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Bis(monoacylglycero)phosphate inhibits TLR4-dependent RANTES production in macrophages

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ABSTRACT

Toll-like receptor 4 (TLR4) is the receptor for bacterial lipopolysaccharide (LPS) triggering production of pro-inflammatory cytokines which help eradicate the bacteria but could also be harmful when overproduced. The signaling activity of TLR4 is modulated by cholesterol level in cellular membranes, which in turn is affected by bis(monoacylglycero)phosphate (BMP), a phospholipid enriched in late endosomes. We found that exogenously added BMP isomers become incorporated into the plasma membrane and intracellular vesicles of macrophages and strongly reduced LPS-stimulated production of a chemokine RANTES, which was correlated with inhibition of interferon regulatory factor 3 (IRF3) controlling *Rantes* expression. To investigate the mechanism underlying the influence of BMP on TLR4 signaling we applied Laurdan and studied the impact of BMP incorporation on lipid packing, a measure for membrane order. Enrichment of model and cellular membranes with BMP significantly reduced their order and the reduction was maintained during stimulation of cells with LPS. This effect of BMP was abolished by enrichment of macrophages with cholesterol. In parallel, the inhibitory effect of BMP exerted on the TLR4-dependent phosphorylation of IRF3 was also reversed. Taken together our results indicate that BMP reduces the order of macrophage membranes which contributes to the inhibition of TLR4-dependent RANTES production.

1. Introduction

Lipopolysaccharide (LPS), the main component of the outer membrane of Gram-negative bacteria, activates Toll-like receptor 4 (TLR4) which is present on monocytes, macrophages, dendritic cells and some non-immune cells. Activation of TLR4 is preceded by interaction of LPS aggregates released from bacteria with a serum protein LBP and subsequent binding of monomeric LPS to a plasma membrane protein CD14. Then, CD14 transfers the LPS to a complex of TLR4 and MD2 protein (da Silva Correia et al., 2001; Gioannini et al., 2005). Upon LPS binding the TLR4/MD2 complexes dimerize allowing adaptor proteins to bind at the cytoplasmic signaling domain of TLR4. Depending on the involvement of the MyD88 or the TRIF adaptor protein the activated TLR4 triggers either of two pro-inflammatory signaling pathways. MyD88 binds TLR4 at the plasma membrane and initiates a signaling cascade leading to early-phase activation of NFκB which controls the production of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Kawai and Akira, 2011; Bjorkbacka et al., 2004). The TRIF-dependent pathway, in contrast, is initiated after CD14-dependent endocytosis of activated TLR4 (Husebye et al., 2006; Kagan et al., 2008; Zanoni et al., 2011). This pathway engages interferon regulatory factor 3 (IRF3) and drives expression of genes encoding type I interferons and some cytokines such as CCL5/RANTES chemokine. Late phase activation of NFκB is also induced in this pathway (Yamamoto et al., 2003; Bjorkbacka et al., 2004). The pro-inflammatory responses triggered by TLR4 aim at eradication of invading bacteria, but exaggerated or prolonged activation of the receptor can cause, respectively, a potentially fatal systemic inflammatory condition named sepsis or low-grade inflammation.

Abbreviations: ABC, ATP-binding cassette transporter; BMP, bis(monoacylglycerophosphate); IL-6, interleukin 6; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; oxLDL, oxidized low-density lipoprotein; MBCD, methyl-β-cyclodextrin; MyD88, myeloid differentiation primary response 88; PUFA, polyunsaturated fatty acids; RANTES, regulated on activated normal T-cell expressed and secreted; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α; TRIF, TIR-domain-containing adaptor-inducing interferon-β.

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Owing to the dynamic redistribution of activated TLR4 in the plasma membrane and its internalization, changes in the organization of the membrane affect the immunostimulatory properties of TLR4 (Ciesielska and Kwiatkowska, 2015). A key component determining lateral plasma membrane heterogeneity, or domain separation, and membrane fluidity is cholesterol. Interactions of cholesterol with saturated sphingolipids and phospholipids result in the assembly of liquid ordered domains characterized by tight packing of lipids (Ahmed et al., 1997). These forces can also drive formation of rafts in cell membranes. Rafts separate from the more disordered and fluid membrane milieu and accommodate distinct proteins which reciprocally also contribute to raft assembly (Kaiser et al., 2009; Owen et al., 2012). Upon cell stimulation, the highly dynamic nanoscopic rafts merge serving as signaling platforms for plasma membrane receptors, including TLR4 (Plociennikowska et al., 2015). Similar raft-based platforms also play a role in the Golgi-plasma membrane trafficking, endocytosis and several other cellular processes (Lingwood and Simons, 2010).

As cholesterol is the main component determining lipid packing and lateral heterogeneity of membranes, enrichment or depletion of the plasma membrane in cholesterol is correlated with an increase or decrease of raft content in macrophage membranes. Furthermore, accumulation of cholesterol results in enhanced translocation of TLR4 to rafts and its hyperactivity leading to increased expression of pro-inflammatory cytokines (Yvan-Charvet et al., 2008). TLR4 signaling is also affected by intracellular cholesterol content. In human skin fibroblasts derived from patients with Niemann-Pick type C disease an excess of cholesterol in endosomes leads to abnormal storage of TLR4 in the endosomes and constitutive secretion of IFN-β, IL-6, and IL-8 (Suzuki et al., 2007). This example indicates that the pro-inflammatory activity of TLR4 underlies several inflammatory disorders associated with abnormal cholesterol metabolism. In accordance, recent studies indicate that TLR4-dependent production of inflammatory mediators can contribute to the development and progression of atherosclerosis (Lundberg et al., 2013; Michelsen et al., 2004). A hallmark of atherosclerosis is the uptake of an excess of oxidized LDL (oxLDL) by macrophages and their conversion to foam cells. The concomitant local production of cytokines causes activation and migration of immune cells to the cholesterol-driven site of inflammation in blood vessels and facilitates development of atherosclerotic plaques. Recently, chemokines such as RANTES draw special attention as important factors in atherogenesis (Braunersreuther et al., 2008; Podolec et al., 2016; Veillard et al., 2004). Notably, an elevated level of RANTES found in patient with acute coronary syndromes was associated with an increased risk of adverse cardiovascular outcomes (de Jager et al., 2012).

At the cellular level, cholesterol homeostasis is an outcome of the balance between cholesterol synthesis, uptake and efflux. Recent data indicate that one of the factors controlling cholesterol metabolism and trafficking is the anionic phospholipid bis(monoacylglycero)phosphate (BMP, also named lysobisphosphatidic acid). BMP with the characteristic *sn*-1,*sn*-1′ glycerophosphate stereoconfiguration (S,S) and usually enriched in mono- and polyunsaturated acyl chains occurs intracellularly primarily in the membranes of late endosomes/multivesicular bodies where LDL-delivered cholesterol is de-esterified prior to its redistribution to other cellular membranes (Chevallier et al., 2008). BMP has also been detected in the plasma of patients with lysosomal storage disorders (Meikle et al., 2008), and an increased level of BMP was found in the serum and urine of individuals treated with drugs inducing tissue phospholipidosis (Liu et al., 2014; Mortuza et al., 2003).

An elevated level of BMP in endolysosomal membranes correlates with local cholesterol accumulation as a consequence of high-fat diet (Rodriguez-Navarro et al., 2012) or some lysosomal storage disorders (Meikle et al., 2008). The engagement of BMP in cholesterol trafficking and its role in atherogenesis have been addressed by few studies (Kobayashi et al., 1999; Luquain-Costaz et al., 2013). Enrichment of cells with BMP facilitates redistribution of free cholesterol to the endoplasmic reticulum at the expense of its delivery to the plasma membrane and reduces the efflux of cholesterol. In consequence, loading of BMP-enriched macrophages with oxLDL increases their conversion into foam cells (Delton-Vandenbroucke et al., 2007; Luquain-Costaz et al., 2013) promoting their pro-inflammatory activity. On the other hand, lowering of the cholesterol content in the plasma membrane as a result of BMP accumulation in cells could be considered as a factor attenuating the pro-inflammatory activity of plasma membrane receptors, including TLR4.

Taking into account the role of BMP in cholesterol trafficking we examined the influence of BMP on TLR4 activation in macrophages. We aimed to check whether BMP can incorporate into lipid membranes and modify their properties leading to changes in the inflammatory response of macrophages. Our results indicate an inhibition of TLR4-dependent RANTES production in macrophages exposed to BMP and show that this reduction is at least partially due to the effect of BMP on the organization of cell membranes.

2. Materials and methods

2.1. Cell culture and stimulation

Murine peritoneal macrophages were isolated from C57BL/6 mice and cultured in RPMI-1604 medium with 10% FBS according to the procedure described earlier (Jozefowski et al., 2010). The experiments have been reviewed and approved by a Local Animal Ethics Committee (permission No. 696/2015). Raw264.7 (Sigma-Aldrich, St. Louis, USA), J774 (ATCC, Teddington, UK) and J774-Dual cells (InvivoGen, San Diego, USA) were cultured in DMEM with 10% FBS.

For stimulation with LPS the cells were preincubated in DMEM or RPMI-1604 with 2% FBS for 2 h and next incubated with 10 or 50 μM BMP for 30 min. BMP isomers: (S,S), (S,R), (R,R) were purchased from Avanti Polar Lipids (Alabaster, USA). After BMP treatment the cells were exposed to 100 ng/ml LPS (*E. coli* O111:B4, List Biological Laboratories, Campbell, USA) for indicated time. In Laurdan-labeled cells (see 2.9) the 2-h preincubation was omitted and the cells were stimulated with 100 ng/ml LPS in the presence of 2% charcoal-stripped FBS (Thermo Fisher Scientific).

2.2. Cytokine assays

Concentrations of TNF-α, IL-6 and RANTES were determined in culture supernatants (0.7×10^5 cells/well) with the use of murine ELISA kits (BioLegend, San Diego, CA, USA; R&D Systems, Minneapolis, USA) according to the manufacturers' protocols, using a Sunrise plate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.3. RT-qPCR analysis

Total RNA was extracted from cells using TRI-Reagent (Sigma-Aldrich) or GeneMATRIX universal RNA purification kit (EURX, Poland). First-strand cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the supplier's procedure. qPCR was performed in a StepOnePlus instrument using fast SYBR Green Master Mix (Thermo Fisher Scientific). The following primers were used: sense 5′GCTCCAATCTTGCAGTCGTGT3′ and antisense 5′CCATTTTCCCAGGACCGAGT3′ for murine *Rantes* gene, and sense 5′CAGTCCCAGCGTCGTGA3′ and antisense 5′GCCTCCCATCTCCTTCAT3′ for the murine *Hprt* gene. The PCR