Dynamic Modulation of Innate Immune Response by Varying Dosages of Lipopolysaccharide (LPS) in Human Monocytic Cells*

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Background: Super-low-dose endotoxin causes mild but significant pro-inflammatory skewing.
Results: Inhibition of GSK3 or activation of CREB ablates preferential induction of pro-inflammatory genes by super-low-dose LPS.
Conclusion: GSK3 is necessary for pro-inflammatory gene induction in response to super-low-dose LPS, acting by activating FoxO1 and suppressing CREB.
Significance: Persistent, non-resolving inflammation is a characteristic of many chronic diseases, and this study points toward potential therapeutic targets.

Innate monocytes and macrophages can be dynamically programmed into distinct states depending upon the strength of external stimuli. Innate programming may bear significant relevance to the pathogenesis and resolution of human inflammatory diseases. However, systems analyses with regard to the dynamic programming of innate leukocytes are lacking. In this study, we focused on the dynamic responses of human promonocytic THP-1 cells to lipopolysaccharide (LPS). We observed that varying dosages of LPS differentially modulate the expression of selected pro- and anti-inflammatory mediators such as IL-6 and IL-33. Super-low dosages of LPS preferentially induced the pro-inflammatory mediator IL-6, while higher dosages of LPS induced both IL-6 and IL-33. Mechanistically, we demonstrated that super-low and high doses of LPS cause differential activation of GSK3 and Akt, as well as the transcription factors FoxO1 and CREB. Inhibition of GSK3 enabled THP-1 cells to express IL-33 when challenged with super-low dose LPS. On the other hand, activation of CREB with adenosine suppressed IL-6 expression. Taken together, our study reveals a dynamic modulation of monocytic cells in response to varying dosages of endotoxin, and may shed light on our understanding of the dynamic balance that controls pathogenesis and resolution of inflammatory diseases.

Increasing evidence from both clinical and laboratory studies indicate that innate immune cells can be programmed into diverse states with varying degrees of pro- and anti-inflammatory phenotypes (1, 2), with consequences for host defense and inflammation (3). Despite its clinical relevance, mechanistic studies with regard to innate cell programming are scant. To fill this critical void, we have examined the dynamic responses of human THP-1 monocytic cells challenged with a model stimulant, bacterial endotoxin lipopolysaccharide (LPS), a major component of the cell walls of Gram-negative bacteria. It is a ubiquitous environmental toxin (4, 5). High doses of LPS are responsible most prominently for septic shock (6, 7). On the other hand, low doses of circulating LPS are common in chronic disease settings, and may contribute to the development of persistent, low-grade, non-resolving inflammation (8–10). The distinct pathological effects of varying dosages of LPS may reflect differential programming of innate leukocytes.

At the biochemical level, LPS is recognized by the Toll-like receptor (TLR) 4. High dosages of LPS can activate multiple pathways capable of inducing both pro- and anti-inflammatory genes (11, 12). Of particular note, the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway induced by high-dose LPS serves as a negative mechanism to down-regulate inflammatory processes, and is also responsible for the expression of anti-inflammatory mediators, partly through the activation of CREB (13). In contrast, super-low dose LPS fails to induce anti-inflammatory mediators, and preferentially induces low-grade inflammatory mediators (14). Studies from other groups in other cellular systems indicate that GSK3 and Akt may form a mutually inhibitory circuit (12, 15). Akt was shown to inhibit the function of GSK3 (16), while GSK3 may in turn inhibit Akt.

Other studies also suggest that GSK3 is a critical molecule involved in various inflammatory processes in vitro and in vivo. Inhibition, knockdown, or knock-out of GSK3 has been shown to inhibit the expression of pro-inflammatory mediators in response to LPS (17, 18). Inhibitory phosphorylation of GSK3 mediated by PI3K/Akt is necessary for the protective induction of anti-inflammatory IL-10 following ischemia/reperfusion injury (19), which triggers inflammation through TLR4. Pharmacological activation of PI3K also suppresses toxicity-induced apoptosis in neurons in a GSK3-dependent fashion (20).

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2 The abbreviations used are: LPS, lipopolysaccharide; TLR, Toll-like receptor; FoxO1, forkhead box O1; CREB, cAMP response element-binding protein; IL, interleukin; TNF, tumor necrosis factor.
These findings suggest that the overall role of GSK3 in the LPS response consists of the promotion of pro-inflammatory cytokine production and suppression of anti-inflammatory mediators (21, 22).

Downstream, GSK3 appears to play a role in the regulation of the transcription factors fork head box O1 (FoxO1) and cAMP response-element-binding protein (CREB). The anti-inflammatory effects of Akt on TLR4 signaling appear to be mediated through FoxO1 (23), suggesting that GSK3 may be important for the activation of FoxO1. GSK3 can potently suppress CREB (24). Recently, it was shown that the anti-inflammatory effects of JAK3 in the context of TLR4 stimulation are exerted by suppressing GSK3, which enables increased CREB activity (13). In cells stimulated by the TLR2 ligand zymosan, the activation status of CREB corresponds closely with heightened production of IL-10 (25). Suppression of CREB by GSK3 may result in the pro-inflammatory skewing of immune responses. Circumstantially, genes suppressed by GSK3 tend to be regulated by CREB (26), and the suppression of IL-10 production by interferon-γ is due to its activation of GSK3 and ensuing suppression of CREB (15). CREB seems to oppose the pro-inflammatory effects of GSK3 by augmenting anti-inflammatory gene expression.

Akt (also known as protein kinase B) is regulated by the PI3K and mammalian target of rapamycin (mTOR) pathways (12, 27). Activation of Akt by rapamycin blunts the sensitivity of pro-inflammatory genes to LPS and increases CREB activity (28), and mTOR/Akt signaling additionally suppresses inflammation by inactivating the transcription factor forkhead box O1 (FoxO1) (23). The role of FoxO1 in TLR4 signaling is predominantly pro-inflammatory. Overexpression of FoxO1 results in increased expression of TLR4 and pro-inflammatory cytokine genes in response to LPS, and knockdown or removal blunts the ability of TLR4 stimulation to induce these genes (29). FoxO1 is also necessary for pro-inflammatory cytokine production by memory T cells (30), pointing to a generalized role for FoxO1 in the regulation of inflammatory gene transcription. Blocking of inhibitory phosphorylation of FoxO1 results in increased pro-inflammatory cytokine production by macrophages upon challenge with a TLR2 ligand (31). Defective Akt activation results in increased FoxO1 activity and pro-inflammatory cytokine production (23). The broadly anti-inflammatory effects of PI3K signaling in TLR4 stimulation (32) can thus be ascribed to the tandem activation of CREB and suppression of FoxO1 by Akt, but the competition between GSK3 and Akt has not been well characterized in the context of LPS challenge.

In this study, we tested the hypothesis that monocytes may be dynamically programmed by varying dosages of LPS through the competing circuits of GSK3 and Akt. To test this, we examined the expression profiles of selected pro- and anti-inflammatory mediators in human monocyte THP-1 cells challenged with varying dosages of LPS. We observed distinct expression patterns of IL-6 and IL-33 in THP-1 cells treated with sub-low or high dose LPS, and this pattern correlated with distinct activation statuses of GSK3 and Akt, as well as the transcription factors FoxO1 and CREB. We further demonstrated that pharmacological intervention by either GSK3 inhibition or CREB activation is sufficient to reverse the pro-inflammatory skewing characteristic of cells stimulated by super-low-dose LPS. Our study reveals a unique network responsible for the dynamic programming of innate leukocytes by varying dosages of bacterial endotoxin.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—** THP-1 cells were grown in RPMI 1640 (Invitrogen 11875–119) supplemented with 2 mm l-glutamine, 1% penicillin/streptomycin (Invitrogen 15140–122), and 1% fetal bovine serum (FBS) (Seradigm 1600–500). Before performing experiments, cells were seeded overnight at a density of 10⁶ cells/ml in RPMI containing 2 mm l-glutamine, 1% penicillin/streptomycin, and 1% FBS. LPS from *Escherichia coli* O114:B4 (Sigma L2630) was dissolved in PBS. The GSK3 inhibitors SB216763 (Sigma S3442) and indirubin-3'-oxime (Sigma I0404) were dissolved in DMSO. Adenosine (Sigma A4036) was prepared in ddH₂O.

**Real-time PCR—** RNA was extracted using TRIzol (Invitrogen 15596–026). Samples were then treated with 20% chloroform by volume at room temperature for 3 min, followed by centrifugation at 12,000 rpm for 15 min at 4 °C. Aqueous phase was transferred to new tubes and isopropanol (50% initial volume) was added, then samples were incubated for 10 min at room temperature. Samples were then centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was discarded. The RNA pellet was then washed with 75% ethanol in DEPC-treated water (100% initial volume), and centrifuged for an additional 10 min at 12,000 rpm, 4 °C. Supernatant was discarded and RNA pellets were resuspended in 30 μl of DEPC-treated water. DNA digestion was performed at 37 °C for 30 min (Invitrogen AM2222), followed by 5 min treatment with 85 °C to degrade DNase. 1500 ng of RNA was reverse-transcribed using a high-capacity kit (Invitrogen 4368813) at 37 °C for 2 h, followed by enzyme inactivation for 5 min at 85 °C. Real-time PCR was performed on a Bio-Rad CFX96 machine using a 2X SYBR Green mix (Bio-Rad 172-5271); the PCR protocol was denaturation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Primers for human IL-6 (F 5'-GGCCACTTCCTCTT-TCGAAACGAA, R 5'-AGTGCTCTTTGCTGTCTTTCAGG), TNFα (F 5'-TCAATCGGCCGACATACGCT, R 5'-CAGGGCATTGATCCAAAGT), IL-10 (F 5'-TCTCTTGGTGGAGGACTTTAAGGGT, R 5'-CTGTCTGGGTCTTGGTTCA-CAC), FNα (F 5'-TGTCAGCCCGGCGGCTACTGC, R 5'-CGAATGAGGCTTCTCTCAG), and IL-33 (F 5'-GGAAAGGACAGCAGCAGTTC, R 5'-TAAGGCCAGAGCGAGCTTCATAA) were purchased from IDT. Readouts were analyzed by the ΔΔCQ method.

**Western Blots—** Whole-cell lysates were harvested using lysis buffer consisting of 2% SDS, 5% Tris-HCl, pH 6.8, and 10% glycerol, placed on ice for 20 min, boiled for 5 min, then centrifuged at 12,000 rpm for 4 min at room temperature for removal of intracellular debris. Protein concentration was assessed by Bradford assay. Protein samples were run on 10% acrylamide gels at 100 V, followed by transfer at 110 V before blocking in 5% milk in TBS-T. Antibodies against pCREB-S133 (Cell Signaling 9191) were used at a concentration of 1:4000 in 5% milk in TBS-T, and CREB (Cell Signaling 9197S), pFoxO1-S256 (Cell Signaling 9461S), FoxO1 (Cell Signaling 9454S), Akt (Santa Cruz Biotechnology sc-8312), GSK3β (Santa Cruz Biotechnol-
ogy sc-9166), and GAPDH (Santa Cruz Biotechnology sc-25778) at 1:1000. pAkt-S473 (Cell Signaling 9271S) and pGSK3/H9252-Y216 (Santa Cruz Biotechnology sc-135653) antibodies were diluted 1:4000 in 5% BSA in TBS-T.

**Statistics**—Statistical analysis by Holm-Sidak pairwise comparison or Student’s t test was performed using SigmaPlot 11 software (SigmaPlot) as detailed in the figure legends. Results were considered to be statistically significant at \( p < 0.05 \).

**RESULTS**

**Differential Regulation of Inflammatory Genes by LPS**—The phenomena of endotoxin tolerance and priming, in which stimulation with LPS alters the nature of the inflammatory response to a subsequent challenge, have been extensively documented (33–36). We therefore sought to characterize the differences between the inflammatory response to super-low (<1 ng/ml) and high (>10 ng/ml) doses of LPS. Stimulation of THP-1 cells with varying dosages of LPS for 4 h reveals that the pro-inflammatory genes IL-6 and tumor necrosis factor \( \alpha \) (TNF\( \alpha \)) are more sensitive to LPS than IL-33, as transcription of the latter is significantly up-regulated only by high doses of LPS (Fig. 1). In a separate experiment, closer analysis employing different statistical methods reveals that IL-6 transcription is mildly but significantly up-regulated by LPS doses as low as 50 pg/ml, while IL-33 is not (Fig. 2), suggesting that stimulation with super-low-dose LPS results in skewing of the TLR4 response toward pro-inflammatory gene expression.

**Mechanistic Effects of Different LPS Dosages**—The signaling kinases GSK3 and Akt are known to compete in the regulation of inflammation (22, 37). This led us to explore whether the differential regulation of pro- and anti-inflammatory genes by varying dosages of LPS could be explained by differential effects on signal transduction through GSK3 and Akt. Stimulation of THP-1 cells with 100 ng/ml LPS for 60 min resulted in robust phosphorylation of Akt at Ser-473, a marker of its activation (38). In contrast, super-low-dose LPS failed to increase, but rather decreased Akt-Ser-473 phosphorylation (Fig. 3A).

Our data suggest that GSK3 may be differentially modulated by super-low and high doses LPS. Given that GSK3 activation is facilitated by tyrosine 216 phosphorylation, we further probed the pY216 GSK3 levels in cells treated with super-low-dose LPS. As shown in Fig. 3B, 50 pg/ml LPS caused a rapid increase of pY216-GSK3 in THP-1 cells. Since Pyk2 is the upstream kinase responsible for the activating Tyr-216 phosphorylation of GSK3 (39), we proceeded to test the activation status of Pyk2 by Western blot. The tyrosine kinase activity of Pyk2 is inhibited by phosphorylation at Tyr-402 (40). We found that 50 pg/ml LPS caused a rapid and dramatic drop in the levels of inhibitory Tyr-402 phosphorylation of Pyk2, indicating an increase in its activity (Fig. 3C). In contrast, higher dose LPS (100 ng/ml) caused a robust elevation of Tyr-402 phosphorylation, indicating its inhibition (Fig. 3C). This correlates with our above finding that super-low dose LPS activates GSK3 through increasing its Tyr-216 phosphorylation.

Downstream of GSK3 and Akt lie two competing transcription factors: FoxO1 and CREB (13, 29). While FoxO1 contributes to the expression of pro-inflammatory mediators (23, 29), CREB is largely involved in the expression of anti-inflammatory mediators (25, 41). Pharmacological activation of Akt corresponds with suppression of FoxO1 (42), which, in light of GSK3 suppression by Akt (26), points to a connection between GSK3 and FoxO1 activity. Akt is also important for the activation of CREB (13, 15).

Since we observed opposing effects of super-low and high dose LPS on GSK3 and Akt, we further tested the activation...
status of FoxO1 and CREB. We found that treatment with 50 pg/ml LPS caused an increase in total FoxO1 proteins levels, while treatment with 100 ng/ml LPS led to a decrease in the total FoxO1 level (Fig. 4A). Since the decrease in FoxO1 may be attributed to its inhibitory phosphorylation by Akt at Ser-256 (43), we also tested the level of Ser-256 phosphorylation of FoxO1. As shown in Fig. 4, only the high dose LPS triggered FOXO1 phosphorylation at Ser-256, while super-low dose LPS had no effect. The effects of LPS on the activation of CREB followed an opposite pattern. Phosphorylation of CREB at Ser-133 results in its activation (24), and this phosphorylation was only induced by high-dose LPS, with low-dose LPS not triggering appreciable Ser-133 phosphorylation of CREB (Fig. 4B).

Inhibition of GSK3 Reverses Pro-inflammatory Skewing—In vivo studies indicate that GSK3 is a key kinase controlling chronic inflammation (12, 13, 44). Given our above findings, we tested whether selective inhibition of GSK3 may alter the inflammatory skewing of innate monocytes by super-low dose LPS. We first tested indirubin, a GSK3 inhibitor derived from natural compounds (18), and found that costimulation of THP-1 cells with 50 pg/ml LPS and 10 μM indirubin abolished the preferential IL-6 induction typically triggered by this dosage (Fig. 5A). Mechanistically, we observed that the suppression of IL-6 by indirubin corresponds with prevention of FoxO1 up-regulation (Fig. 5B). We observed that SB216763, a synthetic compound which potently and selectively inhibits GSK3 (45), sensitized IL-33 to super-low dose LPS, resulting in significant IL-33 induction by 50 pg/ml LPS (Fig. 5C). The effect of SB216763 on IL-33 correlated with increased CREB activation (Fig. 5D).

Activation of CREB Reverses Pro-inflammatory Skewing—We hypothesized that the pro-inflammatory effects of GSK3 may be caused by its inhibition of CREB. We tested this with adenosine, an Akt/CREB agonist known to exert anti-inflammatory effects in macrophages (46–48). Stimulation of THP-1 cells for 4 h with a range of LPS concentrations in the presence of 500 nM adenosine significantly suppressed the transcription of IL-6 and TNFα while promoting the transcription of IL-10 and IL-33 (Fig. 6, A–D). We further tested the levels of CREB in cell nucleus. Co-stimulation of cells with 50 pg/ml LPS and adenosine dramatically elevated the nuclear levels of pCREB-S133 (Fig. 6E). Taken together, our data reveal a potential competitive circuit responsible for the dynamic sensing of varying dosages of endotoxin by THP-1 cells (Fig. 7).

**DISCUSSION**

We investigated the pro-inflammatory skewing effects of super-low-dose LPS and found that the mild and preferential induction of pro-inflammatory genes by 50 pg/ml LPS. This phenomenon was correlated with preferential activation of GSK3 and inhibition of Akt. This was in contrast to the effect of high dose LPS, which induced both pro- and anti-inflammatory mediators. The proper regulation and resolution of inflammation requires activation of both pro- and anti-inflammatory mediators (49, 50), and the non-responsiveness of these resolving circuits to very low doses of LPS may be key to the pro-inflammatory skewing effects of these dosages, contributing to chronically dysregulated low-grade inflammation. By inhibiting GSK3, we were able to abolish the mild, preferential induction of pro-inflammatory IL-6 usually caused by super-low-dose LPS, and confer sensitivity to IL-33, which is not ordinarily induced by super-low dose LPS. The balanced expression of IL-6 and IL-33 could also be achieved by the addition of adenosine, an agonist capable of augmenting Akt and CREB. In the...
context of TLR4 activation, IL-6 and TNFα are both activated by FoxO1 (29), while CREB drives the transcription of IL-10 and IL-33 (15, 41). Our finding that adenosine potentiated the response of IL-10 and IL-33 to LPS while blunting induction of IL-6 and TNFα is therefore strongly suggestive of a competitive link between FoxO1 and CREB.

There is increasing appreciation for the role of GSK3 in inflammation (17, 44, 51). Inhibition of GSK3, in particular, is becoming an appealing strategy for the alleviation of inflammatory symptoms (51–53). Our finding that pharmacological inhibition of GSK3 reverses the pro-inflammatory skewing effects of super-low-dose LPS is thus in line with recent developments in the field. The inhibitors we employed are not necessarily selective for the β or α isoform of GSK3 (45). In particular, indirubin may have inhibitory effects on other kinases such as cyclin-dependent kinases (18). These pleiotropic effects may explain the failure of indirubin to significantly alter the expression of IL-33 (data not shown). Likewise, SB216763 may have differential effects on various isoforms of GSK3, and this may explain its selective effects on IL-33 instead of IL-6. Further study is necessary to determine the precise contribution of the different GSK3 isoforms to the pro-inflammatory skewing of monocytes. The anti-inflammatory effects of GSK3 inhibition appear to be mediated through differential activation of FoxO1 and CREB. This relationship is further buttressed by our observation that CREB activation by adenosine recapitulates the suppression of pro-inflammatory skewing in monocytes brought about by GSK3 inhibition.

We posit that super-low-dose LPS preferentially activates pro-inflammatory genes through selective activation of GSK3,
leading to heightened activation of FoxO1 at the expense of Akt (Fig. 7). Competitive inhibition between these kinases may be a compelling explanation for the phenomena herein described, as we were able to reverse it either by inhibiting GSK3 or activating Akt/CREB. This is consistent with previous observations that reveal an anti-inflammatory role of Akt in the context of TLR4 stimulation (12, 54).

We realize that further biochemical studies are needed to tease out the detailed mechanisms responsible for the competitive circuitry between GSK3 and Akt, as well as the consequences of this relationship. Missing links include membrane receptor combinations, distinct adaptor molecules (e.g. MyD88, TRIF, etc), and a myriad of kinases and phosphatases, as well as intracellular trafficking of signaling molecules responsible for the sensing of varying doses of LPS. Nevertheless, this study is among the first to elucidate a fundamental principle and a key functional motif that may be responsible for the dynamic balance of pro- and anti-inflammatory responses in innate leukocytes.

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