ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MOLECULAR CHARACTERIZATION OF *HELICOBACTER*-ACTIVATED B CELLS

M.Sc. THESIS

Nesteren MANSUR

The Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Program

AUGUST 2014

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

HELICOBACTER-AKTİVE B HÜCRELERİNİN MOLEKÜLER KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

Nesteren MANSUR
(521111120)

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Doç. Dr. Ayça SAYI YAZGAN

AĞUSTOS 2014

Nesteren Mansur, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID **521111120**, successfully defended the **thesis** entitled "**MOLECULAR CHARACTERIZATION OF *HELICOBACTER*-ACTIVATED B CELLS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : Assoc. Prof. Dr. Ayça SAYI YAZGAN .....
Istanbul Technical University

Jury Members : Prof. Dr. Arzu KARABAY KORKMAZ .....
Istanbul Technical University

Assoc. Prof. Dr. Nesrin ÖZÖREN .....
Boğaziçi University

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FOREWORD

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August 2014 Nesteren MANSUR

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ABBREVIATIONS

µg : Microgram
µl : Microliter
µM : Micromolar
µm : Micrometer
AF-488 : Alexa Fluor-488
APC : Allophycocyanin
BCA : Bicinchoninic acid
BCR : B Cell Receptor
Blimp-1 : B lymphocyte-induced maturation protein-1
BM : Bone marrow
bp : Base pair
Breg : Regulatory B cell
Cag : Cytotoxin-associated gene
CD : Cluster of differentiation
CD40L : CD40 ligand
cDNA : Complementary DNA
CIA : Collagen-induced arthritis
CpG : Cytosine-phosphate-Guanine
CTL : Cytotoxic T lymphocyte
DAPI : 4',6-diamidino-2-phenylindole
DC : Dentritic cell
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide
EAE : Experimental autoimmune encephalomyelitis
EDTA : Ethylenediaminetetraacetic acid
ELISA : Enzyme-linked immunosorbent assay
ERK : Extracellular-signal-regulated kinase
Fab : Fragment-antigen binding
FBS : Fetal bovine serum
Fc : Fragment, crystallizable
FcγR : Fc γ receptor
FlaA/B : Flagella A/B
FITC : Fluorescein isothiocyanate
FO : Follicular
g : Gram
GAPDH : Glyceraldehyde 3-phosphate dehydrogenase
GC : Germinal center
***H*act** : *Helicobacter*-activated
H.felis : *Helicobacter felis*
H.pylori : *Helicobacter pylori*
HRP : Horseradish peroxidase

IFN-γ : Interferon gamma
Ig : Immunoglobulin
IKK : IκB kinase complex
IL-10 : Interleukin-10
LPS : Lipopolysaccharide
M : Molar
MAPK : Mitogen-activated protein kinases
Mg : Miligram
MHC : Major histocompatibility complex
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 B
NIK : NF-κB-inducing kinase
NKT : Natural killer T cell
OipA : Outer membrane protein A
PAI : Pathogenicity island
PBS : Phosphate buffered saline
PCR : Polymerase chain reaction
PDTC : Pyrrolidine dithiocarbamate
PE : Phycoerythrin
PGN : Peptidoglycan
pH : Power of Hydrogen
PMA : Phorbol myristate acetate
rcf : Relative centrifugal force
RNA : Ribonucleic acid
rpm : Revolutions per minute
RPMI : Roswell Park Memorial Institute
rRNA : Ribosomal RNA
T4SS : Type IV secretion system
TCR : T cell receptor
TGF-β : Transforming Growth Factor-beta
Th1 : T helper 1
Th2 : T helper 2
Th-3 : T regulatory type 3
Tim-1 : T cell membrane protein 1
T_m : Melting temperature
TLR : Toll-Like Receptor
TNF : Tumor necrosis factor
Tr-1 : T regulatory cell 1
Treg : Regulatory T cell
Vac :Vacuolating toxin

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MOLECULAR CHARACTERIZATION OF *HELICOBACTER*-ACTIVATED REGULATORY B CELLS

SUMMARY

Helicobacter pylori (*H. pylori*) is a gram negative, microaerophilic bacterium which is localized in the stomach. Around %50 of individuals are infected with *H. pylori* in the world. However, the percentage of prevalence goes up to 80% or more in developing countries compared to the developed countries where it is around 40% or less. Most of infected individuals remain asymptomatic. On the other hand, some of infected people may develop gastric malignancies such as gastric atrophy, intestinal metaplasia, dysplasia and it may possibly progress to gastric adenocarcinoma.

H. pylori can exist for years in a dynamic equilibrium with its host. In order to achieve this, *H. pylori* uses its virulence factors. In order to survive under acidic pH of stomach, they produce urease enzyme to neutralize acidity. Also, its cytotoxin-associated gene (Cag) – pathogenicity island (PAI) encodes type IV secretion system. Cag-PAI helps to translocate one of the main virulence factors, CagA, into host cells. Another virulence factor is vacuolating gene A (VacA), which interacts with host cell to manipulate it, activate immune system, disrupt cell-to-cell junctions etc. Outer membrane proteins are necessary to adhere host cells.

In mouse studies, *Helicobacter felis* (*H. felis*) is mainly used because it is more immunogenic than *H.pylori* in mice. It has been shown that *Helicobacter* infected C57BL/6 mice had gastric atrophy and lost specialized cells. These mice also showed decreased level of colonization whereas increased level of a T helper 1 (Th1) cell driven pro-inflammatory cytokine, interferon gamma-γ (IFN-γ). Even if there is high inflammation after *Helicobacter* infection, most of infected individuals stay asymptomatic. It indicates presence of some regulatory mechanisms, which limits high pathology formation. It was shown that $Foxp3^+$ natural regulatory T cells (Tregs) have a role in repression of the immune response against *H. pylori.* This suppressive effect leads to chronic gastric infection and inflammation. In addition to Tregs, a subset of B cells has been recently shown to have a role in immune regulation against *Helicobacter* infection in mice models. These *Helicobacter*-activated B cells (H_{act}-B cells) produce interleukin-10 (IL-10) through Toll-like receptor-2 (TLR-2) and MyD88 signaling. These cells were found to interact with T cells through co-receptors. Therefore, they lead the conversion of T cells into IL-10 producing Tr-1 type cells. These B cells were also shown to produce IgM and IgG2b antibodies. However, it is still not known whether *Helicobacter*-activated $IL\text{-}10^+$ or $IL\text{-}10^-$ B cell subsets are the source of produced antibodies.

In different mouse autoimmune disease and infection models, the presence of functional regulatory B cell subsets has been identified for their phenotypic characteristics. Different groups described B cell subsets with regulatory roles.

$CD1d^+CD5^+$ regulatory B cells, which are named as B10 cells, were found in colitis, contact hypersensitivity and systemic lupus erythematosus mice models. The other subset of regulatory B cells was $CD21^{hi}CD23^+$ B cells. This subset was shown in collagen-induced arthritis and lupus model. Recently, Tim-1^+ regulatory B cells were shown to produce IL-10. However, it is not known whether these subsets are present in IL-10 producing H_{act}-B cells.

Besides IL-10, transforming growth factor-β (TGF- β), another cytokine has been shown to suppress immune response. TGF-β is found to be responsible on cell proliferation, differentiation and immune regulation. TGF- β induce T cells to become natural $Foxp3^+$ Treg cells. Also, it has a function on inhibition of Th1, Th2 and cytotoxic T lymphocytes. It helps for the conversion of naïve IgM^+IgD^+ B cell into IgA producing memory B cell. As it has been shown in T cells, B cells also produce TGF-β. These TGF- β producing B cells were named as Br3 cells. These regulatory B cells were reported in different disease models such as systemic lupus erythematosus, diabetes, non-IgE mediated food allergy in atopic dermatitis. However, there is not any knowledge about TGF-β expression from *Helicobacter*-activated B cells. In this study, IL-10^+ and IL-10^- subsets were compared for TGF-β relative expression and production.

IL-10 is found to be produced by monocytes and myeloid dendritic cells through different signaling pathways. Induction of Toll-like receptor-2 (TLR-2) or TLR-4 with their ligands may lead to p38, extracellular-signal-regulated kinase (ERK) or nuclear factor kappa B (NF-κB) activation in a myeloid differentiation primary response gene-88 (MyD88)-dependent manner. Nuclear factor kappa B (NF-κB) is one of transcription factors, which has a role in immune response against infection, proliferation, differentiation and survival. This pathway can be activated through canonical or non-canonical ways. In previous work, *Helicobacter*-activated B cells were identified as TLR-2 and MyD88 dependent for production of IL-10. However, the role of NF-κB pathway on downstream regulation of *Helicobacter*–activated B cells through TLR-2 and MyD88 needs to be understood.

Therefore, in our project we first aimed to characterize H_{act}-Bregs in more detail. *Helicobacter*-activated B cells were enriched as IL-10^+ and IL-10^- B cells. Previously reported phenotypic markers of IL-10 producing regulatory B cells were characterized in our B cell. H_{act} IL-10^+ and IL-10^- B cells were analyzed by flow cytometry for CD1d-CD5 and CD21-CD23 double staining. Our data suggests that around 55% of *Helicobacter*-activated IL-10 producing B cells are $CD1d^+CD5^+$ and 60% are $CD21^+CD23^+$. Only 8-10 % of IL-10 negative subset of *Helicobacter*-activated B cells are expressing either $CD1d^+CD5^+$ or $CD21^+CD23^+$. Moreover, antibody production levels of *Helicobacter*-activated IL-10^+ and IL-10^- B cells were measured with antibody isotyping ELISA. IgM and IgG2b antibodies, which have been shown to be produced by H_{act} - B cells, are produced only by *Helicobacter*-activated IL-10^- B cell subset but not from IL-10^+ B cells.

Similarly, another defined cytokine with a regulatory role, TGF- β was examined for *Helicobacter*-activated B cells. TGF-β was examined with Real - time PCR for its relative expression level. Moreover, secreted level of TGF- β was analyzed with ELISA. It is found TGF- β is produced from H_{act} B cell population. Also, TGF- β was only produced from *Helicobacter*-activated IL-10^- B cells but not from IL-10^+ B cells.

Another aim of the experiment was to assess the role of NF-κB canonical pathway on the induction of IL-10 expression from H_{act} B cells. In order to understand NF-κB activation in *Helicobacter*-activated B cells, translocation of NF-κB p65 subunit from cytoplasm to nucleus was analyzed with staining of NF-κB in immunofluorescence assay. Also, THP-1 cell line was used to assess NF-κB activation through LPS induction and inhibition of NF-κB pathway through NF-κB inhibitor PTDC. After that, inhibition of NF-κB pathway was used to understand its effect on IL-10 secretion from *Helicobacter*-activated B cells. IL-10 secretion levels were analyzed with IL-10 specific ELISA. We showed that NF-κB canonical pathway may not be the responsible of inducing IL-10 expression from *Helicobacter*-activated B cells.

Taken together this data suggests that H_{act} IL-10 producing B cells are mostly $CD1d^{+}CD5^{+}$ or $CD21^{+}CD23^{+}$ phenotype. On the other hand, *Helicobacter*-activated $IL\text{-}10^{-}$ B cells secrete IgM and IgG2b antibodies and TGF-β, as well. However, $IL\text{-}10^{-}$ B cells are not the source of these antibodies and TGF-β production. Furthermore, it has been suggested that NF-κB p65 has not a role on IL-10 production from *Helicobacter*-activated B cells.

HELICOBACTER-AKTİVE REGÜLATÖR B HÜCRELERİNİN MOLEKÜLER KARAKTERİZASYONU

ÖZET

Helicobacter pylori (*H. pylori*), gram-negatif ve mikroaerofilik bir bakteri olup midede lokalize olur. Konak organizma ile bir denge kurarak uzun yıllar boyunca varlığını sürdürebilir. Dünya genelinde bireylerin %50'si bu bakteri ile enfekte iken, az gelişmiş veya gelişmekte olan ülkelerde bu oran %80'leri bulmaktadır. Enfekte bireylerin çoğu ise semptom göstermez. Ancak bir kısmında gastrik atrofi, displezi, mide kanseri gibi ciddi gastrik komplikasyonlar gelişebilir. *H. pylori*, konakta kolonize olmak ve varlığını sürdürmek için virülans faktörlerini kullanır. Asidik bir ortam olan midede yaşamını sürdürmesi, ürettiği üreaz enzimi sayesinde gerçekleşir. *H. pylori*, Cag (Cytotoxin-associated gene) - PAI (patogenite adası) gen bölgesinde tip IV sekresyon sistemini kodlar. Bu sayede, önemli bir virülans faktörü olan CagA proteini hücre içine aktarılır. VacA (vacuolating cytotoxin A) virülans faktörü ise konak epithel hücresi ile olan etkileşimleri, onların manipüle edilmesi, hücre arası bağlantıların bozulması, immün sistemin aktivasyonu gibi çeşitli etkilerde bulunur. Üzerinde bulunan adhezyon molekülleri konak hücreye tutunmak için gereklidir.

Hayvan çalışmalarında, *Helicobacter pylori*'den immunojenliği daha yüksek olan *Helicobacter felis* kullanılmaktadır. C57BL/6 adı verilen farelerde yapılan enfeksiyon çalışmalarında özelleşmiş hücre kaybı ile gastrik atrofi gözlenmiştir. Kolonizasyonun azalmasına ters orantılı olarak Th1 hücre gruplarınca üretilen ve pro-inflamatuvar bir sitokin olan IFN-γ oranı artmaktadır. Yüksek patoloji oluşmasına karşın, enfekte bireylerin çoğunun semptom göstermeden yaşamını sürdürmesi regülatör bir grubun varlığıyla alakalıdır. *Helicobacter* enfeksiyon modelinde $Foxp3^+$ üreten doğal regülatör T hücrelerinin dokuya göç ederek aşırı immun cevabı baskıladığı gösterilmiştir. T hücrelerinin yanı sıra regülatör özelliği gösterilmiş olan B hücrelerinin *Helicobacter*-enfeksiyonundaki olası rölü yakın zamanda yapılan bir çalışmada gösterilmiştir. *Helicobacter*-aktive edilmiş B hücreleri Interlökin-10 (IL-10) üretimini artırmaktadır. Bu B hücrelerinin T hücreleri ile eş-reseptörler ile hücre-hücre etkileşimi ve IL-10 üretimi ile dolaylı etkileşimleri onlatı Tr-1 adı verilen IL-10 üreten T hücre gruplarına dönüştürür. Ayrıca aynı çalışmada, B hücrelerinin Toll benzeri reseptör (TLR)-2 aracılığı ve myeloid farklılaşma proteini 88 (Myd88) aracılığı ile IL-10 ürettiği gösterilmiştir. *Helicobacter*-aktive edilmiş B hücrelerinden Immunoglobulin M (IgM) ve IgG2b üretildiği gösterilmiştir. Ancak *Helicobacter*-aktive B hücrelerinden, IL-10 üreten ya da üretmeyen gruplardan hangisinin bu antikor üretiminde rolü olduğu bilinmemektedir.

Literatürde regülatör B hücrelerinin varlığı birçok hastalık modelinde gösterilmiştir. Bunlar kolit, deneysel otoimmün ensefalomiyelit, lupus-benzeri hastalık, *Leishmania major* enfeksiyonu gibi farklı hastalık modelleridir. Regülatör B hücrelerinin ayırt edilebilmesi açısından tanımlanabilmeleri oldukça önemlidir. Bazı çalışmalarda,

yüzey belirteçlerinin ekspresyon düzeylerine göre karakterize edilmeye çalışılmıştır. $CD1d^{yük}CD5^{+}$ grubu B10 hücreleri olarak kabul edilmiş, bu grubun arttığı kolit, kontakt hipersensitivite modeli, sistemik lupus eritematozus çalışmalarında gösterilmiştir. $CD21^{yük}CD23^{+}$ grubun varlığı da kolajen indüklenmiş artirit ve lupus modelinde gözlenmiştir. Yakın zamanda Tim-1^{+} IL-10 üreten B hücreleri rapor edilmiştir. Bu gibi çalışmalar, regülatör B hücrelerinin tanımlanması, onların tipik bir/birden fazla belirteç ile ayrılmalarını sağlamak için oldukça önem taşımaktadır.

Regülatör hücrelerde varlığı gösterilen diğer bir sitokin ise transforme edici büyüme faktörü-β (TGF-β)'dır. Bu sitokin, immüne cevap, T hücre proliferasyonu, farklılaşma gibi birçok hücre mekanizmasında regülatör rol oynadığı bilinmektedir. TGF-β, Th1 ve Th2 tip cevabı, ayrıca sitotoksik T lenfositlerinin de aktivasyonunu baskılamaktadır. Ayrıca, naive B hücrelerinin IgA üreten hafıza hücrelerine dönüşümünde rolü vardır. TGF-β, T hücrelerinin $Foxp3^{+}$ doğal regülatör T hücrelerine dönüşümünü sağlar. B hücrelerinde üretilen TGF-β'nın da varlığı bazı çalışmalarda gösterilmiştir. Sistemik lupus eritematozus, diyabet ve ayrıca, atopik dermatitde IgE'den bağımsız olarak gıda allerjisine karşı geliştirilen cevapta etkili olduğu gösterilmiştir. *Helicobacter*-aktive B hücrelerinin TGF-β immün cevabı bilinmemektedir. Çalışmada IL-10 üreten ve üretmeyen gruplar karşılaştırılmıştır.

Daha önce makrofajlarda ve myeloid detritik hücrelerde TLR-2 ve TLR-4 ile uyarılan hücreler, MyD88 adaptor protein aktivasyonundan sonra p38, ERK veya nuclear faktör kappa-B (NF-κB) yolakları aracılığı ile IL-10 ürettikleri gösterilmiştir. *Helicobacter*-aktive B hücrelerinin ürettiği IL-10'un hangi yolakla üretildiği bilinmemektedir. Belirtilen çalışmada, NF-κB aktivasyonunun rolü incelenmiştir.

Bu çalışmada, *Helicobacter*-aktive B hücrelerinin moleküler karakterizasyonunu tanımlamak hedeflenmiştir. *Helicobacter*-aktive B hücreleri IL-10^{+} ve IL-10^{-} gruplara ayrılmıştır. Bu gruplar fenotipik belirteçlerinin analizi için kullanılmıştır. Daha önce belirtilen CD1d-CD5 ve CD21-CD23 yüzey moleküllerinin ekpresyonları, bu belirteçlere özgü antikorlar kullanılarak flürosan bir molekülle işaretlenmiş, sonuçlar akım sitometri ile analiz edilmiştir. Buna göre, IL-10 üreten hücrelerin yaklaşık olarak %55'i $CD1d^{+}CD5^{+}$ ve %60'ı $CD21^{+}CD23^{+}$ bulunmuştur. IL-10^{-} B hücrelerinin ise yaklaşık %8-10 kadarı $CD1d^{+}CD5^{+}$ veya $CD21^{+}CD23^{+}$ olarak bulunmuştur.

Antikor salınımları hakkında da bilgi edinmek adına, IL-10 üretimlerine göre ayrılan bu iki grup antikor profilleme ELIZA'sı yardımı ile analiz edilmiştir. Daha önce gösterilen *Helicobacter*-aktive B hücrelerinden IgM ve IgG2b salınımının daha çok IL-10 üretmeyen grup tarafından yapıldığı görülmüştür. Diğer antikorların salınımı da bu hücrelerde mevcut değildir. *Helicobacter*-aktive IL-10 üreten B hücrelerinden ise herhangi bir antikor salınımı gerçekleşmemiştir.

Diğer bir regülatör sitokin olan TGF-β'nın belirtilen IL-10 üreten ve üretmeyen B hücrelerinde ekspresyonunun ve onlardan salınımın analizi yapılmıştır. TGF-β üretimleri gerçek zamanlı PZR kullanılarak gerçekleştirilmiştir. Salınan TGF-β miktarları ise ELIZA yöntemi ile tayin edilmiştir. Sonuçlara göre, TGF-β üretimi IL-10^{-} B hücreleri tarafından gerçekleşmektedir. *Helicobacter*-aktive IL-10 üreten B hücreleri neredeyse hiç TGF-β eksprese etmemiş ve üretmemiştir.

IL-10 üretimlerinde olası sinyal yolaklarından NF-κB p65 yolağı incelenmiştir. Aktive olduğunda sitoplazmadan çekirdeğe geçen bu alt birim, immunoflorasan boyama ile analiz edilmiştir. *Helicobacter felis (H. f.)* ile muamele edilmiş B hücreleri, herhangi bir muameleye maruz kalmamış kontrol B hücreleri ile benzer

sonuç göstermiştir. *Helicobacter* varlığı herhangi bir aktivasyona sebep olmamıştır. Bunu desteklemek için NF-κB inhibitörü varlığında *H. f* . ile muamele edilmiş ve edilmemiş B hücreleri karşılaştırılmıştır. Daha sonra, IL-10 üretim miktarlarına ELIZA yöntemi ile bakılmıştır. NF-κB inhibitörü varlığında üretilen IL-10 miktarları arasında bir fark gözlenmemiştir. IL-10 aktivasyon yolağının NF-κB p65 yolağı olmadığı düşünülmektedir.

Sonuç olarak, *Helicobacter*-aktive IL-10 üreten hücreler çoğunlukla $CD1d^{+}CD5^{+}$ ve $CD21^{+}CD23^{+}$ pozitif bulunurken, $IL\text{-}10^{-}$ hücreler IgM ve IgG2b, ve de TGF-β üreten hücreler olarak gözlenmiştir. Salınan bu IL-10'un üretiminde NF-κB p65 yolağının bir etkisi olmadığı gözlenmiştir.

1. INTRODUCTION

1.1. *Helicobacter* Species

1.1.1. *Helicobacter pylori*

Helicobacter pylori (H. pylori) is a gram-negative, spiral shaped bacterium which colonizes in the stomach (Figure 1.1). It can continue to exist for years in a dynamic equilibrium with its host. Around %50 of individuals is infected with *H. pylori* in the world. However, the percentage of prevalence goes up to 80% or more in developing countries compared to the developed countries where it is around 40% or less. Most of infected individual remain asymptomatic (Bauer & Meyer, 2011).

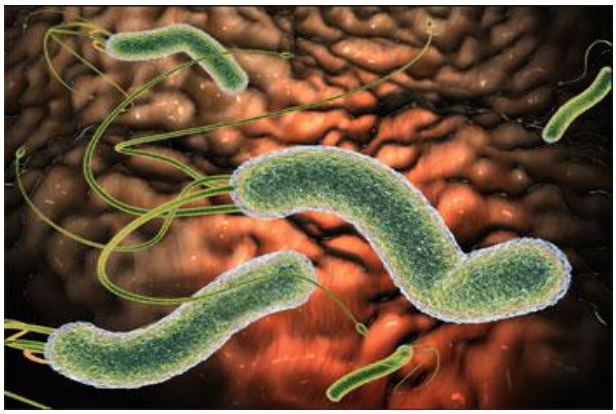

Figure 1.1 : *Helicobacter pylori* in gastric mucosa (adapted from Cancer Information and Support Network [CISN], 2013).

Nearly all infected individuals develop gastritis. However, long-term colonization may lead to some other consequences such as increased risk of gastric atrophy, intestinal metaplasia, dysplasia and it may possibly progress to gastric adenocarcinoma. Because of that, *H. pylori* is classified as type I carcinogen for stomach malignancies (Peek, Fiske & Wilson, 2010; Blaser, Atherton, 2004).

Virulence factors of varying strains of *H. pylori* are important hallmarks for developing different responses in the progression of stomach abnormalities. Stomach's low pH value inhibits growth of most ingested bacteria. It is ranging from

pH 2 to 6 from luminal surface to epithelial lining. Though acidity is a strong biological barrier of host, it is overcomed by *H. pylori*'s cytosolic or cell-surface related urease activity (Bauer & Meyer, 2011). Urease works by neutralizing the acidic environment by breaking down urea to release ammonia and carbonate. Therefore, the bacteria can persist in the stomach (Schoep et al., 2010)

H. pylori has different pathogenic factors in order to adhere and colonize in the first step, to evade the immune system at the second step, and invade into mucosa and damage at the last step (Sheu et al., 2010). Outer membrane proteins (e.g. Blood group antigen binding adhesin-BabA; Outer membrane inflammatory protein-OipA) help in the adherence of bacteria to the gastric epithelium. Flagellins (FlaA and FlaB) of *H. pylori* give bacterium its motility to move into gastric mucosa. After settling down, *H. pylori* leads to immune response attenuation and so persists with the help of other virulence factors such as cell wall component peptidoglycan (PGN) and lipopolysaccharide (LPS) (C. He et al., 2014). For invasion and damage to host, translocated effector proteins especially vacuolating cytotoxin A (VacA), and cytotoxin-associated gene A (CagA) work on modulation of host cell for the benefit of bacterium (Kim & Blanke, 2012).

H. pylori VacA virulence protein is the main cause of vacuolation of host cell. After it is internalized from membrane, it results in endosomal structures as vacuoles, which may interrupt trafficking inside host cell (Palframan et al., 2012). Moreover, VacA leads to apoptosis of host cells by interacting with mitochondria. It inhibits T cell response by directly binding of T cell integrin proteins (Polk & Peek, 2010).

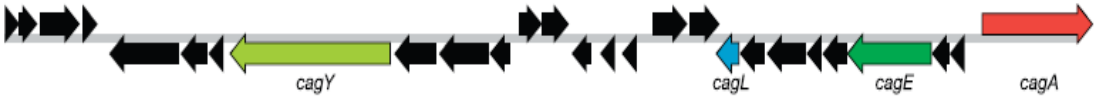

Figure 1.2 : Cag virulence factors on the cag-PAI region (adapted from Peek, Fiske & Wilson, 2010).

H. pylori virulence is mainly dependent to presence of cag pathogenicity island (cag-PAI) which encodes type IV secretion system (T4SS) (Figure 1.2). It constitutes a pilus from CagY protein, which interacts with host integrin β_1 protein together with CagL virulence factor, and cause conformational changes to inject CagA into host cell. CagE gives the energy needed for translocation of CagA by acting as an NTPase (Tegtmeyer et al., 2011; Peek et al., 2010).

CagA is an important virulence factor of *H. pylori*. More than half of the strains in the world have this factor. After CagA is translocated into the host cell, it can directly affect cellular responses such as; interacting with tight junctions, increasing cell number, changing polarity of cells, and activating inflammatory response. Also, it can go its phosphorylation state by Abl or Src family kinases, which they phosphorylate, from tyrosine aminoacids on Glu-Pro-Ile-Tyr-Ala (called as EPIYA) motifs. It ends with the change of host cell shape and motility (Jones, Whitmire &Merrell, 2010).

1.1.2. *Helicobacter felis*

Helicobacter felis (H. felis) is also a gram-negative and spiral shaped bacterium. It is first observed in the stomach of cats but the bacterium is capable of infecting other domestic animals, as well (Fritz et al., 2006). *H. felis* lacks of VacA and CagA virulence factors and has urease activity. It is highly used *Helicobacter* strain to study infection in mice to understand the effect of *Helicobacter* in gastric pathogenesis and role in other responses (Schmitz et al., 2011).

Helicobacter felis is used as an infection model in different studies to understand underlying mechanisms. Long term colonization of specific pathogen-free BALB/c mice with *H. felis* leads to stomach lesions similar to *H. pylori* related gastric B-cell lymphoma in humans (Enno et al., 1995). It has been shown with C57BL/6 mice that *H. felis* infection induces Th1 type response with IFN-γ production. This IFN-γ release from Th1 type cells is reversely correlated with bacterial colonization in the stomach (Sayi et al., 2009).

1.2 B Cells

B cells are one type of the immune player with a role in the respond to an antigen. They are also antigen - presenting cells (APCs), which interact and activate T cell by presenting antigen to T cell receptor (TCR). B cells express different surface receptors to make this interaction possible (Coico & Sunshine, 2009).

B lymphocytes are activated by antigens and primarily differentiate into plasma cells, which are known as antibody-producing cells, with the help of T cell engagement. Antigen is recognized by B cell receptor (BCR) which is composed of surface Immunoglobulin (Ig), two heavy chains and two light chains, together with Igα and

Igβ heterodimer (Chaturvedi et al., 2012). After that, downstream of BCR signaling is activated to induce gene expression related with antigen presentation. Major histocompatibility complex class II (MHC-II) molecules present specifically degraded antigen to T cell receptor (TCR). Activated T cell then triggers complete B cell activation through co-stimulatory interaction which is via CD40, TNF receptor superfamily member, on B cells and CD40L, TNF superfamily member, on T cells, and cytokine secretion (Figure 1.3) (Pierce, 2002).

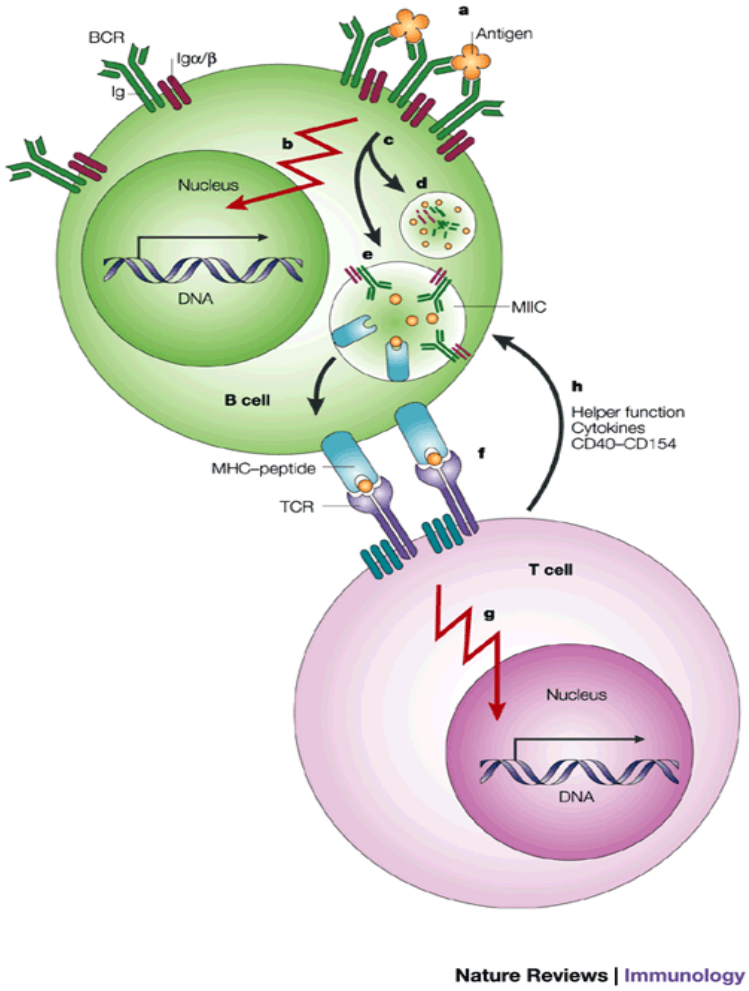

Figure 1.3 : B cell activation through T cell-dependent manner after antigen BCR ligation (adapted from Pierce, 2002).

B cells can also be activated without T cell engagement. T-cell independent type I antigens (e.g. LPS, CpG) bind Toll-like receptors (TLR) to activate B cells. On the other hand, T-cell independent type II antigens (e.g. repetitive sequences) are cross - linked to BCR to activate B cells. Primary B cell response and memory B cell response occur after type II antigens. B-1 and marginal zone (MZ) B cells are

responding to these T-cell independent antigens and produces IgM and/or IgG3 antibodies (Moresco, 2010).

1.3 B Cell Development and Types of B Cells

B cells can be divided into mainly B-1 and B-2 cell lineages (Figure 1.4). In order to protect from infection, B cells work with or without T cell help to differentiate into antibody - producing plasma cells or memory B cells (Dörnar, Radbruch & Burmester, 2009).

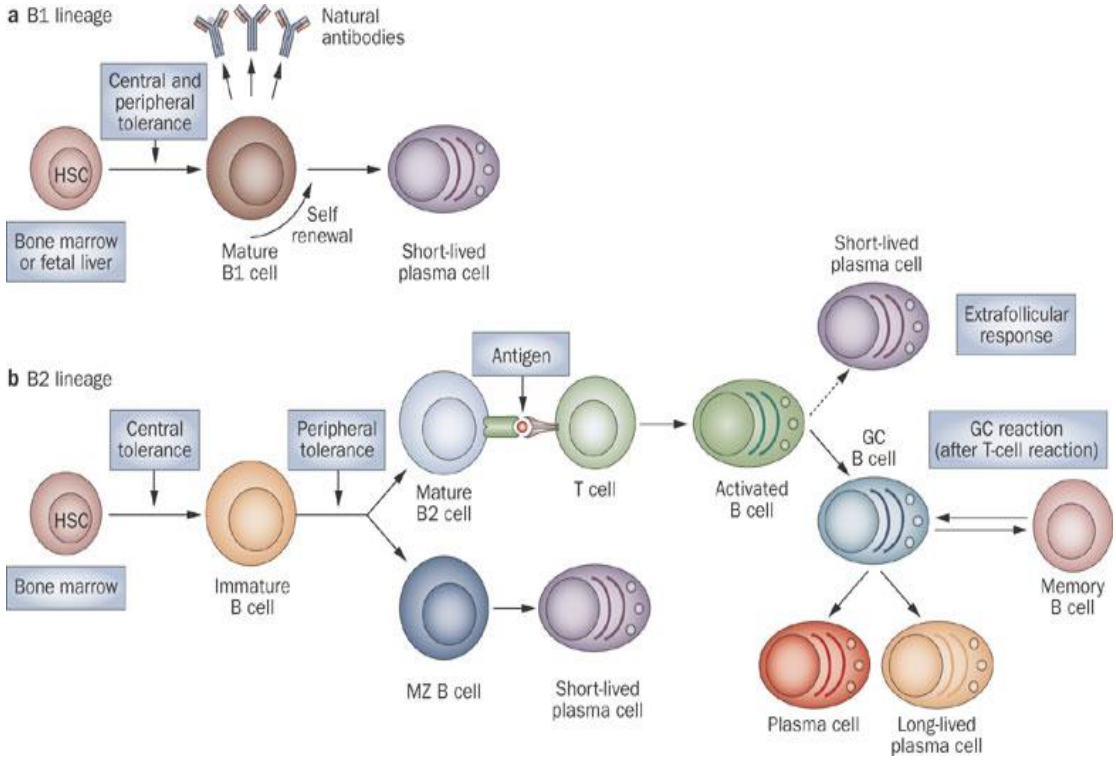

Figure 1.4 : A schematic drawing of B cell development (adapted from Dörnar, Radbruch & Burmester, 2009).

1.3.1 B-1 cells

B-1 cells are one of the sub-classes of B cells. Mouse B1 cells are mainly found in pleural and peritoneal cavities. They are also present in the spleen with a low frequency but have distinct expression molecules. B-1 cells are separated from B-2 type (marginal zone and follicular B cells) B cells by the expression of CD5 molecule (B-1a cells are $CD5^{+}$, B-1b cells are $CD5^{-}$) (Tumang et al., 2004). It is suggested that B-1 cells develop from fetal/neonatal precursors. Also these cells, but not B-2 cells can keep their frequency in the periphery in the lack of hematopoiesis in the bone marrow. B-1 cells produce natural IgM which is needed to protect from

microbial inflections e.g. influenza virus (Choi, 2008). Splenic B-1 cells show $CD19^{hi}CD1d^{mid}CD23^{-}IgM^{hi}IgD^{lo}$ phenotype and constitute around 2% of splenic B cells (Baumgarth, 2011).

1.3.2 Marginal zone B cells

Marginal zone (MZ) B cells are mainly non-circulating B cells which reside in marginal zone of spleen. They can be activated with polysaccharides of blood borne pathogens such as LPS through T cell independent manner (Zouali & Richard, 2011). Whereas they can be also induced by T cell - dependent manner. Binding of antigen to BCR and/or TLR induces MZ B cells to migrate into T cell zone where they can be activated in a T cell dependent manner. Because of the region that MZ B cells are present, they immediately respond to antigens and become mature to produce antibodies especially IgM and IgG3 (in mouse). Therefore, they are important first-defense players of the immune response (Figure 1.5) (DeFranco, Locksley & Robertson, 2007).

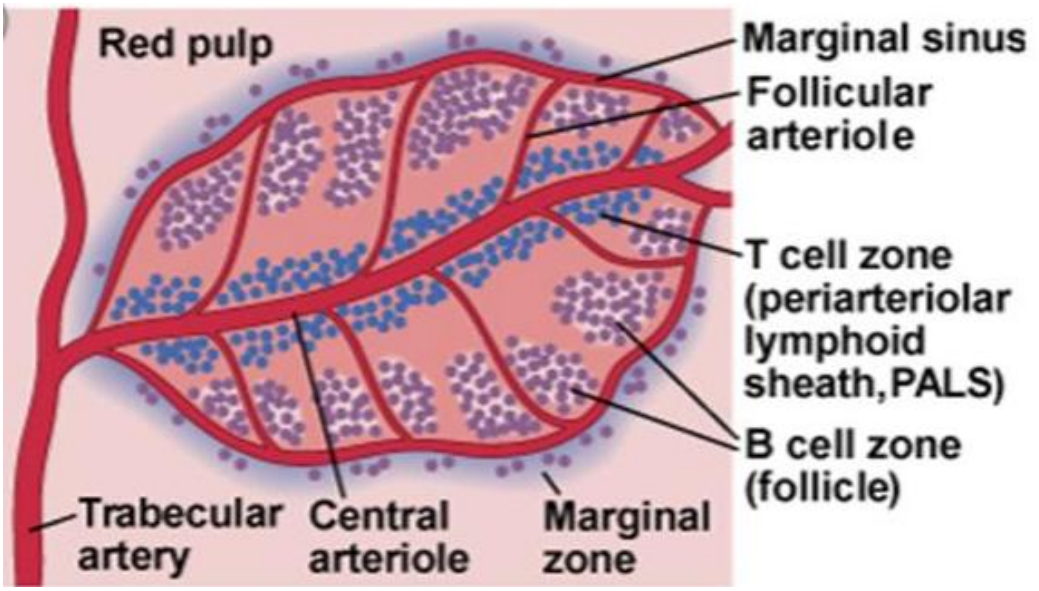

Figure 1.5 : A schematic illustration of spleen. (adapted from Url-1).

Newly emerged immature B cells are moved from bone marrow to spleen as transitional stage 1 (T1) B cells. Their characteristic phenotypes are identified as $CD19^{+}IgM^{hi}IgD^{-}CD21^{-}CD23^{-}$. T1 B cells are precursor of immature T2 B cells in the progression of mature B cells. T2 B cells have $CD19^{+}IgM^{hi}IgD^{hi}CD21^{int}CD23^{+}$

phenotype. In the maturation process, T2 B cells can differentiate into T2-MZ precursor B cells or follicular (FO) B cells. These T2-MZ precursor B cells have $CD19^{+}IgM^{hi}IgD^{hi}CD21^{hi}CD23^{+}$ phenotypic characteristics. After all, mature MZ B cells are distinguished with surface markers as $CD19^{+}IgM^{hi}CIgD^{lo}D21^{hi}CD23^{-}$ (Figure 1.6) (Khan et al., 2009). Moreover, they do not express CD5 but express CD1d surface molecules (DeFranco, Locksley & Robertson, 2007).

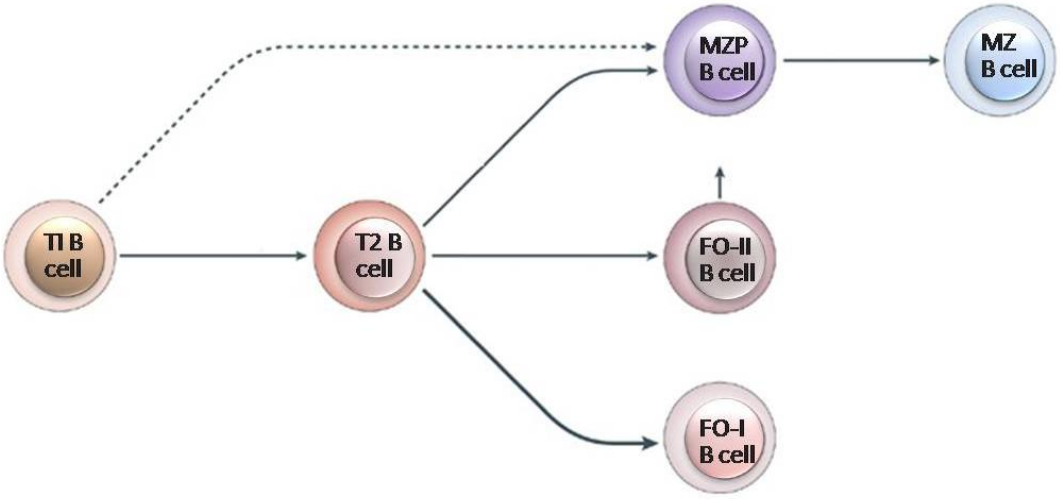

Figure 1.6 : MZ and FO B cell development (modified from Pillai & Cariappa, 2009).

1.3.3 Follicular B cells

Follicular (FO) B cells are one of the B cell types, which can circulate after maturation. They can migrate to their follicular niches in lymph nodes, Peyer's patches and spleen by re-circulating (Cheadle et al., 2012). T2 B cells can differentiate into FO B cells when they enter into follicles in the lymphoid organs. On the border of germinal center (GC), B cells are activated by antigen. By CD40-CD40L interaction with antigen activated T helper cells, FO B cells undergo differentiation process (Fillatreau & Gray, 2003). Afterwards, antigen engagement and T cell interaction lead to affinity maturation and isotype switching of B cells and also memory B cell differentiation. These cells become long-term plasma cells with high-affinity antibody production (Schneider et al., 2001).

FO B cells constitute around 75% of B cells in spleen. In the maturation process, transitional 2 B cells decrease IgM production and increase IgD production. To characterize FO B cells with their surface marker, it can be represented as

$CD19^{+}IgM^{mid}IgD^{hi}CD21^{int}CD23^{+}$. They do not produce CD5 marker, whereas they produce intermediate level of CD1d (Baumgarth, 2005).

1.3.4 Plasma cells

One of the main B cell players is terminally differentiated plasma cell. These cells play a significant role in the maintenance of long-term immunization against infection (Fooksman et al., 2010). In the beginning of plasma cell maturation, antibody-producing plasma cells are named as plasmablasts, which are given rise in lymph nodes (Caraux et al., 2010). They are immature and capable of division. After maturation of plasmablast with signals around, they become non-proliferating short-lived plasma cells in secondary lymphoid organs. These plasma cells are needed for immune response against acute infection and die after (Auner et al., 2010; Hoyer et al., 2004). On the other side, long-lived plasma cells are generated in the germinal center through the interaction of T cells and move to bone marrow (BM) to reside. These cells produce high amount of antibodies with strong affinity to its antigen (Fooksman et al., 2010).

Differentiation of plasma cells increases proteoglycan Syndecan-1 that is used as a marker to identify them. Also, B lymphocyte-induced maturation protein-1 (Blimp-1) transcription factor increases in plasma cells. However, some B cell development markers and transcriptional factors are downregulated to reach terminally differentiated form of these cells (Calame, 2001).

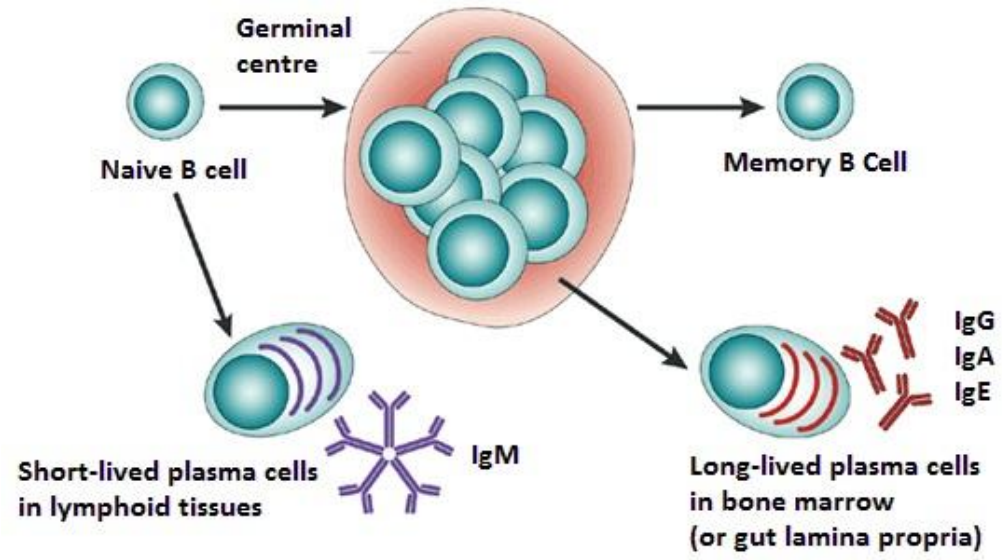

Figure 1.7 : Generation of memory and plasma cells (adapted from Gray, 2002).

In the plasma cell differentiation progress, naive B cells leave bone marrow and come splenic niche. Marginal zone B cells are activated with antigen differentiated toward plasma cells and move to extra follicular regions of periarteriolar sheath. These plasma cells are short-lived and die in a few days. They mostly produce low-affinity IgM antibodies. In long living plasma cells, follicular B cells are recruited GC of lymph nodes and spleen and so then undergo affinity maturation to produce high-affinity antibodies (Figure 1.7). They can live even after months after immunization (Calame, 2001; Wols, 2005).

1.3.5 Memory B cells

These B cell subsets are formed in germinal centers. After first infection, B cells differentiate into either plasma cells or memory cells. They are termed as long-lived IgD^- B cells. After activation of naive follicular B cells as T-cell dependent manner, they become memory B cells. These cells are important especially in second challenge of infection. They help to response quicker (Tangye & Tarlinton, 2009).

1.4 Immunoglobulins

Immunoglobulins (Igs) are types of glycoproteins, which are secreted from plasma cells. After activation of B cells with antigen, they go a differentiation process to produce low affinity or high affinity antigen-specific antibodies. Immunoglobulins are made of two heavy chains (α, δ, γ, ε, μ) and two light chains (κ, λ). They are named with their heavy chain structures (Figure 1.9) (Fleisher & Bleesing, 2002).

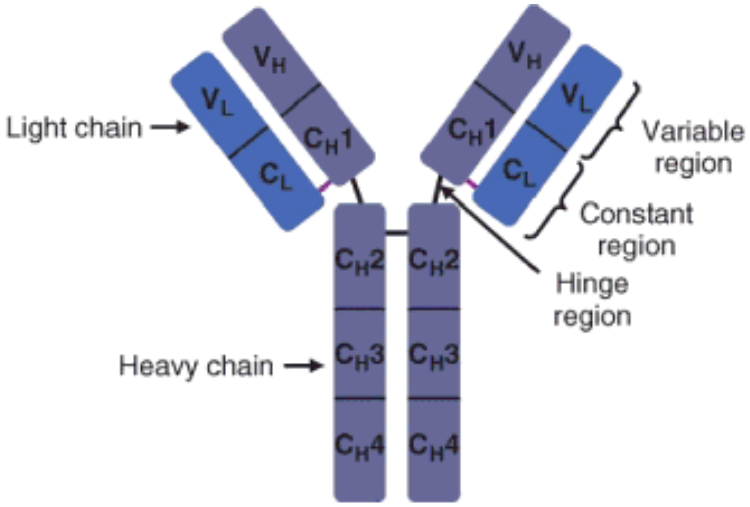

Figure 1.8 : Imunoglobulin structure (adapted from Williams, 2011).

Antigens are recognized specifically by immunoglobulin. Fab (fragment, antigen-binding) part of the immunoglobulins consists of one variable and one constant region. Fc (Fragment, crystallizable) region of immunoglobulins includes two heavy chains with 2 or 3 constant region parts. Fc parts lead to binding of antibodies its Fc receptors on immune cells (Figure 1.8) (Janewat et al., 2001).

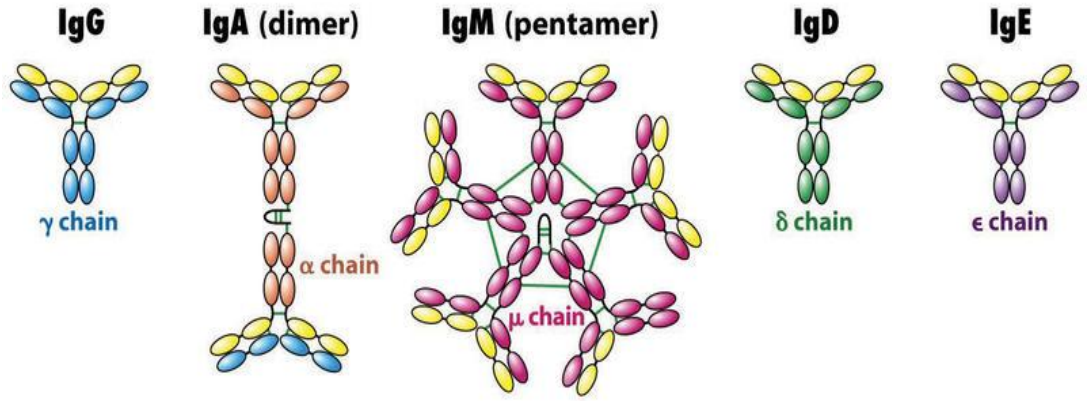

Figure 1.9 : Different types of immunoglobulins (adapted from Berg et al., 2007).

1.4.1 Immunoglobulin A

Immunoglobulin A (IgA) is one of the most found Ig in humans. It has a critical role in mucosal immunity. Secreted form of IgA is mostly dimeric or polymeric. Most of the IgA is produced by B-2 cells from germinal center of mucosa associated lymphoid tissues in a T-cell dependent manner. Others are derived from B-1 cells in peritoneal cavity (Kerr, 2000). IgA can also be in the serum. FcαR, which is specific for Fc regions of IgA, helps to obtain effector response against microbial infections (Snoeck, Peters & Cox, 2006).

1.4.2 Immunoglobulin D

Function of Immunoglobulin D (IgD) is not well studied yet. It is found on the surface of mature B cells together with IgM. Also, it can be secreted and its secreted form has roles on mucosal immunity and on innate immune cells. It is mentioned that IgD has specific function other than IgM. It has more tolerogenic roles according to the data that has been found. Additionally, IgD has microbial activity on respiratory mucosa associated microbes (Chen & Cerutti, 2011).

1.4.3 Immunoglobulin E

Immunoglobulin E (IgE) is found quite related with allergy and asthma. It is also responsible to respond against parasitic helminth infections (Bell, 1996). It binds to its receptor, FcεR. In allergy, it can activate mast cells to induce pro-inflammatory cytokines (Mayr et al., 2003).

1.4.4 Immunoglobulin G

IgG is one of the most effective immunoglobulin among the others. It can stay longer time after it is produced. IgGs have a role in activation of complement system for opsonization. There are four types of IgGs; IgG1, IgG2, IgG3, IgG4. These IgG subtypes have variable affinity to 3 classes of FcγR (I, II, III). IgG1 and IgG3 can bind all three classes. On the other hand, IgG4 can interact with FcγRII and FcγRIII and IgG2 can bind FcγRII (Schroeder & Cavacini, 2010)

Mice can produce IgG1, IgG2a, IgG2b and IgG3. A few strains can produce IgG2c, as well. IgG2b uses classical pathway for the activation of complement system, whereas IgG1 and IgG2a use alternative way (Quimby & Luong, 2006).

1.4.5 Immunoglobulin M

In the development of B cell process, Immunoglobulin M (IgM) is the first antibody present on B cells. IgM is found as its monomer form on the surface of B cells. However, it gathers together to its pentameric form to be secreted. Its role on antigen is opsonization of antigen and complement fixation (Firestein et al., 2012). These antibodies are secreted shortly after antigen activation. Natural IgMs are also found in first line defense of immune response. In mouse, around 80% of IgM is derived from B-1 cells (Grönwall et al., 2012).

1.5 Phenotypic Markers

1.5.1 CD1d surface marker

Antigen - presenting cells (APCs) can express CD1d to present exogenous or endogenous lipid antigens (Bosma et al, 2012). It is found that $CD19^{+}$ IL-10 producing B cells can express high levels of CD1d surface marker. However, both marginal zone B cells and transitional 2 marginal zone precursor B cells can include $CD1d^{hi}$ subpopulations (Kalampokis et al., 2013).

1.5.2 CD5 surface marker

CD5 is a glycoprotein, which is mainly found on T lymphocytes. In B cells, activation of CD5 occurs in the presence of antigens. It defines a distinct B cell population. B-1 (B-1a cells are $CD5^+$, B-1b cells are $CD5^-$) and B-2 ($CD5^-$) cell lineages differ from each other with CD5 expression. Surface expression of CD5 is thought to be an activation marker for B cells (Kaplan et al., 2001).

1.5.3 CD21 surface marker

It is a part of complement system of B cells. CD21 have a role in B cell activation and maturation. It is only found on mature B cells not pre-/pro- B cells. It is found on different types of immune cells such as B cells, T cells, follicular dendritic cells etc (Schwab & Illges, 2001).

1.5.4 CD23 surface marker

CD23 is a glycoprotein, which is a low-affinity receptor for immunoglobulin E. It is expressed by T cells, B cells, polymorphonuclear cells, monocytes, follicular dentritic cells etc. It has a role on differentiation step of B cells with different stimuli (Acharya et al, 2010).

1.6 Regulatory B Cells

Initially, B cells have been thought only to have a role on activation of immune response. In 1974, it was first mentioned that B cells have suppression capability on delayed-type hypersensitivity in guinea pig. After 30 years, regulatory B cell (Breg) concept was defined by Mizoguchi and Bhan (Yang et al., 2013). These regulatory cells can be compared to T cells with regulatory phenotype by the production of Interleukin-10 (Tr-1) and Transforming growth factor-β (Th-3). Similarly, B cells are also identified with the production of these two cytokines (Noh & Lee, 2011).

1.7 Interleukin-10 and IL-10 Producing Regulatory B Cells

Interleukin-10 (IL-10) is a cytokine, which has been shown to inhibit pro-inflammatory responses. IL-10 can be produced by Th2 cells, monocytes, macrophages, B cells, eosinophils, and mast cells (Asadullah, Sterry and Volk, 2003).

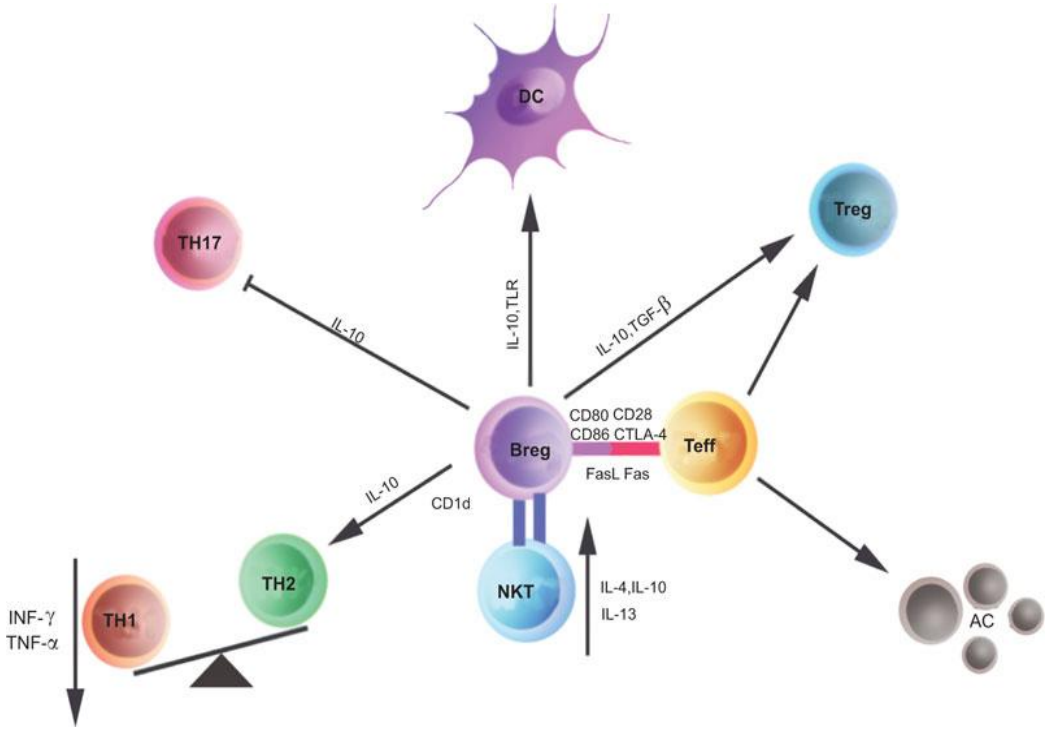

Figure 1.10 : Immune functions of regulatory B cells (adapted from Yang et al., 2013).

Regulatory B cells works on Th1/Th2 balance with the help of IL-10. It also inhibits Th1 and Th17 response while inducing $Foxp3^{+}$ Treg cells and IL-10 producing Tr1 cells. In addition, they inhibit induction of dendritic cells and macrophages (Figure 1.10) (Yang et al., 2013).

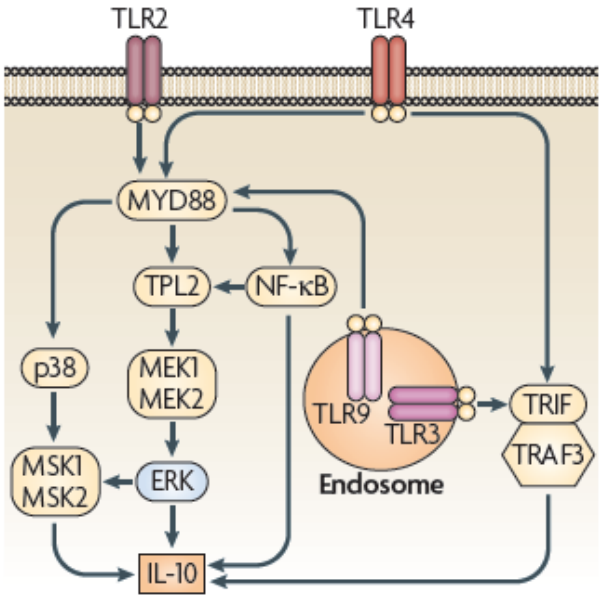

Figure 1.11 : Signal pathways to induce IL-10 production from macrophages and myeloid dendritic cells (adapted from Saraiva & O'Garra, 2010).

IL-10 production from macrophages and myeloid dendritic cells after TLR2 and TLR4 ligand engagement were found to be through p38, ERK or NF-κB according to Figure 1.11 (Saraiva & O'Garra, 2010).

Regulatory B cells, which are producing IL-10, were studied in different disease models. It has been suggested that they suppress progression of diseases such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), colitis, asthma etc. and ameliorate them in murine models (Y. He et al., 2014).

1.8 Phenotypic Identification of IL-10 Producing Regulatory B Cells

B cells with regulatory function in various disease models have been shown to have different phenotypic characters. These subsets of regulatory B cells have been assessed to understand IL-10 producing B cells with specific marker in mice and human (Mauri & Bosma, 2012).

In mice studies, $CD19^{+}CD1d^{hi}CD5^{+}$ regulatory B cell group was shown in colitis (Mizoguchi et al., 2002), systemic lupus erythematosus (Watanebe et al., 2010) and contact hypersensitivity models. This B cell subset was named as B10 cells (Yanaba et al., 2008). Moreover, another subset of B cells has been shown by Mauri and co-workers. CD40 stimulation induced IL-10 secretion from $CD21^{hi}CD23^{+}$ splenic B cells, which are similar to transitional type 2 characteristics (Gray & Gray, 2010). This subset has been also shown in lupus model (Blair et al., 2009). In arthritis mice, number of T2-MZP B cells increase in the remission phase. Additionally, in collagen-induced arthritis DBA/1 mice model, adoptive transfer of T2-MZP B cells decreased disease symptoms and prevented the disease (Evans et al., 2007). Another B cell subset, MZ B cells have been shown to produce IL-10 *in vitro* in the presence of CD40 or TLR9 ligand CpG (C-phosphate-G). However, transfer of MZ B cells to CIA or lupus-like disease mice model did not ameliorate disease (Mauri & Bosma, 2012). On the other hand, TIM-1, a surface marker, has been shown to be enriched for IL-10 production on B cells of BALB/c mice (Ding et al., 2011). As an infection model, *Leishmania major (L. major)* were used to identify regulatory B cells in BALB/c mice. IL-10 producing B cell was isolated after *L. major* stimulation. It has been shown that they were mainly $CD1d^{+}CD5^{+}CD21^{lo}CD23^{lo}$ (Ronet et al., 2010).

In humans, two main subsets of IL-10 producing B cells were found to have suppressive effect. $CD38^{hi}CD24^{hi}$ transitional B cells and $CD24^{hi}CD27^{+}$ memory B cells have capability to inhibition through IL-10 production (Y. He et al., 2014).

1.9 Regulatory B Cell Activation and Differentiation

TLRs are important molecules for activation of immune response against infection and autoimmunity. They have shown to induce regulatory response in different disease types. CpG and LPS treatment could suppress diabetes, arthritis and EAE in mice models (Buenafe & Bourdette, 2007; Quintana et al., 2000; Wu et al., 2007). In the course of regulatory B cell function through TLRs, *in vitro* and *in vivo* studies explain importance of these molecules. MZ B cells can produce IL-10 when induced with TLR2 and TLR4 ligands and increase IL-10 production with CD40 engagement. On the other side, FO B cells can produce pro-inflammatory cytokine by these TLR2 and TLR4 stimulation (Gray et al, 2007). Moreover, $CD1d^{hi}CD5^{+}$ B10 cell population arose after LPS treatment of B cells (Yanaba et al., 2008). CpG induced B cells increased IL-10 expression by TLR9 engagement (Barr et al., 2007). In order to understand the role of TLRs on the suppression effect of regulatory B cells, B cells, which were deficient for TLRs' adaptor protein MyD88 and TLR2 or TLR4, were studied. Mice showed exacerbated EAE (Lampropoulou et al., 2008). In addition, *Helicobacter felis* activates B cells through TLR2 and MyD88 pathway suppress Th1 cell responses in *Helicobacter* mice infection model (Sayi et al., 2011).

CD40-CD40L interaction between B cell and T cell are also found as required for regulatory B cell activation in some models. In a mice model, B cell - deficient for CD40 could not suppress EAE and showed increased level of Th1 and Th17 response (Fillatreau, 2002). Additionally, MRL/lpr mice, which is a model for systemic lupus erythematosus, have a decreased number of regulatory B cells. Disease was ameliorated by administration of agonistic CD40 mAb (Mauri & Bosma, 2012).

B cell receptor signaling is critical for regulatory B cell development. In a transgenic mice model with a deficient BCR activity, number of Breg cells are reduced reduced. Also, CD19 - deficient murine model that lowers BCR activity, showed decreased regulatory B cell amount (Kalampokis et al., 2013)

1.10 Immune Function of *Helicobacter*-Activated B cells

In the previous studies, it has been shown that, *Helicobacter*-induced gastric pathologies are diminished with B cells through their regulatory function on T cells. IL-10 - producing *Helicobacter*-activated B cells have a distinct role on IL-10 producing Tr-1 cell conversion. These Tr-1 cells together with activated B cells are responsible for suppression of inflammation. It occurs with direct interaction of B and T cells and also with the effect of released cytokines (Sayi et al., 2011).

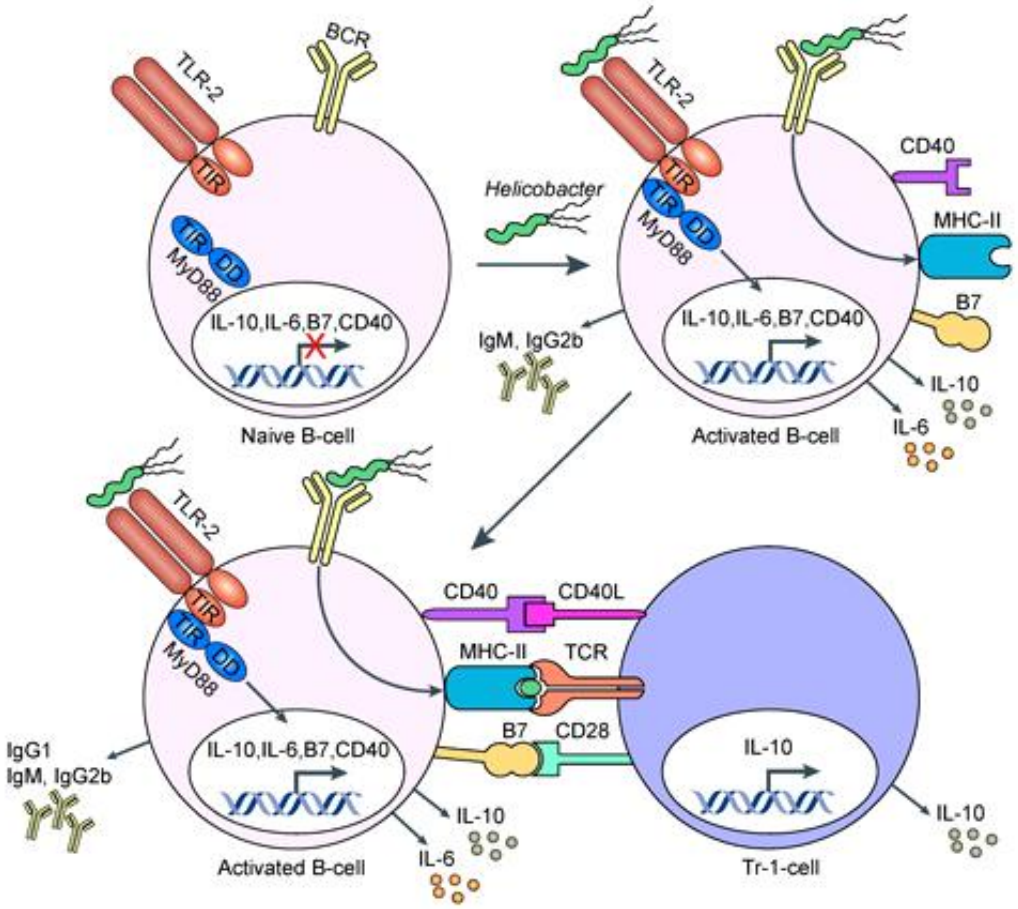

Figure 1.12 : Schematic representation of Tr-1 cell activation through *Helicobacter*-activated B cells (adapted from Sayi et al., 2011).

Helicobacter felis activates B cells through TLR2 and MyD88-dependent manner. These activated B cells up-regulate CD40, MHC-II and other co-stimulatory molecules and also production of IL-10, IL-6, IgM and IgG2b (Figure 1.12) (Sayi et al., 2011).

1.11 Transforming Growth Factor-β and TGF-β Producing Regulatory B cells

Transforming growth factor-β (TGF-β) is another cytokine, which is shown to have regulatory function on cell response, proliferation and differentiation. It mainly has a role on inflammation, and tolerance. It was first identified with the growth of fibroblasts. After that, it is defined as a suppressor molecule for lymphocyte activation. Its activation occurs by converting its latent form to its active form. Therefore, it is an important regulatory factor for survival (Taylor, 2009).

It has been reported that T cell proliferation is suppressed by regulated cell cycle activity through TGF-β. Furthermore, pro-inflammatory cytokines such as IFN-γ was regulated by this cytokine. It helps to differentiation of $Foxp3^+$ Treg cells, as well (Wrzesinski et al., 2007). Therefore, TGF-β leads to inhibition of Th1 and Th2 cells and also blockade of cytotoxic T cell activation. On the other hand, it triggers Treg differentiation together with Th17 activation. The other effect of this cytokine together with IL-10 and IL-21 is induction of CD40-activated B cells to IgA^+ plasma cells and memory B cells (Figure 1.13) (Banchereau, Pascual & O'Garra, 2012)

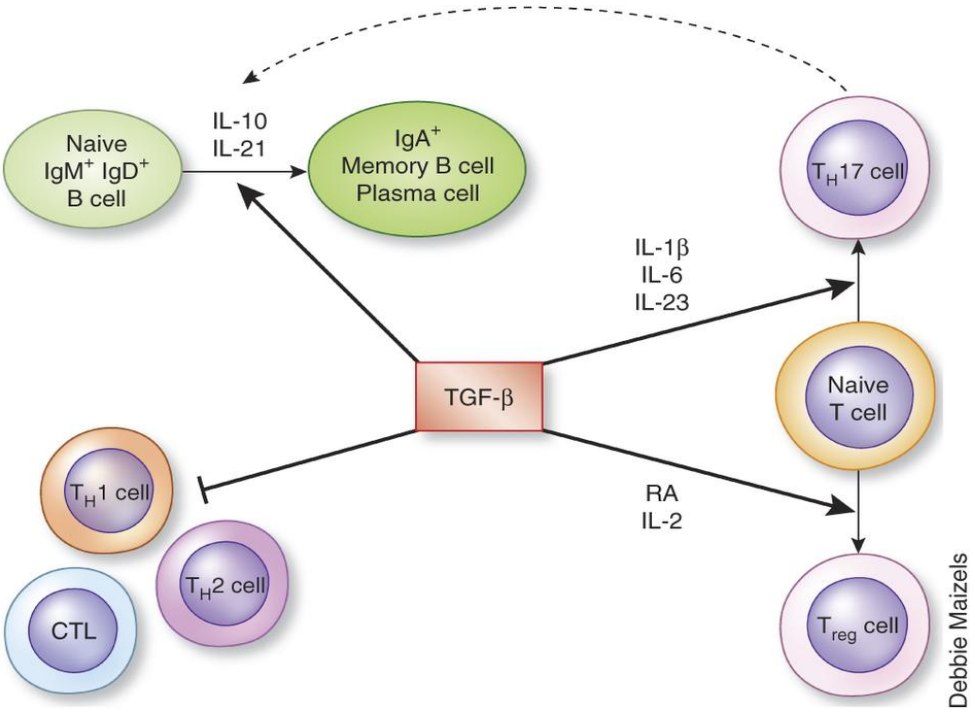

Figure 1.13 : Role of TGF-β on immune cell function (adapted from Banchereau, Pascual & O'Garra, 2012).

In order to assess the role of TGF-β, TGF-β - knockout mice were used in different studies. In the absence of this cytokine, mice showed multifocal inflammatory

disease with the production of high levels of inflammatory cytokine. Also, T cell derived TGF-β - deficient mice resulted with lethal immunopathology with increased T cell activity (Wan & Flavell, 2010). Similar to T cells, B cells can also produce TGF-β. These TGF-β - producing B cells are named as Br3 cells. Regulatory roles of Br3 cells have been reported in systemic lupus erythematosus (Raymond et al., 1997), diabetes (Tian et al, 2001), and it has been also reported in non-IgE mediated allergy response in atopic dermatitis (Noh & Lee, 2011).

1.12 Signaling through Nuclear Factor kappa B

Nuclear factor kappa B (NF-κB) is a transcription factor, which regulates genes. Its activation is closely linked to inflammation, cancer development and autoimmunity. Various viral and microbial ligands can activate NF-κB to induce cytokine secretion and other immunological functions. Therefore, it is an important player of innate and adaptive immune response (Oeckinghaus & Ghosh, 2009).

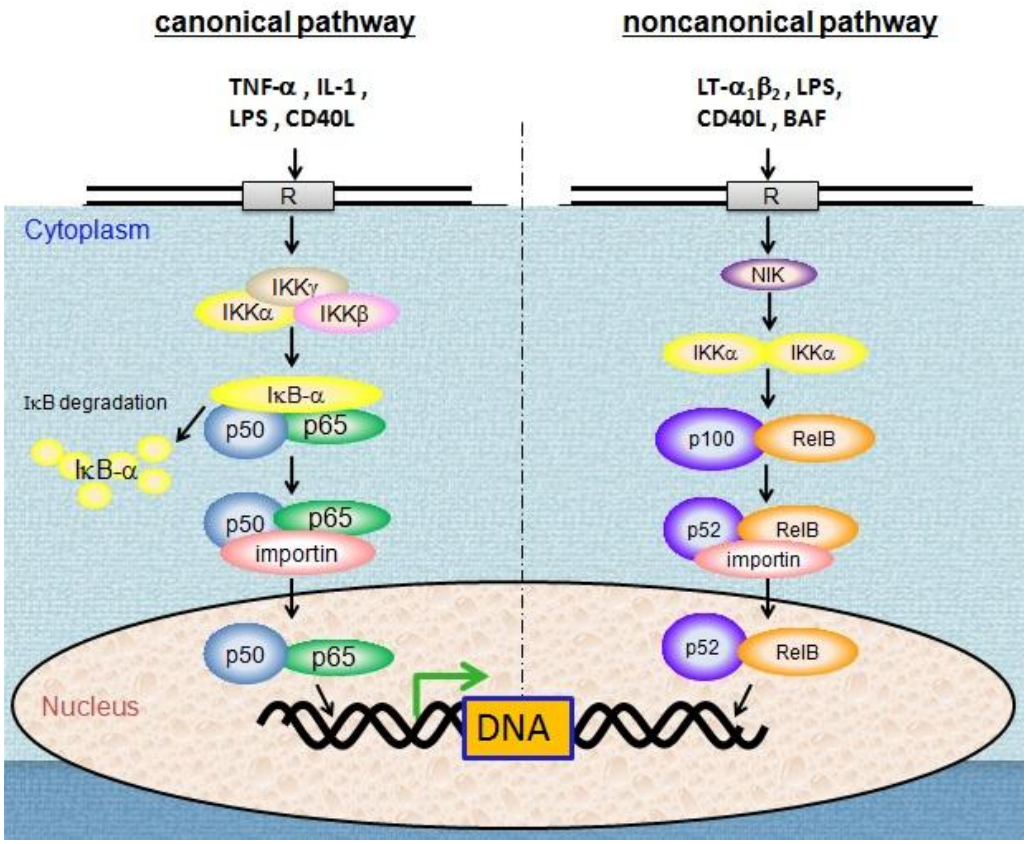

Figure 1.14 : Activation of two NF-κB pathways (adapted from Horie & Umezawa, 2012).

Canonical pathway includes p65/p50 activation through different signals. This complex is kept its inactive form by IκB inhibitory molecule. After induction with a stimuli, IκB kinase complex (IKK) α and β, phosphorylate IκB to induce its degradation. Released p65/p50 complex translocate to nucleus for the activation of inflammatory response (Figure 1.14) (Mincheva et al., 2011).

In non-canonical pathway, p100 molecule is degraded to activate p52/RelB complex. After induction of TNF family members, NF-κB-inducing kinase (NIK) activates IκB kinase-α (IKKα) to induce p100 phosphorylation. After that, it is processed and leads to release of p52/RelB. It goes to nucleus to activate its related genes. This type of activation is slow and persistent (Figure 1.14) (Sun, 2011).

Induction of NF-κB leads to production of IL-1beta, IL-2, IL-6, IL10 cytokines, some cell adhesion and activation molecules (Horie & Umezawa, 2012).

1.13 Aim of the Study

Helicobacter pylori can persist in a balance with its host in the stomach for long time. This long-term relation leads to activation of immune response and changes in the characteristics of epithelial cells. *Helicobacter* can induce regulatory cells, as well, which has been shown in a few years. B cells can produce IL-10 after *Helicobacter* induction, and these B cells with regulatory role can induce T cells to Tr-1 cells. Therefore, suppression of pro-inflammatory response becomes an important subject to understand role of regulatory cells in chronic inflammations.

In our study, *Helicobacter*-activated IL-10^{+} and also IL-10^{-} B cells will be examined in detail. Their phenotypic characterization through surface marker expression analysis, and possible immunoglobulin secretions will be analyzed. Also, TGF-β expressions and productions from these two subsets will be examined. Finally, role of NF-κB p65 subunit on IL-10 production will be studied to characterize these regulatory B cells.

2. MATERIALS & METHODS

2.1 Materials

2.1.1 Bacteria

Helicobacter felis (H. felis) strain was kindly provided by Prof. Dr. Anne Müller from University of Zurich. Bacteria were spreaded on Columbia Agar plates supplemented with 1000X antibiotic cocktail. Ingredients of Columbia Agar (BD, U.S.A.) plate and 1000X antibiotic cocktail were given in Table 1 and Table 2, respectively. Solutions and chemicals that are used in maintenance of *Helicobacter felis* are given in Tables 2.1 and 2.2.

Table 2.1 : Components of Columbia Agar Plates.

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

Table 2.2 : Components of 1000X Antibiotic Cocktail.

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

2.1.1.1 Antibiotics

Antibiotics that are used in *Helicobacter felis* Columbia agar plate are listed in Table 2.3.

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

Content	*Supplier Company*
Trimethoprim	HiMedia
Amphotericin B	HiMedia

2.1.1.2. Liquid culture

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

Table 2.4 : Liquid culture of *Helicobacter felis*

Content	*Amount*
Brucella Broth Media	100 ml
FBS [10% (v/v)]	10 ml
Vancomycin (1000X)	10 µl

2.1.1.3. Freezing of *Helicobacter felis*

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

Table 2.5 : Freezing medium for *Helicobacter felis*.

Component	*Amount*
Brucella Broth	25 ml
Glycerol	25 ml

2.1.2. Primary Cells

$CD19^+$ splenic naive B cells were obtained from C57BL/6 mice via magnetic separation (Macs Miltenyi, Germany). Primary B cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, 1% Penicillin/Streptomycin.

2.1.3 Cell lines

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

2.1.4 Cell Culture

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

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

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

Table 2.7 : Buffers used in cell culture studies.

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

2.1.5. ELISA & Immunofluorescence

Solutions that were used in IL-10 ELISA, Antibody isotyping, TGF-β ELISA and immunofluorescence studies are given in Table 2.8.

Table 2.8 : Solutions used in ELISA and immunofluorescence experiments.

Solution	*App.*	*Amount*
PBS/T 1X (0.05%)	IL-10 ELISA	0.05% Tween-20 in PBS 1X
Stop Solution	IL-10 ELISA	2N H_2SO_4 in ddH_2O
Wash Buffer (1X)	Ab Isotyping	30X wash buffer in ddH_2O
PBS/T 1X (0.1%)	Immunofluorescence	0.01% Tween-20 in PBS 1X
Permeabilization Solution	Immunofluorescence	0.5% Triton X-100 in PBS 1X
Blocking Solution	Immunofluorescence	10% goat serum in PBS 1X
Wash Buffer (1X)	TGF-β ELISA	25 X wash buffer in ddH_2O
Activation reagents	TGF-β ELISA	1N HCL 1.2N NaOH/0.5M HEPES

2.1.6 Equipment

Laboratory equipments, which were used in this study, are shown in Table 2.9 with their suppliers.

Table 2.9 : Laboratory equipment used in this study.

Equipment	*Supplier Company*
Laminar Air Flow Cabinets	FASTER BH-EN 2003
Pipettes	10 µl, 20 µl, 100 µl, 200 µl, 1000 µl

	Socorex and 10 µl, 100 µl, 1000 µl Biohit
Electronic Pipette	CappAid
Centrifuges	Beckman Coulter Allegra ™ 25 R Centrifuge Scanspeed 1730 R Labogene Scanspeed mini
Incubator with CO_2	BINDER
Vortex	Mixer Uzusio VTX-3000L,LMS
Quick spin	LMS
Magnetic stirrer	WiseStir MSH-20D, Wisd Laboratory Equipment
Light Microscope	Olympus CH30
Hemacytometer	Isolab
Ice Machine	Scotsman AF10
Freezers	Altus (+ 4 ^{0}C) Siemens (-20 ^{0}C) Haier (- 80 ^{0}C)
Nanodrop 2000	Thermo Scientific
Shakers	Heidolp Duomax 1030
Flow Cytometer	BD Accuri C6
Step One Real Time Systems	Applied Biosystem
Confocal Microscopy	Leica SP2
Sonicator	Bandelin Sonopuls
Nitrogen Tank	Air Liquid
Microplate Spectrophotometer	BIO-RAD Benchmark Plus
Tissue flask	Sarstedt
Serologic pipettes	Dispenser
Centrifuge tubes	Interlab
Eppendorf tubes (0.6 ml, 1.5 ml, 2 ml)	Interlab
Scale	Precisa
Examination Gloves	Beybi
Cell strainer (70 µm)	BD
Tissue culture flasks (25 cm^2, 75 cm^2)	Sarstedt
Culture plate (6-well, 12-well, 96-well U bottom, 96-well F bottom)	TPP
Syringe filter (0.2 µm pore size)	Millipore
Serological pipette (5 ml, 10 ml, 25 ml)	Sarstedt
Syringe	Set Inject
Anaerobic Jar	Anaerocult
Erlens	Isolab
Falcons (15 ml, 50 ml)	Isolab
Slides	Knittel Glass Interlab
Coverslips	Knittel Glass Interlab
Cotton Swap	Interlab
96-well F plate (for ELISA studies)	Nunc

2.1.7 Commercial Kits

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

Table 2.10 : Commercial kits used in this study.

Kit	*Supplier Company*
Mouse B Cell Isolation Kit	MACS, Miltenyi Biotec
Mouse Regulatory B cell Isolation Kit	MACS, Miltenyi Biotec
Innu-Prep RNA Mini Isolation Kit	Analytic jena, Biometra
High capacity cDNA syntesis Kit, 200 rxns	Applied Biosystems
Power SYBR® Green PCR Master Mix	Applied Biosystems
BCA™ Protein Assay Reagent Assay	Thermo Scientific
Pierce Rapid ELISA Mouse Antibody Isotyping Kit	Thermo Scientific
Mouse IL-10 Deluxe Max ELISA	Biolegend
Mouse TGF-beta ELISA	R&D

2.1.8 General Chemicals

General chemicals used in this study are listed with their supplier companies in Table 2.11.

Table 2.11 : General chemicals used in this study.

Chemical	*Supplier Company*
EDTA	Applichem
Ethanol (absolute)	Merck
NaCl	Merck
Glycerol	Merck
Phosphate-Buffered Saline (PBS)	Biochrome
Tween-20	Fisher-Scientific
Bovine Serum Albumin (BSA)	Santa Cruz
DMSO	Fisher Scientific
β-Mercaptoethanol	Sigma-Aldrich
DAPI	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
HCl	Sigma-Aldrich
NaOH	Sigma-Aldrich
Mounting media	Sigma-Aldrich
Triton-X 100	Sigma-Aldrich
Poly-L-Lysine	Sigma-Aldrich
Columbia Agar	BD

Brucella Broth	BD
CampyGen 2.5L	Oxoid
Fixation buffer	Biolegend

2.1.9 Primers

Primers that were used in this study are given in Table 2.12.

Table 2.12 : Primers and their sequences used in this study.

Primer Name	*Sequence (5'-3')*	*Spec.*	*Tm*	*Exp. size*
IL-10 fw	GAGGCGCTGTCATCGATTTCT	m	64°C	103 bp
IL-10 rv	GGCCTTGTAGACACCTTGGTC	m		
TGF-β fw	TACAGGGCTTTCGATTCAGC	m	60°C	247 bp
TGF-β rv	CGCACACAGCAGTTCTTCTC	m		
18s rRNA fw	GGCCCTGTAATTGGAATGAGTC	m/h	59°C	146 bp
18s rRNA rv	CCAAGATCCAACTACGAGCTT	m/h		
GAPDH fw	CCACATCGCTCAGACACCAT	m/h	55°C	114 bp
GAPDH rv	GGCAACAATATCCACTTTACCA GAGT	m/h		
Oligo dT	TTTTTTTTTTTTTTTTTTT	-	-	-

2.1.10 Antibodies

Antibodies that were used in this study are given in Table 2.13.

Table 2.13 : Antibodies and inhibitors used in this study.

Antibody/Inhibitor	*Clone*	*Supplier Company*	*Application*
Rat anti-mouse CD19-FITC	6D5	Biolegend	FACS
Rat anti-mouse IL-10-PE	Mouse Regulatory B Cell Isolation kit component	MACS Miltenyi	FACS
Rat anti-mouse CD1d-FITC	1B1	Biolegend	FACS
Rat anti-mouse CD5- Alexa Fluor® 647	53-7.3	Biolegend	FACS
Rat anti-mouse CD21/CD35-APC	7E9	Biolegend	FACS
Rat anti-mouse CD23-PE/Cy7	B3B4	Biolegend	FACS
Rabbit anti-NF-κB	93H1	Cell Signaling	IF
Anti-rabbit- Alexa Fluor® 488	-	Abcam	IF
PDTC	-	Tocris	Cell Culture

2.1.11 NF-κB inhibitor (PDTC)

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

2.2. Methods

2.2.1. Maintenance of *Helicobacter felis*

Helicobacter felis was seeded on a blood agar containing appropriate antibiotics and incubated at 37°C under microaerophilic conditions in an anaerobic jar for 3-4 days. Microaerophilic conditions in anaerobic jar were maintained by utilization of CampyGen packs. After 3-4 days, the grown bacteria was checked under light microscope for their viability and mobility and transferred into liquid Brucella Broth containing 10.000 X Vancomycin (working concentration : 1X) with necessary dilutions for optimal growth.

2.2.2. Sonication of *H. felis*

Sonication procedure was started with 120-200 ml liquid culture of *Helicobacter* strains. Before sonication *Helicobacter felis* (10 μl)'s mobility was checked under light microscope. 120- 200 ml liquid culture of *Helicobacter felis* was aliqouted to 15 ml falcons. Falcons were centrifuged at 3000 rpm for 10 minutes. Supernatant was discarded. 10 ml PBS was used to wash bacteria. 15 ml falcon was centrifuged at 3000 rpm for 7 minutes. Supernatant was discarded. 3.5 ml PBS was added on pellet and mixed. Tube was taken to sonication with ice.

Sonication was performed as 30 sec pulse on, 50 sec pulse off for 6.30 minutes at 50 watt. (MS 72 probe of the sonicator was used.) They were aliquoted to 1.5 ml eppendorfs at 500 μl for each. They were centrifuged at 4 °C, 5000 rcf (3000 rpm) for 10 minutes. Supernatant was taken to new eppendorf tubes and labeled. Sonicate concentration was measured with BCA assay.

2.2.3. BCA assay

The determination of protein concentration was performed using Thermo Scientific's Protein BCA Assay. The Bradford dye was diluted with distilled water at 1:4 ratio.

Bovine Serum Albumin (BSA) Standard Set was chosen for microassay. 1X BCA working reagent was prepared from Solution B and Solution A as 1:50 ratio, respectively and was warmed to ambient temperature. 200 µl of working reagent was distributed into each assayed well of a 96-well plate, and all the samples were duplicated to confirm linear range of standards and to get more accurate results. 10 µL of diluted BSA standards in duplicates were put into working reagent-containing wells with the concentrations of 0,025; 0,125; 0,25; 0,5; 1; 1,5; 2 mg/mL, respectively. Dilution scheme for BSA standards are given in Table 2.15. 10 µL of protein samples (diluted of undiluted) with unknown concentrations were put into working reagent-containing wells, and microplate was incubated at 37°C for at least 30 min. After 30 min-long incubation, absorbances were measured at 562 nm on microplate reader.

Table 2.14 : Dilution scheme for BCA Assay standards.

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

2.2.4. Treatment of cells with *Helicobacter felis* sonicates

Purified B cells were treated with *H. felis* sonicates with a final concentration of 10 µg/ml. They were incubated with or without *H. felis* sonicate for 24 h. After 24 h-long incubation, IL-10 producing regulatory B cell subset was magnetically labeled and separated from *H. felis* sonicate-treated cells by using Mouse Regulatory B Cell Isolation Kit (MACS Miltenyi, Germany).

2.2.5 MACS Mouse B cell isolation

2.2.5.1 Cell Preparation

Spleen of C57BL/6 mice was meshed on a 70µm filter mesh with the plunger of a syringe and single cell suspension of the spleen was prepared in a 50 ml falcon tube.

Spleen from C57BL/6 mice was put in the center of filter carefully by the help of a tip. Incomplete RPMI medium was used to soak filter and wash cells from filter to the tube. Procedure was repeated for all the spleens. Tube was filled with incomplete medium. Cell suspension was centrifuged at 1480 rpm (200g) for 10 min. Supernatant was discarded. Cell pellet was dissolved in 1 ml MACS buffer per spleen. Cell number was determined. Cells were counted by diluting with MACS buffer (~1:200). Cell viability was checked with trypan blue.

2.2.5.2 Pre-enrichment of B cells

100 µl of B Cell Biotin-Antibody Cocktail was added per 10^8 cells. Tube was mixed well and incubated at refrigerator for 15 min. 300 µl of MACS buffer and 200 µl of Anti-Biotin MicroBeads were added per 10^8 total cells. Tube was mixed well and incubated in refrigerator for 15 min. Cells were washed with 1-2 ml per 10^7 cells. Cell pellet was resuspended in 500 µl of MACS buffer per 10^8 cells.

2.2.5.3 Magnetic Separation: Depletion of non-B Cells

LS column was placed in suitable MACS separator (Midi) on magnetic field. Column was prepared by rinsing with 3 ml of cold MACS buffer. Cell suspension was applied onto the column. Column was washed by 3x3 ml of MACS buffer and it was always waited until reservoir is empty between washing steps. Flow through was unlabeled B cells. Column was removed from magnetic field and put in a 15 ml falcon tube. The magnetically labeled non-B cells were flushed out with 5 ml of MACS buffer by firmly pushing the plunger (Figure 2.1). Cell number was determined by diluting 1:10 with MACS buffer and cell viability was checked with trypan blue under light microscope. Flow cytometer analysis for CD19 surface marker was performed in order to determine purity.

2.2.5.4 CD19 staining

Purity of purified splenic B cells was determined using flow cytometer. $5x10^5$ B cells and depleted non-B cells were stained with 0.2 µl FITC conjugated anti-CD19 antibody (Clone 6D5, Biolegend) in 50 µl FACS Buffer in the dark on ice for at least 45 minutes. A fraction of B cells were left as unstained controls. Then, cells were washed once with 1 ml FACS Buffer by centrifugation at 3000 rpm for 8 minutes.

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

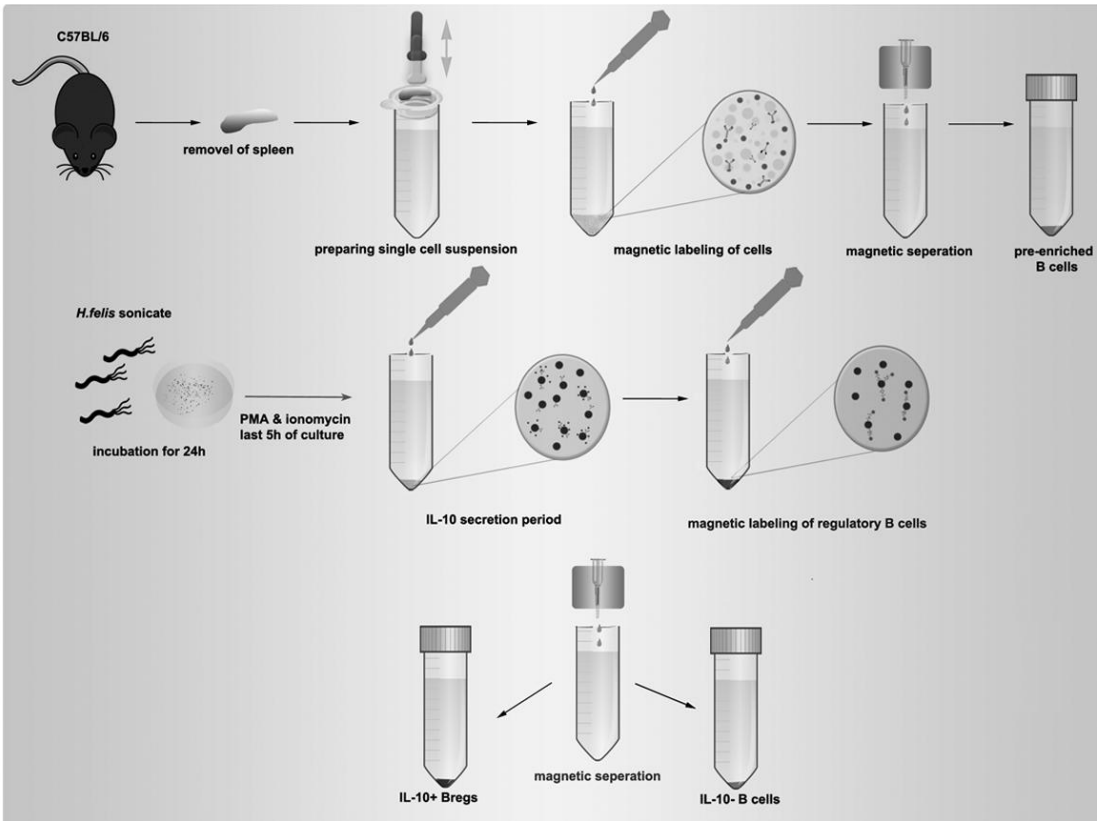

Figure 2.1 : Steps for purification of murine splenic B cells and subsequent separation of B cells according to their IL-10-production capacities.

2.2.6 IL-10 producing regulatory B cell isolation

2.2.6.1 *In vitro* stimulation

Isolated $CD19^+$ B cells were centrifuged at 1480 rpm for 8 min. Cell pellet was re-suspended in proper amount of medium (2.5 x 10^6 cells/ml). Cells were incubated in 96-well U bottom plates with stimulation agent (*Helicobacter felis* sonicate) for 24 h long incubation (Final concentration: 10 µg/ml). Cells which were not stimulated with sonicate were kept as control B cells. 50 ng/ml PMA and 500 ng/ml ionomycin were added for the last 5 hours of incubation.

2.2.6.2 Labeling cells with regulatory B cell catch reagent

Cells were harvested by collecting into a falcon tube by pipetting up and down and checked if any cell were remained. Cell number was determined by diluting 1:10 with MACS Buffer. For cell viability check, trypan blue staining was done. Cells

were washed by adding cold buffer and centrifuged at 1480 rpm for 8 min. Supernatant was discarded carefully. Cell pellet was re-suspended in 90 µl of cold medium per 10^7 total cells. 10 µl of Regulatory B Cell Catch Reagent was added per 10^7 total cells. Tube was mixed well and incubated on ice for 5 min.

2.2.6.3 IL-10 secretion period

10 ml warm medium was added per 10^7 cells. Cells were incubated in closed tube for 45 min at 37 ^{0}C. Tube was turned upside down every 5 min to re-suspend cells.

2.2.6.4 Labeling cells with regulatory B cell detection antibody (PE)

The tube was filled with cold MACS buffer. The tube was incubated on ice for 5 min. in order to prevent non-specific antibody binding. Cells were centrifuged at 1780rpm for 8 min. Supernatant was discarded carefully. Cell pellet was re-suspended in 90 µl of cold buffer per 10^7 total cells. Unlabeled cells were separated as unstained control. 10 µl of Regulatory B Cell Detection Antibody (PE) was added per 10^7 total cells. Tube was mixed well and incubated for 15 min on ice. 10 ml of cold MACS buffer was added and centrifuged at 1780 rpm for 8 min. Supernatant was discarded carefully.

2.2.6.5 Magnetic labeling with Anti-PE microbeads

Cell pellet was re-suspended in 80 µl of cold buffer per 10^7total cells. 20 µl of Anti-PE MicroBeads were added per 10^7 total cells. Tube was mixed well and incubated for 15 min in the refrigerator. Cells were washed by adding 10 ml of cold MACS buffer per 107 total cells. Tube was centrifuged at 1780rpm for 8 min. Supernatant was discarded carefully. Cell pellet was re-suspended in 500 µl of cold MACS buffer.

2.2.6.6 Magnetic separation using MS column

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

out by plunger. Cell numbers were determined by diluting 1:10 with MACS buffer and cell viability was checked with trypan blue under light microscope. Flow cytometer analysis was performed for IL-10-PE in order to check the purity of IL-10 positive and IL-10 negative fractions. A fraction of B+*H.f.* cells before addition of PE-coupled IL-10 detection antibody was separated as unstained. A small fraction of cells were pelleted for further analysis (Figure 2.1).

2.2.7 Flow staining of surface markers

Isolated IL-10 positive and IL-10 negative fractions were stained for surface molecules. Three small fraction of each cell group was put new eppendorf tubes. One of them was stained for CD1d-CD5 and the other one stained for CD21-CD23.One tube was left as unstained control. Cells were re-suspended in 50 µl of 2% FACS buffer.0.2 µl of each anti-mouse CD1d and anti-mouse CD5 antibodies were added one tube of each fraction., 0.2 µl of anti-mouseCD21/CD35 and anti-mouse CD23 antibodies were added to second fraction. Cells were stained for 1h at 4°C. After that, cells were washed with 2% FACS buffer. They dissolved in 150 µl of 2% FACS buffer. Analyzed with flow cytometry.

2.2.8 Analysis of relative expression levels

2.2.8.1 RNA isolation

To determine relative expression levels of IL-10 and TGF-β, B cell pellets were used to isolate their RNA. 400 µl of RL lysis buffer was added to cell pellets and incubated at room temperature for 2 min. After, pellet was re-suspended and incubated for additional 3 min. Samples were taken to spin filter D tubes and centrifuged at 10.000 x g for 2 min. 70% of ethanol was added to flow through. After that, samples were taken to spin filter R. Tubes were centrifuged at 10.000 x g for 2 min. 500 µl of HS solution was added and centrifuged at 10.000 x g for 1 min. Then, 750 µl of LS solution was put on filter and centrifuged at 10.000 x g for 1 min. In order to get rid of ethanol from samples, tubes were centrifuged at 10.000 x g for 3 min. Spin filters were settled into new eppendorf tubes. 35 µl of RNAse-free water was added on filter and incubated for 1 min at room temperature. Finally, tubes were centrifuged at 6.000 x g for 1 min. Flow through was kept as isolated RNA. Their concentrations were measured with NanoDrop.

2.2.8.2 cDNA synthesis

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

Table 2.15 : Components of cDNA synthesis reaction.

Component	*Amount*
RNA (1µg)	depends on concentration
ddH_20	15.075 µl – amount of RNA
10 X RT Buffer	2 µL
Oligo dT (10µM)	1 µl
Ribolock Rnase Inh.	0.125 µl
Reverse Transcriptase	1 µl
25 X dNTP mix	0.8 µl

Table 2.16 : Reaction conditions of cDNA synthesis

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

2.2.8.3 Real time PCR

Relative expression levels of IL-10 and TGF-β was analyzed with Real - time PCR. Amounts used in reaction are given in Table 2.17. PCR conditions performed are given in Table 2.18.

Table 2.17 : PCR reaction components.

Component	*Amount*
Power Sybr Master Mix (2X)	5 µl
Frw Primer (10 µM)	0.5 µl
Rev Primer (10 µM)	0.5 µl
PCR Grade water	1.5 µl

Table 2.18 : Reaction conditions of real time PCR.

Temperature	*Time*
95°C	5 min.
95°C	30 sec.
Depends on primer	1 min. (45 cycles)

72°C	1 min.
72°C	5 min.
4°C	∞

2.2.9 Antibody isotyping

In order to determine antibody production from IL-10^{+} and IL-10^{-} B cells, supernatants of cultured cells were examined with anti-IgG1, IgG2a, IgG2b, IgG3, IgA and IgM pre-coated antibody isotyping kit. First of all, 50 µl of samples were added into wells. After that, 50 µl of the Goat Anti-Mouse IgG+IgA+IgM HRP conjugate was put to each well of the strips. Plate was mixed by gently tapping and covered with parafilm. It was incubated for 1h at room temperature. Contents in plate were discarded. Wells were washed with 1X wash buffer 3 times. Plate was blotted onto paper towels after each washing steps. Then, 75 µl of TMB Substrate was added to each well. After 15 minutes, 75 µl of Stop Solution was used to stop reaction. The stop solution changes the color from blue to yellow. Therefore, plates measured with the spectrophotometer at 450 nm. An absorbance reading ≥ 0.2 was accepted as a positive response.

2.2.10 IL-10 ELISA

For quantitative determination of IL-10 protein in culture medium of B cells, Nunc 96-well plates were coated with IL-10 capture antibody 1:200 diluted in coating buffer 1X and plate was incubated at 4°C overnight. Following day, plate was washed four times with PBS/T. Then, 100 µl of Assay Diluent A 1X (diluted from 5X with PBS 1X) was added into assayed wells and the plate was incubated at room temperature for 1h. Recombinant IL-10 standards were prepared by serial dilution according to manufacturer's instructions. After that, plate was again washed four times with PBS/T. Following washing steps; diluted recombinant IL-10 standards and culture media supernatants (50 µl) were added as biological duplicates. Plate was incubated at room temperature for 2 h. Following 2 h-long incubation, plate was washed four times with PBS/T. Biotinylated IL-10 detection antibody 1:200 diluted in Assay Diluent 1X was added into each assayed well and plate was incubated at room temperature for 1 h. After washing of assayed wells with PBS/T for three times, Avidin-HRP solution 1:1000 diluted in Assay Diluent A 1X was added into each assayed well as 50 µl and plate was incubated in the dark for 30 min at room temperature. After 30 min-long incubation, plate was washed for five times with

PBS/T and 50 µl of TMB Substrate Solution Mixture (1:1 of TMB Substrate A and TMB Substrate B) was added into each assayed well. The plate was incubated for at least 30 minutes at room temperature. High concentration standards and samples turned into blue. After that, the reaction was stopped with 50 µl stop solution (2N H_2SO_4). Then, the absorbances of the samples were measured at 450 nm on a microplate reader.

2.2.11 TGF-β ELISA

To determine produced amount of TGF-β from IL-10^+ and IL-10^- B cells, supernatants of cells were examined with ELISA method. Primarily, 100 µl of samples were activated with 20 µl of 1N HCl solution. After 10 min incubation at room temperature, 20 µl of 1.2 N NaOH/0.5 M HEPES was added to samples to neutralize acidity. Standard solutions were prepared from 2000 pg/ml sample according to manufacturer's instructions. Then, 50 µl of assay diluent was added into pre-coated wells. 50 µl of samples, standards and control sample was put into wells and plate was sealed with adhesive strip. Samples were incubated for 2 hours at room temperature. After that, content of wells were discarded. Wells were washed 4 times with prepared 1X wash buffer. Plates were blotted on paper towels after each wash. Following the washing, 100 µl of TGF-β1 conjugate was added into wells. Wash step was repeated again. 100 µl of substrate solution was added to each well. Plate was incubated for 30 min. at room temperature. In order to stop reaction, 100 µl of stop solution was added to wells. After 30 min. incubation, absorbances of samples were measured at 450nm.

2.2.12 Immunofluorescence (IF)

2.2.12.1 Preparation of poly-L-lysine coated coverslips

In order to attach cells on coverslips, poly-L-lysine treatment were performed. Stock poly-L-lysine (0.1 mg/ml) was diluted to working concentration (200 µg/ml) with distilled water. 18mm round coverslips were settled into 12-well plates. 400 µl of diluted poly-L-lysine solution were put into wells. After 1h, coverslips were cleaned from poly-L-lysine solution and washed 4 times (1 x 1 min, 3 x 5min) with distilled water. Coverslips were left to dry in air.

2.2.12.2 Preparation of cells

In order to analyze NF-κB translocation, THP-1 cells were treated with LPS (1μg/ml) for 1h or left untreated in the presence or absence of PDTC (NF-κB inhibitor). Splenic B cells were treated with *H.f.* sonicate (10μg/ml) for 1h or left untreated. Cells were collected from wells and fixed with 50 μl 1x Fixation buffer for 20min. at room temperature. After that, cells were washed with 300 μl of distilled water. Pellet was dissolved in 15 μl distilled water. Cell suspension was added on poly-L-lysine covered coverslips until it dried.

2.2.12.3 Fluorescence labeling of NF-κB p65

Cells were permeabilized with 75 μl 0.5% Triton X-100 in PBS at room temperature for 12 min. Coverslips were washed 3 x 5 min with washing buffer 0.1% Tween-20 in PBS. For blocking of cells, 75 μl of 10% of goat serum in PBS 1X was put on a piece of parafilm. Coverslip was covered on the spot. Blocking was performed for 1h at room temperature. 75 μl primary antibody (1:75 diluted rabbit anti-mouse/human NF-κB Ab in blocking solution) was replaced with blocking solution. Coverslips were kept at 4°C overnight. Next day, coverslips were washed 3 x 5min in washing buffer 0.1% Tween-20 in PBS 1X. 75 μl of secondary antibody (1:300 diluted anti-rabbit-AF-488 Ab in blocking solution) was used to stain cells. Coverslips were incubated for 1 hour-1.5 hour at room temperature. Coverslips were washed 1 x 5 min in washing buffer 0.1% Tween-20 in PBS 1X. 1:1000 diluted DAPI solution diluted in PBS 1X was used to stain cells. Coverslips were washed once. 15 μl of mounting media was added on slides as a spot. Coverslips were inverted on solution. Edges of coverslips were sealed with clear nail polish. Coverslips were examined under confocal microscopy.

3. RESULTS

3.1 Regulatory B Cell Isolation

3.1.1 B cell isolation from spleen of C57BL/6 mice

In order to characterize *Helicobacter*-activated IL-10-producing B cells, first of all mouse splenic B cells were isolated from freshly isolated C57BL/6 mice spleens by using Regulatory B Cell Isolation Kit (Mat. & Met. part, 2.2.5). Pre-enrichment of B cells was performed with magnetic labeling of cells other than B cells. After that, negatively selected B cell fraction was collected. Following the separation, B cells were labeled for FITC-coupled CD19 antibody and compared to unstained control. Flow cytometer analysis was performed to measure the change in fluorescence intensity. Representative image of $CD19^+$ B cell purity was shown in Figure 3.1.

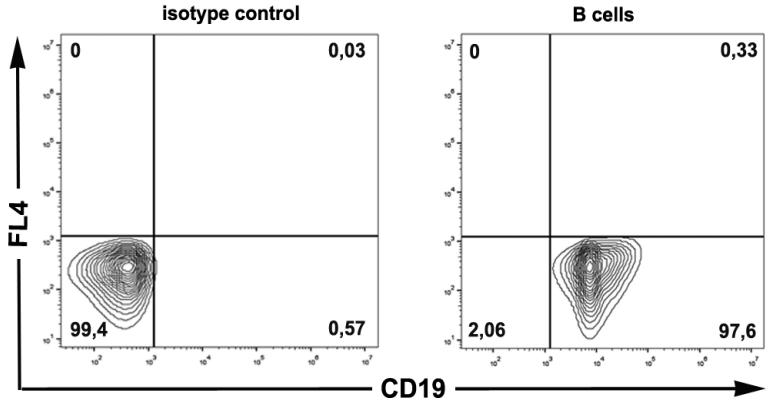

Figure 3.1 : Pure splenic B cells were isolated from C57BL6 mice spleen. B cells were isolated with a B cell isolation kit which magnetically labels non-B cells. After magnetic separation, B cell fraction was obtained with negative selection. In order to examine B cell specific marker, CD19, expressing cell percentage in B cell fraction, around 5x10^4 B cells were stained with FITC-coupled anti-CD19 antibody (right) or same amount of cells were left as unstained control (left). Representative contour plots of staining were shown above for all experiments.

Flow cytometry analysis was performed with FlowJo software with drawing of contour plots. According to figures, $CD19^+$ B cells were isolated with more than 95% purity (right image) compared to the unstained control group (left image). For all B

cell isolation processes, purity of B cells was assessed to confirm enriched cell population.

3.1.2 *Helicobacter*-activated IL-10 producing B cell isolation

Helicobacter-activated IL-10 producing B cells were characterized in detail. For this purpose, isolated mouse splenic B cells were incubated with 10µg/ml *H.f.* sonicate for 24h. Cells were collected and labeled with catch reagent to hold secreted IL-10 in the secretion period. After this process, PE-conjugated IL-10 antibody was used to label cells. At the end, IL-10 producing cells were magnetically labeled and separated with positive selection (Mat. & Met. part, 2.2.6). Flow analysis was performed for PE-conjugated IL-10 antibody intensity for labeled cells. Representative plots for IL-10 - producing B cells, IL-10$^-$ B cells and unstained control for IL-10 expression was shown in Figure 3.2.a. Average of two individual experiments was also shown in Figure 3.2.b.

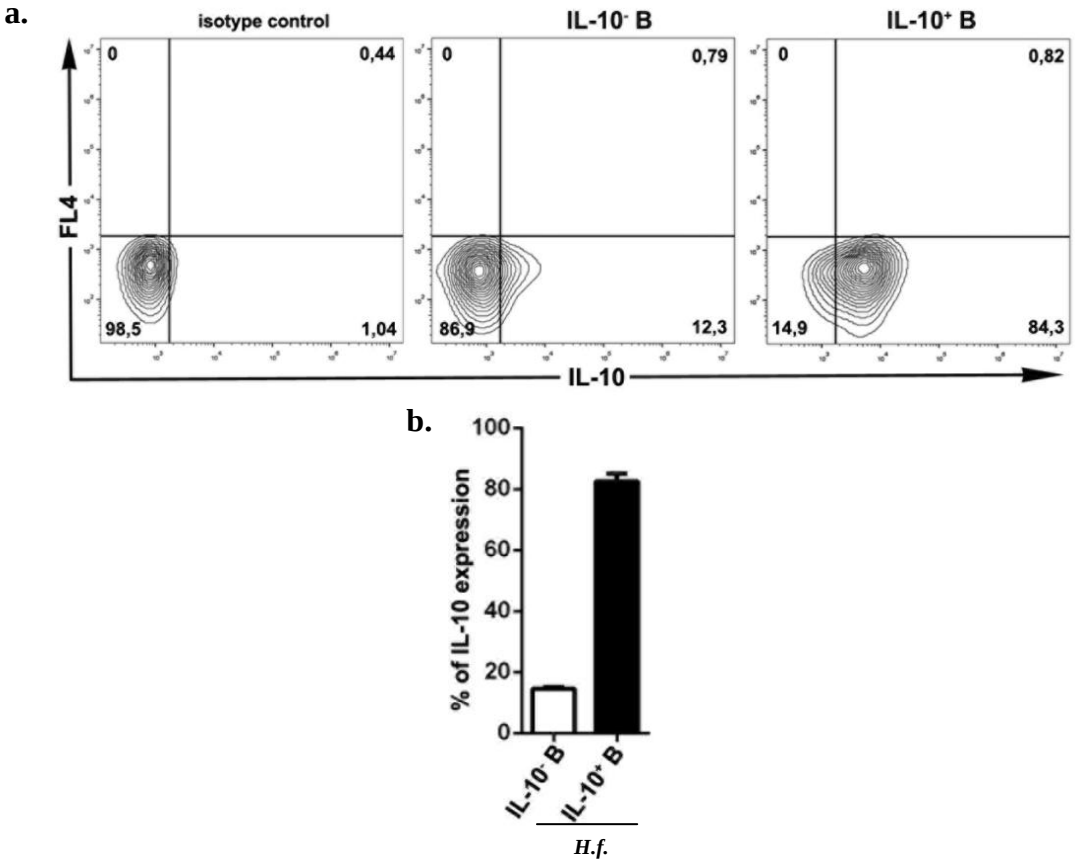

Figure 3.2 : *Helicobacter*-activated IL-10$^+$ B cells and IL-10$^-$ B cells were enriched to around 85% purity. *a*, Isolated B cells were cultured with 10µg/ml *H.f.* sonicate for 24h. PMA (50ng/ml) and ionomycin (500ng/ml) were added to culture last 5h of the incubation. *Helicobacter*-activated IL-10$^+$ and IL-10$^-$ B cells were enriched by magnetic labeling and separation of

Figure 3.2 (cont'd) : IL-10^{+} B cells from IL-10^{-} B cells. Representative contour plots of IL-10 producing (right image), IL-10^{-} (middle image) and unstained (left image) B cells were analyzed with PE-coupled anti-IL-10 antibody which was added in the isolation process after catching secreted IL-10. *b,* Average percentages of two independent experiments for IL-10^{+} and IL-10^{-} B cells after isolation were shown as bar graph. Images were drawn with GraphPad Prism program with indicated fractions of IL-10^{+} (black bar) and IL-10^{-} (white bar) B cells.

Thus, IL-10 positivity of *Helicobacter*-activated IL-10^{+} and IL-10^{-} B cells were compared to unstained control group. Flow results were analyzed with FlowJo software. Analysis of flow cytometer data shows that IL-10^{+} B cell fraction (positively selected after magnetic separation) was around 82-84% pure. Also, IL-10^{-} B cell fraction (negatively selected after magnetic separation) is around 84% pure. IL-10 positivity of these cells was found around 14%. It means that IL-10^{+} cells were mostly depleted from IL-10^{-} cells and vice versa. Bar graph was prepared with GraphPad Prism software. After all isolation process, purity of IL-10^{+} and IL-10^{-} cells were checked by flow cytometry.

3.2 Identification of Surface Marker Expressions

Characterization of *Helicobacter*-activated IL-10 producing B cells were performed primarily with surface marker analysis. Previously, IL-10 producing CD1d^{hi}CD5^{+} B10 cell subsets were identified in colitis (Mizoguchi et al., 2002), systemic lupus erythematosus (Watanebe et al., 2010) and contact hypersensitivity (Yanaba et al., 2008) models. Also, CD21^{hi}CD23^{+} IL-10 producing B cell subsets were reported in collagen-induced arthritis (Evans et al., 2007) and lupus (Blair et al., 2009). However, which type of/types of surface markers are expressed on *Helicobacter*-activated IL-10 producing B cells is still needed to be examined. Therefore, specified possible cluster of differentiation molecules were analyzed with flow staining for *Helicobacter* case.

After magnetic separation, small fraction of IL-10^{+} and IL-10^{-} B cells were directly stained for their surface molecules. Experiments were carried out with fluorochrome tagged antibodies; FITC-coupled CD1d and AF-647-coupled CD5 antibodies or APC-coupled CD21 and PE-Cy7-coupled CD23 antibodies.

3.2.1 CD1dCD5 surface marker analysis

Isolated IL-10^{+} and IL-10^{-} B cells were analyzed for surface molecules CD1d and CD5 single positivity (CD1d^{+}CD5^{-} or CD1d^{-}CD5^{+}), double positivity (CD1d^{+}CD5$^{+)}$ or double negativity (CD1d^{-}CD5^{-}) with flow cytometry.

Representative figure of CD1dCD5 staining was shown in Figure 3.3.a. *Helicobacter*-activated IL-10^{+} B cells and IL-10^{-} B cells were analyzed by comparing to unstained control group. Average percentages was shown in Figure 3.3.b. Flow cytometry analysis was performed with FlowJo software by drawing quadrant on contour plots. Moreover, bar graphs were drawn with GraphPad Prism software. Significance evaluations were done using unpaired t test. The level of 0.05> was accepted as significant.

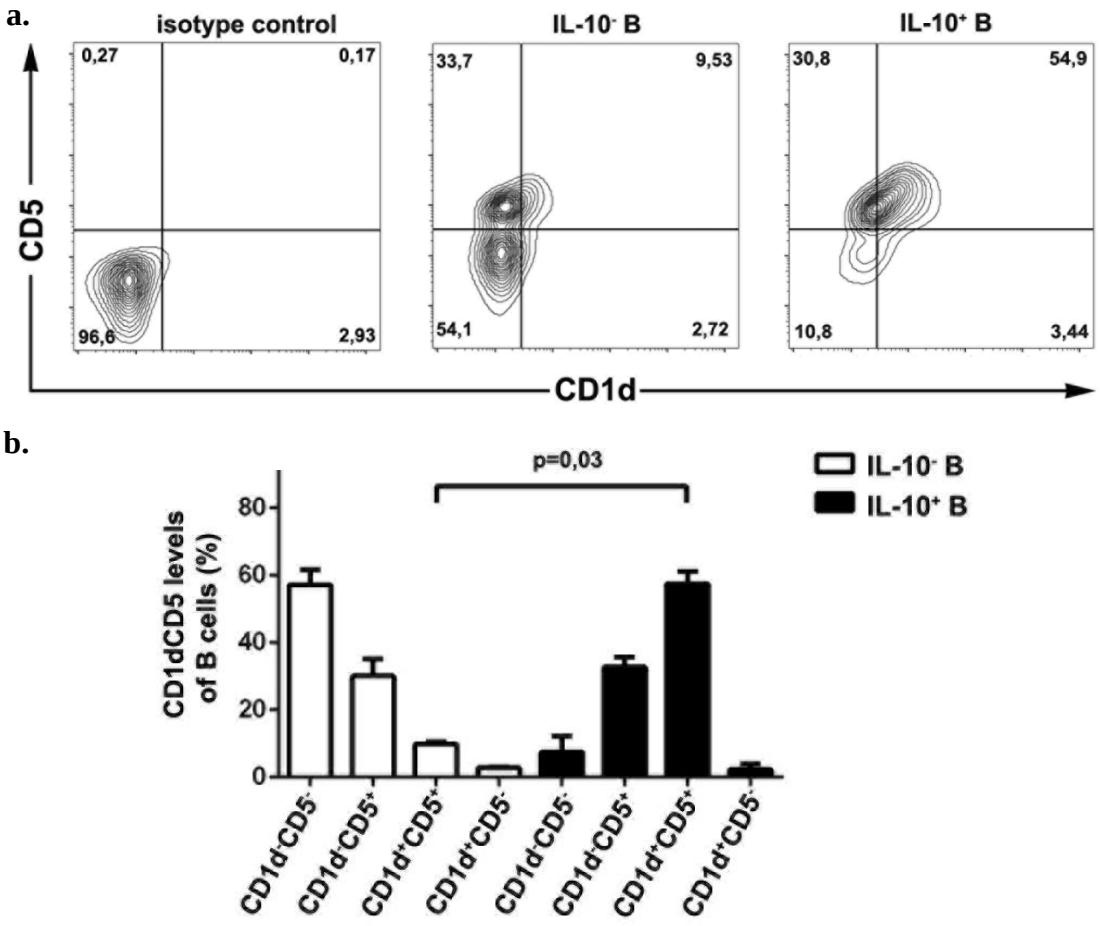

Figure 3.3 : H_{act} IL-10^{+} B cells are mostly CD1d^{+}CD5^{+} but IL-10^{-} B cells are mostly CD1d^{-}CD5^{-}. *a*, After enrichment, H_{act} IL-10^{+} B cells and IL-10^{-} B cells were directly analyzed for their surface marker expressions. Around 5x10^4 cells from two fractions were double stained with FITC-coupled anti-CD1d and AF-647-coupled anti-CD5 antibodies. Same amount of cells were left as unstained control. Representative images of

Figure 3.3 (cont'd) : contour plot analysis for CD1d and CD5 expression were prepared with FlowJo software. IL-10^+ B cells (right image) and IL-10^- B cells (middle image) were analyzed according to unstained control (left image). *b,* Average percentages of two independent experiments were shown for $CD1d^-CD5^-$, $CD1d^+CD5^-$, $CD1d^+CD5^+$ and $CD1d^-CD5^+$ subsets of IL-10^+ (black bars) and IL-10^- (white bars) B cells. Bar graph was drawn with GraphPad Prism program. Significancy evaluation was performed with Student's t test ($p<0.05$ was significant).

Isolated H_{act} IL-10 producing B cells were compared to IL-10^- B cells for their CD1dCD5 expressions. As it was shown in Figure 3.3, around 55% of IL-10 producing B cells were $CD1d^+CD5^+$. Whereas, this value is significantly decreased to 10% for IL-10^-B cells. On the other hand, percentage of $CD1d^-CD5^+$ and $CD1d^+CD5^-$ groups represented similarities for both IL-10^+ and IL-10^- B cells. Only 10% of IL-10^+B cells have $CD1d^-CD5^-$ group, while it is around 55% for IL-10^- B cell group.

As a result, around 10% of *Helicobacter*-activated IL-10^- B cells are $CD1d^+CD5^+$ whereas more than 50% of *Helicobacter*-activated IL-10^+ B cells are $CD1d^+CD5^+$ which is significantly higher (~5.5 fold).

3.2.2 CD21CD23 surface marker analysis

Isolated IL-10^+ and IL-10^- B cells were stained for surface markers CD21 and CD23 and compared for their single positive ($CD21^+CD23^-$ or $CD21^-CD23^+$), double positive ($CD21^+CD23^{+)}$ or negative ($CD21^-CD23^-$) groups with flow cytometry. Representative figure of CD21CD23 staining was shown in Figure 3.4.a. H_{act} IL-10^+ B cells and IL-10^- B cells were analyzed comparing to unstained control. Average percentages was shown in Figure 3.4.b. Flow cytometry analysis was examined with FlowJo software by drawing quadrant on contour plots. Additionally, column graphs were plotted with GraphPad Prism software. Significance calculations were done using Student's t test which $0.05>p$ was accepted as significant.

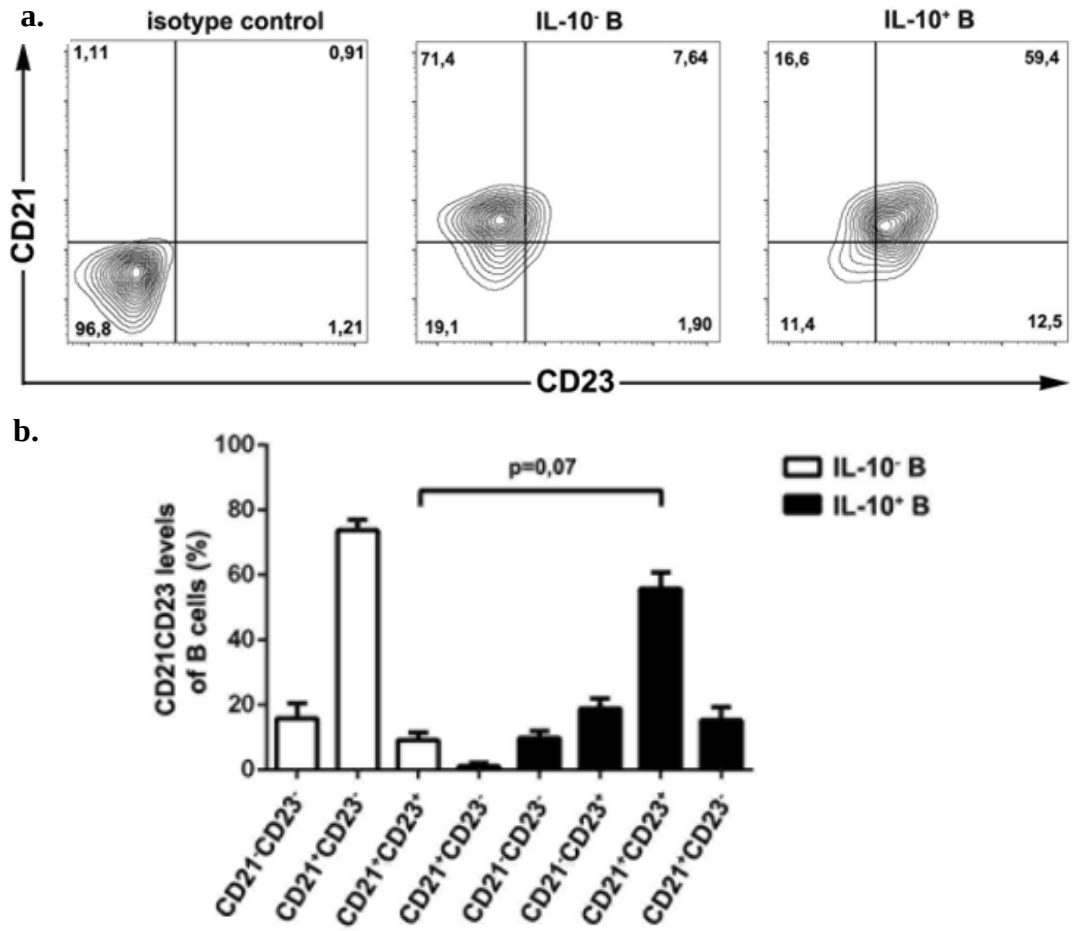

Figure 3.4 : H_{act} IL-10$^+$ B cells are mostly CD21$^+$CD23$^+$ but IL-10$^-$ B cells are mostly CD21$^+$CD23$^-$. *a*, After enrichment, 5x10^4 H_{act} IL-10$^+$ B cells and IL-10$^-$ B cells were directly analyzed for their CD21 and CD23 surface marker expressions. Cells were double stained with APC-coupled anti-CD21 and PE-Cy7-coupled anti-CD23 antibodies FITC. Same amount of cells were left as unstained control. Representative images of contour plot analysis for CD21 and CD23 expression were prepared with FlowJo software. IL-10$^+$ B cells (right image) and IL-10$^-$ B cells (middle image) were analyzed according to unstained control (left image). *b*, Average percentages of two independent experiments were represented for CD21$^-$CD23$^-$, CD21$^+$CD23$^-$, CD21$^+$CD23$^+$ and CD21$^-$CD23$^+$ subsets of IL-10$^-$ B cells (white bars) and IL-10$^+$ B cells (black bars). Bar graph was drawn with GraphPad Prism program. Significancy evaluation was performed with Student's t test ($p<0.05$ was significant).

After isolation of H_{act} IL-10 producing B cells from IL-10$^-$ B cells, their CD21CD23 expressions were analyzed. Unstained control was used to draw quadrants. As it is presented in Figure 3.4., around 60% of IL-10 producing B cells were CD21$^+$CD23$^+$. Whereas, it was significantly at lower level for IL-10$^-$B cells, which is around 8%. Additionally, percentage of CD21$^+$CD23$^-$ cells was around 70% in IL-10$^-$ B cells. It

is almost 16% for IL-10^+ B cells. On the other side, level of $CD21^-CD23^+$ cells was higher in IL-10^+ B cell population than IL-10^- B cells which is around 12% and 2%, respectively.

As a result, around 8% of *Helicobacter*-activated IL-10^- B cells are $CD21^+CD23^+$ whereas more than 50% of *Helicobacter*-activated IL-10^+ B cells are $CD21^+CD23^+$ which is significantly higher (~7 fold).

3.3 Antibody Isotyping of *Helicobacter*-Activated IL-10^+ and IL-10^- B Cells

Activated B cells can produce different antibodies. Previous studies showed that *H.f.* activated total B cells can produce IgM and IgG2b antibodies (Sayi et al., 2011). However, it is not known whether IL-10^+ B cells or IL-10^- B cells are the source of these antibodies. Therefore, the study aimed to characterize these two groups for their antibody production capabilities.

Isolated H_{act} IL-10^+ B cells and IL-10^- B cells were checked for their IL-10 expression with flow cytometry. Then, they were treated with *H.f.* sonicate for additional 2 days. Their supernatant was collected and directly subjected to antibody isotyping process (Mat. & Met. Part 2.2.9). In addition, supernatant of B cells without any treatment and with *H.f.* sonicate treatment after 2 days culture were used as control groups.

Antibody isotyping kit includes IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM detection antibodies. Final measurement was performed at 450nm. According to the protocol, 0.2< absorbance value was accepted as positive response. Bar graphs of data were drawn with GraphPad Prizm software. Significance evaluations were done with unpaired student t test. After that, 0.05> p value was regarded as significant.

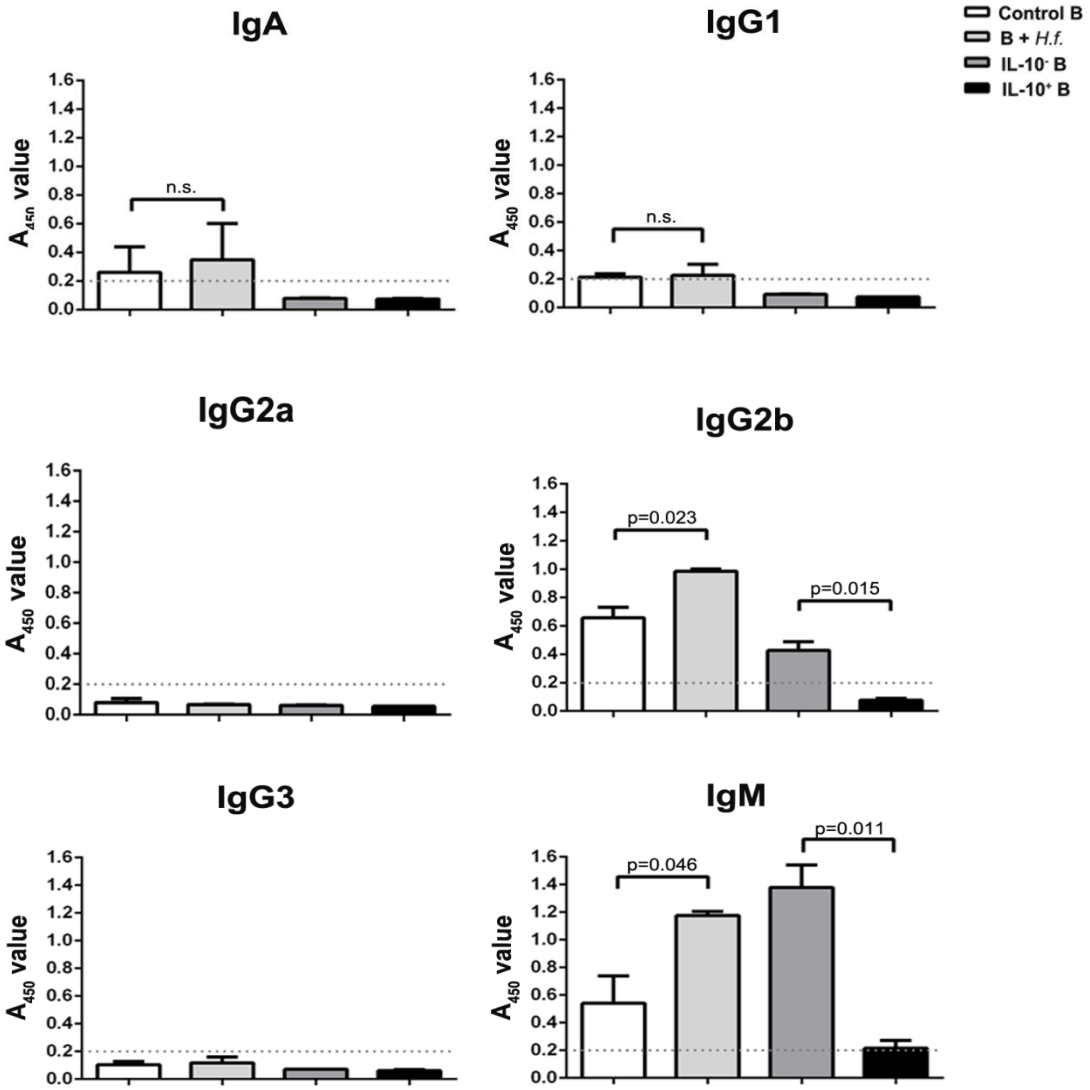

Figure 3.5 : *Helicobacter*-activated IL-10$^-$ B cells are the source of produced IgG2b and IgM antibodies. Isolated B cells were cultured with *H.f.* sonicate (10μg/ml) for 48h. PMA (50ng/ml) and ionomycin (500ng/ml) were added last 5h of culture. After enrichment of IL-10$^+$ and IL-10$^-$ B cells, cells were cultured with *H.f.* sonicate (10μg/ml) for additional 48h. Supernatants of cultures were collected and examined for production of several antibodies with ELISA based antibody isotyping kit. *H.f.* sonicate (10μg/ml) treated or untreated total B cells were used as positive controls. Column graphs were drawn with GraphPad Prism program. They show IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM levels for untreated B (white bar), and *Helicobacter* treated (light grey bar) B cells and also IL-10$^-$ (dark grey bar) and IL-10$^+$ (black bar) B cells. Data represent average of two independent experiments. If absorbance level at 450nm was higher than 0.2, it was accepted as positive response according to manufacturer's protocol.

Analysis of control B cells, *H.f.* treated B cells, *H.f.* treated IL-10$^+$ B cells and *H.f.* treated IL-10$^-$ B cells for antibody production was represented in Figure 3.5. It is

shown that IgG1 and IgG3 levels are not detectable in the supernatant of any sample. Although IgA and IgG1 levels were detected in some degree for control B and *H.f.* treated B cells, difference was not significant. However, these antibodies were not present in the supernatant of *H.f.* treated IL-10^+ B cells and *H.f.* treated IL-10^- B cells. On the other hand, IgG2b and IgM levels were significantly higher in the supernatant of *H.f.* treated B cells compared to the control B cells. When supernatant of *H.f.* treated IL-10^+ B cells and *H.f.* treated IL-10^- B cells were examined for IgG2b and IgM antibodies, only *H.f.* treated IL-10^- B cells showed detectable levels of these antibodies. *H.f.* treated IL-10^+ B cells did not produce IgG2b and IgM. They were under detection level. Data suggest that *Helicobacter*-activated B cells produce IgG2b and IgM antibodies and these antibodies are only produced from *Helicobacter*-activated IL-10^- B cells.

3.4 IL-10 Analysis of *Helicobacter*-Activated B Cells

IL-10 secretion from B cells has an important role on anti-inflammatory response. In order to determine level of IL-10 in both mRNA and protein level, B cells were treated with *H.f.* sonicate (10μg/ml) for 24h. Then, *Helicobacter*-activated B cells were magnetically separated into *Helicobacter*-activated IL-10^+ and IL-10^- B cells. After that, these two fractions were cultured in the presence of *H.f.* sonicate (10μg/ml) for 48h. Their pellets were collected for Real - time PCR for IL-10 relative expression level and their supernatants were collected for ELISA to measure IL-10 secretion level.

3.4.1 Relative expression levels of IL-10 in *Helicobacter*-activated B cells

IL-10 expression was determined with relative expression analysis. Cell pellets were used to isolate RNA and then convert to cDNA. IL-10 expression levels were determined with Real - time PCR and normalized to 18s rRNA which was used as an endogenous control. Column graph was drawn in GraphPad Prism program. Significance evaluations were done with student t test. P value 0.05> was significant.

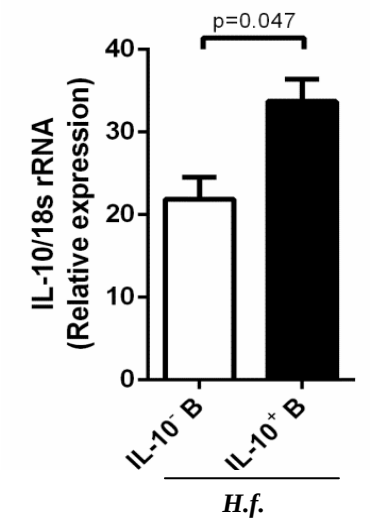

Figure 3.6 : *H.f.* treated IL-10$^+$ B cells expressed higher levels of IL-10 compared to IL-10$^-$ B cells. Isolated splenic B cells were cultured with *H.f.* sonicate (10μg/ml) for 24h. PMA (50ng/ml) and ionomycin (500ng/ml) was added last 5h of culture. Magnetically separated IL-10$^+$ and IL-10$^-$ B cells were cultured for 48h in the presence of *H.f.* sonicate (10μg/ml). Relative expression level analysis of IL-10$^-$ B cells and IL-10$^+$ B cells was performed with real time PCR. Bar graph was drawn with GraphPad Prism program. IL-10$^+$ B cells (black bar) compared to the IL-10$^-$ B cells (white bar) for IL-10 expression levels.

As it is represented in Figure 3.6, IL-10 expression level was significantly higher in H_{act} IL-10$^+$ B cells in comparison to IL-10$^-$B cells. Difference between expression levels of IL-10 between two groups was found around 1.5 fold higher in IL-10$^+$ B cells. The reason of IL-10 expression from IL-10$^-$ B cells may be the expansion of 12% IL-10$^+$ B cell population in IL-10$^-$ B cell fraction (Figure 3.2).

3.4.2 Secretion level of IL-10 from *Helicobacter*-activated B cells

Secreted IL-10 amounts were measured by IL-10 ELISA (Method 2.2.10). Isolated H_{act} IL-10$^+$ B cells and IL-10$^-$B cells were cultured in the presence of *H.f.* sonicate (10μg/ml) for 24h. Supernatants were collected to determine IL-10 production level of *Helicobacter*-activated IL-10$^+$ and IL-10$^-$ B cells by using IL-10 ELISA. Column graph was generated with GraphPad Prism program. Significancy evaluation was performed with Student's t test. $P<0.05$ was accepted as significant.

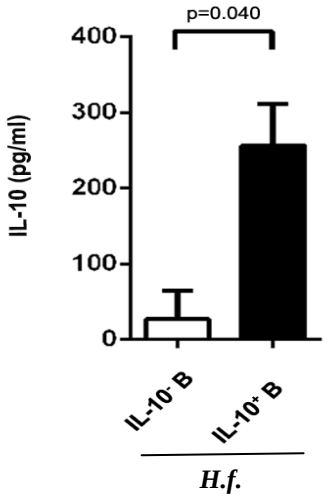

Figure 3.7 : *H.f.* treated IL-10$^+$ B cells secreted higher levels of IL-10 compared to IL-10$^-$ B cells. Enriched *Helicobacter*-activated IL-10$^+$ and IL-10$^-$ B cells were cultured for 24h in the presence of *H.f.* sonicate (10μg/ml). Secreted amounts of IL-10$^-$ B cells and IL-10$^+$ B cells were measured with IL-10 ELISA. Bar graph was drawn with GraphPad Prism program. IL-10$^+$ B cells (black bar) compared to the IL-10$^-$B cells (white bar) for IL-10 secretion levels.

H_{act} IL-10 producing B cells were found as the source of produced IL-10 when it was compared with IL-10$^-$B cells. According to Figure 3.7, secreted IL-10 amount was measured around 250 pg/ml for IL-10$^+$ B cells, whereas it was quite low for IL-10$^-$ B cells. It was measured less than 50 pg/ml.

It suggests that IL-10 secretion from *H.f.* treated IL-10$^+$ B cells was more than 5 fold higher than *H.f.* treated IL-10$^-$B cells.

3.5 TGF-β Analysis of *Helicobacter*-Activated B Cells

TGF-β is another anti-inflammatory cytokine, which is produced by activated regulatory B cells (Br3 cells). Regulatory roles of Br3 cells have been reported in different disease types such as systemic lupus erythematosus (Raymond et al., 1997), diabetes (Tian et al, 2001), and it has been reported in non-IgE mediated allergy response in atopic dermatitis (Noh & Lee, 2011). On the other hand, it is still not known whether TGF-β is produced from *Helicobacter*-activated B cells. Therefore, expression and production level of TGF-β from H_{act} IL-10 producing B cell and IL-10$^-$ B cells are examined to understand possible roles of these two distinct groups. Enriched IL-10$^+$ and IL-10$^-$ B cells were cultured in the presence of *H.f.* sonicate

(10μg/ml) for 48h. Pellet of two subsets were collected for TGF-β expression, supernatants of two subsets were collected for TGF- β ELISA.

3.5.1 Relative expression levels of TFG-β in *Helicobacter*-activated B cells

Isolated H_{act} IL-10$^+$ B cell and IL-10$^-$ B cells were analyzed for relative expression level of TGF-β by using Real - time PCR. Pelleted cells were subjected to RNA isolation and it continued with cDNA synthesis. TFG-β was used as a target gene; GAPDH was used as an endogenous control. Column graph was drawn with GraphPad Prism software. Significance test was performed with student's t test. P value which is 0.05> was accepted as significant change.

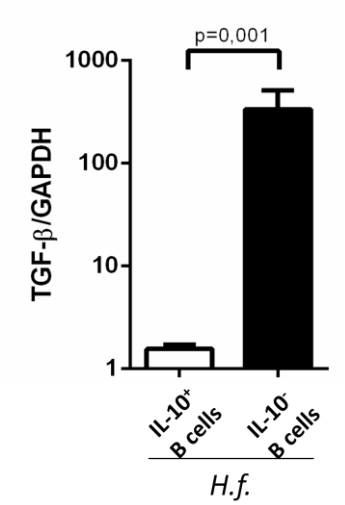

Figure 3.8 : TGF-β was highly expressed in *Helicobacter*-activated IL-10$^-$ B cells. B cells were cultured in the presence of *H.f.* sonicate (10μg/ml) for 48h. In the last 5h of culture PMA (50ng/ml) and ionomycin (500ng/ml) was added. Total B cells were magnetically labeled to separate IL-10$^+$ and IL-10$^-$ B cells. Isolated *Helicobacter*-activated IL-10$^+$ and IL-10$^-$ B cells were cultured with *H.f.* sonicate (10μg/ml) for additional 48h. Cell pellets were used to analyze relative expression level of TGF-β in IL-10$^+$ (left column) and IL-10$^-$ B cells (right column) by using Real - time PCR. TGF-β data were normalized to GAPDH. Bar graph was prepared with GraphPad Prism program and shows average of two independent experiments. Significancy evaluation was performed with Student's t test ($p< 0.05$ was significant).

According to the Figure 3.8, TGF-β expression is significantly increased (more than 250 fold) in *Helicobacter*-activated IL-10$^-$ B cells compared to *Helicobacter*-activated IL-10$^+$ B cells .

Therefore, data suggest that TGF-β is expressed from *Helicobacter*-activated B cells, and IL-10$^-$ B cells highly express TGF-β but IL-10$^+$ B cells hardly express this anti-inflammatory cytokine.

3.5.2 TGF-β secretion level from *Helicobacter*-activated B cells

Isolated *Helicobacter*-activated IL-10$^+$ B cells and IL-10$^-$ B cells were cultured in the presence of *H.f.* sonicate (10μg/ml) for 48h. Supernatants were collected and examined for secreted amount of TGF-β by using TGF-β specific ELISA (Method 2.2.11). Column graph was drawn with GraphPad Prism software. Significance test was performed with student's t test. P<0.05 was accepted as significant change.

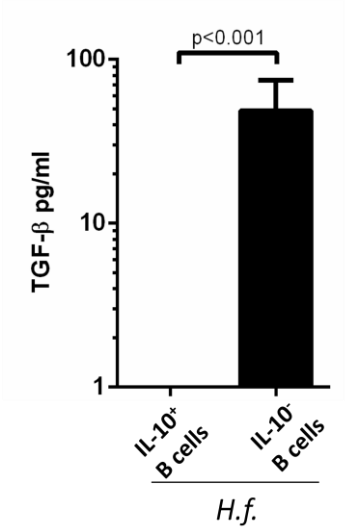

Figure 3.9 : TGF-β only secreted from *Helicobacter*-activated IL-10$^-$ B cells but not from IL-10$^+$ B cells. Enriched IL-10$^+$ and IL-10$^-$ B cells were cultured in the presence of *H. f.* sonicate for 48h. Supernatants of H_{act} IL-10 producing B cells (left column) and IL-10$^-$ B cells (right column) after incubation were collected and examined to measure secreted amount of TGF-β with TGF-β ELISA. Data show average of two independent experiments and bar graph was drawn with GraphPad Prism sofware. Significancy evaluation was performed with student's t test ($p< 0.05$ was significant).

TGF-β cytokine secretion analysis was represented in Figure 3.9. It was previously not known that *Helicobacter*-activated B cells could produce TGF-β. Results show that TGF-β was produced only from *Helicobacter*-activated B cells. Also, this production was only measured from IL-10$^-$ B cells but not from IL-10$^+$ B cells.

3.6 Role of NF-κB Canonical Pathway on IL-10 Production

IL-10 production through TLR-2 and TLR-4 stimuli was shown to result with the activation of p38, ERK and NF-κB activation through MyD-88 dependent manner in macrophages and myeloid dendritic cells (Saraiva & O'Garra, 2010). In our study, importance of NF-κB p65 subunit on IL-10 production through *H.f.* induction was

examined. *Helicobacter*-activated B cells were analyzed for NF-κB p65 subunit activation with translocation from cytosol to nucleus. IL-10 production after inhibition of NF-κB translocation was analyzed to assess the role of this pathway.

3.6.1 Analysis of p65 NF-κB translocation in *H.f.* treated B cells

B cells were isolated from spleen. Pure B cell population was cultured in the presence of *H.f* sonicate (10μg/ml) for 1h or left untreated as control B cells. After incubation, cells were collected and fixed for indirect immunofluorescence procedure. For the detection of translocation of NF-κB p65, rabbit anti-mouse NF-κB p65 antibody was used as primary antibody. Secondary antibody was chosen anti-rabbit-AF488. DAPI was used to dye nuclei.

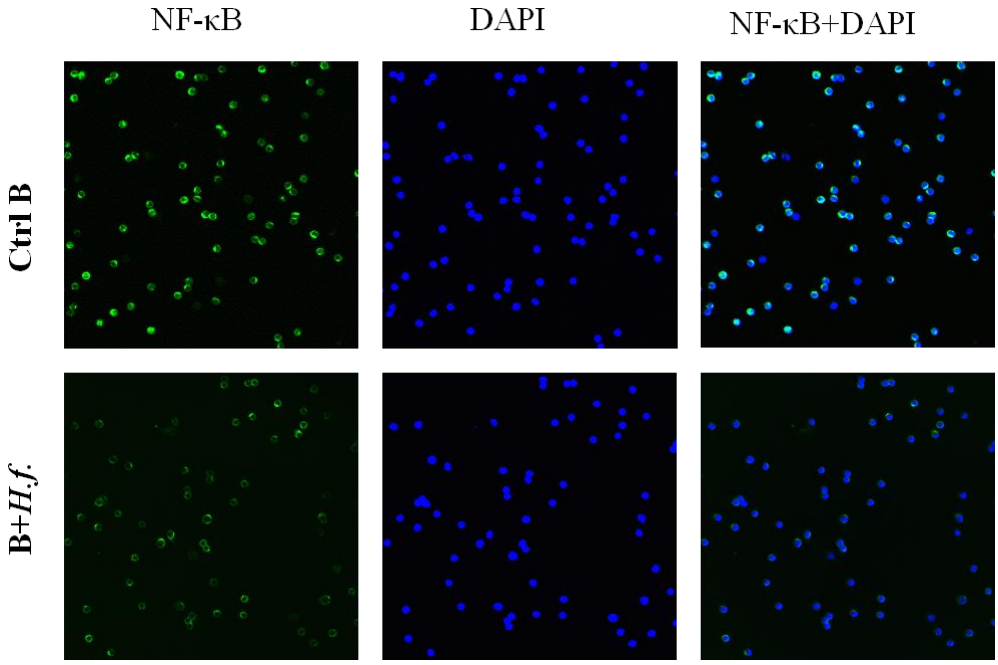

Figure 3.10 : NF-κB p65 subunit was not translocated into nucleus in *Helicobacter*-activated B cells. B cells were treated with *H.f.* sonicate (10μg/ml) for 1h or left untreated. Collected cells were incubated with rabbit anti-NF-κB p65 antibody overnight. NF-κB p65 subunit was labeled as green with AF-488-coupled anti-rabbit antibody in control B cells or *H.f.* treated B cells (left panel). DAPI staining of nuclei was represented for control B cells and *H.f.* treated B cells (middle panel) with blue. Merged images of NF-κB and DAPI control B and *H.f.* treated B (right panel) cell samples were shown on the right panel.

According to Figure 3.10, control B cells and *H.f.* treated B cells kept NF-κB p65 subunit in the cytoplasm, p65 subunit was not translocated into nucleus even in the *H.f.* treated B cells. It may suggest that, *Helicobacter* activation does not lead the induction of canonical pathway of NF-κB through p65 translocation.

3.6.2 Inhibition study of NF-κB p65 translocation

In order to understand NF-κB p65 activation and so translocation, a positive control was used. LPS stimulation induce NF-κB p65 translocation in THP-1 cell line (Wang et al., 2011). Therefore, THP-1 cell line was incubated with 100ng/ml LPS for 1h. Additionally, inhibition of NF-κB p65 translocation was accomplished by using NF-κB inhibitor PDTC (30 μM). It is because *H.f.* treated B cells did not show translocation of p65; inhibitory molecule could not be tested for its inhibitory capacity in B cells. Immunofluorescence images of NF-κB p65, DAPI and merged samples were shown in Figure 3.11.

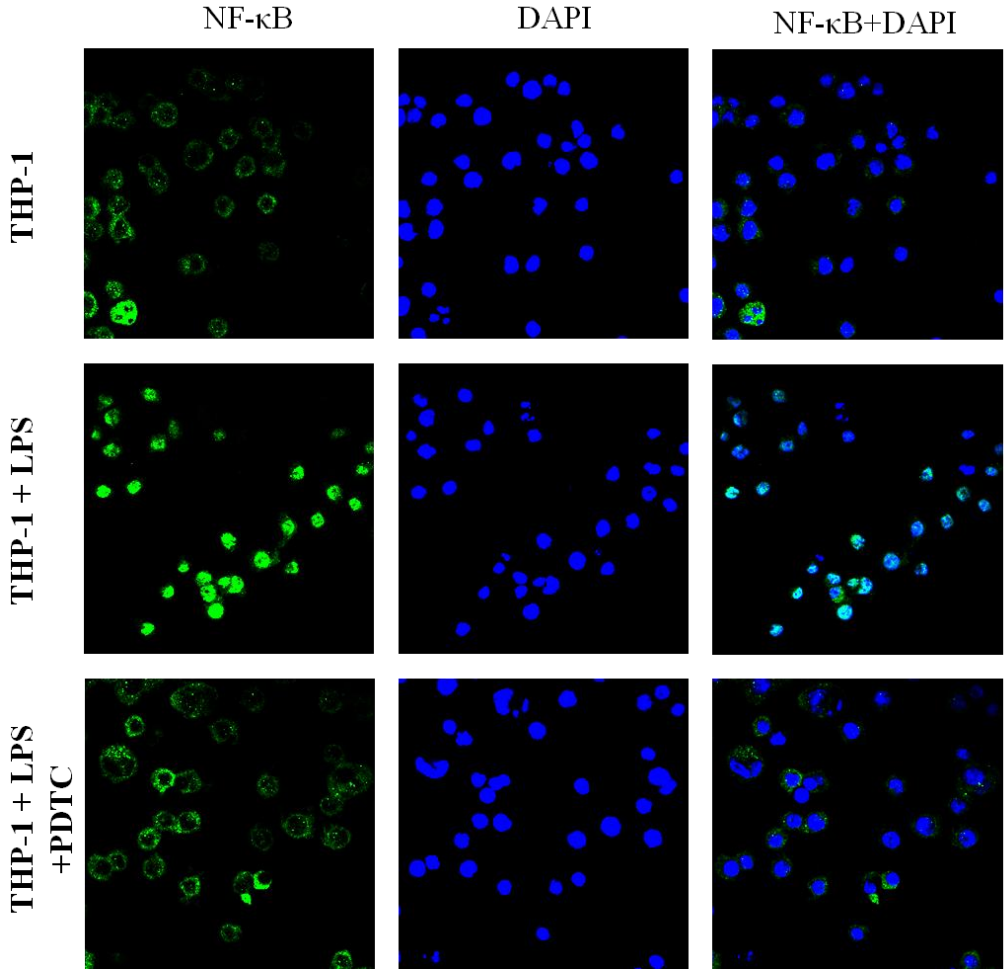

Figure 3.11 : LPS induced NF-κB p65 translocation in THP-1 cell, PDTC inhibited transocation of NF-κB p65 in LPS treated THP-1 cells. THP-1 cells were treated with LPS (100ng/ml), pre-treated with PDTC (30μM) 1h before LPS stimulation (100ng/ml) or left untreated. NF-κB p65 staining (on the left panel), DAPI staining (on the middle panel) and merged samples (on the right panel) was shown for untreated, LPS treated and PDTC used LPS treated samples, respectively. Primary rabbit anti-NF-κB p65 antibody incubated overnight, and labeled with

Figure 3.11 (cont'd) : anti-rabbit AF-488 coupled antibody. DAPI staining of nuclei was shown (on the middle panel). Merged images of NF-κB and DAPI samples were represented (on the right panel).

As it was shown in Figure 3.11, NF-κB p65 was kept in the cytoplasm of untreated THP-1 cells. However, as it is expected LPS treatment induced NF-κB p65 translocation from cytoplasm to nucleus in THP-1 cells. Merged samples indicate translocation with double staining of cells with AF-488 and DAPI. On the other hand, PDTC treatment before LPS induction altered translocation of NF-κB p65 in these cells. It can be said that, our primary antibody and PDTC work well.

3.6.3 Analysis of IL-10 secretion on NF-κB inhibited *H.f* treated B cells

IL-10 secretion from *H.f* treated B cells was analyzed in the presence of NF-κB inhibitor PDTC in order to understand role of NF-κB on IL-10 signaling pathway. For this reason, B cells were treated with NF-κB inhibitor 1h before *H.f* induction or left untreated. After 24h, supernatant of B cells were collected and subjected to IL-10 ELISA. Column graph was drawn with GraphPad Prism software. Significancy evaluation was performed with Student's t test. $P<0.05$ was accepted as significant change.

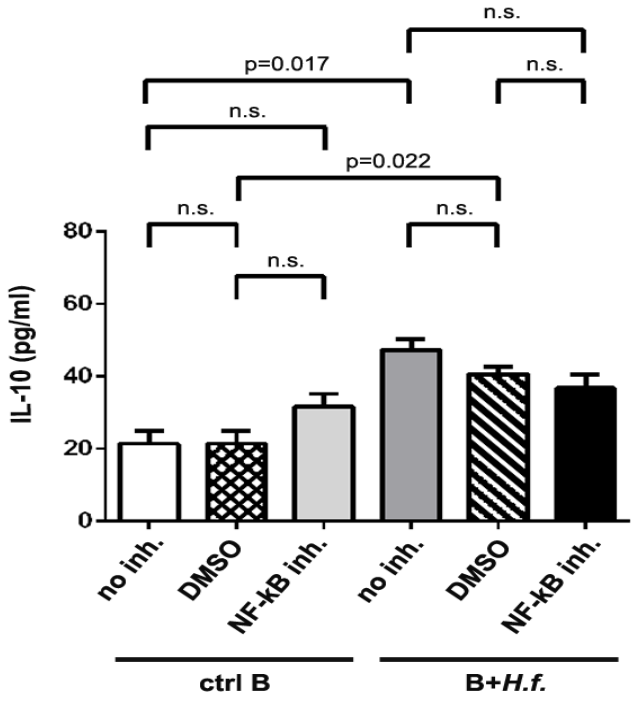

Figure 3.12 : IL-10 production level didn't change with addition of NF-κB inhibitor in the presence or absence of *H.f*. Mouse splenic B cells were isolated and cultured with or without *H. f.* sonicate. For inhibiton of NF-κB

Figure 3.12 (cont'd) : p65 activation, PDTC was used 1h before *H.f.* stimulation. DMSO was used as control. After 24h incubation, supernatants were collected and analyzed for IL-10 production with IL-10 ELISA. *H.f* treated B cells were compared to untreated B cells after inhibition of NF-κB activation. Bar graph was drawn with GraphPad Prism program. Significancy was measured with student's t test ($p<0.05$ was significant).

According to Figure 3.12, IL-10 secretion from *H.f* treated B cells were significantly increased compared to the untreated B cells control. Treatment of B cells with PDTC before addition of *H.f.* did not change secretion level of IL-10 significantly when comparing to DMSO control or untreated control sample.

Data suggest that, IL-10 secretion may not be related with activation of NF-κB p65 in *Helicobacte*r-activated B cells.

4. DISCUSSION & CONCLUSION

In this study, *Helicobacter*-activated IL-10 producing B cells were characterized in detail. *Hf*-activated IL-10^+ B cells showed increased level of $CD1d^+CD5^+$ and $CD21^+CD23^+$ surface marker expressions, which they are at quite low levels in IL-10^- B cells. On the other hand, antibody secretion was detected for IgM and IgG2b only from IL-10^- B cells but not IL-10^+ B cells. Similarly, TGF-β expression and production were mostly found to be increased in IL-10^- B cells. Also, canonical pathway of NF-kB activation through p65 subunit translocation was not induced with *Helicobacter*-activation on IL-10 production. Our findings may help to identify characteristics of IL-10 producing B cells in the case of *Helicobacter* infection to understand role of these cells in asymptomatic long-term *Helicobacter* persistence without clearance in infected individuals (Bauer & Meyer, 2011).

Discovery of regulatory function of B cells increased the studies to understand their possible role on different disease models. They have been shown to produce IL-10 with suppressive function. Presence of IL-10 producing B cells have been identified in various types of infectious and autoimmune diseases such as experimental autoimmune disease, collagen-induced arthritis, colitis, asthma, *Leishmania major* infection, *Helicobacter felis* infection etc (Yang et al., 2013; Y. He et al., 2014). These IL-10 producing B cells are shown to be activated through mainly TLRs. TLR4 ligand LPS or TLR9 ligand CpG can induce B cells to produce IL-10 *in vitro*. Also, CD40-CD40L interaction between B and T cells were also required for induction of these regulatory cell population (Gray et al, 2007; Gray et al, 2007). BCR engagement is one of the inducing ways of differentiation of B cells into IL-10 producing cells. In addition to their activation molecules, phenotypic identification of these cells was also needed to find a unique marker to characterize them. Different study groups defined regulatory B cell subsets within different disease model. In colitis (Mizoguchi et al., 2002), systemic lupus erythematosus (Watanebe et al., 2010) and contact hypersensitivity model IL-10 producing $CD19^+CD1d^{hi}CD5^+$ B cell

population was arisen and named as B10 cells (Yanaba et al., 2008). Another subset of IL-10 producing B cells was shown in lupus model (Blair et al., 2009) and collagen-induced arthritis mice model (Evans et al., 2007), which was $CD21^{hi}CD23^{+}$ B cell group. The molecular characteristics of IL-10 producing *Helicobacter*-activated B cells were not clear. In our study, it is aimed to be identified in *Helicobacter* case. *Helicobacter*-activated IL-10 producing B cells were found as $CD1d^{+}CD5^{+}$ (Figure 3.3, Figure 4.1) and $CD21^{+}CD23^{+}$ (Figure 3.4, Figure 4.1). These IL-10 producing B cells shared similar characteristics of T2-MZP B cells which have $CD19^{+}IgM^{hi}IgD^{hi}CD21^{hi}CD23^{+}$ phenotype (Khan et al., 2009) and B10 cell phenotype which is $CD1d^{+}CD5^{+}$ (Yanaba et al., 2008). However, these indicated regulatory B cell subsets are not unique for $IL\text{-}10^{+}$ B cells. Therefore, other possible surface molecules should be examined to identify IL-10 producing regulatory B cells in detail. Additionally, a newly reported data shows that TIM-1 surface marker is expressed by IL-10 producing B cells (Ding et al., 2011). Thus, *Helicobacter*-activated B cells can be analyzed for Tim-1 surface molecule in order to characterize our regulatory B cells.

B cells differentiate into short-lived or long-lived plasma cells with different stimuli and produce antibodies specific to antigen. These antibodies are needed for proper immune response against pathogens or self-reactive molecules. MZ B cells mainly differentiate into short-lived plasma cells and produce IgM and IgG3 in mouse whereas FO B cells differentiate into long-lived plasma cells and produce other antibodies after class-switching (Auner et al., 2010; Hoyer et al., 2004; Fooksman et al., 2010). It is previously shown by Sayi and her co-workers that *Helicobacter*-induced B cells can produce IL-10 together with IgM and IgG2b antibodies (2011). Our data suggests that *Helicobacter*-activated B cells produced IgM and IgG2b and $IL\text{-}10^{-}$ B cells are the source of these produced antibodies *Helicobacter*-activated $IL\text{-}10^{+}$ B cells did not produce any types of antibodies (Figure 3.5, Figure 4.1). It is shown for the first time that *Helicobacter*-activated $IL\text{-}10^{-}$ B cell secreted IgM and IgG2b antibodies. However, the role of these produced antibodies from *Helicobacter*-activated $IL\text{-}10^{-}$ B cell is still needed to be studied.

Regulatory function of immune cells is also defined with the production of TGF-β. This cytokine has been shown to prevent excessive inflammatory response by inducing $Foxp3^{+}$Treg cell proliferation. It inhibits Th1 and Th2 type responses

together with cytotoxic T cell suppression. TGF-β production from T cells is essential for survival (Taylor, 2009; Wrzesinski et al., 2007). On the other side, its production from B cells is also fundamental for suppression of immune response. TGF-β producing T regulatory cells are names as a T cell subset Th3 cells. Similarly, TGF-β producing B cells are a subset of B cells, which named as Br3 cells (Noh & Lee, 2011). Br3 cells were reported in systemic lupus erythematosus (Raymond et al., 1997), diabetes (Tian et al, 2001) and non-IgE mediated allergy response in atopic dermatitis (Noh & Lee, 2011). However, there was not any information about TGF-β production from *Helicobacter*-activated B cells. In the present study it has been shown that *Helicobacter*-activated IL-10$^-$ B cells can express and produce higher levels of TGF-β whereas IL-10$^+$ B cells expressed low levels of TGF-β, and these cells couldn't produce this cytokine (Figure 3.8, Figure 3.9, Figure 4.1). It is reported for the first time that *Helicobacter*-activated IL-10$^-$ B cell can express and secrete TGF-β. The importance of these produced TGF-β from *Helicobacter*-activated B cells is required to enlighten its role on immune cells.

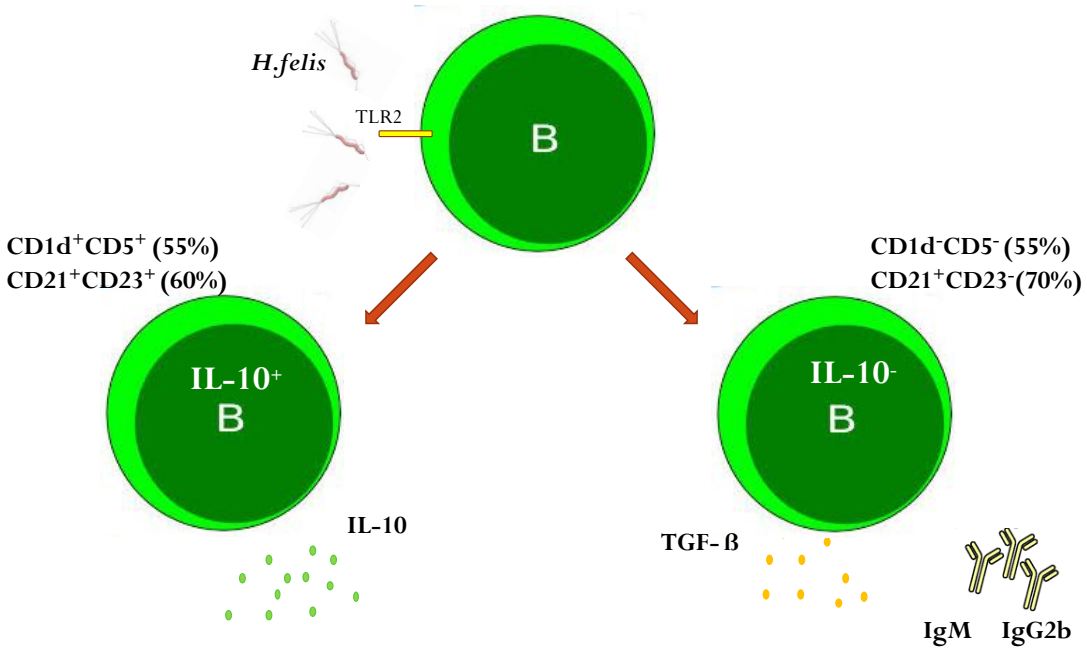

Figure 4.1 : *Helicobacter*-activated IL-10$^+$ and IL-10$^-$ B cells and their characterized properties (modified from Sayi et al., 2011). *Helicobacter*-activated B cells were induced by TLR-2 and MyD88 dependent-manner. Especially, IL-10$^+$ B cell subsets were found mostly CD1d$^+$CD5$^+$ and CD21$^+$CD23$^+$. On the other hand, IL-10$^-$ B cells produced TGF-β, IgM and IgG2b antibodies.

IL-10 production is induced with different stimuli and mainly TLR dependent. As it mentioned before, TLR2, TLR4 and TLR9 ligands could trigger IL-10 production from B cells. In *Helicobacter*-activated B cells, it has been shown that TLR2 is required for the production of IL-10. Also, this induction was found to be MyD88 dependent. However, downstream of this adaptor protein on the production IL-10 is still needed to be identified. In macrophages and myeloid dendritic cells, IL-10 production is shown to occur through TLR2 and TLR4 activation and MyD88 dependent or independent pathway. After that, ERK, NF-κB or p38 pathways are activated to induce IL-10 transcription from TLR2 activated innate cells (Saraiva & O'Garra, 2010). However, it is not known whether activation of these pathways has a role on IL-10 production from *Helicobacter*-activated B cells. In the present study, role of NF-κB p65 subunit on IL-10 production form *Helicobacter*-activated B cells were examined. It has been presented that *H.f.* sonicate could not induce translocation of NF-κB p65 subunit as a part of activation process (Figure 3.10). In order to check our primary antibody and NF-κB p65 inhibitory molecule PDTC, THP-1 cell line was used. THP-1 cells are known to be activated in the presence of LPS (Wang et al., 2011). The data show that LPS activated THP-1 cell line and inhibitory molecule PDTC could suppress this activation (Figure 3.11). The role of NF-κB p65 subunit on IL-10 producing *Helicobacter*-activated B cells was examined with an inhibitor study. Treatment of B cells with *H.f.* sonicates significantly increased IL-10 production. Prevention of NF-κB p65 activation by an inhibitory molecule did not lead to reduction of IL-10 production from *H.f.* sonicate treated B cells (Figure 3.12). It suggests that *H.f.* does not induce activation of canonical pathway of NF-κB while promoting IL-10 production. Other signaling pathways should be triggered for the induction of this regulatory cytokine expression and release. Therefore, other reported pathways should be studied with inhibitor studies to understand their role on IL-10 production from *Helicobacter*-activated B cells.

In conclusion, it has been shown that *Helicobacter*-activated IL-10^+ B cells showed $CD1d^+CD5^+$ or $CD21^+CD23^+$ phenotype. They do not produce antibody and TGF-β. IL-10 production seems to be independent from NF-κB p65 pathway in *Helicobacter*-activated B cells. These data may implicate that characterization of regulatory B cells through a unique surface marker is not possible, yet. Additionally, important transcription factors, which are required for the differentiation of IL-10

producing B cells and expression of IL-10 are needed to be understood. Antigen dependent responses lead to variable expressions of surface markers and therefore different subsets of B cells are present in the regulatory response. Expression of surface bound Igs and production of antibodies from activated B cells helps to identify them in detail. Intracellular signaling mechanism for IL-10 production needs to be understood. These findings may support that induction of B cells with *Helicobacter* ends up with IL-10 production in one way or TGF- β production in other way. Dual suppressive effect of B cells may be the reason of prolonged survival of *Helicobacter* in individuals without showing symptoms or cleared from their niches.

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CURRICULUM VITAE

Name Surname: Nesteren MANSUR

Place and Date of Birth: Istanbul, 28.02.1989

Address: Ferit Selim Paşa Cd. Güzide Hanım Sk. No:20 D:11 Bahçelievler/İstanbul

E-Mail: nesterenmansur@gmail.com

B.Sc.: Istanbul Technical University, Science & Letters Faculty, Department of Molecular Biology and Genetics, 2011