INVESTIGATION OF FAS/FAS-LIGAND INTERACTION IN HELPER T 17 CELL FUNCTIONS

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ABSTRACT

INVESTIGATION OF FAS/FAS-LIGAND INTERACTION IN HELPER T 17 CELL FUNCTIONS

Th17 cells are key players of the adaptive immune system. They mainly take part in responses to extracellular parasites and neutrophil recruitment. They can infiltrate into the inflammation sites and survive for long periods of time. Their malfunction leads to the manifestation of several autoimmune diseases, such as Multiple Sclerosis and Rheumatoid Arthritis. The main reason behind their roles in autoimmune pathogenicity is thought to be their longevity and resistance to apoptosis. One of the main players of apoptosis, Fas, has been found to have non-apoptotic roles and is a candidate for the survival mechanisms of Th17 cells. This study aims to discover possible non-apoptotic roles of the Fas signaling pathway in Th17 cell functions. For this purpose, buffy coats of healthy individuals were used to isolate PBMCs and $CD4^{+}CD45RA^{+}$ naive T cells were sorted from the PBMCs. Obtained naive T cells were cultured under Th17 polarizing conditions and the expressions of Fas, FasL, TNFR1, and TNF-α have been monitored along with apoptosis. The expression of Fas has been found to significantly increase in the cells cultured under Th17 polarizing conditions. However, there were no FasL and TNFR1 expressions observed. The expression of TNF-α was observed on both the negative culture and Th17 polarizing culture, however, there was no significant difference found. In addition, there was no increase in apoptosis in neither culture. In summary, Fas expression has been found to increase in the cells cultured under Th17 polarizing conditions. Further investigation of possible survival mechanisms, such as NFkB, in these cells can shed light on the effects of the Fas signaling pathway on the longevity of Th17 cells.

ÖZET

YARDIMCI T 17 HÜCRE FONKSİYONLARINDA FAS/FAS-LİGAND ETKİLEŞİMİNİN ARAŞTIRILMASI

Th17 hücreleri edinilmiş bağışıklık sisteminde önemli rollere sahiplerdir. Bu hücrelerin ana rolleri hücredışı parazitlerine karşı bağışıklık tepkisi göstermek ve nötrofil hücrelerinin enfeksiyon alanlarına getirilmesini ve tepkilerinin yönetilmesidir. Ayrıca, bu hücreler enfeksiyon alanlarına girebilirler ve uzun süre hayatta kalabilirler. Ancak bu hücreler, çeşitli otoimmün hastalıkların gerçekleşmesinde de rol alabilmektedirler. Bunun sebeplerinden birisi olarak Th17 hücrelerinin uzun ömürlü olmaları mümkündür. Hücre ölümündeki ana elemanlardan birisi olan Fas'in hücre ölümü dışındaki hücresel olaylarda da rol alabildiği son zamanlarda ortaya çıkmıştır ve Fas, Th17 hücrelerinin uzun ömürlülüklerinde rol alması muhtemel adaylardan birisi olarak değerlendirilmektedir. Bunu incelemek için sağlıklı bağıltılardan alınan kanlardan PBMC hücreleri izole edilmiştir ve bu PBMC hücrelerinden $CD4^{+}CD45RA^{+}$ naif T hücreleri ayrıştırılmıştır. Bu hücreler, Th17 fenotipine itilecekleri kültür koşullarında yetiştirilmişlerdir. Kültür süresinde bu hücrelerde Fas, FasL, TNFR1 ve TNF-α ifadeleri ile birlikte hücre ölümü incelenmiştir. İncelemeler sonucunda Th17 kültür koşullarında yetiştirilen hücrelerde Fas ifadesinin önemli bir artış gösterdiği gözlemlenmiş, FasL ve TNFR1 ifade etmedikleri görülmüştür. Ayrıca, TNF-α ifadesinin hem Th17 kültür koşullarında yetiştirilen hücrelerde hem de negatif kontrol hücrelerinde ifade edildiği, ancak herhangi bir istatistiksel fark olmadığı görülmüştür. Fas üretiminin arttığı hücrelerde herhangi bir hücre ölümü artışı gözlemlenmemiştir. Th17 hücrelerindeki uzun ömürlülüğün muhtemel mekanizmalarının daha derinlemesine araştırılması için Th17 hücrelerinde NFkB gibi hayatta kalım mekanizmalarının incelenmesi ileriye dönük araştırmalar için faydalı olacaktır.

"Even if the open windows of science at first make us shiver after the cozy indoor warmth of traditional humanizing myths, in the end the fresh air brings vigor, and the great spaces have a splendor of their own."

— *Bertrand Russell, What I Believe*

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CHAPTER 1

INTRODUCTION

1.1 T Lymphocytes

T lymphocytes are key players of adaptive immunity. Overall the roles of T lymphocytes are to kill host cells that have been infected or became tumor cells, regulate the immune response by taking part in the activation of other immune cells and producing cytokines for a properly coordinated defense against pathogens. Each T lymphocyte will respond to a specific antigen. The specificity comes from the T Cell Receptor (TCR) that has α and β chains on the antigen-binding site. The α and β chains are generated by somatic V(D)J recombination which results in unique formations that are specific for an antigen. However, TCR is also accompanied by an adaptor protein, CD3, since the cytoplasmic tail of TCR is too short to transduce any signals. CD3 contains the motifs required for the transduction of the activation signals downstream of TCR. However, even though it is found on all T lymphocytes, CD3 is not the only co-stimulatory molecule that is found on T lymphocytes. (Abbas & Lichtman, 2016; Murphy & Weaver, 2017)

There are two main types of T lymphocytes; CD4+ T cells and CD8+ T cells. At the beginning of their development in the thymus, T cells are termed as double-negative (DN) as they express neither CD4 nor CD8 molecules. These cells interact with the thymus stromal cells and begin their development. These interactions lead to changes in the expression of several molecules and rearrangements in ß and α chains of the T cell receptor (TCR), respectively. After the rearrangement of the α chain is completed, these cells will express both the CD4 and CD8 molecules and will become double-positive (DP) cells. DPs are exposed to self-antigens. This exposure aims to select the cells that have form proper major histocompatibility complexes and the DP cells that fail to form a strong bind are eliminated via apoptosis. Following this selection comes another selection that aims to eliminate the T cells that interact with self-antigens strongly. This negative selection is very crucial because failure in this process will lead to autoimmune diseases. The DP cells that survive the negative selection will down-regulate either CD4 (helper T

cells) or CD8 (cytotoxic T cells) molecules to become single positive and leave the thymus to circulate the peripheral blood. (Abbas & Lichtman, 2016; Murphy & Weaver, 2017; J. E. Park et al., 2020)

1.2 T Helper T Cells

Helper T cells are a subset of T cells that are responsible for the regulation of the innate immune response and antibody production. These cells are characterized by their CD4 surface marker, which is a co-receptor and it helps with the communication with the antigen-presenting cells (Geginat et al., 2013). These cells need to be activated and become effector cells to do their work. They require the presentation of appropriate antigen through TCR-MHC Class II interactions and co-stimulations. The cytokines in the environment direct the activated helper T cells into one of many subtypes like Th1, Th2, Th17. Each subtype of helper T cell expresses a specific set of cytokines that define the phenotype and role of that subtype. For example, Th1 cells express IFN-γ and Th2 cells express IL-4. The helper T cell phenotype develops depending on the signals coming from the microenvironment to adjust the immune response in the best way possible. This also results in a difference in the roles of each helper T cell type (Abbas & Lichtman, 2016; Murphy & Weaver, 2017; Saravia, Chapman, & Chi, 2019).

1.3 Helper T 17 Cells

Helper T 17 cells are a specific subset of helper T cells. These cells are characterized by their ability to secrete IL-17 family cytokines (IL-17A and IL-17F) (Harrington et al., 2005) and also secrete IL-6, IL-21, and IL-22. IL17A, first discovered in 1993 (Rouvier, Luciani, Mattéi, Denizot, & Golstein, 1993), is responsible for the induction of proinflammatory responses by inducing the production of proinflammatory cytokines, such as IL-1ß, TNFα, and IL-6, on the target cells, for example, fibroblasts, epithelial cells, and macrophages (Chiricozzi et al., 2011; Gu, Wu, & Li, 2013; Kolls & Lindén, 2004). Production of IL-21 is initiated by IL-6 binding to the IL-6R through signal transducer and activator of transcription 3 (STAT-3). The secreted IL-21 manifests

itself through autocrine signaling and leads to the production of Tumor Growth Factor-ß (TGF-ß) (Harris et al., 2007; Korn et al., 2007; Nurieva et al., 2007). IL-22 takes part in the induction production of antimicrobial peptides on the target cells upon binding to the IL-22R (Li et al., 2015). Another signature product of Th17 cells is RAR-related orphan receptor 2 (RORγt, RORC2), which is a transcription factor. RORC2 is identified as the master regulator of Th17 differentiation and its transcription is upregulated by IL-6 through STAT-3 (Ivanov et al., 2006; Nurieva et al., 2007).

1.3.2 Pathology

In numerous studies, Th17 cells have been shown to be involved in the pathogenesis of various autoimmune diseases. One of the autoimmune diseases affiliated with Th17 that comes to mind is Rheumatoid Arthritis (RA). RA is the inflammation of synovial fluids. The pathogenicity behind RA is the infiltration of immune cells, mostly $CD4^+$ T cells (M., D.M., & J., 2013; Srivastava, Dar, & Mishra, 2018). In the early phases of RA, the levels of IL-17 were found to be high in the synovial fluid of RA patients (Raza, 2019). Similarly, the levels of IL-22 and IL-23 were found to be high in the serum of RA patients (Rasmussen et al., 2010). The pro-inflammatory cytokines secreted by Th17 cells are the main driving force of RA inflammation, the inflammation is further increased by the stimulation of the tissue-resident macrophages towards inflammatory responses (Hirota et al., 2018; Zizzo et al., 2011).

Another one of the most studied Th17-related autoimmune diseases is Multiple Sclerosis (MS). In MS patients, the myelin on the nerve cells in the brain and spinal cord are damaged and this causes disruption in the signal transmission along with the nervous system. MS manifests itself with lesions on the brain. These lesions are concentrated sites of inflammation that contain immune cell infiltrates. In the autopsy results of deceased MS patients, these lesions were found to be crowded with Th17 cells and IL-17 family cytokines. On par with these findings, the levels of IL-17 in the peripheral blood of MS patients were higher compared to healthy individuals as well as the expression of IL-17 (Langrish et al., 2005; Lock et al., 2002; H. Park et al., 2005). Another interesting point regarding MS is that in the studies done on the mice model of MS, experimental autoimmune encephalomyelitis (EAE), the mutations of Fas or FasL that impair the

functions of either, resulted in milder progression of the disease (Waldner, Sobel, Howard, & Kuchroo, 1997). In another study done with MS patients, the serum levels of sFasL were found to be significantly higher in the patients compared to healthy individuals (Zipp, Krammer, & Weller, 1999). These findings might suggest a correlation between the pathogenicity and longevity of Th17 cells and the Fas signaling pathway.

1.4 Fas

Fas, also termed as Cluster of differentiation 95 (CD95), Apo-1, tumor necrosis factor receptor superfamily member 6 (TNFRSF6), is a surface receptor that is mainly responsible for receptor-mediated apoptosis. First discovered in 1984, Fas is a type-1 transmembrane protein and found in homotrimers (Biggin et al., 1984). It contains a characteristic death domain (DD) which is 80-100 amino acids long. This DD allows Fas to be a death receptor (DR) (Kischkel et al., 1995). Other than that, Fas has recently been found to have a calcium-inducing domain on its intracellular tail, which can induce signaling via calcium concentration changes in the cell (Poissonnier et al., 2016; Tauzin et al., 2011).

Upon binding of its ligand, Fas Ligand (FasL), the signal transduction begins. There are several mechanisms downstream of Fas-FasL binding. (Chen, Sun, & Nabel, 1998; Kang et al., 1997; Letellier et al., 2010; Suda, Hashimoto, Tanaka, Ochi, & Nagata, 1997) on the cell, depending on the current state of the cell, duration of the interaction, and the status of the ligand. Whether FasL bound to the cellular membrane (membrane-bound FasL, mFasL) or cleaved with several types of Membrane Metalloproteases (MMPs) (soluble FasL, sFasL) (Kiaei et al., 2007; Kirkin et al., 2007; Matsuno et al., 2001; Vargo-Gogola, Crawford, Fingleton, & Matrisian, 2002).

1.4.1 Fas in Apoptosis

As mentioned earlier, Fas is mainly responsible for receptor-mediated apoptosis, also known as extrinsic apoptosis. Extrinsic apoptosis begins with the binding of mFasL to the extracellular N-terminus of the Fas which is rich in cysteine. The ligand-binding site of Fas has CRD2 and CRD3 domains that are adjacent. The binding causes the aggregation of other Fas molecules and leads to oligomerization of these molecules. The

oligomerization is followed by the recruitment of adaptor protein FADD, Fas Associated Death Domain. The DD on the N-terminus of FADD binds to the DD on the C-terminus of Fas molecules. FADD also contains another member of the DD superfamily, Death Effector Domain, DED. This domain on the FADD allows it to recruit procaspase-8, which also contains a DED and can bind to FADD through DED-DED interaction, forming the complex known as Death Inducing Signaling Complex, DISC. The formation of DISC brings two procaspase-8 together, allowing them to be activated through autocleavage resulting in the production of the active form of procaspase-8, caspase-8 (Li-Weber & Krammer, 2003). However, this cleavage takes place in two steps. The first cleavage takes place on the D374 and produces p43/p41 and p12 subunits and the second step of cleavage occurs on D216 and D384 of these subunits and results in the production of p24/p26, p18 and, p10 subunits. p18 and p10 subunits come together and form a $p18_2$-$p10_2$ tetramer, which is the active form of caspase 8 and leaves the DISC and is released to the cytosol (Boatright et al., 2003; Hoffmann, Pappa, Krammer, & Lavrik, 2009; Li-Weber & Krammer, 2003; Mandal, Barrón, Kostova, Becker, & Strebhardt, 2020; Mandal et al., 2014; Zhao, Sui, & Hong, 2010)

The activated caspase 8 can either cleave effector caspases, procaspase 3 and procaspase 7, to complete the extrinsic pathway of apoptosis or it can cleave BID to initiate the intrinsic pathway of apoptosis. The selection between these two ways depends on the cell types of Fas-expressing tissues; if the caspase 8 directly cleaves effector caspases, the cell is the type I, if it is required for the intrinsic pathway to amplify the caspase cascade, the cell is type II (Hao & Mak, 2010; Scaffidi et al., 1998; Sprick & Walczak, 2004). Regarding the apoptotic sensitivities of both of these two types of cells, there are no differences. In type I cells, the activated caspase 8, $p18_2$-$p10_2$, targets the procaspase-3 and cleaves it into p17 and p19 fragments, and activates it. However, this is blocked by the X-linked Inhibitor of Apoptosis Protein (XIAP) and apoptosis occurs through the intrinsic pathway. The caspase 8 cleaves the BID protein, making it truncated BID (tBID) which allows the dimerization of Bax and Bak that leads to mitochondrial outer membrane permeabilization (MOMP). The MOMP causes the release of several factors from inside of the mitochondria, such as cytochrome c (Cyt c) and SMAC (Scaffidi et al., 1998). The released Cyt c binds to the Apoptotic Protease Activating Factor 1 (Apaf 1), seven Cyt c molecules and seven Apaf 1 molecules come together and form the complex known as Apoptosome. The apoptosome recruits procaspase 9 and

cleaves it to activate it. Then, the activated caspase 9 cleaves the procaspase 3 (Yuan et al., 2010).

1.4.2 Roles of Fas-mediated Apoptosis in Immune System

Fas signaling pathway is the main player in apoptosis, however, it also takes part in nonapoptotic cellular events. Apoptotic or nonapoptotic, its roles are crucial for the cells and organisms and it is utilized in different types of events. One of the most important events that utilize the Fas signaling pathway is the maintenance of immune homeostasis. In healthy individuals, the lymphocytes circulate the peripheral blood in their naive states, waiting for the presentation of their specific antigen. To be presented with the antigen, they go into lymph nodes and if their specific antigen is available and presented to them by an antigen-presenting cell (APC), they will be activated and will proliferate and differentiate into effector cells. Once they become effector cells, the lymphocytes can infiltrate into the infected tissues and accomplish their effector functions. After effector cells accomplish their functions, they are eliminated via specialized apoptosis termed as Activation-induced Cell Death (AICD) by the Fas signaling pathway (Castro et al., 1996). Even though several conditions lead to apoptosis of activated T cells, this mode of death is seen after the repeated stimulation of the T Cell Receptor. In response to this repeated stimulation, Fas and FasL expressions are upregulated (Brunner et al., 1995; Ju et al., 1995; Zhang, Xu, & Liu, 2004). The sensitivity of the T cells towards AICD increases over time and differs between different T helper subsets (Varadhachary, Perdow, Hu, Ramanarayanan, & Salgame, 1997). The increased expression of Fas and FasL prevents an excessive amount of immune response and it also plays a major role in peripheral tolerance, the deletion of autoreactive T cell clones. It was also shown that exposure to IL-2, which requires activation of T cells and maturation of IL-2R (CD25), increases the sensitivity of T lymphocytes by affecting the growth cycle of T cells. This is due to the fact that the stimulation of T lymphocytes with IL-2 causes the cell cycle to shift into late G1 and S phases. In the late G1 and S phases, T lymphocytes will have increased susceptibility towards the apoptosis. This is because the expression of Fas is upregulated and the expression of FLIP molecules is downregulated (Fortner, Bouillet, Strasser, & Budd, 2010; Krammer, Arnold, & Lavrik, 2007; Lenardo,

1991). The immune response often consists of the killing of the self-cells to prevent the spread of pathogens or the growth of the tumor. There are several sites termed immune-privileged sites that are not accessible by most immune cells. The reason for this inaccessibility is to protect the cells and tissues that cannot be renewed if they are lost or damaged. To prevent this from happening, said sites have a physical barrier or immune suppression. These sites are mainly the eyes, testicles, placenta, fetus, and nervous system. Recent studies have shown that one of the mechanisms of this phenomenon is the induction of apoptosis through the Fas signaling pathway (Griffith, Brunner, Fletcher, Green, & Ferguson, 1995). Similar to CD8+ T cells, a certain type of CD4+ helper T cells, identified as cytotoxic CD4+ T cells (ThCTLs), are shown to use Fas-FasL interactions to eliminate virally infected target cells (Malyshkina et al., 2017; Stalder, Hahn, & Erb, 1994).

1.4.3 Non-Apoptotic Roles of Fas

Recent findings in the literature have indicated that the Fas signaling pathway can be involved in non-apoptotic functions. This was first shown in 1993, as the proliferation and cytokine production of human T cells was stronger if the Fas signaling pathway is functional (Alderson et al., 1993) and in the same year, it was reported that the activation of T lymphocytes prevents the signal transduction through Fas pathway (Klas, Debatin, Jonker, & Krammer, 1993). These two findings showed that there is more to Fas than just mediating apoptosis. 5 years later as the studies about non-apoptotic roles of Fas continued, deficiency of one of the key components of the DISC, FADD, not only caused impairment of apoptosis but also impaired the TCR induced activation in mice T lymphocytes (Walsh et al., 1998). In the next year, 1999, upon the coupled stimulation of Fas, with FasL, and TCR, with anti-CD3, the caspase-8 levels were found to increase compared to resting cells but there was no increase in terms of apoptosis. Also, the inhibition of caspase activity is shown to block proliferation on activated cells. On top of these findings, the FasL expression was also increased in activated cells, suggesting a situation similar to a feedback loop that depends on the stimulation of Fas during the activation of T cells (Kennedy, Kataoka, Tschopp, & Budd, 1999). The mechanism of how Fas stimulation and caspase inhibition affects activation and proliferation is not fully

understood yet. The first candidate found responsible for such a mechanism was the c-FLIP protein. In 2000, it was revealed that when the proliferation was increased by FasL co-stimulation, the NF-κB and Activator Protein-1 (AP1) levels increased and the c-FLIP, which is almost identical to the procaspase 8 and acts as a caspase inhibitor, was found to be recruited to DISC (T. Kataoka et al., 2000), later to be found to cause the threshold for TCR-mediated proliferation through the inhibition of caspase-8. The increased activation was thought to be due to the induction of NF-κB signaling by c-FLIP (Lens et al., 2002). Then c- FLIPL was shown to be cleaved by the caspase-8 interact with TRAF 2 and induce the activation of the NF-κB signaling pathway (Takao Kataoka & Tschopp, 2004). The same year, it was shown that the inhibition of caspases during activation caused a failure in the initiation of cell cycle progression by the blockage of upregulation of several cyclins and cyclin-dependent kinases via inhibition of downregulation of a cyclin-dependent kinase inhibitor p27KIP1, which is downregulated after CD3 stimulation.

1.4.4 Non-Apoptotic Roles of Fas in Helper T Cells

Whether the Fas stimulation affects the biology of helper T cell subsets or not is not discovered completely. However, findings are suggesting that the Fas signaling pathway is important for helper T cell subsets in various ways. In 1999, it was revealed that Fas ligation on naive CD4+ T cells in the presence of CD3 leads to death of these cells, however, the same ligation leads to the proliferation of memory cells, and the stimulation of Fas inhibits the proliferative response of naive cells but costimulates the proliferation of memory cells. Differentiation cytokines, IL-12 for Th1 and IL-4 for Th2 cells, and costimulation with CD3 and CD28 rescue the naive cells from Fas-induced death and render them receptive to Fas-mediated costimulation. In short, it was shown that the effects of Fas stimulation on T helper cells depend on their status, whether they are naive or memory (Desbarats, Wade, Wade, & Newell, 1999). Fas is also responsible for the expression of Th17-related genes according to a 2013 article. The cells were activated with PHA and treated with IL-2, to mimic Th17-polarizing conditions. The cells showed increased levels of Fas expression and processed caspase-1, however, low levels of apoptosis. 3 days after the stimulation, the cells expressed RORγt, FoxP3, Tbet, and

GATA3. Stimulation of these cells through Fas FasL-expressing glioma cells causes the prolonged expressions of IL-17A, IL-17F, and RORγt. PHA and IL-2 treatment also caused phosphorylation of STAT3, which has a crucial role in Th17 phenotype development, and the stimulation with Fas prolonged the period of phosphorylation from 3 days to 6 days (Su, Lin, Lin, Shan, & Yang, 2013). It was shown to be a positive regulator of the differentiation and targeted by Stat3, which is the target of IL-6 signaling that is crucial for Th17 differentiation. The key genes for Th17 differentiation were observed to be repressed in the Fas knock-down experiments (Yosef et al., 2013).

1.5 Aim of the Study

This study aims to discover whether the Fas signaling pathway plays any role during the development of Th17 cells. For this purpose, buffy coats of healthy donors were taken from the Dokuz Eylul Blood Bank with ethics approval and were used to isolate Peripheral Blood Mononuclear Cells (PBMCs) via the Ficoll-Paque gradient density method. Then $CD4^+$ $CD45RA^+$ naive T cells were sorted from the isolated PBMCs and cultured under Th17 polarizing conditions for seven days. On the critical time points of the culture, which are day 3, day 5, and day 7, expression of Fas, TNFR1, FasL, and TNFα have been investigated as well as the apoptotic membrane changes, via Annexin V and 7AAD. Put together, this study will shed light on the effects of Fas and alike on the Th17 development and will provide insight on whether the Fas signaling pathway has a role in the longevity of Th17 cells in pathological conditions. Any links discovered about this topic will help towards a better understanding of pathologies linked with Th17 longevity, such as Multiple Sclerosis and Rheumatoid Arthritis.

CHAPTER 2

MATERIALS AND METHODS

2.1 PBMC Isolation

The peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples of healthy volunteers that were obtained from the Dokuz Eylul Blood Bank with ethics approval. The Ficoll-Paque density gradient method was used for the isolation.(BØYUM, LØVHAUG, TRESLAND, & NORDLIE, 1991) This method utilizes the density difference between the PBMCs and other components of the blood. First, the samples were centrifuged at 1500 rpm for 5 minutes. This process separated the serum from the blood and the serum was removed. The rest of the blood was diluted 1:1 with 1X PBS and then the diluted blood was laid on 10 ml of Ficoll slowly to prevent the mixing of diluted blood with the Ficoll. The samples were then centrifuged for 45 minutes at 2500 rpm with the lowest acceleration and deceleration settings. After the centrifugation, four phases appear in the tubes, from top to bottom these phases are plasma, PBMC, Ficoll, and erythrocytes. The PBMCs that are found between the plasma and the Ficoll is collected and treated with Red Blood Cell Lysis Solution (Miltenyi Biotech) according to the manufacturer's instructions and washed twice with 1X PBS for 10 minutes at 1200 rpm. The number of cells in the isolated PBMC samples were counted in the counting chamber with Trypan Blue staining. 10µl of cell suspension was taken from the samples and mixed with 90µl Trypan Blue. 10µl of cell-Trypan Blue mixture was taken and placed on the cell counting chamber and the number of cells on the chamber was counted on a compound microscope. The total number of cells was calculated from the number of cells obtained from the counting chamber.

2.2 PBMC Characterization

The isolated PBMCs were characterized with flow cytometry by following surface staining BD Pharmagen Stain Protocol to stain the specific surface markers of the cell types. Fluorescent-conjugated antibodies that target CD4, CD8, CD14, CD19, CD25, CD45RA, CD45RO, CD69, and Annexin V. The blood sample contains 25-30% of $CD4^+$ $CD45RA^+$ T cells is chosen.

2.3 Naive T Cell Enrichment

The $CD4^+$ $CD45RA^+$ naive T cells were sorted by using the Human Naive $CD4^+$ T cell isolation kit II (Miltenyi Biotech) and manufacturers' instructions are followed. The kit uses negative selection to isolate the $CD4^+$ $CD45RA^+$ naive T cells by tagging the PBMC cells with an antibody cocktail targeting CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR, and CD235a molecules. The volumes of antibodies used were determined based on the cell number. The PBMCs were centrifuged at 1200 rpm for 10 minutes and the pellet was resuspended in Variomax Buffer (40µl/10^7 cells). Then the cells were treated with Naive $CD4^+$ T Cell Biotin-Antibody Cocktail II (10µl/10^7 cells) and incubated at 4°C for 5 minutes. After the incubation, the cells were washed with Variomax Buffer (30µl/10^7 cells) and treated with Naive $CD4^+$ T Cell Microbread Cocktail II (20µl/10^7 cells), followed by incubation at 4°C for 10 minutes. During the incubation, the VarioMACS separator column was placed into the magnetic field and prepared by rinsing with 3 mL of Variomax Buffer. The incubated cells then were flown through the column, $CD4^+$ Naive T Cells flew through the column and collected in a 14 ml Falcon Tube whilst the rest of the cells, that were tagged with microbeads, were held in the magnetic field. To increase the efficiency, the column was rinsed again with 3 mL of Variomax Buffer. After all of the $CD4^+$ Naive T cells were collected, the column was removed from the magnetic field and placed into a Falcon tube and the remaining cells, the depleted cells, were flushed into the tube.

2.4 Cell Culture

The CD^+ 4 naive T cells are cultured in T helper 17 polarizing conditions, according to Veldhoen's culture conditions. (Stockinger & Veldhoen, 2007) For the culturing, complete IMDM was used. Complete IMDM is composed of BioWhittaker® IMDM (Lonza), 5% FBS, 2% Penicillin (100µl/ml) and Streptomycin (100µl/ml) (Gibco) and with 0.1% ß-mercaptoethanol. The naive $CD4^+$ T cells are treated with stimulants and neutralizers to be pushed into the T helper 17 phenotypes. The cells were activated via TCR stimulation with anti-CD3 (5µg/mL) and anti-CD28 (5µg/mL) (Miltenyi Biotech). For the differentiation of activated cells into the T helper 17 phenotype, IL-1ß (10ng/ml), IL-23 (10µg/mL), IL-6 (30µgl/mL) and TGF-ß (0,5µg/mL) were used. T helper cells have high plasticity and their phenotype can shift easily depending on the chemical signals from the environment. To prevent Th17 cells to shift into Th1 or Th2 cells, anti-IFNγ (10µg/mL) and anti-IL-4 (10µg/mL) were used, respectively. On day 3, day 5, and day 7 of the culture, cells were analyzed with flow cytometry for the screenings of activation, differentiation, and apoptosis. On day 5, the culture media was refreshed according to the cell number and $CD4^+$ naive T cells that were being polarized into Th17 phenotype were supplied with IL-23 (10ng/mL) for the maintenance of Th17 phenotype and survival. On day 7 of the culture, depending on the IL-17 level, the culture was stoped.

2.5 Cell Staining and Flow Cytometry

2.5.1 Surface Staining

For the monitoring of molecules on the cell surface, cells were stained with fluorescent-tagged antibodies. These specific antibodies target various surface markers and receptors. The cells to be stained were first distributed on a 96-well plate and washed with 200µl 1X PBS at 400g for 6 minutes. The supernatant was removed and 10µl of diluted antibodies (1:10 with 1X PBS) were added to the cells and incubated at 4 ℃ for 15 minutes in the dark. Incubated cells were then washed with 200 µl 1X PBS at 400g for

6 minutes. The supernatant was removed and stained cells were dissolved in 400 µl 1X PBS and collected in flow cytometry tubes.

2.5.2 Intracellular Staining

The staining of intracellular molecules requires extra steps. If any surface staining was required, it was done beforehand. First, 50 µl of 1X FOXP3 fix/perm (BD) buffer was added to the cells and the cells were incubated at room temperature for 20 minutes in the dark. After the incubation, the cells were washed with 100 µl 1X PBS at 800g for 10 minutes. The supernatant was removed and 50 µl of 1X FOXP3 perm buffer (BD) was added to the cells and cells were washed with 800 µl 1X PBS for 10 minutes and the supernatant was removed. 50 µl of FOXP3 perm buffer was added to the cells and the cells were incubated at room temperature for 10 minutes in dark. After the incubation, fluorescent-conjugated antibodies were added to the cells and incubated for 30 minutes at room temperature in dark. Stained cells were washed with 100 µl 1X PBS at 800g for 10 minutes, the supernatant was removed and cells were dissolved in 400 µl of 1X PBS and collected in flow cytometry tubes.

Cytokines are small proteins of cellular communication that are secreted from the immune cells. These cytokines were monitored to determine the phenotype of the helper T cells. Since these molecules are secreted out of the cells, the secretion process was halted for the staining of cytokines. The cells were treated with GolgiStop™ Protein Transport Inhibitor (Containing Monensin) (BD) for 4-6 hours before the staining procedure began. After the incubation for inhibition of secretion was completed, the intracellular staining procedure was followed for the staining of the cytokines.

2.5.3 Detection of Apoptotic Membrane Changes

The changes in the plasma membrane caused by apoptosis were monitored by using Annexin V and 7AAD. The staining of Annexin V requires the usage of Binding Buffer for Annexin V (eBioscience) as staining buffer. If any surface staining was

necessary, it was done beforehand. 50 µl of 1X staining buffer was added to the cells and 10 µl of diluted Annexin V stain (1:10 with 1X Binding Buffer for Annexin V) and 10 µl of diluted 7-Aminoactinomycin D (7AAD) (1:10 with 1X Binding Buffer for Annexin V) were added on the cells. Annexin V stains were conjugated with fluorescent molecules and the 7AAD molecule gives a fluorescent signal by its nature. The stained cells were incubated at room temperature for 10 minutes in dark. Incubated cells were washed with 100 µl of Binding Buffer for Annexin V at 400g for 6 minutes. The supernatant was removed and the cells were dissolved in 400 µl of 1X Binding Buffer for Annexin V and collected in flow cytometry tubes.

2.5.4 Flow Cytometric Analysis

Stained cells were analyzed by using Guava® easyCyte™ (Millipore) flow cytometry instrument and the data analysis was done with guavaSoft® software. Staining antibodies had conjugants like AlexaFluor 488 (eBioscience), Fluorescein isothiocyanate (FITC, BD or eBioscience) for Green channel, Peridinin-Chlorophyll-protein (PerCP, eBioscience) and 7AAD for Red I channel, Allophycocyanin (APC, BD or eBioscience), and AlexaFluor 647 (BD) for Red II channel and Phycoerythrin (PE, eBioscience) for Yellow channel.

2.6 Monitoring of Activation and Differentiation

The activation of naive T cells cultured in the Th17 polarizing conditions was monitored on day 3 and day 5 of the culture. The expression levels of surface molecules CD69, for early stages of activation, and CD25 (IL-2R), for late stages of activation, were stained with surface staining procedure and monitored with flow cytometry. The differentiation of the naive T cells cultured in Th17 polarizing conditions was monitored on day 5 and day 7 of the culture. For the determination of differentiation, presence, and activation of RORC transcription factor, presence of IL-22 and IL-17A cytokines and expression of CCR6 surface molecule were monitored. RORC was stained by using

intracellular staining, IL-17A and IL-22 were stained by using cytokine staining and CCR6 was stained by following surface staining procedures.

2.7 Determination of Apoptosis

The percentage of apoptotic cells in the culture was monitored at every time point of the culture (day zero, day 3, day 5 and, day 7). The apoptosis was determined by using two molecules, Annexin V and 7AAD. Annexin V bind to the phosphatidylserine molecules in the environments with the correct Ca^{+2} concentration. Phosphatidylserine molecules are found on the plasma membrane and normally face cytoplasm. In the early stages of apoptosis, these molecules flip over the plasma membrane and face outside the cell, thus being available for the binding of Annexin V. 7AAD molecule binds to the GC-rich regions of the double-stranded DNA. In the late stages of apoptosis, the genome of the cells can be found inside the cytoplasm and the plasma membrane is damaged. 7AAD, normally not permeable through the plasma membrane, can get inside of the cell through the damaged plasma membrane and can bind to the double-stranded DNA inside the cytoplasm. However, 7AAD alone does not indicate late apoptosis since other cell death types can result in similar consequences, double positivity for Annexin V and 7AAD is interpreted as late apoptosis. For the monitoring of apoptosis, the cells were stained by following the Annexin V staining procedure.

2.8 ELISA

The expression of sFasL was determined by sandwich ELISA method using Human sFas Ligand ELISA Kit (Invitrogen). The cell culture supernatants of both the cells cultured under Th17 polarizing conditions and negative culture were obtained on day 3, day 5 and, day 7 of the culture and stored at -20°C, and the instructions of the manufacturer were followed. First, the antibody-coated strips were washed with 400 µl 1X Wash buffer twice. 100 µl of diluted standards were then added in duplicates on the plate (Diluted with sample diluent). 100 µl of sample diluent was added as blanks and 50 µl of sample diluent was added on the sample wells and 50 µl of samples were added on

the sample diluents in duplicates. Then 50 µl of Biotin-conjugate was added to all wells and incubated at room temperature for 2 hours. After the incubation, the wells were washed 4 times with 400 µl 1X Wash buffer. 100 µl of Streptavidin-HRP was added to all wells and incubated at room temperature for 1 hour. After the incubation, the wells were washed 4 times with 400 µl 1X Wash buffer. Then 100 µl of TMB Substrate Solution was added to all wells to start the reaction and incubated for 10 minutes, until the most concentrated well-turned dark blue, then stopped with 100 µl of Stop Solution. After the reaction was stopped, absorbance values at 450 nm were read on a microplate reader.

2.8 Statistical Analysis

The statistical analysis of data was done with 2-Way ANOVA by using GraphPad. The graphics were drawn with GraphPad, $p<0.05$ was accepted and the error bars represent standard deviation.

CHAPTER 3

RESULTS

3.1 Characterization of PBMC

The Ficoll-Paque density gradient separation method was used to isolate the PBMCs from the buffy coats. After the separation of PBMCs, the percentages of different populations of cells in the PBMCs were analyzed by using flow cytometry. The samples were stained with specific surface markers of each cell type; anti-CD4 PerCP for helper T cells, anti-CD8 APC for cytotoxic T cells, anti-CD14 FITC for monocytes, anti-CD19 PE for B cells, anti-CD69 PE for activated cells, anti-CD45RA FITC for naive cells, anti-CD45RO APC for memory cells, Annexin V-FITC and 7AAD for apoptosis and anti-Fas APC for the detection of Fas expression. The samples were stained with the aforementioned antibodies in appropriate combinations with three replicates (Figure 1).

3.2 Determination of Sorting Efficiency

The $CD4^+$ $CD45RA^+$ T cells were separated from the PBMC by sorting with the usage of Human Naive CD4+ T cell isolation Kit II (Miltenyi Biotech). To determine the efficiency of the sorting procedure, the percentages of $CD4^+$ $CD45RA^+$ T cells were analyzed in the samples before and after the sorting procedure. The main aim of this procedure was to enrich the amounnt of naive T cells in the samples. The samples were stained with anti-CD4 PE and anti-CD45RA FITC fluorochromes and analyzed with flow cytometry in three replicates and the data shown are representative. The efficiency of the sorting procedure was expected to be between 80% and 98% as the manufacturers instructed. The experiments were not continued if the percentage was low. The data below is representative and shows 71.30% efficiency (Figure 2).

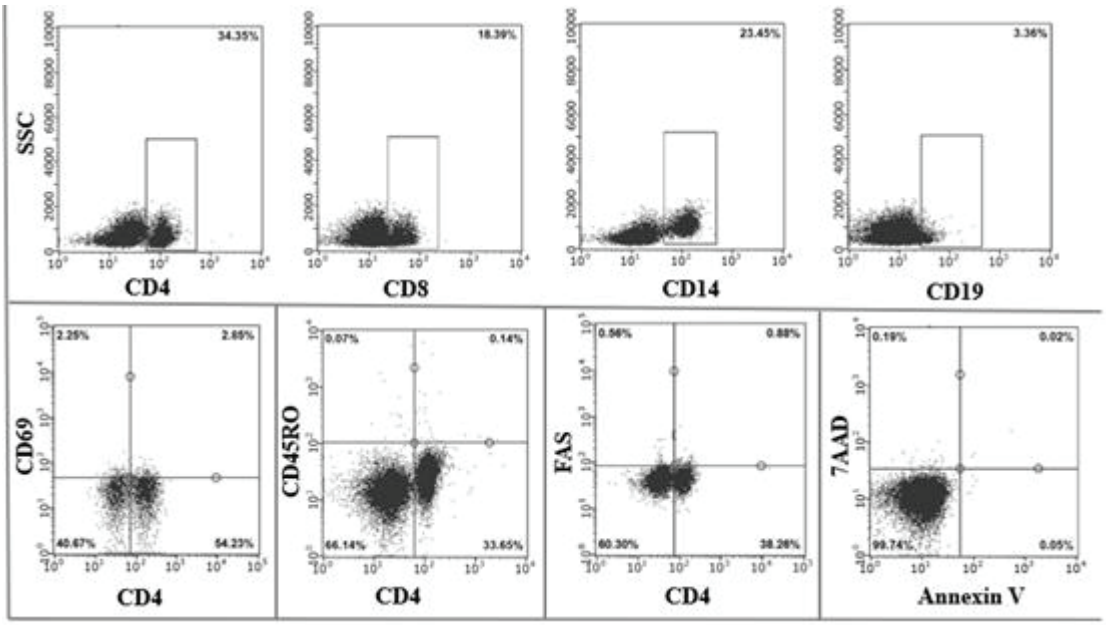

Figure 1: Characterization of PBMC. The PBMC populations were separated from healthy donors buffy coats by using the Ficoll-Paque method. Separated cells were stained combinations of antibodies and each sample was stained in three replicates. The data shown are representative. The percentages of cell populations were analyzed by using flow cytometry. The percentages of $CD4^+$ T cells (A), $CD8^+$ T cells (B), $CD14^+$ monocytes (C), $CD19^+$ B cells (D), $CD4^+$ $CD69^+$ activated T cells (E), $CD4^+$ Fas^+ T cells (F), $CD4^+$ $CD45RO^+$ memory T cells (G) and overall apoptosis via Annexin V versus 7AAD (H) were investigated.

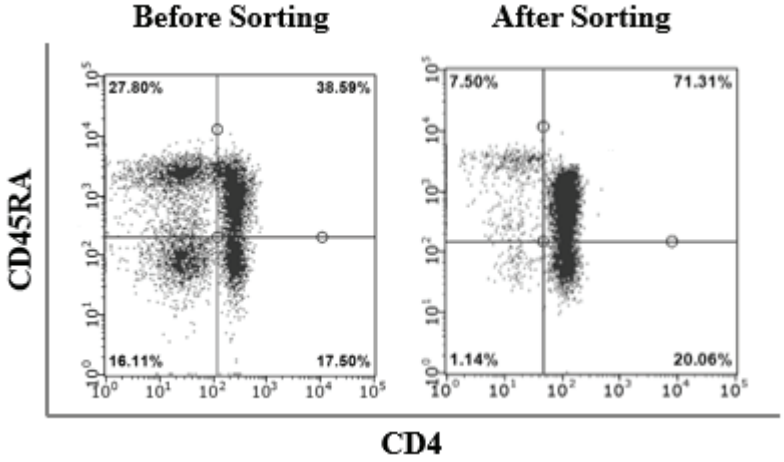

Figure 2: Determination of sorting efficiency. PBMC cells were isolated from the buffy coats of healthy donors and CD4+CD45RA+ cells were sorted from the isolated PBMCs. The samples were stained with anti-CD4 and anti-CD45RA antibodies in three replicates and the data shown are representative. The percentages of CD4+CD45RA+ cells were determined by using flow cytometry

3.3 Monitoring of Activation

The sorted $CD4^+$ $CD45RA^+$ T cells were cultured in Th17 polarizing conditions and complete IMDM media was used for the culturing of negative control culture. The activation status of the cultured cells was monitored on day 3 and day 5 of the culture. On day 3 of the culture, the marker for early activation, CD69 was stained with anti-CD69 PE fluorescent stain and analyzed with flow cytometry. Each sample was done with three replicates. In the naive T cells cultured in Th17 polarizing conditions, the expression of CD69 was averaged as 17.17% for the three replicates done and, the CD69 expression in the negative control was averaged as 0.17% for the three replicates done. The expression of CD69 in the negative control was negligible, meaning the CD69 was not expressed in the negative control, thus no activation. The 17.17% average expression of CD69 in naive T cells cultured in Th17 polarizing conditions indicates that the cells were in the early phase of activation at day 3 of the culture (Figure 3.A). On day 5 of the culture, the marker for late activation, CD25 (IL-2R) was stained with anti-CD25 APC stain and analyzed with flow cytometry. Each sample was done with three replicates. In the naive T cells cultured in Th17 polarizing conditions, the expression of CD25 was 41.11% and the CD25 expression in the negative control was 2.95%. The expression difference of CD25 between naive T cells cultured in Th17 polarizing conditions and negative cells was significant and the data shows that naive T cells cultured in Th17 polarizing conditions were in the late phase of activation at day 5 of the culture (Figure 3.C).

3.4 Th17 Phenotype Detection

The sorted $CD4^+CD45RA^+$ T cells were cultured in Th17 polarizing conditions and complete IMDM media was used for negative control. The activation status of these cells was monitored on day 3 and day 5 of the culture as mentioned in the previous part. Th17 cells are characterized by several different cytokines and transcription factors as mentioned earlier. In this part, two of them were utilized as markers to determine the progression of the stimulated cells into the Th17 phenotype. The transcription factor RORC and the cytokine IL-17A are examples of these markers.Being a transcription

factor, RORC binds to the DNA and regulates the master regulator of Th17 phenotype, IL-17A. IL-17A is the signature cytokine of the Th17 and its detectiom is directly associated with Th17 phenotype. The determination of Th17 polarization was done via flow cytometry on day 7 of the culture. The cells were stained with anti-CD4 PerCP by using surface staining and anti-RORC PE and anti-IL-17A A.647 by using cytokine staining. The stained cells were analyzed with flow cytometry. The data shows that the average expression of RORC in the cells cultured in Th17 polarizing conditions was 6.02% and 0.18% in the negative control (Figure 4.A). The p-values were obtained by 2-way ANOVA in GraphPad and there was a significant difference in the expression of RORc in the 7th day between the two cultures ($p<0.0001$) (Figure 4.B). The average expression of IL-17A in cells cultured in Th17 polarizing conditions was 1.09% and 0.18% in the negative culture (Figure 4.C). The p-values were obtained by 2-way ANOVA in GraphPad and there was a significant difference in the expression of IL-17A in the 7th day between the two cultures ($p<0.001$) (Figure 4.D).

3.5 Determination of Fas Expression

During the culture, the development of the naive T cells cultured under Th17 polarizing conditions was monitored. On top of that, the Fas expression of these cells was monitored at the critical time points of day zero, day 3, day 5, and day 7 of the culture. The levels of Fas at day zero differs from person to person, ergo from culture to culture. Regardless, the expression of Fas was observed to increase in the naive T cells cultured under Th17 conditions starting from the 3rd day, compared to the negative culture. Over the period of 7 days, the Fas expression of Fas was monitored with CD4. The cells were stained with anti-CD4 and anti-Fas fluorochromes and the percentage of $CD4^+$ Fas^+ cells were analyzed with flow cytometry. As seen in Figure 5, the percentages of $CD4^+$ Fas^+ cells in the negative cultures remained relatively the same. On the contrary, in the Th17 polarizing cultures, the percentages of $CD4^+$ Fas^+ cells shown significant increases. Compared to zero-day, the increase in the percentages of $CD4^+$ Fas^+ cells was significant. When compared to the negative cultures, Th17 polarizing cultures also shown significant differences. Comparisons made for each time point shown significant differences between the negative cultures and Th17 polarizing cultures. In summary, the data in

Figure 5 shows that the expression of Fas significantly increased over the 7 days of culture in the $CD4^+$ $CD45RA^+$ cells cultured under Th17 polarizing culture. This increase was not observed in the negative cultures, and at every time point, the levels of Fas were significantly higher in the Th17 polarizing culture compared to the negative culture.

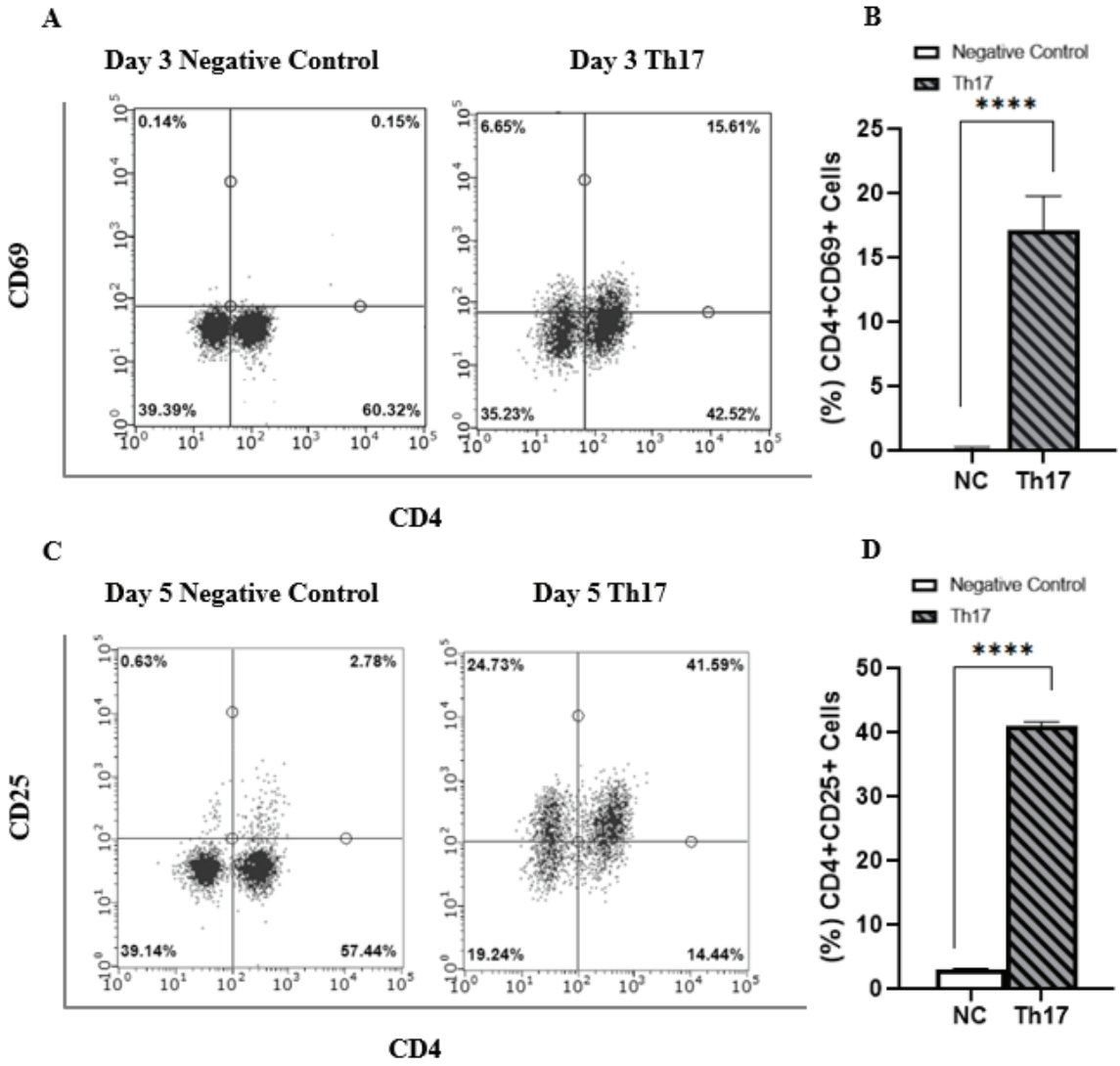

Figure 3: Monitoring of Activation. PBMC cells were isolated from the buffy coats of healthy donors and CD4+CD45RA+ cells were sorted from the isolated PBMCs. The sorted CD4+CD45RA+ cells were cultured under Th17 polarizing conditions, and complete IMDM media was used as the negative control. For the detection of early activation, the expression of CD69 was monitored on day 3 (A), for the detection of late activation, the expression of CD25 was monitored on day 5 (C). 2-way ANOVA was used to obtain p-values by using GraphPad. (n=3; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001) (B, D).

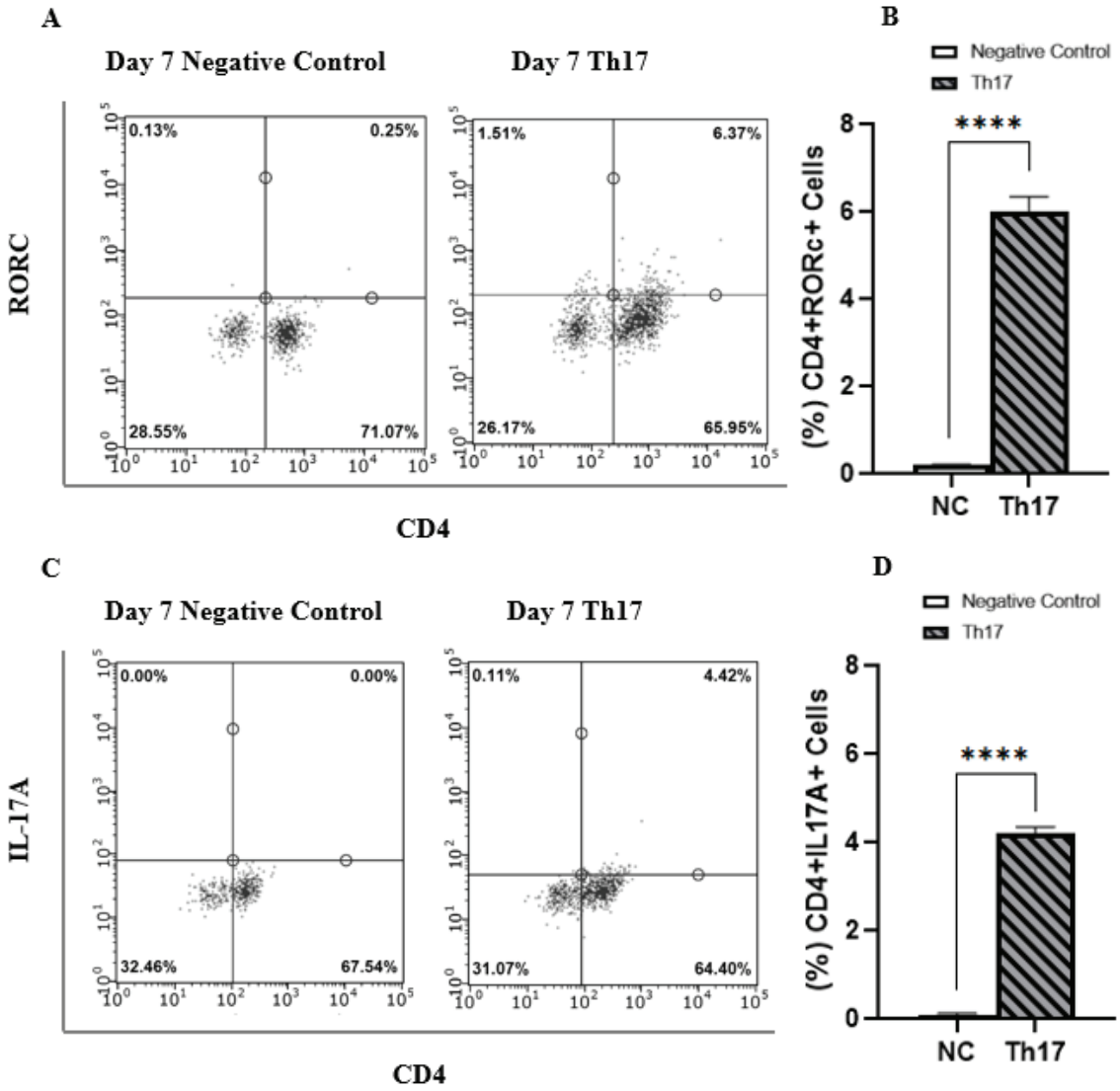

Figure 4: Th17 Phenotype Detection. PBMC cells were isolated from the buffy coats of healthy donors and CD4+CD45RA+ cells were sorted from the isolated PBMCs. The sorted CD4+CD45RA+ cells were cultured under Th17 polarizing conditions, and complete IMDM media was used as the negative control. On day 7 of the culture, the differentiation status of both cultures was investigated by cytokine staining of RORc (A) and IL-17A (B). The data are representative. The data were analyzed with 2-way ANOVA by using GraphPad. (n=3; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001) (B,D).

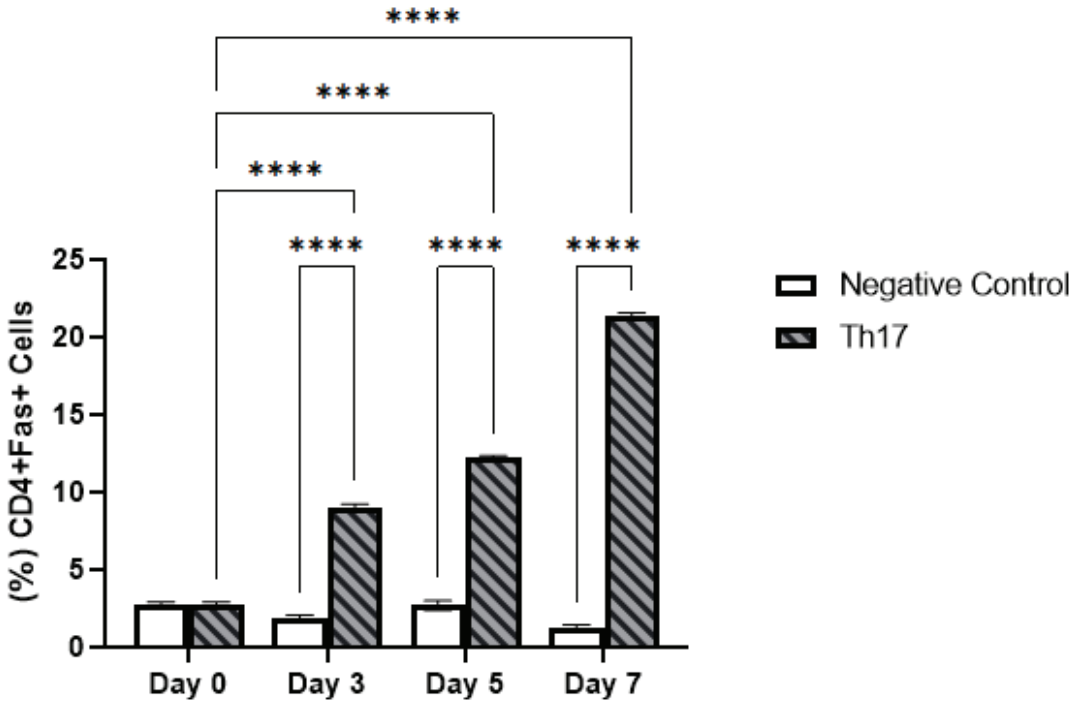

Figure 5: Determination of Fas Expression. The PBMCs were isolated from the buffy coats of healthy donors and CD4+ CD45RA+ cells were sorted from the PBMCs and cultured under Th17 polarizing conditions for a period of 7 days, complete IMDM media was used for negative control conditions. In specific time points, the expression of Fas in the cultured cells was analyzed with flow cytometry on day zero (PBMC), day 3, day 5, and day 7 of the culture. The cells were stained with anti-CD4 and anti-Fas fluorochromes and the percentages of CD4+ Fas+ cells were obtained. The data were analyzed with 2-way ANOVA by using GraphPad (n=3; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001).

3.6 Determination of Apoptosis

As mentioned earlier, Fas is a death receptor and is one of the main players of receptor-mediated apoptosis. Hence, the apoptotic changes in the cultured cells had to be monitored in both cultures. In normal conditions, the increase of Fas expression might suggest an escalation in the sensitivity towards apoptosis in the cell populations. In order to investigate whether the increased Fas expression resulted in increased apoptosis or not, the levels of apoptotic cells were analyzed in both cultures. For the detection of apoptosis, the changes on the cell membrane were monitored with flow cytometry by using Annexin V and 7AAD. Over the 7 days of the culture, in the specific time points, the percentages of apoptotic cells were monitored. The time points were day zero, day 3, day 5, and day 7. Figure 6 shows the percentages of Annexin V and 7AAD double-positive cells in both cultures on day zero, day 3, day 5, and day 7. In Figure 6, it can be seen that the levels of apoptosis did not show any significant differences between the Th17 polarizing culture and the negative control. The levels of apoptosis did not show any significant differences between the two cultures. The data were analyzed with 2-way ANOVA by using GraphPad (n=3).

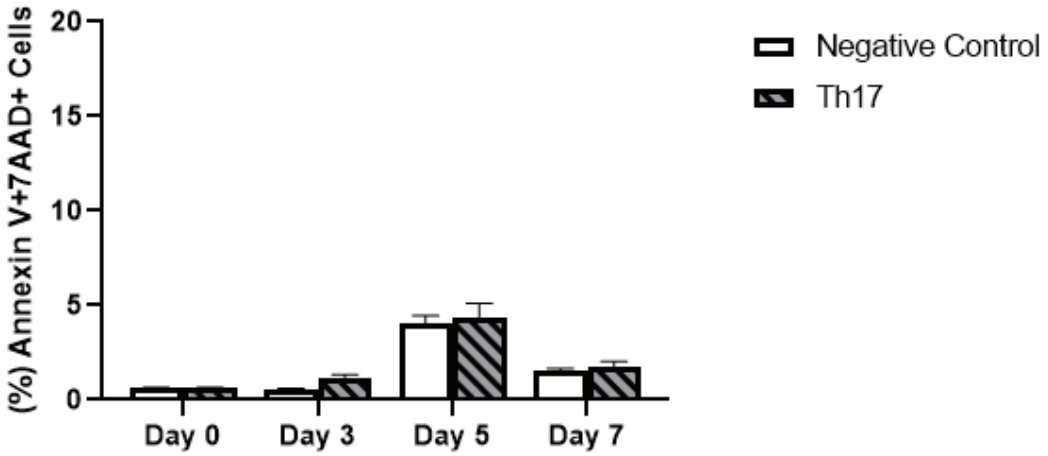

Figure 6: Determination of Apoptosis. The PBMCs were isolated from the buffy coats of healthy donors and CD4+ CD45RA+ cells were sorted from the PBMCs and cultured under Th17 polarizing conditions for a period of 7 days, complete IMDM media was used for negative control conditions. The level of apoptosis was monitored by using Annexin V and 7AAD via flow cytometry on day zero, day 3, day 5, and day 7 of the culture. The data were analyzed by 2-way ANOVA by using GraphPad. (n=3).

3.7 Determination of Apoptosis in the $CD4^+Fas^+$ Gated Cells

The overall level of apoptosis in both the Th17 polarizing culture and the negative culture did not show any significant difference. However, when the apoptosis was analyzed in a narrower scope, there were differences. The cells from both cultures were stained with a fluorochrome combination of anti-CD4, anti-Fas, Annexin V and 7AAD and analyzed with flow cytometry at specific time points of day zero, day 3, day 5, and day 7. First, $CD4^+$ Fas^+ cells were selected from the rest of the culture, and apoptosis was investigated only the selected cells, this procedure is called gating. Figure 7 shows the percentage of apoptotic cells in the $CD4^+$ Fas^+ gated cell population. The apoptosis levels did not show any significant differences on day 3. However, on day 5 and day 7, the levels of apoptosis in the $CD4^+$ Fas^+ gated cells were significantly lower in the cells cultured under Th17 polarizing conditions The data were analyzed with 2-way ANOVA by using GraphPad (n=3).

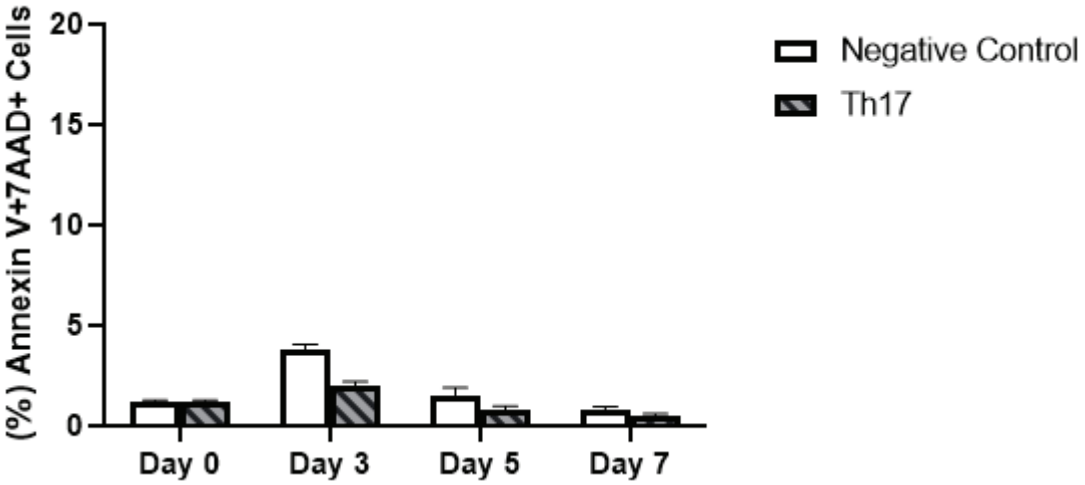

Figure 7: Detection of Apoptosis in $CD4^+$ Fas^+ Cells. The PBMCs were isolated from the buffy coats of healthy donors and $CD4^+$ $CD45RA^+$ cells were sorted from the PBMCs and cultured under Th17 polarizing conditions for a period of 7 days, complete IMDM media was used for negative control conditions. The level of apoptosis was monitored by using Annexin V and 7AAD via flow cytometry on day zero, day 3, day 5 and, day 7 of the culture. The data were analyzed with 2-way ANOVA by using GraphPad. (n=3).

3.8 Determination of FasL Expression

The increase of Fas expression on the stimulated cells was previously shown. However, the sole increase in the expression of Fas would not be effective if there were no ligand to stimulate it. On that note, the expression of Fas Ligand was investigated to determine whether the expression of FasL change at all. For this purpose, the cells were stained with anti-FasL on day 5 and day 7 of the culture. In Figure 8, it can be seen that there was no expression of FasL in both cultures. However, the staining protocol of FasL involves the usage of the inhibitors of Membrane Metalloproteases (MMP), to prevent the cleavage of FasL from the membrane. During the staining of the cells, no MMP inhibitors were used. The data were analyzed with 2-way ANOVA by using GraphPad (n=3).

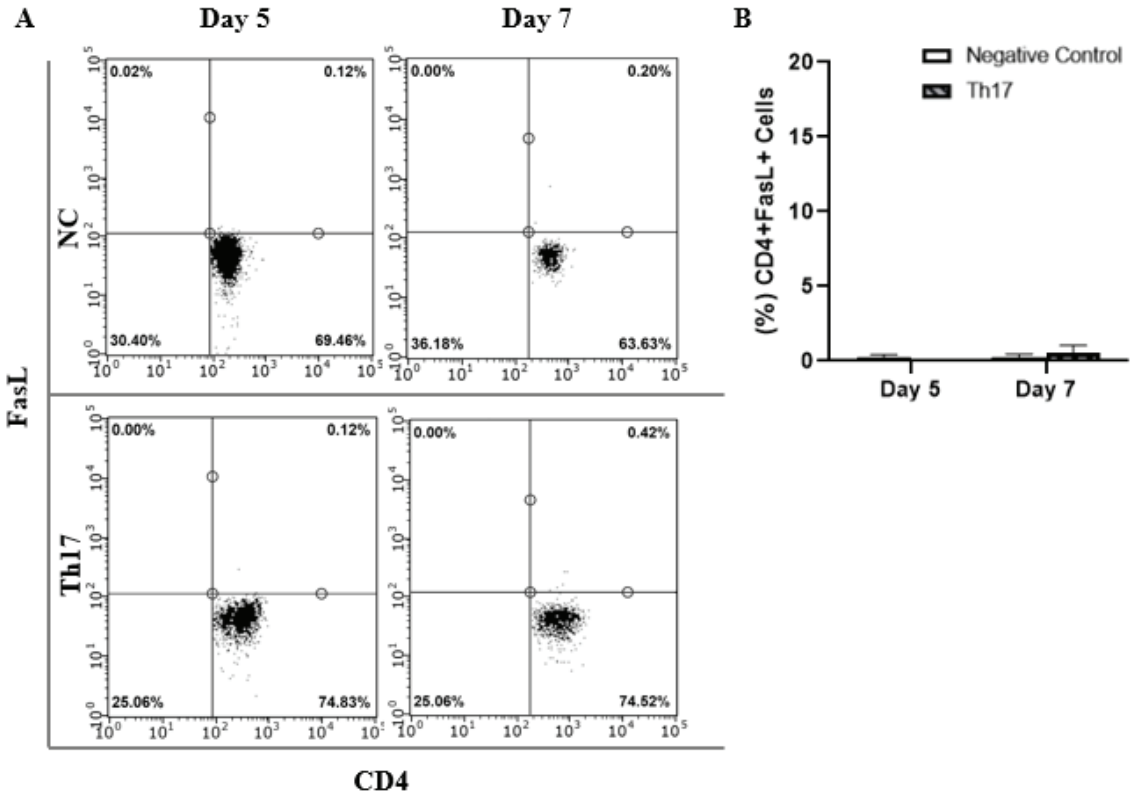

Figure 8: Determination of FasL Expression. The PBMCs were isolated from the buffy coats of healthy donors and $CD4^+$ $CD45RA^+$ cells were sorted from the PBMCs and cultured under Th17 polarizing conditions for a period of 7 days, complete IMDM media was used for negative control conditions. The cells were stained with anti-CD4 and anti-FasL fluorochromes and analyzed with flow cytometry at day 5 and, day 7 (A). The data are representative and were analyzed with 2-way ANOVA by using GraphPad. (n=3) (B).

For the detection of the sFasL molecules that have been possibly cleaved from the membrane, the cell culture supernatants have been collected on day 3, day 5 and, day 7 of the culture. The supernatant samples were then analyzed by using the Human sFas Ligand ELISA Kit. The cell culture supernatants were prepared in accordance with the kit guidelines and the absorbance values at 450 nm were read in a microplate reader. The absorbance values for the samples were almost the same as the blank wells and the calculated concentrations were less than 1 ng/mL for every sample and did not show any significant differences (Figure 9).

Table 1: Absorbance Values of ELISA Experiment**.** The PBMCs were isolated from the buffy coats of healthy donors and CD4+ CD45RA+ cells were sorted from the PBMCs and cultured under Th17 polarizing conditions for a period of 7 days, complete IMDM media was used for negative control conditions. The cell culture supernatants were collected on day 3, day 5 and, day 7. The absorbance values were measured at 450 nm and are means of triplicates. The absorbance value for blank was 0.0565.

OD450	**Day 3**	**Day 5**	**Day 7**
Negative Control	0.067	0.0605	0.053
Th17	0.067	0.0595	0.0475
Negative Control - Blanked	0.0105	0.004	-0.0035
Th17 - Blanked	0.0105	0.003	-0.0035

Table 2: Calculated Concentrations of sFasL. The PBMCs were isolated from the buffy coats of healthy donors and CD4+ CD45RA+ cells were sorted from the PBMCs and cultured under Th17 polarizing conditions for a period of 7 days, complete IMDM media was used for negative control conditions. The cell culture supernatants were collected on day 3, day 5 and, day 7. The absorbance values were measured at 450 nm and were used with the standard curve to interpolate the concentrations. The concentrations are in nanograms per milliliter (ng/mL)

ng/mL	**Day 3**	**Day 5**	**Day 7**
Negative Control	0.501	0.473	0.387
Th17	0.465	0.387	0.371

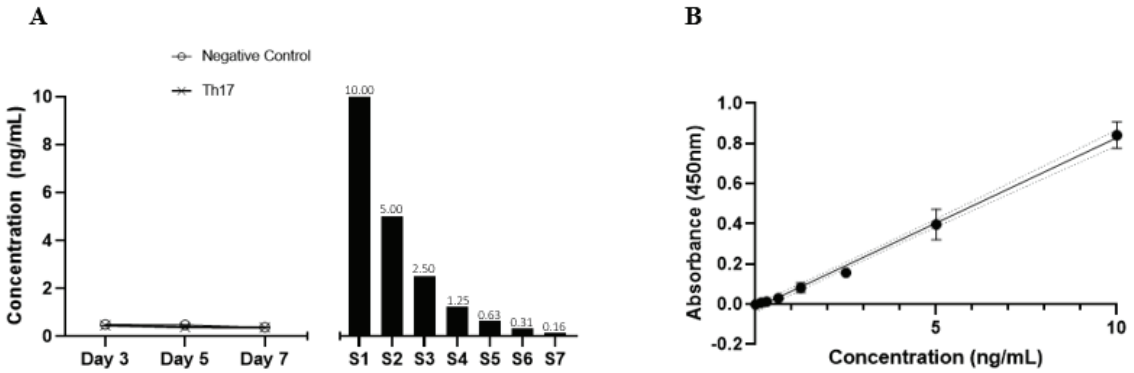

Figure 9: Soluble Fas Ligand Concentrations. The cell culture supernatants were collected in day 3, day 5 and, day 7. The absorbance values were measured at 450 nm and were used with the standart curve to interpolate the concentrations. The concentrations are in nanograms per mililiter (ng/mL). The line graph shows the calculated concentrations of sFasL and the bar garph shows the concentrations of the standarts (A). The standart graph was used to interpolate the concentrations of the samples (R-squared: 98,36)(B). The data are representative and were analyzed with 2-way ANOVA by using GraphPad. (n=3).

3.9 Determination of TNFR1 Expression

To examine whether other members of the tumor necrosis factor receptor superfamily show changes in the expression in a similar fashion to Fas, the expression of Tumor Necrosis Factor Receptor 1 (TNFR1) was also monitored throughout the culture at day 3, day 5 and, day 7. TNFR1 is known to take part in survival mechanisms via ligation of its receptor TNFα, which was also monitored. The cells were stained with anti-CD4 and anti-TNFR1 fluorochromes and analyzed with flow cytometry at day 3, day 5 and, day 7. In Figure 9, the flow cytometry data shows that there was no TNFR1 expression in neither the $CD4^{+}CD45RA^{+}$ T cells cultured under Th17 polarizing conditions nor the negative control cells. The data were analyzed with 2-way ANOVA by using GraphPad (n=3).

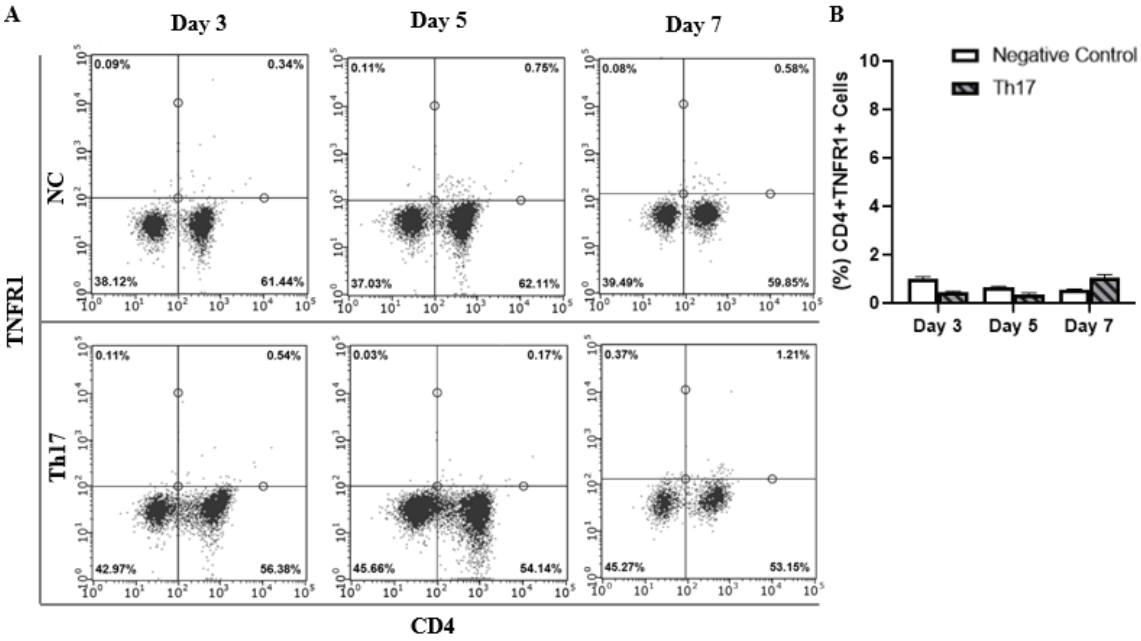

Figure 10: Determination of TNFR1 Expression. The PBMCs were isolated from the buffy coats of healthy donors and $CD4^+$ $CD45RA^+$ cells were sorted from the PBMCs and cultured under Th17 polarizing conditions for a period of 7 days, complete IMDM media was used for negative control conditions. The cells were stained with anti-CD4 and anti-TNFR1 fluorochromes and analyzed with flow cytometry at day 3, day 5 and, day 7 (A). The data are representative and were analyzed with 2-way ANOVA by using GraphPad. (n=3) (B).

3.10 Determination of TNFα Expression

Similar to the investigation of Fas and FasL expression, the expression of TNFα was also investigated along with TNFR1 on day 5 and day 7 of the culture to determine whether TNF signaling pathway takes part in the longevity of Th17 cells or not. The secretion pathway of the cells was inhibited with GolgiStop™ and the cells were stained with anti- TNFα fluorochrome to determine the expression of TNFα. In Figure 10, it can be seen that there were expressions on both the cells cultured under Th17 polarizing conditions and the negative culture. However, the statistical analysis showed that there

were no significant differences between the two cultures. The data were analyzed with 2-way ANOVA by using GraphPad (n=3).

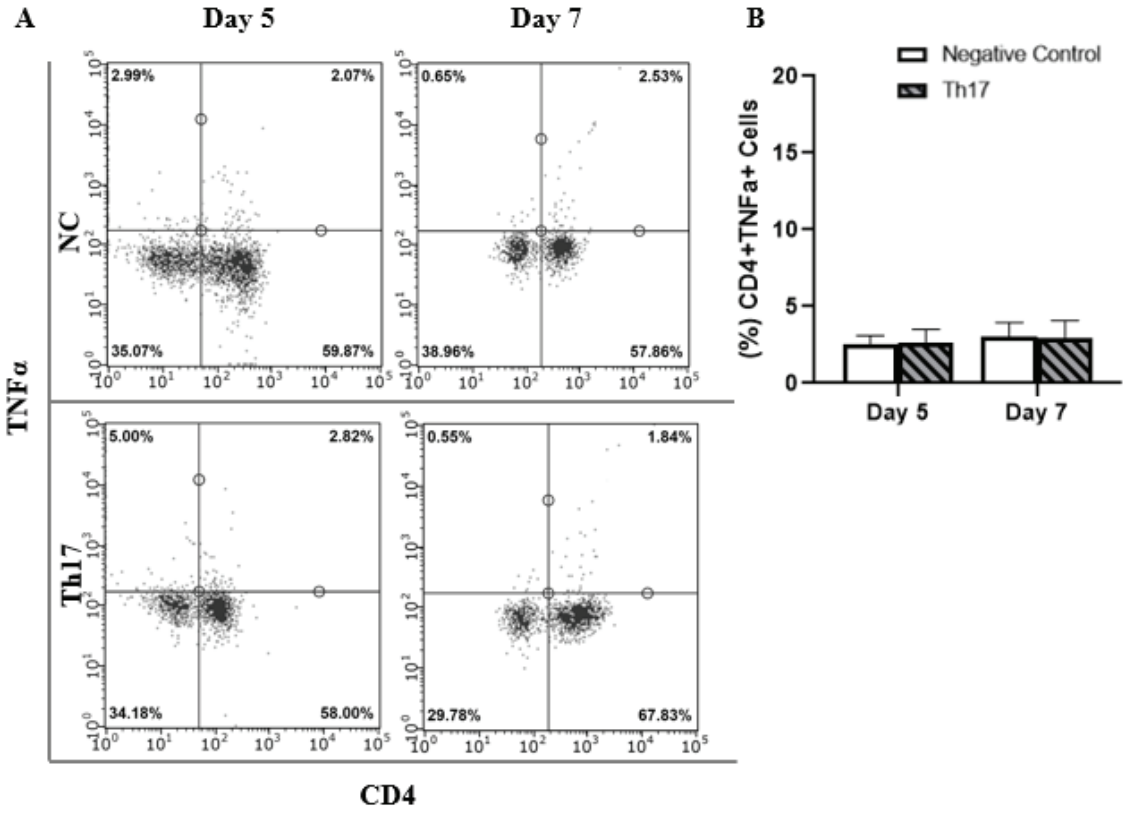

Figure 11: Determination of TNFα. The PBMCs were isolated from the buffy coats of healthy donors and $CD4^+$ $CD45RA^+$ cells were sorted from the PBMCs and cultured under Th17 polarizing conditions for a period of 7 days, complete IMDM media was used as negative control conditions. The cells were stained with anti-CD4 and anti-TNFα fluorochromes and analyzed with flow cytometry at day 3, day 5 and, day 7 (A). The data are representitive and were analyzed with 2-way ANOVA by using GraphPad (n=3)(B).

CHAPTER 4

DISCUSSION

Th17 cells are one of the most important key players of the immune system. They take part in various adaptive immune responses, pro-inflammatory responses, recruitment of neutrophils and regulation of neutrophil functions, responses to extracellular parasites, and allergic reactions. Th17 cells are characterized by their signature cytokine, IL-17. IL-17 is responsible for the production of pro-inflammatory cytokines on the target cells and also acts as a chemoattractant in the recruitment of neutrophils. Th17 cells were also found to play role in the pathologies of several autoimmune diseases, for example, Multiple Sclerosis and Rheumatoid Arthritis. Other lymphocytes are eliminated after they complete their role as effector cells, but it is not the case for Th17 cells. Th17 cells can infiltrate into the inflammation zone and can survive for long periods of time. Their role in the pathologies of autoimmune diseases is thought to be caused by their longevity. However, the reason behind their longevity is unknown. In the pursuit of this knowledge, the Fas signaling pathway was found to be a possible player of this mechanism. When first discovered, Fas was identified as a death receptor, a manifester of cell death. Upon binding to its ligand, FasL, transduces the signals that eventually lead to the death of the cell. However, the findings of the researches done over the decades have shown that cell death is not the only role of the Fas signaling pathway. Various studies have shown the activation of the Fas signaling pathway was found to increase cytokine production in helper T cells and the impairment of the DISC formation would prevent TCR-mediated activation of mice lymphocytes. The stimulation of the Fas signaling pathway in specific concentrations would increase cell cycle progression, increase the expression of activation markers and, the production of IL-4, IFN-γ, and TNF-α, which are signature cytokines of Th1 and Th2 cells, respectively. However, how the Fas signaling pathway affects the Th17 cell development is not. This thesis aims to investigate the effects of Fas-Fas Ligand interactions on Th17 cells and their functions.

In order to determine the effects of the Fas signaling pathway on Th17 cells, the PBMCs were isolated from the buffy coats of healthy individuals. PBMCs consist of various cell types and populations, as seen in Figure 1, and were sorted to obtain $CD4^+$ $CD45RA^+$ naive T cells, as seen in Figure 2. These $CD4^+$ $CD45RA^+$ naive T cells are cultured under Th17 polarizing conditions for seven days. These conditions include anti-CD3 and anti-CD28 to simulate TCR-mediated activation, IL-6, IL-23, TGF-ß, and IL-1ß to provide the required signals for Th17 polarization, and anti-IL-4 and anti-IFN-γ neutralizers to prevent Th1 and Th2 polarization. During the culture, on days 3, 5, and 7, the cells were stained with various fluorochromes to determine the expressions of several proteins are markers. On day 3, the expression of CD69 was determined to track the early activation of the cells and on day 5, the expression of CD25 was determined to track the late activation of the cells, as seen in Figure 3. On day 7, the expressions of RORC and IL-17A were investigated to determine whether the cells achieved the Th17 phenotype or not, as seen in Figure 4. Also, on these days, the expressions of Fas, FasL, TNFR1, and TNF-α were analyzed along with the monitoring of apoptosis with Annexin V and 7AAD, since Fas and TNFR1 are death receptors.

The results have provided precious information regarding the expression levels of Fas, TNFR1, and TNF- α. The expression of Fas was monitored during the culture via flow cytometry. The cells were stained with anti-CD4 and anti-Fas antibodies that were conjugated with fluorochromes. In Figure 5, it can be seen that the expression of Fas increases over time in the cells cultured under Th17 polarizing conditions. This was expected and on par with the previous studies done on this field (Kryczek et al., 2011). Compared to the negative culture, which consists of $CD4^+CD45RA^+$ naive T cells that were cultured in the only medium, the increase of Fas expression was significantly high on every time point investigated. Also, between days zero, 3, 5 and, 7, the expression of Fas in the cells cultured under Th17 polarizing conditions showed a significant increase compared to the previous time point. Then the apoptosis levels in the cultures were monitored since the increased expression of Fas could have meant increased apoptosis, since Fas is a death receptor. However, as seen in Figure 6, on days 3, 5, and 7, the levels of apoptosis did not show any difference between the negative culture and Th17 polarizing culture. This meant there was no effect of increased Fas expression on the apoptosis, hence, Fas increase was due to something else. This trend was also observed when only the apoptosis levels of cells that express both CD4 and Fas were monitored,

as seen in Figure 7. There was no difference between the two cultures either. The increase of Fas does not prove anything if there were nothing to stimulate it. To determine the expression levels of FasL, the cells were stained with anti-FasL antibody conjugated with fluorochrome on days 5 and 7. However, the staining procedure required the inhibition of Membrane Metalloproteases to prevent the cleavage of FasL from the cell membrane, which was not used during the experiment. As seen in Figure 8, there was no FasL expression observed. This could have meant two things, there were no FasL expression on the cells or the FasL was being expressed but cleaved with MMPs and provided signaling as sFasL molecules. To determine that, the culture supernatants were gathered and were tested for the presence of sFasL with ELISA. However, the results of ELISA have shown that the levels of sFasL found in the cell culture supernatant were very low. In Table 1, it can be seen that the absorbance values are very close to the blank value and when blanked, almost zero or below zero. In Table 2, it can be seen that the calculated concentrations of sFasL were also low. It was not a surprise that Th17 cells express very low levels of FasL and sFasL, as it was discovered that the murine Th17 cells had very low sFasL in both the mRNA level and the protein level. (Cencioni et al., 2015) To determine whether the lack of apoptosis was due to survival mechanisms that were activated through TNFR1 or not, the expressions of TNFR1 and TNFα were monitored on days 3, 5, and 7. As seen in Figure 9, neither the cells cultured under Th17 polarizing conditions nor the negative control cells expressed TNFR1. However, TNFα expression was detected on both of the cultures, as seen in Figure 10. However, there was no significant difference between the two cultures in terms of TNFα expression.

Overall, the data obtained from this research shows that the expression of Fas increases in the $CD4^+CD45RA^+$ naive T cells cultured under Th17 polarizing conditions, and this does not affect the apoptosis levels and this is not due to the survival mechanisms activated by TNFR1 since there was no TNFR1 expression, even though there was TNFα expression.

4.1 Future Perspective

To further investigate the possible non-apoptotic functions of Fas in Th17 cells, there are several points to consider. First of all, the results have shown that there were no FasL, so the lack of apoptosis could be due to a lack of Fas stimulation. The cells can be stimulated

with FasL artificially and can be investigated on similar terms. Second, to determine how these cells do not undergo apoptosis, the expression of TNFR2 on Th17 cells can be monitored. Similar to TNFR1, TNFR2 can be stimulated with TNF-α, which was shown to be expressed on Th17 cells, and also with TNF-ß, which can also be monitored. Third, it was shown that the levels of caspase-8 increase during the activation of lymphocytes, which can lead to an increase in the levels of c-FLIP that can activate the NFkB pathway (T. Kataoka et al., 2000; Kennedy et al., 1999). For that, the levels of NFkB can be investigated. Lastly, the proliferation of Fas^+ cells can be monitored to determine whether the expression of Fas affects the proliferation or not.

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