Correlation Between Tumor Necrosis Factor-A (TNF-A) And Bacteria Endotoxin An In Vitro Study

Sawsan Mohsen Salih Al Taei[1*], Muhamed Ali Salman Al Kabe[2]

[1*2] College of Medicine, Department of Medical Microbiology, Wasit University, Iraq
sawsanm102@uowasit.edu.iq[1], Malkaabi@uowasit.edu.iq[2]

***Corressponding Author:** Sawsan Mohsen Salih Al Taei
*College of Medicine, Department of Medical Microbiology, Wasit University, Iraq
sawsanm102@uowasit.edu.iq[1],

Abstract
Gram-negative bacterial lipopolysaccharide (LPS) which has been demonstrated to induce inflammatory reactions in vitro as well as in vivo, including lethal shock. A great number of different cells have been documented to be reactive to LPS, e.g. monocytes/macrophages, vascular cells, polymorph nuclear cells, and even B lymphocytes. We have now established that T lymphocytes could also contribute to an inflammatory reaction to LPS. LPS is a potent inducer of human T-lymphocyte proliferation and cytokine production, macrophages by interacting with Toll-like receptor 4 (TLR4) and triggers the production of various pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and clearance of infectious organisms. Systemic inflammation is implicated in the pathophysiology of many diseases, including chronic inflammatory diseases, infections, sepsis, atherosclerosis, and obesity. This experimental study was performed to find out the effect of healthy person on peripheral blood mononuclear cells(PBMCs) namely T-cells and natural killer cells(NK cells) function through challenging their ability for cytokines production in a comparative study by using of standardized inoculum of Bacterial endotoxins.

Key words: Lipopolysaccharides, in vitro study, peripheral blood mononuclear cells, tumor necrosis factor-α (TNF-α), immune response, Cell culture, Elisa.

1. Introduction

Septic shock (SS) is the last and most severe stage of sepsis. Sepsis occurs when your immune system has an extreme reaction to an infection. The inflammation throughout your body can cause dangerously low blood pressure[1]. Any type of bacteria can cause the infection. Fungi such as candida and viruses can also be a cause, although this is rare. At first the infection can lead to a reaction called sepsis. Pathogenesis refers to the biological processes that lead to the development of a disease or a pathological condition. Disease can arise from pathogens that secrete toxins, from dysregulation of the immune system. Organisms are constantly exposed to microbial pathogens in their environments[2].

The immune system encompasses the protection of the body from invasion by microorganisms (bacteria, viruses, toxins and fungi) and other internal and external threats that may lead to abysmal functioning of the body and disease conditions[3]. Human peripheral blood mononuclear cells (PBMCs) are used to investigate the effect of immune modulatory effects on various immune cells[4]. Cytokines are small proteins secreted by immune cells that mediate communication between different cells of the immune system and regulate immune responses. They are produced by cells of the immune system, such as T cells, B cells, and macrophages, and can act on others cells to stimulate an immune response. Cytokines can be classified into different groups based on their functions, such as pro-inflammatory cytokines, which promote inflammation and the activation of the immune system, and anti-inflammatory cytokines, which help to down regulate the immune response and reduce inflammation[5]. The immune system is basically composed of two compartments, innate and adaptive immunity. The innate immunity provides immediate but less-specific responses, while the adaptive immunity, which is activated by the innate response, gives stronger and very specific responses and develops a memory, by which it remembers specific invaders and mounts a faster and stronger response in later challenges[6]. Exposure to LPS can stimulate the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1(IL-1), and interleukin-6(IL-6). Theses cytokines play a role in activating the immune system and promoting inflammation. In addition to the production of pro-inflammatory cytokines, exposure to LPS can also stimulate production of anti-inflammatory cytokines, such as interleukin-10(IL-10)[7]. Toll- like receptor 4 (TLR-4): is primarily expressed on immune cells, such as monocytes, macrophages, dendritic cells, and certain epithelial cells[8]. The main function of TLR-4 is to recognize and respond to lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. Lipopolysaccharide (LPS) is a large molecule composed of lipid and polysaccharide portions. It is a major component of the outer membrane of gram-negative bacteria. LPS is also referred to as endotoxin because its release from bacterial cells during infection can trigger strong immune responses in mammals[9]. In most Gram-negative bacteria, the outer membrane is composed predominantly of the glycolipid known as lipopolysaccharide (LPS). In E. coli, this complex

molecule is composed of three structurally distinct regions; the hydrophobic anchor called lipid A, a core oligosaccharide, and the long-chain polysaccharide called O antigen (or O-polysaccharide; O-PS). When gram-negative bacteria replicate or undergo lysis, LPS is released into the surrounding environment, such as the bloodstream during an infection. The presence of LPS activates the innate immune system through interactions with Toll-like receptor 4 (TLR4) on immune cells, particularly macrophages and dendritic cells. These immune responses are crucial for mounting an effective defense against bacterial infections[10].

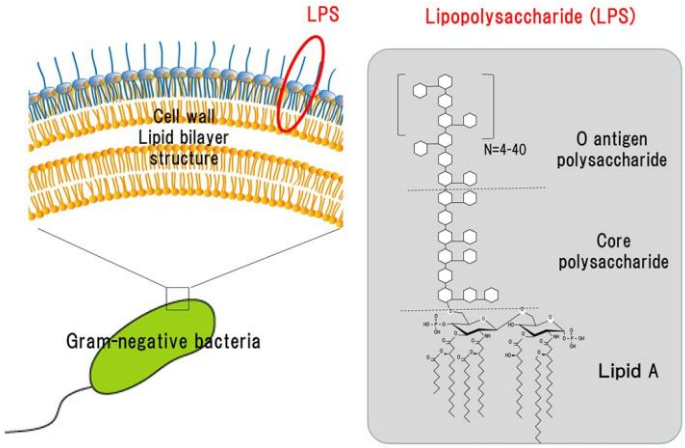

Figure 1 : Lipopolysaccharide (LPS) Structure and function[11].

2. Materials and Methods

2.1 Samples collection

Five ml of venous blood was collected from 40 each Healthy persons participant, aged 20–30 years male and female. The blood has been almost equally divided into (2ml) in EDTA tubes and soon treated for peripheral blood mononuclear cells (PBMCs) separation, (2ml) in EDTA tubes that has been utilized for performing complete blood counting including WBCs (Neutrophils, monocytes, Lymphocytes, Hemoglobin and Platelet) by Cell- DYN Ruby Hematology Abbott ,also (1ml) in the Gel tubes and then labeled [12]

2.2 Peripheral Blood Mononuclear Cells (PBMCs) separation from Whole Blood:

All the specimens of two-ml fresh anticoagulant venous blood were treated first to separate PBMCs by density-gradient centrifugation. Gently layer the blood sample (2 ml)on the top of lymphocytes separation medium (2 ml). Take care not to mix the two layers [13]. The separation process is performed by Centrifuge at 1000 x g for 20 minutes without brake. Carefully harvest the cells by inserting the pipette directly through the upper plasma layer to the mononuclear cells at the interface. Alternatively, you can first remove the upper layer and then collect the cells. The separated PBMCs from each blood specimens were then transferred to a sterile conical tube and volume corrected into (2 ml) by adding of phosphate buffer saline (PBS). Centrifuged at 1000 x g for 10 minutes for cell washing, supernatant removed and the procedure repeated twice. The final cell count for each PBMCs pool was about 400 000 cell /ml cRPMI ensured by slide chamber method of cell counting.

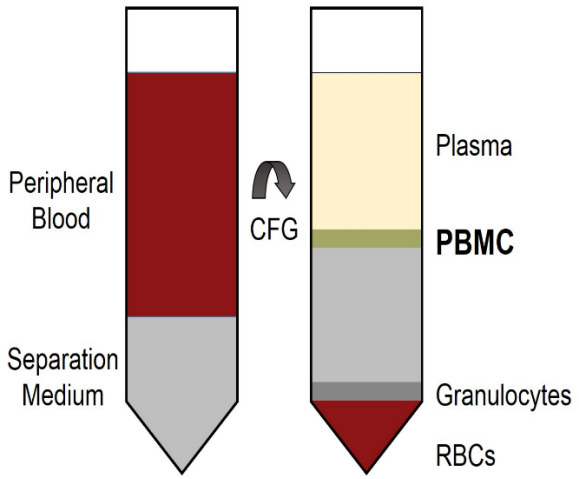

Figure 2 : Peripheral Blood Mononuclear Cells (PBMCs) separation from Whole Blood [14].

2.3 Cell culture:
Human peripheral blood mononuclear cells (PBMCs) were isolated, the cell pellet were then divided into two equal volume and cultured in appropriate media Roswell park Memorial Institute Media(RPMI -1640) incubate both glass tube for 3–5 days in an incubator at 37°C.

2.4 Lipopolysaccharides (LPS) 10 mg :
Source : Escherichia coli 055:b5
Solubility: it can be dissolved in water , shaker by vortex oscillation and heated warm up in water bath to obtain a higher concentration of LPS solution.

2.5 Laboratory Experiments
2.5.1 Estimation of the base level (TNF-α) (that expressed by PBMCs in complete RPMI medium in absence of the LPS antigen):
To detect the base level of (**(**TNF-α) the first 40 samples of PBMCs that prepared from the previous step were incubated in cRPMI medium at 37 degree centigrade for 5 days in the fifth day the tubes of cRPMI were centrifuged at 1000 x g for 10 minutes and then (40 micro litters) of the sample supernatant was aspirated to be tested by ELISA method to determined level of TNF-α (which was regarded as the base level) for each member of the test group[15].

2.5.2 Detection of the stimulation level of (TNF-α) (that expressed in response to LPS along in cRPMI medium as stimulating antigen):-
To detect the stimulation level of TNF-α the second 40 samples of PBMCs that prepared from the previous step were incubated in cRPMI medium at 37 degree centigrade along after adding of 50 µL of LPS for each cRPMI tube. This incubated for five days .Thereafter, each sample was centrifuged at 1000 x g for 10 minutes and the supernatant (40 micro litters) was aspirated to be tested by ELISA method for detecting the level of TNF-α secreted in culture media in response to the stimulating Antigen (i.e. LPS). And this level was regarded as the stimulation level of TNF-α one for each member of the test group[16].

2.6 The ELISA Method
Enzyme- linked immunosorbent assay kit were used to measure the levels of human Tumor necrosis factor-α (TNF-α) in the both group with & without bacteria endotoxin lipopolysaccharide(LPS).

2.6.1 Human Tumor necrosis factor-alpha(TNF-alpha)ELISA Kit:
The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of TNF-alpha in human serum, plasma, Urine, tissue homogenates and other biological fluids[17].

2.6.1.a Test principle
This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Tumor necrosis factor-alpha (TNF-alpha). Add Tumor necrosis factor-alpha(TNF-alpha) to the wells, which are pre-coated with Tumor necrosis factor-alpha (TNF-alpha) monoclonal antibody and then incubate. After that, add anti TNF-alpha antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Tumor necrosis factor-alpha (TNF-alpha) are positively correlated.

2.6.1.b.Assay procedure:
1.Dilution of standard solutions: (This kit has a standard of original concentration, which could be diluted in small tubes by user independently following the instruction the below chart as follows in figure (3):

480 ng/L	Standard No.5	120µl Original Standard + 120µl Standard diluents
240 ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
120 ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
60 ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
30 ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

Standard solution	No5	No4	No3	No2	No1
960 ng/L	480	240	120	60	30

Figure 3: Serial dilution of TNF-alpha standard solution.

2.The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3.Sample injection:
1) Blank well: Add only Chromogen solution A and B, and stop solution.
2) Standard solution well: Add 50µl standard and streptavidin-HRP 50µl.
3) Sample well to be tested: Add 40µl sample and then 10µl TNF-alpha antibodies, 50µl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃ for 60 minutes.
4.Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.
5.Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.
6. Color development: Add 50µl chromogen solution A firstly to each well and then add 50µl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.
7.Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).
8.Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

2.6.1.c.Calculation of result:-
Make concentration of standards the abscissa and OD value the ordinate. Draw the standard curve on the coordinate paper. According to the OD value of the sample, locate its corresponding concentration (which is the concentration of the sample); or calculate the linear regression equation of standard curve according to the concentration of the standard and the OD value. Then substitute with the OD value of the sample to calculate its concentration in figure (4):

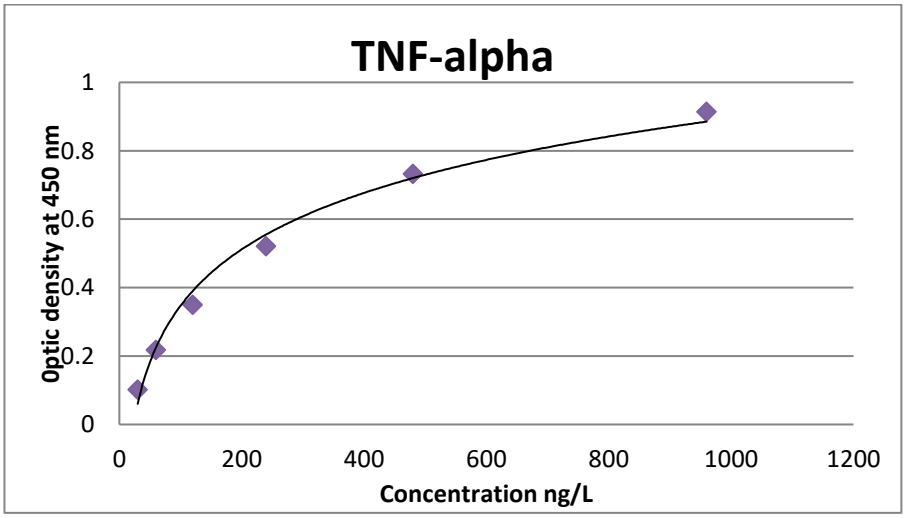

Figure 4:- Standard Curve of TNF-alpha

2.7 Statistical analysis
The mean and standard deviation were used to describe the continuous variables. Both Paired samples t-test and independent samples t-test were used accordingly for the assessment of the mean differences between continuous

variables. Pearson correlation coefficient was used to assess the presence of correlation between the study variables. A P-value equal to or less than 0.05 was considered significant

3. Results

This in vitro study aimed to investigate the factors influencing the tumor necrosis factor-alpha (TNF-α) response to bacterial endotoxin stimulation. TNF-α is a potent pro-inflammatory cytokine involved in immune responses and inflammatory processes. Understanding the drivers of TNF-α production in response to bacterial endotoxin can provide valuable insights into the modulation of inflammatory cascades. In this study, we utilized an in vitro model to examine the factors influencing TNF-α production in response to bacterial endotoxin. Concentration-dependent TNF-α response: Our results demonstrated a concentration-dependent increase in TNF-α production upon stimulation with bacterial endotoxin. Higher concentrations of LPS resulted in greater TNF-α secretion by immune cells.

Time-dependent kinetics: The production of TNF-α showed time-dependent kinetics following LPS stimulation. TNF-α levels increased gradually over time, with a peak observed at a specific time point.

Synergistic effects: Co-stimulation with other immune stimuli, such as cytokines or toll-like receptor (TLR) ligands, enhanced TNF-α production in response to bacterial endotoxin. This suggests the presence of cross-talk between different immune signaling pathways and the potential amplification of the TNF-α response.

Cell-specific responses: Different immune cell types exhibited variations in TNF-α response to bacterial endotoxin. For example, monocytes may display a more robust TNF-α production compared to macrophages.

3.1 Detection Tumor Necrosis Factor Alpha (TNF-α) in experimental vitro by using ELISA method:

Table 1: Mean differences between the baseline and post-stimulation Tumor Necrosis Factor Alpha (TNF-α) as show in figure 5.

TNF-α (ng/L)	Baseline	Post stimulation	P-value
Mean	233.288	461.357	<0.001
Standard deviation	95.559	205.504	

Table 2: Descriptive statistics for the mean difference (post-stimulation – baseline level) TNF-α in the study parameter.

Variables	Mean difference	Standard Deviation
TNF-α (ng/L)	228.06	207.66

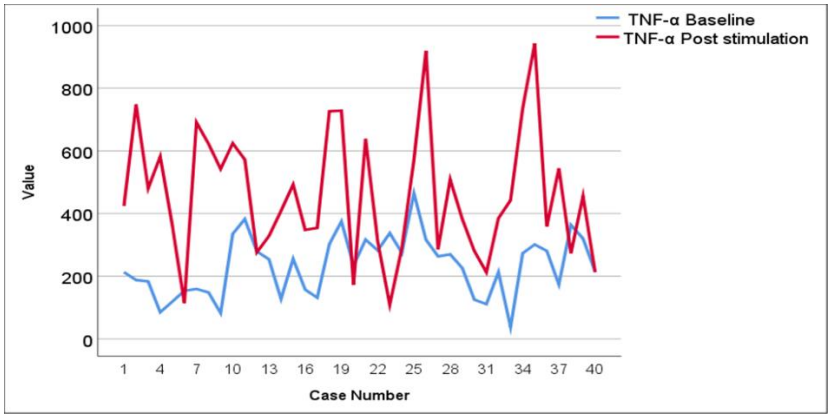

Figure 5: Histogram of the distribution of baseline and post-stimulation TNF-α among the 40 study participants.

4. Discussion

Tumor necrosis factor alpha (TNF-α) is a cytokine that has pleiotropic effects on various cell types. It has been identified as a major regulator of inflammatory responses and is known to be involved in the pathogenesis of some inflammatory and autoimmune *Jang et al*., 2021 [18].

Theses results show that the TNF-α level was significantly elevated after stimulation with a P-value less than 0.001. The mean pre-stimulation TNF-α was 233.288±95.559 ng/L then changed to 461.357±205.504 ng/L post-stimulation and the show in figur 5 Our experiment study appear to agree with *Rahimi et al*., 2020 [19] in the study present effects LPS induced inflammation both *in vivo* and *in-vitro* models and dose-dependently reduced the level of inflammation,and the spatial memory.

Ghanavatinejad et al., 2021 [20] disagreed that the because of the small size of samples, doing experiments all together on some samples was not feasible.

Also *Shih et al.*, 2012 [21] agreement that found endotoxin can activate macrophages, lymphocytes, fibroblasts, and endothelial cells to produce pro-inflammatory cytokines e.g. IL-I, TNFa, IL-6, IL-8. These mediators act as paracrines and/or autocrines to cause a vicious reaction by activating more inflammatory cells.

Our results agree with *Üçeyler et al*., 2019 [22] that found LPS are necessary for further increase. Indeed, these stimuli do increase TNF (and other pro-inflammatory mediators) in PBMC, and the reason Positive controls are an important feature in experimental studies as they show the responsiveness of the model under investigation. An often applied reagent for a pro-inflammatory stimulus is the endotoxin lipopolysaccharide (LPS), which has been shown to induce a cytokine release by various cell cultures *Maldonado et al*., 2016 [23].

5. Conclusions

1. the correlation analysis was conducted to examine the relationships between the mean differences (post-stimulation - baseline level) of the five study parameter. The results are presented in Table 1.
2. Tumor Necrosis Factor Alpha (TNF-α): The mean TNF-α level significantly increased from a baseline of 233.288 ng/L to 461.357 ng/L after stimulation. TNF-α is a major regulator of inflammatory responses and is involved in the pathogenesis of inflammatory and autoimmune diseases.
3. our study sheds light on the factors driving TNF-α production in response to bacterial endotoxin stimulation. Targeting IL-6 signaling pathways may hold therapeutic potential for diseases characterized by dysregulated inflammation.

6. References

1. Nakamori, Y., E.J. Park, and M.J.F.i.i. Shimaoka, *Immune deregulation in sepsis and septic shock: reversing immune paralysis by targeting PD-1/PD-L1 pathway.* 2021. **11**: p. 624279.
2. Jean Beltran, P.M., et al., *Proteomics and integrative omic approaches for understanding host–pathogen interactions and infectious diseases.* 2017. **13**(3): p. 922.
3. Aleruchi, C., et al., *African Journal of Biological Sciences.* 2022.
4. Kleiveland, C.R.J.T.I.o.F.B.o.H.i.v. and e.v. models, *Peripheral blood mononuclear cells* :2015 .p. 161-167.
5. Li, L., et al., *Effects of immune cells and cytokines on inflammation and immunosuppression in the tumor microenvironment.* 2020. **88**: p. 106939.
6. Marshall, J.S., et al., *An introduction to immunology and immunopathology.* 2018. **14**(2 :(p. 1-10.
7. Qian, L., et al., *Safinamide prevents lipopolysaccharide (LPS)-induced inflammation in macrophages by suppressing TLR4/NF-κB signaling.* 2021. **96**: p. 107712.
8. Bueno, B.G., et al., *Innate immune receptor Toll-like receptor 4 signalling in neuropsychiatric diseases.* 2016. **64**: p. 134-147.
9. Gorman, A., A.P.J.E.J.o.P. Golovanov, and Biopharmaceutics, *Lipopolysaccharide Structure and the Phenomenon of Low Endotoxin Recovery.* 2022.
10. .Di Lorenzo, F., et al., *Lipopolysaccharide structures of Gram-negative populations in the gut microbiota and effects on host interactions.* 2019. **43**(3): p. 257-272.
11. Mohammad, S. and C.J.F.i.i. Thiemermann, *Role of metabolic endotoxemia in systemic inflammation and potential interventions.* 2021. **11**: p. 594150.
12. Giavarina, D. and G.J.C.b. Lippi, *Blood venous sample collection: Recommendations overview and a checklist to improve quality.* 2017. **50**(10-11): p. 568-573.
13. David, C.A., et al., *In vitro determination of the immunogenic impact of nanomaterials on primary peripheral blood mononuclear cells.* 2020. **21**(16): p. 5610.
14. Patrone, D., et al., *Optimization of Peripheral Blood Mononuclear Cell Extraction from Small Volume of Blood Samples: Potential Implications for Children-Related Diseases.* 2022. **5**(2): p.20 .
15. Saferding, V., et al., *A6. 42 The role of micro-RNA-146a in inflammatory arthritis*. 2015, BMJ Publishing Group Ltd.
16. Bercusson, A., *Defining the impact of Ibrutinib therapy and CFTR dysfunction on macrophage function in the innate immune response to Aspergillus fumigatus.* 2019.
17. Prado, M.S., et al., *Biological anti-TNF drugs: immunogenicity underlying treatment failure and adverse events.* 2017. **13**(9): p. 985-995.
18. Jang, D.-i., et al., *The role of tumor necrosis factor alpha (TNF-α) in autoimmune disease and current TNF-α inhibitors in therapeutics.* 2021. **22**(5): p. 2719.
19. Rahimi, V.B., et al., *The effects of hydro-ethanolic extract of Capparis spinosa (C. spinosa) on lipopolysaccharide (LPS)-induced inflammation and cognitive impairment: evidence from in vivo and in vitro studies.* 2020. **256**: p. 112706.
20. Ghanavatinejad, A., et al., *Vitamin D3 controls TLR4-and TLR2-mediated inflammatory responses of endometrial cells.* 2021. **86**(1): p. 139-148.
21. Shih, M., et al., *In vitro and in vivo therapeutics of β-thujaplicin on LPS-induced inflammation in macrophages and septic shock in mice.* 2012. **25**(1): p. 39-48.
22. Üçeyler, N., et al., *Tumor necrosis factor-α links heat and inflammation with Fabry pain.* 2019. **127**(3): p. 200-206.
23. R.F., I. Sá-Correia, and M.A.J.F.m.r. Valvano, *Lipopolysaccharide modification in Gram-negative bacteria during chronic infection.* 2016. **40**(4): p. 480-493.