

**IMMUNOPATHOGENESIS OF BEHÇET UVEITIS
AND IMMUNE RESPONSE TO IFN- α -2a TREATMENT**

by

Özgür Albayrak

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IMMUNOPATHOGENESIS OF BEHÇET UVEITIS
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Koç University

Graduate School of Health Sciences

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Özgür Albayrak

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Prof. Dr. Füsün Can (Advisor)

Prof. Dr. Önder Ergönül

Prof. Dr. Kenan Midilli

Prof. Dr. Cansın Saçkesen

Prof. Dr. Özlem Kurt Azap

Date: 14 May 2020

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ABSTRACT

This study was conducted to investigate a) The immunopathogenesis of BD and BD Uveitis and b) the effect of interferon alfa-2a, on adaptive and innate immunity in patients with Behçet uveitis. For the adaptive immune responses, peripheral blood regulatory T Cells and TH17 cells were analyzed. For the innate immune responses, mRNA expression of TLRs 2, 3, 4, 8 and 9 on monocytes were analyzed.

Nineteen patients with active Behçet uveitis who were refractory to conventional immunomodulatory therapy and were administered interferon alfa-2a; and age- and sex-matched healthy control subjects were consecutively enrolled from August 2016 to September 2017. Peripheral blood from patients was collected before treatment and after angiographic remission was achieved with interferon alfa-2a. Treg and TH17 percentages were determined as CD3⁺CD4⁺FoxP3⁺ and CD3⁺CD4⁺IL17A⁺ (after in vitro stimulation) respectively by flow-cytometry. TH17 ROR γ T mRNA expression was verified by real-time PCR (RT PCR). Cytokines released from Treg and TH17 cells were detected by ELISA in culture supernatants. RT PCR was used to measure mRNA levels of TLRs 2, 3, 4, 8 and 9 from monocytes. R Statistical Software v.3.5.1 was used for statistics and Mann-Whitney U and Wilcoxon tests were used for analyses. Treg and TH17 percentages and TH17 ROR γ T mRNA expression were found significantly elevated in BU patients. Patients had significantly lower Treg IL-10 concentration than controls. IL-17A, IL-6, IL-21, IL-22, IL-23, IFN- γ , and TNF- α concentrations from Th17 Cells were found to be significantly higher in patients than in controls. All TLR expressions were found significantly elevated in monocytes of BU patients. Interferon alfa-2a (median total dose: 330 million international units, median duration: 5 months) led to remission in BU patients. A significant reversal of Treg and TH17 percentage, TH17 ROR γ T mRNA expression, Treg and TH17 cytokine release, and TLR expressions from monocytes were observed in BU patients after interferon therapy. Despite a paradoxical increase in Treg population, impaired IL-10 production suggests Treg dysfunction in patients with active BU. Interferon alfa-2a restored Treg functions, induces IL-10 production from Tregs, suppresses TH17s and downregulates TLR expression in monocytes.

ÖZETÇE

Bu çalışma Behçet üveiti hastalarında a) BD ve BD Üveitinin immünopatogenezi araştırmak ve b) interferon alfa-2a'nın adaptif ve doğal bağışıklık üzerine etkisini araştırmak için yapılmıştır. Adaptif bağışıklık yanıtları için periferik kan regülatör T Hücreleri ve TH17 hücreleri analiz edilmiştir. Doğal bağışıklık tepkileri için ise, monositler üzerinde TLR 2, 3, 4, 8 ve 9'un mRNA ekspresyonu analiz edilmiştir.

Konvansiyonel immünomodülatör tedaviye dirençli olan ve interferon alfa-2a tedavisi başlanılan aktif Behçet üveitli 19 hasta, ve yaş ve cinsiyet uyumlu 19 sağlıklı kontrol denekleri, Ağustos 2016'dan Eylül 2017'ye kadar ardışık olarak çalışmaya alınmıştır. Hastaların periferik kanı, interferon alfa-2a ile başlanmadan önce ve tedavi sonrası klinik ve anjiyografik remisyon sağlandıktan sonra toplanmıştır. Treg ve TH17 yüzdeleri akım sitometri ile sırasıyla $CD3^+CD4^+FoxP3^+$ ve (*in vitro* stimülasyondan sonra) $CD3^+CD4^+IL17A^+$ olarak belirlenmiştir. TH17 ROR γ T mRNA ifadesi, gerçek zamanlı PZR (GZ PZR) ile doğrulanmıştır. Treg ve TH17 hücrelerinden salınan sitokinler, kültür süpernatantlarında ELISA ile tespit edilmiştir. Monositlerden TLR 2, 3, 4, 8 ve 9'un mRNA seviyelerini ölçmek için GZ-PZR kullanılmıştır. İstatistikler için R İstatistik Yazılımı (Sürüm 3.5.1), analizler için de Mann-Whitney U ve Wilcoxon testleri kullanılmıştır. Behçet Üveiti hastalarında Treg ve TH17 yüzdeleri ve TH17 ROR γ T mRNA ekspresyonu anlamlı derecede yüksek bulunmuştur. Hastaların Treg IL-10 konsantrasyonları kontrollere göre anlamlı derecede düşük ölçülmüştür. TH17 Hücrelerinden salınan IL-17A, IL-6, IL-21, IL-22, IL-23, IFN- γ ve TNF- α konsantrasyonları hastalarda, kontrollere göre anlamlı derecede yüksek bulunmuştur. Tüm TLR ekspresyonları Behçet Üveiti hastalarının monositlerinde anlamlı derecede yüksek bulunmuştur. İnterferon alfa-2a (ortalama toplam doz: 330 milyon uluslararası birim, ortalama süre: 5 ay) Behçet Üveiti hastalarında remisyona sebep olmuştur. İnterferon tedavisinden sonra Behçet Üveiti hastalarında, Treg ve TH17 yüzdelinde, Th17 ROR γ T mRNA ifadelerinde, Treg ve TH17 sitokin salınımında ve monosit TLR ifadelerinde önemli bir geri dönüş saptanmıştır. Treg popülasyonlarındaki paradoksal bir artışa rağmen IL-10 üretim bozukluğu, aktif Behçet Üveiti hastalarında Treg disfonksiyonunu düşündürmektedir. İnterferon alfa-2a, regülatör T hücrelerinin fonksiyonlarını geri kazanmalarını sağlar, Treg IL-10 üretimini indükler, TH17'leri baskılar ve monositlerdeki TLR ekspresyonunu azaltıcı yönde düzenler.

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1. INTRODUCTION

1.1. Behçet's Disease

Behçet's disease is a systemic vasculitis of obstructive and necrotic type and characterized by multiple organ involvement (Evereklioglu, C, 2005; Yazici, H., I. Fresko, and S. Yurdakul, 2007). This disease was first reported in 1937 by Prof. Dr. Hulusi Behçet and described as hypopyoned iritis and a triple symptom complex consisting of orogenital aphthous lesions. While orogenital ulcers, Erythematousum and ocular uveitis are the four cardinal findings of the disease; neurological and gastrointestinal involvement, arthritis / arthralgia, thrombophlebitis, orchitis / epididymitis, cardiovascular and renal involvement with large vessels can also lead to temporary / permanent dysfunction in the organs, and even death (Evereklioglu, C, 2005). Although the disease is observed all over the world, it is most often observed in the Mediterranean countries and in the Middle and Far East. The highest prevalence was reported from Istanbul with 420/100000 (Azizlerli, G., et al, 2003). The prevalence in other parts of Turkey is ranged between 20-370 over 100000 (Cakir, N., et al., 2004; Idil, A., et al, 2002). In northern and western Europe and the United States, the risk of illness is considerably lower (Khairallah, M., et al., 2012; Calamia, K.T., et al, 2009; Zouboulis, C.C., et al., 2003).

1.2. Behçet's Uveitis

Behçet's is the most common form of disease associated with ocular involvement (Evereklioglu, C, 2005; Deuter, C.M., et al, 2008). Ocular involvement is characterized by explosive and recurrent uveitis attacks with spontaneous remission. Although reported 50% in the hospitals, eye involvement is more common in male patients and reported as 70-90% (Evereklioglu, C, 2005). Uveitis due to Behçet's disease is the most common cause of non-infectious uveitis observed in Turkey and constitutes one third of uveitis in Turkish hospitals (Kazokoglu, H., et al., 2008). Behçet uveitis is capable of involving the front or rear segment of the eye, and more frequently both. The involvement of anterior or posterior segment is important both in terms of treatment and prognosis. Posterior segment involvement is persistent and is associated with significant visual loss (Kitaichi, N., et al., 2007). The most common presentation of BD Uveitis is bilateral nongranulomatous retinal vasculitis with panuveitis (Tugal-Tutkun, I., et al., 2004).

1.3. Treatment of Behçet's Uveitis

Although severe uveitis may result in permanent visual loss, the primary cause of visual impairment is the cumulative damage caused by recurrent attacks (Kacmaz, R.O., et al., 2008; Takeuchi, M., et al., 2005). Therefore, it is very important to provide treatment for the prevention of uveitis attacks. In recent years, improvement in visual prognosis has been reported with the use of conventional immunomodulatory agents rather than long-term, high-dose systemic corticosteroid therapy (Tugal-Tutkun, I., et al., 2004). In particular biological agents including interferon alpha-2a and anti-tumor necrosis factor alpha therapies are being used over the last 10 years (Cingu, A.K., et al., 2012; Taylor, S.R.J., et al., 2011). There are reports of a large number of patients on interferon use in our country, especially those who are unresponsive to conventional immunomodulatory agents including azathioprine and / or cyclosporin (Yalcindag, F.N. and A. Uzun, 2012; Onal, S., et al., 2011; Sobaci, G., et al., 2010; Tugal-Tutkun, I., E. Güney-Tefekli, and M. Urgancioglu, 2006).

IFN- α is one of the type I interferon family of proteins that have strong anti-tumor and antiviral activities (Ferrantini, M., I. Capone, and F. Belardelli, 2007; George, J. and J.J. Mattapallil, 2018). In recent decades, due to the immunoregulatory effects, IFN- α -2a is also being used for the treatment of several autoimmune diseases such as vitilligo (Guney, E., et al., 2012), Behçet's Disease (Alpsoy, E., et al., 2002) and Behçet Associated Uveitis (Warde, N., 2010). However, the effects of IFN- α -2a on adaptive and innate immune responses in Behçet Uveitis is still unclear.

Despite new treatment options, blindness is reported in 25% of Behcet's uveitis patients in a large collaborative study (Kitaichi, N., et al., 2007). Therefore, the understanding of the etiopathogenesis of Behcet's uveitis and the immunopathological effects of the treatment has great importance for preventing visual loss.

1.4. Immunopathogenesis of Behçet's Disease

The Immunopathogenesis of Behçet's disease is still unknown. The generally accepted view is that over-responsive or unregulated immunological responses are triggered by environmental factors, including infectious agents, in immunogenically predisposed individuals (Gul, A., 2005). Studies on the immunopathogenesis of Behçet's disease have focused on cellular immunity, suggesting that T lymphocytes play a central role in pathogenesis (Evereklioglu, C., 2005).

1.4.1. Role of Innate Immunity in Behçet Uveitis

It is claimed that microorganisms play a role in triggering immunological mechanisms in the development of Behçet's disease (Evereklioglu, C., 2005). Toll-like receptors (TLRs) have been described in recent years and are the evolutionarily conserved receptors of the innate immune system (Papadimitraki, E.D., G.K. Bertsias, and D.T. Boumpas, 2007). TLRs located on/in antigen presenting cells are involved in the autoimmune response following infection. The interaction of Toll-like receptor with their ligands causes the release of proinflammatory cytokines from antigen presenting cells such as IL1- β , IL-6, IL-23 and TNF- α and induces the differentiation of naive CD4 + T cells into Th17 cells (Acosta-Rodriguez, E.V., et al., 2007). Toll-like receptors are expressed on/in antigen presenting cells including dendritic cells, monocytes / macrophages, and endothelial and epithelial cells related to innate immunity (Takeda, K., T. Kaisho, and S. Akira, 2003).

In a study of ocular Behçet's patients, the expression of TLR2, 3, 4 and 8 in peripheral blood mononuclear cells (PBMC), CD4 + T cells and monocytes of active Behçet uveitis patients was significantly higher than in healthy controls (Liu, X., et al., 2013). The mechanism of TLR-mediated Treg regulation in the pathogenesis of inflammatory / autoimmune disease has also been reported (Papadimitraki, E.D., G.K. Bertsias, and D.T. Boumpas, 2007).

Figure 1.4.1 shows the TLR signaling in autoimmune Systemic Lupus Erythematosus (SLE) and an SLE associated condition Lupus Nephritis (LN) (Devarapu, S.K. and H.-J. Anders, 2018). TLR-2 / TLR-4 mRNA and surface expressions and related cytokine secretions (Komatsuda, A., et al., 2008; Kirchner, M., et al., 2013; Tsao, J.-T., et al., 2012 ; Yamamoto, M., et al., 2003); and TLR-3 / TLR-9 gene expressions (Klonowska-Szymczyk, A., et al., 2014; Lyn-Cook, B.D., et al., 2014) are increased in SLE patients, whereas TLR-8 has an immunoregulatory effect on SLE (Demaria, O., et al., 2010).

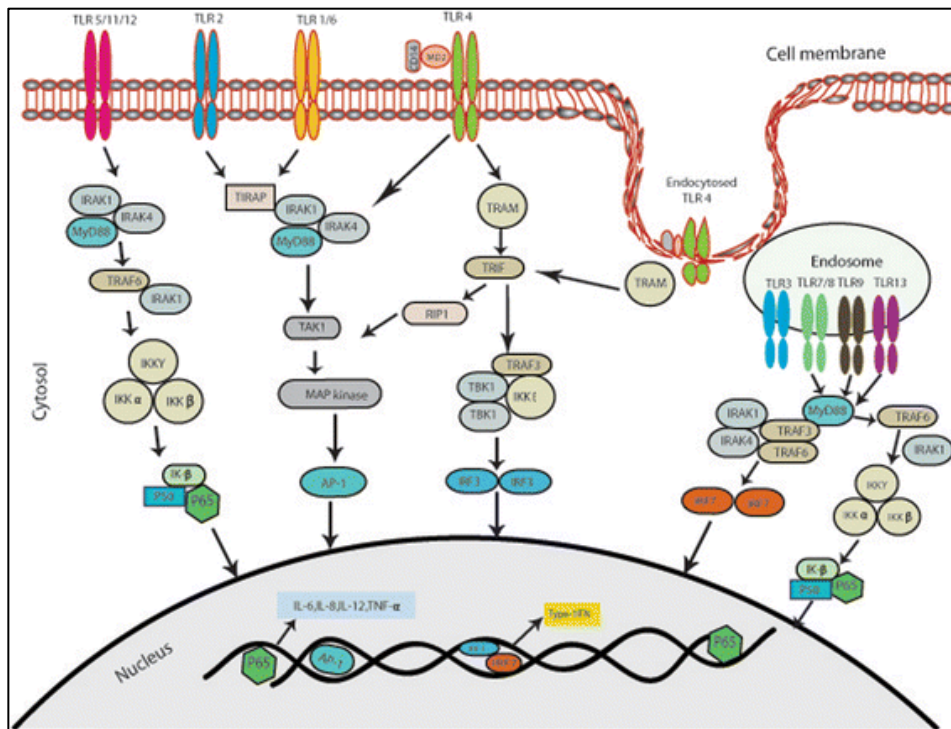


Figure 1.4.1. TLR signaling and Function in SLE and LN.

1.4.2. Role of Adaptive Immunity in Behçet Uveitis

1.4.2.1. Regulatory T-Cells

Regulatory T-Cells (Tregs) are a distinct subset of CD4⁺ Helper T cells that are specialized in suppressing the proinflammatory immune response. Tregs are differentiated from CD4⁺ T Cells in Thymus that are undergoing negative selection. Tregs are determined in peripheral blood or tissues via Flow Cytometry by surface and intracellular immunophenotyping and characterized as surface CD3⁺CD4⁺CD25⁺CD127⁻ and intracellularly FoxP3⁺ cells. FoxP3 is a master transcription factor for CD4⁺CD25⁺ regulatory T-Cells that promotes the expression of anti-proliferative genes CD2S, Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), glucocorticoid-induced TNF receptor family gene (GITR) and folate receptor 4. Regulatory T Cells are proliferated through autocrine IL-2 mechanism.

Immune suppression by Tregs are achieved through four distinct pathways:

- i) Suppression of effector T-Cells via release of IL-10, TGF-β, IL-35 cytokines,
- ii) Cytolysis of effector T-Cells by Granzyme A or B secretion.

iii) Metabolic disruption by IL-2 deprivation through CD25,

iv) Conditioning of Dendritic Cells (DC) to secrete Indolamine 2,3 – dioxygenase via CTLA - 4 secretion (Vignali, D.A.A., L.W. Collison, and C.J. Workman, 2008).

Figure 1.4.2.1 shows the mechanisms of immune suppression by Regulatory T Cells.

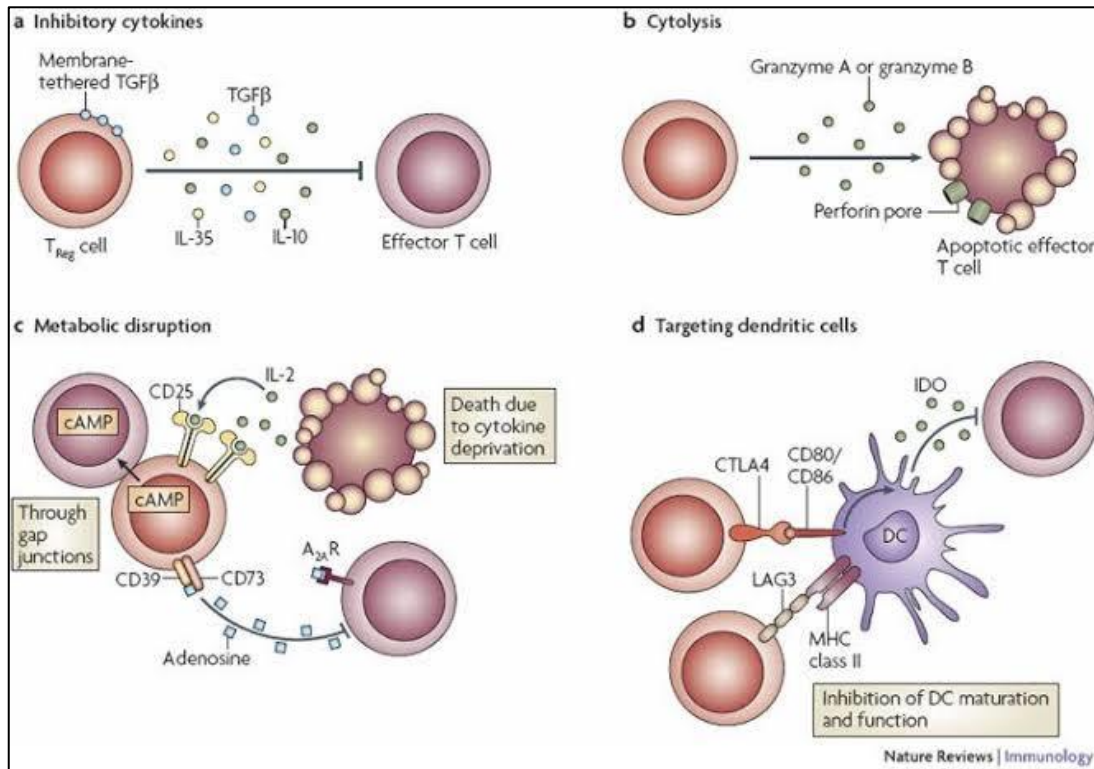


Figure 1.4.2.1. Mechanism of Immune Suppression by Regulatory T Cells

Regulatory T-Cells in Autoimmune Diseases

In autoimmune diseases it has been determined that Treg population was decreased or that its repressive functions were inadequate (Viglietta, V., et al., 2004). Treg cells have been reported to be functionally impaired in multiple sclerosis, rheumatoid arthritis (Viglietta, V., et al., 2004) and systemic lupus erythematosus patients (Alvarado-Sanchez, B., et al., 2006). On the other hand, Treg cells were observed to be reduced in number in patients compared to healthy individuals in type 1 diabetes (Kukreja, A., et al., 2002), autoimmune lymphoproliferative syndrome (Bleesing, J.J., et al., 2001) and Vogt-Koyanagi-Harada disease (Chen, L., et al., 2008). In another systemic inflammatory disease, sarcoidosis, peripheral Treg cell levels have been reported to be increased (Valencia, X. and P.E. Lipsky, 2007; Miyara, M., et al., 2006).

Regulatory T-Cells in Behçet's Disease

Changes in CD4 + CD25 + Treg cell levels were also observed in studies in Behçet's disease. Hamzaoui et al. reported that CD4 + CD25 + Treg levels in patients with active systemic Behçet's findings such as active uveitis, oral ulcers, skin lesions and arthritis were higher than those in BD patients in remission and healthy controls (Hamzaoui, K., A. Hamzaoui, and H. Houman, 2006). In another study involving active uveitis, orogenital ulcer and arthritic Behçet patients and the control group, Treg cells were found to be higher in the active Behçet's disease group than in the control group and CTLA-4 level, which was shown as a Treg cell marker, was found to be high in patients with Behçet's disease (Demir, N., et al., 2012). In both studies, high levels of Treg cells in the active phase of Behçet's disease support the idea that the function of this cell group may be impaired.

Regulatory T-Cells in Behçet Uveitis

In studies conducted with Behçet uveitis patients, peripheral blood Treg cells were found to be low in active uveitis phase (Sugita, S., et al., 2011; Nanke, Y., et al., 2008). Sugita et al. found that CD4 + T cells expressing FoxP3 were found to be lower in healthy individuals and patients with Behçet's disease, Vogt-Koyanagi-Harada disease and active uveitis due to ocular toxoplasmosis (<5%). Foxp3 expression in patients with active Behçet uveitis was also lower than in patients with inactive Behçet uveitis. The same study showed that FoxP3 + Treg cell levels were similar in healthy controls and Behçet's patients treated with colchicine and cyclosporin, whereas FoxP3 + Treg ratio was increased in the group treated with infliximab, an anti-tumor necrosis factor alpha (Sugita, S., et al., 2011).

1.4.2.2. T Helper 17 (TH17) Cells

T Helper 17 Cells (TH17) are a subset of CD4⁺ Helper T Cells amongst TH1 and TH2 subsets and determined by secretion of cytokines IL-17A, IL-17F, IL-21, IL-22, TNF- α and IFN- γ and transcription factor Ror- γ T. IL-17A and IL-17F induce epithelial cells, endothelial cells, fibroblasts and macrophages to synthesize CXCL8 which is the primary cytokine for neutrophil recruitment to inflammation site, while IL-21 stimulates B Cells, CD8⁺ T Cells and natural killer cells which is shown in **Figure 1.4.2.2** (Annunziato, F., et al., 2012). TH17 cells are polarized from Naive CD4⁺ T cells in the presence of IL-6, IL-4, IFN- γ , IL-1 β and IL-23 and stimulation of T Cell Receptor (CD3) and co-stimulation with CD28 Ligand via antigen presenting cells. They are characterized *in vitro* as CD3⁺CD4⁺CD25⁻IL17A⁺ cells via flow cytometry and as Ror- γ T⁺ cells by PCR. Although TH17 cells are responsible for the initiation of inflammation in tissues in bacterial and viral infections, in recent years TH17 cells are associated with autoimmune diseases as well (Bedoya, S.K., et al., 2013).

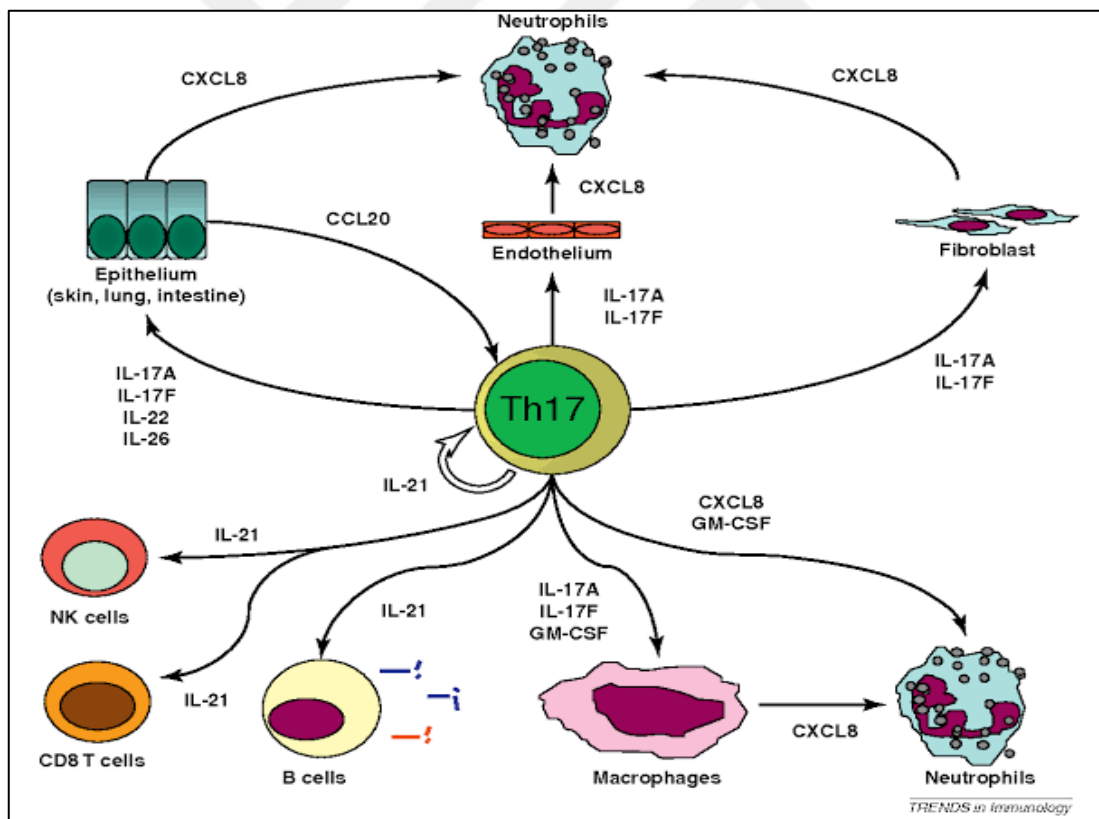


Figure 1.4.2.2. T Helper 17 Cells

TH17 Cells in Autoimmune Diseases

The main proinflammatory T cell population that shapes autoimmune diseases is considered to be the Th17 cell group (Afzali, B., et al., 2007). In the pathogenesis of Behçet's disease, Th1 and Th2 cells and recently Th17 cells have been shown to play a major role (Hamzaoui, K., et al., 2011). Individual susceptibility is modulated by genetic variations of the genes encoding the cytokines of these cell groups (Hamzaoui, K., et al., 2011; Zhou, W., et al., 2012). T helper 1 and Th17 cells play a role in the active phase disease and Th2 cells appear to have the characteristic of determining the development and severity of the disease (Sojka, D.K. and D.J. Fowell, Regulatory, 2011). It has been shown that T helper 17 response is controlled by Treg cells in rheumatoid arthritis patients receiving infliximab therapy (McGovern, J.L., et al., 2012) and anti-TNF alpha therapy induces Treg cells in rheumatoid arthritis (Nadkarni, S., C. Mauri, and M.R. Ehrenstein, 2007).

TH17 Cells in Behçet Uveitis

In patients with Behçet's uveitis, infliximab inhibits Th17 differentiation in ocular fluid (anterior chamber fluid and vitreous) via CD4 + cells. These studies show that Treg cells modulate the Th17 response and thus reduce inflammatory activity of the disease (Sugita, S., et al., 2012). It is also known that IL-17, the major cytokine of T helper 17 cells, coordinates the relationship between lymphocytes and neutrophils in Behçet's disease (Lightman, S., et al., 2015).

1.4.3. Cytokine response in Autoimmune/inflammatory Diseases

1.4.3.1. Interleukin-2

IL-2 is one of the most important cytokines in T- Cell biology for its multiple functions one of them for example is that IL-2 promotes the differentiation of certain T Lymphocytes that undergone negative selection into regulatory T cells to prevent autoimmunity. The other major function of IL-2 is the autocrine T-Cell proliferation where stimulated T-Cell synthesizes both IL-2 and IL-2 Receptor to proliferate (Malek, T.R., 2003). **Figure 1.4.3.1** shows the role of IL-2 in the homeostasis of TH1, TH2, TH17 cells and Tregs (Boyman, O. and J. Sprent, 2012).

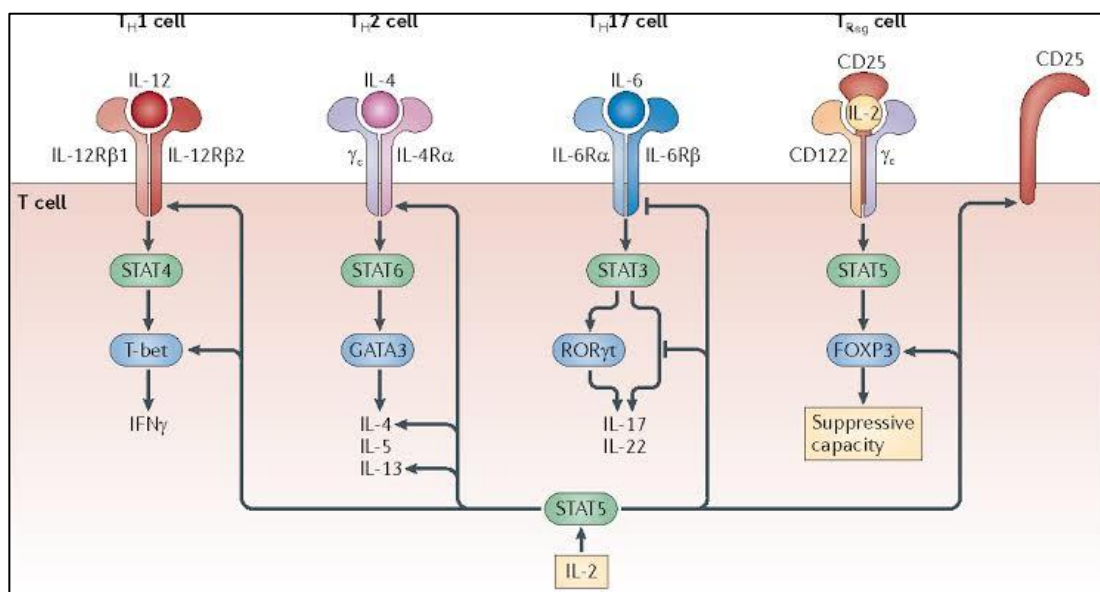


Figure 1.4.3.1. The Role of IL-2 in T Cell Homeostasis

1.4.3.2. Interleukin 10

IL-10 is an anti-inflammatory cytokine that inhibits the inflammatory activity of Helper T-1 cells (Th1), Natural Killer Cells (NK) and Macrophages (Mφ). During infection, activity of inflammatory cells are required for the clearance of infectious agents however, uncontrolled activity of these cells lead to tissue damage. Therefore Regulatory T-Cells secrete IL-10 during infection and inflammation to regulate immune response and prevent tissue damage (Couper, K.N., D.G. Blount, and E.M. Riley, 2008). Diminished IL-10 production from Regulatory T-Cells can lead to autoimmunity (Cho, M.J., C.T. Ellebrecht, and A.S. Payne, 2015). **Figure 1.4.3.2** shows the immunosuppression mechanism of IL-10 on CD4⁺, CD8⁺ T cells, NK cells, macrophages / monocytes, dendritic cells, and B cells (Beebe, A.M., D.J. Cua, and R. de Waal Malefyt, 2002).

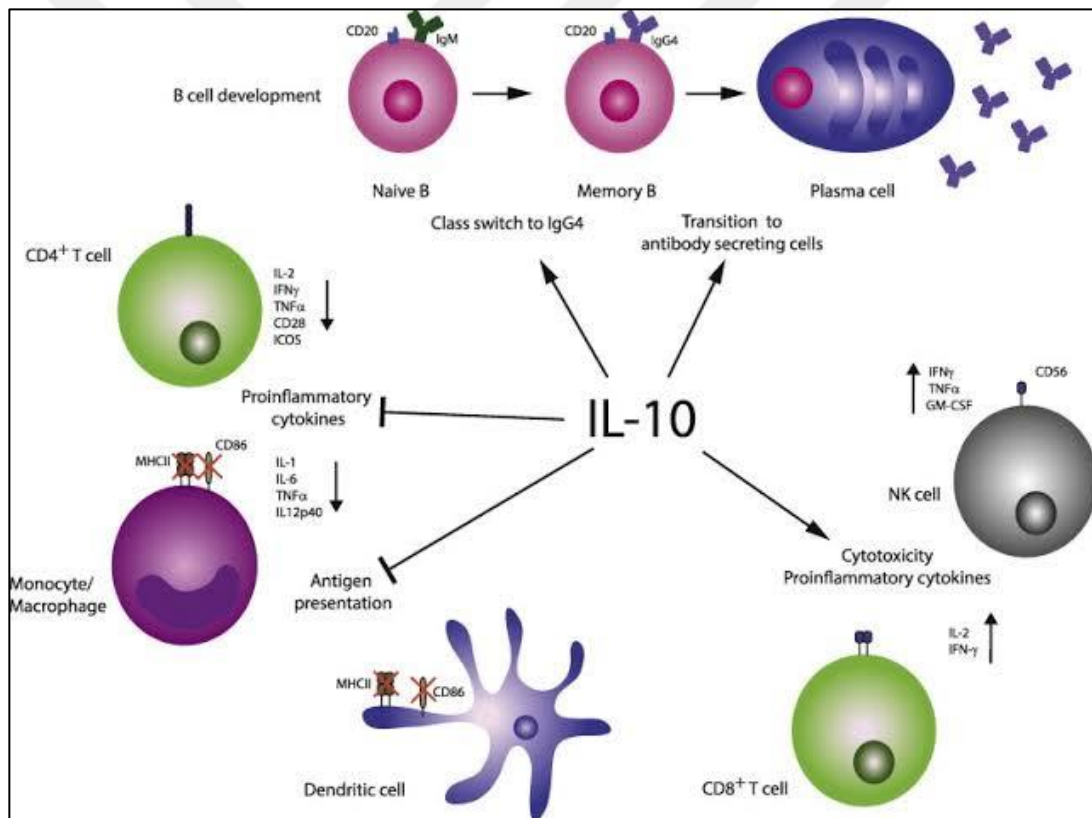


Figure 1.4.3.2. Interleukin-10 Mechanism of Action

1.4.3.3. Interleukin 35

IL-35 is a newly discovered anti-inflammatory cytokine of IL-12 family and is released from Regulatory T-Cells. Its main functions are to regulate the differentiation and function of T-Cells, B-Cells, Macrophages and Dendritic Cells and found to be related with autoimmune diseases such as Diabetes, Multiple Sclerosis and Inflammatory Bowel Disease (Su, L.-C., et al., 2018). As shown in **Figure 1.4.3.3**, IL-35 is responsible for immunosuppressive cell proliferation and suppression of effector T Cells and TH17 cells via p35 pathway (Pylyayeva-Gupta, Y., 2016).

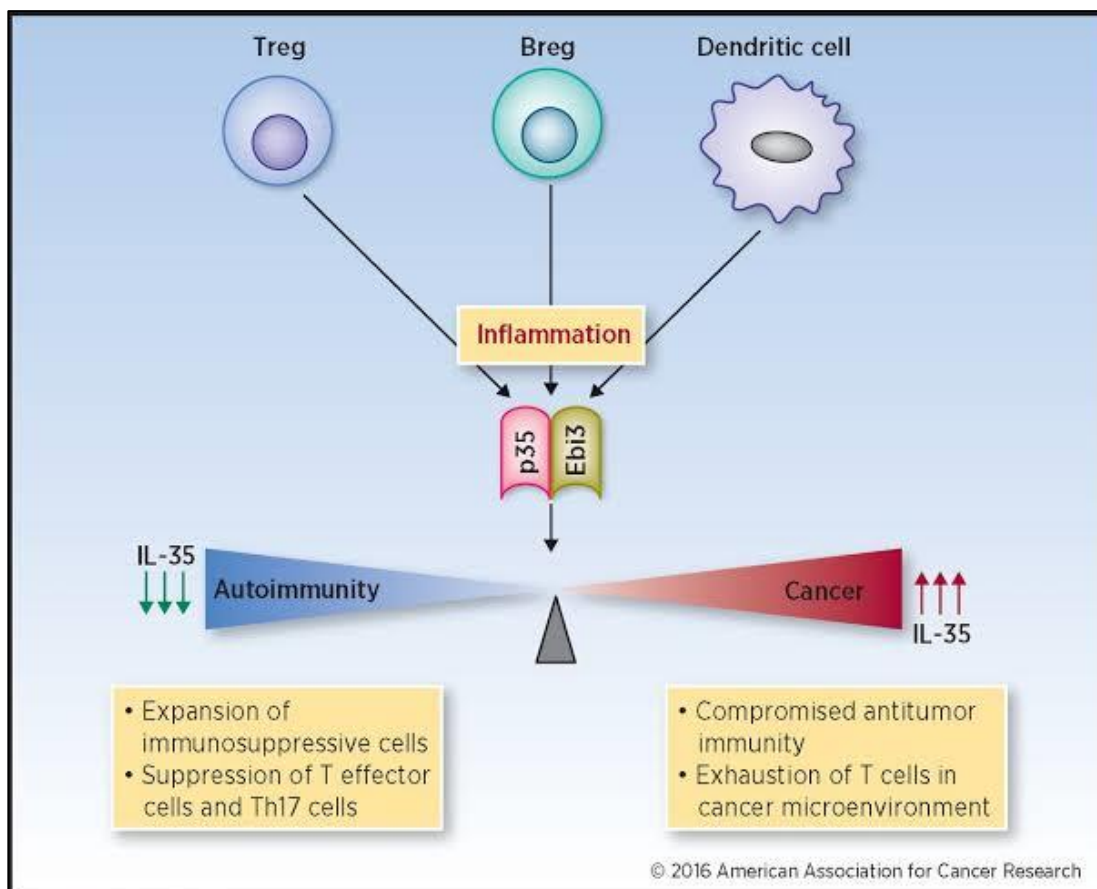


Figure 1.4.3.3. Interleukin 35 in Autoimmunity

1.4.3.4. Interleukin 17-A

IL-17A is a proinflammatory cytokine released from distinct subset of T cell called TH-17 cells. IL-17A is responsible for the clearance of extracellular bacterial infections, fungal infections and cancer. However, there are studies linking IL-17A to autoimmune diseases (Miossec, P., 2017). IL-17A receptor is widely expressed on epithelial and endothelial cells and fibroblasts. In the presence of inflammation, IL-17A regulates the immune function by binding to these cells and inducing proinflammatory cytokine and chemokine secretion which recruit innate immune cells macrophages and neutrophils to the site of inflammation which is shown in **Figure 1.4.3.4** (Qian, Y., et al., 2010).

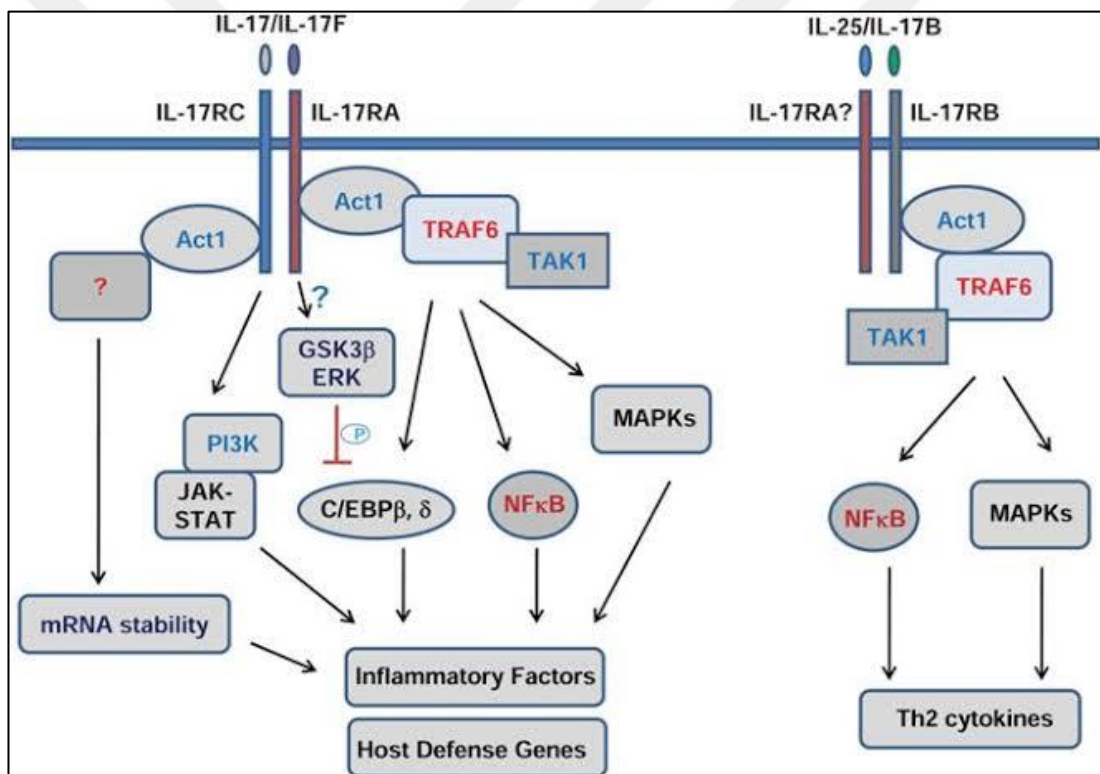


Figure 1.4.3.4.. IL-17A signaling in Host Defense and Inflammation

1.4.3.5. Interleukin 21

IL-21 is a pleiotropic cytokine that effects the differentiation and maturation of cells with lymphoid and myeloid origin. It has a broad effect on regulating both innate and adaptive immune responses and therefore is a key anti-tumor, anti-viral and anti-bacterial cytokine. As shown in **Figure 1.4.3.5**, IL-21 induces apoptosis, IFN- γ signaling and T and B cells differentiation and maturation via STAT1 and STAT3 pathways (Leonard, W. and C. Wan, 2016). However, studies show IL-21 has a promoting effect on autoimmune diseases (Spolski, R. and W.J. Leonard, 2014).

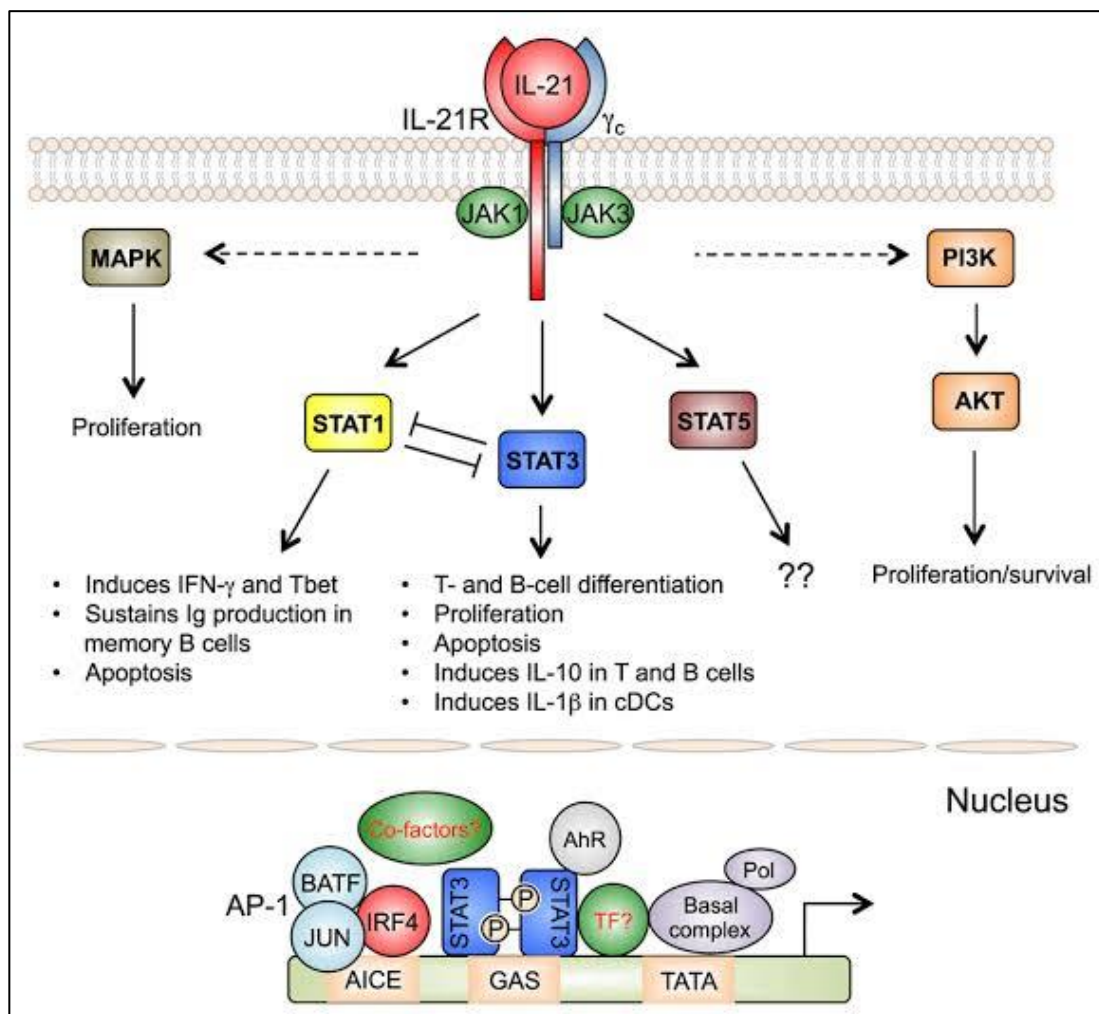


Figure 1.4.3.5. IL-21 Signaling in Immunity

1.4.3.6. Interleukin 22

As a member of IL-10 family of cytokines, IL-22 is a pro-inflammatory cytokine that is expressed by variety of cells like CD4⁺ and CD8⁺ T cells, subsets of NK cells, $\gamma\delta$ T cells and TH-17 cells. As shown in **Figure 1.4.3.6** after binding to its receptor, IL-22 induces the activation of STAT3 via tyrosine kinase receptor-2 (TYK2) and Janus kinase-1 (JAK-1). This leads to antimicrobial immunity, inflammation and tissue repair (Yang, X. and S.G. Zheng, 2014). IL-22 is efficient at its maximum when it is co expressed with IL-17, which makes it a potent cytokine on infections but also autoimmunity (Rutz, S., C. Eidenschenk, and W. Ouyang, 2014).

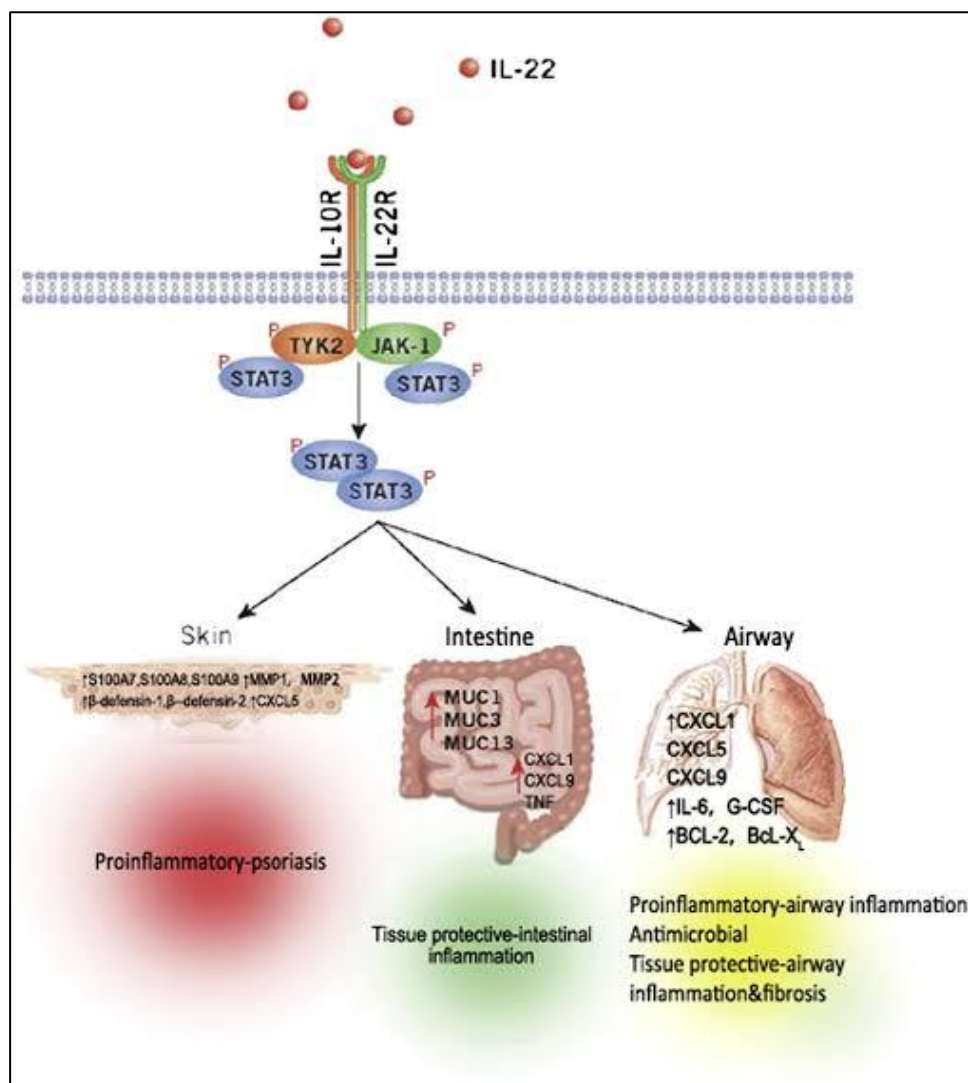


Figure 1.4.3.6. IL-22 Signaling in Autoimmunity

1.4.3.7. Interleukin 23

IL-23 is a proinflammatory cytokine with P19 and P40 subunits which shares common resemblance with IL-12 P40 subunit. As shown in **Figure 1.4.3.7**, IL-23 is produced by inflammatory macrophages and naive $CD4^+$ T cells and one of the cytokines along IL-6 and IL-1b to differentiate naive $CD4^+$ T cells into TH-17 cells, $\gamma\delta$ -T Cells to induced Tregs and osteoblasts to osteoclast precursors (Duvallet, E., et al., 2011). Studies reveal that IL-23 plays a major role in autoimmunity and therefore is a frequent for autoimmune disease therapy (Tang, C., et al., 2012).

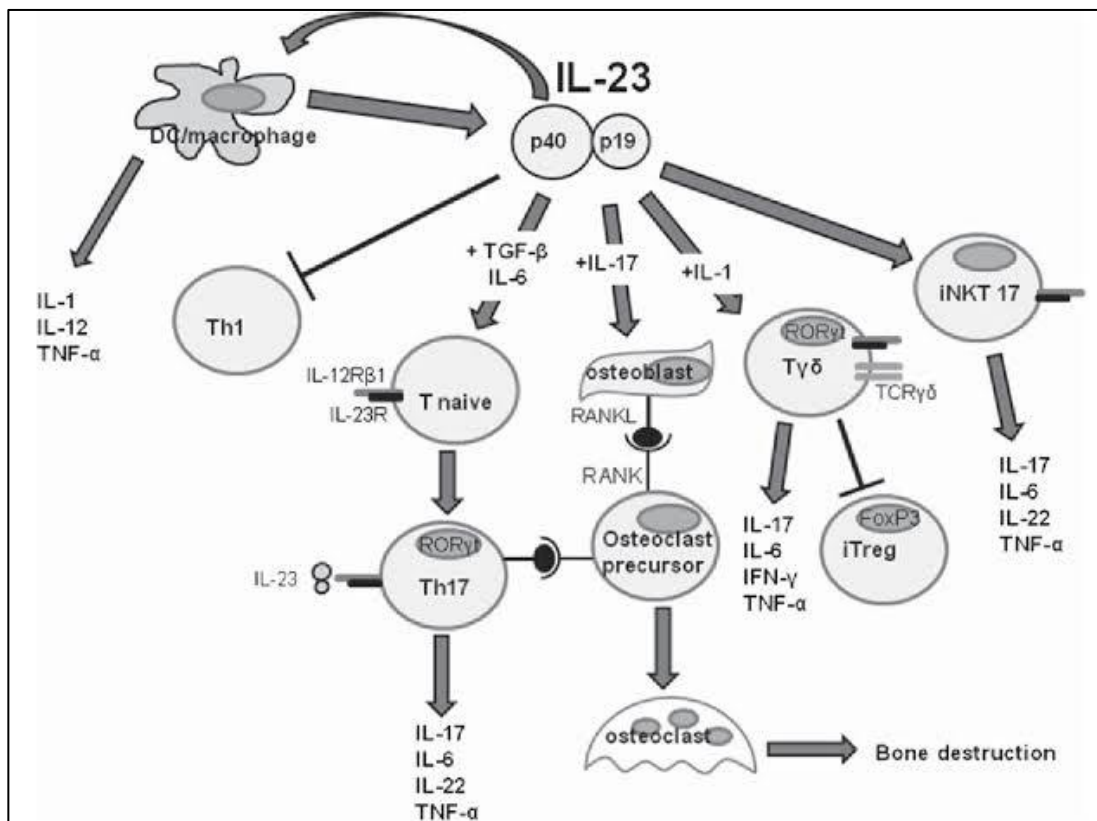


Figure 1.4.3.7. IL-23 Cytokine in Inflammation

1.4.3.8. Interferon Gamma

IFN- γ belongs to the interferon family of cytokines and belongs to Type II Interferons. As shown in **Figure 1.4.3.8**, IFN- γ is a pleiotropic cytokine and is responsible for macrophage and neutrophil activation, upregulation of MHC class I and II molecules, Th-1 polarization, apoptosis, B-Cell maturation among many other processes (Kelchtermans, H., A. Billiau, and P. Matthys, 2008). TH-17 cells express IFN- α to combat viral and fungal infections. However, IFN- γ expressing TH-17 cells also contribute to the promotion of autoimmunity (Chen, Y., et al., 2017).

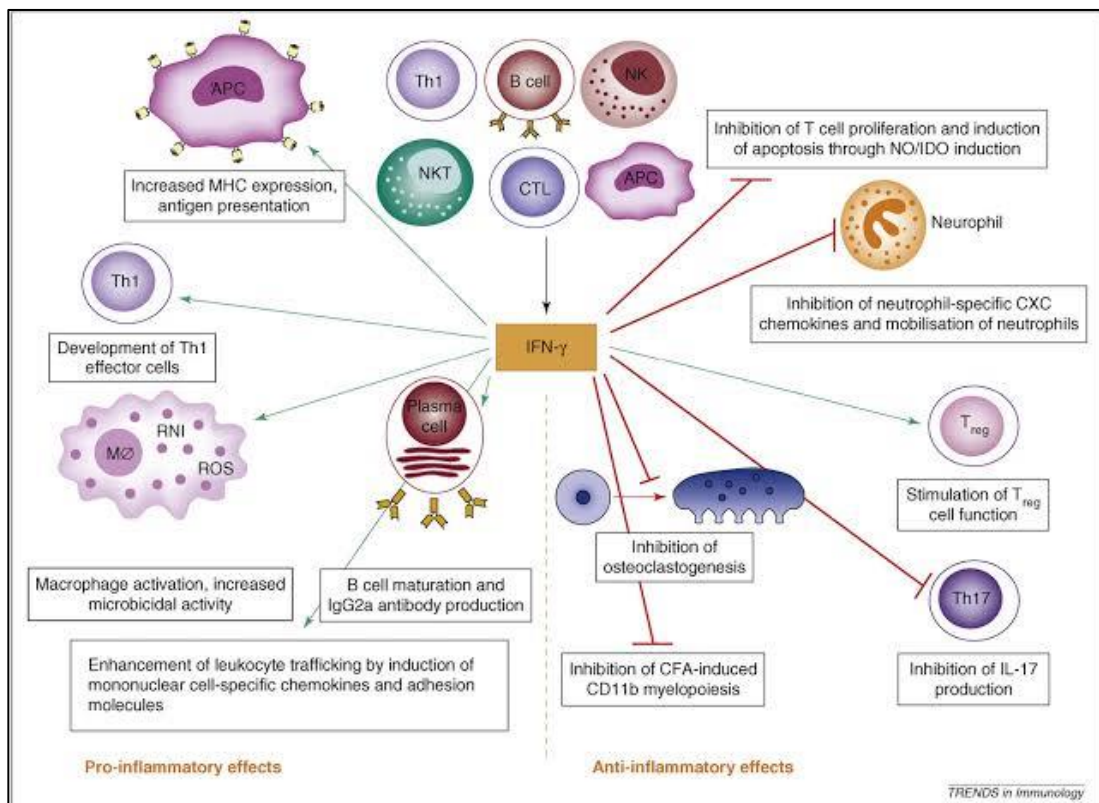


Figure 1.4.3.8. Pro- and Anti- Inflammatory Effects of IFN- γ

1.4.3.9. Tumor Necrosis Factor Alpha

TNF- α is a member of the Tumor Necrosis Factor superfamily and is an potent proinflammatory cytokine that has a role in vasodilation, edema formation and ROS production during inflammation. **Figure 1.4.3.9** shows the multiple effects of TNF- α (Zelová, H. and J. Hošek, 2013).

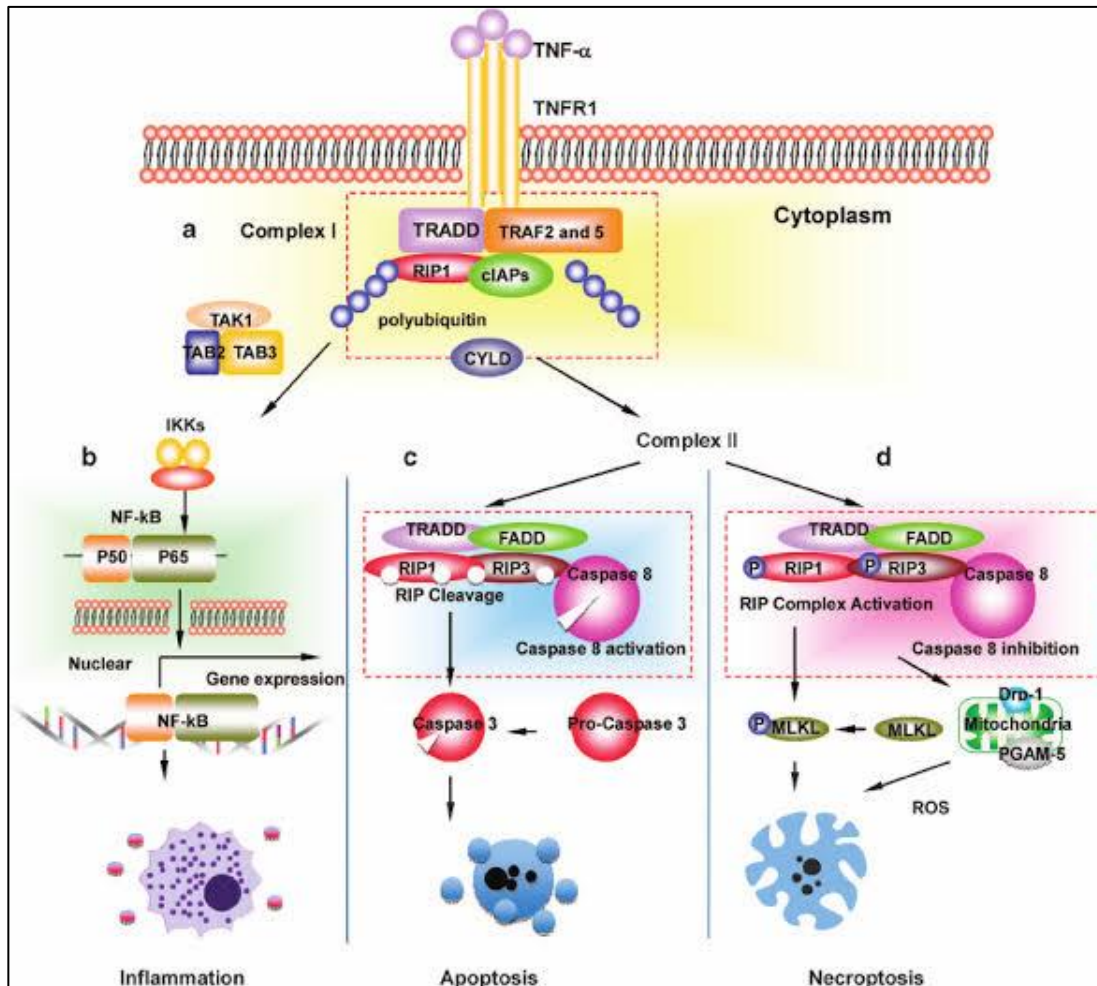


Figure 1.4.3.9. TNF- α Signaling Leads to Inflammation, Apoptosis and Necroptosis

1.5. Aim of The Study

The aim of our study was to

1. To determine the immunopathogenesis of Behcet Uveitis.
2. To demonstrate the effects of IFN- α -2a treatment on adaptive and innate immune responses in Behçet Uveitis Patients.

For this purposes, 19 consecutive Behçet Uveitis patients in the active phase of the disease with resistance to conventional immunomodulatory therapy and 19 age and sex matched healthy controls were included in the study.

1. Immunopathogenesis of Behcet Uveitis (BU)

To determine the immunopathogenesis of BU, we looked into both adaptive and innate immune responses from patients and healthy controls.

1.a. Adaptive Immune Responses

For the adaptive immune responses in BU, we determined the peripheral Treg and TH-17 cell populations in BU patients and healthy controls via flow cytometry and compared the results between two groups. After this, we isolated Tregs and TH17 cells via Magnetic Cell Sorting and stimulated these cells in *in vitro* environment for cytokine release to examine the functionality of Tregs and TH17 cells. We determined; for Treg functions IL-2, IL-10, TGF- β and IL-35 concentrations and for TH17 functions, IL-17A, IL-21, IL-22, IL-23, IFN- γ and TNF- α concentrations from culture supernatants. Also we compared the Ror- γ t gene expression levels between BU patients and healthy controls.

1.b. Innate Immune Responses

For the innate immune responses, we compared the gene expression levels of and Monocytes Toll – Like Receptors 2, 3, 4, 8 and 9 from BU patients and Healthy Controls.

2. The effects of IFN- α -2a treatment on adaptive and innate immune responses in Behçet Uveitis Patients.

For this purpose, we collected blood from same BU patients after IFN- α -2a treatment when the remission of uveitis is achieved and applied the same procedures to the newly acquired samples. Then, we compared the results between BU patients before and after treatment.



2. MATERIALS AND METHODS

2.1. Study population

Nineteen consecutive Behçet disease patients who attended two tertiary referral uveitis clinics and who had an acute Behçet uveitis attack despite conventional immunomodulatory therapy and who were switched to interferon alfa-2a therapy were included from August 1, 2016 to September 30, 2017. Diagnosis of Behçet disease was based on the criteria established by the International Study Group for Behçet's Disease. Patients with a comorbidity such as diabetes mellitus, malignancy, coexistent autoimmune disease, and allergies were not included in the study. Age- and sex-matched healthy subjects were recruited from consecutive patients who were scheduled for routine ocular examination for refractive error correction at two tertiary centers. Laboratory Workflow Chart is given in **Figure 2.1**.

This prospective cross-sectional study was approved by the institutional review board of Koç University (approval number: 2015.017.IRB1.003). All patients and control subjects were Turkish descent. The study was conducted according to the tenets of the Declaration of Helsinki and an informed consent was obtained from the patients and control subjects.

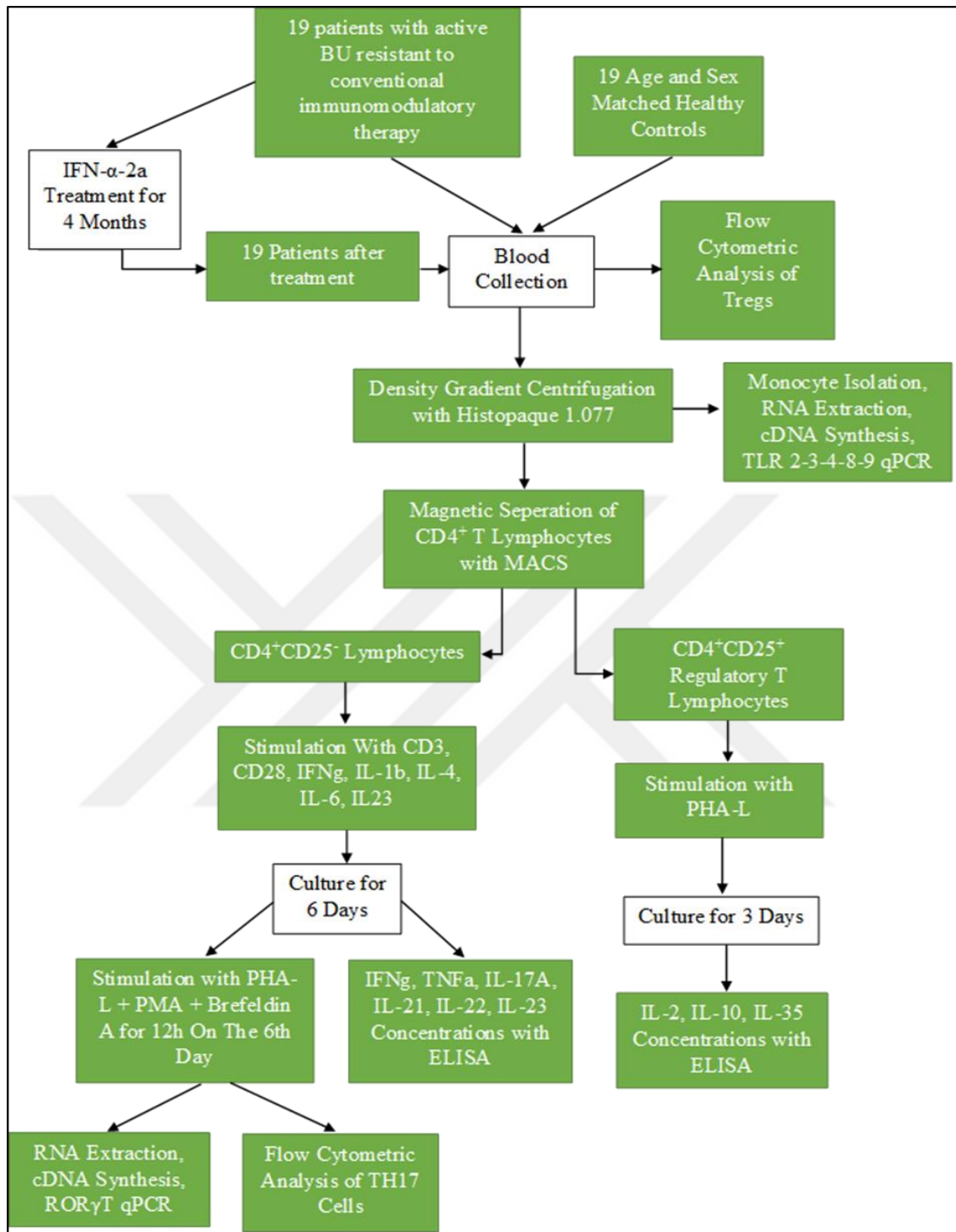


Figure 2.1. Laboratory workflow chart

2.2. Isolation and activation of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells

2.2.1 Determination of regulatory T-Cells by Flow Cytometry

Regulatory T-Cells were determined as CD4⁺CD25⁺FoxP3⁺ cells by flow cytometry using human FoxP3 Buffer Set (BD Biosciences, USA). 100ul of whole blood was stained with 20ul Mouse-Anti Human CD4-FITC, Mouse-Anti Human 5ul CD3-PCY7 and 25ul Mouse-Anti Human CD25-APC antibodies (BD Biosciences, USA) and incubated for 15 minutes at room temperature in dark. After incubation, erythrocytes were lysed with 2ml 1X PharmLyse Erythrocyte Lysing Solution (BD Pharmingen, USA) for 10 minutes and centrifuged at 2800 rpm for 5 minutes. Cells were washed twice with Staining Buffer (PBS + 0.5% BSA) at 2800 rpm for 5 minutes. Supernatants were discarded and cells were fixed with Buffer A for 10 minutes at room temperature in dark and permeabilized with Buffer C (1x Buffer A + 50x Buffer B) for 30 minutes at room temperature in dark. One auto fluorescence tube was used for every sample to discriminate positive staining. 20ul mouse Anti-Human Foxp3-PE antibody (BD Biosciences, USA) was added to the staining tube and samples were incubated for 30 minutes at room temperature in dark. Cells were washed with Staining Buffer and pellet was resuspended with 500ul of staining buffer for analysis. Cells were run under BD Accuri C6 Flow Cytometer (BD Biosciences, USA) and analyzed with FlowJo Software (TreeStar, USA). 50000 cells were counted in Lymphocyte gate and T Lymphocytes were gated in CD3⁺ population. From CD3⁺ gate, Tregs were determined as CD4⁺FoxP3⁺ cells. Gating strategy for Regulatory T-Cell detection is given in **Figure 2.2.1**.

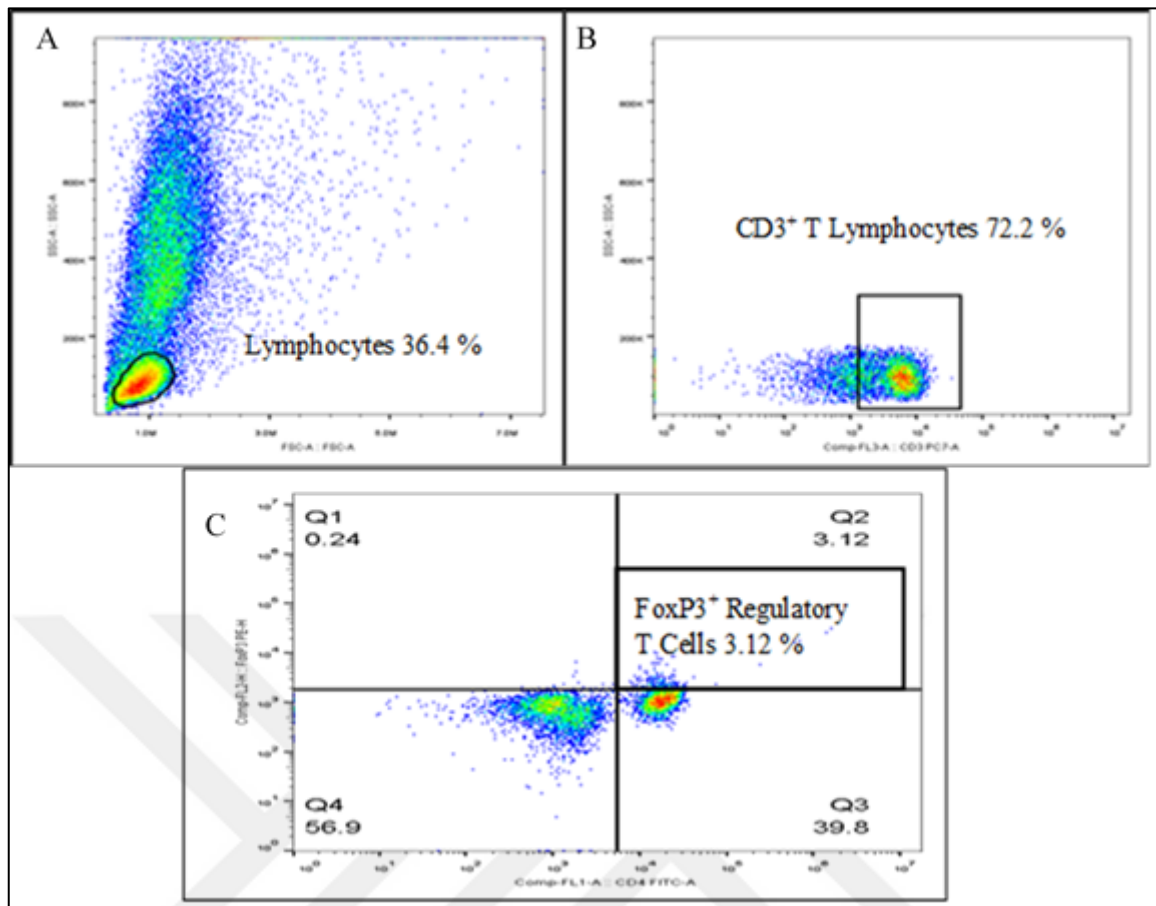


Figure 2.2.1. Flow Cytometric determination of FoxP3⁺ Cells from peripheral blood. Total lymphocytes were gated (A) and CD3⁺ cells were selected (B). Treg Cells were determined as CD3⁺CD4⁺FoxP3⁺ cells (C).

2.2.2. Magnetic Separation of Tregs and CD4+CD25- T-Cells

MACS Human CD4+CD25+ Regulatory T Cell isolation kit (Miltenyi Biotech GMBH, Germany) was used to isolate Tregs from peripheral blood of BD Uveitis patients, healthy controls and after treatment group. PBMCs were counted and 1×10^9 cells were resuspended with 90ul Magnetic Buffer (PBS + 0.5% BSA + 2mM EDTA). 10ul of Biotinylated Mouse Anti-Human CD4 Antibody cocktail containing all leukocyte markers except for CD4 was added onto the samples and vortexed and incubated for 5 minutes at 4°C. 20ul of Anti-Biotin Microbeads were added onto the samples and vortexed and incubated for 10 minutes at 4°C. After incubation, cells were passed through LD magnetic columns attached to MACS magnetic stand with midi separator (Miltenyi Biotech, Germany) and unlabeled CD4+ cells were collected in a separate tube and centrifuged at 300 xg for 10 minutes. Supernatant was aspirated and cells were resuspended with 90ul Magnetic Buffer and 10ul of anti-CD25 microbeads were added onto the cells and vortexed and incubated for 15 minutes at 4°C. 500 µl of Magnetic solution was added onto the cells and cells were centrifuged at 300 x g for 10 minutes. Supernatants were aspirated and pellet was resuspended with 500ul Magnetic solution. Cells were passed through MS column attached to MACS magnetic stand with mini separator. CD4+CD25- cells were collected in the effluent. MS column was separated from the magnet and magnetized CD4+CD25+ Tregs were flushed into a separate sterile tube. CD4+CD25- T cells and CD4+CD25+ Tregs were centrifuged at 500 x g for 5 minutes. Supernatants were discarded and cells were prepared for *in vitro* stimulation.

2.2.3. *In vitro* Stimulation of Tregs and CD4+CD25- Cells

Both Tregs and CD4+CD25- cells were centrifuged at 300xg for 5 minutes. Supernatants were discarded. For the stimulation of Tregs, 10ug/ml Phytohemagglutinin-L (Roche, USA) was prepared in 3ml of RPMI medium containing 10% FBS and 1% Penicillin+Streptomycin (Gibco, USA). Treg pellets were re-suspended with this medium and seeded into 6-well plates for 72 hours in 37⁰C 5% CO₂ incubator. After 72 hours supernatants were collected to be used in ELISA Cytokine assays. 3ml of RPMI medium with 10% FBS and 1% penicillin-streptomycin (GIBCO, USA) containing 2ug/ml anti-CD3, 2ug/ml anti-CD28, 5ug/ml anti-IFN γ , 1ug/ml anti-IL4, 5ug/ml anti-IL1b, 1ug/ml anti-IL6 and 10ug/ml anti-IL23 cytokines (BD Biosciences, USA) were added onto CD4+CD25- cells and cells were seeded into 6-well plates for 6 days in 37⁰C 5% CO₂ incubator. Fresh medium containing 10ug/ml IL-23 were added onto the cells in 2nd and 5th days. On the 6th day, supernatants were collected for ELISA studies and cells were suspended with 3ml RPMI medium containing 20ng/ml PMA (Sigma-Aldrich, Germany), 5ug/ml PHA-L and 3ul Brefeldin-A Protein Transport Inhibitor (BD Biosciences) to stimulate IL-17A synthesis and incubated for 12 hours in 37⁰C 5% CO₂ incubator. After incubation, cells were collected for IL-17A staining and RNA isolation for Ror γ T gene expression studies.

2.3. Determination of TH17 activity

2.3.1. IL-17A Staining with Flow Cytometry

IL-17A studies were done with Cytoperm/Cytofix Kit (BD Biosciences) according to manufacturer's instructions. Briefly CD4⁺CD25⁻ cells were stained with surface antibodies anti-CD3PECY5 and anti-CD4FITC. After staining cells were washed twice with staining solution incubated with 250ul of Cytofix/Cytoperm reagent for 20 minutes at 4⁰C for fixation/permeabilization. Without removing the fixative, cells were washed twice with 2ml 1X PermWash solution at 500 xg for 5 minutes. Supernatant was removed and Anti-Human IL-17A PE antibody (BD Biosciences) was added only to the staining tube and cells were incubated for 30 minutes at 4⁰C. After incubation, cells were washed twice with Staining Buffer and pellet was resuspended with 500ul Staining Buffer and cells were run under BD Accuri C6 Flow Cytometry and analyzed with FlowJo software V.10). 50000 cells were counted in Lymphocyte gate and T Lymphocytes were gated in CD3⁺ population. From CD3⁺ gate, TH17 cells and IL-17A were determined as CD4⁺IL17A⁺ cells. Gating strategy for determining TH-17 subpopulation was given in **Figure 2.3**.

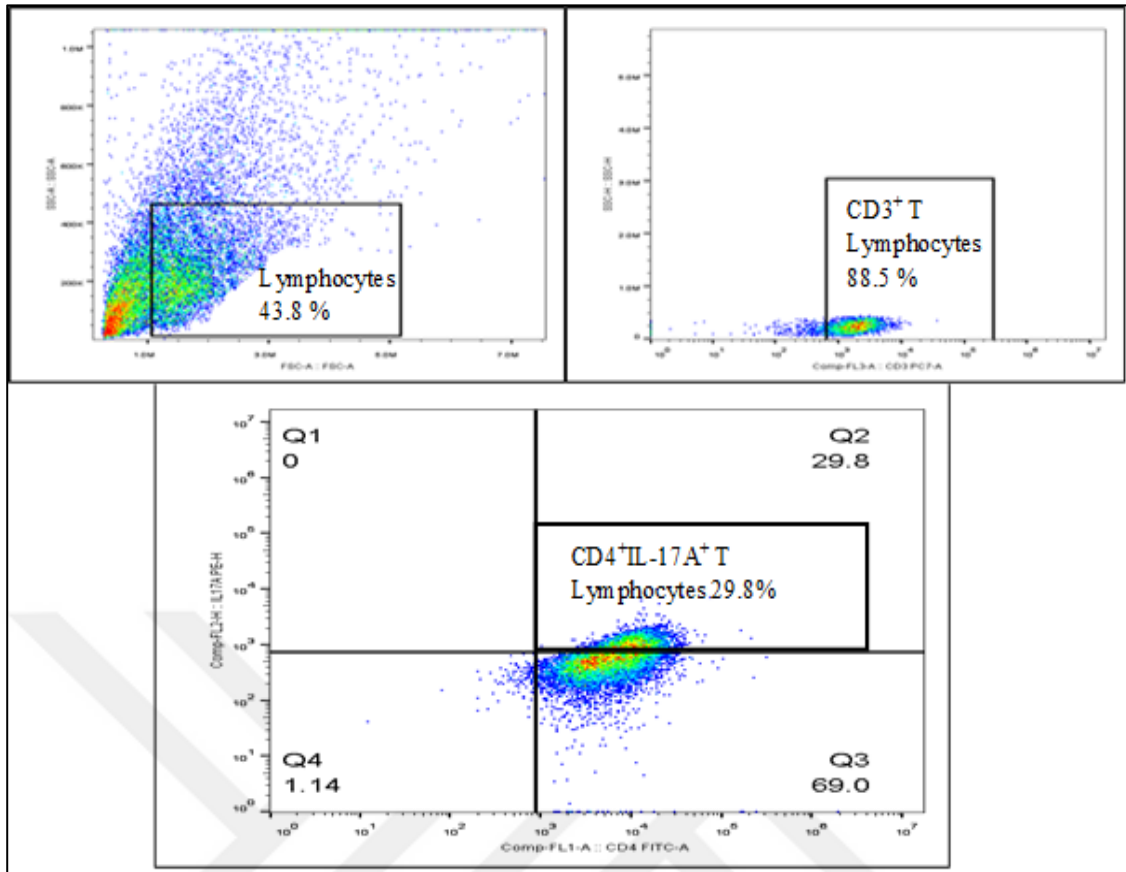


Figure 2.3. IL-17A Staining of Stimulated CD4⁺CD25⁻ Lymphocytes

2.3.2. Cytokine Production of TH17 Cells

IL-17A, IL-21, IL-22, IL-23, TNF α and IFN γ (Thermo Scientific, USA) concentrations of TH17 Cell supernatants were determined by ELISA. Samples and standards were studied as duplicates. Standards were serially diluted according to the concentrations based on the kit's detection limits. Microplates were read with Multiskan Go Microplate Reader (Thermo Scientific, USA). A standard curve was drawn between Absorbance and Concentrations. Concentrations of samples were calculated according to the standard curve.

2.3.3. Expression of ROR- γ T by qPCR

RNAs were isolated from TH17 Cells of patients before and after treatment and healthy controls.

RNAs were isolated by using NucleoSpin RNA Isolation Kit (Macherey-Nagel, Germany) and cDNAs were synthesized by using M-MLV Reverse Transcription Kit (Thermo Scientific, USA) according to manufacturer's instructions.

Gene expression experiments were done by using LightCycler 480 Probes Master Mix (Roche Diagnostics, Germany) according to manufacturer's instructions. Primer and Probes sets were acquired from Roche Diagnostics as TaqMan Real-Time Ready Primer Probe Sets specific to ROR- γ T genes. PCR conditions were 95 $^{\circ}$ C 5 minutes as initial denaturation; 95 $^{\circ}$ C 10 seconds, 60 $^{\circ}$ C 30 seconds and 72 $^{\circ}$ C 1 seconds for 45 cycles; 40 $^{\circ}$ C 10 seconds for cooling. B-2 Microglobulin Real-Time Ready assay (Roche Diagnostics, Germany) was used as endogenous control. QPCR were run with LightCycler 480 Instrument (Roche Diagnostics, Germany). Gene expression was calculated by using 2^{ddCP} comparative CP method.

2.4. Determination of Treg activity

IL-2, IL-10 (Thermo Scientific, USA) and IL-35 (Sunred Biolabs, China) Cytokine concentrations from Treg Cell supernatants were determined by ELISA. Samples and standards were studied as duplicates. Standards were serially diluted according to the concentrations based on the kit's detection limits. Microplates were read with Multiskan Go Microplate Reader (Thermo Scientific, USA). A standard curve was drawn between Absorbance and Concentrations. Concentrations of samples were calculated according to the standard curve.

2.5. Determination of Mononuclear Cell Response

RNAs were isolated from monocytes of patients before and after treatment and healthy controls.

5ml of remaining PBMCs from the first isolation were seeded into a 6 well plate and incubated for 2 hours at 37°C 5% incubator to induce monocyte adherence to the plate. RNAs were isolated by using NucleoSpin RNA Isolation Kit (Macherey-Nagel, Germany) and cDNAs were synthesized by using M-MLV Reverse Transcription Kit (Thermo Scientific, USA) as described in the previous section.

2.5.1. Expression of TLR 2-3-4-8-9 and ROR- γ T Genes

Gene expression experiments were done by using LightCycler 480 Probes Master Mix (Roche Diagnostics, Germany) according to manufacturer's instructions. Primer and Probes sets were acquired from Roche Diagnostics as TaqMan Real-Time Ready Primer Probe Sets specific to TLR 2-3-4-8-9 and ROR- γ T genes. PCR conditions were 95°C 5 minutes as initial denaturation; 95°C 10 seconds, 60°C 30 seconds and 72°C 1 seconds for 45 cycles; 40°C 10 seconds for cooling. B-2 Microglobulin Real-Time Ready assay (Roche Diagnostics, Germany) was used as endogenous control. QPCR were run with LightCycler 480 Instrument (Roche Diagnostics, Germany). Gene expression was calculated by using 2^{ddCP} comparative CP method.

2.6. Statistical Analysis

Non-parametric Independent Samples Wilcoxon Test (Mann-Whitney U) and Paired Sample Wilcoxon Tests were used to calculate statistics because of the data not normally distributed. Results <0.05 were considered statistically significant. Statistical Analysis were done by using R Statistical Analysis Software v3.5.1.

3. RESULTS

3.1. Peripheral Blood CD4⁺FoxP3⁺ Regulatory T Cell Population before and after treatment

BD Uveitis patients at the ocular attack period had significantly increased peripheral blood CD4⁺FoxP3⁺ regulatory T-cells ($6,74 \% \pm 0,26 \%$, $n=19$) when compared to healthy control ($2,45 \% \pm 0,07 \%$, $n=19$) subjects ($p < 0,0001$), however; MFI of FoxP3 expression was significantly lower in BD Uveitis patients ($2008,84 \pm 181,85$, $n=19$) than healthy controls ($2733,68 \pm 246,50$ $n=19$) subjects ($p < 0,0001$).

After IFN- α -2a treatment, we have found that CD4⁺FoxP3⁺ regulatory T-cell population ($2,93 \% \pm 0,19 \%$, $n=19$) were decreased and FoxP3 MFI ($3387,31 \pm 562,57$, $n=19$) were increased significantly ($p < 0,01$).

Figure 3.1A shows the change of regulatory T-Cell population in the patient before and after IFN- α -2a treatment and corresponding healthy control subject. Also statistical graphics are given in **Figure 3.1B**.

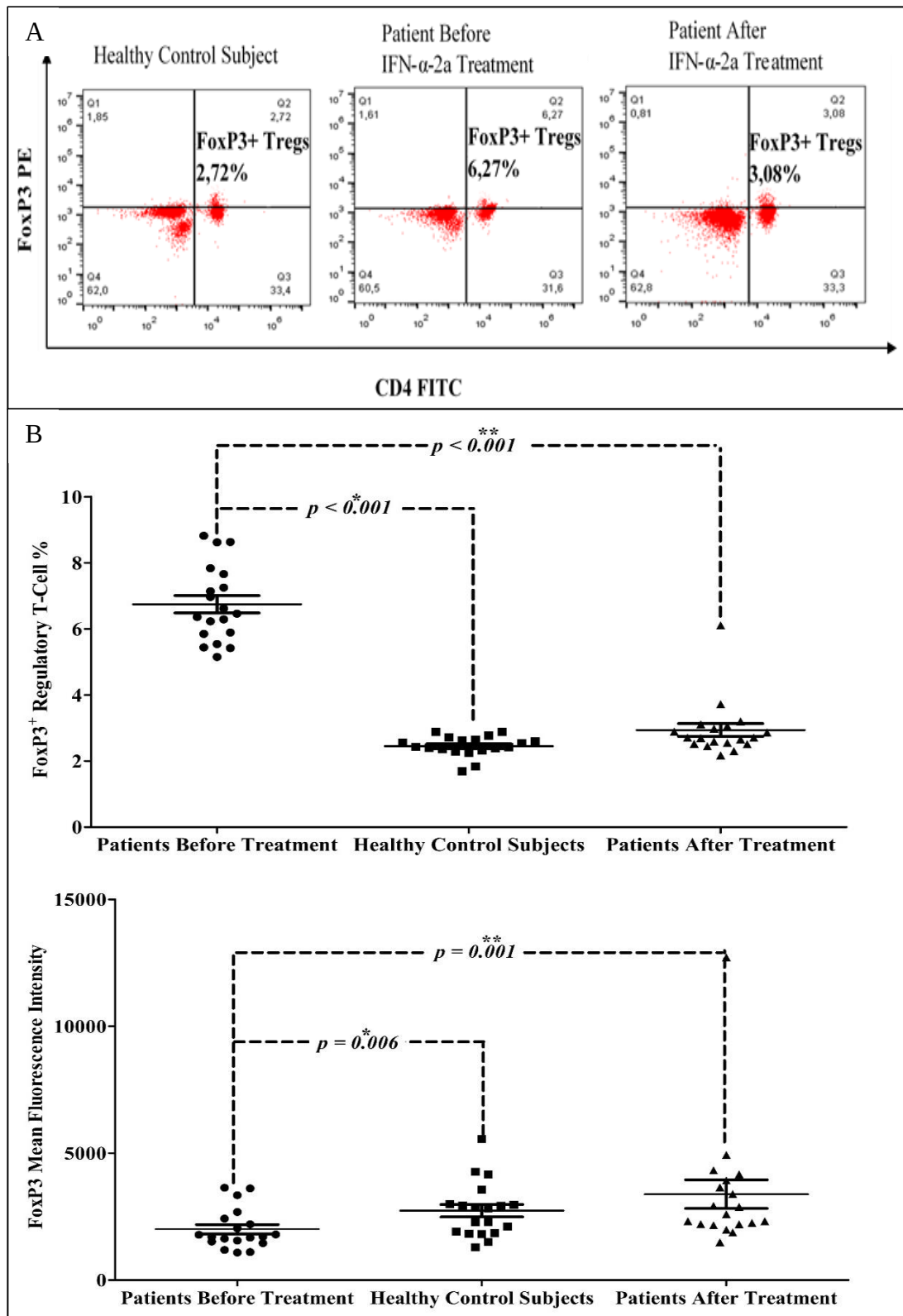


Figure 3.1. A. FoxP3 Expression in Regulatory T-Cells of BD Uveitis Patients before and after treatment and healthy controls. **B.** Comparison of percentage and MFI of Treg FoxP3 expression

3.2. *In vitro* Polarization of CD4⁺CD25⁻ T Cells to TH-17 Cells Were Increased in BD Uveitis Patients

IL-17A producing TH-17 cell population significantly increased in BD Uveitis Patients (33,45 % $\pm$ 0,95 %, n=19), than healthy control (13,74 %, $\pm$ 0,72 %, n=19) subjects ($p < 0,00001$). We also observed that mean fluorescence intensity of IL-17A was significantly higher in BD Uveitis Patients (3297,42 $\pm$ 254,76, n=19) when compared to healthy control (2418,94 $\pm$ 185,51, n=19) subjects ($p < 0,005$). After IFN- α -2a treatment, both IL-17A expression (13,27 % $\pm$ 1,38, n=19) and mean fluorescence intensity (1710,63 $\pm$ 93,33, n=19) was declined to healthy control levels ($p < 0,001$). **Figure 3.2A** shows the change of TH-17 population in the patient before and after IFN- α -2a treatment and corresponding healthy control subject. Also, statistical graphics are given in **Figure 3.2B**.

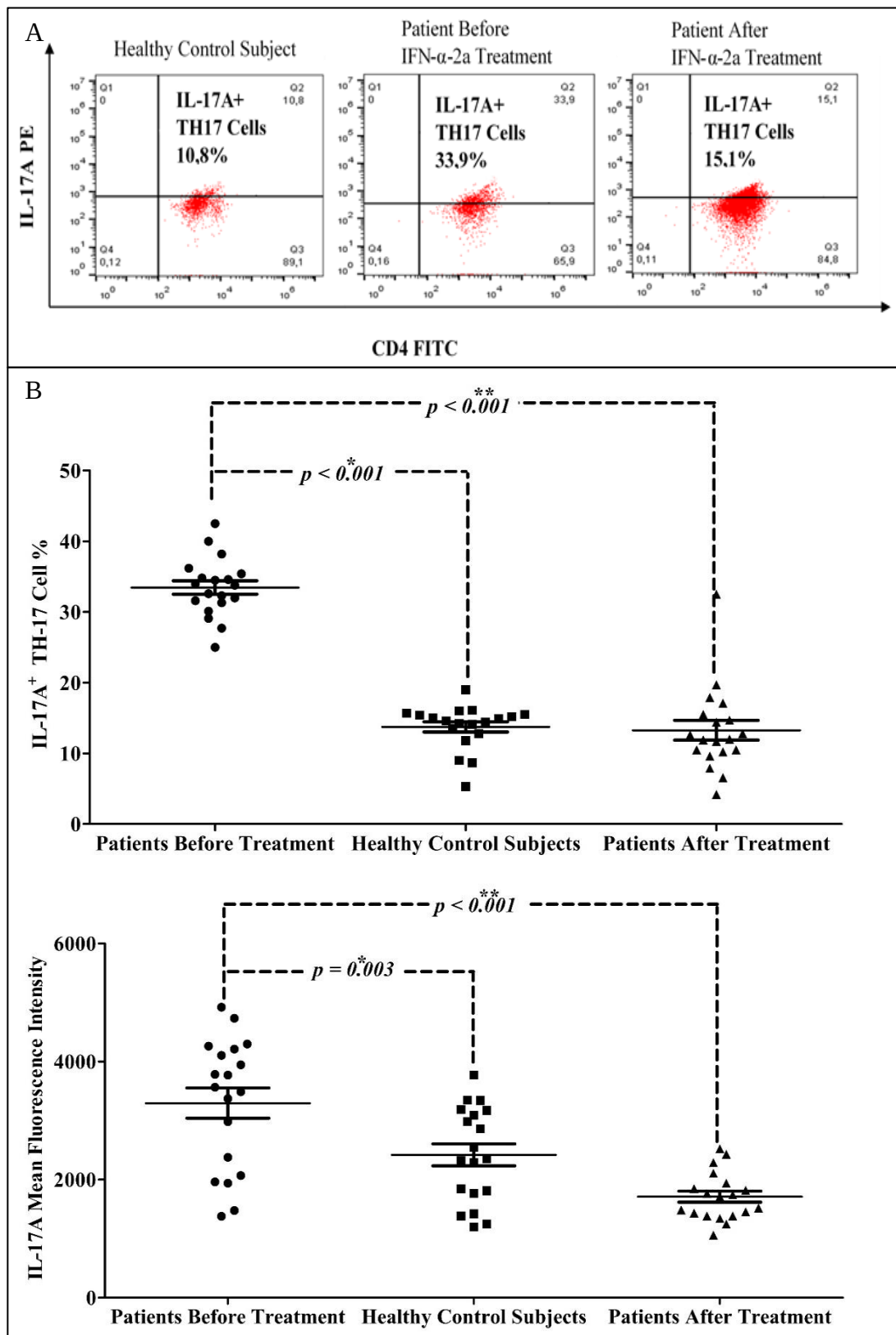


Figure 3.2. A. IL-17A Expression in Polarized CD4⁺CD25⁺ T-Cells of BD Uveitis Patients before and after treatment and healthy controls. B. Statistical analysis results of TH-17 IL-17A expression.

3.3. Results of Cytokine Secretion of Stimulated TH-17 Cells Differentiated From CD4⁺CD25⁻ T Cells

3.3.1. A Significant Increase In IL-17A, IL-21, IL22, IL23, IFN- γ and TNF- α Levels Was Observed In BD Uveitis Patients Compared To Healthy Controls But Decreased To Normal Levels After IFN- α -2a Treatment

3.3.1.1. IL-17A Levels

IL-17A levels released from TH-17 cells of BD uveitis patients ($40,22 \pm 2,95$ pg/ml n=19) were significantly higher than healthy control subjects ($12,45 \pm 1,93$ pg/ml, n=19) ($p < 0,001$). After IFN- α -2a treatment, IL-17A levels were reduced to healthy control levels ($9,09 \pm 2,08$ pg/ml, n=19) ($p < 0,001$). Statistical graphic is given in **Figure 3.3.1.1.**

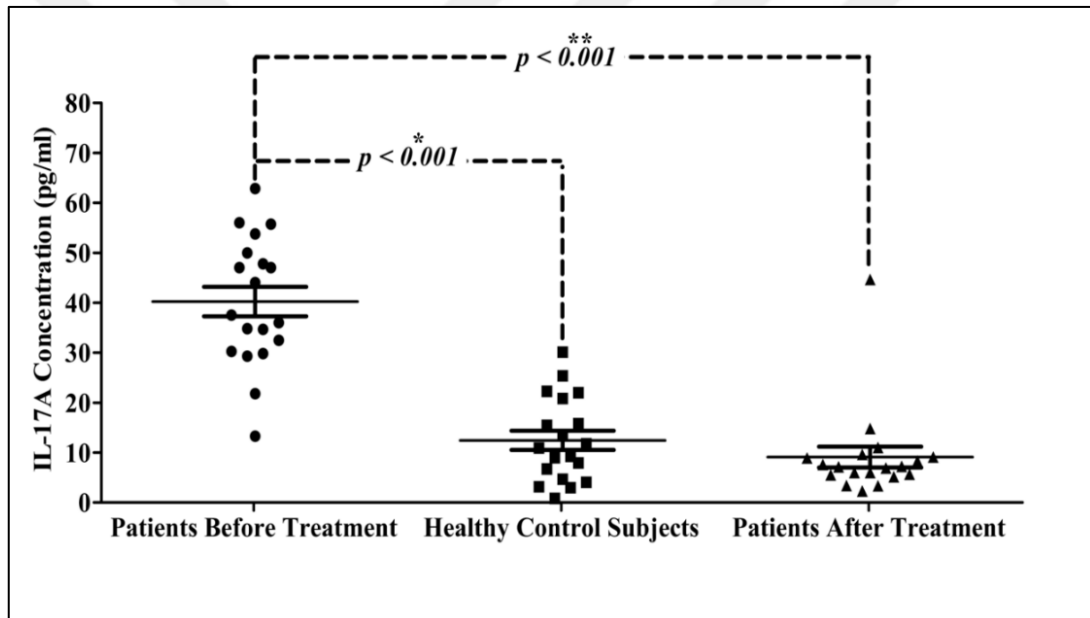


Figure 3.3.1.1. Statistical Analysis of IL-17A concentration from culture supernatants of TH-17 Cells.

3.3.1.2. IL-21 Levels

Significantly elevated levels of IL-21 were observed in BD Uveitis patients ($173,76 \pm 28,87$ pg/ml, $n=19$) compared to healthy control subjects ($42,43 \pm 8,06$ pg/ml, $n=19$) ($p < 0,001$) After IFN- α -2a treatment, IL-21 levels returned to normal levels ($38,64 \pm 7,46$ pg/ml, $n=19$) ($p < 0,001$). Statistical graphic is given in **Figure 3.3.1.2**.

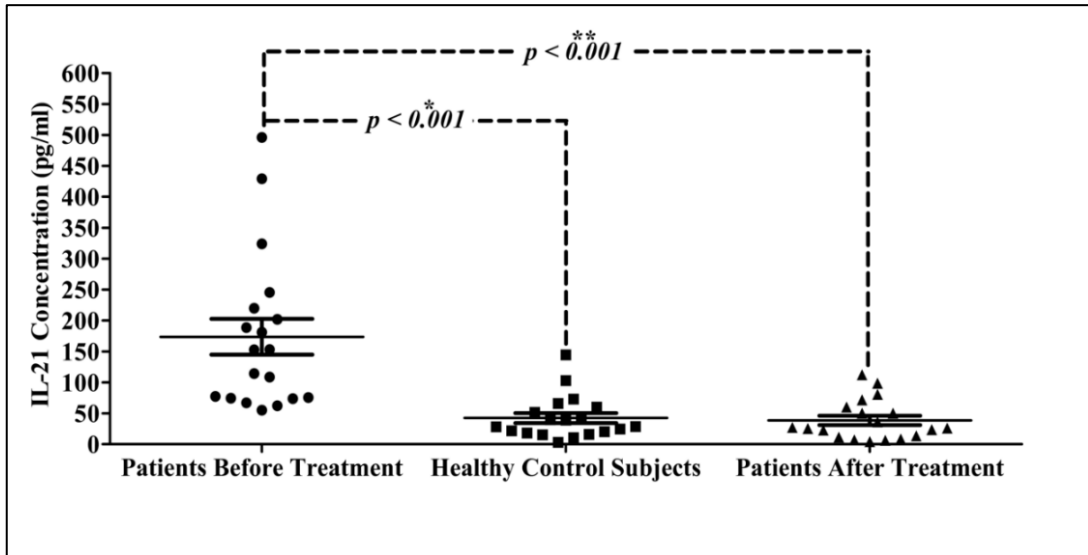


Figure 3.3.1.2. Statistical Analysis of IL-21 concentration from culture supernatants of TH-17 Cells.

3.3.1.3. IL-22 Levels

In our study, with IL-17A we have observed a significant increase in BD Uveitis patients ($510,00 \pm 38,72$ pg/ml, $n=19$) when compared to healthy controls ($145,76 \pm 19,58$, $n=19$) ($p < 0,001$). After IFN- α -2a treatment, IL-22 levels were significantly decreased ($79,47 \pm 24,04$ pg/ml, $n=19$) ($p < 0,001$). Statistical graphic is given in **Figure 3.3.1.3**.

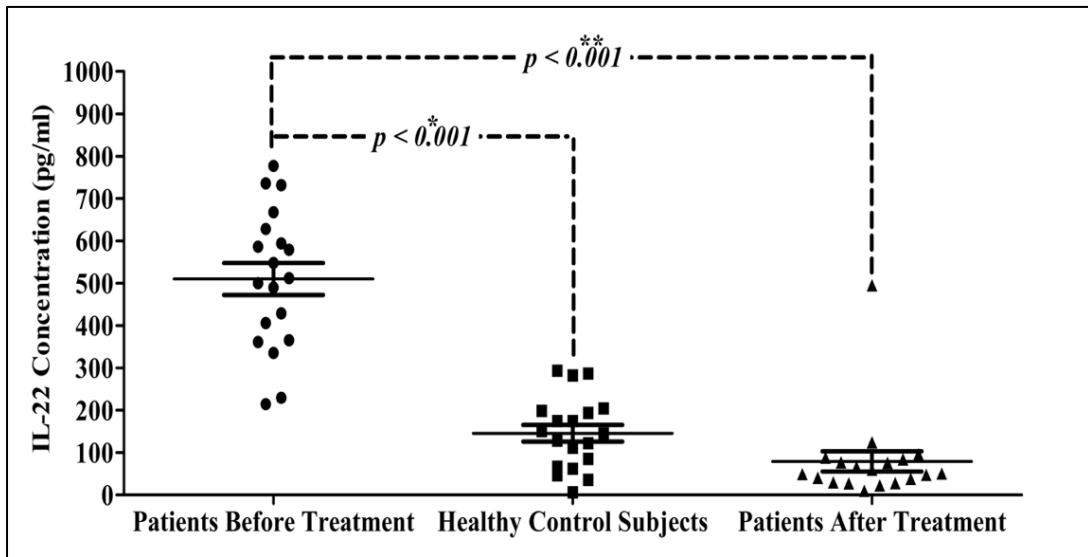


Figure 3.3.1.3. Statistical Analysis of IL-22 concentration from culture supernatants of TH-17 Cells.

3.3.1.4. IL-23 Levels

Increased levels of IL-23 were found in BD Uveitis patients ($4,07 \pm 0,71$ pg/ml, $n=19$) when compared to healthy control subjects ($3,19 \pm 1,23$ pg/ml, $n=19$) which contributes to the higher percentage of TH-17 cells in BD Uveitis patients ($p = 0,007$). After IFN- α -2a treatment, IL-23 levels were significantly decreased ($1,16 \pm 0,45$ pg/ml, $n=19$) which is correlated with the decrease of TH-17 cell population in BD Uveitis patients after treatment ($p < 0,001$). Statistical graphic is given in **Figure 3.3.1.4**.

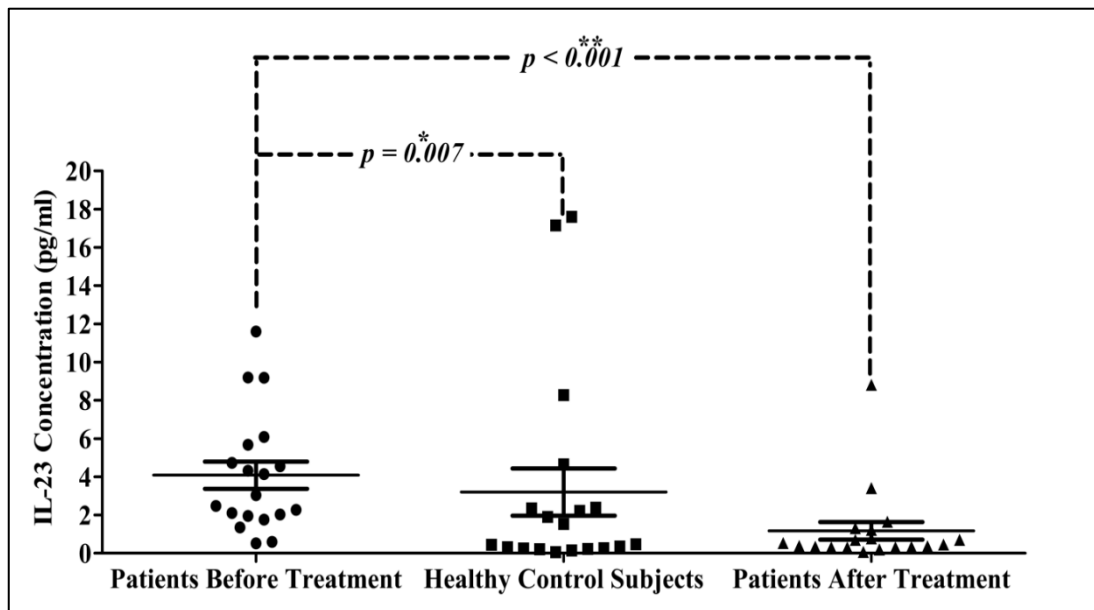


Figure 3.3.1.4. Statistical Analysis of IL-23 concentration from culture supernatants of TH-17 Cells.

3.3.1.5. IFN- γ Levels

IFN- γ levels in BD Uveitis patients were significantly increased ($45,24 \pm 3,5$ pg/ml, n=19) when compared to healthy control subjects ($14,37 \pm 1,43$ pg/ml, n=19) ($p < 0,001$). After IFN- α -2a treatment, IFN- γ levels were significantly reduced to healthy control levels ($10,55 \pm 2,04$ pg/ml, n=19). Statistical graphic is given in **Figure 3.3.1.5**.

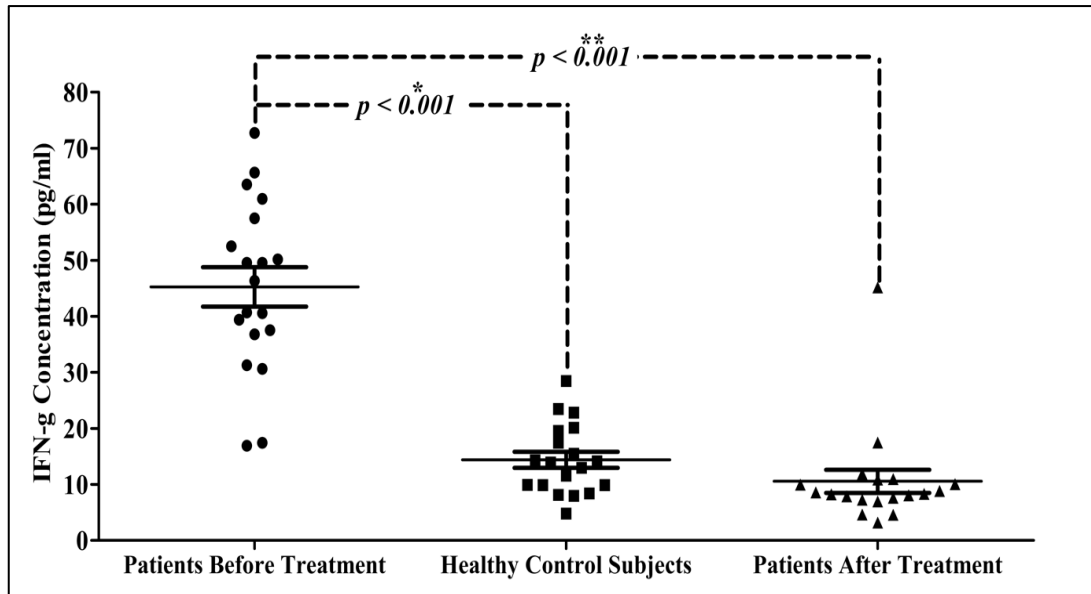


Figure 3.3.1.5. Statistical Analysis of IFN- γ concentration from culture supernatants of TH-17 Cells

3.3.1.6. TNF- α Levels

TNF- α levels were significantly increased in BD Uveitis patients ($30,69 \pm 4,20$ pg/ml, $n=19$) compared to healthy controls ($8,59 \pm 1,34$ pg/ml, $n=19$) ($p < 0,001$). After IFN- α -2a treatment, we observed a drastic decline in TNF- α levels ($2,57 \pm 0,76$ pg/ml, $n=19$) ($p < 0,001$). Statistical graphic is given in **Figure 3.3.1.6**.

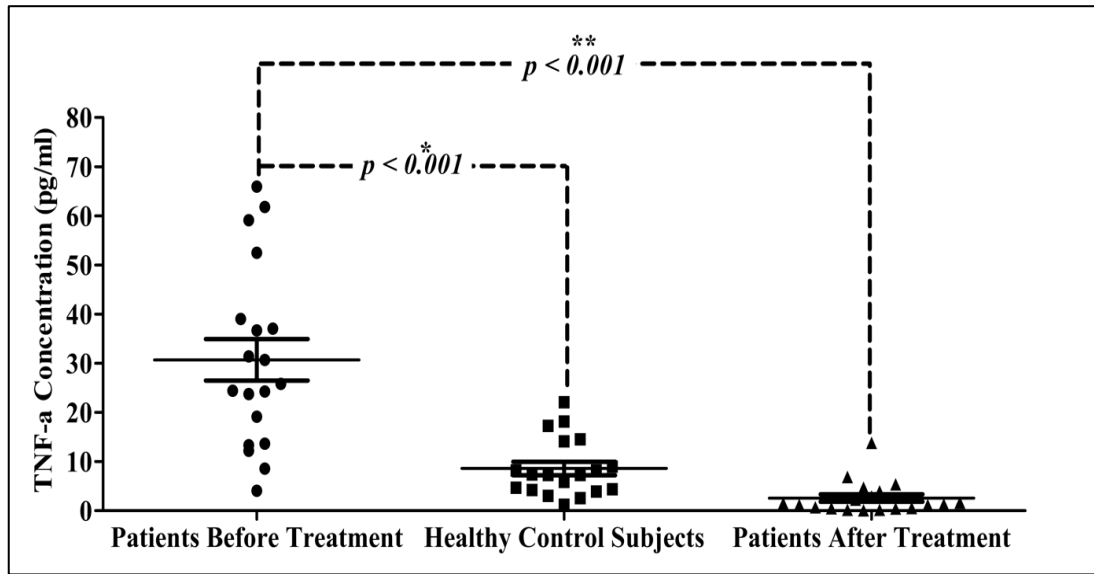


Figure 3.3.1.6. Statistical Analysis of TNF- α concentration from culture supernatants of TH-17 Cells

3.3.2. Upregulated ROR- γ T Gene Expression Was Observed In BD Uveitis Patients. IFN- α -2a Treatment Caused Downregulation On ROR- γ T Expression

Significant upregulation of ROR- γ T was observed in TH-17 cells of BD Uveitis patients ($18,81 \pm 6,34$ fold, $n=19$) ($p < 0,001$) against healthy control subjects. After IFN- α -2a treatment, ROR- γ T expression was significantly downregulated ($0,20 \pm 0,11$ fold, $n=19$) ($p < 0,001$). Statistical graphic is given in **Figure 3.3.2**.

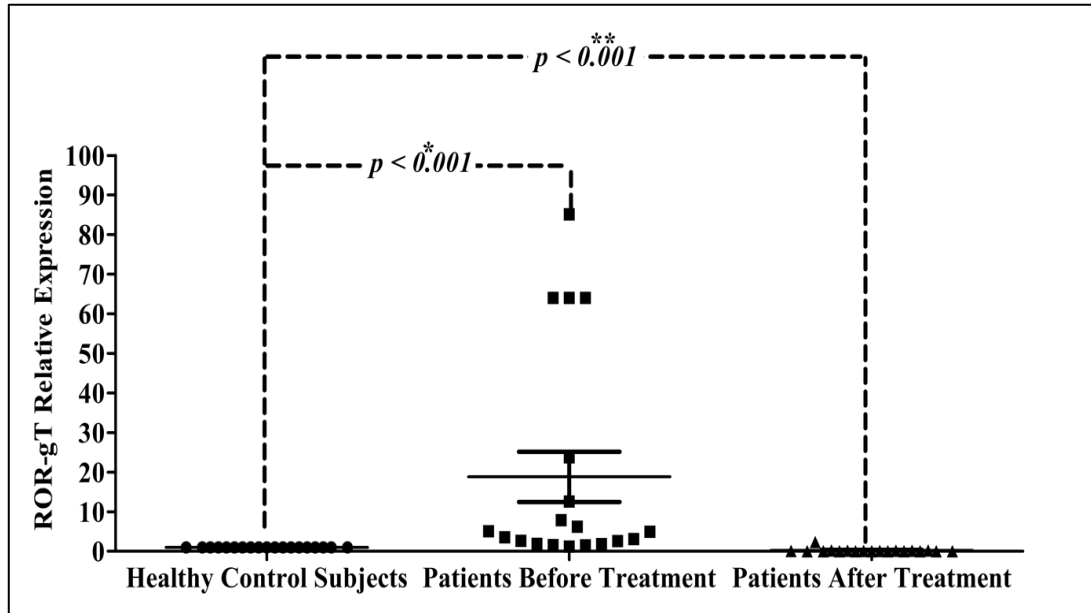


Figure 3.3.2. Statistical Analysis of ROR- γ T Gene Expression TH-17 Cells.

3.4. Results of Cytokine Secretion of Stimulated Regulatory T Cells

3.4.1. IL-2 Secretion From Stimulated Regulatory T Lymphocytes of BD Uveitis Patients Before Treatment Were Increased Compared to Healthy Controls But Decreased After IFN- α -2a Treatment

After 72 hours of stimulation with PHA-L, IL-2 concentrations secreted from Regulatory T-Cells were significantly increased in BD Uveitis Patients ($37,70 \text{ pg/ml} \pm 2,47 \text{ pg/ml}$, $n=19$) compared to healthy control ($31,93 \text{ pg/ml} \pm 1,91 \text{ pg/ml}$, $n=19$) subjects ($p = 0,039$). After IFN- α -2a treatment, IL-2 concentrations were decreased significantly ($20,95 \text{ pg/ml} \pm 2,47 \text{ pg/ml}$, $n=19$) ($p < 0,001$). Statistical graphic is given in **Figure 3.4.1**.

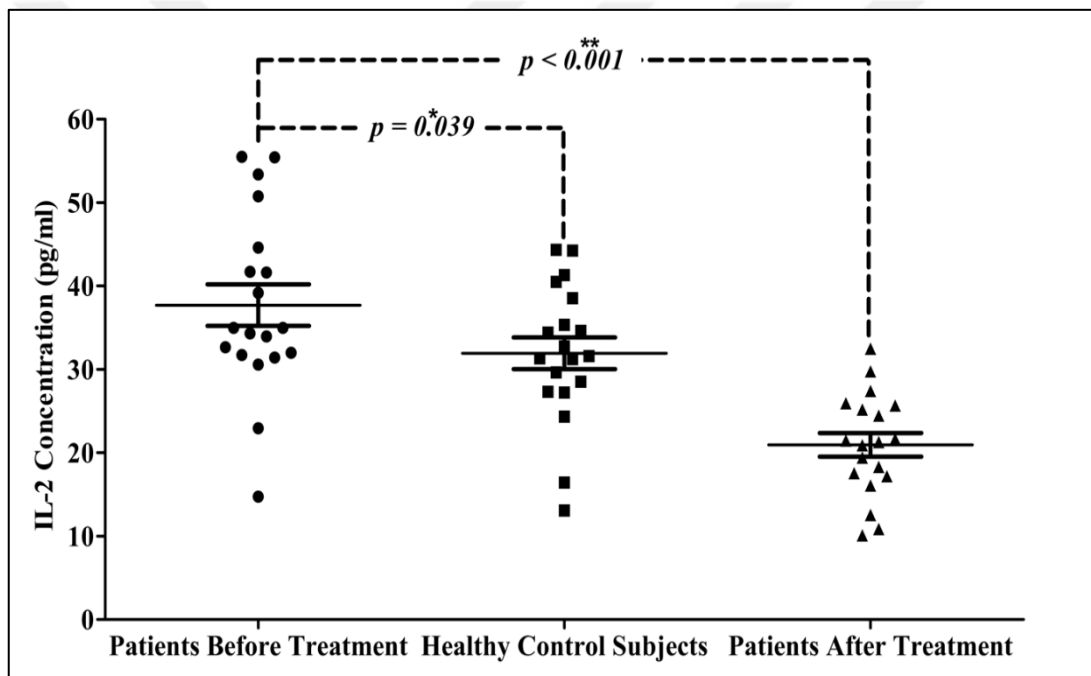


Figure 3.4.1. Statistical analysis of IL-2 concentration from culture supernatants of Regulatory T Cells.

3.4.2. IL-10 Secretion From Stimulated Regulatory T Lymphocytes of BD Uveitis Patients Before Treatment Were Decreased Compared to Healthy Controls But Increased After IFN- α -2a Treatment

IL-10 production was significantly decreased in BD Uveitis Patients ($4,26 \text{ pg/ml} \pm 0,28 \text{ pg/ml}$, $n=19$) when compared to healthy control subjects ($5,15 \text{ pg/ml} \pm 0,32 \text{ pg/ml}$, $n=19$) ($p = 0,016$). After IFN- α -2a treatment, IL-10 levels of BD Uveitis patients were significantly increased ($7,05 \text{ pg/ml} \pm 0,37 \text{ pg/ml}$, $n=19$) ($p < 0,001$). Statistical graphic is given in **Figure 3.4.2**.

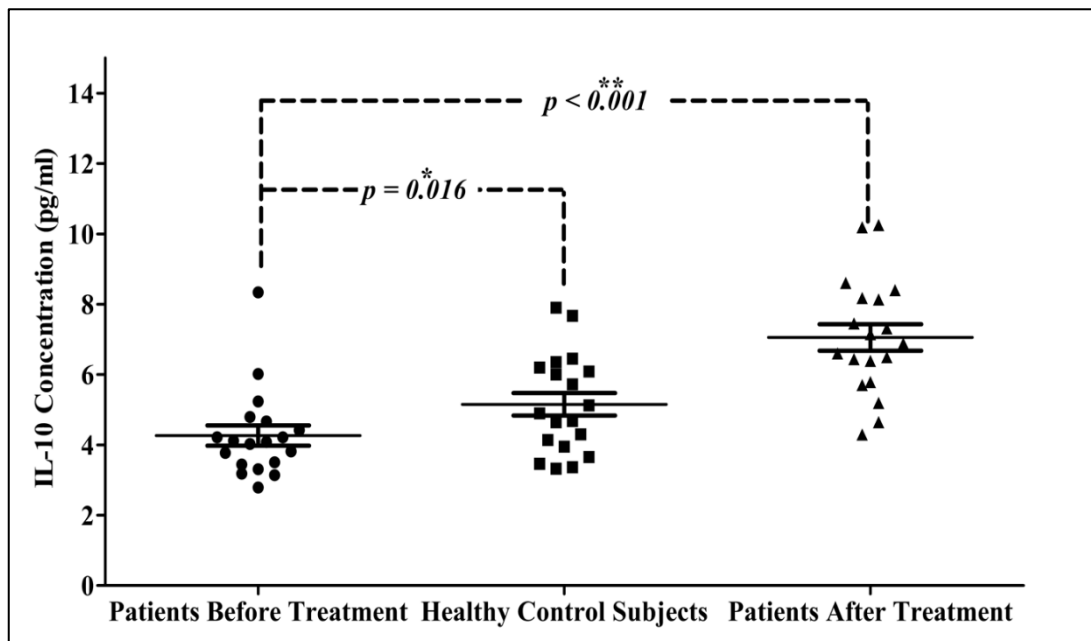


Figure 3.4.2. Statistical Analysis of IL-10 concentration from culture supernatants of Regulatory T Cells.

3.4.3. IL-35 Secretion From Stimulated Regulatory T Lymphocytes of BD Uveitis Patients Before Treatment Was Similar Compared To Healthy Controls But Increased After IFN- α -2a Treatment

There was no significant difference of IL-35 secretion between BD Uveitis Patients (289,74 pg/ml $\pm$ 11,20 pg/ml, n=19) and Healthy Control Subjects (276,15 pg/ml $\pm$ 9,65 pg/ml, n=19) (p = 0,563). After IFN- α -2a treatment, IL-35 concentrations were significantly increased in BD Uveitis Patients (363,51 pg/ml $\pm$ 15,15 pg/ml, n=19) (p < 0,001). Statistical graphic is given in **Figure 3.4.3**.

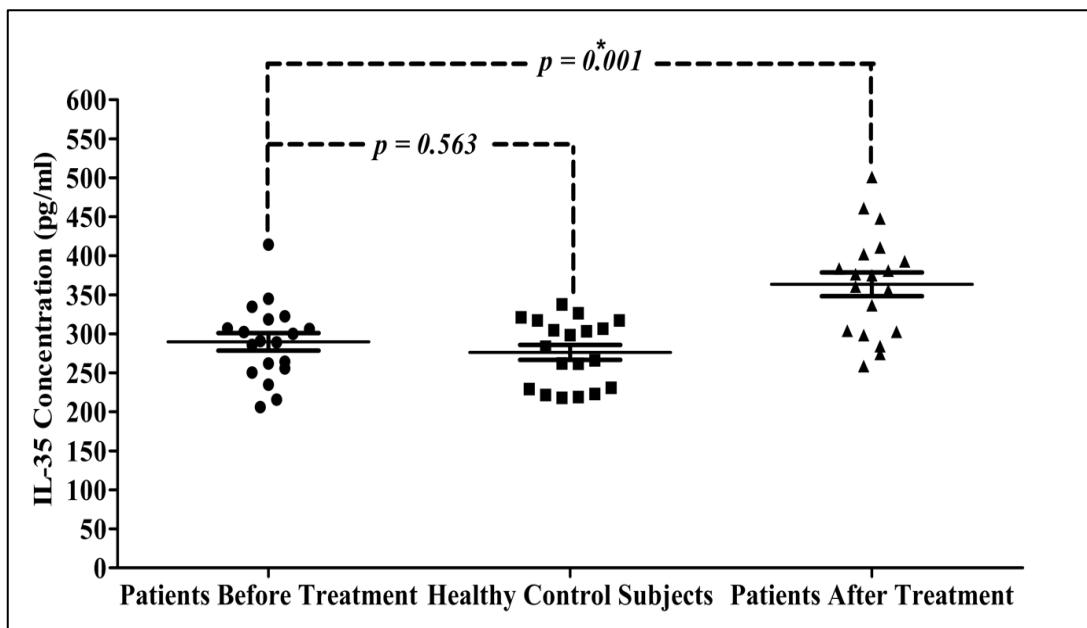


Figure 3.4.3. Statistical Analysis of IL-35 concentration from culture supernatants of Regulatory T Cells.

3.5. Innate Immune Response

3.5.1. Upregulated Toll Like Receptors TLR -2, 3, 4, 8 and 9 Gene Expressions Were Found in Monocytes of BD Uveitis Patients. Downregulation in TLR Genes Were Observed After IFN- α -2a Treatment

According to qPCR gene expression data, when we compared the gene expression of TLRs between monocytes of patients and healthy controls, we have found a significant upregulation in BD Uveitis Patients' Toll Like Receptors TLR-2 ($2,601 \pm 0,54$ fold, $p < 0,001$), TLR-3 ($2,731 \pm 0,40$ fold, $p < 0,001$), TLR-4 ($2,663 \pm 0,40$ fold, $p < 0,001$), TLR-8 ($2,464 \pm 0,34$ fold, $p < 0,001$) and TLR-9 ($3,226 \pm 0,83$ fold, $p < 0,001$). After IFN- α -2a treatment, significant downregulation was observed. Statistical graphic is given in **Figure 3.5.1.**

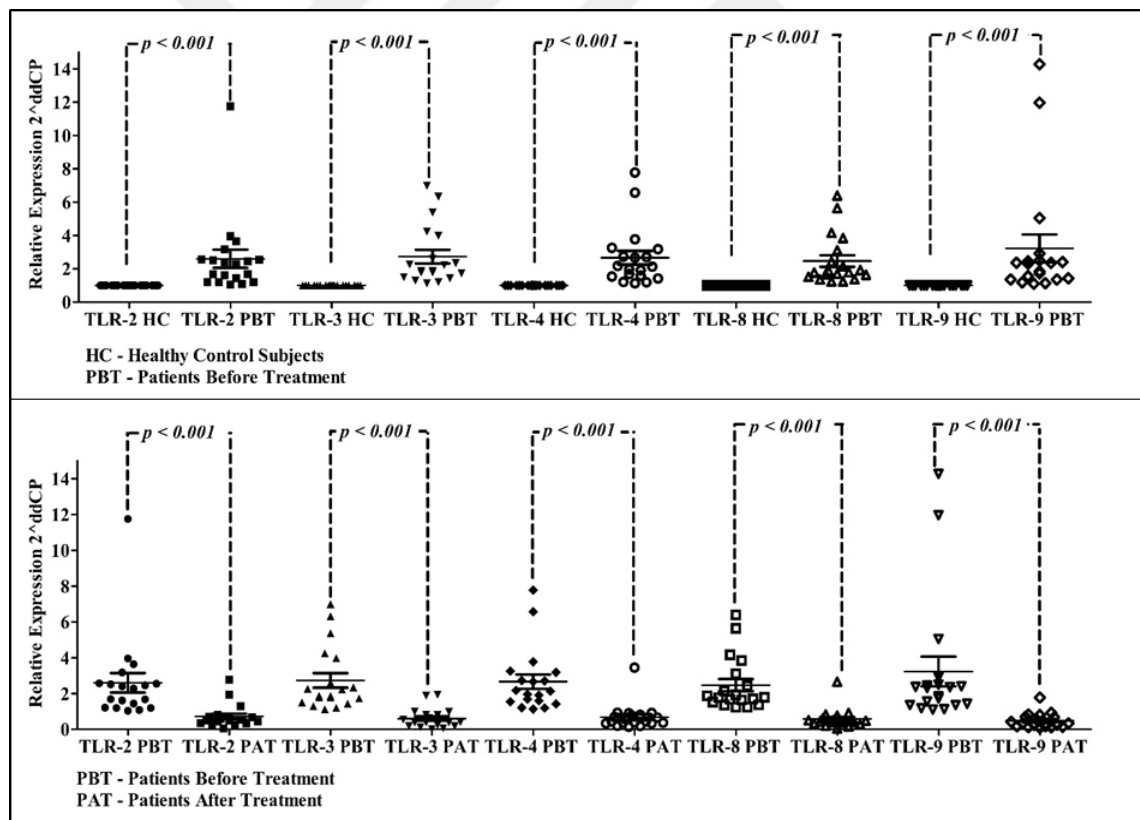


Figure 3.5.1. Statistical Analysis of TLR Gene Expression from Monocytes.

4. DISCUSSION

In this study, we aimed to determine i) Immunopathogenesis of Behçet's Disease (BD) and BD associated Uveitis and ii) Demonstrate the effects of IFN- α -2a treatment on adaptive and innate immune responses in Behçet Uveitis Patients resistant to conventional immunomodulatory treatments.

To determine the immunopathogenesis of BD and BD Uveitis during innate immune responses, we analyzed the gene expressions of Toll Like Receptors (TLR) 2, 3, 4, 8, and 9 in monocytes of 19 patients and healthy controls. TLRs play a critical role in innate immunity against pathogens. *Streptococcus spp.* and herpes simplex virus type 1 (HSV-1) have long been considered as a triggering factor in the pathogenesis of BD (Evereklioglu, C., 2005; Papadimitraki, E.D., G.K. Bertsias, and D.T. Boumpas, 2007). We have found significant upregulation in the means of fold change in BD Uveitis patients than healthy controls. For TLRs 2, 3, 4, and 8, our results are correlated with the results of Liu et. al who also found a significant increase of aforementioned TLRs in the means of fold change in monocytes of active BD Uveitis patients (Liu, X., et al., 2013). Do et. al and Seoudi et. al also reported significant increase for TLRs 2 and 4 gene expressions in monocytes of BD patients which correlates with our results (Do, J.E., et al., 2008; Seoudi, N., et al., 2014). Although there are studies which analyzed the TLR9 polymorphisms on BD (Ben Dhifallah, I., et al., 2009) and effects of TLR9 agonists on murine BD Uveitis (Allensworth, J.J., 2011), ours is the first study to show the gene expression increase of TLR9 in BD Uveitis.

To evaluate the effects of IFN- α -2a treatment on innate immune responses, we looked at the gene expressions of same TLRs in monocytes of follow-up patients after they received IFN- α -2a treatment. We observed that IFN- α -2a led to a significant decrease in expression of studied TLRs on monocytes after clinical remission of uveitis was achieved.

Monocytes are one of the most important part of innate immunity and a major contributor to host defense. These results show us that i) TLRs 2, 3, 4, 8 and 9 expressions in monocytes are upregulated in BD and BD Uveitis and this upregulation may contribute to the pathogenesis of BD and BD Uveitis and ii) IFN- α -2a mediates the innate immune responses in BD Uveitis via regulating monocyte TLR signaling.

Regulatory T-Cells are one of the most important parts of adaptive immune system as a loss of function or scarcity of these cells leads to autoimmunity and systemic organ damage via overactive immune response (Vignali, D.A.A., L.W. Collison, and C.J. Workman, 2008). In our flow cytometric analysis we found a significant increase of CD3⁺CD4⁺FoxP3⁺ Tregs in peripheral blood of BD Uveitis patients than healthy controls. IL-2 concentrations were also significantly increased in stimulated Treg supernatants of these patients which correlates with our flow cytometric results that regulatory T Cell population is increased in BD Uveitis patients in ocular attack period. Interestingly we have observed a decrease in Mean Fluorescence Intensity (MFI) of FoxP3 surface expression on Tregs of BD Uveitis patients. MFI corresponds to a relative quantification of number of antigens on the cell surface. Increased cell surface expression and decreased MFI of FoxP3 led us to think a functional loss of Tregs of BD Uveitis patients. To analyze this, we looked at the anti-inflammatory cytokines IL-10 and IL-35 in supernatants of stimulated Tregs and indeed we have observed a significant decrease in IL-10 concentrations and insignificance in IL-35 concentrations. Peripheral Treg populations were reverted back to normal values in the means of FoxP3 expression and MFI. IL-2 concentrations were also reduced and increased IL-10 and IL-35 concentrations were observed in Treg supernatants of BD Uveitis patients after IFN- α -2a treatment.

Hamzaoui et. al found increased CD4⁺CD25⁺ Treg cell percentage and Foxp3 gene expression in BD patients who had active systemic and intraocular inflammation when compared to healthy control subjects and to patients with BD who were in remission (Hamzaoui, K., A. Hamzaoui, and H. Houman, 2006). Demir et. al also showed significantly higher CD4⁺CD25⁺Foxp3⁺ Treg cell counts in 25 patients with systemically active BD, 13 of them having active uveitis, and in 25 patients with BD in remission stage when compared to controls (Demir, N., et al., 2012). However, Liu et. al showed a dysfunction and decrease in the frequency of CD4⁺CD25⁺Foxp3⁺ cells in active BD patients and increased population of CD4⁺CD25^{hi}Foxp3⁺ Tregs after IFN- α -2a treatment (Liu, X., M. Diedrichs-Möhring, and G. Wildner, 2019). A relative increase in the number of Tregs would be expected in inflammatory diseases due to an increase in polarized effector T cells, and we suggest that it is not their scarcity but their dysfunctionality leads to a pathologic state in patients with BD uveitis. There may also be a disruption in the recruitment of Treg cells to the inflamed tissues and the eye in patients with BD. Altogether our data suggest that significantly lower Foxp3 MFI by Treg cells, despite a

relative increase in Treg cell frequency and decreased IL-10 and IL-35 expressions implicate a functional impairment in Treg cells in patients with BD uveitis and IFN- α -2a treatment leads to a decline in the number of dysfunctional Treg cells in patients with refractory BD uveitis, along with control of disease activity.

TH17 cells are a specialized subtype of CD4⁺ helper T Cells that are responsible for the initiation of inflammation in tissues in bacterial and viral infections. However in recent years TH17 cells are associated with several autoimmune diseases (Bedoya, S.K., et al. 2013). TH17 cells are mainly determined as CD3⁺CD4⁺CD25⁻IL-17A⁺ cells by flow cytometry. In our study, we have found significantly increased expression of intracellular IL-17A expression in BD Uveitis patients than healthy control subjects and IL-17A MFI was also correlated with the increase of IL-17A expression. To confirm our findings, we looked at the gene expression levels of ROR- γ T master transcription factor of TH-17 cells and found a significant upregulation in BD Uveitis patients as well. We then analyzed the pro-inflammatory cytokines expressed by TH17 cells and observed significant elevation of IL-17A, IL-21, IL-22, IL-23, TNF- α and IFN- γ concentrations from polarized and stimulated TH-17 supernatants.

IFN- α -2a treatment resulted in the reversal of all pathologic TH17 immune responses observed in patients with active BD uveitis despite conventional immunomodulatory therapies. We found a significant reversal of the increased frequency of polarized CD3⁺CD4⁺IL17A⁺ TH17 cells, increased expression ROR- γ T mRNA, and their proinflammatory cytokine production potential.

In previous studies, Hamzaoui et. al showed a significant increase in IL-17A concentration and ROR- γ T gene expression in BD patients (Hamzaoui, K., et al., 2011), whereas Chi et. al found increased concentrations of IL-17A and IL-23 in BD patients with active Uveitis (Chi, W., et al., 2008). Sonmez et. al showed significant increase and in IL-17A and correlation with IL-23 in active BD patients (Sonmez, C., et al., 2018). Puccetti et. al also showed increased percentage of CD4⁺IL-17A⁺ cells in active BD patients (Puccetti, A., et al., 2018).

Altogether, these data suggest that i) increased frequency of TH17 cells and their increased expression of proinflammatory cytokines in patients with active BD Uveitis play a significant role in TH17 immune responses in BD and ii) IFN- α -2a treatment prevents inflammation by reducing the polarization of CD4⁺CD25⁻ cells into IL-17A

producing TH-17 cells and inhibiting their pro-inflammatory cytokine production potential.

Ward et.al observed significant response to IFN- α -2a treatment in the means of improved long term visual prognosis and long lasting remissions (Warde, N., 2010). Yalcindag et.al showed that IFN- α -2a treatment is effective in Behcet uveitis and provides significant improvement in visual prognosis (Yalcindag, F.N. and A. Uzun, 2012). Eser-Öztürk and Sullu also showed significant resolve in inflammation in the first month of IFN- α -2a treatment in 84% of their patient population (Eser-Ozturk, H. and Y. Sullu, 2020). Our study is the first to analyze the effects of IFN- α -2a on both innate and adaptive immune responses in the means of Treg and TH17 cell frequency and cytokine production, ROR- γ T expression and TLR expression on monocytes of BD Uveitis patients resistant to conventional immunomodulatory therapy.

Limitations of this study is the lack of a diseased control group. Further studies are required to assess the effect of type 1 interferon therapy on immunologic features of ocular fluids and tissues in patients with BD uveitis and to investigate their correlation with the findings observed in the peripheral blood.

In conclusion, this study shows that BD Uveitis patients in ocular attack periods have increased number of dysfunctional regulatory T Cells that have attenuated levels of IL-10 and IL-35 secretion. Also *in vitro* polarization of CD4⁺CD25⁻ T Cells to TH-17 cells and their secreted proinflammatory cytokine levels are increased in these patients. Combined with the upregulation in TLR expressions on monocytes suggest that both innate and adaptive immune system cells, especially TH-17 cells, play significant roles in the pathogenesis of BD Uveitis. IFN- α -2a treatment reduces the number of dysfunctional regulatory T cells while restoring their function in the means of increased IL-10 and IL-35 secretions. IFN- α -2a also decreases polarization of CD4⁺CD25⁻ T Cells to TH-17 cells and suppresses proinflammatory cytokine secretion from these cells. Downregulation in TLR expression on monocytes after IFN- α -2a treatment combined with the adaptive immune responses suggest that IFN- α -2a have the ability to restore balance and homeostasis in the means of orchestrating innate and adaptive immune responses.

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