

Inhibitory Effects of Acetylsalicylic Acid and Ibuprofen on Interleukin-17 Production

Asetilsalisilik Asit ve İbuprofenin İnterlökin-17 Üretimi Üzerindeki İnhibitör Etkileri

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Objectives: In this study, we aimed to investigate *in vitro* effects of acetylsalicylic acid (ASA) and ibuprofen on interleukin (IL)-17, interferon gamma (IFN- γ), IL-4 and tumor necrosis factor alpha (TNF- α) production.

Patients and methods: Blood samples were obtained from 10 healthy volunteers between May 2007 and May 2009. Acetylsalicylic acid and ibuprofen were dissolved in a phosphate buffered saline (PBS) solution in clinically relevant blood concentrations. Phytohemagglutinin (PHA) and crude bacterial extracts of *Streptococcus sanguis* were used to stimulate cells. The cytokine response was measured by an ELISPOT reader.

Results: Both ASA and ibuprofen significantly inhibited the IL-17 response at all concentrations in a dose-dependent manner. No significant effect of the drugs on IL-4 and IFN- γ response was observed.

Conclusion: Inhibition of IL-17 and TNF- α production by therapeutically reachable concentrations of ASA and ibuprofen can be a novel underlying mechanism for their well-known anti-inflammatory actions.

Key words: Acetylsalicylic acid; ibuprofen; interleukin-17; inhibition.

Amaç: Bu çalışmada asetilsalisilik asit (ASA) ve ibuprofenin interlökin (IL)-17, interferon gama (IFN- γ), IL-4 ve tümör nekroz faktör alfa (TNF- α) üretimi üzerindeki *in vitro* etkileri araştırıldı.

Hastalar ve yöntemler: Mayıs 2007 - Mayıs 2009 tarihleri arasında 10 sağlıklı gönüllüden kan örnekleri alındı. Asetilsalisilik asit ve ibuprofen, klinik düzeyde etkin kan konsantrasyonunda fosfat tamponlu salin (PBS) solüsyon içerisinde çözdürüldü. Hücreleri uyarmak için fitohemaglutinin (PHA) ve ham *Streptococcus sanguis* bakteriyel özütü kullanıldı. Sitokin yanıtı, ELISPOT okuyucusu ile ölçüldü.

Bulgular: Hem ASA hem de ibuprofen, tüm konsantrasyonlarda doza bağımlı bir şekilde IL-17 yanıtını anlamlı düzeyde inhibe etti. Her iki ilacın IL-4 ve IFN- γ yanıtları üzerinde anlamlı bir etkisi gözlenmedi.

Sonuç: Tedavi edici ASA ve ibuprofen konsantrasyonları ile IL-17 ve TNF- α üretiminin inhibe olması, bu ilaçların çok iyi bilinen antienflamatuvar etkileri için yeni bir altta yatan mekanizma olabilir.

Anahtar sözcükler: Asetilsalisilik asit; ibuprofen; interlökin-17; inhibisyon.

Recently a new subset of T helper cells, known as Th17 cells, has been recognized for its high production of interleukin (IL)-17. Transforming growth factor beta (TGF- β), IL-1, IL-6, and IL-21 are the inducer cytokines for this subset, with IL-23 being essential for the survival and proliferation of these cells. In experimental mice models, the Th17 cells and IL-17 have played important roles in the defense against

extracellular pathogens and the pathogenesis of several autoimmune inflammatory conditions, such as collagen induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE), autoimmune uveitis, and inflammatory bowel disease (IBD). Anti IL-17 treatment has shown preventative or beneficial effects in these disease models. Mice lacking IL-23 or IL-17 have been found to be resistant to EAE, CIA,

and IBD induction. Furthermore, in IL-17 receptor knockout mice, erosion observed in streptococcal cell wall-induced arthritis was significantly decreased and the expression of matrix metalloproteinases (MMPs) was inhibited.^[1-3]

In humans, increased IL-17 levels have been reported in multiple sclerosis (MS), Behçet's disease (BD), uveitis, and the synovial fluid of rheumatoid arthritis (RA) patients, suggesting that this cytokine and Th17 cells also play a part in these diseases. In addition, inhibiting production of this cytokine may have therapeutic effects.^[1-6] In human synoviocytes, a positive feedback mechanism has been described in which prostaglandin E2 (PGE2) stimulates both IL-23 and IL-17 production, and the increased IL-17 then stimulates the PGE2 production. Retinoid acid or one of its analogs (resolvin E1, simvastatin, blocking IL-15, and tumor necrosis factor (TNF) receptor fusion protein) has been used for the inhibition of IL-17 production, but thus far, specific inhibitors of these cytokines have not been identified apart from the monoclonal antibodies raised against IL-17 or IL-23.^[6-11]

In this study, using the enzyme-linked immunosorbent spot (ELISPOT) assay, we aimed to investigate the *in vitro* effects of clinically relevant, therapeutic concentrations of two well-known inhibitors of PGE2, aspirin and ibuprofen, on IL-17, IFN- γ , IL-4, and TNF- α production.

PATIENTS AND METHODS

Blood samples from 10 healthy volunteers were used for this study between May 2007 and May 2009 in Department of Pediatric Immunology-Allergy, Medicine Faculty of Akdeniz University, and any individuals with a history of inflammatory or allergic diseases or a history of recent infection were excluded. In addition, individuals who had taken any kind of drugs or consumed alcohol within the week prior to donating blood were also not included.

Aspirin (Bayer AG, Leverkusen, Germany) and ibuprofen (Pfizer, Inc., New York City, NY, USA) were dissolved in phosphate buffer saline solution (PBS) in clinically relevant blood concentrations (50 and 25 $\mu\text{g}/\text{mL}$ for aspirin and (100, 50, and 25 $\mu\text{g}/\text{mL}$ for ibuprofen).

Stimulants

To stimulate the cells, phytohemagglutinin (PHA) and crude bacterial extracts of *Streptococcus sanguis* (*S. sanguis*) were used (PHA at 0.05 $\mu\text{g}/\text{mL}$ concentration and *S. sanguis* at 1/100 dilution). Our previous experiments showed that these stimulants led

to significant IL-17A, IL-4, TNF- α and IFN- γ responses when the ELISPOT method was used.

ELISPOT assay

Mononuclear cells were isolated by density gradient and were plated at a concentration of 2×10^5 cells/well for the IL-17A, IFN- γ , and IL-4 determination. However, 5×10^4 cells/well were used for the TNF- α measurement. The cells were kept in an RPMI-1640 (Roswell Park Memorial Institute, Sigma-Aldrich) medium supplemented with 10% fetal calf serum (FCS) and 2% penicillin/streptomycin. The ELISPOT plates were obtained from eBioscience, Inc. (San Diego, CA, USA) and were processed according to the manufacturer's instructions. For IL-17A, IFN- γ and IL-4 determination, the plates were incubated for 48 hours at 37 °C in incubators containing 5% carbon dioxide (CO₂). Tumor necrosis factor- α determination was performed following a 16-hour incubation period. Afterwards, the cell viabilities were determined using a trypan blue exclusion test, and the spots were counted using an ELISPOT reader (Cellular Technology Limited (CTL)-Europe GmbH, Bonn, Germany). A positive response required there to be more than a two-fold increase in the number of spots when compared with unstimulated wells and at least 10 spots in the stimulated samples. The inhibition percent was calculated as the number of spots at the stimulated well containing the drug/the number of spots at the stimulated well x 100.

A typical protocol of the ELISPOT test was as follows*

Mononuclear cells + medium
 Mononuclear cells + diluent (PBS)
 Mononuclear cells + stimulants
 Mononuclear cells + stimulants + drug

* The tests were conducted in duplicate or triplicate, and the mean values of these wells were used to calculate inhibition. Five healthy controls were analyzed for TNF- α , IFN- γ and IL-4 while 10 healthy controls were tested for IL-17A.

Statistical analysis

The Wilcoxon test was used for the statistical evaluation of the pairs, and *p* values of less than 0.05 was considered to be significant.

RESULTS

After the incubation period, the cell viabilities were determined to be less than 10% in all of the samples. However, these wells containing drugs were not significantly affected by this.

The cytokines that were investigated in the samples responded strongly to PHA (Figure 1) and *S. sanguis*.

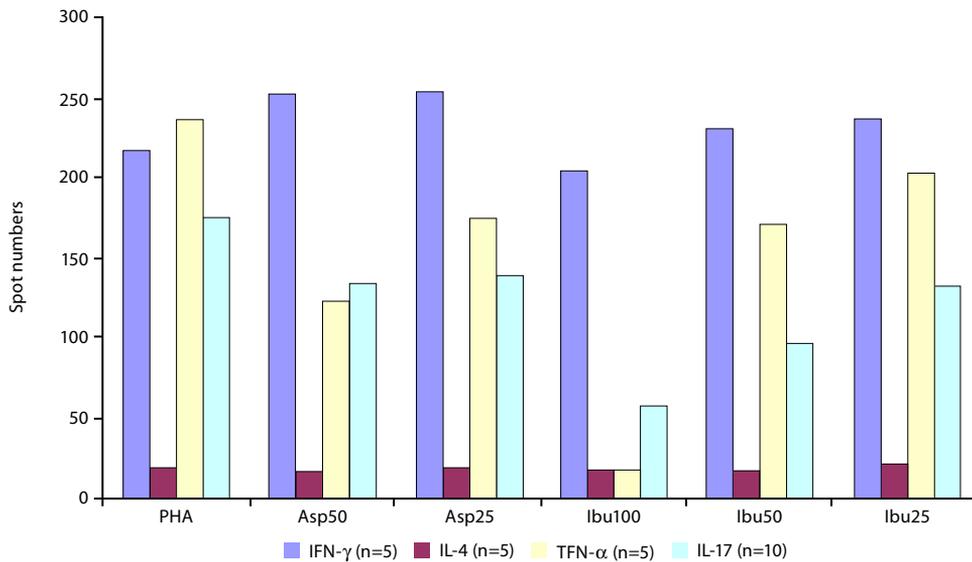


Figure 1. The effects of aspirin and ibuprofen on cytokine response. PHA: Phytohemagglutinin; Asp: Aspirin; Ibu: Ibuprofen.

The inhibition percentages are shown in Figure 2. None of the drugs significantly affected the IL-4 and IFN- γ response ($p > 0.05$), but an increase in spot counts was observed, especially in the wells containing aspirin for the IFN- γ response, but this was not statistically significant. At low concentrations (25 $\mu\text{g}/\text{mL}$), aspirin did not have any effect on the response; however, a significant inhibition in the TNF- α response was observed at 50 $\mu\text{g}/\text{mL}$ concentration. Ibuprofen, on the other hand, significantly decreased the TNF- α response in all concentrations, and this effect was dose-dependent ($p < 0.05$ at 25 $\mu\text{g}/\text{mL}$ and $p < 0.001$ at 100 $\mu\text{g}/\text{mL}$ concentrations). We also determined that both drugs significantly inhibited the IL-17A response

at all concentrations, and this was also dose-dependent (Figures 1 and 2).

DISCUSSION

The stimulation of granulopoiesis, osteoclastogenesis, and upregulation of nitric oxide (NO) synthesis are among the most important biological effects of the IL-17 cytokine family (A-F). In addition, being a pro-inflammatory cytokine, IL-17 acts as a trigger for the production of IL-6, IL-8, IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF), defensins, CxCL1, CCL11, ICAM-1, MMP-9, and PGE2, all of which are important inflammatory mediators. The vital role of Th17 cells along with IL-23 and

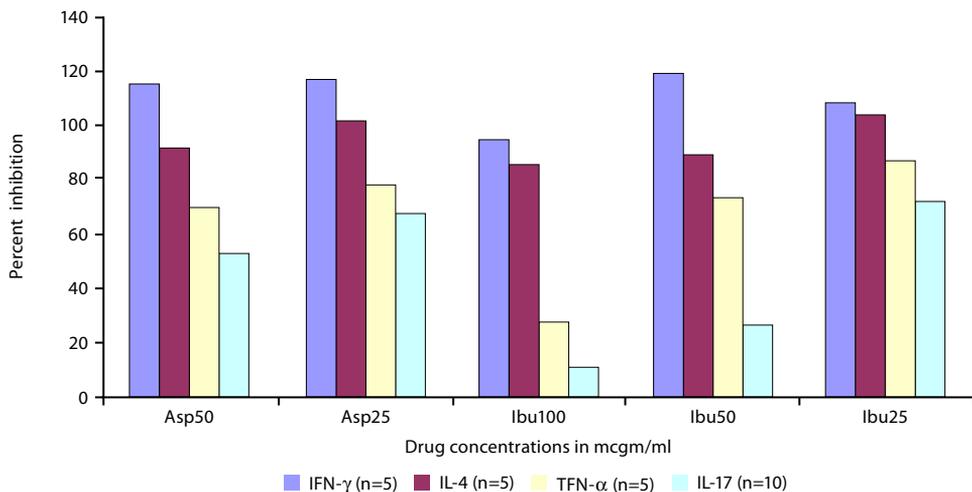


Figure 2. The effects of aspirin and ibuprofen on cytokine response were given as percent of inhibition. Asp: Aspirin; Ibu: Ibuprofen.

IL-17 in autoimmune inflammatory diseases in mice has been well documented.^[2] Evidence from human studies also supports that this cytokine family and Th17 cells have important roles in the pathogenesis of many autoimmune and inflammatory diseases, such as MS, BD, RA, and IBD.^[1-5] Hence, pharmacological manipulation of the effects or production of these IL-17 cytokine family and Th17 cells have potential clinical significance.^[6]

We showed for the first time in this study that aspirin and ibuprofen strongly inhibit the production of IL-17A. This inhibition must be specific since under the same experimental conditions, neither of the drugs had an effect on the IL-4 or IFN- γ responses. At high concentrations (50 $\mu\text{g/ml}$), aspirin caused a significant decrease in TNF- α production, whereas lower doses (25 $\mu\text{g/ml}$) had no effect. On the other hand, ibuprofen caused a significant decrease in the TNF- α response at all of the tested concentrations. The mutual interaction between TNF- α and PGE2 along with the effect of nonsteroid anti-inflammatory drugs (NSAIDs) on this axis is not well documented. Recently, Alvarez-Soria et al.^[12] showed an inhibition in the TNF- α gene expression level in osteoarthritic patients who were being treated by NSAIDs *in vivo*, but they did not observe a similar effect *in vitro*. In addition, Turnbull et al.^[13] showed that aspirin can significantly inhibit the release of monocyte TNF- α . Our findings agree with these studies.

No specific inhibitor of IL-17 or IL-23 has been identified yet. Although, Ziolkowska et al.^[14] showed that methylprednisolone and cyclosporine A can inhibit the IL-17 response, their specificity was a bit in doubt because both of those drugs are known to be general immunosuppressors and can inhibit almost all of the lymphokines and monokines. Chizzolini et al.^[15] recently demonstrated that PGE2 can increase the production of Th17 cells and IL-17, and they also reported that indomethacine has an inhibitory effect on the IL-17 response; however, no effect was observed on IFN- γ . Our observations agree with this study, but we went a step further by showing that the inhibitory effect of these drugs does not involve the IFN- γ and IL-4 response, at least under *in vitro* conditions.

Aspirin and ibuprofen are well-known inhibitors of PGE2 synthesis and have been used in the treatment of inflammatory diseases for more than 30 years. Prostaglandin E2 is the most widely produced prostanoid in the body, and it reduces the production of IL-12p35, IL-6, and TNF- α while increasing the production of IL-10, IL-12/23 (p40), and IL-23. Therefore, PGE2 can affect the phenotype of responding T helper cell subsets.^[7-11]

Our study did not attempt to show how these drugs affected IL-17 production. However, it is known that the development of Th17 cells and IL-17 production is negatively regulated by IL-4 and IFN- γ . When considering the data from the aforementioned studies along with our own results, it is conceivable that the inhibition of IL-17 could be secondary to the inhibition of IL-23 because PGE2 is a known inducer of IL-23 production.^[9,10,13] Furthermore, these drugs had no significant effect on IL-4 or IFN- γ production. The well-known inhibitory effects of these drugs on PGE2 synthesis may be the essential cause of this effect, but we have no evidence to support this conclusion. Thus, further studies are needed to elucidate the exact mechanism of action and determine which types of IL-17A-producing cells (Th17 cells, NK cells) have this effect exerted upon them.

Conclusion

Our data suggests that the inhibition of IL-17 and TNF- α production by therapeutically reachable concentrations of aspirin and ibuprofen can be an additional underlying mechanism for their well-known anti-inflammatory actions. Nevertheless, further studies are needed regarding the *in vivo* effects of these drugs on IL-17 and Th17 levels and their relationship to the clinical parameters in order to clarify the biological and clinical relevance of our findings.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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