Comparison of Peripheral Blood Th17 Cells and Associated Cytokines in Fingolimod-Receiving and Untreated Multiple Sclerosis Patients

Fingolimod Alan ve Almayın Multiple Skleroz Hastalarının Periferik Kanında Th17 Hücrelerinin Karşılaştırılması

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Abstract

Introduction: Th17 cells are critical mediators of pathology in several autoimmune diseases including multiple sclerosis (MS). The aim of this study was to quantify Th17 cells and-associated cytokines in the peripheral blood of relapsing remitting multiple sclerosis patients (RRMS). We also aimed to compare those levels in fingolimod-treated, and untreated patients.

Material and Methods: Fifteen fingolimod administered RRMS, 9 untreated-RRMS patients and 6 healthy controls were evaluated. Their peripheral blood mononuclear cells (PBMCs) were isolated and sera separated. IL-17A +, IL-22 + and GM-CSF + T-cells were quantified via intracellular cytokine staining after stimulation using fluoresecenc ecell sorter. Serum cytokine levels from all groups were measured via enzyme-linked immunosorbent assay (ELISA).

Results: Fingolimod-treated RRMS patients had reduced number of IL-17A + (p=0.02), IL-22 + (p=0.05), and GM-CSF + (p=0.003) T cells in their peripheral blood compared to those of untreated RRMS patients. This is consistent with sequestration of lymphocytes in the secondary lymphoid organs after fingolimod use. However, the levels of same cytokines in the serum were statistically not different.

Conclusions: Fingolimod treatment reduced circulating IL-17A +, IL-22 + or GM-CSF + T cells in RRMS patients.

Keywords: Fingolimod, Multiple sclerosis, IL-17A, IL-22, GM-CSF

Öz

Giriş: Th17 hücreleri, multipl sklerozun da dahil olduğu birçok otoimmün hastalığın patogenezinde rol alan kritik bir hücre popülasyonudur. Bu çalışmada, fingolimod tedavisi alan ve almayın multipl skleroz hastalarının periferik kanında Th17 hücrelerinin karşılaştırılması amaçlanmıştır.

Gereç ve Yöntemler: On beş fingolimod tedavisi alan RRMS hastası, ve tedavi almayın 9 RRMS hastası ile 6 sağlıklı kontrol çapraz olarak edildi. Kanlı izole edilen periferik kan mononükleer hücrelerinin stimülasyonu, intraselüler hücre boyamasi ve FACSAriaII kullanılarak IL-17A, IL-22 ve GM-CSF üreten T hücreleri sayılır ve yüzdeleri ölçülür. Serum sitokin seviyeleri ELISA yöntemleri ile saplandığına dair saptanmıştır.

Bulgular: Fingolimod tedavisi alan RRMS hastalarının kanındaki IL-17A + (p=0.02), IL-22 + (p=0.05), GM-CSF + (p=0.003) T hücrelerinin mutlak sayılardında, fingolimod almayın TSM hastalarına göre, önemli ölçüde azaldığı gözlandı. Bu sonuç, fingolimod’un lenfositlerin sekonder lenfoid organlarda tutma mekanizması ile uyumluştur. ELISA ile serumdan ölçülen aynı sitokinlerin seviyelerinde anlamlı azalma gözlenmiştir.

Sonuç: Fingolimod dolaşımdaki IL-17A +, IL-22 + ve GM-CSF + üreten T hücre sayısını azaltmaktadır.

Anahtar Kelimeler: Fingolimod, Multipl skleroz, IL-17A, IL-22, GM-CSF

Introduction

Th17 cells are a lineage of CD4+ helper T cells that differentiate from naïve CD4+ T cells in the presence of proinflammatory cytokines IL-6, IL-1β, IL-23 along with TGF-β. After their discovery, pathogenesis of many chronic inflammatory diseases including multiple sclerosis (MS), have been shown to associated with Th17 cells. Th17 cell development is regulated by the master regulator transcription factor retinoic acid related
(RAR) orphan receptor gamma t (Rorγt), or Retinoic Acid Related Orphan Receptor C (RORC), in mice and humans, respectively. Other transcription factors such as Stat3, Irf-4, Batf and AhR have also been shown to be critical for Th17 development in mice, however only Stat3 among those have been shown be required for human Th17 development or function. Loss of function mutations in Stat3 is associated with immunodeficiency and reduced Th17 cell response and function. In mice, deletion of Th17 cells by genetic means, or blockade of Th17 cells through biochemical means resulted in resistance to development of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. In this regard, Rorγt deficient mice, Batf, Ahr, similarly, Il6-/-mice, Il23r-/-mice, Il23p19/-/- mice or il23p40 deficient mice all have become resistant fully or partly to EAE development. Although a critical role for Th17 cells in EAE pathogenesis have been established by targeting genes required for Th17 cell development or function, the identities of Th17 cell-derived cytokines responsible for the pathogenesis remained somewhat controversial. In fact, IL-17A-/- and IL-17F-/- mice still developed EAE. Similarly, neutralization of IL-17A with antibodies did not confer resistance to EAE. Likewise, IL-22-/- mice also develop EAE. More recently, GM-CSF was reported to be essential for EAE pathogenesis; however, this was challenged by more recent studies suggesting a more supportive role for GM-CSF. Nevertheless, Th17 cells and associated cytokines have been shown to be elevated in the peripheral blood of MS patients, and post-mortem analyzed CNS tissues.

Fingolimod is the first orally administered agent approved for the treatment of MS. Fingolimod is derived from myriocin, produced naturally by the fungus Isaria sinclairii. It is an analog of sphingosine 1 phosphate (SIP). SIP is endogenously produced by endothelial cells and is found at high concentrations in blood and lymph, and at low levels in the tissues. Immune cells express five receptors for SIP, SIPR1-5. Through SIPR1, T cells can migrate from secondary lymphoid and non-lymphoid tissues into lymph and blood. SIP-SIPR1 binding triggers internalization of the receptor, thus cells are unable to sense the ligand when exposed to high levels of SIP. Similarly, fingolimod, after being phosphorylated in vivo, causes SIPR1 internalization, therefore when cells enter the lymph nodes they are trapped within and their egress is blocked as high fingolimod levels are maintained in the tissues through its intake by the patient.

In this study we aimed to quantify Th17 cell numbers and IL-17A, IL-22 and GM-CSF cytokine levels in the peripheral blood and serum of relapsing remitting MS (RRMS) patients treated in Neurology Clinic at Erciyes University School of Medicine, Kayseri, Turkey. Our results show that consistent with the previous reports in animal models and humans, fingolimod reduced IL-17A, IL-22 and GM-CSF T cells in the RRMS patients’ blood.

**Methods**

**Patient information**

After the informed consent was obtained from donors peripheral blood samples were drawn from patients at Gevher Nesibe Hospital, Erciyes University School of Medicine, Department of Neurology. The diagnosis of the patients was conducted based on the Revised McDonald Diagnostic Criteria for MS (Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria). All subjects in the RRMS patient/no treatment group were recently diagnosed and off immunomodulatory or immunosuppressive drugs at the time of study for about 3 months before the blood was drawn. Fingolimod-receiving RRMS patients were under Fingolimod treatment for at least 3 months and were in remission not in relapse. Control subjects are free-of known autoimmune condition or a family history of autoimmune disease. The research protocols were approved by the Ethics Committee at Erciyes University (2015/346). All methods for human studies involving human samples were performed in accordance with the relevant guidelines and regulations.

**Intracellular cytokine staining**

Peripheral blood mononuclear cells (PBMCs) were isolated from 5 to 10 ml blood via Ficoll Paque (GE Healthcare) according to manufacturer’s protocols. The cells were counted under hemocytometer using Trypan Blue exclusion before freezing and were stored in liquid nitrogen in 10% DMSO containing Fetal Bovine Serum. After thawing and counting PBMCs were resuspended in complete RPMI-1640 medium (containing 10% FBS and essential and non-essential amino acids, and Anti-Anti (Antibiotic-Antimycotic, Gibco)). PBMCs were plated at 10^6 cells/well density into 96-well round bottom plates and stimulated with Phorbol-Myristate-Acetate (PMA) / Ionomycin / Golgi Plug (50 ng/mL / 1 ug/mL / 1 ul/mL) for 4 hours at 37°C. The cells were surface stained...
with anti-human FITC-TCRαβ (BioLegend) for 30 min following Fc block with Human TrueStain FcX™. Cells were washed twice at 400 g, 5 min with PBS containing 2% FBS (Staining Buffer). The cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Plus kit. Anti-human PE-GM-CSF, APC-IL-17A, PerCP-Cy5.5-IL-22 (all from BioLegend) were added for 30 min. Cells were washed and run on a FACS_Aria III (BD Biosciences). Data analysis was performed using FlowJo and FACSDiva software (BD Biosciences).

ELISA
Biolegend Human IL-17A ELISA MAX, Human IL-22 ELISA MAX and Human GM-CSF ELISA MAX kits were used to run ELISA from sera collected from patient blood. Samples were diluted twice before use. The assays were performed based on the manufacturer's specified protocols.

Statistics
Th17-associated cytokines and cells in MS patient peripheral blood, PBMCs were isolated and serum samples and the cells were frozen until sample collection was ended. The PBMCs were then thawed before use and stimulated with PMA/Ionomycin/Golgi Plug for 4 hours. FITC TCRαβ, APC-IL-17A, PerCP-Cy5.5-IL-22 and PE-GM-CSF antibodies were used to stain intracellular cytokines GraphPad Prism 6 program and ANOVA and Student's t test was used to calculate the statistics. The tests defined as statistically significant at p value <0.05.

Results
The gating strategy to define IL-17, IL-22 or GM-CSF producing T cells and a representative flow graph for each group is shown in Figure 1A. The patients' demographics (number of patients, age, gender, and mean age) were given in Figure 1B. To test whether fingolimod treatment reduced Th17-associated cytokines and cells in MS patient peripheral blood. We detected significantly reduced absolute number of lymphocytes in the blood of RRMS patients treated with fingolimod compared to those not treated (Figure 2). Similarly, the absolute number of IL-17A+TCRαβ+ as well as GM-CSF+TCRαβ+ cells were significantly reduced (Figure 2). IL-22+TCRαβ+ cell number was also reduced (p=0.05), however, possibly due to small sample size used for this study the significance was border line. Reduced percentage of IL-17A+TCRαβ+cells among lymphocytes was also noted in Fingolimod-receiving RRMS patients.

![Figure 1. Gating strategy and Patient Information. Representative flow plots are shown. Lymphocytes were gated and charted as cytokine versus TCRαβ+ plots (a). Patient information summary table (b).](image-url)
compared with those not treated (Figure 2). Percentages of IL-22+ TCRαβ+ cells were comparable between Fingolimod-receiving and untreated RRMS groups (Figure 2). The reduction in absolute number of lymphocytes was also evident in complete blood count of RRMS patients treated with fingolimod compared to those untreated or healthy controls (Figure 3). Additionally, absolute numbers of neutrophils and basophils in the peripheral blood were significantly reduced after fingolimod use whereas monocytes and eosinophils remained unchanged (Figure 3). We also wanted to test serum cytokine levels. To this end we performed ELISA for IL-17A, IL-22, and GM-CSF using serum samples from healthy controls, Fingolimod-treated or untreated RRMS patients (Figure 4). Despite the reduction observed in the absolute number of Th17 cells with intracellular cytokine...
staining, none of the cytokines were significantly different across the groups in the serum in our hands.

**Discussion**

MS is a neuroinflammatory and neurodegenerative disease. Immune-mediated pathology is partly mediated by Th17 cells. In humans, Th17 cells are increased in MS patients’ blood as well as inflamed plaques in the CNS.[5,24,26,27,32] Fingolimod blocks T and B cells egress from secondary lymphoid organs and is used in MS treatment. Fingolimod also has immunomodulatory properties particularly on Th17 and Treg cells.[33–36]

In this study we compared Th17 cell number, and Th17-associated cytokines in the peripheral blood of untreated RRMS patients to those treated with fingolimod via flow cytometry-based assays. Previous studies have shown a reduction in IL-17 producing cells in the peripheral blood after Fingolimod treatment.[37,38] Our results from the current study are in line with those reports. Lymphocyte absolute numbers in our patients under Fingolimod treatment show reduction in the peripheral blood as evident in both CBC and flow cytometric assessment. Differences in CBC and flow cytometric counts could be attributed to loss of cells during freezing thawing for flow cytometric tests. Importantly CBC results also point to a significant reduction in neutrophils and basophil counts in patients treated with fingolimod. Eighty (80%) of the patients treated with fingolimod in this study were lymphopenic (lymphocyte counts were between 200 and 800/µL), however, none of the patients had less than 200/µL lymphocyte counts. These numbers are in line with the previously published values.[39–42] Up to 60% of patients have been reported to have lymphocyte counts below 600/µL.[40] Additionally, both absolute number and percentage of IL-17A+ T cells were reduced in the peripheral blood after fingolimod treatment. Moreover, IL-22+ as well as GM-CSF+ T cell absolute numbers show a reduction in the peripheral blood. Percentages of these cells however did not show a significant difference (p=0.05). Although the absolute number of total lymphocytes, or IL-17A+ cells were assessed and explicitly reported in the previous literature, IL-22+ T cells and GM-CSF+ T cell absolute numbers have not. Our study revealed that these cells also reduced in number. The reduction in the absolute number of IL-17+ T cells in the peripheral blood might be due to sequestration of cells in the lymph nodes or due to immunomodulatory effects of fingolimod.[36,37,43,44]

Reduction of central memory Th17 cells in the peripheral blood after fingolimod treatment have been reported previously although others have shown a subset of patients that had increased Th17 cells.[37,43] Fingolimod may also reduce polarization to Th17 by reducing Th17-polarizing cytokine production by dendritic cells[36] or suppressing IL-17 production by CD4+ T cells.[44]

Lastly, we also compared the serum levels of IL-17A, IL-22, and GM-CSF between the fingolimod-receiving and untreated RRMS patient groups. Interestingly we did not observe a difference between healthy controls, fingolimod treated and untreated RRMS group. Previous studies showed an increase in MS patients’ serum of cytokines IL-17A and IL-22.[24,26] Our sample size for each group

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Figure 4. Serum IL-17A, IL-22 and GM-CSF levels in the peripheral blood of fingolimod-receiving (n=16) and untreated RRMS patients (n=9) (n.s, not significant).
was modest, thus this is a limitation of our study. The lack of a significant difference in cytokine levels in the sera between RRMS patients and the healthy controls could be attributed to the small sample size.

In conclusion, our data show that fingolimod treatment decreases the number of circulating IL-17⁺ T cells, IL-22⁺ T cells and GM-CSF⁺ T cells in RRMS patients’ peripheral blood.

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**References**

4. Patel DD, Kuchroo VK. Th17 Cell Pathway in Human Immunology: Lessons from Genetics and Therapeutic Interventions. Immunology 2015;43:1040–51. [CrossRef]
23. Pierson ER, Goverman JM. GM-CSF is not essential for experimental autoimmune encephalomyelitis but promotes brain-targeted disease. JCI Insight 2017;2:e92362. [CrossRef]


33. Liu G, Yang K, Burns S, Shrestha S, Chi H. The S1P(1)-mTOR axis directs the reciprocal differentiation of T (H1) and T (reg) cells. Nat Immunol 2010;11:1047–56. [CrossRef]


