INVESTIGATION OF BCL-2 PROTEINS IN TH17 CELL FUNCTIONS

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ABSTRACT

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Interleukin 17 producing T helper 17 cells are the distinct subset of $CD4^+$ T cells. Th17 cells are an important part of the immune response of host defense. Dysregulation of Th17 cells plays a role in various pathologies including autoimmune diseases and cancer types. Bcl-2 family proteins are mostly known regulators of apoptotic cell death. The apoptotic and survival mechanisms of Th17 cells are not well known yet. Therefore, this study aims to investigate Bcl-2 protein family functions in Th17 cell survival and to understand the regulation network of apoptotic mechanisms of Th17 cells. To do that, Peripheral Blood Mononuclear Cells were isolated from a healthy buffy coat by Ficoll separation. Naive T cells were sorted from PBMC and cultured under Th17 polarizing conditions. Th17 cells were phenotypically characterized by flow cytometry. Afterward, cell lysates were obtained from Th0 and Th17 cells at different time points. The expressions of human transcription factor RORC2, proapoptotic Bik, Bid, Puma and Bim and, antiapoptotic Mcl-1 and Bcl-xL at cell groups were detected by Westernblotting. The increased expressions of Bcl-xL and Mcl-1 were detected where the diminished expressions of Bim and Puma were detected in proportional with Th17 differentiation by increased RORC2 and elevated RORC/IL17A levels. Bik was undetectable in both cell groups while non-truncated isoform of Bid was barely decreased among cell groups. Outputs of this study allow us to understand the dynamics of Bcl-2 family proteins in human Th17 cell survival. The understanding roles of Bcl-2 proteins in Th17 cells may help to develop different therapeutics for Th17 associated diseases in the future.

ÖZET

BCL-2 PROTEİNLERİNİN TH17 HÜCRELERİNDEKİ FONKSİYONLARININ ARAŞTIRILMASI

Interleukin 17 sitokinini üreten T yardımcı hücreleri , CD4+ T hücrelerinin özelleşmiş bir alt türüdür. T yardımcı 17 hücreleri, konak savunma mekanizmasında bağışıklık yanıtı oluşmasında önemli bir yere sahiptir. Th17 hücrelerinin disregülasyonu çeşitli otoimmün hastalıkları ve kanser türlerinin patolojilerinde rol oynamaktadır. Bcl-2 ailesi proteinleri apoptotik hücre ölümünün en iyi bilinen regülatorleridir. Th17 hücrelerinin apoptotik ve yaşamsal mekanizmaları da yeterince bilinmemektedir. Bu sebeple bu çalışmanın amaçları, Bcl-2 protein ailesinin Th17 hücrelerinin hücresel yaşamındaki fonksiyonlarının araştırılması ve Th17 hücrelerinin apoptoz mekanizmalarındaki regülasyon sisteminin anlaşılmasıdır. Bu kapsamda da sağlıklı insan kanından Ficoll ile periferik kan mononükleer hücreler elde edilmiş, naif T hücreleri bu hücrelerden ayrıştırılmış ve Th17 polarize koşullar altında kültüre konmuştur. Th17 hücreleri fenotipik akış sitometrisi ile karakterize edilmiştir. Ardından hücre lizatları, Th0 ile Th17 hücre kültürlerden farklı zamanlarda elde edilmiştir. Ardından insan RORC2 transkripsiyon faktörünün , Bcl-2 ailesinin pro-apoptotik üyeleri Bik, Bid, Bim ve Puma ile anti-apoptotik üyeleri Mcl-1 ve Bcl-xL'in hücre gruplarındaki ifadeleri Western blotlama yöntemi ile belirlenmiştir. Çalışmalar doğrultusunda artan RORC2 ve artan RORC/IL17A seviyelerindeki sitokin fenotipiyle saptanan Th17 farklılaşmasıyla birlikte orantılı şekilde anti-apoptotik Bcl-2 ailesi üyeleri Bcl-xL ve Mcl-1 proteinin ifadelerinde artış gözlemlenirken, pro-apoptotik Bcl-2 ailesi üyelerinden Puma ve Bim ifadelerinde azalış gözlemlennmiştir. Bik ifadesine her iki hücre grubunda da rastlanamamıştır. Bid proteinin de kesilmiş versiyonu olan apoptotik t-Bid ifadesi belirlenememiştir ve kesilmemiş Bid ifadesinde belirgin bir farklılık saptanamamıştır. Bu çalışmanın çıktıları Bcl-2 ailesi proteinlerinin insan Th17 hücresel yaşamındaki dinamiklerini anlamamıza olanak sağlamıştır. Bcl-2 proteinlerinin Th17 hücrelerindeki rollerini anlamak, Th17 ilişkili hastalıklarına gelecekte çeşitli terapötik yaklaşımların geliştirilmesine yardımcı olacaktır.

‘’ My spiritual heritage is science and reason. Those people who want to follow me, if they accept the guidance of science and reason on this fundamental axis, will become my true spiritual heirs. ‘’

- Mustafa Kemal ATATÜRK -

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ABBREVIATIONS

Bcl-2	B-cell lymphoma 2
BH	Bcl-2 Homology
Mcl-1	Induced myeloid leukemia cell differentiation protein
Bcl-xL	B-cell lymphoma-extra large
Bcl-2a1	Bcl-2-related protein A1
Bcl-W	Bcl-2-like protein 2
Bad	Bcl-2-associated agonist of cell death
Bik	Bcl-2-interacting killer
Puma	p53 upregulated modulator of apoptosis
Bim	Bcl-2-like protein 11
Bax	Bcl-2-like protein 4
Bok	Bcl-2-related ovarian killer protein
Bak	Bcl-2 homologous antagonist/killer
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
RORγ	RAR-related orphan receptor gamma
β-Actin	Actin, cytoplasmic 1
PBMC	Peripheral Blood Mononuclear Cells
CD	Cluster of Differentiation
IL	Interleukin
Th	T helper
Th 17	T helper 17 cells
IFNγ	Interferon-gamma
TGFβ	Transforming Growth Factor Beta
ANN-V	Annexin-V
7AAD	7-Amino-actinomycin D
IMDM	Iscove's Modified Dulbecco's Medium
FBS	Fetal Bovine Serum
Caspase	Cysteine aspartic protease
APAF- 1	Apoptotic Protease-Activating Factor 1
MOMP	Mitochondrial Outer Membrane Permeabilization

CHAPTER 1

INTRODUCTION

1.1 T Helper 17 Cells

T lymphocytes are fundamentally classified as CD8+ cytotoxic T cells and CD4+ T helper cells which are activated via antigen presentation by engagement with co-partner cell groups to create an effective immune response against invader pathogens. In this study, the CD4+ T helper 17 cell subset is the main subject as intensively described in depth.

Among the CD4+ T helper cell subsets, T helper 17 lymphocytes also known as IL-17 producing T helper cells which are the most important and intensively studied the cause of its roles on host adaptive immunity and different pathologies (Nalbant A., 2019). The signature cytokine of Th17 cell, IL17-A shows pathological impacts in inflammation (Gu *et al.,* 2013). This subset was newly discovered and the functions and properties of Th17 cells are still under investigation (Harrington *et. al.,* 2005; Park *et al.,* 2005).

Th17 cells contribute to defined autoimmune diseases to fill remarkable niches in various pathologies covering rheumatoid arthritis, psoriasis, inflammatory bowel disease covering ulcerative colitis and Crohn's disease, and multiple sclerosis. Th17 cells also have main roles in asthmatic reaction and differentiation of osteoclast (Zhao *et. al*. 2021). Th17 cells have a dual function in cancer types being like tumor suppressors as well as acting tumor-promoting. For instance, Th17 cell phenotype as IL-17A+, RORC+ contributes to tumor progression concerning poor survival in colorectal cancer (Tosolini *et al.*, 2011).

In contrast, Th17 cells play a role in long-term anti-tumor immunity in ulcerative colitis, graft versus host disease (GVHD), and colon cancer. According to the resemblance between effector memory cells and Th17 cells, human Th17 cells are proliferative to maintain long-term anti-apoptotic impact for autoimmune disorders. Hypoxia-inducible factor 1α is found to have a crucial role in apoptosis and survival of

Th17 cells by controlling gene expression of Bcl-2 family proteins via the Notch signaling pathway. By the way, external impacts of this key molecule and related signaling cascade could provide treatment for respective diseases. According to their immuno-histochemistry experiments and flow cytometric analysis on the tissue sections that are taken from defined patients´ groups, elevated levels of IL17+ demonstrated increased numbers of Th17 cells in sites of chronic disease. Besides these, many Notch binding domains exist in promoter regions of the BCL2 gene, and obstruction of Notch signaling results in diminished expressions of BCL2 and BCLXL genes at Annexin V+ Th17 cells. HIF-1 α could affect activation of the Notch signaling cascade. In a conclusion, it can be said that HIF-1 α controlled Notch signaling is important for the survival of Th17 cells stimulating the expression of Bcl-2 proteins and (Kryzcek *et al*. 2009).

On the other hand, there is a link between the angiotensin-converting enzyme ACE-2 receptor and the IL-17A cytokine. Some research groups detected high levels of Th17 cells in the periphery of SARS CoV-2 infected patients (Wu & Yang, 2020). According to the newly published study, activation of STAT-3 by blocking ACE-2 and inducing IL-17A could reduce the neutrophil infiltration in the lungs (Sodhi *et al.,* 2019). Another study emphasized that IL-17A is disinhibited when ACE-2 expression is downregulated. Besides this, severe acute respiratory syndrome coronavirus leads to hypoxia within severe lung injury. Thus, hypoxic conditions and diminished ACE-2 expression by human coronavirus may promote Th17 cell differentiation (Glowacka *et al.,* 2010).

Consequently, to obtain a more in-depth understanding of Th17 cell-driven pathologies, the underlying cell survival mechanisms of Th17 cells and dysregulation of Th17 cells would be figured out which are critical decisive mechanisms through Th17 cell-mediated immune exposure in the system.

Th17 cells produce pro-inflammatory cytokines which are IL17-A, IL17-B, IL17-C, IL17-D, IL17-E, IL17-F, IL-22, IL-21, IL-26, TNF-α. Mainly produced by cytokines of Th17 cell, IL17-A shows homology with IL17-F in the highest degree among the IL17 family cytokines driving autoimmune exposure and inflammation. Th17 cells are differentiated from classic Th1/Th2 that is characterized by these IL-17A and IL-17F productions. Furthermore, these cytokines and their relevant receptors IL-17RA-RE play crucial roles in the regulation of adaptive and innate immune systems besides being responsible for monitoring defined cell groups to the inflammation site like antigen-

presenting cells, fibroblasts, endothelial cells, and tumor cells. As a result of recent studies, the effect of IL-17A on the inflammatory mediators covering NF-kβ and MAPK related signaling cascades is higher than IL-17F in terms of downstream molecules (Wright *et al.,* 2008; Nalbant A., 2016).

Cytokine milieu, signaling cascades, and regulator transcription factors are responsible for the differentiation of T helper cell subgroups. To create a defined effector T helper cell subgroup, naive CD4+ T cells are activated upon antigenic stimulation, proliferated by clonal expansion, and differentiated, respectively. In this manner, respective cytokines interleukin-23 (IL-23), interleukin-6 (IL-6), interleukin-21(IL-21), transforming growth factor-β (TGF-β) and interleukin-1β (IL-1β) are essential for the development and polarizing of human Th 17 cells (Volpe *et al.,* 2008; Raza *et al.,* 2014).

IL-23 is a crucial stimulant for Th17 differentiation and proliferation which was discovered in 2000. IL-23R is a receptor of IL-23 and their binding activates the JAK2-Try2 related signaling pathway to induce expression of RORC, IL17-A, and IL23-R via downstream effectors. Under the stimulation of IL-6 and IL-23 mainly, Th17 cells could be differentiated from naive CD4+ T cells. (Korn *et al*., 2009) .

Another main cytokine involved in Th17 cell differentiation, IL-6 prevents the inhibition of FoxP3 on RORγt and maintains the preferential production of Th17 cells rather than Treg cells (Chen et. al., 2011). IL-6 and its soluble receptor IL-6R form a complex which activates gp130 in lymphocytes. Then, JAK/STAT signaling cascade could be activated to promote anti-apoptotic exposure via Bcl-2 and Bcl-xL (Jones & Rose-John, 2002). IL-1β and IL-6 are collaborative effects downstream signaling molecules in Th17 cell differentiation that also promotes INF-γ. Additionally, the IRF-4 transcription factor induces RORγt expression with the help of IL-1β (Chung *et. al.,* 2009).

The concentration of TGF-β is critical for its inhibitory activity on RORγt expression. Because the high concentration of TGF-β blocks RORC gene expression through induction of FoxP3 transcription factor expression while low level of TGF-β triggers signaling cascade concerning RORγt transcription (Tsai *et al.,* 2013).

IL-21 is a member of the gamma chain family and it is responsible for the regulation of Th17 differentiation. Triggered IL-6 signaling by activation of STAT3 leads to expression of IL21 in Th17 cells which have an autocrine property with IL21-R. This signaling cascade also combines with TGF-β involved in the production of IL23-R and

RORγt for Th17 cell differentiation. According to a recent study, IL-21 deficiency in T cells leads to a lack of Th17 cell-mediated immune response (Korn *et al*., 2007).

IL-22 is one of the cytokines produced by CD4+ Th17 cells and also by CD4+ Th22 cells. To exert its function, IL-22 binds its receptor IL-22R results in the induction of inflammatory response. Elevated IL-22 level is generally associated with tumor progression. The presence of IL-22 and IL-17 was detected in aggressive phenotypes of defined cancer types like colon and gastric (Doulabi *et al*., 2018; Kempski *et al*., 2017).

In addition to the cytokine milieu, transcription factors have crucial regulatory impacts on Th17 cell differentiation such as STAT1, STAT3, IRF-4, BATF, RUNX1, and RORγ. The nuclear hormone receptors superfamily covers nuclear orphan receptors which are RORα and RORγ bounded DNA as heterodimer or homodimer. These receptors especially bind to the specific DNA sequences that are hormone response elements as monomers. Multiple isoforms of RORα exist containing common DNA and putative ligand-binding domain, but variable amino-terminal domains are created by alternative RNA processing. RORγ consists of 560 amino acid proteins and there is a 50% similarity of amino acid identity with RORα. Besides these, RORγ generally is expressed in skeletal muscle. Because of the lack of receptor-specific ligands, these receptors are considered orphan receptors, but recent studies showed that melatonin could be defined as a ligand for orphan receptors. The genes are encoded within a defined genetic locus which maps to chromosome 15q22.2 for RORα and chromosome 1q21.3 for RORγ (Carlberg & Wiesenberg, 1995).

Th17 cell differentiation is mainly driven by the RORγ variants which are orphan nuclear receptor ROR gamma t (RORγt) in mice and retinoic acid related-orphan receptor-gamma C splice variant 2 (RORC2) in humans as master transcription factors. The RORC gene locus encodes the main regulatory transcription factor RORγt/RORC2 (mice/human) involved in the modulation of Th17 cell polarization. In primary human T cells, the expression of RORC2 promotes IL-17 production. RORC2 maintains partial resistance to suppress FoxP3 within the differentiation of Tregs and provides the preferential differentiation of Th17 cells rather than Tregs. (Unutmaz *et al.,* 2009; Manel *et al.,* 2008). In addition, the expression of RORγ is enhanced by hypoxia-inducible factor 1- alpha while FoxP3 expression is suppressed (Kryzcek *et al.,* 2011).

STAT3 directly could modulate RORC2 expression and IL-17 production. Because the absence of STAT3 leads to preventing the differentiation of Th17 cells. STAT3 is also associated with other critical transcription factors for Th17 cell polarizing which are IRF-4, RORγ, BATF, and RUNX1 (Nurieva *et al.,* 2007). Besides this, STAT1 and STAT4 have roles on Th17 differentiation relevant to the presence of IL-23 cytokine which can induce expansion of IFN-γ-producing Th17 cells, independently (Duhen *et al.,* 2013).

Consequently, the expression of RORC2 promoter in the human RORC locus remains low in naive CD4+ and CD8+ T cells from peripheral blood. However, RORC2 expression can be re-occurred under Th17 polarizing conditions in peripheral CD4+ T cells with help of IL-17 secretion. (Ruan *et al.,* 2011; Yahia-Cherbal *et al.,* 2019).

1.2 Bcl-2 Protein Family Members

Bcl-2 (B cell lymphoma 2) protein family members are the major regulators of programmed cell death type 1 which is known as apoptosis. Through apoptosis, membrane degradation and DNA fragmentation take place with cell shrinkage and fragments are engulfed by neighboring cells. Apoptosis is initiated extrinsically by ligation of cell death receptors via ligands or intrinsically by deprivation stimuli and cell stress. Through Th17 cell development, the regulation of apoptosis is crucial to cell survival and the intrinsic pathway of apoptosis is controlled mainly by Bcl-2 proteins to respond to internal cellular stimuli. Besides apoptosis, necrosis and autophagy are also regulated by Bcl-2 proteins among the cell death modalities (Banuelos *et al*., 2016).

Bcl-2 protein is the founding member of the Bcl-2 protein family which is firstly observed in acute B-cell leukemia cell lines. The genomic translocation on t (14;18) between the BCL2 gene on chromosome 18 (q21) and the immunoglobulin heavy chain joining region on chromosome 14 (q32) results in upregulation of BCL2 oncogene expression in B cell follicular lymphoma (Tsujimoto *et al.* 1984). Despite being an oncogene that generally promotes uncontrolled proliferation, Bcl-2 protein maintains cell survival signals by inhibiting cell death in lymphoid cells which do not carry IL-3 (Vaux *et al.* 1988).

After the discovery of Bcl-2 protein, other Bcl-2 family proteins were identified according to the conserved Bcl-2 homology (BH) domains which are BH1, BH2, BH3, and BH4. These domains control the dimerization ability and regulatory functions of related proteins. Bcl-2 family proteins are consist of globular proteins that composed of α helices. Besides the BH domain, all members carry 23 amino acid-long hydrophobic transmembrane anchoring (TM) domain at the C terminus which is responsible for localizing to the intracellular membrane of relevant organelle. All Bcl-2 proteins introduce similar three-dimensional structures. Based on BH and TM domains, Bcl-2 family proteins are classified into three distinct subgroups. Firstly, anti-apoptotic multidomain members are Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bcl-2a1 (Bfl-1), Bcl-B (Nrh), and Bpr containing BH1, BH2, BH3, and BH4 domains. Secondly, pro-apoptotic multidomain members are Bak, Bok (Mtd), Box, Bcl-wav, Bfk, Bcl-G, and Mil1(Bcl-rambo) containing BH1, BH2, and BH3 domains. Thirdly, pro-apoptotic BH3-only members are Bad, Bik (Nbk), Bid, Bim (Bod), Bmf, Noxa, Puma, and Hrk containing only BH3 (Youle & Strasser, 2008).

Except for Bcl-2a1, all multidomain Bcl-2 proteins have a similar TM domain structure which is conserved through evolution even though existing various Bcl-2 homologs. TM domain provides docking of Bcl-2 proteins into intracellular membranes while Bcl-2 proteins become cytosolic if the hydrophobic TM domain is deleted (Popgeorgiev *et al.* 2018).

During life cycles, many cells are produced for maintaining the survival activities of organisms. To maintain homeostasis, a healthy cycle, and progressive developmental stages, cells are removed when newly produced cells come. This process can be performed by different cell death modalities which two of which can take place as physiological cell death by apoptosis and pathological cell death by necrosis. For getting this output, genetic and molecular examinations from nematode to human were done. Then these studies emphasized that cell suicide is managed by a highly conserved pathway in a cellular manner. On the other hand, the ability to cause apoptosis is inheritable but susceptibility to apoptosis depends on external and cell-autonomous events. The Bcl-2 protein family plays different roles in cell growth and cell death. Apoptosis that is known as programmed cell death is preceded by two ways which are the intrinsic apoptosis pathway and extrinsic apoptosis pathway (Zhan *et al.,* 2017).

Through an intrinsic pathway of the non-return execution phase of apoptosis, pro-apoptotic Bak and Bax form oligomers to promote mitochondrial outer membrane permeabilization which affects mitochondrial outer membrane integrity. After leading mitochondrial outer membrane permeabilization, apoptogenic factors cytochrome c, caspase 9, and APAFs cause assembly of holoenzyme apoptosome structure which maintains activation of caspase 3 through apoptosis. Besides this, Bcl-2 proteins also regulate some proteins at the mitochondrial inner membrane-like cytochrome c oxidase Va, cyclophilin D. Anti-apoptotic members can prevent MOMP by directly binding to Bax and Bak while BH3-only pro-apoptotic members can trigger by activating Bax and Bak or repressing anti-apoptotic members. In contrast, anti-apoptotic Bcl-2 proteins inhibit apoptosis by either prohibiting the release of mitochondrial apoptotic factors or sequestering proforms of caspases. Furthermore, if the cell does not undergo apoptosis, it will be uncontrolled and it can not be sensitive against external stimuli to respond. Thus, expressions of pro-apoptotic Bcl-2 proteins can trigger the cell to not respond to an external stimulus by prolonging cell survival (Nakai *et al.,* 1993; Nguyen *et al.,* 1993; Tsujimoto *et al.* 1984).

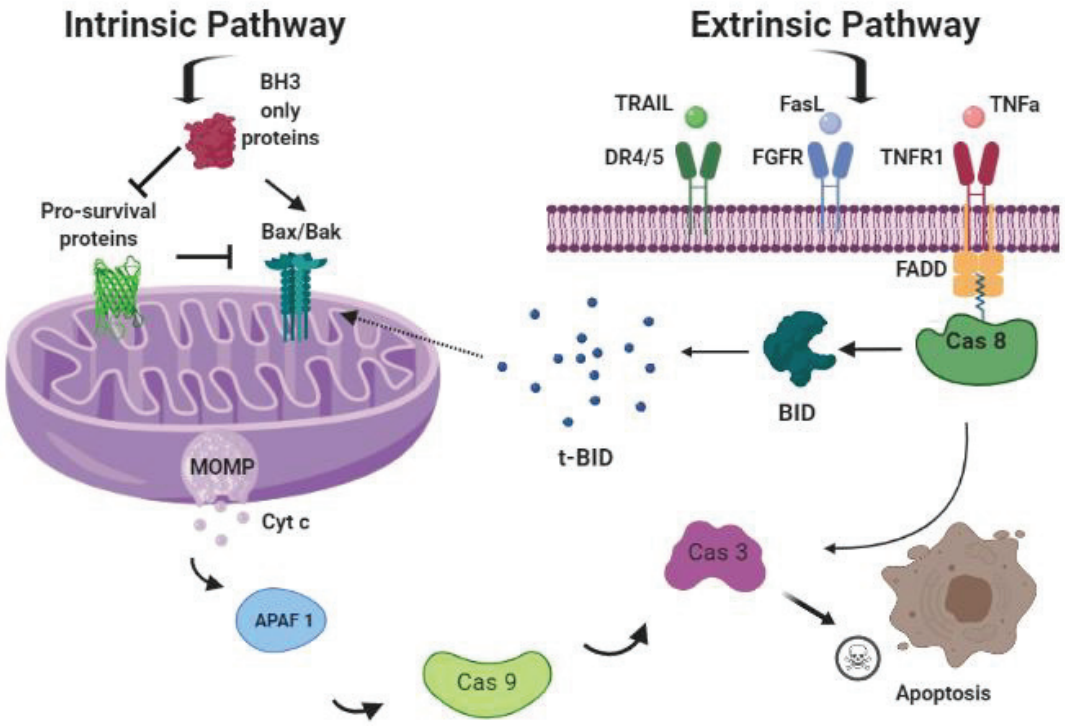

Figure 1. Bcl-2 family proteins involved in the regulation of apoptosis.

Bcl-2 proteins directly localize to mitochondria that regulate mitochondrial outer membrane permeabilization (MOMP). Besides the mitochondria, Bcl-2 proteins also associated with non-apoptotic processes like calcium homeostasis, cell cycle controlling, cell-cell interactions by locating in different intracellular compartments of the cell which are Golgi apparatus, endoplasmic reticulum, peroxisome, and nucleus. Thus, the subcellular distribution of Bcl-2 proteins determines the dynamics of cell fate which affects the function of intracellular proteins (Popgeorgiev *et al.* 2018).

Bcl-2 proteins exist at the cytosolic leaflet of intracellular membranes like a mitochondrial outer membrane (MOM), mitochondrial inner membrane (MIM), nuclear outer membrane (NOM), and ER membranes. In terms of membrane localization, Bcl-2 was firstly identified in cellular membranes while it was also detected at cytosolic and nuclear fractions. At ER levels, Bcl-2 family proteins are responsible for Ca+2 homeostasis by directly binding Ca+2 transporters and mediators of the UPR. Bcl-2 and Bcl-xL are found at MIM while Mcl-1 localizes at the MOM and mitochondrial matrix to inhibit apoptosis. In addition, if the N terminal mitochondrial targeting sequence of Mcl-1 is proteolytically cleaved, Mcl-1 can localize to the mitochondrial matrix (Perciavalle *et al.*, 2012).

Bcl-xL is found at MOM, ER, and cytosolic fractions while its homolog Bcl-2 is found in ER and NOM. Although both of these are anti-apoptotic and homolog proteins, their subcellular distributions are different because their residues are flanked containing hydrophobic α-helix. Bcl-xL possesses two positively charged residues at each end of the α-helix but Bcl-2 does not have these residues which cause interaction with other intracellular membranes (Kaufmann *et al.,* 2003). Bcl-xL residues at TM domain provide MOM targeting signal and homodimerization ability even being homologous of Bcl-2 protein in the cytosol. In addition to the TM domain, the BH4 domain is crucial for subcellular targeting among the BH domains. In the absence of the BH4 domain, Bcl-2 protein can not interact with human FKBP38 chaperone which is required for Bcl-2 localization to the mitochondria and is localized to the nucleus results in induction of apoptosis (Portier & Taglialatela, 2006).

Besides the regulatory role to control apoptosis at the level of mitochondria, Bcl-2 proteins are involved in Ca^{+2} fluxes through the ER-mitochondria border sites in respect to Bcl-2 family subcellular localizations. Mitochondria provide uptake of Ca^{+2} which is

released from ER and excessive amounts of Ca^{+2} cause cell death signaling in mitochondria by swelling. Thus, Bcl-2 proteins regulate this Ca^{+2} flux by communicating with ER Ca^{+2} transporters (Popgeorgiev *et al.* 2018).

Basic differences between BH4 domain amino acid compositions of Bcl-2 and Bcl-xL identify their distinct subcellular localizations. Bcl-2 carries Lys17 on the BH4 domain and it provides binding to the inositol triphosphate receptor in the endoplasmic reticulum and it can inhibit Ca^{+2} release from ER. On the other hand, Bcl-xL does not carry Lys17 on the BH4 domain, so it directly binds mitochondrial voltage-dependent ion channel (VDAC) instead of ER and its hydrophobic groove maintains opening the channel against low levels of IP3. VDAC isoform VDAC2 is also important for Bak activity because it can compromise Bak oligomerization by affecting mitochondrial localization of Bak (Monaco *et al.*, 2012; Roy *et al.*, 2009).

Among the pro-apoptotic members of the Bcl-2 protein family, only multi-domain Bok can directly interact with IP3R1 and IP3R2 thanks to its BH4 domain, it can just protect the channel from proteolytic cleavage but the Ca^{+2} release can not be altered (Schulman *et al.,* 2013).

All BH3-only pro-apoptotic members except for Bad, show canonical properties containing functional hydrophobic C terminus TM domain which regulate Bax localization by direct interaction with cytosolic Bax to recruit into the outer mitochondrial membrane (Kim *et al.,* 2009).

When apoptotic stress has occurred in the cell, Bax, Bak and Bcl-xL are translocated to mitochondria from the cytosol. C terminal hydrophobic TM domain tail is required for pro-apoptotic activity and re-distribution of Bax before apoptotic cell shrinkage. Furthermore, after membrane integration of TM domains of Bax and Bak, other conformational changes are needed to induce apoptosis until they are diffused back to the cytosol. These crucial changes are transient BH3 exposure, α1 helix exposure, avoidance from the membrane-embedded Bcl-2 core region, and elevated proximity of other membrane-embedded monomers (George *et al.,* 2007).

After an apoptotic induction process, Bcl-xL has a role to diffuse back the Bax even though there is no competition between Bax and Bcl-xL for binding to MOM. Conformational changes covering α1 and α2 helices of Bak are required for activation of Bax tethering with the BH3 domain of BH3 only pro-apoptotic members to integrate into

MOM. In this manner, Bcl-xL affects this tethered structure of Bax to deactivate Bax by doing a conformational change on C terminal residue to unmask the BH3 domain by a hydrophobic groove of Bcl-xL. Other apoptosis inhibitor members Bcl-2 and Mcl-1 also provide the translocation of Bax to the cytosol from mitochondria acting as Bcl-xL. Additionally, the hydrophobicity impact is more important than being different sequences on the TM domain for this shuttling process (Edlich *et al.,* 2011).

Besides trafficking through mitochondria and cytosol, Bax and Bak also ER-residents and they play roles in peroxisome and lysosome-related signaling pathways. Both the structures of ER and mitochondria are altered morphologically along the cytoskeleton. Peroxisome and mitochondria-originated vesicles play a role in this manner to transport proteins and phospholipids through ER and mitochondria trafficking. Especially, pro-apoptotic Bak is diverted to interact with peroxisome results in altering membrane integrity of peroxisome in the absence of VDAC2 which provides integration with MOM (Hosoi *et al.,* 2017).

Bcl-2 protein family member Bok, which is found upstream of Bax and Bak, regulates the communication between compartments of ER and Golgi apparatus. When apoptotic stimuli are taken, Bax translocates from the Golgi apparatus to mitochondria concerning p53 dependent signaling cascade. As well as Golgi, Bax localization in lysosome also affects the lysosome permeabilization by releasing lysosomal cathepsin B in a DRAM1 dependent manner and it causes Bid cleavage to truncate Bid (Guan *et al.,* 2015).

At the nucleus aspect, many members of the Bcl-2 protein family members exist in the nucleus for numerous processes. The Bid is one of them by taking place in DNA damage response. During DNA damage, respective kinases ATM and ATR maintain cell cycle arrest, DNA repair, or promoting apoptosis according to the status of the cell. Phosphorylation of Bid is important to stabilize relevant protein complexes or trigger apoptotic processes at the S phase of the cell cycle (Zinkel *et al.,* 2005).

Besides these, Bcl-2 can degrade inhibitor $I_k\beta\alpha$ results in the activation of $NF_K\beta$. Furthermore, BCL2 gene expression is regulated by TNF-α/ $NF_K\beta$ related signaling cascade in human carcinoma cells (Catz & Johnson, 2001). Nuclear localization of Bcl-xL is found to be associated with endometrial and squamous carcinoma types as well as metastasis formation (Choi *et al.,* 2016).

According to the results of an immunoblot analysis of cellular expressions of Bcl-2, Bax, Bcl-xL, and Mcl-1 in human peripheral blood samples and lymphoid tissues, Bax and Bcl-xL expressed in variable levels while Mcl-1 and Bcl-2 showed constant expression levels. Then, all these proteins are expressed in the PBMC population. After the PBMC population was fractionated into T cell subpopulation by sorting, Bcl-2 was highly expressed while Mcl-1, Bcl-xL, and Bax were barely expressed. Furthermore, sorted T cell subpopulations from PBMCs were cultured for 12 days and activated via IL-2 afterward for more than 3 days to activate T cells. Then, all four members showed increased expressions. Markedly, Bcl-xL and Mcl-1 showed diminished expressions in time even though activation. Bcl-2 expression was decreased in the absence of activation while Bax still showed prominent expression despite de-activation by removal of IL-2 (Ohta *et al.,* 1995).

Bcl-2 has been known with long-lived and surviving cell populations since its discovery. In this manner, the expression level of Bcl-2 is high at self-renewing bone marrow cells, long-lived mantle zone B cells, and medulla T cells while Bcl-2 is barely or never expressed at short-lived germinal B cells, neutrophils showing short life span, monocytes, and cortical thymocytes destined to apoptotic death. Bcl-2 protein creates heterodimer or heteromultimeric structures with other members of the Bcl-2 protein family to regulate and activate hematolymphoid cells (Iwai *et al.,* 1994). Bcl-2 increases cell proliferation and longevity. Bcl-2 gene deletion causes a reduction in the cell number of T cells in the periphery and, the capacity of response increases against external apoptotic stimulants. Bcl-2 overexpression is associated with various malignancies and solid tumors (Chen *et. al.,* 2019).

Bcl-xL is an anti-apoptotic member of the Bcl-2 protein family which was isolated by low-level stringency hybridization and sequence similarity with the BCL2 gene. Two isoforms were found for Bcl-xL as a result of alternative splicing as Bcl-xL and Bcl-XS with dual functions. The longer one Bcl-xL prevents promoting apoptosis while shorter isoform Bcl-XS can induce apoptosis by inhibiting anti-apoptotic Bcl-2 protein. It forms a heterodimer structure with any apoptotic Bcl-2 family member to inhibit its pro-apoptotic activity. Compared to Bcl-2, Bcl-xL shows fewer effects on T cell survival while newly produced plasma cells can be survived with help of Bcl-xL (Loo *et al.,* 2020).

Mcl-1 is one of the anti-apoptotic members of the Bcl-2 protein family which was extracted from differentiated human ML-1 human myeloid leukemia cell line which is induced with a phorbol-ester involved in the monocyte-macrophage pathway. Parallel to other members of the Bcl-2 protein family, Mcl-1 is also localized onto the mitochondrial membrane and could antagonize pro-apoptotic members of the Bcl-2 protein family to maintain cell viability and inhibit programmed cell death which is induced by various cytotoxic stimulations. Transcriptional and translational regulations proceed for Mcl-1 differently from other Bcl-2 protein family members. PEST region which is an extended amino-terminal provides a short half-life of Mcl-1 and the transcription of Mcl-1 depends on activation of the PI3K/Akt signaling pathway. As a result of this transcriptional activity, Mcl-1 is highly expressed in differentiated myeloid cells via cytokine stimulation. Mcl-1 is responsible for the early developmental stages of lymphoid and in occurring mature lymphocytes (Kozopas *et al.,* 1993). Mcl-1 enhances cell survival cause its deletion inhibits the DN2/3 stage of T lymphocyte development in the thymus results in a reduction of T cell numbers (Opferman *et. al.,* 2003).

Under the stress conditions in a cell as DNA damage or oncogene activation, BH3-only proteins of the Bcl-2 family covering Bid, Puma, Bim, and Bik are activated through transcriptional and post-translational modifications to interact with BH3 domains of other multidomain Bcl-2 pro-apoptotic proteins to initiate apoptosis. These pro-apoptotic members can induce apoptosis by antagonizing anti-apoptotic members of the Bcl-2 protein family. Bim alters the development of lymphocytes. Cell numbers of lymphocytes are increased when the BIM gene is deleted. CD4+ T cells are able to live longer when Bim expression is suppressed. Thus, regulation of T cell survival, Bim has effective roles. The prolonged life span of CD4+ T cells by diminished expression of Bim causes weaker defense mechanisms against invader pathogens. Reactive lymphocytes could not be eliminated because of this Bim expression manner defectiveness. Bim has three main isoforms by alternative splicing which are named Bim_{EL}, Bim_{L}, and Bim_{S}. Bim_{S} is the shortest one known as being cytotoxic; it is expressed barely through apoptosis. The second one Bim_{EL} and the third one Bim_{L} are released from the dynein motor complex involved in apoptosis. Phosphorylation of Bim_{EL} and Bim_{L} is required for apoptotic activity and external apoptotic stimulants trigger the phosphorylation by JNK leading to dissociation from dynein motor complex.. (Tsukamoto *et. al.,* 2010).

Besides Bim, other BH3-only Bcl-2 members Puma and Bid are combined to resist apoptotic stimuli. In this manner deletions of Puma and Bid are also associated with evading apoptosis. Puma is named as p53 upregulated modulator of apoptosis because Puma mainly is monitored by p53 tumor-suppressing activity. Genomic sequence outputs indicate that Puma is located on chromosome 19 by starting the second exon as initial to transcript covering four exons. Puma promotes apoptosis in a cell with Bim by targeting anti-apoptotic Bcl-2 proteins as much more potent killers and by monitoring T cell activation for creating an immune exposure. In the situation of cell damage, p53 controls the cell cycle to stop and repair the DNA damage during cell stress. Puma maintains cell death, so a lack of Puma can promote cell survival by creating apoptotic resistance. Thus, it can be emphasized that BH3-only members of the Bcl-2 family act like tumor-suppressors because of deletion or loss of Puma and Bim could be seen in many types of human cancers (Yu *et al.,* 2001).

Bid is a death agonist, cytosolic pro-apoptotic member of the Bcl-2 protein family including the BH3 domain in which its cleavaged form t-Bid is located to the mitochondrial outer membrane causing Bax and Bax oligomerization. Bid is cleaved by Caspase-8 mediated after activation of cell death receptors like Fas-associated death domain of tumor necrosis factor family receptors. By the way, pores on membranes are created to trigger induction of apoptosis involved in caspase activation by releasing cytochrome c, Smac, and Diablo caspase activators into the cytosol through induction of apoptosis (Kim *et al.,* 2009).

Among the BH3-only members, Bik is associated with induction of apoptosis by triggering Cas-9 activation and release of cytochrome c in epithelial cancer cell lines. According to the studies which were performed with small animal models, it could be seen that Bik deficiency did not alter the pathological functions and development even though the loss of Bim as well as Bik. Antiviral studies by using the silenced BIK gene emphasized that Bik also acts as a tumor suppressor involved in p53 dependent pathways. Besides this, proteasome inhibitors can induce the overexpression of Bik (Chinnadurai *et al.,* 2008).

To sum up, the Bcl-2 protein family provides cell homeostasis by controlling proper cell numbers and eliminating damaged cells through the regulation of the intrinsic pathway of apoptosis. The proliferation balance of the Th17 lymphocytes is operated by co-operation between pro-apoptotic and anti-apoptotic members of the Bcl-2 protein family at the organelle level. This is crucial to decide if the lymphocyte will live or die. Besides this, structural and copy-number-based changes in the genome of Bcl-2 family products cause various human cancer types and pathologies of numerous autoimmune diseases like graft versus host disease, rheumatoid arthritis, psoriasis, inflammatory bowel disease covering ulcerative colitis and Crohn's disease, and multiple sclerosis. Thus, the dynamics of Bcl-2 proteins in Th17 cells must be highlighted to understand the mechanisms involved in these dysregulations in humans.

1.3 Aim of the Study

Regulatory Bcl-2 protein family members show up impacts on giving life or death decisions of CD4+ T helper 17 cell subset. The main aim of this study is to figure out the dynamics of anti-apoptotic proteins Bcl-xL, Mcl-1, and pro-apoptotic proteins of the Bcl-2 family in human T helper 17 cell functions as differentiation and cell survival. In line with this thesis, molecular expressions of previously defined Bcl-2 family proteins were aimed to analyze protein levels in Th17 cells compared to Th0 cells.

Expected results of this study were planned to insight the dynamics of Bcl-2 family proteins in human Th17 cell survival in a scientific value manner. Because of regulatory roles in cell survival of Th17 cells, understanding of Bcl-2 driven mechanisms is crucial to explain dysregulation of Th17 cells in numerous autoimmune pathologies. Besides this, the understanding roles of the Bcl-2 family may help to develop anti-cancer therapeutics for tumor pathogenesis by acting tumor progressive like pro-survival Bcl-2 proteins or tumor suppressors like pro-apoptotic Bcl-2 proteins for society benefit.

CHAPTER 2

MATERIALS AND METHODS

2.1 PBMC Isolation

Buffy coats were purchased from Dokuz Eylül university based on the Noninvasive Ethics Committee. Ficoll – Hypaque Density Gradient Centrifugation (Boyum *et al.*, 1991) was used for PBMC isolation. Buffy coat was centrifuged at 1500 rpm for 5 minutes and the serum layer was discarded. The pellet was diluted with 1x PBS (Sigma-Aldrich, #D8537) as 1:1 and mixed gently. Diluted buffy coat was overlaid gently onto the 10 mL Ficoll- Paque (GE Healthcare Life Science, #17-1440-03) gently by Pasteur pipette. For this aim, a diluted buffy coat flowed down through the side of the tube to collect on top of them without breaking the layer. Then the sample was centrifuged at 400 g for 45 minutes. PBMC layer was taken and diluted with 1x PBS gently and the supernatant was discarded after centrifugation at 300 g for 10 minutes. Afterward, the red blood cell lysis process was done by 1X Red Blood Cell Lysis buffer (Miltenyi Biotec, 130-094-183). After 10 minutes of incubation, centrifugation was applied at 300 g for 10 minutes. The supernatant was discarded and the pellet was resuspended with 5 mL RPMI 1640 medium (Gibco, with L-Glutamine, #2062175) which was supplemented with 10% FBS serum (Thermo Fisher Scientific, #26140079) and 1% Penicillin-Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin, Invitrogen, #150-063).

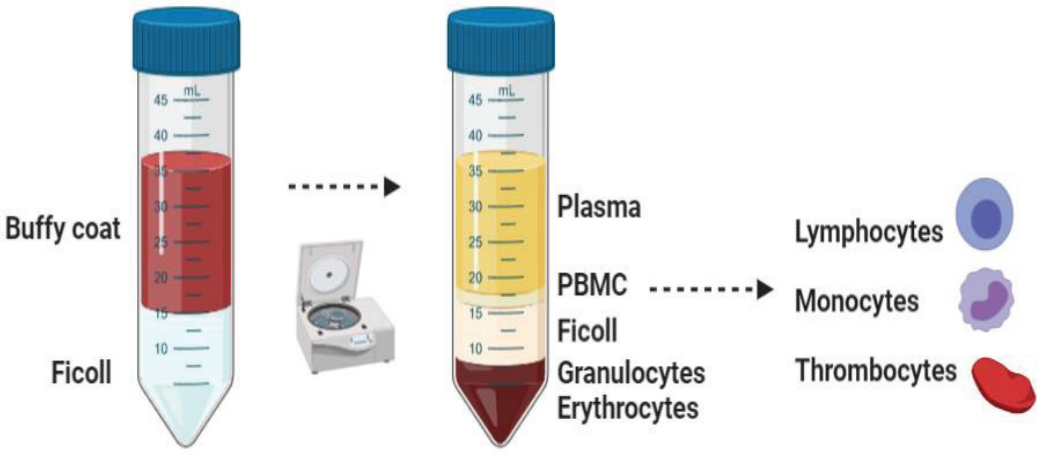

Figure 2. PBMC isolation from buffy coat; Ficoll–Hypaque density gradient centrifugation

2.2 PBMC Characterization with Flow Cytometry

After isolation, obtained PBMC cell populations were characterized with monitoring cell surface markers by flow cytometry method. First of all, the number of isolated PBMC populations was determined by cell counting. For the counting process, 10 µl cell samples from the PBMC containing tube and 90 µl Trypan Blue (Sigma-Aldrich, #T6146) dye were mixed properly. Then counting Thoma chamber was used to count viable cells by using light microscopy. According to the calculation process, the PBMC population was resolved in the falcon tube via RPMI 1640 medium (Gibco, with L-Glutamine, #2062175) to use in the next steps.

Isolated PBMCs were stained via defined cell surface markers tagged with fluorochromes for flow cytometric analysis which were anti-human CD45RA-PE (eBioscience, #E13995-102), CD4-PC5 (Beckman Coulter, #A07752), anti-human CD14-FITC (Pharmingen, #M074000), anti-human FAS-Alexa Fluor®488 (BioLegend, #16895), 7AAD Viability Staining Solution (eBioscience, # E14695-105), ANNV-FITC (Beckman Coulter, #F105016), anti-human CD45RO-FITC (eBioscience, #11-0477-73), anti-human CD25-APC (BD, #555434), CD19-PE (Beckman Coulter, #A07769), anti-human CD8-APC (BD Pharmingen, #54798). For this flow cytometry process, 50 µl cells were taken and mixed with 150 µl PBS (Sigma-Aldrich, #D8537) in each well of the plate as being completed to 200 µl. Then the plate was centrifuged at 400 g for 6 minutes. The supernatant was removed and 10 µl of each dye was added by mixing gently. The plate was covered and incubated at room temperature for 15 minutes. Then it was centrifuged again at 400 g for 6 minutes and the supernatant was removed again. Each well was resuspended with 200 µl PBS and transferred into the flow tubes for flow cytometric analysis. Besides these, AnnexinV staining requires AnnexinV staining buffer (eBioscience, #E00002-1632) instead of PBS as an exception for the staining procedure. For flow data reading and analysis Guava easyCyte (Millipore) system flow cytometer was used.

2.3 Naive CD4+ T Cell Sorting

The isolation of naive CD4+ T cells from PBMCs was done by indirect magnetic labeling sorting with MACS Naive CD4+ T Cell Isolation Kit II according to the manufacturer's protocol (Miltenyi Biotec). LS column (Miltenyi Biotec, #130-042-401) matrix which contains ferromagnetic spheres with a coating that maintains separation of cells with the help of magnetic field of MACS® Separator. Magnetically labeled cells

with MACS® MicroBeads were held onto the column and target cells flowed through the column. First of all, MACS Separation Buffer was prepared as containing 5% final BSA concentration. For this aim, 75 mL MACS BSA Stock Solution was diluted in a 1.45 L autoMACS Rinsing Solution (#130-091-222). It was also pre-cooled for use.

Isolated PBMC cells were counted and the cell suspension was aliquoted as being 10^8 total cells in each falcon tube. Then, the cell suspension was centrifuged at 300 g for 10 minutes and the supernatant was discarded. Pellet was resuspended with 400 µl autoMACS Separation Buffer, then 100 µl Naive CD4+ T Cell Biotin-Antibody Cocktail II, human was added which contains a cocktail of biotin-conjugated monoclonal antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR, and CD235ab. After mixing gently, the mixture was incubated on ice for 5 minutes. Then 300 µl autoMACS Separation Buffer was added then, 200 µl Naive CD4+ T Cell MicroBead Cocktail II, human was added which contains MicroBeads conjugated to a monoclonal anti-CD61 antibody and anti-biotin antibody. These were mixed and incubated on ice for 10 minutes.

After that LS columns and MACS® Separator were set up to use for magnetic separation. The LS column was replaced with the magnetic part of the MACS® Separator. Replaced LS column was washed with 3 mL autoMACS Separation Buffer and the cell suspension was poured into the column. Collected fluid was labeled as enriched naive T cells. Then the column was washed again with a 3 mL autoMACS Separation Buffer and collected fluid was added into the same tube. Another tube was replaced under the column and 5 mL of autoMACS Separation Buffer was added again. Plunger was immediately pushed into the column and non-naive T cells could be collected into the tube that was labeled as depleted cells. By the way, the sorting process could be completed.

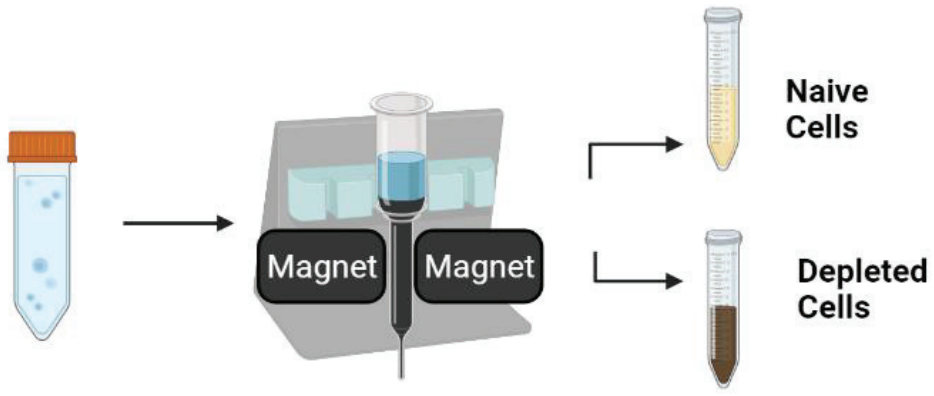

Figure 3. The illustration of magnetically naive CD4+ T cell isolation

2.3.1 Determination of Sorting Efficiency with Flow Cytometry

The sorting efficiency was checked to culture enriched naive CD4+/CD45RA+ T cells which were sorted from the PBMC population. Flow Cytometry method was used for this aim by using anti-human CD14 (BioLegend, #301808), human CD4-PERCP (Miltenyi Biotec, #130-113-217), CD45RA-APC (Beckman Coulter, #IM2473), anti-human CD45RO-FITC (eBioscience, #11-0477-73).

2.4 Human Th17 Cell Differentiation in vitro

Properly sorted Naive CD4+ T cells were cultured under Th17 polarizing conditions with defined cytokines to obtain Th17 cell subset where only cells with the medium were a control group. Sorted cells were taken from +4ºC storage and centrifuged at 1200 rpm for 10 minutes. At the same time, IMDM medium (Lonza BioWhittoker®, with Hepes and L-Glutamine, #12-722F) which was supplemented with 5% FBS serum (Thermo Fisher Scientific, #26140079) and 1% Penicillin-Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin, Invitrogen/Life Technologies, #15070-063) as a culture medium was prepared in a falcon tube and was heated in the water bath. Then, the supernatant was discarded after centrifugation. Pellet was resolved in 1 mL IMDM medium (Lonza BioWhittoker®, with Hepes and L-Glutamine, #12-722F). Stimulant cytokines were added to the stimulated Th17 polarizing group and unstimulated cells were cultured only with the medium. Stimulated cells were treated to induce Th17 cells by specially prepared culture medium that includes Th17 polarizing cytokines which were used as 5 ug/mL CD3 pure functiınal grade human (BioLegend, #B203713), 5 ug/mL CD28 ultra leaf pure anti-human (BioLegend, #302934), 10 ng/mL IL-1β human research grade (Miltenyi, #130-093-895), 10 ng/mL IL-23 leaf purified anti-human (Miltenyi, #130-095-757), 30 ng/mL IL-6 human research-grade (RD Systems, #0003021), 0,5 ng/mL TGF-β1 human research-grade (Invitrogen, #4332400), 10 ng/mL anti-IFNγ pure functional grade human (Miltenyi, #130-095-743), 10 ng/mL anti-IL4 pure anti-human (Miltenyi, #130-095-753) to stimulate 2 x10^6/ mL(medium) cells. Then stimulated and unstimulated cells were incubated for up to seven days at 37 °C, in an atmosphere of 5% CO_2. During this culturing process, CD25 was added on the 5th day, differently. Two experimental cell groups were observed under stereo-microscope on the 3rd, 5th, and 7th days of the experiment, and the media were renewed on the 5th day of the experiment for cell passages.

2.5 Th17 Cell Characterization with Flowcytometry

During cell culture processes, control group and stimulated group cells were characterized phenotypically on the 3rd, 5th, and 7th days of the experiment with monitoring cell surface markers by flow cytometry method. The experimental groups were stained via defined cell surface markers which were tagged fluorochromes for flow cytometric analysis as anti-human CD4-APC/Cy7 (BioLegend, #317418), anti-human FAS-Alexa Fluor®488 (BioLegend, #16895), 7AAD Viability Staining Solution (eBioscience, # E14695-105), ANNV-FITC (Beckman Coulter, #F105016), anti-human CD25-PE (BD Pharmingen™, #555432), anti-human CD69-PC5 (Beckman , #IM2656).

2.5.1 Intracellular Staining

In addition to cell surface staining IL17-A, BCL-2, BCLXL, IL22, and RORC cytokines' profiling is required to do intracellular staining, differently. Anti-human IL17A-AF647 (BioLegend, #B225856), anti-human BCL2-Alexa Fluor®647 (BioLegend, #658706), human ROR gamma /RORC/NR1F3-PERCP (RD Biotechne, #P514492), human IL22-APC (RD Biotechne, #Q9GZX6), and BCLXL-Alexa Fluor®488 (Cell Signaling #2767S). To start, GolgiStop™ (BD Biosciences, #554724) was used as a protein transport inhibitor. 4 µl of was added onto every 10^6 cells and mixed thoroughly. The cells were incubated for 6 hours to increase the detection ability of the target cytokine. Then, 50 µl FoxP3 Fixation/Permeabilization solution (Miltenyi Biotec, #130-093-142) was added onto the cells and mixed gently to safely fix and permeabilize the cells. Mixed cells were incubated at dark, room temperature for 20 minutes. Then, the cells were washed with 100 µl PBS and were centrifuged at 800 g for 10 minutes, then the supernatant was discarded. Afterward, 50 µl FoxP3 Permeabilization buffer (Miltenyi Biotec, #130-093-142) was added onto the cells and mixed gently. Mixed cells were centrifuged at 800 g for 10 minutes, then the supernatant was discarded. Again, 50 µl FoxP3 Permeabilization buffer was added onto the cells and mixed gently. Mixed cells were incubated at dark, room temperature for 10 minutes. Then, the dyes were added respectively by pipetting. Stained cells were incubated at dark, room temperature for 30 minutes. Then, the cells were washed with 100 µl PBS and were centrifuged at 800 g for 10 minutes, then the supernatant was discarded. Stained cells were dissolved with 200 µl PBS and transferred to flow tubes containing 200 µl PBS. For flow data reading and analysis Guava easyCyte (Millipore) system flow cytometer was used.

2.6 Determination of Bcl-2 family proteins with Western Blot

2.6.1 Sample Preparation

To determine Bcl-2 family proteins by Western Blot, cell lysates were obtained from stimulated cells and control group cells at the end of the 7th day of cell culture. To get cell lysate from cultured cells, cell suspensions in flasks were collected and were centrifuged at 1200 rpm for 10 minutes and repeated. Then, for 10 million cells, 4 µl Protease Inhibitor and 396 µl cold RIPA buffer (Cell Signaling, #9806) were added onto each pellet. Then cell suspension was incubated on ice for 30 minutes. After that, the sonication process was done to homogenize cells and disrupted cells were centrifuged at 14000 RCF and 4ºC for 10 minutes. Then, supernatant fluid was taken as total cell lysate. It was frozen at -80 ºC for long storage. To load and run proteins on SDS gel, the amount of the proteins were determined by Bradford (Coomassie Plus) Assay. Bovine serum albumin (BSA, Pierce, #23209) was used in this method as a protein standard to create a protein standard curve for calculations. The blank and relevant protein sample was prepared by mixing 800 µl Bradford Assay reagent (Thermo Scientific, #23238) with 200 µl dH_2O for blank and, 800 µl Bradford Assay reagent (Thermo Scientific, #23238) with 190 µl dH_2O and 10 µl extracted protein. These samples were measured at 595 nm and results that referred to ug extracted protein in 100 µl were calculated by using the BSA standard curve formula. Then, the final volume of the respective protein was calculated to have 20 ug protein as a final concentration to load on SDS gel. After calculations, a total 25 µl sample was prepared for each well and 5 µl of this sample was Non-Reducing Lane Marker Sample Buffer (Thermo Scientific, #UA278539) activated with ß-mercaptoethanol (Millipore Corporation, #S7056540-522). Each sample of protein was diluted with distilled water according to the protein concentration calculations by Bradford Assay to include 20 ug protein in each well. Afterward, samples were covered with parafilm and heated at 95ºC for 6 minutes.

2.6.2 Protein Blotting

Prepared samples were loaded on SDS gel. BioRad gel system was used for this study. To start, two glass plates were clamped in the casting frame placed on the casting stand. Then, 5 mL, 10% Separating gel solution (pH 6.8) was prepared by mixing 1250 µl 4x Separating buffer, 1650 µl 40% Acrylamide/BİS solution 29:1 (BioRad Laboratories, #1610146), 2050 µl dH_2O, 75 µl 10% Ammonium Persulfate (Sigma Life

Science, #MKBK1247) and 5 µl TEMED (AppliChem, #75010367). Separating gel solution was poured into the gap of the glass plates and it was freezing. After that, 5 mL, 5% Stacking gel solution (pH 8.8) was prepared by mixing 1250 µl 4x Stacking buffer, 850 µl 40% Acrylamide/BİS solution 29:1, 2850 µl dH_2O, 75 µl 10% Ammonium Persulfate and 5 µl TEMED. 1 mL of Stacking gel solution was poured onto the separating gel. A Well-forming comb was placed and the Stacking gel was frozen. Then, the comb was taken out and a glass plate including gelated gel was separated from the casting frame and was set in the cell buffer dam. The running tank was filled up with 1X SDS Running buffer (TrisBase BioShop #77861, Glycine BioShop #56406, SDS BioShop #151213). Then, prepared samples and Page Ruler^{TM} Prestained Protein Ladder (Thermo Scientific, #26619) were loaded into the wells of the gel, carefully. The tank was connected to the power source to create an electric field within. As a final step, samples were run at 80 V for 2 hours until samples reached the end of the gel. After gel running, the transfer tank was filled with a pre-cooled 1X Transfer buffer (Tris BioShop #77861, Glycine BioShop #56406) and it was supported with ice accus. 4 Blotting papers and a nitrocellulose membrane were cut in the same size with gel. The SDS gel and membrane were sandwiched between blotting papers and sponges to maintain tight contact. Then, the stack was clamped between solid materials in the transfer tank. The dry ice was also put around the solid materials before giving electrical power. Then the transfer process was done at 325 Amper for 2 hours. After the wet transfer of proteins to the blot, proteins were visualized by using Ponceau S staining solution (Cell Signalling, #59803) to check the success of the wet transfer. For this step, the membrane was washed with TBS-T and it was incubated with Ponceau S dye for 5 minutes on an agitator. Then, the images of the membrane were taken and protein bands were seen as pink colored.

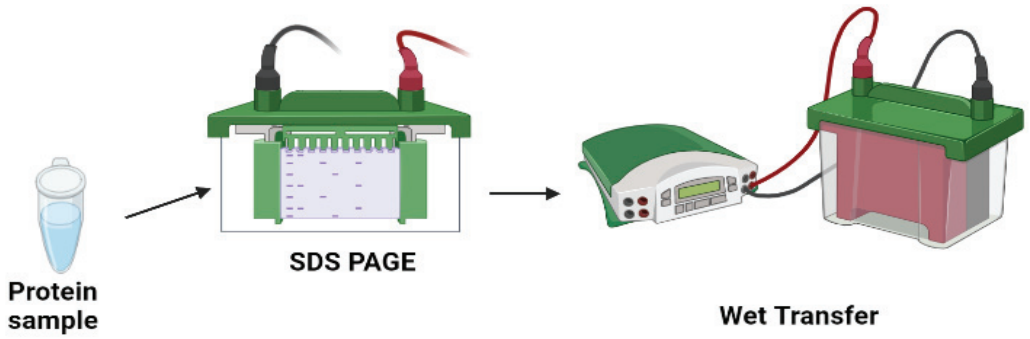

Figure 4. The illustration of Western protein blotting

2.6.3 Antibody Incubations

The transferred nitrocellulose membrane was washed three times for 5 minutes each with 15 mL TBS-T (TBS-Tween 510.100). Then, it was incubated with 25 mL TBS-T milk (TBS-T, Non-Fat Dry Milk #0017) solution to block the membrane for 1 hour on the agitator. The membrane was washed again three times for 5 minutes each with 15 mL TBS-T. After the blocking process, the blot was firstly incubated overnight at 4 °C with a 10 mL primary antibody. The primary antibodies were used at the appropriate dilution according to the recommendations in the product datasheet like 1:1000, 1:2500, or 1:5000 in this study. Pro-Apoptosis Bcl-2 Family Antibody Sampler Kit (Cell Signaling Technology, #9942) was used in this section. The primary antibodies which were Bcl-xL (54H6) Rabbit mAb (Cell Signaling, #2764), BID Human-specific antibody (Cell Signaling, #2002), Bik antibody (Cell Signaling, #4592), Puma (D30C10) Rabbit mAb (Cell Signaling, #12450), Bim (C34C5) Rabbit mAb (Cell Signaling, #2933), Bik antibody (Cell Signaling, #4592), RORy (D-4) Rabbit mAb (Santa Cruz Biotechnology, #365476), β-Actin (13E5) Rabbit mAb (Cell Signaling, #4970). After primary incubation, the membrane was washed three times for 5 minutes each with 15 mL TBS-T. Afterward, the blot was incubated with 10 mL anti-mouse or anti-rabbit, species-appropriate HRP linked secondary antibody by 1:10000 dilution with TBS-T milk for 1 hour on the agitator, at room temperature. The secondary antibody was Anti-rabbit IgG, HRP-linked antibody (Cell Signaling, #7074) used in this study. Then the blot was washed three times for 5 minutes each with 15 mL TBS-T. As a result of antibody incubations and washing process of the blot, the membrane was incubated with 1 mL Luminata Crescendo Western HRP Substrate Chemiluminescence (Millipore^{TM} Corporation, #160815) by gentle agitation for 5 minutes at room temperature, dark condition. After incubation, the membrane was drained of excess chemiluminescence solution without allowing it to be dry. As a final step, the membrane was exposed to X-ray film at the BioRad machine, manually according to the detection of signals from proteins on the membrane. Image Lab software was used in the BioRad machine to take the blot image.

2.7 Statistical Data Analysis

To analyze flow cytometry data, the software of the Guava easyCyte (Millipore) system was used. After obtaining flow data, these were statistically evaluated by using GraphPad Prism software.

To analyze western blot data, Image J software was used to calculate the band density of defined protein by calculating the peak area of relevant protein. Afterward, the data were evaluated by GraphPad Prism. The relative protein levels were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin. Shapiro-Wilk test was applied for normality. Parametric tests were applied because of getting normal distributions on data sets. Thus, Ordinary two-way ANOVA was used to compare three or more groups with multiple variables and column statistics were compared with each other with Tukey's and Sidak's multiple comparisons test. Unpaired t-test was used to compare two groups with Welch's correction regarding not assuming equal SDs. As a result of the statistical results, the data were defined as significantly different and were expressed on graphs according to the significance levels.

CHAPTER 3

RESULTS

3. 1 Cytokine Profiling of Th17 Polarized Human CD4+ T Cells

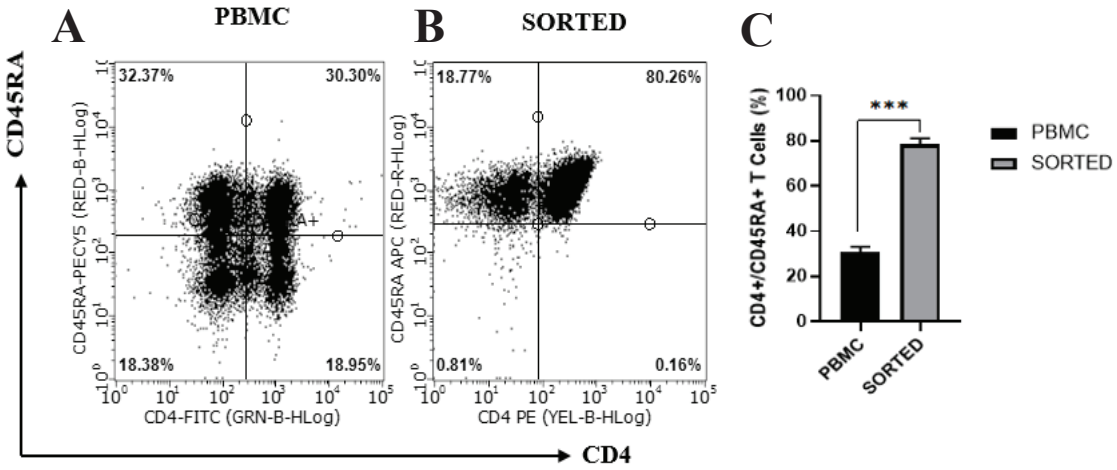

Figure 5. Determination of CD4+/CD45RA+ T cells. Either human PBMCs(A) and sorted cells from human PBMCs by MACS Naive CD4+ T Cell Isolation Kit II (B) were stained with anti-human CD4 and anti-human CD45-RA monoclonal antibodies respectively. Quadrant markers were set based on control antibody staining. Results were expressed as the mean ± SEM for each group from three separate experiments and Shapiro-Wilk test was applied for normality (C) (n=3; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 by Unpaired t-test).

As a result of separation using MACS Naive CD4+ T Cell Isolation Kit II, non-CD4+ T cells and memory T cells could be magnetically labeled to deplete for getting more purified naive CD45RA+ and CD4+ T cells. The sorting efficiency was checked to culture enriched naive CD4+/CD45RA+ T cells which were sorted from the PBMC population. By the way, the sorting efficiency could be calculated. The percentage of double-positive CD4+/CD45RA+ T cells is 30.30% in the human PBMC population before sorting (Fig3.A) and it was increased to 80.206% (Fig3.B) in isolated T cell population from human PBMC after naive CD4+ T cell sorting. When the results were expressed as the mean ± SEM for each group from three separate experiments by Unpaired student t-test, there was a significant difference between cell groups before sorting (A) and after sorting (B) as p-value = 0.0002.

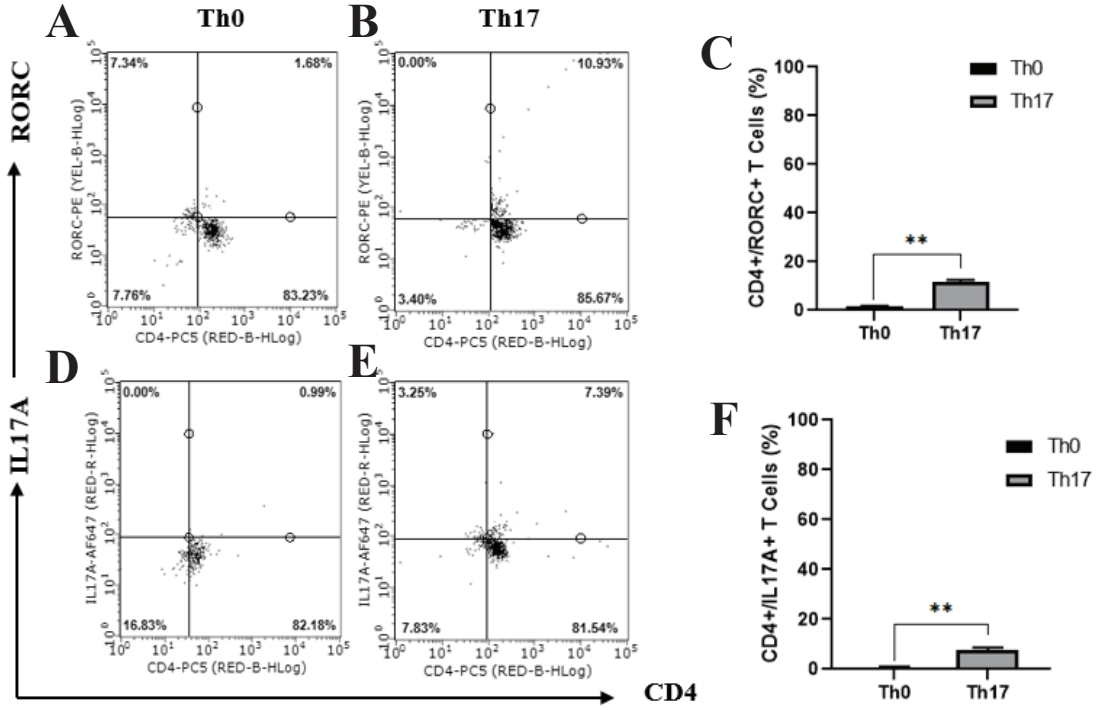

Figure 6. Detection of RORC and IL17A. Either human Th0(A) and Th17(B) cells were stained with anti-human CD4, ant,-human IL-17A, and anti-human ROR gamma /RORC/NR1F3 monoclonal antibodies. Quadrant markers were set based on control antibody staining. Results were expressed as the mean ± SEM for each group from three separate experiments and Shapiro-Wilk test was applied for normality (C, F) (n=3; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 by Unpaired t-test).

Results of the intracellular examination of RORC transcription factor and IL17A cytokine in Th0 cells compared to Th17 cells at a 7-day time point, significantly elevated expression levels were observed for both of these molecules (Fig4). According to the results, 1.68% of CD4+ Th0 cells produced RORC (Fig4.A) while 10.93% of CD4+ Th17 cells produced RORC (Fig4.B). Thus, there was a significant increase in RORC production through Th17 cell differentiation, p-value = 0.0072 (Fig4.C). Simultaneously, CD4+ Th0 cells were expressed at the rate of 0.99% IL17A cytokine (Fig4.D) while CD4+ Th17 cells were expressed at the rate of 7.39% IL17A cytokine (Fig4.E). There also significant increase could be seen in intracellular IL17A cytokine production through Th17 cell differentiation at the 7-day time point of Th17 polarizing cell culture, p-value = 0.0058 (Fig4.F). Besides these, CD4 cell surface marker was examined to understand the naive rate of the relevant population which was sorted previously. Then, it could be seen that the percentage of CD4+ Th0 cell population is 84.91% (Fig4.A), 83.17% (Fig4.D), and the percentage of CD4+ Th17 cell population is 96.6% (Fig4.B), 88.93% (Fig4.E) at the 7-day time point.

3.2 RORC2

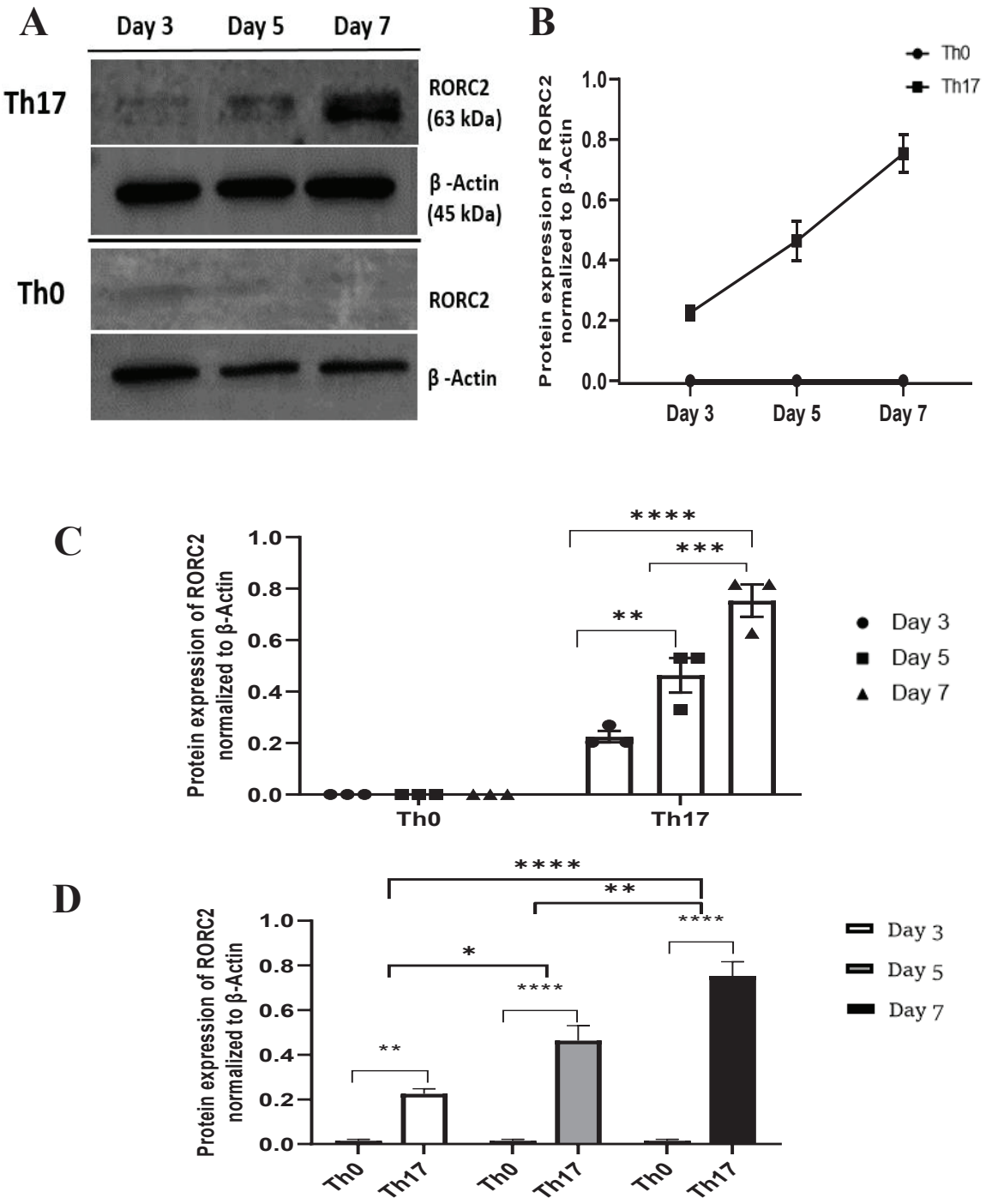

Figure 7. Determination of RORC2**.** Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups at different time points as 3rd, 5th, and 7th day. The molecular weight of human RORC2 is 63 kDa and cytosolic control β-Actin is 45 kDa. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality. (n=3;*p<0,05, **p<0,01,***p<0,001,****p<0,0001 by two-way ANOVA test and Sidak's and Tukey's multiple comparisons test).

The protein lysates were examined by Western blotting belongs to the Th0 control group and Th17 polarizing cell group which was taken from different time points of cell culture as day 3, day 5, and day 7. There was no expression of human RORC2 protein in Th0 cell group lysates at 3rd, 5th, and 7th days of cell culture even though analyzing three different biological samples from donors. However, in line with the Th17 polarized group, there was a bare expression of RORC2 on the 3rd day and there was an increased expression of RORC2 on the 5th day compared to the 3rd day, and the blatantly elevated level of RORC2 expression could be detected at the 7th day of the culture (Fig4.A). When the expression levels were calculated and normalized to cytosolic control, it can be seen that there was a linear increment of RORC2 expression in the Th17 cell group through 7 days of culture even though there was no change or expression of RORC2 level in the Th0 cell group during 7 days of cell culture (Fig4.B). If the expression levels of RORC2 at different time points were compared according to be in Th0 or Th17, there was no significant change in the Th0 cell group in time points manner (p= ns/>0,9999). Despite Th0, there were significant differences between days in the Th17 cell group.

According to the statistical analysis with Tukey's multiple comparison test with two-way ANOVA by examining individual values, the most significant difference was seen between 3rd and 7th-day expressions of RORC2 in the Th17 cell group (p= <0,0001) while the significant difference between 5th and 7th day (p=0.0005) is higher than the significant difference between 3rd and 5th day(p=0.0025) expressions of RORC2 in Th17 cell group (Fig4.C). If the expression levels of RORC2 were examined at cell group manner on the same day, there were significant differences between Th0 and Th17 cell groups in all 3rd, 5th, and 7th days of the experiment (p= <0,0001). In that manner, if the days were compared with each other there was a significant difference between 3rd and 7th days (p=<0,0001) more than between 5th and 7th days(p=0,0087) and between 3rd and 5th days (p=0,0292) according to the statistical analysis with Sidak's multiple comparison test with two-way ANOVA (Fig4.D).

As a result of overall data (Fig4.A-D), RORC2 showed differential expression through Th17 cell polarizing conditions up to seven days which were applied on magnetically sorted naive CD4+ T cells known as Th0 cell group samples.

3.3 Bcl-xL

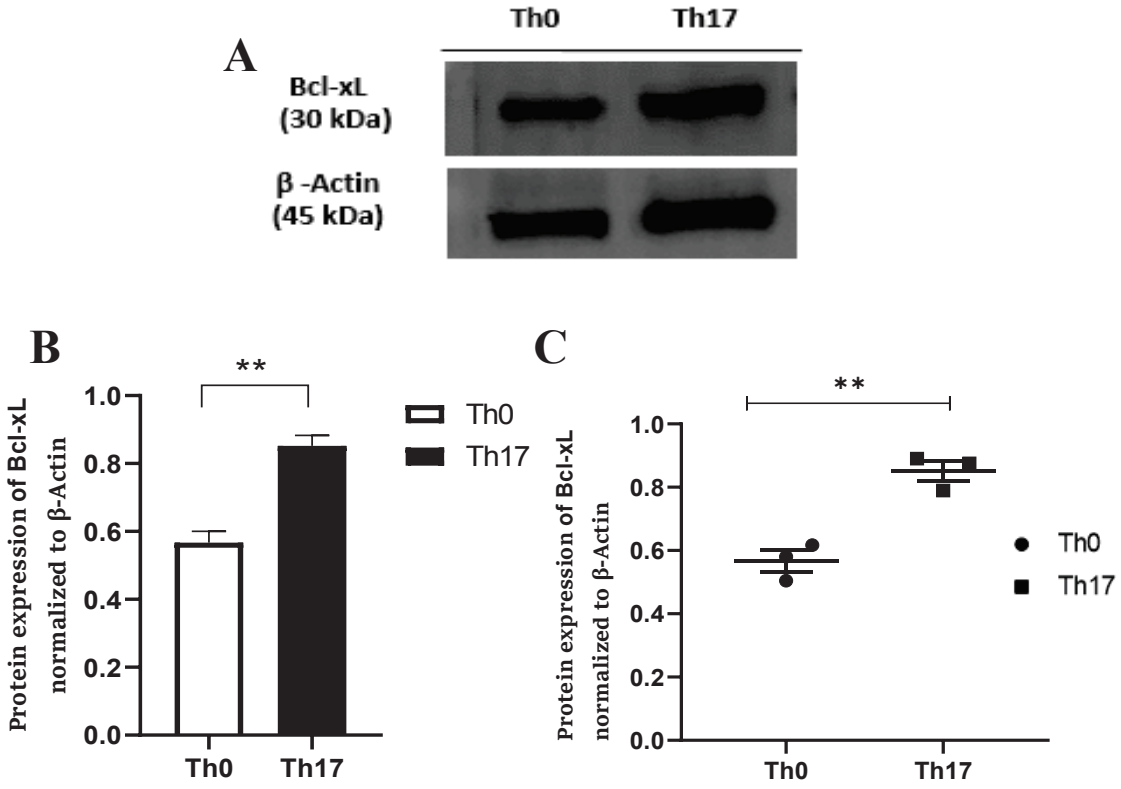

Figure 8. Determination of Bcl-xL. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups at the 7th-day time point. The molecular weight of human Bcl-xL is 30 kDa and cytosolic control β-Actin is 45 kDa. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality. (n=3;*p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 by Unpaired t-test).

The expression of Bcl-xL was detected at the protein level by analyzing protein lysate which was taken on the 7th day of cell culture to insight the dynamic of Bcl-xL in Th17 cells compared to control group Th0 cells. In this manner, it could be seen that the expression of Bcl-xL barely increased in Th17 cells through differentiation rather than control Th0 cells. The relevant protein band of Th17 cell lysate was seen thicker than the protein band of Th0 cell lysate (Fig6.A). According to the Unpaired t-test statistical analysis, there was a significant difference between Th0 and Th17 cell groups in line with Bcl-xL protein expression (p = 0,0034) (Fig6.B). When the individual values were observed from three biological donors, it could be denoted that the normalized individual values by using cytosolic control showed up an associated dispersion with significant difference which was identified by Unpaired t-test analysis (Fig6.C).

3.4 Mcl-1

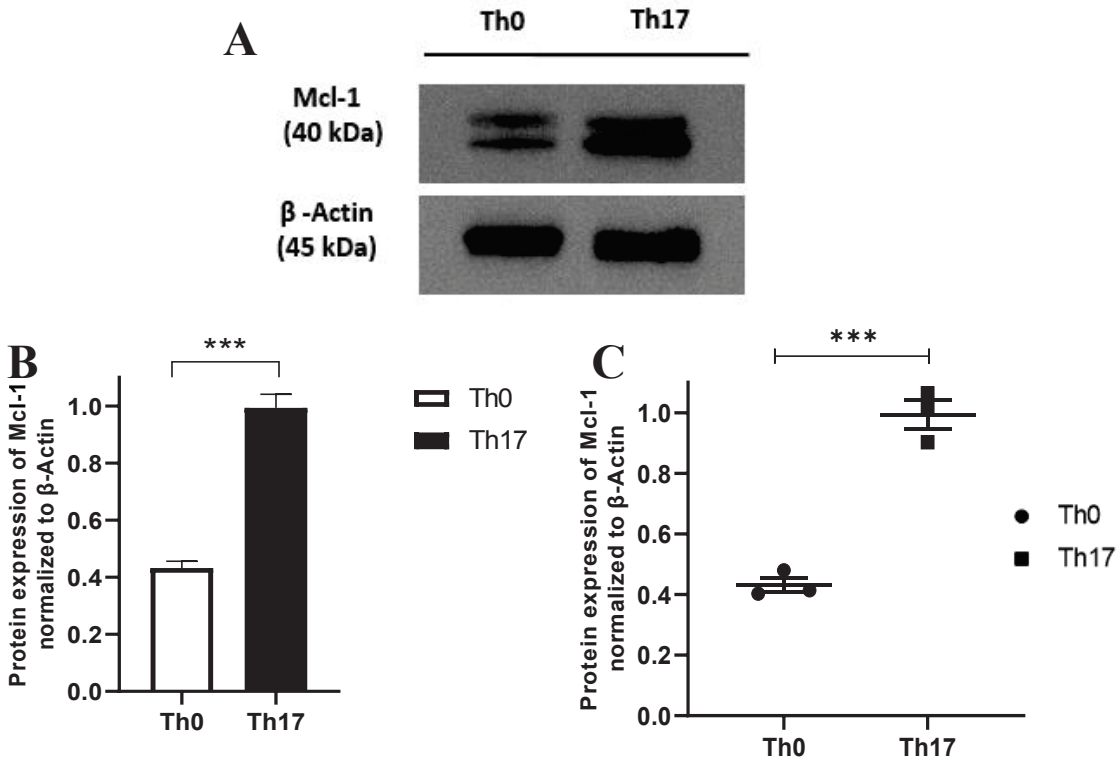

Figure 9. Determination of Mcl-1. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups at the 7th-day time point. The molecular weight of human Mcl-1 is 40 kDa and cytosolic control β-Actin is 45 kDa. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality. (n=3;*p<0,05,**p<0,01,***p<0,001,****p<0,0001 by Unpaired t-test).

The expression of Mcl-1 was detected at the protein level by analyzing protein lysate which was taken on the 7th day of cell culture to insight the dynamic of Mcl-1 in Th17 cells compared to control group Th0 cells. In this manner, it could be seen that the expression of Mcl-1 blatantly increased in Th17 cells through differentiation rather than control Th0 cells. The relevant protein band of Th17 cell lysate was seen thicker than the protein band of Th0 cell lysate (Fig7.A). According to the Unpaired t-test statistical analysis, there was a significant difference between Th0 and Th17 cell groups in line with Mcl-1 protein expression (p = 0,0005) (Fig7.B). When the individual values were observed from three biological donors, it could be denoted that the normalized individual values by using cytosolic control showed up an associated dispersion with significant difference which was identified by Unpaired t-test analysis (Fig7.C).

3.5 Bid and t-Bid

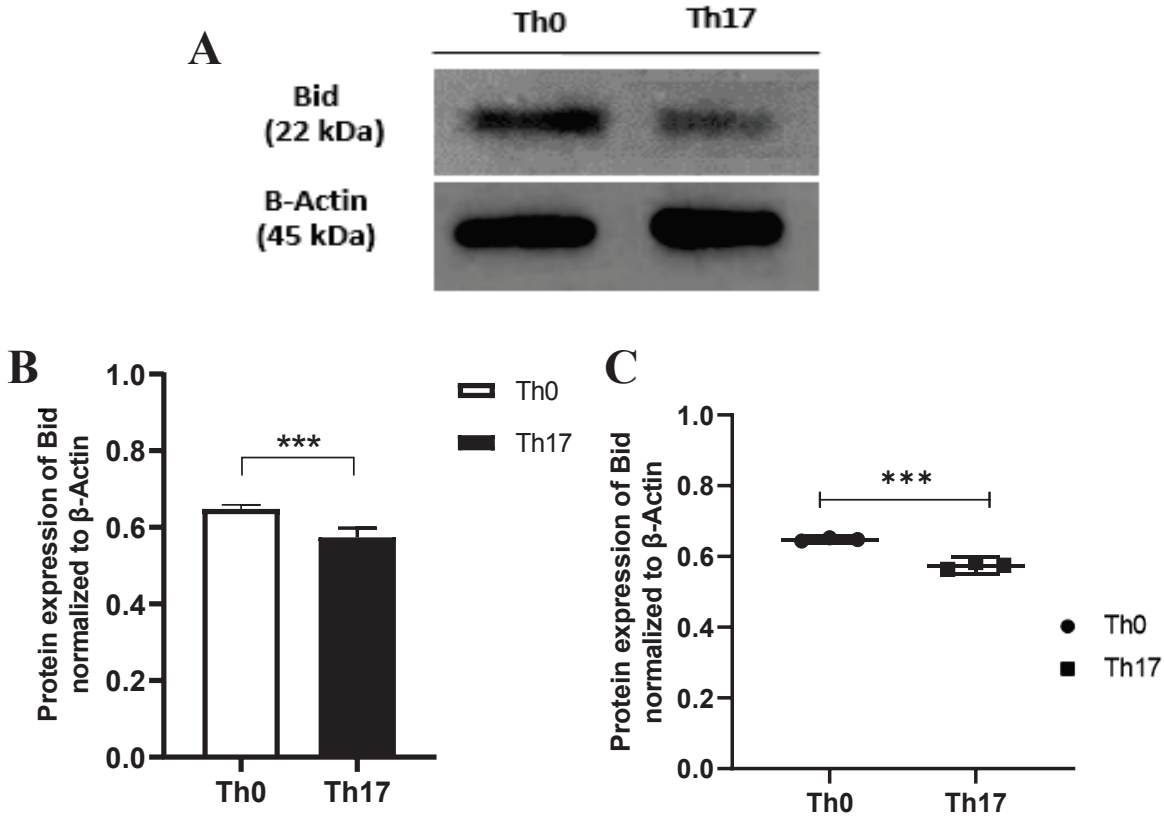

Figure 10. Determination of Bid and t-Bid. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups at the 7th-day time point. The molecular weight of human non truncated Bid is 22 kDa and cytosolic control β-Actin is 45 kDa. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality.
(n=3;*p<0,05,**p<0,01,***p<0,001,****p<0,0001 by Unpaired t-test).

The expression of Bid was detected at the protein level by analyzing protein lysate which was taken on the 7th day of cell culture to insight the dynamic of Bid in Th17 cells compared to control group Th0 cells. In this manner, non truncated version of Bid was expressed in either Th0 and Th17 cells while the truncated version of Bid (t-Bid) was not detectable in both cell groups. Besides this, the expression of Bid was a bit increased in Th17 cells through differentiation rather than control Th0 cells (Fig8.A). According to the Unpaired t-test statistical analysis, there was a significant difference between Th0 and Th17 cell groups in line with Bid protein expression (p=0,0003) (Fig8.B). When the individual values were observed from three biological donors, it could be denoted that the normalized individual values by using cytosolic control showed up an associated dispersion with significant difference which was identified by Unpaired t-test analysis (Fig8.C).

3.6 Puma

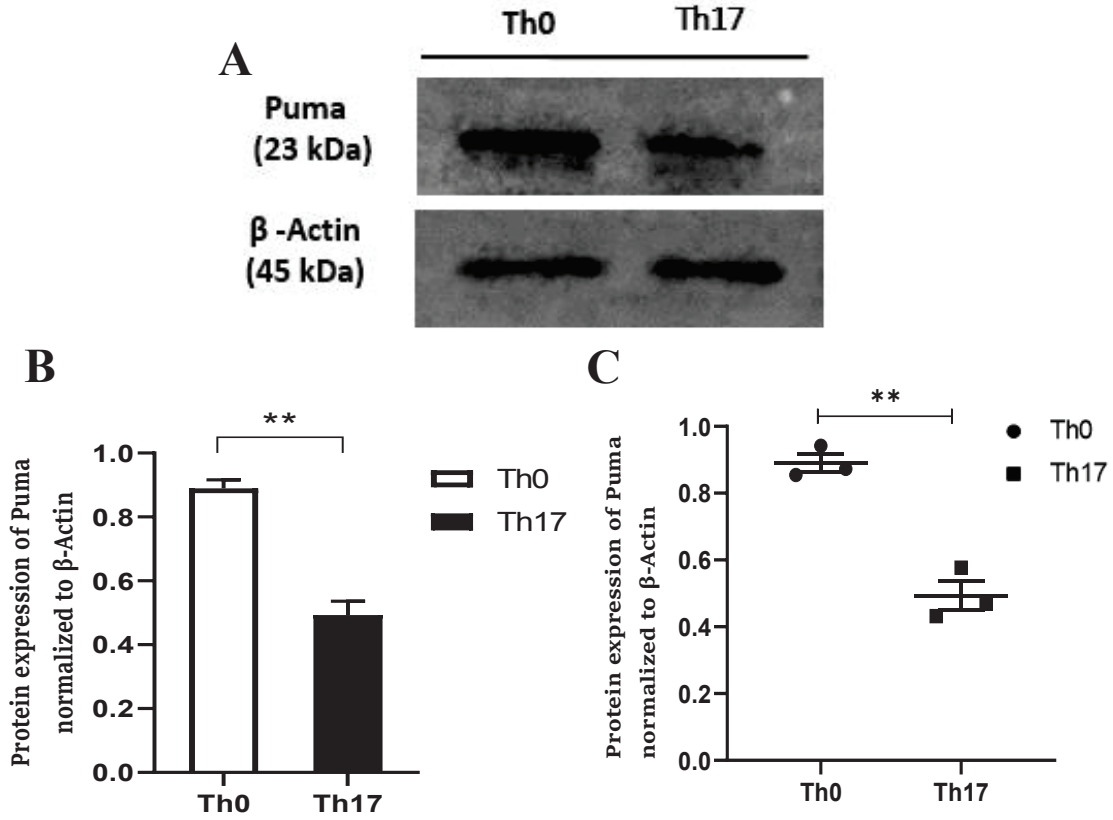

Figure 11. Determination of Puma. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups at the 7th-day time point. The molecular weight of human Puma is 23 kDa and cytosolic control β-Actin is 45 kDa. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality. (n=3;*p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 by Unpaired t-test).

The expression of Puma was detected at the protein level by analyzing protein lysate which was taken on the 7th day of cell culture to insight the dynamic of Puma in Th17 cells compared to control group Th0 cells. In this manner, it could be seen that the expression of Puma substantially decreased in Th17 cells through differentiation rather than control Th0 cells. The relevant protein band of Th17 cell lysate was seen thinner than the protein band of Th0 cell lysate (Fig9.A). According to the Unpaired t-test statistical analysis, there was a significant difference between Th0 and Th17 cell groups in line with Puma protein expression (p = 0,0015) (Fig9.B). When the individual values were observed from three biological donors, it could be denoted that the normalized individual values by using cytosolic control showed up an associated dispersion with significant difference which was identified by Unpaired t-test analysis (Fig9.C).

3.7 Bim protein Bim_{EL}

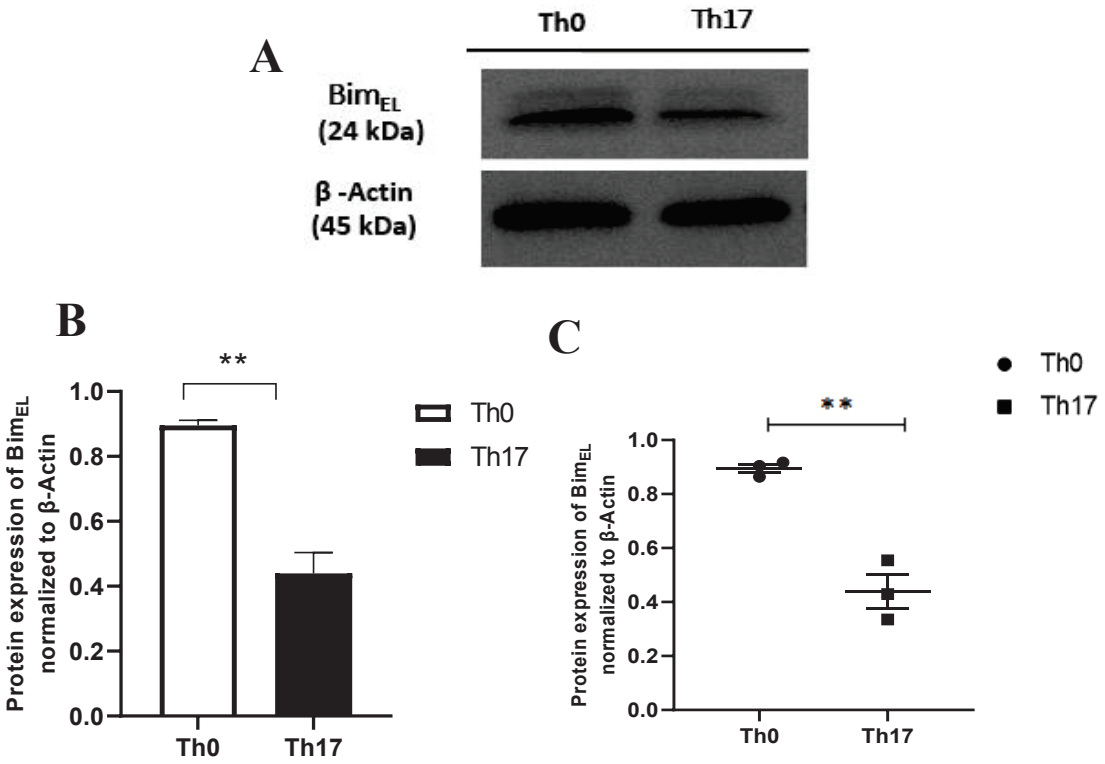

Figure 12. Determination of Bim protein Bim_{EL}. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups at the 7th-day time point. The molecular weight of human Bim_{EL} is 24 kDa and cytosolic control β-Actin is 45 kDa. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality. (n=3;*p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 by Unpaired t-test).

The expression of Bim was detected at the protein level by analyzing protein lysate which was taken on the 7th day of cell culture to insight the dynamic of Bim in Th17 cells compared to control group Th0 cells. It could be seen that the expression of Bim_{EL} is one of three isoforms of Bim. Therefore, Bim_{EL} expression was detected as partly decreased in Th17 cells through differentiation rather than control Th0 cells. The relevant protein band of Th17 cell lysate was seen less than the protein band of Th0 cell lysate (Fig10.A). According to the Unpaired t-test statistical analysis, there was a significant difference between Th0 and Th17 cell groups in line with Bim_{EL} protein expression ($p = 0,0005$) (Fig10.B). When the individual values were observed from three biological donors, it could be denoted that the normalized individual values by using cytosolic control showed up an associated dispersion with significant difference which was identified by Unpaired t-test analysis (Fig10.C).

3.8 Bik

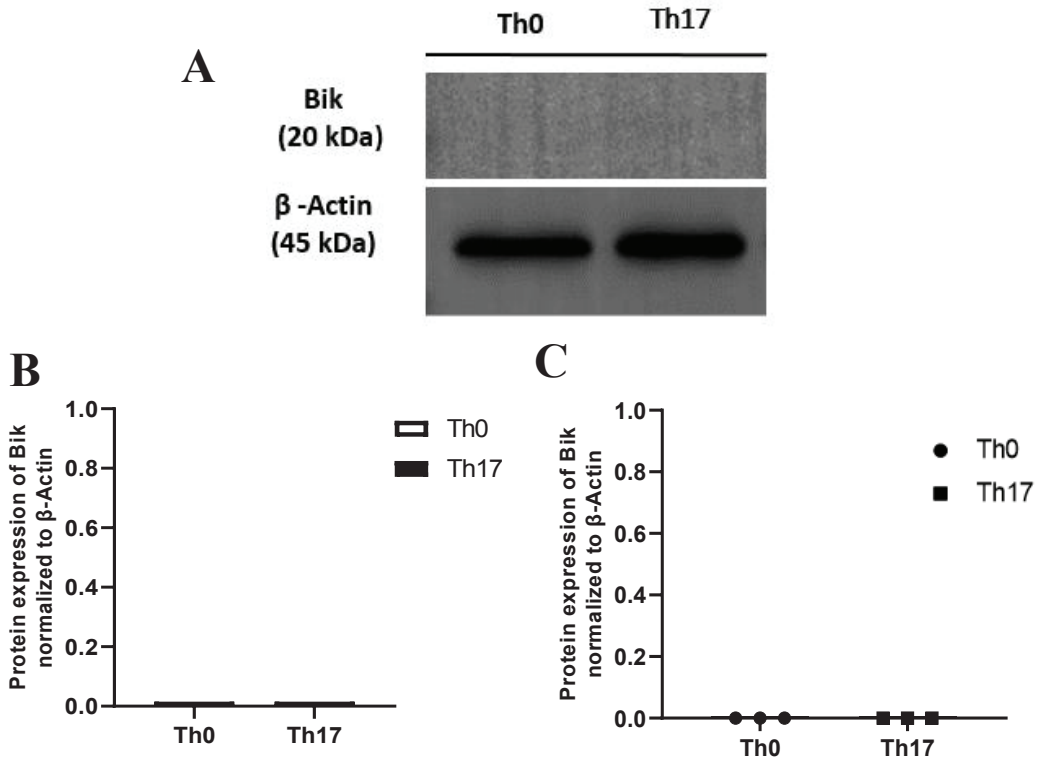

Figure 13. Determination of Bik. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups at the 7th-day time point with different antibody dilutions as 1:1000, 1:1250, 1:1500. The molecular weight of human Bik is 20 kDa and cytosolic control β-Actin is 45 kDa. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality. (n=3;*p<0,05,**p<0,01,***p<0,001,****p<0,0001 by Unpaired t-test).

The expression of Bik was measured at the protein level by analyzing protein lysate which was taken on the 7th day of cell culture to insight the dynamic of Bik in Th17 cells compared to control group Th0 cells. In this manner, it could be examined that the expression of Bik could not be detected either in Th17 cells and control Th0 cells. However, cytosolic control β-actin expressions could be detected properly in both Th17 cells and Th0 cells to confirm the existence of total protein on the blot (Fig11.A). So the significance between Th0 and Th17 cell groups in line with Bik protein expression could not be evaluated (p= ns/>0,9999) (Fig11.B). When the individual values were observed from three biological donors, it could not be denoted any output on the normalized individual values (Fig11.C). The cause of Bik expression could be detected neither in Th0 cells nor in Th17 cells, different dilutions were tried to insight the expression dynamics of Bik (Fig.12-14).

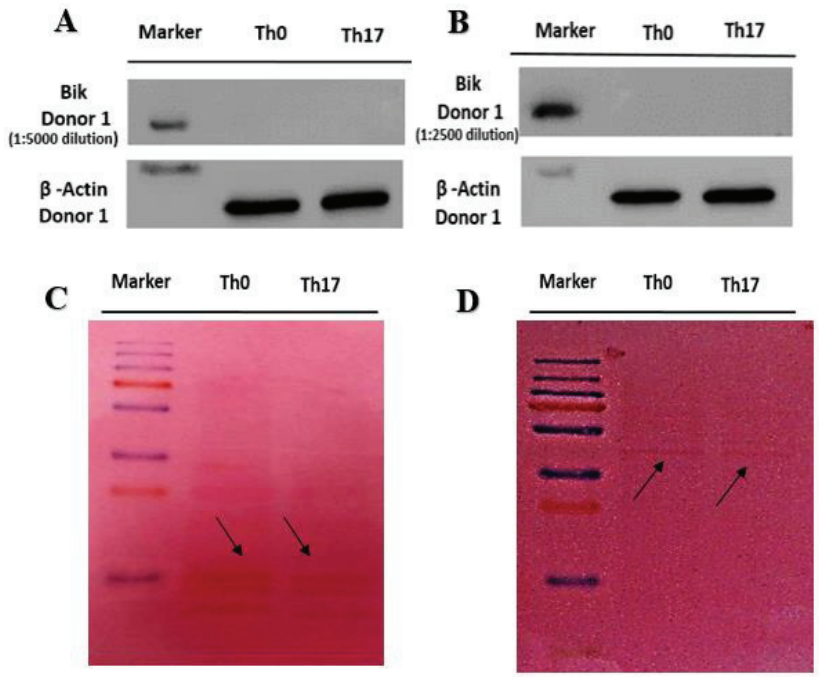

Figure 14. Determination of Bik expression at Donor 1. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups of donor 1 at different 7th-day time points incubated with different Bik antibody dilutions as 1:1250, 1:1500. The molecular weight of human Bik is 20 kDa and cytosolic control β-Actin is 45 kDa.

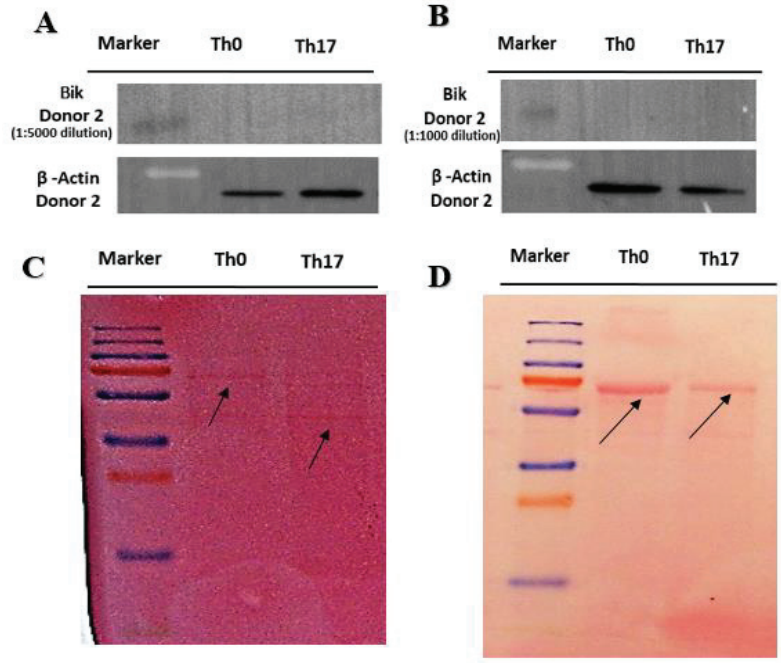

Figure 15. Determination of Bik expression at Donor 2. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups of donor 1 at different 7th-day time points incubated with different Bik antibody dilutions as 1:1000, 1:1500. The molecular weight of human Bik is 20 kDa and cytosolic control β-Actin is 45 kDa.

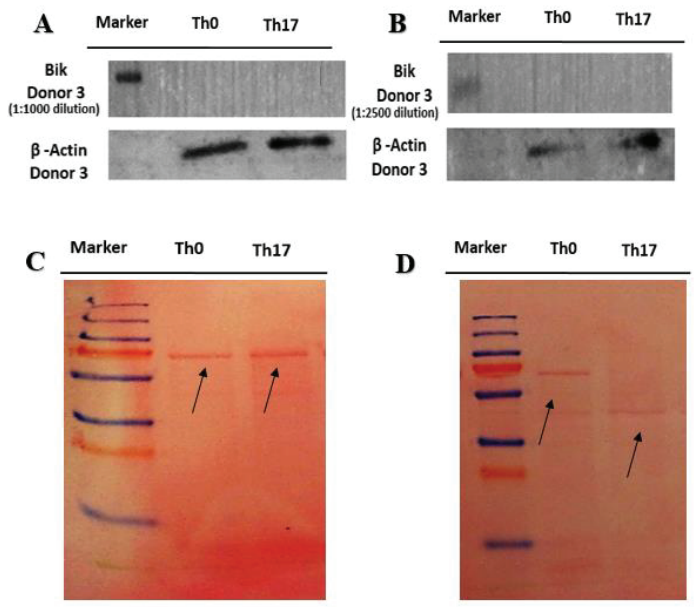

Figure 16. Determination of Bik expression at Donor 3. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels were evaluated by Western blot analysis in Th0 and Th17 cell groups of donor 1 at different 7th-day time points incubated with different Bik antibody dilutions as 1:1000, 1:1250. The molecular weight of human Bik is 20 kDa and cytosolic control β-Actin is 45 kDa.

The expression of Bik protein could not be determined in Th0 and Th17 cell group lysates which were taken from the 7th-day time point. In general, a 1:5000 ratio was used for primary antibody dilution to express relevant protein but different dilutions were used in this case to get Bik expression. Despite using different dilutions and three separate biological donors, Bik expression could not be obtained in Th0 and Th17 cell groups. The membrane images were introduced to show up protein loading onto nitrocellulose membrane after the transfer. Because Ponceau S stain was used to dye protein lines as red while membrane blot was dyed pink. This process was used to confirm the loading of proteins properly onto the membrane by transfer whether not. As a result of performing different dilutions of Bik antibody on three different biological donor protein lysates which were Donor 1 with 1:5000 dilution (Fig12.A), Donor 1 with 1:2500 dilution (Fig12.B), Donor 2 with 1:5000 dilution (Fig13.A), Donor 2 with 1:1000 dilution (Fig13.B), Donor 3 with 1:1000 dilution (Fig14.A), Donor 1 with 1:2500 dilution (Fig14.B) at the 7th-day time point of control Th0 and polarized Th17 cell groups, Bik protein could not be determined. It could be seen that the total protein was properly loaded and transferred onto the related blot for each sample as the lines of proteins were shown by arrows in Panceou S dyed blot images (Fig12.C-D, Fig13.C-D, Fig14.C-D).

3.9 Comparison of anti-apoptotic Bcl-2 family proteins

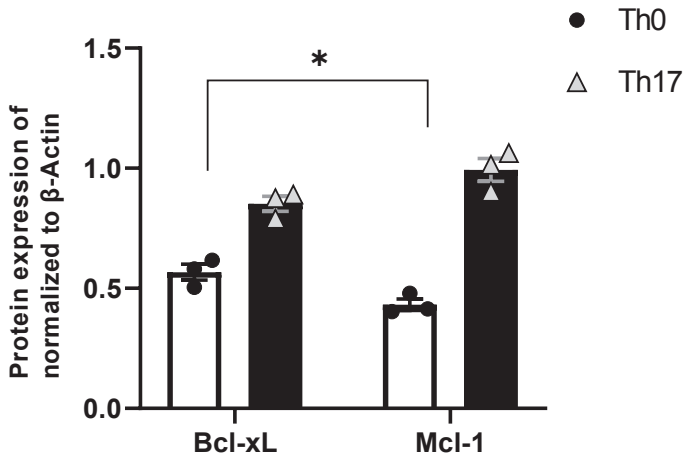

Figure 17. Dynamics of anti-apoptotic Bcl-2 members in Th17 cell survival. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels of Bcl-xL and Mcl-1 were evaluated by Western blot analysis in Th0 and Th17 cell groups at the 7th-day time point. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality. (n=3;*p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 by Unpaired t-test).

Table 1: Tukey's multiple comparisons test of anti-apoptotic Bcl-2 members Bcl-xL and Mcl-1 performed by two-way ANOVA

	Significance	**Adj. P-Value**
Bcl-xL vs. Mcl-1	*	0,0434

To observe the relationship between Th17 differentiation and expression levels of anti-apoptotic Bcl-2 proteins, Tukey's comparison test was applied. Then, the significance levels were calculated between anti-apoptotic Bcl-2 members Bcl-xL and Mcl-1which represented during Th17 cell differentiation in terms of cell survival (Table 1). According to the multiple comparisons by statistical analysis, there was a significant difference of expression fold change in Th0 cells rather than Th17 cells concerning either Bcl-xL and Mcl-1 expression fold changes in Th0 cells rather Th17 cells (p= 0.0434). Thus, it could be seen that the expression changes of Bcl-xL and Mcl-1 showed up proportionally increase through Th17 cell differentiation. The normalized individual values by using cytosolic control showed up an associated dispersion with a significant difference. The significant difference between Bcl-xL and Mcl-1 increments separately (p = 0,0434) was identified as a result of statistical analysis (Fig15).

3.10 Comparison of pro-apoptotic Bcl-2 family proteins

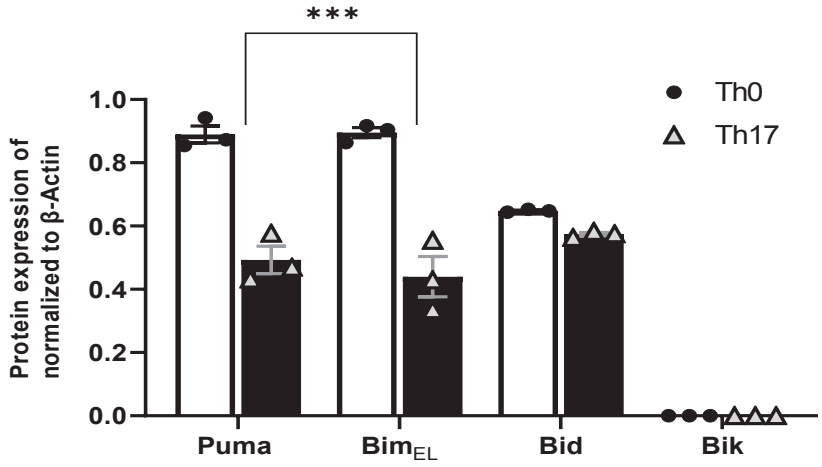

Figure 18. Dynamics of pro-apoptotic Bcl-2 members in Th17 cell survival. Naive CD4+ T (Th0) cells were stimulated under Th17 polarizing conditions for 7 days. The relative protein levels of Puma, Bim$_{EL}$, Bid, and Bik were evaluated by Western blot analysis in Th0 and Th17 cell groups on the 7th day. Results were expressed as the mean ± SEM for each group from three separate experiments normalized for β-Actin and Shapiro-Wilk test was applied for normality. (n=3;*p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 by Unpaired t-test).

Table 2: Tukey's multiple comparisons test of pro-apoptotic Bcl-2 members Puma, Bim, Bid, and Bik performed by two-way ANOVA

	Significance	**Adj. P-Value**
Puma vs. Bim	***	<0,001
Puma vs. Bid	ns	0,1487
Puma vs. Bik	ns	0,0601
Bim vs. Bid	ns	0,4503
Bim vs. Bik	ns	0,7546
Bid vs. Bik	ns	0,5271

To observe the relationship between differential expression fold changes of pro-apoptotic Bcl-2 proteins, which represented during Th17 differentiation, ordinary two-way ANOVA via Tukey's multiple comparison test was applied (Table 1). As a result of overall data, there was a strongly significant difference between decrements of Puma and Bim$_{EL}$ protein expressions separately through Th17 cell differentiation (p= <0.001). Thus, it could be indicated that there was a proportional relationship between diminished expressions of Puma and Bim$_{EL}$ in survived Th17 cells (Fig16). However there was no significant difference between Puma and Bid (p=0.1487), Puma and Bik (p=0.0601), Bim and Bik(p=0.7546), Bim and Bid(p=0.4503), Bid and Bik(p=0.5271) as indicated (Table 2).

CHAPTER 4

DISCUSSION

Th17 cell-driven immunity carries out a vigorous immune fight against invader pathogens by creating memorial, selective and rapid immune exposure. Dysregulation and loss of Th17 cells are seen in various pathologies of autoimmune diseases and cancer types in humans. Thus, the underlying mechanisms involved in Th17 regulation and cell survival are so crucial to deal with these pathologies. However, human Th17 cell survival has not been well known yet and the knowledge concerning regulation of human Th17 cell-driven immunity is limited.

Regarding regulation of T cell survival, the Bcl-2 protein family has been proposed to be regulated by monitoring apoptotic cell death with pro-apoptotic and anti-apoptotic members. Nevertheless, the regulatory roles of Bcl-2 protein involved in human Th17 cell survival relevance to apoptotic regulation would be clarified in depth. Thus, we focused on the investigation of Bcl-2 protein family members in Th17 cell functions and our study revealed the dynamics of defined Bcl-2 proteins in survived human Th17 cells.

To do that, Th17 cells would be examined in humans, we used venous buffy coats drawn from three different healthy donors. Because the buffy coat is an anticoagulated blood fraction that covers most of white blood cells and platelets. PBMC cell population was obtained from these samples which PBMCs mainly contain lymphocytes, monocytes, and thrombocytes as known mononuclear cell layers. Throughout PBMC isolation, buffy coats were measured to sample selection and it could be concluded that the weight of the relevant buffy coat was positively correlated with the number of isolated PBMC cells according to the calculations. Besides this, taking the PBMC layer without breaking was so crucial to avoid other layers of the buffy coat covering plasma, granulocytes, and erythrocytes. Using red blood cell lysis buffer and repeating washing steps made it easier. After getting the PBMC cell population, the sorting step of Naive CD4+ T cells was so critical to creating a more selected Th17 cell population for proper examinations in further steps of our experimental design. So, the PBMC population was characterized phenotypically by flow cytometry to calculate approximate naive CD4+ T cell numbers for the sorting step.

Also, we applied this characterization step to determine the presence ratios of cell surface markers for other cell populations like CD8 carrying cytotoxic T cells, CD14 carrying monocytes, and CD18 carrying B cells in isolated PBMCs. It could be concluded that the 30% percentage of human PBMC composed of CD4+ T cells despite existing different donor profiles. To get this 30% naive CD4+ T cell population from PBMCs, the sorting step was applied with the help of using biotin-conjugated antibodies and anti-biotin microbeads. Through this method, naive T cells could be sorted by getting eluted from the column and non-native T cells were stuck onto the column by magnetic labeling effects. At this level, sorting efficiency was so critical to obtain properly sorted naive CD4+ T cells from PBMCs. After sorting we could get approximately 80% CD4+ T cell-containing cell population by phenotypically characterizing with naive marker CD45RA by flow cytometry. We emphasized that slow progressions were crucial during the sorting mechanism to get a more purified target cell population. Furthermore, previous proportional calculations were so important for better sorting which were done for discrimination of existing cell populations thanks to flow cytometric analyses. However, it can be denoted that using a dead cell removal kit may increase the sorting efficiency in addition to the previous washing and red blood cell lysis steps.

Afterward, naive CD4+T cells could be used to obtain Th17 cells under Th17 polarizing cell culture conditions. Besides this Th17 cell group, naive CD4+ T cells were used as a control group with cultured only medium without any polarization which this group was named as Th0 in our experimental design. Th17 polarizing conditions were optimized in our laboratory before which lasts during 7 days of cell culture with defined stimulatory cytokines in the method section. This optimization was obtained by phenotypic characterizations by monitoring activation with CD25, CD69 activation markers at 3rd and 5th days of culture, by monitoring cell survival with FAS, ANNV, 7AAD apoptotic markers, and by monitoring Th17 cell phenotype with RORC, CCR6, IL17A cell surface/ intracellular markers.

According to the flow cytometry results of this study, IL17A cytokine production was increased in the Th17 cell group by 7.39% compared to the Th0 cell group by 0.99% which IL17A is a mainly produced cytokine by Th17 cells. Then, the master transcription factor of human Th17 cells which is RORC2 production is increased in the Th17 cell group by 10.93% than Th0 cell group by 1.68%. The ratios may be seen low as a percentage of the population but these cytokines are released from intracellular

compartments of the cell, so their intracellular staining step makes it hard to determine them by flow cytometry. The significant differences were important to prove expression changes in the relevant molecule.

To conclude, our data showed that naive $CD4^+$ T (Th0) cells were activated and differentiated into Th17 cells for up to 7 days under Th17 polarizing cell culture conditions based on our optimizations. In addition to this differentiation process, these Th17 cells could survive. In this manner, regulatory proteins play decisive roles during the differentiation of Th17 cells from Th0 cells to drive Th17 cell survival, apoptotic regulation of Th17 cells, and Th17 cell-mediated pathologies. Therefore, we analyzed the expression levels of Bcl-2 protein family members in a protein manner by taking lysates from Th0 and Th17 cell groups at the end of the 7th day of cell culture to maintain a comparative examination.

Before the investigation of Bcl-2 proteins, we examined the RORC2 transcription factor at the protein level. Throughout this aim, we analyzed RORC2 protein expression at the different time points of cell culture which were 3rd, 5th, and 7th days. Our data emphasized that the expression of the human RORC2 transcription factor significantly elevated through Th17 cell differentiation at the protein level and human RORC2 strongly express in survived Th17 cells. To identify differentiated Th17 cells that were originated from Th0 cells, the detection of RORC2 could be evaluated. Thus, we could describe human RORC2 as a key for the initiation of Th17 cell functions.

Through literature review, we were aware that dysregulated expressions of Bcl-2 family proteins lead to inhibition of apoptosis in T cells to protect against pathological disorders. However, these kinds of protective mechanisms and regulatory signaling pathways of apoptosis in T cells would be more examined regards T cell subgroups, especially for human Th17 cells.

Concerning this, we first examined anti-apoptotic members Bcl-xL and Mcl-1 as anti-apoptotic members of the Bcl-2 family to promote cell survival. According to the comparative results between Th0 and Th17 cell groups, either Bcl-xL and Mcl-1 proteins significantly increased within survived human Th17 cells. Diminished Mcl-1 in human Th0 cells blatantly increased in survived human Th17 cells while Bcl-xL expression barely increased in survived human Th17 cells compared to Th0 cells. It means, Bcl-xL did not show a higher amount of increment like Mcl-1 in survived active Th17 cells.

Thus, we concluded that Mcl-1 is more efficient to maintain cell survival in human Th17 cells compared to Bcl-xL. The elevated expression of Mcl-1 in human Th17 cells was proven before based on our previous study (Çimen & Nalbant, 2018). In addition, the less effect of Bcl-xL compared to Bcl-2 on T cell survival was also proven before (Loo *et al.,* 2020) and Bcl-xL expressed in variable levels while Mcl-1 and Bcl-2 showed constant expression levels in T lymphocytes (Ohta *et al.*, 1995).

After studying the anti-apoptotic members of the Bcl-2 family, we focused on the pro-apoptotic members of the Bcl-2 protein family which were Puma, Bim, Bik, and Bid. Among them, Puma expression was substantially decreased in survived Th17 cells compared to Th0 cells. In correlation with the reduction of Puma, Bim isoform results of alternative splicing Bim_{EL} could be detected as partly decreased in Th17 cells compared to Th0 cells. It can be concluded that the significant differences concerning diminished expressions of Puma and Bim_{EL} were determined. According to the literature, BH3-only Bcl-2 members Puma and Bim act like tumor-suppressors because of deletion or loss of Puma and Bim could be seen in many types of human cancers. In this manner, deletions of Puma and Bim are associated with evading from apoptosis because the combined loss of them makes the cell more resistant to apoptotic stimuli. Furthermore, Puma and Bim are combined to be more potent killers rather than other pro-apoptotic BH3 only Bcl-2 proteins which target anti-apoptotic Bcl-2 proteins (Yu *et al.,* 2001). So, we emphasized that pro-apoptotic Puma and Bim_{EL} proteins were combined by decreasing significantly within survived human Th17 cells as a concluding remark of this study.

Besides Bim and Puma, another pro-apoptotic member Bid was investigated in survived Th17 cells. Bid is a death agonist and cytosolic protein which its cleaved form t-Bid is located to the mitochondrial outer membrane causing Bax and Bax oligomerization. So Bid may have dual functions within the regulation of cell death. To clarify, Bid was observed in our study. Then, our results demonstrated that a non-truncated/cleaved form of Bid was detected and there was a significant difference between Th0 and Th17 cell groups in line with Bid protein expression. We concluded that the expression of Bid was a bit increased in Th17 cells through differentiation rather than control Th0 cells. So, non truncated form of Bid may not promote cell survival as the main player like existing anti-apoptotic proteins or loss of pro-apoptotic proteins within human survived Th17 cell functions.

Among the analyzed BH3-only pro-apoptotic members, Bik was analyzed in-depth with different trials and antibody dilutions. Because Bik was undetectable in Th0 and Th17 cells at protein level where cell lysates were taken from three different donors with applying 1:5000 diluted Bik primary antibody. To handle this, confirmation experiments were done by applying cytosolic control β-Actin to confirm the existence of total protein on the membrane. The Western blot efficiency was also confirmed with staining transferred membranes with Ponceau S dye after wet transfer of relevant SDS gels. Because after running, SDS gel was transferred to a nitrocellulose membrane which could be seen as dyed with Ponceau S to visualize proteins for confirmation before antibody incubations. The figures (Fig 13-15) represent transferred SDS gels onto Nitrocellulose membrane belongs to the Bik experiments. Membrane images were loaded up with protein samples from 7th-day time points covering Th0 and Th17 cell groups, respectively.

In a conclusion, β-Actin and Ponceau S dye confirmations demonstrated that proteins existed on the relevant membrane even though Bik expression could not be detected in both Th0 and Th17 cell groups. As another parameter, primary antibody dilution was discussed and different dilutions were applied for Bik primary antibody on all three donors. However, Bik expression still was not observed in any of them. Other parameters concerning Western blot were examined. To do that; compatibility of primary antibody and secondary antibody were checked, excessive washing or too much blocking of the membrane were prevented and cross-reaction with a blocking agent was avoided. According to the literature, Bik is related to promoting apoptosis by triggering Cas-9 activation and release of cytochrome c as an apoptogenic factor. In previous studies which were performed with small animal models, it could be seen that Bik deficiency did not alter the pathological functions and development (Chinnadurai *et al.,* 2008). Overall our data and trials indicated that there was no Bik expression in survived human Th0 and Th17 cells at the protein level. In addition, we emphasized that localization of Bik on nuclear outer membrane and nucleus could be a reason for being undetectable. The expression level of Bik may be checked in nuclear compartments of the human Th0 and Th17 cells.

To sum up, Th17 cells have been demonstrated as crucial players in the pathogenesis of numerous autoimmune disorders and cancer types. The investigation of Bcl-2 protein family dynamics in terms of Th17 cell functions has biological importance to understand regulatory networks and interactions accounting for Th17 cell-driven protective mechanisms and pathologies.

Our study emphasized that anti-apoptotic Bcl-2 protein family members Bik, Bid, Puma, and BimEL decrease when pro-survival Bcl-2 proteins family members Bcl-xL and Mcl-1 increase in terms of promoting cell survival of human Th17 cells (Fig19). In line with this output, our study identified decisive roles of anti-apoptotic Bcl-2 members Bcl-xL and Mcl-1 compared to the pro-apoptotic Bcl-2 members Bim, Puma, Bid and Bik in human survived Th17 cells through differentiation and cell survival of Th17 cells originated from Th0 cells in a scientific value manner.

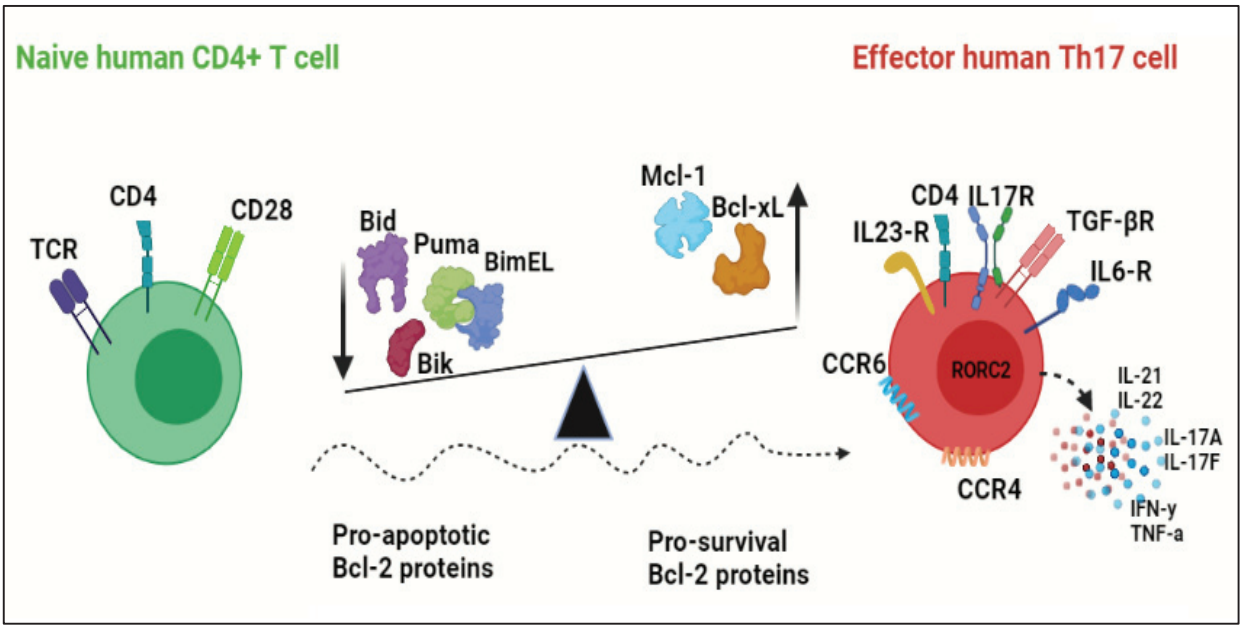

Figure 19. Balance of Bcl-2 proteins in human Th17 cells

CHAPTER 5

FUTURE PERSPECTIVES

In the future directions, other pro-apoptotic family members could be investigated to obtain more comparative results which could be Bad, Bak and Bax. These members are associated with Bax/ Bak oligomerization directly to induce MOMP related induction of apoptosis. In addition, Bcl-2 founding members would be examined to understand the regulation network of the Bcl-2 family members which were studied during this thesis.

As a result of this study, Mcl-1 and Bl-xL showed up significant increment in survived human Th17 cells in terms of cell survival. Then, it can be demonstrated that Puma and Bim were combined to be more potent in terms of monitoring Th17 cell survival. So, the interactions between BH3-only domain pro-apoptotic Bcl-2 proteins and pro-survival Bcl-2 proteins concerning Th17 cell survival might be examined in future directions.

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