

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**FUNCTIONAL INTERACTIONS BETWEEN *HELICOBACTER*-ACTIVATED
B (H_{ACT}-B) CELLS AND CD4⁺ T CELLS**

M.Sc. THESIS

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Department of Molecular Biology-Genetics and Biotechnology
Molecular Biology-Genetics and Biotechnology Programme

DECEMBER 2015

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

***HELICOBACTER* –AKTİVE B (H_{AKT}- B) HÜCRELERİNİN CD4⁺ T
HÜCRELERİ İLE FONKSİYONEL ETKİLEŞİMLERİ**

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To my family and friends,

FOREWORD

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October 2015

Güliz Tuba BARUT

Bioengineer

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ABBREVIATIONS

µg	: Microgram
µm	: Micrometer
µM	: Micromolar
APC	: Antigen presenting cell
BCA	: Bicinchoninic Acid
BCR	: B Cell Receptor
Breg	: Regulatory B cell
BSA	: Bovine Serum Albumin
CagA	: Cytotoxin-associated gene A
CD	: Cluster of differentiation
CD40L	: CD40 ligand
cDNA	: Complementary DNA
CIA	: Collagen-induced arthritis
DC	: Dendritic cell
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
EAE	: Experimental autoimmune encephalomyelitis
EDTA	: Ethylenediaminetetraacetic acid
ELISA	: Enzyme-linked immunosorbent assay
FBS	: Fetal Bovine Serum
GTP	: Guanosine-5'-triphosphate
h	: Hour
Hact	: <i>Helicobacter</i> -activated
<i>H.felis</i>	: <i>Helicobacter felis</i>
<i>H.pylori</i>	: <i>Helicobacter pylori</i>
HRP	: Horseradish peroxidase
IFN-γ	: Interferon gamma
Ig	: Immunoglobulin
iTreg	: induced regulatory T cell
IL-10	: Interleukin-10

kDa : Kilodalton
L : Liter
LPS : Lipopolysaccharide
MALT: Mucosa-associated lymphoid tissue
MHC-I: Major histocompatibility complex class I
MHC-II: Major histocompatibility complex class II
min : Minute
ml : Mililiter
mM : Milimolar
mRNA : Messenger ribonucleic acid
MyD88: Myeloid differentiation primary response gene 88
NF- κ B : Nuclear Factor kappa B
nTreg : natural regulatory T cell
PBS : Phosphate buffered saline
PCR : Polymerase chain reaction
pg : Picogram
PMA : Phorbol 12-myristate 13-acetate
PE : Phycoerythrin
rpm : Revolutions per minute
RPMI : Roswell Park Memorial Institute
STAT : Signal transducer and activator of transcription
T4SS : Type IV secretion system
TCR : T cell receptor
TGF- β : Transforming Growth Factor-beta
Th1 : T helper 1
Th2 : T helper 2
Th3 : T regulatory type 3
Th9 : T helper 9
Th17 : T helper 17
Th22 : T helper 22
TIR : Toll-IL-1R
TLR : Toll-like receptor
TNF- α : Tumor necrosis factor-alpha
Tr-1 : T regulatory-1
Treg : Regulatory T cell

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FUNCTIONAL INTERACTIONS BETWEEN *HELICOBACTER*- ACTIVATED B (H_{ACT}-B) CELLS AND CD4⁺ T CELLS

SUMMARY

Helicobacter pylori is a spiral-shaped, gram-negative bacterium that infects the gastric mucosa of more than half of the world's population. The infection initially occurs in childhood, becomes persistent and the chronic infection leads to gastric inflammation. A major virulence factor of *H. pylori* is the cytotoxin-associated gene A (CagA) protein and this CagA protein interacts with several intracellular components of signal transduction and activates some crucial signaling pathways. *Helicobacter* has developed a variety of mechanisms to persist in the gastric mucosa. Gastric epithelial cells (GECs) are primary target for *H. pylori* infection, therefore they are the first point of contact for *H. pylori* and activate an innate immune response through TLRs. Rather than being a strong TLR4 ligand, *H. pylori* LPS is thought to activate TLR2 on gastric epithelial cells. Animal and cell culture experiments suggested that ligands in *Helicobacter* species can bind to TLR2 and activate NF- κ B in epithelial cells. In mouse studies, *Helicobacter felis* (*H. felis*) is mainly used because it is more immunogenic than *H. pylori* in mice.

B cells and their cytokines have important roles in *Helicobacter* infections by balancing between the infection and T cell driven gastric immunopathology. The ability of B cells to interact with pathogenic T cells and to produce anti-inflammatory cytokines such as IL-10 is crucial to dampen harmful immune responses. It has been found that B-cells exposed to *Helicobacter* sonicate produced large amounts of the regulatory cytokine IL-10. Using mouse models of *Helicobacter*-induced gastric premalignant pathology, it is shown that IL-10 secretion by purified B cells absolutely required MyD88 signaling and TLR-2.

The TLR-2- dependent *Helicobacter* activation of B cells differentiates them into IL-10 and TGF- β producing regulatory B cells. Both IL-10 and TGF- β have crucial effects on T cell differentiation. When co-cultured with *Helicobacter*-activated B cells, naive CD4⁺ T-cells are shown to produce IL-10 and differentiate into T regulatory 1 (Tr1)-like cells. In addition to that, it is suggested that Breg cells contribute to regulatory T-cell induction by producing TGF- β . At the same time, studies have shown convincingly that TGF- β is required for Th17 differentiation *in vitro* and *in vivo*.

In previous studies in our laboratory, *Helicobacter*-activated total B cells were separated into two subgroups: IL-10⁺ B cells and IL-10⁻ B cells. The experimental results showed that *Helicobacter*-activated IL-10⁺ B cells are the source of the IL-10 production while *Helicobacter*-activated IL-10⁻ B cells are mostly TGF- β positive. However, it was not clear if the *Helicobacter*-activated-IL-10⁺ B cells or the *Helicobacter*-activated-IL-10⁻ B were specifically causing the Tr1 differentiation. Taking account that Bregs are known for producing IL-10 and TGF- β which are key

cytokines in T cell differentiation, the interaction between *Helicobacter*-activated B cell subgroups and CD4⁺ T cell differentiation was investigated.

By magnetic isolation techniques, CD19⁺B cells and CD4⁺T cells were isolated from the spleens of C57BL/6 mice with high purities (with an average of 90% and 93%, respectively). Following the B cell isolation, cells were treated with *Helicobacter felis* sonicate (10 µg/ml) for 24 hours. For the last 5 hours of incubation, to induce an optimal IL-10 production and secretion, PMA (50 ng/ml) and ionomycin (500 ng/ml) were added. After the *in vitro* stimulation of B cells, IL-10 producing B cells were labeled and the IL-10⁺B and IL-10⁻B cells were separated. To observe the interaction between the *Helicobacter*-activated-B cell subgroups and CD4⁺ T cells, isolated CD4⁺ T cells were put on co-culture in 1:1 ratio with the *Helicobacter*-activated-IL-10⁺ B cells and *Helicobacter*-activated-IL-10⁻ B cells, for 24 hours. The cell surface markers and intracellular cytokine productions were examined by flow cytometry. While the supernatants of the co-culture groups were subjected to ELISA tests, the cell pellets were used for gene expression analyses.

The intracellular staining of IL-10 of T cells co-cultured with *Helicobacter*-activated B cell subgroups showed that about 15% of T cells co-cultured with *H_{ACT}*-IL-10⁺ B cells produced IL-10 while almost 20% of the T cell population was IL-10 positive when T cells were co-cultured with *H_{ACT}*-IL-10⁻ B cells. In addition, according to IL-10 ELISA results, CD4⁺T cells co-cultured with IL-10⁺B cells and IL-10⁻B cells secreted twice IL-10 when compared to only T cells. For IL-10⁺B cell and T cell co-culture, a part of secreted IL-10 came from B cells while most of the IL-10 secreted from IL-10⁻B cell and T cell co-culture originated is suggested to be from T cells.

CD25 has been used as a marker to identify activated T cells as well as some regulatory T cell subsets in mice; while CD62L has been known to rapidly shed from lymphocytes upon cellular activation. Both CD25 and CD62L levels in co-culture groups showed significant differences compared to T only groups. Furthermore, the differences in T cell CD25 and CD62L levels together might indicate that T cells co-cultured with *Helicobacter*-activated IL-10⁻B cells are more activated/differentiated compared to the T cells interacting with IL-10⁺B cells.

In literature, it has been shown that B cells which were activated by *Helicobacter* induce IL-10-producing CD4⁺CD25⁺ Tr1-like cells *in vitro*. In addition, it has been identified that the co-expression of CD49b and LAG-3 distinguishes Tr1 cells from Th1, Th2 and Th17 cells. Flow cytometry results of CD4-CD49b-LAG3 stainings showed that almost 20% of T cells co-cultured with IL-10⁺B cells express CD4-CD49b-LAG3 surface markers while only around 10% of CD4⁺ T cells co-cultured with IL-10⁻B cells express CD49b and LAG3 together.

CD4⁺ T cells obtain distinct functional properties in response to signals sent by commensal and pathogenic microbe-activated cells of the innate immune system. Th17 cells secrete interleukin-17 (IL-17), IL-17F, and IL-22 and have significant roles in protecting the host from bacterial and fungal infections, particularly at mucosal surfaces. Data of four independent experiments showed that the approximately 15% of CD4⁺ T cells co-cultured with IL10⁻B cells turned into IL17 producing CD4⁺ T cells while surprisingly about 8% of T cells also produced IL-17 when co-cultured with IL10⁺B cells. IL-17 ELISA results were parallel with the IL-17 intracellular cytokine staining analyses. Ror gamma T and IL-17 relative gene expression levels in co-culture groups showed similar results with flow cytometry and ELISA results.

Main research focus of this study was to investigate the interaction between H_{ACT}-B cell subgroups and CD4⁺ T cells and understand the effects of these B cell subgroups on T cell differentiation *ex vivo*. The results revealed that both *Helicobacter*-activated IL-10⁺B and IL-10⁻B cells induce IL-10 production from CD4⁺T cells and it might suggest that T cells differentiate into Tr1-like cells in both co-culture conditions. Although the CD25 and CD62L expression on T cell surfaces show significantly higher activation/differentiation in *Helicobacter*-activated IL-10⁻B and T cell co-cultures, regulatory type indicator CD4-CD49b-LAG3 surface marker co-expressions were higher on T cells in the *Helicobacter*-activated IL-10⁺B and T cell co-cultures. Furthermore, intracellular IL-17A levels and relative gene expression experiments of IL-17A and RorgammaT showed significantly higher results in IL-10⁻ B and T cell co-culture groups, as expected. On the other hand, interestingly IL-17 production was also observed in T cells co-cultured with IL-10⁻ B cells. The reason behind the IL-17 and RorgammaT expressions in IL-10⁺ B-T cell co-culture groups was suggested to be the IL-6 and TGF- β produced by IL-10 negative B cell population in the IL-10 positive co-culture group. This study has contributed to the literature through providing a first step to show the *ex vivo* interactions of *Helicobacter*-activated B cells subgroups, IL10⁺ H_{ACT} B cells and IL-10⁻ H_{ACT} B cells, with CD4⁺ T cells.

HELICOBACTER-AKTİVE (H_{AKT}-B) HÜCRELERİNİN CD4⁺ T HÜCRELERİ İLE FONKSİYONEL ETKİLEŞİMLERİ

ÖZET

Helicobacter pylori, dünya popülasyonunun yarısından fazlasını enfekte etmiş bulunan, gram-negatif, spiral yapıda, mikraerofilik bir bakteri olmakla beraber gastritten mide kanserine kadar uzanan gastrik patolojilerin temel risk faktörü olarak tanımlanmıştır. Enfeksiyonlar genelde çocukluk çağlarında meydana geldiğinden, dünya genelinde enfekte olan kişi sayısı oldukça fazla olmasına karşın, enfekte bireylerin büyük bir kısmı herhangi bir semptom göstermezken, popülasyonun yalnızca % 20'sinde gastrite, gastrik ve duodenal ülserlere ve ilerleyen zamanda gastrik adenokarsinoma sebep olmaktadır. *Helicobacter*'in etkisini incelemek için fare modelleri kullanılarak yapılan çalışmalarda, *Helicobacter pylori*'ye yakınlığıyla bilinen ve CagA virülans faktörü bulundurmamasına rağmen enfeksiyonlarda *pylori*'ye oldukça benzer sonuçlar veren *Helicobacter felis* kullanılmaktadır. *Helicobacter felis* de gram negatif, helikal şekilli ve mikraerofilik bir bakteridir. İmmün sistem hücreleri, mikroçevrelerinde bulunan patojenleri tanımak için hücre yüzeylerinde bazı reseptörlere ihtiyaç duymaktadır.

Toll benzeri reseptörler (Toll-like receptors -TLR), birçok patojenin varlığında sinyal üreterek doğal immün cevabın oluşmasını sağlayan bir grup tip 1 transmembran proteindir. Hayvan ve hücre kültürü deneyleri, *H.pylori* kaynaklı ligandların B hücrelerinde aktivasyonu TLR2 yolu ile yaptığı ortaya konmuştur. Antijen sunucu hücreler (APC- antiger presenting cell) olan B hücrelerinin ve T hücrelerinin IL-10 üreten regülatör alt tipleri olduğu bilinmektedir. Güncel araştırmalar, *Helicobacter felis* (*H.felis*) enfeksiyonlu fare modellerinde TLR-2 yoluyla IL-10 üreten B hücrelerinin immün cevabı baskılayıcı ve düzenleyici rolü olduğunu ortaya koymuştur.

Yardımcı T (Th) hücrelerinin bir alt kümesi olan Th17 (T helper 17) hücrelerinin ve salgıladıkları interlökin 17 (IL-17) sitokininin, *Helicobacter* gibi bakterilerin temizlenmesinde kritik rolü vardır. Th17 dönüşümü için transforme edici büyüme faktörü- β (TGF- β), IL-21, IL-6 gibi sitokinleri etkili olduğu önerilmiştir. Th17 için oldukça önemli bir transkripsiyon faktörü olan ROR γ t, IL-17 gen transkripsiyonunu tetikler. Fakat kronik gastrik enflamasyon sırasında, üretilen IL-17 seviyesinin enfeksiyonu temizlemeye yeterli olmadığı görülmektedir. Buna sebep olarak, bakterinin kendi devamlılığını sağlamak amacıyla diğer immün hücreleri aracılığıyla, T hücrelerini, Th17 etkisini bastıran ve düzenleyici etkiler gösteren regülatör T hücrelerine dönüştürdüğü düşünülmektedir. Doğal regülatör T hücreleri olarak bilinen nTreg'ler (CD4⁺CD25⁺FoxP3⁺) timustaki gelişim sırasında fonksiyon kazanırken, indüklenebilir Treg (iTreg) periferde naif CD4⁺ T hücrelerin farklılaşması sırasında gelişir. Fare ve insan Tr1 hücreleri ile yapılan çalışmalarda, hücre yüzeyinde bulunan CD49b ve lenfosit aktivasyon geni 3 (LAG-3)'ün her

ikisinin de aynı anda yüzeyde eksprese edilmesinin IL-10 salgılayan Tr1 hücrelerini Th1, Th2 ve Th17 hücrelerinden ayırdığı ortaya atılmıştır.

Helicobacter felis ile yapılan fare enfeksiyon modeli çalışmalarında, *Helicobacter* ile aktive edilen B hücrelerinin *in vivo* ve *in vitro* koşullarda T hücrelerini IL-10 üreten CD4⁺CD25⁺ Tr1-benzeri hücrelere dönüştürdükleri gösterilmiştir. Bu çalışmalarda, B hücreleri tarafından indüklenen Tr1 hücrelerinin *in vitro* koşullarda baskılayıcı aktivite gösterdiği ve *in vivo* koşullarda *Helicobacter*'e bağlı immünopatolojiyi baskıladığı ortaya konmuştur. Ayrıca, daha önce laboratuvarımızda yapılan çalışmalarda, *Helicobacter*-aktive B hücrelerinin TGF- β ürettiği gösterilmiştir. Ancak *Helicobacter*-aktive B hücrelerinden, IL-10 üreten ya da üretmeyen gruplardan hangisinin T hücrelerini IL-10 üreten Tr1-benzeri hücrelere dönüştürmede rolü olduğu bilinmemektedir. Bu nedenle, bu çalışmada *Helicobacter*-aktive B hücre alt gruplarının CD4⁺ T hücre farklılaşmaları (Tr1 veya Th17) üzerine olan etkilerinin incelenmesi amaçlanmıştır.

Manyetik izolasyon teknikleri kullanılarak, CD19⁺ B hücreleri ve CD4⁺ T hücreleri, C57BL/6 farelerin dalaklarından %90'dan yüksek saflıklarla izole edilmiştir. B hücre izolasyonunu takiben hücreler *Helicobacter felis* sonikatu ile 24 saat muamele edilmiştir. Optimum bir IL-10 üretimi için inkübasyonun son 5 saatinde PMA ve iyonomisin hücrelere eklenmiştir. B hücrelerinin *Helicobacter felis* sonikatu ile *in vivo* tetiklenmesinin ardından IL-10 üreten hücreler manyetik olarak etiketlenerek bu sitokini üretmeyen IL-10 negatif B hücrelerinden manyetik ayırım yöntemi ile ayrılmıştır. *Helicobacter*-aktive B hücre alt grupları olan IL-10⁺ H_{AKT}-B hücreleri ve IL-10⁻ H_{AKT}-B hücrelerinin CD4⁺ T hücreleri üzerindeki etkilerinin incelenmesi için bu hücreler 24 saat boyunca ko-kültüre konmuştur. *Helicobacter*-aktive B hücre alt gruplarının CD4⁺ T hücre farklılaşmaları (Tr1 veya Th17) üzerine olan etkileri RNA (kantitatif PZR) ve protein düzeyinde (ELIZA, hücre içi akan hücre ölçer analizi) incelenmiştir.

Hücre içinde özgün antikorlar ile boyanan IL-10 analiz sonuçları göstermektedir ki, hem IL-10⁺ H_{AKT}-B hücreleri hem de IL-10⁻ H_{AKT}-B hücreleri CD4⁺ T hücrelerinde IL-10 üretimine yol açarak bu hücrelerin Tr1-benzeri hücrelere dönüşmesinde rol almaktadır. 24 saat ko-kültür boyunca hücre dışına salgılanan IL-10'un ölçümünde kullanılan IL-10 ELISA sonuçları da akan hücre ölçer sonuçlarını desteklemektedir. Aktive olmuş ve regülatör T hücrelerinin yüzeyinde bulunan CD25'in ve T hücrelerinde aktive olduktan sonra ekspresyonu azalan CD62L'nin özgün antikorlarla etiketlenerek akan hücre ölçerde analizlerinin sonucunda IL-10⁻ H_{AKT}-B hücreleri ile ko-kültürde tutulan CD4⁺ T hücrelerinin daha fazla aktive olduğu ya da farklılaştığı önerilmektedir. Literatürde regülatör T hücre belirteci olarak kullanılan CD49b ve LAG-3 yüzey belirteçlerinin birlikte eksprese edilmesinin incelenmesi sonucunda, IL-10⁺ H_{AKT}-B hücreleri ile birlikte tutulan T hücrelerinin yaklaşık %20'si bu belirteçleri bir arada gösterirken, IL-10⁻ H_{AKT}-B hücreleri ile ko-kültürde tutulan T hücrelerinin ancak %10'u bu belirteci aynı anda eksprese etmektedir.

İkincil olarak, *Helicobacter*-aktive B hücreleri ile etkileşime giren T hücrelerindeki IL-17 üretimi incelenmiştir. Hücre dışına salınımı engellenen IL-17 sitokininin hücre içi boyama sonuçlarına göre IL-10⁻ H_{AKT}-B hücreleri ile ko-kültürde tutulan T hücrelerinin %15'i IL-17 üretimi gerçekleştirirken, IL-10⁺ H_{AKT}-B hücreleri T hücrelerinin yaklaşık %8'ini IL-17 üretmesi için tetiklemiştir. IL-17 ELISA sonuçlarının yanı sıra IL-17 üretiminde önemli bir transkripsiyon faktörü olan Ror

gamma T ve IL-17'nin gen düzeyinde kantitatif PZR ile incelenmesinin sonuçları da akan hücre ölçer sonuçları ile paralellik göstermiştir.

Bütün bu sonuçlar bir araya getirildiğinde, *Helicobacter*-aktif B hücre alt grupları her ikisinin de CD4⁺ T hücrelerinin IL-10 ürettiğini göstermiştir. Bu sonuçlara dayanarak, IL-10⁺ H_{AKT}-B ve IL-10⁻ H_{AKT}-B hücrelerinin T hücrelerinin Tr-1 benzeri hücrelere dönüşmesinde etkisinin olduğu ortaya atılmıştır. Regülatör T hücre belirteci olarak kabul edilen CD49b-LAG3 yüzey belirteçlerinin ko-ekspresyonunun, IL-10⁺ H_{AKT}-B hücreleri ile birlikte tutulan CD4⁺ T hücrelerinde daha yüksek olması bu hücrelerin regülatör profile daha yakın olduğunu önerirken, CD25 ve CD62L sonuçları IL-10⁻ H_{AKT}-B hücreleri ile ko-kültürde tutulan T hücrelerinin daha aktif olmuş/farklılaşmış olduğunu desteklemektedir. Fakat bu aktivasyonun ya da farklılaşmanın özgün olup olmadığı belli değildir. Bununla beraber, IL-17'nin hem protein düzeyinde hem de gen düzeyindeki incelemeleri CD4⁺ T hücrelerini Th17 benzeri hücrelere farklılaşmasında IL-10⁻ H_{AKT}-B hücrelerinin IL-10⁺ H_{AKT}-B hücrelerine kıyasla daha fazla rolü olduğunu göstermiştir. Th17 farklılaşmasında TGF-beta'nın önemi göz önünde bulundurulduğunda, bu sonuçlar, daha önceden IL-10⁻ H_{AKT}-B hücrelerinin, IL-10 pozitif gruba göre daha fazla TGF-beta ve IL-6 ürettiğinin gösterilmesi ile tutarlıdır. Bu çalışma literatüre, *Helicobacter*-aktif B hücre alt grupları, IL10⁺ H_{AKT} B ve IL10⁻ H_{AKT} B hücrelerinin, CD4⁺ T hücreleri ile *ex vivo* ilişkisini gösteren ilk çalışma olarak katkıda bulunmaktadır.

1. INTRODUCTION

1.1 *Helicobacter* Species

1.1.1 *Helicobacter pylori* (*H. pylori*)

Helicobacter pylori is a spiral-shaped, gram-negative bacterium that infects the gastric mucosa of more than half of the world's population (Figure 1.1). The infection initially occurs in childhood, becomes persistent and the chronic infection leads to gastric inflammation (Marshall, 1994). It is the major cause of gastritis, gastric and duodenal ulcers, as well as gastric adenocarcinoma (Parsonnet, 1991; Coghlan, 1987).

A major virulence factor of *H. pylori* is the cytotoxin-associated gene A (CagA) protein and it has been shown that patients infected with *cagA*⁺ strains have higher levels of inflammatory responses and they have a higher risk of developing peptic ulcer or gastric cancer (Uemura, 2001; Blaser, 1996). CagA is encoded by the *cagA* gene within the *cag* pathogenicity island, a 40-kb chromosomal region that encodes for a type IV secretion system (T4SS). This CagA protein interacts with several intracellular components of signal transduction and activates NF- κ B, MAPK, STAT3, and PI3K/Akt pathways (Churin, 2013; Lee, 2012). Previous reports have highlighted the fact that *H. pylori* activates STAT3 to modulate host immune responses (Lee et al., 2012).

Gastric epithelial cells (GECs) are the primary target for *H. pylori* infection. After *H. pylori* adheres to GECs, the CagA protein is translocated into their cytosol via a T4SS (Asahi, 2000). Once inside GECs, CagA becomes phosphorylated and elicits multiple cell responses, including disruption of epithelial tight junctions, cytoskeleton rearrangement, changes in cellular adhesion properties and polarity, as well as secretion of proinflammatory mediators (Bourzac, 2005; Hatakeyama, 2004). Despite the marked inflammatory response within the *H. pylori*-infected gastric mucosa, the host immune response is unable to clear *H. pylori*, resulting in

persistent infection and development of chronic gastric inflammation (Beswick, 2005; Müller, 2011).

1.1.2 *Helicobacter felis* (*H. felis*)

Helicobacter felis, which does not express CagA or VacA, causes chronic infection and inflammation in a well-characterized mouse model. Being closely related to *Helicobacter pylori*, *Helicobacter felis* is also a gram negative, helical shaped, microaerophilic bacterium that colonizes in the stomach of cats and mice (Figure 1.1). Lee and his colleagues first isolated *H. felis* from stomach of cats in 1988 (Lee, 1988). Even though, *H.felis* does not have CagA or VacA virulence factors, the *Helicobacter felis* infection and prognosis of disease in mouse models have a striking resemblance to *Helicobacter pylori* infection in humans (Mohammadi, 1996; Mc Cracken, 2005).

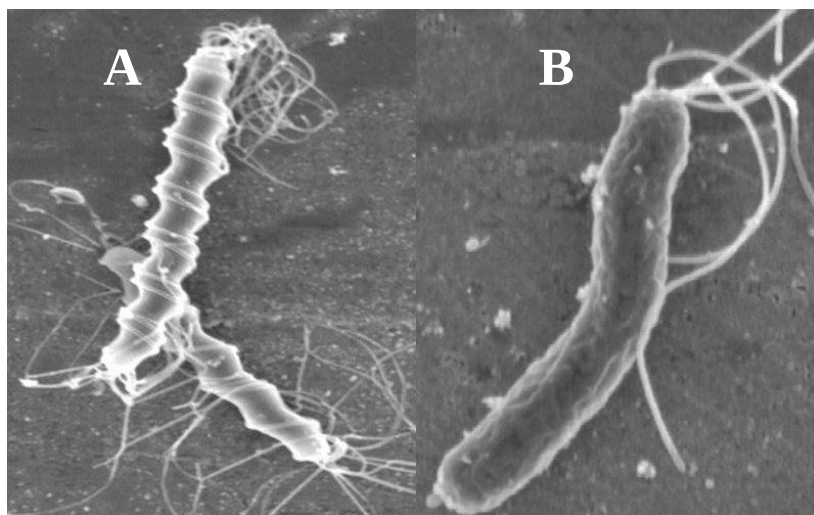


Figure 1.1: *Helicobacter pylori* and *Helicobacter felis*. (A) Scanning electron micrograph of *Helicobacter felis* isolated from the gastric mucosa of a cat. The characteristic spiral morphology and periplasmic fibers are shown. (B) Scanning electron micrograph of *H.pylori* from human gastric epithelium. The gently curved morphology, multiple polar flagella, and absence of periplasmic fibers are shown. Micrographs courtesy of Lucinda Thompson, presently at Stanford University, USA.

1.2 B Cells

In 1965, Max Cooper and Robert Good published a landmark study in *Nature* that led to the birth of the B cell field (Cooper, 1965). Working with chickens, they showed that cells that develop in the bursa of Fabricius (B cells) are responsible for antibody production, whereas those cells that develop in the thymus (T cells)

are necessary for delayed-type hypersensitivity responses. B lymphocytes are essential components of the humoral immune response. Produced in the bone marrow, B cells migrate to the spleen and other secondary lymphoid tissues where they mature and differentiate into immunocompetent B cells (Figure 1.2).

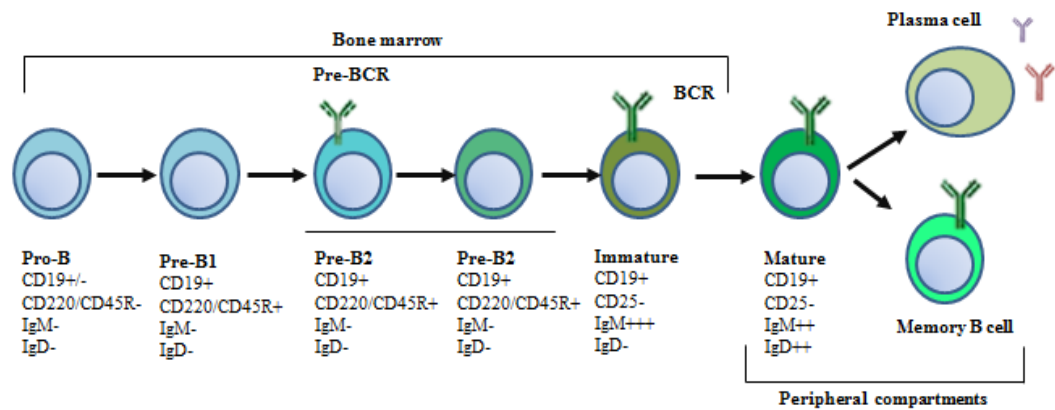


Figure 1.2: B cell development. Stages of B-cell maturation are indicated by their anatomical site and the expression of cell-surface markers.

The initial part of B cell development occurs in the bone marrow and this antigen-independent step generates mature and immunocompetent B cells that bind to a unique antigen. The antigen-independent maturation into immunocompetent naïve, mature follicular B cells is completed in the bone marrow and spleen. Following maturation in the bone marrow and spleen, immunocompetent B cells remain in peripheral tissues until they encounter an antigen. The first activation signal occurs upon antigen binding to B cell receptors (BCRs). Upon binding to the BCR, the antigen is internalized by receptor-mediated endocytosis, digested, and complexed with Major histocompatibility complex class II (MHC II) molecules on the B cell surface.

The second activation signal occurs via either a thymus-dependent or a thymus-independent mechanism. The thymus-dependent activation requires the interaction of B cells with T helper cells. B cells acting as antigen-presenting cells (APCs) to T cells, T cell receptors (TCR) on T helper cells bind to the antigen-complexed MHC class II molecule on the B cell surface resulting in T cell activation. The second activation signal to the B cells is then provided by these activated T cells via CD40-CD40L (CD154) interaction and cytokine secretion (Figure 1.3) (Pierce, 2002).

Alternatively, there are a few thymus-independent activation types where antigens can directly provide the second B cell activation signal. These antigens include components of some bacterial cell wall components (e.g., lipopolysaccharide) or antigens containing highly repetitious molecules (e.g., bacterial flagellin). 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).

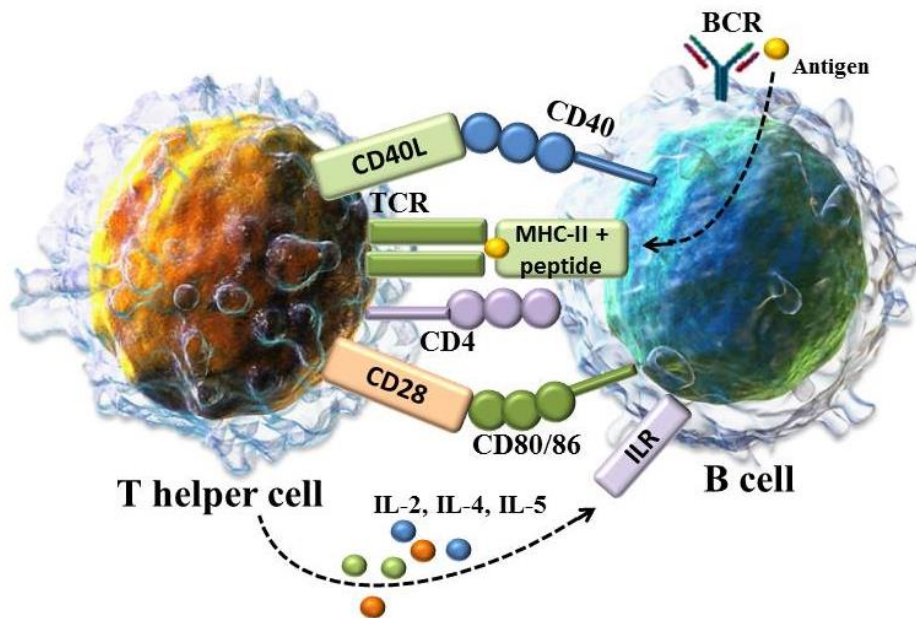


Figure 1.3: T cell-dependent B cell activation. B cells will capture antigen through their B cell receptor and display peptides on their MHC II molecules. This attracts T cells specific for the peptide. These T cells can then provide costimulatory molecules such as CD40L on a T cell that binds to CD40 on the B cell that help the B cell to mature (adapted from Biolegend).

As an important B cell surface marker, CD19 is a 95 kD type I transmembrane glycoprotein also known as B4. It is expressed on B-cells from pro-B to blastoid B cells, absent on plasma cells (Kumar, 2010). CD19 is involved in B cell development, activation, and differentiation.

1.2.1 Plasma and memory B cells

Upon activation, B cells proliferate and form germinal centers where they differentiate into memory B cells or plasma (effector) B cells. Following differentiation into plasma cells, additional signals initiate plasma cell antibody class switching and regulate antibody secretion. The primary function of plasma cells is the secretion of B cell clone-specific antibodies. Each plasma cell secretes antibodies containing a clonally-unique antigen-binding region joined to a constant immunoglobulin (Ig) isotype-defining region.

The success of humoral memory depends on at least two layers of defense. If the antibody concentration at the site is sufficiently high, the pre-existing protective antibodies secreted by long-lived plasma cells function as a first line of defense. If this constitutive memory is not sufficient, the pathogen-experienced memory B cells are rapidly reactivated to produce antibodies by reactive humoral memory. Compared with the primary antibody response, the reactive humoral memory response is typically faster, of greater magnitude and consists of antibodies of switched isotypes and higher affinity (Ahmed&Gray, 1996).

1.2.2 Regulatory B cells

The immune response has evolved to protect the host from a wide range of potentially pathogenic microorganisms, but parallel mechanisms to control over extreme immune responses to limit host damage. Because of their capability to produce antibodies, including autoantibodies, B cells are generally considered to be positive regulators of the immune response. As antigen-presenting cells, B cells facilitate optimal CD4⁺ T cell activation and exert other modulatory functions in immune responses. On the other hand, certain B cells can also negatively regulate the immune response by producing regulatory cytokines and directly interacting with pathogenic T cells via cell-to-cell contact. The hypothesis that suppressor or regulatory B (Bregs) cells control the immune system was proposed in the 1970s and maintains that the suppressive function of B cells was mainly restricted to their ability to produce 'inhibitory' antibodies (Morris&Moller, 1968). Bhan and Mizoguchi were the first to introduce the term 'regulatory B cells' (Mizoguchi et al.,1997). The regulatory function of Breg cells has been demonstrated in mouse models of inflammation, cancer, transplantation, and particularly in autoimmunity

(Yang et al., 2013). Regulatory B cells have immunosuppressive roles that support immunological tolerance. Janeway and colleagues first observed that transgenic mice lacking B cells suffered an unusually severe and chronic form of experimental autoimmune encephalomyelitis (EAE), indicating that B cells have regulatory properties in a mouse model of EAE (Wolf et al., 1996). After a while, it was found that B cells affected this autoimmune disease by regulating interleukin-10 (IL-10) (Fillatreau et al., 2002). By producing IL-10, IL-35, and transforming growth factor β (TGF- β), Breg cells suppress immunopathology by prohibiting the expansion of pathogenic T cells and other pro-inflammatory lymphocytes (Rosser, 2015). In several animal models, Breg stimulation is effective in correcting a variety of autoimmune disorders, most notably those initiated in the mucous membranes (Berthelot, 2013). Nonetheless, these cells can be phenotypically difficult to identify, as they bear many common markers found on other B cell types. The most well studied Bregs may be the B10 with known surface markers CD19, CD1d, CD24, CD5, CD21, CD23 and Transitional 2 Marginal Zone Precursor (T2-MZP) (surface markers CD19, CD1d, CD24 and CD93) population (Mauri, 2010).

1.2.3 Activation and differentiation of regulatory B cells

Through their B cell receptors (BCR), B cells can respond directly to naïve antigen, which also enables an earlier involvement of Bregs in an immune response, when compared to regulatory T cells. It has been suggested that factors present in the microenvironment may play a crucial role in the induction of Breg cells (Gray, 2010). Toll-Like Receptors (TLRs) play a critical role in the early innate immune response to invading pathogens by sensing microorganisms in these microenvironments.

Toll receptor was first identified in *Drosophila melanogaster* as an essential receptor for fungal infection by Christiane Nüsslein-Volhard in 1985 (Lemaitre, 1996). After the discovery of Toll, its homologue Toll like-receptor (TLR4) was identified in mammals. Toll-like receptors can be activated via pathogen-associated molecular patterns (PAMPs) and danger-associated molecular pattern molecules (DAMPs). Cytoplasmic domain of Toll resembles IL-1 receptor so it's named as TIR (Toll-IL-1 receptor) domain. TIR domain mediates intracellular

signaling. TLRs have leucine- rich repeats, which are required for ligand binding. There are 5 TIR adaptor molecules that play a role in signaling pathway: MyD88 (myeloid differentiation primary response gene -88), Mal (MyD88-adaptor -like), TRIF (TIR domain- containing adapter-inducing interferon-), TRAM (Toll-like receptor 4 adaptor protein), and SARM (sterile alpha and HEAT/Armadillo motif). These adaptor proteins activate kinases and induce the secretion of cytokines by NF- B and MAPK pathways. TLR stimulation also leads to activation of JAK/STAT signaling pathway, which is important in cytokine secretion (Schindler, 2007).

Lampropoulou et al. (2008) demonstrated that certain TLR agonists to be potent inducers of B cells with suppressive functions. In a mouse model of experimental autoimmune encephalomyelitis (EAE), LPS from Gram-negative bacteria and CpG-containing oligonucleotides that mimic bacterial DNA have been shown to inhibit disease progression by inducing IL-10-producing B cells whereas mice containing B-cell deletions of Tlr2, Tlr4 or the TLR adaptor myeloid differentiation primary-response gene 88 (MyD88). As a result, the mice could not recover from EAE (Lampropoulou et al., 2008). This showed that TLRs are directly involved in modulating the regulatory function of B cells. TLR-signaling has been shown to initiate IL-10 production, however B cell receptor signaling is also critical for regulatory B cell development. In a study with CD19-deficient murine model, BCR activity was downregulated and results showed decreased regulatory B cell population (Kalampokis et al., 2013). Accumulating data support a two-step model for the establishment of B-cell-mediated suppression. During the initial stage, TLR stimulation induces only a few IL-10-producing B cells. During the second phase, BCR and CD40 ligation, which are classically involved in B-cell survival and expansion, further amplifies the population of IL-10-producing B cells, which results in sufficient IL-10 production for effective suppression (Lampropoulou et al., 2008). While IL-10 expression is considered central to the mechanism of Breg function, suggested that TGF- β may be equally important in the function of Bregs (Lee et al., 2014).

1.2.3.1 IL-10 producing regulatory B cells

Regulatory B cells are present in several murine models of chronic inflammation, including collagen-induced arthritis (CIA), inflammatory bowel disease, and experimental autoimmune encephalomyelitis (Mauri, 2003; Mizoguchi, 2002; Fillatreau, 2002). Their regulatory function appears to be directly mediated by the production of IL-10 and by the ability of B cells to interact with pathogenic T cells to dampen harmful immune responses (Mauri, 2008). IL-10 is a major anti-inflammatory mediator that can be produced by numerous cell-types (Moore et al., 2001). In the early 90's, a particular subset of mouse B cells expressing CD5 was shown to be an important producer of IL-10 in response to LPS (O'Garra et al., 1992). Mainly located in the peritoneum, B1 cells largely contribute to the production of IgM antibodies, which play an important role as a first line of defense against bacterial and viral pathogens (Ochsenbein et al., 1999). Therefore, B1 cells represent a double edge sword with an innate arm against infection by producing T cell independent antibodies and with a potential regulatory role through the production of IL-10. Later, a unique CD1d^{hi}CD5⁺CD19^{hi} subset, identified in spleens of naïve wild-type mice, is suggested as the Breg lineage that contributes to the majority of B cell IL-10 production, once activated by stimulation *in vitro* (Matsushita, 2011). Due to the fact that this novel B-cell subset only produces IL-10, they have been named B10 cells. Expression of IL-10 and CD19 are the most widely used markers for IL-10 producing Bregs. The importance of regulatory B cells is demonstrated by the discovery of parallel B cells in humans. Miyagaki et al. reviewed the significance of regulatory B cells in mouse models of disease and extend these findings to what has been described in humans (Miyagaki et al., 2015). Nevertheless, in comparison to the wealth of data relating to Tregs, little is known regarding the possible existence of Bregs in humans. It is clear that activated human B cells, similar to the mouse B cells, can produce significant quantities of cytokines that might influence the pathological environment (Pistoia, 1997; Harris et al., 2000). Depending on the signals B cells receive, pro- or anti-inflammatory cytokines can be produced. The cell-surface markers generally associated with Bregs are depicted in Table 1.1.

Table 1.1: Different Bregs with their surface markers and mechanisms of suppression.

Subtype	phenotype	Mechanism of suppression	References
Phenotypes of mouse Breg subsets:			
T2-MZP B cells	CD19 ⁺ CD21 ^{hi} CD23 ^{hi} CD24 ^{hi} IgM ^{hi} IgD ^{hi} CD1d ^{hi}	IL-10	Evans et al., 2007
MZ B cells	CD19 ⁺ CD21 ^{hi} CD23 ⁻ CD24 ^{hi} IgM ^{hi} IgD ^{lo} CD1d ^{hi}	IL-10	Gray et al., 2007
B10 cells	CD19 ^{hi} CD1d ^{hi} CD5 ⁺	IL-10	Yanaba et al., 2008
B-1a cells	CD5 ⁺	IL-10	O'Garra et al., 1992
Killer B cells	CD5 ⁺ CD178 ⁺	FasL	Lundy, et al., 2009
GIFT-15 B cells	B220 ⁺ CD21 ⁺ CD22 ⁺ CD23 ⁺ CD24 ⁺ CD1d ⁺ CD138 ⁺ IgD ⁺ IgM ⁺	IL-10	Rafei et al., 2009
Plasma cells	CD138 ^{hi} IgM ⁺ TACI ⁺ CXCR4 ⁺ CD1d ^{hi} Tim1 _{int}	IL-10, IL-35	Shen et al., 2015
Plasmablasts	CD138 ⁺ CD44 ^{hi}	IL-10	Matsumoto, et al., 2014
TIM-1 ⁺ B cells	-	IL-10	Ding et al., 2011
PD-L1 ^{hi} B cells	CD19 ⁺ PD-L1 ^{hi}	PD-L1	Khan et al., 2015
-	B220 ⁺ CD39 ⁺ CD73 ⁺	Adenosine	Kaku et al., 2014
Phenotypes of human Breg subsets:			
Immature B cells	CD19 ⁺ CD24 ^{hi} CD38 ^{hi}	IL-10, PD-L1	Blair et al., 2010
B10 cells	CD19 ⁺ CD24 ^{hi} CD27 ⁺	IL-10	Iwata, et al., 2011
GrB ⁺ B cells	CD19 ⁺ CD38 ⁺ CD1d ⁺ IgM ⁺ CD147 ⁺	GrB, IL-10, IDO	Lindner et al., 2013
Br1 cells	CD25 ^{hi} CD71 ^{hi} CD73 ^{lo}	IL-10, IgG4	van de Veen et al., 2013
Plasmablasts	CD27 ^{int} CD38 ^{hi}	IL-10	Matsumoto et al., 2014
-	CD39 ⁺ CD73 ⁺	Adenosine	Saze et al., 2013
iBregs	-	TGF-β, IDO	Nouël et al., 2015

Tim-1: T cell Ig domain and mucin domain protein 1; PD-L1: Programmed death-ligand 1; GrB: granzyme B; IDO: indoleamine-2,3-dioxygenase.

In contrast to autoimmune processes, these Breg are innate cells, which are activated and mobilized through the recognition of pathogen associated molecular patterns by TLR. The capacity of LPS to engage innate B cells with anti-inflammatory properties raises the possibility of a regulatory role of B cells in

bacterial infections. It has been shown that CD5⁺ B cells produce IL-10 in response to LPS, to agonists of TLR2, 5, 7/8 and 9 (Sun et al., 2005; Zhang et al., 2007). It has been suggested that TLR2 agonists are specialized in inducing IL-10 expression by antigen-presenting cells (APCs) (Agrawal et al., 2003; Dillon et al., 2004; Netea et al., 2004; Hu et al., 2006). Following TLR ligation, signaling cascades are activated through Toll/IL-1 receptor (TIR)-domain-containing adaptor molecules, such as MYD88 and TIR-domain containing adaptor protein inducing IFN β (TRIF; also known as TICAM1), leading to the production of IL-10 and pro-inflammatory cytokines (Boonstra et al., 2006; Dillon et al., 2004; Hacker et al., 2006). TLR signaling through MYD88 leads to the activation of mitogen activated protein kinases (MAPKs) and NF- κ B (Akira&Takeda, 2004). The MAPK cascade is composed of three major groups of kinases: extracellular signal-regulated kinases (ERKs) (comprising ERK1 (also known as MAPK3) and ERK2 (also known as MAPK1), which are collectively referred to here as ERK); Jun N-terminal kinases (JNKs) (comprising JNK1 (also known as MAPK8) and JNK2 (also known as MAPK9)); and p38 (Symons et al., 2006). Following TLR stimulation, activation of ERK modulates IL-10 expression, and in the presence of chemical inhibitors of ERK or in ERK-deficient cells IL-10 production by TLR-activated DCs is decreased (Agrawal et al., 2006).

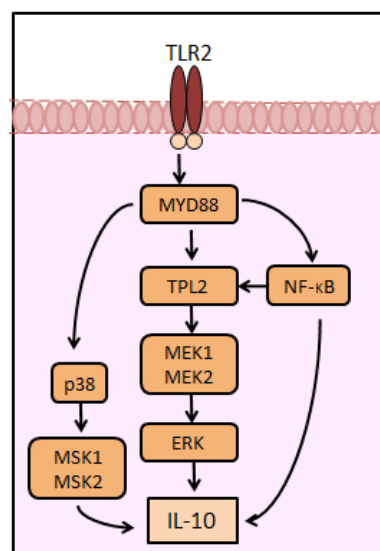


Figure 1.4: TLR2 signaling pathways that induce IL-10 production (adapted from Saraiva & O'Garra, 2010).

There are several direct and indirect mechanisms by which Breg cells exert their regulatory functions during the immune response (Figure 1.5). Regulatory B cells (Bregs) suppress activation and differentiation of CD4⁺, CD8⁺, and NK T cells primarily via the release of the anti-inflammatory cytokines IL-10 and TGF- β . Engagement of costimulatory pathways including CD154-CD40 and CD28-B7 are required for the release of IL-10 by Bregs. A Breg-T cell interaction results in an inhibition of T-cell activation and Th1 differentiation; induction of Tregs (Tr1); inhibition of DC activation, and hence suppression of T-cell activation; and clearance of apoptotic cells mediated via the release of autoreactive antibodies. B cells can also promote DCs to not only secrete IL-4 but also downregulate IL-12, which affects the Th1/Th2 balance (Moulin et al., 2000). The suppressive effect that Bregs exert on T cells might also provoke immune responses against autologous tumor cells. Moreover, transfer of a relatively low number of *in vitro* expanded Breg cells maintains long-term protection against several autoimmune diseases in animal models, which suggests that Breg cells can either further proliferate *in vivo* or initiate an efficient immunosuppressive cascade with other immune suppressive cells (Mauri, 2010). Breg cells can not only suppress effector T cell mediated immune responses but also convert effector T cells into regulatory Tr1 cells (Grammer et al., 2003; Gray et al., 2007; Carter et al., 2011). Gray et al. (2007) have clearly shown that apoptotic cells induce B and T cells to produce IL-10.

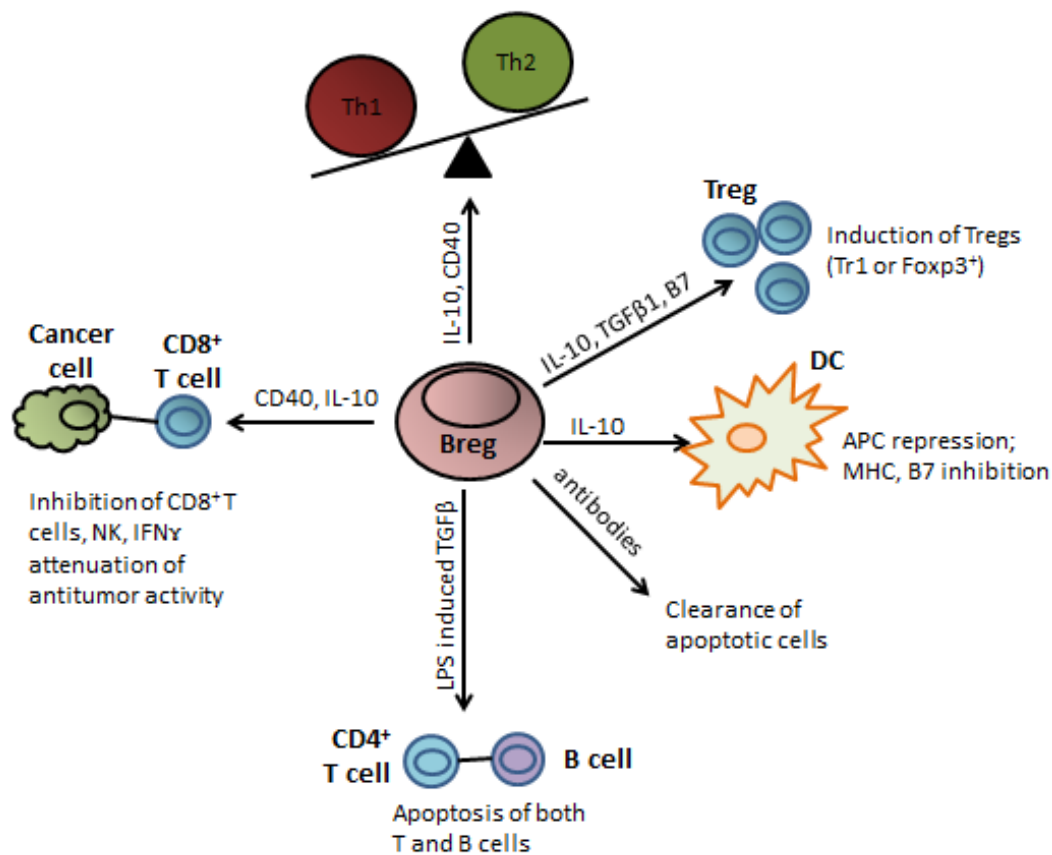


Figure 1.5: Suppressive regulatory B-cell-mediated effector mechanisms in the immune response. Tr1, Type 1 regulatory cell (adapted from Mauri & Ehrenstein, 2007).

1.2.3.2 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. TGF- β is first synthesized as pro-TGF- β and then it is cleaved by furin proprotein convertase in the Golgi apparatus to produce the dimeric propeptides called latency-associated peptide (LAP) that non-covalently associates with the dimeric mature TGF- β to prevent its activity. This complex can further associate with latent-TGF- β -binding protein (LTBP) to produce a large latent form for deposition onto the extracellular matrix (Taylor, 2009). Upon cell-cell contact with a potential target cell, cell surface LAP is stripped away and functionally active TGF- β becomes available for suppression.

While IL-10 expression is considered central to the mechanism of Breg function, it is suggested that TGF- β may be equally important in the function of Bregs (Lee

et al., 2014). In a model of antibody-induced transplantation tolerance, Lee et al. (2014) showed that, Breg cells promote graft survival by promoting Treg-cell development, possibly via TGF- β production. According to their results, Breg cells express the TGF- β with latency-associated peptide (LAP) and Breg-cell mediated graft prolongation post-adoptive transfer is abrogated by neutralization of TGF- β activity. In addition to secreted TGF- β , the membrane-bound LAP/TGF- β complex is shown to exert suppressive function as well (Xiao et al., 2012). It is hypothesized that the surface complex can stimulate TGF- β signaling in the target cell in a contact-dependent manner.

In addition to IL-10-producing Breg cells, TGF- β 1-producing Breg cells have been identified in response to LPS stimulation *in vitro* (Tian et al., 2011; Parekh et al., 2003). These B cells can trigger pathogenic Th1 cells to undergo apoptosis through Fas–FasL interactions and/or the inhibition of antigen-presenting cell activity via the secretion of TGF- β 1 (Tian et al., 2011). Another study using a mouse model of prostate cancer has identified that IgA⁺ plasmocytes in the tumors can prevent tumor-directed cytotoxic T-cell functions, via the expression of IL-10 and PD-L1 (Shalapour et al., 2015). Transforming growth factor (TGF)- β signaling and IgA class switch recombination were required for the development of this immune-suppressive Breg subset (Shalapour et al., 2015). As summarized in Table 1.1, iBreg suppression involves the production of TGF- β and IDO and induces the expansion of IL-10⁺ and TGF- β ⁺ Tregs (Nouël et al., 2015). The expansion of iBregs requires stimulation with CTLA-4 by T cells. Collectively, the emerging roles of human Bregs in maintaining homeostasis emphasize the importance of this population in limiting inflammatory responses.

1.3 T Cells

T cell-mediated immunity is an adaptive process of developing antigen (Ag)-specific T lymphocytes to defeat viral, bacterial, or parasitic infections. In addition, it may also involve aberrant recognition of self-antigens, leading to autoimmune inflammatory diseases. The antigen recognition through the T cell receptor (TCR) of unique antigenic peptides presented by MHC molecules on Ag-presenting cells (APC) such as dendritic cells, macrophages and B cells gives the antigen specificity to the T cells (Figure 1.6). T cell-mediated immunity includes a

primary response by naïve T cells, effector functions by activated T cells, and persistence of Ag-specific memory T cells. The coordinated immune response includes other effector cells such as macrophages, natural killer cells, mast cells, basophils, eosinophils, and neutrophils (Broere et al., 2011). All T lymphocytes carry the cluster of differentiation markers (CD) CD45 and CD3. In addition, two commonly used CD molecules to indicate helper and cytotoxic T cells are CD4 and CD8, respectively.

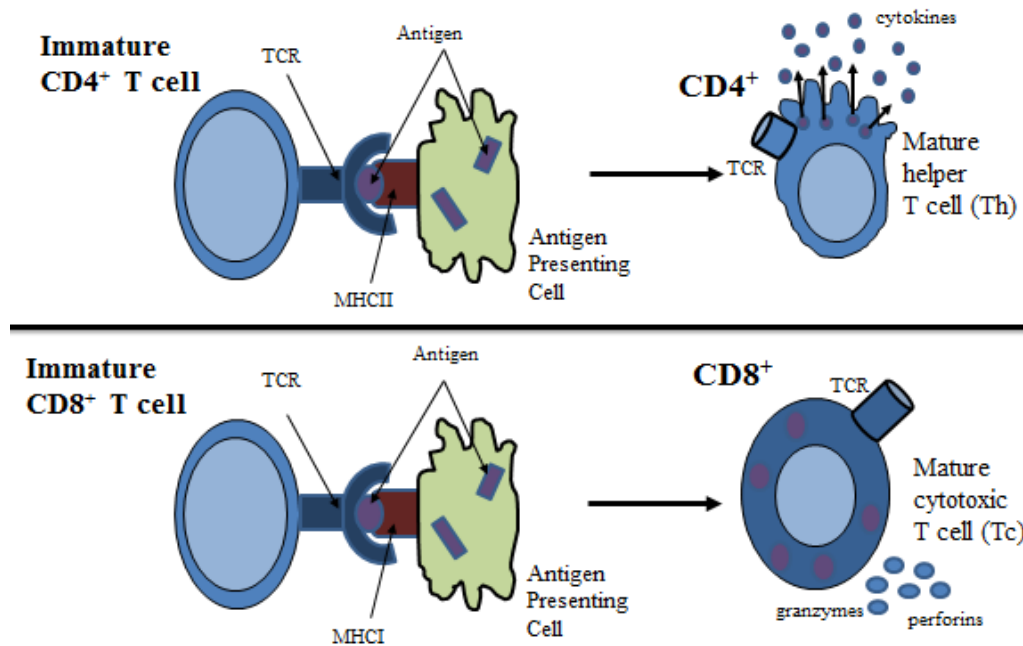


Figure1.6: Antigen presentation to T cells. Antigen presentation stimulates T cells to become either "cytotoxic" CD8⁺ cells or "helper" CD4⁺ cells. Cytotoxic cells directly attack other cells carrying certain foreign or abnormal molecules on their surfaces. Helper T cells, or Th cells, coordinate immune responses by communicating with other cells. In most cases, T cells only recognize an antigen if it is carried on the surface of a cell by one of the body's own MHC, or major histocompatibility complex, molecules.

Every effective immune response involves T cell activation. T cells require at least two signals to become fully activated. The first occurs after engagement of the T cell antigen-specific receptor (TCR) by the antigen-major histocompatibility complex (MHC), and the second by subsequent engagement of co-stimulatory molecules. The most potent T cell co-stimulator is CD28.

TCR activation is regulated by various co-stimulatory receptors. One of the co-stimulatory receptors, CD28 is the receptor for CD80 (B7.1) and CD86 (B7.2) proteins and provides an essential co-stimulatory signal during T-cell activation, which augments the production of IL-2 (Interleukin-2). The 28 co-stimulation increases T-cell proliferation and at the same time prevents the induction of

anergy and cell death. When CD28 is ligated by B7-1 or B7-2, it provides the T-cell with an initial adhesion capable of approximating the T cell and antigen presenting cell membranes. Other than CD28, many other transmembrane receptors also modulate specific elements of TCR signaling. CD45 is one such receptor which regulates TCR signaling by antagonizing the inhibitory impact of inhibitory proteins, thereby favoring T-cell activation (Leupina et al., 2000).

In order to keep a check on the hyperactivation of immune response associated with the pathway, negative regulation is also important. The transmembrane protein cytotoxic T-lymphocyte antigen-4 (CTLA4) serves as a natural inhibitor and negatively regulates T-cell activation. Since CTLA4 has a greater affinity for its B7-1/B7-2 ligands in comparison to CD28, after T-cell activation, CTLA4 is rapidly endocytosed, thus removing it rapidly from the cell surface. When a need to control TCR signaling arises, CTLA4 is again translocated to the membrane. At the membrane, CTLA4 inhibits the phosphorylation of TCR. Another mechanism by which CTLA4 might antagonize T-cell function is through inhibition of CD28 signaling by competing for their shared ligands B7-1 and B7-2 (Gough&Walker,2005).

1.3.1 Naïve T cells

After differentiating in bone marrow, and successfully undergoing the positive and negative processes of central selection in the thymus, T cells enter the bloodstream and migrate between blood and peripheral lymphoid tissue until they encounter their specific antigen. Mature recirculating T cells that have not yet encountered their antigens are known as naïve T cells. Naïve T cells are commonly characterized by the surface expression of L-selectin (CD62L); the absence of the activation markers CD25, CD44 or CD69; and the absence of memory CD45RO isoform (De Rosa et al., 2001). On recognition of specific antigen, they undergo activation. Through their antigen-specific T cell receptor, they recognize specific antigenic peptides in the context of major histocompatibility (MHC) molecules. However, in addition to the cognate signal of the peptide-MHC complex interaction, T cells require additional co-stimulatory signals to complete T cell activation. APCs also provide co-stimulatory signals for effective T cell activation.

1.3.2 Activated T cells

The activated T cells undergo clonal expansion and rapidly proliferate, migrate to the sites of antigen, and perform effector functions such as cell-mediated cytotoxicity and production of various cytokines.

1.3.2.1 Cytotoxic CD8⁺ T cells

Cytotoxic CD8⁺ T cells (often called cytotoxic T lymphocytes, or CTLs) are very effective in direct lysis of infected or malignant cells bearing the antigen, and in tumor surveillance. CD8⁺ T cells secrete anti-tumor and anti-viral cytokines such as TNF- α and IFN- γ . A second role of CD8⁺ T cells is that the production and release of cytotoxic granules which contain perforin and granzymes. Perforin forms a pore in the membrane of the target cell, similar to the membrane attack complex of complement. This pore allows the granzymes also contained in the cytotoxic granules to enter the infected or malignant cell. Granzymes are serine proteases which cleave the proteins inside the cell, shutting down the production of viral proteins and ultimately resulting in apoptosis of the target cell. Another important role of CD8⁺ T cells is the cell destruction of infected cells is via Fas/FasL interactions. Activated CD8⁺ T cells express FasL on the cell surface, which binds to its receptor, Fas, on the surface of the target cell. This binding causes the Fas molecules on the surface of the target cell which results in the activation of the caspase cascade, which also results in apoptosis of the target cell. In addition to their critical role in immune defense against viruses, intracellular bacteria, and tumours, CD8⁺ T cells can also contribute to an excessive immune response that leads to immunopathology, or immune-mediated damage (Liu & Lefrançois, 2004).

1.3.2.2 CD4⁺ T cells

CD4⁺ T cells are commonly divided into conventional T helper (Th) cells and regulatory T (Treg) cells. Th cells control adaptive immunity against pathogens and cancer by activating other effector immune cells; while Tregs are defined as CD4⁺ T cells in charge of suppressing potentially deleterious activities of Th cells.

CD4⁺ T helper cells

The T helper cells (Th cells) play an important role in the adaptive immune system, by helping the activity of other immune cells by releasing T cell cytokines. At first, based on transcription factors and cytokine production, Mossmann and Coffman depicted two main classes of helper cells: Th1 and Th2 (Mossmann *et al.* 1986). While investigating the role of IL-12, a Th1 promoting cytokine in a murine model of EAE, Cua *et al.* surprisingly found that elimination of IL-12 exacerbated disease. IL-12 is composed of p35 and p40 subunits. IL-23, an IL-12 family member, also utilizes p40, but is completed by the p19 subunit. Realizing this, Cua demonstrated the elimination of IL-23 specific p19, but not p35 (IL-12), protected the mice from disease onset. Thus, a third T Helper class was discovered and referred as Th17 Cells (Cua *et al.* 2003).

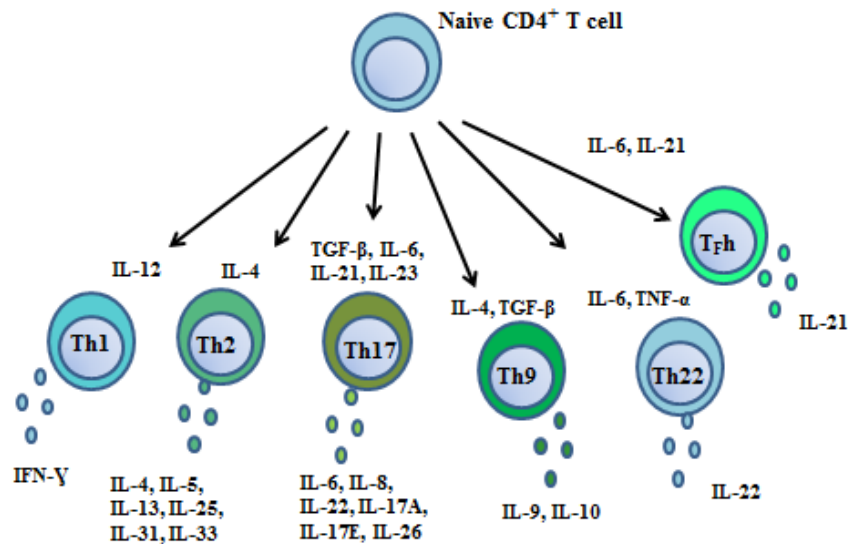


Figure 1.7: CD4⁺ T helper subsets with their effector cytokines. CD4⁺ T cells can differentiate into different subsets depending on the cytokine milieu present during T cell activation.

Th1 cells are involved with intracellular defense, essential to cell-mediated immunity, that drive the killing efficacy of macrophages and proliferation of cytotoxic CD8⁺ T cells in response to intracellular pathogens. Th1 cells are characterized by the expression of the transcription factor T-bet and produce cytokines: IL-2, IFN- γ , TNF, and LT- α . Th1 cells are associated with several chronic inflammatory diseases, including multiple sclerosis, diabetes, and rheumatoid arthritis (Romagnani, 1999).

Th2 cells are polarized by IL-4 cytokine and stimulate the humoral response to infections, including B cell proliferation, class-switching, and increased antibody production. Th2 cells express the transcription factor GATA3 and produce IL-4, IL-5, IL-6, IL-10, and IL-13. Th2 cells are associated with several inflammatory conditions, including allergy and asthma (Romagnani, 1999).

Th17 cells are a unique subset of activated T helper cells that have been found to be vital in extracellular pathogen defense at epithelial and mucosal barriers. In a *Helicobacter felis* infection model, MyD88 was required for Th17 development (Obonyo, 2011). MyD88 is a universal adapter protein used by TLRs to activate NF- κ B signaling, suggesting a role for TLR activation in Th17 development. Studies have shown convincingly that TGF- β is required for Th17 differentiation *in vitro* and *in vivo*. The development of a Th17 response is promoted by TGF- β , IL-1 β , and IL-6 and further expanded and activated by IL-23. IL-23 regulates the secretion of IL-17 through a STAT3 dependent pathway (Caruso et al., 2008). IL-23 induced activation of STAT3 leads to direct binding of phosphorylated STAT3 to IL-17A and IL-17F promoters (Chen et al., 2006). Moreover, STAT3 up-regulates the expression of Retinoic Acid Receptor-Related Orphan Receptor Gamma-T (ROR- γ), a Th17 specific transcriptional regulator that is critical for the expression of two members of IL-17A and IL-17F (Chen et al., 2007b; Laurence and O'Shea, 2007; Yang et al., 2007). IL-17A is associated with gastric inflammation during *H. pylori* infection and, when the infection becomes chronic, this cytokine may contribute to the inflammation-associated carcinogenesis (Kabir, 2011; Resende et al., 2011). Th17 cells have been associated with the pathogenesis of several chronic inflammatory disorders, including rheumatoid arthritis and multiple sclerosis (Langrish, 2005; Hirota et al. 2007). However, there are some reports that Th17s can maintain a protective effect, particularly in the gut (Annunziato et al., 2008). It was shown that although TGF- β and IL-6 drive initial Th17 lineage commitment, the pathogenic potential of Th17 cells are restrained by the coproduction of IL-10. Only when the Th17 cells are exposed to IL-23 they cease IL-10 production and attain their full pathogenic function (McGeachy et al., 2007). Esplugues et al. found that Th17 cells are controlled by two different mechanisms in the small intestine: first, they are eliminated via the intestinal lumen; second, pro-inflammatory TH17 cells simultaneously acquire a

regulatory phenotype with *in vitro* and *in vivo* immune-suppressive properties (rTH17) (Esplugues et al., 2011).

A brief information about the other helper T cells can be found in Table 1.2.

Table 1.2: T helper cell subsets with their effector cytokines and functions.

Name	Effector Cytokine	Function
T helper 9 (Th9)	IL-9, IL-10	Host defense against extracellular parasites, primarily nematodes. Despite their production of anti-inflammatory IL-10, they promote allergic inflammation. Their role in other inflammatory diseases still remains unclear as this subset has only recently been characterized.
T helper 22 (Th22)	IL-22	Identified in inflammatory skin diseases. Their role in host defense remains unclear as this subset has only recently been characterized. Their identity as an independent TH cell subset needs to be confirmed.
T follicular helper (Tfh)	IL-21	Involved in promotion of germinal center responses and provide help for B cell class switching.

Regulatory CD4⁺ T cells

The idea of a naturally occurring T cell subset with suppressive function that limits the outcome of autoimmune responses was first described in the 1970s by Gershon (Gershon, 1975). The role of regulatory T cells is to maintain the balance between self-tolerance, pathogen clearance, and autoimmunity. These cells generally suppress or downregulate induction and proliferation of effector T cells. The discovery of Foxp3 as a specific marker of natural T regulatory cells and adaptive/induced T regulatory cells (a iTregs) gave a molecular anchor to the population of regulatory T cells (Tregs), previously identified by non-specific markers such as CD25 or CD45RB (Hori et al., 2003; Fontenot et al., 2003, 2005).

FOXP3 (Forkhead box protein P3) is a 50-55 kD transcription factor and is proposed to be a master regulatory gene and is considered as a specific marker of T regulatory cells more than most cell surface markers (Ramsdell&Ziegler, 2014). Genome-wide analysis has shown that Foxp3 binds to the promoter region of 700-1000 genes, many of those genes being associated with TCR signaling (Marson et al., 2007; Zheng et al., 2007). Although it was thought to be a critical factor for

Treg function, it was observed that Foxp3 was not expressed by T regulatory cells 1 (Tr1), a Treg subset which is induced by IL-10 and which produces IL-10 (Groux et al., 1997; Levings et al., 2005). Two major classes of CD4⁺ Treg cells have been described: FOXP3⁺ Treg cells and FOXP3⁻ Treg cells. On the other hand, in some studies it is suggested that presently used Treg markers (CD25, CTLA-4, LAG-3 and Foxp3) represent general T cell activation markers rather than being truly Treg-specific. Recent reports have demonstrated that Foxp3⁺ Tregs may differentiate *in vivo* into conventional effector Th cells, with or without concomitant downregulation of Foxp3. Treg cells induced by TGF- β *in vitro* were shown to lose Foxp3 expression and suppressive activity upon restimulation in the absence of TGF- β (Floess et al., 2007). Furthermore, it is suggested that Bregs could increase Treg numbers by expanding existing Treg populations or converting naïve Foxp3⁻ T cells into Foxp3⁺ Tregs (Lee et al., 2014).

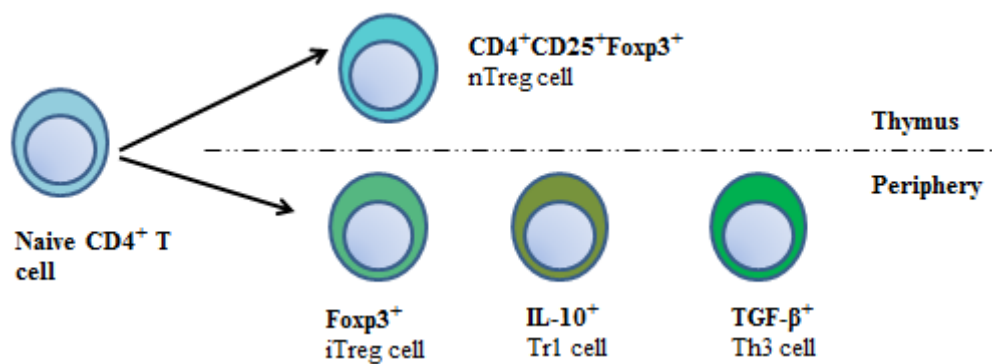


Figure 1.8: Different subsets of Treg cells. The naturally occurring CD4⁺CD25⁺FOXP3⁺ Treg (nTreg) cells are generated in the thymus, whereas three different subsets of inducible Treg (iTreg) cells can be generated in the periphery: (i) FoxP3⁺Treg cells, (ii) CD4⁺FoxP3⁺ IL-10–producing Tr1 cells and (iii) TGF- β -expressing T_H3 cells.

Natural Regulatory T cells

Naturally occurring Tregs (nTregs) are characterized by the expression of CD4, CD25 and Foxp3, which is a transcription factor important in the development of Tregs. nTregs (CD4⁺CD25⁺FoxP3⁺) are mainly derived as a functionally mature subset from the thymus. Studies suggest that relatively higher avidity thymic stromal interactions between the TCR and self-peptide of nTregs as well as FoxP3 play crucial roles in nTreg development. Suppressive mechanisms of nTregs which is thought to be by cell to cell contact involve cytotoxic killing, induction of

other regulatory cells, APC function downregulation, transduction of suppressive signals through B7 molecules on target T cells, as well as other negative signals produced by inhibitory nTreg surface molecules. Subsequent *in vitro* studies showed that this population, now referred to as CD4⁺CD25⁺ T regulatory cells, is both anergic and suppressive (Thornton, 1998; Takahashi et al., 1998; Read et al., 1988).

Induced Regulatory T cells

In addition to nTregs, there are several distinct subsets of induced regulatory T cells (iTregs). The well-known iTregs are Tr1 and Th3, but studies reveal other induced regulatory T cells continuously. Not recently was shown the existence of IL-10 and IL-17 co-producing T cell subset. The inducible regulatory T cells are generated from naive T cells in the periphery. Tr1 cells mediate their suppression mainly through the production of IL-10, while Th3 cells mediate suppression through TGF- β production (Lan, 2003).

Experiments showed that orally immunized mice in whom oral tolerance has been induced manifest T cells that produce TGF- β 1, co-called Th3 cells and that T cells that lines that produce IL-10, so-called Tr1 T cells, can prevent development of colitis in SCID-transfer mouse model (Chen, 1994; Groux, 1997). Additionally, *in vivo* studies using conditional knock-out mouse strains revealed that IL-10 production by T cells, but not B cells, was essential for the suppression of excessive gastric immunopathology (Sayi et al, 2011).

Foxp3⁺ iTreg cells

While natural Foxp3⁺ nTregs are directly released from the thymus independent of APCs (Kyewski and Klein, 2006; Ohkura and Sakaguchi, 2010), conversion of Foxp3⁺ iTregs occurs in peripheral lymphatic organs with interaction with APCs. The basal requirements for the development of Foxp3⁺ iTregs *in vitro* and *in vivo* are defined by TCR signalling and the presence of the cytokines IL-2 and TGF- β (Fantini et al., 2004; Davidson et al., 2007; Curotto de Lafaille and Lafaille, 2009).

Th3 cells

Th3 iTregs, which are characterized by the production of TGF- β , occur primarily after ingestion of a foreign antigen via the oral route. The presence of TGF- β , which is found at high levels in the bowel, helps induce the differentiation of naive T cells into Th3 cells (Berthelot et al., 2004). Th3 cells, like nTregs, express CTLA-4 on their surface, the triggering of which results in the secretion of TGF- β . FoxP3 and CD25 expression is also upregulated after restimulation of TGF- β induced Th3 cells. Unlike nTregs, the main suppressive mechanism of Th3 cells is dependent on the production of TGF- β , which suppresses the proliferation of Th1 and Th2 cells.

Type 1 regulatory T cells (Tr1)

In 1997 so-called type 1 regulatory T cells (Tr1) were identified as a subset of CD4⁺ cells that produces high levels of IL-10, low levels of IL-2 and no IL-4 (Groux et al., 1997). In contrast to nTregs, Tr1 cells do not express high levels of CD25 or FoxP3, and do not mediate suppression via cell–cell contact (Berthelot et al., 2004). The cytokine profile of Tr1 cells include high levels of IL-10 and lower levels of TGF- β and IFN- γ (Levings, 2004). IL-10, and debatably TGF- β , take place in Tr1 mediated suppression of the proliferation and cytokine production of other T cell subsets (Mills, 2004; Roncarolo, 2001; Stassen, 2004).

As mentioned before, *H. pylori* uses a variety of mechanisms to inhibit the T-cell response and persist in the gastric mucosa. Tregs are induced during infection, which express the inhibit other T-cell responses by producing IL-10 and TGF- β (Beswick, 2007; Oertli, 2012). Using mouse models of infection with *Helicobacter felis*, a close relative of the human gastrointestinal pathogen *H. pylori*, Sayı *et al.* showed that B cells activated by *Helicobacter* TLR-2 ligands induce IL-10–producing CD4⁺CD25⁺ T regulatory-1 (Tr-1)–like cells *in vitro* and *in vivo*. According to their results, Tr-1 conversion depended on TCR signaling and a direct T-/B-interaction through CD40/CD40L and CD80/CD28. They suggested that B cell-induced Tr-1 cells acquire suppressive activity *in vitro* and suppress excessive gastric *Helicobacter*-associated immunopathology *in vivo*.

Furthermore, Gagliani and her research group identified the surface markers CD49b and lymphocyte activation gene 3 (LAG-3) as being stably and selectively

co-expressed on mouse and human Tr1 cells (Gagliani et al., 2013). CD49b is expressed by Tr1 cells irrespective of their activation, whereas LAG-3 is expressed on Tr1 cells when they produce IL-10 and have suppressive activity. Co-expression of CD49b and LAG-3 distinguishes Tr1 cells from Th1, Th2 and Th17 cells (Gagliani et al., 2013).

1.3.3 Effects of IL-10 on T Cell Differentiation

IL-10 was first described as a cytokine that is produced by Th2 cell clones (Fiorentino, 1989). It inhibits IFN- γ synthesis in Th1 cell, and therefore it was initially called cytokine synthesis inhibiting factor (CSIF) (Delves et al., 1998). Later, evidences suggested that IL-10 production was associated with many immune cell types including Tregs and Bregs.

As summarized in Figure 1.9, IL-10 produced and secreted by regulatory B cells work on many cellular regulation. It is suggested that the balance between Th1 and Th2 response is accomplished by IL-10. This cytokine also claimed to inhibit Th1 and Th17 response while inducing Foxp3⁺ Treg cells and IL-10 producing Tr1 cells. Another aspect of IL-10 influence is the repression of antigen presentation. Causing a decrease in the expression of MHC-II molecules or co-stimulation molecules such as CD86, IL-10 indirectly suppresses antigen presentation to the T cells (Figure 1.9) (Yang et al., 2013). IL-10 produced by TLR2-activated B cells has also been implicated with their potency to convert CD4⁺ T cells into IL-10 producing Tr-1 cells, *in vitro* in a contact-dependent manner (Sayi et al., 2011).

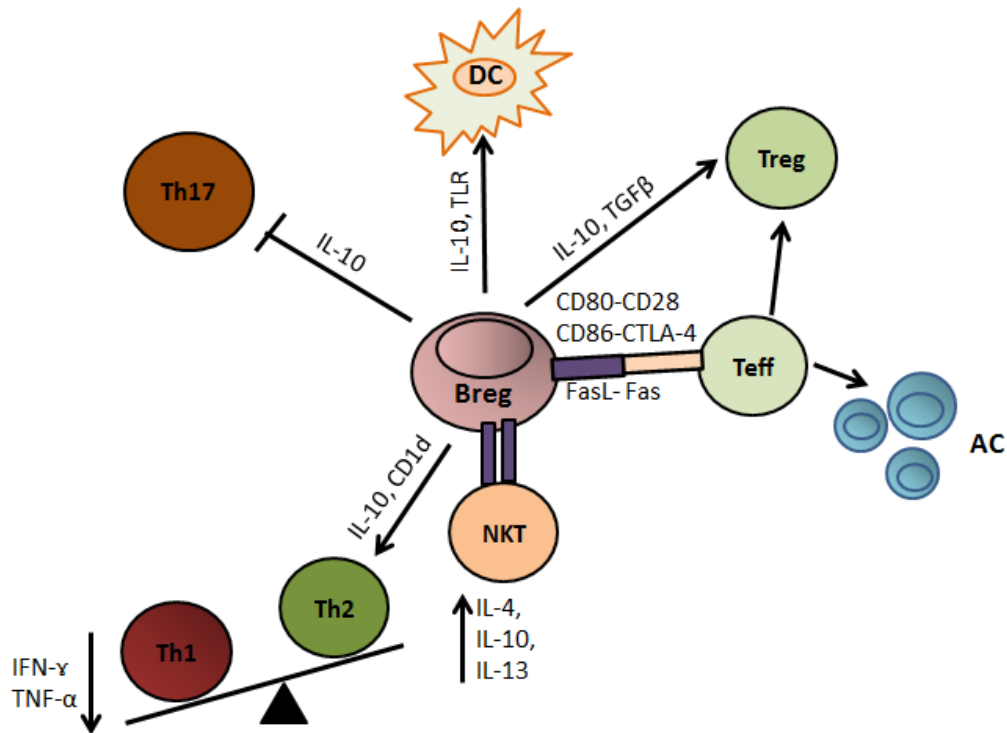


Figure 1.9: Immune functions of IL-10 secreted by regulatory B cells (adapted from Yang et al., 2013).

Although in the presence of IL-10, $CD4^+$ T cells are induced to develop a regulatory phenotype *in vitro* that suppress antigen-specific effector T cell responses via a cytokine-dependent mechanism, it is suggested that the effects of IL-10 on APCs play a more important role in the development of Tr1 than the direct influence on the $CD4^+$ T cells (Groux et al., 1997). Furthermore, IL-10 is also capable of limiting Th1- and Th17-mediated immune responses by reducing the secretion of IL-12 and IL-23 from APCs, respectively. IL-12 is the cytokine responsible for Th1- type T cell conversion from naive $CD4^+$ T cells whereas IL-23 is known to be pivotal for Th17 immune response. As mentioned above, independent of its inhibitory effect on APCs, IL-10 inhibits directly both the proliferation and the cytokine synthesis of $CD4^+$ T cells, including the production of IL-2 and IFN- γ by Th1 and of IL-4 and IL-5 by Th2 (Sabat et al., 2010).

Breg cells have been shown to affect the balance between $Foxp3^+$ and IL-17-producing T cells (Carter et al., 2011; Tedder et al., 2010). Carter et al. have demonstrated that endogenous IL-10-producing B cell-deficient mice develop an exacerbated case of arthritis and exhibit an increased frequency of Th1/Th17 pro-

inflammatory cells, but a decreased frequency of Treg cells (Carter et al., 2011; Carter et al., 2012). Consistent with these findings, Yang et al. have also demonstrated that B10 cells induced *in vitro* can suppress Th17 cell differentiation by decreasing the phosphorylation levels of Stat3, which subsequently reduces the levels of ROR γ t, and partially inhibits the Th17 cell population in an IL-10-dependent manner (Yang et al., 2012). Based on these current findings, it has been shown that Breg cells play an important role in T-cell plasticity.

1.3.4 Effects of TGF- β on T Cell Differentiation

TGF- β promotes T-cell survival by inhibiting activation-induced cell death and blocks T-cell proliferation by inhibiting IL-2 production (Brabletz, 1993; Li, 2006). Through its effects on T-helper (Th)-cell differentiation, TGF- β modulates T-cell activation (Li, 2006). TGF- β also promotes Treg-cell development while inhibiting Th1- and Th2-cell development (Chen, 2003; Horwitz 2003). In a model of transplant tolerance, Lee et al. (2014) aimed to identify soluble factors produced by B cells that might explain their Treg-cell inducing activity and they hypothesized that regulatory B (Breg) cells could contribute to regulatory T-cell induction by producing TGF- β . Starting with sorted Foxp3⁺ T cells in an allergic airway disease model, Singh et al. (2008) demonstrated *in vitro* that co-culture with Breg cells could induce Foxp3 expression upon activation by anti-CD3 and anti-CD28 in a TGF- β -dependent manner. Another important TGF- β activity for Tregs is to mediate the immunosuppression to maintain the peripheral tolerance (Nakamura et al., 2004; Bommireddy et al., 2007). It has also 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 (Figure 1.10) (Banchereau, Pascual & O'Garra, 2012). Studies have shown convincingly that TGF- β is required for Th17 differentiation *in vitro* and *in vivo*. The development of aTh17 response is promoted by TGF- β , IL-1 β , and IL-6 and further expanded and activated by IL-

23. IL-23 regulates the secretion of IL-17 through at STAT3 dependent pathway (Caruso et al., 2008).

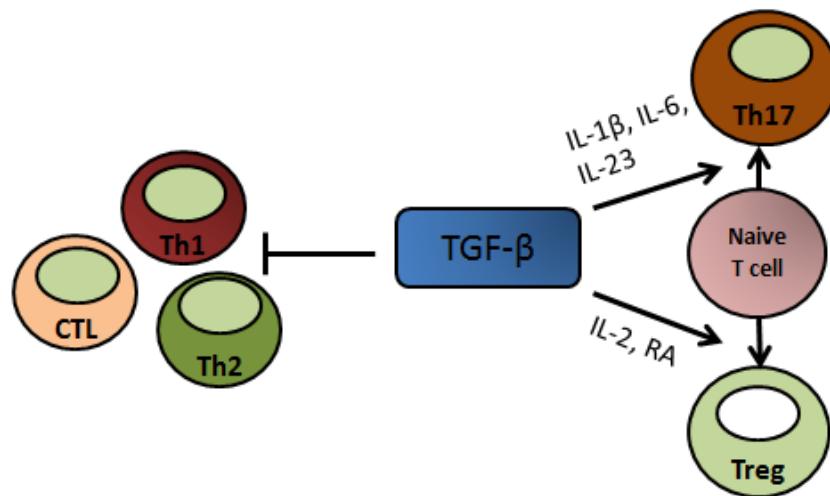


Figure 1.10: Role of TGF- β on immune cells. In order to assess the role of TGF- β , TGF- β - knockout mice were used in different studies. In the absence of this cytokine, mice showed multifocal inflammatory function (adapted from Banchereau, Pascual & O'Garra, 2012).

1.4 Innate and adaptive immunity against *Helicobacter*

Helicobacter induces both humoral and cellular immune responses. Local and systemic antibody responses include IgA, IgM, and IgG isotypes (Crabtree et al., 1991). Early mouse model studies demonstrated that immunization with *H. pylori* antigens could produce protective immunity (Marchetti et al., 1995). *H. pylori* causes an inflammatory reaction with both polymorphonuclear and mononuclear cells (Goodwin et al., 1986), and gastric mucosa of infected patients contains increased levels of proinflammatory cytokines such as IL-1 β , tumor necrosis factor (TNF)- α , IL-8, and IL-6 (Crabtree et al., 1993). On the other hand, *Helicobacter* has developed a variety of mechanisms to persist in the gastric mucosa. Gastric epithelial cells (GECs) are primary target for *H. pylori* infection, therefore they are the first point of contact for *H. pylori* and activate an innate immune response through TLRs. Though, *H. pylori* differs from other gastrointestinal pathogens. Due to mutations in the TLR5 recognition site of the N-terminal D1 domain of flagellin (Andersen-Nissen et al., 2005), *H. pylori* flagellin is a poor ligand of TLR5 (Gewirtz et al., 2004). Furthermore, rather than being a strong TLR4 ligand, *H. pylori* LPS is thought to activate TLR2 on gastric epithelial cells (Smith et al., 2003; Chaouche-Drider et al., 2009). Animal and cell

culture experiments suggested that ligands in *Helicobacter* species can bind to TLR2 and activate NF- κ B in epithelial cells (Chaouche-Drider et al., 2009; Sayi et al., 2011).

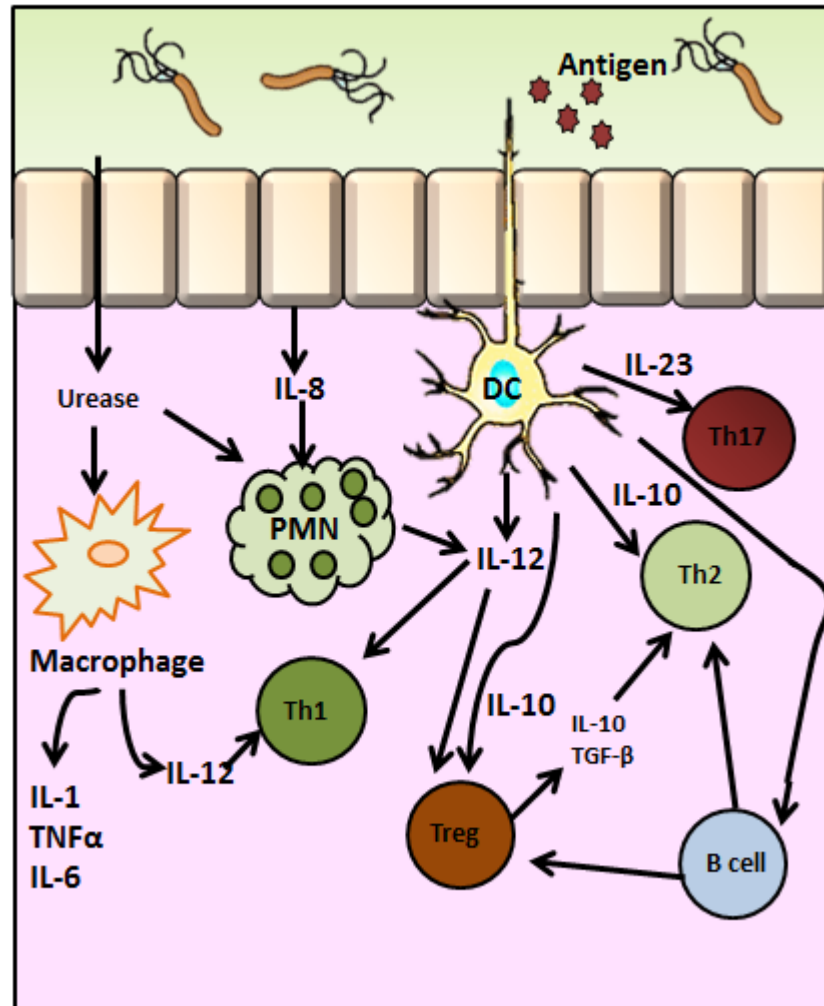


Figure 1.11: Schematic demonstrating how dendritic cells may bridge the innate and adaptive immune response directed against *H. pylori* within the gastric mucosa (adapted from Peek et al., 2010).

H. pylori infection stimulates the activation of epithelial cell, macrophage, and DC, as well as a Th1 predominant lymphocyte response. The line separating the innate from the adaptive response is vague. B cells can respond to *H. pylori* directly, or via the interaction of activated T cells, illustrates the complexity of the host immune response. Colonization of *H. pylori* can be abrogated by immunization with bacterial components such as urease (Panchal et al., 2003), indicating activation of the adaptive response, but urease is also a major inducer of

innate responses in monocytes and macrophages, stimulating cytokine and nitric oxide generation (Gobert et al., 2002; Mai et al., 1992).

As a characteristic feature of *Helicobacter* infection, neutrophils or polymorphonuclear leukocytes (PMNs) migrate to the site of the infection and trigger an inflammation which still cannot clear the infection due to the ability of the bacteria to survive by manipulating phagocytosis and the subsequent oxidative burst. Direct interactions between gastric epithelial cells and *H. pylori* or *H. pylori* urease can also activate PMN cells and/or macrophages, which further amplifies the T-cell response. The neutrophil-activating protein (NAP) of *H. pylori* contributes to Th1 polarization by stimulating both IL-12 and IL-23 secretion from neutrophils and monocytes (Amedei et al., 2006).

Other essential innate responders to *H. pylori* related products are the epithelial cells that are in direct contact with the bacterium on the surface of the mucosa and the macrophages which also function as effector cells in host defense by their nitric oxide secretion. Monocytes and macrophages, along with DCs, coordinate the immune responses to *H. pylori* as activators of adaptive immunity by producing factors such as IL-12 (Haeberle et al., 1997; Meyer et al., 2003). IL-12 stimulates Th1 cells, resulting in production of cytokines such as IFN- γ which is linked to the development of peptic ulcers in infection with *H. pylori* *cag*⁺ strains (Hida et al., 1999). Macrophages are also involved in amplification of the inflammatory response by production of cytokines such as IL-1, TNF- α , and IL-6 (Harris et al., 1998), and IL-6 activation has been linked to activation of TLR4, MAPK, and NF- κ B signaling events (Pathak et al., 2006).

It has been shown that DCs are primary responders to stimuli including bacterial products and have an important duty as APCs. DCs can engulf bacteria directly by penetrating epithelial monolayers *in vitro* and the intestinal epithelial barrier *in vivo* (Chieppa et al., 2006; Niess et al., 2005; Rescigno et al., 2001). Thus, being capable of inducing either a Th1, Th2/regulatory T cell (Treg), or a Th17 response by generation of interleukin (IL)-12, IL-10, or IL-23, respectively, they activate T cells in different ways.

Furthermore, B cells also contribute to the immunopathogenesis of *H. pylori* infection. In studies conducted in B cell-deficient (μ MT) mice infected with *H.*

pylori, when compared with wild-type mice, there was no difference in colonization at 2 wk after infection, but a 2 log-fold reduction developed at 8 and 16 wk postinoculation, that was associated with increased gastric inflammation and infiltration of CD4⁺ T cells (Akhiani et al., 2004). While IgG and IgA responses to *H. pylori* in the serum and gastric mucosa may be involved in protective immunity, the latter study, and another by the same group implicating the negative effect of IgA antibodies (Akhiani et al., 2005), suggests that B cell-mediated antibody responses may be counterproductive.

The importance of IL-12 that may be produced from monocytes, macrophages, or DCs in the induction of Th1 lymphocyte response (Guiney et al., 2003; Hafsi et al., 2004) and the role of gastric epithelial cells as antigen presenting cells in activation of CD4⁺Th cells (Ye et al., 1997) has been shown. Moreover, mucosal T cells harvested from *H. pylori*-infected hosts produce abundant levels of the Th1 cytokines IFN- γ and IL-2 and low levels of the Th2 cytokines IL-4 and IL-5 (Bamford et al., 1998).

As another T cell response, IL-17 has been linked to chemokine-mediated neutrophil infiltration, and IL-17 levels are increased in *H. pylori*-infected human (Luzza et al., 2008) and mouse (Scott et al., 2007) gastritis tissues. Recently, it has been shown that immunization of mice with *H. pylori* lysate markedly enhanced IL-17 expression in the gastric mucosa and in CD4⁺T cells isolated from spleens and co-cultured with *H. pylori*-stimulated DCs or macrophages. The results indicated increased gastric inflammation and decreased colonization (DeLyria et al., 2009). Taken together, it is suggested that the attenuated cytokine/chemokine response in unimmunized mice suggest that the IL-17/Th17 response may be defective in a normal host, thereby contributing to chronic persistence of the bacterium.

Beside effector T cell responses, Tregs are shown to be crucial in the pathogenesis of *H. pylori* infection. In an study of *H. pylori*-infected humans, Lundgren et al. (2003) showed that *H. pylori*-specific Tregs suppress memory T-cell responses and could thus contribute to the persistence of the infection. It has been reported that *H. pylori*-infected individuals have increased levels of CD4⁺CD25^{high} T cells in the gastric and duodenal mucosa that express mRNA of FOXP3, a gene

involved in the development of Tregs, and high levels of the CTLA-4 protein. Meanwhile, more CD4⁺CD25^{high} T cells were observed in *H. pylori*-associated gastric adenocarcinoma tissues than the adjacent tissue (Lundgren et al., 2005). Along with gastric cancer, differences in the number of regulatory T cells have been identified in patients with and without peptic ulcer disease. Robinson et al. (Robinson et al., 2008) reported that *H. pylori*-infected persons with peptic ulceration had significantly less gastric regulatory T cells but increased Th1 and Th2 responses compared with infected subjects without ulcers, suggesting that imbalances within the regulatory T-cell network may predispose to diseases that develop within the context of *H. pylori* infection.

1.5 Interaction between *Helicobacter*-activated B cells and T cells

Helicobacter has developed a variety of mechanisms to persist in the gastric mucosa. Gastric epithelial cells (GECs) are primary target for *H. pylori* infection, therefore they are the first point of contact for *H. pylori* and activate an innate immune response through TLRs. Though, *H. pylori* differs from other gastrointestinal pathogens. Due to mutations in the TLR5 recognition site of the N-terminal D1 domain of flagellin (Andersen-Nissen et al., 2005), *H. pylori* flagellin is a poor ligand of TLR5 (Gewirtz et al., 2004). Furthermore, rather than being a strong TLR4 ligand, *H. pylori* LPS is thought to activate TLR2 on gastric epithelial cells (Smith et al., 2003; Chaouche-Drider et al., 2009). Animal and cell culture experiments suggested that ligands in *Helicobacter* species can bind to TLR2 and activate NF- κ B in epithelial cells (Chaouche-Drider et al., 2009; Sayi et al., 2011).

B cells and their cytokines have important roles in *Helicobacter* infections by balancing between the infection and T cell driven gastric immunopathology. The ability of B cells to interact with pathogenic T cells and to produce anti-inflammatory cytokines such as IL-10 is crucial to dampen harmful immune responses (Mauri, 2008). It has been found that B-cells exposed to *Helicobacter* extract produced large amounts of the regulatory cytokine IL-10 (Sayi et al., 2011). Using mouse models of *Helicobacter*-induced gastric premalignant pathology, Sayi et al.(2011) showed that IL-10 secretion by purified B cells absolutely required MyD88 signaling and TLR-2, as B cells isolated from the

respective MyD88^{-/-} and TLR-2^{-/-} animals failed to respond to the stimulations. In addition, their results indicated that the upregulation of surface CD80 and CD86 on B cells and the antibody secretion, was largely dependent on MyD88 and TLR-2 (Figure 1.12).

The TLR-2 dependent *Helicobacter* activation of B cells differentiates them into IL-10 and TGF- β producing regulatory B cells. As explained in the previous sections, both IL-10 and TGF- β have crucial effects on T cell differentiation. When co-cultured with *Helicobacter*-activated B cells, naive CD4⁺ T-cells are shown to produce IL-10 and differentiate into T regulatory 1 (Tr1)-like cells (Sayi et al, 2011). In addition to that, Lee et al. (2014) suggested that Breg cells contribute to regulatory T-cell induction by producing TGF- β . At the same time, studies have shown convincingly that TGF- β is required for Th17 differentiation *in vitro* and *in vivo* (Caruso et al.,2008).

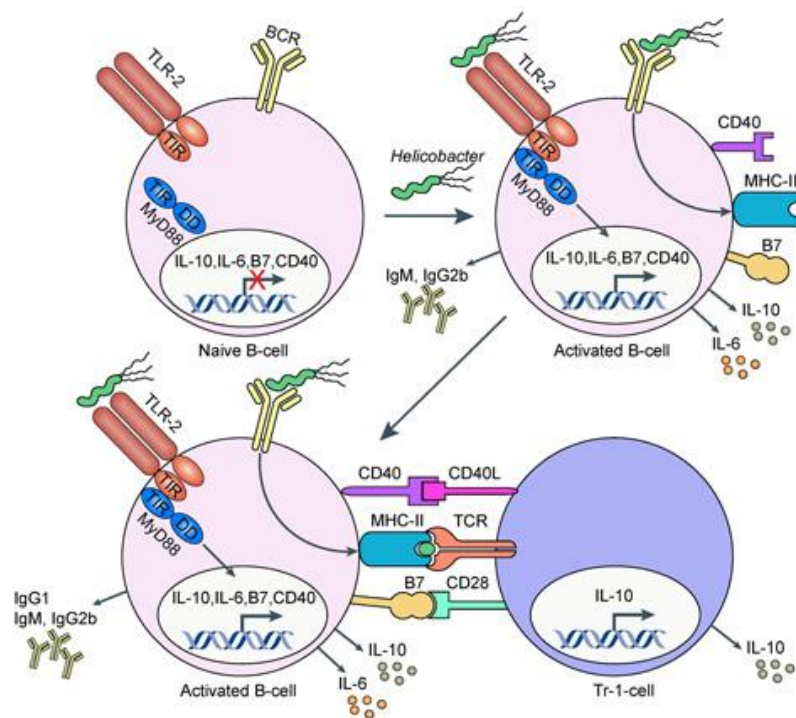


Figure 1.12: One suggested interaction between *Helicobacter*-activated B cell and T cell. *Helicobacter* TLR-2 ligands activate B-cells in a MyD88-dependent manner, which leads to the expression and surface exposure of CD80, CD86, and CD40, and the secretion of IL-10, IL-6 and TNF- α as well as antibodies of the IgM and IgG2b subclasses. The interaction of activated B-cells and naive T-cells induces T-cellular IL-10 expression and suppressive activity in a manner dependent on a direct interaction between both cell types via CD40/CD40L, B7/CD28 and MHCII/TCR. IL-10 secreting T-cells are essential players in the prevention of *Helicobacter*-associated immunopathology (Sayi et al, 2011).

1.6 Aim of the study

The immune response mechanisms against *Helicobacter pylori* involve both protective and damaging effects for the host. Results of many studies suggested a crucial role for B cells in *Helicobacter* infection that balances between the infection and T cell mediated pathology. *Helicobacter* induces B cells to produce and secrete IL-10 which is suggested to have a role in the differentiation of naïve CD4⁺ T cells into suppressive IL-10 producing Tr-1-like cells (Sayi et al.,2011); while other studies revealed the importance of TGF- β in both Tr-1 and Th17 differentiation (Lee et al., 2014; Caruso et al., 2008).

In previous studies in our laboratory, colleagues separated the *Helicobacter*-activated B cells into two subgroups: *Helicobacter*-activated IL-10⁺ B cells and *Helicobacter*-activated IL-10⁻ B cells. The experimental results showed that *Helicobacter*-activated IL-10⁺ B cells are the source of the IL-10 production while *Helicobacter*-activated IL-10⁻ B cells are mostly TGF- β positive. However, it was not clear if the *Helicobacter*-activated-IL-10⁺ B cells or the *Helicobacter*-activated-IL-10⁻ B cells were specifically causing the Tr1 differentiation. Taking account that Bregs are known for producing IL-10 and TGF- β which are key cytokines in T cell differentiation, and focusing on these cytokines produced by *Helicobacter*-activated B cells as well as the cell-to-cell contact, in this study the role of *Helicobacter*-activated-IL-10⁺ B cells and the *Helicobacter*-activated-IL-10⁻ B cells on CD4⁺ T cell differentiation was investigated.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

Helicobacter felis (*H. felis*) strain was kindly provided by Prof. Dr. Anne Müller from University of Zurich. Bacteria are planted 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 2.1 and Table 2.2, respectively.

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* culture are listed in Table 2.3.

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

Content	Supplier Company
Trimethoprim	HiMedia
Amphotericin B	HiMedia

2.1.1.2 Liquid culture

Ingredients of liquid culture of *Helicobacter felis* are shown in Table 2.4 with a representative volume of 50 ml. The volumes of the ingredients may change with the same ratio.

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

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

2.1.1.3 Freezing of *Helicobacter felis*

Helicobacter felis freezing medium ingredients are given in Table 2.5 with its ingredients. Upon preparation the medium can be stored at 4°C.

Table 2.5: Freezing medium for *Helicobacter felis*.

Component	Amount
Brucella Broth	25 ml
Glycerol	25 ml

2.1.2 Primary cell lines

2.1.2.1 CD19⁺ B cells

Splenic CD19⁺ B cells were isolated 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. A part of the purified CD19⁺ B cells were stimulated with *Helicobacter felis* sonicate.

2.1.2.2 CD4⁺ T cells

Splenic CD4⁺ T cells were isolated from the spleens of C57BL/6 mice via magnetic separation (Macs Miltenyi, Germany). To maintain the viability of CD4⁺ T cells in the culture, recombinant IL-2 (10 ng/ml, MACS, Miltenyi) and anti-CD3ε antibody (1 mg/ml, 17A2, Miltenyi Biotech) is added inside the completed (10% FBS, 1% Pen/Strep) RPMI culture media.

2.1.3 Cell culture

Buffers that were used in cell culture studies are given in Table 2.6 while culture media and solutions that have been used in cell culture studies are listed in Table 2.7.

Table 2.6: Buffers used in cell culture studies.

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

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

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

2.1.4 ELISA

Solutions that are required for IL-10 and IL-17 ELISA studies are given in Table 2.8.

Table 2.8: Solutions used in IL-10 and IL-17 ELISA experiments.

Solution	Content
PBS/T 1X	0.05% Tween-20 in PBS 1X
Stop Solution	2N H ₂ SO ₄ in ddH ₂ O

2.1.5 Equipment and Materials

Laboratory equipments necessary for the study and their suppliers are shown in Table 2.9 and the materials are provided in Table 2.10.

Table 2.9: Laboratory equipment used in this study.

Equipment	Supplier Company
Laminar Air Flow Cabinets	FASTER BH-EN 2003

Table 2.9(cont'd): Laboratory equipment used in this study.

Equipment	Supplier Company
Laminar Air Flow Cabinets	FASTER BH-EN 2003
Centrifuges	Beckman Coulter Allegra TM 25 R Centrifuge Scanspeed 1730 R Labogene Scanspeed mini
Freezers	Altus (+ 4°C) Siemens (-20°C) Haier (- 80°C)
Microplate Spectrophotometer	BIO-RAD Benchmark Plus
Magnetic stirrer	WiseStir MSH-20D, Wids Laboratory Equipment
Light Microscope	Olympus CH30
Ice Machine	Scotsman AF10
Flow Cytometer	BD Accuri C6
Nitrogen Tank	Air Liquid
Incubator with CO ₂	BINDER
Nanodrop 2000	Thermo Scientific
Shakers	Heidolp Duomax 1030
Step One Real Time Systems	Applied Biosystem
Sonicator	Bandelin Sonopuls
Vortex	Mixer Uzusio VTX-3000L,LMS
Quick spin	LMS
Scale	Precisa

Table 2.10: Materials used in this study.

Material	Supplier Company
Tissue flask	Sarstedt
Serologic pipettes	Dispenser
Centrifuge tubes	Interlab
Eppendorf tubes (0.6 ml, 1.5 ml, 2 ml)	Interlab
Nitrocellulose membrane (0.2 µm pore size)	Santa Cruz
Examination Gloves	Beybi
Cell strainer (70 µm)	BD

Table 2.10 (cont'd): Materials used in this study.

Material	Supplier Company
Anaerobic Jar	Anaerocult
Erlenmeyer	Isolab
Falcon (15 ml, 50 ml)	Isolab
Slides	Interlab
Coverslips	Interlab
Cotton Swap	Interlab
96 well F plate (for ELISA studies)	Nunc
Hemocytometer	Isolab
Pipettes	10 µl, 20 µl, 100 µl, 200 µl, 1000 µl Socorex and 10 µl, 100 µl, 1000 µl Biohit
Electronic Pipette	CappAid
Tissue culture flasks (25 cm ² , 75 cm ²)	Sarstedt

2.1.6 Commercial kits

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

Table 2.11: Commercial kits used in this study.

Kit	Supplier Company
Mouse B Cell Isolation Kit	MACS, Miltenyi Biotec
Mouse Regulatory B cell Isolation Kit	MACS, Miltenyi Biotec
Mouse CD4 ⁺ T cell Isolation Kit	MACS, Miltenyi Biotec
NucleoSpin RNA Isolation Kit	Macherey-Nagel
High capacity cDNA synthesis Kit, 200 rxns	Applied Biosystems
Power SYBR® Green PCR Master Mix	Applied Biosystems
Mouse IL-10 ELISA Max Deluxe	Biolegend
Mouse IL-17 ELISA Max Standard	Biolegend
BCA™ Protein Assay Reagent Assay	Thermo Scientific

2.1.7 General chemicals

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

Table 2.12: General chemicals used in this study.

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

2.1.8 Primers

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

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

Primer Name	Sequence (5'-3')	Spec.	Tm	Exp. size
IL-17 forward	GGACTCTCCACCGCAATGAA	m	60°C	94 bp
IL-17 reverse	TTTCCCTCCGCATTGACACA	m	60°C	94 bp
Ror gamma T fw.	AGCAATGGAAGTCGTCCTAGTCA	m	60°C	110 bp
Ror gamma T rv.	AGCCCAAGGCTCGAAACAG	m	60°C	110 bp
18s rRNA fw.	GGCCCTGTAATTGGAATGAGTC	m/h	55°C	114bp
18s rRNA rv.	CCAAGATCCAACACTACGAGCTT	m/h	55°C	114bp

2.1.9 Antibodies

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

Table 2.14: Antibodies used in this study.

Antibody	Clone	Supplier Company	Application
Recombinant IL-2	-	Miltenyi Biotech	Cell Culture
Anti-mouse Anti-CD3 ϵ	17A2	Miltenyi Biotech	Cell Culture
Rat anti-mouse CD4-APC	Rm-4-5	BD	FACS
Rat anti-mouse CD19-FITC	6D5	Biolegend	FACS

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

Antibody	Clone	Supplier Company	Application
Rat anti-mouse CD62L-APC	MEL-14	Biolegend	FACS
Anti-mouse IL-17-FITC	TC11-18H10.1	Biolegend	FACS
Rat anti-mouse IL-10-PE	JES5-16E3	BD	FACS
Anti-mouse CD25-biotin	# 280406	R&D	FACS
Streptavidin-FITC	-	Biolegend	FACS
Anti-mouse CD49b-FITC	DX5	Biolegend	FACS
Anti-mouse LAG-3-PerCpCy5.5	C9B7W	Biolegend	FACS

2.2 Methods

2.2.1 Maintenance of *Helicobacter felis*

Helicobacter felis was seeded on a Columbia blood agar containing suitable amounts of the antibiotics and incubated at 37°C under microaerophilic conditions in an anaerobic jar for 3-4 days. Microaerophilic conditions in anaerobic jar were maintained by utilization of CampyGen packs. For preparation of Columbia agars, 42,5 g Columbia agar was dissolved in 1000 ml water. Liquid was autoclaved. The bottle was put in 56 °C water for 1 hour. Afterwards, 50 ml horseblood was added to agar. For *H. felis* growth 10 ml β -cyclodextrin and 1 ml of 1000 X antibiotic cocktail (Table 2.2) was added. After 3-4 days of incubation, the bacteria were checked under light microscope for viability and mobility and transferred into liquid Brucella Broth containing 10.000 X Vancomycin (final concentration: 1X) with necessary dilutions for optimal growth. For preparation of Brucella Broth medium, 28 g of brucella broth powder was resuspended in 1L of sterile distilled water (ddH₂O). Following resuspension, the liquid medium was autoclaved at 121°C for 15 min for sterilization.

2.2.2 Sonication of *H. felis* strain

120-200 ml liquid culture of *Helicobacter* strains was used for sonication. Before sonication, the mobility and viability of *Helicobacter felis* (10 μ l) were checked under light microscope. 120- 200 ml liquid culture of *Helicobacter felis* was divided into aliquotes in 15 ml falcons. Falcons were centrifuged at 1500 rpm for 10 minutes. Supernatant was discarded. 10 ml PBS was used to wash bacteria. 15 ml falcons were centrifuged at 1500 rpm for 10 minutes. Supernatant was discarded. 3.5 ml PBS was added on pellet and mixed. Sonication was performed as 30 sec pulse on; 50 sec pulse off for 6.30 minutes at 50 watt on ice to prevent excessive heat formation. MS 72 probe of the sonicator was used. Sonicate was aliquoted to

as 100 µl for each 0.6 ml eppendorfs. They were centrifuged at 15,000 rpm for 20 minutes at 4°C. Sonicate concentration was measured with BCA assay.

2.2.3 Protein bicinchoninic acid (BCA) assay

For determination of *Helicobacter felis* sonicates after sonication and determination of protein samples after cell lysis, Thermo Scientific's Pierce Protein BCA Assay Kit was used. Bovine Serum Albumin (BSA) Standard Set was chosen for microassay. According to the total number of samples and Bovine Serum Albumin (BSA) standards, working reagent (200 µl per sample) was prepared from Solution B and Solution A as 1:50 ratio, respectively and was warmed to ambient temperature. 200 µl of working reagent was distributed into each assayed well of a 96-well F-bottom plate, and all the samples and standards were used in duplicates in order to obtain more accurate results. 10 µl of diluted BSA standards in duplicates were added into corresponding working reagent-containing wells with the concentrations of 0,025; 0,125; 0,25; 0,5; 1; 1,5; 2 mg/mL, respectively. Dilution scheme for BSA standards are given in Table 2.15. 10 µl of protein samples (diluted or undiluted) with unknown concentrations were added to corresponding working reagent-containing wells, and plate was covered and incubated at 37°C for at least 30 min. After 30 min- long incubation, the plate was cooled to room temperature and absorbances were measured at 562 nm on microplate reader.

Table 2.15: Dilution scheme for BCA Assay standards.

Vial	Volume of diluents ddH ₂ O (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 B cells with *Helicobacter felis*

For the separation of *Helicobacter*-activated-IL10⁺B cells and *Helicobacter*-activated-IL10⁻B cells, purified B cells were treated with *H. felis* sonicate (10 µg/ml). They were incubated with or without *H. felis* sonicate for 24 hours. After 24 hours long incubation, IL-10 producing regulatory B cell subset was magnetically labelled and separated from *H. felis*

sonicate-treated cells by using Mouse Regulatory B Cell Isolation Kit (MACS, Miltenyi). The procedures for both B cell and regulatory B cell, isolations are explained in detail in upcoming sections.

2.2.5 MACS Mouse B cell isolation

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

2.2.5.1 Preparation of the cell suspension

Spleen obtained from C57BL/6 mouse was put in the center of 70 μm filter mesh carefully by the help of a tip. Spleen was meshed on the filter mesh with the plunger of a syringe. Single cell suspension was prepared in a 50 ml falcon tube. Incomplete RPMI medium was used to soak the filter and rinse cells from filter to the tube. Procedure was repeated for all obtained spleens. Tube was filled with incomplete medium. Cell suspension was centrifuged at 1480 rpm (200g) for 10 minutes. Supernatant was discarded. Cell pellet was dissolved in 1 ml MACS buffer per spleen. Cell number was determined. Cells were counted by diluting with MACS buffer (~1:200). Cell viability was checked with trypan blue.

2.2.5.2 Pre-enrichment of B cells

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

2.2.5.3 Magnetic separation: depletion of non-B cells

LS column (if total cell number is less than 2×10^8 MS column is more suitable for purification) was placed in suitable MACS separator (Midi) on magnetic field. Column was activated by rinsing with 3 ml of cold MACS buffer. Cell suspension was applied onto the column. Column was washed by 3x3 ml of MACS buffer and time was always given for the reservoir to be emptied between washing steps. Flowthrough was unlabeled B cells. Column

was removed from magnetic field and put in a 15 ml falcon tube. The magnetically labeled non-B cells were flushed out with 5 ml of MACS buffer by firmly pushing the plunger of the column. Cell number was determined by diluting 1:10 with MACS buffer and cell viability was checked with trypan blue under light microscope. Flow cytometer analysis for CD19 surface marker was performed in order to determine purity of B cells.

2.2.5.4 Flow staining for CD19 surface marker

Purity of freshly purified splenic B cells was determined using flow cytometer. 5×10^5 B cells and depleted non-B cells were stained with 0.2 μ l FITC conjugated anti- CD19 antibody in 50 μ l FACS Buffer in the dark on ice for at least 45 minutes. A fraction of B and non-B cells were left as unstained controls. Then, cells were washed once with 1 ml FACS Buffer by centrifugation at 2000 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended in 150 μ l of FACS Buffer and samples (both unstained and stained) were analyzed on flow cytometer.

2.2.6 IL-10 producing regulatory B cell isolation

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

2.2.6.1 *In vitro* stimulation

Isolated CD19⁺ B cells were centrifuged at 1780 rpm for 8 minutes. Cell pellet was resuspended in proper amount of medium (2.5×10^6 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 μ g/ml). Cells which were not stimulated with sonicate were kept as control B cells. Yanaba et al. (2009) demonstrated that splenic B cells treated with PMA and ionomycin in vitro for 5 hours result in optimal IL-10 production. Therefore, PMA (50 ng/ml) and ionomycin (500 ng/ml) were added for the last 5 hours of incubation in order to induce IL-10 secretion from *Helicobacter*-stimulated B cells.

2.2.6.2 Labeling cells with regulatory B cell catch reagent

Cells were harvested by collecting into a falcon tube by pipetting up and down. The wells also washed with MACS Buffer in order to ensure harvesting of all B cells. Cell number was determined by diluting 1:10 with MACS Buffer. For cell viability check, trypan blue staining

was performed. Cells were washed by adding MACS buffer and centrifuged at 1780 rpm for 8 minutes. Supernatant was discarded carefully. Cell pellet was resuspended in 90 μ l of cold medium per 10^7 total cells. 10 μ l of Regulatory B Cell Catch Reagent was added per 10^7 total cells. Regulatory B Cell Catch Reagent is a specific molecule which has two binding domains one of which recognizes CD45 on B lymphocytes and the other recognizing IL-10. Tube was mixed well and incubated on ice for 5 min.

2.2.6.3 IL-10 secretion period

10 ml warm medium was added per 10^7 cells. Cells were incubated in closed tube for 45 min at 37°C. Tube was turned upside down every 5 minutes to resuspend settled cells. In this period, the aim was basically to catch IL-10 secreted from *Helicobacter*-stimulated B cells on cell surface following binding of catch reagent to CD45 surface marker on B cells.

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

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

2.2.6.5 Magnetic labeling with anti-PE microbeads

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

2.2.6.6 Magnetic separation using MS column

MS column (up to 2×10^8 total cells) was placed on mini MACS Separator in the magnetic field. Column was activated by rinsing with the 500 μ l of MACS buffer. Cell suspension was applied onto the column. Unlabeled cells (IL-10 negative B cell fraction) were collected as flow-through and column was washed 3 times with 500 μ l MACS buffer. Column was removed from separator and put on a falcon tube. 1 ml of MACS buffer was pipetted onto the column and magnetically labeled cells (IL-10 positive B cell fraction) were flushed out by plunger. Cell numbers were determined by diluting 1:10 with MACS buffer and cell viability was checked with trypan blue under light microscope. Flow cytometer analysis was performed for IL-10-PE in order to check the purity of IL-10 positive and IL-10 negative fractions. A fraction of B+*H.f.* cells before addition of PE-coupled IL-10 detection antibody was separated as unstained control. The steps of procedures including splenic murine CD19⁺ B cell isolation and separation of B cells according to their IL-10 production capacity following stimulation with *H. felis* sonicate for 24 hours are displayed in Figure 2.1.

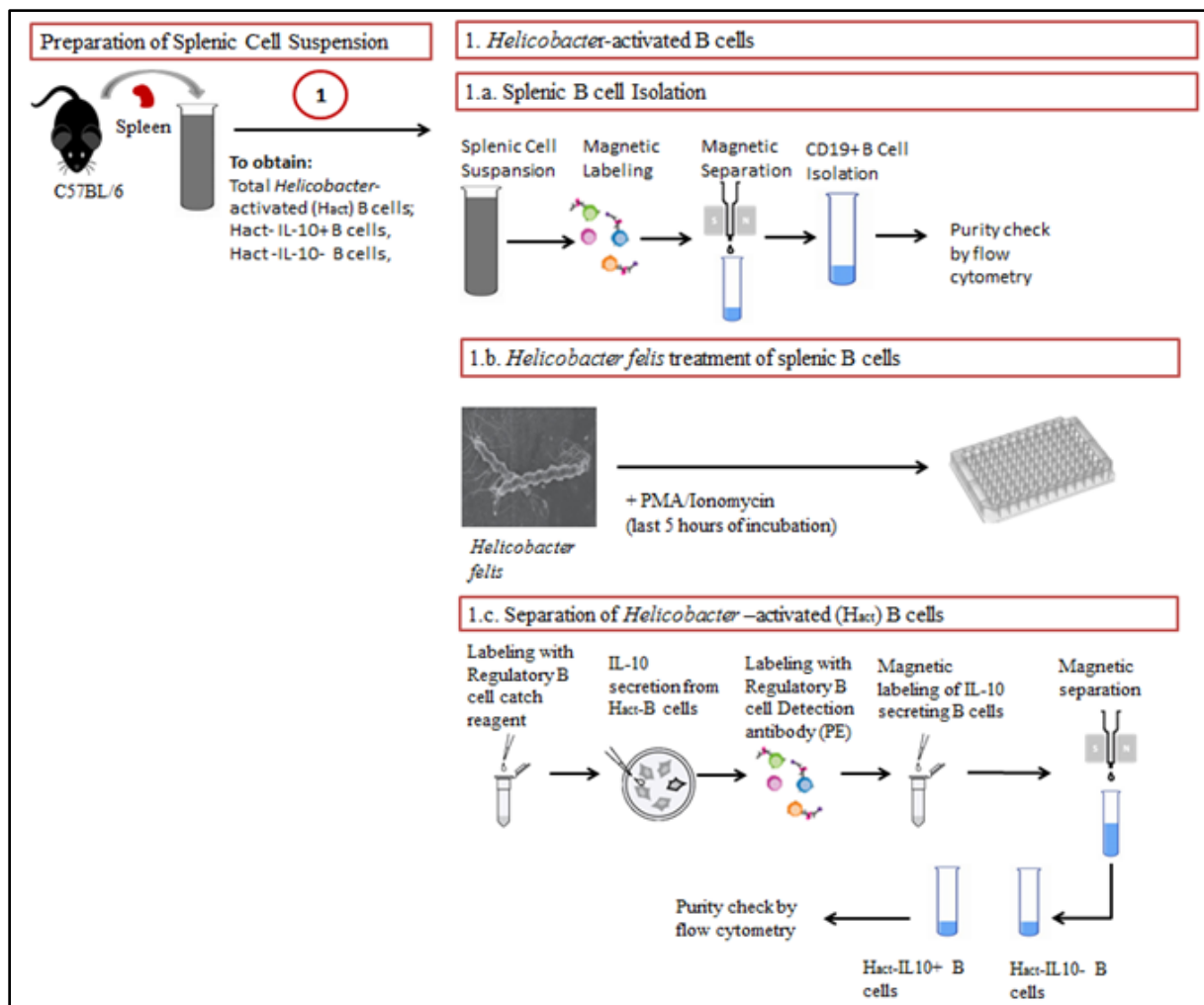


Figure 2.1: Purification of murine splenic B cells and subsequent separation of B cells according to their IL-10-production capacities. CD19⁺ B cells were purified from murine splenic cells (splenocytes) by negative selection (Macs Miltenyi). All splenocytes except for CD19⁺ cells were magnetically labeled by an antibody cocktail and CD19⁺ B cells were acquired as flow through in a magnetic field. Purified B cells were cultured (2.5x10⁶ cells/ml) in the presence of *H.felis* sonicate (10µg/ml) for 24 h. PMA (50 ng/ml) and ionomycin (500 ng/ml) were added into the culture during the last 5 h in order to induce IL-10 secretion. Following 24 h-long incubation, cells were harvested and labeled with a compound called Regulatory B Cell Catch Reagent (Macs Miltenyi) that was designed to capture secreted IL-10 at IL-10 secretion period on the cell surface. Then, cells were magnetically labeled with an PE- conjugated IL-10 detection antibody. Upon application of a magnetic field, IL-10 producing B cells (IL-10⁺ B) were kept in the column (Macs Miltenyi) whereas IL-10 negative B cells (IL-10⁻ B) were acquired as flow-through. IL-10⁺ B cells were obtained by subsequent flushing-out of cells from the column.

2.2.7 MACS Mouse CD4⁺T cell isolation

CD4⁺ T cells were isolated using Mouse CD4⁺ T Cell Isolation Kit (MACS Miltenyi). The procedure is explained in detail in sections 2.2.6.1-2.2.6.4.

2.2.7.1 Preparation of the cell suspension

Spleen obtained from C57BL/6 mouse was put in the center of 70 μm filter mesh carefully by the help of a tip. Spleen was meshed on the filter mesh with the plunger of a syringe. Single cell suspension of the spleen was prepared in a 50 ml falcon tube. Incomplete RPMI medium was used to soak filter and rinse cells from filter to the tube. Procedure was repeated for all obtained spleens. Tube was filled with incomplete medium. Cell suspension was centrifuged at 1480 rpm (200g) for 10 minutes. Supernatant was discarded. Cell pellet was dissolved in 1 ml MACS buffer per spleen. Cell number was determined. Cells were counted by diluting with MACS buffer (~1:200). Cell viability was checked with trypan blue.

2.2.7.2 Magnetic labeling of non-CD4⁺ T cells

After cell number and viability was determined, cell suspension was centrifuged at 1780 rpm for 8 minutes. Cell pellet was resuspended in 40 μl of MACS buffer per 10^7 cells. Then 10 μl of T Cell Biotin-Antibody Cocktail (containing antibodies targeted against CD8a, CD11b, CD11c, CD19, CD45 (B220), CD49b (DX5), CD105, anti-MHC Class II, Ter-119, TCR γ/δ) was added per 10^7 cells. Tube was mixed well and incubated at refrigerator for 5 min. 30 μl of MACS buffer and 20 μl of Anti-Biotin MicroBeads were added per 10^7 total cells. Tube was mixed well and incubated in refrigerator for another 10 min. Cells were washed with 1-2 ml of MACS Buffer per 10^7 cells. Cell pellet was resuspended in 500 μl of MACS buffer per 10^8 cells.

2.2.7.3 Magnetic separation: depletion of non-T cells

LS column (if total cell number is less than 2×10^8 MS column is more suitable for purification) was placed in suitable MACS separator (Midi) on magnetic field. Column was activated by rinsing with 3 ml of cold MACS buffer. Cell suspension was applied onto the column. Column was washed by 3x3 ml of MACS buffer and time was always given for the reservoir to be emptied between washing steps. Flowthrough was unlabeled T cells. Column was removed from magnetic field and put in a 15 ml falcon tube. The magnetically labeled non-T cells were flushed out with 5 ml of MACS buffer by firmly pushing the plunger of the column. Cell number was determined by diluting 1:10 with MACS buffer and cell viability was checked with trypan blue under light microscope. Flow cytometer analysis for CD4 surface marker was performed in order to determine purity of T cells.

2.2.7.4 Flow staining for CD4 surface marker

Purity of freshly purified splenic T cells was determined using flow cytometer. 5×10^5 T cells and depleted non-T cells were stained with 0.2 μ l APC conjugated anti- CD4 antibody in 50 μ l FACS Buffer in the dark on ice for at least 45 minutes. A fraction of T and non-T cells were left as unstained controls. Then, cells were washed once with 1 ml FACS Buffer by centrifugation at 2000 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended in 150 μ l of FACS Buffer and samples (both unstained and stained) were analyzed on flow cytometer.

2.2.8 Co-culture of *Helicobacter*-activated-B cells and T cells

To observe the interaction between the *Helicobacter*-activated-B cell subgroups and CD4⁺ T cells, isolated CD4⁺ T cells were put on co-culture in 1:1 ratio with the *Helicobacter*-activated-IL-10⁺ B cells and *Helicobacter*-activated-IL-10⁻ B cells (separation is explained in section 2.2.6) for 24 hours.

2.2.9 Antibody stainings for flow cytometry

2.2.9.1 Surface marker stainings

After 24 hour incubation (37°C, 5% CO₂) of the co-cultures of B and T cells, the cells were harvested, centrifuged at 2000 rpm for 5 minutes, resuspended in FACS Buffer and separated into 0,5 ml eppendorf tubes for different stainings. One sample for each different co-culture group was left as unstained control. Cells were re-suspended in 50 μ l of 2% FACS buffer, antibodies for surface stainings are added into the tubes with appropriate concentrations. Cells were incubated for 1 hour at 4°C for staining. Afterwards, cells were washed with 2% FACS buffer and centrifuged at 2000 rpm for 5 minutes. Supernatants were discarded and the pellets were resuspended in 150 μ l of 2% FACS buffer for flow cytometry analysis.

2.2.9.2 Intracellular stainings for IL-10 and IL-17

For *in vitro* stimulation of cells, it is necessary to block secretion of cytokines with protein transport inhibitors, such as the Monensin or Brefeldin A Solution. In order to determine intracellular IL-10 and IL-17 production levels via flow cytometry, the IL-10 and IL-17 cytokine secretion was blocked by Monensin (3 μ g/ml, Calbiochem) 5 hours before harvesting of the cells. Monensin is a protein transport inhibitor commonly used to enhance intracellular cytokine staining signals by blocking transport processes during cell activation. It leads to the

accumulation of most cytokines at the Golgi Complex/Endoplasmic Reticulum (Jung et al., 1993). A fraction of the co-culture cells were left as unstained controls. After harvesting of the cells by centrifugation at 2000 rpm for 5 minutes, cells were fixed with 100 µl of 2% Fixation Buffer (Biolegend) containing paraformaldehyde that cross-links the proteins, for 15 minutes on ice. Following fixation, cells were washed with 500 µl of FACS Buffer once by centrifugation at 2000 rpm for 5 minutes. Supernatant was discarded and pellets were resuspended in 175 µl of 1X Permeabilization Buffer (diluted from 10X with ddH₂O) to provide access to intracellular antigens. Saponin in the permeabilization buffer interacts with membrane cholesterol, selectively removing it and leaving holes in the membrane. Cells were centrifuged at 2000 rpm for 5 minutes. Pellets were resuspended in 175 µl of 1X Permeabilization Buffer once more, then they were centrifuged at 2000 rpm for 5 minutes. This results in the creation of pores in the cellular membrane that require the continuous presence of the permeabilization buffer during all subsequent steps to allow antibodies to have access to the cytoplasm of the cell. The cells are resuspended in 50 µl of permeabilization buffer containing 0.4 µl of PE-coupled anti-mouse IL-10 detection antibody (MACS Miltenyi) and 0.4 µl of FITC-coupled anti-mouse IL-17 detection antibody (MACS Miltenyi). Afterwards, samples were stained in the dark at 4°C for more than 1 hour. Following the incubation, cells were washed with 175 µl permeabilization buffer by centrifugation. Pellets were washed once in 500 µl of FACS Buffer by centrifugation and pellets were resuspended in 150 µl of FACS Buffer and analyzed on flow cytometer.

2.2.10 ELISA

2.2.10.1 IL-10 ELISA

IL-10 protein levels in supernatant of samples were determined by Biolegend's Mouse IL-10 ELISA Deluxe Max Kit. For quantitative determination of IL-10 protein in culture medium of B cells, Nunc 96-well plates were coated with IL-10 capture antibody 1:200 diluted in coating buffer 1X and plate was incubated at 4°C overnight. Following day, plate was washed four times with PBS/T. Then, 100 µl of Assay Diluent A 1X (diluted from 5X with PBS 1X) was added into assayed wells and the plate was incubated at room temperature for 1h. Recombinant IL-10 standards were prepared by serial dilution according to manufacturer's instructions. After that, plate was again washed four times with PBS/T. Following washing steps, diluted recombinant IL-10 standards and culture media supernatants (50 µl) were added as biological duplicates. Plate was incubated at room temperature for 2 h. Following 2 h-long

incubation, plate was washed four times with PBS/T. Biotinylated IL-10 detection antibody diluted 1:200 in Assay Diluent 1X was added into each assayed well and plate was incubated at room temperature for 1 h. After washing of assayed wells with PBS/T for three times, Avidin-HRP solution 1:1000 diluted in Assay Diluent A 1X was added into each assayed well as 50 µl and plate was incubated in the dark for 30 min at room temperature. After 30 min-long incubation, plate was washed for five times with PBS/T and 50 µl of TMB Substrate Solution Mixture (1:1 of TMB Substrate A and TMB Substrate B) was added into each assayed well. The plate was incubated for at least 30 minutes at room temperature. High concentration standards and samples turned into blue. After that, the reaction was stopped with 50 µl stop solution (2N H₂SO₄). Then, the absorbances of the samples were measured at 450 nm on a microplate reader.

2.2.10.2 IL-17 ELISA

IL-17 protein levels in supernatant of samples were determined by Biolegend's Mouse IL-17 ELISA Kit. For quantitative determination of IL-17 protein in culture medium of B and T cells, Nunc 96-well plates were coated with IL-17 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-17 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-17 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-17 detection antibody diluted 1:200 in Assay Diluent 1X was added into each assayed well and plate was incubated at room temperature for 1 h. After washing of assayed wells with PBS/T for three times, Avidin-HRP solution 1:1000 diluted in Assay Diluent A 1X was added into each assayed well as 50 µl and plate was incubated in the dark for 30 min at room temperature. After 30 min-long incubation, plate was washed for five times with PBS/T and 50 µl of TMB Substrate Solution Mixture (1:1 of TMB Substrate A and TMB Substrate B) was added into each assayed well. The plate was incubated for at least 30 minutes at room temperature. High concentration standards and samples turned into blue. After that, the reaction was stopped

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

2.2.11 Analysis of relative expression levels

2.2.11.1 RNA isolation

To determine relative expression levels of IL-10 and IL-17, T cell, B cell and T&B co-culture pellets were subjected to RNA isolation. To lyse the cells, 350 µl of RA1 lysis buffer and 3.5 µL β-mercaptoethanol were added to cell pellets and then they were vortexed vigorously. For the filtration of the lysates, NucleoSpin® Filter was placed in a Collection Tube (2 mL) and the mixture is applied on the filter, then centrifuged for 1 min at 11,000 x g. The NucleoSpin® Filter is discarded. 350 µl of 70% ethanol was added to flowthrough and the solution is homogenized by pipetting up and down at least 5 times. After homogenization, lysates are loaded on the NucleoSpin® RNA Column (blue ring) and centrifuged at 11,000 x g for 30 seconds. To digest the DNA, DNase reaction mixture is prepared in a sterile 1,5 mL eppendorf tube. For each isolation, 10 µL reconstituted rDNase was added to 90 µL Reaction Buffer for rDNase. The solution was mixed by flicking the tube. 95 µL DNase reaction mixture was directly applied onto the center of the silica membrane of the column. The tubes are incubated at room temperature for 15 minutes. To inactivate the rDNase, 200 µL Buffer RAW2 was added to the NucleoSpin® RNA Column. Tubes were centrifuge for 30 s at 11,000 x g. The column was placed into a new Collection Tube (2 mL). For efficient washing of the inner rim, 600 µL Buffer RA3 was added to the NucleoSpin® RNA Column. The tubes were centrifuge for 30 s at 11,000 x g. The flowthrough was discarded and the column was placed back into the Collection Tube. For a final washing, 250 µL Buffer RA3 was added to the NucleoSpin® RNA Column, centrifuged for 2 min at 11,000 x g to dry the membrane completely. Then the column was placed into a nuclease-free Collection Tube (1.5 mL). 35 µl of RNase-free water was added on filter and incubated for 1 min at room temperature. Finally, tubes were centrifuged at 11,000 x g for 1 min. Flow through was kept as isolated RNA. The RNA concentrations were measured with NanoDrop.

2.2.11.2 cDNA synthesis

Synthesis of cDNA was performed according to manufacturer's instructions. Amounts used in synthesis reaction are given in Table 2.16. Synthesis conditions are given in Table 2.17.

Table 2.16: Components of cDNA synthesis reaction.

Component	Amount
RNA (1µg)	depends on concentration
ddH ₂ O	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 2.17: Reaction conditions of cDNA synthesis.

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

2.2.11.3 Real time PCR

Relative expression levels of IL-17 and Ror gamma T was analyzed with Real - time PCR. Amounts used in reaction are given in Table 2.18. PCR conditions performed are given in Table 2.19.

Table 2.18: 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 2.19: Reaction conditions of real time PCR.

Temperature	Time
95°C	5 min.
95°C	30 sec.
Depends on primer	1 min. (45 cycles)
2°C	1 min.
72°C	5 min.
4°C	∞

2.2.12 Flow cytometry analyses

Flow cytometry analyses were performed using FlowJo and Accuri C6 software.

2.2.13 Statistical analyses

All p values were calculated using GraphPad Prism 6.0 software. Significancies were determined by Student t test. In all analyses, a two-tailed p-value of less than 0.05 was considered statistically significant. In column bar graphs, vertical bars indicate standard deviations of the mean, n.d. stands for not determined, and n.s. denotes not significant.

3. RESULTS

3.1 CD19⁺ B cell isolation from spleens of C57BL/6 mice

B cells were isolated from spleens of C57BL/6 mice by using B Cell Isolation Kit (see Materials&Methods, Section 2.2.5). Pre- enrichment of B cells was performed by negative selection using magnetic separation method. Following the separation, to detect their purity, B cells were labeled with FITC-coupled anti-CD19 antibody and compared to unstained control. Flow cytometry analyses showed that the B cells were isolated with purities higher than 83% with an average purity of almost 90% (Figure 3.1).

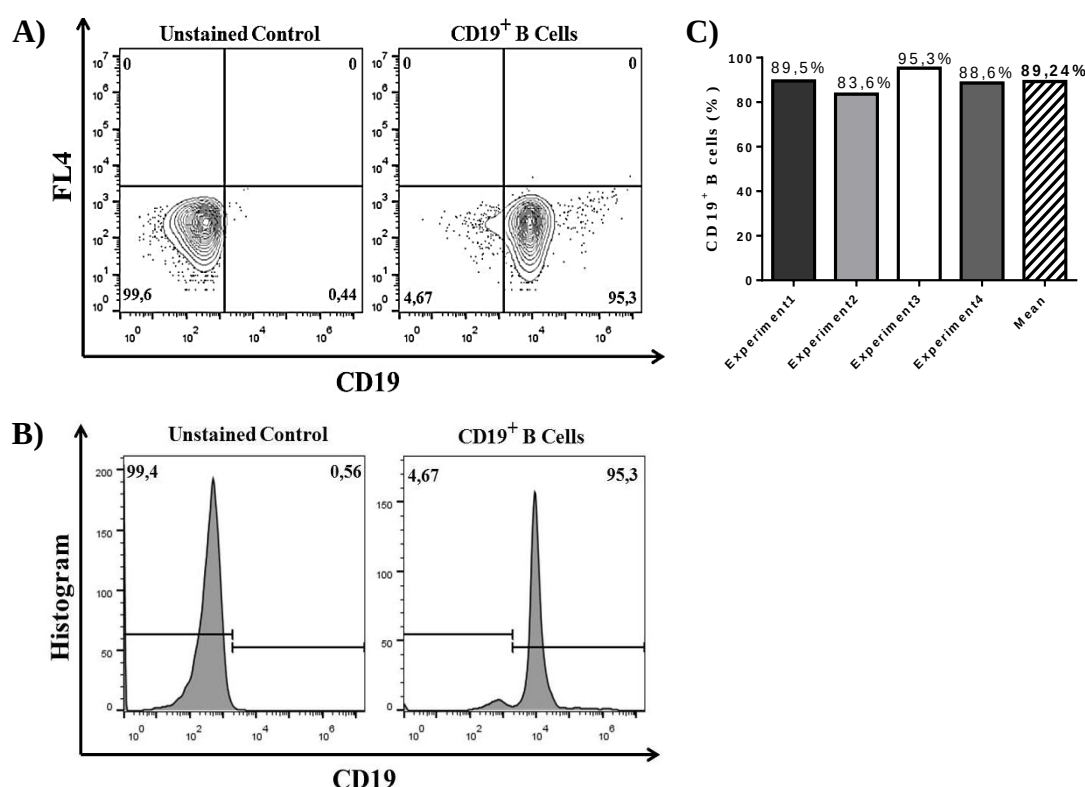


Figure 3.1: Purity of B cells isolated from spleen of C57BL/6. Representative flow cytometry quadrant plot (A) and histogram plot (B) of purified B cells compared to unstained control. B cells were magnetically isolated from spleen of C57BL/6 mice by negative selection. Cells were either stained with FITC-coupled anti-CD19 antibody (0.5 µg/µl) or left unstained (unstained control) in order to detect the percentage of cells expressing B cell-specific surface marker CD19. (C) B cell purities of each experiment and a mean value are shown in the graph. For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software is used. Graphs are prepared with Graphpad Prism 6 software.

3.2 Separation of *Helicobacter*-activated-IL-10⁺ B cells and *Helicobacter*-activated-IL-10⁻ B cells

To obtain IL-10-producing B cells upon *Helicobacter felis* sonicate activation, after 24 hours of *H.felis* stimulation, B cells were subjected to magnetic separation (see Materials&Methods, Section 2.2.6). The basis of the separation is to capture secreted IL-10 on the surface of IL-10 secreting B cells with an anti-IL-10-PE antibody. Thus, after magnetic separation of the IL-10⁺ and IL-10⁻ B cells, the purity of the cells is determined by assessing the intensity of PE fluorochrome (Figure 3.2).

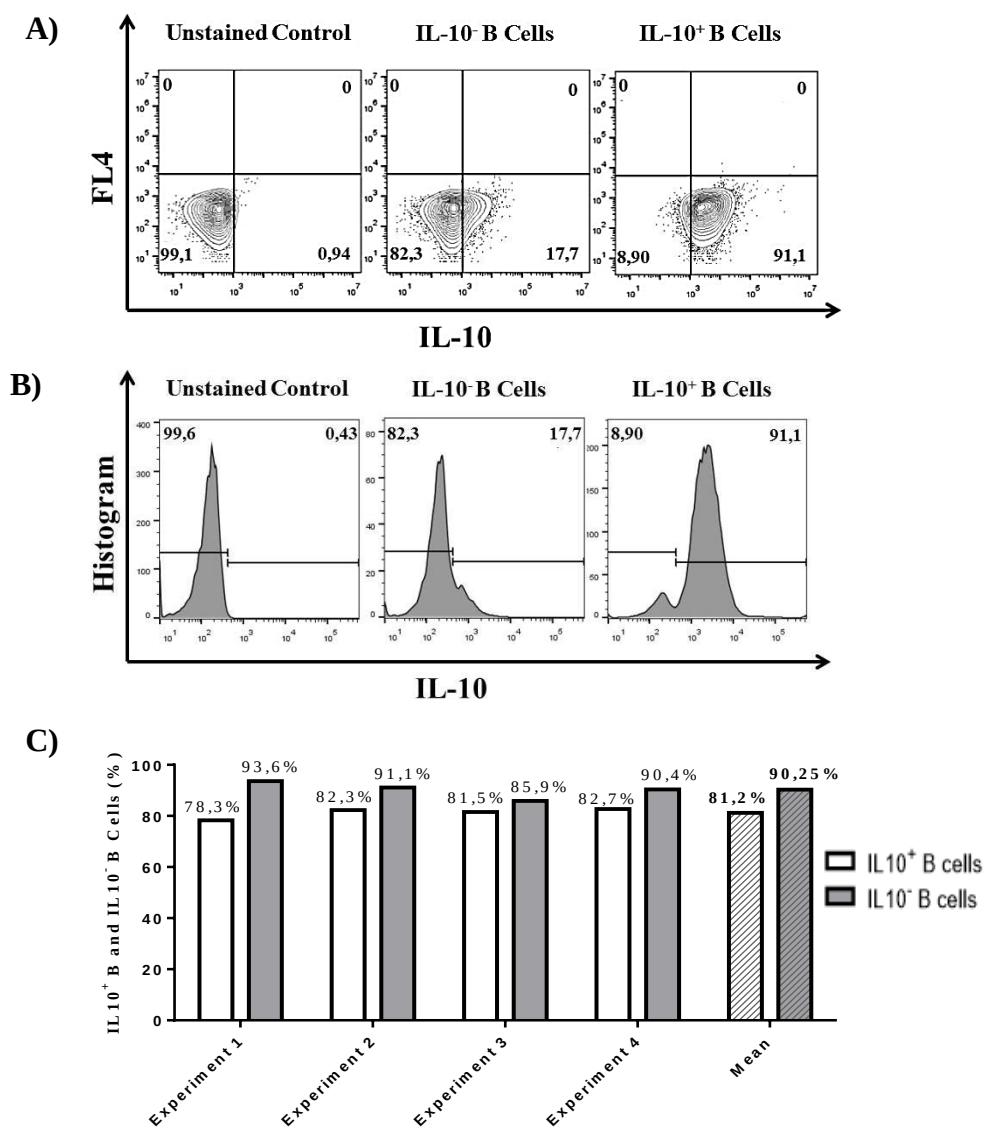


Figure 3.2: Percentages of IL-10 positive and IL-10 negative B cells after *Helicobacter felis* treatment for 24 hours. Representative flow cytometry quadrat plot (A) and histogram plot (B) of separated *Helicobacter*-activated IL-10⁺ and IL-10⁻ B cells compared to unstained control. (C) IL-10⁺ and IL-10⁻ B cell purities of all experiments and a mean value are shown in the graph. For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software was used. Graphs were prepared with Graphpad Prism 6 software.

3.3 CD4⁺ T cell isolation from C57BL/6 mice

Splenic CD4⁺ T cells magnetically isolated with CD4⁺ T Cell Isolation Kit (MACS, Miltenyi) from C57BL/6 mice (see Materials&Methods, Section 2.2.6). Negatively selected CD4⁺ T cell fractions were collected. Following the separation, CD4⁺ T cells were labeled with APC-coupled anti-CD4 antibody and compared to unstained control. Flow cytometer analysis was performed to measure the purity (Figure 3.3).

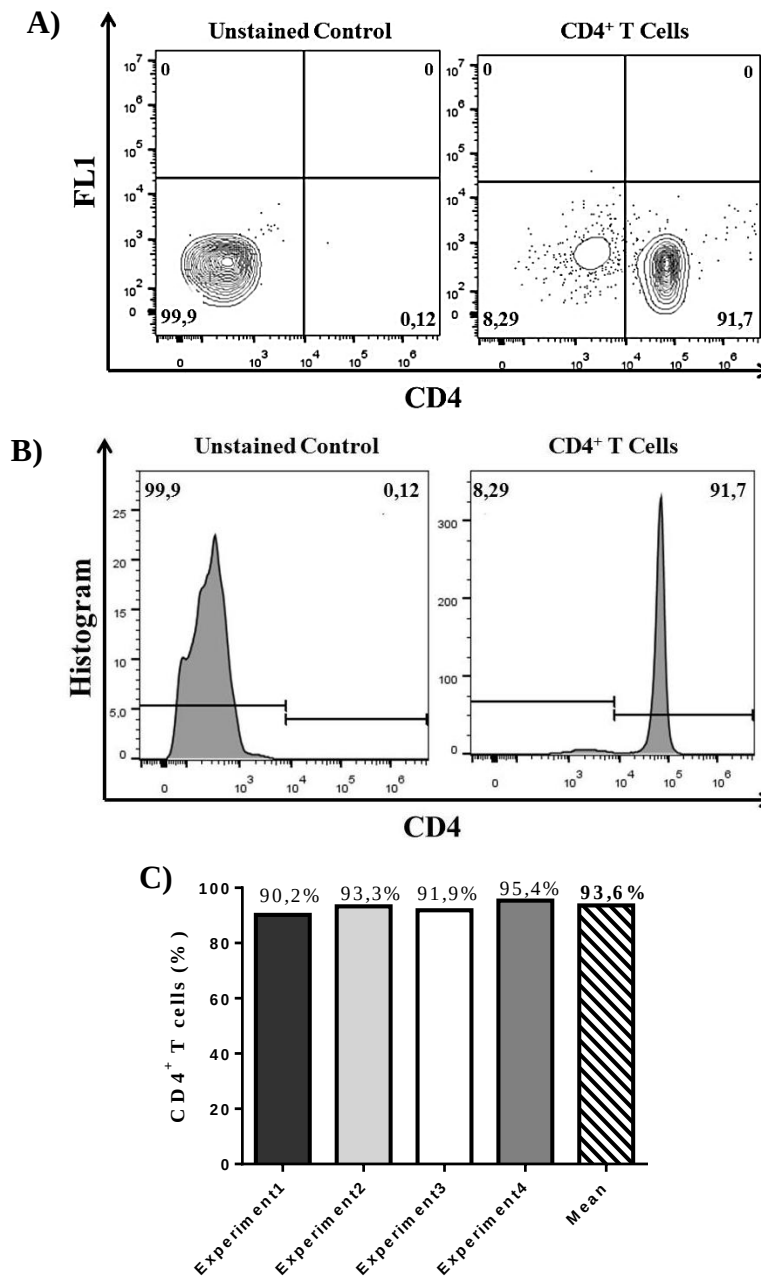


Figure 3.3: Purity of CD4⁺T cells isolated from C57BL/6 mice. Representative flow cytometry quadrant plot (A) and histogram plot (B) of purified CD4⁺T cells compared to unstained control. CD4⁺T cells were magnetically isolated from spleen of C57BL/6 mice by negative selection. Cells were either stained with APC-coupled anti-CD4 antibody (0.5 µg/µl) or left unstained (unstained control) in order to detect the percentage of cells expressing CD4⁺T cell- specific surface marker CD4. (C) CD4⁺T cell purities of all experiments and a mean value is shown in the graph. For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software was used. Graphs were prepared with Graphpad Prism 6 software.

Addition to CD4, another surface marker, CD25, was labeled to observe the activated T cell ratio in the CD4⁺ T cell population. CD25 is the alpha chain of the IL-2 receptor. It is a type I transmembrane protein present on activated T cells. CD25 has been also used as a marker to identify CD4⁺FoxP3⁺ regulatory T cells in mice. Right after the T cell isolation, a surface antibody staining was applied with FITC coupled anti-CD25 antibody and APC coupled anti-CD4 antibody (Figure 3.4).

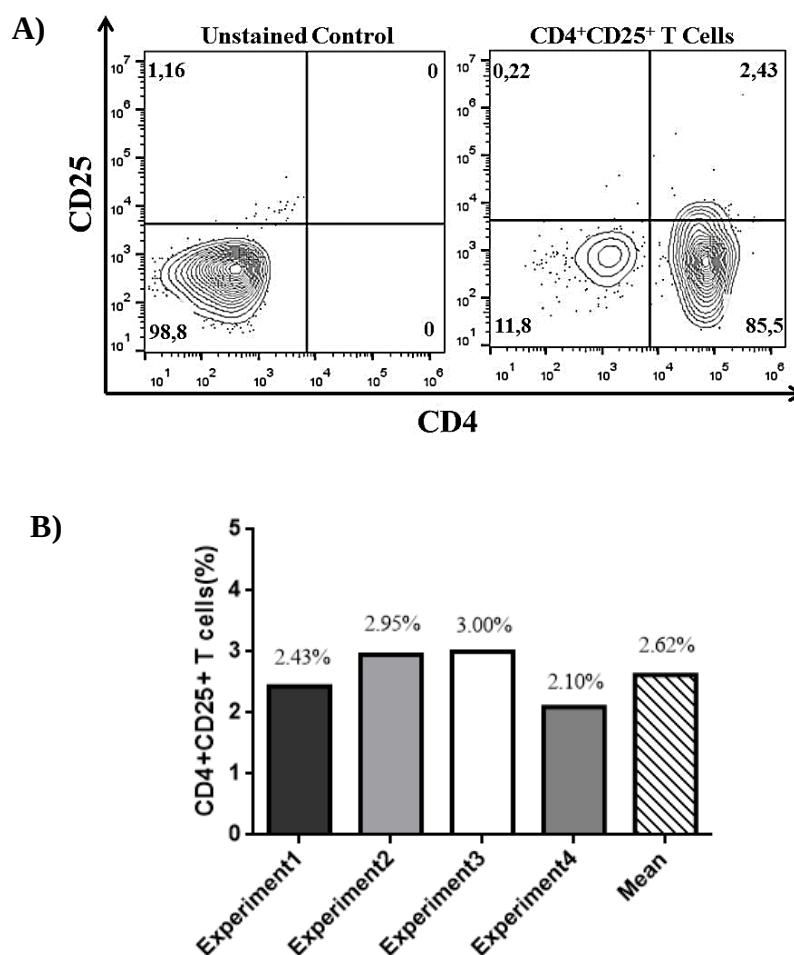


Figure 3.4: CD25 levels of freshly isolated CD4⁺T cells. (A) Representative flow cytometry plot of CD25 and CD4 surface stainings of CD4⁺T cells compared to unstained control. Directly after isolation of CD4⁺T cells, cells were stained both with APC-coupled anti-CD4 antibody (0.5 µg/µl) and FITC coupled anti-CD25 antibody or left unstained (unstained control). (B) CD4⁺CD25⁺ T cell percentages of all experiments and a mean value is shown in the graph (4 experiments). For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software is used. Graphs are prepared with Graphpad Prism 6 software.

To sum up, CD19⁺ B cells as well as CD4⁺ T cells were isolated with high purities, while the level of CD4⁺CD25⁺ T cells were low. After 24 hour treatment of B cells with *Helicobacter felis*, the *Helicobacter*-activated-IL-10⁺ B cells (*H_{ACT}*-IL-10⁺ B) and *Helicobacter*-activated-IL-10⁻ B (*H_{ACT}* -IL-10⁻ B) cells were successfully separated with purities higher than 80% and 90% respectively.

3.4 Effects of H_{ACT} -IL-10⁺ B cells and H_{ACT} -IL-10⁻ B cells on Tr1 cell differentiation

By using mouse models of *Helicobacter*-induced gastric pathology, Sayı *et al.* showed regulatory function of B cells depended on their ability to sense and respond to *Helicobacter* TLR-2 ligand and to directly interact with naive T cells, which are converted to Tr-1-like, CD4⁺CD25⁺IL-10⁺ cells in a CD40L- and CD28-dependent manner (Sayı *et al.*, 2011). Based on this information, it was known that *Helicobacter*-activated B cells could differentiate T cells to Tr1 cells; however, it was not clear which subset of these *Helicobacter*-activated B cells took part in this differentiation. For this reason, after the separation of H_{ACT} -IL-10⁺ B cells and H_{ACT} -IL-10⁻ B cells, CD4⁺ T cells were placed into co-culture with these *Helicobacter*-activated B cell subsets. They were cultured in 1:1 ratio, for 24 hour (37°C, 5% CO₂). As a control group, only T cell group without B cells were included in the experiments.

3.4.1 IL-10 levels of CD4⁺ T cells in co-culture with H_{ACT} -IL-10⁺ B cells and H_{ACT} -IL-10⁻ B cells

The IL-10 production of CD4⁺T cells was examined by intracellular IL-10 antibody staining which was afterwards detected by flow cytometry (Figure 3.5). For this purpose, the cells in the co-cultures were treated with a protein transport inhibitor, Monensin (3µg/ml, Calbiochem) to keep the cytokines accumulated inside the Golgi complex. T cells were labeled with APC-coupled anti-CD4 antibody. Secondly, the cells were fixed with 2% fixation buffer which contains paraformaldehyde that cross-links proteins. Next, by using a saponin-based Permeabilization Buffer (Biolegend), holes were created in the membrane thereby allowing the intracellular staining antibodies to enter the cell effectively. After the cells were permeabilized, they were incubated with PE-coupled anti-IL-10 antibody that was prepared inside the permeabilization buffer.

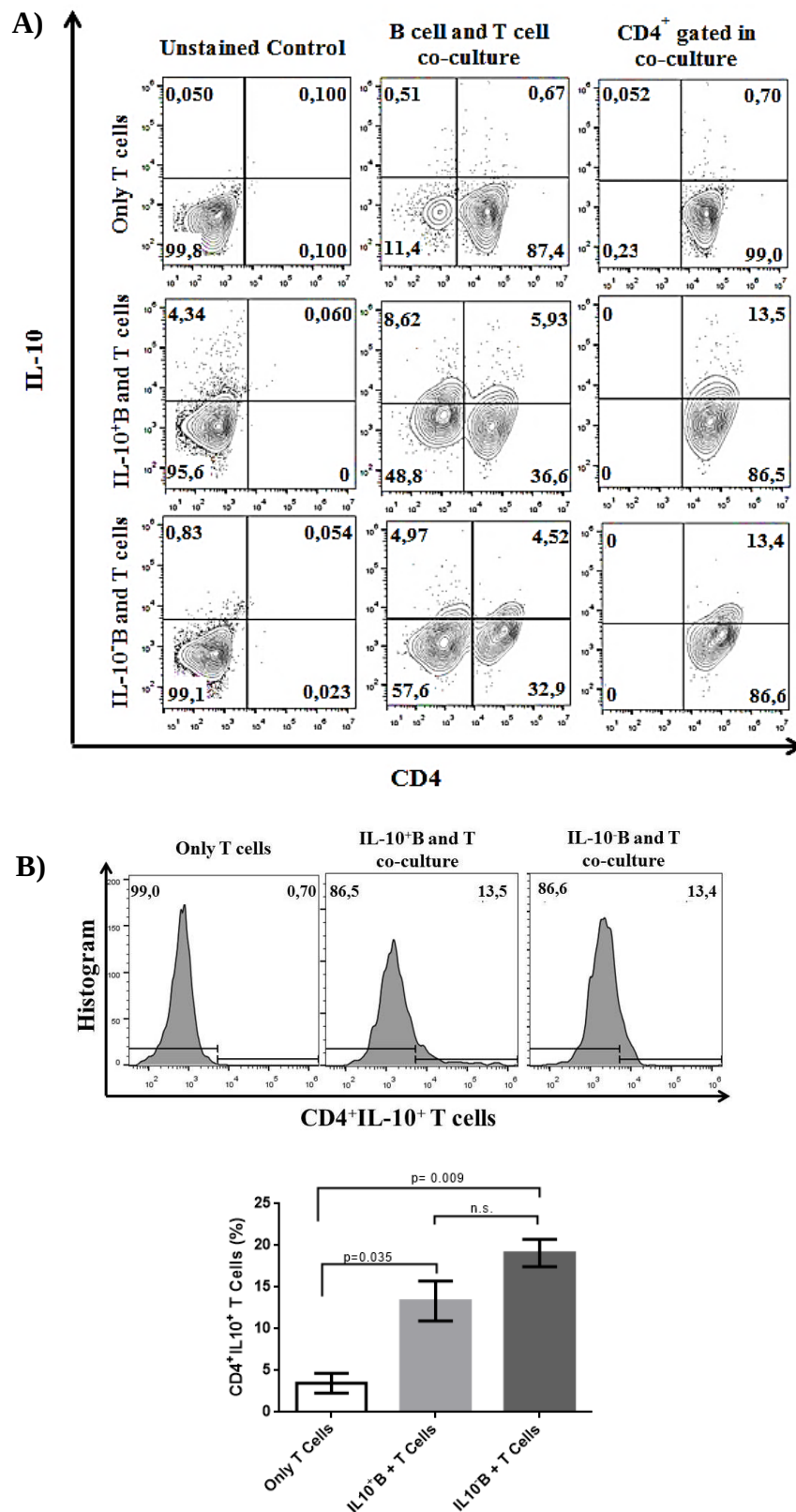


Figure 3.5: IL-10 Levels of CD4⁺ T cells co-cultured with *Helicobacter*-activated IL-10⁺B and IL-10⁻B cells. Representative flow cytometry quadrant plot (A) and a histogram plot (B) of CD4⁺IL-10⁺ T cells in co-culture with B cell subsets compared to unstained control is given. Cells were stained with anti-CD4-APC and intracellular staining was done for IL-10 cytokine using anti-IL10-PE antibody. To detect the IL-10 levels for T cells were shown in , CD4gate. (C) A graphical expression of the CD4⁺IL-10⁺ T cells within the co-culture groups (4 independent experiments). For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software was used. Graphs were prepared with Graphpad Prism 6 software. Significance evaluation was performed with Student's t test ($p < 0.05$ was significant).

As shown in Figure 3.5, when compared with only T cell groups, both co-culture groups had significantly higher levels of IL-10 producing T cells. About 15% of T cells co-cultured with H_{ACT} -IL-10⁺ B cells produced IL-10 while almost 20% of the T cell population was IL-10 positive when T cells were co-cultured with H_{ACT} -IL-10⁻ B cells. Even though there were some deviations within the four experimental data sets that led to relatively high standard deviations in the result graph (Figure 3.5(C)), the IL-10 cytokine production levels of T cells in two co-culture groups were comparable for all experiments. Altogether, it could be suggested that in both co-culture groups there was a significant differentiation of CD4⁺ T cells into IL10⁺CD4⁺ Tr1-like cells.

3.4.2 IL-10 secretion levels in co-culture groups of T cell with H_{ACT} -IL-10⁺ B cells and H_{ACT} -IL-10⁻ B cells

To proceed the intracellular staining of IL-10, the cells were treated with Monensin for the cytokine accumulation inside the cells for 5 hours; however the overall IL-10 which was secreted out of the cells could not be measured by that method. For a better understanding of the amount of secreted IL-10 levels from the co-cultures, IL-10 ELISA was performed with the 24 hour co-culture supernatants (Figure 3.6).

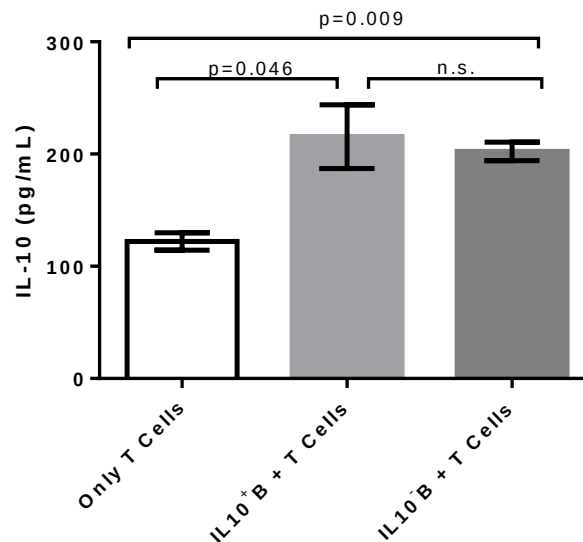


Figure 3.6: IL-10 Secretion Levels in co-culture groups of CD4⁺ T cells with *Helicobacter*-activated IL-10⁺B and IL-10⁻ B cells. CD4⁺ T cells were either co-cultured with *Helicobacter*-activated IL-10⁺ B and IL-10⁻ B cells or left alone. Supernatants were collected after 24 hour co-culture and the amounts of secreted IL-10 were measured with IL-10 ELISA. Bar graph was prepared with GraphPad Prism 6 software.

According to IL-10 ELISA results, CD4⁺T cells co-cultured with IL-10⁺B cells and IL-10⁻B cells secreted twice IL-10 when compared to only T cells. For IL-10⁺B cell and T cell co-culture, a part of secreted IL-10 came from B cells while all the IL-10 secreted from IL-10⁻B cell and T cell co-culture originated from T cells. All in all, it is suggested that compared to IL-10⁺B cells, IL-10⁻B cells drive more IL-10 secretion from CD4⁺T cells.

3.4.3 CD25 expression levels of CD4⁺ T cells in co-culture with H_{ACT}-IL-10⁺ B cells and H_{ACT} -IL-10⁻ B cells

Following the activation of T cells, CD25 is expressed on these cells and IL-2 is immediately synthesized (Kuniyasu et al., 2000). In addition, it is known to be expressed by regulatory T cell types (natural Tregs and CD4⁺CD25⁺Foxp3⁺ induced Tregs) (Sakaguchi et al., 2006). After the isolation of CD4⁺ T cells from mice spleens, these cells express low levels of CD25. The comparison of the CD25 levels after T cells were cultured with *Helicobacter*-activated B cell subgroups would give an opinion whether the T cell activation could alternate when co-cultured with different subgroups of *Helicobacter*-activated B cells. Therefore, after 24 hours of co-culture, anti-CD4-APC and anti-CD25-FITC antibodies were used for the labeling of those surface markers and analyzed by flow cytometry.

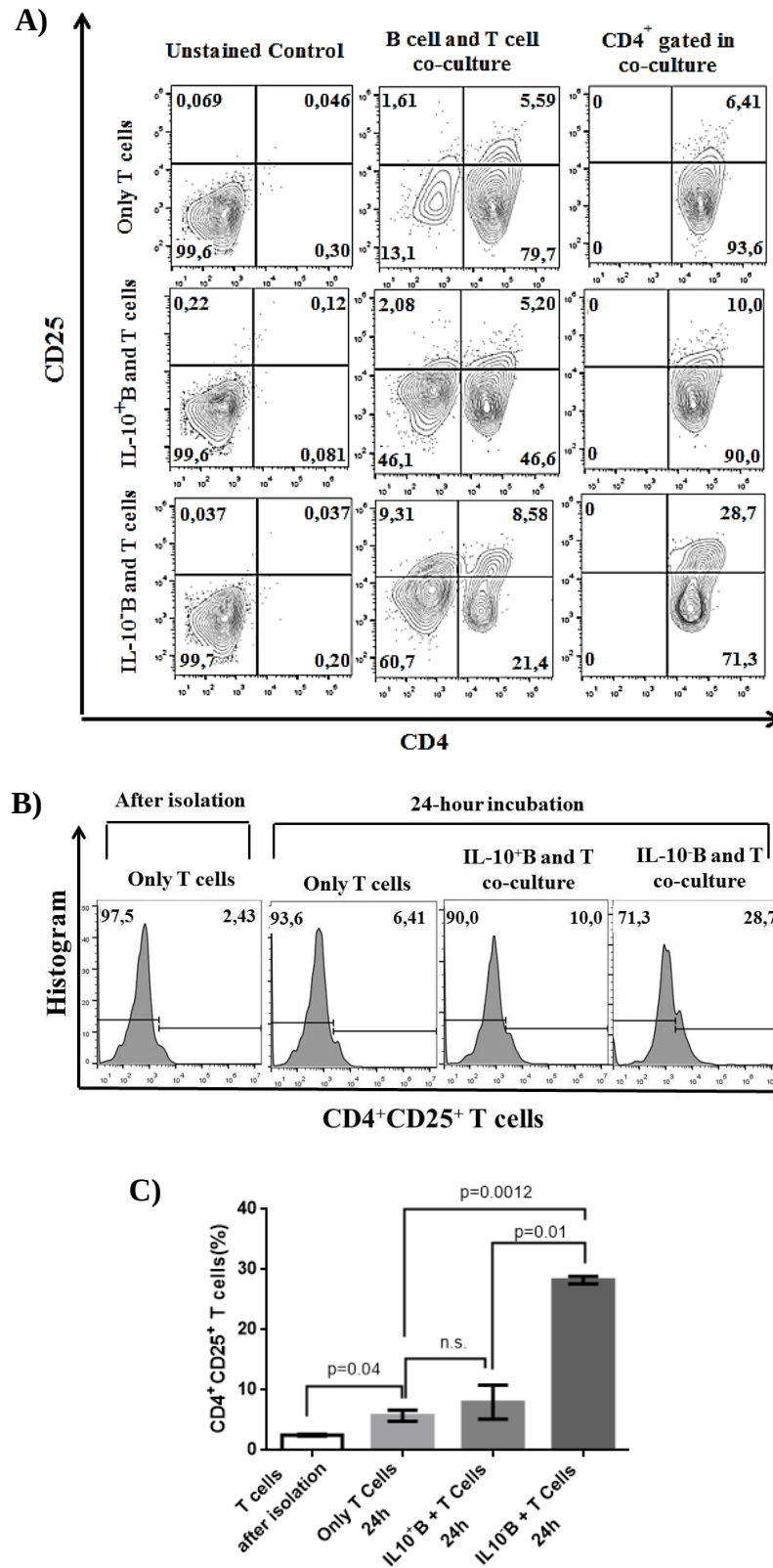


Figure 3.7: Increase in CD25 levels in CD4⁺ T cells co-cultured with *Helicobacter*-activated IL-10⁺B and IL-10⁻B cells. Representative flow cytometry quadrant plot (A) and histogram plot (B) of CD25⁺CD4⁺T cells in co-culture groups compared to unstained control. Cells were labeled with anti-CD4-APC and anti-CD25-FITC and to see the CD25 levels only for T cells, a CD4 gate was selected. (C) A graphical expression of the CD25⁺CD4⁺ T cells within the co-culture groups (4 experiments). For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software was used. Graphs were prepared with Graphpad Prism 6 software. Significance evaluation was performed with Student's t test ($p < 0.05$ was significant).

As shown by Thornton et al. (1998) $CD4^+CD25^+$ T cells expanded when they were cultured with anti-CD3 and IL-2. Being consistent with this knowledge, in only T cell groups, when compared to the CD25 levels in freshly isolated T cells, an increase in CD25 positive T cells was observed after 24 hour incubation (compared with the results from Figure 3.4). Furthermore, the increase in CD25 expression in co-culture groups could be explained in two ways: the increased activation of T cells or the induction of T cells to differentiate into other regulatory T cell subtypes. Even though these results need further experiments to reveal the reason behind the increase in CD25 expression on T cells, they indicated a significant difference in the CD25 expression in T cell co-cultures with IL-10⁻B cells compared to the T and IL-10⁺B cell co-cultures.

3.4.4 CD62L expression levels of $CD4^+$ T cells in co-culture with $H_{ACT}-IL-10^+$ B cells and $H_{ACT}-IL-10^-$ B cells

CD62L, also known as L-selectin, is a 74-95 kD glycoprotein which is expressed on the majority of B and naïve T cells, and a subset of memory T cells. It is rapidly shed from lymphocytes upon cellular activation (Gerberick et al., 1997). Therefore, the expression levels of CD62L in conjunction with other markers have been used to distinguish naïve, effector, and memory T cells. The anti-CD62L antibody is also coupled with APC as anti-CD4 antibody. Consequently, the staining was done with anti-CD62L-APC and anti-CD19-FITC antibodies. To observe the CD62L levels in $CD4^+$ T cells, anti-CD19-FITC negative gate was selected (Figure 3.8).

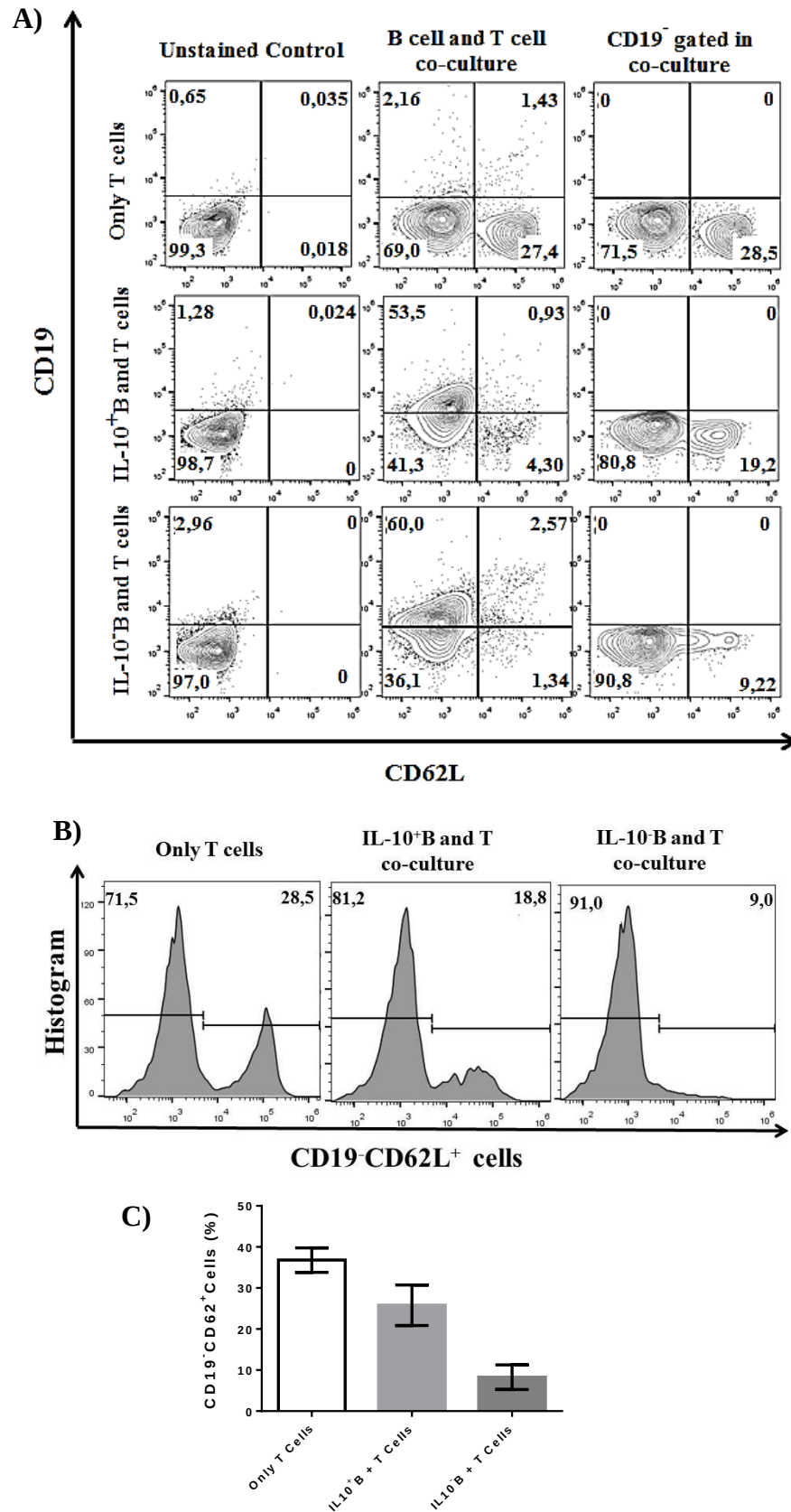


Figure 3.8: CD62L Levels of CD4⁺ T cells co-cultured with *Helicobacter*-activated IL-10⁺B and IL-10⁻ B cells. Representative flow cytometry quadrant plot (A) and histogram plot (B) of anti-CD62L-APC and anti-CD19-FITC staining. Since the B cells were labeled with anti-CD19 antibody, a negative gate to CD19 was selected to see the CD62L levels in T cells. (B) CD19⁻CD62L⁺ cell ratios for co-culture groups are representatively shown on histogram. (C) A graphical expression of the CD4⁺CD62L⁺ T cells within the co-culture groups. For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software was used. Graphs were prepared with Graphpad Prism 6 software.

T-cell activation provokes the shedding of CD62L from the membrane (Mascarell et al., 2004). Together with increased CD25 levels (Result 3.4.3), the significant difference in CD62L expression on T cells suggest that T cells interacting with *Helicobacter*-activated IL-10⁻B cells show higher activation compared to the co-culture group with *Helicobacter*-activated IL-10⁺B cells.

3.4.5 CD49b-LAG3 co-expression levels of CD4⁺ T cells in co-culture with H_{ACT}-IL-10⁺ B cells and H_{ACT}-IL-10⁻ B cells

Sayı et. al showed that B cells which were activated by *Helicobacter* induce IL-10–producing CD4⁺CD25⁺ Tr1–like cells *in vitro*. According to their results, it was suggested that B cell-induced Tr-1 cells acquire suppressive activity both *in vitro* and *in vivo* (Sayi et al., 2011). In addition, Gagliani and colleagues identified that the coexpression of CD49b and LAG-3 distinguishes Tr1 cells from Th1, Th2 and Th17 cells (Gagliani et al., 2013). Based on this information, CD4⁺ T cells cultured with H_{ACT}-IL-10⁺ B cells and H_{ACT}-IL-10⁻ B cells were examined for their LAG-3 and CD49b co-expressions by flow cytometry (Figure 3.9).

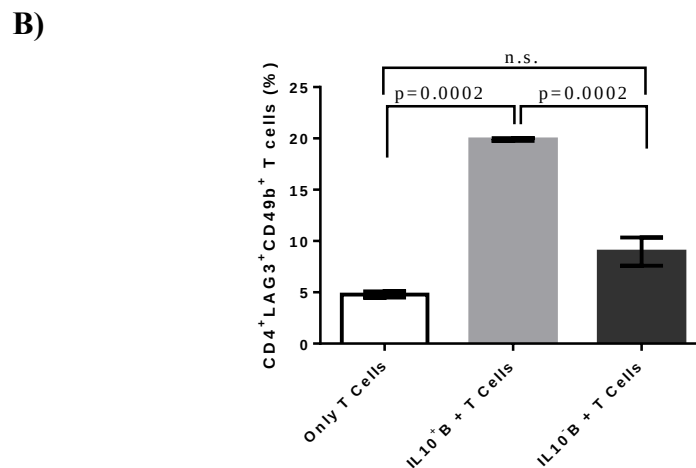
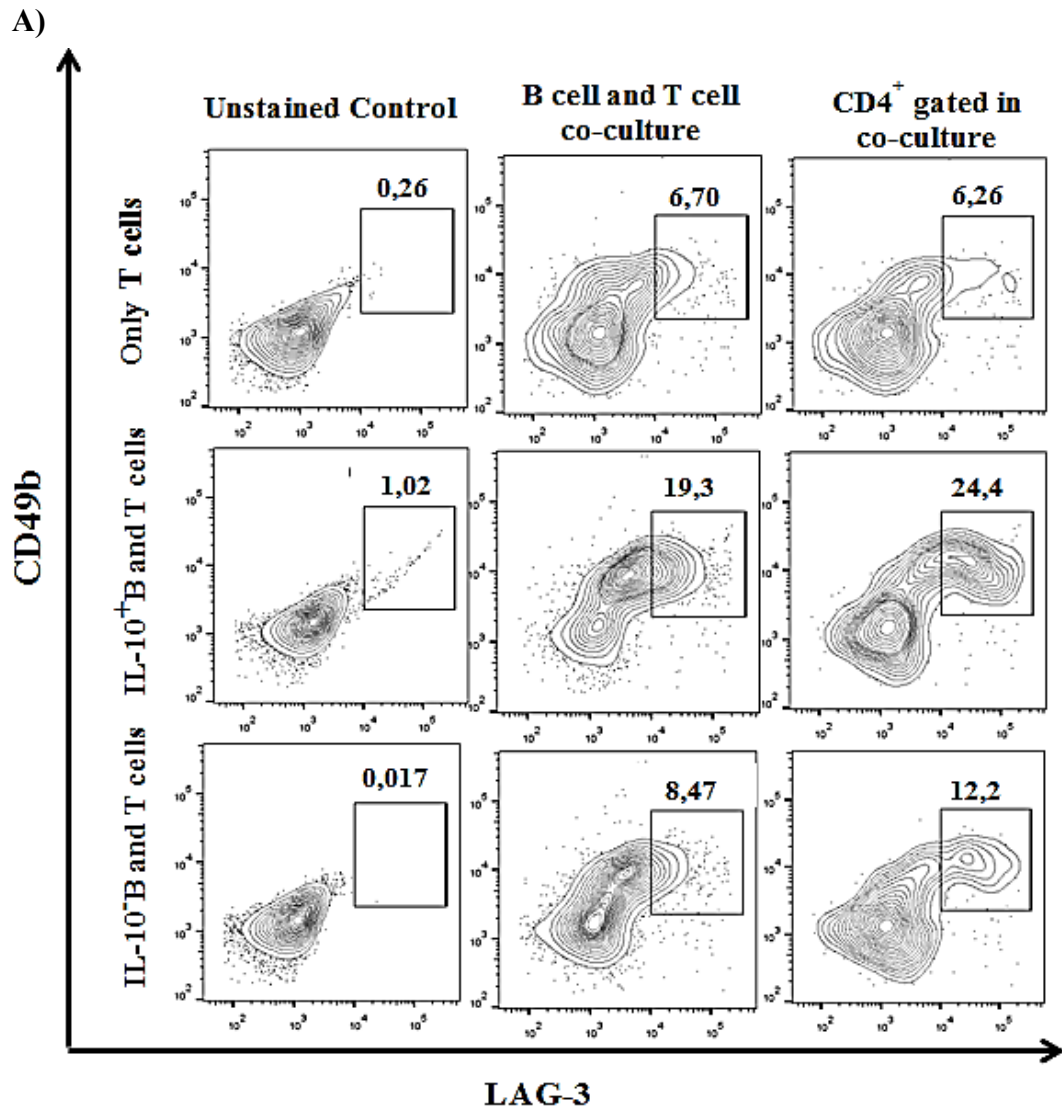


Figure 3.9: LAG3-CD49b co-expression levels of CD4⁺ T cells co-cultured with *Helicobacter*-activated IL-10⁺B and IL-10⁻ B cells. (A) Representative flow cytometry plot of LAG3⁺CD49b⁺CD4⁺T cells compared to unstained control. Cells were labeled with anti-CD4-APC, anti-LAG3-PerCpCy5.5 and CD49b-FITC. To see the co-expression levels only for T cells, a CD4 gate was selected. B) A graphical expression of the LAG3⁺CD49b⁺CD4⁺T cells within the co-culture groups. For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software was used. Graphs were prepared with Graphpad Prism 6 software. Significance evaluation was performed with Student's t test (p<0.05 was significant).

Flow cytometry results of CD4-CD49b-LAG3 stainings showed that almost 20% of T cells co-cultured with IL-10⁺B cells express those three surface markers while only around 10% of CD4⁺ T cells co-cultured with IL-10⁻B cells express CD49b and LAG3 together. According to those results, it could be suggested that CD4⁺ T cells differentiate into Tr1-like cells when co-cultured with both of the *Helicobacter*-activated B cell subgroups; however, IL-10⁺B cells seemed to have more potency on CD49b-LAG3 co-expression on CD4⁺ T cell surfaces.

3.5 Effects of H_{ACT} -IL-10⁺ B cells and H_{ACT} -IL-10⁻ B cells in Th17 cell differentiation

CD4⁺ T cells obtain distinct functional properties in response to signals sent by commensal and pathogenic microbe-activated cells of the innate immune system (Seder and Paul, 1994). Th1 and Th2 cells control intracellular microorganisms and helminths, respectively (Abbas et al., 1996; Glimcher and Murphy, 2000), whereas Th17 cells secrete interleukin-17 (IL-17), IL-17F, and IL-22 and have significant roles in protecting the host from bacterial and fungal infections, particularly at mucosal surfaces (Ivanov et al., 2009). At appropriate concentrations of TGF- β and IL-6, antigen-activated CD4⁺ T cells upregulate ROR γ t and express Th17 cell cytokines (Zhou et al., 2008). Sayı et al. showed that *Helicobacter*-activated B cells produce high amounts of IL-6 (Sayı et al., 2011). Moreover, *Helicobacter*-activated IL-10-B cells were shown to produce IgM and IgG2b antibodies and secrete TGF- β (unpublished data). Based on this data, the direct effect of *Helicobacter*-activated IL-10⁺B cells and *Helicobacter*-activated IL-10⁻TGF- β ⁺ B cells on Th17 cell differentiation was investigated.

3.5.1 IL-17 production levels of CD4⁺ T cells in co-culture with H_{ACT} -IL-10⁺ B cells and H_{ACT} -IL-10⁻ B cells

The IL-17 production of CD4⁺ T cells was examined by intracellular antibody staining that was detected by flow cytometry (Figure 3.10). For this purpose, the cells in the co-cultures were treated with Monensin (3 μ g/ml, Calbiochem) to keep the cytokines accumulated inside the Golgi complex. T cells were labeled with APC-coupled anti-CD4 antibody. Secondly, the cells were fixed with 2% Fixation buffer which cross-links proteins. Next, by using the Permeabilization Buffer (Biolegend),

holes were created in the membrane thereby allowing the intracellular staining antibodies to enter the cell effectively. After the cells were permeabilized, FITC-coupled anti-IL-17 antibody was used for the staining of the IL-17 that accumulated in the cells.

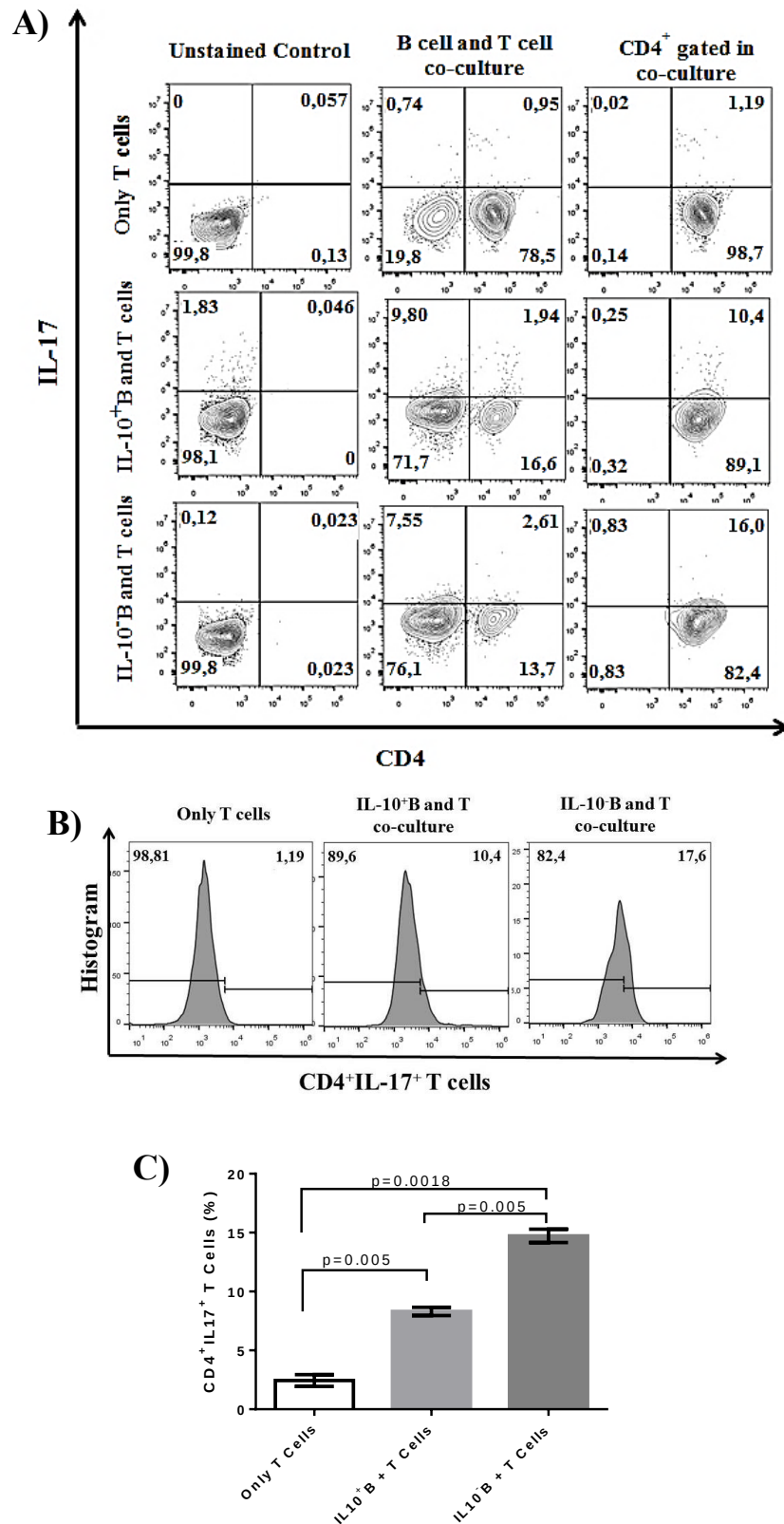


Figure 3.10: IL-17 Levels of CD4⁺ T cells co-cultured with *Helicobacter*-activated IL-10⁺B and IL-10⁻ B cells. Representative flow cytometry quadrant plot (A) and histogram plot (B) of CD4⁺IL-17⁺ T cells in co-culture groups compared to unstained control. Cells were labeled with anti-CD4-APC and intracellular staining was done for IL-17 cytokine detection. To detect the IL-17 levels for T cells, a CD4 gate was selected. (C) A graphical expression of the CD4⁺IL-17⁺ T cells within the co-culture groups (4 experiments). For the measurements BD ACCURI C6 flow cytometer and for the analyses FlowJo Software was used. Graphs were prepared with Graphpad Prism 6 software. Significance evaluation was performed with Student's t test ($p < 0.05$ was significant).

Results indicated that in both co-culture groups of CD4⁺ T cells with *Helicobacter*-activated B cell subgroups, T cells differentiated into Th17 cells. Data of four different experiments showed that the percentage of CD4⁺ T cells co-cultured with IL10⁻B cells turned into IL17 producing CD4⁺ T cells approximately 15% while with IL10⁺B cells the ratio was about 8% of T cells. Altogether, it could be suggested that in both co-culture groups there was a significant differentiation of CD4⁺ T cells into Th17 cells.

3.5.2 IL-17 secretion levels of CD4⁺ T cells in co-culture with H_{ACT}-IL-10⁺ B cells and H_{ACT}-IL-10⁻ B cells

For a better understanding of Th17 differentiation, the amounts of secreted IL-17 levels were measured by IL-17 ELISA with the 24 hour co-culture supernatants (Figure 3.11).

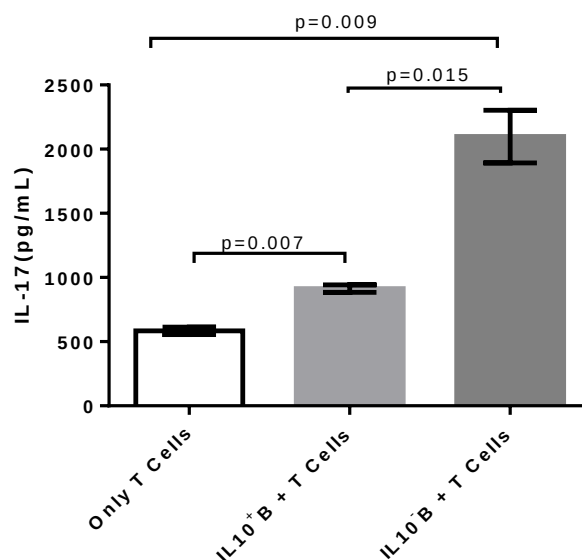


Figure 3.11: IL-17 Secretion Levels in co-culture groups of CD4⁺ T cells with *Helicobacter*-activated IL-10⁺B and IL-10⁻ B cells. CD4⁺ T cells were either co-cultured with *Helicobacter*-activated IL-10⁺B and IL-10⁻ B cells or left alone. Supernatants were collected after 24 hour co-culture and the amounts of secreted IL-17 were measured with IL-17 ELISA (4 independent experiments). Bar graph was prepared with GraphPad Prism 6 software.

IL-17 ELISA results were parallel with the IL-17 intracellular cytokine staining analyses. Accordingly, T cells co-cultured with IL-10⁺B cells secreted more IL-17 cytokine than only T cells (~ 1,5 fold). However, T cells that were cultured together with IL-10⁻B cells secreted almost 4 fold more IL-17 when compared to only T cells as well as almost 2,5 fold more IL-17 compared to IL-10⁺B and T cell co-

culture. This would suggest that even both B cell subgroups have role in Th17 differentiation, IL-10⁻B cells are more effective than IL-10⁺B cells in differentiating CD4⁺ T cells into Th17 cells.

3.5.3 Relative Ror gamma T and IL-17 expression levels of CD4⁺ T cells in co-culture with *H_{ACT}*-IL-10⁺ B cells and *H_{ACT}* -IL-10⁻ B cells

Interleukin (IL)-17A was originally described and cloned by Rouvier et al. (1993) and named CTLA8, was subsequently renamed IL-17, and more recently, IL-17A. Since it is the most significant indicator of Th17 cell presence, the gene expression levels of IL-17A were examined by Quantitative Polymerase Chain Reaction (Q-PCR). The IL-17A levels in co-culture groups were normalized to 18sRNA results and divided to the results of only T cell groups to acquire a relative change in the gene expression levels (Figure 3.12).

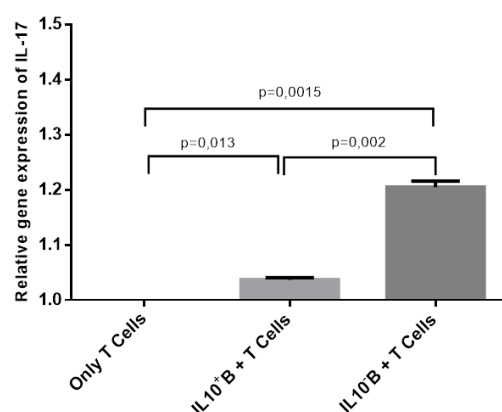


Figure 3.12: Gene expression of IL-17A in CD4⁺ T cells co-cultured with *Helicobacter*-activated IL-10⁺ B and IL-10⁻ B cells. The average of four different experimental results was graphed. Only T cell groups were set as base level and the co-culture results were divided to only T cell results to acquire a relative change. Bar graph was prepared with GraphPad Prism 6 software.

Furthermore, the nuclear hormone receptor RA-related orphan receptor γ t (ROR γ t), originally defined as a thymic specific isoform of ROR γ (He et al., 1998), has been identified as the lineage-specific transcription factor for Th17 cells, required for their generation (Ivanov et al., 2006; and Acosta-Rodriguez et al., 2007) (Figure 3.13).

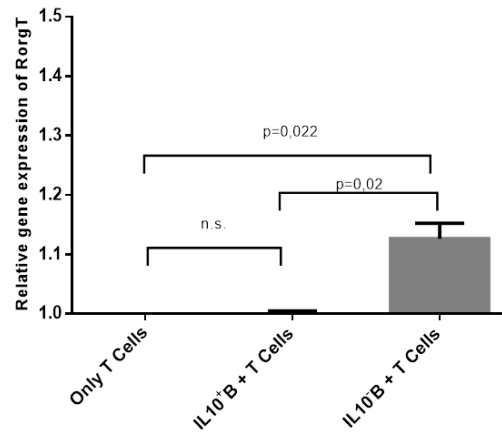


Figure 3.13: Gene expression of Ror gamma T in CD4⁺ T cells co-cultured with *Helicobacter*-activated IL-10⁺ B and IL-10⁻ B cells. The average of four different experimental results was graphed. Only T cell groups were set as base level and the co-culture results were divided to only T cell results to acquire a relative change. Bar graph was prepared with GraphPad Prism 6 software.

As shown in Figure 3.12 and 3.13, IL-17A and Ror gamma T showed similar results for gene expression in co-culture groups. Only T cells being the basis for the comparison, IL-10⁻B cells induce T cells to express IL-17A and Ror gamma T almost 1.2 fold higher than IL10⁺B cells which would explain the significant differences in IL-17 intracellular staining and secreted IL-17 levels in these co-culture groups (Figures 3.10 and 3.11).

4. DISCUSSION AND CONCLUSION

The immune response mechanisms against *Helicobacter pylori* involve both protective and damaging effects for the host. Results of many studies suggested a crucial role for B cells in *Helicobacter* infection that balances between the infection and T cell mediated pathology. *Helicobacter* induces B cells to produce and secrete IL-10 which is suggested to have a role in the differentiation of naïve CD4⁺ T cells into suppressive IL-10 producing Tr-1-like cells (Sayi et al.,2011); while other studies revealed the importance of TGF- β in both Tr-1 and Th17 differentiation (Lee et al., 2014; Caruso et al., 2008).

In previous studies in our laboratory, *Helicobacter*-activated B cells are separated into two subgroups: *Helicobacter*-activated IL-10⁺ B cells and *Helicobacter*-activated IL-10⁻ B cells. The experimental results showed that *Helicobacter*-activated IL-10⁺ B cells are the source of the IL-10 production while *Helicobacter*-activated IL-10⁻ B cells are mostly TGF- β positive (unpublished data). However, it was not clear if the *Helicobacter*-activated-IL-10⁺ B cells or the *Helicobacter*-activated-IL-10⁻ B were specifically causing the Tr1 differentiation. Taking account that Bregs are known for producing IL-10 and TGF- β which are key cytokines in T cell differentiation, and focusing on these cytokines produced by *Helicobacter*-activated B cells as well as the cell-to-cell contact, in this study the role of *Helicobacter*-activated-IL-10⁺ B cells and the *Helicobacter*-activated-IL-10⁻ B cells on CD4⁺ T cell differentiation was investigated.

Using magnetic isolation techniques, CD19⁺B cells and CD4⁺T cells were isolated from the spleens of C57BL/6 mice. The average B and T cell purities of four independent experiments were 90% and 93%, respectively (Figure 3.1; Figure 3.3). Following the B cell isolation, cells were treated with *Helicobacter felis* sonicate (10 μ g/ml) for 24 hours. To induce an optimal interleukin-10 (IL-10) production and secretion, PMA (50 ng/ml) and ionomycin (500 ng/ml) were added for the last 5 hours of incubation. After the *in vitro* stimulation of B cells, IL-10 producing B cells were labeled and the IL-10⁺B and IL-10⁻B cells were separated. To observe the

interaction between the *Helicobacter*-activated-B cell subgroups and CD4⁺ T cells, isolated CD4⁺ T cells were put on co-culture in 1:1 ratio with the *Helicobacter*-activated-IL-10⁺ B cells and *Helicobacter*-activated-IL-10⁻ B cells. After 24-hour incubation of the co-cultures of B and T cells, the cell surface markers and intracellular cytokine productions were examined by flow cytometry. While the supernatants of the co-culture groups were subjected to ELISA tests, the cell pellets were used for gene expression analyses.

The intracellular staining of IL-10 of T cells co-cultured with *Helicobacter*-activated B cell subgroups resulted in significant differences when the co-culture groups were compared with only T cells (control group). About 15% of T cells co-cultured with H_{ACT}-IL-10⁺ B cells produced IL-10 while almost 20% of the T cell population was IL-10 positive when T cells were co-cultured with H_{ACT}-IL-10⁻ B cells (Figure 3.5). Altogether, it could be suggested that in both co-culture groups there was a significant differentiation of CD4⁺ T cells into IL10⁺CD4⁺ Tr1-like cells. In addition, according to IL-10 ELISA results, the co-culture groups showed twice IL-10 levels when compared to only T cells (Figure 3.6). For IL-10⁺B cell and T cell co-culture, a part of secreted IL-10 came from B cells while most of the IL-10 secreted from IL-10⁻B cell and T cell co-culture originated from T cells. One possibility is that compared to IL-10⁺B cells, IL-10⁻B cells drive more IL-10 secretion from CD4⁺T cells. On the other hand, it could also explained by the IL-10 production of IL-10⁻B cells when they interact with T cells. The purities of IL-10⁻B cells would also effect the Tr1 differentiation since 80% pure IL-10⁻B cell population would contain 20% IL-10⁺B cells that would stimulate CD4⁺ T cells in the co-culture to produce IL-10 cytokine at some levels.

For CD25 levels, in only T cell control groups, when compared to the freshly isolated T cells, an increase in CD25 positive T cells was observed after 24-hour incubation (Figure 3.7). It could be justified by the recombinant IL-2 added into the culture to maintain the viability of T cells for 24 hours, since it has been reported that CD4⁺CD25⁺ T cells expand when they were are cultured with anti-CD3 and IL-2 (Thornton et al., 1998). Furthermore, the increase in CD25 expression in co-culture groups could be explained in two ways: the increased activation of T cells or the induction of T cells to differentiate into other regulatory T cell subtypes. Even though these results could not clarify whether the cells were just activated or

differentiated into a regulatory T cell subtype, the CD62L results showed the same pattern. It has been known that CD62L rapidly shed from lymphocytes upon cellular activation (Gerberick et al., 1997). Both CD25 and CD62L levels were higher in T cell co-cultured with *Helicobacter*-activated IL-10⁻B cells (Figure 3.8). These results together might indicate that T cells co-cultured with *Helicobacter*-activated IL-10⁻B cells are more activated/differentiated compared to the T cells interacting with IL-10⁺B cells. Still, the question whether the cells were activated into effector cells or differentiated into a suppressive, regulatory T cell subtype should be answered by functional analyses.

In the literature, it has been shown that B cells, which were activated by *Helicobacter*, induce IL-10-producing CD4⁺CD25⁺ Tr1-like cells *in vitro* (Sayi et al., 2011). In addition, it has been identified that the co-expression of CD49b and LAG-3 distinguishes Tr1 cells from Th1, Th2 and Th17 cells (Gagliani et al., 2013). Flow cytometry results of CD4-CD49b-LAG3 stainings showed that almost 20% of T cells co-cultured with IL-10⁺B cells express CD4-CD49b-LAG3 surface markers while only around 10% of CD4⁺ T cells co-cultured with IL-10⁻B cells express CD49b and LAG3 together (Figure 3.9). According to those results, it could be suggested that CD4⁺ T cells differentiate into Tr1-like cells when co-cultured with both of the *Helicobacter*-activated B cell subgroups; however, IL-10⁺B cells seemed to have more potency on CD49b-LAG3 co-expression on CD4⁺ T cell surfaces. Considering the CD49b-LAG3 co-expression being a regulatory cell indicator, to suggest that the cells expression both of the surface markers together show regulatory/suppressive functions, the regulatory/suppressive effects of the T cells need to be demonstrated on other effector cell types by *in vitro* suppression assays (CFSE dilution assay or 3H-thymidine incorporation assay).

CD4⁺ T cells obtain distinct functional properties in response to signals sent by commensal and pathogenic microbe-activated cells of the innate immune system. Th17 cells secrete interleukin-17 and have significant roles in protecting the host from bacterial and fungal infections, particularly at mucosal surfaces (Ivanov et al., 2009). At appropriate concentrations of TGF- β and IL-6, antigen-activated CD4⁺ T cells upregulate ROR γ t and express Th17 cell cytokines.

As *Helicobacter*-activated B cells produce high amounts of IL-6 and *Helicobacter*-activated IL-10⁻B cells were shown to produce and secrete TGF- β , it was

hypothesized that T cells co-cultured with *Helicobacter*-activated IL-10⁻B cells would differentiate into Th17. Data of four independent experiments showed that the approximately 15% of CD4⁺ T cells co-cultured with IL10⁻ B cells turned into IL-17 producing CD4⁺ T cells while surprisingly about 8% of T cells also produced IL-17 when co-cultured with IL10⁺B cells (Figure 3.10). One possible explanation could be that the purity of IL-10⁺B cells affected the IL-17 results. 80% IL-10 positive B cell population contains 20% IL-10 negative cells, which could have been inducing the IL-17 production from T cells and cause the 8% of Th17 differentiation in IL-10⁺ B and T cell co-culture groups. IL-17 ELISA results were parallel with the IL-17 intracellular cytokine staining analyses (Figure 3.11). Accordingly, T cells co-cultured with IL-10⁺ B cells secreted more IL-17 cytokine than only T cells (~ 1,5 fold). However, T cells that were cultured together with IL-10⁻ B cells secreted almost 4 fold more IL-17 when compared to only T cells as well as almost 2,5 fold more IL-17 compared to IL-10⁺ B and T cell co-culture (Figure 3.12). ROR γ t showed similar results for gene expression in co-culture groups (Figure 3.13). Only T cells being the basis for the comparison, IL-10⁻B cells induce T cells to express IL-17A and ROR γ t almost 1.2 fold higher than IL10⁺B cells which would explain the significant differences in IL-17 intracellular staining and secreted IL-17 levels in these co-culture groups. Again, the IL-17 and ROR γ t results could be caused by the IL-6 and TGF- β coming from IL-10 negative B cell population in IL-10⁺ B–T cell co-culture groups. Further experiments with higher purities in the separation of IL-10 positive and negative groups would give a clearer picture in the Th17 response for *ex vivo Helicobacter felis* infection case.

A schematic overview of the results obtained in this study is depicted in Figure 4.1.

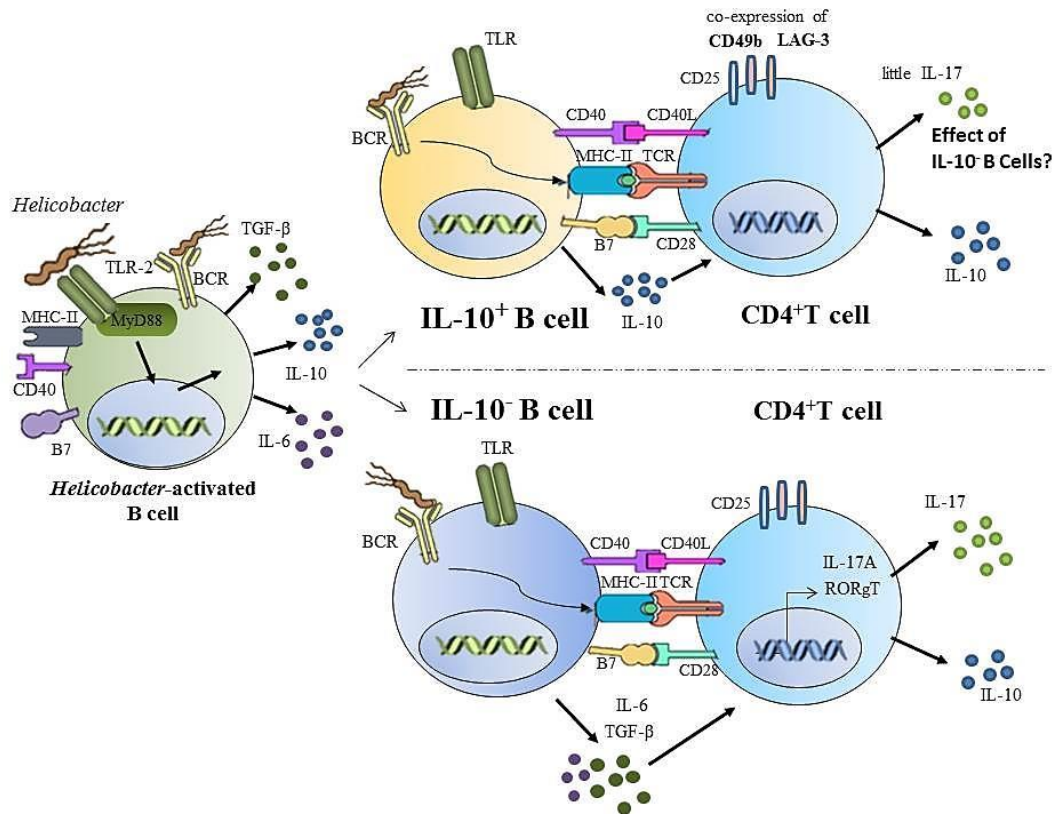


Figure 4.1: Proposed schematic explanation of the *ex vivo* interaction of *Helicobacter*-activated B cells subgroups, IL10⁺ H_{ACT} B cells and IL-10⁻ H_{ACT} B cells, with CD4⁺T cells.

The results of this study regarding the investigation of *Helicobacter*-activated B cell subgroup interactions with CD4⁺ T cells revealed that both *Helicobacter*-activated IL-10⁺B and IL-10⁻B cells induce IL-10 production from CD4⁺T cells and it might suggest that T cells differentiate into Tr1-like cells in both co-culture conditions. Although the CD25 and CD62L expression on T cell surfaces show significantly higher activation in *Helicobacter*-activated IL-10⁻B and T cell co-cultures, regulatory type indicator CD4-CD49b-LAG3 surface marker co-expressions were higher on T cells in the *Helicobacter*-activated IL-10⁺B and T cell co-cultures. In the meantime, intracellular cytokine analyses and relative gene expression experiments of IL-17A and RORγt showed significantly higher results in IL-10⁻B and T cell co-culture groups, as expected. However, interestingly IL-17 production was also observed in T cells co-cultured with IL-10⁻B cells. The reason behind the IL-17 and RORγt expressions in IL-10⁺B–T cell co-culture groups was suggested to be the IL-6 and TGF-β produced by IL-10 negative B cell population in the IL-10 positive co-culture group since these cytokines induce the Th17 differentiation.

Even though the whole picture is far to be clear yet and answers give rise to more questions, this study has contributed to the literature through providing a first step to show the *ex vivo* interactions of *Helicobacter*-activated B cells subgroups, IL10⁺ H_{ACT} B cells and IL-10⁻ H_{ACT} B cells, with CD4⁺T cells.

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- 1) Barut, G.T., Korkmaz, A., Esencan, Z., **Sayı A.**, (2015): *Helicobacter*-aktive B(Hakt-B) hücrelerinin CD4⁺ T hücreleri ile Fonksiyonel Etkileşimleri. 23th National Immunology Congress, 26-30 April 2015, Antalya/TURKEY.
- 2) Barut, G.T., Korkmaz, A., Esencan, Z., **Sayı A.**, (2015): Functional Interactions between *Helicobacter*-Activated B (*H_{act}*-B) cells and CD4⁺ T Cells. IV. International Congress of the Molecular Biology Association of Turkey, 27-29 November 2015, Ankara/TURKEY.