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The Effects of Inactivated Lipopolysaccharide (LPS) from *Escherichia coli* on Intact LPS in Relation to Toll-Like Receptor- 4 (TLR-4)

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Abstract

Background: Lipopolysaccharide (LPS) is a powerful activator of the innate immune system. Among other effects, it binds to the Toll-like receptor- 4 (TLR-4) expressed by a number of cell types including blood mononuclear cells that result in the activation of signaling pathways leading to the production and release of pro-inflammatory cytokines and Type I interferons. If produced in excessive amounts some of the cytokines can contribute to septic shock.

Aim: To determine the effects of heat-inactivated LPS (HI-LPS) and Intact LPS from *E. coli* (LPS-EB) each alone or in combination on TLR-4 by assessing levels of cytokine release (TNF-α and IL-12) in vivo and in vitro. Moreover, to assess the survival of treated mice that received different injections.

Methods: Mononuclear cells isolated from blood collected from a group of syngeneic mice were cultured in the presence of different concentrations of Intact LPS (LPS-EB) and heat-inactivated LPS (HI-LPS), and TNF-α and IL-12 levels were determined in the supernatants by ELISA. One hundred and twenty-six Balb/c mice divided into groups were used for the *in vivo* experiments. They received intraperitoneal injections of different combinations of Intact and heat-inactivated LPS. Sixty three mice from the different groups were bled at set time intervals post-injection and serum levels of IL-12 and TNF-α were determined. The rest of the mice were used to monitor survival rate for a period of 7 days.

Results: Statistically significant results were observed at the *in vivo* level, when TNF-α and IL-12 levels decreased in the group of mice that received intact LPS followed by HI- LPS injections. There was a 11.1% survival rate in the group of mice that received an injection of Intact LPS followed by inactive LPS compared to 0% survival in mice that did not receive heat- inactivated LPS. There was a statistically non-significant decrease in levels of IL-12 and TNF-α in supernatants of cells treated *in vitro*.

Conclusion: The *in vivo* results indicate that heat-inactivated LPS plays a curative role possibly by reducing or blocking the engagement of intact LPS to TLR-4. This could be of potential benefit in blocking and preventing septic shock.

Keywords: Lipopolysaccharide(LPS); Intact LPS; TLR-4; Heat-inactivated LPS; Septic shock; Mononuclear cells (MNCs); TNF-α; IL-12; ELISA

Introduction

Invasion of the body by bacteria, and especially Gram negative bacteria, can result in an overdrive of the body's defense leading to a dysregulation in the inflammatory response. The key players in this reaction are one of the constituents of the outer membrane of the cell wall which is the lipopolysaccharide (LPS), also referred to as endotoxin. LPS plays an important role in the physiological membrane functions and is, therefore, crucial for bacterial viability and growth [1]. It consists of a highly variable O-specific chain made up of oligosaccharides, a less variable core oligosaccharide, and a lipid component, termed lipid A, with low structural variability [2]. LPS is a highly effective activator of the innate immune system [2]. It interacts with several types of cells [3] that leads to the release of a wide spectrum of inflammatory mediators that are important for the early innate followed by the adaptive response [4]. One of its mechanisms of action is by its role as a ligand for Toll-Like receptor-4 (TLR-4) [5], the first in the TLR family to be recognized as a human homologue of Drosophila Toll receptors [6]. Engagement of a TLR by its ligand would lead to the activation of signaling pathways, activation of nuclear factors (NF-κB and IRF3) followed by the production of cytokines that are involved in innate, adaptive and inflammatory responses [7].

Poltorak et al. [1,8] analyzed positional cloning of the LPS-nonresponsive mouse strain, C3H/HeJ, and reported that a point mutation in the TIR domain of TLR4 was responsible for the defect in LPS signal transduction. Hoshino et al. [9] further confirmed that a group of mice were unresponsive to LPS when they were induced with a targeted deletion of the TLR4 gene. All these findings demonstrated the essential role for TLR4 in recognition of a major component of Gram-negative bacteria, the Lipopolysaccharide.

TLR-4 signaling involves the downstream recruitment of protein kinases by a family of adaptor proteins which activate transcription factors such as nuclear factor-κB (NF-κB) and members of the interferon (IFN)-regulatory factor (IRF) family [10]. Four adaptor molecules have been identified so far: MyD88, TIR-associated protein (TIRAP), TIR domain-containing adaptor protein-inducing IFN-b (TRIF), and TRIF-related adaptor molecules (TRAM) [7]. MyD88 and TIRAP are responsible for the induction of pro-inflammatory genes and the expression of pro-inflammatory cytokines and chemokines such as tumor necrosis factor- alpha (TNF-α), IL-1, IL-6, and IL-12 by activation of the Myeloid Differentiation Factor 88 (MyD88)-dependent pathway; while TRIF and TRAM induce α and β IFNs by the activation of the MyD88-independent pathway [11].

If activation of both MyD88 dependent and independent pathways by LPS are not controlled properly, imbalanced inflammatory response and the excess production of inflammatory cytokines such as Tumor Necrosis Factor-alpha (TNF-α) and Interlukin-12(IL-12) will be released. Tumor Necrosis Factor- α (TNF-α) regulates immune and inflammatory responses and is involved in systemic inflammation [12,13]. It is produced by several cell types including macrophages, lymphocytes and neutrophils [12] and is a marker to assess septic shock and inflammatory conditions [13]. On the other hand, Interlukin-12 is produced by several cell types including macrophages to stimulate an adaptive response by lymphocytes, thus being the mediator in linking the innate and adaptive immune response upon activation of TLR-4 by LPS [14]. Increased production of cytokines by LPS can be partially the cause of septic shock. Hence the aim of this study was to attempt to neutralize the LPS-TLR-4 engagement effect by heat-inactivated LPS.

Materials and Methods

Reagents and preparation of injections

Intact LPS (LPS-EB) from *E. coli* 0111:B4 strain was purchased from InvivoGen (5, rue Jean Rodier, F-31400 Toulouse, France). The TLR-4 blocker used as a control in the experiments was LPS-RS Ultrapure lipopolysaccharide from *Rhodobacter sphaeroides* (a TLR-4 antagonist) which was purchased from InvivoGen (5, rue Jean Rodier, F-31400 Toulouse, France). All substances were prepared in sterile pyrogen free distilled water.

Determination of LD50 of LPS

For the determination of LD50, 5 groups of 5 mice each were used and were injected intraperitoneally (IP) with different concentrations of LPS-EB (Intact) (500μg/ml, 1000μg/ml, 2000μg/ml, 3000μg/ml, 4000μg/ml) in 0.2ml/mouse correspondingly. The mice were monitored for survival for a week. Based on the survival rates a linear curve was obtained and LD50 of LPS was calculated.

LPS inactivation

LPS was inactivated by dry heating in an oven at 200 °C degrees for 1.5 hours to reduce the LPS activity. Desired concentrations of inactive LPS were prepared by diluting LPS in Sterile Pyrogen free distilled water. To confirm the inactivation of LPS, one group of five mice received IP injections of 800 μg/0.2ml of the inactivated LPS preparation and was monitored for one month.

Measurement of endotoxin activity

LPS activity was determined using the Limulus Amoebocyte Lysate Assay (LAL) Kit (Lonza, 8830 Biggls Ford Rd., Wakerville, MD, USA). Procedure was performed according to the manufacturer's instructions.

Animals

Hundred and seventy-six female BALB/c mice (6-8 weeks old), were used. Mice were maintained on 12-h light/dark cycle and were provided food and water ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Medicine, American University of Beirut (AUB).

Procurement of specimens

To determine the effect of Intact LPS and Heat-Inactivated LPS on levels of TNF-α and IL-12 in serum, 63 mice were divided into seven groups of 9 mice each and received different IP injections as shown in Table 1. LPS-EB (Intact) was administered at a dose of 300μg/0.2ml/mice, H-I LPS at 300μg/0.2ml/mice, and *Rhodobacter sphaeroides* LPS (a TLR-4 antagonist) at 100μg/0.2ml/mice. At 2, 4 and 6 hours post-injections, three mice from each group were euthanized then dissected and blood was collected by cardiac puncture. Blood from each group was pooled and serum was separated and used for TNF-α and IL-12 quantification.

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Table 1: Protocol followed for mice injection for sacrifice and survival.

Group Description	Treatment Protocol	
	T_0	T_{30}
Control Group	Sterile Water	-
Positive Control	LPS-EB	-
Negative Control	HI-LPS	-
Test Group 1	LPS-RS	-
Test Group 2	LPS-EB	HI-LPS
Test Group 3	HI-LPS	LPS-EB
Test Group 4	LPS-EB	TLR-4 Blocker

LPS- EB(Intact): Lipopolysaccharide extracted from *E. coli* 0111:B4. (300µg/0.2ml/mice); HI-LPS: Heat-Inactivated LPS-EB. (300µg/0.2ml/mice); TLR-4 Blocker: LPS extracted from *Rhodobacter sphaeroides* (100µg/0.2ml/mice).

Survival studies

To assess survival rates, another group of 63 mice were used and were divided into seven groups of 9 mice each and received the same protocol of IP injections as shown in Table 1. Mice were then monitored for survival for a week.

Cell culture

Ten mice were sacrificed and blood was collected and pooled. Mononuclear cells were separated from whole blood using Ficoll Isopaque gradient separation medium. The obtained cells were then treated with RBC Lysis solution and washed before culturing. MNCs were counted and seeded in 48-well plates, in RPMI medium supplemented with 1% L-Glutamine, 1% Pen-strep antibiotics, and 10% of heat inactivated fetal bovine serum (FBS) with a seeding density of $2x10^6$/200µl per well. Cells were incubated with varying concentrations and combinations of 200µl of either LPS-EB (Intact), HI-LPS (Heat-Inactivated) or TLR-4 Blocker (Figure 1). All wells were run in duplicates and were incubated at 37 °C in a 5% CO_2. Supernatant was collected after 4 hours of incubation.

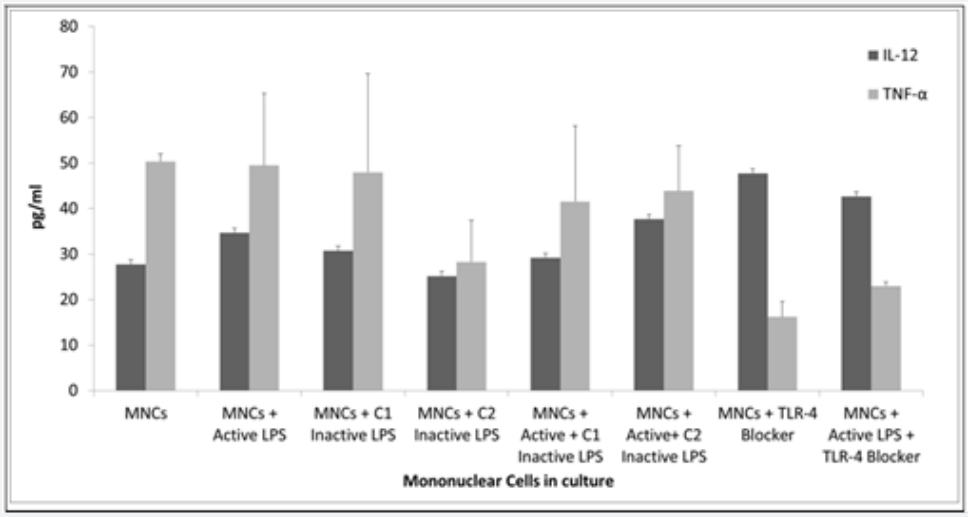

Figure 1: IL-12 and TNF-α levels of cell supernatant of the in-vitro study as detected by ELISA. (C LPS-EB: 50ng/ml, C1 HI-LPS: 50ng/ml, C2HI-LPS: 100ng/ml).

TNF-α and IL-12 quantification

TNF-α ELISA Kit (Qiagen, Germany) and Interlukin-12 p70 Mouse ELISA kit (Abcam, ab119531, USA) were used to determine serum and supernatant levels of TNF-α and IL-12, respectively. The procedures were performed according to the manufacturer's protocol. All experiments were run in duplicates.

Statistical analysis

Whenever applicable, results were expressed as Mean±SD. Mice survival was evaluated by generating Kaplan-Meier survival curves and percentage curves. The unpaired student T-test was implemented to assess the sample variations between groups using the online Graph pad software. Results were considered to be statistically significant when P value was < 0.05.

Results

Lethal dose 50 (LD-50) of intact LPS

LD50 of LPS was determined by plotting a linear curve based on the number of mice from each group that died on Day 1. The LD-50 for intact LPS was 300.2µg/0.2ml per mouse.

Presence and activity of endotoxin

Presence of LPS molecule in the LPS-EB (Intact) and heat-inactivated LPS (H-I LPS) suspensions was determined by the Limulus Amebocyte Lysate (LAL) assay. Clotting was obtained in both suspensions whereas no clotting was observed in the negative control. The five mice given an injection of inactive LPS remained alive for a month (100% survival), indicating the

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complete inactivation of the LPS toxicity whereas the same dose of active LPS was lethal to mice.

TNF-α serum levels

The serum levels of TNF-α increased significantly at 2 hours followed by a decrease at 4 and 6 hours in the group that received intact LPS followed by inactive LPS, as compared to the group that received injections of only intact LPS. However, there was a significant increase in TNF-α levels at 2, 4 and 6 hours post injections in the group that received injections of inactive LPS first followed by intact LPS (Figure 2).

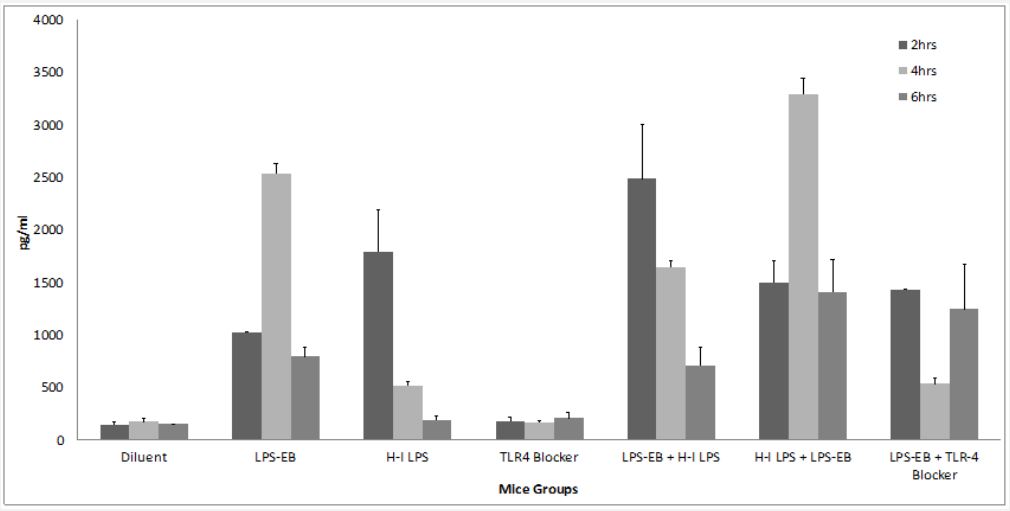

Figure 2: Serum TNF-α levels of the 7 different groups of Balb/c mice at 2, 4 and 6 hours after injections as detected by ELISA. LPS-EB: Intact LPS; H-I LPS: Heat-Inactivated LPS; TLR-4 Blocker: LPS Extracted from *Rhodobacter sphaeroides*.

IL-12 serum levels

The serum levels of IL-12 increased significantly at 2 hours followed by a decrease at 4 and 6 hours in the group that received intact followed by inactive LPS, as compared to the group that received injections of only intact LPS. A minor decrease in IL-12 levels was observed at 2 hours followed by a significant increase at 4 and 6 hrs post injections in the group that received injections of inactive LPS first followed by intact LPS (Figure 3).

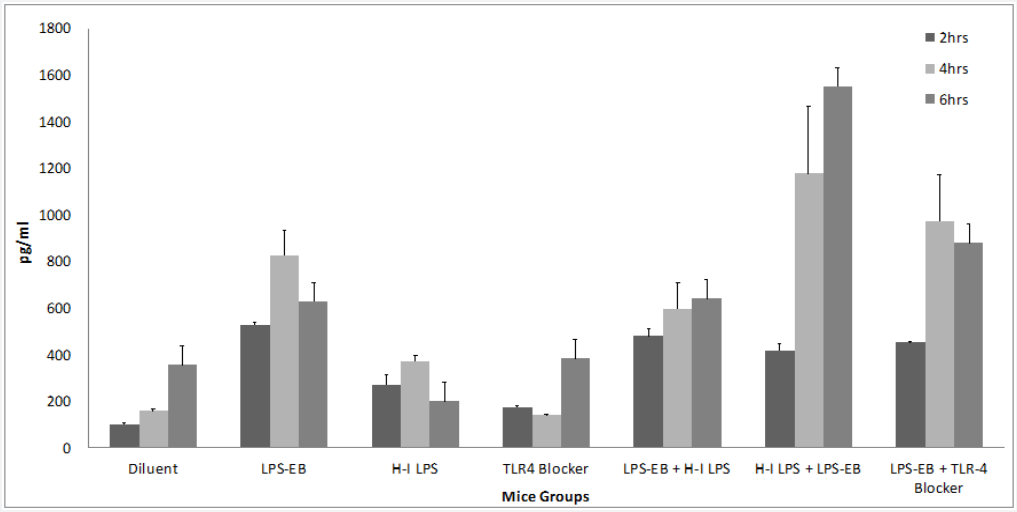

Figure 3: Serum IL-12 levels of the 7 different groups of Balb/c mice at 2, 4 and 6 hours after injections as detected by ELISA. LPS-EB: Intact LPS; HI-LPS: Heat Inactivated LPS; TLR4 blocker: LPS extracted from *Rhodobacter sphaeroides*.

Mice survival

By day 7, all mice in the 3 control groups, the group that received either the diluent, the inactive LPS or the TLR-4 Blocker showed a 100% survival. As opposed to the groups that received intact LPS (LPS-EB) alone or heat-inactivated LPS followed by intact LPS (LPS-EB) and had 0% survival rate by day 2, and the group that received intact LPS followed by TLR-4 blocker with 0% survival rate by day 3. However, the only group apart from

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the control groups that had an 11.1% survival rate was the group that received inactive LPS as a prophylactic treatment post intact LPS (LPS-EB) injections. The remaining one mouse could overcome the LPS infection (Figure 4A).

The survival results were further evaluated by generating the Kaplan-Meier survival curves showing the probability of survival in a given period of time. The statistical significance of the results obtained was assessed by determining the p-value; p-value≤0.05 was considered statistically significant. All groups showed significant survival results (Figure 4B).

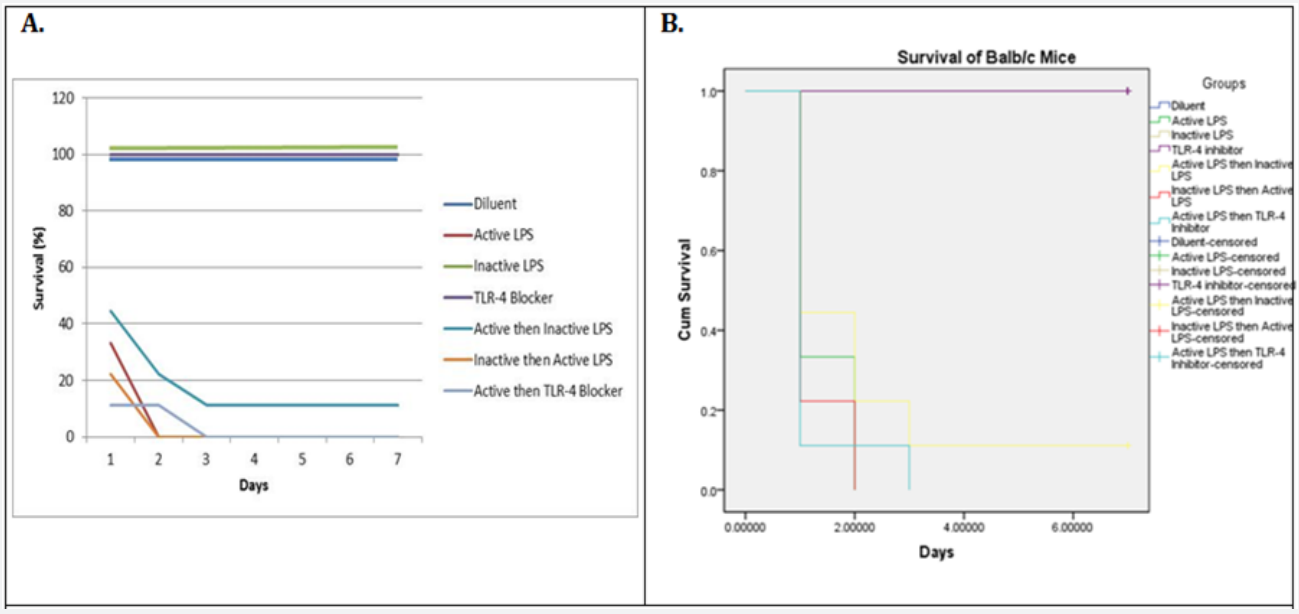

Figure 4: Survival Results of the 7 groups followed over 7 days. (4A) shows the percent survival curve of the seven groups that received different IP injections, and only the mice that received inactive LPS as a prophylactic treatment post intact LPS injection showed an 11.1% survival rate (4B) Kaplan Meier survival curve, for the seven groups. Significance was considered when P value < 0.05.

TNF-α level in supernatants of cell culture

Incubation of MNCs with 100ng of heat-inactivated LPS (H-I LPS) alone caused a significant decrease in TNF-α level as compared to the groups that received Intact LPS alone or lower dose of H-I LPS (50ng) alone. However, when cells were cultured with two different separate doses of H-I LPS and active LPS, it was the lower dose of HI-LPS (50ng) that caused a more and significant decrease in TNF-α levels as compared to the cells cultured with intact LPS alone or with higher (100ng) H-I LPS. The TLR-4 blocker additionally caused a decrease in TNF-α levels when used alone with the cells or when added to the intact LPS (Figure 1).

IL-12in cell culture supernatants Levels

Il-12 levels were elevated when MNCs were incubated with the intact LPS as compared to the control group. However, when H-I LPS was used alone either at 50ng or 100ng, it caused a decrease in IL-12 levels as compared to the intact LPS alone, the lowest decrease was best seen at the 100ng of H-I LPS concentration. When 100ng H-I LPS was used in combination with the intact LPS, IL-12 levels were decreased; however when equal amount of H-I LPS (50ng) was used with intact LPS, IL-12 levels were additional decreased. TLR-4 blocker alone or in combination with intact LPS, resulted in higher levels of Il-12 when compared to all groups (Figure 1).

Discussion

The worldwide incidence of sepsis is estimated to be up to 19 million per year and accounts for more than one third of intensive care unit (ICU) admissions. Despite improvement in patients' care, and a decrease in mortality rates throughout the last years it still remains high, accounting for 5.3 million deaths every year [18]. Constructing treatment strategies which aim to improve the survival of septic patients should be a chief goal in research.

The aim of this study was to use heat-inactivated LPS as a means of blocking TLR-4, inhibiting the signaling pathways and suppressing over-production of pro-inflammatory cytokines. Inactivation or depyrogenation processes indicate that bacterial LPS activity is reduced to an accepted level [19]. A study by Gao et al. [20] where LPS inactivation was achieved by boiling, suggests that heat treatment was sufficient to inactivate LPS [20]. They also found that boiling reduced the size of aggregates of LPS. However, the physical and chemical basis of heat-induced changes in LPS aggregate sizes and LPS activity are not clear. Mueller et al. [21] have explained that aggregated LPS are more active than monomeric LPS, which might clarify why heat inactivation decreases the activity of LPS. Consistent with these reports our results indicated that heat inactivation of LPS had a non-toxic and non-lethal effect on Balb/c mice. However,

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it appeared that results of the Limulus Lysate Assay did not correlate with the results of the lethal effects of LPS in mice since both intact and heat-inactivated LPS were Limulus Lysate-positive.

Although there was a decrease in the production of both TNF-α and IL-12 by MNCs treated with intact LPS followed by heat-inactivated LPS *in vitro*, the difference was not statistically significant. It might be possible that significance could be attained if the amounts of reagents are manipulated [22-24].

In the *in-vivo* studies, a significant decrease in serum TNF-α levels was observed after 4 hours of administering intact LPS followed by heat-inactivated (H-I LPS). Moreover, a significant decrease in serum IL-12 levels at 2 and 4 hours post injections was observed. However, when mice received H-I LPS first as a prophylactic measure followed by intact LPS, both TNF-α and IL-12 levels were significantly elevated, confirming that the administration of inactive prior to the intact LPS does not manipulate the activity of intact LPS. The cytokine levels obtained concurred with the survival results, as the group of mice that received H-I LPS as a treatment post-intact LPS injections had a better survival rate than all other experimental groups (11.1%). Based on these observations it can be suggested that inactive LPS plays a curative rather than a preventive role when infected by a Gram negative bacteria, possibly by blocking the engagement of intact LPS to TLR-4,or by other means by which intracellular signals are blocked. Finally, it is worth noting that only one aspect of LPS involvement in innate immunity was investigated. Other aspects include the Complement, Clotting, Fibrinolytic and Kinin systems [25,26].

Conclusion

In conclusion, heat-inactivated LPS (H-I LPS) suppresses the effect of intact LPS (LPS-EB) by decreasing the production of pro- inflammatory cytokines, only when administered it after intact LPS and not as a prophylactic measure. However, this study concentrated on the effect of inactive LPS on production of cytokines and not on the mechanism of action of inactive LPS. It would be interesting to carry out further studies that can assess the molecular structure of the inactive LPS in relation to the intact LPS and compare the method by which they bind to TLR-4 and neutralize the downstream reaction. This might be achieved by using molecular and immune fluorescent techniques. Moreover, this study could be used as a starting point to consider using inactive LPS for treating septic shock.

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Authors' Contributions

Gharin Haidossian and Nayla S Al Akl: contributed to the conception and design of this study, acquisition of laboratory data, data analysis and/or interpretation, drafting and /or critical revision of the manuscript and approved this final version. Yara Khalifeh: aided in the experimental part of this study, Alexander M Abdelnoor, Principle Investigator: Funded and introduced the conception and design of this study. He drafted and made a critical revision of the manuscript and approved this final version.

Conflict of Interest

The authors declare that they have no conflict of interests.

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