ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

THE ROLE OF CHEMERIN ON *HELICOBACTER PYLORI* INDUCED GASTRIC PATHOGENESIS

M.Sc. THESIS

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Department of Molecular Biology- Genetics and Biotechnology

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Thesis Advisor: Assoc. Prof. Ayça SAYI YAZGAN

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

HELİCOBACTER PYLORİ İLE İNDÜKLENEN GASTRİK PATOGENEZDE KEMERİNİN ROLÜ

YÜKSEK LİSANS TEZİ

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Tez Danışmanı: Doç. Dr. Ayça SAYI YAZGAN

ARALIK 2016

Mantasha TABASSUM, a **M.Sc** student of İTU **Graduate School of Science Engineering and Technology** student ID **521121162**, successfully defended the thesis entitled "**THE ROLE OF CHEMERIN ON *HELICOBACTER PYLORI* INDUCED GASTRIC PATHOGENESIS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family and friends,

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Mantasha Tabassum
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ABBREVIATIONS

µg : Microgram
µm : Micrometer
µM : Micromolar
1,25D3 : 1,25 Dihydroxyvitamin D3
APC : Antigen Presenting Cell
AlpA : Adherence associated Lipoprotein A
BCA : Bicinchoninic Acid
BSA : Bovine Serum Albumin
BabA : Blood group antigen binding Adhesion
BAT cells : Brown Adipocyte Tissue derived cells
BMSCs : Bone Marrow Mesenchymal Stem Cells
CagA : Cytotoxin- Associated Gene A
CagE : Cytotoxin- Associated Gene E
CagL : Cytotoxin- Associated Gene L
CagY : Cytotoxin- Associated Gene Y
CagI : Cytotoxin- Associated Gene I
CCRL2 : CC-motif chemokine Receptor-Like 2
CD : Cluster of Differentiation
CD40L : CD40 Ligand
CSK : C-terminal Src Kinase
CRP : C-Reactive Protein
CMKLR1 : Chemokine like Receptor-1
ChemR23 : Chemerin Receptor 23
COPD : Cigarette smoke- induced chronic Obstructive Pulmonary Disease
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
DupA : Duodenal Ulcer Promoting Gene A
EAE : Experimental Autoimmune Encaphalomyelitis
EDTA : Ethylenediaminetetraacetic Acid
ELISA : Enzyme-Linked Immunosorbent Assay
EPIYA : Glutamine- Proline- Isoleucine- Tyrosine- Alanine
FAK : Focal Adhesion Kinase
FBS : Fetal Bovine Serum
FFA : Free Fatty Acids
FITC : Fluorescein Isothiocyanate
FOXP3 : Forkhead Box P3
FXR : Farnesoid X Receptor
g : Gram
GGT : Gamma Glutamyl Transpeptidase
GM-CSF : Granulocyte Macrophage Stimulating Factor

GPR1 : G Protein-coupled Receptor 1
GRB5 : Growth factor Receptor-Bound protein 5
h : Hour
H. felis : *Helicobacter felis*
H. pylori : *Helicobacter pylori*
HRP : Horseradish Peroxidase
HopZ : Homeodomain-only protein Z
HP-NAP : H. pylori Neutrophil Activating Protein
ICAM3 : Intercellular Adhesion Molecule 3
IBD : Inflammatory Bowel Disease
IceA : Epithelium gene A
IFN-γ : Interferon Gamma
IFN-β : Interferon-Beta
Ig : Immunoglobulin
IL-1β : Interleukin-1 Beta
IL-4 : Interleukin-4
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
KO : Knock Out
KSR1 : Kinase Suppressor of Ras
L : Liter
LFA1 : Leukocyte Function associated Antigen-1
LPS : Lipopolysaccharide
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
MET : Mesenchymal to Epithelial Transition
M-CFU : Macrophage Colony Factor Unit
MHC-I : Major Histocompatibility Complex Class I
MHC-II : Major Histocompatibility Complex Class II
min : Minute
ml : Mililiter
mM : Milimolar
mm : Milimeter
mRNA : Messenger Ribonucleic Acid
MyD88 : Myeloid Differentiation Primary Response Gene 88
NFAT : Nuclear Factor of Activated T cells
NF-κB : Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells

NK : Natural Killer cell
NKT : Natural Killer T cell
NLRP3 : NOD-like Receptor Protein 3
OA : OsteoArthritis
OipA : Outer Membrane Protein A
PAR1 : Prader-Willi/Angelman region-1
PUD : Peptic Ulcer Disease
PAI : Pathogenicity Island
PAMP : Pathogen Associated Molecular Pattern
PBS : Phosphate Buffered Saline
PCOS : Polycystic Ovary Syndrome
PCR : Polymerase Chain Reaction
pDCs : Plasmacytoid Dendritic Cells
pg : Picogram
PGN : Peptidoglycan
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
PMNs : Polymorphonuclear leukocytes
PRR : Pattern Recognition Receptor
RAF1 : Rapidly Accelerated Fibrosarcoma
RAS : Retrovirus Associated DNA sequences
RARRES2 : Retinoid Acid Receptor Responder 2
RARβ/γ : Retinoic Acid Receptors β and γ
RGD motif : Arginine-Glycine-Aspartate motif
ROS : Reactive Oxygen Species
rpm : Revolutions Per Minute
RPMI : Roswell Park Memorial Institute
rRNA : Ribosomal RNA
SabA : Sialic acid binding Adhesion
SHP2 : Protein Tyrosine Phosphatase 2
STAT : Signal Transducer and Activator of Transcription
TAM : Tumor Associated Macrophages
TF : Transcription Factor
T4SS : Type IV Secretion System
TGF-β : Transforming Growth Factor-beta
TIG2 : Tazarotene-Induced Gene 2
Th1 : T Helper 1
Th2 : T Helper 2
Th17 : T Helper 17
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
VCAM-1 : Vascular Cell Adhesion Molecule 1

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THE ROLE OF CHEMERIN IN *HELICOBACTER PYLORI* INDUCED GASTRIC PATHOGENESIS

SUMMARY

Helicobacter pylori (H. pylori) is a gram negative, spiral –shaped, microaerophilic, flagellated bacterium which colonize in the human gastric epithelium and is classified as type I carcinogen for association with the development of gastric malignancies such as chronic gastritis, gastric adenocarcinoma, peptic ulcers and gastric lymphoma. Although 50% of the world's population is infected with *Helicobacter pylori,* the majority of infections do not lead to symptoms or gastrointestinal disease due to the pathogen's unique ability to avoid strong innate and adaptive immune responses. The bacteria are rarely eliminated, colonization usually persists throughout life, and infection involves both innate and adaptive immune responses.

Chemerin is a protein from cathelicidine/cystatin family and the major ligand of G protein coupled receptor CMKLR1 (chemokine like receptor 1). It is present in various tissues including epithelial cells, liver, spleen, lymph nodes etc. and promotes chemotaxis of leukocyte populations that express CMKLR1, such as macrophages, plasmacytoid dendritic cells and natural killer (NK) cells to site of inflammation. It is ubiquitously found in plasma as 143 amino acids prochemerin at nanomolar concentrations and becomes activated into various isoforms through proteolytic cleavage by a myriad of serine and cysteine proteases as well as carboxypeptidases. Chemerin promotes both pro- and anti-inflammatory immune response based on the stimuli provided and the disease being investigated. Inflammatory diseases, such as erythematosus, rheumatoid arthritis, psoriasis, Crohn's disease showed association of elevated chemerin in inflammation development which is also observed in several mice and human studies. CMKLR1 knockout mice showed reduced CNS inflammation in EAE and pulmonary inflammation in COPD (cigarette smoke- induced chronic obstructive pulmonary disease) model, suggesting a pro-inflammatory role of chemerin. However, anti-inflammatory response was also observed when chemerin was discarded and in CMKLR1 knockout mice in LPS-induced lung injury and acute viral pneumonia with reduced expression of pro-inflammatory markers.

Macrophages are plastic and heterogenic group of cells, which can polarize according to stimuli and the local microenvironment and shape the local inflammatory status to adapt to outside stimuli. Macrophages can be polarized into the classically activated -M1 type- macrophages and the alternatively activated -M2 type- macrophage subsets. M1 type macrophages are the pro-inflammatory effector cells in innate immune response produced by granulocyte macrophage stimulating factor (GM-CSF), lipopolysaccharide (LPS) and IFN-γ and secrete 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.

M2 type macrophages work in immunosuppression and tissue repair, produced by IL-4/IL-13 or M-CSF and secrete IL-10 and IL-1RA and low expression of IL-12/ IL-23. They do not produce NO. M2 macrophages can be further divided into subsets based on their cytokine expression profiles; M2a, M2b, and M2c.

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, alternatively activated M2 macrophages were detected in human gastric biopsy specimens from *H. pylori* positive individuals. 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. 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*. Macrophage mediated pro-inflammatory Th1 and Th17 response was observed as the pre-dominant adaptive response against *H. pylori* in humans.

Chemerin/chemerin receptor signaling mediates chemotaxis and adhesion of macrophages to extracellular matrix proteins and endothelial cells through increased promotion of integrins clustering (VLA-5 and VLA-4). ChemR23-dependent anti-inflammatory and protective effects of chemerin was found in mice, whereas in humans LPS, TNF-alpha and IFN-gamma stimulation increased ChemR23 expression in monocytes and macrophages which is associated with chronic and systemic inflammation. Macrophages may also exert pro- or anti-inflammatory response to different chemerin isoforms. Chemerin was observed to suppress M2 macrophage polarization with increased pro-inflammatory cytokine profile in DSS induced colitis model in vivo and in vitro. Another study found increased chemerin expression on M1 macrophages with distinct IL-10 expression, explaining the inflammatory effect of chemerin on M1 macrophages. Marked expression of chemerin was shown to be produced by intestinal epithelial cells (IECS) which is involved in the continuous recruitment of macrophage precursors to 10-24 week fetal intestine and boosted the host defence mechanism and mucosal immunity in neonates. However, only fetal IECs but not mature IECs, produces chemerin.

Many studies work on the effects of *H. pylori* on macrophages and gave valuable insights, there are studies that investigated the inter-relation between chemerin and macrophages as well. However, no studies have yet investigated the effect of chemerin on the polarization status of macrophages in *H. pylori* induced pathogenesis. It is known from the literature that *H. pylori* could potentially induce both M1 and M2 macrophages in human but how/whether chemerin could effect this inter-relation is not known. Therefore, in this study, we investigated the effect of both chemerin and *H. pylori* on the monocytic cell line THP-1 and observe the differences on its polarization status according to cytokine profiles.

It was known from the literature that epithelial cells secrete chemerin. In order to observe the effect of chemerin on gastric immunopathology, it's expression was first investigated on three gastric epithelial cell lines; KATO III, AGS and MKN45. Conventional PCR results revealed expression of chemerin in different levels by these cell lines. Next we evaluated the effect of *H. pylori* on chemerin expression by these cell lines. In order to do that, KATO III. AGS and MKN45 cells were treated with two *H. pylori* sonicates, the wild type G27 strain sonicate and its ΔcagA mutant

sonicate. The *H. pylori* virulence factor Cag A (cytotoxin- associated gene-A) was chosen since its association with development of gastric malignancies have been reported. Chemerin expression was signifantly increased in KATO III and AGS cell line when they were treated with wild type G27 and ΔcagA mutant sonicate for 6 and 24 hours. After 24 hours, the expression level of chemerin reduced. No significant expression was observed on MKN45 cell line. Therefore, the following experiments were performed on KATO III and AGS cell lines. Next, we assess if chemerin protein expression was also increased along with the mRNA expression upon *H. pylori* treatment. Repeated attempts of western blot optimization experiments failed to show any expected band of chemerin in any treated or untreated groups of any cell line. To clarify if the lack of detection is real or due to inefficient antibody binding to chemerin, human chemerin was cloned and transfected into HEK293T cell line. Western blot was performed using various samples from different cell lines with different treatments and chemerin-expressing HEK293T cells. Chemerin was only detected in the whole cell extract of chemerin-transfected HEK293T cell lines but not in other samples. As a conclusion, we could detect elavated chemerin in mRNA levels upon *H. pylori* treatment but not in protein level.

Next, we investigated expression of chemerin in gastric biopsy specimens of *H. pylori* positive patients of acute gastritis and chronic ulcer and compared it to uninfected patients without pathology. Real-time PCR experiments revealed presence of significantly higher levels of chemerin in ulcer patients. Of note, chemerin mRNA expression in samples from patients with gastritis was not significantly different when compared to control group. These data was supported by the results obtained from immunohistochemistry analysis performed on gastric tissues of *H. pylori* postive patients of acute gastritis, chronic ulcer and *H. pylori* negative healthy individiuals. Chemerin was shown to be highly expressed in gastric tissues of ulcer patients.

We investigated effects of chemerin on the polarization status of macrophages. For that, a monocytic cell line THP-1 was used and PMA (phorbol 12-myristate 13-acetate) was administered to induce differentiation of monocytes into macrophages. The differentiated macrophages were then exposed to different stimuli such as LPS and recombinant IL-4 to polarize them into M1 and M2 subtypes respectively, with or without the presence of chemerin. The presence of macrophage specific markers were used to show the differentiation of monocytes into macrophages. Also, cytokine levels were measured by conventional PCR. Two pro-inflammatory cytokines namely IL-1β and IL-6 were used as a marker for M1 macrophages. Both of them were significantly increased in LPS- treated samples. When chemerin was administered with LPS, a significantly high expression of IL-1β and low expression of IL-6 observed when compared to LPS- treated sample only in the first set. Initially it was thought chemerin might have a role in the polarization of M1 macrophages but three repeated experiments showed no difference in the expression of IL-1β and IL6 in samples treated with LPS alone or with chemerin. This disproves the possible effect of chemerin on M1 polarization. IL-10 expression was assessed to detect M2 polarization. IL-4 treatment resulted in elevated IL-10 expression compared to control group and treatment of chemerin along with IL-4 diminished this expression. This is parallel with the study performed by Lin et al. (2014) where chemerin reduced the expression of M2 associated genes.

Finally, we investigated the effect of *H. pylori* on the polarization of macrophages with or without the presence of chemerin to check whether chemerin may alter the

effect exerted by *H. pylori* on macrophages. The THP-1 macrophages were treated with wild type G27 and ΔcagA mutant sonicate with/without chemerin for 24 hours. The sonicate treatment polarized macrophages to M1 subtype with high profiles of pro-inflammatory IL-1β and IL6 and also increased IL-10. M1 macrophages are known to produce IL-10 in response to bacterial stimulation, this was also observed in a study on mice model stimulated by *H. felis* in our laboratory (unpublished data). However, no change of expression in IL-1β, IL6 and IL-10 was observed when chemerin was added with *H. pylori* sonicates. Therefore our results suggested chemerin may not have a role on effect of *H. pylori* induced M1 macrophage polarization, however M2 polarization was seen to be inhibited upon chemerin treatment which is *H. pylori* independent.

HELİCOBACTER PYLORİ İLE İNDÜKLENEN GASTRİK PATOGENEZDE KEMERİNİN ROLÜ

ÖZET

Helicobacter pylori (*H. pylori*), insan gastik epitelinde kolonize olan, gram negatif, spiral-şekilli, mikroaerofilik, kamçılı bir bakteridir. Bu bakteri, kronik gastrit, gastrik adenokarsinom, peptik ülserler ve gastrik lenfomalar gibi gastrik hastalıkların gelişimiyle olan bağlantısından dolayı Tip I karsinojen olarak sınıflandırılmaktadır. Dünya nüfusunun %50'si *H. pylori* ile enfekte olduğu halde, enfeksiyonların büyük bir çoğunluğu, patojenin güçlü doğal ve edinsel bağışıklık cevaplarından kaçınabilme yeteneğinden ötürü, semptomlara veya gastrointestinal hastalıklara yol açmamaktadır. Bakteriler nadiren tamamen yok edilir, kolonizasyon genel olarak ömür boyu sürer, ve enfeksiyon hem doğal, hem de edinsel bağışıklı cevaplarını içerir.

Kemerin (chemerin), katelisidin/sistatin ailesinden bir proteindir ve G proteinine bağlı reseptör CMKLR1 (kemokin benzeri reseptör 1)'in ana ligandıdır. Epitel hücreler, karaciğer, dalak, lenf düğümleri vb. dahil birçok dokuda mevcuttur. Bu şekilde, makrofajlar, plazmositoid dendritik hücreler ve NK hücreleri gibi CMKLR1 ifade eden lökosit popülasyonlarının enflamasyon bölgesine kemotaksisini başlatır. Plazmada her yerde, sürekli, nanomolar konsantrasyonlarda 143 aa prokemerin olarak bulunan protein, serin ve sistein proteazlarının yanı sıra karboksipeptidazlar tarafından proteolitik kesime uğrayarak, farklı formlar halinde aktive olur. Kemerin, ortamdaki uyarıcıya ve incelenen hastalığa göre, hem pro-enflamatuvar, hem de anti-enflamatuvar cevaplara sebep olabilmektedir. Eritematöz, romatoid artrit, psöriyazis (sedef hastalığı), Crohn hastalığı gibi enflamatuvar hastalıklarda, birçok kobay ve insan çalışmalarında da gözlemlendiği gibi, enflamasyon gelişiminde artan kemerin seviyelerinin hastalıklarla ilişkisi gösterilmiştir. CMKLR1 nakavt farelerde, EAE'de merkezi sinir sistemi (MSS) enflamasyonunun ve sigara içmeye bağlı kronik obstrüktif akciğer hastalığı (KOAH) modelinde pulmoner enflamasyonun azaldığı gösterilmiştir. Bu şekilde, kemerinin pro-enflamatuvar rolü ortaya konmuştur. Fakat, LPS ile akciğer hasarlı modelde ve pro-enflamatuvar markerlerin ifadesi azaltılmış akut viral pönomi modelinde, CMKLR1 nakavt farelerde ve kemerinin baskılandığı durumlarda, kemerinin anti-enflamatuvar rolü de gözlemlenmiştir.

Makrofajlar, dışarıdan gelen uyarılara adapte olmak için yerel enflamatuvar durumlarını şekillendirebilen, uyarana ve yerel mikroçevreye göre polarize olabilen, plastik ve heterojenik bir hücre grubudur. Makrofajlar, klasik yollardan aktive olan-M1 tipi-makrofajlara ve alternatif yollardan aktive olan –M2 tipi-makrofaj alt gruplarına polarize olabilmektedir. M1 tipi makrofajlar, granülosit makrofaj uyarıcı faktör (GM-CSF), lipopolisakkarit (LPS) ve IFN-γ tarafından üretilen doğal bağışıklık cevapta görülen, pro-enflamatuvar efektör hücrelerdir ve TNF-α, IL-1β, IL-6, IL-12/ IL-23 gibi pro-enflamatuvar sitokinler salgılarlar. M1 tipi makrofajların antimikrobiyal fonksiyonları, L-argininden nitrik oksit ve çok miktarda NO üreten, indüklenebilir niktik oksit sentazın (iNOS) ifadesinin artmasına bağlıdır. M2 tipi

makrofajlar ise, IL-4/IL-13 veya M-CSF ile üretilen, immün baskılama ve doku tamirinde görev alan, IL-10 ve IL-1RA salgılayıp, düşük seviyelerde IL-12/ IL-23 ifade eden hücre gruplarıdır. Bu hücreler, NO üretmezler. M2 tipi makrofajlar, sitokin ifade profillerine göre kendi içlerinde M2a, M2b, ve M2c olmak üzere alt gruplara ayrılırlar.

H. pylori'nin makrofajlardaki etkileri ile ilgili birkaç çalışma mevcuttur. İlk çalışmalarda, *H. pylori*'nin makrofajlarda IL-6, IL-8, TNF-α, IL-1-β gibi pro-enflamatuvar sitokinlerle birlikte indüklenebilir NO sentetaz (iNOS) enziminin ekspresyonunu indüklediği gösterilmiştir. *H. pylori* pozitif bireylerden alınan gastrik biyopsi örneklerinde alternatif olarak aktive olan M2 makrofajlar gözlenmiştir. Ayrıca, *H. pylori* enfeksiyonu durumunda insan monositleri, IL-1β, IL-6, IL-10 ve IL-12p40 (kısmen IL-23 olarak salgılanan) salgılarken IL-12p70 sitokini salgılamamıştır. Bununla beraber, doğal immün sistem hücreleri tarafından salgılanan sitokinler, makrofajların ve dendritik hücrelerin antijen sunması ve mikroçevredeki değişimler, *H. pylori'*ye karşı sonradan kazanılmış immün cevabı aktive eder. İnsanlarda *H. pylori'*ye karşı makrofaj aracılı pro-enflamatuvar Th1 ve Th17 immün cevap hakimdir.

Kemerin/kemerin reseptör sinyalleşmesi, artan integrin kümelenmesi yoluyla (VLA-5 ve VLA-4) kemotaksise ve makrofajların hücre dışı matriks proteinlerine ve endotelyal hücrelere adhezyonuna aracılık eder. Farelerde kemerinin ChemR23-bağımlı anti-enflamatuvar ve koruyucu etkileri gözlenmiştir. İnsanlarda ise LPS, TNF-a and IFN-g uyarımı, kronik ve sistemik enflamasyonla ilişkili olan monositlerde ve makrofajlarda ChemR23 ekspresyonunu artırmıştır. Ayrıca, makrofajlar farklı kemerin izoformlarına cevap olarak pro- ve anti-enflamatuvar yanıt oluşturabilir. Kemerinin DSS ile indüklenen kolit modelinde *in vivo* and *in vitro* koşullarda artan pro-enflamatuvar sitokin profilinin yardımıyla M2 makrofaj polarizasyonunu baskıladığı gözlenmiştir. Bir başka çalışmada M1 makrofajlarda belirgin IL-10 ekspresyonu ile birlikte artan kemerin ekspresyonun gözlenmesi kemerinin M1 makrofajlardaki enflamatuvar etkisini açıklamaktadır. Belirgin kemerin ekspresyonunun, makrofaj öncüllerinin 10-24 haftalık fetal bağırsakta sürekli olarak toplanmasında görev alan ve yeni doğanlarda konak savunma mekanizmasını ve mukozal bağışıklığı artıran intestinal epitel hücreler (IECler) tarafından gerçekleştirildiği gösterilmiştir. Fakat, olgun IEC'lerin aksine sadece fetal IEC'ler kemerin üretir.

Makrofajlarda *H. pylori*'nin etkilerini araştıran birçok çalışma değerli görüşler ortaya koymuştur. Ayrıca, kemerin ve makrofajlar arasındaki ilişkiyi araştıran çalışmalar da mevcuttur. Fakat, *H. pylori* ile indüklenen patogenezde kemerinin makrofaj polarizasyonuna etkisini araştıran çalışmalar henüz yoktur. *H. pylori*'nin insanda potansiyel olarak M1 ve M2 tipi makrofajları indükleyebildiği bilinirken; kemerinin bu ilişkiyi nasıl etkileyebildiği bilinmemektedir. Dolayısıyla bu çalışmada, kemerin ve *H. pylori*'nin monositik hücre hattı olan THP-1 hücreleri üzerindeki etki ve bu hücrelerin sitokin profillerine göre polarizasyon durumları arasındaki farklılıklar araştırılmıştır.

Literatürden epiteliyal hücrelerin kemerin salgıladıkları bilinmektedir. Kemerinin gastrik patolojideki etkisini gözlemlemek için, ilk olarak 3 gastrik epiteliyal hücre hattında (KATO III, AGS ve MKN45) kemerin ekspresyonu araştırılmıştır. Klasik PCR sonuçları kemerinin bu hücreler tarafından eksprese edildiğini göstermiştir. Daha sonra, *H. pylori*'nin bu hücre hatlarında kemerin ekspresyonuna etkisi

incelenmiştir. Bunu gerçekleştirmek için, KATO III, AGS ve MKN45 hücreleri yabani tip G27 suş sonikatı ve bu suşun ΔcagA mutant sonikatı ile olmak üzere 2 ayrı *H. pylori* sonikatı ile muamele edilmiştir. *H. pylori* virulans faktörü CagA'nın (sitotoksin-ilişkili gen-A) gastrik kötü huylu tümörlerle ilişkisi gösterildiğinden, bu çalışma için CagA virulans faktörü seçilmiştir. Kemerin ekspresyonu, KATO III and AGS hücre hatlarında yabani tip G27 ve ΔcagA mutant sonikatları ile 6 ve 24 saat muamele edildiğinde önemli ölçüde artmıştır. Bundan sonra ise kemerin ifadesi azalmıştır. Ancak MKN45 hücre hattında anlamlı bir ifade gözlenmemiştir. Bu nedenle diğer deneylere KATO III and AGS hücre hatları ile devam edilmiştir. Daha sonra, *H. pylori* muamelesi ile mRNA ekspresyonunun yanı sıra protein ifadesinin de artıp artmadığı kontrol edilmek istenmiştir. Tekrarlı western blot optimizasyon deneyleri sonucunda, herhangi bir hücre hattının muamele edilen ve edilmeyen gruplarında kemerin bandı görülmemiştir. Bu sonuçların nedeninin protenin olmaması veya antikorun proteine yetersiz bağlanması olduğunun kararlaştırılması amacıyla, insan kemerini klonlanmış ve HEK293T hücre hattına transfekte edilmiştir. Tüm örnekler kullanılarak western blot gerçekleştirilmiştir ve kemerin sadece insan kemerin genini içeren plasmid ile transfekte edilen HEK293T nin tüm hücre ekstraktında tespit edilmiştir. Sonuç olarak, *H. pylori* muamelesinden sonra kemerin artışı mRNA seviyesinde tespit edilirken, protein seviyesinde görülememiştir. Daha sonra, *H. pylori* pozitif akut gastrit ve kronik ülser hastalarında da kemerin ekspresyonunu incelenmiştir ve patoloji göstermeyen enfekte olmamış hastalarla karşılaştırılmıştır. Real-time PCR deneyler, ülser hastalarında kemerinin anlamlı ölçüde fazla bulunduğunu göstermiştir. Gastrit hastalarının numunelerinde, kemerin mRNA ifadesinin, kontrol grubuyla anlamlı farklılıklar göstermediğine dikkat edilmelidir. Bu veriler, akut gastrit ve kronik ülserli *H. pylori* pozitif hastaların ve *H. pylori* negatif sağlıklı bireylerin gastrik dokuları üzerinde yürütülen immünohistokimya analizlerinden elde edilen sonuçlar tarafından desteklenmektedir.

Daha sonra, kemerinin makrofajların polarizasyon durumu üzerindeki etkilerini incelenmiştir. Bu amaçla, monositik bir hücre hattı olan THP-1 kullanılmış ve monositlerin makrofajlara farklılaşmasını indüklemek için PMA (phorbol 12-myristate 13-acetate) ile muamele edilmiştir. Farklılaşan makrofajların M1 and M2 alt tiplerine polarize olması için kemerin varlığında ve yokluğunda sırasıyla LPS ve rekombinant IL-4 gibi farklı uyaranlar eklenmiştir. Monositlerin makrofajlara farklılaştığını göstermek için makrofajlara özgü belirteçlerin varlığına bakılmıştır. Aynı zamanda, klasik PZR ile sitokin seviyeleri ölçülmüştür. IL-1β ve IL-6 pro-enflamatuvar sitokinleri M1 tipi makrofajların belirteci olarak kullanılmıştır. Her ikisinin de LPS ile muamele edilen örneklerde anlamlı ölçüde arttığı gözlemlenmiştir. Kemerin, LPS ile uygulandığı zaman, yalnızca ilk setteki LPS ile muamele edilen örnekler ile karşılaştırıldığında IL-1β'nın anlamlı ölçüde yüksek ekspresyonu ve IL-6'nın düşük ekspresyonu gözlenmiştir. Başlangıçta, kemerinin M1 tipi makrofajlara polarizasyonunda bir etkisinin olabileceği düşünülmüştür, ancak tekrarlanan üç deney sadece LPS ile veya kemerin ile muamele edilen örneklerde IL-1β ve IL6 ekspresyonunda bir değişim olmadığını göstermiştir. Bu da, kemerinin M1 tipi hücrelere polarizasyona bir etkisinin olma olasılığını ortadan kaldırmıştır. M2 polarizasyonunu tespit etmek için IL-10 ekspresyonu değerlendirilmiştir. Kontrol grubu ile karşılaştırıldığında, IL-4 muamele edilen gruplarda IL-10'un ekspresyonu artış göstermiştir ve kemerinin IL-4 ile birlikte uygulanması bu etkiyi ortadan kaldırmıştır. Bu da, Lin vd. (2014) tarafından yapılan kemerinin M2 ile ilişkili genlerin ekspresyonunu azalttığı çalışmayı desteklemektedir.

Sonuç olarak, yapılan çalışmada, *H. pylori*'nin makrofajlar üzerindeki etkisini değiştirip değiştirmediğini kontrol etmek için, kemerin varlığında ve yokluğunda, *H. pylori*'nin makrofaj polarizasyonu üzerideki etkisi incelenmiştir. THP-1 makrofajları kemerinin varlığı ve yokluğunda yabani tip G27 ve ΔcagA mutant sonikatları ile 24 saat muamele edilmiştir. Sonikat muamelesi makrofajları yüksek IL-1β ve IL6 üreten, pro-enflamatuvar profilindeki M1 alt tipine polarize ederken IL-10'u da arttırmıştır. M1 makrofajlarının bakteriyal uyarımına cevap olarak IL-10 ürettiği bilinmektedir, bu durum ayrıca laboratuvarımızdaki diğer bir çalışmada *H. felis* ile uyarılan bir fare modelinde de gözlenmiştir (yayımlanmamış veri). Ancak, *H. pylori* sonikatlar ile birlikte kemerin muamele edildiğinde IL-1β, IL6 ve IL-10 ekspresyonunda hiçbir değişiklik gözlenmemiştir. Bu nedenle, sonuçlarımız, *H. pylori* ile indüklenen M1 makrofaj polarizasyonunda kemerinin bir rolü olmayabileceğini önermektedir, ancak M2 polarizasyonunun kemerin muamelesi ile *H. pylori*'den bağımsız şekilde engellendiği gözlemlenmiştir.

1. INTRODUCTION

1.1 *Helicobacter Pylori*

Helicobacter pylori, previously known as *Campylobacter pylori*, is a gram-negative, microaerophilic, slow-growing, spiral-shaped and flagellated bacterium usually reside in the gastric epithelium (Figure 1.1). In 1983, two Australian scientists named Barry Marshall and Robin Warren first discovered this human pathogen in gastric biopsies of a patient with chronic gastritis and gastric ulcers, conditions not previously believed to have a microbial cause [1].

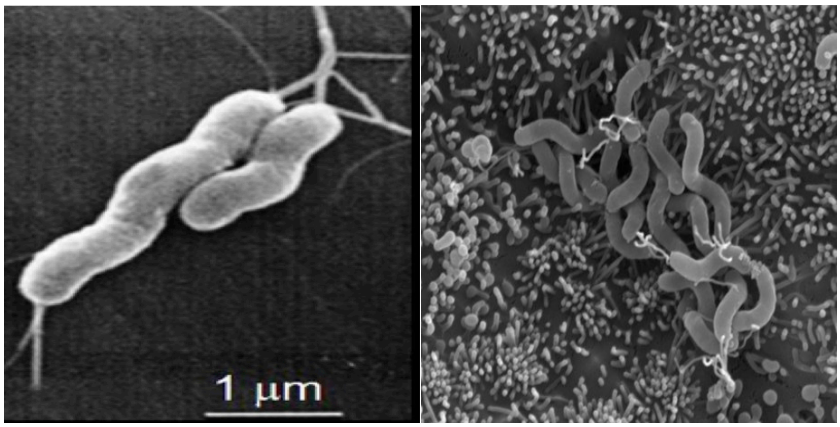

Figure 1.1 : Scanning electron microscopy of *Helicobacter pylori* (from Yoshiyama & Nakazawa, 2000).

Approximately 50% of the world's population is infected with *Helicobacter pylori,* rendering it the most prevalent chronic bacterial infection of humans. It is typically transmitted via oral-oral, faecal-oral or gastro-oral exposures [2]. This pathogen can persevere for years in the gastric mucosa irrespective of prompting strong innate and adaptive immune responses via characteristic urease production, motility, and adherence to gastric epithelium. All these features enable it to neutralize gastric acid, penetrate through the mucus layer to the gastric epithelium, and colonize.

H. pylori infection causes diseases of the upper gastrointestinal tract such as chronic gastritis, poses a major threat for the development of peptic ulcer disease (PUD) and gastric malignancies such as gastric marginal zone/mucosa-associated lymphoid

tissue (MALT) and gastric adenocarcinoma (Figure 1.2) [3]. In addition to its association with cancer, this organism has also been recently found to be involved with extraintestinal diseases, such as immune thrombocytopenic purpura, refractory iron deficiency anaemia and vitamin B12 deficiency [4]. While infection with *H. pylori* perseveres without treatment, the majority of infections do not lead to symptoms or gastrointestinal disease.

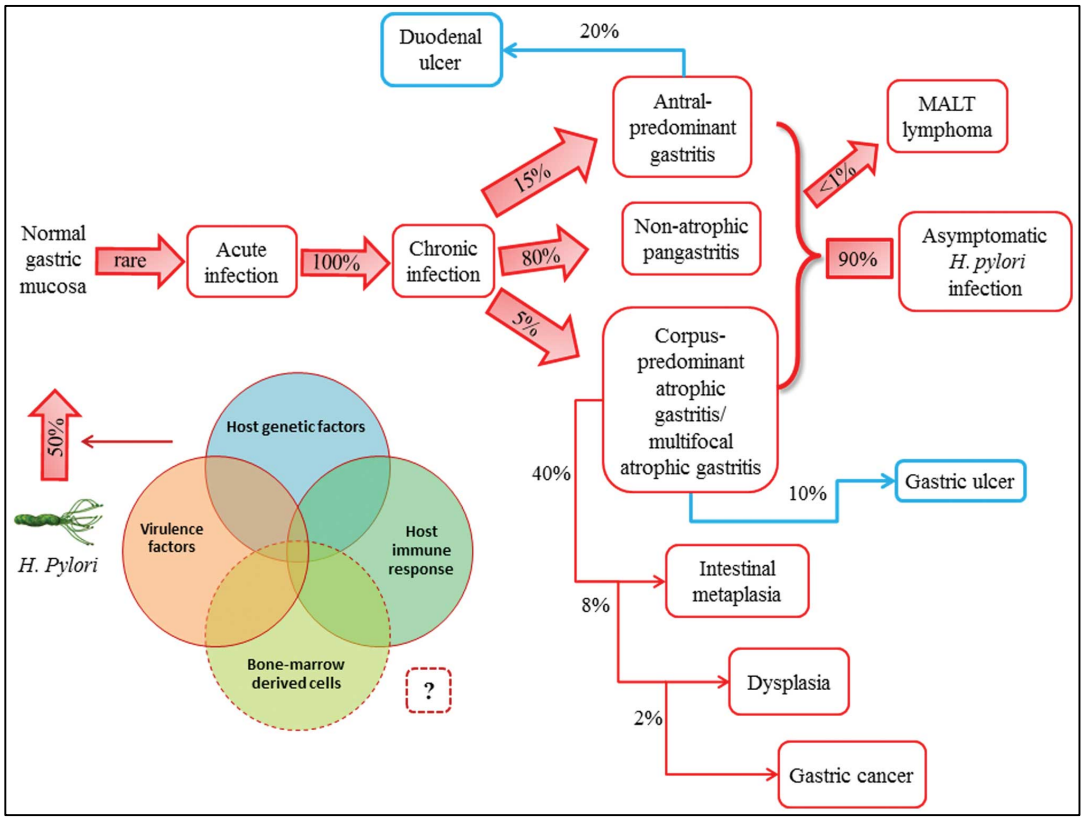

Figure 1.2 : Progression of *H. pylori* infection towards carcinoma (Adapted from Vincenza et al., 2013).

The growing rate of antibiotic resistance and the declining rates of antimicrobial eradication pose an alarmingly critical situation worldwide, from 80% in the early 1990s to as low as 60% in some countries at present [5]. There is a yearly occurrence of 0.3- 0.7% and 6-14% *H. pylori* infection estimated in developed and developing countries respectively [6]. A cohort study in 207 *H. pylori*-negative individuals recognized four new infections (2.5%), comparing to an annually 0.25% (0.10-0.63) prevalence rate [7].

1.1.1 Genome

The two independent *H. pylori* genomes that have been completely sequenced have distinct origins, *H. pylori* 26695 isolated from a gastritis patient and *H. pylori* J99 isolated from a patient suffering from a duodenal ulcer and duodenitis [8]. The *H. pylori* 26695 strain is a circular chromosome with 1,667,867 base pairs (bp) that has 39% average G+C content. Within the genome different G+C composition is shown in five regions (Table 1.1) . Two of these regions encompass genes involved in DNA processing. One region has 2 orthologues of the *virB4/ptl* gene, which is required for the transfer of oncogenic T-DNA of *Agrobacterium* and the secretion of the *pertussis* toxin by *Bordetella pertussis* [9] and therefore crucial. Another region is known as *cag* pathogenicity island (PAI), which is flanked by 31-bp direct repeats, and appears to be the product of lateral transfer [10].

Table 1.1 : G + C Regions present in *H. pylori* genome with associated genes (Adapted from Jean-F. & Owen et al., 1997).

Distinct G+C Regions	Associated Genes
Region 1 (33% G+C) 452-479 kb	IS605, 5SRNA and repeat 7; virB4
Region 2 (35% G+C) 539-579 kb	cagPAI
Region 3 (33% G+C) 1,049-1,071 kb	IS605, 5SRNA and repeat 7
Region 4 (33% G+C) 1,264-1,276 kb	β and β' RNA Polymerase, EF-G (fusA)
Region 5 (33% G+C) 1,590-1,602 kb	Two restriction/modification systems

1.1.2 Virulence factors of *Helicobacter pylori*

The major virulence factors of *H. pylori* are the cytotoxin-associated gene (*cag)* pathogenicity island (PAI)-encoded virulence factors, such as the cytotoxin-associated antigen (CagA) protein, the vacuolating toxin-A (VacA), the blood group antigen-binding adhesin (BabA) and the outer inflammatory protein (OipA). This would be further discussed into the next section.

Adhesion molecules are necessary for the adherence of bacteria to host cells as well as for the colonization in human stomac. *H. pylori* adherence molecules belong to Hop family and are the members of outer membrane protein (OMP) family. Hop family includes BabA (blood group antigen binding adhesion), OipA (the outer inflammatory protein A), SabA & B (sialic acid-binding adhesion), HopZ and AlpA/B (Figure 1.3) [11]. Apart from *H. pylori* virulence factors and adhesion molecules, flagella, LPS (lipopolysaccharides), peptidoglycan (PGN), HP-NAP,

IceA, DupA, superoxide dismutase, catalase, and protease enzymes may play a role in progression and severity of diseases.

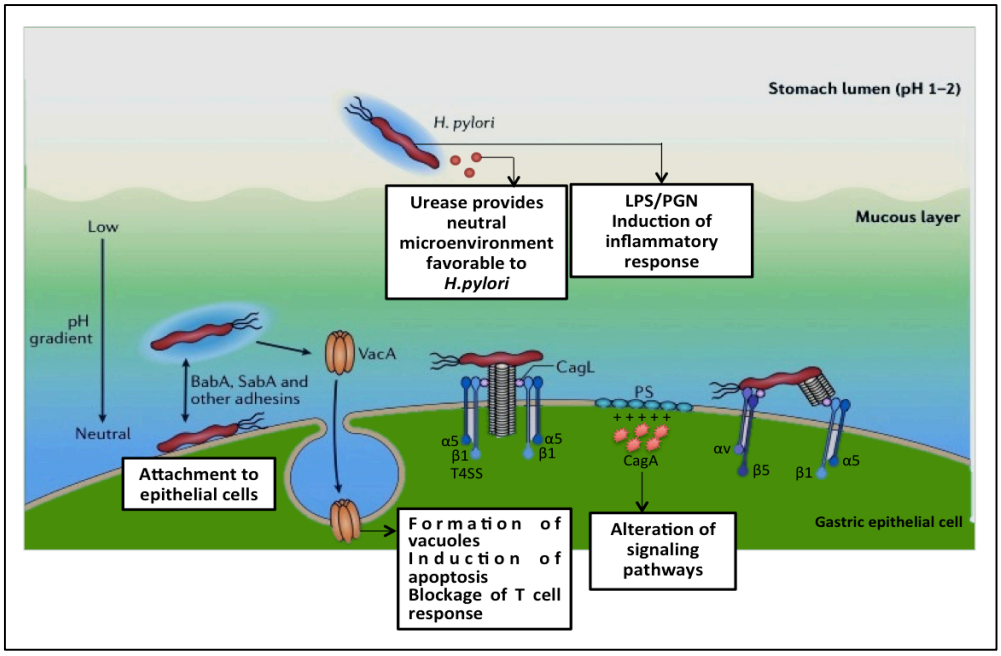

Figure 1.3 : Virulence factors of *H. pylori* and their functions (adapted from Nina et al., 2013).

1.1.2.1 The Helicobacter pylori cagPAI

The cag Pathogenicity Island (cagPAI) was first identified in 1996. cagPAI is a 40 kb DNA insertion element and includes approximately 31 genes. cagPAI is present in 60-70% of Western *H. pylori* strains and approximately 100% of East –Asian *H. pylori* strains [12]. This island encodes a type IV secretion system (T4SS) which is required for bacterial conjugation and translocation of bacterial effector proteins into the host gastric epithelial cells [13,14]. *Helicobacter pylori* uses T4SS injection apparatus for translocation of Cag A protein and peptidoglycans (PGNs) into the host cell [15]. CagPAI contains several virulence factors such as Cag A, Cag E, Cag L, Cag Y, and Cag I [16].

Cag E helps in translocation and phosphorylation of Cag A protein and mediates the production of proinflammatory cytokines such as IL-8 (Interleukin-8) (neutrophil-activating chemokine) through NF-κB (Nuclear Factor -kappa B) pathway [17]. Cag L, located at the surface of the pilus, has a specific Arginine-Glycine-Aspartate (RGD) motif which is recognized by the integrin receptor on the target epithelial cell. CagL helps translocation of Cag A protein into the cytoplasm of host cell [18]. Also,

CagL activates host Src and host focal adhesion kinase (FAK) for phosphorylation of Cag A protein. Cag Y virulence factor is found at the pilus and both Cag Y and Cag I are required for Cag A translocation.

1.1.2.2 Secreted toxins of Helicobacter pylori

H. pylori strains secret several toxins to perform various functions necessary for prolonged persistence. VacA (Vacuolating Cytotoxin A), a pore-forming toxin, is carried by all *H. pylori* strains that interrupts cell polarity, stimulates apoptosis of epithelial cells and prevents T cell proliferation and effector functions. Sequence variation in several domains of VacA results in varying levels of expression, cell type-specific toxicity as well as disease severity [19].

CagA, another important toxin of *H. pylori*, has been regarded as a bacterial oncoprotein for its association with an increased risk of cancer and inducing gastric carcinoma and other malignancies in transgenic mice. This 120-140 kDa protein was initially isolated as an immunodominant antigen from patients infected with highly virulent *vacA* alleles [41]. CagA translocates into host cells with the aid of Cag T4SS which is encoded on the *cag* pathogenicity island (PAI) [1,20] (Figure 1.3).

CagA oncoprotein has repeated sequences in its 3' region consisting of Glutamine-Proline- Isoleucine- Tyrosine- Alanine (EPIYA) motifs and these motifs include a tyrosine phosphorylation site. After translocation into host cells, CagA can be tyrosine phosphorylated and interacts with SHP2 tyrosine phosphatase and CSK kinase. Unphosphorylated CagA is known to interact with CRK adaptor, MET, growth factor receptor-bound protein 5 (GRB5), PAR1 (also known as MARK) and E-cadherin (Figure 1.4). These interactions lead to altered cell signalling and changes in cell polarity, extrusion, motility, proliferation and pro-inflammatory cytokine secretion [1,20].

1.1.3 *Helicobacter pylori* colonization in gastric mucosa

Unlike any other pathogen, *H. pylori* has a distinct ability to inhabit in an extremely acidic environment. After entering into gastric milieu by oral ingestion, it is promptly exposed to the extreme pH in the lumen side of the gastric mucous layer (pH ≅2). The survival of *H. pylori* in such adverse environment depends on the following mechanism.

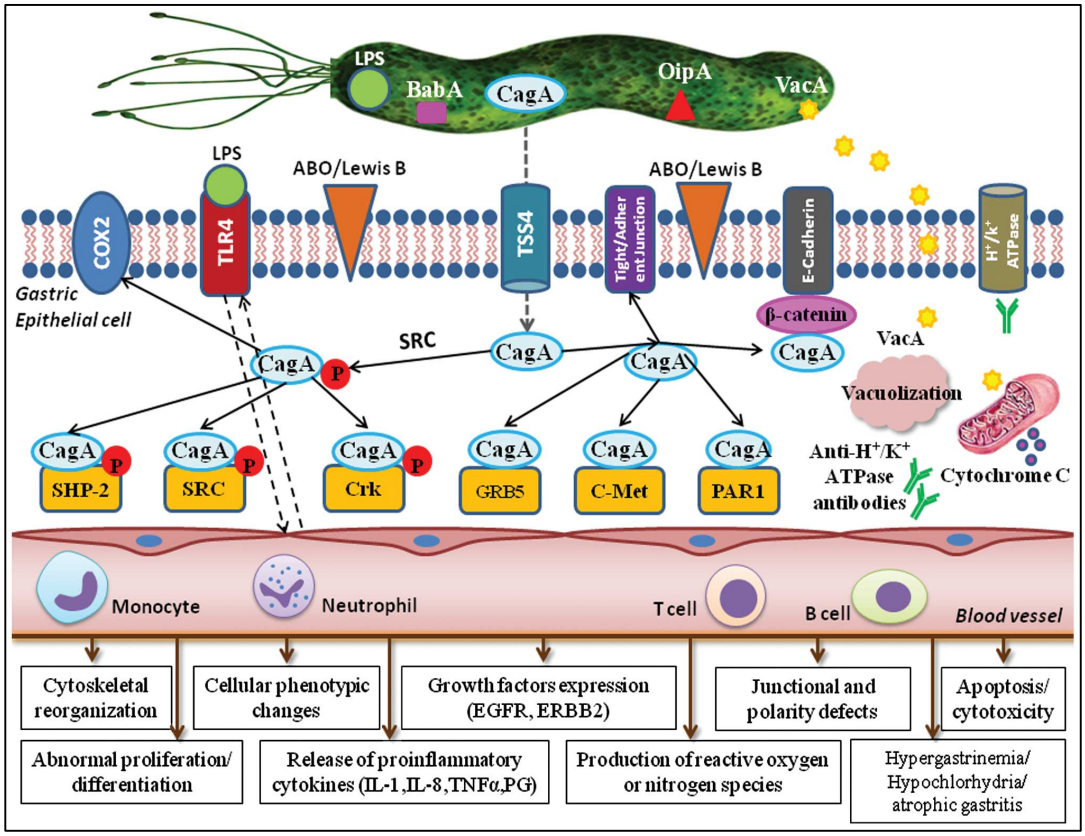

Figure 1.4 : Interaction of CagA and VacA virulence factors with gastric epithelial cells and the immune system, resulting in an inflammatory response and mucosal damage (Adapted from Vincenza et al., 2013).

1.1.3.1 Escape from the acidic lumen

In order to survive *H. pylori* must escape from the acidic stomach lumen and migrate to its usual habitation in the gastric epithelium. This is achieved by bacterial urease that aids in acid resistance through the localized production of ammonium ions. Gastric mucins form a gel at low ph to trap microbes efficiently in the lumen. These ammonium ions aid in flagellar motility via raising the pH to near neutral and the mucous gel transitions to a viscoelastic solution through which *H. pylori* can swim [21,22].

Other than assisting in clearance, *H. pylori* infection associates with less inflammation, less $CD4^+$ T cells recruitment and a lack of T helper 17 (T_H17) response [23] hence supporting stable infection while at the same time aggravating more inflammation. Based on the observation that increased inflammation often associates with lower bacterial loads [24], it is suggested that *H. pylori* must actively maintain its interaction with the host epithelium to avoid clearance and survive.

1.1.3.2 Persistent colonization of the gastric mucosa

Detoxification of reactive oxygen species (ROS) is a crucial step for the survival of *H. pylori*. *H. pylori* derived catalase, superoxide dismutase and arginase control nitric oxide produced by nitric oxide synthase from macrophages, neutrophils and epithelial cells [25]. Furthermore, DNA repair pathways have also shown to be able to support bacterial colonization efficiently even when the neighboring host tissue accrues DNA lesions [26].

Additionally, *H. pylori* strains constitutively express DNA repair proteins such as RecA. After DNA damage, *H. pylori* upregulates natural competence instead, promoting chronic perseverance possibly via enhanced genetic diversification [27].

The *H. pylori* genome encompasses numerous intragenic and extragenic repeat sequence. For example, cell surface protein CagY, which promotes type IV secretion system (T4SS)-mediated translocation of the effector CagA, can undergo recombination between internal repeat motifs that generally preserve the reading frame [28].

1.1.4 Evasion Of innate immune recognition

The key to the success of the bacterium for prolonged inhabitation in host gastric mucosa lies in the development of intricate tactics to avoid and undermine host immune defense mechanism. The first defense barrier against *H. pylori* is the mucus produced by the epithelial cells lining the gastric mucosa and the innate immune cells that either dwell in the gastric lamina propria under steady state conditions or are recruited there during infection. Epithelial cells as well as innate immune cells detect conserved pathogen-derived molecular structures or PAMPs (pathogen-associated molecular patterns) on pathogent via pattern recognition receptors (PRRs). *H. pylori* evades recognition by several types of PRR critical for the identification of other Gram-negative enteropathogens.

1.1.4.1 *H. pylori* mediated evasion and manipulation of Toll-like receptors (TLRs) recognition

Toll-like receptors (TLRs) are single, membrane-spanning, non-catalytic receptors usually expressed by macrophages and dendritic cells that recognize PAMPs from microbes.

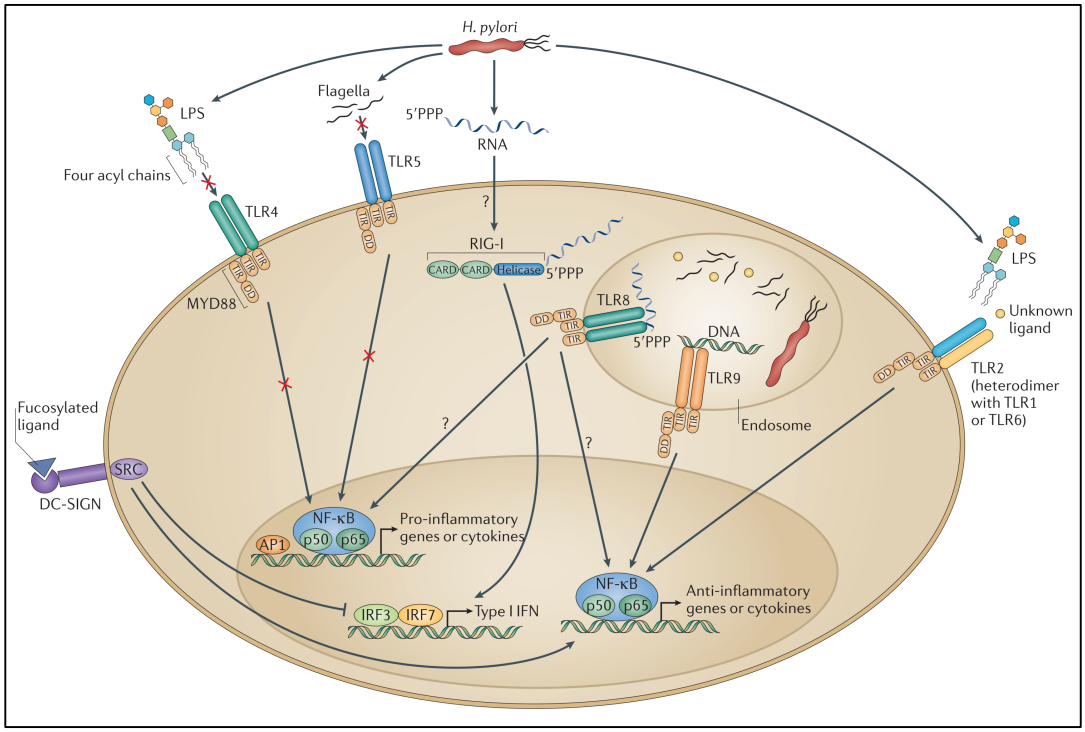

Figure 1.5 : *H. pylori* mediated evasion from Innate immune response (Adapted from Nina et al.; 2013).

H. pylori mainly escapes from recognition by TLR4, which recognizes Lipopolysaccharides or LPS found in outer membrane of Gram- negative bacteria (Figure 1.5). *H. pylori* produces 1,000-fold less active LPS than hexa-acylated E. coli LPS via removal of phosphate groups from the backbone of lipid A. The outcome is tetra-acylated LPS with less negative charge that resists antimicrobial peptides attachment (such as polymyxin B) and avoids detection by TLRs [29]. They frequently express LPS O antigens structurally similar to human Lewis blood group antigens (Monteiro et al., 1998; Aspinall & Monteiro, 1996). Such resemblance to "self" antigens enables them in molecular mimicry or immune tolerance and protection from immune recognition [30].

It also escapes recognition by TLR5 through the modifications in the N-terminal TLR5 recognition domain of Flagellin [31]. *H. pylori* infection activates endosomal TLR9 in an anti-inflammatory fashion has been observed in a mouse model (Figure 1.5) [32].

This has been supported in a study conducted by Sayi et al. (2011) on Tlr2 knock out mice, when infected with *H. felis* (pathogen of domestic animals) showed stronger T cell responses and T cell-driven immune response with better infection control. The

effects of TLR2 gene deletion are phenocopied by Myd88$^{-/-}$ mice, indicating that the absence of anti- inflammatory signals induced by *Helicobacter* spp. is phenotypically dominant over the simultaneous lack of MYD88-dependent pro-inflammatory signals that are induced by other TLRs [33].

1.1.4.2 Suppression of CLR-mediated signalling

H. pylori infection also mediates an anti-inflammatory response by suppressing DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), or CD209, a C-type lectin receptors (Figure 1.5). The fucose residues present on the ligand for DC-SIGN efficiently remove a signaling complex from DC-SIGN [34]. The signaling complex consists of scaffold proteins LSP1, KSR1 and CNK and the kinase RAF1, causing DC-SIGN- and RAF1-mediated activation of NF-κB, which prolongs and increases IL-10 transcription [35]

Briefly, *H. pylori* suppresses strong pro-inflammatory response and escapes from consequential adaptive immunity and clearance, via two main mechanisms: by avoiding pro-inflammatory TLRs driven innate immune detection and the superior activation and manipulation of anti-inflammatory TLRs and CLRs. Together, these approaches promote the persistence of the organism.

1.1.5 Modulation of effector T cell responses

1.1.5.1 Suppression of Th1- and Th17-mediated immunity

The association of two virulence factors, VacA and GGT in the manipulation and inhibition of human T cell response was reported. VacA impedes IL-2 mediated T cell proliferation pathway. It inhibits Ca^{2+}/calmodulin-dependent phosphatase calcineurin [36], necessary for the nuclear import of NFAT, an important transcription factor for T cell mediated immune response. An adhesion molecule LFA1 (αLβ2 integrin) present on the T cell is exploited by *H. pylori* to promote uptake of VacA. β2 integrin, a component of the LFA1 has been identified as a receptor for VacA [37] that undergoes serine/threonine phosphorylation on the cytoplasmic tail and facilitates VacA uptake [38].

GGT interrupts the RAS signalling pathway and inhibits cyclin-dependent kinase activity in the G1 phase of the cell cycle, therefore blocks T cell proliferation [39].

1.1.5.2 Skewing of T cell responses

In order to respond to a pathogenic infection, 'tolerogenic' dendritic cells have to encounter first to the pathogen and then initiate differentiation in naive T to mediate T_H1 and T_H17 effector T cell response. However, *H. pylori* exposed Dendritic cells fail to produce effector T cell responses both *in vitro* and *in vivo.* Instead they stimulate the expression of regulatory T cell specific transcription factor FOXP3, the surface marker CD25 and the anti-inflammatory cytokine IL-10 in naive T cells [40]. *H. pylori*-specific memory T cell responses has been severely compromised by such T_{Reg} cells accumulated in *H. pylori*- infected human gastric mucosa [41].

Such *H. pylori*-specific tolerance to dendritic cells requires the activity of both VacA and GGT [42], although the exact mechanism by which VacA- and GGT- achieved such tolerance remains unclear.

1.2 Chemerin

Chemerin, primarily known for its chemotactic and adipokine properties, is structurally related to the cathelicidin/cystatin family of proteins, was first discovered in psoriatic lesions of the skin in the late nineties. This multifunctional protein was transcriptionally upregulated by the anti- psoriatic synthetic retinoid tazarotene, therefore initially named as tazarotene-induced gene 2 protein (TIG2) or retinoid acid receptor responder 2 (RARRES2)[43].

A few years later chemerin was rediscovered as a ligand for the orphan seven-pass transmembrane receptor chemokine-like receptor 1 or CMKLR1 through G protein-associated receptor screening assays [44]. CMKLR1 (also known as ChemR23, DEZ, and finally renamed "chemerin receptor" by the International Union of Basic and Clinical Pharmacology) is expressed by several immune cell subsets (plasmacytoid dendritic cells (pDCs), macrophages and NK cells) that respond to chemerin either through chemotaxis or modulation of their defense function [45].

It is widely expressed and secreted as a 143-aa (chemerin 20–163) precursor protein (prochemerin) with low biological activity and ubiquitously found in plasma at nanomolar concentrations. It can be converted to a variety of isoforms with different functions through proteolytic cleavage by a myriad of serine and cysteine proteases as well as carboxypeptidases [46]. Several tissues, also release Prochemerin

including liver, spleen, lymph nodes, epithelia, endothelia, adrenal glands, pancreas, placenta, lung, skin, platelets etc.

Various studies showed circulating chemerin was elevated in inflammatory diseases, such as erythematosus, rheumatoid arthritis, psoriasis, Crohn's disease associated with markers of inflammation and found to regulate leucocyte recruitment towards the site of inflammation. It was also found to play a role as an important adipocyte-signaling molecule in adipogenesis, angiogenesis, osteo-blastogenesis, myogenesis, glucose homeostasis or even proposed a role in inhibiting bacteria growth. Therefore, the original notion of chemerin mainly as a leukocyte chemoattractant has been substantially challenged and broadened over the years. Taken together, chemerin emerges as a protein of interest for a number of disciplines, including immunology, dermatology, metabolism and development.

1.2.1 Regulation

The expression, secretion and processing of chemerin is tightly coordinated through a number of mechanisms which is essential for establishing chemerin levels, localization and, ultimately, activity. The regulation occurs both transcriptionally and post translationally.

1.2.1.1 Transriptional regulation of chemerin activity

Chemerin is regulated a by variety of inflammatory and metabolic mediators on a transcriptional level. They can be broadly classified as

i) Nuclear receptors agonists (retinoids, vitamin D, glucocorticoids),

ii) Metabolic factors (e.g. fatty acids, insulin, glucose) and

iii) Immunomodulatory mediators (e.g. cytokines of acute or chronic inflammation and LPS)

Regulation of chemerin expression by nuclear receptors agonists

Tazarotene (a synthetic retinoid acid analog), an agonist of the nuclear retinoic acid receptors β and γ (RARβ/γ), was the first identified factor to induce chemerin expression in psoriatic lesions and skin rafts (Table 1.2) [43].

Agonists of the steroid/thyroid/vitamin D3 nuclear receptor family, such as 1,25 dihydroxyvitamin D3 (1,25D3/calcitriol) and synthetic glucocorticoid-derivative dexamethasone (DEX) markedly induced TIG2 mRNA in the stromal cell line ST2

during osteoclast differentiation [47]. Significant chemerin expression was also observed when 1,25D3 administered to primary cultures of fibroblasts from healthy or psoriatic skin. [48].

Another member of the nuclear hormone receptor superfamily, peroxisome proliferator-activated receptor γ (PPARγ) was reported to elevate chemerin expression in BMSCs (Bone marrow mesenchymal stem cells) treated with Rosiglitazone (PPARγ agonist) [49]. Silencing of PPARγ in these cells resulted into almost loss of rosiglitazone-induced TIG2 expression [50]. Troglitazone, another agonist of PPARγ, increased TIG2 mRNA in 3T3-L1 cells during their differentiation [51].

In human and mouse liver hepatocytes, a synthetic ligand of nuclear receptor farnesoid X (FXR), GW4064 induced chemerin expression dose dependently, suggesting a correlation between chemerin and FXR (Table 1.2) [52].

Regulation of chemerin expression by metabolic factors

Chemerin is also under regulation of metabolic factors, such as free fatty acids (FFA) and insulin. FFA in 3T3-L1 cells up regulates chemerin synthesis during their differentiation into adipocytes. Plasma chemerin levels were significantly increased following insulin infusion in healthy individuals and adipocyte tissue explants. Metformin, an antidiabetic drug suppressed chemerin synthesis and secretion in adipocyte tissue explants in PCOS patients, suggesting a communication between chemerin and insulin/glucose (Table 1.2) [53].

Regulation of chemerin expression by cytokines

TNFα (Tumor necrosis factor alpha) is a cell signaling protein (cytokine) that is found to have an ambiguous role in controlling chemerin expression. *In vitro* study showed that TNFα significantly increased TIG2 mRNA expression in 3T3-L1 adipocytes, but suppressed TIG2 transcription and chemerin secretion in renal proximal tubular epithelial cells [54].

Interleukin 1 beta (IL-1β), a member of the interleukin 1 family of cytokines, was reported to increase TIG2 mRNA and chemerin protein concentrations significantly in 3T3-L1 adipocytes derived conditioned medium and immortalized brown adipocyte tissue (BAT)-derived cells (Table 1.2) [55]. Interferon gamma (IFNγ), the only member of the type II class of interferons was reported to enhance robust

chemerin expression by fibroblast like synoviocytes [56]. An elevated serum level of IFNγ has been detected in diseases postulated to be involved with chemerin such as psoriasis or rheumatoid arthritis [57].

The mechanism or pathways involved in the regulations of chemerin expression/secretion by IL-1β or IFNγ have yet to be defined.

Table 1.2 : Regulation of chemerin expression by inflammatory and metabolic factors (Adapted from Zabel et al., 2014).

Cell type	Nuclear receptors/ nuclear receptor ligands	Metabolic factors	Immunomodulatory mediators
Epithelial cells			
Human skin keratinocytes	Tazarotene (RARβ/γ), SR 11217/ SR 11237 (RXR)-, 1,25D3-		
Fetal human intestinal cells	ATRA (RARβ)		TNFα
Human intestinal enterocyte-like Caco2 cells			TNFα
Human renal tubular epithelial cells	1,25D3-		TNFα, IFNγ, IL1β, TGFβ
Fibroblasts			
Human skin fibroblasts	1,25D3-		IFNγ, IFNα, TNFα, CXCL8, TGFβ
Human synoviocytes			TNFα, IFNγ, IL1β, IL6, TGFβ
Chondrocytes			
T/C-28a2, human immortalized chondrocytes	IL1β	Adiponectin, leptin	LPS
ATDC-5 mouse chondrogenic cell line	DEX	Adiponectin, leptin	IL1β, LPS
Osteoclasts-suppurting mouse stromal ST2 cell line	1,25D3, DEX		
Adipocytes			
Human adipocytes/ adipocyte tissue explants	PPARγ (troglitazone) (mRNA, protein), gonadal and adrenal steroids	Adiponectin, insulin, metformin, FFA (SREBP2)	TNFα
Mouse adipogenic cell line 3T3-L1	PPARγ (troglitazone)		TNFα, IL1β
Bone marrow mouse mesenchymal stem cell	PPARγ (rosiglitazone)		
hepatocytes			
Human hepatoma HepG2 cell line	FXR (GW4064)		
Mouse hepatocytes	FXR (GW4064)		TNFα

1.2.1.2 Posttranslational regulation/ proteolytic processing

Total circulating chemerin in a healthy population is 90-200 ng mL^{-1} in both serum and plasma. Females and older adults have higher total circulating chemerin than males and younger adults reportedly, although not evident by all studies [58].

Human chemerin is initially synthesized as a 163 amino acid precursor, preprochemerin (chemerin 1–163) along with an N-terminal signal sequence (20 aa), which is cleaved off before secretion into an inactive 18-kDa precursor, prochemerin (chemerin 20–163 or Chem-163) [59]. When this cleavage occurs, either co- or post-translationally during translocation to the endoplasmic reticulum, is still not clear.

The majority of chemerin in the circulation is functionally inactive prochemerin, bearing a cystatin-like fold-containing domain and a labile C-terminus that can be cleaved at multiple distinct sites by a variety of extracellular proteases. This releases a terminal inhibitory peptide resulting into a number of bioactive isoforms with varying length and biological activity [60].

Several isoforms have been isolated from several human biological specimens (Table 1.4). Chem-157 lacks six amino acids from C-terminus is the most biologically active isoform (approximately 100-fold higher than prochemerin. Chem-156 (not yet reported in human biological fluids) is slightly less active than Chem-157, Chem-155 and -158 showed low activity, and Chem-152 and -154 are relatively inactive (Table 1.3) [59].

Extracellular proteases that process chemerin include; serine proteases of the coagulation, fibrinolytic and inflammatory cascades such as neutrophil elastase and cathepsin G, mast cell tryptase, plasma-derived factor XIIa, VIIa and plasmin, as well as host cysteine protease such as cathepsins K and L (Table 1.3) [60]

Among those proteases, some can process prochemerin at more than one site (e.g. elastase, tryptase [61], while some can process an already precessed isoform. For example, carboxypeptidases B and N process relatively low active chemerin isoforms into more active forms [62]. Chemerin can also be controlled by proteases from human pathogens other than host ones. Staphopain B, a potent cysteine protease derived from *Staphylococcus aureus* activates the chem-157 variant [46].

Table 1.3 : A: In the human chemerin primary amino acid sequence (NCBI Reference Sequence: NP_002880.1) the following regions are shown: functionally-evaluated chemerin peptides displaying antibacterial activity (green) or chemotactic activity (blue), inhibitory C-terminal region (red). B: Processing of prochemerin by different proteases. (Adapted from Zabel et al., 2014 and Rourke et al., 2012).

(A) human Chemerin primary amino acid sequence

1.
MRRLLIPLALWLGAVGVGVAELTEAQRRGLQVALEEFHKHPPVQWAFQETSVESAVDTPF
61. PAGIFVRLEFKLQQTSCRKRDWKKPECKVRPNGRKRKCLACIKLGSEDKVLGRLVHCPIE
121. TQVLREAEEHQETQCLRVQRAGEDPHSF^{149}YFPGQF^{154}AFS^{157}KALPRS^{163}

(B) Processing of prochemerin by different proteses

Protease	Cleaves	Generates	C-terminal sequence	Resulting activity
Prochemerin ($chem_{21-163}$)			... PGQFAFSKALPRS	Inactive
Carboxypeptidase N or B	Chem-158	Chem-157	... PGQFAFS	Converts high to highest
Cathepsin G	Chem-163	Chem-156	... PGQFAF	High
Cathepsin K, L	Chem-163	Chem-157	... PGQFAFS	Highest
Human leucocyte elastase	Chem-163	Chem-157	... PGQFAFS	Highest
		Chem-155	... PGQFA	Low
		Chem-152	... PG	Inactive
Mast cell chymase	Chem-156 Chem-157	Chem-154	... PGQF	Converts high/ highest to inactive
Mast cell tryptase	Chem-163	Chem-158	... PGQFAFSK	Low
		Chem-155	... PGQFA	Low
Plasmin	Chem-163	Chem-158	... PGQFAFSK	Low
Proteinase 3	Chem-163	Chem-155	... PGQFA	Low
Staphopain B	Chem-163	Chem-157	... PGQFAFS	Highest

Table 1.4 : Biological sources of chemerin isoforms ((Adapted from Rourke et al., 2012).

Biological source	Identified isoforms
Ascites (20)	Chem-157
Cerebrospinal fluid (23,25)	Chem-158
Hemofiltrate (22)	Chem-154
Plasma (24,25)	Chem-155, -157, -158, -163
Synovial fluid (25)	Chem-158

Extracellular proteases are also engaged in inactivating or degrading the attractant, therefore limiting the extent of chemerin activity. Tissue-recruited neutrophils secrete chemerin-activating elastase and cathepsin G, but also release proteinase 3 that cleaves chem-163 to chemotactically inactive chem-155 [63]. Mast cell chymase converts bioactive chemerin into relatively inactive Chem-155 [64]. This Chem-155 might act as a weak antagonist in the presence of highly active chemerin isoforms [65].

In chemerin isoform specific ELISAs, it was found that majority of chemerin in inflammatory milieus is proteolytically processed, pointing towards extensive proteolytic processing of chemerin during inflammation *in vivo* [66].

1.2.2 Receptors and signalling

There are three known heptahelical receptors for chemerin: CMKLR1, GPR1, and CCRL2. These receptors bind to chemerin with similar low nanomolar affinities but with substantially different functional outcomes.

CMKLR1, or ChemR23 or Chemerin receptor, is the seven-pass transmembrane G protein-coupled receptor located on chromosome 12q24.1. It shares homology with some chemoattractant receptors, such as somatostatin receptors, anaphylatoxin C3a and C5a receptors, and formyl peptide receptors [67].

CMKLR1 is highly expressed in leukocyte populations, particularly immature dendritic cells (DCs), resident macrophages and cytotoxic natural killer (NK) cells. It has also been located in adipose tissue, bone, lung, brain, heart, placenta [68,69], recently in human endothelial cells (ECs) and cultured human venous smooth muscle cells [66].

CMKLR1 activation by chemerin triggers several biological responses. These include; intracellular calcium mobilization, β-arrestin2 association and receptor internalization, up-regulation of the phosphatidylinositol 3-kinase/Akt signalling pathway, down-regulation of NF-kB and inhibition of cAMP and MAPK ERK1/2-mediated signalling [6].

GPR1, G protein-coupled receptor 1, a member of the G protein-coupled receptor family of transmembrane receptors, is expressed in adipose tissue, central nervous system (CNS), skeletal muscle and in a limited manner in leukocytes [32]. Chemerin binding to GPR1 triggers β-arrestin2 association and receptor internalization. It may involve in regulating local chemerin level as well by functioning as a chemerin-binding ‘interceptor’ that binds, internalizes, and degrades chemerin [70].

CCRL2, CC-motif chemokine receptor-like 2, is an atypical chemerin receptor expressed by mast cells, activated macrophages and dendritic cells, and vascular endothelial cells. *In vivo* studies showed CCRL2 regulation in circulating chemerin

levels and its proteolytic processing during systemic inflammation. It mediates chemerin localization, presents it to nearby cells and thereby contributing to CMKLR1 and potentially GPR1-mediated processes [71].

1.2.3 Chemerin initiates an inflammatory response in inflammation and diseases

Polymorphonuclear leukocytes or PMNs (Neutrophils, eosinophils, and basophils) are the first cells recruited early at the sites of active tissue injury or inflammation, which release a set of proteases that generate bioactive chemerin. Blood coagulation and human pathogen-derived proteases may also contribute to the increased chemerin activity. After activation, chemerin mediates the recruitment of CMKLR1-expressing Antigen-Presenting Cells (APC) such as macrophages, immature DCs and several other types of immune cells at inflammatory sites; thereby triggering an early immune response (Figure 1.6) [72].

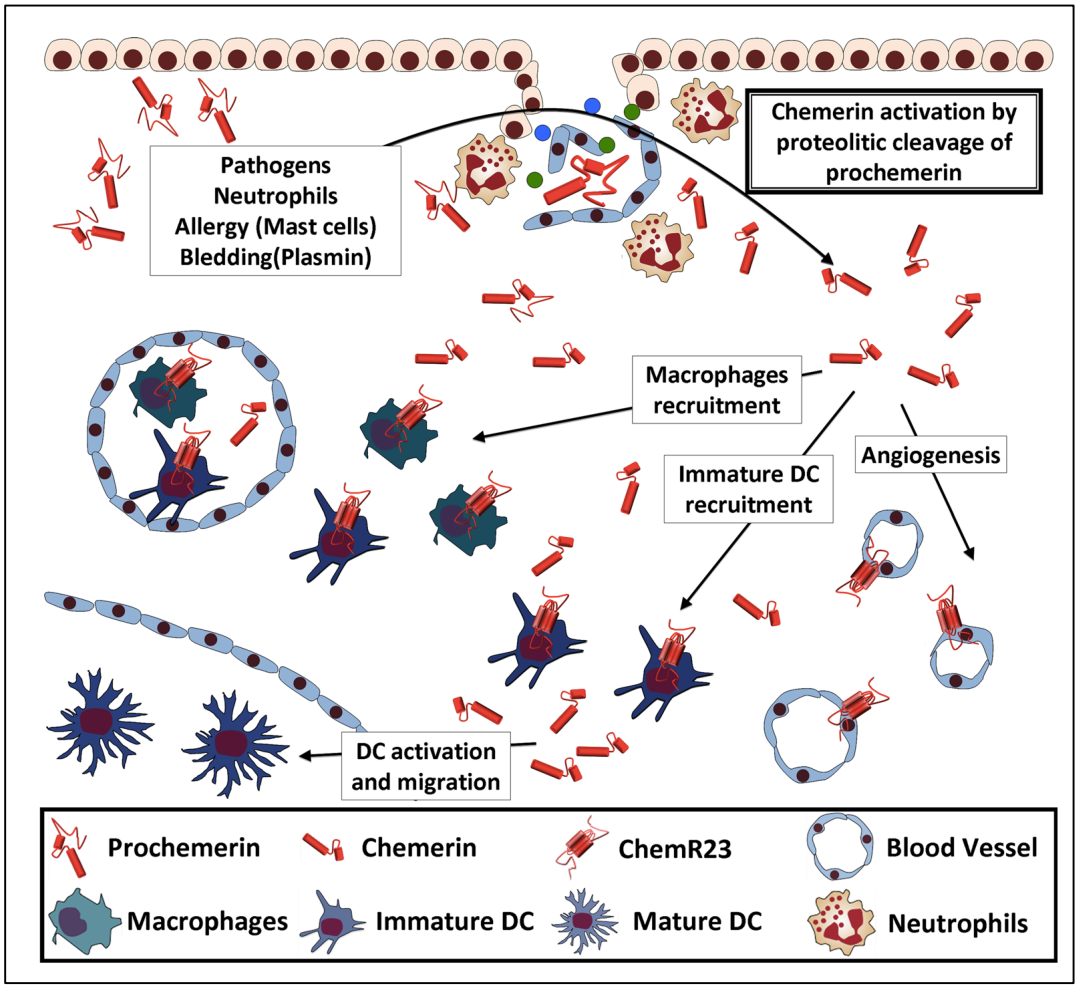

Figure 1.6 : Chemerin driven development of inflammatory response (Adapted from Francesco & luca et al., 2014).

Serum chemerin levels are significantly elevated in patients with chronic inflammation, such as Crohn's disease, ulcerative colitis, chronic kidney disease, chronic pancreatitis, pre-eclampsia, polycystic ovary syndrome and liver disease with higher inflammatory markers such as C-reactive protein (CRP), IL-6 and TNFa, as well as the pro-inflammatory adipokines leptin and resistin [73].

Upregulated chemerin levels are often observed in diseased tissues in both mice and humans, such as psoriasis, cancer, arthritis, lupus and multiple sclerosis. Human studies on rheumatoid arthritis, osteoarthritis (OA) and psoriatic arthritis showed twofold increase of chemerin in localized synovial fluid but little or no change in serum level. This point out an idea that elevated chemerin level is often associated with inflamed tissue that may or may not correspond to a similar change in circulating chemerin levels [74].

Furthermore, the ratio of cleaved to total chemerin is dramatically increased and is often associated with unique isoform profiles that differ considerably from the distribution of isoforms in circulation [75].

1.2.4 Chemerin/Chemerin receptor signaling in inflammation and diseases

Numerous in vitro and animal studies have demonstrated the involvement of chemerin in chemotaxis of CMKLR1-expressing leukocytes such as human macrophages, immature DCs and natural killer (NK) cells to sites of localized inflammation, tissue damage or bleeding. These leukocyte populations evaluate local conditions and initiate an appropriate immune response. Thus, based on the stimuli confronted by recruited CMKLR1^{+} cells and importantly, the tissues and disease being investigated, chemerin may aggravate or ameliorate the local inflammatory response.

The role of chemerin and CMKLR1 signaling has been investigated in a handful of inflammatory disease models, usually with the use of conventional CMKLR1 deficient mice or exogenous administration of chemerin to recruit cells to a particular anatomic location.

The recruitment and retention of leukocytes at sites of infiltration by chemerin was supported by a study on murine macrophages where chemerin facilitates the adhesion of murine macrophages to extracellular matrix proteins and endothelial cells [76].

Chemerin signaling has been found to be required for advancing optimal tissue swelling and leukocyte infiltration in various mouse inflammation models such as IgE-mediated anaphylaxis and lipopolysaccharide (LPS)-induced pulmonary inflammation [71].

Consisting with this, CMKLR1 knockout mice exhibited reduced CNS inflammation in EAE (experimental autoimmune encephalomyelitis), a model of autoimmune demyelinating disease [77]. This finding is also supported by a study that used COPD (cigarette smoke- induced chronic obstructive pulmonary disease) model. CMKLR1 KO mice developed less severe pulmonary inflammation associated with a reduction in PMN, DCs and CD4+ T cells infiltration into the airways [78]. All these clinical and experimental data suggest a pro-inflammatory role of chemerin/CMKLR1 signaling.

However, several inflammation models experimented on animals such as peritoneal inflammation, LPS-induced lung injury and acute viral pneumonia exhibit exacerbated inflammation and decreased leukocyte infiltration when endogenous chemerin activity is inhibited or CMKLR1 expression is lost. *In vitro* study showed marked inhibition of TNFa, IL-1b, IL-6, IL-12 and RANTES like pro-inflammatory mediators by chemerin through classically activated murine macrophages [79]. In a transplantable melanoma model, tumor-expressed chemerin inhibited *in vivo* tumor growth by NK cell depletion or CMKLR1 deficiency [80].

Additionally, CMKLR1 KO mice developed more severe pulmonary inflammation associated with an increased leukocyte infiltration into the lung, high viral burden and delayed viral clearance in PVM model (an infectious model of viral pneumonia) study [81], suggesting an anti-inflammatory role of the chemerin/CMKLR1 signaling.

Therefore, both pro- and anti-inflammatory role for chemerin has been suggested by various studies. It may be due to different isoforms of chemerin that could potentially play contrasting roles in various stages of inflammation, on different population in a particular condition.

1.2.5 Chemerin/Chemerin receptor signaling in macrophages

As macrophages in both human and mouse predominantly expresses ChemR23, they most likely play a central role in chemerin-mediated development of inflammatory diseases.

After activation, as stated before, chemerin facilitates the chemotaxis and adhesion of macrophages to extracellular matrix proteins and endothelial cells through increased adhesion to fibronectin and VCAM-1 and promotion of integrins clustering (VLA-5 and VLA-4) in a ChemR23-dependent manner [76]

Although ChemR23 expression is predominantly observed both in human and mouse macrophages, the regulation and eventual function of ChemR23 can be varied in these two species. A previous study on mouse macrophages showed that inflammatory stimuli, such as TLR ligands (LPS, CpG) and inflammatory cytokines like IFN-g and TNF-α down regulated Chem23 expression whereas anti-inflammatory cytokines like TGF-β caused upregulation, suggesting the role of ChemR23 in naive and anti-inflammatory macrophages only [76, 82].

However, LPS, TNF-a and IFN-g stimulation increased ChemR23 expression in human monocytes and macrophages [83]. This concept is further supported by some studies on mouse inflammation models that found ChemR23-dependent anti-inflammatory and protective effects of chemerin, whereas in humans, chemerin was found to be directly associated with chronic and systemic inflammation [84]. Different isoforms of Chemerin can also function differently in modulating macrophages. It was found that active chemerin-156 induced proinflammatory cytokines expression on macrophages whereas alternative digestion products of chemerin increased anti-inflammatory cytokines such as IL-10. [85].

The differential expression pattern of ChemR23 on stimulated macrophages and the possible opposite effect of chemerin on inflammation between mouse and human may indicate that the role of ChemR23 signaling differs between the two species.

Chemerin/ ChemR23 signaling also plays a potential role in the polarization of macrophages. In an Inflammatory Bowel Disease (IBD) study on DSS (Dextran sulfate sodium) induced colitis model, exogenously administered chemerin is shown to significantly decrease colonic expression of all the M2 associated genes, Arg-1, Ym1, FIZZ1 and IL-10. This is further supported by an in vitro study done by the

same group, proposing the involvement of chemerin in the resolution of IBD by suppressing M2 macrophage polarization [86].

Interestingly another study showed functional CMKLR1 expression on human M1 macrophages but not on M2 macrophages *in vitro.* This classically activated human M1 macrophages are prone to chemotaxis by chemerin and produce increased IL10 expression. This finding provides another aspect to the study performed by lin et al group and would be interesting to assess the effect of chemerin on M1/M2 polarization in other models as well [87].

Chemerin has been found to play a role in the recruitment of macrophages to the developing fetal intestine, indicating a much more complex and vast relation with macrophages not only limited to inflammation. Intestinal macrophages are the first phagocytic cells of the innate immune system to combat luminal bacteria in fetal intestine and have a unique adaptive mechanism to prevent unnecessary inflammation in the gut mucosa. These macrophages do not undergo clonal expansion and continuous recruitment from blood monocytes is necessary for development and maintenance of gut macrophages. While chemerin contributes to the shaping of mucosal immunity by continuous recruitment of macrophages in the developing fetal intestine, the expression of chemerin is low in the mature intestine [88].

1.3 Macrophages

Macrophages, also known as 'big eaters' or 'phagocytes', were first discovered and presented by Ilya Metchnikoff which had led him earn the Nobel Prize for Medicine in 1905 [89]. The name came from two Greek words, *'makros'* meaning 'large' and '*phagein'* meaning 'to eat'. Macrophages are heterogeneous and dynamic immune cells that are present in almost all tissues in human under different names, forms and different functions. They engulfs and digests cellular debris, foreign substances, pathogens, cancer cells, and anything else that does not have the types of proteins specific of healthy body cells on its surface through the process phagocytosis, hence the name macrophages. Macrophages are the first line defense of innate immunity system against pathogens and play a critical role in initiating the adaptive immune response via antigen processing and presenting to other leucocytes [90]. They take part in both generation and resolution of inflammation, tissue repair and wound

healing [91]. Although macrophages are very effective in initiating immune response and phagocytosis, some microbes develop strategies to escape and interfere with macrophage activation and modulate host responses [92].

1.3.1 Monocyte / macrophage development

Monocytes are generated by bone marrow from myeloid hematopoietic progenitor cells (Figure 1.7). After generation, they circulate into the peripheral blood stream, and within three days or so, typically migrate into different tissues. Half of the population migrates into the spleen, which serves as a reservoir for immature monocytes [93] and the rests mature into different types of macrophages and/or dendritic cells in different tissues when exposed to local growth factors, cytokines, pathogens or pathogen related compounds [94]. The entire process is known as "mononuclear phagocyte system". The variety of undifferentiated monocytes in the circulation could affect their polarization status in various tissues [95]. Monocytes are primarily involved in eliminating invading organisms (bacteria, virus, fungi, and protozoans) although could potentially bring about negative effects on the pathogenesis of inflammatory and degenerative diseases [96].

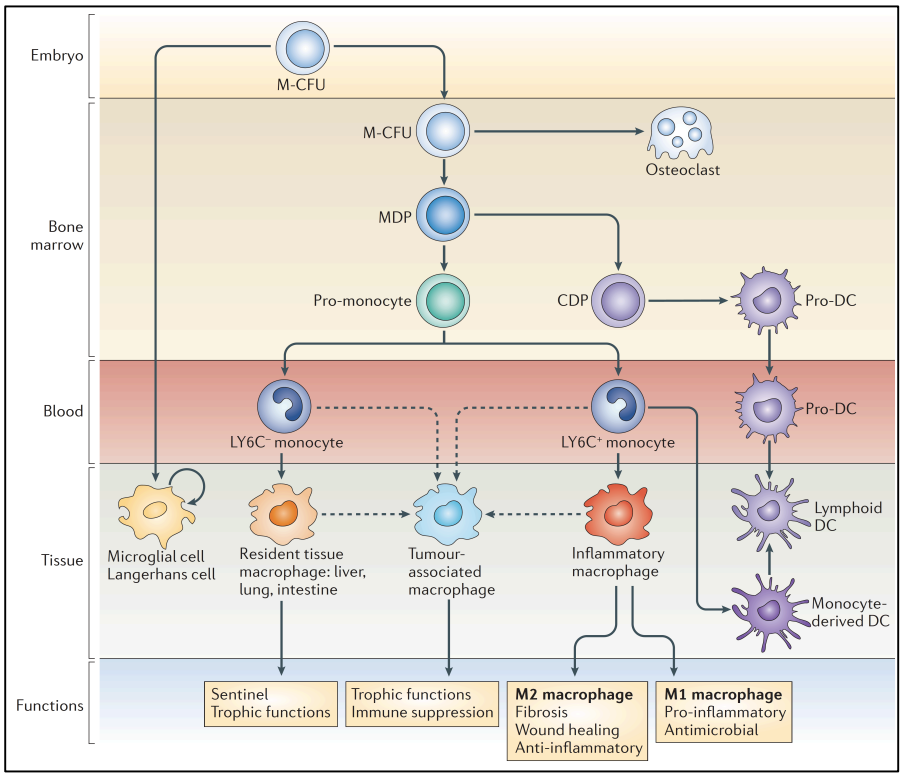

Figure 1.7 : The mononuclear phagocyte system (adapted from Lawrance & Natoli, 2011) (solid lines represent common pathway of development and dashed lines represent alternative pathway of development).

Macrophages can be divided according to their anatomical location with specific names; such as osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue), Kupffer cells (liver) etc (Figure 1.8) [95]. These tissue- resident specific macrophages patrol for potential pathogens, engulf foreign substances and recruit additional macrophages from circulation during an infection or injury with other specified functions.

Such as, macrophages present in the gut work as groups to maintain tolerance to the gut flora and the food ingested. Macrophages present in the spleen (marginal zone macrophages) inhibit innate and adaptive immune response against apoptotic cells [97], macrophages of lymph nodes (subcapsular sinus) eliminate viruses and activate antiviral humoral immune responses [98,99]. Nonetheless, macrophages present in the immune-privileged sites, such as the brain (microglia), eye and testis focus mainly on tissue remodeling and homeostasis.

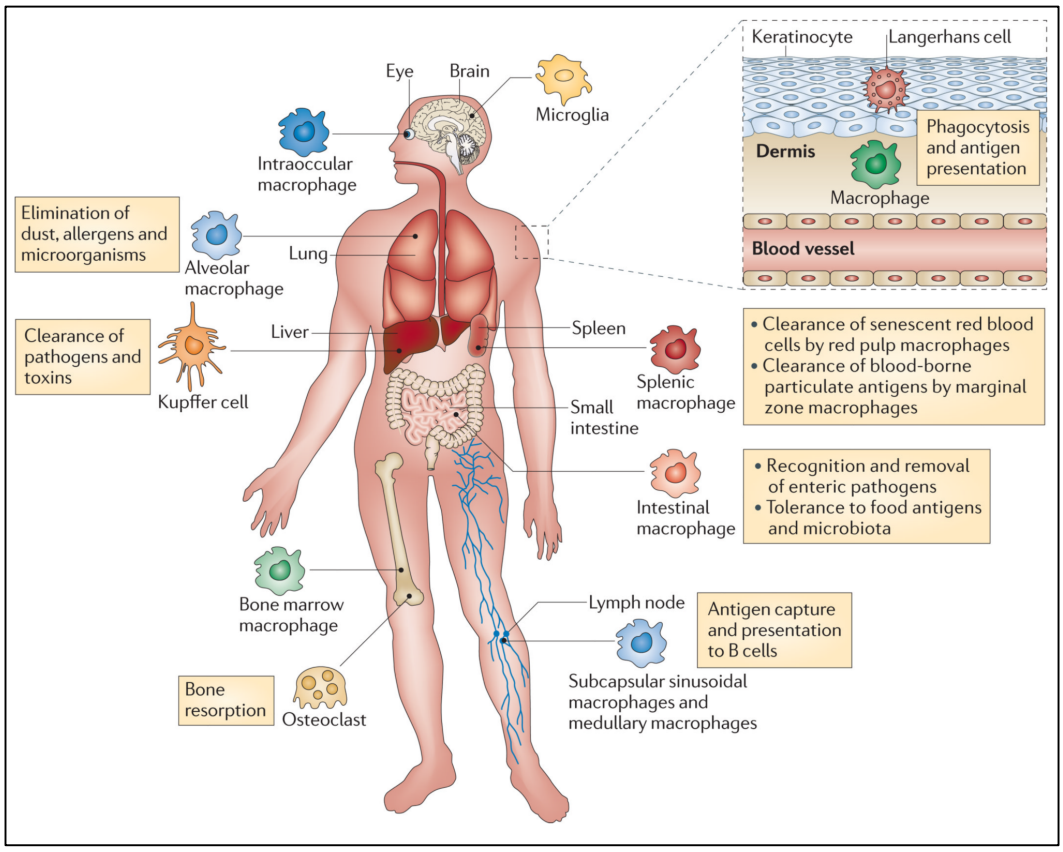

Figure 1.8 : Different tissue macrophages (adapted from Murray & Wynn, 2011).

However, not all tissue resident macrophages are differentiated from monocytes. For example, Langerhans cells in the skin and microglial cells in the CNS proliferate

locally. Recent studies indicate development of these cells from M-CFU in the yolk sac of the developing embryo [100].

Macrophages are distinguished from dendritic cells (DCs) or other mononuclear cells by differential expression of specific surface makers such as CD11a, CD11b/CD18, CD11c, CD14, and CD68.

1.3.2 Pathogen recognition of macrophages via Toll-like receptors

The toll-like receptors (TLRs) on macrophages recognize PAMPs derived from microbes and danger signals; initiate inflammatory responses; activate microbial killing and clearance mechanisms [101]. They have also been described as 'necessary' or 'required' for an adaptive immune response. There are 13 known paralogous of TLRs present; among which 10 is present in humans and 12 in mice (Figure 1.9).

TLR4 present on macrophages identifies LPS from cell walls of Gram-negative bacteria and initiate two separate signaling pathways regulated by two separate adaptor proteins, MyD88 or Toll/IL-1R domain–containing adapter inducing IFN-β (TRIF) [102]. TLR4 stimulation activates inflammatory cytokines production such as tumor necrosis factor TNF-α, IL-1 and IL-6. Flagellin of *H. pylori* were found to be recognized by TLR5 [79].

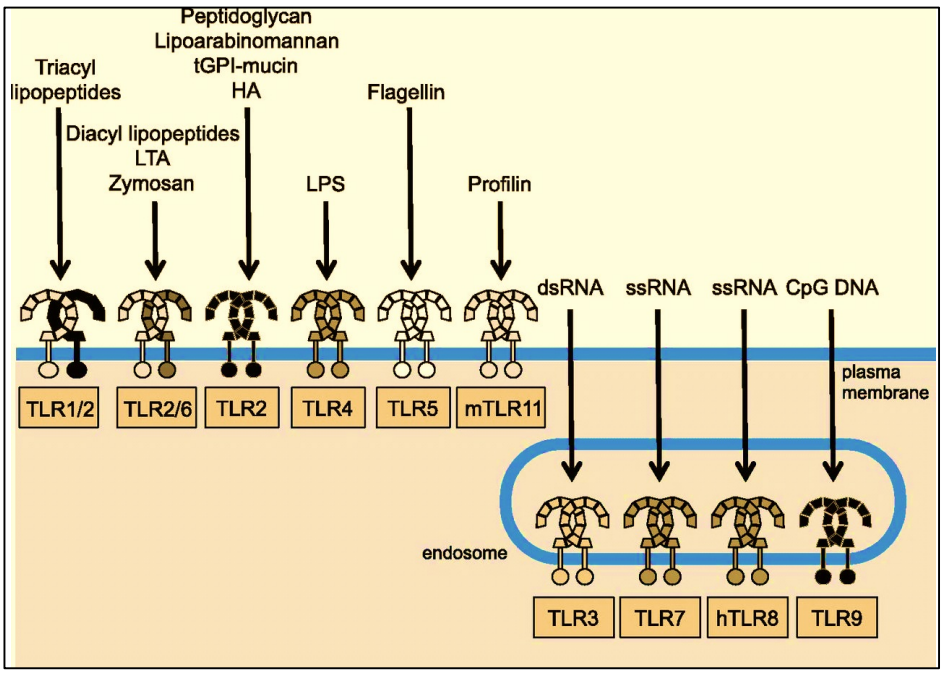

Figure 1.9 : Toll like receptors and their ligands (adapted from styliani et al., 2016).

However, LPS and flagellin of *H. felis* were identified by TLR2, and it's signaling depends on MyD88 adapter protein, unlike typical LPS and flagellin whose recognition is not dependent on TLR4 or 5 as (Figure 1.5).

Study conducted by *Sayi et al.* (2011) [33] showed that *H. felis* activates B lymphocytes through TLR2 with up-regulated CD40, MHC-II and other co-stimulatory molecules and production of IL-10, IL-6, IgM and IgG2b. (Figure 1.8). TLR2 identify *helicobacter* mosty due to the uncommon lipid composition of the bacterial envelope with high concentration of lysophospholipids and cholesteryl glucosides [103] (Figure 1.5).

1.3.3 Macrophage polarization and plasticity

Macrophages can be polarize into different phenotypes under the influence of different stimuli. During tumor progression, phenotypical change of macrophages from classically (M1) to alternatively activated (M2) has been observed [104] whereas in obesity, it changes to M2 from M1 [105]. These findings suggest the notion that polarization of macrophages alters according to stimuli given and the local microenvironment as well, enabling them to form the local inflammatory status to adjust to external stimuli [91]. However, These two subtypes do not exist as distinct populations excluding each other out, rather they often coexist. The balance of activatory and inhibitory activities and the tissue environment often determine the resulting mixed phenotype.

1.3.3.1 Classically activated macrophages (M1)

The M1 macrophage phenotype is typically described by elevated production of pro-inflammatory cytokines, marked resistance to pathogens, strong microbicidal characteristics, marked production of reactive nitrogen and oxygen intermediates, and promotion of Th1 or TH17 immune responses (Figure 1.12). Stimulation with LPS, IFN-gamma and granulocyte-macrophage colony stimulating factor (GM-CSF) assist in polarizing M1 macrophages with secretion of high amounts of pro-inflammatory cytokines such as IL-1-beta, tumor necrosis factor (TNF), IL-12, IL-18 and IL-23 (Figure 1.12) [106,107]. These macrophages express high levels of major histocompatibility complex class I and II (MHC I & II), the CD68 marker, and co-stimulatory molecules CD80 and CD86, various pro-inflammatory chemokines and secrete complement factors for complement-mediated phagocytosis (Table 1.5)

[108]. Increased expression of the intracellular protein suppressor of cytokine signaling 3 (SOCS3) has also been observed, as well as nitric oxide synthase (*NOS2* or iNOS) to produce NO from L-arginine [109] (Figure 1.10).

Different stimuli could potentiate different responses on M1 type macrophages. For instance, Stimulation of macrophages with IFN-γ results in higher levels of IL-12 and IL-23 with low levels of IL-10 [95,110]. While secreted IL-12 facilitates Th1 response with improved phagocytosis [111], IL-23 promotes the development and expansion of Th17 cells with high levels of IL-17, causing inflammatory autoimmune pathologies [112,113].

Various transcription factors and post-transcriptional regulators form a network that is involved in M1 polarization. Upon activation by IFNs and toll-like receptor (TLR), The IRF/STAT pathways initiate M1 polarization via STAT1 [104]. On the other hand,The LPS/TLR4 pathway activates STAT1-alpha/beta in a MyD88 independent fashion to polarize M1. In the presence of LPS, Bruton's tyrosine Kinase (Btk) has been implicated in macrophage polarization to M1. Induction of NO production by P2Y(2)R, a G protein coupled receptor via *NOS2* and SOCS3 via NF-κB/PI-3 kinase pathways promotes M1 markers (Figure 1.10) [114].

M1 type macrophages can be identified by F4/80, CD11b, and $CD11c^{+}$ surface marker expressions in a recent obesity study (Table 1.5) [115,116]. They secure resistance to intracellular bacteria and control the acute phase of infection. *Listeria monocytogenes* infection in immunocompromised patients and pregnant women, M1 type macrophages were shown activated and mediated intracellular killing of bacteria is in vitro and in vivo [117]. M1 macrophages have also play a role in infections caused by *L. monocytogenes* [118], *Salmonella typhi*, *Salmonella typhimurium* [119] and *Mycobacterium tuberculosis* [120].

M1 macrophages are responsible for sustained inflammation in various diseases including gastroenteritis, urinary tract infections, neonatal meningitis, and sepsis, and can therefore be detrimental to health [121].

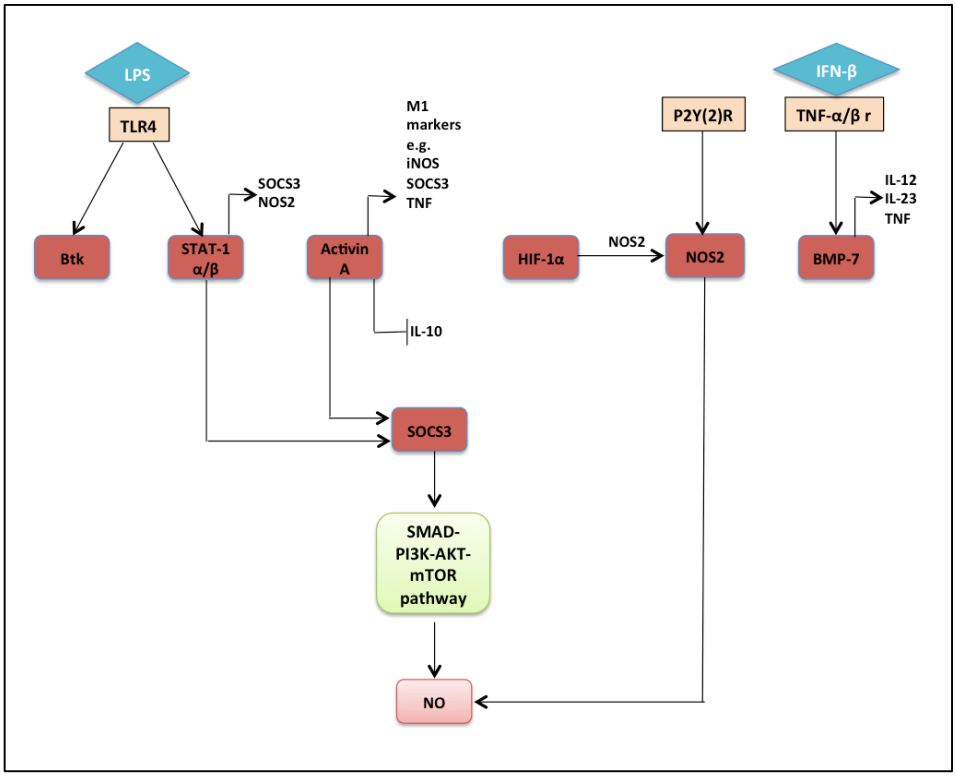

Figure 1.10 : Signaling molecules involved in M1 polarization.

1.3.3.2 Alternatively activated, M2 type macrophages

Alternatively activated macrophages are mainly described by their contribution to parasite control, reduction of inflammation, immune regulation, tissue remodeling, tumor progression and efficient phagocytic activity (figure 1.12) [122]. M2 macrophage is activated by various stimuli including fungal cells, immune complexes, helminth infections, complement components, apoptotic cells, macrophage colony stimulating factor (MCSF), IL-4, IL-13, IL-10 and TGF-beta. They secrete high amounts of IL-10 and low levels of IL-12 and IL23 [110, 123]. M2 macrophage embraces a functionally diverse group of populations, which can be further subdivided into M2a, M2b, M2c and M2d according to distinct gene expression profiles [108]. The M2a macrophage is stimulated by IL-4, IL-13 or fungal and helminth infections. IL-1 receptor ligands, immune complexes and LPS induce M2b subtype. M2c is activated by IL-10, TGF-beta and glucocorticoids. The fourth type, M2d is stimulated by IL-6 and adenosine. M2d macrophages are similar to ovarian TAMs bot phenotypically and functionally but are different from M2a-c (Table 1.5) [124, 125].

Table 1.5 : Classically activated (M1) and alternatively activated (M2) subset phenotypes (Adapted from Rőszer T 2015 and Duluc D et al. 2007).

	M1	M2a	M2b	M2c	M2d
Stimulation/activation	IFN-gamma LPS GM-CSF	IL-4 IL-13 Fungal and Helminth infection	ICs IL-1R	IL-10 TGF-beta GCs	IL-6 LIF Adenosine
Marker expression	CD68 CD86 CD80 MHC II IL-1R TLR2 TLR4 iNOS SOCS3	CD163 MHC II SR MMR/CD206 CD200R TGM2 DecoyR IL-1R II *Mouse only:* *Ym1/2* *Fizz1* *Arg-1*	CD86 MHC II	CD163 TLR1 TLR8	VEGF
Cytokine secretion	TNF IL-1beta IL-6 IL-12 IL-23	IL-10 TGF-beta IL-1ra	IL-1 IL-6 IL-10 TNF-alpha	IL-10 TGF-beta	IL-10 IL-12 TNF-alpha TGF-beta
Chemokine secretion	CCL10 CCL11 CCL5 CCL8 CCL9 CCL2 CCL3 CCL4	CCL17 CCL22 CCL24I	CCL1	CCR2I	CCL5 CXCL10 CXCL16

M2 macrophage embraces a functionally diverse group of populations, which can be further subdivided into M2a, M2b, M2c and M2d according to distinct gene expression profiles [108]. The M2a macrophage is stimulated by IL-4, IL-13 or fungal and helminth infections. IL-1 receptor ligands, immune complexes and LPS induce M2b subtype. M2c is activated by IL-10, TGF-beta and glucocorticoids. The fourth type, M2d is stimulated by IL-6 and adenosine. M2d macrophages are similar to ovarian TAMs bot phenotypically and functionally but are different from M2a-c (Table 1.5) [124, 125].

The signalling molecule involved in macrophage polarization is STAT 6 (Figure 1.11) [126]. IL-4/IL-13 receptor signaling activates STAT6 mediated upregulation of Arginase 1, a specific trademark of M2 polarization. Krüppel-like factor 4 (KLF-4) coordinates with STAT6 to induce M2 genes such as Arg-1, Mrc1,

Fizz1 and PPARγ, and inhibit M1 genes such as TNF-alpha, Cox-2, CCL5 and NOS2 [127]. The NF-κB

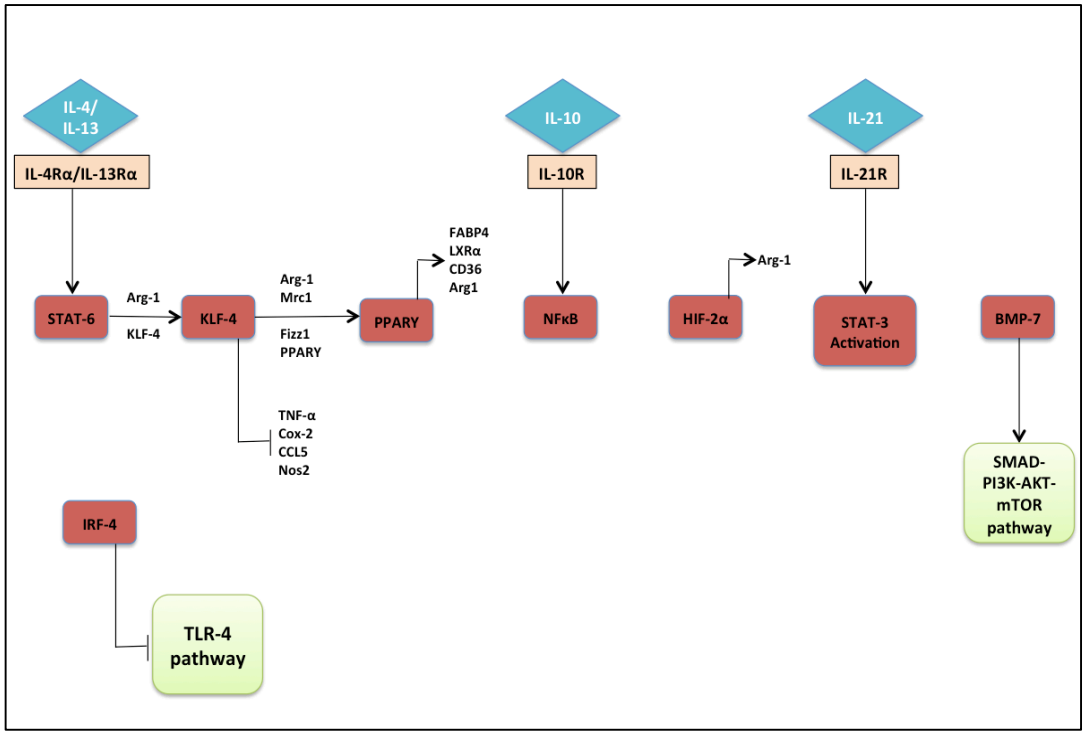

Figure 1.11 : Signaling molecules involved in M2 polarization.

p50 subunit (as homodimers) also plays an important role in M2 polarization *in vitro* and *in vivo* [128] as well the nuclear receptor, peroxisome proliferator-activated receptor γ (PPARγ) [129]. The hypoxia inducible factors HIF-2α controls arginase 1 expression and the M2 state [130]. IRF4 negatively regulates M2 polarization in a MyD88 independent fashion [131] and BMP-7 positively regulates M2 polarization *in vitro* through SMAD-PI3K-Akt-mTOR pathway (Figure 1.11) [132].

M2 macrophages have distinct chemokine and chemokine receptor profiles, secreting CCL17, CCL22 and CCL24 (Table 1.5). Recent *in vitro* studies showed that, macrophages can completely repolarize between themselves, from M2 to M1 and vice versa, depending on the chemokine environment [133].

1.3.3.3 Tumor associated macrophages (TAMs)

Studies in mice models exhibited that macrophages aid in surviving malignant tumor cells to survive and growing in distant sites, potentially involve in metastasis which is one of the major causes of patient's death. Macrophages are one of the major populations of infiltrating leukocytes associated with solid tumors [95]. Circulating

monocytes can be recruited into the tumor microenvironment through secretion of chemotactic molecules by the tumor itself and mature into TAMs [134]. Mice model study suggested such tumor infiltrating monocyte pool to be primarily of Ly-$6C^{+}CX_3CR_1^{low}$, and TAM monocyte precursors to be Ly-$6C^{high}$ cells exclusively [135].

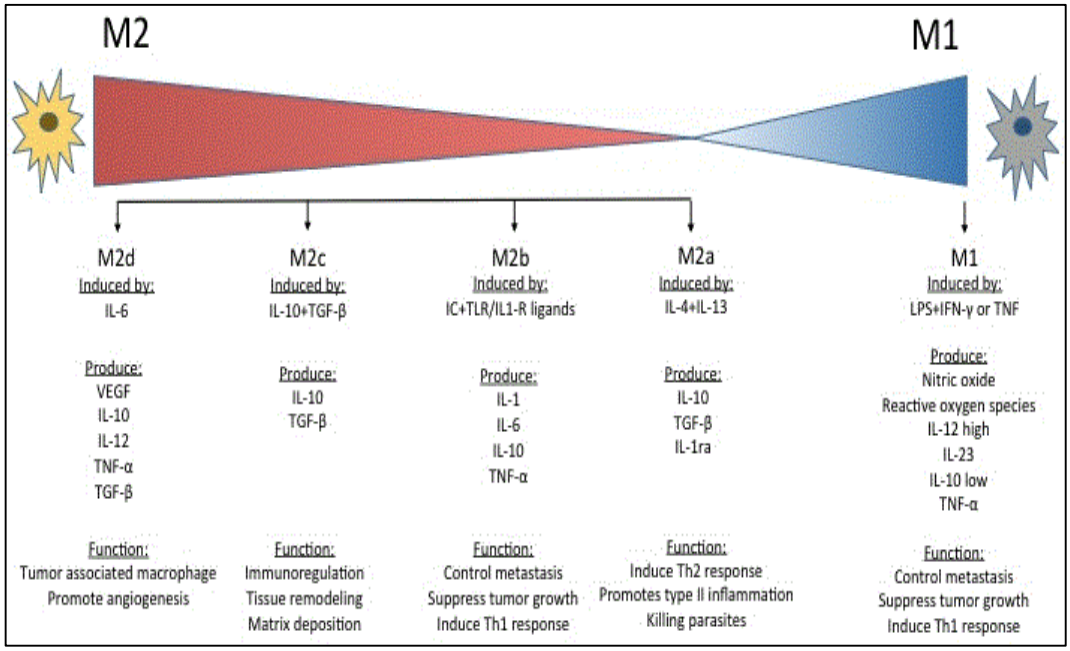

Figure 1.12 : Inducers and selected functional properties of classically (M1) and alternatively (M2) activated macrophage populations (adapted from Evita et al., 2015).

TAMs actually stands for a population of various macrophage subsets, primarily of M2-like phenotype [136] although studies have shown the presence of both M1 and M2 subsets [137,138]. TAMs are known to promote tumor progression and are associated with poor prognosis. They facilitate angiogenesis, lymphogenesis, stroma remodeling and immune suppression and promote tumor invasion and metastasis [139] via secretion of the enzymes plasmin, uPA, matrix metalloproteinases (MMPs) and cathepsin B (Figure 1.13) [140,141].

The transcriptional profile of TAMs is distinct from M1 and M2 macrophages [142]. They are phenotypically categorized as $CCL2^{hi}CCL5^{hi}IL\text{-}10^{hi}$, express MGL-1, Dectin-1, CD68, CD206, VEGF-A, *NOS2*, CD81, MHC II and scavenger receptor A [143-44]. In addition, they exhibit enhanced IRF-3/STAT-1 activation and defective NF-κB signaling [142].

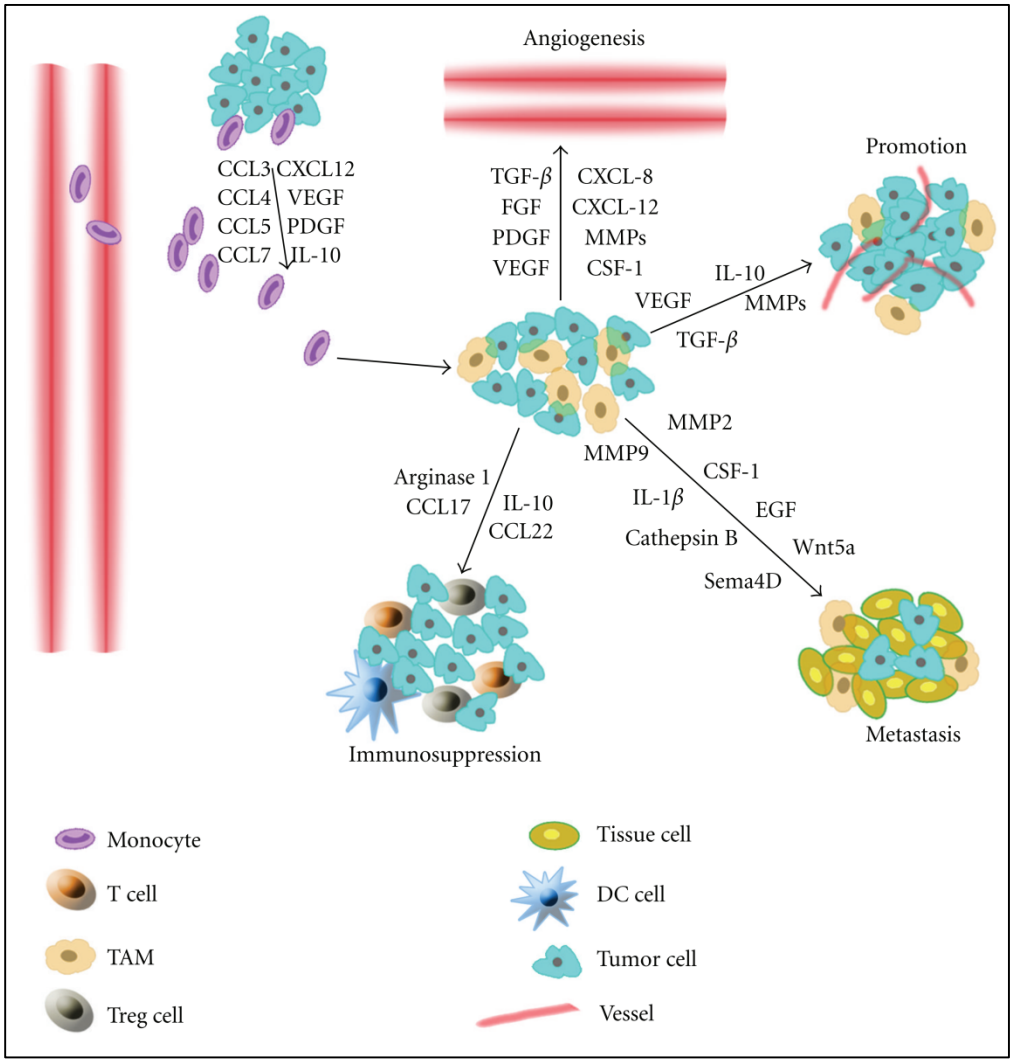

Figure 1.13 : TAM functions in tumor progression (adapted from Hao et al., 2012).

Infiltration with TAM has been assoaciated with poor prognosis observed in Hodgkin disease, glioma, cholangiocarcinoma, and breast carcinoma [145-46]. For the identification of TAMs, CD163 surface marker generally is used [147-48].

1.3.4 Immune response against *Helicobacter pylori*

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 [149] indicating activation of the adaptive response. However, urease is a major activator of macrophages, stimulating cytokine and NO generation [150]. 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 [151].

Chronic infection of *H. pylori* named as "chronic superficial gastritis" [152] in which T cell, B cell, macrophage, neutrophil, mast cell, and dendritic cell (DCs) infiltration is seen [153-54]. $CD4^+$ T cells present more abundantly than $CD8^+$ T cells. $CD4^+/CD25^{hi}/$ $FOXP3^+$ 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 [155]. 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.

1.3.4.1 Innate immune response against *Helicobacter pylori*

Epithelial cells

TLRs present on Gastric epithelial cells can identify pathogen-associated molecular patterns (PAMPs). When bacteria penetrate into the gastric epithelial barrier, the alternative complement pathway is activated, and macrophages and neutrophils are recruited to infected area. However, *H. pylori* do not invade gastric tissue, it localize within the gastric mucus layer. Therefore, contact between *H. pylori* and phagocytic cells do not take place unless gastric epithelial barrier disrupts [156].

High levels of pro-inflammatory cytokines including IFN-γ, TNF-α, IL-1β, IL-6, IL-7, IL-8, IL-18 had been observed in the stomachs of patients infected with *H. pylori* compared to uninfected humans as well as IL-10, which may limit the inflammatory response [157]. Polymorphisms, which reduce the production of anti-inflammatory cytokines such as IL-10, increase the risk of gastric cancer. Also, polymorphisms of IL-1β, TNF-α, and IL-10 combined, increase the risk of cancer 27- fold over baseline [158].

In 2004 Graham et al. conducted a study with 20 human volunteers who were infected with 10^4 to 10^{10} CFU of *H. pylori* to observe course of infection. More than 50% of subjects displayed symptoms including dyspepsia, headaches, anorexia, abdominal pain, belching, and halitosis, during the second week following infection. Lymphocyte and monocyte infiltration was examined in gastric biopsies with significant increase of IL-1β, IL-8, and IL-6 cytokine expression in the gastric

antrum [159]. The numbers of gastric $CD4^+$ and $CD8^+$ T cells were higher following four weeks of infection. These observations indicated development of gastric inflammation a short while after *H. pylori* infection. The upper gastro-intestinal symptoms of acute infection are resulted by initial colonization of the bacteria in the stomach. If *H. pylori* infection persists, it becomes chronic and unless cleared, with the help of the immune response, may lead to gastric carcinoma.

Dendritic cells (DCs)

Dendritic cells are the professional antigen-presenting cells (APCs) and act as a bridge between innate and adaptive immune responses (Figure 1.14) [160-61]. These cells can penetrate epithelial monolayers and engulf bacteria directly [162-63]. Upon recognition of PAMPs by the surface TLRs, dendritic cells activate T cells either in a Th1 or Th2/ regulatory T-cell (Treg) response through exhibiting bacterial products to T cells [164]. *Ex vivo* stimulation of human peripheral blood mononuclear cell-derived DCs with *H. pylori* led to higher production of IL-12 and IL-10 [165-66]. Moreover, activated DCs co-cultured with naïve T cells induced TNF-α, IFN-γ, and IL-2 secretion, initiating Th1 response [167].

E*x vivo* stimulation of mouse bone marrow- derived DCs with *H. pylori* led to higher production of pro- inflammatory cytokines such as IL-1α, IL-1β, and IL-6 b with improved phagocytosis [168]. However, an *Ex vivo* study observed skewing of Th17/Treg balance towards Tregs by activated DCs, thereby contributing to bacterial immune evasion [169] and leading to chronic inflammation and cancer risk.

Neutrophils

Neutrophils are the first-responders of immune response that migrate to the site of inflammation and clear invading pathogen through phagocytosis and releasing enzymes that kill microorganisms. During the onset of *H. pylori* infection, neutrophils migrate into the gastric mucosa and initiate inflammation (Figure 1.14), although bacteria can persist even when neutrophils are present in the localized environment by manipulating phagocytosis and oxidative reactions. *H. pylori* secrete neutrophil-activating protein (HP-NAP), a virulance factor that promotes neutrophil recruitment and induces production of reactive oxygen radicals [170-71]. Recognized by TLR2, HP-NAP upregulates IL-12, IL-23, and TNF-α [172], therefore promoting Th1 cells, increased production of IFN-γ, TNF-α and cytolytic activity.

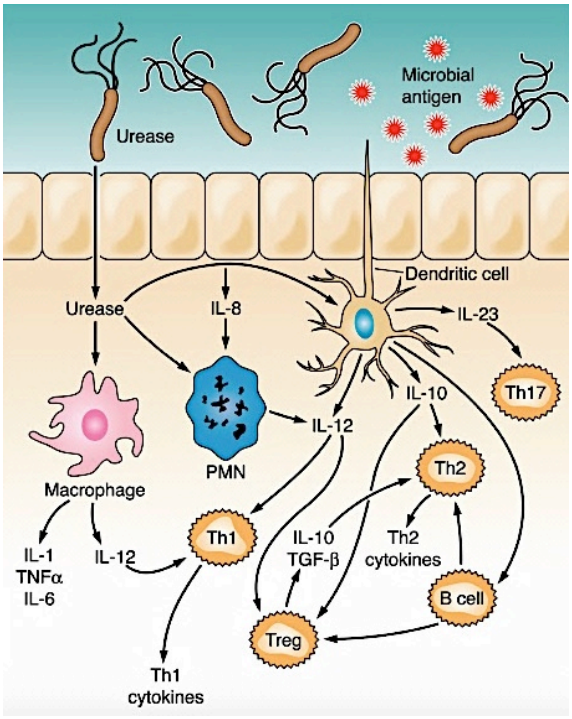

Figure 1.14 : Innate and adaptive immune response stimulated by *Helicobacter pylori* (adapted from Peek et al., 2010).

Macrophages

Monocytes, macrophages and dendritic cells present in the lamina propria of the gastric mucosa play important roles in antigen presentation [153,164]. During the onset of *H. pylori* infection, macrophages and DCs activate adaptive immune response through production of IL-12 [173], initiating Th1 response and IFN-γ production. The virulence factor HP-NAP mediated IL-12 and IL-23 secretion by neutrophils and monocytes contributes to Th1 polarization as well (Figure 1.14) [172]. Macrophages also amplify the inflammatory response by production of cytokines such as IL-1β, TNF-α, and IL-6 [174].

H. pylori stimulates the expression of inducible NO synthetase (iNOS) in gastric mucosal epithelial cells, vascular endothelial cells or infiltrated inflammatory immune cells. Increased iNOS mediates NO production using L-arginine as a substrate in these cells, macrophages being the chief source among them all [175]. Although *in vitro* experiments observed that NO produced by macrophages could potentially kill *H. pylori* [176] but not in human stomach, hence the chronic inflammation persists in the gastric mucosa. The bacterial LPS also suppresses NO production by stimulating polyamine-mediated apoptosis of macrophages [177] and

iNOS expression by inducing arginase and ornithine decarboxylase (ODC) (Figure 1.13) [178].

The gastric biopsy specimens taken from *H. pylori* positive individuals showed $CD163^+$ alternatively activated (M2 type) macrophages. Human monocytes also secreted IL-1β, IL-6, IL-10, and IL-12p40 upon infection (partially secreted as IL-23) but not IL-12p70. Upregulation CD14 and CD32 and downregulation of CD11b and HLA-DR are observed. Human M2 macrophages exhibited CD14 and CD206 upregulation with increased IL-10 but less pro-inflammatory cytokines than M1 macrophages [179].

B7-H1(CD80) and B7-H2 (CD86) expressions were markedly increased on T cells, macrophages, and dendritic cells after activation [180] upon *H. pylori* infection. In vitro studies showed *H. pylori* stimulation increased expression of co-stimulatory molecules and MHC-II in human monocytes [181] but failed to up-regulate on gastric mucosa. This could be due to the prevalence of Tregs and IL-10 in the *H. pylori* infected gastric mucosa that inhibits the up-regulation of co-stimulatory molecules and MHC-II on macrophages [182].

In mice, vaccination against *H. pylori* polarizes gastric macrophages to M1. Also, in human atrophic gastritis where the mixed M1/M2 subsets present is replaced by an M1 phenotype, creating a tumor-promoting inflammation. Therefore, shifting macrophage polarization from M1 to M2 could be a therapeutic target in chronic *H. pylori* infection.

Although *H. pylori* can be internalized into phagosomes by macrophages, they still can escape from phagocytosis by fusing phagosomes into "megasomes" that contain large numbers of live bacteria. The CAG+ strains of *H. pylori* secretes VacA toxin that prevents the fusion of phagosomes to lysosomes necessary for bacterial killing. This disruption of phagosome maturation is lost when cells are infected with isogenic vacA− mutant strains [183].

1.3.4.2 Humoral immune response against *Helicobacter pylori*

H. pylori-specific serum IgM antibodies present in the infected ones turns on humoral Immune response concomitantly with the adaptive response, usually observed after 4-weeks of post-infection approximately [184]. Serum IgA and IgG antibodies were directed toward many different *H. pylori* antigens [185-86]. These

H. pylori-specific IgA or IgM antibodies secreted from activated plasma were directed toward many different *H. pylori* antigens in the gastric mucosa [185]. This is further supported by the evidence found in gastric juice where secretory IgA antibodies were present, indicating the onset of local secretory IgA response in the stomach [187].

1.3.4.3 Adaptive immune response against *Helicobacter pylori*

T cells

IFN-γ, the key defining cytokine of Th1 response was seen to be highly expressed from gastric T cells of *H. pylori*-infected individual [188-89]. IFN-γ could potentially reduce bacterial colonization but augment the severity of *H. pylori*- induced gastric inflammation, supported by the study performed on IFN-$\gamma^{-/-}$ knockout mice [190-91]. IFN-γ also activates macrophages indirectly to secrete more pro-inflammatory cytokines and reduces expression of anti-inflammatory cytokines (e.g TGF-β) (Figure 1.14), together of which may increase the severity of gastritis [192].

In order to investigate the Th2 response on *Helicobacter* infection, mice co-infected with nematode (Th2 response inducer) and *H. felis* had sufficiently reduced Th1 cytokine profile (IFN-γ, TNF, and IL-1β), boosted Th2 cytokines expression (IL-4, IL-10, and TGF-β) with less gastric inflammatory scores than only *H. felis* infected mice [193]. However, Th2 response may not be required for protection from *H. pylori* infection as seen in the study where IL-4 and IL-5 knockout mice were successfully protected after immunization [194]. Another study on MHC class $I^{-/-}$ mice model revealed that $CD4^+$ T, but not $CD8^+$ T cells cells are necessary for protection against *Helicobacter* infection [195].

Th17 response (IL-17 expression) was observed when mice were immunized with *H. pylori* lysate and when $CD4^+$ T cells isolated from spleens were co-cultured with *H. pylori*-treated DCs or macrophages, with augmented gastric inflammation and reduced colonization [196].

H. pylori infected individuals have increased levels of $CD4^+$ $CD25^{high}$ FOXP3 expressing Tregs in the gastric and duodenal mucosa [197], depletion of which may cause increased gastritis severity, higher cytokine profiles as well as serum IgG1 and IgG2c levels and reduced bacterial colonization in a mice model [198]. This study suggests that an important role of Treg response to uphold a sense of balance

between host and bacterium, allowing *H. pylori* to survive while at the same time preventing the risk of detrimental inflammation.

B cells

B-cell-deficient (μ-MT) mice progress to severe gastritis upon *H. pylori* with eventual clearance of infection due to absence of antibodies produced by wild-type mice which block the inhibitory IgG receptor (FcγRIIb) on leukocytes and increase anti-inflammatory cytokines such as IL-10 [199]. IL-10 is a potent anti-inflammatory and immunoregulatory cytokine that reduces *H. pylori*-induced inflammation, supported by a study conducted on *Helicobacter*-infected IL-$10^{-/-}$ mice that develop severe gastritis [200]. Similar results have also been observed in *H. pylori*-infected IL-$4^{-/-}$ mice, suggesting a role of both IL-10 and IL-4 in down-regulating gastric inflammation [201-02].

In the study of gastric MALT lymphoma, chronic infection of *H. pylori* was observed to protect splenic B cells from apoptosis, indicating development of B-cell activation/survival phenotype associated in MALT lymphoma [203].

In mouse, B cells recognized *Helicobacter* by TLR2 and produce anti-inflammatory IL-10 through activation of myeloid differentiation primary response gene 88 (MyD88), both *in vitro* and *in vivo.* Decreased IL-10 expression had observed in both TLR2 or MyD88 knock-out mice (TLR$2^{-/-}$or MyD$88^{-/-}$) with accelerated gastric histopathology. IL-10 producing regulatory B cells inhibit strong Th1-mediated immune response via suppression and conversion of $CD4^{+}$ T cells into IL-10-producing T regulatory 1 (Tr-1) (Figure 1.14) [33]. Bregs work in harmony in order to restore the immune balance in *Helicobacter*-infection by minimizing excessive gastric immunopathology while preventing bacterial clearance in the gastric mucosa [33]. This study was significant for demonstrating a B cell subset with regulatory function in a bacteria-associated disease model for the first time.

1.4 Purpose of Thesis

Chemerin has been regarded as one of the major attractants for macrophages observed in many disease studies. The effect of *Helicobacter pylori (H. pylori)* on macrophage polarization is somewhat investigated in both human and mice studies as

well as the role of chemerin on macrophage polarization. However, no studies have yet investigated the effect of chemerin on the polarization status of macrophages in *H. pylori* induced pathogenesis. Therefore, in this study, we investigated the expression of chemerin on *H. pylori* treated gastric epithelial cell lines first to observe the role of chemerin and *H. pylori* on gastric immunity. On the second part, we investigated the role of both chemerin and *H. pylori* on monocytic cell line THP-1 and observe the differences on its polarization status according to cytokine profiles. Expression levels of M1 type specific cytokines (IL-1β and IL-6) and M2 type specific cytokine (IL-10) were assessed.

2. MATERIALS AND METHODS

1.5 Materials

1.5.1 Bacteria

Helicobacter pylori Wild type (G27) and its ΔCagA mutant were kindly provided by Prof. Dr. Anne Müller from University of Zurich. Bacteria were spreaded on Columbia Agar plates supplemented with 1000X and 200X antibiotic cocktails. Ingredients of Columbia Agar (BD, U.S.A.) plate, 1000X and 200X antibiotic cocktails were given in Table 1 and Table 2, respectively. Solutions and chemicals used in the maintenance of *Helicobacter pylori* are given in Tables 2.1, 2.2 and 2.3.

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

Table 2.3 : Components of 200X Antibiotic Cocktail.

Content	Amount
Vancomycin	100 mg
Cefsulodin	50 mg
Polymixin B	3.3 mg
H_2O	50 ml

1.5.1.1 Antibiotics

Antibiotics used in *Helicobacter pylori* Columbia agar plate are listed in Table 2.4.

Table 2.4 : Antibiotics used in *Helicobacter pylori* culture.

Content	Supplier company
Trimethoprim	HiMedia
Amphotericin B	HiMedia
Vancomycin	HiMedia
Cefsulodin	HiMedia
Polymixin B	HiMedia

1.5.1.2 Liquid culture

Ingredients of liquid culture of *Helicobacter pylori* are shown in Table 2.5 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 2.5 : Components of *Helicobacter pylori* liquid culture.

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

1.5.1.3 Freezing Helicobacter pylori

The medium suitable for freezing *Helicobacter pylori* for stock purposes is depicted in Table 2.6 with its ingredients. Upon preparation the medium can be stored at 4°C.

Table 2.6 : Freezing medium for *Helicobacter pylori*.

Component	Amount
Brucella Broth	25 ml
Glycerol	25 ml

1.5.2 Cell lines

KATO III, AGS, MKN 45 and THP-1 cell lines were used for this study. KATO-III human gastric adenocarcinoma cell line was kindly provided by Doç.Dr. Rengül Çetin Atalay of Bilkent University, Ankara, Turkey.

1.5.3 Cell culture

Culture media and solutions used in cell culture studies can be seen in Table 2.7 and buffers used in cell culture studies are listed in Table 2.8.

Table 2.7 : 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
DMSO	Fisher-Scientific

Table 2.8 : Buffers and media used in cell culture studies.

Buffers	Content and Amount
1X PBS	9,55g in 1L ddH_2O
Complete RPMI growth medium	RPMI medium with 10% FBS, 1% Penicillin/Streptomycin
Complete DMEM growth medium	DMEM medium with 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)
Trypsin-EDTA	0.25% (1X)
Detaching buffer	10 mM EDTA in 1X PBS

1.5.4 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
Incubator with CO_2	BINDE
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

Table 2.9 (cont'd): Laboratory equipments used in the study.

Equipment	Company
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 ˆ
Flow Cytometer	BD Accuri C6
Nitrogen Tank	Air Liquid
Microplate Spectrophotometer	BIO-RAD Benchmark Plus
Fluorescent Microscope	Zeiss Axiovert A1 Inverted
Confocal Microscope	Leica SP2

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
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 for Real time)	Nunc
6-well and 24-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

1.5.5 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

Table 2.11 (cont'd) : Commercial kits used in this study.

Kit	Supplier Company
PCR kit	New England Biolabs
Power SYBR® Green PCR Master	Applied Biosystems

1.5.6 Reagents used in cloning

Necessary reagents for molecular cloning was kindly provided by Associate Professor Doc Asli Kumbasar.

Table 2.12 : Reagents for cloning.

Reagents	Supplier Company
HindIII-HF and XhoI Restriction enzymes	New England Biolabs
CutSmart Buffer	New England Biolabs
Nucleospin Gel and PCR Clean-up, 50 preps	Macherey Nagel
DNA Ligation Kit	Clonetech
Zyppy Plasmid Miniprep Kit	Zymoresearch
PEI (Polyethylenimine)	Sigma-Aldrich

Table 2.13 : Reagents for Luria Broth agar and medium.

Reagents	Amount (For 500 ml)	Supplier Company
Tryptone	5 g	Lab M Neogen
Yeast	2.5 g	Lab M Neogen
Nacl	5 g	Merck Millipore
For medium, Agar no. 2 (Bacteriological)	7.5g	Lab M Neogen
Water	Upto 500 ml	

1.5.7 General chemicals

General chemicals used in this study are listed with their supplier companies in Table 2.14.

Table 2.14 : General chemicals used in this study.

Chemical	Supplier Company
EDTA	Applichem
Ethanol (absolute)	Merck
NaCl	Merck
Glycerol	Merck
Phosphate-Buffered Saline (PBS) 10X	Lonza
Tween-20	Fisher-Scientific

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

Chemical	Supplier Company
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
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Fixation Buffer (4%)	Biolegend
Permeabilization Buffer (10X)	Biolegend
Isopropanol	Sigma-Aldrich
HCl	Sigma-Aldrich
NaOH	Sigma-Aldrich
Xylol	Sigma-Aldrich
Sodium Citrate	Sigma-Aldrich
Triton-X 100	Sigma-Aldrich
Donkey Serum	Sigma-Aldrich

1.5.8 Primers

Primers used in this study are given in table 2.15.

Table 2.15 : Primers used in the study and their sequences.

Primer Name	Sequence (5'-3')	Spec.	Tm	Exp. Size
Chemerin Fw	GGAATATTTGTGAGGCTGGAAT	h	60°C	139 bp
Chemerin Rv	CAGGCATTTCCGTTTCCTC			
Chemerin Fw (for cloning)	TATAAGCTTATGCGACGGCTGCTGATCCCT	h	68°C	105 bp
Chemerin Rv (for cloning)	ATACTCGAGTTAGCTGCGGGGCAGGGCCTT			
Chem Rec Fw	GAGAGATGGGCTTCCCTCTGGTC	h	65.3°C	103 bp
Chem Rec Rv	TGAATGAACTGCTTTCTGGGCA			
IL-1β Fw	GGCTTATTACAGTGGCAAT	h	57.1°C	124 bp
IL-1β Rv	GAAGCCCTTGCTGTAGTG			
IL-6 Fw	CCTGAGAAAGGAGACATGTAA	h	54°C	103 bp
IL-6 Rv	AGTCTCCTCATTGAATCCA			
IL-10 Fw	AAGCCTGACCACGCTTTCTA	h	60°C	120 bp
IL-10 Rv	GCTCCCTGGTTTCTCTTCCT			
18s rRNA Fw	GGCCCTGTAATTGGAATGAGTC	m/h	60°C	146 bp
18s rRNA Rv	CCAAGATCCAACTACGAGCTT			
Oligo dT	TTTTTTTTTTTTTTTTTTT	-	-	-

1.5.9 Antibodies

Antibodies used in this study are given in table 2.16.

Table 2.16 : Antibodies used in this study.

Antibody	Supplier Company	Application
Rat anti-mouse/human CD11b-biotin	Biolegend	FACS
Rat anti-mouse/human CD14-PE	Biolegend	FACS
Rat anti-mouse/human CD68-biotin	Biolegend	FACS
Anti-mouse Alexa Fluor 488	Biolegend	FACS
streptavidin-PE	Biolegend	FACS
streptavidin-APC	Biolegend	FACS
Goat anti-human Chemerin	RnD	WB
Rabbit anti-human GAPDH	Cell Signaling	WB
Anti-Goat IgG-HRP	Cell Signaling	WB
Anti-Rabbit IgG-HRP	Cell Signaling	WB
Donkey anti-Goat AF488	Invitrogen	FACS, IF, IHC

1.6 Methods

1.6.1 Maintenance of Helicobacter pylori

Helicobacter pylori 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. pylori* 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.

1.6.2 Sonication of Helicobacter pylori

Sonication procedure was started with 120-200 ml liquid culture of *Helicobacter* strains. Before sonication *Helicobacter pylori* (10 µl)'s mobility was checked under light microscope. 120- 200 ml liquid culture of *Helicobacter pylori* was aliqouted to 15 ml falcons. Falcons were centrifuged at 3000 rpm for 10 minutes, 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.

1.6.3 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 2.17 : Dilutions of BCA assay standards.

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
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

1.6.4 KATO III, AGS, MKN 45, THP-1 and HEK293T cell culture

Three gastric cancer cell lines KATOIII, AGS and MKN45 were used in this study. All cell lines except HEK293T were cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin under a humidified atmosphere containing 5% CO_2 at 37 ° C. HEK293T cells were cultured in DMEM under the same condition mentioned above. Confluent cells were passed routinely at a split ratio recommended by the distributor after trypsin/EDTA digestion for attached/mixed population. All cell lines are adherent in nature except THP-1 cells. cells were frozen in freezing medium containing complete DMEM/RPMI with %50 FBS and %10 DMSO.

1.6.4.1 Cell thawing

9 ml required RPMI/DMEM medium was added to a 15 ml falcon tube. Frozen cells were thawed with the warmth of hand briefly. The cells were dissolved with completely in medium and centrifuged at 1000 rpm for 5 min to remove DMSO (which is toxic for cells) from cells. The supernatant was discarded carefully following centrifugation. ~5 ml fresh medium for T25 flask or ~12 ml fresh medium for T75 flask was added to the pellet and the pellet was dissolved in the medium. The viability of cells was checked under the microscope before placing the flask to the incubator (37°C + 5% CO_2).

1.6.4.2 Cell passage

Cell density was determined on light microscope. When reached to almost 80% confluence, suspension cells were put on a 15 ml falcon tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was dissolved in 4

ml culture medium and 1 ml cells (2×10^6 cells) were taken in a new flask with a fresh culture medium. For adherent cells, the flask was washed once with 12 ml 1X PBS and 1500 µl of trypsin-EDTA 10X was added to flask. The flask was incubated at 37^0 C for 4 minutes. Then the flask was taken and mechanic force was applied to flask. 3-4 ml of culture medium were added to inhibit the effect of trypsin. 1ml cells ($2x10^6$ cells) were taken in a new flask with a fresh culture medium

1.6.4.3 Cell freezing

The medium was discarded from the flask. PBS was added to flask to wash cells and then discarded. 1,5 ml trypsin was added to T75 flask and incubate for 4 min. at 37°C. Attachment of cells were controlled under the microscope. Approximately 9 ml medium was added to T75 flask to inactivate trypsin and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and 1 ml freezing medium was added (supplemented with growth media and 10% DMSO) to the pellet and dissolve the pellet in the freezing medium. Frozen cells were stored at -80°C.

1.6.4.4 Cell counting

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

1.6.4.5 Treatment of KATO III, AGS and MKN 45 cell lines with *Helicobacter pylori* sonicate

When cells were fully-grown in culture media, cells were detached, counted and seeded into 6 well plates as $4x10^5$ cells/well. The cells were rested for 24 hours before treatment started so that they can gain stability and attach to the surface of the well. After 24 hours of resting, each well was treated with 10 ug sonicates of *H. pylori* strain, G27 which is the wild type and G27-ΔCagA strain along with the control group. After designated period of incubation, in this case 6,24,48 and 72 hours cells, were harvested.

1.6.4.6 Cell harvesting

After designated time frame of incubation cells were harvested. At first medium/supernatants were collected from each group. After removing the supernatant, each well was washed with PBS 1X (0.5-1 ml/well) and then PBS was discarded. After washing, 400 µl of Trypsin-EDTA was added to wells for the detachment of cells and plates were incubated at 37ºC for 2-3 minutes. The plates were then checked under the microscope to confirm detachment of the cells and adding same amount of fresh medium neutralized trypsin. After that, the cells were collected and pelleted by centrifugation at 3000 x rpm for 8 min. Cell pellets were stored in -800C for further experiments.

1.6.5 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.

1.6.6 cDNA synthesis

Synthesis of cDNA was performed according to manufacturer's instructions. Amounts used in synthesis reaction are given in Table 2.18. Synthesis conditions are given in Table 2.19.

Table 2.18 : 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 2.19 : cDNA synthesis reaction conditions.

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

1.6.7 Conventional PCR

In order to check the cDNA, 18S rRNA primers were used for the conventional PCR. Apart from that, the mRNA expression of chemerin, chemerin receptor, IL-1β, IL-6 and IL-10 was measured. Required PCR reagents are shown in below Table 2.20 and Conventional PCR conditions are indicated in Table 2.21.

Table 2.20 : Reagents for PCR.

Component	Amount
10X Buffer	2.5 ul
10 mM dNTP	0.5 ul
10 uM Forward primer	0.5 µl
10 uM Reverse primer	0.5 µl
Taq Polymerase	0,125 µl
ddH_2O	19.875 µl
Template	1 µl

Table 2.21 : Conventional PCR reaction conditions.

	Temperature	Time	
Initial Denaturation	94°C	30 sec	1 cycle
Denaturation	94°C	30 sec	
Annealing	54-68°C	45 sec	35 cycle
Extension	72°C	45 sec	
Final Extension	72°C	5 min	1 cycle
Hold	4°C	∞	

1.6.8 Agarose gel electrophoresis

2% agarose gel is sufficient for the observation of our expected PCR products. The agarose gel contains 2 g agarose in 100 ml 1X TAE. The gel solution was boiled in the microwave until the agarose was dissolved. After the cooling, the solution was poured in to the gel cassette. Marker was loaded as 2.5 µl always in the first well of the gel to evaluate the length of the PCR products. 7.5 µl PCR products were mixed with 1.5 µl 6X loading dye. Loading dye was used to facilitate the observation. 1 ul Tibo Gold was used for every samples along with the marker for detection. Gel was run at 90V for 15 minutes and then 110 V for another 15 minutes. The bands were observed with UV trans-illuminator by the help of UV PhotoMW software.

1.6.9 Real time PCR

Relative expression levels of 18s RNA, chemerin and IL-10 were analyzed with Real-time PCR. Amounts used in reaction are given in Table 2.22. PCR conditions performed are given in Table 2.23.

Table 2.22 : 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 2.23 : 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	∞

1.6.10 Protein isolation

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

1.6.11 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting

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

In order to detect the specific proteins Western Blot technique is used in this study. After SDS-PAGE, proteins were transferred from gel to the nitrocellulose membrane using Wet Transfer blotting (at 160V for 1h at 4°C). Transferred proteins were stained with Ponceau S. After visualizing the proteins, membrane was blocked at 4°C overnight in blocking solution (TBS/T containing 5% non-fat dry milk) to prevent non-specific binding. Then its incubated with 1:1000-1:2000 diluted primary antibodies (in TBS/T containing 5% BSA) at 4°C overnight with gentle shaking. The day after, membrane was washed with TBS-T buffer 3 times for 10 minutes in the shaker. 1:3000 diluted secondary antibody which was conjugated with HRP enzyme prepared again in blocking solution and then membrane was incubated with secondary antibody solution for 1-2 hour at 4°C in the shaker. After washing steps of membrane 3 times with TBS/T, the HRP substrate 20X LumiGLO ® Reagent (Cell Signaling) was diluted in to 1X with distilled sterile water (ddH_2O). This substrate is required for the visualization of the proteins. The membrane was exposed to X-ray film and then developed in Kodak Medical X-ray Processor according to manufacturer's instruction. For the detection of other proteins in the same membrane, membrane can wash with TBS/T and can be incubated in other primary antibody.

1.6.12 Molecular cloning of hChemerin

Molecular cloning refers to the process by which recombinant DNA molecules are produced and transformed into a host organism, where they are replicated. A molecular cloning reaction is usually comprised of two components: 1) The DNA fragment of interest to be replicated and 2) A vector/plasmid backbone that contains all the components for replication in the host. Steps in molecular cloning of Chemerin are as followed.

1.6.12.1 cDNA synthesis

Intact RNA of high purity is essential for generating cDNA for cloning applications. So for the optimum results, High purity sample RNA was converted to cDNA according to the cDNA synthesis mentioned before.

1.6.12.2 Conventional PCR

Conventional PCR was performed for amplification of Chemerin using prepared cDNA and primers designed for cloning purposes. The sequence of primers was given to primer sections. The reagents and PCR set up was given before.

1.6.12.3 Vector and insert restriction digest

For the purpose of cloning, pcDNA3.1 (+) vector was used with restriction sites HindIII (911) and XhoI (985). Vector was digested with the mentioned restriction enzymes to prepare circular DNA. The insert was digested with the same restriction enzymes. The reagents used in this step are followed.

Briefly, vector and insert was incubated with all the reagents for 60 minutes at 37^0C as required. At the end of insert restriction digest, enzymes need to be deactivated so they don't interfere with ligation. In most cases it could be done by heat deactivation. The gel purification kit can also serve as an enzyme removal kit. Amounts used in reaction are given in Table 2.24.

Table 2.24 : Reagents for vector and insert restriction digest.

Vector restriction digest			**Insert restriction digest**		
Reagents	**50 ul**	**Final conc.**	**Reagents**	**50 ul**	**Final conc.**
10X Cutsmart buffer	5 ul	1X	10X Cutsmart buffer	5 ul	1X
Vector DNA	5 ul	Upto 2 ug	cDNA insert	5 ul	Upto 5 ug
Restriction enzyme HindIII	0.5 ul (5 units)	0.05 unit	Restriction enzyme HindIII	1 ul (10 units)	0.1 unit
Restriction enzyme XhoI	0.5 ul (5 units)	0.05 unit	Restriction enzyme XhoI	1 ul (10 units)	0.1 unit
Nuclease free water	Upto 50 ul		Nuclease free water		Upto 50 ul

1.6.12.4 Gel purification

Gel purification allows us to separate vector DNA from uncut fraction, clean PCR fragment from unspecific PCR products, primers and uncut fragments, and also serves as a quality control for the molecular weight of vector and insert. Briefly, both digested vector and insert were electrophoresed using 1% agarose gel prepared by the mentioned way. The uncut vector and insert were electrophoresed as well to distinguish cut fragments and ensure digestion was done successfully. Then the

desired DNA fragments were cut from the gel and purified by Nucleospin Gel and PCR Clean-up, 50 preps (Macherey Nagel). NanoDrop measured the amount of purified DNA.

1.6.12.5 Ligation

During the ligation step the linerized vector and the insert is mixed together and a DNA ligase is added. The ligase will fuse their ends forming a circular plasmid. Ligation was performed using Clonetech DNA Ligation Kit, version 1 (50 reactions). The target backbone was 5428 bp long and the insert was 510 bp (both linerized and gel purified). According to the protocols 150 ng backbone vector should be ligated with 3-fold excess of insert but bp length should also be considered.

So regarding length,

3 fold insert= (510 bp/ 5428 bp) x 150 ng =14.09 ng

All the components were incubated for 5-10 minutes. A sample of vector with no insert has also been ligated to use as a control. Reagents and amounts used in reaction are given in Table 2.25.

Table 2.25 : Reagents used for Ligation.

Reagents	Final volume 20 ul
Ligation buffer	10 ul
Target backbone	150 ng
Insert	14.09 ng
T4 DNA Ligase	1 ul
Nuclease free water	To 20 ul

1.6.12.6 Transformation

During this step ligated plasmid is added to chemically competent E.coli cells, and expose the cell suspension to a heat shock. The heat shock will introduce the DNA inside the cells. At first, the chemically competent *E. coli* cells were thawed on ice for 15 min and ligation mix was added to the competent cells by tapping gently on the tube and incubated on ice for 30 minutes. The cells were then subjected to heat shock at at 42^0C for 60 seconds in water bath and placed them again on ice for 5 minutes. After that 4X (approx. 280 ul) volume of LB medium containing antibiotics was added and incubated for 45 min at 37^0C on a shaker to develop antibiotic resistance. Then the cells were centrifuged at 13000 rpm for 1 min, supernatant was discarded and pellet was resuspended in the remaining LB medium (200 ul). The

approximate volume of LB medium containing cells was transferred to LB plates with the help of a spreader in front of a gas burner. The plates were incubated in the lid up position for 30 minutes at 37^0C to dry the liquid on the plate surface and flipped them upside down and waited for 24 hours for bacterial growth. After 24 hours, single colonies were picked from the plate, resuspended in LB medium and incubated for 24-48 hours at 37^0C on shaker and then proceed to the plasmid purification step (miniprep).

1.6.12.7 Plasmid miniprep

Briefly, The bacterial culture grown on LB medium was centrifuged to maximum speed for 30s, supernatant was discarded and the pellet was resuspended in 600 ul of water. Then,100 ul 7x lysis buffer was added to each 600 ul and mix by inverting the tube by 4-6 times. 350 ul of cold neutralization buffer was added, mixed thoroughly and centrifuged to 11,000-16,000 g for 2-4 minutes. The supernatant was collected and transferred to zymospin column on a new collection tube and centrifuged for 15 seconds. Flow through was discarded and 200 ul of Endo-wash buffer was added to the column and centrifuged for 30 seconds followed by 400 ul of zappy wash buffer and centrifuged for 1 minute. After that the column was transferred to a clean 1.5 ml microcentrifuge tube then 30 µl of elution Buffer was added directly to the column matrix and let stand for one minute at room temperature followed by centrifuge for 30 seconds to elute the plasmid DNA.

1.6.12.8 Transfection

Hek293T cell line was transfected with plasmid DNA using PEI (Polyethylenimine). Briefly Hek293T cell line was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were split one day before transfection in DMEM/10% FBS medium and seeded in a 6 well plate. On the day of transfection, 3ug of plasmid DNA was diluted in serum free DMEM and 9 ug of PEI was added to the diluted DNA solution as a 3:1 ratio of PEI (ug):total DNA (ug). The mixture was immediately mixed by vortexing and incubated for 15 minutes at room temperature. The DNA/PEI mixture was added to cells and transfected cells were harvested at 48 hours post-transfection.

1.6.13 Immunohistochemistry

Immunohistochemistry was performed on formalin fixed paraffin embedded human gastric tissue samples. Briefly, the frozen slides were incubated for 1 hour at 60^0C on water bath. After that, samples were deparaffinized by washing 3 times in xylene for 10 minutes followed by washing 2 times for 10 minutes in 100%, 80% and finally 70% ethanol consequently. Finally samples were rinsed in water thoroughly for 10 minutes. Antigen retrieval was achieved by immersing the slides in sodium citrate solution and boiled for 1 minute with maximum heat and then reduced to 20% for 3 minute. The slides were cooled at room temperature followed by washing in 1x PBS for 10 minutes. Afterwards, slides were incubated with 0.2% Triton X-100 solution for 15 minutes followed by subsequent washing with 1x PBS 3 times for 10 minutes. Non-specific binding was prevented by incubated slides in a blocking solution (contains 10% donkey serum and 3% BSA in 1x PBS) for 1 hour at RT. Slides were again washed with PBS 3 times and incubated with human anti-chemerin antibody (1:100 dilution) and Pan-cytokeratin (10 ul/sample) overnight. After overnight incubation, slides were thoroughly washed with PBS 3 times and then incubated in anti-goat AF488 for 1 hour. Slides were rinsed 3 times with PBS, dried and mounting medium containing DAPI was added and covered with coverslips.

1.6.14 Macrophage polarization performed on THP-1 cell line

Thp-1 cells were cultured according to the protocol mentioned before. When cells are fully grown $3x10^5$ were seeded into 12 well plates one day before treatment. Next day, cells were differentiated into macrophages by stimulation with 20 nM PMA (phorbol 12-myristate 13-acetate) for 48 hrs. A group without PMA treatment was also kept as control. Differentiation into macrophages was determined by increased cell attachment to the flasks and by changes in cell morphology: mainly larger cell size, number of pseudopodia highly increased. After 48 hours of stimulation, medium was discarded, cells were washed with PBS 1X and cultured for another 24 hours in fresh medium without PMA to induce further efficient differentiation. After that Cells were stimulated for 24 hours with different stimulants: LPS (100ng/mL) recombinant IL-4 (20 ng/mL) and chemerin (50ng/mL) along with or without *H. pylori* sonicates (G27 wild type and G27-ΔCagA, 10 ug/ml). After 24 hours of incubation cells were harvested and preceded to other experiments.

1.6.15 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 CD11b, CD14, CD68 and a fraction of cells were left unstained.

given in Table 2.16.

1.6.16 Flow cytometry analysis

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

1.6.17 Densitometric analyses

Densitometric analyses were performed to detect the densities of protein bands of the target proteins or to determine semi quantitative mRNA expression levels which are normalized to the density of housekeeping protein (GAPDH). Densitometric analyses were performed using Adobe PhotoShop CS5 Software. Densitometric values of bands were determined by multiplying index and mean levels of each protein band and these values were normalized to their corresponding housekeeping controls (GAPDH levels).

1.6.18 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

1.7 Expression of Chemerin in Gastric Epithelial Cells

It was known from the literature that tumor epithelial cells secret chemerin [204,205] and increased plasma chemerin has been observed in gastric cancer patients [206]. To further understand the role of chemerin on gastric immune response to infection, firstly, the expression level of chemerin was investigated in three gastric epithelial adenocarcinoma cell lines, namely KATO III, AGS and MKN45. Conventional PCR results performed on these three cell lines revealed the presence of chemerin. Moreover, KATO III and AGS cells prominently express chemerin compared to MKN45 cell line as shown in figure 3.1.

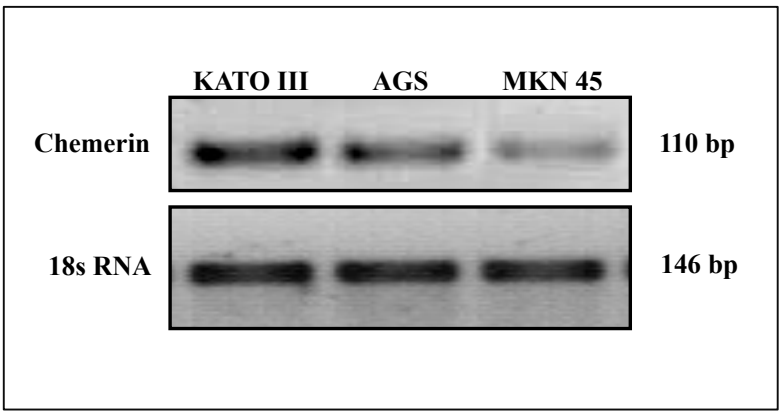

Figure 3.1 : Expression levels of chemerin in KATO III, AGS and MKN45 cell lines performed by conventional PCR. RNAs were isolated from KATO-III, AGS and MKN45 cells (5×10^5 cells), converted to cDNA and Chemerin and 18S rRNA expression levels were investigated. Figure is representative of three independent experiments (n=3).

1.7.1 Chemerin expression in *H. pylori* -treated gastric epithelial cells

Various inflammatory disease models were shown to be closely associated with increased chemerin expression according to many literatures [48, 54, 57, 84]. Therefore, the expression status of chemerin on *H. pylori* induced gastric pathogenesis was evaluated. Firstly, the expression level of chemerin in KATO III and AGS cells treated with *H. felis* sonicate and various *H. pylori* sonicates,

including wild type G27 strain, along with its single virulence factors mutants, for example CagA deletion mutant G27-ΔCagA, PAI deletion mutant G27-ΔPAI and CagE deletion mutant G27-ΔCagE (Figure 3.2). As shown in the figure 3.2, the level of chemerin expression in *H. pylori* sonicate treated KATOIII (Figure 3.2 (A)) and AGS cells (Figure 3.2 (B)) was increased compared to their control groups. Elevated expression level of chemerin was more prominent in KATO III cell line, although both cell lines exhibited higher level of expression in wild type and ΔCagA treated samples. AGS cells did not show noticeable chemerin expression in G27-ΔPAI and G27-ΔCagE treated groups (Figure 3.2 (B)) as shown in densitometric analysis. As *H*. *pylori* virulence factor CagA was associated with the development of several gastric malignancies, the rest of the experiments were carried out using wild type and ΔCagA mutant sonicates.

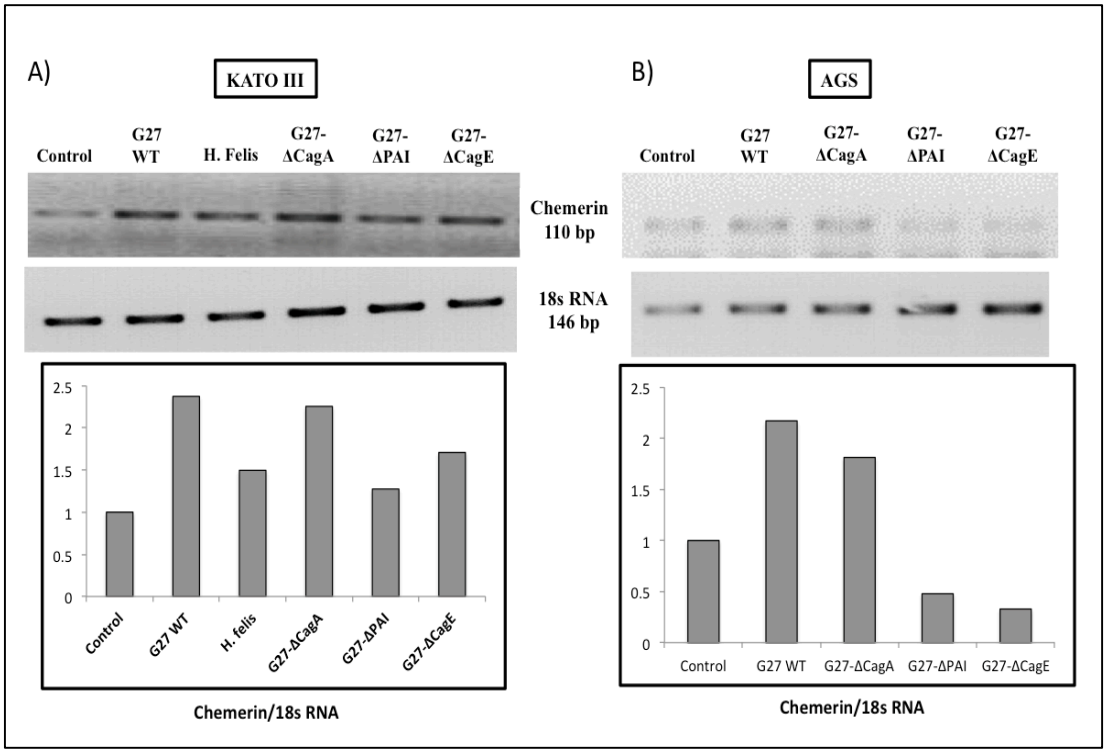

Figure 3.2 : Expression of chemerin in *H. pylori* sonicates -treated KATO III (A) and AGS (B) gastric epithelial cell lines. KATO-III and AGS cells were seeded on 6-well plates as 5×10^5 cells and each group was treated with 10 ug/ml of different *helicobacter* sonicates or left unstimulated (medium) for 6 hours. Then, cells were harvested and RNA isolations were performed. Following RNA isolation, they were converted to cDNA and analysed for Chemerin and 18S rRNA expressions using conventional PCR. Densitometric analysis of chemerin was performed using ImageJ software. Images were prepared using Excel Office. All the graphs were normalized to 18S rRNA. Figure represents 3 independent experiments (n=3).

Next, we investigated quantitatively the expression levels of chemerin, in *H. pylori* sonicates (G27 wild type and its ΔCagA mutant) - treated KATO III and AGS cell lines in a time course experiment. The mRNA expression was significantly increased in both G27 wild type treated cell lines at 6 hours time point. The increased expression of chemerin was also detected at 24 hours time point in G27 wild type - treated KATO III cells (Figure 3.3 (A)), but not in AGS cells (Figure 3.3 (B)). No chemerin expression was detected in G27-ΔCagA -treated samples (Figure 3.3).

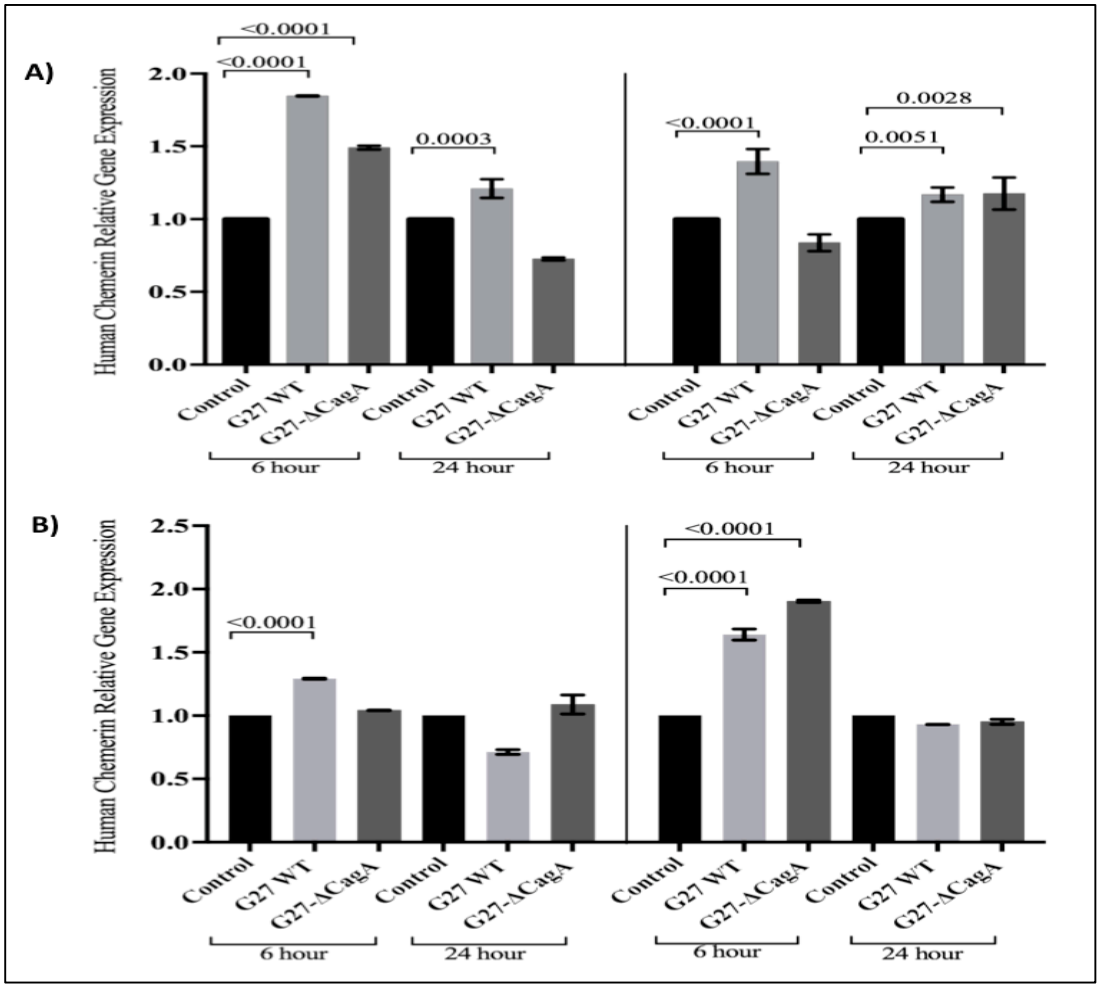

Figure 3.3 : Expression of chemerin in *H. pylori* G27 WT and G27 ΔCagA sonicates -treated KATO III (A) and AGS (B) gastric epithelial cell lines. 5×10^5 cells were treated with G27 wild type and G27-ΔCagA sonicates (10ug/ml) or left untreated (medium) for 6 and 24 hours. After that, cells were harvested and RNA isolations were performed. Isolated RNAs were converted to cDNA and evaluated for Chemerin and 18S rRNA expressions using qRT-PCR. Levels of 18S rRNA were used as endogenous control. Chemerin mRNA expression levels were normalized to endogenous control. Graphs were prepared with Graphpad Prism 6 software. Statistical analysis was conducted using student's *t*-test. The experiment was performed three times (n=3).

Moreover, the level of chemerin expression was assessed in gastric biopsies of 30 patients with chronic ulcer and acute gastritis infected with *H. pylori* and compared to normal gastric tissue without any infection Interestingly, we have detected a significantly higher expression of chemerin in 10 ulcer samples when compared to gastritis and negative samples (Figure 3.4). Even though, more samples needs to be evaluated before coming to a conclusion, our result may suggest an association of chemerin with gastric pathology development.

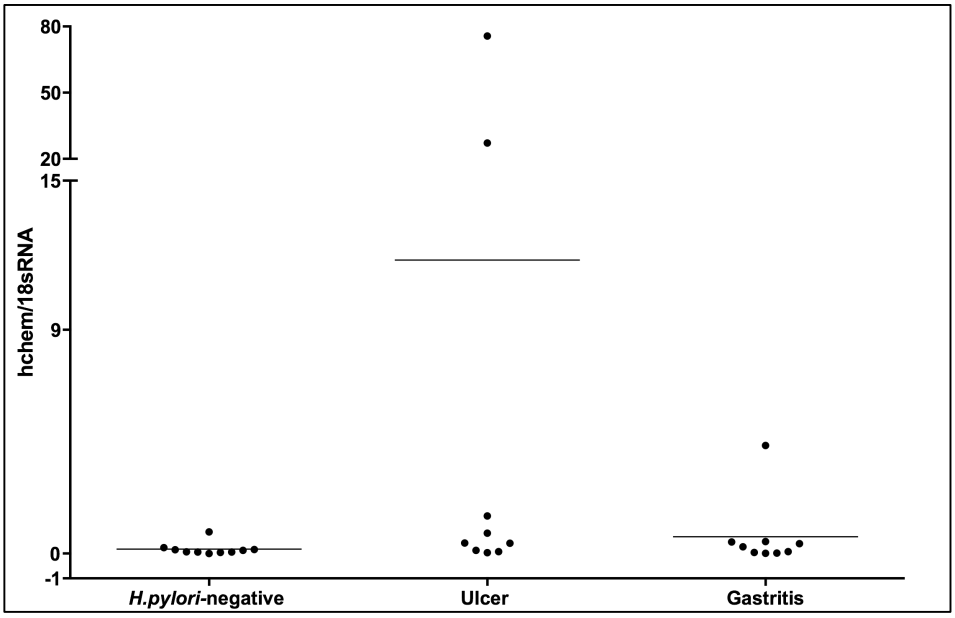

Figure 3.4 : Expression level of chemerin in 10 patients each with chronic ulcer and acute gastritis compared to 10 normal gastric biopsies (negative), evaluated by real-time PCR. Levels of 18S rRNA were used for normalization. Graphs were prepared using Graphpad Prism 6 software. * represents p value less than 0.01. Statistical analysis was conducted using student's *t*-test. Data is representative of two independent experiments (n=2).

1.8 Chemerin Expression on Protein Level in Gastric Epithelial Cells

After detecting increased expression level of chemerin in *Helicobacter* treated gastric cells, western blot was performed to investigate whether chemerin was also elevated on protein level in response to *H. pylori* treatment. Initially we evaluated levels of chemerin in whole cell extract and cell culture supernatants of KATO-III cells by western blot analysis at 6 and 24 hour time points (Figure 3.5). These time points were the ones that we detected the highest expression levels of chemerin in mRNA level. Chemerin is secreted as an inactive 18 kDa protein prochemerin, which

becomes activated by various proteases into isoforms of varying sizes, ranging from 15 to 17 kDa [60-66].

Although, the expected size of chemerin in western blot analysis was between 15-17 kDa, we did not detect any bands in that range. Interestingly, prominent bands were observed on around 50 kDa. Initially it was thought the bands possibly represented another isoforms of chemerin or chemerin in a bound form.

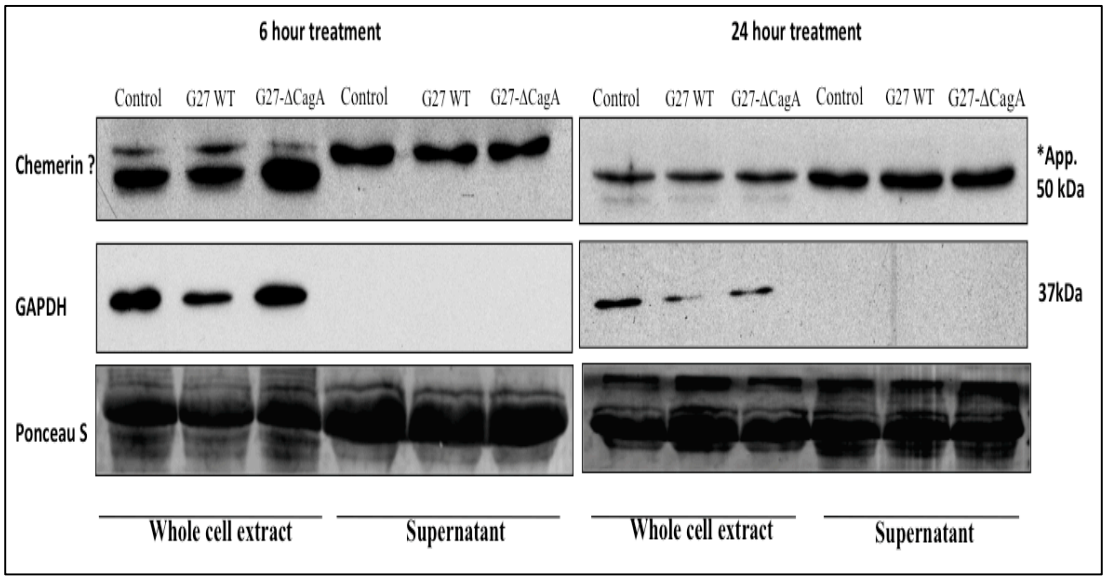

Figure 3.5 : Western blot was performed to assess protein level of chemerin in *H. pylori* G27 wt and G27-ΔCag A sonicate treated KATO-III cells at 6 and 24h. Cells were stimulated with *H. pylori* G27 WT sonicate and H. pylori G27 ΔCag A at 10 ug/ml final concentration or left untreated (medium) for 6 and 24 h. KATO-III cells were harvested for protein isolations and culture supernatants were aliquoted for detection of chemerin on SDS-PAGE. Cells were lysed and immunoblotted for membrane-bound chemerin (18 kDa). Supernatants were immunoblotted for soluble chemerin (18 kDa). GAPDH (37 kDa) and Ponceau S were used as loading control. Figure represents 3 independent experiments (n=3).

However, all the isoforms of chemerin should be lower than 18 kDa size supported by numerous journals [88,207,208]. Repeated performance of western blot analysis of chemerin results into the similar outcome with no bands in the expected size. In order to investigat if antibody against chemerin detects any expected band, chemerin was cloned and transfected in HEK293T cells. Thereafter, western blot performed on HEK293T cells transfected with plasmid containing human chemerin gives bands at the expected18 kDa size (Figure 3.6 A). When we performed western blot analysis with *Helicobacter* -treated KATO III samples and chemerin over-expressed HEK293T cells together, band was only observed in the chemerin

over-expressed HEK293T cells. Similar results were observed in the western blot performed on AGS samples too (Figure 3.6 B).

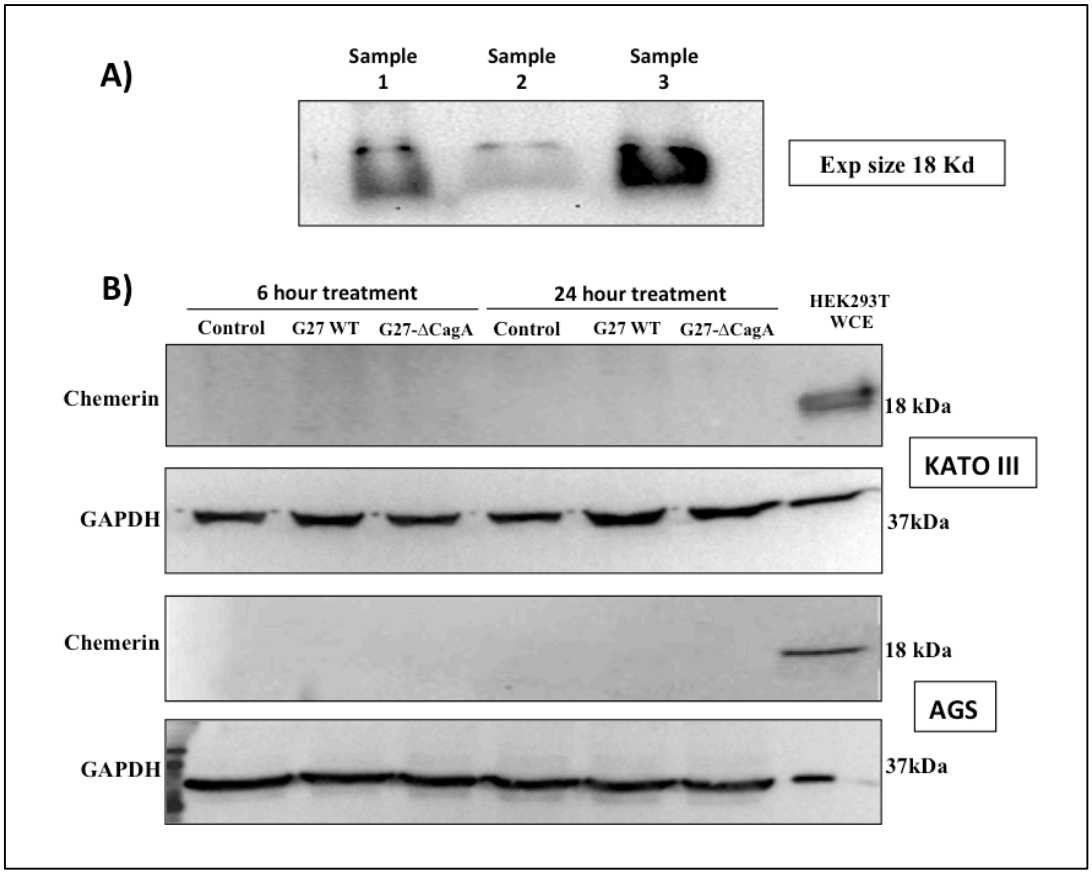

Figure 3.6 : Molecular cloning of chemerin using pCDNA3.1(+) plasmid was performed and transfected into HEK293T cell line (A). Western blot performed on the HEK293T protein lysates ensured the presence of chemerin on 18 kDa size. (B) Immunoblot was performed again using *H. pylori* G27 WT and G27-ΔCag A sonicate treated KATO-III and AGS cells at 6h and 24h along with the Chemerin expressing HEK293T protein lysates. GAPDH house-keeping proiten were used as loading controls respectively. Figure represents 2 independent experiments (n=2).

We may conclude from our results that, chemerin protein level can not be detected in *Helicobacter* sonicate –treated gastric epithelial cells regardless of its elevated mRNA level.

1.9 Immunohistochemical Analysis of Human Samples with Ulcer and Gastritis

Previously an elevated levels of chemerin mRNA expression was measured in patients with chronic ulcer biopsy samples. In order to investigate the levels of chemerin protein in biopsy specimens, as a preliminary study, we performed an immunohistochemical staining of chemerin on paraffin- embedded gastric tissue

sections of patients with acute gastritis and chronic ulcer (Figure 3.7). Even though, we experienced a high background staining with our chemerin antibody, we could detect a higher staining of chemerin in ulcer specimens compared to gastritis and normal gastric tissues. We performed our studies using both flourescence (Zeiss) and confocal microscopes (Leica).

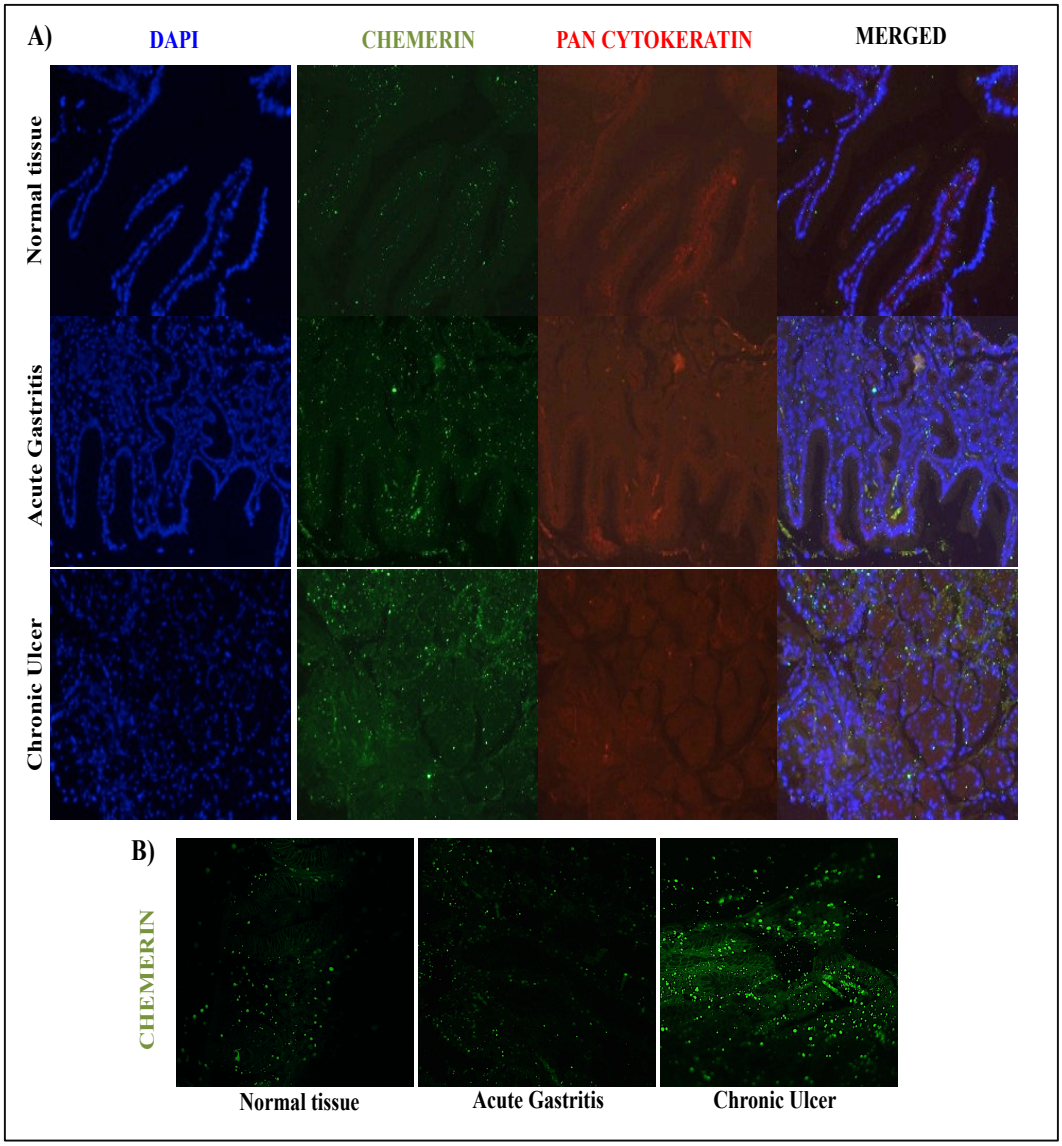

Figure 3.7 : Chemerin immunohistochemistry. Photomicrographs showing chemerin protein expression (green) in human gastric tissues collected from patients with *H. pylori* positive acute gastritis and chronic ulcer. Gastric tissue of a healthy individual was used as a control. Nuclear staining was obtained with 4,6-diamidino-2-phenylindole (DAPI; blue) and pancytokeratin was used to stain cytokeratin proteins of epithelial tissues. Chemerin immunoreactivity was more prominent in ulcer tissues. Photographs were taken in Fluorescence (Zeiss Axiovert A1 Inverted) (A) and Confocal (Leica SP2 (B) microscope. The experiment was performed two times (n=2).

1.10 The Expression Level of Chemerin Receptor in Thp-1 and U937 Cells

One study suggested that, chemerin stimulates macrophages to differentiate into M1 subtype and elevates pro-inflammatory cytokines associated, while it inhibits M2 differentiation in various disease models [86]. To further explore this hypothesis, recombinant human chemerin was used to test its effect on macrophages, with or without the presence of *H. pylori* sonicate. Two human monocytic cell lines, U937 and THP-1 were initially sought for this purpose but as we did not detect expression of chemerin receptor in U937 cells, all the experiments were performed only on THP-1 cell line (Figure 3.8).

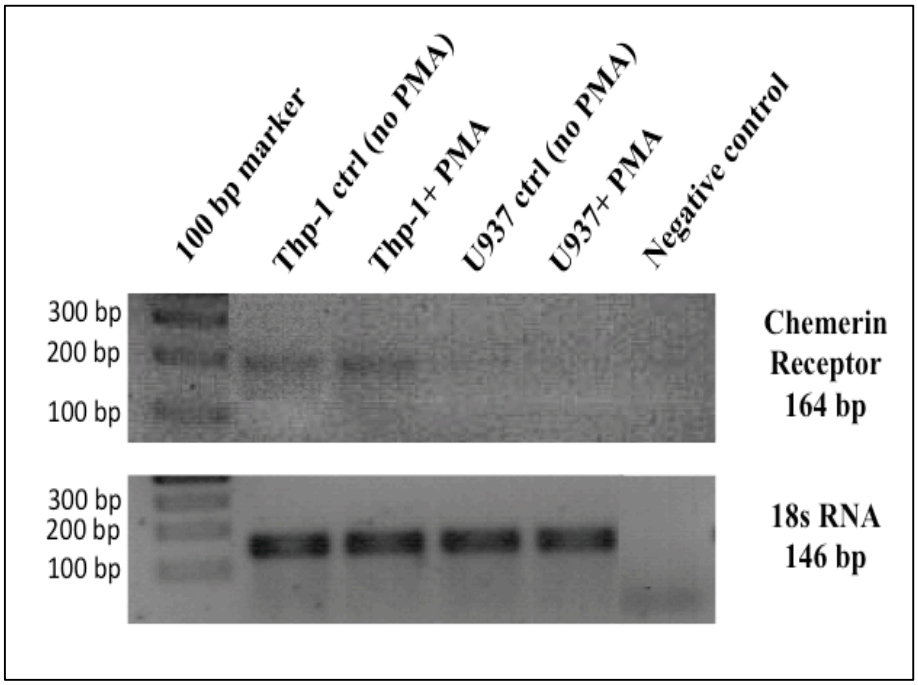

Figure 3.8 : Chemerin receptor expression in PMA differentiated THP-1 and U937 macrophages. Conventional PCR revealed the presence of chemerin receptor in THP-1 macrophages only but no fchemerin receptor expression was observed on U937 differentiated macrophages. The experiment was performed two times (n=2).

1.10.1 Monocytes to macrophage differentiation

PMA (phorbol 12-myristate 13-acetate) was used to induce differentiation of monocytes into macrophages. PMA is an activator of Protein Kinase C (PKC) due to its structural resemblance to PKC activator diacylglycerol (DAG) and has a key role in monocyte adhesion. It exerts effect through inducing adherence and cell cycle arrest followed by differentiation of monocytes into macrophages via activation of PKC due to activation of transcriptional factors such as activator protein-1 (AP1),

resulting in the upregulated expression of cell adhesion receptors (β2-integrin family). Differentiated macrophages were further exposed to LPS and human recombinant IL4 to polarize them into M1 and M2 subtypes respectively, with or without the presence of chemerin. PMA differentiated macrophages were first analyzed for macrophage specific surface markers; CD11b, CD14 and CD68 by flow cytometer (Figure 3.9).

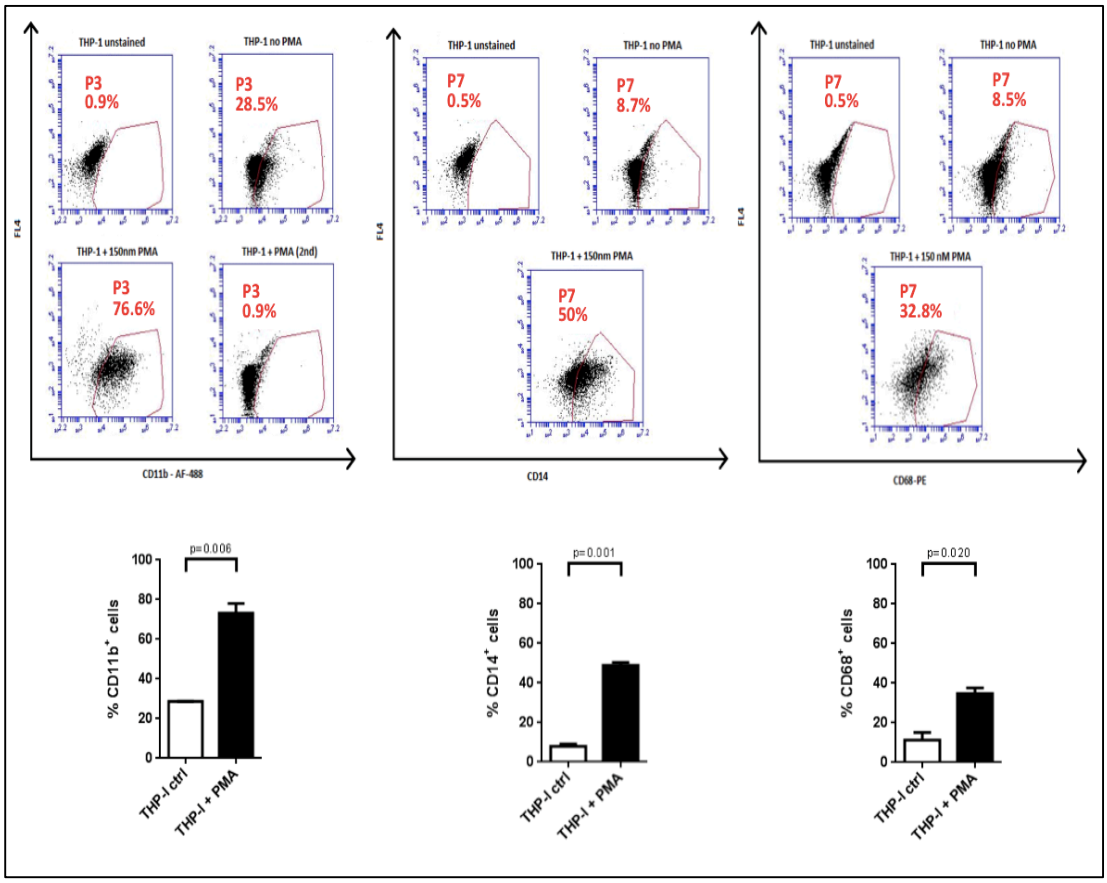

Figure 3.9 : Percentages of PMA differentiated THP-1 macrophages. Flow cytometry, dot blot graphs represents staining of approximately $5x10^4$ with a specific macrophage marker, biotin-coupled anti-CD11b, PE coupled anti-CD14 and biotin coupled anti CD-68 antibody (upper panel). Independent percentages and average percentage values are expressed as bar graph (Lower panel). Images were prepared using GraphPad Prism program. Data is representative of two independent experiments (n=2).

All CD (Cluster of Differenttiation) surface markers were significantly upregulated in response to PMA stimulation compared to no PMA treated group. CD11b is an integrin family member that is expressed on the surface of both monocytes and macrophages and regulates leukocyte adhesion and migration to mediate the inflammatory response. Though it is not a specific macrophage marker *per se* , PMA

treatment significantly upregulated CD11b expression (76.6%) on differentiated macrophages detected by flow cytometer (Figure 3.9).

CD14 is a specific macrophage marker that mainly detects bacterial lipopolysaccharide as well as other PAMPs. Significant upregulation was again observed on THP-1 macrophages, half of the population was found to be expressing CD14 on their surface, whereas 8.7% of unstimulated control cells (THP-I no PMA) were found to express CD14. CD68 is another monocyte/macrophage marker; it is a member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family that binds to low-density lipoprotein. Flow cytometer analysis showed 32.8% population expressing CD68 over PMA stimulation compared to 8.5% on control population (Figure 3.9). All the data confirmed successful macrophage differentiation from monocytes.

1.10.2 Effect of Chemerin on macrophage polarization

After monocytes were differentiated into macrophages, they were further subjected to different stimuli to obtain M1 and M2 subtype with or without the presence of human recombinant chemerin. Briefly, macrophages were stimulated with 100 ng/mL LPS to obtain M1 macrophages and 20 ng/mL recombinant IL4 to obtain M2 subtype with or without 50 ng/mL recombinant chemerin. After cell harvesting, RNA was isolated and samples were converted to cDNA. M1 and M2 associated cytokines were measured by conventional PCR (Figure 3.10).

IL1β and IL6 are two M1 macrophage associated pro-inflammatory cytokines and IL10 is a M2 macrophage associated anti-inflammatory cytokine. Conventional PCR analysis indicated increased IL1β expression in LPS treated samples with or without chemerin, rendering M1 subtype polarization. IL-6 expression was also observed in those samples. IL10 expression was only observed in recombinant IL4 treated population. However, no IL10 expression observed when IL4 administered with recombinant chemerin, showing chemerin could potentially inhibit macrophage polarization into M2 subtype. This is also supported by other studies such as *Lin et al* where chemerin was detected to inhibit M2 associated genes in DSS induced colitis model. Although IL1β was elevated in response to LPS and chemerin stimulation but such effect was not observed on IL6 expression, which depicts that chemerin may not have potential effects on M1 subtype polarization.

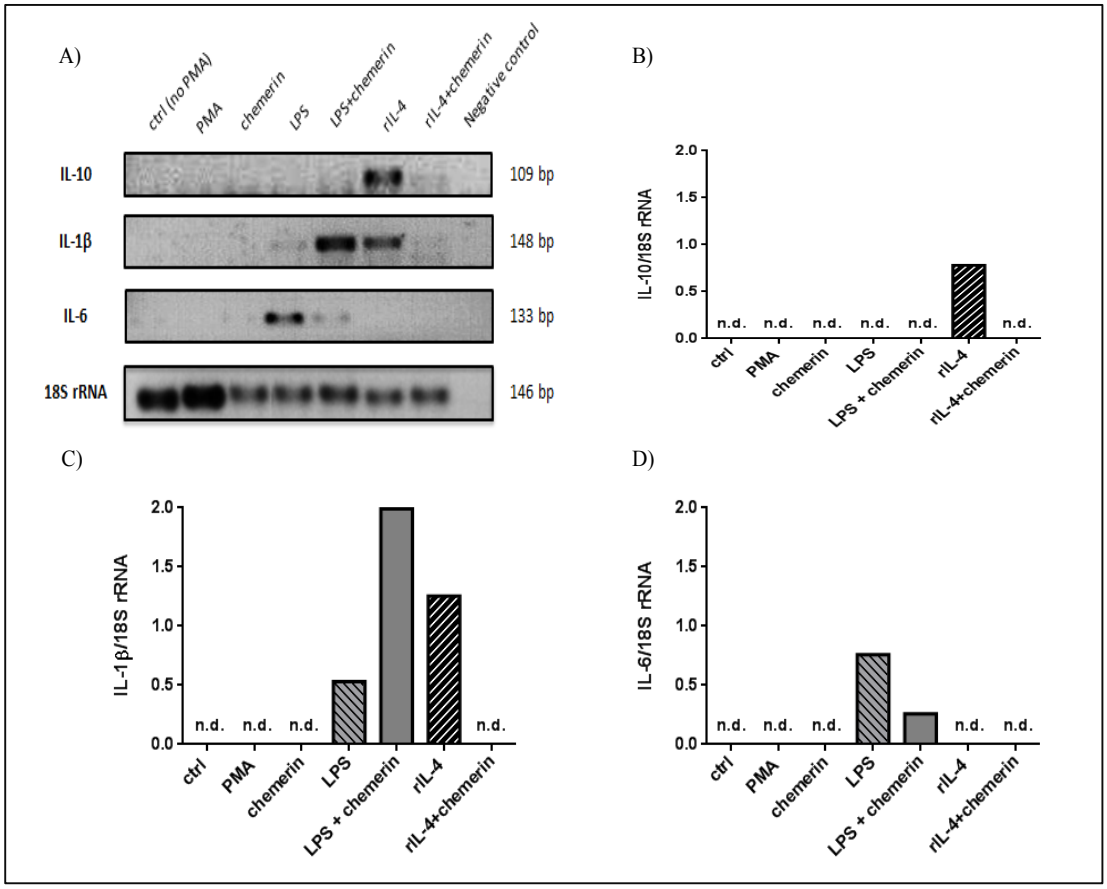

Figure 3.10 : M1 and M2 specific cytokine expression in THP-1 differentiated macrophages treated with various stimuli, e.g LPS (100 ng/ml), recombinant IL-4 (20 ng/ml) and recombinant chemerin (50 ng/ml) for 24 hours. After RNA isolation IL-1β, IL-6 and IL1-10 expression was observed by conventional PCR (A). Densitometric analysis of IL-10 (B), IL-1β (C) and IL-6 (D) was performed using ImageJ software. Images were prepared using Excel Office. All the graphs were normalized to 18S rRNA. Data is representative of three independent experiments (n=3).

1.10.3 Effect of *Helicobacter pylori* and Chemerin on macrophage polarization

Next, we investigated the potential effect of *Helicobacter pylori* on macrophage polarization. Briefly, PMA differentiated macrophages were treated with sonicates of G27 wild type and G27-ΔCagA with or without the presence of recombinant human chemerin for 24 hours. After incubation, RNA isolation was performed followed by cDNA synthesis. IL1β, IL-6 and IL-10 expression was determined by conventional PCR and normalized to 18s RNA (Figure 3.11).

We did not detect any change of IL-1β expression in presence of *H. pylori* or chemerin stimulation as we show overall elevated expression in all samples except the control group. However, expression of IL-6 levels increased in all sonicate treated samples compared to the LPS group although such elevation was unaffected

by chemerin. This again support the idea that chemerin may not exert its effect on M1 type macrophage polarization (Figure 3.11).

We detected a very low expression of IL-10 in the group treated with recombinant IL-4 with chemerin by conventional PCR (Figure 3.11 A). Moreover, we asssesed the expression level of IL-10 by real-time PCR.

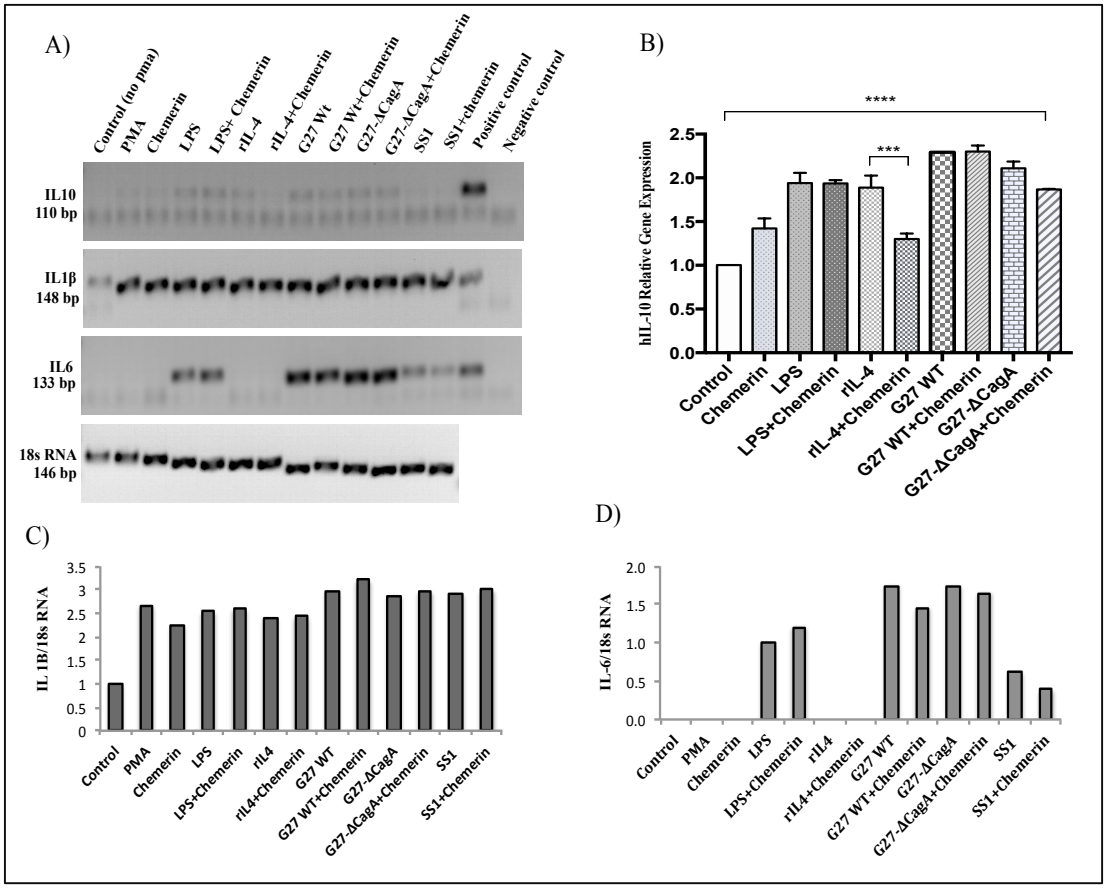

Figure 3.11 : M1 and M2 specific cytokine expression in THP-1 differentiated macrophages treated with *Helicobacter* sonicates along with various stimuli, e.g LPS (100 ng/ml), recombinant IL-4 (20 ng/ml) and recombinant chemerin (50 ng/ml) for 24 hours. After RNA isolation IL-1β, IL-6 and IL1-10 expression was observed by conventional PCR (A). IL-10 expression was observed by RT pcr and graphs were prepared with Graphpad Prism 6 software (B). Densitometric analysis of IL-1β (C) and IL-6 (D) was performed using ImageJ software. Images were prepared using Excel Office. All the graphs were normalized to 18S rRNA. Figure is representative of three independent experiments (n=3).

Although, IL-10 expression was observed in treated groups, the expression was significantly reduced when chemerin was treated along with recombinant IL-4, suggesting the inhibition of M2 macrophages by chemerin (Figure 3.11 B). Interestingly, treatment of *H. pylori* sonicates triggered IL-10 expression which was slightly reduced with an addition of chemerin.

4. DISCUSSION AND CONCLUSION

During an inflammatory process, damaged tissues release mediators that contribute to the mounting of immune responses by regulating the trafficking of leukocyte populations. Chemokines and other chemoattractant molecules therefore play fundamental roles in the physiology of inflammatory events, as well as in the pathological dysregulations of these processes. *Helicobacter pylori* is a gram negative pathogen that resides in the human gastric epithelium and has been known for its association with severe gastric inflammation and malignancies, which ultimately leads to carcinoma. Chemerin is a 143 aa novel extracellular mediator, primarily known as a chemokine, is found in high amounts in various inflammatory diseases and expressed by many epithelial cells. However, its influence on *Helicobacter pylori* induced gastric inflammation is unknown. Therefore, we investigated the effect of chemerin which is potentially expressed and secreted by *Helicobacter pylori* - induced gastric epithelial cells.

Macrophages are the members of the first line immune defence that can polarize according to stimuli and the local microenvironment and shape the local inflammatory status to adapt to outside stimuli. They express chemerin receptor CMKLR1 (also known as chemR23). They can be polarized into the classically activated -M1 type- macrophages and the alternatively activated -M2 type- macrophage subsets. 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 and M2 macrophages were detected in human gastric biopsy specimens from *H. pylori* positive individuals. Macrophage mediated pro-inflammatory Th1 and Th17 response was observed as the pre-dominant adaptive response against *H. pylori* in humans. Chemerin was shown to suppress M2 macrophage polarization with increased pro-inflammatory cytokine profile in DSS induced colitis model *in vivo* and *in vitro*. Another study found increased chemerin expression on M1 macrophages with distinct IL-10 expression, explaining the inflammatory effect of chemerin on M1 macrophages. However, no

studies have yet investigated the effect of chemerin on the polarization status of *H. pylori* induced macrophages. Therefore, in this study, we investigated the effect of both chemerin and *H. pylori* on the monocytic cell line THP-1 and assess the differences on its polarization status according to cytokine profiles.

In order to evaluate the effect of chemerin on gastric immunopathology, we first investigate mRNA expression level of chemerin in three gastric epithelial cell lines, KATO III, AGS and MKN45, by conventional PCR. Conventional PCR confirmed chemerin expression in all three cell lines, with comparatively less expression in MKN45 cell line. Next, we examine the expression of chemerin in KATO III and AGS cell line treated with different *H. pylori* sonicates, the wild type G27 strain and its ΔcagA, ΔcagE, ΔPAI mutant sonicates. We observed increased chemerin expression in the sonicate treated groups compared to control in both cell lines, which was more prominent in KATO III cell line. However, increased expression was observed only in wild type G27 and its ΔcagA mutant sonicate treated groups in AGS cell line. Since the *H. pylori* virulence factor Cag A (cytotoxin- associated gene-A) has been associated with the development of gastric malignancies as reported by many studies, we were interested to assess its effect on chemerin expression level.

Real-time PCR was performed to study chemerin expression in KATO III and AGS cell lines treated with wild type G27 and ΔcagA mutant sonicate for 6 and 24 hours Chemerin expression was elevated after 6 hours of treatment with wild type G27 strain sonicate in both cell lines. However, chemerin was elevated in 24 hour treatment with G27 wild type sonicate only in KATO III cells. Therefore, we presumed that chemerin may potentially have a role and associated with immune response in gastric epithelial cell lines which were infected with *H. pylori* strain.

Infection with *H. pylori* has been one of the major reasons of developing gastritis and ulcer in humans [204]. In accordance with that, we have also checked chemerin expression in gastric biopsy speciments collected from patients with *H. pylori* infected chronic ulcer and acute gastritis and patients without pathology and infection. Chemerin was detected to be elevated significantly in ulcer patients compared to uninfected normal tissue. Interestingly, such increase was not detected in the gastric biopsy of patients with acute gastritis.

Next, western blot analysis was performed to investigate the level of chemerin protein upon *H. pylori* sonicates treatment. However, chemerin could not be detected by western blot in repeated attempts. Therefore, human chemerin was cloned, transfected into HEK293T cell line and immunoblot was performed. We observed chemerin by western blot in the chemerin-transfected HEK293T cell lines but not in other samples. Therefore, we presumed that, even if chemerin is significantly elevated upon *H. pylori* treatment on mRNA level, the protein level was not biologically significant enough to be detected by western blot. We also know from the literature that the various isoforms of active and inactive chemerin were present in different biological source [60-66]. So that, it could also be possible that the chemerin expressed in the KATO and AGS samples have undergone different proteolytic cleavage, forming different isoform of chemerin that probably lacked the epitope to be recognized by the antibody used for the immunoblot. Further experiments need to be performed to resolve this hypothesis.

We showed previously that chemerin is highly expressed in inflamed gastric tissues of ulcer patients. As next, we performed immunohistochemistry to visualize the distribution and localization of chemerin in gastric specimens. Immunohistochemistry performed on paraffin embedded gastric tissue samples collected from *H. pylori* - infected patients of acute gastritis and chronic ulcer and patients who are not infected with no pathology . Similar to the mRNA expression, chemerin was shown to be highly expressed in gastric tissues of ulcer patients compared to tissues from gastritis patients and uninfected control.

In order to understand the effects of chemerin on the polarization status of macrophages *in vitro*, a monocytic cell line THP-1 was used. In order to polarize THP-1 monocytes to macrophages, PMA (phorbol 12-myristate 13-acetate) was administered. Differentiation was checked by assessing expression of the cell surface markers specific for macrophages, such as CD11b, CD14 and CD68. PMA treatment significantly upregulated CD11b (76.6%), CD14 (50%) and CD68 (32.8%) expression on differentiated macrophages compared to monocytes The differentiated macrophages were then exposed to different stimuli such as LPS and recombinant IL-4 to polarize them into M1 and M2 subtypes respectively, with or without the presence of chemerin. Two pro-inflammatory cytokines, IL-1β and IL-6, were measured as markers for M1 macrophages. Both of them were significantly increased

in LPS- treated samples. When chemerin was administered with LPS, a significantly high expression of IL-1β and low expression of IL-6 observed when compared to LPS- treated sample only in the first experimental set. Therefore, initially it was thought that, chemerin might have a role in the polarization of M1 macrophages but repeated three experiments showed no difference in the expression of IL-1β and IL6 in samples treated with LPS alone or with chemerin. This disprove our hypothesis that chemerin may have an effect on M1 polarization. IL-10 expression levels were assessed as a marker for M2 polarization. IL-4 treatment resulted in elevated IL-10 expression compared to control group. When chemerin is added along with IL-4, IL-10 expression significantly reduced. The similar effect of chemerin was also mentioned in the study performed by Lin et al. (2014) where chemerin reduced the expression of M2 associated genes.

Next, we investigated the effect of *H. pylori* on the polarization of macrophages with or without the presence of chemerin to assess whether chemerin may alter the effect exerted by *H. pylori* on macrophages. The THP-1 macrophages were treated with wild type G27 and ΔcagA mutant sonicate with/without chemerin for 24 hours. The sonicate treatment polarized macrophages to M1 subtype with high expression levels of pro-inflammatory IL-1β and IL6 and also increased IL-10 compared to control group. The pro-inflammatory IL-1β expression was observed in the monocytes which is well recognized by the literature, since monocytes constitutively activate caspase-1 which is necessary for IL-1beta processing and secretion, while macrophages require a second stimulus such as IL-4/IL-13 to activate caspase-1 [205,206].

M1 macrophages are known to produce IL-10 in response to bacterial stimulation, immune complexes and LPS [207,208]. This was also shown in a study on mouse macrophages which were treated by *H. felis* in our laboratory (unpublished data). We did not detect a prominent change of expression in IL-1β, IL6 and IL-10 when chemerin was added with sonicates. Therefore, our results suggested that, chemerin may not have a role on *H. pylori* induced M1 macrophage polarization. Moreover, IL-4 mediated M2 polarization was detected to be inhibited upon chemerin treatment. As IL-10 expression in the sonicate - treated group did not change in presence of chemerin, it could be said that these IL-10 was expressed by the M1 macrophages and chemerin had no effect on polarizing or even changing the cytokine profile expressed by the M1 macrophages.

In conclusion, even though chemerin protein level could not be detected in gastric epithelial cells regardless of elevated mRNA expression, further investigation is required in order to understand and detect if different isoforms of chemerin are present. One plausible idea would be to detect chemerin protein using different antibody that recognize other epitopes present on the protein. Since chemerin is a secretory protein, identification of the protein level using chemerin specific ELISA can also deem logical. According to results found in RT-PCR and immunohistochemistry performed on human tissue samples, chemerin could have a role in the development of gastric pathology although further investigation should be carried out on more patients to confirm this preliminary finding. Our findings showed that chemerin did not affect the polarization of macrophages induced by *H. pylori* in vitro , this should be further investigated in animal studies, since in vitro studies may not always reproduce similar effects as observed in *in vitro*. As observed in the literatures, chmerin potentiate both pro [85] and anti-inflmmatory [79, 85] response through altering macrophage polarization conducted in animal study, this would be an interesting idea to detect chemerin's effect on *Helicobacter* induced macrophage polarization status in animal models.

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