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The role of interleukin-6 as an early predictor of sepsis in a murine sepsis model

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Abstract

Aim: Evaluating the role of interleukin-6 (IL-6) as an early predictor of sepsis in a murine model. *Materials and Methods*: The study divided 26 Wistar rats into two experimental groups in which sepsis was induced through the intraperitoneal injection of different *Escherichia coli* cultures [Group 1: Extended-spectrum beta-lactamase (ESBL)-producing culture and Group 2: Standardized ATCC35218 culture] and a control group. IL-6 levels were determined at 5 and 24 hours post-inoculation and immunohistochemistry (IHC) was performed on tissue samples from the sacrificed animals. *Results*: Mean plasma IL-6 levels in Group 1 peaked at 5 hours [37.4 pg/mL; standard deviation (SD) = 2.4 pg/mL] and decreased at 24 hours (34 pg/mL; SD=3.2 pg/mL) after inoculation. IL-6 levels in Group 1 were elevated compared to Group 2, at 5 hours (33.7 pg/mL; SD=3.3 pg/mL; p=0.019) and non-significantly so at 24 hours (32.5 pg/mL; SD=2.4 pg/mL; p=0.233). The results did not show an increase over control levels at either 5 hours (37.6 pg/mL; SD=3.4 pg/mL) or 24 hours (40.8 pg/mL; SD=2.9 pg/mL) after inoculation. The IHC shows a varying degree of IL-6 expression across all organ types studied. No statistically significant correlations were found between the tissue level quantification of IL-6 and serum values at 24 hours in either group. *Conclusions*: For an early stage of infection/inflammation, serum levels of IL-6 are not correlated with tissue-level inflammation disproving a potential role of IL-6 as a very precocious diagnostic and predictor test. Accumulation of IL-6 in lung, kidney and spleen tissue can be observed from the beginning of inflammation.

Keywords: interleukin-6, sepsis, immunohistochemistry, infection, inflammatory cascade.

Introduction

Sepsis represents a systemic host response to an infection in the organism. Septic shock is the most frequent cause of death in intensive care units (ICUs) with mortality reaching 70% in cases of septic shock [1–3]. Due to the potential of sepsis to rapidly evolve into septic shock and cause death, it is imperative that fast and specific ways to diagnose sepsis are developed [4, 5]. Immune dysfunction is the principal physiopathological process underlying sepsis. After an infection is manifested, microbial antigens can trigger an inflammatory cascade with an overexpression of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6). IL-6 is a cytokine with an important role in acute-phase response [6]. Its secretion by T-cells and macrophages can be stimulated by bacterial lipopolysaccharides and viruses, which makes it an attractive target for sepsis research [4, 7, 8]. IL-6 serves a key role in the systemic inflammatory response having a series of biological roles including the activation of B- and T-lymphocytes, inducing acute-phase protein synthesis in the liver, modulating hematopoiesis and activating coagulation. Elevated levels of IL-6 are identified in severe forms of sepsis and correlated with increased mortality, potentially uncovering a role for IL-6 as both a diagnostic and prognostic test for sepsis [9].

The role of immunohistochemistry (IHC) in sepsis is well understood, allowing for the *in situ* identification and visualization of specific cellular antigens [10–13].

Inducing sepsis through injections of *Escherichia coli* (*E. coli*) leads to an increase in neutrophil infiltrates and increased immunoreactivity [14]. In most tissue types, IL-6 is predominantly expressed in the cytoplasm [12]. One of the organs most susceptible to modifications is the lung, where immunohistochemical studies evidence the presence of macrophages and neutrophils at the bronchial epithelium level and a thickening of the alveolar wall [14]; molecules such as TNF-α, IL-1β and IL-6 are expressed intermittently, for short periods of time, as a response to the sepsis inflammatory reaction [15, 16]. At a renal level, the periarteriolar lymphoid network shows cellular depletion, while IL-6 expression is upregulated in the mesangial cells and downregulated in tubular and interstitial epithelial cells [17]. At a hepatic level, IHC shows an accumulation of neutrophils with an overexpression of IL-6 and TNF-α cytokines in sepsis cases [18, 19]; in cardiac tissue, inflammatory infiltrates of mononucleate cells appear transiently and are linked directly to the activity of cytokines [20].

The binding of IL-6 to its cellular receptor (IL-6R), will trigger the association of the IL-6R domain of the receptor to the gp130 domain that can transduce the signal intracellularly [21]. Elevated concentrations of IL-6 and IL-6R can be found in the plasma in different pathologies, such as sepsis and tumors, as they are released by cells expressing them on their surface [22]. An immediate, but transitory, increase of IL-6 occurs as a result of a wide variety of stress factors [22]. This expression triggers and activates the host's defense mechanisms. Upon cessation of the stressors, the IL-6 mediated inflammatory cascade ceases through the internalization and degradation of gp130 [23, 24].

The objective of this study was to evaluate the levels of circulating levels of IL-6, its cellular expression levels in organs and correlate them to sepsis severity.

Materials and Methods

Rats

Female Wistar rats (300–350 g) were sourced from the "Cantacuzino" National Medico-Military Research and Development Institute, Bucharest, Romania. The subjects were acclimated to standard laboratory conditions for 14 days, at the University Experimental Station, with free access to water and food. The subjects were randomly assigned to two experimental groups and one control group. All experiments were performed in accordance to established international ethical animal research guidelines and approved by the Ethics Commission of the University of Medicine, Pharmacy, Science and Technology of Târgu Mureş, Romania (Approval No. 75/27.04.2018).

Induction of sepsis

Two *E. coli* cultures, a standardized ATCC35218 culture and an extended-spectrum beta-lactamase (ESBL)-producing culture, were used to induce sepsis. Analgesia was provided through an intraperitoneal (i.p.) injection of 0.3 mL of Ketamine–Xylazine (2:1) mixture. Each rat was given an i.p. injection of 0.5 mL of either bacterial culture or normal saline (NS). Group 1 (n=10) received a 0.5 mL i.p. injection of 0.5 McFarland (MF) units of ESBL-producing culture; Group 2 (n=10) received a 0.5 mL i.p. injection of 0.5 MF units of ATCC35218 culture. The control group (n=6) received a 0.5 mL i.p. injection of NS.

The Murine Sepsis Score (MSS) was determined at 5 hours, by analyzing clinical signs of infection [25]. To determine plasma levels of IL-6, mice were provided analgesia using Xylazine 5 mg/kg and blood samples were obtained from the caudal vein, 5 hours after inoculation. At 24 hours, additional blood samples were obtained through the cannulation of the abdominal aorta, after which the animals were sacrificed using a Sodium Pentobarbital injection (>100 mg/kg). After euthanasia, tissues (heart, lung, spleen and kidney) were harvested for immunohistochemical analysis.

Blood culture

Arterial blood samples were obtained at 24 hours after inoculation under sterile conditions. Samples were processed through the automated blood culture system BacT/Alert3D (bioMérieux, France).

Determination of IL-6 plasma levels

Plasma was obtained by the centrifugation of whole blood samples (2000 rpm, 3 minutes) and stored at -70^{0}C. Plasma levels of IL-6 were determined using the Rat IL-6 Quantikine enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, Inc., Minneapolis, USA), following the manufacturer's instructions.

Immunohistochemistry

Biopsy samples were processed using standard processing and storage methods. After sampling, the formalin-fixed paraffin-embedded tissues were sectioned at 5 μm. After deparaffinization, the immunohistochemical stainings were manually performed using the EnVision Flex detection kit (Agilent Technologies, California, USA), in accordance to the manufacturer's recommendations. Antigen unmasking was done by microwave incubation of slides in a citrate buffer (pH 6) solution for 40 minutes. Overnight antibody incubation, at room temperature, was done. We have used mouse monoclonal IL-6 antibody (Abcam), with 1:50 dilution. Developing was performed with 3,3'-diaminobenzidine (DAB).

The rats used for positive control were healthy Wistar rats weighing 300–350 g. The normal rat lymph nodes and spleen were used for positive external control, where lymphocytes were considered the positive internal control. Post-operatively, the IL-6 expression was checked in the heart, lung, spleen and kidney of rats from the two experimental groups.

The analysis of the samples was performed using optical microscopy and digital morphometry [26].

Digital morphometry

The degree of inflammation and IL-6 expression at a tissue level were determined using digital morphometry [26]. Digital images were obtained using light microscopy (Nikon Eclipse E600) at a 40× magnification. Repre-

sentative images of each organ were obtained for each individual using the 3DHistech Panoramic Viewer program and saved in the Joint Photographic Experts Group (*JPG*) format. For image analysis, the Java (1.6.0_20 32 bit) cell counter plugin was used to manually calculate the percentage of cells positive for the signal out of the total number of cells in the image, neutrophils included.

IHC quantification

To quantify the immunohistochemical reaction, each image was scored for both the intensity of the reaction (weak – 1, intense – 2) and the density (percentage) of the positive cells (0% – 0; 1–50% – 1; 51–75% – 2; 76–100% – 3). The IHC Image Score was calculated for each image by multiplying the intensity with the density [27].

Statistical analysis

The normality of the data was analyzed using the Shapiro–Wilk normality test, in all groups and time intervals. Mean values of MSS and plasma IL-6 levels were compared using the Mann–Whitney test. To compare all groups at the different time intervals the Kruskal–Wallis test was used. The immunohistochemical image scores and the plasma values of IL-6 at 24 hours were correlated using the Spearman's test.

Results

Survival rate and type of bacterial culture

The survival rate was monitored across the study, all groups showing a 100% survival rate. The bacterial culture did not influence the survival rate at 24 hours.

Clinical symptoms in infected rats

Murine Sepsis Score – mean MSS values of Group 1 (5.6) and Group 2 (5.5) at 5 hours were significantly increased compared to the control group (0, $p<0.001$). However, there was no significant difference between the two septic groups ($p=0.702$) (Figure 1).

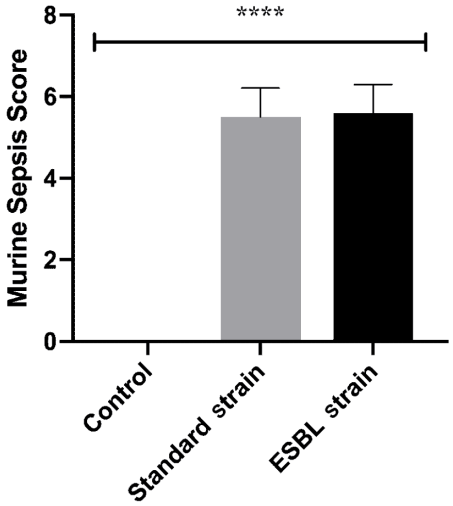

Figure 1 – ***MSS: mean values of Group 1 (Standard strain) and Group 2 (ESBL strain) compared to the control group (p<0.001). No significant difference between the two septic groups (p=0.702). MSS: Murine Sepsis Score; ESBL: Extended-spectrum beta-lactamase.***

Plasma IL-6 levels

Mean plasma IL-6 levels in Group 1 peaked at 5 hours [37.4 pg/mL; standard deviation (SD) = 2.4 pg/mL] and decreased at 24 hours (34 pg/mL; SD=3.2 pg/mL) after inoculation. IL-6 levels in Group 1 were elevated compared to Group 2, at 5 hours (33.7 pg/mL; SD=3.3 pg/mL; $p=0.019$) and no significantly so at 24 hours (32.5 pg/mL; SD=2.4 pg/mL; $p=0.233$). However, the results did not show an increase over control levels at either 5 hours (37.6 pg/mL; SD=3.4 pg/mL) or 24 hours (40.8 pg/mL; SD=2.9 pg/mL) after inoculation.

Bacteremia

Blood samples taken at 24 hours after inoculation did not show any evidence of bacteremia in any of the groups.

Histopathology

After 24 hours from inoculation, organs were harvested for histological analysis. Macroscopically, areas of punctiform hemorrhage and subcapsular abscesses in the pulmonary and renal regions as well as sub-capsular abscesses at the splenic level can be visualized. Hematoxylin–Eosin (HE) staining recapitulates the macroscopic findings of infection by evidencing neutrophilic infiltrates in the red pulp of the spleen and non-specific inflammatory infiltrate with mono- and polymorphonuclear leukocytes in the renal tubular epithelium (Figure 2).

IHC and digital morphometry

IHC shows a brown cytoplasmic staining, representing a positive expression of IL-6, across all organ types studied.

IL-6 expression in lung tissue was diffuse, localizing to the cytoplasm of alveolar epithelial cells in concert with an inflammatory infiltrate (Figure 3, A–C). The intensity of the staining reaction was reduced in both groups; regardless, more than 75% of cells showed a positive signal in both Group 1 (score 2.8; SD=0.63) and Group 2 (score 2.9; SD=0.57) at a significantly higher level than in the control group (score 1; SD=0; $p<0.001$) (Figure 4).

In renal tissue, IL-6 showed cytoplasmic expression (Figure 3, D–F). The signal was absent in the renal corpuscle (Bowman's capsule, glomerulus, juxtaglomerular apparatus). The signal was strong in proximal tubule epithelial cells and lower in intensity in the Henle loop and distal tubule epithelial cells. Despite an identical percentage of cells positive for IL-6 (76%), Group 1 (score 5.6; SD=0.84) showed an increase in staining intensity and overall morphometry score, compared to Group 2 (score 2.7; SD=0.48; $p<0.001$). The results from Group 1 showed a significant increase compared to the controls (score 2.8; SD=0.41; $p<0.001$) (Figure 4).

The expression of IL-6 was evident in cellular conglomerates in the splenic red pulp but absent in the white pulp, with few positive cells at its periphery. There was a clear demarcation between the two histological areas. Positive cells are also found in the Billroth cords (Figure 3, G–I). In the ESBL-producing culture, the expression of IL-6 (score 3.6; SD=0.84) was significantly

increased compared to both the standard culture (score 1.2; SD=0.42, $p<0.001$) and controls (score 1; SD=0; $p<0.001$) (Figure 4).

In the cardiac tissue, the expression of IL-6 was weak and diffusely distributed in the cytoplasm of atrial and ventricular myocytes, being present in a large number of the examined cells (Figure 3, J–L). The expression in Group 1 (score 1.3; SD=0.48), Group 2 (score 1.2; SD=0.42) and controls (score 1; SD=0) was much more reduced compared to the other tissue types (Figure 4).

Correlation between IHC results and serum IL-6 levels

Analyzing the results of the digital morphometry with the serum values of IL-6 at 24 hours in the two groups, no statistically significant correlations were found.

Discussions

Previous work has showed that circulating levels of IL-6 are elevated in sepsis and septic shock [9]. Our experiment used a simple sepsis model, the i.p. injection of two *E. coli* strains. We determined the circulating levels of IL-6 at 5 and 24 hours to observe the dynamic response of this cytokine; additionally, we examined the tissue-specific expression of IL-6 at 24 hours. Experimental endotoxemia models are popular due to their reproducibility [28]. A single injection of endotoxin or lipopolysaccharide is most commonly used. Endotoxin, a component of the external membrane of Gram-negative bacteria has been implicated in the pathogenesis of sepsis and triggers a rise in inflammatory cytokines and a systemic inflammatory response mimicking sepsis, but without causing bacteremia [29].

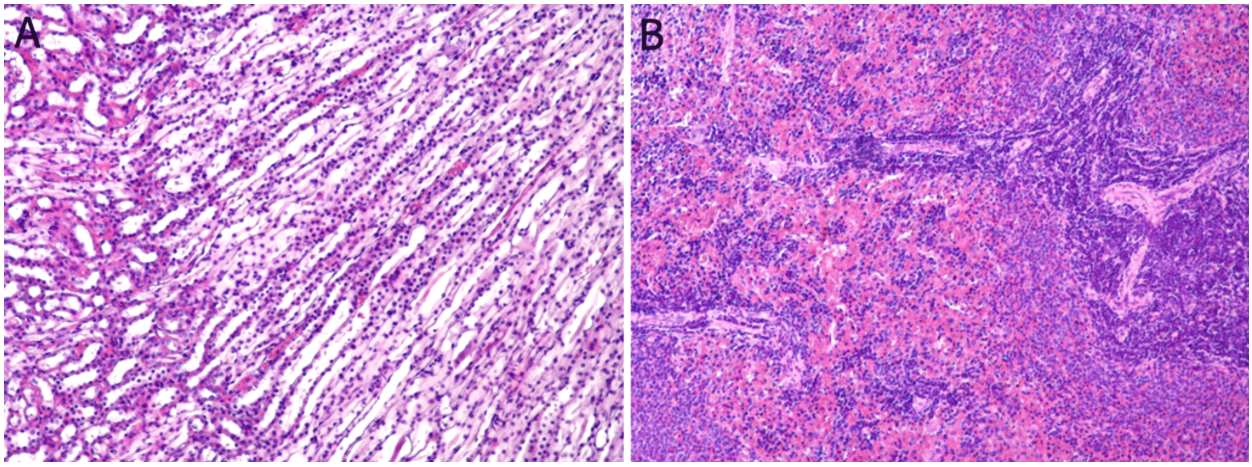

Figure 2 – ***Hematoxylin–Eosin (HE) staining (×100) of kidney (A) and spleen (B) showing inflammation in both organs.***

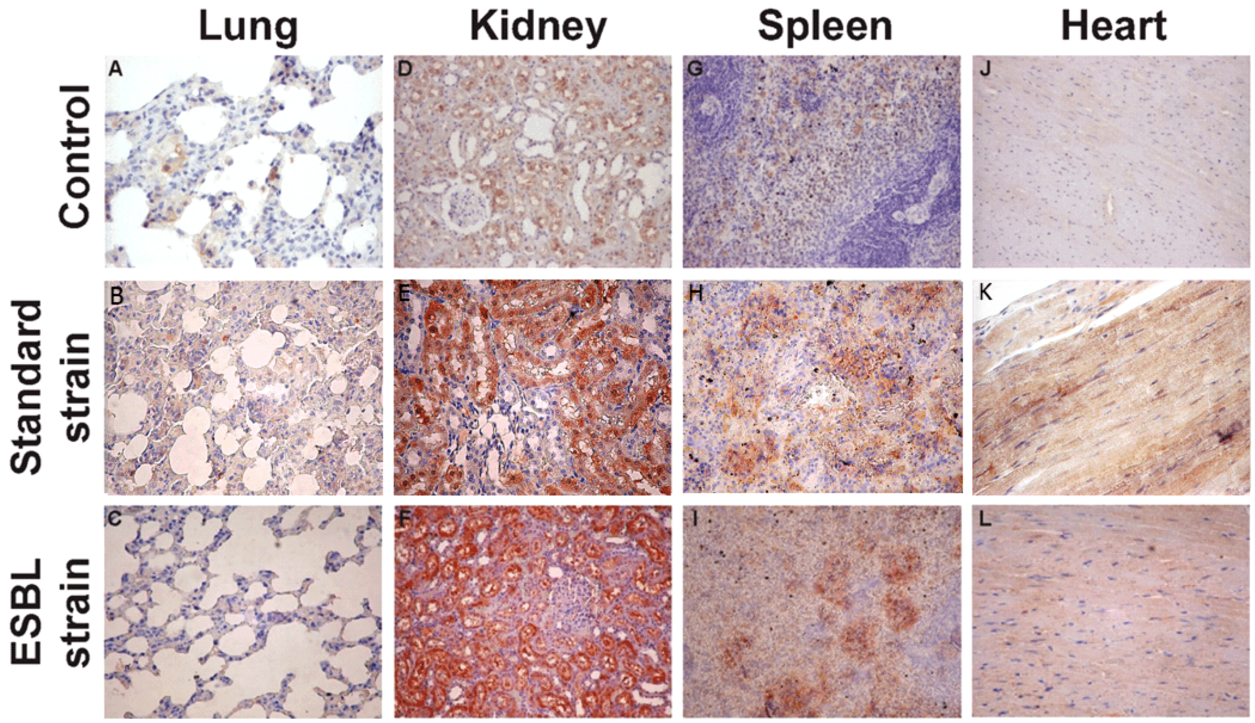

Figure 3 – ***IL-6 expression in lung: (A) Control group; (B) Group 2; (C) Group 1. IL-6 expression in the kidney: (D) Control group; (E) Group 2; (F) Group 1. IL-6 expression in the spleen: (G) Control group; (H) Group 2; (I) Group 1. IL-6 expression in the heart: (J) Control group; (K) Group 2; (L) Group 1. Anti-IL-6 antibody immunomarking: (A–L) ×200. IL-6: Interleukin-6; ESBL: Extended-spectrum beta-lactamase.***

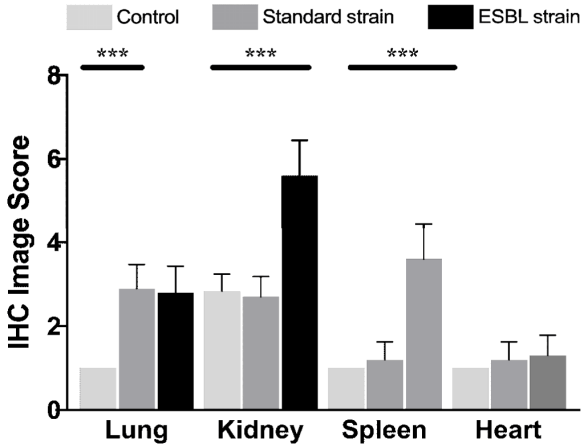

Figure 4 – ***IHC Image Score for IL-6 expression in the studied organs: mean values of Group 1 (Standard strain) and Group 2 (ESBL strain) compared to the control group. IHC: Immunohistochemistry; IL-6: Interleukin-6; ESBL: Extended-spectrum beta-lactamase.***

After induction of sepsis, the first clinical signs appear and progress rapidly and can be quantified using the murine sepsis score [25, 30–34]. An intravenous injection of *E. coli* causes a rapid increase in serum cytokine levels, while an intraperitoneal injection leads to a more limited response [35, 36]. The increase in cytokine levels starts 1.5 hours after inoculation, peaks at 5 hours and decreases afterwards [30, 37, 38]. Some authors are suggesting that a single large dose of bacteria can produce similar effects to that of an intravenous injection [37]. Our study used virulent *E. coli* strains due to their capacity to multiply *in vivo* after the injection of a small volume. To replicate in an organism, the strain must be able to avoid the host defense systems and survive in an unfavorable environment [39].

The inflammatory response in each tissue reflects the severity of sepsis. Histological modifications, the presence of inflammatory cells, abscess and zones of hemorrhage and necrosis confirm the presence of infection and are similar to the results described in existing literature [17, 40, 41]. Soresi *et al.* described the existence of a reactive an increased cytoplasmic and membrane reactivity in the large hepatic cell [22], while Wu *et al.* found varying degrees of alveolar hemorrhages, pulmonary necrosis areas and multiple renal abscesses [42]. Benli *et al.* identified glomerular modifications at the renal level and inflammatory cellular infiltrates in both sepsis and sham experimental conditions [40]. Wu *et al.* evidenced a correlation between TNF-α and IL-6 levels in the serum, lungs and kidneys [42]. While we were able to recapitulate the histological results, we were unable to correlate the results with changes in levels of serum IL-6.

Our study evidenced a disconnect between early clinical infection signs (MSS), histological modifications and circulating IL-6 values. Low doses of virulent pathogens, while causing a mild increase in clinical symptoms, do not seem to cause a significant increase in circulating IL-6 levels at either 5 or 24 hours after inoculation. Interestingly, despite the stagnant serum cytokine levels, accumulation of IL-6 in lung, kidney and spleen tissue can be observed even at low levels of infection. Kidney and spleen tissue samples show an enhanced IL-6 response when exposed to bacteria producing extended-spectrum β-lactamase, but this change is not fully recapitulated in circulatory levels. The most likely explanation for the disconnect is that the mice were not given enough time to progress from localized tissue-level inflammation to a more systemic process or bacteremia, which is absent in our disease model. This is consistent with other works in the field that associated massive release of cytokines with Gram-negative bacteremia and septic shock, but not with more moderate Gram-negative infections in which the regulatory system is still intact [37, 43].

The low bacterial density combined with a route of administration that limits the intensity of the immune response and severity of the disease provided an excellent platform to test whether IL-6 can be used as an extremely precocious marker of sepsis. Our results suggest that circulating levels of IL-6 do not have a strong response to mild inflammatory conditions, despite an overexpression in a wide range of tissue types. Therefore, normal levels of IL-6 are not indicative of the absence of sepsis or an inflammation process, and all results should be correlated with existing clinical signs.

Like most animal studies, our set of experiments has several limitations. While the endotoxemia model is well characterized in rats, the sepsis inflammatory reaction is more subdued than in human subjects. The less pronounced immune response would require a higher dose in order to achieve a comparable degree of immune response to that of human subjects.

Future research should focus on the validation of IL-6 as a sepsis diagnostic and prognostic biomarker in a more severe animal sepsis model. Furthermore, the dose of bacterial culture used for the induction of sepsis should be modulated to achieve an appropriate immune response that is appropriate for the discrimination of the pathogen virulence through serum measures.

Conclusions

Our study showed that for an early stage of infection/ inflammation, serum levels of IL-6 are not correlated with tissue-level inflammation. Mild increases in clinical symptoms caused by low doses of virulent pathogens do significantly increase circulating IL-6 levels at either 5 or 24 hours after inoculation. However, despite stagnant serum cytokine levels, accumulation of IL-6 in lung, kidney and spleen tissue can be observed even from the onset of inflammation. Circulating levels of IL-6 do not have a strong response to mild inflammatory conditions, despite an overexpression in a wide range of tissue types, disproving a potential role of IL-6 as a very precocious diagnostic and predictor test.

Conflict of interests

The authors report no conflict of interests.

Acknowledgments

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