Levels of Proteasome Subunit Expression Provide Information about Host's Immune System Status

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ABSTRACT

Mortality due to bacterial septic shock is a major healthcare issue and incidence is increasing worldwide. The objective of the present study was to evaluate expression levels of proteasome subunits as circulatory markers, in plasma and blood RNA obtained from severe septic patients and control volunteers, in order to differentiate patients in a pro-inflammatory phase from those in a subsequent tolerant phase. As an in vitro model, blood $CD14^+$ monocytes were treated with lipopolysaccharide (LPS) to investigate the mechanism of inflammation /tolerance. We evaluated plasma biomarkers from control and septic patients using ELISA or bead assays, and blood RNA samples by using real time qPCR. We identified that plasma levels of CRP, VCAM1, ICAM1, resistin, bilirubin and creatinine, were robustly upregulated in septic shock patients, as compared to controls. Whole blood total RNA analysis revealed that expression of immuno-proteasome LMP subunits, cytokines such as IL-8 were downregulated, while autophagy gene atg7 and resistin were upregulated in severely septic patients, as compared to controls, suggesting that these severe septic patients were in a state of tolerance (unresponsive to LPS). However, treatment of tolerant $CD14^+$ monocytes with IFNγ followed by LPS, reversed the tolerance response by upregulating gene expression of proteasome subunits and the other biomarkers determined in this study. Overall, our data define a novel group of clinical biomarkers that can differentiate septic shock patients that are in an inflammatory phase or a tolerant phase, which can be strongly correlated with high or low expression of proteasome subunits. These findings will be vital in designing effective novel therapeutic approaches for sepsis, and other diseases based on inflammation.

Keywords: Inflammation, proteasome subunits, macrophages, cytokines, VCAM1, Resistin, IL-8

INTRODUCTION

For decades, severe sepsis and septic shock has been thought to result from the development of an exaggerated inflammatory response to LPS (most potent) or other agonists. Bacteria invade the cells or tissues and cause infection, and bacterial cell death caused by antibiotic administration can lead to release of LPS. This initiates a strong inflammatory cellular response to an overall systemic inflammation response syndrome (SIRS), leading to blood coagulation and leaky endothelial cells. Severe sepsis can follow, characterized by hypoperfusion, organ dysfunction and septic shock via excessive NO production and vasodilation [1,2]. The underlying mechanisms for SIRS and shock are not understood, however an elevated inflammatory response is believed to stem subsequent responses. A number of potentially promising interventions based upon targeting of inflammatory mediators have been evaluated in costly and time-consuming randomized clinical trials[3]. Relatively little improvement in treatment strategies have been described because patients are treated with the same drug regardless of the stage, sepsis, severe sepsis, or septic shock. The disappointment resulting from such clinical trial failures has significantly dampened enthusiasm for the ultimate promise of immunology-based interventions [3]. Anti-TNF-α therapies, IL-1 receptor antagonist, and several other anti-inflammatory therapies have been tested in trials, without success, because blocking one cytokine is not sufficient to prevent mortality.

After inflammation subsides, the host goes into a tolerant phase, where host macrophages/monocytes do not respond to further treatments with LPS [4,5]. Although, tolerance in human subjects has been observed; the mechanisms underlying tolerance are unclear. Tolerance to LPS and other agonists could be beneficial to the host because the cells can restore the inflammation system, but it may result in susceptibility to infections. Crucial mechanisms underlying inflammation/ tolerance in human sepsis need better understanding [3].

Several biomarkers for sepsis, septic shock have been reported using both murine and human models [6-7]. A crucial issue is differentiation between pro-inflammatory phase and tolerant phase of clinical sepsis (refractoriness, unresponsiveness to LPS). Although, most early studies were carried out in mouse models, tolerance has been shown to occur in humans. There are reports that monocytes isolated from sepsis patients are tolerant and refractory to treatments with LPS (4). A panel of biomarkers should differentiate between the two, thus predicting sepsis complications and subsequent organ dysfunction.

Our recent work has established the role of Ubiquitination Proteasome System (UPS) as a pivotal regulator of LPS-induced inflammation and tolerance [8-11]. We have shown that expression of hundreds of pro-inflammatory and anti-inflammatory genes are upregulated in murine macrophages in response to bacterial cell components such as LPS, CpG DNA, and peptidoglycan that can be modulated by murine macrophage proteasome [8, 11]. Proteasomes are present in all cells and regulate cytokine gene expression, as well as degradation of ubiquitinated signaling proteins (multiple pathways) that are modulated by LPS [8, 9]. The 26S proteasome complex is a barrel-shaped organelle that is composed of 14 different subunits that are present in duplicate. The β-subunits X (β5), Y (β1), Z (β2) and their corresponding immuno-

proteasome subunits LMP7 (β5ι), LMP2 (β1ι) and LMP10 (β2ι) are the proteases that have the chymotrypsin-like, post-acidic activities and trypsin-like, respectively. The role of the proteasome in LPS-induced inflammation was first reported by us (reviewed in [11]). The proteasome degrades transcription factors such as NF-κB [12], hypoxia-inducing factor 1α[13], P-interferon-regulatory factor (P-IRF3) [14], other key regulatory and mediator proteins such as Toll-like receptor 4 (TLR4), TLR9, TNF receptor associated factor-6 (TRAF6), interferon regulatory factor 3 (IRF3), phosphorylated Interleukin-1 receptor-associated kinase 1 (P-IRAK), Mitogen-activated protein kinase (MAPK) precursors and NF-κB precursors are also degraded by the proteasome. The change in subtypes and expression levels of proteasome subunits in cells is crucial in controlling the signal transduction of LPS [8, 9,10].

Previously, we discovered that proteasome subunits have specific functions [15, 16] using peritoneal macrophages from mice; and human monocytes during LPS-induced inflammation/tolerance. Human monocytes (contain only LMP subunits) do not induce NO, while peritoneal mouse monocytes (contain both XYZ and LMP subunits) can effectively induce NO, in response to LPS. Regardless, LPS upregulates gene expression of proteasome subunits during the inflammatory phase, but in contrast, LMP subunits and TNF-α gene expression are both downregulated during tolerance [10]. We have also recently shown that IFN-γ reverses LPS-induced tolerance with a concomitant rise in expression of LMP subunits of proteasomes in both human monocytes and mouse macrophages. These findings underscore the importance of UPS in both inflammation and tolerance in response to LPS by analyzing TNF-α and NO induction [10, 15]. We have also shown that a combination of proteasome inhibitors (to reduce inflammation) and antibiotics (to kill bacteria) works effectively in a cecal-ligation and puncture mouse model of sepsis when administered early during infection [17].

Our overall hypothesis is that gene expression levels of proteasome subunits, biomarkers, resistin, and IL-8 in whole blood RNA determine whether these severely septic/septic shock subjects are in a state of inflammation or tolerance. We compared levels of select inflammatory, anti-inflammatory markers, adhesion molecules (proteasome-dependent) and proteasome subunits in plasma/serum; and in RNA extracted from whole blood from terminally sick patients or those that remained in ICU, as compared to control volunteers. The results provide strong evidence that level of adhesion molecules and other proteins such as vascular cell adhesion molecule1 (VCAM1), intercellular adhesion molecule 1 (ICAM1) (proteasome-dependent genes, resistin, plasminogen activator inhibitor-1 (PAI-1), and C-reactive protein (CRP) was significantly upregulated even during the late-stages of shock. In contrast, gene expression of proteasome's LMP subunits and IL-8 were strongly down-regulated in septic shock patients, while resistin remained upregulated, as observed by qRT-PCR. These data suggest that proteasome subunits, IL-8 and resistin may diagnose severe sepsis, and that septic shock patients enter a state of tolerance due to very low levels of proteasome subunits.

MATERIALS AND METHODS

Reagents: Highly purified, deep rough chemotype LPS (Re-LPS) from *E. coli* D31m4 was prepared, as described by Qureshi *et al.* [18]. For cell culture studies, RPMI-1640, heat-inactivated low-endotoxin

fetal bovine serum (FBS), and gentamicin were purchased from Lonza, Basel, Switzerland.

Study Subjects and Design:

Two preliminary studies were carried out to determine appropriate biomarkers for inflammation/tolerance from blood samples of sepsis and septic shock patients. We were able to obtain several subjects for this study carried out in Pakistan, because of unusually high prevalence of sepsis in that country.

Study 1 Blood samples were obtained from healthy adult volunteers and the patient population. This study was approved by the University of Missouri Kansas City (UMKC), institutional review board and informed consent was obtained from all controls, and patients or next of kin. The study was conducted in accordance with current Good Clinical Practices and declaration of Helsinki. Blood samples (40 ml) were collected in EDTA containing tubes and diluted two-fold with wash buffer, Dulbecco's phosphate-buffered saline with calcium and magnesium containing fetal calf serum (heat inactivated 2%), and EDTA (1 mM). Then, 50 µl and 25 µl/ml of human monocyte enrichment cocktail and granulocyte depletion cocktail (Stem Cell technologies, Canada), respectively, were added and contents incubated at room temperature for 20 min. This mixture was layered onto Ficoll-Paque PLUS gradients, and centrifuged to obtain enriched CD14 positive cells ($CD14^+$), and plasmas according to manufacturer's instructions.

Study 2 Blood samples were obtain-ned from healthy adult volunteers with a sex age distribution that matches the patient population (16-86 years). This study included Asian men, and women. We studied controls and septic shock patients living in villages near Rawalpindi, Pakistan. The study was conducted in accordance with current Good Clinical Practices and declaration of Helsinki. This study was also approved by the institutional review board of Pakistan Ordinance Factory (POF) Hospital, Wah Cant, Pakistan. Adult controls (n=20) and septic shock patients (n=26) were recruited for the study according to guidelines provided. All subjects were selected based on a clinical history examination by the consultant physician. This study recruited some patients with severe sepsis and septic shock.

Inclusion criteria for both studies: confirmed or suspected infection, abnormal temperature (>38°C or <36°C), Respiratory compromised (respiratory rate >20, PCO_2 <32 mm of Hg, or mechanical ventilation), increased heart rate (>90 beats/min), and abnormal white blood cell count (>12,000 or <4,000 cells/mm^3). Patients were selected only if they met at least 3 out of 4 criteria. Some patients progressed into septic shock. These are recognized as clinical criteria for SIRS/sepsis/septic shock patients [2]. Some of the severe septic patients also had organ dysfunction in study 2.

Exclusion criteria for both studies: included AIDS, cancer, and corticosteroid use before study entry, or recent major surgery. Prisoners and pregnant women were excluded from the study.

Blood Sample Collection and Biochemical assays:

Blood was drawn in tubes for complete blood count and biochemical analysis. For these studies, cell lysates were prepared from serum using Norgen (ON, Canada) total RNA purification kit (product # 17200), according to the manufacturer's instructions. Briefly, serum (200 µl) was treated with lysis buffer (600 µl) in an RNase-free micro-centrifuge tube. Contents were mixed by vortexing for 10 sec, and 400

µl of 95-100% ethanol was added to every 400 µl of lysate. RNA was purified by binding RNA to column as described in the kit directions. Complete blood count assessment including hemoglobin and platelets were carried out on a hematology analyzer (SYXMEX KX-21, Japan). The routine liver functions tests including serum total bilirubin, Alanine aminotransferase test (ALT), Alkaline phosphatase (ALP) and serum creatinine were determined by using Roche diagnostic kits (Switzerland) on P-800 auto analyzer according to manufacturer's protocol.

Biomarker Analysis Using Milliplex Assays and Immunometric Assays:

For study 1, biomarker immunoassays for cytokines including TNF-α, IL-12, IL-8, IFN-γ, IL-6, IL-2, IL-10, IL-4, IL-1α, IL-17α, adhesion molecules, such as VCAM1, and ICAM1, other proteins such as resistin, C-reactive protein (CRP), monocyte chemotactic protein-1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1), and growth factors such as fibroblast growth factor (FGF-b) were performed. ELISA kits were purchased from Signosis (special order) and were based on chemiluminescence. Serum TNF-α, IFN-γ, VCAM1, ICAM1 and resistin were also determined quantitatively by ELISA kits (R & D) for study 1. Milliplex Human Magnetic bead multiplex assay kit (EMD Millipore, USA) using Luminex 200^{TM} analyzer (EMD Millipore, USA), according to manufacturer's instructions in study 2. Serum hsCRP was analyzed by two-site sequential chemiluminescent immunometric assay kit (Siemens, USA) on Immulite 1000.

Inflammation/Tolerance experiment:

Isolated blood CD14^{+} monocytes were treated in 6-well plates as follows: Monocytes were first treated with medium (M) or LPS (L10 ng/ml) for 24 h, followed by washing and replacement with M or L for an additional 4 h. The four combinations are represented as MM, ML, LM and LL (L 10 ng/ml). The first LPS treatment renders cells tolerant to second LPS treatment, as described previously [10]. In some experiments, tolerized (LM) cells were washed with medium, treated with IFN-γ (50 units) for 4h (LM+IFNγ), washed, and then followed by another 4h treatment with LPS (LM+IFNγ+LPS). After the indicated treatment, all cells were washed with PBS and total RNA was extracted by using RNeasy mini kit (Qiagen) as per manufacturer's instructions. Real time RT-PCR was performed by using TaqMan RNA-to-Ct one step kit.

Real-time RT-PCR:

Quantitative Real-time qRT-PCR was performed on total RNA isolated from plasma samples of septic shock patients and control subjects, as well as treated CD14^{+} monocytes. Before performing real time RT-PCR, quality of RNA was assessed by spectrophotometric measurements. Taqman primer-probes and One-Step qRT-PCR kit were obtained from Life Technologies (Foster City, California). All reactions were performed in triplicate using equal amount of mRNA per reaction. Reverse-transcriptase step involved incubation at 48°C for 15 min. The PCR cycling conditions included an initial denaturation of 95° C for 10 min, followed by 40 cycles of 95° C for 15 sec, and 60° C for 60 sec. Real-time PCR assays were completed using a Step-one plus Real time PCR system. Relative gene expression from septic patients and control subjects was normalized ($2^{-\Delta CT}$ analysis) to GAPDH.

Statistical Analysis:

All statistical procedures and graphs were performed with GraphPad Prism 5 (v5.01, San Diego, CA). Data are presented as means + SEM. Data were compared using an unpaired t-test, 2 tailed and significance was set at the $P \leq 0.05$ level for the control and septic groups. A one-factor ANOVA with Tukey's multiple-comparison *post hoc* tests were used for analyzing gene expression ($2^{-\Delta\Delta CT}$) between treated $CD14^{+}$ monocytes.

RESULTS

We wanted to diagnose septic patients based on whether they were in initial inflammatory mode or in the late tolerant phase. We first compared the clinical characteristics of subjects used in this study and results obtained for different cytokines, growth factors and adhesion molecules from blood obtained from controls and septic patients. These results are summarized in Tables 1-3.

For **study 1**, biomarker levels from 7 control samples (day 1) and 7 patient samples (days 1, 4, and 7) were analyzed. Biomarkers that showed significant changes (p values <0.05) were evaluated in the larger study. Patients 1, 4 and 5 were very ill, and later two died from shock (Table 1). We were interested in finding biomarkers that were present in plasmas from patients that were severely septic and those that died from septic shock. Initially, we studied the levels of TNF-α and IL-1β, which are considered excellent early biomarkers in mice. There was no significant difference in expression levels from controls and patients (data not presented). However, we detected other biomarkers that were present in plasma.

Table 1 Comparison of physical and biochemical parameters for controls and septic patients (Study 1).

	AGE	SEX	Race	Fever	Cultures	Description
Pt1-D1	82	M	AA*	Yes	negative	Scrotal and pelvic abscess, Survived,
Pt2-D1	56	F	W*	No	negative	Altered level of consciousness. Survived.
Pt3-D1	71	F	AA	Yes	negative	Liver cancer. Survived.
Pt4-D1	70	M	AA	Yes	positive	Subarachnoid hemorrhage and respiratory failure. Deceased.
Pt5-D1	66	F	AA	No	negative	CHF exacerbation, Para pneumonic effusion. Deceased.
Pt6-D1	49	M	AA	No	negative	Acute hypoxic respiratory failure. Survived.
Pt7-D1	51	F	AA	Yes	positive	Pancreatic mass, survived
C1	72	M	W	No	negative	
C2	28	F	W	No	negative	
C3	29	F	W	No	negative	
C4	60	F	W	No	negative	
C5	70	M	W	No	negative	
C6	31	M	W	No	negative	
C7	21	M	Asian	No	negative	

***AA denotes African American, and W denotes whites. Most of these patients had confirmed or suspected bacterial infections. Although the races of the controls and patients were different, we think that sepsis (and other afflictions such as diabetes, heart disease, etc.) would radically change the induction of cytokines and outcomes in study 1.**

In this study, we observed 1646 fold difference between plasma CRP levels in controls vs. septic patients on day 1, as shown in Tables 2-3. Moreover, there were only five biomarkers whose levels were significantly (p values < 0.05) upregulated or downregulated in plasmas from patients vs. controls. These included CRP, VCAM1, ICAM1, PAI-1 and resistin which were robustly upregulated in patients, as compared to controls. Other biomarkers studied, including IL-10, VEGF, MCP-1 that were upregulated in some patients, but not in all septic patients. There were less significant differences in levels of IL-10 and VEGF (p values >0.05) in plasma from controls and patients. We found differences in plasma levels of GCSF, VEGF, IL-4, IL-6, IL-1α and IL1-RA; as shown in Table 3.

Table 2 Comparison of physical and biochemical parameters for controls and septic patients (Study 1).

	μg/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	pg/mL	pg/mL
	CRP	**VCAM1**	**ICAM1**	**PAI-1**	**Resistin**	**IL-2**	**VEGF**	**IL-4**
Pt1-D1	191.5	180.01	360	2.66	60.9	7.59	96	841
Pt2-D1	119.82	179.68	441.94	3.34	49.772	6.35	96	90.5
Pt2-D4	75.12	258.98	228.38	0.5	20.194	6.13	116	85.5
Pt2-D7	44.63	383.43	503.17	1.41	45.392	7.09	92	103.5
Pt3-D1	116.2	53.84	266.15	2.6	38.244	6.41	84	241
Pt3-D4	118.59	92.02	307.65	1.77	21.422	6.23	114	104
Pt3-D7	97.33	58.43	313.96	4.67	26.946	6.27	118	105
Pt4-D1	124.95	144.43	156.89	11.21	190.824	5.72	102	221.5
Pt4-D4	117.71	226.03	119.02	0.49	85.068	7.06	166	1242
Pt5-D1	127.61	56.48	143.23	7.57	34.594	7.21	102	81
Pt6-D1	115.04	200.36	302.47	3.81	23.432	7.37	122	14.5
Pt6-D4	112.65	262.2	1504.08	3.35	65.196	6.99	84	236.5
Pt6-D7	115.38	182.35	1453.77	3.38	41.118	6.45	84	680
Pt7-D1	123.24	46.69	358.41	5.6	18.33	6.11	146	24.5
Pt7-D4	101.03	17.74	210.79	3.86	10.608	6.75	96	119.5
C1	0	0	117.76	4.27	8.122	10.1	92	77.5
C2	0	0	ND	0.65	ND	7.83	100	24
C3	0.22	0	130.33	0.36	4.65	7.23	84	59.5
C4	0	0	160.06	1.87	5.856	7.11	0	93.5
C5	0.17	1.77	160.06	2.65	7.53	6.39	114	65
C6			123.11		2.112			
C7			118.55		6.78			

All subject samples were run on the same day for comparison purposes and have been standardized. ND not determined.

Table 3 Comparison of acute inflammatory proteins and cytokines in controls vs septic shock patients' plasma on Day 1 (study 1).

	CONTROLS	SEPTIC PATIENTS	p values
CRP μg/mL	0.08±0.05	131.8±10.2	0.0001
VCAM1 ng/mL	0.35±0.35	123.2±26	0.0027
ICAM1 ng/mL	135.5±8.14	289.9±41.59	0.006
RESISTIN ng/mL	5.8±1.06	59.44±22.58	0.05
PAI-1 ng/mL	1.96±0.90	5.26±1.2	0.06
IL-2 ng/mL	7.73±0.64	6.68±0.27	0.11
VEGF pg/mL	78.00±20.12	106.9±7.82	0.16
IL-4 pg/mL	63.90±11.56	216.3±109.4	0.27
IL-1RA pg/mL	20.00±10.51	102.9±62.30	0.3
IL-8 pg/mL	13.20±3.94	20.68±5.55	0.34
IL-1α pg/mL	2.62±0.47	44.24±35.20	0.36
MCP-1 pg/mL	17.20±17.2	37.71±17.20	0.36
FGF-b ng/ml	2.15±0.97	7.75±5.97	0.46
IL-6 pg/mL	39.21±5.51	83.40±54.83	0.49
IL-10 ng/mL	1.03±0.28	1.38±0.87	0.77

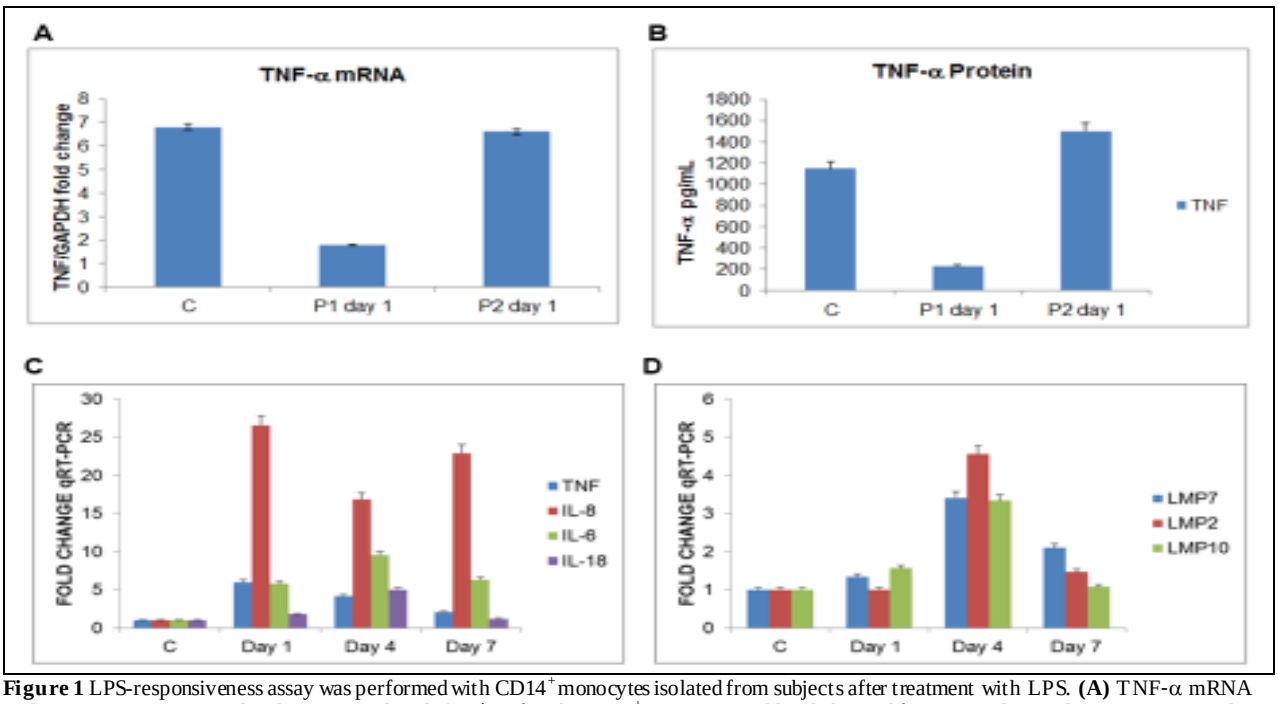

Figure 1 LPS-responsiveness assay was performed with CD14$^+$ monocytes isolated from subjects after treatment with LPS. **(A)** TNF-α mRNA and **(B)** protein expression levels in LPS-induced (1ng/mL for 6h) CD14$^+$ monocytes in blood obtained from control C1, tolerant patient (P1) day 1, and non-tolerant patient (P2) day 1. **(C)** TNF-α, IL-8, IL-6, and IL-18 mRNA expression in LPS-induced (1ng/mL for 6h) CD14$^+$ monocytes obtained from non-tolerant patient (P2) on days 1, 4, and 7 after admission to hospital. **(D)** LMP7, LMP2 and LMP10 mRNA expression in LPS-induced (10ng/mL for 6h) CD14$^+$ monocytes obtained from non-tolerant patient (P2) on different days after admission to hospital. Error bars denote SEM values. However, statistical significant results were not obtained in CD14$^+$ monocytes obtained from tolerant patient (P1, data not presented) and treated with LPS.

To distinguish between LPS-responsive or tolerant patients, we tested $CD14^+$ monocytes isolated from blood of patient 1 (day 1) and patient 2 (day 1) for tolerance to LPS, using a TNF-α standard ELISA assay for determining tolerance. We found that mRNA and protein expression of TNF-α protein were robustly downregulated in patient 1 (tolerant), as compared to control subjects; thus suggesting that patient 1 was tolerant to further stimulation by LPS, whereas monocytes of patient 2 responded normally to LPS (Figure 1A, B). We also studied $CD14^+$ monocytes ex-vivo from patient 2 (not tolerant) obtained on days 1, 4 and 7 (Figure 1C, D), in response to LPS by qRT-PCR. IL-8 was the most expressed cytokine induced on all three days, but TNF-α, IL-6 and IL-18 were also expressed. VCAM-1 and ICAM-1 were also elevated as observed by ELISA (data not shown). We also assayed gene expression of LMP subunits in response to LPS by qRT-PCR, and determined that LMP7, LMP2 and LMP10 subunits of proteasomes were upregulated on day 4, while downregulated on day 7. Collectively, monocytes obtained from some patients were tolerant, while others could respond to LPS. The monocytes isolated from different patients in different stages of shock also responded to LPS differentially. To confirm this finding, we carried out a larger study (study #2).

We carried out **study 2** with 20 control samples and 23 patient samples (Table 4). We evaluated only those biomarkers that showed distinct differences between controls and late stage septic shock patients in our preliminary study, Table 4. Most of the septic shock patients were very ill and 17 out the 23 patients died within 4 days of care in this present study, so only day 1 data could be obtained. The controls and septic patients differed with respect to clinical and biochemical parameters as shown in Table 5. Pulse rate, total leukocyte counts, bilirubin and creatinine levels were elevated, while hemoglobin level, blood pressure and platelet counts were lower in septic shock patients, as compared to controls as expected (Table 4). Septic shock patients had mean arterial pressures in range of 60 to 70 mm Hg at time of admission in hospital, as compared to 88 and 112 mm Hg in control group ($p< 0.001$). Most of the patients had initial empirical multiple antibiotic therapies from the doctors in OPD and were transferred to hospitals during late stage of their illness. Some of the female patients developed sepsis related to childbirth. Complications due to Gram-negative bacterial infections were followed by Gram-positive bacteria and mixed bacterial microorganisms, including *Escherichia coli, Acinetobacter baumannii, Methicillin-resistant Staphylococcus aureus* and *Providencia species.* The patients were ill with infections and their sepsis then progressed on to septic shock. Most patients had either confirmed or suspected infection and organ failure; and later 17 out of the 23 patients died from septic shock. In study 2, we studied the biomarkers that were statistically significant in a few septic patients observed in study 1, and observed >70 fold difference between plasma CRP levels in controls vs. septic patients (Table 5). There were five biomarkers whose levels were significantly upregulated in patients vs. control plasmas (study 1) and these were CRP, VCAM1, ICAM1, resistin, and IFN-γ, while TNF-α was down-regulated in study 2. Mortality could not be related to any marker or symptoms.

Table 4. Comparison of physical characteristics and biomarkers of controls vs septic shock patients' on Day 1 (study 2)

SNo.	Age	Gender	Pulse	Temp °C	Hb g/L	TLC 10^9/L	Platelets 10^9/L	Bilirubin µmol/L	Creatinine µmol/L	*Death
1	40	F	130	39.3	7.0	36500	42000	77	225	SS
2	50	F	124	38.6	7.1	35800	43004	100	462	SS
3	28	F	110	39.0	15.1	39400	113000	21	177	SS
4	20	F	120	38.6	11.6	39900	215000	17	179	A
5	65	M	108	39.1	9.9	19000	149100	46	461	SS
6	51	M	90	37.6	6.4	3600	60050	38	360	SS
7	86	F	118	39.4	7.4	19100	43600	8	408	SS
8	25	F	102	38.4	10.6	37900	186000	56	401	A
9	26	M	106	39.1	11.2	27500	65000	5	291	SS
10	65	M	100	38.1	10.7	13800	111000	4	136	SS
11	55	M	120	39.6	7.0	13500	18600	126	96	SS
12	50	F	97	38.6	10.2	33300	160000	68	250	A
13	50	F	110	38.0	10.9	10500	127000	12	159	SS
14	42	M	116	39.2	10.0	24500	245000	15	203	A
15	40	F	112	38.2	12.2	5600	53000	83	111	SS
16	25	F	115	39.3	15.3	11100	85100	28	359	SS
17	50	F	122	39.6	7.9	18700	113000	67	169	SS
18	28	F	94	37.8	6.9	6400	27000	34	154	SS
19	51	M	114	39.8	7.4	22200	110000	58	153	A
20	32	F	120	39.2	8.6	27000	80000	13	112	SS
21	35	F	116	38.3	5.1	18000	35000	5	204	SS
22	38	M	104	39.5	10.7	15000	126000	0	191	A
23	52	F	100	38.1	6.2	17000	68000	12	411	SS
Patients	**43.65 ±3.30**	**15F/8 M**	**110.8 ±2.16**	**38.8 ±1.3**	**9.37 ±0.57**	**21535 ±2361**	**98933 ±12749**	**38.83 ±7.26**	**246.6 ±24.99**	**6A/17 SS**
Controls	**39.55 ±3.17**	**11F/9 M**	**74.70 ±1.11**	**37.4 ±0.09**	**14.3 ±0.37**	**7109 ±480.5**	**188690 ±15583**	**10.75± 1.06**	**80±7**	**A**
p values	**0.38**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

*SS denotes patient died from septic shock. A stands for patients with septic shock that survived

Table 5 Comparison of acute inflammatory proteins and cytokines in controls vs septic shock patients' serum on Day 1 (study 2)

SNo.	CRP μg/mL	ICAM-1 pg/mL	VCAM-1 pg/mL	RESISTIN pg/mL	TNF-α pg/mL	IFN-γ pg/mL	Death*
1	110.00	13.20	12.42	670.28	18.59	1.92	SS
2	55.00	4.47	8.90	812.17	2.67	0.35	SS
3	100.00	15.40	24.05	478.99	8.15	0.47	SS
4	50.00	17.04	21.31	396.40	9.47	0.58	A
5	100.00	3.31	7.84	201.87	2.50	0.32	SS
6	56.00	5.53	9.08	595.31	9.85	0.90	SS
7	100.00	5.26	10.40	240.82	5.36	0.63	SS
8	100.00	5.38	9.34	112.11	4.14	0.70	A
9	110.00	15.81	19.09	553.87	6.84	2.84	SS
10	100.00	5.42	10.60	279.73	10.19	0.38	SS
11	78.00	10.26	15.92	443.74	16.46	0.32	SS
12	48.00	8.71	15.56	678.68	7.82	0.54	A
13	124.00	10.12	6.89	861.23	6.21	0.85	SS
14	24.00	5.69	26.33	224.59	5.70	2.81	A
15	24.00	6.64	12.53	166.87	10.75	3.13	SS
16	97.50	13.29	12.12	827.12	10.95	1.00	SS
17	100.00	2.09	5.38	170.34	5.70	1.86	SS
18	84.00	2.23	5.62	44.20	2.29	1.00	SS
19	53.30	6.83	10.55	503.79	9.59	0.47	A
20	83.00	8.07	23.03	458.60	10.70	1.37	SS
21	91.00	5.54	12.74	215.34	5.66	0.84	SS
22	57.00	7.34	24.31	94.82	17.25	0.71	A
23	48.00	8.42	9.21	324.84	15.17	0.40	SS
Patients	**77.95±5.99**	**8.09±0.9**	**13.62±1.33**	**406.8±51.81**	**8.78±0.97**	**1.06±0.18**	**6A/19 SS**
Controls	**2.46±0.24**	**2.36±0.20**	**5.76±0.57**	**65.68±4.55**	**17.62±2.33**	**0.63±0.74**	**A**
p values	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0005**	**<0.0430**	

*SS denotes patient died from septic shock. A stands for patients with septic shock that survived

Next, we evaluated the expression of IL-1RN, IL-8, IL-10, IL-12, resistin, IFN-γ and TNF-α at the gene level by using qRT-PCR of mRNA from controls and patient's whole blood samples. TNF-α, IL-8, IL-12, IL-RN, were all downregulated; however, the only cytokine that was robustly downregulated was IL-8 (p value of <0.001) (Figure 2). IL-8 was also the most upregulated in LPS-induced monocytes ex-vivo, as shown in Figure 1. Further, we evaluated the gene expression of the proteasome's subunits; X, Y, Z, LMP7, LMP2, and LMP10. Initially, in control subjects (RNA samples), LMP2 was predominantly expressed, compared to all other proteasome subunits.

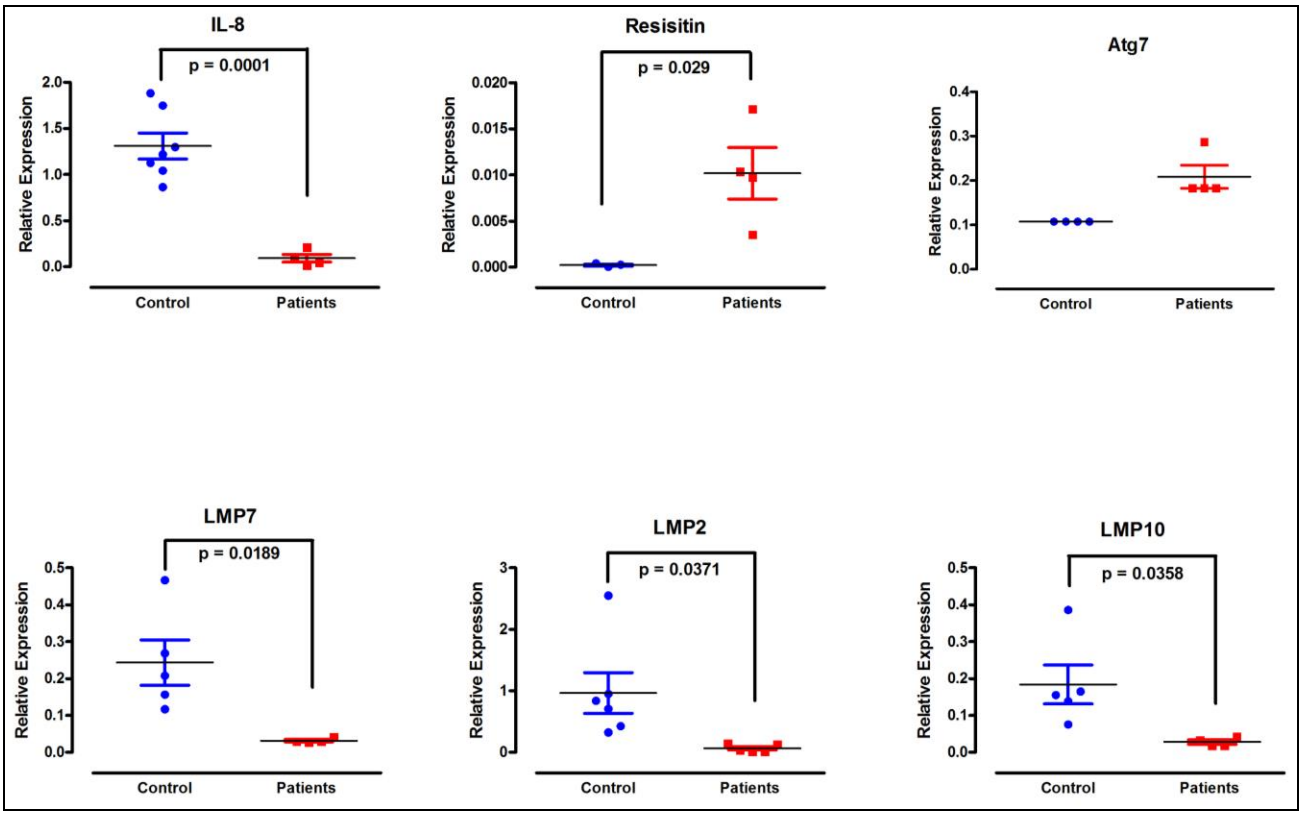

Figure 2 Proteasome subunit expression levels in RNA obtained from whole blood cells of septic shock patients versus healthy controls by real time RT-PCR. IL-8, RETN, and atg7 represent gene symbols and LMP7, LMP2 and LMP10 represent proteasome subunits. Significant differences (P<0.05) from septic shock patients versus controls was observed.

However, immunoproteasome subunits (LMP7, LMP2 and LMP10) expression was significantly downregulated (p values <0.05) in septic shock patients, compared to controls (Figure 2). The ubiquitinated proteins are degraded by the proteasome but excessive downregulation of proteasome's subunits leads to a process called autophagy (self-eating) where the bacteria, big particles, ubiquitinated, and aggregated proteins are degraded to conserve nutrients as discussed in the next section. We investigated the gene expression of autophagy genes atg7 (E1 enzyme for activation of the ubiquitination of proteins), and atg5 (E3 ligases, that introduce ubiquitin groups onto proteins). The atg7 autophagy gene expression was upregulated in cells of septic patients, whereas atg5 was downregulated, compared to controls (Figure 2). This suggested that whole blood cells obtained from septic shock patients

were in state of LPS-induced tolerance, as compared to controls. LPS is the main agonist of Gram-negative bacteria and causes inflammation/tolerance in human sepsis. Process of inflammation leading to tolerance process has not been well-studied in human sepsis. However monocytes isolated from sepsis patients have been observed to be tolerant in one study [18], but the mechanisms underlying tolerance during sepsis are not known. Therefore, we performed *in vitro* LPS tolerance experiments using $CD14^+$ monocytes isolated from blood samples from control subjects. We detected increased expression of pro-inflammatory cytokines after 4h LPS (ML) treatment of $CD14^+$ monocytes, while inhibition of pro-inflammatory cytokines after 24h LPS treatment (LM). There was a robust increase in expression of pro-inflammatory cytokines such as TNF-α, IL-8, IL-12, and IL-1RN after 4h LPS treatment (ML), while suppression of these cytokines (tolerance) after 24h treatment (LM) with LPS (Figure 3) except for resistin gene. There was no change in levels of resistin expression after 4h LPS (ML), but at 24h LPS treatment (LM, tolerant cells) resulted in enhanced expression (Figure 3). Interestingly, treatment with IFNγ overcame this tolerance (LM), and monocytes started responding normally to LPS again. Similarly, expression of ICAM1 was also upregulated after 4h LPS treatment, but suppressed after 24h, and this suppression was reversed after IFNγ and LPS treatment (Figure 3). As discussed previously, all proteasome subunits were downregulated in these tolerant monocytes [10]. Altogether, our results show that severely septic patients and LPS treated monocytes are in a state of tolerance as detected by reduced levels of LMP subunits present in these cells.

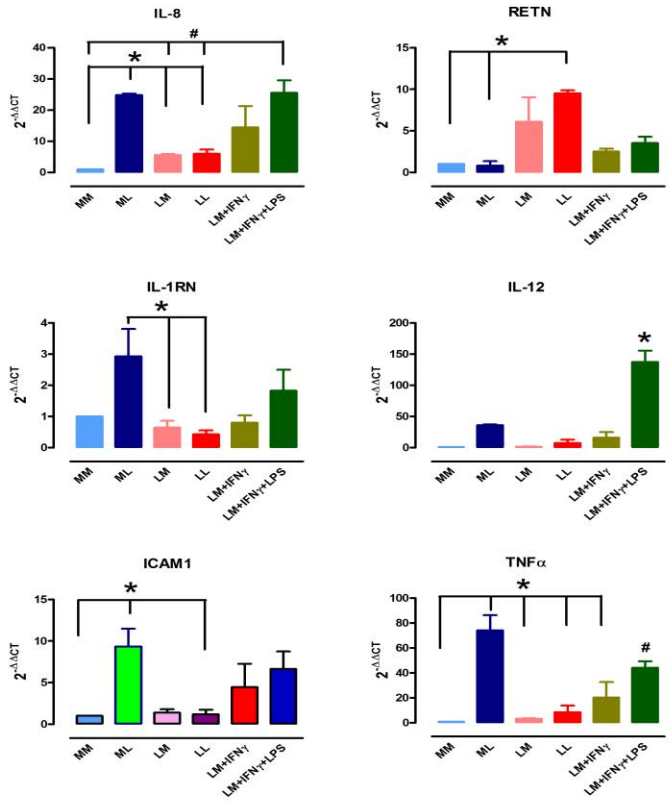

Figure 3 Human $CD14^+$ monocytes ($5x10^6$ cells/well) were first grown for 1 day and then treated for MM, ML, LM, and LL conditions, as described in methods and results sections. Cells were treated with medium or LPS (first LPS treatment) for 24h, followed by medium or LPS for 4h (second LPS treatment). Tolerized (LM cells) were washed with medium, treated with IFNγ for 4h (LM+IFNγ), followed by another 4h with LPS (LM+IFNγ+LPS). After treatment, cells were washed with PBS and total RNA was extracted by using RNeasy mini kit (Qiagen), as per manufacturer's instructions. Real-time RT-PCR was performed by using TaqMan RNA-to-Ct one step kit. IL-8, IL-1RN, RETN, IL-12, ICAM1 and TNFα represent the gene symbols. *and # shows significant difference ($P<0.05$) from ML treatment and LM+IFNγ+LPS treatment respectively.

DISCUSSION

This is a first study that provides strong evidence that supports the conclusion that RNA obtained from whole blood of severe septic shock patients (study 2) after admission to ICU revealed a robust downregulation in expression of all three LMP subunits of proteasome and IL8, with upregulation in atg7 and resistin, as summarized in Table 6. This would be consistent with tolerance in these patients. Even though, there was a significant increase in plasma/serum levels of CRP, VCAM1, ICAM1, PAI-1, IFN-γ and resistin (markers associated with inflammation), Some increases in cytokines were seen in sera obtained from severe late-stage septic patients (study 2), presumably because of persistence of the inflammatory cytokine storm. However, there was a significant decrease in TNF-α and most cytokines in septic shock patients, compared to control subjects in studies 1 and 2. While most investigations emphasize the identification of biomarkers at early stage of sepsis, septic shock patients often arrive in hospital at late stages, when much of peak inflammatory response has already waned [19]. Importantly, whole blood cells from terminally ill septic shock patients were in a state of tolerance (refractory to LPS), even though adhesion molecules, resistin, and CRP were still present in their sera/plasmas. Tolerance due to low levels of proteasome activity can lead to autophagy (self-eating, to conserve nutrients) of cells. The atg7 autophagy gene expression was upregulated in cells of septic patients, whereas atg5 gene was downregulated, as compared to controls. Collectively, these results provide strong support for the hypothesis that patients in critical condition are in a state of tolerance which could be strongly correlated with severely decreased expression of LMP subunits and perhaps autophagy of cells [20].

Table 6: Expected gene expression in human CD14+ monocytes and PBMCs during various phases of sepsis and septic shock.

Sepsis/LPS stage	XYZ	LMP	Mediators	*TF	Autophagy
Human PBMCs					
Early	XY	↑ LMP	↑TNF-α, IL-8, IL-6	↑ NF-κB	
Late		↓ LMP	↓TNF-α, IL-8, IL-6		↑ATG7
Human CD14 Monocytes					
Early	Y	↑ LMP	↑ TNF-α, IL-8, IL-6	↑NF-κB	
Late		↓ LMP	↓ TNF-α, IL-8, IL-6		↑ATG7

- TF Denotes transcription factors.

Biomarkers CRP, VCAM1 and ICAM1 can also be considered important in diagnosis of septic shock. CRP is predominantly produced and secreted by hepatocytes and other cells including macrophages in response to IL-6 [21]. In

this study, there was a robust upregulation in levels of CRP in septic shock patients vs controls. Recent studies have provided evidence to suggest that CRP is also elevated during shock, cardiovascular crisis, and trauma [22]. Other biomarkers such as VCAM1 and ICAM1 that mediate the adhesion of monocytes, lymphocytes and other cells to vascular endothelium play an important role in prediction of neonatal sepsis [23]. Taken together, CRP, and to a lesser extent ICAM1 can be considered excellent biomarkers for inflammation.

Other tolerance biomarkers such as high levels of resistin and low levels of proteasome subunits are also very useful in determining if a person is in a critical condition and at the late-stages of sepsis. Resistin is a protein known for causing the condition of "insulin resistance" when introduced into animals [24]. Resistin has also been shown to be associated with biomarkers of inflammation, organ dysfunction and mortality in severe sepsis and septic shock. Serum resistin levels are reported to be elevated in all critical care patients as compared with healthy controls and more significantly in sepsis patients with symptoms of insulin resistance. We also detected increased levels of resistin in septic shock patients, and therefore this could be used as a septic shock biomarker for diagnosis of tolerance (gene expression upregulated during tolerance) observed in these ill patients. In contrast, LMP7 is a key beta subunit of the proteasome complex that has robust chymotrypsin-like activity was downregulated in patients in critical condition (data not provided). Our previous work focused on proteasomes as a key regulator of cellular inflammation [9]. We and others have shown that LPS upregulates LMP7 gene expression in both mouse macrophages and human monocytes *in vitro* [10, 16, 25] however; we found that levels of LMP7 are significantly lower in plasmas from severe sepsis patients, as compared to controls. This finding is significant because after LPS-induced inflammation caused by cytokines subsides, the process of tolerance sets in patients, and this may be due to low levels of proteasome subunit expression in cells under these conditions.

Most researchers have focused on plasma/serum biomarkers [26, 27]. Instead, we have now established that cellular whole blood RNA provides a better measure of determining cellular mechanisms in patients' cells. We showed that gene expression of IL-8 was severely downregulated in patient's RNA, as compared to controls. IL-8, a chemotactic cytokine, elicits a massive neutrophil accumulation at the injection site [28]. Expression of other cytokines such as IL-12, IL1-RN, IFN-γ and TNF-α was downregulated, but not as much as IL-8, and expression of IL-10 was slightly elevated (data not shown). Similarly, our *in vitro* results obtained after treating $CD14^+$ monocytes with LPS also supports data obtained from septic patients. No increase in expression of proinflammatory cytokine levels after second LPS treatment to tolerized cells (LM) was observed. IFN-γ treatment reversed the LPS-induced tolerance. This is consistent with other studies where IFN-γ has been previously used by researchers to revive tolerant monocytes, as well as in septic patients [29, 30], but the mechanisms had not been determined. In our study, treatment with IFNγ followed by LPS was able to revive tolerant monocytes to induce pro-inflammatory cytokines again by upregulating proteasome LMP subunits, which renders the monocytes active again [10].

Most of the sepsis drug trials carried out have used anti-inflammatory drugs in all

patients, regardless of whether they were in a state of tolerance or in inflammatory cytokine inducing mode. Therefore, perhaps this could be one of the reasons why some of these trials have not been successful in the past. Collectively, a group of biomarkers are necessary for prognostic and diagnostic purposes to distinguish patients that have high levels of cytokines in blood cells, from patients who are in the tolerance phase. Therefore, while resistin, CRP, VCAM1, ICAM1, IL-4, IFN-γ, creatinine, and bilirubin are good biomarkers for plasma levels and organ function, we show evidence for the first time that low levels of expression of LMP subunits, IL-8, but high expression of resistin and atg 7 may be indicative of a major weakness in immune system that is incapable of mounting an effective immune response against bacteria, leading to cell death and organ failure upon subsequent infection. We have now provided evidence to suggest that some patients that died from severe late-stage septic shock (study 2) exhibited low expression of proteasome subunits, and thus were in a state of tolerance and this ultimately leads to autophagy/apoptosis of cells, as discussed previously. Although, endotoxin-induced tolerance has been previously described in mouse models [3, 31] and in monocytes from sepsis patients treated with LPS ex-vivo [19, 26], the mechanisms for this tolerance had not been fully elucidated [elegantly reviewed in 5, 32-35]. Each patient is an individual, and we were interested in biomarkers that can distinguish patients in the inflammation and tolerant modes, so appropriate custom therapy can be planned. However, this is the first clinical study that describes the role of proteasome subunits, IL-8, and resistin in tolerance in severe sepsis and septic shock patients.

Mechanistically, this can be explained by the fact that proteasomes have many functions, and are involved in multiple signaling pathways at gene expression level via activation of transcriptional factors and signaling proteins in an ordered fashion [8, 9, 11]. Proteasomes also degrade a number of important regulatory ubiquitinated proteins, hormones, enzymes, as well as modulation of cell-cycle components and autophagy. Therefore the proteasome would be an important therapeutic target for sepsis/septic shock. Our working model is presented in Figure 4, where priming of cells occur due to changes in types of proteasome's subunits upon activation by agonists; most tissue cells that exist in XYZ form initially and upon exposure to agonists, LPS, TNFα and IFN-γ, new proteasomes are biosynthesized that contain predominantly LMP subunits and cells are primed. However, we have shown that most of the human $CD14^+$ monocytes [10], B-cells, and Jurkat T cells, already contain predominantly LMP subunits [data not shown]. Thus a cytokine and adhesion molecule storm is created to clear up infection during the inflammation phase (upregulation of proteasome subunits) that is dependent on the activation of transcription factors, such as NF-κB via degradation of phosphorylated ubiquitinated IκB (inhibitor); and TLR4 (also degraded by the proteasome) mediated signaling. Nitric oxide and oxygen radicals are induced which may regulate the proteasomes via feedback mechanism and the oxygen radicals may destroy the proteasomes during severe sepsis. When the proteasome activity is turned down; the reverse occurs and other transcription factors such as HIF-1α, PPAR's, and NrF2 [13, 14, 36] (also degraded by the proteasomes) are activated and this leads to tolerance. These changes affect histone acetylation/deacetylation and

changes in gene transcription. During tolerance phase cells become refractory to future insults by LPS, but these cells can be rescued by IFN-γ *in vitro*, as described previously [9]. LPS-induced tolerance could also be due to degradation of regulatory proteins, since LPS treatment causes a rapid ubiquitination of proteasome's subunits (Qureshi et al, unpublished data). These ubiquitinated proteins may be degraded either by the proteasome, itself or by autophagy. The proteasome degrades the short-lived proteins, while autophagy degrades the bacteria and bigger particles.

This study has clinical relevance. Antibiotics are useful for killing bacteria, but most antibiotics cannot eliminate the inflammatory processes resulting from action of excessive LPS (released from bacteria) and other agonists such as CpG DNA and peptidoglycan that eventually lead to tolerance (Figure 4). We observed that monocytes isolated from Patient 1 (study 1) were tolerant to LPS, while Patient 2 was not tolerant to LPS, based on in vitro induction of TNF-α. Therefore, proteasome inhibitors or nutritional supplements (such as resveratrol) in combination with antibiotics may be useful in patients that show excessive biomarker activity [16], since proteasome inhibitors can regulate both the inflammatory and anti-inflammatory markers [9, 37-39]. During late stages of shock when proteasome's subunit expression is decreased during tolerance, and there is excessive cell death and it may be necessary to use fresh plasma, leukopaks, proteasome activators or nutritional supplements for activating expression of proteasome subunits; because inhibition of all proteasome activities may lead to cell death, or excessive autophagy. Autophagy may be beneficial to the host in some cases, because it recycles the body's nutrients and gets rid of bacteria and toxins [20]. However, excessive autophagy may be harmful to the host. Collectively, we were able to find new biomarkers, and the study also suggests that those patients with severe sepsis/shock cannot all be treated with same drug. Our study highlights the need for personalized medicine approaches, which may include customized proteasome-based treatment [38, 39] that controls inflammation and/or tolerance [9] dependent on the individual patient clinical course and biochemical markers.

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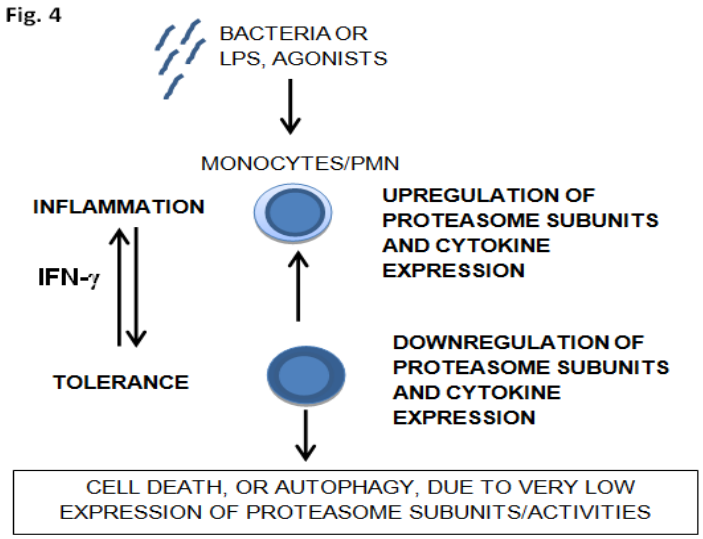

Figure4: Bacteria, LPS and other agonists interact with cells and leads to an upregulation of proteasome's subunits, followed by cytokine induction (Inflammation phase). This is followed by tolerance phase where the proteasome's activities and some cytokines are downregulated (tolerance phase). If the proteasome activities are severely downregulated then the cells die.

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