB inhibitor)	30 µM	Tocris Bioscience

2.1.8.1 MEK-1/2 inhibitor (U0126)

5 mg of lyophilized MEK-1/2 inhibitor (U0126) was reconstituted in 1.31 ml of DMSO prior to use in order to get 10 mM stock solution. Stock solution was aliquoted and stored at -20°C. Working concentration of U0126 was 15 µM.

2.1.8.2 p38 MAPK inhibitor (SB203580)

5 mg of lyophilized p38 MAPK inhibitor (SB203580) was reconstituted in 1.32 ml of DMSO prior to use in order to get 10 mM stock solution. Stock solution was aliquoted and stored at -20°C. Working concentration of SB203580 was 10 μM.

2.1.8.3 PI3K inhibitor (LY294002)

1.5 mg of lyophilized PI3K inhibitor (LY294002) was reconstituted in 488 μL of DMSO prior to use in order to get 10 mM stock solution. Stock solution was aliquoted and stored at -20°C. Working concentration of LY294002 was 15 μM.

2.1.8.4 NF-κB inhibitor (PDTC)

50 mg of lyophilized NF-κB inhibitor (PDTC) was reconstituted in 3.04 ml of DMSO prior to use in order to get 100 mM stock solution. Stock solution was aliquoted and stored at -20°C. Working concentration of PDTC was 30 μM.

2.2 Methods

2.2.1 Maintenance of *Helicobacter felis*

Helicobacter felis was seeded on a Columbia blood agar containing appropriate antibiotics and incubated at 37°C under microaerophilic conditions in an anaerobic jar for 3-4 days. Microaerophilic conditions in anaerobic jar were maintained by utilization of CampyGen packs. For preparation of Columbia agars, 42,5 g Columbia agar was dissolved in 1000 ml water. Liquid was autoclaved. The bottle was put in 50 ^{0}C water for 1 hour. 50 ml horseblood was added to agar. 10 ml β-cyclodexin and 1 ml of 1000 X antibiotic cocktail (see Table 2.2) was added for *H. felis* growth. After 3-4 days, the grown bacteria was checked under light microscope for their viability and mobility and transferred into liquid Brucella Broth containing 10.000 X Vancomysin (final concentration: 1X) with necessary dilutions for optimal growth. For preparation of Brucella Broth medium, 28 g of brucella broth powder was resuspended in 1L of sterile distilled water (ddH_2O). Following resuspension, the liquid medium was autoclaved at 121°C for 15 min for sterilization.

2.2.2 Sonication of *H. felis* strain

Sonication procedure was initiated with 120-200 ml liquid culture of *Helicobacter* strains. Before sonication mobility and viability of *Helicobacter felis* (10 µl) were checked under light microscope. 120- 200 ml liquid culture of *Helicobacter felis* was aliqouted into 15 ml falcons. Falcons were centrifuged at 1500 rpm for 10 minutes. Supernatant was discarded. 10 ml PBS was used to wash bacteria. 15 ml falcons were centrifuged at 1500 rpm for 10 minutes. Supernatant was discarded. 3.5 ml PBS was added on pellet and mixed. Sonication was performed as 30 sec pulse on; 50 sec pulse off for 6.30 minutes at 50 watt on ice to prevent excessive heat formation. (MS 72 probe of the sonicator was used.) Sonicate was aliquoted to 1.5 ml eppendorfs at 500 µl for each. They were centrifuged at 15,000 rpm for 20 minutes at 4°C. Supernatants were transferred into new eppendorf tubes and labeled. Sonicate concentration was measured with BCA assay.

2.2.3 Protein bicinchoninic acid (BCA) assay

For determination of *Helicobacter felis* sonicates after sonication and determination of protein samples after cell lysis, Thermo Scientific's Pierce Protein BCA Assay Kit was used. Bovine Serum Albumin (BSA) Standard Set was chosen for microassay. According to the total number of samples and Bovine Serum Albumin (BSA) standards, working reagent (200 µl per sample) was prepared from Solution B and Solution A as 1:50 ratio, respectively and was warmed to ambient temperature. 200 µl of working reagent was distributed into each assayed well of a 96-well F-bottom plate, and all the samples and standards were used in duplicates in order to obtain more accurate results. 10 µl of diluted BSA standards in duplicates were added into corresponding working reagent-containing wells with the concentrations of 0,025; 0,125; 0,25; 0,5; 1; 1,5; 2 mg/mL, respectively. Dilution scheme for BSA standards are given in Table 2.16. 10 µl of protein samples (diluted of undiluted) with unknown concentrations were added to corresponding working reagent-containing wells, and plate was covered and incubated at 37°C for at least 30 min. After 30 min-long incubation, the plate was cooled to room temperature and absorbances were measured at 562 nm on microplate reader.

Table 2.16: 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

2.2.4 Treatment of purified B cells

Purified B cells were treated with *H. felis* sonicates with a final concentration of 10 µg/ml. They were incubated with or without *H. felis* sonicate for 24 h. After 24 h-long incubation, IL-10 producing regulatory B cell subset was magnetically labelled and separated from *H. felis* sonicate-treated cells by using Mouse Regulatory B Cell Isolation Kit (MACS Miltenyi, Germany) The procedures for both B cell and regulatory B cell isolations are explained in detail in upcoming sections. LPS and CpG were also used as stimulation agents for $CD19^+$ B cells for 48h-long stimulation. LPS was used at 10 µg/ml final concentration whereas CpG was used at 5 µg/ml final concentration in cell culture studies.

2.2.5 Mouse B cell isolation

Splenic B cells were isolated using Mouse B Cell Isolation Kit (MACS Miltenyi). The procedure is explained in detail in sections 2.2.5.1-2.2.5.4.

2.2.5.1 Cell preparation

Spleen obtained from a sacrificed C57BL/6 mouse was put in the center of 70 µm filter mesh carefully by the help of a tip. Spleen was meshed on the filter mesh with the plunger of a syringe single cell suspension of the spleen was prepared in a 50 ml falcon tube. Incomplete RPMI medium was used to soak filter and rinse cells from filter to the tube. Procedure was repeated for all obtained spleens. Tube was filled with incomplete medium. Cell suspension was centrifuged at 1480 rpm (200g) for 10

minutes. Supernatant was discarded. Cell pellet was dissolved in 1 ml MACS buffer per spleen. Cell number was determined. Cells were counted by diluting with MACS buffer (~1:200). Cell viability was checked with trypan blue.

2.2.5.2 Pre-enrichment of B cells

After cell number and viability was determined, cell suspension was centrifuged at 1780 rpm for 8 minutes. Cell pellet was resuspended in 40 µl of MACS buffer per 10^7 cells. Then 10 µl of B Cell Biotin-Antibody Cocktail (containing antibodies targeted against CD4, CD43 and Ter-119) was added per 10^7 cells. Tube was mixed well and incubated at refrigerator for 15 min. 30 µl of MACS buffer and 20 µl of Anti-Biotin MicroBeads were added per 10^7 total cells. Tube was mixed well and incubated in refrigerator for another 15 min. Cells were washed with 1-2 ml of MACS Buffer per 10^7 cells. Cell pellet was resuspended in 500 µl of MACS buffer per 10^8 cells.

2.2.5.3 Magnetic separation: depletion of non-B cells

LS column (if total cell number is less than $2x10^8$ MS column is more suitable for purification) was placed in suitable MACS separator (Midi) on magnetic field. Column was activated by rinsing with 3 ml of cold MACS buffer. Cell suspension was applied onto the column. Column was washed by 3x3 ml of MACS buffer and time was always given for the reservoir to be emptied between washing steps. Flowthrough was unlabeled B cells. Column was removed from magnetic field and put in a 15 ml falcon tube. The magnetically labeled non-B cells were flushed out with 5 ml of MACS buffer by firmly pushing the plunger of the column. Cell number was determined by diluting 1:10 with MACS buffer and cell viability was checked with trypan blue under light microscope. Flow cytometer analysis for CD19 surface marker was performed in order to determine purity of B cells.

2.2.5.4 Flow staining for CD19 surface marker

Purity of freshly purified splenic B cells was determined using flow cytometer. $5x10^5$ B cells and depleted non-B cells were stained with 0.2 µl FITC conjugated anti-CD19 antibody in 50 µl FACS Buffer in the dark on ice for at least 45 minutes. A fraction of B and non-B cells were left as unstained controls. Then, cells were washed once with 1 ml FACS Buffer by centrifugation at 3000 rpm for 8 minutes.

Supernatant was discarded and pellet was resuspended in 150 µl of FACS Buffer and samples (both unstained and stained) were analyzed on flow cytometer.

2.2.6 IL-10 producing regulatory B cell isolation

IL-10 producing subset of B cells was magnetically separated by Mouse Regulatory B Cell Isolation Kit (MACS Miltenyi). The detailed procedure for isolation of IL-10 producing regulatory B cells is explained in sections 2.2.6.1-2.2.6.6.

2.2.6.1 *In vitro* stimulation

Isolated $CD19^+$ B cells were centrifuged at 1780 rpm for 8 minutes. Cell pellet was resuspended in proper amount of medium ($2.5x10^6$ cells/ml). Cells were incubated in 96-well U bottom plates with stimulation agent (*Helicobacter felis* sonicate) for 24 h long incubation (Final concentration: 10 µg/ml). Cells which were not stimulated with sonicate were kept as control B cells. PMA (50 ng/ml) and ionomycin (500 ng/ml) were added for the last 5 hours of incubation in order to induce IL-10 secretion from *Helicobacter*-stimulated B cells.

2.2.6.2 Labeling cells with Regulatory B Cell Catch Reagent

Cells were harvested by collecting into a falcon tube by pipetting up and down. The wells also washed with MACS Buffer in order to ensure harvesting of all B cells. Cell number was determined by diluting 1:10 with MACS Buffer. For cell viability check, trypan blue staining was performed. Cells were washed by adding MACS buffer and centrifuged at 1780 rpm for 8 minutes. Supernatant was discarded carefully. Cell pellet was resuspended in 90 µl of cold medium per 10^7 total cells. 10 µl of Regulatory B Cell Catch Reagent was added per 10^7 total cells. Regulatory B Cell Catch Reagent is a specific molecule which has two binding domains one of which recognizes CD45 on B lymphocytes and the other recognizing IL-10. Tube was mixed well and incubated on ice for 5 min.

2.2.6.3 IL-10 secretion period

10 ml warm medium was added per 10^7 cells. Cells were incubated in closed tube for 45 min at 37 ^{0}C. Tube was turned upside down every 5 minutes to resuspend settled cells. In this period, the aim was basically to catch IL-10 secreted from *Helicobacter*-

stimulated B cells on cell surface following binding of catch reagent to CD45 surface marker on B cells.

2.2.6.4 Labeling cells with Regulatory B Cell Detection Antibody (PE)

The tube was filled with cold MACS buffer. The tube was incubated on ice for 5 min. in order to prevent non-specific antibody binding. Cells were centrifuged at 1780 rpm for 8 minutes. Supernatant was discarded carefully. Cell pellet was resuspended in 90 µl of MACS buffer per 10^7 total cells. A fraction of unlabeled cells were separated as unstained control. 10 µl of Regulatory B Cell Detection Antibody (PE) was added per 10^7 total cells. Tube was mixed well and incubated for 15 min on ice. In this step, IL-10 which was secreted from *Helicobacter*-stimulated B cells and caught on cell surface via catch reagent was labeled by a PE-conjugated IL-10 detection antibody. 10 ml of cold MACS buffer was added for washing and the tube was centrifuged at 1780 rpm for 8 minutes. Supernatant was discarded carefully.

2.2.6.5 Magnetic labeling with Anti-PE MicroBeads

Cell pellet was resuspended in 80 µl of cold buffer per 10^7 total cells. 20 µl of Anti-PE MicroBeads were added per 10^7 total cells. Tube was mixed well and incubated for 15 min in the refrigerator. This step enabled magnetic labeling of IL-10 producing *Helicobacter*-activated B cells by anti-PE Microbeads which is bound to PE-conjugated IL-10 detection antibody. Cells were washed by adding 10 ml of cold MACS buffer per 10^7 total cells. Tube was centrifuged at 1780 rpm for 8 minutes. Supernatant was discarded carefully. Cell pellet was resuspended in 500 µl of cold MACS buffer (up to $5x10^7$ cells, the working dilution was 10^8 cells/ml for higher cell numbers).

2.2.6.6 Magnetic separation using MS Column

MS column (up to $2x10^8$ total cells) was placed on mini MACS Separator in the magnetic field. Column was activated by rinsing with the 500 µl of MACS buffer. Cell suspension was applied onto the column. Unlabeled cells (IL-10 negative B cell fraction) were collected as flow-through and column was washed 3 times with 500 µl MACS buffer. Column was removed from separator and put on a falcon tube. 1 ml of MACS buffer was pipetted onto the column and magnetically labeled cells (IL-10 positive B cell fraction) were flushed out by plunger. Cell numbers were determined

by diluting 1:10 with MACS buffer and cell viability was checked with trypan blue under light microscope. Flow cytometer analysis was performed for IL-10-PE in order to check the purity of IL-10 positive and IL-10 negative fractions. A fraction of B+*H.f.* cells before addition of PE-coupled IL-10 detection antibody was separated as unstained control. The steps of procedures including splenic murine $CD19^+$ B cell isolation and separation of B cells according to their IL-10 production capacity following stimulation with *H. felis* sonicate for 24 hours are displayed in Figure 2.1.

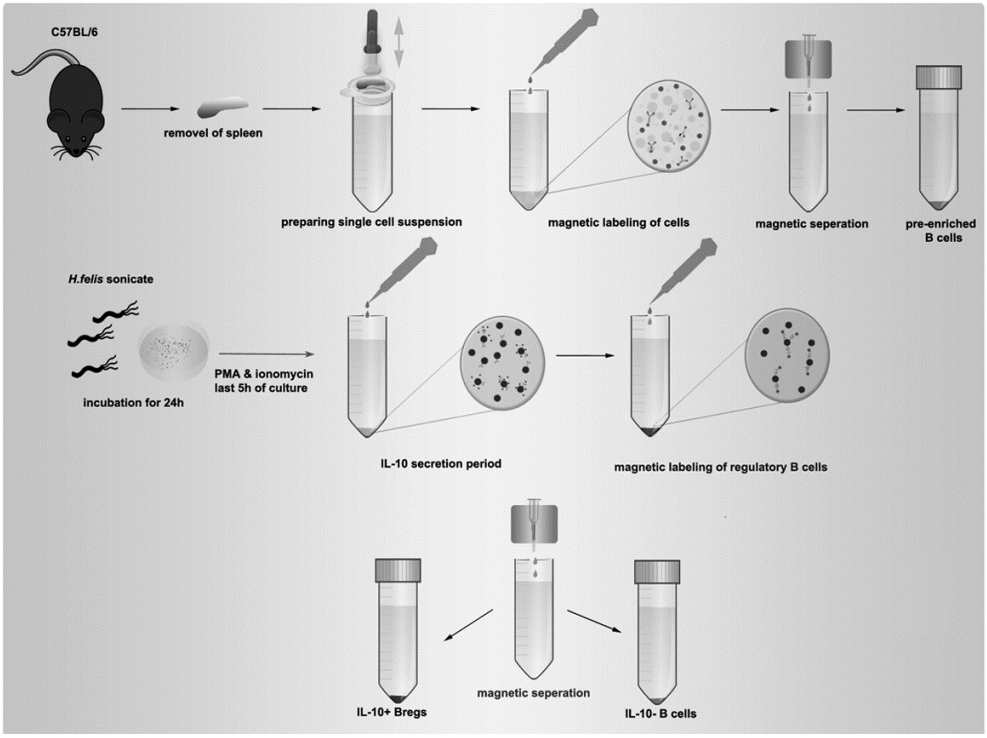

Figure 2.1: *Purification of murine splenic B cells and subsequent separation of B cells according to their IL-10-production capacities.* $CD19^+$ B cells were purified from murine splenic cells (splenocytes) by negative selection (*Macs Miltenyi*). All splenocytes except for $CD19^+$ cells were magnetically labeled by an antibody cocktail and $CD19^+$ B cells were acquired as flow through in a magnetic field. Purified B cells were cultured ($2{,}5x10^6$ cells/ml) in the presence of *H.felis* sonicate (10µg/ml) for 24 h. PMA (50 ng/ml) and ionomycin (500 ng/ml) were added into the culture during the last 5 h in order to induce IL-10 secretion. Following 24 h-long incubation, cells were harvested and labeled with a compound called Regulatory B Cell Catch Reagent (*Macs Miltenyi*) that was designed to capture secreted IL-10 at IL-10 secretion period on the cell surface. Then, cells were magnetically labeled with an PE-conjugated IL-10 detection antibody. Upon application of a magnetic field, IL-10 producing B cells ($IL\text{-}10^+$ B) were kept in the column (*Macs Miltenyi*) whereas IL-10 negative B cells ($IL\text{-}10^-B$) were acquired as flow-through. $IL\text{-}10^+$ B cells were obtained by subsequent flushing-out of cells from the column.

2.2.7 Intracellular flow staining for IL-10

In order to determine intracellular IL-10 production levels via flow cytometry, purified $CD19^+$ B cells were either treated with H. felis sonicate for 24 hours or left untreated. IL-10 secretion was blocked by 2 µM monensin 5 hours before harvesting of the cells. A fraction of ctrl B, B+*H.f.* cells were left as unstained controls. After harvesting of the cells by centrifugation at 3000 rpm for 8 minutes, cells were fixed with 100 µl of Fixation Buffer (Biolegend) for 20 minutes on ice. Following fixation, cells were washed with 500 µl of FACS Buffer once by centrifugation at 3000 rpm for 8 minutes. Supernatant was discarded and pellets were resuspended in 175 µl of 1X Permeabilization Buffer (diluted from 10X with ddH_2O). Cells were centrifuged at 3000 rpm for 8 minutes. Pellets were reconstituted in 175 µl of 1X Permeabilization Buffer once more. Cells were centrifuged at 3000 rpm for 8 minutes. Pellets were resuspended in 50 µl of permeabilization buffer containing 0.2 µl of PE-coupled anti-mouse IL-10 detection antibody (MACS Miltenyi) and samples were stained in the dark on ice for 1 hour. Following 1 hour long incubation on ice, cells were washed with 175 µl permeabilization buffer by centrifugation. Pellets were washed once in 500 µl of FACS Buffer by centrifugation and pellets were resuspended in 150 µl of FACS Buffer and analyzed on flow cytometer.

2.2.8 Protein extraction

For whole cell lysis, after snap-freezing cell pellets in liquid nitrogen, pellets were resuspended in appropriate amounts of RIPA Buffer containing freshly added 0.1 M PMSF (final concentration: 0.5 mM), 100X HALT Protease Inhibitor Cocktail (final concentration: 1X) and 10X Roche Phosphatase Inhibitor Cocktail (final concentration: 1X). After complete resuspension, samples were incubated on ice for at least 30 minutes. Then, they were centrifuged at 14,000 rpm for 10 min. Supernatants were aliquoted as total protein samples. Protein concentrations were determined by Protein BCA Assay and protein samples were stored at -80°C.

2.2.9 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis is a technique widely used for separation of proteins. The proteins are separated in SDS gel according to their molecular weight. In this study, 5% stacking gel and 10%

separating gels were used. Prepared gel mixtures were poured in between short plates and spacer plates (1 mm) and polymerized. Isopropanol was used to overlay separating gel in order to facilitate polymerization and ensure flattening the surface of the gel. For phosphorylation studies, since many of the phosphorylated and total forms of proteins gave bands at approximately at the same region, two separate gels were cast, one for total protein detection and the other for phospho-protein detection. After gels were polymerized, samples, that were mixed with 5X Laemmli Buffer and boiled at 100°C for 5 minutes, were loaded in corresponding wells of SDS gel. The amounts of loaded protein sample were ensured to be equal for each well. The gels were placed into the electrophoresis tank and protein samples were run in running buffer 1X. The running voltage was kept at 90V during the samples were running in the stacking gel (for approximately 30 minutes). After the samples were stacked, the voltage was increased up to 120V or 150V and samples were run for approximately 2 more hours ensuring the target protein would not run off the gel.

2.2.10 Western blotting

Western blotting is used to detect specific proteins from total protein repertoir of a cell. Following SDS-PAGE, SDS gels were removed from the tank and stripped off from short and spacer plates. Transfer of proteins from gel to the nitrocellulose membrane was performed by wet transfer (at 160V for 1h at 4°C). Following transfer, the transferred proteins were visualized by Ponceau S staining. Ponceau S staining was performed by incubating the newly-transferred nitrocellulose membrane in Ponceau S solution for at least 5 minutes with gentle shaking. Reversibly bound Ponceau S bands (reddish) were made visible following rinsing of the membrane in distilled water. After destaining in distilled water, the blots were blocked at 4°C for 1h in blocking solution (5% nonfat dry milk in TBS/T) to prevent non-specific binding.

Blocked membranes were then incubated overnight with relevant primary antibodies appropriately diluted in TBS/T containing 5% BSA at 4°C with gentle shaking. Following day, membranes were washed with TBS/T buffer 3 times for a total of 30 minutes with gentle shaking. Appropriately diluted secondary antibody was prepared in blocking solution, and membranes were incubated with relative secondary antibody for 1-2 hour at 4°C. After washing of the membrane with TBS/T for 3 x 10

miutes, 20X LumiGLO® Reagent was used as 1X diluted in distilled sterile water (ddH_2O) for the detection of proteins according to manufacturer's instructions. The membrane was exposed to X-ray film for a certain amount of time and then following enough exposure, the X-ray film was developed in Kodak Medical X-ray Processor according to manufacturer's instruction.

The antibodies that were used in western blotting studies are listed in Table 2.17 along with their working dilutions and supplier companies.

Table 2.17: Antibodies used in Western Blotting.

Antibody	Clone	Dilution	Supplier Company
Rabbit anti-CREB mAb	48H2	1:1000	Cell Signaling
Rabbit anti-Phospho-CREB (Ser133) mAb	87G3	1:1000	Cell Signaling
Mouse anti-GSK-3β mAb	3D10	1:1000	Cell Signaling
Rabbit anti-Phospho-GSK-3β (Ser9) mAb	D85E12	1:1000	Cell Signaling
Rabbit anti-p44/42 MAPK (Erk1/2) mAb	137F5	1:1000	Cell Signaling
Rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mAb	D13.14.4E	1:2000	Cell Signaling
Rabbit anti-Akt (Pan) mAb	C67E7	1:1000	Cell Signaling
Rabbit anti-Phospho-Akt (Thr308) mAb	C31E5E	1:1000	Cell Signaling
Rabbit anti-p38 MAPK mAb	D13E1	1:1000	Cell Signaling
Rabbit anti-Phospho-p38 MAPK (Thr180/Tyr182) mAb	D3F9	1:1000	Cell Signaling
Mouse anti-Vinculin mAb	hVIN-1	1:1000	Sigma
Anti-rabbit IgG, HRP-linked Antibody	2° Ab	1:3000	Cell Signaling
Anti-mouse IgG, HRP-linked Antibody	2° Ab	1:3000	Cell Signaling

2.2.11 Inhibitor studies

For inhibitor studies, purified $CD19^+$ splenic B cells ($2{,}5x10^6$ cells/ml) were pre-incubated with or without following inhibitors with indicated concentrations for duration of 1 h at 37°C. DMSO was also used at indicated concentrations as a vehicle control for each inhibitor.

- U0126 (MEK-1/2 inhibitor) (final concentration: 15 μM)

- SB203580 (p38 MAPK inhibitor) (final concentration: 10 μM)
- LY294002 (PI3K inhibitor) (final concentration: 15 μM)
- PDTC (NF-κB inhibitor) (final concentration: 30 μM)

Following 1 h-long incubation, B cells were either stimulated with *Helicobacter felis* sonicate (final conc: 10μg/ml) or left unstimulated for 24 h. After 24 h-long incubation, cells pellets were harvested and stored at -80°C for further protein isolation, and cell culture supernatants were aliqouted for further IL-10 ELISA experiments.

2.2.12 IL-10 ELISA

IL-10 protein levels in supernatant of samples were determined by Biolegend's Mouse IL-10 ELISA Deluxe Max Kit. For quantitative determination of IL-10 protein in culture medium of B cells, Nunc 96-well plates were coated with IL-10 capture antibody 1:200 diluted in coating buffer 1X and plate was incubated at 4°C overnight. Following day, plate was washed four times with PBS/T. Then, 100 μl of Assay Diluent A 1X (diluted from 5X with PBS 1X) was added into assayed wells and the plate was incubated at room temperature for 1h. Recombinant IL-10 standards were prepared by serial dilution according to manufacturer's instructions. After that, plate was again washed four times with PBS/T. Following washing steps, diluted recombinant IL-10 standards and culture media supernatants (50 μl) were added as biological duplicates. Plate was incubated at room temperature for 2 h. Following 2 h-long incubation, plate was washed four times with PBS/T. Biotinylated IL-10 detection antibody diluted 1:200 in Assay Diluent 1X was added into each assayed well and plate was incubated at room temperature for 1 h. After washing of assayed wells with PBS/T for three times, Avidin-HRP solution 1:1000 diluted in Assay Diluent A 1X was added into each assayed well as 50 μl and plate was incubated in the dark for 30 min at room temperature. After 30 min-long incubation, plate was washed for five times with PBS/T and 50 μl of TMB Substrate Solution Mixture (1:1 of TMB Substrate A and TMB Substrate B) was added into each assayed well. The plate was incubated for at least 30 minutes at room temperature. High concentration standards and samples turned into blue. After that, the reaction was stopped with 50 μl stop solution (2N H_2SO_4). Then, the absorbances of the samples were measured at 450 nm on a microplate reader.

2.2.13 PathScan® Signaling Nodes Multi-Target Sandwich ELISA

Cell Signaling's Pathscan Signaling Nodes Multi-Target Sandwich ELISA Kit is a complex kit which enables detection of many key signaling molecules such as phospho-Akt, phospho-p38 MAPK, phospho-MEK-1/2, phospho-STAT3, phospho-NF-κB p65. Only p-STAT3 and phospho-NF-κB p65 detection was utilized for this study. The wells on strips of the microplate were pre-coated with either anti-STAT3 antibody or anti-phospho-NF-κB p65 antibody. After the microwells reached room temperature, required strips were separated and the rest of the strips were sealed and incubated at 4°C. The protein samples of ctrl B, B+*H.f.*, *Helicobacter*-activated IL-10^- B and IL-10^+ B were diluted with sample diluent provided by the kit. They were assayed at protein concentration of approximately 0.15 mg/ml. 75 µl of diluted protein samples were added into appropriate wells as duplicates. The wells were sealed with the sealing tape. The plate was incubated overnight at 4°C. Following overnight incubation, the plate was washed 4 times with 1X wash buffer (diluted from 20X wash buffer provided with ddH_2O). After each wash, the plate was tapped upside down on fresh towels, but the wells were not allowed to dry between wash steps. After washing step, 50 µl of detection antibody solution was added into corresponding wells. Matching of the color code on the cap of the detection antibody and color code of the 8-well strip was ensured. After that, the plate was sealed again and incubated at 37°C for 1 hour. Following 1-hour-long incubation, the plate was washed again for 4 times. After washing steps, 50 µl of HRP-linked secondary antibody was added into corresponding wells. Matching of the color code on the cap of the HRP-linked secondary antibody and color code of the 8-well strip was ensured once again. The wells were sealed with tape and the plate was incubated at 37°C for 30 minutes. Following 30-minutes- long incubation, 50 µl of TMB substrate was added into each well. The wells were sealed with tape and incubated at 25°C for 30 minutes. After 30 minutes, the reaction was stopped by addition of 50 µl of stop solution provided by the kit. Color changing from blue to yellow was observed upon stop solution addition. After stopping of the reaction, the absorbance values of the samples were determined at 450 nm on a microplate reader.

2.2.14 Flow cytometry analyses

Flow cytometry analyses were performed using *FlowJo* software.

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 (vinculin). 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 (vinculin 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, n.d. denotes not determined.

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.d. stands for not determined, and n.s. denotes not significant.

3. RESULTS

3.1 Isolation of Splenic Naive B Cells From C57BL/6 Mice

B cells were purified from spleen of C57BL/6 mice by negative selection. Negative selection involves magnetic labeling of total splenic cells (splenocytes) except for B cells and acquiring unlabeled B cells as flow-through upon exposure of splenocytes to magnetic field. Splenic B cell purity was determined by B cell-specific surface marker CD19 surface staining upon isolation of $CD19^+$ B cells. Figure 3.1 shows a representative flow staining of purified B cells compared to unstained control. Purity of isolated and cultured B cells was more than 97 %.

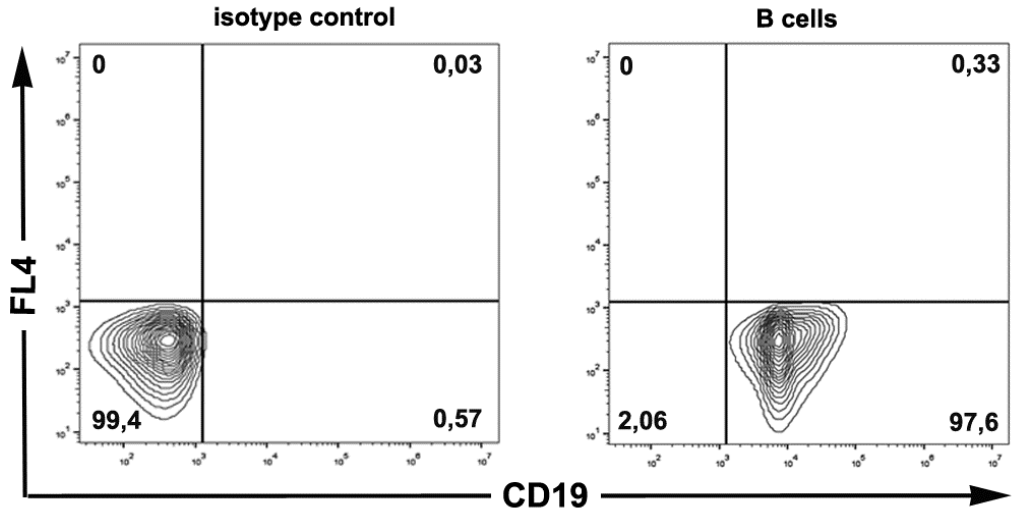

Figure 3.1: *Purity of B cells isolated from spleen of C57BL/6.* Representative flow cytometry plot of purified B cells compared to unstained control. B cells were magnetically isolated from spleen of C57BL/6 mice by negative selection. $5x10^4$ of isolated cells were either stained with FITC-coupled anti-CD19 antibody (0.5 µg/µl) or left unstained (isotype control) in order to detect the percentage of cells expressing B cell-specific surface marker CD19. Values in quadrants indicate percentages of cells.

3.2 Purity of IL-10$^-$ B and IL-10$^+$ B Cells Magnetically Separated Upon *Helicobacter felis (H. felis)* Sonicate Treatment

In order to obtain IL-10-producing B cells upon *Helicobacter*-sonicate treatment, B cells were subjected to magnetic separation (explained in detail at section 2.2.6) following 24h-long incubation with *H.felis* sonicate treatment. Since the fundamental logic of the separation was capturing secreted IL-10 on the surface of IL-10 secreting B cells with an anti-IL-10-PE antibody, determining the purity of separated fractions (IL-10 negative B cells and IL-10 positive B cells) depended upon PE flow readings (Fig. 3.2). Purity of magnetically separated *Helicobacter*-activated IL-10$^+$ B cells and IL-10$^-$ B cells were >84% and >86%, respectively.

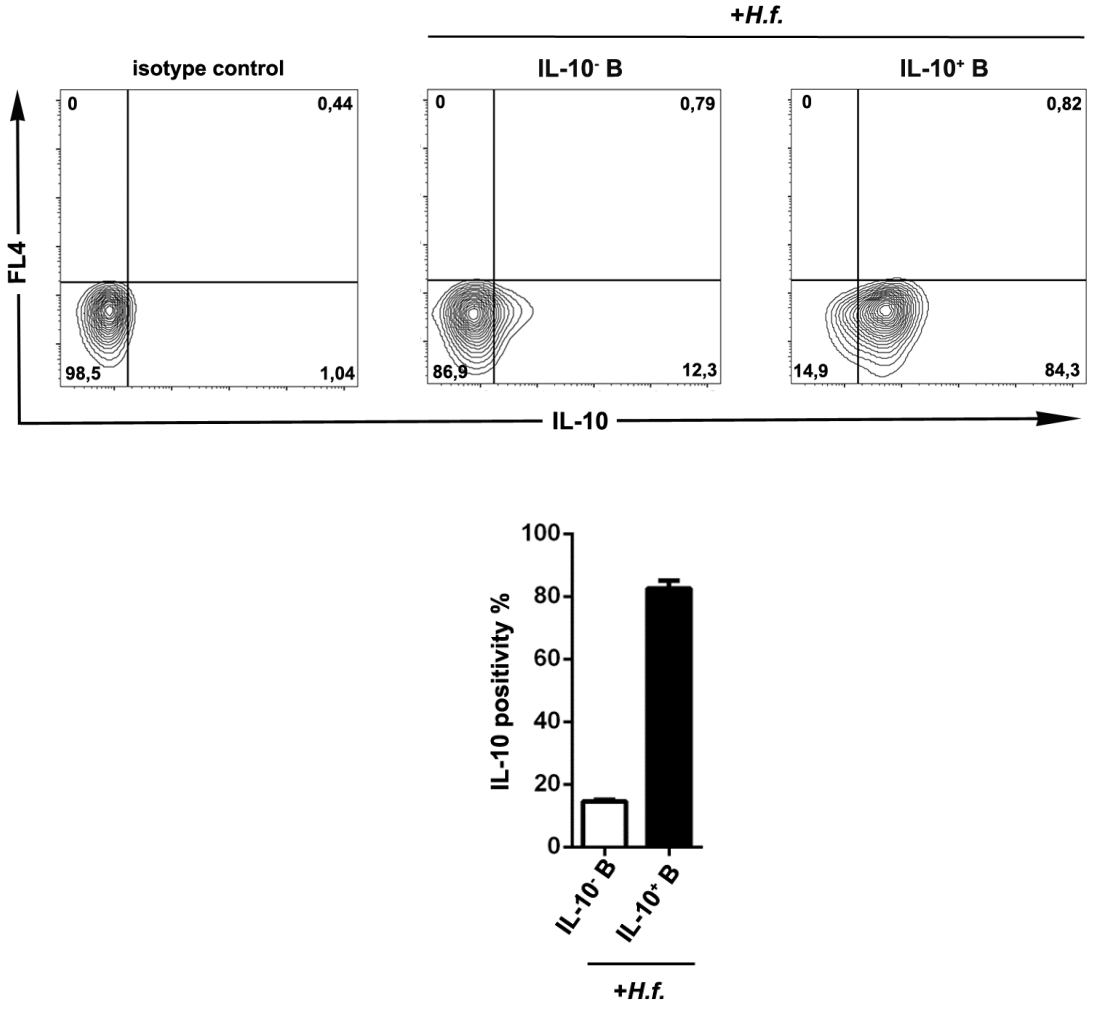

Figure 3.2: *Purity of IL-10$^-$ B and IL-10$^+$ B cells magnetically separated from splenic B cells following H. felis sonicate treatment.*

Figure 3.2 (cont'd.): *Purity of IL-10$^-$ B and IL-10$^+$ B cells magnetically separated from splenic B cells following H. felis sonicate treatment.* IL-10 secretion from B cells was induced by addition of PMA (50 ng/ml) and ionomycin (500 ng/ml) during the last 5 h of 24 h-long incubation with *H.felis* sonicate at 10 µg/ml final concentration. *H.felis* sonicate-stimulated B cells were then magnetically separated into IL-10$^-$ B and IL-10$^+$ B cell fractions and IL-10 levels of these fractions compared to unstained isotype control were determined by flow cytometry. *Upper panel* shows representative flow cytometry plots of three independent experiments (n=3). Values in quadrants indicate percentages of cells. *GraphPad Prism 5.0* was used to graph average percentage for IL-10 positivity of each fraction (*lower panel*).

3.3 Assessment of IL-10 Production From *Helicobacter felis* - treated B Cells

In order to determine IL-10 production levels of *H. felis* - treated B cells compared to untreated control, CD19$^+$ splenic B cells, were cultured as untreated (ctrl B) or treated with *H. felis* sonicate (B+*H.f.*) for 24 h. IL-10 production levels were determined by intracellular flow staining (Fig. 3.3).

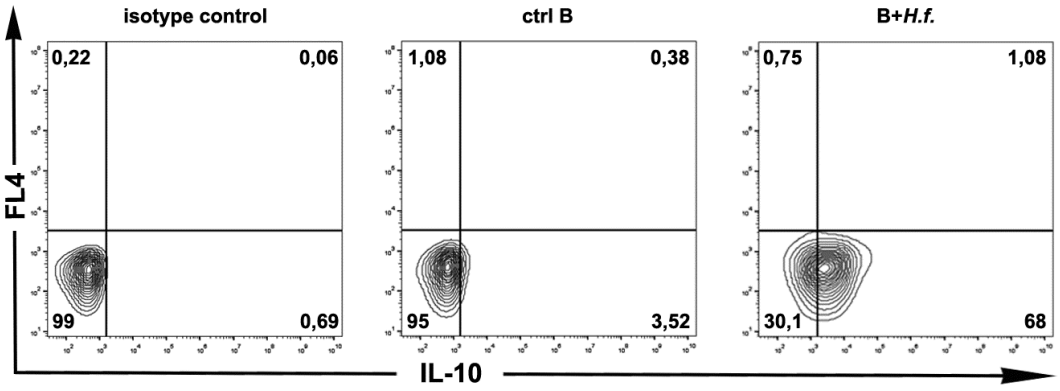

Figure 3.3: *IL-10 production levels of untreated splenic B cells and B cells treated with H. felis sonicate.* B cells were either treated with *H.felis* sonicate at 10 µg/ml final concentration (B+*H.f.*) or left untreated (ctrl B) for 24 h. Monensin (2 µM) was added into culture medium during last 5 h of incubation. Following fixation and permeabilization, cells were either stained with PE-coupled anti-IL-10 antibody (0.2 µg/µl) or left unstained (isotype control) in order to detect percentage of cells producing IL-10. Values in quadrants indicate percentages of cells.

According to flow readings, 67,08% of *H. felis* sonicate–treated B cells were found to be positive for IL-10 whereas 3,8% untreated B cells were positive for IL-10 after

24 hour-long incubation. These results suggest that B cells produce IL-10 upon stimulation with *Helicobacter felis* sonicate.

3.4 Assessment of IL-10 Secretion From *Helicobacter felis* - treated B Cells

B cells were shown to produce IL-10 upon *Helicobacter*-stimulation (Fig. 3.3). In order to determine IL-10 secretion levels of *H. felis* sonicate - stimulated B cells compared to untreated control, $CD19^+$ splenic B cells were cultured as untreated (ctrl B) or treated with *H. felis* sonicate (B+*H.f.*) for 24h. Supernatants of cultured B cells were then investigated for secreted amounts of IL-10 (Fig. 3.4).

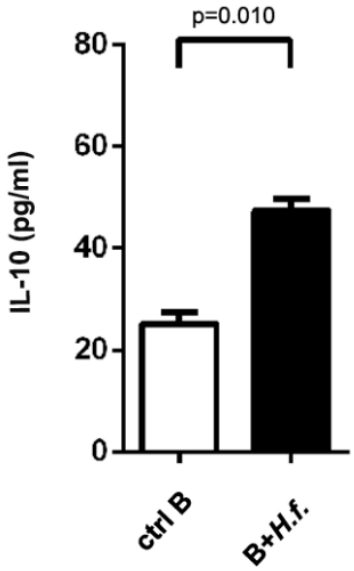

Figure 3.4: *IL-10 secretion levels of untreated splenic B cells and B cells treated with H. felis sonicate.* B cells were either treated with *H.felis* sonicate at 10 µg/ml final concentration (B+*H.f.*) or left untreated (ctrl B) for 24 h. Following 24 h-long incubation, cell culture supernatants were probed for IL-10 by IL-10 ELISA. Data are representative of three independent experiments (n=3). *GraphPad Prism 5.0* was used to graph mean levels of secreted IL-10 amount. Statistical analysis was conducted using Student's *t* test.

IL-10 ELISA results indicated that B cells treated with *H. felis* sonicate secreted significantly more IL-10 (2-fold) than untreated B cells. Results of intracellular staining for IL-10 and IL-10 ELISA collectively demonstrated that splenic B cells could be induced to produce and secrete IL-10 by stimulation with *Helicobacter felis* sonicate.

3.5 Assessment of IL-10 Secretion Levels of *Helicobacter*-activated IL-10$^-$ B and IL-10$^+$ B Cells

In order to determine IL-10 secretion levels of *Helicobacter*-activated IL-10$^-$ B and IL-10$^+$ B cells, magnetically separated IL-10$^-$ B cells and IL-10$^+$ B cells were further cultured with *H. felis* sonicate for 48h. Supernatants of cultured cells were then investigated for secreted amounts of IL-10 (Fig. 3.5).

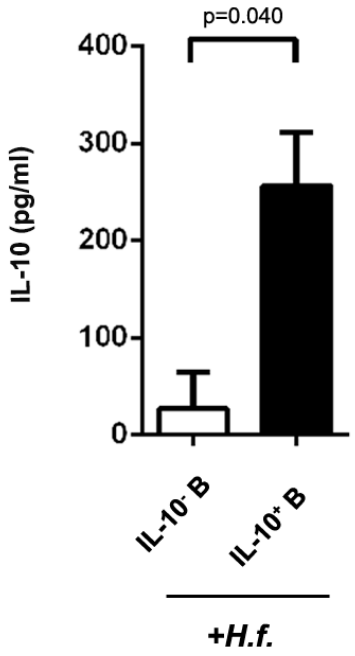

Figure 3.5: *IL-10 secretion levels of Helicobacter-activated IL-10$^-$ B and IL-10$^+$ B cells treated with H. felis sonicate for 48 h.* B cells were treated with *H.felis* sonicate at 10 µg/ml final concentration for 24 h. Following 24 h, *Helicobacter*-activated B cells were magnetically separated into IL-10$^-$ B cells and IL-10$^+$ B cells. IL-10$^-$ B and IL-10$^+$ B cells were cultured in the presence of *H.felis* sonicate (10µg/ml) for 48 h. Following 48 h-long incubation, cell culture supernatants were probed for IL-10 by IL-10 ELISA. Data are representative of three independent experiments (n=3). *GraphPad Prism 5.0* was used to graph mean levels of secreted IL-10 amount. Statistical analysis was conducted using Student's *t* test.

ELISA results indicated that secreted IL-10 was significantly more (8-fold) in IL-10$^+$ B cells compared to IL-10$^-$ B cells. This result was expected since magnetic separation of *Helicobacter*-activated B cells was performed depending on IL-10 production capacity of these subsets. Secretion of IL-10 from IL-10$^-$ B cells upon stimulation with *H.felis* sonicate may be attributed to expansion of 12% IL-10 producing B cells in IL-10$^-$ B fraction that was observed as impurity (Fig. 3.2).

3.6 The Role of MAPK/ERK Pathway on IL-10 Production From *Helicobacter*-stimulated B Cells

3.6.1 The role of p38 MAPK on IL-10 production

Phosphorylation, therefore activation of p38 MAPK was found to be required for IL-10 production from TLR-stimulated macrophages, DCs and TLR4- or TLR9-stimulated primary murine B cells [75, 80]. In order to determine the role of p38 MAPK on induction of IL-10 production from *Helicobacter*-activated B cells, protein samples of ctrl B, B+*H.f., Helicobacter*-activated IL-10$^-$ B cells and IL-10$^+$ B cells were investigated for phosphorylation of p38 MAPK by Western Blotting (Fig. 3.6).

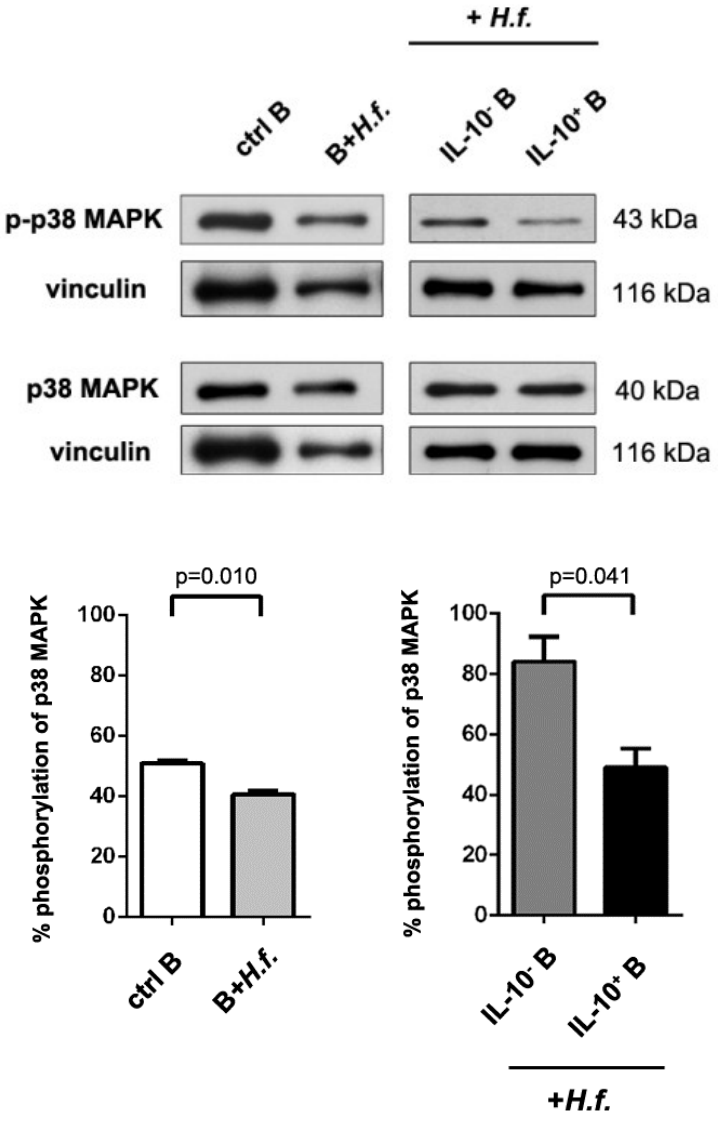

Figure 3.6: *Assessment of phosphorylation levels of p38 MAPK.*

Figure 3.6 (cont'd.): *Assessment of phosphorylation levels of p38 MAPK.* Splenic B cells were stimulated with *H. felis* sonicate for 24 h (B+*H.f.*). Control B (ctrl B) cells did not receive any treatment. Upon addition of PMA (50ng/ml) and ionomycin (500ng/ml) during last 5h of 24h-long incubation, *H. felis*-stimulated cells were magnetically separated as IL-10^- B and IL-10^+ B cells. Cells were lysed and whole cell extracts were immunoblotted for p-p38 MAPK and p38 MAPK. Vinculin was used as loading control. Western Blotting results (*upper panel*) are representative of three independent experiments (n=3). *GraphPad Prism 5.0* was used to graph densitometric analysis results (*lower panel*). Statistical analysis was conducted using Student's *t* test.

Western Blotting results indicated that p38 MAPK was significantly less phosphorylated (~40%) in *H.felis*-stimulated B (B+*H.f.*) cells when compared to unstimulated B (ctrl B) cells (~50%). Comparison of p38 MAPK phosphorylation between *Helicobacter*-activated IL-10^- B and IL-10^+ B cells revealed that IL-10^- B had significantly more phosphorylated p38 MAPK (~80%) than IL-10^+ B cells (~50%). Phosphorylation percentages were found to be higher in *Helicobacter*-activated IL-10^- B and IL-10^+ B fractions when compared to B+*H.f.* sample possibly due to the induction of these fractions with PMA and ionomycin. This result suggests that activation of p38 MAPK may play a more prominent role in *Helicobacter*-activated IL-10^- B cells rather than IL-10^+ B.

3.6.2 The role of MEK-1/2 on IL-10 production

ERK-1/2 activation was shown to be required for induction of IL-10 production from macrophages, DCs and T helper subsets (Th1, Th2, Th17) [75]. MEK-1/2 is upstream activator of ERK-1/2 in ERK-1/2 signaling module of MAPK pathway [52]. In order to determine the role of MEK-1/2 on induction of IL-10 production from *Helicobacter*-activated B cells, whole cells extracts of ctrl B, B+*H.f.*, *Helicobacter*-activated IL-10^- B cells and IL-10^+ B cells were examined for phosphorylation of MEK-1/2 by Western Blotting (Fig. 3.7).

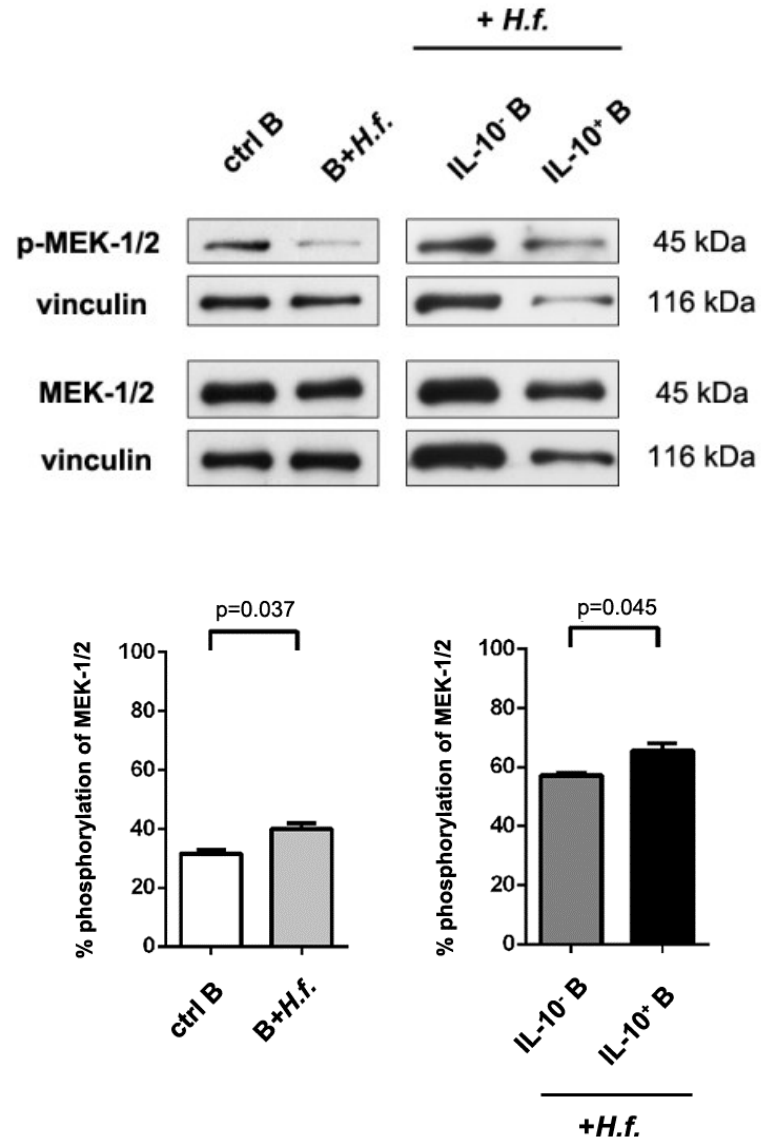

Figure 3.7: *Investigation of phosphorylation levels of MEK-1/2.* Splenic B cells were stimulated with *H. felis* sonicate for 24 h (B+*H.f.*). Control B (ctrl B) cells did not receive any treatment. Upon addition of PMA (50ng/ml) and ionomycin (500ng/ml) during last 5h of 24h-long incubation, *H. felis*-stimulated cells were magnetically separated as IL-10^{-} B and IL-10^{+} B cells. Cells were lysed and whole cell extracts were immunoblotted for p-MEK-1/2 and MEK-1/2. Vinculin was used as loading control. Western Blotting results (*upper panel*) are representative of three independent experiments (n=3). *GraphPad Prism 5.0* was used to graph mean levels of MEK-1/2 phosphorylation (*lower panel*). Statistical analysis was conducted using Student's *t* test.

Western Blotting results indicated phosphorylation of MEK-1/2 was significantly more B+*H.f.* cells when compared to ctrl B cells (40% and 30%, respectively). Furthermore, MEK-1/2 was significantly more phosphorylated in *Helicobacter*-

activated IL-10$^+$ B cells compared to IL-10$^-$ B cells (65% and 56%, respectively). Phosphorylation percentages were found to be higher in *Helicobacter*-activated IL-10$^-$ B and IL-10$^+$ B fractions when compared to B+*H.f.* sample possibly due to the induction of these fractions with PMA and ionomycin. This result suggests that activation of MEK-1/2 may play a more prominent role in induction of IL-10 production from *Helicobacter*-activated B cells.

3.6.3 The role of p44/42 MAPK (ERK-1/2) on IL-10 production

Many immune cells such as antigen presenting cells of innate immune system (DCs and macrophages), T helper cells and TLR7/8-stimulated primary human B cells were found to utilize ERK-1/2 signaling module of MAPK pathway in IL-10 production [75, 100]. In order to elucidate the role of ERK-1/2 on induction of IL-10 production from *Helicobacter*-activated B cells, whole cells extracts of ctrl B, B+*H.f., Helicobacter*-activated IL-10$^-$ B cells and IL-10$^+$ B cells were investigated for phosphorylation of ERK-1/2 by Western Blotting upon utilizing antibodies against total- and phosphorylated forms of ERK-1/2 (Fig. 3.8).

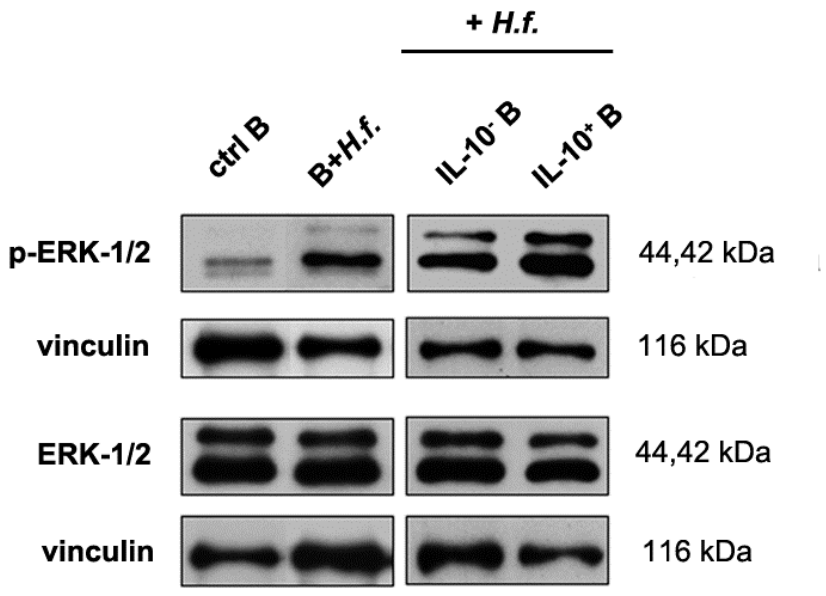

Figure 3.8: *Assessment of phosphorylation levels of ERK-1/2.*

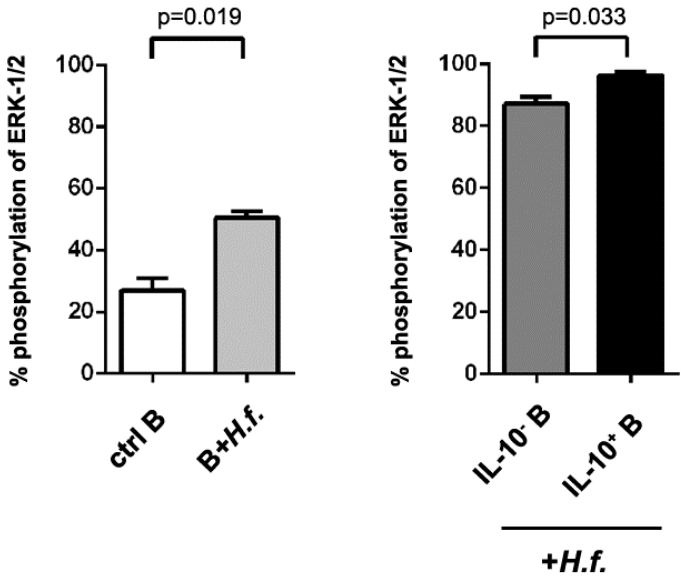

Figure 3.8 (cont'd.): *Assessment of phosphorylation levels of ERK-1/2.* Splenic B cells were stimulated with *H. felis* sonicate for 24 h (B+*H.f.*). Control B cells (ctrl B) did not receive any treatment. Upon addition of PMA (50 ng/ml) and ionomycin (500 ng/ml) during last 5 h of 24 h-long incubation, *H. felis*-stimulated cells were magnetically separated as IL-10^{-} B and IL-10^{+} B cells. Cells were lysed and whole cell extracts were immunoblotted for phospho- and total-ERK-1/2. Vinculin was used as loading control. Western Blotting data (*upper panel*) from one of three independently conducted experiments are shown (n=3). *GraphPad Prism 5.0* was used to graph mean levels of ERK-1/2 phosphorylation obtained by densitometric analysis (*lower panel*). Statistical analysis was conducted using Student's *t* test.

According to total- and phospho-ERK-1/2 Western Blotting results, ERK-1/2 phosphorylation was found to be significantly elevated in B+*H.f.* cells when compared to unstimulated control (from 25% to 50%, respectively). ERK-1/2 was found to be highly phosphorylated in both fractions of PMA and ionomycin-stimulated *Helicobacter*-activated B cells. Comparison of ERK-1/2 phosphorylation between *Helicobacter*-activated IL-10^{-} B and IL-10^{+} B samples demonstrated that IL-10^{+} B cells had significantly more phosphorylated ERK-1/2 than IL-10^{-} B cells (85% and 97%, respectively). PMA and ionomycin induction was the possible reason underlying the increase in phosphorylation percentages of *Helicobacter*-activated IL-10^{-} B and IL-10^{+} B compared to B+*H.f.* cells.

Considering that ERK-1/2 is a downstream effector of MEK-1/2, the similar trend of MEK-1/2 and ERK-1/2 phosphorylation for same samples was expected. And taken

together, these results further suggest that activation of MEK-ERK pathway is required for induction of IL-10 production from *Helicobacter*-activated B cells.

3.6.4 The role of CREB as a downstream effector of MAPK signaling pathway on IL-10 production

As a transcription factor, CREB is known to be a downstream effector of both signaling modules (p38 MAPK and ERK-1/2) of MAPK signaling pathway which plays an important role in transcription of IL-10 in TLR-stimulated macrophages. Activated CREB gets translocated into the nucleus with AP-1 and binds to its response element cAMP response element (CRE) to initiate the transcription of IL-10 gene [75]. In order to determine the role of CREB on induction of IL-10 production from *Helicobacter*-activated B cells, whole cell extracts of ctrl B, B+*H.f.*, *Helicobacter*-activated IL-10$^-$ B cells and IL-10$^+$ B cells were investigated for CREB phosphorylation via Western Blotting by using antibodies targeted against total- and phospho-CREB (phosphorylated at Ser133 residue) (Fig. 3.9).

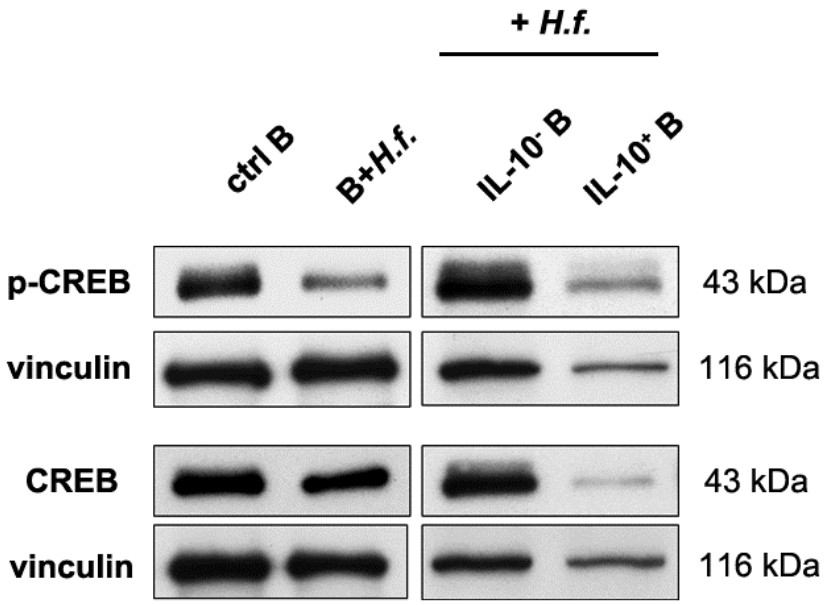

Figure 3.9: *Investigation for phosphorylation levels of CREB.*

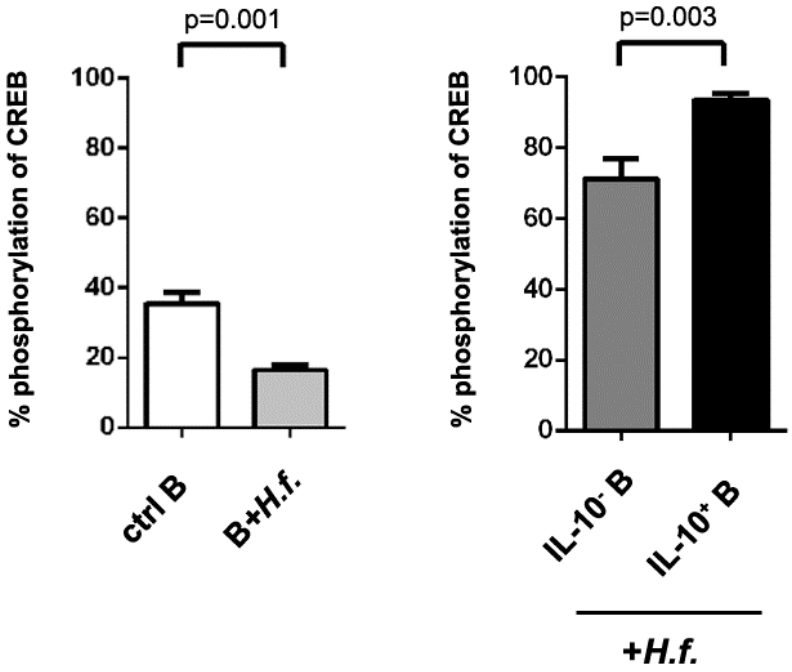

Figure 3.9 (cont'd.): *Investigation for phosphorylation levels of CREB.* Splenic B cells were stimulated with *H. felis* sonicate for 24 h (B+*H.f.*). Control B cells (ctrl B) did not receive any treatment. Upon addition of PMA (50ng/ml) and ionomycin (500ng/ml) during last 5 h of 24 h-long incubation, *H. felis*-stimulated cells were magnetically separated as IL-10^{-} B and IL-10^{+} B cells. Cells were lysed and whole cell extracts were immunoblotted for phospho- and total-CREB. Vinculin was used as loading control. Western Blotting data (*upper panel*) from one of three independently conducted experiments are shown (n=3). *GraphPad Prism 5.0* was used to graph mean levels of CREB phosphorylation obtained by densitometric analysis (*lower panel*). Statistical analysis was conducted using Student's *t* test.

Western Blotting results indicated that phosphorylation of CREB was significantly decreased in *H.felis* sonicate-stimulated B cells when compared to untreated control (from 40% to 20%, respectively). Comparison of CREB phosphorylation between *Helicobacter*-activated IL-10^{-} B and IL-10^{+} B samples revealed that CREB phosphorylation was significantly elevated from 70% to 95% in IL-10^{+} B cells. Phosphorylation percentages were found to be higher in *Helicobacter*-activated IL-10^{-} B and IL-10^{+} B fractions when compared to B+*H.f.* sample possibly due to the induction of these fractions with PMA and ionomycin prior to magnetic separation. The role of additional intracellular signaling pathways acting on activation status of CREB may be speculated regarding the reduction of CREB phosphorylation in

Helicobacter-stimulated B cells compared to untreated control. These results overall suggest involvement of CREB activation in induction of IL-10 production from *Helicobacter*-activated B cells.

3.6.5 The effect of MEK-1/2 inhibitor (U0126)

3.6.5.1 The effect of MEK-1/2 inhibitor (U0126) on ERK-1/2 phosphorylation

MEK-ERK pathway was found to be central for induction of IL-10 production upon TLR-stimulation from many immune cell types [75]. Western Blotting results for ERK-1/2 phosphorylation (Fig. 3.8) suggested involvement of ERK-1/2 signaling module on induction of IL-10 production from *Helicobacter*-activated B cells. In order to identify the role of MEK-ERK pathway on IL-10 production in *Helicobacter*-activated B cells, a specific inhibitor of MEK-1/2 (U0126) was utilized. For validating the efficiency of MEK-1/2 inhibitor, its effect on ERK-1/2 phosphorylation was investigated (Fig. 3.10).

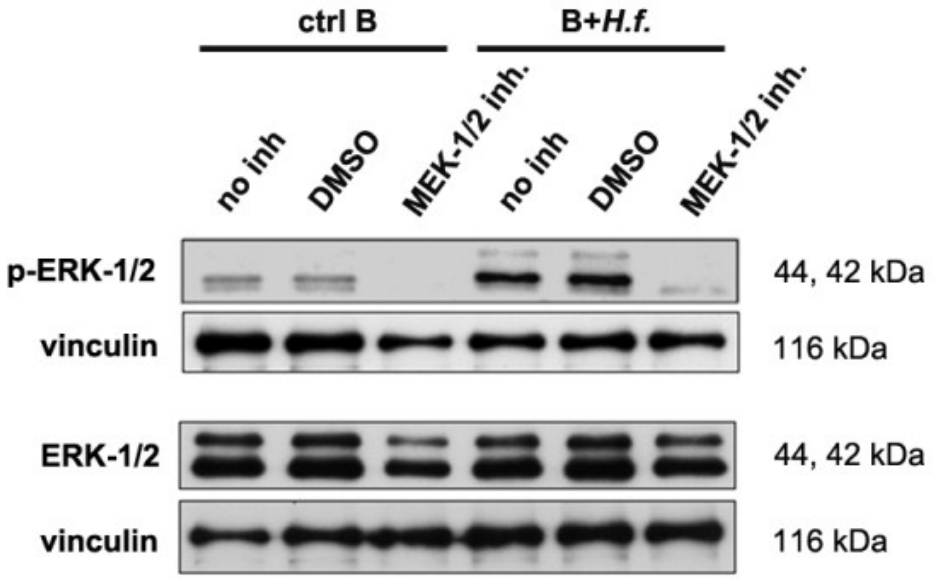

Figure 3.10: *Effect of MEK-1/2 inhibitor on phosphorylation of ERK-1/2.*

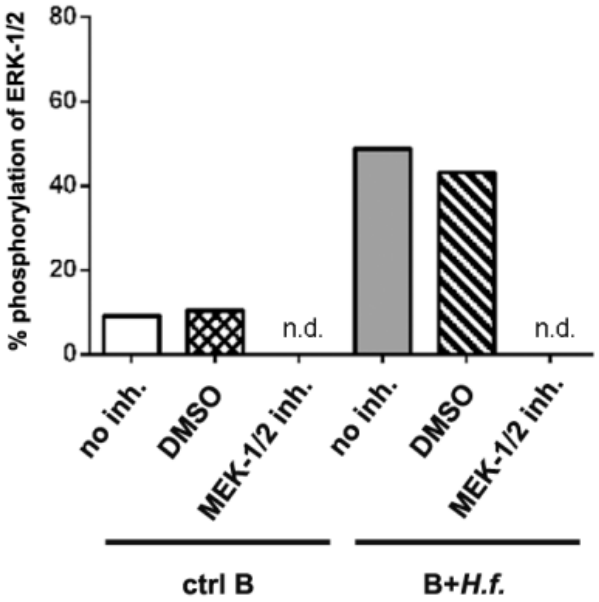

Figure 3.10 (cont'd.): *Effect of MEK-1/2 inhibitor on phosphorylation of ERK-1/2.* Murine splenic B cells were either pre-treated with MEK-1/2 inhibitor (15 μM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 μg/ml *H.felis* sonicate (B+*H.f.*) or left unstimulated (ctrl B) for 24 h. Cells were lysed and whole cell extracts were immunoblotted for phospho- and total-ERK-1/2. Vinculin was used as loading control. Western Blotting data (*upper panel*) from one of three independently conducted experiments are shown (n=3). *GraphPad Prism 5.0* was used to graph mean levels of ERK-1/2 phosphorylation obtained by densitometric analysis (*lower panel*). n.d. denotes not determined.

Western Blotting results demonstrated that MEK-1/2 inhibitor totally abrogated phosphorylation of ERK-1/2 in *H.felis*-sonicate treated B cells and untreated controls. Since vehicle control (DMSO) did not alter the phosphorylation of ERK-1/2, it can be claimed that the abrogation of ERK-1/2 phosphorylation was merely the effect of used inhibitor. Furthermore, in parallel with previous findings (Fig. 3.8), phosphorylation of ERK-1/2 was found to be elevated from 10% to 50% in *H.felis* sonicate treated B cells when compared to untreated controls. These results suggest that MEK-1/2 inhibitor was very efficient in blocking MEK-ERK signaling in *Helicobacter*-activated B cells.

3.6.5.2 The effect of MEK1/2 inhibitor (U0126) on CREB phosphorylation

Since CREB was known to be a downstream effector of ERK-1/2 [75], the effect of MEK-1/2 inhibitor on phosphorylation of CREB was also investigated (Fig. 3.11).

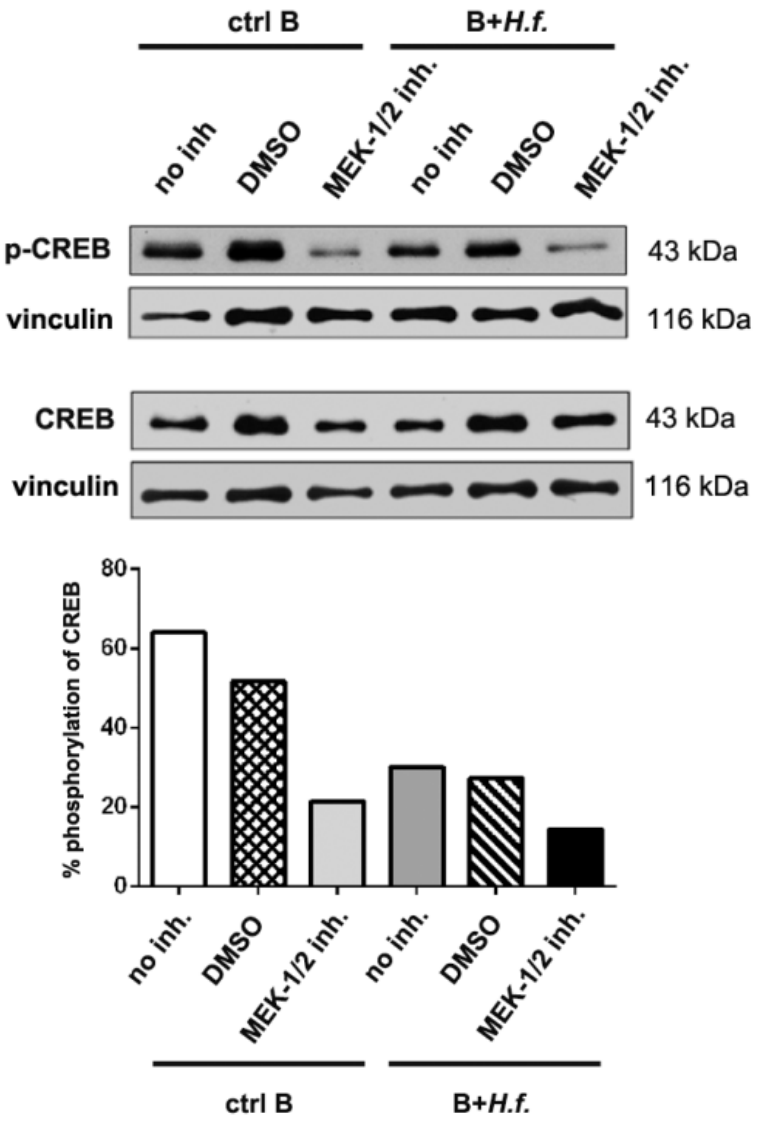

Figure 3.11: *Effect of MEK-1/2 inhibitor on phosphorylation of CREB.* Murine splenic B cells were either pre-treated with MEK-1/2 inhibitor (15 µM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 µg/ml *H.felis* sonicate (B+*H.f.*) or left unstimulated (ctrl B) for 24 h. Cells were lysed and whole cell extracts were immunoblotted for phospho- and total-CREB. Vinculin was used as loading control. Western Blotting data (*upper panel*) from one of three independently conducted experiments are shown (n=3). *GraphPad Prism 5.0* was used to graph mean levels of CREB phosphorylation obtained by densitometric analysis (*lower panel*).

According to immunoblotting results, CREB phosphorylation was decreased for both ctrl B and B+*H.f.* samples with the use of MEK-1/2 inhibitor whereas DMSO application did not bring about a noticeable change in phosphorylation status of CREB except for a slight decrease in ctrl B samples. CREB phosphorylation was not totally abrogated with the use of MEK-1/2 inhibitor. This result was expected since it

was known that signaling modules other than ERK-1/2 such as p38 MAPK or PI3K may act on CREB for activation of this transcription factor [75]. Nevertheless, these results also portray the efficiency of MEK-1/2 inhibitor on signaling molecules downstream of ERK-1/2.

3.6.5.3 The effect of MEK-1/2 inhibitor (U0126) on IL-10 secretion

Following assessment of the efficiency for MEK-1/2 inhibitor at 15 µM concentration on blocking MEK-ERK signaling pathway in *Helicobacter*-activated murine splenic B cells, the effect of MEK-1/2 inhibitor on IL-10 production and secretion was investigated. In order to determine the role of MEK-ERK pathway on IL-10 secretion, culture supernatants of B cells were probed for secreted amounts of IL-10 by ELISA (Fig. 3.12).

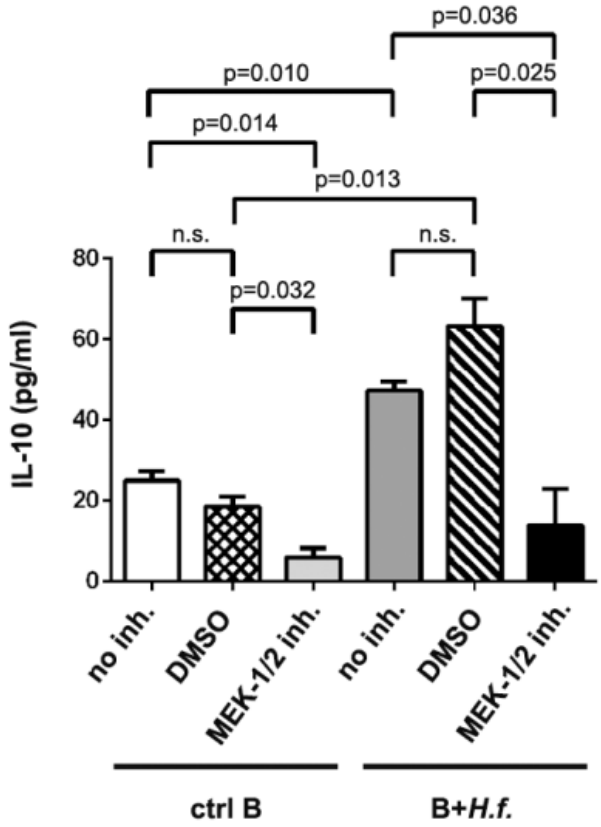

Figure 3.12: *Effect of MEK-1/2 inhibitor on IL-10 secretion.* B cells were either pre-treated with MEK-1/2 inhibitor (15 µM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 µg/ml *H.felis* sonicate (B+*H.f.*) or left unstimulated (ctrl B). Following 24h-long incubation, culture supernatants were probed for secreted IL-10 by ELISA. Data are representative of three independent experiments (n=3). Results are graphed using *GraphPad Prism 5.0*. Statistical analysis was conducted using Student's *t* test. n.s. stands for not significant.

IL-10 ELISA results (Fig. 3.12) demonstrated that IL-10 secretion was significantly reduced in samples treated with MEK-1/2 inhibitor when compared to untreated or vehicle (DMSO)-treated controls. No significant change in secreted IL-10 levels was observed between samples without inhibitor treatment and samples that were treated with vehicle control (DMSO). Moreover, as expected, IL-10 secretion levels were found to be significantly elevated (2.5-fold) in B cells stimulated with *H.felis* sonicate when compared to ctrl B samples. DMSO-treated samples displayed a similar pattern (with 3-fold increase) for IL-10 secretion. These results suggest the requirement of MEK-ERK signaling module of MAPK pathway for IL-10 production and secretion from *Helicobacter*-activated B cells.

3.6.6 The effect of p38 MAPK inhibitor (SB203580)

3.6.6.1 The effect of p38 MAPK inhibitor (SB203580) on CREB phosphorylation

p38 MAPK pathway was found to be central for induction of IL-10 production upon TLR-stimulation from many immune cell types such as macrophages or DCs [75]. Moreover, the requirement for p38 MAPK signaling for induction of IL-10 production from TLR4- or TLR9-stimulated murine B cells was recently shown. Even though Western Blotting results for p38 MAPK phosphorylation (Fig. 3.6) suggested involvement of p38 MAPK signaling module in *Helicobacter*-activated IL-10$^-$ B cells rather than IL-10$^+$ B cells, the role of p38 MAPK on induction of IL-10 production from *Helicobacter*-activated B cells was further investigated with the use of specific chemical inhibitor (SB203580) of p38 MAPK signaling. The effect of p38 MAPK inhibitor (SB203580) on CREB phosphorylation was investigated since SB203580 did not affect the phosphorylation status of p38 MAPK but was known to block signaling downstream of p38 MAPK and CREB was known to be a downstream effector of p38 MAPK module of MAPK signaling pathway [51, 75] (Fig. 3.13).

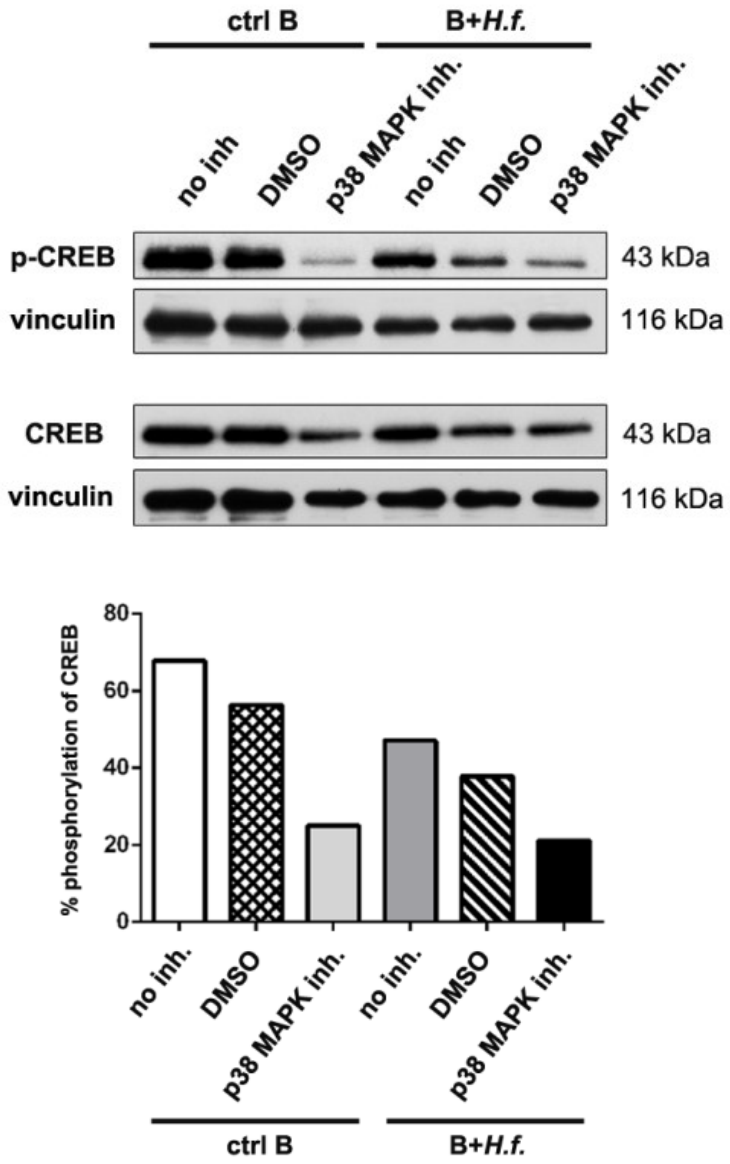

Figure 3.13: *Effect of p38 MAPK inhibitor on phosphorylation of CREB.* Murine splenic B cells were either pre-treated with p38 MAPK inhibitor (15 μM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 μg/ml *H.felis* sonicate (B+*H.f.*) or left unstimulated (ctrl B) for 24 h. Cells were lysed and whole cell extracts were immunoblotted for phospho- and total-CREB. Vinculin was used as loading control. Western Blotting data (*upper panel*) from one of three independently conducted experiments are shown (n=3). *GraphPad Prism 5.0* was used to graph mean levels of CREB phosphorylation obtained by densitometric analysis (*lower panel*). Statistical analysis was conducted using Student's *t* test.

According to Western Blotting results, p38 MAPK inhibitor was found to reduce phosphorylation of CREB in both ctrl B (from 60% to 25%) and B+*H.f* (from 40% to 20%) samples whereas DMSO control did not alter the phosphorylation of CREB

noticeably (Fig. 3.13). Moreover, in parallel with previous findings (Fig. 3.9), compared to untreated control, CREB phosphorylation level was decreased in *H.felis* sonicate-treated B cells (from 60% to 40%, respectively). Samples pre-treated with DMSO were found to follow the same trend for CREB phosphorylation with slight decrease compared to inhibitor-free samples. CREB phosphorylation was not totally abrogated with the use of p38 MAPK inhibitor. This result was expected since it was known that signaling modules other than p38 MAPK such as ERK-1/2 or PI3K may act on activation of CREB [75]. These results suggest that p38 MAPK inhibitor was efficient enough to interfere with p38 MAPK downstream signaling.

3.6.6.2 The effect of p38 MAPK inhibitor (SB203580) on IL-10 secretion

Following validation of p38 MAPK inhibitor's efficiency at 10 µM concentration on murine splenic B cells, culture supernatants of B cell samples were probed for quantitative determination of IL-10 secretion by ELISA (Fig. 3.14).

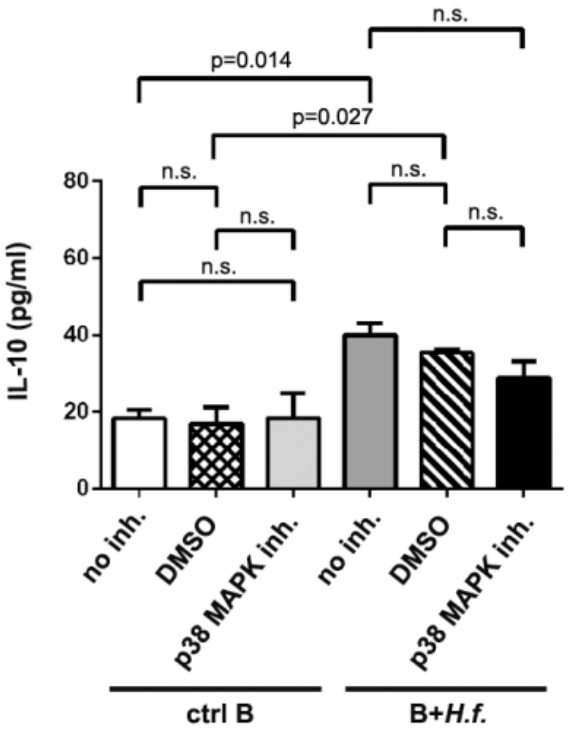

Figure 3.14: *Effect of p38 MAPK inhibitor on IL-10 secretion*. B cells were either pre-treated with p38 MAPK inhibitor (10 µM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 µg/ml *H.felis* sonicate (B+*H.f.*) or left unstimulated (ctrl B). Following 24h-long incubation, culture supernatants were probed for secreted IL-10 by ELISA. Data are representative of three independent experiments (n=3). Results are graphed using *GraphPad Prism 5.0*. Statistical analysis was conducted using Student's *t* test. n.s. denotes not significant.

IL-10 ELISA results demonstrated that IL-10 secretion was not significantly altered in samples treated with p38 MAPK inhibitor when compared to their inhibitor-free control samples either pre-treated with vehicle control (DMSO) or not. A comparison between ctrl B and B+*H.f.* samples revealed significant elevation (2-fold) of secreted IL-10 levels in B+*H.f.* samples either pre-treated with DMSO or not. These results suggested that p38 MAPK signaling module of MAPK pathway was not required for IL-10 production and secretion from *Helicobacter*-activated B cells.

3.7 The Role of PI3K Pathway on IL-10 Production From *Helicobacter*-stimulated B Cells

3.7.1 The role of Akt on IL-10 production

PI3K pathway was found to be involved in induction of IL-10 from macrophages. Phosphorylation, therefore activation of Akt, the key player protein kinase of PI3K pathway, was found to be required for IL-10 production from TLR-stimulated macrophages [75]. In order to elucidate the role of PI3K pathway on induction of IL-10 production from *Helicobacter*-activated B cells, phosphorylation of Akt was investigated in whole cell extracts of ctrl B, B+*H.f., Helicobacter*-activated IL-10$^-$ B cells and IL-10$^+$ B samples using antibodies against total and phosphorylated forms of Akt (Fig. 3.15).

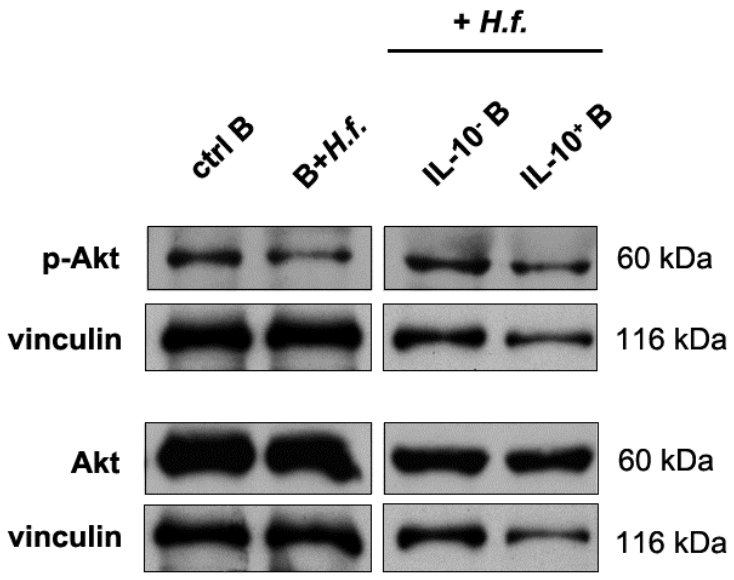

Figure 3.15: *Assessment of phosphorylation levels of Akt.*

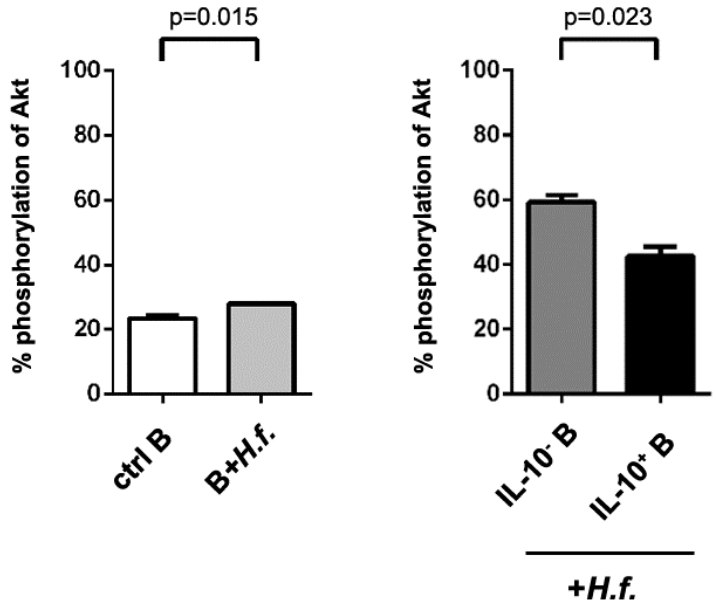

Figure 3.15 (cont'd.): *Assessment of phosphorylation levels of Akt.* Splenic B cells were stimulated with *H.felis* sonicate for 24 h (B+*H.f.*). Control B cells (ctrl B) did not receive any treatment. Upon addition of PMA (50 ng/ml) and ionomycin (500 ng/ml) during last 5 h of 24 h-long incubation, *H.felis*-stimulated cells were magnetically separated as IL-10^{-} B and IL-10^{+} B cells. Cells were lysed and whole cell extracts were immunoblotted for phospho- and total-Akt. Vinculin was used as loading control. Western Blotting data (*upper panel*) from one of three independently conducted experiments are shown (n=3). *GraphPad Prism 5.0* was used to graph mean levels of ERK-1/2 phosphorylation obtained by densitometric analysis (*lower panel*). Statistical analysis was conducted using Student's *t* test.

Western Blotting results indicated that Akt was significantly more phosphorylated in *H.felis*-stimulated B cells (B+*H.f.*) cells when compared to unstimulated ctrl B cells (30% and 20%, respectively). Comparison of Akt phosphorylation between *Helicobacter*-activated IL-10^{-} B and IL-10^{+} B cells revealed that Akt was significantly more phosphorylated in IL-10^{-} B than IL-10^{+} B cells (60% and 40%, respectively). Phosphorylation percentages were found to be higher in *Helicobacter*-activated IL-10^{-} B and IL-10^{+} B fractions when compared to B+*H.f.* sample possibly due to the induction of these fractions with PMA and ionomycin. This result suggests that, similar to p38 MAPK, activation of Akt may play a more prominent role in *Helicobacter*-activated IL-10^{-} B cells rather than IL-10^{+} B.

3.7.2 The role of GSK-3β on IL-10 production

Activated Akt phosphorylates, therefore inactivates GSK-3β. Active GSK-3β is known to inhibit binding of CREB and AP-1 to DNA and initiation of IL-10 expression [75]. In order to figure out the role of PI3K pathway on induction of IL-10 production from *Helicobacter*-activated B cells, phosphorylation of GSK-3β, downstream effector of Akt, was also investigated (Fig. 3.16).

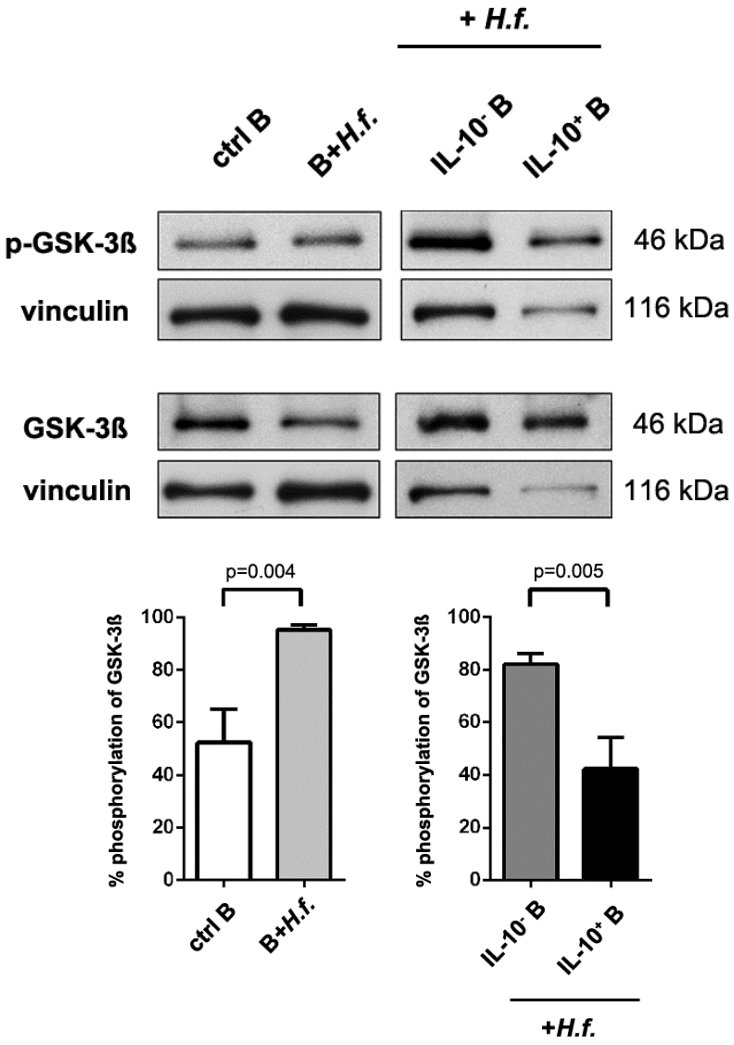

Figure 3.16: *Investigation for phosphorylation levels of GSK-3β.* Splenic B cells were stimulated with *H. felis* sonicate for 24 h (B+*H.f.*). Control B cells (ctrl B) did not receive any treatment. Upon addition of PMA (50 ng/ml) and ionomycin (500 ng/ml) during last 5 h of 24 h-long incubation, *H. felis*-stimulated cells were magnetically separated as IL-10^{-} B and IL-10^{+} B cells. Cells were lysed and whole cell extracts were immunoblotted for phospho- and total-GSK-3β. Vinculin was used as loading control. Western Blotting data (*upper panel*) from one of three independently conducted experiments are shown (n=3). *GraphPad Prism 5.0* was used to graph mean levels of GSK-3β phosphorylation obtained by densitometric analysis (*lower panel*). Statistical analysis was conducted using Student's *t* test.

According to Western Blotting results, phosphorylation of GSK-3β was significantly elevated in *H.felis*-stimulated B cells (B+*H.f.*) cells when compared to unstimulated ctrl B cells (90% and 50%, respectively). Phosphorylation of GSK-3β was significantly reduced from 80% to 40% in *Helicobacter*-activated IL-10^{+} B cells (~40%) when compared to IL-10^{-} B cells. Phosphorylation status of GSK-3β was found to be similar to Akt phosphorylation status. This was expected since GSK-3β is a downstream effector of Akt. These results suggest that GSK-3β gets inactivated in B cells upon *Helicobacter felis* sonicate-stimulation. On the other hand, *Helicobacter*-activated IL-10^{+} B cells were found to have more active GSK-3β compared to IL-10^{-} B cells. Taken together with Akt phosphorylation status (Fig. 3.15), these results collectively suggest involvement of PI3K signaling pathway in *Helicobacter*-activated IL-10^{-} B cells rather than IL-10^{+} B cells.

3.7.3 The role of CREB as a downstream effector of PI3K signaling pathway on IL-10 production

CREB is a downstream effector of both MAPK and PI3K signaling pathways. CREB is known to play an important role in expression of IL-10 from TLR-stimulated macrophages [75]. Western Blotting data on phosphorylation of CREB is shown in Figure 3.9. Western Blotting results indicated that phosphorylation of CREB was significantly decreased from 40% to 20% in *H.felis* sonicate-stimulated B cells when compared to untreated control. Comparison of CREB phosphorylation between *Helicobacter*-activated IL-10^{-} B and IL-10^{+} B samples revealed that CREB phosphorylation was significantly elevated from approximately 70% to 90% in IL-10^{+} B cells.

GSK-3β is rendered inactive when phosphorylated and when unphosphorylated, in its active form, it is known to inhibit binding of CREB to DNA and initiate transcription of related response genes [75, 88]. Hence, phosphorylation of GSK-3β means inactivation of GSK-3β, which in turn leads to activation and phosphorylation of CREB. According to Western Blotting results of GSK-3β phosphorylation (Fig. 3.16), GSK-3β seems to be more active in *Helicobacter*-activated IL-10^{+} B cell subset since it is less phosphorylated when compared to IL-10^{-} B subset. However, contrary to expectations based on GSK-3β phosphorylation results, phosphorylation of CREB is significantly elevated in *Helicobacter*-activated IL-10^{+} B subset. This

discrepancy may be explained by CREB's inhibitory second phosphorylation at Ser129 by active GSK-3β [87]. Since the antibody that was used in this study was produced to recognize CREB phosphorylated merely at Ser133, but inactive CREB is also phosphorylated at Ser133 along with Ser129, investigating phosphorylation of CREB by only checking for p-CREB (Ser133) may not be enough to assess CREB activity. Moreover, many other signaling pathways may regulate activation of CREB other than GSK-3β such as ERK-1/2 module. It may be speculated that the effect of ERK-1/2 on activation status of CREB is dominant over that of GSK-3β.

3.7.4 The effect of PI3K inhibitor (LY294002)

3.7.4.1 The effect of PI3K inhibitor (LY294002) on Akt phosphorylation

PI3K pathway was found to be one of the signaling pathways utilized in induction of IL-10 from TLR-stimulated macrophages [75]. Even though Western Blotting results for phosphorylation of Akt and GSK-3β (Figures 3.15 and 3.16, respectively) suggested involvement of PI3K signaling pathway in *Helicobacter*-activated IL-10$^-$ B cells rather than IL-10$^+$ B cells, the role of PI3K signal transduction pathway on induction of IL-10 production from *Helicobacter*-activated B cells was further investigated with the use of specific chemical inhibitor (LY294002) of PI3K signaling. For this purpose, initially the effect of PI3K inhibitor (LY294002) on Akt phosphorylation was investigated (Fig. 3.17).

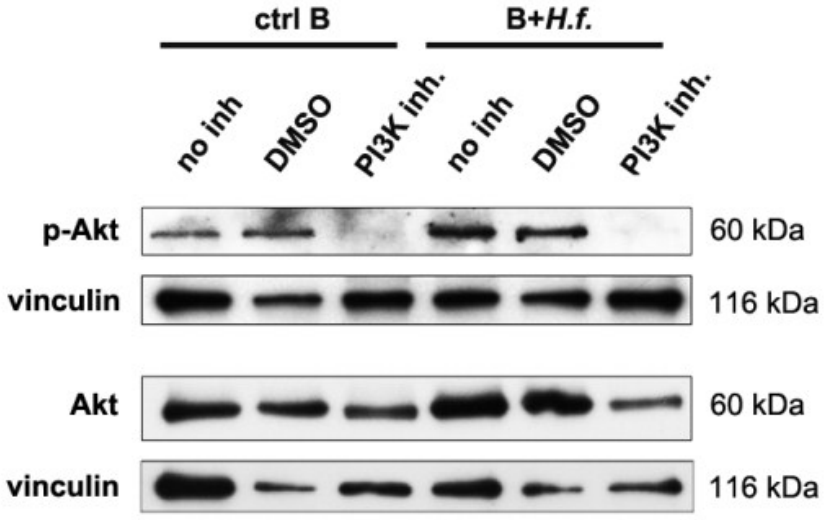

Figure 3.17: *Effect of PI3K inhibitor on phosphorylation of Akt.*

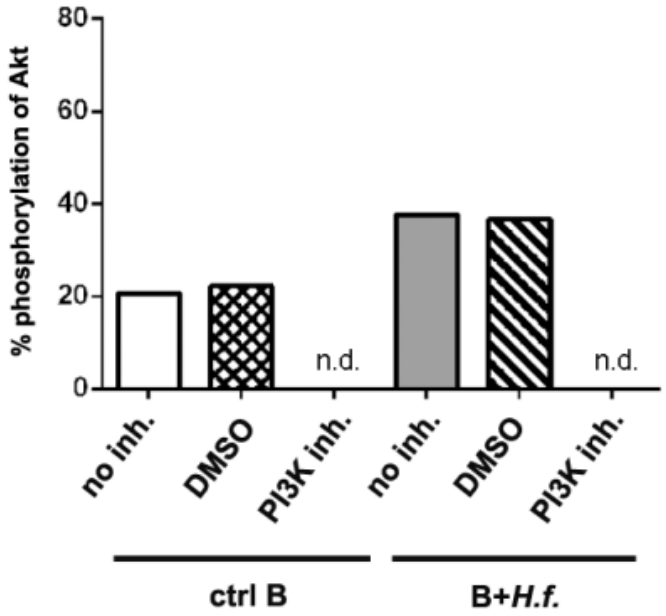

Figure 3.17 (cont'd.): *Effect of PI3K inhibitor on phosphorylation of Akt.* Murine splenic B cells were either pre-treated with PI3K inhibitor (15 μM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 μg/ml *H.felis* sonicate (B+*H.f.*) or left unstimulated (ctrl B) for 24 h. Cells were lysed and whole cell extracts were immunoblotted for phospho- and total-Akt. Vinculin was used as loading control. Western Blotting data (*upper panel*) from one of three independently conducted experiments are shown (n=3). *GraphPad Prism 5.0* was used to graph mean levels of Akt phosphorylation obtained by densitometric analysis (*lower panel*). n.d. stands for not determined.

Total abrogation of Akt phosphorylation was observed in PI3K inhibitor-treated samples whereas phosphorylation of Akt was not affected in samples pre-treated with vehicle control (DMSO). Moreover, in parallel with previous findings, an elevation in Akt phosphorylation from 20% to 40% was evident for B+*H.f.* samples when compared to ctrl B samples. A similar trend in phosphorylation of Akt was also observed in samples pre-treated with DMSO. These results suggest that PI3K inhibitor was very efficient in blocking PI3K signaling in *Helicobacter*-activated B cells.

3.7.4.2 The effect of PI3K inhibitor (LY294002) on IL-10 secretion

Following validation of the efficiency for PI3K inhibitor at 15 μM concentration on blocking PI3K signaling pathway in *Helicobacter*-activated murine splenic B cells,

the effect of PI3K inhibitor on IL-10 production and secretion was investigated. In order to elucidate the role of PI3K pathway on IL-10 secretion, culture supernatants of B cells were probed for secreted amounts of IL-10 by ELISA (Fig. 3.18).

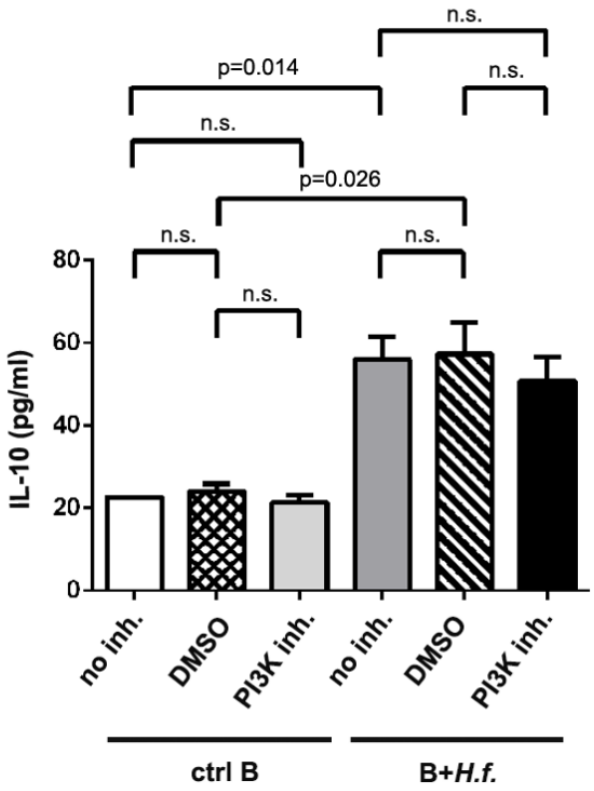

Figure 3.18: *Effect of PI3K inhibitor on IL-10 secretion.* B cells were either pre-treated with PI3K inhibitor (15 µM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 µg/ml *H.felis* sonicate (B+*H.f.*) or left unstimulated (ctrl B). Following 24 h-long incubation, culture supernatants were probed for secreted IL-10 by ELISA. Data are representative of three independent experiments (n=3). Results are graphed using *GraphPad Prism 5.0*. Statistical analysis was conducted using Student's *t* test. n.s. stands for not significant.

IL-10 ELISA results demonstrated that secreted IL-10 levels did not significantly change in *Helicobacter*-activated B cells pre-treated with PI3K inhibitor when compared to untreated or DMSO pre-treated B cells. Furthermore, compared to ctrl B samples, significant increase (2.5-fold) of secreted IL-10 levels was observed in B+*H.f.* samples either pre-treated with DMSO or not. These results suggest that PI3K signaling pathway was not required for induction of IL-10 production from *Helicobacter*-activated B cells.

3.8 The Role of Canonical NF-κB Pathway on IL-10 Production From *Helicobacter*-stimulated B Cells

3.8.1 The role of p65 subunit of NF-κB on IL-10 production

p65 subunit of NF-κB was found to interact with IL-10 coding DNA region in murine macrophages stimulated by LPS for IL-10 production [92]. The role of p65 subunit on IL-10 production from *Helicobacter*-activated B cells was not known. In order to determine the phosphorylation of p65 subunit of NF-κB signaling pathway, whole cell extracts of ctrl B, B+*H.f., Helicobacter*-activated IL-10$^-$ B and IL-10$^+$ B cells were used as samples for Signaling Nodes PathScan ELISA (explained in detail in section 2.2.13) which detects p65 protein when phosphorylated at Ser536 residue (Fig 3.19).

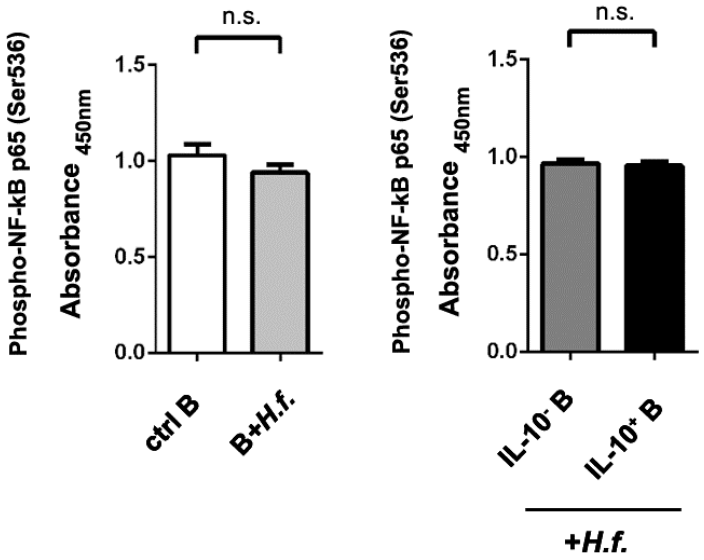

Figure 3.19: *Investigation for phosphorylation of p65 subunit of canonical NF-κB signaling pathway.* Splenic B cells were stimulated with *H. felis* sonicate for 24 h (B+*H.f.*). Control B cells (ctrl B) did not receive any treatment. Upon addition of PMA (50 ng/ml) and ionomycin (500 ng/ml) during last 5 h of 24 h-long incubation, *H. felis*-stimulated cells were magnetically separated as IL-10$^-$ B and IL-10$^+$ B cells. Cells lysates were assayed at a protein concentration of 0.15 mg/ml using the PathScan Signaling Nodes Multi-Target Sandwich ELISA Kit #7272 (Cell Signaling Technology, Inc.) as per manufacturer's instructions. The absorbance readings of biological duplicate samples at 450 nm were graphed using *GraphPad Prism 5.0*. Statistical analysis was conducted using Student's *t* test. n.s. denotes not significant.

According to preliminary PathScan ELISA results, no significant change in phosphorylation status of p65 subunit among ctrl B, B+*H.f., Helicobacter*-activated IL-10$^-$ B cell or IL-10$^+$ B cell samples was observed. These results suggest *Helicobacter*--stimulated B cells do not utilize canonical pathway of NF-κB for induction of IL-10 production.

3.8.2 The effect of NF-κB inhibitor (PDTC)

3.8.2.1 The effect of NF-κB inhibitor (PDTC) on IL-10 secretion

In order to further determine the role of canonical pathway of NF-κB on induction of IL-10 production from *Helicobacter*-activated B cells, a chemical inhibitor of canonical NF-κB pathway (PDTC) was used. The effect of blockage of NF-κB canonical pathway on IL-10 secretion was determined by IL-10 ELISA (Fig. 3.20).

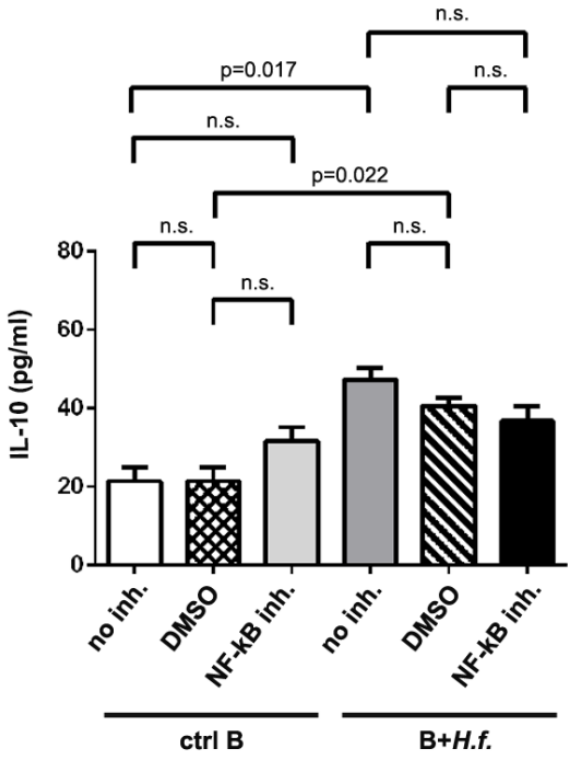

Figure 3.20: *Effect of NF-κB inhibitor on IL-10 secretion.* B cells were either pre-treated with NF-κB inhibitor (30 μM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 μg/ml *H.felis* sonicate (B+*H.f.*) or left unstimulated (ctrl B). Following 24 h-long incubation, culture supernatants were probed for secreted IL-10 by ELISA. Data are representative of three independent experiments (n=3). Results are graphed using *GraphPad Prism 5.0*. Statistical analysis was conducted using Student's *t* test. n.s. stands for not significant.

IL-10 ELISA results demonstrated that IL-10 secretion levels did not significantly change in the samples treated with PDTC when compared to their inhibitor-free control samples either DMSO-treated or not. A comparison between ctrl B and B+*H.f.* samples revealed significant increase (2-fold) of secreted IL-10 levels in B+*H.f.* samples either pre-treated with DMSO or not. These results suggest that canonical pathway of NF-κB was not required for IL-10 production and secretion from *Helicobacter*-activated B cells.

3.9 The Effect of MEK-1/2 Inhibitor (U0126) on TLR4 or TLR9-stimulated IL-10 Production

It was recently shown that ERK-1/2 module of MAPK signaling pathway was not required for IL-10 production from TLR4- or TLR9-stimulated murine B cells [80]. In order to investigate the involvement of ERK-1/2 module of MAPK signaling in IL-10 production and secretion from TLR4 or TLR9-stimulated B cells, MEK-1/2 inhibitor (U0126) was used and culture supernatants of B cells were probed for IL-10 by ELISA (Fig.3.21).

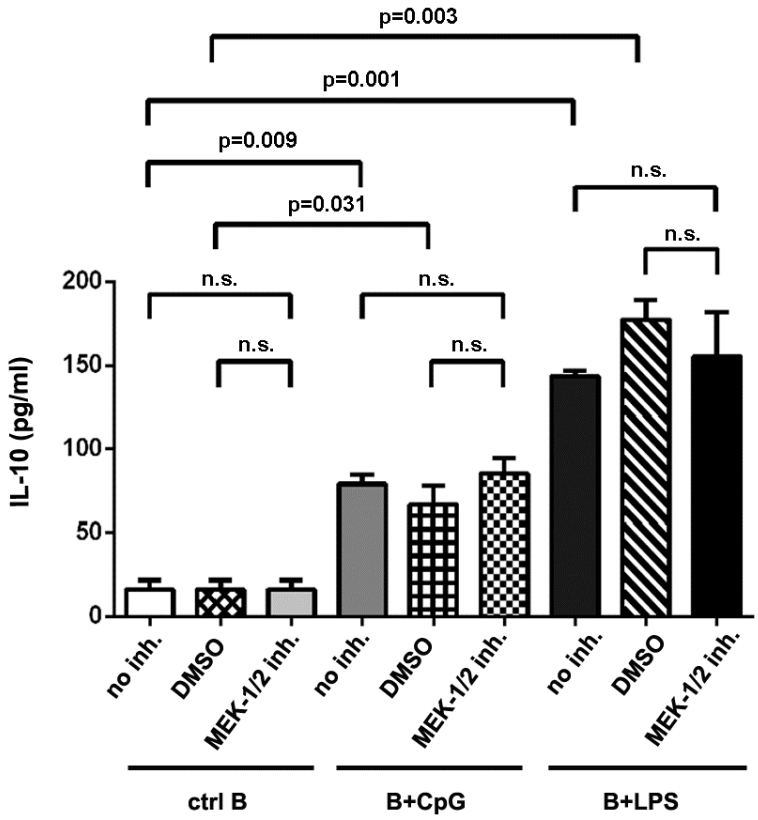

Figure 3.21: *Effect of MEK-1/2 inhibitor on TLR4- or TLR9-stimulated IL-10 secretion.*

Figure 3.21 (cont'd.): *Effect of MEK-1/2 inhibitor on TLR4- or TLR9-stimulated IL-10 secretion.* B cells were either pre-treated with MEK-1/2 inhibitor (15 μM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 μg/ml LPS (B+LPS), 5 μg/ml CpG (B+CpG) or left unstimulated (ctrl B). Following 48 h-long incubation, culture supernatants were probed for secreted IL-10 by ELISA. All samples were used as biological duplicates. Results are graphed using *GraphPad Prism 5.0*. Statistical analysis was conducted using Student's *t* test. n.s. stands for not significant.

Preliminary IL-10 ELISA results indicated that IL-10 secretion levels did not significantly change in MEK-1/2 inhibitor-treated LPS or CpG-stimulated samples when compared to untreated or DMSO-treated samples. Besides, IL-10 secretion levels were found to be significantly increased in both TLR4-stimulated and TLR9-stimulated B cells when compared to unstimulated controls either DMSO-treated or not. LPS stimulation elicited more IL-10 secretion (15-fold) from B cells when compared to CpG stimulation (8-fold). These results are correlated with the findings of Mion and colleagues [80] and they suggest that IL-10 production from TLR4- or TLR9-stimulated B cells did not require ERK-1/2 module of MAPK pathway.

3.10 The Effect of p38 MAPK Inhibitor (SB203580) on TLR4 or TLR9-stimulated IL-10 Production

Recently, the involvement of p38 MAPK signaling in IL-10 production from TLR4- or TLR9-stimulated B cells has been shown [80]. In order to confirm the involvement of p38 MAPK module of MAPK signaling in TLR-mediated IL-10 production and secretion, TLR4 ligand LPS and TLR9 ligand CpG were chosen as stimulants which were known to induce IL-10 production from B cells [80]. For investigation of the role of p38 MAPK signaling module in IL-10 production from LPS or CpG-stimulated B cells, p38 MAPK inhibitor (SB203580) was used and culture supernatants were monitored for IL-10 secretion by ELISA (Fig. 3.22).

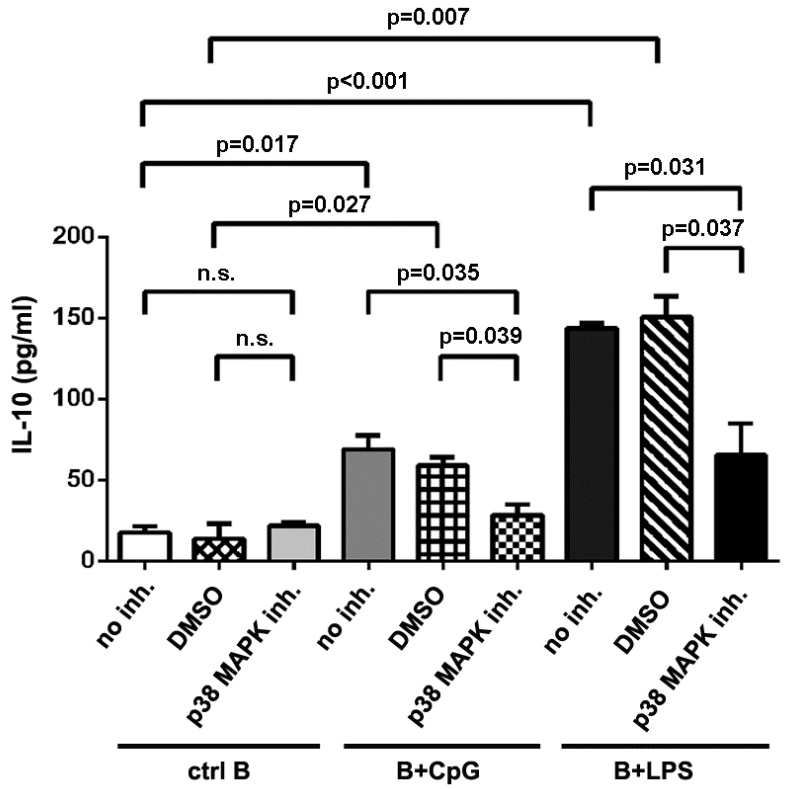

Figure 3.22: *Effect of p38 MAPK inhibitor on TLR4- or TLR9-stimulated IL-10 secretion.* B cells were either pre-treated with p38 MAPK inhibitor (10 μM) or vehicle control (DMSO) for 1 hour or left untreated (no inh.). After 1 h, cells were either stimulated with 10 μg/ml LPS (B+LPS), 5 μg/ml CpG (B+CpG) or left unstimulated (ctrl B). Following 48 h-long incubation, culture supernatants were probed for secreted IL-10 by ELISA. All samples were used as biological duplicates. Results are graphed using *GraphPad Prism 5.0*. Statistical analysis was conducted using Student's *t* test. n.s. denotes not significant.

According to preliminary IL-10 ELISA results, both TLR4- and TLR9-stimulation resulted in significantly elevated IL-10 secretion from B cells when compared to unstimulated control. Furthermore, IL-10 secretion was found to be significantly reduced in TLR4 and TLR9-stimulated samples which were pre-treated with p38 MAPK inhibitor when compared to untreated and vehicle control (DMSO)-treated ones. When compared to CpG stimulation (8-fold), more IL-10 was found to be secreted from B cells as a result of LPS stimulation (15-fold).

Taken together with the results for the effect of MEK-1/2 inhibitor on TLR4- or TLR9- stimulated IL-10 production (Fig. 3.21), these findings were in line with recent findings of Mion and colleagues and suggest the involvement of p38 MAPK module rather than ERK-1/2 module of MAPK signaling pathway in induction of IL-

10 production from TLR4- or TLR9-stimulated B cells. Considering that ERK-1/2 was found to be required for induction of IL-10 production from TLR2-stimulated B cells, these findings collectively can be interpreted as IL-10 production from B cells stimulated by different TLR ligands may require differential intracellular signaling pathways.

3.11 The Role of STAT3 on IL-10 Production

IL-10 exerts its effects on immune cells by binding to its receptor complex. Binding of IL-10 to its receptor complex brings about phosphorylation of STAT3 via JAK/STAT signaling pathway [23, 61-63]. IL-10 is known to induce its own production via interaction with its own receptor complex and activation of STAT3 by phosphorylation in TLR-stimulated macrophages [75]. Moreover, activation of STAT3 along with ERK is also found to be required for IL-10 production from T helper 17 (Th17) cells [75] and from TLR7/8-stimulated human primary B cells [100]. The involvement of STAT3 in IL-10 production from *Helicobacter*-stimulated B cells was not known. In order to determine the role of STAT3 on induction of IL-10 production from *Helicobacter*-stimulated B cells, whole cell extracts of ctrl B, B+*H.f., Helicobacter*-activated IL-10$^-$ B cells and IL-10$^+$ B cells were used as samples for Cell Signaling Signaling Nodes PathScan ELISA which detects STAT3 protein when phosphorylated at Tyr705 residue (Fig. 3.23).

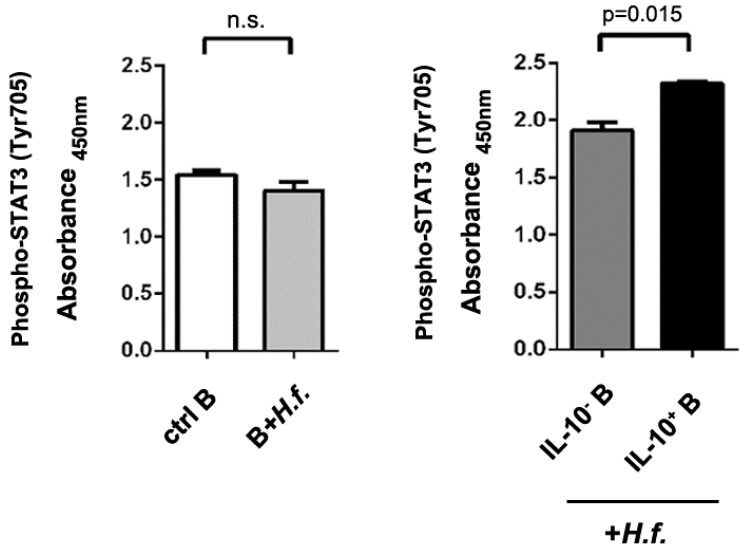

Figure 3.23: *Investigation for phosphorylation of STAT3.*

Figure 3.23 (cont'd.): *Investigation for phosphorylation of STAT3.* Splenic B cells were stimulated with *H. felis* sonicate for 24 h (B+*H.f.*). Control B cells (ctrl B) did not receive any treatment. Upon addition of PMA (50 ng/ml) and ionomycin (500 ng/ml) during last 5 h of 24 h-long incubation, *H. felis*-stimulated cells were magnetically separated as IL-10^- B and IL-10^+ B cells. Cells lysates were assayed at a protein concentration of 0.15 mg/ml using the PathScan Signaling Nodes Multi-Target Sandwich ELISA Kit #7272 (Cell Signaling Technology, Inc.) as per manufacturer's instructions. The absorbance readings of biological duplicate samples at 450 nm were graphed using *GraphPad Prism 5.0*. Statistical analysis was conducted using Student's *t* test. n.s. denotes not significant.

According to preliminary PathScan ELISA results, no significant change in STAT3 phosphorylation status was observed between ctrl B and B+*H.f.* samples. On the other hand, comparison between *Helicobacter*-activated IL-10^- B and IL-10^+ B cell samples revealed that STAT3 was significantly more phosphorylated, therefore activated in *Helicobacter*-activated IL-10^+ B cells. These preliminary results suggested involvement of STAT3 signaling in IL-10 production and a potential role for IL-10 in inducing its own production via STAT3-dependent pathway in an autocrine/paracrine manner.

4. DISCUSSION AND CONCLUSION

Examination of potential intracellular signal transduction pathways that may have a role in induction of IL-10 production from *Helicobacter*-stimulated B cells was the main research focus of this study.

Helicobacter pylori has causative role in many gastric malignancies such as chronic gastritis, peptic ulcer and gastric adenocarcinoma. Even though half of the world population is infected with the pathogen, only a minority develop gastric malignancies. The asymptomacity of majority of infected population can be attributed to existence of regulatory cells which suppress excessive pro-inflammatory response elicited against bacteria. An anti-inflammatory cytokine, IL-10 has been shown to possess suppressive and immune regulatory functions. A subset of B cells has recently been shown to produce IL-10 in response to *Helicobacter*-infection. Murine B cells sense *Helicobacter felis* (a counterpart of *Helicobacter pylori* which has higher immunogenicity for mice) in TLR2-dependent manner. TLR2-MyD88-dependent pathway has designated role in IL-10 production from *Helicobacter*-activated B cells [18]. Even though TLR-mediated IL-10 production pathways were well-characterized for macrophages, dendritic cells and T cells [75], intracellular signal transduction pathway(s) leading to IL-10 production and secretion from TLR2-stimulated B cells were not elucidated.

For examination of intracellular pathways, naive B cells obtained from spleen of C57BL/6 were stimulated with *Helicobacter felis*-sonicate in order to elicit IL-10 production and secretion from B cells as implicated by intracellular IL-10 flow staining (Fig. 3.3) and IL-10 ELISA (Fig. 3.4). Fractions of IL-10^{-} B cells and IL-10^{+} B cells from *Helicobacter*-stimulated B cells were obtained by magnetic separation upon PMA and ionomycin addition. Then, phosphorylation of certain kinases that take part in fundamental intracellular signaling pathways were investigated in naive B cells (ctrl B), *Helicobacter felis* sonicate-treated B cells (B+*H.f.*), *Helicobacter*-activated IL-10^{-} B cells and IL-10^{+} B cells. Investigated kinases were p38 MAPK,

MEK-1/2, ERK-1/2 of MAPK pathway, Akt, GSK-3β of PI3K pathway, CREB of both pathways and p65 of NF-κB canonical signaling pathway. These kinases were chosen for investigation since previous studies indicated their involvement in TLR-mediated IL-10 production from many immune cell types such as macrophages, DCs and T helper subsets [75].

A comparison between *Helicobacter*-activated IL-10$^-$ B and IL-10$^+$ B cells regarding phosphorylation of signaling molecules of MAPK pathway indicated significantly elevated phosphorylation of both MEK-1/2 (Fig. 3.7) and its downstream effector ERK-1/2 (Fig. 3.8) in IL-10$^+$ B cells. On the other hand, phosphorylation of p38 MAPK was found to be decreased in IL-10$^+$ B cells (Fig. 3.6). Significant increase in phosphorylation of CREB was observed for IL-10$^+$ B cells (Fig. 3.9). Since MEK-1/2 and ERK-1/2 are kinases of ERK-1/2 module of MAPK pathway, increase in phosphorylation, therefore activation of both kinases suggested a prominent role for MEK-ERK signaling pathway in IL-10 production. Moreover, it is worth noting that comparison of the ctrl B and B+*H.f.* samples demonstrated similar trends in phosphorylation of relevant kinases. Significant increase in phosphorylation of both MEK-1/2 and ERK-1/2 and a significant decrease of p38 MAPK phosphorylation was observed for B+*H.f.* This similarity in phosphorylation trend was expected given the results of intracellular staining for IL-10 in ctrl B and B+*H.f.* samples (Fig. 3.3). According to those results, more than 60% of *Helicobacter*-activated B cells were found to be producing IL-10. Furthermore, higher phosphorylation levels of *Helicobacter*-activated IL-10$^-$ B and IL-10$^+$ B cells compared to total B cells stimulated by *H.felis* sonicate (B+*H.f.*) may be attributed to PMA and ionomycin treatment these cells received before magnetic separation.

Use of inhibitors blocking MAPK pathway-specific kinases (p38 MAPK inhibitor and MEK-1/2 inhibitor) revealed that inhibition of p38 MAPK signaling did not result in reduction of IL-10 secretion from *Helicobacter*-activated B cells (Fig. 3.14), whereas IL-10 secretion was significantly decreased when MEK-ERK signaling was blocked with MEK-1/2 inhibitor (Fig. 3.12). Taken together with phosphorylation status of MAPK-pathway-specific kinases, these results suggest a more prominent role for p38 MAPK module in IL-10$^-$ B cells rather than IL-10$^+$ B. Most importantly, these results also indicate the involvement of MEK-ERK signaling module of MAPK pathway in IL-10 production and secretion from *Helicobacter*-activated B cells.

When phosphorylation of signaling molecules of PI3K pathway were scrutinized, significant decrease in phosphorylation of both Akt and its downstream effector GSK-3β in *Helicobacter*-activated IL-10^{+} B cells compared to IL-10^{-} B cells was observed (Figures 3.15 and 3.16, respectively). Phosphorylation of both Akt and GSK-3β was found to be significantly increased in B+*H.f.* samples compared to unstimulated controls. Based on these results, it can be claimed that *Helicobacter felis* sonicate brings about an activation of PI3K pathway through phosphorylation of both Akt and its downstream effector GSK-3β. However, this activation seems to be more prominent in IL-10^{-} B subset when compared to IL-10^{+} B cell subsets. Since the most eminent distinction between these subsets is their IL-10 production capacity, it would not be wrong to assume that PI3K/Akt pathway seems to play a rather more significant role in IL-10^{-} B cell subset. Consistent with these findings, abrogation of PI3K/Akt signaling with PI3K inhibitor did not significantly alter IL-10 secretion from *Helicobacter*-activated B cells. Taken together, these results suggest phosphorylation Akt was not required for IL-10 production and secretion from *Helicobacter*-activated B cells.

As a downstream effector for both MAPK and PI3K signaling pathways, phosphorylation of CREB was found to be significantly increased in *Helicobacter*-activated IL-10^{+} B cell subset when compared to IL-10^{-} B cells. Induction of IL-10 production by CREB activation was shown in literature in many immune cell types such as macrophages and DCs (Fig. 1.13). Thus, elevated phosphorylation of CREB in *Helicobacter*-activated IL-10^{+} B cell subset may affirm CREB's designated role in IL-10 production also from TLR2-stimulated B cells. However, significant decrease of CREB phosphorylation in total B cells stimulated with *Helicobacter felis* sonicate compared to unstimulated B cells was observed (Fig. 3.9) Since GSK-3β is significantly more phosphorylated, therefore inactivated in B+*H.f.* compared to ctrl B samples, decrease of CREB phosphorylation in this sample cannot be attributed to GSK-3β activity. This result suggests involvement of other signaling pathways acting on activation status of CREB other than MAPK and PI3K pathways.

GSK-3β seems to be more active in *Helicobacter*-activated IL-10^{+} B cell subset since it is less phosphorylated when compared to IL-10^{-} B subset (Fig. 3.16). In contrast, phosphorylation of CREB is found to be elevated in *Helicobacter*-activated IL-10^{+} B subset (Fig. 3.9). CREB's inhibitory second phosphorylation at Ser129 by active

GSK-3β may be proposed as an explanation for this discrepancy. CREB gets activated by phosphorylation at Ser133 residue, but gets inactivated by a secondary phosphorylation at Ser129 by active GSK-3β [87]. The antibody used in this study, which only detects CREB when phosphorylated at Ser133, may not truly reflect activation status of CREB. Moreover, the effect of other signaling pathways such as ERK-1/2 signaling module on CREB activation may be dominant over the effect of GSK-3β. Moreover, activated CREB is known to be translocated into nucleus upon phosphorylation at Ser133 [75]. Translocation of phosphorylated CREB can be monitored by immunofluorescence in order to confirm involvement of active CREB in induction of IL-10 production.

Besides, there are many transcription factors downstream of fundamental intracellular pathways [75]. One or more of these transcription factors other than CREB together or alone can induce transcription of IL-10 from *Helicobacter*-activated IL-10^{+} B cells. Phosphorylation of other transcription factors such as ELK-1, AP-1, c-FOS, c-JUN can also be investigated in order to determine their contribution in IL-10 production. Furthermore, MAPKAPKs activated exclusively by ERK-1/2 signaling module (RSKs) and downstream transcription factor MAF (which was shown to be of critical importance in IL-10 induction from macrophages and T helper subsets [75]) can also be investigated (Fig. 1.7).

Phosphorylation of p65 subunit was found to be unaffected in murine B cells upon *Helicobacter felis* sonicate stimulation (Fig. 3.19). Accordingly, inhibition of IκB degradation by a chemical inhibitor did not bring about a fluctuation in IL-10 secretion (Fig. 3.20). These results suggest that TLR2-stimulated IL-10 induction was not maintained through canonical pathway of NF-κB. These findings are in line with association of pro-inflammatory cytokine production by canonical pathway of NF-κB and IL-10's inhibitory effect on p50/p65 heterodimer [74]. These results also correlate with recent findings of Liu and colleagues [100] that attribute a dispensable role for canonical NF-κB pathway in IL-10 induction from human B cells upon TLR7/8 stimulation. Investigating the involvement of p50 subunit or non-canonical pathway can be intriguing since recent research on LPS-stimulated primary murine macrophages indicated the role of p50/p50 homodimer in IL-10 production [93].

Although it needs to be further confirmed with at least two more independent experiments, preliminary findings regarding STAT3 phosphorylation revealed a

significant increase of STAT3 phosphorylation in *Helicobacter*-activated IL-10^{+} B compared to IL-10^{-} B cell subset even though there was no significant change in STAT3 phosphorylation between ctrl B and B+*H.f.* samples (Fig. 3.23). Elevation of STAT3 phosphorylation can suggest involvement of STAT3 activation in induction of IL-10 production. Moreover, IL-10 is known to induce its own production through IL-10R-STAT3-dependent signaling [75]. Hence, a mechanism can be proposed for secreted IL-10 inducing its own production from *Helicobacter*-activated IL-10^{+} B cell subset in an autocrine-paracrine manner. Further studies to elucidate the role of STAT3 signaling on induction of IL-10 production from *Helicobacter*-activated B cells with the use of STAT3-specific inhibitors may be of importance.

The role of MAPK signaling pathway modules in IL-10 production from B cells upon stimulation of TLRs other than TLR2 was also investigated in this study. Utilization of specific inhibitors of p38 MAPK and MEK-1/2 revealed that TLR4- and TLR9-stimulated IL-10 induction from murine B cells was associated with activation of p38 MAPK module rather than ERK-1/2 module (Fig 3.21 and Fig. 3.22). This preliminary finding was in line with recent findings of Mion and colleagues [80] which suggested the involvement of p38 MAPK module MAPK signaling pathway rather than ERK-1/2 module in induction of IL-10 production from TLR4- or TLR9-stimulated B cells. When the requirement of MEK-ERK signaling for induction of IL-10 production from *Helicobacter*-stimulated B cells was taken into consideration, these findings could be interpreted as utilization of differential intracellular signaling pathways in IL-10 induction depends on the type of ligand, therefore stimulated TLR.

Significance of this research underlies in its contribution to the understanding of unexplored intrinsic signal transduction pathways of *Helicobacter*-activated IL-10^{+} B cell subset leading to IL-10 induction. B cells belong to a group of immune cells that are collectively called APCs along with macrophages and DCs which take active role in innate immune response. B cells are also important players of adaptive immune response along with T cells. B cells are unique that they resemble both APCs and T lymphocytes with their various characteristics. Common signaling pathway leading to IL-10 production pathway was found to be ERK module in previous studies [75]. Results of this work suggest involvement of ERK-1/2 module of MAPK pathway in IL-10 induction and secretion from *Helicobacter*-activated B cells (Fig. 4.1).

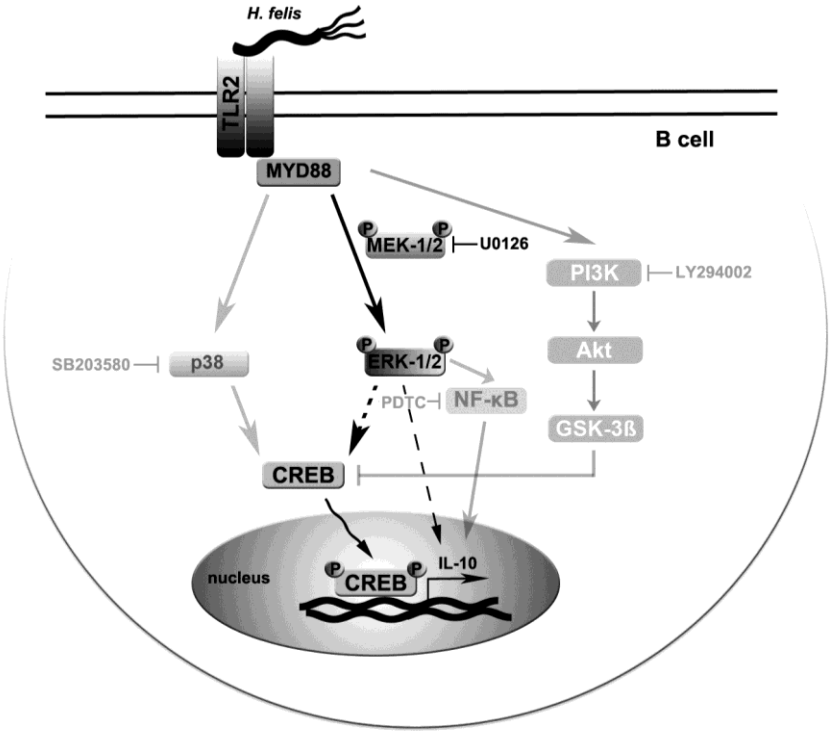

Figure 4.1: *Proposed model of intracellular signaling pathway for IL-10 production from Helicobacter-activated B cells.* Following investigation for activation status of specific kinases that take part in fundamental intracellular signaling pathways and monitoring the effect of chemical inhibitors which block relevant pathways on IL-10 secretion, ERK signaling module of MAPK pathway is found to be involved in induction of IL-10 production from *Helicobacter*-stimulated B cells rather than p38 MAPK signaling module of MAPK pathway, PI3K pathway or canonical pathway of NF-κB.

This work has been performed only in protein level. Expression levels of IL-10 in mRNA level also need to be validated in order to determine a complete scheme for the role of designated intracellular pathways on IL-10 induction upon TLR2-stimulation. The involvement of p38 MAPK and PI3K signaling pathways in *Helicobacter*-activated IL-10$^-$ B cells can also be investigated further. Moreover, the involvement of ERK-1/2 module of MAPK pathway on IL-10 production capacity and function of IL-10 *Helicobacter*-activated IL-10$^+$ B cells can also be investigated.

In conclusion, this study has contributed to the literature through providing molecular investigation of pathways regulating IL-10 production and describing the involvement of ERK-1/2 phosphorylation in IL-10 induction from *Helicobacter*-activated B cells for the first time.

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