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Research article

# Lipopolysaccharide Stimulates Peripheral Blood Lymphocytes of Patients with Rheumatoid Arthritis to Produce IL-10 *in vitro*

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**ABSTRACT**

Lipopolysaccharide (LPS) plays an important role in stimulating the pro-inflammatory immune response, especially in patients with rheumatoid arthritis (RA). Production of IL-10 in the host plays an important role in reducing the effect of the pro-inflammatory immune response. The role of LPS of Pseudomonas aeruginosa in stimulating IL-10 in patients with RA is not clear in the literature. The current study aims to determine the ability of the lymphocytes of patients with RA to produce IL-10 after being stimulated with LPS of *P. aeruginosa in vitro*. The human peripheral blood lymphocytes (PBLs) were isolated from patients with RA (test group A) and healthy cohorts (HC) (test group B), and they were cultured in vitro and stimulated with 1 µg of LPS of *P. aeruginosa* and PBS. The folds of the IL-10 gene expression of IL-10 were estimated in the PBLs, while the concentration of IL-10 was measured in the cell culture supernatants. The results of the folds of the gene expression and the concentrations of Il-10 of test groups were compared with control groups [PBLs of patients with RA (control groups A) and HC (control groups B) stimulated with PBS]. The significant elevations of IL-10 gene expression and IL-10 concentrations were seen in the PBLs cultures of test group A as compared with control groups A and B. The results showed no significant difference in the IL-10 gene expression and IL-10 concentrations of cell culture of test group B as compared with control groups A and B. It can be concluded that the LPS stimulates the lymphocytes of patients with RA to produce IL-10 and help in balance of the effect of pro-inflammatory immune reaction.

**Keywords**: Interleukin-10, Lipopolysaccharide, Peripheral blood lymphocytes, Tissue culture.

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## 1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that primarily affects the joints, particularly the hand, wrist, and knee joints. It can also impact other organs and tissues in the body, including the lungs, heart, blood vessels, eyes, kidneys, and nerve tissue bone marrow [1, 2]. The disease is characterized by inflammation, which leads to the production of cytokines, chemokines, and inflammatory reactants, causing oxidative stress and damage to joint linings [3]. Risk factors for RA include age, gender, race, ethnicity, obesity, smoking, gut microbiota, viral infections, and diet [2]. The progression of RA occurs in four stages: triggering, maturation, targeting, and fulminant. People with RA have higher levels of oxidative stress and lower levels

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of antioxidants, leading to increased lipid peroxidation, protein oxidation, and DNA damage [4]. Early detection and proper treatment are crucial to minimize pain and slow the progression of the disease. Disease-modifying anti-rheumatic drugs (DMARDs) are commonly used to inhibit joint destruction and achieve remission [5].

Interleukin-10 (IL-10) is a cytokine that has important roles in regulating immune responses. It is known to be a potent anti-inflammatory and immunosuppressive cytokine [6]. IL-10 is produced by various immune cells, including dendritic cells, macrophages, B cells, and T cells [7]. IL-10 plays a crucial role in preventing inflammation-mediated tissue damage and is involved in the outcome of infections, allergies, autoimmune reactions, tumor development, and transplant tolerance [8]. IL-10 acts as a negative regulator of immune responses and helps maintain the balance between effective immunity and tissue protection [9].

Lipopolysaccharide (LPS) induces a pro-inflammatory reaction by up-regulating the expression levels of pro-inflammatory cytokines. In a study by Liu et al., intravenous injection of LPS in rabbits resulted in an immune response state, with certain doses of LPS leading to the death of some rabbits [10]. Another study by Li et al. showed that L-Arginine (L-Arg), a critical substrate for nitric oxide (NO) production, can protect against LPS-induced acute lung injury by inhibiting NF-kappa B activation and the release of inflammatory factors [11].

LPS and IL-10 interact with each other in several ways. IL-10 prevents LPS-induced activation of neutrophils, secretion of TNF-α and IL-8, and reactive oxygen species (ROS) generation [12]. IL-10 also inhibits LPS-induced tissue factor (TF) upregulation on monocytes, potentially limiting the development of disseminated intravascular coagulation (DIC) [13]. Furthermore, IL-10 inhibits LPS-induced tumor necrosis factor (TNF)-α [14].

LPS is known to stimulate the production of IL-10 in monocytes and macrophages [15]. IL-10 is a cytokine that has been investigated for its potential therapeutic use in RA [16]. In RA, activated CD4 T helper cells and macrophages play a role in joint inflammation, and synovial macrophages produce pro-inflammatory cytokines. IL-10 has the ability to downregulate the production of multiple pro-inflammatory cytokines, making it a potential treatment for RA [16]. Additionally, studies have investigated the association between IL-10 gene polymorphisms and RA [17], but no significant associations have been found. Overall, LPS stimulates IL-10 production, and IL-10 has potential therapeutic use in RA, but further research is needed on that.

# 2. MATERIALS and METHODS

## 2.1. LPS extraction and purification

The *Pseudomonas aeruginosa* isolate was procured from the Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq. The isolate was stored for the short term by inoculating it on the nutrient agar slant (stored at 4 °C for 2- 4 weeks) and for the long term by inoculation into the nutrient broth (20 % glycerol) and stored at – 20 °C for many months. The standard method of sobhi et al. (2016) was followed to extract and purified the LPS from *P. aeruginosa*. The hot EDTA method was followed to extract the LPS of *P. aeruginosa*. The supernatant of LPS extract was passed through a gel filtration column (Sephadex G-200, Sigma-Aldrich) for partial purification of LPS [10]. The sample was eluted and the absorbency was measured at 280 nm (protein), 490 nm (carbohydrates), 260 nm (nucleic acids), and 525 nm (lipids) [18].

## 2.2. Patients' samples

Blood samples were obtained from a cohort consisting of 10 patients diagnosed with RA, along with 8 age and sex-matched healthy controls. The classification criteria for RA as outlined in the 2010 guidelines were strictly adhered to by the rheumatologist's guidelines at Central Public Health Laboratories (CPHL), Baghdad, Iraq [19]. All necessary laboratory tests were conducted and based on the results, the rheumatologists were able to accurately identify the patients suffering from RA. The Disease Activity Score 28 (DAS28) was calculated to be 3.6 ± 1.4. The volunteer patients were undergoing different treatments. The blood samples were carefully collected in tubes containing anticoagulants (heparinized tubes).

## 2.3. Cell collection

The collection of peripheral blood lymphocytes (PBLs) was done according to a previous study [20] that described the buoyant density gradient technique using Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals Inc., Piscataway, N.J., Hypaque, Winthrop Laboratories, Sterling Drug Co., New York) that was used to separate lymphocytes from fresh heparinized venous blood. The trypan blue exclusion test was used to assess the lymphocytes' vitality [21]. The standard RBMI 1640 tissue culture media supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate (pH 7.2), 4.5 g/l glucose, and 10 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) (37°C and a $CO_2$ concentration of 5%,)

## 2.4. PBLs culture groups

In the current study, four groups of cell cultures were prepared. 1st group (test group A), 2 ml of complete tissue culture media [RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate (pH, 7.2), 4.5 g/l glucose and 10 mM HEPES, and supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Sigma)] containing $10^6$ PBLs (95 % viable cells) collected from patients with RA and stimulated with 1 µg of purified LPS. 2nd group (test group B), 2 ml of complete tissue culture media (same media of groups A) containing $10^6$ PBLs (95 % viable cells) obtained from healthy cohorts (HC) and stimulated with 1 µg of purified LPS. 3rd group (control group A), 2 ml of complete tissue culture media (same media of groups A) containing $10^6$ PBLs (95 % viable cells) obtained RA patients and exposed to 20 µl of sterile PBS (pH, 7.2, 0.1 M). 4th group (control group B), 2 ml of complete tissue culture media (same media of groups A) containing $10^6$ PBLs (95 % viable cells) obtained from HC and exposed to 20 µl of PBS (pH, 7.2, 0.1 M). Five patients with RA and 5 healthy volunteers participated in the experiment. All tissue culture tubes were incubated at 37 °C and 5 % $CO_2$.

## 2.5. Levels of IL-10 in cell cultures

In this experiment, 400 µl were collected in micro-centrifuge tubes from each cell culture tube (test group A, test group B, control A, control B) at different intervals of time (1 h, 24h, 48 h, and 72 h). The micro-centrifuge tubes were centrifuged at 1500 g for 10 min. The supernatants were then collected and stored at -20 °C until used for measuring the IL-10 using enzyme linkage immune sorbent assay (ELISA) [human IL-1β ELISA kit (BD Biosciences, USA)].

## 2.6. IL-1β mRNA expression in cultured PBLs

The pellets that remained in the micro-centrifuge tubes in the step of IL-10 measurement were mixed with 600 µl of Trizol

reagent (Invitrogen, CA) using an Ultra Turrax homogenizer and stored at -80 ºC. The total RNA was extracted using an RNA isolation kit (Dongsheng Biotech, China). The IL-10 mRNA levels were measured using a real-time polymerase chain reaction (RT-PCR). The total RNA was implemented as the template in the RT-PCR. The RNA was reversely transcribed to complementary DNA (cDNA) using WizScript™ RT FDmix Kit. The PCR primers used were as described previously for IL-10 and G3PDH [22,23] and the reactions were performed as described previously [24]. The relative expression of IL-10 with G3PDH as the reference gene was determined using the $2^{-\Delta\Delta_T}$ (Livak) method.

## 2.7. Statistical analysis

Every value was utilized to determine the standard deviation and provide a mean value. Using Origin version 8.0 software, the Student's t-test and Chi-square test were used to assess the differences. $P < 0.05$ was the threshold for statistical significance.

# 3. RESULTS

## 3.1. Levels of IL-10 mRNA expression in cell cultures

The results of the fold of IL-10 mRNA expression in PBLs that were cultured in vitro and exposed to 1µ of LPS extracted from *P. aeruginosa* isolated from infected wounds at different time intervals (1h and 24 h) were shown in **Fig 1**. A significant increase in the folds of IL-10 mRNA expression was seen in the test group A and control group A at both time points (cultures of PBLs that were isolated from patients with RA that were exposed to either LPS or PBS). The results showed that no effect of exposing PBLs (obtained from either patient with RA or HC) at both time points.

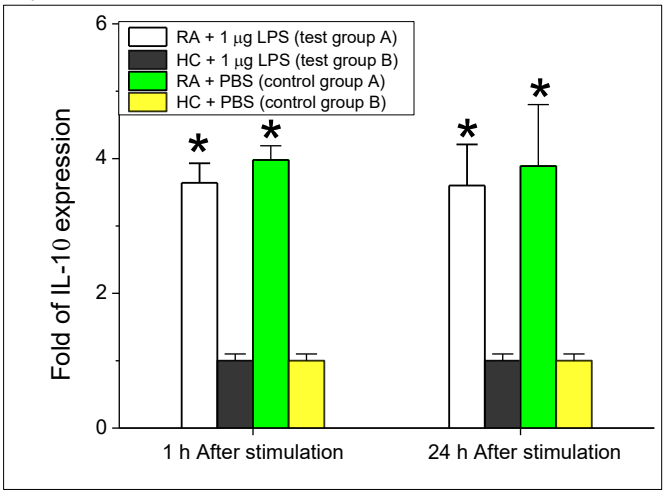


**Fig 1**. Folds of gene expression of IL-10 into PBLs obtained from Rheumatoid arthritis (RA) patients and healthy control (HC) after stimulation with either 1 µg of *P. aeruginosa* LPS or PBS (pH, 7.2; 0.1 M) at two time points (1 and 24 h). *, indicates of significant difference from control groups [PBLs obtained from RA patients (control group A) and HC (control group B) stimulated with PBS (pH 7.2, 0.1 M)]. A p-value less than 0.05 is considered a significant difference.

## Levels of IL-10 in cell cultures

In the current study, the level of IL-10 was measured in PBL culture post-stimulating either with 1µ of P. aeruginous LPS extracted at different intervals of time (24, 48, 72h). The results showed that a significant increase ($P<0.05$) in IL-10 levels was seen in the cultures of PBLs that were collected from patients with RA and stimulated with LPS at all-time points (24, 48,72 h) (Fig. 2) as compared with test group B, control group A, and control group B. Although the level of IL-10 was high in tissue cultures of control group A (PBLs collected from patients with RA and stimulated with PBS), these increases were not significant ($P>0.05$).

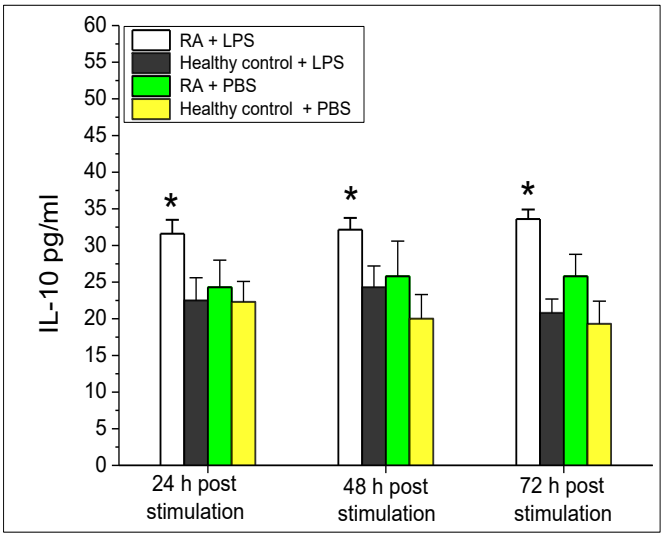


**Fig 2**. Levels of IL-10 into PBLs obtained from Rheumatoid arthritis (RA) patients and healthy control (HC) after stimulation with either 1 µg of *P. aeruginosa* LPS or PBS (pH, 7.2; 0.1 M) at different time points (24, 48, 72 h). *, indicates of significant difference from control groups [PBLs obtained from RA patients (control group A) and HC (control group B) stimulated with PBS (pH 7.2, 0.1 M)]. A P-value less than 0.05 is considered a significant difference.

# 4. DISCUSSION

Lipopolysaccharide (LPS) stimulation leads to an increase in interleukin 10 (IL-10) production in patients with RA [25]. IL-10 is produced by monocytes and macrophages when they are activated with LPS [26]. The initial burst of pro-inflammatory cytokines by an increase in IL-10 synthesis [26]. IL-10 release from LPS-stimulated monocytes can be increased by factors such as TGFβ, IFNα, IFNβ, histamine, and ligation of the Fcγ receptor I. On the other hand, IL-10 production may be inhibited by factors such as IL4, IFNγ, and ligation of CD23 [27]. The relationship between LPS and IL-10 levels in RA patients suggests that IL-10 plays a role in downregulating the production of pro-inflammatory cytokines and dampening the inflammatory response in RA.

In the present study, LPS was extracted from *P. aeruginosa* isolated from infected wounds. Lymphocytes were obtained from patients with RA and HC and then stimulated with LPS *in vitro*. It was found that only the PBLs collected from patients with RA and stimulated with LPS produced high levels of IL-10 as compared with other groups (PBLs collected from RA patients and stimulated with LPS, PBLs collected from HC and stimulated with either LPS or PBS). This finding supports the concept of the role of LPS in stimulating IL-10 in patients with RA and the role of this cytokine in balancing the inflammation reaction in patients with RA.

LPS affects the immune response to IL-10 in various ways. In malnourished mice, LPS stimulation resulted in increased levels of IL-10 [28]. In sensitized animals, exposure to LPS reversed the changes in nitric oxide synthase (NOS) isoenzyme activities

induced by ovalbumin (OVA) challenge and also increased IL-10 release [29]. IL-10 reduced the migration of monocytes and T lymphocytes across endothelium stimulated by LPS, and decreased endothelial production of chemokines in response to LPS [30]. IL-10 also negatively influenced protein tyrosine phosphorylation in LPS-treated human polymorphonuclear leukocytes (PMN) [31]. These findings suggest that LPS can modulate the immune response to IL-10 by altering the production and activity of IL-10, as well as its downstream signaling pathways.

The production of pro-inflammation will produce tissue damage and the effect negatively on the host, which is why preventing this damage may help the host tissue to heal soon. Interleukin-10 (IL-10) has been shown to prevent tissue damage through various mechanisms. IL-10 can facilitate tissue repair by delivering immunomodulatory plasmids to the injured site, leading to an accelerated tissue repair outcome. It also contributes to the regulation of inflammation without compromising the healing response, as observed in murine full-thickness excisional wounds [32]. IL-10 treatment has been found to reduce pathological signs of tissue damage caused by organophosphate poisoning, such as liver, kidney, and lung damage. Additionally, IL-10 protects against blood-induced joint damage and reduces the production of inflammatory cytokines in hemophilic synovial tissue, suggesting its potential as a candidate for the treatment of joint hemorrhages. Overall, IL-10 plays a crucial role in preventing tissue damage by promoting tissue repair, regulating inflammation, and protecting against toxic insults [33]. For this reason, the body's production of IL-10 in response to exposure to LPS plays an important role in maintaining the balance between stimulating and suppressing pro-inflammation.

## 5. CONCLUSION

The current study showed that stimulation of peripheral blood lymphocytes (PBLS) obtained from patients with RA with 1 µg of *P. aeruginosa* LPS results significant increase in the production of IL-10 in vitro, and this increase was not observed significantly when stimulating PBLs obtained from healthy cohort (HC) people, with 1 µg of *P. aeruginosa* LPS in vitro. This confirms the ability of peripheral blood lymphocytes in patients with RA to produce IL-10 when exposed to bacterial infection, and this secretion contributes to maintaining a balance in the pro-inflammatory response, which plays an important role in the activity (severity) of RA.

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**Conflict of interest**

The authors declare that they have no conflict of interests.

**Ethical Approval**

This review was approved by the Ministry of Health, Baghdad, Iraq (No 212, 2023).

**Author contributions**

**Israa M. Subhi.** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Supervision; Validation, Roles/Writing - original draft., Software; Visualization; Writing - review & editing

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