

# The Effect of Denatured Flagellin on Toll-Like Receptor-5 (TLR-5) in Mice



Soha Mcheik, Nayla S. Al-Akl and Alexander M. Abdelnoor\*

Department of Experimental Pathology, Immunology and Microbiology, Faculty of Medicine, American University of Beirut, Lebanon

**Abstract: Background:** Previous studies have demonstrated that flagellin, a component of bacterial flagella, engages Toll-Like receptor 5 (TLR-5) causing the activation of the Myeloid Differentiation Factor-88 (MYD-88) pathway that leads to the production of pro-inflammatory cytokines including Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) and Interleukin-12 (IL-12). In physiological levels, cytokines can aid in protection against infectious agents. However, excessive production of cytokines can lead to septic shock during sepsis.

**Objective:** In this study, we aimed at investigating the effect of denatured flagellin on hindering the effects induced by intact flagellin or flagellated *Pseudomonas aeruginosa* on the Toll-Like Receptor-5 (TLR-5) in mice.

**Methods:** Mouse mononuclear cells (MNCs) were cultured with intact flagellin, heat-denatured flagellin, TLR-5 antagonist, *Pseudomonas aeruginosa*, and TLR-4 antagonist each alone or in combinations. Supernatants were collected at 4 hours post incubation to assess the levels of IL-12 and TNF- $\alpha$  by Enzyme-Linked ImmunoAssay (ELISA). Furthermore, groups of BALB/c mice were injected intraperitoneally (IP) with *Pseudomonas aeruginosa*, LPS-RS, intact flagellin, and denatured flagellin, each alone or in different combinations. Serum levels of IL-12 and TNF- $\alpha$  were measured at 2, 4, and 6 hours post injections of *Pseudomonas aeruginosa* or intact flagellin.

**Results:** Pretreatment with denatured flagellin significantly reduced the amount of TNF- $\alpha$  and IL-12 produced both *in vitro* and *in vivo* by intact flagellin or *Pseudomonas aeruginosa*.

**Conclusion:** Denatured flagellin suppressed the production of the pro-inflammatory cytokines induced by intact flagellin or *Pseudomonas aeruginosa* both *in vitro* and *in vivo*, probably by blocking TLR5. Denatured flagellin might be considered as an anti-septic shock agent.

**Keywords:** TLR-5, flagellin, Anti-mTLR5-IgG, TLR-4, LPS-RS, cytokines.

## 1. INTRODUCTION

The engagement of Flagellin with Toll Like Receptor-5 (TLR-5), expressed by different cell types including immunocompetent cells, results in the initiation of a signaling pathway that leads to the activation of the nuclear factor NF- $\kappa$ B and the production of pro-inflammatory cytokines including Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) and Interleukin-12 (IL-12) [1-3]. TNF- $\alpha$ , mainly produced by macrophages, is considered as an endogenous pyrogen and contributes to the promotion of sepsis [4]. IL-12, also produced by macrophages, is implicated in the development of an adaptive immune response during sepsis [5].

Since *Pseudomonas aeruginosa* (*P. aeruginosa*) is a flagellated bacterium that has Lipopolysaccharide (LPS) in

its outer membrane, it can activate two signaling pathways; one initiated by flagellin-TLR-5 engagement and the other by LPS-TLR-4 engagement. In the former case, pro-inflammatory cytokines are produced and in the latter both pro-inflammatory cytokines and Type I interferon's are produced [6].

The presence of an elevated concentration of flagellin in the plasma of human patients suffering from Gram-negative sepsis suggests that it might be responsible for the inflammatory response. Moreover, it was implicated that flagellin/TLR-5 signaling promoted the pathophysiology of sepsis. The pro-inflammatory potency of flagellin appears in the development of cardiovascular collapse and acute lung inflammation in mice models. Additionally, it was reported that *Salmonella* flagellin was responsible for cytokine release from intestinal epithelial cells in culture, and when it was injected intraperitoneally into lipopolysaccharide (LPS)-resistant C3H/HeJ mice, it caused shock-like syndrome [7, 8].

\*Address correspondence to this author at the Department of Experimental Pathology Immunology and Microbiology, Faculty of Medicine, American University of Beirut, P.O. Box: 11-0236, Riad EL Solh, Beirut, Lebanon 1107-2020; Tel/Fax: +961-1-350000, ext 5124; E-mail: aanoor@aub.edu.lb

Knowing that bacterial flagellin elicits a sepsis-like systemic inflammatory response in mice *via* the production of pro-inflammatory cytokines, we hypothesized that denatured flagellin might decrease pro-inflammatory cytokines production; thus, alleviating septic shock. In this study, we investigated the effect of denatured flagellin on hindering intact flagellin and *Pseudomonas aeruginosa* from inducing the production of the pro-inflammatory cytokines TNF- $\alpha$  and IL-12.

## 2. MATERIALS AND METHODS

### 2.1. Reagents and Preparations

Flagellin extracted from *Salmonella typhimurium* (FLA\_ST) (Invivogen 3950 sorrento Valley Blvd. suite 100, San Diego CA-92121-USA) was used by preparing a suspension of 50 $\mu$ g of the lyophilized powder in 0.5 ml endotoxin-free water and will be referred to as intact flagellin. Denatured flagellin was prepared by heating at 70 °C in a water bath for 20 minutes since it is thermolabile. Anti-mTLR-5-IgG (Invivogen 3950 Sorrento Valley Blvd. suite 100, San Diego CA-92121-USA) was suspended in sterile distilled water to a final concentration 100 $\mu$ g/ml, Lipopolysaccharide extracted from *Rhodobacter sphaeroides* (TLR-4 antagonist, Invivogen 3950 Sorrento Valley Blvd. suite 100, San Diego CA-92121-USA), was prepared by reconstituting 5 mg of the lyophilized powder in 1ml endotoxin-free water. Desired dilutions were used to reach final concentrations for each experiment.

### 2.2. *Pseudomonas aeruginosa* Strain PAK

*P. aeruginosa* strain PAK was grown overnight in Luria Bertani (LB) agar. The colonies were then suspended in sterile distilled water; for the *in vitro* studies, a concentration of 2.5x10<sup>5</sup>CFU/ml was used (each 0.2 ml containing 5x10<sup>4</sup>CFU), and for the *in vivo* studies, a concentration of 5x10<sup>6</sup>CFU/ml was used (each 0.2 ml containing 10<sup>6</sup>CFU).

### 2.3. Treatment of Mice and Procurement of Specimens

Sixty three female Balb/c mice were obtained from the Animal Care Facility at the American University of Beirut, all procedures followed were reviewed and approved by the Institutional Animal Care and Usage Committee of the

American University of Beirut. Mice were divided into 7 groups of 9 mice each and received intraperitoneal (IP) injections of *P. aeruginosa*, LPS-RS (TLR-4 antagonist), intact flagellin, and denatured flagellin, each alone or in different combinations at altered time points as shown in Table 1. Two, four and six hours post-injection, 3 mice of each group were bled by cardiac puncture, pooled and serum was separated for the measurement of TNF-alpha and IL-12 levels.

### 2.4. Provision of Mononuclear Cells (MNCs) and Cell Culture

Blood was collected from 20 Balb/c mice by cardiac puncture and mononuclear cells (MNCs) were isolated using Ficoll -Isopaque. Collected cells were suspended in culture medium, (RPMI, 10% FBS and 1% L-Glutamine) and then seeded in a 48 well plate, at 2x10<sup>5</sup>cells /well/0.2ml. Cells were incubated at 37°C and 5% CO<sub>2</sub> for 4 hours with varying concentrations and combinations of intact flagellin, heat-denatured flagellin, Anti-mTLR5IgG (TLR-5 antagonist), *P. aeruginosa*, and LPS-RS as shown in Table 2. Denatured flagellin, Anti-mTLR5IgG and LPS-RS were added to the cells at 1 hour prior to the addition of other reagents. Following incubation for 4 hours, supernatants were collected for the assessment of IL-12 and TNF- $\alpha$  levels.

### 2.5. TNF- $\alpha$ and IL-12 Levels

Serum and supernatants TNF- $\alpha$  and IL-12 Levels were determined using ELISAKits (Abcam company, Moscow, Russia) according to the manufacturer's protocol.

### 2.6. Statistical Analysis

The unpaired student T-test was implemented to assess the sample variations between groups using the Graphpad online software. Results were considered to be statistically significant when p value was <0.05.

## 3. RESULTS

### 3.1. TNF- $\alpha$ Levels in Cells Supernatants

TNF- $\alpha$  levels of supernatants from cells treated with heat denatured flagellin were significantly lower than levels in

Table 1. The protocol used for the treatment of mice.

Group Description	Treatment Challenge	
	T(-1hr)	T (0hr)
Negative Control Group		Saline
Positive Control Group		Intact Flagellin (0.2 ml of 5 $\mu$ g/ml)
Test Group 1		Denatured Flagellin (0.2 ml of 5 $\mu$ g/ml)
Test Group 2		<i>P. aeruginosa</i> (0.2 ml of 5x10 <sup>6</sup> CFU/ml)
Test Group 3	LPS-RS (0.1 ml of 100 $\mu$ g/ml)	<i>P. aeruginosa</i> (0.2 ml of 5x10 <sup>6</sup> CFU/ml)
Test Group 4	Denatured Flagellin (0.2 ml of 5 $\mu$ g/ml) LPS-RS (0.1 ml of 100 $\mu$ g/ml)	<i>P. aeruginosa</i> (0.2 ml of 5x10 <sup>6</sup> CFU/ml)
Test Group 5	Denatured Flagellin	<i>P. aeruginosa</i> (0.2 ml of 5x10 <sup>6</sup> CFU/ml)

**Table 2.** Protocol for determining the *in vitro* effect of denatured flagellin on intact Flagellin and *Pseudomonas aeruginosa*.

Well Description	Cells	Preparations	
		T(-2hr)	T (0hr)
Negative Control Well	MNCs		
Positive Control Well	MNCs		Intact Flagellin (0.2 ml of 0.1 µg/ml)
Test Well 1	MNCs	Denatured Flagellin (0.2 ml of 0.1 µg/ml)	
Test Well 2	MNCs	Denatured Flagellin (0.2 ml of 0.1 µg/ml)	Intact Flagellin (0.2 ml of 0.1 µg/ml)
Test Well 3	MNCs		<i>P. aeruginosa</i> (0.2 ml of 2.5x10 <sup>5</sup> CFU/ml)
Test Well 4	MNCs	Anti-mTLR5IgG (0.2 ml of 0.5 µg/ml) + LPS_RS (0.2 ml of 1 µg/ml)	<i>P. aeruginosa</i> (0.2 ml of 2.5x10 <sup>5</sup> CFU/ml)
Test Well 5	MNCs	Denatured Flagellin + LPS_RS	<i>P. aeruginosa</i> (0.2 ml of 2.5x10 <sup>5</sup> CFU/ml)
Test Well 6	MNCs	LPS_RS (0.2 ml of 1 µg/ml)	<i>P. aeruginosa</i> (0.2 ml of 2.5x10 <sup>5</sup> CFU/ml)
Test Well 7	MNCs	Anti-mTLR5IgG (0.2 ml of 0.5 µg/ml)	<i>P. aeruginosa</i> (0.2 ml of 2.5x10 <sup>5</sup> CFU/ml)

supernatants obtained from cells treated with intact flagellin (p=0.028) (Fig. 1).

### 3.2. TNF- $\alpha$ Levels in Sera

When only denatured flagellin was administered to mice before the infection with *P. aeruginosa*, it caused a significant decrease in TNF- $\alpha$  levels at 6 hours as compared to mice only infected with *P. aeruginosa* (p=0.012). The treatment of mice with LPS-RS in addition to heat denatured flagellin prior to *P. aeruginosa* infection led to a significant decrease at 2 hours (p = 0.0007) and 6 hours (p=0.004) post-challenge with *P. aeruginosa*. Hence, the decrease was further pronounced at 2 hours and 6 hours when LPS-RS was added as compared to the group treated only with denatured flagellin and infected with *P. aeruginosa* (Fig. 2).

### 3.3. IL-12 Levels in Cells Supernatants

IL-12 levels of supernatants from cells treated with heat-denatured flagellin were significantly lower than levels in supernatants obtained from cells treated with intact flagellin. Cells pretreated with heat-denatured flagellin and LPS-RS prior to *P. aeruginosa* infection showed a significant decrease of IL-12 levels as compared to levels in supernatants from cells pretreated with LPS-RS followed by infection with *P. aeruginosa* (p=0.0004) (Fig. 3).

### 3.4. IL-12 Levels in Sera

At 6 hours post infection, mice that received heat denatured flagellin and LPS-RS prior to administration of *P. aeruginosa*, had a significant lower IL-12 serum level at 6 hours as compared to mice infected only with *P. aeruginosa* (p=0.0113). Mice given denatured flagellin and challenged with *P. aeruginosa* had a significant decrease in serum levels of IL-12 at 2 and 4 hours post-infection as compared to the group that had received only *P. aeruginosa* (p=0.0017, p=0.0039) (Fig. 4).

## 4. DISCUSSION

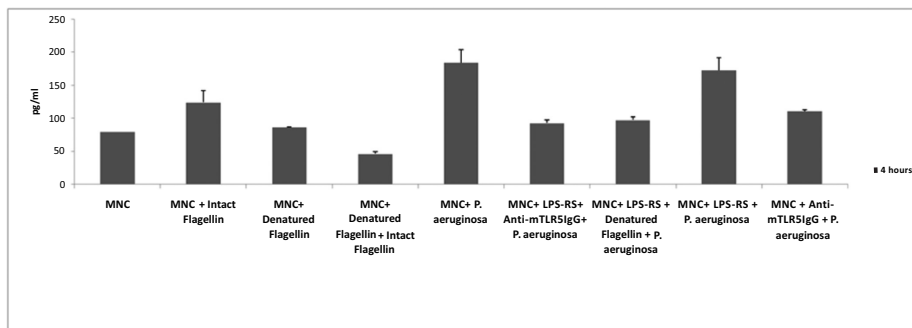
During infection, the binding of flagellin to TLR-5 contributes to the production of pro-inflammatory cytokines including TNF- $\alpha$  and IL-12 [2, 9]. These cytokines, when produced in physiological amounts, play a protective role against infectious agents. However, if produced in excessive amounts they can contribute to septic shock. And this has been reported by Krakauer who showed that the binding of Super antigens to MHC class II molecules caused the activation of both antigen-presenting cells and T lymphocytes. Hence, the resulting excessive production of pro-inflammatory cytokines may lead to fever, hypotension, and shock [10].

Reports have indicated that flagellin can induce systemic inflammation [7, 9, 10] and that flagellin/TLR5 signaling elicits several mechanisms that are instrumental in the pathophysiology of sepsis [11]. Moreover, flagellin was shown to be responsible for myocardial inflammation and contractile dysfunction during sepsis and that pro-inflammatory cytokine TNF- $\alpha$  can decrease heart rate and contractility [12, 13].

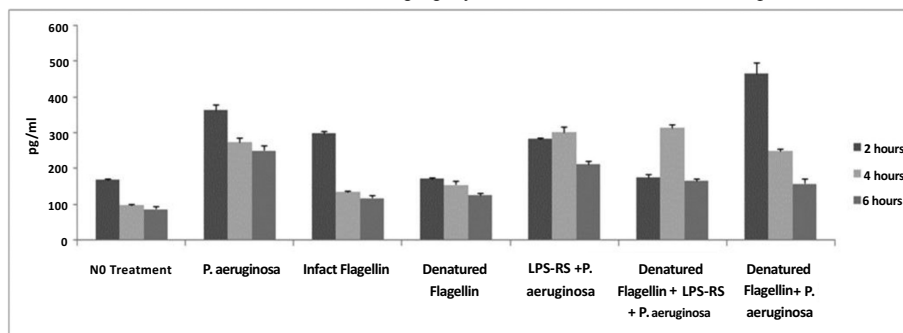
This study focused on detecting methods of blocking the interaction of intact flagellin and TLR-5 and or the signaling pathway that occurs as a result of flagellin engaging TLR-5. To rule out the effect of LPS as confounding factor, LPS-RS, a TLR-4 antagonist was used, when experiments made use of a flagellated Gram-negative organism (*P. aeruginosa*).

The approach was to decrease the levels of pro-inflammatory cytokines produced *in vitro* and *in vivo* by pretreatment of MNCs with heat-denatured flagellin prior to administration of intact flagellin or Gram-negative flagellated *Pseudomonas aeruginosa*. The results indicated that the pre-treatment of MNCs did significantly decrease the levels of TNF- $\alpha$  and IL-12.

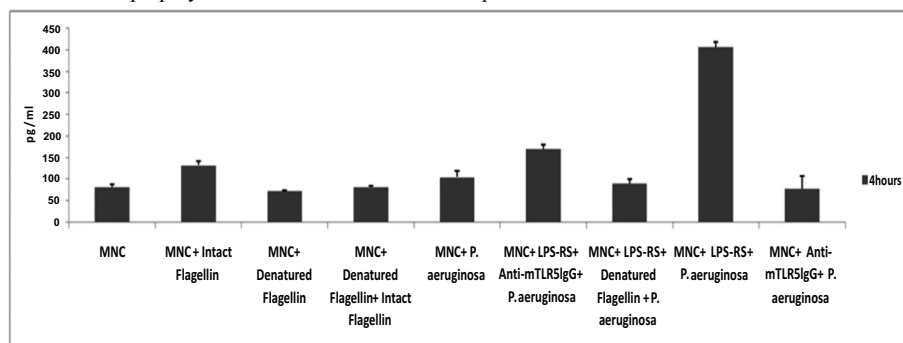
The *in vitro* results concurred with the *in vivo* results when the groups of mice that were pretreated with denatured



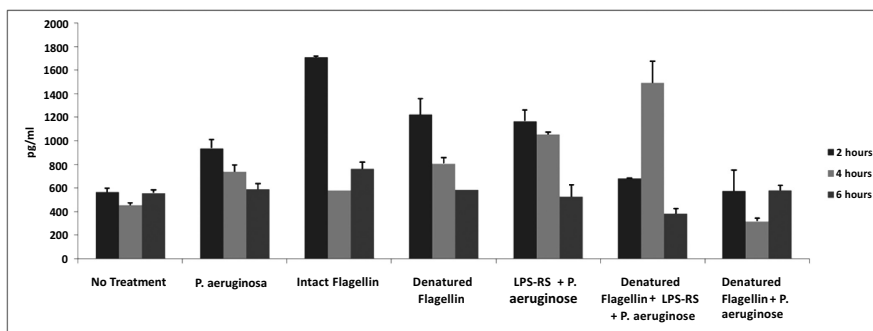
**Fig. (1).** TNF- $\alpha$  levels in MNCs supernatants after 4 hours of incubation with different combinations of intact flagellin, and /or denatured flagellin, Anti-mTLR-5IgG, *P. aeruginosa*, and LPS-RS. The average of duplicate run for each sample is shown along with the standard deviations\*. Abbreviations: MNC: mononuclear cells, LPS-RS: Lipopolysaccharide from *Rhodobacter Spheroides*, Anti-TLR5IgG.



**Fig. (2).** Serum TNF- $\alpha$  levels at 2, 4 and 6 hours post- injection in the different groups of mice. The average of duplicate runs for each sample is shown along with the standard deviations. Denatured flagellin decreased levels of TNF- $\alpha$  at 4 and 6 hours post- injection with *P. aeruginosa*. Abbreviations: LPS-RS: Lipopolysaccharide from *Rhodobacter Spheroides*.



**Fig. (3).** IL-12 levels in MNCs supernatants after 4 hours of incubation with different combinations of intact flagellin, and/ or denatured flagellin, anti-mTLR-5IgG, *P. aeruginosa*, and LPS-RS. The average of duplicate run for each sample is shown along with the standard deviations\*. Abbreviations: MNC: mononuclear cells. LPS-RS: Lipopolysaccharide from *Rhodobacter Spheroides* , Anti-TLR5IgG.



**Fig. (4).** Serum IL-12 levels at 2, 4 and 6 hours post injection of the different groups of mice. The average of duplicate run for each sample is shown along with the standard deviations. Denatured flagellin decreased levels of TNF- $\alpha$  at 4 and 6 hours post injection with *P. aeruginosa*. Abbreviations are indicated as: LPS-RS: Lipopolysaccharide from *Rhodobacter Spheroides*.

flagellin prior to intact flagellin or *P. aeruginosa* challenge showed significantly decreased levels of TNF- $\alpha$  and IL-12.

It is of note that the levels of TNF- $\alpha$  and IL-12 after 4 hours were higher in the group of mice that have received LPS-RS and *P. aeruginosa* than the group that received only *P. aeruginosa*. Likewise, IL-12 level was higher in the group of mononuclear cells treated with LPS-RS and *P. aeruginosa* than that treated only with *P. aeruginosa*. This could be attributed to the fact that LPS-RS contains lipopeptide contaminants that can stimulate Toll-like Receptor-2 (TLR-2), and could be responsible for the high levels of TNF- $\alpha$  and IL-12 produced after 4 hours [14].

## CONCLUSION

In conclusion, heat denatured flagellin appeared to block the engagement of flagellin to Toll-Like Receptor-5, or binds elsewhere on the cell to antagonize the signaling pathway generated by flagellin-TLR-5 interaction. In doing so, it suppressed excessive production of pro-inflammatory cytokines. Overproduction of pro-inflammatory cytokines may in part be the cause of septic shock. The use of heat-denatured flagellin along with other modified bacterial constituents could be considered in the treatment of septic shock. In future studies using x-ray crystallography and spectroscopic techniques, the structure of denatured flagellin can be compared to intact flagellin and structure-function relationships can be properly defined.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The approval was obtained from the Institutional Animal Care and Usage Committee at AUB (American University of Beirut).

## HUMAN AND ANIMAL RIGHTS

No humans were involved in this study. The research was performed in animals in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals ([http:// grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf](http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf)) published by the National Academy of Sciences, The National Academies Press, Washington DC, United States of America.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

## ACKNOWLEDGEMENTS

All individuals listed as authors must have contributed substantially to the design, performance, analysis, or reporting of the work and are required to indicate their specific contribution. Anyone (individual/company/institution) who has substantially contributed to the study for important intellectual content, or who was involved in the article's drafting the manuscript or revising must also be acknowledged.

Guest or honorary authorship based solely on position (e.g. research supervisor, departmental head) is discouraged. The Lebanese National Council for Scientific research for partial support of this project.

## REFERENCES

- [1] Hajam, I.A.; Dar, P.A.; Shah Nawaz, I.; Jaume, J.C.; Lee, J.H. Bacterial flagellin-A potent immunomodulatory agent. *Exp. Mol. Med.*, **2017**, *49*(9), e373.
- [2] Gupta, S.K.; Bajwa, P.; Deb, R.; Chellappa, M.M.; Deya, S. Flagellin A toll-like receptor 5 agonist as an adjuvant in chicken vaccines. *Clin. Vaccine Immunol.*, **2014**, *21*(3), 261-270.
- [3] Song, W.S.; Jeon, Y.J.; Namgung, B.; Hong, M.; Yoon, S.I. A conserved TLR5 binding and activation hot spot on flagellin. *Scientific Reports*, **2017**, *7*, 40878.
- [4] Spooner CE, Markowitz NP, Saravolatz LD. The role of tumor necrosis factor in sepsis. *Clin. Immunol. Immunopathol.*, **1992**, *62*(1), 11-17.
- [5] Jezierska, A.; Kolosova, I.A.; Verin, A.D. Toll Like receptors signaling pathways as a target for therapeutic interventions. *Curr. Signal. Transduct. Ther.*, **2011**, *6*(3), 428-440.
- [6] Kothari, N.; Bogra, J.; Abbas, H.; Kohli, M.; Malik, A.; Kothari, D.; Srivastava, S.; Singh, P.K. Tumor necrosis factor gene polymorphism results in high TNF level in sepsis and septic shock. *Cytokine*, **2013**, *61*(2), 676-681.
- [7] Liaudet, L.; Szabó, C.; Evgenov, O.V.; Murthy, K.G.; Pacher, P.; Virág, L.; Mabley, J.G.; Marton, A.; Soriano, F.G.; Kirov, M.Y.; Bjertnaes, L.J.; Salzman, A.L. Flagellin from gram-negative bacteria is a potent mediator of acute pulmonary inflammation in sepsis. *Shock*, **2003**, *19*(2), 131-137.
- [8] Eaves-Pyles, T.; Murthy, K.; Liaudet, L.; Virág, L.; Ross, G.; Soriano, F.G.; Szabó, C.; Salzman, A.L. Flagellin, a novel mediator of Salmonella-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J. Immunol.*, **2001**, *166*(2), 1248-1260.
- [9] Kowalski, E.J.A.; Li, L. Toll-Interacting Protein in Resolving and Non-Resolving Inflammation. *Front. Immunol.*, **2017**, *8*, 511.
- [10] Bosmann, M.; Ward, P.A. The inflammatory response in sepsis. *Trends Immunol.*, **2013**, *34*(3), 129-136.
- [11] Rolli, J.; Loukili, N.; Levrant, S.; Rosenblatt-Velin, N.; Rignault-Clerc, S.; Waeber, B.; Feihl, F.; Pacher, P.; Liaudet, L. Bacterial flagellin elicits widespread innate immune defense mechanisms, apoptotic signaling, and a sepsis-like systemic inflammatory response in mice. *Crit. Care*, **2010**, *14*(4), R160.
- [12] Rolli, J.; Rosenblatt-Velin, N.; Li, J.; Loukili, N.; Levrant, S.; Pacher, P.; Waeber, B.; Feihl, F.; Ruchat, P.; Liaudet, L. Bacterial flagellin triggers cardiac innate immune responses and acute contractile dysfunction. *PLoS One*, **2010**, *5*(9), e12687.
- [13] Merx, M.W.; Weber, C. Sepsis and the heart. *Circulation*, **2007**, *116*(7), 793-802.
- [14] LPS\_RS. Available from: [http://www.invivogen.com/PDF/LPS\\_RS\\_TDS.pdf](http://www.invivogen.com/PDF/LPS_RS_TDS.pdf). [Accessed August 16, 2017].