Short-term effect of IFN-β therapy on the expression of IL23A, FOXP3 and IL10 in CD4+ and CD25+ T cells of MS patients

Short title: IFN-β changes IL23A, FOXP3 and IL10 expression in MS patients

**Hazal Gezmis[a*], **Tansu Doran[a], Fusun Domac Mayda[b], Deniz Yucel[c], Rahsan Oz[b], Deniz Kirac[a]

[a]*Department of Medical Biology, Faculty of Medicine, Yeditepe University, Istanbul, Turkey;*

[b]*Department of Neurology, Erenkoy Mental and Nervous Diseases Training and Research Hospital, Istanbul, Turkey;*

[c]*Department of Histology and Embryology, Faculty of Medicine, Acibadem Mehmet Ali Aydinlar University, Istanbul, Turkey.*

**Correspondence:* Department of Medical Biology, Faculty of Medicine, Yeditepe University, 26 Agustos Yerlesimi, Kayisdagi Cad. 34755 Atasehir, Istanbul-Turkey

hazalgezmis@gmail.com (Hazal Gezmis)

**These authors contributed equally to this work.

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Abbreviations: Central nervous system (CNS), cerebrospinal fluid (CSF), experimental autoimmune encephalomyelitis (EAE), expanded disability status scale (EDSS), fluorescence activated cell sorting (FACS), forkhead box P3 (FOXP3), interferon beta (IFN-β), interleukin (IL), multiple sclerosis (MS), peripheral blood mononuclear cells (PBMC), relapsing-remitting multiple sclerosis (RRMS), signal transducers and activators of transcription 4 (STAT4), T helper (Th), T regulatory cells (TREGs), visual evoked potential (VEP), water soluble tetrazolium-1 (WST-1), optimum drug concentration (ODC).

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Summary

Multiple sclerosis (MS) is an autoimmune disorder causing demyelination in axons. Available therapies target different molecules, but not all have therapeutic effects on disease progression, and this effect can only be seen after a long-time administration. By the time, the disease progresses, and its outcomes become unbearable for the patient. IFN-β has been used in MS therapy for many years. It slows down the disease progression, also reduces disease symptoms by targeting T cells. Yet, a considerable portion of the patient has experienced no therapeutic response to IFN-β. Therefore, it is necessary to determine disease-specific biomarkers which allow early diagnosis or treatment of MS. Here, it was aimed to determine the effects of IL10, IL23A and FOXP3 genes on the therapeutic response to MS after IFN-β administration. PBMCs were extracted from blood samples to isolate CD4+ and CD25+ T cells. Cytotoxicity assays were performed on each cell type for determining optimum drug concentration. Then cells were cultured again and determined drug concentration was administered to the cells to measure gene expressions with RT-PCR. At the end of the study, it was found that the cytotoxic effect of IFN-β was more efficient as the exposure time was expanded regardless of drug concentration. Moreover, CD25+ T lymphocytes were more resistant to IFN-β. IL23A was down-regulated, whereas FOXP3 was up-regulated at 48h in CD4+ T cells. For CD25+ T cells, the graded increase of FOXP3 was obtained while IL10 expression was gradually decreased throughout the drug intake, which both were statistically significant.

Introduction

Multiple sclerosis (MS) is a neurodegenerative disease of the central nervous system (CNS), and characterized by autoimmune demyelination. It generally affects young individuals (20-40 ages), the most commonly in females (1). Considering the heterogeneity of MS patients (genetics, environmental factors, etc.), neither a fully effective treatment has been developed nor the molecular mechanism of disease has been understood yet (2-4). Recent treatment approaches depend on immune-modulatory drugs, one of them is interferons. Interferons are cytokines with anti-inflammatory properties and have been used for the treatment of MS for years. IFN-β is a first injectable drug for the first-line treatment of relapsing-remitting multiple sclerosis (RRMS) patients (5-7). It can reduce clinical and radiological activity in MS patients. On the other hand, the desired therapeutic response to IFN-β cannot be obtained in most RRMS patients (8, 9). For better understanding of whether patients respond to the IFN-β, a treatment period of at least 1 year is usually required (10, 11). During this term, the harder life conditions following the progression of MS disease are delayed. Individuals experiencing non-desired therapeutic response (non-responder 10-50%) may have an adverse side effects of the drug while increasing the level of advanced disability (12, 13). Therefore, it is of importance to identify specific biomarkers that will be able to determine if individuals receive positive response from treatment or not. Although many biomarkers have been identified throughout other studies (14, 15), the molecular mechanism that can effectively detect the therapeutic responses to IFN-β has not been totally elucidated (16).

IL10 is thought to have pleiotropic effects in immune regulation and inflammation, and its relationship with IFN-β metabolism and MS progression has been demonstrated in many studies (17). IL23 gene encodes the subunit of the interleukin 23 heterodimeric cytokine. The IL23 gene is located in the p40 subunit of both this protein and the IL12 cytokine. A recent study showed that lower IL23 serum level was obtained in MS patients treated with IFN-β (18).

IL23 cytokine also plays an important role in CD4 + T cells (19, 20). FOXP3 belongs to transcriptional regulatory genes family and encodes an important protein with immune deficiency in case of any defect (21). It has been reported that FOXP3 expression is essential for Treg development and plays a suppressive role in CD4+ CD25+ Treg cells. Studies with MS patients have shown that the number of Tregs expressing FOXP3 may change as the disease progresses (22). Therefore, to identify some specific biomarkers, it was aimed to determine the effects of IL10, IL23A and FOXP3 genes on the therapeutic response in the treatment of MS. Thus, in this *in vitro* study, it was aimed to evaluate these inflammatory cytokines and investigate their relationships for further understanding the pathogenesis of MS and possible detection of therapeutic markers.

Materials and Methods

Study Population

Forty-two patients who applied to Erenkoy Mental and Neurological Diseases Training and Research Hospital, Neurology Polyclinic within 1 year were enrolled to the study. Clinical history, neurological examination, brain and spinal cord MRIs, CSF analysis, and the number of clinical episodes were used for the diagnosis of RRMS, according to McDonald 2017 criteria. VEP test, patient's family history, EDSS value and first relapse finding were noted for further examination. Patients with RRMS who have not received any immunoregulatory drug for at least 3 months and whose EDSS is less than 2.5 were included. The written consents of each patient were provided prior to the study.

PBMC Isolation

Blood was collected into heparinized tubes for each patient, and PBMCs were separated by Ficoll-Paque density gradient centrifugation within the first 4 hours following the blood sampling (4-10 mL). Firstly, the blood was diluted with 1:1 volume of DPBS (Thermo Fisher,

USA) at 37°C. These suspensions were then added gently onto the pre-heated Histopaque (Sigma-Aldrich, Germany) (4:3 v/v ratio) from the edge of the falcon tube. The mixture was centrifuged at 400 xg for 35 minutes at room temperature. The obtained mononuclear cell layer was gently rinsed by adding 1:1 volume of DPBS, then centrifuged at 300 xg for 10 minutes. The pellet was resuspended in the same ratio of DPBS to remove the platelets and centrifuged at 200 xg for twice. The cells were resuspended in cryo-fluid solution (50% FBS, 40% RPMI 1640 and 1X Penicillin/Streptomycin (100 U/mL, 100 µg/mL) (all from Thermo Fisher, USA)) and 10% DMSO (Sigma Aldrich, Germany) at -80°C for 24 hours then transferred to -150°C until the isolation of naïve CD4+ T cells.

Isolation of Naïve CD4+ T Cells

Naive CD4+ T Cell Isolation Kit and its separating apparatus MidiMACS Starting Kit (both from Miltenyi Biotec, Germany) were used to isolate naïve CD4+ T cells from PBMC according to the manufacturer's instructions. Following isolation, final flow-through solution highly contained unlabeled naïve CD4+ T lymphocytes. Negatively selected naïve CD4+ T cells were cultured in 24 well plates with the RPMI 1640 complete media (10% FBS, 1X Pen/Strep) containing 10 ng/mL interleukin-2 (IL2) (Miltenyi Biotec, Germany). Subsequently, magnetic labelled cells were cultured in the same culture conditions by adding Human T Cell Activator CD28/CD3 (2.5%) (ProMab Biotechnologies, USA). The medium was renewed every other day for one week in order to reach the appropriate cell number for secondary characterization.

Flow Cytometry

150,000 cells/tube were determined for the secondary characterization of CD4+ T cells by flow cytometer (FACSVerse™, BD Biosciences, USA). Naïve CD4+ T cells (passage 0) of each patient were dissociated and centrifuged at 400xg for 10 minutes at +4°C. The cell pellets were

dissolved in MACS buffer solution (Miltenyi Biotec, Germany) and were counted. 25 µL DPBS with 2% FBS was added onto cell suspensions, then mixed with 1 µL FITC-conjugated CD4+ human antibody (BioLegend, USA) followed by incubation at +4°C for 30 minutes. Labelled cells were centrifuged at 2000 rpm for 5 minutes for two times and completed with DPBS.

CD25+ T lymphocyte Cell Sorting

CD25+ T lymphocytes were initially isolated by fluorescence activated cell sorting (FACSAria™ III, BD Biosciences, USA) from PBMC. Magnetic labelled cells following naïve CD4+ T cell isolation were dissociated from well plates. CD3/CD28 magnetic beads were discarded from cell suspension through magnetic separator. Cells were centrifuged at 400 xg for 3 minutes, re-suspended and counted. Cells were incubated with mouse anti-human CD25 antibody (FITC conjugated) (BD Biosciences, USA) for 30 min at +4°C then rinsed with DPBS containing 2% FBS. CD25+ regulatory T cells were sorted by laser scattering. After scattering, cells were cultured in the RPMI 1640 complete media for further studies and medium was renewed every other day.

Cytotoxicity Assay

Cells were pooled on the basis of cell type in order to eliminate immunity differences among patients as well as to increase cell number. IFN-β drug concentrations for cytotoxic effect analysis were determined as 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 300, 400, 500 IU/mL. Initially, cells at passage 7 were cultured on 96 well plate with RPMI 1640 basal media (with 1X Pen/Strep only) in a density of 10,000 cell/well and applied IFN-β for 4, 16, 24 and 48 hours, respectively. Following incubation, WST-1 (Water Soluble Tetrazolium-1) cytotoxicity assay (Roche, UK) was conducted by simply adding 10 µl/well WST-1 solution and incubating at 37°C for 2.5 hours. The absorbance was measured at 405 nm wavelength in ELISA plate

reader. Analysis was worked as triplicates. Relative drug concentration was determined as minimum 500 IU/mL according to result.

Real-Time Polymerase Chain Reaction (RT-PCR)

Both CD4+ and CD25+ T cells at passage 8 were cultured on 96 well plates with RPMI 1640 basal media (1X Pen/Strep only) containing IFN-β in the concentration of 500 IU/mL for 4, 16, 24 and 48 hours, respectively. Cells (P8) without any drug administration were used as control groups for each time period. mRNA samples were extracted by PureLink RNA mini kit (Thermo Fisher, USA) according to the manufacturer's instructions. mRNAs were reverse transcribed into cDNA using high capacity cDNA reverse transcription kit (Thermo Fisher, USA) and PCR amplification of the cDNA was performed by T100^{TM} Thermal Cycler (BioRad, USA). Syber Green conjugated RT-PCR analysis was conducted by Universal Master Mix (Thermo Fisher, USA). Comparative C_T values of IL23A (Hs00372324_m1), IL10 (Hs00174086_m1) and FOXP3 (Hs00203958_m1) were determined in 7500 Fast Real-Time PCR System (Applied Biosystems, USA). GAPDH (Hs99999905_m1) was used as an endogenous control. Three replicate samples were processed at each time point.

Statistical Analysis

Student t-test was used to compare the mean of continues variables between two groups. One-way ANOVA was used to compare the mean values of RT-PCR. p values less than 0.05 were considered statistically significant.

Results

Study Population

Patients diagnosed with RRMS according to the neurological and biochemical parameters were characterized as follows: age(s) (39.83±12.21), duration of disease (5.62±5.51), number of episodes (2.62±0.99), EDSS (1.40±0.80).

Characterization of T Lymphocytes

We performed flow cytometry analysis for the secondary characterization of freshly isolated naïve CD4+ T cells from each patient. We found that each cell suspension had CD4+ T cell subpopulation, but some colonies had a population that those represent 70% and below of targeted population (data not shown). In order to eliminate any decrease in confluence of naïve CD4+ T cell population, we did not involve CD+ T cell subpopulation with under 70 %. According to that, thirty-two patients' naïve CD4+ T cells out of forty-two were mixed to increase the cell number as well as the viability since we observed that these cells had better growth profile when they were more confluent (Fig 1a). Cell suspensions containing CD25+ T cell subpopulations of each patients were directly mixed and cultured after the negative selection of CD4+ T cells. Following several passaging, mixed suspension was used for the isolation of CD25+ T cells (Tregs) subpopulation by FACS (Fig 1c). We obtained Treg cell subpopulation with a percentage of 8.4 of total. Sorted cells were cultured in certain conditions to increase viability (Fig 1b).

Cytotoxicity Assay

WST-1 assay was preferred to observe the cytotoxic effect of IFN-β. Our results showed that 20 IU/mL of drug dropped the CD4+ T cell viability to 60% after the first 4 hours and this decrease continued until the latest observation hours, as the drug concentration increases (Table 1a). We observed that the same concentration was more effective when the administration time was expanded. As the exposure time increased, the viability moderately went down and this decrease was more obvious in the early time of administration. Moreover, each drug

concentration had similar pattern after 16h of administration. Eventually cytotoxic effect of IFN-β killed all the CD4+ T lymphocytes within culture plate. We showed that CD25+ T lymphocytes were more resistance to IFN-β since the first interaction with the drug dropped viability to 80% within 4 hours and around 50% within the following hours when the drug concentration was 20 IU/mL (Table 1b). The decrease in viability was experienced more dramatically when the concentration was even higher. Consequently, for CD4+ T cells, we found that IFN-β had cytotoxic effect as the exposure time was extended even if the concentration was lower. We also noted that the exposure time should be supported by the higher drug concentration to see clear cytotoxic effect on CD25+ T cells. There was also no difference between 24h and 48h administration for the viability of CD4+ T cells.

RT-PCR

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 of CD4+ T cells, it was observed that gene was significantly down-regulated at the 48h of drug exposure (Table 2a). However, this regulation was not significant in CD25+ T 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 CD25+ T cells throughout the drug intake ($p=0.0002$). On the contemporary, IL10 gene expression of CD25+ T cells was gradually decreased, which was also statistically significant ($p<0.0001$).

Discussion

MS is one of the common neurodegenerative disease of CNS in worldwide. Current challenges have been to find an appropriate therapeutic approach for MS since there is a diversity of factors involving in the disease progression. Interferons which are induced cytokines with its highly anti-proliferative effects have been widely used as one of the immune-modulatory approach to

treat MS (23). It is reported that IFN-β can reduce the number of episodes and slow down the progression of disability in MS patients (24-26). However, it is concluded that expected therapeutic response has not been experienced in the majority of MS patients treated with IFN-β. Moreover, the cause of disease conserves its mystery and unfortunately at least one-year of treatment is needed to understand if IFN-β is efficient on the course of the disease or not. Besides, the disease related disabilities can worsen during this term. Therefore, the disease-specific biomarkers which can determine therapeutic failure or success in early stages or those can enlighten the role in disease mechanism can be used for the treatment of RRMS. There are a few biomarkers claimed by several studies but not for the disease mechanism. IL23 is known as critical immune modulatory factor mainly produced by macrophages and dendritic cells; and plays an important role in the production of Th-17 (T helper type 17) which is a cytokine synthesized by CD4+ T cell subsets (27, 28). It is also showed that the accumulation of CD4+ T cell can be reduced by IL10 cytokines which can also support the proliferation of Tregs (29, 30). The progression of Tregs are highly controlled by FOXP3 and any functional loss of it has been reported as a reduction in the activity of Tregs in MS (31). There are several studies that investigated the relationship between the IFN- β treatment and different types of cytokines; however, those results are still on dispute (32-36). Therefore, here, we focused on how *in vitro* IFN-β treatment has affected the expression of IL23A, FOXP3 and IL10 genes in the CD4+ and CD25+ T cells of MS patients.

We first examined the short-term cytotoxic effect of IFN-β for two cell subsets by WST-1 assay. We administrated the drug to the cells for different time points. In the first 4 hours, our results indicated that the viability has decreased significantly to 60% and 80% on CD4+ and CD25+ T cells, respectively. This decrease has continued throughout the administration and it was even more dramatic when the drug concentration was higher. We also observed that the drug exposure time was especially effective regardless of IFN-β concentration for both cell subset,

more obvious in CD25+ T cells. It is known that interferons directly target CD4+ T cells to accumulate immune response (37, 38). It is also shown that these immune-regulators can play role in differentiation of naïve CD4+ T cells to Th-1 like cells (38, 39). In a similar manner of our goal, Ruuls *et al*. investigated the long-time effects of IFN-beta on experimental autoimmune encephalomyelitis (EAE) which is a preclinical animal model of human MS. (40). When group introduced the pharmaceutical active to the animals for 3 weeks, the inhibition and complete prevention of disease were dose dependent. The treatment efficiency also had close association with the administration time and duration of therapy.

To understand how short-term *in vitro* drug administration can change the gene expression levels of IL23A, IL10 and FOXP3, we introduced 500 IU/mL IFN-β-1a (Betaferon) to CD4+ and CD25+ T cells for 4, 16, 24 and 48 hours. IL23A was reported one of the cell-mediators in immune response and can be a specific marker to treat MS (41, 42). 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 (Table 2a). However, for IL23A, there was no significant change in the gene expression of CD25+ T cells. Increased mRNA expression of IL23 in MS patients were detected in several studies (28, 41, 43), and more, it is shown that anti-IL23 therapy may have impact on MS inflammation (44). One particular study presented that short-term administration of IFN-β has reduced the IL23 levels in patients with MS (45). Here we demonstrated reduction in IL23A gene expression after 48h of drug intake, only for CD4+ T cells, remained unchanged for CD25+ T cells. Since CD25+ T cell are more resistant to drug, expansion of follow-up for these cells is required and may change the significance.

We observed considerable up-regulation at 48h in both cell type in terms of FOXP3 gene expression, more obvious for CD25+ T cells (Table 2d). It is well known that FOXP3 has a critical importance in CD25+ T cell immune response (31, 46, 47). It is significantly down-regulated during MS disease; therefore up-regulation of the gene may imply recovery in MS

(48). However, similar regulation is generally not obtained for CD4+ T cells (49). Surprisingly, we detected a slight 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.

IL10 is one of the important cytokines in the immunopathogenesis of MS (50). It is believed that induction of IL10 by immune-modulatory drugs may be beneficial in the disease therapy (51, 52). The major result obtained by this study was IL10 gene regulation in CD25+ T regulatory 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 (53). 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 (54). A short-term study showed an increase IL10 serum level at 2 and 12h of post-IFN-β injection (55). Another study followed up three MS patients who have been injected IFN-β weekly for 6 months (56). 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 significantly after 6 months of treatment (57). One particular study investigating the relation between CD25+ T regulatory cells and IL10 level depicted that IL10 secretion was reduced by 9-fold in monkeys with active MS (29). 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.

Here we showed the earliest therapeutic effect of IFN-β treatment on CD4+ and CD25+ T cell immunity. The influence of IL23A, FOXP3 and IL10 genes on MS pathogenesis needs to be further studied. To determine the long-term effects of IFN-β on the expression level of these genes within cells, follow-up for up to 1 year of therapy is still needed .

Conflict of Interest

No conflict of interest was declared by the authors.

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Hazal Gezmis and Tansu Doran performed the experiments and wrote this paper. Dr Deniz Kirac and Hazal Gezmis designed the study. Dr Fusun Domac Mayda determined the eligibility of MS patients and collected the blood samples. Dr Rahsan Oz contributed to writing this paper. Dr Deniz Yucel collaborated on *in vitro* study design and the evaluation of these results. This work was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK), Grant No: SBAG-216S828. We are grateful to Dr Gamze Torun Kose for their support on study design, Dr Omer Faruk Bayrak and Emre Can Tuysuz for performing FACS.

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Tables

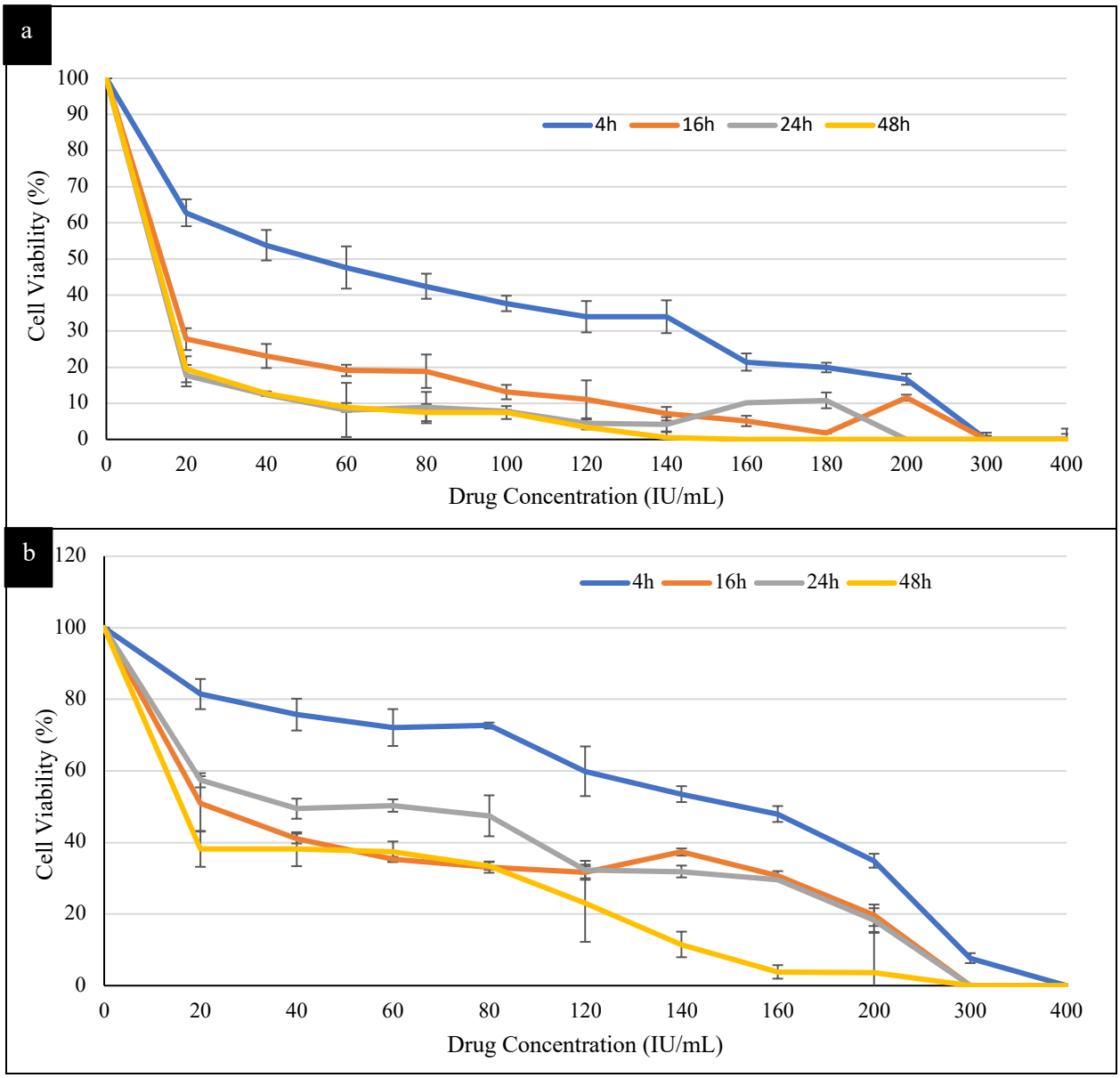

Table 1. Cytotoxicity assay results. WST-1 assay determined cytotoxicity profile of IFN-β after 4, 16, 24 and 48h incubation with a) CD4+ T cells and b) CD25+ T cells at varied concentrations (n = 3).

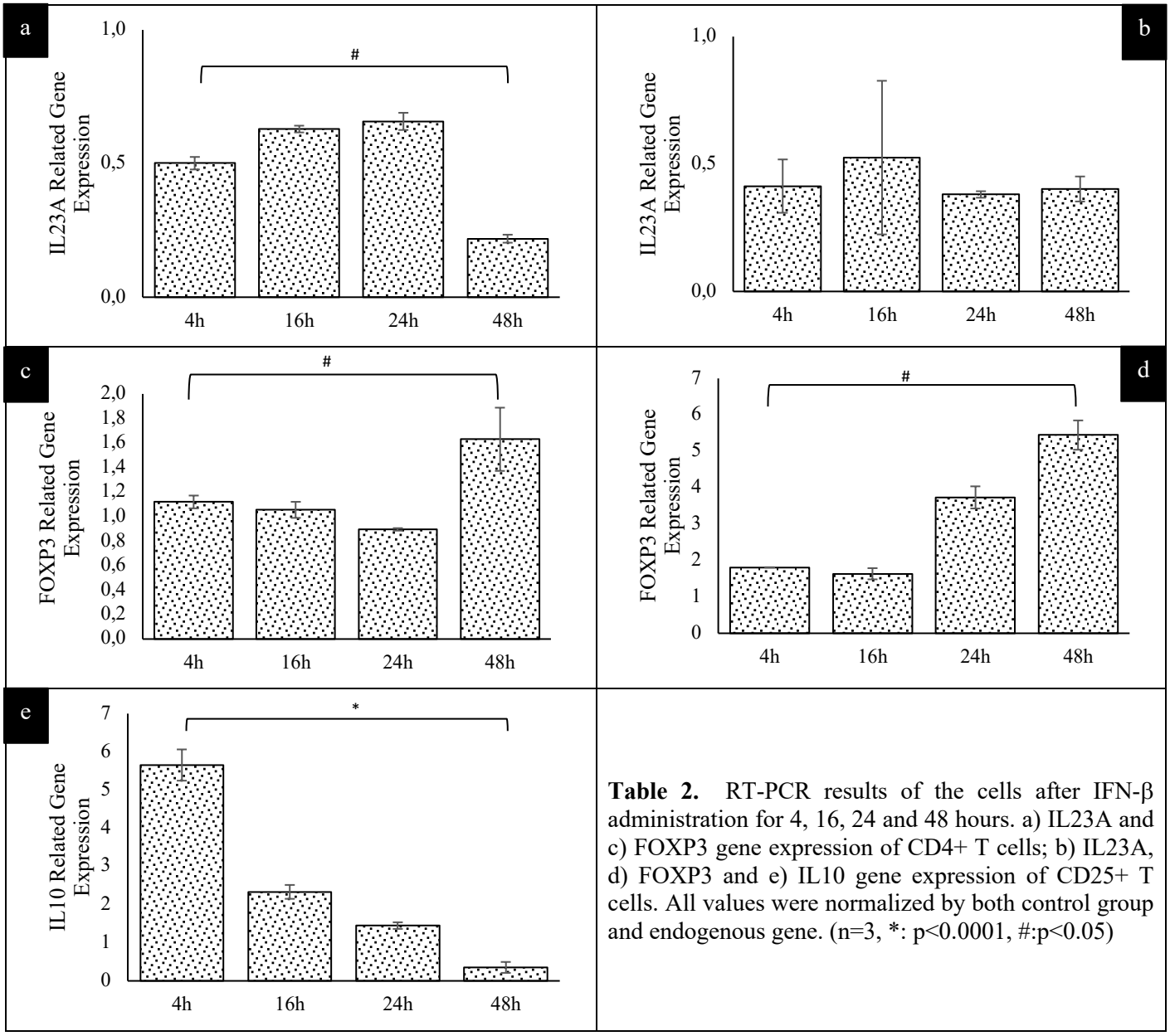

Table 2. RT-PCR results of the cells after IFN-β administration for 4, 16, 24 and 48 hours. a) IL23A and c) FOXP3 gene expression of CD4+ T cells; b) IL23A, d) FOXP3 and e) IL10 gene expression of CD25+ T cells. All values were normalized by both control group and endogenous gene. (n=3, *: $p<0.0001$, #:$p<0.05$)

Figures

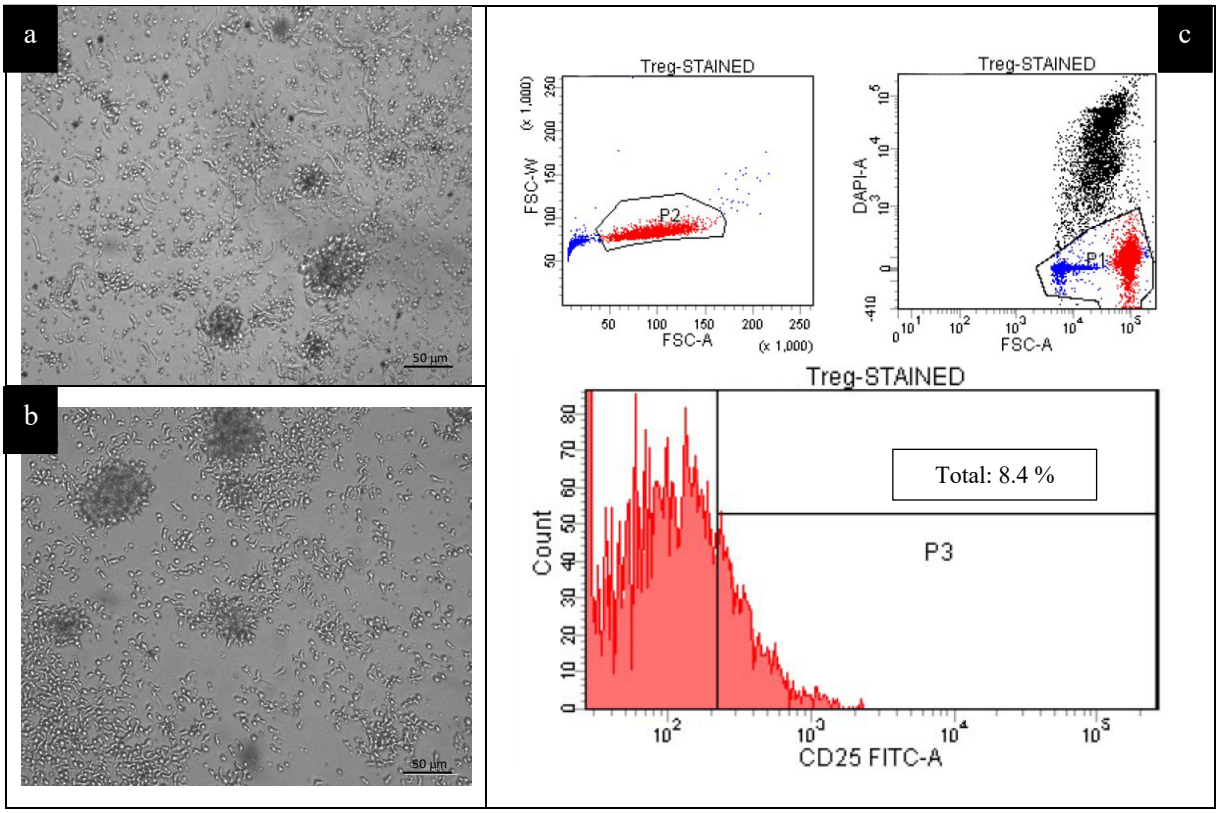

Fig 1. Images of a) CD4+ T cells and b) CD25+ T cells (Tregs) in culture plates (Passage 2) Mag. 50 µm. c) Fluorescence activated cell sorting result of CD25+ T cells after negative selection of CD4+ T cells.