ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

FUNCTIONAL INTERACTION BETWEEN *HELICOBACTER*-STIMULATED B (H_{STIM}-B) CELLS AND DENDRITIC CELLS

M.Sc. THESIS

Doğuş ALTUNÖZ

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

HELICOBACTER-STİMÜLE B (H_{STiM}-B) HÜCRELERİ İLE DENDRİTİK HÜCRELER ARASINDAKİ FONKSİYONEL ETKİLEŞİM

YÜKSEK LİSANS TEZİ

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Doğuş Altunöz, a M.Sc. student of ITU Graduate School of Science Engineering and Technology student ID 521141106, successfully defended the thesis/dissertation entitled "FUNCTIONAL INTERACTION BETWEEN *HELICOBACTER*-STIMULATED B (H_{STIM}-B) CELLS AND DENDRITIC CELLS", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family and friends,

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June 2017 Doğuş ALTUNÖZ

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ABBREVIATIONS

µg : Microgram
µm : Micrometer
µM : Micromolar
1,25D3 : 1,25 Dihydroxyvitamin D3
APC : Antigen Presenting Cell
BAFF : B-Cell Activating Factor
BAFF-R : B-Cell Activating Factor Receptor
BCA : Bicinchoninic Acid
BCR : B-Cell Receptor
BSA : Bovine Serum Albumin
BM-DC : Bone Marrow-Derived Dendritic Cell
Br1 : B Regulatory 1
Breg : Regulatory B cell
Cag : Cytotoxin- Associated Gene
CagA : Cytotoxin- Associated Gene A
CD : Cluster of Differentiation
cDC : Conventional Dendritic Cell
cDNA : Complementary Deoxyribonucleic Acid
CD40L : CD40 Ligand
DAMP : Danger-Associated Molecular Pattern
DC : Dendritic Cell
DC-SIGN : DC-Specific ICAM3-Grabbing Non-Integrin
DMSO : Dimethyl Sulfoxide
DNA : Deoxyribonucleic Acid
dNTP : Deoxyribonucleotide
EAE : Experimental Autoimmune Encaphalomyelitis
EDTA : Ethylenediaminetetraacetic Acid
ELISA : Enzyme-Linked Immunosorbent Assay
FBS : Fetal Bovine Serum
FITC : Fluorescein Isothiocyanate
FOXP3 : Forkhead Box P3
g : Gram
GM-CSF : Granulocyte Macrophage Colony-Stimulating Factor
h : Hour
H. felis : *Helicobacter felis*
H. pylori : *Helicobacter pylori*
HRP : Horseradish Peroxidase
H_{stim} : *Helicobacter*-stimulated
Hf_{stim} : *Helicobacter felis*-stimulated
IFN-α : Interferon-Alpha
IFN-β : Interferon-Beta
IFNγ : Interferon Gamma
Ig : Immunoglobulin
IL-1β : Interleukin-1 Beta

IL-4 : Interleukin-4
IL-6 : Interleukin-6
IL-8 : Interleukin-8
IL-10 : Interleukin-10
IL-18 : Interleukin-18
IL-10R : IL-10 Receptor
IRF : Interferon- Regulatory Factors
JAK : Janus kinase
kDa : Kilodalton
L : Liter
LPS : Lipopolysaccharide
M : Molar
MALT : Mucosa- Associated Lymphoid Tissue
MAPK : Mitogen-activated protein kinase
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
MZ : Marginal Zone
NF-κB : Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
ng : Nanogram
NK : Natural Killer Cell
NKT : Natural Killer T cell
NLR : Nod-Like Receptor
NLRP3 : NOD-like Receptor Protein 3
PAI : Pathogenicity Island
PAMP : Pathogen Associated Molecular Pattern
PBS : Phosphate Buffered Saline
PCR : Polymerase Chain Reaction
pDCs : Plasmacytoid Dendritic Cells
PD-1 : Programmed Cell Death Protein 1
PD-L1 : Programmed Death-Ligand 1
PE : Phycoerythrin
pg : Picogram
pH : Power of Hydrogen
PMA : Phorbol-12-Myristate-13-Acetate
PRR : Pattern Recognition Receptor
rpm : Revolutions Per Minute
RPMI : Roswell Park Memorial Institute
RNA : Ribonucleic Acid
rRNA : Ribosomal RNA
STAT : Signal Transducer and Activator of Transcription
T2-MZP : Transitional 2 Marginal-Zone Precursor
TCR : T-cell receptor
TGF-β : Transforming Growth Factor-Beta
Th1 : T Helper 1

Th2 **:** T Helper 2
Th17 **:** T Helper 17
Tim-1 **:** T Cell Ig Domain And Mucin Domain Protein 1
TLR **:** Toll-Like Receptor
Tm **:** Melting Temperature
TNF-α **:** Tumor Necrosis Factor-Alpha
Tr-1 **:** T Regulatory-1
Treg **:** Regulatory T cell
VacA **:** Vacuolating Cytotoxin A

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FUNCTIONAL INTERACTION BETWEEN *HELICOBACTER*-STIMULATED B (H_{STIM}-B) CELLS AND DENDRITIC CELLS

SUMMARY

Helicobacter pylori (H. pylori) is a microaerophilic gram negative and spiral-shaped bacterium which is firstly identified by Robbin Warren and Barry Marshall. About more than 50% of the people in the world have infected with this bacterium; however, the bacteria cause serious gastrointestinal diseases only in 10-20% of infected people, whereas the remaining infected people has only asymptomatic gastritis. *H. pylori* can lead to development of gastric cancer and categorized as a group-I carcinogen by World Health Organization. Due to lack of efficient immune response against *H.pylori* in murines; mice has been infected with *Helicobacter felis (H. felis*) to create mouse model of *Helicobacter* infection.

Dendritic cells (DCs) which are firstly identified by Steinmann, are the member of antigen presenting cells (APCs) and provide a connection between innate and adaptive immune response due to having a crucial role for activation of specific immune response against foreign antigens. Regulatory DCs express low levels of IL-12 and co-stimulatory molecules such as CD40, CD80 and CD86. Instead, expression of inhibitory molecules such as IL-10, PD-L1, CD95L and indoleamine 2,3 deoxygenase (IDO) are upregulated in regulatory DCs. Moreover, in addition to immature DCs, also semi-mature DCs characterized with MHC-II^{hi}CD80/CD86^{hi}IL-12^{-}TNF-α^{-}IL-10^{+} phentotype are thought as tolerogenic DCs and inducer of type-1 regulatory T (Tr1) cell-mediated immune suppression.

B cells have an important role for regulation of immune response in addition to their effector roles such as antibody production and antigen presentation. Regulatory role of B cells was shown with the use of mice lacking B cells which was not able to overcome autoimmune encephalitis. *Helicobacter felis* activates B cells via TLR2 and Myd88 and suppress immune responses *in vivo* and *in vitro* by triggering IL-10-producing Tr1-like cells. This suppression results in persistency of bacteria in gastric mucosa and decrease in pathology score. Therefore, balance between regulatory and effector response could determine the fate of infection. As demonstrated by our previous laboratory studies, *H. felis*-mediated activation of B cells induce two different B cell subgroups: IL-10-producing IL-10^{+} B cells and TGF-β-producing IL-10^{-} B cells.

Our first aim was to understand the effects of *H. felis* on bone marrow-derived dendritic cells (BM-DCs). Second aim was to determine effects of *Helicobacter*-stimulated B (Hf_{stim}-B) cells on bone marrow-derived dendritic cells (BM-DCs). To perform these experiments; C57BL/6 mouse bone marrow cells were differentiated into immature dendritic cells in the presence of IL-4 and GM-CSF. B cells were magnetically isolated from spleens of C57BL/6 mice and treated with *H. felis* sonicate in order to obtain *Helicobacter*-stimulated IL-10^{+} B and IL-10^{-} B cells

through magnetic separation. Afterwards, these B cell subgroups were treated with *H.felis* sonicate, their supernatant were collected and applied onto immature BM-DCs. Following incubation and washing of the cells, BM-DCs were treated with LPS or *H. felis* sonicate. Cytokine profile and activation status of DCs were determined on protein and expression level. Moreover; for functional analysis, these DCs were co-cultured with naive $CD4^+$ T cells at a ratio of 1:2 (DC:T) in the presence of 10 ng/ml IL-2 and 1 µg/ml anti-CD3Ɛ. Then, frequency of IL-10 secreting $CD4^+$ T cells in different groups were analyzed by flow cytometry.

H. felis-induced BM-DCs secrete IL-12/IL-23 (p40), IL-1β, TNF- α and IL-10 into the culture medium. Consistently, expression of IL-12/IL-23 (p40), TNF-α and IL-10 in addition to IL-6 was also induced. Flow cytometric analysis showed that *H. felis* activates DCs and causes upregulation of CD86 expression.

All Hf_{stim}-B cell groups including IL-10^+ B and IL-10^- B cells, induced DCs to express CD86 molecules in the absence of additional antigenic stimulation. However, there was no difference between DC groups stimulated with supernatant of IL-10^- B and IL-10^+ B in terms of expression of CD86 in untreated and LPS- or *H. felis*-treated groups. Moreover, IL-10 secretion was not induced upon stimulation of DCs with supernatant of unstimulated B, *Helicobacter felis*-stimulated B (Hf_{stim}) or IL-10^+ B cell followed by LPS or *H. felis* sonicate treatment. Nevertheless, DCs secreted significantly more IL-10 upon stimulation with IL-10^+ B cell followed by LPS or *H.felis* treatment compared to IL-10^- B cell supernatant stimulation. IL-12 secretion from DCs was induced by supernatant of all *Helicobacter*-stimulated B cell types. Strikingly, TNF-α secretion from DCs was suppressed upon stimulation of DCs with Hf_{stim}-B cell supernatant prior to LPS or *H. felis* treatment.

Interaction of BM-DCs with supernatant of Hf_{stim}-IL-10^+ B cells in the absence of subsequent LPS or *H. felis* treatment retained the frequency of IL-10-producing $CD4^+$ T cells generated by immature DCs. Moreover, LPS treatment of DCs which had been previously stimulated with supernatant of unstimulated B cells, Hf_{stim}-B cells or Hf_{stim}-IL-10^- B cells, but not Hf_{stim}-IL-10^+ B cells, resulted in the decreased frequency of IL-10-producing $CD4^+$ T cells compared to LPS-treated control group. However, *H. felis* treatment of DCs following stimulation with supernatant of B cells have no effect on DCs to induce IL-10-producing $CD4^+$ population. Therefore, it is possible that in addition to IL-10, also some other factors may be involved in differentiation of IL-10-producing $CD4^+$ T cells by DCs stimulated with supernatant of different Hf_{stim}-B cells subgroups.

Furthermore, immature BM-DCs were co-cocultured with Hf_{stim}-B cells at a ratio of 1:1 and 1:2 (DC:B) to evaluate the role of cell-to cell contact on this interaction. Accordingly, direct contact between Hf_{stim}-B cells and immature DCs activates DCs and upregulates CD86 expression. B cells alone did not induce any IL-10 and TNF-α secretion regardless of the ratio between DC and B cell. However LPS or *H. felis* treatment of DCs after interaction with B cells resulted in decreased IL-10 production when the ratio between these cells was 1:1. Importantly, interaction of immature BM-DCs with Hf_{stim}-B cells prior to LPS treatment induced production of IL-10 by DCs; whereas, unstimulated B cells exhibited inhibitory effect on IL-10 production when the ratio between these cells was 1:2 (DC:B). Moreover, TNF-α production by DCs was inhibited efficiently by Hf_{stim}-B cells and unstimulated B cells when BM-DCs were co-cultured with these B cells at a ratio of 1:2 (DC:B) prior to LPS or *H. felis* treatment. It seems that the number of B cells co-cultured

with immature DCs could be a critical factor to exhibit their regulatory function on DCs. Interestingly, Hf_{stim}-IL-10^{+} B cells and Hf_{stim}-IL-10^{-} B cells did not affect the production of TNF-α by DCs.

Collectively; some molecules secreted from Hf_{stim}-B cells in addition to IL-10 synergistically with cell-to-cell contact have a regulatory role on activation and function of DCs.

Consequently; first, DCs were activated and secreted both pro-inflammatory and anti-infammatory cytokines in response to *H. felis* antigens *in vitro*. Second, *Helicobacter felis*-stimulated B cells converts immature BM-DCs into IL-10-secreting tolerogenic form. Therefore, this study includes comprehensive analysis for the effects of *H. felis* on mouse BM-DCs and the first investigation that clarifies the *ex vivo* interaction of Hf_{stim}-B cells subgroups with BM-DCs.

HELICOBACTER-STİMÜLE B ($H_{STİM}$-B) HÜCRELERİ İLE DENDRİTİK HÜCRELER ARASINDAKİ FONKSİYONEL ETKİLEŞİM

ÖZET

Helicobacter pylori (*H. pylori*) ilk olarak Robbin Warren ve Barry Marshall tarafından tanımlanan mikroaerofilik, spiral şekilli ve gram negatif bir bakteridir. Dünya populasyonun yaklaşık yarısından fazlası bu bakteri ile enfekte durumdadır; fakat, enfekte bireylerin sadece %10-20'sinde ciddi gastrointestinal hastalıklar gözlenmektedir. Geriye kalan enfekte bireylerde semptom vermeyen gastrit gözlenmektedir. *H. pylori* mide kanserine neden olabilen bir bakteridir; dolayısıyla, Dünya Sağlık Örgütü tarafından tip-1 karsinojen olarak sınıflandırılmıştır. Farelerde *H. pylori'*ye karşı etkin bir immün yanıt oluşmadığından; fare hastalık modelinin oluşturulmasında *Helicobacter felis* (*H. felis)* kullanılmaktadır.

İlk olarak Steinmann tarafından tanımlanan dendritik hücreler (DC), antijen sunan hücrelerden biridir ve yabancı antijenlere karşı spesifik immün yanıt oluşturulmasında etkili olduklarından doğal ve kazanılmış immün yanıt arasındaki bağlantının oluşturulmasında önemlidir. Regülatör dendritik hücreler IL-12 ve eşuyarıcı proteinleri (CD40, CD80 ve CD86 gibi) düşük düzeyde eksprese ederken PD-L1, CD95L ve indolamin 2,3 dioksijenaz (IDO) gibi baskılayıcı molekülleri yüksek düzeyde eksprese ederler. Öte yandan, olgun olmayan DC'lerin yanı sıra $CD11c^{+}MHCII^{yüksek}CD80/CD86^{yüksek}IL\text{-}12^{-}TNF\text{-}\alpha^{-}IL\text{-}10^{+}$ fenotipine sahip olan yarı olgun DC'lerin tolerojenik rollerinin olduğu düşünülmektedir.

B hücreleri, antikor üretimi ve antijen sunmak gibi efektör görevlerinin yanı sıra immün sistemin regüle edilmesinde önemli role sahiptir. B hücrelerinin regülatör özellikleri, B hücrelerden yoksun farelerin otoimmün ensefalopati hastalığının üstesinden gelememeleri sonucu ortaya çıkmıştır. *H. felis*, B hücrelerini TLR2 ve MyD88 aracılığıyla aktive eder ve tip-1 regülatör T (Tr1) benzeri hücrelerin farklılaşmasını indükleyerek immün yanıtı *in vivo* ve *in vitro*'da baskılar. Bu baskılama hastalığın iyileşmesinde arttırıcı etki gösterirken bakterinin mide mukozasında kronik bir biçimde bulunmasına neden olmaktadır. Dolayısıyla, regülatör ve efektör immün yanıt arasındaki denge enfeksiyonun nasıl sonuçlanacağını belirleyebilmektedir. *H. felis*-aracılı B hücre aktivasyonu sonucunda IL-10 üreten $IL\text{-}10^{+}$ B hücreleri ve TGF-β üreten $IL\text{-}10^{-}$ B hücreleri olmak üzere iki farklı B hücre alt grubunun farklılaştığı laboratuvarımızdaki önceki çalışmalar ile gösterilmiştir.

Bu çalışmadaki ilk amaç, *H. felis*'in fare kemik iliği kökenli DC'ler üzerine olan etkilerini belirlemektir. İkinci amaç ise, *Helicobacter felis*-stimüle B (Hf_{stim}-B) hücrelerinin kemik iliği kökenli DC'ler (BM-DCs) üzerine etkilerini araştırmaktır. Bu deneylerin gerçekleştirilmesi için; C57BL/6 farelerden alınan kemik iliği hücreleri IL-4 ve GM-CSF varlığında olgun olmayan DC'lere farklılaştırılmıştır. B hücreleri ise, C57BL/6 farelerin dalaklarından manyetik ayırım aracılığıyla izole edilmiştir. Daha sonra, $IL\text{-}10^{+}$ B ve $IL\text{-}10^{-}$ B hücre alt gruplarının yine manyetik ayırım ile elde

edilebilmesi için, B hücreleri *H. felis* sonikatı ile muamele edilmiştir. IL-10^{+} B ve IL-10^{-} B hücre alt gruplarının manyetik ayırım yöntemiyle izolasyonunun ardından; hücrelerin salgıladıkları moleküllerin elde edilebilmesi için bu hücreler *H. felis* sonikatı ile uyarılmıştır. Salgıladıkları molekülleri içeren medyumun DC'ler üzerine uygulanmasından sonra hücreler yıkanmış ve BM-DC'ler LPS veya *H. felis* sonikatı ile muamele edilmiştir. DC'lerin sitokin profilleri ve aktivasyon durumları protein ve gen düzeyinde belirlenmiştir. Bununla beraber, DC'lerin fonksiyonel analizleri için DC'ler naif CD4^{+} T hücreleri ile 1:2 oranında (DC:T) 10 ng/ml IL-2 and 1 μg/ml anti-CD3Ɛ varlığında kültürde tutulmuştur. Daha sonra, farklı gruplardaki IL-10 salgılayan CD4^{+} T hücrelerinin oranları akan hücre ölçer analizleri ile belirlenmiştir.

H. felis, DC'lerden IL-12/IL-23 (p40), IL-1β, TNF-α ve IL-10 salgılamalarını indüklemiştir. Bununla uyumlu olarak, IL-12/IL-23 (p40), TNF-α, IL-10 ve IL-6'nın gen ifade düzeyleri indüklenmiştir. Ayrıca, akan hücre ölçer analizleri *H. felis*'in DC'lerin CD86 ifadesindeki artış ile aktive ettiğini göstermiştir.

IL-10^{+} B ve IL-10^{-} B hücreleri de dahil olmak üzere Hf_{stim}-B hücre grupları, herhangi bir antijenik uyarımın yokluğunda DC'lerin CD86 ifadesini indüklemiştir. Fakat, IL-10^{+} B hücre salgıları ile uyarılan ve IL-10^{-} B hücre salgıları ile uyarılan DC'ler arasında CD86 ifadesi açısından anlamlı bir fark gözlenmemiştir. Bununla beraber, Hf_{stim}-B, IL-10^{+} B ve stimüle edilmeyen B hücrelerinin salgıları ile uyarılan DC'lerde LPS veya *H. felis* sonikatı ile muamelenin ardından kontol grubuna göre IL-10 salgılanması farklılık göstermemiştir. Fakat, LPS ile muamele edilen grupta IL-10^{-} B hücre salgıları ile uyarılan DC'lerde IL-10 salgılanması azalmıştır ve ayrıca LPS veya *H. felis* ile muamele edilen gruplarda, IL-10^{+} B hücre salgıları ile uyarılan DC'lerin IL-10^{-} B hücre salgıları ile uyarılan DC'lere göre daha fazla IL-10 salgıladıkları gözlenmiştir. Bütün Hf_{stim}-B hücre gruplarının salgıları DC'lerden IL-12 salgılanmasını aynı oranda indüklerken; LPS veya *H. felis* ile muamele edilen gruplarda TNF-α salgılanmasında baskılanma gözlenmiştir.

Hf_{stim}-IL-10^{+} B hücre salgıları ile uyarılan ve sonradan LPS veya *H. felis* sonikatı ile muamele edilmeyen DC'ler olgun olmayan kontrol DC grubu ile benzer oranda IL-10 üreten CD4^{+} T hücrelerinin farklılaşmasını sağlamıştır. Ayrıca, önceden stimüle edilmeyen B hücreleri, Hf_{stim}-B hücreleri veya Hf_{stim}-IL-10^{-} B hücreleri ile kültürde tutulup ardından LPS ile muamele edilen DC gruplarında sadece LPS ile muamele edilen DC'lere göre daha az IL-10 üreten CD4^{+} T hücreleri gözlenmiştir. B hücre salgılarıyla stimüle edildikten sonra *H. felis* sonikatı ile muamele edilen DC gruplarında IL-10 salgılayan CD4^{+} T hücre oranları açısından bir fark gözlenmemiştir. Dolayısıyla, IL-10'a ek olarak farklı Hf_{stim}-B hücre altgruplarının salgıları ile uyarılmış DC'lerden üretilen başka faktörler IL-10 üreten CD4^{+} T hücrelerinin farklılaşmasında etkili olmaktadır.

Bu analizlere ek olarak, hücreler arasındaki direkt etkileşimin rolünün değerlendirilmesi için olgun olmayan fare kemik iliği kökenli DC'ler Hf_{stim}-B hücreleri ile 1:1 ve 1:2 oranında (DC:B) kültürde tutulmuştur ve sonrasında LPS ve *H. felis* sonikatı ile muamele edilmiştir. Hf_{stim}-B hücreleri ve olgun olmayan DC'ler arasındaki hücreler arasındaki direkt etkileşim DC'lerin CD86 ekspresyonlarını arttırarak DC'leri aktive etmektedir. B hücreleri tek başına DC'lerin IL-10 ve TNF-α salgılamalarını indüklememektedir. Fakat, 1:1 oranında kültürde B hücreleri ile etkileşimin ardından, DC'lerin LPS veya *H. felis* sonikatı ile muamelesi DC'lerin IL-10 salgılamalarının azalmasına neden olmuştur. Bununla beraber, LPS ile muamelenin öncesinde DC'lerin B hücreleriyle 1:2 (DC:B) oranında olacak şekilde

tutulması DC'lerin IL-10 üretimini indüklemiştir; bunun tersine, stimüle edilmeyen B hücreleri aynı koşullarda DC'lerin IL-10 salgılamasını baskılamıştır. Ayrıca, LPS veya *H. felis* sonikatı ile muamelenin öncesinde stimüle edilmeyen veya Hf_{stim}-B hücreleri ile 1:2 (DC:B) oranında etkileşen DC'lerin TNF-α salgılamalarında etkili bir baskılanma gözlenmiştir. Dolayısıyla, DC'ler ile kültürde tutulan B hücrelerinin sayısı, B hücrelerinin DC'ler üzerindeki regülatör etkilerini göstermelerinde etkili bir faktör olabilir. İlginç bir şekilde, Hf_{stim}-IL-10^{+} B veya Hf_{stim}-IL-10^{-} B hücreleri direkt etkileşimde oldukları DC'lerin TNF-α salgılamalarını etkilememiştir.

Dolayısıyla; IL-10'a ek olarak Hf_{stim}-B hücrelerinden salgılanan diğer moleküller direkt hücre etkileşimi ile birlikte DC'lerin aktivasyonu ve fonksiyonu üzerine regülatör role sahiptir.

Sonuç olarak; ilk olarak, DC'lerin *in vitro*'da *H. felis* antijenlerine yanıt olarak aktive oldukları ve pro-enflamatuvar ve anti-enflamatuvar sitokinleri salgıladıkları söylenebilir. İkinci olarak da; Hf_{stim}-B hücrelerinin olgun olmayan fare kemik iliği kökenli DC'leri IL-10 salgılayan tolerojenik forma dönüştürdüğü önerilmektedir. Bu çalışmada, *H. felis* antijenlerinin kemik iliği kökenli DC'ler üzerine etkileri ayrıntılı bir şekilde gösterilmiştir. Ayrıca, çalışmamız Hf_{stim}-B hücre alt gruplarıyla kemik iliği kökenli DC'lerin *ex vivo* ortamdaki etkileşimlerini araştıran ilk çalışmadır.

1. INTRODUCTION

1.1 *Helicobacter* Species

1.1.1 *Helicobacter pylori*

Helicobacter pylori (*H. pylori*), first identified by Robbin Warren and Barry Marshall, is a microaerophilic gram negative and spiral-shaped bacteria (Figure 1.1). About more than 50% of the people in the world have been infected with this bacteria; however, the bacteria cause serious gastrointestinal diseases only in 10-20% of the infected people, whereas the remaining infected people have only asymptomatic gastritis. *H.pylori* can lead to development of gastric cancer and categorised as a group-I carcinogen by World Healh Organization (Bauer and Meyer, 2011).

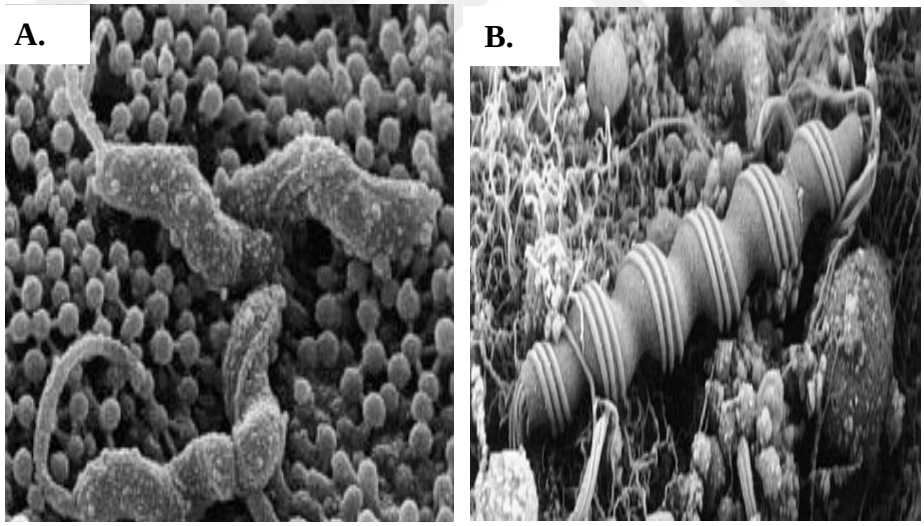

Figure 1.1 : Electron micrograph of *H.pylori* and *H. felis.* A) *H.pylori* in human gastric mucosa B) *H. felis* in infected mice, adapted from (Stoffel et al., 2000).

H. pylori has the ability to live and multiply in gastric mucosa, whose pH is between 4 and 6.5, thanks to bacterial urease activity, which increases the pH value by producing ammonia and carbamate from urea (Clyne et al., 2007). Chronic exposure to *H.pylori* in the gastric environment might lead to peptic ulcer or gastric cancer following atrophic gastritis disease depending on environmental and host factors

such as alcohol, smoking and immune response (Kusters et al., 2006) (Figure 1.2). Major virulance factors which are important for progression of serious complications are cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) (Blaser and Atherton, 2004).

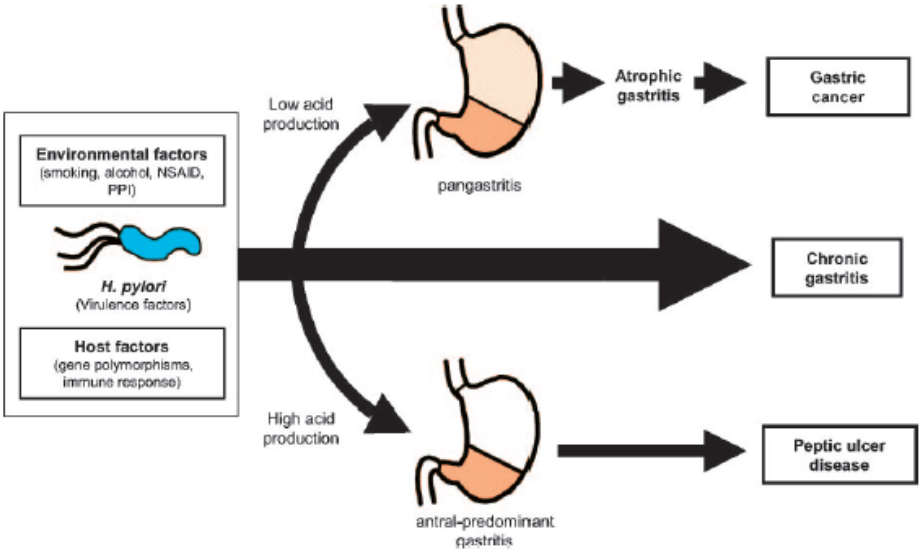

Figure 1.2 : The effect of factors on progression of *H.pylori*-associated gastric diseases, adapted from (Kusters et al., 2006).

1.1.2 *Helicobacter felis*

Helicobacter felis (*H. felis*) was isolated from gastric mucosa of a cat for the first time and is classified in the same family with *H. pylori*. *H. felis* which has a spiral and helical shape exhibits high motility in the culture (Lee et al., 1988) (Figure 1.1B). Gastritis and epithelial cell proliferation have been observed in *H.felis*-infected murines. *H. felis* infection in mice induces inflammation via predominantly activating mononuclear cells and might cause atrophic gastritis. As *H. pylori* has reduced the capacity to induce efficient immune response in mice (Philpott et al., 2002), *H. felis* is used to develop a mouse model of *Helicobacter* infection. However, unlike *H. pylori*, since *H. felis* insufficiently respond to genetic methods that have been performed to clarify its physiology, metabolism or virulence genes, there are not much knowledge about *H. felis* (Kusters et al., 2006). Similar to the other *Helicobacter* species, *H. felis* has urease activity to adapt to the acidic environment of the stomach (Ferrero and Labigne, 1993).

1.2 Dendritic Cells

There are mainly two types of immune responses in the organism; first one is innate immune response which is not antigen-specific and promptly activated upon encountering with a foreign antigen; and latter is antigen-specific adaptive immune response (Banchereau et al., 2000). Dendritic cells (DCs) which were first identified by Steinmann, are professional antigen presenting cells (APCs) and provide a connection between innate and adaptive immune response due to having a crucial role for activation of specific immune response against the foreign antigens (Steinman and Witmer, 1978).

1.2.1 Dendritic cell subsets

Dendritic cells are categorized into two groups based on their function and morphology: conventional DCs and plasmacytoid DCs (pDCs). Both of these cell groups originate from the same bone marrow precursors which is named as common DC progenitors (CDP). Conventional DCs (cDCs) have classical DC morphology and highly express integrin CD11c and MHC-II on their surface. pDCs are found mostly in blood and lymph tissues, chacterized by their capacity to produce large amount of type I interferons. Also, pDCs have the ability to activate $CD4^+$ T cells and $CD8^+$ T cells (Merad et al., 2013, Reizis et al., 2011). The type of cDCs in tissues are called migratory DC whereas the ones in the lymph tissues are called lymphoid DCs. Migratory DCs which comprise $CD103^+$ DCs and $CD103^-$ DCs, are found in skin (Langerhans cells), gut and liver (interstitial DCs). $CD103^+$ DCs and $CD103^-$ DCs recognize and take up foreign antigens from tissues and present them on their surface to activate T cells following their migration to lymph nodes (Liu and Cao, 2015). Lymphoid DCs have $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets depending on the CD8 expression. $CD8\alpha^+$ lymphoid DCs activate $CD8^+$ T cells via MHC-I molecule through cross-presentation; thereby they contribute to immune defence against viral infection or tumor. On the other hand, $CD8\alpha^-$ DCs activate $CD4^+$ T cells by presenting the antigen on the cell surface with MHC-II molecule (Grajales-Reyes et al., 2015, Liu and Cao, 2015) (Figure 1.3) .

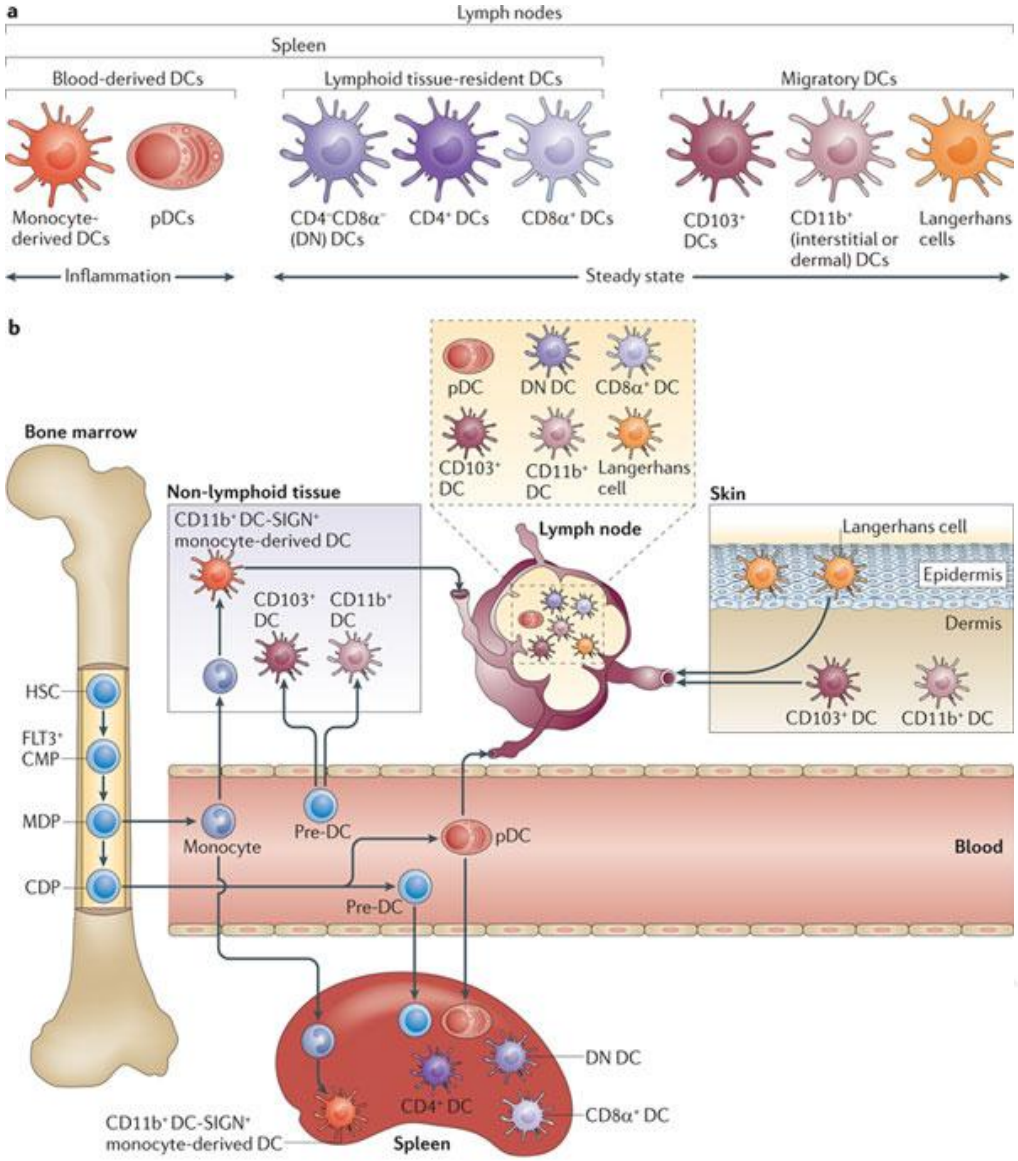

Figure 1.3 : Dendritic cell subsets a) The localization and phenotype of lymphoid and myeloid DCs. b) Fate of different subsets of DCs originated from mouse bone marrow progenitors. DCs originate from the same bone marrow precursors (CDP) and migrate to various lymphoid or peripheral tissues, adapted from (Belz and Nutt, 2012).

1.2.2 Activation and maturation of dendritic cells

The stimuli coming from a pathogen are called pathogen associated molecular patterns (PAMPs) whereas the signals coming from damaged tissues or dead cells are named as danger associated molecular patterns (DAMPs). These molecules activate pattern recognition receptors (PRRs) found on the immune cells related to innate immune response. One of the most studied PRRs are Toll-like receptors (TLRs) which localize on the surface of plasma membrane or intracellular membranes.

Different TLRs are capable of recognizing different molecules; for instance, TLR4 and TLR5 found on the cell surface sense bacterial LPS and flagellin, respectively. On the other hand, TLR-3 and TLR-9, which localize on the surface of endosomal membranes, are activated with dsRNA and CpG DNA, respectively (Abbas et al., 2012) (Figure 1.4).

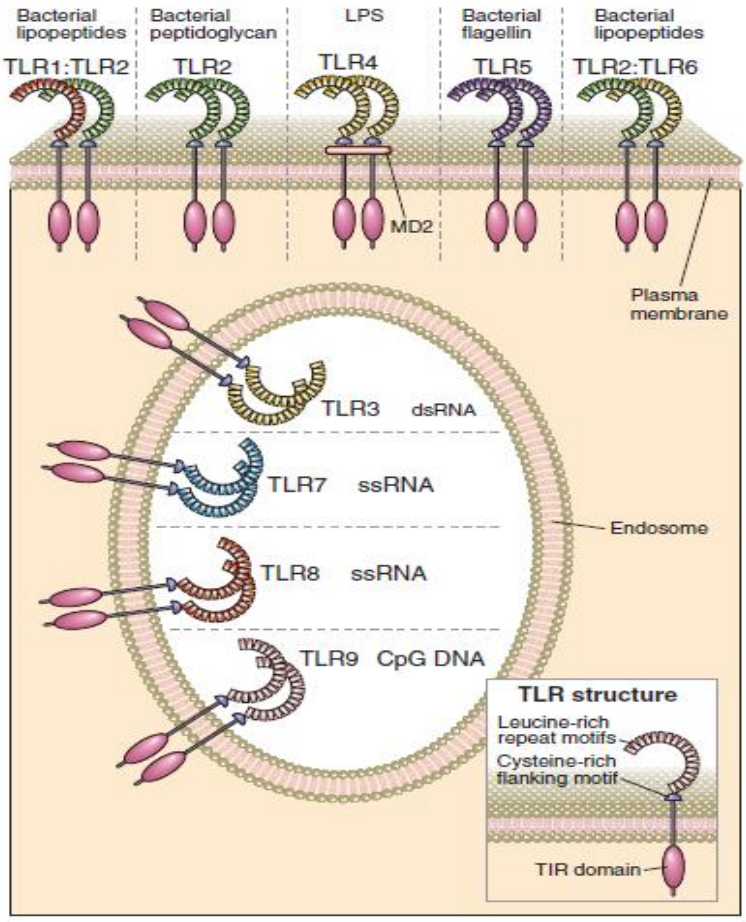

Figure 1.4 : Localization, specifity and structure of mammalian TLRs, adapted from (Abbas et al., 2012).

In addition to PRRs localized on the membranes, Nod-like receptors (NLRs) and Rig-like receptors are the cytosolic PRRs and recognize intracellular foreign antigens such as viral DNA, viral RNA and muramyl dipeptide (Kawai and Akira, 2009).

Immature DCs, which highly express PRRs, are generated in bone marrow and migrate to different tissues. After immature DCs recognize foreign antigens with their PRRs, they become activated and secrete interferons which activate macrophages, natural killer cells (NKs) and eosinophils (Banchereau et al., 2000).

Also, stimulation of PRRs activates intracellular machinery such as MAPK, NF-κB and IRF pathway and results in secretion of various cytokines and chemokines, upregulation of MHC and co-stimulatory molecules such as CD40, CD80 and CD86 which are crucial for activation of T cells (Liu and Cao, 2015). Then, activated DCs migrate to lymph nodes in order to present captured foreign antigens with MHC molecules on their cell surface to naive T cells by delivering three signals. Mature DCs present antigens as MHC-peptide complex and this provides "first signal" to naive T cells. Co-stimulatory molecules such as CD40, CD80 and CD86 expressed on DCs after activation, provide "signal two" which is crucial for T cell function. Finally, the cytokines such as IL-6, IL-12, and transforming growth factor-beta (TGF-β) secreted from DCs induce "signal three" which affects the differentiation of naive T cells (Maldonado and von Andrian, 2010) (Figure 1.5).

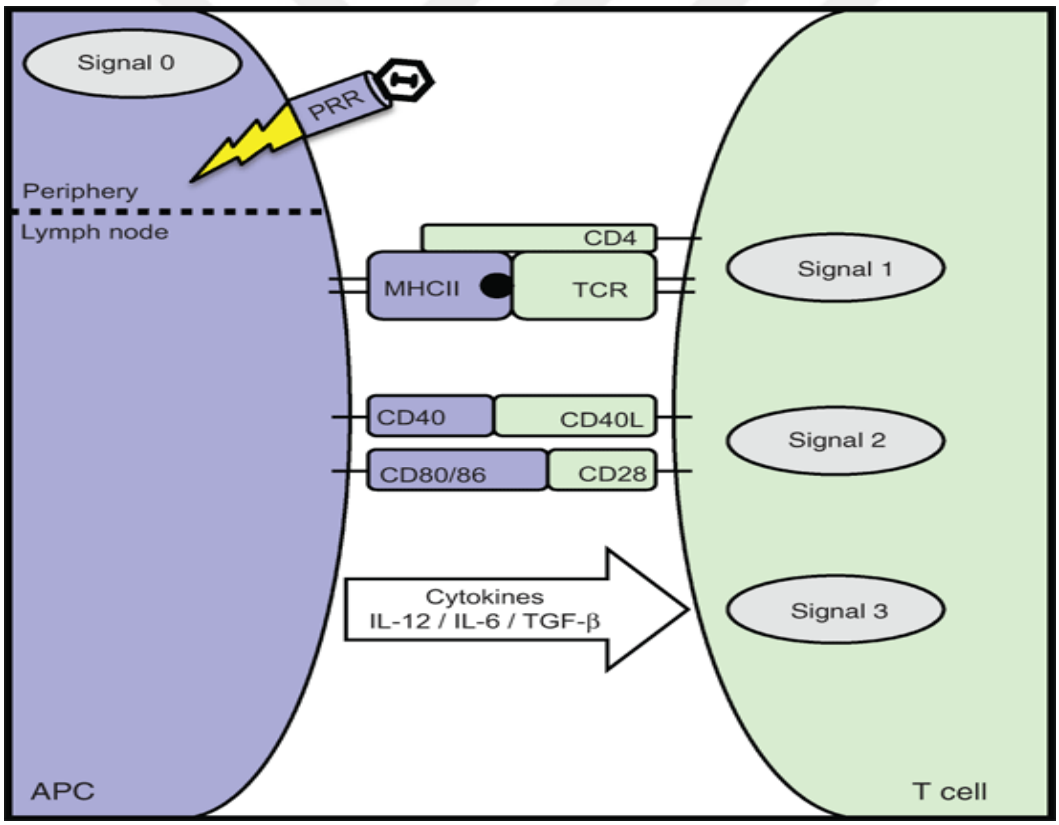

Figure 1.5 : Activation of naive T cells by APCs through three signals, adapted from (Mays and Wilson, 2011).

1.2.3 DCs and immune tolerance

Immune response against harmful organisms leads to inflammation and is important for removing these organisms. However, if this effective response is not regulated well or occurs against self-antigens, excessive response might cause autoimmune diseases and abolish immune homeostasis (Medzhitov, 2008). Regulatory cells suppress and control immune response by secreting anti-inflammatory cytokines such as IL-10 and TGF-β (Nathan and Ding, 2010). Regulatory DCs express low levels of IL-12 and co-stimulatory molecules such as CD40, CD80 and CD86. Instead, expression of inhibitory molecules such as PD-L1, CD95L and indoleamine 2,3 deoxygenase (IDO) are upregulated in regulatory DCs (Morelli and Thomson, 2007). Moreover, in addition to immature DCs, also semi-mature DCs characterized with MHC-II^{hi}CD80/CD86^{hi}IL-12^{-}TNF-α^{-}IL-10^{+} phentotype are thought as tolerogenic DCs and inducer of type-1 regulatory T (Tr1) cell-mediated immune suppression (Rutella et al., 2006). Pathogens and their interactions with PRRs found on DCs, determine the form of DCs which play a critical role for the activation or suppression of T cell response. Each activated mature DCs have specific cytokine profile which is important for induction of T cells via signal three. Together with signal one and two, IL-12 and IFN-γ secreted from DCs induce type 1 T helper (Th1) cells which are responsible cellular immune response with subsequently activated CD8^{+} T cells (Mays and Wilson, 2011).

IL-4, IL-6 and IL-10 secreted from DCs generate type 2 T helper cell (Th2) response which contribute to humoral response. In the case of immune tolerance, mature DCs secrete anti-inflammatory cytokines such as IL-10 and TGF-β, together with the induction of T cells with first two signal. These mature DCs direct naive CD4^{+} T cells to differantiate into Foxp3-expressing regulatory T cells (Tregs) which suppress immune response. Moreover, T cell anergy and immune tolerance can be induced by immature DCs which are unable to deliver signal one and signal two to T cells strongly (Mays and Wilson, 2011) (Figure 1.6).

1.2.3.1 Natural tolerogenic DCs

Tissue microenvironment might lead to generation of tolerogenic DCs. For instance, thymic microenvironment enables pDCs to induce Tregs by means of IL-7-related thymic stromal lymphopoietin found in thymic medulla (Watanabe et al., 2005).

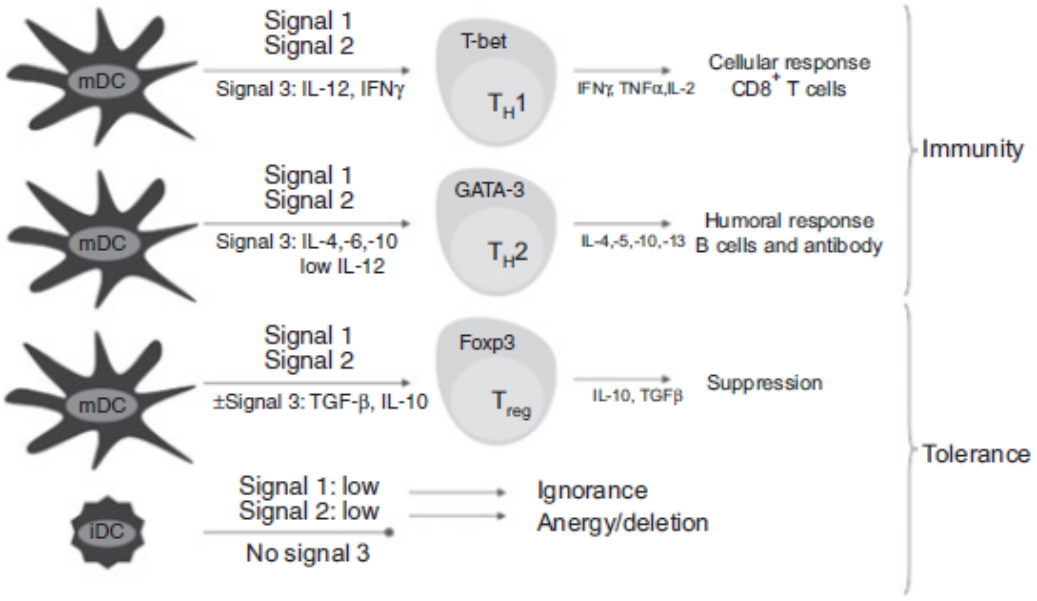

Figure 1.6 : The effect of activation state of dendritic cells on immunity and tolerance, adapted from (Mays and Wilson, 2011).

Tolerance against food and harmless commensal microorganisms in intestine is required and maintained by $CD103^+$ DCs which are capable of inducing Tregs in the presence of retinoic acid and TGF-β secreted from epithelial cells (Artis, 2008). Similar to intestine, tolerance against inhaled molecules in lung are provided by lung $CD103^+$ DCs. In the skin, 1,25-dihydroxyvitamin D3 produced from vitamin D3 by ultraviolet light affect DC function. Triggering of vitamin D3 receptor on DC leads to induction of Tregs in the skin (Maldonado and von Andrian, 2010).

1.2.3.2 Induced tolerogenic DCs

Tolerogenic DCs can be induced by immunosuppressive molecules, genetic engineering, pathogens, other immune cells and tumor microenvironment (Steinman and Banchereau, 2007). Some microorganisms induce tolerogenic DCs in order to escape from immune response. For instance; *Mycobacterium* (Geijtenbeek et al., 2003), *H. pylori* and Yersinia pestis (Depaolo et al., 2008) stimulate tolerogenic DCs which in turn activate Treg mediated response. *H. pylori*-infected bone-marrow derived DCs induce $CD4^+CD25^+Foxp3^+$ regulatory T cells and decrease the Th17:Treg ratio *in vitro* (Kao et al., 2010, Oertli et al., 2012, Zhang et al., 2010). Tumor microenvironment might induce tolerogenic DCs via production of IL-10,

TGF-β, vascular endothelial growth factor (VEGF) and other regulatory molecules (Maldonado and von Andrian, 2010).

$CD4^+CD25^+$ regulatory T cells inhibit IL-6 but induce IL-10 secretion from bone marrow-derived dendritic cells (BM-DCs) contrary to memory or activated $CD4^+$ T cells (Veldhoen et al., 2006). Genetic engineering methods allow modification of DCs by the upregulation of anti-inflammatory cytokines or suppressive molecules such as IL-10, TGF-β, CTLA-4, programmed death ligand-1 (PD-L1) or downregulation of effector molecules such as IL-12, co-stimulatory molecules (CD80 and CD86) (Morelli and Thomson, 2007). This genetically engineered DCs may be used for therapeutic strategies for autoimmune diseases.

1.3 B Cells

Hematopoietic stem cells in bone marrow differentiate into immature B cells after formation of pro-B and pre-B cells, respectively. In the bone marrow, B cell development continues without the effect of any antigens contrary to peripheral lymphoid tissue and brain where development of B cell is antigen-dependent (Dalakas, 2008). During this maturation, B cell receptor is produced on the cell surface through somatic recombination of the antibody gene segments called V, D and J. Immature B cells express immunoglobulin M (IgM) on their cell surface and migrate to spleen from bone marrow via blood stream to form transitional B cells which highly express IgM and immunoglobulin D (IgD). In spleen, survival of these cells are supported by the activation of B cell activating factor receptor (BAFF-R) signaling and transitional B cells are converted to $IgM^{hi}IgD^{lo}CD21^+CD23^-BAFFR^+$ marginal zone B cells or $IgM^{lo}IgD^{high}CD21^+CD23^+BAFFR^+$ follicular B cells. Marginal zone B cells are converted into plasma cells upon their encounter with an antigen whereas follicular B cells differentiate to memory or plasma cells in germinal center following their interaction with an antigen. During this development there are checkpoints to prevent development of self-reactive B cells (Pieper et al., 2013) (Figure 1.7).

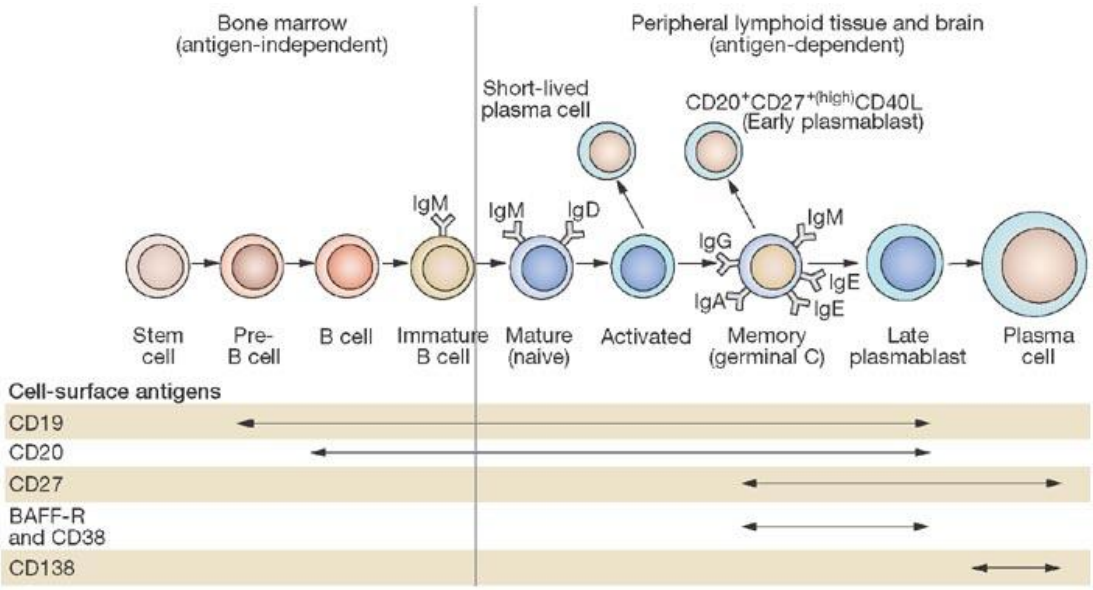

Figure 1.7 : Development of B cells from bone marrow-derived stem cells. At different maturation stages, B cells express distinct surface antigens, adapted from (Dalakas, 2008).

1.3.1 Activation of B cells

Extracellular region is suitable environment for the extracellular pathogens to multiply in the host. On the other hand, intracellular pathogens exploit extracellular region to reach and infect the other cells as well. Humoral immune response which is carried out with antigen-specific antibodies produced by B cells is vital for clearence of these pathogens found in extracellular space. For thymus-dependent antigens, B cell should receive two signals. First; B cell recognizes foreign antigens through B cell receptor (BCR) which delivers first signal to B cell. Second; B cell captures and presents these antigens as MHC-II-peptide complex to previously activated helper-II T cell (Th2) which delivers the second signal to B cell. B cell contacts with Th2 cell by means of CD40-CD40L interaction and T cell secretes cytokines such as IL-4, IL-5 and IL-6 which stimulate B cell to proliferate and differentiate into antibody-releasing plasma cell. Moreover, differentiation of B cells results in generation of memory cells as well (Figure 1.8A and B). On the other hand, sometimes B cell receives second signal from thymus-independent antigens through TLR signaling (Figure 1.8C) (Murphy, 2012).

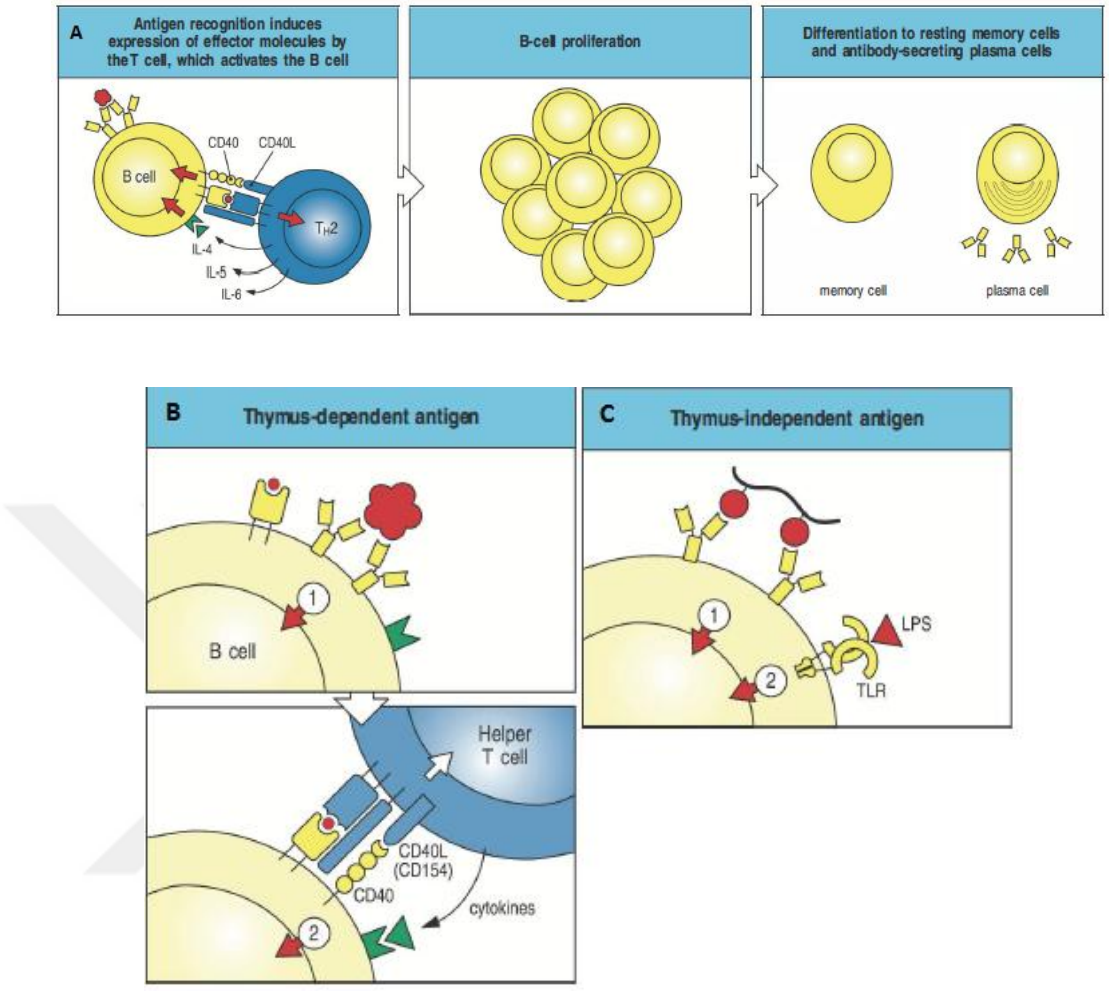

Figure 1.8 : Activation of B cells through two signals A) Th2 induce B cells to proliferate and differentiate into plasma and memory B cells. B) B cells receive second signal from Th2 cells for thymus-dependent antigens. C) Thymus-independent antigens deliver second signal to B cells by triggering TLR signaling, adapted from (Murphy, 2012).

1.3.2 Regulatory B cells

B cells have an important role in the regulation of immune response in addition to their effector roles such as antibody production and antigen presentation (Figure 1.9). Regulatory role of B cells was shown by using mice lacking B cells which was not able to overcome autoimmune encephalitis (Susan D. Wolf et al., 1996). Moreover, B cell-secreted IL-10 which is an anti-inflmmatory cytokine, is responsible for suppression of chronic inflammation since the chimeric mice, having IL-10-deficient B cell, was not able to recover from experimental autoimmune encaphalomyelitis (EAE) (Fillatreau et al., 2002).

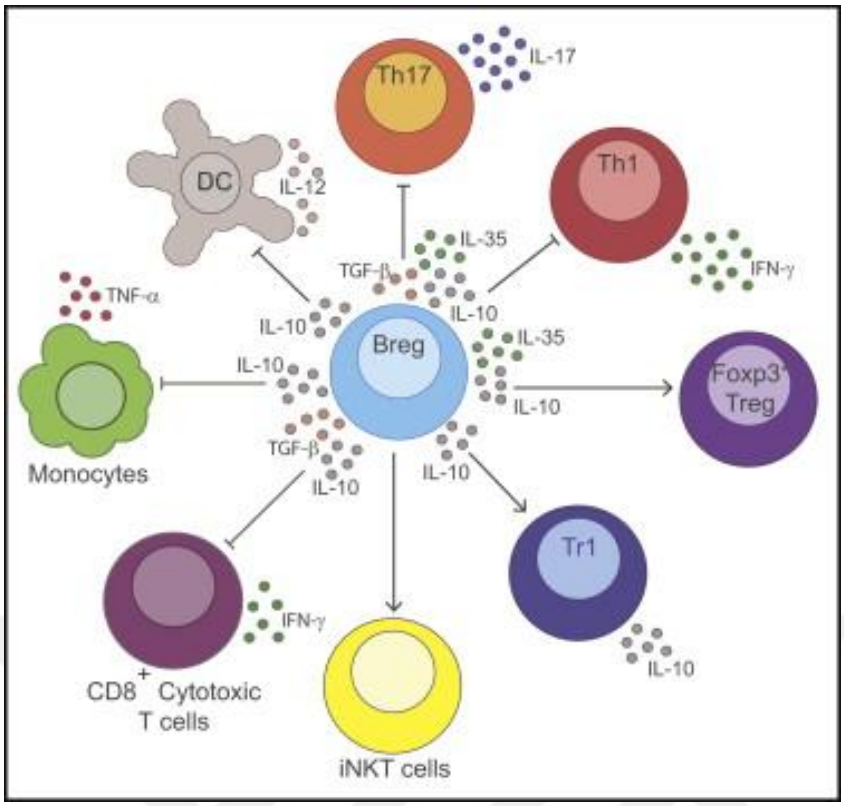

Figure 1.9 : Breg-mediated regulation of immune response, adapted from (Rosser and Mauri, 2015).

Arthritis induction in IL-$10^{-/-}$ B cell mice resulted in an increase in Th1 and Th17 responses but a decrease in exacerbated disease whereas the frequency of both type 1 regulatory T (Tr1) cells and $Foxp3^+$ Tregs were decreased (Carter et al., 2012, Carter et al., 2011). Strikingly, when B cells were transfered to B cell deficient mice, Treg response and immune tolerance was restored (Sun et al., 2008). Regulatory B cells (Bregs) induce Tregs in contact dependent manner via MHC-II and co-stimulatory molecules. Expression of Fas ligand (FasL) and TGF-β by LPS-activated B cells promotes apoptosis of mononuclear cells and lymphocytes (Tian et al., 2001). The other way for suppression of effector T cell response by Bregs is through the inhibition of the IL-6 and IL-12 expression in DCs which gives rise to suppression of Th1 and Th17 responses (Matsumoto et al., 2014). Bregs also produce other anti-inflammatory cytokines in addition to IL-10 such as TGF-β and IL-35. Deficiency of IL-35 in B cells gives rise to failure of suppression of Th1 response while TGF-β production by B cells activated by LPS leads to $CD4^+$ T cell apoptosis and $CD8^+$ T cell anergy. Pro-inflammatory response mediated by IL-1β and IL-6 enhances IL-10-secreting Bregs in the presence of CD40-stimulation in arthritis-induced mice (Rosser et al., 2014). In addition, *H.felis* stimulates B cells via TLR2/Myd88

pathway and this stimulation results in formation of IL-10-producing Breg which converts naive T cells into Tr1 cells through cell-to-cell contact. These complex interactions increase immune tolerance and decrease pathology score (Sayi et al., 2010).

Different Breg subsets in mouse and human including IL-10-producing B10 cells, transitional-2 marginal zone-precursor, plasmablast, plasma cells were identified (Table 1.1).

Table 1.1 : Phenotype of human and mouse Breg subsets.

Breg Subset	Mouse	Human	References
T2-MZP cells	$CD19^{+}CD21^{hi}$ $CD23^{hi}CD24^{hi}$	-	(Evans et al., 2007)
MZ cells	$CD19^{+}CD21^{hi}CD23^{-}$	-	(Bankoti et al., 2012)
B10 cells	$CD5^{+}CD1d^{hi}$	$CD24^{hi}CD27^{+}$	(Iwata et al., 2011, Yanaba et al., 2008)
Plasma cells	$CD138^{+}MHC\text{-}11^{lo}B220^{+}$	-	(Shen et al., 2014)
Tim-1^{+} B cells	$CD19^{+}$Tim-1^{+}	-	(Ding et al., 2011)
Plasmablast	$CD138^{+}CD44^{hi}$	$CD19^{+}CD24^{hi}$ $CD27^{int}$	(Matsumoto et al., 2014)
Immature cells	-	$CD19^{+}CD24^{hi}$ $CD38^{hi}$	(Blair et al., 2010)
Br1 cells	-	$CD19^{+}CD25^{hi}$ $CD71^{hi}$	(van de Veen et al., 2013)
PD-$L1^{hi}$ B cells	$CD19^{+}$PD-$L1^{hi}$	-	(Khan et al., 2015)

Br1: B regulatory 1, MZ: marginal zone; T2-MZP: transitional 2 marginal-zone precursor, Tim-1: T cell Ig domain and mucin domain protein 1, PD-L1: Programmed death-ligand 1.

1.4 T Cells

T cell precursors which migrate to thymus for cellular development are differentiated from hematopoietic stem cells in bone marrow. Thymic selection and production of unique T cell receptor (TCR) on the surface of each T cell through rearrangement of TCR genes enable T cells to recognize infinite numbers of foreign antigens (Luckheeram et al., 2012). Mature T lymphocytes that are not encountered with an antigen are called naive cells and enter secondary lymphoid organs via blood and lymphatic vessels. In secondary lymphoid organs, dendritic cells activate T cells through presentation of a foreign antigen as MHC-peptide complex and co-

stimulatory molecules that bind to CD28 expressed on naive T cells as explained in Section 1.2.2. Activated T cells proliferate quickly and migrate to the place where antigen of interest is present. T cells exhibit effector functions against foreign antigens by $CD8^+$ cytotoxic T cells which directly kills cells carrying foreign antigen, or by $CD4^+$ helper T cells which secrete various cytokines that affect other immune cells and inflammatory mechanisms (Broere et al., 2011).

1.4.1 $CD4^+$ T cells

Naive T cells can differentiate into different types of $CD4^+$ T cells upon activation signals depending on cytokine profile of the microenvironment, type of antigen presenting cells and antigen and co-stimulatory molecules. IL-12 and IFNγ are critical molecules that contribute to differentiation of Th1 cells. These Th1 cells secrete IFNγ to elevate pro-inflammatory response against intracellular pathogens. Whereas, IL-4 is required for differentiation of IL-4-secreting Th2 cells which are critical for elimination of extracellular parasites. Furthermore, effector Th17 response is activated against extracellular pathogens in the presence of TGF-β and IL-6. On the other hand, presence of TGF-β in the microenvironment together with other factors might result in Treg differentiation that regulates immune response (Luckheeram et al., 2012). Differentation status of $CD4^+$ T cells are controlled by expression of different transcription factors in each cells. In this context; Tbet, GATA3, RORγt and Foxp3 are major transcription factors for Th1, Th2, Th17 and Treg; respectively (Zou and Restifo, 2010) (Figure 1.10).

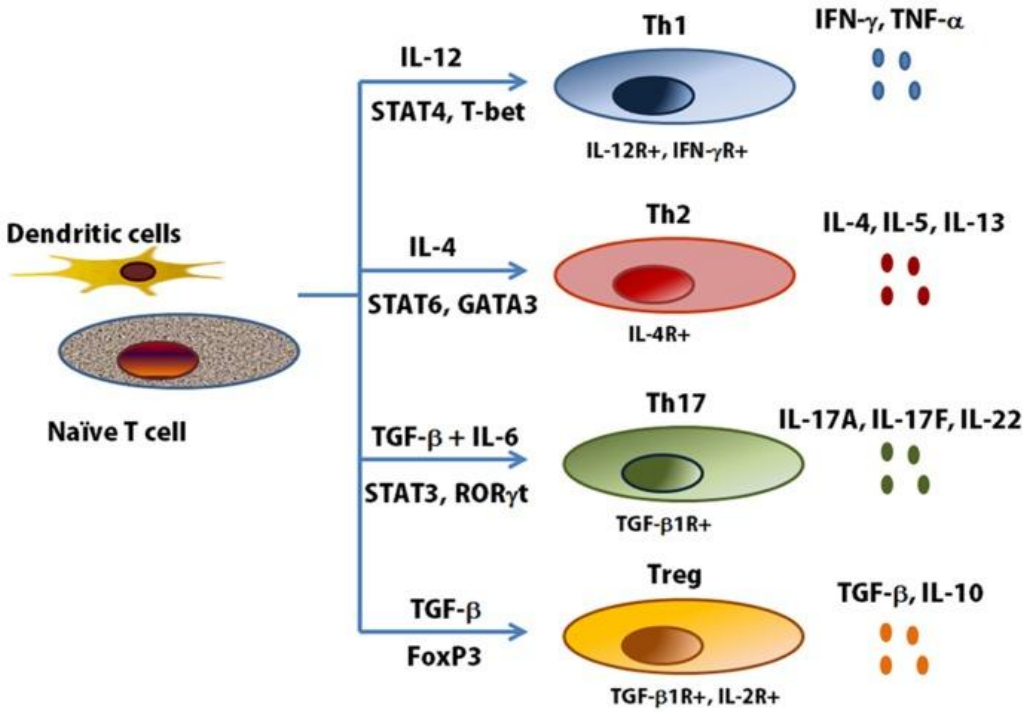

Figure 1.10 : Differentiation of naive $CD4^+$ T cells into different T cell subsets, adapted from (Kudo et al., 2013).

1.4.2 Regulatory T cells

There are two main types of regulatory T cells: natural and induced regulatory T cells (Figure 1.11). Natural regulatory T cells (nTregs) are formed in thymus upon encountering self antigens and then migrate to periphery tissues to prevent the functions of effector T cells that react to self antigens. nTregs express α chain of IL-2 receptor called CD25 and Foxp3 transciption factor; thereby; they are chacterized as $CD4^+CD25^+Foxp3^+$ T cells (Bluestone and Abbas, 2003). Foxp3 transciption factor is critical for the function of Tregs since in the absence of Foxp3, human and mice developed severe systemic autoimmune diseases. Consistently, it has been demonstrated that Foxp3 expression in Tregs results in upregulation of inhibitory molecules such as CD25 and CTLA-4. On the other hand, inducible Tregs (iTregs) characterized as $CD4^+CD25^-$ are differentiated from naive T cells in periphery in the presence of TGF-β and IL-10 or in the case of antigen presentation without co-stimulation (Broere et al., 2011). Among iTregs; type 1 regulatory T cells (Tr1) which are $Foxp3^-$ generated via IL-10 suppress immune responses by secreting IL-10, whereas the suppressive function of T helper 3 (Th3) cell is performed by TGF-β. Other than $CD4^+$ T cells, there are inducible $CD8^+$ regulatory T cells differentiated

from naïve $CD8^{+}CD25^{-}$ T cells. iTregs suppress effector immune response via cell-to-cell contact or secretion of anti-inflammatory cytokines. Direct contact for suppression is provided by CTLA-4 which is an inhibitory molecule that binds to B7 proteins (CD80/CD86) on antigen presenting cells and prevent CD28 signaling. On the other hand; IL-10 has a suppressive role on DCs and macrophages by preventing secretion of TNF and IL-12, whereas TGF-β affects the expression of T-bet and IL-12R which are crucial for the activation of Th1 response. Although Tregs prevent excessive immune response and autoimmune diseases, sometimes pathogens induce Treg-mediated immune regulation that results in persistent infection (Mills, 2004). For instance, *Helicobacter felis* activates B cells via TLR2 and Myd88 which suppress immune response *in vivo* and *in vitro* by triggering IL-10-producing Tr1-like cells (Sayi et al., 2010).

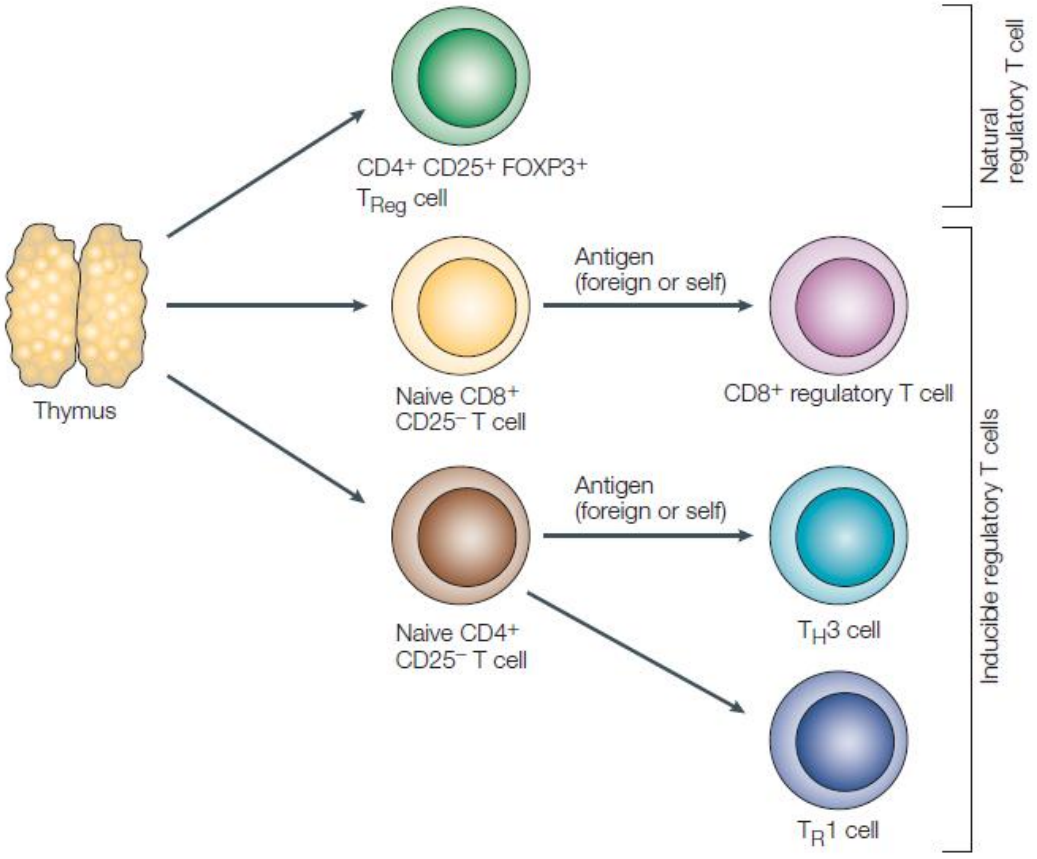

Figure 1.11 : Natural and inducible regulatory T cells, adapted from (Mills, 2014).

1.5 Immune Response Against *Helicobacter pylori*

H. pylori is a non-invasive bacterium due to its inability to cross epithelial barrier. However; thanks to flagella, bacterial shape, and virulance factors such as *vacA* and *cagA*, they can penetrate to mucus layer (Moyat and Velin, 2014). Although *H. pylori* has LPS which is normally sensed by TLR4, it has been shown that *H. pylori* activates immune response via TLR2-mediated signaling (Fredrik Backhed et al., 2003, Smith et al., 2003). Complex immune response against *H. pylori* contains both humoral and cellular immune responses. Gastric biopsies from persistently infected individuals have increased number of infiltrated T cells, B cells, monocytes, macrophages, eosinophils, mast cells and dendritic cells compared to uninfected individuals (Moyat and Velin, 2014). Moreover; infected individuals, but not uninfected individuals, have *H. pylori*-specific $CD4^+$ T cells in the gastric mucosa (Annalisa Di Tommaso et al., 1995). Significantly increased level of IFNγ, TNF-α, IL-1, IL-6, IL-7, IL-8, IL-10, and IL-18 were found in the stomach of the infected people (C. Lindholm et al., 1998) together with the increase of IL-17A in the gastric mucosa (Luzza et al., 2000). Collectively, it seems that Th1- and Th17- mediated immune responses are activated upon *H. pylori* infection. Consistently, IFN-γ production from Th1 cells or Th17- mediated immune response against *H. pylori* leads to gastric inflammation together with the increased clearance of the bacteria (Kao et al., 2010, Sayi et al., 2009). Other than the effector immune response, *H. pylori* infection might induce Treg response which facilitates persistency of the bacteria in the host. Besides, elimination of Tregs in mice using anti-CD25 before infection have resulted in inreased bacterial clearence but promoted gastritis (Rad et al., 2006).

Humoral immune response is activated in all individuals infected with *H. pylori*. IgA and IgG target the antigens of *H. pylori* in chronically infected individuals. Furthermore, local antibody response with increased number of IgA- and IgM-secreting cells have been observed in infected people. *H. pylori* might also lead to production of autoreactive antibodies that target host epithelial cells resulting in tissue injury (Moyat and Velin, 2014).

Direct contact of *H. pylori* with gastric epithelial cells or presence of some *H. pylori*-derived molecules such as urease result in activation of polymorphonuclear cells and

macrophages which induce T cell response. Dendritic cells play a critical role in immune response against *H. pylori* within gastric mucosa (Figure 1.12). DCs can penetrate to epithelial barrier and capture *H. pylori* antigens. Also, disruption of epithelial organization might facilitate DCs to take up these antigens. Then these activated DCs via TLR-signaling direct the activation of different T cell subsets with different manners. Accordingly; IL-10, IL-12 or IL-23 production by DCs following the encounter with the *H. pylori* antigens induce Th2/Treg, Th1 or Th17 response; respectively (Peek et al., 2010).

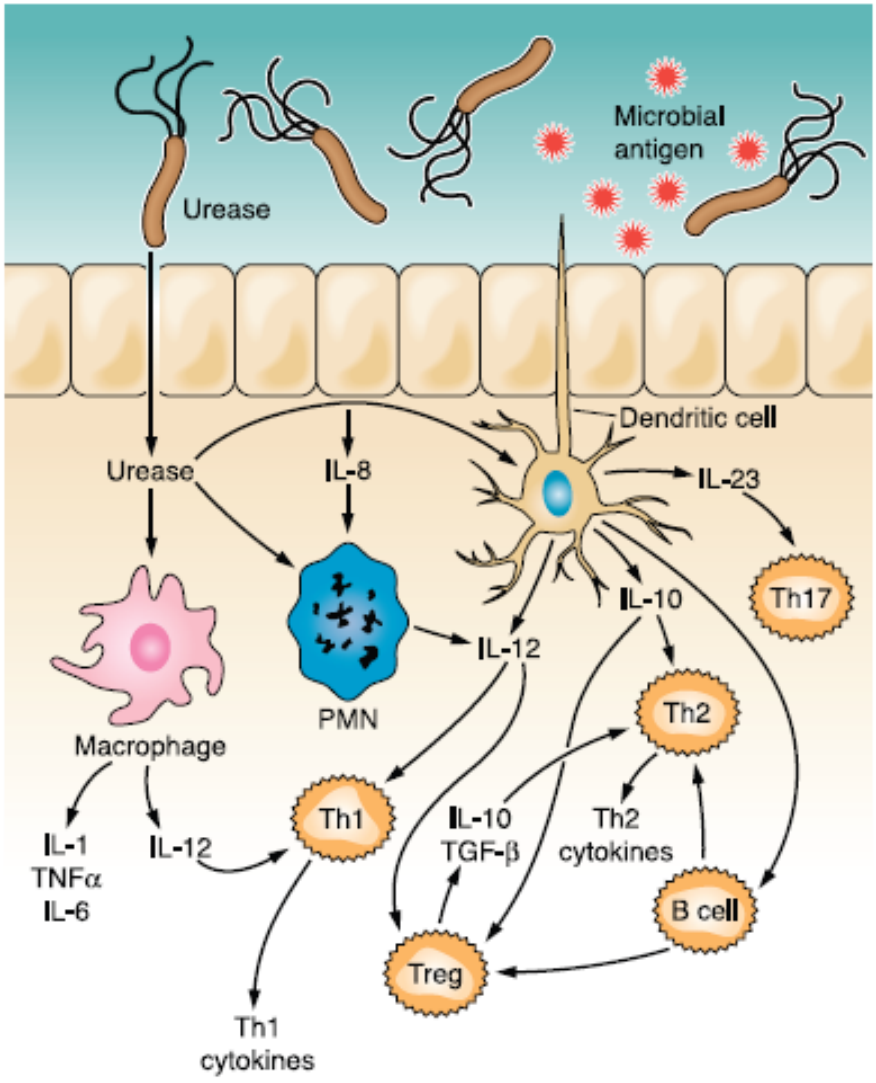

Figure 1.12 : Immune response against *Helicobacter pylori* within the gastric mucosa, adapted from (Peek et al., 2010).

H.pylori activates human monocyte-derived DCs by inducing co-stimulatory molecules and cytokine secretion. Moreover, *H.pylori*-pulsed human DCs induce Th1 differentiation since IL-2, TNF-α and IFNγ were detected upon co-culture of DCs with naive T cells. Supportively, these T cells expressed T-bet, whereas their GATA-3 expression was suppressed (Hafsi et al., 2004). Activation and maturation of human monocyte-derived DCs are independent of *vacA* and *cag* PAI virulance factors and induced by formalin-inactivated *H. pylori*, culture supernatant or sonicated bacteria (Kranzer et al., 2005). *H. pylori*-stimulated BM-DCs internalize the bacteria and express IL-1α, IL-1β and IL-6; however, they express low levels of IL-12 (Kao et al., 2006). However, Oertli et al. (2012) has demonstrated that live *H. pylori* induce BM-DCs to express CD86 and secrete IL-10 and IL-12 but IL-6 secretion is not induced. Moreover, *H. pylori*-stimulated DCs inhibit LPS-mediated DC maturation and leads to immune tolerance by induction of Treg cells or lowering the Th17/Treg ratio (Kao et al., 2010, Oertli et al., 2012). Besides, as a result of chronic exposure of DCs to *H. pylori*, DCs become incapable of inducing Th1 response (Mitchell et al., 2007).

1.6 Interaction Between DCs and B Cells

There is a complex interplay between DCs and B cells. Maturation and differentiation of monocyte-derived DCs are regulated by B cells upon BCR and TLR9 signaling since these B cells downregulate the expression of maturation markers such as CD80, CD86, CD40, HLA-DR and DC-SIGN compared to DCs differentiated without B cell stimulation. Moreover, B cells activated in this way induce apoptosis in differentiating DCs (Maddur et al., 2012, Morva et al., 2012). In addition, activated B cells have inhibitory effects on mature DCs by lowering IL-12 secretion and downregulating maturation markers in contact dependent-manner and via soluble factors secreted from B cells (Morva et al., 2012). IL-10 is crucial for inhibition of the effector functions of DCs since $CD5^+$ B cells, which receive TLR9 signaling produce IL-10 and negatively regulate the function of neonatal DCs by suppressing IL-12 production and DC-mediated Th1-activation (Sun et al., 2005). Also, IL-10-producing plasmablasts inhibit the expression of IL-12 and IL-6 in DCs along with the suppression ability of DCs to activate Th1 and Th17 responses (Matsumoto et al., 2014). In addition to regulatory effect of B cells on DCs,

maturation of monocyte-derived DCs are induced by B cells activated via BCR-signaling upon cell-to-cell contact (Maddur et al., 2014). As a result, it seems that different signaling pathways in B cells play a crucial role for the status of DCs.

DCs have a role in proliferation and survival of B cells. Accordingly, DCs induce proliferation of B cells independent of CD40 signaling; whereas survival signals from DCs are conveyed to B cells in CD40-dependent manner (Wykes and Macpherson, 2000). Moreover, secretion of a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF) molecules by DCs contribute to development, survival and activation of B cells (MacLennan and Vinuesa, 2002). Dendritic cells can present the captured unprocessed foreign antigen to naive B cells *in vivo* and *in vitro* resulting in antigen-specific immune response (Wykes et al., 1998). Regulatory DCs obtained through IL-10 induce B cells to differentiate into IL-10-producing $CD19^{hi}FcRIIb^{hi}$ B cells via CD40-CD40L and DC-derived IFN-β dependent manner (Qian et al., 2012). Dexamethasone and 1a,25-dihydroxyvitamin D3 (DexVD3)-induced DCs have tolerogenic properties and can promote IL-10-secreting $CD19^{+}$ B cells (Volchenkov et al., 2013).

1.7 Aim of the Study

More than 50% of the people in the world have been infected with *H. pylori*, although most of them developed asymptomatic gastritis (Bauer and Meyer, 2011). Moreover, it is known that Th1- and Th17- mediated effector immune response against *H. pylori* is responsible for clearance of bacteria along with gastric inflammation (Kao et al., 2010, Sayi et al., 2009). On the other hand, Tregs contribute to persistency of the bacteria since elimination of Tregs leads to aggravated gastric disease (Rad et al., 2006). In addition, the protective and immune-suppressive role of regulatory B cells for *Helicobacter* infection has been shown *in vivo* and *in vitro* (Sayi et al., 2010). Moreover, it is known that IL-10-producing plasmablasts suppress effector functions of dendritic cells (Matsumoto et al., 2014). Collectively, balance between effector and regulator immune response is critical for the outcome of infection and interaction between Bregs and DCs might play a role for this regulatory mechanisms against *H. pylori*.

In this study, *H. felis* is used instead of *H. pylori* which is incapable of inducing efficient immune response in mice. First, we aimed to determine the effects of *H.*

felis on mouse bone marrow-derived dendritic cells. In literature, most investigations have been conducted using *H. pylori*. Nevertheless, Drakes et al. (2006) used *H. felis* antigen for stimulation of BM-DCs; however, they have not checked CD86, IL-12 and TNF-α. Henceforth, we wanted to make comprehensive analysis for *H. felis*-stimulated BM-DCs by investigating CD86, IL-1β, IL-6, IL-10, IL-12 and TNF-α.

Previous studies in our laboratory showed that *H. felis* sonicate activates B cells and we determined two B cell subgroups: IL-10-producing *Helicobacter*-stimulated IL-10^+ B cells and TGF-β-producing *Helicobacter*-stimulated IL-10^- B cells. Accordingly, our second aim was to investigate the regulatory effects of *Helicobacter*-stimulated B cell subgroups on BM-DCs.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

Helicobacter felis (*H.felis*) was provided by Prof. Dr. Anne Müller from University of Zurich. The bacteria was plated on Columbia Agar plate containing 1000 X antibiotic cocktail. Columbia Agar medium and 1000 X antibiotic cocktail were prepared as shown in Table 2.1 and Table 2.2, respectively.

Table 2.1 : The contents of Columbia Agar medium.

Constituent	Amount
BD Columbia Agar	42,5 g
Horse Blood	50 ml
β-cyclodextrin	10 ml
1000X Antibiotic Cocktail	1 ml

Table 2.2 : The contents of 1000X Antibiotic Cocktail.

Constituent	Amount	Company
Trimethoprim	100 mg	HiMedium
Amphotericin B	160 mg	HiMedium
DMSO	20 ml	Fisher-Scientific

2.1.2 Liquid culture for *Helicobacter felis*

Liquid medium for *H. felis* was prepared as 50 ml total volume and contents and volume of constituents were given in Table 2.3.

Table 2.3 : The contents of liquid medium for *H. felis*.

Constituent	Amount	Company
Brucella Broth	50 ml	BD
FBS [%10 (v/v)]	5 ml	Lonza
Vancomysin	5 µl	HiMedium

2.1.3 Freezing of *H. felis*

The constituents and their volume for preparation of freezing medium were given in Table 2.4. Freezing medium was stored in 4 ^{0}C refrigerator.

Table 2.4 : The contents of Freezing Medium for *H. felis*.

Constituent	Amount
Brucella Broth	25 ml
Gliserol	25 ml

2.1.4 Primary cells

2.1.4.1 Bone marrow-derived dendritic cells (BM-DCs)

Mouse bone marrow cells were cultured for 7 days in DC Differentiation Medium to differentiate them into dendritic cells. DC Differentiation Medium was prepared as shown in Table 2.5.

Table 2.5 : The contents of DC Differentiation Medium.

Constituent	Concentration	Company
RPMI Medium	50 ml	Lonza
FBS	10 %	Lonza
Penicillin-Streptomycin	1 %	Gibco
Recombinant GM-CSF	10 ng/ml	Biolegend
Recombinant IL-4	10 ng/ml	PeproTech
Beta-mercaptoethanol	50 µM	Sigma-Aldrich

2.1.4.2 CD19$^+$ B cells

CD19$^+$ B cells were isolated from the spleen of C57BL/6 mouse through magnetic separation (MACS, Miltenyi Biotec) and cultured in Complete RPMI (containing 10% FBS, 1 % Penicillin-Streptomycin) medium in 96-well plate.

2.1.4.3 CD4$^+$ T cells

CD4$^+$ T cells were isolated from the spleen of C57BL/6 mouse through magnetic separation (MACS, Miltenyi Biotec) and cultured in Complete RPMI (containing 10% FBS, 1 % Penicillin-Streptomycin) medium in 96-well plate. In addition, 10 ng/ml recombinant IL-2 (MACS, Miltenyi) and 1 µg/ml anti-CD3Ɛ (17A2, Miltenyi Biotec) were added to culture medium for maintenance of the cells in culture.

2.1.5 Cell culture

Reagents that were used in cell culture studies were shown in Table 2.6. Moreover, the preparation of the buffers and medium were given in Table 2.7.

Table 2.6 : Reagents for cell culture studies.

Solution	Company
Roswell Park Memorial Institute Medium (RPMI)	Lonza
Fetal Bovine Serum (FBS)	Lonza
Penicillin-Streptomycin	Gibco
Trypan blue	Lonza

Table 2.7 : Preparation of solutions used in cell culture studies.

Solution	Preparation
PBS 1 X	9,55 g in 1L ddH_2O
MACS Buffer	0.5 % BSA and 2 mM EDTA in 1 X PBS
FACS Buffer	2% FBS in 1 X PBS
Complete RPMI	Contains 10% FBS and 1% Penicillin-Streptomycin
10 mM PBS/EDTA	10 mM EDTA in 1 X PBS

2.1.6 ELISA

Solutions used in ELISA experiments and their preparations were given in Table 2.8.

Table 2.8 : Solutions used in ELISA experiments.

Solution	Preparation
PBS/T (0.05 %)	0.05 % Tween in 1 X PBS
Stop Solution	2N H_2SO_4 (in ddH_2O)

2.1.7 Laboratory equipments and materials

Laboratory equipments and materials that were used in the experiments were shown in the Table 2.9 and Table 2.10, respectively.

Table 2.9 : Laboratory equipments used in the experiments.

Equipment	Company
Laminar Air Flow Cabinets	FASTER BH-EN 2003
Flow Cytometer	BD Accuri C6
Pipettes	10 µl, 20 µl, 100 µl, 200 µl, 1000 µl Socorex and 10 µl, 100 µl, 1000 µl Biohit
Electronic pipette	CappAid
Centrifuges	Beckman Coulter Allegra ™ 25 R Centrifuge Scanspeed 1730 R Labogene Scanspeed mini
CO_2 Incubator	BINDER
Nanodrop 2000	Thermo Scientific
Shaker	Heidolp Duomax 1030
Step One Real Time PCR	Applied Biosystem
Sonicator	Bandelin Sonopuls
Vortex	Mixer Uzusio VTX-3000L,LMS
Ouick Spin	LMS
Magnetic Stirrer	WiseStir MSH-20D, Wisd Laboratory Equipment
Light Microscope	Olympus CH30
Hemocytometer	Isolab
Ice Machine	Scotsman AF10

Table 2.9 (continued) : Laboratory equipments used in the experiments.

Equipment	Company
Refrigerators	Altus (+4 ^{0}C)
	Siemens (-20 ^{0}C)
	Haier (-80 ^{0}C)
Spectrophotometer	BIO-RAD Benchmark Plus
Weight	Precisa

Table 2.10 : General laboratory materials used in the experiments.

Material	Company
Gloves	Tenty
Anaerobic Jar	Anaerocult
Erlenmeyers	Isolab
Falcon Tubes (15 ml, 50 ml)	Isolab
Slide	Interlab
Coverglass	Interlab
Cotton swab	Interlab
96-well plate for ELISA	Nunc
Cell Strainer (70 μm)	BD
Serological pipettes	Sarstedt
96-well plates	TPP
Eppendorf tubes (0,6 ml, 1,5 ml, 2 ml)	Interlab
Columns for Magnetic Separation (MS, LS)	Miltenyi Biotec.
CampyGen 2.5L	Oxoid

2.1.8 General chemicals

General chemicals used in the experiments were given in the Table 2.11.

Table 2.11 : General chemicals used in the experiments.

Material	Company
EDTA	Applichem
Absolute ethanol	Merck
Phosphate Buffered Saline (PBS)	Lonza
Gliserol	Merck

Table 2.11 (continued) : Laboratory equipments used in the experiments.

Material	Company
Bovine Serum Albumin (BSA)	Santa Cruz
DMSO	Fisher Scientific
β-Mercaptoethanol	Sigma-Aldrich
Columbia Agar	BD
Brucella Broth	BD
Lipopolysaccharide (LPS)	Sigma-Aldrich
Fixation Buffer (4%)	Biolegend
Permeabilization Buffer (10 X)	Biolegend
Monensin	Calbiochem
PMA	Sigma
Ionomycin	Calbiochem
Tween 20	Fisher-Scientific
HCl	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
NaOH	Sigma-Aldrich

2.1.9 Primers

Primers, which were used in the experiments, and their properties were given in the Table 2.12.

Table 2.12 : Primers used in the experiments.

Primer	Sequence (5'-3')	Species	Tm	Product size
TNF-α Fw	GTCGTAGCAAACCACCAAGT	m	58 ^{0}C	139 bp
TNF-α Rv	CCTGGGAGTAGACAAGGTACAA			
IL-12 Fw	GCACTCCCCATTCCTACTTC	m	60 ^{0}C	103 bp
IL-12 Rv	AACGCACCTTTCTGGTTACA			
IL-10 Fw	GAGGCGCTGTCATCGATTTCT	m	60 ^{0}C	103 bp
IL-10 Rv	GGCCTTGTAGACACCTTGGTC			
IL-6 Fw	CACAGAGGATACCACTCCCAACA	m	60 ^{0}C	120 bp
IL-6 Rv	TCAGAATTGCCATTGCACAACT			
18s rRNA Fw	GGCCCTGTAATTGGAATGAGTC	m/h	60 ^{0}C	146 bp
18s rRNA Rv	CCAAGATCCAACTACGAGCTT			

2.1.10 Antibodies

Anti used in the experiments and their properties were given in the Table 2.13.

Table 2.13 : Antibodies used in the experiments.

Antibody	Clone	Experiment	Company
Recombinant IL-2	-	Cell culture	Militenyi Biotec
Anti-mouse Anti-CD3Ɛ	17A2	Cell culture	Militenyi Biotec
Rat anti-mouse CD4-APC	Rm-4-5	Flow Cytometry	BD
Rat anti-mouse CD19-FITC	6D5	Flow Cytometry	Biolegend
Rat anti-mouse IL-10-PE	JES5-16E3	Flow Cytometry	BD
Armanian hamster anti-mouse CD11c-APC	N418	Flow Cytometry	Biolegend
Rat anti-mouse CD86-PE	GL-1	Flow Cytometry	Biolegend

2.2 Methods

2.2.1 Culture of *H .felis*

H. felis was cultured on Columbia blood agar which contains appropriate amount of antibiotics and incubated at 37 ^{0}C under microaerophilic condition in anaerobic jar for 3-4 days. The anaerobic condition in the jar is provided by using CampyGen packets. 42,5 gram Columbia agar was dissolved in 1 liter distilled water and then bottle containing this solution was autoclaved. Afterwards, the bottle was incubated in 56 ^{0}C water for 1 hour and following incubation 50 ml horse blood was added into the agar solution. Also, for growth of *H. felis* in the culture 10 ml β-cyclodextrin and 1 ml 1000 X antibiotic cocktail were added. After 3-4 days incubation, the viability and motility of the bacteria were checked under light microscope. According to dilution rate which is required for optimum growth, the bacteria were cultured in Brucella liquid medium containing 10 µg/ml Vancomycin. For preparation of Brucella medium, 28 gram Brucella Broth powder was dissolved in 1 L sterile distilled water and after that, for sterilization, liquid medium was autocloved at 121 ^{0}C for 15 minutes.

2.2.2 Sonication of *H. felis*

When the liquid culture were reached to 120-200 ml, sonication process were performed. Before sonication, viability and motility of the bacteria were checked under light microscope and liquid culture were shared to 15 ml falcon tubes. These

falcon tubes were centrifuged at 1500 rpm for 10 minutes and supernatants were discarded. For washing step, 10 ml PBS was added onto the pellets and after that, the falcon tubes were centrifuged at 1500 rpm for 10 minutes. Supernatants were discarded and 3.5 ml PBS solution was added onto the pellets. Sonicator was set as 30 seconds *pulse on* and 50 seconds *pulse off* for 6.5 minutes at 50 watt. Then, the falcon tube was placed to machine and the process was performed on ice to prevent overheating. For this process, MS 72 prob was used. After sonication, sonicates was shared to eppendorf tubes as 100 µl for each tube and the tubes were centrifuged at 15.000 rpm, at +4 ^{0}C for 20 minutes. Concentration of sonicates were measured by BCA assay.

2.2.3 Protein bicinchoninic acid (BCA) Assay

After sonication, the concentration of the *H. felis* sonicates were determined using Thermo Scientific's Pierce Protein BCA Assay Kit. For this method, bovine serum albumin (BCA) standarts set was used. Solution B and Solution B were mixed at 1:50 ratio according to number of standarts and samples in duplicate. The mixture were put to the proper wells in 96 well plate (flat bottom) as 200 µl for each well. Diluted BSA standarts, whose details were given in the Table 2.14**,** and the samples were put as 10 µl into the proper wells containing indicator. Then, the plate was covered and incubated at 37 ^{0}C at least for 30 minutes. After incubation, the plate was cooled to room temperature and absorbance of samples were measured at 562 nm using Microplate Reader.

Table 2.14 : Preperation of BSA standarts for BCA Assay.

Standart	ddH2O (µl)	BSA source ve and volumes (µl)	Final BSA Concentration (µg/ml)
A	0	300 µl from stock	2,000
B	125	375 µl from stock	1,500
C	325	325 µl from stock	1,000
D	175	175 µl from Tube B	750
E	325	325 µl Tube C	500
F	325	325 µl Tube E	250
G	325	325 µl Tube F	125
H	400	100 µl Tube G	25
I	400	0	0=Blank

2.2.4 Isolation of mouse B cell

B cells were isolated from the spleen of C57BL/6 mouse using Mouse B Cell Isolation Kit (MACS Miltenyi). The isolation protocol was performed according to manufacturer's instructions and explained in details in the following sections.

2.2.4.1 Preparation of cell suspension

The spleen was taken from sacrified mouse and put on the 70 μm cell strainer by means of pipette tip. Then, the spleen was smushed using plunger of syringe and filtered single cell suspension were prepared in 50 ml falcon. For filtration and washing of cell strainer, incomplete RPMI medium was used. These steps were performed for each spleen that were taken. After that, falcon tube was filled with incomplete RPMI and the cell suspension was centrifuged at 1780 rpm for 8 minutes. Supernatant were discarded and cell pellet was dissolved in proper amount of MACS Buffer which was used as 1 ml for each spleen. Then, the cells were diluted at 1:200 ratio with MACS Buffer and counted by hemacytometer under ligh microscope. Cell viability were determined by trypan blue staining.

2.2.4.2 Magnetic labelling of non-B cells

The cells were centrifuged at 1780 rpm for 8 minutes. 40 μl of MACS Buffer for each 10^7 cells was used to resuspend the cell pellet. After that, for each 10^7 cells, 10 μl of Biotin-Antibody Cocktail was added onto the cell suspension. Tube were mixed well by pipetting and incubated at 4 ^{0}C refrigerator for 10 minutes. Then, 30 μl of MACS Buffer and 20 μl of Anti-Biotin Microbeads for each 10^7 cells was added onto the cell suspension and the falcon tube was put 4 ^{0}C refrigerator for 15 minutes again. After this incubation, the cells were washed with 1-2 ml of MACS Buffer for each 10^8 cells and centrifuged at 1780 rpm for 8 minutes. Then, cell pellet was dissolved in 500 μl MACS Buffer for each 10^8 cells.

2.2.4.3 Magnetic separation: depletion of non-B cells

LS column was placed to MACS Midi magnetic seperator and activated 3 ml of cold MACS Buffer. Then, cell suspension was applied onto the column and after solution had passed through the column completely, column was washed with 3 ml of MACS

Buffer for 3 times. After being added buffer to column, it is crucial to wait for the buffer to completely pass through the column. The cells passing through the column were unlabeled B cells; whereas the cells which were bound to the column were non-B cells. To obtain the non-B cells, the column was removed from seperator and placed on a 15 ml falcon tube. Then, 5 ml of MACS Buffer was added to column and the non-B cells were collected in this falcon tube by strongly pushing the plunger into the column. Cell numbers were determined and cell viability were checked by trypan blue staining under light microscope.

2.2.4.4 Determination of B cell purity

Purity of the isolated splenic B cells was determined by using flow cytometer. $5x10^4$ B and non-B cells were taken to eppendorf tubes and dissolved in 50 µl FACS Buffer containing 0,2 µl 0.5 mg/ml FITC-conjugated CD19 antibody. The eppendorf tubes were incubated at dark for about 1 hour. B and non-B cell dissolved in FACS Buffer without the stain were used as unstained control. Afterwards, the cells were washed by adding 1 ml of FACS Buffer and centrifuged at 2000 rpm for 5 minutes. Then, supernatants were discarded and pellets were dissolved in 150 µl of FACS Buffer. All prepared samples were analyzed using flow cytometer.

2.2.4.5 Isolation of IL-10-producing regulatory B cell

IL-10-producing B cells were magnetically isolated using Mouse Regulatory B Cell Isolation Kit (MACS Miltenyi). The isolation protocol was performed according to manufacturer's instructions and explained in details in the following sections.

2.2.4.6 *in vitro* stimulation

Isolated $CD19^+$ B cells were centrifuged at 1780 rpm for 8 minutes and cell pellet was dissolved in 1 ml of complete RPMI for each $2.5x10^6$ cells. Then, the cells were plated in 96 well plate (U bottom) as 500.000 cells for each well. For stimulation, the cells were incubated with 5 µg/ml *Helicobacter felis* sonicate for 16 hours. Unstimulated B cells were used as a control group. For inducing of B cells to secrete IL-10, at 11th hour of incubation, 50 ng/ml PMA and 500 ng/ml Ionomycin were added to the wells. Additionally, enough number of *Helicobacter felis*- stimulated B cells and unstimulated B cells and their supernatant were kept and used for further experiments.

2.2.4.7 Labelling cells with Regulatory B Cell Catch Reagent

The cells were collected in a falcon tube and counted by hemacytometry. Cell viability were controlled by trypan blue staining. The cells were washed with cold MACS Buffer and centrifuged at 1780 rpm for 8 minutes. Supernatant was discarded and pellet was dissolved in 90 µl of cold MACS Buffer for each 10^7 cells. Then, 10 µl of Regulatory B Cell Catch Reagent for each 10^7 cells was added to falcon tube. Catch reagent containing anti-IL-10 and anti-CD45 monoclonal antibodies, is attached to the cell surface. After these steps, the tube was mixed well and incubated for 5 minutes on ice.

2.2.4.8 IL-10 secretion period

10 ml of warm complete RPMI for each 10^7 cells was added in the tube and the cells were incubated for 45 minutes at 37 ^{0}C under slow continuous rotation. In this period, the secreted IL-10 binds to the catch reagent that is found on cell surface.

2.2.4.9 Labelling cells with Regulatory B Cell Detection Antibody (PE)

The tube was filled with MACS Buffer and put on ice for 5 minutes. This step prevents unspecific labelling. 10 µl of Regulatory B Cell Detection Antibody (PE) for each 10^7 cells was added in the tube. The tube was mixed well and cells were incubated on ice for 10 minutes. After incubation, the cells were washed by adding 10 ml of cold MACS Buffer and centrifuged at 1780 rpm for 8 minutes. Supernatant was carefully removed. At this step, IL-10-specific and PE-conjugated detection antibody binds to *Helicobacter*-stimulated IL-10-producing B cells and make possible to check the purity of these cells by flow cytometry.

2.2.4.10 Magnetic labelling with Anti-PE Microbeads

80 µl of cold MACS Buffer and 20 µl Anti-PE Microbeads for each 10^7 cells was added onto the cell pellet and the tube was mixed well. Then, the cells were incubated for 15 minutes at 4 ^{0}C. After incubation, the cells were washed with 10 ml cold MACS Buffer for each 10^7 cells and centrifuged at 1780 rpm for 8 minutes. Supernatant was removed and up to $5x10^7$ cells were dissolved in 500 µl cold MACS Buffer. At the end of this step, the IL-10 producing B cells are magnetically labeled and ready for magnetic separation using MACS seperator.

2.2.4.11 Magnetic separation through MS Column

MS column was placed to MACS Mini magnetic seperator and activated 500 µl of cold MACS Buffer. Then, cell suspension was applied onto the column and after solution had passed through the column completely, column was washed with 500 µl of MACS Buffer for 3 times. After being added buffer to column, it is crucial to wait for the buffer to completely pass through the column. The cells passing through the column were unlabeled IL-10^- B cells; whereas the cells which were bound to the column were IL-10^+ B cells. To obtain IL-10^+ B cells, column was removed from seperator and placed on a 15 ml falcon tube. Then, 1 ml of MACS Buffer was added to column, IL-10^+ B cells were collected in this falcon tube by strongly pushing the plunger into the column. Cell numbers were determined and cell viability were checked by trypan blue staining under light microscope. The purity of IL-10^- and IL-10^+ B cell was determined by flow cytometry.

2.2.5 Isolation of mouse T cell

$CD4^+$ T cells were isolated from the spleen of C57BL/6 mouse using Mouse $CD4^+$ T Cell Isolation Kit (MACS Miltenyi). The isolation protocol was performed according to manufacturer's instructions and explained in details in the following sections.

2.2.5.1 Preparation of cell suspension

The spleen was taken from sacrified mouse and put on the 70 µl cell strainer by means of pipette tip. Then, the spleen was smushed using plunger of syringe and filtered single cell suspension were prepared in a 50 ml falcon. For filtration and washing of cell strainer, incomplete RPMI medium was used. These steps were performed for each spleen that were taken. After that, falcon tube was filled with incomplete RPMI and the cell suspension was centrifuged at 1780 rpm for 8 minutes. Supernatant were discarded and cell pellet was dissolved in proper amount of MACS Buffer which was used as 1 ml for each spleen. Then, the cells were counted by hemacytometer under ligh microscope. Cell viability were determined by trypan blue staining.

2.2.5.2 Magnetic labelling of non-T cells

The cells were centrifuged at 1780 rpm for 8 minutes. 40 µl of MACS Buffer for each 10^7 cells was used to resuspend the cell pellet. After that, for each 10^7 cells, 10

µl of T Cell Biotin-Antibody Cocktail was added onto the cell suspension. Tube were mixed well by pipetting and incubated at 4 ^{0}C refrigerator for 5 minutes. Then, 30 µl of MACS Buffer and 20 µl of Anti-Biotin Microbeads for each 10^7 cells was added onto the cell suspension and the falcon tube was put 4 ^{0}C refrigerator for 10 minutes. After this incubation, the cells were washed with 1-2 ml of MACS Buffer for each 10^8 cells and centrifuged at 1780 rpm for 8 minutes. Then, cell pellet was dissolved in 500 µl MACS Buffer for each 10^8 cells.

2.2.5.3 Magnetic separation: depletion of non-T cells

LS column was placed to MACS Midi Magnetic Seperator and activated 3 ml of cold MACS Buffer. Then, cell suspension was applied onto the column and after solution had passed through the column completely, column was washed with 3 ml of MACS Buffer for 3 times. After being added buffer to column, it is crucial to wait for the buffer to completely pass through the column. The cells passing through the column were unlabeled T cells; whereas the cells which were bound to the column were non-T cells. To obtain the non-T cells, the column was removed from seperator and placed on 15 ml falcon tube. Then, 5 ml of MACS Buffer was added to column and the non-T cells were collected in this falcon tube by strongly pushing the plunger into the column. Cell numbers were determined and cell viability were checked by trypan blue staining under light microscope. T cells were resuspended in complete RPMI containing 10 ng/ml IL-2 and 1 µg/ml anti-CD3Ɛ and seeded in 96-well plate.

2.2.5.4 Determination of T cell purity

Purity of the isolated splenic B cells was determined by using flow cytometer. $5x10^4$ T and non-T cells were taken to eppendorf tubes and dissolved in 50 µl FACS Buffer containing 0,2 µl 0.2 mg/ml APC-conjugated CD4 antibody. The eppendorf tubes were incubated at dark for about 1 hour. T and non-T cell dissolved in FACS Buffer without stain were used as unstained control. Afterwards, the cells were washed by adding 1 ml of FACS Buffer and centrifuged at 2000 rpm for 5 minutes. Then, supernatants were discarded and pellets were dissolved in 150 µl of FACS Buffer. All prepared samples were analyzed using flow cytometer.

2.2.6 Generation of mouse bone marrow-derived dendritic cells

Bone marrow cells were isolated from tibia and femur bones of C57BL/6 mouse. To perform that, after C57BL/6 mouse had been sacrificed, the hind legs of mouse were taken by the help of sterile scissor. The tissue and muscles around the bones were removed carefully using sterile gauze. After the bones had been cleaned, the tips of bones were cut sterile needle were placed into the end of bones. Then, bone marrow cells were collected in 50 ml falcon tube by injecting complete RPMI into the bones. To obtain single cell suspension, the cells were filtrated using 70 μm cell strainer. After that, the cells were centrifuged at 1780 rpm for 8 minutes and cell pellet was dissolved in 1 ml of complete RPMI. Cell number was determined using hematocytometer under light microscope. Moreover, cell viability was checked by trypan blue staining. For differentiation to dendritic cells, Dendritic Cell Differentiation Medium, which is complete RPMI containing 10 ng/ml recombinant GM-CSF, 10 ng/ml recombinant IL-4 and 50 μM Beta-mercaptoethanol, was prepared. Bone marrow cells were plated in 96 well plate (flat bottom) as $25x10^4$ cells for each well for 7 days. Then, proper number of cells were dissolved in proper volume of DC Differentiation Medium. Every 2 days, half of the medium was refreshed with freshly prepared DC Differentiation Medium. Immature DCs were generated after 7 days and cells were detached by 1X PBS containing 10 mM EDTA. Then, for enrichment of immature $CD11c^+$ DCs, magnetic separation using anti-CD11c microbeads was performed according to manifacturer's instructions.

2.2.6.1 Magnetic separation through CD11c Microbeads

$CD11c^+$ bone marrow-derived dendritic cells were magnetically seperated based on positive selection. The prosedure was explained in details in the following sections.

2.2.6.2 Magnetic labeling

At day 7, the medium of the wells and adherent cells were collected in a falcon tube. Adherent cells were detached by adding 200 μl 1X PBS containing 10 mM EDTA. Then, the cells were centrifuged at 1780 rpm for 8 min and cell pellet was dissolved in 1 ml of complete RPMI. After cell number had been determined, the cells were centrifuged again at 1780 rpm for 8 min. Supernatant was removed completely and cell pellet was dissolved in 400 μl of MACS Buffer for each 10^8 total cells. After that, 100 μl of CD11c Microbeads for each 10^8 total cells was added and the falcon

tube was mixed well. Then, the cells were incubated for 15 minutes in the 4^0C refrigerator. When the cell number was fewer than 10^8, same volumes were used as recommended in the protocol. After incubation, the cells were washed with 1-2 ml of MACS Buffer for each 10^7 cells and centrifuged at 1780 rpm for 8 min. Up to 10^8 cells were resuspended in 500 µl of MACS Buffer.

2.2.6.3 Magnetic separation through MS Column

MS column was placed to MACS Mini magnetic seperator and activated 500 µl of cold MACS Buffer. Then, cell suspension was applied onto the column and after solution had passed through the column completely, column was washed with 500 µl of MACS Buffer for 3 times. After being added buffer to column, it is crucial to wait for the buffer to completely pass through the column. The cells passing through the column were unlabeled non-DCs; whereas the cells which were bound to the column were DCs. To obtain the DCs, column was removed from seperator and placed on 15 ml falcon tube. Then, 1 ml of MACS Buffer was added to column, DCs were collected in this falcon tube by strongly pushing the plunger into the column. Cell numbers were determined and cell viability were checked by trypan blue staining under light microscope. Immature DCs were plated in 96 well plate (flat bottom) as $5x10^4$ cells for each well. The purity of non-DC and DC was determined by flow cytometry.

2.2.6.4 Determination of DC purity

Purity of the isolated splenic B cells was determined by flow cytometry. $5x10^4$ DC and non-DC were taken to eppendorf tubes and dissolved in 50 µl FACS Buffer containing 0,2 µl 0.2 mg/ml APC-conjugated CD11c antibody. The eppendorf tubes were incubated at dark for about 1 hour. DC and non-DC dissolved in FACS Buffer without stain were used as unstained control. Afterwards, the cells were washed by adding 1 ml of FACS Buffer and centrifuged at 2000 rpm for 5 minutes. Then, supernatants were discarded and pellets were dissolved in 150 µl of FACS Buffer. DC and non-DC dissolved in FACS Buffer without stain were used as unstained control. All prepared samples were analyzed using flow cytometer. Schematic representation of interaction *Helicobacter*-stimulated B cells and dendritic cells is shown in Figure 2.1.

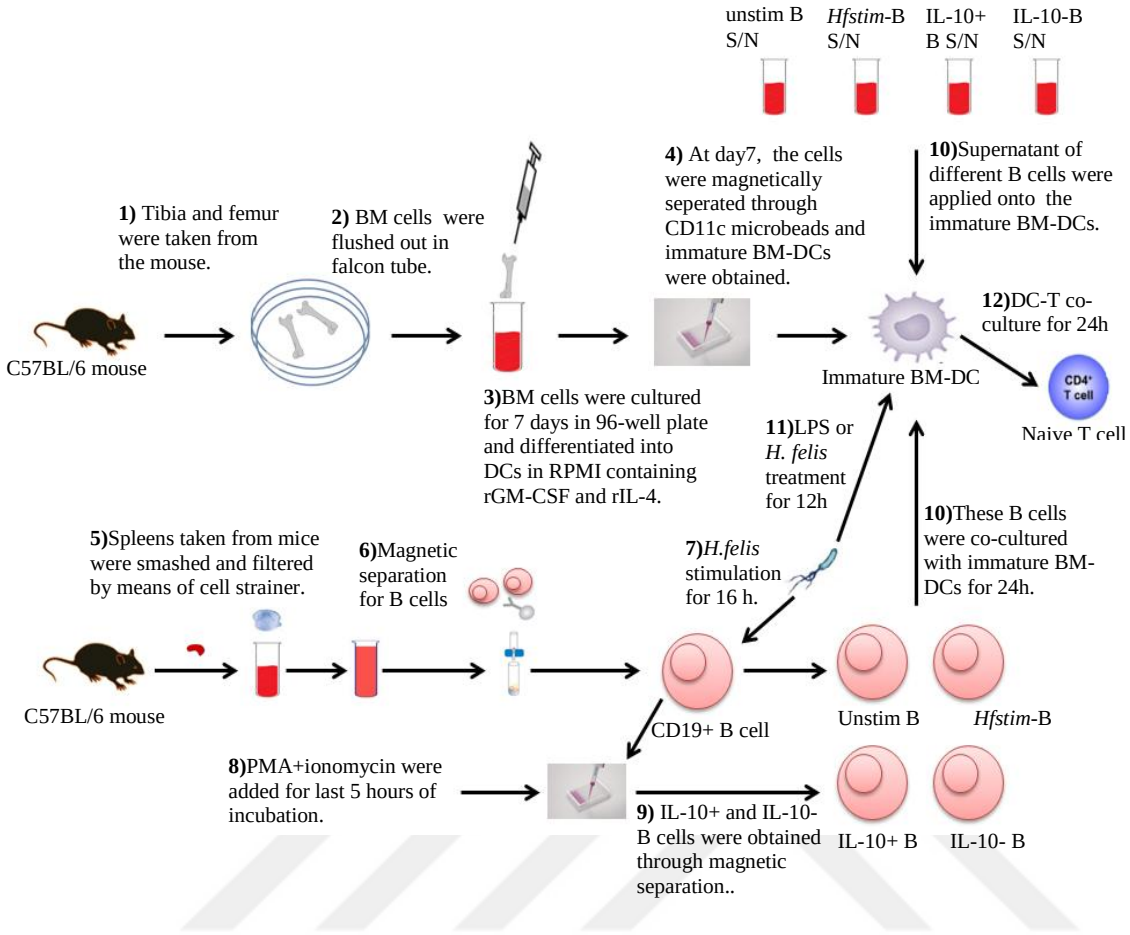

Figure 2.1 : Schematic representation of interaction *Helicobacter*-stimulated B cells and dendritic cells.

2.2.7 Stimulation of BM-DCs with B cell supernatant

After isolated B cells had been treated with *H. felis* sonicate for 16 hours, the cells were harvested and centrifuged at 1780 rpm for 8 min. Then, supernatant were collected. These supernatants contain cytokines that are secreted from B cells. *H. felis*-stimulated B cells (Hf_{stim}-B cells) were used for isolation IL-10^+ B and IL-10^- B cells as mentioned before (Section 2.2.4.5). After IL-10^+ B and IL-10^- B cells had been treated with 5 µg/ml *H. felis* sonicate for 8 hours, their supernatant were collected in the same way with treated B cells. On the other hand, enough number of B cells were left as untreated (unstimulated B cells) and their supernatant were taken at the same time with supernatant of Hf_{stim}-B cells. Then, the medium from the 96 well plate, that DCs had been plated before, was discarded and to check the effect of molecules secreted from given B cells, immature BM-DCs were stimulated with 100 µl of supernatant of unstimulated B cells, Hf_{stim}-B cells, IL-10^+ B cells or IL-10^- B

cells per well for 24 hours. Also, 100 µl complete RPMI was applied to some wells instead of the supernatants and these groups were used as unstimulated control groups.

2.2.8 BM-DC-B Cell co-culture

BM-DCs were cultured with unstimulated B cells, Hf_{stim}-B cells, IL-10^+ B cells or IL-10^- B cells at a ratio of 1:1 (DC:B; $5x10^4$ DC: $5x10^4$ B cells) or 1:2 (DC:B; $5x10^4$ DC: $1x10^5$ B cells) for 24 hours in 96 well-plates (flat bottom) in order to assess the effect of these B cell groups on BM-DCs. Also, some groups without B cells were used as control groups.

2.2.9 Treatment of BM-DCs with LPS or *H. felis* sonicate

BM-DCs were treated with 100 ng/ ml LPS or 10 µg/ml *H. felis* sonicate for 12 hours after stimulation with supernatants from B cells or DC-B co-culture. Also, untreated BM-DCs were used as control groups. After treatments, the supernatant of DCs were collected and used for ELISA while DC pellets were used for flow cytometry and real time PCR. Additionally, immature BM-DCs were treated with 100 ng/ ml LPS or 10 µg/ml *H. felis* sonicate for 12 hours in the absence of B cells or their supernatant. For IL-1β secretion, BM-DCs were treated with different doses of silica crystals (50 µg/cm^2, 25 µg/cm^2, 10 µg/cm^2, 5 µg/cm^2) during the last 8 hours of LPS or *H. felis* sonicate treatment.

2.2.10 BM-DC-T Cell co-culture

For funtional analysis of BM-DCs that were stimulated with supernatants from unstimulated B, Hf_{stim}-B, IL-10^+ B or IL-10^- B cells or cultured with these B cells, followed by *H. felis* sonicate or LPS treatments, were cultured with naive $CD4^+$ T cells at a ratio of 1:2 (DC:T; $5x10^4$ DC: $1x10^5$ T cells) for 24 hours in 96 well-plates (flat bottom).

2.2.11 ELISA

2.2.11.1 IL-10 ELISA

Mouse IL-10 ELISA Deluxe Max Kit (Biolegend) was used for determination of IL-10 secretion level that was secreted from BM-DCs to the culture medium during incubation. Firstly, 200 X Capture Antibody was diluted to 1 X using appropriate

amount of 1 X Coating Buffer which was also diluted from 5 X concentration with distilled water in a falcon tube. The total volumes were calculated according to number of wells for the samples and standarts. Then, Nunc 96-well plate was coated with the solution containing Capture Antibody in Coating Buffer as 50 µl per well. The plate was sealed with parafilm and incubated overnight at 4 ^{0}C refrigerator. Next day, the plate was washed with PBS/T Solution for 4 times. Then, 100 µl of 1X Assay Diluent A, which was diluted with 1X PBS from 5X, was added for each well for blocking step and the plate was incubated at room temperature for 1 hour with shaking. Standarts were prepared by serial dilutions according to manifacturer's instructions. After incubation, the plate was washed with PBS/T for 4 times and prepared standart solutions and samples, which were supernatant of BM-DCs, were added to proper wells as 50 µl per well. The plate was incubated at room temperature for 1 hour with shaking. Then, the plate washed with PBS/T for 4 times. 200 X biotinylated Detection Antibody was diluted to 1 X using appropriate amount of 1 X Assay Diuent A and added as 50 µl per well. The plate was incubated at room temperature for 1 hour with shaking. Next, the plate washed with PBS/T for 4 times. 1000 X Avidin-HRP was diluted to 1 X using appropriate amount of 1 X Assay Diuent A and added as 50 µl per well. The plate was incubated at room temperature for 30 minutes with shaking. After incubation, the plate was washed with PBS/T for 5 times. The TMB Substrat Solution was prepared by mixing Solution A and Solution B at a ratio of 1:1. Then, 50 µl TMB Substrate Solution was added for each well and the plate was incubated at room temperature for 20-30 min. TMB Substrate Solution was converted to a blue product by HRP enzyme in the positive wells. Stop Solution, 2 N H2SO4, was prepared and added into the wells as 50 µl per well in order to stop reaction. The absorbance of the standarts and samples were measured at 450 nm and 570 nm using Microplate Reader.

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

Mouse IL-12 ELISA Deluxe Max Kit (Biolegend) was used for determination of IL-12 secretion level that was secreted from BM-DCs to the culture medium during incubation. Firstly, 200 X Capture Antibody was diluted to 1 X using appropriate amount of 1 X Coating Buffer which was also diluted from 5 X concentration with distilled water in a falcon tube. The total volumes were calculated according to number of wells for the samples and standarts. Then, Nunc 96-well plate was coated

with the solution containing Capture Antibody in Coating Buffer as 50 µl per well. The plate was sealed with parafilm and incubated overnight at 4 ^{0}C refrigerator. Next day, the plate was washed with PBS/T Solution for 4 times. Then, 100 µl of 1X Assay Diluent A, which was diluted with 1X PBS from 5X, was added for each well for blocking step and the plate was incubated at room temperature for 1 hour with shaking. Standarts were prepared by serial dilutions according to manifacturer's instructions. After incubation, the plate was washed with PBS/T for 4 times and prepared standart solutions and samples, which were supernatant of BM-DCs, were added to proper wells as 50 µl per well. The plate was incubated at room temperature for 1 hour with shaking. Then, the plate was washed with PBS/T for 4 times. 200 X biotinylated Detection Antibody was diluted to 1 X using appropriate amount of 1 X Assay Diuent A and added as 50 µl per well. The plate was incubated at room temperature for 1 hour with shaking. Next, the plate washed with PBS/T for 4 times. 1000 X Avidin-HRP was diluted to 1 X using appropriate amount of 1 X Assay Diuent A and added as 50 µl per well. The plate was incubated at room temperature for 30 minutes with shaking. After incubation, the plate was washed with PBS/T for 5 times. The TMB Substrat Solution was prepared by mixing Solution A and Solution B at a ratio of 1:1. Then, 50 µl TMB Substrate Solution was added for each well and the plate was incubated at room temperature for 20-30 min. TMB Substrate Solution was converted to a blue product by HRP enzyme in the positive wells. Stop Solution, 2 N H_2SO_4, was prepared and added into the wells as 50 µl per well in order to stop reaction. The absorbance of the standarts and samples were measured at 450 nm and 570 nm using Microplate Reader.

2.2.11.3 TNF-α ELISA

Mouse TNF-α ELISA Deluxe Max Kit (Biolegend) was used for determination of TNF-α secretion level that was secreted from BM-DCs to the culture medium during incubation. Firstly, 200 X Capture Antibody was diluted to 1 X using appropriate amount of 1 X Coating Buffer which was also diluted from 5 X concentration with distilled water in a falcon tube. The total volumes were calculated according to number of wells for the samples and standarts. Then, Nunc 96-well plate was coated with the solution containing Capture Antibody in Coating Buffer as 50 µl per well. The plate was sealed with parafilm and incubated overnight at 4 ^{0}C refrigerator. Next day, the plate was washed with PBS/T Solution for 4 times. Then, 100 µl of 1X

Assay Diluent A, which was diluted with 1X PBS from 5X, was added for each well for blocking step and the plate was incubated at room temperature for 1 hour with shaking. Standarts were prepared by serial dilutions according to manifacturer's instructions. After incubation, the plate was washed with PBS/T for 4 times and prepared standart solutions and samples, which were supernatant of BM-DCs, were added to proper wells as 50 µl per well. The plate was incubated at room temperature for 1 hour with shaking. Then, the plate was washed with PBS/T for 4 times. 200 X biotinylated Detection Antibody was diluted to 1 X using appropriate amount of 1 X Assay Diuent A and added as 50 µl per well. The plate was incubated at room temperature for 1 hour with shaking. Next, the plate washed with PBS/T for 4 times. 1000 X Avidin-HRP was diluted to 1 X using appropriate amount of 1 X Assay Diuent A and added as 50 µl per well. The plate was incubated at room temperature for 30 minutes with shaking. After incubation, the plate was washed with PBS/T for 5 times. The TMB Substrat Solution was prepared by mixing Solution A and Solution B at a ratio of 1:1. Then, 50 µl TMB Substrate Solution was added for each well and the plate was incubated at room temperature for 20-30 min. TMB Substrate Solution was converted to a blue product by HRP enzyme in the positive wells. Stop Solution, which is 2 N H_2SO_4, was prepared and added into the wells as 50 µl per well in order to stop reaction. The absorbance of the standarts and samples were measured at 450 nm and 570 nm using Microplate Reader.

2.2.11.4 IL-1β ELISA

Mouse IL-1β ELISA Deluxe Max Kit (Biolegend) was used for determination of IL-1β secretion level that was secreted from BM-DCs to the culture medium during incubation. Firstly, 200 X Capture Antibody was diluted to 1 X using appropriate amount of 1 X Coating Buffer which was also diluted from 5 X concentration with distilled water in a falcon tube. The total volumes were calculated according to number of wells for the samples and standarts. Then, Nunc 96-well plate was coated with the solution containing Capture Antibody in Coating Buffer as 50 µl per well. The plate was sealed with parafilm and incubated overnight at 4 ^{0}C refrigerator. Next day, the plate was washed with PBS/T Solution for 4 times. Then, 100 µl of 1X Assay Diluent A, which was diluted with 1X PBS from 5X, was added for each well for blocking step and the plate was incubated at room temperature for 1 hour with shaking. Standarts were prepared by serial dilutions according to manifacturer's

instructions. After incubation, the plate was washed with PBS/T for 4 times and prepared standart solutions and samples, which were supernatant of BM-DCs, were added to proper wells as 50 µl per well. The plate was incubated at room temperature for 1 hour with shaking. Then, the plate was washed with PBS/T for 4 times. 200 X biotinylated Detection Antibody was diluted to 1 X using appropriate amount of 1 X Assay Diuent A and added as 50 µl per well. The plate was incubated at room temperature for 1 hour with shaking. Next, the plate washed with PBS/T for 4 times. 1000 X Avidin-HRP was diluted to 1 X using appropriate amount of 1 X Assay Diuent A and added as 50 µl per well. he plate was incubated at room temperature for 30 minutes with shaking. After incubation, the plate was washed with PBS/T for 5 times. Then, 50 µl Solution D (substrate) was added for each well and the plate was incubated at room temperature for 20-30 min. Solution D was converted to a blue product by HRP enzyme in the positive wells. Stop Solution, which is 2 N H_2SO_4, was prepared and added into the wells as 50 µl per well in order to stop reaction. The absorbance of the standarts and samples were measured at 450 nm and 570 nm using Microplate Reader.

2.2.12 Gene expression analysis

2.2.12.1 RNA isolation

For analysis of expression level of IL-10, IL-12 / IL-23 (p40), TNF-alpha and IL-6 genes, RNA isolation from BM-DC pellets were carried out using MN Nucleospin® RNA Kit. Firstly, 350 µl of RA1 solution and 3.5 µl Beta-mercaptoethanol was added to each tube and the tubes were mixed strongly using vortex. Then, liquid nitrogen was applied to the tubes for facilitation of lysis of cells. Next, for the filtration of the lysates, Nucleospin Filter® was placed onto a Collection Tube (2 mL) and lysate was applied to the filter. This step was performed for all lysates. Then, lysates were centrifuged at 11.000 g for 1 minute and Nucleospin® Filters were removed from the tubes. Afterwards, for each tube, 350 µl 70% ethanol was added to filtrated solution and the mixture was pipetted up and down for 5 times. Each lysate was applied onto a Nucleospin® RNA. Column placed in a Collection Tube and centrifuged at 11.000 g for 30 seconds. Then, for desalting of the membrane, which makes the rDNase more effective, 350 µl of MDB (Membran Desalting Buffer) was added onto each column and the the mixture was centrifuged

at 11.000 g for 1 minute. rDNAse reaction mixture was prepared by mixing 10 µl reconstituted rDNase and 90 µl Reaction Buffer for rDNase per tube in 1,5 ml eppendorf. For digesting of DNA, 95 µl of this rDNAse reaction mixture was applied onto each column and tubes were incubated at room temperature for 15 minutes. 200 µl of RAW2, which inactivates rDNase, was added onto each column and the tubes were centrifuged at 11.000 g for 30 seconds. The columns were placed to the new collection tubes. For washing of the membrane, 600 µl of RA3 was added to each column and centrifuged at 11.000 g for 30 seconds was performed. Flow through was removed and the columns were placed to the collection tubes again. Then, the membranes were washed again by adding 250 µl of RA3 onto each column and the tubes were centrifuged at 11.000 g for 2 minutes. After centrifuge, each column were placed to a Nuclease-free Collection Tube and 32 µl of RNase-free H_2O was added onto each column for elution of RNA. Centrifuge at 11.000 g for 1 min was performed. Finally, RNA solution was obtained in Nuclease-free Collection Tube and kept in -80 ^{0}C. RNA concentrations were determined using NanoDrop.

2.2.12.2 cDNA synthesis

For synthesis of cDNA from RNA, High Capacity cDNA Reverse Transcription Kit was used and 2 X master mix were prepared according to manifacturer's instruction. The volume of components and thermal cycler condition for the reaction were given at Table 2.15 and Table 2.16, respectively.

Table 2.15 : The volume of the components for cDNA synthesis reaction.

Component	Volume
RNA	Depending on RNA concentration
Nuclease-free H_2O	15.075 µl - RNA volume
10 X RT Buffer	2 µl
Oligo dT (10 µM)	1 µl
Riboblock RNase Inhibitor	0.125 µl
Reverse Transcriptase	1 µl
25 X dNTP Mix (100 mM)	0.8 µl
Total volume per reaction	20 µl

Table 2.16 : Thermal cycler conditions for cDNA synthesis.

Step	Temperature	Time
Step 1	25 ^{0}C	10 min
Step 2	37 ^{0}C	120 min
Step 3	85 ^{0}C	5 min
Step 4	4 ^{0}C	∞

2.2.12.3 Conventional PCR

Conventional PCR reaction was performed using ThermoPol PCR Kit and master mix were prepared according to manifacturer's instruction. The volume of components and thermal cycler condition for the reaction were given at Table 2.17 and Table 2.18, respectively.

Table 2.17 : The volume of the components for PCR reaction.

Component	Volume	Final Concentration
10 X RT Reaction Buffer	2.5 µl	1 X
dNTP (10 mM)	0.5 µl	200 µM
10 µM Forward Primer	0.5 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	0.2 µM
5000 U/ml *Taq* DNA Polymerase	0.125 µl	0.625 unit
Template DNA (cDNA)	2 µl	-
Nuclease-free H_2O	18,875 µl	-
Total volume per reaction	25 µl	-

Table 2.18 : Thermal cycler conditions for PCR reaction.

Step	Temperature	Time
Initial denaturation	95 ^{0}C	30 seconds
	95 ^{0}C	30 seconds
32 cycles	Depending on primers	45 seconds
	68 ^{0}C	45 seconds

Table 2.18 (continued) : Laboratory equipments used in the experiments.

Step	Temperature	Time
Final extension	68 ^{0}C	5 minutes
Hold	4 ^{0}C	∞

2.2.12.4 Real Time PCR (qPCR)

For quantitative analysis of relative expression level of IL-10, IL-12 / IL-23 (p40), TNF-alpha and IL-6 in BM-DCs, real time PCR was performed using Power SYBR® Green PCR Master. The volume of components and thermal cycler condition for the reaction were given at Table 2.19 and Table 2.20, respectively.

Table 2.19 : The volume of the components for qPCR reaction.

Component	Volume
Power Sybr Master Mix (2X)	5 µl
Forward Primer (10 µM)	0.5 µl
Reverse Primer (10 µM)	0.5 µl
Molecular Grade H_2O	1.5 µl
cDNA	2.5 µl
Total volume per reaction	10 µl

Table 2.20 : Reaction conditions for qPCR reaction.

Step	Temperature	Time
Holding stage	95 ^{0}C	10 minutes
	95 ^{0}C	15 seconds
Cycling Stage (40	Depending on primers	30 seconds
cycles)	68 ^{0}C	1 minute
	95 ^{0}C	15 seconds
Melt Curve Stage	60 ^{0}C	1 minute
	95 ^{0}C	15 seconds
Holding	72 ^{0}C	5 minutes

2.2.13 Flow cytometry

2.2.13.1 Surface staining

At day 7 of BM culture, BM-DCs were stained with APC-conjugated anti CD11c. To do that, 50.000 BM-DCs were prepared as pellet through centrifugation at 3000 rpm for 8 min at 4 ^{0}C in the 1.5 ml eppendorf tubes and the cells were dissolved in 50 µl of FACS Buffer containing 0,2 µl 0.2 mg/ml APC-conjugated anti-CD11c. At day 9 of the culture, BM-DCs, that were stimulated with supernatants from given B cells or co-cultured with given B cells, followed by *H. felis* sonicate or LPS treatments, were stained with 0,2 µl 0.2 mg/ml PE-conjugated anti-CD86. After BM-DC-T cell co-culture, T cells were harvested and stained with 0,2 µl 0.2 mg/ml APC-conjugated anti-CD4 in the same way with staining of BM-DCs in order to test the effect of BM-DCs on naive CD4$^+$ T cells. The stained tubes were incubated for 1 hour at 4 ^{0}C and the cells, which were not stained, used as unstained control group. After incubation, the cells were washed by adding 300 µl of FACS Buffer per tube and centrifuged at 3000 rpm for 8 min. Then, the cells were dissolved in 150 of FACS Buffer per tube and analyzed using flow cytometer. In addition, for purity check, B cells were stained with 0,2 µl 0.5 mg/ml FITC-conjugated CD19 antibody whereas T cells were stained with 0,2 µl 0.2 mg/ml anti-CD4 as explained before. Centrifuge for obtaining B and T cells pellet was performed at 2000 rpm for 5 min at 4 ^{0}C.

2.2.13.2 Intracellular staining

After staining of T cells with APC-conjugated anti-CD4, these cells were stained with PE-conjugated anti-IL-10. 50 ng/ml PMA, 500 ng/ml Ionomycin and 3 µg/ml monensin were added to the appropriate wells for the last 5 hours of BM-DC-T cell co-culture. Monensin is a molecule that prevent the transport process of the cell and gives rise to accumulation of the cytokines at Golgi/Endoplasmic Retikulum complex. On the other hand, PMA and ionomycin are used for the induction of the cells to secrete cytokines. After washing step for CD4 surface staining, the cells were dissolved in 100 µl of 1 X Fixation Buffer (diluted to 1 X with 1 X PBS) containing paraformaldehyde in order to fix the cells. The cells were incubated on ice in dark for 15 min. Then, the cells were washed with 500 µl of FACS Buffer and centrifuged at 2000 rpm for 5 min. The pellets were dissolved in 300 µl of 1 X Permeabilization Buffer (diluted to 1 X from 10 X with distilled water). Permeabilization Buffer

contains saponin and leads to formation of pores on the membrane which facilitate the capture of intracellular molecule by the antibody. The cells were incubated for 5-10 minutes on ice in dark. Next, the cells were centrifuged at 2000 rpm for 5 min and supernatants were discarded. Afterwards, the cells were dissolved in 50 µl of Permeabilization Buffer containing 0,3 µl 0.2 mg/ml PE-conjugated anti-IL-10 per tube and incubated for 2 hours in dark at 4 ^{0}C. After incubation, the cells were washed with 300 µl of Permeabilization Buffer and centrifuged at 2000 rpm for 5 min. Finally, each pellets were dissolved in 150 µl of FACS Buffer and analyzed using flow cytometer. In addition, the cells which were not stained, used as unstained control group.

2.2.14 Analysis for flow cytometry

The results were analyzed by using FlowJo and Accuri C6 softwares.

2.2.15 Statistical analysis

GraphPad Prism 6.0 was used for calculation of *p* values and significancy was determined by Student *t* test. For all analysis, the difference betweeen the groups was accepted as significant when $p<0.05$. In column bar graphs, the stars indicate the significancy level and n.s. means "non-significant" whereas vertical bars indicate standard deviations of the mean.

3. RESULTS

3.1 Differentiation of Bone Marrow Cells Into Bone-Marrow-Derived Dendritic Cells

Mouse bone marrow cells were differentiated into dendritic cells (DCs) and at day 7 for enrichment of $CD11c^+$ DCs, magnetic separation by using CD11c-microbeads were performed as explained in Section 2.2.6. At day 7, bone marrow-derived dendritic cells (BM-DCs) were stained with APC-coupled anti-CD11c and their purity was determined by flow cytometry. Average purity of BM-DCs was 67.13%, whereas after magnetic separation highly pure immature $CD11c^+$ BM-DCs were obtained (93.24%) (Figure 3.1).

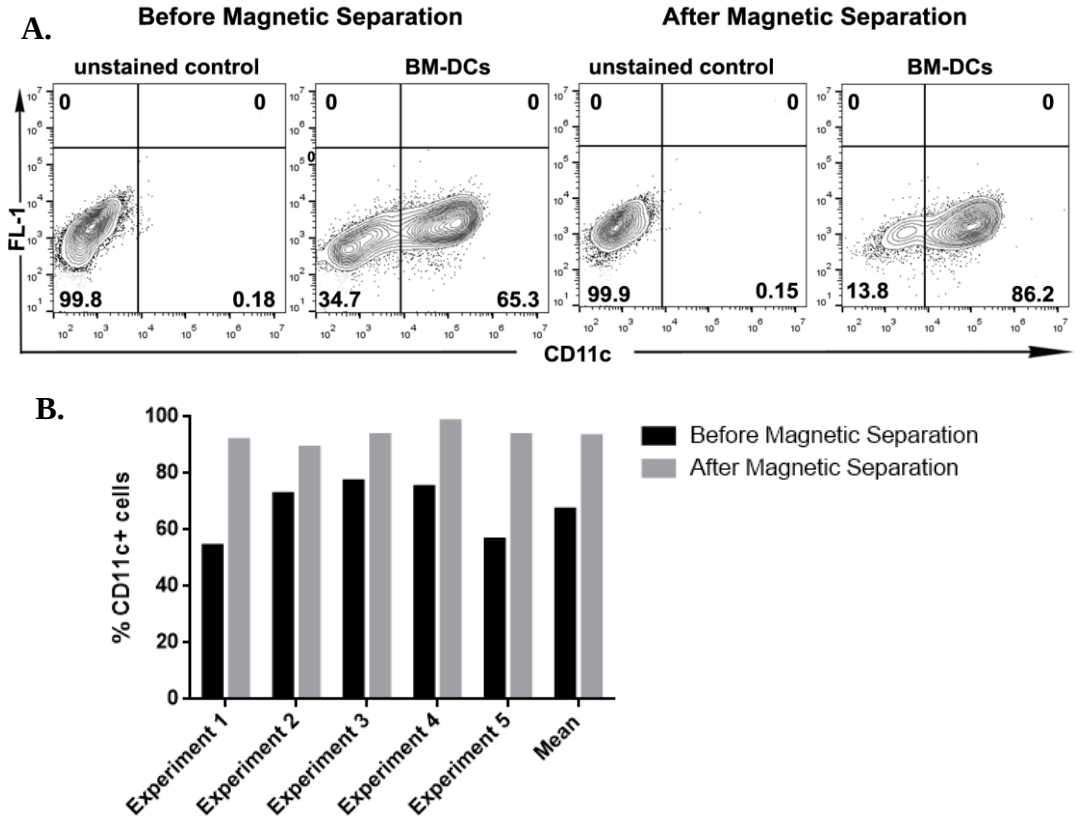

Figure 3.1 : Purity of differentiated BM-DCs at day 7 A) The purity of BM-DCs before and after magnetic separation were shown as a representative Cytometry plot. B) Purity of BM-DCs in five independent experiments and average purity was given in the graph. At day 7, BM-DCs were stained with APC-coupled anti-CD11c and the percentage of stained cells were determined using flow cytometer. Graphs were prepared using Graphpad Prism 6.

3.2 Determination of the Effects of *H. felis* Sonicate on BM-DCs

Various pathogen-derived foreign antigens such as LPS regulate the maturation of DCs (Banchereau et al., 2000). LPS provides maturation and activation signals to DCs by inducing the expression of co-stimulatory molecules including CD40, CD80 and CD86 and secretion of various pro-inflammatory cytokines such as IL-6 and IL-12 that affect the differentiation of naive T cells. Also, LPS induces DCs to secrete IL-10 which is an immune-suppressive cytokine (Hui-Rong Jiang et al., 2002, Langenkamp et al., 2000). In addition, it has been shown that stimulation of BM-DCs with *H. felis* antigens resulted in induction of IL-6 and IL-10 (Drakes et al., 2006). In this part of the study, effects of *H. felis* sonicate on immature BM-DCs were investigated on protein and mRNA expression levels. After treatments of DCs with *H. felis* sonicate, CD86 expression was analyzed by flow cytometry and cytokine profile of BM-DCs were analyzed by ELISA and/or qPCR. *E. coli*-derived LPS was used as positive control.

3.2.1 *H .felis* activates BM-DCs by upregulating CD86 expression.

CD86 (B7.2) is one of the co-stimulatory molecules and maturation marker expressed on the activated DCs. Interaction of CD80/CD86 with CD28 which is found on surface of naive T cells is critical for T cell activation and survival (Mays and Wilson, 2011). The expression of CD86 in BM-DCs was induced significantly by *H. felis* sonicate (52.17 %) at least as much as LPS (45.4 %) compared to untreated control in which only 8.87 % of BM-DCs exhibited CD86 on their cell surface (Figure 3.2).

3.2.2 *H. felis* induces BM-DCs to secrete IL-12, TNF-α, IL-1β and IL-10.

IL-12 is a pro-inflammatory cytokine which induces effector type-I helper T cell (Th1) response, whereas IL-10 is involved in induction of regulatory T cell-mediated immune tolerance (Hui-Rong Jiang et al., 2002). Also, upon recieving TLR signals provided by bacterial products such as LPS, DCs secrete TNF-α and IL-1β that both contribute to effective host defense in various ways including inducing fever, increasing vascular permeability etc. Moreover, these activated DCs also produce another pro-inflammatory cytokine IL-6, which is crucial for lymphocyte activation and increased antibody production (Murphy, 2012). Cytokine profile of BM-DCs

were determined by ELISA and qPCR. For ELISA analysis, the supernatants from control and treatments group were collected.

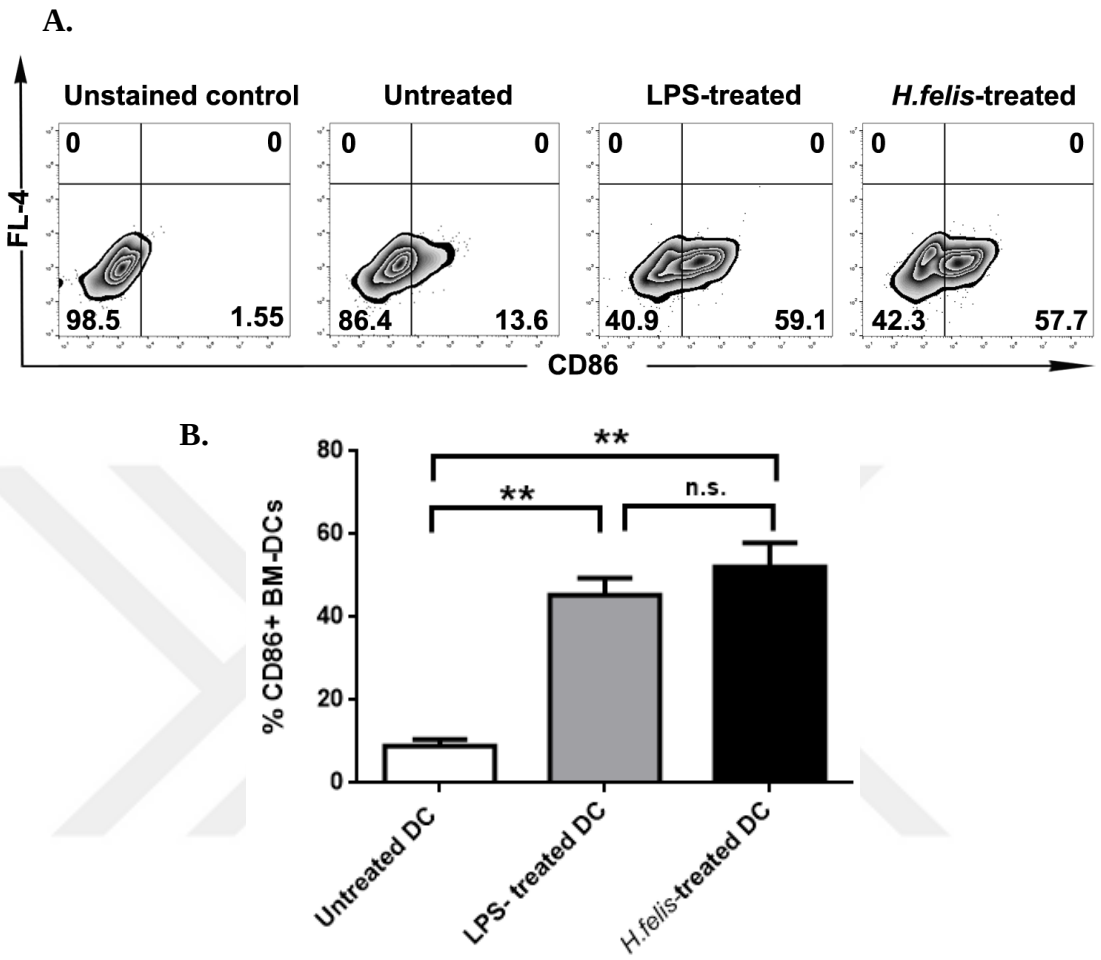

Figure 3.2 : *H .felis* activates BM-DCs by upregulating CD86 expression. A) The percentage of $CD86^{+}$ BM-DCs were shown as a representative Cytometry plot before and after treatments. B) Each value represents the mean ± SEM of two biological replicates analyzed by Student *t* test. After treatments, BM-DCs were stained with anti-CD86-PE and the percentage of stained cells were determined using flow cytometer. Graphs were prepared using Graphpad Prism 6. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, n.s.: non-significant.

Secretion of IL-12/IL-23 (p40), TNF-α, IL-1β and IL-10 from DCs upon LPS or *H. felis* treatment were analyzed by ELISA, whereas IL-6 levels were checked on mRNA expression level. Secretion of IL-12/IL-23 (p40), TNF- α and IL-10 was induced by *H. felis*-treated BM-DCs compared to untreated BM-DCs by which none of these cytokines were secreted. On the other hand, *H.felis* induced BM-DCs to secrete IL-12 and TNF-α more than LPS (Figure 3.3A,B,C); however, there was no difference for IL-10 secretion when LPS and *H.felis*-treated BM-DCs were compared (Figure 3.3A). Secretion of expressed IL-1β requires activation of NLRP3 inflammasome which leads to maturation of pro-IL-1β to active-IL-1β. Moreover, it

has been shown that silica crystals activate NLRP3 inflammasome (Peeters et al., 2013). Therefore, BM-DCs were treated with different doses of silica crystals (50 μg/cm^2, 25 μg/cm^2, 10 μg/cm^2, 5 μg/cm^2) during the last 8 hours of LPS or *H. felis* treatment. IL-1β secretion was induced upon *H. felis* or LPS treatment. Furthermore, LPS-treated DCs secreted more IL-1β than *H. felis*-treated DCs only in the case of 5 μg/cm^2 silica treatment; whereas with the other doses of silica crystals, LPS-treated and *H. felis*-treated DCs secreted comparable concentrations of IL-1β (Figure 3.3D).

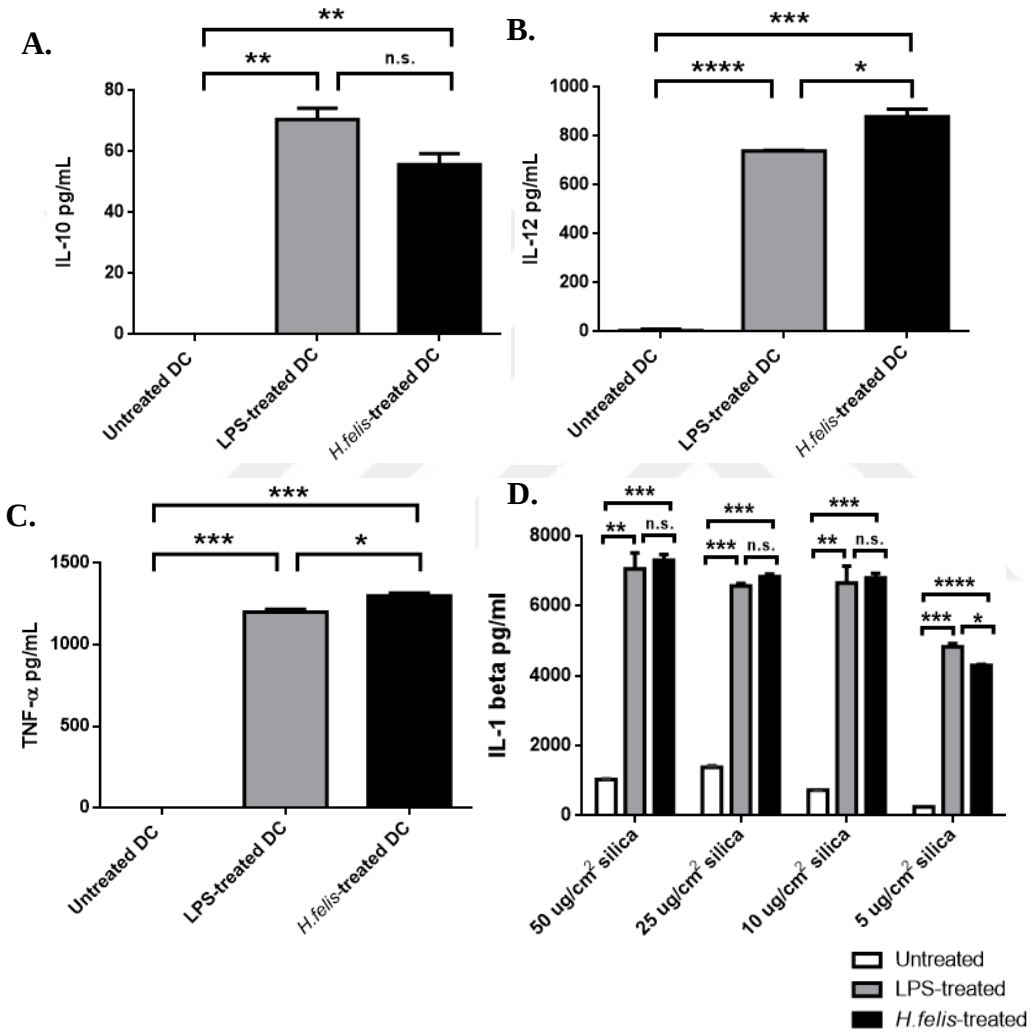

Figure 3.3 : *H. felis* induces BM-DCs to secrete IL-12, TNF-α, IL-1β and IL-10. ELISA analysis for A) IL-10, B) IL-12/IL-23 (p40), C) TNF-α, D) IL-1β. Supernatants from control and treated groups were collected and analyzed by ELISA. For IL-1β, DCs were kept with indicated doses of silica crystals for last 8 hours. Graphs except for the one regarding IL-1β are representative of three independent experiments. Graphs were prepared using Graphpad Prism 6. Each value represents the mean ± SEM of two biological replicates analyzed by Student *t* test. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, n.s.: non-significant.

3.2.3 *H. felis* upregulates mRNA expression of IL-12, TNF-α, IL-6 and IL-10 in BM-DCs.

After determination of secretion level, the expression level of IL-6 in addition to IL-10, IL-12 and TNF-α were assessed by qPCR. According to the results, in *H.felis* sonicate-treated BM-DCs; 2-fold induction in IL-12 expression, 6.7-fold induction in IL-6 expression, 2.9-fold induction in TNF-α and IL-10 expression were observed compared to untreated DCs (Figure 3.4).

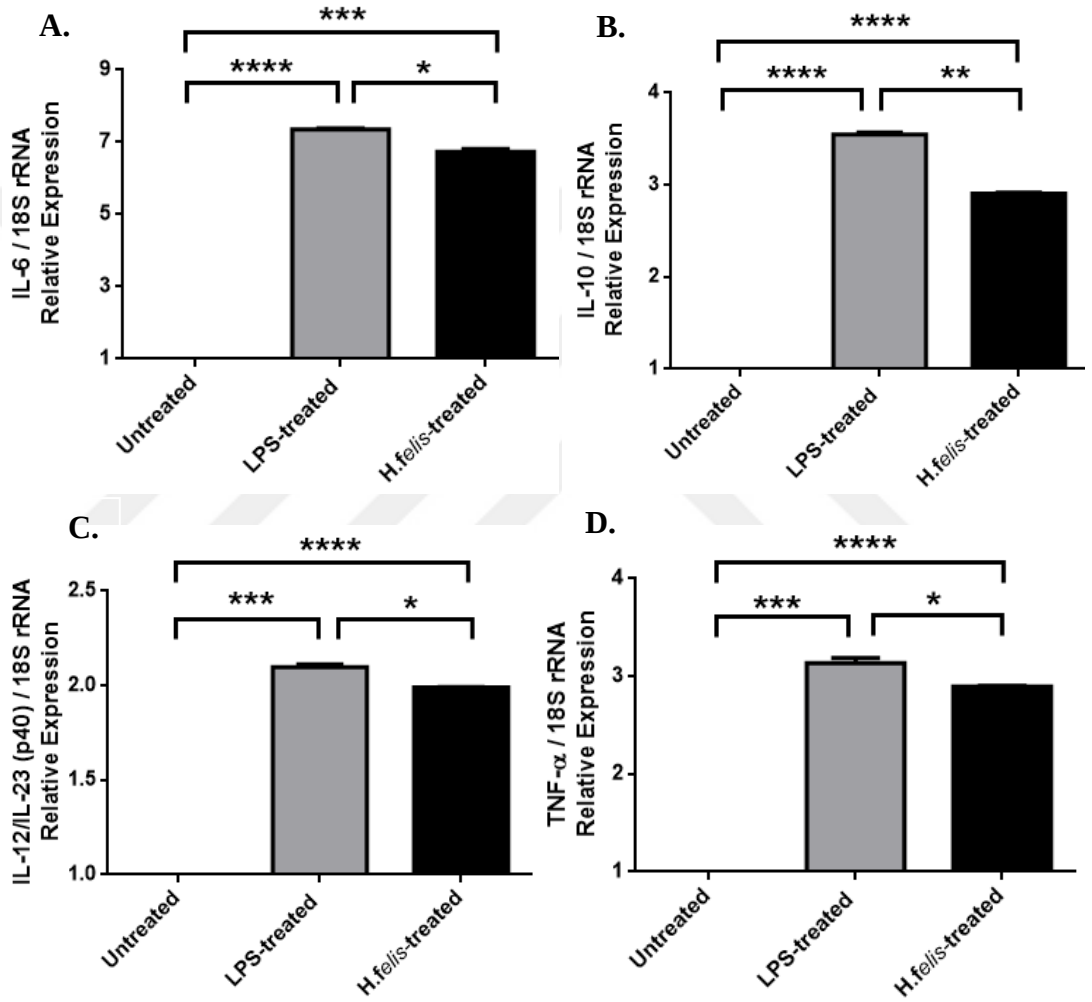

Figure 3.4 : *H. felis* upregulates mRNA expression of IL-12, TNF-α, IL-6 and IL-10 in BM-DCs. qPCR analysis for A) IL-6, B) IL-10, C) IL-12/IL-23 (p40), D) TNF-α. Cell pellets from control and treated groups were collected and analyzed by qPCR. Expressions were normalized according to expression of 18S rRNA and fold change were shown in graphs. Graphs were prepared using Graphpad Prism 6. Each value represents the mean ± SEM of two replicates analyzed by Student *t* test. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, n.s.: non-significant.

3.3 Isolation of *Helicobacter felis*-Stimulated (Hf_{stim}) IL-10^+ And IL-10^- B Cells

Previous studies in our laboratory showed that treatment of *H. felis* sonicate activates B cells and leads to differentiation of two B cell subgroups: IL-10-producing *Helicobacter*-stimulated IL-10^+ B cells and TGF-β-producing *Helicobacter*-stimulated IL-10^- B cells. Therefore; before isolation of IL-10^+ B and IL-10^- B cells through magnetic separation, first $CD19^+$ B cells were isolated from mouse spleen and stimulated with *H. felis* sonicate (Section 2.2.4.5). $CD19^+$ B cells were stained with FITC coupled anti-CD19 and IL-10^+ B cells were labeled with PE-coupled anti-IL-10 and assessed to determine their regulatory B cell purity by flow cytometry. According to average of three independent experiments, purity of $CD19^+$ B cells, *Helicobacter*-stimulated (Hf_{stim})-IL-10^+ and IL-10^- B cells were 85,3%, 83.6 % and 84.8 %, respectively (Figure 3.5).

3.4 Effects of Supernatant of Hf_{stim}-B cells on BM-DCs

It has been shown that IL-10-producing *Helicobacter*-stimulated B cells has regulatory roles on immune response by inducing IL-10-producing T regulatory-1 (Tr1)-like cells. (Sayi et al., 2010). Regulatory DCs produce anti-inflammatory cytokines and express low level of co-stimulatory and pro-inflammatory molecules (Morelli and Thomson, 2007). Matsumoto et al. (2014) have shown that supernatant of IL-10-secreting plasmablasts inhibit effector functions of DCs by suppressing IL-6 and IL-12 expression. Therefore, supernatant of unstimulated B cells, Hf_{stim}-B cells, Hf_{stim}-IL-10^- and Hf_{stim}-IL-10^+ B cells were obtained and applied onto the immature DCs in order to determine the regulatory effect of secreted molecules from Hf_{stim}-B cells on BM-DCs. After treatments; CD86 expression, cytokine profile and functional properties of BM-DCs were analyzed.

3.4.1 Supernatant of Hf_{stim}-B cells activates immature BM-DCs.

All Hf_{stim}-B cell groups including IL-10^+ B and IL-10^- B cells, induced DCs to express CD86 molecules in the absence of antigenic stimulation. However, exhibition of CD86 on surface of DCs were not changed by supernatant of unstimulated B cells. Moreover; expression of CD86 was induced in DCs which were treated with LPS following stimulation with supernatant of Hf_{stim}-B or IL-10^+ B but not with supernatant of unstimulated B and IL-10^- B cells. Additionally, when

DCs were treated with *H. felis* sonicate, the expression of CD86 was not induced in any supernatant-stimulated groups compared with unstimulated DCs. Furthermore, there was no difference between DC groups stimulated with supernatant of IL-10$^-$ B and IL-10$^+$ B cells in terms of the expression of CD86 in untreated and treated groups. (Figure 3.6).

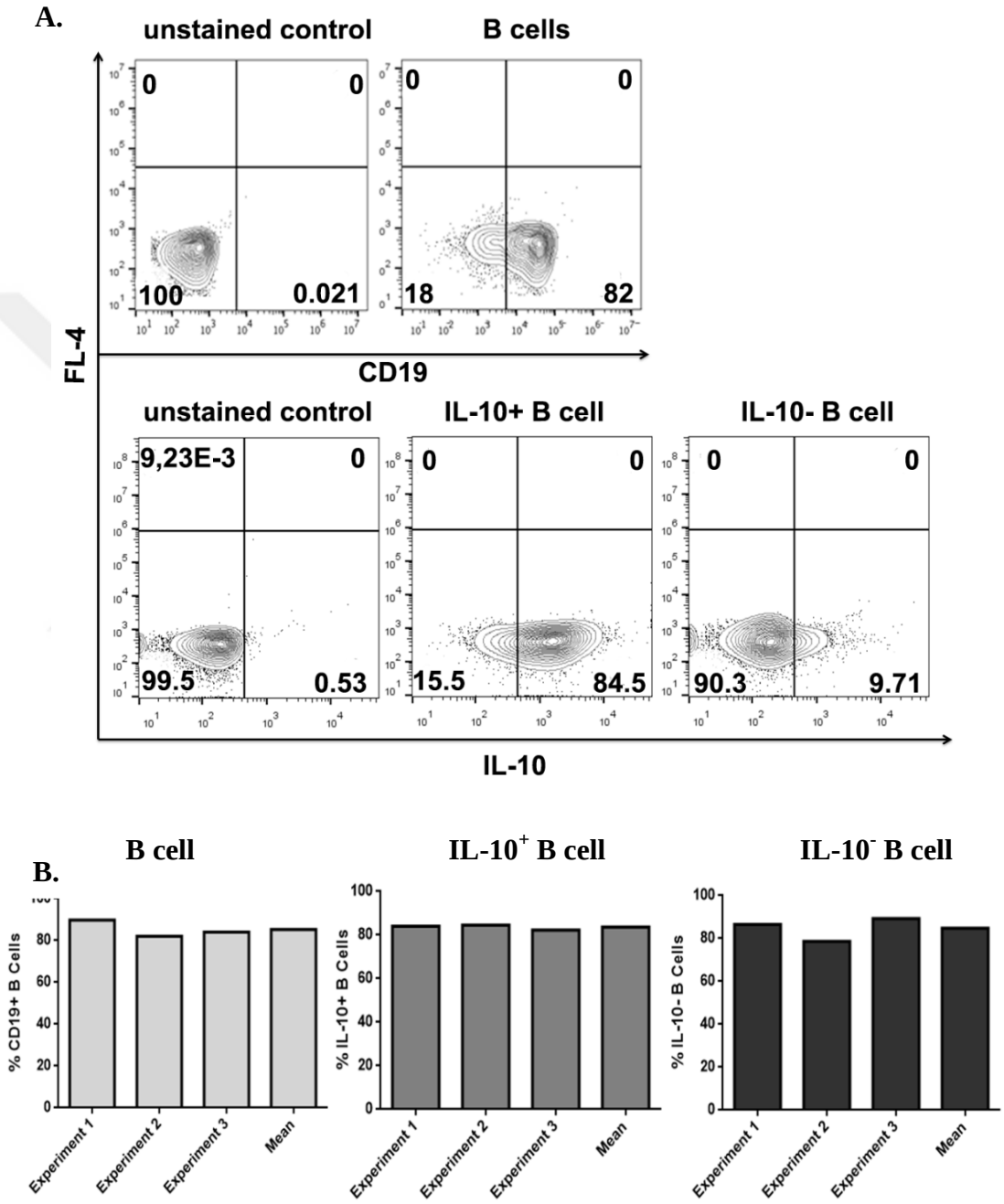

Figure 3.5 : Purity of CD19$^+$ B and Hf_{stim}-B cell subgroups A) Purity of CD19$^+$ B cells, Hf_{stim}-IL-10$^+$ and IL-10$^-$ B cells were shown as a representative flow cytometry plot. B) Purity of B cells in three independent experiments and average purity was given in the graphs. B cell were stained with FITC-coupled anti-CD19 and Hf_{stim}-B cells were isolated through PE-coupled anti-IL-10. Percentage of stained cells were determined using flow cytometer. Graphs were prepared usnig Graphpad Prism 6.

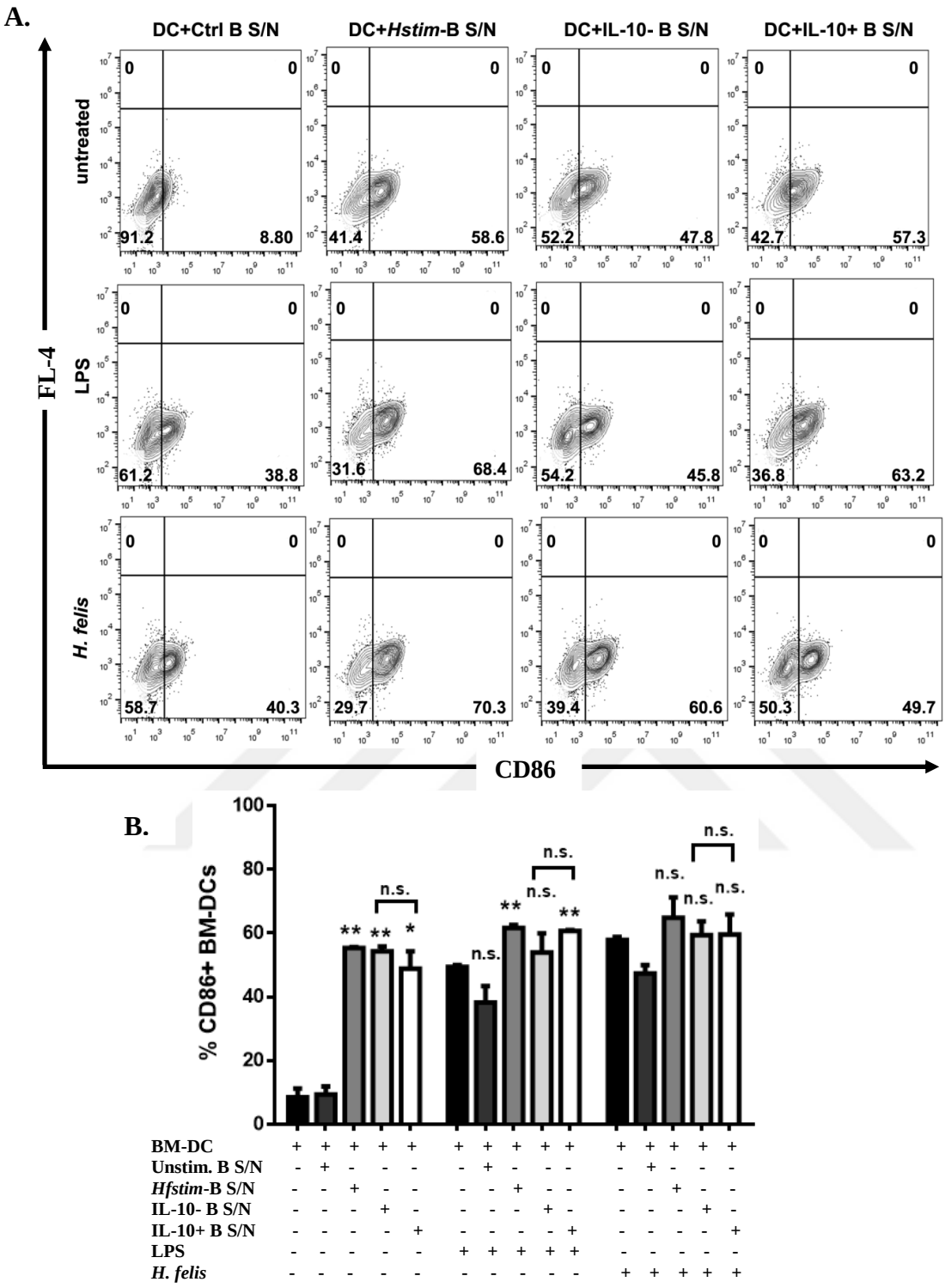

Figure 3.6 : Supernatant of Hf_{stim}-B cells activates immature BM-DCs. A) The percentage of $CD86^{+}$ BM-DCs with indicated conditions were shown as a representative flow cytometry plot. B) Supernatant of different B cells were applied onto immature BM-DCs followed by LPS or *H. felis* treatment or no treatment. BM-DCs were stained with anti-CD86-PE and the percentage of stained cells were determined using flow cytometer. Graphs were prepared using Graphpad Prism 6. Each value represents the mean ± SEM of two independent experiments analyzed by Student *t* test. Statistical results indicated on each bar represent comparisons within each group (untreated, LPS- or *H.felis*- treated DCs) according to its control group (untreated, LPS- or *H.f.*- treated DCs without B cell supernatant). **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, n.s.: non-significant.

3.4.2 Supernatant of Hf_{stim}-IL-10$^+$ B cells does not affect IL-10 secretion but inhibits TNF-α secretion from BM-DCs.

Hf_{stim}-B cell supernatant did not induce secretion of IL-10 from DCs in the absence of antigenic stimulation. Concentration of IL-10 secreted from DCs in response to supernatant of IL-10$^-$ B and IL-10$^+$ B cells was comparable although DCs secreted more IL-10 upon stimulation with supernatant of IL-10$^+$ B cells compared to IL-10$^-$ B cells. On the other hand; IL-10 secretion from DCs decreased upon stimulation with supernatant of IL-10$^-$ B cells followed by LPS treatment but not by *H. felis* sonicate treatment. IL-10 secretion was not induced upon stimulation of DCs with supernatant of unstimulated B cell, Hf_{stim}-B cell or IL-10$^+$ B cell followed by LPS or *H. felis* sonicate treatment. Nevertheless, DCs secreted more IL-10 upon stimulation with supernatant of IL-10$^+$ B cell followed by LPS or *H.felis* treatment compared to IL-10$^-$ B cell supernatant stimulation (Figure 3.7A).

Secretion of IL-12 and TNF-α by DCs was induced only by all Hf_{stim}-B cell groups in the absence of antigenic stimulation (Figure 3.7B-C). However, comparable concentration of IL-12 was secreted from DCs upon stimulation with supernatant of unstimulated B and all Hf_{stim}-B cell groups followed by LPS or *H. felis* sonicate treatment. Furthermore, there was no difference between DC groups stimulated with supernatant of IL-10$^-$ B and IL-10$^+$ B cells in terms of secretion of IL-12 and TNF-α in untreated and treated groups. DCs stimulated with supernatant of Hf_{stim}-B cell secreted more TNF-α than DCs stimulated with supernatant of IL-10$^-$ B or IL-10$^+$ B cell in the absence of antigenic stimulation. In all treated DC groups stimulated with supernatant of Hf_{stim}-B, IL-10$^-$ B or IL-10$^+$ B cells, but not unstimulated B cells; TNF-α secretion from DCs was decreased significantly compared to LPS- or *H. felis*-treated DCs without stimulation of B cell supernatant (Figure 3.7C).

3.4.3 Supernatant of Hf_{stim}-IL-10$^+$ B and IL-10$^-$ B cells inhibits mRNA expression of pro-inflammatory and anti-inflammatory cytokines in BM-DCs.

In addition to determination cytokine profile of DCs by ELISA, mRNA expression analysis of IL-6 in addition to these cytokines was performed for DCs that stimulated supernatant of Hf_{stim}-IL-10$^+$ B cell or Hf_{stim}-IL-10$^-$ B cell.

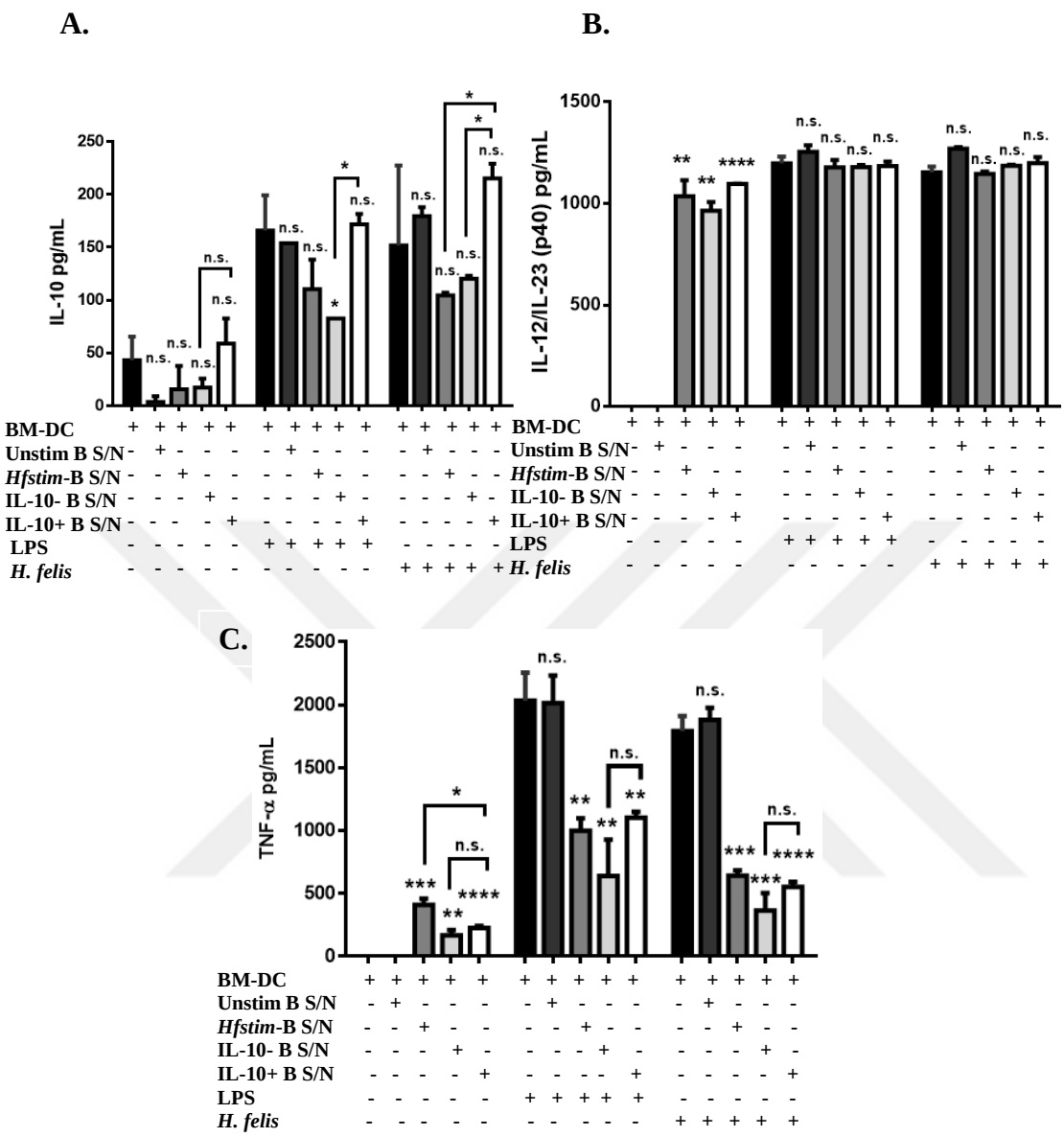

Figure 3.7 : Supernatant of Hf_{stim}-IL-10^{+} B cells does not affect IL-10 secretion but inhibits TNF-α secretion from BM-DCs. ELISA analysis for A) IL-10, B) IL-12/IL-23 (p40), C) TNF-α. Supernatant of different B cells were applied onto immature BM-DCs followed by LPS or *H. felis* treatment or no treatment. Supernatants from samples of different groups were collected and analyzed by ELISA. Graphs are representative of two independent experiments. Graphpad Prism 6 was used for preparation of graphs. Each value represents the mean ± SEM of two biological replicates analyzed by Student *t* test. Statistical results indicated on each bar represent comparisons within each group (untreated, LPS- or *H. felis*- treated DCs) according to its control group (untreated, LPS- or *H.f.*-treated DCs without B cell supernatant). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s.: non-significant.

Relative expression of IL-10, IL-12, TNF-α and IL-6 decreased in DCs stimulated with supernatant of IL-10^+ B or IL-10^- B cells in treated groups compared to only LPS- or *H. felis*-treated DCs whose cytokine expressions were induced. Moreover; in DCs stimulated with supernatant of IL-10^- B cells; more expression of these cytokines were observed compared to IL-10^+ B cell supernatant-stimulated DCs in LPS-treated groups (1.2-fold for IL-10 and IL-12, 0.9-fold for TNF-α, 1.3-fold for IL-6). In contrast; in *H.felis*-treated groups, more expression of these cytokines were observed in IL-10^+ B cell supernatant-stimulated DCs compared to IL-10^- B cells supernatant-stimulated DCs (0.4-fold for IL-10, 0.3-fold for IL-12 and TNF-α, 0.4-fold for IL-6) (Figure 3.8).

3.4.4 Isolation of naive $CD4^+$ T cells

$CD4^+$ T cells were isolated from C57BL/6 mice through magnetic separation as explained in Section 2.2.5. $CD4^+$ T cells were collected and stained with APC-coupled anti-CD4 in order to determine the purity by flow cytometry. Purity of T cells was 87.9 % according to average of two independent experiments (Figure 3.9).

3.4.5 LPS-treated BM-DCs that have been previously stimulated with supernatant of Hf_{stim}-IL-10^- B cells, but not IL-10^+ B cells, lead to a decrease in the frequency of IL-10-producing $CD4^+$ T cells.

IL-10-producing regulatory B cells prevent effector functions of DCs required for induction of Th1 and Th17 response (Matsumoto et al., 2014). In order to determine the effect of Hf_{stim}-B cell supernatant on the function of DCs to induce T cells, BM-DCs were cultured with naive $CD4^+$ T cells at a ratio of 1:2 (DC:T). After incubation, to check whether T cells were converted to IL-10-secreting regulatory type (Tr1), T cells were harvested and stained with APC-coupled anti-CD4 and PE-coupled anti-IL-10.

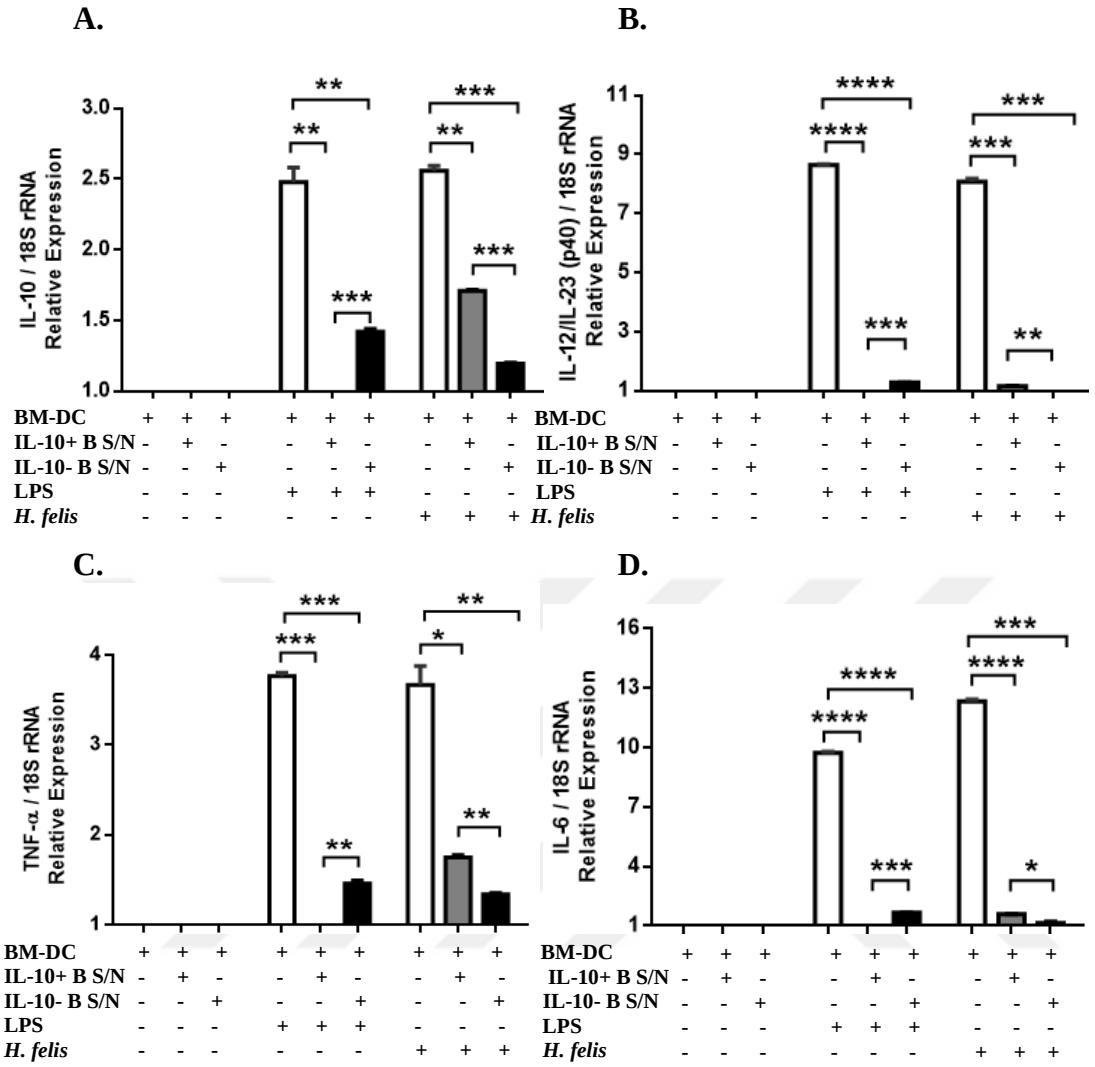

Figure 3.8 : Supernatant of Hf_{stim}-IL-10$^+$ or IL-10$^-$ B cells inhibits mRNA expression of pro-inflammatory and anti-inflammatory cytokines in BM-DCs. qPCR analysis for A) IL-10, B) IL-12/IL-23 (p40), C) TNF-α, D) IL-6. Cell pellets from indicated groups were collected and analyzed by qPCR. Supernatant of different B cells were applied onto immature BM-DCs followed by LPS or *H. felis* treatment or no treatment. Expressions were normalized according to expression of 18S rRNA. Fold change between treated groups and corresponding untreated groups were calculated. Expressions were normalized according to expression of 18S rRNA and fold change were shown in graphs. Graphs were prepared using Graphpad Prism 6. Each value represents the mean ± SEM of two replicates analyzed by Student *t* test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s.: non-significant.

In the first experiment; untreated DC groups had comparable frequency of IL-10-expressing T cells whereas in the second experiment; frequency of IL-10-expressing CD4$^+$ T cell in IL-10$^+$ B cell supernatant-stimulated DC group observed 3.9% which is 1.4 fold more than the frequency of IL-10-expressing CD4$^+$ T cell in Hf_{stim}-B cell supernatant-stimulated DC group (1.6%) or IL-10$^-$ B cell supernatant-stimulated DC group (2.8%). In both experiments, the frequency of CD4$^+$IL-10$^+$ cells was almost same in LPS or *H. felis* sonicate treated control groups and IL-10$^+$ B cell supernatant-

stimulated DC groups. Moreover; in LPS- or *H. felis* sonicate-treated DC groups; same trend was observed between IL-10$^-$ B cell and IL-10$^+$ B cell supernatant-stimulated DCs in terms of the frequency of CD4$^+$IL-10$^+$ cells. Especially, in the first experiment; the frequency of CD4$^+$IL-10$^+$ cells in IL-10$^+$ B cell supernatant-stimulated DC group was 5.4 % which was ~1.6 and 1.4 fold more than the frequency of CD4$^+$IL-10$^+$ cells in the Hf_{stim}-B and IL-10$^-$ B cell supernatant-stimulated DC groups, respectively (Figure 3.10).

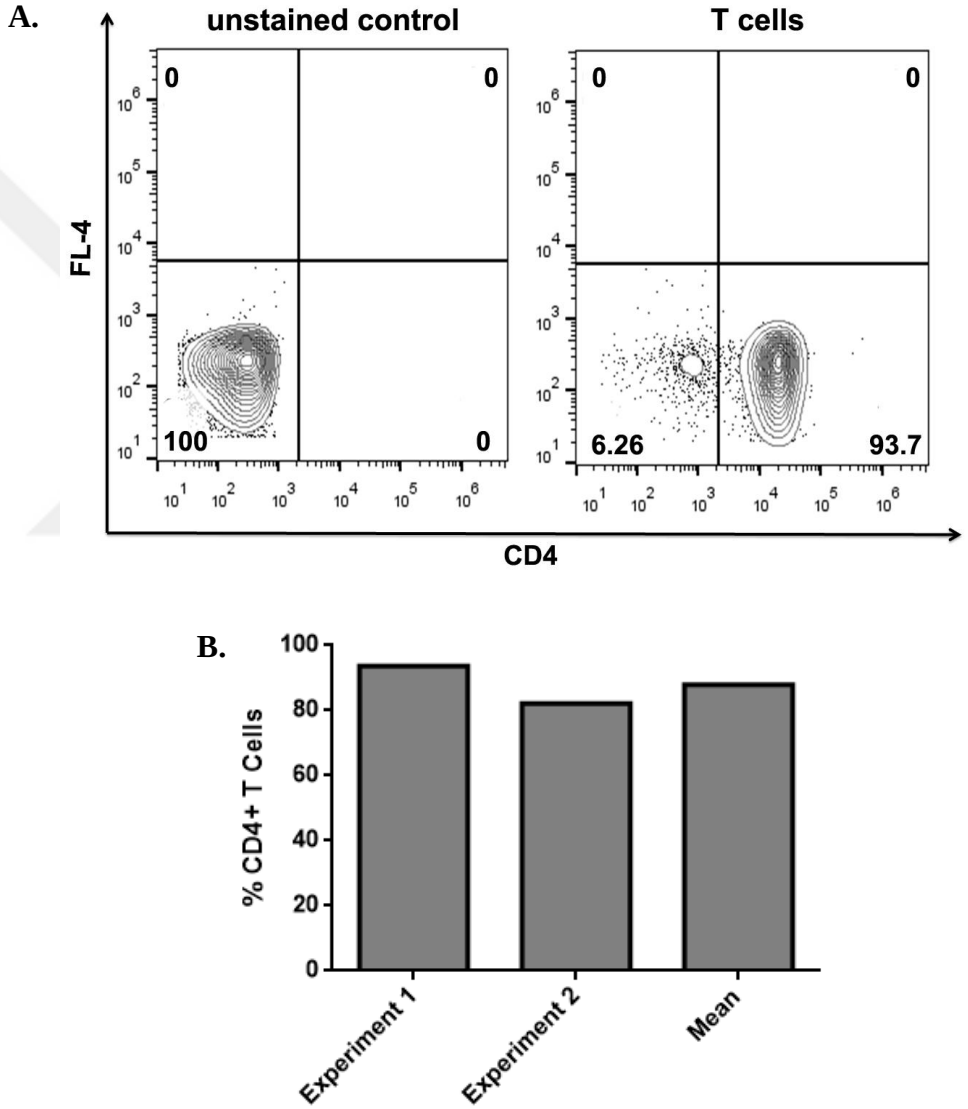

Figure 3.9 : Purity of CD4$^+$ T cells A) Purity of CD4$^+$ T cells were shown as a representative flow cytometry plot. B) Purity of T cells in two independent experiments and average purity was given in the graphs. T cell were stained with APC-coupled anti-CD4. Percentage of stained cells were determined using flow cytometer. Graphs were prepared using Graphpad Prism 6.

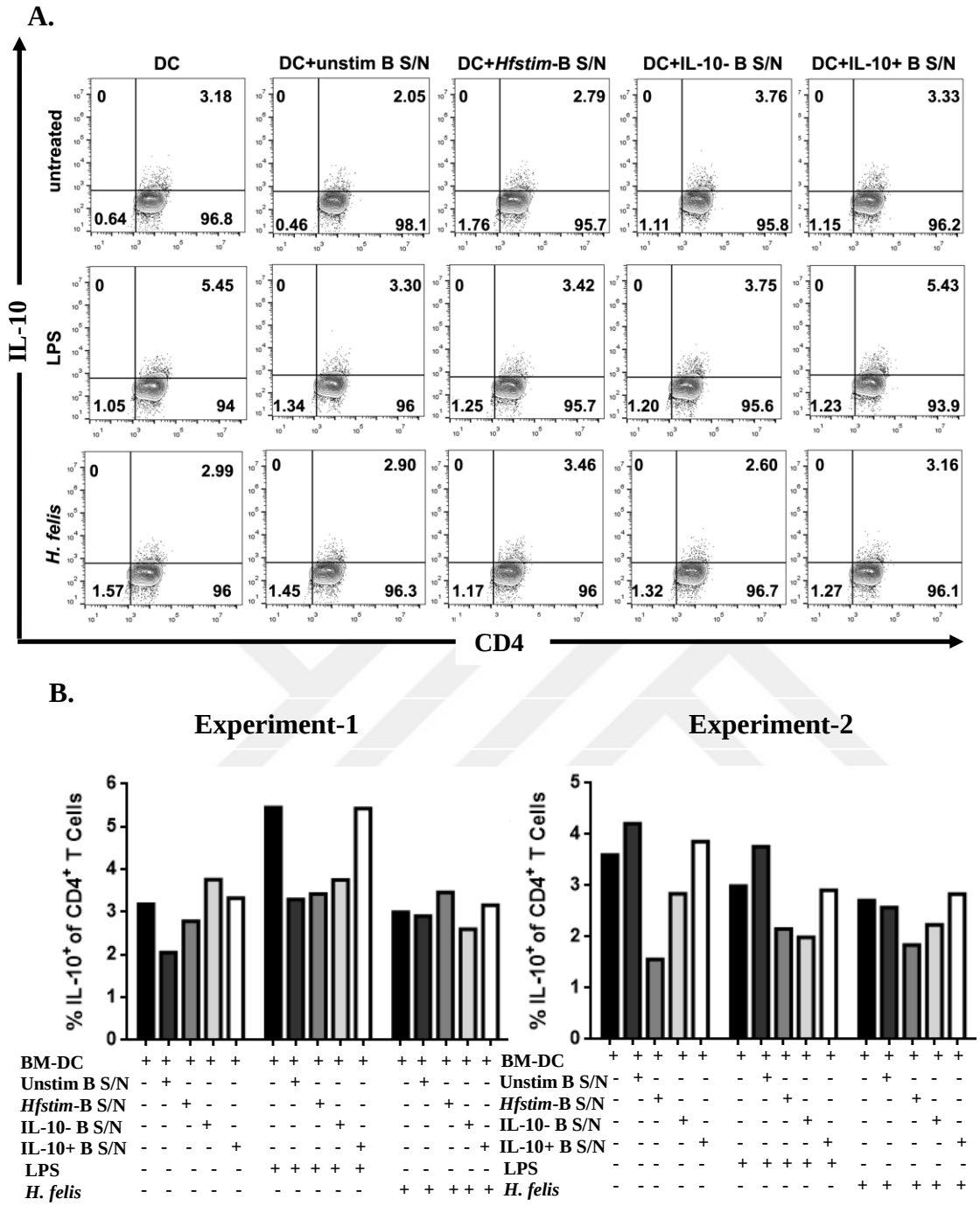

Figure 3.10 : LPS-treated BM-DCs that have been previously stimulated with supernatant of Hf_{stim}-IL-10$^-$ B cells, but not IL-10$^+$ B cells, lead to a decrease in frequency of IL-10-producing CD4$^+$ T cells. A) The percentage of IL-10$^+$ of CD4$^+$ cells in indicated conditions were shown as a representative flow cytometry plot. B) Percentage of IL-10$^+$ of CD4$^+$ cells in different groups from two independent experiments was shown in graphs. Naive CD4$^+$ T cells were co-cultured with Hf_{stim}-B cells supernatant-stimulated BM--DCs at a ratio of 1:2 (DC:T) for 24h in the presence of α-CD3 and IL-2. Afterwards, T cells were harvested and stained with APC-coupled anti-CD4 and PE-coupled anti-IL-10. Percentage of stained cells were determined using flow cytometer. Graphs were prepared using Graphpad Prism 6.

3.5 Direct Effects of Hf_{stim}-B Cells on BM-DCs

Hf_{stim}-B cells were co-cultured with BM-DCs at a ratio of 1:1 or 1:2 in order to determine the role of direct contact on the phenotype of BM-DCs. After co-culture, B cells which are suspension cells were discarded and adherent DCs were treated with LPS or *H. felis* sonicate to observe how DCs respond to the foreign antigens following interaction with Hf_{stim}-B cells. Activation status and cytokine profile of DCs were analyzed by flow cytometry and ELISA; respectively.

3.5.1 Hf_{stim}-B cells activate BM-DCs

Hf_{stim}-B cells induced the expression of CD86 in BM-DCs (10.1%) in the absence of antigenic stimulus after co-culture with B cells at 1:1 ratio; however, IL-10^+ B or IL-10^- B cells did not have a significant effect on DCs (11.1% and 8.65%; respectively). They led to a slight increase in exhibition of CD86 on the surface of DCs compared to unstimulated control group (7.2%). Besides; B cell to DC ratio were increased to 2:1, CD86-expressing DCs were induced stronger by Hf_{stim}-B cells (46.85%) without LPS or *H. felis* treatment. Contrary to this, unstimulated B cells were unable to show this effect on DCs under the same condition regardless of the ratio between DCs and B cells.

Treatment of DCs with LPS or *H. felis* sonicate induced the frequency of CD86-expressing DCs as indicated before (Section 3.2.1). However; when the DCs and B cells were kept together at a ratio of 1:1 or 1:2 (DC:B) followed by LPS treatment, all B cell groups containing IL-10^+ B or IL-10^- B cells had no significant effects on CD86-expressing DCs compared to unstimulated control groups. Contrary, Hf_{stim}-B cells led to a decrease in the frequency of CD86-expressing DCs. Moreover, upon *H. felis* treatment following DC-B cell co-culture at a ratio of 1:1, Hf_{stim}-B cell, but not IL-10^+ B and IL-10^- B cell, had a suppressive effect on activation of DCs by decreasing $CD86^+$ DCs from 21.65 % to 11.63 %. Also same trend was observed when B cell to DC ratio were increased to 2:1. Strikingly, when unstimulated B cells were cultured with DCs in 2:1 ratio, and later DCs were treated with LPS or *H. felis*, the ability of DCs to exhibit CD86 were decreased. In addition to that, a similar trend without significancy was detected in DC-B co-culture at a ratio of 1:1. Furthermore, IL-10^+ B and IL-10^- B cells had similar effects on activation of DCs. Accordingly, these two Hf_{stim}-B cell subgroups did not affect the frequency of CD86-expressing

DCs. Nevertheless, they led to a decrease in the frequency of CD86-expressing DCs without significancy when DCs were treated with *H. felis* following DC-B cell co-culture (from 21.65 % to 17.2 % and 17.6 %, respectively). Also, it is important to take in consideration that in Experiment-2, LPS and *H. felis* induced more CD86-expressing DCs than in Experiment-1 (Figure 3.11).

3.5.2 Interaction of BM-DCs with Hf_{stim}-B cells affects cytokine profile of BM-DCs

B cell groups did not have the capability to induce DCs to secrete IL-10 and TNF-α even if their number were increased (Figure 3.12). However; when DCs were co-cultured with unstimulated and all Hf_{stim}-B cell subgroups at a ratio of 1:1 followed by LPS treatment; secretion of IL-10 from DCs were decreased significantly. In contrast, DCs which were co-cultured with Hf_{stim}-B cells, did not have a significant decrease in their IL-10 level compared to DCs that were only treated with LPS. Moreover, a similar trend was observed for DCs co-cultured with B cells that was followed by *H. felis* treatment. Of note, no IL-10 was secreted from DCs which were co-cultured with IL-10^{+} B cells followed by *H. felis* treatment. In addition, there was no significant difference of IL-10 secretion level between DCs co-cultured with unstimulated B cells and DCs co-cocultured with Hf_{stim}-B cells in LPS or *H. felis* treted groups (Figure 3.12A, Experiment-1).

Strikingly, when the number of B cells co-cultured with BM-DCs, were increased to obtain the ratio of 1:2 between DC and B cell, Hf_{stim}-B cells, but not unstimulated B cells which cause a 1-fold decrease in IL-10 secretion from DCs, induced DCs to secrete 0.4-fold more IL-10 followed by LPS treatment compared to DCs treated only with LPS. Moreover, upon *H. felis*- treatment after DC-B cell interaction, the secretion of IL-10 by DCs was not affected by Hf_{stim}-B cells. In addition, no IL-10 was secreted from DCs that were treated with *H. felis* sonicate following interaction with unstimulated B cells (Figure 3.12A, Experiment-2).

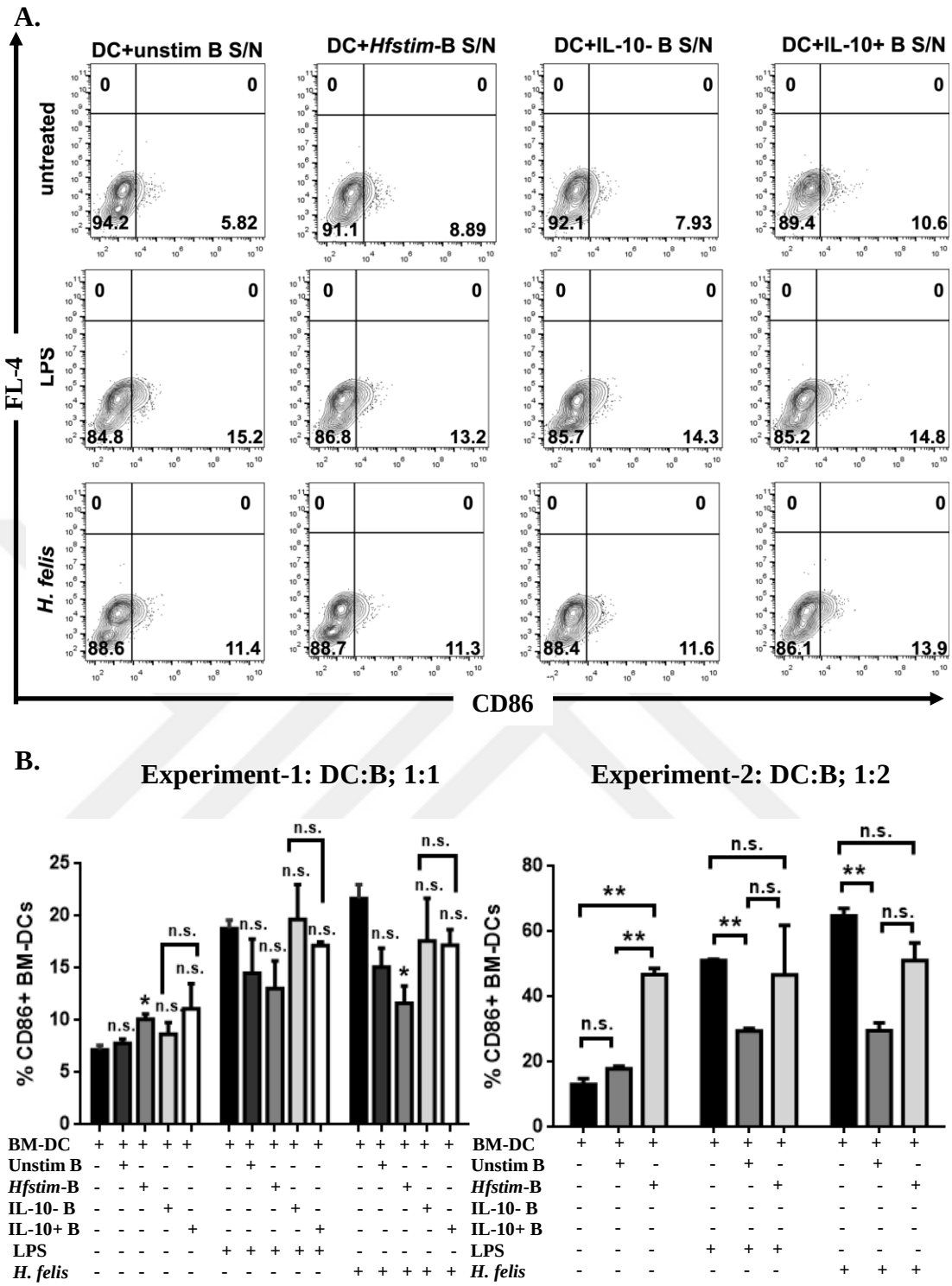

Figure 3.11 : Hf_{stim}-B cells activate BM-DCs. A) The percentage of $CD86^{+}$ BM-DCs with indicated conditions were shown as a representative flow cytometry plot. B) Effect of Hf_{stim}-B cells on activation of BM-DCs at indicated DC:B ratio. BM-DCs were co-cultured with unstimulated and *H. felis*-stimulated B cells at a ratio of 1:1 or :1:2 followed by LPS- or *H. felis*- treatment or no treatment. BM-DCs were stained with anti-CD86-PE and the percentage of stained cells were determined using flow cytometer. Graphs were prepared using Graphpad Prism 6. Each value represents the mean ± SEM of two biological replicates analyzed by Student *t* test. Statistical results indicated on each bar represent comparisons within each group (untreated, LPS- or *H.f.*- treated DCs) according to its control group (untreated, LPS- or *H.f.*- treated DCs without B cell). **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, n.s.: non-significant.

LPS-treated BM-DCs which previously had been kept with unstimulated B cells or Hf_{stim}-B cells at a ratio of 1:2 (DC:B) secreted significantly less TNF-α compared to LPS- treated DCs; on the other hand, a similar trend was also observed when DCs were kept with these B cell groups at a ratio of 1:1. Moreover, when DCs were treated with LPS following DC-B cell co-culture, the concentration of TNF-α secretion from DCs co-cultured with unstimulated B cells was comparable with the secretion of DCs co-cultured with Hf_{stim}-B cells regardless of the ratio between DC and B cell. IL-10^{+} B and IL-10^{-} B cells did not affect the secretion of TNF-α from DCs upon LPS or *H. felis* treatment. However; after LPS treatment TNF-α secretion from DCs, that had been kept with IL-10^{-} B cells before, was observed to be more than that DCs co-cultured with unstimulated B cells or Hf_{stim}-B cells.

In the case of *H. felis* treatment, interaction of DCs with Hf_{stim}-B cells led to significantly less TNF-α secretion than control group when DC:B cell ratio was 1:1 or 1:2. However, when ratio between the cells was 1:2, DCs which were kept with Hf_{stim}-B cells secreted more IL-10 than DCs kept with unstimulated B cells following *H. felis* treatment (Figure 3.12B).

Collectively; the ratio between DC and B cell affect the phenotype of DCs since interaction of DCs with Hf_{stim}-B cells at a ratio of 1:2 (DC:B) result in strong suppression of TNF-α but promoted IL-10 upon stimulation with LPS or *H. felis*.

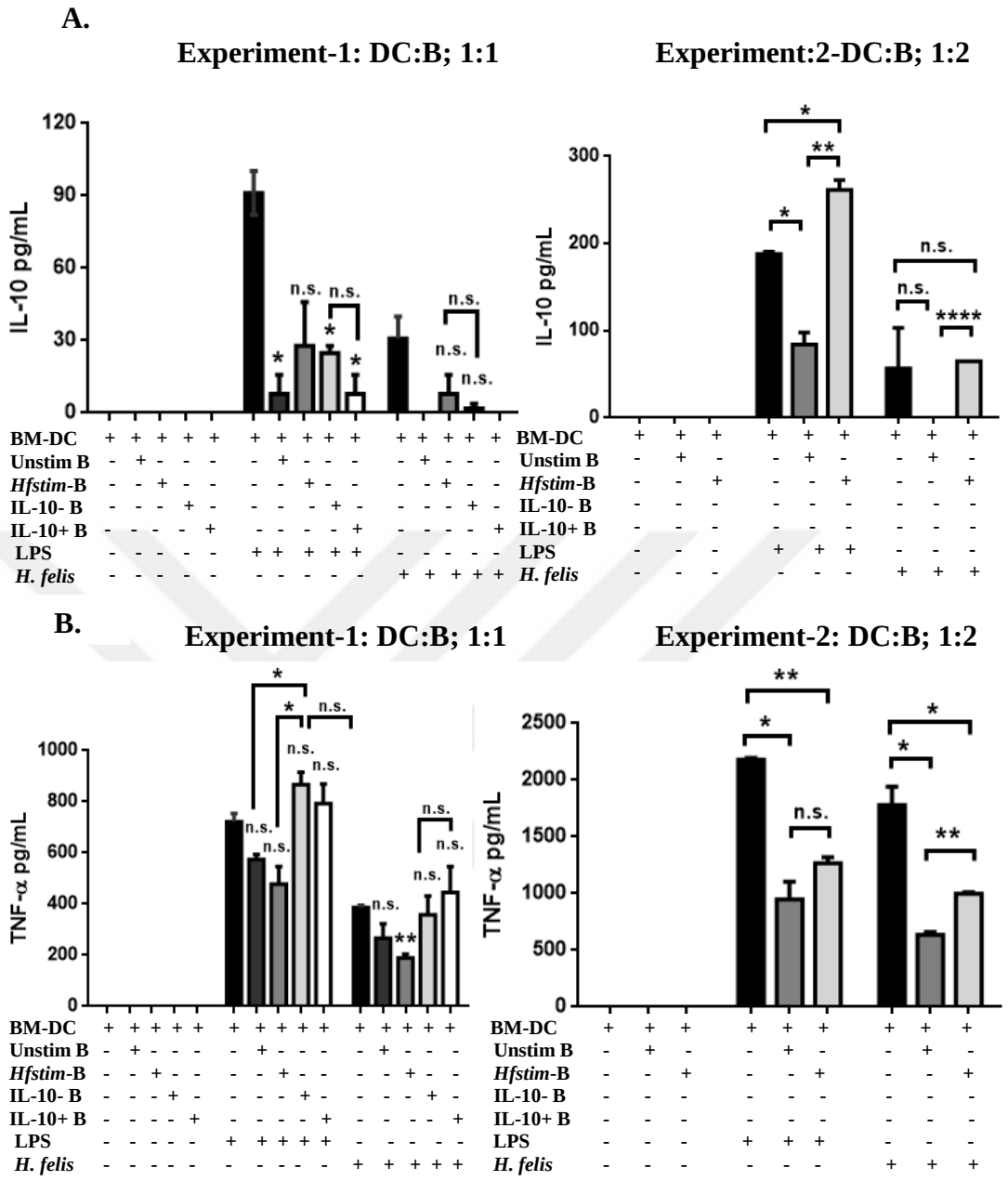

Figure 3.12 : Interaction of BM-DCs with Hf_{stim}-B cells affects cytokine profile of DCs. ELISA analysis for A) IL-10, B) IL-12/IL-23 (p40), C) TNF-α. BM-DCs were co-cultured with unstimulated and *H. felis*-stimulated B cells at a ratio of 1:1 or :1:2 followed by LPS- or *H. felis*- treatment or no treatment. Supernatants from samples of different groups were collected and analyzed by ELISA. Graphs were prepared using Graphpad Prism 6. Each value represents the mean ± SEM of two biological replicates analyzed by Student *t* test. Statistical results indicated on each bar represent comparisons within each group (untreated, LPS- or *H.f.*- treated DCs) according to its control group (untreated, LPS- or *H.f.*- treated DCs without B cell supernatant). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s.: non-significant.

4. DISCUSSION AND CONCLUSION

H. pylori infection is responsible for serious gastric complications which might result in gastric cancer. More than 50% of people are infected with this bacteria although only 10-20 % of infected people have serious complications. Therefore; this might indicate regulatory immune responses against bacteria that facilitates persistent *H. pylori* infection. Consistently, Sayi et al. (2010) has shown that *Helicobacter felis* activates B cells via TLR2/Myd88 pathway, which suppresses immune response *in vivo* and *in vitro* by triggering IL-10-producing type-1 regulatory T cell (Tr1)-like cells. This suppression result in persistency of bacteria in gastric mucosa and a decrease in pathology score. Moreover, IFN-γ production from Th1 cells or Th17-mediated immune response against *H. pylori* leads to gastric inflammation together with increase in clearance of bacteria (Kao et al., 2010, Sayi et al., 2009). Therefore; immune balance between effector and regulatory immune cells during infection determine the seriousness of infection. Other than B cells; also direct effects of *H. pylori* on modulation of dendritic cells were demonstrated.

In this study; *H. felis* was used due to lack of efficient immune response against *H. pylori* in mice. Our first aim was to understand the effects of *H. felis* on bone marrow-derived dendritic cells (BM-DCs). Mouse bone marrow cells were diffentiated into $CD11c^+$ DCs using 10 ng/ml GM-CSF and 10 ng/ml IL-4 (P. Matheu et al., 2008). However; we could not obtain highly pure immature DCs (average purity: 67.13%). Hence, $CD11c^+$ DC population was enriched by magnetic separation and highly pure $CD11c^+$ immature DCs were obtained (average purity: 93.24%) (Figure 3.1). Then, immature BM-DCs were treated with LPS and *H. felis* sonicate. Due to known effects of LPS on DCs, it was used as positive control in these experiments (Hui-Rong Jiang et al., 2002, Langenkamp et al., 2000). We observed BM-DC phenotype with induced level of CD86 and pro-inflammatory cytokines upon LPS treatment which are consistent with previous observations (Figure 3.2 and Figure 3.3) (Jiang et al., 2002, Oertli et al., 2012). However; contrary to observation of Oertli et al. (2012), we also demostrated significant induction of IL-10 production in LPS-treated BM-DCs as demonstrated previously by Jiang et al.

(2002) (Figure 3.3A). Secretion of IL-10, IL-12 and TNF-α by *H. felis*-treated DCs were significantly induced and was comparable with LPS-treated DCs (Figure 3.3A,B,C).

Secretion of expressed IL-1β to outside of the cell requires activation of NLRP3 inflammasome by silica crystals etc., which leads to maturation of pro-IL-1β to mature IL-1β (Peeters et al., 2013). Therefore, BM-DCs were treated with different doses of silica crystals (50 μg/cm^2, 25 μg/cm^2, 10 μg/cm^2, 5 μg/cm^2) during the last 8 hours of LPS or *H. felis* treatment. IL-1β secretion was induced upon *H. felis* or LPS treatment. Furthermore, LPS-treated DCs secreted more IL-1β than *H. felis*-treated DCs only in the case of 5 μg/cm^2 silica treatment; whereas with the other doses of silica crystals, LPS-treated and *H. felis*-treated DCs secreted comparable concentration of IL-1β (Figure 3.3D).

Consistently, LPS- or *H. felis*- treated DCs have significantly induced expression of CD86 and IL-10, IL-12 and TNF-α cytokines in addition to IL-6 compared to untreated DCs; whereas expression of these cytokines in *H. felis*-treated DCs were less than LPS-treated DCs (Figure 3.4). In literature; it has been shown that stimulation of BM-DCs with *H. felis* resulted in induction of IL-6 and IL-10 (Drakes et al., 2006). However; they did not observe an induction in terms of CD86 molecule in *H.felis* antigen-exposed DCs. Oertli et al. (2012) has demonstrated that live *H. pylori* induce BM-DCs to express CD86 and secrete IL-10 and IL-12. In addition, they did not observe any induction for secretion of IL-6 upon *H. pylori*-stimulation. Collectively, our findings are first comprehensive analysis of BM-DC phenotype after stimulation with *H. felis* demonstrating that *H. felis* induce significantly both pro-inflammatory and anti-inflammatory molecules by BM-DCs (Figure 4.1).

As previously demonstrated by our laboratory studies, *H. felis*-mediated activation of B cells induce two different B cell subgroups: IL-10-producing IL-10$^+$ B cells and TGF-β-producing IL-10$^-$ B cells (unpublished data). Accordingly, our second aim was to investigate the potential regulatory effects of *H. felis*-stimulated B (Hf_{stim}-B) cells on immature BM-DCs. To perform that CD19$^+$ B cells were isolated from spleen of C57BL/6 mouse through magnetic separation. Then, CD19$^+$ B cells were stimulated with *H. felis* sonicate for 16 hours and Hf_{stim}-IL-10$^+$ and Hf_{stim}-IL-10$^-$ B cells subgroups were seperated using magnetic isolation techniques. According to average of three independent experiments, purity of CD19$^+$ B cells, Hf_{stim}-IL-10$^+$ and

Hf_{stim}-IL-10$^-$ B cells were 85,3%, 83.6 % and 84.8 %, respectively (Figure 3.5). First we investigated the effects of cytokines secreted from Hf_{stim}-B cell subgroups on immature BM-DCs. To perform that, supernatant of Hf_{stim}-B cell subgroups and unstimulated B cells were collected and applied onto immature BM-DCs. Second, immature BM-DCs were co-cocultured with Hf_{stim}-B cells at a ratio of 1:1 and 1:2 (DC:B) to evaluate the role of cell-to cell contact on this interaction. Afterwards, BM-DCs were treated with 100 ng/ml LPS or 10 μg/ml *H. felis* sonicate in order to determine the phenotype of given DCs that had been previously stimulated with unstimulated and Hf_{stim}-B cells.

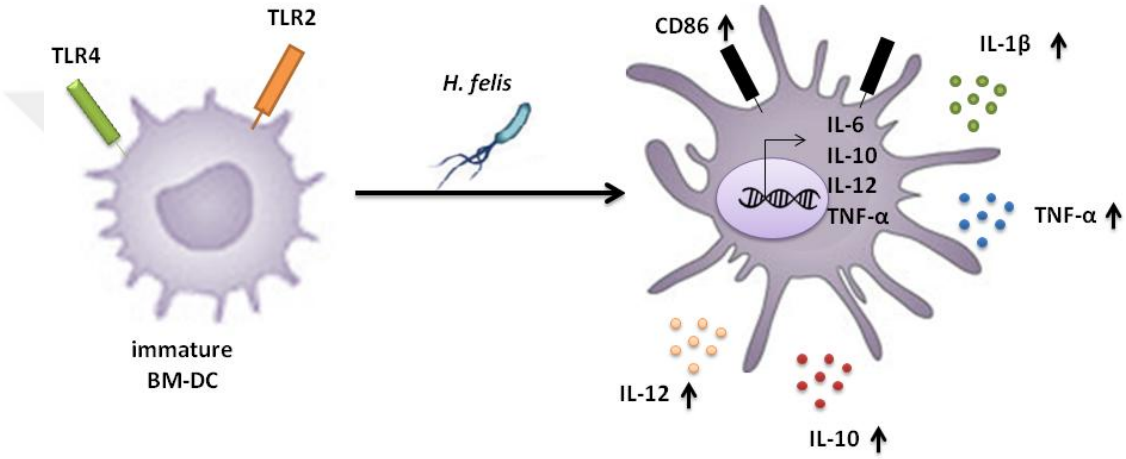

Figure 4.1 : Proposed model of the effect of *H. felis* antigens on immature bone marrow-derived dendritic cells (BM-DCs).

Supernatant of unstimulated B cells alone did not induce maturation of DCs since these DCs express low levels of CD86 and did not secrete any cytokines except for little amount of IL-10. Moreover, as expected, secretions of unstimulated B cells did not affect the cytokine profile of DCs induced by LPS or *H. felis* (Figure 3.6 and Figure 3.7). On the other hand, cytokines from all Hf_{stim}-B cell subgroups induced the maturation of DCs by increasing secretion of TNF-α, IL-12 and upregulating CD86 expression even in the absence of any subsequent antigenic stimulation. However, cytokines from IL-10$^-$ B cells, but not from IL-10$^+$ B cells, inhibited the secretion of IL-10 from DCs stimulated with LPS. In addition, similar inhibition trend was observed also upon treatment of DCs with *H. felis.* Furthermore, all Hf_{stim}-B cell subgroups, but not unstimulated B cells, suppressed BM-DCs to secrete TNF-α which was normally induced by LPS or *H. felis* strongly (Figure 3.7).

Then, we decided to analyze IL-6, IL-10, IL-12 and TNF-α mRNA expression levels of DCs that were stimulated with supernatant of IL-10^+ B or IL-10^- B cells. Strikingly, mRNA expression analysis indicates that expression of IL-6, IL-12, TNF-α and IL-10 were inhibited by cytokines secreted from Hf_{stim}-IL-10^+ B or Hf_{stim}-IL-10^- B cells. However, in LPS-treated DC groups, IL-10^- B cell supernatant-stimulated DCs have more expression of these cytokines as opposed to *H.felis*-treated DC groups. In this context, contrary to analysis on protein level, expression level of IL-10 was decreased in DCs that were stimulated with the secretions of IL-10^+ B cells. Moreover, also there is a contradiction between IL-12 secretion and expression in DCs that were stimulated with the supernatant of Hf_{stim}-IL-10^+ B cell or Hf_{stim}-IL-10^- B cell. Actually, it is possible that DCs might have secreted IL-12 and IL-10 during certain period of 12 hours incubation after LPS or *H. felis* treatment. However; at the end of the incubation, *de novo* mRNA expression of these cytokines were not induced by cytokines from Hf_{stim}-IL-10^+ B cell or Hf_{stim}-IL-10^- B cell (Figure 3.8).

Immature DCs express low levels of MHC-II and co-stimulatory molecules (CD80/CD86) and are incapable to secrete any cytokine which provides signal-3 to naive T cells. In addition to immature DCs, also semi-mature DCs were chacterized with MHC-II^{hi}CD80/CD86^{hi}IL-12^-TNF-α^-IL-10^+ phentotype are thought as inducer of Tr1 cell-mediated immune suppression (Rutella et al., 2006). Accordingly; it can be suggested that the cytokines from Hf_{stim}-IL-10^+ B cells have regulatory effects on immature BM-DCs more than Hf_{stim}-IL-10^- B cells and lead to generation of semi-mature-like DCs unlike LPS or *H. felis*-treated mature DCs. Matsumoto et al. (2014) demonstrated that stimulation of EAE-induced mice-derived DCs with supernatant of IL-10-producing wild type plasmablasts, but not IL-$10^{-/-}$ plasmablast, results in decreased mRNA expression of IL-6 and IL-12 but unchanged CD86 expression. However, according to cytokine profile of DCs, it seems that not only IL-10 but also different cytokines might contribute to generation of tolerogenic DCs since also some cytokines, but not IL-10, secreted from Hf_{stim}-IL-10^- B cells inhibit some pro-inflammatory properties of DCs. Supportively, it has been demostrated that IL-10 from activated B cells does not have a significant role on expression of maturation markers and production of IL-12p70 by mature human DCs (Morva et al., 2012). Our previous study demonstrated that Hf_{stim}-IL-10^- B cells secrete TGF-β (unpublished

data) which has regulatory effects on DCs (Kobie et al., 2003, Schmidt et al., 2012). Therefore, Hf_{stim}-IL-10$^-$ B cell-derived TGF-β might be responsible for this regulatory effect on BM-DCs. The mechanism and cytokines that are involved in induction of tolerogenic DCs by Hf_{stim}-B cells remain to be elucidated.

Although it is believed that antigen presentation by immature DCs in the absence of co-stimulatory molecules and signal-3 provided by secreted cytokines is important for induction of Tregs, also tolerogenic factors that are produced by DCs such as IL-10, retinoic acid, programmed death ligands contribute to Treg differentiation and proliferation (Maldonado and von Andrian, 2010). Nevertheless, it has been shown that mature DCs also direct expansion of CD4$^+$CD25$^+$ regulatory T cells (Yamazaki et al., 2003). Therefore maturation status is not sufficent alone to classify DCs as tolerogenic or immunogenic. In our study, highly pure naive CD4$^+$ T cells were isolated from mouse spleen through magnetic separation (average purity: 87.9%) (Figure 3.9). To evaluate functional properties of given DCs regarding induction of Tr1-like cells, they were co-cultured with naive CD4$^+$ T cells at a ratio of 1:2 (DC:T) in the presence of 10 ng/ml IL-2 and 1 μg/ml anti-CD3Ɛ. Interaction of BM-DCs with supernatant of Hf_{stim}-IL-10$^+$ B cells in the absence of subsequent LPS or *H. felis* treatment retained the frequency of IL-10-producing CD4$^+$ T cells generated by immature DCs (Figure 3.10, Experiment-2). In the first experiment, LPS treatment of DCs which had been previously stimulated with supernatant of unstimulated B cells, Hf_{stim}-B cells or Hf_{stim}-IL-10$^-$ B cells, but not Hf_{stim}-IL-10$^+$ B cells, resulted in the decreased frequency of IL-10-producing CD4$^+$ T cells compared to LPS-treated control group. On the other hand, in the second experiment in spite of observation of similar trend in LPS-treated DC group; supernatant of unstimulated B cell gave rise to a slight increase in IL-10$^+$ CD4$^+$ T cell population. Moreover, also in the absence of any LPS or *H. felis* treatment, supernatant of unstimulated B cells or Hf_{stim}-IL-10$^+$ B cells retained the frequency of IL-10-producing CD4$^+$ T cells unlike DCs stimulated with supernatant of Hf_{stim}-B cell or Hf_{stim}-IL-10$^-$ B cell. However, *H. felis* treatment of DCs following stimulation with supernatant of B cells have no effect on DCs to induce IL-10-producing CD4$^+$ population (Figure 3.10B). Unfortunately, we could not perform this experiment in duplicate for each group due to insufficient number of naive T cells we obtained. Also, the frequency of IL-10-producing CD4$^+$ population under all conditions was observed at low level. It may be due to the

inefficiency of intracellular staining which was used for the analysis of IL-10 production by $CD4^+$ T cells. Apart from intracellular staining, other methods such as IL-10 secretion assay can be performed to overcome this problem.

Plasmablast-derived IL-10 is crucial for suppressing autoimmune EAE by inhibiting differentiation of DC-mediated effector Th1 and Th17 cells (Matsumoto et al., 2014). Moreover, studies indicate that in addition to IL-10 secreted from DCs, other DC-derived molecules such as IL-27, TGF-β1, ICOSL and PD-L1 involve in differentiation of Tr1 cells (Kushwah and Hu, 2011). Taking these observations into consideration, it seems that in LPS-treated DC groups induction of IL-10-producing T cells might be due to action of IL-10; however it contradicts with the result observed in *H. felis*-treated DC groups. Therefore, it is possible that in addition to IL-10, also other factors may be involved in differentiation of IL-10-producing $CD4^+$ T cells by DCs stimulated with supernatant of different Hf_{stim}-B cells subgroups. In future, together with IL-10, regulatory T cell-specific molecules such as Foxp3 transcription factor can be analyzed to identify the exact type of the regulatory T cell. Also, the role of H_{stim}-B cell-induced BM-DC on effector T cell differentiation such as Th1 and Th17 cell or T cell proliferation can be investigated.

Regulatory effect of activated tonsillar B cells on function of human DCs requires cell-to-cell contact (Morva et al., 2012). Hence, we wanted to investigate the effect of Hf_{stim}-B cells on immature BM-DCs. To perform that, DCs were co-cultured with B cells at a ratio of 1:1 or 1:2 (DC:B) before LPS or *H. felis* treatment. Unlike unstimulated B cells, direct contact between Hf_{stim}-B cells and immature DCs activates DCs by upregulating CD86 expression in the absence of subsequent antigenic stimulation. This effect promoted when the number of B cells co-cultured with DCs were increased (Figure 3.11B). Strikingly, interaction of immature BM-DCs with Hf_{stim}-B cells prior to *H. felis* treatment inhibited activation of DCs only when DCs were co-cultured with B cells at a ratio of 1:1. It seems that increased activation of BM-DCs depends on the number of Hf_{stim}-B cells co-cultured with DCs. Interestingly, not in the first experiment but in the second experiment unstimulated B cells inhibited the activation of BM-DCs which were treated with LPS or *H. felis* following co-culture at a ratio of 1:2 (DC:B). Moreover, Hf_{stim}-IL-10^+ B and Hf_{stim}-IL-10^- B cells did not have any effect on activation of DCs by LPS or *H. felis*.

In addition, also cytokine profile of BM-DCs after interaction with unstimulated, Hf_{stim}-B cells, Hf_{stim}-IL-10^{+} B cells and Hf_{stim}-IL-10^{-} B cells were checked. B cells alone did not induce any IL-10 and TNF-α secretion regardless of the ratio between DC and B cell; however LPS or *H. felis* treatment of DCs after interaction of B cells resulted in decreased IL-10 production when the ratio between the cells was 1:1 (Figure 3.12; Experiment-1). Moreover, TNF-α production by DCs was inhibited efficiently by Hf_{stim}-B cells or unstimulated B cells when BM-DCs were co-cultured with these B cells at a ratio of 1:2 (DC:B) prior to LPS or *H. felis* treatment (Figure 3.12B). Interestingly, Hf_{stim}-IL-10^{+} B cells and Hf_{stim}-IL-10^{-} B cells did not affect the production of TNF-α by DCs. Importantly, interaction of immature BM-DCs with Hf_{stim}-B cells (DC:B; 1:2) prior to LPS treatment induced production of IL-10 by DCs; whereas unstimulated B cells exhibited inhibitory effect on IL-10 production (Figure 3.12A, Experiment-2). It seems that the number of B cells co-cultured with immature DCs might be a critical factor to exhibit regulatory function on DCs. In addition to cytokines secreted from Hf_{stim}-B cells also cellular contact via surface molecules might have a regulatory role on DCs. In respect to that, they showed that CD62L-selectin expressed on activated B cells might be responsible for modulation of human DC function independent of IL-10 (Morva et al., 2012). Neverheless, it is not obvious since we performed this experiment only once. Therefore, the experiment must be repeated to make an exact deduction for the role of cellular contact between DCs and B cells.

Collectively; some molecules secreted from Hf_{stim}-B cells in addition to IL-10 synergistically with cell-to-cell contact may have a regulatory role on activation and function of DCs (Figure 4.2).

As a result; first, *H. felis* antigens activate immature BM-DCs and induce production of pro-inflammatory cytokines as well as IL-10 *in vitro*. Second, *Helicobacter felis*-stimulated B cells converts immature BM-DCs into IL-10-secreting tolerogenic form. Therefore, this study includes comprehensive analysis for the effects of *H. felis* on mouse BM-DCs and the first investigation that clarifies the *ex vivo* interaction of Hf_{stim}-B cells subgroups with BM-DCs.

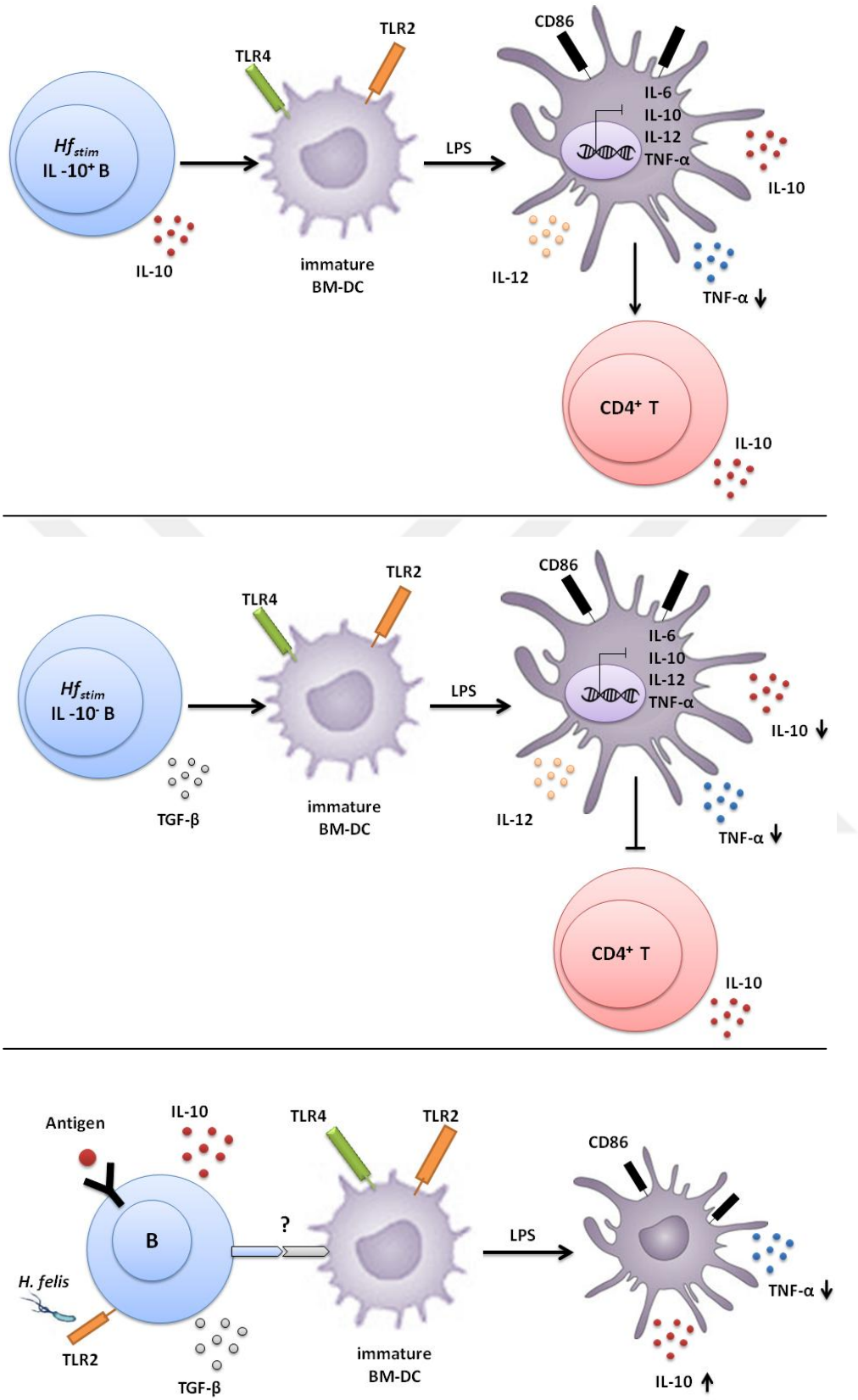

Figure 4.2 : Proposed model of *ex vivo* interaction between *H. felis*-stimulated B (Hf_{stim}-B) cell and bone marrow-derived dendritic cell (BM-DC).

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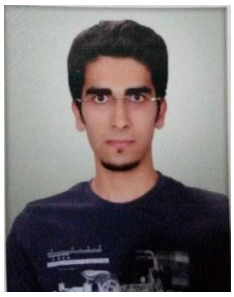
CURRICULUM VITAE



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