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LPS-Induced Formation of Immunoproteasomes: TNF-α and Nitric Oxide Production are Regulated by Altered Composition of Proteasome-Active Sites

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Abstract

Stimulation of mouse macrophages with LPS leads to tumor necrosis factor (TNF-α) secretion and nitric oxide (NO) release at different times through independent signaling pathways. While the precise regulatory mechanisms responsible for these distinct phenotypic responses have not been

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fully delineated, results of our recent studies strongly implicate the cellular cytoplasmic ubiquitin–proteasome pathway as a key regulator of LPS-induced macrophage inflammatory responses. Our objective in this study was to define the relative contribution of specific proteasomal active-sites in induction of TNF-α and NO after LPS treatment of RAW 264.7 macrophages using selective inhibitors of these active sites. Our data provide evidence that LPS stimulation of mouse macrophages triggers a selective increase in the levels of gene and protein expression of the immunoproteasomes, resulting in a modulation of specific functional activities of the proteasome and a corresponding increase in NO production as compared to untreated controls. These findings suggest the LPS-dependent induction of immunoproteasome. In contrast, we also demonstrate that TNF-α expression is primarily dependent on both the chymotrypsin- and the trypsin-like activities of X, Y, Z subunits of the proteasome. Proteasome-associated post-acidic activity alone also contributes to LPS-induced expression of TNF-α. Taken together; our results indicate that LPS-induced TNF-α in macrophages is differentially regulated by each of the three proteasome activities. Since addition of proteasome inhibitors to mouse macrophages profoundly affects the degradation of proteins involved in signal transduction, we conclude that proteasome-specific degradation of several signaling proteins is likely involved in differential regulation of LPS-dependent secretion of proinflammatory mediators.

Keywords

Inflammation; Endotoxic shock; Cytokines; Nitric oxide

Introduction

Gram-negative bacteria-derived lipopolysaccharide (LPS) is the prototype stimulus for triggering an inflammatory cascade in macrophages through Toll-like receptor 4 (TLR4). This response is characterized by production of TNF-α, NO, and a variety of additional inflammatory mediators [1–3]. Uncontrolled inflammatory responses to infection with Gram-negative microorganisms often lead to the development of vasodilation, shock, and ultimately, death. We previously demonstrated that TNF-α and NO induction, in response to stimulation of macrophages with LPS, are regulated by independent signaling pathways [4, 5]. By carefully controlling conditions of LPS stimulation, distinct phenotypic responses (i.e., preferential induction of TNF-α vs. NO) can be readily elicited in macrophages [4], though the precise regulatory mechanisms underlying these distinct phenotypes have not been fully delineated.

Results from our more recent studies have strongly implicated the cytoplasmic proteasome as a key regulator of LPS-induced macrophage inflammatory responses [6–9]. Structurally, proteasomes exist as multi-subunit complexes, consisting of a number of distinct, well-characterized, proteins. The 26S proteasome (2.5 MDa) is a barrel-shaped multiprotein complex comprised a 20S proteasome with 19S “caps” on either side. The 20S proteasome, by itself, has potent proteolytic activity, whereas the 19S caps provide key regulatory functions. Structurally, the constitutively expressed 20S proteasome is a hollow, cylindrical, multi-protein complex containing three proteolytic subunits (X, Y, and Z). Each of these subunits provide catalytic residues for distinct catalytic sites, specifically subunits X, Y, and Z have chymotrypsin-like, post-acidic (post-glutamate), and trypsin-like activities, respectively [10–13]. IFN-γ, which is well-recognized for its capacity to amplify the response of macrophages to inflammatory stimuli [14], has been shown to induce alterations in the subunit composition of proteasomes. In this respect, in response to IFN-γ, subunits X, Y, and Z of newly assembled immunoproteasomes are partially replaced by proteolytic LMP7, LMP2, and MECL-1 (LMP10), subunits, respectively [10, 11, 15]. While the relative enzymatic specificity of these newly synthesized protease-sites bear similarities to X, Y, and

Z, their enzymatic characteristics (e.g., V_{max}, K_m) are distinctly different. The overall contribution of these immunoproteasome subunit substitutions to IFN-γ-induced amplification of inflammatory responses is currently unclear. Given the increasing appreciation for the involvement of the proteasome in LPS-dependent macrophage signaling, however, it is important to dissect the potential contribution of immunoproteasomes in LPS-dependent cytokine expression.

Our previously published findings provide compelling evidence supporting the conclusion that the proteasome functions as a key regulator of LPS-induced signaling in mouse macrophages through Toll-like receptor 4 (TLR4) [6–9]. In the course of those studies, we found that pre-treatment of LPS-stimulated macrophages with low doses of lactacystin, a potent proteasome inhibitor that binds to proteasomal subunits, profoundly affected (>90%) the levels of gene expression of inducible nitric oxide synthase (iNOS) and many proinflammatory cytokines implicated in the pathogenesis of septic shock. Surprisingly, TNF-α gene expression was found to be a major exception to this general finding; LPS-induced gene expression of TNF-α was only minimally affected (<25%) by low-dose (2.5–5 μM) lactacystin pretreatment. The observed differential effects of lactacystin pretreatment on production of TNF-α vs. iNOS and other proinflammatory cytokines led us to hypothesize that, since inhibition of chymotrypsin-like activity alone (the primary target of lactacystin) did not modulate LPS-induced TNF-α gene expression, other proteasome-dependent proteases must also be involved. To test this hypothesis regarding potential function and their contribution to innate immune responses, our overall strategy was to delineate the role of specific proteasomal proteases in LPS-induced TNF-α vs. NO production in mouse macrophages using selective proteasomal protease inhibitors.

In this report, we provide data to support the conclusion that RAW 264.7 macrophages express proteasome subunits X, Y, and Z subunits predominantly and that LPS stimulates TNF-α. LPS then triggers selective increases in mRNA and protein expression of LMP2, LMP7, and MECL-1 immunoproteasome subunits, leading to a differential activation of the inducible chymotrypsin-like activity of the proteasome and a corresponding increase in NO production, as compared to untreated controls in RAW 264.7 macrophages. Moreover, we found that inhibition of at least two proteasome activities, specifically, the chymotrypsin-like, and trypsin-like activities, are required to block LPS-induced TNF-α gene and protein expression. Our data suggest the post-acidic activity of the proteasome may also contribute to TNF-α production. Based upon these findings, we postulate that, in addition to modulation of LPS-induced gene expression that we observed previously, the proteasome regulates signal transduction pathways in a very precise fashion via selective degradation of select LPS-induced signaling mediator proteins.

Materials and Methods

Reagents

Highly purified, deep rough chemotype LPS (Re LPS) from *E. coli* D31m4 was prepared as described by Qureshi et al. [16]. For tissue culture experiments, Dulbecco's Modified Eagle Medium (DMEM), heat-inactivated low-endotoxin fetal bovine serum (FBS), and gentamicin were purchased from Cambrex (Walkersville, MD). RNeasy mini kit was purchased from QIAGEN sciences (Germantown, MD). Lactacystin was purchased from Boston Biochem (Cambridge, MA) and used as described previously [8]. "Proteasome-Glo" assays kits for the chymotrypsin-like activity (substrate, Suc LLVY-Glo, succinyl-leucine-leucine-valine-tyrosine-aminoluciferin), trypsin-like activity (Z-LRR aminoluciferin, Z-leucine-arginine-arginine-aminoluciferin), and the post-acidic (caspase) activities (ZnLPnLD-Glo, Z-norleucine-proline-norleucine-aspartate-aminoluciferin) of the proteasome were purchased from Promega (Madison, WI). Antibodies specific to mouse X,

Y, LMP7 and GAPDH were purchased from Boston Biochem and the secondary antibodies (goat and rabbit) were purchased from Santa Cruz. Antibodies to Z, LMP2, LMP10 (MECL-1) and the secondary antibodies (donkey and goat) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Maintenance

The mouse macrophage cell line, RAW 264.7, was used throughout these studies and was maintained in DMEM supplemented with 10% heat inactivated low endotoxin FBS and 10 mg/500 ml gentamicin at 37°C in a humidified atmosphere with 5% CO_2 as described previously [8]. Cells were cultured in 6-well plates as described in the legends to the figures. Viability of RAW 264.7 cells treated with LPS and/or lactacystin was determined by trypan blue dye exclusion or by a quantitative colorimetric assay with 3-(4, 5)-dimethylthiozol-2,5-diphenyltetrazolium bromide (MTT) as described previously [17].

Sample Preparation and Real-Time PCR

For the isolation of RNA and protein, cells were plated at a concentration of 3×10^6 cells/60 mm plate. Three hours after plating, fresh media was added containing LPS at a final concentration of 25 ng/ml. For mock-treated cells, an equivalent volume of 0.91% saline was added in place of the LPS. RNA was subsequently isolated using an RNeasyPlus Mini kit from Qiagen (Valencia, CA). cDNA was generated from total RNA (2 μg) using MuLV reverse transcriptase and random primers (High Capacity cDNA Reverse Transcription kit; Applied Biosystems, Foster City, CA). Amplification reactions were performed using TaqMan Fast Universal PCR Master Mix and TaqMan gene expression assays for the following mouse genes: Z, PSMB7 (Mm00650844_g1); Y, PSMB6 (Mm00833555_g1); X, PSMB5 (Mm01615821_g1); MECL-1, PSMB10 (Mm0 0479052_g1); LMP-7, PSMB8 (Mm00440207_m1); LMP-2, PSMB9 (Mm00479004_m1); or GAPDH (Mm999999 15_g1). For LMP-2, LMP-7, MECL-1 (LMP-10), Y, X, and Z, amplification reactions contained cDNA generated from 15 ng of RNA. All reactions were performed using an Applied Biosystems Step One thermal cycler. Relative quantification of gene expression was performed with GAPDH as the endogenous control using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

RAW 264.7 cells were treated as described in the legends to the figures. After stimulation, macrophages were washed with PBS, and cytoplasmic extracts were prepared from cells using cell extraction buffer (Biosource, Camarillo, CA) supplemented with a protease inhibitor cocktail, containing phenylmethylsulfonyl fluoride and phosphatase inhibitors, according to the manufacturer's directions. Protein concentrations were measured with BCA protein assay kits (Thermo Fisher Scientific, Rockford, IL). Western blots were used to semiquantitatively assess the relative proportions of the LMP7, LMP2, MECL-1, X, Y, and Z subunits of the proteasome. Each well of the gel was loaded with 20–40 μg of protein and samples were subjected to electrophoresis at a constant 150 V in 1× Tris glycine buffer for 50 min. Proteins in the gels were transferred onto the Immobilon Transfer Membranes (IPVH 15150; Millipore, Bedford, MA) using the semidry transfer cell and, after the appropriate antibody treatments, bands were visualized with an enhanced chemiluminescence detection kit (Pierce) as described previously [9].

Measurement of the Chymotrypsin-Like, Trypsin-Like, and Post-Glutamase Activities of the Proteasome

RAW 264.7 at 10^4 cells/100 μl in each well of white colored plates, 96-well (Fisher, 0877126, for luminescence studies) were used. After the addition of medium and/or inhibitors (various concentrations), cells were incubated in the 37° incubator at 5% CO_2, for

30 min. After the incubation period, the cells in the 96-well plates were taken out of the incubator 20 min prior to the addition of the reagents to detect chymotrypsin-like, trypsin-like, and post-acidic protease enzymatic activity. At room temperature, Proteasome-Glo reagent (100 μl) (for detection of chymotrypsin-like activity (Suc LLVY-Glo), ZnLPnLD-Glo (for detection of post-acidic activity) or Z-LRR-Glo (for detection of trypsin-like activity) was added to each well, and a total volume of up to 200 μl. The plates were covered with a plate sealer and incubated at room temperature for 30 min. The relative luminescence units (RLU) of assays were read in the plate luminometer according to manufacturer's directions (Promega, WI) in the linear dose response profiles.

Measurement of TNF-*α* and NO

The levels of TNF-*α* in cell culture supernatants were determined "in triplicate" by Quantikine M ELISA kit (R&D System, Minneapolis, MN) according to manufacturer's instructions as described previously [6]. The lower limit of detection for TNF-*α* in this method is approximately 5.0 pg/ml. NO in the supernatants was assayed using the Griess reagent kit (Sigma) also in triplicate, and the data are presented as the mean ± SEM. All experiments for determination of TNF-*α* and NO in cell-supernatants were carried out at least twice.

Results

LPS Stimulation of the Mouse Macrophage Cell Line, RAW 264.7, Triggers Activation of mRNA and Protein Expression of LMP Subunits of the Proteasome

IFN-*γ* has previously been shown to alter significantly the level of expression of newly formed LMP subunits in immunoproteasomes [13, 14] of several carcinoma cell lines. It is also now well established that LPS induces IFN-*γ* synthesis in macrophages [18], and that exogenous IFN-*γ* synergizes with LPS to induce production of NO and other proinflammatory cytokines in mouse macrophages [18]. Consequently, we sought to determine the extent to which LPS, like IFN-*γ*, would modify the composition of macrophage immunoproteasome protease-specific LMP subunits. For these studies, RAW 264.7 macrophages were treated with medium (control) or LPS for 4, 8, 12, or 24 h. RNA was then extracted and subjected to real-time PCR. The data in Fig. 1a provide evidence that relative levels of expression for LMP2, LMP7, and MECL-1 macrophage proteasomal subunit-specific mRNA were increased 2–4-fold, 4–12 h after LPS treatment relative to control untreated cells, while there was little detectable effect in the level of expression of X, Y, or Z mRNA. Western blot results of the proteasomal protein subunits obtained from LPS-treated RAW 264.7 macrophage-like cells are shown in Fig. 1b. Proteasomes of RAW 264.7 cells had predominantly X, Y, and Z at 0 time and indicate changes at the significant levels of proteasome protease subunit expression. Significant increases in protein expression levels were observed at 10 h for LMP2 and MECL-1 and at 20 h for LMP7. Collectively, LPS treatment of cells upregulates the proteasomal LMP inducible subunits in RAW 264.7 cells, at both the mRNA and the protein levels.

LPS Stimulation of RAW 264.7 Macrophages Modulates Proteasome Activities and Regulates NO Production Exogenously

Exogenous IFN-*γ* stimulation of HeLa cells in vitro has been reported to trigger the synthesis and insertion of LMP subunits into newly synthesized proteasomes [13–15]. Such newly formed proteasomes have also been reported to manifest enhanced chymotrypsin-like activity [13–15]. Our own results, shown in Fig. 1, provide evidence that LPS also has the capacity to induce expression of LMP subunits, and led us to explore how LPS treatment alters the enzymatic activities of the LMP subunits, thereby altering the chymotrypsin-like or post-glutamate activities of macrophage proteasomes. For these studies, RAW 264.7 cells

were treated with or without LPS (10 ng/ml) for up to 32 h, followed by incubation with a synthetic peptide substrate covalently conjugated to luciferin to quantify the chymotrypsin-like and post-glutamate enzymatic activities of the proteasome as described previously [19, 20]. Briefly, cells are permeabilized by digitonin to allow for the entrance of peptide substrates which are acted upon by the proteasome, and aminoluciferin is released from the cells, which can then be quantified using a luciferase-based luminescent assay (see "Materials and methods"). As shown in Fig. 2a, stimulation of control untreated macrophages and macrophages treated with LPS produced a significant increase in induced chymotrypsin-like activity by 4 h in the latter cells relative to controls, and the magnitude of this increase, compared to controls, remained relatively constant throughout the 32-h period of stimulation. In contrast, stimulation of macrophages with LPS resulted in decreased relative levels of post-acidic activity of the proteasome as compared to controls (Fig. 2b). Interestingly, the post-acidic activity in controls increased several fold over this time frame, although the reasons for this are currently not clear. Overall, the ratio of the chymotrypsin-like/post-acidic activities of LPS/control rose significantly (4.4-fold) at 32 h (Fig. 2c). The LPS-induced increase in chymotrypsin-like activity and decrease in post-acidic activity of macrophage proteasomes demonstrate the profound effects that LPS has on the functional activity of macrophage proteasomal proteases. Trypsin-like activity was also measured in this experiment and it was upregulated at the same time as the chymotrypsin-like activity (data not presented). In contrast, this activity was not affected by LPS treatment, compared to the untreated controls.

To determine the consequences of LPS- and/or IFN-γ-induced altered macrophage proteasome enzymatic activities on NO or TNF-α production, we first measured levels of NO and TNF-α in culture supernatants of RAW 264.7 cells treated for up to 36 h with or without LPS alone, IFN-γ alone, or LPS plus IFN-γ (Fig. 3). Upon stimulation with LPS and IFN-γ alone we observed an increase in NO in 24–36 h. However, with LPS plus IFN-γ, we observed the expected synergistic increase in levels of NO generated that was initially detected at ~12 h, and increased steadily up to 36 h (Fig. 3a). Thus, the kinetics of increased NO release approximates that of LPS-induced alterations in LMP subunits levels and chymotrypsin- and post-acidic activities presented in Fig. 2.

To determine more specifically which activities of the proteasome are essential for the LPS-dependent increases in macrophage production of TNF-α, we used lactacystin, which primarily inhibits chymotrypsin-like activity of the proteasome at low concentrations [8], as well as two additional selective inhibitors of the proteasome, NC-005 and NC-001 [18], to block the chymotrypsin-like and post-acidic activities, of the proteasome, respectively. NC-005 is a Nac-methyl-Tyr-Phe-Leu-epoxyketone, while NC-001 is an Ac-Ala-Pro-nLeu-Leu-epoxyketone. These inhibitors are the most specific inhibitors of the respective active sites available and have been reported to function to inhibit the primary target active site by >90%, and produces <10% decrease in activity of other proteasome-sites in vitro [21] in human cells.

We first undertook these studies to confirm the relative specificity of these inhibitors in RAW 264.7 cells (Fig. 4). At low concentrations, lactacystin (up to 2.5 μM) preferentially inhibited the chymotrypsin-like activity, but at concentrations of 10–20 μM it inhibited the trypsin-like activity (Fig. 4a). Similarly, NC-005 preferentially inhibited the chymotrypsin-like activity induced by LPS; at higher concentrations, however, this inhibitor also blocked trypsin-like and post-acidic activities (Fig. 4b). As expected, NC-001 preferentially inhibited post-acidic activity, with little effect on trypsin-like or chymotrypsin-like activity at any of the concentrations tested (Fig. 4c). Our findings confirmed previous results on the specificity of these inhibitors [8, 18].

In the data shown in Fig. 5, we have compared the relative inhibitory activities of lactacystin, NC-005, and NC-001 on LPS-induced TNF-α secretion in RAW 264.7 cells. Lactacystin failed to block TNF-α secretion at 1 μM, consistent with our previous observation [6, 8], while the chymotrypsin-specific inhibitor, NC-005, inhibited LPS-induced TNF-α secretion at a concentration of only 0.1 μM. NC-001, a selective post-glutamase inhibitor, inhibited LPS-induced TNF-α < 20% at most of the concentrations tested. Importantly, the extent to which LPS-induced TNF-α production was inhibited by NC-005 continued to increase at concentrations well beyond the concentration our data suggest are required to produce maximal inhibition of chymotrypsin-like enzymatic activity. At these higher concentrations, NC-005 also demonstrated dose-dependent inhibition of trypsin-like and post-acidic proteasomal activities, suggesting that these proteasome protease enzymatic activities may be important to TNF-α production. Since NC-001, which inhibits caspase-like proteasomal enzymatic activity, only modestly inhibited TNF-α production, it would appear that NC-005 exerts its inhibitory effect on LPS-induced TNF-α production by inhibiting both chymotrypsin- and at higher concentrations, trypsin-like proteasomal activities, and that post-acidic activity is modestly important. At low concentrations, NC-005 acts primarily through its inhibitory effect on chymotrypsin activity. At higher concentrations of NC-005, after chymotrypsin is maximally inhibited, dose-dependent inhibition of trypsin-like activity results in further inhibition of LPS-induced TNF-α production. At low concentrations of NC-005 and NC-001 all cells died within 4–8 h. Thus, blocking all three proteasome-protease sites leads to cell death.

We have previously shown that LPS-induced TNF-α is inhibited <25% by 5 μM lactacystin pretreatment of mouse macrophages conditions that result in >90% inhibition of IL-1, IL-12, and iNOS [8]. In contrast, TNF-α production in response to CpG-DNA is blocked by 5 μM lactacystin [22] suggesting that lactacystin primarily affects TNF-α induced by the MyD88-dependent pathway. This suggests the possibility that LPS-induced TNF-α is not totally dependent on the MyD88 pathway, but also is induced by the TRIF-dependent pathway or through some other signaling pathway. Therefore, we next sought to determine the effect of 5 μM lactacystin pretreatment on a ligand that induces TNF-α solely via the endosomal TRIF pathway; poly I:C (double-stranded RNA) was used for this purpose [23]. Like LPS, TNF-α produced in response to poly I:C was not significantly inhibited by pretreatment of cells with 5 μM lactacystin (Fig. 6). At higher concentrations, however, lactacystin inhibited poly-IC-induced TNF-α. Thus, we conclude that a relatively low dose of lactacystin does not block production of TNF-α induced by the TRIF pathway (e.g., 5 μM lactacystin fails to inhibit the TNF-α response to poly I:C), but does block CpG DNA-induced production of TNF-α induced solely via the MyD88 pathway. Since LPS-mediated responses employ both the MyD88 and the TRIF pathways for TNF-α production, low doses of lactacystin fail to inhibit LPS-induced TNF-α production totally because the MyD88 pathway is inhibited, but the TRIF pathway is still functional. At higher concentrations, however, lactacystin inhibits both the MyD88 and the TRIF pathways, resulting in almost complete inhibition of LPS-induced TNF-α production.

It is well known that activation of NF-κB requires the processing of the p105 processing and the degradation of IκB by the proteasomal proteases [24]. Treatment of cells with lactacystin has been shown to inhibit TNF-induced NF-κB activation by accumulating IκB-α, and preventing the translocation of NF-κB to the nucleus. However, since LPS-induced TNF-α gene expression and protein expression in macrophages was not greatly affected by low doses lactacystin, we had proposed that several LPS-induced signaling mediators, besides NF-κB, may be likely substrates for the proteasome [8]. Therefore, we sought to determine if other signaling proteins may be affected similarly by proteasome inhibition. We determined the time-course and consequence of lactacystin pretreatment on RAW 264.7 macrophages (with predominantly X, Y, and Z subunits) on LPS-induced signaling

mediators. Macrophages were first treated in vitro with LPS for 0, 5, 15, 30 min, 1, 2, and 4 h. Select proteins such as IκB-α, IRAK-1, and TRAF6, important for induction of TNF-α and NO; and phosphorylated-IRF3 (P-IRF3), and phosphorylated STAT-1 (P-STAT-1), important for induction of NO were monitored at various time points. Our findings indicate that time course of degradation of the various signaling proteins is different. Then, after determining the optimal time for analyzing the effect on each signaling protein, cells were pretreated with medium or lactacystin for 1 h (low dose 2.5 μM), and then treated with LPS or medium at indicated times 0–4 h. Protein extracts from these cell cultures were analyzed by Western Blotting, using specific antibodies to IκB-α, IRAK-1, P-IRF3, P-STAT-1, and TRAF6 (Fig. 7). Our findings with RAW 264.7 macrophages were as follows: IκB-α was present at zero time, but was rapidly degraded within 15–30 min after LPS treatment 200 ng; however, in cells first treated with lactacystin, followed by LPS 25 ng, IκB-α was not degraded by the proteasome, resulting in some accumulation of IκB. IRF3 was phosphorylated by LPS treatment at time points between 30 min and 2 h, and lactacystin pretreatment of cells resulted in greater accumulation of LPS-induced P-IRF3 at 1–2 h. STAT-1P (S727) was also activated by LPS, with detectable accumulation at 30–60 min; lactacystin pretreatment resulted in increased levels at 30 min, and longer persistence (e.g., 2 h). Levels of LPS-induced IRAK-1, present at time 0, gradually diminished after LPS stimulation, with discernible decreases between 1 and 4 h. Lactacystin pretreatment of macrophages markedly suppressed this LPS-mediated diminution of levels of IRAK-1 at 2 h. TRAF6 was also present at time 0, but was down-regulated after LPS treatment of 4 h, but accumulated in response to a lactacystin pretreatment followed by LPS (Fig. 7). Although the relative intracellular levels all of these signaling proteins were sensitive to LPS, the concentration of some increased while others decreased following LPS stimulation. The time required to detect an initial response, and the duration of the response, also differed among the different proteins. Regardless of the kinetics, or direction of the changes in levels of these signaling proteins in response to LPS, however, levels of each of these proteins, e.g., P-IκB, P-IRF3, STAT-1P, TRAF6, and IRAK1, increased when LPS-stimulated RAW 264.7 cells were pretreated with lactacystin (low dose, 2.5 μM, affects X, and LMP7 if present). These findings reveal that several LPS-induced signaling proteins are degraded by the proteasome and these proteins can be either negatively or positively regulated by proteasome proteolytic-sites.

Discussion

It is well established that NO and TNF-α play a critical role in the development of innate immunity during septic shock, and inflammation induced by LPS [25–29]. LPS-induced NO and TNF-α in RAW 264.7 macrophages are differentially regulated by the proteasome. In this article, our results have provided strong evidence to support the conclusion that the proteolytic subunits of the proteasome play an active role in cells to determine the LPS-induced response in macrophages, as suggested previously [6–9]. In this respect, we have previously shown that the proteasome is a central regulator of LPS-induced gene expression and inflammation in macrophages [6–9]. Proteasomes have previously been shown to play a passive role and the general conception is that any signaling protein that is ubiquitinated is degraded by the proteasome. We have now demonstrated that the proteasomes and immunoproteasomes have specific functions. Unlike most cell types that contain only X, Y, and Z subunits, murine macrophages have been shown to possess all six subunits of the proteasome (X, Y, Z, LMP2, LMP7, and MECL-1) [30]. Our study demonstrates for the first time that RAW 264.7 macrophages are unique and initially contain only constitutive proteasomes, but no immunoproteasomes. We have established that LPS-induced TNF-α (4 h) in RAW 264.7 macrophages is not dependent on the immunoproteasomes of the macrophages, while LPS-induced NO occurs much later, and is dependent on the immunoproteasomes.

In this study, results of real-time PCR studies demonstrate that levels of expression of the LMP2, LMP7, and MECL-1 proteasomal subunit genes are increased by treatment of RAW 264.7 macrophages with LPS. These changes in the proteolytic subunits of newly formed proteasomes correlate with increases in the chymotrypsin-like activity and decreases in the post-acidic activity of proteasomes from macrophages treated with LPS. Our findings suggest that by treatment of RAW 264.7 cells with LPS, proteasome subunits predominantly X, Y, and Z are replaced by LMP7, LMP2, and MECL-1 in proteasomes. Results of our experiments provide direct evidence that, after replacement of X, Y, and Z, with LMP7, LMP2, and MECL-1 subunits, there are changes in the specificity of proteolytic profiles. These results are consistent with previous results observed with IFN-γ, in that replacement of X and Z with LMP7 and MECL-1, respectively, results in increased chymotrypsin-like and trypsin-like activities. In contrast, replacement of Y with LMP2 serves to decrease post-glutamase activity [15].

We have also partially defined, for the first time, the extent to which each of the proteolytic activities (X, Y, and Z) of the proteasome contributes to LPS-induced TNF-α secretion in macrophages. We accomplished this using the selective inhibitors NC-005 and NC-001 [21]. We have shown that, relative to lactacystin, NC-005 is a significantly more effective inhibitor of chymotrypsin-like activity of the proteasome. We have also demonstrated that inhibition of either chymotrypsin-like activity (with low concentrations of lactacystin or NC-005) or post-acidic activity alone (with NC-001) did not result in a detectable decrease in production of TNF-α in RAW 264.7 macrophage in response to LPS. In contrast, inhibition of all three proteasomal activities, achieved with high concentrations of NC-005, markedly reduced LPS-induced TNF-α. The viability of cells in tissue cultures was not detectably affected in 4 h by lactacystin or other proteasome inhibitors as assessed by the MTT assay. These results suggest that the chymotrypsin- and trypsin-like proteolytic activities of subunits of X and Z (LMP subunits are not present at 4 h) must be simultaneously suppressed to inhibit LPS-induced TNF-α production through the proteasome (Table 1).

Our data demonstrate that LPS-induced degradation of IκB (resulting in increased NF-κB activation) is observed at 15–30 min, whereas induction of P-IRF3 and P-STAT1 occurred between 30 min and 2 h. Blockage of macrophage proteasomal chymotrypsin-like activity with low level lactacystin leads to increased levels of P-IRF3 and P-STAT1, and to a decrease in NF-κB activity, in LPS-stimulated macrophages relative to control cells. Consequently, LPS-induced NO production would be predicted to be highly susceptible to proteasome inhibitors, because both NF-κB activation and P-IRF3 activation are known to be required for LPS-induced NO [5]. In contrast, while generation of TNF-α in LPS-stimulated macrophages has been most often associated with activation of NF-κB following degradation of IκB-α [31, 32], results of other studies have provided evidence that generation of TNF-α in LPS-stimulated macrophages can be induced in an NF-κB-independent fashion [33, 34]. Recently, degradation of IκB-β, instead of IκB-α, has also been implicated in the induction of TNF-α induction in response to LPS [35]. It is possible that when IκB is not degraded (as a result of presence of lactacystin), thus precluding NF-κB activation, compensatory signaling occurs that enables NF-κB-independent TNF-α induction. Other mechanisms for TNF-α induction involving the mitogen-activated protein kinases (MAPK) may also be operating in macrophages. We have also demonstrated a role of MAPK, ERK, and JNK in LPS-induced TNF-α expression using RAW 264.7 cells [36].

Currently, two signaling pathways have been established to be required for the induction of cytokines in mouse macrophages in response to LPS as shown in Fig. 8. LPS-induced cytokines are known to be dependent, in part, on the MyD88 pathway and/or the MyD88-independent TRIF/TRAM pathway, both of which are triggered through LPS-dependent

activation of TLR4. We have recently shown that CpG DNA-induced TNF-α, which occurs almost exclusively through activation of the MyD88-dependent pathway, can be inhibited by a relatively low dose of lactacystin [22], whereas poly I:C-induced TNF-α (triggered by TLR3, and a solely a TRIF-dependent pathway) requires a much higher dose of lactacystin. These experiments suggest the possibility that proteasome inhibitors may ultimately be used to therapeutically block selective agonist-induced pathways depending on the dose of the inhibitor.

Based upon current and earlier results, we propose a model in which LPS interaction with RAW 264.7 macrophages leads to activation of the proteasome from its basal state (predominantly X, Y, and Z) to one characteristic of the immunoproteasome LMP subunits. Over time, the relative composition and distribution of the three proteasome proteolytic activities changes, along with their specific proteolytic cleavage profiles. The X, Y, and Z protease subunits are likely to be important for generating the early, LPS-induced, TNF-α response in RAW 264.7 cells (at 4 h) because the LMP subunits are not present at 4 h. The development of the immunoproteasome phenotype in macrophages in response to LPS results in significant changes in the activity profiles of transcription factors, and selective degradation of mediator signaling proteins, thus affecting levels of a number of inflammatory mediators, including the late stage mediator, NO as discussed in the accompanying article. In this regard, the profile of cytokine responses of the macrophage to LPS can be significantly modified by using selective proteasome inhibitors. Inhibiting one NF-κB pathway may be activating the P-IRF3 pathway. In addition, such experimental approaches potentially provide insights into the unique contribution of the cellular proteasome for regulation of synthesis and secretion of inflammatory mediators.

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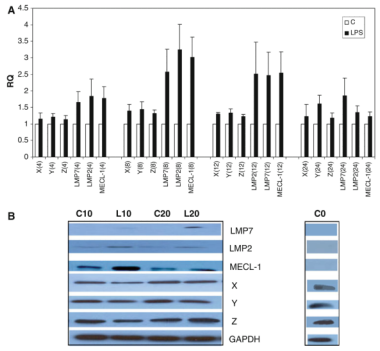

Fig. 1.

LPS activates gene and protein expression of the LMP subunits of the proteasome. **a** Real-time PCR analysis. RAW 264.7 cells were treated medium (DMEM) or LPS for 0, 4, 8, 12, and 24 h. For the isolation of RNA and protein, cells were plated at a concentration of 3×10^6 cells/60 mm plate. Three hours after plating, fresh media was added containing LPS at a final concentration of 25 ng/ml. For mock-treated cells, an equivalent volume of 0.91% saline was added in place of the LPS. Cells were extracted and analyzed by real-time PCR and Taqman probes were used for these analyses. The vertical axis (RQ, relative quantity) was determined by comparison of the expression levels of the LPS-versus medium-treated samples harvested at the same time. *Error bars* denote standard deviation obtained with data from three separate experiments. **b** LPS treatment of macrophages causes a decrease in X, Y, and Z subunits, and a corresponding increase in LMP7, LMP2, MECL-1 subunits of the proteasome. RAW 264.7 cells were treated with medium for 0 time (C0), medium or 100 ng/ml LPS for 10 or 20 h (C10, control at 10 h; C20, control at 20 h; L10, LPS at 10 h; L20, LPS at 20 h). The cell lysate was purified by SDS-PAGE and subjected to Western blotting using antibodies to X, Y, Z, LMP7, LMP2, and MECL-1. GAPDH was used as a standard protein. Subunits X, Y, and Z were present predominantly at 0 time point in RAW 264.7 cells. We also observed a band above LMP2 at 0 time also (data not presented)

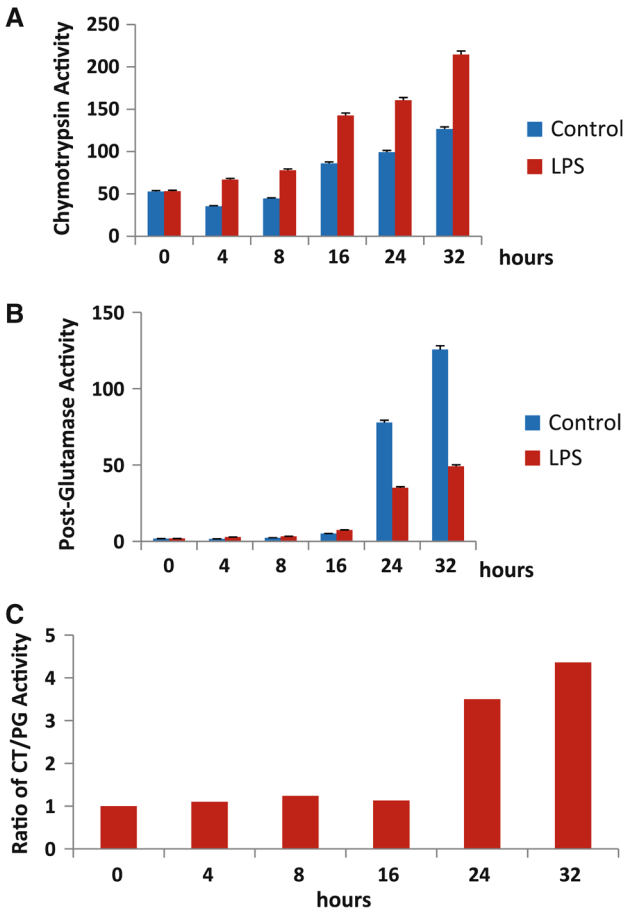

Fig. 2.

LPS activates the inducible chymotrypsin-like, and inhibits the post-glutamase activity, of proteasomes. The effect of LPS treatment on chymotrypsin-like activity of the proteasome, in macrophages was investigated. RAW 264.7 cells were treated with or without LPS (10 ng/ml) for 0, 4, 8, 16, 24, and 32 h. **a** Total chymotrypsin-like activity of proteasomes. **b** Post-glutamase activity of the proteasomes. **c** The ratio of LPS chymotrypsin/control chymotrypsin to LPS post glutamase/control post glutamase

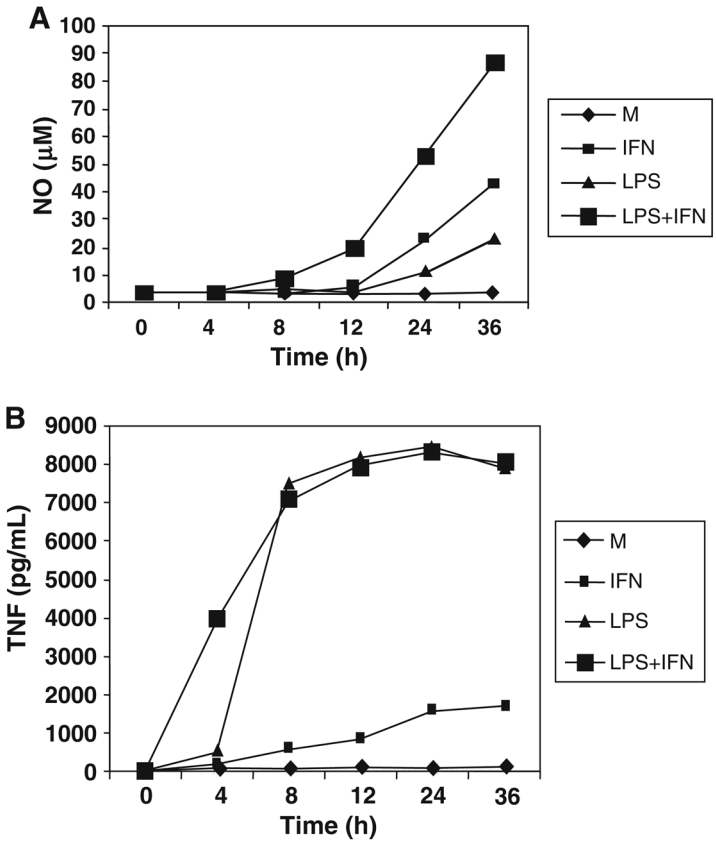

Fig. 3.

LPS and IFN-*γ* have a synergistic effect on NO release and TNF-*α* induction. **a** The effect of LPS treatment on NO release in RAW 264.7 cells. RAW 264.7 cells were treated with medium, LPS alone (10 ng/ml), LPS (10 ng/ml) plus IFN-*γ* (50 U/ml), and IFN-*γ* (50 U/ml) alone. NO was analyzed by Greiss reagent kit. **b** RAW 264.7 cells were treated as described in the legend of (**a**), except that the supernatants were analyzed for TNF-*α* by ELISA

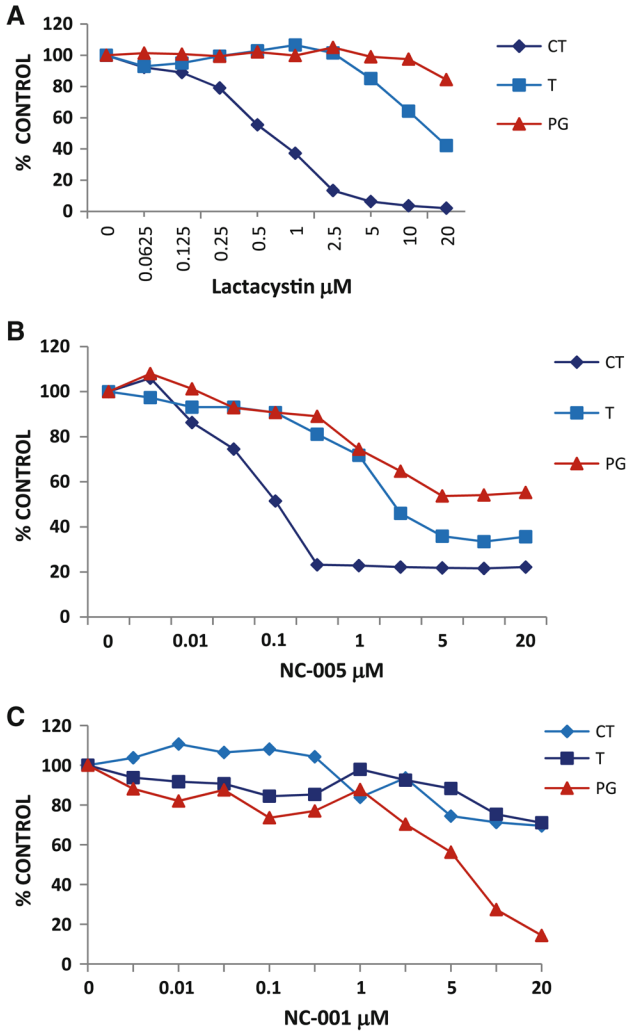

Fig. 4.

Effect of lactacystin, NC-005, and NC-001 treatment on the three proteasomal proteases. RAW 264.7 cells were treated with medium or various concentrations (μM) of the inhibitors lactacystin, NC-005, and NC-001 for 30 min, and the proteasomal activities were monitored using the Proteasome-Glo assays in a luminometer. Results represent mean of two separate experiments. **a** Lactacystin, **b** NC-005, and **c** NC-001

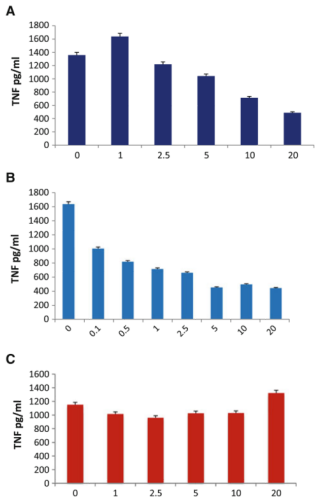

Fig. 5.

Effect of lactacystin, NC-005, and NC-001 treatment on the LPS-induced TNF-*α* in macrophages. RAW 264.7 cells were pretreated with various concentrations of the inhibitors lactacystin, NC-005, or NC-001 for 1 h, followed by stimulation with LPS for 4 h. Supernatants were collected and assayed for TNF-*α* using an ELISA assay. The *error bars* denote standard error of mean for two independent experiments. **a** Lactacystin (μM), **b** NC-005 (μM), and **c** NC-001 (μM)

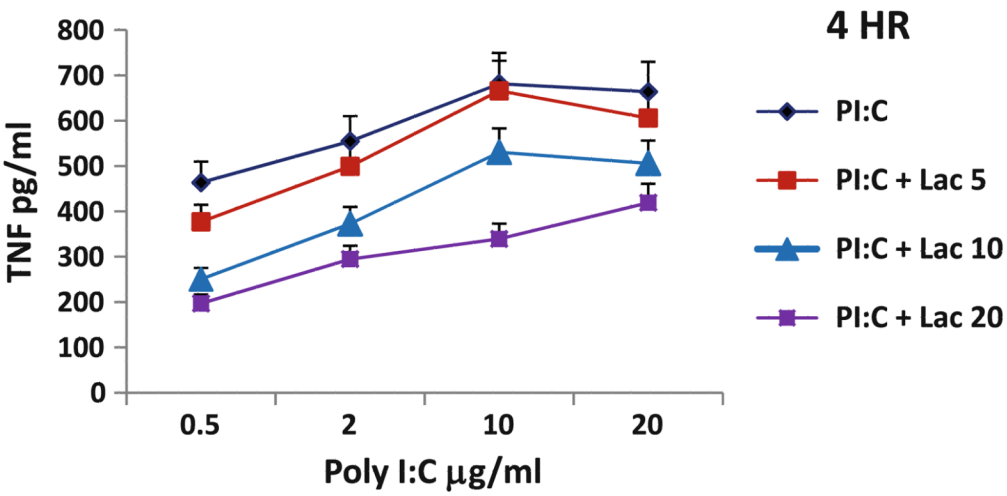

Fig. 6.

Effect of lactacystin, on the poly I:C-induced TNF in macrophages. RAW 264.7 cells were pretreated with various concentrations of the inhibitor lactacystin, for 1 h, followed by stimulation with various doses of poly I:C (0.5, 2, 10, and 20 µg/ml) for 4 h. Supernatants were collected and assayed for TNF-*α* using an ELISA assay. The *error bars* denote standard error of mean for two independent experiments

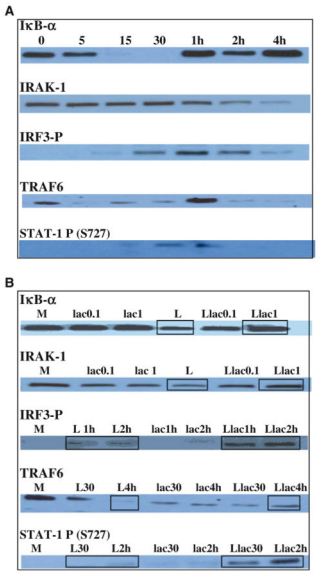

Fig. 7.

Time course and effect of lactacystin treatment on the LPS-modulated signaling proteins. RAW 264.7 cells were pretreated with the proteasome inhibitor, lactacystin (lac, low dose 2.5 μM), or medium for 1 h, and then treated with LPS (L, 200 ng/ml) or medium (M) at indicated times 0–4 h. LPS (L, 25 ng/ml and lactacystin was used for I*κ*B determination and L200 ng/ml was used for NF-*κ*B time course experiment). Proteins in lysates were analyzed by Western analysis. **a** The first Western analysis for each protein shows the time course after LPS treatment at indicated time, **b** the second Western analysis shows the effect of lactacystin pretreatment on the LPS-induced modulation of signaling proteins. *L* LPS, *lac* lactacystin, and *Llac* LPS plus lactacystin. Compare L4h and Llac4h for TRAF6; L30, L2h, and Llac30, Llac2 for P-STAT-1; L1h, L2h, and Llac1, Llac2 for P-IRF3; L and Llac1 for I*κ*B-*α* and IRAK1 at 15 min and 2 h, respectively. Experiments were carried out at optimal time points for each signaling protein

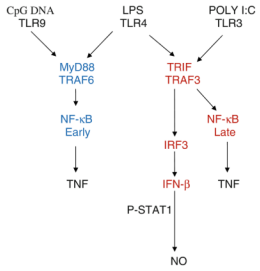

Fig. 8.

Two pathways for the LPS-induced cytokines in macrophages. LPS-induced cytokines are triggered by TLR4 and are dependent in part on the MyD88 pathway and/or the MyD88-independent TRIF pathway. Low-dose lactacystin pretreatment of cells blocks primarily the LPS-induced and the CpG-DNA-induced MyD88 pathway, because it leads to the inhibition of the activation of NF-κB pathway, thus causing a partial block in the gene expression of TNF-α, while a higher-dose lactacystin is required for inhibition of the LPS-induced and poly I:C-induced TRIF pathway that leads to the expression of TNF-α

Table 1

Effect of inhibitors on proteasomal subunits in cells

Lactacystin		
Low dose	X	LMP7
High dose	All subunits	
NC-005		
Low dose	X	LMP7
High dose	Z	MECL-1
NC-001		
High dose	Y	LMP2