Original Research

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Intracytoplasmic and Plasma Cytokine Levels of Natural Killer Cells in Patients with Asthma

Astımlı Hastalarda Doğal Öldürücü (NK) Hücre İntrasitoplazmik ve Plazma Sitokin Seviyeleri

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Abstract

Objective: Natural killer cells play immunomodulatory role the pathogenesis of chronic inflammatory airway diseases. NK cells may induce allergic airway inflammation by increasing the production of type 2 cytokines.
Materials and Methods: Intracytoplasmic cytokine levels of IL-13, IL-4, IFN-γ and IL-10 in NK cells and plasma levels of IFN-γ, TNF-α, IL-2, IL-8, IL-4, IL-5, IL-6, IL-17, IL12, IL-13, IL-1β and IL-10 in patients with newly diagnosed asthma, asthmatic patients under treatment and controls by using flow cytometry and ProcartaPlex multiplex immunoassays kit were measured.
Results: The numbers of IFN-γ$^+$ NK1 cells were decreased in both patient groups; comparing both asthmatic groups, IFN-γ$^+$ NK1 cells were higher in asthmatic patients under treatment. Intracellular IL-4 levels of NK cells were increased, however IL-10$^+$ NK cells were diminished in both patient groups vs. controls. Increased number of IL-13$^+$ NK2 cells in newly-diagnosed asthmatics vs. asthmatics patients under treatment and controls were obtained. Increased levels of plasma IL-1β levels in newly-diagnosed asthmatic patients compared to that of asthmatics under treatment were found. Newly-diagnosed asthmatics showed higher IL-8 levels compared to asthmatics under treatment and controls. Increased IL-5 levels were measured in newly-diagnosed asthmatic patients compared to healthy subjects and higher IL-13 levels were detected in newly-diagnosed asthmatic patients vs. other groups and also in controls vs. patients with asthma patients under treatment. IL-10 and IL-12 levels were decreased in newly-diagnosed asthmatic patients compared to healthy subjects. Compared to healthy subjects in both asthmatic patients' also diminished IL-2 levels were obtained.
Conclusion: In order to use NK cells as a therapeutic target, a strategy must be established to regulate its cytokine secretion functions with respect to their role of these cells in each disease.

Keywords: Asthma, flow cytometry, natural killer cells, cytokines

Öz

Amaç: Doğal öldürücü hücreler, kronik enflamatuvar havayolu hastalıklarının patogenezinde immünomodülatör rol oynamaktadır. NK hücreleri, tip 2 sitokinlerin üretimini artırarak alerjik havayolu iltihabına neden olabilmektedir.
Gereç ve Yöntem: Yeni teşhis ve tedavi altındaki astım hastaları, sağlıklı kontrol örneklerinde, NK hücre IL-13, IL-4, IFN-γ ve IL-10 intrasitoplazmik sitokin ve plazma IFN-γ, TNF-α, IL-2, IL-8, IL-4, IL-5, IL-6, IL-17, IL12, IL-13, IL-1β ve IL-10 seviyeleri akan hücre ölçer ve ProcartaPlex multipleks kiti kullanılarak değerlendirilmiştir.
Bulgular: IFN-γ$^+$ NK1 hücreleri her iki hasta grubunda da azalmıştır, her iki astımlı grup karşılaştırıldığında IFN-γ$^+$ NK1 hücreleri tedavi altındaki astım hastalarında daha yüksek bulunmuştur. Kontrollere göre her iki hasta grubunda NK hücre içi IL-4 seviyelerindeki artışa karşılık, IL-10$^+$ NK hücrelerinde azalma saptanmıştır. Tedavi altındaki astım hastaları ve kontrollere göre, yeni teşhis astımlı hastalarda IL-13$^+$ NK2 hücrelerinin sayısında artış gözlenmiştir. Yeni tanılı astım hastaları tedavi altındaki hastalar ile karşılaştırıldığında, plazma IL-1β seviyelerinde artış, yeni teşhis astım/tedavi altındaki astım hastaları ve sağlıklı kontroller ile karşılaştırıldığında IL-8 seviyelerinde de artış gözlenmiştir. Yeni teşhis astımlı hastalar sağlıklı bireylerle karşılaştırıldığında, yüksek IL-5, yeni teşhisli astım hastaları diğer gruplar ve tedavi altındaki astım hastaları ile karşılaştırıldığında kontrol grubunda daha yüksek IL-13 seviyeleri tespit edilmiştir. IL-10 ve IL-12 seviyeleri, yeni teşhis astım hastalarında kontrollere göre azalmıştır. Sağlıklı kontroller ile karşılaştırıldığında, her iki astım grubunda IL-2 seviyeleri de düşük saptanmıştır.
Sonuç: Farklı hastalıklarda NK hücre sitokin seviyelerinin fonksiyonlarının saptanması, NK hücrelerinin tedavi amaçlı kullanılmasında yol gösterici olabilir.

Anahtar kelimeler: Astım, akan hücre ölçer, doğal öldürücü hücre, sitokinler

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Introduction

Asthma is a chronic inflammatory disease of the airways and characterized by airway hyperreactivity and accompanying episodes of dyspnea, wheezing and symptoms of shortness of breath, and coughing.[1] These attacks are accompanied by airway obstruction that resolves spontaneously or by treatment. Inflammation in the bronchial mucosa in allergic asthma is caused cytokines and mediators that are secreted by active T cells, eosinophils, mast cells.[2] It is known that more than 100 different mediators are involved in asthma leading to mixed inflammatory response in the airways.[3] Cytokines being a group of these mediators govern the inflammatory response in asthma and determine the severity of the disease. Various inflammatory cells such as macrophages, mast cells, eosinophils and lymphocytes, as well as structural elements including epithelial cells, endothelium and airway smooth muscle cells have the ability to synthesize and secrete cytokines. Mediators such as histamine and cysteine leukotrienes play important roles in acute and subacute inflammatory responses and asthma attacks, but cytokines appear to be predominantly involved in chronic inflammation.[4] Th2 type cytokines including IL-4, IL-13 and IL-5 have important functions in asthma. While IL-4 and IL-13 play a key role in the initiation of allergic inflammation, by leading to the transformation of B lymphocytes into IgE expressing plasma cells; IL-4, unlike IL-13, plays role in the Th2 polarization of the immune response and thus is effective in atopy development. IL-13 seems to be more important in chronic inflammation.[5] Bronchial biopsy specimens from patients with asthma showed an increase in IL-5 gene expression in lymphocytes. It is known that IL-5 leads to the maturation of eosinophils in bone marrow, and to prolongation of their life span, and it also plays crucial roles in the activation and migration of the eosinophils from the blood to the airways.[6] A significant decrease was observed in induced sputum and blood of patients with asthma following the administration of anti-IL-5 indicated to the important role of IL-5 in eosinophil chemotaxis. But anti-IL-5 antibody administration showed no impact on the symptoms of asthma and the early/late phase response to allergen challenge, and these findings led to questions about the role of eosinophils in asthma pathogenesis.[7] IL-3, another Th2 lymphocyte-derived cytokine, is considered to be effective in the persistence of tissue mast cells.[8] IL-1β, IL-6, TNF-α and GM-CSF released from macrophages and epithelial cells contribute to the strengthening of the inflammatory response. Even the unresponsiveness of $CD4^+$ cells of asthmatic patients receiving successful subcutaneous immunotherapy was broken by IL-1β stimulation.[9] TNF-α inhalation therapy leads to increased airway hyperresponsiveness in healthy individuals, and increased TNF-α levels were detected in the airways of asthmatic patients. TNF-α and IL-1β are known to activate transcription factors playing role in the synthesis of many inflammatory cytokines. Other cytokines, such as IFN-γ, IL-10, IL-12 and IL-18, play regulatory roles and suppress allergic inflammation.[10]

Many studies showed evidence of their role in T cell priming, maturation of dendritic cell, and inflammation, all of which have the ability to enhance or reduce allergic responses.[11,12] The potential role of NK cells in the production T helper (Th)2-type cytokines leads to the contribution of these cells in the immunopathogenesis of allergic airway disease.[13] Previous studies revealed that NK cells from patients with asthma demonstrated increased cytotoxic activity. It decreased shortly after allergen challenge suggesting either migration of NK cells from circulation to the lung, or suppression of NK cell cytotoxicity by an allergen or allergic inflammatory milieu[14] NK cells were found to be in interaction with many cell types such as macrophages and dendritic cells. There are many examples showing their role in the regulation of T cell responses in many inflammatory diseases including asthma.[15] Human NK cell function differs according to their CD56 expression.[16] The $CD56^{bright}$ subset expresses low levels of CD16, has low cytotoxic activity, and has high cytokine secretion capacity. The $CD56^{dim}$ subset expresses high levels of CD16, has high cytotoxic activity and low cytokine secretion capacity. The majority of human NK cells are $CD56^{dim}$, and only 10% of cells were $CD56^{bright}$ and they could produce IFN-γ, TNF-β, GM-CSF, IL-10, and IL-13.[13] NK cells were recently divided into NK1, NK2, and regulatory NK subsets.[17] Type 2 cytokines are known to play important roles in allergic asthma immunopathogenesis that are not only produced by $CD4^+$ T lymphocytes but also by NK2 cells.[18] In this study, the levels of intracytoplasmic cytokines in NK cells and plasma cytokine levels were evaluated and were compared with cytokine levels to determine their role in the pathogenesis of asthma.

Materials and Methods

Study Population

Newly diagnosed asthmatic patients (n:10), asthmatic patients under treatment (n:9) and healthy subjects (n:9)

were included in the study. All the patients with asthma fulfilled the Global Initiative of Asthma (GINA) criteria. The allergic status was demonstrated by means of positive immediate skin prick test responses to at least one of 10 common inhaled allergens (skin wheal response of >5 mm at 15 minutes) and they all had well controlled asthma. Newly diagnosed patients with mild-moderate asthma were not receiving any treatment (anti-inflammatory) for at least one month except beta agonists if necessary. Healthy control subjects exhibited no evidence of atopy and had negative skin prick tests or no bronchial hyperresponsiveness. They had no history of asthma or any other allergic diseases (e.g, rhinitis) or smoking. This study was approved by the Ethics Review Committee of the Istanbul University-Cerrahpasa in accordance with the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects (Date: 11.04.2007, Approval Number: 9149).

Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood by density gradient centrifugation over Ficoll (Sigma Chem. Co., St. Louis, MO). NK cells were purified by magnet-activated cell separation (MACS, Miltenyi Biotec AG, Bergisch Gladbach, Germany). Briefly, NK cells were isolated from PBMC by immunomagnetic depletion of T cells, B cells, monocytes and other myeloid cells such as basophils and dendritic cells according to expression of CD3, CD4, CD19 and CD33. The purity of NK cells was >98% as assessed by flow-cytometric analysis of cells stained with FITC-labeled anti-CD3, PE-labeled anti CD16+56 (BD Bioscience, San Jose, CA). CD3 positive T cell contamination in purified NK cells was <1%[9].

Intracytoplasmic Cytokine Staining

Freshly isolated NK cells resuspended in medium were stimulated with PMA (50 ng/ml) and Ionomycin (250 ng/ml). Monensin (1μL) was added to culture at the last 4 h. After the incubation, NK cells were washed with PBS and stained with anti-CD56-FITC (BD Bioscience, San Jose, CA) monoclonal antibody for 30 minutes. Samples were fixed and permeabilized according to manufacturerís directions (Caltag Laboratories, Austria) and cells were stained with intracellular anti- IL-13-PE, IL-4-PE, IFNγ-PE, IL-10-PE, RPE-conjugated isotype control (IC) antibodies (all from Caltag, Laboratories, Austria) for an additional 30 minutes. After washing, cells were fixed in 1% paraformaldehyde (Sigma Chem. Co., St Louis, MA) and stored at 4°C before flow-cytometric analysis.[19] Data acquisition and analysis were performed by BD FACSCalibur with CellQuest software (BD Bioscience, San Jose, CA) and WinMDI programs. Results are presented as mean values of percentages and standard deviation.

Multiplex Bead Immunoassay

Plasma samples were stored at -80°C immediately until laboratory analysis. Plasma levels of IFN-γ, TNF-α, IL-2, IL-8, IL-4, IL-5, IL-6, IL-17, IL12, IL-13, IL-1β and IL-10 were analyzed by using ProcartaPlex multiplex immunoassays panels, (Affymetrix, Luminex 100/200, eBioscience Austin, TX). All samples were analyzed in duplicate using 96-well plates according to manufacturer's instructions. Sample volume was 25 μL, and all samples were run neat (not diluted) per manufacturer's instructions. Plates were read using a Luminex MagPix instrumentation (Luminex Corporation, Austin, TX). Minimum bead count was 50 for each cytokine and data were analyzed by Milliplex Analyst 5.1 software (Luminex Corporation, Austin, TX).

Statistical Analyses

Statistical analyses were performed with SPSS (version 21.0; SPSS Inc., Chicago, IL, USA). A comparison of variables within the same group was performed with paired Student t-test or Wilcoxon test, whichever suitable. Variables between groups were compared by independent sample t-test or Mann Whitney U test according to the distribution of the data. Differences were considered statistically significant for p values less than 0.05.

Results

Intracytoplasmic Cytokine Levels

Intracytoplasmic cytokine levels of isolated NK cells were analyzed in newly diagnosed asthmatic patients, asthmatic patients under treatment and healthy control subjects by flow cytometry (Figure 1). Comparing to healthy controls the intracytoplasmic IL-4 levels of isolated NK cells, was found significantly higher in newly diagnosed and under treatment asthmatic groups (p=0.001 and p=0.0076), but IL-10 and IFN-γ (Figure 1) were significantly decreased for newly diagnosed and under treatment asthmatics (p=0.0007 and p=0.0172 for IL-10 and p=0.0001 and p=0.0435, respectively for IFN-γ). NK cells of newly diagnosed asthmatic patients had lower levels of intracytoplasmic IFN-γ compared to that of asthmatics under treatment (Figure 1, p=0.0007). Intracytoplasmic IL-13 was significantly higher in newly diagnosed asthmatic patients compared to both healthy

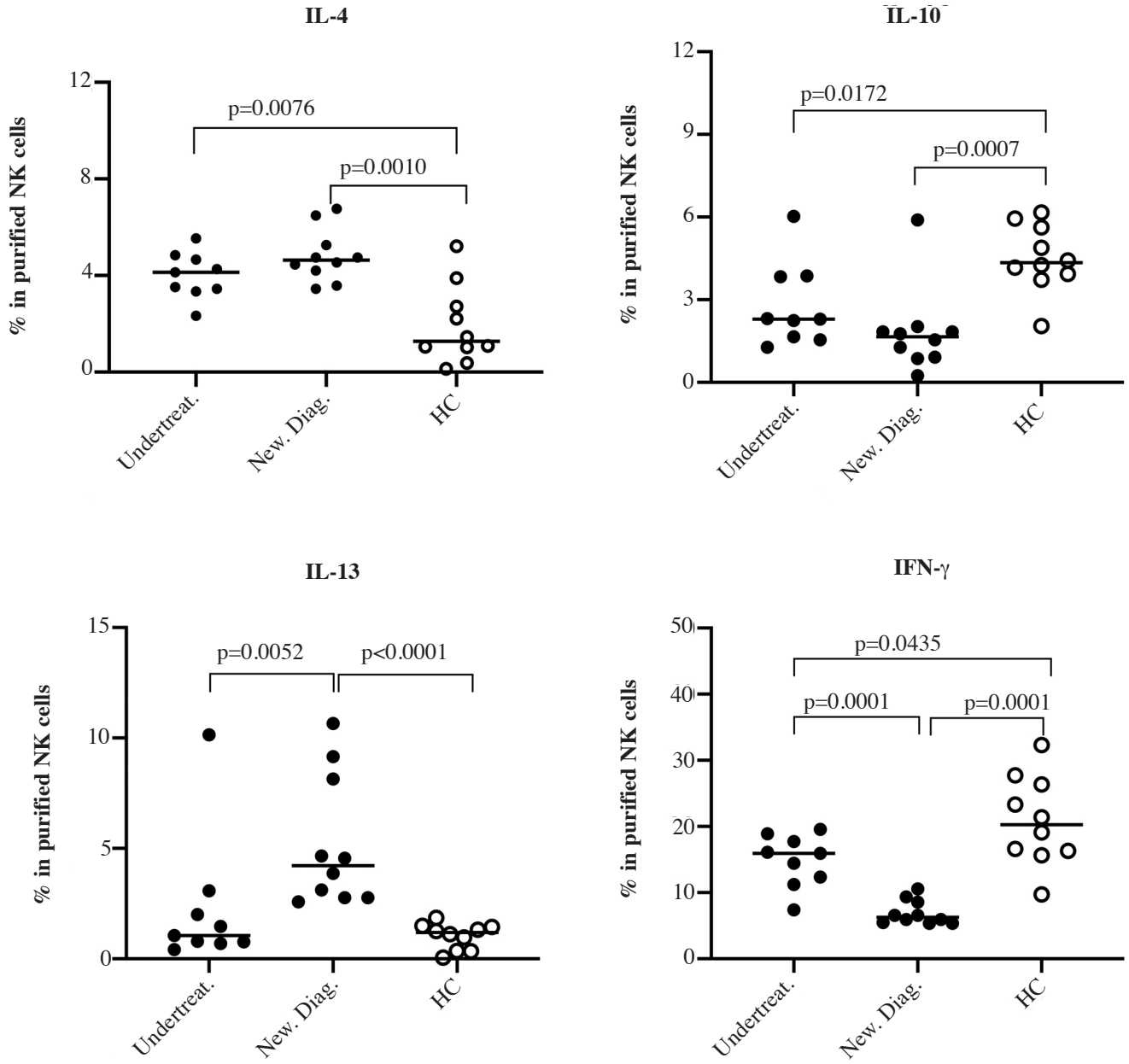

Figure 1. Intracytoplasmic cytokine levels of isolated NK cells in newly diagnosed asthmatic patients, asthmatic patients under treatment and healthy control subjects. HC: Healthy control.

subjects and asthma under treatment group (Figure 1, p<0.0001 and p=0.0052, respectively).

Plasma Cytokine Levels

The mean fluorescence intensity (MFI) values for IL-5, IL-8, IL-12, IL-13 plasma levels were significantly higher in newly diagnosed asthmatic patients compared to healthy subjects (p=0.00034, p=0.0109, p=0.00021 and p=0.0020, respectively), but the MFI value for IL-10 showed a decrease in newly diagnosed asthmatic patients compared to healthy controls (p=0.00094) (Figure 2).

MFI values of IL-2 plasma levels in both asthmatic groups were found to be lower compared to that of healthy controls (p=0.0198 for newly diagnosed and p=0.0319 for under treatment asthmatic patients).

IL-8 plasma levels measured by MFI values were

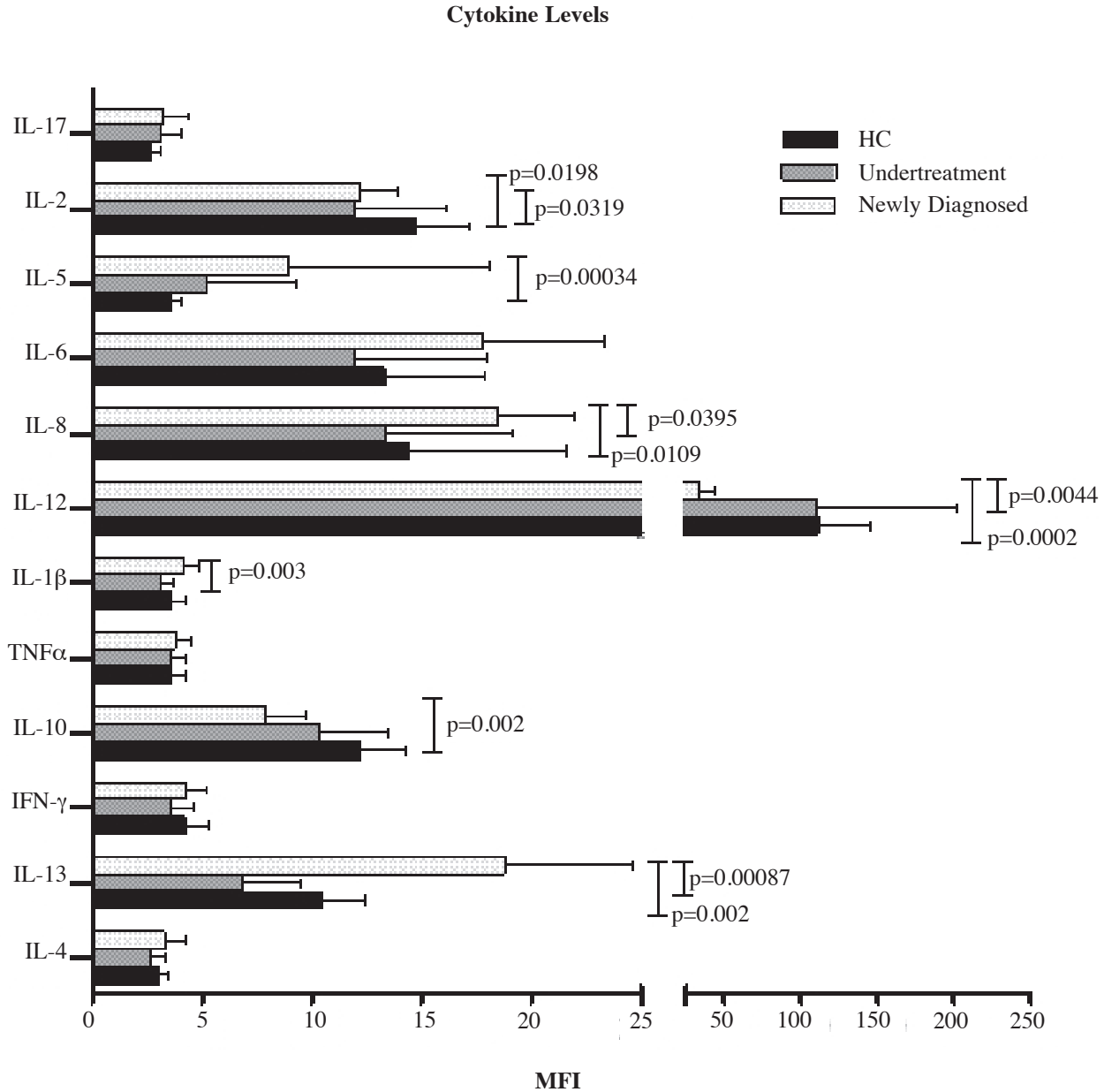

Figure 2. Plasma cytokine levels in newly diagnosed asthmatic patients, asthmatic patients under treatment and healthy control subjects.

increased in newly diagnosed asthmatic patients compared to those of asthmatic patients under treatment (p=0.0395), which was the opposite for IL-12 plasma levels (p=0.0044) (Figure 2).

MFI values for IL-1β and IL-13 plasma levels in newly diagnosed asthmatic patients' group were significantly higher compared to that of the asthmatic patients under treatment (p=0.003 and p=0.00067, respectively). Plasma levels of IL-4, IL-6, IL-17, IFN-γ, TNF-α did not show any difference among the three study groups (Figure 2).

Discussion

It has been shown that, elevated plasma TNF-α in late phase of severe asthma is suppressed by oral steroid therapy,[20] but TNF-α level did not show any difference in mild-moderate asthma.[21] Similar TNF-α levels in both asthmatic groups and healthy subjects were detected in our study, which may be due to the fact that the newly diagnosed patients consisted of mild to moderate asthmatics and the group under treatment consisted of individuals

using steroids or anti-inflammatory agents being similar to the previous findings.[20] The same was true for IL-6. Similar to the literature, elevated levels of IL-1β in newly diagnosed asthmatics compared to asthmatics under treatment were observed.[22]

IL-8 was also found to be elevated in plasma and bronchial tissues especially in cases of severe asthma.[21] The newly diagnosed asthmatic patients in our study also showed significantly higher IL-8 levels compared to that of asthmatic patients having treatments and healthy subjects. This elevation in newly diagnosed asthmatics suggests that providing it might induce neutrophils to migrate to the area of inflammation at the beginning of the disease.

IL-4 has been found to be increased in plasma of atopic cases.[23] But we were not able to detect any significant difference in IL-4 levels between our study groups. This may probably be due to the fact that the newly diagnosed asthmatic patients in our study have mild-moderate asthma severity and the other asthmatic group was having steroid treatment. On the contrary, IL-5 was detected in higher levels even in patients with mild or moderate asthma,[24] and reduction of IL-5 by steroid therapy has been shown to reduce the number of eosinophils. Similarly, the present study showed that higher levels of IL-5 was found in newly diagnosed asthmatic patients, but IL-5 levels did not show any statistical difference between the asthmatics under treatment and healthy controls.

Many studies have shown that not only IL-13 plasma levels but also mRNA expression in the mucosal tissue is increased in asthma.[25] In our study, we also detected high levels of IL-13 in newly diagnosed asthma cases compared to that of those under treatment and controls, and in control subjects compared to patients with asthma under treatment. The decreased IL-13 levels after treatment supports the idea that the important contribution of IL-13 to asthma pathogenesis, which suggests effective individualized therapy approaches blocking specific inflammatory pathways using monoclonal antibodies against IL-13 and IL-4.

IL-10 has been shown to reduce in BALF at mRNA and protein levels in asthma.[26] In contrast, there are studies reporting higher plasma IL-10 and mRNA levels in asthma.[27] In this study, IL-10 levels were significantly decreased in newly diagnosed asthmatic group compared to healthy controls, but there was no difference between asthmatics under treatment and control subjects. In general, airway inflammation is expected to increase in cases with decreased IL-10 levels known to be inversely correlated with the severity of allergic asthma.[28] Similar to the reported finding, IL-10 levels in allergic asthma after treatment were similar to healthy individuals in our study.

In particular, IFN-γ secreted by Th1 cells was found to be low in Th2-dominant allergic asthma, but there was no association in terms of IFN-γ gene polymorphisms in asthma.[29] Exogenous IFN-γ or pulmonary epithelial IFN-γ gene transfer was demonstrated to suppress allergic response in animal studies.[30] This finding was reported to be due to the enhancing effect of IFN-γ on IL-10 production. IFN-γ synthesis by T cells has been shown to increase after immunotherapy or steroid treatment in asthma.[31,32] The lack of difference in IFN-γ between groups of new-onset asthma, asthma under treatment and control groups in our study might be explained by the inverse correlation of IFN-γ with the severity of the disease.[33] This finding may also be explained by the disease groups consisting of mild moderate asthmatics and by the medication of the asthmatics under treatment.

IL-12 production was expected to be low in allergic patients with Th2 polarization compared to that of non-allergic asthmatic patients.[34]

In our study, IL-12 levels were decreased in newly-diagnosed asthmatic group compared to controls, but they were found to be similar in asthmatics under treatment and that of controls. The increase of IL-12 levels after treatment and the Th2 shift of the immune response in new-onset asthma were in accordance with the previous finding.

This has been shown in animal studies and it was demonstrated that IL-12 could inhibit eosinophilia, IgE synthesis and airway reactivity in recurrent allergen exposure.[35]

IL-2, as a lymphocyte growth factor, was reported to be especially increased in BALF of asthmatic patients with steroid-resistant asthma compared to steroid sensitive asthmatics, and lymphocytes expressing IL-2 receptor were elevated in severe acute asthma.[11] IL-2 levels in our study were low in both asthmatic groups compared to controls. This decrease might be attributable to the fact that our patients consist of allergic asthmatics with Th2 predominance.

IL-17 has been shown to be associated with many inflammatory diseases.[36] This finding supports the idea that neutrophils could participate the inflammation and the number of neutrophils increased in BALF of resistant asthmatic patients. Plasma IL-17 levels was found to be correlated with the severity of the airway hyperresponsiveness,[37] and for this reason IL-17 levels

are suggested to be a good marker to differentiate asthma phenotypes.[36] The lack of any difference of IL-17 levels in our study groups might be explained by the fact that the disease groups consisted of mild to moderate asthmatic patients with allergic background.

In this study we also determined intracellular levels of IFN-γ, IL-4, IL-10 and IL-13 in NK cells in newly-onset asthmatic patients, asthmatics under treatment and healthy controls.

IFN-γ^+ NK1 cells were decreased in both groups with asthma, but comparing both asthmatic groups, the number of IFN-γ^+ NK1 cells was higher in asthmatics under treatment possibly due to anti-inflammatory medication. Another study reported similar results indicating an increase of IFN-γ^+ $CD56^+$ NK1 cells and a decrease of IL-4^+ $CD56^+$ NK2 cells in peripheral blood of asthmatic patients after treatment.[38] The intracellular IL-4 level of NK cells was higher in both patients' groups with asthma compared to healthy controls and this finding was similar to the results those of Mesdaghi et al.[39] In our study, the number of NK cells secreting the immunoregulatory cytokine IL-10, was found to be lower in both patient groups with asthma compared to those of healthy controls. The decrease in the release of the regulatory cytokine IL-10 from NK cells in both asthmatic groups might be explained by the possible broken tolerance to allergens.

IL-13 secreting NK2 cells are shown in PBMCs from patients with allergic asthma following allergen exposure.[40] Similar to the literature, we also detected increased numbers of IL-13+ NK2 cells in newly-onset asthmatic group compared to those of asthmatics under treatment and healthy subjects. This finding is parallel to the study performed by Katsumoto T et al. indicating that in vitro differentiated NK2 cells secreted significant amounts of IL-13 and IL-5, however, they did not produce IL-4.[41] They also found that NKr1 (NK1 regulatory) cells secrete IL-10 and IFN-γ.[41]

In conclusion, the importance of NK function in allergic diseases including asthma is far from completion. Further investigations especially on the cytokines of NK cells modifying immune responses in allergic asthma are needed to fill this gap of knowledge.

Ethics Committee Approval: This study was approved by the Ethics Review Committee of the Istanbul University-Cerrahpaşa in accordance with the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects (Date: 11.04.2007, Approval Number: 9149).

Conflict of Interest: None of the authors has any potential financial conflict of interest related to this manuscript. On behalf of all authors, the corresponding author states that there is no conflict of interest.

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