RESEARCH ARTICLE OPEN ACCESS

Temporal Dynamics of TNF-α Expression and Cell Viability in LPS-Stimulated Peripheral Blood Mononuclear Cells

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Submission November 29, 2024
Accepted December 10, 2024
Available online on December 13, 2024

ABSTRACT

Background: Lipopolysaccharide (LPS), a key component of Gram-negative bacterial membranes, activates innate immune responses through Toll-like receptor 4 (TLR4) signaling in peripheral blood mononuclear cells (PBMCs). This study aimed to evaluate the temporal dynamics of TNF-α expression and cell viability in LPS-stimulated PBMCs to understand the inflammatory and cytotoxic effects of prolonged LPS exposure. **Methods:** Human PBMCs were treated with increasing concentrations of LPS (10, 30, and 50 ng/mL) for 4, 8, 12, and 24 hours. TNF-α mRNA expression was analyzed using quantitative PCR, while cell viability was assessed via CCK-8 assay and microscopic imaging. **Results:** LPS stimulation induced a robust, dose-dependent upregulation of TNF-α expression, peaking at 4 hours and gradually declining over time. Concurrently, PBMC viability remained stable up to 12 hours post-stimulation but significantly decreased at 24 hours, particularly at higher LPS concentrations (30–50 ng/mL). Microscopic analysis revealed increased cellular aggregation and morphological changes consistent with immune activation and cytotoxic stress. **Conclusion:** LPS triggers early TNF-α expression in PBMCs through TLR4-mediated activation of the NF-κB pathway. However, prolonged exposure to LPS results in decreased cell viability, likely due to sustained inflammatory signaling and oxidative stress. These findings provide insight into the dual-phase response of PBMCs to LPS and underscore the importance of tightly regulated inflammation in innate immunity.

Keywords : Immune system, PBMC, LPS, TNF-α

INTRODUCTION

The immune system is a mechanism that allows a living organism to discriminate between “self” and “nonself”[1]. The immune system can be simplistically viewed as having two lines of defense, which is innate immunity and adaptive immunity. Innate immunity represents the first line of defense to an intruding pathogen. Antigen non-specific defense mechanism that is used by the host immediately or within hours of encountering an antigen. They are response has no immunologic memory and, therefore, it is unable to recognize the same pathogen should the body be exposed to it in the future. Adaptive immunity is antigen-dependent and antigen-specific, which is can memorize enables the host to improve rapid and efficient immune response upon subsequent exposure to the antigen. Therefore, involves a lag time between exposure to the antigen and maximal response [2].

Lipopolysaccharides (LPS) are the molecular constituents of the so-called endotoxins. LPS are present in the outer leaflet of the external membrane of Gram-negative bacteria. Lipopolysaccharides are large amphipathic glycoconjugates that typically consist of a lipid domain (hydrophobic) attached to a core oligosaccharide and a distal polysaccharide. These molecules are also known as lipoglycans due to the presence of lipid and sugar molecules. The lipopolysaccharides are composed of: Lipid A, O-antigen, and the hydrophilic core polysaccharide[3].

Peripheral Blood Mononuclear Cells (PBMC) consists of lymphocytes, monocytes and dendritic cells obtained from human blood or buffy coats. In humans, the frequencies of these populations vary across individuals, but lymphocytes are most abundant, constituting in the range 70%-90%. Types of lymphocytes can be distinguished to B cell produce antibodies, T cells support cellular imunity, and natural killer have their own cytolytic activity[4].

The study showed that co-culture of MSCs with LPS-stimulated PBMCs resulted in a significant decrease in TNF-α secretion, an increase in IL-10, and an increase in IL-6. These effects were observed within 24 hours and demonstrated the potential of MSCs in modulating the inflammatory response of PBMCs[5].In previous study shows that conditioned medium from LPS-activated PBMCs can enhance IL-10 secretion by MSCs. This suggests the potential use of conditioned medium to modulate MSC immune activity[6].

MATERIAL AND METHODS

Isolation of Human PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood obtained from healthy donors using Ficoll-Paque™ density gradient centrifugation. Blood was diluted with an equal volume of phosphate-buffered saline (PBS) and layered carefully over Ficoll-Paque solution in a sterile 15 mL conical tube, followed by centrifugation at 400 × g for 30 minutes at room temperature without brake. The mononuclear cell layer was collected, washed twice with PBS, and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

LPS Treatment

PBMCs were plated at a density of 1×10^4 cells/well in 96-well culture plates and 1×10^6 cells/well in 24-well culture plates and allowed to rest for 3 hours at 37°C in a humidified incubator with 5% CO_2. Cells were then stimulated with lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich) at final concentrations of 10, 30, or 50 ng/mL. Untreated cells served as negative control. Cells were harvested at 4, 8, 12, and 24 hours post-treatment for analysis.

RNA Extraction and Quantitative PCR for TNF-α Expression

Total RNA was extracted from PBMCs using an RNA isolation kit (Qiagen RNeasy Mini Kit) according to the manufacturer's instructions. RNA concentration and purity were determined using a Nanodrop spectrophotometer. One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) using a reverse transcription kit (TOYOBO, Japan), following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was then carried out using SensiFAST™ SYBR® Green Master Mix (Bioline) and TNF-α primer (F 5'- ACT GAACTTCGGGGGATTG-3' and 5'-GCTTGGTGGTTTGCTACGAC-3') and β-actin primer (F 5'-ACAGCTACGAGCTGCCTGAC-3'and R 5'- GGATGCCACAGGACTCCA-3') on an Illumina real-time PCR system to quantify gene expression levels. The expression level of TNF-α was normalized to β-actin as a housekeeping gene and analyzed using the $2^{-\Delta\Delta Ct}$ method. All reactions were performed in triplicate.

Cell Viability Assay (CCK-8)

PBMC viability was measured using the Cell Counting Kit-8 (CCK-8; Dojindo) according to the manufacturer's protocol. Briefly, at each time point, 10 µL of CCK-8 solution was added to each well containing 100 µL of cell suspension in a 96-well plate. After 2 hours of incubation at 37°C, absorbance was measured at 450 nm using a microplate reader. Cell viability was calculated as a percentage relative to the untreated control.

Microscopic Observation

Cell morphology and density were monitored using an inverted phase-contrast microscope at 100× magnification. Representative images were captured at each time point to evaluate morphological changes, aggregation, and signs of cytotoxicity. Scale bars were standardized at 50 µm.

Data Analysis

Data were presented as the means ± SD. All calculations were carried out using IBM SPSS 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The statistical significance of the differences between the groups was assessed using one way-ANOVA and continued with Duncan post-hoc analysis. P values: **, $P < 0.001$.

RESULTS

LPS priming affect PBMC viability

The viability of PBMCs without LPS treatment (control) remained stable at 100% throughout the incubation period (4–24 h), indicating that the culture conditions did not have a toxic effect on the cells. In contrast, LPS administration showed a decrease in cell viability that depended on the concentration and duration of incubation. At a concentration of 10 ng/mL, the decrease in viability was minimal (97.3% at 24 h), but concentrations of 30 ng/mL and 50 ng/mL caused a greater decrease in viability, reaching 83.2% and 78.7% at 24 h, respectively. This indicates that LPS has a significant cytotoxic effect on PBMCs, especially at high doses and longer incubation periods. This effect is most likely related to the activation of inflammatory pathways and oxidative stress that can trigger apoptosis.

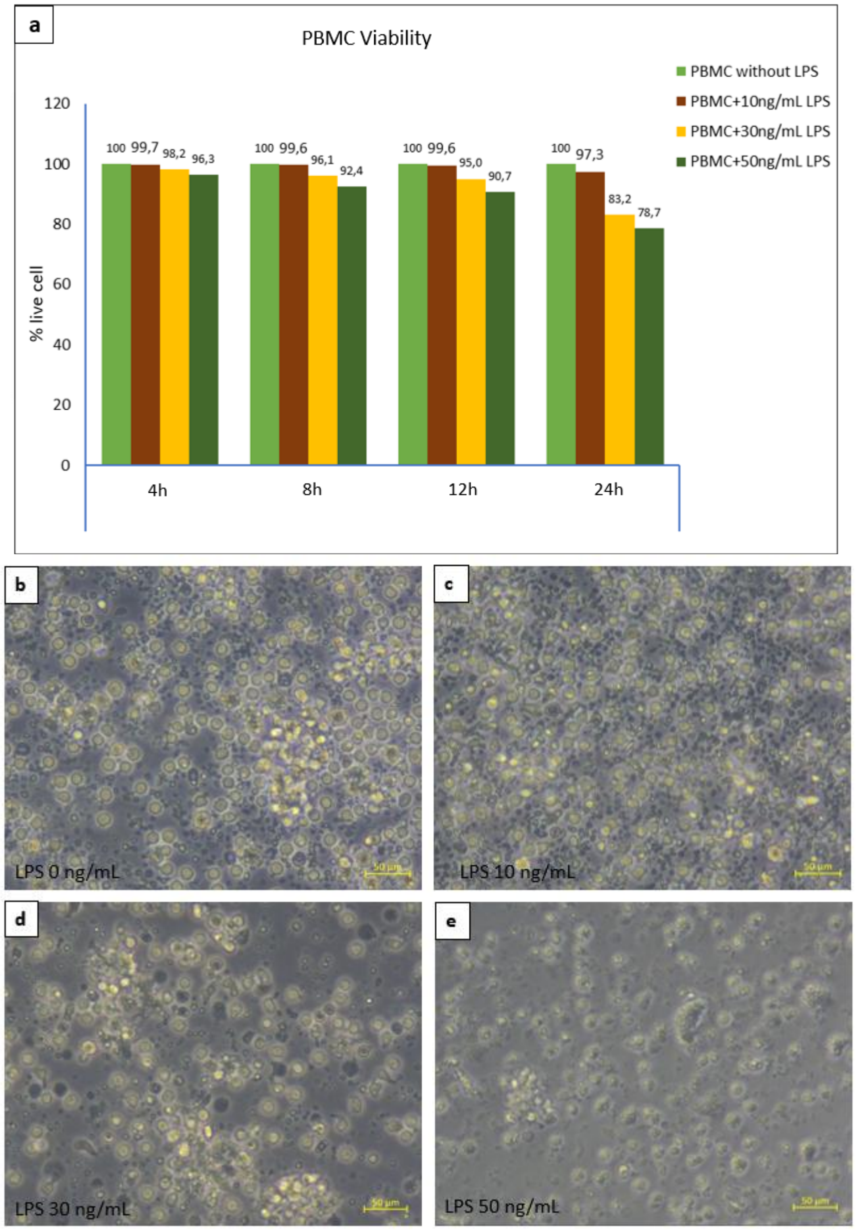

Figure 1. *Viability of PBMCs after LPS stimulation over different incubation periods*. Cell viability was assessed and expressed as the percentage of live cells relative to untreated controls (set as 100%) at several time periods (a). PBMCs were cultured for 24 hours in the absence of LPS (b) or in the presence of 10 ng/mL (c), 30 ng/mL (d), or 50 ng/mL (e) of LPS. Images were captured using an inverted microscope at 100× magnification. Cells without LPS (b) appear mostly rounded and evenly distributed. Upon LPS treatment, especially at higher concentrations (d–e), PBMCs showed increased aggregation, altered morphology, and decreased cell density, indicative of LPS-induced cytotoxicity or activation. Scale bar = 50 µm.

LPS priming induce TNF-a expression in PBMC

TNF-α mRNA expression increased sharply in the first 4 hours after LPS stimulation, especially at high concentrations (30 and 50 ng/mL), with fold changes reaching 74.3 and 89.3. The increase in TNF-α expression in this early phase reflects the rapid activation of PBMCs in response to LPS as an immunostimulant agent, through activation of the TLR4 receptor that stimulates the NF-κB pathway, which in turn induces the expression of proinflammatory cytokines such as TNF-α. Interestingly, TNF-α expression decreased drastically after 8 hours of incubation and tended to fluctuate at 12 and 24 hours. For example, at 8 hours, TNF-α expression dropped to 18.1 (fold change) at 30 ng/mL LPS and only 2.4 at 50 ng/mL LPS. However, expression increased again at 12 hours with a peak of 24.08 at 30 ng/mL LPS. At 24 h, TNF-α expression was again high at concentrations of 10 and 30 ng/mL (40.8 and 46.9, respectively), but remained low at 50 ng/mL (0.5), likely due to cytotoxic effects inhibiting further gene transcriptional activity.

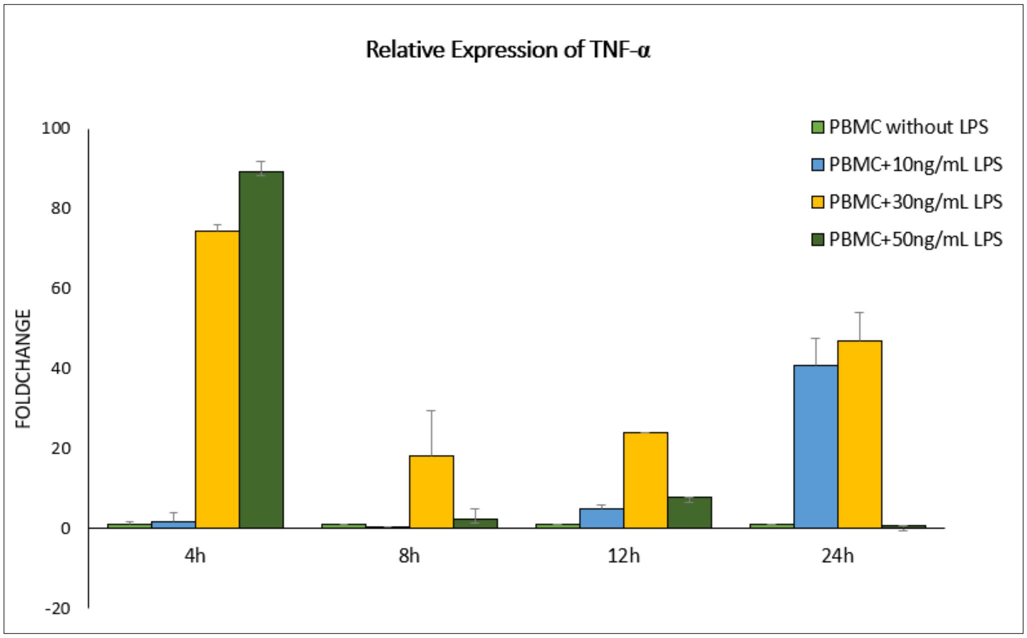

Figure 2. *Relative expression of TNF-α mRNA in PBMCs stimulated with varying concentrations of LPS over time.* Peripheral Blood Mononuclear Cells (PBMCs) were stimulated with LPS at concentrations of 10 ng/mL (blue), 30 ng/mL (yellow), and 50 ng/mL (dark green), while unstimulated PBMCs served as a control (light green). TNF-α mRNA expression was measured at 4-, 8-, 12-, and 24-hours post-stimulation using quantitative PCR and expressed as fold change relative to unstimulated control (β-actin) at each time point. The peak expression occurred at 4 hours post-stimulation, particularly at the highest LPS concentration. Data are presented as mean ± standard deviation (n = 3, specify number of replicates).

DISCUSSION

This study demonstrates that stimulation of PBMCs with lipopolysaccharide (LPS) induces significant changes in both cell viability and pro-inflammatory TNF-α gene expression. Based on the CCK-8 assay, PBMCs without LPS treatment (control) maintained 100% viability up to 24 hours, indicating that the culture conditions did not induce cytotoxic stress on the cells. In contrast, LPS treatment at various concentrations resulted in a time- and dose-dependent decrease in cell viability. At a concentration of 10 ng/mL, cell viability remained relatively high up to 24 hours (97.3%), whereas at 30 ng/mL and 50 ng/mL, viability significantly declined to 83.2% and 78.7%, respectively, after 24 hours of incubation. These findings are consistent with previous researches which showed that LPS reduces PBMC viability through activation of inflammatory signalling and the release of reactive oxygen species that promote apoptosis[1–3].

The decline in viability was closely associated with inflammatory activation, as reflected by TNF-α mRNA expression. TNF-α expression peaked at 4 hours post-stimulation, particularly in

PBMCs treated with 30 ng/mL and 50 ng/mL LPS, showing fold changes of 74.3 and 89.3, respectively. This reflects an acute cellular response to LPS through Toll-like receptor 4 (TLR4) activation and downstream NF-κB signalling, which transcriptionally upregulates pro-inflammatory genes such as TNF-α. This pattern is supported by previous study, who reported that LPS induces TNF-α expression within 4–6 hours via the canonical inflammatory pathway[4]. Interestingly, TNF-α expression dropped dramatically by 8 hours, with fold changes declining to 18.1 (30 ng/mL LPS) and 2.4 (50 ng/mL LPS). This fluctuation may indicate the initiation of negative feedback mechanisms or endotoxin tolerance, whereby PBMCs reduce cytokine expression in response to prolonged or repeated LPS exposure. A study by Page (2022) describes such endotoxin tolerance in immune cells as a homeostatic mechanism to prevent excessive inflammatory damage, characterized by downregulation of TNF-α and other cytokines[5]. By 12 hours, TNF-α expression re-elevated in PBMCs treated with 30 ng/mL LPS (24.08 fold change), while cells treated with 50 ng/mL showed continued suppression (7.67 fold change). This could be attributed to reduced cell viability or impaired transcriptional function due to oxidative stress[1,5,6]. At 24 hours, TNF-α expression remained high in 10 and 30 ng/mL LPS-treated cells (40.8- and 46.9-fold change, respectively), but was markedly low (0.5-fold change) in the 50 ng/mL group, corresponding with the most pronounced loss of cell viability. These findings align with previous studies, which emphasized the dependency of inflammatory gene expression on cellular metabolic status and mitochondrial integrity—both of which are compromised during sustained oxidative stress and cell death[4,7,8].

Lipopolysaccharide (LPS) induces an innate immune response in PBMCs through activation of the Toll-like receptor 4 (TLR4) signaling pathway[8,9]. Upon LPS recognition, TLR4 recruits adaptor molecules such as MyD88 and TRIF, initiating downstream signaling cascades that activate nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs)[4]. This leads to the rapid transcriptional upregulation of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α)[10], with peak expression observed at 4 hours post-stimulation. Prolonged exposure to LPS, especially at higher concentrations, results in sustained activation of inflammatory pathways, which can disrupt cellular homeostasis[11]. Chronic NF-κB signaling and excessive production of reactive oxygen species (ROS) and inflammatory mediators may induce endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and activation of apoptotic or pyroptotic pathways[10,12]. These molecular events contribute to reduced PBMC viability observed at 24 hours, suggesting that persistent inflammatory stress exceeds the cell's adaptive capacity, leading to cell death.

CONCLUSIONS

Overall, the data support the conclusion that LPS induces an innate immune response in PBMCs characterized by early TNF-α upregulation, followed by reduced viability due to prolonged inflammatory stress. Therefore, in *in vitro* inflammation models, both LPS dosage and incubation time must be carefully optimized to elicit a representative immune response without excessive cytotoxicity. These results are consistent with previous studies and contribute to a better understanding of PBMC dynamics under inflammatory conditions, providing a basis for evaluating immunomodulatory or anti-inflammatory interventions.

Acknowledgement

The authors would like to acknowledge the support of the Institute Karya Mulia Bangsa for providing the facilities and resources necessary to conduct this research. Special thanks to the donors who provided umbilical cord samples for the isolation of MSCs.

Competing Interests

The authors declare that there is no conflict of interest.

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