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# EFFECTS OF IL-17 ON FUNCTIONAL ACTIVITY OF PERIPHERAL BLOOD CELLS

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*Interleukin-17 (IL-17) is a proinflammatory cytokine produced mainly by activated $CD4^+$ and $CD8^+$ T cells, while its specific receptor is ubiquitously distributed. The inflammatory capacity of IL-17 is based on its ability to stimulate a wide range of stromal cells to produce and release a number of proinflammatory mediators, some with a known impact on hematopoiesis, particularly granulopoiesis. Recent data indicate a role for IL-17 in the pathogenesis of several inflammatory diseases, transplant rejection and tumor growth. The purpose of this study was to determine functional responses, including the respiratory burst, nitric oxide (NO) production, adhesiveness and metabolical activity/viability of human peripheral blood leukocytes (total white blood cells, mononuclear cells and granulocytes) from healthy donors in the presence of recombinant human (rh)IL-17. The obtained results showed that rhIL-17 did not induce significant changes in the respiratory burst, NO production, and metabolical activity of each peripheral blood cell fraction the tested, while a slight increase in phorbol-12-myristate-13-acetate (PMA) stimulated adhesiveness of granulocytes and mononuclear cells was noted. The absence of significant changes in tested functional activities of various peripheral blood cells suggests that IL-17 does not express its proinflammatory ability in steady-state, since the requirement for its action really does not exist.*

*Key words: cytokines, IL-17, inflammation, leukocyte functional activity*

## INTRODUCTION

IL-17 is a newly described cytokine, first identified as the human homolog of cytotoxic T lymphocyte-associated antigen 8, CTLA-8 (Rouvier *et al.*, 1993). Since CTLA-8 displayed some of the features of cytokine genes, such as the predicted hydrophobic $NH_2$-terminal sequence which could correspond to a signal peptide, and the presence of a 3' untranslated region of AU rich repeats associated with mRNA instability, it was subsequently renamed as IL-17. IL-17 is recognized as the prototype member of an emerging cytokine family, comprising least six members (IL-17 A, B, C, D, E, F), which together with the cognate receptors represent a

unique signaling system that appears to be highly conserved across vertebrate evolution (Aggarwal and Gurney*,* 2002). Human IL-17 is a 20-30 kDa homodimeric, variably glycosylated polypeptide mainly produced by activated $CD4^+$ and $CD8^+$ T cells, mostly of the memory $CD45RO^+$subset (Fossiez *et al.*, 1998). In contrast to its apparently restricted expression, the receptor for IL-17 shows an almost ubiquitous cellular distribution (Yao *et al.,* 1997).

IL-17 exhibits pleiotropic biological activities, attributed mostly to its ability to stimulate production of numerous biologically active molecules by a variety of cells, among which are hematopoietic and proinflammatory cytokines (Fossiez *et al.*, 1996). Accumulating evidence has shown that IL-17 is an important mediator of inflammation, especially in neutrophil-dominated responses to bacterial challenge (Witowski *et al.,* 2004). Nevertheless, even the first data indicated that IL-17 provides a link between T-cell immune responses and hematopoietic system. In their seminal paper Fossiez *et al.* (1996) demonstrated that IL-17 sustained the proliferation of human $CD34^+$ hematopoietic progenitors and their maturation into neutrophils *in vitro*, only in the presence of the fibroblast feeder layer. In addition, IL-17 overexpression *in vivo* induced granulopoiesis in mice (Schwarzenberger *et al.,* 1998).

Recent data support a role for IL-17 in the pathogenesis of several inflammatory diseases, transplant rejection and tumor growth (Schwarzenberger and Kolls*,* 2002). Elevated levels of IL-17 have been found in the synovial fluid from rheumatoid arthritis patients, in sputum and bronchoalveolar fluid in asthma as well as in colorectal tissues and serum of subjects with active inflammatory bowel disease (Kotake *et al*., 1999; Molet *et al*., 2001; Fujino *et al*., 2003). Its mRNA expression was increased in mononuclear cells in multiple sclerosis (Matusevicius *et al*., 1999) and in T lymphocytes isolated from psoriatic and atopic contact dermatitis lesions (Albanesi *et al*., 1999; Teunissen *et al*., 1998). It has been postulated that its contribution to these inflammatory processes is exerted by stimulation of a wide range of stromal cells to produce and release a number of proinflammatory mediators (Witowski *et al.,* 2004; Aggarwal and Gurney*,* 2002), among which are those that attract leukocytes, predominantly neutrophils, to the site of inflammation (IL-8, GRO$\alpha$, GCP-2, MCP-1), activate their effector functions (IL-1$\beta$, TNF$\alpha$) or stimulate granulopoiesis in bone marrow (IL-6, G-CSF, GM-CSF).

It is well known that upon stimulation the functional responses of activated leukocytes differ depending on the leukocyte cell subset and in addition to production of cytokines and other inflammatory mediators, may include shape change, adhesion, aggregation, chemotaxis, degranulation, phagocytosis and respiratory burst. The aim of this study was to analyze the effects of IL-17 on human peripheral blood leukocytes obtained from healthy volunteers. The respiratory burst, nitric oxide (NO) production, adhesiveness and metabolical activity/viability of total white blood cells (WBC), as well as granulocytes and mononuclear cell populations were investigated.

## MATERIAL AND METHODS

### *Separation of peripheral blood cells*

Peripheral blood was obtained from healthy persons. Separation of WBC was performed by mixing 6% HES (Hydroxyethylstarke, Plasmasteril, Frasenius, Hamburg, Germany) with heparinized venous peripheral blood at 1:7 ratio, followed by centrifugation at 2700rpm for 15 min. Peripheral blood mononuclear cells and granulocytes were separated using an isotonic discontinuous Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). Heparinized peripheral blood was diluted with 0.15 M NaCl at the ratio of 1:1 and over layered on Percoll solutions of specific gravity 1.09 and 1.07 g/ml. After centrifugation at 1300 rpm for 30 min at room temperature, mononuclear cell and granulocyte layers were recovered and subsequently removed. The remaining erythrocytes in the isolated cell fractions were lysed using isotonic $NH_4Cl$ solution (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4). Separated WBC, mononuclear cells and granulocytes were washed twice in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemicals Co., St. Louis, Mo., USA) and suspended in DMEM supplemented with 10% fetal calf serum (FCS, Sigma Chemicals Co., St. Louis, Mo., USA) to a concentration of $5x10^6$ cells/ml. The purity of mononuclear and granulocyte cell fractions was more than 98%, as determined by May-Grünwald-Giemsa staining.

### *NBT reduction assay*

The activation of each isolated peripheral blood cell fraction was evaluated by cytochemical assay for the respiratory burst (Monboisse *et al.*, 1991), measured by the intracellular reduction of nitroblue tetrazolium salt (NBT, Merck, Germany). Increasing concentrations of rhIL-17 (0, 50, 100, 500 ng/ml) (R&D Systems, Minneapolis, MN, USA) and NBT (10 μl, 5 mg/ml) were added to each cell suspension tested ($5x10^5$ cells/well, 100 μl), plated in 96-well plates and incubated for 30 min. For the measurement of spontaneous or stimulated NBT reduction, cells were incubated in medium only or in the presence of 50 ng/ml PMA (Sigma Chemicals Co., St. Louis, Mo., USA), respectively. Formazan produced by the cells was extracted overnight in 10% SDS-0.1N HCl at 37°C and was measured spectrophotometrically at 540 nm in an ELISA 96-well plate reader (Labsystems Multiskan PLUS, Finland).

### *Measurement of nitric oxide production*

WBC, mononuclear cells or granulocytes were plated, in 96-well plates ($5x10^5$ cells/well, 100 μl) along with increasing concentrations of rhIL-17 (0, 50, 100, 500 ng/ml) and cultivated for 24h in the presence or absence of 1000 ng/ml LPS (lipopolysaccharide from *E. coli*, serotype 055: B5 strain; Sigma Chemicals Co., St. Louis, Mo., USA). Production of nitric oxide, quantified by the accumulation of nitrite in the 24h culture medium, was measured spectrophotometrically using the Griess reaction with sodium nitrite as standard (Green *et al.*, 1982). Briefly, 50 μl of culture supernatants were mixed with equal

volumes of 1% sulfanilamide (Sigma Chemicals Co., St. Louis, Mo., USA) in 5% $H_3PO_4$ and 0.1% *N*-1-naphthylethylene diamine dihydrochloride (Sigma Chemicals Co., St. Louis, Mo., USA) in distilled water and after 10 min at room temperature, the absorbance at 540 nm was measured.

*Measurement of adhesion*

Adhesion of peripheral blood cells to plastic was assessed using a modified assay initially described by Oez *et al.* (1990). Peripheral blood cells were plated ($5x10^5$ cells/well, 100 μl) in 96-well plates in the presence of rhIL-17 (0, 50, 100, 500 ng/ml) and incubated for 60 min. For the measurement of spontaneous or stimulated adhesion, cells were incubated in medium only (spontaneous adhesion), or in the presence of 50 ng/ml PMA (stimulated adhesion). After this incubation, non-adherent cells were carefully removed by sequentially adding and aspirating the saline three times. Cells adhering to the plastic surface were fixed with methanol for 8 min and then stained with 0.1% crystal violet for 10 min at room temperature. The plates were washed three times in running water and left to air dry. The dye was dissolved in 200 μl of 33% acetic acid and the absorbance was measured at 540 nm in an ELISA 96-well plate reader.

*MTT assay*

Metabolic activity/viability of the peripheral blood cells was analyzed by a quantitative colorimetric assay in which 3-(4,5-dimethyl-thiazol-2yl)-2,5 diphenyl-tetrazolium bromide (MTT, ICN Biomedicals, Ohio, USA) was metabolically reduced to the colored end product, formazan (Mosmann *et al*., 1983). The MTT assay was conducted with freshly isolated cells in the presence of increasing concentrations of rhIL-17 (0, 50, 100, 500 ng/ml). WBC, mononuclear cells or granulocytes were added to the wells ($5x10^5$ cells/well, 100 μl) of 96-well plates. Immediately, MTT (10 μl, 5 mg/ml) was added and the cultures were incubated for 3h at 37°C in a humidified atmosphere. Formazan produced by the cells was dissolved during overnight incubation in SDS-HCl (10%SDS-0.1N HCl) and subsequently its absorbance was measured at 540 nm in an ELISA 96-well plate reader.

*Data display and statistical analysis*

Each test for every isolated peripheral blood cell fraction was performed at least three times. Statistical analysis was performed by Student's t-test, using the Origin PC Program with the actual numbers of each investigated parameter. A p value less than 0.05 was considered significant.

## RESULTS

*Effect of IL-17 on NBT reduction by peripheral blood cells*

The respiratory burst activity of peripheral blood cells upon stimulation with rhIL-17 (0, 50, 100, 500 ng/ml) was evaluated by a cytochemical test for respiratory

burst based upon the capacity of cells to reduce NBT (Monboisse *et al.,* 1991). NBT reduction occurs by a chemical reaction between the dye and the superoxide anion ($O_2^-$) generated by the activated respiratory burst NADPH oxidase. No significant changes in spontaneous NBT reduction by WBC, peripheral granulocytes and mononuclear cells were detected. in the presence of increasing concentrations of rhIL-17. However, the responses to IL-17 given by mononuclear cells and granulocytes were up to 20% higher in comparison to the spontaneous NBT reduction observed in medium only. PMA-stimulated NBT reduction by all tested peripheral blood cells was not affected significanlty in the presence of rhIL-17 (Figure 1).

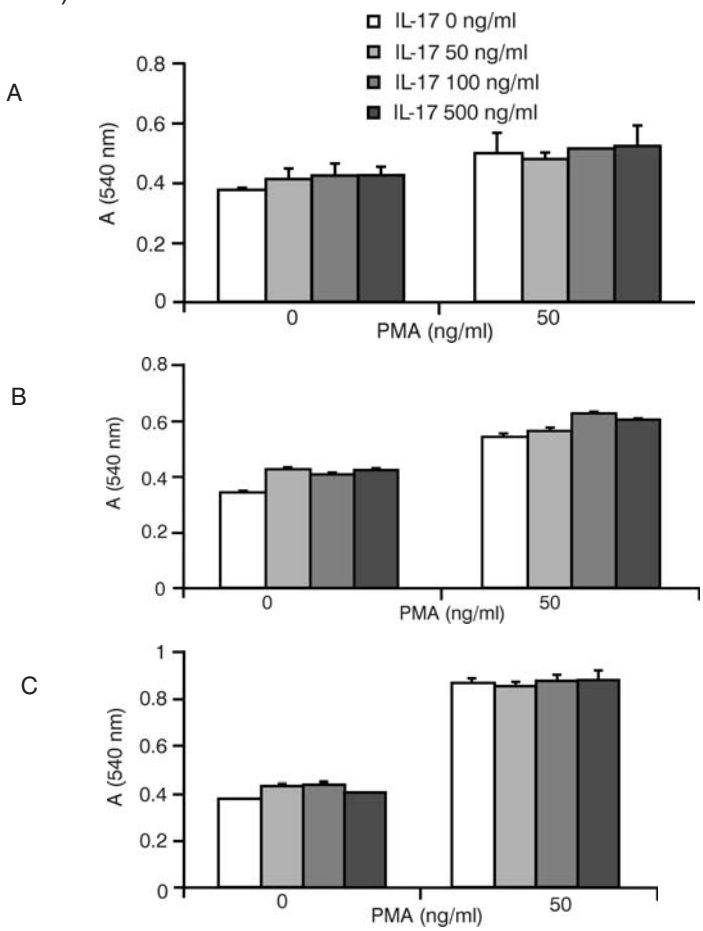


Figure 1. The effect of rhIL-17 on NBT reduction by human peripheral blood cells. WBC (A), mononuclear cells (B) and granulocytes (C) were incubated with increasing concentrations of rhIL-17 in the absence or presence of PMA. NBT reduction is expressed as absorbance at 540 nm. The results are presented as the mean ± SE of triplicate samples from one experiment representative of four.

*Effect of IL-17 on NO production by peripheral blood cells*

It is well known that inflammatory cytokines can stimulate peripheral blood leukocytes, predominantly mononuclear phagocytes and neutrophils, to produce NO. In order to evaluate NO production by human peripheral blood cells in the presence of rhIL-17, nitrite accumulation in 24h culture supernatants of WBC, granulocytes or mononuclear cells was determined. As presented in Figure 2, IL-17 did not influence spontaneous or LPS-stimulated NO production by WBC. Moreover, rhIL-17 did not induce changes in spontaneous and LPS-stimulated NO production by peripheral blood mononuclear cells and granulocytes either (data not shown).

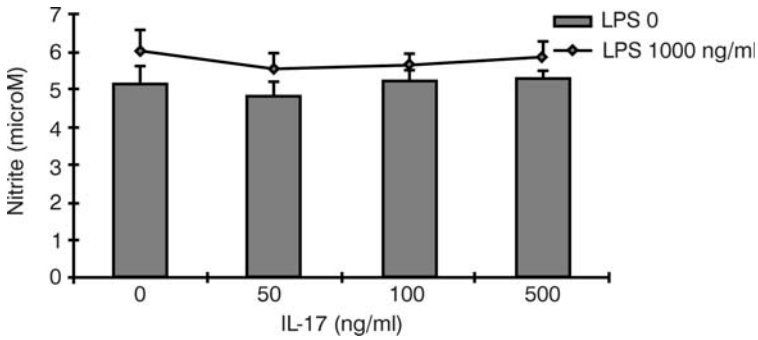


Figure 2. The effect of rhIL-17 on NO production by human peripheral blood cells. WBC were incubated with increasing concentrations of rhIL-17 in the absence or presence of LPS and nitrite accumulation in cell culture supernatants was determined after 24h. NO production is expressed as nitrite concentration (μM). The results are presented as the mean ± SE of three experiments each performed in triplicate.

*Effect of IL-17 on peripheral blood cell adhesion to plastic*

Plastic microtitre plates are widely used in the assessment of leukocyte function since the plastic surface behaves physiologically with respect to leukocyte adhesion (Forsyth and Levinsky, 1989; Oez *et al.,* 1990). The adhesion to plastic of WBC, as well as mononuclear cells and granulocytes was determined in the presence of increasing concentrations of rhIL-17. No rhIL-17 concentration used induced changes in spontaneous adhesion of WBC, mononuclear cells or granulocytes (Figure 3). A slight, but statistically insignificant increase in PMA-stimulated granulocyte adhesion was detected in the presence of rhIL-17 at 100 and 500 ng/ml (approximately 27% of the response determined with PMA alone). A similar increase in adhesiveness was observed in the population of mononuclear cells, but only at the highest concentration of rhIL-17 used (500 ng/ml). However, in WBC cultures no effect of rhIL-17 on PMA- stimulated adhesion was noted.

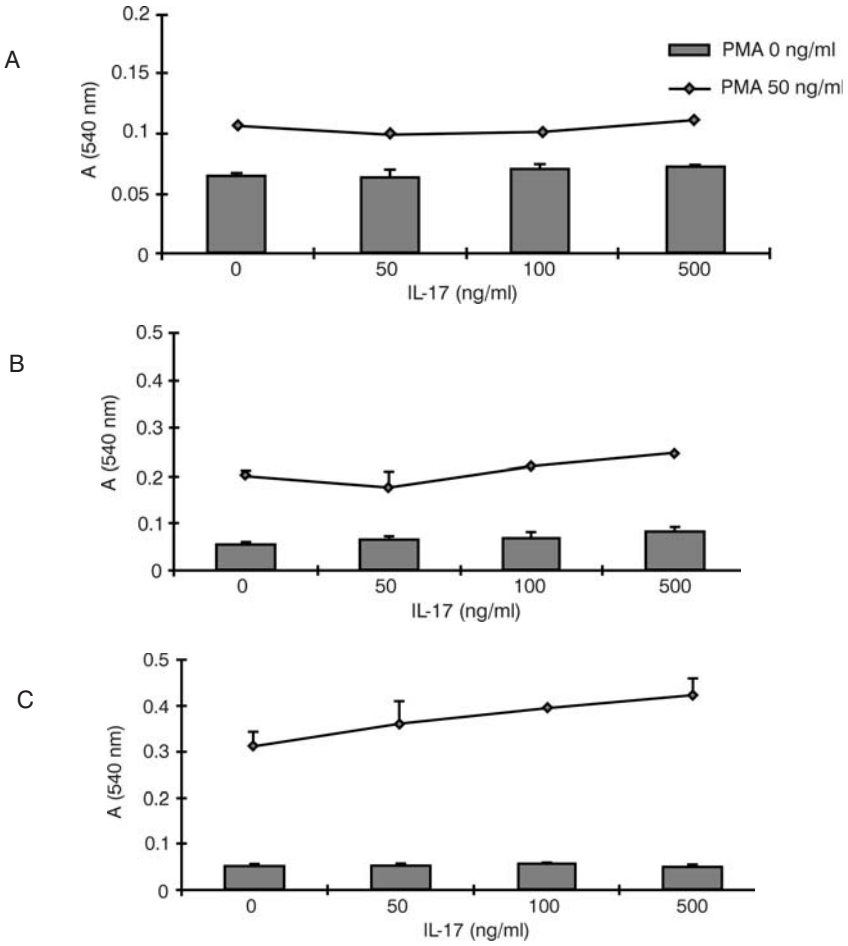


Figure 3. Figure 3. The effect of rhIL-17 on human peripheral blood cell adhesion to plastic. WBC (A), mononuclear cells (B) and granulocytes (C) were cultured with increasing concentrations of rhIL-17 in the absence or presence of PMA and adherence to plastic was measured after 60 min incubation at 37$^{o}$C. Adhesion is expressed as absorbance at 540 nm. The results are presented as the mean ± SE of triplicate samples from one experiment representative of four.

*Effect of IL-17 on MTT reduction by peripheral blood cells*

MTT is metabolically reduced exclusively by living cells and therefore it is recommended as a quantitative colorimetric assay for the measurement of cellular proliferation, viability and cytotoxicity (Mosmann *et al*., 1983). To estimate if rhIL-17 affected the metabolic activity/viability of peripheral white blood cells, we

conducted the MTT assay with freshly isolated total WBC, granulocytes or mononuclear cells in the presence of increasing concentrations of rhIL-17, since only metabolically viable cells reduce soluble tetrazolim salt-MTT to insoluble formazan (Jabbar *et al*., 1989). None of the rhIL-17 concentrations tested induced any significant changes in the levels of MTT reduction by either examined peripheral blood cell fraction (Figure 4). The estimated levels of MTT reduction in the presence of rhIL-17 were no more than 10% different from the response observed with medium alone.

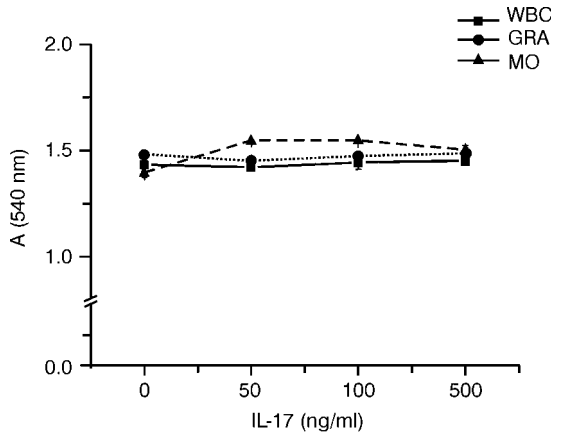


Figure 4. Figure 4. The effect of rhIL-17 on MTT reduction by human peripheral blood cells. Freshly isolated human peripheral WBC (■), mononuclear cells (▲) and granulocytes (●) were exposed to increasing concentrations of rhIL-17 and MTT reduction was measured after 3h incubation at 37$^{o}$C. MTT reduction is expressed as absorbance at 540 nm. The results are presented as the mean ± SE of triplicate samples from one experiment representative of three.

## DISCUSSION

Peripheral blood leukocytes circulate in the vasculature in a passive, quiescent state. In the course of inflammation they are recruited to the site of inflammation, where they become activated by cytokines and inflammatory mediators to express their effector functions. IL-17 has been classified as a proinflammatory cytokine because of its ability to induce expression of many mediators of inflammation, but the data concerning its activity on peripheral blood leukocytes are limited. In this study we have examined if the proinflammatory activity of IL-17 can be extended to the functional activation of peripheral blood leukocytes, since this property of IL-17 may be of importance in the pathogenesis of chronic inflammatory diseases.

Macrophages, neutrophils and other phagocytic cells are key components of the antimicrobial and tumoricidal immune responses, due to their capability to generate large amounts of highly toxic molecules - reactive oxygen (ROIs) and nitrogen intermediates (RNIs). The production of the oxygen radical, superoxide ($O_2^-$), is mediated by activation of respiratory burst NADPH oxidase. It is known that in resting cells NADPH oxidase is dormant and is activated upon stimulation by different mechanisms depending on the agonist used, either various cytokines or different microbial products, such as LPS, PMA, fMLP (N-formyl-methionyl-leucyl-phenylalanine). Proinflammatory cytokines, IL-1$\alpha/\beta$, TNF$\alpha$, GM-CSF, IL-6 and IL-8, have been shown to interact with polymorphonuclear neutrophils (PMN) by stimulating the oxidative burst and/or exerting a priming effect, i.e. enhancing the PMN response to a secondary applied stimulus (Elbim *et al*., 1994). However, in our study IL-17 did not provoke any significant changes in respiratory burst activity of the total peripheral blood leukocytes, or granulocytes or mononuclear cells, either at the level of spontaneous or PMA-stimulated NBT reduction.

The production of NO, by inducible NO synthase (iNOS) enzyme, is also regulated by various inflammatory cytokines (TNF$\alpha$, IFN$\gamma$, IL-18, IL-1$\alpha/\beta$) and bacterial products (e.g. LPS) at transcriptional and posttranscriptional levels (Bogdan *et al.*, 2000). IL-17 was previously reported to induce NO production in various cells, such as human chondrocytes, mouse osteoblasts, rodent astrocytes and endothelial cells (Miljković and Trajković, 2004). However, in our experiments no changes were detected in the basal levels of NO production by total WBC, granulocytes or mononuclear cells after 24h incubation with rhIL-17. This is in agreement with the data demonstrating that human peripheral blood mononuclear phagocytes, neutrophils and T lymphocytes, produce no or little NO after various treatments *in vitro* (Weinberg *et al.,* 1995; Webb *et al.,* 2000; Arcos *et al.,* 2003). Moreover, it was reported that IL-17 was unable to stimulate significant NO release in cultured human monocytes or rodent macrophages (Jovanovic *et al*., 1998; Trajković *et al.,* 2001).

Circulating peripheral blood leukocytes are known to become more adhesive upon stimulation at the sites of inflammation. The adhesiveness of leukocytes to various matrices is used as an *in vitro* correlate of leukocyte adherence to endothelium or connective tissue (Oez *et al*., 1990). Numerous leukocyte functions dependent on adhesion are mediated by $\beta_2$ intergrins (CD11/CD18), normally expressed on the cell surface, although the relative abundance and the mode of CD11/CD18 avidity varies depending on the cell type and the state of cell activation and differentiation (Arnaout, 1990). The critical importance of the CD11/CD18 complex was also demonstrated for leukocyte adhesion to plastic (Schleiffenbaum *et al.,* 1989). IL-17 in our experiments did not induce significant changes in the spontaneous adhesion of peripheral mononuclear cells and granulocytes, but a slight stimulation of adhesiveness was observed during the simultaneous presence of IL-17 and PMA. This finding pointed to a possible contribution of IL-17 in the priming of these cells. Previous data showed that various inflammatory mediators, such as TNF$\alpha$, IL-8, GM-CSF and C5a prime neutrophils to respond more effectively to a subsequent stimulation (Daniels *et al.,* 1994; Hallet and Loyds, 1995). In this regard, it is

possible that IL-17 can also prime peripheral blood granulocytes and mononuclear cells, probably by inducing changes at the level of signal transduction, enabling them to respond to PMA, as a secondary stimulus, with stronger adhesiveness.

The level of MTT reduction directly correlates with the number of metabolically active cells in the culture (Mosmann, 1983; Carmichael *et al.,* 1987). Cellular bioreduction of MTT is associated with the activity of NADPH- and NADH-dependent enzymes of the endoplasmatic reticulum, as well as mitochondrial succinate dehydrogenase. The production of reduced pyridine nucleotides during glucose metabolism was reported to be rate limiting for cellular MTT reduction (Berridge *et al*., 1996). Also, the critical role of glucose-6-phosphate dehydrogenase (G6PDH) activity, the rate-limiting enzyme of the glycolytic and pentose phosphate pathway, was emphasized in the regulation of cell death/survival (Tian *et al*., 1999). In this regard changes in MTT reduction were recommended as an assay for the assessment of the overall functional state of human polymorphonuclear leukocytes. According to our results obtained from the MTT assay it seems that rhIL-17 does not affect the overall metabolical activity of WBC, peripheral blood granulocytes and mononuclear cells from healthy persons.

The presented results demonstrate that IL-17 induced no or little changes in various functional activities of total peripheral blood leukocytes, granulocytes and mononuclear cells from normal subjects, suggesting that IL-17 does not express any proinflammatory ability in steady-state, since the requirement for its action really does not exist. This is consistent with data showing that IL-17 had no effect on the secretion of proinflammatory cytokines by blood monocytes (Fossiez *et al*., 1996), but stimulated the production and expression of IL-1$\beta$ and TNF$\alpha$ only by activated macrophages (Jovanovic *et al.,* 1998). In addition, this finding is in agreement with other studies reporting that leukocyte responsiveness to cytokines requires additional stimulation, such as adherence or combined action with other cytokines (Jovanovic *et al.,*1998; Nathan *et al.,* 1989). This also may be a reason why circulating leukocytes are not routinely activated by the low level of circulating cytokines. Moreover, numerous reports have documented that IL-17 alone induce weak responses in various stromal cells, but emphasized its synergistic action with other proinflammatory cytokines, implying a possible role as a "fine-tuning" element in the complex cytokine network (Katz *et al.,* 2001; LeGrand *et al.,* 2001; Chabaud *et al.,* 2001). In our previous study we demonstrated that the influence of IL-17 on hematopoietic progenitor cell growth and cytokine release was dependent on the physiological/pathological status of the organism and that IL-17 exerted a more prominent effect especially on cytokine secretion in post-irradiated murine bone marrow cells (Jovčić *et al*, 2001). Increased levels of IL-17 have been associated with some chronic inflammatory conditions, such as rheumatoid arthritis, (RA), in which circulating leukocytes exhibit several features indicative of partial activation (Edwards and Hallett, 1997). Since prior *in vivo* priming could alter subsequent leukocyte functions, our future studies will consider if IL-17 can modulate the functional

activity of peripheral blood leukocytes obtained from patients with RA or other chronic inflammatory diseases.

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## EFEKTI IL-17 NA FUNKCIONALNU AKTIVNOST ĆELIJA PERIFERNE KRVI

VLAŠKI MARIJA, KRSTIĆ ALEKSANDRA, JOVČIĆ GORDANA, BUGARSKI DIANA, PETAKOV MARIJANA, STOJANOVIĆ NEVENKA i MILENKOVIĆ P

SADRŽAJ

Interleukin 17 (IL-17) je proinflamatorni citokin koga produkuju aktivirane CD4+ i CD8+ T ćelije, dok je njegov receptor ubikvitarno distribuiran. Inflamatorni kapacitet IL-17 se zasniva na njegovoj sposobnosti da stimuliše širok spektar stromalnih ćelija da produkuju i oslobađaju različite proinflamatorne medijatore, među kojima neki imaju efekte na hematopoezu, posebno granulopoezu. Dosadašnji podaci ukazuju na ulogu IL-17 u patogenezi različitih inflamatornih bolesti, odbacivanju transplanta i razvoju tumora. Cilj ovog rada je bio da se odrede funkcionalni odgovori, uključujući respiratorni prasak, produkciju azot monoksida (NO), adhezivnost i metaboličku aktivnost/vijabilnost različitih ćelija periferne krvi (ukupnih leukocita, mononuklearnih ćelija i granulocita) zdravih donora, u prisustvu IL-17. Dobijeni rezultati su ukazali da IL-17 ne dovodi do značajnih promena respiratornog praska, produkcije NO i metaboličke aktivnosti ćelija periferne krvi, ali da uzrokuje blago povećanje forbol-12-miristat-13-acetat (PMA) stimulisane adhezivnosti granulocita i mononuklearnih ćelija. Odsustvo značajnih promena u ispitivanim funkcionalnim aktivnostima različitih ćelija periferne krvi, ukazuje da IL-17 ne eksprimira proinflamatorno dejstvo kod zdravih osoba, jer najverovatnije i ne postoji potreba za njegovim delovanjem.