EXPRESSION OF AQUAPORIN 1, 3, AND 4 IN T CELL ACTIVATION AND APOPTOSIS

A Thesis Submitted to
the Graduate School of Engineering and Science of
Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

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June 2018

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ACKNOWLEDGMENTS

I would like to express my gratitude to my advisor Assoc. Prof. Dr. NALBANT ALDANMAZ for his suggestions, guidance, encouragement and support throughout my Msc. study. I also would like to thank her for his generosity for providing materials and laboratory facilities.

This work was supported by a grant from the Scientific and Technological Research Council of Turkey (TÜBİTAK) (Project #215Z127 to Dr. Ayten NALBANT). Ayşe Bengisu GELMEZ received monthly stipend from this funded project.

I also want to thank the committee members, Prof. Dr.Volkan SEYRANTEPE and Assoc. Prof. Dr. Ayşe NALBANTSOY due to their time and support for my thesis.

I have a great appreciation for Dr. Günel ALİZADE, who play a irreplaceable part in my education in the way of being scientist and also support me in any time as my friend. I want to thank my co-worker and friend Seminay GÜLER for sharing my good and bad times in this journey. I would express my appriation to my precious friends Dr. İprk ERDOĞAN, Bilge YAYLAK, Osama SWEEF, and Cansu GÜRER for their supports and friendships. Each one of them is always with my side to support me and to share their knowledge and love every time. I also thank to our research student, Tuğçe ÇIMEN to asist me in my experiments during last four mounts of my thesis. I also appreciate many of my friends that came into my life, bring their love and provide me with their support and encouragement to continue on my path.

Finally, I want to express special thanks to my mother Tülay KAYA, my grand mother Hatice KAYA, my grand father Haydar KAYA, and my father Muhammet GELMEZ for their love, encouragement, patience and support during my education.

ABSTRACT

EXPRESSION OF AQUAPORIN 1, 3, AND 4 IN T CELL ACTIVATION AND APOPTOSIS

Aquaporins (AQPs) are membrane proteins responsible for transporting water, some gases and small solutes such as CO_2 and glycerol. Until now, it has been shown that AQP1, 3 and 5 expressed in both B and T lymphocytes of mice, regulate cell volume. However, aquaporin expression involved in activation, proliferation, and differentiation as well as apoptosis of T cells are not well known yet. The goal of this study is to detect the expression level of AQP1, AQP3, and AQP4 in activated and apoptotic T cells. In order to do that, two types of T cells cultured in both condition were utilized. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from Human peripheral blood drawn from healthy donors by ficoll density gradient Centrifugation method. Naive $CD4^+$ T cells were sorted from PBMC. The stimulants generating Th17 were chosen for activation and differentiation of naïve CD4 T cells. Jurkat cell line as a second cell type were activated by PMA/Ionomycin as well as treated by camptothecin for apoptotic processing. Th17 and Jurkat cell cultures were analysed by flow cytometry to measure the rate of both activation and apoptosis. Western Blot was performed to identify expression of AQP 1, 3 and 4. We found a significance between increased expression level of AQP1, 3, and 4 in activated T cells as well as decreased expression level of each three AQPs in apoptotic T cell populations. According to our findings, tested aquaporin proteins may play roles in T cell activation, differentiation, and apoptosis. The scientific significance of this research is that it can fill the gaps about these three functional processes of T cells. Besides, all findings can contribute to treatment of many autoimmune disease like MS which Th17 cells involve in pathogeny.

ÖZET

AQUAPORIN 1, 3 VE 4 PROTEİNLERİNİN T HÜCRESİ AKTİVASYONU VE APOPTOZUNDA EKSPRESYONLARI

Aquaporinler suyun, CO_2 gibi bazı gazların ve gliserol çözünmüş küçük maddelerin transferinden sorumlu olan hücre zarı proteinleridir. Şu zamana kadar, AQP1, 3 ve 5 proteinlerinin hücre hacmini düzenlemek için B ve T lenfositlerinin ikisinde de ekspresyonun olduğu farelerde gösterilmiştir. Bununla birlikte, bu aquaporinlerin T hücrelerinin aktivasyonundaki, çoğalmasındaki, farklılaşmasındaki ve apoptozdaki katkısı henüz açıklığa kavuşmamıştır. Bu çalışmanın amacı, AQP1, AQP3 ve AQP4 proteinlerinin aktive edilmiş ve apoptotik T hücrelerindeki ekspresyon seviyelerini saptamaktır. Bunu yapabilmek için farktı koşullarda bulunan iki tip T hücresi kullanılmıştır. Sağlıklı donörlerden alınan kanlardan PBMC, ficoll yoğunluk tırmanışında çeperleme yöntemi ile izole edilmiştir. Naif $CD4^+$ T hücreleri bu PBMC' den ayrılmıştır. Naif $CD4^+$ T hücrelerinin aktivasyonu ve farklılaşması için Th17 oluşturan uyaranlar seçilmiştir. İkinci hücre tipi olarak Jurkat hücre hattı PMA/Ionomycin ile aktive edilmiştir ve apoptotik süreç için camptothecin ile muamele edilmiştir. Aktivasyon ve apoptoz oranlarını ölçmek için Th17 ve Jurkat hücre kültürleri flow sitometride analiz edilmiştir. AQP1, 3 ve 4 proteinlerinin ekspresyonunu belirlemek için western blot yapılmıştır. AQP1, 3 ve 4'ün artan ekspresyon seviyeleri ile aktive edilmiş T hücreler ve azalan ekspresyon seviyeleri ile apoptotik T hücre popülasyonu arasında anlamlılık bulduk. Bulgularımıza göre, test edilen aquaporin proteinleri T hücrelerinin aktivasyonunda, farklılaşmasında ve apoptozunda rolü olabilir. Bu araştırmanın bilimsel önemi, T hücrelerinin aktivasyon ve farklılaşma süreçleri hakkındaki boşlukları doldurabilir olmasıdır. Bunla birlikte bütün bulgular Th17 hücrelerinin de dahil olduğu MS gibi otoimmün hastalıklarının tedavisine katkı sağlayabilir.

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CHAPTER 1

INTRODUCTION

1.1. T lymphocytes

Human body comprises a powerful system to defence against components of microbes, as well as chemicals, proteins, and polysaccharides considered as foreign substances called immune system. The initial step of this protection starts with the recognition of these pathogens by the cells of innate immune system. After presentation of particles of foreigners to the lymphocytes by antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages, second part of immune response, called as antigen specific response, begins. Binding a specific antigen to the own receptors results in clonal expansion, and differentiation of lymphocytes self from non-self to eliminate self-reactive lymphocytes. This system is especially important for choosing the direct target and preventing the uncontrolled reaction to the other cells or tissue. (Abbas and Lichtman, 2015; Chaplin, 2010).

Under the obligation of the recognition a large pool of antigens by the immune system while distinguishing foreign from self has a fundamental role in the defence of the body. All mononuclear blood cells express certain surface glycoproteins which are generally utilized for characterization such as CD14 and CD19 for monocytes and B cells, respectively. T lymphocytes (T Cells) which generally express the CD3 glycoprotein as a signature, contain antigen receptors on the surface to provide response specificity for harmful substances. The ability of responding to wide range of antigens confers a master degree on T cells as a main regulators of cell-mediated immunity. The major subsets of T Lymphocytes are $CD8^+$ T cells (CTLs) and $CD4^+$ T helper cells which both have antigen receptors called T cell receptors (TCRs). The activation of T cells starts with binding of an antigen to the specific TCRs, and continues with the signal transduction cascades necessary for the activation of transcription factors responsible for differentiation, proliferation and producing different cytokines which are needed for activation and regulation of other members of immune system. (Abbas and Lichtman, 2015; Broere et al., 2011).

1.1.1. $CD8^+$ Cytotoxic T Cells

Cytotoxic T Cells which are characterized by expressing the CD8 glycoprotein as a co-receptor for activation. After effector – target cell connection, CTLs kill infected cells and tumours by expressing cell death receptors (FAS ligand, Apo1L) and releasing some cytotoxins and cytokines such as IFN-γ and tumour necrosis factor-α (TNF - α) that induce apoptosis. Major histocompatibility complex 1 (MHC – 1) found in all eukaryotic cells presents the antigen of invaders such as viruses or cancerogenic peptides to CTLs. This process results in the activation of CTLs through T cell receptor (TCR). That's why destroying mechanisms of CTLs have target specificity. (Andersen M.H. et al., 2006)

1.1.2. $CD4^+$ T Helper Cells

T helper (Th) cells which also express the CD4 glycoprotein in addition to CD3, take a crucial part in establishing and maximizing the capabilities of both innate and adaptive immune response**.** The main part of their duties consist in activating macrophages, directing neutrophils, basophils, and eosinophils to the site of the inflammation as well as helping B cells to produce antibodies. By the secretion of definite cytokines and chemokines, helper T cells act like a maestro to coordinate immune response. (Geginat et al., 2013)

Administration of immune response by $CD4^+$ helper T cells requires their TCRs to engage with class II major histocompatibility complex (MHC II) which contains antigen is situated on APCs. The cytokines existing in the environment guide naïve T cells to turn into effector T cells expressing CD45RA glycoprotein and memory T cells expressing CD45RO glycoprotein during the activation process. In the presence of different cytokines naïve T helper cells can turn into specific subunits which have their unique cytokine profiles and functional properties, including Th1, Th2, Th17, and Treg, as well as Tfh, Th22, and Th9 cells. Each Th cell subtypes is destined to express own surface markers and to release characteristic cytokines that determine their effector cell function (Zhu and Paul, 2008; Raphael et al., 2015).

One of the first discovered Th cell subsets is Th1 cell which is an exemplary cell type participate in cell mediated immune response and delayed – type

hypersensitivity reactions. The main subject of these cells is promoting cellular immune mechanism against intracellular pathogens such as viruses and intracellular bacteria. In addition to the main secreted cytokines of Th1 cells are IFN-γ and IL-2, it is found that TNF, lymphotoxin, and granulocyte and macrophage colony stimulating factor (GM-CSF) are produced to activation of B cells and macrophages. The transcription factor expressed by Th1 effector cells is T-bet working with other factors and signals such as IFN-c/STAT1 signalling, IL-2/STAT5 signalling, IL-12/STAT4 signalling and strong T cell receptor (TCR) signals throughout differentiation (Szabo et al., 2000). After discovery of Th1, Th2 has been identified. The essential targets of these cells includes parasites effecting several cells and their allergenic particles that cause atopic illnesses. The identifier cytokines produced by Th2 are IL-4, IL-5 and IL-13, as well as IL-9 and IL-10. Th2 cells differentiation is mainly promoted by IL-4 which in turn initiates the phosphorylation of STAT6. The expression of nuclear transcription factor GATA-3 is generally considered as the main marker of Th2 cells (Ho et al., 2009). However Th1 and Th2 cells have own specific targets as well as own products, both of them are responsible for enrichment of $CD8^+$ cytotoxic T cell (CTLs) number and their functions counter to Treg cells. Treg cells known as regulatory or suppressor T cells function as a protector the body against unnecessary presence of other defenders by increasing immune tolerance and providing homeostasis of immune system. In order to do that Tregs suppress activity of $CD4^+$ T cells and $CD8^+$ T cells as they overpower B and dendritic cells. The characteristic expression marker of Treg cells is forkhead box P3 (FOXP3) which is necessary for Treg development and function. Tregs is able to produce soluble messengers which play roles as suppressors, including TGF-beta, IL-10 and adenosine (Kondělková et al., 2010).

Another subset of T helpers is Th17 which produces IL-17 cytokine family. It is known that Th17 cells involves in host defence against fungi and other eukaryotic pathogens, as well as extracellular bacteria. In addition to its protective functions, Th17 accompanied with IL-17 cytokines are discovered in the wide range of inflammatory conditions and the pathology of autoimmune diseases (Stockinger et al., 2007*)*. Follicular helper T cell (Tfh) is another $CD4^+$ T cell subset which ıts identification has been recently done. Tfh cells are focussed on supporting B cells, essential for a different of procedures including producing antibody, affinity maturing, class exchanging, and differentiation of effectors into memory B cells. Tfh cells migrate as a result of the expression of chemokine receptor called CXCR5 to the site of B cells, and initiation of

the main regulatory protein Bcl6 is necessary for Tfh differentiation process (Crotty 2014). Lastly, two unusual subsets, Th22 and Th9 have also recently joined in $CD4^+$ T helper group. In addition to sharing some mutual properties with others such as expressing IL-22 and IL-9, they also have own role especially participating in certain diseases (Raphael et al., 2015).

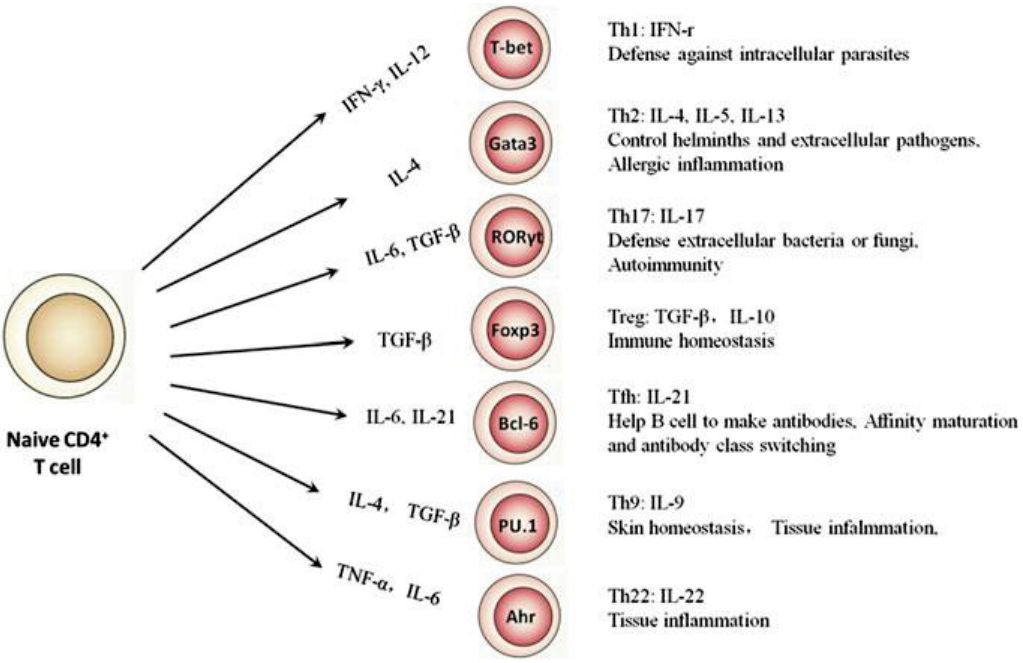

Figure 1.1 Differentiation of naive CD4+ T cells into different T-helper-cell subsets (Source: Sun et al., 2014)

1.1.3. T Cell Activation

T lymphocytes are main players in acquired immune response against different pathogens such as bacteria, viruses, and fungi as well as other eukaryotic parasites. In order to become effectors, T cell activation is needed. The activation process initiates different responses such as differentiation, proliferation, cytokine production, migration as well as apoptosis. Because of the presence of inappropriate and uncontrolled activation resulting in autoimmune diseases and an incorrect response against invaders, this mechanism needs to consist of complex signalling cascades.

Two main signalling pathways are required for complete T cell activation. The first signal comes from antigen bounded MHC II displayed on the surface of APCs. The TCR complex is a heterodimer protein composed of six polypeptides. The specific ligand binding is provided by α and β chains as a result of genetic rearrangement making available to numerous receptor variants. After the engagement between MHC II and external part of TCR, the activation signal is transmitted to initiate internal signal transduction by the constant part of the complex: the γ, δ, ζ chains, and the CD3 complex. The signal is started by the activation of tyrosine kinases protein family called Src. This kinases phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) placed on the ζ and CD3 subunits. The phosphorylation cascade continue with the activation of ZAP-70 which is another tyrosine kinase. ZAP-70 promotes the activation of LAT and SLP-76 respectively. The activation of these proteins leads to the phosphorylation of phospholipase Cγ1 (PLCγ1) which is responsible for the formation of IP_3 and DAG proteins, in turn initiating Ca^{+2} flux, PKC and Ras cascades. In order to provide second signal, it is necessary that co-receptors, CD28 or CD4 bind with their counterparts on APCs. The stimulation of these two receptors support the phosphorylation cascades such as PIP2 – PIP3 – Akt signalling which activates NF-kB transcription factor (Figure 1.2) (Cantrell 2015; Smith-Garvin 2009).

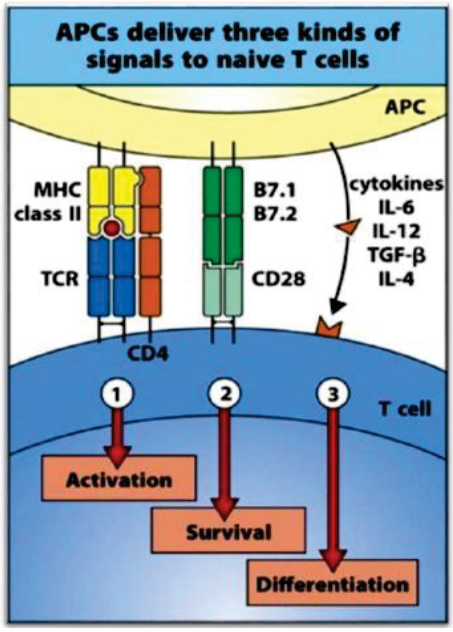

Figure 1.2. T cell activation through TCR ligation with MHC II and co-stimulatory pathway. (Source: Janeway's Immunobiology, 8th Edition)

1.1.4. Th17 cell Differentiation

However the presence of IL-17 cytokine family, especially IL-17A and IL-17 F, has been discovered for many years, the existence of IL-17 – producing T cells was identified few years ago under the pathological conditions. The main function of Th17 cells is providing host defence against extracellular bacteria, fungi and parasites by bridging innate and acquired immunity. In addition to this, it is shown that Th17 cells are involve numerous autoimmune diseases such as encephalomyelitis (EAE), collagen-induced arthritis as well as neuromyelitis optica (NMO) (Annunziato et al. 2007).

The differentiation of naïve $CD4^+$ T helper cells into Th17 takes place under the presence of several cytokines such as TGF-β, IL- 6, and IL-23. After stimulation, specific genes and protein expression are also needed to complete Th17 transformation (Harrington et al. 2005).

TGF-β is one of the essential cytokine that involves Th17 differentiation. It is a member of tumour growth factors which is also relevant with the cancer development. It is demonstrated that TGF-β levels is directly correlated with the expression rate of RORγt which is main transcription factor of Th17 cells. RORγt expression reaches the highest level in the existence of TGF-β together with IL-21. On the other hand less amount of TGF-β inhibits the differentiation of Th17 as a result of the induction of FOXP3 which is antagonist of RORγt (Ivanov et al. 2006; Yang et al, 2008).

Second important cytokine participating in Th17 formation is IL-6. According to researches, only TGF-β is not enough to induce Th 17 differentiation. In order to obtain complete differentiation, the teamwork is necessary between TGF-β and IL-6. In the presence of TGF-β, IL-6 is responsible for the suppressing of FOXP3 expression that leads to the generation of Treg (Lim et al. 2008).

IL-23, generally known as one of pro-inflammatory cytokines, is also take place in Th27 differentiation. The main function of IL-23 is expansion and proliferation of Th17 cells instead of involving the polarization process. IL-23 belongs to IL-6 cytokine family and contains p19 subunit coupled with p40 unit of IL-12. The expression of IL-17 is also induced after IL-23 is activated during Th17 formation. As a result of the increased level of IL-17, monocytes and neutrophils are recruited to the inflammation site. In addition to this, IL-23 also stimulates the production of GM-CSF

which is responsible for the accumulation of more innate immune cells (Stockinger et al. 2007).

1.1.5. Jurkat Cells

Jurkat cells also called JM are specific subtypes of T lymphocytes first collected from the peripheral blood of a boy suffering from leukaemia. Jurkat cells have an ability for the production of interleukin-2 (IL-2). As a result of its properties, Jurkat cells are preferred to investigate TCR signalling and T cell activation. Jurkat cells also become useful in other areas such patterns of gene expression, viral interactions, and cancer biochemistry, among others (Schneider et al. 1977).

1.2. Apoptosis: Programed Cell Death

1.2.1. Description and Morphological Characterization of Apoptosis

Programmed cell death, also named as apoptosis, regulates the number of cells in the tissues and also plays an important role in development and aging in normal physiology. The initiation of apoptosis is depended on the activation of cysteine Proteases known as Caspases triggered by receptor-ligand binding externally and/or toxic drugs or radiation internally (Blank and Shiloh 2007).

Characteristic morphological changes has been demonstrated in the cells that undergo apoptotic process such as phosphatidylserine accumulation on membrane surface, physiological changes of membrane such as blebbing, and cellular shrinkage, as well as chromatin condensation and fragmentation of nucleus (Jin and El-Deiry 2005; Blank and Shiloh 2007).

1.2.2. Pathways of Apoptosis

The induction of apoptosis depends on two main pathway: activating the apoptotic receptors (the extrinsic pathway) and/or mitochondrial stimuli (the intrinsic

pathway). Apoptotic cell death is initiated externally by the connection between death receptors and their ligands. The members of tumour necrosis factor (TNF) superfamily such as Fas, TRAMP, TNFR 1 and 2, TRAILR 1 and 2 are activated by binding of their specific ligands and start the caspase phosphorylation cascades, including the activation of initiator caspases 2, 8, 10 and effector caspases 3, 6, and 7, leading to apoptosis (Blank and Shiloh 2007).

Stress-induced apoptosis as an intrinsic pathway depends on cytochrome c releasing from mitochondria and the release of Ca^{+2} from endoplasmic reticulum (ER). In mitochondrial pathway, the unleashed cytochrome c binds and activates apoptotic protease-activating factor 1 (Apaf1) complex resulting in the activation of caspase 9. Caspase 9 is one of the initiator caspase that activates effector caspases the mitochondrial pathway is controlled by Bcl 2 protein family consisting of pro-apoptotic, anti-apoptotic, and BH3-only proteins. The BH3-only subsets, including BAD, BID, BIM, NOXA, BIK, HRK, and PUMA promotes the pro-apoptotic effectors BAX and BAK resulting in release of cytochrome c from mitochondrial intermembrane. On the contrary, the anti-apoptotic members (Bcl-2, Bcl-XL, Bcl-X, and BAG) prevent apoptosis by blocking BAX-BAK activation on pore formation (Figure 1.3) (Gross et al. 1999; Hardwick and Soane 2013; Czabotar et al. 2014).

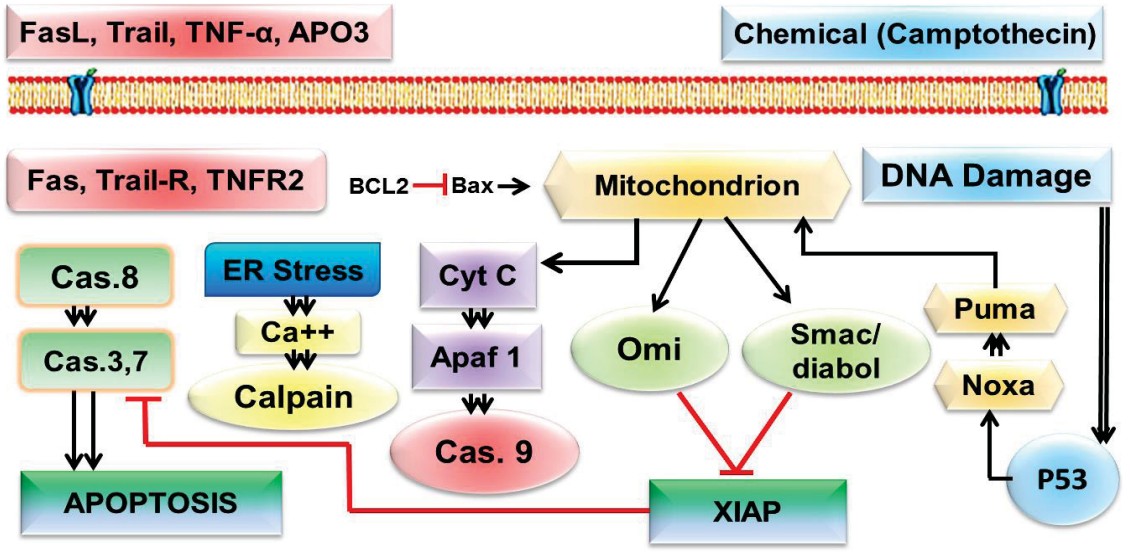

Figure 1.3. Pathways of apoptosis regulation. Binding of specific ligands to their death receptor initiates the extrinsic pathway. On the other hand, Bcl-2 family proteins are induced by several reasons and trigger the intrinsic pathway.

Camptothecin (CPT) is an antitumor extract of plant *Camptotheca acuminate*, used as topoisomerase 1 (TOP1) inhibitor in human. This chemical is generally utilized for triggering apoptosis in immune cells. As a result of inhibition of topoisomerase 1 which is responsible for the relaxation of DNA supercoils by single strand breaks allowing the rotation of DNA double helix during transcription and chromatin replication, the regeneration of double helix is blocked and the production of p53 is elevated. The p53 protein, known as a tumour suppressor, regulates the activation of pro-apoptotic Bcl 2 family and BH-3 only members. Together with the activator role in apoptotic Bcl-2 proteins, p53 also inhibits anti-apoptotic Bcl 2 protein family and induces the mitochondrial pathway of apoptosis (Pommier et al. 2003; Pommier 2006; Sanchez-Alcazar 2000).

Apoptosis plays a crucial role in immune system regulation, especially in T lymphocyte homeostasis after activation. All activated T cell subsets must be eliminated from the circulation in order to prevent uncontrolled function resulting in autoimmune disease and malignance. It is also known as activation-induced cell death (AICD) which is the main mechanism of apoptosis in T cell. AICD is initiated as soon as activation process by the ligation of several apoptotic receptors and their ligands such as Fas/FasL (Alderson et al. 1995).

1.3. Aquaporin Proteins

Aquaporins (AQPs) are transmembrane proteins from a family of major intrinsic proteins (MIP) which are divided into two subfamilies; Aquaporins as water channels and Glp (also called aquaglyceroporins in mammals) as glycerol facilitators. The presence of these water channels increase the water permeability of the cell membrane. The primarily importance of aquaporins is the maintenance of osmotic balance which is in parallel with cell survival. That is the reason that they are found in all kingdoms of life including bacteria, yeasts, plants and animals (Finn R. N. and Cerdà J. 2015). Up to now, 13 different AQPs playing a part in many cellular events during normal or pathological condition, have been identified in mammals. Transport of water through a channel are discovered in the early 1980s. In the mid-1980s Peter Agre and colleagues (1991) specifically isolated these channels from the red blood cells and the kidney epithelial cells. Then, in 1991 they resolved the structure of this protein and

called Aquaporin. This discovery brought the Nobel Prize to Peter Agre in 2003 (Agre P 2006; Kitchen et al. 2015).

1.3.1. Structure and Subtypes of Aquaporins

Aquaporins are about 30 kDa proteins embedded in the cell membrane to regulate the flow of water. The high-resolution structures of aquaporin family have been identified by using electron diffraction and X-ray diffraction. Studies showed that aquaporins are homotetrameric assemblies of four identical monomers. Each of them behaves as a water channel. The monomers interacts with other two monomers near them. All of four contiguous monomers form the tetramer structure called central pore that has a role of removing extra water molecules out. Supercomputer simulations showed that the central pore also transmits some gases through cell membrane and is a potential transporter for certain cations. As a result of further analyses, it has been identified that the central pore facilitates the conduction of some gases such as CO_2 and O_2 (Figure 1.4) (Agre et al. 1993; Kreida and Törnroth-Horsefield 2015).

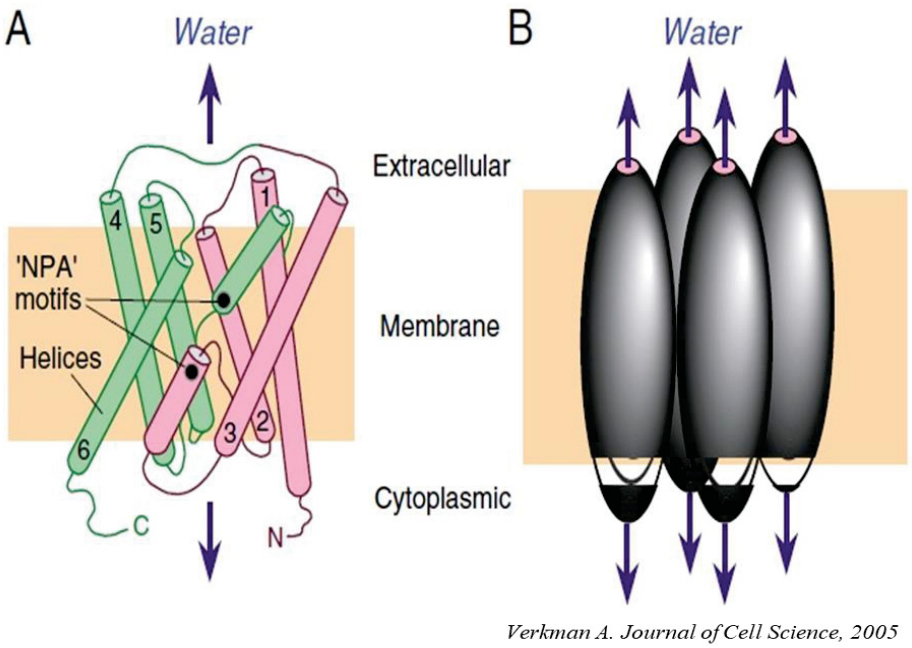

Figure 1.4. Structure of Aquaporin. A. Monomer of aquaporin composed of six transmembrane α helices and two hemi-helices consist NPA motifs. B. Aquaporin tetramer. Four monomers forming homotetramer, each consist of a pore which is selective water. The pore at the centre of homotetramer allows the passage of ions such as CO_2. (Source: Verkman A. 2005).

Mammalian AQPs have been grouped in three subclasses; pure AQPs including AQP0, 1, 2, 4, 5 and 8 which mainly select water, glycerol-selective aquaporins AQP3, 7, 9 and 10 called aquaglyceroporins, and Super-aquaporins (subcellular AQP) called AQP11 and 12. As a result of having structure like S-aquaporin class, AQP6 belongs to this group, however it is shown that AQP6 also transport some ions. (Gomes et al. 2009).

1.3.2. Mechanism of Water Passage and Selectivity

The channel selectivity and passage of the water are based upon the property of size and electrostatic field of the channel. The water-selective AQPs have a pore size of ~2.8Å which is nearly same with a water molecule. Substrate specificity and size restriction are determined by the loops B and E which consist of Gly, Ala and His side chains. This loops preceding the NPA (asparagine-proline-alanine) motif on the carbonyl group creates the hydrophobic domain which enables rapid conveyance of water molecules as a hydrogen-bonded single series. The former strong interaction between the water molecule and the pore occurs both at the site of two asparagine of NPA motif as well as at the hydrophobic residues (Phe, Val and Ile). Under the NPA motif, next contacting area formed by the aromatic side chains together with Arg (ar/R) which has positive charge. As a result of these two interaction sites, the influx of protons was prevented. Due to the positively charged area in the pore, the oxygen existing in the water molecule passes the channel close to the Asn-Ala-Pro side causing the water dipoles to pass the channel with 180^{o} rotation. (Wang and Tajkhorshid 2007; Kreida and Törnroth-Horsefield 2015).

1.3.3. Aquaporin Regulation by Gating and Trafficking

Structural analyses and knockout models showed that aquaporins are regulated by intracellular and/or extracellular signals. In this way, water transport can be controlled at the protein level by conformational change or by changing the distribution of aquaporins in the plasma membrane. Aquaporins can also be regulated at the transcriptional and translational level as well as protein trafficking between intracellular vesicles and the plasma membrane. The regulation mechanisms of mammalian AQPs

are proceeded by both gating and trafficking which control the passage of solutes. In addition to post-transcriptionally pathways, the mechanisms of aquaporin regulation are driven by intracellular and extracellular signals depending on changes in the environment or cytoplasm, such as pH rate or Ca^{+2} content. The gating mechanisms differ depending on the aquaporin types (Wang and Tajkhorshid 2007; Kreida and Törnroth-Horsefield 2015).

1.3.4. Aquaporin Function and Distribution in Human Body

The body expresses AQPs in diverse organs and cell types to utilize for different events such as homeostasis of brain water, secretion from glands, urine concentration, and skin moisturization, fat metabolism as well as neural signal transduction under normal physiology. For example, the well-studied organ about this is kidney utilizing AQPs to regulate urine production, AQP5 was needed for salvia secretion from salvia glands or AQP9 plays a role in glycogenesis in liver. On the other hand, it is understood that AQPs involve numerous processes under pathological situations. For instance, AQP4 is the main aquaporin expressed in brain which is responsible for water balance between brain and blood – brain barrier together with being involved in nerve plasticity and memory development. However, the over-expression of AQP4 in the conditions causing brain oedema results in worse outcomes (Day et al. 2014). There are also increasing evidence about AQPs placing in cancer development and metastasis. Studies with AQP1 knockout mice improve that up-regulation of AQPs support angiogenesis and the development of new blood vessels which effect tumour growth positively as a result of increasing the migration ability of cancer cells. With the ability for transporting glycerol, aquaglyceroporins also act as metalloid channels. It has been discovered that AQP7 and AQP9 are permeable to arsenic in the condition of arsenic poisoning. Another hypothesis about the functions of AQPs is the participation in the process of cellular death and apoptosis. In addition to their functions in different conditions and pathologies, AQPs are under investigation as potential drug targets to modulate the effect of AQPs on diseases (Day et al. 2014; Verkman 2005; Verkman et. al. 2014).

In this thesis, the main focus has been AQP1, AQP3, and AQP4, because it is predicted that these aquaporins may involve in T cell functions such as activation and apoptosis by direct or indirect ways. The presence of AQP1 and AQP3 in lymphocytes has been shown by Chulso Moon and his colleagues in 2004. Besides, it is known that AQP4 involves in different autoimmune diseases occurring in central nervous system such as Neuromyelitis Optica (Varrin-Doyer et al. 2012; Chulso et al. 2004). AQP1 is the predominant water channel distributed among almost every part of the body. In addition to water selectivity, AQP1 also facilitates CO_2, NH, H_2O_2, and NH_3. It is known that tumour angiogenesis and metastasis, nephrogenic diabetes insipidus, brain swelling, and glaucoma are related to AQP1 dysfunction. It has been shown that AQP1 takes a part in many physiological function such as cell cycle and proliferation as well as apoptosis (Galan-Cobo et al. 2015; Li et al. 2015) AQP3 primarily transporting glycerol was detected in kidney, brain, trachea, colon, urinary bladder, skin epidermis, red blood cells and immune system. AQP3, are also permeable to urea. Studies shows that AQP3 has function in lipid biosynthesis and the deficiency of AQP3 effects on some metabolic signal transduction such as MAPK which plays a role in the activation of NF-κB resulting in T cell activation. AQP4 is expressed predominantly in CNS, however, it is reported that AQP4 is expressed in mouse thymus, spleen, and lymph nodes and researches about autoimmune diseases especially in CNS proves that AQP4 acts like an autoantigen in autoimmune diseases or effects immune cells by changing their cell progresses (Day et al. 2014; Verkman 2005; Verkman et al. 2014).

The aim of this study is the determination of AQP1, 3, and 4 expression changes in both activated and apoptosis induced T cells. The expression levels of these three aquaporins were also investigated in Th17 cells compared with naïve $CD4^+$ T helper cells as s pre-information for future studies.

CHAPTER 2

MATERIALS AND METHODS

2.1. PBMC Isolation

The blood samples taken from healthy individuals were supplied by blood bank in Dokuz Eylül University with the permission of Ethical Committee. The technique of ficoll-hypaque density gradient centrifugation (Boyum et al., 1991) was utilized for the separation PBMCs from total blood. PBMCs contain complex populations of T cells, B cells, NK cells, monocytes, and dendritic cells. Furthermore, there are untypical cells such as NKT cells that share many properties with T lymphocytes as well as NK cells. Obtaining PBMCs is based on gradient difference between cell types in the blood. It is proved that ficoll-hypaque density gradient centrifugation is one of the effective method used for obtaining PBMCs.

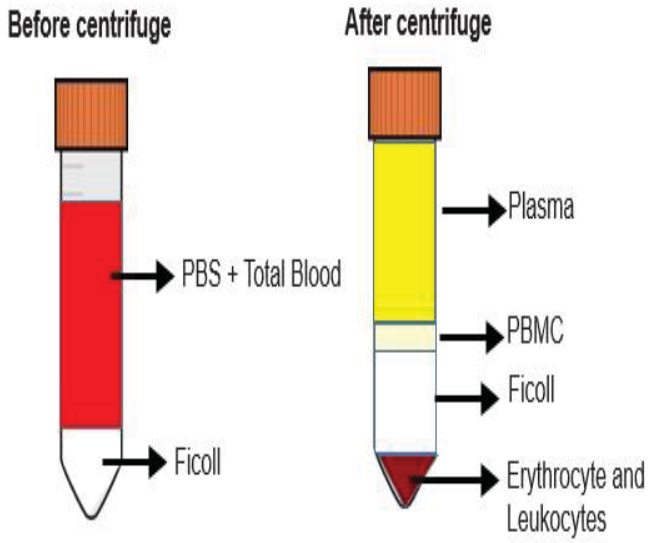

Figure 2.1. PBMC isolation before and after centrifuge. PBMC placed in the interphase of the tube after centrifuge.

For starting, blood samples taken freshly was separated in the amount of 20 ml in each falcon. To get rid of plasma they were centrifuged at 400 g for 5 minutes. After removing plasma, the rest of blood was mixed with PBS in 1:1 dilution. 30 ml of the mixture was spread on 10 ml ficoll gently, and to make a gradient they were centrifuged at 700 g for 45 minutes. At the end of this process, the blood was divided according to the cell density. The owner of highest densities such as erythrocytes and leukocytes placed in the down part of the tube. In the upper part, the lightest ones, thrombocytes, cytokines, and hormones, as well as electrolytes were collected. The middle grade comprised of PBMCs was collected by Pasteur pipette (Figure 2.1).

2.2. PBMC Characterization and Sample Selection

The characterization of PBMC profile is necessary for choosing the sample which is abundant in $CD4^+$ Naïve T cells. In order to do that, flow cytometer was performed by utilizing surface staining BD Pharmingen Stain Protocol. Different antibodies were used for deciding suitable sample. Presence of CD69 and CD25 activation markers gave information about infected samples. CD4, CD45RA and CD45RO were other markers to be used for understanding the percentage of memory and effector naïve T cells. Annexin V and 7AAD were stained for measuring apoptotic rate, while CD8, CD14 and CD19 carried out to confirm the amount of CTLs, monocytes, and B Cells, in the total PBMC sample. Finally, the sample was chosen according to the one that contained 20-30% $CD45RA^+$ and 30-40% $CD4^+$.

2.3. $CD4^+$ $CD45RA^+$ Naïve T Cell Sorting

Isolation of Naïve T cells was achieved by **"**Human Naive CD4+ T cell isolation Kit II (Miltenyi Biotec, Bergisch Gladbach) based on the negative selection method. The Kit comprises of beads conjugated with CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR, and CD235a antibodies as well as anti-biotin microbeads for magnetic labelling. Before starting the sorting procedure, PBMC was treated by 1x Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach) in order to remove remaining erythrocytes. 10^7 cells were used for each sorting. After determination of cell number, PBMC in media was

centrifuged at 300 g for 10 min to move away the supernatant. The pellet was re-suspended with 40 µl of AutoMacs buffer containing 5% BSA (Miltenyi Biotec) and 10 µl of Naive CD4+ T Cell Biotin-Antibody Cocktail II was added. As soon as addition of both solution, the mixture was incubated at 4° C for 5 minutes. Subsequent to the incubation, 30 µl of buffer and 20 µl of Naive CD4+ T Cell MicroBead Coctail II were accreted in the solution that was waited at 4° C for 10 minutes. During the incubation, VarioMacs separator column was prepared by placing in the magnetic field and washing with 3 mL of buffer. No sooner was incubation process finished than the mixture was put in the column. The labelled cells by beads were adhered on the internal surface of the column magnetically as well as Naive $CD4^+$ T cells were taken as flow through. In order to increasing the efficiency of the separation, 3 mL of buffer was loaded into the column and collected inside the tube for sorted cells. Finally, the column was removed from the separator unit. Together with the addition of 5 mL of buffer and pushing the plunge into the column, depleted cells were taken.

2.4. Th17 Cell Culture

The culture media for Naive $CD4^+$ T cells was composed of IMDM (Lonza) with 5% FBS, 2% Penicillin (100µl/mL) and Streptomycin (100µl/mL) and 0.1% β-mercaptoethanol. The differentiation of Naïve $CD4^+$ T cells into effector Th17 cells depended on eight different stimulants. 5 µg/ml anti-CD3 and 5 µg/ml anti-CD28 (BD Biosciences) which bound TCR complex, were used for cell activation. It was aimed to obtain a complete Th17 differentiation by utilizing 10ng/ml IL-1β, 10ng/ml IL-23, 30ng/ml IL-6 and 0,5ng/ml TGF-β (eBioscience), ın order to prevent the naïve cells differentiation into other subsets of T helper cells such as Th1, Th2, and Treg cells, 10µg/ml anti-IFNγ and 10µg/ml anti-IL4 (eBioscience) were used. Cells were analysed by flow cytometer at 3th, 5th, and 7th day of the culture to observe the differentiation process. The culture were stopped at 7^{th} or 9^{th} days according to IL-17 levels. The culture were refreshed with 10ng/ml IL-23 in every 5 days to keep cells alive and to continue proliferation. Culture contents of Th17 cells were modified from Veldhoen's culture conditions (Veldhoen et al., 2006) in Nalbant Lab.

2.5. Jurkat Cell Culture

Jurkat cells were cultured in full RPMI 1640 comprising of 10% FBS, and 1% Penicillin (100 µl/mL) and Streptomycin (100 µl/mL). Jurkat cell culture were adjusted as so being 10^6 cell/ml and refreshed in every 2-3 days.

2.6. Cell Counting

Jurkat cells and naive CD4 T cells were counted by trypan blue staining. 10 µl of cell culture was added in micro tubes containing 90 µl of trypan blue dye, and was mixed well. After that, 10 µl of cell culture/trypan blue mix was placed on a haemocytometer and cell were counted. The result was calculated by using specific formula belonging to haemocytometer to find the exact number of cells.

2.7. Jurkat Cell Activation

The activation of Jurkat cells were carried out by stimulation with PMA (25 ng/ml) and Ionomycin (1 µg/ml). Jurkat cells were cultured at a concentration of 1×10^6 cells/mL for 24h. Cells were incubated with or without stimulants at 37°C in a humidified incubator with 5% CO_2. The medium included full RPMI-1640 enriched with 10% fetal bovine serum (FBS) (Gibco), 1% mixture of 100 µl/mL penicillin and 100 µl/mL streptomycin.

2.8. Camptothecin Treatment of Jurkat Cell

Jurkat cells cultured in RPMI enriched with 5% fetal calf serum in an incubator containing 5% CO_2 at 37° C were treated with 20 µM CPT (SantaCruz) solved in DMSO for 24 hour (at 37° C under 5% CO_2) to induce apoptosis. Same amount of Jurkat cells were also cultured without Camptothecin as a negative control.

2.9. Cell Staining and Flow Cytometric Analysis

2.9.1. Determination of Activation

Cell surface staining was practiced on Th17 cells in both 3rd and 5th days as well as Jurkat cells treated with PMA/Ionomycin. Cell types were identified by staining CD4 conjugated fluorescence for Th17s and CD3 conjugated one for Jurkat cells. The early activation marker CD69 for both cell type and activation marker CD25 for Th17 cell were used in order to detect activation rates. For each samples, 50 µl of negative and stimulated cells were placed in 96 well plate. After centrifuging at 400 g for 6 minutes, supernatant removed cells were stained 10µl fluorescent dye for each marker. The stained cells were incubated at 4° C for 15 minutes. In the end of the incubation time, cells were washed with 1x PBS at 400 g 6 minutes. Finally, samples were prepared for flow cytometer analysis by dissolving in 400 µl PBS.

2.9.2. Determination of Differentiation

The detection of Th17 cell differentiation in 5th and 7th days was proceeded by both surface and intracellular staining. Anti - CCR6 and anti –CD4 fluorescent dyes were applied as a differentiation marker by surface staining protocol. In order to determination transcription factor, RORγt and the production levels of IL-17, intracellular staining was performed. Cells were stimulated with golgi stop (BD Bioscience) for 4 – 6 hours. 6 µl golgi stop was added in every 44 µl of cell culture and mixed thoroughly. After membrane staining for cell surface markers, 200 µl 1x Fix/Perm Buffer (BD Bioscience) was put in each well and incubated for 30 minutes at room temperature. After washing cells with PBS at 500 g for 5 minutes. 200 µl 1x Perm Buffer (BD Bioscience) was added and cell with buffer were directly centrifuged again at 500 g for 5 minutes. After removing the supernatant, cells were incubated for 30 min with 200 µl 1x Perm Buffer. At the end of the incubation, the solution was centrifuged at 500 g for 5 minutes to get rid of extra buffer. Cells were stained with 10 µl of each

intracellular dye and incubated for 30 minutes at room temperature. Lastly, the media was removed from cells by washing with PBS and analysed by flow cytometer.

2.9.3. Determination of Cell Death

Cell surface staining procedure was performed to determine apoptotic cell death. Annexin V and 7AAD membrane markers were utilized to investigate the rate and type of cell death in both negative controls and stimulated cells. 50 µl of samples were taken from both negative control and CPT treated cells and washed with PBS at 400 g for 5 min. Cells were dissolved in 1x Annexin V binding buffer (BD). Annexin V and 7AAD fluorescent dyes were applied. After 15 min incubation, cells were washed with PBS at 400g for 5 min. Then they were analysed by flow cytometer.

2.9.4. Flow Cytometric Analysis

Cells were analysed by using GUAVA Flow Cytometry (Millipore). Different antibody conjugated dyes were used for luminescence. Markers conjugated with AlexaFluor 488 (eBioscience) and FITC (BD or eBioscience) dyes were screened in green channel while PE dye used for yellow one. Detection in Red I Channel was done by 7AAD (BD), PerCP (eBioscience), and PE – Cy5 (BD) as well as PE – Cy5.5 (BD). Lastly, Alexa 647 (eBioscience) and APC (BD or eBioscience) dyes were utilized to display markers in Red II Channel.

2.10. Western Blot Analysis

2.10.1. Determination of Apoptosis by Caspase 3 and Caspase 9 Expression

CPT-treated Jurkat cells were with 1x PBS and 400 µl radio immunoprecipitation assay (RIPA) solution (CST) together with protease inhibitor cocktail (CST) in the 1:100 ratio of RIPA were put in the pellet. The Bradford protein assay was used for measuring the concentration of total protein in samples. Different

gels were prepared for determination of each aquaporin proteins. 20 µg total protein samples were loaded in each well. Protein ladder (ThermoFisher) was also loaded to estimate the molecular weight of proteins. SDS-PAGE was proceed to separate proteins according to their molecular weights. After separation of proteins, gels were transferred onto different PVDF (Millipore) membrane. Membranes were blocked with 5% non-fat dry milk (CST-E) in TBS-T composed of 1x tris buffered saline (TBS) and 1% Tween 20 (FISHER) to prevent antibodies binding non-specifically to the membrane for 1 hour at room temperature. After washing with TBS-T solution for 4 times in 30 minutes, membranes were incubated overnight at 4°C with monoclonal primer mouse antibodies, anti-caspase3 (CST) and anti-caspase 9 (CST) used separately.

After removal of primer antibody solutions, membranes were washed with TBS-T solution for 4 times in 30 minutes. Anti-mouse IgG-HRP conjugate secondary antibody (CST-E) for caspase 3 and caspase 9 primers in blocking solution were applied for 1 hour at room temperature for visualization of proteins. After all, membranes were washed with TBS-T for 3 times for 10 minutes. All washing and incubation process were performed on the shaker. Membranes treated by chemiluminescent substrate (ThermoFisher) solution for 2 minutes were scanned by BIO-RAD, VERSADOC 4000 MP.

2.10.2. Determination of Aquaporin Proteins (AQP1, AQP3, and AQP4)

Cell lysates were taken from 3rd, 5th, and 7th days of Th17 cell cultures and Jurkat cells stimulated by PMA/Ionomycin as well as treated by camptothecin as a beginning. Cells were washed with 1x PBS and 400 µl radio immunoprecipitation assay (RIPA) solution (CST) together with protease inhibitor cocktail (CST) in the 1:100 ratio of RIPA were put in the pellet. The Bradford protein assay was used for measuring the concentration of total protein in samples. Different gels were prepared for determination of each aquaporin proteins. 20 µg total protein samples were loaded in each well. Protein ladder (ThermoFisher) was also loaded to estimate the molecular weight of proteins. SDS-PAGE was proceed to separate proteins according to their molecular weights. After separation of proteins, gels were transferred onto different PVDF (Millipore) membrane. Membranes were blocked with 5% non-fat dry milk (CST-E) in

TBS-T composed of 1x tris buffered saline (TBS) and 1% Tween 20 (FISHER) to prevent antibodies binding non-specifically to the membrane for 1 hour at room temperature. After washing with TBS-T solution for 4 times in 30 minutes, membranes were incubated overnight at 4°C with monoclonal primer mouse antibodies AQP1, and AQP4 (ThermoFisher) as well as polyclonal rabbit antibody AQP3 (SantaCruz Biotech) used separately.

After removal of primer antibody solutions, membranes were washed with TBS-T solution for 4 times in 30 minutes. Anti-mouse IgG-HRP conjugate secondary antibody (CST-E) for AQP1 and AQP4 primers and anti-rabbit IgG-HRP conjugate secondary antibody (CST-T) for AQP3 in blocking solution were applied for 1 hour at room temperature for visualization of proteins. After all, membranes were washed with TBS-T for 3 times for 10 minutes. All washing and incubation process were performed on the shaker. Membranes treated by chemiluminescent substrate (ThermoFisher) solution for 2 minutes were scanned by BIO-RAD, VERSADOC 4000 MP.

2.11. Statistical Analysis

The data were analysed by GUAVA Flow Cytometry. Microsoft office excel was used for further analysis of these data. Flow Cytometric experiments on Jurkat cells were performed in triplicates and the average was taken at each time point and were compared to control groups (unstimulated Jurkat cells) using student's T test and $P<0.05$ accepted scientifically significant. Error bars represent the standard deviation.

The western blot results were also analysed by ImageJ programme. Microsoft office excel was used for further analysis of these data. Western blotting was performed in triplicates with lysates taken from Jurkat cells and the average was calculated at each time point and were compared to control groups (unstimulated Jurkat cells) using student's T test and $P<0.05$ accepted scientifically significant. Error bars represent the standard deviation.

CHAPTER 3

RESULTS

3.1. Identification of PBMC Contents.

The first Flow Cytometric analysis was done to determine the rate of cell contents in PBMC. Firstly, CD4, CD8, CD14, and CD19 markers were used for the identification of Th cells, CTLs, monocytes and B lymphocytes rates, respectively. Results show that 31.86% CD4, 18.76% CD8, 12.66% CD24, and 0.88% CD19 exist in PBMC (Figure 3.1. A). After that, as a second part, CD4, CD45RA, and CD45RO as well as CD69 staining was performed to realise the percentage of effector and memory CD4 T cells as well as if there are infected cells or not. The data enlightened that 35.57% of $CD4^+$ T cells were effector (CD45RA positive), however, the presence of $CD45RO^+$ cells were not detected. In addition to this, the absence of CD69 proved that PBMC contained only uninfected cells (Figure 3.1. B)

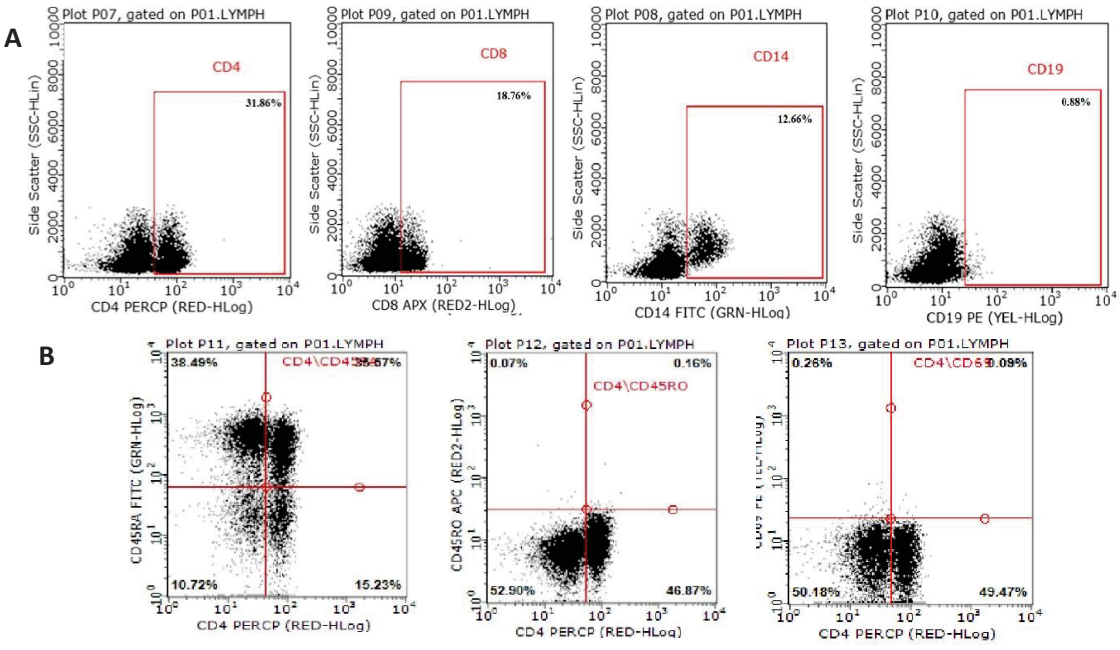

Figure 3.1 A. Determination of cell components in PBMC. B. The percentage of effector, memory and infected $CD4^+$T cells.

3.2. Determination of Naive $CD4^+$ T cell Sorting Efficiency

PBMC was sorted to collect $CD45RA^-$ $CD4^+$ T cells. In order to understand sorting efficiency, depleted and sorted cells were stained with CD4, CD45RA, and CD14 dyes. These markers shows that the sorting efficiency was 74.70%. Besides, sorted cell population did not contain any $CD4^+$ $CD14^+$ cells.

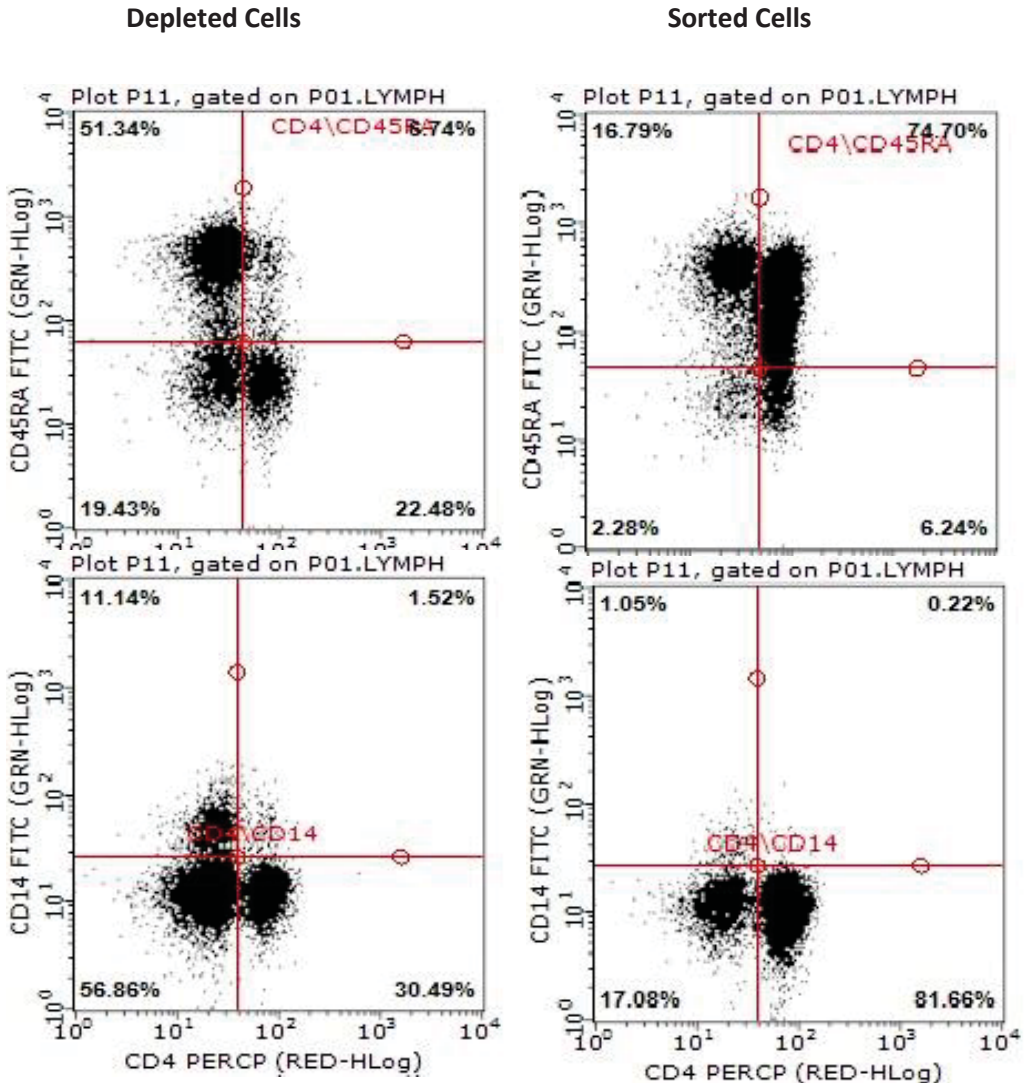

Figure 3.2. The determination of sorting efficiency. a) The rate of $CD4^+$ $CD45RA^+$ cells in depleted and sorted cells. b) $CD14^+$ cells staining.

3.3. Activation of CD4$^+$ T cells and Expression of RORγt Transcription Factor in CD4$^+$ T cells

Naïve CD4$^+$T cells were differentiated into Th17 cells by expressing CD69 (early activation) in 3rd day, CD25 (activation marker) and RORγt (transcription factor) in 5th day under the Th17 culture condition. Double positive CD4 and CD69 was detected 28.03% of expression at the 3rd day (Figure 3.3.A). At the 5th day, the increased levels of CD25 and RORγt expression were observed as 78.88% and 53.55%, respectively (Figure3.3.B and C). However, no expression was detected in the negative control.

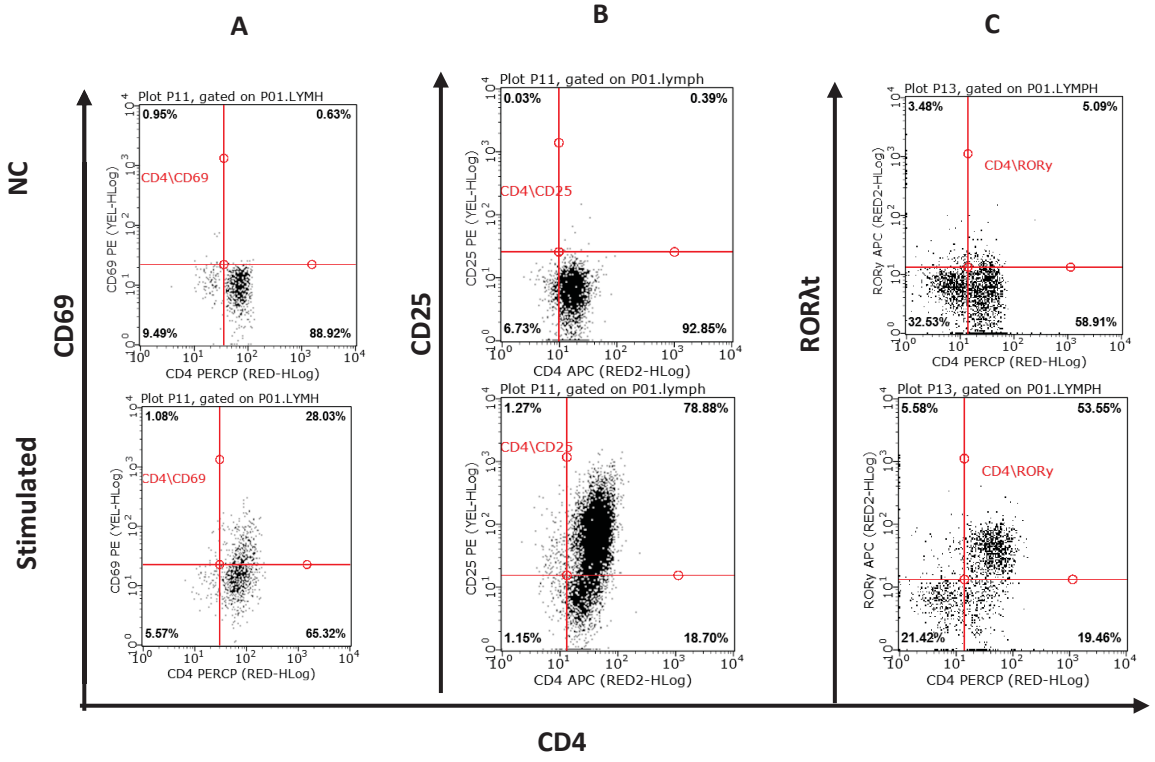

Figure 3.3. Flow data of activation markers, CD69 and CD25 as well as transcription factor, RORγt. A. CD69 expression at the 3rd day. B. CD25 expression at the 5th day. C. RORγt expression at the 5th day.

3.4. Determination of AQP1, AQP3, and AQP4 in Th17 cells.

Western blot analysis was applied to check AQP1, AQP3, and AQP4 expression. Naïve $CD4^+$ T cells were stimulated with the Th17 culture condition. Culture was stopped at the 7^{th} day, and protein lysate was taken.

According to results AQP1 and AQP4 expression were increased in Th17 cells compared with negative control while there was no expression of AQP3 in Th17 cells (Figure 3.4).

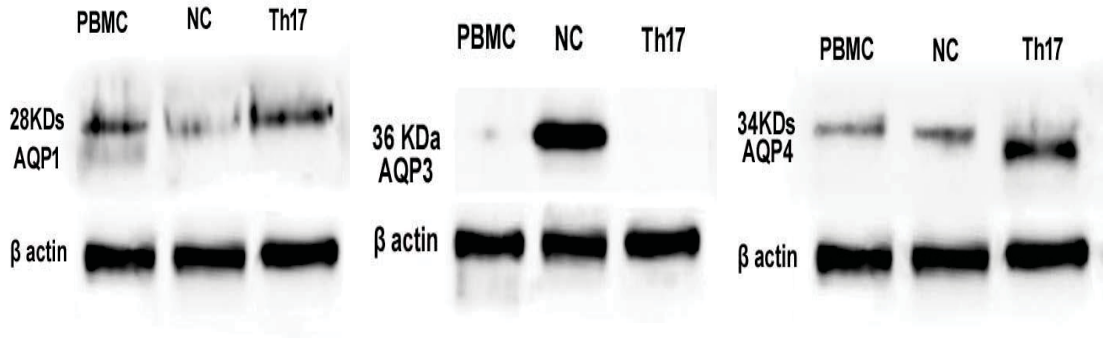

Figure 3.4. Western Blot results for AQP1, AQP3, and AQP4 expression in Th17. AQP1 and AQP4 expression were increased in Th17 but AQP3 was not detected.

3.5. Jurkat Cell Activation by PMA/Ionomycin

Jurkat cells were stimulated by PMA (25 ng/ml) and Ionomycin (1 μg/ml) for 24 hours. At the end of the 24 hours, the cells were stained with CD69 marker to visualize the activation rate. Stimulated Jurkat cell were expressed at the rate of 97.85% CD69. There was no CD69 expression in unstimulated cells used as a negative control (Figure 3.5. A). Statistical analysis indicate that the activation rate of PMA/Ionomycin stimulated cells are significant (Figure 3.3. B)

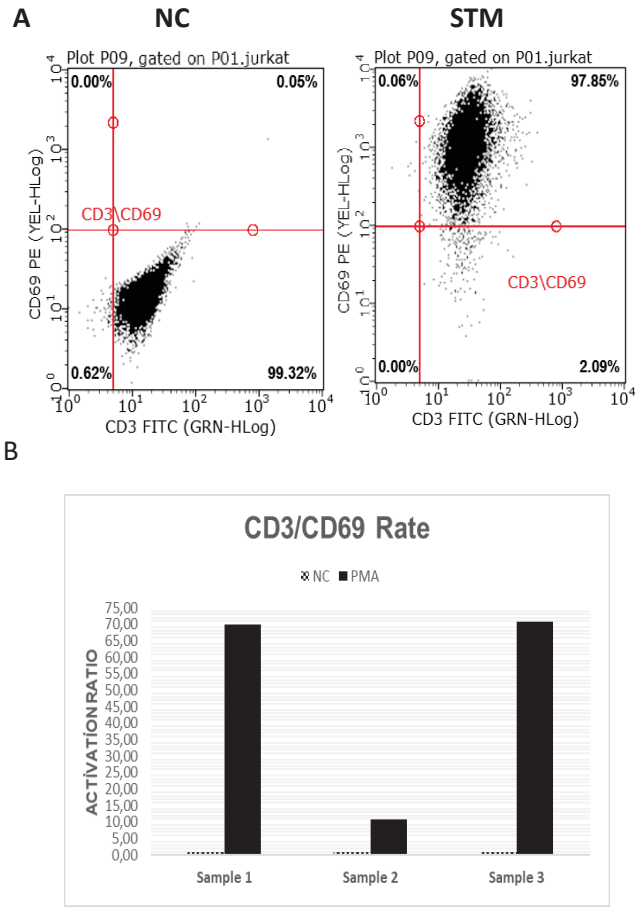

Figure 3.5. A. Activation of Jurkat cells with PMA/Ionomycin. 97.85% CD69 expression was detected in stimulated cells while no expression in negative control. B. Graph presentation of activated Jurkat cell rates.

Table 3.1. Activation rates of Jurkat cells in terms of CD3/CD69 expression after stimulation with PMA/Ionomycin for 24 hr.

CD3/CD69	*Rep.1*	*Rep.2*	*Rep.3*	*CD3/CD69*	*Rep.1*	*Rep. 2*	*Rep.3*
NEG 1	1.42	7.44	1.50	**PMA 1**	79.66	91.96	98.02
NEG'2	1.09	8.20	1.58	**PMA 2**	87.46	92.34	97.84
NEG 3	1.14	9.56	1.08	**PMA 3**	89.02	88.43	98.12
Mean	1.22	8.40	1.39	**Mean**	85.38	90.92	98.00
SD	0.170	1.070	0.260	**SD**	5.015	2.154	0.139

N=3, P value < 0.05

3.6. Detection of AQP1, AQP3, and AQP4 in Activated Jurkat Cells

Western blot analysis was performed to check AQP1, AQP3, and AQP4 expression in activated Jurkat cells. Protein lysates were obtained both unstimulated Jurkat cells and stimulated Jurkat cells by PMA/Ionomycin for 24 hours. Results showed that AQP1, AQP3 as well as AQP4 were expressed in both unstimulated and stimulated Jurkat cells (Figure 3.6 A). In addition to this, statistical analysis of each band were applied to determine the expression levels of AQP1, AQP3, and AQP4. (Figure 3.6 B and C).

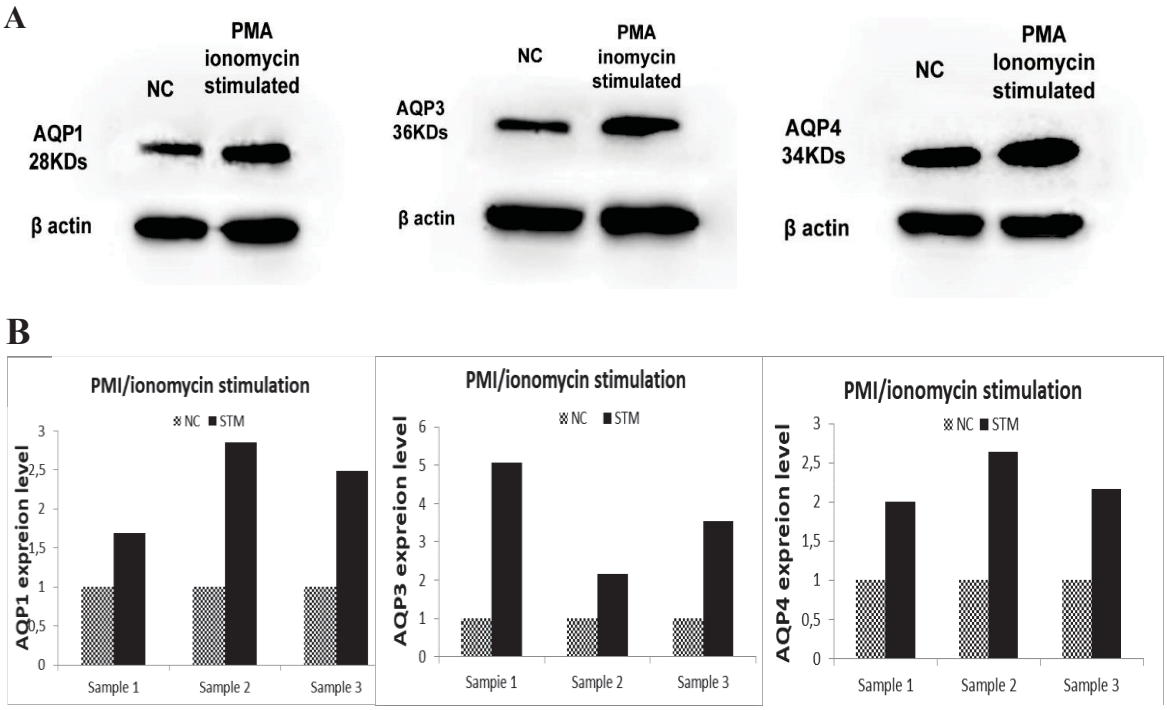

Figure 3.6. Western Blot results for AQP1, AQP3, and AQP4 expression in activated Jurkat cells. A. Western Blot images for each of three AQPs. The expression of AQP1, AQP3, and AQP4 were detected both negative control and activated Jurkat cells. B. Graph presentation of the AQP expression for each replicate. C. Graph presentation of fold increase representing three replicates for each AQP type. **(Continue on next page)**

C

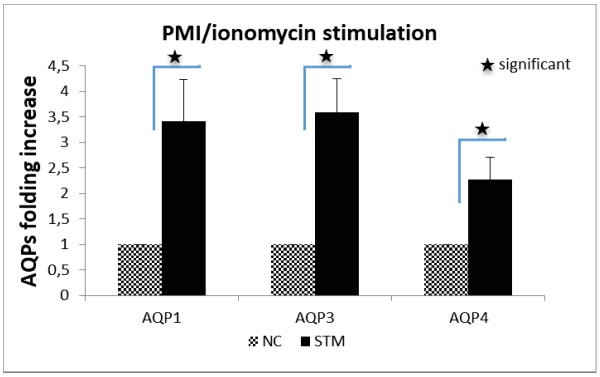

Figure 3.6. **(Cont.)**

Table 3.2. Western Blot analysis to determine expression levels of AQP1, AQP3, and AQP4.in activated cell population.

A	***NC***	***STM***	***Fold Increase Ratio***
AQP1	0.597±0.01	1.012±0.01	1.69±0.8
AQP1	0.411±0.01	0.846±0.01	2.85±0.8
AQP1	0.297±0.01	2.24±0.01	5.71±0.8
B	*NC*	*STM*	*Fold Increase Ratio*
AQP3	0.147±0.10	0.747±0.07	1.69±0.9
AQP3	0.296±0.10	0.639±0.07	2.85±0.9
AQP3	0.482±0.10	1.710±0.07	5.71±0.9
C	*NC*	*STM*	*Fold Increase Ratio*
AQP4	0.660±0.05	1.325±0.06	2.007±0.10
AQP4	0.740±0.05	1.422±0.06	2.160±0.10
AQP4	0.700±0.05	1.374±0.06	2.084±0.10
	AQP1	**AQP3**	**AQP4**
P value	0,017269	0,036955	0,002628

N=3, P value < 0.05

3.7. The Expression Levels of Annexin V and 7AAD in Jurkat Cells Treated by Camptothecin

Jurkat cells were treated by 20 µM CPT to induce apoptotic cell death for 24 hours. In order to determine apoptotic cell death Annexin V and 7AAD markers were checked. At the end of the 24 hours, 7AAD levels was increased at the rate of 20-30% in CPT treated Jurkat cell population opposite to negative control. These values showed that there was DNA fragmentation in CPT treated cells (Figure 3.7).

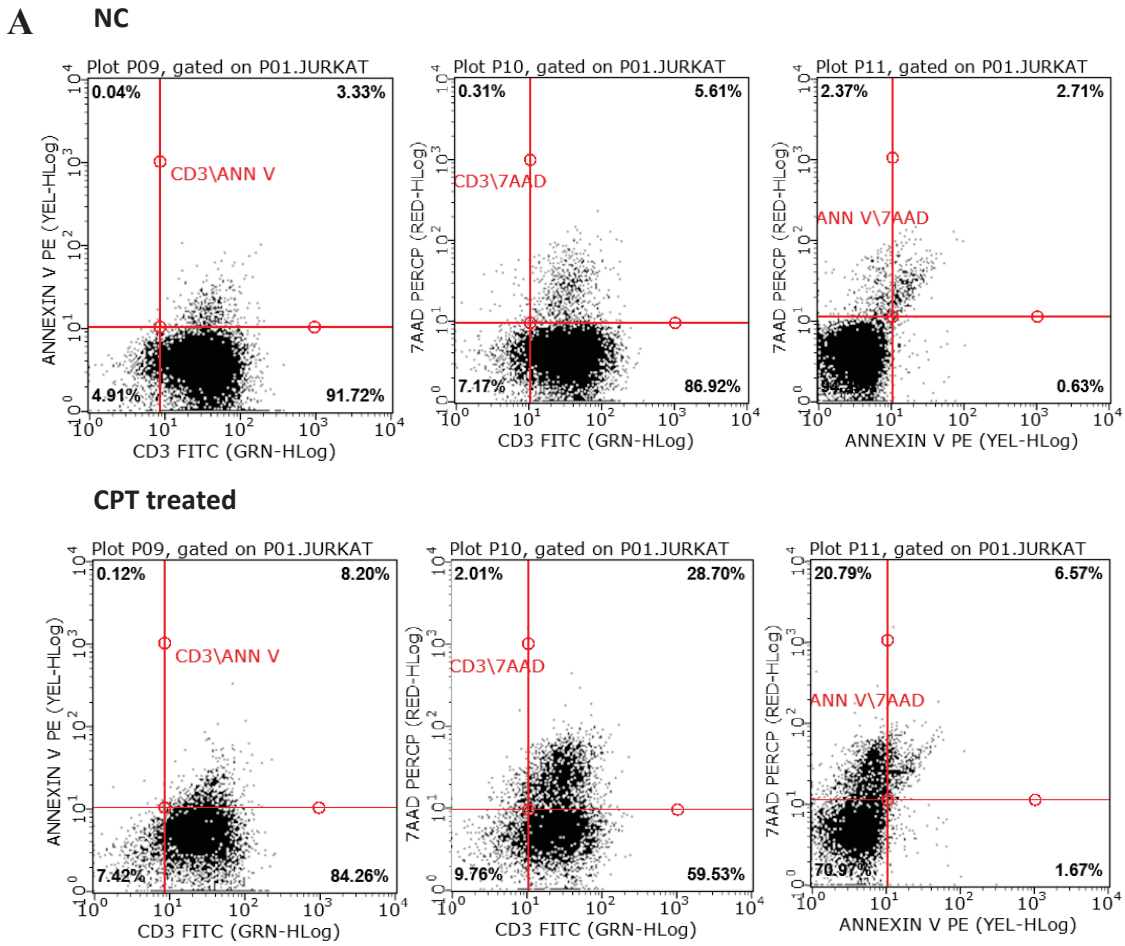

Figure 3.7. A. The measurement of apoptosis rates in CPT treated Jurkat cells by Flow Cytometry. To detect apoptosis, the rate of CD3 –Annexin V, CD3-7AAD, and Annexin V-7AAD markers were used. B. Graph presentation of apoptotic Jurkat cell rates. **(Continue on next page)**

B

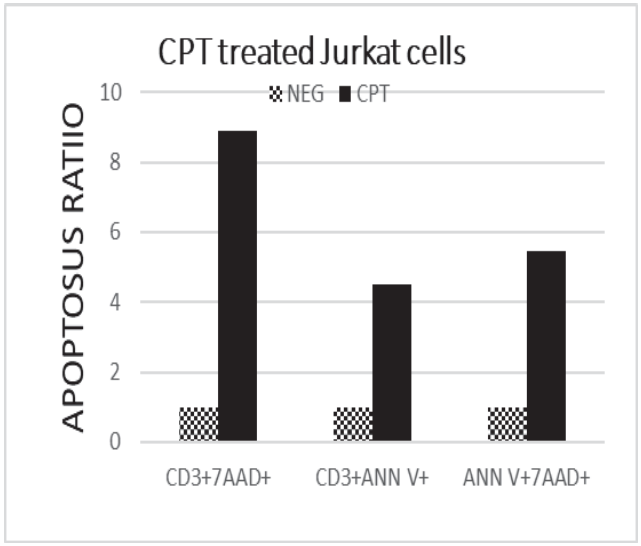

Figure 3.7. **(Cont.)**

Table 3.3. Apoptosis rates of Jurkat cells in terms of $CD3^+7AAD^+$, $CD3^+ANN\ V^+$ and $ANN\ V^+7AAD^+$ after treatment with CPT for 24 hr.

Sample	$CD3^+7AAD^+$	$CD3^+ANN\ V^+$	$ANN\ V^+7AAD^+$
NEG 1	3,40	0,34	0,04
NEG 2	3,08	0,39	0,03
NEG 3	2,91	0,44	0,00
CPT 1	29,15	2,83	0,25
CPT 2	31,42	1,40	0,06
CPT 3	22,98	1,11	0,16
Mean NEG	3,13	0,39	0,02
Mean CPT	27,85	1,78	0,16
SD NEG	0,24	0,04	0,01
SD CPT	4,36	1,16	0,11
P value	0,0003	0,0294	0,0391

N=3, P value < 0.05

3.8. Detection of Caspase 3 and Caspase 9 in Camptothecin Treated Jurkat Cells

Further analysis was performed by using western blotting to confirm apoptosis in Jurkat cells. Caspase-3 and -9 cleavages were observed in the lysates of CPT treated Jurkat cells. Western blot images showed that caspase-3 generated a 17 kDa cleavage as Caspase-9 generated cleavages of 35 kDa and 37kDa. These results proved that camptothecin-treated Jurkat cells underwent apoptosis.

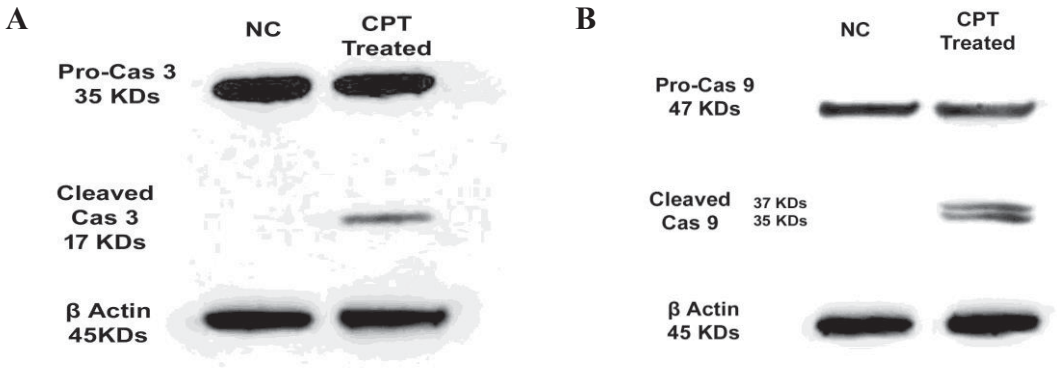

Figure 3.8. Western blot results for caspase 3 and caspase 9 in CPT-treated Jurkat cells. A. Caspase 3 results indicating that the generation of 17 kDa cleavage. B. Caspase 9 generates two cleavages at the site of 35kDa and 37 kDa, respectively.

3.9. Detection of AQP1, AQP3, and AQP4 in Camptothecin Treated Jurkat Cells

In order to understand the expression of AQP1, 3 and 4 in apoptotic T cells, total protein lysates of Jurkat cells treated with Camptothecin and without Camptothecin were taken. In the light of western blotting results, AQP1, AQP3, and AQP4 were expressed in both negative control and CPT treated Jurkat cells (Figure 3.8. A). Statistical analysis of each band were applied to determine the expression levels of

AQP1, AQP3, and AQP4. According to results, the expression of each AQPs was decrease significantly in Jurkat cells under the apoptotic conditions (Figure 3.8. B).

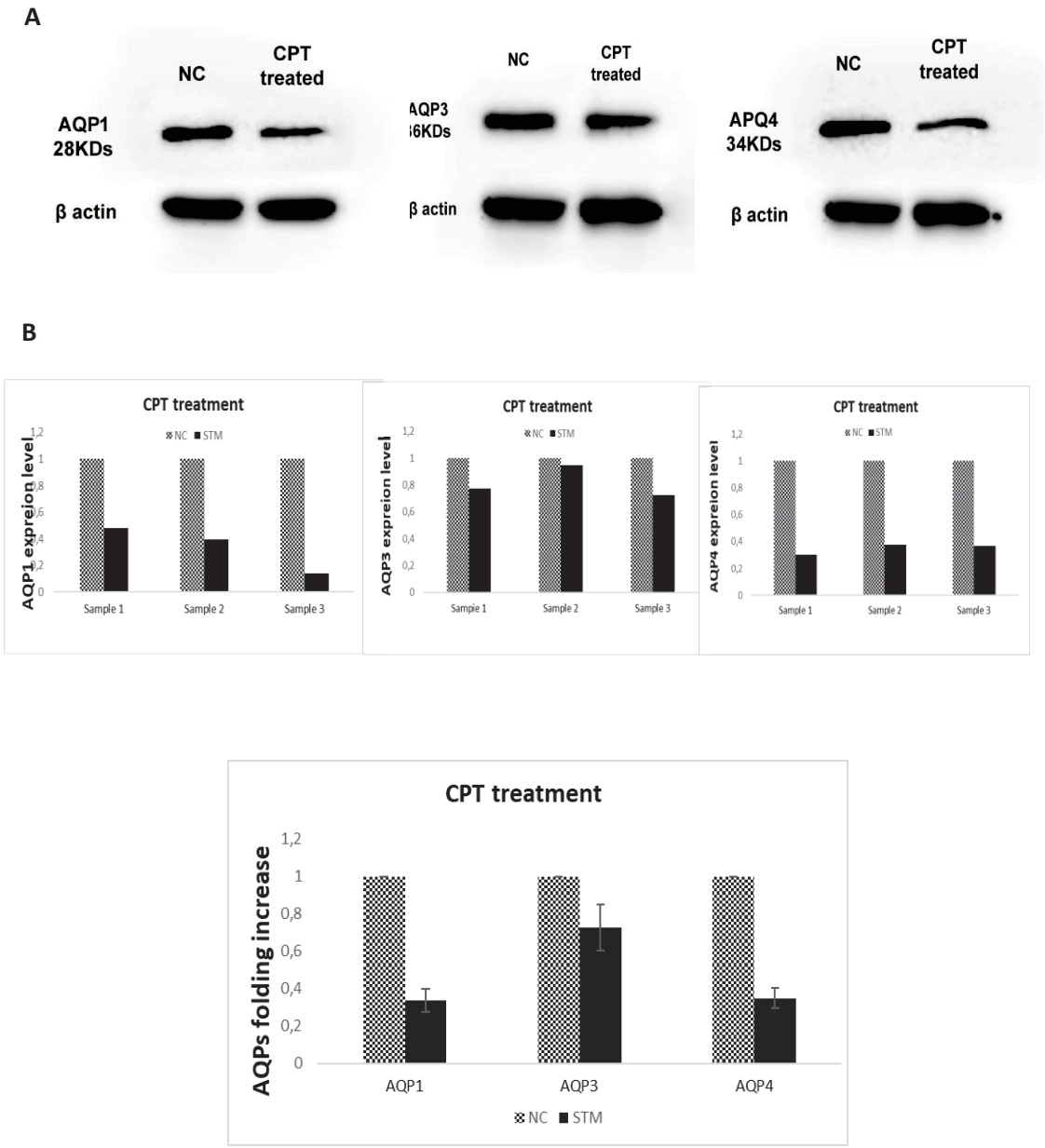

Figure 3.9. A. Western Blot results for AQP1, AQP3, and AQP4 expression in CPT treated Jurkat cells. The expression of AQP1, AQP3, and AQP4 were detected both negative control and CPT treated Jurkat cells. B. Graph presentation of the AQP expression for each replicate of CPT-treated Jurkat cells. C. Graph presentation of fold increase representing three replicates for each AQP type.

Table 3.4. Western Blot analysis to determine expression levels of AQP1, AQP3, and AQP4 in CPT treated cell population.

A	***NC***	***STM***	***Fold Increase Ratio***
AQP1	1.159 ±0.3	0.558±0.1	0.481±0.6
AQP1	1.112±0.3	0.438±0.1	0.394±0.6
AQP1	1.727±0.3	0.236±0.1	0.137±0.6
B	***NC***	***STM***	***Fold Increase Ratio***
AQP3	0.954±0.01	0.739±0.09	0.774±0.12
AQP3	0.930±0.01	0.880±0.09	0.946±0.12
AQP3	1.571±0.01	1.140±0.09	0.726±0.12
C	***NC***	***STM***	***Fold Increase Ratio***
AQP4	1.629±0.3	0.493±0.05	0.302±0.05
AQP4	1.112±0.3	0.418±0.05	0.376±0.05
AQP4	1.004±0.3	0.369±0.05	0.367±0.05
	AQP1	**AQP3**	**AQP4**
P value	0,003046	0,050617	0,001250

N=3, P value < 0.05

CHAPTER 4

DISCUSSION

T lymphocytes are fundamental players of adaptive immune system. They are required for defending the body against invaders by responding in favourable ways. Specifically, helper T cells play a critical role in adaptive immune response. They are not only essential for the activation of B cells to produce antibodies and macrophages to destroy ingested pathogens, but also directing cytotoxic T cells to the target cells. In order to accomplish these functions. T cells must turn into effectors from their naïve forms by activation. Together with the activation, helper T cells undergo differentiation process depending on the presence of specific cytokines which determine the proper subset of T helper cells. The activation process also induces apoptosis to maintain homeostasis and to prevent undesirable effects resulting in autoimmune diseases (Murali and Mehrotra 2011, Broere et al., 2011)

Water is one of the keystones of all living organisms. It is needed for not only complex metabolic processes at the extracellular level but also many biochemical reactions at the intracellular level. In addition of water, cells also need to transport other some solutes or gases for their vital activities or as a result of these activities. Aquaporins are transmembrane proteins of which main function is transferring a huge amount of water in a short time. In addition to this, they have abilities to transport some solutes and ions such as glycerol, H_2O_2, and CO_2. After the identification of AQP subtypes and the resolution of their structures, scientists have focused on the diverse functions of AQPs in the cells. Up to now, it is revealed that AQPs have involved in cell proliferation and survival, migration, and death process in normal condition, as well as pathology.

In this thesis, I focused on the expression of three AQP subtypes; AQP1, AQP3, and AQP4 and their effects on human T cells during the activation and apoptosis processes. Previous studies showed that AQP1 and AQP3 are expressed in B and T lymphocytes in mice, but the expression of these AQPs in T cells in different conditions has not been clear yet. AQP4 is also chosen for investigating, because it is revealed that AQP4 involves in the process of several autoimmune diseases related with T cells.

Because of the importance of helper T cells in immune response as well as autoimmune disorders, naïve $CD4^+$ T cells was used for the investigation of AQPs in activation and differentiation. Jurkat cell line was also utilized to understand activation and apoptotic process.

First of all, PBMC was isolated from the blood samples obtained from healthy donors. The cell profile of PBMC detected by flow cytometric analysis. As every sample has its own T helper cell population ranged between 25-60% of PBMC we first determined the percentage of CD4, CD45RA and CD45RO surface markers (Autissier P. et al. 2010).Combined with these three markers, CD69 staining was also performed to eliminate infected samples. The samples were chosen according to the percentage of these four markers. The PBMC candidates composed of 30-40% of $CD4^+$ $CD45RA^+$ T cells and generally CD69 ranged between 0-5 percent of the total cells. After enrichment of $CD4^+$ T helper cells by negative selection obtained sorting, the efficiency was checked by utilizing same surface markers. According to results, we enriched the $CD4^+$ $CD45RA^+$ T cell population up to 74.70%. However, there are $CD4^-CD45^+$ cells in the sorted cell population. In order to detect this population we applied further flow staining for CD8, CD14, and CD19 but none of them was detected in the sorted population. That is why we decided to continue with these samples.

Sorted $CD4^+$ population was stimulated to generate Th17 cells with eight different stimulants having been optimized in our laboratory before. The activation process was observed on 3^{rd} and 5^{th} days by staining with CD69 and CD25 activation markers. Increased level of CD4 CD69 double positive cells up to 28.03% and $CD4^+$ $CD25^+$ cells up to 78.88% showed that the activation process was accomplished. We also used RORγt transcription factor to identify Th17 differentiation on 5^{th} day. 53.55% of the increment in RORγt levels was observed. These results proved that naïve $CD4^+$cells were activated and underwent Th17 differentiation.

Another T cell type, Jurkat cell line was stimulated by PMA/Ionomycin to initiate activation. Flow cytometric analysis indicate that 97.85% of Jurkat cell population were successfully activated in 24 hours. Jurkat cells were also treated with camptothecin to induce apoptosis. 29% of the population was observed as 7AAD positive. In order to confirm Jurkat cells undergone apoptosis, caspase 3 and caspase 9 were checked by western blotting. It was observed that caspase 3 had one cleavage at the place of 17 kDa and two cleavages, 35 kDa and 37 kDa, were placed in the PVDF

membrane for caspase 9. These observations referred to the CPT treatment resulted in the apoptosis.

Second part of our experimental design was the investigation of AQP expression at protein level by Western Blotting. We performed Western Blotting for AQP1, AQP3, and AQP4 proteins. The aim was the determination of AQP expression levels in both activated and apoptotic cells.

Firstly, lysates taken from activated Jurkat cells and differentiated $CD4^+$ T cells was used for detection of AQP1, AQP3, and AQP4 expression. In the literature, it is known that AQP1 expression is upregulated during the cell proliferation and migration (Galan-Cobo et al. 2015). Researches claimed that AQP1 upregulates by NF-kB which is the one of transcription factors in T cell activation (Vassiliou et al. 2013; Mezzasoma et al. 2013). In addition to this, subcellular localization of AQP1 is correlated with the mechanism dependent on t extracellular calcium influx and calmodulin which participate in T cell activation (Conner et al. 2012; Leggett et al. 2012). AQP 3 also plays a role in cell lipid metabolism and activation. Direct correlation has been shown between AQP3 expression and PI3K/Akt signalling pathway due to the energy and lipid production (Li et al. 2016). AQP4 has also similar function with both AQP1 and AQP3 about involving in activation via synergistically working with Ca^+ channels and NF-kB mechanism. In addition to this, it also effects inflammation processes as well as acts like an autoantigen (Fukuda and Badaut 2012; Thranea et al. 2011). Our western blot analysis results have been in accordance with the findings in the related literature. It is detected that the expression of each AQP subtypes was increased significantly in activated and differentiated T cell population when compared with negative control.

Secondly, we used western blot technique to find out the expression levels of AQP1, AQP3, and AQP4 in apoptotic cells. According to the previous researches, AQP1 activity was decreasing in apoptotic cells. It was also found that AQP1 and tumour necrosis factor receptor 1 (TNF-R1) are co-localized in the cell membrane during apoptotic processes. Water loss mediated by AQPs is essential for apoptotic volume decrease (AVD) as well as downstream mechanisms of apoptosis because of the correlation between the water permeability of the membrane and the rate of apoptosis (Jablonski et al. 2004). Researches focused on AQP3 in apoptosis claimed that deficiency of AQP3 decreased glycerol uptake resulting in damaged lipid synthesis which affected in apoptosis (Chen et al. 2017). It is also shown that AQP4 decrease

contributes to increase apoptosis under pathological conditions such as intracerebral haemorrhage and it is suggested that the underlying mechanism correlated with the initiation of apoptosis through cytokines such as TNF-α and IL-1β (Chu et al. 2014). Our findings are also correlated with the related literature. We found that AQP1, AQP3, and AQP4 expressions were decrease significantly in CPT treated cell populations.

CHAPTER 5

CONCLUSION

The objective of this study is the investigation of AQP1, 3, and 4 expression in both activated and apoptotic T cells. The expression of AQP1 and 3 was identified in mice T and B lymphocytes as well as dendritic cells. However, it has not observed in different T cell functions such as activation and apoptosis. Both function of T cells are crucial for maintaining proper immune response and homeostasis of immunity. There is also no information about AQP4 expression in human T cells. As a result of this, we examined the expression levels of AQP1, 3, and 4 in both activated $CD4^+$ T helper cells and Jurkat cells as well as apoptotic Jurkat cells. As we discussed in previous chapter our findings are correlated with literature. The expression levels are increasing in activated cells and decreasing apoptotic ones for each of three AQPs. In the light of this research, the relations between the signalling pathways involving in activation or apoptosis processes and AQP functions can be investigated as further studies. The scientific significance of this research is that it can fill the gaps about activation and differentiation processes of T cells existing in the literature. Besides, all findings can contribute to design new drug targets for controlling T cell functions which can be used for the treatment of many autoimmune disease like MS which Th17 cells involve in its pathogeny. As a result, many people having autoimmune disease can be cured and have more comfortable life both economically and psychologically.

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