Alteration of Lysosome Fusion and Low-grade Inflammation Mediated by Super-low-dose Endotoxin*

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Subclinical super-low-dose endotoxin LPS is a risk factor for the establishment of low-grade inflammation during the pathogenesis and progression of chronic diseases. However, the underlying mechanisms are not well understood. At the cellular level, a disruption of lysosome fusion with endosomes or autophagosomes may contribute to the potentiation of low-grade inflammation. In this study, we identified that subclinical super-low-dose endotoxin LPS can potently inhibit the process of endosome acidification and lysosome fusion with endosomes or autophagosomes in primary macrophages. Super-low-dose LPS induced the inhibitory phosphorylation of VPS34, thus leading to the disruption of endosome-lysosome fusion. This effect may depend upon the clearance and relocation of Tollip in macrophages by super-low-dose LPS. Consistent with this notion, Tollip-deficient macrophages had constitutively elevated levels of VPS34 inhibitory phosphorylation and constitutive disruption of endosome-lysosome fusion. By employing a skin excision wound-healing model, we observed that Tollip-deficient mice had significantly elevated levels of cell stress and reduced wound repair. This study reveals a novel mechanism responsible for the modulation of endosome-lysosome fusion and low-grade inflammation in innate macrophages.

Background: Super-low-dose endotoxemia contributes to cell stress and low-grade inflammation.

Results: Super-low-dose LPS removed Tollip from late endosomes/lysosomes and blocked lysosome fusion with endosomes or autophagosomes. Tollip knock-out mice had impaired wound healing.

Conclusion: Super-low-dose LPS leads to cell stress through clearing Tollip and blocking lysosome fusion.

Significance: Our data reveal molecular dynamics of innate immunity regulation.

At the cellular level, alterations of intracellular trafficking processes, such as endosome acidification and autophagy initiation and completion, as well as fusion of lysosomes with endosomes or autophagosomes, are critically involved in the propagation of low-grade inflammation (6, 7, 13, 14). Of particular interest, timely and orderly fusion of lysosomes with endosomes or autophagosomes would enable efficient clearance of cell debris and resolution of inflammation (15). Limited studies suggest that the disruption of endosome acidification and/or endosome-lysosome fusion may be correlated with the modulation of inflammatory phenotypes in monocytes and macrophages (16, 17).

At the biochemical level, several distinct gate-keeping enzymes are responsible for the initiation of autophagy and the fusion of lysosomes with autophagosomes and/or endosomes. The cell stress-sensing protein kinase adenosine monophosphate-activated protein kinase (AMPK) is responsible for autophagy initiation through the activation of ULK1 (unc-51-like autophagy-activating kinase 1) (18). Upon phosphorylation, AMPK becomes activated and is able to phosphorylate and thus activate ULK1, triggering the autophagic cascade (18–20). Activated ULK1 is recruited to the phagophore, where it binds to regulatory proteins and begins the assembly process of the autophagosome.

Activation of the class III PI3K VPS34, also known as PI3KC3, plays a key role during the completion of autophagy and fusion of lysosomes with endosomes, phagosomes, or autophagosomes. VPS34 is responsible for the phosphorylation of phosphatidylinositol to generate phosphatidylinositol 3-phosphate (PI(3)P), an essential signaling lipid in the regulation of lysosome fusion with endosomes/autophagosomes (21–24). VPS34 is negatively regulated via phosphorylation by various kinases, including cyclin-dependent kinase (25, 26). The phosphorylation of VPS34 inhibits the proper fusion of endosomes and/or autophagosomes with lysosomes (27).

Chronic low-grade inflammation is a common feature of numerous debilitating diseases and disorders (1–8). Subclinical super-low-dose endotoxin in the blood circulation is one of the emerging risk factors that can cause and sustain chronic low-grade inflammation in humans and animals (9–11). Super-low-dose endotoxin selectively induces mild inflammatory responses in innate leukocytes without triggering compensatory anti-inflammatory responses (12). As a consequence, this may explain the nonresolving nature of low-grade inflammation that often accompanies low-grade endotoxemia.

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¶ The abbreviations used are: AMPK, AMP-activated protein kinase; PI(3)P, phosphatidylinositol 3-phosphate; BMDM, bone marrow-derived macrophage; MEF, murine embryonic fibroblast.
Although subclinical low-grade endotoxemia has been associated with nonresolving low-grade inflammation in humans and experimental animals, limited studies are available to clarify the underlying mechanisms. Previous studies suggest that Tollip (Toll-interacting protein), one of the key signaling molecules downstream of the TLR4 (Toll-like receptor 4) pathway, can interact with Tom1 and may facilitate lysosome fusion with lysosomes (28). However, the role of Tollip in the context of innate immunity is not well understood and may be highly context-dependent. High-dose LPS induces the expression of Tollip and may facilitate the resolution of inflammation (29, 30). Tollip may also be subjected to ubiquitination and degradation (31). In contrast, we previously reported that super-low-dose LPS effectively relocates Tollip away from late endosomes/lysosomes to mitochondria (see Fig. 4) (12). As a consequence, super-low-dose LPS induces mitochondrial reactive oxygen species and low-grade inflammation (12).

In this study, we further tested the hypothesis that the clearance and relocation of Tollip by super-low-dose LPS may be responsible for the disruption of lysosome fusion with endosomes/autophagosomes and cell stress in innate leukocytes. Using macrophages harvested from WT and Tollip-deficient mice, we examined the role of Tollip during the process of lysosome fusion. We determined that super-low-dose LPS caused cell stress in macrophages by reducing the total levels of Tollip and by clearing Tollip away from late endosomes/lysosomes and thus hindering lysosome fusion with endosomes/autophagosomes. We further examined the pathological relevance by using a skin incision wound repair model in WT and Tollip-deficient mice. Collectively, our results define a novel mechanism responsible for the potentiation of low-grade inflammation through the disruption of endosome-lysosome fusion.

**EXPERIMENTAL PROCEDURES**

Reagents—LPS (Escherichia coli O111:B4) was obtained from Sigma-Aldrich. Anti-GAPDH antibody was obtained from Santa Cruz Biotechnology. Antibodies against phospho-JNK, total JNK, phospho-ERK, total ERK, phospho-p38, total p38, SQSTM1/p62, phospho-AMPK, and total AMPK were obtained from Santa Cruz Biotechnology and Cell Signaling Technology. Antibodies against phospho-Tollip (Toll-interacting protein), one of the key signaling molecules downstream of the TLR4 (Toll-like receptor 4) pathway, can interact with Tom1 and may facilitate lysosome fusion with lysosomes (28). However, the role of Tollip in the context of innate immunity is not well understood and may be highly context-dependent. High-dose LPS induces the expression of Tollip and may facilitate the resolution of inflammation (29, 30). Tollip may also be subjected to ubiquitination and degradation (31). In contrast, we previously reported that super-low-dose LPS effectively relocates Tollip away from late endosomes/lysosomes to mitochondria (see Fig. 4) (12). As a consequence, super-low-dose LPS induces mitochondrial reactive oxygen species and low-grade inflammation (12).

**Analysis of Lysosome Fusion with Endosomes or Autophagosomes**—BMDMs harvested from WT and Tollip-deficient mice were incubated with Alexa Fluor 546-tagged dextran (1 mg/ml) for 1 h. Cells were then washed several times and reincubated in fresh medium for 3 h to allow lysosome translocation of dextran. Cells were subsequently incubated with FITC-conjugated zymosan for 1 h, followed by washing and fixation with 4% paraformaldehyde in PBS for 15 min at room temperature. Data were collected using a Zeiss LSM 510 laser-scanning confocal microscope. Experiments were conducted at 37 °C. Phagosomes (intracellular zymosan, green) fused with lysosomes (red) appeared yellow.

To determine whether the Tollip mutation affects lysosome fusion, we employed the Tollip mutant MEFs stably expressing the Tollip M240A/F241A mutant in a Tollip-deficient MEF background. These MEF cell lines were kindly provided by Drs. Kimberly Burns (University of Lausanne, Lausanne, Switzerland) and Jürg Tschopp. Unlike macrophages, which are efficient phagocytes and proficient in endosome acidification, MEFs are not efficient in uptake of zymosan and phagosomal/endosomal trafficking. Thus, we specifically examined the fusion of lysosomes with intracellular autophagosomes, as represented by the co-localization of LC3 and LAMP1 by confocal microscopy.

**Electron Microscopy**—WT and Tollip-deficient BMDMs were harvested after the specified treatments and washed with PBS. The cells were pelleted in a microcentrifuge tube, and 1 ml of 2% glutaraldehyde with 0.1 m cacodylate buffer (pH 7.4) fixative was placed on top of the cells without causing resuspension. Samples were sectioned and prepared on grids to be visualized under a JEOL JEM-1400 transmission electron microscope.

**Immunoblotting**—Cells were washed with PBS, harvested in SDS lysis buffer containing protease inhibitor mixture, and subjected to SDS-PAGE. The protein bands were transferred to an Immob-Blot PVDF membrane (Bio-Rad). Western blot analyses were performed with the specified antibodies as described previously (12).

**Measuring Endosome Acidification**—The effect of LPS on endosome acidification was measured using pH-sensitive dextran double-labeled with FITC and tetramethylrhodamine. Cells treated with LPS for the indicated times were then pulsed with dextran (1 mg/ml) for 10 min at 37 °C. Cells were extensively washed with cold PBS and immediately analyzed by FACS. The ratio of the mean fluorescence intensity emission between both dyes was determined. An increase in mean fluorescence intensity represents a decrease in acidification.

**Confocal Microscopy**—BMDMs harvested from WT mice were seeded on coverslips and treated with or without super-low-dose LPS (50 pg/ml) for 1 or 24 h. Some samples were labeled with MitoTracker Red (200 nM; Invitrogen) for 20 min. After fixation with 4% paraformaldehyde and permeabilization with 0.2% saponin, the cells were stained with goat anti-mouse Tollip antibody (Santa Cruz Biotechnology), followed by Alexa Fluor 488-labeled rabbit anti-goat IgG (Invitrogen). In some
experiments, cells were also co-stained with Cy3-conjugated rabbit anti-mouse LAMP1 antibody (Abcam). WT and Tollip M240A/F241A mutant MEF cells were seeded on coverslips and treated with or without super-low-dose LPS (50 pg/ml). After 4 h, cells were starved with 1% FBS for 20 h to induce autophagy with or without LPS treatment. After fixation with 4% paraformaldehyde and permeabilization with methanol, the cells were stained with Alexa Fluor 488-labeled rabbit anti-mouse LC3 antibody (Novus Biologicals) together with Cy3-conjugated rabbit anti-mouse LAMP1 antibody. The samples were analyzed under the Zeiss LSM 510 confocal microscope.

Wound-healing Assays—Male 8-week-old Tollip−/− and WT mice were injected intraperitoneally with either PBS or LPS (5 ng/kg of body weight) every 3 days for 10 days. At day 10, mice were anesthetized as recommended. The backs of the mice were shaved with an Oster Mark II animal clipper (Sunbeam-Oster Co., Fort Lauderdale, FL), followed by treatment with cooling hair removal gel (Church & Dwight Co., Ewing, NJ). After disinfection with 7.5% iodine, followed by 70% ethanol to remove the iodine, four full-thickness punch biopsies were obtained using an AcuPunch (6 mm; Acuderm Inc., Fort Lauderdale, FL). The skin incision was immediately covered with a transparent dressing (3.8 × 3.8 cm; Systagenix, North Yorkshire, United Kingdom). The mice were monitored and photographed on a daily basis. The area of the wound was measured daily using NIH ImageJ. Wound sizes at day 9 post-wounding were expressed as ratio of the initial (day 0) wound area. Wound tissues were collected at day 9 post-wounding and processed for Western blotting.

Statistical Analysis—Results are expressed as mean ± S.D. Statistical significances between groups were determined using a two-tailed Student’s t test and are indicated by asterisks in the figures; p values <0.05 were considered statistically significant.

RESULTS

Super-low-dose LPS Disrupts Lysosome Fusion and Causes Low-grade Stress in Macrophages—We first tested the endosome acidification process in primary macrophages harvested from WT mice treated with super-low-dose LPS. As shown in Fig. 1A, a higher dose of LPS (100 ng/ml) induced significant acidification of late endosomes as determined by flow cytometry analyses of the phagocytosed pH-sensitive dextran. In contrast, super-low-dose LPS (50 pg/ml) failed to induce any noticeable late endosome acidification.
compared with control macrophages. To further corroborate our conclusion, we performed electron microscopy analyses of WT macrophages treated with and without 50 pg/ml LPS. As shown in Fig. 1C, macrophages treated with 50 pg/ml LPS exhibited elevated levels of densely stained lysosome structures compared with fused gray-colored endolysosomes. We further examined the cellular levels of p62, the accumulation of which is correlated with the disruption of lysosome fusion with late endosomes or autophagosomes (32). As shown in Fig. 1D, super-low-dose LPS induced a rapid accumulation of p62. Next, we determined the activation status of key stress kinases such as JNK and p38. As shown in Fig. 1E, 50 pg/ml LPS induced mild yet sustained phosphorylation of JNK and p38. In contrast, 100 ng/ml LPS triggered robust and transient phosphorylation of JNK and p38. Intriguingly, 50 pg/ml LPS not only failed to induce ERK phosphorylation, but also caused a slight reduction.

**Tollip Facilitates the Process of Lysosome Fusion**—Given previous findings that Tollip interacts with PI(3)P and Tom1, and localizes at late endosomes/lysosomes (29, 33), we tested the hypothesis that Tollip may be involved in the process of lysosome fusion. As shown in Fig. 2A, Tollip-deficient macrophages had a constitutive defect in the acidification of late endosomes. Furthermore, the lysosome fusion with endosomes/autophagosomes was constitutively disrupted in Tollip-deficient macrophages, as visualized by the clear separation of dextran and zymosan in Tollip-deficient cells under a confocal microscope (Fig. 2B). Western blot analyses demonstrated that cellular p62 levels were constitutively and significantly elevated in resting macrophages from Tollip-deficient mice, a clear sign
of disrupted fusion of lysosomes with autophagosomes (Fig. 2C). We observed that super-low-dose LPS has no further effects on p62 in Tollip-deficient macrophages. Electron microscopy analyses also revealed elevated lysosomes in resting Tollip-deficient macrophages (Fig. 2D).

**Suppression of AMPK and VPS34 by Super-low-dose LPS Is Dependent on Tollip**—To further examine the underlying molecular mechanisms, we determined the phosphorylation status of AMPK and VPS34. AMPK phosphorylation is required for the initial assembly of autophagosomes (19), whereas VPS34 phosphorylation inhibits lysosome fusion with endosomes/autophagosomes (25). As shown in Fig. 3, WT macrophages treated with 50 pg/ml LPS exhibited a rapid and dramatic reduction in AMPK phosphorylation. On the other hand, 50 pg/ml LPS significantly induced the inhibitory phosphorylation of VPS34 (Fig. 3).

Because we determined that Tollip is involved in the process of lysosome fusion with endosomes/autophagosomes, we further determined the phosphorylation status of AMPK and VPS34 in Tollip-deficient cells. As shown in Fig. 3, Tollip-deficient cells had constitutively reduced levels of AMPK phosphorylation compared with WT cells. LPS failed to further alter the phosphorylation status in Tollip-deficient cells. On the other hand, the inhibitory phosphorylation of VPS34 was constitutively elevated in Tollip-deficient macrophages compared with WT macrophages. LPS failed to further alter the phosphorylation status of VPS34 in Tollip-deficient cells.

**Clearance of Tollip by Super low-dose LPS**—Our data suggest that super-low-dose LPS may disrupt lysosome fusion by compromising the function of Tollip. We previously reported that super-low-dose LPS causes translocation of Tollip to mitochondria (12). A separate study reported that Tollip may undergo ubiquitination and degradation (31). On the basis of these studies, we further tested whether super-low-dose LPS reduces and/or clears Tollip away from late endosomes/lysosomes and subsequently disrupts lysosome fusion with endosomes/autophagosomes. Indeed, as shown in Fig. 4A, we observed that macrophages treated with 50 pg/ml LPS reduced Tollip protein levels in WT macrophages. Furthermore, we tested whether LPS treatment affects Tollip subcellular localization. To test this, WT BMDMs treated with PBS or 50 pg/ml LPS were stained with specific antibodies against Tollip, the late endosome/lysosome marker LAMP1, or the mitochondrial dye MitoTracker Red. Consistent with previous findings (33, 34), Tollip co-localized with late endosomes/lysosomes in resting cells as shown by confocal microscopy, enabling lysosome fusion and cellular homeostasis (Fig. 4B). In sharp contrast, a clear separation of Tollip from LAMP1-positive endosomes was observed in cells treated with 50 pg/ml LPS. Instead, we observed increased co-localization of Tollip with mitochondria in LPS-treated cells (Fig. 4C), consistent with our previous report (12).

Next, we aimed to further confirm that the disruption of lysosome fusion by super-low-dose LPS is mediated by Tollip removal from late endosomes/lysosomes. To achieve this objective, we employed a Tollip mutant MEF cell line. Tollip fulfills its function in lysosome fusion through its critical...
PI(3)P-binding potential of the C2 domain (35, 36). Notably, the Tollip CUE domain compromises the PI(3)P-binding potential of the Tollip C2 domain (35). The Tollip M240A/F241A mutant lacks a functional CUE domain and thus may have constitutive lysosome localization. We hypothesized that super-low-dose LPS may fail to translocate the Tollip mutant away from lysosomes to mitochondria and fail to block the fusion of lysosomes with endosomes/autophagosomes.

To test this hypothesis, we treated WT and Tollip mutant MEFs with either PBS or 50 pg/ml LPS. The distribution of WT and mutant Tollip MEFs was visualized by immunohistochemical staining and confocal analyses. As shown in Fig. 5A, super-low-dose LPS treatment caused translocation of WT Tollip away from late endosomes/lysosomes to mitochondria, as manifested by the separation of Tollip staining from LAMP1 staining. In contrast, mutant Tollip retained its co-localization with LAMP1 in the presence or absence of super-low-dose LPS (Fig. 5A and B).

We functionally tested the fusion of lysosomes with autophagosomes in WT and Tollip mutant MEF cells because MEFs are not proficient in endocytic uptake of zymosan particles. To test the fusion of lysosomes with autophagosomes, we co-stained the cells with the autophagosome marker LC3 and the lysosome marker LAMP1. Indeed, we observed that super-low-dose LPS caused a separation of LC3 and LAMP1 in WT MEFs, an indication of the disruption of lysosome fusion with autophagosomes (Fig. 5B). In contrast, LC3 and LAMP1 were co-localized in Tollip mutant MEF cells with or without LPS treatment (Fig. 5C).

**Impaired Wound Healing and Elevated Inflammation in Tollip-deficient Mice**—We next tested the in vivo relevance of our above observation by using a full skin-deep excision wound-healing model in WT and Tollip-deficient mice. Mice injected with super-low-dose LPS had delayed wound closure compared with mice injected with PBS. Furthermore, Tollip-deficient mice demonstrated significantly reduced wound closure compared with WT mice (Fig. 6A).

To determine whether the in vitro mechanisms remain valid in vivo, we examined the levels of phospho-JNK, p62, and phospho-VPS34 in wound tissues. As shown in Fig. 6B, wound tis-
sues in mice injected with super-low-dose LPS had elevated levels of phospho-JNK and p62 compared with wound tissues in mice injected with PBS, suggesting a disruption of lysosome fusion with autophagosomes and elevated inflammation \textit{in vivo}. Super-low-dose LPS also reduced the total protein levels of Tollip in wound tissues (Fig. 6B). Consistent with our \textit{in vitro} observation, wound tissues from Tollip knock-out mice exhibited elevated levels of phospho-JNK and p62.

This study reveals novel mechanisms that underlie low-grade chronic inflammation induced by super-low-dose endotoxin. Our findings suggest that super-low-dose LPS may selectively induce cell stress and propagate nonresolving low-grade inflammation through the disruption of lysosome fusion with endosomes/autophagosomes. We have demonstrated that super-low-dose LPS inhibited lysosome fusion through the clearance of Tollip from late endosomes/lysosomes and the subsequent suppression of AMPK and VPS34 (Fig. 7).

**DISCUSSION**

Emerging studies point to the concept that an orderly fusion of lysosomes with endosomes/autophagosomes is essential for resolving inflammation (14, 15). Disruption of this process has been shown to cause elevated activation of inflammatory macrophages through exacerbated activation of stress kinases and chronic inflammatory diseases such as atherosclerosis (37). Our data reveal that super-low-dose LPS is highly potent in disrupting lysosome fusion and causing low-grade inflammation. Given the emerging recognition of low-grade endotoxemia in chronic diseases, our work provides a novel mechanism reconciling the pathological impacts of super-low-dose endotoxemia in humans and experimental animals.

Our study reveals a critical role of Tollip in maintaining proper homeostasis in resting cells through facilitating the fusion of lysosomes with endosomes/autophagosomes. We demonstrated that a reduction in Tollip through either gene knock-out or TLR-mediated clearance renders macrophages deficient in lysosome fusion. Our data provide functional relevance to previous biochemical studies reporting the interaction of Tollip with PI(3)P near late endosome/lysosome compartments (29, 33). Tollip was also shown to interact with Tom1, a key molecule involved in lysosome fusion (34). Consistent with our observation, a recent study suggests that Tollip may be involved in autophagy (36). Our study not only extends these previous works, but also provides a clear role of Tollip in the
completion of lysosome fusion with endosomes/autophagosomes in the context of innate macrophages. Our data further reveal that Tollip facilitates lysosome fusion through affecting the activation status of the lipid phosphatase VPS34. Proper lysosome fusion is critically important during the resolution of inflammation through facilitating the clearance of cell debris, microorganisms, and other stress signals (6, 15, 37, 38). Our findings suggest that Tollip may serve as a key target for the modulation of cell stress and inflammation. In the context of super-low-grade endotoxemia, a phenomenon widely present in humans with chronic infection or inflammation (39), we speculate that Tollip may be degraded and/or cleared away from late endosomes/lysosomes. Supporting this hypothesis, our data reveal that super-low-dose LPS can indeed reduce cellular Tollip levels, clear Tollip away from late endosomes/lysosomes, and disrupt lysosome fusion. Thus, depending on its cellular location and binding partner, Tollip may serve as a double-edged sword in inflammation through its lipid-binding and ubiquitin-binding domains (40). Our biochemical data further clarify the inhibitory nature of the Tollip ubiquitin-binding CUE domain in lysosome fusion. We found that the Tollip mutant protein with a modified CUE domain preferentially localizes at late endosomes/lysosomes, and cells carrying this Tollip mutant can maintain proper lysosome fusion when challenged with super-low-dose LPS. Future strategies that explore compounds capable of stabilizing Tollip or retaining Tollip at late endosomes/lysosomes may hold therapeutic potential.

Our work provides a mechanistic explanation for recent clinical studies regarding Tollip polymorphisms in humans (41, 42). For example, humans with a rare Tollip haplotype have reduced Tollip levels and elevated risks for infection and tuberculosis (41). Other studies suggest that Tollip variants may be associated with elevated risks of sepsis, dermatitis, and pulmonary fibrosis (43–45). Our wound-healing model clearly demonstrates a key role for Tollip in the resolution of inflammation during tissue repair. Collectively, our results suggest that reduced lysosome fusion due to Tollip deficiency may be responsible for reduced bacterial clearance and/or inflammatory complications.

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