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Group X Secretory Phospholipase A2 Enhances TLR4 Signaling in Macrophages

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Secretory phospholipase A2 (sPLA2) hydrolyze glycerophospholipids to liberate lysophospholipids and free fatty acids. Although group X (GX) sPLA2 is recognized as the most potent mammalian sPLA2 in vitro, its precise physiological function(s) remains unclear. We recently reported that GX sPLA2 suppresses activation of the liver X receptor in macrophages, resulting in reduced expression of liver X receptor-responsive genes including ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1), and a consequent decrease in cellular cholesterol efflux and increase in cellular cholesterol content (Shridas et al. 2010. Arterioscler. Thromb. Vasc. Biol. 30: 2014–2021). In this study, we provide evidence that GX sPLA2 modulates macrophage inflammatory responses by altering cellular cholesterol homeostasis. Transgenic expression or exogenous addition of GX sPLA2 resulted in significantly dampened response to LPS. Plasma concentrations of inflammatory cytokines were significantly lower in GX sPLA2-deficient mice compared with wild-type mice after LPS administration. Thus, GX sPLA2 amplifies signaling through TLR4 by a mechanism that is dependent on its catalytic activity. Our data indicate this effect is mediated through alterations in plasma membrane free cholesterol and lipid raft content. The Journal of Immunology, 2011, 187: 482–489.

Atherosclerosis is a disorder of lipid metabolism, as well as a chronic inflammatory disease. Macrophages play a central role in the development of atherosclerosis through the cellular accumulation of lipid and the production of inflammatory mediators (1, 2). By multiple mechanisms, cellular cholesterol content plays an important role in modulating macrophage inflammatory responses. Free cholesterol loading leads to excess cholesterol in the normally cholesterol-poor endoplasmic reticulum membrane, which stimulates a stress pathway known as the unfolded protein response and subsequent activation of NF-κB and MAPK inflammatory signaling pathways (3). This pathway has been suggested to underlie the observed relationship between free cholesterol accumulation and inflammation in advanced atherosclerotic lesions (3). In contrast, increases in plasma membrane free cholesterol have also been shown to enhance macrophage inflammatory responses through an MyD88-dependent signaling pathway that is independent of an endoplasmic reticulum (ER) stress response (4, 5). This pathway has been identified in macrophages that are deficient in ATP-binding cassette transporter A1 (ABCA1) and/or G1 (ABCG1), and hence have increased plasma membrane free cholesterol and lipid raft content because of defects in cellular cholesterol efflux. Such alterations in plasma membrane free cholesterol result in increased signaling through TLR and enhanced inflammatory responses to LPS (4, 5), a known ligand for TLR4.

The possibility that secretory phospholipase A2 (sPLA2) enzymes modulate macrophage inflammatory responses has also been the focus of investigation. Of the 10 sPLA2s described in mammals, group X (GX) sPLA2 has the highest capacity to hydrolyze phosphatidylcholine and is, therefore, the most potent in hydrolyzing intact mammalian membranes in vitro (6). The generation of arachidonic acid by GX sPLA2 has the potential to create a number of bioactive lipid mediators, including PGs, leukotrienes, thromboxanes, and lipoxins, which are known to exert both proinflammatory and anti-inflammatory effects. The ability of GX sPLA2 to modulate inflammatory responses in vitro and in vivo has been investigated in a number of studies, with disparate results. Treatment of primary human lung macrophages with GX sPLA2 produces a concentration-dependent increase in TNF-α and IL-6 release. Interestingly, this activity was attributed to GX sPLA2’s ability to serve as a high-affinity ligand for the M-type receptor rather than through the hydrolysis of membrane phospholipids (7). In contrast, overexpression of GX sPLA2 in murine macrophage-like RAW264.7 cells was reported to inhibit macrophage activation on stimulation with LPS as evidenced by reduced cell adhesion, NO production, and TNF-α secretion (8). These effects were attributed to an increased production of PGE₂.
and PGJ2, and enhanced release of IL-6. However, in the same study, the authors also reported that transgenic expression of GX sPLA2 in macrophages leads to fatal pulmonary defects, suggesting this enzyme plays a critical role in inflammatory lung disease (8). A recent report suggested a role for GX sPLA2 in human asthma, possibly through dysregulation of eicosanoid production (9). In a murine model of asthma, GX sPLA2 deficiency leads to significantly reduced OVA-induced eicosanoid production, inflammatory cell infiltration, smooth muscle cell layer thickening, and subepithelial fibrosis (10). Mice that lack GX sPLA2 also exhibit reduced myocardial infarct size in response to ischemia/reperfusion injury, which has been attributed to attenuated neutrophil cytotoxic activities (11). More recently, GX sPLA2 was shown to induce the production of vascular endothelial growth factors by human lung macrophages through a mechanism that is independent of its catalytic activity, suggesting a role in inflammatory angiogenesis (12). Thus, available data concerning the impact of GX sPLA2 on macrophage inflammatory responses and the role of enzymatic activity and eicosanoid production on such effects have been contradictory.

Recent studies in our laboratory provide an alternative mechanism whereby GX sPLA2 may regulate macrophage inflammatory responses. We have shown that GX sPLA2 suppresses macrophage expression of ABCA1 and ABCG1, leading to reduced cellular cholesterol efflux and increased cellular cholesterol content (13). In this study, we conducted comprehensive gain-of-function and loss-of-function studies to more clearly define the role of GX sPLA2 in macrophage inflammatory responses. Because TLR4 signaling pathways have been shown to be modulated by plasma membrane free cholesterol and are implicated in atherosclerosis (14), we investigated whether GX sPLA2 modulates macrophage responses to LPS.

Materials and Methods

Animals and experimental treatments

Targeted deletion of the GX sPLA2 gene was performed by InGenious Targeting Laboratory (Stony Brook, NY) using embryonic stem cells derived from C57BL/6 mice (15). Heterozygous GX sPLA2+/− mice were bred to produce GX sPLA2−/− mice (GX knockout [KO]) mice that were used for the experiments. Mice deficient in TLR4 and MyD88 (16) were generously provided by Dr. L. Curtiss (Scripps Research Institute) and K. Moore (Harvard Medical School), respectively. Male and female mice (3–5 mo old) were maintained on a 10-h light/14-h dark cycle and received standard mouse chow and water ad libitum. For experiments with LPS, J774 cells were incubated with 100 ng/ml LPS in the presence or absence of 0.1 μg/ml mGX sPLA2 in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) before analysis. For other experiments, J774 cells were incubated with 100 ng/ml LPS in the presence or absence of 0.1 μg/ml mGX sPLA2 in lipoprotein-deficient medium (DMEM supplemented with 10% lipoprotein-deficient serum, 100 U/ml penicillin, 100 μg/ml streptomycin) for 8 h before analysis. J774 cells were treated with 10 μM lysophosphatidylcholine (Sigma, St. Louis, MO), 10 μM arachidonic acid (Sigma, St. Louis, MO), or vehicle (ethanol) in DMEM containing 1% fatty acid-free BSA for 16 h before treatment with 100 ng/ml LPS for 8 h.

Confocal microscopy and flow cytometry (FACS) to visualize lipid rafts

J774 cells were seeded on glass coverslips and grown until confluent in complete medium. All subsequent procedures were performed using pre-chilled solutions according to the manufacturer’s directions (Vybrant Lipid Raft Labeling Kit; Invitrogen). Cells were washed once with complete medium before the addition of Alexa Fluor 488-labeled cholera toxin B (CT-B) conjugate (1 μg/ml). Cells were incubated for 15 min at 4˚C, washed with PBS, and then incubated for 15 min at 4˚C with anti–CT-B Ab to cross-link CT-B and lipid rafts. After washing with PBS, cells were fixed in 4% parafomaldehyde for 20 min at 4˚C. Fixed cells were mounted with a Prolong Antifade Kit (Molecular Probes). Confocal microscopy was performed at the University of Kentucky Imaging Facility using a Leica laser-scanning confocal microscope with argon (488 nm) and krypton (568 nm) lasers. For FACS analysis, J774-C and J774-GX cells (50–70% confluent) were detached from plates, washed with chilled complete medium, and then incubated for 10 min at 4˚C with 10 ng/ml Alexa Fluor 488-labeled CT-B. After several washes in chilled PBS, cells were resuspended in chilled anti–CT-B Ab for 15 min at 4˚C, washed, and then analyzed by flow cytometry to assess lipid rafts.

RNA extraction and quantitative PCR

Total RNA was isolated from J774 cells or MPMs using RNeasy Mini kit (Promega). RNA (0.2–1 μg) was reverse transcribed into cDNA using a reverse transcription system (Promega). After 4-fold dilution, 3 μl was used as a template for real-time PCR. Amplification was done for 40 cycles using Power SYBR Green PCR master mix Kit (Applied Biosystem) and IQ 5 real-time PCR machine (I Cycler; Bio-Rad). Quantification was performed in duplicate using the standard curve method and normalized to 18s. Sequences of primers are as follows: 18s, 5′-CCG CTA CCA CAT CCA AGG AA-3′ (forward) and 5′-GCT GGA ATT ACC GCC GGC GCT-3′ (reverse); TNF-α, 5′-GCC AGG TCT ACT TTG GAG TCA TTG-3′ (forward) and 5′-GTG AGT ATG GTA CTC CAG AAG-3′ (reverse); IL-6, 5′-CAA CGA TGA TGC ACT TGC AGC-3′ (forward) and 5′-GTA GCT ATG ATG CTC CAG AAG-3′ (reverse); cyclooxygenase-2 (COX-2), 5′-CCA GCA TTT CAC CCA TCA GTT-3′ and 5′-ACC CAG GTC CTC GCT TAT GA-3′ (reverse).

Cholesterol depletion and repletion

Cholesterol depletion and repletion of J774 cells was conducted as described by Zhu et al. (4). In brief, cells were incubated with or without prewarmed 10 mM methyl-β-cyclodextrin (MβCD; Sigma) at 37˚C for 30 min to deplete cholesterol. Macrophages were then washed with PBS and incubated in the presence or absence of cholesterol coupled with MβCD (80 μg/ml; Sigma) at 37˚C for 1 h to replete cholesterol. The cells were then incubated for 8 h with 100 ng/ml LPS before collection of culture media and cells.

Biochemical reagents and assays

FLAG-tagged fusion protein (GX sPLA2) was detected by Western blot analysis using anti-FLAG M2 antibody (Stratagene, La Jolla, CA). Total JNK Ab was from R&D Systems (Minneapolis, MN); pErk, total Erk, and pJNK Abs were from Cell Signaling (Danvers, MA); TNF-α and IL-6 protein concentrations in culture media were determined by ELISA (R&D Systems). The cholesterol content of J774 cells was quantitated as described by Boyanovsky et al. (17).

Statistical analyses

Data are expressed as mean ± SEM. Results were analyzed by Student t test or one-way ANOVA followed by Bonferroni’s posttest. The p values <0.05 were considered statistically significant. Data were tested for normality and equal variance before analysis.
**Results**

GX sPLA₂ promotes macrophage inflammatory responses

To determine whether GX sPLA₂ modulates macrophage inflammatory responses, MPMs isolated from WT and GX sPLA₂-deficient (GX KO) mice were treated with 100 ng/ml LPS for 6 h. Notably, GX KO MPMs exhibited a significantly blunted upregulation (∼60% reduction) of TNF-α, IL-6, and COX-2 mRNAs in response to LPS compared with WT cells (Fig. 1A). There was no significant difference in the expression of these genes when WT and GX KO cells were incubated in the absence of LPS (not shown).

As another approach to define the role of GX sPLA₂ in macrophage inflammatory responses, we performed gain-of-function studies in J774 macrophage-like cells. J774 cells were stably transfected with C-terminally FLAG-tagged GX sPLA₂ (J774-GX cells). J774-GX cells secreted significantly more phospholipase activity compared with J774-C cells (4- to 5-fold increase; data not shown). Overexpression of GX sPLA₂ did not alter the basal expression of the inflammatory mediators TNF-α, IL-6, or COX-2 in J774 cells (data not shown). However, incubations with 100 ng/ml LPS resulted in a significantly increased induction of TNF-α (1.7-fold), IL-6 (2.2-fold), and COX-2 (4-fold) in J774-GX cells compared with J774-C cells (Fig. 1B). Similarly, J774 cells treated with LPS in the presence of recombinant mGX sPLA₂ (0.1 μg/ml; ∼7 nM) exhibited a significant increase in TNF-α (2.7-fold), IL-6 (1.6-fold), and COX-2 (3.6-fold) mRNA expression compared with cells treated with LPS in the absence of GX sPLA₂ (Fig. 1C).

GX sPLA₂ promotes macrophage inflammatory responses through a mechanism that is dependent on its catalytic activity

GX sPLA₂ is known to elicit biological effects that are independent of its catalytic function (7, 12). To determine whether GX sPLA₂ catalytic activity is required for its effect on macrophage responses to LPS, we developed J774 cells stably overexpressing a catalytically inactive GX sPLA₂ mutant (J774-H46Q cells). As we previously reported, the amount of FLAG-tagged recombinant GX sPLA₂ secreted into the media was similar for J774-H46Q and J774-GX cells, whereas the phospholipase activity in conditioned media from J774-H46Q cells was not increased compared with J774-C cells (13). Consistent with data from the analysis of mRNA (Fig. 1C), J774-GX cells treated with 100 ng/ml LPS secreted significantly more TNF-α (1.7-fold) and IL-6 (1.7-fold) protein compared with J774-C cells (Fig. 2A, 2B). In contrast, there was no significant difference in cytokine secretion between LPS-treated J774-H46Q and J774-C cells. To verify that the hyperresponsiveness of J774-GX cells was a specific effect of the transgene, we treated cells with LPS in the presence of indoxam, a specific inhibitor of sPLA₂ activity (18). Indoxam significantly reduced LPS-induced TNF-α secretion by J774-GX cells to a level that was similar to LPS-induced secretion by J774-C cells (Fig. 2C). These results indicate that GX sPLA₂ catalytic activity is required for its ability to produce hypersensitivity to inflammatory stimuli. Although there was a trend for a blunted inflammatory response when J774-C cells were treated with indoxam, this did not reach statistical significance. Previously published studies indicate that lipoprotein particles are substrates for GX sPLA₂ hydrolysis (19). Therefore, to rule out the possibility that GX sPLA₂ modulates inflammation by modifying lipoproteins in the media, we conducted additional experiments with media supplemented with lipoprotein-deficient serum. Under these conditions, TNF-α and IL-6 expression by J774 cells treated with LPS was blunted compared with the same cells incubated with media supplemented with FBS. Nevertheless, the addition of GX sPLA₂ significantly increased the induction of cytokine expression, suggesting that lipoprotein hydrolysis was not required for this effect (Supplemental Fig. 1). Although our data are consistent with the conclusion that GX sPLA₂ amplifies the effect of LPS by generating a lipid mediator, we were unable to recapitulate the enzyme’s effect by exogenous addition of 10 μM arachidonic acid or lysophosphatidylcholine, the two major products of GX sPLA₂ hydrolysis (Supplemental Fig. 2).

GX sPLA₂ augments TLR4-dependent signaling in macrophages

TLR4/CD14/MD2 act as coreceptors that mediate LPS-induced signaling in macrophages, which is initiated by the recruitment of cytosolic adapter proteins such as MyD88 to the coreceptor complex (20, 21). Thus, it was of interest to define the role of TLR4 and MyD88 and downstream signaling pathways in GX sPLA₂-mediated...
hyperresponsiveness. MPMs from TLR4+/+ and TLR4−/− and MyD88+/+ and MyD88−/− mice were treated with LPS in the presence or absence of 0.1 mg/ml mGX sPLA2. Consistent with the data in Fig. 1C, the addition of GX sPLA2 resulted in significantly increased cytokine expression in WT MPMs treated with LPS (Fig. 3A,3B). As expected, the expression of the inflammatory mediators was markedly blunted in TLR4−/− and MyD88−/− MPMs treated with LPS in the absence of GX sPLA2. Notably, LPS also failed to induce inflammatory mediators in TLR4−/− and MyD88−/− MPMs in the presence of GX sPLA2, indicating that GX sPLA2 enhances inflammatory responses through a TLR4- and MyD88-dependent mechanism.

NF-κB and MAPK signaling are major signaling pathways involved in TLR4-mediated inflammatory cytokine induction (22). Using an NF-κB promoter luciferase reporter assay, we previously reported that LPS-induced NF-κB activation is enhanced in cells overexpressing GX sPLA2 (13). Interestingly, deficiency of GX sPLA2 in MPMs was associated with a considerable reduction in LPS-induced phosphorylation of Erk1/2 and JNK that was restored by the addition of recombinant GX sPLA2 (Fig. 3C), indicating that GX sPLA2 enhances the activation of the MAPK pathway downstream of TLR4. Taken together, our data indicate that GX sPLA2 increases macrophage inflammatory responses by augmenting TLR4-dependent signaling.

**FIGURE 2.** GX sPLA2 enhancement of macrophage inflammatory responses is dependent on its catalytic activity. A, TNF-α concentrations in conditioned media from cells treated with 100 ng/ml LPS for 20 h. TNF-α was not detected in media from untreated cells. B, IL-6 concentrations in conditioned media from cells treated with 100 ng/ml LPS for 20 h. IL-6 was not detected in media from untreated cells. C, TNF-α concentrations in conditioned media from cells treated for 20 h with 100 ng/ml LPS in the presence of either vehicle or 20 μM indoxam, an sPLA2 inhibitor. A–C, Data are means ± SEM (n = 4) and are representative of three independent experiments. **p < 0.01; ***p < 0.001.

**FIGURE 3.** GX sPLA2 augments TLR4-dependent signaling in macrophages. A, Relative expression of TNF-α, IL-6, and COX-2 mRNAs in MPMs isolated from TLR4+/+ and TLR4−/− mice treated for 8 h with 100 ng/ml LPS in the absence (UT) or presence (+GX) of 0.1 μg/ml GX sPLA2. Values are presented relative to TLR4+/+ cells treated with LPS alone after normalization internally to 18S RNA. **p < 0.01; ***p < 0.001. B, Relative expression of TNF-α, IL-6, and COX-2 mRNAs in MPMs isolated from MyD88+/+ and MyD88−/− mice treated for 8 h with 100 ng/ml LPS in the absence (UT) or presence (+GX) of 0.1 μg/ml GX sPLA2. Values are presented relative to MyD88+/+ cells treated with LPS alone after normalization internally to 18S RNA. Data are means ± SEM (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001. C, Phosphorylation of Erk1/2 and JNK in WT and GX KO MPMs treated for 0 or 30 min with 100 ng/ml LPS after 18 h pretreatment with 0 or 100 ng/ml mGX sPLA2, as indicated. Cell extracts (10 μg protein) were immunoblotted with the indicated Abs. ns, not statistically significant compared with cells treated with LPS alone.

GX sPLA2 modulates cellular free cholesterol and lipid raft content

We previously demonstrated that hydrolytic products generated by GX sPLA2 negatively regulate liver X receptor (LXR) activation in macrophages, and thus significantly reduce the expression of LXR target genes ABCA1 and ABCG1. As a consequence, cholesterol efflux to extracellular acceptors is blunted in macrophages by GX sPLA2 (13). Membrane free cholesterol and lipid raft content are known to modulate TLR signaling in macrophages (23–26), and the absence of ABCA1 and ABCG1 leads to the accumulation of cell membrane free cholesterol and enhanced macrophage inflammatory responses. These observations prompted us to consider whether GX sPLA2 regulates TLR4 signaling by modulating macrophage free cholesterol and lipid raft content.
Overexpression of GX sPLA2 in J774 cells was associated with a significant (30%) increase in cellular free cholesterol (Fig. 4A), which compares with the 12–14% increase reported for ABCA1-deficient macrophages (4). Other published studies reported that similar changes in cellular free cholesterol content cause physiologically relevant differences in inflammatory responses (5, 24). Similarly, treatment with recombinant GX sPLA2 significantly increased the free cholesterol content of nontransfected J774 cells (Fig. 4B). Alterations in GX sPLA2 did not affect cellular cholesterol ester content in either model system (data not shown). These findings are consistent with our previous report that MPMs deficient in GX sPLA2 have significantly reduced free cholesterol compared with WT MPMs (13). Cell surface lipid rafts were assessed by staining cells with Alexa Fluor-488 labeled CT-B, which binds to a ganglioside GM1 in lipid rafts (27). When viewed by confocal laser microscopy, J774-GX cells stained with CT-B showed increased fluorescence and more punctuate staining compared with control cells (Fig. 4C). Analysis by flow cytometry confirmed significantly increased CT-B labeling of J774-GX cells, consistent with increased lipid rafts and plasma membrane free cholesterol (Fig. 4D) (28). Thus, our data point to a previously unrecognized role for GX sPLA2 in regulating plasma membrane lipid raft content.

The hyperresponsiveness to LPS mediated by GX sPLA2 is related to altered cellular free cholesterol and lipid raft content

To determine whether increased inflammatory responses by J774-GX cells was related to increased membrane free cholesterol and lipid rafts, we manipulated plasma membrane free cholesterol content by treating cells with MβCD before LPS exposure. Treatment with 10 mM MβCD for 30 min reduced free cholesterol content in J774-C and J774-GX cells by 54 and 73%, respectively (Fig. 5A). Subsequent incubation of cholesterol-depleted cells for 60 min with cholesterol-loaded MβCD increased free cholesterol content in both cell types (Fig. 5A). Notably, cholesterol depletion and repletion resulted in free cholesterol levels that were no longer significantly different in the two cell types (Fig. 5A). After each of these cholesterol manipulations, cells were treated for 8 h with LPS and the amount of TNF-α in the media was quantified. As previously shown, in the absence of cholesterol depletion/repletion, LPS induced significantly more TNF-α secretion in J774-GX cells compared with J774-C cells (Fig. 5B). After cholesterol depletion, the response to LPS in both cell types was significantly reduced and the hyperresponsiveness of J774-GX cells was no longer evident. In the case of cholesterol-repleted cells, the responsiveness to LPS was restored to a level that was similar for the two cell types (Fig. 5B). These data demonstrate a direct relationship between plasma membrane free cholesterol and the response to LPS, and also indicate that the hyperresponsiveness of J774-GX cells is secondary to increased plasma membrane free cholesterol content.

GX KO mice exhibit reduced response to LPS

To determine whether GX sPLA2 impacts inflammatory responses in vivo, we injected WT and GX KO mice with LPS (3 μg/kg body weight) or saline, and plasma cytokine levels were determined 3 h after treatment. Plasma levels of IL-6, IL-1β, and TNF-α were below the level of detection in both strains of mice after saline

**FIGURE 4.** GX sPLA2 enhances macrophage free cholesterol and lipid raft content. A, Free cholesterol content of J774-C and J774-GX cells normalized to cell protein. B, Free cholesterol content of untreated J774 cells and cells treated with 0.1 μg/ml GX sPLA2 for 20 h normalized to cell protein. C, J774-C and J774-GX cells were labeled with Alexa Fluor 488-CT-B to visualize lipid rafts. Original magnification ×100. D, J774-C and J774-GX cells were labeled with Alexa Fluor 488-CT-B and analyzed by flow cytometry to determine mean fluorescence intensity of cells. A, B, and D, Data (means ± SEM; n = 4) are representative of three independent experiments. *p < 0.05; ***p < 0.001.

**FIGURE 5.** GX sPLA2-mediated hyperresponsiveness to LPS is reversed by plasma membrane cholesterol depletion and repletion. A, Free cholesterol content of J774-C and J774-GX cells normalized to cell protein. Cells were incubated in the presence or absence of 10 mM MβCD at 37°C for 30 min to deplete cholesterol. Subsets of cholesterol-depleted cells were then incubated for 60 min with 1 mM MβCD-loaded cholesterol to replete cholesterol before LPS treatments. B, TNF-α in conditioned media from cells depicted in A after treatment with 100 ng/ml LPS for 8 h. A and B, Data are means ± SEM (n ≥ 3). **p < 0.01; ***p < 0.001. The analysis of IL-6 secretion produced similar results.
injection (data not shown). Although LPS injection evoked a marked increase in plasma cytokine levels in both WT and GX KO mice, the increase in GX KO mice was significantly blunted compared with similarly treated WT mice (25–30% decrease; Fig. 6). We interpret this data to suggest that GX sPLA₂ expression augments inflammatory responses to LPS stimulation in vivo. Together with our previously published results (13), the current data suggest that GX sPLA₂ modulates inflammatory processes in macrophages by generating lipolytic products that suppress LXR target gene expression and thus promote plasma membrane cholesterol accumulation (Fig. 7).

**Discussion**

Macrophages play a key role in atherosclerosis by influencing the extent of lipid deposition and inflammation in the vessel wall (29). GX sPLA₂ is expressed by macrophages and is present in atherosclerotic lesions, where it has been implicated in proatherogenic and proinflammatory processes (19, 30, 31). We recently reported that GX sPLA₂ negatively regulates ABCA1 and ABCG1 expression, and hence reduces cellular cholesterol efflux in macrophages. These effects were dependent on GX sPLA₂ hydrolytic activity and the expression of LXRα/β (13). In this study, we extend these findings to show that GX sPLA₂ modulation of cholesterol homeostasis in macrophages is associated with an altered response to inflammatory stimuli. Using gain-of-function and loss-of-function approaches, we demonstrated that GX sPLA₂ enhances macrophage responses to LPS, a known ligand for TLR4. This hyperresponsiveness is associated with reduced ABCA1 and ABCG1 expression, reduced cellular cholesterol efflux, and increased plasma membrane free cholesterol and lipid rafts. The finding that GX KO mice exhibit a blunted response to LPS provides compelling evidence that GX sPLA₂ plays an important role in modulating inflammation in vivo.

Our conclusion that the enhancement of TLR4 signaling by GX sPLA₂ is secondary to alterations in plasma membrane cholesterol and lipid raft content is consistent with previous reports that cholesterol accumulation in macrophages is associated with a proinflammatory phenotype (4, 5, 24, 32). Li et al. (3) concluded that free cholesterol accumulation in macrophages leads to the induction and secretion of TNF-α and IL-6 by inducing an ER stress response, which activates several inflammatory pathways including IκB kinase/NF-κB and MAPK. More recent studies (4, 5) suggest that changes in plasma membrane free cholesterol/lipid raft content modulate macrophage inflammatory responses through an MyD88-dependent signaling pathway that is independent of an ER stress response. Lipid rafts also play an important role in trafficking and secretion of TNF-α (23). In this study, we show that increased expression of GX sPLA₂ leads to significantly increased cellular free cholesterol and lipid raft content, consistent with our previous finding that GX sPLA₂ deficiency is associated with reduced cholesterol content in MPMs (13). We further demonstrated that the hyperresponsiveness to LPS mediated by GX sPLA₂ is completely abrogated when cellular free cholesterol is normalized using cyclodextrin, a watersoluble compound capable of transferring cholesterol directly to and from the plasma membrane (28, 33). Thus, our findings are analogous to previous studies in ABCG1- and/or ABCA1-deficient macrophages, where augmented TLR4 signaling was related to increases in plasma membrane lipid rafts (4, 5). Although we did not directly test whether GX sPLA₂ leads to enhanced signaling through other TLRs, increased inflammatory gene responses to TLR2, TLR3, and TLR4 ligands have been demonstrated for bone marrow-derived macrophages deficient in both ABCA1 and ABCG1 (5).

According to our model (Fig. 7), GX sPLA₂ modulates macrophage free cholesterol content by suppressing the activation of LXRα/β and hence the expression of LXR target genes ABCA1 and ABCG1. This altered uptake of cholesterol by ABCA1 and ABCG1 results in increased intracellular cholesterol and lipid rafts, which in turn enhances TLR4 signaling. Whether GX sPLA₂ hydrolyzes extracellular or intracellular phospholipid membranes, or both, is unclear.

**FIGURE 6.** LPS-induced cytokine production is significantly decreased in GX KO mice compared with WT mice. Plasma IL-6 (A), TNF-α (B), and IL-1β (C) concentrations 3 h after administration of LPS (3 μg/g body weight; n = 6). Data are means ± SEM. *p < 0.05; **p < 0.01. Cytokines were undetectable in both genotypes after treatment with saline (not shown). Data are representative of two independent experiments.

**FIGURE 7.** Model for GX sPLA₂ regulation of macrophage inflammatory responses. GX sPLA₂ enhances macrophage free cholesterol and lipid raft content, most likely by suppressing the expression of LXR targets ABCA1 and ABCG1. Increased plasma membrane free cholesterol and lipid raft content enhances TLR4 signaling. Whether GX sPLA₂ hydrolyzes extracellular or intracellular phospholipid membranes, or both, is unclear.
and ABCG1. Interestingly, in addition to being cholesterol sensors, LXR are known to act as negative regulators of inflammatory signaling in macrophages through a mechanism that is independent of its ability to trans-activate target genes (34). On activation by some ligands, LXR are capable of blunting macrophage responses to inflammatory stimuli through trans-repression of NF-kB (35, 36). Importantly, in our previous study using an NF-kB promoter luciferase construct, we ruled out the possibility that GX sPLA2 alters the trans-repressive effect of the LXR ligand T0901317 (13). Likewise, the ability of T0901317 to suppress LPS-mediated induction of IL-6 and TNF-α is similar in J774-C and J774-GX cells, indicating that GX sPLA2 does not modulate the trans-repressive effect of LXR activation on inflammatory cytokine expression (data not shown).

GX sPLA2 is speculated to exhibit properties independent of its catalytic function by binding to sPLA2 receptors including the M-type receptor (12, 37), although direct evidence is lacking. Granata et al. (7) reported that catalytically inactive GX sPLA2 was equally effective as WT GX sPLA2 in enhancing cytokine production in human lung macrophages. Our data that GX sPLA2, but not the catalytically inactive mutant H46Q, enhances LPS-induced cytokine production and that indomethin blocks the effect provides strong evidence that phospholipid hydrolysis is required. However, exogenous treatment of J774 cells with hydrolytic products of GX sPLA2, arachidonic acid or lysophosphatidylcholine, did not significantly increase cytokine production in response to LPS treatment. This does not rule out the possibility that arachidonic acid or lysophosphatidylcholine mediates GX sPLA2’s effect, however, because it is possible that supplementing arachidonic acid or lysophosphatidylcholine in the media complexed to BSA does not recapitulate what occurs when GX sPLA2 is continuously hydrolyzing cellular membranes. Real-time PCR analysis of J774 cells and MPMs indicated absence of M-type receptor expression (data not shown), further excluding a role for this sPLA2 receptor.

Studies in gene-targeted mice support the conclusion that GX sPLA2 promotes inflammatory processes in the setting of acute and chronic asthma (10) and in inflammatory lung disease (8). Most recently, Sato et al. (38) reported that pharmacological inhibition of sPLA2 significantly attenuates the acute lung inflammation and injury induced by LPS in C57BL/6J mice. The authors concluded that the protective effect was most likely due to inhibition of group V sPLA2 and GX sPLA2 activities.

In a recently published study, we determined that GX sPLA2 deficiency significantly reduces abdominal aortic aneurysm formation in apolipoprotein E-deficient mice infected with angiostatin II (39). This protective effect was associated with a significantly blunted induction of inflammatory mediators in the aortas of GX sPLA2-deficient apolipoprotein E-deficient mice after angiostatin II infusion. Although GX sPLA2 is generally considered to induce proinflammatory responses in macrophages in vitro (7), one study concluded that overexpression of GX sPLA2 in RAW264.7 cells has an anti-inflammatory effect (8). Although GX sPLA2 enhanced LPS-stimulated IL-6 production in RAW264.7 cells in line with our study, TNF production was significantly suppressed (8). The basis for the discrepant effect of GX sPLA2 on TNF induction in the previously published study and this study is not clear, but it may be related to the different macrophage-like cell lines used. Our data indicate that GX sPLA2 is not transcriptionally regulated by LPS in macrophages (data not shown). Current evidence suggests that GX sPLA2 is regulated posttranscriptionally through proteolytic cleavage of an inactive proenzyme. Although the pro tease(s) responsible for its proteolytic activation have yet to be identified, it is known that GX sPLA2 is expressed in an inactive form that requires removal of 11 aa residues at the N terminus for catalytic activity (40). Studies using transgenic mice indicate that GX sPLA2 enzymatic activity is under tight regulation and suggest that during inflammation the inactive zymogen is proteolytically activated (41). These data and our current study provide the interesting possibility that GX sPLA2 acts in a feed-forward loop to augment macrophage responses to inflammatory stimuli.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure Legends

Figure 1. Relative expression of TNFα and IL-6 mRNAs in J774 cells treated for 8 h with 100 ng/ml LPS in the absence (J774-UT) or presence of 0.1 ug/ml GX sPLA₂ (J774-GX) in lipoprotein-deficient medium. Values are presented relative to control cells treated only with LPS after normalization internally to 18S RNA. **, p < 0.01 compared to control cells treated with LPS.

Figure 2. Relative expression of TNFα and IL-6 mRNAs in J774 cells treated with 10 μM arachidonic acid or 10 μM lysophosphatidylcholine for 16 h prior to treatment with 100 ng/ml LPS for 8 h as described in the ‘Materials and Methods’. Values are normalized internally to 18S RNA.
Supplementary Figure 1

Relative mRNA expression of IL-6 and TNFα in J774-C and J774-GX.
Supplementary Figure 2

![Graph showing relative mRNA expression (fold increase over baseline) for TNFα and IL-6 under different conditions: C, AA, and LPC.](image-url)