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BASIC MEDICAL SCIENCES / TEMEL TIP BİLİMLERİ

Effects of Interleukin-33 on Neopterin and IP-10 Production in Macrophages

Makrofajlarda Neopterin ve IP-10 Üretimine İnterlökin-33'ün Etkileri

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Abstract

Objectives: Interleukin-33 (IL-33) is a pleiotropic cytokine from the IL-1 family. It is known to cause plasticity in the Th2 direction. The macrophages, important cells of the innate immune system, express the IL-33 receptor ST2L and exhibit M2 macrophage polarity under the influence of IL-33. Macrophages produce interferon gamma-stimulated protein 10 (IP-10), an interferon-γ-induced chemokine, and neopterin which exhibits cellular immunoreaction. The aim of this study was to investigate the effects of IL-33 on neopterin and IP-10 production in unstimulated and pre-stimulated macrophages with different cytokines.

Materials and Methods: The J774.1 macrophage cell line was used. J774.1 macrophages were stimulated by IFN-γ (40 U/mL) + LPS (10 ng/mL) after pretreatment with IL-33 (100 ng/mL). Nitrit levels were measured by the Griess reaction. To examine the effect of IL-33 on polarized macrophages, J774.1 macrophages were stimulated with IL-4, IL-10 ve IL-13 cytokines to provide M1, M2a and M2c macrophage polarization. Neopterin and IP-10 measurements were carried out by ELISA method. Statistical analysis was performed with SPSS 18 software.

Results: IL-33 was found to significantly increase neopterin production in unstimulated and plasticity-affected J774.1 cells. There was no change in IP-10 levels in all experiments. No correlation was found between neopterin and IP-10 levels.

Conclusion: Our present study is the first study on the effects of IL-33 cytokine on neopterin production. IL-33 causes a significant increase in neopterin production in J774.1 cells.

Key Words: Interleukin-33, IP-10, Neopterin, Macrophage, ELISA

Öz

Amaç: İnterleukin-33 (IL-33), IL-1 ailesinden pleiotropik bir sitokindir. Th2 yönünde plastisiteye yol açtığı bilinmektedir. Doğal bağışıklık sisteminin önemli hücreleri olan makrofajlar, IL-33 reseptörü ST2L'yi ifade eder ve IL-33 etkisi altında M2 makrofaj polaritesi sergiler. Makrofajlar, interferon-γ ile indüklenen bir kemokin olan interferon gama ile uyarılan protein 10 (IP-10) ve hücresel immünoreaksiyon gösteren neopterin üretir. Bu çalışmanın amacı, uyarılmamış ve farklı sitokinlerle önceden uyarılmış makrofajlarda IL-33'ün neopterin ve IP-10 üretimi üzerindeki etkilerini araştırmaktır.

Gereç ve Yöntem: J774.1 makrofaj hücre dizisi kullanıldı. J774.1 makrofajları, IL-33 (100 ng/mL) ile ön işlemden sonra IFN-γ (40 U/mL) + LPS (10 ng/mL) ile uyarıldı. Nitrit seviyeleri Griess reaksiyonu ile ölçüldü. IL-33'ün polarize makrofajlar üzerindeki etkisini incelemek için J774.1 makrofajları, M1, M2a ve M2c makrofaj polarizasyonu sağlamak üzere IL-4, IL-10 ve IL-13 sitokinleri ile uyarıldı. Neopterin ve IP-10 ölçümleri ELISA yöntemi ile yapıldı. İstatistiksel analiz SPSS 18 yazılımı ile yapıldı.

Bulgular: IL-33'ün, uyarılmamış ve plastisitesi etkilenmiş J774.1 hücrelerinde neopterin üretimini önemli ölçüde artırdığı bulundu. Tüm deneylerde IP-10 seviyelerinde değişiklik olmadı. Neopterin ve IP-10 seviyeleri arasında bir korelasyon bulunmadı.

Sonuç: Bu çalışmamız, IL-33 sitokinin neopterin üretimi üzerindeki etkileri üzerine yapılan ilk çalışmadır. IL-33, J774.1 hücrelerinde neopterin üretiminde önemli artışa neden olmaktadır.

Anahtar Kelimeler: İnterlökin-33, IP-10, Neopterin, Makrofaj, ELİZA

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Introduction

Macrophages are the main effector cells of the innate immune system that participate in the inflammatory response against microbial pathogens. Tissue macrophages contribute to tissue development, metabolism, homeostasis, and repair. Tissue-resident macrophages are polarized by tissue cytokines, chemokines, and hormonal stimuli. Macrophages are classified into 2 subgroups termed classically activated macrophages (M1) and alternatively activated macrophages (M2) (1). Macrophages are also stimulated by interleukin-33 (IL-33). This interaction is mediated by the macrophage-expressed IL-33 ligand suppression of tumorigenicity 2 (ST2). IL-33/ST2 signaling is involved in alternative activation of type 2 macrophage (M2) polarization (2,3).

IL-33 was first identified in 2005 and classified as a new member of the IL-1 family. While IL-1α and IL-18 from these family members are immunomodulatory agents for Type I immune response, IL-33 leads to Th2 plasticity towards Type II response (4). IL-33 is quickly released upon tissue damage, exposure to pathogens, stress, or in necrosis-induced death, and subsequently mediates immune response as an alarmin cytokine. IL-33 plays critical roles in maintaining host homeostasis and in pathological conditions, such as allergy, infectious diseases, and cancer, by acting on multiple types of immune cells and promoting type 1 and 2 immune responses (3,5).

The C-X-C motif chemokine10, also known as interferon-γ inducible protein 10 (IP-10), is a chemokine that modulates innate and adaptive immune responses by recruiting inflammatory cells to the sites of inflammation. IP-10 is secreted by several cell types, such as leukocytes, monocytes, neutrophils, eosinophils, and epithelial cells, in response to IFN-γ (6). IP-10 is exhibits pleiotropic effects on a wide range of biological processes, including immunity, angiogenesis, and tumor metastasis (7).

Neopterin is a guanosine triphosphate metabolite with a low molecular weight, which is a member of the pteridine family. It is produced in human macrophages and dendritic cells (8,9). IFN-γ is the strongest stimulus of the GTP cyclohydrolase-I enzyme. For this reason, neopterin concentrations are an important indicator of IFN-γ activity (10). Therefore, examination of neopterin is important to detect cellular inflammatory response and Type-1 inflammatory events.

In this study, we aimed to elucidate the effect of IL-33 on neopterin and IP-10 production in the macrophage cell line J774.1. The effects of IL-33 on macrophages on neopterin production have not been investigated previously and this is the first study in this area. Therefore, mrIL-33 (recombinant murine) cytokine was added to cell cultures with or without stimulation of IFN-γ and LPS. Subsequently, neopterin, IP-10, and nitrite production of macrophages were measured. In addition, it has been investigated whether there is a relationship between these productions.

Materials and Methods

Cell and Cell Culture

J774.1 macrophage cell line (The American Type Culture Collection - ATCC) which was frozen in a liquid nitrogen tank at approximately -196 °C, in RPMI containing 10% DMSO (Gibco, USA), was used. Serial passage of J774.1 macrophage cell line was performed in RPMI-1640 (Gibco, USA) medium supplemented with 10% fetal bovine serum (FCS, Gibco, USA) and penicillin/streptomycin (Pen Strep, Gibco, USA) at 37 °C in a humidified atmosphere of 5% CO_2.

For stimulation experiments, mouse J774.1 macrophage cells grown in a 37 °C, 5% CO_2 incubator in cell culture flasks were excised with cell scrapers for fresh use in experiments. Before the experiments, the trypan blue deprivation test was performed for cell viability and the cells were counted in a thoma counting chamber. The 90% cell viability was considered sufficient for the next stage of experiments. For the experiments, dilutions were carried out to be $0.5x10^6$ viable cells per 1 mL. The cells were transferred to 24-well cell culture plates. A triplicate experiment was performed in order to evaluate the reproducibility of the data. mrIL-33 (100 ng/mL) (Ebioscience, USA), mrIL-4 (10 ng/mL) (Ebioscience, USA), mrIL-13 (10 ng/mL) (Life Sciences, USA), mrIL-10 (100 ng/mL) (Ebioscience, USA), mrIFN-γ (40 U/mL) (Ebioscience, USA), LPS (10 ng/mL) (Escherichia coli. Santa Cruz, USA) were determined as working concentrations by preliminary experiments.

Nitrite Measurement

Nitrite production was evaluated to check for stimulation in J7741 macrophages. 100 µL of cell culture supernatant was mixed with 100 µL of Griess reagent. At the end of the required study incubations, the absorbance at 540 nm was measured in a spectrophotometric microplate reader. The macrophage nitric oxide response was determined by measuring the level of nitrite accumulating in the culture supernatant with the Griess reagent (Sigma-Aldrich, USA).

Neopterin ELISA

We used a commercial neopterin enzyme immunoassay kit (Immuno Guide. Turkey) for quantitative analysis of the neopterin levels in the supernatants. Briefly, Neopterin had been pre-coated onto a microplate. Assay Buffer was added to the 96-well plate at a rate of 100 µL/well. Standards and samples were pipetted into the wells. It was covered with black adhesive film. The plate was incubated for 90 minutes at room temperature in the dark. Any Neopterin was bound by the

immobilized antibody and then any unbound substances were eliminated by washing three times. After washing, 100 µL of TMB Substrate was added to each well and incubated for 15 minutes at room temperature in the dark. Stop solution (100 µL) was added and the color development was stopped. Finally, the plate was evaluated spectrophotometrically using an ELISA reader at 450 nm wavelength within 15 minutes (BioTek ELx800, USA). The concentration in nmol/L was calculated from the standard curve. Sensitivity was 1 nmol/L.

IP-10 ELISA

Ready-to-use commercial sandwich ELISA kit was used for IP-10 (pg/mL) chemokine measurement (Mouse IP-10, YH Biosearch, China). Briefly, 120 µL of original standard + 120 µL of serial dilution from 120 µL of a standard diluent tube were prepared. Only the dilution solution was added to the blank wells. 50 µL of standard and 50 µL of conjugate were added to the standards. 40 µL of sample + 10 µL of IP-10 antibody + 50 µL of the conjugate was added to the wells of the samples. It was shaken slightly to mix and covered with adhesive film, the plate was incubated for 1 hour in an incubator at 37 °C. The plate, whose liquid content was emptied, was washed 5x with 350 µL of hand-washing solution. After washing, 50 µL of chromogen A + 50 µL of chromogen B solution was added to each well. It was incubated for 10 minutes in an incubator at 37 °C. The reaction was stopped by adding 50 µL/well stop solution to the plate and the color of the liquid in the wells turned from blue to yellow. The plate was evaluated spectrophotometrically using an ELISA reader (BioTek ELx800, USA) at 450 nm wavelength within 15 minutes. Sensitivity was 2.42 pg/mL.

Statistical Analysis

All groups were subjected to Levene's test of homogeneity within themselves, and ANOVA and Student's t-tests were performed among the groups as parametric tests if the data were homogeneous. For comparison of parametric data, ANOVA was used amongst multiple groups, and Student's t-tests were used between two groups. SPSS 18 software was used in evaluating the statistics of the data. The significance threshold value was accepted as $p<0.05$.

Results

Nitrite, neopterin, and IP-10 production of the J774.1 cell line at different stimulation doses and times

In our results, IFN-γ (4 U/mL) + LPS (10 ng/mL) and IFN-γ (40 U/mL) + LPS (10 ng/mL) stimulations increased the level of neopterin after 18 hours. A significant increase was established when the other groups were compared (15 min, 30 min, 60 min, 4 h) ($p<0.001$) (Figure 1a). It also caused an increase in neopterin production in 4 hour-treatment compared to 15 minute-treatment ($p<0.05$) (Figure 1a). There was no significant difference between IP-10 concentrations (Figure 1b). No significant correlation was observed between IP-10 production and neopterin levels ($r2=0.032$). In the high dose (1 µg/mL) LPS-treated groups, nitrite production increased during 18 hours of incubation ($p<0.05$). A significant increase in nitrite levels were observed in the IFN-γ (4 U/mL) + LPS (10 ng/mL) and IFN-γ (40 U/mL) + LPS (10 ng/mL) treatments during overnight incubation compared with the other times ($p<0.001$) (Figure 1c).

Evaluation of nitrite production stimulated by IFN-γ (40 U/mL) + LPS (10 ng/mL) and IL-33 (100 ng/mL) in J774.1 macrophages

When IL-33 was administered 18 hours before IFN-γ (40 U/mL) + LPS (10 ng/mL) stimulation, nitrite production was significantly inhibited compared to at the same time and after 4 hours (0 and +4) administration ($p<0.001$). When IL-33 was administered -1 and -4 hours before IFN-γ (40 U/mL) + LPS

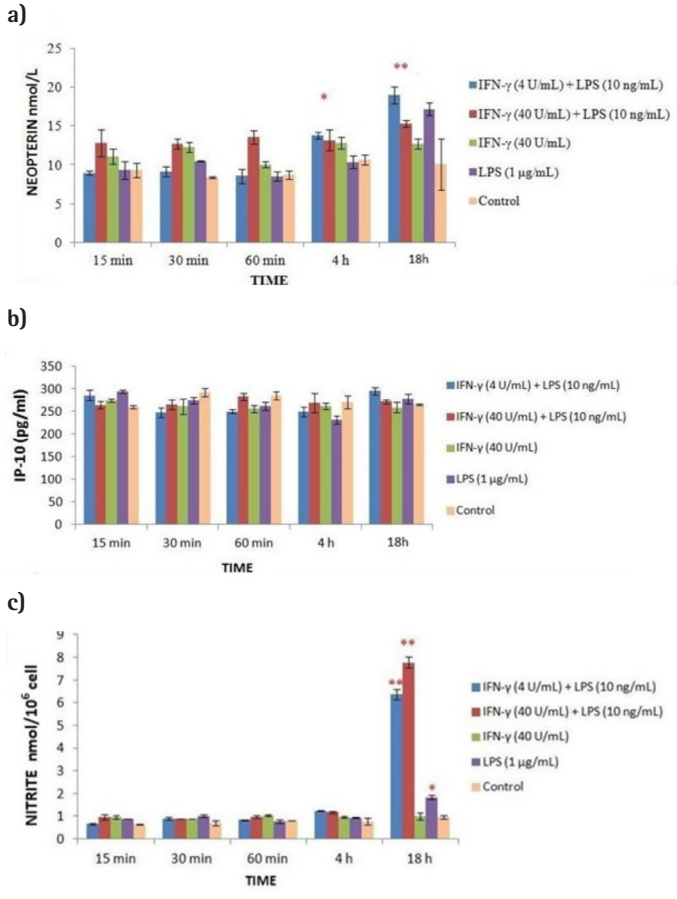

Figure 1: Neopterin, IP-10, and nitrite production in J774.1 macrophages at different stimulation doses and incubation times

a) Time-dependent neopterin concentration (*: $p<0.05$ compared with 15 min. time group, **: $p<0.001$ compared with the all-time groups) **b)** Time-dependent IP-10 concentration. **c)** Time dependent nitrit concentration (*: $p<0.05$ compared with control, **: $p<0.001$ compared with control)

(10 ng/mL) stimulation, nitrite production was significantly inhibited compared to 0 and +4 hour administrations (p<0.05). IL-33 pretreatment has been shown to inhibit nitrite production in J774.1 macrophages. It was observed that only IL-33 cytokine administration did not affect nitrite levels (Figure 2).

Neopterin production stimulated with IFN-γ (40 U/mL) + LPS (10 ng/mL), LPS (1 µg/mL), and IL-33 (100 ng/mL) after pretreatment IL-33 (100 ng/mL) in J774.1 macrophages

When IL-33 was administered 18 hours ago, it caused a significant increase in neopterin production in all groups IFN-γ (40 U/mL) + LPS (10 ng/mL), IL-33, LPS (1 µg/mL) (p<0.001) (Figure 3a). When IL-33 was administered 18 hours ago, there was no significant difference in IP-10 concentrations amongst groups in a time-dependent manner (Figure 3b). The stimulation didn't change the IP-10 level in the cells.

Neopterin production in IFN-γ (40 U/mL) + LPS (10 ng/mL)-stimulated J774.1 macrophages after IL-33 (100 ng/mL) pre-treatments

When IL-33 was treated 18 hours before IFN-γ (40 U/mL) + LPS (10 ng/mL) stimulation, it caused a significant increase in neopterin production (p<0.001) (Figure 4). This effect was observed many times in our experiments in IL-33 treatments. Therefore, we observed the neopterin-enhancing effect of IL-33 cytokine in culture for 36 hours and longer.

The effects of IL-33 on nitrite, neopterin, and IP-10 levels in the J774.1 macrophages

IL-33 treatment alone did not affect nitrite production after 2 overnight incubations (p>0.05) (Figure 5a). IL-33 cytokine showed a neopterin-increasing effect in culture treatments of 36 hours or more. It was observed that neopterin concentrations increased significantly after two overnight (42 hour) incubations (p<0.001) (Figure 5b). IP-10 production was not affected by long-term IL-33 stimulation (p>0.05) (Figure 5c).

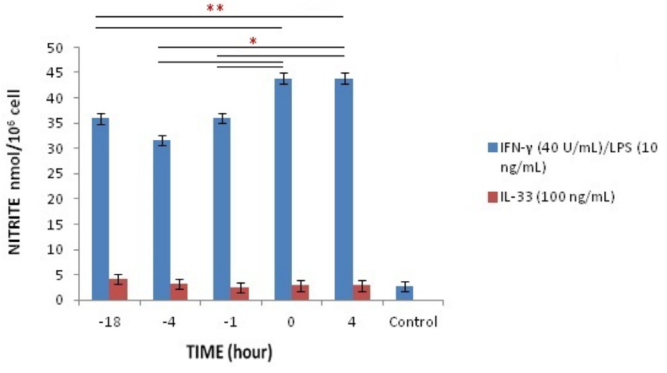

Figure 2: Nitrite production in J774.1 macrophages stimulated with IFN-γ (40 U/mL) + LPS (10 ng/mL) after preliminary administration of IL-33 (100 ng/mL) (*: p<0.05 compared with -4 and -1, time group, **: p<0.001 compared with -18 time group). Control: Untreated (-18= 18 hours before stimulation) (-4= 4 hours before stimulation) (-1= 1 hour before stimulation) (0= stimulation time) (+4= 4 hours after stimulation)

The effects of IL-4, IL-13, IL-4 + IL-13 and IL-10 on neopterin and IP-10 levels in the J774.1 macrophages

J7741 macrophages were primarily stimulated with appropriate cytokines such as IL-4, IL-10, and IL-13 so that they could be polarized towards M1, M2a, and M2c. Compared with control groups with no stimulation at 0 and 48 hours, neopterin levels were not affected by IL-4 (p>0.05). A statistically significant difference was found between 4-hour stimulation and 48-hour stimulation in neopterin production of IL-4 cytokine (p<0.001). This suggested that inhibition of the IL-4 cytokine on neopterin production might be temporary (Figure 6a). It was observed that

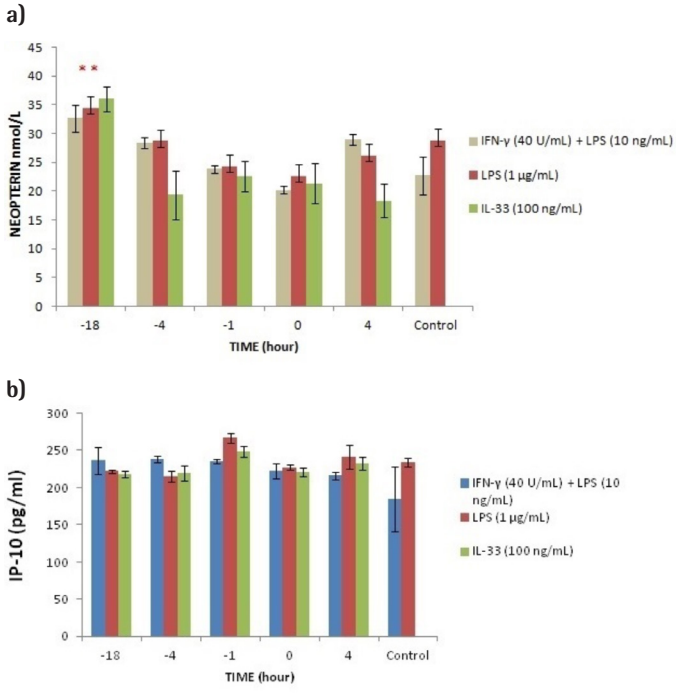

Figure 3: a) Neopterin production in J774.1 macrophages stimulated with IFN-γ (40 U/mL) + LPS (10 ng/mL) and LPS (1 µg/mL) after preliminary administration of IL-33 (100 ng/mL) (**: p<0.001 compared with control). Control: Untreated. **b)** IP-10 production in J774.1 macrophages stimulated with IFN-γ (40 U/mL) + LPS (10 ng/mL) and LPS (1 µg/mL) after preliminary administration of IL-33 (100 ng/mL) Control: Untreated

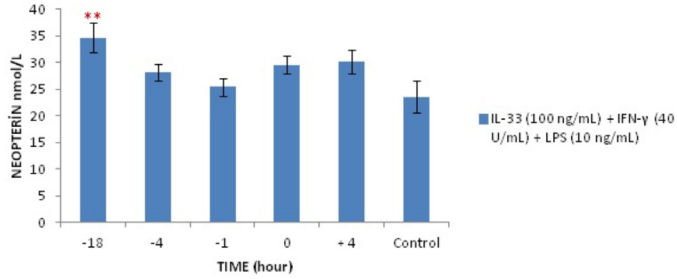

Figure 4: Neopterin production in J774.1 macrophages stimulated with IFN-γ (40 U/mL) + LPS (10 ng/mL) after preliminary administration of IL-33 (100 ng/mL). A statistically significant increase was found in the 18-hour incubation treatment when compared to the other stimulated groups (**: p<0.001 compared with control.) Control: Untreated

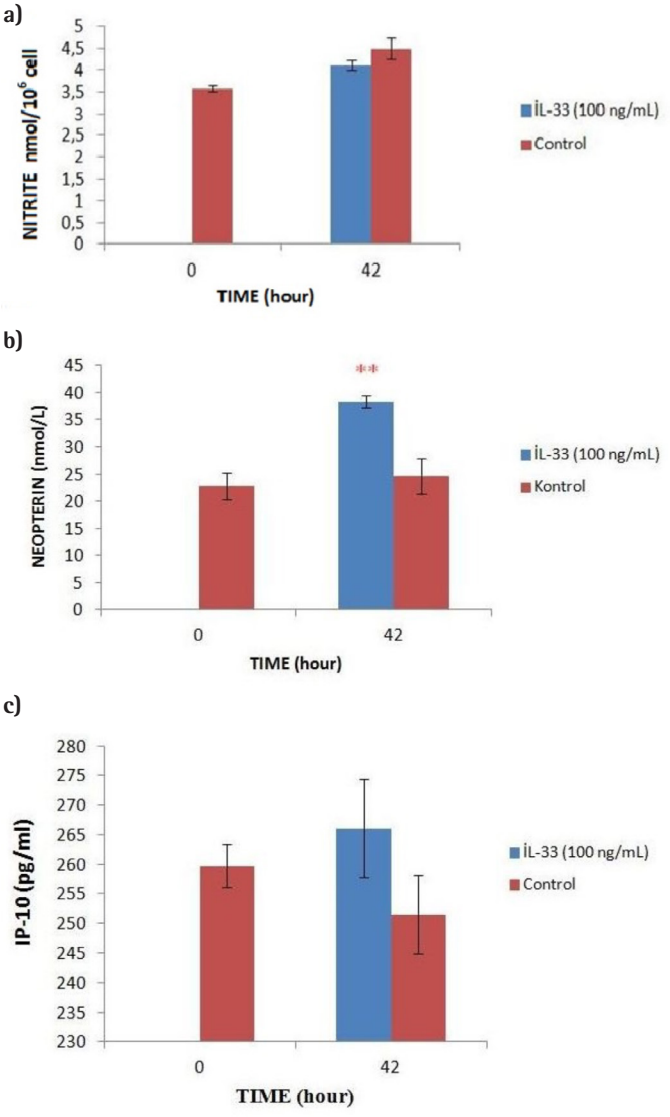

Figure 5: Nitrite, neopterin, and IP-10 production after 42 hours in J774.1 macrophages stimulated with IL-33 (100 ng/mL). **a)** Nitrit concentration. **b)** Neopterin concentration (**: p<0.001 compared with control.) **c)** IP-10 concentration

IL-10, IL-13, and IL-4 + IL-13 cytokine administrations did not affect dependent to time the neopterin levels (p>0.05). It was observed that IL-4, IL-13, IL-4 + IL-13, and IL-10 did not affect dependent to time the IP-10 levels (p>0.05).

The effects of IL-33 on neopterin and IP-10 levels in the IL-10 and IL-4 + IL-13 pre-stimulated J774.1 macrophages

When J774.1 macrophages had been pre-stimulated by IL-10 and IL-4 + IL-13 for 12 hours, they significantly increased neopterin levels compared to the control after 24 hours of stimulation with IL-33 (p<0.01). We observed that after 48 h neopterin was more elevated similar to our first observed results (p<0.001) (Figure 6b). IP-10 levels were not affected compared to the control (p>0.05).

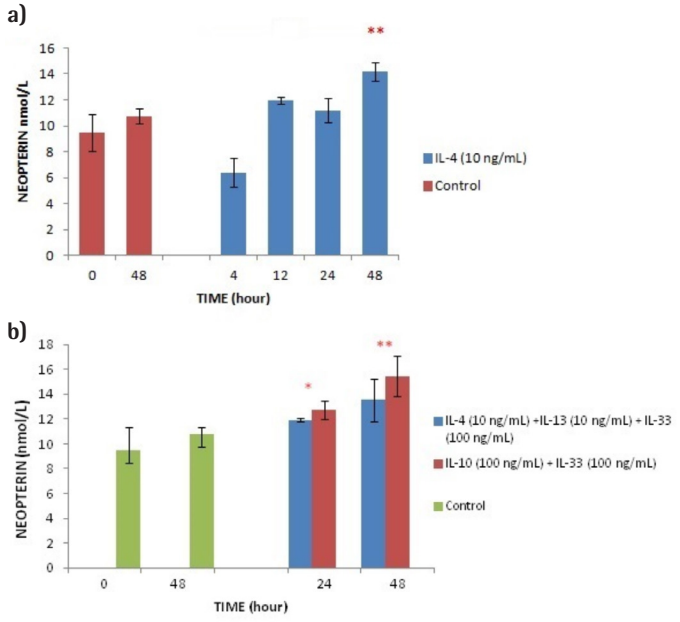

Figure 6: Neopterin production in J774.1 macrophages stimulated with IL-4, IL-10, and IL-4 + IL-13. **a)** Neopterin concentrations in J774.1 macrophages stimulated with IL-4 (10 ng/mL) (**: p<0.001 compared with 4 hours). **b)** Neopterin concentrations in J774.1 macrophages stimulated with IL-33 (100 ng/mL) after preliminary administration of IL-4 (10 ng/mL) + IL-13 (10 ng/mL) and IL-10 (100 ng/mL). (*: p<0.01 compared with control) (**: p<0.001 compared with control)

Discussion

In this study, the effect of IL-33 on neopterin and IP-10 production was investigated in macrophage cell line J774.1. Although there were many studies showing neopterin production in human macrophages, neopterin production in mouse macrophages was a controversial issue. Neopterin production was shown in the THP-1 human monocyte cell line stimulated with IFN-γ and LPS, and NO production was shown in the RAW 264.7 and J774.1 mouse macrophage cell lines (10,11). It has been reported that neopterin was produced in mouse macrophages. Production of NO and biopterin has also been demonstrated in the RAW 264.7 mouse macrophage cell line (12). In our study, it was detected that J774.1 macrophages produced neopterin. In addition, it was established that the production of neopterin increased after 18 hour-stimulation with IFN-γ + LPS of J774.1 macrophages.

IFN-γ was also known to stimulate the production of IP-10, another inflammatory marker (7,13). In a study by Tighe et al. (14), IFN-γ was found to stimulate IP-10 production in macrophages. It was also shown that treatment of LPS to peritoneal macrophages stimulated IP-10 *in vivo* as well as *in vitro*. Viral dsRNA analog poly I:C had been shown to increase IP-10 production in THP-1 cells and RAW264.7 macrophages (15). In our study, we showed that the J774.1 macrophage cell

line produced IP-10, but we found no significant difference in IP-10 concentrations amongst the groups of the time-dependent treatments. The difference might be due to the longer incubations were not used in this study.

Macrophages have been reported to constitutively express ST2, supporting the idea that IL-33 could stimulate and polarize macrophages towards M2, dependent on IL-13 or IL-4. When responding to LPS, IL-33-primed macrophages expressed increased levels of MD2 and TLR-4, thereby elevating its pro-inflammatory effect (16). Macrophages pre-stimulated with IL-33 have been reported to act in the Th1 direction (17). Classical information was that IL-33 was a cytokine that had effects on Th2 immunity. In recent studies, IL-33 had emerged as a cytokine with too many pleiotropic properties. It had been reported that IL-33 did not only increase Th2 immunity but also induced Th1 immunity, depending on the immune cells targeted by IL-33 (3,18). In our study IL-33 cytokine increased neopterin production in cells to which it was administered 18 hours ago. In addition, we found that IL-33 influenced the Type I immune response and that this effect was time-dependent. In our experimental results, it was revealed that IL-33 influenced the macrophages in favor of Type I immunoreactivity and stimulated IFN-γ induced neopterin production.

It had been shown in experimental autoimmune encephalitis that cells in IL-33-treated mice produced less IFN-γ and IL-17, but more IL-5 and IL-13, than cells in control mice. In the same study, cytokines such as IL-10, TNF-α, IL-6, and IL-4 and chemokines such as IP-10, MCP-1, MIG, and VEGF were detected at low levels and no significant difference was found between the two groups (19). The response to treatment with different concentrations of IL-33 in serum and Fibroblast-like synoviocytes of patients with RA was evaluated *in vitro*. In the synovium of patients with RA, a decrease in IL-6, IL-8, IL-1β, MIG, and IP-10 cytokines and chemokines was observed as the dose of IL-33 increased, but not in the serum (20). Although IL-33 had effects altering according to the cytokine environment it is also a cytokine with effects in Th2 direction in macrophages. These results suggested that IL-33 did not affect IP-10 production among Th1 chemokines in naive macrophages. IP-10 values were not affected depending on treatments with IL-33 made at different times. In humans, IL-33 might potentially act as a critical regulator of innate immune responses. However, IL-33 might not have the same effects in mouse macrophages.

Pre-polarized M1 cells have been reported to exhibit the M2 phenotype after exposure to IL-13. Similarly, cells previously in the M2 phenotype were reported to exhibit the M1 phenotype after LPS + IFN-γ treatment (21). Another study found that IL-33 strongly increased mannose receptor (MR) expression, but not TLR2, in the presence of IL-13. It has been shown that IL-33 and IL-4 have a similar synergistic effect on MR expression (22). The plasticity of macrophages indicated that different phenotypes could synthesize products different from their phenotypic characteristics (23). The findings showed that macrophages polarized in the M2a direction were forced from the M2a direction towards the M1 direction when they encounter IL-33. Our study also showed that the synthesis of neopterin produced by M1 macrophages was also stimulated by a cytokine in the M2 phenotype.

Study Limitations

The main limitation of our study, in which we evaluated the effects of IL-33 on neopterin and IP-10 production in macrophages, was that it was performed only in the J7741 macrophage cell line. It would be appropriate to perform the same experiments on RAW 264 and THP-1 macrophages.

Conclusion

Our study demonstrated that IL-33 potentiated alternative-activated macrophage development during innate and adaptive immune responses and could achieve classically activated macrophage activation in different immune conditions. IL-33 caused a significant increase in neopterin production in J774.1 cells. No correlation was observed between neopterin levels and IP-10 inflammatory markers induced by IFN-γ in J774.1 macrophages. Future *in vivo* studies are needed to elucidate the immunological significance of the effects of IL-33 on neopterin and IP-10 production in macrophages.

Ethics

Ethics Committee Approval: The study was performed using a cell line. Does not include human and animal testing. Therefore, ethical committee approval is not required.

Informed Consent: Patient samples were not used in this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: R.A., C.A., H.T., V.B., Design: R.A., C.A., H.T., V.B., Data Collection or Processing: R.A., C.A., V.B., Analysis or Interpretation: R.A., H.T., V.B., Literature Search: R.A., V.B., Writing: R.A.

Conflict of Interest: There is no conflicts of interest with respect to the authorship and/or publication of this article.

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