Research Article

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The Effect of the Administration of Interferon and Steroids on Regulatory T-cells in the Liver, Spleen, and Bone Marrow of Mice

Farelerde İnterferon ve Steroid Uygulamasının Karaciğer, Dalak ve Kemik İliğindeki Regülatuvar T-hücrelerine Etkisi

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ABSTRACT

Objectives: Regulatory T-cells (T-regs) maintain immune tolerance by affecting other cells of the immune system. They play an important role in autoimmune diseases and the prevention of graft rejection. Steroids suppress the immune system, especially inhibiting cytokine secretion of T-lymphocytes, initiation of the cell-mediated immune response, and stimulation of T-regs. Interferons (IFN) also have immunomodulatory, antiviral, and anti-proliferative effects. They activate macrophages and cytotoxic T-cells and stimulate the differentiation of T-regs. The aim of this study was to evaluate the effects of IFN and steroids on T-regs in the liver, spleen, and bone marrow in a mouse model, and to determine if they exert their immunosuppresive/immunomodulatory effects through T-regs.

Materials and Methods: A total of 24 mice were randomly separated into 3 groups and administered an intraperitoneal injection for five days. The control group received 0.1 mL saline every day, the IFN group received IFN-alpha-2b 20,000 IU on the first, third, and fifth days, and only 0.1 mL saline on the other days, and the steroids group received 5 mg/kg dexamethasone in 0.1 mL

ÖZ

Amaç: Regülatuvar T-hücreleri (T-reg) immün sistemde görevli birçok hücre çeşidine etki ederek immünolojik toleransı sağlayan hücrelerdir. Otoimmün hastalıklar, greft rejeksiyonunun önlenmesi ve enfeksiyon hastalıklarında önemli role sahiptirler. Steroidler, immün sistemi baskılarlar; özellikle T-lenfositlerin sitokin salgılamasını ve hücresel immünolojik yanıtın başlamasını önlerler ve T-reg'leri de stimüle ederler. Diğer yandan interferonlar (İFN) immünomodülatör, antiviral ve anti-proliferatif etkiye sahiptirler. Makrofajları ve sitotoksik T-hücrelerini aktive ederler ve T-reg'lerin diferansiyasyonunu uyarırlar. Biz bu çalışmamızda İFN ve steroidin karaciğer, dalak ve kemik iliğindeki T-reg'lere etkisini, bilinen immünosüpresif/immün düzenleyici etkilerini T-reg'ler üzerinden yapıp yapmadıklarını değerlendirmeyi amaçladık.

Gereç ve Yöntemler: Bunun için 24 fareye 5 gün boyunca intraperitoneal enjeksiyon yapıldı. Kontrol grubuna 0,1 cc serum fizyolojik her gün uygulandı. İFN grubuna İFN-alfa-2b 20.000 IU 0,1 cc olacak şekilde serum fizyolojik ile sulandırılarak gün aşırı 3 kez diğer günler 0,1 mL serum fizyolojik uygulandı. Steroid grubuna deksametazon 5 mg/kg 0,1 mL olacak şekilde serum fizyolojik

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saline every day. Two days after the end of therapy, each mouse was anesthetized, the portal vein was explored via laparotomy, and 5 mL bovine serum albumin (BSA) was administered through the portal vein. The inferior vena cava was cut to allow BSA perfusion of the liver, and then the mice were sacrificed. The liver, spleen, and bone marrow were removed for analysis. T-regs were identified and counted using flow cytometry.

Results: The flow cytometry count results showed no significant difference between the IFN, steroid, and control groups.

Conclusion: IFN and steroid use do not seem to affect the quantity of T-regs.

Keywords: Regulatory T-cells, interferon, steroid, immunology

ile sulandırılarak her gün uygulandı. Enjeksiyonlar bittikten sonra 2 gün beklendi. Farelere genel anestezi uygulandı, laparotomi yapılıp portal ven açığa çıkarıldı, portal venden 5 mL bovine serum albümin (BSA) verildi, inferior vena kava kesilerek karaciğerin BSA ile perfüzyonu sağlandı, fareler feda edilmiş oldu. Karaciğer, dalak ve kemik ilikleri elde edildikten sonra T-reg'lerin ayrımı yapıldı ve akım sitometrisi ile sayıldı.

Bulgular: Akım sitometrisi ile sayımda İFN, steroid ve kontrol grubunda T-reg sayılarında istatiksel olarak anlamlı bir farlılık bulunamadı.

Sonuç: Sağlıklı farelerde İFN ve steroid kullanımının karaciğer, dalak ve kemik iliğindeki T-reg'lerin miktarına etki etmediği düşünüldü.

Anahtar Kelimeler: Regülatuvar T-hücresi, interferon, steroid, immünoloji

Introduction

Lymphocytes are a single cell group in the immune system that carry receptors specific to antigens. Non-response of lymphocytes to self-antigens is known as immune tolerance. The absence of immunological tolerance results in autoimmune diseases. In 1969, Nizhizuka and Sakakura (1) demonstrated that one cell type was especially affected in autoimmune diseases. In 1995, Sakaguchi et al. (2) demonstrated the role of CD4(+) T-cells that show CD25 positivity in immunological tolerance. Regulatory T-cells (T-regs) are a heterogeneous cell group which carry CD4(+) CD25(+) surface molecules expressing forkhead box P3 (FOXP3) transcription factor (3,4). This is indispensable for the reliable functioning of the immune system. T-regs are located among CD4(+) and CD8(+) T-cells, B-cells, natural killer cells, natural killer T-cells, and dendritic cells, and suppress the activation, proliferation, differentiation, and effect functions of many cell types. In this way, they control the immune responses that develop against pathogens, alloantigens, and tumors, with self-antigens (5).

Interferons (IFN) have antiviral, immunomodulatory, and antiproliferative effects and stimulate the differentiation of T-regs (6).

Corticosteroids are the most frequently used drugs because of their anti-inflammatory, antiallergic, and immunosuppressive effects. Glucocorticoids have been shown to upregulate FOXP3 expression and regulatory T-cells (7,8,9).

The aim of this study was to evaluate the effect of IFN and steroids on T-regs in the liver, spleen, and bone marrow and whether they produce their immunosuppressor/immunomodulatory effects through T-regs.

Materials and Methods

Mouse Model

Approval for this study was granted by the Animal Experiments Local Ethics Committee of İstanbul University. Adult male Balb-C mice at least 8 weeks old and each weighing 25-35 gr were obtained from İstanbul University Experimental Medical Research Institute. Throughout the experiment, the mice were kept in a cage at standard room temperature and humidity with a daily provision of food and drinking water.

The protocol was approved by the Committee on the Ethics of Animal Experiments of İstanbul University (approval number: 119).

Applications

The animals were separated into 3 groups and intraperitoneal (ip) injections were performed daily for 5 days. The control group (n=6) received 0.1 cc isotonic saline (0.9% NaCl) (IS) each day. The IFN group (n=9) received IFN-alpha (α) 2b 20,000 IU (Intron-A flacon, 10 M IU/1 mL) diluted in saline to a dose of 0.1 cc on days 1, 3, 5 and 0.1 cc IS only on days 2 and 4 (10,11,12). The steroid group (n=9) received 5 mg/kg dexamethasone (Dekort-im/iv ampoule 8 mg/mL) diluted in IS to a dose of 0.1 cc, every day for 5 days (1). At 2 days after completion of all the injections, general anesthesia was administered ip to all the mice with atropine sulfate 0.3 mg/kg (atropine ampoule), xylazine 10 mg/kg (rompun flacon), and ketamine 200 mg/kg (ketalar flacon). Laparotomy was performed, and the portal vein was exposed. Bovine serum albumin (BSA) 5 mL was administered, and by cutting the inferior vena cava, perfusion of BSA to the liver was achieved. All mice were then euthanized.

Obtaining the T-regs

After euthanasia of the mice, the liver, spleen, and bone marrow were removed from each animal for evaluation of T-regs. The liver was removed and weighed, then placed in Roswell Park Memorial Institute (RPMI) 1640 medium solution and cut into small pieces with surgical scissors. The pieces were made homogenous by crushing with a syringe piston and then passed through a tea strainer. After leaving for 1 min in a flacon, the supernatant was withdrawn. This was made up to 40 cc with RPMI, then centrifuged at 500 rpm for 3 mins and the supernatant was withdrawn. Thus, leukocytes within the liver were obtained. The spleen was removed and placed in RPMI solution. RPMI solution was administered between the capsule and the spleen, and when the capsule was swollen, the spleen was separated from the capsule using the tip of an insulin syringe with gentle movements. This was made homogenous by crushing with a syringe piston and

after leaving for 1 min in a flacon, the supernatant was withdrawn. This was made up to 40 cc with RPMI, then centrifuged at 500 rpm for 3 mins and the supernatant was withdrawn and passed through a tea strainer. Thus, leukocytes in the spleen were obtained.

To obtain bone marrow, the tibia and femur bones were removed, the surrounding muscle tissues were cleaned, and the bones were placed in an RPMI solution. The RPMI solution was administered with a syringe from one end of the bone. The bone marrow was separated from the bone with the pressure of injection. This was made homogenous by crushing with a syringe piston, and after leaving for 1 min in a flacon, the supernatant was withdrawn and passed through a tea strainer. Thus, leukocytes in the bone marrow were obtained.

All procedures were performed over a dish of ice. For separation of the T-regs from the lymphocytes obtained, the CD4(+) CD25(+) Regulatory T-cell Isolation Kit (Miltenyi Biotec), MS column (Miltenyi Biotec) and MiniMACS separator (Miltenyi Biotec) were used.

The leukocytes obtained were counted on a Thoma slide separately for each organ, and the cell count was determined as millimeters cubed, the ratio of cells in the suspension was calculated, and the total leukocyte count was determined. First, the CD4(+) lymphocytes was separated. For this, the cell suspension was centrifuged at 1393 rpm for 10 min. The supernatant was completely aspirated, and the precipitate remaining at the bottom was made a suspension again with 90 µL buffer for every 10^7 cells, then 10 µL CD4 microbead for every 10^7 cells was added, mixed well, and then cooled at 4-8 °C for 15 mins. The cells were washed with 1-2 mL buffer for every 10^7 cells, then centrifuged at 1393 rpm for 10 mins, the supernatant was completely aspirated and the precipitate remaining at the bottom was made a suspension again with 500 µL buffer for every 10^8 cells. The MS column was washed with 500 µL buffer and the cell suspension was applied to the column, then the column was washed 3 times with 500 µL buffer, the column was removed from the separator and placed in an appropriate collection tube, then 1 mL buffer was applied to the column. The cells adhering to the column were shed and thus the CD4(+) cells were obtained, and the CD25(+) cells within these cells were marked to be counted in flow cytometry. For this, cells shed from the column were counted on a Thoma slide. The cell suspension was centrifuged at 1393 rpm for 10 min. The supernatant was completely aspirated. The precipitate remaining at the bottom was made a suspension again with 100 µL buffer for every 10^7 cells, then 10 µL CD25 antibody for every 10^7 cells was added, mixed well, and then left in the dark for 10 min at 2-8 °C. The cells were then washed with 1-2 mL buffer for every 10^7 cells, then centrifuged at 1393 rpm for 10 mins, the supernatant was completely aspirated and then made a suspension again with 1 mL buffer for flow cytometry counting. Thus the CD4(+) CD25(+) cells (T-regs) were prepared for counting with flow cytometry. After counting a total of 10,000 cells on flow cytometry, the lymphocytes were gated and the CD4(+) CD25(+) cells within the gated lymphocytes were counted (Figure 1).

Statistical Analysis

Data obtained in the study were analyzed using SPSS 16.0 software. For the comparison of categorical variables, the One-Way ANOVA (post-hoc Scheffe) test was used.

Results

All parameters examined in the IFN, steroids, and control groups were calculated as mean ± standard deviations values. The results are shown in Table 1.

No statistically significant difference was determined between the IFN, steroid, and control groups regarding the leukocyte count in the liver, spleen, and bone marrow, the CD4(+) cell count, the CD4(+) CD25(+) cell count, lymphocyte count gated on flow cytometry, ratio of CD4(+) CD25(+) cells to lymphocytes gated on flow cytometry, and ratio of CD4(+) CD25(+) cells to cells counted on flow cytometry.

Discussion

T-regs were first identified in 1969, and especially after their role in immunological tolerance was shown in 1995, questions were asked about T-regs and an increase was seen in research (1,2,3). It is understood that T-regs have been studied in healthy individuals and in peripheral blood samples of patients reflecting a specific disease model (autoimmune disease, after transplant, etc). There are few studies in the literature that have evaluated T-regs within tissue. A previous study that investigated T-regs in tissue compared the intrahepatic T-reg distribution in 43 patients with chronic hepatitis C, a control group of 31 healthy adults, and 8 organ donors. Extensive leukocyte infiltration containing CD4(+) FOXP3 (+) T-regs was determined at a high level in livers infected with hepatitis C virus (HCV), and almost no T-regs were determined in healthy livers (13). In another study, a high level of CD4(+) FOXP3 (+) T-regs was determined in livers infected with hepatitis B virus (HBV) (14).

Claassen et al. (15) evaluated the T-reg count in the liver during and after IFN-α and ribavirin treatment in 22 naive chronic hepatitis C patients and showed that the intrahepatic T-reg count was inversely proportional to the level of fibrosis. It was determined that the intrahepatic T-regs examined during antiviral treatment were relatively increased compared to the CD4(+) T-cell count in 10 patients and to the CD8(+) T-cell count in 12 patients. It was reported that this increase in the T-reg ratio could be due to an increase in the T-reg count or a decrease in the effector cells. Of these patients, 20 completed the treatment, and the treatment resulted in success in 13 patients. Of the 13 successfully treated patients, the intrahepatic T-reg count examined 4 weeks after the end of treatment was seen to be increased compared with the total liver lymphocyte, CD4(+), and CD8(+) T-cell counts. In 3 successfully treated patients, no change was determined in the intrahepatic CD4(+) CD25(+) FOXP3 (+) T-regs examined 24 weeks after the end of treatment, and although there was a decrease in the T-reg count in other patients, it was seen to be higher than that of healthy livers (15).

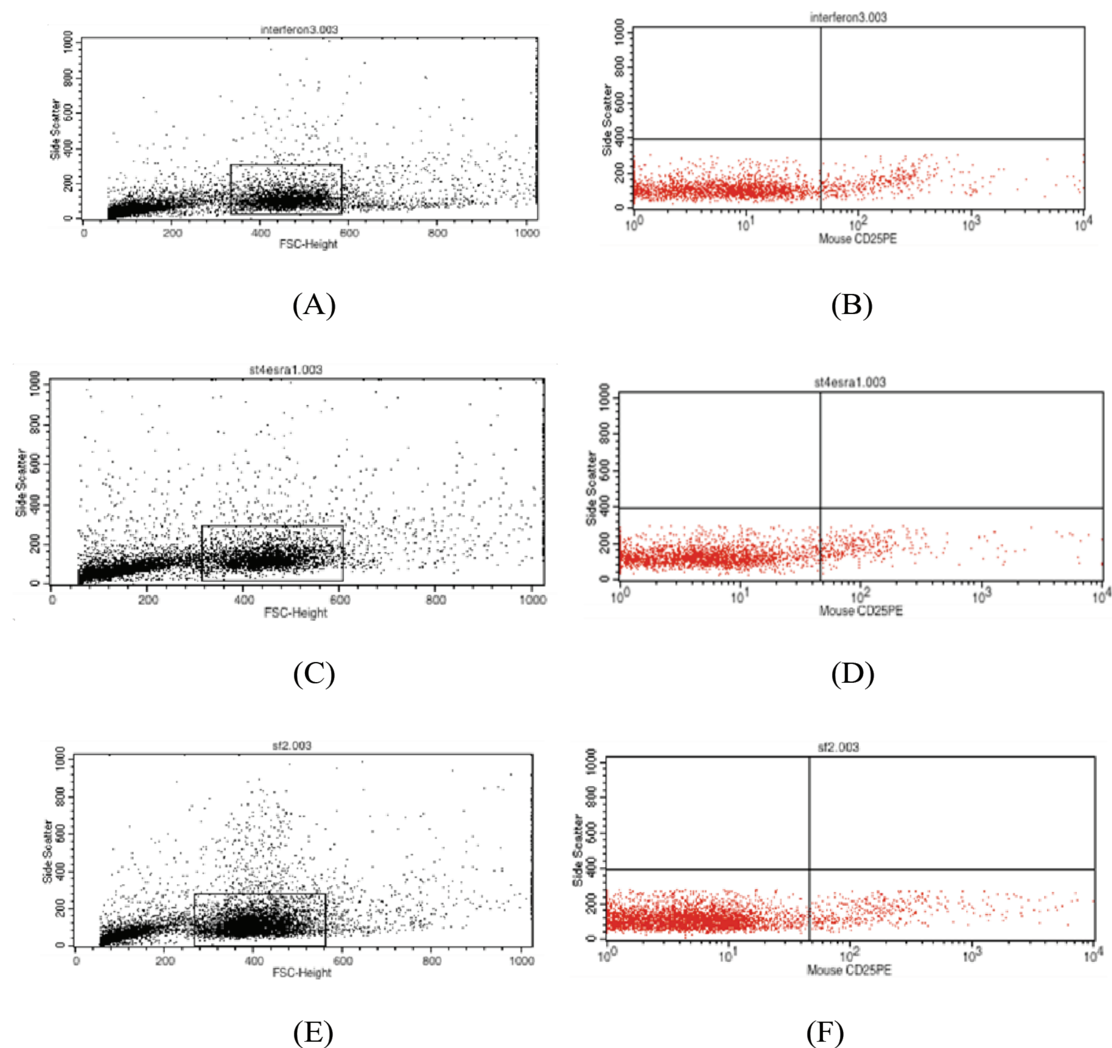

Figure 1. Counts and gating of lymphocytes on flow cytometry. Gating of lymphocytes in the liver on flow cytometry of the interferon, steroid, and control groups (A, C, E, respectively) and the CD4(+) CD25 (+) cell count on flow cytometry (B, D, F, respectively)

In a study by Demirkiran et al. (16), the intrahepatic T-reg level was examined in liver transplantation patients, and immunosuppressive treatment of anti-CD25 monoclonal antibody (basiliximab), calcineurin inhibitor (cyclosporin A or tacrolimus) and prednisone was applied to the patients. FOXP3 was examined with polymerase chain reaction in liver tissue obtained with fine-needle aspiration biopsy at 6 or 12 months after transplantation and an increase in FOXP3 expression was detected. Recurrence occurred in 5 HCV (+) recipients within the first 3 months, and in these patients, FOXP3 expression was found to be higher compared to HCV (-) patients. Of the 15 HCV (-) recipients, acute rejection developed in 3 within the first 3 months, and FOXP3 expression was determined to be higher in these patients than in those without rejection (16).

In another study that evaluated intrahepatic T-regs in 24 liver transplantation patients who developed cirrhosis associated with chronic HCV or HBV infection, immunosuppressive treatment of steroids and tacrolimus was administered, and early acute rejection developed in 10 patients. The T-reg levels of patients who developed acute rejection were found to be lower than those of patients who did not. Although the reason for the decrease in T-reg level could not be explained, it was thought that it could have been related to the immunosuppressive treatment or genetic factors of the recipient (17).

In an experimental mouse model study that examined the T-reg level in the spleen tissue, adult male mice were treated with dexamethasone and/or interleukin-2 (IL-2). After the mice were euthanized, the spleens were removed and spleen cell culture was performed, and then ip was administered to adult female mice that had undergone total-body irradiation. The development of graft-versus-host disease was followed up. The results determined an increase in CD4(+) CD25(+) spleen T-cells and in the ratio of CD4(+) cells to CD25(+) FOXP3 (+) T-cells gated on flow cytometry in the group given dexamethasone and IL-2. When dexamethasone or

IL-2 were administered separately, it was reported that although an increase was observed compared to the control group, it did not result in an increase as great as in the group where they were administered together (18).

In another experimental mouse model study that evaluated the effect of immunosuppressive drugs on T-regs in the spleen and lymph nodes, the spleen and lymph nodes were removed from adult male mice, CD+(+) CD25(+) T-cells were obtained, and cell culture was performed. Cell cultures of T-cells obtained from another lineage of mice were treated with suppressed splenocytes. When there was no immunosuppressive drug in the medium and methylprednisolone was given, an increase was determined in the T-reg level. The rate of increase in the methylprednisolone group was determined to be less compared to the group not given any immunosuppressive drug (19).

Starting from the hypothesis that glucocorticoids act as a co-stimulant in increasing IL-2-mediated selective T-reg expansion, a previous study used healthy mice and an experimental autoimmune encephalomyelitis (EAE) model formed of mice. These patients were given dexamethasone and/or IL-2, and then the rate of T-regs in the spleen and inguinal and mesenteric lymph nodes were compared. An increase was observed in the T-reg percentage in the spleen and in the inguinal and mesenteric lymph nodes both daily and at the end of 5 days of treatment in healthy mice given dexamethasone and IL-2 together. When the effects of the administration of dexamethasone alone or IL-2 alone or the two together were evaluated after 3 days, there was seen to be an increase in the ratio of CD25(+) T-cells in the CD4(+) cell population and an increase in the ratio of CD4(+) CD25(+) T-cells to CD4(+) CD25(-) T-cells, and despite the increase in this ratio, there was observed to be a decrease in splenic CD4(+) CD25(+) T-cell count in the group given dexamethasone alone. Unexpectedly, a decrease was determined in the CD4(+) FOXP3 (+) cell percentage in the lymph nodes, spleen, and peripheral blood in the group applied with dexamethasone alone. Similar results were obtained in mice forming the EAE model. It was demonstrated that dexamethasone significantly strengthened IL-2-mediated FOXP3 (4) T-reg cell growth in both normal healthy mice and the mice forming the EAE model (20).

Another study also showed that dexamethasone treatment in normal naive mice increased the T-reg ratio in lymphoid tissues (21).

In an experimental animal study using New Zealand Black mice, which evaluated the effect of IFN-on the T-reg level in splenic tissue, spleen cell cultures were performed with 48-h 1000 U/mL IFN-α, and IFN-gamma (γ), and phosphate buffer solution was applied to the control group. In the flow cytometry analysis, it was seen that IFN-γ led to a lesser change in the mean T-reg ratio, while IFN-α resulted in a 35% decrease. In addition, of the major cytokines produced by T-regs, the IL-10 level was examined, while there was no change in the IL-10 mRNA level in the group given IFN-γ, there was an 18% decrease in the IFN-α group (22).

Table 1. The leukocytes, CD4(+) cells, CD4(4) CD25 (+) cells, and lymphocyte counts gated on flow cytometry, and the ratios to each other in the liver, spleen, and bone marrow, in the interferon, steroid, and control groups

	Steroid group, (n=9)	Interferon group, (n=9)	Control group (n=6)
Liver: leukocyte count (x10^6):	8.83±3.94	12.1±6.92	9.3±2.4
Liver: CD4+ cell count	250,000±62,449.98	560,000±364,965.75	70,000±28,284.27
Liver: lymphocyte count gated on flow cytometry	3783.33±1912.76	4628.67±1846.57	3676±776.4
Liver: CD4+ CD25+ cell count	343.67±199.2	131.67±219.4	405.5±125.16
Liver: ratio of CD4+ CD25+ cells to lymphocytes gated on flow cytometry	10.97±9	4.18±7.06	11.65±5.87
Liver: ratio of CD4+ CD25+ cells to cells counted on flow cytometry	3.44±1.99	1.32±2.19	4.04±1.22
Spleen: leukocyte count (x10^6)	15.07±4.37	47.87±18.08	46.3±30.69
Spleen: CD4+ cell count	166,666.67±109,696.55	696,666.67±667,108.19	400,000±14,1421.36
Spleen: lymphocyte count gated on flow cytometry	3730.67±2291.36	5022.67±914.12	3966.5±649.83
Spleen: CD4+ CD25+ cell count	222.33±88.58	87.67±135.4	144±38.18
Spleen: ratio of CD4+ CD25+ cells to lymphocytes gated on flow cytometry	6.45±1.45	1.8±2.78	3.76±1.58
Spleen: ratio of CD4+ CD25+ cells to cells counted on flow cytometry	2.22±0.89	0.88±1.35	1.44±0.38
Bone marrow: leukocyte count (x10^6):	5.43±1.1	13.03±1.89	8.4±3.68
Bone marrow: CD4+ cell count	116,666.67±66,583.28	83,333.33±66,583.28	160,000±113,137.08
Bone marrow: lymphocyte count gated on flow cytometry	2384±1545.88	1539.67±610.68	2327±671.75
Bone marrow: CD4+ CD25+ cell count	132±156.12	82±63.55	89±60.81
Bone marrow: ratio of CD4+ CD25+ cells to lymphocytes gated on flow cytometry	4.65±3.2	6.9±6.11	4.39±3.88
Bone marrow: ratio of CD4+ CD25+ cells to cells counted on flow cytometry	1.45±1.44	0.82±0.64	0.89±0.61

In multiple sclerosis patients treated with IFN-beta (β), an increase in T-reg levels was determined in the 3rd-6th months of treatment, but this increase could not be fully explained (23,24). An experimental animal study was conducted with mouse EAE model to explain this increase. One group was administered ip IFN-β treatment every other day for 2 months, and no treatment was given to the control group. At the end of the treatment, the T-regs in the spleen were examined and an increase was determined in the CD4(+) CD25(+) FOXP3 (+) T-regs in the treated group compared to the control group (25).

Prasad et al. (26) examined patients aged 2-16 years with a diagnosis of childhood idiopathic nephrotic syndrome. The frequency of CD4(+) CD25(+) FOXP3 (+) T-regs was examined in peripheral blood before treatment in patients given prednisolone treatment, at 4 weeks after the treatment was stopped as remission had been entered, and before re-starting immunosuppressive treatment because of relapse. Compared with pre-treatment, it was seen that T-regs increased in remission and decreased during relapse (26).

The increase in T-reg activity and function in autoimmune diseases is discussed as one of the potential treatment options. Although IFN-β treatment did not increase the T-reg frequency in the peripheral blood of multiple sclerosis patients, it increases the suppressing function of T-regs. Therefore, further research is needed to explore the mechanism of this effect (27).

In an experimental animal study (Balb-C mice) by Prenek et al. (28), the response of T-regs to apoptosis caused by glucocorticoid hormone was evaluated. After 4 weeks of dexamethasone treatment, the mice were euthanized and thymic and splenic T-regs were obtained. The rate of thymic T-regs was seen to increase but this was a relative increase (as thymocytes are sensitive to glucocorticoid) and there was no change in the absolute T-reg count (28).

Study Limitations

The limitation of the study is that the disease model was not used in the study.

Conclusion

In the current study, IFN-α and dexamethasone did not change the T-reg count in the liver, spleen, and bone marrow of mice. No disease model was used in the study, and the effect was investigated in healthy mice. Repeating this study with a disease model (especially autoimmune disease) would be able to more clearly reveal the relationship between the immunosuppression and immunomodulatory effects of IFN and dexamethasone and T-regs.

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Ethics

Ethics Committee Approval: The protocol was approved by the Committee on the Ethics of Animal Experiments of İstanbul University (approval number: 119).

Informed Consent: Patient approval has not been obtained as it is performed on animals.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.Z., E.Ö., E.A., R.Ö., Concept: E.Z., E.Ö., E.A., B.M., N.S., F.T., R.Ö., Design: E.Z., E.Ö., E.A., B.M., N.S., F.T., R.Ö., Data Collection or Processing: E.Z., E.Ö., E.A., B.M., N.S., F.T., R.Ö., Analysis or Interpretation: E.Z., E.Ö., E.A., B.M., N.S., F.T., R.Ö., Literature Search: E.Z., E.Ö., E.A., B.M., N.S., F.T., R.Ö., Writing: E.Z., E.Ö., E.A., B.M., N.S., F.T., R.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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