ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

THE EFFECT OF *HELICOBACTER FELIS* ON MACROPHAGE POLARIZATION

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

HELICOBACTER FELIS'İN MAKROFAJ POLARİZASYONU ÜZERİNE ETKİSİ

YÜKSEK LİSANS TEZİ

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ARALIK 2015

Aslı KORKMAZ a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID **521121165**, successfully defended the **thesis** entitled "**THE EFFECT OF *HELICOBACTER FELIS* ON MACROPHAGE POLARIZATION**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my beloved family and friends,

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December 2015

Aslı KORKMAZ
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ABBREVIATIONS

µg : Microgram
µm : Micrometer
µM : Micromolar
AMP : Adenosine Monophosphate
AP-1 : Activator Protein-1
APC : Antigen Presenting Cell
BCA : Bicinchoninic Acid
BM : Bone Marrow
Breg : Regulatory B cell
BSA : Bovine Serum Albumin
CagA : Cytotoxin- Associated Gene A
CD : Cluster of Differentiation
CD40L : CD40 Ligand
CIA : Collagen- Induced Arthritis
Cm^2 : Centimeter Square
CpG ODN : CpG Oligodeoxynucleotides
CTL : Cytotoxic T lymphocyte
DC : Dendritic Cell
DC-SIGN : DC-Specific ICAM3-Grabbing Non-Integrin
DMEM : Dulbecco's Modified Eagle Medium
DMSO : Dimethyl Sulfoxide
DNA : Deoxyribonucleic Acid
dNTP : Deoxyribonucleotide
EAE : Experimental Autoimmune Encaphalomyelitis
EDTA : Ethylenediaminetetraacetic Acid
ELISA : Enzyme-Linked Immunosorbent Assay
FBS : Fetal Bovine Serum
FITC : Fluorescein Isothiocyanate
g : Gram
GM-CSF : Granulocyte Macrophage Stimulating Factor
GTP : Guanosine-5'-Triphosphate
h : Hour
H. felis : *Helicobacter felis*
H. pylori : *Helicobacter pylori*
ICAM3 : Intercellular Adhesion Molecule 3
HRP : Horseradish Peroxidase
IFN-γ : Interferon Gamma
IFN-β : Interferon-Beta
Ig : Immunoglobulin
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-18 : Interleukin-18
IL-10R : IL-10 Receptor
IRF : Interferon- Regulatory Factors
JAK : Janus kinase
kDa : Kilodalton
L : Liter
LPS : Lipopolysaccharide
LTβ : Lymphotoxin-β
M : Molar
M1 : Macrophages type 1 (classically activated macrophages)
M2 : Macrophages type 2 (alternatively activated macrophages)
M2a : Macrophages type 2a
M2b : Macrophages type 2b
M2c : Macrophages type 2c
M2d : Macrophages type 2d
MALT : Mucosa- Associated Lymphoid Tissue
M-CFU : Macrophage Colony Factor Unit
MHC-I : Major Histocompatibility Complex Class I
MHC-II : Major Histocompatibility Complex Class II
min : Minute
MIP-2α : Macrophage Inflammatory Protein 2-Alpha
ml : Mililiter
mM : Milimolar
mm : Milimeter
mRNA : Messenger Ribonucleic Acid
MyD88 : Myeloid Differentiation Primary Response Gene 88
NF-κB : Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NKT : Natural Killer T cell
NLRP3 : NOD-like Receptor Protein 3
OipA : Outer Membrane Protein A
PAI : Pathogenicity Island
PAMP : Pathogen Associated Molecular Pattern
PBS : Phosphate Buffered Saline
PCR : Polymerase Chain Reaction
pg : Picogram
pH : Power of Hydrogen
PI3K : Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2 : Phosphatidylinositol 4,5-bisphosphate
PIP3 : Phosphatidylinositol (3,4,5)-trisphosphate
PKB : Protein Kinase B
PM : Peritoneal Macrophages
PRR : Pattern Recognition Receptor
rpm : Revolutions Per Minute
RPMI : Roswell Park Memorial Institute
rRNA : Ribosomal RNA

STAT : Signal Transducer and Activator of Transcription
TAM : Tumor Associated Macrophages
TF : Transcription Factor
T4SS : Type IV Secretion System
TCR : T cell Receptor
TGF-β : Transforming Growth Factor-beta
Th1 : T Helper 1
Th2 : T Helper 2
Th17 : T Helper 17
TIR : Toll-IL-1R
TLR : Toll-like receptor
Tm : Melting Temperature
TNF-α : Tumor Necrosis Factor-alpha
Tr-1 : T Regulatory-1
Treg : Regulatory T cell
VacA : Vacuolating Cytotoxin A

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THE EFFECT OF *HELICOBACTER FELIS* ON MACROPHAGE POLARIZATION

SUMMARY

Helicobacter pylori (*H. pylori*) has been identified and classified as type I carcinogen for gastric malignancies such as chronic gastritis, peptic ulcer and gastric adenocarcinoma. Even though more than half of the world's population is infected with the *H. pylori*, only a minority develops gastric complications and/or remain asymptomatic. The bacteria are rarely eliminated, colonization usually persists throughout life, and infection involves both innate and adaptive immune responses.

Helicobacter felis (H. felis) is a gram-negative, spiral- shaped bacterium which was first isolated from the stomach of a domestic cat. It is more immunogenic on mice and zoonotic species of *H. pylori.* Hence, it is widely used in murine *Helicobacter* studies because it causes similar pathogenic effect on mice as *H. pylori* on humans. However, *H. felis* lacks Vacuolating cytotoxin A (VacA) and Cytotoxin-associated gene A (CagA) virulence factors. Despite lacking important *H. pylori* virulence factors, it causes more severe gastric inflammation than *H. pylori* does on mice.

Neutrophils, dendritic cells and macrophages mediate innate immune response against *H. pylori.* Macrophages are plastic and heterogenic group of cells, which can polarize to different types under different stimuli. Polarization status of macrophages changes according to stimuli and the local microenvironment, allowing them to shape the local inflammatory status to adapt to outside stimuli. However, different stimuli do not exist alone in tissues and macrophages may not form clear-cut activated subsets or expand clonally. The various macrophage functions are associated with the stimuli variety, receptor recognition on the macrophage upon stimuli and the presence of cytokines. There are two distinct states of polarized activation for macrophages: the classically activated -M1 type- macrophages and the alternatively activated -M2 type- macrophage subsets. Cell markers alone do not fully define the many subpopulations of macrophages. Therefore, macrophages should be defined based on their specific functional activities.

M1 type macrophages are the pro-inflammatory effector cells in innate immune response. Granulocyte macrophage stimulating factor (GM-CSF), lipopolysaccharide (LPS) and IFN-γ polarize macrophages towards the M1 phenotype which induces the macrophage to produce large amounts of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12/ IL-23. The antimicrobial functions of M1 macrophages are linked to up-regulation of inducible nitric oxide synthase (iNOS) that generates nitric oxide from L-arginine and substantial production of NO. M1 type macrophages are professional antigen presenting cells and phagocytes, they express high levels of MHC I and class II antigens, CD40, CD80 (B7.1), CD86 (B7.2) co-stimulatory molecules and secrete complement factors to facilitate complement-mediated phagocytosis. Even though cell markers alone are not enough to fully define the subpopulations of

macrophages, it has been shown that M1 type macrophages express CD11c surface marker along with the specific macrophage identification markers such as CD11b and F4/80.

M2 type macrophages function in immunosuppression and tissue repair. Exposure to IL-4 or treatment with M-CSF produces an "anti-inflammatory" meaning, alternatively activated M2 macrophages. M2 type macrophages are generally characterized by production of high levels of IL-10 and IL-1RA and low expression of IL-12/ IL-23, combined with high levels of scavenger, mannose (CD206). They repurpose arginine metabolism to ornithine and polyamine by arginase, which promotes growth. Meaning that, they do not produce NO. However, M2 macrophages can be further divided into subsets. M2a, M2b, and M2c based on their cytokine expression profiles. The M2a subtype is induced by IL-4 or IL-13. The M2b is elicited by IL-1R ligands or exposure to immune complexes plus LPS. The M2c subtype is characterized by production of IL-10, TGF-β and glucocorticoid hormones.

The fourth type of macrophage M2d (or TAMs) is characterized by an IL-10^{high} IL-12^{low} M2 profile. M2d's have phenotypic and functional attributes distinct from M2a-c. Their functions are immunosuppression, low tumoricidal activity and promotion of tissue remodeling and angiogenesis.

There are several studies regarding effects of *H. pylori* on macrophages. In early studies, it has been shown that *H. pylori* induces the expression of inducible NO synthetase (iNOS) from macrophages along with pro- inflammatory cytokines such as IL-6, IL-8, TNF-α, IL-1-β. However, in recent studies, it has been shown that, in human gastric biopsy specimens from *H. pylori* positive individuals, $CD163^{+}$ (alternatively activated; M2) macrophages were detected. Also, upon *H. pylori* infection, human monocytes secreted IL-1β, IL-6, IL-10, and IL-12p40 (partially secreted as IL-23), but not IL-12p70, meaning that, M2 macrophages were up-regulated and secreted IL-10 but produced less of the pro-inflammatory cytokines than M1 macrophages.

Furthermore, cytokines secreted from innate immune cells, antigen presentation from macrophages and dendritic cells and changes in the microenvironment, also activates the adaptive immune response against *H. pylori*. Pro-inflammatory Th1 and Th17 $CD4^{+}$T cells mediate the pre-dominant adaptive response against *H. pylori*, the latter being induced by IL-23, IL-1β, and IL-6. In spite of evidence for $CD4^{+}$ T-cell-mediated protection against *H. pylori* infection in mouse models, Th1 and Th17 responses in humans are also present in chronic *H. pylori* infection as is IL-17. In mice, the induction of regulatory T (Treg) cells with the simultaneous suppression of Th17 cells may contribute to bacterial persistence.

Also, recent findings revealed that murine splenic B cells produce and secrete IL-10 upon *Helicobacter*-infection *in vitro* as well as *in vivo*. 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. The interaction between regulatory B cells and T cells were also denoted as required for the function of regulatory B cells (Bregs). 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.

Furthermore, in humoral response, *H. pylori*- specific serum IgM antibodies were present in *H. pylori* infected humans. Also, Serum IgA and IgG antibodies were directed toward many different *H. pylori* antigens.

Above information shows that there is some information about the effects of *H. pylori* on macrophages but not definite characterization of *H. pylori* or *H. felis* on murine or human studies. Therefore, in this study, we investigated the effect of *H. felis* on polarization of two types of macrophages: bone marrow- derived macrophages and peritoneal macrophages to show the polarization status and their differences according to their surface receptor expressions and cytokine profiles.

For that purpose, firstly bone marrow cells were isolated from leg bones of C57BL/6 mice and were differentiated to bone marrow- derived macrophages in the presence of M-CSF derived from L929 cell line. Peritoneal macrophages were collected from peritoneal cavity of thioglycollate induced C56BL/6 mice. After determining the percentage of differentiation and purity of isolation, cells were treated with LPS, *H. felis* sonicate, or left untreated as an internal control for 24 hours. Small portion of cell pellets were used for surface marker stainings of activation markers (CD40, CD80 and CD86) and of M1 and M2- type specific surface markers (CD11c and CD206, respectively). Also, their supernatants were collected for ELISA to measure IL-12/IL-23 (p40), TNF-α, IL-1β, IL-10, and NO secretion levels via griess reagent protocol. In addition to that, their pellets were collected for relative gene expression analysis of IL-12/IL-23 (p40), TNF-α, IL-1β, IL-10, IL-6 and iNOS using real-time PCR assay.

The results of this study indicated that both LPS and *H. felis* sonicate- treated cells express high levels of activation markers of CD40 and CD80. CD86, which also is an activation marker, was the determining marker in identifying the activation status. CD86 marker expression suggesting the activation status, did not affect the polarization status of both bone marrow- derived and peritoneal macrophages. LPS treated bone marrow- derived macrophages were categorized as M1 type macrophages with the high CD11c and low CD206 expression, and *H. felis* sonicate- treated bone marrow -derived macrophages were categorized as M2 type macrophages with the high CD206 and low CD11c expression. However, high levels of CD11c surface marker expression was detected in LPS and *H. felis* sonicate- treated, thioglycollate induced peritoneal macrophages. Despite the high levels of CD11c expression, there was higher CD206 expression among *H. felis* sonicate- treated peritoneal macrophages when compared to LPS treated group. Therefore, as a result of surface marker expressions, there seems to be no correlation between the activation status and polarization status of peritoneal macrophages.

After that, cytokine profiles of activated macrophages were also examined. Firstly, activation status of bone marrow- derived and peritoneal macrophages were assessed via CD86 marker expression. After that, cytokine profiles of activated macrophages were identified. As a result, LPS- treated BM- derived and peritoneal macrophages were polarized to M1 phenotype with high IL-12/IL-23 (p40), TNF-α, IL-1β, IL-6 cytokine expression and secretion, NO production and iNOS expression. Furthermore, *H. felis* sonicate- treated bone marrow -derived and peritoneal macrophages categorized as M2b phenotype with the high anti-inflammatory IL-10 production along with TNF-α, IL-1β, and IL-6 production. Also, IL-10 secretion observed from LPS- treated bone marrow- derived and peritoneal macrophages was thought to be the protective effect of IL-10 against LPS toxicity. However, production of pro-inflammatory IL-12/IL23 (p40) cytokine lead us to the conclusion that *H. felis* does

not drive macrophages to polarize into only one phenotype of M2b, but there is probably M1 type macrophages mixed in the population.

In conclusion, this study has contributed to the literature through providing definitive characterization of *H. felis* infected bone marrow- derived and peritoneal macrophages polarization, describing the surface marker and cytokine profiles for the first time.

HELICOBACTER FELIS'İN MAKROFAJ POLARİZASYONU ÜZERİNE ETKİSİ

ÖZET

Helicobacter pylori (H. pylori), mide kanserinin gelişimine neden olan en önemli risk faktörlerinden birisi olarak tanımlanmıştır. Türkiye'nin dahil olduğu gelişmekte olan ülkelerde bireylerin %80'i *H. pylori* ile enfekte durumdadır. Ancak, enfekte bireylerin sadece %20'si gastrit, ülser veya mide kanseri gibi gastrik komplikasyonlar göstermektedir. *H. pylori* enfeksiyon patogenezi ile ilgili birçok durum hücre kültürü deneyleri ve fare modelleri kullanılarak aydınlatılmaya çalışılmaktadır.

Helicobacter felis (H. felis), spiral şekilli, gram negatif bir bakteridir. Fare modellerinde, *H. pylori*'nin yanı sıra onunla aynı aileden ve farelerde immünojenliği daha yüksek olan *H. felis* yaygın şekilde kullanılmaktadır. C57BL/6 fare modellerinde *H. felis*'in kronik enfeksiyonunun pre-neoplastik patolojiyi tetiklediği gösterilmiştir. Histolojik olarak mide atrofisi, pit hücre hiperplazisi ve intestinal metaplazi ile karakterizedir. *H. felis* normalde *H. pylori*'nin bulundurduğu önemli virulans faktörlerini taşımamaktadır. Buna rağmen, fare modellerinde *H. pylori*'den daha ağır mide inflamasyonlarına sebep olmaktadır.

H. pylori'ye karşı proinflamatuar doğal immün cevap makrofajlar, nötrofiller ve dendritik hücreler tarafından sağlanmaktadır. Makrofajlar heterojenik ve dinamik hücrelerdir ve gerekli durumlarda, farklı uyaranlara cevap olarak farklı tiplere polariza olabilirler. Polarizasyon durumu, uyaranlara ve yerel mikro-çevrenin durumuna göre değişiklik gösterir ve makrofajların değişen ortama uyum sağlamasına olanak sağlar. Farklı uyaranlar tek dokularda tek başlarına var olmazlar ve makrofajlar sadece tek bir tipe polarize olup, tek tipte klonal olarak çoğalmayabilirler. Makrofajların farklı fonksiyonları; uyaranlara, mikro-çevreki sitokin profiline göre ve uyaranların farklı reseptörler ile tanınması ile ilişkilidir. Klasik olarak aktive edilmiş makrofajlar (M1 tip) ve alternatif olarak aktive edilmiş makrofajlar (M2 tip) olmak üzere iki genel makrofaj polarizasyon fenotipi bulunmaktadır. Bu fenotipleri sadece hücre yüzey belirteçleri sayesinde karakterize etmek mümkün değildir. Bu yüzden, makrofajlar hücre yüzey belirteçlerinin yanı sıra, spesifik fonksiyonları ve ürettikleri sitokin profilleri incelenerek kategorize edilmelidirler.

Klasik olarak aktive edilmiş (M1 tip) makrofajlar proinflamatuar efektör doğal immün cevap hücreleridir. Bu hücreler GM-CSF, LPS ve IFN-γ ile aktive olurlar ve yüksek miktarlarda TNF-α, IL-1β, IL-6, IL-12/ IL-23 proinflamatuar sitokinlerini salgılarlar. Anti mikrobiyal fonksiyonları, iNOS enzimi tarafından üretilen nitrik oksit (NO) ile ilişkilidir. M1 tipi makrofajlar profesyonel antijen sunan hücrelerdir ve yüksek miktarlarda MHC sınıf I ,sınıf II antijenleri ile beraber, CD40, CD80 (B7.1), CD86 (B7.2) yardımcı uyaran molekülleri ve fagositoz aktivasyonu için kompleman sistemi proteinlerini eksprese ederler. Aynı zamanda, hücre yüzey belirteçlerinden CD11c'nin M1 tipi makrofajlarda eksprese edildiği obesite çalışmasında kanıtlanmıştır.

Alternatif olarak aktive edilmiş (M2 tip) makrofajlar immün baskılama ve doku onarımında görev alırlar. Bu hücreler IL-4 veya M-CSF ile aktive olurlar. Genelde yüksek miktarda anti-inflamatuar IL-10 sitokin salgılaması, IL-1RA ekspresyonu, CD206 yüzey belirteci ekspresyonu ve düşük miktarda IL-12/ IL-23 sitokini salgılaması ile karakterizedirler. M2 tip makrofajlar, M2a, M2b ve M2c olarak üç alt gruba ayrılırlar. M2a alt tipi IL-4 veya IL-13 sitokinleri ile uyarılırlar. M2b alt tipi IL-1R ligandı ve/veya LPS ve immünkompleksleri ile uyarılırlar. M2c alt tipi ise IL-10, TGF-β ve glukokortikoid hormonları ile uyarılırlar.

Dördüncü M2d (TAM) tipi makrofajlar ise yüksek IL-10 ve düşük IL-12 sekresyonu ile karakterizedirler. Bu makrofajların fenotipik and fonksiyonel özellikleri M2a-c tipi makrofajlardan farklıdır. Fonksiyonları arasında immün cevabın baskılanması, düşük tümörisidal aktivite, doku yenilenmesi ve anjiyogenez bulunmaktadır.

Literatürde bazı çalışmalarda *H. pylori*'nin makrofajlar üzerindeki bazı etkileri gösterilmiştir. İlk çalışmalarda, *H. pylori*'nin makrofajlarda iNOS ekspresyonunu indüklediği, aynı zamanda IL-6, IL-8, TNF-α, IL-1-β gibi proinflamatuar sitokinlerin salgılanmasını sağladığı gösterilmiştir. Fakat, yakın zamandaki insan mide biyopsileri ile gerçekleştirilen çalışmalarda, *H. pylori* pozitif hastaların dokularında CD163+ (alternatif olarak aktive edilmiş (M2 tip) makrofaj) makrofajlara rastlanmıştır. Ayrıca, *H. pylori* enfekte insanların monositlerinden IL-1β, IL-6, ve IL-12p40 proinflamatuar sitokinlerin yanı sıra, anti-inflamatuar IL-10 salgılandığı gösterilmiştir. Başka bir deyişle, M2 tipi makrofajlar yukarı regüle edilmiştir ve yüksek miktarda anti-inflamatuar sitokinler ile M1 tipi makrofajlara kıyasla daha düşük miktarda pro-inflamatuar sitokinlerin salgılandığı gözlemlenmiştir.

H. pylori'ye karşı edinsel immün cevap, doğal bağışıklık hücrelerinin salgıladıkları sitokinler, antijen sunan hücrelerinin edinsel bağışıklık hücreleri ile etkileşimleri ve mikro-çevredeki değişimler sayesinde aktive olur. Pre-neoplastik patoloji, sadece enfeksiyon tarafından değil (örneğin 'bakteriyel onkoprotein' olarak davranan *Helicobacter* virulans faktörleri aracılığıyla), aynı zamanda local $CD4^+$ efektör T hücrelerce (Th-1) üretilen interferon-γ aracılığıyla oluşur. Th-1 hücrelerinin yanı sıra proinflamatuar Th-17 hücreler de *Helicobacter* patolojisinde rol almaktadır. Th-17 hücre grubu IL-23, IL-1β, ve IL-6 sitokinleri tarafından uyarılır. *Helicobacter*- enfekte farelerde, $CD4^+$ efektör T hücreleri regülatör T hücre (Treg) popülasyonunun sıkı kontrolü altındadır. Treg'lerin eliminasyonu mide patolojisini kötüleştirir.

Treg hücre grubunun yanı sıra, yakın zamanlarda, fare dalağından izole edilen B hücrelerinin *Helicobacter* enfeksiyonunda IL-10 ürettiği ve salgıladığı *in vivo* ve *in vitro* çalışmalarda gösterilmiştir. *Helicobacter* –asosiye mide patolojisinin baskılanmasında *Helicobacter* –aktive regulatör B hücrelerinin (H_{sps} Breg) rolü gösterilmiştir. Bu IL-10 üreten regülatör B (Breg) hücreleri, C57BL/6 faresindeki *Helicobacter* kaynaklı yüksek Th1 dominant immün cevabı baskılamaktadır. Uyarılan *Helicobacter* spesifik- Breg hücre grubu, naif T hücrelerinin IL-10 üreten $CD4^+CD25^+$ T regülatör-1 (Tr-1) hücrelerine farklılaşmasını sağlayarak, *Helicobacter*- kaynaklı gastrik patolojiyi *in vivo* ve *in vitro*'da baskılamaktadır.

Aynı zamanda, *Helicobacter* enfekte hastaların serumlarında, *Helicobacter*- spesific IgM, IgA ve IgG antikorlarına rastlanmıştır.

Yukarıda bahsi geçen bilgiler *H. pylori*'nin makrofajlar üzerindeki bazı etkilerini ortaya koymaktadır. Fakat, halen *H. pylori* ya da *H. felis*'in makrofaj üzerindeki etkilerinin kesin olarak karakterize edildiği bir bilgi bulunmamaktadır. Bu sebeple,

çalışmamız, *Helicobacter felis*'in kemik iliği kökenli makrofajların (Kİ- kökenli makrofajlar) ve peritoneal makrofajların polarizasyonu üzerindeki etkisini incelemeyi amaçlamaktadır. Bu etki, M1 ve M2 tipi makrofajlara özgü yüzey belirteçlerinin ve sitokin profillerinin aralarındaki farklılıklar baz alınarak açıklanmıştır.

Bu sebeple, C57BL/6 tipi farelerin uzuv kemiklerinden elde edilen kemik iliği hücreleri, L929 hücresince üretilen M-CSF varlığında *ex vivo* ortamda Kİ- kökenli makrofajlarına çevrilmiştir. Peritoneal makrofajlar ise thioglycollate enjekte edilmiş C57BL/6 farenin periton boşluğundan izole edilmiştir. Kİ- kökenli makrofajlarına farklılaşmanın teyidi ve peritoneal makrofajların saflığının teyidi için, makrofaj spesifik anti-F4/80 ve anti-CD11b antikorlarıyla sağlanmıştır. Ardından, elde edilen makrofajlar, bakteriyel lipopolisakkarit (LPS) ve *Helicobacter felis (H. felis)* sonikatı ile, 24 saat süresince muamele edilmiştir. Hücre peletleri hücre aktivasyon belirteci (CD40, CD80 ve CD86) boyamaları ve M1 ve M2 tipi spesifik belirteçleri (sırasıyla, CD11c ve CD206) boyamaları için ve IL-12/IL-23 (p40), TNF-α, IL-1β, IL-10, IL-6 ve iNOS sitokinleri için gerçek zamanlı PZR testleri için kullanılmıştır. Hücre süpernatanları ise IL-12/IL-23(p40), TNF-α, IL-1β ve IL-10 spesifik ELIZA testleri ile NO sekresyonu tayini için griess bileşim protokolü testi için kullanılmıştır.

Gerçekleştirilen deneylerin sonucunda, kemik iliği hücrelerinin *ex vivo* ortamda, %90'ın üzerinde saflıkla Kİ- kökenli makrofajlarına dönüştüğü, ve peritoneal makrofajların yine %90 üzerinde saflıkla izole edildiği, makrofaj spesifik F4/80 ve CD11b yüzey belirteçleri ile teyit edilmiştir. Aktivasyon belirteci boyamaları sonucunda, Kİ- makrofajları ile peritoneal makrofajlar, muameleden bağımsız olarak yüksek miktarlarda CD40 ve CD80 yüzey belirteci eksprese ettikleri gözlemlenmiştir. Bunun yanında, belirleyici olarak CD86 belirteci, muamele görmeyen gruba kıyasla, *H. felis* sonikatı ile muamele gören Kİ- kökenli makrofajlarından ve peritoneal makrofajlarından yüksek miktarda eksprese edilmiştir. Yapılan deneylerde, CD86 antikoru ile belirlenen aktivasyon statüsünün, CD11c ve CD206 antikorları ile belirlenen sırasıyla M1 tipi ve M2 tipi makrofajlara polarizasyonu statüsünün arasında bir ilişki gözlemlenememiştir. polarizasyon statüsünün karakterizasyonu için, yüzey belirteçlerinin yanı sıra aktif makrofajların sitokin profilleri de incelenmiştir. Bu incelemeler sonucunda, *Helicobacter felis* sonikatı ile muamele edilen kemik iliği ve peritoneal makrofajlarının yüksek düzeyde IL-10 sitokini üretmeleri ve NO üretmemelerinden dolayı, M2 tipine benzer fenotip sergilediği gösterilmiştir. Aynı zamanda, *Helicobacter felis* sonikatı ile muamele edilen bu makrofajların, muamele edilmeyen gruba göre, yüksek oranda IL-12/IL-23(p40), TNF-α, IL-1β ve IL-6 sitokinlerini salgıladıkları ve eksprese ettikleri gözlemlenmiştir.

Sonuç olarak, LPS ile muamele edilen Kİ- kökenli makrofajları, *ex vivo* ortamda, yüzeylerinde bulundurdukları yüksek miktardaki CD86 ile daha aktif, CD11c belirteci ve salgıladıkları IL-12/IL-23(p40), TNF-α, IL-1β, IL-6 sitokinleri ve NO üretmeleri dolayısıyla tarafımızdan M1 tipi makrofaj olarak sınıflandırılmıştır. Öte yandan, *Helicobacter felis* sonikatı ile muamele edilmiş Kİ-makrofajlarının, M2 tipi makrofajlara özgü CD206 yüzey belirteci bulundurmaları ve IL-10 sitokini salgılamaları dolayısıyla, M2 tipine benzer bir fenotip sergilediği önerilmektedir. Aktiflik statüsünün, makrofajların polarizasyonuna etki etmediği de yüzey belirteçleri ile belirlenmiştir. Ayrıca makrofajların TNF-α ve IL-1β sitokinleri ile beraber IL-6 sitokinini salgılamaları ile beraber literatürde bilinen M2b fenotipi makrofajlara polarize oldukları gözlemlenmiştir. Bu makrofajların aynı zamanda M1 tipi makrofajlara özgü IL-12/IL-23(p40) sitokini de ürettikleri gözlemlenmiştir.

Çalışmalarımızın sonucunda, *Helicobacter felis* sonikatı ile muamele edilmiş Kİ-makrofajlarının, M2b ve M1 tipine benzeyen hücrelere farklılaştıkları önerilmektedir.

1. INTRODUCTION

1.1 *Helicobacter* Strains

1.1.1 *Helicobacter pylori*

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral shaped bacterium which colonizes in the stomach (Figure 1.1). It can persist for long time periods within the host without being extinguished by the immune system or by the frequent gastric environmental changes. Around %50 of individuals is infected with *H. pylori* around the world. The prevalence of *H. pylori* is decreasing in the Western world; however, it is still a significant medical issue in less industrialized countries, with constant higher infection rates and more widespread distribution. Most of infected individual remain asymptomatic (Bauer & Meyer, 2011).

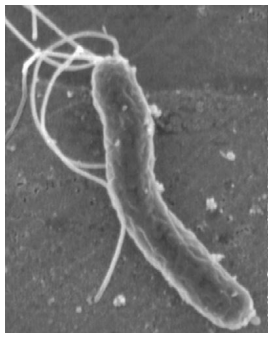


Figure 1.1 : *Helicobacter pylori* (Electron Micrographs courtesy of Lucy Thompson, school of Biotechnology & Biomolecular Sciences, University of New South Wales).

H. pylori is typically acquired in childhood and can persist for many decades despite the development of an adaptive immune response (Algood & Cover,2006). Nearly all infected individuals develop chronic active gastritis, which in turn may progress to other conditions; especially peptic ulcer disease, distal gastric adenocarcinomas and gastric lymphomas (Figure 1.2.) (Ernst & Gold, 2000). Therefore, *H. pylori* is classified as type I carcinogen for gastric malignancies (Blaser, Atherton, 2004).

Survival of *H. pylori* in host depends on how the bacteria elicit effective escape mechanisms, which often causing cellular damage and inflammation (Petersson, Forsberg, and Aspholm et al, 2006). These escape mechanisms are mostly virulence factors of varying strains of *H. pylori* and its enzymatic activities (Figure 1.2.). For example, stomach's low pH value, which is ranging from pH 2 to 6 from luminal surface to epithelial lining, inhibits growth of most ingested bacteria. However, this strong acidity is defeated by *H. pylori's* cytosolic or cell-surface related urease activity. Urease is highly conserved among *Helicobacter* strains and breaks down urea to release ammonia and carbonate which neutralizes the acidic environment. Therefore, the bacteria can persist in the gastric epithelium (Graham. Go &Evans, 1992; Solnick et al., 1995).

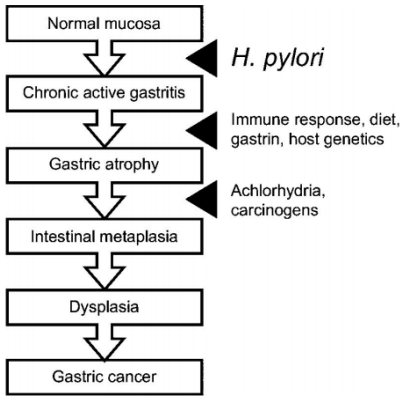


Figure 1.2 : Role of *Helicobacter pylori* on gastric malignancies (adapted from Johannes et al., 2006).

H. pylori infection starts with adherance and colonization of the bacteria, then bacteria evade the immune system, finally invade into mucosa and cause damage at the last step (Sheu et al., 2010). Outer membrane proteins (e.g. Blood group antigen binding adhesin-BabA; Outer membrane inflammatory protein-OipA) help bacteria to adhere to the gastric epithelium. Also, different adhesion protein expression within a single strain over time leads to the dynamic adaption capacities by switching of gene expression on/off, gene inactivation, or recombination (Aspholm-Hurtig et al., 2004; Bâckstrôm et al., 2004; Solnick, 2004). Flagellins (FlaA and FlaB) give motility to bacteria (Gewirtz et al., 2004). After invading, *H. pylori* leads to immune response attenuation and persists with the help of their other virulence factors such as cell wall component peptidoglycan (PGN) and lipopolysaccharide (LPS) (He et al., 2014). For efficient invasion and damage, *H. pylori* translocates effector proteins such as

vacuolating cytotoxin A (VacA), and cytotoxin-associated gene A (CagA), which modulate host cell immune response for the benefit of bacterium (Kim & Blanke, 2012) (Figure 1.3).

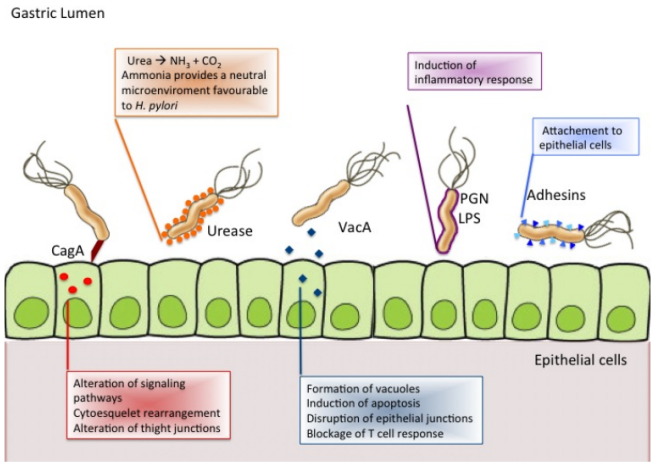


Figure 1.3 : *Helicobacter pylori* virulence factors (adapted from Morales-Guerrero et al., 2013).

VacA virulence protein causes vacuolation of host cell. After it is translocated to host and internalized, vacuole formation occurs, which may interrupt trafficking inside host cell (Palframan et al., 2012). Also, VacA leads to apoptosis of host cells by interacting with mitochondria. It inhibits T cell response by directly binding to T cell integrin proteins (Peek, Fiske &Wilson, 2010) (Figure 1.3).

Cag pathogenicity island (cag- PAI) is mostly the main cause for *H. pylori* pathogenicity. Cag- PAI encodes type IV secretion system (T4SS) that is necessary for the translocation of the virulence proteins into host cell. Translocation starts with a pilus from CagY protein, which interacts with host integrin β1 protein together with CagL virulence factor, and cause conformational changes to inject CagA into host cell. Also, CagE gives the energy needed for translocation of CagA by acting as an NTPase (Odenberit et al., 2000; Bauer et al., 2005).

After CagA is translocated into the host cell, it can directly affect cellular responses such as; interacting with tight junctions, increasing cell number, changing polarity of cells, and activating inflammatory response. Also, it can phosphorylate itself by Abl or Src family kinases, which starts from tyrosine aminoacids on Glu-Pro-Ile-Tyr-Ala (called as EPIYA) motifs. This process results with host cell shape change and motility (Jones, Whitmire &Merrell, 2010).

1.1.2 *Helicobacter felis*

The spiral-shaped, gram-negative bacteria, *Helicobacter felis (H. felis)* was first isolated from the stomach of a cat and was later also found in dogs (Lee, Hazell, O'Rourke & Kouprach, 1988) (Figure 1.4). Meaning that, it is one of the *Helicobacter* species with zoonotic potential. Also, *H. felis* is highly motile; on agar plates, it does not really form colonies, but rather grows as a lawn (Gottwein et al., 2001).

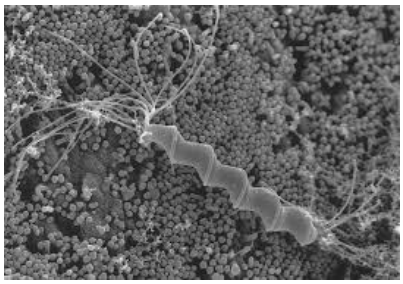


Figure 1.4 : *Helicobacter felis* (adapted from Neiger&Simpson, 2000).

The bacterium contains a urease enzyme and two flagellin genes (*flaA* and *flaB*). Flagellin genes are inactivated, also mutation of *flaA* gene results in the inability to colonize a murine model of infection (Josenhans et al., 1999). However, *H. felis* lacks VacA and CagA virulence factors. Despite lacking important *H. pylori* virulance factors, it causes more severe gastric inflammation *than H. pylori* (Sakagami et al., 1996). *H. felis* may induce gastritis, epithelial cell proliferation, and apoptosis in murine models, therefore, it is widely used in murine *Helicobacter* studies. (Court et al., 2002; Schmitz et al., 2011). It has been shown with C57BL/6 mice that *H. felis* infection induces Th1 type response with IFN-γ production. This IFN-γ release from Th1 type cells is reversely correlated with bacterial colonization in the stomach (Sayi et al., 2009).

1.2 Macrophages

Macrophages were first identified by Ilya Metchnikoff and his identification and description of phagocytosis brought him the Nobel Prize for Medicine in 1905 (Nathan, 2008). Macrophages are heterogeneous and dynamic cells. They are found almost in all tissues with different functions. However, their main functions are to respond to pathogens for shaping the adaptive immune response via antigen processing and presentation and to be first line of defense for innate immune response (Hoebe, Janssen & Beutler, 2004). Moreover, generation and resolution of inflammation and

tissue repair are among the functions of macrophages (Mosser and Edwards 2008). Although macrophages are very effective in antigen presentation and microbicidal effects, several pathogens have evolved strategies to escape and interfere with macrophage activation and to modulate host responses (Russell, 2007).

1.3 Monocyte / Macrophage Development

Monocytes originate from myeloid hematopoietic progenitors located in bone marrow. They enter the peripheral blood circulation, and within three days or so, circulating monocytes migrate into different tissues, such as the spleen, which serves as a storage reservoir for immature monocytes (Geissmann, 2010). After exposure to local growth factors, cytokines, pathogens, pathogen related compounds in migrated tissues, monocytes differentiate into macrophages and/or dendritic cells (Tacke & Randolph, 2006) This process is called "mononuclear phagocyte system" (Figure 1.5). The variety of undifferentiated circulating monocytes may affect their polarization status in different tissues (Gordon & Taylor, 2005). Mainly, monocytes play a key role in eliminating invading bacteria, virus, fungi, and protozoans. However, they also may have negative effects on the pathogenesis of inflammatory and degenerative diseases (Strauss-Ayali, Conrad & Mosser, 2007).

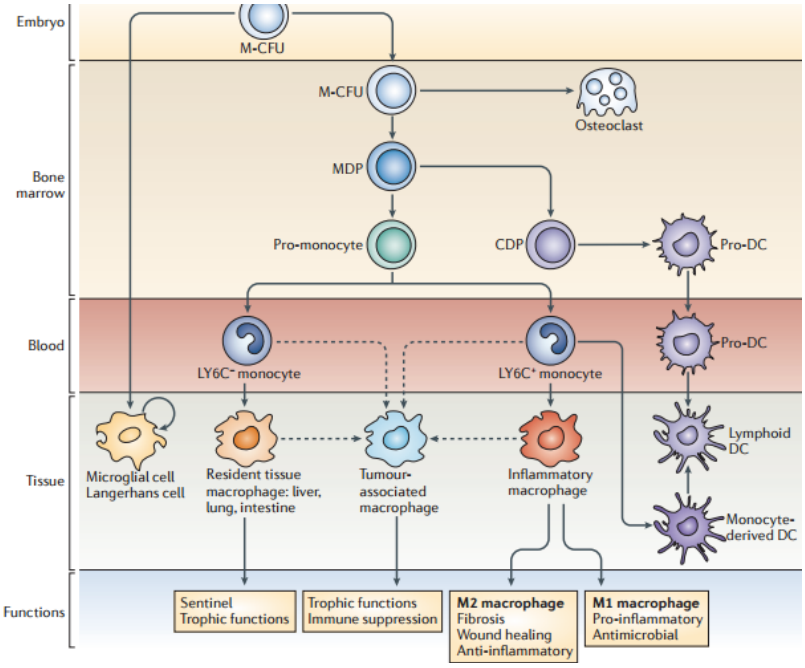


Figure 1.5 : The mononuclear phagocyte system (adapted from Lawrance & Natoli, 2011) (solid lines represent common pathway of development and dashed lines represent alternative pathway of development).

Macrophages are sorted based on their anatomical location and function; such as specialized tissue-resident macrophages include osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue) and Kupffer cells (liver) (Gordon & Taylor, 2005). The functions of tissue-specific macrophages are to ingest foreign materials and recruit additional macrophages from circulation during an infection or following injury (Figure 1.6).

For example, gut is populated with various types of macrophages, which work together to maintain tolerance to the gut flora and to food. Also, secondary lymphoid organs contain different macrophage populations, such as marginal zone macrophages in the spleen, which suppress innate and adaptive immunity to apoptotic cells (McGaha et al., 2011), and subcapsular sinus macrophages of lymph nodes, which clear viruses from the lymph and activate antiviral humoral immune responses (Jannacone et al., 2010; Junt et al., 2007). Moreover, macrophages are also present at immune-privileged sites, such as the brain (microglia), eye and testes. They function mainly in tissue remodelling and homeostasis.

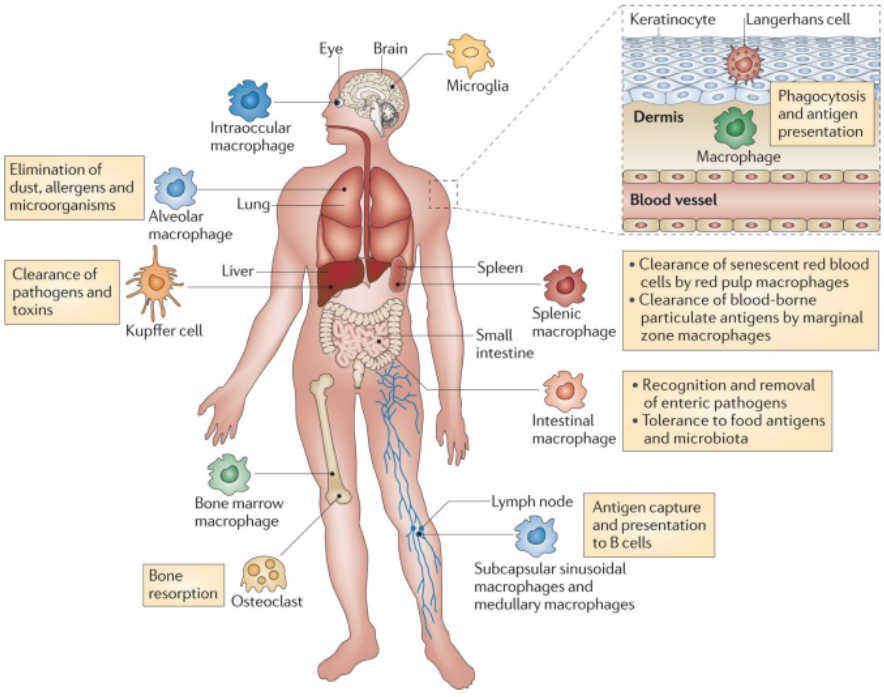


Figure 1.6 : Different tissue macrophages (adapted from Murray & Wynn, 2011).

However, not all tissue macrophages are differentiated from monocytes. For instance, Langerhans cells in the skin and microglial cells in the brain proliferate locally. Also,

recent studies indicate that these cells initially develop from M-CFU in the yolk sac of the developing embryo (Lawrance & Natoli, 2011).

The peritoneal cavity is a membrane-bound and fluid-filled abdominal cavity of mammals. The liver, spleen, most of the gastro-intestinal tract and other organs are located in peritoneal cavity. It also contains different immune cells including macrophages, B1 cells and T cells. The presence of a high number of naïve macrophages in the peritoneal cavity makes it a preferred site for the collection of naïve tissue resident macrophages. Typically, 5-10 million peritoneal cavity cells can be obtained from an unmanipulated mouse and among them, 50-60% are B1 cells, ~30% are peritoneal macrophages and 5-10% are T cells (Zhang, Goncalves, & Mosser, 2008). Moreover, peritoneal macrophages (PMs) have been widely used as a macrophage source in mice since the 1960s (Rous, 1925; Kaufmann & Schaible, 2005).

Macrophages are distinguished from dendritic cells (DCs) and from other mononuclear cells by differential expression of specific surface makers such as CD11b and F4/80 (which is encoded by EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (*Emr1*) (Austyn & Gordon, 1981). Also, CD18 (also known as MAC1), CD68 and Fc receptors may be used.

1.4 Pathogen Recognition of Macrophages Via Toll-like Receptors

The toll-like receptors (TLRs) are type I transmembrane family proteins expressed by antigen presenting cells (macrophages, B lymphocytes, dendritic cells) and neutrophils. Their primary function is to recognize structurally conserved molecules or pathogen associated molecular patterns (PAMPs) derived from microbes. Also, TLRs function to recognize infectious organisms and danger signals; initiate inflammatory responses; activate microbial killing and clearance mechanisms (Akira & Takeda, 2004). There are known 13 paralogous TLRs; 10 in humans and 12 in mice (Figure 1.7).

TLRs act principally to initiate an innate immune response, and inflammation is the central hallmark of this response. TLRs have also been described as ‘necessary’ or ‘required’ for an adaptive immune response (Janeway et al., 2007).

TLR4 responds to LPS from Gram-negative bacterial cell walls. TLR4 can signal through two different pathways dependent on, and modulated by, separate adaptor proteins MyD88 or Toll/IL-1R domain–containing adapter inducing IFN-β (TRIF) (Rossol et al., 2011). Hence, TLR4 signalling causes macrophages to produce inflammatory cytokines such as tumor necrosis factor TNF-α, IL-1 and IL-6.

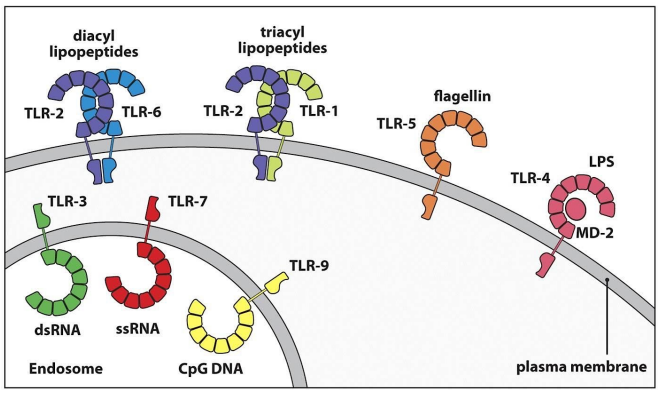


Figure 1.7 : Toll like receptors and their ligands (adapted from Janeways Immunobiology 7th edition, 2007).

LPS and flagellin of *H. felis* were found to be recognized by TLR2, and its signaling depends on MyD88 adapter protein. However, their recognition is not dependent on TLR4 or 5 as typical LPS and flagellin (Figure 1.8). *Helicobacter*'s LPS is less proinflammatory than LPSs from many other gram-negative species (Khamri et al., 2005). For instance, *Escherichia coli* or *Salmonella enterica serovar Typhimurium's* LPS has 500-fold-higher endotoxic activity than *Helicobacter* LPS. Also, macrophage production of proinflammatory cytokines, nitric oxide, and prostaglandins stimulation by *Helicobacter* is weak (Bliss et al., 1998; Perez- Perez et al., 1995). The low biological activity of *H. pylori* LPS is caused by modifications of its lipid A component (Moran & Aspinall, 1998, Muotiala et al., 1992) and *H. pylori* expresses tetra-acylated LPS, which is less biologically active than the hexa-acylated form that is typical of other gram-negative pathogens (Salama, Hartung & Müller, 2013) (Figure 1.8). *Helicobacter* commonly express LPS O antigens that have similar structure to Lewis blood group antigens found on human cells (Monteiro et al., 1998; Aspinall & Monteiro, 1996). This similarity causes molecular mimicry or immune tolerance that allows *Helicobacter* LPS antigens to be protected from immune recognition because of similarity to "self" antigens. Also, *H. pylori* flagella are not detected by TLR5 owing to mutations in the TLR5 binding site of flagellin.

Study conducted by Sayi et al. (2011) showed that *H. felis* activates B lymphocytes through TLR2 and cells up-regulate CD40, MHC-II and other co-stimulatory molecules and also production of IL-10, IL-6, IgM and IgG2b. TLR2 recognition of *helicobacter* mosty caused by its unusual lipid composition of the bacterial envelope which consits of high concentration of lysophospholipids and cholesteryl glucosides (Tannaes and Bukholm, 2005) (Figure 1.8).

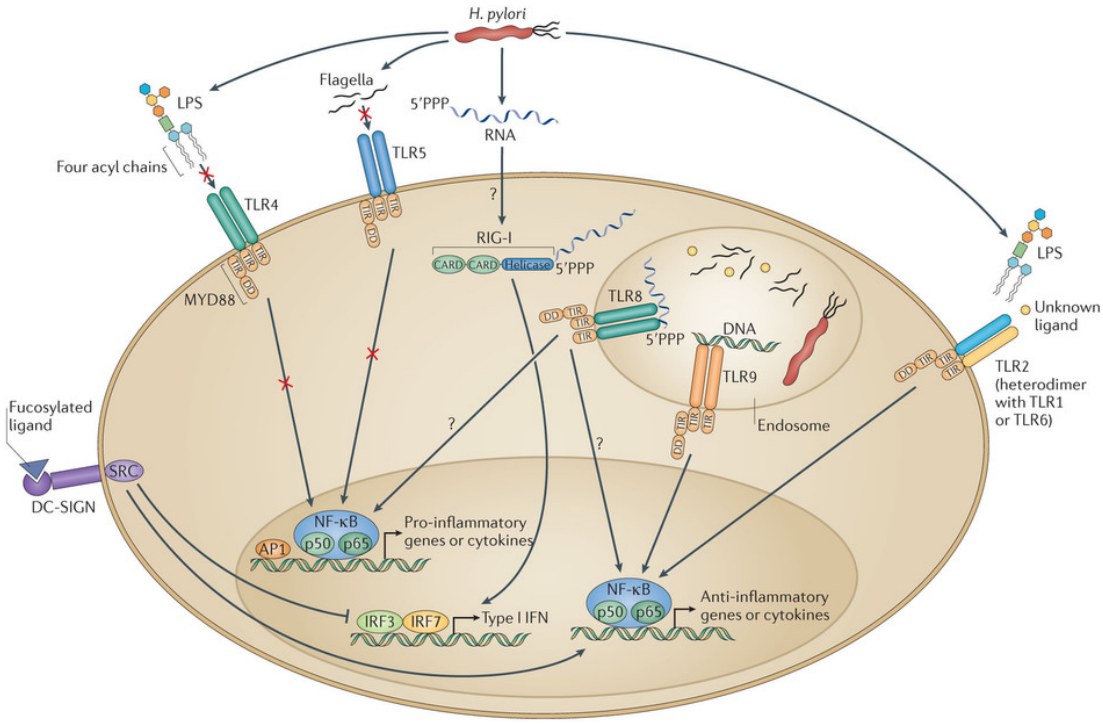


Figure 1.8 : *Helicobacter pylori* recognition by Toll- like receptors (adapted from Salama, Hartung &Müller, 2013).

1.5 Macrophage Polarization and Plasticity

Under different stimuli, macrophages can polarize into different phenotypes. However, these phenotypes are not stable. For instance, the macrophage changes phenotype from classically to alternatively activated during tumor progression (Sica, Larghi & Mancino et al., 2008). However, in obesity case, the macrophage phenotype changes from M2 to M1 (Suganami & Ogawa, 2010). These data clearly suggest that polarization status of macrophages changes according to stimuli and the local microenvironment, allowing them to shape the local inflammatory status to adapt to outside stimuli (Mosser & Edwards, 2008). However, the main limitations of the current strict classifications are: First, it ignores the source and context of the stimuli;

second, the M1 and M2 stimuli do not exist alone in tissues; and, third, macrophages may not form clear-cut activation subsets nor expand clonally.

1.5.1 Classically activated, M1 type macrophages

There are two distinct states of polarized activation for macrophages: the classically activated (M1) macrophages and the alternatively activated (M2) macrophage subsets (Murray & Wynn, 2011). M1 polarization occurs either wtih human Granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN- γ) stimulation alone or together with bacterial components, such as lipopolysaccharide (LPS) or cytokines (e.g., tumor necrosis factor-α (TNF-α)) (Mantovani et al., 2002; Jaguin et al. 2013) (Figure 1.7). Classically activated M1 type macrophages secrete high levels of pro-inflammatory cytokines, which are TNF-α, IL-1β, IL-6, IL-12, and IL-23, and increase their concentrations of superoxide anions, oxygen radicals, and nitrogen radicals, which helps them to exert killing functions (Fairweather & Cihakova, 2009; Sindrilaru, Peters & Wieschalka et al., 2011) (Table 1.1). Moreover, since macrophages are professional antigen presenting cells and phagocytes, they express high levels of MHC I and class II antigens, CD40, CD80 (B7.1), CD86 (B7.2) co-stimulatory molecules and secrete complement factors to facilitate complement-mediated phagocytosis (Mantovani et al., 2004). In order to accurately identify the M1 subtype, inducible nitric oxide synthase (iNOS; NOS2) activity must be observed. iNOS degrades arginine into nitric oxide (NO) and citrulline which in turn initiate killing functions of macrophages (Modolell et al., 1995).

The secretion of cytokines and chemokines occurs after TLR signaling; it does not require an additional stimulation except for IL-1β. IL-1β is an inflammatory cytokine whose secretion depends on processing after expression (Mariathasan et al., 2006). Cytokine is expressed as pro– IL-1β protein, 35 kDa, and after that is cleaved to active IL-1β, 17 kDa, by caspase-1 or other polymorphonuclear neutrophil proteases, such as proteinase-3, elastase, chymase, and granzyme A (Martinon et al., 2006; Kanneganti et all., 2007). Caspase activation occurs via inflammazome NLRP3 which is in turn activated by silica crystals or ATP, active IL-1β is then secreted into the extracellular environment (Netea et al., 2008; Peeters et al., 2013)

Furthermore, different inducers of M1 type macrophages may exert different properties. For example, IFN-γ stimulation results in high secretion levels of IL-12 and

IL-23 but low levels of IL-10 (Gordon & Taylor, 2005; Marie et al., 2008; Solinas et al., 2009). As a result, while secreted IL-12 promotes differentiation of Th1 cells, which improve antigen phagocytosis [Marie et al., 2008; Cassol et al., 2010), secreted IL-23 triggers the development and expansion of Th17 cells, which results in secretion high levels of IL-17 and contribute to inflammatory autoimmune pathologies (Verreck, de Boer, & Langenberg et al. 2004; Kolls & Linden, 2004).

Classically activated M1 type macrophages also express pro-inflammatory chemokines such as CCL10, CCL11, CCL5, CCL8, CCL9, CCL2, CCL3, CCL4 and also CXCL9 and CXCL10 (Duluc et al., 2007; Hao et al., 2012). These proinflammatory chemokines improve intracellular pathogen killing and attract Th1 cells (Mantovani et al., 2004).

In addition to cytokine and chemokine profiles, M1 type macrophages were identified by F4/80, CD11b, and $CD11c^{+}$ surface marker expressions in a recent obesity study (Lumeng et al., 2007; Fujisaka et al., 2009).

Classically activated M1 type macrophages exert resistance to intracellular bacteria and controls the acute phase of infection. For example, during *Listeria monocytogenes* infection, disease in immunocompromised patients and pregnant women, M1 type macrophages are activated. Therefore, bacterial phagosome escape is prevented and intracellular killing of bacteria is achieved by M1 type macrophages in vitro and in vivo (Shaughnessy & Swanson, 2007). Also, mice without IFN-γ and TNF and their respective receptors die from *L. monocytogenes* infection, since M1 type macrophages cannot destroy the pathogen effectively without IFN-γ and TNF-α (Pfeffer, 1993). Similarly, the M1 type polarization of human and murine macrophages is exerted by *Salmonella typhi*, which is the agent of typhoid fever, and *Salmonella typhimurium*, which is a gastroenteritis agent. Control of the infection is associated with the M1 type macrophages (Benoit et al., 2008).

Moreover, during the early phase of *Mycobacterium tuberculosis* infection, macrophages are polarized to M1 type according to the clinical data collected from active tuberculosis patients (Chacon-Salinas, 2005). Also, other mycobacterial diseases such as Buruli disease caused by *Mycobacterium ulcerans* and opportunistic infections by *Mycobacterium avium* are also characterized and controlled by M1 polarization of macrophages (Murphy et al., 2006; Kiszewski et al., 2006).

Even though M1 type macrophages control the infection against intracellular

pathogens, an excessive and/or prolonged M1 type macrophage effect can be deleterious for the host during acute infections with *Escherichia coli (E. coli)* or *Streptococcus sp.,* which causes many diseases, including gastroenteritis, urinary tract infections, neonatal meningitis, and sepsis. Sepsis causes systemic inflammatory response with immune dysregulation, leading to tissue damage and multiple organ failure caused my prolonged M1 macrophage response (O'Reilly, Newcomb & Remick, 1999).

1.5.2 Alternatively activated, M2 type macrophages

Aside from classical activation, exposure to IL-4 or treatment with M-CSF produces an "anti-inflammatory" meaning, alternatively activated M2 macrophages (Jaguin et al. 2013). M2 type macrophages generally characterized by production of high levels of IL-10 and IL-1RA and low expression of IL-12 and IL-23 (Solinas et al., 2009; Condeelis & Pollard, 2006). Generally, functions of M2 type macrophages include: parasite clearance, reduction of inflammation, immuneregulation, tissue remodeling and tumor progression (Sindrilaru, Peters, Wieschalka et al., 2011). M2 macrophages also express high levels of scavenger (SR), mannose (MR or CD206) and galactose receptors (GR) (Verreck et al., 2006).

M2 macrophages inhibit CXCL9, CXCL10, and CXCL5 expression by downregulating NF-κB and STAT1, which are the M1 type macrophage pathways for production of pro-inflammatory cytokines (Li & Verma, 2002; Hu et al., 2003).

IL-4 is a major promotor of wound healing because it can activate arginase, which contributes to the production of the extracellular matrix. The differential metabolism of L-arginine provides a means of distinguishing the two macrophage activation states. M1 macrophages upregulate iNOS to catabolize L-arginine to nitric oxide (NO) and citrulline, but M2 macrophages induce arginase 1, which metabolizes arginine to ornithine and polyamines, which are precursors necessary for collagen synthesis and cellular proliferation (Odegaard & Chawla, 2008).

M2 macrophages are polarized by distinct stimuli and can be further subdivided into M2a, M2b, and M2c macrophages (Figure 1.9).

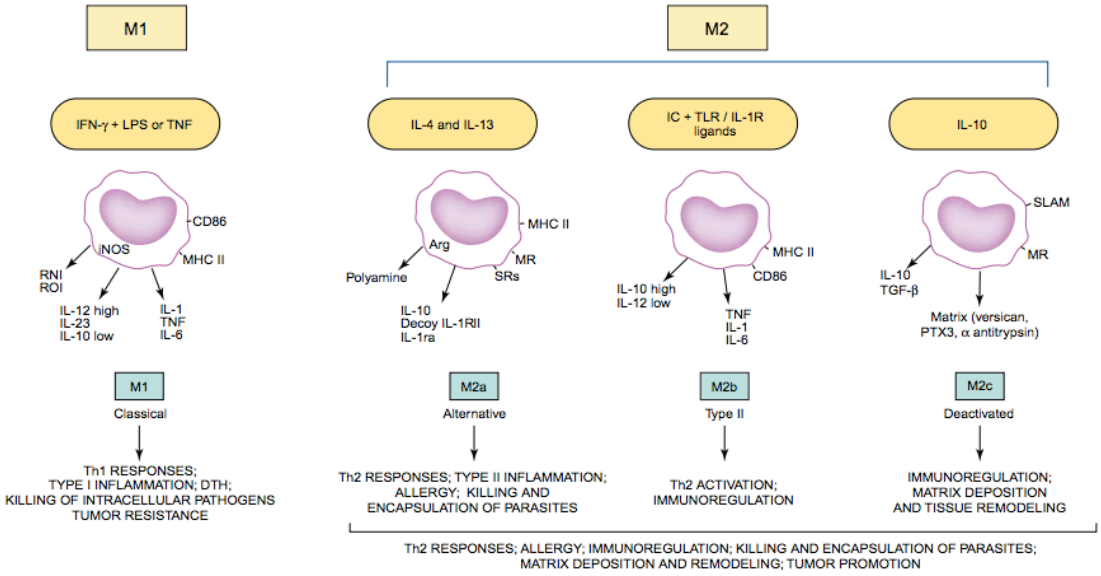


Figure 1.9 : Inducers and selected functional properties of classically (M1) and alternatively (M2) activated macrophage populations (adapted from Mantovani et al., 2004).

1.5.2.1 M2a type macrophages

M2a macrophages are stimulated by the IL-4 or IL-13 cytokines and secrete IL-10, TGF-β and IL-1 receptor antagonist (IL-1ra) cytokines (Figure 1.9) (Martinez, Helming & Gordon, 2009) and chemokine (C–C motif) ligand 24 (CCL24), CCL17, and CCL22. These chemokines can specifically combine with Chemokine (C–C motif) receptor 3 (CCR3) and CCR4, which accelerate the recruitment of eosinophils, basophils, and T helper 2 type (Th2) cells, to lead to a type II response that is associated with asthma and allergy (Romagnani, 1999). In a study conducted by Lu et al. (2013), it has been shown that M2a type macrophages exhibit anti-inflammatory functions in vitro and protect against renal injury in vivo.

1.5.2.2 M2b type macrophages

M2 cells are generally characterized by low production of pro-inflammatory cytokines (IL-1, TNF and IL-6). M2b macrophages, which are induced by immune complexes (ICs), LPS, TLRs, and/or the IL-1 receptor antagonist (IL-1ra), are the exception (Martinez, Helming & Gordon, 2009). Because, they retain high levels of inflammatory cytokine production with concomitant high IL-10 and low IL-12 levels (Mosser, 2003) (Figure 1.9). In spite of their high production of inflammatory cytokines and toxic molecules, M2b cells protect mice against LPS toxicity because of

IL-10 secretion (Mosser et al., 1999). IL-10 alone or in concert with LPS activates four distinct transcriptional programs: (i) control and deactivation of immunity and inflammation, including suppression of inflammatory cytokines and induction of signaling lymphocytic activation molecule; (ii) tuning of cytokine or G protein-coupled receptor signaling, (iii) remodeling of the extracellular matrix; (iv) B-cell function and lymphoid tissue neogenesis. Thus, IL-10 is more than a mere deactivator of macrophage and DC function (Mantovani et al., 2004).

The only difference seperates M2b type macrophages from other M2 subtypes is the IL-6 secretion (Duluc et al., 2007). Furthermore, M2b cells always secrete CCL1, which combines with CCR1 to promote the infiltration of eosinophils, Th2, and regulatory T cells. These cells exert immune regulation and drive the Th2 response.

1.5.2.3 M2c type macrophages

M2c macrophages are induced by IL-10, transforming growth factor-β (TGF-β), or glucocorticoids (GCs) and secrete also IL-10 and TGF-β (Martinez, Helming & Gordon, 2009) (Figure 1.9). IL-10 induces CXCL13, CCL16, and CCL18, which can combine with CXCR5, CCR1, and CCR8 to promote the accumulation of eosinophils and naive T cells, which play a prominent role in suppressing immune responses and promoting tissue remodeling (Mantovani et al., 2004). Also, in a recent study conducted about murine adriamycin nephrosis, it has been found that M2c macrophages effectively reduced glomerulosclerosis, tubular atrophy, interstitial expansion and proteinuria. They also reduced the effector $CD4^+$ Tcells infiltration and induced Treg induction and proliferation in kidney. Thus, M2c are potent in protecting against renal injury due to their ability to induce Tregs via their secretion of IL-10 and TGF-β.

1.5.2.4 Tumor associated macrophages (TAMs)

Studies conducted in mice models showed that different macrophage populations help to disperse malignant tumor cells to survive and grow in distant sites. The process is called metastasis of tumor cells and one of the major causes of patient's death. Metastasis relies on the help of macrophages and removal of these macrophages inhibits tumor growth and reduces cancer mortality.

Macrophages are one of the major populations of infiltrating leukocytes associated with solid tumors (Gordon & Taylor, 2005). Initially, classically activated M1-polarized macrophages can exhibit antitumor activity and to elicit tumor tissue disruption (Mantovani et al., 2002). However, in some models of carcinogenesis in the mouse, macrophage polarization switches from M1 to M2 (Zaynagetdinov et al., 2011). At later stages of cancer in mice and humans, tumor associated macrophages (TAMs) show similar functions to M2 type macrophages, having an IL-10high IL-12low phenotype (Mantovani *et al.* 2002) with potent immunosuppressive functions, low tumoricidal activity and promotion of tissue remodeling and angiogenesis (Figure 1.10).

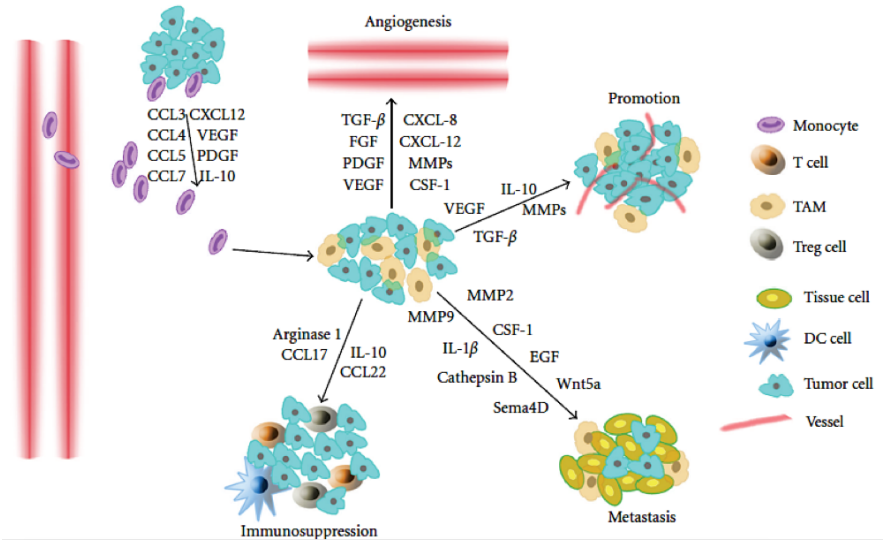


Figure 1.10 : TAM functions in tumor progression (adapted from Hao et al., 2012).

TAM infiltration, associated with poor prognosis, has been shown in Hodgkin disease, glioma, cholangiocarcinoma, and breast carcinoma (Steidl et al., 2010; Chen et al., 2011). Lymphocytes are key orchestrators of TAM function with different pathways in different tumors (Biswas & Mantovani, 2010). For example, macrophages were polarized to M2 type mediated by IL-4–producing Th2 cells in skin (Schioppa et al., 2011; Andreu et al., 2010), also by B1 cells (Biswas & Mantovani, 2010) and by antibody-producing B cells (plasma cells) in breast cancer (DeNardo et al., 2009; Pedroza-Gonzalez et al., 2011). Moreover, fibroblasts contribute to drive macrophage polarization into M2 type and tumor promotion (Erez et al., 2010).

Tumor cell produce extracellular matrix components, IL-10, CSF-1, and chemokines (CCL2, CCL18, CCL17, and CXCL4). These products polarize macrophages in a M2-

like, cancer-promoting mode (Mantovani, Allavena, Sica & Balkwill, 2008; Roca et al., 2009). TAMs also interact with and promote the tumorigenicity of cancer stem cells (CSCs) (Jinushi et al., 2011). Moreover, CD163 surface marker is used for identification of TAMs sensitively and accurately (Lau *et al.,* 2004; Pettersen *et al.,* 2011).

1.6 Intreactions Between *Helicobacter* and Immune Cells

H. pylori- induced inflammation is stimulated by different bacterial factors that stimulate epithelial cells, macrophages, and DCs activation, also pre-dominant Th1 response. Actually, colonization of *H. pylori* can be prevented by immunizing the host with bacterial components such as urease (Panchal et al., 2003) indicating activation of the adaptive response. However, urease is a major activator of macrophages, stimulating cytokine and NO generation (Gobert et al., 2002). Therefore, distinguishing between purely an innate or adaptive response is difficult, and the recognition that cells such as B cells can respond to *H. pylori* directly or via the interaction of activated T cells illustrates the complexity of the host immune response (Peek et al., 2010).

Chronic infection of *H. pylori* named as "chronic superficial gastritis" (Warren, 2000) in which T cell, B cell, macrophage, neutrophil, mast cell, and dendritic cell (DCs) infiltration is seen (Suzuki et al., 2002; Bergman et al., 2004). $CD4^+$ T cells present more abundantly than $CD8^+$ T cells (Lundgren et al., 2005; Quiding-Jarbrink et al., 2001). $CD4^+/CD25^{hi}/ FOXP3^+$ natural regulatory T cells (Tregs) also seen in higher numbers in the gastric mucosa of *H. pylori*- infected individuals, hence, they seemed to have an important role in the regulation of inflammatory response (Lundren et al., 2005). Therefore, *H. pylori* specific- chronic gastric mucosal inflammatory response occurs in a combination of cellular immune response and an ongoing stimulation of an innate immune response.

1.6.1 Innate immune response against *Helicobacter pylori*

1.6.1.1 Epithelial cells

Gastric epithelial cells express TLRs and can recognize pathogen-associated molecular patterns (PAMPs). When bacteria invade and penetrate the gastric epithelial barrier,

the alternate pathway of complement is activated, and macrophages and neutrophils recruited to infection area upon recognition. However, *H. pylori* localize within the gastric mucus layer, do not invade gastric tissue. Therefore, contact between *H. pylori* and phagocytic cells do not occur unless there are disruptions in the gastric epithelial barrier (Backhed et al., 2003; Schmausser et al., 2004).

Levels of numerous cytokines, including IFN-γ, TNF-α, IL-1β, IL-6, IL-7, IL-8, IL-18, which have pro-inflammatory effects, and IL-10, which is an immune-regulatory cytokine that may limit the inflammatory response, increased in the stomachs of *H. pylori* infected humans compared to uninfected humans (Lindholm et al., 1998). Polymorphisms which reduce the production of anti-inflammatory cytokines such as IL-10 increases the gastric cancer risk (El-Omar et al., 2003). Also, polymorphisms of IL-1β, TNF-α, and IL-10 combined, increase the risk of cancer 27- fold over baseline (El-Omar et al., 2003).

In a study conducted in 2004 by Graham et al., 20 human volunteers were experimentally infected with 10^4 to 10^{10} CFU of an *H. pylori* and course of infection was observed. Symptoms which were seen in >50% of subjects including dyspepsia, headaches, anorexia, abdominal pain, belching, and halitosis, started during the second week after infection. After that, lymphocyte and monocyte infiltration was observed in gastric biopsies along with significantly increased IL-1β, IL-8, and IL-6 cytokine expression in the gastric antrum (Graham et al., 2004). Also, after four weeks of infection, the numbers of gastric $CD4^+$ and $CD8^+$ T cells were higher than pre-infection levels. These observations showed that a short while after *H. pylori* infection, gastric inflammation develops. The initial colonization of the stomach by *H. pylori* results in upper gastro-intestinal symptoms of acute infection. However, in persistent *H. pylori* infection becomes chronic and unless cleared, with the help of the immune response, may lead to gastric carcinoma (Figure 1.11).

1.6.1.2 Dendritic cells (DCs)

Dendritic cells are the primary responders to signals and professional antigen-present cells (APCs), which act as a bridge between innate and adaptive immune responses (Figure 1.11) (Pulendran et al., 2001; Sallusto & Lanzavecchia, 1999). They are able to penetrate between epithelial monolayers *in vitro* and *in vivo* to take up bacteria directly directly (Chieppa et al., 2006; Niess et al., 2005; Rescigno et al., 2001).

Recoginition by the TLRs on their surface, after that, they present bacterial products to T cells to activate them in different ways, inducing either a Th1 or Th2/ regulatory T-cell (Treg) response by generation of IL-12 or IL-10 (Banchereau et al., 2000). Recoginition by the TLRs on their surface, after that, they present bacterial products to T cells to activate them in different ways, inducing either a Th1 or Th2/ regulatory T-cell (Treg) response by generation of IL-12 or IL-10 (Banchereau et al., 2000). When stimulated with *H. pylori ex vivo,* human peripheral blood mononuclear cell-derived DCs produce IL-12 and IL-10 (Kranzer et al., 2004; Guiney et al., 2003). Also, after the co-culture of activated DCs with naïve T cells, TNF-α, IFN-γ, and IL-2 secretion is observed, indicating Th1 response (Hafsi et al., 2004). However, Lewis antigen expression by *H. pylori* LPS can suppress Th1 responses by binding to DC-SIGN (CD209) on DCs, hence suggesting immunosuppression (Appelmelk et al., 2003).

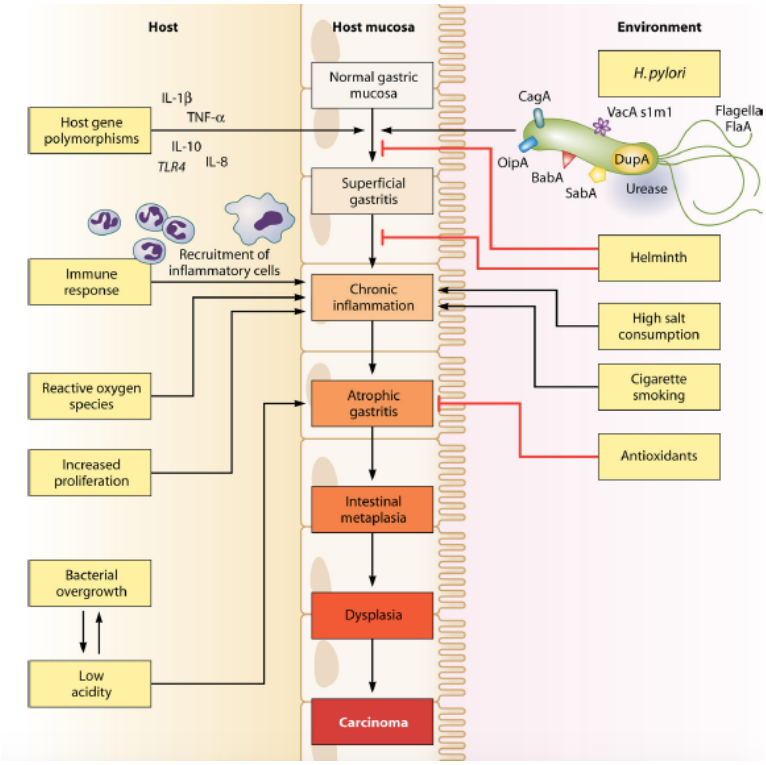


Figure 1.11 : Multifactorial pathway leading to gastric carcinoma (adapted from Wroblewski et al., 2010).

When mouse bone marrow- derived DCs stimulated with *H. pylori ex vivo,* bacteria are phagocytized and pro- inflammatory cytokines such as IL-1α, IL-1β, and IL-6 are secreted. However, another pro- inflammatory cytokine IL-12 secretion is low (Zavros et al., 2002). Also, when 8 hour long and 48 hour long stimulation of DCs by *H. pylori*

were examined, it is seen that 48 hour long stimulation induce more IFN-γ production upon co-culture with naïve T cells than 8 hour long stimulation. However, there was loss of response to CD40 ligation (Mitchell et al., 2007). Therefore, this suggests that long time *H. pylori* exposure of DCs results in a loss of ability to induce a Th1 response that could help to the persistence of the infection. *Ex vivo* activation of DCs by *H. pylori* skew the Th17/Treg balance toward Tregs contributing to bacterial immune evasion (Kao et al., 2009) and hence leading to chronic inflammation and cancer risk.

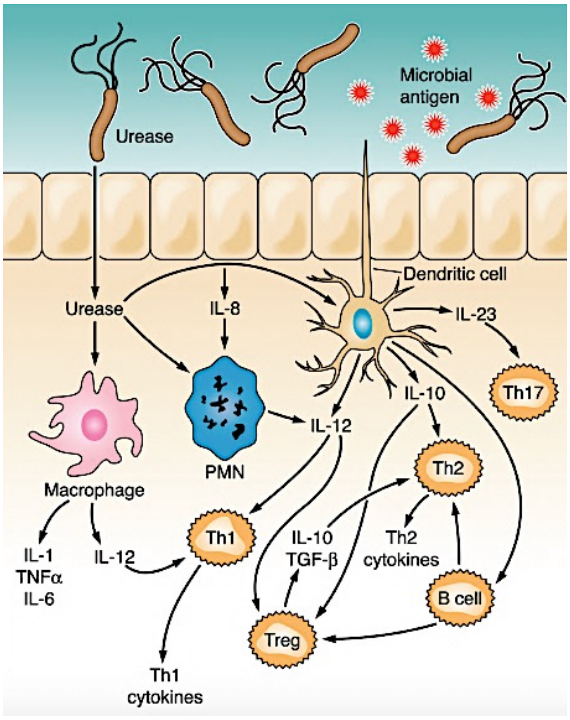


Figure 1.12 : Innate and adaptive immune response stimulated by *Helicobacter pylori* (adapted from Peek et al., 2010).

1.6.1.3 Neutrophils

Phagocytosis is one of the most important features of the innate immune response in clearing pathogens. During a typical *H. pylori* infection, neutrophils migrate into the gastric mucosa and then inflammation starts (Figure 1.12). Even though neutrophils are present in the *H. pylori* infected environment, bacteria can survive by manipulating phagocytosis and oxidative reactions. *H. pylori* secretes neutrophil-activating protein (HP-NAP) which is a virulance factor that promotes neutrophil recruitment and induces production of reactive oxygen radicals (Polenghi et al., 2007; Satin et al., 2000). HP-NAP recognized by TLR2 and in downstream upregulates IL-12, IL-23, and TNF-α (Amedei et al., 2006). Also, HP-NAP stimulates production ofTh1 cells,

resulting in increased production of IFN-γ, TNF-α and increased cytolytic activity.

In order to survive, *H. pylori* escape opsonization with the help of low pH and mucins, which are the natural local gastric environment and they prevent complement system antibody binding to the bacterial surface (Berstad et al., 1997). Also, *H. pylori* urease prevents deposition of Complement antibody 3 (Rokita et al., 1998). Moreover, *H. pylori* uprregulates decay-accelerating factor and CD59, both of which inhibit complement-mediated opsonization (Sasaki et al., 1998).

Furthermore, *H. pylori* can escape neutrophilic phagocytosis by disrupting genes in the cagPAI, which enhances engulfment. This shows the important function of type IV secretion system in preventing phagocytosis (Ramarao et al., 2000).

H. pylori can also survive within neutrophils by disrupting the synthesis of reactive oxygen species (ROS). *H. pylori* engulfed neutrophils produce a significant amount of ROS. However, they do not accumulate inside the phagosomes, and neutrophil releases ROS into the extracellular space, resulting in increased local inflammation (Allen et al., 2005).

Therefore, to promote its survival, the *H. pylori* modulate phagocytosis through production of bacterial constituents and dysregulation of host signaling pathways and diverts ROS formation away from the phagosome and into the extracellular space (Peek et al, 2010).

1.6.1.4 Macrophages

Monocytes, macrophages and dendritic cells in the lamina propria of the gastric mucosa play important roles in antigen presentation (Kranzer et al., 2004, Suzuki et al., 2002). Priming of the immune response to *H. pylori* may occur within lymph nodes draining the stomach or may occur at intestinal sites in response to *H. pylori* antigens or intact organisms that are shed from the stomach.

In *H. pylori* infection, macrophages along with DCs activate adaptive immunity by producing factors such as IL-12 (Meyer et al., 2003) that stimulate Th1 cells and production of IFN-γ. Also, the of *H. pylori* neutrophil-activating protein (HP-NAP) cause IL-12 and IL-23 secretion from neutrophils and monocytes, both of which contributes to Th1 polarization (Amedei et al., 2006). Macrophages are also involved

in amplification of the inflammatory response by production of cytokines such as IL-1β, TNF-α, and IL-6 (Gobert et al., 2004).

In the first stages of acute inflammation, *H. pylori* induces the expression of inducible NO synthetase (iNOS) in gastric mucosal epithelial cells, vascular endothelial cells or infiltrated inflammatory immune cells. Increased iNOS induces NO production using L-arginine as a substrate in these cells, where the main source of NO production is macrophages (Tsuji et al., 1996). NO production by macrophages is a normal host immune response against *H. pylori*, and this NO can kill *H. pylori* in *in vitro* experiments (Kuwahara et al., 2000). However, in human stomach, NO cannot eliminate *H. pylori* and therefore the chronic inflammation persists in the gastric mucosa. However, in chronic inflammation, *H. pylori* also suppresses NO production by inducing apoptosis of macrophages. Its LPS stimulates macrophages to produce polyamine, which induces apoptosis of macrophages (Bussiere et al., 2005) and suppresses their iNOS expression, by inducing arginase and ornithine decarboxylase (ODC) (Figure 1.13) (Gobert et al., 2001).

Studies of iNOS-deficient mice have shown that iNOS promotes development of atrophy and cancer in the gastric mucosa during *Helicobacter* infection (Nam et al., 2004; Ihrig et al., 2005). Moreover, clearance of *H. pylori* after vaccination occurs independently of iNOS (Garhart et al., 2003). Thus, iNOS appears to contribute to host pathology rather than protection during infection with *H. pylori.*

IL-1β and TNF are acid-suppressive pro-inflammatory cytokines, which are significantly increased within *H. pylori*-colonized human gastric mucosa (Crabtree et al., 1991; Noach et al., 1994). Also, increase in pro-inflammatory cytokines, such as IL-6, IL-8, TNF-α, IL-1-β, and granulocyte macrophage colony-stimulating factor is seen in the gastric biopsy sections of *H. pylori* infected individuals and resulting in the recruitment of granulocytes, mononuclear phagocytes and lymphocytes to the gastric mucosa (Lindholm et al., 1998).

H. pylori is able to stimulate IL-6 secretion in human blood- derived macrophages nearly to the same extent as *E. coli* LPS, which is known being a potent inducer of IL-6. However, in contrast to *E. coli*, *H. pylori* must be ingested by macrophages to induce a substantial amount of IL-6 secretion in these cells (Odenbreit et al., 2006).

In human gastric biopsy speciments from *H. pylori* positive individuals, $CD163^{+}$ alternatively activated (M2 type) macrophages were detected (Fehlings et al., 2012). Also, Upon *H. pylori* infection, human monocytes secreted IL-1β, IL-6, IL-10, and IL-12p40 (partially secreted as IL-23) but not IL-12p70. Also, CD14 and CD32 up- and CD11b and HLA-DR down-regulations are observed (Fehlings et al., 2012). Human M2 macrophages up-regulated CD14 and CD206 and secreted IL-10 but produced less of the pro-inflammatory cytokines than M1 macrophages (Fehlings et al., 2012).

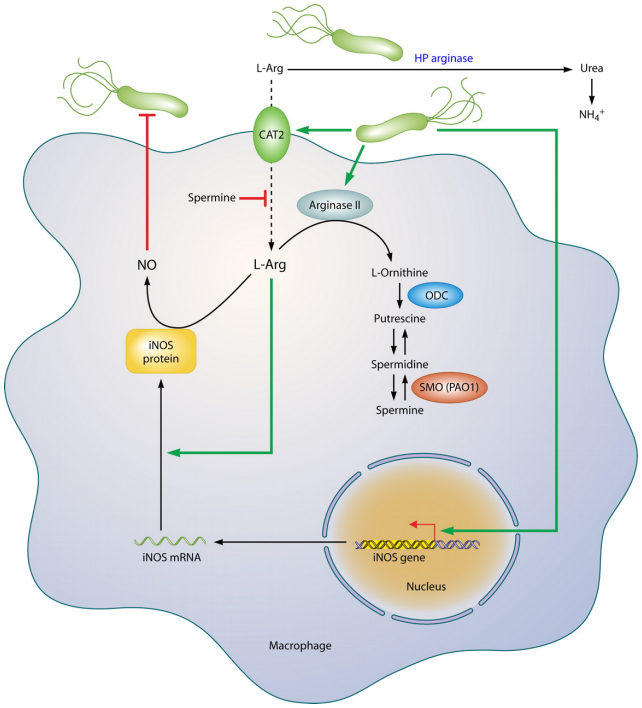


Figure 1.13 : Regulation of iNOS and NO production (adapted from Wroblewski et al., 2010).

B7-H1(CD80) and B7-H2 (CD86) expressions were significantly induced following *H. pylori* infection. CD80 is constitutively expressed on freshly isolated murine splenic T cells, B cells, macrophages and dendritic cells, and is up-regulated on T cells, macrophages, and dendritic cells after activation (Liang et al., 2003).

In vitro treatment of human monocytes with *H. pylori* stimulates co-stimulatory molecules and MHC-II (Mai et al., 1991). Therefore, the failure to up-regulate MHC-II and CD86 on gastric macrophages in chronic *H. pylori* infection may be caused by the inflammatory milieu. For instance, Tregs are prevalent in the *H. pylori* infected gastric mucosa (Lundgren et al., 2005; Rad et al., 2006) and are able to prevent the up-regulation of co-stimulatory molecules and MHC-II (Yamazaki et al., 2006). In

addition, IL-10 can prevent the up-regulation of co-stimulatory molecules and MHC-II on macrophages (Moore et al., 2001).

Vaccination of mice against *H. pylori* amplifies M1 polarization of gastric macrophages (Quiding-Jârbrink et al., 2010). Also, in human atrophic gastritis where the mixed M1/M2 polarization present in uncomplicated gastritis is replaced by an M1 dominated polarization. This may induce a tumor-promoting inflammation, and shifting macrophage polarization from M1 to M2 could therefore represent a therapeutic target in chronic *H. pylori* infection (Quiding-Jârbrink et al., 2010).

Also, *H. pylori* can escape from effective phagocytosis by macrophages (Allen et al., 2000; Zheng & Jones, 2003). Although *H. pylori* can be internalized into phagosomes by macrophages, phagosomes fuse and form "megasomes" containing large numbers of live bacteria. Also, *H. pylori* CAG+ strains that produce VacA toxin prevent the fusion of phagosomes with lysosomes that is needed for bacterial killing. Therefore, this disruption of phagosome maturation is lost when cells are infected with isogenic vacA− mutant strains (Zheng & Jones, 2003).

1.6.2 Humoral immune response against *Helicobacter pylori*

Simultaneously with adaptive response, humoral immune response was initiated and resulted in *H. pylori*-specific serum IgM antibodies were present in *H. pylori* infected humans. This situation was seen approximately after 4-weeks post-infection (Nurgalieva et al., 2005). Serum IgA and IgG antibodies were directed toward many different *H. pylori* antigens (Mattsson et al., 1998; Perez- Perez et al., 1988). *H. pylori*-specific IgA or IgM antibodies secreted from activated plasma cells were detected in the gastric mucosae of *H. pylori*-infected individuals (Mattsson et al., 1998). Also, secretory IgA antibodies were present in gastric juice, which suggests that local secretory IgA response in the stomach was initiated (Hayashi et al., 1998).

1.6.3 Adaptive immune response against *Helicobacter pylori*

1.6.3.1 T cells

IFN-γ, which is the defining Th1 cytokine, is expressed in higher levels from gastric T cells of *H. pylori*-infected individuals than uninfected individuals (Lindholm et al., 1998; Bamford et al., 1998). Also, in the experiments carried out with *H. pylori* infected IFN-$\gamma^{-/-}$ mice, it has been seen that mice develop less severe gastric

inflammation but have higher bacterial colonization densities compared to wild type mice (Akhiani et al., 2002; Sommer et al., 2001). These results can be interpreted as that IFN-γ increase severity of *H. pylori*- induced gastric inflammation however reduces bacterial colonization. Also, *H. pylori*- infected SCID (severe combined immunodeficient) mice grafted with splenocytes that express IFN-γ, developed more severe gastritis than did mice grafted with IFN-γ- deficient splenocytes (Eaton, Mefford & Thevenot, 2001). IFN-γ functions indirectly by activating macrophages to secrete pro-inflammatory cytokines and also down-regulates the expression of anti-inflammatory cytokines (e.g TGF-β) to affect the severity of gastritis (Strober et al., 1997).

Furthermore, to investigate the effects of Th2 response, C57BL/6 mice were initially infected with a nematode that induces a strong Th2 response and then challenged with *H. felis* (Fox et al., 2000). The mice co-infected with *H. felis* and the nematode had reduced gastric Th1 cytokines expression (IFN-γ, TNF, and IL-1β), increased gastric Th2 cytokines expression (IL-4, IL-10, and TGF-β) and reduced gastric inflammatory scores than mice infected with only *H. felis* (Fox et al., 2000). These data prove that the Th2 response decreases the severity of *H. pylori*-induced gastric inflammation. However, there is also evidence that Th2 response may not be required for protection from *H. pylori* infection. In the study conducted with IL-4 and IL-5 knockout C57BL/6 mice, after immunization; these mice were successfully protected from *H. pylori* infection (Garhart et al., 2003). Moreover, IL-4 receptor α-chain-deficient BALB/c mice (mice deficient of both IL-4 and IL-13 signaling) were successfully protected from *H. pylori* infection (Lucas et al., 2001). However, adoptive transfer of Th2 cells from *H. felis*-infected C57BL/6 mice into infected C57BL/6 mice significantly reduced the bacterial load compared to when Th1 cells were adoptively transferred (Mohammadi et al., 1997). The data described above, involving experiments with different knockout or wild type mice, shows that expression of IFN-γ (or Th1 response) contributes to enhanced gastric inflammation, whereas expression of IL-10 and IL-4 (or Th2 response) contributes to diminished inflammation.

Furthermore, mice lack in $CD8^+$ T cells (MHC class $I^{-/-}$ mice) were immunized and protected against colonization of *H. pylori* (Pappo et al., 1999), but mice lack $CD4^+$ T cells (MHC class$II^{-/-}$ mice) were not protected by prophylactic immunization against

H. pylori (Pappo et al., 1999). $CD4^+$ T cells from *H. felis*-immunized mice can mediate protective immunity if adoptively transferred into immunodeficient $Rag1^{-/-}$ mice (Gottwein et al., 2001). These data suggest that $CD4^+$ T cells, but not $CD8^+$ cells, are necessary for protection.

Immunization of mice with *H. pylori* lysate increases IL-17 (Th17 response) expression in the gastric mucosa and in $CD4^+$ T cells isolated from spleens and co-cultured with *H. pylori*-treated DCs or macrophages. These data were associated with increased gastric inflammation and decreased colonization (DeLyria et al., 2009). Therefore, defective IL-17/Th17 response contributes to chronic persistence of the bacterium. In *H. felis* infection, stimulation of Th17 response with increased IL-17 cytokine production was shown by Velin et al. (2009). Also, it has been proved that IL-1 receptor deficient mice presented less *H. felis*– associated pathology and showed reduced Th1 and Th17 responses (Hitzler et al., 2012).

In addition, Wang et al. (2001) reported that, *H. pylori* itself causes immune tolerance by selecting *H. pylori* non-reactive T cells via induction of T-cell apoptosis. Also, there are several investigations exist, which implicate Treg role in the *H. pylori* infection. Circulating memory T cells from *H. pylori*-infected individuals have less proliferation and IFN-γ production in response to *H. pylori*-treated DCs than do T cells from naïve donors. This defect can be rescued by depletion of $CD4^+$ $CD25^{high}$ Tregs. This means that *H. pylori*-specific Tregs suppress memory T-cell responses and contribute to the continuity of the infection (Lundgren et al., 2003).

H. pylori infected individuals have increased levels of $CD4^+$ $CD25^{high}$ FOXP3 expressing Tregs in the gastric and duodenal mucosa (Lundgren et al., 2005). The anti-CD25 monoclonal antibody PC61 depletes $Foxp3^+$ Treg cells and results in increased *H. pylori* gastritis severity, gastric cytokine levels, and serum IgG1 and IgG2c levels and a decrease in bacterial colonization in C57BL/6 mice (Rad et al., 2006). Together with findings that *H. pylori*- infected patients express increased levels of FOXP3 mRNA and protein in gastric lymphocytes (Rad et al., 2006). This study suggests that the induction of a Treg response helps to set up an equilibrium between host and bacterium, allowing *H. pylori* to survive, but also preventing the risk of destructive inflammation.

1.6.3.2 B cells

B-cell-deficient (μ-MT) mice infected with *H. pylori*, develop gastritis that is more severe than wild-type mice, and eventually *H. pylori* infection is cleared (Akhiani et al., 2004). One of the reasons why *H. pylori*-induced gastritis can be more severe in μ-MT mice than in wild-type mice is that antibodies produced by wild-type mice engage the inhibitory IgG receptor (FcγRIIb) on leukocytes and increase expression of anti-inflammatory cytokines such as IL-10 (Akhiani et al., 2004).

Helicobacter-infected IL-$10^{-/-}$ mice develop more severe gastritis than wild type mice (Chen et al., 2001). IL-10 is known to be a potent anti-inflammatory and immunoregulatory cytokine, and therefore it seems likely that IL-10 has a role in decreasing the *H. pylori*-induced inflammation (Chen et al., 2001). Also, *H. pylori*-infected IL-$4^{-/-}$ C57BL/6 mice developed more severe gastritis than *H. pylori*-infected wild-type C57BL/6 mice (Smythies et al., 2000). Similarly, *H. felis*-infected IL-$4^{-/-}$ mice developed significantly more severe gastric inflammation than did *H. felis*-infected IL-$4^{+/+}$ mice. These data suggest that both IL-10 and IL-4 have a role in down-regulating gastric inflammation (Smythies et al., 2000; Zavros et al., 2003).

A major focus of investigation has been related to the development of gastric MALT lymphoma, which arises from activated B cells. Naïve mouse splenocytes exposed to *H. pylori* are protected from spontaneous apoptosis and exhibit proliferation in response to low, but not high, multiplicities of infection, and the responding cells are derived from B-cell populations (Bussiere et al., 2006). Furthermore, chronic infection with *H. pylori* protects splenic B cells from apoptosis, indicating a B-cell activation/survival phenotype that may have implications for MALT lymphoma (Bussiere et al., 2006).

Recent findings revealed that murine splenic B cells produce and secrete IL-10 upon *Helicobacter*-infection *in vitro* as well as *in vivo*. *H. 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 (TLR$2^{-/-}$or MyD$88^{-/-}$) had significantly less IL-10 secretion when compared to wild type (WT) mice. Gastric pathology developed upon *Helicobacter* infection was similar to WT in TLR$4^{-/-}$ and TLR$9^{-/-}$ mice. Moreover, both TLR$2^{-/-}$ and MyD$88^{-/-}$ 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 (Sayi et al., 2011). 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 (Sayi et al., 2011). This study was significant for demonstrating a B cell subset with regulatory function in a bacteria-associated disease model for the first time.

1.7 Interactions Between *Helicobacter felis* and Macrophages

In literature, most of the studies were conducted using *H. pylori*. Even in mouse studies, mouse adapted strain of *H. pylori* were used instead of *H. felis*. However, *H. felis* causes more similar pathology on mice comparing to humans. Therefore, regarding the *H. felis* effects on macrophages, there is little amount of data present. *H. felis* stimulates murine macrophages through TLR2 to produce IL-6 more than E. coli LPS (Mandell et al. 2004). Also, macrophages produce IL-1β upon Helicobacter felis treatment (Tu et al., 2011).

1.8 Aim of The Study

Macrophages exist as heterogeneous and plastic group, consisting of M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes. Depending on the conditions, M1 and M2 phenotypes polarize to one another. Even though *Helicobacter pylori (H. pylori)* effects on macrophage polarization is somewhat investigated in both human and mice studies, there is not a definite characterization regarding effects of *Helicobacter felis (H. felis*) on macrophages. Therefore, we aim to investigate the effect of *H. felis* on the polarization of macrophages to either M1 or M2 type. We examined the macrophage polarization by utilizing activation specific surface markers (CD40, CD80 and CD86) and cell-type specific surface markers of M1 and M2 type macrophages (CD11c and CD206 respectively). Furthermore, different secretion and expression levels of M1 type specific cytokines (IL-12, TNF-α, IL-1β, and IL-6), M1 type specific enzyme inducible nitric oxide synthase (iNOS) M1 type specific production of nitric oxide (NO), M2 type specific cytokine (IL-10) were assessed.

2. MATERIALS AND METHOD

2.1 Materials

2.1.1 Bacteria

Helicobacter felis (H. felis) strain was kindly provided by Prof. Dr. Anne Müller from University of Zurich. Bacteria were spreaded on 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 used in the maintenance of Helicobacter felis are given in Tables 2.1 and 2.2.

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 used in *Helicobacter felis* Columbia agar plate 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 *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 Cell lines

L929 cell line was kindly provided by Assoc. Prof. Dr. Nesrin Özören from Boğaziçi University. Cells were maintained and cultured in DMEM medium supplemented with 10% FBS, 1% Penicillin/Streptomycin.

2.1.3 Cell culture

Culture media and solutions used in cell culture studies can be seen in Table 2.6 and buffers 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
Dulbecco's Modified Eagle Medium (DMEM)	Lonza
Fetal Bovine Serum (FBS) (10%)	Lonza
Penicillin/Streptomycin (1%)	Gibco
Trypan Blue	Lonza

Table 2.6 (cont'd.): Solutions and media used in cell culture studies.

Solution	Supplier company
DMSO	Fisher-Scientific

Table 2.7 : Buffers and media used in cell culture studies.

Buffers	Content and Amount
1X PBS	9,55g in 1L ddH_2O
MACS Buffer	0,5% BSA and 2mM EDTA in 1X PBS
FACS Buffer	2% FBS in 1X PBS
Complete RPMI growth medium	RPMI medium with 10% FBS, 1% Penicillin/Streptomycin
Complete DMEM growth medium	DMEM medium with 10% FBS, 1% Penicillin/Streptomycin
L929 cell line derived macrophage condition medium (LCCM)	Contains M-CSF
Thioglycollate medium	3% (w/v) in ddH_2O
Complete Macrophage growth medium	RPMI medium with 10% LCCM, 10% FBS, 1% Penicillin/Streptomycin
RPMI Freezing medium	FBS:RPMI:DMSO (5:4:1 ratio, v/v)
DMEM Freezing medium	FBS:DMEM:DMSO (5:4:1 ratio, v/v)
Detaching buffer	10 mM EDTA in 1X PBS

2.1.4 Primary cells

2.1.4.1 Bone marrow- derived macrophages

Bone marrow cells were isolated from C57BL/6 mice's leg bones (femur and tibia) and cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% LCCM, 10% FBS, 1% Penicillin/Streptomycin for differentiation into macrophages for 7 days.

2.1.4.2 Peritoneal macrophages

Peritoneal macrophages were isolated from thioglycollate induced C57BL/6 mice's peritoneal cavity and cultured overnight in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% Penicillin/Streptomycin for separation from B1 cells. After incubation, adherent cells are peritoneal macrophages.

2.1.5 ELISA

Solutions that were used in IL-12 / IL-23 (p40), IL-1β, TNF-α, IL-10 ELISA are given in Table 2.8.

Table 2.8 : Solutions used in ELISA experiments.

Solution	App.	Amount
PBS/T 1X (0.05%)	All ELISA experiments	0.05% Tween-20 in 1X PBS
Stop Solution	All ELISA experiments	2N H_2SO_4 in ddH_2O

2.1.6 Equipments and supplies

Laboratory equipment and supplies used in this study are shown in Table 2.9 and Table 2.10 with their companies, respectively.

Table 2.9 : Laboratory equipments used in the study.

Equipment	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
Nanodrop 2000	Thermo Scientific
Shakers	Heidolp Duomax 1030
Step One Real Time Systems	Applied Biosystem
Sonicator	Bandelin Sonopuls
Vortex	Mixer Uzusio VTX-3000L,LMS
Quick spin	LMS
Magnetic stirrer	WiseStir MSH-20D, Wisd Laboratory Equipment
Light Microscope	Olympus CH30
Hemacytometer	Isolab
Ice Machine	Scotsman AF10
Freezers	Altus (+ 4 ^{0}C) Siemens (-20 ^{0}C) Haier (- 80 ^{0}C)
Flow Cytometer	BD Accuri C6
Nitrogen Tank	Air Liquid
Microplate Spectrophotometer	BIO-RAD Benchmark Plus

Table 2.10 : Laboratory supplies used in the study.

Supplies	Company
Nitrocellulose membrane (0.2 µm pore size)	Santa Cruz
Scale	Precisa
Examination Gloves	Beybi

Table 2.10 (cont'd.) : Laboratory supplies used in the study.

Supplies	Company
Tissue culture flasks (25 cm^2, 75 cm^2)	Sarstedt
Anaerobic Jar	Anaerocult
Erlens	Isolab
Falcons (15 ml, 50 ml)	Isolab
Slides	Interlab
Coverslips	Interlab
Cotton Swap	Interlab
96-well F plate (for ELISA studies)	Nunc
6-well F plate	Sarstedt
Tissue flasks	Sarstedt
Serological pipettes	Sarstedt
Centrifuge tubes	Sarstedt
Eppendorf tubes (0,6ml, 1,5ml, 2ml)	Interlab
Cell strainer (70 μm)	BD

2.1.7 Commercial kits

Commercial kits used in this study are listed with their supplier companies in the table below (Table 2.11).

Table 2.11 : Commercial kits used in this study

Kit	Supplier Company
BCA™ Protein Assay Reagent Assay	Thermo Scientific
NucleoSpin RNA Isolation Kit	Macherey-Nagel
High capacity cDNA syntesis Kit, 200 rxns	Applied Biosystems
Power SYBR® Green PCR Master	Applied Biosystems
Mouse IL-12/IL-23 (p40) ELISA Max Deluxe	Biolegend
Mouse IL-1β ELISA Max Deluxe	Biolegend
Mouse TNF-α ELISA Max Deluxe	Biolegend
Mouse IL-10 ELISA Max Deluxe	Biolegend
Griess Reagent Kit for Nitrite Determination	Molecular Probes

2.1.8 General chemicals

General chemicals used in this study are listed with their supplier companies in Table 2.12.

Table 2.12 : General chemicals used in this study.

Chemical	Supplier Company
EDTA	Applichem
Ethanol (absolute)	Merck
NaCl	Merck

Table 2.12 (cont'd.) : General chemicals used in this study.

Chemical	Supplier Company
Glycerol	Merck
Phosphate-Buffered Saline (PBS) 10X	Lonza
Tween-20	Fisher-Scientific
Bovine Serum Albumin (BSA)	Santa Cruz
DMSO	Fisher Scientific
β-Mercaptoethanol	Sigma-Aldrich
Columbia Agar	BD
Brucella Broth	BD
CampyGen 2.5L	Oxoid
Lipopolysaccharide (LPS)	Sigma-Aldrich
Fixation Buffer (4%)	Biolegend
Permeabilization Buffer (10X)	Biolegend
Isopropanol	Sigma-Aldrich
HCl	Sigma-Aldrich
NaOH	Sigma-Aldrich

2.1.9 Primers

Primers used in this study are given in table 2.13.

Table 2.13 : Primers used in the study and their sequences.

Primer Name	Sequence (5'-3')	Spec.	Tm	Exp. Size
TNF-α Fw	GTCGTAGCAAACCACCAAGT	**m**	58°C	139 bp
TNF-α Rv	CCTGGGAGTAGACAAGGTACAA			
IL-1β Fw	GAGGACATGAGCACCTTCTTT	**m**	59°C	105 bp
IL-1β Rv	TCTAATGGGAACGTCACACAC			
IL-12 Fw	GCACTCCCCATTCCTACTTC	**m**	60°C	103 bp
IL-12 Rv	AACGCACCTTTCTGGTTACA			
iNOS Fw	CTTGGAAGAGGAGCAACTACTG	**m**	59°C	124 bp
iNOS Rv	CCTGAAGGTGTGGTTGAGTT			
IL-10 Fw	GAGGCGCTGTCATCGATTTCT	**m**	60°C	103 bp
IL-10 Rv	GGCCTTGTAGACACCTTGGTC			
IL-6 Fw	CACAGAGGATACCACTCCCAACA	**m**	60°C	120 bp
IL-6 Rv	TCAGAATTGCCATTGCACAACT			
18s rRNA Fw	GGCCCTGTAATTGGAATGAGTC	**m/h**	60°C	146 bp
18s rRNA Rv	CCAAGATCCAACTACGAGCTT			
Oligo dT	TTTTTTTTTTTTTTTTTTTT	**-**	**-**	**-**

2.1.10 Antibodies

Antibodies used in this study are given in table 2.14.

Table 2.14 : Antibodies used in this study.

Antibody	Clone	Supplier Company	Application
Rat anti-mouse/human CD11b-biotin	M1/70	Biolegend	FACS
Rat anti-mouse F4/80-biotin	BM8	Biolegend	FACS
Armenian hamster anti-mouse CD11c-APC	N418	Biolegend	FACS
Rat anti-mouse CD206-APC	C068C2	Biolegend	FACS
Rat anti-mouse CD86-PE	GL-1	Biolegend	FACS
Rat anti-mouse CD40	FGK4.5	BioXcell	FACS
Armenian hamster anti-mouse CD80-APC	16-10A1	Biolegend	FACS
Rat anti-mouse IL-10-PE	JES5-16E3	Biolegend	FACS
anti-rat PE	-	BioXcell	FACS
streptavidin-PE	-	Biolegend	FACS
streptavidin-APC	-	Biolegend	FACS

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 560C water bath for an hour. 50 ml horse blood was added to the agar. 10 ml β-cyclodexin and 1 ml of 1000X antibiotic cocktail (see table 2.2) was added for *H.felis* growth. After 3-4 days, the grown bacteria were checked under light microscope for their viability and mobility, and transferred into liquid Brucella Broth containing 10.000 X Vancomycin (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 (ddH2O). Following resuspension, the liquid medium was autoclaved at 121°C for 15 min for sterilization.

2.2.2 Sonication of *Helicobacter felis*

Sonication procedure was started with 120-200 ml liquid culture of *Helicobacter* strains. Before sonication *Helicobacter felis* (10 μl)'s mobility was checked under light

microscope. 120- 200 ml liquid culture of *Helicobacter felis* was aliqouted to 15 ml falcons. Falcons were centrifuged at 3000 rpm for 10 minutes, and supernatant was discarded. 10 ml PBS was used to wash bacteria. 15 ml falcon was centrifuged at 3000 rpm for 7 minutes, and supernatant was discarded. 3.5 ml PBS was added on pellet and mixed. Tube was taken to sonication with ice. Sonication was performed as 30 second pulse on, 50 second pulse off for 6 minutes 30 seconds at 50 watts (MS 72 probe of the sonicator was used). They were aliquoted to 1.5 ml eppendorfs at 500 µl for each. They were centrifuged at 4 °C, 5000 rcf (3000 rpm) for 10 minutes. Supernatant was taken to new eppendorf tubes and labeled. Sonicate concentration was measured with BCA assay.

2.2.3 Protein bicinchoninic acid (BCA) assay

The determination of protein concentration was performed using Thermo Scientific's Protein BCA Assay. The Bradford dye was diluted with distilled water at 1:4 ratios. Bovine Serum Albumin (BSA) Standard Set was chosen for microassay. 1X BCA working reagent 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 plate, and all the samples were duplicated to confirm linear range of standards and to get more accurate results. 10 µL of diluted BSA standards in duplicates were put into 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 2.15. 10 µL of protein samples (diluted of undiluted) with unknown concentrations were put into working reagent-containing wells, and microplate was incubated at 37°C for at least 30 minutes. After 30-minute-long incubation, absorbances were measured at 562 nm on microplate reader.

Table 2.15 : Dilutions of BCA assay standarts.

Vial	Volume of diluents ddH2O (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

Table 2.15 (cont'd.) : Dilutions of BCA assay standarts.

Vial	Volume of diluents ddH2O (µl)	Volume & source of BSA (µL)	Final BSA Concentration (µg/ml)
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 Preparation of thioglycollate medium

Thioglycollate medium was kindly provided by Prof. Dr. İhsan Gürsel from Bilkent University. For preparation of the medium, 30 g thioglycollate medium was dissolved in 1000 ml distilled water. Solution was brought to boil to dissolve all solids and autoclaved to sterilize. Autoclaved thioglycollate medium was stored in the dark for up to 3 months (or longer) at room temperature.

2.2.5 Preparation of L929 cell line derived macrophage condition medium (LCCM)

L929 cell line was kindly provided by Assoc. Prof. Dr. Nesrin Özören from Boğaziçi University. L929 cells are naturally capable of producing macrophage colony stimulating factor (M-CSF), which is used in differentiation of bone marrow cells into macrophages. After cells were fully grown in culture media, cells were detached and $6x10^6$ of L929 cells were put into 40ml complete DMEM medium. After 7 days of continuous incubation, cells were discarded. Medium, which contains M-CSF, was taken, filtered with 0,22µm filter, aliquoted and stored at -20^0C until further use (Figure 2.1).

2.2.6 Differentiation of bone marrow cells into bone marrow- derived macrophages

Macrophages were differentiated from bone marrow cells of C57BL/6 mice. The procedure is explained in detail in section. 2.2.6.1

2.2.6.1 Bone marrow cells isolation and differentiation into bone marrow-derived macrophages

Leg bones of a sacrificed C57BL/6 mouse were cut without damaging the tips of the bones by surgical scissors. Tissue and muscles around the bones were carefully

cleaned by pinching with gauze soaked in 70% ethanol. After proper cleaning, tips of the bones were cut and bone marrow cells were flushed out into a falcon tube with complete RPMI medium. Procedure was done for all bones. To obtain single cell suspension and to get rid of the any impurities, cells were put through 70-µm filters. Filter was washed with complete RPMI and cell suspension was centrifuged at 1780 rpm (300g) for 8 minutes. Supernatant was discarded. Cell pellet was dissolved in 1 ml complete RPMI and counted in hemacytometer by diluting with complete RPMI (1:200). Cell viability checked with trypan blue dye.

For differentiation, cell suspension is adjusted to $1x10^6$ cells/ml and placed into complete macrophage growth medium. Cells were incubated in 6 well F bottomed plates, $2x10^6$ cells per well, for 7 days at 37^0C and 5% CO_2. At every other day, medium is discarded, cells were washed gently with pre-warmed 1X PBS, and complete macrophage growth medium was renewed. After 7 days, bone marrow-derived macrophages were thought to be fully differentiated. After washing cells with pre-warmed 1X PBS, ~1 ml of detaching buffer was put onto cells and incubated on ice for ~10 minutes. Then, gentle scraping and pipetting; cells were harvested, centrifuged at 3000 RPM for 8 minutes. Supernatant was discarded. Then, flow cytometer analysis for CD11b and F4/80 surface markers were performed in order to determine the purity of differentiated cells. The steps of procedures including preparation of L929 cell line derived macrophage condition medium (LCCM) and bone marrow cells isolation and differentiation into bone marrow- derived macrophages are displayed in Figure 2.1.

2.2.6.2 Flow staining for CD11b – F4/80 surface markers for purity

Purity of bone marrow- derived macrophages was determined using flow cytometer. 5x105 cells were stained with 0.2-µl biotin conjugated anti- CD11b antibody and biotin conjugated anti-F4/80 antibody in 50-µl FACS Buffer in the dark on ice for at least 45 minutes. A fraction of bone marrow derived- macrophages was left as unstained controls. Then, cells were washed once with 1 ml FACS Buffer by centrifugation at 3000 rpm for 8 minutes. For secondary staining, 0.2-µl APC conjugated anti- biotin (streptavidin) antibody was used to stain same cells in 50 µl FACS Buffer in the dark on ice for at least 45 minutes. Then, the 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.

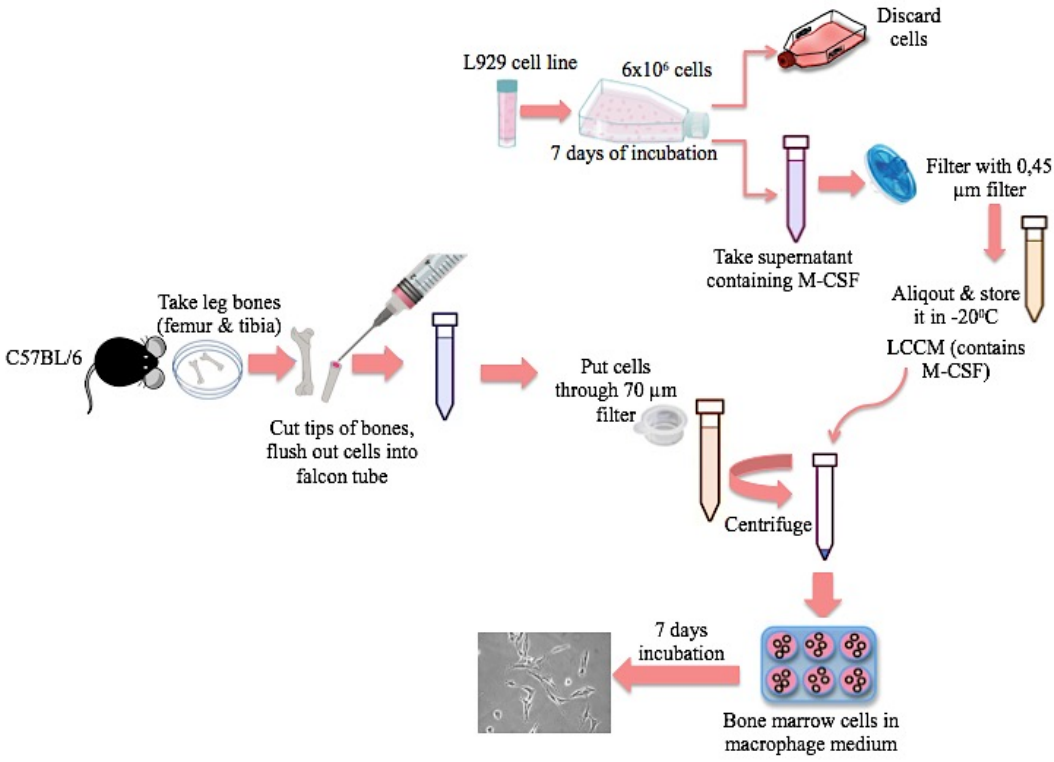


Figure 2.1 : Isolation of bone marrow- derived macrophages.

2.2.7 Isolation of peritoneal macrophages

Peritoneal macrophages were isolated from C57BL/6 mice. The procedure is explained in detail in section. 2.2.8.1 – 2.2.8.3.

2.2.7.1 Thioglycollate injection to C57BL/6 mice

~2ml previously prepared (Mat. & Met. part, 2.5.4.) thioglycollate medium was injected to living C57BL/6 mice intraperitoneally. After 4-5 days of incubation, mice were sacrificed. Peritoneal cavity was opened without damaging peritoneal membrane. ~4-5 ml of sterile complete DMEM medium was injected into peritoneal cavity with small needle. After gentle palpation of peritoneum, medium was withdrawn without damaging peritoneal membrane.

2.2.7.2 Collecting peritoneal cells from C57BL/6 mice

Peritoneal cell number was determined and cells were incubated in 6 well F bottomed plates, $5x10^6$ cells per well in 2 ml of complete DMEM medium, for 24 hours in 37^0C,

5% CO_2. Peritoneal cavity provides two different population of cells: peritoneal macrophages which are adherent in nature, and B1 cells which are suspension in nature. After 24-hour incubation, B1 cells were removed from the suspension and peritoneal macrophages were attached to bottom of the plate. To harvest peritoneal macrophages, cells were washed with pre-warmed 1X PBS, and ~1ml of detaching buffer was put onto cells and incubated on ice for ~10 minutes. Then, with gentle scraping and pipetting, cells were harvested, centrifuged at 3000 RPM for 8 minutes. Supernatant was discarded. Then, flow cytometer analysis for CD11b and F4/80 surface markers were performed in order to determine the purity of cells.

2.2.7.3 Flow staining for CD11b – F4/80 surface markers for purity

Purity of peritoneal macrophages was determined using flow cytometer. 5x105 cells were stained with 0.2-µl biotin conjugated anti- CD11b antibody and biotin conjugated anti-F4/80 antibody in 50-µl FACS Buffer in the dark on ice for at least 45 minutes. A fraction of peritoneal macrophages was left as unstained controls. Then, cells were washed once with 1 ml FACS Buffer by centrifugation at 3000 rpm for 8 minutes. For secondary staining, 0.2-µl APC conjugated anti- biotin (streptavidin) antibody was used to stain same cells in 50 µl FACS Buffer in the dark on ice for at least 45 minutes. Then, the 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.

2.2.8 Treatments

2.2.8.1 Lipopolysaccharide (LPS)

Cells were treated with *H. felis* sonicates with a final concentration of 100 ng/ml when needed for 24-hours. However, when combined with silica treatment for IL-1β secretion, 4 hours of incubation, followed by 8 hours of silica treatment.

2.2.8.2 *Helicobacter felis (H. felis)* sonicate

Cells were treated with *H. felis* sonicates with a final concentration of 10 µg/ml when needed for 24-hours. However, when combined with silica treatment for IL-1β secretion, 4 hours of incubation, followed by 8 hours of silica treatment.

2.2.9 Antibody stainings for flow cytometry (for surface markers)

After treatments, for harvesting, cells were washed with pre-warmed 1X PBS, and ~1ml of detaching buffer was put onto cells and incubated on ice for ~10 minutes. Then, with gentle scraping and pipetting, cells were harvested, centrifuged at 3000 RPM for 8 minutes. Supernatant was discarded. Cells were resuspended in FACS Buffer and divided to 0,5 ml eppendorf tubes for different stainings. Stainings were only F4/80, F4/80-CD11c, F4/80-CD206 and a fraction of cells were left unstained.

2.2.10 IL-12 / IL-23 (p40) ELISA

IL-12 / IL-23 (p40) protein levels in supernatants were analyzed via Biolegend Mouse IL-12 / IL-23 (p40) ELISA Max Deluxe Kit. For quantitative determination of secreted IL-12 / IL-23 (p40) protein levels in culture medium of bone marrow derived and peritoneal macrophages, Nunc 96-well plates were coated with IL-12 / IL-23 (p40) 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 1 hour with shaking in order to block non-specific binding. Recombinant IL-12 / IL-23 (p40) protein 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-12 / IL-23 (p40) standards and culture media supernatants (50-µl) were added as biological duplicates. Plate was incubated at room temperature for 2 hours with shaking. Following 2 hours of incubation, plate was washed four times with PBS/T. Biotinylated IL-12 / IL-23 (p40) detection antibody diluted 1:200 in Assay Diluent 1X was added into each assayed well and plate was incubated at room temperature for 1 hour with shaking. After washing of assayed wells with PBS/T for four times, Avidin conjugated HRP enzyme, 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 minutes with shaking at room temperature. After 30 minutes of incubation, plate was washed for five times with PBS/T and 50-µl of TMB Substrate Solution Mixture (1:1 v/v 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 in dark. High concentration standards and samples turned into blue. After that, the reaction was

stopped with 50-µl stop solution (2N H2SO4). Then, the absorbances of the samples were measured at 450 nm on a microplate reader.

2.2.11 IL-1β ELISA

IL-1β protein levels in supernatants were analyzed via Biolegend Mouse IL-1β ELISA Max Deluxe Kit. For quantitative determination of secreted IL-1β protein levels in culture medium of bone marrow derived and peritoneal macrophages, Nunc 96-well plates were coated with IL-1β 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 1 hour with shaking in order to block non-specific binding. Recombinant IL-1β protein 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-1β standards and culture media supernatants (50-µl) were added as biological duplicates. Plate was incubated at room temperature for 2 hours with shaking. Following 2 hours of incubation, plate was washed four times with PBS/T. Biotinylated IL-1β detection antibody diluted 1:200 in Assay Diluent 1X was added into each assayed well and plate was incubated at room temperature for 1 hour with shaking. After washing of assayed wells with PBS/T for four times, Avidin conjugated HRP enzyme, 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 minutes with shaking at room temperature. After 30 minutes of incubation, plate was washed for five times with PBS/T and 50-µl of Solution D (substrate) was added into each assayed well. The plate was incubated for at least 30 minutes at room temperature in dark. High concentration standards and samples turned into blue. After that, the reaction was stopped with 50-µl stop solution (2N H2SO4). Then, the absorbances of the samples were measured at 450 nm on a microplate reader.

2.2.12 TNF-α ELISA

TNF-α protein levels in supernatants were analyzed via Biolegend Mouse TNF-α ELISA Max Deluxe Kit. For quantitative determination of secreted TNF-α protein levels in culture medium of bone marrow derived and peritoneal macrophages, Nunc

96-well plates were coated with TNF-α 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 1 hour with shaking in order to block non-specific binding. Recombinant TNF-α protein 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 TNF-α standards and culture media supernatants (50-µl) were added as biological duplicates. Plate was incubated at room temperature for 2 hours with shaking. Following 2 hours of incubation, plate was washed four times with PBS/T. Biotinylated TNF-α detection antibody diluted 1:200 in Assay Diluent 1X was added into each assayed well and plate was incubated at room temperature for 1 hour with shaking. After washing of assayed wells with PBS/T for four times, Avidin conjugated HRP enzyme, 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 minutes with shaking at room temperature. After 30 minutes of incubation, plate was washed for five times with PBS/T and 50-µl of TMB Substrate Solution Mixture (1:1 v/v 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 in dark. High concentration standards and samples turned into blue. After that, the reaction was stopped with 50-µl stop solution (2N H2SO4). Then, the absorbances of the samples were measured at 450 nm on a microplate reader.

2.2.13 Griess reagent protocol for nitrite determination

Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) enzyme was measured by griess reagent kit. In principle, sulfanilic acid was quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt was then coupled to N- (1-naphthyl) ethylenediamine, forming an azo dye that can be spectrophotometrically quantitated based on its absorbance at 548 nm. Firstly, equal volumes of N-(1-naphthyl) ethylenediamine (Component A) and sulfanilic acid (Component B) was mixed together to form the Griess Reagent. Nitrite standards were prepared with concentrations between 1-100 µM by diluting the nitrite standart solution (component C) with deionized water. After that, in a microplate, 20-µl of griess reagent, 150-µl of sample and standarts, and 130-µl deionized water was mixed

and incubated at room temperature for 30 minutes. Also, photometric reference sample (negative control) was prepared by mixing 20-µl of griess reagent and 280-µl of deionized water. Absorbances were measured relative to the reference samples (standarts) in spectrophotometric microplate reader at 548 nm.

2.2.14 IL-10 ELISA

IL-10 protein levels in supernatants were analyzed via Biolegend Mouse IL-10 ELISA Max Deluxe Kit. For quantitative determination of secreted IL-10 protein levels in culture medium of bone marrow- derived and peritoneal macrophages, 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 1 hour with shaking in order to block non-specific binding. Recombinant IL-10 protein 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 hours with shaking. Following 2 hours of 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 hour with shaking. After washing of assayed wells with PBS/T for four times, Avidin conjugated HRP enzyme, 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 minutes with shaking at room temperature. After 30 minutes of incubation, plate was washed for five times with PBS/T and 50-µl of TMB Substrate Solution Mixture (1:1 v/v 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 in dark. High concentration standards and samples turned into blue. After that, the reaction was stopped with 50-µl stop solution (2N H2SO4). Then, the absorbances of the samples were measured at 450 nm on a microplate reader.

2.2.15 Analysis of relative expression levels

2.2.15.1 RNA isolation

To determine relative expression levels, bone marrow derived and peritoneal macrophages pellets were used to isolate their RNA. 350 µl of RA1 lysis buffer and 3,5 µl β-mercaptoethanol was added to cell pellets and vortex vigorously. Samples were taken to nucleospin filter (violet ring) tubes and centrifuged at 11,000 x g for 1 minute. Nucleospin filter (violet ring) was discarded and 350 µl of 70% of ethanol was added to lysate and mixed by pipetting up and down (5 times). For each preparation, one nucleospin RNA column (light blue ring) was placed into a collection tube and lysates were pipetted up and down 2-3 times more and loaded to the column. Columns were centrifuged at 11,000 x g for 30 seconds. 350 µl of MBD (membrane desalting buffer) was added and centrifuged at 11,000 x g for 1 minute to dry the membrane. Then, DNase reaction mixture was prepared. For each isolation, in a sterile 1,5ml microcentrifuge tube, 10 µl reconstituted rDNase (reconstituted by manifacturer's guides) was added to 90 µl reaction buffer for rDNase, and mixed by flicking by the tube. Then, 95 µl DNase reaction mixture was applied directly onto the center of the silica membrane of the column. Column was incubated at room temperature for 15 minutes. After incubation, 200 µl of RAW2 buffer was added and centrifuged at 11,000 x g for 30 seconds to inactivate the rDNase. Then, 600 µl of RA3 buffer was added to column and centrifuged at 11,000 x g for 30 seconds. Lastly, columns were placed onto new collection tubes, 250 µl of RA3 buffer was added onto the column and centrifuged at 11,000 x g for 2 minutes to dry the membrane. Finally, columns were settled into new eppendorf tubes. To elute the RNA, 35 µl of RNAse-free water was added on columns and columns were centrifuged at 11,000 x g for 1 minute. Flow through was kept as isolated RNA. To get more concentrated RNA, flow through was put onto the center of the column once and centrifuged again at 11,000 x g for 1 minute. RNA concentrations were measured with NanoDrop.

2.2.15.2 cDNA synthesis

Synthesis of cDNA was performed according to manufacturer's instructions. Amounts used in synthesis reaction are given in Table 2.15. Synthesis conditions are given in Table 2.16.

Table 2.16 : cDNA synthesis reaction components.

Component	Amount
RNA (1µg)	Depends on concentration
ddH_2O	15.075 µl – amount of RNA
10X RT Buffer	2 µl
Oligo dT (10µM)	1 µl
Ribolack Rnase inhibitor	0,125 µl
Reverse transcriptase enzyme	1 µl
25X dNTP mix	0,8 µl

Table 2.17 : cDNA synthesis reaction conditions.

Temperature	Time
25°C	10 min
37°C	120 min
85°C	5 min
4°C	∞

2.2.15.3 Real-time PCR

Relative expression levels of IL-12 / IL-23 (p40), IL-1β, TNF-α, iNOS, IL-6, IL-10, and arginase-1 were analyzed with Real-time PCR. Amounts used in reaction are given in Table 2.17. PCR conditions performed are given in Table 2.18.

Table 2.18 : Components of real time PCR.

Component	Amount
Power Sybr Master Mix (2X)	5 µl
Forward Primer (10 µM)	0,5 µl
Reverse Primer (10 µM)	0,5 µl
PCR Grade water	1,5 µl

Table 2.19 : Real time PCR reaction conditions.

Temperature	Time
95°C	5 min
95°C	30 sec
Depending on primer sets	1 min (45 cycle)
72°C	1 min
72°C	5 min
4°C	∞

2.2.16 Flow cytometry analysis

Flow cytometry analyses were performed using BD accuri C6 software and FlowJo software.

2.2.17 Statistical analysis

All p values were calculated using GraphPad Prism 5.0 software and determined by Student t test. In all analyses, a two-tailed t-test was applied and p-value of more than 0.05 was considered statistically not 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

Macrophages undergo specific differentiation depending on the local tissue environment. Two distinct states of polarized activation for macrophages have been defined: the classically activated (M1) macrophage phenotype and the alternatively activated (M2) macrophage phenotype (Gordon and Taylor 2005; Mantovani et al. 2002). Cell markers alone do not fully define the many subpopulations of macrophages therefore; macrophages should be defined based on their specific functional activities (Biswas & Mantovani 2010 and Geissmann et al. 2010). Functions of polarized macrophage subsets and their cytokine profile have been summarized in Table 3.1.

Table 3.1 : Inducers, produced cytokines and functions of macrophage subtypes.

	M1	M2a	M2b	M2c
Activation	IFN-γ TNF-α LPS	IL-4/IL-13	ICs LPS TLR/IL-1R	IL-10 TGF-β GCs
Produced cytokines	TNF-α IL-1β IL-6 IL-12/IL-23 NO	IL-10 TGF-β IL-1ra	IL-1β TNF-α IL-10 IL-6	IL-10 TGF-β
Enzyme	iNOS			
Function	Pro-inflammatory	Pro-fibrotic	Immune regulation	Anti-inflammatory, Tissue repair and regeneration

3.1 Isolation of Bone Marrow- Derived Macrophages

In order to obtain bone marrow- derived macrophages; first of all, mouse bone marrow cells were extracted from leg bones of C57BL/6 mice, and incubated in complete macrophage growth medium for 7 days (Mat. & Met. Part, 2.2.6). Following incubation, bone marrow- derived macrophages were harvested and labelled with APC-coupled CD11b and F4/80 antibodies, and compared to unstained control. Flow

cytometer analysis was performed for the quantitative analysis of stained cells. Representative images of the $CD11b^+$ and $F4/80^+$ bone marrow- derived macrophages were shown in Figure 3.1.a and histogram plots were shown in Figure 3.1.b. Besides, the percentages of positivity of four independent experiments and their averages were given in Figure 3.1.c.

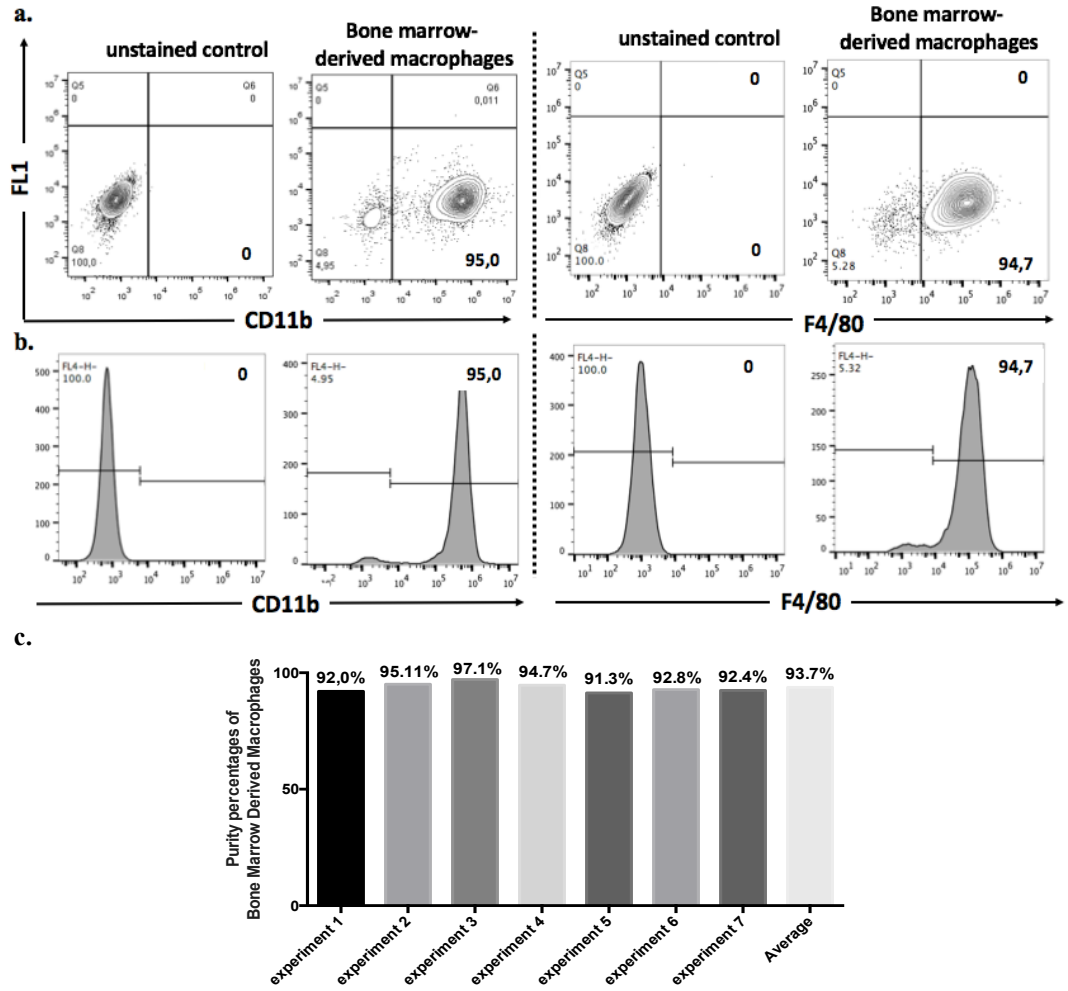


Figure 3.1 : *Purity of differentiated bone marrow- derived macrophages.* **a.** Approximately $5x10^4$ cells were stained with a specific macrophage marker, CD11b and F4/80 (APC-coupled anti-CD11b antibody and APC-coupled anti-F4/80 antibody) (right) or left as unstained control (left). Representative contour plots were shown above for all experiments. **b.** Representative histograms were shown above for all experiments. **c.** Independent percentages and average percentage values are expressed as bar graph. Images were prepared using GraphPad Prism program.

According to figure 3.1, bone marrow- derived macrophages were 95% $CD11b^+$ and similarly 94,7% $F4/80^+$. After all isolation processes, purities were checked by flow

cytometry. Since, both CD11b and F4/80 positivity were parallel to each other, in the following experiments cells were stained only with F4/80 antibody. As a result, bone marrow- derived macrophages differentiation was established with more than 90 %.

Experiments 1,2 and 3 were used to identify the activation status via CD40, CD80, and CD86 surface marker expressions and to determine the correalation between activation status (CD86 surface marker expression) and polarization status, which was investigated via CD11c (for M1 type) and CD206 (for M2 type) surface marker expressions. Furthermore, experiments 4,5,6, and 7 were used to identify the cytokine profiles of activated macrophages.

3.2 Isolation of Peritoneal Macrophages

In order to obtain peritoneal macrophages, firstly, prepared thioglycollate medium (Mat. & Met. part, 2.5.4.) was injected into peritoneal cavity of C57BL/6. After 4-5 days, mice were sacrificed and peritoneal cells are harvested and incubated in cell culture conditions for 24 hours. 5-10 million peritoneal cavity cells can be obtained from an unmanipulated mice and among them, only ~30% (about 1-2 million cells) peritoneal macrophages (Zhang, Goncalves, & Mosser, 2008). However, this number was not enough for the experiment sets. So, the peritoneal macrophages were induced with thioglycollate medium, which enriches their numbers up to 15-20 million of peritoneal macrophages. Peritoneal cell population consists of two types of cells: B1 cells which are suspension in nature and peritoneal macrophages which are adherent in nature. Therefore, after incubation of peritoneal cells, peritoneal macrophages were attached to plate bottom and harvested (Mat. & Met. part, 2.2.7). After harvesting, peritoneal macrophages were labelled with APC-coupled CD11b and F4/80 antibodies, and compared to unstained control. Representative contour plots of $CD11b^+$ and $F4/80^+$ peritoneal macrophages purity was shown in Figure 3.2.a. Representative histogram plots of $CD11b^+$ and $F4/80^+$ peritoneal macrophages were shown in Figure 3.2.b. and purities of three individual experiments and their average was given in Figure 3.2.c. Since, both CD11b and F4/80 positivity were parallel to each other, following experiments were stained only with F4/80 antibody.

According to figure 3.2, peritoneal macrophages were 91% $CD11b^+$ and 97% $F4/80^+$. After all isolation processes, purities were checked by flow cytometry. Since, both CD11b and F4/80 positivity were parallel to each other, following experiments were

stained only with F4/80 antibody. Therefore, according to average, peritoneal macrophages isolated more than 94% purity compared to the unstained group.

In order to investigate the effect of *H. felis* on both bone marrow- derived and peritoneal macrophages, they were treated with 10 µg/ml *H. felis* sonicate and 100 ng/ml LPS. In a study conducted in vitro using *E. coli* showed that LPS induces a typical M1 profile through TLR4 recognition (Liang et al., 2005; Pinheiro de Silva et al., 2007). For that reason, LPS was used such as an internal M1 phenotype control, and also used in comparison with *H. felis* results. Then, polarization status was examined according to their surface receptor expressions and cytokine profiles.

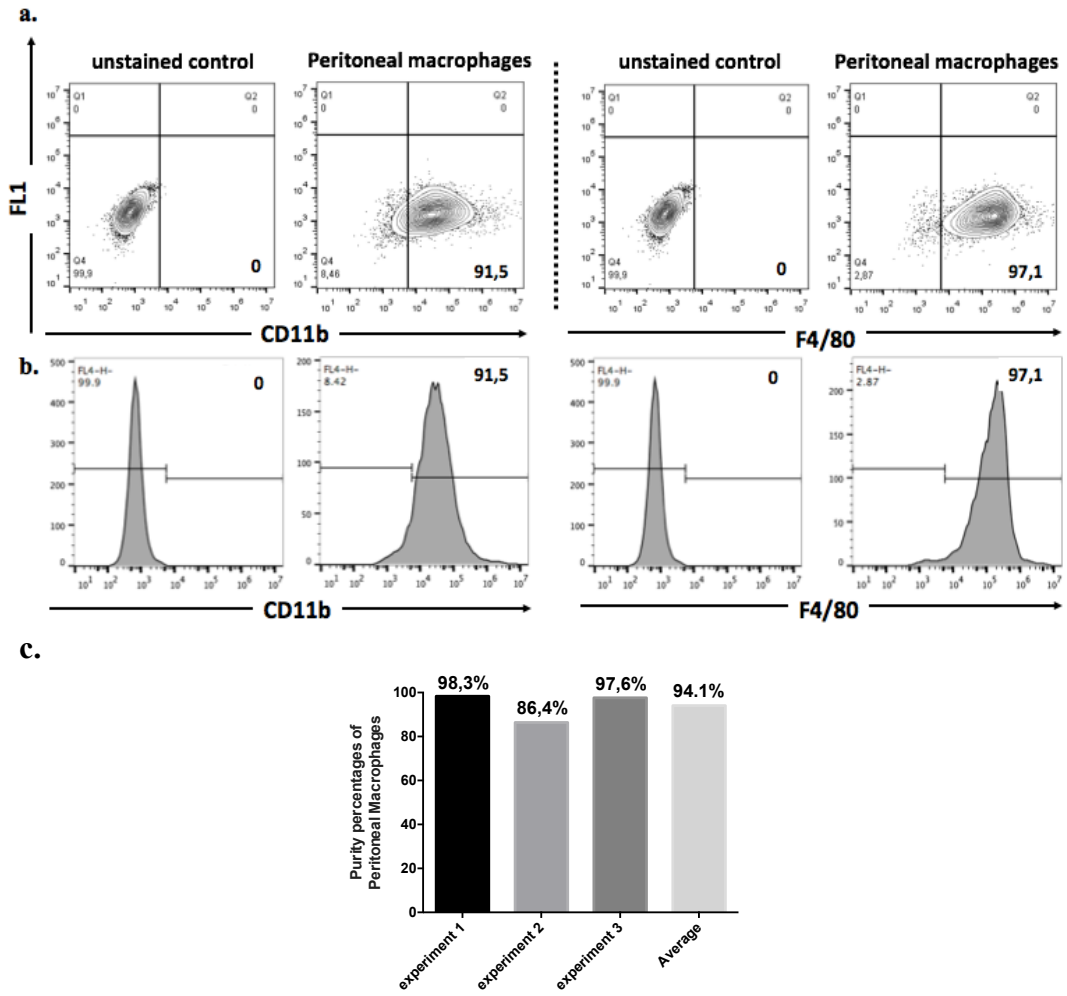


Figure 3.2 : *Purity of isolated peritoneal macrophages.* **a.** Approximately $5x10^4$ cells were stained with a specific macrophage marker, APC-coupled anti-CD11b antibody and APC-coupled anti-F4/80 antibody (right) or left as unstained control (left). Representative contour plots and representative histograms **(b.)** were shown above for all experiments. **c.** Independent percentages and average percentage values are expressed as bar graph. Images were prepared using GraphPad Prism program.

3.3 Identifying the Activation Status of Macrophages Through Analysis of CD40 and CD80 Surface Markers

CD40 is a co-stimulatory protein, which is encoded by this gene is a member of the TNF- receptor superfamily, found on antigen presenting cells and is required for their activation and cell-to-cell contact with T cells. CD80 (B7-1) protein found on activated B cells and monocytes that provides a co-stimulatory signal necessary for T cell activation and survival. They are considered as important activation markers on macrophages. As a result of activation, the macrophage expresses more CD40 and CD80 on its surface which helps increase the level of activation.

For the activation investigation of macrophages, LPS and *H. felis* sonicate- treated bone marrow-derived macrophages and peritoneal macrophages were stained with APC or FITC-conjugated F4/80, PE-conjugated CD40 and APC-conjugated CD80 antibodies. CD40 and CD80 positivity among F4/80^{+} gated cells were compared to unstained control and untreated group. Flow cytometer analysis was performed for the quantitative analysis of stained cells. Representative contour plots, histograms and averages of four independent experiments of CD40^{+} were shown in figure 3.3. and CD80^{+} bone marrow- derived macrophages were shown in figure 3.4.

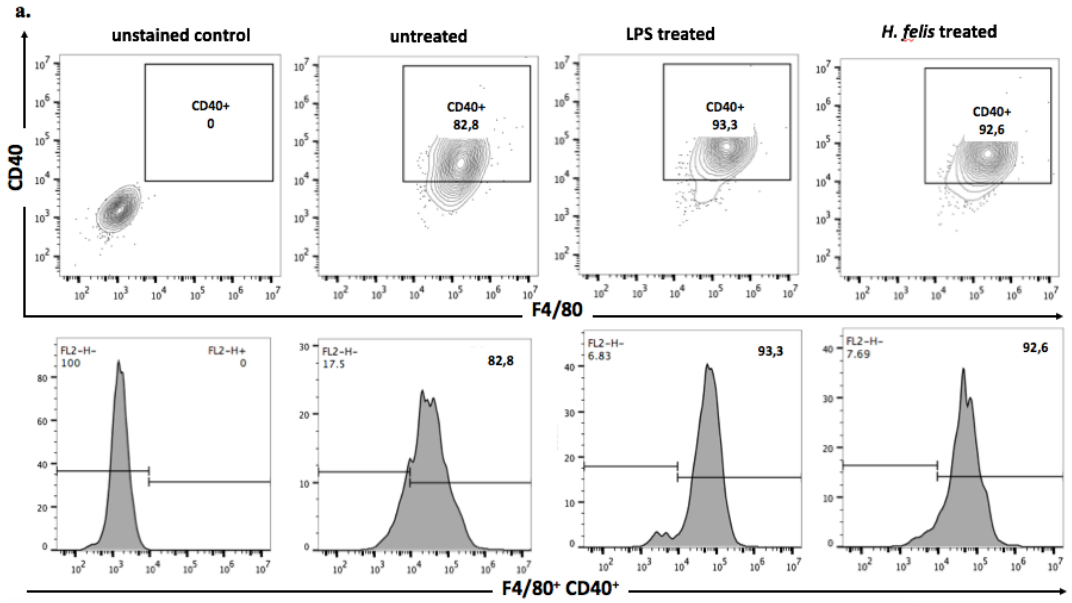


Figure 3.3 : *CD40 positivity of differentiated F4/80^{+} bone marrow- derived macrophages.*

b.

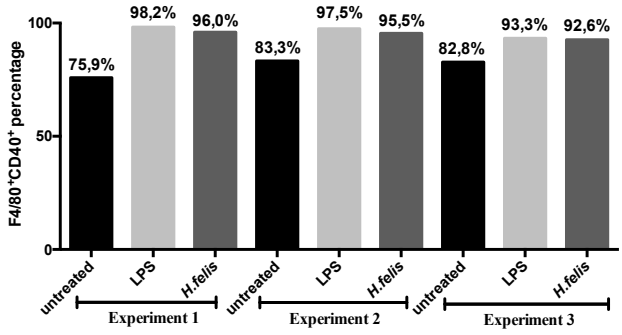


Figure 3.3 (cont'd.) : *CD40 positivity of differentiated F4/80$^+$ bone marrow- derived macrophages.* **a.** Approximately 5x10^4 cells were stained with APC-coupled anti-F4/80 antibody and PE-coupled CD40 or were left as unstained control. Representative contour plots and histograms were shown above for all experiments. **b.** Independent percentages are expressed as bar graph. Images were prepared using GraphPad Prism program.

As a result, according to Figure 3.3 and 3.4, untreated bone marrow- derived macrophages expressed approximately 80% CD40 and 70% CD80. This percentage increased to above 90% for both markers in LPS- and *H. felis* sonicate- treated cells. When compared to untreated group, there was activation in LPS and *H. felis* sonicate-treated bone marrow- derived macrophages. However, CD40 and CD80 expression between LPS- and *H. felis* sonicate- treated cells were not different than each other. Therefore, CD40 and CD80 stainings were not performed on peritoneal macrophages.

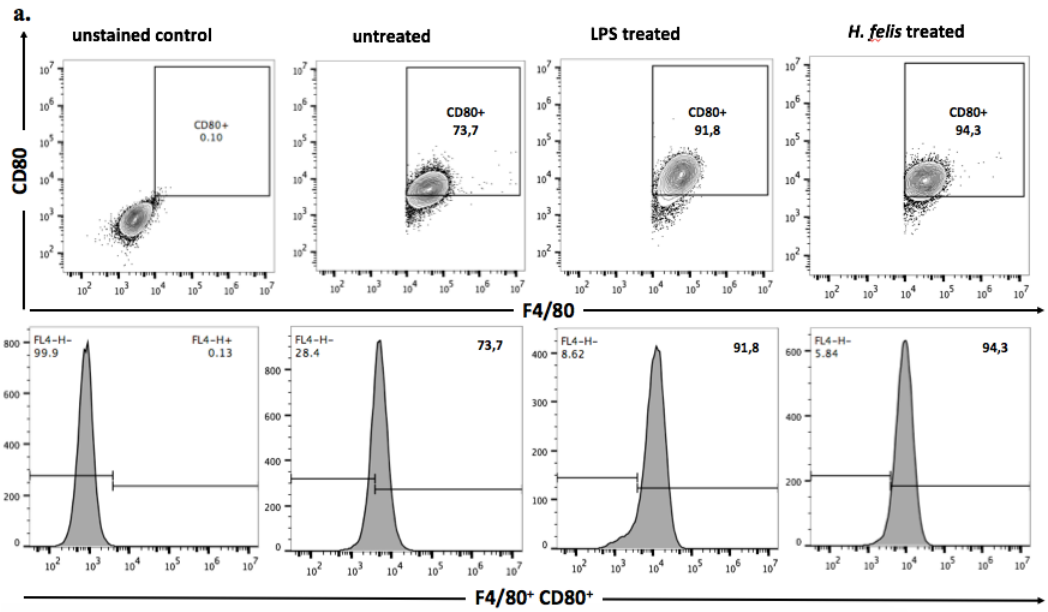


Figure 3.4 : *CD80 positivity of differentiated F4/80$^+$ bone marrow- derived macrophages.*

b.

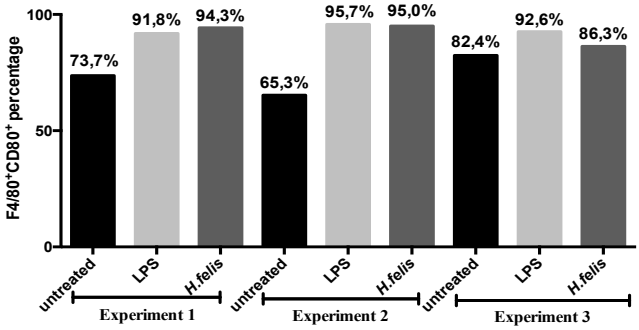


Figure 3.4 (cont'd.) : *CD80 positivity of differentiated F4/80$^+$ bone marrow- derived macrophages.* **a.** Approximately 5x10^4 cells were stained with FITC-coupled anti-F4/80 antibody and APC-coupled CD80 or were left as unstained control. Representative contour plots and histograms were shown above for all experiments. **b.** Independent percentages are expressed as bar graph. Images were prepared using GraphPad Prism program.

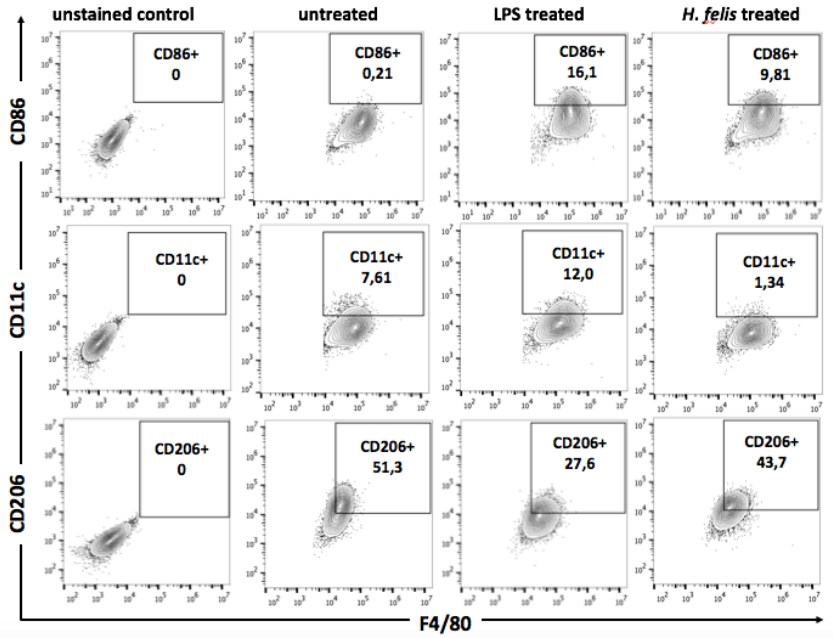
3.4 Identifying the Correlation Between Activation and Polarization Status of Bone Marrow- Derived and Peritoneal Macrophages

CD86 (B7-2) protein, as CD40 and CD80(B7-1), found on activated B cells and monocytes that provide a co-stimulatory signal necessary for T cell activation and survival. CD86 works in tandem with CD80 to prime T cells. As a result of activation, the macrophage expresses more CD86 on its surface which helps determine the level of activation. Recently, M1 type macrophages were identified by F4/80, CD11b, and CD11c+ surface marker expressions (Lumeng et al., 2007; Fujisaka et al., 2009) and M2 macrophages were shown to express high levels of mannose receptor (MR or CD206) (Verreck et al., 2006). However, there was no definite information about the effect of *H. felis* on macrophage polarization. Also, it has been shown that CD86 surface marker was expressed on M1 type and M2b type macrophages (Mantovani et al., 2004).

Therefore, we aimed to characterize the activation status of *H. felis* sonicate- treated bone marrow- derived macrophages and peritoneal macrophages via analysis of CD86 surface marker expression, polarization status via analysis of CD11c and CD206 surface marker expressions and determine whether there is a correlation between activation and polarization status of *H. felis* sonicate- treated bone marrow- derived macrophages and peritoneal macrophages. Representative flow cytometry analysis image and histogram plots of F4/80$^+$CD86$^+$, F4/80$^+$CD11c$^+$ and F4/80$^+$CD206$^+$ bone

marrow- derived macrophages were shown in Figure 3.5.a and 3.5.c, respectively, and positivities of three independent experiments were given in Figure 3.5.b. Also, representative flow cytometry analysis contour plot and histogram plots of $F4/80^{+}CD86^{+}$, $F4/80^{+}CD11c^{+}$ and $F4/80^{+}CD206^{+}$ peritoneal macrophages were shown in Figure 3.6.a and 3.6.c, respectively, and positivities of three independent experiments were given in Figure 3.6.b.

a. Experiment 1



Experiment 2

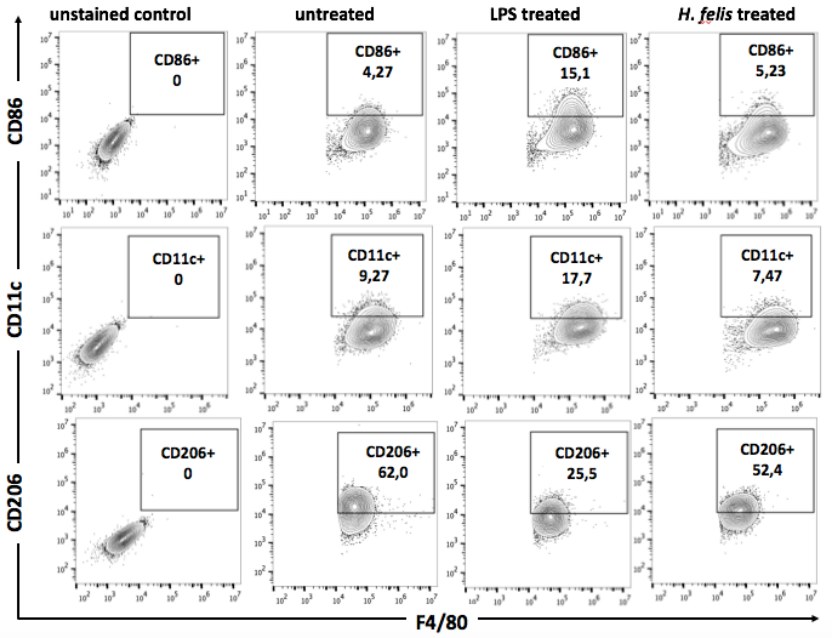
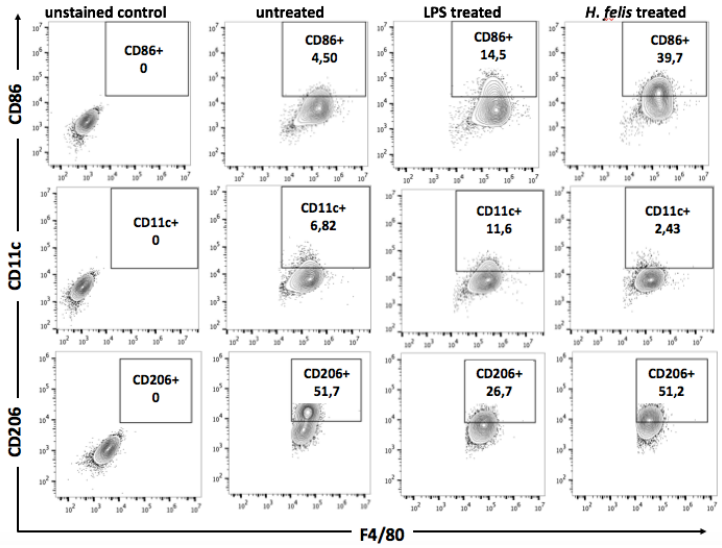
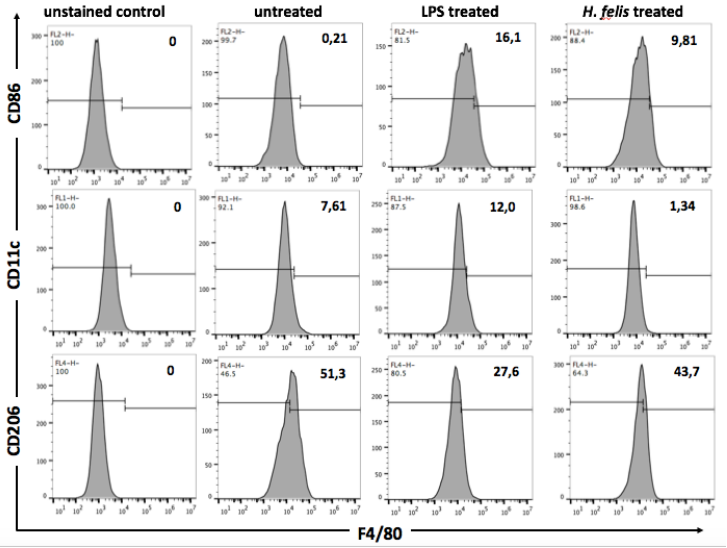


Figure 3.5: *CD86, CD11c and CD206 positivity of differentiated $F4/80^{+}$ bone marrow- derived macrophages.*

Experiment 3



b. Experiment 1



Experiment 2

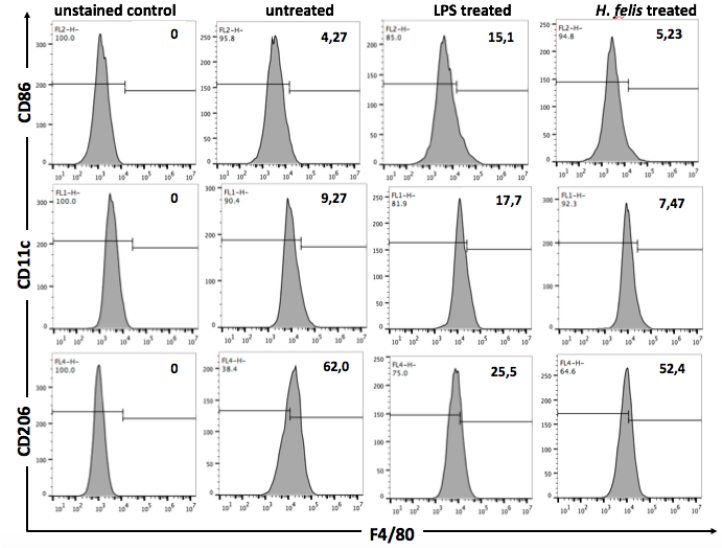
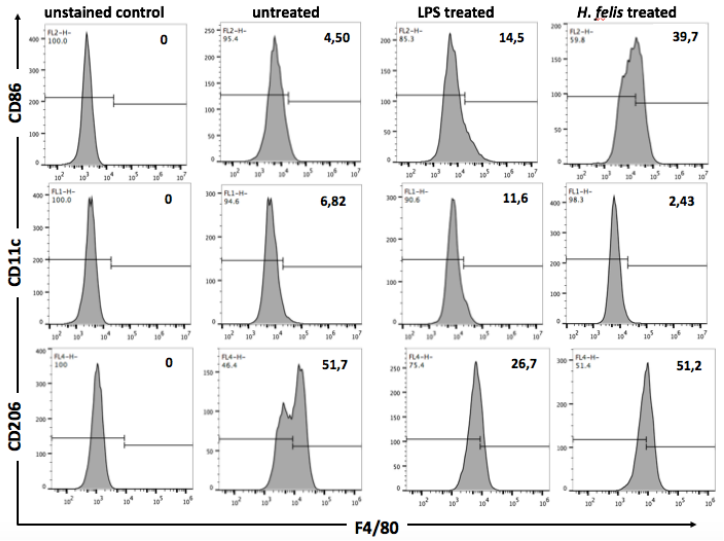


Figure 3.5 (cont'd.) : *CD86, CD11c and CD206 positivity of differentiated F4/80$^+$ bone marrow- derived macrophages.*

Experiment 3



c.

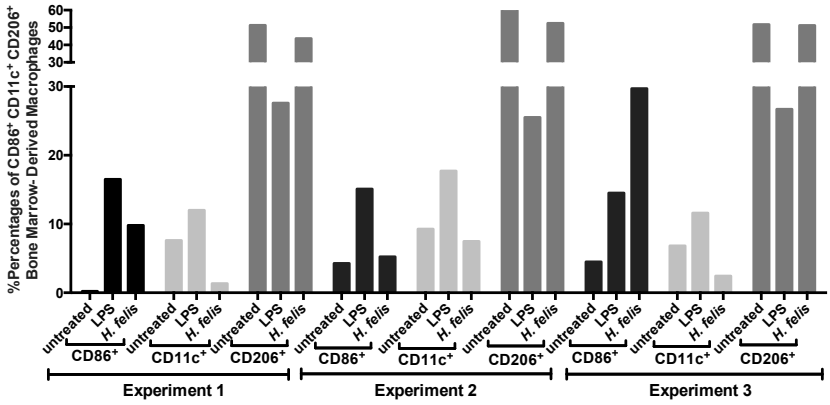


Figure 3.5 (cont'd.) : *CD86, CD11c and CD206 positivity of differentiated F4/80$^+$ bone marrow- derived macrophages.* **a.** Approximately 5x10^4 cells were stained with FITC or APC-coupled anti-F4/80 antibody and PE-coupled CD86, APC-coupled CD11c and CD206 or were left as unstained control. Contour plots **(a.)** and histogram plots (**b.)** were shown above for all experiments. **c.** Independent percentages are expressed as bar graph. Image was prepared using GraphPad Prism program.

According to Figure 3.5, in experiment 1 and 2, CD86 positivity patterns of LPS- and *H. felis* sonicate- treated BM-derived macrophages were similar. Meaning that, LPS-treated BM-derived macrophages were expressed approximately 2 times higher levels of CD86 than *H. felis* sonicate- treated group, indicating that LPS- treated BM-derived macrophages were more activated than *H. felis* sonicate- treated cells. When expression of CD11c surface marker (for M1 type) and CD206 surface marker (for M2 type) were examined, it has been seen that, in both experiment 1 and 2, LPS- treated BM-derived macrophages expressed approximately 2 times higher levels of CD11c

and 2 times lower levels of CD206 than *H. felis* sonicate- treated group, which were similar to untreated group. Higher expression of CD206 seen in untreated bone marrow-derived macrophages was a result of M-CSF directed differentiation (Jaguin et al., 2013). Therefore, LPS- treated BM-derived macrophages were categorized as M1 type macrophages with the high CD11c (Liang et al., 2005 and Pinheiro de Silva et al., 2007) and low CD206 expression. Also, *H. felis* sonicate- treated BM-derived macrophages were categorized as M2 type macrophages with the high CD206 and low CD11c expression. However, in experiment 3, CD86 surface marker expression levels of *H. felis* sonicate- treated BM- derived macrophages were approximately 2-times higher than LPS- treated cells, unlike experiment 1 and 2. Nevertheless, CD11c and CD206 surface marker expression results of experiment 3 were the same as experiment 1 and 2. As a result, regardless of activation status, examined via CD86 surface marker analysis, LPS- treated BM-derived macrophages were categorized as M1 type macrophages with the high CD11c and low CD206 expression, and *H. felis* sonicate-treated BM-derived macrophages were categorized as M2 type macrophages with the high CD206 and low CD11c expression. As a result, whether, *H. felis* sonicate treated BM- derived macrophages were more or less activated than LPS- treated BM- derived macrophages, polarization status was not affected by activation status. Also, since both M1 type and M2b type macrophages expressed CD86 (Mantovani et al., 2004), CD86 could not gave any information about the polarization status, whether M1 or M2b, according to the surface marker expression results.

a. Experiment 1

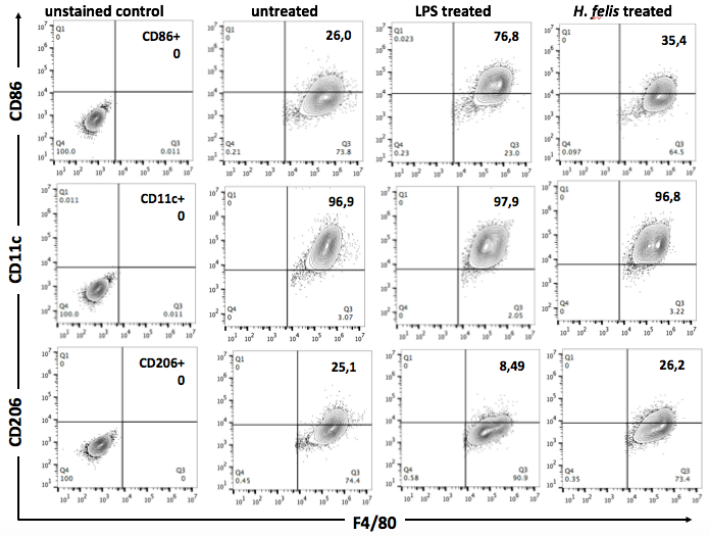
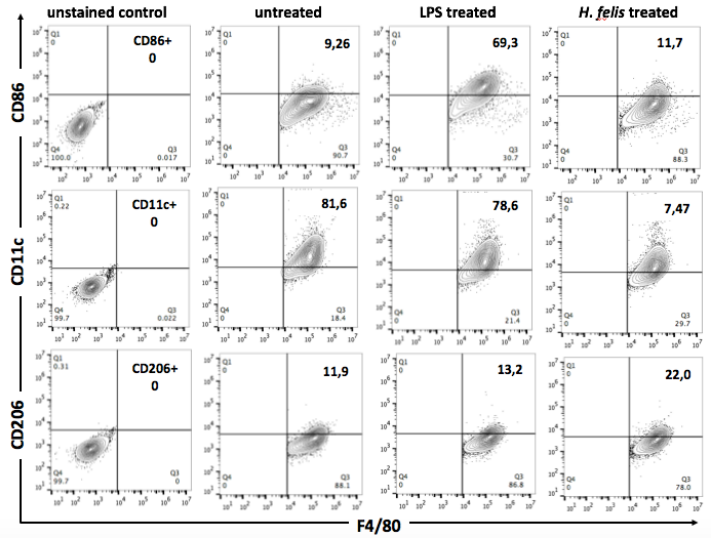
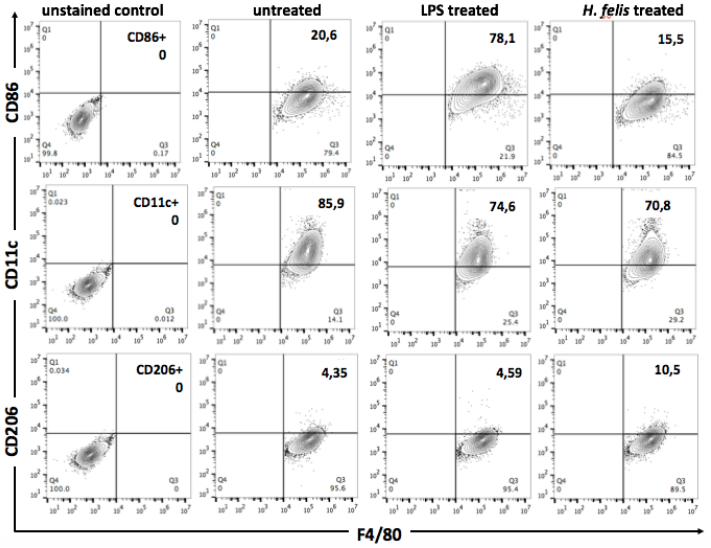


Figure 3.6 : *CD86, CD11c and CD206 positivity of differentiated F4/80^{+} peritoneal macrophages.*

Experiment 2



Experiment 3



b. Experiment 1

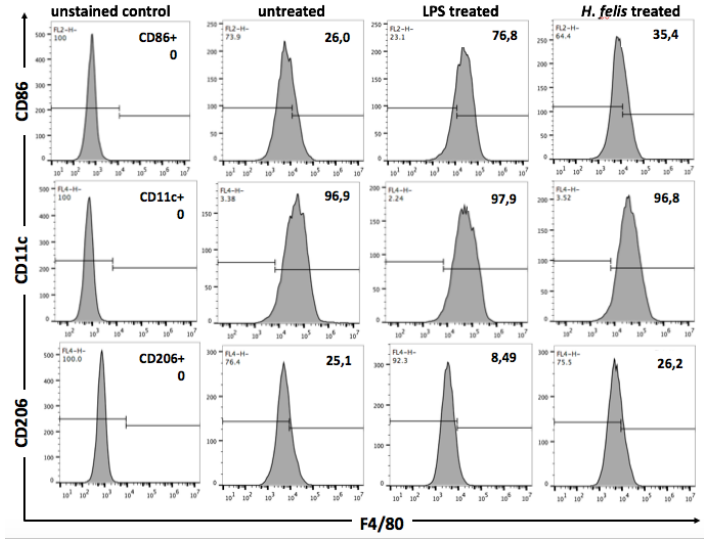
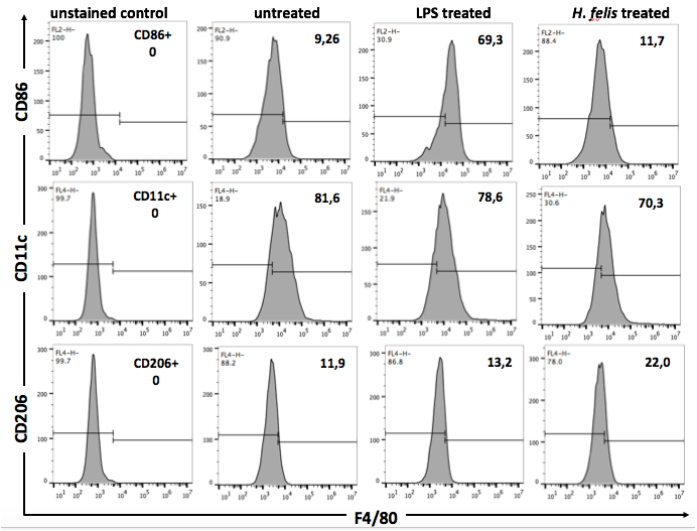
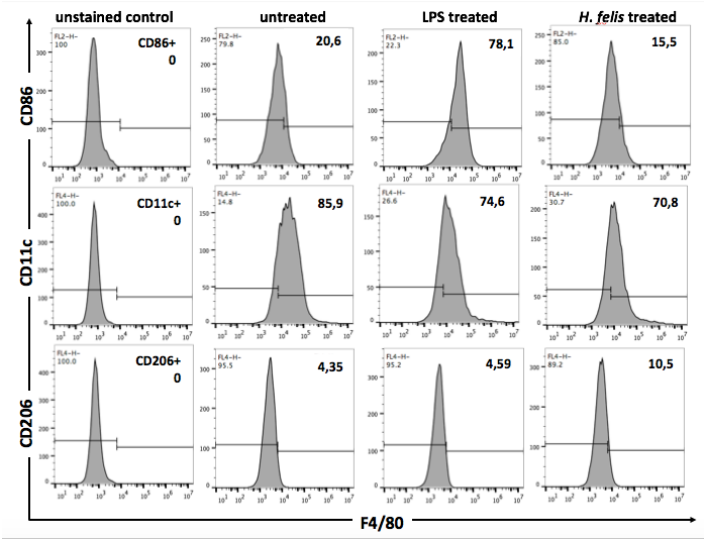


Figure 3.6 (cont'd.) : *CD86, CD11c and CD206 positivity of differentiated F4/80$^+$ peritoneal macrophages.*

Experiment 2



Experiment 3



c.

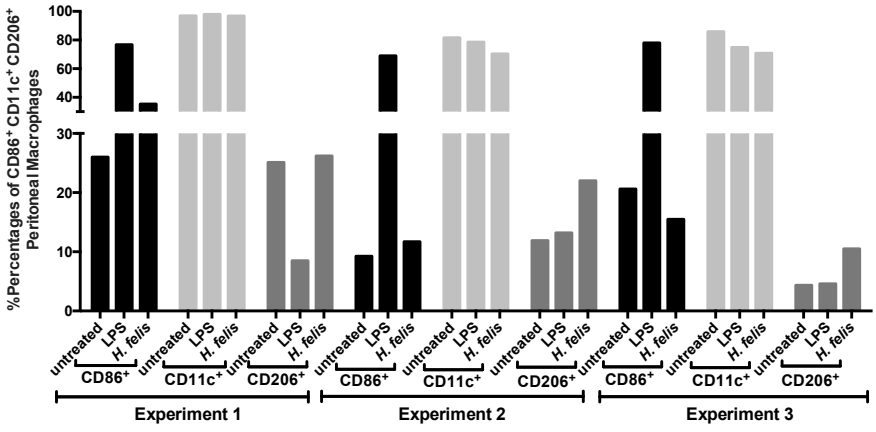


Figure 3.6 (cont'd.) : *CD86, CD11c and CD206 positivity of differentiated F4/80^{+} peritoneal macrophages.*

Figure 3.6 (cont'd.) : *CD86, CD11c and CD206 positivity of differentiated F4/80$^+$ peritoneal macrophages.* **a.** Approximately 5x10^4 cells were stained with FITC or APC-coupled anti-F4/80 antibody and PE-coupled CD86, APC-coupled CD11c and CD206 or were left as unstained control. Contour plots **(a.)** and histogram plots (**b.)** were shown above for all experiments. **c.** Independent percentages are expressed as bar graph. Image was prepared using GraphPad Prism program.

According to Figure 3.6 showing peritoneal macrophages' anti-F4/80 and anti-CD86 double staining results demonstrated approximately 80% CD86$^+$ LPS- treated peritoneal macrophages, in all three experiments. This percentage decreased to 35% in *H. felis* sonicate- treated peritoneal macrophages in experiment 1, and in experiment 2 and 3, percentage was even lower at approximately 15%. Therefore, it seemed that LPS activates the peritoneal macrophages more than *H. felis* sonicate. Also, in all experiments, activation of *H. felis* sonicate- treated peritoneal macrophages was similar to untreated group of cells. Furthermore, when M1 type polarization was examined, it has been shown that, untreated, LPS- and *H. felis* sonicate- treated peritoneal macrophages, expressed more than 70% CD11c surface marker. Therefore, it is suggested that the LPS- treated BM- derived and peritoneal macrophages were polarized to M1 type, as it was showed in the study conducted by Liang et al., 2005 and Pinheiro de Silva et al., 2007. The reason for high CD11c expression in peritoneal macrophages was suggested that thioglycollate injected and isolated peritoneal macrophages are M1 type macrophages since thioglycollate media primes peritoneal macrophages because mostly it is contaminated with LPS endotoxin. In other words, thioglycollate induced peritoneal macrophages behaves as if they were treated with IFN-γ which polarize them into M1 type macrophages (Zhang, Goncalves & Mosser, 2008). Also, for the investigation of M2 type polarization, CD206 surface marker was analyzed in peritoneal macrophages. In all experiments, *H. felis* sonicate- treated peritoneal macrophages expressed approximately 2 times higher levels of CD206 surface marker when compared to LPS- treated peritoneal macrophages. As a result, despite the high levels of CD11c expression, there was higher CD206 expression among *H. felis* sonicate- treated peritoneal macrophages. Also, according to the surface marker analysis, there seems to be no correlation between the activation status and polarization status of peritoneal macrophages.

3.5 Cytokine Profiles of Activated Bone Marrow- Derived and Peritoneal Macrophages

Surface markers alone are not sufficient to determine the polarization status of macrophages, which changes according to stimuli and the local microenvironment, allowing them to shape the local inflammatory status to adapt to outside stimuli by producing different cytokines and having different functions (Table 3.1.) (Mosser & Edwards, 2008). In this study, four new independent experiments were conducted in order to examine the cytokine profiles to identify the different polarization types and subtypes of activated macrophages. For this purpose, firstly activation status of four independent experiments (experiments 4,5,6, and 7) were assessed via the examination of CD86 surface marker expression. After that, cytokine profiles were investigated.

3.5.1 Identification of activation status for cytokine profiling via CD86 surface marker analysis

For the activation investigation of macrophages, LPS- and *H. felis* treated- bone marrow- derived macrophages and peritoneal macrophages were stained with APC-conjugated F4/80 and PE-conjugated CD86 antibodies. CD86 positivity among $F4/80^+$ gated cells were compared to unstained control and untreated group. Flow cytometer analysis was performed for the quantitative analysis of stained cells. Flow cytometer analysis images and individual percentages of four independent experiments of $CD86^+$ bone marrow- derived macrophages and of three independent experiments of peritoneal macrophages were shown in Figure 3.7.a, and 3.7.b respectively.

According to anti-F4/80 and anti-CD86 double staining results of BM- derived macrophages indicated in the figure 3.7.a., in experiment 4,5 and 7, LPS- treated bone marrow- derived macrophages expressed approximately 2 times higher CD86 than *H. felis* sonicate- treated BM- derived macrophages. It seemed that LPS activates these macrophages more than *H. felis* sonicate. However, in experiment 6, activation of LPS- and *H. felis* sonicate- treated BM-derived macrophages were similar. When compared to untreated group, LPS- and *H. felis* sonicate- treated BM- derived macrophages were activated in all experiments (Figure 3.7.a). Furthermore, peritoneal macrophages anti-F4/80 and anti-CD86 double staining results demonstrated approximately 75% $CD86^+$ LPS treated peritoneal macrophages in all experiments. This percentage dropped to 20% in *H. felis* sonicate- treated peritoneal macrophages (Figure 3.7.b.). As a result, it

seemed that LPS activates the peritoneal macrophages more than *H. felis* sonicate. Also, when compared to untreated group, activation of *H. felis* sonicate- treated peritoneal macrophages were similar.

a. Bone Marrow- Derived Macrophages

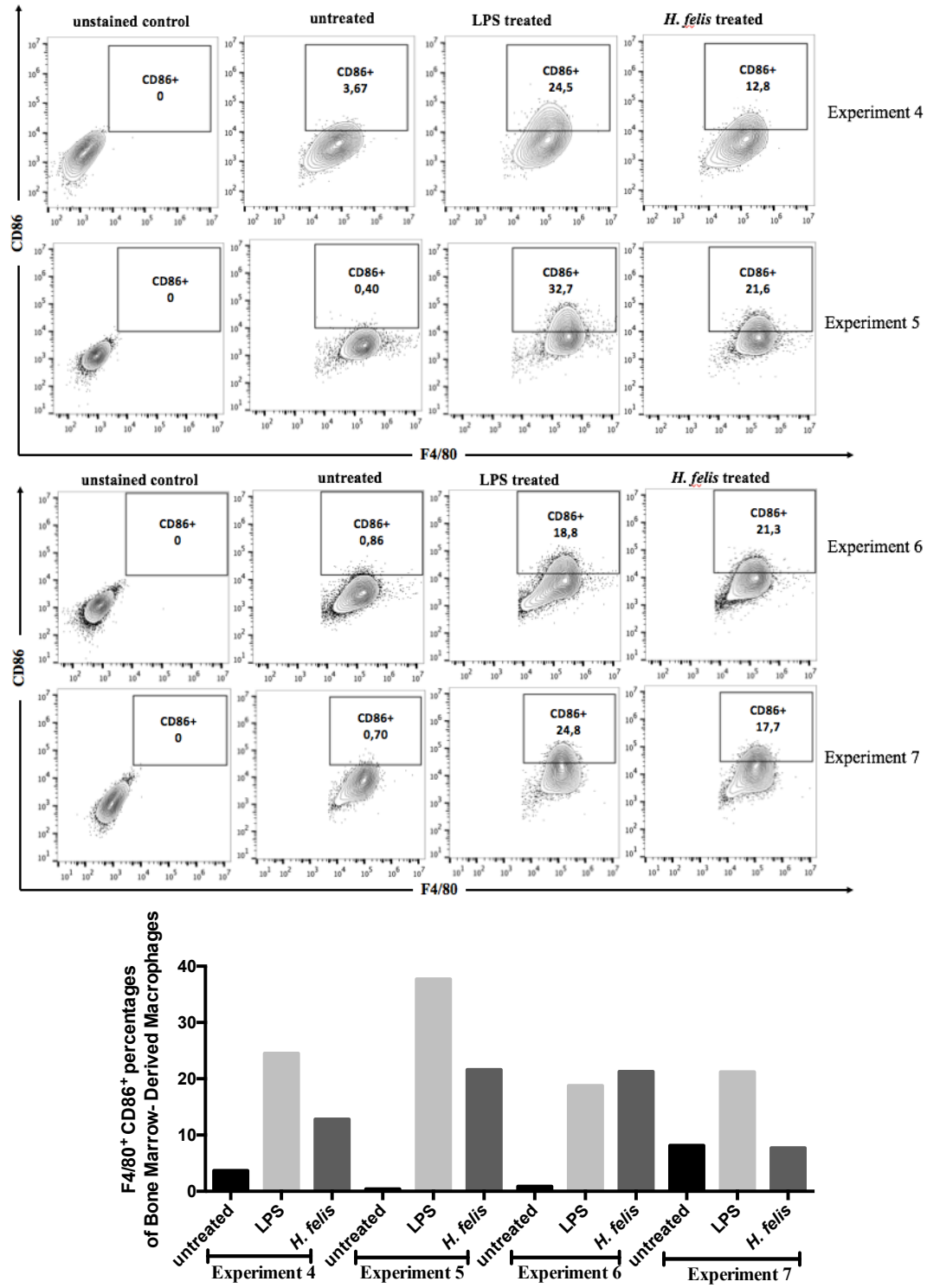


Figure 3.7 : *CD86 positivity of differentiated F4/80$^+$ bone marrow- derived and isolated peritoneal macrophages.*

b. Peritoneal Macrophages

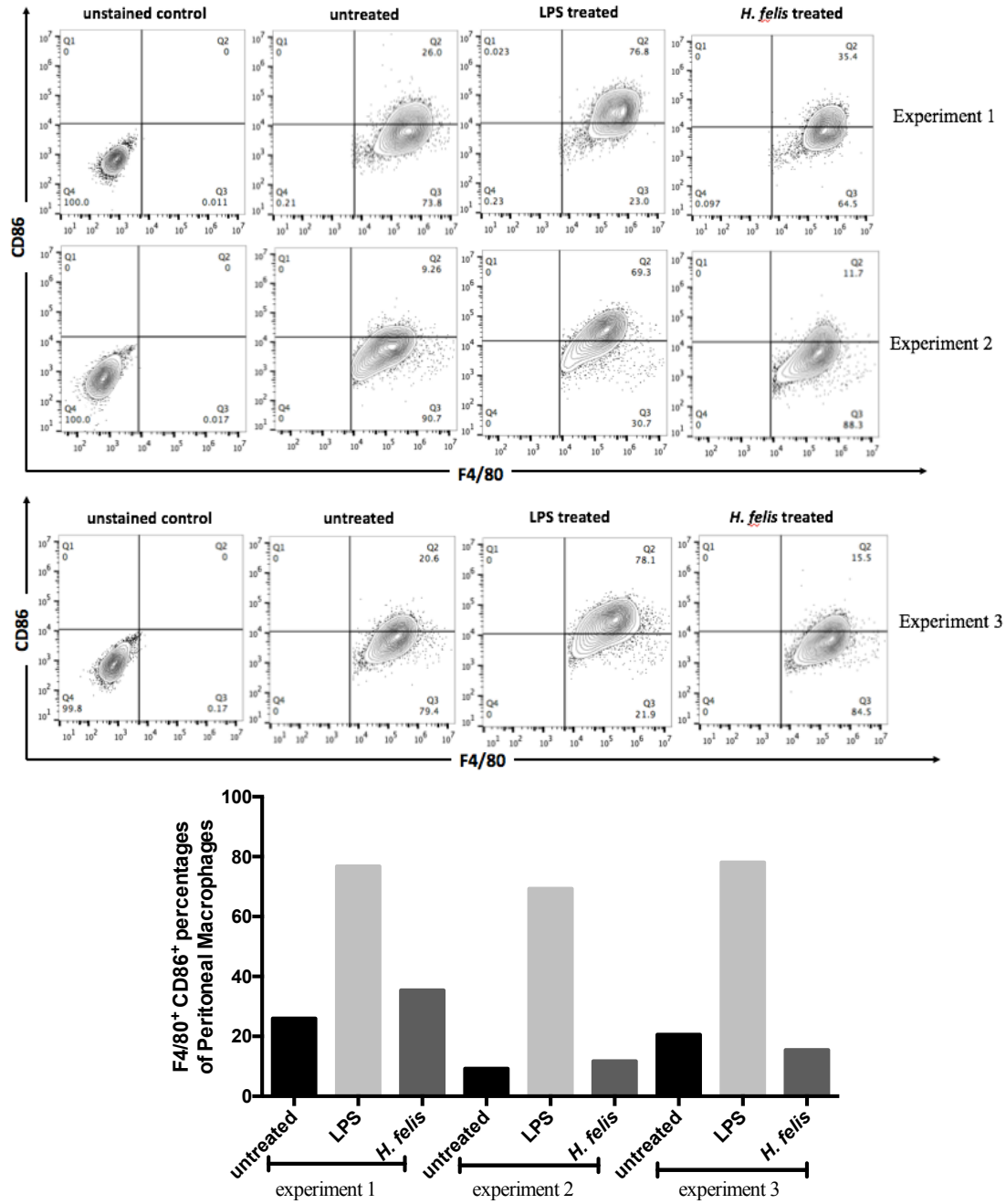


Figure 3.7 (cont'd.) : *CD86 positivity of F4/80$^+$ differentiated bone marrow- derived and isolated peritoneal macrophages.* Approximately 5x10^4 cells were stained with APC-coupled anti-F4/80 antibody and PE-coupled CD86 antibody or were left as unstained control. Contour plots were shown above for all experiments of BM-derived macrophages **(a.)** and peritoneal macrophages **(b.).** Independent percentages and expressed as bar graph for BM-derived macrophages and peritoneal macrophages. Images were prepared using GraphPad Prism program.

3.5.2 IL-12 / IL-23 (p40) cytokine analysis in activated macrophages

IL-12 is the key pro-inflammatory cytokine in determining the polarization status of macrophages and it is mostly secreted from M1 type macrophages. In order to determine the level of IL-12/IL-23 (p40) cytokine in both mRNA and protein level, bone marrow cells were differentiated to bone marrow- derived macrophages in presence of M-CSF derived from L929 cell line and peritoneal macrophages were collected from peritoneal cavity of thioglycollate induced C56BL/6 mice. After determining the percentage of differentiation and purity of isolation, cells were treated with LPS (100 ng/ml), *H. felis* sonicate (10 µg/ml), or left untreated as an internal control for 24 hours. After that, cells were harvested, their supernatants were collected for ELISA to measure IL-12/IL-23 (p40) secretion level and their pellets were collected for Real-time PCR for IL-12/IL-23 (p40) relative expression level. Figure 3.8.a and 3.8.b indicates the IL-12/IL-23 (p40) cytokine analysis results of bone marrow- derived macrophages and peritoneal macrophages, respectively.

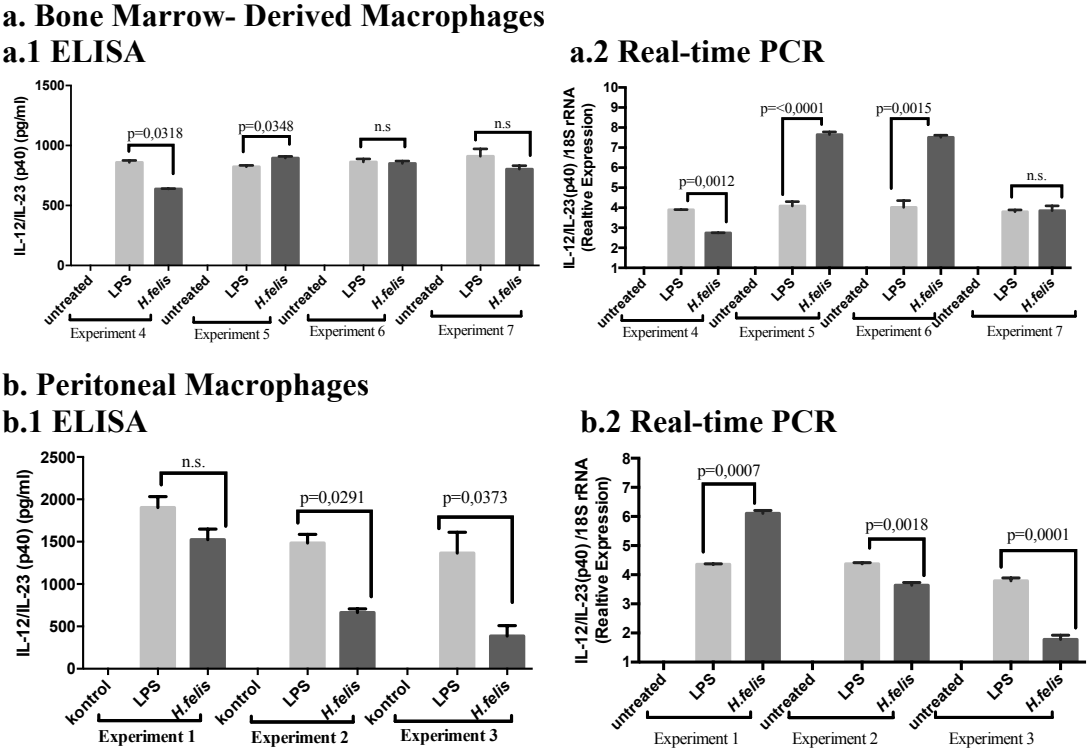


Figure 3.8 : *IL-12/IL-23 (p40) cytokine analysis of bone marrow- derived and peritoneal macrophages.* LPS treated cells (light gray bar) and *H. felis* sonicate treated (dark gray bar) compared to untreated control cells and to each other. Bar graph was drawn with GraphPad Prism program. **(a.)** indicates bone marrow- derived macrophages ELISA (a.1) and Real time PCR (a.2) results. **(b.)** indicates peritoneal macrophages ELISA (b.1) and Real-time PCR (b.2) results.

IL-12/IL-23 (p40) ELISA results indicated that, LPS- and *H. felis* sonicate- treated bone marrow- derived and peritoneal macrophages secreted significantly higher levels of IL-12/IL-23 (p40) when compared to untreated control cells since there was no IL-12/IL-23 (p40) secretion from them (Figure 3.8.a & 3.8.b). Also, both BM-derived and peritoneal macrophages showed *de novo* expression of IL-12/IL-23 (p40). Furthermore, even though LPS- treated BM- derived and peritoneal macrophages were more activated than *H. felis* sonicate treated macrophages (Figure 3.7); *H. felis* sonicate- treated bone marrow- derived macrophages had similar levels of IL-12/IL-23 (p40) secretion when compared to LPS- treated cells. However, LPS treated-peritoneal macrophages secreted significantly higher levels of IL-12/IL-23 (p40) than *H. felis* sonicate treated peritoneal macrophages (Figure 3.8.b.1); but the expression of IL-12/IL-23 (p40) was similar. Therefore, these results suggest that *H. felis* may drive the bone marrow- derived and peritoneal macrophages into M1-like phenotype.

3.5.3 TNF-α cytokine analysis in activated macrophages

TNF-α is a pro-inflammatory cytokine and one of the key cytokines in determining the polarization status of macrophages. It is mostly secreted from M1 type macrophages. However recent data suggest that TNF-α is present by the M2b type macrophages (Mantovani et al., 2004).

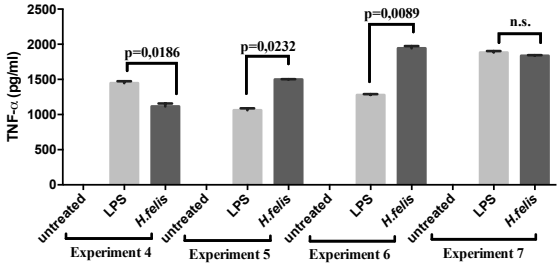
In order to determine the level of TNF-α cytokine in both mRNA and protein level, bone marrow cells differentiated to bone marrow- derived macrophages in presence of M-CSF derived from L929 cell line and peritoneal macrophages were collected from peritoneal cavity of thioglycollate induced C56BL/6 mice. After determining the percentage of differentiation and purity of isolation, cells were treated with LPS (100 ng/ml), *H. felis* sonicate (10 μg/ml), or left untreated for internal control for 24 hours. After that, cells were harvested, their supernatants were collected for performing TNF-α ELISA to analyze their secretion level and their pellets were collected for Real-time PCR to analyze TNF-α relative expression level. Figure 3.9.a and 3.9.b indicates the TNF-α cytokine analysis results of bone marrow- derived macrophages and peritoneal macrophages, respectively.

TNF-α cytokine results shown in the Figure 3.9 indicated that, LPS- and *H. felis* sonicate- treated bone marrow- derived and peritoneal macrophages secreted significantly higher levels of TNF-α when compared to untreated control cells since

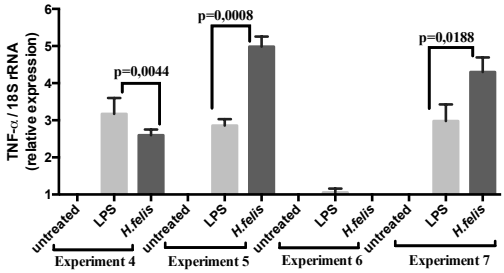
there was no TNF-α secretion from them (Figure 3.9.a & 3.9.b). Also, both BM-derived and peritoneal macrophages showed *de novo* expression of TNF-α cytokine**.** Moreover, even though LPS- treated BM- derived and peritoneal macrophages were more activated than *H. felis* sonicate treated macrophages (Figure 3.7), TNF-α secretion from BM- derived and peritoneal macrophages were similar. *De novo* expression of TNF-α from *H. felis* sonicate- treated BM- derived macrophages were higher than LPS- treated BM- derived macrophages. However, the same result was similar in the case of peritoneal macrophages. In experiment 6 of BM-derived macrophages, despite TNF-α secretion was present, TNF-α expression was not detected. The reason for this setback could not be detected. Hence, both relative expression data and ELISA data suggests that *H. felis* may drive the BM- derived and peritoneal macrophages into M1-like and/or M2b-like phenotype.

a. Bone Marrow- Derived Macrophages

a.1 ELISA

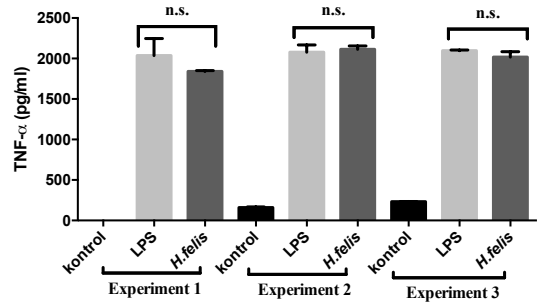


a.2 Real-time PCR



b. Peritoneal Macrophages

b.1 ELISA



b.2 Real-time PCR

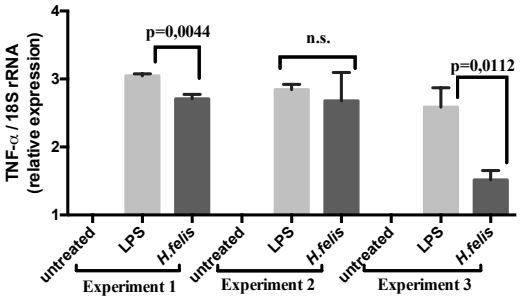


Figure 3.9 : *TNF-α cytokine analysis of bone marrow- derived and peritoneal macrophages.* LPS treated cells (light gray bar) and *H. felis* sonicate treated (dark gray bar) compared to untreated control cells and to each other. Bar graph was drawn with GraphPad Prism program. **(a.)** indicates bone marrow- derived macrophages ELISA (a.1) and Real-time PCR (a.2) results. **(b.)** indicates peritoneal macrophages ELISA (b.1) and Real-time PCR (b.2) results.

3.5.4 IL-1β cytokine analysis in activated macrophages

IL-1β is a pro-inflammatory cytokine and one of the key cytokines in determining the polarization status of macrophages. It is mostly secreted from M1 type macrophages. However recent data suggest that IL-1β is secreted by the M2b type macrophages (Mantovani et al, 2004; Mosser, 2003).

Aside from other secreted cytokines, IL-1β secretion depends on the cleaving of expressed pro- IL-1β by caspase-1 by inflammazome NLRP3 activation via silica crystals or ATP (Netea et al., 2008; Peeters et al., 2013). Therefore, determining the secreted IL-1β requires secondary activation through silica crystals or ATP.

In order to determine the level of IL-1β cytokine in both mRNA and protein level, bone marrow cells were differentiated to bone marrow- derived macrophages in presence of M-CSF derived from L929 cell line and peritoneal macrophages were collected from peritoneal cavity of thioglycollate induced C56BL/6 mice. After determining the percentage of differentiation and purity of isolation, cells were treated with LPS (100 ng/ml), *H. felis* sonicate (10 μg/ml), or left untreated for internal control for 4 hours. Then, silica treatment in different doses (25 μg/cm^2, 10 μg/cm^2, 5 μg/cm^2) was done which is necessary for IL-1β secretion and incubated in cell culture conditions for 8 hours. After that, cells were harvested, their supernatants were collected for ELISA to measure IL-1β secretion level and their pellets were collected for Real-time PCR for IL-1β relative expression level. Figure 3.10.a and 3.10.b indicates the IL-1β cytokine analysis results of bone marrow- derived macrophages and peritoneal macrophages, respectively.

a. Bone Marrow- Derived Macrophages

a.1 Real-time PCR **a.2 ELISA**

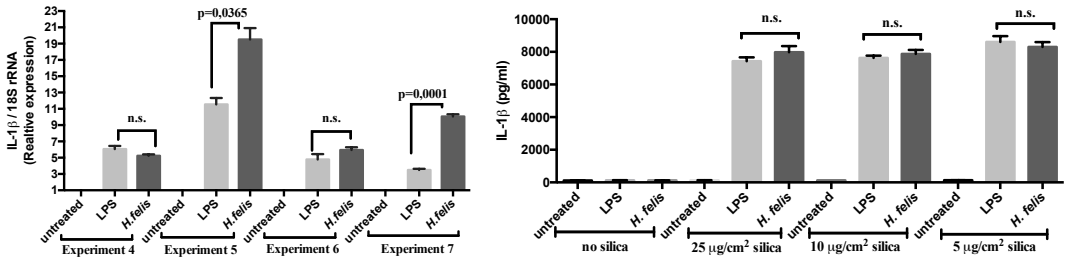


Figure 3.10 : *IL-1β cytokine analysis of bone marrow- derived and peritoneal macrophages.*

b. Peritoneal Macrophages

Real-time PCR

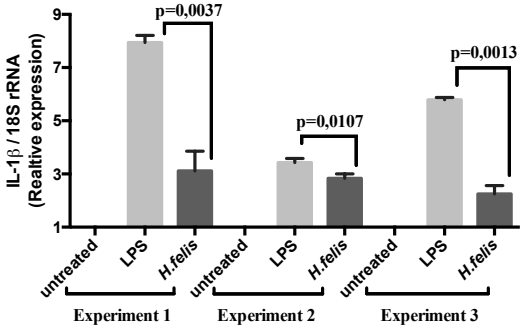


Figure 3.10 (cont'd.) : *IL-1β cytokine analysis of bone marrow- derived and peritoneal macrophages.* LPS treated cells (light gray bar) and *H. felis* sonicate treated (dark gray bar) compared to untreated control cells and to each other. Bar graph was drawn with GraphPad Prism program. **(a.)** indicates bone marrow- derived macrophages Real-time PCR (a.1) and ELISA (a.2) results. **(b.)** indicates peritoneal macrophages Real-time PCR results.

As it is represented in Figure 3.10, LPS- treated and *H. felis* sonicate- treated bone marrow- derived and peritoneal macrophages expressed higher levels of IL-1β. *H. felis* sonicate- treated BM- derived macrophages expressed higher levels of IL-1β compared to LPS- treated BM-derived macrophages, while *H. felis* sonicate- treated peritoneal macrophages expressed lower levels of IL-1β compared to LPS treated peritoneal macrophages. Also, since IL-1β secretion requires secondary activation via silica crystals or ATP, in order to cleave expressed pro-IL-1β by caspase-1 by inflammazome NLRP3 activation (Netea et al., 2008; Peeters et al., 2013), different concentrations of silica treatments were examined on untreated, LPS- treated and *H. felis* sonicate- treated bone marrow- derived macrophages, and secretion levels were investigated via ELISA. Results indicated in Figure 3.10.a.2 showed that, untreated, LPS- and *H. felis* sonicate- treated BM-derived macrophages did not secrete IL-1β without silica treatment. However, when silica was added to the environment, there was high levels of IL-1β secretion from LPS- and *H. felis* sonicate- treated BM-derived macrophages, but not from untreated group of cells. Also, 25 μg/cm^2, 10 μg/cm^2 and 5 μg/cm^2 of silica, triggered similar amounts of IL-1β secretion from both LPS- and *H. felis* sonicate- treated BM- derived macrophages; even though LPS-treated BM- derived were more activated than *H. felis* sonicate treated macrophages (Figure 3.7). As a result, both ELISA and relative expression data suggests that *H. felis*

may drive the bone marrow- derived and peritoneal macrophages into M1-like and/or M2b-like phenotype.

3.5.5 iNOS analysis in activated macrophages

Inducible nitric oxide synthase (iNOS) enzyme must be expressed by macrophages in order to truly characterize the macrophages as M1 type. iNOS enzyme activity is increased as a result of anti-microbial effects of macrophages, this enzymatic reaction causes nitric oxide (NO) production (MacMicking et al, 1997). In order to elucidate the role of nitric oxide (NO), mRNA expression of inducible nitric oxide synthase (iNOS), and production of NO were determined in bone marrow- derived macrophages. The most suitable way to investigate NO production, is to measure nitrite production, which is a stable degradation product of NO, from supernatants of bone marrow derived macrophages. Basically, griess reagent protocol is a chemical reaction between sulfanilic acid (phosphoric acid) and N- (1-naphthyl) ethylenediamine. As a result, spectrophotometrically measureable azo dye is formed (Mat. & Met. Part, 2.2.13). Supernatants of LPS- and *H. felis* sonicate- treated bone marrow- derived macrophages were collected to determine NO production level via griess reagent. After determining the percentage of differentiation, BM- macrophages were treated with LPS (100 ng/ml), *H. felis* sonicate (10 µg/ml), or left untreated for internal control for 24 hours. After that, cells were harvested, their supernatants were collected for griess reagent protocol to measure NO production and cell pellets were collected for Real-time PCR for determining the mRNA expression of iNOS. The griess reagent protocol results and iNOS expression results of bone marrow- derived macrophages and peritoneal macrophages were shown in Figure 3.11.a and 3.11.b, respectively.

a. Bone Marrow- Derived Macrophages

a.1 Griess Reagent Protocol **a.2 Real-time PCR**

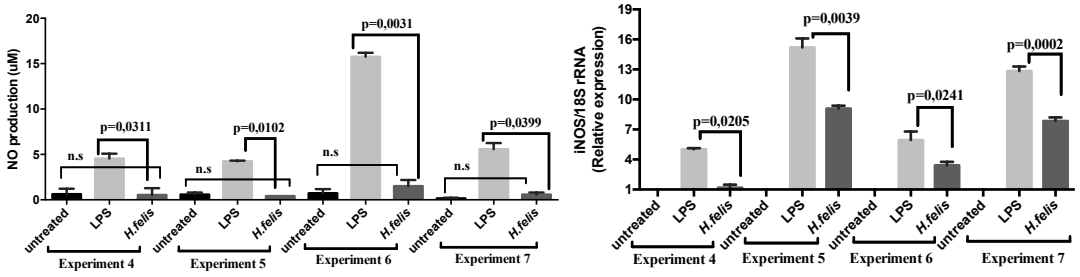


Figure 3.11 : *NO production and iNOS analysis of bone marrow- derived and peritoneal macrophages.*

b. Peritoneal Macrophages

b.1 Griess Reagent Protocol **b.2 Real-time PCR**

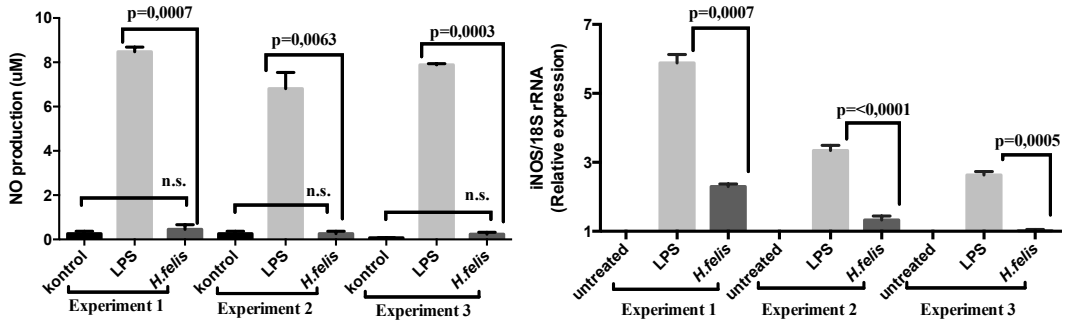


Figure 3.11 (cont'd.) : *NO production and iNOS analysis of bone marrow- derived and peritoneal macrophages.* LPS treated cells (light gray bar) and *H. felis* sonicate treated (dark gray bar) compared to untreated control cells and to each other. Bar graph was drawn with GraphPad Prism program. **(a.)** indicates bone marrow-derived macrophages griess reagent protocol (a.1) and Real-time PCR (a.2) results. **(b.)** indicates peritoneal macrophages griess reagent protocol (b.1) and Real-time PCR (b.2) results.

According to the griess reagent protocol results showed in Figure 3.11, significantly higher levels of nitrite was detected from LPS- treated bone marrow- derived and peritoneal macrophages when compared to the untreated and *H. felis* sonicate- treated macrophages. Since nitrite was the indication of NO production caused by iNOS activity, our results proved that the LPS- treated bone marrow- derived macrophages had high iNOS activity; therefore, concordant with common literature knowledge; these cells were categorized as M1 type macrophages. And since nitrite presence was not detected from *H. felis* sonicate- treated bone marrow- derived and peritoneal macrophages, these results suggest that *H. felis* sonicate treatment may polarize the BM- derived and peritoneal macrophages into M2-like phenotype. Also, relative iNOS expression of LPS- treated BM- derived and peritoneal macrophages were higher than *H. felis* sonicate- treated and untreated cells. However, increase in relative iNOS expression was detected on *H. felis* sonicate- treated BM- derived and peritoneal macrophages when compared to untreated cells. However, according to griess reagent protocol results, NO production from *H. felis* sonicate- treated macrophages were at the same level as untreated cells, indicating that there was no iNOS activity (Figure 3.11.a.1 & 3.11.b.1). Therefore, these data suggested that, although iNOS enzyme was detected to be inactive on *H. felis* sonicate- treated macrophages, there was little amount of iNOS enzyme expression present. Since, macrophages are a plastic population, they polarize from one type to another in certain environments, expression

of some enzymes and cytokines are not lost in one type or another. In other words, *H. felis* sonicate- treated BM-derived and peritoneal macrophages were polarized to M2-like phenotype, however, iNOS expression was not lost entirely in order to adapt quickly to any changing environment.

3.5.6 IL-10 cytokine analysis in activated macrophages

IL-10 is one of the key cytokines in determining the polarization status of macrophages and it is mostly secreted from M2 type macrophages. In order to determine the level of IL-10 cytokine in both mRNA and protein level, bone marrow cells differentiated to bone marrow derived macrophages in presence of M-CSF derived from L929 cell line and peritoneal macrophages were collected from peritoneal cavity of thioglycollate induced C56BL/6 mice. After determining the percentage of differentiation and purity of isolation, cells were treated with LPS (100 ng/ml), *H. felis* sonicate (10 μg/ml), or left untreated for internal control for 24 hours. After that, cells were harvested, their supernatants were collected for performing IL-10 ELISA to analyze their secretion level and their pellets were collected for Real-time PCR to analyze IL-10 relative expression level. Figure 3.12.a and 3.12.b indicates the IL-10 cytokine analysis results of bone marrow- derived macrophages and peritoneal macrophages, respectively.

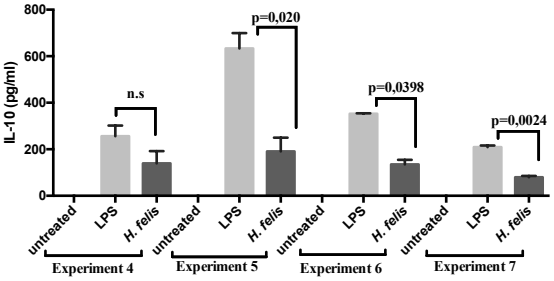
IL-10 cytokine analysis results of bone marrow- derived macrophages indicated that, *H. felis* sonicate- treated cells secreted and expressed higher levels of IL-10, approximately 2 times higher, compared to untreated control cells (Figure 3.12.a). However, in experiment 6, despite IL-10 secretion was present, IL-10 expression was not detected. The reason for this setback could not be detected. These results indicated that *H. felis* sonicate- treated bone marrow- derived macrophages were polarized to M2 phenotype. Furthermore, IL-10 secretion and expression from LPS- treated bone marrow- derived macrophages was significantly higher when compared to *H. felis* sonicate- treated and untreated cells. This data was in concordance with the literature knowledge about LPS triggering IL-10 secretion (Duluc et al, 2007; Hao et al, 2012). And also, M2 cells protect mice against LPS toxicity because of IL-10 secretion (Mosser et al., 1999).

However, IL-10 ELISA results of peritoneal macrophages, showed in the Figure 3.12.b.1, showed that, there was no measurable IL-10 secretion in untreated and *H.*

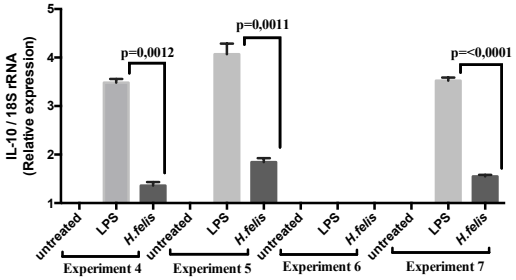
felis sonicate- treated peritoneal macrophages. IL-10 secretion from LPS- treated peritoneal macrophages was observed only in experiment 1. This result couldn't have replicated in other two experiments. Also, in concordance with IL-10 ELISA results, there was no IL-10 expression from *H. felis* sonicate- treated peritoneal macrophages either. Since IL-10 secretion or expression in *H. felis* sonicate- treated peritoneal macrophages was not measured, it has been showed that peritoneal macrophages, which were collected by thioglycollate injection, are polarized to M1 type regardless of the environment.

a. Bone Marrow- Derived Macrophages

a.1 ELISA

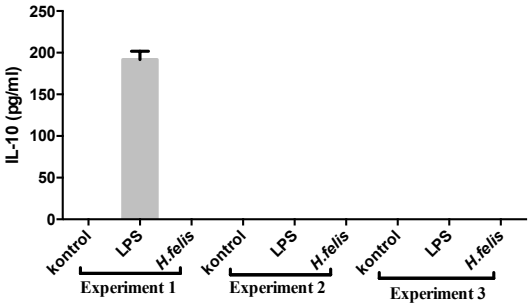


a.2 Real-time PCR



b. Peritoneal Macrophages

b.1 ELISA



b.2 Real-time PCR

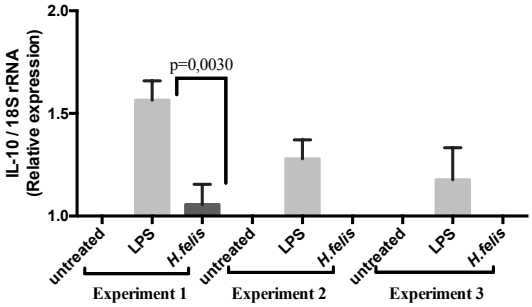


Figure 3.12 : *IL-10 cytokine analysis of bone marrow- derived and peritoneal macrophages.* LPS treated cells (light gray bar) and *H. felis* sonicate treated (dark gray bar) compared to untreated control cells and to each other. Bar graph was drawn with GraphPad Prism program. **(a.)** indicates bone marrow- derived macrophages ELISA (a.1) and Real-time PCR (a.2) results. **(b.)** indicates peritoneal macrophages ELISA (b.1) and Real-time PCR (b.2) results.

3.5.7 IL-6 cytokine analysis in activated macrophages

IL-6 is one of the key cytokines in determining the polarization status of macrophages and it is mostly expressed from M1 type macrophages. However, recent studies suggest that M2 type macrophages subtype M2b type macrophages also express IL-6

cytokine, and it is also a unique cytokine in determining the M2b subtype. Because, among the M2 phenotype macrophages, only M2b uniquely express IL-6 (Duluc et al, 2007; Hao et al, 2012, Murry & Wynn, 2011).

In order to determine the level of IL-6 cytokine in mRNA level, bone marrow cells differentiated to bone marrow derived macrophages in presence of M-CSF derived from L929 cell line and peritoneal macrophages were collected from peritoneal cavity of thioglycollate induced C56BL/6 mice. After determining the percentage of differentiation and purity of isolation, cells were treated with LPS (100 ng/ml), *H. felis* sonicate (10 μg/ml), or left untreated for internal control for 24 hours. After that, cells were harvested and their pellets were collected for Real-time PCR for IL-6 relative expression level in order to examine the M2b type polarization. Cell pellets were used to isolate RNA and then convert to cDNA. IL-6 expression levels were determined with Real - time PCR via IL-16 specific primers (Table 2.12) and normalized to 18s rRNA which was used as an endogenous control. Figure 3.13.a. and 3.13.b. shows the IL-6 Real-time PCR results of bone marrow- derived macrophages and peritoneal macrophages, respectively.

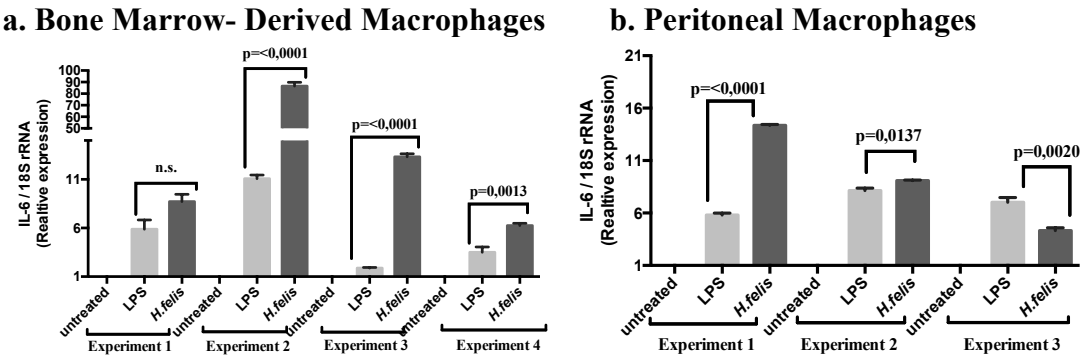


Figure 3.13 : *IL-6 cytokine gene expression analysis of bone marrow- derived and peritoneal macrophages.* LPS treated cells (light gray bar) and *H. felis* sonicate treated (dark gray bar) compared to untreated control cells and to each other. Bar graph was drawn with GraphPad Prism program. **(a.)** indicates bone marrow- derived macrophages results. **(b.)** indicates peritoneal macrophages results.

As it is represented in Figure 3.13, LPS- treated and *H. felis* sonicate- treated bone marrow- derived and peritoneal macrophages expressed higher levels of IL-6 when compared to untreated control cells. Also, *H. felis* sonicate- treated macrophages expressed higher levels of IL-6 when compared to LPS treated cells. Therefore, this data suggests that while LPS drives the bone marrow- derived and peritoneal

macrophages into M1 phenotype, *H. felis* drives the bone marrow macrophages into M1 and/or M2b-like phenotype.

4. DISCUSSION AND CONCLUSION

Helicobacter pylori (H. pylori) has been identified and classified as type I carcinogen for gastric malignancies (Blaser & Atherton, 2004) such as chronic gastritis, peptic ulcer and gastric adenocarcinoma. Even though more than half of the world's population is infected with the *H. pylori*, only a minority develops gastric complications and/or remains asymptomatic.

Helicobacter felis (H. felis) is the more immunogenic on mice and zoonotic species of *Helicobacter*. Hence, it is widely used in murine *Helicobacter* studies because it causes similar pathogenic effect on mice as *H. pylori* on humans (Court et al., 2002; Schmitz et al., 2011).

Innate immune response to *H. pylori* is mediated by pro-inflammatory neutrophils (Amedei et al., 2006), dendritic cells (DCs) (Zavros et al., 2002) and macrophages (Gobert et al., 2004). Macrophages are plastic and heterogenic group of cells, which can polarize to different types under different stimuli. Polarization status of macrophages changes according to stimuli and the local microenvironment, allowing them to shape the local inflammatory status to adapt to outside stimuli (Mosser & Edwards, 2008). There are two distinct states of polarized activation for macrophages: the classically activated (M1) macrophages and the alternatively activated (M2) macrophage subsets (Murray & Wynn, 2011). Cell markers alone do not fully define the many subpopulations of macrophages (Geissmann et al., 2010a). Therefore, macrophages should be defined based on their specific functional activities which can be determined by their produced cytokine profiles and enzymes (Biswas and Mantovani 2010).

In the literature, there are some studies on the *H. pylori* effecting macrophages. In early studies, *H. pylori* was shown to induce the expression of inducible NO synthetase (iNOS) from macrophages (Kuwahara et al., 2000) along with pro- inflammatory cytokines such as IL-6, IL-8, TNF-α, IL-1-β (Lindholm et al., 1998). However, recent studies conducted with human gastric biopsy speciments from *H. pylori* positive

individuals, showed that $CD163^+$ (alternatively activated; M2) macrophages were detected (Fehlings et al., 2012). Moreover, human monocytes secreted IL-1β, IL-6, IL-10, and IL-12p40 (partially secreted as IL-23) but not IL-12p70, upon *H. pylori* infection, suggesting that, M2 macrophages were up-regulated and secreted IL-10 but produced less of the pro-inflammatory cytokines than M1 macrophages (Fehlings et al., 2012).

Above information shows that there are some infromation about *H. pylori* effect on macrophages but not definite chracterization of *H. pylori* or *H. felis* on murine studies. Therefore, in this study, we investigated the effect of *H. felis* on polarization of two types of macrophages: bone marrow- derived macrophages (BM- derived macrophages) and peritoneal macrophages to show the polarization status and their differences of both types of macrophages according to their surface receptor expressions and cytokine profiles.

In order to investigate the effect of *H. felis* on both BM- derived and peritoneal macrophages, *H. felis* sonicate was used in our study. Also, LPS (derived from *E. coli*) was used as an internal M1 phenotype control. Because, in a study conducted *in vitro* using *E. coli* showed that LPS induces a typical M1 profile through TLR4 recognition (Liang et al., 2005; Pinheiro de Silva et al., 2007; Jaguin et al., 2013).

BM- derived macrophages were differentiated by M-CSF obtained from L929 cell line and peritoneal macrophages were isolated via thioglycollate induction from C56BL/6 mice. BM- derived macrophages differentiation (Figure 3.1) and peritoneal macrophages isolation (Figure 3.2) was established with more then 90 % purity, which was determined with CD11b and F4/80 antibody surface staninigs.

Examination of surface marker expression of CD40 and CD80 (B7.1) co-stimulatory molecules, which indicates activation, on LPS- and *H. felis* sonicate- treated BM-derived macrophages revealed that either with or without treatment, BM- derived macrophages expressed 90% for both markers (Figure 3.3 and Figure 3.4). Hence, CD40 and CD80 expressions did not indicate the activation difference between LPS and *H. felis* sonicate- treated cells.

Furthermore, for determining the activation and whether activation and polarization status have correlation between them; CD86 (B7.2) co-stimulatory molecule was identified, along with the CD11c and CD206 surface marker expressions for

characterization of polarization status of activated BM- derived and peritoneal macrophages. M1 type macrophages were identified by F4/80, CD11b, and CD11c+ surface marker expressions (Lumeng et al., 2007; Fujisaka et al., 2009) and M2 macrophages were shown to express high levels of mannose receptor (MR or CD206) (Verreck et al., 2006). For this purpose, three different experiments were conducted.

In experiment 1 and 2 of BM- derived macrophages, LPS- treated BM-derived macrophages were expressed approximately 2 times higher levels of CD86 than *H. felis* sonicate- treated group, indicating that LPS treated BM-derived macrophages were more activated than *H. felis* sonicate- treated cells (Figure 3.5). Also, LPS-treated BM-derived macrophages expressed approximately 2 times higher levels of CD11c and 2 times lower levels of CD206 than *H. felis* sonicate- treated group, which were similar to untreated group (Figure 3.5). Higher expression of CD206 seen in untreated BM- derived macrophages was a result of M-CSF directed differentiation (Jaguin et al., 2013). Since, CD11c was considered as M1 type macrophage and CD206 was considered as M2 type macrophage indicator, LPS- treated BM-derived macrophages were categorized as M1 type macrophages with the high CD11c (Liang et al., 2005 and Pinheiro de Silva et al., 2007) and low CD206 expression. Furthermore, *H. felis* sonicate- treated BM-derived macrophages were categorized as M2 type macrophages with the high CD206 and low CD11c expression. Although, in experiment 3, CD86 expression levels of *H. felis* sonicate- treated BM- derived macrophages were approximately 2-times higher than LPS treated cells; CD11c and CD206 expression results were the same as experiment 1 and 2 (Figure 3.5). Therefore, LPS- treated BM-derived macrophages were categorized as M1 type macrophages with the high CD11c and low CD206 expression, and *H. felis* sonicate- treated BM-derived macrophages were categorized as M2 type macrophages with the high CD206 and low CD11c expression in all experiments. It can be concluded that the activation status did not affect the polarization status.

Moreover, LPS treated peritoneal macrophages were approximately 80% $CD86^{+}$ in all three experiments. CD86 expression levels decreased to 35% in *H. felis* sonicate-treated peritoneal macrophages in experiment 1 and to 15% in experiment 2 and 3 (Figure 3.6). Hence, it seemed that LPS- activates the peritoneal macrophages more than *H. felis* sonicate. When M1 type polarization was examined; untreated, LPS- and *H. felis* sonicate- treated peritoneal macrophages expressed more than 70% CD11c

surface marker. Therefore, it is suggested that the LPS- treated peritoneal macrophages were polarized to M1 type (Liang et al., 2005 and Pinheiro de Silva et al., 2007) (Figure 3.6). The reason for high CD11c expression in peritoneal macrophages was caused from thioglycollate injection in order to isolate peritoneal macrophages. Because, thioglycollate media primes peritoneal macrophages; since, it is mostly contaminated with LPS endotoxin. Hence, thioglycollate induced peritoneal macrophages behaves as if they were treated with IFN-γ which polarize them into M1 type macrophages (Zhang, Goncalves & Mosser, 2008). Also, for the investigation of M2 type polarization, in all experiments, *H. felis* sonicate- treated peritoneal macrophages expressed approximately 2 times higher levels of CD206 surface marker when compared to LPS- treated peritoneal macrophages (Figure 3.6). Hence, despite the high levels of CD11c expression, there was higher CD206 expression among *H. felis* sonicate treated peritoneal macrophages. As a result, according to the surface marker analysis, there seems to be no correlation between the activation status and polarization status of peritoneal macrophages (Figure 3.5 and 3.6).

Surface marker expressions of CD11c (for M1 type polarization) and CD206 (for M2 type polarization) gave us some idea about the polarization status of BM- derived and peritoneal macrophages, as it was indicated before, it was not sufficient. Therefore, after confirming that CD86 surface marker expression could not give any ideas about the polarization status about of macrophages, it can only identify the activation status; we decided to further characterize the BM- derived and peritoneal macrophages through their cytokine profiles in both mRNA and protein level. For this purpose, firstly activation status of four independent experiments (experiments 4,5,6, and 7) were assessed via the examination of CD86 surface marker expression. After that, cytokine profiles were investigated.

In experiments 4,5 and 7, LPS- treated BM- derived macrophages expressed approximately 2 times higher CD86 than *H. felis* sonicate- treated BM- derived macrophages. It seemed that LPS activates these macrophages more than *H. felis* sonicate. However, in experiment 6, activation of LPS- and *H. felis* sonicate- treated BM-derived macrophages were similar, around 20% CD86 expression. Therefore, when compared to untreated BM- derived macrophages, LPS and *H. felis* sonicate-treated BM- derived macrophages were activated in all experiments (Figure 3.7.a). Furthermore, peritoneal macrophages demonstrated approximately 75% $CD86^{+}$ in

LPS- treated peritoneal macrophages in all experiments. This percentage decreased to 20% in *H. felis* sonicate- treated peritoneal macrophages (Figure 3.7.b.). Hence, it seemed that LPS activates the peritoneal macrophages more than *H. felis* sonicate. Also, when compared to untreated group, activation of *H. felis* sonicate- treated peritoneal macrophages were similar. Therefore, in BM- derived macrophages *H. felis* sonicate- treated cells were activated; however, in peritoneal macrophages, *H. felis* sonicate- treated cells were not activated when compared to untreated groups of BM-derived and peritoneal macrophages.

After determination of activation status, cytokine profiles of activated macrophages were assessed. IL-12/IL-23 (p40) cytokine was examined to investigate the M1 type polarization since it is the key pro-inflammatory cytokine secreted from M1 type macrophages (Fairweather & Cihakova, 2009; Sindrilaru, Peters & Wieschalka et al., 2011). Investigation of IL-12/IL-23 (p40) in both mRNA and protein level results indicated that, LPS- and *H. felis* sonicate- treated BM- derived and peritoneal macrophages secreted high levels of IL-12/IL-23 (p40). Also, they showed *de novo* expression of IL-12/IL-23 (p40) (Figure 3.8.a & 3.8.b). High secretion and expression of IL-12/IL-23 (p40) from LPS- treated macrophages is parallel with literature knowledge. Even though LPS- treated BM- derived and peritoneal macrophages were more activated than *H. felis* sonicate- treated macrophages (Figure 3.7), this activation status was not seemed to affect the IL-12/IL-23 (p40) cytokine secretion or expression. Therefore, these results suggest that *H. felis* may drive the BM- derived and peritoneal macrophages into M1-like phenotype.

Furthermore, TNF-α cytokine was examined. It is mostly secreted from M1 type macrophages. However recent data suggest that TNF-α is secreted by the M2b type macrophages (Mantovani et al, 2004). Since, *H. felis* sonicate- treated macrophages showed high levels of CD206 (mannose receptor) expression, the question was that maybe *H. felis* drive macrophages into M2b type, which is an immune-regulatory functioning phenotype and very similar cytokine profile to M1 phenotype. LPS and *H. felis* sonicate- treated BM- derived and peritoneal macrophages secreted significantly higher levels of TNF-α when compared to untreated control cells since there was no TNF-α secretion from them (Figure 3.9.a & 3.9.b). Also, both BM-derived and peritoneal macrophages showed *de novo* expression of TNF-α cytokine**.** Hence, both relative expression data and ELISA data suggests that *H. felis* may drive the BM-

derived and peritoneal macrophages into M1 and/or M2b phenotype. Also, similar to IL-12/IL-23 (p40) results, activation status, examined by CD86 surface marker expression, did not seemed to have any correlation to the TNF-α cytokine secretion or expression.

Similar results were obtained in the examination of IL-1β cytokine profile in both mRNA and protein level. As TNF-α, IL-1β is also expressed by both M1 type and M2b type macrophages (Mantovani et al., 2004; Mosser, 2003). Since, IL-1β secretion depends on the cleaving of expressed pro- IL-1β by caspase-1 by inflammazome NLRP3 activation via, silica crystals or ATP (Netea et al., 2008; Peeters et al., 2013), different doses of silica treatments were investigated in BM- derived macrophages. There was high levels of IL-1β secretion from LPS- and *H. felis* sonicate- treated BM-derived macrophages, but not from untreated group of cells (Figure 3.10.a.2). Different doses of silica triggered similar amounts of IL-1β secretion from both LPS- and *H. felis* sonicate- treated BM- derived macrophages (Figure 3.10.a.2); even though LPS-treated BM- derived macrophages were more activated than *H. felis* sonicate- treated BM- derived macrophages (Figure 3.7). Furthermore, IL-1β expression was determined from LPS- treated and H. felis sonicate- treated BM- derived and peritoneal macrophages (Figure 3.10.a.1 and 3.10.b). When compared to each other, *H. felis* sonicate- treated BM- derived macrophages expressed higher levels of IL-1β compared to LPS- treated BM-derived macrophages (Figure 3.10.a.1); while *H. felis* sonicate- treated peritoneal macrophages expressed lower levels of IL-1β compared to LPS- treated peritoneal macrophages (Figure 3.10.b). As a result, it can be concluded that *H. felis* may drive BM- derived and peritoneal macrophages into M1-like and/or M2b-like phenotype.

Inducible nitric oxide synthase (iNOS) enzyme must be expressed by macrophages in order to truly and uniquely characterize the macrophages as M1 type. Macrophages exert their anti-microbial functions through nitric oxide (NO) synthesis, which is the end product of enzymatic reaction of iNOS. For that reason, we examined both BM-derived and peritoneal macrophages for NO production and iNOS gene expression. NO production was examined via griess reagent protocol (Mat. & Met. Part, 2.2.13). As a result, higher levels of NO production were seen from LPS- treated BM- derived and peritoneal macrophages. Hence, concordant with common literature knowledge,

these cells were categorized as M1 type macrophages (Figure 3.11). However, nitrite presence, which is the indicative of NO production as a result of griess protocol, was not detected from *H. felis* sonicate- treated BM-derived and peritoneal macrophages. Therefore, these results suggest that *H. felis* polarize the BM- derived macrophages into M2-like phenotype (Figure 3.11). Similar results were obtained from the relative gene expression results of iNOS. LPS- treated both BM- derived and peritoneal macrophages, expressed higher levels of iNOS, as the indicative of M1 type polarization (Figure 3.11). However, in *H. felis* sonicate- treated BM-derived and peritoneal macrophages showed higher iNOS expression than untreated group of cells. However, there was no NO production. Therefore, we can conclude that iNOS enzyme was detected to be inactive on *H. felis* sonicate- treated macrophages, there was little amount of iNOS enzyme expression present. The reason is that since, macrophages polarize from one type to another in certain environments, expression of some enzymes and cytokines are not fully lost in one type or another. In other words, *H. felis* sonicate- treated macrophages were polarized to M2-like phenotype, however, iNOS expression was not lost entirely in order to adapt quickly to any changing environment.

For further categorization of effect of *H. felis* on macrophage polarization, we examined IL-10 cytokine, which is the anti-inflammatory cytokine secreted from M2 type macrophages. IL-10 ELISA and real-time PCR results of BM- derived macrophages indicated that, *H. felis* sonicate- treated cells secreted and expressed significantly higher levels of IL-10, when compared to untreated control cells (Figure 3.12.a.). Hence, we concluded that *H. felis* sonicate- treated BM- derived macrophages were polarized to M2 phenotype. Furthermore, IL-10 secretion from LPS treated BM-derived macrophages was approximately 3 times higher when compared to *H. felis* sonicate- treated and untreated cells. This data was in concordance with the literature knowledge about LPS triggering IL-10 secretion (Duluc et al, 2007; Hao et al, 2012). And also, M2 cells protect mice against LPS toxicity because of IL-10 secretion (Mosser et al., 1999). However, IL-10 ELISA and real-time PCR results of peritoneal macrophages, showed in the Figure 3.12.b., showed that, there was no measurable IL-10 secretion in untreated and *H. felis* sonicate- treated peritoneal macrophages. Also, in one experiment only, IL-10 secretion of LPS treated peritoneal macrophages was observed. This result couldn't be replicated in other two experiments. Therefore, we concluded that, even though IL-10 secretion in *H. felis* sonicate- treated peritoneal

macrophages was not measured, they did not produce NO (Figure 3.11) and there were very low amounts of iNOS expression (Figure3.11). Thus, we can conclude that peritoneal macrophages, which were collected by thioglycollate injection, are polarized to M2-like type phenotype. They cannot completely show M2 phenotype, probably because of the thioglycollate injection (Zhang, Goncalves & Mosser, 2008)

So far, we understood that *H. felis* drove BM- derived and peritoneal macrophages into mostly M2-like phenotype with the high expression of CD206 surface marker, low expression of CD11c surface marker, no production of NO, and production of an anti-inflammatory cytokine IL-10. However, these macrophages also produce, IL-12/IL23 (p40), IL-1β, and TNF-α cytokines. Since we know that M2 type macrophages further categorized into 3 different subtypes (Figure 1.9), we examined whether they have a unique property to help us categorize *H. felis* sonicate- treated BM- derived and peritoneal macrophages. Hence, we have found that M2 type macrophages subtype M2b type macrophages express IL-6 cytokine, and it is also a unique cytokine in determining the M2b subtype. Because, among the M2 phenotype macrophages, only M2b-type uniquely express IL-6 (Duluc et al, 2007; Hao et al, 2012, Murry & Wynn, 2011). As it is represented in Figure 3.13, LPS- treated and *H. felis* sonicate- treated BM- derived and peritoneal macrophages expressed high levels of IL-6 when compared to untreated control cells. Also, IL-6 expression from *H. felis* sonicate-treated macrophages was approximately 2 times higher than LPS- treated macrophages. Therefore, we concluded that *H. felis* sonicate- treated BM- derived and peritoneal macrophages were polarized to M2b phenotype (Table 4.1).

In the light of the above findings, we finally categorized our BM-derived and peritoneal macrophages as M2b phenotype with the high CD40 and CD80, moderate CD86, high CD206 and low CD11c expression, also, anti-inflammatory IL-10 secretion along with TNF-α, IL-1β, and IL-6 production. Also, we have found that there was no correlation between the activation and polarization status. However, production of pro-inflammatory IL-12/IL23 (p40) cytokine lead us to the conclusion that *H. felis* does not drive macrophages to polarize into only one phenotype of M2, but there is probably M1-like type macrophages mixed in the population (Figure 4.1).

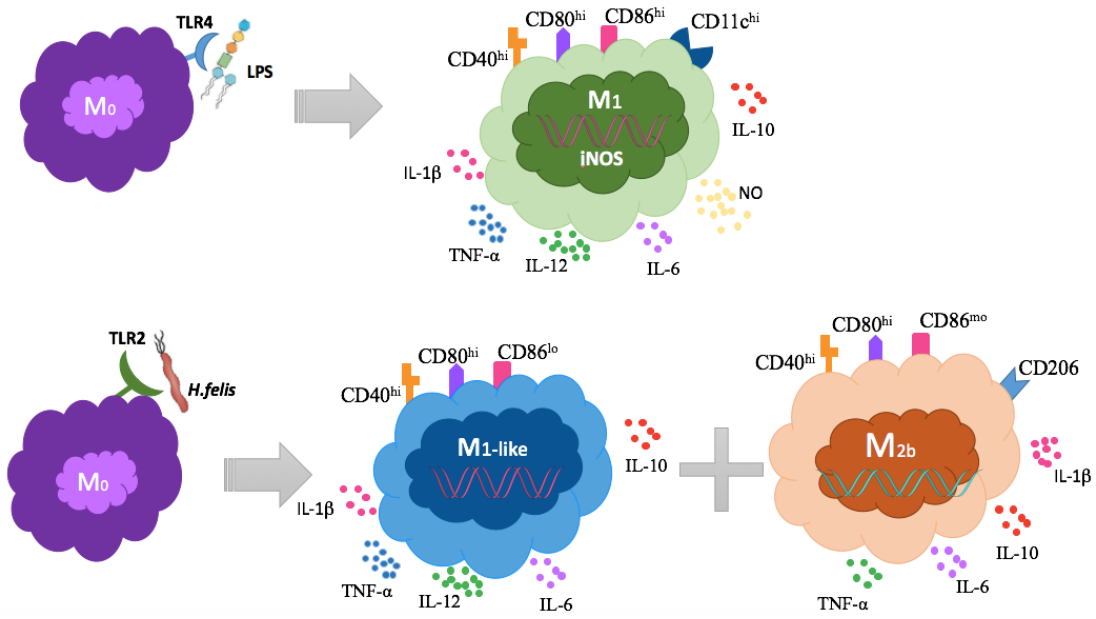


Figure 4.1: Proposed model of the effect of LPS and *Helicobacter felis* on macrophage polarization.

Significance of this research lies in its contribution to the understanding of the effect of *H. felis* on macrophage polarization for the first time. However, for the characterization of M1 and M2 type macrophages, we examined only one enzyme which was iNOS activated in M1 type macrophages. Also, for future perspective, arginase enzyme, which becomes active in M2 type macrophages instead of iNOS (Odegaard & Chawla, 2008) should be further investigated.

These results contribute just to the one side of the question that we need to give answers to. Macrophages are the gateways between innate and adaptive immunity. Recent findings revealed that regulatory B cell (Breg) involvement in the murine *H. felis* infection. They proved that 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 (Sayi et al., 2011). From that point, since macrophages function as effector cells and to help to shape the adaptive immune response, possible interactions between Bregs and macrophages should be investigated in order to be able to understand the *H. felis* infection consequences and try to find some therapeutic approaches to the problem.

In conclusion, this study has contributed to the literature through providing definitive characterization of *H. felis* infected bone marrow- derived and peritoneal macrophages polarization, describing the surface marker and cytokine profiles for the first time.

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- **Korkmaz, A**., Barut, G.T., Esencan, Z., and Sayi-Yazgan, A. 2015: *Helicobacter felis*'in Kemik İliği Kökenli Makrofaj Polarizasyonu Üzerine Etkisi. *23th National Immunology Congress* – April 26- 30, 2015 Antalya, Turkey.

- **Korkmaz, A**., Barut, G.T., Esencan, Z., and Sayi-Yazgan, A. 2015: Effect of Helicobacter felis on Bone Marrow- Derived Macrophage Polarization. *4th International Congress of Molecular Biology Association of Turkey*, November 27-30, 2015 METU, Ankara, Turkey.