IDENTIFICATION OF IFN-BETA THERAPY RELATED BIOMARKERS IN CD4+ T CELLS OF MULTIPLE SCLEROSIS PATIENTS

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IDENTIFICATION OF IFN-BETA THERAPY RELATED BIOMARKERS IN CD4+ T CELLS OF MULTIPLE SCLEROSIS PATIENTS

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to my family…

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ABSTRACT

IDENTIFICATION OF IFN-BETA THERAPY RELATED BIOMARKERS IN CD4+ T CELLS OF MULTIPLE SCLEROSIS PATIENTS

Multiple sclerosis (MS) is neurodegenerative disease characterized by autoimmune demyelination of the central nervous system (CNS). Human interferon-β (IFN-β) treatment is generally used for relapsing-remitting form of MS (RRMS). Nevertheless, IFN-β does not show expected effects in some MS patients. This study aims to identify specific biomarkers related with IFN-β therapy. Forty-two RRMS patients (32 female, 10 male) were included in the study according to the McDonald 2017 criteria. These patients did not take any immunoregulatory drugs for at least three months and their EDSS score was below two point five. Patients were separated into two groups as female and male. After obtaining the blood samples from each patient, CD4+ T cells were isolated. CD4+ CD25+ regulatory T cells (Treg cells) were isolated from CD4+ T cells populations by fluorescence-activated cell sorting system (FACS). Cytotoxicity assays were performed to optimize drug concentrations which can affect viability of cell. Treg and CD4+ T cells treated with optimum drug concentration and control cells with no drug supplementation were used for investigation of *IL-6*, *IL-10*, *IL-17*, *IL-23* and *FOXP3* gene expressions by using Real-Time Polymerase Chain Reaction (RT-PCR). At the end of the study, it was observed that *FOXP3* gene expression level was found to decrease approximately one point seven fold after 16 hours drug treatment in CD4+ T cells. In Treg cells, *FOXP3* gene expression levels were observed to increase depending on time. The *IL-10* gene expression levels were found lower in Treg cells in comparison to the control group as a response to drug administration in depending on time ($p<0.0001$). The results of our study show that *FOXP3* and *IL-10* genes could be used as biomarkers for CD25 + T cells. It is thought that the results obtained from this study may help to develop a personalized treatment method in the future.

ÖZET

MULTİPL SKLEROZ HASTALARININ CD4+ T HÜCRELERİNDE IFN-BETA TEDAVİSİYLE İLİŞKİLİ BİYOBELİRTEÇLERİN BELİRLENMESİ

Multipl skleroz (MS), merkezi sinir sisteminde (MSS) gözlenen otoimmün demiyelizasyonuyla karakterize edilen nörodejeneratif bir hastalıktır. İnsan interferon (IFN-β) ilacı genellikle MS'in ataklarla seyreden formunun tedavisinde kullanılmaktadır. Ancak IFN-β, bazı MS hastalarında yeterince etki gösterememektedir. Bu çalışmanın amacı, IFN-β tedavisiyle ilişkili biyobelirteçlerin belirlenmesidir. 42 RRMS hastası (32 kadın, 10 erkek) McDonald 2017 kriterlerine göre çalışmaya dahil edilmiştir. Bu hastalar en az 3 ay boyunca herhangi bir immünoregülatör ilaç kullanmamış olup EDSS skorları iki buçuğun altındadır. Hastalar cinsiyetlerine göre iki gruba ayrılmıştır. Hasta bireylerden periferik kan örneği alındıktan sonra CD4+ T hücreleri izole edilmiştir. CD4+ T hücre popülasyonundan spesifik olarak CD4+ CD25+ düzenleyici T hücreleri (Treg) ise floresan-aktifleştirilmiş hücre ayırma sistemi (FACS) kullanılarak ayrıştırılmıştır. Hücre popülasyonunun canlılığını etkileyen optimum ilaç konsantrasyonunun belirlenebilmesi için sitotoksisite deneyleri gerçekleştirilmiştir. Optimum ilaç konsantrasyonu ile muamele edilen Treg ve CD4+ T hücreleri ve herhangi bir ilaç uygulaması yapılmamış kontrol hücrelerinde *IL-6*, *IL-10*, *IL-17*, *IL-23* ve *FOXP3* genlerinin anlatımlarında meydana gelen değişiklikler Gerçek Zamanlı Polimeraz Zincir Reaksiyonu (GZ-PZR) kullanılarak incelenmiştir. Çalışma sonunda CD4+ T hücrelerinde *FOXP3* geninin kontrol grubuna göre gen anlatımında özellikle 16 saat sonunda yaklaşık bir nokta yedi katlık bir azalma görülmüştür. Treg hücreleri ise ilaç uygulamasına cevap olarak *FOXP3* gen anlatımında zamana bağlı bir artış göstermiştir. Treg hücrelerinin *IL-10* gen anlatım düzeyleri ise kontrol grubu ile kıyaslandığında ilaç uygulanan saatlere göre anlamlı düzeyde kademeli olarak azaldığı tespit edilmiştir ($p<0.0001$). Çalışmamızın sonuçları *FOXP3* ve *IL-10* genlerinin CD25+ T hücreleri için biyobelirteç olarak kullanılabileceğini göstermektedir. Bu çalışmadan elde edilen sonuçların ileride kişiye özgü bir tedavi yöntemi geliştirilmesine yardımcı olabileceği düşünülmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

Abs	Absorbance
APC	Antigen presenting cells
BBB	Blood-brain barrier
CD4+ T Cell	T helper cells
CD4+ CD25+ T Cell	Treg cells
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
DMSO	Dimethylsulfoxide
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovin serum
FOXP3	Forkhead box P3
GWAS	Genome-wide association studies
IFN	Interferon
IFNγ	Interferon gamma
IFNβ	Interferon beta
IFNβ-1α	Interferon beta 1 alpha
IL	Interleukin
IMT	Immunomodulatory drugs
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
Nabs	Neutralizing antibodies
PBS	Phosphate buffered saline
RRMS	Relapsing-Remitting Multiple sclerosis
SPSS	Statistical Packages of Social Sciences
TGF	Transforming growth factor

TNF	Tumor necrosis factor
VEP	Visual evoked potential

1. INTRODUCTION

1.1. MULTIPLE SCLEROSIS

It is known to be a neurodegenerative disease associated with T cells that affects the CNS. It is charactarized by inflammation, chronic autoimmune demyelization and primary or secondary axon damage [1,2]. Affecting one point five million adults in worldwide, it is a very common CNS disease whose prevalence varies between two and 200 individuals per 100,000 people depending on geographical characteristics [2]. Visual symptoms such as weakness in the extremities, fatigue, bladder problems, memory-concentration-attention disorder, sensory symptoms, diplopia, ataxia, blurred vision, and dysarthria are common symptoms and signs associated with somatic nervous system damage in MS patients. Epileptic seizures, headache, movement disorders, cognitive impairment at the level of dementia, amyotrophy, hearing loss and cortical symptoms are the rare symptoms [3]. In this disorder, unexpected recurrences in the CNS and stages of regression in the nervous system are observed [4, 5, 6]. The disease is widely distributed among adults and especially women, and is affected by various immune-related conditions. It is known that various factors (environmental, genetic) play a role in the emergence of this disease [2]. To date, more than 100 polymorphisms have been identified as susceptible to MS by large-scale genome wide association studies (GWAS). Pathway analyzes on MS-associated genes indicate that the identified polymorphisms are associated with functions, in particular the formation of antiviral responses, and interferon (IFN) signaling [7].

1.2. TYPES OF MS

1.2.1. Clinically Isolated Syndrome

This condition is characterized by demyelination in the spinal cord, optic nerves, or brain stem. Most patients may develop neurological disabilities over time. [8]. This syndrome is not included in the initial MS clinical forms [9]. Magnetic resonance image (MRI) is very

important radiological scanning technique in diagnosis of MS. In patients, attacks should last at least 24 hours and are considered MS characteristic [10].

1.2.2. Relapsing-Remitting MS (RRMS)

This is the most prevalant form of MS, almost 90 per cent of cases begin in this way. It is majorly seen at early age. In the treatment of RRMS with attacks, it may be possible to treat it through immunomodulatory drugs (IMT) and acute anti-inflammatory agents such as immune-modifying therapies or steroids [3, 11, 12]. There is an unknown future for RRMS patients because severity of the disease is highly variable. Repeated inflammations in the nerves damage to myelin and axons. It leads to neurological symptoms first and then disability in the disease. Most patients with RRMS go to the secondary progressive disease related with constant physical and neuropsychological decline [13].

1.2.3. Primary Progressive MS

It occurs in almost 10 per cent of total patients and characterized by recurrent or gradual recurrence from the beginning, and PPMS patients presents progressive neurological deficits [14]. It typically occurs in the male population with age-progressing myelopathy [15]. The progression of the disease is quite variable: some of patients have significant disability for a short time, while others are more than a few decades. The most obvious and common progression in PPMS patients is spinal cord function disorder [16].

1.2.4. Secondary Progressive MS

It is diagnosed during the developing course with a gradual worsening history after the first course of recurrent disease. The methods of imaging and analyzing biomarker features that can understand this progress are limited [9]. Axonal losses are the most important disorder seen in secondary progressive multiple sclerosis [17]. There is no defined clinical criterion that allow clinicians to assign where it is converted from RRMS to this type of disorder. It is recommended to consider the clinical data and the patient's MS history in order to determine disease level [11].

1.2.5. Progressive-Relapsing MS

This is the rare form of the disease. It is characterized by different acute relapses and neuron dysfunction from the beginning [18, 19]. There is not much information about the clinical features of this type of MS as it makes up a small proportion of the MS population (about five per cent). Mostly, it is difficult to distinguish PRMS from PPMS, considering similar clinical properties, and some clinicians may use the similar terminology for both [19].

1.3. EPIDEMIOLOGY OF MS

It is known that almost two-three million people have this disease globally (50-300 per 100,000 people). The global distribution of MS is generally known to rise as it moves away from the equator line [20]. It is known that Epstein-Barr virus (EBV) infection and vitamin D deficiency are related with MS [21, 22]. Low vitamin D levels may be a factor in increasing susceptibility to MS in humans due to decreased sun exposure in these areas. Some researchers demonstrated that high vitamin D level may be protective in susceptible patients. People with EBV infection in childhood are approximately 15 times more related with MS, and about 30 times more likely to develop EBV at later ages [15]. In addition, the relationship between smoking and MS risk has been demonstrated by scientists. The occurrence of MS disease is about one point five times more in individuals who smoke [23].

There is also difference ratio of MS disease risk in gender. MS incidence is higher in women than in men (two:one ratio). Gender differences show that it may be related to genes, especially on X and Y chromosomes. As an example, it was observed that MS was relatively stagnant during pregnancy, but its frequency increased after birth [24].

1.4. INTERFERON BETA (IFN-β)

Interferon is a recombinant protein produced in beta-1a glycosylated form and contains the same sequence and number of amino acids as its native human form. Biological activity for many recombinant human IFN-β-1a is regulated through interactions of heterodimeric interferon receptors [25,26]. IFN-β is generally used for RRMS treatment [7]. IFN-β is

routinely administered subcutaneously at 22 μg, three times a week in patients [27]. Although the whole effect of IFN-β in patients is unknown; it plays a role in the blockage of T cell activity, apoptosis of T cells, interferon-gamma (IFNγ) antagonism, regulation of the activity of Treg cells, expression of neurotrophic factors, cytokine movement, also on the blood-brain barrier (BBB) or antiviral effects [28]. For this reason, it is thought that clarifying the effects of the drug in the treatment will contribute significantly to the treatment process [29].

IFN-β and other drugs used in therapy are only low-impact first-line treatment modalities and relapse rates are observed in 30-50 per cent of RRMS patients and/or sustained disability progresses [28, 30, 31]. This is thought to be due to immune and genetic differences observed among individuals [32]. Unfortunately, whether an individual responds to IFN-β therapeutically can be determined at least one year of treatment. If an increase in the expanded disability status scale (EDSS) of the patient is not detected and any episode during the follow-up period is not found, it is accepted that the patient has a therapeutic response to this drug. However, IFN-β treatment does not produce an effective therapeutic response for an individual if at least two controls at six-month intervals showed an increase in EDSS of at least 1 point and one or more episodes occurred [33]. The identification of predictive biomarkers has become of great importance nowadays, since neutralizing antibodies (Nabs) appear in these individuals where the effective therapeutic response can not be obtained, as these antibodies are observed to disappear over time and other possible mechanisms are predicted to cause no therapeutic response [32].

1.5. ETHIOLOGY OF MS

MS's etiology is unknown, but immune reactions directed to the CNS reveal that the myelin structure is important in pathogenesis [34]. It is known that Th cells play an crucial role in this process [35]. T helper lymphocytes are subdivided into two groups: effector T cells (Th1/Th2/Th17) and T regulatory cells (Treg/Tr1/Th3), each of which play a role in MS [36]. T cells are differentiated by antigen-presenting cells (APC) due to certain cytokines present in the medium to Th1, Th1, Th2, or Th17. Interleukin-10 (IL-10)/transforming

growth factor β (TGF-β), IL-2, IL-4, or IL-1/6/23) is known to be effective differentiation of CD4+ T cells (Figure 1.1).

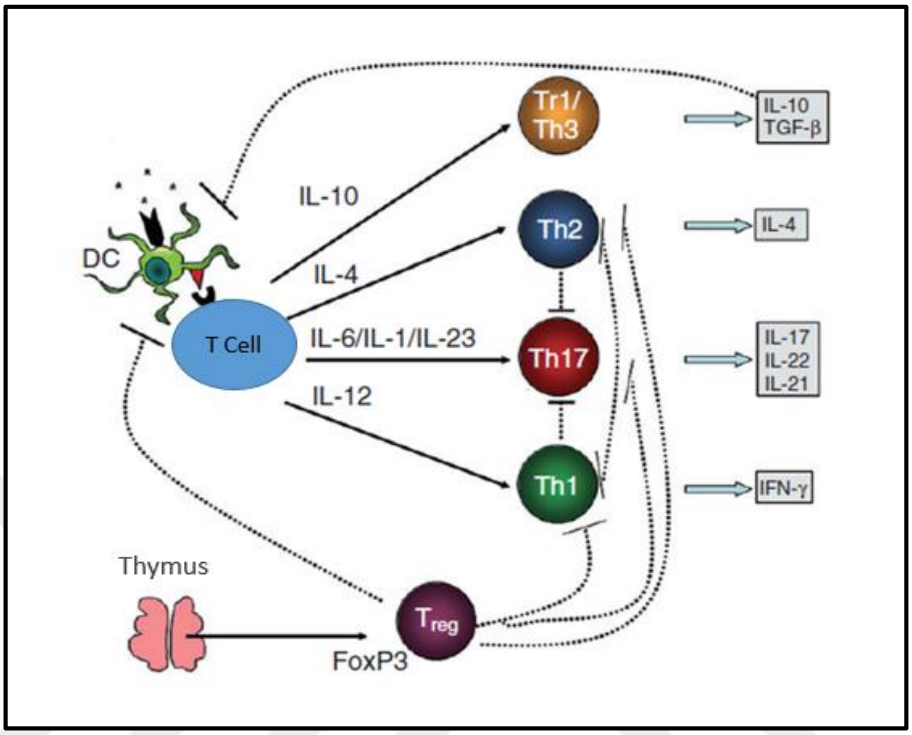


Figure 1.1. Regulation and differentiation of CD4+ T cells.

T cells differentiate into T helper and Treg cells during a process induced by dendritic cells (DC) and antigen-presenting cells (APC). This process is driven by various cytokines in the environment, and different cytokines or combinations of cytokines can induce differentiation into different cell types. Natural Treg cells are derived from thymus and can suppress effector T cell response via APCs [37].

MS is a progressive, inflammatory and chronic disease of the brain and spinal cord. The hypothesis of that "The disease is associated with the pathogenic T cell response to myelin antigens" is generally accepted, considering the studies in the brain lesions of patients and biological products generated by these cells. Following this cellular response, a neurodegenerative process begins [37]. The apoptotic process of T cells is under strict control with immune homeostasis. In the thymus, commonly T cells die, the other cells go the lymphoid organs and create T cell pool. The continuity of peripheral CD4+ T cells is ensured by the homeostatic balance between elimination and production. If an antigen is detected, CD4+ T cells first proliferate and then differentiate to effector T cells for immune

response. When the immune response decreases, significant pathways involving cell death are observed. Autonomous deaths inactive T cells in these pathways suppress the effector T cells activity. Since the expression of the signal regulators cannot be done correctly, the death of CD4+ T cells cannot be controlled, preventing the autoreactive CD4+ T cells from going into apoptosis or prolonging the CD4+ T cell immune response. This disrupts the homeostasis of T cells and as a result autoimmune disorders such as MS occur [38].

Studies have demonstrated that myelin-reactive CD4+ T lymphocytes that secrete Th1, and tumor necrosis factor (TNF) and cytokines (IFNγ) can be induced in CNS diseases that are histopathologically similar to MS [21]. Besides, Th17 cells play an important role in triggering the disease [39]. Th17 cells secrete IL-6, TNF, IL-17 and a small amount of IFN-γ. Many studies have demonstrated that IFN-β reduces the Th1 cells number and inhibits the differentiation of Th17 cells. Although reduced inflammation, IFN-β cause a high production of IL-10 [31, 34]. In addition, IFN-β application have an inhibitory effect on Th17 cells, in contrast to Th1 cell type. This is thought to result from an IFN-αR1 receptor protein induced by IL-23 and other cytokines on the surface of Th17 cells [40]. IL-23 cytokine has been reported to be used as a biomarker for Th17 cells [41]. Despite Th17 cells inhibition and the numerical increase of cytokines, it is not clear why the IFN-β treatment method cannot produce a therapeutic response in a large majority of RRMS patients [31].

The progression of the disease is caused by the autoreactive lymphocytes migration along the BBB [42]. Autoreactive T cells cross the BBB and cause damage to the myelin sheaths and central neurons and axons of these neurons via secreted cytokines [37, 42, 43, 44]. The progression from physiological control to pathological cascade increases regulatory failures in the brain that produce an immune response to autoreactive T cells. Regulatory lymphocytes are not sufficient to suppress effector cells in MS patients. These autoreactive cells cannot enter apoptosis during stimulation. This is thought to be owing to overexpression of β-arrestin 1, the promoter molecule for the survival of CD4+ T cells [42]. Primary demyelination is the most prominent morphological feature of MS, which leads to blocking of signal transduction or slowing of signal transduction at the demyelination site [37].

Additionally, Treg cells play a significant role in MS progression. Treg cells suppress the detrimental immune response developed against external antigens or the individual's own antigens [45, 46]. Disruption of Treg cells leads to the emergence of many autoimmune

disorders in humans. The suppression mechanism of regulatory T cells is subdivided into cell-to-cell interaction-dependent suppression, inhibition of cytokine release, regulation of the function of antigen-presenting cells, cytolysis, metabolic lysis, triggering or infectious tolerance of suppressor cells. Cells are classified dur to cytokine secretion profiles or surface phenotypes; thus the best-characterized type is native Treg cells. Treg cells can be selected from other T cells by the expression of the forkhead box protein 3 (*FOXP3*) and IL-2 receptor (CD25) [47]. However, there is a doubt that FOXP3 is a unique biomarker to detect natural Treg cells. Indeed, the *FOXP3* expression in humans has also been demonstrated to be a natural result of T helper cell activation [48, 49, 50, 51]. It is known that autoreactive T cells play a key role in the regulation of autoimmunity by regulating autoreactive T cells [45]. It has previously been shown that human Treg cells can induce the expression of cytokines such as IL-10 to suppress the function of T cells and DCs [52]. In another study, Treg cells were shown to be able to secrete IL-10 *in vitro* [47]. Also, Treg cells in active systemic lupus erythematosus patients have been shown to decrease numerically, cell number at the end of the treatment process has been shown to increase [53]. Because of these findings, numerical changes observed in Treg cells are thought to play an important role in neuroinflammatory diseases [27].

The MS progression and the mechanism of drug treatment are multifactorial and controlled by many metabolic pathways. Also, many different T lymphocyte cells have been implicated in this process. Therefore, many candidates may be specific biomarkers that may indicate that IFN-β drug content does not provide an adequate therapeutic response in MS patients.

1.6. IL-6

This gene produces cytokines that have a functional role in maturation and inflammation of B cells, and it is produced by T cells and macrophages [54], also known as IFNβ-2 [55]. These cytokines play a potential role in pathology of MS [56]. The protein produced is primarily synthesized at the site of acute and chronic inflammation [54]. In a study, IL-6 levels were measured, assuming that high IL-6 levels may be responsible of B cell response in the cerebrospinal fluid (CSF) of MS patients. Researchers indicated that MS patients have high level of IL-6 in plasma [57]. In another study, the temporary increase of IL-6 level in serum was associated with pre-treatment headache at the beginning of MS treatment, with a

low relapse rate. Increased levels of IL-6 in CSF and blood samples of patients have been reported [55].

1.7. IL-10

IL-10 encodes cytokines synthesized primarily by monocytes, and in small amounts by lymphocytes. The coded protein is thought to have pleitropic effects in immune regulation and inflammation. Th1 cytokines negatively regulate expression. Several studies have demonstrated its association with IFN-β metabolism and MS progression [58]. It is thought that measuring the level of IL-10, which has anti-inflammatory effects, may be useful in evaluating IFN-β therapy response [59]. Some studies have demonstrated that MS patients have lower IL-10 levels than controls (healthy individuals) [60, 61]. Researchers found that IL-10 levels are high in plasma of MS patients [59].

1.8. IL-17

IL-17 is the gene encoding proinflammatory cytokines synthesized by activated T cells. These cytokines are responsible for the regulation of mitogen-activated protein kinases and Nuclear Factor kappa Band. It is also known that IL-17 cytokines regulate *IL-6* expression and increase nitric oxide production. Excess cytokine production is thought to be related with various chronic inflammatory diseases, including MS [62]. In a study it was found that Th17 cells number increases in active MS lesion sites [63]. In another study, it has been demonstrated that concentrations of IL-17 have increased in MS patients. In patients, increased concentrations of IL-17 have a crucial role in the pathogenesis of MS in men or women, according to results obtained at higher levels compared to healthy individuals [64].

1.9. IL-23

The *IL-23* gene encodes the subunit of IL-23 heterodimeric cytokine. The *IL-23* gene is located in the p40 subunit of both this protein and IL-12. IL-23 and IL-12 can activate the STAT4 transcription activator and regulate IFN-γ production. In contrast to IL-12, which acts mainly in CD4 + T cells, IL-23 plays a major role in memory CD4+ T cells [65]. IL-23

is secreted by DCs and active macrophages found in peripheral tissues such as lung, skin and intestinal mucosa [66]. IL-23, created by activation of dendritic and phagocytic cells, activates memory cells and increases the number of CD4 + cells that produce IL-17. Th17 cells and IL-23 are required for the induction of experimental models of MS. In a study, it was observed that *IL-17* and *IL-23* expression levels increases in MS patients [67].

1.10. FOXP3

FOXP3 belongs to the transcriptional regulatory gene family and encodes an crucial protein causing immunodeficiency in any defect [68]. *FOXP3* expression is essential for Treg development and plays a suppressive role in Treg cells. In a study, it is shown that it is a major decrease in *FOXP3* expression of RRMS and SPMS patients [69]. FOXP3 is a unique marker of Treg lymphocytes. Studies with MS patients have demonstrated that the number of Treg cells expressing *FOXP3* may change during disease processes [70].

In this study, especially cytokines IL-6, IL-10, IL-17 and IL-23 which are believed to play an important role in IFN-β metabolism and T cell differentiation and the FOXP3 transcriptional promoter is emphasized to be crucial in the characterization of Treg cells.

2. MATERIALS

2.1. MATERIALS

Histopaque, Sigma-Aldrich, Germany. DPBS, Thermo Fisher, U.S.A. FBS, Thermo Fisher, U.S.A. RPMI 1640, Gibco, Thermo Fisher, U.S.A. Penicillin/Streptomycin, Thermo Fisher, U.S.A. DMSO, Sigma Aldrich, Germany. autoMACS Rinsing Solution, Miltenyi-Biotech, Germany. MACS BSA Stock Solution, Miltenyi-Biotech, Germany. LS Columns, Miltenyi-Biotech, Germany. Naive CD4+ T Cell Isolation Kit, Miltenyi-Biotech, Germany. MidiMACS Starting Kit, Miltenyi Biotec, Germany. Interleukin-2 (IL2), Miltenyi-Biotech, Germany. Human T Cell Activator CD28/CD3, ProMab Biotechnologies, U.S.A. MACS Buffer Solution, Miltenyi Biotec, Germany. FITC-conjugated CD4+ Human Antibody, BioLegend, U.S.A. Mouse anti-human CD25 antibody (FITC conjugated), BD Biosciences, U.S.A. Corning Costar Cell Culture Plates, 96 Well and 6 Well, Sigma, U.S.A. Cellstar Filter Cap Cell Culture Flasks, Greiner, Germany. CryoStar CryoTube, Greiner, Germany. WST-1 Cytotoxicity Assay, Roche, U.K. PureLink RNA Mini Kit, Thermo Fisher, U.S.A. cDNA Reverse Transcription Kit, Thermo Fisher, U.S.A. 2-Merkaptoetanol, Thermo Fisher, U.S.A. Ethanol, Absolute, Sigma Aldrich, St. Louis, U.S.A. MicroAmp Fast Optical 96-Well Reaction Plate, Applied Biosystems™, CA, U.S.A. Optical Adhesive Covers, Applied Biosystems™, CA, U.S.A. Proteinase K, QIAGEN GmBH, Hilden, Germany. QIAamp DNA Blood Mini Kit, QIAGEN GmBH, Hilden, Germany.

TaqMan Gene Expression Assays, Thermo Fisher, U.S.A., *IL6* (Hs00174131_m1), *IL10* (Hs00174086_m1), *FOXP3* (Hs00203958_m1), *IL17A* (Hs00174383_m1), *IL23A* (Hs00372324_m1), *GAPDH* (Hs99999905_m1). Universal Master Mix, Thermo Fisher, U.S.A. Water Nuclease Free, Thermo Scientific, Rockford, IL, U.S.A.

2.2. INSTRUMENTS

Real-Time PCR System, Applied Biosystems, CA, U.S.A. Centrifuge, Spectrafuge 24D, Labnet International, Inc. ELISA plate reader, Biotek, Vermont, U.S.A. Flow Cytomery, FACSVerse™, BD Biosciences, U.S.A. FACS, FACSAria™ III, BD Biosciences, U.S.A. T100™ Thermal Cycler, BioRad, U.S.A. Heater, Bioer. Nanodrop™ 2000/C Spectrophotometer, Thermo Scientific, Rockford, IL, U.S.A. Vortex, IKA. Water Bath.

3. METHODS

3.1. DETERMINATION OF VOLUNTEER MS PATIENTS WITH SUITABLE CRITERIA AND BLOOD SAMPLING

Forty-two volunteer MS patients who applied to the Neurology Clinic of Erenköy Mental and Neurological Diseases Hospital were recruited into the study. Neurological examination, clinical history, CSF examination, brain and spinal cord MRI and the number of clinical attacks were evaluated. Multiple sclerosis was diagnosed by using 2017 McDonald criteria [71].

Visual evoked potential (VEP) test was carried out as auxiliary examination method. The disability status was evaluated with EDSS. Patient history, pedigree, smoking and alcohol habits, first episode findings, number of attacks, EDSS values, MRI results, CSF examinations and VEP tests were performed.

Patients with a diagnosis of RRMS without any immunoregulatory drug treatment for at least three months and EDSS score below two point five were included in the study considering the information obtained from neurological and biochemical parameters. Although it was stated in the thesis proposal that equal number of patients were included into this study, the incidence of the disease more frequently among women in the population made it difficult to collect enough male patients with the appropriate criteria in the project plan. Due to the fact that the project is a time-dependent funded project, the duration of the patient collection is relatively short compared to the large-scale projects. There is no additional time in the rapid support projects, 10 male and 32 female patients based on gender are recruited into the study due to the difficulty of finding MS patients who have not received any drug treatment voluntarily was included.

Patients with other inflammatory, rheumatologic, neurological, psychiatric diseases and malignancies that were not associated with MS, and those with primary progression were excluded from the study. MS patients who had attacks according to the 2017 McDonald criteria were enrolled into the study.

3.2. CD4+ T CELLS ISOLATION FROM BLOOD SAMPLES

Cell isolation, cell culture and molecular analyzes of the study were performed by using the technical equipment in Acıbadem University R&D Laboratories and Yeditepe University, Faculty of Medicine, Department of Medical Biology Laboratory.

3.2.1. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Blood of MS Patients

PBMCs were collected by using the "Ficoll-Paque density gradient centrifugation method" within the first four hours following the collection of two EDTA tubes (four-ten mL) from the patients. Firstly, blood samples were diluted with phosphate-buffered saline (PBS) (37ºC) in one:one ratio. This cell-PBS suspension was then added onto Histopaque (Sigma Aldrich) (four:three v/v) at room temperature. The mixture was centrifuged at 400 xg for 35 minutes at room temperature. After centrifugation, mononuclear cells were directly transferred to a new tube (Figure 3.1).

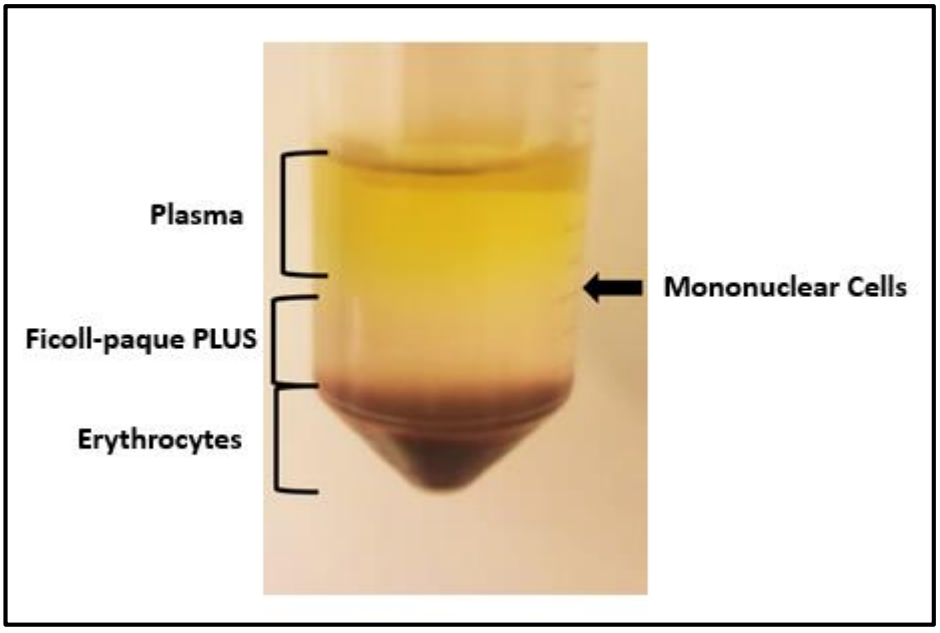


Figure 3.1. Plasma and cell layers after density gradient centrifugation

PBS was added gently in a one:one ratio to wash the cells. The suspension cells were centrifuged at 300 xg for ten minutes at RT and the supernatant was carefully removed. To

remove platelets, the pellet was resuspended in a one:one ratio with PBS. The suspended cells were centrifuged at 200 xg for 10 minutes at room temperature and again the supernatant was completely removed. Finally, the cells were washed again with a one:one ratio of PBS and centrifuged at 200 xg to remove the supernatant. The cell pellet was resuspended in a cell freezing solution (cryofluid solution) containing 50 per cent FBS (fetal bovine serum), 40 per cent RPMI 1640 (main media), and 1X Penicillin and Streptomycin antibiotic mixture. This cell mixture was transferred to cryotubes and 10 per cent dimethylsulfoxide (DMSO) was added and stored at -80 ºC for one day. PBMCs from each sample were then transferred to liquid nitrogen (-150 ºC) to keep until further Naïve CD4+ T cell isolation.

3.2.2. Thawing of PBMCs and CD4+ T Cell Isolation

"Naïve CD4+ T Cell Isolation Kit" and the separation apparatus "MidiMACS Starting Kit" and the specific columns of the kit were used to obtain CD4+ T cells from the total PBMCs of each patient. The primary characterization of the cells was made possible by the antibody cocktail of CD8/14/15/16/19/25/34/36/45RO/56/123,235a, TCRγ/δ, HLA-DR in the kit. The main feature of the kit is the magnetic beads which cause the CD4+ T cells to collapse to a great extent owing to the effect of the magnetic area in it. The magnetic beads used for isolation are specific to Naïve CD4+ T cells and allow isolation of only this cell type. In addition, the isolation kit ensures that the cells are pure, alive and functional, but does not cause any stress.

PBMCs of each MS patient were thawed by transferring them to 15 mL conical tubes containing RPMI 1640 complementary medium (10 per cent FBS, 1X penicillin/streptomycin (100 U/mL, 100 µg/mL) at room temperature to dilute DMSO in the cryotube. Cells dissolved in the medium were then precipitated by centrifugation at 400xg for 10 minutes at +4 ºC. The supernatant was completely removed. 10 µL "Naïve CD4+ T Cell Biotin-Antibody Cocktail II" was added onto the pellet and incubated for five minutes at +4 ºC. After incubation, 30 µL of cold MACS buffer solution (obtained by mixing MACS BSA (Bovine Serum Albumin) solution and autoMACS rinsing solution vials) 20 µL of Naïve CD4+ T Cell MicroBead Cocktail II was then added and incubated for ten minutes at +4 ºC. The LS column was placed on the magnetic separator and dissolved in three mL

MACS buffer. Column was prepared to allow the passing of cells comfortably. This cell mixture was slowly passed through the column by adding 400 µL of MACS buffer solution onto the incubated cell suspension. During the magnetic isolation that allows negative selection, this cell mixture containing non-magnetic labeled CD4+ T cells was collected from the column into 15 mL conical tubes (Figure 3.2).

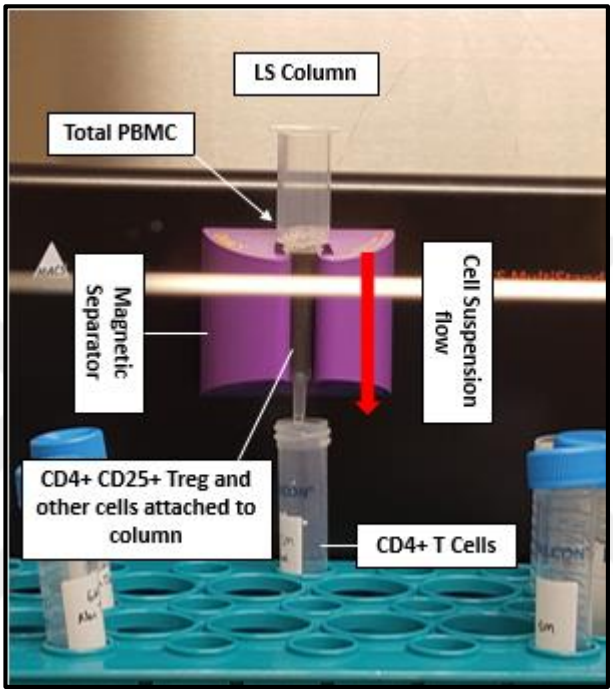


Figure 3.2. CD4 + T cell isolation apparatus

Three mL MACS buffer solution was used to wash the column to isolate CD4+ T cells. A new 15 mL conical tube was placed under the column and the LS column was pushed down with a syringe to collect cells labeled with the corresponding non-CD4+ T magnetically labeled antibodies. These cell suspensions were precipitated by centrifugation at 400 xg for five minutes. CD4+ T cells were added into 24-well plates with one mL RPMI 1640 complementary medium, and cultured in incubator at 5 per cent CO_2 and 37 °C conditions. Positive selective cells isolated with magnetic beads were cultured in RPMI 1640 complementary medium by adding Human T Cell Activator CD3/CD28 (2.5 per cent) (ProMab). The media were renewed every day for a period of a week until the isolation of T cells from PBMCs for all patients was expected to reach the appropriate cell number for CD4+ T cell characterization by using flow cytometry.

3.2.3. Secondary Characterization of Cells by Flow Cytometry Method

CD4+ T cells (passage 0) in 24-well plates were cultured in incubator at five per cent CO_2 and 37 ºC conditions. These cells were collected into tubes and then centrifuged at 400 xg for four minutes at +4 ºC. By removing the supernatant, cell pellets were dissolved in one mL MACS buffer and cells were counted. Cells were prepared for flow cytometry with 150,000 cells/tube in 400 µL cell solution. For this purpose, the cells were centrifuged at 2000 rpm for five minutes. The supernatant was removed and 25 µL PBS containing two per cent FBS was added. Cell suspensions were then mixed with one µL CD4+ Human Antibody (FITC labeled) and the cells were incubated in the dark at +4 ºC for thirty minutes. The incubated cells were centrifuged at 2000 rpm for five minutes and the supernatant discarded and washed with one mL PBS. The cells were again centrifuged at 2000 rpm for five minutes. The supernatant was discarded and then PBS containing 400 µL of two per cent FBS was added to the cells for flow cytometry (BD FacsVerse).

3.3. SEPARATION OF TREG CELLS WITH FLUORESCENT-ACTIVE CELL SEPARATION (FACS) METHOD

After isolation of CD4+ T cells from the total cell population, the separation of Treg cells from total cells was performed by FACS, a type of flow cytometry. Total cell colonies isolated from PBMCs with positive selection were cultured in 24-well plates for some time and then transferred to 15 mL conical tubes for FACS isolation. Removal of CD3/CD28 magnetic beads from cell populations is critical. Otherwise, the FACS device is clogged by magnetic beads, adversely affecting the performance of the insulation. Therefore, 15 mL conical tubes containing cell suspensions were stored for five minutes in a special rack forming magnetic field and the cell suspensions were transferred to another 15 mL conical tubes without being removed from the magnetic separator. These cell suspensions were precipitated by centrifugation at 400 xg for three minutes. After this step, the supernatant was removed and the cell pellet was thawed with one mL complementary medium, and then the cells were counted on a Thoma slide. The supernatant was discarded and the cells were thawed in 800 µL of complement medium, and 200 µL of the Treg cells were labeled with a specific antibody, “Mouse Anti-Human CD25 Antibody (FITC labeled) (BD

Pharmingen)", and added in the dark at +4 ºC for 30 minutes. The incubated cells were washed with PBS containing two per cent FBS and centrifuged at 400 xg for three minutes. The supernatant was removed and the pellet was washed with one mL PBS containing two per cent FBS. While the total cell suspension was flowing through the special column, the cells labeled with CD25 antibody were affected by the laser scattering of the device and collected in a separate column, and Treg cells were isolated.

3.4. DETERMINATION OF IFNβ DRUG CONCENTRATION AND CELL BEHAVIOR ANALYSIS WITH WST-1 CYTOTOXICITY TESTS

T cell groups obtained from different individuals were pooled as male and female based on gender and individual differences arising due to gender and immunity differences between individuals were minimized. Thus, both the number of cells increased significantly and T cells were prevented from being affected negatively by long term culture conditions. Populations containing CD4+ helper T cells in female individuals with MS and Treg cells in male subjects could not be established despite all attempts. The experimental groups characterized and studied are indicated below.

I. Population of CD4+ T helper cells in male individuals with MS

II. Population of female individuals with MS containing Treg cells

Two groups of self-pooled cells (passage six-seven) were determined at concentrations of 0-20-40-60-80-100-120-140-160-180-200-300-400-500 IU/ml (Table 3.1) It is stated that the IFN-β drug administration will be carried out for 48 hours in this study proposal. In order to enrich the method of the project and to examine the changes that may occur in other time points, drug application was carried out for 4, 16, 24, and 48 hours.

Table 3.1. Determination of drug concentrations

Last Concentration (IU/mL)	Stock Concentration (IU/mL)	Last Volume (µL)	Volume (µL)	Medium Volume (µL)
500	1200	1000	417	583
400	500	1000	800	200
300	500	1000	600	400
200	500	1000	400	600
180	500	1000	360	640
160	500	1000	320	680
140	500	1000	280	720
120	500	1000	240	760
100	500	1000	200	800
80	500	1000	160	840
60	500	1000	120	880
40	500	1000	80	920
20	500	1000	40	960

In order to prepare the drug at different concentrations, it was taken care not to lose the main stock as a result of any error by storing the stock IFN-β drug content in appropriate dilutions (with sterile dH_2O). Cells were seeded in plates at RPMI 1640 complementary medium and the indicated drug concentrations. Investigation of cell proliferation or death at the end of the period following drug administration. and cytotoxicity analysis (WST-1) was performed to determine the drug dose (50 per cent inhibition concentration / IC50) acting on more than 50 per cent of the cells in group 2. Cell Proliferation Reactive WST-1 proliferation and

viability in cell populations using 96-well plate format WST-1 containing stable tetrazolium salt is broken down into formazan primarily by a complex cellular mechanism on the surface of the cell. This bioreductive process takes place due to the glycolytic production of NAD(P)H in living cells. Tetrazolium salts are particularly appropriate for this type of research. These salts are broken down into formazan using the succinate-tetrazolium reductase system belonging to the respiratory chain of mitochondria which can only be observed in living cells. Thus, the number of metabolically active cells in culture is directly related to color formation resulting from formazan degradation. WST-1 was used to determine cell viability and cytotoxicity. For this assay, two groups of cells were first removed from 24-well plates and centrifuged at 400 xg for three minutes. After the cells were precipitated by centrifugation, the supernatant was discarded and cell counting was performed on the Thoma slide to calculate the required cell number. CD4+ T and Treg cells (passage 7) were seeded at 10,000 cells / well in each 96-well plate. Then, these cells were treated with 0, 20, 40, 60, 80, 100, 120, 140, 160 IU/ml, respectively. IFN-β drug concentrations of 180, 200, 300, 400 and 500 IU/ml were added and incubated at specific hour intervals. After 4, 16, 24 and 48 hours of drug incubations, 10 µl/well of WST-1 solution was added directly and incubated in a five per cent CO_2 incubator at 37 °C for two point five hours. At the end of the two point five hour incubation period, absorbance values were measured spectrophotometrically by ELISA plate reader (Biotek). The absorbance values measured were directly related to the number of viable cells in culture. Optimum drug concentrations were determined as 524 IU/mL in CD4+ T cells and 492 IU/mL in Treg cells for 4, 16, 24 and 48 hours. Proliferation analyzes were performed in three replicates to support the statistical significance and minimize errors caused by seeding.

3.5. DETERMINATION OF DIFFERENCES IN GENE EXPRESSION LEVELS ARISING FROM IFNβ DRUG APPLICATION

CD4+ T cells (passage eight) and Treg cells (passage eight) were cultured in 96-well plates to examine the changes caused by IFNβ drug administration. Cells treated with IFNβ at the concentrations of 524 IU/mL in CD4+ T cells and 492 IU/mL in Treg cells were cultured for 4, 16, 24 and 48 hours. Control cell groups (without any drug) were used as control. At the end of each time point, culture was terminated and total RNA isolation was performed

using appropriate kit for control cells without drug administration and cell groups with the optimum drug dose.

3.5.1. RNA Isolation from Cells

For RNA isolation, cells were first taken from 96-well containers into RNAase-free centrifuge tubes, and cells were centrifuged at +4 ºC for five minutes to remove the growth medium. Lysis buffer containing 300 µL of one per cent 2-mercaptoethanol was added to the cell pellets and the cell solution was vortexed until the cell pellets dispersed. Cell lysates were passed through the 21-gauge syringe needle at least 10 times for the homogenization step. After homogenization, 70 per cent ethanol (prepared using 100 per cent ethanol) was added to the homogenates in a one:one ratio. The lysate mixture containing the cell suspension was transferred to special centrifuge tubes with a filter to hold the RNA so as not to exceed 700 µL. Samples were centrifuged at 12,000 xg for 15 seconds at room temperature. The collected solution in the collection tubes was removed. 700 µL Wash Buffer I was added to the filtered tubes and the samples were centrifuged at 12,000 xg for fifteen seconds. The solution and collection tubes collected in the collection tube were removed and new collection tubes were placed. 500 µL Wash Buffer II was added to the centrifuge tubes with filter and the samples were centrifuged at 12,000 xg for 15 seconds. The last two stages were repeated. Samples were centrifuged dry at 12,000 xg for two minutes. The collection tubes were removed and the filtered section was placed in new centrifuge tubes to elute the respective RNA. The tubes were incubated for one minute at room temperature by adding 30 µL nuclease-free H_2O. Finally, the samples were centrifuged for two minutes at 12,000 xg.

3.5.2. cDNA Synthesis

Isolated RNAs were transformed into cDNA by cDNA Reverse Transcription kit. The kit uses cDNA synthesis using random primer sequences. Firstly, a reverse transcriptase master mix was prepared at a concentration of 2X to effect the appropriate PCR reaction. mRNA samples with appropriate concentrations were added to the prepared mixture and “BioRad T100™ Thermal Cycler” was used for the reverse transcription reaction.

3.5.3. Real-Time Polymerase Chain Reaction (RT-PCR)

The comparative analysis of the gene expression levels of *IL6, IL10, IL17, IL23*, and *FOXP3* genes was performed by RT-PCR. Each gene region was studied as triplicates. In the real-time PCR analysis, the desired target mRNA expression was performed by the comparative ΔΔCT method. This method was used to normalize the amount of mRNA expression obtained from the endogenous gene (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), the number of copies of the mRNA of the target gene. Normalization rate (NO);

$$NO = 2^{-\Delta\Delta CT} \tag{3.5}$$

calculated with this formula. ΔΔCT value;

$$\Delta\Delta CT = (CT_{(\text{target gene / sample})} - CT_{(\text{target gene / pool})}) - (CT_{(\text{GAPDH / pool})} - CT_{(\text{GAPDH / pool})}) \tag{3.6}$$

calculated with the formula.

TaqMan analysis codes used in real-time PCR analysis are given in the Section 2.1. The purity of the isolated RNAs belonging to each group was measured with the NanoDrop device (Thermo Scientific).

3.6. STATISTICAL ANALYSIS

Statistical packages of social sciences (SPSS) 23.0 programme was used for statistical analysis. For comparing the mean values of continuous variables with normal distribution between the groups two independent samples t-tests were used. When a statistically significant difference was detected, Mann-Whitney U-test was used for pair comparison. One way ANOVA test was used to compare the mean expression levels of RT-PCR. p-value smaller than zero point zero five was considered as statistically significant.

4. RESULTS

4.1. DETERMINATION OF VOLUNTEER MS PATIENTS AND COLLECTING RELATED DATA

The basic features of patients are shown in Table 4.1.

Table 4.1. Baseline characteristics of patients.

Patient Number	Age	Age of disease	Number of attacks	EDSS	MRI	CSF	VEP
1W	40	18	3	2.5	1;3	1	0
2W	25	1	2	1	1;2	1	1
3W	27	2	2	0	1	0	1
4W	37	1	2	1	1	0	0
5W	55	5	2	1	1	0	1
6W	33	1	2	1	1	0	1
7W	60	4	2	0	1	0	1
8W	70	21	4	2.5	1	0	0
9W	33	2	2	1	1	0	0
10W	27	5	3	0	1;2	1	1
11W	21	2	2	0	1;2	0	0
12W	26	1	2	1	1	0	0
13W	33	0	2	1	1;2;3	0	0
14W	46	1	2	1	1	1	0

15W	42	19	3	1.5	1;2;3	1	0
16W	31	1	2	0	1	0	0
17W	52	19	5	1	1	1	1
18W	31	7	5	1.5	1;2	1	1
19W	33	1	2	1.5	1	0	1
20W	63	18	3	1.5	1	1	0
21W	52	7	3	1.5	1;2	1	0
22W	35	13	5	2.5	1	0	0
23W	41	6	2	2.5	1	0	0
24W	40	5	2	2	1;2	0	0
25W	26	1	2	1.5	1	0	0
26W	41	5	2	2.5	1;2	1	0
27W	46	2	5	2	1;2	1	0
28W	52	1	2	2	1;2	0	0
29W	58	8	5	2.5	1;2	1	0
30W	42	2	2	1	1;2	0	0
31W	20	3	2	1	1;2	1	1
32W	22	5	3	2	1	1	0
1M	31	5	3	2.5	1;2;3	1	0
2M	61	12	3	2.5	1	1	0
3M	38	2	2	2	1;2	1	0
4M	27	0	2	1	1	0	1
5M	47	11	2	2	1	0	1

6M	51	13	3	2.5	1	0	0
7M	38	1	2	1	1	0	0
8M	47	1	2	0	1	0	0
9M	36	2	2	1	1;3	1	1
10M	37	2	2	1.5	1	0	1

W: Women; M: Men; for MRI 1: Cranial lesion, 2: Cervical lesion, 3: Thoracic lesion; for CSF and VEP 0: No, 1: Yes

Data on patient's age, disease age, number of attacks, and EDSS value calculated as mean ± standard deviation according to Table 4.2 are shown below.

Table 4.2. Standard deviation of patient's data

Patient Age	39,83±12,21
Age of Disease	5,62±5,98
Number of attacks	2,62±0,99
EDSS	1,40±0,80

4.2. DISSOLUTION OF PBMCS AND CD4+ T CELL ISOLATION

The "Naïve CD4+ T Cell Isolation Kit" and the separation apparatus "MidiMACS Starting Kit" that allows the use of this kit, and the special columns of the kit, the cells cultured in 24-well plates with CD4+ T cells and magnetic beads after performing the pure acquisition of cells of interest. CD4+ T cells were reproduced in the complementary media. After about three days, it was observed that the relevant cells multiplied by forming cell colonies and covered the entire surface (Figure 4.1).

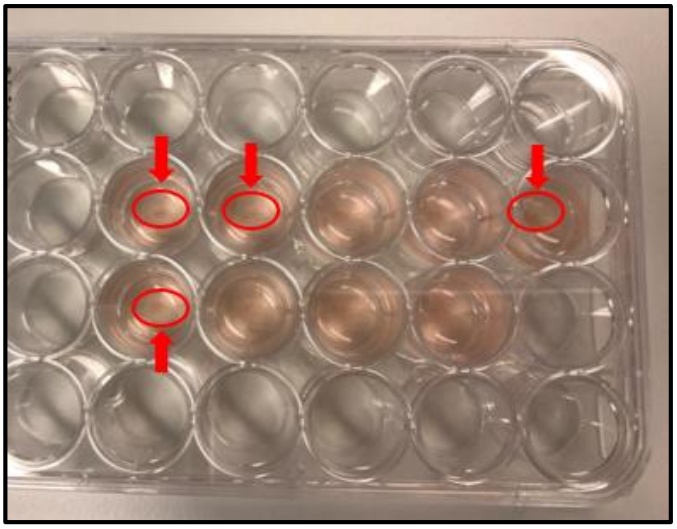


Figure 4.1. Cell colonies formed by culturing cells in 24-well plates (Red circles indicate cell colonies.)

4.3. SECONDARY CHARACTERIZATION OF CD4+ T CELLS BY FLOW CYTOMETRY

After T cell isolation with the help of magnetic beads, secondary characterization of the cells by flow cytometry was carried out to see the CD4+ T cell percentage in total cell colonies. Figure 4.2 shows the reference population boundaries (gate) determined by the total population of unstained cells during analysis. Flow cytometry results showing the population CD4+ T cell percentage in cell colonies of each female MS patient individual in 24-well plates. The flow cytometry results showing the population CD4+ T cell percentage in the cell colonies of each male MS patient in 24-well plates are shown in Figure 4.4.

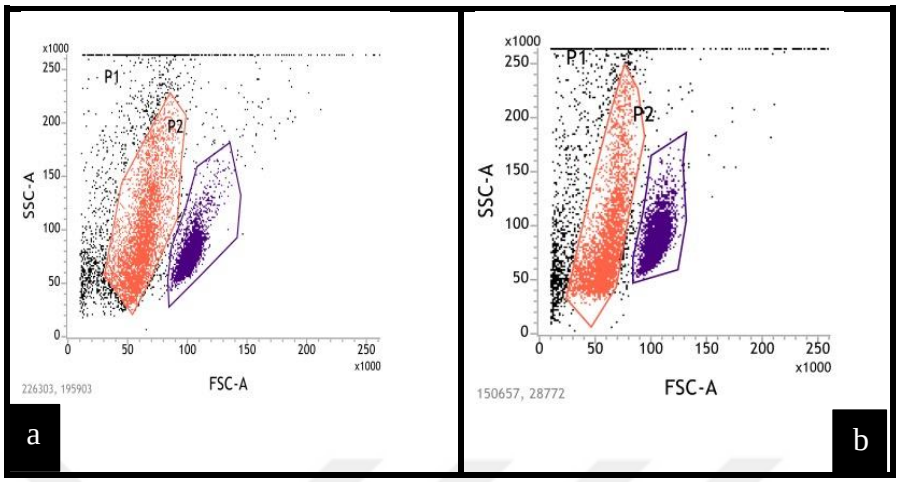
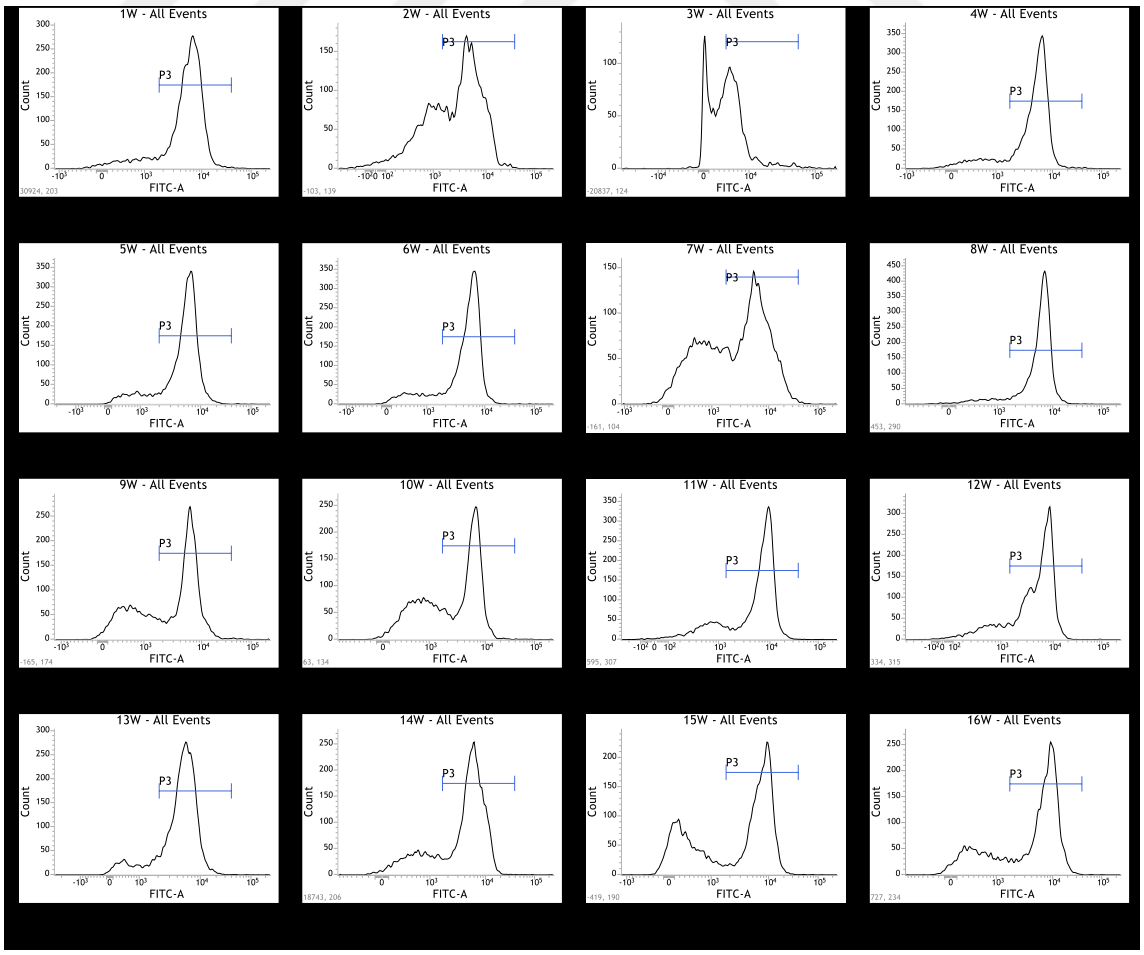


Figure 4.2. Determination of cell populations by flow cytometry. (a) female RRMS (b) distribution of cell populations isolated from male RRMS patients. (P1: Population 1; P2: Population 2)



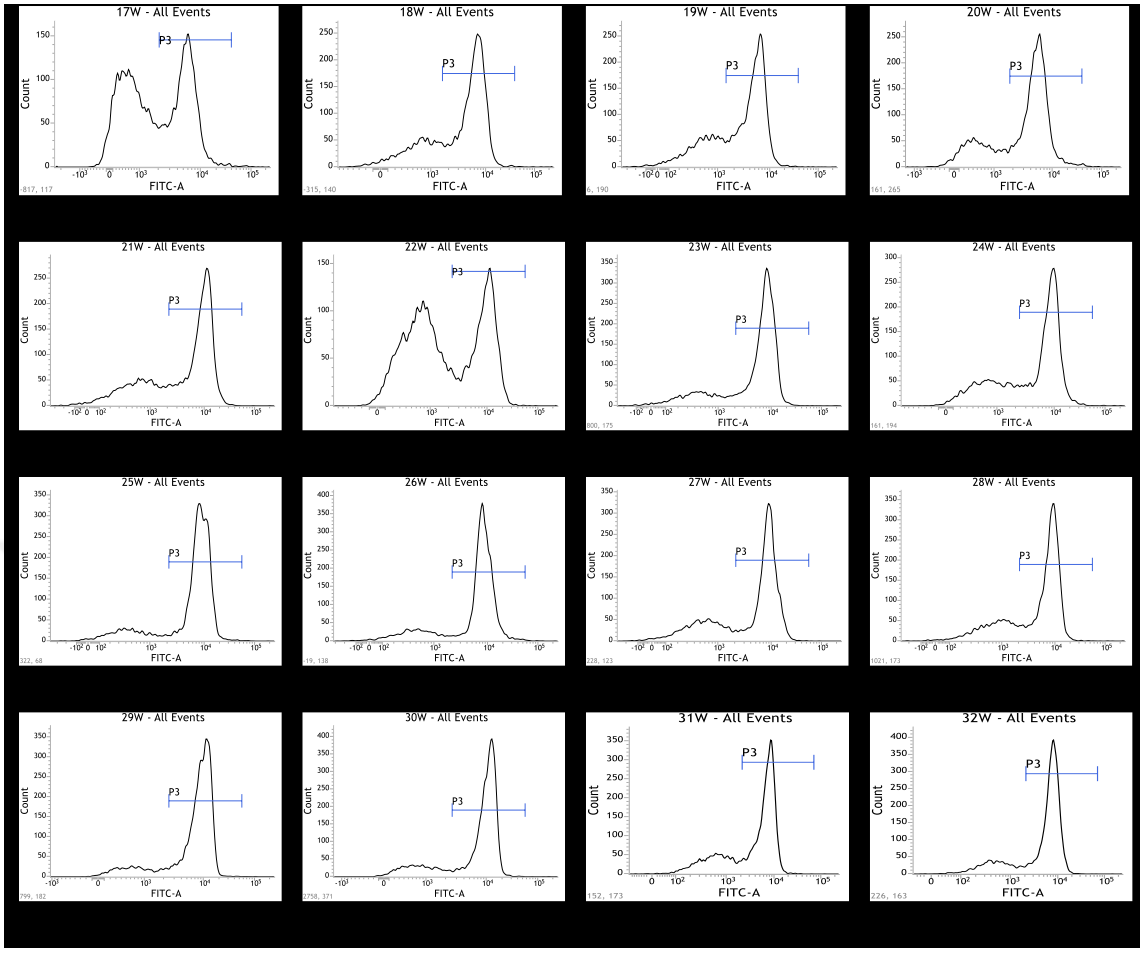
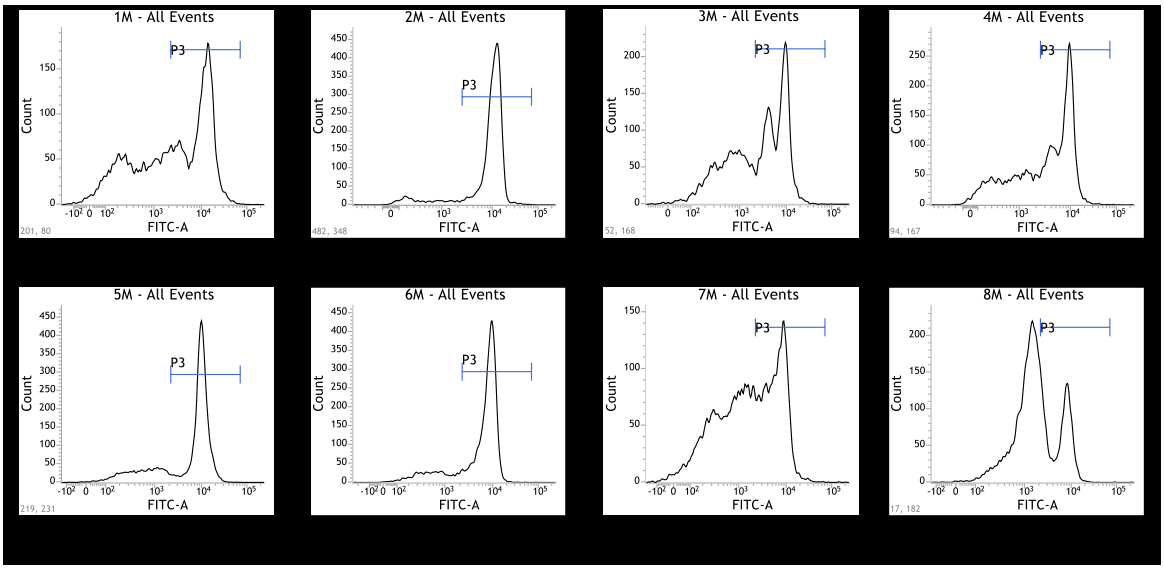


Figure 4.3. Secondary characterization of CD4+ T cells with flow cytometry after magnetic isolation from PBMCs in female MS patients. (W: Female)



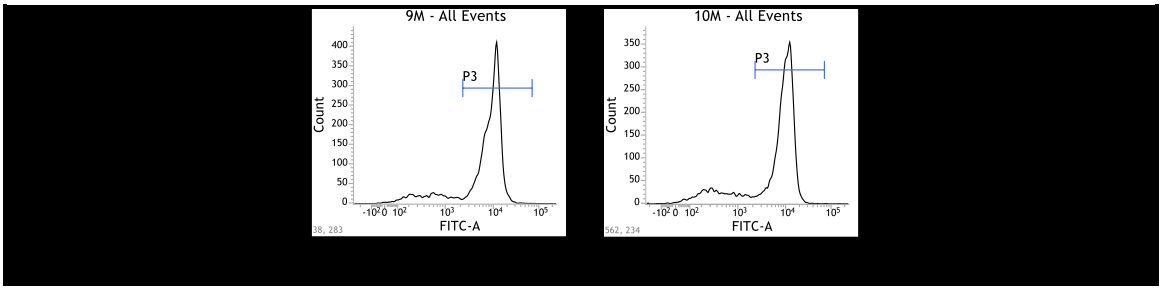


Figure 4.4. Secondary characterization of CD4+ T cells with flow cytometry after magnetic isolation from PBMCs of male MS patients. (M: Male)

The population percentage of cells of interest as a result of labeling with the CD4 antibody on flow cytometry are shown in Table 4.3. According to these results, CD4+ T cells isolated from 1M, 3M, 4M, 7M, 8M samples with 7W, 9W, 10W, 15W, 17W, 21W, 22W, 24W, 27W with a CD4+ T cell percentage in the population were below 70 per cent. These cells did not involve in the pool. Thus, it was aimed to keep the CD4+ T cell ratio high in the pool.

Table 4.3. CD4+ T cell percentages in patients after CD4 antibody labeling on flow cytometry. (W: Female; M: Male)

Sample	**CD4+ T Percentage (%)**	**Sample**	**CD4+ T Percentage (%)**
1W	88,38	22W	40,57
2W	72,66	23W	75,33
3W	63,04	24W	63,75
4W	85,85	25W	81,09
4W	88,02	26W	79,60
6W	86,13	27W	68,15
7W	64,63	28W	69,55
8W	91,72	29W	82,77

9W	67,06	30W	79,48
10W	65,42	31W	71,12
11W	83,30	32W	78,77
12W	85,02	1M	56,49
13W	88,68	2M	89,23
14W	78,70	3M	57,25
15W	63,22	4M	62,04
16W	73,29	5M	75,05
17W	51,22	6M	81,80
18W	75,58	7M	45,84
19W	73,82	8M	36,02
20W	74,10	9M	83,76
21W	64,40	10M	79,60

4.4. SORTING OF TREG CELLS WITH FACS METHOD

After CD4+ T cell isolation from the total cell population, Treg cells were isolated from total cells by FACS, a type of flow cytometry. Negative controlled isolation of CD4+ helper T cells was also achieved when the separation was successfully performed. In order to proliferate the cells, the same medium was used. However, despite the addition of CD3/CD28 cytokines to cell culture plates for activation, cell proliferation could not be observed in 96-well plates because the cells could not interact adequately with each other. Therefore, cell populations including Treg cells which were stocked following the CD4+ T cell isolation were re-cultured and FACS method was applied again following the same protocol. Isolation and characterization of the desired Treg cells was achieved from female

MS patients with sufficient cell numbers (approximately $8x10^6$ cells). Within the scope of the project, regulatory T cells of both male and female individuals would be isolated and characterized by FACS method. However, the isolation of Treg cells from the pooled total cell population from the peripheral blood of 10 male subjects could not be performed for a second time due to the insufficient number of cells. When DAPI core staining was performed, the majority of the cells were observed as dead (Figure 4.5).

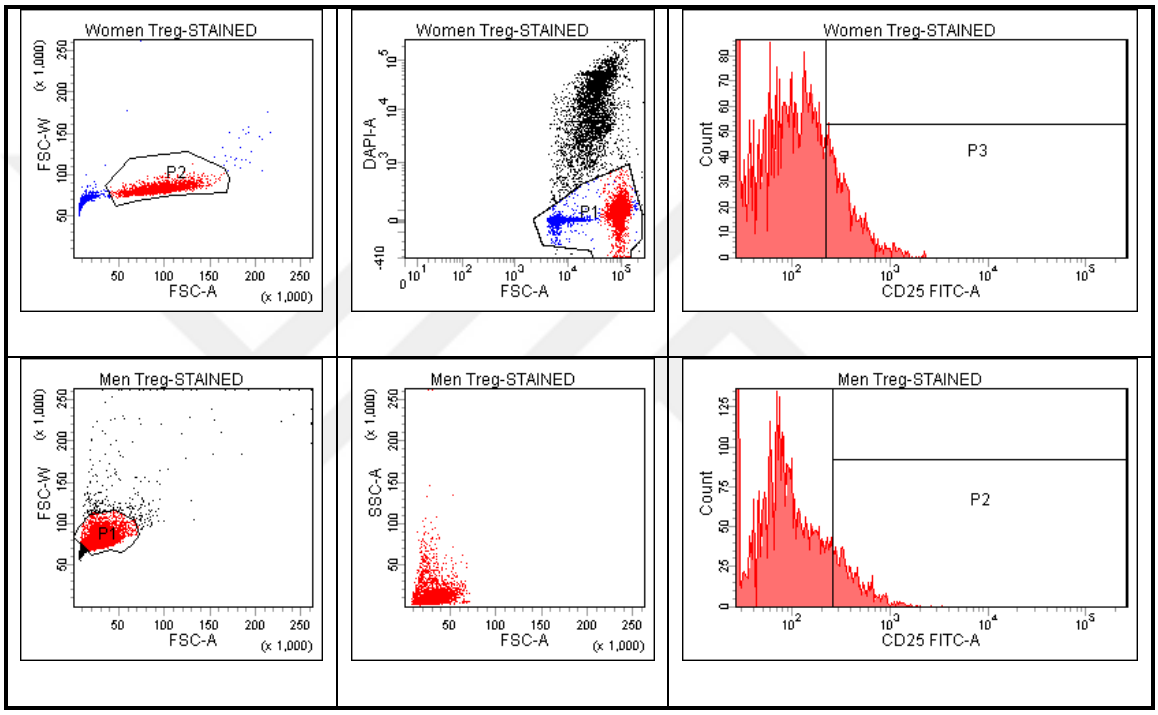
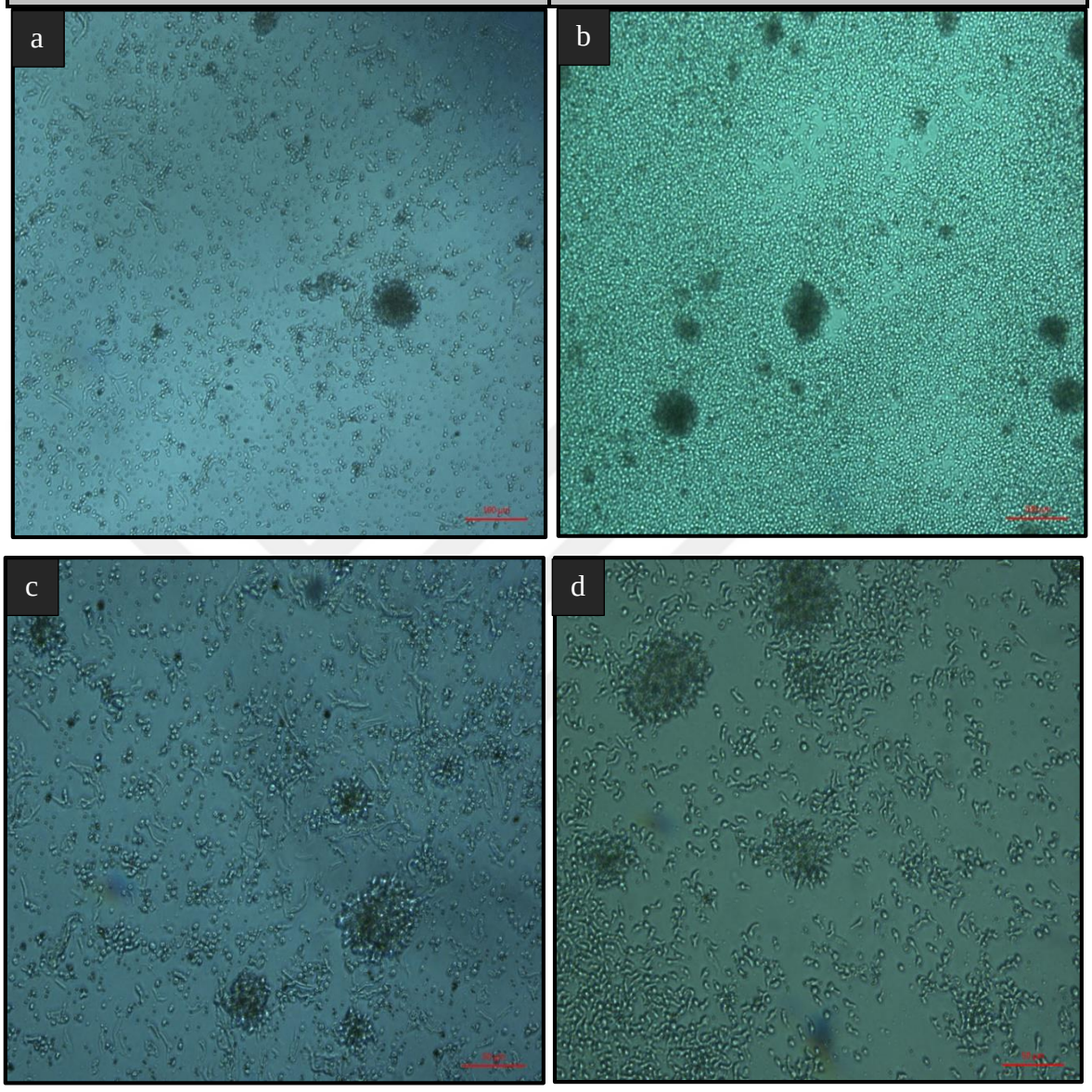


Figure 4.5. Isolation and characterization of Treg cells by FACS method.

The resulting cells were grown in culture in order to collect a sufficient number of cells at a later stage. Light microscopy images of the cells in growth media are shown in Figure 4.6.

CD4+ T Cells	Treg Cells



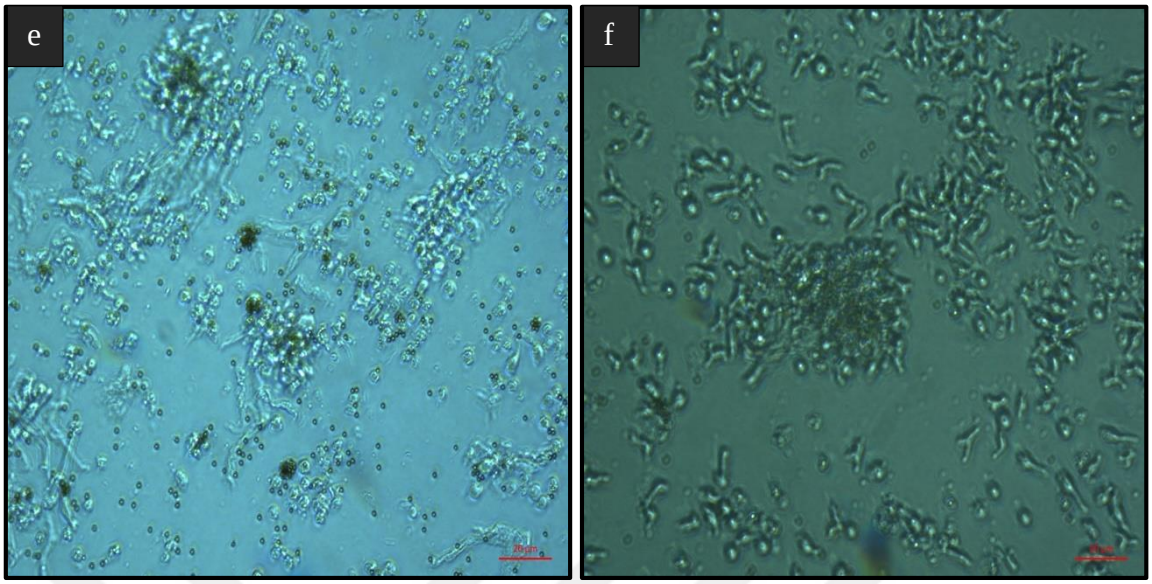


Figure 4.6. Magnified images of 10X (a, b), 20X (c, d) and 40X (e, f) under light microscopy of CD4+T and Treg cells grown under conditions specified in cell culture and in optimal growth medium.

4.5. DETERMINATION OF IFN-B DRUG CONCENTRATION AND CELL BEHAVIOR ANALYSIS WITH WST-1 CYTOTOXICITY TESTS

T cell groups obtained from different individuals were pooled as male and female based on gender and individual differences arising due to gender and immunity differences between individuals were minimized. Thus, both the number of cells increased significantly and T cells were prevented from being affected negatively by long term culture conditions. Populations containing CD4+ helper T cells in female individuals with MS and Treg cells in male subjects could not be established despite all attempts. The experimental groups characterized and studied are indicated below.

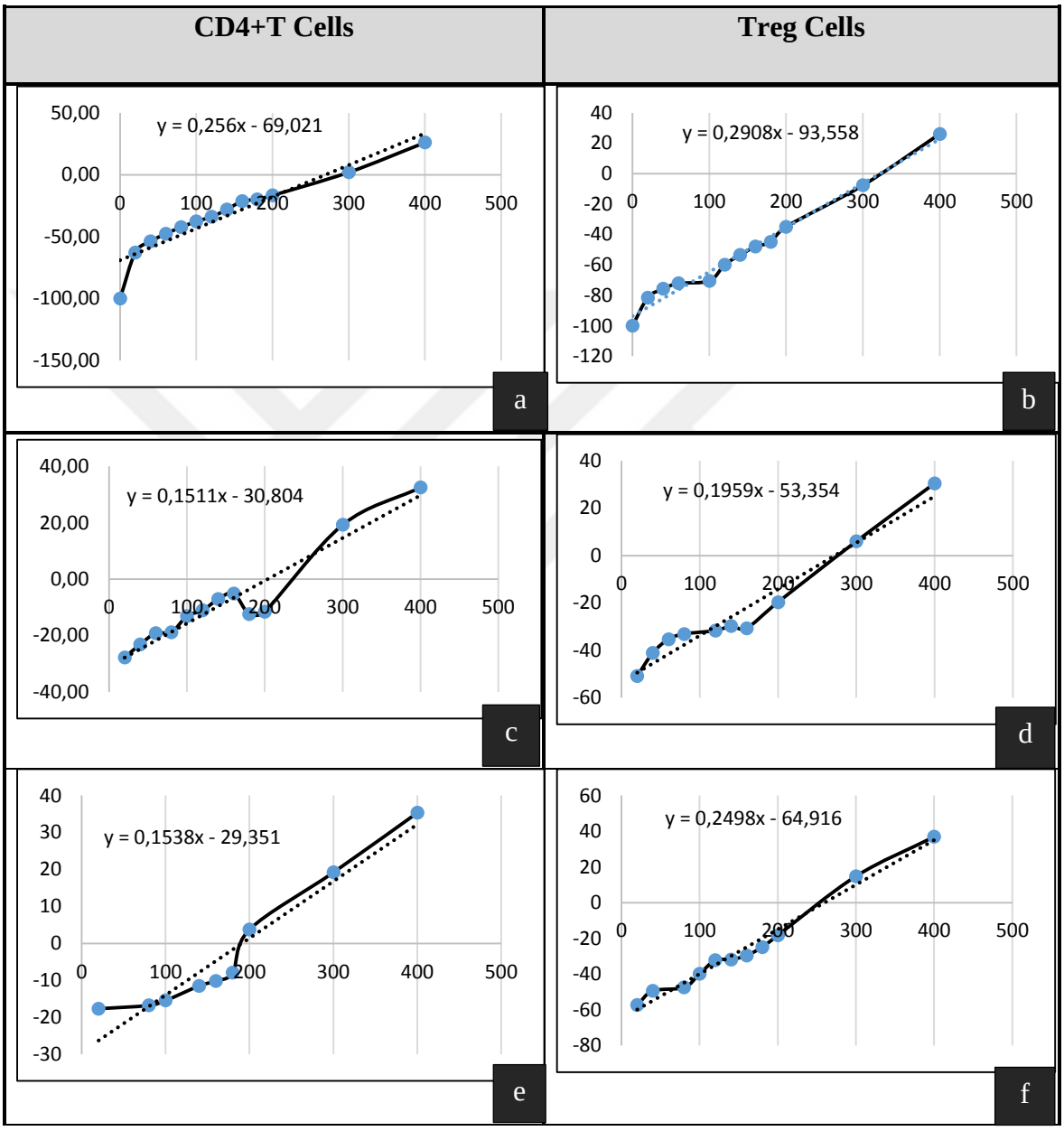
I. Population of CD4 + T helper cells in male individuals with MS

II. Population of female individuals with MS containing Treg cells

In this study, IFN-β was administered as 0-20-40-60-80-100-120-140-160-180-200-300-400-500 IU/ml for two groups of cells pooled for 48 hours. In order to examine the changes that may occur in other time points, drug application was carried out for 4, 16, 24, and 48 hours.

When the concentration and the percent inhibition are drawn on the X and Y-axis respectively, the slope of the graphic gives the IC50 value.

When the values obtained as [(Abs. Control-Abs. Sample)/Abs. Control]x100 are reflected in the graphic, graphics presenting the data obtained from WST-1 cytotoxicity analysis of 4, 16, 24, and 48 hours are shown in Figure 4.7. (Abs: Absorbance)



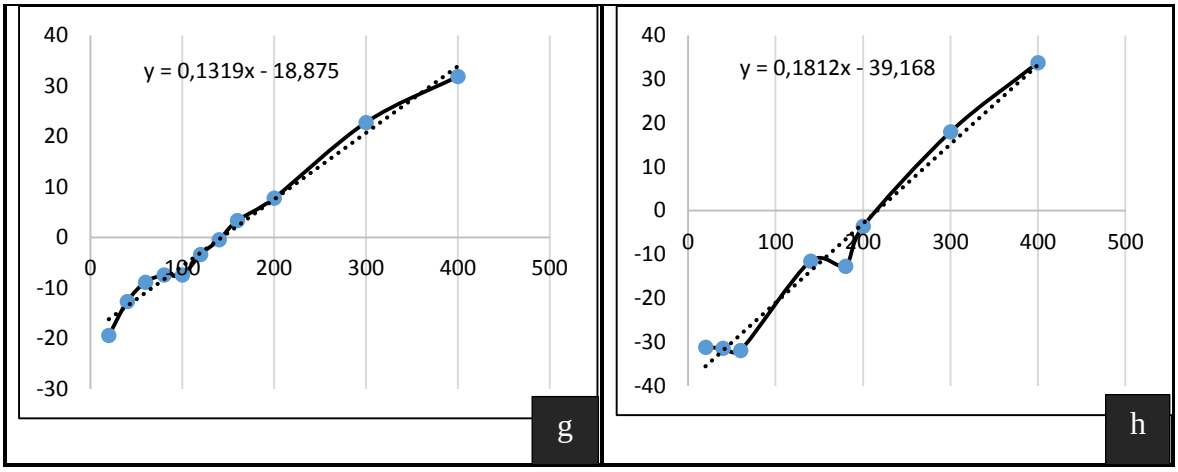


Figure 4.7. CD4+ T and Treg cells for 4 (a, b), 16 (c, d), 24 (e, f) and 48 (g, h) hours for different concentrations of IFN-β following drug administration results of cytotoxicity analysis.

Drug concentrations causing 50 per cent inhibition using the obtained slope values are shown in Table 4.4. (where y = 50, x represents the concentration that causes appropriate cell inhibition.)

Table 4.4. Drug concentrations thought to cause 50 per cent inhibition of T cells from WST-1 cytotoxicity analysis.

Time (h)	CD4+ T Cells	Treg Cells
4	464,9 IU/mL	493,7 IU/mL
16	534,8 IU/mL	527,6 IU/mL
24	515,9 IU/mL	460 IU/mL
48	522,2 IU/mL	492,1 IU/mL

Because the obtained IFNβ drug concentrations exhibit similar profiles for CD4+ T cells and Treg cells at the same periods, the optimum concentration of drug concentrations for each subsequent cell type is also considered for each cell type. Considering the proximity of the optimum drug concentration values 4, 16, 24 and 48 hours of concentration values averaged to be examined at the level of gene expression was decided. As a result, IFNβ drug concentration was 524 and 492 IU / mL for CD4+ T cells and Treg cells respectively.

4.6. DETERMINATION OF DIFFERENCES IN THE EXPRESSION LEVEL ARISING FROM IFNB DRUG APPLICATION

WST-1 analysis of IFNβ drug administration to determine drug concentrations causing 50 per cent death of the total population on CD4+ T and Treg cells determined the optimum drug concentration for CD4+ T cells to be 524 IU / mL while Treg cells 492 IU / mL. Determined drug concentrations were applied to 96-well plates at $1x10^4$/well for 4, 16, 24, and 48 hours in the seeded cells, and culture was terminated at the end of the specified time. mRNA isolation was performed according to the protocol detailed in Section 3.5. The mRNAs were reverse transcribed into the cDNA within the first 24 hours following isolation, considering the overall stability of the obtained mRNAs and their rapid degradation. The time-dependent expression change of each gene normalized to the reference gene (*GAPDH*) as a result of RT-PCR results are shown in Figure 4.8 for CD4+ T and Treg cells.

Here, we demonstrated that the genes in interest were up- or down-regulated by IFN-β administration. When we performed RT-PCR for *IL23A* gene expression profile for CD4+ T cells, it was observed that gene was significantly down-regulated at the 48h of drug exposure (Figure 4.8a). However this regulation was not significant in Treg cells ($p=0.917$). *FOXP3* gene expression in CD4+ was significantly increased at 48h post-administration ($p=0.009$). This upregulation was gradually increased in Treg cells throughout the drug intake ($p=0.0002$). Contrary to this finding, *IL10* gene expression of Treg cells was gradually decreased, which was also found statistically significant ($p<0.0001$).

IL10, IL17A and *IL6* expressions were not detected in CD4+ T cells. Also, *IL17A* and *IL6* expressions were not detected in Treg cells. For this reason, primers specific to the gene regions of interest were redesigned by the company concerned and the studies were repeated. Despite all attempts, the expression data of the above genes could not be obtained.

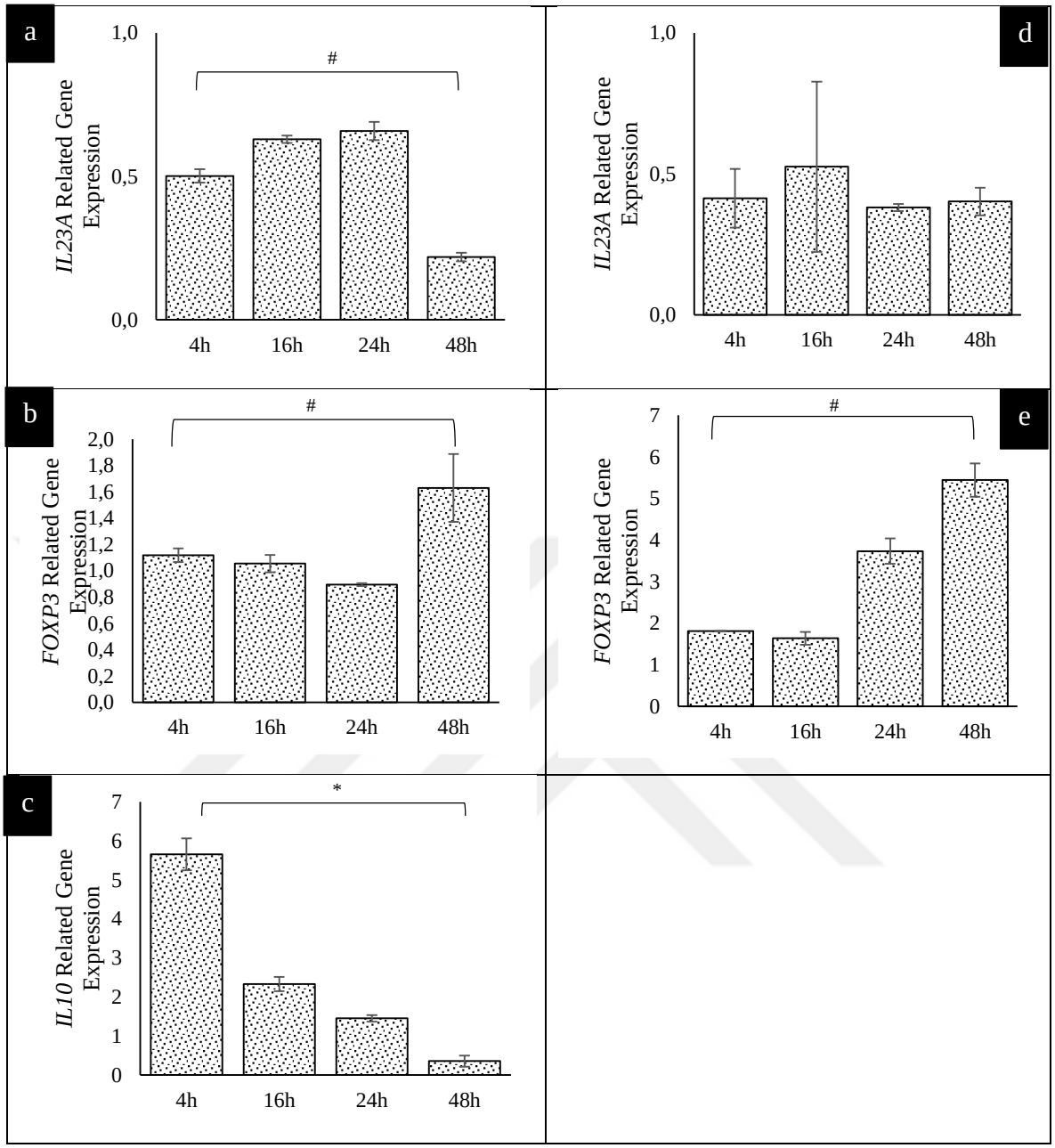


Figure 4.8. RT-PCR results of the cells after IFN-β administration for 4, 16, 24 and 48 hours. a) *IL23A* and b) *FOXP3* gene expression of CD4+ T cells; c) *IL10*, d) *IL23A* and e) *FOXP3* gene expression of Treg cells. All values were normalized by both control group and endogenous gene. (n=3, *: $p<0.0001$, #:$p<0.05$)

5. DISCUSSION

MS is known to be a common neurodegenerative disease in CNS. The presence of many factors in the progression of MS disease makes it difficult to create an effective treatment for the disease. Generally, IFN-β is used for treatment of the disease and belongs to the class of inducible cytokines with high antiproliferative effects. It was reported that the drug can reduce the rate of attacks observed in MS patients by 30 per cent, decrease MRI activities, and slow down the progression of disability [72]. However, it is not possible to obtain the desired therapeutic response to the treatment in some patients who undergo this treatment. It is significant to assign specific biomarkers that can identify the failure status of the treatment early, to determine whether individuals can get positive results from the treatment, or to explain the various molecular mechanisms that will allow the identification of related biomarkers for further studies. Although a number of biomarkers have been identified by studies conducted for this purpose, molecular mechanism has not been fully understood. Therefore, in this study, *IL-6*, *IL-10*, *IL-17*, *IL-23*, and *FOXP3* genes were thought to be effective for the detection of therapeutic response in the treatment of MS. In our study, it was aimed to study the impact of IFN-β on diverse CD4+ T cell subsets, and cytokines. T cells were collected from PBMCs of MS patients who did not use any immunomodulatory drug. *In vitro* drug administration was carried out for 4, 16, 24, and 48 hours. The possible changes in the mRNA level occurring thoughout the process has been investigated to identify specific biomarkers.

As a result of the analysis, optimum drug concentration was determined for both cell types causing inhibition/death of 50 per cent of the cell population due to concentration increase at 4, 16, 24 and 48 hours. The concentration profile obtained for both cell types exhibits an increased-decreased values as shown in Figure 4.8. For this reason, taking the average of the drug concentrations obtained from different time points based on the cell type, it was decided to use the mean value as the optimum drug concentration. It was not included into the average considering that the first four hours of drug administration may have a misleading result.

When the changes in the expression level of *IL23A, IL17A, IL6, IL10* and *FOXP3* genes in cells cultured for 4, 16, 24 and 48 hours at optimum drug concentrations were examined by RT-PCR, it was found that CD4+ T and Treg cells showed a different profile.

In vitro studies of T helper cells into Th17 cells have been informed to add TGF-β and IL-6 to culture medium [73]. In this thesis, although TGF-β and IL-6 were not involved, it was thought that the addition of IL-2 could suppress the differentiation of Th cells. In addition, the fact that *IL-6* gene expression was not detected in this study supports the hypothesis that cells may be differentiated into cells other than Th17 helper cells as a result of stimulation with IL-2 and CD3/CD28.

IL10 is one of the important cytokines in the immunopathogenesis of MS [74]. It is believed that induction of IL10 by immune-modulatory drugs may be beneficial in the disease therapy [75, 76]. The major result obtained by this study was *IL10* gene regulation in Treg cells. Study revealed that IL10 was significantly down regulated throughout the experiment. It was more dramatic in the first 16h, which dropped by 1/3 of that at 4h and reached almost zero at 48h. A study showed that high IL10 production was associated with lower disability in MS patients and it could reduce the MRI lesion load [77]. When a clinical study compared Betaferon and Avonex which both contain pharmaceutically active form of IFN-β, it is found that higher dose and frequently drug intake for a week would change IL10 production compare to one with lower and single dose [78]. A short-term study showed an increase IL10 serum level at two and 12h of post-IFN-β injection [79]. Another study followed up three MS patients who have been injected IFN-β weekly for six months [80]. It was revealed that the serum IL10 level was lowered during the treatment compare to those in pre-treatment. Another long-time followed-up study showed that IL10 mRNA levels lowered dramatically after 6 months of treatment [81]. One particular study investigating the relation between T regulatory cells and IL10 level depicted that IL10 secretion was reduced by nine-fold in monkeys with active MS [82]. Keeping all in mind, here we administrated a single dose of IFN-β for the duration of experiment, therefore dramatic increase in the first 4h compared to control group and constant decrease in expression were not unexpected. However, long time follow-up and comparison of each time points will probably clarify this outcome.

Studies have demonstrated that various gene *IL23A* variants are closely related with MS [83]. In this study, we found that *IL23A* expression decreased significantly at 48h post administration in CD4+ T cells, even after it slightly increased in the first 24h follow-up (Figure 4.8a). However, for *IL23A*, there was no major change in the gene expression of Treg cells. Increased mRNA expression of *IL23* in MS patients were detected in several studies [67, 83, 84], and more, it is shown that anti-IL23 therapy may have impact on MS inflammation [85]. One particular study presented that short-term administration of IFN-β

has reduced the IL23 levels in patients with MS [86]. Here we demonstrated reduction in *IL23A* gene expression after 48h of drug intake, only for CD4+ T cells, remained unchanged for Treg cells. Since Treg cells are more resistant to drug, expansion of follow-up for these cells is required and may change the significance.

FOXP3 gene expression was shown as a key element for Treg cell function. However, the literature on using of *FOXP3* expression similarly as a marker in human Treg cells is limited [48]. We observed considerable up-regulation at 48h in both cell type in terms of *FOXP3* gene expression, more obvious for Treg cells (Figure 3.8e). It is well known that FOXP3 has a critical importance in Treg cell immune response [87, 88, 89]. It is significantly down-regulated during MS disease; therefore up-regulation of the gene may imply recovery in MS [69]. However, similar regulation is generally not obtained for CD4+ T cells [90]. Surprisingly, we detected a low increase in the gene expression for CD4+ T cells after 48h. This made us think that *in vitro* drug exposure may show single cell type effect of IFN-β, but complex relationship among immune cells can change final result. Yet, these findings require further studies to enlighten the disease mechanism.

6. CONCLUSION

Here we showed therapeutic effect of IFN-β drug treatment on CD4+ T and Treg cell immunity. The influence of *IL23A, FOXP3* and *IL10* genes on MS pathogenesis should be further studied. It is still needed to follow-up for up to one year of therapy, to determine the longtime effects of IFN-β on the expression level of these genes within cells.

In our study, it was found that IL-10 and FOXP3 may be used as a biomarker for Treg cells. This study may help to understand the molecular mechanisms of MS and to develop a personalized treatment technique in the future.

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