INVESTIGATION OF THE EFFECTS OF IL-7 ON THE TH-17 T CELL APOPTOSIS

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF IL-7 ON THE TH-17 T CELL APOPTOSIS

Th17 cells known as Interleukin-17 (Inflammatory Cytokine) producing cells are differentiated subsets from naïve CD4+ T cells and have crucial roles in regulation of inflammation, host defense and autoimmunity. TCR (T Cell Receptor) activation is triggered under Th17 cell culture conditions and resulting naïve CD4+ T cells are induced to differentiate through Th17 cells. In the life time of activated T cells, the activation process also induces an apoptotic mechanism which is called activation-induced cell death (AICD) for elimination of activated cells from the environment for maintenance of homeostasis. AICD is known as the main programmed cell death mechanism for T cells by Fas-FasL signaling resulting activation of early and late apoptotic caspase proteins such as caspase-3 and caspase-8. Moreover, Interleukin-7, which is a member of Interleukin-2 family, has a survival mechanism in T cells by the activation and maintenance of anti-apoptotic proteins mainly Bcl-2 and inhibition of pro-apoptotic proteins such as Bax and Bim. This research analyzes apoptosis mechanism in Th17 cells in terms of AICD and the effects of IL-7 on that apoptosis signaling pathway. Our results showed that IL-7 did not have any effect to AICD throughout Fas-FasL signaling and activation of caspase-3 and caspase-8 protein.

ÖZET

İNTERLÖKİN 7'NİN TH17 T HÜCRELERİNİN APOPTOZUNDAKİ ETKİLERİNİN ARAŞTIRILMASI

Th17 hücreleri, ayrıca İnterlökin-17 (enflamatuar sitokinini) salgılayan hücreler, naif CD4+ T hücrelerinin farklılaşmış bir alt grubudur ve bağışıklık sistemde, özellikle konak savunması ve otoimmunitede çok önemli bir yere sahiptir. Naif CD4+ T hücreleri, T hücresi reseptörünün (THC) aktifleşmesiyle beraber aktif Th17 hücresini oluştururlar ve bu aktif T hücreleri, kendi fonksiyonları gerçekleştirdikten sonra çevreden yok edilmeye tabi tutulur. Bundan dolayı, aktivasyondan hemen sonra, aktivasyon işlemi aktivasyonun tetiklediği hücre ölümü (ATHÖ) denilen bir apoptoz mekanizması aktif eder. ATHÖ, Fas-FasL sinyal yolağını tetikleyerek son apoptoz protein olan caspase-3 proteini aktive eder ve bununla beraber Th17 hücreleri özdengeden elenmiş olur. Bunun dışında İnterlökin-2 ailesin bir üyesi olan İnterlökin-7'nin T hücreleri üzerinde yaşamsal kurtarma ektisi olduğu bilinmektedir. Bu işlem Bcl-2 aktivasyonu ya da var olan Bcl-2 aktivasyonun devamlılığını sağlamak üzere kurulur. Bunun yanı sıra apoptoz tetikleyici protein olarak bilinen Bax ve Bim proteinlerinin durdurulmasında rol alır. Bu tezde ATHÖ'nün Th17 hücreleri üzerindeki apoptoz etkisini ve IL-7'nin bunu nasıl etkilediğini araştırdık. Bu bilgilerin ışığında yapılan araştırmalarla, her ne kadar literatürde IL-7'nin Th17 hücrelerinde Bcl-2 proteinin aktifleşmesini sağladığı bilinse de, IL- 7'nin ATHÖ üzerinde Fas-Fas sinyal yolağı ve caspase-3 ve 8 proteinin aktifleşmesinde her hangi bir etkisi yoktur.

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ABBREVIATIONS

Treg	Regulatory T Cell
DC	Dendritic Cell
APC	Antigen Presenting Cells
CD4+	Helper T Cell
CD	Cluster of Differentiation
IL	Interleukin
Th	T Helper
RORC2	Retinoic Acid Receptor-Related Orphan Receptor C2
GMCSF	Granulocyte-macrophage Colony-Stimulating Factor
GATA3	GATA Binding Protein 3
IFNγ	Interferon Gamma
Foxp3	Forkhead box P3
TGFβ	Transforming Growth Factor Beta
CD25	Alpha Chain of the IL-2 Receptor
STAT	Signal Transducer and Activator of Transcription
T-bet	T-box Expressed in T cells
ANN-V	Annexin-V
IMDM	Iscove's Modified Dulbecco's Medium

CHAPTER 1

INTRODUCTION

1.1. T Cells and Activation

In the immune system, Thymus derived lymphocytes (T Cells) have a very important role against pathogens. In order to respond to pathogens, T cells must differentiate to effectors cells via their activation. Differentiation of CD4+T cells into activated T cells is a very complex process that includes many signal transduction events resulting transcription activation, also cytokine production and finally proliferation (Valle-Rios et al., 2009).

In T cell stimulation, T Cell Receptor (TCR) and CD3 activation are required. TCR is found on T lymphocyte membrane and it recognizes the MHC molecules on Antigen Presenting Cells (APC). TCR is a kind of heterodimer protein with disulfide bonds, it has subunits; alpha and gamma. Together with these subunits, TCR produces a complex that includes the CD3 co-receptor. Binding an antigen to TCR triggers the phosphorylation of tyrosine residues on immunoreceptor tyrosine-based activation motif (ITAMs). This phoshorylation event is needed for recruitment of several proteins such as ZAP-70. Together with the recruitment of ZAP-70, Src protein is activated. The conformational change in the structure of CD3 can activate the intracellular protein activation but the mechanism is not known very well. In addition to that, aggregation of TCR is also important for activating CD3 signaling. Together with the integration of pMHC to TCR-CD3 complex causes a direct contact between CD3 and protein tyrosine kinase (PTKs) and transphosphorylation (Yasuda et. al, 2007).

Moreover, in T cell membrane there are CD28 inducer molecules that regulate the T cell activation. The intracellular domain of CD28 is conjugated with phosphatidylinositide 3-kinases (PI3K) and this interaction catalyzes the conversion of Phosphatidylinositol 4,5-bisphosphate (PIP2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on membrane. PIP3 then activates Akt protein that is important for the phoshorylation of several proteins. Activation of Akt also induces the location of NFkB to the nucleus. This regulates the survival genes in cells (Liu et al., 1999).

1.2. T Helper Cell Subsets (Th1, Th2, Th9, Th17,Th22,Th3 and Treg)

After the activation of T cells, several effector T cell subsets are differentiated from naïve CD4+ T Cells. Based on different cytokine production, those differentiated effector T cells have distinct functions in the immune system. According to recent researches, Th1, Th2, Th9, Treg, Th22, Th3 and finally Th17 were identified as T helper phenotypes (Jager and Kuchroo, 2010).

Th1 cells are essential for clearance of intracellular pathogens and involved in cell-mediated immune responses. Basically, Th1 cells secrete interferon (IFN)-γ and lymphotoxin that cause activation of macrophages and trigger B cells to produce isotypes of immunoglobulins such as; IgG2a (Coffman, 2006). Moreover, according to researches, Th1 cells are related with the cytotoxicity genes expression such as; apoptotic signaling genes (Yu et al., 2003). Generation of Th1 cells depends on TCR activation and STAT1 signaling pathway. This pathway is induced of IFN-γR with IFNs. Then kinase activity phosphorylates STAT1 and it increases the expression of the transcription factor T-bet. T-bet enhances Th1 cells specific cytokine and its receptor: IL-12 and IL-12Rβ2 (Yang et al., 1999).

Th2 cells play an important role in allergies and response to some parasites. Also Th2 cells have related in IgE production in inflammation (Murphy and Reiner, 2002). Th2 cells are differentiated from naïve CD4+ T Cells via activation of TCR and as well as IL-4 which cause several phosphorylation events especially STAT6. Phosphorylated STAT6 induces the transcription factor of GATA3 in Th2 and several Th2 specific cytokines such as IL-4, IL-5 and IL-13. On the other hand, it causes downregulation Th1- related factors such as STAT4 (Cua et al., 2003).

Regulatory T cells or known as suppressor T cells are involved in abrogation of autoimmune disease. Regulatory T cells are crucial in the immune system because, they can suppress the immune response via different mechanisms that give rise to the production of several cytokines. In addition, those regulatory T cells are known as Foxp3 expressing cells. Foxp3 is a kind of transcription factor for Treg cell development and prevent the immune homeostasis. In absent of Foxp3 transcription factor, cells can produce IL-2, IL-4, IL-17 and IFN-γ which are related with Th1 and Th2 subtypes (Curotto de Lafaille and Lafaille, 2009).

Treg and Th17 cells have been accepted as the same cell lineage for many years. However, Th17 cells are mostly linked to the pathogenesis of autoimmune diseases and immune responses to bacterial and fungal infections (Wilson et al., 2007). Th17 cells are known as IL-17, IL-17F and IL-22 cytokine producing cells. Basically those cells can provide a protection in the probable infection but also most importantly they are related with autoimmune disease such as; multiple sclerosis and rheumatoid arthritis (Wilson et al., 2007). Together with IL-17 cytokine, cells can induce pathogenesis to several autoimmune diseases as well as cancer. RORC2 is the human transcription factor in the Th17 cells and have homologue of mouse RORγt. The combination effect of TGF-β, IL-21 and IL-6 induce high level of RORC2 that regulates the expression of IL-17 (Yang et al., 2008).

1.3. Th17 Cell Differentiation

Differentiation of naïve CD4+ T cells to Th17 cells requires several gene and protein expression. Mainly TGF-β, IL-6 and IL-23 are involved in the development of Th17 cells (Wilson et al., 2007).

Researches demonstrated that IL-23 is required for the expansion and proliferation of Th17 cells rather than the polarization of Th17 cells. IL-23 is a member of IL-6 family of cytokines and heterodimeric cytokine that have unique p19 subunit which is coupled to the p40 subunit of IL-12. The main founding of IL-23 in memory T cell is that it can promote the proliferation together with p19 activation resulting induction of systemic inflammation and premature death. Together with the activation of IL-23, the expression of IL-17 is induced and it leads to Th17 cell differentiation. As a result of this, IL-23 promotes adaptive immune response to any infection or pathogens via activation of IL-17 because IL-17 activation can trigger the rapid recruitment of monocytes and neutrophil. Moreover, IL-23 can induce Granulocyte-macrophage colony-stimulating factor (GM-CST) production that induces more additional myeloid cells (Aggarwal et al., 2002).

For the differentiation of naïve CD4+ into Th17 Cells, TGF-β is essential. This is kind of growth factor and type of cytokine which has relevant with immunity as well as cancer. The result of quantitative PCR and RT-PCR of the expression level of RORC2 (the transcription factor of Th17) are affected by the level of TGF-β.The

presence of TGF-β or the interaction of TGF-β with IL-21 cause high level of RORC2 (Yang et al., 2008). If TGF-β concentration is in high level from the normal amount, it inhibits the Th17 differentiation by inducing Foxp3 via antagonizing RORC2 function (Zhou et al., 2008).

IL-6 is also known about the responsible of the differentiation through Th17 cells from naïve CD4+ T cells. According to researches, IL-17 producing T cells from naïve CD4+ T cell generation is not properly induced by alone TGF-β or alone IL-6. However the combination of TGF-β and IL-6 induce most naïve T cells to produce IL-17. In addition, IL-6 has the suppressing effect Foxp3 expression and Treg generation by induction of TGF-β (Bettelli et al, 2006).

1.4. Th17 Culture Condition

Differentiation of CD4+ T cell to Th17 cells and maintenance of those cells are based on the specific Th17 culture condition which includes anti-CD3 and anti-CD28 for the activation of T Cells through TCR activation. For maintenance and survival of Th17 cells in the culture, IL-23 is essential. For trigger of differentiation of naïve CD4+ T cells to Th17 cells, main component is IL-6. Also addition of TGF-β induces the transcription factor RORC2 and regulates the activation of Th17 specific genes. Also TGF-β suppresses STAT4 and GATA-3 expression, therefore Th1 and Th2 cells cannot differentiate from naïve CD4+ T cells. It is well known that Th17 propagation also requires IFN-γ and IL-4 and IL1β (Veldhoen et al., 2006). IFN-γ and IL-4 were also shown as a player in murine Th17 differentiation. Their absence in mice results in reduced RORC2 expression increased Foxp3 expression, loss of IL-17 production, (Brustle et al., 2007).

Another important issue in the culture condition is the specific medium for Th17 cells. IMDM (Iscove's Modified Dulbecco's Medium) is used as a medium for CD4+ T Cells. Basically the difference between DMEM (Dulbecco's Modified Eagle Medium) is having additional L-glutamine and HEPES. In order to get more efficient medium for culture, IMDM is treated with FBS (Fetal Bovine Serum), penicillin, streptomycin and finally β-mercaptoethanol is added.

1.5. Th17 Produces Cytokines and Function of Those Cytokines

Th17 Cells are also known as IL-17 producing cell lines. This is because that they express IL-17A and IL-17F cytokine and they express IL-22, IL-26, TNF and GM-CSF as effector cells. The activation IL-17 in Th17 cells are considered as a potential target for the cure of several disorders. Together with the inhibition of IL-17 in the expressing cells, there would be a treatment area in the several chronic inflammatory diseases such as; ankylosing spondylitis and psoriasis. (Miossec, 2012) Beside of IL-17, IL-21 and IL-22 have role in induction of tissue inflammation and effectors function of Th17 in different tissues. Transcription factors such as TRAF6, JNK, Erk1/2, p38, AP-1 and NF-κB have been implicated in IL-17 mediated signaling in a stimulation-dependent, tissue-specific manner (Korn, 2009).

The first receptor of IL-17 is IL-17RA but after all, IL-17RC is identified as another receptor for IL-17. The ligand of IL-17 (IL-17A and IL-17F) binds to its receptor complex which is composed of IL-RA and IL-17RC. Together with the activation of receptor, signaling leads to activate ACT1 protein that results in the activation of NF-kB. This activates IL-6 and IL-8 genes transcription (Miossec, 2012).

1.6. Apoptosis Definition and General Properties in Immune System

Apoptosis is known as programmed cell death that occurs normally during development and aging also known as homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease (Elmore et al, 2007).

In apoptosis, major players can be categorized into four different classes. One of them is Caspase family. The term caspases is derived from cysteine-dependent aspartate-specific proteases and those cascades of caspase are activated in most cases of apoptotic cell death. According to researches, there are twelve caspases that are found in humans subcategorized in two caspases subfamilies: initiator caspases are -2, -8, -9, -10 and effector caspases are -7, -6 and -3. The rest of caspases are identified as the inflammatory caspases for the reasons of structural (Labbe and Saleh, 2008).

The other class is Bcl-2 Family proteins. The members of Bcl-2 family are a group of crucial regulatory factors in apoptosis. Those can be pro-apoptotic or anti-apoptotic proteins examined in 1.7.3 Section in a detail (Jain et al., 2014).

The last two players are Tumor Necrosis Factor Receptor Superfamily and Adaptor Molecules. Extrinsic apoptosis signaling is mediated by the activation of "death receptors" (DRs) which belong to Tumor necrosis factor receptor (TNFR) superfamily. They transmit signals that come with binding of specific ligands (Jain et al., 2014).

Together with those major players, apoptosis can be triggered by different signaling pathways. One of them is extrinsic or death receptor activation pathway. Signaling is started by binding of ligands which can trigger the death receptors. The most well characterized ligands of these receptors are FasL, TNF-alpha, Apo3L, and Apo2L. Corresponding receptors are FasR, TNFR1, DR3, and DR4/DR5 (Ashkenazi et al., 2008). The other signaling includes intrinsic or mitochondrial pathway. This pathway is often activated in response to signals resulting from DNA damage, loss of cell-survival factors, or other types of severe cell stress. Together with mitochondrial outer membrane permeabilization (MOMP), mitochondrial death proteins consisting in cytochome c and Smac/DIABLO are released into cytosol. In addition to that, pro-apoptotic proteins are released from the mitochondria to activate caspase proteases and trigger apoptosis (Labbe and Saleh, 2008).

1.6.1. AICD (Activation- induced cell death) Mechanism in Th17 Cells

The balance of presence or absence of Th17 cells in the homeostasis is very critical for several immunologic or neurotically diseases. The proper elimination of Th17 cells from the environment is therefore crucial for immune homeostasis. That is why many recent studies focus on the apoptosis of Th17 cells. Activation-induced cell death (AICD) is the major mechanism in T cells apoptosis. This is also accepted kind of programmed cell death mechanism. After the activation through TCR signaling of naive CD4+ T cells, activated T cells perform their function against to pathogens then those cells must be eliminated in the circulation. Similar events take place in Th17 apoptosis condition. Th17 cells go to apoptosis just after activation by mainly mediated Fas/FasL pathways (Li et al., 2013).

1.6.2. Fas and FasL Mediated Apoptosis

Recent studies show that, Th17 cells express Fas and its ligand FasL after the TCR activation. Also it is indicated that, Th17 cells are very sensitive to agonistic anti-Fas-induced apoptosis without any reactivations (Zhang et al., 2008).

Fas is a type of cell surface receptor also known APO-1 or CD95 was originally discovered in the human tumor cell lines. Main known about Fas is that it can trigger the apoptotic cell death (Trauth et al., 1989). It is relieved that Fas is member of the tumor necrosis factor receptor (TNF-R) family, and its ligand is Fas ligand (FasL or CD95L)

The activation of Fas receptor is provided by binding of ligand (FasL) and this interaction can trigger the apoptosis. According to several studies, FasL is found as membrane-bound form (mFasL) and complemented antibodies in researches shows the interaction between receptor and ligands. The activation of Fas causes several intracellular events. The activation assembly "death inducing signaling complex" (DISC) consists of the aspartate specific cysteine protease, caspase-8, its adaptor or activator FADD and finally its modulator c-FLIP. The interaction between Fas and FADD has to have death domain (DD) and this DD is crucial for the proteolytic activity of caspase-8 which destines the cell to apoptosis. According to researches, in lymphoid cells and hepatocytes, this proteolytic activity of caspase-8 is essential then it results in access to critical proteolytic substrates. As a result of this section, it is important to note that, FasL and Fas signaling is very crucial for the elimination of unwanted cells in order to maintenance of homeostasis (Strasser et al., 2009).

1.6.3. Bcl-2 Family Proteins in Apoptosis

Bcl-2 family proteins are categorized into anti-apoptotic or pro-apoptotic effects in the cells. Mainly Bcl-2 and Bcl-xl proteins are known as anti-apoptotic proteins on the other hand Bax, Bak, Bim, Bad are known as pro-apoptotic proteins. Those members of Bcl-2 family have several conserved domain named BH domains that is important for the proper function proteins. Each member has different domains combination therefore they have different function in the apoptosis mechanism (Chao et al., 1998).

Beside of the brief information about the Bcl-2 family proteins, several articles shows that, anti-apoptotic Bcl-2 proteins are relevant with the rescuing T lymphocytes in survival threat conditions (Koenen, 2013). The main player of this situation is Bcl-2 protein. They state that, the rescuing mechanism by Bcl-2 protein is induced by the IL-7 signaling pathway. After the activation by IL-7 signaling, Bcl-2 causes the increase in several survival protein expression level and arrest the apoptosis mechanism (Akashi et al., 1997). Moreover in the Th17 cells, the switching mechanism between downregulation of Bcl-2 and induction of Bim is essential for the survival of Th17 cells. According to studies, several pro-apoptotic proteins (Bim and Bax) are increased and anti-apoptotic proteins (Bcl-2) are inhibited after the activation by TCR. In addition, STAT5 is the transcription factor that is activated by the IL7 signaling in Th17 cells and causes the expression of Bcl-2 as a survival protein. However, together with TCR activation, STAT5 activation is inhibited therefore Bcl-2 does not rescue Th17 anymore (Koenen, 2013).

1.6.4. IL-7 as a Survival Mechanism in Apoptosis of Th17 Cells

IL-7 was discovered in the induction of proliferation of murine pro-B cells (Namen, 1988). However, it is recently understood that IL-7 is more related with the T cells in human. Together with the loss of IL-7 receptor signaling, causes several reductions such as; thymic cellularity and T-cell development blockage together with depleting CD3, CD4 and CD8 precursor populations (Grabstein, 1993). According to this information, IL-7 signaling mechanism is required for the survival. Based on the Hofmeister et al, IL-7R signaling has a role in the induction Bcl-2 expression that is very important for the cell alive (Hofmeister, 1999). Basically IL-7 signaling mechanism is started by the binding of IL-7 to its receptor IL-7R and activates Jak-STAT pathway. This pathways regulates the family of Bcl-2 proteins which has own balance between pro-apoptotic and anti-apoptotic proteins. Beside of the influence on the Bcl-2 family protein, articles state that IL-7 survival mechanism do not have so much influence at Fas/FasL ligand pathway or p53 pathway in T cells (Kim, 1998).

In the Th17 Cells survival mechanism, IL-7 mediates the survival homeostasis. Bcl-2 family proteins also have a crucial role in the survival mechanism in Th17 cells. Together with the inhibition of IL-7 signaling in Th17 cells, it causes to increase in the

pro-apoptotic proteins and decrease in the anti-apoptotic proteins. Together with the binding of ligand to IL-7R, it activates STAT5 signaling pathway caused the increase of Bcl-2 protein level. Together with the T cell activation, this IL-7 survival mechanism is blocked through increasing pro-apoptotic proteins such as; Bim or Bax (Koenen, 2013). This is the point of the relationship between AICD and IL-7 survival mechanism, because together with the activation of T cells, apoptosis can be induced and IL-7 survival mechanism that provides maintenance of T cells in circulation is inhibited.

1.7. Th17 Cells in Disease

Th17 Cells are related with several chronic inflammatory diseases such as; Multiple Sclerosis, Crohn's disease and Rheumatoid arthritis.

Rheumatoid arthritis is most common type of chronic inflammatory disease and it leads to bone and cartilage destruction. Th17 cells were the first thing that is demonstrated with this disorder. Together with the inhibition of IL-17, the development and consequences of arthritis are protected. In addition, mice lacking IL-17RA shows very mild arthritis therefore IL-17 has role all different stage of chronic diseases (Miossec, 2012).

Multiple Sclerosis is characterized by brain inflammation and myelin destruction. Together with the TNF inhibition, the number and the activity of brain lesions are increased in the multiple sclerosis patients. On the other hand, the accumulation of cells that can produce IL-17 contributes to the pathogenesis of experimental autoimmune encephalomyelitis. Moreover, overexpression of IL-17 is found in the biopsy samples from brain with multiple sclerosis (Miossec, 2012).

In Crohn's disease which is an inflammatory bowel disease, TNF has also contribution together with the high expression of IL-17, IL-23, IL-22 and IL-6. This states that there are several therapeutic targets for this disease. In the bowel disease, IL-17 has both protective roles and pro-inflammatory roles (Miossec, 2012).

CHAPTER 2

MATERIAL AND METHODS

2.1. PBMC (Peripheral Blood Mononuclear Cells) Isolation

The whole blood samples were taken from healthy volunteers from Dokuz Eylül Hospital based on Ethical Committee. Ficoll – Hypaque Density Gradient Centrifugation (Boyum et al., 1991) was used for PBMC isolation.

2.2. PBMC Characterization by Flow Cytometer and Sample Selection

PBMC characterization by flow cytometer was done by surface staining BD Pharmingen Stain Protocol. In order to understand the cell types, different antibodies were used such as; CD4, CD45RA, CD45RO, CD25, CD69, ANNEXIN-V, 7AAD, PI and CD14. There are several fluorescence such as; PE, PECy7, Alexa 488, Alexa 647, PECy5, APC and APCCy7 conjugated those antibodies analyzed by flow cytometer. In order to get rid of the infected samples, samples were selected according to CD25 and CD69 activation markers and Annexin-5,7AAD, PI apoptosis markers. Also for naïve CD4+ isolation, samples that have ~25-30% CD45RA and ~ 30-35% CD4 were selected.

2.3. Naïve CD4+ Cells Isolation

Together with "Human Naive CD4+ T cell isolation Kit II (Miltenyi Biotec, Bergisch Gladbach)" naïve CD4+ T cells were isolated based on the negative selection manner with beads conjugated CD8, CD15, CD16, CD25, CD45RO, CD34, CD36 antibodies. There would be no selection for CD45RA (naïve cells marker) and CD4. Based on the cell number (10^8 cells for each experiment), sorting procedure was applied. At the beginning of the procedure, cells were centrifuged for 10 min at 300g at 4ºC and supernatants were taken. 400 µL of variomax buffer and 100 µL Naïve CD4+ T Cell Biotin-Antibody Coctail II were added for each 10^8 cells. Cells were incubated for

5 min at 4ºC. 300 µL of variomax buffer and 200 µL Naïve CD4+ T Cell Microbead Coctail II was added for each 10^8 cells. Cells were incubated for 10 min at 4ºC. After that, VarioMacs separation unit was used in order to get rid of unwanted antibodies-bound cells. This unit has extremely magnetic field power therefore cells can be separated. Naïve CD4+ T Cells were taken as flow through. 3 ml of variomax buffer is loaded into the column in order to increase the efficiency of the separation. Then column was taken from the VarioMacs separation unit. 5 ml variomax Buffer was added into the column and together with push power, depleted cells were collected.

2.4. Th17 Cells Culture

In order to culture Naïve CD4+ T Cells, IMDM (Lonza) with 5% FBS 2% Penicillin (100IU/mL) and Streptomycin (100ul/mL) and 0.1% β-mercaptoethanol was used. It was named as FULL IMDM. For differentiation from naïve CD4+ to Th17 cells, eight different stimulants were used. 5µg/ml anti-CD3 and 5µg/ml anti-CD28 (BD Biosciences) were used in order to activate cell through TCR. 10ng/ml IL-1β, 10ng/ml IL-23, 30ng/ml IL-6 and 0,5ng/ml TGFβ (eBioscience) were used for the differentiation to Th17 Cells. 10µg/ml anti-IFNγ and 10µg/ml anti-IL4 (eBioscience) were used for inhibition of differentiation the other subtypes of T cells such as; Th1, Th2 or Treg. Cultures were stopped according to expression level of IL-17 (7^{th} or 9^{th} days) and for every 5 day, cells were stimulated by 10ng/ml IL-23 in order to refresh the culture condition. In addition, in order to investigate the effects of IL-7 to the culture, additional 10µg/ml anti-IL7 (eBioscience) was added to the culture (Veldhoen et al., 2006).

Table 2.1. Culture conditions.

CYTOKINES	AMOUNT PER 1ml CULTURE
Anti-CD3	5μg/ml
Anti-CD8	5μg/ml
IL-1β	10ng/ml
IL-23	10ng/ml
IL-6	30ng/ml
TGFβ	0,5ng/ml
Anti-IFNγ	10μg/ml
Anti-IL4	10μg/ml
IL-7	10μg/ml

2.5. Th17 Characterization by Flow Cytometer

2.5.1. Cell Surface Staining

Cell surface staining by flow cytometer was done by BD Pharmingen Stain Protocol. In order to understand Th17 characterization, membrane surface antibody was used such as; CCR6 conjugated PECy7 fluorescence. For each samples, 50 μl of cells are treated with 10 μl fluorescence and incubated 15 min at 4ºC. After the incubation, cells were added 150 μl PBS then centrifuged for 10 min at 1500 rpm at 4ºC. After supernatants were removed, cells were analyzed by Flow Cytometer by adding 400 μl of PBS for each sample.

2.5.2. Intracellular Staining

Cells were stimulated for 4 hour with GolgiStop (BD Bioscience). 27 μl cells were stimulated with 3 μl GolgiStop. Membrane staining procedure was done for those cells surface markers. After centrifuge 1x 80 μl Fix/Perm Buffer was added to each well cell. Cells were incubated for 20 min at the room temperature in the dark. Cells were centrifuged for 5 min at the 300g. 80 μl 1X Perm was added then cells were centrifuged

for 5 min at the 300g. 80ul 1x Perm was added and cells incubated for 15 min at the room temperature in the dark. Cells were centrifuged for 5 min at the 300g. Together with 50 µl 1x Perm, µl intracellular cell marker antibodies were added then cells were incubated for 30 min at the room temperature in the dark. Cells were washed two times with PBS and cells were analyzed by flow Cytometer.

2.6.Determination Apoptosis Markers of Th17 Cells by FlowCytometer

Cells were stimulated for 4 hours with GolgiStop (BD Bioscience). 27 µl cells were stimulated with 3 µl GolgiStop. Membrane staining procedure was done for FAS, FASL, CD25, CD69 cell surface markers. After centrifugation, 1x 80 µl Fix/Perm Buffer was added to each well. Cells were incubated for 20 min at room temperature in the dark. Cells were centrifuged for 5 min at 300g. 80 µl 1X Perm was added then cells were centrifuged for 5 min at 300g. 80ul 1x Perm was added and cells were incubated for 15 min at the room temperature in the dark. Cells were centrifuged for 5 min at the 300g. Together with 50 µl 1x Perm, 5µl IL-17, Bcl-2, RORC2 antibodies were added then cells were incubated for 30 min at the room temperature in the dark. Cells were washed two times with PBS and cells were analyzed by flow Cytometer.

2.7. Determination Apoptosis Markers of Th17 Cell by Western Blotting

For Western Blotting, Th17 cells culture was stopped at either 7^{th} day or 9^{th} day of culture and cells were washed with 1X PBS. 400 µl 1X RIPA solution (CST) and 1:100 dilution of protease inhibitor cocktail (CST) were added into pellet. The total protein concentration was determined by BCA Protein Assay Kit (Pierce) Samples (10µg) were loaded for each well. Proteins were separated together with 10% SDS-PAGE. In order to determine the molecular weight of proteins, protein standard (NEB) was used. Proteins separated into gel were transferred onto PVDF (MILLIPORE) membrane. In order to get rid of non-specific binding of antibodies on to membrane, membrane were shaken with 5% of dry milk (CST-E PRODUCT), 1X Tris-Buffered Saline (TBS)(FISHER) and 0.1% Tween 20 (FISHER) for 1 hour at room temperature.

Membrane was incubated for overnight at 4ºC together with monoclonal primary antibody CASPASE-3, CASPASE-8 and CASPASE-9. Primary antibody solution was reserved in order to use again. Membrane was washed with TBS-T (1X TBS+0.1% Tween-20) solution for 5 times for 5 min. Membrane was incubated with secondary antibody (anti-mouse IgG-HRP conjugate, CST-E PRODUCT) in blocking solution (1X TBS+ 0.1% Tween-20 + 5% dry milk) for 1 hour at room temperature. After all, membrane was washed with 1X TBS-T for 3 times for 10 min at the shaker. Distilled water was used for washing for 2 times. Then membrane was subjected by membrane Chemiluminescent substrate (Thermo) solution for 3 min. Finally, membrane was visualized by BIO-RAD, VERSADOC 4000MP.

2.8. Cell counting

In order to count cells before and after naive CD4+ isolation, and also before Th17 culture, cells were subjected to tyrphan blue staining (90 µl for each 10 µl sample) then examined in hemocytometer. This determined live and death cells in the samples. In order to confirm the data in hemocytometer, Countess Automated Cell Counter (Invitrogen) was used. 10 µl tyrphan blue was used for each 10 µl samples.

2.9. Data analysis

Flow Cytometer result was analyzed by Attune Software. Western Blotting results were visualized by BIO-RAD, VERSADOC 4000MP. In order to make figures Excel and Power Point Office Program were used. For statistical analysis, standard two-tailed t test was performed. p values equal to or smaller than 0.05 were considered as significant. For Flow Cytometer analysis, each sample had three replicas.

CHAPTER 3

RESULTS

3.1. CD25 Activation and IL-17 Expression in CD4+ T Cells

Naïve CD4+ T cells were differentiated through to Th17 cells by expressing CD25 (activation marker) and then IL-17 (Th17 identification marker) under Th17 cell culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4). The interaction between CD25 activation and IL-17 producing were parallel to each other. CD25 activation and IL-17 expression were increased from 5^{th} day to 7^{th} day and reached the maxima level at the 7^{th}. Analyses were based on 20 different donors and approximately they had similar expression of CD25 and IL-17. At 5^{th} day, double IL-17+ CD25+ had average 30.2% of expression; on the other hand it had 41.0% of expression at 7^{th} day. There was no expression in the negative control.

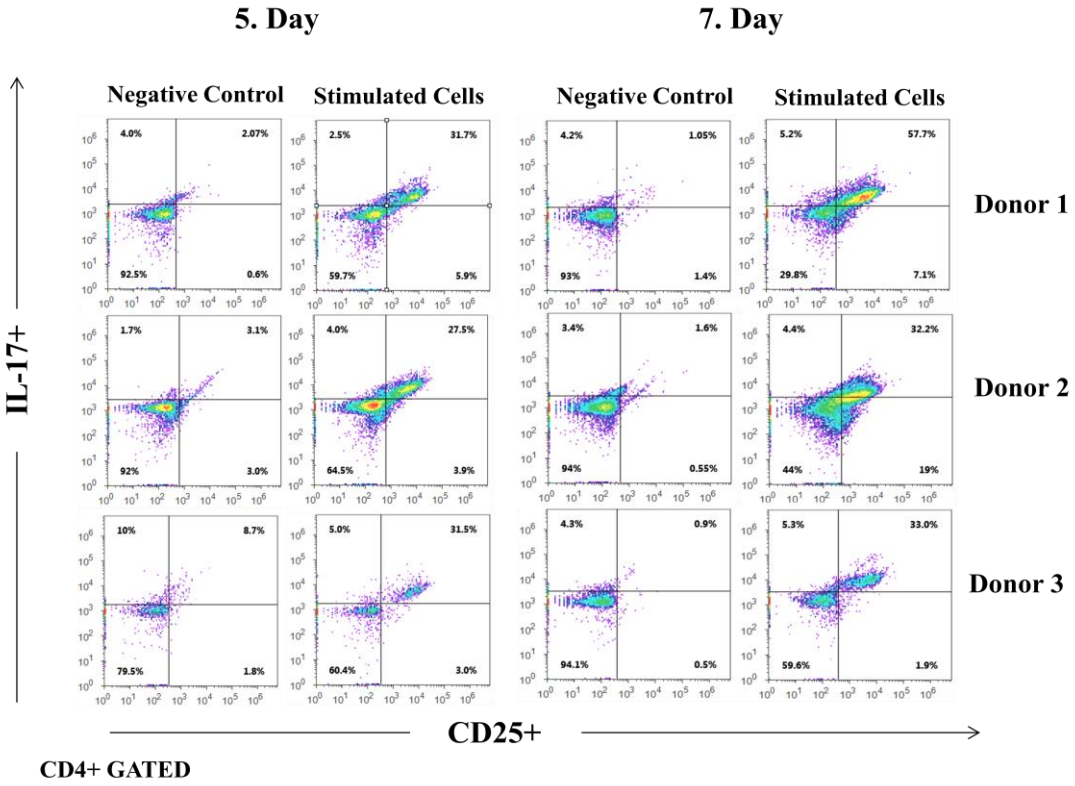

Figure 3.1. CD25 expression in Th17 Cells. CD25 activation and IL-17 expression are parallel to each other. Culture condition was based on different stimulants (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4). Culture was refreshed by adding IL-23 at 5th day. For intracellular staining cells were treated with Golgi Stop and incubated for 4 hours. Membrane surface staining was done for CD4 and CD25. After membrane staining, intracellular staining process was done by using fix and perm solution for IL-17 expression. 20 donor samples were analyzed by flow cytometer. As a negative control, just IMDM and cells were used.

3.2. The Expression of RORC2 Transcription Factor

RORC2 is the transcription factor for Th17 cells. Together with the Th17 culture condition, RORC2 expression is needed for IL-17 producing cells.

3.2.1. The Interaction between Activation and Expression of RORC2

Naïve CD4+ T cells were stimulated with Th17 culture stimulants. (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4). Stimulants activate the expression of RORC2 and it leads to activation of naïve CD4+ T cells to Th17 cells in 7 days. Activation was determined with the marker of CD25. Differentiated cells had both CD25 expression and RORC2 expression at from 5^{th} (13.2%) day to 7^{th} (30.7%) day by increasing.

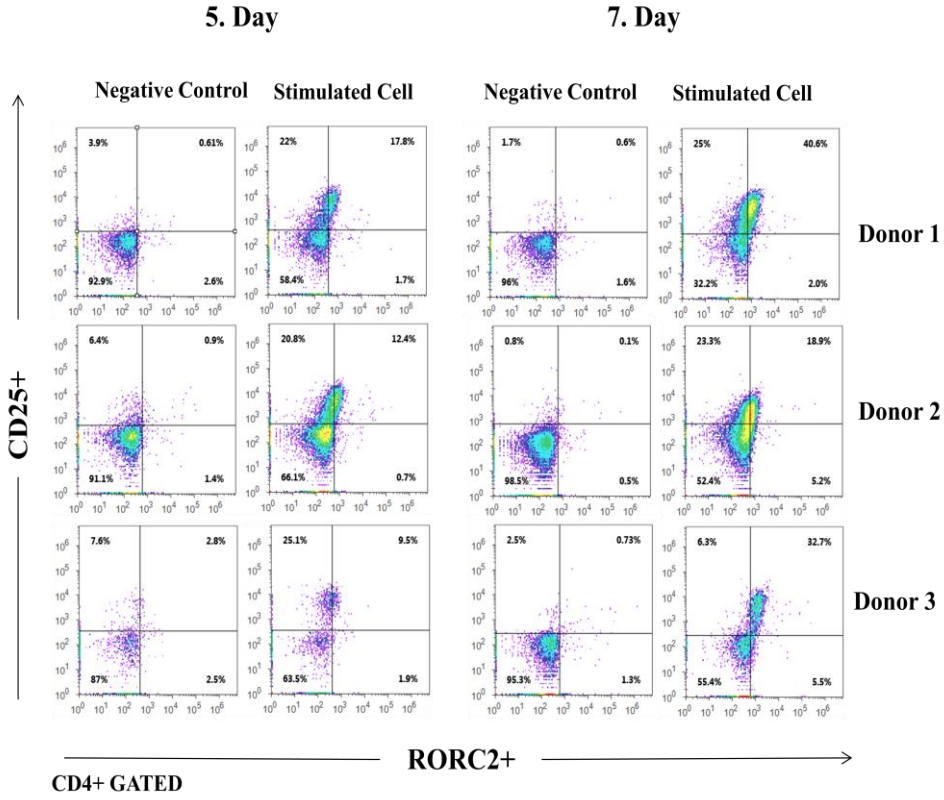

Figure 3.2. CD25 activation increased the expression of RORC2 transcription factor. Together with Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4), activation at 5th day gave rise the expression of RORC2 and the both expression was increased until 7th day. Culture was refreshed by adding IL-23 at 5th day. For intracellular staining cells were treated with Golgi Stop and incubated for 4 hours. Membrane surface staining was done for CD4 and CD25. After membrane staining, intracellular staining process was done by using fix and perm solution for RORC2 expression. Analyses were done by flow cytometer. As a negative control, just IMDM and cells were used.

3.2.2. The Interaction between IL-17 and RORC2

Naïve CD4+ T cells were cultured for 9 days with Th17 cell culture conditions (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4). With presence of Th17 differentiation stimulants, RORC2 transcription factor expression was started to increase from 3rd (4.5%) day. After 5 day, IL-17 expression was increased as parallel to RORC2 expression (10.2%). At the day of 7, double expression of RORC and IL-17 reached the maximum level (51.0%). Finally, their expression level decreased at 9th (0.30%). There was no expression in the negative control.

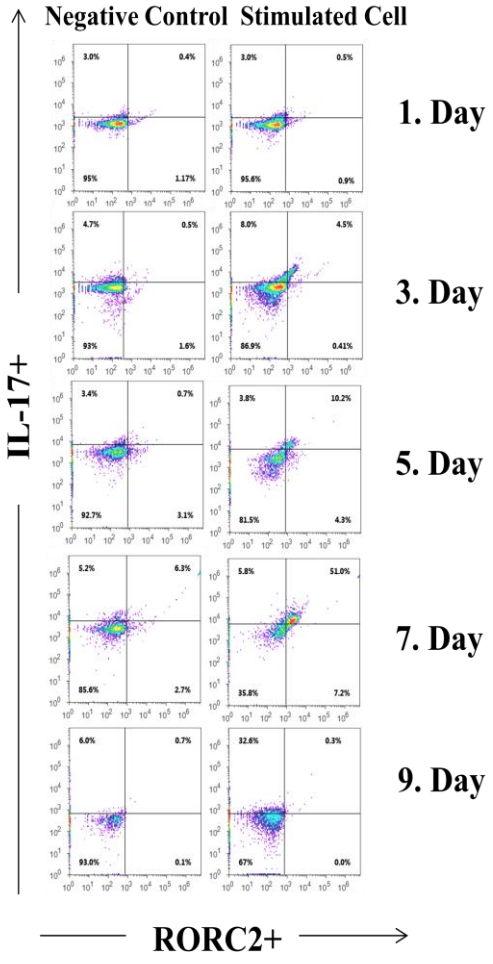

Figure 3.3. RORC2 expression increased the production of IL-17. In cell culture, RORC2 transcription factor expression was started at the day of 3 with Th17 stimulants (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) and gave rise the expression of IL-17 at the 5th day. At the 7th day, double expression reached the maximum level and started to decrease at the 9th day. Culture was refreshed by adding IL-23 at 5th day. For intracellular staining cells were treated with Golgi Stop and incubated for 4 hours. Membrane surface staining was done for CD4 and CD25 and CCR6. After membrane staining, intracellular staining process was done by using fix and perm solution for IL-17 and RORC2 expression. Analyses were done by flow cytometer. As a negative control, just IMDM and cells were used. Results based on CD4+ gated.

3.3. Apoptosis Is Induced in Activated T Cells

Apoptosis can be determined by the expression level of Fas and as well as Annexin-V in activated T cells and Th17 cells. Analyses were done by Flow Cytometer.

3.3.1. Fas Expression Is Increased Both Activated T Cells and IL-17 Producing Cells

Together with the activation, Fas expression was also induced and Fas expression level was increased parallel with activation throughout days. Double expression of FAS+ CD25+ was increased after day 3 and reached 19.8% at the 5th day. At the 7th day, cells had 27.3 % double expression. There was no expression in the negative control. Culture was based on Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4)

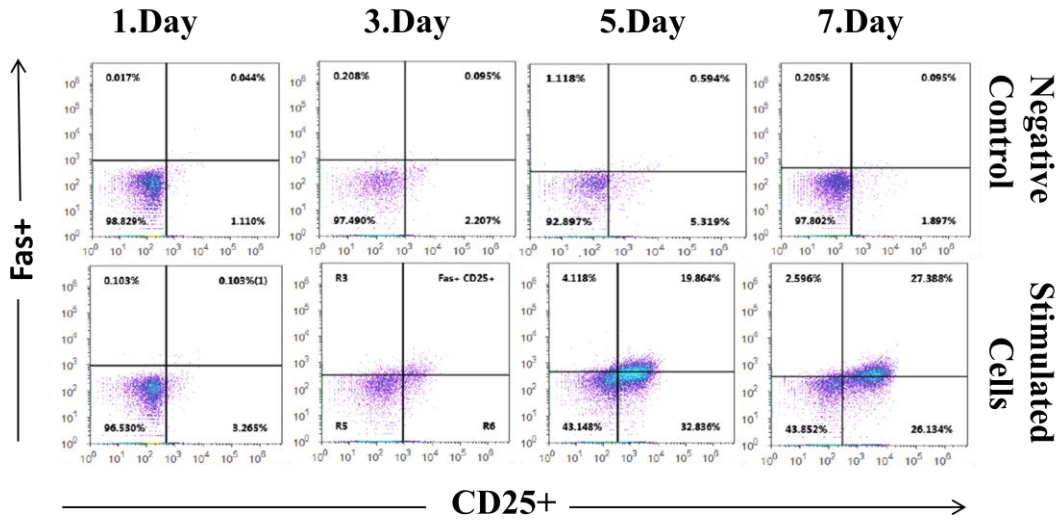

Figure 3.4. Fas expression was increased together with the activation. Fas expression was started at 3rd day as parallel to CD25 activation and according to ongoing day, double expression of CD25 and Fas were increased and reached the maximum level of 27.3%. Culture was refreshed by adding IL-23 at 5th day. Membrane surface staining was done for CD4, Fas and CD25. As a negative control, just IMDM and cells were used. Results based on CD4+ gated.

Together with the activation, IL-17 expression was induced. Like CD25 activation, Fas expression showed similar phenotypes together with expression of IL-17. At the 3rd day, only Fas expression was seen approximately 11.1% of culture. Double expression of FAS+ IL-17+ was seen at 5th (18.5%) and increased at 7th (25.6%) day. There was no expression in the negative control. Culture was based on Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4)

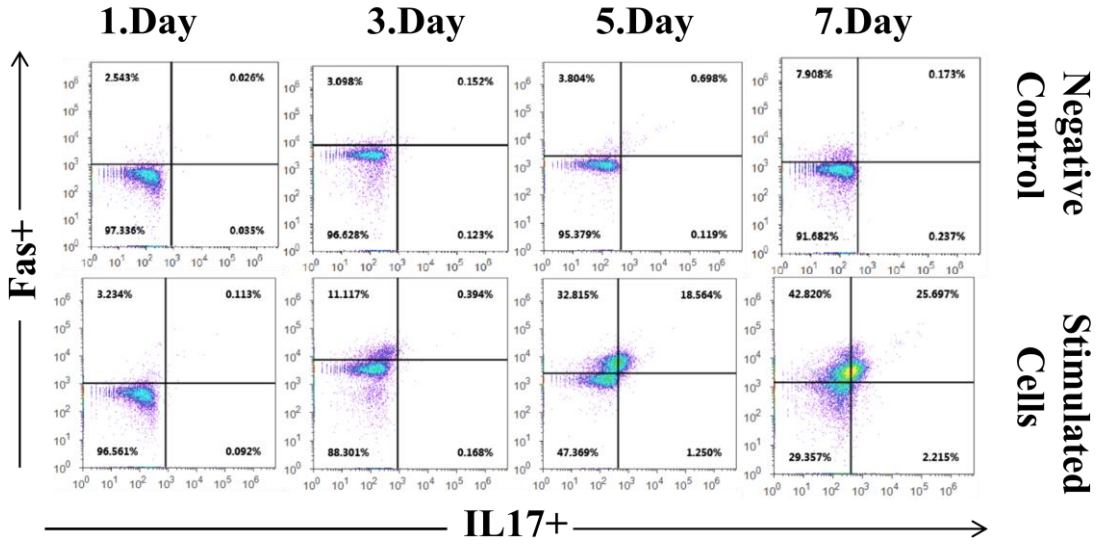

Figure 3.5. Fas expression in Th17 cells. Fas expression was just started at 3rd day and ongoing days, parallel increases were seen on IL-17 expression and Fas expression. At the day of 7, cells have 25.6 % double expression of Fas+ IL-17+. Culture was refreshed by adding IL-23 at 5th day. Membrane surface staining was done for CD4 and Fas. For intracellular staining cells were treated with Golgi Stop and incubated for 4 hours. Intracellular staining process was done by using fix and perm solution for IL-17 expression. As a negative control, just IMDM and cells were used. Results based on CD4+ gated.

3.3.2. Annexin-V Expression Starts After Fas Expression

Expression of Fas was started at the 3rd (12.8%) day of culture and increased day by day. 5th day- 33.4%, 7th day- 47.1%, 9th day- 56.6% However Annexin-V and Fas double positive cell were seen at the 9th (24.3%) day of culture. Naïve CD4+ cell were stimulated by Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) There was no expression in the negative control.

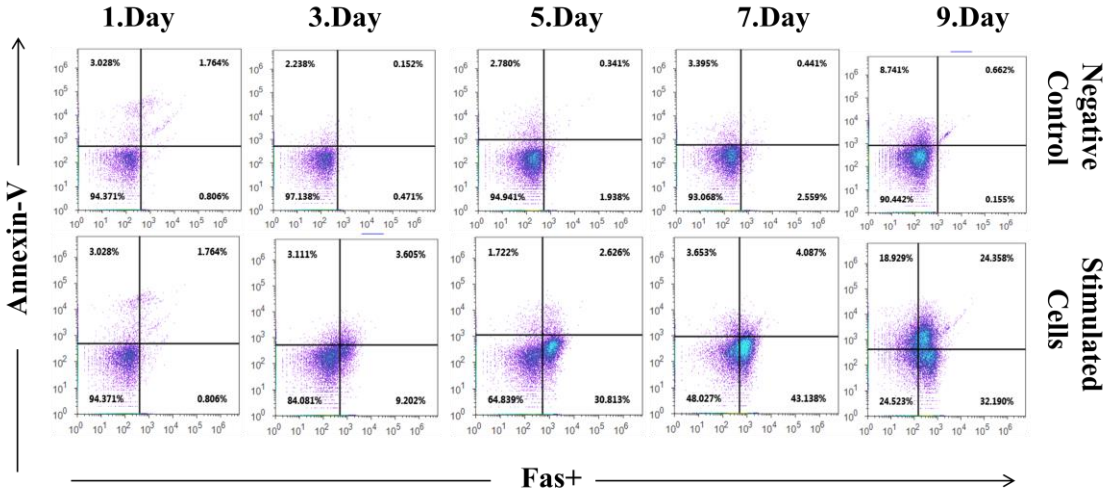

Figure 3.6. Fas expression induced Annexin-V expression at the 9th day of culture. Fas expression was started at 3rd day and increased day by day. Reaching to a huge level of Fas expression increased the expression of Annexin-V at the 9th day of culture. Double Annexin-V and Fas positive cells were reached 24.3% of culture. Culture was refreshed by adding IL-23 at 5th day. Membrane surface staining was done for CD4, Fas, CD25 and Annexin-V. As a negative control, just IMDM and cells were used. Results based on CD4+ gated.

3.4. Bcl-2 Expression Is Maintained During Th17 Differentiation and Expressions of Fas Receptor-FasL Are Independent From Bcl-2 Expression

During Th17 culture condition, there was no big difference in the expression of Bcl-2. Expression of Bcl-2 remained the same at almost 98.0 % of culture. (Figure 3.6.) However, CD4+ Bcl-2+ gated cells started to express Fas at the day of 3 and increase the expression level to almost 60.0 %. On the other hand, FasL expression was not seen so much until 7th of they and it reached 15.4 % expression level. (Figure 3.7.)

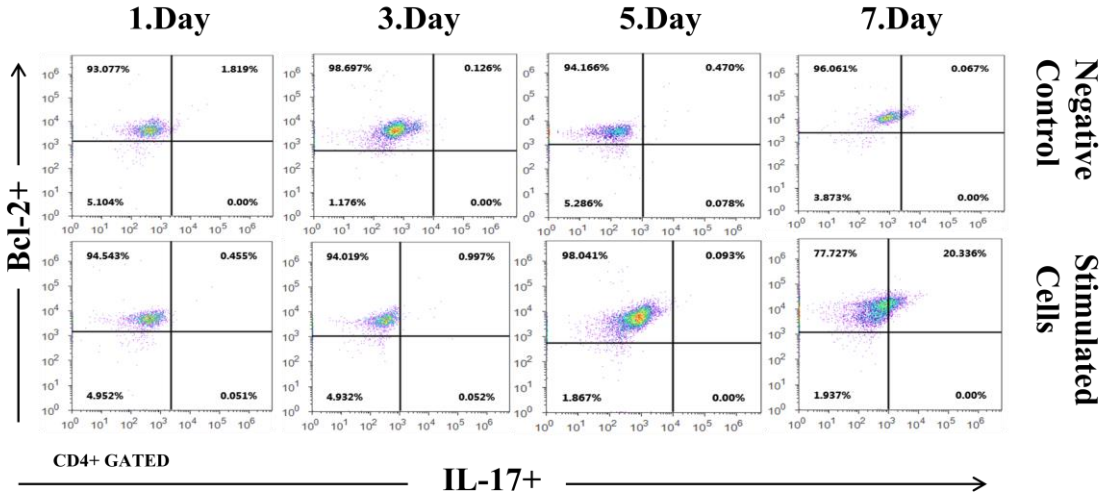

Figure 3.7. Bcl-2 expression remained the same during the Th17 differentiation. During the differentiation and apoptosis of T17 cell process, Bcl-2 expression had the same value around 98 percent of cells. Culture was refreshed by adding IL-23 at 5th day. Membrane surface staining was done for CD4. For intracellular staining cells were treated with Golgi Stop and incubated for 4 hours. Intracellular staining process was done by using fix and perm solution for Bcl-2 and IL-17 expression. As a negative control, just IMDM and cells were used. Results based on CD4+ gated.

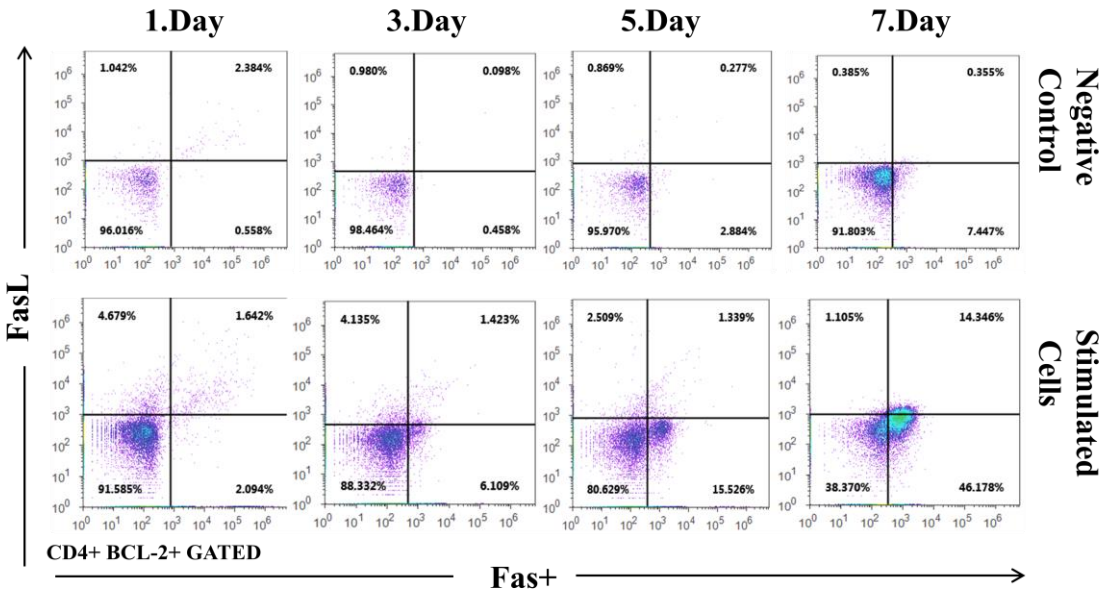

Figure 3.8. Activation-induced cell death is independent from Bcl-2 expression. While Fas expression was increasing from 3rd day of culture to 7th day of culture, there was no significantly difference in FasL expression until day 7. At 7th day of culture, Fas and FasL double positive expression reached 14.4 % of culture. Culture was refreshed by adding IL-23 at 5th day. Membrane surface staining was done for CD4, Fas and FasL. For intracellular staining cells were treated with Golgi Stop and incubated for 4 hours. Intracellular staining process was done by using fix and perm solution for Bcl-2 and IL-17 expression. As a negative control, just IMDM and cells were used. Results based on CD4+ Bcl-2+ gated.

3.5. Th17 Cells Express Caspase-8

Naïve CD4+ T Cells were stimulated with the Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) during 7 days of culture. Culture was stopped at the 7th day and checked the caspase-8 activity in stimulated cells and negative control (non-stimulated). Stimulated cells expressed two caspase-8 bands which include pro-caspase-8 (57 kDa) and activated caspase-8 (46 kDa). The other activated caspase-8 band was not seen (18 kDa). There was just pro-caspase-8 activity in negative control.

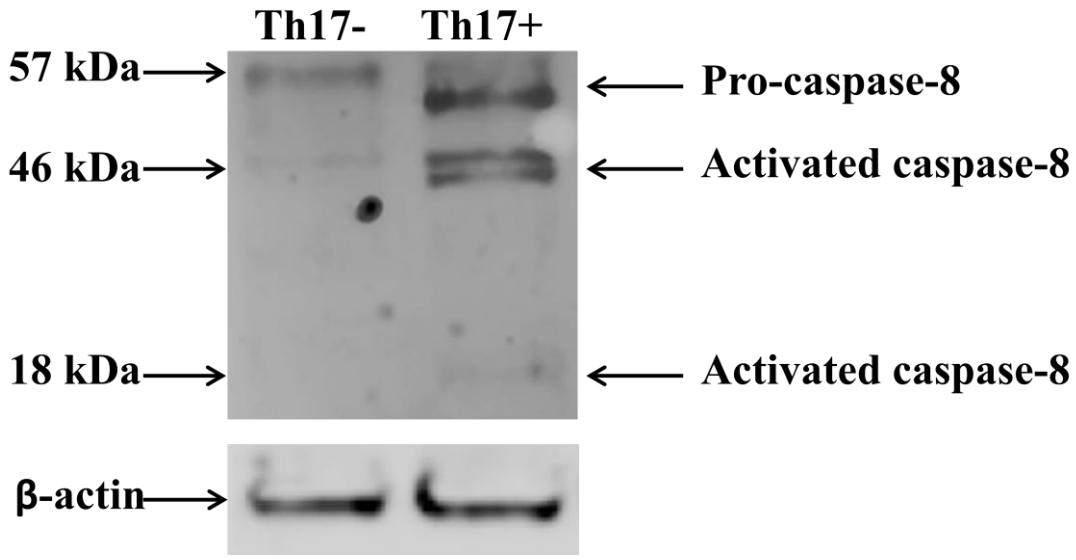

Figure 3.9. Caspase-8 was activated after the activation-induced cell death. Both stimulated cells and negative cells expressed pro-caspase-8. Stimulated cells also expressed activated caspase-8 at 46 kDa. Cultures were stopped at the end of 7th day and analyzed with western blotting. Western membrane was visualized by BIO-RAD, VERSADOC 4000MP.

3.6. The Expression of Caspase-9 Is Detected In Th17 Cells

Naïve CD4+ T Cells were stimulated with the Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) during 7 days of culture. Culture was stopped at the 7th day and checked the caspase-9 activity in stimulated cells and negative control (non-stimulated). Stimulated cells and negative control expressed pro-caspase-9 bands (50 kDa). There was no activated caspase-9 activity (35kDa) in negative control but in stimulated cells.

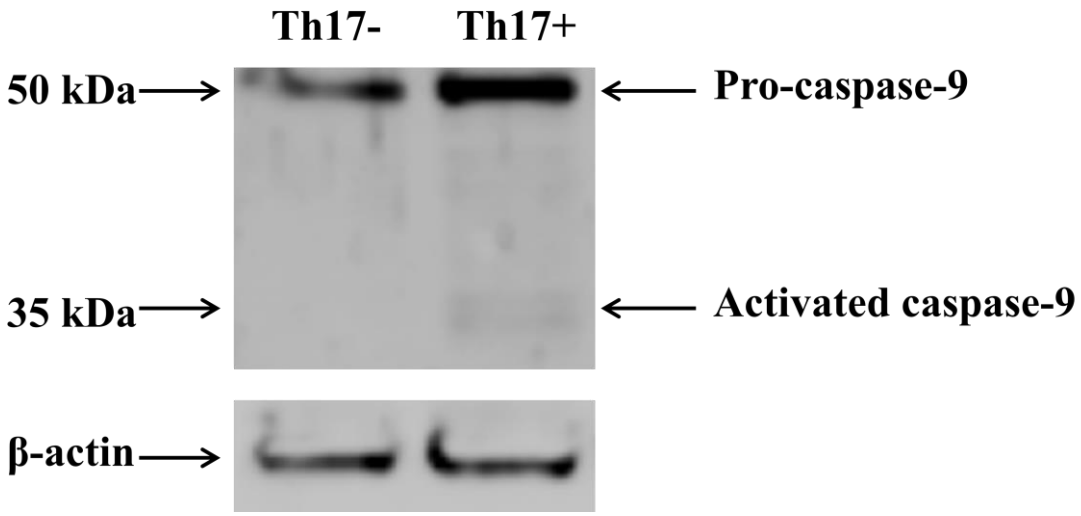

Figure 3.10. Caspase-9 expression was detected in Th17 apoptosis. Both stimulated cells and negative cells expressed pro-caspase-9. There was no expression of activated caspase-9 in negative control but in stimulated cells. Cultures were stopped at the end of 7th day and analyzed with western blotting. Western membrane was visualized by BIO-RAD, VERSADOC 4000MP.

3.7. Th17 Cells Express Caspase-3 as a Result of Activation-Induced Cell Death

Naïve CD4+ T Cells were stimulated with the Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) during 7 days of culture. Culture was stopped at the 7th day and checked the caspase-3 activity in stimulated cells and negative control (non-stimulated). Stimulated cells expressed three caspase-3 bands which include pro-caspase-3 (35 kDa) and activated caspase-3 (19 and 17 kDa), however 17 kDa band was weak. Stimulated cells expressed pro- caspase-3 and as well as activated caspase-3 at 19 kDa. There was no pro- and activated caspase-3 activity in negative control.

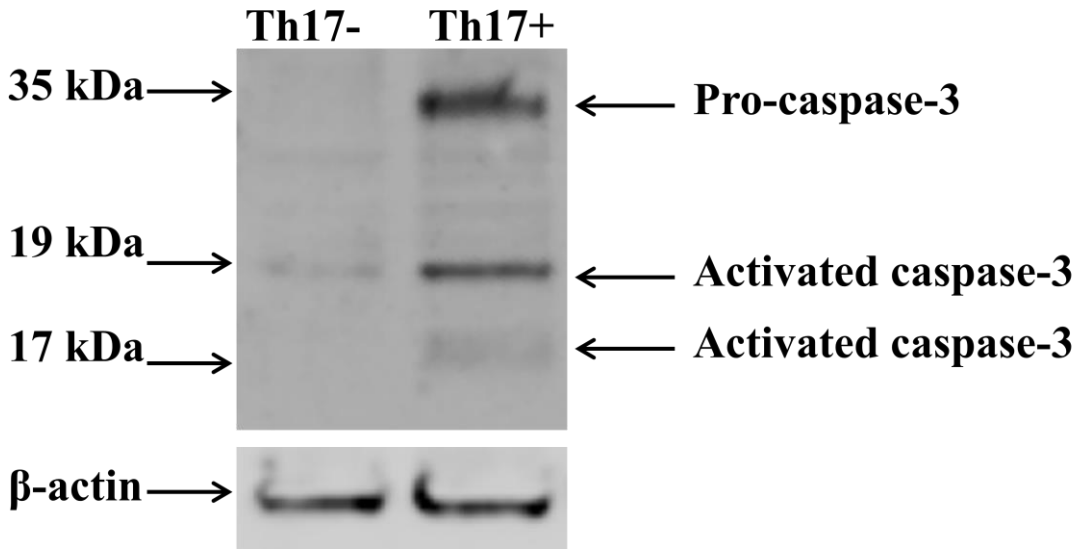

Figure 3.11. Caspase-3 was activated in Th17 apoptosis. Stimulated cells expressed pro-caspase-3 and activated caspase-3 (19kDa). There was no expression of pro- and activated caspase-3 in negative control. Cultures were stopped at the end of 7th day and analyzed with western blotting. Western membrane was visualized by BIO-RAD, VERSADOC 4000MP.

3.8. Addition of IL-7 Does Not Influence of Fas Expression

Naïve CD4+ T Cells were differentiated to Th17 cells with Th17 culture conditions (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) In order to understand the effect of IL-7, Th17 differentiation cultures were stimulated with IL-7 (1μg/ml). Stimulated cells and IL-7 culture had quite similar result in terms of Fas expression increased by AICD. At the 3rd day, only Fas expression was seen approximately 11.1% of stimulated cells and 14.6% of IL-7 culture. Double expression of Fas+ IL-17+ was seen at 5th day 18.5% of expression in stimulated cells and 16.8% of expression in IL-7 culture. There was increase both stimulated cells and IL-7 culture at 7th day, respectively 25.6% and 38.1%. There was no expression in negative control.

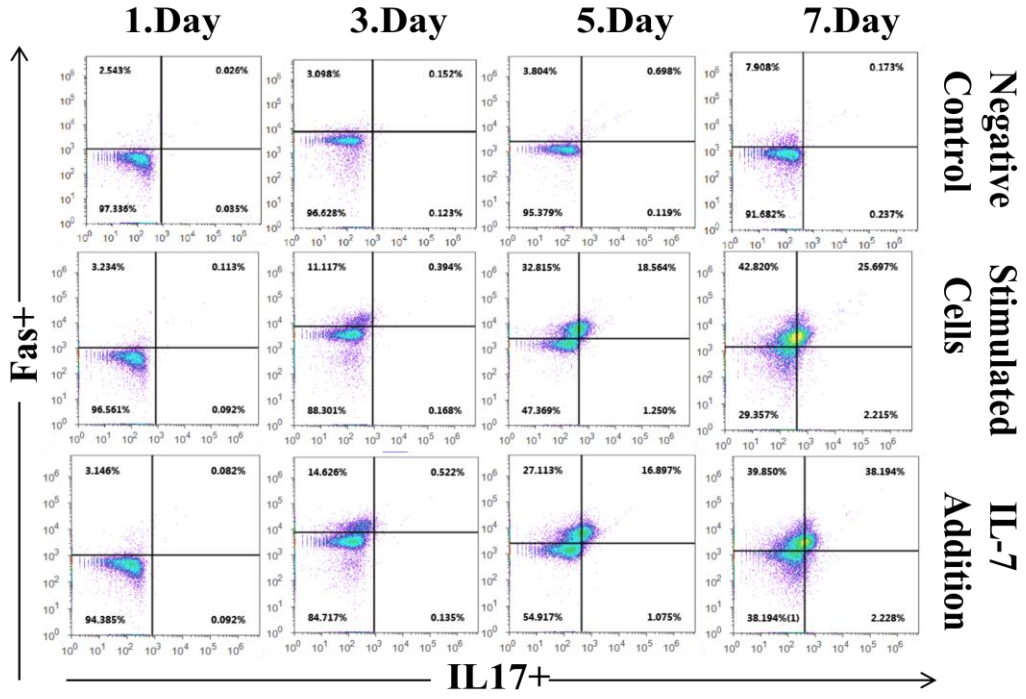

Figure 3.12. IL-7 did not affect the expression of Fas increased by AICD. Fas expression was just started at 3rd day in both stimulated cells and IL-7 culture (1μg/ml) and reached maxima level at 7th day for both cultures. Throughout days, parallel increases were seen on IL-17 expression in stimulated cells and IL-7 culture. Culture was refreshed by adding IL-23 at 5th day. Membrane surface staining was done for CD4 and Fas. For intracellular staining cells were treated with Golgi Stop and incubated for 4 hours. Intracellular staining process was done by using fix and perm solution for IL-17 expression. As a negative control, just IMDM and cells were used. Results based on CD4+ gated.

3.9. Bcl-2 Expression Level Remains the Same in the Presence of IL7

Bcl-2 expression was stabile during the Th17 differentiation and apoptosis. Adding of IL-7 did not make any difference in the level of expression of Bcl-2.

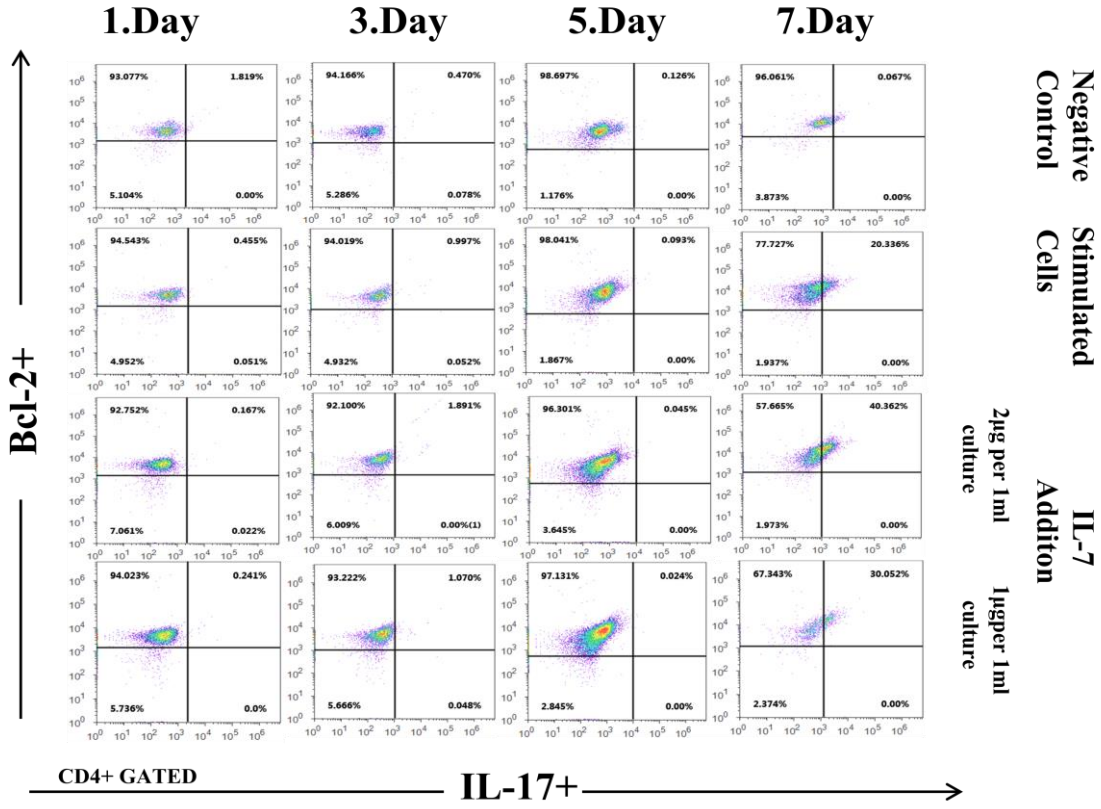

Figure 3.13. Bcl-2 expression level remained the same in treatment with IL-7. During the differentiation and apoptosis of Th17 cell process, Bcl-2 expression remained the same value around 98.0 percent of cells. Addition of IL-7 did not change the expression level of Bcl-2. Culture was refreshed by adding IL-23 at 5th day. Membrane surface staining was done for CD4. For intracellular staining cells were treated with Golgi Stop and incubated for 4 hours. Intracellular staining process was done by using fix and perm solution for Bcl-2 and IL-17 expression. As a negative control, just IMDM and cells were used. Results based on CD4+ gated.

3.10. Caspase-8 Is Expressed Despite IL-7 Addition

Naïve CD4+ T Cells were stimulated with the Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) during 7 days of culture. In addition, IL-7 was added to cell culture in two different amounts. (2μg/ml and 1μg/ml) Culture was stopped at the 7th day and checked the caspase-8 activity in stimulated cells, negative control (non-stimulated) and IL-7 added culture. Stimulated cells,IL-7(2μg/ml) and (1μg/ml) expressed two caspase-8 bands which include pro-caspase-8 (57 kDa) and activated caspase-8 (46 kDa). The other activated caspase-8 band was not seen or seen but so weak. (18 kDa). There was just weak pro-caspase-8 activity in negative control.

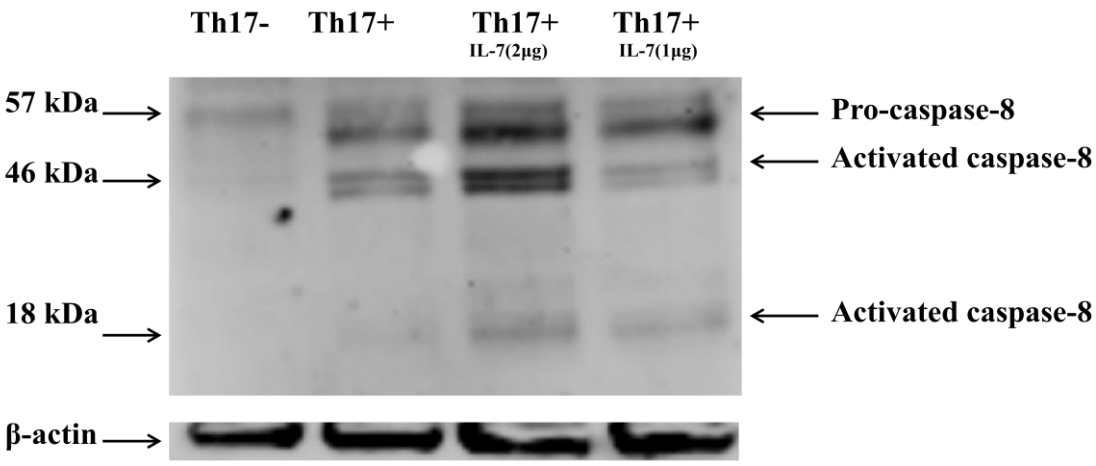

Figure 3.14. Caspase-8 was activated after the activation-induced cell death despite IL-7 addition. Stimulated cells, two different IL-7 amount cultures and negative control expressed pro-caspase-8. Stimulated cells and IL-7 added culture also expressed activated caspase-8 at 46 kDa. IL-7(2μg/ml) had more expression of pro-caspase-8 than IL-7(1μg/ml). Cultures were stopped at the end of 7th day and analyzed with western blotting. Western membrane was visualized by BIO-RAD, VERSADOC 4000MP.

3.11. Caspase-9 Expression Is Seen in Th17 Apoptosis Despite IL-7 Addition

Naïve CD4+ T Cells were stimulated with the Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) during 7 days of culture. In addition, IL-7 was added to cell culture in two different amounts. (2μg/ml and 1μg/ml). Culture was stopped at the 7th day and checked the caspase-9 activity in stimulated cells, negative control (non-stimulated) and IL-7 added culture. Stimulated cells, IL-7(2μg/ml) and (1μg/ml) expressed pro-caspase-9 bands (50 kDa). Negative control also expressed pro-caspase-9 but weaker. There was weak activated caspase-9 activity in stimulated cells however negative control did not have any expression. Moreover, IL-7(2μg/ml) and (1μg/ml) had activated- caspase-9 expression.

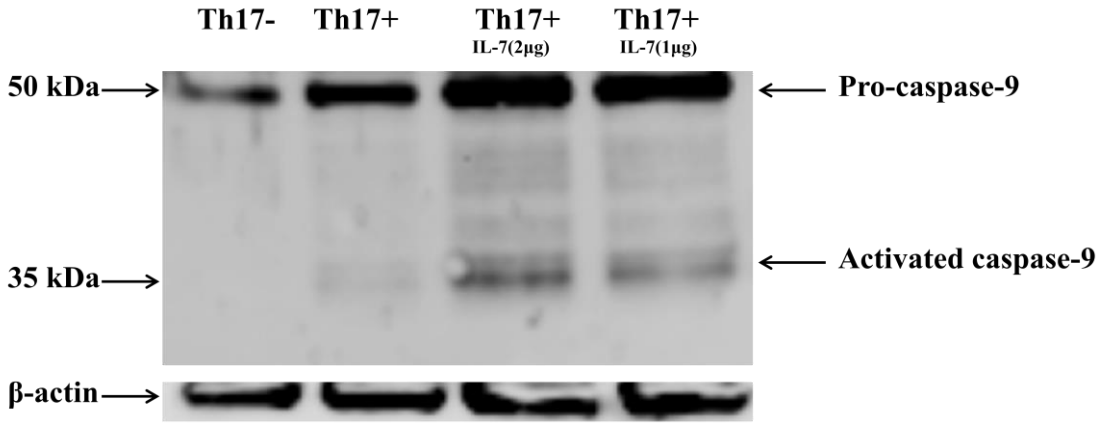

Figure 3.15. Caspase-9 expression was determined in Th17 despite of presence of IL-7. Both stimulated cells, IL-7(2μg/ml) and (1μg/ml) and negative cells expressed pro-caspase-9. There was no expression of activated caspase-9 in negative control. However, stimulated cells, IL-7(2μg/ml) and (1μg/ml) had activated- caspase-9 expression. Cultures were stopped at the end of 7th day and analyzed with western blotting. Western membrane was visualized by BIO-RAD, VERSADOC 4000MP.

3.12. Caspase-3 Is Activated as a Result of Activation-Induced Cell Death Despite IL-7 Addition

Naïve CD4+ T Cells were stimulated with the Th17 culture condition (anti-CD3, anti-CD28, TGF-β, IL-6, IL1-β, IL-23, INF-γ, IL-4) during 7 days of culture. In addition, IL-7 was added to cell culture in two different amounts. (2μg/ml and 1μg/ml). Culture was stopped at the 7th day and checked the caspase-3 activity in stimulated cells, negative control (non-stimulated) and IL-7 added cultures. Stimulated cells and IL-17 added culture expressed pro-caspase-3 band (35 kDa) and activated caspase-3 (19 kDa). 2μg/ml and 1μg/ml of IL-7 cultures had also 17 kDa bands but they were weak. There was no pro- and activated caspase-3 activity in negative control.

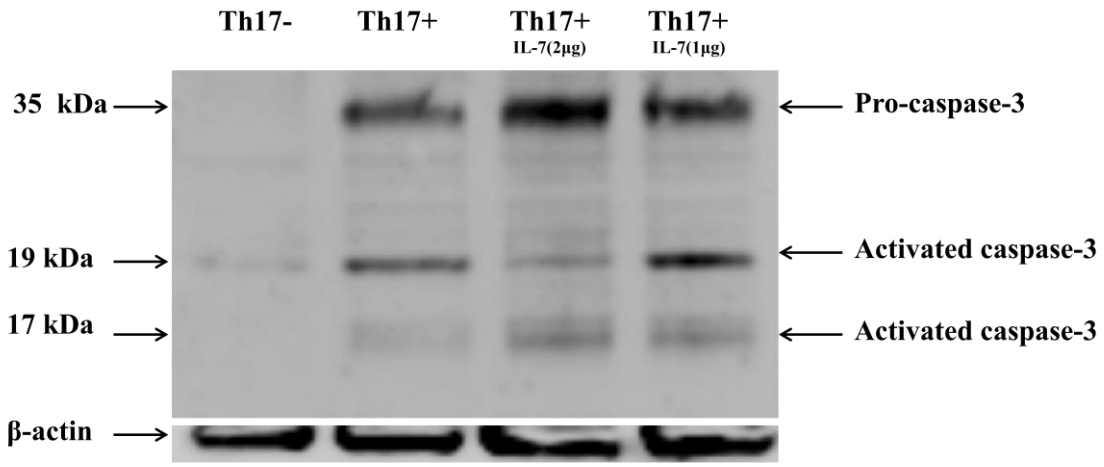

Figure 3.16. Caspase-3 was activated as a result of activation-induced cell death despite IL-7 addition. Stimulated cells, 2μg/ml and 1μg/ml of IL-7 culture expressed pro-caspase-3 and activated caspase-3 (19kDa). Stimulated cells, 2μg/ml and 1μg/ml of IL-7 cultures had also 17 kDa bands but they were weak. There was no expression of pro- and activated caspase-3 in negative control. Cultures were stopped at the end of 7th day and analyzed with western blotting. Western membrane was visualized by BIO-RAD, VERSADOC 4000MP.

CHAPTER 4

DISCUSSION

Th17 cells are essential for the immune system in order to response to pathogens and also very crucial for some diseases especially neuroimmunological ones (Miossec, 2012). Therefore investigation presence of Th17 in the homeostasis and its maintenance mechanism are so important. Activation-induced cell death is known apoptosis mechanism in T cells by inducing Fas signaling pathway however the steps of signaling pathway of AICD in Th17 cells are not known very well. (Li et al., 2013) On the other hand, IL-7 is known survival mechanism in T cells but the effects of IL-7 on AICD still is interesting (Koenen, 2013). In the light of the aims of this thesis, it was tried to understand the apoptosis of Th17 in terms of AICD and the effect of IL-7 on that mechanism.

Thesis focused firstly getting differentiated Th17 cells from naïve CD4+ T cells. There are different T helper cell types and some of them are similar to each other (Wilson et al., 2007). Therefore, blocking of differentiation of others cell types are essential for Th17 cell culture. There were eight different stimulants and most important ones are IL-6, TGF-β and IL-23. IL-23 is for proliferation and correlated effects of IL-6 and TGF-β is for differentiation of naïve CD4+ T cells to Th17 cells (Veldhoen et al., 2006). It is worth to mention that, Treg and Th17 cells were known same subtypes until these days, however, today Treg is known as Foxp3+ Cells and Foxp3 producing cells can be inhibited by adding IL-6 induced TGF-β in the culture condition.

After setting up the Th17 culture condition, the activation of naïve CD4+ T cells was determined and the expression of IL-17 was checked. As mentioned before, differentiation of naïve CD4+ T cells were occurred by the activation of TCR. Hence CD25 is very convenient for the determination of the TCR activation, CD25 was used. In the Figure 3.1., it was observed that there was a parallel increase in both CD25 activation and IL-17 expression from day of 5^{th} to day of 7^{th}. 41.0% of CD25+ IL-17+ double positive cells were reached. In order to confirm the presence of Th17 in the culture, we examined RORC2 which is the transcription factor of Th17 cells. First of all, we investigated the interaction between activation (expression of CD25). In the

Figure 3.2., we observed that CD25 activation increased the expression of RORC2. Previous figure, we stated that CD25 expression and IL-17 expression both are found at the same time. However, we observed that, CD25 activation came first and induce the RORC2 transcription factor. Therefore we aimed to examine both RORC2 and IL-17 expression. According to Figure 3.3, we observed that RORC2 expression increased the production of IL-17. Double positive RORC2 and IL-17 expression reached the maximum level at the 7^{th} day and decreased after day 9^{th}. This told us, the expression of transcription factor of RORC2 causes the increase of IL-17. Together with those data, we confirmed that CD25 and RORC2 were needed for the expression of IL-17 as well in literature (Yang et al., 2008).

Afterwards, apoptosis of Th17 cells was examined in terms of activation-induced cell death. According to researches, AICD is very crucial in the maintenance of T cells in the homeostasis. They stated that after activation of TCR, apoptosis is induced by Fas and FasL signaling (Li et al., 2013). Therefore we started to investigate Fas activation in activated Th17 cells. Firstly, we investigated the relationship between CD25 activation and Fas expression. According to Figure 3.4., we stated that, CD25 expression caused an increase of level of Fas expression. After 5^{th} day, both increases of CD25 and Fas were seen until 7^{th} day of culture. Then, we wondered whether there is a same interaction between Fas and IL-17 expression. IL-17 expression indicated that the differentiation of Th17 cells by activation and the presence of Fas proved the AICD mechanism. In the Figure 3.5.; we served the interaction between of Fas and IL-17. Together with differentiated to Th17 cells, activation triggered the expression of Fas that causes the apoptosis in Th17 cells by using mechanism of AICD. For both activated cells and Th17 cell culture, we saw the maximum level of Fas expression in the 7^{th} day of culture. On the other hand, until the 9^{th} culture, we did not observe any significant increase at the expression of Annexin-V. It is another marker of apoptosis and it refers the changing in the cell membrane and finally induces the apoptosis in a cell. According to our Figure 3.6., Fas expression induced Anenxin-V expression at the day of 9^{th}.This may be related with the timing of apoptosis events and Annexin-V activation maybe comes after the activation of Fas receptor.

Moreover, in order to understand the survival protein effect on activation-induced cell death mechanism, we chose Bcl-2 protein. Bcl-2 is known as pro-apoptotic protein and takes place both in extrinsic and intrinsic apoptosis signaling (Koenen, 2013). As it is dictated in Chapter 3.4., Bcl-2 expression was maintained during the

differentiation and apoptosis of Th17 cells. While Fas expression was increasing, there was no change in the expression level of Bcl-2. Therefore we conclude that, Bcl-2 expression was stabile during the activation and Fas expression is independent from Bcl-2 protein level.

According to literature, activation Fas receptor triggers the activation of caspase-8 from pro-caspase-8 by its cleavage (Strasser et al., 2009). We aimed to reveal the steps of activation-induced cell death mechanism in Th17 cells therefore we analyzed the expression level of caspase-8. Both stimulated cells and negative cells expressed pro-caspase-8. Stimulated cells also expressed activated caspase-8 at 46 kDa in Figure 3.9. This means that, Fas expression upon activation caused the cleavage of pro-caspase-8 and increased the level of activated caspase-8. Caspase-9 has a role in the mitochondrial apoptosis in general hence we examined whether there is a relationship between mitochondria disruption and AICD. According to figure 3.10., there was weak activated caspase-9 expression in Th17 cells. We showed that, Bcl-2 expression level was stable and activated caspase-9 was so weak. Therefore we concluded that AICD steps do not include mitochondrial events. Based on known information, caspase-3 is late apoptotic marker and activated caspase-8 causes the increase in level of activated caspase-3. Stimulated cells expressed pro-caspase-3 and activated caspase-3 (19kDa) in Figure 3.11. There was no expression of pro- and activated caspase-3 in negative control. Absent of pro-caspase-3 in negative showed pro-caspase-3 stabilization of Th17 differentiated cells.

In the light of the apoptosis events in Th17 cells, we wanted to investigate the role of IL-7 as a survival signaling. Based on the literature, we knew that, IL-7 causes the stability of Bcl-2 expression and inhibits the pro-apoptotic proteins (Hofmeister, 1999). Therefore, we wanted to investigate the interaction between Fas expression and IL-7 survival so we added different amount of IL-7 to our Th17 Cell cultures and monitored the interaction. According to Figure 3.12., IL-7 did not affect the expression of Fas increased by AICD. According to Koenen et al, they state that, TCR activation block the IL-7 signaling caused Bcl-2 expression. However, adding more IL-7 to the culture do not interfere the AICD mechanism. As figure 3.7, we observed Bcl-2 expression in IL-7 cultures and saw that adding of IL-7 to the Th17 cells culture did not change the expression level of Bcl-2 in Figure 3.13.

Finally, we wanted to investigate caspase activity together with western blotting analysis for cell cultures with treatment IL-7. Stimulated cells and IL-7 added cultures

expressed two caspase-8 bands which include pro-caspase-8 (57 kDa) and activated caspase-8 (46 kDa) in Figure 3.14. The other activated caspase-8 band was not seen or seen but so weak.(18 kDa). This resulted that, adding of IL-7 to the culture did not interfere the cleavage of pro-caspase-8 to activated caspase-8. Interestingly, in terms of caspase-9, we expected similar result with stimulated cells however, adding IL-7 to the culture trigger the activation of caspase-9. (Figure 3.15) Pro-caspase-3 was seen both IL-7 addition culture and as well as in Th17 culture means that IL-7 did not influence the pro-caspase-3 stabilization in apoptosis. On the other hand, activated caspase-3 was seen in Th17 cell culture, but also in the IL-7 addition culture. (Figure 3.16)

CHAPTER 5

CONCLUSION

The investigation of Th17 cells apoptosis is very crucial for maintenance of homeostasis in disease also in neuronal systems. According to our researches throughout whole thesis, Fas signaling by activation-induced cell death is crucial for the apoptosis result in the activation of early apoptotic marker caspase-8 and late apoptotic marker of caspse-3. Together with these steps we can say that, In the Th17 cells apoptosis, mainly extrinsic apoptotic pathway is used by activation of Fas receptor and respectively activation of caspase-8 and -3. AICD is independent from Bcl-2 expression in Th17 cells and probably there is no interaction between mitochondria and AICD in Th17 cells because of waek caspase-9 expression. (Figure 5.1.) Additional IL-7 to the culture did not any affect to block AICD as expected. IL-7 signaling is known as a survival mechanism in Th17 cells that induces Bcl-2 expression however, still further analysis are needed in order to understand the exact interaction between IL-7 and AICD because although Th17 cells have Bcl-2 activity, they have also Fas expression that causes AICD in cells. (Figure 5.2.) Also according to Young et al., Stat3 and Stat5 transcription factor s are essential for the differentiation of Th17 cells as well as survival mechanism. The investigation the relationship between IL-7 and STAT proteins is essential for the future experiments.

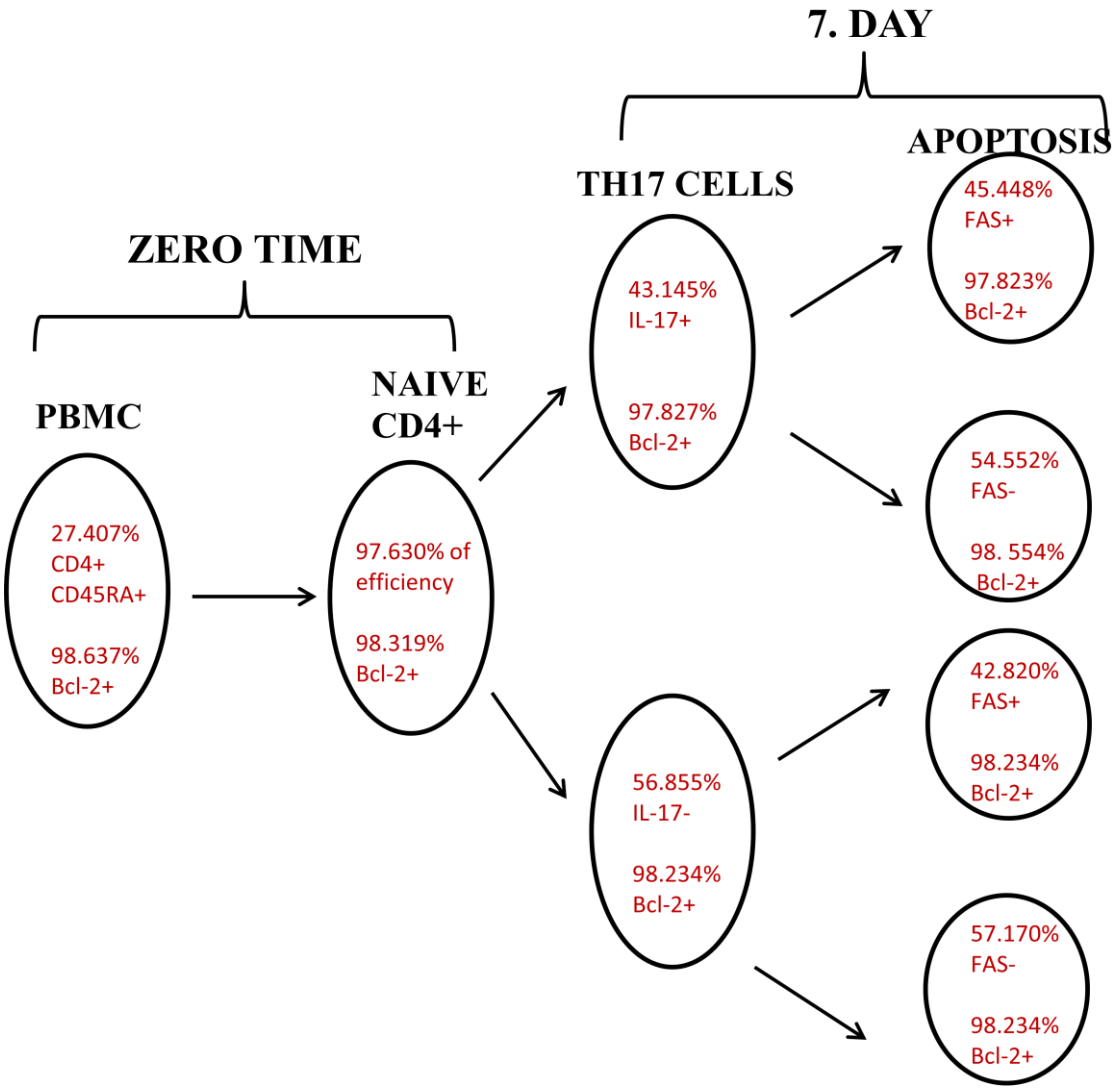

Figure 5.1. Overall view of Bcl-2 expression during differentiation and apoptosis of Th17 cells

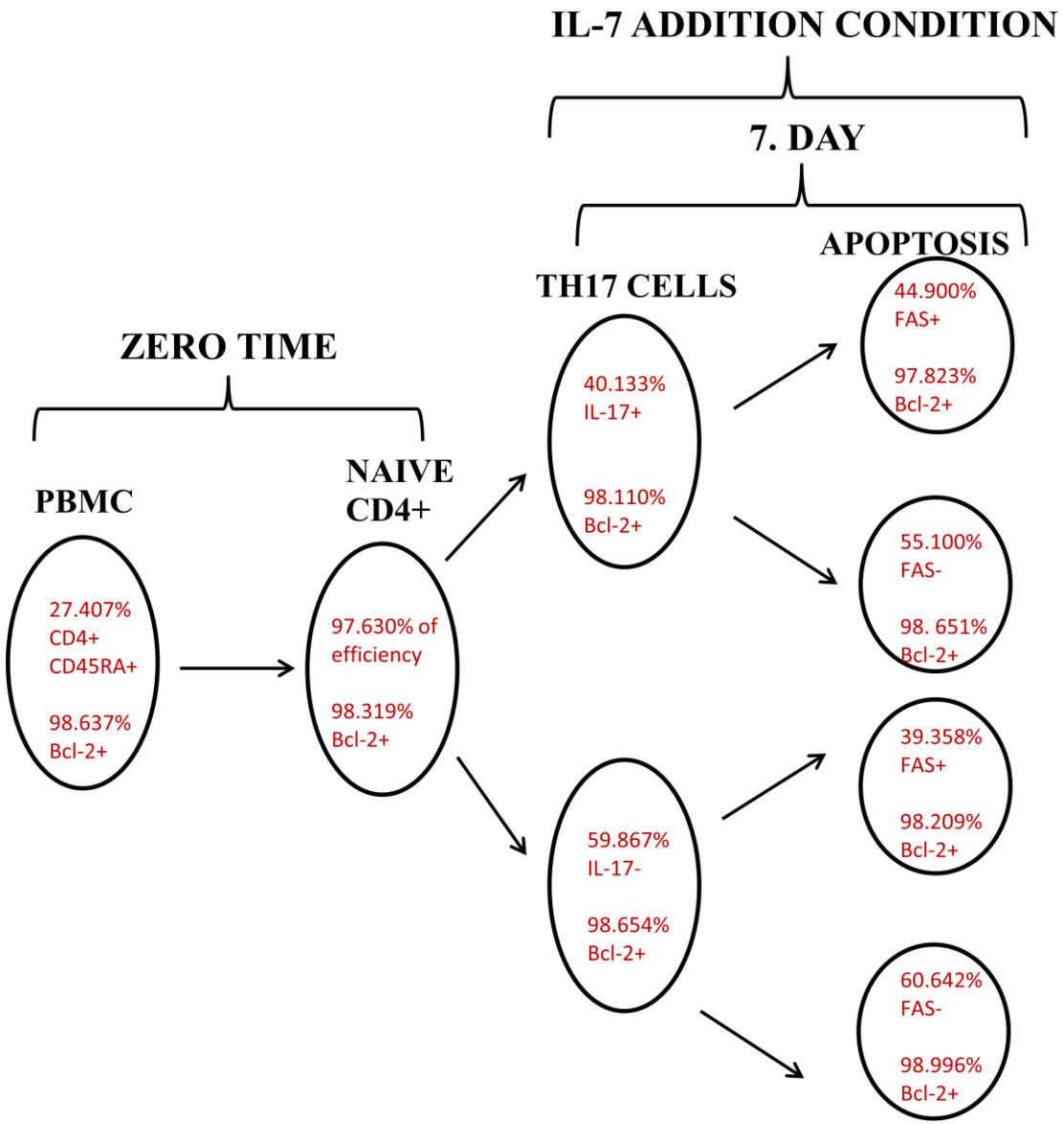

Figure 5.2. Overall view of Bcl-2 expression during differentiation and apoptosis of Th17 cells in presence of IL-7

DONOR NAME	%CD4+ CD45RA+ in PBMC	Efficiency of naive CD4+	CD4 (7.Day)	CD25 (7.Day)	IL-17 (7. Day)	Fas (7.Day)	Bcl-2 at PBMC	Bcl-2 in naive CD4+	Bcl-2 (7. Day)
DONOR 1	21.427	98.549	97.114	61.405	56.314	41.444	98.678	98.112	97.543
DONOR 2	12.495	96.912	95.961	44.504	50.532	55.431	98.987	98.876	98.433
DONOR 3	36.221	98.321	98.047	42.598	51.865	25.615	98.432	97.432	96.745
DONOR 4	20.151	98.021	94.032	19.300	25.224	44.211	97.432	98.543	98.765
DONOR 5	12.908	97.654	82.362	29.815	39.369	60.541	99.654	98.632	97.651
DONOR 6	28.904	98.114	87.071	33.374	30.233				
DONOR 7	30.804	95.765	86.223	42.226	57.071				
DONOR 8	27.018	96.884	78.081	38.812	30.303				
DONOR 9	39.567	98.081	88.320	43.318	49.567				
DONOR 10	32.464	97.999	89.028	47.089	40.967				
DONOR 11	39.464								
Average	27.402	97.630	89.624	40.244	43.145	45.448	98.637	98.319	97.827

Table 2. Overview of Quantitative Data of Th17 Differentiation and Apoptosis.

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