Toll-like receptor 4 senses oxidative stress mediated by the oxidation of phospholipids in extracellular vesicles

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Oxidative stress produced in response to infection or sterile injury activates the innate immune response. We found that extracellular vesicles (EVs) isolated from the plasma of patients with rheumatoid arthritis or secreted from cells subjected to oxidative stress contained oxidized phospholipids that stimulated cells expressing Toll-like receptor 4 (TLR4) in a manner dependent on its co-receptor MD-2. EVs from healthy subjects or reconstituted synthetic EVs subjected to limited oxidation gained the ability to stimulate TLR4-expressing cells, whereas prolonged oxidation abrogated this property. Furthermore, we found that 15-lipoxygenase generated hydro(pero)xylated phospholipids that stimulated TLR4-expressing cells. Molecular modeling suggested that the mechanism of activation of TLR4 by oxidized phospholipids in EVs was structurally similar to that of the TLR4 ligand lipopolysaccharide (LPS). This was supported by experiments showing that EV-mediated stimulation of cells required MD-2, that mutations that block LPS binding to TLR4 abrogated the stimulatory effect of EVs, and that EVs induced TLR4 dimerization. On the other hand, analysis of gene expression profiles showed that genes encoding factors that resolve inflammation were more abundantly expressed in responses to EVs than in response to LPS. Together, these data suggest that EVs act as an oxidative stress–induced endogenous danger signal that underlies the pervasive role of TLR4 in inflammatory diseases.

INTRODUCTION

Toll-like receptor 4 (TLR4) was the first vertebrate TLR to be characterized with a role in the immune response (1, 2). TLR4 is the only TLR that stimulates signaling pathways mediated by both of the adaptor proteins myeloid differentiation marker 88 (MyD88) and Toll/interleukin-1 (IL-1) receptor (TIR) domain–containing adapter-inducing interferon-β (TRIF), and it requires the presence of a co-receptor, MD-2, for its activation by the pathogen-associated molecular pattern lipopolysaccharide (LPS), a potent immunostimulatory component of Gram-negative bacteria. Although the specific molecular mechanism of TLR4 activation by LPS was revealed by the determination of the crystal structure of the activated complex (3), TLR4 plays roles in a large number of diseases that are not linked to Gram-negative bacterial infections. The requirement for TLR4 for defense against infection by Gram-positive bacteria, fungi, and viruses, and also for the progression of sterile inflammation, including atherosclerosis, rheumatoid arthritis (RA), hemorrhage, ischemia and reperfusion, alcohol-induced neuroinflammation, and neuropathic pain, suggests the participation of endogenous TLR4 agonists that are released or generated under different pathological conditions. Mice with a defect in TLR4 signaling are protected from RA, and TLR4 antagonists provide therapeutic protection in experimental arthritis (4–7). Many endogenous agonists of TLR4 have been proposed (8, 9); however, the molecular mechanisms by which they activate TLR4 and any potential role of the TLR4 co-receptor MD-2 in this process are unclear.

Reactive oxygen species (ROS) are mediators of oxidative stress, which plays a role in homeostasis and defense against infection, but can cause damage to cellular components. Lipids with unsaturated fatty acid chains are particularly prone to oxidation by ROS (10). Atherosclerosis and RA are chronic diseases with inflammatory characteristics (11, 12) involving oxidative stress (13, 14). Oxidized phospholipids have been reported to activate TLR4, although other reports observed inhibition of TLR4 activity (15–19), and the mechanism by which oxidized phospholipids interact with TLR4 has not been clarified.

Extracellular vesicles (EVs) are submicrometer-sized particles, which are composed of a phospholipid membrane bilayer that encapsulates its contents. Included among EVs are large microvesicles, which are shed from the plasma membrane (20), and small exosomes, which are released from intracellular multivesicular bodies (21). The amount of EVs that are released from cells increases under stressful conditions, such as hypoxia, irradiation, oxidative injury, and apoptosis (22, 23). An increased concentration of EVs is detected in the peripheral blood of patients with chronic inflammatory diseases, such as atherosclerosis, RA, thrombembolic events, cancer, and infection (24). Most of the EVs in the peripheral blood originate from platelets, whereas the remaining pool is mainly derived from endothelial cells and lymphocytes (25). EVs serve as a readily available substrate for lipid peroxidation (26). EVs elaborated by activated platelets in joint fluid from patients with RA amplify inflammation in arthritis, and the inflammatory cytokine IL-1β is proposed as the responsible mediator (27).
Here, we report that oxidized hydro(pero)xylated phospholipids, for example, hydroperoxy eicosatetraenoic-phosphatidylethanolamine [H(p)ETE-PEs], produced by 15-LO or by a biological Fenton reaction (FR), a combination of peroxide and iron catalyst, are the active molecular species in EVs that activate the TLR4–MD-2 complex in human embryonic kidney (HEK) 293T cells. Oxidative stress–derived EVs (stressEVs) were identified as agonists of TLR4–MD-2 in cultured cells and in vivo. We suggest that the molecular mechanism by which EVs activate the TLR4–MD-2 complex closely mimics that of LPS, which therefore presents a potential mechanism of receptor activation by both pathogenic and ubiquitous endogenous danger signals that shares the similar structural determinants of the activators. This similarity was corroborated by the effects of point mutations in the LPS recognition site of the TLR4 ectodomain on activation by oxidized phospholipids, by the effects of inhibitors, and by receptor complex dimerization. Despite having a similar mechanism of TLR4 activation to that of LPS, the stressEVs [which we consider as endogenous danger-associated molecular patterns] induced a transcriptional response in bone marrow–derived macrophages (BMDMs) that differed in some aspects from that induced by LPS. The stressEVs differentially increased the expression of several genes whose products contribute to the resolution of inflammation. Together, these results suggest that similar structural principles underlie the activation of TLR4 by a microbial signal (LPS) and an endogenous danger signal (EVs).

RESULTS

EVs from the plasma of RA patients activate nuclear factor-κB signaling in HEK 293T cells in an MD-2–dependent manner

The concentration of EVs in the plasma (native EVs) of RA patients is increased compared to that in the plasma of healthy subjects (28). We found that EVs (fig. S1) from RA patients, but not EVs from healthy subjects, activated nuclear factor-κB in transfected HEK 293T cells expressing TLR4 and its co-receptor MD-2 (Fig. 1A), but did not activate NF-κB signaling in transfected cells expressing TLR4 in the absence of MD-2 (Fig. 1B) or in cells expressing other cell surface TLRs, and plasmids encoding (A) hTLR4, hMD-2 (human MD-2), and hCD14 (human CD14), (B) hTLR4 alone, (C) hTLR1 and hTLR2, or (D) hTLR5 alone were left untreated (control) or were treated for 20 hours with EVs (2000 EV/ml) isolated from the plasma of RA patients (p1 and p2) or healthy donors (h1 and h2). As positive controls, cells were stimulated with LPS (100 ng/ml), Pam3CSK4 (100 ng/ml), or flagellin (20 ng/ml). Dual luciferase assays for NF-κB activity were then performed. Three technical replicates were performed per experiment. Data are representative of three (for A and E) or two (for B to D, F, and G) independent experiments. RLU, relative luminescence unit.
such as TLR1 and TLR2 (Fig. 1C) or TLR5 (Fig. 1D). Incubation of the human monocytic cell line MonoMac6 with EVs from RA patients resulted in an increase in the amounts of IL-1A and IL-6 mRNAs (Fig. 1E), and led to the rapid phosphorylation of the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase 1 (ERK1) and ERK2 in RAW264.7 cells (Fig. 1F).

EVs are composed of a phospholipid bilayer, proteins that are embedded in the membrane, and cellular content that is trapped inside the membrane. The EVs from RA patients retained the ability to activate NF-κB signaling after protease K-dependent digestion of any protein components (Fig. 1G), which suggests that the nonprotein fraction of EVs was the active component. The addition of the human TLR4 (hTLR4–MD-2) antagonist lipid IVα before stimulation with EVs from RA patients inhibited the activation of NF-κB in HEK 293T cells expressing TLR4 and MD-2 (Fig. S2A), which suggests that there is overlap in TLR4 of the binding site for LPS and that for the active component of the EVs. The capacity of plasma samples from healthy subjects and RA patients to neutralize endotoxin in the endotoxin neutralization capacity (ENC) assay was the same for EVs from RA patients and from healthy subjects (Fig. S2B), excluding the possibility that circulating LPS in the plasma samples activated TLR4–MD-2 in the treated cells.

**Oxidative stress induces the generation of TLR4-stimulatory EVs**

Next, we wanted to identify the underlying mechanism by which EVs had to be modified to function as stimulators of signaling in TLR4-expressing cells. Oxidative stress is a common hallmark of chronic diseases. The calcium ionophore A23187 causes oxidative stress and stimulated the shedding of EVs (stressEVs) from HEK 293 cells (fig. S1B) (29). StressEVs from HEK 293 cells (Fig. 2A, and B) and from human umbilical vein endothelial cells (HUVECs) (Fig. S3A) activated NF-κB signaling in BMDMs from C3H/HeN mice and in HEK 293T cells transplanted with plasmids encoding TLR4 and MD-2, but not in BMDMs from the TLR4-deficient C3H/HeJ mouse strain or in HEK 293T cells transplanted with plasmid encoding TLR4 alone. Whereas stressEVs that were injected intravenously into wild-type mice induced IL-6 production after 4 hours, they induced the production of little or low amounts of the cytokine when they were injected into TLR4*/C57BL/6 mice (Fig. 2C), suggesting the in vivo relevance of EVs as endogenous TLR4 agonists. Although TLR6 and CD36 have been proposed as receptors that are activated by oxidized low-density lipoprotein (LDL) (17), we did not observe any stressEV-induced NF-κB activation in transplanted HEK 293T cells expressing combinations of TLR2, TLR6, or CD36 (fig. S3, B and C).

Similar to EVs from healthy subjects, EVs released from HEK 293 cells stimulated with tumor necrosis factor–α (TNF-α) induced little NF-κB activation in transplanted HEK 293T cells expressing TLR4 and MD-2 (fig. S3D). Additionally, quenching ROS by incubating HEK 293 cells with 15 mM N-acetyl cysteine (NAC) before treating them with the calcium ionophore A23187 resulted in a fivefold reduction in the amount of stressEVs that the cells released. Signaling by the TLR4–MD-2 complex is initiated by receptor dimerization. We found that stressEVs induced TLR4 dimerization that was detectable by coimmunoprecipitation, although it was less substantial than that induced by LPS (Fig. 2D). In addition to the essential role of the co-receptor MD-2, membrane-anchored or soluble CD14 enhances LPS-stimulated signaling (30). We found that CD14 also augmented NF-κB activation by stressEVs in TLR4-expressing cells, whereas the addition of serum slightly decreased the extent of activation induced by stressEVs (Fig. 2E), as was observed previously (31), presumably because of the lipid-binding components of the serum. LPS stimulates TLR4 to use either MyD88- or TRIF-dependent signaling pathways, which activate NF-κB signaling or the production of type I interferon (IFN), respectively. We found that stimulation of TLR4-expressing cells by stressEVs exhibited a dose-dependent activation of both the NF-κB reporter (Fig. 2F) and a reporter containing the IFN-β response element (IFN-β RE) (Fig. 2G).

**15-LO generates stimulatory EVs**

The oxidation of phospholipids may occur in an organism through several mechanisms. Lipoxygenases generate hydro(pero)xylated phospholipids by inserting molecular oxygen into polyunsaturated fatty acids. For example, 15-LO produces 15-Hp(ETE)-PEs (32). Lipoxygenase activity is induced in cells stimulated by the calcium ionophore A23187. The phospholipid AAPE (1,2-diacyl-OH-sn-glycero-3-phosphoethanolamine) was oxidized in vitro with 15-LO, which was isolated from soybeans to avoid contamination with LPS. The presence of oxidation compounds was confirmed by mass spectrometry (MS) analysis, which identified compounds with one hydroxyl group [average molecular weight (M₀) 805], two hydroxyl groups on each fatty acid chain, or one hydroperoxy group (M₀ 821), and with one hydroxyl and one hydroperoxy group on each chain (M₀ 837) (fig. S4A). The same 15-Hp(ETE)-PE compounds were also identified in stressEVs (fig. S4B), as detected previously on the outer leaflet of the plasma membrane (32). The 15-LO–generated oxidation compounds of AAPE [15-Hp(ETE)-PE] activated NF-κB signaling and target gene expression in HEK 293T and MonoMac6 cells expressing TLR4 and MD-2 (Fig. 3A and fig. S4C), whereas the addition of a specific 15-LO, inhibitor I, before 15-LO blocked this effect (Fig. 3A), demonstrating the requirement for enzymatically active 15-LO. 15-Hp(ETE)-PE also induced the expression of IL6 in BMDMs from C3H/HeN mice, but not from C3H/HeJ mice, which have defective TLR4 (Fig. 3B).

To exclude the possibility that the EVs that we used were contaminated with LPS, we prepared synthetic EVs (synEVs) (fig. S1C) composed of the same phospholipid mixture as that found in the native EVs (33). The 15-LO–treated synEVs activated NF-κB signaling and target gene expression in HEK 293T and MonoMac6 cells (Fig. 3C and fig. S4D), suggesting that the enzymatic activity of 15-LO was required for the generation of stimulatory EVs. To determine whether 15-LO activity could directly generate stimulatory phospholipids, we added the enzyme to the culture medium of BMDMs. Low concentrations of 15-LO resulted in increases in the amounts of Il6 and Il1b mRNAs in BMDMs from C3H/HeN mice, but not in BMDMs from C3H/HeJ mice (Fig. 3D). Both inhibitors of 15-LO (inhibitor I and ebetol) reduced the activation of NF-κB signaling in TLR4 and MD-2–expressing HEK 293T cells exposed to 15-LO (fig. S4E), confirming the importance of 15-LO activity. Although we did not observe NF-κB–dependent gene expression in 15-Hp(ETE)-PE–treated BMDMs from C3H/HeJ mice, we tested their effects on HEK 293T cells expressing the same combinations of TLRs and CD36 as were tested with stressEVs. Cells showed some activation of NF-κB signaling in response to either AAPE or the 15-LO oxidation compounds (fig. S5).

Another strong and physiologically relevant producer of ROS is the FR, which is catalyzed by ferrous ion. This reaction causes lipid peroxidation in atherosclerosis and in injuries to the central nervous system in which heme containing iron as the peroxidation catalyst may be reoxidized (34). Initially, inactive EVs from healthy subjects and synEVs were converted to activators of human monocytes and mice BMDMs from C3H/HeN mice, but not the TLR4-deficient C3H/HeJ mice, by the FR, as determined by measuring the extent of expression of NF-κB target genes (Fig. 3, E and F). This resulted in the ability to increase the abundance of Il1b mRNAs after 30 min (Fig. 3, E and F), demonstrating that oxidative processes other than those dependent on 15-LO can lead to the production of endogenous TLR4 agonists.
**LPS and EVs differ in the transcriptional profiles that they induce**

The physiological response of an organism to bacterial infection should be distinct from that to sterile injury, because the latter response does not need to block replicative pathogens. We investigated the differences in the transcriptional responses of mouse BMDMs to incubation with LPS or stressEVs through genome-wide transcriptional microarray analysis. Pairwise signal comparisons (Pearson’s correlation) of all nine arrays (LPS, stressEVs, and controls in triplicate) demonstrated a high concordance within treatment arrays (that is, there was low biological replicate variance) (fig. S6A). Stimulation of BMDMs by stressEVs or LPS resulted in 9169 or 9150 differentially expressed transcripts [false discovery rate (FDR) < 0.01], **Fig. 2. EVs generated under conditions of oxidative stress have immunostimulatory activity.** (A to C) StressEVs were isolated from HEK 293 cells that were stimulated with 12 μM A23187 for 1 hour. (A) HEK 293T cells transfected with luciferase and Renilla reporter plasmids and plasmids encoding hTLR4, hMD-2, and CD14 or hTLR4 alone, as indicated, were left untreated (control) or were treated for 20 hours with LPS (100 ng/ml) or with stressEVs (5000 or 20,000 EVs/ml). Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. (B) BMDMs from C3H/HeN mice or C3H/HeJ mice were left untreated (control) or were treated for 8 hours with LPS (100 ng/ml) or with stressEVs (5000 or 10,000 EVs/ml). The amounts of IL-6 secreted by the cells were determined by enzyme-linked immunosorbent assay (ELISA). Three technical replicates were performed per experiment. (C) Wild-type (WT) C57BL/6 mice and TLR4−/− mice of the same strain were injected intravenously with phosphate-buffered saline (PBS) or stressEVs (90,000 EVs). Four hours later, the concentrations of IL-6 in the serum were measured by ELISA. Each symbol represents an individual mouse. Data are means ± SD from eight or six mice for each condition. **P < 0.05. (D) HEK 293T cells transfected with plasmids encoding hMyc (human Myc)–TLR4, hTLR4–green fluorescent protein (GFP), hMD-2, and hCD14 were left untreated or were treated with stressEVs (20,000 EVs/ml) or with LPS (1 μg/ml) for 4 hours. Cell lysates were then subjected to immunoprecipitation with an anti-GFP antibody to pull down hTLR4-GFP, and samples were analyzed by Western blotting with the indicated antibodies. Lysates labeled + ctrl.” were subjected to IP with an anti-Myc antibody. Lysates labeled as “− ctrl.” were from cells transfected with empty vector. The blot was stripped between incubations with different primary antibodies. The molecular mass standard (stand.) corresponds to 130 kD. The vertical line indicates noncontiguous blots. (E) HEK 293T cells transfected with luciferase and Renilla reporter plasmids and plasmids encoding hTLR4 and hMD-2 with or without plasmid encoding hCD14, as indicated, were left untreated (control) or were treated with LPS (100 ng/ml) or with stressEVs (5000 EVs/ml) in the presence or absence of fetal bovine serum (FBS). Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. (F and G) HEK 293T cells transfected with plasmids encoding hTLR4, hMD-2, hCD14, and either (F) an NF-κB luciferase reporter plasmid or (G) an IFN-β RE luciferase plasmid were left untreated (control) or were treated with the indicated concentrations of LPS (ng/ml) or stressEVs (EVs/ml). Dual luciferase tests for (F) NF-κB or (G) IFN-β RE activity were then performed. Three technical replicates were performed per experiment. Data are representative of three (for A and E) or two (for B, C, D, F, and G) independent experiments.
respectively, in comparison to the control cells. This finding is similar to that of a previous report on transcriptional activation by LPS (35), confirming that our array detected known LPS-induced global transcriptional changes. A considerable proportion (~25%) of the differentially expressed genes was unique to either stressEV -treated cells (2288) or LPS-treated cells (2242) (Fig. 4A). Nevertheless, even with the increased stringency of an FDR < 0.001, the pairwise comparison between treatments with stressEVs and LPS produced 777 differentially expressed targets, supporting the conclusion that determination of a large number of distinct transcriptional responses. Gene Set Enrichment Analysis (GSEA) (36) was performed to identify sets of genes with statistically significant differences in expression between stressEV- and LPS-treated cells. In the GSEA analysis, Gene Ontology was used to annotate gene sets. Three functionally related gene sets (Fig. 4B) identified in cells stimulated with stressEVs belong to processes of regulation of immune response, cytokine receptor binding, and regulation of metabolic processes by phosphorylation. A singleton gene set contained genes enriched in the process of cell proliferation regulation. These results suggest that stressEVs as the potential danger signal induce a subset of distinct transcriptional changes.

Because we showed that the responses of HEK 293T and MonoMac6 cells to stressEVs and EVs isolated from RA patients were similar, we next examined whether the genes with the largest change in expression in response to stressEV displayed enrichment for genes involved in the pathology of RA (based on the Ingenuity Knowledge Database). At the level of the relevant individual genes, stressEVs statistically significantly enhanced the expression of genes involved in inflammation-resolving functional pathways, which were not induced by LPS. The expression of a group of genes encoding cytokines and chemokines, notably Il1b, correlated with exposure to stressEVs (fig. S6B). The connection of the genes, whose expression was most significantly differentially transcribed between stressEVs and LPS, with regulation either by IL-4 [for Il5, Il13, Ccl24, Ccl11, and Ccl11 (37)] or by TLR4 [Il23a and
Ifnb1 \(^{(38)}\) has been previously established. All of these cytokines and chemokines are associated with RA, providing an independent indication of the physiological relevance of our stressEV-inducible BMDM transcriptome analysis. StressEVs, but not LPS, induced an increase in Il4 mRNA abundance in BMDMs from wild-type mice, but not TLR2\(^{-/-}\)/TLR4\(^{-/-}\) C57BL/6 mice were treated with LPS (1 ng/ml) or with stressEVs (5000 EVs/ml) for 4 hours. Samples were subjected to qPCR analysis to determine the relative abundances of Il4 mRNA, which are expressed as fold increases compared to the mRNA amounts in untreated cells. Three technical replicates were performed per experiment. (D) BMDMs from WT C57BL/6 mice were left untreated (control) or were treated for the indicated times with LPS (1 ng/ml) or stressEVs (10,000 EVs/ml). The amount of IL-4 secreted by the indicated cells was determined by high-sensitivity ELISA. Three technical replicates were performed per experiment. Data in (C) and (D) are representative of two independent experiments.
The extent of phospholipid oxidation determines the stimulatory capacity of EVs

There are conflicting data regarding the effect of oxidized phospholipids on the activation of TLR4. Whereas some reports show that oxidized phospholipids activate TLR4 (39, 40), others indicate that oxidized phospholipids suppress TLR4 activation (16, 41). To attempt to resolve this controversy, we analyzed the effect of the time of phospholipid oxidation on TLR4 activation. When EVs from healthy subjects or synEVs underwent 30 min of oxidation, they stimulated increased IL8 and Il1b mRNA abundance in MonoMac6 cells (Fig. 5A), whereas if the EVs underwent 24 hours of oxidation, this ability was inhibited (Fig. 5A). Similarly, 24 hours of oxidation of active stressEVs reduced their ability to stimulate IL-6 production (Fig. 5B). Furthermore, prolonged oxidation of EVs decreased their ability to stimulate IL-6 secretion when injected into mice (Fig. 5C), although not statistically significant. Additional confirmation of the role of oxidation in generating TLR4-activating phospholipids was provided by the effect of the antioxidant NAC. Addition of NAC before the oxidation of synEVs with the FR completely blocked their ability to stimulate IL6 secretion (Fig. 5D), suggesting that, once formed, the TLR4-activating molecular species are stable unless they are further oxidized.

The molecular mechanism of TLR4 activation by partially, but not fully, oxidized phospholipids mimics LPS recognition

To provide mechanistic insight into the activation of TLR4 by oxidized phospholipids, we performed a modeling study of the docking of oxidized phospholipids onto the TLR4–MD-2 complex. 15-HETE-PE docked into the MD-2 pocket even when the hydrophobic pocket of MD-2 was partially occupied by resident fatty acids or other phospholipids (Fig. 6A), which have been observed in the crystal structures of MD-2 (42) and MD-1 (43, 44). Docking of 15-HETE-PE was similar to that of LPS observed in the LPS–MD-2–TLR4 complex (Fig. 6B) (45). Phospholipids in mammalian cells are preferentially oxidatively modified at unsaturated acyl chains at the sn-2 position, which increases the polarity of the modified chain (fig. S7A, left), directing docking of the phospholipid molecule onto MD-2. In the most favorable docking arrangement, the end of the hydroxyl acyl chain of the phospholipid protruded out of the MD-2 pocket and was poised to interact with the secondary binding site of the TLR4 ectodomain, thus mediating receptor dimerization (Fig. 6C). To mediate the interaction with this secondary hydrophobic binding site in the TLR4 ectodomain (residues Phe430 and Phe463 of hTLR4) (46), the solvent-exposed acyl chain of lipid A

Fig. 5. The extent of phospholipid oxidation determines the potency of TLR4 activation. (A) EVs from healthy donors and synEVs were left unoxidized or were oxidized for 30 min or 24 hours through the FR. MonoMac6 cells (left) and BMDMs from C3H/HeN mice (right) were treated for 4 hours with LPS (100 ng/ml), the indicated unoxidized or oxidized EVs (5000 EVs/ml), or buffer from the FR (buf FR). Samples were then subjected to qPCR analysis to determine the relative abundances of Il6 and Il1b mRNAs, as indicated, which are expressed as fold increases compared to the mRNA amounts in untreated cells. Technical replicates were performed per experiment. (B) BMDMs from C3H/HeN mice were left untreated (control) or were treated with LPS (1 ng/ml), stressEVs (5000 EVs/ml), or stressEVs that were subjected to 24-hour oxidation by FR (stressEVs + FR, 5000 EVs/ml). The amounts of IL-6 that were secreted by the BMDMs were determined by ELISA. Three technical replicates were performed per experiment. (C) C57BL/6 mice were injected intravenously with PBS, stressEVs (300,000 EVs), or stressEVs that were oxidized for 24 hours by FR (300,000 EVs). Twenty-four hours later, the concentrations of IL-6 in the serum were determined by ELISA. Each symbol represents an individual mouse. Data are means ± SD from four mice for each condition from a single experiment. (D) SynEVs were left unoxidized or were oxidized for 30 min or 24 hours through the FR. In addition, to prevent oxidation, NAC was added before (NAC + FR) 30 min of oxidation to protect oxidized compounds, and samples were further incubated for 24 hours. RAW264.7 cells were then treated for 4 hours with LPS (100 ng/ml) or the indicated synEVs (25,000 EVs/ml). Samples were subjected to qPCR analysis to determine the relative abundances of Il6 mRNA, which are expressed as fold increases compared to the mRNA amounts in untreated cells. Technical replicates were performed per experiment. Data are representative of two (for A and B) or three (for D) independent experiments.
that is bound to MD-2 must be sufficiently long. On the other hand, the extended oxidation of phospholipid results in the truncation of the acyl chain at the position of the double bond (fig. S7A, right), which can thus no longer provide the interaction with the TLR4 ectodomain that is required for receptor dimerization. This may explain why the extended oxidation of phospholipid resulted in EVs that failed to activate TLR4. We tested the involvement of the secondary hydrophobic binding site on the TLR4 ectodomain, which mediates interactions with the acyl chain of the lipid A or, according to the molecular model, the oxidized acyl chain of the phospholipids, by analyzing the activity of mutants within this site. The mutant F463A strongly reduced and the mutant F440A completely abolished the activation of hTLR4 by both EVs and LPS (Fig. 6D), confirming the molecular model of TLR4 activation by oxidized phospholipids.

According to the docking study, the binding of one molecule of LPS or two hydro(pero)xyl phospholipid molecules is required to generate the TLR4-activating complex [MD-2–LPS or MD-2–(oxPL)2], because each combination exposes an acyl chain at the edge of the binding pocket of MD-2 (fig. S7B, left and right). Different anionic phospholipids were found to bind to MD-2 (fig. S8). On the other hand, a single hydro(pero)xyl phospholipid molecule can be completely buried in the hydrophobic pocket of MD-2 (fig. S7B, middle), resulting in an inactive MD-2 complex, thus inhibiting the binding of LPS to TLR4. This predicted biphasic, concentration-dependent response model of stimulation of TLR4 by oxidized phospholipid in the presence of LPS was confirmed experimentally (Fig. 6E). Reduced concentrations of EVs inhibited the stimulation of TLR4 in HEK 293T cells by LPS, whereas in the presence of increased concentrations of EVs, LPS regained the ability to activate TLR4 (Fig. 6F, right).

**DISCUSSION**

Oxidative stress is an almost universal hallmark of the response to infection and different types of sterile tissue injury. Whereas the effect of activation of different TLRs on the generation of ROS is well characterized, the effect of oxidative stress on the activation of TLR4 and the molecular

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**Fig. 6.** Molecular models of the docking of hydro(pero)xylated phospholipids onto the TLR4–MD-2 complex and simulation of TLR4 response. (A and B) 15-HETE-PE (red) docks into the MD-2 (yellow) structure with residential fatty acids observed in the crystal structure (A, magenta). The hydroxylated sn-2 chain protrudes from the MD-2 binding pocket and is available for interaction with the hydrophobic patch of the TLR4 ectodomain (A and B, green) (residues Phe440 and Phe463, cyan) and hydrophobic solvent-exposed residues of MD-2, similar as lipid A (B, blue). (C) Modeling suggests that formation of a complex between hydroxylated phospholipid, MD-2, and TLR4 causes dimerization of the TLR4 ectodomain and receptor activation by the same types of interactions and geometry that occur during the activation of TLR4 by lipid A. (D) HEK 293T cells transfected with luciferase and Renilla reporter plasmids and plasmids encoding WT hTLR4, the F440A mutant hTLR4, or the F463A mutant TLR4 together with plasmids encoding hMD-2 and hCD14 were left untreated (control) or were treated with LPS (100 ng/ml) or stressEVs (5000 EVs/ml) for 20 hours. Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. (E) Simulation of the response of TLR4 activation in the presence of a fixed amount of LPS titrated by the increasing concentration of oxidized phospholipid (oxPLs). (F) HEK 293T cells transfected with luciferase and Renilla reporter plasmids and plasmids encoding WT hTLR4, hMD-2, and hCD14 were left untreated (control) or were treated for 20 hours with the indicated concentrations of stressEVs (EVs/ml) in the absence (left) or presence (right) of LPS (1 ng/ml). Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. Data in (D) and (F) are representative of three independent experiments.
mechanism involved are less well understood. We present a potential explanation for the prominent role of TLR4 in sensing oxidative stress that is mediated through the oxidation of phospholipids. Oxidized phospholipids can be generated directly within cells, or they can be disseminated to other cells through EVs. In a previous report on the role of EVs in RA, it was suggested that IL-1β contained within EVs derived from platelets may underlie joint inflammation in patients with RA (27). Our results suggest that oxidative stress is a prerequisite for the formation of EVs that can then activate the inflammatory response by stimulating TLR4. Enhanced lipid peroxidation compounds in chronic diseases and trauma are recognized as important markers of disease conditions (47, 48). Oxidized phospholipids represent a physiologically relevant endogenous signal for the innate immune response because they are formed and released under various pathological conditions. We found that cellular activation by EVs required both TLR4 and MD-2, but not other TLRs (Figs. 1 and 3, C and D), including the CD36-TLR4-TLR6 complex, which was previously proposed to be activated by oxidized LDL in an MD-2-independent manner (17). The inhibitory effect of a ROS quencher during, but not after, oxidation of EVs demonstrated that this type of TLR4 activator is stable, which enables active EVs to have a systemic effect, remote from the initial site of oxidative stress.

We demonstrated the activation of EVs by oxidation through 15-LO and by the FR, which both play roles under different pathological conditions. Furthermore, we identified a biphasic role of phospholipid oxidation: partial oxidation led to the generation of TLR4-stimulatory EVs; however, extensive oxidation led diminished activity. Oxidation may also lead to the generation of TLR4 antagonists as shown previously (16, 18). This biphasic oxidation effect potentially resolves the controversy on the mediation of the innate immune response by oxidized phospholipids (19, 39, 49). Different methods were used to prepare the active EVs, including synthetic phospholipids and the subsequent characterization of the EVs as activators of TLR4 in mouse models and cell lines excluded the presence of any other TLR4-activating contaminants.

MS analysis indicated that the presence of TLR4 agonists in the EVs depended on the catalytic activity of 15-LO. A deficiency in 12/15-LO reduces oxidative stress in the brain (50) and alleviates inflammatory arthritis (51) and atherosclerosis (52). Additionally, collagen-induced arthritis in mice stimulates the production of 12-HETE-PE in platelets (53). Nunemaker et al. showed that 12-LO-deficient mice are resistant to the inflammatory effects of obesity induced by a Western diet (54), similar to results from experiments with C3H/HeJ mice with a loss-of-function mutation in TLR4 (55). We demonstrated that a mixture of 15-Hp(ETE)-PE compounds produced by 15-LO and the direct addition of 15-LO to HEK293T cells and BMDCs stimulated NF-κB activation and cytokine production. Although most of the 15-H(2)ETE from activated monocytes belong to the PE phospholipid class (53), it is likely that 15-(p)ETE compounds with other phospholipid head groups can act as TLR4 agonists, because we detected the binding of several anionic phospholipids to MD-2. Also, we speculate that structurally similar 15-ketoecosatetraenoic acid-phosphatidylethanolamines (15-KETE-PEs), which activate peroxisome proliferator-activated receptor-γ (36), might act as TLR4 agonists. Our results suggest that lipooxygenases may act as a link between oxidative stress and the (patho)physiological role of TLR4.

Despite seeming to activate the same receptor through a similar molecular mechanism, LPS and stressEVs were distinguished by their ability to stimulate the expression of different genes. This suggests that cells of the innate immune system may differentially interpret pathogenic from endogenous sources of TLR4 agonists. Both types of stimulus induced the expression of genes encoding proinflammatory cytokines; however, stressEVs also increased the expression of genes encoding cytokines that stimulate the differentiation of M2 (alternative) macrophages, including IL-4. M2 macrophages mediate resolution of inflammation, promotion of wound repair, and tissue healing. This difference in signaling between LPS and EVs could be a result of different stabilities of the activated ligand-receptor complex and the resulting kinetics of TLR4 activation, or could be caused by interactions with additional components of the signaling complex that differentially affect different downstream signaling pathways.

In conclusion, our results suggest that hydro(pero)xylated phospholipids act as endogenous TLR4-activating danger signals, which suggests that TLR4 can function as a sensor of oxidative stress. A ligand-binding model provides an explanation for the unexpected biphasic, concentration-dependent response to LPS and oxidized phospholipids also observed by others (19, 32). Our results suggest that EVs produced during the establishment of chronic inflammation, accompanied by oxidative stress, contribute to the innate immune response as endogenous agonists of TLR4. In this way, EVs may be involved in many other inflammatory processes that involve TLR4 activation besides RA. On the other hand, the inhibition of TLR4–MD-2 signaling by advanced oxidation compounds of phospholipids (16, 18, 19) may be an endogenous mechanism to restrict excessive stimulation of TLR4. Our results suggest that TLR4 antagonists should be considered as therapeutics for chronic inflammatory diseases. As an example, the TLR4 antagonist eritoran decreases the mouse mortality in influenza infection (57) in which oxidative stress may have stimulated TLR4-dependent inflammation.

MATERIALS AND METHODS

Reagents

LPS (from Salmonella abortus equi HL83) was provided by K. Brandenburg (Forschungszentrum Borstel). Pam3CSK4 and flagellin were obtained from InvivoGen. Antibodies specific for phosphorylated pERK1/2 and α- and β-tubulin were from Cell Signaling Technology; horseradish peroxidase (HRP)-conjugated anti-mouse antibody was from Santa Cruz Biotechnology; HRP-conjugated anti-rabbit antibody and anti–c-Myc antibody were from Sigma; and an anti-GFP antibody was from Life Technologies. LPS from Escherichia coli O55:B5, proteinase K, 1-α-lysoPC, sphingomyelin, 1-α-PC, 3-sn-lysoPE, A23187, and 3-sn-PE were obtained from Sigma. Recombinant human TNF-α (hTNF-α) protein was obtained from Life Technologies, and lipid IVa was from Peptide Institute Inc. 1-α-PS, 1-α-PI, 1-oleoyl-2-hydroxy-sn-glycerol-3-PS, tetra-oleoyl CL, tetramyristoyl CL, and 1,2-diarachidonyl-sn-glycero-3-phosphoethanolamine (AAPE) were purchased from Avanti. 15-LO, 15-LO, and inhibitor 1 were obtained from Cayman.

Isolation, preparation, and oxidation of EVs and phospholipids

Blood samples were mixed with sodium citrate and centrifuged. Plasma was separated from the cells, and 250 μl of plasma was centrifuged twice at 17,570g for 30 min at 20°C to isolate EVs. All studies were approved by the ethical committee of the Medical faculty of the University of Ljubljana. SynEVs were prepared by mixing determined phospholipids in proposed ratios, as described previously (33). The phospholipid mixture was dried, reconstituted, and hydrated in PBS at 65°C for 2 hours. SynEVs were prepared by pressing the mixture through a 400-μm filter (Whatman). StressEVs were produced by HEK 293 cells or HUVECs that were stimulated with 12 μM A23187 for 1 hour. EVsTNFα were produced by stimulation of HEK 293 cells with hTNF-α (50 ng/ml) for 24 hours. EVs were isolated from culture medium by ultracentrifugation twice at 100,000g for 1 hour and then were resuspended in PBS. The concentrations of EVs were determined on a Coulter EPICS Alfa flow cytometer.
Molecular docking experiments
The structures of 15-HETE-PE and LPS were built and docked into the structure of hMD-2 with TLR4. The tertiary structure of MD-2 from the complex with TLR4 and Ra-LPS [Protein Data Bank (PDB) ID: 3FXI] was selected. Part of its hydrophobic pocket was filled with the residing fatty acids observed in PDB # 2E56 (42). Docking was performed with the Autodock Vina algorithm (60). The recommended scoring function from the PDBeBind set and default optimization parameters for the iterated local search global optimizer of Vina were used. The program was run in a multithreaded mode [central processing units (CPUs) = 4]. Docked solutions were evaluated for the formation of the complex with the TLR4 ectodomain based on superposition of the TLR4-MD-2 complex on LPS (3FXI).

MD-2–LPS–oxPL binding equilibrium simulation
The equilibrium between different species to MD-2, free, with bound LPS and one or two partially oxidized phospholipid molecules was solved on the basis of the single assumption that the concentration of free oxidized phospholipids is constant, which is based on the addition of a more than 100-fold excess of the amount of oxidized phospholipid compared to that of MD-2 and LPS to achieve the inhibition of TLR4 activation by partially oxidized phospholipids of EVs. Equilibrium was defined between the species MD-2, MD-2–LPS, MD-2–oxPL, and MD-2–oxPL₂, where only MD-2–LPS and MD-2–oxPL₂ led to TLR4 activation.

Microarray experiments
BMDMs from C57BL/6 mice were left untreated (control) or were treated for 4 hours with stressEVs or LPS. RNA was extracted from the BMDMs with the PureLink RNA mini kit (Life Technologies), and RNA integrity was estimated with an Agilent 2100 bioanalyzer (Agilent Technologies). The RNA integrity numbers of all samples were between 8.2 and 9.1. RNA was hybridized to Mouse Affymetrix Genechip 2.0 arrays according to standardized protocols at the Ark Genomics Centre. All arrays passed the quality control assessed by the Affymetrix Expression Console software. Expression values were summarized with the Robust Multi-array Average method (61). The number of differentially expressed genes in BMDMs treated with stressEVs or LPS compared to control BMDMs was determined with the Bioconductor package limma (62). The FDR was computed with the Benjamini-Hochberg method for multiple hypothesis testing. The affected biological processes were analyzed by the GSEA method (36) under high-stringency statistical analyses (FDR < 0.001, P < 0.001). Sets of genes that are correlated with stressEVs formed clusters of sets—each cluster contained gene sets that shared some genes. We manually identified four clusters, one of them was a singleton. Therefore, three clusters of gene sets and one singleton gene set were correlated with stressEVs when compared to LPS treatment. Data were visualized by Enrichment Map framework (63).

Preparation of recombinant MD-2
Recombinant MD-2 protein was produced in E. coli as previously described (64), through the solubilization of inclusion bodies and their purification and refolding with reversed-phase column chromatography. The biological activity of MD-2 was tested in experiments with HEK 293 cells that were transfected with luciferase and Remilla reporter plasmids and plasmid encoding TLR4, and then stimulated with LPS.

ELISA assays
Polyclonal chicken antibodies against hMD-2 were used to coat a 96-well microtiter plate (Nunc). MD-2 wild type recombinant protein (1 μm) was preincubated with individual phospholipid compounds (in a range of concentrations).
concentrations from 0 to 50 μM) for 2 hours at 25°C. As a negative control, compounds were preincubated in buffer. MD-2 that was bound to the chicken anti-MD-2 antibodies was detected with a mouse anti–hMD-2 monoclonal antibody (9B4) followed by an HRP-conjugated secondary goat anti-mouse immunoglobulin G antibody (Santa Cruz Biotechnology). HRP was detected with ABTS substrate, and color intensity was measured at 420 nm.

**Statistical analysis**

Data were analyzed by analysis of variance (ANOVA) to determine whether the differences between the group means were greater than expected by chance and to define the 95% confidence interval for the difference between the pairs of group means (post tests). To determine which treatment group was statistically significantly different from the control group, Dunnett’s multiple comparisons test was used. When all possible pairs of means were compared, we applied Tukey’s multiple comparisons test. Representative experiments are shown. Experiments were performed in triplicate and the results are presented as means ± SD. *P < 0.1; **P < 0.05; ***P < 0.01; ****P < 0.005; n.s., not significant.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Determination of the numbers of various EVs, stressEVs, and synEVs.

Fig. S2. Inhibition of LPS signaling also inhibits the activity of EVs.

Fig. S3. StressEVs activate the TLR4-MD-2 complex.

Fig. S4. 15-LO-hydroperoxidized PE compounds stimulate cytokine production, and 15-LO stimulates NF-κB signaling in a TLR4- and MD-2-dependent manner.

Fig. S5. 15-LO-hydroperoxidized PE compounds stimulate NF-κB activity in a TLR2-dependent manner.

Fig. S6. Heat maps of the pairwise signal comparison (Pearson’s correlation) and the 50 genes whose expression was most affected by stressEVs.

Fig. S7. Schematic representation of different phospholipid oxidation compounds, molecular complexes, and equilibria between MD-2 and different molecular species.

Fig. S8. Phospholipids bind to MD-2 at a region that overlaps with its LPS-binding site.

**REFERENCES AND NOTES**


Toll-like receptor 4 senses oxidative stress mediated by the oxidation of phospholipids in extracellular vesicles
Science Signaling 8 (381), ra60. [doi: 10.1126/scisignal.2005860]

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