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TLR-4 Surface Display on Human Monocytes is Increased in Septic Patients

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Abstract

Background: Sepsis is a serious condition, most often occurring as a complication of bacterial infections. The Toll-like receptors (TLR)-2 and TLR-4 have been identified as key molecules in response to Gram-positive and Gram-negative bacteria. This study aimed to assess possible alterations of the surface display of TLR-2 and TLR-4 on monocytes and granulocytes derived from patients with sepsis in comparison with healthy controls.

Methods: We have utilized flow-cytometry to determine the presence of TLR-2 and TLR-4 on the cell surface at baseline and in response to LPS (40 ng/ml) *in vitro*.

Results: We found no significant differences of TLR-2 display on monocytes and granulocytes from septic patients compared to controls. Surface display of TLR-4 on monocytes from septic patients at baseline was significantly higher than in healthy controls but there was no further response to LPS, whereas controls showed a significant increase of TLR-4 display on the cell surface after LPS stimulation. In contrast, TLR-4 baseline cell surface display on granulocytes was significantly lower in septic patients than in controls and there was no response to LPS in both groups.

Conclusion: Our data suggest a complex relationship between TLR-4 display and bacterial challenge *in vivo* and *in vitro*.

Key words: TLR2, TLR4, sepsis, monocytes, granulocytes

Abbreviations:

Allophycocyanin, APC; American College of Chest Physicians/Society of Critical Care Medicine, ACCP/SSCM; Blood culture, BC; bronchoalveolar lavage, BAL; Bronchial aspirate, BA; deoxyribonucleic acid, DNA; fluorescence, FL; forward- and sideward scatter FSC/SSC; Focal infection, FI; infectious diseases I.D; interleukin-1 receptor, IL-1R; lipopolysaccharide, LPS; molecules of equivalent soluble fluorochrome, MESF; myeloid differentiation-2 MD-2; ribonucleic acid, RNA; Phycoerythrin, PE; simplified acute physiology score, SAPS; Toll-like receptor, TLR; Toll interleukin-1 receptor, TIR; Urine culture, UC; Urinary tract infection, UTI

Introduction

Although our understanding of the pathogenesis of sepsis as the worst scenario of bacterial infection has increased continuously over the past years, the mechanisms of this very often fatal condition are not fully understood. The mortality rate of sepsis varies from 30% to 50% [1,2], pointing to the limited benefit of current treatment strategies such as antibiotic therapy, activated protein C and support of failing organ systems. To understand how the inflammatory processes in sepsis are initiated it is necessary to identify specific molecules of microbial origin that act as inducers of inflammation and the host receptor molecules that detect them. A large number of molecules derived from bacteria have been identified as inducers of inflammatory responses in the innate immune system that in turn activates adaptive immunity. Among these are, most important, lipopolysaccharide (LPS), the principle glycolipid component of the outer-membrane of Gram-negative bacteria, but also cord factor (trehalose dimycolate), peptidoglycan, double stranded RNA, and unmethylated DNA [3-8].

All of these molecules are recognized by members of the family of Toll-like receptors (TLRs) ten of which have been identified in humans to date. All are type I integral membrane receptors with extracellular leucine-rich regions and a highly homologous Toll/IL-1R (TIR) cytoplasmic domain, which is important for signal transmission into the cell [9]. The differences in the leucine-rich repeats of the extracellular domains determine the specificity of the interaction with the microbial molecules [10].

TLR-2 and TLR-4 have been identified as key molecules in response to Gram-positive and Gram-negative infections. TLR-4 together with MD-2 forms the LPS recognition complex; TLR-2 in concert with TLR-1 or TLR-6 is essential for recognizing Gram-positive cell wall structures [11]. Both of these TLRs are displayed on the cellular surface and ligation by the microbial molecules leads to receptor aggregation, which was clearly shown for TLR-4 and is suggested also for TLR-2 [12]. As a consequence of cluster formation signalling occurs.

This study was conducted to assess possible alterations of the surface display of TLR-2 and TLR-4 on monocytes derived from patients with severe sepsis in comparison with healthy controls as well as to deter-

mine whether there is a relationship between these alterations and the severity and outcome of sepsis, respectively.

Patients and Methods

Patients

The study was approved by the ethical committee of the University of Regensburg and carried out in accordance to the Declaration of Helsinki. Twenty-two patients who suffered from sepsis according to the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SSCM) consensus definition [13] were recruited from intensive care units of different services (Surgery, Internal Medicine, Anesthesiology) in a tertiary care university hospital. 19 age-matched healthy donors served as controls. Patients were subdivided into Gram-positive (n = 4), Gram-negative (n = 7), polymicrobial (n = 8) or culture-negative infection (n = 3) according to bacterial growth in specimens. The sites of infection as well as the causative organisms were determined according to standard criteria by a clinical evaluation committee consisting of three experienced I.D. specialists. In brief, every positive microbiological culture was classified as „definite ", „possible" or „unlikely" focal or generalized infection. In the case of a new chest x-ray finding consistent with pneumonia, a negative sputum culture, but a positive diagnostic microbial antigen serology, a „definite" infection was considered. The clinical picture of a respiratory infection with a positive new chest x-ray finding with a negative sputum culture in the absence of any other possible focus was considered as a culture negative, but „possible" focal infection. The presence of *Candida, Enterococci* or coagulase negative *Staphylococci* in respiratory cultures were considered as "unlikely" focal infection. An observed aspiration with positive chest x-ray finding and typical bronchoscopy was considered as a „possible" polymicrobial focal infection, even if BAL was negative or not performed. Perforated bowel or other intra-abdominal leakage with negative microbiological cultures, but with evidence of free intra-abdominal air, evidence of perforation at surgical intervention and signs of ascitic fluid infection was considered a „possible" polymicrobial focal infection. The clinical status of patients was evaluated by the new simplified acute physiology score (SAPS II).

Ex-vivo Whole Blood Assay

Blood for analysis was obtained by venepuncture within 48hrs after the diagnosis of sepsis was established, collected into sterile Li-heparin tubes (Sarstedt, Nürnbrecht, Germany) and immediately processed. 1 ml of whole blood was incubated for 15 minutes at 37 °C at a concentration of 40 ng/ml of LPS from *Salmonella minnesota* (Sigma, Steinheim, Germany) versus pyrogen-free 0,9% NaCl (Braun, Melsungen, Germany). Cells were washed in Dulbecco's Phophate Buffered Saline (Biochrom, Berlin, Germany).

Staining Procedure

Monocytes were identified using Allophycocyanin (APC)-labeled anti–CD14-antibodies (Becton-Dickinson, San Jose, USA). TLR-2 was analyzed using a TL2.1 clone monoclonal anti-human antibody (Ebioscience, San Diego, USA). For secondary staining, a goat anti-mouse IgG 2a Phycoerythrin (PE)-labeled antibody (Southern Biotechnology Associates, Birmingham, USA) was applied. TLR-4 was measured using a PE-conjugate anti-human TLR-4 antibody (clone HTA 125, Ebioscience, San Diego, USA). All antibodies were titrated against plain and LPS-stimulated cells from healthy controls and septic patients to ensure adequate antibody concentration.

Cells in the presence of antibodies were incubated on ice for 15 minutes. Antibodies were washed off in Dulbecco's PBS without Ca^{++} Mg^{++}/0.5 % albumin (Biochrom Berlin, Germany) before lysis of red blood cells was performed using FACS™ Lysing Solution (Becton Dickinson Biosciences, San Jose, USA) according to the manufacturer's instructions.

Flow Cytometry

Surface display of TLR-2, TLR-4 and CD14 was measured by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg). The flow cytometer was calibrated with fluorescent microbeads (Quantum 27 R-PE, Bangs Laboratories, Fishers, USA) to allow quantification of the samples fluorescence on a standard curve. After establishing the calibration plot, no further adjustments to the instruments settings were done.

The red fluorescence (Fl 2) of PE-labeled TLR-antibodies and the orange fluorescence (FL 4) of the APC-labeled anti–CD14-antibodies was analyzed simultaneously with the FSC/SSC with a dual argon laser system tuned to 488 nm and 635 nm respectively. The PE fluorescence (FL 2) was obtained through a 585-nm band pass filter, compensated by 23.3 % from the signal of FL 1. The APC fluorescence (FL4) was obtained through a 661 nm band pass filter and was compensated by 9,8% from the signal of FL3.

Both red (FL 2) and orange (FL 4) fluorescence were measured on a four decade logarithmic scale. TLR display was considered as positive if FL 2 above that of the negative control (autofluorescence of plain cells) and thus cells negative and positive for TLR display were discriminated by two markers M1 and M2 respectively. Statistical evaluation of the measurements was obtained using Cell Quest 3.1 f.

The mean fluorescence was converted into molecules of equivalent soluble fluorochrome (MESF) using QuickCal software (Flow Cytometry Standards Corp, Fishers, USA).

As the percentage of cells registered with M2 was variable, TLR-4 display was determined as mean FL2 x M2 (%). TLR-4 display after LPS challenge was determined by the stimulation index calculated as the ratio of FL2 x M2 (%) upon LPS and at baseline respectively ($FL2_{LPS}$ / $FL2_{baseline}$).

Statistics

Statistical analysis was performed using SPSS 11.0 (SPSS Inc, Chicago, Ill.). TLR-2 and TLR-4 display on granulocytes and monocytes as well as the stimulation index (LPS stimulation/baseline) were analyzed by ANOVA analysis between subgroups. Predefined contrasts were between controls and all patients with sepsis, differences between controls and subgroups of septic patients were analyzed with post hoc contrasts and Tanhames T2-tests without assuming equal variance.

Results

Patients

The demographic data and characteristics of sepsis in these patients are given in Table 1. The median age of patients was 65.5 years (range 27-85). The median SAPS II was 22 (range 12-44).

Table 1. Patients characteristics at time of sepsis development.

Patient no	Age (year)	Sex (M/F)	SAPS II	Died (Y/N)	**Underlying condition**	FI[1]	Bacteriology
Gram-negative sepsis							
1	78	F	32	N	Pancreatitis	d	Klebsiella pneumonia (BC)
2	68	M	27	N	Pancreatitis	d	*Escherichia coli* (BC)
3	37	M	21	Y	Pneumonia	d	Rising Chlamydia titers
4	49	M	20	N	Peritonitis	d	*Escherichia coli* (aszites)
5	75	M	19	N	Pneumonia	d	*Pseudomonas aeruginosa* (BA)
6	63	F	32	N	Pneumonia, urinary tract infection	d	*Pseudomonas aeruginosa* (BA), *Morganella morganii* (UC)
7	75	M	20	N	Pneumonia	d	*Escherichia coli* (BA)
Gram-positive sepsis							
1	52	F	21	Y	Wegener's Granulomatosis	u	Enterococcus faecium (lung biopsy)
2	27	F	12	N	Pneumococcal sepsis	d	*Streptococcus pneumonia* (BC)
3	81	F	12	Y	Infected intramedullary pin	d	Methicillin resistant *Staphylococcus aureus* (BC)
4	55	F	16	N	Infected arterial graft	d	*Staphylococcus aureus* (BC)
Polymicrobial sepsis							
1	85	M	26	Y	Pneumonia	d	*Escherichia coli*, *Staphylococcus aureus* (BA)
2	72	F	32	Y	Perforated colon, peritonitis	p	Culture negative[2]
3	58	F	22	N	Catheter related sepsis, UTI	d	*Staphylococcus aureus* (BC and central venous catheter), Klebsiella pneumoniae (UC)
4	80	M	20	N	Aspiration pneumonia	p	Culture negative
5	73	M	44	Y	Aspiration pneumonia	p	Coagulase negative Staphylococci and Enterococcus species (BA)
6	62	M	44	Y	Aspiration pneumonia	p	Culture negative
7	50	F	19	Y	Bacterial peritonitis following cholecystectomy	p	Culture negative
8	81	F	39	N	Cholecystitis	p	Culture negative
Culture-negative							
1	75	M	24	Y	Pneumonia	p	Culture negative[3]
2	48	M	22	N	Pneumonia	p	Culture negative[3]
3	59	M	28	N	Pneumonia	p	Culture negative

(1) For Classification see Material and Methods (d=definite, p=probable, u=unlikely). (2) intraoperative swab under broad spectrum antibiotic therapy (3) under antibiotic therapy
Bronchial aspirate (BA); Blood culture (BC); Urine culture (UC); Focal infection (FI); Urinary tract infection (UTI)

Surface Display of TLR-2 on Monocytes and Granulocytes

Baseline TLR-2 surface display on monocytes and granulocytes was not significantly different between septic patients and healthy controls. For both groups evaluated there was no significant increase in surface display, neither on monocytes nor on granulocytes, upon LPS challenge. Accordingly, there was no difference regarding the stimulation index between patients with sepsis and healthy controls (data not shown).

Surface Display of TLR-4 on Monocytes and Granulocytes

Baseline display of TLR-4 on monocytes was significantly higher in septic patients than in controls ($p < 0.001$) (Fig.1A). This difference was significant for all subgroups of septic patients compared to healthy controls. However, subgroup analysis of septic patients did not show significant differences in baseline display of TLR-4 ($p < 0.001$) (Fig. 1B).

The stimulation index of TLR-4 on monocytes was significantly higher in controls compared to all patients with sepsis and each subgroup of patients ($p < 0.001$ for overall sepsis, $p < 0.05$ for patients with gram- positive sepsis and $p < 0.01$ for all other subgroups) (Fig. 2)

TLR-4 display on granulocytes was significantly higher in controls than in patients with sepsis (p =

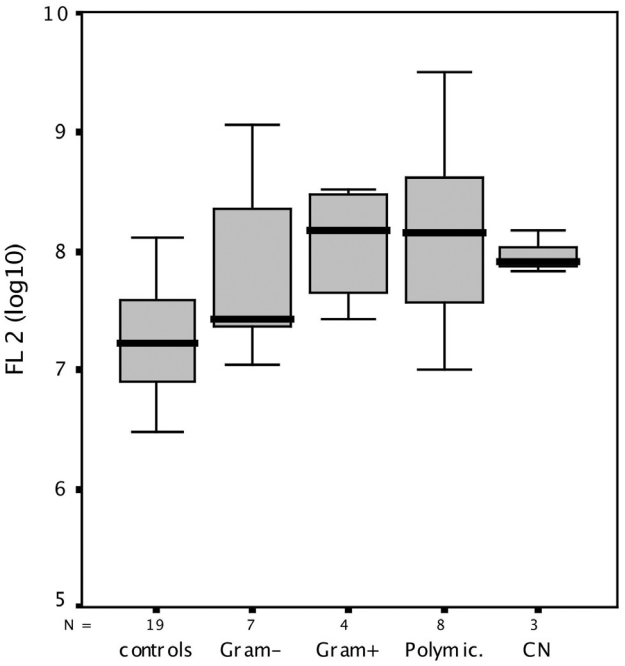

Fig. 1B. Baseline display of TLR-4 on monocytes for all subgroups of sepsis patients compared to healthy controls was significantly higher in septic patients than in controls ($p < 0.001$). *Abbreviations:* Gram- = Gram negative, Gram+ = Gram positive, Polymic. = polymicrobial, CN = culture negative

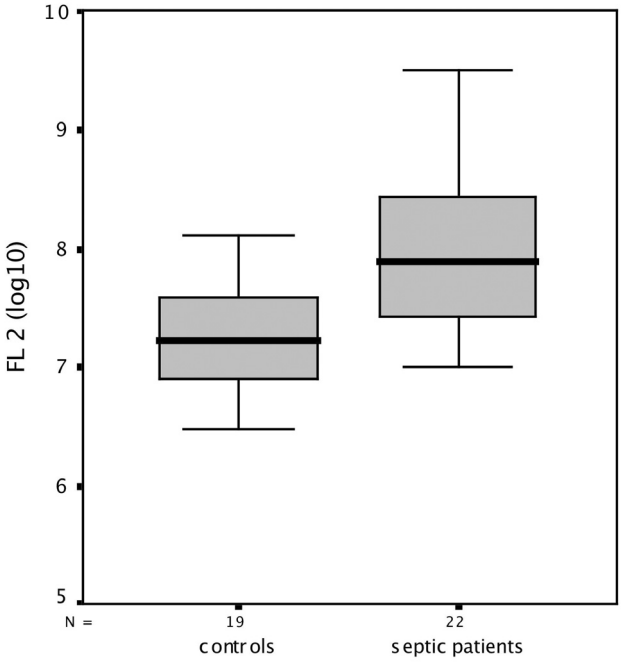

Fig. 1A. Baseline display of TLR-4 on monocytes was significantly higher in septic patients than in control subjects ($p < 0.001$). Monocytes were identified using APC-labeled anti–CD14-antibodies. TLR-4 was measured using a PE-conjugate anti-human TLR-4 antibody detected by flow cytometry. All box plot figures show median, interquartile range and extremes. *Abbreviations:* Gram- = Gram negative, Gram+ = Gram positive, Polymic. = polymicrobial, CN = culture negative

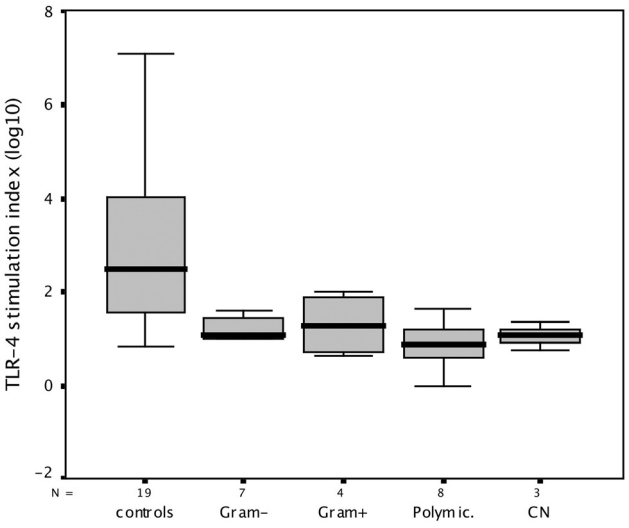

Fig. 2. The TLR-4 stimulation index on monocytes was significantly higher in controls compared to all patients with sepsis and to each subgroup of patients ($p < 0.001$ for overall sepsis, $p < 0.05$ for patients with gram- positive sepsis and $p < 0.01$ for all other subgroups). For Ex-vivo LPS-challenge, 1 ml whole blood was incubated for 15 minutes at 37 °C at a concentration of 40 ng/ml of LPS from *Salmonella minnesota*, Cells were washed in Dulbecco's Phophate Buffered Saline and monocytes identified using APC-labeled anti–CD14-antibodies. TLR-4 was measured using a PE-conjugate anti-human TLR-4 antibody detected by flow cytometry. Abbreviations: Gram- = Gram negative, Gram+ = Gram positive, Polymic. = polymicrobial, CN = culture negative

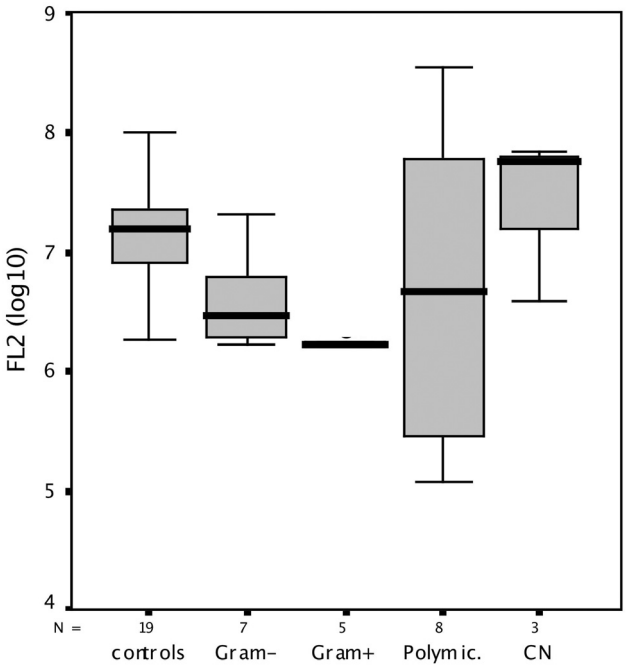

Fig. 3. Baseline display of TLR-4 on granulocytes was significantly higher in control subjects than in patients with sepsis ($p = 0.02$). Granulocytes were gated on the basis of forward- and sideward scatter (FSC/SSC) with an argon laser tuned to 488 nm and 635nm, respectively. TLR-4 was measured using a PE-conjugate anti-human TLR-4 antibody. Abbreviations: Gram- = Gram negative, Gram+ = Gram positive, Polymic. = polymicrobial, CN = culture negative

0.02). However, analyzing single differences between subgroups did only show a significant difference between controls and patients with gram-positive sepsis ($p= 0.006$) (Fig 3). Upon LPS challenge there was no difference for the stimulation index of TLR 4 on granulocytes between controls and patients with sepsis.

No correlation could be obtained between TLR surface display and SAPS or clinical outcome, respectively.

Discussion

TLR-2 and TLR-4 have been identified as key molecules in response of mammals to Gram-positive and Gram-negative infections [11,12]. We conducted an *in vitro* study to assess possible alterations of TLR-2 and TLR-4 surface display on monocytes and granulocytes derived from patients with severe sepsis in comparison with healthy controls.

Our data suggest a relationship between TLR-4 display on monocytes with bacterial challenge. Patients with sepsis per se showed a significantly higher presence of TLR-4 on the cell surface in comparison with healthy controls. Furthermore ex vivo stimulation with LPS showed a significant increase in TLR-4 surface display on monocytes derived from healthy controls. These observations are supported by two recent studies, which described a significant increase of TLR-4 mRNA expression of healthy control monocytes cultured with 1 μg/ml LPS for 2 hrs and 100ng/ml for 4 hrs, respectively [14,15]. However, in contrast to our findings, this increase could not be appreciated on the protein level and it was speculated by the authors that the majority of mRNA transcripts was not translated into protein [14]. Contradicting this hypothesis, but confirming our data, Marsik et al. described a marked upregulation of TLR-4 on monocytes in response to a low dose stimulation with 5 pg/ml LPS after 2 hrs [16]. In our study the increased surface display of TLR-4 in septic patients was independent from the subgroup causative agents. TLR-4 is recognized as the receptor mediating LPS signal transduction as a cell-wall structure component of Gram-negative bacteria. We also found an increased surface display of TLR-4 on monocytes from patients with Gram-positive bacteria as the suspected causative agent. However, this group was rather small with only 4 patients and a possible polymicrobial infection including Gram-negative bacteria, even if not confirmed by culture results, cannot be excluded.

In comparison with monocytes from healthy donors, which showed a significant increase of TLR-4 surface display upon LPS stimulation, no further increase of TLR-4 was observed on monocytes from patients suffering from sepsis after stimulation with LPS, indicating that there is a ceiling effect in TLR-4 surface display as a response to LPS.

Investigating TLR-4 surface display on granulocytes at baseline, we observed a converse pattern in comparison with the results described for monocytes. Here the presence of TLR-4 on granulocytes of septic patients was significantly lower than in healthy controls. No change of TLR-4 surface display was observed on granulocytes of septic patients or controls after stimulation with LPS for 15 min. The meaning of this finding is not clear. Not much is known about TLR-4 regulation, how and when it occurs neither in the natural time course of sepsis nor *in vitro*. Possible factors of influence may be the dosage of LPS, the stimulation time and the interval in which the stimulus is applied. Marsik et al. found downregulation in TLR-4 expression in neutrophils of healthy individuals upon LPS challenge *in vivo* and *in vitro* [16]. Assuming that our patients experienced a natural LPS challenge in the setting of sepsis our data are consistent with these findings. The reason why we did not see a change in TLR-4 presence after LPS stimulation *in vitro* may be the short stimulation time of 15 min. in comparison with Marsik who applied LPS *in vitro* for a minimum of 2 hrs.

We found no differences between septic patients and controls in TLR-2 display on monocytes and granulocytes neither at baseline nor after LPS challenge. In contrast, Armstrong et al. found a significantly higher TLR-2 mRNA expression as well as protein in septic patients compared to non-septic control patients [14]. The latter was also confirmed by another study showing increased TLR-2 expression in monocytes after LPS infusions in healthy controls. However, in this study, consistent with our results, there was no change of TLR-2 surface display on granulocytes after stimulation with LPS [16].

Again these incoherent results may be explained by the different stimulation times and LPS doses used in these studies.

We compared surface display of TLR-2 und TLR-4 on monocytes and granulocytes derived from patients with sepsis and healthy donors. The comparative analysis of donors, cell type and LPS challenge did not reveal a consistent pattern in TLR surface display.

In summary we demonstrated an increase of TLR-4 presence on monocytes upon bacterial challenge in the setting of sepsis and *in vitro*. We showed that TLR-4 display on monocytes upon further LPS stimulation *in vitro* is limited in patients with sepsis, which may have a clinical implication that deserves further studies. Furthermore we saw that TLR-4 on granulocytes from septic patients is low in comparison with controls. There was no correlation of TLR display to severity of sepsis as defined by the SAPS, nor to the outcome of patients.

The contradictory results of the studies investigating TLR-2 and TLR-4 regulation in sepsis demonstrate the complexity of the regulatory mechanism of TLR expression in human sepsis. Further studies with a larger number of patients as well as more homogenous subgroups that would provide a higher statistical power, will be necessary to explore the factors involved in TLR regulation in sepsis in order to asses their possible clinical applications.

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