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*J Immunol* published online 15 June 2012
http://www.jimmunol.org/content/early/2012/06/15/jimmunol.1200857

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Molecular Mechanisms Responsible for the Selective and Low-Grade Induction of Proinflammatory Mediators in Murine Macrophages by Lipopolysaccharide

Urmila Maitra,*1 Hui Deng,*1 Trevor Glaros,*1 Bianca Baker,* Daniel G. S. Capelluto,* Zihai Li,† and Liwu Li*

Low-dose endotoxemia is prevalent in humans with adverse health conditions, and it correlates with the pathogenesis of chronic inflammatory diseases such as atherosclerosis, diabetes, and neurologic inflammation. However, the underlying molecular mechanisms are poorly understood. In this study, we demonstrate that subclinical low-dose LPS skews macrophages into a mild proinflammatory state, through cell surface TLR4, IL-1R–associated kinase-1, and the Toll-interacting protein. Unlike high-dose LPS, low-dose LPS does not induce robust activation of NF-κB, MAPKs, PI3K, or anti-inflammatory mediators. Instead, low-dose LPS induces activating transcription factor 2 through Toll-interacting protein–mediated generation of mitochondrial reactive oxygen species, allowing mild induction of proinflammatory mediators. Low-dose LPS also suppresses PI3K and related negative regulators of inflammatory genes. Our data reveal novel mechanisms responsible for skewed and persistent low-grade inflammation, a cardinal feature of chronic inflammatory diseases. The Journal of Immunology, 2012, 189: 000–000.

Slightly elevated levels of circulating bacterial endotoxin (˜1–100 pg/ml) persists in humans with adverse health conditions and/or lifestyles such as chronic infections, obesity, aging, chronic smoking, and drinking (1–7). Compromised mucosal barriers, altered commensal microbiota, and vasculature leakage collectively contribute to the mild and persistent elevation of plasma endotoxin in these individuals. In contrast to high-dose endotoxin that induces a robust yet transient inflammatory response, “subclinical” low-dose endotoxin causes low-grade yet persistent inflammatory responses from the host, as reflected in the mildly sustained levels of inflammatory mediators (1, 8–13). This may underlie the initiation and propagation of chronic pathologic diseases, including cardiovascular diseases, diabetes, Parkinson’s disease, and other chronic inflammatory syndromes (8, 14–16). Given the prevalence of low-dose endotoxemia and low-grade inflammation at humans at various stages of life, the economic and health tolls are reaching a pandemic level (17–22). As a consequence, strategies targeting low-dose endotoxemia and low-grade inflammation may hold significant promise in not only the treatment, but also the prevention or reversal, of these debilitating diseases. Despite its significance, the molecular mechanism underlying the effect of low-grade endotoxemia is neither well studied nor properly understood.

Host macrophages are the most potent responders to the bacterial endotoxin LPS (23). Given the fact that a high dose of circulating LPS (>10 ng/ml) can cause acute septic shock (24–26), almost all available mechanistic studies regarding cellular responses to endotoxin used high dosages of LPS (>10 ng/ml). High doses of LPS cause a robust induction of various proinflammatory mediators in macrophages through the TLR4 pathway (27). In the meantime, high-dose LPS is also capable of inducing the expression of anti-inflammatory mediators such as IL-10. This serves as a compensatory mechanism for the host to dampen excessive inflammation (28, 29).

Molecular mechanisms responsible for the effect of LPS were largely obtained in the context of high-dose LPS. Through TLR4 and other coreceptors, high-dose LPS opens up a flood gate of intracellular pathways that eventually lead to the activation of diverse transcription factors such as NF-κB and AP-1 (30, 31). Collectively, these transcription factors contribute to the robust induction of proinflammatory mediators (31). The pathway leading to the activation of NF-κB is the most extensively studied and is relatively well defined (32, 33). In this classical pathway, TLR4 activates the IL-1R–associated kinases (IRAK)-4, -2, and -1 via the MyD88 adaptor molecule (32, 33). IRAKs then recruit TNFR-associated factor (TRAF)6/2 and contribute to the activation of MAPKKs (e.g., TAK1, MLK3, and Tpl2), the phosphorylation of the IκB kinase (IKK)α/β complex leading to the phosphorylation and degradation of IκB, and the eventual release and nuclear translocation of p65/RelA. The IRAK-1/TRAF complex is also responsible for the activation of MAPKs and downstream transcription factors such as AP-1. Although the potent activation of the TLR4 pathway is capable of robustly inducing the expression of proinflamatory mediators, it also strongly induces negative regulators at multiple levels, including IκBα (the nega-
tive regulator of NF-κB), PI3K, MKP-1, as well as the inactivation of IRAK-1 (33–39). Of particular note, the PI3K pathway attenuates the expression of proinflammatory mediators and facilitates the expression of anti-inflammatory mediators (40). CREB activation by the PI3K pathway facilitates the expression of IL-10 (41), whereas induction of MKP-1 by PI3K contributes to the suppression of proinflammatory mediators (42, 43). This may serve to dampen excessive inflammation and reduce collateral damage to the host.

In contrast, low levels of circulating LPS (∼1–100 pg/ml) in experimental animals and humans with adverse health conditions can induce mild, yet selective expression of host proinflammatory mediators (1, 8–13). In well-controlled in vitro studies, low dosages of LPS (10–100 pg/ml) cause a distinct effect by priming cells for more robust expression of proinflammatory mediators in response to a second LPS challenge (44–47). Furthermore, chronic microbial infections or injections of low-level TLR agonists can exacerbate inflammatory diseases such as atherosclerosis (48, 49). Despite emerging recognition of the chronic and pathological effects of subclinical low-dose endotoxin, no report is available regarding the underlying molecular mechanism. To our knowledge, we provided the first evidence indicating that subclinical doses of LPS (<100 pg/ml) fail to activate the classical NF-κB pathway and its ensuing negative feedback loops (50).

The present study aims to reveal the intracellular mechanisms responsible for the mild yet selective expression of proinflammatory mediators induced by low-dose endotoxin. Based on previous observations that IRAK-1 and Toll-interacting protein (Tollip) are selectively involved in mediating macrophage responses to low-dose endotoxin (50, 51), we examined the molecular networks involving IRAK-1 and Tollip that are responsible for the selective induction of proinflammatory mediators by low-dose endotoxin. Our study reveals that mitochondria reactive oxygen species (ROS), induced by low-dose LPS through IRAK-1 and Tollip, contribute to the expression of proinflammatory mediators through activating transcription factor (ATF)2. Furthermore, our data indicate that low-dose LPS suppresses PI3K pathway involved in the expression of anti-inflammatory mediators.

Materials and Methods

Reagents

LPS (Escherichia coli 0111:B4) was purchased from Sigma-Aldrich. Anti-IgG (no. 9242), p-JNK (no. 9251), p-ERK (no. 4370), p-p38 (no. 9211), and p-ATF2 (#9225) Abs were obtained from Cell Signaling Technology. Anti-lamin B (ab-16048) was purchased from Abcam. Anti-C/EBPβ (M-17), anti-MKP-1 (M-18), anti-IRAK-1 (F-4), anti-GPSH (FL-335), anti-IRAK-M, anti-GSK3β, and anti-ATF2 (C-19) Abs were from Santa Cruz Biotechnology. Anti-mouse IgG and anti-rabbit IgG HRP-linked Abs were purchased from Cell Signaling Technology.

Mice and cell culture

Wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories. IRAK-1−/− mice from C57BL/6 background were provided by Dr. James Thomas from the University of Texas Southwestern Medical School. Tollip−/− mice from C57BL/6 background were provided by Dr. Jürg Tschopp from the University of Lausanne at Switzerland. All mice were housed and bred at the Virginia Polytechnic Institute and State University Animal Facility in compliance with approved Animal Care and Use Committee protocols of Virginia Polytechnic Institute and State University. Bone marrow–derived macrophages (BMDMs) were isolated from the tibias and femurs of WT, IRAK-1−/−, and Tollip−/− mice by flushing the bone marrow with DMEM. The cells were cultured in untreated tissue culture dishes with 50 ml DMEM containing 30% L929 cell supernatant. On the third day of culture, the cells were fed with an additional 20 ml fresh medium and cultured for another additional 3 d. Cells were harvested with PBS, resuspended in DMEM supplemented with 1% FBS, and allowed to rest overnight before further treatments. WT Raw264.7 and GP96 knocked-down (GP96KD) Raw264.7 cells defective in cell surface TLR4 were maintained as described previously (52).

Cell transfection

MEF cells were cultured in complete DMEM medium, as previously described. The endotoxin levels within the culture media were below the detectable range. Transfections with enhanced GFP-Tollip expression plasmid were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after transfection, the stably transfected cells were selected using G418 anti-biotics (400 μg/ml) for 4 wk before use in subsequent experiments.

Confocal microscopy

The GFP-Tollip–transfected MEF cells were plated in 35-mm glass bottom petri dishes (MatTek). For staining of mitochondria, cells were incubated with 75 nM MitoTracker Red (Invitrogen) for 25 min at 37°C in darkness. After washing three times with PBS, cells were fixed with paraformaldehyde (4%) in PBS for 15 min at room temperature and then washed three times with PBS. The nuclei were stained using DAPI. Fluorescence images were obtained with a Zeiss LSM510 laser-scanning confocal microscope. MitoTracker Red was excited with a 543-nm laser line and its emission was collected between 590 and 640 nm.

Analysis of protein and mRNA

Cells were harvested after specified treatments and washed with PBS. The cells were resuspended in a lysis buffer containing protease inhibitor mixture (Sigma-Aldrich) and subjected to SDS-PAGE. The protein bands were transferred to an immunoblot polyvinylidene difluoride membrane (Bio-Rad) and subjected to immunoblot analysis with the indicated Abs. Total RNA was extracted using an Isol-RNA lysis reagent (Invitrogen) and

**FIGURE 1.** Low-dose LPS selectively induces mild and prolonged expression of proinflammatory mediators. (A) WT BMDMs were treated with either low-dose LPS (50 pg/ml) or high-dose LPS (100 ng/ml) for the specified time periods. Total RNA was isolated, and real-time RT-PCR assays were performed to determine the expression levels of proinflammatory mediators such as Il10 and Tnfα. (B) WT BMDMs were treated with either low-dose LPS (30 pg/ml) or high-dose LPS (200 ng/ml) for the specified time periods. The levels of anti-inflammatory Il10 and Cc22 were measured by real-time RT-PCR. The relative transcript levels were standardized against Gapdh levels. Data were representative of at least three independent experiments. *p < 0.05.
cDNA was generated with a high-capacity cDNA reverse transcription kit (Applied Biosystems) followed by analysis using SYBR Green Supermix on an IQ5 thermocycler (Bio-Rad). The relative levels of different transcripts were calculated using the ΔΔCt method, and results were normalized based on the expression of Gapdh within the same experimental setting. The relative level of mRNA in untreated WT cells was adjusted to 1 and served as the basal reference value. The following primer sets were used: mouse Gapdh forward, 5'-AAC TTT GGC ATT GTG GAA GGG CTC-3', reverse, 5'-AGG CTC AGC AAG CCC TAT TCT TCT-3'; mouse Il10 forward, 5'-ATC CAG TTG CCT TCT TGG GAC TGA-3', reverse, 5'-TAA GCC TCC GAC TGT TGA AGT GGT-3'; mouse Ccl2 forward, 5'-AGG CTC AGC AAG CCC TAT TCT TCT-3', reverse, 5'-GAA TGT GTT CTA CCA AAG CCA CAA-3'; mouse Il6 forward, 5'-GCT CTA CCA AAG CCA CAA-3', reverse, 5'-AGG CAG AGC AGG CAG CAT AGC AGT-3'; mouse Tnf forward, 5'-AGC CGA TGG GTT GTA-3', reverse, 5'-AGC CGA TGG GTT GTA-3'.

**Analysis of mitochondrial proteins and ROS**

Mitochondrial protein fractions were prepared using a mitochondria isolation kit from Thermo Scientific. Briefly, BMDMs were grown on 150-mm² tissue culture plates and treated with LPS as indicated prior to the isolation procedure as per the manufacturer’s instructions. The purity of the mitochondrial fraction was determined using Western blot analysis for mitochondrial resident protein cyclooxygenase IV and cytosolic protein, GAPDH.

For the detection of mitochondrial ROS production, we used the mitochondrial-sensitive dye MitoSOX Red (Invitrogen). BMDMs and MEF cells were plated in DMEM without phenol red prior to the start of the experiment. Cells were then stained with 1 µM Hoechst 34580 (Invitrogen) for 10 min at 37°C for normalization of fluorescence data. After washing three times with 1 ml HBSS (Invitrogen), cells were stained with 5 µM MitoSOX Red for 20 min at 37°C. Cells were then washed with HBSS as mentioned above prior to LPS treatment for the specified time periods. Fluorescent wavelength pairs for the individual dyes were 510/580 nm for MitoSOX Red and 392/440 nm for Hoechst 34580 on a Spectromax M2e plate reader (Molecular Devices).

**Chromatin immunoprecipitation analysis**

BMDMs were cross-linked using 1% formaldehyde solution in complete media for 15 min with gentle rocking every 3 min at room temperature. Cells were washed twice with cold PBS and treated with a glycine solution for 5 min to stop the cross-linking reaction. Cells were then lysed in an ice-cold buffer containing SDS and protease inhibitor mixture. The samples were harvested and the levels of GAPDH were measured from cells treated with either low- or high-dose LPS. The total levels of Akt were used as the loading controls. (F) Low-dose LPS fails to activate the MAPK pathways. Whole-cell lysates from WT cells treated with either low-dose (50 pg/ml) or high-dose (200 ng/ml) LPS were measured by Western blot. The levels of lamin B were used as a loading control. Panels are representative of three independent experiments. (H) ChIP analysis to detect the binding of RelB to the Il6 promoter in response to high- and low-dose LPS. WT BMDMs were treated with either low- or high-dose LPS for the indicated time periods. The samples were immunoprecipitated using a RelB-specific Ab and analyzed by PCR using primers spanning the promoter region of murine Il6.
were then sonicated in an ice water bath to shear the chromatin for 10 min (30 s on and 30 s off). The sheared chromatin was processed using the Chip-IT Express Kit (Active Motif). The immunoprecipitated chromatin was analyzed by PCR using primer pairs that span the proximal promoter regions of mouse Il6. The primer sequences used to amplify the enriched chromatin samples with the mouse Il6 promoter are as follows: forward primer, 5′-TCC CAT CAA GAC ATG CTC AAG TGC-3′, reverse, 5′-AGC AGA ATG AGC TAC AGA CAT CCC-3′.

Statistical analysis

Results are expressed as means ± SD. Statistical significances between groups were determined using a two-tailed Student t test. A p value of <0.05 was considered statistically significant.

Results

Mild and selective induction of proinflammatory mediators by subclinical low-dose LPS

We first examined the expression profile of various pro- and anti-inflammatory genes in macrophages challenged with either low- or high-dose LPS. WT BMDMs were treated with low-dose (50 pg/ml) or high-dose LPS (100 ng/ml) for the specified time periods. As expected, high-dose LPS robustly induced the expression of both proinflammatory (Il6, Tfna) and anti-inflammatory (Il10, Ccl2) mediators (Fig. 1). In contrast, low-dose LPS only induced mild expression of proinflammatory mediators, but failed to induce any noticeable expression of anti-inflammatory mediators (Fig. 1). The levels of proinflammatory Tfna and Il6 induced by high-dose LPS peaked transiently at 4–6 h and dropped significantly at 8–10 h. In contrast, the expression of Tfna and Il6 induced by low-dose LPS was prolonged and persisted throughout the time course.

Subclinical-dose LPS fails to induce robust activation of NF-κB and MAPK as well as negative regulators

To clarify the underlying molecular mechanisms, we examined the activation status of key TLR4 downstream components, including NF-κB and MAPKs. As shown in Fig. 2A, high-dose LPS induced a rapid degradation of IκB, an indication of the activation of the classical NF-κB pathway. In contrast, low-dose LPS failed to induce noticeable degradation of IκB. High-dose LPS also triggered robust activation of MAPKs, including p38, ERK, and JNK (Fig. 2B). In contrast, low-dose LPS failed to induce noticeable activation of all three MAPKs.

As well noticed in the field, high-dose LPS also induces negative regulators, including MKP-1 and IRAK-M, that serve to dampen the expression of proinflammatory mediators (43, 53). We then examined the expression of MKP-1 and IRAK-M by high- versus low-dose LPS. As shown in Fig. 2C, high-dose LPS induced significant expression of MKP-1. In contrast, low-dose LPS failed to induce MKP-1. Intriguingly, low-dose LPS reduced the levels of IRAK-M (Fig. 2D). Other mechanisms of compensatory suppression/tolerance triggered by high-dose LPS include degradation of IRAK-1 (33). As shown in Fig. 2E, high-dose LPS led to rapid modification and degradation of IRAK-1, as reflected in the occurrence of an upper band typical of ubiquitinated IRAK-1, and later disappearance of IRAK-1. In contrast, low-dose LPS failed to induce IRAK-1 ubiquitination and degradation.

Activation of the PI3K pathway by high-dose LPS was shown to be responsible for the induction of negative regulators, including MKP-1 and degradation of IRAK-1 (42, 54). Additionally, PI3K activation also leads to the expression of anti-inflammatory mediators such as IL-10 (40, 41). Thus, we evaluated the activation status of the PI3K pathway in cells treated with either low- or high-dose LPS. As shown in Fig. 2F, high-dose LPS led to robust phosphorylation of Akt, an indication of PI3K activation. Strikingly, low-dose LPS not only failed to induce Akt phosphorylation, but rather reduced the residual levels of phosphorylated Akt. As a consequence, the nuclear levels of GSK3β were induced by low-dose LPS and reduced by high-dose LPS (Fig. 2G).

Previous reports also indicate that high-dose LPS recruits the suppressive RelB to the promoter of inflammatory genes and accounts for the endotoxin tolerance (55, 56). After an initial transient decrease in RelB, high-dose LPS is well documented to induce a late-phase RelB through NF-κB as well as PI3K-mediated expression (56, 57). However, no report is available regarding the status of RelB in cells challenged with low-dose LPS. To further test the involvement of RelB in differential gene expression induced by low- and high-dose LPS, we performed chromatin immunoprecipitation (ChIP) analysis on the promoter of the Il6 gene. As shown in Fig. 2H, high-dose LPS (100 ng/ml) caused a transient decrease of suppressive RelB from the Il6 promoter (2 h time point), allowing for transient induction of Il6 expression. The RelB levels at the Il6 promoter subsequently rose significantly at the 4 h time point, corresponding to the development of endotoxin tolerance (56). In stark contrast, low-dose LPS caused prolonged removal of RelB from the Il6 promoter at both time points (Fig. 2H).

Subclinical-dose LPS preferentially activates ATF2 and C/EBPβ

AP-1 family proteins are among potential transcription factors responsible for the mild expression of Tfna and Il6. We noticed a rapid and significant induction of ATF2 protein by low-dose LPS. As well noticed in the field, low-dose LPS fails to induce robust activation of NF-κB and MAPK as well as negative regulators. WT BMDMs were treated with low-dose (50 pg/ml) or high-dose (100 ng/ml) LPS for the indicated times. Total ATF2 protein levels were determined by Western blot, and GAPDH levels were used as loading controls. (A) Low-dose LPS induces a rapid increase in ATF2 protein levels in WT BMDMs. WT cells were treated with low-dose (50 pg/ml) or high-dose (100 ng/ml) LPS for the indicated times. Total ATF2 protein levels were determined by Western blot, and GAPDH levels were used as loading controls. (B) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (C) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (D) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (E) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (F) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (G) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (H) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (I) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (J) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (K) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (L) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (M) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (N) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (O) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (P) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (Q) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (R) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (S) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (T) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (U) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (V) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (W) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (X) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (Y) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs. (Z) Low-dose LPS recruits ATF2 to the proximal promoter of the proinflammatory gene IL-6 in WT BMDMs.
LPS. The protein levels of ATF2 were similarly and significantly induced after 30 min stimulation with high-dose LPS (Fig. 3A). To determine whether the induction of ATF2 may be due to its gene expression, we measured mRNA levels of Atf2 and did not detect any noticeable increase in Atf2 message within 2 h LPS stimulation (data not shown). This suggests that LPS may selectively stabilize ATF2 protein through a novel mechanism independent of gene transcription.

To confirm that ATF2 contributes to the expression of Il6 induced by low-dose LPS, we performed ChIP analyses to examine the recruitment of ATF2 to the Il6 promoter. As shown in Fig. 3B, low-dose LPS challenge led to significant recruitment of ATF2 to the proximal promoter of Il6.

We and others also reported that a late-phase C/EBPδ induction mediated by IKKe contributes to prolonged expression of proinflammatory mediators such as Il6 (50, 58). To further confirm this phenomenon, we measured the levels of C/ebpδ in cells treated/ stimulated with low-dose LPS. As shown in Fig. 3C, a late induction of C/ebpδ (4–8 h) was observed following low-dose LPS stimulation. In contrast, high-dose LPS led to a transient induction of C/ebpδ.

**IRAK-1 and Tollip are involved in the induction of ATF2**

To further determine the upstream signaling pathways responsible for the mild expression of Il6 and Tnfα, we examined the role of IRAK-1 and Tollip using macrophages harvested from IRAK-1– and Tollip-deficient mice. This is based on previous reports suggesting that IRAK-1 and Tollip are involved in mediating the effect of low-dose LPS (50, 51). Inconsistent with previous reports, the induction of Il6 and Tnfα by low-dose LPS was significantly reduced in IRAK-1– and Tollip-deficient cells (Fig. 4A).

We further examined the protein levels of ATF2 in various cells treated with low-dose LPS. As shown in Fig. 4B, ATF2 protein levels were induced in WT cells treated with low-dose LPS. In contrast, low-dose LPS failed to increase ATF2 protein levels in either IRAK-1– or Tollip-deficient cells. Alternatively, the late induction of C/ebpδ by low-dose LPS is only dependent on IRAK-1, but not Tollip (Fig. 4C).

**Tollip-mediated ROS generation in mitochondria**

Tollip is a novel TLR intracellular adaptor molecule with poorly defined function (59). It contains a lipid-binding C2 domain and an ubiquitin-binding CUE domain (60). Limited studies suggest that

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4**. Low-dose LPS-mediated activation of ATF2 requires IRAK-1 and Tollip. (A) Reduced induction of Il6 and Tnfα in IRAK-1– and Tollip-deficient BMDMs. WT and IRAK-1– and Tollip-deficient BMDMs were treated with low-dose LPS (50 pg/ml) for 4 h and the transcript levels of Il6 and Tnfα were analyzed by real-time RT-PCR. *p < 0.05. (B) Induction of ATF2 in response to low-dose LPS depends on IRAK-1 and Tollip. Whole-cell lysates were prepared from WT and IRAK-1– and Tollip-deficient BMDMs treated with low-dose LPS for the indicated time points and analyzed by Western blot. The same blots were probed with GAPDH as loading controls. The adjusted resting levels of ATF2 in each cell type were set as 1, and the ATF2 levels in cells treated with LPS were compared and plotted (n = 3). *p < 0.05. (C) Low-dose LPS-mediated induction of C/ebpδ message is dependent on IRAK-1. WT and IRAK-1– and Tollip-deficient BMDMs were treated with low-dose LPS (50 pg/ml) for 6 h and the C/ebpδ message levels were analyzed and plotted as shown. *p < 0.05. (D) Tollip and IRAK-1 are required for low-dose LPS-mediated recruitment of ATF2 to the promoter of Il6. WT and IRAK-1– and Tollip-deficient BMDMs were either untreated or treated with 50 pg/ml LPS for 2 h and subjected to ChIP assay using Abs specific to ATF2 or C/EBPδ and primers specific to the proximal promoter of Il6. The same samples were immunoprecipitated using IgG as specificity control and input DNA was analyzed as the loading control. Arrow points to the specific amplification product. *Nonspecific band.
Tollip might be involved in modulation of intracellular traffic (61, 62). To reveal the molecular mechanism responsible for Tollip-mediated activation of ATF2, we first examined cellular distribution of Tollip following LPS challenge. First, we fractionated cellular lysates from WT BMDMs into soluble and mitochondrial fractions. Western blot analyses revealed that low-dose LPS stimulation led to increased Tollip levels in the mitochondrial fraction (Fig. 5A). We realize that subcellular fractionation may contain potential contamination from other membrane sources. Thus, we also employed a confocal microscopy approach to further confirm the mitochondria translocation of Tollip. To achieve this, we generated primary murine embryonic fibroblasts stably transfected with enhanced GFP-Tollip fusion protein. Through this, we generated primary murine embryonic fibroblasts stably transfected with enhanced GFP-Tollip fusion protein. Through confocal microscopy, we detected that low-dose LPS induces Tollip translocation to mitochondria (Fig. 5B).

Recent studies indicate that mitochondria play a key role in TLR signaling processes (63, 64). ROS generated from mitochondria induced by LPS plays a key role in the activation of the TLR downstream pathway. It is particularly relevant that mitochondrial ROS was shown to contribute to the stabilization of transcription factors, including HIF1α and ATF2 (65, 66). To test whether Tollip is involved in the generation of mitochondrial ROS, we measured the levels of mitochondrial ROS using MitoSOX Red, a selective dye for mitochondrial ROS. As shown in Fig. 5C, low-dose LPS induced mitochondrial ROS in WT cells but failed to do so in either IRAK-1– or Tollip-deficient cells.

*Inhibition of mitochondrial ROS reduces low-grade induction of proinflammatory mediators*

We further tested the role of ROS in the induction of IL-6 by low-dose LPS by applying a selective ROS inhibitor, N-acetyl cysteine (NAC). As shown in Fig. 6A, NAC effectively blocked the induction of ATF2 by low-dose LPS (Fig. 6A). Furthermore, NAC significantly reduced the expression of Il6 induced by low-dose LPS (Fig. 6B). As C/EBPβ is reported to be induced by IRAK-1 through IKKe (50, 58), we demonstrated that joint inhibition of mitochondrial ROS and IKKe completely ablated the induction of IL-6 by low-dose LPS (Fig 6B). Taken together, our data provide a mechanism responsible for low-grade inflammation induced by low-dose LPS (Fig. 7).

**Discussion**

Our study reveals novel intracellular mechanisms used by sub-clinical low-dose LPS in the skewed and low-grade induction of proinflammatory mediators. First, low-dose LPS fails to activate either the classical NF-κB pathway or pathways activating MAPKs. Second, low-dose LPS suppresses, instead of activating, the compensatory and anti-inflammatory PI3K pathway. Third, low-dose LPS utilizes IRAK-1 and Tollip to induce mitochondrial ROS, which subsequently activates ATF2. The lack of negative feedbacks in cells challenged with low-dose LPS may enable prolonged expression of proinflammatory mediators, a potential culprit for chronic inflammatory diseases often associated with low-grade endotoxemia.

These mechanisms reconcile recent clinical findings that low-dose endotoxemia closely correlates with low-grade inflammation and the pathogenesis of chronic human diseases, including atherosclerosis, diabetes, and neurologic diseases (67–70). Cardinal features of chronic low-grade inflammation include mild and persistent expression of inflammatory mediators as well as skewed proinflammatory profiles. For example, aged individuals tend to have elevated plasma levels of proinflammatory mediators including CRP and IL-6 (71, 72), reduced Ag-presentation capabilities (73, 74), as well as reduced levels of anti-inflammatory TGF-β and IL-10 (75). Our finding that low-dose LPS fails to

**FIGURE 5.** Low-dose LPS translocates Tollip to the mitochondria and induces ROS. (A) Mitochondrial localization of Tollip in response to low-dose LPS using subcellular fractionation. WT BMDMs were treated with low-dose LPS and mitochondrial protein fractions were prepared. The purity of the mitochondrial fraction was determined using Western blot analysis for mitochondrial-resident protein cyclooxygenase IV and cytosolic protein, GAPDH. (B) Mitochondrial localization of Tollip in response to low-dose LPS using confocal microscopy. The GFP-Tollip–transfected MEF cells were stained with MitoTracker Red to stain the mitochondria. The nuclei were stained using DAPI (blue). The cells were visualized under a laser-scanning confocal microscope (original magnification ×400). The merged images were magnified and shown on the right panel and the colocalization is indicated with an arrow. (C) Involvement of Tollip in mitochondrial ROS generation. WT and IRAK-1– and Tollip-deficient BMDMs were stained with mitochondrial ROS selective dye, MitoSOX Red. Cells were then washed with HBSS and treated with low-dose LPS for 60 min. The cells were then stained with 1 μM Hoescht 34580 for normalization of fluorescent data. Fluorescent intensities were measured and quantified (n = 3). *p < 0.05.
induce the PI3K pathway and other negative regulators of inflammatory pathways (MKP-1, SOCS1, and degradation of IRAK-1) provides a potential explanation for skewed proinflammatory profiles associated with low-grade inflammation. When faced with a higher dose LPS, multiple pathways in macrophages are activated that lead to robust induction of both pro- and anti-inflammatory mediators. As a consequence, the coordinated and balanced portfolio of pro- and anti-inflammatory pathways ensures transient inflammatory responses and subsequent resolution. Perhaps the levels of endotoxin during low-grade inflammation may fall below the “threshold of alarm” that can effectively invoke an anti-inflammatory resolution, thus undermining the long-term danger of chronic inflammatory diseases (Fig. 7).

The lack of robust NF-κB activation and limited activation of ATF2 and C/EBPδ identified in this study may underlie the mild expression pattern of proinflammatory mediators. Although ATF2 and C/EBPδ may not be capable of robustly inducing the expression of proinflammatory mediators, they are also not capable of evoking negative feedbacks. Instead, both ATF2 and C/EBPδ have been shown to induce a positive feedback that leads to their own expression (30, 76). The selective presence of a positive feedback implies a novel “digital” or “bi-stable” toggle switch controlling the activation of macrophages by low-dose endotoxin. In contrast to a linear or “analog” switch due to the activation of a linear pathway, a digital switch owing to a positive feedback circuit can build up an “energy reservoir” and only be turned on when the concentration of stimulant reaches a critical threshold (77–79). Alternatively, once the cells are activated, the presence of the energy reservoir would allow the cells to stay activated with significantly less stimulant. This phenomenon, called “hysteresis,” is well documented in relevant biological contexts, such as cell cycle and DNA annealing (77–79). This hysteresis, which has not been applied in leukocyte biology, would nicely explain several key features of leukocyte physiology and pathology. For example, our immune response is built to withstand challenges without easily getting inflammatory diseases. However, once humans are challenged with a sufficient amount of LPS, due to chronic infection, obesity, or other inflammatory conditions, the inflammatory state will be easy to maintain and difficult to get rid of. Indeed, we collected data indicating the critical LPS concentration (~5 pg/ml) is able to maintain the expression of IL-6. We realize that human plasma environments in vivo as well as cell culture medium in vitro include a plethora of reagents and stimulants. The exact LPS concentration that may elicit a digital activation of macrophages would most likely vary under different conditions. Nevertheless, our experiments with precisely added LPS in vitro reveal a fundamental principle for the novel nature of macrophage activation.

Our data reinforce the notion that mitochondrial ROS plays a key role in innate immunity regulation by LPS. Recent studies
indicate that mitochondrial ROS induced by LPS participate in the expression of proinflammatory mediators (64, 80). Mechanistically, LPS is shown to induce the translocation of TRAF6 to mitochondria, ubiquitination of ECSIT, and disruption of the electron transport system (63, 64). These events may collectively lead to the generation of mitochondrial ROS. Our data reveal that IRAK-1 and Tollip are key signaling molecules responsible for the generation of mitochondrial ROS. To our knowledge, Tollip is the first TLR4 intracellular adaptor molecule shown to translocate into mitochondria following LPS challenge. Note that Tollip is a novel TLR adaptor molecule capable of binding to phosphoinositides, a phenomenon first reported by our group (81). Additionally, Tollip contains an ubiquitin-binding CUE domain that can potentially facilitate protein ubiquitination and degradation (82). Based on our data, future studies are clearly warranted to determine the role and mechanism of Tollip-mediated generation of mitochondrial ROS. Importantly, note that the role and regulation of Tollip are highly complex. Despite its proinflammatory role in cells stimulated with low-dose LPS, Tollip may dampen proinflammatory responses in cells when the NF-κB pathway is activated (83). Although the mechanisms are far from clear, we hypothesize that Tollip may distribute to distinct subcellular compartments and form distinct protein complexes in cells treated with varying dosages of TLR agonists.

Our study reveals an intriguing paradigm of PI3K modulation by low- versus high-dose LPS. Although high-dose LPS is known to activate PI3K, to our knowledge, this is the first study to reveal that low-dose LPS potently suppresses basal PI3K activation. The opposing effects of low- versus high-dose LPS on PI3K may be responsible for multiple unexplained paradigms. In addition to the skewed proinflammatory profiles in cells challenged with low-dose LPS, macrophages/monocytes can be programmed to exhibit either “LPS tolerance” or “LPS priming” dependent on the initial dosage of LPS challenge. High-dose LPS causes tolerance whereas low-dose LPS causes priming (84). The differential modulation of PI3K by different dosages of LPS may contribute to these opposing effects. Further complementing this claim, our published data indicate that low-dose LPS activates GSKβ, whereas high-dose LPS is known to reduce GSKβ activity (41). Consistent with our finding, a recent study reported that brain tissues harvested from mice injected with low-dose LPS have increased GSKβ activity (85). This is associated with low-grade inflammation and the pathogenesis of Alzheimer’s disease (85). Several other independent studies also support the conclusion that low-dose endotoxemia directly contributes to chronic low-grade neuroinflammation and chronic diseases (16, 67). The molecular mechanisms responsible for the opposing effect of LPS on PI3K are not clear. High-dose LPS activates PI3K through active recruitment of p85 to MyD88 (86). An intriguing recent study indicates that protein kinases D and C can potentially suppress basal PI3K activation by causing a distinct phosphorylation pattern of p85 (87). LPS is known to activate protein kinase C (88). The balance and coordination between the activation of protein kinase C and PI3K by LPS may finely modulate the pro- versus anti-inflammatory outcomes. Future studies are clearly warranted to dissect the differential regulation of PI3K by low- versus high-dose LPS.

We also demonstrated that low-dose LPS exerts an opposing regulation of key negative regulators in TLR4 pathway as compared with high-dose LPS. Specifically, we demonstrated that low-dose LPS potently suppresses IRAK-M and fails to induce MKP-1. Furthermore, low-dose LPS decreases the suppressive RelB on the promoter of Il6. This is in stark contrast to increased levels of IRAK-M, MKP-1, and increased RelB recruitment to the Il6 promoter in tolerant macrophages challenged with high-dose LPS. This mechanism underlies the skewed and low-grade inflammation elicited by low-dose LPS.

Although it is clear that low- and high-dose LPS induces distinct downstream signaling networks, the responsible mechanisms at the receptor level are not well understood. TLR4 traffics between endosome and plasma membrane, and it can mediate LPS signaling at both locations (89). Our data indicate that the cell surface TLR4 is essential for the effect of low-dose LPS (Supplemental Fig. 4). Given the fact that TLR4 may heterodimerize with multiple other receptors, including CD36 and MAC1 (90–92), we hypothesize that low-dose LPS may engage a unique set of receptor configuration at the cell surface, and it may prevent further receptor internalization. Future studies are needed to determine the receptor configuration and related proximal signaling network responsible for mediating the opposing effects of low- versus high-dose LPS.

In summary, our study reveals novel mechanisms responsible for low-grade inflammation induced by low-dose LPS. Key features identified include IRAK-1- and Tollip-mediated generation of mitochondrial ROS as well as selective suppression of PI3K. The opposing effects of low- versus high-dose LPS identified in this study explain the intriguing paradigm of endotoxin priming and tolerance, and provoke future studies paying close attention to the dosages as well as history of LPS challenges experienced by the host. Intervention of the unique cellular network activated by low-dose LPS may hold significance promise in designing effective therapies for low-grade and chronic inflammatory diseases.

Acknowledgments
We thank Matt Morris for assistance in the harvest and culture of macrophages, and members of the Li Laboratory for discussions.

Disclosures
The authors have no conflicts of interest.

References


Supplementary Figure S1. Minimum dosage of LPS required for the induction of Il-6 in macrophages. WT BMDM were treated with varying dosages of LPS for 6 hrs. The levels of expressed Il-6 mRNA were measured by real-time RT-PCR. n=3. * p<0.05.
Supplementary Figure S2. Minimum dosage of LPS required to sustain the expression of *Il-6*. WT BMDM were treated with 50 pg/ml LPS for 4 hrs to induce the expression of *Il-6*. The cells were then washed twice with PBS, and re-incubated with fresh medium supplemented with varying dosages of LPS for an additional 6 hrs. The levels of *Il-6* were measured by real-time RT-PCR.
**Supplementary Figure S3.** An illustration of the bi-stable “digital” activation of macrophages by low dose LPS. Macrophages have to be challenged with a critical threshold amount of LPS (Ct) in order to be turned on and express pro-inflammatory mediators. Once the cells are turned on, a significantly lower LPS concentration (Cm) can still maintain its activation and expression of proinflammatory mediators.
Supplementary Figure S4. Surface TLR4 is required for low-grade induction of pro-inflammatory mediators

LPS has been shown to signal both through cellular surface and endosomal TLR4 (64). Thus, we examined whether cell surface or endosomal TLR4 is required for the effect of low dose LPS. To test this, we employed both wild type Raw264.7 murine macrophages (expressing cell surface TLR4), as well as Raw264.7 cells with GP96 knocked-down (GP96KD) (52). GP96 is required for the assembly and trafficking of TLR4 from the endosome to the cell membrane. GP96KD cells only have endosomal TLR4 and lack cell surface TLR4 (52). Indeed, as determined by flow cytometry, cell surface TLR4 was only detectable in WT Raw264.7 cells but not GP96KD cells (Fig. 7A). Following treatment with a low dose LPS, wild type Raw264.7 cells expressed IL-6 (Fig. 7B). In contrast, the induction of IL-6 was completely ablated in GP96KD cells. In agreement with previous findings that show high dose LPS can signal through intra-cellular TLR4, we observed that a high dose LPS was still capable of inducing IL-6 expression in GP96KD cells. This observation indicates that cell surface TLR4 is critically required for the effect of low dose LPS.