

Research Paper

Lipopolysaccharide enhances FcεRI-mediated mast cell degranulation by increasing Ca²⁺ entry through store-operated Ca²⁺ channels: implications for lipopolysaccharide exacerbating allergic asthma

Chengbin Yang^{1,2}, Xucheng Mo¹, Jingzhang Lv³, Xiaoyu Liu¹, Meichun Yuan³, Ming Dong⁴, Li Li², Xinping Luo¹, Xinmin Fan⁵, Zhe Jin⁵, Zhigang Liu¹ and Jie Liu⁴

¹State Key Laboratory of Respiratory Disease for Allergy at Shengzhen University, Shenzhen 518060, China

²College of Life Science, Shenzhen University, Shenzhen 518060, China

³Shenzhen Entry-Exit Inspection and Quarantine Bureau, Shenzhen 518045, China

⁴Departments of Pathophysiology and ⁵Pathology, School of Medicine, Shenzhen University, Shenzhen 518060, China

Lipopolysaccharide (LPS) can exacerbate asthma; however, the mechanisms are not fully understood. This study investigated the effect of LPS on antigen-stimulated mast cell degranulation and the underlying mechanisms. We found that LPS enhanced degranulation in RBL-2H3 cells and mouse peritoneal mast cells upon FcεRI activation, in a dose- and time-dependent manner. Parallel to the alteration of degranulation, LPS increased FcεRI-activated Ca²⁺ mobilization, as well as Ca²⁺ entry through store-operated calcium channels (SOCs) evoked by thapsigargin. Blocking Ca²⁺ entry through SOCs completely abolished LPS enhancement of mast cell degranulation. Consistent with functional alteration of SOCs, LPS increased mRNA and protein levels of Orai1 and STIM1, two major subunits of SOCs, in a time-dependent manner. In addition, LPS increased the mRNA level of Toll-like receptor 4 (TLR4) in a time-dependent manner. Blocking TLR4 with Cli-095 inhibited LPS, increasing transcription and expression of SOC subunits. Concomitantly, the effect of LPS enhancement of Ca²⁺ mobilization and mast cell degranulation was largely reduced by Cli-095. Administration of LPS (1 μg) *in vivo* aggravated airway hyperreactivity and inflammatory reactions in allergic asthmatic mice. Histamine levels in serum and bronchoalveolar lavage fluid were increased by LPS treatment. In addition, Ca²⁺ mobilization was enhanced in peritoneal mast cells isolated from LPS-treated asthmatic mice. Taken together, these results imply that LPS enhances mast cell degranulation, which potentially contributes to LPS exacerbating allergic asthma. Lipopolysaccharide increases Ca²⁺ entry through SOCs by upregulating transcription and expression of SOC subunits, mainly through interacting with TLR4 in mast cells, resulting in enhancement of mast cell degranulation upon antigen stimulation.

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Corresponding author J. Liu: Department of Pathophysiology, Medical School of Shenzhen University, Shenzhen 518060, China. Email: ljljz@yahoo.com

Z. Liu: Department of Immunology, Medical School of Shenzhen University, Shenzhen 518060, China. Email: lzg@szu.edu.cn

C.Y., X.M. and J.L. contributed equally to this work.

Allergic asthma is a complex airway inflammatory disease characterized by allergen-induced early and late bronchoconstriction, structural changes to the airway wall associated with progressive decline in lung function, airway hyperresponsiveness (AHR) and excessive production of mucus (Nauta *et al.* 2008; Boyce *et al.* 2009; Murdoch & Lloyd, 2010). It has been suggested that the respiratory manifestation of asthma may be exacerbated by respiratory infections. Lipopolysaccharide (LPS) is a major component of Gram-negative bacteria ubiquitously distributed in the environment. It is well recognized that in humans, LPS is a risk factor for asthma (Michel *et al.* 1991; Rizzo *et al.* 1997), although LPS may exacerbate, protect against or have no effect on allergic asthma, depending on the timing and level of LPS exposure (Tulic *et al.* 2000; Schwartz, 2001; Liu, 2002). Early exposure to endotoxin may minimize the risk of allergen sensitization by inducing T helper cell type 1 (Th1) responses; however, exposure to endotoxin during the usual time course of allergic reactions can enhance the airway response to allergens by enhancing T helper cell type 2 (Th2) responses (Tulic *et al.* 2000; Eisenbarth *et al.* 2002; Liu, 2002; Kim *et al.* 2007).

Mast cells play a central role in the pathogenesis of allergic asthma. Activation of mast cells by antigen results in degranulation, leukotriene generation and cytokine synthesis. The mediators released from mast cells induce a range of responses, such as bronchoconstriction, mucus secretion, mucosal oedema and inflammatory responses, which largely account for the symptoms of asthma (Knol, 2006; Yamashita *et al.* 2010). It has been suggested that the autacoid mediators, such as histamine, prostaglandin D₂, leukotriene C₄ and eotaxin, are released from activated mast cells by degranulation, while most of the cytokines are synthesized and released by a mode unrelated to degranulation. The degranulation is Ca²⁺ dependent and can only be activated by an increase in intracellular Ca²⁺ characterized by Ca²⁺ entry through store-operated calcium channels (SOCs; Fewtrell & Sherman, 1987; Ma *et al.* 2008; Sanchez-Miranda *et al.* 2010), while the degranulation-unrelated cytokine release is independent of intracellular Ca²⁺ increase.

Recent progress has revealed that LPS synergistically increases cytokine production with antigen, enhancing allergic airway eosinophilic inflammation and thus exacerbating allergic asthma (Masuda *et al.* 2002; Nigo *et al.* 2006). However, LPS has been suggested to have no effect on antigen-stimulated Ca²⁺ mobilization and mast cell degranulation (Qiao *et al.* 2006). It is noteworthy that only the acute effect of LPS (LPS applied for <1 h) was investigated by Qiao *et al.* (2006). No study heretofore has investigated the chronic effect of LPS on antigen-stimulated mast cell degranulation, a situation more relevant to respiratory infection complicated with asthma *in vivo*.

In the present study, we aimed to investigate whether chronic exposure to LPS regulates IgE-mediated mast cell degranulation and the role in LPS exacerbation of asthma. Furthermore, we explored the molecular mechanisms underlying regulation of mast cell degranulation by LPS.

Methods

Mice

Female BALB/c mice, 4–6 weeks old and weighing 20–22 g, were housed in groups of four per cage in a controlled environment with a photoperiod of 12 h light–12 h dark and a temperature of 20 ± 2°C. Sanitary controls were performed for all major rodent pathogens, and the results of these tests were uniformly negative. Animals were given free access to water and a nutritionally balanced diet. All animal experiments complied with ethical standards as formulated in the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of Shenzhen University.

Cell culture

RBL-2H3 cell lines (China Center for Type Culture Collection, Wuhan, China) were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Paisley, UK), 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Sigma). Trypsinized cells were plated into 96-well tissue culture plates (at a density of 2 × 10⁴ cells per well) or Petri dishes (at a density of 2 × 10⁵ cells per dish) and cultured for 24 h before preparing for detection of degranulation (shown in Fig. 1A–D) or Ca²⁺ signalling (shown in Figs 2A, C, D and E–H).

Isolation and culture of peritoneal mast cells

Mice were anaesthetized by inhalation of ether and killed by decapitation. Mouse peritoneal mast cells (MPMCs) were obtained by peritoneal lavage and purified by density gradient fractionation as described previously (Login *et al.* 1997; Swindle *et al.* 2004). Isolated MPMC were at least 98% pure, and at least 98% of these cells were viable. The cells were cultured in 96-well tissue culture plates (at a density of 2 × 10⁴ cells per well) or Petri dishes (at a density of 2 × 10⁵ cells per dish) with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. After being cultured for 24 h, the cells were prepared for detection of degranulation (shown in Fig. 1E) or Ca²⁺

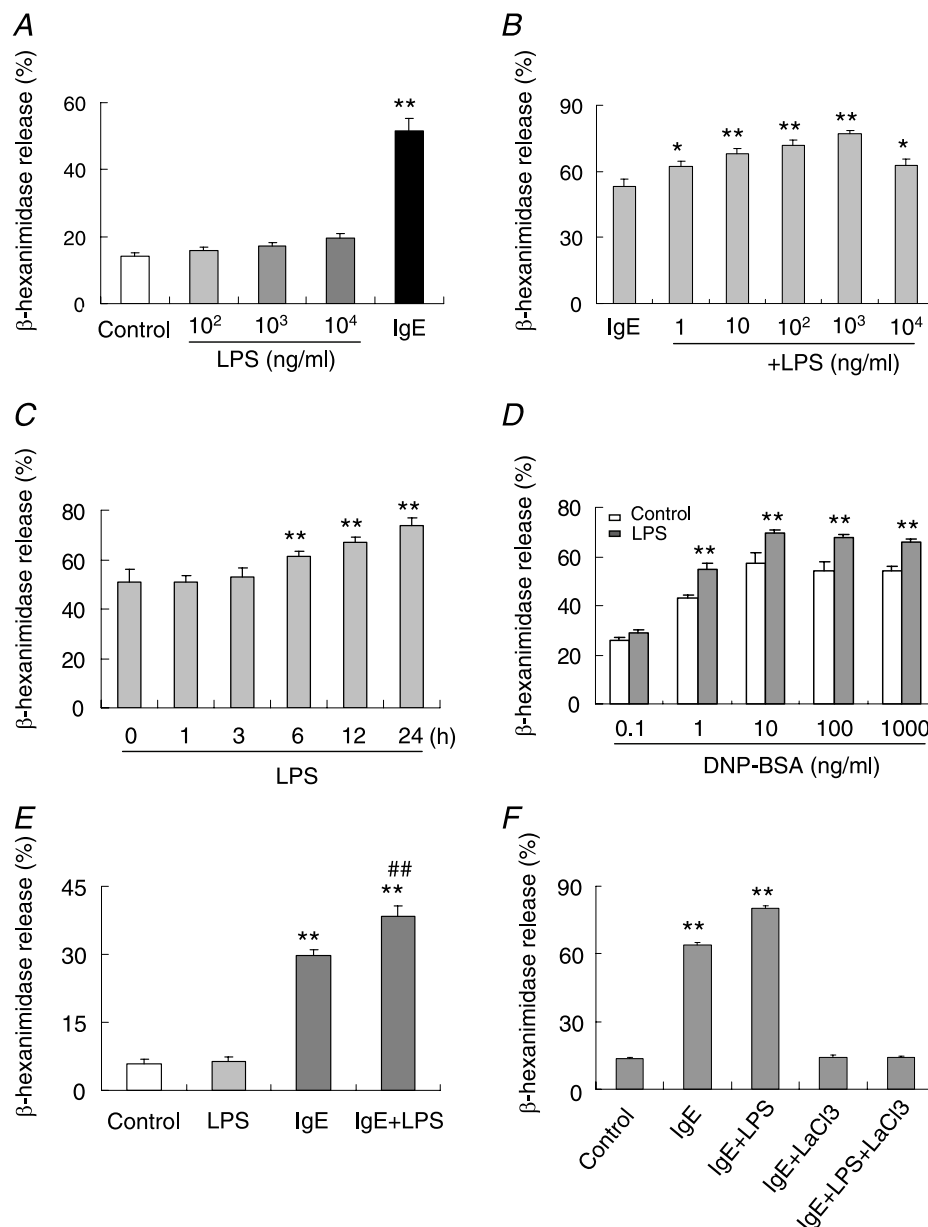


Figure 1. Lipopolysaccharide (LPS) enhanced Fc ϵ RI aggregation-activated mast cell degranulation Mast cell degranulation was assessed by measuring the relative release of β -hexosaminidase. Data are means $\pm$ SEM from triplicate samples in four independent experiments. *A* and *B*, effect of different concentrations of LPS on baseline (*A*) and Fc ϵ RI-activated mast cell degranulation (*B*). RBL-2H3 cells or IgE-sensitized RBL-2H3 cells were incubated with 0.01, 0.1, 1 or 10 μ g ml⁻¹ LPS for 24 h, and then stimulated with 1 μ g ml⁻¹ dinitrophenol-bovine serum albumin (DNP-BSA; antigen) for 30 min at 37°C. *C*, time-dependent effect of LPS (1 μ g ml⁻¹) on Fc ϵ RI-mediated mast cell degranulation in RBL-2H3 cells. *D*, effects of LPS on mast cell degranulation in response to stimulation of different doses of antigen. The IgE-sensitized RBL-2H3 cells were incubated with 1 μ g ml⁻¹ LPS for 24 h, and then stimulated with 1–1000 ng ml⁻¹ antigen for 30 min at 37°C. *E*, effect of LPS (1 μ g ml⁻¹) on baseline and Fc ϵ RI-mediated mast cell degranulation in primary cultured mouse peritoneal mast cells (MPMCs). *F*, effects of blocking store-operated calcium channels (SOCs) with LaCl₃ on LPS-induced enhancement of Fc ϵ RI-mediated mast cell degranulation. The LaCl₃ was applied at 10 μ M, which completely abolished Fc ϵ RI-mediated mast cell degranulation and LPS-induced enhancement of Fc ϵ RI-mediated mast cell degranulation. **P* < 0.05, ***P* < 0.01 versus respective control; and ##*P* < 0.01 versus IgE in *E* (by repeated-measures one-way ANOVA and Student–Newman–Keuls test).

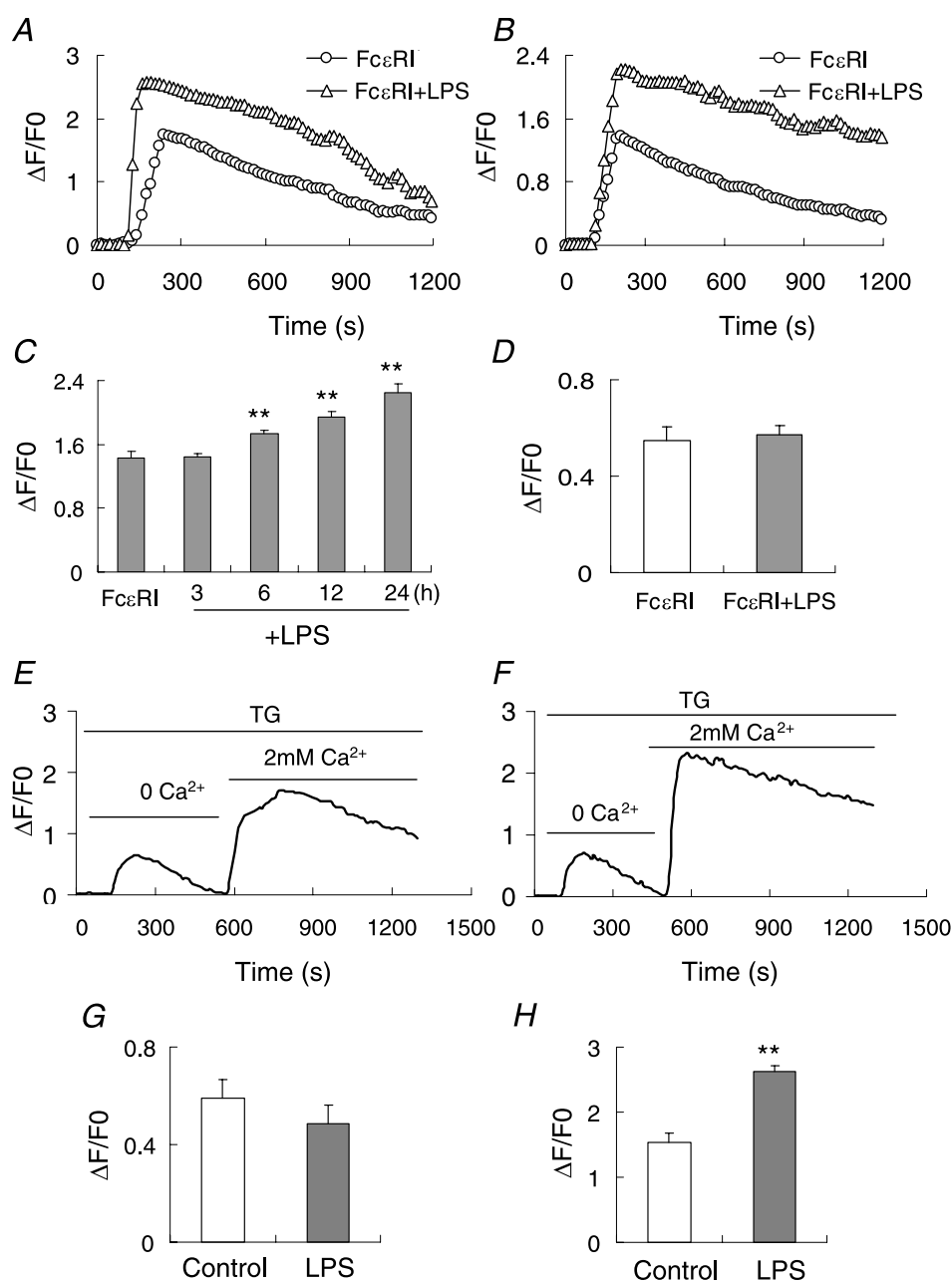


Figure 2. Lipopolysaccharide upregulated Ca $^{2+}$ entry through SOCs

A and B, typical Fc ϵ RI-activated Ca $^{2+}$ signals in a RBL-2H3 cell (A) or a MPMC (B) with or without LPS treatment. Intracellular Ca $^{2+}$ was indicated by fluo-4 fluorescence. Lipopolysaccharide increased the Fc ϵ RI-activated Ca $^{2+}$ increase in both types of cells. C, time-dependent effect of LPS (1 μ g ml $^{-1}$) on Fc ϵ RI-activated Ca $^{2+}$ increase in RBL-2H3 cells. D, effects of LPS on Fc ϵ RI-activated Ca $^{2+}$ increase in the presence of LaCl $_3$ to block SOCs in RBL-2H3 cells. The LaCl $_3$ completely abolished the LPS-induced increase of Fc ϵ RI-activated Ca $^{2+}$ signalling. E and F, representative Ca $^{2+}$ response stimulated by the Ca $^{2+}$ add-back protocol in a control (E) and a LPS-treated RBL-2H3 cell (F). The Ca $^{2+}$ add-back protocol stimulated two peaks of Ca $^{2+}$ increase, where the first one represents intracellular Ca $^{2+}$ release from the endoplasmic reticulum and the second one represents Ca $^{2+}$ entry through SOCs. G and H, averaged peak amplitude of the first and second Ca $^{2+}$ increase as recorded in E and F. $^{**}P < 0.01$ versus respective control (by repeated-measures one-way ANOVA and Student–Newman–Keuls test in C and by Student's unpaired *t* test in D, G and H). Total cell numbers are 40–50 for each group, and the cells were from four independent experiments.

signalling (shown in Fig. 2B). Acutely isolated MPMCs from animal models were used directly for examining the Ca^{2+} response to thapsigargin (TG; shown in Fig. 6C and D).

Degranulation assay

Degranulation was monitored by the release of β -hexosaminidase, as previously described (Itoh *et al.* 2010). Briefly, cultured RBL-2H3 cells or MPMCs in 96-well plates were sensitized overnight with $1 \mu\text{g ml}^{-1}$ dinitrophenol (DNP)-specific IgE (Sigma). The cells were subsequently washed twice with PBS and stimulated with various amounts of DNP-bovine serum albumin (BSA; Sigma) in Tyrode buffer at 37°C for 30 min, and mixed with an equal volume of substrate solution (*p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, 2 mM in 0.1 M citrate, pH 4.5) and incubated for 1 h at 37°C . The reaction was then terminated by adding 200 μl of stopping buffer (0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.0) and the absorbance measured with a microplate reader (Bio-Rad, Hercules, CA, USA). Cell were lysed with 0.1% Triton X-100, and the β -hexosaminidase activity

of the extracts was measured (total). The percentage degranulation in response to each stimulus was calculated as a portion of β -hexosaminidase release in the culture medium to the whole β -hexosaminidase release in the culture medium and in the cell lysate.

Confocal Ca^{2+} signalling detection

The intracellular Ca^{2+} signal was measured as described previously with minor revision (De Jonge *et al.* 2004). The RBL-2H3 cells or MPMCs were loaded with $5 \mu\text{mol l}^{-1}$ Ca^{2+} indicator fluo-4 AM (Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C . Cells were washed three times with Tyrode buffer, and dye was allowed to de-esterify for 30 min at 37°C . Approximately 95% of the dye was confined to the cytoplasm as determined by the signal remaining after saponin permeabilization (Venkatachalam *et al.* 2001). Fluorescence images of Ca^{2+} were observed using an Olympus 1000 confocal microscope with a $\times 40$ oil immersion lens (NA 1.3). The fluo-4 was excited by 488 nm light and the fluorescence collected at >505 nm. Frame-scan images were acquired at sampling rate of 15 ms per frame and 20 s per interval.

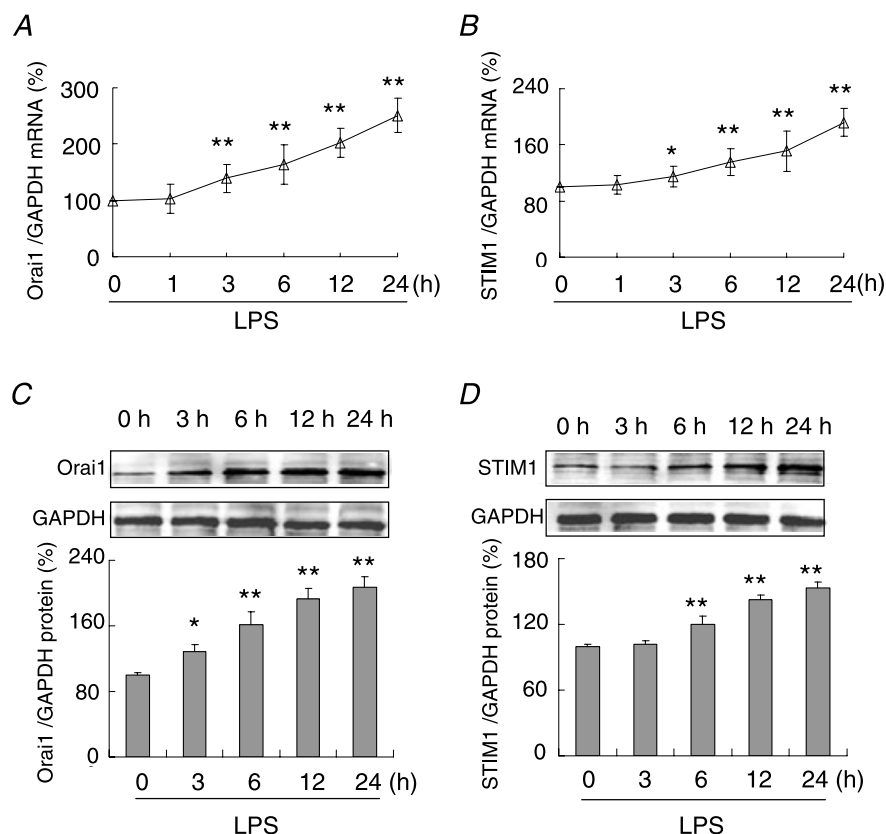


Figure 3. Lipopolysaccharide increased mRNA transcription and protein expression of Orai1 and STIM1. A and B, effect of LPS on mRNA levels of Orai1 (A) and STIM1 (B). C and D, representative images and quantification of protein levels of Orai1 (C) and STIM1 (D). The RBL-2H3 cells were incubated with $1 \mu\text{g ml}^{-1}$ LPS for 0–24 h. $n = 6$ for each group. * $P < 0.05$, ** $P < 0.01$ versus the value at 0 h (by repeated-measures one-way ANOVA and Student–Newman–Keuls test).

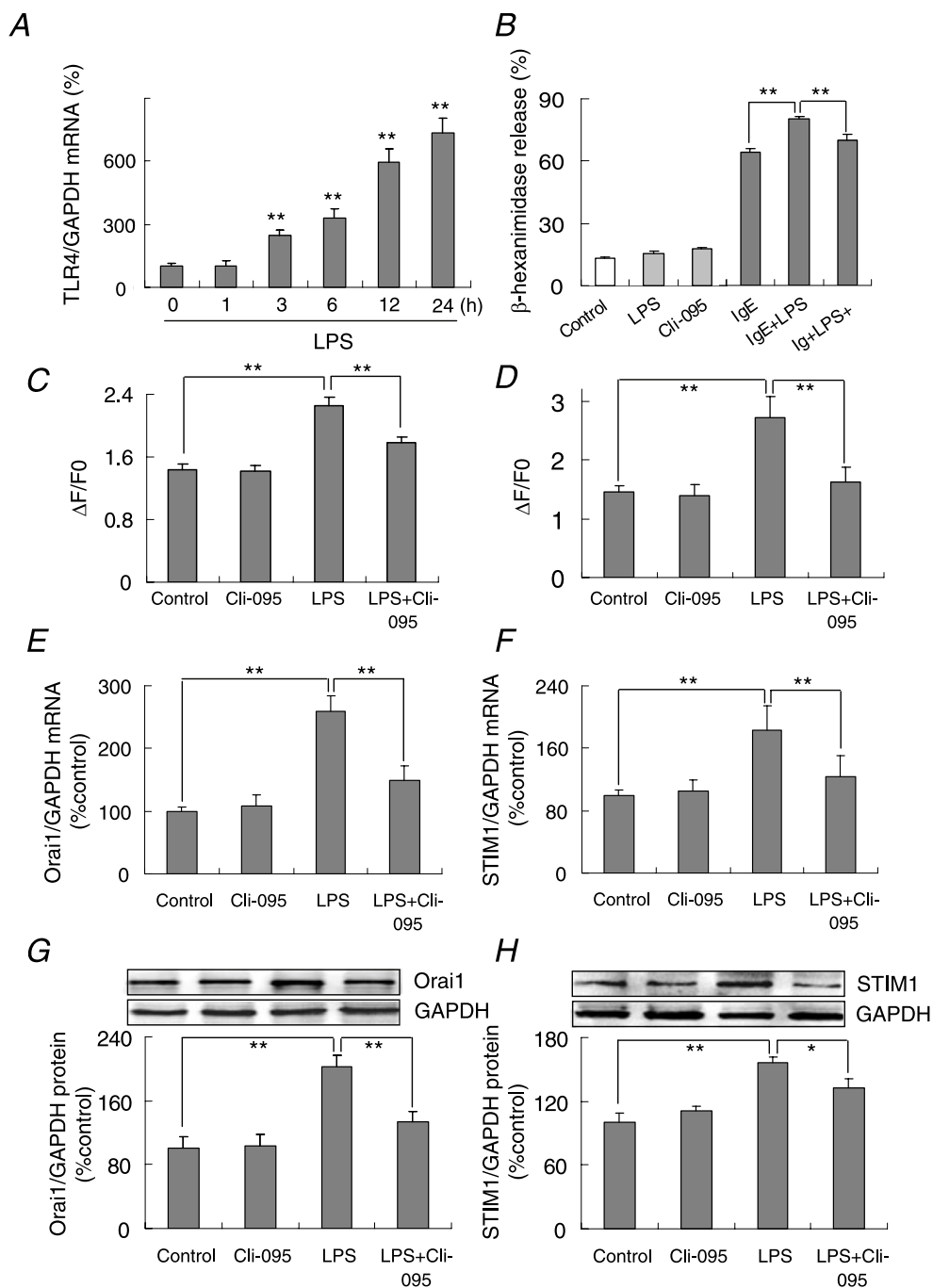


Figure 4. Lipopolysaccharide regulated Ca^{2+} mobilization and degranulation in mast cells through Toll-like receptor 4 (TLR4)

A, quantification of mRNA level of TLR4. The RBL-2H3 cells were incubated with $1 \mu\text{g ml}^{-1}$ LPS for 0–24 h. $**P < 0.01$ versus the value at 0 h (by repeated-measures one-way ANOVA and Student–Newman–Keuls test; $n = 4$ for each time point). B and C, effect of Cli-095 on mast cell degranulation (B) and Ca^{2+} mobilization (C) with or without LPS treatment. Cli-095 is a specific inhibitor of TLR4. Lipopolysaccharide-induced enhancement of IgE-dependent Ca^{2+} mobilization and degranulation were inhibited by blocking TLR4 with Cli-095. D, effects of Cli-095 on thapsigargin (TG)-stimulated Ca^{2+} entry through SOCs with or without LPS treatment. E and F, effects of Cli-095 on LPS-induced modulation of mRNA levels of Orai1 (E) and STIM1 (F). G and H, effects of Cli-095 on LPS-induced modulation of protein levels of Orai1 (G) and STIM1 (H). Cli-095 inhibited LPS-induced upregulation of mRNA transcription and protein expression of both subunits of SOCs. $*P < 0.05$, $**P < 0.01$ (by repeated-measures one-way ANOVA and Student–Newman–Keuls test; $n = 30$ –40 cells from four independent experiments for each group in B–D, and $n = 6$ for each group in E–H).

For detection of IgE receptor (FcεRI)-mediated Ca^{2+} mobilization, the cells were sensitized overnight with $1 \mu\text{g ml}^{-1}$ DNP-specific IgE before loading with fluo-4 AM. The FcεRI-mediated Ca^{2+} mobilization was stimulated with $1 \mu\text{g ml}^{-1}$ DNP-BSA.

Real-time quantitative RT-PCR

Total RNA from RBL-2H3 cells was extracted using TRIzol[®] Reagent (Invitrogen). Reverse transcription was conducted in a $20 \mu\text{l}$ reaction mixture and cDNA was synthesized from $2 \mu\text{g}$ of total RNA. The RT products were then used as templates for subsequent PCR with gene-specific primers (Marie *et al.* 2009). The primer sequences for different genes were as follows: *Orail*, forwards 5'-ACGTCCACAACCTCAACTCC-3' and reverse 5'-GGTATTCTGCCTGGCTGTCA-3'; *STIM1*, forwards 5'-GGCCAGAGTCTCAGCCATAG-3' and reverse 5'-TAGTCGCACCTCCTGGATAC-3'; Toll-like receptor 4 (*TLR4*), forwards 5'-TGCTCAGACATGGCAGTTTC-3' and reverse 5'-TCAAGGCTTTTCCATCCAAC-3'; and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), forwards 5'-TCACCATCTTCCAGGAGCGA-3' and reverse 5'-TGCTGGTGAAGCCGTAACAC-3'. Real-time

PCR was performed using Bio-Rad SsoFast[™] EvaGreen[®] Supermix, with the following cycling conditions: 5 min at 94°C , followed by 45 cycles of 94°C for 30 s, 54.3°C for 30 s and 72°C for 45 s. The RNA abundance was expressed as $\Delta\Delta C_t$, and the fluorescence signals of target gene expression were normalized to that of the internal control (*GADPH*).

Western blotting

Total cellular extracts from RBL-2H3 cells were lysed in Laemmli buffer. Equal amounts of proteins were loaded, separated by 10% SDS-PAGE before being transferred to polyvinylidene difluoride membranes, and then probed with the following primary antibodies: anti-Orail (1:1000 dilution; Abcam, Cambridge, UK), anti-STIM1 (1:1000 dilution; Abcam) and anti-GADPH (1:1500 dilution; Abcam), followed by HRP-conjugated secondary antibody (Abcam). Bound antibodies were visualized using the enhanced chemiluminescence (ECL) detection kit (Invitrogen). Equal loading was confirmed by immunoblotting for GADPH and subjected to SDS-PAGE in an identical manner.

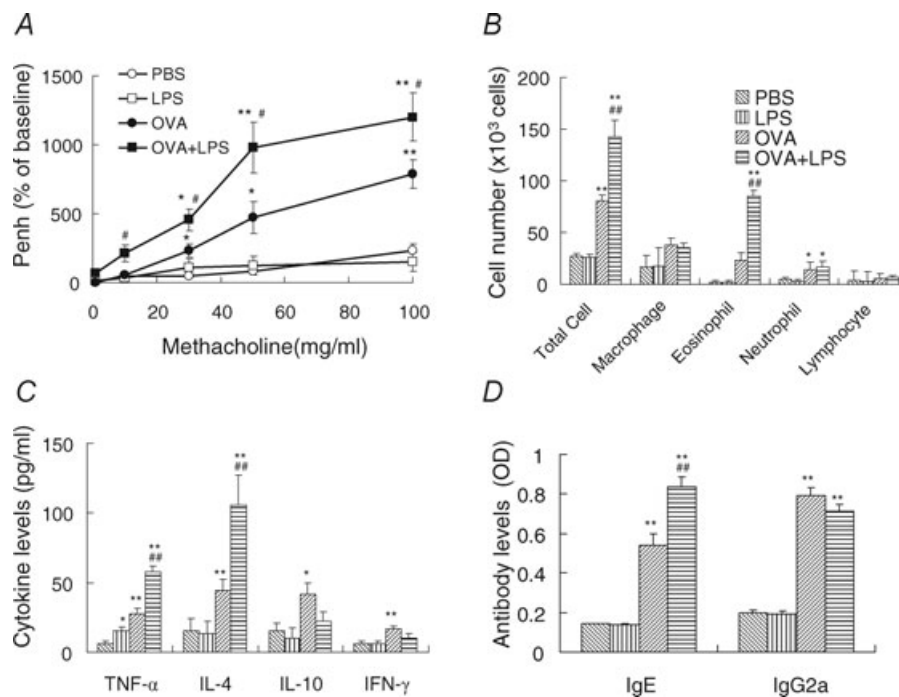


Figure 5. Lipopolysaccharide exacerbated allergic asthma

A, average of airway hyperresponsiveness (AHR) in PBS, LPS, ovalbumin (OVA) and OVA+LPS groups. Airway hyperresponsiveness was measured using plethysmography 24 h after the last OVA challenge. $n = 4$ animals for each group. B and C, statistics of inflammatory cell numbers (B) and cytokine levels [including tumour necrosis factor- α (TNF α), interleukin-4 (IL-4), interleukin-10 (IL-10) and interferon- γ (IFN- γ)] in bronchoalveolar lavage fluid (BALF; C) in four groups. D, average antibody levels of IgE and IgG2a in serum in four groups. * $P < 0.05$, ** $P < 0.01$ versus PBS; and # $P < 0.05$, ## $P < 0.01$ versus OVA (by repeated-measures one-way ANOVA and Student–Newman–Keuls test).

Allergen sensitization and challenge

Allergen sensitization and challenge was performed as described previously with minor revision (Eisenbarth *et al.* 2002). The mice were anaesthetized with pentobarbital sodium (60 mg kg^{-1} i.p.) and sensitized intranasally with $100 \mu\text{g}$ of ovalbumin (OVA; Sigma) and $1 \mu\text{g}$ of LPS (Sigma) in $35 \mu\text{l}$ of PBS on days 0, 1, 2 and 3. The sensitized mice were challenged on days 14, 15, 18 and 19 intranasally with $50 \mu\text{g}$ of OVA in $35 \mu\text{l}$ of PBS under general anaesthesia with pentobarbital sodium (60 mg kg^{-1} i.p.). Methacholine AHR and lung inflammation were evaluated on days 20 and 21, respectively. Mouse peritoneal mast cells were isolated on day 21 for detection of Ca^{2+} signalling.

Measurement of airway hyperresponsiveness

Twenty-four hours after the final aerosol challenge, AHR was assessed in conscious and unrestrained mice by means of whole-body plethysmography (Buxco, San Diego, CA,

USA), as described previously (Albertine *et al.* 2002; Lee *et al.* 2012). The enhanced minute pause (Penh) value was evaluated from bronchoconstriction-induced changes in box pressure during expiration and changes in box pressure during inspiration. Mice were exposed to increasing concentrations of nebulized methacholine (from 0 to 100 mg ml^{-1} , dissolved in PBS). The exposure time for each dose was 2 min. The highest Penh value obtained during each methacholine challenge was expressed as a proportion of the basal Penh value seen in response to PBS challenge.

Inflammatory cell counts in bronchoalveolar lavage fluid (BALF)

Mice were anaesthetized by inhalation of ether and killed by decapitation, and tracheotomies were performed. After ice-cold PBS (0.5 ml) was instilled into the lungs, BALF was obtained by three aspirations via tracheal cannulation. The BALF was centrifuged at $500g$, 37°C for 5 min. The

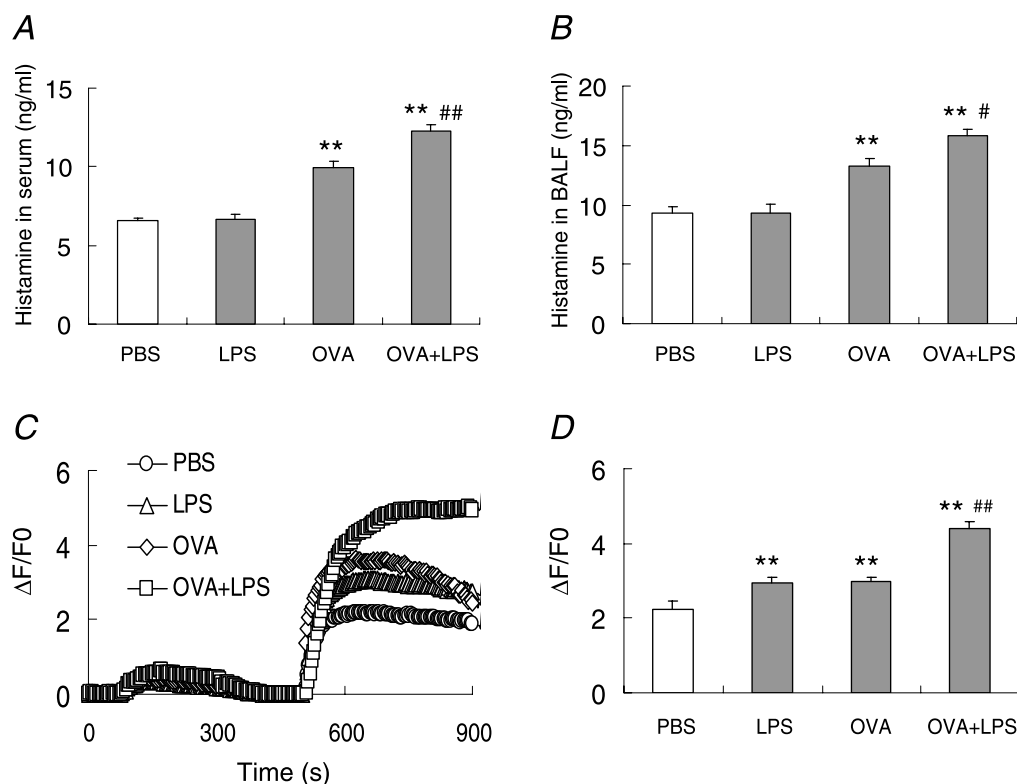


Figure 6. Lipopolysaccharide treatment increased histamine release and mast cell Ca^{2+} entry in OVA-challenged mice

A and B, statistical analysis of histamine concentration in serum (A) and in BALF (B) in PBS, OVA, LPS and OVA+LPS groups. $n = 4$ animals for each group. C, typical responses of TG-evoked Ca^{2+} entry through SOC in peritoneal mast cells from mice in each of the four groups. D, average of the peak amplitude of Ca^{2+} entry in different groups ($n = 60$ – 100 cells for each group). $*P < 0.05$, $**P < 0.01$ versus PBS; and $\#P < 0.05$, $\##P < 0.01$ versus OVA (by repeated-measures one-way ANOVA and Student–Newman–Keuls test).

cell pellet was resuspended in 1 ml PBS for counting inflammatory cell numbers, while the supernatant was collected and stored at -80°C for measurement of cytokines and histamine in BALF.

Enzyme-linked immunosorbent assay (ELISA)

The contents of tumour necrosis factor- α (TNF α), interleukin-4 (IL-4), interleukin-10 (IL-10), interferon- γ (IFN- γ) and histamine in BALF and serum were assayed by commercial ELISA kits using paired antibodies according to the manufacturer's instructions. The kits for detecting TNF α , IL-4, IL-10 and IFN- γ were bought from eBioscience (San Jose, CA, USA) and the kit for detecting histamine was bought from R&D Inc. (Wiesbaden, Germany). Serum IgE and IgG2a levels were also detected using a commercial ELISA kit (BD Pharmingen, San Diego, CA, USA), following the manufacturer's instructions.

Statistics

Data are presented as means $\pm$ SEM. When two comparisons were obtained, Student's unpaired two-tailed *t* test was used. When multiple comparisons were obtained, the analyses consisted of one-way ANOVA for repeated measures and Student–Newman–Keuls multiple-comparison test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Lipopolysaccharide enhanced Fc ϵ RI-mediated mast cell degranulation

We first examined the effect of LPS on mast cell degranulation with or without Fc ϵ RI activation. Treatment of RBL-2H3 cells with 0.1, 1 or $10\text{ }\mu\text{g ml}^{-1}$ LPS for 24 h did not induce mast cell degranulation (Fig. 1A). In contrast, LPS increased Fc ϵ RI-activated degranulation in RBL-2H3 cells. At concentrations of 0.001 – $1\text{ }\mu\text{g ml}^{-1}$, LPS treatment for 24 h significantly enhanced IgE-dependent degranulation in a dose-dependent manner (Fig. 1B). However, this effect was decreased when the LPS concentration was further increased (Fig. 1B). Furthermore, LPS ($1\text{ }\mu\text{g ml}^{-1}$) regulated mast cell degranulation in a time-dependent manner. A significant increase of degranulation appeared 6 h after LPS treatment (Fig. 1C).

We next explored the effect of LPS on different concentrations of antigen. As shown in Fig. 1D, LPS ($1\text{ }\mu\text{g ml}^{-1}$) treatment for 24 h significantly enhanced the degranulation by 26.3, 21.7, 24.4 and 22.4% with 1, 10, 100 and 1000 ng ml^{-1} DNP–BSA, respectively, indicating

that LPS has a similar effect on degranulation with different concentrations of antigen. In primary cultures of MPMCs, LPS increased IgE-dependent degranulation by 30% (Fig. 1E). These results collectively indicate that chronic exposure to LPS enhances mast cell degranulation upon antigen stimulation.

Lipopolysaccharide increased Ca^{2+} entry through SOCs in mast cells

It has been suggested that an increase in intracellular Ca^{2+} is essential for Fc ϵ RI-mediated mast cell degranulation (Fewtrell *et al.* 1987). We therefore examined whether LPS modulates Fc ϵ RI-stimulated Ca^{2+} mobilization. As reported previously, Fc ϵ RI cross-linking evoked an increase in intracellular Ca^{2+} in RBL-2H3 cells (Fig. 2A) and MPMCs (Fig. 2B). Parallel to the alteration of degranulation, LPS treatment significantly increased the Fc ϵ RI-elicited Ca^{2+} rise in both types of mast cells (Fig. 2A–C). Lipopolysaccharide treatment for 24 h increased the peak amplitude of the Ca^{2+} rise (indexed by the alteration of Fluo 4 fluorescence, $\Delta F/F_0$) by 57.3% in RBL-2H3 cells ($P < 0.01$) and by 89.9% in cultured peritoneal mast cells ($P < 0.01$). Furthermore, LPS increased the Fc ϵ RI-elicited Ca^{2+} rise in RBL-2H3 cells in a time-dependent manner (Fig. 2C), consistent with the time-dependent enhancement of degranulation by LPS.

To investigate the contribution of Ca^{2+} entry through SOCs to the LPS-induced increase of the Fc ϵ RI-activated Ca^{2+} rise, we examined the effect of blocking SOCs with LaCl_3 ($10\text{ }\mu\text{M}$; Parekh & Putney, 2005) on the Ca^{2+} signalling. The result (Fig. 2D) shows that LaCl_3 completely abolished the LPS-induced increase of Fc ϵ RI-activated Ca^{2+} mobilization, suggesting that LPS acts on Ca^{2+} entry through SOCs to upregulate Fc ϵ RI-activated Ca^{2+} mobilization.

The direct effect of LPS on Ca^{2+} entry through SOCs was further examined with a Ca^{2+} add-back protocol to distinguish Ca^{2+} entry from endoplasmic reticulum Ca^{2+} release, as described previously (Hoth & Penner, 1993; Ma *et al.* 2008). The cells were first stimulated by $1\text{ }\mu\text{M}$ TG, a sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA; Ca^{2+} pump) blocker (Putney, 1989; Janczewski & Lakatta, 1993) in the presence of 0 mM extracellular Ca^{2+} to deplete the endoplasmic reticulum Ca^{2+} store completely. Entry of Ca^{2+} through SOCs was then induced by adding back 2 mM Ca^{2+} . Figure 2E and F shows the typical Ca^{2+} response to the Ca^{2+} add-back protocol with or without LPS treatment. Lipopolysaccharide significantly increased the peak amplitude of Ca^{2+} entry through SOCs ($P < 0.01$; Fig. 2E–H).

To explore the causal relationship between the LPS-induced increase of Ca^{2+} entry through SOCs

and LPS enhancement of Fc ϵ RI-activated mast cell degranulation, we examined Fc ϵ RI-activated mast cell degranulation while the SOC α s were blocked. The results show that LaCl $_3$ completely abolished Fc ϵ RI-activated mast cell degranulation with or without LPS treatment (Fig. 1F), indicating that LPS enhancement of mast cell degranulation is dependent on SOC-mediated Ca $^{2+}$ entry.

Lipopolysaccharide increased mRNA and protein levels of Orai1 and STIM1

It has recently been discovered that STIM1 and Orai1 proteins are two major players in both the signalling and permeation mechanisms for SOC α s (James & Putney, 2007; Deng *et al.* 2009). Overexpression of STIM1 together with Orai1 causes a massive increase in store-operated Ca $^{2+}$ entry in rat basophilic leukemia (RBL) cells (Soboloff *et al.* 2006). To test whether this is the case for LPS increasing store-operated Ca $^{2+}$ entry, we measured mRNA transcription and protein expression of both subunits during stimulation with LPS. The results show that LPS increased mRNA and protein levels of both subunits in a time-dependent manner (Fig. 3). The mRNA levels of Orai1 and STIM1 were significantly increased 3 h after LPS treatment (Fig. 3A and B), while protein levels were significantly increased 3 h (Orai1; Fig. 3C) or 6 h after LPS treatment (STIM1; Fig. 3D). The PBS control had no effect on transcription and protein expression of both subunits in 24 h (data not shown). The data indicate that LPS increases SOC activity mainly by increasing transcription and expression of SOC subunits.

The role of TLR4 in LPS regulation of Ca $^{2+}$ mobilization and degranulation

It has been suggested that LPS is a TLR4 ligand and stimulates mast cells in a TLR4-dependent manner to produce a series of cytokines and chemokines (McCurdy *et al.* 2001; Supajatura *et al.* 2002; Qiao *et al.* 2006). We therefore investigated whether TLR4 is the molecule responsible for the effects of LPS on mast cells. The results show that LPS increased TLR4 mRNA levels in a time-dependent manner (Fig. 4A). Blocking TLR4 with its specific inhibitor, Cli-095 (1 μ g ml $^{-1}$, applied 6 h before addition of LPS), significantly diminished the LPS enhancement of Fc ϵ RI-mediated degranulation and Ca $^{2+}$ mobilization (Fig. 4B and C), as well as TG-elicited Ca $^{2+}$ entry (Fig. 4D). Concomitantly, Cli-095 decreased LPS-mediated upregulation of Orai1 and STIM1 mRNA and protein levels (Fig. 4E–H). These results collectively indicate that TLR4 is an important molecule for LPS regulation of Ca $^{2+}$ mobilization and degranulation in mast cells.

Lipopolysaccharide exacerbated allergic asthma

Lipopolysaccharide enhanced AHR. The 24 h OVA challenge remarkably increased AHR. The average Penh values at 30, 50 and 100 mg ml $^{-1}$ methacholine in OVA-treated mice were significantly higher than that in PBS-treated control mice ($P < 0.05$ at 30 and 50 mg ml $^{-1}$ methacholine and $P < 0.01$ at 100 mg ml $^{-1}$ methacholine; Fig. 5A). Lipopolysaccharide treatment further increased the average Penh values in OVA-challenged mice ($P < 0.05$ versus OVA; Fig. 5A).

Lipopolysaccharide aggravated inflammatory reactions.

A summary of infiltrating cells and cytokine levels in BALF is presented in Fig. 5B and C, respectively. The OVA challenge resulted in significant eosinophilic infiltration and an increase of cytokine levels of TNF- α , IL-4, IL-10 and IFN- γ . Lipopolysaccharide treatment in combination with the OVA challenge further increased eosinophilic infiltration, as well as the production of TNF- α and the Th2 response-associated cytokine IL-4 (Fig. 5B and C). Moreover, LPS plus OVA synergistically increased the serum IgE level related to Th2-type responses (Fig. 5D), suggesting that LPS aggravates the allergen-associated Th2 response.

Lipopolysaccharide increased histamine release and mast cell Ca $^{2+}$ entry in OVA-challenged mice.

Mast cell degranulation *in vivo* was estimated by histamine release to BALF and serum. The OVA challenge caused a significant increase of histamine level in both serum and BALF, indicating a burst of mast cell degranulation (Fig. 6A and B). Although LPS alone had no effect on histamine release, it synergistically increased histamine release with OVA challenge. Histamine concentrations in both serum and BALF in LPS-treated allergic mice were significantly higher than those in LPS-untreated allergic mice ($P < 0.05$).

The *in vitro* data suggest that the LPS-induced increase in Ca $^{2+}$ entry through SOC α s contributes largely to enhancement of IgE-dependent mast cell degranulation. To test whether this is the case *in vivo*, we examined Ca $^{2+}$ entry through SOC α s elicited by TG stimulation in MPMCs. The results show that either OVA challenge or LPS stimulation increased Ca $^{2+}$ entry through SOC α s in MPMCs, and LPS in combination with OVA has a synergistic effect on it (Fig. 6C and D).

Taken together, the results indicate that LPS enhanced mast cell degranulation in OVA-challenged mice, which is likely to be attributed to upregulation of Ca $^{2+}$ entry through SOC α s in mast cells.

Discussion

Lipopolysaccharide enhances IgE-dependent mast cell degranulation: a potential mechanism for LPS exacerbating allergic asthma

Previous study has revealed that the acute effect of LPS is related to an increase of cytokine synthesis, without interference with mast cell degranulation upon FcεRI activation (Qiao *et al.* 2006). It is therefore proposed that increased inflammatory cytokine release, rather than enhancement of degranulation in mast cells, accounts for the LPS-induced exacerbation of allergic asthma. However, in the present study, we challenged this notion by the evidence that chronic exposure to LPS enhanced antigen-stimulated mast cell degranulation in RBL-2H3 cells and MPMCs, as well as in an allergic asthmatic mouse model. Our finding is not contradictory to previous research, because this effect can only be induced 6 h after LPS stimulation, while previous study only investigated the acute effect of LPS (applied within 1 h). In the allergic asthmatic mouse model, we found that LPS treatment aggravated OVA challenge-induced airway hyperreactivity and enhanced eosinophilic inflammation. At the same time, Ca²⁺ entry in mast cells and mast cell degranulation (demonstrated as histamine levels in both serum and BALF) were increased. As mediators degranulated from mast cells make a large contribution to the major symptoms of asthma, it is rational to propose that enhancement of mast cell degranulation plays an important role in LPS exacerbating allergic asthma.

It is noteworthy that the enhancement of FcεRI-stimulated mast cell degranulation by LPS is relatively small in comparison with the significant increase of airway responses, suggesting that LPS exacerbating allergic asthma is only partly attributed to enhancement of mast cell degranulation. This is in agreement with previous reports that LPS augmentation of airway inflammatory reactions also participates in LPS aggravating allergic asthma (Masuda *et al.* 2002; Nigo *et al.* 2006). In the present study, we found that LPS applied *in vitro* significantly increased SOC activity, indexed by TG-elicited Ca²⁺ entry, through increasing expression of SOC subunits (Fig. 2H). However, the increase of TG-elicited Ca²⁺ entry in MPMCs isolated from LPS-treated mice (Fig. 6D) is relatively smaller. The disparity between the effects of LPS *in vivo* and *in vitro* might be due to a lower LPS concentration *in vivo* compared with that *in vitro*. Furthermore, the isolation procedure for the MPMCs might change some of the properties of SOCs, and the acutely isolated cells cannot completely reflect complicated situations *in vivo*. Nevertheless, the data indicate that LPS applied *in vivo* increases Ca²⁺ entry through SOCs in mast cells, which contributes to LPS-induced enhancement of mast cell degranulation.

In addition, the present findings should be interpreted with caution, because we employed the method of measurement of Penh by unrestrained plethysmography to assess airway responsiveness, which does not provide direct assessment of a specific physiological variable (Drazen *et al.* 1999; Lundblad *et al.* 2002).

Lipopolysaccharide enhances mast cell degranulation by upregulating SOCs via increasing transcription and protein expression of Orai1 and STIM1

It is well established that an increase of intracellular Ca²⁺ characterized by Ca²⁺ entry through SOCs is essential to mast cell degranulation (Fewtrell & Sherman, 1987; Ma *et al.* 2008; Sanchez-Miranda *et al.* 2010). With regard to IgE-mediated mast cell degranulation, FcεRI aggregation activates phospholipase Cγ to increase IP₃ generation. The IP₃ causes Ca²⁺ release from the endoplasmic reticulum through IP₃ receptors, which consequently results in a large amount of Ca²⁺ influx via SOCs, leading to mast cell degranulation. In the present study, we found that, parallel to enhancement of FcεRI-mediated mast cell degranulation, LPS increased FcεRI-mediated Ca²⁺ entry through SOCs. Blocking SOCs with La³⁺ completely abolished the LPS-induced enhancement of mast cell degranulation. Furthermore, inhibition of LPS-induced enhancement of FcεRI-activated Ca²⁺ increase and TG-stimulated Ca²⁺ entry through SOCs by blocking TLR4 with Cli-095 suppressed FcεRI-mediated mast cell

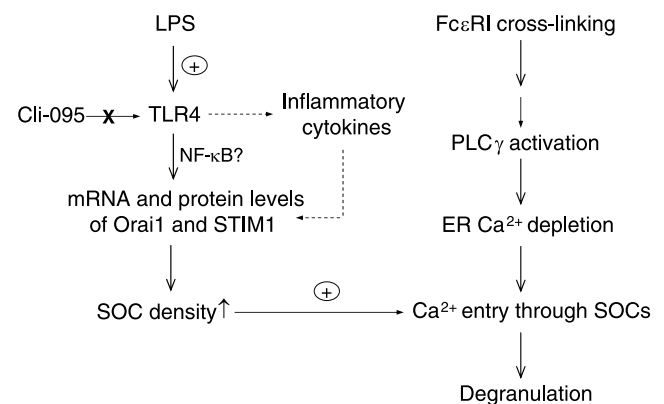


Figure 7. Proposed model for LPS enhancing mast cell degranulation upon antigen stimulation

In this model, LPS increases Orai1 and STIM1 transcripts and protein expression levels through interacting with Toll-like receptor 4 (TLR4). The increased SOC density due to upregulation of both subunits results in enhancement of FcεRI-activated mast cell degranulation. Blocking TLR4 with Cli-095 inhibits the effects of LPS on mast cells. Activation of the TLR4–nuclear factor-κB (NF-κB) pathway might be involved in LPS-induced upregulation of the transcription and expression of SOC subunits. Abbreviations: ER, endoplasmic reticulum; and PLCγ, phospholipase Cγ.

degranulation concomitantly. We propose, therefore, that an increase of Ca^{2+} entry through SOC_s contributes to enhancement of Fc ϵ RI-mediated mast cell degranulation by LPS.

It has recently been discovered that STIM1 and Orai proteins are two major players in both the signalling and the permeation mechanisms for SOC_s. Overexpression of STIM1 together with Orai1 causes a massive increase in store-operated Ca^{2+} entry in RBL cells (Soboloff *et al.* 2006). In the present study, we found that LPS increased mRNA and protein levels of Orai1 and STIM1 in a time-dependent manner. Furthermore, blocking TLR4 simultaneously inhibited the LPS-induced increase of mRNA and protein levels of both subunits of SOC_s and diminished the LPS-induced enhancement of Ca^{2+} mobilization. Therefore, we propose that an increase of transcription and expression of SOC subunits contributes to LPS-induced upregulation of SOC activity. The time-consuming course of transcription and expression of SOC subunits determines that enhancement of mast cell degranulation appears 6 h after LPS stimulation.

Lipopolysaccharide is the ligand of TLR4, which is widely expressed in mast cells, including RBL-2H3 cells (Gon *et al.* 2005; Passante *et al.* 2009). Previous study has shown that LPS acts through TLR4 in mast cells to increase Fc ϵ RI-activated cytokine production, enhancing eosinophilic inflammation in allergic asthmatic mice (Nigo *et al.* 2006; Qiao *et al.* 2006). In the present study, we found that LPS-induced enhancement of mast cell degranulation and Ca^{2+} mobilization was largely suppressed by blocking TLR4 with its specific inhibitor, Cli-095. Concomitantly, LPS-induced upregulation of Orai1 and Stim1 transcription and expression was also significantly inhibited by Cli-095. These results collectively suggest that TLR4 plays an important role in LPS-induced modulation of mast cell Ca^{2+} mobilization and consequent degranulation. A schematic diagram of the proposed model for LPS enhancing mast cell degranulation upon antigen stimulation is shown in Fig. 7.

In summary, the present study, for the first time, that LPS enhances Fc ϵ RI-activated mast cell degranulation, which potentially plays an important role in LPS exacerbating allergic asthma. An increase of Ca^{2+} entry through SOC_s upon Fc ϵ RI activation is accounted for by upregulation of both Orai1 and Stim1 transcription and expression, which contributes to enhancement of mast cell degranulation. Toll-like receptor 4 on mast cells is a crucial molecule in LPS regulation of Ca^{2+} mobilization and degranulation.

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