NUCLEOFECTION EFFECTS ON HUMAN T CELLS AS A NON-VIRAL TRANSFECTION TECHNIQUE

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by

Seminay GÜLER

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İZMİR

We approve the thesis of **Seminay GÜLER**

Examining Committee Members:

Assist. Prof. Dr. Ayten NALBANT ALDANMAZ

Department of Molecular Biology and Genetics, İzmir Institute of Technology

---

Prof. Dr. Volkan SEYRANTEPE

Department of Molecular Biology and Genetics, İzmir Institute of Technology

---

Prof. Dr. Semra KOÇTÜRK

Department of Medical Biochemistry, Dokuz Eylül University

19 December 2016

Assist. Prof. Dr. Ayten NALBANT ALDANMAZ

Supervisor, Department of Molecular Biology and Genetics,

İzmir Institute of Technology

Prof. Dr. Volkan SEYRANTEPE

Head of the Department of

Molecular Biology and Genetics

Prof. Dr. Bilge KARAÇALI

Dean of the Graduate School of

Engineering and Sciences

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ABSTRACT

NUCLEOFECTION EFFECTS ON HUMAN T CELLS AS A NON-VIRAL TRANSFECTION TECHNIQUE

In times of quickly developing ways of immunological approaches applying on cells, nucleic acid delivery becomes a fundamental process. The ways of delivering nucleic acids mainly affects the consequence of immunological response. The nucleofection technology which is a modified form of electroporation is reliable method for transfection of hard to transfect cells. In the current study, we compared naive CD4 T cells and Jurkat cells in terms of nucleofection efficiency and cell viability. Naive CD4 T cells and Jurkat cells were transfected with pmax GFP at different time points. Out of 5 different nucleofection programs, one for naive CD4 T cells was identified. Jurkat cells were also transfected with one nucleofection program. Flow cytometric analyses of nucleofected naive CD4 T cells and Jurkat cells indicated that transfection efficiency significantly increased at 48h post transfection. We also differentiated naive CD4 T cells into T helper 17 cell phenotype by using certain stimulants and we activated Jurkat cells by using PMA and Ionomycin. We found that GFP was efficiently taken up by both T helper 17 cells and activated Jurkat cells. Therefore, nucleofection significantly holds important place in transfecting of hard to transfect cells including primary human CD4 T cells.

ÖZET

VİRAL OLMAYAN TRANSFEKSİYON TEKNİĞİ OLARAK NÜKLEOFEKSİYONUN İNSAN T HÜCRELERİ ÜZERİNDEKİ ETKİLERİ

Hücreler üzerine uygulanan immünolojik yaklaşımların hızla arttığı bu dönemde nükleik asitlerin hücre içine gönderilmesi önemli bir işlem haline gelmiştir. Elektroporasyona dayalı bir metod olan nükleofeksiyon, transfeksiyonu zor olan hücreler için güvenilir bir teknolojidir. Bu çalışmada naive CD4 T hücreleri ve Jurkat hücreleri transfeksiyon verimliliği ve hücre yaşayabilirliği yönünden karşılaştırıldı. Naive CD4 T hücreleri ve Jurkat hücreleri farklı zaman dilimlerinde pmax GFP ile transfekte edildi. 5 farklı nükleofeksiyon programından bir tanesi naive CD4 T hücreleri için tanımlandı ve yapılan deneylerle doğrulandı. Ayrıca Jurkat hücreleri de onlara özgü nükleofeksiyon programı uygulanarak transfekte edildi. Akım sitometrisi analizleri sonucunda hem naive CD4 T hücrelerinde hemde Jurkat hücrelerinde GFP ifadesinin transfeksiyondan 48 saat sonra önemli derecede arttığı gözlemlendi. Bu çalışmada T helper 17 hücrelerinin ve aktifleştirilmiş Jurkat hücrelerinin nükleofeksiyonu da araştırıldı. Bu hücrelerin de GFP'yi verimli bir şekilde hücre içine aldıkları görüldü. Böylece, nükleofeksiyonun transfeksiyonu zor olan hücrelerin transfeksiyonunda kullanılabilecek yararlı bir metod olduğu saptandı.

TABLE OF CONTENTS

LIST OF FIGURES viii

LIST OF TABLES ix

ABBREVIATIONS x

CHAPTER 1. INTRODUCTION 1

1.1. T Cells 1

1.1.1. Naive CD4 T Cells 1

1.1.2. T Helper Cells 3

1.1.3. T Helper 17 Cells 4

1.1.4. Jurkat Cells 5

1.2. Plasma Membrane Changes 6

1.3. Transfection 6

1.3.1. Biological Transfection Methods 7

1.3.2. Chemical Transfection Methods 8

1.3.3. Physical Transfection Methods 8

1.3.4. Nucleofection 9

CHAPTER 2. MATERIALS AND METHODS 11

2.1. PBMC Isolation 11

2.2. Naive CD4 T Cells Sorting 12

2.3. Th17 Cells Culture 13

2.4. Jurkat Cells Culture 13

2.5. Cells Counting 14

2.6. Jurkat Cells Activation 14

2.7. Nucleofection 14

2.8. Cell Surface Staining 15

2.9. Cell Viability Determination 15

2.10. Flow Cytometric Analysis 15

2.11. Fluorescence Microscope Analysis 16
2.12. Statistical Analysis 16

CHAPTER 3. RESULTS 17
3.1. Nucleofection of Unstimulated Human T cells 17
3.1.1. Nucleofection of PBMC 17
3.1.2. Nucleofection of Naive CD4 T Cells 20
3.1.3. Nucleofection of Jurkat Cells 23
3.2. Activation of Jurkat Cells 26
3.3. Nucleofection of Stimulated Human T Cells 31
3.3.1. Nucleofection of Activated Jurkat Cells 31
3.3.2. Nucleofection of T helper 17 Cells 32

CHAPTER 4. DISCUSSION 36

CHAPTER 5. CONCLUSION 39

REFERENCES 40

LIST OF FIGURES

Figures **Page**

Figure 1. T cell activation 3

Figure 2. T helper cell differentiation 4

Figure 3. Jurkat cells 5

Figure 4. Transfection methods 7

Figure 5. PBMC isolation 12

Figure 6. Untransfected PBMCs 18

Figure 7. PBMC transfection with program EO-115 18

Figure 8. PBMC transfection with program EA-113 19

Figure 9. PBMC transfection with program FI-115 19

Figure 10. PBMC transfection with program FF-115 19

Figure 11. PBMC transfection with program FA-115 20

Figure 12. Transfection efficiency for naive CD4 T cells 24h post nucleofection 21

Figure 13. Cell viability results for naive CD4 T cells 24h post nucleofection 21

Figure 14. Transfection efficiency for naive CD4 T cells 48h post nucleofection 22

Figure 15. Cell viability results for naive CD4 T cells 48h post nucleofection 23

Figure 16. Transfection efficiency for Jurkat cells 24h post nucleofection 24

Figure 17. Cell Viability results for Jurkat cells 24h post nucleofection 25

Figure 18. Transfection efficiency for Jurkat cells 48h post nucleofection 25

Figure 19. Cell Viability results for Jurkat cells 48h post nucleofection 26

Figure 20. Non-activated (control) Jurkat cells 27

Figure 21. 24h activation results of Jurkat cells 28

Figure 22. 48h activation results of Jurkat cells 29

Figure 23. 72h activation results of Jurkat cells 30

Figure 24. Nucleofection of activated Jurkat cells 32

Figure 25. Nucleofection of 3th day of Th17 cell culture 33

Figure 26. Nucleofection of 5th day of Th17 cell culture 34

Figure 27. Nucleofection of 7th day of Th17 cell culture 35

LIST OF TABLES

Tables **Page**

Table 1. Culture contents of Th17 cells 13

ABBREVIATIONS

APC	Antigen Presenting Cells
MHC	Major Histocompatibility Complex
CD	Cluster of Differentiation
Th	T helper
Treg	Regulatory T cells
IL	Interleukin
ROR	RAR-related Orphan Receptor
CCR6	Chemokine receptor 6
TGF-β	Transforming Growth Factor β
STAT4	Signal Transducer and Activator of Transcription 4
GATA3	GATA Binding protein 3
IFN-γ	Interferon gamma
IMDM	Iscove's Modified Dulbecco's Medium
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
PS	Phosphatidylserine
PMA	Phorbol 12-myristate 13-acetate
7AAD	7- aminoactinomycin D
24h	24 hours
48h	48 hours
72h	72 hours
FOXP3	Forkhead box P3
PI	Propidium Iodide

CHAPTER 1

INTRODUCTION

1.1. T Cells

The immune system is a specialized system to protect the body aganist a variety of pathogens. The defense mechanism is very powerful. In order to destroy pathogens, immune system cells must recognize pathogens. Recognizing pathogens by receptors on the immune cells is an antigen specific process. Binding of antigens to immune cell receptors causes clonal expansion of lymphocytes. The initiation of immunological response depends on the ability of lymphocytes to differentiate self from non-self. Because of random joining of gene segments, lymphocytes has a large scale of receptors so that the self reactive immune cells which lead to autoimmune diseases can be eliminated by these receptors (Jager and Kuchroo, 2010).

T cells have important roles in regulation of immune response aganist different pathogens. A complete immune response is started by the activation of T cells. T cell activation is a complicated process. Naive CD4 T cells can be activated by a signal transduction network that causes the activation of transcription factors, the production of many cytokines, and the proliferation and differentiation of T cells. T cell activation causes transcription of some molecules, and transcripted molecules have major potentials in the formation of immune responses (Valle-Rios et al., 2009).

1.1.1. Naive CD4 T Cells

CD4 T cells take a prominent part in immune protection. They have important tasks such as helping B cells and directing them to make antibodies, activating macrophages, directing neutrophils, basophils and eosinophils to the inflammation site. They secrete certain cytokines and chemokines to administer immune response so naive

CD4 T cells can be accepted as the chef that regulate immune responses (Zhu and Paul, 2008).

Naive CD4 T cell activation requires interaction between T cell receptors and MHC class II molecules located on Antigen presenting cells (APCs). Naive CD4 T cells receive 3 different signals from APCs (Figure 1). The first signal includes the engagement of T cell receptors with MHC class II molecules. The signal is antigen specific. It is prominent for the initiation of T cell activation. There must also be a co-stimulatory molecule, CD4 for Naive CD4 T cells, to complete the activation process. The second signal provides the survival of T cells. The second signal includes co-stimulatory molecules such as B7 molecules. B7 molecules are one of the member of immunoglobulin superfamily whose structure is homodimeric. B7 molecules are involved in the initiation of T cell proliferation. CD28 molecules, another member of immunoglobulin superfamily, located on T cells interact with B7 molecules. The interaction has a significant role to ensure the clonal expansion of naive CD4 T cells. The third signal includes the cytokines which are secreted from APCs to provide the differentiation of naive CD4 T cells into different T helper cell subsets (Tseng and Dustin, 2002).

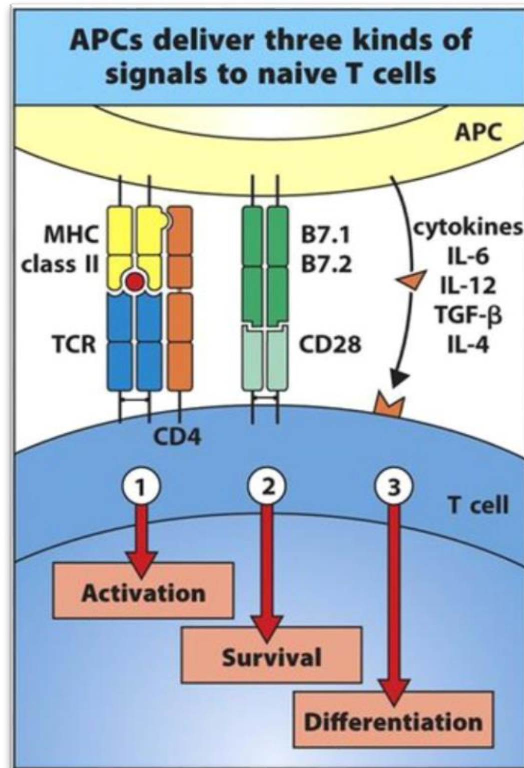

Figure 1. T cell activation
(Source: Janeway's Immunobiology, 8th Edition)

1.1.2. T Helper Cells

Naive CD4 T cells, which are the main players of the immune system regulation, can differentiate into diverse T helper (Th) cell subsets with different biological functions (Muranski and Refisto, 2009, Zhu et al., 2010) (Figure 2). The first two T helper cell subsets, Th1 and Th2 cells, were described in 1989. Th1 cells are known to produce IFN-γ. Th1 cell's master transcription factor is T-bet. Th1 cells are involved in cell mediated immunity. Th2 cells are characterized by the production of IL-4. Th2 differentiation is regulated by master transcription factor GATA3. Th2 cells provide humoral immune responses (Mosmann and Coffman, 1989). Although two helper cell subsets are different in terms of their cytokine production and their functions, both subsets are recognized to increase antitumor immunity by the induction of cytotoxic CD8 T cell (CTL) enlargement (Chamoto et al., 2003, Ankathatti et al., 2012). On the contrary, regulatory T cells (Treg) prevents the induction of CTLs and repress antitumor immunity. Treg cells are known to

produce TGF-β. Treg cell differentiation is driven by master transcription factor FOXP3. Treg cells increase immune tolerance and keep the homeostasis of T lymphocytes (Antony and Refisto, 2005, Turk et al., 2004). Th17 cells are another T cell subset. Identification of Th17 cells has important part in understanding regulator mechanisms in many inflammatory diseases (Steinman, 2007). While the importance of Th17 cells in inflammation and autoimmune diseases are well defined, their role in cancer is still not clear.

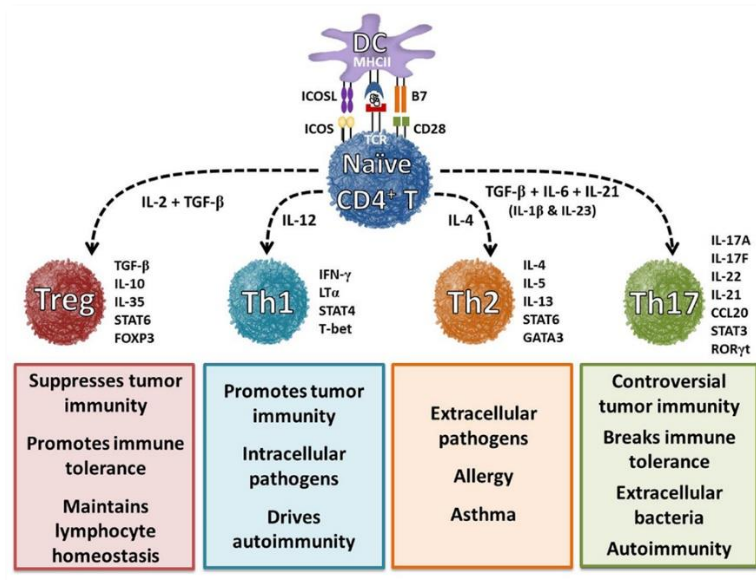

Figure 2. T helper cell differentiation. Naive CD4 T cells have the ability to differentiate into distinct T helper cell subsets in the presence of certain cytokines (Source: Bailey et al. 2014).

1.1.3. T helper 17 Cells

CD4 T helper cells play critical role in regulating cellular immune response. For many years, it was considered that there was only two T helper cell subsets, Th1 and Th2 cells. However, the case changed in the mid-2000s with the identification of Th17 cells. These cells were named as Th17 cells because their cytokine profile is IL-17. (Harrington et al., 2005, Park et al., 2005). Th17 cells differ from other T helper cell subsets in terms of their regulation and functions. Th17 cells are considered to play in pro-inflammation process and have important part in providing host defense aganist infection, by promoting

neutrophils and macrophages to inflammation site. Th17 cells produce IL-17A, IL-17F, and IL-22 cytokines (Annunziato et al., 2007, Wilson et al., 2007) and chemokine receptor CCR6 (Acosta-Rodriguez et al.,2007b, Annunziato et al., 2007, Hirota et al., 2007, Lim et al., 2008, Singh et al., 2008). Th17 cell differentiation is mediated by the master transcription factors RORγt (RORC in human) and RORα (Ivanov et al., 2006).

1.1.4. Jurkat cells

Jurkat cells are a subset of human T cells (Figure 3). Jurkat cells were firstly isolated from peripheral blood of a boy who has T cell leukemia. Jurkat cells are effectively used for the study of leukemia, signaling pathways of T cells, and many other purposes. They have ability to produce IL-2. Jurkat cells are one of the ideal cell line to investigate the answers the cell gives to cancer drugs and radiation effects (Schneider et al., 1977).

Figure 3. Jurkat cells (40X magnification light microscope image. Data from our experiments).

1.2. Plasma Membrane Changes

Because the working principle of nucleofection is to apply electrical voltage to the cells and to make pores throughout the cell membrane, it changes the phospholipid structure of the cell membrane and affects the continuity of cell function so the cells can undergo apoptosis. Apoptosis is a term used to describe the programmed cell death. Apoptosis is known as an important component of different processes including cell cycle, improvement of immune system, embriyonic development. During early apoptosis, different morphological changes occur. Cell shrinkage and pyknosis are two of them which can be seen in the light microscope. In cell shrinkage, the cells size become smaller, the cytoplasm becomes intense. One of the earliest change during apoptosis is the change the localization of phosphatidylserine (PS). Under normal viability conditions, phosphatidylserine (PS) is mainly found in the inner membrane leaflet. After apoptosis begins, PS is transferred to the outer leaflet of the plasma membrane (Fink and Cookson, 2005). The changes can be detected by using Annexin V. Annexin V is a Calcium dependent phospholipid binding protein used for detecting plasma membrane changes in early apoptosis. Annexin V has high affinity for PS. Annexin V binds to PS in the outer leaflet of the plasma membrane that are in early apoptotic process. However, in the late apoptosis, plasma membrane loses its normal physiological properties of the plasma membrane and allows the passage of some dyes such as 7AAD, PI. 7AAD and PI binds to DNA. So, 7AAD and PI are used to investigate late apoptotic cells.

1.3. Transfection

Transfection is a process that delivers molecules into different cell types to obtain genetically altered cells. It is an important method for the investigation of genes and proteins regulative mechanisms. Transfected molecules can exist in two ways in the cells: stably or transiently. The existance depends on the nature of transfected molecules. In stable transfection, introduced molecules always include a distinctive gene part for selection. Distinctive gene is a marker gene. Stable transfection can be characterized by the integration of genetic molecules into host genome. As long as host cell replication is continued, marker genes can replicate and express as the same time. Conversely, transient

transfection includes limited period of time to express genes. Transfected molecules can not integrate into host genome and can be lost due to different reasons, including environmental reasons, cell division. The choice for stable or transient transfection only depends on the purpose of your study. Many transfection methods have been improved. These methods can extensively be categorized into 3 groups: biological, chemical, and physical transfection methods (Figure 4). Each method has its own approach that apply on different cell type with different purposes (Kim and Eberwine, 2010).

Class	Methods	Advantages	Disadvantages
Biological	• Virus-mediated	- High-efficiency	- Potential hazard to laboratory personnel
		- Easy to use	
		- Effective on dissociated cells, slices, and in vivo	- Insertional mutagenesis
			- Immunogenicity
			- DNA package size limit
Chemical	• Cationic polymer	- No viral vector	- Chemical toxicity to some cell types
	• Calcium phosphate	- High-efficiency	- Variable transfection efficiency by cell type or condition
	• Cationic lipid	- Easy to use	- Hard to target specific cells
		- Effective on dissociated cells and slices	
		- Plenty of commercially available products	
		- No package size limit	
Physical	• Direct injection	- Simple principle and straightforward	- Needs special instruments
	• Biolistic particle delivery	- Physical relocation of nucleic acids into cell	- Vulnerable nucleic acids
	• Electroporation	- No need for vector	- Demands experimenter skill, laborious procedure
	• Laser-irradiation	- Less dependent on cell type and condition	
	• Sonoporation	- Single-cell transfection	
	• Magnetic nanoparticle		

Figure 4. Transfection methods
(Source: Kim et al. 2010)

1.3.1. Biological Transfection Methods

Virus based transfection is one of the most used methods in clinical research. This method is also known as transduction. Transduction provides high transfection efficiency and its simple to obtain continuous transfected gene expression. Biological transfection method contains viral vectors. The viral vectors can be Herpes simplex virus, Adeno virus, Adeno associated virus and many others. These vectors are used for providing the

integration of transfected genes into host genome. The main disadvantage of biological transfection method is the requirement of extra laboratory safety systems. There is also another considerable point. When a viral vector is integrated into host genome, the vector can trigger some of the important genes including immune system genes, oncogenes. So the immune response network can change (Kim and Eberwine, 2010).

1.3.2. Chemical Transfection Methods

Chemical based methods are initially used for transfection of molecules into mammalian cells. Chemical methods include the use of some chemicals such as calcium phosphate, cationic lipid. The main mechanism is based on the formation of a complex between chemical substances and nucleic acids. Then the chemical/nucleic acid complex binds to the cell membrane and passes through pores in the cell membrane and goes into the cell. Transfection efficiency can be affected by the rate of nucleic acids and chemicals used, the pH value of solutions, cell membrane permeability. When these reasons are taken into account, chemical transfection methods have low transfection efficiency than biological transfection methods. However, chemical methods have some benefits when compared to biological methods. The benefits can be low cytotoxicity, the absence of mutagenesis, the absence of nucleic acid package restriction (Kim and Eberwine, 2010).

1.3.3. Physical Transfection Methods

Physical methods have recently known the most preferred methods. These methods use a variety of physical tools to send molecules into the cell. These method contains biolistic particle delivery, micro-injection, laser mediated transfection, electroporation and nucleofection. Biolistic particle delivery contains gold molecules. Gold molecules bind to nucleic acids. Then, the gold/nucleic acid complex is sent into the cell with a high speed. The process can also be named as gene gun. Biolistic particle delivery is trustworthy but expensive laboratory tools are necessary. Micro-injection method injects molecules into cells. Micro-injection method requires skill. And the disadvantage is that cell survival is not maintained after transfection. Laser based

transfection utilize laser to create pores in the cell membrane. So the molecules can go into cells through these pores. Because it utilizes laser, laser mediated transfection can applied on very small cells. Electroporation is widely preferred method in physical methods. Electroporation gives electrical pulses to form pores in the cell membrane. So molecules can pass through these holes and go into cell cytoplasm. Electroporation is quick and simple. Once optimal transfection conditions are established, it is possible to transfect different cells efficiently (Kim and Eberwine, 2010).

1.3.4. Nucleofection

Nucleofection which is a modified form of electroporation is an important technology for transfection of molecules into cells. Nucleofection is a combination of nucleofection solutions and specific electrical parameters for each cell type. Nucleofection is used to transfect different cell types. Nucleofection has some benefits over standard electroporation. Nucleofection provides effectively transfection results in hard to transfect cells. It allows high transfection efficiency by using much lower substrate amounts. Nucleofection method was used to transfect DNA into natural killer cells in 2003 (Trompeter et al., 2003). The study wanted to show a simple and efficient method for transfection of natural killer cells. In 2005, nucleofection was applied on neuron cells (Leclere et al., 2005). The study especially focused on transfection of retinal ganglion cells. Another study in 2006 aimed to investigate the effects of nucleofection on stem cells (Aluigi et al., 2006). Since little information was available in the literature about stem cell transfection, the study demonstrated that stem cells could be transfected by using nucleofection. Few studies have been available in the literature that focused on T cell nucleofection. In 2003, resting and activated mouse T cells were transfected by nucleofection (Lai et al., 2003). Nucleofection efficiency of resting mouse CD4 T cells was found nearly 14% in a representative experiment, and the efficiency increased to 35% after resting CD4 T cells were activated. In a study carried out in 2014, the effects of nucleofection on the activation state of human CD4 T cells morphology,, intracellular Ca++ levels, cell surface activation markers, and transcriptional activity were investigated (Zhang et al., 2014).

The aim of this thesis is to transfect primary human CD4 T cells with high efficiency, to investigate the effects of activation on T cells transfection efficiency and the effects of nucleofection on T cells apoptosis.

CHAPTER 2

MATERIALS AND METHODS

2.1. PBMC Isolation

The blood samples were obtained from healthy donors with the ethical permission of Dokuz Eylul University. PBMC isolation was achieved by Ficoll-Hypaque density gradient centrifugation (Boyum et al., 1991). Peripheral blood was the main resource of lymphocytes for studying of human immune regulation. Peripheral blood mononuclear cells (PBMC) were obtained by using Ficoll-Hypaque density gradient centrifugation. Ficoll centrigugation method was an effective method for obtaining PBMCs. This method used the density differences between lymphocytes and other cells. 20 ml whole blood sample was put into falcon tube and was centrifugated at 1500 RPM for 5 minutes. After centrifugation, the top of blood sample was removed because this part was serum. Then, the blood sample was diluted 1:1 with PBS and was mixed well. 20 ml blood/PBS mix was placed on 10 ml Ficoll. Then, tubes were centrifugated at 2500 RPM for 45 minutes. After centrifugation process, erythrocytes were collected on the bottom of the tube because their density was higher than ficoll. Trombocytes, hormones, cytokines and electrolytes were collected on the top of the tube because their density was lower than ficoll. PBMC was layered on the interphase of the tube just above the ficoll (Figure 5).

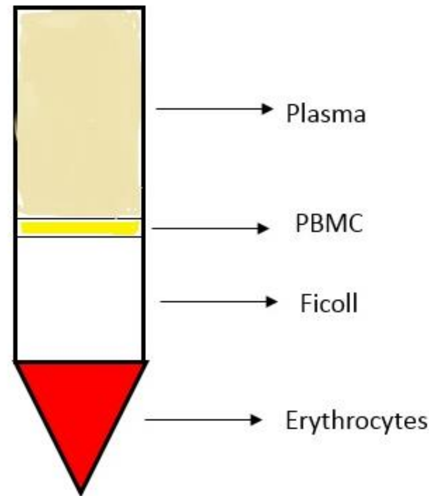

Figure 5. PBMC isolation. PBMC placed in the interphase of the tube.

2.2. Naive CD4 T Cells Sorting

Naive CD4 T cells were sorted by using "Human Naive CD4+ T cell isolation Kit II (Miltenyi Biotec, Bergisch Gladbach). The isolation of these cells was based on a negative selection manner. The sorting process was started with the manufacturer's procedure, 10^7 cells for each experiment. Firstly, cell were washed at 300g for 10 minutes and the supernatant was removed. Cell pellet was resuspended with 40 µl of variomax buffer and 10 µl of Naive CD4+ T Cell Biotin-Antibody Coctail II and then was incubated at 4 ^{0}C for 5 minutes. 30 µl of buffer and 20 µl of Naive CD4+ T Cell MicroBead Coctail II were added. Cells were incubated at 4 ^{0}C for 10 minutes. After incubation, VARIOMACS separator column was placed in the magnetic field. The this column was washed with 3 mL of buffer. After that, antibodies and cell pellet mix were placed in this column and the unwanted antibodies were hold in the column so Naive CD4 T cells were taken as flow through. For increasing sorting efficiency, the column was loaded again with 3 mL buffer. Then, the column was removed from the separator and was loaded with 5 mL of buffer and by pushing the plung into the column depleted cells were taken.

2.3. Th17 Cells Culture

Naive CD4 T cells were cultured in IMDM (Lonza) with 5% FBS, 2% Penicillin (100µl/mL) and Streptomycin (100µl/mL) and 0.1% β-mercaptoethanol. Naive CD4 T cells were differentiated into Th17 cells in the presence of different stimulants. For T cell receptor (TCR) activation, 5µg/ml anti-CD3 and 5µg/ml anti-CD28 (BD Biosciences) were used. In order to achieve a complete Th17 differentiation, 10ng/ml IL-1β, 10ng/ml IL-23, 30ng/ml IL-6 and 0,5ng/ml TGF-β (eBioscience) were used. Also some of other stimulants such as 10µg/ml anti-IFNγ and 10µg/ml anti-IL4 (eBioscience) were used to prevent the differentiation to another T helper cell subsets including Th1, Th2 or Treg. Cells were transfected at 3th, 5th, and 7th day of the culture. For 7 days culture, the cells were refreshed with 10ng/ml IL-23 at 5th day of the culture (Table 1).

Table 1. Culture contents of Th17 cells. (Modified from Veldhoen's culture conditions (Veldhoen et al., 2006) in Nalbant Lab).

STIMULANTS	AMOUNT	FUNCTIONS
Anti-CD3	5µg/ml	for TCR activation
Anti-CD28	5µg/ml	for TCR activation
IL-1β	10ng/ml	for Th17 polarization
IL-6	30ng/ml	for Th17 polarization
TGF-β	0,5ng/ml	for Th17 polarization
IL-23	10ng/ml	for Th17 polarization
Anti-IFN-γ	10µg/ml	to block Th1differentiation
Anti-IL-4	10µg/ml	to block Th2 differentiation

2.4. Jurkat Cells Culture

Jurkat cells were cultured in 90% RPMI 1640 with 10% FBS + 2 mM L-glutamine and 1% Penicillin (100 µl/mL) and Streptomycin (100 µl/mL). Jurkat cell culture were refreshed in every 2-3 days.

2.5. Cells Counting

Jurkat cells and naive CD4 T cells were counted by Trypan blue staining. 10 µl cell culture was put in ependorf. And 90 µl Trypan Blue dye was added to this culture, and was mixed well. Then 10 µl of cell culture/Trypan Blue mix was put in a hemocytometer and cell were counted.

2.6. Jurkat Cells Activation

For Jurkat cells activation, PMA (25 ng/ml) and Ionomycin (1 µg/ml) were used. Jurkat cells were cultured at a concentration of 1×10^6 cells/mL. Cells were incubated at different time points (24, 48, 72h) with or without stimulants at 37°C in a humidified incubator with 5% CO_2. The medium included 90% RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 µl/mL penicillin, 100 µl/mL streptomycin.

2.7. Nucleofection

Nucleofection process was achieved by using Amaxa 4D-Nucleofector. Firstly, cells were counted with trypan blue staining. Then cells were centrifugated at 1200 RPM for 10 minutes. The supernatant was removed and the cell pellet was mixed with mastermix. The nucleofection experiment was fixed at 1 million cells for each of the nucleofector strip well. This mastermix contained 16,4 µl Nucleofector Solution, 3,6 µl Nucleofector Supplement, and 0,4 µg pmax GFP. Cell pellet and mastermix was then loaded in nucleofector strip well and the strip was placed in Nucleofector X unit. After that, the appropriate program was chosen according to the cell type. After nucleofection, 80 µl media was placed in the strips which contained transfected cells and were incubated for 10 minutes. After incubation, nucleofected cells were placed in plates with media.

2.8. Cell Surface Staining

After nucleofection process, the cells were analyzed with flow cytometer and flourescence microscope at the certain time points. For analyzing in flow cytometer, the cells should have stained with different cell surface antibodies. Naive CD4 T cells were stained with CD4 antibody. Jurkat cells were stained with CD69 antibody. 50 µl of nucleofected cells were placed in 96 well plate. Then, 10 µl of CD4 and CD69 antibody was placed in cell culture and the cells were incubated at 4 ^{0}C for 15 minutes. after incubation the cells were washed with PBS at 300g for 10 minutes. The supernatant was removed and the cells were mixed with PBS and analyzed in flow cytometer and fluorescence microscope.

2.9. Cell Viability Determination

The cells were also stained with cell death markers including Annexin V, 7AAD and PI. 50 µl of cell culture was placed in 96 well plate. Then, 50 µl of 1X Annexin Binding Buffer was placed in cell culture. After that, 10 µl Annexin, 10 µl 7AAD or 10 µl PI was put in wells and was incubated at the dark for 15 minutes. After incubation, the cells were washed with PBS at 300g for 10 minutes and the cells were mixed with PBS and were analyzed in flow cytometer and fluorescence microscope.

2.10. Flow Cytometric Analysis

After cell were stained with different antibody conjugated dyes, they were analyzed in flow cytometry. Flow cytometric analysis were performed with ATTUNE Flow Cytometry. GFP was analyzed in BL1 channel. Annexin V was analyzed in BL2 channel. 7AAD was analyzed in BL3 channel. CD4 was analyzed in RL2 or BL4 channels.

2.11. Fluorescence Microscope Analysis

After cells were stained with different antibody conjugated dyes, they were also analyzed in fluorescence microscope (Olympus IX70). GFP+ cells were analyzed in Filter 1 (Blue light). CD69+ cells were also analyzed in Filter 1 (Blue light). PI+ cells were analyzed in Filter 2 (Green light).

2.12. Statistical Analysis

The data were analyzed by ATTUNE Flow Cytometry. Microsoft Office Excel was used for further analysis of the flow cytometry data. Flow cytometric experiments on naive CD4 T cells and Jurkat cells were performed in triplicates and the average was taken at each time point and were compared to control groups (untransfected cells) using student’s t test and $P<0.05$ accepted scientifically significant. Error bars represent the standart deviation.

CHAPTER 3

RESULTS

3.1. Nucleofection of Unstimulated Human T cells

3.1.1. Nucleofection of PBMC

The first nucleofection experiment was focused on PBMCs. Because PBMC contains different cells, including CD3 T cells, CD4 T cells, CD14 cells also known as monocytes. In this first experiment, 5 different nucleofection programs were applied on PBMCs. The purpose of this experiment was to create an overlook for the effect of nucleofection on PBMC. The applied programs were; EO-115, EA-113, FI-115, FF-115, and FA-115. PBMC cells were transfected with pmax GFP. There was also untransfected cells (Figure 6). These programs had different voltage properties. CD3+GFP+ cells represented transfection efficiency for CD3 cells. CD4+GFP+ cells represented transfection efficiency for naive CD4 T cells. CD14+ GFP+ cells represented transfection efficiency for monocytes. All programs used except one had high transfection efficiency on CD3 T cells. Analysis were carried out in flow cytometer 48h post nucleofection. In FI-115, CD3+GFP+ cells rate was 19% (Figure 9.A). This was the highest rate for CD3 T cells. This rate decreased to 16% in FF-115 (Figure 10.A). The lowest 3 programs for CD3 T cells were EO-115 (Figure 7.A), EA-113 (Figure 8.A), and FA-115 (Figure 11.A), and the rates of CD3+ GFP+ cells were 10%, 10%, and 2%, respectively. These programs had different effect on CD4 T cells. The rate of CD4+GFP+ cells was 17% in FI-115 (Figure 9.B). This is the highest rate for CD4 T cells. The second high rate for CD4+ GFP+ cells was 13% in FF-115 (Figure 10.B). This rate was decreased to 8% in both EO-115 (Figure 7.B) and EA-113 (Figure 8.B). The lowest rate for CD4+GFP+ cells were 1% in FA-115 (Figure 11.B). These programs were also applied on CD14 cells. However, the transfection efficiency is very very low. The rate of CD14+GFP+ cells was 0% in all programs. This meaned that all applied program did not have the appropriate voltages for

nucleofection of CD14 cells. Among the 5 different nucleofection programs, one provided high transfection efficiency for CD4 T cells. This program was FI-115 (Figure 9.B). FI-115 was a specific program for unstimulated human T cells. So the next nucleofection experiments on naive CD4 T cells were carried out by using FI-115.

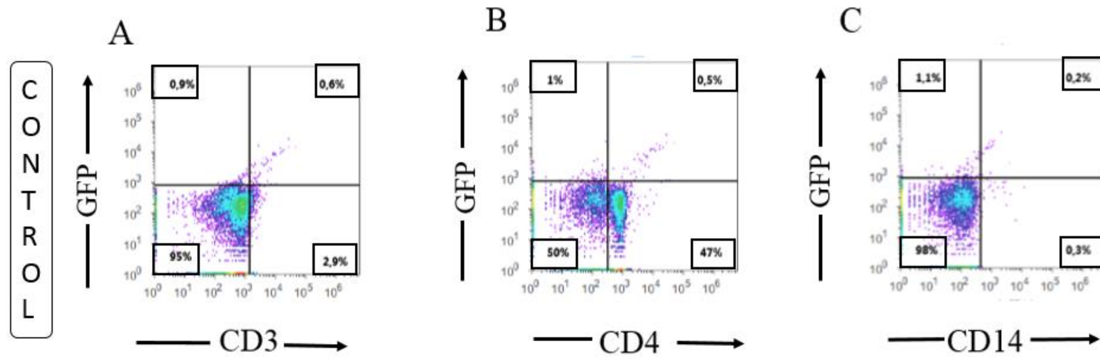

Figure 6. Untransfected PBMCs. (A) Transfection efficiency for CD3 T cells. (B) Transfection efficiency for naive CD4 T cells. (C) Transfection efficiency for monocytes.

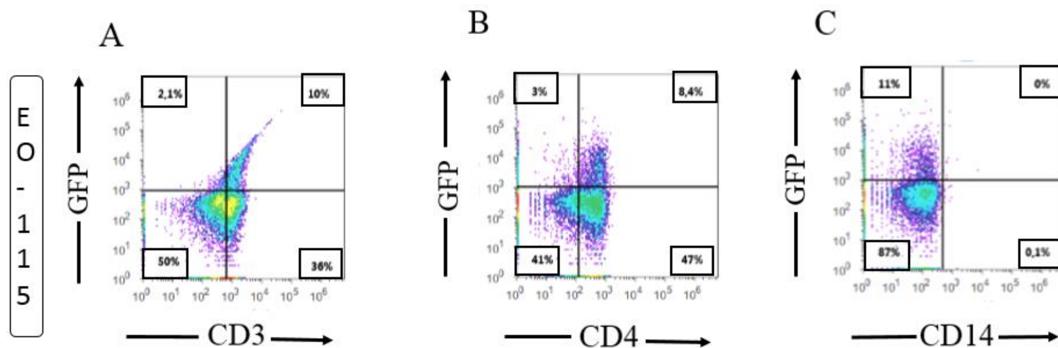

Figure 7. PBMC transfection with program EO-115. (A) Transfection efficiency for CD3 T cells. (B) Transfection efficiency for naive CD4 T cells. (C) Transfection efficiency for monocytes.

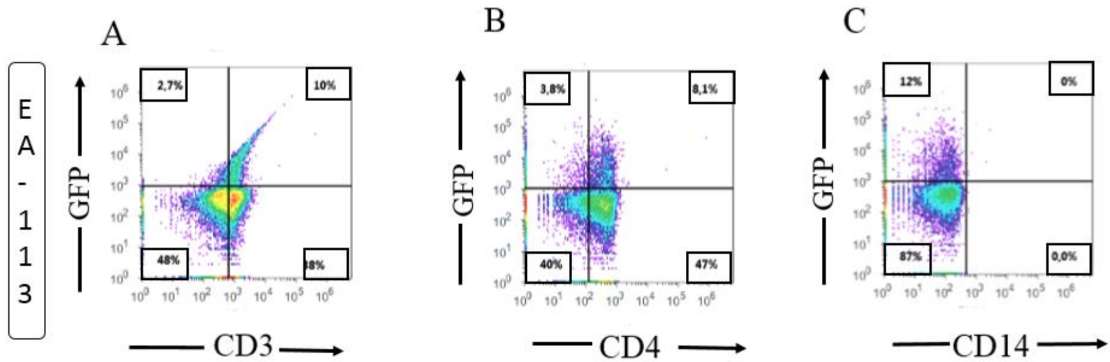

Figure 8. PBMC transfection with program EA-113. (A) Transfection efficiency for CD3 T cells. (B) Transfection efficiency for naive CD4 T cells. (C) Transfection efficiency for monocytes.

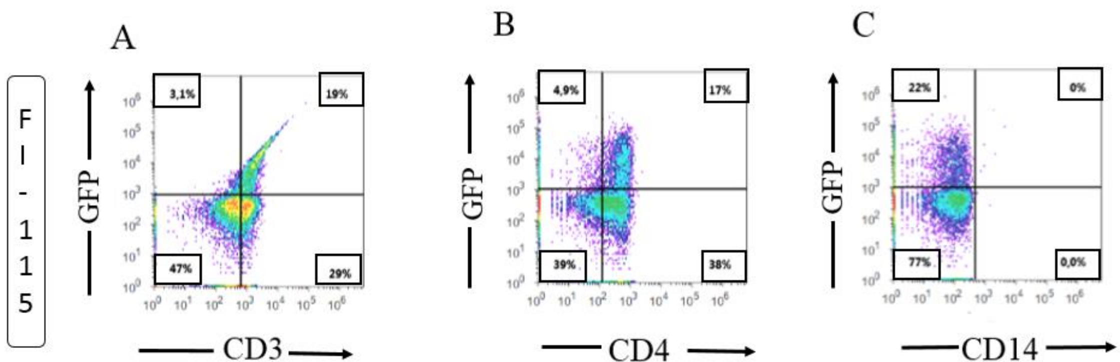

Figure 9. PBMC transfection with program FI-115. (A) Transfection efficiency for CD3 T cells. (B) Transfection efficiency for naive CD4 T cells. (C) Transfection efficiency for monocytes.

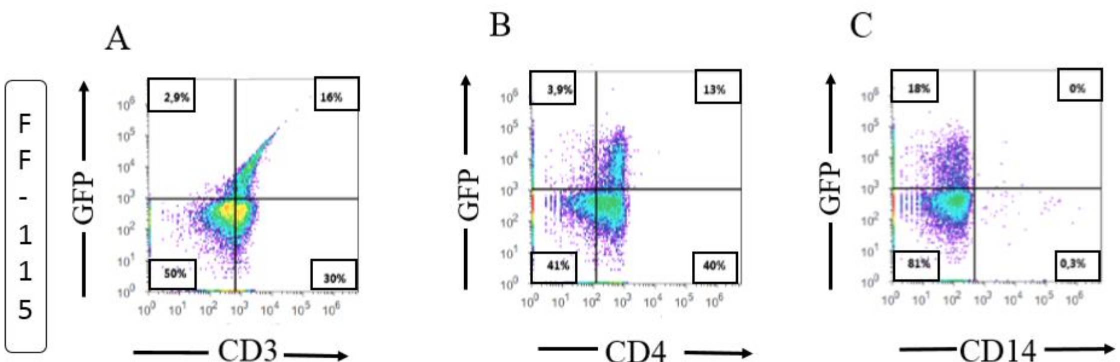

Figure 10. PBMC transfection with program FF-115. (A) Transfection efficiency for CD3 T cells. (B) Transfection efficiency for naive CD4 T cells. (C) Transfection efficiency for monocytes.

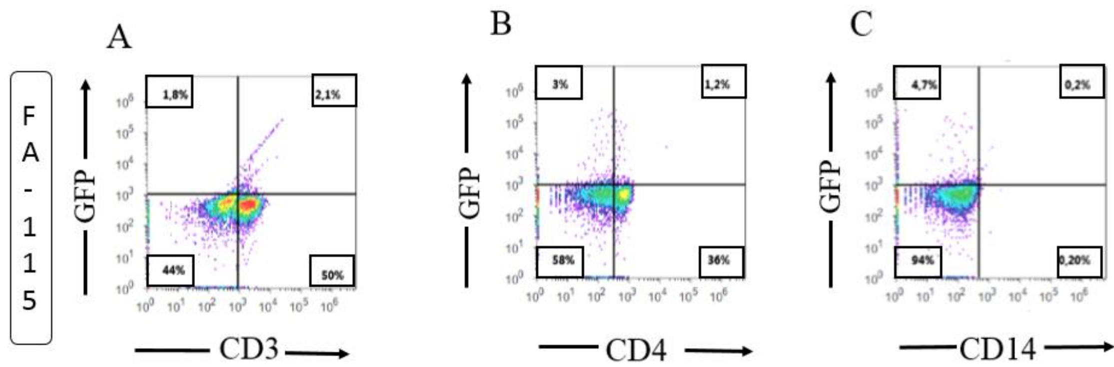

Figure 11. PBMC transfection with program FA-115. (A) Transfection efficiency for CD3 T cells. (B) Transfection efficiency for naive CD4 T cells. (C) Transfection efficiency for monocytes.

3.1.2 Nucleofection of Naive CD4 T cells

After the first nucleofection experiment which was carried out on human PBMCs, the highest transfection efficiency for naive CD4 T cells was found 17% in program FI-115. So the next experiment was designed to find the highest transfection efficiency and the highest cell viability by using FI-115. Naive CD4 T cells were transfected with pmax GFP for 24h and 48h. There were also untransfected cells (Figure 12.A). Post transfection analysis were carried out in flow cytometer. CD4+GFP+ cells represented transfection efficiency. Transfection efficiency was found 12% 24h post nucleofection (Figure 12.B). Cell viability was determined by using Annexin V and 7AAD staining. Annexin V was Ca++ dependent phospholipid binding protein which showed the cells were undergoing to early apoptosis. 7AAD was a nucleic acid binding dye for detecting death cells. Annexin V+GFP+ cells showed early apoptotic cells. GFP+7AAD+ cells showed dead cells. Annexin V+GFP+ cells rate was found 8% (Figure 13.C) and GFP+7AAD+ cells rate was found 5% (Figure 13.D) 24h post nucleofection.

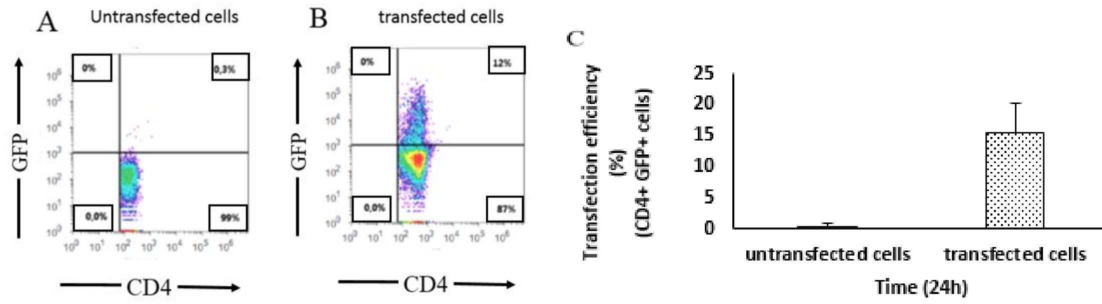

Figure 12. Transfection efficiency for naive CD4 T cells 24h post nucleofection. (A) Untransfected cells. (B) Cells were nucleofected with pmax GFP by using program FI-115. (C). Standart deviations and means were calculated. Error bars show standart deviation and $P<0.05$. Data are representative of three independent experiments.

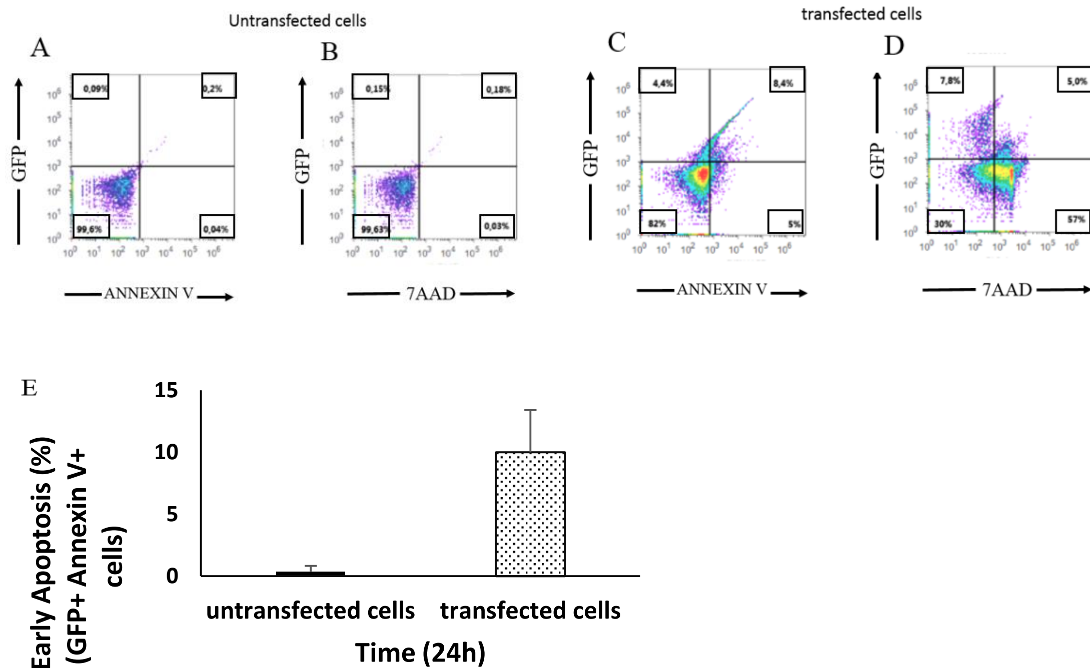

Figure 13. Cell viability results for naive CD4 T cells 24h post nucleofection. (A) Early apoptotic cells rate (GFP+Annexin V+) for untransfected cells. (B) Late apoptotic cells rate (GFP+ 7AAD+) for untransfected cells. (C) Early apoptotic cells rate (GFP+Annexin V+) for transfected cells. (D) Late apoptotic cells rate (GFP+ 7AAD+) for transfected cells. (E) Standart deviations and means were calculated. Error bars show standart deviation and $P<0.05$. Data are representative of three independent experiments.

Naive CD4 T cells were also nucleofected with pmax GFP for 48h. Post transfection analysis were carried out in flow cytometer. CD4+GFP+ cells rate showed transfection efficiency. 48h transfection efficiency was found higher than 24h post nucleofection. CD4+GFP+ cells rate was 45% (Figure 14.B). Cell viability rates were also analyzed. GFP+Annexin V+ cells rate was found 8% (Figure 15.C). This rate was the same as 24h post nucleofection GFP+Annexin V+ cells rate analysis. GFP+7AAD+ cells rate was found 0% (Figure 15.D). 24h post nucleofection analysis had higher cell death rate than 48h post nucleofection analysis but this rate was not at a level that would affect for functional studies. Transfection efficiency was significantly increased to 45% 48h post nucleofection (Figure 14.B).

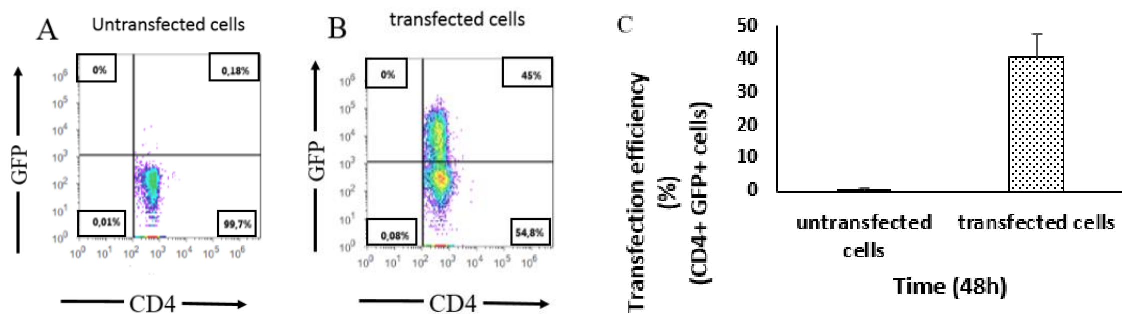

Figure 14. Transfection efficiency for naive CD4 T cells 48h post nucleofection. (A) Untransfected (control) cells. (B) Cells were transfected with pmax GFP by using program FI-115. (C) Standart deviations and means were calculated. Error bars show standart deviation and P<0.05. Data are representative of three independent experiments.

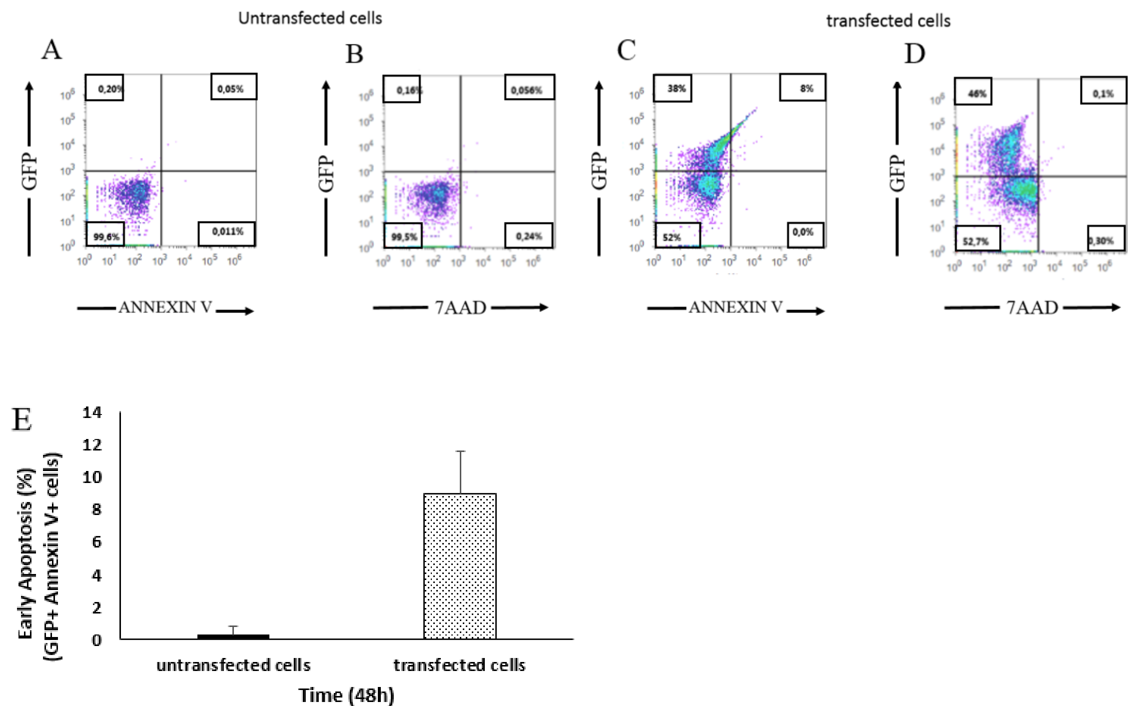

Figure 15. Cell viability results for naive CD4 T cells 48h post nucleofection (A) Early apoptotic cells rate (GFP+ Annexin V+) for untransfected cells. (B) Late apoptotic cells rate (GFP+ 7AAD+) for untransfected cells. (C) Early apoptotic cells rate (GFP+ Annexin V+) for transfected cells. (D) Late apoptotic cells rate (GFP+ 7AAD+) for transfected cells. (E) Standart deviations and means were calculated. Error bars show standart deviation and $P<0.05$. Data are representative of three independent experiments.

3.1.3. Nucleofection of Jurkat Cells

Nucleofection process was also applied on a human T cell line, Jurkat cells. Jurkat cells were nucleofected with pmax GFP for 24h and 48h by using program CL-120. Transfection efficiency and cell viability results were carried out in flow cytometer. Transfection efficiency indicated GFP+ cells rate. The transfection efficiency for Jurkat cells was 14% 24h post nucleofection (Figure 16.B). GFP+ Annexin V+ cells rate showed early apoptotic cells. GFP+Annexin V+ cells rate was found 3% (Figure 17.C). GFP+7AAD+ cells rate showed late apoptotic cells. GFP+ 7AAD+ cells rate was found 1% (Figure 17.D). Jurkat cells were nucleofected with pmax GFP for 48 hours. Transfection efficiency was found to increase 47% (Figure 18.B). GFP+ Annexin V+

cells rate increased to 18% (Figure 19.C). The rate of GFP+7AAD+ cells was found 3% (Figure 19.D).

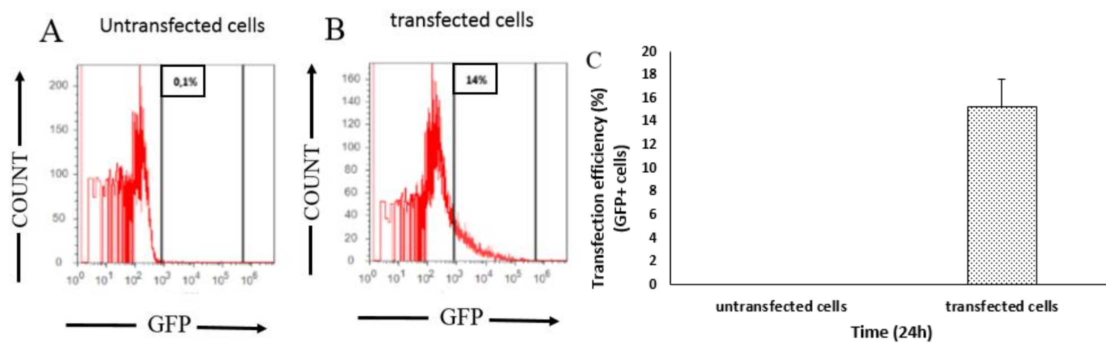

Figure 16. Transfection efficiency for Jurkat cells 24h post nucleofection (A) Untransfected (control) cells. (B) Jurkat cells were transfected with pmax GFP by using program CL-120 (C) Standart deviations and means were calculated. Error bars show standart deviation and $P<0.05$. Data are representative of three independent experiments.

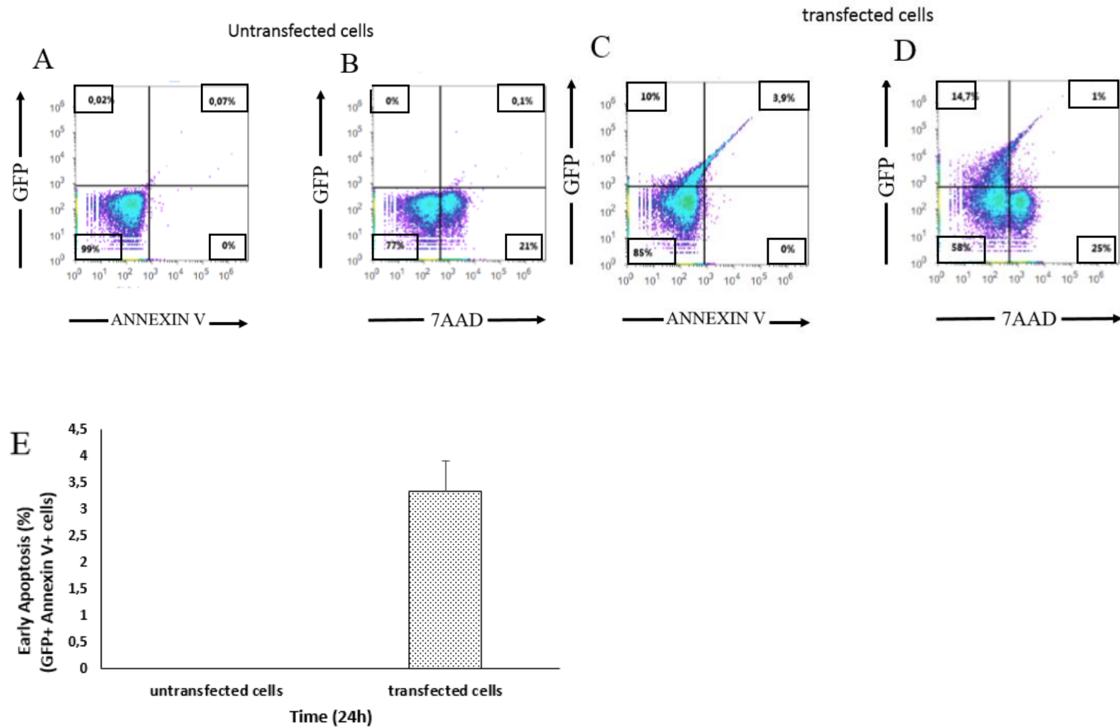

Figure 17. Cell viability results for Jurkat cells 24h post nucleofection (A) Early apoptotic cells rate (GFP+ Annexin V+) for untransfected cells. (B) Late apoptotic cells rate (GFP+ 7AAD+) for untransfected cells. (C) Early apoptotic cells rate (GFP+ Annexin V+) for transfected cells. (D) Late apoptotic cells rate (GFP+ 7AAD+) for transfected cells. (E) Standart deviations and means were calculated. Error bars show standart deviation and P<0.05. Data are representative of three independent experiments.

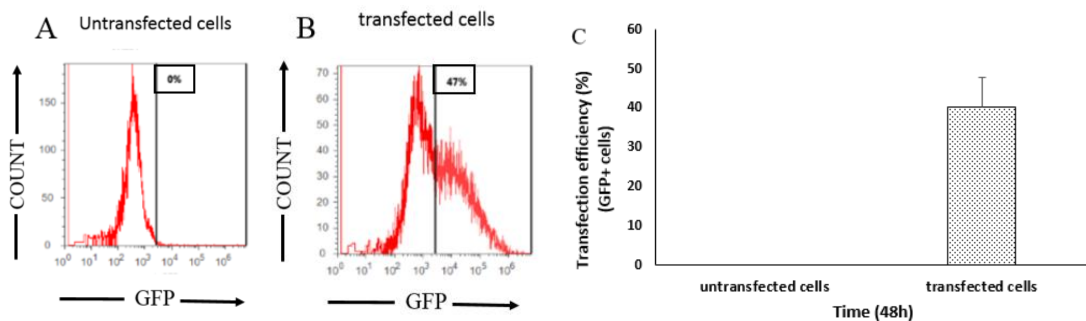

Figure 18. Transfection efficiency for Jurkat cells 48h post nucleofection (A) Untransfected (control) cells. (B) Jurkat cells were transfected with pmax GFP by using program CL-120 (C) Standart deviations and means were calculated. Error bars show standart deviation and P<0.05. Data are representative of three independent experiments.

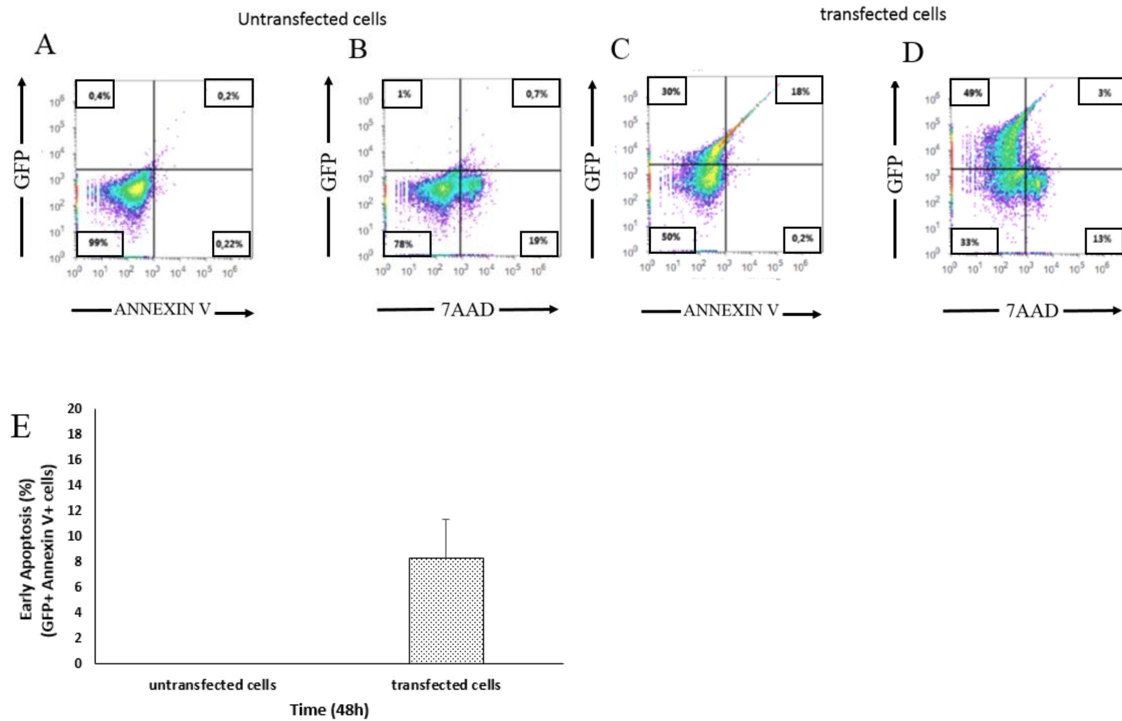

Figure 19.Cell viability results for Jurkat cells 48h post nucleofection (A) Early apoptotic cells rate (GFP+ Annexin V+) for untransfected cells. (B) Late apoptotic cells rate (GFP+ 7AAD+) for untransfected cells. (C) Early apoptotic cells rate (GFP+ Annexin V+) for transfected cells. (D) Late apoptotic cells rate (GFP+ 7AAD+) for transfected cells. (E) Standart deviations and means were calculated. Error bars show standart deviation and $P<0.05$. Data are representative of three independent experiments.

3.2. Activation of Jurkat Cells

Jurkat cells were activated with PMA and Ionomycin. PMA was a small organic molecule. When cells were exposed to PMA, PMA passed through the pores and went to the cytoplasm. PMA activated PKC in the cytoplasm and provided the release of Ca++. Ionomycin was a calcium ionophor. Like PMA, it provided Ca++ release. Control cells were not activated with PMA and Ionomycin (Figure 20). Jurkat cells were activated with PMA and Ionomycin for 24 (Figure 21), 48 (Figure 22), and 72 hours (Figure 23). Both activated and control cells were stained with CD69 and PI. CD69 positive cells represented activated cells. PI positive cells represented dead cells. The activation state and cell viability determination were carried out in fluorescence microscope (Olympus IX70).

Activated Jurkat cells were stained with CD69 and PI. CD69 is an early activation marker. CD69+ stained cells represented activated Jurkat cells. PI+ stained cells represented death cells. CD69+PI+ merged image showed both activated and dead cells. So 24 hours activation experiment showed that Jurkat cells became activated. There were death cells 24h post activation but their rate was not very high. So activation time point was determined as 24 hours.

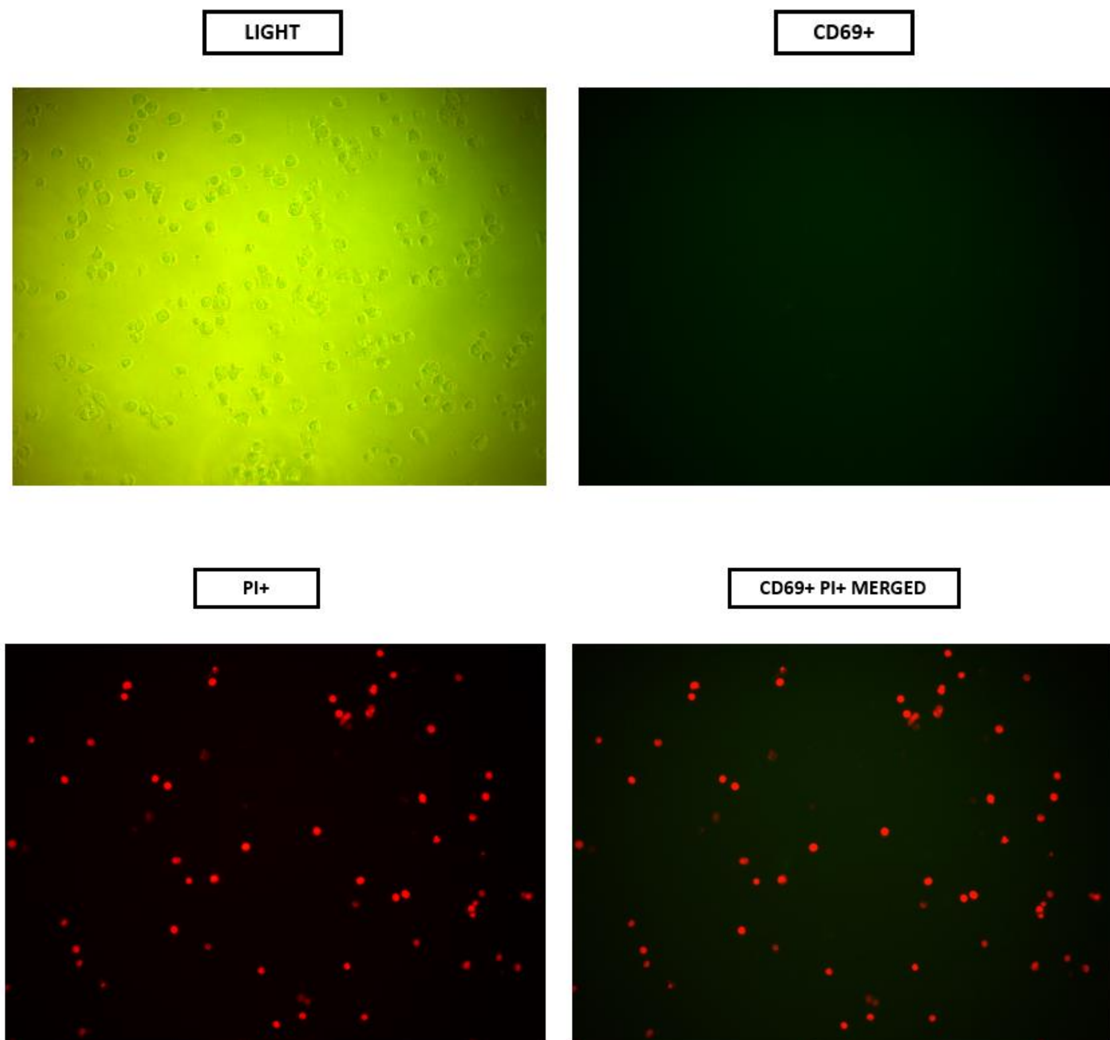

Figure 20. Non-activated (control) Jurkat cells. (40X magnification) CD69+ represented activated Jurkat cells. PI+ represented dead Jurkat cells. CD69+PI+ merged represented both activated and dead Jurkat cells

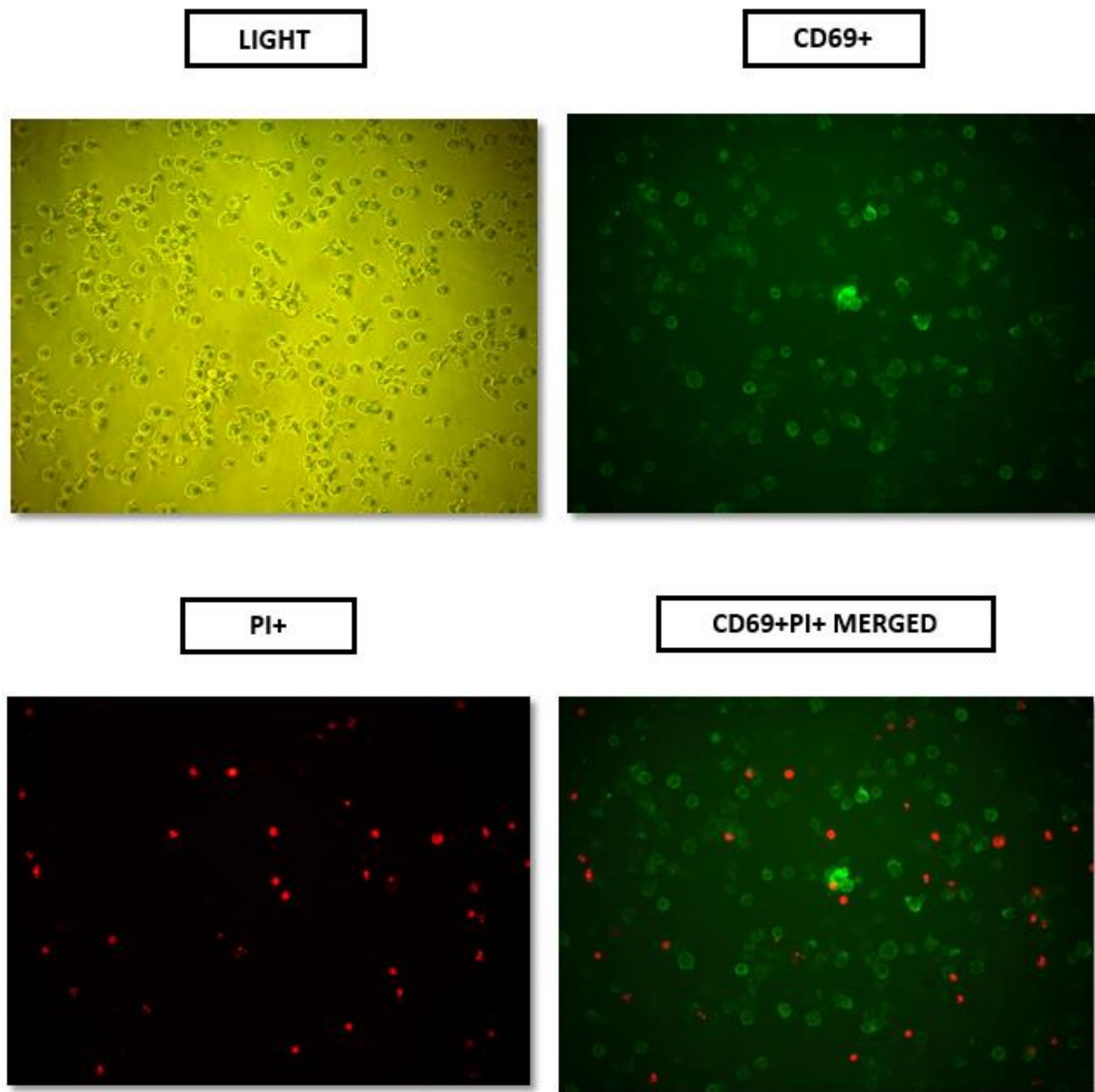

Figure 21. 24h activation results of Jurkat cells. (40X magnification) CD69+ represented activated Jurkat cells. PI+ represented dead Jurkat cells. CD69+PI+ merged represented both activated and dead Jurkat cells

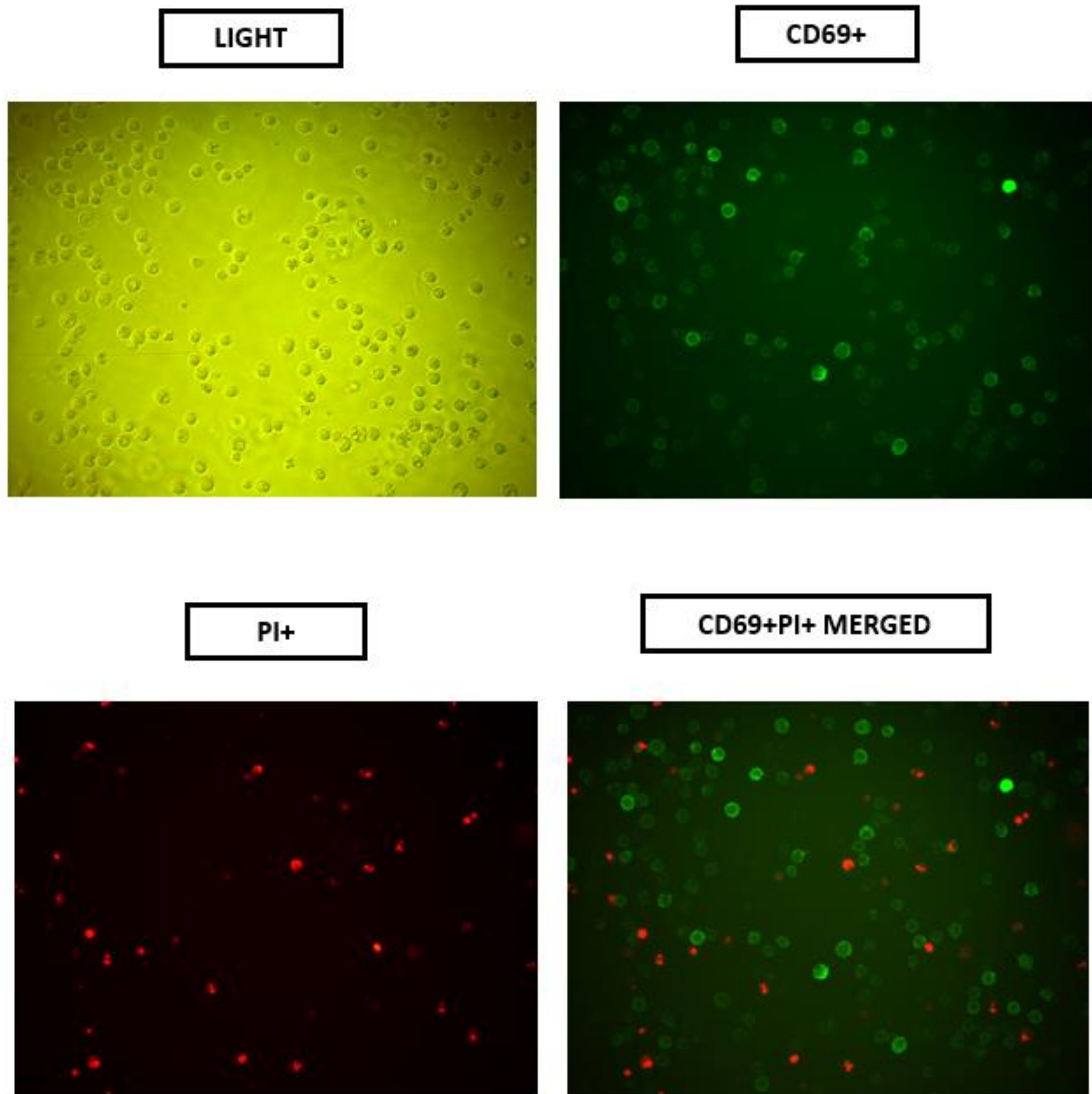

Figure 22. 48h activation results of Jurkat cells. (40X magnification) CD69+ represented activated Jurkat cells. PI+ represented dead Jurkat cells. CD69+PI+ merged represented both activated and dead Jurkat cells

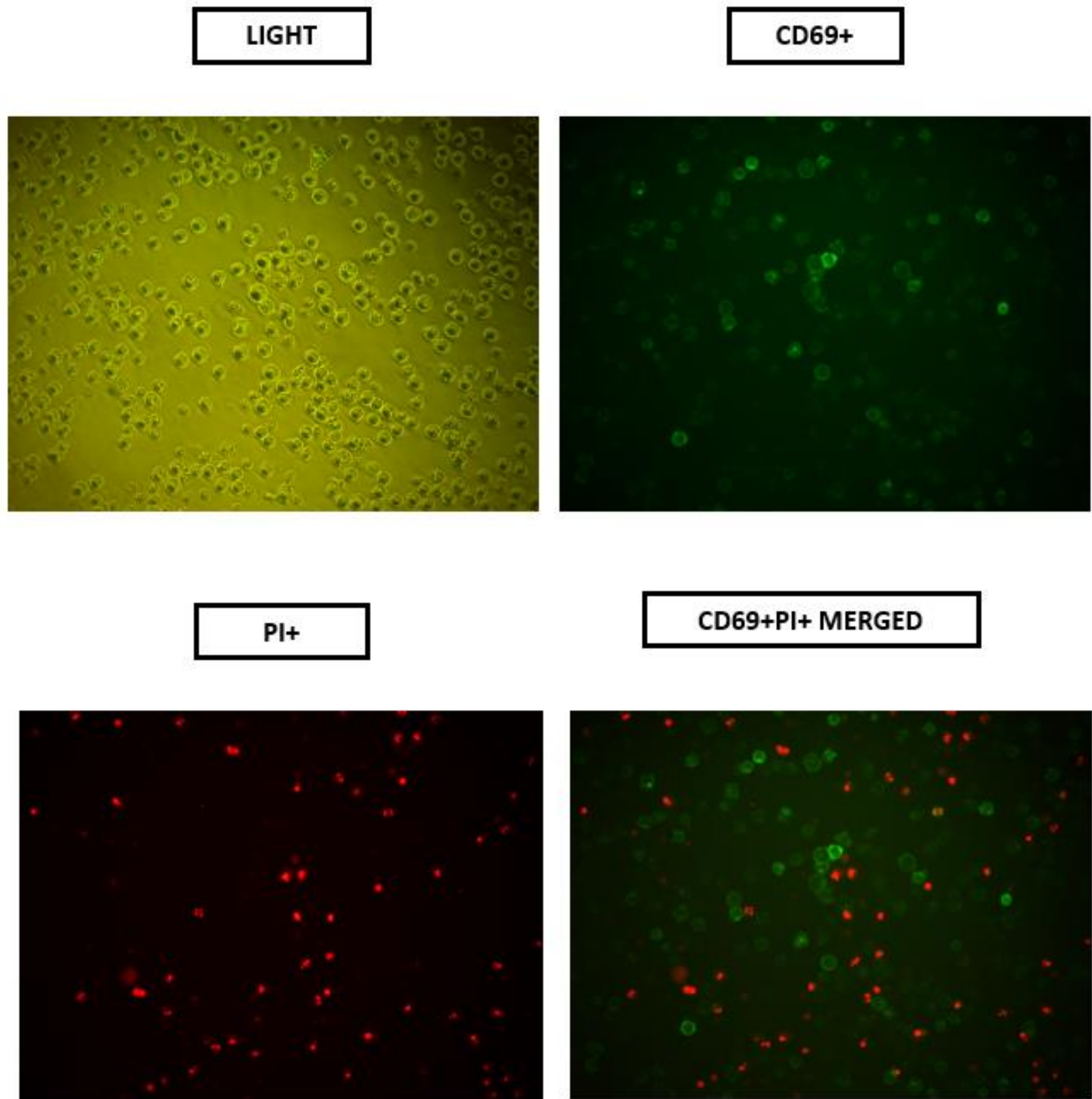

Figure 23. 72h activation results of Jurkat cells. (40X magnification) CD69+ represented activated Jurkat cells. PI+ represented dead Jurkat cells. CD69+PI+ merged represented both activated and dead Jurkat cells

3.3. Nucleofection of Stimulated Human T cells

3.3.1. Nucleofection of Activated Jurkat Cells

After the optimum activation conditions were determined for Jurkat cells, the nucleofection process was applied to these activated jurkat cells. Jurkat cells were activated with PMA and Ionomycin for 24 hours. These 24 hours activated Jurkat cells then were transfected with pmax GFP by using program EO-115 for 48 hours. Cell viability also was determined by PI staining. Analysis were performed in fluorescence microscope (Olympus IX70). GFP+ cells were analyzed in Filter 1(Blue light). PI+ cells were analyzed in Filter 2 (Green light). GFP+PI+ merged image showed both GFP transfected and dead cells. The results showed that activated jurkat cells were transfected successfully with pmaxGFP (Figure 24).

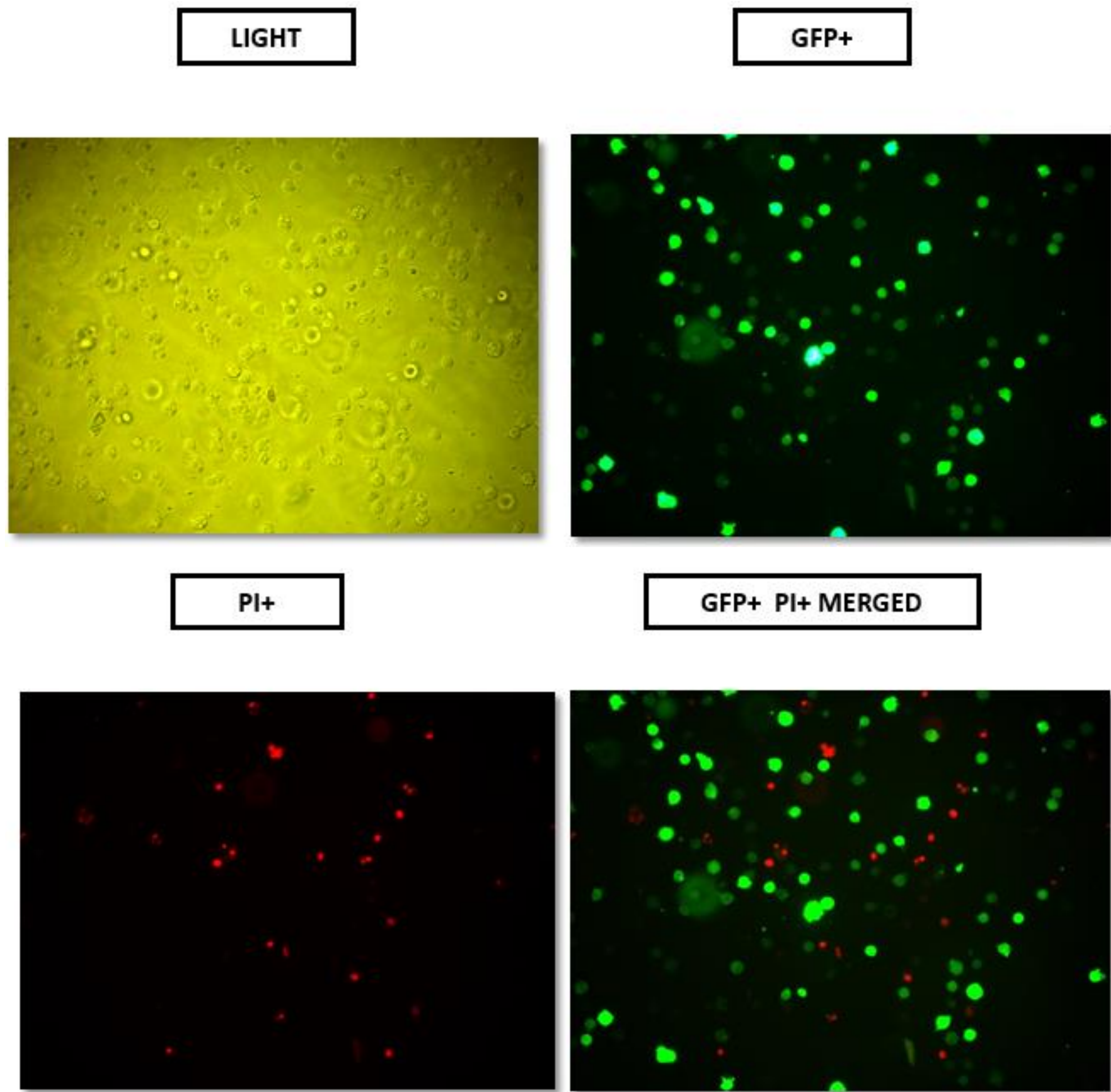

Figure 24. Nucleofection of activated Jurkat cells. (40X magnification) GFP+ represented transfected Jurkat cells. PI+ represented dead Jurkat cells. GFP+PI+ merged represented both transfected and dead Jurkat cells

3.3.2. Nucleofection of T helper 17 Cells

Naive CD4 T cells were differentiated into Th17 cells in the presence of certain cytokines. The differentiated cells were nucleofected at 3th (Figure 25), 5th (Figure 26), and 7th day (Figure 27) of the culture by using program EO-115. The result were analyzed in fluorescence microscope (Olympus IX70). Cells were analyzed in terms of GFP transfection and cell death. The results indicated that Th17 cell culture could be

transfected at 3th, 5th, and 7th day of the culture. GFP+ cells were analyzed in Filter 1 (Blue Light). PI+ cells were analyzed in Filter 2 (Green Light). The GFP+PI+ merged images showed both transfected and dead cells.

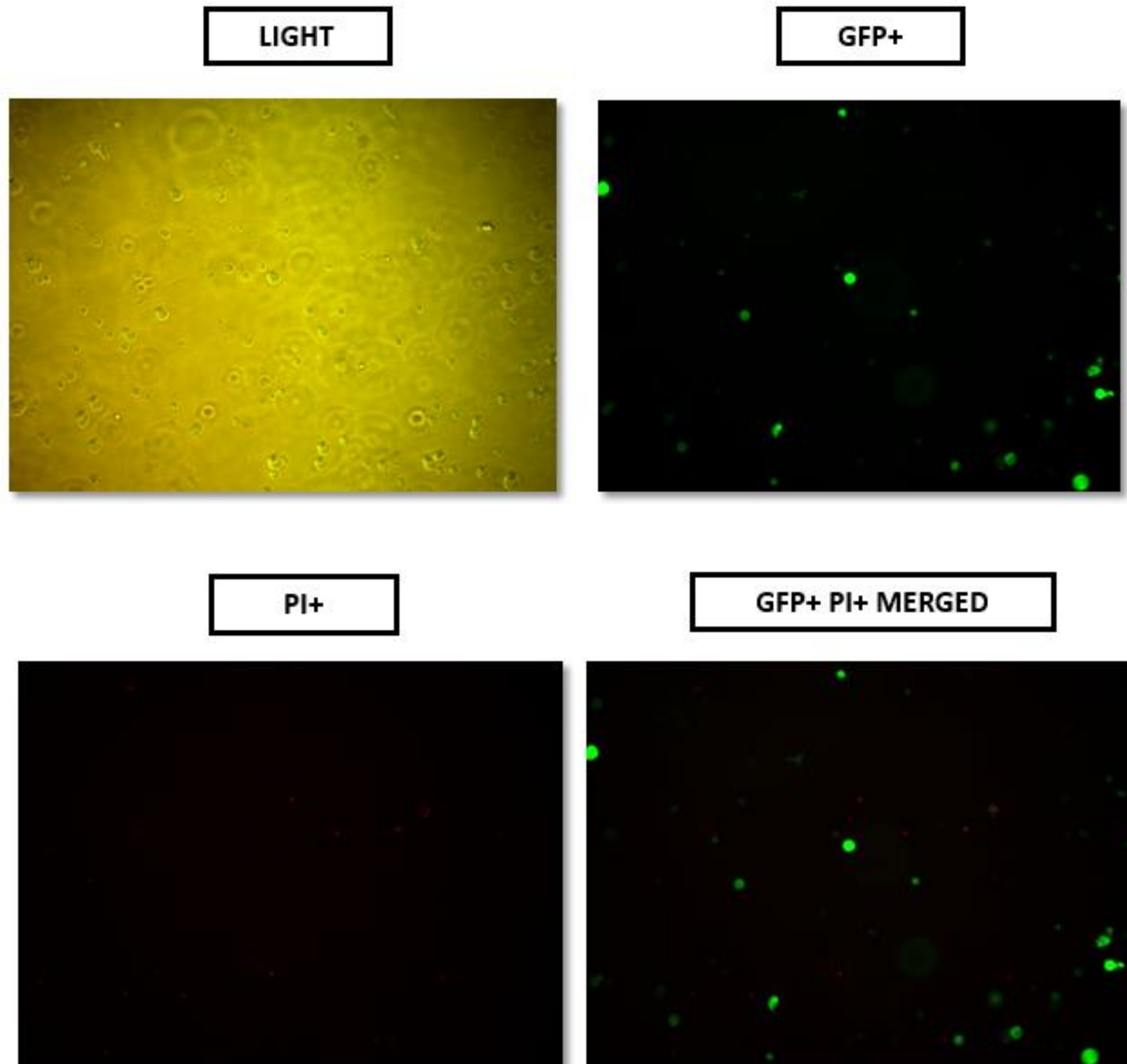

Figure 25. Nucleofection of 3th day of Th17 cell culture. (40X magnification) GFP+ represented transfected cells. PI+ represented dead cells. GFP+PI+ merged represented both transfected and dead cells

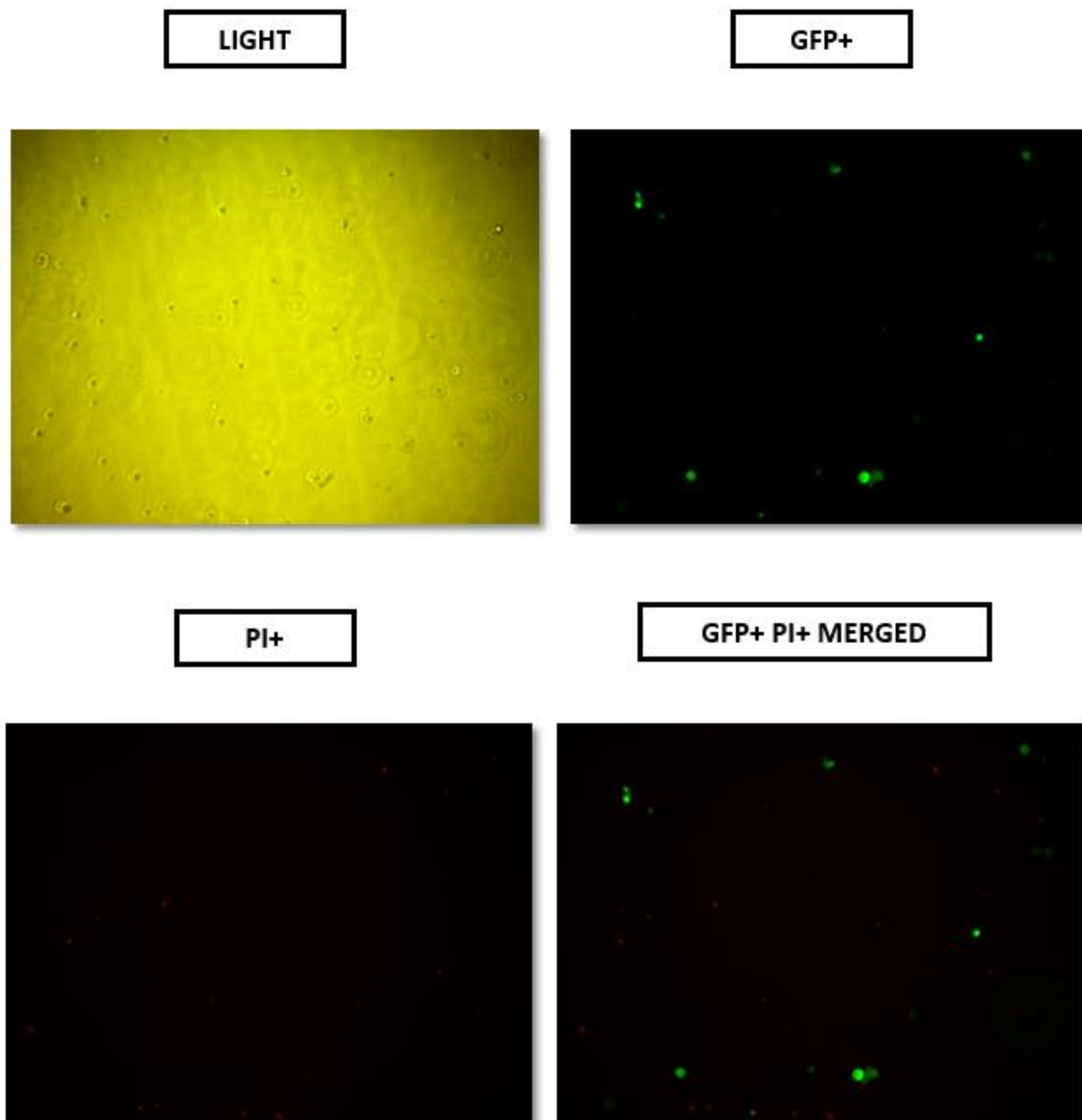

Figure 26. Nucleofection of 5th day of Th17 cell culture. (40X magnification) GFP+ represented transfected cells. PI+ represented dead cells. GFP+PI+ merged represented both transfected and dead cells

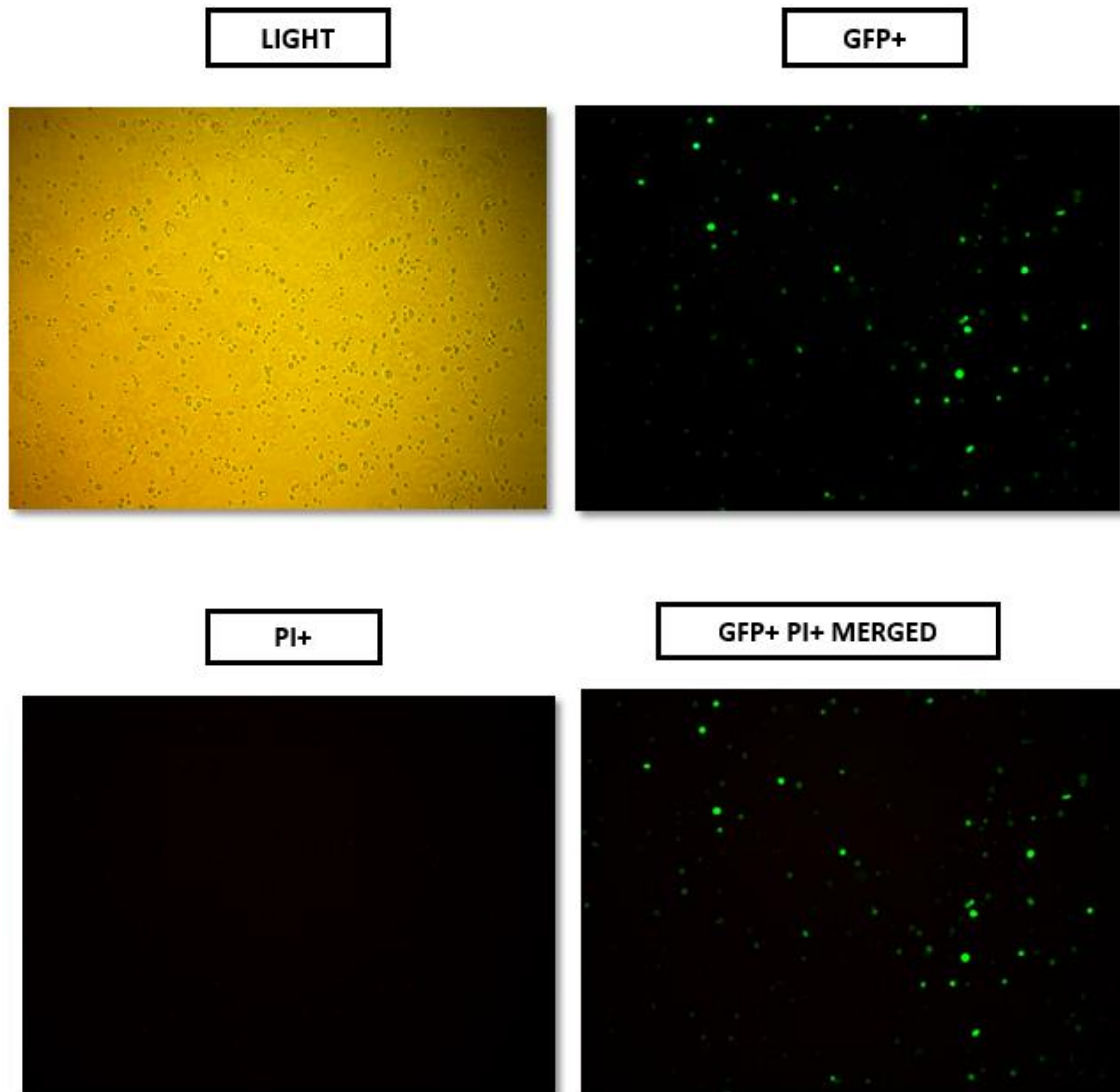

Figure 27. Nucleofection of 7th day of Th17 cell culture. (10X magnification) GFP+ represented transfected cells. PI+ represented dead cells. GFP+PI+ merged represented both transfected and dead cells

CHAPTER 4

DISCUSSION

In this study, we firstly transfected PBMCs which was isolated from peripheral blood of healthy donors. We applied 5 different nucleofection programs. Each program had different voltage properties. These programs included EO-115, EA-113, FI-115, FF-115, and FA-115. None of the 5 nucleofection programs were successful for monocyte transfection. Because nucleofection results showed that CD14+ GFP+ cells rate was 0% in all 5 nucleofection programs. For naive CD4 T cells, one program, FI-115, gave the highest transfection efficiency (17%). Looking at the first PBMC nucleofection experiment, we decided to transfect naive CD4 T cells with pmax GFP by using FI-115 program. Naive CD4 T cells were used as primary cells. Time dependent experiments showed that naive CD4 T cells had low transfection efficiency 24 hours post transfection. This value was found 12% (Figure 12.B). During nucleofection process, cell viability was also investigated. Cells were stained with Annexin V and 7AAD. 24 hours transfection experiments showed that GFP+ Annexin V+ cells rate was found 8% (Figure 13.C) . Another cell death marker was also used in this study. 7AAD is a nucleic acid binding dye so it indicated the rate of non-living cells. GFP+7AAD+ cells rate was found 5% (Figure 13.D). So 5% of GFP+ cells of the population was completely dead. Since 24 hours nucleofection experiments did not give the desired transfection efficiency, 48 hours nucleofection experiments were performed. Transfection efficiency was significantly increased to 45% in 48 hours transfection experiments (Figure 14.B). GFP+Annexin V+ cells rate did not changed. This value remained 8% (Figure 15.C). But surprisingly, nonviable cells rate was decreased. GFP+7AAD+ cells rate was found 0% Figure 15.D).

We also tried nucleofection on Jurkat cell line. Time dependent transfection efficiency and cell viability were investigated as did in naive CD4 T cells. 24 hours transfection results showed that Jurkat cells had 14% transfection efficiency (Figure 16.B) and 3% of the cells were GFP+Annexin V + (Figure 17.C) and 1% of the cells were GFP+7AAD+ (Figure 17.D). 24 hours transfection efficiencies of naive CD4 T cells and Jurkat cells were close to each other. However, GFP+ Annexin V+ cells rate of naive

CD4 T cells (8%) was higher than Jurkat cells (3%). The GFP+7AAD + cells rate of naive CD4 T cells (5%) was higher than Jurkat cells (1%). Because 24 hours transfection experiment did not give high transfection efficiency, 48 hour transfection experiments were applied on Jurkat cells. 48 hour Jurkat cell transfection experiments showed that transfection efficiency was considerably increased to 47% (Figure 18.B). This value was almost the same as 48 hour transfection efficiency of naive CD4 T cells. However, the mortality rate of transfected cells were also increased. GFP+Annexin V+ cells rate was found 18% (Figure 19.C). GFP+7AAD+ cells rate was found 3% (Figure 19.D). All these 48 hours Jurkat cell transfection cell viability rates were higher than 48 hour naive CD4 T cell transfection cell viability rates.

Both naive CD4 T cells and Jurkat cells had low transfection efficiency 24h post nucleofection and their transfection efficiency significantly increased 48h post nucleofection. Cell death rates of 24h transfection for both naive CD4 T cells and Jurkat cells were not very high to affect the subsequent functional assays. However, cell death rate (GFP+ Annexin V+) of 48 hours transfection for Jurkat cells were increased.

Jurkat cells were activated by PMA and Ionomycin (Saygılı et al., 2012). After T cell activation, some of the cell surface expression markers changed. CD69, early activation marker, expression increased after PMA/Ionomycin stimulation. In this study, Jurkat cells were activated for 24, 48, and 72 hours. Cells were analyzed in terms of CD69 expression and cell death. Activation results showed that Jurkat cells were activated after 24h (Figure 21). So the next nucleofection of activated Jurkat cells were decided to be performed on 24h activated Jurkat cells. Then this 24h activated jurkat cells were nucleofected by pmax GFP and 48h nucleofection post 24h activation analysis were carried out in fluorescence microscope (Figure 24). The results indicated that activated Jurkat cells could be nucleofected by pmax GFP.

In the presence of certain cytokines, Naive CD4 T cells differentiated into Th17 cells. In this study, Naive CD4T cells were differentiated into Th17 cells. Cells were transfected at 3th (Figure 25), 5th (Figure 26), and 7th day of the culture (Figure 27) and the analysis were carried out in fluorescense microscope. The purpose of the nucleofection of Th17 cell culture is to analyze the cells could be transfected after stimulation process. This study was useful for the future functional studies. For example,

the last studies includes the nucleofection of microRNAs into naive CD4 T cells. These studies aimed that either certain microRNAs directly led to Th17 cell differentiation or they had helpful effect on Th17 cell differentiation together with distinct cytokines. So the results of the nucleofection of Th17 cell culture was so important. The results showed that differentiated Th17 cells could be transfected by pmax GFP.

CHAPTER 5

CONCLUSION

The introduction of molecules into different cells holds an important place in many studies. Especially in immunological researches, many diseases are studied by transfection of different genes into cells. Electroporation is one of the promising physical transfection method used for nucleic acid delivery to primary T lymphocytes. Nowadays, Nucleofection which is an electroporation based transfection method has been developed to ease transfection of hard to transfect cell lines and primary cells. Nucleofection has some advantages over other transfection methods such as it is quick method, it does not require extra laboratory safety systems. Although, nucleofection has been widely used for different cell types to search the function of genes and proteins, how nucleofection influences the biology of naive CD4 T cells is still under investigation. The information in the literature on this field is very limited. These studies focused on transfection efficiency and the effects of nucleofection on cell activation. Here, we investigate that nucleofection has apoptotic effects on human primary naive CD4 T cells. Also, the effects of nucleofection on activated cells are investigated. We found human primary CD4 T cells, Jurkat cells, and activated T cells could be effectively transfected by nucleofection. Cell death rate was not very high in these cell types.

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